ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti

IJFS

Volume XXXI
Number 3
2019

CHIRIOTTI EDITORI

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded By Paolo Fantozzi under the aegis of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Initially supported in part by the Italian Research Council (CNR) - Rome - Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage - Rome - Italy

Editor-in-Chief:
Paolo Fantozzi - Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università di Perugia
Via S. Costanzo, I-06126 Perugia, Italy - Tel. +39 075 5857910 - Telefax +39 075 5857939-5857943
e-mail: paolo.fantozzi@ijfs.eu

Co-Editors:
Chiavaro Emma - Università degli Studi di Parma, e-mail: emma.chiavaro@unipr.it
Del Caro Alessandra - Università degli Studi di Sassari - e-mail: delcaro@uniss.it
De Noni Ivano - Università degli Studi di Milano, e-mail: ivano.denoni@unimi.it
Hidalgo Alyssa - Università degli Studi di Milano, e-mail: alyssa.hidalgovidal@unimi.it
Lavelli Vera - Università degli Studi di Milano - DeFENS, e-mail: vera.lavelli@unimi.it
Loizzo Monica Rosa - Università della Calabria, e-mail: monica_rosa.loizzo@unical.it
Rantsiou Kalliopi - Università di Torino, e-mail: kalliopi.rantsiou@unito.it
Rolle Luca Giorgio Carlo - Università degli Studi di Torino, e-mail: ijfscoedi@unito.it
Vincenzi Simone - Università degli Studi di Padova, e-mail: simone.vincenzi@unipd.it
Vittadini Elena Giovanna - Università di Camerino, e-mail: elenagiovanna.vittadini@unicam.it

Publisher:
Alberto Chiriotti - Chiriotti Editori srl, Viale Rimembranza 60, I-10064 Pinerolo, Italy - Tel. +39 0121 393127 - Fax +39 0121 794480 e-mail: alberto@chiriottieditori.it - URL: www.chiriottieditori.it

Aim:

The Italian Journal of Food Science is an international journal publishing original, basic and applied papers, reviews, short communications, surveys and opinions on food science and technology with specific reference to the Mediterranean Region. Its expanded scope includes food production, food engineering, food management, food quality, shelf-life, consumer acceptance of foodstuffs. Food safety and nutrition, and environmental aspects of food processing.

Reviews and surveys on specific topics relevant to the advance of the Mediterranean food industry are particularly welcome.

Upon request and free of charge, announcements of congresses, presentations of research institutes, books and proceedings may also be published in a special "News" section.

Review Policy:

The Co-Editors with the Editor-in-Chief will select submitted manuscripts in relationship to their innovative and original content. Referees will be selected from the Advisory Board and/or qualified Italian or foreign scientists. Acceptance of a paper rests with the referees.

Frequency:

Quarterly - One volume in four issues. Guide for Authors is published in each number and annual indices are published in number 4 of each volume.

Impact Factor:

Impact Factor: 0.615 published in 2017 Journal of Citation Reports, Scopus CiteScore 2018: 0.90. IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); UDL-Edge Citations Index (Malaysia); EBSCO Publishing; Index Copernicus Journal Master List (PL).

IJFS has a publication charge of € 350.00 each article.

Subscription Rate: IJFS is now an Open Access Journal and can be read and downloaded free of charge at http://www.ijfs.eu

Journal sponsorship is € 1,210.00

HOUSEHOLD CHARACTERISTICS INFLUENCING FISH CONSUMPTION IN VAN PROVINCE, TURKEY

M. TERİN*
Van Yuzuncu Yil University, Faculty of Agriculture, Department of Agricultural Economics, 65080 Van, Turkey
*Corresponding author: Tel.: +905356478210
E-mail address: mustafaterin@yyu.edu.tr

ABSTRACT

This study investigates the relationship between households' fish consumption frequency and their socio-demographic characteristics and attitudes. Using Chi-square test of independence, the study compares households' fish consumption frequencies of never, once a month, twice a month, once a week and more than once a week. The empirical model was estimated using an ordered probit model to obtain the coefficients applied to the calculation of marginal effects and probabilities. The results indicate that, households' income, children per households, working households' head, households' consumption of aquaculture products other than fish and the surveyed being households' head significantly influence the frequency of fish consumption.

Keywords: fish consumption, socio-demographic characteristics, ordered probit model, Turkey

1. INTRODUCTION

In recent years, healthy nutrition has increasingly been encouraged, as a result, different healthy food consumption tendencies are emerged (GILBERT, 2000; LEEK *et al.*, 2000). Sea food is an important part of healthy nutrition (TRONDSEN *et al.*, 2003). Regular fish consumption reduces the likelihood of many chronic diseases including cardiovascular disease (KORNITZER, 2001; PLEADIN *et al.*, 2017) and contributes significantly to healthy living (VERBEKE and VACKIER, 2005).

Sea food is regarded as one of the most valuable nutrients in terms of the nutrients it contains. Sea food products contribute greatly to human nutrition because of high protein ratio, richness in omega-3 fatty acids, and minerals and vitamins they contain (GÜLYAVUZ and ÜNLÜSAYIN, 1999). Especially, fish has many benefits to human nutrition. Fish meat is easy to digest, contains high protein and is excellent in fat content. In addition, the vitamins and minerals and the low energy of the dietary supplement increase its importance (TATAR, 1995; TURAN *et al.*, 2006; SAYGI *et al.*, 2015). These factors are main causes that led consumers to change their consumption preferences from red meat to chicken meat and fish meat (RICKERTSEN, 1996; MANGEN and BURRELL, 2001).

Aquaculture plays an important role in ensuring nutritional needs and global food security in both developed and developing countries. In the past 50 years, global average supply of fishery products has increased by 3.2% per year on average, and world population has increased by 1.6%, resulting in an increase in average per capita consumption of aquatic products. In the world, the average per capita annual consumption of aquatic products is estimated to be 20.5 kg in 2017, while it was 9.0 kg, 17.0 kg and 20.2 kg in 1961, 2000 and 2015, respectively. This impressive increase in average fish consumption per capita was mainly due to the increase in production, income, population and urbanization, as well as the development of modern distribution channels (FAO, 2018).

As a country surrounded by sea, Turkey has a significant potential for aquatic products with its lakes, dams, streams and spring waters. Fishing in Turkey is an important field of activity in terms of being one of the basic livelihood resources in the coastal regions and human nutrition (ANONYMOUS, 2014). Aquaculture production in Turkey has shown significant fluctuations over the years. In 2017, the production of aquaculture products in Turkey increased by 7.15% to 630 thousand tons compared to the previous year. Out of this production 354 thousand tones (56.2%) was obtained through hunting, and 276 thousand tones (43.8%) were obtained through aquaculture. Van Province with the biggest Lake (Van Lake) accounts for 23.0% (8310 tons) of inland water fish production in Turkey with its *Pearl Mullet* (Tarek) fish unique for Van Lake (TURKSTAT, 2018). In addition, there are 24 trout farms in the province and approximately 200 tons of trout is produced yearly (GÜNGÖR, 2014).

In Turkey, per capita consumption of aquatic products has ranged from 6.3 to 8.6 kg/year in the last 18 years and has been 5.5 kg/year as of 2017 (TURKSTAT, 2018). The amount of consumption of aquatic products per capita differs significantly between regions in Turkey. While per capita consumption was high at regions by seas in Giresun and Trabzon 28.08 kg/year (AYDIN and KARADURMUŞ, 2013), in Mersin 25.8 kg/year (ŞEN, 2011) in Hatay 21.5 kg/year (DEMIRTAŞ *et al.*, 2014) and in İzmir 15 kg/year (ÇAYLAK, 2013), domestic, Eastern and Southeastern regions were below the world average being 13 kg/year in Tokat (ERDAL and ESENGÜN, 2008), 12.4 kg/year in Isparta (HATIRLI *et al.*, 2004), 6.5 kg/year in Erzurum (UZUNDUMLU, 2017), 4.13 kg/year in Kahramanmaraş (ERCAN and ŞAHIN, 2016), 3.8 kg/year in Niğde (BASHIMOV, 2017) and 3.4 kg/year in Ankara (GÜL YAVUZ *et al.*, 2015).

There are many factors affecting fish consumption, including socioeconomic structure, general food consumption structure, personal health status and maritime nature of the living area (MYRLAND *et al.*, 2000; TRONDSEN *et al.*, 2004; VERBEKE and VACKIER, 2005). But, the most determining factor for purchasing fish is nutrition (ADELI *et al.*, 2011). The aim of the study in this context was to determine the socio demographic and behavioral characteristics that affect the frequency of fish consumption of households in urban areas in Van.

2. MATERIAL AND METHODS

The main material of the study is the original data collected through questionnaires from 260 households living in the urban area of Van. Survey was conducted between December 2015 and January 2016. The sample size was determined by ungrouped one stage random likelihood sampling method based on households (COLLINS, 1986; AKBAY *et al.*, 2007).

$$n = t\text{^}2\ [1 + (0.02)(b - 1)] * pq/E\text{^}2 \qquad (1)$$

The statistical relationship between the frequency of fish consumption of households and their socio-demographic and behavioral characteristics was estimated using the Chi square test. On the other hand, the effects of the socio-demographic and behavioral characteristics of the habits on the fish consumption frequency was estimated using "Ordered Probit Model" method. Statistical Package for Social Science (SPPSS 17.0) and LIMDEP 10 programs were used in the analysis of the data.
The ordered probit model is based on the McFadden (1973) utility maximization theory. The utility function in the research indicates the utility of the consumer in terms of the frequency of fish consumption. However, the level of utility provided here cannot be observed. Behind the observable, intermittent and ordered categories (y) in the ordered probit model is assumed to be a continuous, but unobservable, hidden dependent variable. The unobserved, latent dependent variable (y*) is explained by the vector of explanatory variables and the error term. The term error is assumed to have normal distribution (GREENE, 2012).

$$Y^* = x'\beta + \varepsilon \quad \varepsilon \sim N\ [0, 1] \qquad (2)$$

In the study, households chose one of the five alternatives for fish consumption, the dependent variable was classified according to its size (y = 0, 1, 2, 3, 4). Thus, the relationship between the model dependent variable (y) and the unobserved dependent variable (y*) is as follows (CHEN *et al.*, 2002; GREENE, 2012).

$$\begin{array}{ll} \textit{if } y^* \le 0, & y=0 \\ \textit{if } 0 < y^* \le \mu_1, & y=1 \\ \textit{if } \mu_1 < y^* \le \mu_2 & y=2 \\ \textit{if } \mu_2 < y^* \le \mu_3 & y=3 \\ \textit{if } \mu_3 \le y^* & y=4 \end{array}$$

The dependent variable used in the model is one of the following categories: *"y=0"*, *"y=1"*, *"y=2"*, *"y=3"* and *"y=4"* which represents households' non-fish consumers, households consumes fish once per month, households consume fish once per fifteen days,

households consumes fish per week and households consumes fish more than once per week, respectively. In the ordered probit model, the likelihood of the producers selecting one of five alternatives is as follows (GREENE, 2012).

$$\text{Prob}\,(y = 0 \mid \mathbf{x}) = \Phi\,(-\mathbf{x}\beta), \quad (3)$$
$$\text{Prob}\,(y = 1 \mid \mathbf{x}) = \Phi\,(\mu_1 - \mathbf{x}\beta) - \Phi\,(-\mathbf{x}\beta), \quad (4)$$
$$\text{Prob}\,(y = 2 \mid \mathbf{x}) = \Phi\,(\mu_2 - \mathbf{x}\beta) - \Phi\,(\mu_1 - \mathbf{x}\beta), \quad (5)$$
$$\text{Prob}\,(y = 3 \mid \mathbf{x}) = \Phi\,(\mu_3 - \mathbf{x}\beta) - \Phi\,(\mu_2 - \mathbf{x}\beta), \quad (6)$$
$$\text{Prob}\,(y = 4 \mid \mathbf{x}) = 1 - \Phi\,(\mu_3 - \mathbf{x}\beta) \quad (7)$$

For all probabilities to be positive, $0<\mu 1<\mu 2<...<\mu j\text{-}1$ Φ shows the cumulative normal distribution function. The solution of the model can be realized by "logarithmic maximum likelihood" method. The marginal effects of the variables are calculated as follows for each probability (GREENE, 2012).

$$(\partial Prob(y = 0|\boldsymbol{x}))/\partial \boldsymbol{x} = -\emptyset(\boldsymbol{x}\text{^}'\,\beta)\beta, \quad (8)$$
$$(\partial Prob(y = 1|\boldsymbol{x}))/\partial \boldsymbol{x} = [\emptyset(-\boldsymbol{x}\text{^}'\,\beta) - \emptyset(\mu 1 - \boldsymbol{x}\text{^}'\,\beta)]\beta, \quad (9)$$
$$(\partial Prob(y = 2|\boldsymbol{x}))/\partial \boldsymbol{x} = [\emptyset(\mu 1 - \boldsymbol{x}\text{^}'\,\beta) - \emptyset(\mu 2 - \boldsymbol{x}\text{^}'\,\beta)]\beta, \quad (10)$$
$$(\partial Prob(y = 3|\boldsymbol{x}))/\partial \boldsymbol{x} = [\emptyset(\mu 2 - \boldsymbol{x}\text{^}'\,\beta) - \emptyset(\mu 3 - \boldsymbol{x}\text{^}'\,\beta)]\beta, \quad (11)$$
$$(\partial Prob(y = 4|\boldsymbol{x}))/\partial \boldsymbol{x} = \emptyset(\mu 3 - \boldsymbol{x}\text{^}'\,\beta)]\beta \quad (12)$$

3. RESULTS AND DISCUSSION

Out of households surveyed 89.2% consumed fish while the remaining 10.8% did not consume fish. In similar studies conducted in Van province, 88.2% and 78.53% of the consumers consumed fish, respectively (SARI *et al.*, 2000, CEYLAN, 2006). In some other studies, the fish consumption rate of consumers was 95.8% (GÜRGÜN, 2006), 88.3% (ORHAN and YÜKSEL, 2010), 90.6% (BALIK *et al.*, 2013), 98.8% (ONURLUBAŞ, 2013), 84.0% (OLGUNOGLU *et al.*, 2014), 95.0% (CICEK *et al.*, 2014) and 96.52% (DJORDJEVIC *et al.*, 2015). Out of the households who didn't consume fish 35.7% stated the reason as fish odor followed by dislike fish, having no habit of fish consumption, and insufficient purchasing power with 25.0 21.4 and 17.9%, respectively. ORHAN and YÜKSEL (2010) stated that 60.50% of consumers didn't consume fish due to odor followed by 12.12% having no habit of fish consumption in Burdur Province, Turkey.

Out of surveyed households 10.8% didn't consume fish at all. On the other hand, 27.3% of households consumed fish once per two months followed by once per week, once per month and more than once per week with 26.9, 22.7 and 12.3%, respectively (Table 1). In the study conducted by GÜNGÖR (2014), 36.2% of the consumers consumed fish once a month, followed by those who consumed fish once per fifteen days and once per week with 23.0 and 17.8%, respectively. CICEK *et al.* (2014) stated that 28.0% of consumers consumed fish once per fifteen days followed by those who consumed fish once per week and once per month with 25.0 and 23.0%, respectively in Elazığ Province, Turkey. SAYGI *et al.* (2015) pointed out that 25% of consumers in İzmir, Turkey consumed fish once per week while consumers in Ankara generally consumed fish once per fifteen days. In a study conducted in Serbia more than half of consumer at school age consumed fish once per week (52.24%) and 34.33% once per month (DJORDJEVIC *et al.*, 2015). In Mexico one third of consumers (32.51%) consumed fish more than once per week followed by 25.62% once per week, 25.62% once per week, 24.24% once per fifteen days and 17.63% once per month (PEREZ-RAMIREZ *et al.*, 2015). HICKS *et al.* (2008) pointed out in American 22.0% of consumers consumed fish more than once, 24.0% of consumers consumed fish once per

week, 29.02% of consumers consumed fish once per two-three months and 12.0% of consumers consumed fish once per month.
The average monthly income of the households varied between TL 750 and TL 7500 and the monthly average household income was TL 2776.77. While the average monthly income of 7.7% of households was less than 1000 TL, 66.9% of households had a monthly income varied between TL 1001 and TL 3000 and 25.4% of consumers had income above TL 3001. In a study conducted in Van Province, 14.9% of the households had monthly income between TL 0-1000, 43.8% between TL 1001-3000 and 41.3% had income above TL 3001 (GÜNGÖR, 2014).
The average number of children per household was 2.61 (Table 1). Out of surveyed households, 26.9% had one child followed by 23.8, 16.9, 12.0 and 11.9% of households with three, one, five and four child, respectively. In a study conducted by CEYLAN (2006), 55.21% of households in Van, Turkey had more than one child, 12.5% had one child and 2.08% had no child. GÜRGÜN (2006) pointed out that 7.3% of households had no child, 14.6% of households had two and 5.0% had one child in Bitlis, Turkey. In a study by VERBEKE and VACKIER (2005) in Belgium, it was found that 57.3% of the households consisted of families with children.

Table 1. Descriptive statistics of the sample.

Variables		Values
Dependent variable		
Fish consumption frequency		
Never	Y= 0	%10.8
Once a month	Y= 1	%22.7
Twice a month	Y= 2	%27.3
Once a week	Y= 3	%26.9
More than once a week	Y= 4	%12.3
Continuous explanatory variables		**Means**
Income (TL/month)		2776.77 (1453.18)
Child number		2.61 (1.29)
Binary explanatory variables		**Values**
Household head (if 1; otherwise 0)		%75.8
Household head woman (if 1; otherwise 0)		%19.6
House wife working (if 1; otherwise 0)		%13.8
Consumption other seafood except fish (if 1; otherwise 0)		%9.6
Resides in rental house (if 1; otherwise 0)		%33.1
Fish prices high (if 1; otherwise 0)		%62.3
Public spots effect fish consume. positive (if 1; otherwise 0)		%65.0
Household head working (if 1; otherwise 0)		%94.6

Standard deviations are given in brackets.

The average monthly fish consumption quantity per household and per capita was 6.3 kg and 1.4 kg, respectively. Average yearly fish consumption quantity per capita in Turkey is 7 kg (TURKSTAT, 2018). In this case, yearly fish consumption quantity per capita in Van province is more than that of Turkey. Pearl mullet (only caught from Van Lake) consisted 30.0% of the Turkey's inland fish production in 2017 (TURKSTAT, 2018). Considering

factors such as the price of pearl mullet, which is cheaper than the other fish types and being suitable for the taste of the local people, it is expected that the quantity of fish consumption per capita in the province of Van is relatively high. This is also confirmed by the research results.

The statistical relationship between the socio-demographic and behavioral characteristics of the households and the fish consumption frequency is given in Table 2. A statistically significant relationship was found between socio-demographic characteristics such as households' income, working household head, the households' consuming aquatic products other than fish, the high prices of fish, positive effect of public spots and fish consumption frequency of households. It can be said that the results obtained are in accordance with the expectations.

Table 2. Socio-demographic characteristics and several attitudes of the sample and fish consumption frequencies.

Variables	Never	Once a month	Twice a month	Once a week	More than once a week	X^2
Household Head						
Yes	10.2	20.8	26.4	27.9	14.7	5.95
No	12.7	28.6	30.2	23.8	4.8	
Household head woman						
Yes	13.7	21.6	27.5	25.5	11.8	0.61
No	10.0	23.0	27.3	27.3	12.	
House wife working						
Yes	2.8	22.2	27.8	33.3	13.9	3.21
No	12.1	22.8	27.2	25.9	12.1	
Consumption other seafood except fish						
Yes	0.0	16.0	8.0	48.0	28.0	17.40***
No	11.9	23.4	29.4	24.7	10.6	
Renter						
Yes	10.5	24.4	26.7	27.9	10.5	0.60
No	10.9	21.8	27.6	26.4	13.2	
Fish prices high						
Yes	17.3	25.3	28.4	19.8	9.3	29.87***
No	0.0	18.4	25.5	38.8	17.3	
Public spots effects fish consumption positive						
Yes	0.0	24.3	32.5	30.2	13.0	59.47***
No	30.8	19.8	17.6	20.9	11.0	
Household head working						
Yes	9.3	21.5	27.6	28.5	13.0	17.19***
No	35.7	42.9	21.4	0.0	0.0	
Income						
Less than 1000 TL	30.0	35.0	30.0	5.0	0.0	
1001-3000 TL	10.3	24.1	28.2	27.0	10.3	23.57***
3001 TL and over	6.1	15.2	24.2	33.3	21.2	

***: 0,01 significant level.

The ordered probit model results of the socio-demographic and behavioral characteristics that affect the frequency of fish consumption of the households are given in Table 3. The ordered probit model was found to be totally statistically significant using likelihood method ($p < 0.000$). The coefficients of the model were tested using z-rate and standard error. Estimated threshold values in the model indicate the numerical relationship between the utility function of the consumer and the consumption frequency (AKBAY *et al.*, 2007; GUNDUZ and EMIR, 2010). In view of MADDALA (1983), the threshold values should be positive and $\mu 1 < \mu 2 < \mu 3$. The threshold values of the model were positive and statistically significant at 0.01 level. This shows that the consumption frequency categories households are arranged appropriately and the socio-demographic and behavioral characteristics of the households are influential on fish consumption.

The marginal effects of the socio-demographic and behavioral characteristics that affect the frequency of fish consumption of the households are given in Table 4. While model results are interpreted, marginal effects and coefficients are discussed together. In the study, a positive and statistically significant relationship was found between the household income and the fish consumption frequency (Table 3). As a result, the increase in household income will increase the frequency of fish consumption of the households. A TL 1,000 increase in household income will result in reducing the likelihood of no consumption (y=0) and monthly consumption (y=1) by 2.0% and 3.6%, respectively while increase the likelihood of consuming in a weekly (y=3) and more than once a week (y=1), by 3.5% and 2.5%, respectively (Table 4). A positive relationship between household income and the frequency and amount of fish consumption has been found in previous studies (AKINBODE and DIPEOLU, 2012; CAN *et al.*, 2015; DAUDA *et al.*, 2016).

In the study, a positive and statistically significant relationship was found between the number of children of households and the frequency of fish consumption (Table 3). As a result, one more child in the households will reduce the likelihood of no-consumption (y=0) and consumption per month (y=1) by 1.08 and 1.92%, respectively, while it will increase the likelihood of consumption once per week (y=3) and consumption more than once per week (y=4) by 1.85 and 1.31%, respectively (Table 4). In a study by MYRLAND *et al.* (2000), it was found that the frequency of fish consumption increases by having children between 0-7 years and 8-12 years, having children between 0-7 age group reduce the likelihood of no-consumption and consuming per month by 1.9 and 1.4%, respectively while increasing the likelihood of consuming once per week and once per two weeks by 2.9 and 0.9%.

In the study, a positive and statistically significant relationship was found between the heads of households and the frequency of fish consumption (Table 3). As a result, the head of households will reduce the likelihood of no-consumption (y=0) and consumption per month(y=1) by 6.36 and 9.26%, respectively, while it will increase the likelihood of consumption once per week (y=3) and consumption more than once per week (y=4) by 9.39 and 5.68%, respectively.

A positive and statistically significant relationship was found between the consumption of other aquaculture products except fish and the frequency of fish consumption (Table 3). As a result, the households' consumption of aquaculture products except fish will reduce the likelihood of no-consumption (y=0) and consumption per month (y=1) by 5.55 and 13.58%, respectively, while it will increase the likelihood of consumption once per week (y=3) and consumption more than once per week (y=4) by 11.09 and 13.52%, respectively (Table 4). MYRLAND *et al.* (2000) pointed out that households' consumption of aquaculture products would increase the fish consumption frequency, namely would decrease the likelihood of non-consumption and consumption per month frequency by 16.2 and 23.7%, respectively while would increase the likelihood of consumption frequency of once per week and twice per week by 32.7 and 24.9%, respectively.

In the study, a positive and statistically significant relationship was found between the households' finding fish prices high and the frequency of fish consumption (Table 3). As a result, the households' considering the fish prices high will reduce the households' fish consumption frequency. The households' considering the fish prices high will increase the likelihood of no-consumption (y=0) and consumption per month (y=1) by 6.90 and 13.15%, respectively, while it will reduce the likelihood of consumption once per week (y=3) and consumption more than once per week (y=4) by 12.14 and 10.24%, respectively (Table 4).
In the study, a positive and statistically significant relationship was found between the households' thought that public spots had positive effects on fish consumption and the frequency of fish consumption (Table 3). The households' thought that public spots had positive effects on fish consumption will reduce the likelihood of no-consumption (y=0) and consumption per month (y=1) by 9.71 and 13.97%, respectively, while it will increase the likelihood of consumption once per week (y=3) and consumption more than once per week (y=4) by 14.11 and 8.97%, respectively (Table 4).
A positive and statistically significant relationship was found between the working households' head and the frequency of fish consumption (Table 3). As a result, households' head working at any place will increase fish consumption frequency. The working households' head will reduce the likelihood of no-consumption (y=0) and consumption per month (y=1) by 15.28 and 13.84%, respectively, while it will increase the likelihood of consumption once per week (y=3) and consumption more than once per week (y=4) by 16.19 and 7.31%, respectively (Table 4).

Table 3. Estimates ordered probit model for fish consumption frequencies.

Variables	Coefficient	St. Error	z-statistic	p-value
Constant	-0.2056	0.402	-0.51	0.6089
Income	0.00016	0.0000546	2.91	0.0036***
Child number	0.084	0.043	1.94	0.0528*
Household head	0.422	0.168	2.51	0.0120***
Household head woman	-0.158	0.189	-0.83	0.4040
House wife working	-0.105	0.233	-0.45	0.6518
Other seafood consumption	0.630	0.242	2.61	0.0091**
Resides in rental house	-0.147	0.153	-0.96	0.3352
Fish prices high	-0.591	0.142	-4.15	0.0000***
Public spots	0.648	0.143	4.54	0.0000***
Household head working	0.754	0.333	2.26	0.0238**
Threshold parameters				
μ (1)	1.031	0.08262	12.48	0.0000***
μ (2)	1.856	0.08267	22.45	0.0000***
μ (3)	2.879	0.11400	25.26	0.0000***
Log likelihood = -357.910		Restricted log likelihood = -400.952		
Likelihood ratio statistic (LR) = 86.084		$Chi\text{-}square_{(10;0.05)}$=18.307 LR>Chi-square=0.000		

*:0.1, **:0.05 and ***: 0,01 significant level.

Table 4. The marginal effects of factors on the probability of relative frequencies for fish consumption.

Variables	Y(0)	Y(1)	Y(2)	Y(3)	Y(4)
Income	-0.000020***	-0.000036***	-0.0000031	0.000035***	0.000025***
Child number	-0.01078*	-0.01921*	-0.00164	0.01850*	0.01313*
Household head	-0.06366**	-0.09256***	0.00552	0.09392**	0.05678***
Household head woman	0.2171	0.03566	0.00079	-0.03514	-0.02303
House wife working	0.01426	0.02384	0.00084	-0.02338	-0.01556
Other seafood consumption	-0.05501***	-0.13582***	-0.05534	0.11094***	0.13523**
Resides in rental house	0.01955	0.03337	0.00176	-0.03252	-0.02216
Fish prices high	0.06900***	0.13151***	0.02331	-0.12144***	-0.10239***
Public spots	-0.09714***	-0.13973***	0.00609	0.14111***	0.08967***
Household head working	-0.15276	-0.13839***	0.05612	0.16191**	0.07312***

*:0.1, **:0.05 and ***: 0,01 significant level.

4. CONCLUSIONS

Result of this study showed that various socio-economic and demographic factors of households and households' heads significantly influenced the likelihood of consuming fish. There was a positive relationship between the socio-economic and demographic characteristics of households such as households' income, children per households, working households' head, households' consumption of aquaculture products other than fish, households' head and the behavioral variables such as households' thought that public spots affected the fish consumption positively.
Based on the findings of the study, the following recommendations were made; public or private organization should continue to educate the households' heads (parents) on the importance of fish on their health. Price of fish should be reduced so as to increase the fish consumption in the area since it was observed that price of fish and fish consumption are inversely related. As the income of the households' increases, fish consumption also increases, therefore government should provide income opportunities by creating jobs to enhance the household's purchasing power. In this way, it is possible to provide a healthier life for the society by encouraging the households to consume more fish. Educational programs regarding healthy and balanced nutrition in the region, should intended on the development of fish consumption habits of households in the region.

REFERENCES

Adeli A., Hasangholipour T., Hossaini A., Salehi H. and Shabanpour B. 2011. Status of fish consumption per capita of Tehran citizens. Iran J. Fish Sci. 10(4):546-556.

Akbay C., Tiryaki, G.Y. and Gül A. 2007. Consumer characteristics influencing fast food consumption in Turkey. Food Control 18(8):904-913.

Akinbode S.O. and Dipeolu A.O. 2012. Double-Hurdle model of fresh fish consumption among urban households in South-West Nigeria. Current Research Journal of Social Sciences 4(6):431-439.

Anonymous 2014. Aquaculture private commission report. Publication No: 2871, Ankara.

Aydın M. and Karadurmuş U. 2013. Consumer behaviors for seafood in Giresun and Trabzon province. The Black Sea Journal of Sciences 3(9):57-71.

Balık I., Yardımcı C. and Turhan O. 2013. Comparatively investigation of fish consumption habits of people in Fatsa and Aybastı districts of Ordu province. Ordu Univ. J. Sci. Tech. 3(2):18-28

Bashimov G. 2017. Determining fish meat consumption habits in Nigde Province. Turkish Journal of Agricultural and Natural Sciences 4(2):196-204.

Can M.F., Günlü A. and Can H.Y. 2015. Fish consumption preferences and factors influencing it. Food Science and Technology 35(2):339-346.

Çaylak B. 2013. Fishery product consumption and consumer preferences survey in İzmir province Msc.Thesis, Çanakkle Onsekiz Mart University, Institute of Natural and Applied Sciences, Çanakkale, Turkey.

Ceylan M. 2006. The consumption structure of meat and meat products in urban and rural area of van province and the purchase behavior of consumers Msc.Thesis, Yuzuncu Yıl University, Institute of Natural and Applied Sciences, Van, Turkey.

Chen K.Z., Ali M., Veeman M.M., Unterschultz J. and Le T. 2002. Relative importance rankings for pork attributes by Asian-origin consumers in California:Applying an ordered probit model to a choice-based sample. Journal of Agricultural and Applied Economics 34(1):67-79.

Çiçek E., Akgün H. and İlhan S. 2014. Determination of fish meat consumption habits and preferences in Elazığ province. Yunus Research Bulletin 1:3-11.

Collins M. 1986. Sampling (Editors: Worcester R.M. and Downham J.) Consumer Market Research Handbook, London:McGraw-Hill

Dauda A.B., Ojoko E.A. and Fawole B.E. 2016. Economic analysis of frozen fish demand in Katsina metropolis, Katsina State, Nigeria. Journal of Fisheries and Aquatic Science 11(1):93-99.

Demirtaş B., Dağistan E., Akpınar M.G. and Sayın C. 2014. Fish consumption patterns of urban households in Hatay, Turkey. Journal of Academic Documents for Fisheries and Aquaculture 2:69-77.

Djordjevic V., Sarcevic D. and Petronijevic R. 2015. The attitudes and habits of Serbian school children to consumption of fish. Procedia Food Science 5:73-76.

Ercan O. and Şahin A. 2016. Analysis of Fish Meat Consumption at Kahramanmaraş City Centre. KSU J. Nat. Sci. 19(1):51-65.

Erdal G. and Esengün K. 2008. Analysis of factor affecting fish consumption with logit model in Tokat. E.U. Journal of Fisheries & Aquatic Sciences 25(3):203-209.

FAO 2018. The state of World fisheries and aquaculture. ISBN 978-92-5-130562-1 www.fao.org/3/I9540EN/i9540en.pdf (28.08.2018).

Gilbert L.C. 2000. The functional food trend: What's next and what Americans think about eggs. Journal of the American College of Nutrition 19(5):507-512.

Greene W.H. 2012. Econometric Analysis. Seventh Edition. Pearson Prentice-Hall, Upper Saddle River, New Jersey, 07458. ISBN 10:0-13-139538-6

Gül Yavuz G., Yasan Ataseven Z., Gül U. and Gülaç Z.N. 2015. Factor affecting consumer preferences on seafood consumption:The case of Ankara. Yunus Research Bulletin 1:73-82.

Gülyavuz H. and Ünlüsayın M. 1999. Fisheries processing technology. Sahin printing press, Isparta, 366

Gündüz O. and Emir M. 2010. Analysis of factors influencing frozen food consumption: A case study of Samsun, Turkey. J. Agric.Fac.HR.U. 14(3):15-24.

Güngör E.S. 2014. A survey on fish consumption and consumer preference in Erzurum and van provinces Msc.Thesis, Atatürk University, Institute of Natural and Applied Sciences, Erzurum, Turkey.

Gürgün H. 2006. An investigation into the fish consumption in some towns on the shores of Lake Van Msc.Thesis, Ege University, Institute of Natural and Applied Sciences, İzmir, Turkey.

Hatırlı S.A., Demircan V. and Aktaş A.R. 2004. An analysis of households' fish consumption in Isparta province. Süleyman Demirel University Journal of Faculty of Economics and Administrative Sciences 9(1):245-256.

Hicks D., Pivarnik L. and McDermott R. 2008. Consumer perceptions about seafood-an internet survey. Journal of Foodservice 19(4):213-226.

Kornitzer M. 2001. Fish and health among adults. In: Descheemaeker K. and Provoost C. Impact of food on health-Recent developments, Antwerpen:Garant, 53-65.

Leek S., Maddock S. and Foxall G. 2000. Situational determinants of fish consumption. British Food Journal 102(1):18-39.

Maddala G.S. 1983. Limited-dependent and qualitative variables in econometrics. New York: Cambridge University Press.

Mangen M.J. and Burrell A.M. 2001. Decomposing preference shifts for meat and fish in the Netherlands. Journal of Agricultural Economics 52(2):16-28.

McFadden D. 1973. Conditional logit analysis of qualitative choice behaviour. In: P. Zarembka (Ed.), Frontiers in econometrics (pp. 105-142). New York: Academic Press.

Myrland O., Trondsen T., Johnston R.S. and Lund E. 2000. Determinants of seafood consumption in Norway: lifestyle, revealed preference and barriers to consumption. Food Quality and Preference 11(3):169-188.

Olgunoğlu İ.A., Bayhan Y.K., Olgunoğlu M.P., Artar E. and Ukav İ. 2014. Determination of habits of fish meat consumption in the province of Adıyaman. Electronic Journal of Food Technologies 9(1):21-25.

Onurlubaş E. 2013. The factors affecting fish consumption of the consumers in Keşan Township in Edirne. Bulgarian Journal of Agricultural Science 19(6):1346-1350.

Orhan H. and Yüksel O. 2010. Fishery product consumption survey in Burdur province. Süleyman Demirel University Journal of the Faculty of Agriculture 5(1):1-7.

Perez-Ramirez M., Almendarez-Hernandez M.A., Aviles-Polanco G. and Beltran-Morales L.F. 2015. Consumer acceptance of eco-labeled fish:A Mexican case study. Sustainability 7:4625-4642.

Pleadin J., Lesic T., Kresic G., Baric R., Bogdanovic T., Oraic D., Vulic A., Legac A. and Zrncic S. 2017. Nutritional quality of different fish species farmed in the Adriatic Sea. Ital. J. Food Sci. 29:537-549.

Rickertsen K. 1996. Structural change and the demand for meat and fish in Norway. European Review of Agricultural Economics 23(3):316.330.

Sarı M., Demirulus H. and Söğüt B. 2000. A research on determination of fish meat consumption habits of students in Van province. In Proceedings of the East Anatolian Region 4th Aquaculture Symposium, Erzurum, Turkey.

Saygı H., Bayhan B. and Hekimoğlu M.A. 2015. Fishery products consumption in the cities of Ankara and İzmir in Turkey. Turkish Journal of Agriculture-Food Science and Technology 3(5):248-254.

Şen A. 2011. Individuals living in Konya and Mersin provincial centers comparison of fish consumption on the habits and knowledge level Msc. Thesis, Selçuk University, Institute of Natural and Applied Sciences, Konya, Turkey.

Tatar O. 1995. Nutritional properties of fish and healthy respect. E.U. Journal of Fisheries & Aquatic Sciences 12:169-170.

Trondsen T., Braaten T., Lund E. and Eggen A.E. 2004. Health and seafood consumption patterns among women 45-69 years. A Norwegian Fish Consumption Study 1996. Food Quality and Preference 15(2):117-128.

Trondsen T., Scholdere J., Lund E. and Eggen A.E. 2003. Perceived barriers to consumption of fish among Norwegian women. Appetite 41(3):301-314.

Turan H., Kaya Y. and Sönmez G. 2006. Fish meat value and place of human health. E.U. Journal of Fisheries & Aquatic Sciences 23:505-508.

TURKSTAT 2018. Turkish Statistical Institute. Aquaculture statistics. www.tuik.gov.tr/PreTablo.do?alt_id=1005 (28.08.2018).

Uzundumlu A.S. 2017. Determining fish consumption behavior among households and the most suitable type of fish in Erzurum Province. Iran J. Fish Sci. 16(2):684-697.

Verbeke W. and Vackier I. 2005. Individual determinants of fish consumption: application of the theory of planned behavior. Appetite 44(1):67-82.

Paper Received November 29, 2018 Accepted February 24, 2019

PAPER

EFFECT OF STORAGE METHOD: AEROBIC REFRIGERATED, VACUUM REFRIGERATED AND FROZEN STORAGE, ON THE QUALITY OF CHURRA SUCKLING LAMB MEAT

A.R. CALLEJAS-CÁRDENAS[1], S. SOTO[1,2], I. CARO[3], D.E. CARBALLO[1], E.J. QUINTO[3] and J. MATEO*[1]

[1]Departamento de Higiene y Tecnología de los Alimentos, Universidad de León, Campus de Vegazana s/n, 24007, León, Spain

[2]Instituto de Ciencias Agropecuarias. Universidad Autónoma del Estado de Hidalgo, Ave. Universidad km 1 s/n, 43600, Tulancingo, Hidalgo, Mexico

[3]Área de Nutrición y Bromatología, Facultad de Medicina. Universidad de Valladolid, Av. Ramón y Cajal 7, 47005, Valladolid, Spain

*Corresponding author: jmato@unileon.es

ABSTRACT

Suckling lamb meat is typically refrigerated stored for few days before being sold, although the use of vacuum and frozen storage is increasing. The effect of storage: non-stored (NS), 7-day aerobic refrigerated storage (ARS), 21-day vacuum refrigerated storage (VRS) and 3-month frozen storage (FS) on the suckling lamb quality was investigated using 32 legs (8 per method) of Churra-breed lambs. Raw meat pH, colour and water holding capacity (WHC) and cooked meat texture and oxidative stability were evaluated. ARS showed increased discoloration than NS. VRS showed the highest pH increment and the lowest hardness. FS was the lowest in WHC and lightness.

Keywords: meat colour, meat texture, oxidative stability, packaging, suckling lamb

1. INTRODUCTION

In the European Mediterranean region suckling lamb meat is typically produced in sheep farms with local breeds, and characterised by a white to light pink colour, mild flavour and soft texture (GORRAIZ *et al*., 2000; MARTÍNEZ-CEREZO *et al*., 2005a; SANTOS *et al*., 2007; TEIXEIRA *et al*., 2005). The suckling lamb meat produced under selected conditions in Castilla y León region (Spain) was granted by the EU's Protected Geographical Indication (PGI) "*Lechazo de Castilla y León*" (Commission Regulation EEC No. 2107/99). The PGI lambs must belong to one of the authorized breeds – Churra, Castellana or Ojalada – or their crosses, have to be fed on maternal milk and slaughtered under 35 d old. In 2017 the PGI-protected lambs were approximately 300 thousand, being Churra the predominant breed (SÁNCHEZ, 2018).
Suckling lamb meat is usually stored under aerobic refrigerated conditions in retail meat premises during a short period of time before being sold to the consumers (MARM, 2010). The PGI appellation allows aerobic refrigerated storage for a maximum of 8 d, as well as other storage methods in order to extend the meat's shelf-life as long as storage does not negatively affect the meat colour and edible quality. During aerobic refrigerated storage of carcasses or meat joints ageing takes place resulting in more tenderness, although meat becomes unacceptable to the consumer over time due to microbial spoilage (BELLÉS *et al*., 2017). Edible quality changes in suckling lamb joints during aerobic refrigerated storage have been studied (MARTÍNEZ-CEREZO *et al*., 2005a, 2005b; VIEIRA and FERNÁNDEZ, 2014). These studies reported that 4-5-d storage periods resulted in a decrease in meat hardness and fibrosity and that 4 d could be enough time to obtain a high-quality meat.
Retail premises are increasingly offering vacuum packaged suckling lamb joints, or chops obtained from previously vacuum packaged joints. Vacuum packaging provides an anoxic environment between the meat surface and the packaging retarding microbial spoilage. RUBIO *et al*. (2016) studied the shelf-life of vacuum packaged suckling lamb forelegs stored at 4 ºC and found a suitable storage period of up to 16 d. Ageing is not stopped by vacuum storage during the retail period (BELLÉS *et al*., 2017). Freezing prolongs meat shelf-life; however, freezing and thawing can affect juiciness, flavour and colour to a level that depends on the meat characteristics and the freezing, frozen storage and thawing conditions (LEYGONIE *et al*., 2012). No studies have addressed the effect of frozen storage on the quality of suckling lamb meat after thawing; however, there are a number of studies on its effect on the meat of older lambs (3-4 months old), concluding that thawed meat showed small differences in quality as compared to fresh meat, e.g. lower juiciness (BUENO *et al*., 2013; MUELA *et al*., 2016, 2012).
The storage-related changes in suckling lamb meat have been scarcely studied. The aim of the present study is thus to explore the effect of three different storage procedures (7-d aerobic refrigerated storage, 3-week refrigerated storage under vacuum, or 3-month frozen storage) on the colour and water holding capacity of suckling lamb meat, as well as on the texture and oxidative stability of this meat after cooking.

2. MATERIAL AND METHODS

2.1. Meat samples and storage

Thirty-two legs from Churra PGI "*Lechazo de Castilla y León*" lambs were sampled. According to the PGI specifications, the lambs were fed on ewe`s milk, slaughtered between 20 and 35 d of age, and their carcasses weighed between 4.5 to 7.0 kg. Legs were purchased from the local market on 8 different days, up to 6 legs per day, during a 1-

month period. All the sampled legs had to come from a different lamb and the post-mortem time at purchasing had to be one day. Twenty-four legs had to come from males and eight from females in order to resemble the distribution of gender in the market. After sampling all legs were carried to the lab at 0-6 ºC, wrapped with a cling film (polyvinyl chloride, PVC; oxygen permeability of 580 ml m^{-2} h^{-1}), and stored at 3 ºC until the following day.
Afterwards, the covering film, the tail, and the epiploic fat adhered on the leg surface were removed and the legs were weighed. The legs, weighing 785 g±184 g (standard deviation) were randomly assigned to one of the following storage groups, (i) Non-stored (NS), with no further storage; (ii) aerobic refrigerated storage (ARS) at 3 ºC for 7 d with the legs wrapped with PVC cling film; (iii) vacuum-packaged refrigerated storage (VRS) at 3 ºC for 21 d using a 150-μm film (polyamide/polyethylene 30/120; oxygen permeability of 1.25 ml m^{-2} h^{-1}); and (iv) frozen storage (FS), freezing the legs wrapped with PVC cling film in a freezing tunnel (-25 ºC for 60 min) and then stored at -18 ºC for 90 d followed by a 12-h (3 ºC) thawing period. The proportion of male-female samples in the assignment was balanced to adjust for the potential confounding effect of sex (8 legs per group with 2 out of them being from female lambs), which could be expected to be of little or no significant (MIGUÉLEZ *et al*., 2008). The ARS, VRS and FS groups would be representative, respectively, for whole lamb joints stored in a cool room, packaged under vacuum and frozen, for extending shelf-life. The NS group would serve as a control to compare the quality changes due to storage method.

2.2. Sample preparation and meat analysis

2.2.1 Raw meat

After the storage, the covering film was removed from the legs, the legs were weighed, and pH was measured in the *semimembranosus* muscle using a 52-32 puncture pH electrode (Crison Instruments, Barcelona, Spain). Colour was measured at three different points on the external surface of *gracilis* muscle where it showed the thinnest epimysium and lack of visible fat, using a spectrophotometer CM-700d (Konica Minolta Sensing Inc., Osaka, Japan) operating in triplicated with a D65 illuminant, SCI mode, 11 mm aperture for illumination and 8 mm for measurement, and 10° visual angle. The results were expressed according to the CIE *L*a*b** system and the ratio of reflectance at 630 nm and at 580 nm (R_{630}/R_{580}) was calculated as discoloration index in cut meat surfaces exposed to oxygen due to storage, i.e. the lower the ratio, the higher the proportion of metmyoglobin relative to oxymyoglobin plus deoxymyoglobin (AMSA, 1995).
The top-round-cap muscle group – *semimembranosus*, *adductor* and *gracilis* muscles – was then separated from the NS legs on the following day of purchasing or from the rest of the legs after the storage. This muscle group was sliced transversely into two equal-sized parts and 1 h after slicing the water holding capacity (WHC) as assessed in duplicate determining the percentage of expressible juice released by a 300 mg (290-310 mg) meat sample placed on filter paper after a 5 min compression under a 1-kg weight (GRAU and HAMM, 1957), and the colour of the cut surface following the above-mentioned procedure were determined. Chroma (the squared root of $a^{*2}+b^{*2}$) was calculated to better describe the vividness of colour in the meat surface (YOUNG *et al*., 1999).

2.2.2 Cooked meat

The legs, without the top-round-caps, were roasted (180 ºC) in a forced-air oven rotating them every 15 min until the leg core temperature reached 80 ºC, and then tempered at

20 ºC for one h. The *biceps femoris* and *semitendinosus* muscles were separated, individually vacuum packaged and frozen at -25 ºC for up to 15 d until further instrumental texture and oxidative stability analyses, which were carried out after a 12-h 3 ºC thawing period.
The texture profile analysis (TPA), as predictor for sensory texture (RUIZ DE HUIDOBRO *et al*., 2005), was determined in three 1.0-cm thick cubes cut from the long head of the *biceps femoris* muscles after being tempered at 21 °C for 1 h using a TA-XT2i analyser. The operating conditions used were a 25-kg load cell, 0.5 mm/s test speed, 10 mm/s pre- and post-test speeds, compression percentage of the initial height of 80%, perpendicularly to the muscle fibre, and a 10-s elapse between the 2 compression cycles. Oxidative stability was determined in duplicate using the thiobarbituric acid reactive substances (TBARS) test (NAM and AHN, 2003). For this purpose, the *semitendinosus* muscle was transversely cut in two similar parts. One was used for immediate analysis and the other was wrapped with PVC cling film, stored for two days at 3 ºC under darkness and then analyzed. Results were expressed as the increment in TBARS during storage, i.e the difference between TBARS before and after the two days storage.

2.3. Statistical analysis

The experimental data were analyzed using a univariate analysis of variance (ANOVA) with storage method as fixed factor – to assess weight losses the NS group was excluded of the model. When the fixed factor showed significance ($P < 0.05$) the ANOVA was followed by the least square difference (LSD) test. The SPSS Statistics software (version 23; IBM, Somers, NY, USA) was used.

3. RESULTS AND DISCUSSION

3.1. Raw meat

The mean colour values (± standard deviation) of the *gracillis* muscle for the 32 legs before storage were as follows: L^*, 44.0±2.4; a^*, 6.6±1.4; b^*, 11.6±2.3, and the mean pH was 5.74±0.08. No differences were found among the legs assigned to the different storage methods ($P > 0.05$).
The highest weight losses due to storage were found in the VRS (Table 1). The ARS weight losses were comparable to those found in legs submitted to frozen storage and thawing (FS) suggesting the suckling lamb meat to have a good ability to reabsorb water during thawing. In contrast, FS lamb showed the highest expressible juice percentage (Table 1), indicating the lowest WHC. Decreases in meat WHC due to frozen storage have been widely recognized and related to disruption of the muscle fibre structure and denaturation of muscle proteins (LEYGONIE *et al*., 2012). Freezing meat at slow freezing rates, i.e. using air temperatures between -20 and -33 ºC (as done in the present study) results in significant water diffusion from the muscle fibres into the intercellular spaces, fibre separation and myofibril damage (GRUJIĆ *et al*., 1993).
The pH of NS, ARS and FS meat did not differ significantly among them; however, a higher pH was found in the VRS meat (Table 1). The pH increment during refrigerated storage has been attributed to a switch from a glycolytic to an amino acid-degrading microbial metabolism (NYCHAS *et al*., 2008). In contrast to the present results, CALLEJAS-CARDENAS *et al*. (2014) found no significant change in lamb pH after a similar vacuum storage period. The discrepancy among studies could be attributed to the differences between the lamb ages at slaughter, i.e. 3-months vs 3-weeks in the former and present study, respectively, and explained by a faster microbial growth and/or lower muscle

glycogen content in the meat from the younger lambs. In spite of that the pH of thawed meat tends to be lower than prior to freezing (LEYGONIE *et al*., 2012), as seen in this study and in previous studies on light lamb (MUELA *et al*., 2010), frozen storage showed no effect on pH.

Table 1. Effect of storage method on leg weight loss, expressible juice of *adductor* muscle, pH and colour characteristics[z] of *gracilis* muscle surface and *adductor* muscle cut surface (after 1 h of slicing) in suckling lamb legs.

	NS (n=8)	ARS (n=8)	VRS (n=8)	FS (n=8)	SEM	*P*-level
Weight loss (%)	-	0.85[b]	1.29[a]	0.88[b]	0.136	*
Expressible juice (%)	19.9[b]	17.6[b]	21.4[b]	26.7[a]	1.462	**
pH	5.70[b]	5.75[b]	5.96[a]	5.79[b]	0.017	***
Colour of muscle surface						
*L**	42.88[a]	43.11[a]	45.23[a]	39.98[b]	0.990	**
*a**	7.34	8.02	6.76	6.22	0.630	n.s.
*b**	11.60	10.70	8.76	8.80	1.004	n.s.
R_{630}/R_{580}	2.52[a]	2.05[b]	2.43[a]	1.99[b]	0.111	**
Colour of muscle cut surface						
*L**	40.28[a]	37.54[a]	39.06[a]	33.32[b]	1.264	**
*a**	7.62	9.37	9.20	8.01	0.724	n.s.
*b**	15.12	17.42	16.05	16.63	0.611	#
Chroma	16.09	19.81	18.53	18.59	0.785	#

[z]: *L**, lightness; *a**, redness: *b**, yellowness; R_{630}/R_{580}, wavelength reflectance ratio (630 nm and 580 nm wavelengths).
NS: Non-stored, 2-day post-mortem refrigerated legs. ARS: refrigerated legs (2-day post-mortem) wrapped with air-permeable cling film and stored for 7 days at 3 ºC. VRS: refrigerated legs (2-day post-mortem) stored under vacuum for 21 days at 3 ºC. FS: refrigerated legs (2-day post-mortem) wrapped with air-permeable cling film, frozen and stored for 3 months at -18 ºC and then thawed at 3 ºC overnight.
SEM: Standard error of the mean.
P-level: n.s.: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.
[ab]: Means in the same row showing different superscripts are significantly different ($P < 0.05$; least significant difference test).

Colour in the muscle leg surface was affected by storage (Table 1). FS resulted in a significant lower *L** value as compared to NS, ARS and VRS. Accordingly, other studies found frozen storage to decrease *L** in comparison with not-frozen meat (MOORE and YOUNG, 1991; MUELA *et al*., 2010). This difference should be attributed to changes affecting light scattering on the meat surface and could negatively affect the colour preference by lamb consumers, which has been strongly related to *L** (CALLEJAS-CÁRDENAS *et al*., 2014; HOPKINS, 1996). It might specially affect suckling lamb meat, characterized by a bright white to light pink colour (ERASMUS *et al*., 2017). As another adverse effect on leg appearance, freezing tended to produce a more pronounced red-blood colour into the superficial large blood vessels visible on the leg internal face (Fig. 1). The differences found for R_{630}/R_{580} ratio suggest that meat discoloration occurred during those storage methods allowing the higher exposure of meat to oxygen, i.e. ARS and FS, since the R_{630}/R_{580} ratio is inversely related to metmyoglobin formation (AMSA, 1995) during aerobic storage (MANCINI and HUNT, 2005). Discoloration due to oxygen exposure is

considered to be a regular phenomenon (MCKENNA *et al*., 2005; MOORE, 1990). A decrease in that ratio was previously observed during a 10-d aerobic refrigerated storage period in suckling lamb chops (MATEO *et al*., 2018). MUELA *et al*. (2016) also reported discoloration in the meat of 3-month age lambs after one month of frozen (-18 ºC) storage as detected by a trained sensory panel.



Figure 1. Pairs of photographs from four of the legs used in this study (A to D) just before being freezing (top photo of the pair) and just after thawing (bottom); an arrow and ellipse have been drawn in each photo to locate and highlight the main difference in leg appearance due to frozen storage.

The effect of the storage on the colour of cut meat after the 1-hour blooming period was significant for L^* and near-to-significant for b^* and chroma (Table 1). L^* value was the lowest in the FS meat confirming the results obtained for the colour in the whole leg muscle surface and suggesting a lower consumer colour acceptance. Furthermore, a near-to-significant differences suggest b^* higher chrome values in ARS as compared to NS meat, which should not be considered an advantage for ARS meat because suckling lamb meat is not valuated by a more vivid colour (more chroma) but by a white pale pink colour (ERASMUS *et al*., 2017). Other studies have reported an increase in b^* due to ageing as the most relevant change in the colour of sliced ruminant meat (BOAKYE and MITTAL, 1996; VIEIRA and FERNÁNDEZ, 2014). The latter of them, studying specifically suckling lamb, reported a significant increase in the b^* of cut meat surface due to a 5-d dry ageing period. However, none of those studies explained this effect.

3.2. Cooked meat

Table 2 shows the effect of storage on the TPA of cooked lamb. Hardness was significantly affected ($P < 0.05$) by storage, masticability was near-to-significantly affected ($P < 0.1$) and

no effect was detected on elasticity and cohesiveness. Significant differences in hardness were found between NS exhibiting the highest values and VRS lamb with the lowest.
There is a general agreement that refrigerated aerobic storage tends to decrease the instrumental hardness of lamb (MARTÍNEZ-CEREZO *et al*., 2005b; STARKEY *et al*., 2015), with this decrement depending on storage length. The effect of refrigerated storage on suckling lamb hardness has been however scarcely studied. VIEIRA and FERNÁNDEZ (2014) reported a decrease in instrumental and sensory hardness in cooked suckling lamb due to a 5-d storage period of carcasses when carcasses were chilled under a conventional regimen (2 ºC for 24 h); however, hardness did not decrease with storage when carcasses were slowly chilled (12 ºC for 7 h and then 2 ºC for 22 h).

Table 2. Effect of storage method on the texture profile analysis (TPA) in suckling lamb cooked meat and the lipid oxidation calculated as increment in thiobarbituric-acid reactive substances during a two-day aerobic refrigerated storage period.

	NS (n=8)	ARS (n=8)	VRS (n=8)	FS (n=8)	SEM	*P*-level
TPA						
Hardness (N)	18.90^{a}	17.06^{ab}	15.35^{b}	16.45^{ab}	8.814	*
Elasticity	0.45	0.43	0.43	0.43	0.013	n.s.
Cohesiveness	0.45	0.44	0.43	0.44	0.009	n.s.
Masticability (N)	3.87	3.24	2.88	3.10	0.264	#
Lipid oxidation due to storage (Δ mg malonaldehyde/kg of meat)	4.15	3.98	5.48	3.60	0.522	#

NS: Non-stored, 2-day post-mortem refrigerated legs. ARS: refrigerated legs (2-day post-mortem) wrapped with air-permeable cling film and stored for 7 days at 3 ºC. VRS: refrigerated legs (2-day post-mortem) stored under vacuum for 21 days at 3 ºC. FS: refrigerated legs (2-day post-mortem) wrapped with air-permeable cling film, frozen and stored for 3 months at -18 ºC and then thawed at 3 ºC overnight.
SEM: Standard error of the mean.
P-level: n.s.: not significant; # $P < 0.1$; *: $P < 0.05$.
^{ab}: Means in the same row showing different superscripts are significantly different ($P < 0.05$; least significant difference test).

Results from previous studies seem to agree with the decrease in instrumental hardness resulting from VRS. MARTÍNEZ-CEREZO *et al*. (2005b) found in suckling lamb meat from three different breeds, one of them Churra, that a 16-d vacuum storage period significantly decreased the TPA-20%-compression hardness as assessed in raw meat and associated this effect to degradation of muscle structure. Furthermore, using the same meat samples, MARTÍNEZ-CEREZO *et al*. (2005a) reported a clear and steady tenderization effect as assessed in cooked meat by a sensory panel. BÓRNEZ *et al*. (2010) also evaluated the effect of a 21-d refrigerated storage of suckling lamb joints under modified atmospheres on the hardness of meat after cooking and observed a decrease in the shear-force. The meat of 1-month age suckling lambs, as that in this study, is considered to be tenderer and easier to swallow as compared with meat from weaned older lambs, i.e. 2-month age lambs (GORRAIZ *et al*., 2000), and tenderness in suckling lamb seems to be highly valued by consumers (SAÑUDO *et al*., 2007; VIEIRA and FERNÁNDEZ, 2014). Notwithstanding, an excess in tenderness might not be desirable. Studies are needed to order to establish a low-value tenderness threshold for consumers.

In partial agreement with our results, no significant effect of a 3-month -18 ºC storage was found on the sensory tenderness of meat from lambs weighing twice as much as those of this study (BUENO *et al*., 2013; MUELA *et al*., 2012). However, frozen meat, in contrast with non-frozen meat, can show similar or lower instrumental hardness and, at the same time, higher toughness as perceived by consumer panels (LEYGONIE *et al*., 2012). This discrepancy between instrumental and sensory results has been attributed to the lower juiciness in thawed meat after cooking together with the positive relationship between sensory juiciness and tenderness. The effect of frozen storage on the sensory tenderness of suckling lamb meat deserves further study.

The increment in TBARS value during the 2-d aerobic storage period in cooked meat was near-to-significantly affected by the previous storage ($P < 0.1$; Table 2). The mean TBARS values just after cooking were 1.6±0.9 mg of malonaldehyde/kg of cooked meat, with no differences between treatments ($P = 0.610$). The value for the TBARS increment in VRS cooked meat was higher than those from the other storage methods. No studies have been found on the oxidative stability of cooked suckling lamb to compare with our results. In light lamb raw meat, neither an up-to-6-month frozen-storage of meat from 3-4-month age lambs (MUELA *et al*., 2010) nor a 18-d vacuum refrigerated storage of suckling lamb (RUBIO *et al*., 2016) resulted in increased TBARS values. A TBARS value of 2 mg of malonaldehyde/kg was suggested as the threshold for raw beef, just before being cooked, over, which rancidity flavour can be perceived by consumers (CAMPO *et al*., 2006). However, the application of this threshold to the present study should be not reliable because the TBARS thresholds for warmer-over-flavour detection appears to depend on different factors such as species, animal age, or whether the meat is raw or cooked (FERNÁNDEZ *et al*., 1997). Studies should be done to determine the TBARS levels in cooked suckling lamb above which oxidized flavours are detectable.

4. CONCLUSIONS

The use of three commonly used methods for increasing the shelf-life of Churra suckling lamb joints could diminish the meat acceptability with regard to non-stored meat. Aerobic storage for 7 d can result in discoloration at the joint muscle surface and increased colour intensity in the surface of fresh cut chops; therefore, it seems to be not advisable to use longer aerobic storage periods to that already established in the PGI's standard (8 d). Vacuum storage (21 d) would result in increased meat pH and softer meat, and frozen storage would give muscle surface discoloration in thawed meat joint and lower water holding capacity. Sensory studies are required for evaluating the association between L^* value and chroma in suckling lamb chops and consumer purchasing intent, and that of tenderness, WHC and TBARS on the meat edible quality.

ACKNOWLEDGEMENTS

Authors are grateful to the Asociación de Ganaderos de Ganado Ovino de Raza Churra for its support in sampling. They are also grateful for a doctoral grant from CONACYT (MEX/Ref. 288189) and to the University of León who provided general support to this study.

REFERENCES

AMSA. 1995. Research guidelines for cookery, sensory evaluation, and instrumental tenderness measurements of fresh meat. Chicago. IL:American Meat Science Assoc.

Bórnez R., Linares M.B. and Vergara H. 2010. Effect of different gas stunning methods on Manchega suckling lamb meat packed under different modified atmospheres. Meat Sci. 84:727-734.

Bueno M., Resconi V.C., Campo M.M., Cacho J., Ferreira V. and Escudero A. 2013. Effect of freezing method and frozen storage duration on odor-active compounds and sensory perception of lamb. Food Res. Int. 54:772-780.

Callejas-Cárdenas A.R., Caro I., Blanco C., Villalobos-Delgado L.H., Prieto N., Bodas R., Giráldez F.J. and Mateo J. 2014. Effect of vacuum ageing on quality changes of lamb steaks from early fattening lambs during aerobic display. Meat Sci. 98:646-651.

Campo M.M., Nute G.R., Hughes S.I., Enser M., Wood J.D. and Richardson R.I. 2006. Flavour perception of oxidation in beef. Meat Sci. 72:303-311.

Colle M.J., Richard R.P., Killinger K.M., Bohlscheid J.C., Gray A.R., Loucks W.I., Day R.N., Cochran A.S., Nasados J.A. and Doumit M.E. 2015. Influence of extended aging on beef quality characteristics and sensory perception of steaks from the gluteus medius and longissimus lumborum. Meat Sci. 110:32-39.

Erasmus S.W., Muller M. and Hoffman L.C. 2017. Authentic sheep meat in the European Union:Factors influencing and validating its unique meat quality. J. Sci. Food Agric. 97:1979-1996.

Fernández J., Pérez-Álvarez J.A. and Fernández-López J.A. 1997. Thiobarbituric acid test for monitoring lipid oxidation in meat. Food Chem. 59:345-353.

Gorraiz C., Beriain M.J., Chasco J. and Iraizoz M. 2000. Descriptive analysis of meat from young ruminants in mediterranean systems. J. Sens. Stud. 15:137-150.

Grau R. and Hamm R. 1957. Über das Wasserbindungsvermögen des Säugetiermuskels. II. Mitteilung. Über die Bestimmung der Wasserbindung des Muskels. Z. Lebensm. Unters. Forsch. 105:446-460.

Grujić R., Petrović L., Pikula B. and Amidžić L. 1993. Definition of the optimum freezing rate-1. Investigation of structure and ultrastructure of beef M. longissimus dorsi frozen at different freezing rates. Meat Sci. 33:301-318.

Hopkins D.L. 1996. Assessment of lamb meat colour. Meat Focus Int. 5:400-401.

Leygonie C., Britz T.J. and Hoffman L.C. 2012. Impact of freezing and thawing on the quality of meat:Review. Meat Sci. 91:93-98.

Mancini R.A. and Hunt M.C. 2005. Current research in meat color. Meat Sci. 71:100-121.

MARM. 2010. Base de datos de consumo de hogares. Madrid, Spain.

Martínez-Cerezo S., Sañudo C., Medel I. and Olleta J.L. 2005a. Breed, slaughter weight and ageing time effects on sensory characteristics of lamb. Meat Sci. 69:571-578.

Martínez-Cerezo S., Sañudo C., Panea B., Medel I., Delfa R., Sierra I., Beltrán J.A., Cepero R. and Olleta J.L. 2005b. Breed, slaughter weight and ageing time effects on physico-chemical characteristics of lamb meat. Meat Sci. 69:325-333.

Mateo J., Caro I., Carballo D.E., Gutiérrez-Méndez N., Arranz J.J. and Gutiérrez-Gil B. 2018. Carcass and meat quality characteristics of Churra and Assaf suckling lambs. Animal 12:1093-1101.

McKenna D.R., Mies P.D., Baird B.E., Pfeiffer K.D., Ellebracht J.W. and Savell J.W. 2005. Biochemical and physical factors affecting discoloration characteristics of 19 bovine muscles. Meat Sci. 70:665-682.

Miguélez E., Zumalacárregui J.M., Osorio M.T., Figueira A.C., Fonseca B. and Mateo J. 2008. Quality traits of suckling-lamb meat covered by the protected geographical indication "Lechazo de Castilla y León" European quality label. Small Rumin. Res. 77:65-70.

Moore V.J. 1990. Factors influencing frozen display life of lamb chops and steaks: Effect of packaging and temperature. Meat Sci. 27:91-98.

Moore V.J. and Young O.A. 1991. The effects of electrical stimulation, thawing, ageing and packaging on the colour and display life of lamb chops. Meat Sci. 30:131-145.

Muela E., Monge P., Sañudo C., Campo M.M. and Beltrán J.A. 2016. Sensory quality of lamb following long-term frozen storage. Meat Sci. 114:32-37.

Muela E., Sañudo C., Campo M.M., Medel I. and Beltrán J.A. 2012. Effect of freezing method and frozen storage duration on lamb sensory quality. Meat Sci. 90:209-215.

Muela E., Sañudo C., Campo M.M., Medel I. and Beltrán J.A. 2010. Effect of freezing method and frozen storage duration on instrumental quality of lamb throughout display. Meat Sci. 84:662-669.

Nam K.C. and Ahn D.U. 2003. Combination of aerobic and vacuum packaging to control lipid oxidation and off-odor volatiles of irradiated raw turkey breast. Meat Sci. 63:389-95.

Nychas G-J.E., Skandamis P.N., Tassou C.C. and Koutsoumanis K.P. 2008. Meat spoilage during distribution. Meat Sci. 78:77-89.

Rubio B., Vieira C. and Martínez B. 2016. Effect of post mortem temperatures and modified atmospheres packaging on shelf life of suckling lamb meat. LWT - Food Sci. Technol. 69:563-569.

Ruiz de Huidobro F., Miguel E., Blázquez B. and Onega E. 2005. A comparison between two methods (Warner-Bratzler and texture profile analysis) for testing either raw meat or cooked meat. Meat Sci. 69:527-536.

Sánchez B. 2018. Private communication. PGI. Protected Geographical Indication. Zamora, Spain.

Santos V.A.C., Silva S.R., Mena E.G. and Azevedo J.M.T. 2007. Live weight and sex effects on carcass and meat quality of "Borrego terrincho-PDO" suckling lambs. Meat Sci. 77:654-661.

Sañudo C., Alfonso M., San Julián R., Thorkelsson G., Valdimarsdottir T., Zygoyiannis D., Stamataris C., Piasentier E., Mills C., Berge P., Dransfield E., Nute G.R., Enser M. and Fisher A.V. 2007. Regional variation in the hedonic evaluation of lamb meat from diverse production systems by consumers in six European countries. Meat Sci. 75:610-621.

Starkey C.P., Geesink G.H., Oddy V.H. and Hopkins D.L. 2015. Explaining the variation in lamb longissimus shear force across and within ageing periods using protein degradation, sarcomere length and collagen characteristics. Meat Sci. 105:32-37.

Teixeira A., Batista S., Delfa R. and Cadavez V. 2005. Lamb meat quality of two breeds with protected origin designation. Influence of breed, sex and live weight. Meat Sci. 71:530-536.

Vieira C. and Fernández A.M. 2014. Effect of ageing time on suckling lamb meat quality resulting from different carcass chilling regimes. Meat Sci. 96:682-687.

Young O.A., Priolo A., Simmons N.J. and West J. 1999. Effects of rigor attainment temperature on meat blooming and colour on display. Meat Sci. 52:47-56.

Paper Received January18, 2019 Accepted March 12, 2019

PAPER

EFFECTS OF PROBIOTIC FERMENTATION OF SELECTED MILK AND WHEY PROTEIN PREPARATIONS ON BIOACTIVE AND TECHNOLOGICAL PROPERTIES

K. SKRZYPCZAK[1]*, E. FORNAL[2], A. WAŚKO[3] and W. GUSTAW[1]

[1]Department of Plant Food Technology and Gastronomy, Department of Fruits, Vegetables and Mushrooms Technology, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland

[2]Department of Pathophysiology, Medical University of Lublin, Jaczewskiego 8b, 20-090 Lublin, Poland

[3]Department of Biotechnology, Microbiology and Human Nutrition, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland

*Corresponding author: katarzyna.skrzypczak@up.lublin.pl

ABSTRACT

The objective of this study was to compare the effect of probiotic fermentation (conducted by *L. acidophilus* LA-5) of milk containing an addition of selected whey or milk protein preparations (whey protein concentrates, whole milk, whey protein isolate, casein glycomacropeptide or α-lactalbumin) on the technological and functional properties of obtained milk beverages. Determination of the antioxidative activities and identification sequences of biologically active peptides generated in selected protein preparations during probiotic fermentation also constituted the aim of the research. The results obtained indicate that the addition (1%) of α-lactalbumin into regenerated skimmed milk in the highest degree reduced the syneresis level in fermented products. Also, α-lactalbumin hydrolysates exhibited the strongest antioxidative properties (57.57±6.6%), while casein glycomacropeptide hydrolysates allowed us to obtain the highest amount of various biopeptides.

Keywords: biopeptides, lactic acid fermentation, probiotics

1. INTRODUCTION

The area of functional food that becomes a part of an everyday diet (NAGPAL *et al*., 2012) and the significance of many dairy products, especially fermented ones, is dynamically developing. Nowadays, it is observed that the consumers' awareness of the role of diet and beneficial health effects of some food compounds is on the increase, a fact also reflected in higher consumption of fermented milk products. Simultaneously, the requirements of and demand for various the health-promoting features of those kinds of products have been growing too. Therefore, to meet those expectations, scientists and the dairy industry are aiming at improving the technological and functional properties of dairy products as well as creating new ones.

Proteins are the most valuable constituent of whey protein isolates (WPI) and whey protein concentrates (WPC) that supply good nutritional quality and enhance the functionality of many product formulations (GUSTAW and MLEKO, 2007; JEEWANTHI *et al*., 2015). It is suggested that selected whey protein constituents (e.g.α-lactalbumin, lactoferrin, serum albumin lactoperoxidase, β-lactoglobulin, bovine) and peptides deriving from them exhibit, among others, an anticarcinogenic activity, may stimulate the immune system and influence metabolic processes (GOBBETTI *et al.,* 2002). Therefore, they constitute an important source of bioactive ingredients that might find an application, e.g. in the preparation of some of the functional food and therapeutic formulations (KABAŠINSKIENĖ *et al.*, 2015). Furthermore, apart from nutritional aspects, whey (as well as products deriving from it) is also a functional component that might influence some products' properties, including colour, flavour, and texture (LIUTKEVIČIUS *et al.*, 2016). Thus, incorporation of selected milk and whey protein preparation might not only enhance the technological properties of a product but might also positively influence the functional properties. This is largely associated with the fact that milk and whey proteins are precursors of biologically active peptide sequences (biopeptides), which exhibit a wide range of desired beneficial health effects affecting the regulation of the human body's system functions (MOHANTY *et al.*, 2016; LUCARINI, 2017). However, biopeptides enclosed within a native structure of milk and whey proteins are inactive, whereas enzymatic hydrolysis with digestive enzymes as well as fermentation process conducted by a starter culture with an efficient proteolytic enzyme system contributes to the release of various sequences of active peptides (KORHONEN and PIHLANTO, 2003; MICHAELIDOU, 2008). Moreover, bacteria involved in the fermentation processes not only contribute to the formation of bioactive substances that give food product a functional characteristic, but determined strains meeting certain requirements may also constitute a functional food component.

The microorganisms claimed as probiotics constitute another important functional food constituent that might be introduced to milk products. An activity of probiotics contributes to the modification of the microbiota composition in the host's intestines, which influences some of the physiological process leading to favourable health consequences.

The health-promoting effects caused by probiotics concern their desired influence on maintaining proper intestine micrflora (normalization of the bacteria composition, e.g. after antybioticoteraphy), hypocholesterolemic or anticarcinogenesis effects, inhibition growth of some pathogens, stimulating the immunity system and also alleviation of some of the allergy, including food allergies and lactose intolerance (SARKAR *et al.*, 2016).

The objective of this study was to compare the effect of probiotic fermentation (conducted by *L. acidophilus* LA-5) of milk containing the addition of chosen whey and milk protein preparations on technological and functional properties of obtained milk beverages. The study was also focused on evaluation of the antioxidative activities and determination of

the biologically active peptide sequences generated during probiotic fermentation of selected protein preparations.

2. MATERIAL AND METHODS

2.1. Microorganism and growth conditions

The probiotics strain *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Poland) was stored at -80°C in Man- Rogosa- Sharpe broth (BTL, Łódź, Poland) containing 15% (v/v) glycerol stock. For further analysis, the strain culture was activated and routinely cultured (2%v/v of inoculum) in MRS broth and incubated at 37°C for 18h under aerobic conditions.

2.2. Fermentation of milk with the addition of protein preparations

The samples of 13% regenerated skim milk (OSM Krasnystaw, Poland) were enriched by the 1% (w/v) addition of one of the following protein preparations: whole milk powder (OSM Krasnystaw, Poland) - WMP; whey protein concentrates (Polsero, Poland) - WPC 30 and WPC40; whey protein isolate (Milei GmbH, Allgau, Germany) - WPI; Casein glycomacropeptide (Arla Food, Denmark) - CGMP and α-lactalbumin (Arla Food, Denmark) - α-la. Samples of milk without any protein addition (RSM) were used as a control variant.
All obtained sample variants were pasteurized (80°C/30min), then cooled down to ambient temperature and inoculated by 1% (v/v) of *Lactobacillus acidophilus* LA-5 cell suspension, which was previously prepared as follows: an overnight culture of analyzed probiotic strain (grown in MRS broth) was used to inoculate fresh medium (sterile MRS broth) to obtain an optical density at 600 nm (OD_{600}) of 0.05 (Helios Gamma; Thermo Fisher Scientific), subsequently the bacteria strain was cultivated at 37 °C until an OD_{600} of 0.7 (exponential phase). Afterwards, bacteria cells were harvested by centrifugation at 8000g for 10 min at 4 °C (MPW 350-R; MPW). The pellet was washed twice with sterile phosphate-buffered saline (PBS, pH 7.0) and resuspended (also in PBS) to obtain cell suspension of OD_{600} equal to 0.3 that was used for inoculation. Then, inoculated samples of all milk variants were transferred in equal volumes (40mL) into sterile packages and sealed. After the process of fermentation (42°C/12h, thermostatic method), all samples were cooled down up to 4°C and maintained in this temperature for another 12h before further analysis.

2.3. Texture profile analysis

To compare the textural properties of the fermented products received, the texture profile analysis (TPA) was performed, using a TA-XT2i (Stable Micro Systems, Godalming, UK), according to the procedure described by GUSTAW *et al.* (2016). The parameters hardness, fracturability springiness, cohesiveness, gumminess and chewiness were included in the analyses.

2.4. Spontaneous syneresis assay

The acidic milk gels (the fermented final products) obtained were analyzed also in terms of their ability for water binding. Therefore, the level of syneresis in received products was measured according to the method described by AMATAYAKUL *et al.* (2006).

2.5. Hydrolysis of milk and whey protein preparations

Aqueous solutions (1% w/v) of skim milk powder (SMP) and all above-mentioned protein preparations were pasteurized in a water bath (80 °C/30 min). After cooling down up to 35 °C, all variants of solutions were inoculated (1% v/v) by previously prepared inoculum (uninoculated samples were control variants) and incubated at 37 °C for 24 h. Subsequently, all samples were heated at 100 °C for 5 min in water bath to inhibit the hydrolysis process and inactivation of bacterial enzymes. Then, all samples were filtered (syringe filter Ø = 0.45 μm) and subjected to further analysis.

2.6. Antioxidant activity

An antioxidant activity in obtained filtrates (of fermented and nonfermented 1% (w/v) aqueous solutions of skim milk powder [SMP] and all analyzed protein preparations) were determined as an ability for free radical scavenging, using alcoholic solution of DPPH (1,1-Diphenyl-2-picrylhydrazyl, Sigma-Aldrich, Poland) in accordance with the method described by NAMDARI and NEJATI (2016). Briefly, the analyzed samples were diluted with phosphate buffer (0.1 M) in ratio 1:4. Afterwards, to 1 ml of each of the diluted samples 2.5 ml of 0.1 mM DPPH (in 60% methanol) was added and vigorously mixed. After 30 min of sample incubation (in darkness at ambient temperature), the absorbance was measured at λ=517 nm. The determination of the value of the analyzed activity for tested samples was carried out in a five-fold repetition (n=5). Antioxidant activity (radical-scavenging activity) was expressed as % inhibition of DPPH-absorbance that was calculated using the equation:

$$\text{Inhibition } [\%] = [(Ac-As)/Ac] \times 100$$

where, As - absorbance (λ=517 nm) of the test sample, and Ac - the absorbance (λ=517 nm) of the control sample consisting of 1 ml phosphate buffer mixed with 2.5 ml of DPPH (the methanolic solution of free radicals).

2.7. Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) and peptide sequencing

To analyze the biopeptides content, those protein preparations characterized by the highest antioxidative activities and the ones that allowed obtaining the fermented products with the most desirable textural properties were selected.
The analysis was conducted through the previously described procedure (SKRZYPCZAK *et al.*, 2017) using Agilent Mass Hunter acquisition B.05.01 software to acquire data. The data analysis and peptide mapping were performed using the Agilent Mass Hunter qualitative analysis B.07 with integrated Bioconfirm add-on software.

2.8. Determination of the biological activities of peptide sequences

To determine the profiles of potential biological activities, the obtained peptide sequences were subjected to further analysis that was performed according to the procedure included in the databases BIOPEP-UWMP (MINKIEWICZ *et al.*, 2008; DZIUBA *et al.*, 2009; www.uwm.edu.pl/biochemia/index.php/pl/biopep) and BioPepDB (bis.zju.edu.cn/biopepdbr).

2.9. Statistical analysis

Statistical analysis was performed using the Statistica 8.0 (StatSoft, Poland) program. To evaluate the differences between means values, the data were subjected to analysis of variance (ANOVA) using Tukey's test with a level of significance set at $p<0.05$.
The similarities of the textural properties analyzed between obtained fermented final products were determined on the basis of the results of bottom-up hierarchical cluster analysis using average linkage clustering as a linkage criterion (UPGMA). To avoid the effect of the differences in measurement units between the parameters on the values of Euclidean distances, the data were standardized prior to the analysis.

3. RESULTS AND DISCUSSION

3.1. Effect of addition of selected protein preparation on the textural properties and syneresis level of fermented products

The fermented milk products obtained with the addition of analyzed protein preparations exhibited some variety in terms of texture properties as well as level of spontaneous syneresis (Table 1).
Analyzing hardness parameter, there were no statistically significant differences ($p<0.05$) between control variant and products containing the addition of WPC30, WMP or α-la additions. The highest values of measured parameter were exhibited by samples with addition of CGMP, but simultaneously these fermented products were characterized by the most intensified syneresis effect (Table 1). This might be caused by the formation of a strong bond between generated products of hydrolyzed CGMP that influenced the moulding a strong structural network increasing the strength (hardness) of the gels and enhancing water disposal from the structure of curds.
Regarding the values of fracturability as well as cohesiveness (Table 1), there were no differences ($p<0.05$) between analyzed products. However, comparative analysis of all textural parameters indicated that, in terms of similar of texture profiles, the obtained variants of fermented products might be divided into three clusters consisting of pairs of fermented products' variants containing one of the tested protein preparations' addition (WMP with α-la, control variant and WPC30, WPI and CGMP) (Fig. 1).
Moreover, the results of cluster analysis indicated that in terms of values of analyzed texture profile parameters (springiness hardness, fracturability, cohesiveness, gumminess and chewiness), fermented beverages containing a WPI or CGMP additive were distinguished from all tested products and constituted a separate and farthest cluster that represents textural properties (Fig. 1).
The addition of whey protein concentrate (WPC 30 and WPC40) or CGMP to milk increased the intensity of syneresis in fermented products, while acidic milky gels containing the α-la or WMP addition were characterized by the highest water-binding capacity exhibiting the lowest syneresis effect (Table 1). The obtained results are in accordance with JOVANOVIĆ *et al*. (2005), who suggested that α-la influence the increase in the hydrophilic properties of the coaggregates at pH 4.5. Moreover, the study of MATUMOTO-PINTRO *et al*. (2011) indicates that a higher proportion of α -la in the yoghurt ingredients or partial hydrolysis of whey protein polymers inhibit the rate of sedimentation in the fermented product.

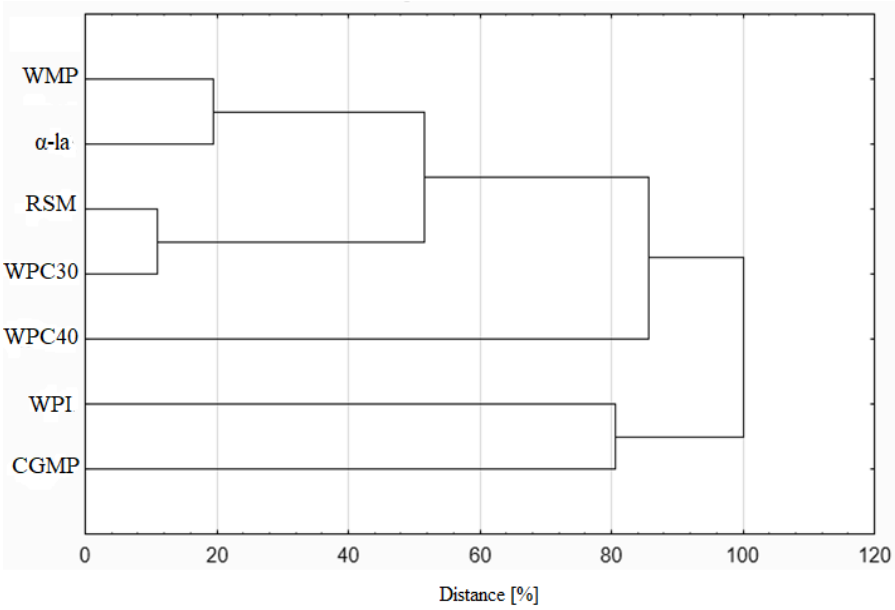


Figure 1. The result of UPGMA analyses.
Diagram expresses the similarity of texture characteristics between fermented products regarding all parameters measured in texture profile analysis; the similarity between particular groups of products was expressed as distance [%]; RSM is the control sample (fermented regenerated skim milk [13 %] without addition of any protein preparation).

It was claimed that the gelation properties of whey proteins are dependent on hydrolysis conditions and the degree of conducted enzymatic process (JEEWANTHI *et al.*, 2015). The process of gel formation is an effect accompanying the fermentation conducted by starter cultures. Therefore, the modification conditions of the hydrolysis process enable the creation of gels with various rheological properties (CREUSOT and GRUPPEN 2007; POULIOT *et al.*, 2009; JEEWANTHI *et al.*, 2015). Moreover, the results obtained might suggest that preferences of the probiotic strain to composition of fermenting medium (RSM samples containing selected protein preparations) and the specificity of bacterial enzymes toward selected milk and whey proteins might also influence the degree of hydrolysis and various characteristics of received gels (fermented products). It was implied that some of the generated peptide sequences might also initiate the aggregation process of whey protein hydrolyzates that influence the textural and rheological characteristic of fermented milk products. In WPI hydrolysates obtained using bacterial enzymes, CREUSOT and GRUPPEN (2007) identified such peptides and determined their sequences: β-Lg [f1-45], β-Lg AB[f(90-108)]-S-S-α-La [f(50-113)], α-La[f(12-49)]-S-S-α-La [f(50-113)], β-Lg AB[f(90-108)]-S-S-α-Lg AB[f(90-108)], β-Lg A[f(90-157)] and β-Lg AB[f(135-157/158)].
It was reported that in the production of yoghurt, the addition of WPI to milk enhances the formation of disulfide bonds during fermentation, which influences the increase in the mechanical resistance of received acidic gel (MATUMOTO-PINTRO *et al.*, 2011). However, in the presented study the results indicate that the 1% addition of WPI (compared with the control sample) has no significant effect on the improvement of textural property in terms of gel hardness.
Study results presented by DĄBROWSKA *et al.* (2017) revealed that the addition of whey protein hydrolysates (instead of other protein preparations like SMP or WPC80) to milk in yoghurt production might contribute to the increase in counts of starter culture bacteria at the initial stage of fermentation. Moreover, the addition of some whey protein

hydrolysates into milk improves the viability of probiotic bacteria in the final products (ZHAO *et al.*, 2006; DĄBROWSKA *et al.*, 2017). Also, the prebiotic-like properties of some whey proteins (including WPC) have been confirmed (GUSTAW *et al.*, 2016). This positive effect of selected milk and whey protein preparations, as well as their hydrolysates on the growth of the desired strains of probiotic bacteria, may strengthen the potential of the health-promoting properties of various fermented products.

3.2. Comparing the antioxidant activity of milk and whey protein preparations' hydrolyzates

The fermentation process conducted using the probiotic strain (*L. acidophilus* LA-5) influenced the increase in the ability of analyzed protein preparation solutions to free radical scavenging (Fig. 2).
Unfermented samples of SMP, WMP, WPC 30 and WPC40 exhibited a similar level of DPPH radical scavenging activity (differences, not statistically significant, $p > 0.05$). Moreover, it was noted that the hydrolysis process carried by tested bacteria improved the antioxidative properties of all analyzed protein preparations (Fig. 2).

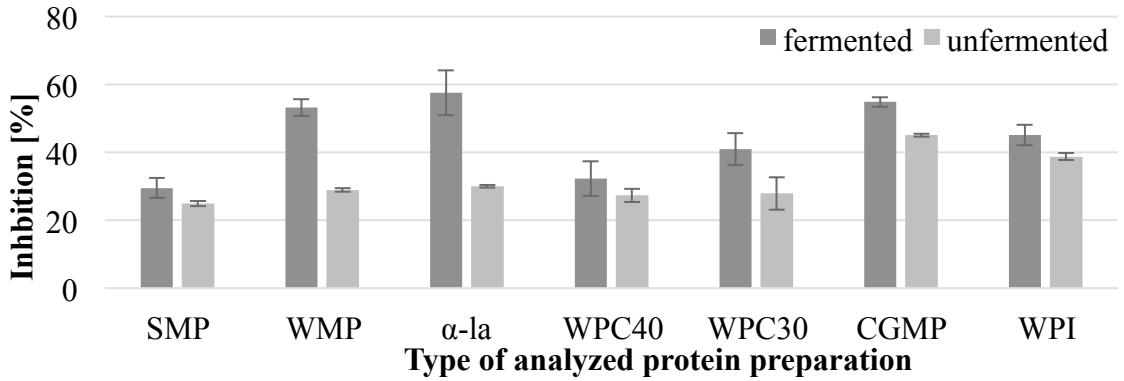


Figure 2. Antioxidant activity of analyzed protein preparations in DPPH assay.
Differences between mean values ($\bar{x}$; n=5) of antioxidant activity in obtained filtrates of tested samples (fermented and unfermented aqueous solutions (1% w/v) of skim milk powder [SMP] as control variant of samples and all analyzed protein preparations) denoted by different letters are statistically significant ($p<0.05$); error bars express the standard deviation (± SD).

The samples of α-la and CGMP fermented by probiotic strain exhibited the strongest hydrogen-donating capacity among analyzed solutions of protein preparations (57.57±6.6% and 54.87±1.3%, respectively). Differences in the value of antioxidative activity recorded for both types of samples were not statistically significant ($p > 0.05$), while the lowest values were noted for samples of SMP before (24.90±0.81%) as well as after hydrolysis (29.53±3.0%). These results are in accordance with RAHMAWATI and SUNTORNSUK (2016), who suggested that increased antioxidant activities in yoghurt (compared to raw milk material) might be associated with the released peptides (antioxidative) by bacterial proteolitic enzymes from protein molecules during the fermentation process. The peptides generated through protein hydrolysis as well as some products of the bacteria metabolism exhibit properties of electron donors and react with free radicals (DPPH) to achieve more stable molecule (KULLISAAR *et al.*, 2002).

3.3. Biological activities of peptide sequences identified in hydrolyzes of selected protein preparations

The analysis of identified biopeptides indicates that diversity of amino acid sequences as well as the quantity of the peptides in tested hydrolyzates depended on the protein matrix, namely the type of protein preparation (Table 2).
The results of liquid chromatography-high-resolution mass spectrometry (LC-HRMS)-and peptide sequencing revealed that most of the identified biopeptides sequences in analyzed hydrolyzates of protein preparations possess potential antihypertensive activities (Table 2) while in only one hydrolyzate (WPI) the presence of the sequence (RELEELNVPGEIVESLSSSEESITR) with potential of mineral-binding was confirmed. Furthermore, some of the biopeptide sequences possess more than one biologically active function, for instance, TTMPLW detected in hydrolyzate of CGMP or AYPS presented in hydrolyzed α-la (Table 2).
The analysis of peptide sequences revealed the presence of different biopeptides with antioxidant properties (IKH, IPNPIGSE, NEN, AYPS, LLR) in all analyzed hydrolyzate samples (Table 2). However, fermented CGMP was characterized by the largest number of different peptides with antioxidative properties. Moreover, within 21 of various biopeptides identified in this hydrolyzate samples, the potential of their biologically active properties also involved the following activities: antihypertensive, antithrombotic, antibacterial, immuno- and cytomodulatory peptide as well as the function of opioid and ACE inhibitor. Furthermore, the antioxidant peptide IPNPIGSE [αS1 -CN, f(182-189)], previously identified by GÚTIEZ *et al.* (2013) as a major peptide fragment in supernatants of *E. faecalis* strains grown in bovine skim milk has also been detected in CGMP hydrolized by the analyzed probiotic *L. acidophilus* strain. These results are of particular importance in the creation of functional food products.
It is claimed that peptide abilities to scavenge free radicals is connected with the presence of hydrophobic amino acid residues like: Met (M), Ile (I), Val (V), Leu (L), Phe (F), Trp (W), Ala (A), Tyr (Y) and Pro (P) (PENA- RAMOS *et al.*, 2004; CHEISON *et al.*, 2007; REN *et al.*, 2008). An example of such peptide sequence is AYPS that was identified in hydrolyzate of α-la. It was also proved that the presence of amino acids with aromatic residues enhances the ability for radical scavenging (RAJAPAKSE *et al.*, 2005).
The findings of the investigations indicate that the probiotic strain *L. acidophilus* LA-5 is also capable of degrading whey and milk proteins and generating peptide sequences exhibited (among others) the ACE-inhibitory activities (Table 2). Obtained results are in accordance with GÚTIEZ *et al.* (2013), who noticed that proline residue is present in most amino acid sequences of ACE-inhibitory peptides. It was also suggested that this residue is favourable for peptide binding to the active site of ACE. Moreover, GÚTIEZ *et al.* (2013) identified the peptide LHLPLP that is a competitive inhibitor of ACE exhibiting resistance toward gastrointestinal enzymes (QUIRÓS *et al.*, 2009). Interestingly, another sequence LPYPYY (identified as angiotensin-I-converting enzyme inhibitory peptide) identified in analyzed CGMP hydrolyzates was detected through ESI-MS/MS in samples of yak milk casein hydrolysates exhibiting a high level of ACE inhibition (83.16±1.37 %) (JIANG *et al.*, 2007). Moreover, HERNÁNDEZ-LEDESMA *et al.* (2007) described the β-lg-derived dipeptide, WY [f(19–20)] as a sequence with ACE-inhibitory bioactivity and radical-scavenging capacity. The same biopeptide was detected in the samples of WPI hydrolyzates obtained using *L. acidophilus* LA-5 (Table 2).

Table 1. Texture profiles and spontaneous syneresis levels exhibited by obtained fermented milk products.

Added protein preparation	Texture parameter						Syneresis [%]
	Hardness [N]	Fracturability [N]	Springiness	Cohesiveness	Gumminess	Chewiness	
Control*	0.153±2.59^{ab}	4.59±0.84^{a}	0.82±0.05^{c}	0.45±0.10^{a}	5.32±0.21^{ab}	4.70±0.44^{bc}	19.79±1.86^{ab}
CGMP	0.192±0.35^{a}	4.23±1.08^{a}	0.92±0.00^{ab}	0.44±0.09^{a}	7.69±0.45^{ab}	7.32±0.07^{a}	31.79±3.17^{a}
WPI	0.128±0.30^{b}	4.25±0.51^{a}	0.99±0.01^{a}	0.64±0.07^{a}	8.26±0.48^{a}	7.59±0.50^{a}	11.27±1.29^{bc}
WMP	0.133±1.02^{ab}	5.66±1.04^{a}	0.94±0.03^{ab}	0.55±0.05^{a}	6.43±0.44^{ab}	6.76±0.69^{ab}	7.33±0.08^{c}
α-la	0.136±2.37^{ab}	4.11±0.52^{a}	0.94±0.01^{ab}	0.51±0.02^{a}	6.95±1.00^{ab}	6.52±0.96^{ab}	6.95±0.66^{c}
WPC30	0.147±2.39^{ab}	3.94±0.40^{a}	0.89±0.01^{b}	0.50±0.07^{a}	5.03±0.86^{ab}	5.91±0.82^{abc}	26.07±3.8^{a}
WPC40	0.096±1.00^{b}	5.14±0.49^{a}	0.92±0.01^{abc}	0.49±0.06^{a}	3.97±0.77^{b}	3.73±0.37^{c}	27.63±6.03^{a}

*Control - samples consisting of 13% regenerated skim milk without any addition of protein preparation.
Differences between mean values (n = 8) ± standard deviation in the same column with the same letter designation are not statistically significant ($p < 0.05$).

Table 2. The sequences of biopeptides identified in analyzed hydrolysates obtained using *Lactobacillus acidophilus* LA-5.

Source of peptides (analyzed hydrolysate)	Identified peptide sequence	Mass (Da)	ID of the bioactive peptide in the data base	Activity/function reported in the data base
CGMP	LPYPYY	814.30	biopep00859/BioPepDB[b]	antihypertensive
CGMP	SPPEIN	655.30	biopep01254/BioPepDB	antihypertensive
CGMP WPI	ERF	450.22	biopep00189/BioPepDB	antihypertensive
CGMP	VRSP	457.26	biopep01460/BioPepDB	antihypertensive
α-la	SRY	424.21	biopep01260/BioPepDB	antihypertensive
CGMP α-la	RPKHPIKHQGLPQEVLNEN	2233.22	biopep01215/BioPepDB	antihypertensive
CGMP	LEQL	501.28	biopep00755/BioPepDB	antihypertensive
WPI	WY	367.15	biopep01540/BioPepDB	antihypertensive
WPI	GKEKV	559.33	biopep00360/BioPepDB	antihypertensive
WPI	VAPFPEVFGKE	1218.63	biopep01336/BioPepDB	antihypertensive
WPI	FVAPFPEV	904.47	biopep00301/BioPepDB	antihypertensive
CGMP	RPK	399.26	biopep01206/BioPepDB	antihypertensive
WPI	VLNENL	700.37	biopep01413/BioPepDB	antihypertensive
WPI	VAPFPEVFGKE	1218.63	biopep01336/BioPepDB	antihypertensive
WPI	LQPEVMGVSK	1086.57	biopep00867/BioPepDB	antihypertensive
WPI	LVYPFPGPI	1001.56	biopep00927/BioPepDB	antihypertensive

CGMP	HKEMPFPKYPVEPF	1744.86	biopep00457/BioPepDB	antihypertensive
CGMP WPI	SQSKVLPVPQ	1081.61	biopep01258/BioPepDB	antihypertensive
WPI	LQSW	532.26	biopep00875/BioPepDB	antihypertensive
CGMP	TQSLVYP	806.42	biopep01306/BioPepDB	antihypertensive
WPI	TEDELQDKIHP	1323.62	biopep01279/BioPepDB	antihypertensive
CGMP WPI	MAIPPKK	783.46	biopep00946/BioPepDB 3294/BIOPEP-UWM[c]	antihypertensive, antithrombotic
CGMP	IPNPIGSE	825.42	biopep00561/BioPepDB	antioxidative
WPI	IKH	396.25	biopep00532/BioPepDB	antioxidative
CGMP	NEN	375.32	9363/BIOPEP-UWM	antioxidative
α-la	AYPS	436.20	8472/BIOPEP-UWM 8380/BIOPEP-UWM	antioxidative, ACE inhibitor
CGMP WPI	LLR	400.28	8484/BIOPEP-UWM biopep00827/BioPepDB	antioxidative, antihypertensive
CGMP	LKTVYQHQKAMKPWIQPKTKVIPYVRYL	3455.96	8337/BIOPEP-UWM	antibacterial
WPI	RELEELNVPGEIVESLSSSEESITR	2801.39	biopep04772/BioPepDB	mineral-binding
CGMP α-la	YQEPVLGPVRGPFPIIV	1880.06	biopep04801/BioPepDB biopep04091/BioPepDB biopep01621/BioPepDB	immuno- and cyto-modulatory peptides antimicrobial antihypertensive
CGMP	NLHLPLP	802.47	2669/BIOPEP-UWM, biopep01010 /BioPepDB	ACE inhibitor antihypertensive
CGMP WPI	VTSTAV	576.30	7481/BIOPEP-UWM biopep01475/BioPepDB	ACE inhibitor, antihypertensive
α-la	LLYQEPVLGPVRGPFPIIV	2106.22	8174/BIOPEP-UWM	immunomodulating
CGMP	TTMPLW	747.30	3530/BIOPEP-UWM, 3127/BIOPEP-UWM, 8172/BIOPEP-UWM, biopep0479 6/ BioPepDB, biopep01313/BioPepDB	ACE inhibitor, opioid, immunomodulating immuno- and cytomodulatory peptide, antihypertensive
CGMP α-la	MAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATL EDSPEVIESPPEINTVQVTSTAV	6703.37	biopep03480/BioPepDB	antibacterial
α-la	YYQQKP	825.40	8383/BIOPEP-UWM	ACE inhibitor
CGMP WPI	VQVTSTAV	803.40	biopep01445/BioPepDB, 8264/BIOPEP-UWM	antihypertensive, antibacterial

[b]data base: bis.zju.edu.cn/biopepdbr/

[c]data base: www.uwm.edu.pl/biochemia/index.php/pl/biopep

Hydrolysis of CGMP conducted with the application of the probiotic *L. acidophilus* strain allowed to receive the sequence TTMPLW exhibited multi-directional biological activities (opioid, ACE inhibitor, antihypertensive, immuno- and cytomodulatory peptide) (Table 2). The results of analysis of ACE-inhibitory and antihypertensive activity in spontaneously hypertensive rats of biopeptides (generated through tripsine hydrolysis of milk proteins) claimed that this sequence was effective in decreasing the level of systolic blood pressure (KARAKI *et al.*, 1990). The concentration of peptide (TTMPLW) needed to inhibit 50% ACE activity (IC50) achieved the value of 16 μM, whereas for sequences LQSW [β-CN, f(155−158)], YQEPVLGPVRGPFPIIV [β-CN, f(208−224)] and MAIPPKK [κ-CN f(106-112)], the IC50 values were 500, 101 and 4785 μM, respectively (KARAKI *et al.*, 1990; MAENO *et al.*, 1996; MIGUEL *et al.*, 2007; HERNÁNDEZ-LEDESMA *et al.*, 2011). All the sequences mentioned above were also identified in whey and milk protein preparations hydrolyzed by *L. acidophilus* LA-5 (LQSW in samples of hydrolyzed WPI; MAIPPKK in CGMP and WPI hydrolyzates; YQEPVLGPVRGPFPIIV in CGMP and α-la hydrolyzates).
The presented results of the research have practical relevance because health-promoting properties provided in food by biopeptides may find an application in personalized nutrition as well as individual dietary practices (KORHONEN and PIHLANTO, 2006). Biologically active peptides are important constituents of food and allow the design of novel foods, including functional food products, supplements, nutraceuticals or even pharmaceuticals (MEISEL, 2005; LIUTKEVIČIUS *et al.*, 2016; LUCARINI *et al.*, 2017).

4. CONCLUSIONS AND FUTURE WORK

The obtained study findings demonstrate that analyzed protein preparations are an important source of dietary antioxidants. Furthermore, due to their influence on fermented product, texture properties may find a wider application in the formulation of new dairy products, especially functional food.
The use of 1% α-la additive into regenerated skimmed milk is conducive to the reduction of the level of syneresis, with 1% addition of CGMP leaded to obtain the strong, hard gels in fermented milk products, but with a weaker capacity to water-binding. In addition, the hydrolysis process of protein preparations carried out by the probiotic strain improved their antioxidative properties. Furthermore, using the *L. acidophilus* LA-5 to hydrolysis of CGMP and α-la seems to be an effective method of obtaining products with high antioxidant properties as well as some various biopeptide sequences. Among all analyzed hydrolysates, the sequences with potential antihypertensive activity were the most numerous biopeptides, whereby fermented (by the probiotic *L. acidophilus* strain) CGMP allowed acquiring the largest amount of different biologically active peptides. However, despite the promising research results, undoubtedly, further investigations are necessary to verify *in vivo* the biological activity of biopeptide sequences identified in presented study. Also, further analyses might yield new biologically active substances or contribute to a more efficient use of *L. acidophilus* LA-5 in the production of functional foods, nutraceuticals or pharmaceuticals.

ACKNOWLEDGEMENTS

The research was funded by the National Science Centre, Poland (Research Grant No. 2014/15/N/NZ9/04042).

REFERENCES

Amatayakul T., Sherkat F. and Shah N.P. 2006. Physical characteristics of set yoghurt made with altered casein to whey protein ratios and EPS-producing starter cultures at 9 and 14% total solids. Food Hydrocolloids 20(2-3):314-332. DOI: doi.org/10.1016/j.foodhyd.2005.02.015.

Cheison S.C., Wang Z. and Xu S.Y. 2007. Preparation of whey protein hydrolysates using a single- and two-stage enzymatic membrane reactor and their immunological and antioxidant properties: characterization by multivariate data analysis. Journal of Agricultural and Food Chemistry 55(10):3896-3904. DOI: doi.org/10.1021/jf062936i.

Creusot N. and Gruppen H. 2007.Protein peptide interactions in mixtures of whey peptides and whey proteins. Journal of Agricultural and Food Chemistry 55(6):2474-2481. DOI: doi.org/10.1021/jf062608i

Dąbrowska A., Babij K., Szołtysik M. and Chrzanowska J. 2017. Viability and growth promotion of starter and probiotic bacteria in yogurt supplemented with whey protein hydrolysate during refrigerated storage. Postępy Higieny i Medycyny Doświadczalnej 71:952-959. DOI: doi.org/10.5604/01.3001.0010.5866.

Dziuba M., Dziuba B. and Iwaniak A. 2009. Milk proteins as precursors of bioactive peptides. Acta Scientorum Polonorum Technologia Alimentaria 8(1):71-90.

Gobbetti M., Stepaniak L., De Angelis M., Corsetti A. and Di Cagno R. 2002. Latent bioactive peptides in milk proteins: Proteolytic activation and significance in dairy processing. Critical Reviews in Food Science and Nutrition 42(3):223-239. DOI: doi.org/10.1080/10408690290825538.

Gustaw W., Kozioł J., Radzki W., Skrzypczak K., Michalak-Majewska M., Sołowiej B., Sławińska A. and Jabłońska-Ryś E. 2016. The effect of addition of selected milk protein preparations on the growth of *Lactobacillus acidophilus* and physicochemical properties of fermented milk.Acta Scientorum Polonorum Technologia Alimentaria 15(1):29-36. DOI: doi.org/10.17306/J.AFS.2016.1.3.

Gustaw W. and Mleko S. 2007. The effect of polysaccharides and sodium chloride on physical properties of processed cheese analogs containing whey proteins. Milchwissenschaft 62(1):59-62.

Gútiez L., Gómez-Sala B., Recio I., del Campo R., Cintas L.M., Herranz C. and Hernández P.E. 2013. *Enterococcus faecalis* strains from food, environmental, and clinical origin produce ACE-inhibitory peptides and other bioactive peptides during growth in bovine skim milk. International Journal of Food Microbiology 166(1):93-101. DOI: doi.org/10.1016/j.ijfoodmicro.2013.06.019.

Hernández-Ledesma B., Amigo L., Recio I. and Bartolomé B. 2007. ACE-inhibitory and radical-scavenging activity of peptides derived from beta-lactoglobulin f(19-25). Interactions with ascorbic acid. Journal of Agricultural and Food Chemistry 55(9):3392-337. DOI: doi.org/10.1021/jf063427j.

Hernández-Ledesma B., del Mar Contreras M. and Recio I. 2011. Antihypertensive peptides: production, bioavailability and incorporation into foods. Advances in Colloid and Interface Science 165(1): 23-35. DOI: doi.org/10.1016/j.cis.2010.11.001.

Jeewanthi R.K., Lee N.K. and Paik H.D. 2015. Improved Functional Characteristics of Whey Protein Hydrolysates in Food Industry. Korean Journal for Food Science of Animal Resources 35(3):350-359. DOI: doi.org/10.5851/kosfa.2015.35.3.350.

Jiang J.L., Chen S.W., Ren F.Z., Luo Z. and Zeng S.S. 2007. Yak milk casein as a functional ingredient: Preparation and identification of angiotensyn I-converting enzyme inhibitory peptides. Journal of Dairy Research 74(1):18-25. DOI: doi.org/10.1017/S0022029906002056.

Jovanović S., Barać M. and Maćej O. 2005. Whey Proteins-Properties and Possibility of Application. Mljekarstvo 55(3):215-233.

Kabašinskienė A., Liutkevičius A., Sekmokien D., Zaborskienė G. and Šlapkauskaitė J. 2015. Evaluation of the physicochemical parameters of functional whey beverages. Food Technology and Biotechnology 53: 110-115. DOI: doi.org/10.17113/ftb.53.01.15.3763.

Karaki H., Doi K., Sugano S., Uchiwa H., Sugai R., Murakami U. and Takemoto S. 1990. Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. Comparative Biochemistry & Physiology969(2):367-371. DOI: doi.org/10.1016/0742-8413(90)90023-3.

Korhonen H. and Pihlanto A. 2006. Bioactive peptides: production and functionality. International Dairy Journal 16(9):945-960. DOI: doi.org/10.1016/j.idairyj.2005.10.012.

Kullisaar T., Zilmer M. and Mikelsaar M. 2002.Two Antioxidative lactobacilli strains as promising probiotics. International Journal of Food Microbiology72(3):215-224. DOI: doi.org/10.1016/S0168-1605(01)00674-2.

Liutkevičius A., Speičienė V., Kaminskas A., Jablonskienė V., Alenčikienė G., Mieželienė A., Bagdonaitė L., Vitkus D. and Garmienė G. 2016. Development of a functional whey beverage, containing calcium, vitamin D, and prebiotic dietary fiber, and its influence on human health. CyTA - Journal of Food 14(2): 309-316. DOI: doi.org.10.1080/19476337.2015.1108366.

Lucarini M. 2017. Bioactive peptides in milk: From encrypted sequences to nutraceutical aspects. Beverages 3(3):41. DOI: doi.org/10.3390/beverages3030041.

Maeno M., Yamamoto N. and Takano T. 1996. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. Journal of Dairy Science 79(8):1316-1321. DOI: doi.org/10.3168/jds.S0022-0302(96)76487-1.

Meisel H. 2005. Biochemical properties of peptides encrypted in bovine milk proteins. Current Medicinal Chemistry 12(16):1905-1919. DOI: doi.org/10.2174/0929867054546618.

Michaelidou A.M. 2008. Factors influencing nutritional and health profile of milk and milk products. Small Ruminant Research 79(1): 42-50. DOI: doi.org/10.1016/j.smallrumres.2008.07.007.

Miguel M., Manso M.A., López-Fandiño R., Alonso M.J. and Salaices M. 2007. Vascular effects and antihypertensive properties of kappa-casein macropeptide. International Dairy Journal 17(12):1473-1477. DOI: doi.org/10.1016/j.idairyj.2007.04.009.

Minkiewicz P., Dziuba J., Iwaniak A., Dziuba M. and Darewicz M. 2008.BIOPEP database and other programs for processing bioactive peptide sequences. Journal of AOAC International 91(4):965-980.

Mohanty D.P., Mohapatra S., Misra S. and Sahu P.S. 2016. Milk derived bioactive peptides and their impact on human health - A review. Saudi Journal of Biological Sciences 23(5):577-583. DOI: doi.org/10.1016/j.sjbs.2015.06.005

Nagpal R., Kumar A., Kumar M.Behare P.V., Jain S. and Yadav H. 2012. Probiotics, their health benefits and applications for developing healthier foods: a review. FEMS Microbiology Letters 334(1):1-15. DOI: doi.org/10.1111/j.1574-6968.2012.02593.x.

Namdari A. and Nejati F. 2016. Development of Antioxidant Activity during Milk Fermentation by Wild Isolates of *Lactobacillus helveticus*. Applied Food Biotechnology 3(3):178-186. DOI: 10.22037/afb.v3i3.11422.

Osuntoki A. and Korie I. 2010. Antioxidant activity of whey from milk fermented with Lactobacillus species isolated from Nigerian fermented foods. Food Technology and Biotechnology 48(4):505-511.

Pena-Ramos E.A., Xiong Y.L. and Arteaga G.E. 2004. Fractionation and characterization for antioxidant activity of hydrolysed whey protein. Journal of the Science of Food and Agriculture 84(14):1908-1918. DOI: doi.org/10.1002/jsfa.1886.

Pouliot Y., Guy M.M., Tremblay M., Gaonac'h A.C., Tin B.P.C.P., Gauthier S. and Voyer N. 2009. Isolation and characterization of an aggregating peptide from a tryptic hydrolysate of whey proteins. Journal of Agricultural and Food Chemistry 57(9):3760- 3764. DOI: 10.1021/jf803539f.

Quirós A., Contreras M.M., Ramos M., Amigo L. and Recio I. 2009. Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties. Peptides 30(10):1848-1853. DOI:10.1016/j.peptides.2009.06.031.

Rahmawati I.S. and Suntornsuk W. 2016. Effects of Fermentation and Storage on Bioactive Activities in Milks and Yoghurts. Procedia Chemistry 18: 53-62. DOI: doi.org/10.1016/j.proche.2016.01.010.

Rajapakse N., Mendis E., Jung W.K., Je J.Y. and Kim S.K. 2005. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. Food Research International 38(2):175-182. DOI: 10.1016/j.foodres.2004.10.002.

Ren J., Zhao M., Shi J., Wang J., Jiang Y. and Cui C. 2008. Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization mass spectrometry. Food Chemistry 108(2):727-736. DOI: doi.org/10.1016/j.foodchem.2007.11.010.

Sarkar S., Sur A., Sarkar K., Majhi R., Basu S., Chatterjee K. and Sikder B. 2016. Probiotics: A Way of Value Addition in Functional Food. International Journal of Food Science, Nutrition and Dietetics 5(4):290-293. DOI: dx.doi.org/10.19070/2326-3350-1600052.

Skrzypczak K., Gustaw W., Szwajgier D., Fornal E. andWaśko A. 2017. κ-Casein as a source of short-chain bioactive peptides generated by *Lactobacillus helveticus*. Journal of Food Science and Technology 54(11):3679-3688. DOI: doi.org/10.1007/s13197-017-2830-2.

Zhao Q.Z., Wang J.S., Zhao M.M., Jiang Y.M. and Chun C. 2006. Effect of casein hydrolysates on yogurt fermentation and texture properties during storage. Food Technology and Biotechnology 44(3):429-434.

Paper Received November 12, 2018 Accepted February 20, 2019

PAPER

THE EFFECT OF AN INCREASE IN PASTE TEMPERATURE BETWEEN MALAXATION AND CENTRIFUGATION ON OLIVE OIL QUALITY AND YIELD: PRELIMINARY RESULTS

L. GUERRINI*[1], P. MASELLA[1], G. ANGELONI[1], B. ZANONI[1], C. BRESCHI[1], L. CALAMAI[2,3] and A. PARENTI[1]

[1]Dipartimento di Gestione dei Sistemi Agrari, Alimentari e Forestali (GESAAF), Università degli Studi di Firenze, Piazzale delle Cascine 15, 50144 Florence, Italy

[2]Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente (DISPAA), Università degli Studi di Firenze, Piazzale delle Cascine 18, 50144 Florence, Italy

[3]Istituto Bioscienze e Biorisorse, (IBBR) CNR, Florence, Via Madonna del Piano 10, Sesto Fiorentino, Italy

*E-mail address: lorenzo.guerrini@unifi.it

ABSTRACT

Olive oil extraction conditions are usually a compromise between quality and yield. Enhancing yield decreases quality, and vice versa. We aim to understand if a change in temperature between malaxation and centrifugation can result in a better compromise. Thus, malaxation is carried out at 20°C and oil is extracted at 20°C, 27°C, and 35°C.

The results show that a moderate increase in temperature between malaxation and centrifugation (i.e. from 20°C to 27°C) increases both yield and quality of the oil. Conversely, an excessive temperature increase (i.e. from 20°C to 35°C) leads to the production of rancid-related compounds.

Keywords: olive oil, phenols, quality, temperature, yield

1. INTRODUCTION

The extra virgin olive oil extraction involves several subsequent steps. Briefly, olives are washed and crushed to obtain a paste. Then, the olive paste is kneaded (i.e. malaxation phase) and centrifuged to separate the olive oil from water and exhaust solids (i.e. pomace) (GUERRINI *et al.*, 2017a).
Temperature control during extraction is a key issue in extra virgin olive oil (EVOO) production, since it affects both yield and quality (BOSELLI *et al.*, 2009).
The effects of temperature on yield are mainly related to the malaxation and centrifugation steps. During malaxation, temperature influences the physic, chemical and enzymatic activity of olive paste. On the one hand, higher temperatures enhance the coalescence of oil droplets, decrease viscosity, and accelerate the diffusion of substances from the aqueous to the oily phase. Usually, higher temperatures increase yield (TRAPANI *et al.*, 2017a). During malaxation, the slow and continuous kneading of the paste merges oil small droplets into larger ones, creating a continuous liquid phase that can easily be separated mechanically (KALUA *et al.*, 2006). Drops with a diameter >30 μm are considered to be separated more easily with the decanter centrifuge (MARTINEZ MORENO *et al.*, 1957). Furthermore, the viscosity of olive oil and the consistency of the olive paste decrease with an increase in temperature, facilitating EVOO extraction in the decanter (GUERRINI *et al.*, 2017b).
The temperature during the extraction process has a marked effect on some EVOO quality parameters. High temperatures enhance enzymatic reactions involving lipolytic, hydrolytic and oxidative degradation, affecting both phenols and aroma. Furthermore, higher temperatures increase the vapor pressure of volatile substances, and may causes aroma losses (TAMBORRINO, 2014).
Phenols are considered indicators of EVOO quality for their functional, nutritional, and sensory properties (RODIS *et al.*, 2002). Their recovery into the EVOO is strongly affected by temperature. High temperatures enhance phenol solubility in the oily phase (RODIS *et al.*, 2002) and their release from olive tissues on the one hand, while, on the other hand, they improve the rate of enzymatic activity involved in phenol consumption and molecular weight reduction (TRAPANI *et al.*, 2017b).
EVOO flavor is mainly described by fruity and defective attributes. The European directive EC 1019/2002 on marketing standards for olive oil states that the fruity attribute must be perceivable, while no defective attributes should be present. The fruity attribute has been related to lipoxygenase (LOX) pathway compounds (ANGEROSA *et al.*, 2001). LOX activity is significantly affected by temperature. In the same way, temperature affects the appearance in the oil of all enzymatic-related defects (ZHU *et al.*, 2014).
Current practice is to select the malaxation temperature to maximize desired effects (i.e. higher yield, phenolic content and LOX-related compounds) and minimize undesirable ones (i.e. phenol degradation and off-flavor biosynthesis).
Recently, heat exchangers have provided a valuable technological solution to improve the thermal control of olive paste. The first study of heat exchangers in olive mills was conducted by AMIRANTE *et al*. (2006). Destoned olive paste was heated between the crusher and the malaxer to obtain the same yield for stoned and destoned paste (otherwise yield is lower for the latter). On the other hand, in two studies, Veneziani *et al*. (2015, 2017) cooled the olive paste between the crusher and malaxer, and found an increase in EVOO quality without a decrease in yield. These studies highlight that malaxation temperature plays a central role for the EVOO quality. However, we want to test if a fast temperature increase after the malaxation can improve yield without decrease the oil quality. In fact, in a theoretical instantaneous temperature increase, we can reasonable suppose that only the physical effects (change in olive oil/paste viscosity, and change in solubility) occurs. On

the contrary, the chemical and enzymatic reactions occur during the time required for the temperature increase.
Hence, given the improved ability to control temperature during the process, in this work we test, the effect of heating olive paste between the malaxer and the centrifuge. Trials explore the possibility of conducting malaxation at one temperature and extraction at another. The aim was to test if such heating has advantages in terms of both EVOO yield and quality.

2. MATERIAL AND METHODS

2.1. Trials

Olives (cv. *Frantoio*) were manually harvested in Tavarnelle Val di Pesa (Italy) at the beginning of December 2017. The following day they were washed and crushed. Processing was designed to reduce oxidative impact and limit any damage to oil quality due to oxygen. Hence, 1.2 kg of the obtained olive paste was weighed and malaxed in sealed conditions for 30 min at 20°C. The result was divided into three portions of 400 g each. Each portion was put in a sealed jar and the temperature was bring at 20°C, 27°C, or 35°C in one of three thermostatic baths. The time required for the temperature increase was 7 min. Preliminary trials established the water temperature needed in each of the thermostatic baths to obtain these three temperatures in 7 min. Then the paste was centrifuged for 2 min at 2000 rpm (TRAPANI *et al.*, 2017a) to separate olive oil from other phases. Three replicates were conducted.

2.2. Yield, water and oil content

The oil extraction yields were calculated both as the actual yield (OY) and as an Extractability Index (EI):

$$OY = \frac{OE_x}{Ol_m} \cdot 100 \quad (1)$$

$$EI = \frac{OE_x}{OC_{om}} \cdot 100 \quad (2)$$

where:
OE_x was the measured extracted olive oil (g);
Ol_m was the measured olive paste (g);
OC_{om} was the oil content of the olive paste (g), which was determined from the oil content of the olive fruits (%).
Water content was measured using the gravimetric method. Total oil content was determined in olive paste and pomace with hexane in an automatic Randall extractor (mod. 148, Velp Scientifica, Milan, Italy), following the analytical technique described in Cherubini *et al*. (2009). Results were expressed as the percentage of total weight.

2.3. Chemical analysis

Olive oils were tested for free fatty acid content, peroxide value, K232, K270 and ΔK based on methods set out in European Commission Regulation EEC/2568/91. Total and individual biophenol content were recorded according to the method described in

IOOC/T.20 Doc. N 29. Volatile compounds were measured with the HS-SPME-GC-MS technique (FORTINI *et al*. 2017).

2.4. Statistical analysis

Data were processed with a one-way ANOVA to assess differences among the three extraction temperatures. Where a significant difference ($p<0.05$) was found, a Tukey Honest Significant Difference post-hoc test was carried out.

3. RESULTS AND DISCUSSION

3.1. The effect of paste temperature increase after a malaxation at low temperature for 30 min

The EVOO samples were analyzed after their extraction by centrifugation of the olive paste, which were malaxed at 20°C for 30 min and then, kept at 20°C for 7 min or heated to 27°C and 35°C in 7 min (Tables 1 and 2).
In the above EVOO samples the oil extraction yields and the EU legal chemical parameters (i.e. able to measure the triglycerides degradation level due the lipase hydrolytic activity and the auto-oxidation and photo-oxidation phenomena) were measured (Table 1).

Table 1. Parameters of EVOO extracted by centrifugation of the olive paste, which had no malaxation (i.e. at time $t = 0$) and were malaxed at 20°C for 30 min and then, kept at 20°C for 7 min or heated to 27 and 35°C in 7 min; a and b represent a statistically significant difference ($p<0.05$) based on the Tukey HSD post-hoc test.

		Final temperature after olive paste heating (°C)			
Parameter	**At time $t = 0$**	**20 °C**	**27 °C**	**35 °C**	**p**
OY (%)	n.d.	20.7±0.8 b	23.3±0.3 a	23.7±0.9 a	0.01
EY (%)*	n.d.	79±4 b	89±2 a	91±6 a	0.01
Free fatty acids content (%)	n.d.	0.43±0.01	0.47±0.04	0.47±0.06	ns
Peroxide number (m_{eq}/kg)	n.d.	11.4±0.7	11±3	11±1	ns
K_{232}	n.d.	1.73±0.03	1.67±0.04	1.74±0.03	ns
K_{270}	n.d.	0.08±0.02	0.08±0.01	0.09±0.01	ns
$\triangle K$	n.d.	0.002±0.001 a	0.003±0.001 ab	0.003±0.001 b	0.03
Total phenolic content (mg/kg)	230±31 b	238±23 b	290±10 a	298±37 a	0.04
3,4-DHPEA-EDA (mg/kg)	21±1 a	23±1 a	29±2 b	46±2 c	0.03
Total LOX compounds (mg/kg)	n.d.	45.36±5.50	46.00±13.45	44.90±6.01	ns
E-2-Hexenal (mg/kg)	n.d.	41.88±4.95	42.20±12.69	41.13±5.15	ns
1-penten-3-ol (mg/kg)	n.d.	0.26±0.03 a	0.31±0.01 b	0.33±0.03 b	0.05
E-2-Heptenal (μg/kg)	n.d.	25±3 a	27±3 a	39±3 b	0.04

*From the oil content of olive paste (%) = 26.2±0.7; n.d. = not determined.

The final temperature after the olive paste heating influenced significantly the extraction yields. The OY values increased approximately from 21% at 20°C to 24% at 35°C and the EY values increased approximately from 79% at 20°C to 91% at 35°C; the OY and EI values had no significant differences between 27°C and 35°C final temperatures. These results

were consistent with the literature data, which showed a positive relationship between temperature and extraction yield (TRAPANI *et al.*, 2017a). However, in all of these works the temperature was kept constant throughout the process, while our data show that a yield increase can be obtained with a temperature increase that is limited to the centrifugation process.

No significant effect of the final temperatures occurred for the free fatty acids content, the peroxide number, the K232 and the K270 UV spectroscopic indexes. Although the ΔK spectroscopic index showed a slight increase between 20°C and 35°C, EVOO samples were in compliance with the EU legal chemical parameters.

The total phenolic content increased significantly with the final temperature (Table 1). The EVOO samples which were extracted at the 20°C final temperature contained a significant lower content (about 25%) of phenols than those extracted at 27°C and 35°C reached temperatures.

The dialdehydic form of decarboxymethyl oleuropein aglycone - 3,4 DHPEA-EDA content (i.e the EVOO's most abundant phenolic compound – ZANONI, 2014) was also found to significantly increase with the final temperature (Table 1); it increased from 23 mg/kg at 20 °C to 46 mg/kg at 35°C. The positive effect of temperature on the phenolic compounds content was already described in the literature. Veneziani *et al*. (2015; 2017) described an increase in both the total phenolic content and the 3,4 DHPEA-EDA content in relation with the malaxation temperature, due to the combined activities of endogenous enzymes. Rodis *et al*. (2002) found that the partition coefficients (oil/water) of phenolic compounds increased with temperature from 25°C to 45°C; hence, high temperatures were associated with high phenolic compounds content in the oily phase.

No significant variation of the total phenolic and 3,4 DHPEA-EDA contents occurred between EVOO samples that were not malaxed (i.e. at time t = 0) and those processed at 20°C.

Volatile composition of EVOO samples was measured to verify the behavior of both the lipoxygenase (LOX) pathway compounds, related to positive sensory attributes, and the compounds related to sensory defects (Table 1). The experimental data of LOX compounds content were similar at all the final temperatures. No significant differences were found for aldehydes and esters and only one alcohol (1-penten-3-ol) was found to increase with the highest temperatures. The olive paste heating after malaxation did not affect the LOX pathway, but the C5 and C6 compounds contents in EVOO samples seemed only related to the malaxation at 20°C for 30 min; Angerosa *et al*. (2002) reported that malaxation conditions with temperature lower than 25°C for 30-45 min are optimal for the LOX pathway.

Only one volatile compound related to sensory defects (i.e. the used method measured 48 defective compounds; FORTINI *et al.*, 2017) was significant in the EVOO samples (Table 1). The (E)-2-Heptenal, which is related to the rancid defect (KALUA *et al.*, 2006; ZHU *et al.*, 2016), occurred in all EVOO samples and it increased with the final temperatures after the heating of malaxed olive paste. No significant differences were found between 20°C and 27 °C, but a significant difference was found at 35°C. The content of the (E)-2-Heptenal varied approximately from 25 to 40 μg/kg, which are values above the relevant literature odor threshold of 0.005 mg/kg (KOTTI *et al.*, 2011); values around 40 μg/kg of the (E)-2-Heptenal content were significantly correlated with olive oil samples with sensory defects (ZANONI *et al.*, 2015).

3.2. A predicted comparison of EVOO yield and quality between the paste temperature increase after a malaxation treatment and the time-temperature malaxation treatment under stationary conditions

In this paragraph we compare the effects of a malaxation at 20° C for 30 min, followed by 7 min of olive paste heating to 20, 27 and 35°C (i.e. the tested theses), with the effects of a malaxation carried out under stationary temperature conditions (i.e. at 20, 27 and 35°C) for the same time (i.e. 37 min).
For this aim we use the mathematical predictions obtained with the computer program (MalaxAction 1.0). The software is the only tool available today in literature (ZANONI *et al.*, 2018) for the comparisons of the effects of different olive paste malaxation conditions at laboratory scale. The computer program is based on the kinetic studies of Trapani *et al.* (2017a; 2017b), which were carried out with the reference Abencor lab equipment under exposure to air. The computer program is able to predict (i) the oil extraction yield as function of the olive paste oil content, expressed as EI%; (ii) the relative variation of the 3,4 DHPEA-EDA in the olive oil as the quality index that best represents the effect on the phenolic compounds in the extractable oil.
Table 2 reports the comparison between our results and the predictive model. To evaluate the EI% during the experimental trials we used the total oil content of the olive paste (i.e. roughly the 26% - Table 1); to evaluate the change in 3,4 DHPEA-EDA content in the olive oil samples we used the concentration in non-malaxed olive paste (i.e at time t = 0) of 22 mg/kg. Finally we report a semi-quantitative evaluation of the rancid defect obtained using the odor threshold of (E)-2-Heptenal in olive oil samples (Table 1).
The comparison between the EI% values and the relative variation of 3.4 DHPEA-EDA content showed similar results between the experimental values and the predicted values at 20 °C. It is important to point out that this is the only case among those studied without olive paste heating (i.e. malaxation at 20°C for 37 min). The results predicted by the computer program are consistent with the measured value (Table 2), and the MalaxAction 1.0 software has been considered suitable for the comparisons.

Table 2. The predicted comparison of EVOO yield and quality between the paste temperature increase after a malaxation treatment and the time-temperature malaxation treatment under stationary conditions.

Parameter	Predictive comparison at 20°C		Predictive comparison at 27°C		Predictive comparison at 35°C	
	Experimental data (final temperature of 20°C)	Predicted data (malaxation at 20°C for 37 min)	Experimental data (final temperature of 27°C)	Predicted data (malaxation at 27°C for 37 min)	Experimental data (final temperature of 35°C)	Predicted data (malaxation at 35°C for 37 min)
Oil Extractability Index - EI (%)	79	80	89	89	91	92
Decrease (-) or increase (+) of 3,4 DHPEA-EDA content in oi (%)	0	0	+30	-61	+110	-4

The values of EI% obtained during our trials were similar those predicted by *MalaxAction 1.0* (Table 2). This result remarks that the paste heating between malaxation and centrifugation allow to obtain the same yield results than the whole process at higher temperature.
The model for EI% described by Trapani and co-workers (2017a) tends to asymptotically reach the maximum value of 100%; low increases in temperature and short increases in

malaxation time have a high impact on EI% when temperature and time values are low. On the other hand, for long times and high temperatures the increase in EI% due to a further increase of such parameters is lower. Consistently with the predictive model, a short heating of the olive paste was sufficient to increase the yield between 20°C and 27°C, while no further yield increase was obtained between 27 and 35°C.
The model was not able to predict the changes in 3,4-DHPEA-EDA content (Table 2). Moreover, the model was built for olive paste under exposure to air, while this test was conducted in a sealed malaxer (i.e. only the oxygen in the malaxer headspace was available). However, the 3,4-DHPEA-EDA contents can be considered consistent with the general kinetic theory of Trapani *et al*. (2017b). It suggested that the phenolic compound transformation phenomenon during a time-temperature treatment of olive paste is caused by two opposing phenomena during olive paste malaxation: (i) a decreasing phenomenon probably due to enzymatic oxidative damage of the phenolic compounds; (ii) an increasing phenomenon probably due to a physical and enzymatic release of phenolic compounds from the cellular tissues. Obviously, the change in the oxygen availability reduces the extent of the decreasing phenomenon. Thus, discrepancies between 3,4-DHPEA-EDA measured and predicted values were found. At 27 °C the model predicted a 3,4-DHPEA-EDA decrease of 61%, while at 35°C of 4%. In the trial data we measured an increase of 30% and 110% of 3,4-DHPEA-EDA at 27°C and 35°C, respectively. In our trial, the above decreasing phenomenon was inhibited by the reduced oxidative conditions, resulting in a 3,4-DHPEA-EDA increase.

4. CONCLUSIONS

The current trend in high-quality EVOO production is to reduce the malaxation time and temperature. Although this enhances quality, it reduces yield. Recent works have aimed to improve control of the temperature of the process by introducing a heat exchanger between the crusher and the malaxer. This is designed to improve the control of the process, allowing to maintain quality and increase yield for shorter malaxation times, or improve quality without decreasing yield.
A further improvement can be obtained by placing a heat exchanger between the malaxer and the centrifuge and increasing the temperature. This approach obtains a remarkable increase in yield and increases phenols in the olive oil. However, an excessive temperature increase appears to be self-defeating, since at higher temperature off-flavour compounds appeared and no further yield increases were measured.
The placement of a heat exchanger between the malaxer and the "decanter" further improves the control of the extraction process and seems to be complementary to the current trend to place a heat exchanger between the crusher and the malaxer.

REFERENCES

Amirante P, Clodoveo M.L., Dugo G., Leone A. and Tamborrino A. 2006. Advance technology in virgin olive oil production from traditional and de-stoned pastes: Influence of the introduction of a heat exchanger on oil quality. Food Chem. 98:797-805.

Angerosa F., Mostallino R., Basti C. and Vito R. 2001. Influence of malaxation temperature and time on the quality of virgin olive oils. Food Chem. 72:19-28.

Boselli E., Di Lecce G., Strabbioli R., Pieralisi G. and Frega N.G. 2009. Are virgin olive oils obtained below 27 °C better than those produced at higher temperatures? LWT - Food Sci. Technol; 42:748-57.

Cherubini C., Migliorini M., Cecchi L., Trapani S. and Zanoni B. 2009. Technological ripening indices for olive oil fruits. J. Sci. Food Agric. 89:671.

EC 1991 Commission Regulation EEC 2568/1991 and later amendments. Official J. Eur. Communities. 1991, L248(9).

Espínola F., Moya M., Fernández D.G. and Castro E. 2011. Modelling of virgin olive oil extraction using response surface methodology. Int. J. Food Sci. Technol. 46:2576-2583.

Fortini M., Migliorini M., Cherubini C., Cecchi L. and Calamai L. 2017. Multiple internal standard normalization for improving HS-SPME-GC-MS quantitation in virgin olive oil volatile organic compounds (VOO-VOCs) profile. Talanta 165:641-652.

Guerrini L., Migliorini M., Giusti M. and Parenti A. 2017a. The influence of crusher speed on extra virgin olive oil characteristics. Eur. J. Lipid Sci. Technol., 119: 1600156.

Guerrini L., Masella P., Angeloni G., Migliorini M. and Parenti A. 2017b. Changes in Olive Paste Composition During Decanter Feeding and Effects on Oil Yield. Eur. J. Lipid Sci. Technol. 119:1700223.

International Olive Oil Council. Determination of Biophenols in Olive Oil by HPLC. IOOC/T.20/Doc No 29. 2009

Kalua C.M., Bedgood D.R., Bishop A.G. and Prenzler P.D. 2006. Changes in volatile and phenolic compounds with malaxation time and temperature during virgin olive oil production. J. Agric. Food Chem. 54:7641-7651.

Kotti F., Cerretani L., Gargouri M., Chiavaro E., Bendini A. 2011. Evaluation of the volatile fraction of commercial virgin olive oils from Tunisia and Italy: relation with olfactory attributes. Journal of Food Biochemistry, 35:681-698.

Martìnez Moreno J.M., Gomez Herrera C. and Janner del Valle C. 1957. Estudios fisico- quìmicos sobre las pastas de aceitunas molidas. IV. Las gotas de aceite. Grasas Aceites 8:112:120.

Rodis P.S., Karathanos V.T. and Mantzavinou A. 2002. Partitioning of olive oil antioxidants between oil and water phases. J. Agric. Food Chem. 50:596-601.

Tamborrino A. 2014. Olive paste malaxation. In: The Extra-Virgin Olive Oil Handbook, John Wiley and Sons.

Trapani S., Guerrini L., Masella P., Parenti A., Canuti V., Picchi M., Caruso G., Gucci R. and Zanoni B. 2017a. A kinetic approach to predict the potential effect of malaxation time-temperature conditions on extra virgin olive oil extraction yield. J Food Eng. 195:182-90.

Trapani S., Breschi C., Cecchi L., Guerrini L., Mulinacci N., Parenti A., Canuti V., Picchi M., Caruso G., Gucci R. and Zanoni B. 2017b. Indirect indices of oxidative damage to phenolic compounds for the implementation of olive paste malaxation optimization charts. J. Food Eng. 207:24-34.

Veneziani G., Esposto S., Taticchi A., Selvaggini R., Urbani S., Di Maio I. *et al*. 2015. Flash thermal conditioning of olive pastes during the oil mechanical extraction process: Cultivar impact on the phenolic and volatile composition of virgin olive oil. J. Agric. Food Chem. 63:6066-6074.

Veneziani G., Esposto S., Taticchi A., Urbani S., Selvaggini R., Di Maio I. *et al*. 2017. Cooling treatment of olive paste during the oil processing: Impact on the yield and extra virgin olive oil quality. Food Chem. 221:107-113.

Zanoni B. 2014. The role of oxygen and water in the extra-virgin olive oil process. In: The Extra-Virgin Olive Oil Handbook, John Wiley and Sons.

Zanoni B., Guerrini S., Mari E., Migliorini M., Cherubini C., Trapani S. and Vincenzini M. 2015. Investigation on microbiology of olive oil extraction process. Ital. J. Food Sci. 27:236-247.

Zanoni B., Breschi C., Canuti V., Guerrini L., Masella P., Picchi M. and Parenti A. 2018. An original computer program (MalaxAction 1.0) to design and control olive paste malaxation under exposure to air. J. Food Eng. 234:57-62.

Zhu H., Wang S.C. and Shoemaker C.F. 2016. Volatile constituents in sensory defective virgin olive oils. Flavour Fragr. J. 31:22-30.

Paper Received September 24, 2019 Accepted January 31, 2019

PAPER

FACTORS AFFECTING THE SPECTROPHOTOMETRIC QUANTIFICATION OF FLAVONOIDS IN WINE

O. CORONA*[1], M. SQUADRITO[1] and A. TIRELLI[2]

[3]Dipartimento Scienze Agrarie, Alimentari e Forestali, Università di Palermo,
Viale delle Scienze 4, 90128 Palermo, Italy

[3]Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università di Milano,
Via Celoria 2, 20133 Milano, Italy

*Corresponding author: Tel.: +3909123897058; Fax: +39091484035
E-mail address: onofrio.corona@unipa.it

ABSTRACT

The quantification of flavonoids in wine and grape skin extract by spectrophotometric evaluation at 280 nm wavelength provides essential information to oenologist concerning wine composition and evolution, and it is commonly applied in wine labs. The measurement of the absorption peak height at 280 nm reported by DI STEFANO and GUIDONI (1989) allows to selectively quantify flavonoids with minor interferences. However, it has proved to be susceptible to SO_2 at low pH or acetone in unpurified grape skin extracts. Moreover, the effect of pH on flavonoids quantification in wine, either containing SO_2 or not, has not been assessed. The effect of SO_2, purification, pH and dilution solvent on spectrophotometric quantification of flavonoids in red wine samples has been evaluated in this work. SO_2 can overrate the flavonoids content in red wine when ethanol and Cl^- ions are contained in acid dilution solvents. A wine sample dilution with a strong acid solvent is mandatory to attain a reliable quantification of flavonoids due to the low anthocyanins absorption at 280 nm in water solution. A minor effect arises from the ethanol content. Eventually, flavonoids can be quantified in SO_2-containing wine diluted with a strong acid solution but a 7% overrating should be expected.

Keywords: polyphenol index, flavonoids, anthocyanins, SO_2; hyperchromic effect, bathochromic effect

1. INTRODUCTION

The amount of polyphenols, especially flavan-3-ols and anthocyanins, affects astringency, bitter taste and green/woody properties of red wine (GIBBINS and CARPENTER, 2013; SOARES *et al*., 2015). Their fast quantification in wine-making and wine ageing is crucial in winery since it makes it possible to carefully address the oenological choices involving the duration and conditions of maceration and ageing. Flavonoids are quantitatively the main phenol fraction in red wine by far; therefore, many analytical methods are based on the quantification of total polyphenols (ALEIXANDRE-TUDO *et al*., 2017). However, many of them are poorly selective or accurate (FOLIN and DENIS, 1912; SINGLETON and ROSSI, 1965) and require quite complex analytical approaches (GARCÍA-GUZMÁN *et al*., 2015) or even expensive analytical instrumentation for quality control laboratories (KENNEDY and JONES, 2001). The spectrophotometric methods are still among the fastest, easy to apply and cheapest for the oenologist; therefore, they are usually applied in the winery laboratories (ALEIXANDRE-TUDO *et al*., 2017). The spectrophotometric analytical approach reported by DI STEFANO and GUIDONI (1989) is widespread and routinely applied in the wineries to achieve a fast and reliable evaluation of flavonoids in grape extract and wine. It is based on the absorption spectrum obtained in the wavelength range 230–700 nm of a diluted sample. The peak height measured at 280 nm (E_{280}) subtracted from the absorbance measured at its valley-to-valley baseline returns the absorbance value mainly due to the flavonoids (E'_{280}). Such an approach allows to avoid the interference due to compounds without an absorption peak at 280 nm like aromatic amino acids, nucleosides and nucleotides (SOMERS and ZIEMELIS, 1985). Wine dilution with strong acid solutions allows to quantify the total anthocyanin content based on the height of the absorbance peak at about 520 nm of the spectra. An easier wine dilution with distilled water is commonly applied to assess the total flavonoids based on the E'_{280}. However, there is a lack of information about the analytical factors affecting the accuracy of this approach, in spite of its widespread use at wine control laboratories. CORONA *et al*. (2015) pointed out the interference exerted by SO_2 (as an undissociated molecular form) in quantifying flavonoids extracted from the grape berry and dissolved in a strong acid solvent like ethanol-hydrochloric acid mixture (EtOH-HCl). A further interference can arise from residual amounts of acetone used as extraction solvent of phenols. Both SO_2 and acetone can be easily removed by solid phase extraction (SPE) packed with a C18 resin (CORONA at al., 2015). It is well-known that sulfites can negatively affect the spectrophotometric quantification of anthocyanins at wine pH and acetaldehyde is needed to effectively remove the SO_2 bound to anthocyanins (USSEGLIO-TOMASSET *et al*., 1982; MAZZA *et al*., 1999). Recently, SO_2 proved capable of forming sulfonated adducts of flavan-3-ols over wine aging (ARAPITSAS *et al*., 2014). The binding involves the C4 position of the flavan ring and only monomeric flavan-3-ols and the terminal flavanol unit of proanthocyanindins are expected to undergo sulfonation in time. Sulfonation of elongation flavanol units has not been reported, possibly owing to steric hindrance issues. The spectrophotometric properties of flavanol-sulfite adducts are unknown, as well as their role in flavonoid quantification. However, their low relative abundance has to be considered, especially in young wine (ARAPITSAS *et al*., 2018). Poor information is available about the role exerted by SO_2 in wine concerning the quantification of flavonoids based on the E'_{280} value, especially when strongly acidic solutions (pH < 1) are used as a dilution solvent to attain the quantification of anthocyanins in the meantime. Moreover, there is a lack of information about how the composition and acidity of the dilution solvent affect the quantification of total flavonoids assessed using the E'_{280} value.

In this work the effects of SO_2, ethanol concentration, acid and pH on the absorbance values E_{280} and E'_{280} assessed in diluted red wine samples were assessed to monitor their role on the quantification of wine flavonoids.

2. MATERIALS AND METHODS

2.1. Chemicals

Methanol, ethanol, sulphuric acid, hydrochloric acid, tartaric acid, ethanol, sodium hydroxide, citric acid monohydrate, potassium phosphate monobasic, sodium phosphate dibasic, hydrogen peroxide solution (30% w/w in water), ethyl acetate, polyvinylpolypyrrolidone (PVPP) and bromocresol green methyl red indicator were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium metabisulphite and phosphoric acid were purchased from J.T. Baker (Deventer, Holland). Seeds and white and red grape tannins were provided by Bono and Ditta s.p.a. Italian Grape Juice from Campobello di Mazara (Trapani, Italy).

2.2. Wine Samples

Sixty different commercial red wine samples produced in the years 2013–2015 were collected at the market and submitted for the evaluation of total anthocyanin and total flavonoid contents. Moreover, the spectrophotometric response obtained following different dilution conditions of eight samples of red wine obtained from *Nero d'Avola* grape (vintages 2013–2015), *Nerello Mascarese* grape (vintage 2015), *Cabernet Sauvignon* grape (vintage 2014 and 2015) and *Merlot* (vintage 2014) was assessed. All measurements were performed in triplicate.

2.3. Purification of flavonoids

Half millilitre of wine sample was diluted with 5 mL H_2SO_4 5 mM and loaded into a 400 mg C18 SPE cartridge (Sep-Pak, Waters, Milan, Italy) previously conditioned with 2 mL methanol and then 3 mL H_2SO_4 5 mM. The polar compounds were eluted with 3 mL H_2SO_4 5 mM to drying and discarded, then the phenols were collected into a 25 mL volumetric flask by eluting with 3 mL methanol and brought to volume with one of the following solvents: H_2O, 0.5 M H_2SO_4, ethanol:H_2O:12 M HCl 70:30:1 (v/v/v) (EtOH-HCl). The same solutions were also used for diluting 0.5 mL of the wine samples to 25 mL in volumetric flasks. Triplicate preparations were carried out.

2.4. Determination of total flavonoids

Flavonoids were purified by treatment with SPE procedure. The UV-visible absorption spectra in the range 230–700 nm wavelength of either unpurified or purified flavonoids were recorded, and the absorption values at 280 nm (E_{280}) were measured. Triplicate preparations were carried out. The E'_{280} value was also measured according to DI STEFANO and GUIDONI (1989) and modified by Corona *et al*. (2015). The total flavonoid content was calculated according to DI STEFANO and GUIDONI (1989) and CORONA *et al*. (2010) as follows:

$$\text{Total flavonoids (as mg/L (+)-catechin equivalent)}: 82.4 \times E'_{280} \times 50.$$

2.5. Absorbance parameters of white grape skin extract

Buffered solutions at pH 1.1, 3.0, 5.0 and 7.0 were prepared according to KÜSTER *et al*. (1979) and used for dissolving 30 mg/L grape skin extract. Their UV-visible absorption spectra in the range 230-400 nm wavelength were recorded and the values of λ_{max}, E_{280} and E'_{280} were measured. Triplicate preparations were carried out.

2.6. Purification of anthocyanins from red grape skin extract

Phenols from red grape skin extract were obtained from the skin of 50 berries by using a tartaric buffer (5 g tartaric acid, 22 mL NaOH 1 N, 2 g $Na_2S_2O_5$, 125 mL ethanol 95–96%, brought to 1 L with H_2O). Anthocyanins were obtained from the extract as follows. Three millilitres of H_2SO_4 0.5 M and 6 g of PVPP were added to 60 mL of skin extract. The mixture was stirred for 2 min, then centrifuged at 2000 *g* × 10 min and the PVPP was recovered and then rinsed with 20 mL of H_2SO_4. The mixture was centrifuged as above and the PVPP was recovered. The anthocyanins absorbed on the PVPP were dissolved by dispersing the PVPP into 15 mL EtOH-HCl solution and centrifuging at 2000 *g* × 10 min. The addition of EtOH-HCl solution and the centrifugation were carried out four times again, and all the five supernatants were collected and blended in a 100 mL evaporation flask. The ethanol contained in the anthocyanins solution was removed by vacuum-drying and the water solution was transferred in a 100 mL extraction funnel. The residual flavan-3-ols were removed by a triplicate extraction with 10 mL ethyl acetate each. The purified anthocyanin extract was transferred in a 100 mL evaporation flask and the residual ethyl acetate was removed by vacuum drying. Finally, the dried anthocyanins were dissolved with 50 mM H_2SO_4 10 mL and recovered.

2.7. Absorbance parameters of anthocyanins

One millilitre of either red skin extract or purified anthocyanins solution was diluted to 25 mL with buffer solutions at pH 1.1, 3.0, 5.0 and 7.0 prepared according to KÜSTER *et al*. (1979) or with 0.1 M HCl solutions containing 10, 20, 40 or 80% ethanol. Their UV-visible absorption spectra in the range 230–700 nm wavelength was recorded, and the values of maximum absorption wavelengths in the range 275-282 nm (λ_{maxUV}) and in the range 510-550 nm (λ_{maxVIS}) were measured, as well as their absorption values (E_{280}, E'_{280}, E_{520}).

2.8. Determination of SO_2 in wine samples

The SO_2 content in wine was carried out according to the Functional EEC in 2376 (1990) standard procedures. Triplicate determinations were carried out.

2.9 Statistical analysis

Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test to calculate significant differences between treatments were carried out. All tests were performed at a significance level of $p < 0.05$ using the statistical program SPSS (ver. 13, IBM, Armonk, NY, USA).

3. RESULTS AND DISCUSSION

A fast quantification of total flavonoids and anthocyanins in wine can be achieved by measuring the spectrophotometric values E'_{280} and E_{520} of the sample diluted with an acid solution. However, such an approach can overrate the flavonoid content owing to the presence of gallic acid. SO_2 has an absorption peak close to 280 nm (276 nm) and diluting wine in strong acid solutions might increase the E'_{280} value, thus inducing a major overrating of the flavanol concentration (CORONA *et al*., 2015). Wine dilution with ethanol-HCl can further increase the absorbance of SO_2 owing to the bathochromic and hyperchromic effects induced by ethanol and Cl^-, respectively. SO_2 can be removed from the wine sample by SPE packed with a C18 resin (CORONA *et al*., 2015). To assess the effect of sample purification on the spectrophotometric quantification of flavonoids, the analytical responses of 61 SPE-treated and untreated red wine samples, both of them diluted in an ethanol-HCl solution, were compared (Fig. 1).

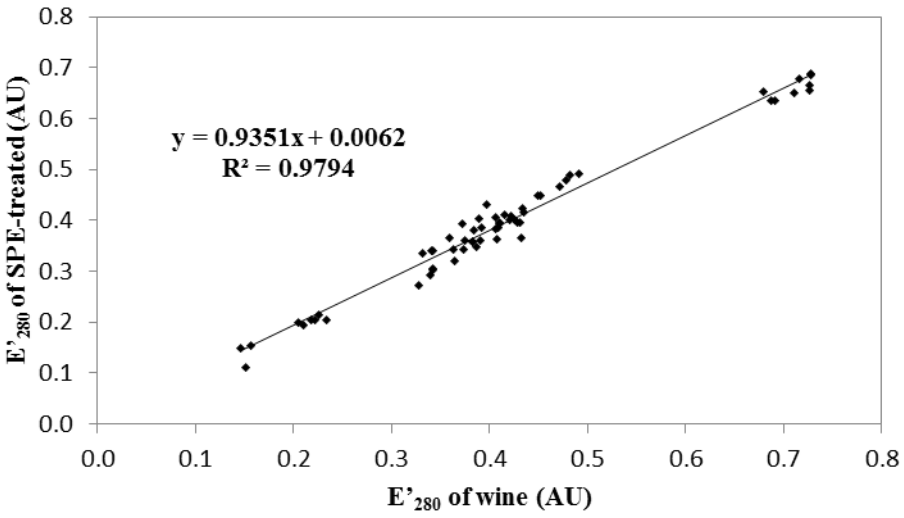


Figure 1. Comparison of E'_{280} values obtained for wine samples and their corresponding SPE-treated wine (n=3). All the samples were diluted with EtOH-HCl solution.

A good correlation (r^2 = 0.979) was obtained; however, the slope of the regression line shows the E'_{280} values of wine were 6-7% higher than the corresponding SPE-treated wine. Such an overrating was expected as SPE purification removes the polar phenols unretained on the SPE resin, namely gallic acid, tyrosine and tyrosol (DI STEFANO and GUIDONI, 1989). However, the role of SO_2 is hard to assess in unknown samples, even though it was proved in the previous work of CORONA *et al*. (2015). Therefore, a known addition of SO_2 in real wine samples is expected to increase the E'_{280} value, but such an interference is hard to quantify owing to the occurrence of different pH values as well as quality and content of SO_2-binding compounds (ethanal, anthocyanins, pyruvate or other carbonyl compounds). To better focus the interference of SO_2 on the quantification of flavonoids, the absorption spectra obtained from red wine samples containing different concentrations of SO_2 either submitted or not to SPE purification of flavonoids and diluted with acid solutions with different pH values were compared (Table 1). Following the purification step, the E_{280} values of the acid-diluted samples decreased by up to -20% in accordance with the work of SOMERS and ZIMELIS (1985) (Table 1).

Table 1. Value of λ_{max}, E_{280} (as AU) E'_{280} (as AU), flavonoids (as mg/L (+)-catechin equivalent) and SO_2 (mg/L) in wine and SPE-treated wine samples diluted with different solutions.

Wine and vintage year		Wine			SPE-treated wine			SO_2 level in wine	
		H_2O	5×10^{-1} M H_2SO_4	Ethanol-HCl	H_2O	5×10^{-1} M H_2SO_4	Ethanol-HCl	Total	Free
Nero d'Avola 13	λ_{max}	276.0±0.00^{a}	277.5±0.71^{b}	278.5±0.71^{b}	278.0±0.00^{A}	279.0±0.00^{A}	280.0±0.00^{A}	59.5±1.2	17.3±0.3
	E_{280}	0.428±0.01^{a}	0.466±0.02^{b}	0.484±0.00^{b}	0.378±0.00^{A}	0.400±0.00^{B}	0.390±0.00^{AB}		
	E'_{280}	0.129±0.00^{a}	0.168±0.00^{b}	0.175±0.00^{b}	0.126±0.00^{A}	0.162±0.00^{B}	0.163±0.00^{B}		
	Total Flavonoids	1068±11^{a}	1384±87^{b}	1445±9^{b}	1037±12^{A}	1337±22^{B}	1345±11^{B}		
Nero d'Avola 13	λ_{max}	277.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{a}	278.5±0.71^{A}	279.0±0.00^{A}	280.0±0.00^{A}	61.1±1.1	17.9±0.1
	E_{280}	0.450±0.00^{a}	0.504±0.01^{b}	0.543±0.00^{c}	0.384±0.07^{A}	0.446±0.01^{B}	0.435±0.00^{B}		
	E'_{280}	0.148±0.00^{a}	0.186±0.01^{b}	0.201±0.01^{b}	0.125±0.00^{A}	0.184±0.01^{B}	0.187±0.00^{B}		
	Total Flavonoids	1219±29^{a}	1533±33^{b}	1660±10^{c}	1034±19^{A}	1514±11^{B}	1542±10^{B}		
Nero d'Avola 14	λ_{max}	276.0±0.00^{a}	276.5±0.71^{b}	278.0±0.00^{b}	278.0±0.00^{A}	278.0±0.00^{A}	280.0±0.00^{A}	1.3±0.0	0.6±0.0
	E_{280}	0.384±0.01^{a}	0.403±0.00^{ab}	0.419±0.01^{b}	0.342±0.00^{A}	0.358±0.00^{B}	0.345±0.00^{A}		
	E'_{280}	0.114±0.00^{a}	0.131±0.00^{ab}	0.140±0.00^{b}	0.106±0.00^{A}	0.136±0.00^{B}	0.130±0.00^{B}		
	Total Flavonoids	938±22^{a}	1076±43^{b}	1154±43^{b}	873±4^{A}	1122±22^{C}	1071±11^{B}		
Nero d'Avola14	λ_{max}	276.0±0.00^{a}	276.5±0.71^{ab}	278.0±0.00^{b}	278.0±0.00^{A}	278.5±0.71^{A}	280.0±0.00^{A}	1.3±0.0	0.6±0.0
	E_{280}	0.415±0.00^{a}	0.431±0.00^{ab}	0.456±0.02^{b}	0.368±0.00^{A}	0.395±0.01^{A}	0.397±0.00^{A}		
	E'_{280}	0.122±0.00^{a}	0.139±0.01^{ab}	0.160±0.01^{b}	0.120±0.00^{A}	0.146±0.01^{B}	0.154±0.01^{B}		
	Total Flavonoids	1007±33^{a}	1145±20^{ab}	1314±120^{b}	992±11^{A}	1203±20^{B}	1273±9^{C}		
Nero d'Avola 15	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{b}	278.0±0.00^{A}	279.0±0.00^{A}	280.0±0.00^{A}	24.3±1.2	8.7±0.5
	E_{280}	0.415±0.00^{a}	0.432±0.01^{a}	0.447±0.01^{a}	0.379±0.00^{A}	0.385±0.00^{B}	0.389±0.02^{B}		
	E'_{280}	0.122±0.01^{a}	0.146±0.01^{ab}	0.157±0.01^{b}	0.124±0.00^{A}	0.151±0.00^{B}	0.155±0.01^{B}		
	Total Flavonoids	1005±11^{a}	1204±20^{b}	1291±43^{b}	1018±4^{A}	1245±21^{B}	1276±22^{B}		
Nero d'Avola 15	λ_{max}	278.0±0.00^{a}	278.5±0.71^{a}	279.0±0.00^{a}	279.0±0.00^{A}	279.0±0.00^{A}	280.0±0.00^{A}	34.3±4.2	11.3±2.4
	E_{280}	0.357±0.00^{a}	0.379±0.00^{a}	0.381±0.01^{a}	0.323±0.00^{A}	0.319±0.00^{A}	0.319±0.00^{A}		
	E'_{280}	0.110±0.00^{a}	0.112±0.00^{a}	0.113±0.01^{a}	0.105±0.00^{A}	0.107±0.00^{A}	0.108±0.00^{A}		
	Total Flavonoids	910±4^{a}	923±15^{a}	930±11^{a}	869±11^{A}	884±11^{A}	892±20^{A}		

Nerello Mascalese 15	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	278.0±0.00^{a}	278.0±0.00^{A}	278.0±0.00^{A}	279.5±0.71^{A}	35.7±4.2	13.0±2.9
	E_{280}	0.344±0.01^{a}	0.346±0.00^{a}	0.360±0.01^{a}	0.285±0.01^{A}	0.292±0.00^{A}	0.286±0.00^{A}		
	E'_{280}	0.125±0.00^{a}	0.130±0.00^{a}	0.144±0.00^{b}	0.119±0.01^{A}	0.118±0.00^{A}	0.126±0.00^{A}		
	Total Flavonoids	1030±22^{a}	1069±10^{a}	1184±22^{b}	982±20^{A}	970±21^{A}	1038±11^{B}		
Cabernet Sauvignon 15	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{b}	278.0±0.00^{A}	277.0±0.00^{A}	279.0±0.00^{A}	86.8±7.2	27.9±4.1
	E_{280}	0.565±0.00^{a}	0.591±0.01^{b}	0.626±0.00^{c}	0.514±0.01^{A}	0.585±0.00^{C}	0.542±0.01^{B}		
	E'_{280}	0.185±0.00^{a}	0.217±0.01^{b}	0.236±0.01^{c}	0.176±0.01^{A}	0.212±0.00^{B}	0.215±0.01^{B}		
	Total Flavonoids	1524±28^{a}	1786±30^{b}	1948±15^{c}	1453±15^{A}	1736±48^{B}	1772±6^{B}		
Nero d'Avola 14	λ_{max}	276.0±0.00^{a}	277.5±0.71^{b}	278.5±0.71^{b}	n.d.	n.d.	n.d.	62.1±1.3	22.5±0.75
	E_{280}	0.416±0.00^{a}	0.473±0.01^{b}	0.491±0.01^{b}					
	E'_{280}	0.127±0.00^{a}	0.171±0.01^{b}	0.174±0.01^{b}					
	Total Flavonoids	1045±15^{b}	1407±23^{b}	1430±17^{b}					
Nero d'Avola 15	λ_{max}	276.0±0.00^{a}	276.5±0.71^{ab}	278.0±0.00^{b}	n.d.	n.d.	n.d.	27.2±1.5	10.8±0.05
	E_{280}	0.398±0.00^{a}	0.432±0.00^{b}	0.479±0.00^{b}					
	E'_{280}	0.107±0.00^{a}	0.137±0.00^{b}	0.141±0.01^{b}					
	Total Flavonoids	884±8^{a}	1130±18^{b}	1161±12^{b}					
Cabernet Sauvignon 14	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	278.0±0.00^{a}	n.d.	n.d.	n.d.	33.4±1.7	15.3±0.61
	E_{280}	0.369±0.00^{a}	0.402±0.00^{b}	0.429±0.00^{b}					
	E'_{280}	0.124±0.00^{a}	0.152±0.00^{b}	0.160±0.00^{b}					
	Total Flavonoids	1022±10^{a}	1253±12^{b}	1322±31^{b}					
Merlot 14	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{b}	n.d.	n.d.	n.d.	89.7±9.1	30.4±0.60
	E_{280}	0.413±0.01^{a}	0.454±0.01^{b}	0.492±0.01^{b}					
	E'_{280}	0.132±0.01^{a}	0.166±0.01^{b}	0.172±0.01^{b}					
	Total Flavonoids	1084±38^{a}	1368±19^{b}	1414±10^{b}					

n = 3 samples; mean value ± standard deviation. n.d.: not determined.
Different letters in the same row indicate significant differences between wine samples or SPE-treated wine samples (Tukey's HSD test, $p < 0.05$).

It is mainly due to the loss of purines, pyrimidines, nucleosides, amino acids and aromatic alcohols occurred following the purification procedure. Therefore, the E_{280} value confirms to be an unsuitable index of the phenol content in wine. The quantification of flavonoids based on the E'_{280} value shows the major role of the dilution solvent on the measured values. The E'_{280} values obtained by water dilution of the wine samples were always significantly lower than the sample diluted with acid solutions. The E'_{280} value increases as the pH decreases, thus suggesting that the SO_2 plays a role. Moreover, the hyperchromic effect detectable only in the wine samples diluted with ethanol-HCl solution further supports such a conclusion. However, a comparable or even higher increase of the E'_{280} value can be detected after the removal of SO_2 by SPE, even in the samples containing negligible SO_2 level (Nero d'Avola 14). Neither the bathochromic nor the hyperchromic effects detected in the wine samples, Nero d'Avola 14, ought to be observed when ethanol-HCl instead of a H_2SO_4 solution is used as dilution solvents. Moreover, no increase of the E'_{280} value should be detected in the SPE-treated samples following dilution with H_2SO_4 if SO_2 had a major role. Nonetheless, the calculated flavonoids content strongly increases as the pH of the dilution solvent decreases, even in the SPE-treated wine samples (Table 1). All these data highlight the minor contribution of SO_2 to the E'_{280} value and quantification of flavonoids in wine, while pH and solvent composition strongly affect the analytical response. Since the variation of E'_{280} values also occurs with the SPE-treated samples where the hydrophobic compounds eluted with methanol from the C18 resin are contained, phenols are likely involved in this behaviour. However, flavan-3-ols, either monomer or polymer, are not expected to be affected by pH values lower than 7, as their pK_a exceeds 9. Therefore, pH variations in the range 0-7 should attain negligible dissociation effects whatever the alcohol content of the adopted diluting solvent (DANILEWICZ, 2003; FRIEDMAN and JÜRGENS, 2000). As expected trials carried out at pH values spanning from 1 to 7 with different ethanol content did not show any significative effect on the E'_{280} values recorded for grape phenols extracted from white skin (Table 2) and comparable results were obtained when phenols extracted from grape seeds were evaluated (data not shown). Therefore, flavan-3-ols and proanthocyanidins, as well as other colourless skin or seed phenols (phenolic acids, flavonols, hydroxystilbenes), can hardly be responsible for the E'_{280} variations induced by pH and solvent differences. Consequently, the role of anthocyanins was investigated. The absorption spectra of anthocyanins are affected by the pH. Moreover, the E_{280} value of their flavilium ion is higher than its neutral form occurring in wine at pH values lower than 6 (MARÇO *et al*., 2011). The E'_{280} value obtained assessing anthocyanins from red grape skin extract treated by SPE packed with PVPP significantly decreases as the pH increases. The E'_{280} values increase more than 15% by wine acidification (pH 3–4) down to pH 1 (Table 1). Such a change is lower than it occurs when the absorption peak at 520–540 nm is considered (see $E_{lmaxVis}$ in table 3); nonetheless, it can strongly affect the spectrophotometric evaluation of flavonoid by the E'_{280}, especially when wines containing a high amount of anthocyanins are considered (Table 3). As the role of ethanol on the absorbance of anthocyanins in diluted wine is well-known (LEE *et al*., 2005), the increase in E_{520} values following the increased ethanol concentration was expected. Ethanol does not affect the E'_{280} value and only an ethanol level as high as 80% (v/v) shows a minor role. Same results were obtained when HCl was replaced with H_2SO_4 (data not shown). If grape skin extract is concerned, increasing E'_{280} values are clearly visible when the ethanol content increases due to the hyperchromic effect arising from the presence of SO_2 in the skin extract (Table 3).

Table 2. Effect of pH and dilution solvent on the analytical response parameters of phenols extracted from white grape skin.

	λ_{max}	E_{280}	E'_{280}
pH			
1.1	283±0.6^{a}	1.193±0.00^{a}	0.279±0.00^{a}
3.0	284±0.6^{a}	1.197±0.01^{a}	0.280±0.00^{a}
5.0	283±0.6^{a}	1.195±0.00^{a}	0.284±0.00^{a}
7.0	283±0.6^{a}	1.195±0.00^{a}	0.284±0.00^{a}
Ethanol %, 0.1 M HCl			
0%	283±0.6^{a}	1.145±0.00^{a}	0.277±0.00^{ab}
10%	283±0.6^{a}	1.146±0.00^{a}	0.279±0.00^{b}
20%	282±0.0^{a}	1.143±0.01^{a}	0.275±0.00^{ab}
40%	282±0.6^{a}	1.143±0.00^{a}	0.274±0.00^{ab}
80%	282±0.0^{a}	1.149±0.00^{a}	0.270±0.00^{a}

n = 3 samples; mean value ± standard deviation.
Different letters in the same column indicate significant difference between dilution solvents (Tukey's HSD test, $p < 0.05$).

Table 3. Effect of pH, dilution solvent and purification on some spectrophotometric parameters of grape skin anthocyanins.

	λ_{maxUV}	E_{280}	E'_{280}	λ_{maxVis}	$E_{\lambda maxVis}$
pH			**purified anthocyanin**		
1.1	277.0±0.0^{b}	0.466±0.01^{c}	0.221±0.02^{c}	519±0.0^{a}	0.618±0.01^{d}
3.0	277.0±0.0^{b}	0.414±0.00^{b}	0.186±0.00^{bc}	519±0.0^{a}	0.464±0.00^{c}
5.0	276.5±0.7^{b}	0.371±0.00^{a}	0.155±0.00^{b}	519±0.0^{a}	0.192±0.00^{b}
7.0	275.0±0.0^{a}	0.351±0.01^{a}	0.099±0.00^{a}	551±0.0^{b}	0.088±0.00^{a}
Ethanol %, 0.1 M HCl			**purified anthocyanin**		
0%	277.0±0.0^{a}	0.436±0.00^{a}	0.241±0.00^{b}	519±0.0^{a}	0.609±0.00^{a}
20%	278.0±0.0^{a}	0.437±0.00^{a}	0.243±0.00^{b}	528±0.0^{b}	0.650±0.00^{b}
40%	279.0±0.0^{a}	0.435±0.00^{a}	0.246±0.00^{b}	536±0.0^{c}	0.684±0.00^{c}
80%	280.0±0.0^{a}	0.431±0.00^{a}	0.229±0.00^{a}	544±0.0^{d}	0.730±0.00^{d}
Ethanol %, 0.1 M HCl			**red grape skin extract**		
0%	278.0±0.0^{a}	0.854±0.04^{a}	0.315±0.00^{a}	520±0.0^{a}	0.305±0.01^{a}
20%	278.0±0.0^{a}	0.882±0.01^{a}	0.331±0.01^{a}	527±0.0^{b}	0.336±0.00^{ab}
40%	279.0±0.0^{a}	0.892±0.02^{a}	0.334±0.01^{a}	535±0.0^{c}	0.358±0.01^{bc}
80%	280.0±0.0^{a}	0.916±0.03^{a}	0.341±0.01^{a}	542±0.0^{d}	0.381±0.01^{c}

n = 3 samples; mean value ± standard deviation.
Different letters in the same column indicate significant difference between treatments (Tukey's HSD test, $p < 0.05$).

4. CONCLUSIONS

Our data highlight that the dilution of wine with water to assess the total flavonoid content by the E'_{280} value prevents from the spectrophotometric interference of SO_2 in the analytical response unless it occurs at concentration values exceeding the permitted

amounts in wine. However, under such conditions the interference exerted by the polar non-flavonoid phenols and acid equilibrium of anthocyanins induces a biased quantification. On the other hand, wine dilution with EtOH-HCl can induce an overrated quantification due to both the interference of SO_2 in low pH solutions and the hyperchromic effect exerted by Cl^-. Such interferences can be avoided by carrying out the SPE purification of wine and then diluting the sample with an acid solution (pH < 1).

ACKNOWLEDGMENTS

The authors thank Prof. Rocco Di Stefano for his valuable technical suggestions and Dr Fabio Ditta of the "Bono and Ditta s.p.a. Italian Grape Juice" Campobello di Mazara (Sicily) for providing grape seed tannins.

REFERENCES

Aleixandre-Tudo J.L., Buica A., Nieuwoudt H., Aleixandre J.L. and du Toit W. 2017. Spectrophotometric analysis of phenolic compounds in grapes and wines. J. Agric. Food Chem. 65:4009-4026.

Arapitsas P., Speri G., Angeli A., Perenzoni D. and Mattivi F. 2014. The influence of storage on the "chemical age" of red wines. Metabolomics 10:816-832.

Arapitsas P., Guella G. and Mattivi F. 2018. The impact of SO_2 on wine flavanols and indoles in relation to wine style and age. Sci. Rep-UK 8:858.

Corona O., Squadrito M., Borsa D. and Di Stefano R. 2010. Behaviour of some compounds with λmax at 280 nm in the determination of total flavonoids of grape skin extracts made from a hydroalcoholic SO_2-rich solvent. Ital. J. Food Sci. 22:347-351.

Corona O., Squadrito M., Vento G., Tirelli A. and Di Stefano R., 2015. Over-evaluation of total flavonoids in grape skin extracts containing sulphur dioxide. Food Chem. 172:537-542.

Danilewicz J.C. 2003. Review of reactions mechanism of oxygen and proposed intermediate reduction products in wine: central role of iron and copper. Am. J. Enol. Vitic. 54:73-85.

Di Stefano, R. and Guidoni S. (1989). La determinazione dei polifenoli totali nei mosti e nei vini. Vignevini 16:47-52.

EEC, 1990. European Communities. Commission Regulation No. 2676/90 on "Community Analysis Methods to Use in Wine Sector". Official Journal European Communities L272/3.10.90.

Folin, O., and Denis, W., 1912. On phosphotungstic-phosphomolybdic compounds as color reagents. J. Biol. Chem. 12:239-243.

Friedman M. and Jürgens H.S., 2000. Effect of pH on the stability of plant phenolic compounds. J. Agric. Food Chem. 48:2101-10.

García-Guzmán J.J., Hernández-Artiga M.P., Palacios-Ponce de León L. and Bellido-Milla D. 2015. Selective methods for polyphenols and sulphur dioxide determination in wines. Food Chem. 182:47-54.

Gibbins H.L. and Carpenter G.H. 2013. Alternative mechanisms of astringency – What is the role of saliva?. J. Text. Stud. 44:364-375.

Kennedy J.A. and Jones G.P. 2001. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. J. Agric. Food Chem. 49:1740-1746.

Küster F.W., Thiel A. and Fischbeck K. 1979. Tabelle logaritmiche per chimici, farmacisti, medici e fisici. 11ª Ediz., Milano, Hoepli.

Lee J., Durst R. W. and Wrolstad R.E. 2005. Determination of total monomeric anthocyanin pigment contentof fruit juices, beverages, natural colorants, and wines by the ph differential method: collaborative study. J. Aoac Int. 88:1269-1278.

Março P.H., Poppi R.J., Scarminio I.S. and Tauler R. 2011. Investigation of the pH effect and UV radiation on kinetic degradation of anthocyanin mixtures extracted from *Hibiscus acetosella*. Food Chem. 125:1020-1027.

Mazza G., Fukumoto L., Delaquis P., Girard B. and Ewert. B. 1999. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. J. Agric. Food Chem. 47:4009-4017.

Usseglio-Tomasset L., Ciolfi G. and Di Stefano R. 1982. The influence of the presence of anthocyanins on the aseptic activity of sulfur dioxide towards yeasts. Vini d'Italia 24:86-94.

Singleton V.L. and Rossi J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-158.

Soares S., Kohl S., Thalmann S., Mateus N., Meyerhof W. and De Freitas V. 2015. Different phenolic compounds activate distinct human bitter taste receptors. J. Agric. Food Chem. 61:1525-1533.

Somers T.C. and Ziemelis G. 1985. Spectral evaluation of total phenolics components in *Vitis vinifera*: grapes and wines. J. Sci. Food Agric. 36:1275-1284.

Paper Received July 21, 2018 Accepted December 18, 2018

PAPER

DEVELOPMENT OF FRUIT LEATHER FROM *ACTINIDIA ARGUTA* BY-PRODUCT: QUALITY ASSESSMENT AND SHELF LIFE STUDIES

G. GIACALONE, T.M. DA SILVA, C. PEANO and N.R. GIUGGIOLI*
Department of Agricultural, Forest and Food Sciences (DISAFA), Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy
*Corresponding author: Tel.: +390116708646; Fax: +390116708658
E-mail address: nicole.giuggioli@unito.it

ABSTRACT

Strategies are needed to reduce the substantial fruit losses of *Actinidia arguta* (speciality fruits) caused by the high perishability of the edible skin. Fruit leathers are considered a valuable opportunity to meet the requirements of today's consumers searching for healthy and time-saving products. This work aimed to (a) develop a novel baby kiwifruit leather product (four formulations) and (b) evaluate the best formulation under different shelf life conditions. Storage at 25°C and polyethylene samples presented the best colour and received acceptable scores for sensory evaluation at the end of the shelf life.

Keywords: *Actinidia arguta*, formulations, leather, shelf life, storage

1. INTRODUCTION

In 2014, the High Level Panel of Experts (HLPE) on Food Security and Nutrition of the United Nations Food and Agriculture Organisation (FAO) affirmed that 'A sustainable food system is a food system that ensures food security and nutrition for all in such a way that the economic, social and environmental bases to generate food security and nutrition of future generations are not compromised'. Today, the topic of the agro-food system sustainability transitions remains high on the agenda of many countries of the European Union and international organisations (International Energy Agency, Organisation for Economic Co-operation and Development, and the World Bank). The scientific community that works with the agro-food industry agrees with solutions about waste and food losses prevention, minimisation and valorisation (PORAT *et al*., 2018).

In the fruit and vegetable sector, this issue represents a critical point for products that belong to speciality foods, such as berry fruits (MURPHY *et al*., 2002; GIRGENTI *et al*., 2016), which need to be marked with high qualitative standards, and small aesthetic defects are enough to compromise the competitivity of these products in the market chain. Although several studies have reported about the opportunity to valorise fruit waste (MA *et al*., 1993; LAUFENBERG *et al*., 2003; FEDERICI *et al*., 2009; PATHAK *et al*., 2017) and suggested approaches to reduce the fresh fruit losses in the supply chain, there are currently limited research publications about the fate of specialities fruits that do not meet market specifications. A related option could be represented by the creation of new processed products with a high nutrient profile to fulfil the increasing demand for innovative and time-saving products (as well as the needs of consumers, who are looking for healthy products to prevent health issues, such as diabetes, obesity and cardiac diseases).

Earlier investigations evaluated the various effects of the drying (KAYA *et al*., 2010), pasteurisation (LESPINARD *et al*., 2012), freezing (CANO and MARÍN, 1992; PARK *et al*., 2016) and canning (CANO and MARÍN, 1992) of processed fruits. Processing of new fruit puree formulations revealed to be a well-performing strategy to prolong shelf life and diversify the market, by creating novel products, which may retain or even improve the nutraceutical profile (SIMAL *et al*., 2005; TORRES *et al*., 2013). Among these strategies, fruit leathers seem to be innovative dried products with high levels of convenience, as they represent a suitable choice for consumers searching for nutritious snacks (SHARMA *et al*., 2013).

Fruit leather processing is characterised by fruit puree dehydration, which is necessary to obtain a solid product. Apple, mango and a range of tropical fruit, for example, can be used to produce fruit leathers. However, the formulation is highly dependent on the type of fruit that is incorporated into the puree mix because of variations in the acid, sugar and pectin contents. Thus, the formulation may contain different types of sugars and, sometimes, additives, such as preservatives (VATTHANAKUL *et al*., 2010; QUINTERO *et al*., 2012; DIAMANTE *et al*., 2014) and hydrocolloids (VATTHANAKUL *et al*., 2010; AL-HINAI *et al*., 2012), which are occasionally included to improve the rheological characteristics or to retain the fruit's natural colour (QUINTERO *et al*., 2012; DIAMANTE *et al*., 2014).

Processed fruit products are overall healthy since they are less caloric than traditional snacks and are also an important source of fibre that promotes a sense of satiety (DIAMANTE *et al*., 2014), so many feasibility studies have been developed to assess fruit leather production (CONCHA-MEYER *et al*., 2016). Given that baby kiwifruit (*Actinidia arguta*) suppliers (due to the high perishability of the edible skin of the berries) have not yet found specific strategies for fruit non-compliant with the fresh market standards, and no prior literature has assessed the shelf life stability of baby kiwifruit leather, this work

had two goals. The first was to develop a novel baby kiwifruit leather product (four formulations) presenting a composition as close as possible to the fresh fruit profile, namely, with low sugar and an attractive bright green appearance, and second, to evaluate the physicochemical quality of the developed product during storage.

2. MATERIAL AND METHODS

2.1. Materials

Frozen baby kiwifruit (*A. arguta*) puree added with 10% sucrose was provided by FAR, a fruit processing industry located in Cuneo (Italy). Fresh apples (*Malus domestica*, cv Granny Smith), glucose syrup 33 dextrose equivalent (DE) (SELCA Srl, Italy), sucrose (Reire, Italy), invert sugar (Zucchero & C., Italy), low-methylated pectin powder (Reire), citric acid powder (Reire), fructose powder (Reire) and fresh lemon (*Citrus limon*) were provided by the Department of Agricultural, Forest and Food Sciences (DISAFA Department). Polyamide/polyethylene (PA/PE) pouches, with a thickness of 0.09 mm, a water vapour rate of 5 g m^{-2} d^{-1} (at 23°C, 85% relative humidity), and an oxygen permeability of than 65 cc m^{-2} d^{-1} atm^{-1} (at 23°C, 75%), were used for shelf life studies.

2.2. Formulation development

Preliminary trials were conducted to select the best formulation. The first trial involved thawing 4 kg of frozen puree that was then weighed and combined with varying proportions of the ingredients mentioned above (section 2.1) to generate four different formulations (Table 1). Whole apples were cut and blended into a puree before being added to all formulations, to improve the texture uniformity of the final product. Direct heat treatment with an open copper pan (100°C, for about 30 min) was applied to allow proper mixing of the product during the concentration process. All formulations were concentrated until the soluble solids content (SCC) reached 70% Brix. Afterwards, the formulations were transferred to four separate trays, covered with baking paper to avoid external contamination, and left at 25°C for almost 24 h, to allow gelatinisation. The definitive formulation was chosen by a sensory evaluation panel of ten semi-trained judges, using a 5-point hedonic scale to describe the overall acceptance (1 = "dislike extremely", 5 = "like extremely").

Table 1. Formulations 1, 2, 3, 4 produced on first trial.

Formulation 1	Formulation 2	Formulation 3	Formulation 4
39% baby kiwi puree			
8% Granny Smith apple	38% baby kiwi puree	71% baby kiwi puree	
33% sucrose	58% sucrose	22% Granny Smith apple	43% baby kiwi puree
14% invert sugar	2% low methylated pectin	5% glucose syrup 33 DE	28% Granny Smith apple
3% water	powder	1% citric acid powder	28% glucose syrup 33 DE
2% low methylated pectin	1% citric acid powder	1% water	1% water
powder	1% water		
1% citric acid powder			

2.3. Shelf life test and fruit quality assessments

The best solid formulation (formulation 3A) was cut into 36 leathers (5.0 cm × 5.0 cm × 0.5 cm) that were divided into four samples: two samples were stored at 25°C (room temperature) and the remaining two samples maintained under an accelerated shelf life condition (37°C) in a static air oven for 21 days, respectively. For both shelf life tests, one of the two samples was packaged in awith polyethylene (HDPA/PE) foils, giving rise to four definitive leather samples: Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE). PA/PE packaging was selected due, owing to its transparent surface, in order to that, alloweding the consumers to appreciate the product's appearancespect. At the start of storage (day 0), Baby baby kiwifruit raw puree (raw puree), raw formulation and the leather samples were analysed for SCC, colour, texture parameters, total phenolic content (TPC), antioxidant capacity (AC) and sensory attributes. Leather samples were assessed every 7 days for 21 days, using the same criteria as applied at the start of storage (0 day) plus weight loss.

2.3.1 Weight loss determination

Leather samples were weighed on an electronic balance (SE622, WVR Science Education) (±0.001 g) at day 0 and every 7th day. The average of triplicate measurements was calculated, and the percentage weight loss was computed as follows:

$$\text{Weight loss } (\%) = \frac{(a-b)}{a} \times 100 \quad (1)$$

where a is the initial weight (day 0), and b is the weight recorded every 7 days for 21 days.

2.3.2 Soluble solids content (SCC)

The SSC was determined using an Atago Pocket PAD-1 digital refractometer for raw puree, raw formulation and leather at day 0, and on every 7th day for the leather sample (AOAC 932.12). Ten grams of each leather sample was homogenised, and the SCC (%) of the filtered pulp was measured in triplicate.

2.3.3 Colour and browning index (BI)

Colour was measured in triplicate at day 0 and every 7th day for leather samples, using a Minolta CR-400 chromameter (Konica Minolta Sensing, Inc., Osaka, Japan) pre-calibrated against a standard white tile. Parameters L^*, a^* and b^* were recorded at three different points on each sample, and browning index (BI), defined as brown-colour purity, which is usually used as an indicator of the extent of browning in sugar-containing food products (PEREZ-GAGO *et al*., 2005) was also calculated (QUINTERO RUIZ *et al*., 2012):

$$\text{BI} = \frac{(x - 0.31)}{0.172} \times 100 \quad (2)$$

where x is the chromaticity coordinate calculated from the X, Y, Z tristimulus values, according to the following formulae:

$$x = \frac{X}{(X + Y + Z)} \quad (3)$$

$$X = Xn \times \left[\left(\frac{a*}{500}\right) + \left(\frac{(L*+16)}{116}\right)\right]^3 \quad (4)$$

$$Y = Yn \times \left(\frac{(L*+16)}{116}\right)^3 \quad (5)$$

$$Z = Zn \times \left[\left(\frac{-b*}{200}\right) + \left(\frac{(L*+16)}{116}\right)\right]^3 \quad (6)$$

where Xn, Yn and Zn correspond to white reference tristimulus values of Xn = 91.97, Yn = 93.8 and Zn = 107.98.

2.3.4 Texture parameters

A texture profile analysis (TPA), acquired using a TA.XTplus texture analyser (Stable Micro Systems, USA) (30 kg load cell), was evaluated to determine some of the most important TPA parameters. A compression test was conducted, using a 75-mm aluminium flat probe to a 55% strain, with a pre-test, test and post-test speed of 5, 1 and 5 mm s^{-1}, respectively, and 5 g trigger force.

2.3.5 Total phenolic content (TPC) determination and antioxidant capacity (AC)

The Folin–Ciocalteu (SINGLETON and ROSSI, 1965) and ferric reducing/antioxidant power (FRAP) tests (BENZIE and STRAIN, 1996) were undertaken to assess the TPC and AC, respectively. For both analyses, 10 g of raw puree, 10 g of raw formulation and 10 g of leather, were each extracted with solvent (500 mL methanol, 1.4 mL concentrated HCl and 23.8 mL of deionised water [DW]) for 60 min under reduced light conditions, followed by homogenisation (15 min) to obtain sample extracts.
For the TPC test, the supernatant of each sample was mixed with 30 mL DW and 2.5 mL Folin–Ciocalteu reagent. After 8 min incubation, the mixture was combined with 10 mL of sodium carbonate solution (15% w/v) and incubated at room temperature (25°C) for 2 h. Absorbance was measured at 765 nm (UV-1600 PC spectrophotometer, VWR), and the results expressed as milligrams of chlorogenic acid equivalents (CAE) per 100 g DW (TORRES *et al*., 2015).
The AC assay was carried out using stock solutions (FRAP) containing 0.3 M acetate buffer (3.1 g sodium acetate, 1 L DW and 16 mL acetic acid), 10 mM 2,4,6-tripyridyl-s-triazine solution in 40 mM HCl, and 20 mM $FeCl_3$ solution. Readings of the coloured product (ferrous–tripyridyltriazine complex) were recorded at 595 nm, and the results expressed as mmol Fe^{2+} kg^{-1}. Triplicate measurements were acquired for both assays.

2.3.6 Sensory analysis

A sensory evaluation was undertaken on leather samples at day 0 and 21, by a panel of ten semi-trained judges (aged 25-65 years) from the DISAFA Department. Leathers were brought to 25°C and presented to panellists coded, who appraised the samples for hardness, sweetness, sourness, flavour and overall acceptance, using a 5-point hedonic scale. Panellists' average responses were considered for each attribute.

2.4. Statistical analysis

All the statistical analyses were computed using SPSS 20 for Windows (SPSS, Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was conducted, using the software IBM-SPSS 22 (2015), followed by Tukey's honestly significance difference test ($P<0.05$).

3. RESULTS AND DISCUSSION

3.1. Formulation development

At the end of the first trial, all formulations differed by colour, texture and taste. Formulations 1 and 2 presented the desired firmness and flexibility, due to low-methylation pectin powder addition and its gelling property, which promotes hydrogen bonds between carboxyl groups and water, and ionic bonds with calcium (Ca^{2+}) and other divalent metals (VALENZUELA and AGUILERA, 2015). Although *Actinidia* species are known to have low-pectin content (REDDY *et al*., 2015), formulation 3 with no added pectin powder presented an acceptable texture, afforded by the endogenous pectic compounds of the Granny Smith apples that comprised 22% of the recipe (Table 1) and are known to have a high pectin content (QUINTERO RUIZ *et al*., 2012). Furthermore, the extremely low amounts of glucose syrup (5%) and fructose (5%) in this formulation favoured concentration and the outcome of a desirable texture. The addition of sugar compounds strengthens pectic gels up to a maximum point, after which, such property may reduce (OREGO *et al*., 2014). This behaviour is explained by the presence of low molecular weight compounds, such as glucose, fructose, sucrose and organic acids, which have a low glass transition temperature. These molecules may affect the product texture because they are highly hygroscopic in their amorphous state and show high molecular mobility at temperatures above the glass transition temperature leading to a less stable, sticky and rubbery leather (DIRIM *et al*., 2015). In this work, this undesirable texture was observed only in formulation 4, which had the greatest glucose syrup content (28 %), in the absence of pectin powder.

Besides texture, sourness was also an important factor in determining the overall acceptance of baby kiwifruit leathers. While formulations 1, 2 and 4 presented a sugary and well-balanced taste, respectively, formulation 3 was considered too sour by the panellists. A second trial was, therefore, necessary to optimise baby kiwifruit formulations. Given the aim was to develop a formulation that produced a leather presenting a composition as similar to that of the fresh fruit, only formulations 3 and 4 were modified in the second trial, generating formulations 3A and 4A, respectively. These formulations were preservative-free, with a relatively less sour taste (created by replacing the citric acid powder with fresh lemon juice) and an appropriate texture (Table 2). Formulation 4A had less glucose syrup than formulation 4, to reduce the low molecular weight compounds while the glucose syrup content of formulation 3A was higher than formulation 3, to improve taste. Fructose power was added to both formulations as a sweetener. Finally, formulation 4A incorporated green apple to enhance the gel flavour.

The same concentration process and sensory analysis of the original formulations were applied to formulations 3A and 4A. Although both formulations presented a desirable texture and taste, formulation 4A did not keep a desirable colour, due to its high sugar content, so only formulation 3A was assessed for shelf life quality.

Table 2. Formulations 3A and 4A, produced during second trial obtained by optimization of formulations 3 and 4.

Formulation 3A	Formulation 4A
66% baby kiwi frozen puree	47% baby kiwi frozen puree
22% apple Granny Smith	31% apple Granny Smith
5% glucose syrup 33 DE	10% glucose syrup 33 DE
5% fructose powder	10% fructose powder
1% lemon juice	1% lemon juice
1% water	1% water

3.2. Shelf life test and fruit quality assessments

3.2.1 Weight loss, soluble solids and acidity

Weight loss was statistically different among all leather samples and did not alter during shelf life (Fig. 1), except sample OPE, which demonstrated a slight increment at 21 days.

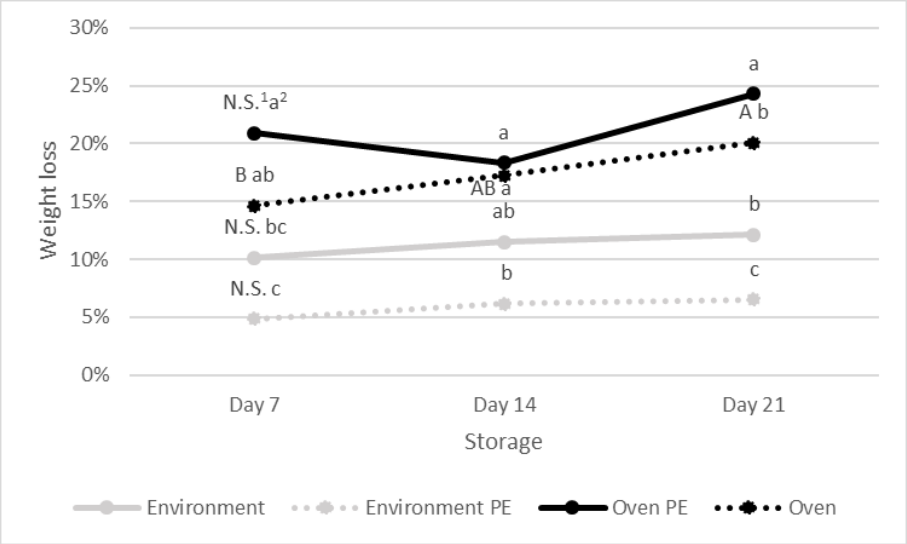


Figure 1. Weight losses (%) of Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

[1]Different capital letters (A–B) show significant differences (P≤ 0.05) within treatment.

[2]Different lower-case letters (a-d) show significant differences among treatments (P ≤ 0.05) for each storage time.

Major weight loss was observed for OPE and O samples. In this work, even though moisture content was not determined, weight loss might be related to the moisture loss during shelf life, as demonstrated in previous trials (DIAMANTE *et al.*, 2014). Enhancement of this parameter might indicate a poor shelf life stability of samples since this trend could change the texture, colour and nutrient composition. Lower weight loss was noted for the PA/PE packaged samples than the unpackaged ones, under both storage conditions, suggesting that PA/PE packaging was efficient to avoid or delay weight loss in OPE, E and PE samples when compared with their unpackaged

counterparts, stored under the same temperature condition. Conversely, the PA/PE packaging was insufficient to circumvent the augmented weight loss trend of OPE sample during simulated long-term shelf life (i.e., the accelerated storage condition).

3.2.2 Soluble solids content (SSC)

Figure 2 shows the SSC of raw puree, raw formulation and leather samples. During leather manufacturing, the addition of glucose syrup increased the SSC from 16% (raw puree) to 25% (raw formulation). Owing to the concentration process, leather samples at day 0 reached 75%, which was higher than the 65% SCC needed for sugar-acid-high-methoxyl pectin gelation (QUINTERO *et al*., 2012). An increase in the SSC is mainly a result of the concentration of the sugars and is important to the gel flavour, as the hydrogen bonded sugar–water molecules will be entrapped in the pectic gel. This phenomenon is possible only at a high sugar concentration of 55 g 100 g^{-1} (TORRES *et al*., 2013). Differences between samples were observed, especially at 14 and 21 days. Oven samples registered significantly higher SSC values than environment samples, irrespective of the storage time. This finding was consistent with the literature that indicates an increase of SSC during storage is a consequence of dehydration and possible conversion of complex carbohydrates into sugars and other soluble components (KUCHI *et al*., 2014). In this work, carbohydrates were probably represented by the starch content since frozen puree was produced from unripe fruit, not suitable for the fresh market, though further analysis of those components should have been done, to provide a better understanding of the product composition. The SSC trend demonstrated in this work also occurred in the simulated long-term storage products (O/OPE samples), corroborating the work of CHAVAN and SHAIK (2015). Differences between packaged and unpackaged samples were also noticed: those with PE packaging presented lower SSC values at the end of shelf life when compared with the unpackaged samples under the same temperature condition. Although, from day 7, the Brix values within samples fluctuated and, at the end of the shelf life, were higher than the initial values (0 day), demonstrating poor stability of the samples, with and without packaging. These results are different from QUINTERO *et al.* (2012), wherein apple leathers packaged with a metalised-plastic material did not show any modifications because of the superior protective property of the packaging material. As far as we know, there are no other shelf life studies of PA/PE packaged fruit leather. Although, the high water vapour transmission rate (5 g m^{-2} d^{-1}) of PA/PE packaging means it is not as efficient at preserving the product's quality as other packaging materials, such as polyester/aluminium/polyamide/polyethylene, which has a water vapour transmission rate of 0.1 g m^{-2} d^{-1} (FU *et al*., 2018).

3.2.3 Colour and browning index (BI)

The colour degradation of leather samples was highly correlated with the evolutions of L^* and BI during shelf life, so only those two parameters are presented in this work. At the end of shelf life, L^* values decreased significantly for O, OPE and E samples, indicating a darkening of the colour. Only EPE samples retained initial L^* values (Table 3), which demonstrates a protective effect of PA/PE packaging but exclusively in simulated short-term shelf life. Parameter L^* expresses the degree of brightness, which can be related to browning reactions and production of dark pigments, such as melanoidins (QUINTERO *et al*., 2012; PATEL *et al*., 2013), caused by oxygen exposure, moisture and an extended shelf life (PATEL *et al*., 2013). Thus, the trend found for OPE when simulating a longer shelf life, illustrated the inadequacy of PA/PE in preventing the quality loss of this sample, as its L^* values were even higher than those of O samples.

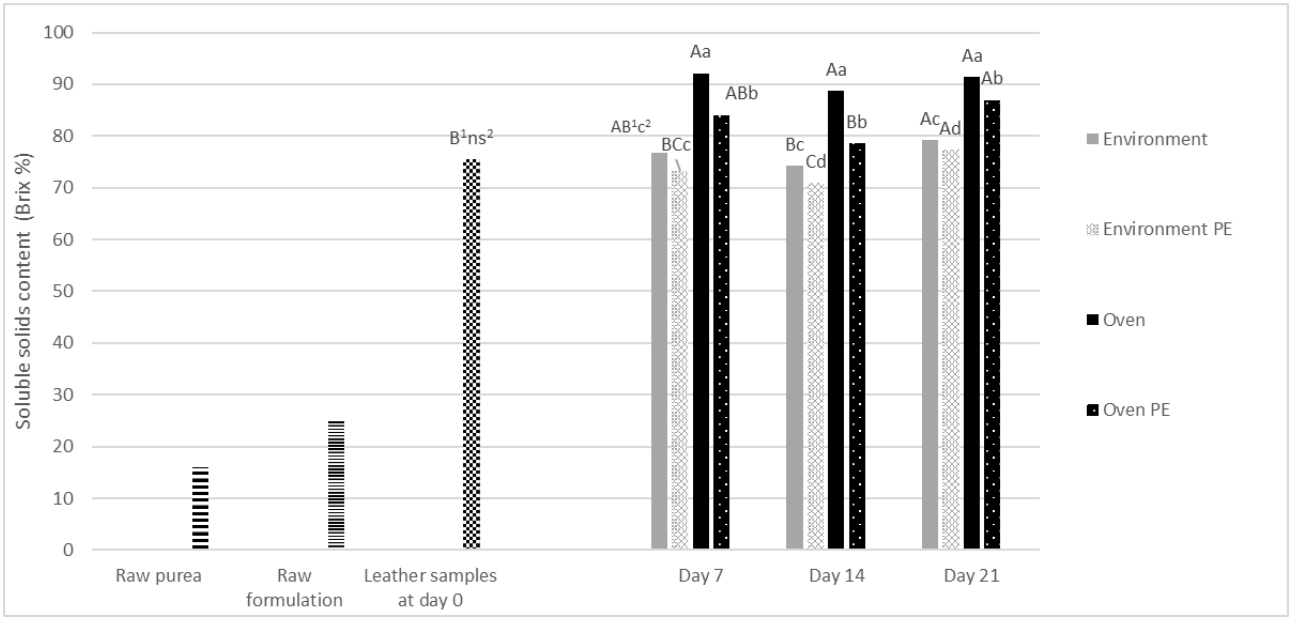


Figure 2. Soluble solids content of raw puree, raw formulation and leather samples at day 0; and brix of Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

[1]Different capital letters (A-C show significant differences ($P \leq 0.05$) within treatment.

[2]Different lower-case letters (a-d) show significant differences among treatments ($P \leq 0.05$) for each storage time.

Table 3. L* evolution of Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

Samples	L*							
	Day 0		Day 7		Day 14		Day 21	
E	26,71	A[1] n.s.[2]	26,04	AB bc	25,85	AB a	25,13	B a
EPE	26,71	N.S. n.s.	26,76	N.S. ab	25,62	N.S. a	25,81	N.S. a
O	26,71	AB n.s.	28,03	A a	25,88	B a	25,68	B a
OPE	26,71	A n.s.	24,78	B c	22,40	C b	23,24	C b

[1]Different capital letters (A-C) in the same row show significant differences ($P \leq 0.05$) within treatment.
[2]Different lower-case letters (a-b) in the same column show significant differences among treatments ($P \leq 0.05$) for each storage time.

The BI (Fig. 3), which is a combination of the *L**, *a** and *b** values, and used as a colour darkening indicator of leathers (QUINTERO RUIZ *et al.*, 2012; TORRES *et al.*, 2013), contrasted with the *L** values and demonstrated colour quality loss only for O samples. For these samples, the BI increased from 54.11 to 89.68. Conversely, E and EPE samples were stable, and OPE displayed only a slight decrease at 14 days of shelf life, probably related to the decline in *b**, from 6.25 to 2.46, as the *b** values of the other samples increased from 6.22 to 4.32 (E), 4.90 (EPE) and 7.52 (O), thereby affecting the estimation of the BI of OPE throughout the shelf life. For this reason, the *L** values were a better indicator of colour quality loss during shelf life. The browning reactions were probably caused by the degradation of ascorbic acid, present at high amounts in the raw material (MCNEILAGE *et al.*, 2001; MCNEILAGE *et al.*, 2004) and probably initially reduced by the

concentration process and preparation of leather samples, but degraded further during shelf life studies. Ascorbic acid is strongly affected by temperature but may incur browning reactions, even at 25°C (PATEL *et al*., 2013). The formation of browning compounds during storage could also be due to the Maillard reaction initiated by the interaction between reducing sugars and amino groups, and ultimately generates brown pigment precursors, such as hydroxymethylfurfural (MASKAN, 2001; LESPINARD *et al*., 2012).

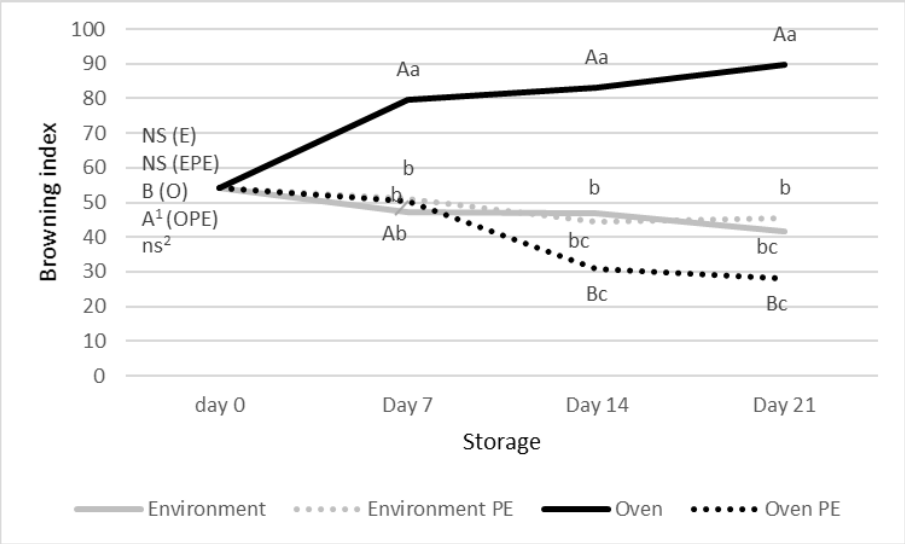


Figure 3. Browning Index evolution for Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

[1]Different capital letters (A-B) show significant differences ($P \leq 0.05$) within treatment.

[2]Different lower-case letters (a-b) show significant differences among treatments ($P \leq 0.05$) for each storage time.

3.2.4 Texture parameters

Adhesiveness and springiness express the rheological and mechanical properties of organic materials (ARANA, 2012) and might indicate the efficiency of the packaging material adopted for stabilising the leather texture, as well as how the endogenous pectin and low molecular weight compounds might affect texture during the concentration process and shelf life. A limited amount of literature has demonstrated the TPA measurements of leather (OREGO *et al*., 2014). Adhesiveness is an important parameter of dried fruit products since it could infer the stickiness of the products (NISHINARI and FANG, 2018). The adhesiveness values (Table 4) of samples enhanced to become closer to 0, indicating a lower sensation of stickiness of samples at the end of shelf life. Samples O and OPE had significantly different values from the other samples during shelf life since they were the least sticky. According to VALENZUELA and AGUILERA (2015), high adhesiveness values of leather at day 0 (-237.68) could be related to the presence of low molecular weight compounds in the leather formulation. It means the high adhesiveness levels found in the current work are probably explained by the use of fructose and glucose syrup 33 DE in the leather formulations. These components contribute a large number of monomers relative to the other ingredients and with lower DE values, lowering the glass transition temperature, which is the temperature at which products may become increasingly sticky. In contrast, glucose syrup 17 DE and 19 DE, used by VALENZUELA and AGUILERA (2015), contribute to relatively high molecular weight polymer

compounds, which can compete with sugars for hydrogen bonding with water, thereby avoiding the product stickiness. During shelf life, the water loss of samples (suggested by the weight loss and SSC concentration) might have reduced the adhesiveness, a phenomenon most frequently apparent for O samples, attributed to the higher temperature condition of 37 versus 25°C. Similarly, Al HINAI *et al*. (2013) verified that adhesiveness was also influenced by the moisture content of leather samples.
Springiness values (Table 5) fluctuated during shelf life. Pectin was found to be the most important factor influencing (increasing) springiness of fruit leather (OREGO *et al*., 2014). Consequently, the decrease in springiness is possibly explained by endogenous pectin gel degradation, leading to the formation of new compounds, such as pectic acids and uronic acid (KUCHI *et al*., 2014) that might have occurred during shelf life. The absence of significant differences between packaged and unpackaged samples for both texture parameters at the end of shelf life reaffirmed the PE packaging was inadequate in maintaining the texture stability of the leathers.

3.2.5 Total phenolic content (TPC) determination and antioxidant capacity (AC)

During leather production, the TPC of the raw puree decreased in the raw formulation but then increased in the leather samples at day 0. The TPC values did not differ significantly among the leather samples or during shelf life (Fig. 4).

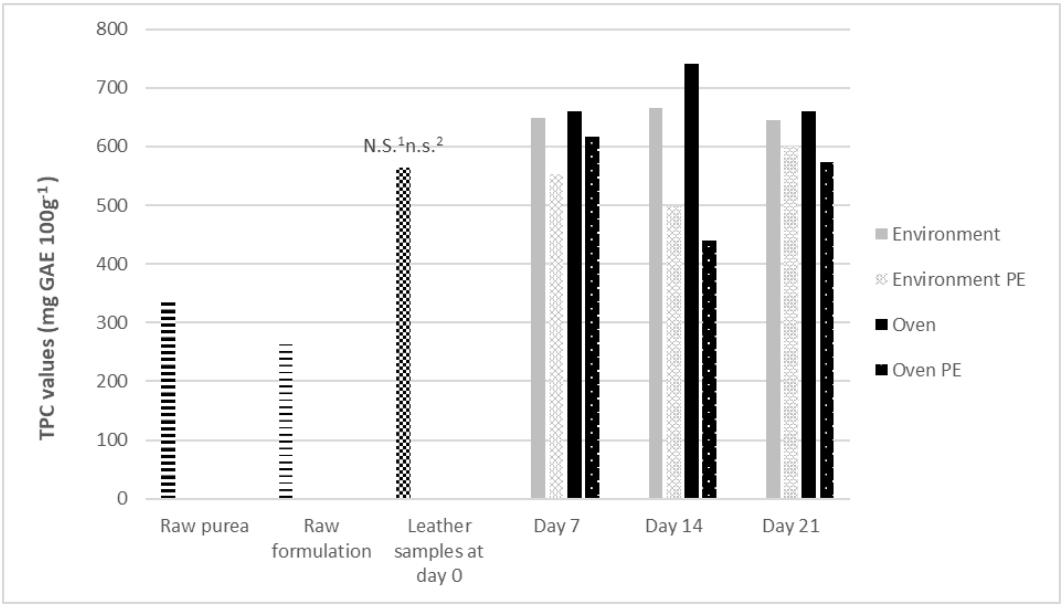


Figure 4. Total polyphenol content of raw puree, raw formulation and leather samples at day start, and total polyphenol content evolution for Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

[1]Capital letters (N.S) show absence of significant differences($P \leq 0.05$) within treatment.

[2]Lower-case letters (n.s.) show absence of significant differences among treatments ($P \leq 0.05$) for each storage time.

The major polyphenol compounds in baby kiwifruit are phenolic acids, tannins and flavanols (LEONTOWICZ *et al*., 2016). In our work, the TPC of raw puree (334.79 mg GAE 100 g^{-1}) was very similar to fresh fruit, as also shown by ZOU *et al*. (2012). Baby kiwifruit is currently marked in Europe as berries, due to its size, commercial availability and

nutraceutical quality. However, even though the TPC values are higher than raw apple puree (LANDL *et al*., 2010), they are still lower than strawberry and blueberry puree (PATRAS *et al*., 2009). Moreover, the addition of apples to the raw puree during the formulation process further lowered the TPC amounts in the raw formulation, thereby deviating the product from the berries nutraceutical profile.

Leather samples at day 0 presented an increased TPC (563.93 GAE 100 g^{-1}), as a result of the concentration process, wherein water loss meant all the compounds present were concentrated, in concurrence with observations by SUNA *et al*. (2014), LUTZ *et al.* (2015) and CONCHA-MEYER *et al*. (2016). In this work, the SSC increased almost three-fold while the TPC values only doubled. Thus, on a dry weight basis, it is suggested that heat treatment would have led to a loss of phenolic compounds, despite the absolute increased values.

No changes within and between leather samples during shelf life affirmed that shelf life and packaging conditions did not affect the TPC, in agreement with QUINTERO *et al*. (2012) and TORRES *et al*. (2013). Although, Fig. 5 emphasises the large variability in the data, especially among O and OPE samples. Considering the substantial fluctuations in the data of almost all analysis, blending of the ingredients must be further improved to ensure homogeneity of the samples.

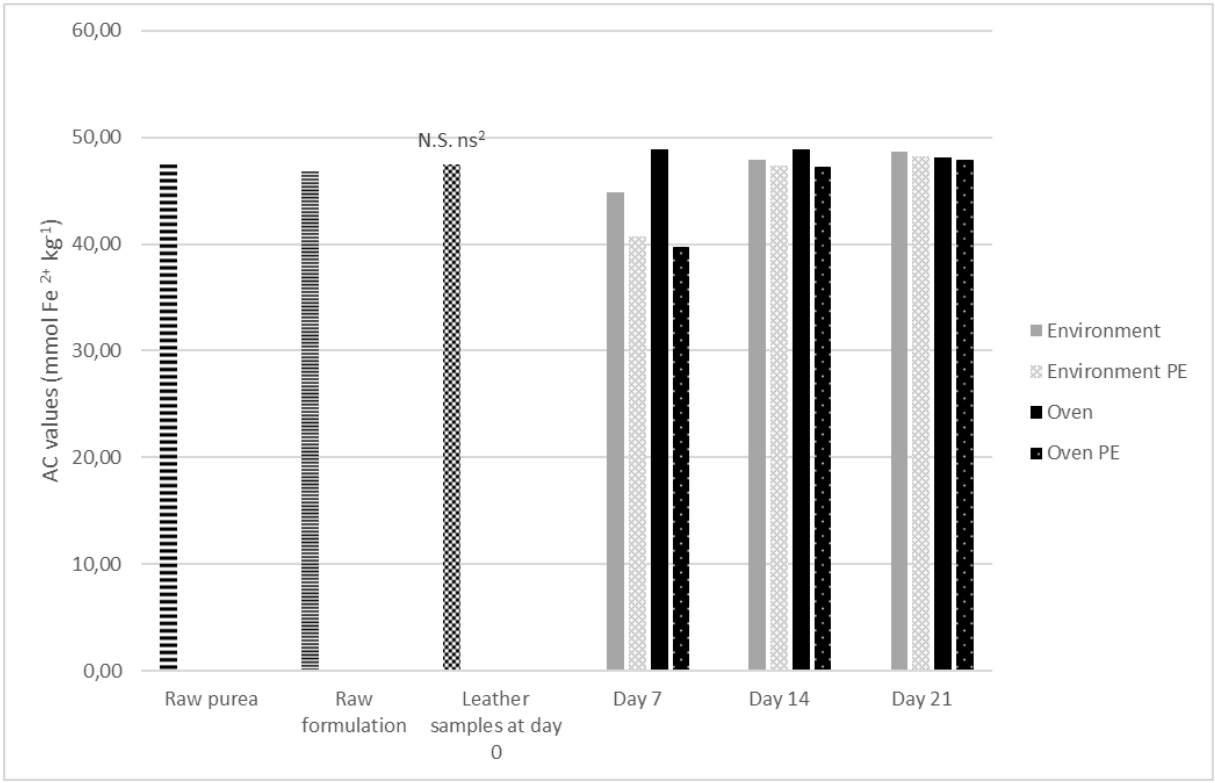


Figure 5. Antioxidant capacity of raw puree, raw formulation and leather samples at day 0. Antioxidant capacity of Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.
[1]Capital letters (N.S).show absence of significant differences ($P \leq 0.05$) within treatment.
[2]Lower-case letters (n.s.) show absence of significant differences among treatments in($P \leq 0.05$) for each storage time.

The AC of raw puree, raw formulation and leather samples at day 0 were similar to each other. This trend was not expected since TPC has been well correlated to AC in fresh, pureed and dried fruits, respectively (KARADAG *et al*., 2009; OREGO *et al*., 2014). The

absence of significant differences within and among samples highlights that PE packaging and storage time did not affect the AC of leathers (Fig. 5). The AC stability after day 7 well correlated with the TPC trend. This pattern was also observed by SACCHETTI *et al.* (2008) and CONCHA-MEYER *et al.* (2016).

3.2.6 Sensory evaluation

Figure 6 illustrates the scores obtained for each leather sample at days 0 and 21. Overall, at day 0 the descriptors hardness (3.2), sweetness (3.2), flavour (4.2) and overall liking (3.5) scored highest while sourness (2.2) scored lowest, for all samples. At the end of storage, flavour decreased, and sourness increased, in all samples. Sweetness decreased for O and OPE samples while hardness remained stable for E and EPE. These results contrasted with the SSC analysis, which showed an increase in SSC content of O and OPE samples, suggesting an increased sweetness. Probably, the SSC increase was accompanied by a similar trend in the acidity content, which was not assessed in this work. Both O and OPE exhibited particularly enhanced hardness values, arising from the intense dehydration of the leathers. Overall acceptance remained stable during the shelf life of E and EPE samples while for O and OPE samples, this descriptor drastically decreased, as a result of hardening.

Table 4. Adhesiveness parameter of TPA for Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

Samples	Adhesiveness (N*s)							
	Day 0		Day 7		Day 14		Day 21	
E	-237,68	B[1] n.s.[2]	-69,91	Aab	-58,02	Aab	-47,98	Aab
EPE	-237,68	B. n.s.	-115,64	Ab	-67,71	Ab	-71,10	Ab
O	-237,68	B n.s.	-1,01	Ab	-21,64	Aa	-1,08	Aa
OPE	-237,68	B n.s.	-70,92	Aab	-59,98	Aab	-17,95	Aa

[1]Different capital letters (A–C) in the same row show significant differences ($P \leq 0.05$) within treatment. [2]Different lower-case letters (a–b) in the same column show significant differences among treatments ($P \leq 0.05$) for each storage time.

Table 5. Springiness parameter of TPA for Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples during storage.

Samples	Springness (s)							
	Day 0		Day 7		Day 14		Day 21	
E	0,49	N.S.[1]n.s.[2]	0,63	N.S. b	0,71	N.S.ab	0,88	N.S.n.s.
EPE	0,49	B n.s.	0,69	A b	0,45	B b	0,72	A n.s.
O	0,49	C n.s.	0,56	B a	0,90	A a	0,88	A n.s.
OPE	0,49	B n.s.	0,56	AB b	0,71	A ab	0,77	A n.s.

[1]Different capital letters (A–C) in the same row show significant differences ($P \leq 0.05$) within treatment. [2]Different lower-case letters (a–b) in the same column show significant differences among treatments ($P \leq 0.05$) for each storage time.

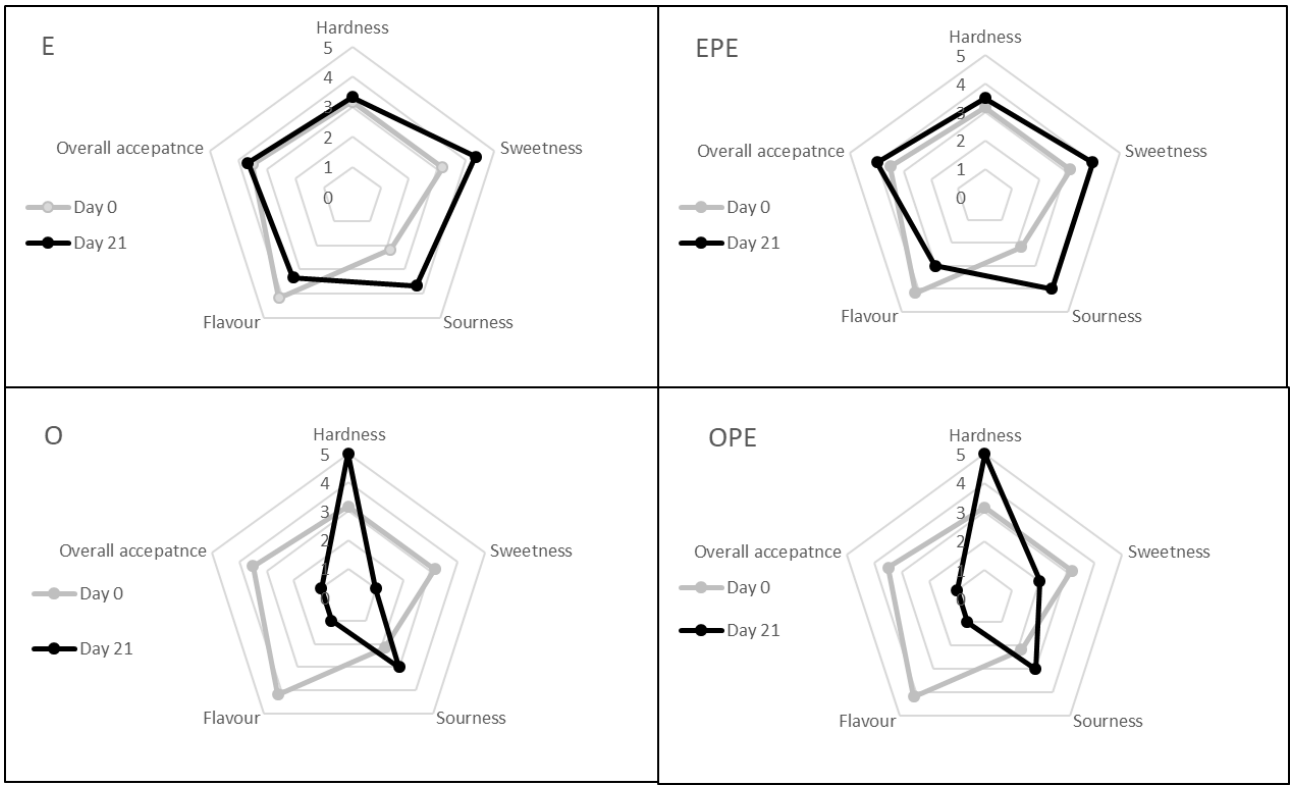


Figure 6. Sensory analysis scores of Environment (E), Environment PE (EPE), Oven (O) and Oven PE (OPE) samples at day 0 and after day 21.

4. CONCLUSIONS

The development of leather formulations could be a promising opportunity to improve the baby kiwifruit supply chain, solving the *A. arguta* losses and offering a processed product that meets the requirements of current consumers seeking healthy and convenient food. Baby kiwifruit formulations considered for obtaining a healthy product with low sugar and a bright green appearance were achieved by adjusting the recipe to offer a balanced content of fructose and glucose syrup. In turn, it reduced the Maillard reaction and favoured formation of a desirable texture, generating a flexible but solid product after the concentration process. In this investigation, preparing baby kiwifruit leather with high fruit content allowed shelf life extension and improved the nutritional properties of the fresh raw material by the concentration-induced reduction in the water content and, consequently, enhanced sugar and polyphenol concentrations. However, the accelerated shelf life experiment (simulating long-term conservation) exposed baby kiwifruit leathers to intense browning reactions and dehydration, leading to the darkening of O and OPE samples and hardening of the texture up to a level that highly compromised the overall acceptance. PA/PE packaging was inconsistent at maintaining leather quality by the end of the shelf life experiment, regardless of a shelf life extension. Additional research is needed to identify new material packaging that is suitable to prevent quality loss during shelf life, and further analysis should be done to characterise the product's composition, including the presence of nutraceutical compounds other than the TPC, such as ascorbic acid and carotenoids.

ACKNOWLEDGEMENTS

This study was supported by the University of Turin-Local Research.

REFERENCES

Al-Hinai K.Z., Guizani N., Singh V., Rahman M. S. and Al-Subhi L. 2013. Instrumental texture profile analysis of date-tamarind fruit leather with different types of hydrocolloids. Food Science and Technology Research 19(4):531.

AOAC. 2005. Official Methods of Analysis of AOAC International (18th ed.). AOAC International, Gaithersburg, Maryland, USA.

Arana, I. 2012. Physical properties of food:novel measurements techniques and applications. Ed. CRC Press. 420 pp.

Benlloch-Tinoco M., Kaulmann, A., Corte-Real J., Rodrigo, D., Martínez-Navarrete N., & Bohn, T. 2015. Chlorophylls and carotenoids of kiwifruit puree are affected similarly or less by microwave than by conventional heat processing and storage. Food Chemistry 187:254-62.

Benzie I. F. F. and Strain J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power":The FRAP assay. Analytical Biochemistry 239:70.

Cano M.P. and Marín M.A. 1992. Pigment composition and colour of frozen and canned kiwi fruit slices. Journal Agricultural Food Chemistry (40):2141.

Chavan U.D. and Shaik J.B. 2015. Standardization and preparation of guava leather. International Journal of Advanced Research in Biological Sciences 2(11):102.

Concha-Meyer A. A., D'ignoti V., Saez B. Diaz RI. and Torres C.A. 2016. Effect of storage on the physical-chemical and antioxidant properties of strawberry and kiwi leathers. Journal of Food Science 81(3):569.

Diamante L.M., Bai X. and Busch J. 2014. Fruit leathers:method of preparation and effect of different conditions on qualities. International Journal of Food Science 2014:1.

Dirim N., Ergum K. and Caliskan G. 2015. Freeze drying of kiwi (*Actinidia deliciosa*) puree and the powder properties. Journal of Food Science (27):385.

Federici F., Fava F., Kalogerakis K, Mantzavinos D. 2009. Valorisation of agro-industrial y-products, effluents and wastes:concept, opportunities and the case of olive oil sector. Journal of Chemical Technology and Biotechnology 84:895.

Fu, X., Xing, S., Xiong, H., Min, H., Zhu, X., He, J., Feng, J., Mu, H., 2018. Effects of packaging materials on storage quality of peanut kernels 1-10.

Girgenti V., Massaglia S., Mosso A., Peano C. and Brun F. 2016. Exploring perceptions of raspberries and blueberries by Italian consumers. Sustainability 8:1027.

Karadag A., Ozcelik B. and Saner S. 2009. Review of methods to determine antioxidant capacities. Food Analytical Methods 2(1):41.

Kaya A., Aydin O. and Kolayli S. 2010. Effect of different drying conditions on the vitamin C (ascorbic acid) content of Hayward kiwifruits (*Actinidia deliciosa* Planch). Food and Bioproducts Processing (88):165.

Kuchi V. S., Gupta R., and Tamang S. 2014. Standardization of recipe for preparation of guava jelly bar. Journal of Crop and Weed 10(2):77-81.

Landl A., Abadias M., Sárraga C., Viñas I. and Picouet P.A. 2010. Effect of high pressure processing on the quality of acidified Granny Smith apple purée product. Innovative Food Science and Emerging Technologies 11(4):557.

Laufenberg G., Kunz B. and Nystroem M. 2003. Transformation of vegetable waste into value added products: (A) the upgrading concept (B) practical implementations. Bioresource Technology 87:167.

Leontowicz H., Leontowicz M., Latocha P., Jesion I., Park Y., Katrich E., Barash D., Nemirovski A. and Gorinstein S. 2016. Bioactivity and nutritional properties of hardy kiwi fruit Actinidia arguta in comparison with *Actinidia deliciosa* 'Hayward' and *Actinidia eriantha* 'Bidan'. Food Chemistry (196):281.

Lespinard A.R., Bambicha R.R. and Mascheroni R.H. 2012. Quality parameters assessment in kiwi jam during pasteurization. Modelling and optimization of the thermal process. Food and Bioproducts Processing 90(4):799.

Lutz M., Hernández J. and Henríquez C. 2015. Phenolic content and antioxidant capacity in fresh and dry fruits and vegetables grown in Chile. CyTA-Journal of Food (6337):37.

Ma E., Cervera Q. and Sánchez G.M.M. 1993. Integrated utilization of orange peel. Bioresource Technology 44:61.

Maskan M. 2001. Drying, shrinkage and rehydration characteristics of kiwifruits during hot air and microwave drying. Journal of Food Engineering 48(2):177.

McNeilage M.A., Beatson R.A. and Macrae E.A. 2001. Patent US 2001/0042258 P1, United States Plant Patent, pp. 1-3.

Mcneilage M.A., Beatson R.A. and Macrae E. A. 2004. Patent N° US PP14,625 P3 United States Plant Patent, pp. 1-6.

Murphy A., Meehan H.F., O'Reilly S. and Bogue J. 2002. A ten-year Delphi forecast of the Irish speciality food market. The International Food and Agribusiness Management Review, 14:67.

Nishinari K., Fang Y. 2018. Perception and measurement of food texture:solid foods. Journal of Texture Studies 49:160.

Orego C., Salgado N. and Botero C. 2014. Developments and trends in fruit bar production and characterization. Critical Reviews in Food Science and Nutrition, 54(1):84.

Park Y.S., Ham K.S., Park Y.K., Leontowicz H., Leontowicz M., Namieśnik J., Katrich E. and Gorinsteine S. 2016. The effects of treatment on quality parameters of smoothie-type 'Hayward' kiwi fruit beverages. Food Control 70:221.

Pathak P.D., Mandavgane S.A. and Kulkarni B.D. 2017. Fruit peel waste:characterization and its potential uses. Current Science 113:444.

Patel K. N., Modi R.B., Patel H.G. and Aparnathi K.D. 2013. Browning, its chemistry and implications in dairy products:a review. Indo-American Journal of Agricultural and Veterinary Sciences, 1.

Patras A., Brunton N.P., Da Pieve S. and Butler F. 2009. Impact of high pressure processing on total antioxidant activity, phenolic, ascorbic acid, anthocyanin content and colour of strawberry and blackberry purées. Innovative Food Science and Emerging Technologies 10(3):308.

Perez-Gago M.B., Serra M., Alonso M., Mateos M. and del Río M.A. 2005. Effect of whey protein- and hydroxypropyl methylcellulose-based edible composite coatings on color change of fresh-cut apples. Postharvest Biology and Technology 36(1):77.

Porat R., Lichter A., Terry Leon A., Harker R. and Buzby J. 2018. Postharvest losses of fruit and vegetables during retail and in consumers' homes:Quantifications, causes, and means of prevention. Postharvest Biology and Technology 139:135.

Quintero Ruiz N.A., Demarchi M.D., Facundo Massolo J., Rodoni L.M. Giner S.A. 2012. Evaluation of quality during storage of apple leather. Food Science and Technology, (47):495.

Reddy D.K., Samala P., and Singh J.K. 2015. Formulation and evaluation of preserved products using an under-exploited fruit [Kiwi Fruit (*Actinidia Deliciosa*)]. International Journal of Basic and Applied Biology, 2:205.

Sharma S.K., Chaudhary S.P., Rao V.K., Yadav V.K. and Bisht T.S. 2013. Standardization of technology for preparation and storage of wild apricot fruit bar. Journal of Food Science and Technology, 50(4):784.

Simal S., Femenia A., Garau M.C. and Rosselló C. 2005. Use of exponential, page's and diffusional models to simulate the drying kinetics of kiwi fruit. Journal of Food Engineering, 66(3):323..

Singleton V.L. and Rossi J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16(3):144.

Sacchetti G., Cocci E., Pinnavaia G., Mastrocola D. and Dalla-Rosa M. 2008. Influence of processing and storage on the antioxidant activity of apple derivatives. International Journal of Food Science & Technology (43):797.

Suna S., Tamer C.E., Incedayi B., Sinir G.Ö. Çopur Ö.U. 2014. Impact of drying methods on physicochemical and sensory properties of apricot pestil. Indian Journal of Traditional Knowledge 13(1):47.

Torres, C.A., Romero L.A. and Diaz R.I. 2013. Quality and sensory attributes of apple and quince leathers made without preservatives and with enhanced antioxidant activity. LWT - Food Science and Technology 62(2):996-1003.

Valenzuela C. and Aguilera J. M. 2015. Effects of different factors on stickiness of apple leathers. Journal of Food Engineering 149:51.

Vatthanakul, S., Jangchud, A., Jangchud, K., Therdthai, N. and Wilkinson, B. 2010. Gold kiwifruit leather product development using Quality function deployment approach. Food Quality and Preference 21(3):339-345.

Zou L., Wang Z., Fan Z., Tian S. and Liu J. 2012. Evaluation of antioxidant and antiproliferative properties of three Actinidia (Actinidia kolomikta, Actinidia arguta, Actinidia chinensis) extracts in vitro. International Journal of Molecular Science (13):5506.

Paper Received September 10, 2018 Accepted February 25, 2019

PAPER

DRYING OF LIME SLICES BY MICROWAVE AND COMBINED MICROWAVE-CONVECTIVE METHODS

N. IZLI*[1], O. TASKIN[1] and G. IZLI[2]

[1]Department of Biosystems Engineering, Faculty of Agriculture, University of Bursa Uludag, Gorukle Campus, 16059, Bursa, Turkey

[2]Department of Food Engineering, Faculty of Natural Sciences, Architecture and Engineering, University of Bursa Technical, 16190, Bursa, Turkey

*Corresponding author: Tel.: +902242941604; Fax: +902242941402

E-mail address: nazmiizli@gmail.com

ABSTRACT

This research deals with lime samples and aims to investigate the impacts of microwave and combined microwave-convective drying applications. According to the statistical outcomes, models of Midilli *et al*. and Page were discovered to give more suitable predictions than the other models. Increasing level of both temperature and microwave power, induced a significant reduction in the drying interval while increasing D_{eff} values. Drying experiment at power 90 W and temperature 55°C ensured the best values in color parameter of a* (greenness) and energy consumption. Experiments made with using different techniques will help to select the appropriate drying technique for the relevant sectors.

Keywords: activation energy, color, drying kinetics, effective moisture diffusivity, specific energy consumption

1. INTRODUCTION

The Persian lime (*Citrus latifolia* tanaka) belongs to the family of Rutaceae (citrus family) and also named as 'Shiraz Limoo', 'Bearss lime', and 'Tahiti lime', which is mainly grown in the Gulf Coast of Mexico (LAMBERT *et al*., 2015; SALIH, 2015; ARREDONDO *et al*., 2015). It is an economically significant horticultural crop (KAEWSUKSAENG *et al*., 2015). The major flavor components are neural, citral, geranial, α-terpineol, β-bisabolene, β-pinene, p-cymene, 1,4-cineole, 1,8-cineole, and 4-terpineol (YADAV *et al*., 2004). It had been used traditionally to treat sinusitis (SALIH, 2015) and stomachache. Also, the cleansing action of mesocarp usage as facial scrub helps in prevention of pimples. Besides, the rind is burnt against mosquitoes in some homes (AIBINU *et al*., 2007). Human benefit from lime to extract its juice, to prepare beverages, concentrates, squashes and other derivative products such as pectin and citric acid etc. (YADAV, 2004).

To preserve food, drying is an ancient and prevalent technique. In the food industry, the primary reason to dry food is to reduce packaging, storage, and transportation costs by downsizing end products in terms of both volume and weight. Hence, in the food industry, developing economical drying techniques have become a prominent research topic (PHOUNGCHANDANG *et al*., 2008; TASIRIN *et al*., 2014).

Long drying intervals or high temperatures in conventional air drying may distort the dried product (DIAZ *et al*., 2003). Many studies have shown that drying with microwave energy usage has many conveniences like shorter drying interval, higher energy efficiency, less space, and faster start-up and shut-down periods (GUO and ZHU, 2014). However, inhomogeneous distribution in the microwave cavity creates an uneven heating problem. To circumvent a number of the disadvantages of single microwave or hot-air dryers, combine microwaves with hot air one is another methodology (DARVISHI *et al*., 2014).

Microwave and/or microwave–air combined driers were carried out by several researchers to illustrate drying attributes of many agricultural products, for example, parsley (SOYSAL, 2004), peach (WANG and SHENG, 2006), mint leaves (ÖZBEK and DADALI, 2007), spinach (KARAASLAN and TUÇER, 2008), tomato pomace (AL-HARAHSHEH *et al*., 2009), onion slices (ARSLAN and ÖZCAN, 2010), seedless grapes (KASSEM *et al*., 2011), sage leaves (ESTURK, 2012), chili flesh (ZHAO *et al*., 2013), okra (KUMAR *et al*., 2014) and apple slices (ZAREIN *et al*., 2015). The aim of conducted research to scrutinize the thin-layer drying behavior of lime slices in both microwave and microwave-convective drying with existing thin-layer drying models. Also, effective moisture diffusivity, activation energy, color, energy consumption and specific energy consumption of the lime slices were studied.

2. MATERIAL AND METHODS

2.1. Drying equipment and drying procedure

Fresh lime samples were obtained from a local grocery one day before the experiments begin and kept at 4±0.5°C temperature. Samples had an average diameter of 40±0.08 mm and were sliced to 5±0.03 mm thickness by using a food slicer (Nicer Dicer, China). In the beginning, the moisture content of the fresh lime samples was identified to be 5.13 (g H_2O · g d.m.$^{-1}$) on a dry basis (d.b.) by using an oven drying at 105°C for 24 hours (ED115 Binder, Tuttlingen, Germany).

The drying experiments were conducted at room temperature. A microwave-convective oven (Whirlpool AMW 545, Italy) that works at ~230 V, 50 Hz with 2450 MHz frequency.

The system worked in microwave mode at 90 W and 160 W microwave output power levels and in combined microwave-convective mode at 90 W – 55 °C, 90 W – 65 °C, 90 W – 75 °C, 160 W – 55 °C, 160 W – 65 °C, and 160 W – 75 °C microwave output power and temperature combinations with 1 m s^{-1} air flow. All drying experiments were performed on a 400 mm diameter and 210 x 450 x 420 mm sized glass plate that rotates at the base of the oven. In order to figure out mass, a digital balance (Baster, Istanbul, Turkey) having ±0.01 g accuracy was positioned below the microwave oven device (Fig. 1). The loss of the moisture of the lime samples was noted down during drying interval in every 5 minutes. For each lime sample (100 g), these were applied three times and their mean was figured out.

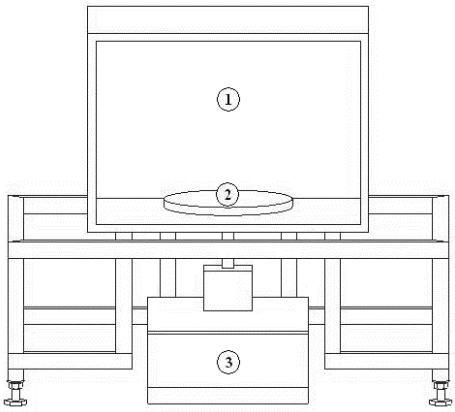


Figure 1. Schematic diagram of experimental microwave-convective drying system (microwave-convective drying chamber (1), rotating glass tray (2) and balance (3)).

2.2. Mathematical modelling of drying data

The data on moisture content were transformed into the moisture ratio (MR) and adapted by the aid of ten thin-layer drying models (Table 1).

Table 1. Selected thin layer drying models used to mathematically model the lime drying kinetics.

No	Model name	Model	References
1	Henderson and Pabis	$MR = a\exp(-kt)$	BHATTACHARYA *et al.* (2015).
2	Newton	$MR = \exp(-kt)$	SHARMA *et al.* (2005)
3	Page	$MR = \exp(-kt^n)$	BHATTACHARYA *et al.* (2015).
4	Logarithmic	$MR = a\exp(-kt) + c$	UNAL and SACILIK (2011)
5	Two Term	$MR = a\exp(-k_0 t) + b\exp(-k_1 t)$	SU *et al.* (2015)
6	Two Term Exponential	$MR = a\exp(-kt) + (1-a)\exp(-kat)$	SHARMA *et al.* (2005)
7	Wang and Singh	$MR = 1 + at + bt^2$	SU *et al.* (2015)
8	Diffusion Approach	$MR = a\exp(-kt) + (1-a)\exp(-kbt)$	UNAL and SACILIK (2011)
9	Verma *et al.*	$MR = a\exp(-kt) + (1-a)\exp(-gt)$	OMOLOLA *et al.* (2014)
10	Midilli *et al.*	$MR = a\exp(-kt^n) + bt$	UNAL and SACILIK (2011)

The moisture ratio was calculated with the equation presented below:

$$MR = \frac{M_t - M_e}{M_o - M_e} \quad (1)$$

Above, M_t represents the moisture content (g water g dry matter^{-1}) at a particular time, M_0 represents the initial moisture content (g water g dry matter^{-1}), represents the equilibrium moisture content (g H_2O · g d. m.$^{-1}$). M_e values are proportionately less than M_t or M_0 values. In consequence, the moisture ratio was made simpler as presented below (ARUMUGANATHAN *et al*., 2009):

$$MR = \frac{M_t}{M_o} \quad (2)$$

2.3. Determination of effective moisture diffusivity

Drying of agricultural products in a falling rate period is embedded into a mass-diffusion equation in accordance with the second law of Fick on diffusion is presented in Eq. (3) below:

$$\frac{\partial M}{\partial t} = \nabla[D_{eff}(\nabla M)] \quad (3)$$

So as to ascertain the moisture ratio in Eq. (4), benefiting from the second law of Fick on unsteady state diffusion provided in Eq. (3) is possible. Crank (1975) put forward the clarification of the diffusion equation for an infinite slab and so uniform moisture distribution at the beginning, negligible shrinkage and external resistance, and constant diffusivity was assumed to be as follows:

$$MR = \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left(-\frac{(2n+1)^2\pi^2 D_{eff}t}{4L^2}\right) \quad (4)$$

Where: D_{eff} symbolizes effective moisture diffusivity in terms of ($m^2 \cdot s^{-1}$); t symbolizes an interval in terms of (s); L symbolizes half-thickness of samples in terms of (m), and n symbolizes a positive integer.
For long drying intervals, only the first term in Eq. (4) is significant and the equation is simplified to the one stated below:

$$MR = \frac{8}{\pi^2}exp\left(-\frac{\pi^2 D_{eff}t}{4L^2}\right)$$

Equation (5) can be articulated in a logarithmic format as can be seen below:

$$\ln(MR) = \ln\left(\frac{8}{\pi^2}\right) - \left(\frac{\pi^2 D_{eff}t}{4L^2}\right) \quad (5)$$

In Eq. (6), effective moisture diffusivity values were identified by plotting experimental drying data in ln (MR) versus drying interval. This plot generates a direct line with a slope calculated below (DOYMAZ *et al*., 2015):

$$Slope = \left(\frac{\pi^2 D_{eff}}{4L^2}\right) \quad (6)$$

2.4. Computation of activation energy

On the grounds that temperature inside the microwave dryer cannot be gauged precisely, modification of the revised Arrhenius equation is used to find the activation energy. In this technique, it is deemed that the effective moisture diffusion and the microwave output power ratio are associated with the sample weight (m p^{-1}) rather than air temperature. In this direction, Eq. (7) can be utilized in an efficient way, as noted below (ÖZBEK and DADALI, 2007):

$$D_{eff} = D_0 \left(-\frac{E_a \mathrm{m}}{P}\right) \quad (7)$$

above E_a symbolizes the activation energy in terms of (W g^{-1}), m symbolizes the mass of raw sample in terms of (g), P symbolizes the microwave power in terms of (W), and lastly, D_0 symbolizes the pre-exponential factor in terms of (m^2 s^{-1}).

2.5. Energy and specific energy consumption

The experimental apparatus was plugged into the electricity via a digital electric counter (Kaan, Type 101, Turkey) that has a precision of 0.01 kWh. With the use of the counter, the total energy consumption (E_t) was measured for whole drying process (KOWALSKI and PAWŁOWSKI, 2011). Energy consumption to dry 100 g fresh lime sample was obtained by applying Equation 8. The E_{kg} and W_0 symbolize the specific energy needed and the primary mass of the sample, respectively (MOTEVALI *et al*., 2012).

$$E_{kg} = \frac{E_t}{w_0} \quad (8)$$

2.6. Colour measurement

The flesh color of fresh and dried lime samples was determined with Hunterlab Color Analyzer (MSEZ-4500L, Reston, Virginia, USA) in the *L*, *a*, *b* color scale. Color measurements were conveyed in a three-dimensional color space namely *L**, *a**, and *b**, where *L** indicates darkness/lightness value, *a** indicates redness value (if positive) and greenness value (if negative), and *b** indicates the yellowness value (if positive) and blueness value (if negative). On the other side L_0^*, a_0^* and b_0^* are color parameters for fresh lime samples. After the calibration of colorimeter against a standard white surface and black one, six replicate measurements were performed for each sample and *L**, *a**, *b**, L_0^*, a_0^* and b_0^* color values were recorded. To illustrate the color changes, Chroma (*C*), Hue angle (α), and total color variance (ΔE) values were defined by the following equations (ARGYROPOULOS *et al*., 2011):

$$C = \sqrt{(a^2 + b^2)} \quad (9)$$

$$\alpha = \tan^{-1}(\frac{b}{a}) \tag{10}$$

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \tag{11}$$

2.7. Statistical analysis

This research was realized by the aid of randomized plots factorial design of experimental type. In the course of calculation of the inspected items, three replicates were utilized. While interpreting the outcomes, MATLAB (MathWorks Inc., Natick, MA) and JMP (Version 7.0, SAS Institute Inc., Cary, NC, USA) software technologies were employed. Significance levels of mean differences were tested and the least significant difference (LSD) test resulted in a 5% significance level. It has been determined that the most convenient model that expresses the drying attributes of lime samples in a thin layer is the one that has lowest reduced chi-squared (χ^2) value, lowest root mean square error (RMSE) value and the highest coefficient of determination (R^2) (ARUMUGANATHAN *et al*., 2009). The statistical figures mentioned above are formulated below:

$$\chi^2 = \frac{\sum_{i=1}^{N} (MR_{\exp,i} - MR_{pre,i})^2}{N - n} \tag{12}$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} (MR_{pre,i} - MR_{\exp,i})}{N}} \tag{13}$$

where:
$MR_{exp,i}$, symbolizes the experimental moisture ratio for test number i,
$MR_{pre,i}$, symbolizes the estimated moisture ratio for test number i,
N symbolizes the observation number,
n symbolizes the number of constants in the drying model (DOYMAZ and ISMAIL, 2011).

3. RESULTS AND DISCUSSION

3.1. Drying kinetics of dried lime

Fig. 2 illustrates the drying curves of the lime samples that are dried under distinct microwave and microwave-convective drying settings. Drying phase of lime samples took 225, 120, 130, 115, 100, 105, 85 and 75 minutes at 90W, 160W, 90 W – 55°C, 90 W – 65°C, 90 W – 75°C, 160 W – 55°C, 160 W – 65°C, and 160 W – 75°C, respectively. Outcomes of the experiments implied that drying intervals of lime samples dried by microwave-convective technique at 90 W – 75°C and 160 W – 75°C reduced 55.6% and 41.7% in comparison to only 90 W and 160 W microwave powers, respectively. The outcomes stated above are in good harmony with former researches. GOWEN *et al*. (2008), MOHANTA *et al*. (2014), CHAYJAN *et al*. (2015) found that the use of combined microwave convective technique ensured considerable time savings in drying interval for soybean, ginger and hawthorn

samples, respectively. In addition, the average total drying interval at 160 W – 75°C become shorter by 46.2% as against the drying interval at 90 W – 55°C. It can be said that increasing the microwave power level and temperature level ended in a significant reduction in the drying interval of lime samples in combined microwave-convective drying technique. In agreement to our study, KARAASLAN and TUNCER (2010) combined fan-assisted convection (100, 180 and 250°C) and microwave (180 and 540 W) drying and the time to reduce the moisture content of banana slices from the initial 80 % (w.b.) to the final 15 % (w.b.) was highest at 180 W – 100°C and lowest at 540 W – 250°C, respectively. Similarly, SADEGHI *et al*. (2013) were measured drying time of lemon slices about 80, 78 and 73 minutes, respectively, when applying 0.97 W g^{-1} microwave power at 50, 55 and 60°C convective drying.

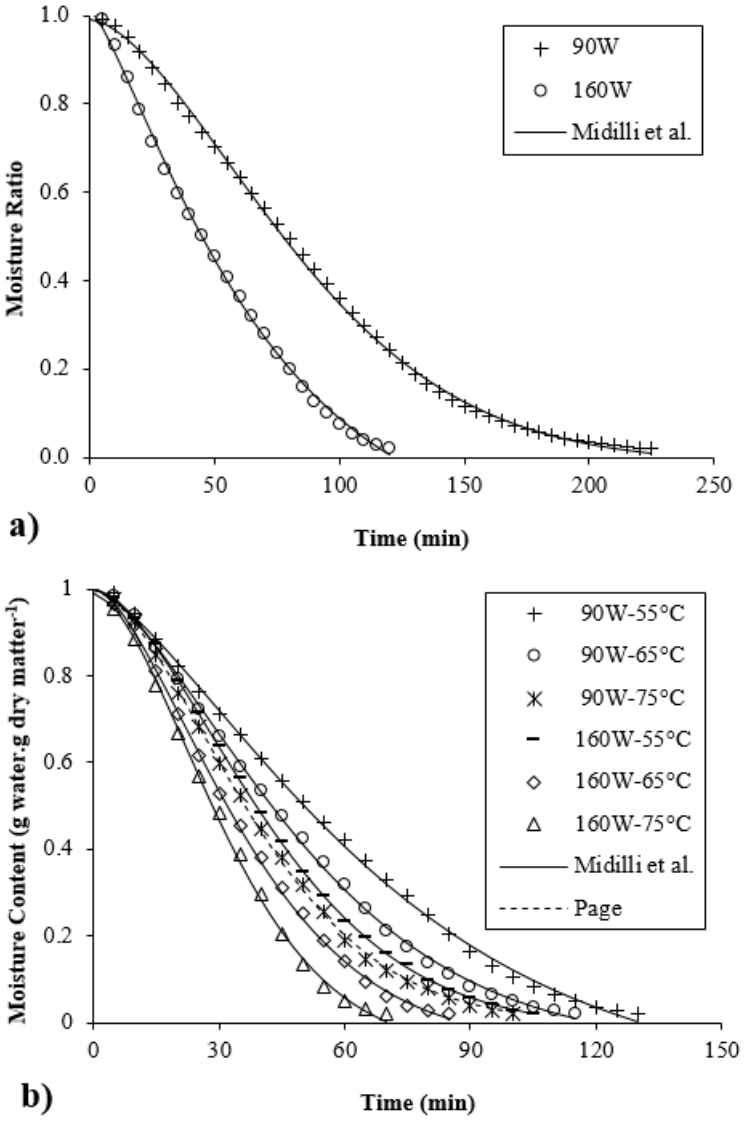


Figure 2. A comparison of the experimental and theoretical moisture ratios predicted by the Midilli *et al*. and Page models at specific drying times under selected drying conditions (microwave (a) and microwave-convective (b)).

3.2. Fitting of drying curves lime

Table 2 reveals the statistical analysis values obtained from the 10 distinct thin layer drying models, covering the drying model coefficients and the comparison criteria that are benefited from to evaluate the congruous quality, R^2, *RMSE*, and χ^2. For the statistical parameters, the values varied between 0.9143 and 0.9998 for R^2, between 0.0042 and 0.1037, 0.1659 x 10^{-4} and 106.3810 x 10^{-4} for *RMSE* and χ^2, respectively. In Table 3, the Page *et al*. model presented greater R^2 and smaller *RMSE* and χ^2 values in comparison to other thin-layer drying models for microwave-convective combinations of 90W – 75°C, while the Midilli *et al*. model displayed more suitable statistical values for all other drying settings. In all cases, values of R^2, *RMSE* and χ^2 in the Midilli *et al*. and Page models were ranged from 0.9984 to 0.9998, 0.0042 to 0.0143 and 0.1659 x 10^{-4} to 1.8507 x 10^{-4}; and 0.9958 to 0.9997, 0.0046 to 0.0211 and 0.1956 x 10^{-4} to 4.3222 x 10^{-4}, in return. Based on the statistical values, the Midilli *et al*. model was the most convenient one for all drying conditions tested, except for drying with the microwave-convective combinations of 90 W – 75°C where the Page model is the best one. Fig. 2 presents the comparison between the predicted values and experimental ones using the most convenient models with drying interval at chosen drying conditions of lime. As can be seen from these figures, the Midilli *et al*. and Page models slightly over-predicted or under-predicted the experimental values, but they are quite close to the experimental results. Accordingly, it could be deduced that models of Midilli *et al*. and Page sufficiently explained the thin layer drying attributes of lime under the experimental conditions. Similar outcomes have been stated by UNAL *et al*. (2011), BHATTACHARYA *et al*. (2015) and SU *et al*. (2015) for Midilli *et al*. and SHARMA *et al*. (2005), DOYMAZ and İSMAIL (2011), THERDTHAI *et al*. (2011) for Page model.

Table 2. Estimated values of coefficients and statistical analyses obtained from various thin layer drying models for drying of lime using microwave (90 and 160 W) method.

No	90 W				160 W			
	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$
1	a=1.164; k=0.01247	0.9632	0.0641	40.4278	a=1.123; k=0.02029	0.9695	0.0566	32.0739
2	k=0.01078	0.9374	0.0836	69.1906	k=0.01803	0.9522	0.0709	50.5494
3	k=0.000555; n=1.641	0.9990	0.0104	1.0139	k=0.002511; n=1.483	0.9958	0.0211	4.3222
4	a=1.403; k=0.00734 c=-0.3089	0.9888	0.0354	12.2592	a=1.505; k=0.01025 c=-0.4523	0.9969	0.0181	3.1788
5	a=-23.08; k_o=0.02414 b=24.08; k_1=0.02306	0.9955	0.0224	4.8956	a=-14.31; k_o=0.03806 b=15.32; k_1=0.03578	0.9929	0.0274	7.3938
6	a=0.0000627; k=171.7	0.9360	0.0845	70.7869	a=0.0000608; k=296.6	0.9501	0.0725	52.7683
7	a=-0.00774; b=0.0000141	0.9861	0.0394	15.2627	a=-0.01276; b=0.0000362	0.9954	0.0221	4.8176
8	a=-21.79; k=0.02327 b=0.9567	0.9943	0.0252	6.0009	a=-30.8; k=0.03781 b=0.9704	0.9934	0.0264	6.5723
9	a=-33.45; k=0.02396 g=0.02321	0.9956	0.0221	4.7390	a=-13.74; k=0.03845 g=0.036	0.9934	0.0265	6.8890
10	a=0.9905; k=0.0005298 n=1.645; b=-0.0000446	0.9992	0.00946	0.8400	a=1.016; k=0.005021 n=1.272; b=-0.000886	0.9987	0.0119	1.2692

Table 3. Estimated values of coefficients and statistical analyses obtained from various thin layer drying models for drying of lime using combined microwave-convective method.

No	90 W – 55°C				90 W – 65°C				90 W – 75°C			
	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$
1	a=1.135; k=0.01856	0.9609	0.0651	43.1892	a=1.142; k=0.02208	0.9617	0.0659	42.9539	a=1.149; k=0.02614	0.9592	0.0696	49.2828
2	k=0.01632	0.9404	0.0804	66.3224	k=0.01936	0.9402	0.0824	67.7189	k=0.02285	0.9367	0.0867	76.2392
3	k=0.001448; n=1.58	0.9962	0.0202	3.9792	k=0.001695; n=1.606	0.9981	0.0145	1.8601	k=0.001792; n=1.66	0.9996	0.0073	0.6060
4	a=1.637; k=0.008336 c=-0.5794	0.9951	0.0231	5.2789	a=1.511; k=0.01135 c=-0.4383	0.9919	0.0302	8.4956	a=1.461; k=0.01441 c=-0.3763	0.9873	0.0388	15.2013
5	a=-46.82; k_o=0.0353 b=47.82; k_1=0.03455	0.9919	0.0297	8.8554	a=-32.98; k_o=0.04255 b=34; k_1=0.04126	0.9943	0.0255	6.1631	a=-35.94; k_o=0.05143 b=36.95; k_1=0.04992	0.9965	0.0203	4.4728
6	a=0.0000609; k=267.5	0.9379	0.0819	68.9980	a=0.0000627; k=308.7	0.9374	0.0842	70.8216	a=0.0000636; k=359.3	0.9333	0.0889	80.2788
7	a=-0.01129; b=0.0000262	0.9931	0.0274	7.7391	a=-0.01364; b=0.0000412	0.9894	0.0347	11.6260	a=-0.01629; b=0.000061	0.9842	0.0432	18.9157
8	a=-24.67; k=0.03574 b=0.9605	0.9921	0.0293	8.2467	a=-20.28; k=0.0433 b=0.9505	0.9949	0.0241	5.2427	a=-40.28; k=0.05181 b=0.9731	0.9970	0.0189	3.7127
9	a=-43.46; k=0.03522 g=0.03443	0.9921	0.0293	8.6181	a=-42.65; k=0.04245 g=0.04144	0.9949	0.0240	5.5011	a=-15.08; k=0.053 g=0.04934	0.9970	0.0190	3.9366
10	a=1.002; k=0.002416 n=1.42; b=-0.0006716	0.9986	0.0121	1.3012	a=1.003; k=0.002283 n=1.514; b=-0.0003645	0.9989	0.0114	0.9357	a=1.001; k=0.001906 n=1.641; b=-0.0000735	0.9995	0.0074	0.6163
No	160 W – 55°C				160 W – 65°C				160 W – 75°C			
	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$	Model coefficients	R^2	RMSE	$\chi^2(10^{-4})$
1	a=1.151; k=0.02433	0.9590	0.0694	49.2496	a=1.143; k=0.0294	0.9560	0.0728	51.7650	a=1.137; k=0.03362	0.9393	0.0872	74.3240
2	k=0.02119	0.9352	0.0873	78.1182	k=0.02579	0.9346	0.0888	76.5298	k=0.02962	0.9204	0.0999	98.7500
3	k=0.001568; n=1.665	0.9997	0.0046	0.1956	k=0.002196; n=1.661	0.9986	0.0131	1.7386	k=0.001827; n=1.779	0.9972	0.0188	3.1235
4	a=1.498; k=0.01294 c=-0.4127	0.9882	0.0373	14.2787	a=1.568; k=0.01441 c=-0.4931	0.9897	0.0352	11.5807	a=1.89; k=0.01257 c=-0.8272	0.9869	0.0405	16.0290
5	a=-29.68; k_o=0.04847 b=30.69; k_1=0.04673	0.9971	0.0183	3.4801	a=-18.37; k_o=0.05935 b=19.38; k_1=0.05599	0.9950	0.0246	5.3362	a=-22.73; k_o=0.06944 b=23.74; k_1=0.06613	0.9872	0.0400	14.7394
6	a=0.0000597; k=354.9	0.9319	0.0894	82.0501	a=0.0000647; k=398.8	0.9305	0.0915	81.3402	a=0.0000758; k=390.9	0.9143	0.1037	106.3810
7	a=-0.015; b=0.0000502	0.9842	0.0431	19.3033	a=-0.01796; b=0.0000689	0.9864	0.0405	15.1585	a=-0.01974; b=0.000069	0.9844	0.0443	19.6322
8	a=-14.75; k=0.04954 b=0.9292	0.9975	0.0171	2.8964	a=-21.74; k=0.05933 b=0.9512	0.9955	0.0233	5.1105	a=-24.99; k=0.06934 b=0.9558	0.9890	0.0372	11.7403
9	a=-15.14; k=0.04946 g=0.04604	0.9975	0.0171	3.0502	a=-14.52; k=0.06004 g=0.05575	0.9955	0.0233	5.4618	a=-20.43; k=0.0702 g=0.06641	0.9890	0.0372	12.7526
10	a=1.002; k=0.001717 n=1.638; b=-0.0000823	0.9998	0.0042	0.1659	a=1.007; k=0.002971 n=1.564; b=-0.0004321	0.9991	0.0104	1.0207	a=0.9896; k=0.002083 n=1.714; b=-0.0006544	0.9984	0.0143	1.8507

3.3. Effective moisture diffusivity

Effective moisture diffusivity of microwave and microwave-convective dried lime slices ranged from 1.95 x 10^{-9} and 5.84 x 10^{-9}. It is clear in Table 4 that D_{eff} values showed an increase with the rise in microwave power and heating temperature. 160 W - 75 °C drying setting has the maximum effective moisture diffusivity value and 90 W has the minimum effective moisture diffusivity value. This can be explained by the higher heat caused the higher mass transfer. The diffusivity values derived from the present study were in agreement with values proposed in the literature. DOYMAZ *et al*. (2015) stated that D_{eff} values for dried agricultural products were generally varied between 10^{-8} and 10^{-12} m^2/s. DARVISHI *et al*. (2014) found that D_{eff} values of lemon slices dried at 180, 360, 540 and 720 W microwave power level were 1.87, 2.48, 3.29 and 3.95 x 10^{-8}, respectively. Similarly, SADEGHI *et al*. (2013) studied on lemon slices dried by using combined microwave - convective drying method at 50, 55 and 60°C inlet hot-air temperatures and microwave powers of 185.5 W and 388.5 W. The lowest and highest values of D_{eff} are 5.45 × 10^{-10} (185.5 W - 50°C) and 1.25 × 10^{-9} m^2 s^{-1} (388.5 W – 60°C), respectively. All of the outcomes above verify the rule that the rise in D_{eff} leads to decline in the drying interval.

3.4. Activation energy

The results indicated that Ea values of lime are 1.05 W g^{-1} during microwave drying and 15.99-19.33 W g^{-1} for microwave-convective drying combinations at 90 W and 160 W, in return. These findings are in congruence with the former researchers. To give an example, ZAREIN *et al*. (2015) found that the Ea of microwave dried apple samples at 200, 400 and 600 W was 12.15 W g^{-1}. The similar trend was determined by AMIRI CHAYJAN *et al*. (2015) for hawthorn fruit drying with microwave-convective drying technique. In general, the obtained results of the current study were well consistent with previous high moisture agricultural and food materials studies (RAVULA *et al*., 2017).

3.5. Energy analysis

Based on the benchmarking of the energy and specific energy consumption figures obtained during drying experiments of lime samples, it is found that in the 90 W – 55 °C combination required the minimum volume of energy and specific energy, whereas 90 W required the maximum volume of energy and specific energy. Out of these drying experiments, the microwave-drying ones consume more energy and they are not cost-effective to dry lime samples, whereas the microwave-convective drying combinations are energy-efficient and they consume less energy to dry lime samples. Taking into consideration that energy costs high all around the world, a combination of microwave-convective drying method seems to be an outstanding alternative. According to Table 5, the specific energy requirement is a function of air temperature and microwave power, such that when the microwave power level is constant, specific energy requirement to dry lime surges with the rise in air temperature. As drying rate is associated with microwave power and air temperature linearly, it could be detected that the surge in the microwave power and the air temperature consequently end up with the decline in drying interval. Similarly, DEMIREL and ISMAIL (2017) stated that total energy consumption depends on the overall drying interval. Out of all the drying techniques, the optimal energy consumption arose through microwave drying at 180 W power level with 0.042 kWh energy consumption. The overall energy consumption in all of the combined microwave-convective drying test processes of nectarine was found between 2.03 - 4.25

kWh. As VARITH *et al*. (2007) investigated the peeled longan drying with microwave-hot air combination. The least required specific energy consumption was found 29.7 MJ kg^{-1} water with MW400-300/40-60 and specific energy consumption can reduce the up to 48.2%.

Table 4. Effective moisture diffusivities of dried lime samples.

Drying method	D_{eff} (m^2 s^{-1})
90 W	1.95×10^{-9}
90 W - 55 °C	3.24×10^{-9}
90 W - 65 °C	3.89×10^{-9}
90 W - 75 °C	4.54×10^{-9}
160 W	3.24×10^{-9}
160 W - 55 °C	3.89×10^{-9}
160 W - 65 °C	5.19×10^{-9}
160 W - 75 °C	5.84×10^{-9}

Table 5. Energy consumption values of dried lime samples.

Drying method	Drying time (min)	Energy consumption (kWh)	Specific energy consumption (kWh kg^{-1})
90 W	269	0.85	10.36
90 W - 55 °C	131	0.57	6.93
90 W - 65 °C	118	0.60	7.40
90 W - 75 °C	97	0.67	8.17
160 W	131	0.57	7
160 W - 55 °C	120	0.61	7.48
160 W - 65 °C	77	0.62	7.5
160 W - 75 °C	33	0.63	7.64

3.6. Colour analysis

Because of to being highly appreciated quality parameter, the effect of drying on the color should be minimized (VEGA-GÁLVEZ *et al*., 2009). The results concerning the color changes under different experimental conditions are compared to the fresh sample in Table 6.

As the increase in drying air temperature at constant microwave power lead to decreases in L* values and increases in values of b*. The decrease of L* value implies darker color for all dried lime samples. While negative a* value (greenness) was seen for fresh lime samples (-3.79), it has turn to positive value (redness) in all dried lime samples. Besides, the drying at 160 W (28.08) has less negative effect than 90 W (29.21) in color value of b*. According to the ΔE parameter, there were no significant differences except 160 W – 65°C and 160 W – 75°C drying conditions ($p<0.05$).

Previous studies such as FADAVI and MEHRABI (2013), who considered the different temperature treatments (Shadow, sun-dried, 40, 105 and 200°C) effect on dried lime and defined that the attractive color is a necessary quality parameter. Similar outcomes have been put forward by DÍAZ *et al*. (2011).

Table 6. Color values of fresh and dried lime samples.

Drying method	Color parameters					
	*L**	*a**	*b**	*C*	*a°*	*ΔE*
Fresh	62.58±0.06^{a}	-3.79±0.08^{a}	26.39±0.05^{a}	26.67±0.06^{a}	98.14±0.17^{a}	-
90 W	57.52±0.34^{d}	5.14±0.06^{cd}	29.21±0.33^{cd}	29.66±0.33^{cd}	80.06±0.01^{c}	16.84±0.19^{a}
90 W - 55 °C	60.78±0.35^{b}	2.90±1.58^{b}	30.81±2.19^{de}	30.96±2.31^{de}	84.80±2.66^{b}	16.64±2.61^{a}
90 W - 65 °C	58.88±0.03^{c}	3.60±0.01^{b}	30.10±0.02^{de}	30.31±0.01^{de}	83.23±0.01^{b}	16.56±0.02^{a}
90 W - 75 °C	52.78±0.19^{g}	5.62±0.91^{cd}	27.10±0.88^{ab}	27.69±0.67^{ab}	78.29±2.25^{cd}	17.59±0.24^{a}
160 W	54.16±0.23^{f}	4.84±0.49^{c}	28.08±1.53^{bc}	28.50±1.42^{bc}	80.21±1.55^{c}	17.18±1.01^{a}
160 W - 55 °C	57.79±0.08^{d}	3.42±0.01^{b}	31.30±0.03^{e}	31.49±0.03^{e}	83.81±0.02^{b}	17.85±0.01^{a}
160 W - 65 °C	56.05±0.04^{e}	5.81±0.02^{cd}	29.97±0.01^{de}	30.53±0.01^{de}	79.08±0.01^{cd}	18.26±0.02^{ab}
160 W - 75 °C	49.24±0.09^{h}	6.19±0.03^{d}	26.98±0.06^{ab}	27.68±0.06^{ab}	77.12±0.02^{d}	19.95±0.03^{b}

$^{a-g}$ mean that different letters in same column with differ significantly ($p < 0.05$).

The rise of drying temperature from 60 to 70 °C without pretreatment resulted color degradation in 3 and 5 mm lime slices. However, the pretreated drying processes end up with fewer color changes and culminate in a product quality analogous to the fresh fruit.

4. CONCLUSIONS

In this research, the influences of microwave and combined microwave-convective drying techniques on the kinetics of drying, color, effective moisture diffusivity, activation energy and energy consumption of lime samples were studied. The obtained outcomes showed that the combined microwave-convective drying technique enabled significantly higher time-saving than microwave drying. The experimental moisture ratio data were tailored to the chosen 10 different thin-layer drying models and the drying attributes are illustrated better in the models of Midilli *et al*. and Page. The optimal color parameter of a* (greenness) and energy consumption values are achieved in 90 W – 55°C. Alternative techniques should be used to save time or energy in the industrial food process.

REFERENCES

Aibinu I., Adenipekun T., Adelowotan T., Ogunsanya T. and Odugbemi, T. 2007. Evaluation of the antimicrobial properties of different parts of Citrus Aurantifolia (Lime Fruit) as used locally. Afr. J. Tradit. Complement. Altern. Med. 4(2):185.

Al-Harahsheh M., Ala'a H. and Magee T.R.A. 2009. Microwave drying kinetics of tomato pomace: Effect of osmotic dehydration. Chem. Eng. Process. Process Intensif. 48(1):524.

Amiri Chayjan R., Kaveh M. and Khayati, S. 2015. Modeling drying characteristics of hawthorn fruit under microwave-convective conditions. J. Food Process. Preserv. 39(3):239.

Argyropoulos D., Heindl A. and Müller J. 2011. Assessment of convection, hot-air combined with microwave-vacuum and freeze-drying methods for mushrooms with regard to product quality. Int. J. Food Sci. Technol. 46(2):333.

Arredondo J., Ruiz L., Lopez G. and Diaz-Fleische, F. 2015. Determination of the host status of the 'Persian' lime (Citrus latifolia Tanaka) for Anastrepha ludens (Loew) (*Diptera*: *Tephritidae*). J. Econ. Entomol. 108(1):1.

Arslan, D. and Özcan M.M. 2010. Study the effect of sun, oven and microwave drying on quality of onion slices. LWT Food Sci. Technol. 43(7):1121.

Arumuganathan T., Manikantan M.R., Rai R.D., Anandakumar S. and Khare, V. 2009. Mathematical modeling of drying kinetics of milky mushroom in a fluidized bed dryer. Int. Agrophys. 23(1):1.

Bhattacharya M., Srivastav P.P. and Mishra, H.N. 2015. Thin-layer modeling of convective and microwave-convective drying of oyster mushroom (*Pleurotus ostreatus*). J. Food Sci. Technol. 52(4):2013.

Darvishi H., Khoshtaghaza H.M. and Minaei S. 2014. Drying kinetics and colour change of lemon slices. Int. Agrophys. 28:1.

Demirel F.M. and Ismail O. 2017. Investigation of the effect of a hybrid drying method on the color quality of nectarine slices and energy consumption. Stud. U. Babes-Bol. Che. 62(1):237.

Díaz D.M., Alcalá O.R., Avilés C.A., De la Cruz Medina J. and Jimenes, G.R. 2011. Drying process design for the third-qualıty persian lime use from Cuitláhuac, Veracrúz. México. Revista Iberoamericana de Tecnología Postcosecha 12(1):69.

Diaz G.R., Martinez-Monzo J., Fito P. and Chiralt A. 2003. Modelling of dehydration-rehydration of orange slices in combined microwave-air drying. Innov. Food Sci. Emerg. Technol. 4:203.

Doymaz İ. and İsmail O. 2011. Drying characteristics of sweet cherry. Food Bioprod. Process. 89(1):31.

Doymaz I., Kipcak A.S. and Piskin S. (2015). Microwave drying of green bean slices: Drying Kinetics and physical quality. Czech J. Food Sci. 33(4):367.

Esturk O. 2012. Intermittent and continuous microwave-convective air-drying characteristics of sage (Salvia officinalis) leaves. Food Bioprocess Technol. 5(5):1664.

Fadavi A. and Mehrabi A.A. 2013. Mechanical processing and temperature effect on lime shrinkage. Agricultural Engineering International: CIGR Journal. 15(3):238.

Gowen A.A., Abu-Ghannam N., Frias J. and Oliveira J. 2008. Modeling dehydration and rehydration of cooked soybeans subjected to combined microwave–hot-air drying. Innovative Food Sci. Emerg. Technol. 9(1):129.

Guo W. and Zhu, X. 2014. Dielectric Properties of Red Pepper Powder Related to Radiofrequency and Microwave Drying. Food Bioprocess Technol. 7:3591.

Kaewsuksaeng S., Tatmala N., Srilaong V. and Pongprasert N. 2015. Postharvest heat treatment delays chlorophyll degradation and maintains quality in Thai lime (Citrus aurantifolia Swingle cv. Paan) fruit. Postharvest Biol. Technol. 100:1.

Karaaslan S.N. and Tuncer I.K. 2008. Development of a drying model for combined microwave–fan-assisted convection drying of spinach. Biosyst. Eng. 100(1):44.

Karaaslan S. and Tunçer İ.K. 2010. Combined microwave fan-assisted convection dehydration of banana [Musa acuminata Colla (Cavendish Group)]. Philipp. Agric. Sci. 93(1):43.

Kassem A.S., Shokr A.Z., El-Mahdy A.R., Aboukarima A.M. and Hamed E.Y. 2011. Comparison of drying characteristics of Thompson seedless grapes using combined microwave oven and hot air drying. J. Saudi Society of Agr. Sci. 10(1):33.

Kowalski S.J. and Pawłowski A. 2011. Energy consumption and quality aspect by intermittent drying. Chem. Eng. Process. Process Intensif. 50(4):384.

Kumar D., Prasad S. and Murthy G.S. 2014. Optimization of microwave-assisted hot air drying conditions of okra using response surface methodology. J. Food Sci. Technol. 51(2):221.

Lambert G.F., Lasserre A.A., Azzaro-Pantel C., Miranda-Ackerman M.A., Vázquez R.P. and Salazar M.D.R.P. 2015. Behavior patterns related to the agricultural practices in the production of Persian lime (Citrus latifolia tanaka) in the seasonal orchard. Comput. Electron. Agric. 116:162.

Mohanta B., Dash S.K., Panda M.K. and Sahoo G. R. 2014. Standardization of process parameters for microwave assisted convective dehydration of ginger. J. Food Sci. Technol. 51(4):673.

Motevali A., Abbaszadeh A., Minaei S., Khoshtaghaza M. H. and Ghobadian B. 2012. Effective moisture diffusivity, activation energy and energy consumption in thin-layer drying of Jujube (*Zizyphus jujube* Mill). J. Agr. Sci. Tech. 14(3):523.

Omolola A.O., Jideani A.I.O. and Kapila P.F. 2014. Modeling microwave drying kinetics and moisture diffusivity of Mabonde banana variety. Int. J. Agric. Biol. Eng. 7(6):107.

Özbek B. and Dadali G. 2007. Thin-layer drying characteristics and modelling of mint leaves undergoing microwave treatment. J. Food Eng. 83(4):541.

Phoungchandang S., Srinukroh W. and Leenanon B. 2008. Kaffir lime leaf (Citrus hystric DC.) drying using tray and heat pump dehumidified drying. Drying Technol. 26(12):1602.

Ravula S.R., Munagala S.R., Arepally D. and Reddy P. 2017. Mathematical modelling and estimation of effective moisture diffusivity, activation energy, energy and exergy analysis of thin layer drying of pineapple. J. Exp. Biol. 5(3):392.

Sadeghi M., Mirzabeigi Kesbi O. and Mireei S.A. 2013. Mass transfer characteristics during convective, microwave and combined microwave–convective drying of lemon slices. J. Agr. Sci. Tech. 93(3):471.

Salih N.D. 2015. Evaluation of the Antimicrobial Effects of Citrus Aurantifolia (Key Lime) Against Different Microbial Species Isolated From Asthma and Sinusitis Patients. World J. Pharm. Pharm. Sci. 4(5):324.

Sharma G.P., Verma R.C. and Pathare P. 2005. Mathematical modeling of infrared radiation thin layer drying of onion slices. J. Food Eng. 71(3):282.

Soysal Y. 2004. Microwave drying characteristics of parsley. Biosyst. Eng. 89(2):167.

Su D., Zhang M., Wei Z., Tang X., Zhang R., Liu L. and Deng Y. 2015. Effect of microwave power on kinetics and characteristics of microwave vacuum-dried longan (*Dimocarpus longan* Lour.) pulp. Food Sci. Technol. Int. 21(2):124.

Tasirin S.M., Puspasari I., Lun A.W., Chai P.V. and Lee, W.T. 2014. Drying of kaffir lime leaves in a fluidized bed dryer with inertparticles: Kinetics and quality determination. Ind. Crops Prod. 61:193.

Therdthai N., Zhou W. and Kanitta Pattanapa K. 2011. Microwave vacuum drying of osmotically dehydrated mandarin cv. (*Sai-Namphaung*). Int. J. Food Sci. Technol. 46(11):2401.

Unal H.G. and Sacilik K. 2011. Drying characteristics of hawthorn fruits in a convective hot-air dryer. J. Food Process. Preserv. 35(2):272.

Varith J., Dijkanarukkul P., Achariyaviriya A. and Achariyaviriya S. 2007. Combined microwave-hot air drying of peeled longan. J. Food Eng. 81(2):454.

Vega-Gálvez A., Di Scala K., Rodríguez K., Lemus-Mondaca R., Miranda M., López J., and Perez-Won M. 2009. Effect of air-drying temperature on physico-chemical properties, antioxidant capacity, colour and total phenolic content of red pepper (*Capsicum annuum*, L. var. Hungarian). Food Chem. 117(4):647-653.

Wang J. and Sheng K. 2006. Far-infrared and microwave drying of peach. LWT Food Sci. Technol. 39(3):247.

Yadav A.R., Chauhan A.S., Rekha M.N., Rao L.J.M. and Ramteke R.S. 2004. Flavour quality of dehydrated lime [Citrus aurantifolia (Christm.) Swingle]. Food Chem. 85:59.

Zarein M., Samadi S.H. and Ghobadian B. 2015. Investigation of microwave dryer effect on energy efficiency during drying of apple slices. J. Saudi Society of Agr. Sci. 14(1):41.

Zhao D., Zhao C., Tao H., An K., Ding S. and Wang Z. 2013. The effect of osmosis pretreatment on hot-air drying and microwave drying characteristics of chili (*Capsicum annuum* L.) flesh. Int. J. Food Sci. Technol. 48(8):1589.

Paper Received June 13, 2018 Accepted February 1, 2019

PAPER

SENSORY QUALITY OF MOLECULAR DISHES AND CONSUMERS' ATTITUDES TOWARDS THEM

A. GŁUCHOWSKI, E. CZARNIECKA-SKUBINA*, M. PIELAK and E. OŁUBIEC-OPATOWSKA
Department of Food Gastronomy and Food Hygiene, Faculty of Human Nutrition and Consumer Sciences, Warsaw Univeristy of Life Sciences (WULS-SGGW), 159c Nowoursynowska St., 02-776 Warsaw, Poland
*Corresponding author: Tel.: +48225937063; Fax: +48225937068
E-mail address: ewa_czarniecka_skubina@sggw.pl

ABSTRACT

The aim of the study was to explore consumers' attitude toward molecular cuisine, specifically the sensory evaluation of these dishes. The experiment was performed in a laboratory among 10 panelists and 150 consumers to compare a sensory profile of molecular courses to their traditional versions.
Sensory profile of traditional dishes had a significantly higher, or comparable intensity in sensory attributes than molecular version and was preferred by consumers ($p \leq 0.05$). The results indicate positive attitude of consumers to modernist dishes and their moderately high sensory-experience ratings. Most of them showed willingness to try other molecular dishes in the future.

Keywords: molecular cuisine, sensory quality, consumer preference, food attitude, innovative technique

1. INTRODUCTION

The progress of civilization, globalization, increasing pace of life, economic and socio-demographic changes have all affected overall consumption pattern of food over the years. Modern consumers look for new experiences and there are fewer new products and services to surprise them.

Catering establishments want to meet consumers' expectations, so they try to expand and adjust their offer by adding new dishes to the menu card. Modernist cuisine also called molecular is an example of such activities. It is a new way of cooking, defined as 'producing food, using new tools, ingredients and methods', which has not been used in the food service so far (THIS, 2013 a, b). Among many of its features are obtaining surprising textures and forms as well as an enhanced ability to add to the original flavor and odor. Molecular cuisine uses both food additives (emulsifiers, stabilizers, thickeners and gelling agents) and new techniques (spherification, gelification, emulsification, foaming, blast freezing, sous-vide, lyophilization, concentration, homogenization, dehydration, and deconstruction) (IVANOVIC *et al*., 2011; LEE and ROGERS, 2012; VEGA and CASTELLS, 2012). The dishes are served as a tasting menu in the form of dozen small portions, typically on a variety of plates and unusual tableware (IVANOVIC et al., 2011). Chefs work to provide an interesting multisensory sensation to arouse emotions and stimulate senses beyond taste to make the consumption of the dishes a unique experience (YEK and STRUWE, 2008).

The preferences and/or food aversions of each individual is always 'colored' with emotions (DEN UIJL *et al*., 2014). Food choices are often associated with emotional states (VAN TRIJP and VAN KLEEF, 2008), expressed in dichotomies: pleasure/displeasure, ingestion/rejection, acceptance/rejection. Accordingly, the food preferences of modern consumers, especially toward food offered at catering establishments, are contradictory. On the one hand, perceptions of new food technologies can be affected by the degree of naturalness in food production (SIEGRIST, 2008). On the other hand, customers go to the restaurants not only to satisfy their nutritional requirements, but also to socialize and try new flavors that are not prepared at their homes (TÜZÜNKAN and ALBAYRAK, 2015).

Consumers are paying more attention to the quality of food products and the health benefits associated with their consumption (BARRENA and SÁNCHEZ, 2013). However they may be reluctant to compromise hedonic pleasure for health benefits (VERBEKE, 2006). New food technologies market success depends on consumers' behavioral responses. As a consequence, negative and suspicious attitude towards food technologies may lead to product failure (CHEN *et al*., 2013).

Only a few studies (YEOMANS *et al*., 2008; MIELBY and FROST, 2010; GUINÉ *et al*., 2012; TRAYNOR *et al*., 2013; FRAAT and ZAINAL, 2016) were dedicated to assess acceptance of molecular dishes and consumers' attitudes. Moreover, any comparison of traditional and molecular cuisine has not been performed so far. For this reason, in this study we:

- explore the knowledge and attitudes toward molecular cuisine and sensory-affective response amongst consumers in a laboratory settings;
- compare the sensory profile of molecular courses and similar traditional dishes to find factors that affect sensory ratings.

2. MATERIAL AND METHODS

2.1. Data collection and methods

2.1.1 Experimental design

The experiment was carried out in a laboratory environment and consisted of consumer tests and descriptive sensory profiling, hence involved both sensory panel and consumers of the academic subpopulation. The assessed dishes were three courses produced using various techniques applied in molecular cuisine (spherification, gelification and foaming) which were compared with three traditional dishes prepared basing on the same main ingredients (Table 1; Figs. 1-3). We decided to use simply dishes to allow comparison of sensory profiles and limit the results to a single technique used in molecular cuisine.

Table 1. Evaluated dishes.

Dishes	Molecular or traditional techniques	Characteristics of the product's appearance	Ingredients
Sample A - Liquid pea ravioli	spherification*	green semi-fluid core closed in an edible spherical coating	green peas, broth, sodium alginate, calcium chloride, seasonings
Sample A-1 - Cream of green pea soup	traditional dish	green creamy soup	green peas, broth, onion, butter, wheat flour, sour cream, seasonings
Sample B - Tomato soup spaghetti	gelification**	orange spaghetti shaped gel	tomatoes, the tomato puree, broth, leek, carrot, agar agar, carob gum, seasonings
Sample B-1- Pure tomato soup	traditional dish	orange creamy soup	tomatoes, the tomato puree, broth, onion, butter, seasonings
Sample C - Spinach foam	foaming***	green, light like whipped cream foam	spinach, broth, onion, sour cream, butter, xanthan gum, seasonings
Sample C-1 - Spinach dip	traditional dish	thick dark green dip	spinach, onion, natural yoghurt 2% of fat, feta cheese, seasonings

*Spherification is a technique of creating liquid filled spheres based on the controlled gelling reaction between calcium chloride and sodium alginate (e.g. caviar, gnocchi and ravioli).
**Gelification is a process of turning a liquid into a gel using gelatin, agar agar, carrageenan Kappa and Iota, gellan, xanthan (e.g. spaghetti, pearls or flexible wraps).
***Foaming is a process of transforming liquids into light foams using culinary siphon and food additives like xanthan gum, carob gum, agar agar, gelatin etc. to stabilize a foam (IVANOVIC *et al*., 2011, LEE and ROGERS, 2012).



Figure 1. Proposition of the pea dishes plating: A. liquid pea ravioli, A-1 cream of green pea soup.



Figure 2. Proposition of the tomato dishes plating: B. tomato soup spaghetti, B-1 pure tomato soup.



Figure 3. Proposition of the spinach dishes plating: C. spinach foam, C-1 spinach dip.

2.1.2 Sample presentation for evaluation

Samples of dishes (30-40g) were placed in plastic, transparent containers, then covered with a lid, coded and delivered for assessment.

Samples were served at a temperature, at which they are normally consumed. Temperature of cream of green pea soup and pure tomato soup was approximately 63 ± 2°C. Due to the low stability of gelling agents in higher temperature, molecular spaghetti and ravioli, as well as spinach dip and foam was were evaluated at temperature of 23 ± 1°C. Differentiation of evaluated samples due to the serving temperature was resulted from specific of dish. Evaluation of samples brought to the same temperature would exhibit a disproportionate sensory intensities and does not reflect actual characteristics of dishes.

2.1.3 Descriptive sensory profiling

Descriptive sensory profiling of dishes using a quantitative descriptive analysis was evaluated triple by a 10 persons assessing panel, which held expert qualifications (PN-EN ISO 8586:2014-03). Conditions of the assessment were determined in accordance with CIVILLE and CARR (2015). Selection of quality descriptors was carried out in accordance with the procedure of PN-ISO 13299:2003 and presented in Table 4.

2.1.4 Consumer testing

Consumer testing consisted of a validated questionnaire survey as well as degree of liking and preference assessment. The questionnaire was validated among 5 people in a pilot study. All problems have been identified. The questionnaire included 8 questions about previous experiences with molecular dishes and their occurrence places (e.g. food service outlets, culinary school), familiarity and understanding of the term 'molecular cuisine', willingness to taste molecular dish in the future as well as consumers gender and age. They were also tasked to indicate their own attitudes towards novel food (novelties enthusiast, traditional consumer who didn't exclude new products, consumer afraid of novelty). Then they received food samples and made sensory assessment. All respondents agreed to participate in research.
An unstructured linear graphical scale, with word anchors (like-dislike) on the edges was used to determine the level of sensory-liking of odor, color, flavor, consistency and overall liking (PN-ISO 4121:1998). The assessors also pointed out the sensory characteristics of the food that influenced their assessment and indicated the feelings accompanying them in relation to the evaluated dishes (liking, surprising, arousing curiosity, innovative, traditional, the challenge for the senses, being skeptical). To determine which version (molecular/traditional) consumers have preferred the most, the paired comparison test (PN-EN ISO 5495:2007) was used. Samples were encoded and served in a pairs (molecular dish vs. traditional).
Consumer tests of dishes were carried out in a group of 150 students and academics of Faculty of Human Nutrition and Consumer Sciences, WULS (SGGW), designed as a study with a convenience sampling. The group was dominated by women (82%) and mainly under 25 years old (86%) (Table 2).The respondents were diverse in their attitude towards food. Among them were novelties enthusiasts (60%), traditional consumers who didn't exclude new products (35.3%), consumers afraid of novelty (low willingness to try new foods) (4.7%).

Table 2. Characteristic of consumers surveyed (n=150).

Characteristic of consumers	Respondents' answers	
	Number	Percent (%)
Gender		
Woman	123	82.0
Man	27	18.0
Age		
<25	129	86.0
25-60	21	14.0
Familiarity with the molecular cuisine term		
Yes	120	80.0
No	30	20.0
Understanding the term of molecular cuisine*		
Cuisine based on science (physico - chemical transformations of food)	85	73.9
Experimental cuisine (using scientific experiments)	67	58.3
Cuisine with new, innovative dishes	55	47.8
Cuisine, wherein dishes are presented in another manner than traditionally	80	69.6
Do not know	2	1.7
I understand it in a different way...	0	0.0
Possibility of earlier tasting of molecular cuisine		
Yes	42	28.0
Therein*		
Cooking workshops	13	31.0**
Catering establishment in Poland	9	21.4**
Catering establishment abroad	8	19.1**
Catering school	9	21.4**
At friends' house	7	16.7**
I make it by myself	5	11.9**
Other (at University, at work)	7	16.7**
No	108	78.0**
Attitudes towards novel food		
I am novelties enthusiast	90	60.0
I am traditional consumer, but do not exclude new products	53	35.3
I am afraid of food novelties	7	4.7

*respondents could choose several answers.
**percent of consumers that responded affirmatively in previous questions (n=42).

2.2. Data analysis

The statistical analysis of the results was performed using Statistica software version 13.1 PL (StatSoft, Krakow, Poland). The mean of results, standard error, one-way analysis of variance (ANOVA), as well as coefficient of correlation according Pearson and Spearman's rank were calculated. The result was considered to be statistically significant at the level of materiality equal to 0.05 or 0.001.

3. RESULTS AND DISCUSSION

3.1. Familiarity with molecular cuisine in group of consumers

Most of consumers (80%) participating in experiment (n=150), were familiar with term of molecular cuisine (Table 2).
Approximately 28% of surveyed consumers had practical experience with molecular dishes, where of these, about one third consumed it at a cooking workshops, and almost every fifth at catering establishment in Poland or culinary school. Quite low percentage of people trying the dishes at gastronomic establishments in Poland might be caused by small amount of those places and limited financial availability. Almost 63% of the interviewed have not tasted molecular cuisine before.
The term of the 'molecular cuisine' was not associated with only one definition. The most respondents indicated that is cuisine based on physio-chemical changes in food (74%) and the cuisine wherein dishes are presented in another manner than traditionally (69%). Many respondents stated, that is experimental cuisine (using scientific experiments) (58%). The least of respondents indicated that these are innovative dishes (48%). Nobody tried to redefine the term again.

3.2. Consumer sensory - affective response

Consumers were tasked with determining the degree of liking of four sensory characteristics and overall liking in each dish. Mean scores for all individual attributes and statistically significant differences are shown in Table 3.

Table 3. Results of consumer sensory evaluation (n=150).

Sample dishes	Degree of liking (scale 1-10 c.u.) - average (± SE)					Paired comparison test
	Odor	Color	Consistency	Flavor	Overall liking	
A – liquid pea ravioli	5.31±0.26a	6.45±0.22a	5.11±0.27a	5.80±0.23a	5.92±0.22a	28
A-1 – cream of green pea	6.53±0.23b	7.04±0.20b	6.73±0.23b	7.34±0.21b	7.12±0.22b	87*
B – tomato soup spaghetti	6.43±0.22a	6.93±0.21a	6.57±0.25a	5.73±0.26a	6.17±0.25a	37
B-1 – pure tomato soup	7.12±0.18b	7.31±0.19a	6.89±0.22a	7.15±0.22b	7.11±0.21b	78*
C – spinach foam	6.56±0.23a	7.27±0.19a	6.52±0.26a	5.39±0.28a	5.88±0.25a	35
C-1 – spinach dip	7.57±0.18b	7.14±0.21a	6.98±0.21a	6.95±0.23b	7.13±0.20b	89*

a, b, values bearing different letters are significantly different; p≤0.05 (differences between molecular and traditional dishes); *samples in pairs differ significantly p≤0.001.

Among the surveyed consumers (n=150), the degree of liking of molecular dishes was moderately high (5.1-7.3 c.u.) (Table 3).
Traditional dishes (A-1, B-1, C-1) were ranked significantly (p≤0.05) higher in attributes such as: odor, flavor, overall liking and in sample A-1 also in color and consistency, when compared to molecular ones: A, B, C (Table 3). Moreover, the result of the paired comparison test showed that most of consumers preferred traditional courses, $p<0.01$ (Table 3). The high intensity of flavor of traditional courses was the most strongly correlated with overall liking from all analyzed characteristics. The highest Pearson's

correlation coefficient between flavor and overall liking for both traditional (0.84-0.92) and molecular (0.87-0,89) dishes has been calculated (α=0.05), while lower for consistency (<0.7), color, (<0.6) and odor (<0.5).

Sensory experience associated with a specific food or beverage does not start with the moment of the consumption. It can begin even before the food is seen, for example by hearing about it from friends, relatives or through mass media (FAVALLI *et al*., 2013). Therefore, the correlation between perception of certain attributes and previous consumption of molecular cuisine with significance level of 0.05 has been observed, but only for liquid pea ravioli. Consumers who tasted molecular cuisine before, ranked color (rho = 0.33), consistency (rho = 0.29) and overall liking (rho = 0.19) of molecular ravioli higher. Dishes which were related to familiar food or had the potential to evoke positive memories were liked the most. The phenomenon can be explained by research results whose, found that acceptance of an unfamiliar food is largely determined by its degree of similarity to familiar food in individual's diet (MIELBY and FROST, 2010).

The literature reported differences between acceptability of novel and familiar foods among consumers who are food neophobes and neophiliacs. Neophobes were less inclined to try novel foods, while neophiliacs had a higher awareness, willingness to try and rated unfamiliar food more favorably KING *et al*. (2008). Consumers participating in experiment were those, who like new products (60%) or the traditional consumers, who do not exclude novelties (35.3%), but 4.7% of them were consumers who are afraid of novelty (low willingness to try new foods).

Consumers indicated the traits of dish, which had effect on their evaluation (not shown in table).

Among consumers, who preferred molecular versions – novelty enthusiasts (e.g. A sample), the highest percentage of them indicated flavor (about 54%) and consistency (about 42%) as a traits affecting on their evaluation, but for those who chose traditional dishes - traditionalists (e.g. B-1 and C-1 sample), the most important was flavor (71-80%) and odor (about 38%). It is proved by results of correlation in the degree of liking assessment.

Traditional and molecular dishes were intentionally differed in consistency using techniques such as spherification, gelification, and foaming, although, for consumers it was less important in perception than flavor. Similarly in FRAAT and ZAINAL's (2016) study, the greatest effect on chef's acceptance of modernist version of Malaysian Asam Pedas (sphere, foam, powder, sous-vide) the degree of overall liking and consistency-liking had. Odor and color was less important in their research. In our study, just a few respondents indicated non-sensory characteristics when explained their choice. This confirms TUORILA (2007) observations, that sensory-liking usually plays a major role in food choice decisions, and flavor had effect on choice of a particular dish. Authors indicated a significant effect of flavor, quality, novelty, expectation, and familiarity (OLABI *et al*., 2015), as well as appearance and odor (FERNQVIST and EKELUND, 2014) on quality and its positive correlation with the degree of liking.

Lower scores of molecular dishes may result from the fact that consumer response is based not only on sensory perception, but also on stimulation of many other senses, evoking various intellectual (THIS, 2013b) and aesthetic experiences (MICHEL *et al*., 2015; YOUSSEF *et al*., 2015). The concept of this cuisine is aimed to create avant-garde, unique dishes that surprise customers. Although the laboratory evaluation is objective, does not fully reflect dining experience. Many authors noticed lower hedonic scores of dishes in laboratory settings when compared to those assessed at the target consumption atmosphere: at the military field (DA GRAAF *et al*., 2005), at high altitude field conditions (PREMAVALLI *et al*., 2009) and on the airplane board (HOLTHUYSEN *et al*., 2017). Similar observation indicated authors (MICHEL *et al*., 2015; YOUSSEF *et al*., 2015). According to

them, visual presentation and the manner of platting, affects its liking and appreciation for example by enhancing a diner's rating of the dish flavor.

3.3. Sensory profile of dishes

To determine more detailed differences between traditional and molecular dishes, quantitative descriptive analysis by panelist was performed. The results of the sensory profiling show that most attributes of odor (sweet, brothy, creamy, and specific for dishes) were significantly more intensive in traditional (A-1, B-1, C-1) than molecular dishes (A, B, C) (Table 4). The opposite relationship was observed in intensity of color. Traditional dishes based on green pea or tomato (A-1 and B-1) were characterized by lower intensity of color than their molecular versions (A and B). Spinach foam (C) had significantly lower intensity of color because of dish form - foam, so the high aeration.
Molecular dishes were significantly differed in consistency compared with traditional ones. Liquid pea ravioli (A) was evaluated significantly higher in attributes such density and smoothness ($p=0.0001$). Spinach foam (C) distinguished itself with high fluffiness and low density ($p=0.001$). Tomato dishes have been evaluated using different consistency attributes because of wide variations in consistency (Table 4).

Table 4. Sensory profiles of evaluated dishes (n=30).

Attributes	Average (0-10 c.u.) Sample		Attributes	Average (0-10 c.u.) Sample		Attributes	Average (0-10 c.u.) Sample	
	A	A-1		B	B-1		C	C-1
green pea o.	8.10a	7.22b	tomato o.	7.31a	7.94b	spinach o.	7.23a	8.00b
sweet o.	2.26a	2.85a	sweet o.	2.57a	3.31b	garlic o.	6.83a	6.28a
brothy o.	2.68a	5.30b	brothy o.	3.32a	4.05b	lemon o.	1.95a	2.04a
creamy o.	1.52a	2,86b	creamy o.	1.64a	2.45b	creamy o.	1.47a	1.93b
other o.	0.46a	0.23a	other o.	0.22a	0.25a	other o.	0.27a	0.16a
green color	5.93a	4.39b	orange color	6.51a	5.85b	green color	6.08a	6.96b
density	7.63a	6.21b	density	-	3.47	density	3.29a	6.99b
			smoothness	-	7.06			
smoothness	7.08a	5.82b	springiness/elasticity	7.72	-	fluffiness	8.69a	4.63b
			fragility	7.55	-			
			ability to fragmentation	7.77	-			
green pea f.	7.82a	7.48a	tomato f.	7.20a	8.16b	spinach f.	7.37a	7.27b
brothy f.	2.58a	5.01b	brothy f.	3.44a	3.56a	garlic f.	5.84a	7.44b
creamy f.	1.71a	4.46b	other f.	0.26a	0.23a	lemon f.	1.92a	2.90a
other f.	0.15a	0.16a				creamy f.	2.81a	2.60a
sweet f.	3.31a	5.22b	sweet f.	2.24a	3.32b			
salty t.	2.10a	1.88a	salty t.	2.22a	2.40a	salty t.	1.85a	1.89a
			sour f.	2.73a	4.20b	pungent f.	1.69a	2.35a
			tart t.	0.85a	1.00a			
overall quality	7.89a	8.29a	overall quality	7.86a	8.63a	overall quality	7.23a	7.85b

o., odor; f., flavor; t., taste; a, b, values bearing different letters are significantly different at $p < 0.001$.
A, liquid pea ravioli; A-1, cream of green pea; B, tomato soup spaghetti; B-1, pure tomato soup; C, spinach foam; C-1, spinach dip

Intensity of many flavor attributes in traditional dishes was estimated significantly higher. Cream of green pea soup (A-1) had higher intensity of brothy, creamy and sweet flavor, tomato soup (B-1) higher notes of tomato, sweet and sour flavor while spinach dip (C-1) had higher intensity of garlic flavor ($p=0.001$) (Table 4). Other attributes differentiated not significantly between traditional and molecular dishes. In the case of green pea dishes (A, A-1) and tomato dishes (B, B-1), the differentiation of individual attributes defining their sensory quality, statistically did not differ in the overall quality of these dishes in both versions ($p \geq 0.05$). Overall quality of traditional spinach dish (sample C) was significantly higher ($p=0.0001$) than the molecular version (C-1) as a result of higher intensity of odor (spinach and creamy smell), green color, consistency (density, fluffiness), as well as flavor (garlic).

The lower liking scores for odor and flavor of the molecular versions may be the result of the interactions between flavor or aroma compounds and polysaccharides used to give the shape or form of dishes (gelling agents in ravioli and spaghetti, stabilizer in foam). Similar observation was made by TRAYNOR *et al*. (2013) who studied various food-pairing combinations and emphasized that complex synergistic or antagonistic interactions between food matrices and volatile compounds may occur and affect the hedonistic rating of the new combination. According to FAVALLI *et al*. (2013) in new developed product it is possible to change the appearance and consistency characteristics as long as it does not change the ingredients and provides the same level of retro-nasal attributes intensity. In this study, molecular dishes were less liked than traditional ones. Moreover, analysis of the sensory profiles revealed significantly higher or comparable intensity of most quality factors relating to odor, color, consistency (density, smoothness, fluffiness), and flavor in traditional dishes. This is related to higher content of ingredients such as butter and cream in those dishes. For example, more intense flavor and odor of pure tomato soup may be caused by presence of fat in it because fat has influence on mouthfeel, forms deliciousness and contributes to the release of a scent from food products.

3.4. Consumers' feelings toward evaluated dishes

Consumers were also asked to indicate the feelings accompanying them in relation to evaluated dishes (Table 5). In general, modernist cuisine evoked in consumers more emotions (35.3%) than traditional one.

Table 5. The feelings of consumers in relation to the assessed dishes.

Sample dishes	The feelings of consumers (Percentage of respondents' answers)						
	liking	surprising	arousing curiosity	innovative	traditional	challenging for sense	being skeptical
A – liquid pea ravioli	37	17	28	13	6	10	28
A-1 – cream of green pea	59	4	16	3	41	4	11
B – tomato soup spaghetti	17	54	59	54	5	22	13
B-1 – pure tomato soup	50	6	5	6	69	4	7
C – spinach foam	21	59	50	51	3	23	20
C-1 – spinach dip	65	3	9	3	47	14	10

Most of the molecular courses were associated by consumers (about 50%) with feelings, like 'surprising', 'arousing curiosity', 'innovative' and 'liking'. A small percentage (about 20%, depending on dish) of consumers indicated feelings as: 'challenging for sense' and 'being skeptical'. As expected, majority of consumers associated traditional dishes with feelings: liking (50-65%) and traditional (41-69%). Molecular dishes that use gelification or foaming process were assessed as 'surprising', 'arousing curiosity', and 'innovative', as 50-59% of surveyed consumers indicated. The dish made by using spherification process was seen by the majority of respondents (28-30%) as 'liking', 'arousing curiosity', and 'being skeptical'. Dishes, which were perceived by largest percentage of consumers (>50%) as arousing curiosity, surprising, and innovative (tomato soup spaghetti, spinach foam), were also less liked.

Almost 88% of respondents (n=150) would like to taste different molecular courses in the future, although, most of them chose traditional ones in the paired comparison test.

It may be explained by the most frequent feelings related to modernist cooking, i.e. arousing curiosity, surprising, as well as liking and innovative. This is all the more interesting because consumers taking part in analogous research conducted by MIELBY and FROST (2010) would not like to consume the same molecular cuisine dishes at another occasion. This indicates that consumers seek a new food experience. Additionally, differences in the responses may be associated with different groups of subjects. Consumers who have participated in this study were young, mainly under 25 years old so more inclined to culinary experiments. MIELBY and FROST (2010) emphasize the relationship between molecular dishes and sensations they evoke.

Today, consumers are in search of something more than solely food, which is no longer considered as satisfying physiological needs, but could also bring emotional benefits, the pleasure of tastes (MĂRKUT *et al*., 2014).

4. CONCLUSIONS

The study has shown significant differences between the sensory quality of molecular and traditional cuisine. Higher degree of liking of traditional dishes, as well as more intense typical flavor and odor has been noted ($p<0.05$). Consumers chose traditional dishes and indicated flavor as the most important sensory trait. The largest percentage of consumers associated modernist cuisine with feelings like: 'arousing curiosity', 'surprising' and 'innovative'. Most of them showed their willingness to try other molecular dishes in the future, although prefer traditional ones. The evaluation of molecular courses is not easy because the idea of creating them is based on constant novelty and uniqueness of dishes, hence, is described as 'haute cuisine'. However, our results revealed a positive attitude of consumers towards molecular dishes, their moderately high sensory-experience ratings and openness towards new ideas. The juxtaposition of molecular cuisine assessment at laboratory environment and restaurant setting, as well as cluster analysis to identify target consumer segment seem to be good idea for the further studies.

4.1. Practical implications

The results of this study emphasize the possibility of molecular cuisine use to vary and extend the range of menu items in restaurants. Modernist cuisine as an element of traditional dish might make its more interesting, surprising and arousing curiosity. Such a dish beside the nutrition role would evoke more emotions and therefore draw attention of more consumers.

4.2. Limitations

Some limitations of our study need to be addressed. Although presented consumer attitude applies to evaluated dishes, the authors tried to choose the most popular techniques of molecular cuisine among chefs. It should be emphasized that the idea and principle of this cuisine is the creation of innovative, unique dishes, which are different at each catering establishment and often rotated in the menu.

ACKNOWLEDGEMENTS

Research financed by Polish Ministry of Science and Higher Education within funds of Faculty of Human Nutrition and Consumer Sciences, Warsaw University of Life Sciences (WULS) for scientific research.

REFERENCES

Barrena R. and Sánchez M. 2013. Neophobia, personal consumer values and novel food acceptance. Food Qual. Pref. 27(1):72-84.

Chen Q., Anders S., and An H. 2013. Measuring consumer resistance to a new food technology: a choice experiment in meat packaging. Food Qual. Pref. 28(2):419-428.

Civille G.V. and Carr B.T. 2015. 'Sensory evaluation techniques'. 5th. ed. Boca Raton: CRC Press, pp. 29-42.

de Graaf C,. Cardello A.V., Kramer F.M., Lesher L.L., Meiselman H.L. and Schutz H.G. 2005. A comparison between liking ratings obtained under laboratory and field conditions: The role of choice. Appetite 44(1):15-22.

Den Uijl L.C., Jager G., De Graaf C., Waddell J., and Kremer S. 2014. It is not just a meal, it is an emotional experience – a segmentation of older persons based on the emotions that they associate with mealtimes. Appetite 83:287-296.

Favalli S., Skov T., and Byrne D.V. 2013. Sensory perception and understanding of food uniqueness: From the traditional to the novel. Food Res. Int. 50(1):176-188.

Fernqvist F. and Ekelund L. 2014. Credence and the effect on consumer liking of food – A review. Food Qual. Prefer. 32:340-353.

Fraat F. and Zainal A. 2016. The Influence of Hedonic Characteristics on Chefs' Acceptance Towards Molecular Asam Pedas. In Regional Conference on Science, Technology and Social Sciences (RCSTSS 2014), pp. 1085-1094. Springer, Singapore.

Guiné R.P., Dias A., Peixoto A., Matos M., Gonzaga M. and Silva M. (2012). Application of molecular gastronomy principles to the development of a powdered olive oil and market study aiming at its commercialization. Int. J. Gastron. Food Sci. 1(2):101-106.

Holthuysen N., Vrijhof M.N., de Wijk R.A. and Kremer S. 2017. Welcome on board: Overall liking and just-about-right ratings of airplane meals in three different consumption contexts-laboratory, re-created airplane, and actual airplane. J. Sens. Stud. 32(2). DOI: doi.org/10.1111/joss.12254.

Ivanovic S., Mikinac K. and Perman L. 2011. Molecular gastronomy in function of scientific implementation in practice. J. Economics 2:139-150.

King S.C., Meiselman H.L. and Henriques A. 2008. The effect of choice and psychographics on the acceptability of novel flavors. Food Qual. Prefer. 19(8):692-696.

Lee P. and Rogers M.A. 2012. Effect of calcium source and exposure time on basic caviar spherification using sodium alginate Int. J. Gastron. Food Sci. 1:96-100.

Mărkut L., Mărkut A. and Mârza B. 2014. Modern Tendencies in Changing the Consumers' Preference. Procedia Econ. Financ. 16:535-539.

Michel C., Velasco C., Fraemohs P. and Spence Ch. 2015. Studying the impact of plating and cutlery on ratings of the food served in naturalistic dining contexts. Appetite 90:45-50.

Mielby L.H. and Frost MB. 2010. Expectations and surprise in a molecular gastronomic meal. Food Qual. Prefer. 21:213-224.

Olabi A., Neuhaus Th., Bustos R., Cook-Camacho M., Corvi T. and Abdouni L. 2015. An investigation of flavor complexity and food neophobia. Food Qual. Prefer. 42:123-129.

PN-EN ISO 5495:2007. Polish Standard. Sensory analysis. Methodology. Paired comparison test.

PN-EN ISO 8586:2014-03. Polish Standard. Sensory analysis. General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors.

PN-ISO 13299:2003. Polish Standard. Sensory analysis. Methodology. General guidelines for determining the sensory profile.

PN-ISO 4121:1998. Polish Standard. Sensory analysis. Methodology. Evaluation of Food Products by Methods Using Scales.

Premavalli K.S., Wadikar D.D. and Nanjappa C. 2009. Comparison of the acceptability ratings of appetizers under laboratory, base level and high altitude field conditions. Appetite 53(1):127-130.

Siegrist M. 2008. Factors influencing public acceptance of innovative food technologies and products. Trends Food Sci. Technol. 19(11):603-608.

This H. 2013a. Solutions are Solutions, and gels are almost solutions. Pure Appl. Chem. 85, 1:257-276.

This H. 2013 b. Molecular gastronomy is a scientific discipline, and note by note cuisine is the next culinary trend. Flavour 2:1.

Traynor M.P., Burke R., O'Sullivan M.G., Hannon J.A. and Barry-Ryan C. 2013. Sensory and chemical interactions of food pairings (basmati rice, bacon and extra virgin olive oil) with banana. Food Res. Int. 54(1):569-577.

Tuorila H. 2007. 'Sensory perception as a basis of food acceptance and consumption. Consumer-led food product development'. pp. 34-65, Woodhead Publishing.

Tüzünkan D. and Albayrak A. 2015. Research About Molecular Cuisine Application As An Innovation Example In Instambul Restaurants. Procedia Soc. Behav. Sci. 195:446-452.

Van Trijp H.C.M. and van Kleef E. 2008. Newness, value and new product performance. Trends Food Sci. Technol. 19:562-573.

Verbeke W. 2006. Functional foods. Consumer willingness to compromise on taste for health. Food Qual. Prefer. 17(1-2):126-131.

Vega C. and Castells P. 2012. 'Spherification - Faux Caviar and skinless Ravioli'. In 'The kitchen reflections on the science of food and cooking'. C. Vega, J. Ubbink and E. Linden (Ed.), pp. 25-32, Columbia University Press. New York.

Yek G.S. and Struwe K. 2008. Deconstructing Molecular gastronomy. Food Technol. 62:34-45.

Yeomans M.R., Chambers L., Blumenthal H. and Blake A. 2008. The role of expectancy in sensory and hedonic evaluation: The case of smoked salmon ice - cream. Food Qual. Prefer. 19:565-573..

Youseff J., Juravle G., Youssef L., Woods A. and Spence Ch. 2015. Aesthetic plating: a preference for oblique lines ascending to the right. Flavour 4:27.

Paper Received October 30, 2018 Accepted January 8, 2019

PAPER

FREEZE DRYING AND MOISTURE ADSORPTION KINETICS OF KEFIR POWDER

H. ISLEROGLU
Tokat Gaziosmanpasa University, Faculty of Natural Sciences and Engineering, Food Engineering Dept., 60150, Tokat, Turkey
E-mail address: hilal.isleroglu@gop.edu.tr

ABSTRACT

Freeze drying and moisture adsorption behaviour of kefir powder were investigated by fitting the experimental drying data to ten thin layer drying and sorption isotherm models. Moisture adsorption isotherms of kefir powder were determined at 5-35°C and within the range of 0.11–0.88 water activity. By statistical comparison of the values, Midilli et al. was found to be the best model describing the freeze drying behaviour. The GAB and Oswin equations gave the closest fit to the adsorption data over the tested range of temperatures and water activities. Additionally, adsorption isotherms data were used to determine the thermodynamic properties such as isosteric heat, sorption entropy and Gibbs free energy. The enthalpy-entropy compensation was valid for the adsorption process and showed that the process was controlled by the enthalpy. Gibbs free energy was negative at all of the tested temperatures, which indicates that moisture adsorption of kefir powder was a spontaneous process.

Keywords: adsorption isotherm, compensation theory, freeze drying, Gibbs free energy, kefir powder

1. INTRODUCTION

Kefir is a fermented milk drink with a unique acidic aromatic taste and it is a complex probiotic containing numerous bacterial species such as acetic acid bacteria, *Leuconstoc, Lactobacillus*, *Lactococcus*, and also yeasts (CHIFIRIUC *et al.*, 2011). Kefir also contains small amounts of acetic acid, ethanol, acetaldehyde, lactic acid, diacetyl, carbon dioxide, and acetoin that characterized its specific flavour (GRONNEVIK *et al.*, 2011; ARSLAN, 2014). Kefir grains or commercially available dairy cultures were used to inoculate milk for producing kefir (TAMIME *et al.*, 2011). Since kefir has several health promoting and therapeutic effects, consumer's interests are increasing about consuming it.
The health beneficial effects are identified with bioactive compounds such as exopolysaccharides and bioactive peptides produced during fermentation process (FARNWORTH, 2005). However, kefir has a short shelf-life even in refrigerator conditions due to its biologically active components. Therefore, improvements in the shelf-life of kefir can be achieved by changing it into a shelf-stable powder. Also, kefir powder enables easier transportation and storage, and it can be used as an ingredient in various formulations. ATALAR and DERVISOGLU (2015) produced kefir powder by spray drying and determined the optimum drying conditions. However, there are no reports related freeze drying characteristics and sorption properties of kefir in the literature.
Compared to spray drying process, freeze drying generally results in better survival of starter culture and improved reconstitution properties (KUMAR and MISHRA, 2004). In freeze drying process, moisture is removed at low temperature which prevents thermal inactivation and material structure is maintained i.e. particles and cells are not damaged (ADAMIEC *et al.*, 2015).
Moisture sorption isotherm (MSI) measured experimentally under isothermal conditions characterizes the relationship between the equilibrium moisture content (EMC) and water activity (a_w) of foods at constant temperature and pressure (KAYMAK-ERTEKIN and GEDIK, 2004). MSI provides information about hygroscopic properties of a product and it can be used in modelling drying process, calculating changes in moisture content during storage and estimating shelf-life by theoretical calculation (RIZVI, 1995). EMC depends on many factors such as physical structure and chemical composition of the product, and the environmental air conditions. Wide range of equations (theoretical, semi-empirical, empirical) have been suggested; however, no one equation provides precise results over the entire range of water activity (WANG *et al.*, 2013). Some of the best known equations are Brunauer-Emmet-Tetter (BET) equation which has been successfully applied to almost all kinds of materials, but especially to hydrophilic polymers for $a_w < 0.5$ and Guggenheim-Anderson-Boer (GAB) equation which is evaluated as the most useful sorption model present in the literature, capable of application to circumstances over a wide range of water activities ($0.1 < a_w < 0.9$) (KOÇ et al., 2010).
Temperature dependence of MSI is very important because the foods are subjected to different temperatures during storage and also processing (KAYMAK-ERTEKIN and SULTANOGLU, 2001). Thermodynamic properties such as the isosteric heat of sorption, differential entropy and Gibbs free energy are important parameters for processing and storage, and they provide a conception about water properties and energy requirements related to sorption behaviour and these properties can be calculated from sorption isotherms (RIZVI, 1995; TOLABA *et al.*, 2004).
The aims of the current study were to examine a proper thin-layer drying model for describing the freeze drying of kefir, to achieve the adsorption isotherms of kefir powder at different temperatures, to determine the best model describing adsorption behaviour and to estimate thermodynamic properties such as net isosteric heat (q_{st}), differential entropy (ΔS) and Gibbs free energy (ΔG) for kefir powder.

2. MATERIAL AND METHODS

2.1. Materials

Ultra-high temperature (UHT) sterilized milk with 3.0 % milkfat was used for kefir production. Milk (3 liters) was fermented with a commercial kefir starter (~1 g of powder) (Maysa A.S., Turkey) and fermentation was performed at 30°C for 20 hours, which were the declared optimal conditions according to producers' information. Final pH value of kefir was 4.55 and it was refrigerated until drying.

2.2. Freeze drying characteristics

Drying experiments were carried out in a laboratory scale freeze dryer (CHRIST, Alpha 1-4 LSC, Germany). Kefir was poured in to drying pans as 3 mm thickness and then frozen at -20°C for a night. Freeze drying processes were performed at 1 mbar absolute pressure and -55°C condenser temperature. Each experiment for incrementing time periods was conducted with incipient samples of equal mass (~30 g of frozen sample), and the moisture loss was established by weighing drying pans using a digital balance. Weight of sample was saved at 2 hours intervals until constant weight was achieved. All drying experiments were duplicated.
The dimensionless moisture ratio (MR) of the samples was determined using Eq. (1).

$$\mathrm{MR} = \frac{M - M_e}{M_0 - M_e} \qquad (1)$$

where, M_0 is the initial moisture content, M_e is the equilibrium moisture content and M is the moisture content at time t. The moisture content of the samples were determined using an infrared moisture analyser (Shimadzu, MOC 63u, Japan) at 90°C as two parallels and the results were confirmed by the standard oven method. To determine drying characteristics the experimental data was fitted to ten different thin layer drying models given in Table 1.
Fick's second law of diffusion for an infinite slab was used to describe moisture diffusion (Eq. 2) and it was assumed that the moisture was migrating only by diffusion; shrinkage and external mass transfer resistance were negligible.

$$\mathrm{MR} = \frac{M - M_e}{M_0 - M_e} = \frac{8}{\pi} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} exp\left[-(2n+1)^2 \frac{\pi^2}{4} \frac{D_{eff} t}{L^2}\right] \qquad (2)$$

where, D_{eff} is the effective diffusion coefficient and L is the thickness of the slab. At sufficiently large drying times, only the first term of the Eq. (2) is taken into consideration to calculate the effective diffusion coefficient (Eq. 3).

$$\ln \mathrm{MR} = In \frac{8}{\pi^2} - \frac{\pi^2 D_{eff} t}{4L^2} \qquad (3)$$

2.3. Adsorption characteristics of kefir powder

Moisture adsorption isotherms of kefir powder were established by a gravimetric method at ten relative humidities between the range of 0.11 and 0.88. To obtain desired relative humidity, hermetic jars containing saturated salt solutions of LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaBr, $SrCl_2$, NaCl, KCl and $BaCl_2$ were prepared (GREENSPAN, 1977; SPIESS and WOLF, 1983). A test tube consisting of 5 ml toluene was put into every jar

which had relative humidity higher than 50 % to avoid microbial growth (LABUZA, 1984). Triplicate samples of ~0.3 g of kefir powder were put into measuring bottles and afterward into glass jars containing the saturated salt solutions. The glass jars were placed in controlled temperature cabinets at selected temperatures of 5, 25 and 35°C. The samples were weighed at certain intervals until a change of less than 0.001 g was observed. The equilibrium moisture content (EMC) of samples was obtained by drying in an oven at 102°C for 2 hours (AOAC, 1990). The experimental sorption data at three temperatures was fitted to the most widely used sorption models given in Table 2.

Table 1. Equations for different thin layer drying models.

Model name	Model equation	Reference
Midilli *et al.*	$\mathrm{MR} = a\,exp(-kt^n) + bt$	MIDILLI *et al.* (2002)
Parabolic	$\mathrm{MR} = a + bt + ct^2$	SHARMA and PRASAD (2004)
Wang and Singh	$\mathrm{MR} = 1 + at + bt^2$	WANG and SINGH (1978)
Logarithmic	$\mathrm{MR} = a\,exp(-kt) + c$	GOYAL *et al.* (2007)
Verma	$\mathrm{MR} = a\,exp(-kt) + (1-a)\exp(-bt)$	VERMA *et al.* (1985)
Page	$\mathrm{MR} = exp(-kt^n)$	ZHU and XINQI (2014)
Two term exponential	$\mathrm{MR} = a\,exp(-kt) + (1-a)\exp(-kat)$	SHARAF-ELDEEN *et al.* (1980)
Henderson and Pabis	$\mathrm{MR} = a\,exp(-kt)$	WANG *et al.* (2007)
Two term	$\mathrm{MR} = a\,exp(-k_0 t) + b\,exp(-k_1 t)$	MADAMBA and DRISCOLL (1996)
Lewis	$\mathrm{MR} = exp(-kt)$	AVHAD and MARCHETTI (2016)

Table 2. Adsorption models for kefir powder.

Model name	Model equation	References
GAB	$\mathrm{M} = \frac{M_0 C K a_w}{[(1-Ka_w)(1-Ka_w+CKa_w)]}$	VAN DEN BERG (1985)
BET	$\mathrm{M} = \frac{M_0 c a_w}{[(1-a_w)+(c-1)(1-a_w)a_w]}$	BRUNAUER *et al.* (1938)
Oswin	$\mathrm{M} = k(a_w/1-a_w)^n$	OSWIN (1946)
Halsey	$a_w = exp(-k/M^n)$	HALSEY (1948)
Modified Halsey	$a_w = exp(-\exp(kT+c)M^{-n})$	IGLESIAS and CHIRIFE (1976c)
Henderson	$1-a_w = exp(-kTM^n)$	HENDERSON (1952)
Modified Henderson	$\mathrm{M} = \{ln(1-a_w)\,/\,[-k(T+c)]\}^{(1/n)}$	HENDERSON (1952)
Chen	$a_w = exp[k - c\,exp(-bM)]$	CHEN (1971)
Peleg	$\mathrm{M} = (k\,{a_w}^n) + (c\,{a_w}^b)$	PELEG (1993)
Iglesias–Chirife	$ln\left[M + (M^2 + M_{0.5})^{1/2}\right] = k\,a_w + c$	IGLESIAS and CHIRIFE (1978)

2.4. Thermodynamic properties of adsorption

The net isosteric heat (differential enthalpy, q_{st}) and differential entropy (ΔS) of sorption can be determined from Clausius-Clapeyron equation (Eq. 4) for constant EMC and with the assumption of heat of vaporization and heat of sorption don't change with temperature (AL-MUHTASEB *et al.*, 2004).

$$\left(\frac{\partial(\ln a_w)}{\partial(1/T)}\right)_{X_e} = -\frac{q_{st}}{R} \quad (4)$$

where, q_{st} is the net isosteric heat of sorption (kJ/mol), T is the absolute temperature (K), and R is the universal gas constant (8.314×10^{-3} kJ/mol K). The Eq. (5) was obtained by integration of the Clausius-Clapeyron equation. Net isosteric heat (q_{st}) and differential entropy (ΔS) were determined by generating ln (a_w) versus $1/T$ graph for a selected EMC (X_e) of material and then q_{st} was calculated from the slope and ΔS was determined from the intercept.

$$\ln a_w = -\left(\frac{q_{st}}{R}\right)\left(\frac{1}{T}\right) + \frac{\Delta S}{R} \quad (5)$$

The enthalpy-entropy compensation theory suggests a linear relationship between the differential entropy (ΔS) and enthalpy (q_{st}) (Eq. 6) for a specific reaction.

$$q_{st} = T_\beta \Delta S + \Delta G_\beta \quad (6)$$

where T_β s the isokinetic temperature and ΔG_β is the Gibbs free energy at the specific temperature of T_β. The isokinetic temperature was compared with the harmonic mean temperature (T_{hm}) in order to validate the enthalpy-entropy compensation theory.

$$T_{hm} = \frac{n}{\sum_{i=1}^{n}(1/T)} \quad (7)$$

where, n is the number of isotherms. The compensation theory only applies if $T_\beta \neq T_{hm}$. If $T_\beta > T_{hm}$ the process is controlled by enthalpy, while if $T_\beta < T_{hm}$ the process is entropy driven (NOSHAD *et al.*, 2012).
Gibbs free energy (ΔG, kJ/mol) was determined using the change in a_w data estimated by the GAB model and was calculated according to Eq. (8) (YAZDANI *et al.*, 2006).

$$\Delta G = R \cdot T \cdot \ln(a_w) \quad (8)$$

2.5. Data analysis

Nonlinear regression analysis was used to evaluate the drying and adsorption model parameters using MATLAB software version 9.1.0 (MathWorks Inc., USA). To evaluate the quality of fit, regression coefficient (R^2), root mean square error (RMSE), chi-square (χ^2) and the mean relative percentage deviation (P %) between predicted and experimental values were calculated using Eq.s (9), (10), (11) and (12), respectively.

$$R^2 = \frac{\sum_{i=1}^{N}(M_i - M_{pre,i}).(M_i - M_{exp,i})}{\sqrt{\left[\sum_{i=1}^{N}(M_i - M_{pre,i})^2\right].\left[\sum_{i=1}^{N}(M_i - M_{exp,i})^2\right]}} \quad (9)$$

$$\text{RMSE} = \left[\frac{1}{N}\sum_{i=1}^{N}(M_{exp,i} - M_{pre,i})^2\right]^{1/2} \quad (10)$$

$$\chi^2 = \frac{\sum_{i=1}^{N}(M_{exp,i} - M_{pre,i})^2}{N-n} \quad (11)$$

$$P\ (\%) = \frac{100}{N}\sum_{i=1}^{N}\frac{\left|M_{exp,i}-M_{pre,i}\right|}{M_{exp,i}} \qquad (12)$$

where, M_{exp} and M_{pre} express the experimental and predicted moisture ratio for drying and moisture content for adsorption process, respectively. N is the number of observations and n is the number of constants of the model. The best models defining the thin-layer drying and adsorption behaviour were chosen as the one with the highest R^2, and the lowest RMSE and χ^2 value. A model was accepted to be valid if the *P* value was lower than 10 % for the adsorption process (KAYMAK-ERTEKIN and GEDIK, 2004).

3. RESULTS AND DISCUSSION

3.1. Drying characteristics

The drying characteristic of kefir during freeze drying was evaluated using the moisture ratio data and the change in the moisture ratio of samples versus time was illustrated in Fig. 1. Obviously, the higher level of weight reduction happens in the beginning periods of the drying and the moisture content decreased extensively with increasing drying time. Total drying time was achieved to be as 10 hours by monitoring the changes in the weight of the samples. The equilibrium moisture content was found to be 4.58 (±0.20) %. It was observed that the constant rate period was absent and the complete drying process of kefir took place during the falling rate period (Fig 1).

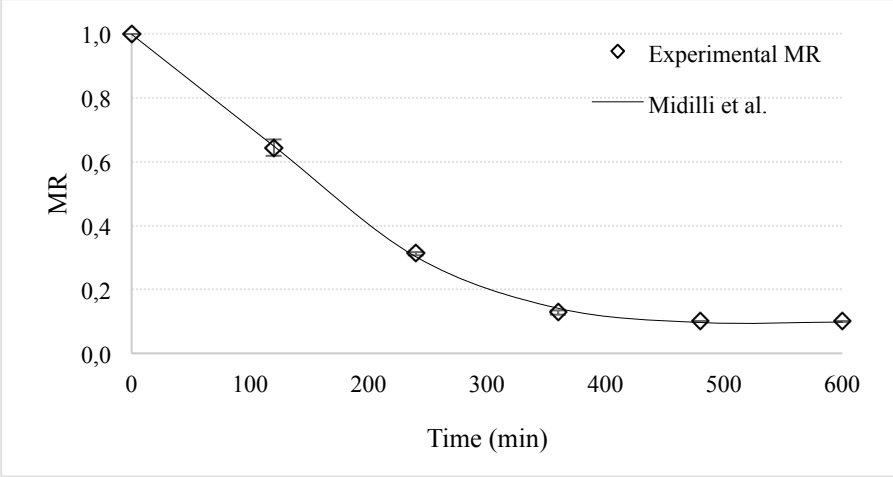


Figure 1. Experimental and predicted moisture ratios for freeze dried kefir.

The curve fitting processes were performed with ten well-known thin layer drying models. Estimated model constants and curve fitting data including regression coefficient (R^2), root mean square error (RMSE) and chi-square (χ^2) values are given in Table 3. The model giving the highest R^2, and the lowest RMSE and χ^2 values was considered as the best model defining the drying characteristic of kefir as in different studies (ERGÜN *et al.*, 2016; İZLI, 2017; ANTAL and KEREKES, 2016). According to the selection criteria, MIDILLI *et al*. model was found to be the best model representing the drying characteristics of kefir with its highest R^2 (0.999), and the lowest RMSE (0.012) and χ^2 ($1.51*10^{-4}$) values. Likewise, MIDILLI *et al*. model was selected as the best model for defining the freeze drying

behaviour of soluble coffee (GHIRISAN *et al.*, 2017) and kiwi (İZLI *et al.*, 2017). Furthermore, Parabolic and Wang and Singh models were also satisfactory for describing the freeze drying characteristics of the kefir owing to high R^2, and low RMSE and χ^2 values (Table 3). Page and Two-term exp. models which gave fairly well statistical results were also reported by some researchers as the most appropriate model for freeze drying processes (İZLI, 2017; MARQUES and FREIRE, 2005; ACAR *et al.*, 2015; İZLI *et al.*, 2018), but they did not exactly match the experimental data in the current study.
The effective moisture diffusivity (D_{eff}) of the kefir was achieved from Fick's Law of Diffusion model and calculated from the slope of the natural logarithm of MR (ln MR) against drying time (s) plot, and found to be as 2.61×10^{-10} m^2/s.

Table 3. Estimated parameters and statistical analysis of drying models.

Model	Parameters	R^2	RMSE	$\chi^2 * 10^4$
Midilli *et al.*	a= 0.999 b= 0.0001558 k= 0.0003095 n= 1.526	0.999	0.012	1.51
Parabolic	a= 1.01 b= -0.003731 c= 3.711*10-6	0.997	0.028	7.69
Wang and Singh	a= -0.00367 b= 3.634*10-6	0.996	0.025	6.08
Two term	a= 0.9523 b= 0.06838 k0= 0.004697 k1= 0.004699	0.985	0.071	49.81
Page	k= 0.001535 n= 1.2	0.990	0.042	17.35
Logarithmic	a= 1.026 c= -0.005806 k= 0.004633	0.985	0.058	33.14
Henderson	a= 1.021 k= 0.004699	0.985	0.050	24.91
Two term exp.	a= 1.819 k= 0.006567	0.991	0.040	15.80
Lewis	k= 0.004611	0.985	0.046	20.90
Verma	a= 1.008 b= 0.004233 k= 0.004608	0.985	0.059	34.83

3.2. Adsorption characteristics

Adsorption isotherms were constructed in which the moisture bound by adsorption per unit weight was plotted against water activity (Fig 2). As seen in Fig. 2, the moisture adsorption isotherms of kefir powder are temperature-dependent and the sorption ability of the samples was lower at higher temperatures. At increasing temperatures, the binding forces decrease; hence less moisture is absorbed at the same water activity at higher temperatures (MULET *et al.*, 2002). The decreasing sorptivity of kefir powder with increasing temperature can be also explained by its rich composition in terms of proteins and polysaccharides which have higher water-binding capacity at low temperatures in comparison with high temperatures (KAYMAK-ERTEKIN and GEDIK, 2004). This demonstrates the kefir powder turned out to be less hygroscopic at high temperatures. These results are similar to those of other studies about the obtaining of sorption isotherms

of yoghurt powder (KOÇ *et al.*, 2010; STENCL, 2004; KUMAR and MISHRA, 2006). The type III isotherm was observed for kefir powder, which indicated that binding energy was so high that water activity was suppressed while water was absorbed. This phenomenon is characteristic for these types of materials and when all the binding sites are filled, the increase in moisture content causes a significant increase in water activity (RAHMAN, 1995). Bioactive peptides and exopolysaccharides are the main components of kefir and their concentration depends on milk and the applied culture type (BARUKČIĆ *et al.*, 2017). Foods rich in soluble components such as kefir were found to exhibit type III behaviour (RAO and RIZVI, 1995).

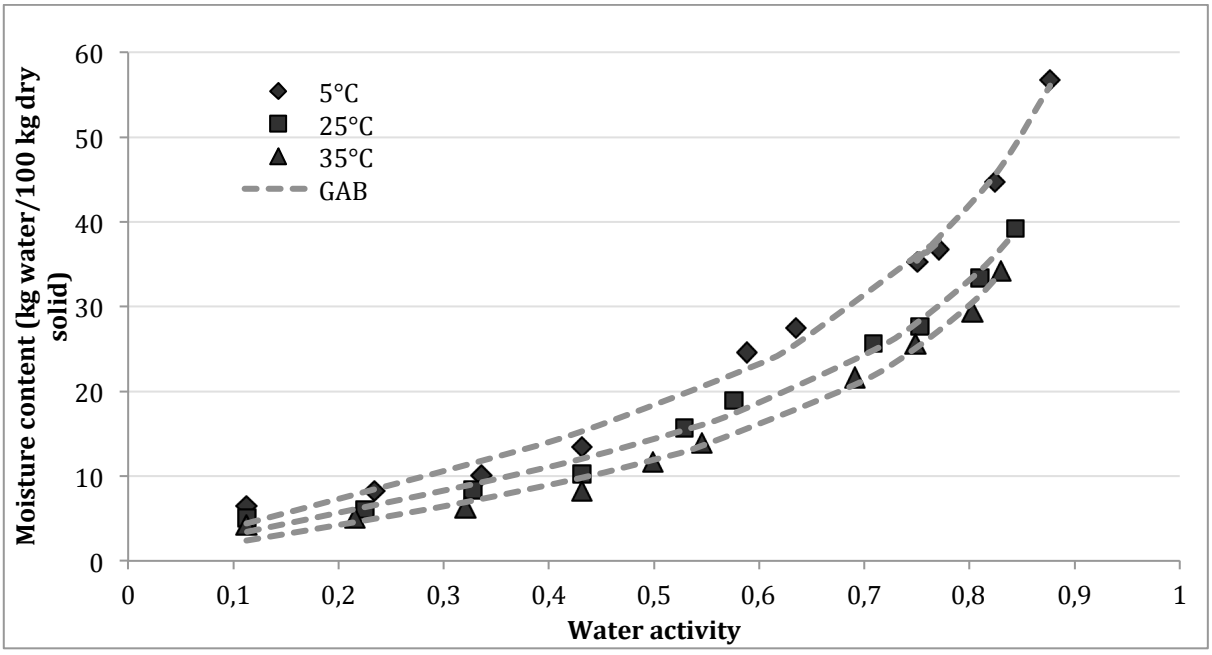


Figure 2. Moisture adsorption isotherms of kefir powder at different temperatures.

To represent water adsorption characteristics of kefir powder, the sorption data were fitted to ten sorption isotherm equations. Non-linear regression analysis was used to determine the best-fitted values of model constants and they are given in Table 4 and Table 5 for the model that account for temperature effect or not. The regression coefficient (R^2), root mean square error (RMSE) and the mean relative percentage deviation (P %) are also represented in Table 4 and Table 5 as the fitting criteria. The selection criteria of the most appropriate adsorption model were the simplicity of the model and the degree of fit to the experimental data. It can be seen that GAB, Oswin and Peleg equations gave the best fit to the experimental data of kefir powder (Table 4).

Peleg equation is a four-parameter, easy-to-solve semi-empirical model, and is based on the explicit denial of water monolayer presence (PELEG, 1993). Although this equation has been successfully applied at all temperatures in a wide range of water activity at all types of isotherms (MOREIRA *et al.*, 2005), it gave higher P (%) value (> 10%) at 5°C (Table 4).

Oswin equation is an empirical model and considered as the best model for defining the isotherms of starchy foods (OSWIN, 1946). Both Oswin and GAB equations showed a high goodness of fit with R^2> 0.99 and low P (< 10%) values for all temperatures (Table 4).

Table 4. Estimated parameters and statistical analysis of the sorption models which are invariant with temperature.

Model	Parameters	5°C	25°C	35°C
GAB	M_0	13.530	10.94	9.776
	C	3.742	3.509	2.510
	K	0.885	0.881	0.899
	R^2	0.992	0.991	0.992
	P (%)	8.635	8.438	9.591
	RMSE	1.714	1.283	1.124
*BET	M_0	7.884	6.146	5.963
	C	17.220	18.000	8.373
	R^2	0.964	0.968	0.921
	P (%)	5.122	4.812	10.439
	RMSE	0.698	0.508	0.971
Oswin	k	18.320	14.290	11.890
	n	0.581	0.601	0.670
	R^2	0.992	0.991	0.992
	P (%)	8.500	8.145	8.116
	RMSE	1.640	1.199	1.050
Halsey	k	15.910	11.86	8.450
	n	1.120	0.601	0.670
	R^2	0.985	0.984	0.988
	P (%)	10.257	9.767	8.330
	RMSE	0.034	0.034	0.030
Chen	k	-0.119	-0.155	-0.206
	c	2.492	2.637	2.718
	b	0.078	0.110	0.148
	R^2	0.976	0.976	0.972
	P (%)	13.894	12.879	15.049
	RMSE	0.046	0.045	0.047
Peleg	k	75.470	8.715	48.110
	n	10.800	0.278	2.859
	c	45.140	47.850	5.040
	b	1.238	2.863	0.097
	R^2	0.991	0.992	0.995
	P (%)	10.832	4.756	2.866
	RMSE	1.996	1.306	0.813
Iglesias–Chirife	$M_{0.5}$	19.113	13.679	11.725
	k	2.785	2.746	2.901
	c	2.211	1.995	1.758
	R^2	0.989	0.989	0.985
	P (%)	7.415	7.450	8.684
	RMSE	0.029	0.508	0.033

*a_w range for BET equation was 0.11-0.50. The first four data points were used.

GAB equation is a semi-theoretical and multi-molecular and homogeneous adsorption model and capable of application to situations over a wide variety of conditions. Therefore, it is the most suitable equation for the design of drying process because of its reliability, simple mathematical form, and the possibility of being used in a wide range (AL-MUHTASEB *et al.*, 2002). GAB equation includes the monolayer moisture content (M_0) as well as the C and K constants associated with the energies of interaction between first and further molecules at the particular sorption sites (BASTIOĞLU *et al.*, 2017); hence the predicted EMC values using GAB model was selected and shown in Fig. 2 because of the physical meaning of the coefficients in the GAB model. The monolayer moisture contents of kefir powder were determined in the range of ~9.8-13.5 kg water/100 kg dry solid and decreased as temperature increased from 5 to 35°C. These M_0 values indicate the theoretical moisture at which kefir powder could present maximum stability during storage. It was observed that the C parameter decreased with increasing temperature (Table 4) and it could be explained by the strong adsorbent-adsorbate interaction at lower temperature. The K parameter assumes that multilayer molecules have interactions with the sorbent and it is in tendency to decrease between energy values of the molecules in the monolayer and liquid water (AL-MUHTASEB *et al.*, 2002). All K values of kefir powder were found to be higher than 0.88, and these high values of K show that the monolayer and multilayer molecules were not very different and that the multilayer molecules act like liquid water. The multilayers have the same properties of liquid water when the K value is equal to 1, hence the sorption behaviour can be modelled by BET equation (GABAS *et al.*, 2007). BET equation also provides an estimation of the monolayer moisture adsorbed on the surface and according to BET equation M_0 values were in the range of ~6.0-7.9 kg water/100 kg dry solid. It was observed that the M_0 values achieved by the GAB model were higher than that achieved by the BET equation (Table 4). BASU *et al.* (2006) stated that the M_0 value estimated by the BET model is always lower than the monolayer value given by GAB and similar results were achieved by KOÇ *et al.* (2010) for yoghurt powder produced using spray drying process. Although BET equation is one of the most effective methods for evaluating the measure of bound water to particular polar sites in dry foods, it gave the lowest R^2 values among the tested models (Table 4).
The Halsey model is favourable for the materials of types I, II, and III and Iglesias-Chirife has been effectively used for type III isotherms (i.e. foods rich in soluble components) (AL-MUHTASEB *et al.*, 2004). Halsey and Iglesias-Chirife equations gave a satisfying estimation of the adsorption for a wide range of water activity (0.11-0.88) (Table 4). Modified Halsey, Henderson and Modified Henderson, which account for temperature effect failed to describe the sorption behaviour at high temperature (Table 5). Furthermore, Chen equation failed to predict the sorption characteristic at all temperatures due to high mean relative percentage deviation values (>10%).

3.3. Thermodynamic properties

Thermodynamic properties of foods give a comprehension of hygroscopic properties and energy requirements related with the sorption behaviour; hence they are important parameters for both processing and storage. Isosteric heat of sorption ensures an indication of the state of the sorbed water and gives information about the stability of the food during handling, storage and processing. Also, the change in heat of sorption with moisture content enables important data for energy consumption calculations and following drying equipment designs (TOLABA *et al.*, 2004). The net isosteric heat of sorption (q_{st}, kJ/mol) values were estimated using Clausius-Clapeyron equation with the equilibrium data at different temperatures and calculated at different equilibrium moisture contents. The variation of the isosteric heat of sorption of the kefir powder with

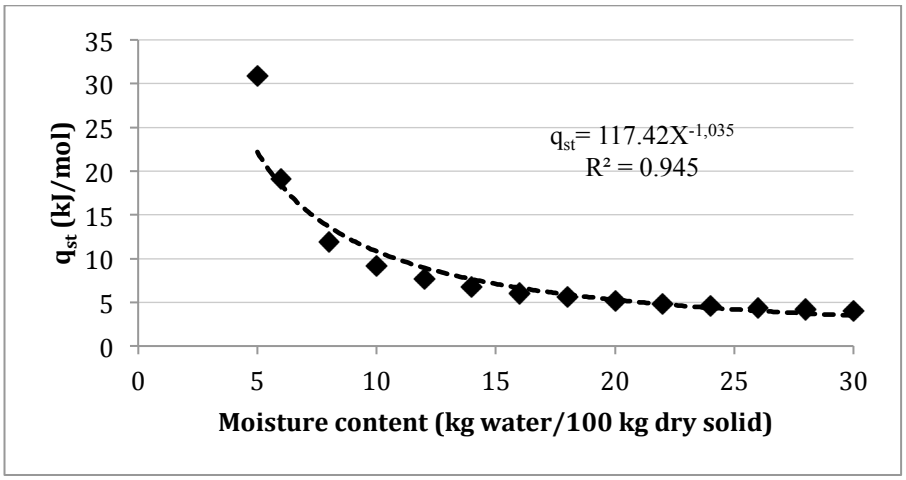
EMC is illustrated in Fig. 3a. The heat of sorption decreased with increasing moisture content. It was observed that the isosteric heat has a strong relationship with moisture content and decreased with a steep slope at lower moisture contents (5-10 kg water/100 dry solid). Sorption initially occurs in highly active polar sites on the surface with the highest interaction energy and pursue by the progressive filling of the less favourable sites with lower binding activation energies (SINIJA and MISHRA, 2008). As the moisture content increases, the net heat of adsorption approaches to zero, hence the heat of adsorption tends to that of pure water (MOREIRA *et al.*, 2008). Similar patterns have been accounted for the net isosteric heats of other milk-based materials (RÜCKOLD *et al.*, 2000; YU and LI, 2012; SAWHNEY *et al.*, 2014; TADAPANENIA *et al.*, 2017).

Table 5. Estimated parameters and statistical analysis of the sorption models that account for temperature effect.

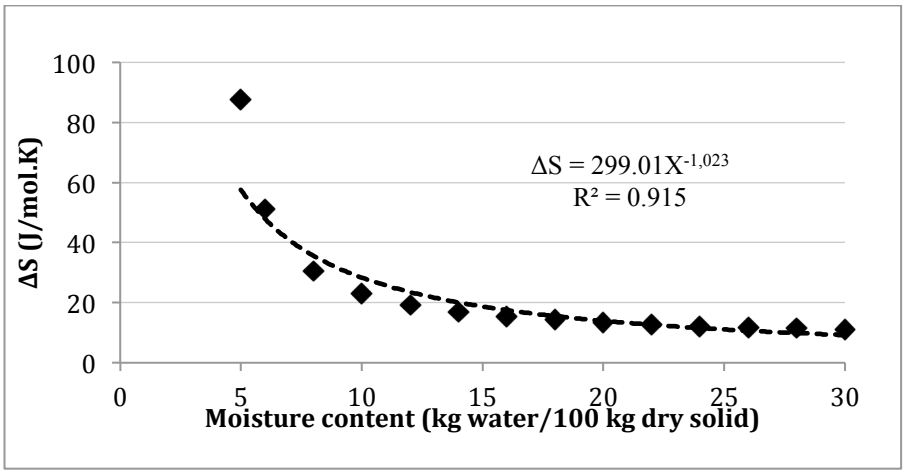
Model	Parameters	5°C	25°C	35°C
Modified Halsey	k	0.130	0.107	-0.092
	c	-33.410	-29.500	30.000
	n	1.150	1.095	0.905
	R^2	0.985	0.984	0.975
	P (%)	8.984	9.749	14.767
	RMSE	0.037	0.036	0.045
Henderson	k	0.0001020	0.0001230	0.0001773
	n	1.087	1.089	1.005
	R^2	0.981	0.979	0.972
	P (%)	9.847	9.218	11.837
	RMSE	0.039	0.039	0.044
Modified Henderson	k	0.0001426	0.0001340	0.0002119
	c	-87.500	-32.590	-46.640
	n	1.100	1.100	1.000
	R^2	0.981	0.979	0.972
	P (%)	9.797	9.451	11.687
	RMSE	0.041	0.041	0.047

The estimated differential entropy values showed a tendency to stabilize at high moisture contents as similar to the isosteric heat of sorption pattern (Fig. 3b).
The entropy of a material is proportional to the quantity of sorption sites present at a given energy level, which gives an indication of the mobility state of water molecules (TELIS *et al.*, 2000). At higher EMC values of kefir powder, the active sites on the surface were occupied by the water molecules, so they had less capacity for sorption and therefore entropy was reduced.
Enthalpy-entropy compensation theory is used to investigate physical and chemical phenomena such as sorption reactions. According to this theory, the relationship between the enthalpy and entropy for a given reaction is linear as a result of the nature of the solute and solvent interaction, which causes the reaction (FASINA, 2006).

a



b



c

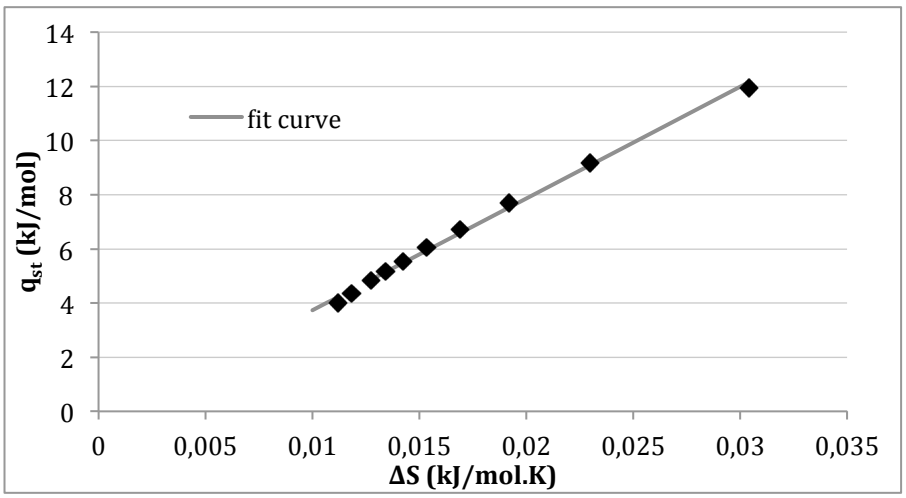


Figure 3. Enthalpy-entropy compensation a) isosteric heat of adsorption, b) differential entropy versus moisture content, c) relationship between enthalpy and entropy. Dashed lines represent the estimated values obtained by a power law relation.

Fig. 3c shows a strong correlation between isosteric heat and differential entropy for the adsorption, which indicates that compensation exists and confirms the enthalpy-entropy compensation theory. The isokinetic temperature (T_β) indicates the temperature at which all reactions in the series continue at the same rate. The isokinetic temperature determined from the data by linear regression for adsorption was calculated as 412.7 K and the harmonic mean temperature (T_{hm}) was calculated as 294.1 K, hence a significant difference was observed, the suitability of the compensation theory was reconfirmed. Furthermore, as the T_β value was higher than T_{hm} the adsorption process of kefir powder was driven by enthalpy, hence the process was controlled by energy interactions in conjunction with chemical composition of the product (AZURA-NIETO and BERISTAIN-GUEVARA, 2007; CORRÊA *et al.*, 2012). The ΔG_β represents the Gibbs free energy at the isokinetic temperature and the negative sign of ΔG_β indicates that the water adsorption reaction is spontaneous, and the positive sign represents that it is a non-spontaneous process. The sign of ΔG_β was determined as negative (-393.3 J/mol) for kefir powder showing a spontaneous adsorption process.

Gibbs free energy is a thermodynamic parameter related to the amount of process free energy at constant temperature and pressure which is an indication of whether the water sorption is a spontaneous process and indicates the affinity of the sorbents to the water (TELIS *et al.*, 2000). Fig. 4 shows the estimated Gibbs free energy values as a function of the moisture content at each temperature during the adsorption process of kefir powder. Gibbs free energy decreased with increasing temperatures and EMC (Fig. 4). Additionally, Gibbs free energy was noticeably negative at all temperatures, thus showing that water adsorption is a spontaneous process. These results corroborate the findings of SOUZA *et al.* (2015), SILVA *et al.* (2014) and MUNIO *et al.* (2015) in their studies of mango skin, rosemary essential oil micro-particles and spray-dried cherimoya puree, respectively.

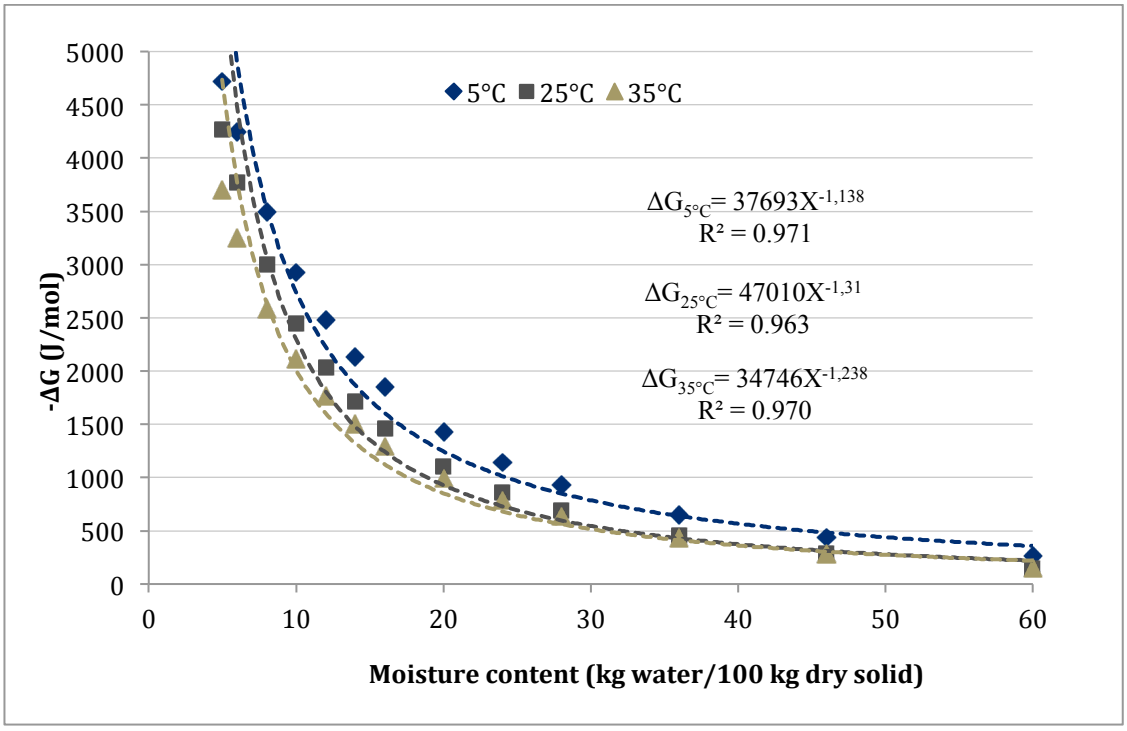


Figure 4. Gibbs free energy of kefir powder at different temperatures. Respective dashed lines indicate the estimated values obtained by a power law relation.

4. CONCLUSIONS

In this study, instant kefir powder was obtained by freeze drying process and drying kinetics of kefir were investigated. Midilli et al. model was chosen to describe the drying behaviour of kefir with great accuracy. To provide information about hygroscopic properties of kefir powder, moisture adsorption isotherms were achieved at different temperatures. The equilibrium moisture content increased with decreasing temperature at constant water activity. Among the sorption models, GAB and Oswin models described well the adsorption behaviour of kefir powder for all experimental conditions. Net isosteric heat and differential entropy estimated using the Clausius-Clapeyron equation increased with decreasing moisture content. Furthermore, compensation enthalpy-entropy theory was confirmed and it can be concluded that the adsorption mechanism for the kefir powder is controlled by enthalpy.
The Gibbs free energy decreased with increasing equilibrium moisture contents and temperatures, and showed that the moisture adsorption of kefir powder is a spontaneous process. It is thought that the data obtained in this study can be used to establish drying and storage conditions of instant kefir powder, which may be a new and alternative product.

REFERENCES

Acar B., Sadikoglu H. and Doymaz I. 2015. Freeze-Drying Kinetics and Diffusion Modeling Of Saffron (*Crocus Sativus* L.). J. Food Proces. Pres. 39:142.

Adamiec J., Kaminski W., Markowski A.S. and Strumillo C. 2015. Drying of Biotechnological Products in Handbook of Industrial Drying, 4th edn. (CRC Press, Taylor&Francis Group)

Al-Muhtaseb A.H., McMinn W.A.M. and Magee T.R.A. 2002. Moisture sorption isotherm characteristics of food products:A review. Food Bioproducts Proces. 80:118.

Al-Muhtaseb A.H., McMinn W.A.M. and Magee T.R.A. 2004. Water sorption isotherms of starch powders. Part 1:Mathematical description of experimental data. J. Food Eng. 61, 297.

Al-Muhtaseb A.H., McMinn W.A.M. and Magee T.R.A. 2004. Water sorption isotherms of starch powders: Part 2: Thermodynamic characteristics. J. Food Eng. 62 (2):135.

Anonymous. 1990. Official Methods for Analysis, Vol. 2, 15th ed.; AOAC. Arlington, VA.

Antal T. and Kerekes B. 2016. Investigation of Hot Air- And Infrared-Assisted freeze-Drying Of Apple. J. Food Process Pres. 40:257.

Arslan S. 2014. A review: chemical, microbiological and nutritional characteristics of kefir. CyTA- J. Food. 13:1.

Atalar I. and Dervisoglu M. 2015. Optimization of spray drying process parameters for kefir powder using response surface methodology. LWT - Food Sci. Technol. 60:751.

Avhad M.R. and Marchetti J.M. 2016. Mathematical modelling of the drying kinetics of Hass avocado seeds. Ind. Crops Prod. 91:76.

Azura-Nieto E. and Beristain-Guevara C.I. 2007. Thermodynamic and kinetic study of water adsorption on whey protein. Rev. Mex. Ing. Quím. 6:359.

Barukčić I., Gracin L., Jambrak A.R. and Božanić R. 2017. Comparison of chemical, rheological and sensory properties of kefir produced by kefir grains and commercial kefir starter. Mljekarstvo. 67 (3):169.

Bastıoğlu A.Z., Koç M. and Kaymak-Ertekin F. 2017. Moisture sorption isotherm of microencapsulated extra virgin olive oil by spray drying. Food Measure. 11:1295.

Basu S., Shivhare U.S. and Mujumdar A.S. 2006. Models for sorption isotherms for foods:A review. Dry. Technol. 24:917.

Brunauer S., Emmett P.H. and Teller E. 1938. Adsorption of gases in multimolecular layer. J. Am. Chem. Soc. 60:309.
Chen, C.S. 1971. Equilibrium moisture curves for biological materials. Transaction of the ASEA. 14:924.

Chifiriuc M.C., Cioaca A.B. and Lazar V. 2011. In vitro assay of the antimicrobial activity of kefir against bacterial and fungal strains. Anaerobe. 17:433.

Corrêa P.C., Oliveira G.H.H. and Santos E.S. 2012. Thermodynamic properties of agricultural products processes. In Physical properties of foods: Novel measurement techniques and applications, ed. by I. Arana, (CRC Press, Boca Raton), p.131-141

Ergün K., Çalıskan G., and Dirim S.N. 2016. Determination of the drying and rehydration kinetics of freeze dried kiwi (*Actinidia deliciosa*) slices. Heat Mass Transf. 52:2697.

Farnworth E.R. 2005. Kefir a complex probiotic. Food Sci. Technol. 2:1.

Fasina O.O. 2006. Thermodynamic properties of sweetpotato. J. Food Eng. 75:149.

Gabas A.L., Telis V.R.N., Sobral P.J.A. and Telis-Romero J. 2007. Effect of maltodextrin and arabic gum in water vapor sorption thermodynamic properties of vacuum dried pineapple pulp powder. J. Food Eng. 82:246.

Ghirisan A., Drăgan S. and Miclăuş V. 2017. Freeze-Drying Kinetics Approach Of Soluble Coffee: Mass Transfer Parameters Estimation. Studia Ubb Chemia. 1:7.

Goyal R.K., Kingsly A.R.P., Manikantan M.R. and Ilyas S.M. 2007. Mathematical modelling of thin layer drying kinetics of plum in a tunnel dryer, J. Food Eng. 79 (1):176.

Greenspan L. 1977. Humidity fixed points of binary saturated aqueous solutions. J. Res. Natl. Bur. Stand. 81:89-112.

Gronnevik H., Falstad M. and Narvhus J.A. 2011. Microbiological and chemical properties of Norwegian kefir during storage. Int. Dairy J. 21:601.

Halsey G. 1948. Physical adsorption on non-uniform surfaces. J. Chem. Phys. 16:931.

Henderson S.M. 1952. A basic concept of equilibrium moisture. Agric. Eng. 33:29.

Iglesias H.A and Chirife J. 1976c. Prediction of the effect of temperature on water sorption isotherms of food material. J. Food Technol. 11:109.

Iglesias H.A. and Chirife J. 1978. An empirical equation for fitting water sorption isotherms of fruits and related products. Can. Inst. Food Sci. Technol. J. 11:12.

İzli G. 2017. Total phenolics, antioxidant capacity, colour and drying characteristics of date fruit dried with different methods. Food Sci. Technol. 37(1):139.

Izli N., Izli G. and Taskin O. 2017. Drying kinetics, colour, total phenolic content and antioxidant capacity properties of kiwi dried by different methods. Food Measure. 11:64.

Izli N., Izli G. and Taskin O. 2018. Impact of different drying methods on the drying kinetics, color, total phenolic content and antioxidant capacity of pineapple. CYTA- J. Food. 16 (1):213.

Kaymak-Ertekin F. and Gedik A. 2004. Sorption isotherms and isosteric heat of sorption for grapes, apricots, apples and potatoes. LWT - Food Sci. Technol. 37(4):429.

Kaymak-Ertekin F. and Sultanoglu M. 2001. Moisture sorption isotherm characteristics of peppers. J. Food Eng. 47:225.

Koç B., Sakin-Yilmazer M., Balkır P. and Kaymak-Ertekin F. 2010. Spray drying of yoghurt: optimization of process conditions for improving viability and other quality attributes. Dry. Technol. 28:495.

Kumar P. and Mishra H.N. 2004. Yogurt powder. A review of process technology, storage and utilization. Food Bioprod. Process. 82 (2):133.

Kumar P. and Mishra H.N. 2006. Moisture sorption characteristics of mango–soy-fortified yogurt powder. Int. J. Dairy Technol. 59:22.

Labuza T.P. 1984. Moisture Sorption: Practical Aspects of Isotherm Measurement and Use, AACC. St. Paul.

Madamba P.S., Driscoll R.H. and Buckle K.A. 1996. The thin-layer drying characteristics of garlic slices. J. Food Eng. 29 (1):75.

Marques L.G. and Freire J.T. 2005. Analysis of Freeze-Drying of Tropical Fruits. Dry. Technol. 23:2169.

Midilli A., Kucuk H. and Yapar Z. 2002. A new model for single-layer drying. Dry. Technol. 20 (7):1503.

Moreira R., Chenlo F., Torres M.D. and Vallejo N. 2008.Thermodynamic analysis of experimental sorption isotherms of loquat and quince fruits. J. Food Eng. 88:514.

Moreira R., Chenlo F., Vázquez M.J. and Cameán P. 2005. Sorption isotherms of turnip top leaves and stems in the temperature range from 298 to 328 K. J. Food Eng. 71:193.

Mulet A., Garcia-Pascual P., Sanjuán N. and Garcia-Reverter J. 2002. Equilibrium isotherms and isosteric heats of morel (*Morchella esculenta*). J. Food Eng. 53 (1):75.

Munio M.M., Guadix E.M. and Guadix A. 2015. Modeling of Water Sorption Isotherms Characteristics of Spray-Dried Cherimoya (*Annona cherimola*) Puree. Particl. Sci. Technol. 33:264.

Noshad M., Mohebbi M., Shahidi F. and Mortazavi S.A. 2012. Effect of osmosis and ultrasound pretreatment on the moisture adsorption isotherms of quince. Food Bioprod. Process. 90:266.

Oswin C.R. 1946. The kinetics of packing life. III. The isotherm. J. Chem. Ind. 65:419.

Peleg M. 1993. Assessment of a semi-empirical four parameter general model for sigmoid moisture sorption isotherms. J. Food Process. Eng. 16:21.

Rahman S. 1995. Water activity and sorption properties of foods, in Food Properties Handbook ed. By S. Rahman, (CRC Press, Boca Raton, FL), pp. 1–86

Rao M.A. and Rizvi S.S.H. 1995. Engineering Properties of Foods (Marcel Dekker Inc, New York, USA).

Rizvi S.S.H. 1995. Thermodynamic properties of foods in dehydration. In Engineering Properties of Foods; ed. by M. Dekker (New York) pp. 223-309.

Rückold S., Grobecker K.H. and Isengard H.D. 2000. Determination of the contents of water and moisture in milk powder. Fresenius J. Anal. Chem. 368:522.

Sawhney I.K., Sarkar B.C., Patil G.R. and Sharma H.K. 2014. Moisture sorptıon isotherms and thermodynamic properties of whey protein concentrate powder from buffalo skim milk. J. Food Proces. Pres. 38:1787.

Sharaf-Eldeen Y.I., Blaisdell J.L. and Hamdy M.Y. 1980. A model for ear corn drying, Transactions of the ASAE. 23 (5):1261.

Sharma G.P. and Prasad S. 2004. Effective moisture diffusivity of garlic cloves undergoing microwave-convective drying. J. Food Eng. 65 (4):609.

Silva E.K., Fernandes R.V.B., Borges S.V., Botrel D.A. and Queiroz F. 2014. Water adsorption in rosemary essential oil microparticles: Kinetics, thermodynamics and storage conditions. J. Food Eng. 140:39.

Sinija V.R. and Mishra H.N. 2008. Moisture sorption isotherms and heat of sorption of instant (soluble) green tea powder and green tea granules. J. Food Eng. 86:494.

Souza S.J.F., Alves A.I., Vieira E.N.R. Vieira J.A.G., Ramos A.M. and Telis-Romero J. 2015. Study of thermodynamic water properties and moisture sorption hysteresis of mango skin. Food Sci. Technol. 35 (1):157.

Spiess W.E.L. and Wolf W. 1983. The results of the COST 90 project on water activity, in Physical properties of foods ed. By R. Jowitt, F. Escher, B. Hallström, H. Mefert, W. Spiess, G. Vos (Elsevier Applied Science, London), pp. 65–91

Stencl J. 2004. Modelling the water sorption isotherms of yoghurt powder spray. Math. Comput. Simul. 65:157.

Tadapanenia R.K., Yanga R., Carterb B. and Tanga J. 2017. A new method to determine the water activity and the net isosteric heats of sorption for low moisture foods at elevated temperatures. Food Res. Int. 102:203.

Tamime A.Y., Wszolek M., Božanić R. and Özer B. 2011. Popular ovine and caprine fermented milks. Small Ruminant Res. 101:2.

Telis V.R.N., Gabas A.L., Menegalli F.C. and Telis-Romero J. 2000. Water sorption thermodynamic properties applied to persimmon skin and pulp. Thermochimica Acta. 343:49.

Tolaba M.P., Peltzer M., Enriquez N. and Pollio M.L. 2004. Grain sorption equilibria of quinoa grains. J. Food Eng. 61:365.

Van den Berg C. 1985. Development of B.E.T. like models for sorption of water of foods; theory and relevance, in Properties of water in foods, ed. by D. Simatos, J.L. Multon (Martinus Nijhoft Publishers, Dordrecht), pp. 119–135.

Verma L.R., Bucklin R.A., Endan J.B. and Wratten F.T. 1985. Effects of drying air parameters on rice drying models, Transactions of the ASAE. 28 (1):296.

Wang C.Y. and Singh R.P. 1978. A single layer drying equation for rough rice, ASAE. 78:3001.

Wang X., Shi Q., Zhao Y., Wang X. and Zheng Y. 2013. Moisture Adsorption Isotherms And Heat Of Sorption Of Agaricus Bisporus. J. Food Process. Preserv. 37:299.

Wang Z., Sun J., Liao X., Chen F., Zhao G., Wu J. and Hu X. 2007. Mathematical modeling on hot air drying of thin layer apple pomace. Food Res. Int. 40 (1):39.

Yazdani M., Sazandehchi P., Azizi M. and Ghobadi P. 2006. Moisture sorption isotherms and isosteric heat for pistachio. Eur. Food Res. Technol. 223:577.

Yu H. and Li Y. 2012. Sorption isotherms and thermodynamic properties of freeze-dried colostral whey powders with different additives. Int. J. Food Sci. Technol. 47:613.

Zhu A. and Xinqi S. 2014. The model and mass transfer characteristics of convection drying of peach slices. Int. J. Heat Mass Transfer. 72:345.

Paper Received September 19, 2018 Accepted January 8, 2019

PAPER

QUALITY OF BROILER CHICKEN MEAT DURING FROZEN STORAGE

A. AUGUSTYŃSKA-PREJSNAR*[1], M. ORMIAN[1] and R. TOBIASZ-SALACH[2]
[1]University of Rzeszow, Faculty of Biology and Agriculture, Department of Animal Production and Poultry Products Evaluation, 35-601 Rzeszów, Poland
[2]University of Rzeszow, Faculty of Biology and Agriculture, Department of Crop Production, Rzeszow 35-601, Poland
*Corresponding author: augusta@ur.edu.pl

ABSTRACT

The objective of the study was to assess the influence of frozen storage (1mth and 8 mth) and thawing methods on the breast meat quality of broiler chickens. Tests revealed significant changes in meat quality in defrosted raw breast muscles as well as in those subjected to thermal treatment. Defrosting by using microwave oven proved to be more useful for breast muscles stored for a period of eight months regarding the volume of drip loss, ash content of the raw breast muscles as well as the cooking loss, desirable aroma, and the brittleness of meat subjected to thermal treatment.

Keywords: breast muscles, broiler chickens, freezing storage, quality, thawing methods

1. INTRODUCTION

The poultry meat market is a classic example of a market displaying increased diversification. Poultry meat remains a valuable component of human diet with its consumption in Europe steadily rising. Fast growing broiler chickens are dominant in poultry meat production due to their efficient feed use (AUGUSTYŃSKA-PREJSNAR *et al.*, 2014). The choice of poultry is greatly influenced by its nutritive and sensorial values, as well as its low price, bountiful supply, varied assortment, price relative to that of red meat and other animal products. The high popularity of poultry meat is also determined by its culinary values and the ease of preparation. In addition, the promotions and advertising methods, as well as the increasing awareness of the nutritive value of foods and proper dietary practice enhance the attractiveness of poultry meat as a food.
Quality issues have attracted greater attention in the much saturated market of meat products. A specific characteristic of meat products is their short shelf life (BENLI, 2016).
Majority of the poultry meat for culinary purposes in home markets is offered as frozen meat. Freezing is the most commonly applied method in managing its excess production. An accruing benefit of freezing in respect of the transportation of chilled meat is its possibility of prolonged storage as well as its greater handling flexibility in both retail and wholesale trade. Freezing of meat effects quality changes that are direct consequences of freezing processes and frozen storage (AKHTAR *et al.*, 2013). The frozen state does not entirely eliminate the bio-physicochemical processes taking place in meat, but only minimizes them. Freezing results in the slowing down or halting of post-slaughtering meat maturity processes, while in-meat water freezing and crystallization processes become intensified (LEYGONIE *et al.*, 2012, GAMBUTEANU *et al.*, 2013). The quality of frozen stored meat depends on on-going changes both during the initial pre-freezing and in later stages of freezing treatment and storage. The quality of frozen products depend on the quality of the primary material, adequate freezing parameters, storage conditions, stability of storage temperature and its duration as well as the applied thawing methods (ALI *et al.*, 2016; CHEN *et al.*, 2017; FERNANDES *et al.*, 2016).
The last stage in the chilling technological process is thawing, aimed at restoring the meat's properties to conditions similar to that of fresh meat. The procedural parameters like temperature and efficient thawing play significant roles during this stage. The thawing process is acclaimed to be more difficult to control than the freezing process. Any improprieties in the thawing process may result in quality deterioration (ZHANG *et al.*, 2017). Thawing in atmospheric air has become a popular method applied in meat thawing. The growing role in industry-led deep freezing of meat products necessitates the application of modern and fast methods of meat thawing in order to shorten the process and have a better control of its parameters. The microwave thawing procedure does fulfil this criteria (CHWASTOWSKA and KONDRATOWICZ, 2005).
The aim of the study was to assess the influence of frozen storage duration and thawing methods on the breast muscles quality of broiler chickens.

2. MATERIAL AND METHODS

The research materials were breast muscles obtained directly after slaughtering of minimum 36-day old ROSS 308 broiler chickens. The chickens were reared on litter and fed on complete standard feed mixtures (Starter, Grower and Finisher), for broiler chickens with full access to water supply. The average weight of slaughtered chickens was 2.30kg. The slaughter and dissection of chickens was carried out under prevailing production specifications at a local slaughterhouse. Breast muscles (*musculus pectoralis*)

were manually cut out from the chilled carcasses (ZIOŁECKI and DORUCHOWSKI, 1989). The samples (n=80 and mean weight of 200±50g) were weighed (balance type ED 423S-0CE, accuracy 0,001g, company Sartorius Mechatronics, Poland) labelled and vacuum packed in polyethylene bags. The meat was subjected to freezing at -20°C and were stored in deep freeze conditions in freezer compartments (GN 3056 of Liebherr Company, Germany) over a 1 month period for group I and 8 months for group II. On completing the storage period the samples from group I (n=40) and group II (n=40) were thawed in atmospheric air and in a microwave oven. The atmospheric air method involved thawing the sample under atmospheric conditions in refrigerated cabinet (FKv 36110, from Liebherr company, Germany) at 4°C without removing them from the bags until the samples achieved internal temperature of 4°C±1°C. The thawing process with a microwave involved placing the samples in a microwave oven (type 29Z013, 800 W, Zelmer Company, Poland) and subsequently subjected electromagnetic wave treatment over a 5 minute period. The samples internal temperature was 0°C±1°C after thawing, but was 5°C ±1°C on its outer Surface. The sample's internal temperature was stabilized at 4°C ±1°C.

The laboratory assessment of raw breast muscles from group I (n=20) and group II (n=20) covered the following: volume of drip loss, pH, water-holding capacity, chemical composition (protein, fat, and mineral salts content) as well as the meat's colouration and brittleness. The volume of drip loss was determined by placing the sample in a cuvette fitted with a 5 mm thick grid spacer to prevent the leakage from getting in contact with the sample. The amount of leakage was calculated by the difference in weight before freezing and after thawing process using the formula:

$$W_r(\%) = \left(\frac{M_1 - M_2}{M_1}\right) \times 100\%$$

where: Wr - the amount of drip loss (%), M_1 - sample weight before freezing (g), M_2 - sample weight after thawing (g) (MARCHEL *et al.*, 2013).

pH measurements were undertaken using a pH-meter fitted with a dagger electrode (HI 99163, Hanna Instruments, USA) calibrated with DuraCal buffer solutions: pH 4.01, pH 7.00. (Hmilton Bonaduz AG, Switzerland). The water-holding capacity (WHC) was determined based on the volume of free water squeezed from sample (280-300mg) using the Whatman no 2 filter papers and 2kg load for 5 minutes (GRAU and HAMM, 1953). WHC was calculated on the basis of the mass difference of the sample before squeezing to the mass of the sample after squeezing in relation to the sample weight before squeezing x 100%. The nitrogen content was determined using Kjeldah method (Foss Tecator, Höganäs, Sweden), and converted to protein using a multiplying factor of 6.25. The fat content was determined using Soxhlet method (Büchi Extraction System B-811 apparatus, Flawil, Switzerland). After drying the samples (5g±0.001g) at 105°C, they were subjected to extraction using n-hexan as the solvent. The fat amount was determined by weighing after the solvent has been separated. Total ash content was measured after the complete mineralization of 5g of the meat sample at 550-6505°C in a muffle Carbolite oven type AAF1100, Hope Valley, UK. The colour of the raw meat's cross-section was assessed using the Chrome Meter colorimeter (Konica Minolta Osaka, Japan), fitted with a CR 400 head (ø=11mm) set at illumination levels compatible with D_{65}, calibrated with Konica Minolta calibration plate (observer 2°, illuminant D_{65}, Y=93.5, x=0.3160, y=0.3324). The readings of measurement results and their conversion in real time was achieved in the CIE LAB (CIE, 1978) colorimetric system, where L* represents brightness, a* - relative redness, on red-green axis, b * - relative yellowness, on yellow-blue axis. Three repetitions were performed for each sample. The brittleness was assessed by measuring the share force (F_{max}), using the

Zwick/Roell BT1-FR1.OTH.D14 resistance machine (Zwick CmbH & Co. KG. Ulm, Germany), using a Warner-Bratzler V-blade knife with a head speed of 100 $mm \cdot \cdot min^{-1}$ and initial force of 0.2 N. Meat portions with cross-sectional diameter of 100 mm^2 and 50 mm in length were subjected to cutting. After the thawing of samples from both group I (n=20) and group II (n=20) they were weighed (balance type ED 423S-0CE, accuracy 0,001g, company Sartorius Mechatronics, Poland) with accuracy of 0.1g and then subjected to boiling at a ratio 1:2 (meat/ water) until the attainment of meat internal temperature of 805°C±25°C. Assessments of physicochemical properties after cooking were conducted in the same way to those applied for the raw breast muscles. Cooking losses were calculated based on the differences in weight prior to and after thermal treatment. The sensory assessment of the quality of thawed breast meat was conducted using the scaling method (BARYŁKO-PIKIELNA and MATUSZEWSKA 2009). A five-point scale assessment was applied, covering such quality indicators as the meat's aroma and flavour (especially its desirability and intensity), juiciness, brittleness and general appearance (1 point being the lowest score, and 5 points being the highest). In order to conduct the sensory assessment, the thermally treated samples were cooled to 205°C±25°C, cut into 1.5 cm thick slices, perpendicular to the run of meat fibers and then placed in plastic containers. The samples were randomly assessed after they had been encoded. The sensory assessment process was conducted, in two repetitions, by a 7-member assessment team with proven sensory sensitivity, trained in accordance with ISO 8586-2:2008 and ISO 8587:2006 standards.
The results obtained were statistically analysed using the Statistica 13.1 (STATSOFT, INC. 2018) program, taking account of the arithmetic mean ($\bar{x}$), standard deviation (SD), standard mean error of measurement (SEM), and the principal effect, namely (C - impact of storage duration, R - influence of thawing method, C x R - impact of storage duration and thawing method), using the two-factor analysis of variance ANOVA. The significance of differences between the mean values within groups was verified using the Tukey test. Statistically significant differences were assumed at a significance level of $p<0.05$, while the lack of significance was designated with „ns" (statistically not significant).

3. RESULTS AND DISCUSSION

The freezing process leads to the denaturation of myofibril proteins, which results in the deterioration of meat functional properties. As a result of the water transformation phase, there is increased concentration of ions, ionic strength, as well as pH changes (ALI *et al.*, 2015). The current studies have indicated significant ($p<0.05$) influence of frozen storage duration on the pH value (Table 1). According to CHWASTOWSKA-SIWIECKA (2011) and LEYGONIE *et al.* (2012) the pH of broiler chicken breast muscles in appropriate conditions decreases along with the prolongation of frozen storage. Likewise, results obtained by ALI *et al.* (2015), CHEN *et al.* (2017) and WEI *et al.* (2017) have indicated significant declines in pH value of breast muscles over subsequent weeks of frozen storage. SANTOS KUMAR *et al.* (2014), however, noted rising pH values of breast muscles of broiler chickens obtained from varied sources, stored frozen. The current studies did not indicate any significant ($p>0.05$) influence of thawing methods on the pH value. Similar results for the thawing methods, but with varying intensity of microwaves, were obtained by KIM *et al.* (2011) for broiler chickens breast muscles, CHWASTOWSKA-SIWIECKA *et al.* (2013) for rabbit meat, while CHWASTOWSKA and KONDARTOWICZ (2005) obtained same for pork meat. Drip loss is a watery solution that issues from frozen meat without the application of any external force serving as a significant indicator of the quality of meat subjected to frozen storage (GAMBUTEANU *et al.*, 2013; LEYGONIE *et al.*, 2012). It is acclaimed that the volume of meat leakages while being thawed using various

methods may serve as one indicator of the degree of damage to meat muscle tissues during freezing and also as an indirect evaluation of various thawing methods (CHWASTOWSKA and KONDRATOWICZ, 2005).

Table 1. Physicochemical properties of frozen raw breast muscles, including the frozen storage duration and thawing methods ($\bar{x}\pm$ s).

Traits	Frozen storage duration, months				SEM	Impact		
	(Group I)		(Group II)					
	Thawing method							
	atmospheric air	microwave oven	atmospheric air	microwave oven		C	R	C x R
pH	6.00±0.02	5.91±0.03	5.84±0.18	5.87±0.17	0.01	*	ns	ns
Drip loss (%)	3.34±0.30	2.78±0.1	5.04±0.28	4.74±0.35	0.14	*	*	*
Water-holding capacity (%)	15.58±1.37	16.11±1.54	18.34±2.47	17.72±1.23	0.20	*	ns	ns
Colour L*- lightness	53.12±2.30	52.07±2.48	50.32±1.85	50.60±2.04	0.49	*	ns	n
a*- redness	1.76±0.66	1.98±0.51	2.21±0.88	3.30±0.79	0.07	*	*	s*
b*- yellowness	6.06±1.07	6.58±1.39	7.32±1.28	8.04±1.62	0.18	*	*	ns
Fmax (N)	13.56±2.02	15.12±1.96	11.86±1.88	13.98±2.44	0.25	*	*	ns
Crude protein (%)	23.84±1.13	23.75±1.04	24.04±0.65	23.92±0.58	0.08	ns	ns	ns
Fat (%)	1.16±0.16	1.19±0.12	1.18±0.20	1.17±0.16	0.04	ns	ns	ns
Ash (%)	1.15±0.10	1.21±0.15	1.13±0.18	1.15±0.12	0.02	*	*	ns

Explanations: ($\bar{x}$ ± s) arithmetic mean±standard deviation, Group I - 1 month freezing storage (n=20) , Group II - 8 months freezing storage (n=20); atmospheric air (n=10), microwave oven (n=10); C - impact of frozen storage duration; R - impact of freezing method; C x R - impact of frozen storage duration and freezing method; *- statistically significant differences $p<0.05$; ns- differences statistically not significant.

The current studies have shown the significant ($p<0.05$) influence of frozen storage duration, thawing methods, including the interplay of these factors on the volume of drip loss (Table 1). The volume of drip loss increases proportionately with frozen storage duration. The study finding corroborates those obtained by ALI *et al.* (2016) with samples thawed in atmospheric air. Studies by WEI *et al.* (2017) indicated lower values for this factor, although with a similar increasing trend of the value for drip loss and with an 8-month long frozen storage. The current studies have shown that the amount of drip loss of breast muscles using the microwave method was significantly ($p<0.05$) lower than in case of meat thawed in atmospheric air. The results obtained are similar to those obtained by OLIVEIRA *et al.* (2015) in studies conducted using the same thawing methods on breast meat of broiler chickens and by CHWASTOWSKA *et al.* (2013) on rabbit meat as well as by CHWASTOWSKA and KONDRATOWICZ (2005) on pork meat. YU *et al.* (2005) has posted that the amount of drip loss is influenced by the thawing temperature, with the amount increasing with the growing temperature.

The current studies have indicated significant ($p<0.05$) impact of frozen storage duration on the water-holding capacity of the breast muscles. The results of measurements for the meat water-holding capacity, determined using the forced leakage method point to the existence of dependency between changes in the meat properties and the amount of drip loss. Greater rate of water loss during frozen storage stage limited the amount of forced

leakage from the meat, which could translate to mean better water absorption during prolonged meat storage, irrespective of the thawing method applied. The findings of the study corroborate those obtained in studies by WEI *et al.* (2017) conducted on the breast muscles of broiler chickens as well as those by CHWASTOWSKA and KONDRATOWICZ (2005) conducted on pork meat.

An important criterion of the meat's technological quality is its colour. The degree of change in colour of frozen meat relies mainly on the availability of atmospheric oxygen and depends on the conditions of frozen storage (AKHTAR *et al.*, 2013). The results of colour assessment of raw breast muscles after the freezing stage, considering the frozen storage duration as well as the thawing methods are illustrated in Table 1. The studies have demonstrated that a 8-month frozen storage duration resulted in a significant ($p<0.05$) reduction of L* (brightness) parameter, which corroborates the study findings of WEI *et al.* (2017). However, ALI *et al.* (2016) and GALOBART and MORAN (2004) demonstrated the impact of shorter frozen storage duration on the increased L*(brightness) and b*(yellowness) parameters. The current study demonstrated significant ($p<0.05$) impacts of both the duration of frozen storage and thawing methods on the colour saturation in favour of a*(redness) and b*(yellowness). A darker colouration was characteristic of breast muscles stored over a 8-month period and thawed using a microwave method in contrast to breast muscles stored for a month duration and thawed using the atmospheric air method. Similar values for the a*(redness) parameter were obtained by KIM *et al.* (2011), using the microwave thawing method on the breast muscles of broiler chickens. A similar trend in colour change during a shorter frozen storage period was posted by CHWASTOWSKA and KONDRATOWICZ (2005) that conducted studies on pork meat thawed using the same methods. Colour change in post thermally treated meat depend on the level of myoglobin denaturation as well as the level of temperature applied for the thermal treatment (ZHUANG and SAVAGE, 2010). The colour assessment of post thermally treated meat indicated that frozen storage lead to increased L*(brightness) but a decline b*(yellowness) parameters (Table 2). Similar results were obtained by GALOBART and MORAN (2004). The studies demonstrated a significant ($p<0.05$) influence of thawing methods on the colour saturation towards redness while retaining the tendency of being similar to raw meat.

The current studies have demonstrated the significant ($p<0.05$) influence of both the frozen storage duration and thawing methods on the share force of raw breast muscles (Table 1). It was demonstrated that the brittleness of raw meat measured by its share force increased with the frozen storage duration. The research findings correspond to those of ŚMIECIŃSKA *et al.* (2015) conducted on turkey breast muscles. In SHANKS *et al.* (2002) and FAROUK *et al.* (2003) opinions, the process of frozen storage increases the brittleness of meat, especially in case of unprocessed meat. Changes in the meat's brittleness during the frozen storage could be attributable to protein changes in the meat tissue (LEE *et al.*, 2008). GAMBUTEANU *et al.* (2103) argue that the brittleness of poultry meat depend on the thawing methods applied. The current study have demonstrated a significantly ($p<0.05$) less share force was characteristic of both raw breast muscles, thawed in atmospheric air and subjected to thermal treatment compared to those thawed using microwave method (Tables 1 and 2). Similar findings were obtained by OLIVEIRA *et al.* (2015), who evaluated the brittleness of breast muscles applying the same thawing methods. The thawing temperature, according to YU *et al.* (2005), has impacts on the brittleness of broiler chickens breast muscles in which lower share force was characteristic of those samples thawed at lower temperatures. The brittleness of post thermally treated meat depends on the quality of the initial raw material, type of thermal treatment as well as the heating duration and temperature (AKHTAR *et al.*, 2013). The current study did not identify any significant ($p>0.05$) impact of frozen storage duration on the brittleness of

post thermally treated breast muscles. However, LEE *et al.* (2008) observed increasing toughness in breast muscles stored frozen and boiled.

Table 2. Physicochemical properties of defrosted breast muscles subjected to thermal treatment, including the frozen storage duration and thawing methods ($\bar{x} \pm s$).

Traits	Frozen storage duration, months				SEM	Impact		
	(Group I)		(Group II)					
	Thawing method							
	atmospheric air	microwave oven	atmospheric air	microwave oven		C	R	C x R
Cooking loss (%)	23.47±1.13	25.98±0.44	26.23±1.05	28.21±1.05	0.15	*	*	*
Colour L*- lightness	80.79±1.04	81.64±0.84	82.71±0.42	82.68±0.77	0.56	*	ns	ns
a*- redness	1.27±0.15	1.45±0.13	1.28±0.09	1.50±0.07	0.02	ns	*	ns
b*- yellowness	13.08±0.20	12.91±0.37	11.13±0.16	10.80±0.35	0.04	*	ns	ns
Fmax (N)	19.58±2.36	20.01±1.14	18.04±1.84	19.36±1.74	0.27	ns	*	ns
Crude protein (%)	30.95±0.71	29.54±0.83	31.02±0.65	30.62±0.51	0.15	ns	ns	ns
Fat (%)	1.26±0.16	1.25±0.12	1.24±0.20	1.23±0.16	0.08	ns	ns	ns
Ash (%)	1.29±0.10	1.30±0.15	1.23±0.20	1.26±0.18	0.03	*	ns	ns

Explanations: ($\bar{x} \pm s$) arithmetic mean±standard deviation, Group I - 1 month freezing storage (n=20), Group II - 8 months freezing storage (n=20); atmospheric air (n=10), microwave oven (n=10); C- impact of frozen storage duration; R- impact of freezing method; C x R - impact of frozen storage duration and freezing method; *- statistically significant differences $p<0.05$; ns- differences statistically not significant.

AKHTAR *et al.* (2013) and GAMBUTEANU *et al.* (2013) postulate that protein metabolism which takes place under specific conditions during the freezing process is not significant and are limited only to minor loss of proteins and amino-acids as a result of drip loss. Frozen storage induces protein carbonylation, carboxylation as well as the formation of Schiff bases in chicken meat (UTRERA and ESTEVEZ, 2013). The current study did not indicate any significant ($p<0.05$) influence of the duration of frozen storage and freezing methods on the total protein, and fat content in the raw meat (Table 1). These findings are corroborated by those from WEI *et al.* (2017) study. KIM *et al.* (2011) did not demonstrate the impact of applied thawing methods on the protein content in raw breast muscles. BUSTAMANTE-VARGAS *et al.* (2016), however, observed that greater protein losses were noted during thawing using microwave than in atmospheric air. Protein losses increased proportionately with increasing freezing temperature. Deep freezing storage can result in muscle proteins forming into gel, which is associated with a higher solubility of meat protein in frozen meat compared to fresh meat (FAROUK *et al.*, 2003). The afore-mentioned dependency was observed in studies by ŚMIECIŃSKA *et al.* (2015), that indicated increased total and soluble protein content following a 6-week long deep freeze storage of turkey breast muscles. Both the deep storage and thawing procedures affect the activity of endogenous proteolytic enzymes responsible for the degradation and relaxation of muscle protein structures of meat tissues (FAROUK *et al.*, 2003). Studies conducted by CHAN *et al.* (2011) noted increased content of soluble protein in frozen vacuum-packed turkey meat, stored for 3 weeks and thawed in atmospheric air at 4°C. Both CHWASTOWSKA and KONDRATOWICZ (2005) demonstrated the effect of freezing storage and both thawing methods on the total protein and ash content of raw pork meat.

Higher protein content, but lower ash content was demonstrated in meat stored for a 3-month period and thawed using the atmospheric air method. The current study also demonstrated the influence of deep freeze storage on the ash content of raw breast muscles (Table 1).

The observed decrease in ash content during the 8-month long deep freeze storage in breast muscles was due to increased meat leaks in the process of thawing, thus resulting in greater loss of minerals. The thermal treatment of raw meat leads to changes in its nutrient content (AKHTAR *et al.*, 2013). The current studies demonstrated similar change tendencies in the nutrients of breast muscles thawed and subjected to thermal treatment as in raw meat (Table 2). A significant ($p<0.05$) influence of the storage duration, thawing methods as well as their interactions on the volume of cooking loss was observed. Higher cooking losses were observed following the 8-month long deep freeze storage in meat thawed using the microwave method. Similar results for an 8-month long deep freeze storage were obtained by WEI *et al.* (2017) in breast muscles thawed using atmospheric air method. CHEN *et al.* (2017) and ŚMIECIŃSKA *et al.* (2015), on the other hand, observed slight decreases in thermal leakages during the last phase of the freezing process. A similar trend was demonstrated in studies conducted by CHWASTOWSKA and KONDRATOWICZ (2005) on pork meat stored deep frozen over a 3-month period. YU *et al.* (2005) found that the volume of thermal leakage varied in relation to the thawing temperature applied. The magnitude of the thermal leakage has a huge influence on the quality characteristics of culinary meat and the yield of the finished product (OLIVIER *et al.*, 2015).

Several changes affecting sensory qualities may take place in meat and its allied products stored frozen. Such changes should be explained by way of the physical (recrystallization, denaturation, freeze-thawing burns), chemical (hydrolysis, auto-oxidation) as well as microbiological and enzymatic (hydrolysis, oxidation, dehydration) transformations. The extent of such transformations depends on the temperature and duration of freezing, including the conditions of storage (AKHTAR *et al.*, 2013; GAMBUTEANU *et al.*, 2013). The sensory properties of breast muscles subjected to thermal treatment following the freezing period, depending on the duration of the deep freeze storage as well as the thawing methods are presented Table 3.

Table 3. Sensory properties of breast muscles after thermal treatment, including the frozen storage duration and thawing methods ($\bar{x} \pm s$).

Traits	Frozen storage duration, months				SEM	Impact		
	(Group I)		(Group II)					
	Thawing metod							
	atmospheric air	microwave oven	atmospheric air	microwave oven		C	R	C x R
Odour intensity	3.67±0.46	3.67±0.48	3.58±0.49	3.42±0.49	0.05	ns	ns	ns
Flavour intensity	4.48±0.47	4.55±0.43	3.37±0.36	3.53±0.50	0.05	*	ns	ns
Odour desirability	3.83±0.37	3.75±0.43	3.67±0.44	3.58±0.49	0.04	ns	ns	ns
Flavour desirability	4.05±0.50	4.80±0.37	3.33±0.48	3.40±0.49	0.05	*	*	ns
Juiciness	4.42±0.49	4.68±0.49	3.17±0.32	3.20±0.37	0.04	*	*	ns
Tenderness	3.88±0.49	3.74±0.47	3.78±0.45	3.68±0.49	0.05	ns	ns	ns

Explanations: ($\bar{x} \pm s$) arithmetic mean±standard deviation, Group I - 1 month freezing storage (n=20) , Group II - 8 months freezing storage (n=20); atmospheric air (n=10), microwave oven (n=10); C- impact of frozen storage duration; R- impact of freezing method; C x R - impact of frozen storage duration and freezing method; *- statistically significant differences $p<0.05$; ns- differences statistically not significant.

Sensory scale:
odour; flavour intensity: 1- negatively changed, 2- moderately changed, 3- typical, weak; 4- typical, strong, 5- typical, very strong; odour; flavour desirability: 1- not desirable, 2- fairly desirable, 3- desirable, 4- very desirable, 5- highly desirable; juiciness: 1- very dry, 2- dry, 3- slightly juicy, 4- juicy, 5- very juicy; tenderness: 1- very hard, 2- hard, 3- slightly tender, 4- tender, 5- very tender.

The current studies have demonstrated that a significantly ($p<0.05$) lower intensity of taste desirability and less juiciness was characteristic of meat following a 8-month deep freeze storage. A similar trend in sensory changes was demonstrated in studies conducted by ŚMIECIŃSKA *et al.* (2015) and SANTOSH KUMAR *et al.* (2014), where the prolongation of the deep freeze storage was associated with the deterioration of the taste and juiciness of broiler chickens' meat. The microwave thawing method has, in the current studies, been shown to be more suitable than the atmospheric air method in respect of taste desirability and juiciness of broiler chickens' meat.

4. CONCLUSIONS

Long deep freeze storage (8 mth) resulted in significantly ($p<0.05$) higher drip loss, pH decline, darker colouration (less value of L* parameter and higher saturation toward redness and yellowness), reduced ash content and improved brittleness, measured by the shear force in broiler chicken meat. Deep freeze storage also resulted in significant ($p<0.05$) changes in the raw meat following the thermal treatment. Long frozen storage contributed to the increased cooking losses, increased saturation of the L*(brightness) parameter and reduction in the b*(yellowness) tone as well as a decrease in ash content and reduced sensory properties compared to the 1-month long frozen storage duration. Raw breast muscles that has been thawed using the microwave method was characterized by a significantly ($p<0.05$) lower drip loss, higher saturation toward redness (a*) and yellowness (b*), higher ash content but with less brittleness compared with breast muscles thawed using atmospheric air. Significantly ($p<0.05$) higher cooking losses, higher saturation toward redness (a*) and higher shear force were characteristics of breast muscles thawed using the microwave method and subjected to thermal treatment compared to meat thawed in atmospheric air. The sensory assessment of breast muscles subjected to thermal treatment following its freezing has demonstrated that microwave is, for reasons of taste desirability and juiciness of meat, a more suitable thawing method ($p<0.05$) than atmospheric air.

ACKNOWLEDGMENTS

The authors would like to thank Faculty Laboratory for Analysis of Environment Health and Materials of Agricultural Origin for technical support and assistance.

REFERENCES

Akhtar S., Khan M.I. and Faiz F. 2013. Effect of thawing on frozen meat quality: a comprehensive review. Pakistan J. Food Sci. 23(4):198- 211.

Ali S., Zhang W., Rajput N., Khan M., Li C.B. and Zhou G. 2015. Effect of multiple freeze-thaw cycles on the quality of chicken breast meat. Food Chem. 18: 808-814.

Ali S., Rajput N., Li C.B., Zhang W. and Zhou G. 2016. Effect of freeze-thaw cycles on lipid oxidation myowater in broiler chickens. Braz. J. Poultry Sci. 1:35-39.

Augustyńska-Prejsnar A., Ormian M. and Gajdek G. 2014. Market choices for broiler chicken meat in the opinion of students (in Polish). Journal of Agribusiness and Rural Development 3(33):5-13.

Benli H. 2016. Consumer attitudes toward storing and thawing chicken and effects of the common thawing practices on some quality characteristics of frozen chicken. Asian- Austral. J. Anim. Sci. 29(1):100-108.

Baryłko-Pikielna N. and Matuszewska I. 2009. Sensory testing of food. Basics - Methods - Application (in Polish), Publishing: Polish Society of Food Technologists.

Bustamante-Vargas C.E., Trentini M.S., Backes G.T., Cansian R.L., Mello R.O., Kubota E.H., Demiate I.M. and Prestes R.C. 2016. Eletrophoretic profile of exudates of chicken breast submitted to different thawing methods. International Food Research Journal 23(1):55-60.

Chan J.T.Y., Omana D.A. and Betti M. 2011. Effect of ultimate pH and freezing on the biochemical properties of proteins in turkey breast meat. Food Chem. 127:109- 117.

Chen T., Zhu Y., Han M., Wang P., Wei R., Xu X. and Zhou G. 2017. Classification of chicken muscle with different freeze- thaw cycles, using impedance and physicochemical properties. J. Food Eng. 196:94-100.

CIE 1976. Supplement No 2 to: Recommendations on uniform color spaces - Color difference equations. Psychometric Color Terms. Commission Internationale de Eclarage (1971) to 1-1. CIE Publication No 15 (E-1.3.1), Paris France.

Chwastowska I. and Kondratowicz J. 2005. Technological properties of a frozen pork meat depending on the storage time period and a thawing method (in Polish). Food. Science. Technology. Quality. vol. 3, no. 44, supl. (44):11-20.

Chwastowska-Siwiecka I. 2011. Effect of freezing and freezing storage conditions on the quality of poultry meat (in Polish). Polish Poultry 1:8-10.

Chwastowska-Siwiecka I., Kondratowicz J., Gugołek A. and Matusevicius P. 2013. Changes in the physiocochemical properties od deep-frozen rabbit meat as dependent on thawing method. Vet. Med. Zoot. 62(84):68- 72.

Farouk M.M., Wieliczko K.J. and Merts I. 2003. Ultra-fast freezing and low storage temperature as are not necessary to maintain the functional properties of manufacturing beef. Meat Sci. 66:171-179.

Fernandes R.T.V., de Arruda A.M.V., de Oliveira Costa M.K., de Oliveira Lima P., dos Santos L.O.G., da Silva Melo A. and Marinho J.B.M. 2016. Physicochemical and microbiological parameters of frozen and chilled chicken meat. Rev. Bras. Zootech. 45(7):417-421.

Galobart J. and Moran J. 2004. Freeze-thaw and cooking effects on broiler breast fillets with extreme initial L* values. Poultry Sci. 83:2093-2097.

Gambuteanu C., Borda D. and Alexe P. 2013. The effect of freezing and thawing on technological properties of meat: review. J. Agroaliment. Proc. Technol. 19(1):88-93.

Grau R. and Hamm R. 1953. A simple method for the determination of water binding in muscles. Naturwissenschaften 40:29-30.

ISO 8587:2006. Sensory analysis. Methodology. www.iso.org/standard/36172.html

ISO 8586-2:2008 Sensory analysis. General guidance for the selection, training and monitoring of assessors. www.iso.org/standard/15875.html

Kim T., Choi J., Choi Y., Kim H., Kim S., Kim H. and Kim C.H. 2011. Physicochemical properties of thawed chicken breast as affected by microwave power levels. Food Sci. Biotechnol. 20(4):971-977.

Lee Y.S., Saha A., Xiong R., Owens C.M. and Meullenet J.F. 2008. Changes in broiler breast fillet tenderness water-holding capacity and colour attributes during long-term frozen storage. J. Food Sci. 73(4):162-168.

Leygonie C., Britz T.J. and Hoffman L.C. 2012. Impact of freezing and thawing on the quality of meat: review. Meat Sci. 91:93- 98.

Marchel J., Żmijewski T., Cierach M. and Malczyk E. 2013. Influence of storing beef meat in frozen state on the amount of thawing and heating losses and on the meat texture. Acta Agrophysica 20(2):377-387.

Oliveira M.R., Gubert G., Roman S.S., Kempka A.P. and Prestes R.C. 2015. Meat quality of chicken breast subjected to different thawing methods. Braz. J. Poultry Sci. 17(2):165-172.

Santosh Kumar H.T., Pal U.K., Sudheer K., Mandal P.K. and Das C.D. 2014. Changes in the quality of dressed chicken obtained from different sources during frozen storage. Exploratory Animal and Medical Research 4(1):95-100.

Shanks B. C., Wulf D. M. and Maddock R.J. 2002. Technical note: The effect of freezing on Warner-Bratzler shear force values of beef longissimus steaks across several postmortem ageing periods. J. Anim. Sci. 80(8):2122-2125.

Śmiecińska K., Hnatyk N., Daszkiewicz T., Kubiak D. and Matusevicius P. 2015. The effect of frozen storage on the quality of vacuum- packaged turkey meat. Vet. Med. Zoot. 71(93):61- 66.

Utrera M. and Estevez M. 2013. Oxidative damage to poultry, pork, and beef during frozen storage throught the analysis of novel protein oxidation markers. J. Agr. Food Chem. 61:7987-7993.

Wei R., Wang P., Han M., Chen T., Xinglian X. and Zhou G. 2017. Effect of freezing on electric al properties and quality of thawed chicken breast meat. Asian-Australas J. Anim. Sci. 30:569-575.

Yu L.H., Lee E.S., Jeong J.Y., Paik H.D., Choi J.H. and Kim C.J. 2005. Effects of thawing temperature on the physicochemical properties of pre-rigor frozen chicken breast and leg muscles. Meat Sci. 71:375-382.

Zhang X., Gao T. Song L., Zhang L. Jiang Y., Li J., Gao F. and Zhou G. 2017. Effects of different thawing methods on the quality of chicken breast. Int. J. Food Sci. Tech. 52:2097-2105.

Zhuang H. and Savage E.M. 2010. Comparisons of sensory descriptive flavor and texture profiles of cooked broiler fillets categorized by raw meat color lightness values. Poultry Sci. 89(5):1049-1055.

Ziołecki J. and Doruchowski W.1989. Evaluation method of the poultry slaughter value (in Polish). COBRD Poznań.

Paper Received June 4, 2019 Accepted February 28, 2019

PAPER

THE EFFECTS OF LOCUST BEAN GUM AND RHUBARB ON THE PHYSICAL AND FUNCTIONAL PROPERTIES OF THE GLUTEN-FREE BISCUITS

H. DOĞAN[a] and R. MERAL*[a]
Van Yüzüncü Yil University, Faculty of Engineering, Department of Food Engineering, 65080 Van, Turkey
*Corresponding author: Tel.: +904322251024; Fax: +904322251104;
E-mail address: raciyemeral@yyu.edu.tr

ABSTRACT

In this study, rhubarb plant, which is known to have functional properties, was added to gluten-free biscuit formulations and the effects of rhubarb were examined on the physical and antioxidant properties of biscuits. From the examination, it was observed that the spread ratio of the biscuits varied between 51.70 and 63.80. Also, the spread ratios of the biscuits decreased as the rhubarb and gum levels increased. Firmness values of the gluten-free biscuits were 29.71-54.02 N. The increase in gum levels of biscuit formulas that did not contain rhubarb led to an increase in the firmness values. Total phenolic content (TPC) of the biscuits varied between 2.00 and 3.18 μg GAE/g. The lowest TPC value was determined in the biscuit formula 2 which did not contain rhubarb and contained the highest level of gum, while the highest TPC value was determined in the biscuit formula 1 which did not contain gum, but contained the highest level of rhubarb. The DPPH scavenging values of the gluten-free biscuits were 42.60% and 57.51%.The addition of rhubarb alone in the formula increased antioxidant activity. However, the addition of rhubarb with gum in the formula decreased antioxidant activity. The aim of this study was to produce biscuits with high nutritional value using functional food components for the consumption of patients with celiac disease. The addition of rhubarb increased the TPC of the biscuits as well as the functional properties of the biscuits by inhibiting free radicals.

Keywords: antioxidant, biscuit, gluten-free, rhubarb, RSM

1. INTRODUCTION

Celiac disease has been identified as an inflammatory intestinal disease induced by gluten in wheat, barley, rye etc. (JNAWALI *et al.,* 2016). Currently, the only treatment for celiac disease is a gluten-free diet. However, gluten-free products have low nutritional quality and are more expensive than gluten-containing food products. Therefore, there is a strong need to develop gluten-free products that have high nutritional and economic value (JNAWALI *et al.,* 2016). The production of gluten-free products will increase with increase in technological developments. Flours from pseudocereals such as buckwheat, quinoa or amaranth, alternative cereals such as white sorghum or pigmented cereal grains and non-cereals sources such as legumes, seeds or nuts are emerging as alternatives to common gluten free flours and they have been used as components in the production of different gluten free cereal based foods (CAPRILES *et al.,* 2016; PADALINO *et al*., 2016; GIUBERTI *et al.,* 2018). In addition, by-products from fruit and vegetable processing have been incorporated into gluten free food formulations (BOUREKOUA *et al*., 2017; DRABINSKA *et al*., 2017). Also, chestnut flour has been incorporated into gluten free biscuit formula by PACIULLI *et al*. (2018) and it was observed that chestnut flour improved the technological and organoleptic quality of gluten-free biscuits. Although, in recent years, the enhancement of common gluten-free cereal-based foods with ingredients rich in phytochemicals such as polyphenols is gaining a growing interest, the number of studies on the enrichment of gluten-free products with components of functional properties is still limited (ROCHETTI *et al.,* 2018).

Functional food is defined as food that possesses health benefits beyond its basic nutritional properties (YETIM *et al.,* 2010). In recent years, the changes in the dietary habits and increasing awareness of healthy eating have increased the production and consumption of functional foods. Functional foods contain physiologically active ingredients that have positive health benefits beyond their basic nutritional value (MERAL and DOGAN, 2009). The enrichment of the gluten-free biscuit formulations with functional components is of great importance as these functional components are also ingested along with the product.

There are various studies investigating the effects of the natural additives on the quality and nutritional properties of the biscuits. However, the number of studies involving the addition of natural components to enrich gluten-free biscuit are limited. Natural components are rich in antioxidant activity and help to increase the antioxidant activity of the foods. Furthermore, these components are found widely in nature and are used in the treatment of various diseases.

Rhubarb (*Rheum ribes*) is a perennial plant from the Polygonaceae family. It grows in rocky areas of mountainous regions. Rhubarb is rich in Vitamin C and grows during spring (ANDİÇ *et al.,* 2009). MERAL (2011) reported that rhubarb had a high phenolic compound content and inhibited the free radicals. It could be used as a functional food component due to its antioxidant properties. In spite of these functional properties, there are limited number of studies on its usage as a functional food component. The aim of this study was to produce functional biscuits that are rich in components such as phenolic and antioxidants. Thus, the study aimed to develop a healthy gluten-free biscuit formula. In this study, rhubarb and locust bean gum were used to produce the gluten-free biscuits. The levels of rhubarb and gum were selected based on preliminary experiments; the optimal amount of rhubarb and gum was obtained by means of preliminary baking tests combined with a consumer test with 10 untrained panelists to evaluate consumers' acceptance of the improved biscuits. While choosing the levels of rhubarb and gum, the firmness of dough and the tastes of the biscuits were taken into account by the untrained panelists.

2. MATERIAL AND METHODS

2.1. Material

Rice flour was obtained from Makeks (İstanbul, Turkey), potato starch was obtained from Smart Chemistry (İzmir, Turkey), locust bean gum was obtained from A&D (İstanbul, Turkey), and rhubarb plant used in the formulations were obtained from a market in Van, Turkey. Chemical substances at analytical grade used in the study were obtained from Merck (Darmstadt, Germany). Rhubarb was manually separated from the bark and dried at 40°C. Then, the dried rhubarb was ground with a laboratory mill (Perten LM 120, Hägersten, Sweden) just before usage.

2.2. Pasting properties of flours

The viscosity properties of starch used in formulations were determined using the Rapid Viscosity Analyser (RVA) (RVA-4500, Perten, Switzerland) depending on temperature and time. The viscosity values were given as cP. For RVA analysis, 3 g samples were taken (corrected according to 14% moisture) and 25 g of deionized water were added. The heating and cooling cycles of the device were adjusted as follows: The sample was kept at 50°C for 1 min, then the temperature was increased to 95°C in 3.5 min. The samples were kept for 2.5 min at 95°C and cooled to 50°C in 3.5 min, and then kept at 50°C for 2 min (SAKIYAN *et al.,* 2009). Values obtained from RVA graph were expressed as peak viscosity, trough viscosity, breakdown viscosity, setback viscosity and final viscosity.

2.3. Preparation, shaping and baking of the dough

The optimization of the formula to produce gluten-free biscuits was conducted using the Response Surface Methodology (RSM) and biscuit doughs were prepared following the formation of design patterns. The formulations of gluten-free biscuits were presented in Table 1.

Table 1. Gluten-free biscuit formula.

Ingredients	(g)
Rice flour	80
Potato starch	20
Sugar	42
Shortening	40
Water	22
Sodium bicarbonate	1.0
Amonium bicarbonate	0.5
Corn syrup	1.5
Salt	1.2
Vanilla	0.3
Rhubarb	0, 1 and 2
Locust bean gum	0, 1.5 and 3

The biscuit dough was prepared according to the AACC (1999) method with some modifications using the KitchenAid Mixer (Model 5KSM45). After the addition of shortening, sugar, salt, vanilla and sodium bicarbonate, the mixture was stirred for 3

minutes at approximately ~60 rpm and then the specific gravity of the obtained cream was determined. Subsequently, high-fructose corn syrup, ammonium bicarbonate and water were added and stirred again for 1 minute at ~90 rpm. Then, flour was added to the formula and the dough preparation process was completed by stirring for another two minutes at ~60 rpm. The AACC (10-50.05) (AACC, 1999) method was used to shape the biscuit dough. The doughs were cooked in the electric oven (Öztiryakiler, İstanbul-Turkey) at 85% humidity and at 185˚C for 12±1 min.

2.4. Determination of baking loss and spread ratio of biscuits

The weight of the biscuit was determined both before and after the cooking of the biscuits and the baking loss was determined. The width (W) and height (H) values of the biscuit samples were determined in mm using the scaled wooden apparatus according to the AACC (10-53.01) method (AACC, 1999). To obtain the average value, four measurements were made by changing the position and location of the biscuits and then the spread ratio of the biscuit was calculated using the formula (W/H)*10.

2.5. Determination of firmness

The firmness of the biscuit samples were determined using the TA.XT plus Texture Analyzer (TA.TX2. Stable Micro Systems Ltd. Godalming Surrey, UK) according to three-point bend rig technique. (Load cell: 5 kg, pre-test speed: 1.0 mm/s, test speed: 5.0 mm/s, post-test speed: 10.0 mm/s, distance: 10 mm, trigger force: 50 g).

2.6. Extraction of the phenolic compounds

The method proposed by MERAL and DOĞAN (2013) was used to extract the phenolic compounds. The 5 g sample collected from the milled samples was placed in centrifuge tubes and, then, 15 mL of methanol was added to the tubes. Using a shaking incubator at 35˚C, the tubes were kept for 22 hours in a shaking incubator at 35˚C and at a rotational speed of 3500 rpm. Thereafter, the tubes were centrifuged at 12,000 *g* for 15 minutes. After centrifugation, the supernatant was transferred to amber bottles and the process continued until the volume of the final supernatant reached 50 mL. Each sample was transferred to amber vials, and kept at -20˚C until analysis.

2.7. Determination of total phenolic content (TPC)

TPC were determined using the Folin-Ciocalteu method. For this purpose, 300 μL sample and 3 mL Na_2CO_3 (2%) were added to test tubes and about 2 minutes later, 150 μL of the Folin-Ciocalteu reagent that was 1:1 diluted with ultra-pure water was added to the tubes. The mixture was vortex mixed and kept for 45 minutes at room temperature in darkness and, then, the absorbance of the mixture was read on a spectrophotometer at 765 nm (T80 UV/VIS, PG Instrument, UK). TPC was calculated using the calibration graph plotted using gallic acid and the results were expressed as gallic acid equivalent (GAE) (MERAL and DOĞAN, 2013).

2.8. Determination of DPPH radical scavenging effects

The extracts containing antioxidant substances were transferred to a test tube and the final volume was brought to 1 mL with methanol. For the samples, 3 mL of the 0.004% DPPH solution that was prepared with methanol on a daily basis was added and the samples

were rapidly vortex-mixed. Upon mixing, the test tubes were incubated in darkness and their absorbance was read against a control sample (the sample containing 1 mL methanol and 3 mL DPPH solution) on a spectrophotometer at 517-nm wavelength using a quartz cuvette. The measurements were performed in two repetitions and the radical scavenging property of each sample was calculated using the following equation (MERAL and DOĞAN, 2013).

$$\% \text{ Inhibition} = A_{control}\text{-}A_{sample} / A_{control}$$

A control: Absorbance of control at 517 nm
A sample: Absorbance of sample at 517 nm

2.9. Experimental design

The experimental design for the production of gluten free biscuits containing rhubarb was carried out using response surface methodology (RSM) (StatGraphics Centurion 15.19) which gave 9 experimental runs as shown in Table 2. Three-level factorial design patterns were used to determine the effects of the factors and interactions on the properties of the biscuits. The variables, A was determined as the gum and the level of gum varied between 0 and 3%. B was determined as the rhubarb and the level of rhubarb varied between 0 and 2%. Significant difference between group averages was determined using Duncan multiple comparison tests at the $P<0.05$ level of significance. The appropriate response values for each formulation were determined.

3. RESULTS AND DISCUSSION

3.1. Pasting properties of biscuit flours

The viscosity analysis results were reported in Table 2.

Table 2. Pasting properties of different biscuit dough formulations.

Trail	Gum (%) (A)	Rhubarb (%) (B)	Peak Viscosity (cP)	Trough Viscosity (cP)	Breakdown Viscosity (cP)	Setback Viscosity (cP)	Final Viscosity (cP)
1	0	2	3022±50.9	1503±61.0	1519±16.2	912±30.6	2415±91.1
2	3	0	4611±55.8	2204±60.8	2406±172.8	1099±62.7	3304±117.8
3	1.5	2	3502±127.4	1665±110.0	1836±52.9	906±25.3	2572±135.0
4	3	1	4199±33.2	1924±151.8	2274±118.9	975±49.7	2899±110.2
5	3	2	4155±37.5	2010±101.6	2145±137.4	874±52.8	2884±48.9
6	1.5	1	3583±42.6	1893±94.5	1690±132.9	926±46.1	2819±56.8
7	0	1	3000±17.8	1503±150.5	1496±133.1	1014±33.4	2517±160.5
8	1.5	0	4053±110.4	2093±33. 6	1960±89.3	1049±38.0	3142±37.8
9	0	0	3660±10.7	1717±123.4	1942±128.2	1192±45.4	2910±89.9

Peak viscosity

The peak viscosity values of the biscuit formulations ranged from 3000 to 4611 cP. The lowest viscosity value was obtained in the biscuit formula 7 (0% gum, 1% rhubarb) and the

highest viscosity value was determined in the biscuit formula 2 (3% gum, 0% rhubarb). The model, which explained the relationship between the peak viscosity of the dough and the independent factors (A and B), is outlined in Equation 1:

$$Peak\ viscosity = 3634{,}07 + 224{,}93\times A - 789{,}94\times B + 36{,}47\times A^2 + 30{,}4444\times A\times B + 230{,}06\times B^2 \quad R^2=0.99 \quad (1)$$

The total difference in terms of the peak viscosity values can be explained with the linear and quadratic effects of the components in the model and the interaction between the components. Peak viscosity values were significantly affected by the linear effects of gum and rhubarb. Furthermore, the interaction effect of the factors was not significant ($P>0.05$). Fig. 1A showed the response surface for peak viscosity. The effects of gum and rhubarb on the peak viscosity values were found to be significant ($P<0.05$). Peak viscosity values increased as the level of gum in formulation increased, while the peak viscosity values decreased with increasing levels of rhubarb (Fig. 1A). High viscosity value indicates that starch granules and starch-gum mixture have higher swelling powers (JYOTHI *et al.*, 2005). Various studies have reported that gum influenced the gelatinization and rheological properties of starch. Gum is widely used in foods containing starch to improve the textural and rheological properties, the water holding capacity as well as the quality of the product (SHI and MILLER, 2002; CHAISIWANG and SUPHANTHARIKA, 2006). In gluten-free products, it is difficult to maintain the desired viscoelastic structure, gas holding capacity and textural properties due to the lack of the gluten protein. Different starch sources, gums and dairy products can be added to the gluten-free formulation to eliminate the problems arising from the lack of gluten in the food. Previous studies have shown that gums increased the peak viscosity due to their water binding properties (CHAISIWANG and SUPHANTHARIKA, 2006). Rhubarb has high water binding capacity due to its high nutritional fiber content (MERAL, 2011). However, in this study, it was determined that rhubarb reduced the peak viscosity. This result was associated with the low pH value of rhubarb. Acids, due to their hydrolyzing effects, form cracks on the starch granules and, as a result, the viscosity decreases (JYOTHI *et al.*, 2005).

Trough viscosity

Trough viscosity varied between 1503 and 2204 cP. The lowest trough viscosity values were determined in the flour formulations containing 0% gum and 1% rhubarb, and containing 0% gum and 2% rhubarb, whereas, the highest trough viscosity value was determined in the formula containing 3% gum and 0% rhubarb (Fig. 1B). Results revealed that gum and rhubarb content had significant effects on the trough viscosity of flours ($P<0.05$). Gums increased the viscosity by binding water, there by making water release from starch-gum systems difficult. The decrease in viscosity of mixtures containing gum was lower than that in mixtures that do not contain gum. Rhubarb, probably due to its hydrolyzing effect, decreased the viscosity value at a higher level compared to gum.
The final equation for the trough viscosity of the dough is shown in equation 2 below.

$$Trough\ viscosity = 1754.8 + 232.56\times A - 321.5\times B - 26.27\times A^2 + 3{,}4444\times A\times B + 84.89\times B^2 \quad R^2=0.83 \quad (2)$$

Breakdown viscosity

Breakdown viscosity is one of the important gelatinization characteristics of starch gel (YILDIZ *et al*., 2013; YILMAZ *et al.,* 2015). The breakdown viscosity values of the flours ranged from 1496 to 2406 cP (Fig. 1C). The model, which explained the relationship between the breakdown viscosity of the dough and the independent factors (A and B), is shown in Equation 3:

$$Breakdown\ viscosity = 1879.28 - 7.62963 \times A - 468.444 \times B + 62.7407 \times A^2 + 27 \times A \times B + 145.17 \times B^2 \quad R^2 = 0.87 \quad (3)$$

It was determined that the effects of gum and rhubarb had significant effects on trough viscosity ($P<0.05$). The decomposition of starch due to high temperature and mechanical stress resulted to a decrease in viscosity. Breakdown viscosity is the difference between the peak viscosity and trough viscosity. Low breakdown viscosity value indicates a final product with good stability (YILDIZ *et al.,* 2013; YILMAZ *et al.,* 2015).

Setback viscosity

The setback viscosity of the flours ranged from 906 to 1192cP. Rhubarb had a significant linear effect, while gum had both significant quadratic and linear effects on the setback viscosity of the flours ($P<0.05$). Equation 4 showed the model, which explained the relationship between the setback viscosity of the dough and the independent factors (A and B).

$$Setback\ viscosity = 117393 - 100.407 \times A - 187.78 \times B + 24.124 \times A^2 + 9{,}11 \times A \times B + 32.11 \times B^2 \quad R^2 = 0.85 \quad (4)$$

Fig. 1D showed the effects of gum and rhubarb on the setback viscosity values of the flours. With increase in the gum, viscosity values of the flours initially decreased for a while, followed by an increase. Setback viscosity is related to the retrogradation that occurs during the cooling of the starch gel. Low setback viscosity value indicates low retrogradation and syneresis rates (YILMAZ *et al*., 2015). YILDIZ *et al.* (2013) stated that fiber type and concentrations significantly affected gelatinization properties in starch-fiber mixtures. It was determined that, with the increase in the fiber content of starch-fiber mixtures, peak viscosity, trough viscosity, breakdown viscosity, setback viscosity and final viscosity values increased, while peak viscosity values decreased.

Final viscosity

Final viscosity of the flours varied between 2415 and 3304 cP. The suggested model including linear, quadratic and interaction effects of gum and rhubarb levels in the formula explains 90% of changes in the final viscosity. The final equation for the final viscosity was given in Equation 5.

$$Final\ viscosity = 2928.72 + 132.148 \times A - 509.278 \times B - 2.14815 \times A^2 + 12.56 \times A \times B + 117 \times B^2 \quad R^2 = 0.90 \quad (5)$$

It was determined that while the linear effects of gum and rhubarb and quadratic effect of rhubarb on the final viscosity values were significant ($P<0.05$), interaction effects of gum

level and rhubarb level were not also significant (*P>0.05*). Final viscosity values decreased as the rhubarb level increased (Fig. 1E).

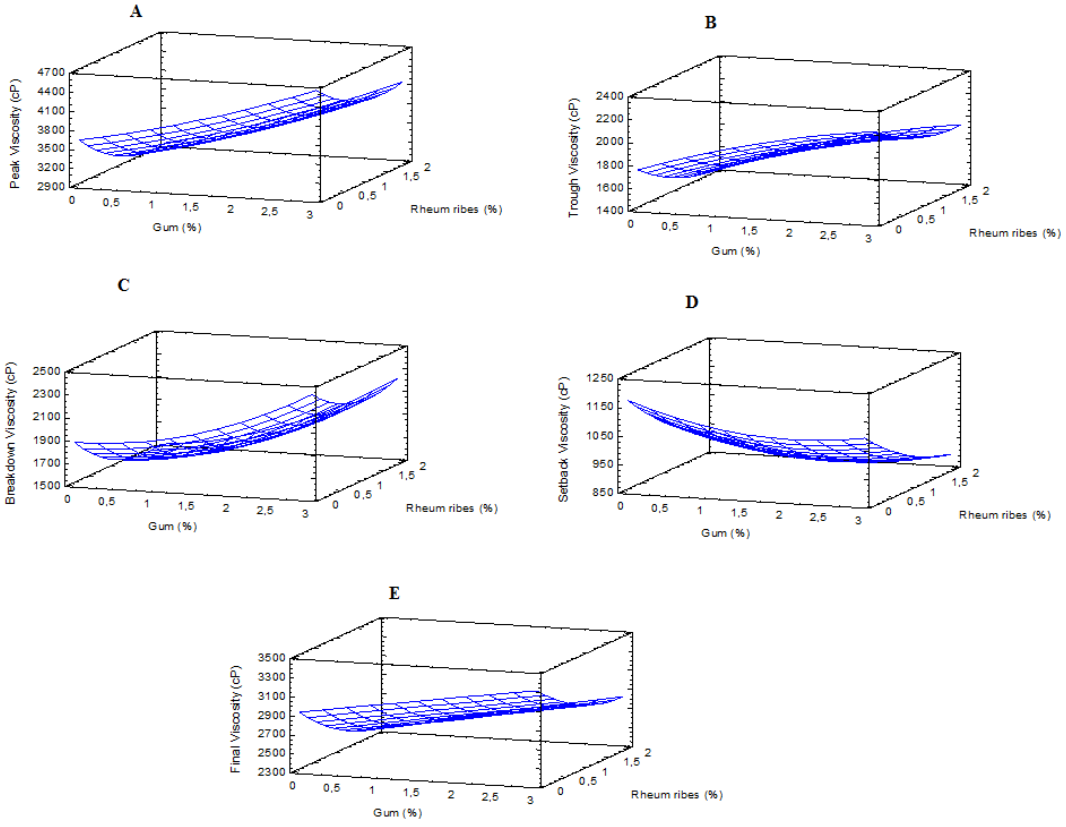


Figure 1. Response surface of viscosity properties of gluten-free biscuit doughs.
A: Peak viscosity, B: Trough viscosity, C: Breakdown viscosity, D: Setback viscosity, E: Final viscosity.

According to YILMAZ *et al*. (2015), low breakdown viscosity value indicates a final product with good stability and low setback viscosity value indicates low retrogradation and syneresis rates. In this study, it was observed that gum and rhubarb affected the pasting properties of flour. While increasing level of gum increased the peak, trough breakdown and final viscosity values of flour, increasing level of rhubarb decreased the peak through breakdown and final viscosity values of flour. The obtained results indicated that rhubarb delayed retrogradation and syneresis.

3.2. Baking loss and spread ratio of biscuits

The physical and functional properties of biscuits were reported in Table 3.

3.2.1. Baking loss

The baking loss values of the biscuits varied between 11.90% and 15.68%. High baking loss values in the biscuits results in the lack of consistency in the actual weight of packaging, subsequent increase in brittleness in the following stages and decrease in appeal. In a study, it has been reported that baking loss values of biscuits varied between 13.3% and 21.4% (DOĞAN and UĞUR, 2005). The quadratic effects of gum and rhubarb and the

interactions of gum and rhubarb on baking loss values of biscuits were insignificant (*P>0.05*).
Equation 6 showed the model, which explained the relationship between the baking loss of the biscuits and the independent factors (A and B).

$$Baking\ loss = 15.2783 - 1.04 \times A - 1.14667 \times B + 0.197778 \times A^2 + 0.0133333 \times A \times B + 0.47 \times B^2 \quad R^2 = 0.21 \quad (6)$$

It was reported that baking loss did not change significantly when the level of gum and rhubarb was increased. A decrease in baking loss indicated a decrease in the shrinkage. The changes in baking loss values were associated with the water binding effects of gum and rhubarb. As known, baking loss occurs due to the evaporation of moisture during the baking process (MALKANTHI and UMADEVI, 2018). RODRIGUEZ-GARCIA *et al.* (2012) stated that lower baking loss is desirable for retaining its components and shape.

Table 3. Physical and functional properties of biscuits.

Trail	Gum (%) (A)	Rhubarb (%) (B)	Baking loss (%)	Spread ratio	Firmness (N)	TPC (μg GAE/g)	DPPH Scavenging (%)
1	0	2	14.83±0.0	52.94±0.1	35.07±5.9	3.18±0.1	57.51±2.4
2	3	0	13.18±0.1	51.70±0.1	54.02±0.7	2.00±0.4	47.74±2.2
3	1.5	2	13.88±0.1	56.00±0.1	40.52±2.2	2.96±0.1	56.67±2.8
4	3	1	14.12±0.5	53.32±0.2	53.35±2.9	2.95±0.3	49.06±2.1
5	3	2	13.56±0.4	52.74±0.0	36.20±3.8	3.10±0.4	42.60±2.3
6	1.5	1	11.90±3.0	54.78±0.7	41.66±0.7	2.91±0.1	52.05±1.9
7	0	1	15.40±1.0	60.17±0.3	41.34±6.5	3.11±0.2	50.26±3.5
8	1.5	0	15.68±1.7	56.99±0.2	29.71±1.6	2.76±0.1	43.23±1.6
9	0	0	14.53±1.0	63.80±0.4	32.12±2.8	2.69±0.1	52.68±4.0

3.2.2. Spread ratio

Spread ratio of the products varied between 51.70 and 63.80. The effect of gum and rhubarb and the interactions between these components on the spread ratio of biscuits were found to be significant (*P<0.05*). The spread ratios of the biscuits decreased as the rhubarb and gum levels increased. The spread ratio values of the biscuits that do not contain rhubarb were higher than those that contained rhubarb and the spread ratio values of the biscuits increased as the gum level decreased. DE SIMAS *et al*. (2009), in their study on gluten-free biscuits, associated the decrease in the spread ratio with the absorption of water added to the dough due to the functional components added to the formula and the consequent lack of water for the dissolution of sugar left in the system during baking, leading to an increase in viscosity.
The final equation for the spread ratio was given in Equation 7.

$$Spread\ ratio = 63.6083 - 3.91389 \times A - 3.98083 \times B - 0.0655556 \times A^2 + 1.98333 \times A \times B - 0.3975 \times B^2 \quad R^2 = 0.92 \quad (7)$$

3.3. Firmness of biscuits

Firmness values of the gluten-free biscuits varied between 29.71 and 54.02 N. The lowest firmness value was obtained in the biscuit formula 8 (1.5% gum, 0% rhubarb) and the highest firmness value was determined in the biscuit formula 2 (3% gum, 0% rhubarb). Equation 8 showed the model which explained the relationship between the hardness of the biscuits and the independent factors (A and B).

$$Firmness = 2973.32 + 108.512 \times A + 1991.38 \times B + 213.805 \times A^2 - 352.943 \times A \times B - 765.401 \times B^2 \quad R^2 = 0.72 \quad (8)$$

Gum, gum-rhubarb interaction and quadratic effects of gum and rhubarb had significant effects on the firmness values ($P<0.05$). The effect of rhubarb on the firmness values was not significant ($P>0.05$). Fig. 2C showed the effects of gum and rhubarb on the firmness values. It was previously mentioned that setback viscosity decreased with the increase in rhubarb levels and the decrease in the setback viscosity value may indicate that the retrogradation may occur at lower levels. The addition of rhubarb alone did not change the firmness values, however, the interaction between rhubarb and gum significantly changed the firmness value. The increase in gum levels in biscuit formulas that did not contain rhubarb led to an increase in the firmness values. As a result of the water binding by gum, free water in the medium decreased, leading to an increase in the firmness values. In biscuits containing 2% rhubarb, with the increase in gum levels, firmness values initially decreased for a while, followed by an increase. HOSENEY and ROGERS (1994) associated the firmness in biscuits with the development of dough and the interaction of starch and proteins during baking. PACIULLI *et al* (2018) demonstrated the fact that a direct link existed between fibre content and firmness of wheat and gluten-free biscuits.

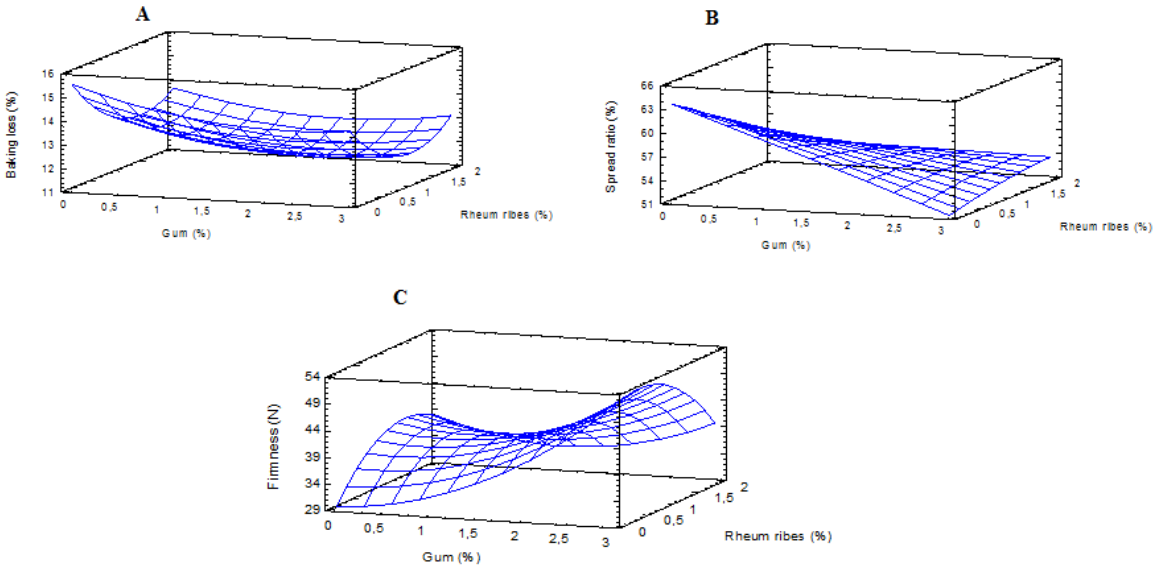


Figure 2. Response surface of physical properties of gluten-free biscuit doughs.
A: Baking loss, B: Spread ratio, C: Firmness.

3.9. Total Phenolic Content

Phenolic compounds possess antioxidant effects. Numerous studies have reported the antioxidant activity of phenolic compounds and the determination of TPC was emphasized as one of the measurement methods for antioxidant activity (MICHALSKA *et al.*, 2008). The TPC contents of the biscuits varied between 2.00 and 3.18 μg GAE/g. The lowest TPC value was determined in the biscuit formula 2 which did not contain rhubarb and contained the highest level of gum, while the highest TPC value was determined in the biscuit formula 1 which did not contain gum but contained the highest level of rhubarb. The model comprising the linear and quadratic effects of the components and their interactions explained 63% of the total changes in the TPC. The increase in the rhubarb contents significantly increased the TPC values. In a study in which rhubarb was used as a functional compound, it was stated that rhubarb contained high levels of phenolic compounds (MERAL, 2011). Furthermore, gum supplementation had a significant effect on the TPC contents of the biscuits ($P<0.05$). TPC decreased with the increase in gum levels (Fig. 3A). Numerous studies were carried out to determine the TPC values of foods and the results obtained in these studies have revealed that composition of foods, the ratio of the food components and the interaction between these components significantly changed the TPC and antioxidant properties of the foods (MERAL, 2011). The decreasing effect of gum on TPC can be associated with the decrease in the TPC dissolved in water due to the binding of the free water by gum. However, further studies should be conducted to verify this hypothesis. The results regarding the TPC obtained in the present study were verified using the results reported by SAKAČ *et al*. (2015) in their study on biscuits produced using rice starch and buckwheat flour at different ratios. The researchers reported that the TPC values increased as the level of buckwheat increased. The final equation for TPC was given in Equation 9.

$$TPC = 2.76468 - 0.145345\times A + 0.57325\times B - 0.0179496\times A^2 + 0.0977287\times A\times B - 0.20917\times B^2 \quad R^2=0.63 \quad (9)$$

3.10. DPPH radical scavenging effect of the biscuits

The DPPH scavenging values of the gluten-free biscuits varied between 42.60% and 57.51%.

$$\%\ scavenging = 48.6729 + 0.221667\times A + 5.45125\times B - 0.299444\times A^2 - 1.6625\times A\times B - 0.385\times B^2 \quad R^2=0.50 \quad (10)$$

Equation 10 showed the model that explained the relationship between the baking loss of the biscuits and the independent factors (A and B).
The highest antioxidant activity was found in the biscuits prepared from the biscuit formula 1 which did not contain gum and contained 2% rhubarb, while the lowest antioxidant activity was found in the biscuits prepared from the biscuit formula 5 which contained the highest levels of gum and rhubarb. The effect of rhubarb and gum used in the formula and gum-rhubarb interaction on inhibition was significant. Fig. 3B showed the response surface the effects of gum and rhubarb have on the antioxidant activity. The DPPH radical scavenging effect increased with increasing rhubarb levels, while the scavenging effect decreased with increasing gum levels. According to the comparisons of the biscuits with the highest and the lowest antioxidant activities, the increase in the antioxidant activity was determined to be 26%. The scavenging effect increased with increasing rhubarb levels and decreasing gum levels. Previous studies have revealed the antioxidant activity of rhubarb and its use as a functional component in food formulas

(ÖZTÜRK *et al.*, 2007; MERAL, 2011). Therefore, the increase in the antioxidant activity due to rhubarb supplementation is an expected result. However, the biscuit formula 5, which was one of the formulas with the highest rhubarb content, had the lowest antioxidant activity. This result was attributed to the lack of dissolved antioxidant agents due to the binding of the free water and/or the binding of some functional groups with antioxidant activity by the gum. However, further studies should be conducted to better understand the reason for this result and the gum-antioxidant relationship.

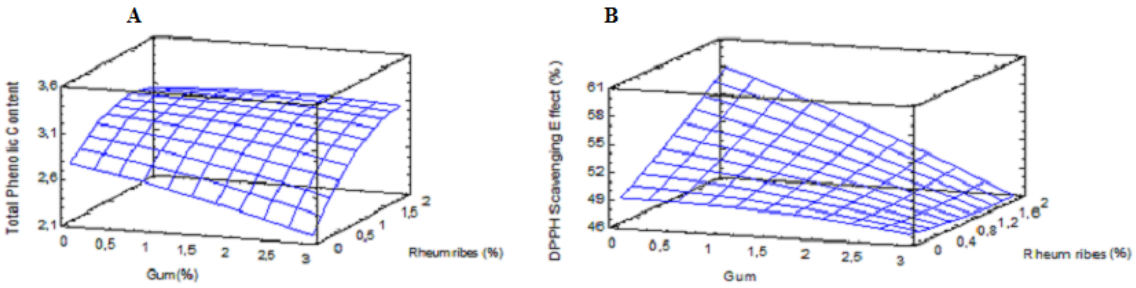


Figure 3. Response surface of functional properties of gluten-free biscuit doughs.
A:TPC, B: DPPH Scavenging.

4. CONCLUSIONS

In this study, rhubarb, which has an antioxidant activity, and is rich in nutritional fiber content, was added to the biscuit composition to increase the functional properties of gluten-free biscuits. Rhubarb was added at two different ratios to the formulas and the effects of this functional component on the biscuit properties, and antioxidant properties of the biscuits were investigated. The peak viscosity, trough viscosity and final viscosity values decreased in biscuits with the addition of rhubarb and increased with the addition of gum. Although, an increase was expected in the spread ratio values with the addition of components containing fiber (with the increase in the rhubarb ratio of the biscuits), there were no significant changes in the spread ratio values of the biscuits. Spread ratio is one of the important quality criteria for biscuits and high spread ratio rate indicates dryer, firmer biscuits with smoother upper surface appearance, pore structure and pore distribution. Therefore, we concluded that the quality was preserved, since rhubarb did not affect the spread ratio. It was determined that rhubarb had no effect on the firmness values of the gluten-free biscuits, while gum had an increasing effect on the firmness. Rhubarb supplementation positively affected the TPC of the gluten-free biscuits. Gum supplementation decreased the TPC of the gluten-free biscuits. Our study showed that by adding natural components to gluten-free biscuit formulations, functional properties can be obtained in biscuits without sacrificing quality and biscuits with positive effects on health can be produced by improving the antioxidant properties.

ACKNOWLEDGEMENTS

This project was financed by the Scientific Research Project Chairmanship of YYU (2015-FBE-YL210).

REFERENCES

AACC, 1999. Approved Methods of the American Association of Cereal Chemists, 9th Ed. American Association of Cereal Chemists, St. Paul, MN, USA.

Andiç S., Tunçtürk Y., Ocak E. and Köse Ş. 2009. Some chemical characteristic of edible wild rhubarb species (*Rheum ribes* L.). Res J. Agri Bio Sci. 5 (6):973-977.

Bourekoua H., Rozylo R., Gawlik-Dziki U., Benatallah L., Zidoune M. N. and Dziki D. 2017. Evaluation of physical, sensorial, and antioxidant properties of gluten-free bread enriched with Moringa oleifera leaf powder. Eur Food Res Technol. 44:189-195.

Capriles VD., dos Santos FG. and Areas J. A. G. 2016. Gluten-free breadmaking: improving nutritional and bioactive compounds. J. Cereal Sci. 67:83-91.

Chaisiwang M. and Suphantharika M. 2006. Pasting and rheological properties of native and anionic tapioca starches as modified by guar gum and xanthan gum. Food Hydrocoll, 20:641-646.

De Simas K. N., Vieira L. N., Podesta R., Müller C. M. O., Veira M. A., Beber R. C., Reis M. S., Barreto P. L. M., Amante E. R. and Amboni, R. D. M. C. 2009. Effects of king palm (*Archontophoenix alexandrae*) flour in corporation on physicochemical and textural characteristics of gluten-free cookies. Int. J. Food Sci. Technol. 44:531-538.

Doğan İ. S. and Uğur, T., 2005. A study on biscuit quality of some wheat grown in Van. YYU J. Agri Sci. 15(2):139-148.

Drabinska N., Ciska E., Szmatowicz B. and Krupa-Kozak U. 2017. Broccoli byproducts improve the nutraceutical potential of gluten-free mini sponge cakes. Food Chem. 267:170-177.

Giuberti G., Rocchetti G., Sigolo A., Fortunati P., Lucini L. and Gallo A. 2018. Exploitation of alfalfa seed (*Medicago sativa* L.) flour into gluten-free rice cookies: nutritional, antioxidant and quality characteristics. Food Chem. 239:679-687.

Hoseney R. C. and Rogers D. E., 1994. Mechanism of sugar functionality in cookies: the science of cookie and cracker production. The Science of Cookie and Cracker Production (Editor: Hamed Faridi). Chapman&Hall, St. Paul, MN. 516.

Jnawali P. Kumar V. and Tanwar B. 2016. Celiac disease: overview and considerations for development of gluten-free foods. Food Sci Human Wellness. 5:169-176.

Jyothi A. N., Korappatti S., Sajeev M. S., Revamma R., Moorthy S. N. 2005. Gelatinisation properties of cassava starch in the presence of salts, acids and oxidising agents. Starch, 57:547-555.

Malkanthi H. H. A. and Umadevi S. H. 2018. Effect of dried pumpkin pulp and seed powder on physical, chemical and sensory properties of biscuits. : Int. J. Sci. Res. 7(8):32-34.

Meral R. and Doğan İ. S. 2009. Use of natural ingredients that have functional importance in the production of bakery products. J. Food 34(3):193-198.

Meral R. 2011. Determination of the effects of natural components having functional properties on dough and bread properties. (phd thesis, unpublished). Yüzüncü Yıl University, Institute of Science, Van-Turkey.

Meral R. and Doğan İ. S. 2013. Grape seed as a functional food ingredient in bread-making. Int. J. Food Sci. Nutri. 64(3):372-379.

Michalska A., Amigo-Benavent M., Zielinski H. and Dolores del Castillo M. 2008. Effect of breadmaking on formation of Maillard reaction products contributing to the over all antioxidant activity of rye bread. J. Cereal Sci. 48:123-132.

Öztürk M., Aydoğmuş-Öztürk F., Duru M. E. and Topçu G. 2007. Antioxidant activity of stem and root extract of Rhubarb (*Rheum ribes*): An edible medicinal plant. Food Chem. 103:623-630.

Paciulli M., Rinaldi M., Cavazza A., Ganino T., Rodolfi M., Chiancone B. and Chiavaro E. 2018. Effect of chestnut flour supplementation on physico-chemical properties and oxidative stability of gluten-free biscuits during storage. LWT, 98:451-457.

Padalino L., Conte A. and Del Nobile MA. 2016. Overview on the general approaches to improve gluten-free pasta and bread. Foods. 5:87.

Rocchetti G., Giuberti G. and Lucini L. 2018. Gluten-free cereal-based food products: The potential of metabolomics to investigate changes in phenolics profile and their in vitro bioaccessibility. Curr. Opin. Food Sci. 22:1-8.

Rodriguez-Garcia J., Laguna L., Puig A., Salvador A. and Hernando I. 2012. Effect of fat replacement by inulin on textural and structural properties of short dough biscuits. Food Bioprocess Tec. 6(10):2739-2750.

Sakač M., Pestorić M., Mišan A., Nedeljković N., Jambrec D, Jovanov P., Banjac V., Torbica, A., Hadnađev M. and Mandić A. 2015. Antioxidant capacity, mineral content and sensory properties of gluten-free rice and buckwheat cookies, Food Technol. Biotechnol. *53*(1):38-47.

Sakiyan O., Sumnu G., Sahin S., Meda V., Koksel H. and Chang P. 2009. A study on degree of starch gelatinization in cakes baked in three different ovens. Food Bioprocess Tech. **4**:1237-1244.

Shi X. and Be Miller J. N. 2002. Effects of food gums on viscosities of starch suspensions during pasting. Carbohydrate Polymers, 50:7-18.

Yetim H., Öztürk İ., Törnük F., Sağdıç O. and Hayta M. 2010. Functional properties of edible plants and seed sprouts. J. Food 35 (3):205-210.

Yıldız Ö., Yurt B., Baştürk A., Toker Ö. S., Yılmaz M. T., Karaman S. and Dağlıoğlu O. 2013. Pasting properties, texture profile and stress-relaxation behavior of wheat starch/dietary fiber systems. Food R. Int. 53:278-290.

Yılmaz M. T., Yıldız Ö., Yurt B., Toker Ö. S., Karaman S. and Baştürk A. 2015. A mixture design study to determine interaction effects of wheat, buckwheat, and rice flours in a aqueous model system. LWT, 61:583-589.

Paper Received June 5, 2018 Accepted February 20, 2019

PAPER

CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *ALOYSIA TRIPHYLLA* L. ESSENTIAL OILS AND METHANOLIC EXTRACT

L. REZIG[1], M. SAADA[2], N. TRABELSI[3], S. TAMMAR[2], H. LIMAM[2], I. BETTAIEB REBEY[2], A. SMAOUI[3], G. SGHAIER[2], G. DEL RE[4], R. KSOURI[2] and K. MSAADA*[2]

[1]High Institute of Food Industries, 58 Alain Savary Street, El Khadra City, Tunis, 1003, Tunisia

[2]Laboratory of Aromatic and Medicinal Plants, Biotechnology Center in Borj Cedria Technopole, BP. 901 Hammam-Lif 2050, Tunisia

[3]Laboratory of Olive Biotechnology, Biotechnology Center of Borj-Cedria, P.O. Box 901, 2050 Hammam-Lif, Tunisia

[4]Università dell'Aquila, Dipartimento di Ingegneria Industriale e dell'Informazione e di Economia, Piazzale Ernesto Pontieri, Monteluco di Roio, 67100 L'Aquila, Italy

*Corresponding author: Tel.: +21622205878; Fax: +21679412638

E-mail address: msaada.kamel@gmail.com

ABSTRACT

The essential oil variability in aerial parts of *Aloysia triphylla,* collected from four different Tunisian regions was assessed. In addition, total polyphenols, flavonoids, and condensed tannins as well as antioxidant, antibacterial, and antifungal activities of methanolic extract and essential oils were determined. Chromatographic analysis of *Aloysia triphylla* essential oils showed the predominance of monoterpene aldehydes represented mainly by neral and geranial. RP-HPLC analysis of *Aloysia triphylla* methanolic extract revealed the predominance of *p*-coumaric acid among the 15 phenolic acids identified. Antiradical activity was region-dependent and the methanolic extract of Kairouan sample had the strongest activity. Concerning the reducing power, the methanolic extract of Kairouan, Siliana, Kairouan, and Gabes samples were less active than the positive control. Siliana sample showed the least ability to prevent the bleaching of β-carotene, whereas Kairouan sample exhibited the strongest activity. Obtained results of antimicrobial and antifungal activities showed that *Aloysia triphylla* essential oil was endowed with important antibacterial and antifungal properties. Overall, based on its methanolic extract and essential oil features, *Aloysia triphylla* may be considered as a valuable source of new multipurpose products for industrial, cosmetic, and pharmaceutical utilization.

Keywords: *Aloysia triphylla* L., regions, phenolic, essential oils, antioxidant activity, antimicrobial activity

1. INTRODUCTION

The lemon verbena, *Aloysia triphylla* (l'Herit), Britt's = *Lippia citriodora* (Lam.), is a perennial shrub belonging to the Verbenaceae family (SHARMA *et al.*, 2016). It grows naturally in South America and was introduced into North Africa (Tunisia) and in Southern Europe in the late seventeenth century (CARNAT *et al.*, 1999; BENSABAH *et al.*, 2014). Leaves of *Aloysia triphylla* are used for culinary purposes as food seasoning and beverage flavoring (DOMINGUEZ-AVILA *et al.*, 2016). Furthermore, due to their aromatic, antispasmodic and digestive properties, leaves of *Aloysia triphylla* are commonly used as infusion or herbal tea (BENSABAH *et al.*, 2014). Traditional applications of *Aloysia triphylla* include its use as herbal remedy for colds, fever, spasms asthma, flatulence, colic, diarrhea, indigestion, insomnia, anxiety, analgesic and sedative (VALENTAO *et al.*, 1999). These therapeutic benefits were attributed to phenolic compounds (mainly flavonoids, phenolic acids, and phenylpropanoids) (PASCUAL *et al.*, 2001; QUIRANTES-PINÉ *et al.*, 2009). Antioxidants acting as radical scavengers are able to protect the human body as well as processed foods from oxidative damage. Presently, much attention has been focused on the antioxidant effect of plant natural compounds due to their wide application in food. Medicinal plants, being a promising source of phenolics, flavonoids, anthocyanins and carotenoids, are usually used to add flavor and improve the shelf life of dishes and processed food products. Regarding these beneficial effects, low cost and properties of plant phenolics, the interest is to increase research on natural antioxidants, in order to improve on their use in the food industry and as preventive medicine (EL BABILI *et al.*, 2013). Antioxidant properties of extracts of *Aloysia triphylla* leaves, as well as chemical composition of their essential oil obtained from different localities were investigated by several authors (PASCUAL *et al.*, 2001; CATALAN and LAMPASONA, 2002; CRABAS *et al.*, 2003; KIM and LEE, 2004; SANTOS GOMEZ *et al.*, 2005; CHOUPANI *et al.*, 2014). The essential oil has been shown to exhibit antimicrobial and anti-Candida activities (DUARTE *et al.*, 2005; TEIXEIRA *et al.*, 2007). According to ALI *et al.* (2011), antimicrobial activity of *Aloysia triphylla* essential oil may be attributed to the presence of high concentration of long-chain alcohols and aldehydes especially citral, citronellol, menthol, and β-caryophyllene. Otherwise, antifungal activity is related to eugenol, camphene, and β-caryophyllene contents (MAGWA *et al.*, 2006). There is hardly any available datum concerning *Aloysia triphylla* cultivated in Tunisia. In our present study, we have studied essential oil and methanolic extracts composition of *Aloysia trihpylla* cultivated in Tunisia and gathered from four distinct regions (Kairouan, Korba, Siliana, and Gabes). Moreover, we have evaluated their antioxidant, antimicrobial, and antifungal activities. This report also stressed on the underlying variability of *Aloysia triphylla* essential oil and methanolic extracts and their biological activities as affected by the collection site.

2. MATERIAL AND METHODS

2.1. Plant material

The aerial parts of four accessions of Tunisian *Aloysia triphylla* were gathered from four different regions in Tunisia, namely Kairouan (center), Korba (northest), Siliana (northwest), and Gabes (southeast) (Table 1). The aerial part samples were air- dried and conserved in a desiccator at room temperature (~25°C) in darkness for further extraction.

Table 1. Geographical coordinates and bioclimatic classification of the collecting zone of aerial parts of *Aloysia triphylla*.

	Longitude	Latitude	Elevation (m)	Bioclimatic zone
Kairouan	10°05'30,93''E	35°40'33,29''N	62	arid superior
Korba	10°51'43,49''E	36°34'50,18''N	12	semi-arid superior
Siliana	9°21'52,32''E	36°05'19,39''N	425	semi-arid superior
Gabes	10°05'51,08''E	33°53'17,08''N	7	arid inferior

2.2. Essential oil extraction

Dried aerial parts (100 g) were subjected to hydro distillation in a Clevenger type apparatus for 3h according to the European Pharmacopoeia (2017).

2.3. Essential oil analysis

Analysis of volatile compounds by GC was carried out on a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and an electronic pressure control (EPC) injector. A polar polyethylene glycol (PEG) HP Innowax capillary column (30 m × 0.25 mm, 0.25 mm film thickness; Hewlett-Packard, CA, 127 USA) was used. The flow of the carrier gas (N_2) was 1.6 mL/min. The split ratio was 60:1. The analysis was performed using the following temperature program: oven temperature kept isothermally at 35°C for 10 min, increased from 35°C to 205°C at the rate of 3°C/min and kept isothermally at 205°C for 10 min. Injector and detector temperatures were held at 250°C and 300°C, respectively. The individual peaks were identified by comparing their relative retention indices to n-alkanes (C6-C22) with those of literature (ADAMS, 2004) and/or with those authentic compounds available in our laboratory. Volatile aroma compounds analysis by GC/MS was performed on a gas chromatograph HP 5890 (II) interfaced with a HP 5972 mass spectrometer (Palo Alto, CA, USA) with electron impact ionization (70 eV is the ionization energy). A HP-5 MS capillary column (30 m × 0.25 mm, coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane, 0.25 mm film thickness; Hewlett-Packard, CA, USA) was used. The column temperature was programmed to rise from 50°C to 240°C at a rate of 5°C/min. The carrier gas was helium with a flow rate of 1.2 mL/min; split ratio was 60:1. Scan time and mass range were 1 s and 40-300 m/z, respectively. Identification of aroma compounds was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC/MS data system and other published mass spectra.

2.4. Polyphenols extraction

The air-dried aerial parts of *Aloysia triphylla* were finely ground with a blade-carbide gringing (IKA-WERK Type: A: 10). Triplicate sub-samples of 1 g of each ground organ were separately extracted by stirring with 10 mL of pure methanol for 30 min. The extracts were then kept for 24 h at 4°C, filtered through a Whatman No. 4 filter paper, evaporated under vacuum to dryness and stored at 4°C until analyzed (MAU *et al.* 2004).

2.5. Total phenolic content

Total phenolic contents were assayed using the Folin-Ciocalteu reagent, following the Singleton's method, which was slightly modified by DEWANTO *et al.* (2002). An aliquot

(0.125 mL) of a suitable diluted methanolic organ extract was added to 0.5 mL of deionized water and 0.125 mL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 1.25 mL of 7% Na_2CO_3 solution. The solution was then adjusted with deionized water to a final volume of 3 mL and mixed thoroughly. After incubation for 90 min at 23°C, the absorbance versus prepared blank was read at 760 nm. Total phenolic contents of aerial parts (three replicates per treatment) were expressed as mg gallic acid equivalents per gram (mg GAE/g of DW) through the calibration curve with gallic acid. The calibration curve range was 50-400 mg/mL (R^2 = 0.99). All experiments were performed in triplicates.

2.6. Total condensed tannins content

The total tannin content was measured using the modified vanillin assay described by SUN *et al.* (1998). A total of 3mL of 4% methanol vanillin solution and 1.5mL of concentrated H_2SO_4 were added to 50 μL of suitably diluted sample. The mixture was kept for 15 min, and the absorbance was measured at 500 nm against the blank (methanol). The amount of total condensed tannins was expressed as milligrams of (+)-catechin equivalent per gram of dry weight (mg of CE/g of DW) through the calibration curve with catechin. Triplicate measurements were taken for all samples.

2.7. Total flavonoid content

Total flavonoid content was measured according to DEWANTO *et al.* (2002). The 250 µL appropriately diluted methanolic aerial extract was mixed with 75 µL $NaNO_2$ (5%). After 6 min, 150 µL of 10% $AlCl_3$ and 500 µL of NaOH (1 M) were added to the mixture. Finally, the mixture was adjusted to 2.5 mL with distilled water. The absorbance versus prepared blank was read at 510 nm. Total flavonoid contents of aerial parts (three replicates per treatment) were expressed as mg catechin equivalents per gram (mg CE/g of DW) through the calibration curve with catechin. The calibration curve range was 50-500 mg/mL.

2.8. Isolation and identification of phenolic compounds

Dried samples from aerial parts of *Aloysia triphylla* were treated and used to hydrolize phenolic compounds following the method of PROESTOS *et al.* (2006). Thereafter, the analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC) coupled with an ultraviolet (UV)-vis multi wavelength detector. The separation was carried out on a 250 × 4.6-mm, and 4 μm Hypersil ODS C18 reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was kept at 0.5 mL/min. The gradient program was as follows: 15% A/85% B 0-12 min, 40% A/60% B 12-14 min, 60% A/40% B 14-18 min, 80% A/20% B 18-20 min, 90% A/10% B 20-24 min, and 100% A 24-28 min. Phenolic compounds were identified based on their retention times and spectral characteristics of their peaks against those of the standards, as well as by spiking the sample with standards. Analyses were performed in triplicate.

2.9. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The scavenging capacity of the obtained extracts was measured by bleaching of the purple-colored solution of the DPPH radical according to the method of HANATO *et al.* (1988). A total of 1 mL of different concentrations of extracts prepared in methanol was

added to 0.5 mL of a 0.2 mmol/L DPPH methanolic solution. The mixture was shaken vigorously and kept at room temperature for 30 min. The absorbance of the resulting solution was then measured at 517 nm after 30 min. The antiradical activity was expressed as IC_{50} (μg/mL), which is the concentration required to cause 50% DPPH inhibition. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect \%} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. BHT was used as a positive control.

2.10. Reducing power assay

The method of OYAIZU (1986) was used to assess the reducing power of different extracts. A total of 1 mL of different concentrations of extracts in methanol was mixed with 2.5 mL of a 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide $[K_3Fe(CN)_6]$ and incubated in a water bath at 50°C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture that was centrifuged at 650g for 10 min. The supernatant (2.5 mL) was then mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. The intensity of the blue-green color was measured at 700 nm. Results were expressed as EC_{50} (mg/mL), which was the extract concentration at which the absorbance was 0.5 for the reducing power and was calculated from the graph of absorbance at 700 nm against the extract concentration. Ascorbic acid was used as a positive control.

2.11. β-Carotene bleaching test

The β-carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (KULISIC *et al*., 2004). A modification of the method described by KOLEVA *et al.* (2002) was employed. β-Carotene (2 mg) was dissolved in 20 mL of chloroform and to 4 mL of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 mL of oxygenated ultra-pure water was added, thereafter, the emulsion was vigorously shaken. Reference compound (BHT), sample extracts were prepared in methanol. The emulsion (3 mL) was added to a tube containing 0.2 mL of different concentrations of extract (1, 10, 100 and 200 μg/mL). The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50°C for 120 min, when the absorbance was measured again. BHT was used as the positive control. For the negative control, the extract was substituted by an equal volume of methanol. The antioxidant activity (%) of the *Aloysia triphylla* aerial parts extracts was evaluated in terms of bleaching of the β-carotene using the following formula:

$$\text{\% Inhibition} = \frac{At - Ct}{C0 - Ct}$$

where At and Ct are the absorbance values measured for the test sample and control, respectively, after incubation for 120 min. C_0 is the absorbance values for the control measured at zero time during the incubation. The results are expressed as IC_{50} values (μg/mL), which is the concentration required to cause a 50% β-carotene bleaching inhibition. Tests were carried out in triplicate.

2.12. Screening of antibacterial and antifungal activities

Antibacterial activity was analyzed by the disc diffusion method against four human pathogenic bacteria including *Escherichia coli (ATCC 35218)*, *Pseudomonas aeruginosa (ATCC 4141), Enterococcus faecalis (ATCC 2212), and Bacillus subtilis (CIP 5265*) according to the method of RIOS and RECIO (2005).
The same agar-disc diffusion method was used for screening the antifungal activity of *Aloysia triphylla* aerial parts extracts. Four yeast strains (*Candida albicans* (ATCC 2091), *Candida kefyr*, *Candida parapsilosis, and Candida glabrata* (ATCC 90030)) were first grown on Sabouraud chloramphenicol agar plate at 30°C for 18-24 h. Several colonies of similar morphology of the clinical yeast were transferred into API suspension medium and adjusted to 2 McFarland turbidity standards with a Densimat. The inocula of the respective yeast were streaked on to Sabouraud chloramphenicol agar plates at 30°C using a sterile swab and then dried. A sterilized 6 mm paper disc was loaded with 20 μL (10 mg/mL) of aerial parts extract. The treated Petri dishes were placed at 4°C for 1-2h and then incubated at 37°C for 18-24h. The inhibition of fungal growth was also evaluated by measuring the diameter of the transparent inhibition zone around each disc. The susceptibility of the standard was determined using a disc paper containing Nystatin.

2.13. Data analysis

All analyses were performed in triplicate and the results expressed as mean values±standard deviations (SD). The data were subjected to statistical analysis using statistical program package STATISTICA (STATSOFT, 1998). The one-way analysis of variance (ANOVA) followed by Duncan multiple range test was employed and the differences between individual mean values were significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Essential oil yields

Essential oil yields were determined by hydrodistillation of 100 g of *Aloysia triphylla* dry aerial parts gathered from different localities. From these results, the optimal yield was observed in the sample of Korba (0.56%) followed by the samples of Kairouan, Siliana and Gabes. Statistical examination revealed significant differences in the essential oil yield of *Aloysia triphylla* originated from Korba, and those from Siliana, Kairouan, and Gabes. These values are closer to those reported by DI LEO LIRA *et al.* (2013) on essential oil yields of *Aloysia triphylla* from Argentina. When compared with other studies reported by MOEIN *et al.* (2014) and EL-HAWARY *et al.*(2011) on *Aloysia citriodora* Palau leaves and on *Lippia citriodora* Kunth fresh leaves, essential oil yields are higher than those of Tunisia region, bearing essential oil contents of 1.3% and 0.9%, respectively. According to TAGHI EBADI *et al.* (2015), EL-HAWARI *et al.* (2015), and BENSABAH *et al.* (2014), variation in the essential oil yield of *Aloysia triphylla* is attributed to cultivation climates, edaphic variables, water quality irrigation, ripening stages, and drying methods.

3.2. Variability in chemical composition of essential oil

Essential oils analyzed are divided into seven classes based on their chemical functional groups (Table 2).

Table 2. Essential oils composition (peak area % w/w±SD) and analysis of variance analysis results (*p*-values) of essential oil *Aloysia triphylla* aerial parts.

Compound*	RRI	Collection region				*P*
		Kairouan	Korba	Siliana	Gabes	
α-Pinene	1031	0.42 ^{c}±0.03	0.55 ^{a}±0.04	0.35 ^{d}±0.02	0.44 ^{b}±0.03	0.002**
Sabinene	1132	0.22 ^{a}±0.01	0.13 ^{d}±0.01	0.20 ^{b}±0.01	0.19 ^{c}±0.02	0.268^{NS}
Myrcene	1176	0.38 ^{d}±0.04	0.41 ^{c}±0.03	0.45 ^{b}±0.05	0.52 ^{a}±0.06	0.001**
Limonene	1202	**7.27** ^{c}±0.54	**6.34** ^{d}±0.43	**7.52** ^{b}±0.74	**7.80** ^{a}±0.81	0.000***
(Z)-β-Ocimene	1245	1.59 ^{d}±0.13	2.00 ^{c}±0.21	2.20 ^{b}±0.19	2.41 ^{a}±0.31	0.309^{NS}
γ-Terpinene	1265	0.21 ^{c}±0.05	0.22 ^{b}±0.03	0.23 ^{a}±0.08	0.19 ^{d}±0.02	0.150^{NS}
cis -Limonene oxide	1450	0.42 ^{b}±0.04	0.44 ^{a}± 0.03	0.39 ^{c}±0.03	0.35 ^{d}±0.03	0.501^{NS}
trans- Sabinene hydrate	1474	0.26 ^{a}±0.02	0.15 ^{d}±0.01	0.19 ^{c}±0.02	0.24 ^{b}±0.01	0.918^{NS}
cis- Sabinene hydrate	1556	0.23 ^{a}±0.02	0.22 ^{b}±0.02	0.20 ^{c}±0.01	0.22 ^{b}±0.02	0.034*
Linalol	1552	0.50 ^{d}±0.04	0.55 ^{c}±0.05	0.58 ^{b}±0.06	0.60 ^{a}±0.05	0.444^{NS}
Terpinene-4-ol	1611	0.12 ^{c}±0.01	0.13 ^{b}±0.01	0.11 ^{d}±0.01	0.15 ^{a}±0.01	0.483^{NS}
α-Cadinol	1620	0.49 ^{d}±0.05	0.54 ^{c}±0.05	0.58 ^{a}±0.06	0.57 ^{b}±0.07	0.009**
trans-Chrysanthenol	1684	0.89 ^{a}±0.07	0.77 ^{b}±0.06	0.69 ^{b}±0.07	0.68 ^{bc}±0.06	0.042*
α-Terpineol	1705	0.74 ^{c}±0.06	0.70 ^{d}±0.05	0.78 ^{a}±0.07	0.75 ^{b}±0.08	0.480^{NS}
cis-Chrysanthenol	1762	0.58 ^{a}±0.06	0.51 ^{b}±0.05	0.40 ^{c}±0.03	0.38 ^{d}±0.03	0.004**
Nerol	1798	0.25 ^{a}±0.02	0.24 ^{b}±0.02	0.20 ^{d}±0.01	0.23 ^{c}±0.02	0.774^{NS}
Geraniol	**1856**	**5.57** ^{d}±0.54	**6.02** ^{b}±0.59	**5.87** ^{c}±0.61	**6.42** ^{a}±0.54	**0.024***
Geranyl acetate	1765	1.79 ^{a}±0.14	1.80 ^{a}±0.12	1.72 ^{b}±0.15	1.70 ^{c}±0.17	0.296^{NS}
Chrysanthenone	1507	0.55 ^{d}±0.05	0.66 ^{a}±0.06	0.65 ^{b}±0.06	0.61 ^{c}±0.06	0.192^{NS}
Neral	**1240**	**17.22** ^{b}±1.80	**14.81** ^{d}±1.25	**15.60** ^{c}±1.44	**18.73** ^{a}±1.75	**0.000*** **
Geranial	**1742**	**25.15** ^{c}±2.15	**26.85** ^{b}±2.17	**27.41** ^{a}±2.45	**24.85** ^{d}±2.51	**0.000*** **
1-8-Cineole	1213	1.62 ^{d}±0.14	1.70 ^{c}±0.15	1.77 ^{b}±0.18	1.78 ^{a}±0.16	0.051^{NS}
α-Cubebene	1350	0.29 ^{b}±0.03	0.33 ^{a}±0.02	0.25 ^{c}±0.03	0.28 ^{bc}±0.01	0.075^{NS}
β-Cubebene	1466	0.11 ^{c}±0.01	0.10 ^{cd}±0.01	0.12 ^{b}±0.01	0.15 ^{a}±0.01	0.223^{NS}
δ-Elemene	1479	0.45 ^{d}±0.03	0.48 ^{c}±0.05	0.50 ^{b}±0.06	0.55 ^{a}±0.06	0.318^{NS}
α-Copaene	1497	0.16 ^{c}±0.01	0.18 ^{b}±0.01	0.19 ^{ab}±0.02	0.20 ^{a}±0.02	0.069^{NS}
β-Bourbonene	1535	0.86 ^{b}±0.07	0.88 ^{a}±0.08	0.85 ^{c}±0.07	0.85 ^{c}±0.08	0.110^{NS}
cis-α-Bergamotene	1560	0.46 ^{a}±0.05	0.38 ^{b}±0.03	0.35 ^{c}±0.03	0.34 ^{c}±0.02	0.003**
β-Copaene	1580	0.30 ^{b}±0.03	0.32 ^{a}±0.03	0.28 ^{c}±0.02	0.25 ^{d}±0.02	0.000***
α-Cedrene	1583	0.52 ^{b}±0.05	0.50 ^{d}±0.04	0.53 ^{a}±0.03	0.51 ^{c}±0.06	0.910^{NS}
β-Gurjunene	1597	0.36 ^{a}±0.03	0.26 ^{b}±0.02	0.21 ^{c}±0.02	0.19 ^{d}±0.01	0.036*
α-Caryophyllene	1610	1.64 ^{d}±0.15	2.21 ^{c}±0.20	2.23 ^{b}±0.21	2.24 ^{a}±0.25	0.244^{NS}
β-Caryophyllene	1612	1.06 ^{a}±0.12	1.00 ^{b}±0.11	0.98 ^{cd}±0.07	0.99 ^{c}±0.08	0.000***
Allo-Aromadendrene	1630	0.48 ^{a}±0.04	0.44 ^{b}±0.03	0.41 ^{c}±0.05	0.48 ^{a}±0.05	0.000***
Aromadendrene	1661	1.03 ^{d}±0.11	1.22 ^{b}±0.10	1.12 ^{c}±0.12	1.25 ^{a}±0.10	0.459^{NS}
α-Amorphene	1679	1.23 ^{d}±0.12	1.40 ^{b}±0.11	1.33 ^{c}±0.10	1.52 ^{a}±0.14	0.279^{NS}
β-Acoradiene	1688	0.41 ^{b}±0.03	0.40 ^{c}±0.05	0.44 ^{a}±0.04	0.38 ^{d}±0.03	0.255^{NS}
α-Zingiberene	1720	0.94 ^{a}±0.08	0.85 ^{b}±0.07	0.84 ^{c}±0.08	0.83 ^{d}±0.09	0.000***
Germacrene-D	1725	0.44 ^{d}±0.05	0.45 ^{c}±0.03	0.47 ^{b}±0.05	0.49 ^{a}±0.05	0.439^{NS}
Bicyclogermacrene	1755	4.54 ^{a}±0.42	4.21 ^{c}±0.41	4.25 ^{b}±0.39	4.12 ^{d}±0.40	0.000***
ar-Curcumene	**1760**	**5.24** ^{a}±0.45	**4.31** ^{d}±0.32	**5.22** ^{b}±0.61	**5.13** ^{c}±0.44	**0.861^{NS}**
α-Cadinene	1773	0.42 ^{c}±0.04	0.40 ^{d}±0.03	0.47 ^{b}±0.05	0.48 ^{a}±0.04	0.344^{NS}
δ-Cadinene	1776	0.63 ^{d}±0.05	0.68 ^{c}±0.06	0.72 ^{b}±0.07	0.77 ^{a}±0.08	0.621^{NS}

Caryophyllene Oxide	2008	0.91 ^{b}±0.08	0.85 ^{d}±0.06	0.92 ^{a}±0.07	0.90 ^{c}±0.08	0.485^{NS}
(E)-Nerolidol	2050	1.41 ^{d}±0.12	1.54 ^{b}±0.14	1.56 ^{a}±0.16	1.47 ^{c}±0.13	0.995^{NS}
Spathulenol	2150	1.06 ^{c}±0.10	1.20 ^{a}±0.11	1.08 ^{b}±0.09	1.09 ^{b}±0.09	0.030*
T-Cadinol	2185	0.53 ^{d}±0.04	0.62 ^{a}±0.05	0.55 ^{c}±0.05	0.59 ^{b}±0.06	0.023*
Isospathulenol	2230	2.60 ^{c}±0.24	2.65 ^{b}±0.25	2.64 ^{bc}±0.26	2.66 ^{a}±0.29	0.995^{NS}
Chemical classes						
Monoterpene hydrocarbons		10.77 ^{c}±1.34	10.46 ^{d}±2.01	11.73 ^{b}±1.32	12.36 ^{a}±2.45	0.000***
Monoterpene alcohols		9.14 ^{d}±0.76	9.46 ^{b}±0.95	9.21 ^{c}±0.83	9.78 ^{a}±0.89	0.000***
Monoterpene esters		1.79 ^{a}±0.14	1.80 ^{a}±0.12	1.72 ^{b}±0.15	1.70 ^{c}±0.17	0.296^{NS}
Monoterpene Ketones		0.55 ^{a}±0.05	0.66 ^{a}±0.06	0.65 ^{a}±0.06	0.61 ^{a}±0.06	0.192^{NS}
Monoterpene aldehydes		42.37 ^{d}±4.37	41.66 ^{c}±3.98	43.01 ^{b}±4.38	43.58 ^{a}±5.12	0.000***
Monoterpene ethers		1.62 ^{d}±0.14	1.70 ^{c}±0.15	1.77 ^{b}±0.18	1.78 ^{a}±0.10	0.051^{NS}
Sesquiterpenes		28.08 ^{d}±2.56	27.86 ^{a}±3.11	28.51 ^{c}±2.73	28.71 ^{b}±3.54	0.000***
Total identified		94.29 ^{c}±7.52	93.60 ^{d}±8.64	96.60 ^{b}±8.72	98.52 ^{a}±7.24	0.000***

RRI: relative retention index; ∗Compounds in order of elution on HP-innowax; values of volatile essential oil percentages are the average of three determinations (n = 3). These values with different letters (a-d) are significantly different at P <0.05. NS: not significant. **P < 0.01. ***P < 0.001. *P*: probability.

A total of 48 compounds were identified representing 94.29%, 93.6%, 96.6%, and 98.52% of total volatiles in the samples of Kairouan, Korba, Siliana, and Gabes, respectively. These different identified compounds vary significantly (P < 0.05) from one region to another and are highly (P <0.001) affected by the regional factor (Table 2). The major contribution was attributed to the monoterpene aldehydes fraction which represents 42.3%, 41.66%, 43.01%, and 43.58% of all compounds detected in Kairouan, Korba, Siliana, and Gabes samples, respectively. Indeed, this latter fraction is dominated by geranial (24.85% in Gabes and 27.41% in Siliana) and neral (14.81% in Korba and 18.73% in Gabes). These results are in agreement with previous studies reported by MOEIN *et al.* (2014) and TAGHI EBADI *et al.* (2015). Our studies have reported that limonene was the third major compound in the essential oil of *Aloysia triphylla* samples with a content ranging between 6.34% and 7.8% for Korba and Gabes samples, respectively.

This result is similar to that reported by MOEIN *et al.* (2014), on essential oil of *Aloysia citriodora* Palau cultivated in gardens where neral, geranial, and limonene rates reached 13.46%, 16.7%, and 12.41%, respectively. Some authors reported citral to be present at a higher percentage than limonene in *L. citriodora* (KIM and LEE, 2004), while others reported the opposite (ÖZEK *et al.*, 1996). SANTOS-GOMES *et al.* (2005) reported that the percentage of citral exceeded that of limonene. The *ar*-curcumene is the main component of sesquiterpene fraction with a rate ranging between 4.31% and 5.24% for the samples of Korba and Kairouan, respectively. From a statistical point of view, this compound seemed not to be affected by any regional factor. Geraniol represented the main constituent in the monoterpene alcohols fraction with a percentage of 5.57%, 6.02%, 5.87%, and 6.42% in the sample of Kairouan, Korba, Siliana, and Gabes, respectively. With regard to the 1-8 cineole recognized for its antimicrobial and antifungal properties, this component showed lower amounts in all localities studied varying between 1.62% and 1.78% for Kairouan and Gabes samples, respectively. TAGHI EBADI *et al.* (2015) reported higher levels of 1-8 cineole in *Lippia citriodora* Kunth essential oil than those reported in our study. The 1-8 Cineole content amounted to 4.5% and 7.3% in freeze dried and oven dried leaves, respectively. It must be pointed out that a variety of geographical and ecological factors can lead to qualitative and quantitative differences in the essential oil produced. At the

same time, essential oil composition can be affected by a number of other factors such as the development stage of the plant, its physiology, the age of leaves, and the growing conditions (SANTOS GOMES *et al.*, 2005) as well as, the conditions and isolation method (CRABAS *et al.*, 2003; KIM and LEE, 2004; SANTOS GOMES *et al.*, 2005).

3.3. Total polyphenols, flavonoids, and condensed tannins

The aerial parts of *Aloysia triphylla* were gathered from different localities, ranging from the center (Kairouan), north (Korba and Siliana), and south (Gabes) in Tunisia characterized by diverse geographic and climatic conditions as mentioned in Table 1. Depending on its geographical origin, total polyphenols, flavonoids, and condensed tannins contents of *Aloysia triphylla* extracts are illustrated in Fig. 1.

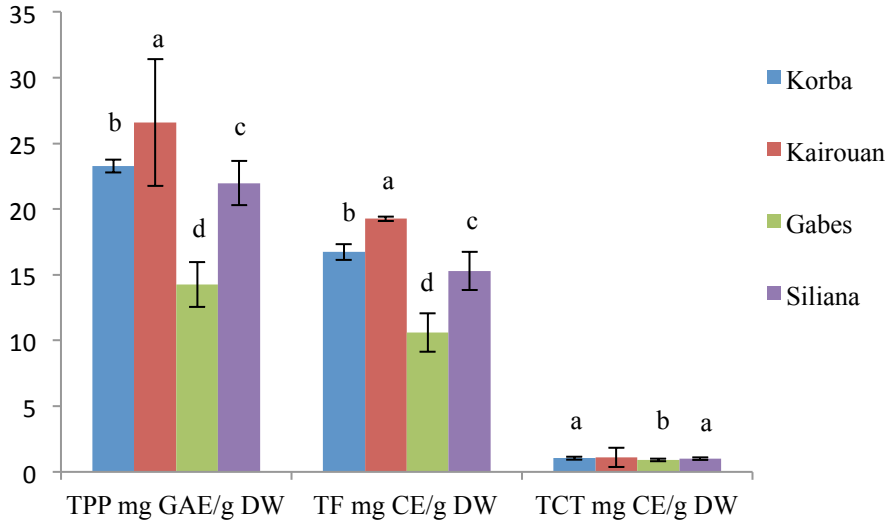


Figure 1. Total polyphenols (TPP), total flavonoids (TF), and total condensed tannin (TCT) contents of different regions of *Aloysia triphylla* aerial parts. GAE: gallic acid equivalents; CE: catechin equivalents. The letters (a-d) indicate significant differences ($p < 0.05$).

The results showed that the plant is a valuable source of phenolics with content ranging from 26.58 mg GAE/g for Kairouan to 14.25 mg GAE/g for Gabes, respectively. These results are lower than those cited by CHEURFA and ALLEM (2016) on hydro-alcoholic and aqueous extracts of Algerian *Aloysia triphylla* leaves. Moreover, ZHANG and WANG (2001) reported a total phenolic content of 1.55 mg GAE/ g of fresh weight on *Aloysia triphylla* herb extracted with phosphate buffer. Statistical analysis revealed that the total polyphenol contents varied significantly ($p < 0.05$) between the four studied localities. Likewise, the importance of the solvent type used in the extraction has been mentioned by CHOUPANI *et al.* (2014) and BETTAIEB REBEY *et al.* (2012). According to VASCO *et al.* (2008), differences in total phenol content may also be attributed to varieties, ripening stage, and post-harvest conditions (MSAADA *et al.*, 2007). Besides, the highest total flavonoid content was observed in Kairouan (19.26±0.74 mg EC/ g) followed by Korba (16.72±0.59 mg EC/g), Siliana (15.3±1.46 mg EC/g), and Gabes 10.59±1.1 mg EC/g), respectively. These results are much higher than those reported by VINHA *et al.* (2012) on the aqueous extract of *Aloysia triphylla*, showing a total flavonoid content of 43.38 mg C/100 g). It is well known that an important function of flavonoids and phenolic acids is

their action in plant defense mechanisms (DIXON and PAIVA, 1995). Indeed, flavonoids have several biological activities such as the inhibition of plasma platelet aggregation and cyclooxygenase activity, the suppression of histamine release, potent nitric oxide radical scavenging activity and exhibiting antibacterial, antiviral, anti-inflammatory and antiallergenic effects (COOK and SAMMAN, 1996).
Among the different localities studied, no significant differences ($p > 0.05$) were found in the total condensed tannins contents of *Aloysia triphylla* methanolic extracts. Kairouan showed the highest total condensed tannins (1.10±0.17 mg CE/g of DW), followed in a descending order by Korba (1.04±0.11 mg CE/g of DW), Siliana (1.01±0.10 g CE/g of DW), and Gabes (0.89±0.09 g CE/g of DW). Interestingly, no condensed tannins were observed in aqueous extracts (infusion and decoction) of *Aloysia citriodora* aerial parts (PORTMANN *et al.*, 2012). This finding was attributed to these authors in the absence of proanthocyanidins. Meanwhile, EL BABILI *et al.* (2013) reported a condensed tannins content of 1.97 g CE/kg dry in aqueous extract of verbena (*Verbena officinalis* L.) belonging to the same botanical family (Verbenacea).

3.4. Individual phenolic compounds

The phenolic compounds in methanol extracts of the aerial parts of *Aloysia triphylla* were identified by a RP-HPLC system. This system is a high resolution chromatographic technique widely used for simultaneous separation and quantification of phenolic substances. The results related to phenolic compounds are summarized in Table 3. It is fair to say that methanol extracts of *Aloysia triphylla* are rich in phenolics. In total, 15 compounds were identified. Statistical analysis revealed that the identified compounds were significantly affected ($P < 0.001$) by the regional factor. The *p*-coumaric acid was the predominant phenolic compound. The highest content of *p*-coumaric acid was observed in Kairouan (54.90%), followed by Korba, Siliana, and Gabes. The second major compound was catechol with a content ranging from 6.00% to 12.23% for Kairouan and Korba, respectively. Likewise, RP-HPLC analysis was used for the identification and quantification of phenolic compounds in leaves of *Aloysia triphylla* after extraction with a mixture of 62.5% aqueous methanol. Authors reported the presence of only four compounds: caffeic acid (0.84%), ferulic acid (0.82%), hydroxytyrosol (0.4%), and apigenin (0.24%). The presence of caffeic and ferulic acids in methanol extracts of *Aloysia triphylla* is worth noting with concentrations ranging between 0.28% and 2.39%, and 0.36% and 3.28% for Korba and Gabes, and Gabes and Siliana, respectively. Furthermore, apigenin and hydroxytyrosol were not identified in our present study. The *O*-hydroxybenzoic acid, hydroxycaffeic acid, and 3-nitro-phthalic acid were also identified by GC-MS after sialylation according to PROESTOS *et al.* (2006). Three phenolic compounds; two phenolic acids, dihydrocaffeic acid and 4-hydroxycinnamic acid and a flavonoid glycoside, luteolin-7-*O*-glucoside, were isolated and identified from the ethyl acetate fraction of the fresh aerial parts of *Lippia citriodora* Kunth cultivated in Egypt (EL HAWARY *et al.*, 2012). Luteolin 7-*O* glucoside was also identified in methanol extracts of the aerial parts of *Aloysia triphylla*, which originated from Korba (2.46%), Siliana (0.16%), Gabes (2.10%), and Kairouan (0.35%). This flavonoid glycoside is one of the main flavonoid constituents in many herbs and is known to possess low oxygen radical absorbance capacity (ORAC) values (ZHANG and WANG, 2001).

3.5. Antioxidant activities of methanol extracts

The results for antioxidant activities from the different accessions are displayed in Table 4. ANOVA analysis (Table 4) showed that the methanol extracts are highly influenced by the

regional effect (antiradical activity was region-dependent). Methanolic extract of Kairouan sample shows the highest antioxidant activity (IC_{50} = 5.78±0.08 μg/mL), which was stronger than that of the positive control: BHT (11.5±1.23 μg/mL). The reduced activity was observed in the sample of Gabes (30.67 μg/mL). CHEURFA and ALLEM (2016) have reported antiradical of aqueous extract of Algerian *Aloysia triphylla* leaves activity with an IC_{50} of 27.4 mg/mL. This value was significantly higher ($P < 0.05$) than that of hydro-alcoholic extract witnessing an IC_{50} of 23.52 mg/mL. On the other hand, the positive control BHT exhibited a significantly lower IC_{50} value ($P < 0.05$) when compared to the two studied extracts (6.96 mg/mL). These antiradical activities are lower than those reported in our present study. Other studies conducted on culinary decoction of verbena (*Verbena officinalis* L.), belonging to the same botanical family of *Aloysia triphylla*, showed an IC_{50} of 15.76 mg/mL (EL BABILI *et al.*, 2013). It is worth mentioning now that there is a linear correlation between total polyphenols, flavonoids, and condensed tannins, and the scavenging activity against DPPH for the methanolic extracts of the aerial parts of *Aloysia triphylla*. In fact, the methanolic extract of Kairouan sample, rich in polyphenols, flavonoids, and condensed tannins, was a more effective scavenger of DPPH radicals than the poor ones observed in Korba, Siliana, and Gabes samples. Consistency can be seen in our results and those obtained by CHEURFA and ALLEM (2016) who analyzed the total polyphenols, flavonoids, and scavenging activity of DPPH in aqueous and hydro-alcoholic extracts of *Aloysia triphylla* leaves. Meanwhile, EL-BABILI *et al.* (2013) failed to show any positive correlation between phenol contents and anti-oxidant activities according to the ABTS/DPPH assays on culinary decoction of *Verbena officinalis* L.

Besides, Table 4 showed that the Fe^{3+} reducing power of *Aloysia triphylla* methanolic extracts differs greatly depending on accession provenance. Gabes sample showed the higher reducing capacity (EC_{50} = 482.00 μg/mL) followed by Siliana (EC_{50} = 371.00 μg/mL), Korba (EC_{50} = 322.66 μg/mL), and Kairouan (EC_{50} = 209.33 μg/mL). Furthermore, in comparison with the positive control: ascorbic acid (EC_{50} = 37.33 μg/mL), Kairouan, Korba, Siliana, and Gabes samples methanolic extracts exhibited 6, 9, 10, and 13 fold lower activities, respectively. These results indicate that the different methanolic extracts are able to act as electron donor and, therefore, react with free radicals, converting them to a more stable products and, thereby, terminating radical chain reactions. On the other hand, statistical analysis revealed a higher region effect ($P < 0.001$) on reducing power (Table 4).

The antioxidant activity of *Aloysia triphylla* methanolic extract was also evaluated by the β-carotene-linoleate bleaching method (Table 4). This method was based on the loss of the yellow color of β-carotene due to its reaction with radicals formed after linoleic acid oxidation in emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (KULISIC *et al.*, 2004). Siliana sample showed the lowest ability to prevent the bleaching of β-carotene (IC_{50} = 4066.67 μg/mL), whereas Kairouan sample exhibited the strongest activity (IC_{50} = 500 μg/mL).

On the other hand, all the methanolic extracts had lower antioxidant activities than BHT with IC_{50} of 75.00 μg/mL (Table 4). In addition, the β-carotene-linoleate bleaching values were highly ($P < 0.001$) affected by the accession provenance.

It is worth mentioning that differences in antioxidant activities according to the DPPH/β-carotene bleaching inhibition/reducing power might reflect differences in the ability of anti-oxidant compounds to act against the different radicals present or formed during each specific reaction.

3.6. Antibacterial activity

The test results of the antibacterial effect are summarized in Table 5. The results showed that the diameter of the inhibition zone (IZ) is highly affected by the region's factor for

Escherichia coli (ATCC 35218) and *Bacillus subtilis (CIP 5265)* strains ($P < 0.001$). On the other hand, essential oils of *Aloysia Triphylla* did not exhibit an antibacterial activity against *Pseudomonas aeruginosa (ATCC 4141) and Enterococcus faecalis (ATCC 2212)* strains. The highest antibacterial activity was observed against *Bacillus subtilis (CIP 5265)* witnessing an inhibition zone equal to 85±7.67 mm for the *Aloysia triphylla* essential oil of Kairouan, followed by that of Siliana (IZ = 32±3 mm), Korba (IZ = 26±4.08 mm), and Gabes (IZ = 25.33±9.62 mm). The lower antibacterial activity of *Aloysia triphylla* essential oils against *Escherichia coli*, when compared to that against *Bacillus subtilis,* was attributed to the cell-wall of the Gram-negative bacteria covered by an outer membrane (Lipopolysaccharide, phospholipid and some proteins) (CHAO *et al.,* 2000). This latter prevents uptake of oils or protect peptidoglycan layer from oils. Hence, Lipopolysaccharide (LPS) membrane of Gram-negative bacteria presents a permeability barrier to hydrophobic substances that can enter and inhibit the Gram-positive bacteria. In Gram-positive bacteria, the peptidoglycan layer is on the outside and more in contact with the oils.
Our results are in compliance with those reported by ALI *et al.* (2011) who reported a very strong antibacterial activity of *Aloysia triphylla* essential oil against *Bacillus subtilis (CAICC)* (IZ ≥ 16 mm) and a negative one against *Escherichia coli (ATCC 25922)* (IZ = 0 mm).
The interesting antibacterial activity against *Bacillus subtilis* was attributed to the presence of a high concentration of long-chain alcohols especially geranial and neral, particularly active against Gram-positive bacteria (DELAQUIS *et al.,* 2002). In addition, ALI *et al.* (2011) reported that the antibacterial nature of *Aloysia triphylla* essential oil was apparently related to the presence of β-caryophyllene, showing in vitro activity against *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* (SACCHETTI *et al.* 2004).

3.7. Antifungal activity

Aloysia triphylla essential oils had a significant antifungal activity against the four strains of *Candida* species studied as shown in Table 5. The highest antifungal activity was recorded for the *Aloysia triphylla* essential oil of Kairouan, showing inhibition diameter zones of 85±8.27 mm, 85±7.89 mm, 85±6.59 mm, and 85±8.57 mm against *Candida albicans (ATCC 2091)*, *Candida kefyr*, *Candida parapsilosis*, and *Candida glabrata (ATCC 90030),* respectively. These antifungal activities were higher than that observed for Nystatin (IZ = 25±2.68 mm) and used as a reference substance. Higher antifungal activities were also observed for the *Aloysia triphylla* essential oil of Siliana with inhibition diameter zones of 85±8.04 mm for *Candida albicans (ATCC 2091)*, 85±8.45 mm for *Candida parapsilosis,* and 85±8.81 mm for *Candida glabrata (ATCC 90030)*, respectively. Moreover, *Aloysia triphylla* essential oil of Gabes, showed an antifungal activity higher than that of Korba against *Candida albicans* (IZ = 85±2.48 mm vs 46.33±2.61 mm) and lower than those observed for the three other strains of *Candida* studied. Our results are higher than those observed by ALI *et al.* (2011) on antifungal activity of *Aloysia triphylla* essential oil against *Candida albicans* CAICC 51, witnessing an inhibition diameter zone between 10 and 15 mm. Antifungal activity presented by *Aloysia tripylla* essential oils could be associated with the presence of citral. In fact, literature points to the fact that citral acts as a fungicidal agent because it is capable of forming a charge transfer complex with an electron donor of fungal cells, resulting in fungal death (KURITA *et al.,* 1981).

Table 3. Phenolic composition (peak area %, w/w) and analysis of variance results (*p*-value) of methanol extracts of *Aloysia tripyllla* aerial parts.

Compound	Collection region				*P*
	Kairouan	Korba	Siliana	Gabes	
Resorcinol	1.19 ^{d}±0.07	1.96 ^{a}±0.07	1.72 ^{b}±0.02	1.25 ^{c}±0.01	0.000***
Catechol	6.00 ^{d}±0.39	12.23 ^{a}±0.34	11.14 ^{b}±0.08	8.23 ^{c}±0.11	0.000***
Epigallocatechin	1.30 ^{b}±0.06	0.09 ^{cd}±0.03	0.10 ^{c}±0.01	1.34 ^{a}±0.02	0.000***
Caffeic acid	1.50 ^{b}±0.10	0.28 ^{d}±0.07	1.11 ^{c}±0.09	2.39 ^{a}±0.03	0.000***
Syringic acid	1.92 ^{b}±0.28	2.10 ^{a}±0.13	1.49 ^{c}±0.13	0.96 ^{d}±0.36	0.000***
p-Coumaric acid	54.90 ^{c}±1.33	38.91 ^{a}±0.53	38.81 ^{a}±2.50	38.23 ^{b}±3.34	0.000***
Sinapic acid	3.97 ^{a}±0.36	1.82 ^{c}±0.18	0.86 ^{d}±0.10	3.73 ^{b}±0.32	0.000***
Ferulic acid	0.38 ^{c}±0.03	1.24 ^{b}±0.04	3.28 ^{a}±0.26	0.36 ^{d}±0.03	0.000***
Luteolin 7-*O*- glucoside	0.35 ^{c}±0.02	2.46 ^{a}±0.10	0.16 ^{d}±0.03	2.10 ^{b}±0.07	0.000***
Coumarin	1.45 ^{c}±0.06	0.22 ^{d}±0.02	1.63 ^{b}±0.05	1.92 ^{a}±0.21	0.000***
Rutin	0.52 ^{b}±0.03	0.25 ^{c}±0.01	1.03 ^{a}±0.10	1.04 ^{a}±0.36	0.000***
Rosmarinic acid	1.20 ^{b}±0.09	0.22 ^{d}±0.01	1.33 ^{a}±0.03	0.49 ^{c}±0.16	0.000***
Resveratrol	0.20 ^{d}±0.01	0.61 ^{b}±0.02	0.44 ^{c}±0.11	0.76 ^{a}±0.06	0.000***
Ellagic acid	0.28 ^{d}±0.13	1.26 ^{a}±0.11	0.31 ^{c}±0.02	0.70 ^{b}±0.06	0.000***
Quercetin	0.26 ^{d}±0.06	0.45 ^{b}±0.05	1.39 ^{a}±0.03	0.38 ^{c}±0.40	0.000***

The values of the levels and percentages of phenolic compounds represent the average of three replicates ($n = 3$). Letters (a-d) indicate significant differences at $P < 0.05$. *** Significant effect at $P < 0.001$. *P*: probability.

Table 4. DPPH scavenging activity (IC_{50} μg/mL), reducing power (EC_{50} μg/mL), and β-carotene bleaching (IC_{50} μg/mL) and analysis of variance results (*p*-value) of methanol extracts of *Aloysia tripyllla* aerial parts.

	Collection region					BHT	Ascorbic acid
	Kairouan	Korba	Siliana	Gabes	*P* value		
DPPH scavenging activity (IC_{50} μg/mL)	5.78 ^{d}±0.08	6.07 ^{c}±0.13	13.23 ^{b}±0.28	30.67 ^{a}±1.31	0.000***	11.5±1.23	
Reducing power (EC_{50} μg/mL)	209.33 ^{d}±1.30	322.66 ^{c}±2.84	371.00 ^{b}±1.13	482.00 ^{a}±2.26	0.000***		37.33±3.41
β-carotene bleaching (IC_{50} μg/mL)	500.00 ^{c}±11.32	3966.67 ^{a}±65.33	4066.67 ^{a}±65.33	1566.67 ^{b}±130.66	0.000***	75±5.12	

Values are means of triplicates±SD. Values in the same row with different superscripts (a–d) are significantly different at $P < 0.05$. *** $P < 0.001$.

Table 5. Antibacterial and antifungal (IZ mm) activities and analysis of variance results (*p*-value) of essential oils of *Aloysia tripyllla* aerial parts.

	Collection region				*P*	Tetracycline 10 μg/mL	Nystatin 10 μg/mL
	Kairouan	Korba	Siliana	Gabes			
bacteria							
Escherichia coli (ATCC 35218)	12 ^{a}±1.96	11 ^{c}±1.13	8.67 ^{d}±0.65	11.33 ^{b}±0.65	0.000***	23±2.55	
Pseudomonas aeruginosa (ATCC 4141)	na	na	na	na	-	22±2.71	
Enterococcus Faecalis (ATCC 2212)	na	na	na	na	-	24±2.64	
Bacillus Subtilis (CIP 5265)	85 ^{a}±7.67	26 ^{c}±4.08	31.67 ^{b}±3.27	25.33 ^{d}±9.62	0.000***	25±2.11	
Fungi							
Candida albicans (ATCC 2091)	85 ^{a}±8.27	46.33 ^{a}±2.61	85 ^{a}±8.04	85 ^{b}±2.48	0.000***		25±2.68
Candida kefyr	85 ^{a}±7.89	21.67 ^{c}±4.71	28.33 ^{b}±1.73	19.33 ^{d}±1.31	0.000***		24±2.18
Candida parapsilosis	85 ^{a}±6.59	28.67 ^{b}±9.15	85 ^{a}±8.45	25 ^{c}±2.56	0.000***		25±3.05
Candida glabrata (ATCC 90030)	85 ^{a}±8.57	18 ^{b}±4.08	85 ^{a}±8.81	14 ^{c}±1.96	0.000***		23±2.33

Results are the mean of three replications. The diameter of disc was 6 mm. Values with different superscripts (a-d) are significantly different at $P < 0.05$. na: not active. NS: not significant. *P*: probability. ** $P < 0.01$. *** $P < 0.001$.

4. CONCLUSIONS

This study has revealed that *Aloysia triphylla* methanolic extracts and essential oils features are markedly influenced by the regional factor. Korba sample have high potential for selecting variety rich in essential oil, while Kairouan sample was suitable for being a valuable source of antioxidants, including condensed tannins, flavonoids, phenolic compounds, and polyphenols.
The high content of these bioactive compounds both highlights their nutritional and medicinal values and provides a high protection against oxidation phenomenon. Essential oil of *Aloysia triphylla,* which originated from Kairouan stood out for presenting the best selective antibacterial and antifungal performances. Additional researches on the bioactivities of *Aloysia triphylla* might pave the way for its broad application in industrial, pharmaceutic, and cosmetic fields.

REFERENCES

Adams R.P. 2004. Identification of essential oil components by gas chromatography/mass spectrometry. Allured, IL: Carol Stream.

Ali H.F.M., El-Beltagi H.S. and Nasr N.F. 2011. Evaluation of antioxidant and antimicrobial activity of *Aloysia Triphylla*. Elec. J. Envir. Agric. Food Chem. 10:2689-2699.

Bensabah F, Lamiri A, and Naja J. 2014. Effect of purified wastewater from the city of Settat (Morocco) on the quality of *Lippia citriodora* essential oil and infusion. J. Saudi Soc. Agric. Sci. 14:101-108.

Bettaieb Rebey, I, Bourgou S, Ben Slimen Debez I, Jabri Karoui I, Hamrouni Sellami I, Msaada K, Limam F, and Marzouk B. 2012. Effects of extraction solvents and provenances on phenolic contents and antioxidant activities of Cumin (*Cuminum cyminum* L.) Seeds. Food Bioprocess Technol. 5:2827–2836.

Carnat A., Carnat A.P., Fraise D. and Lamaison, J.L. 1999. The Aromatic and polyphenolic composition of *Lemon verbena* Tea. *Fitoterapia* 7:44-49.

Catalan C.A.N., de and Lampasona M.E.P. 2002. The chemistry of the Genus Lippia (Verbenaceae). *In*: Kintzios, S.E. (Ed.), Oregano: The genera Origanum and Lippia, first ed. Taylor and Francis Inc. 127-149. pp.

Chao S.C., Young D.G. and Oberg C.J. 2000. Screening of inhibitory activity of essential oils on selected bacteria, fungi and viruses. J. Essent. Oil Res. 12:639-649.

Cheurfa M. and Allem R. 2016. Evaluation of antioxidant activity of different extracts of *Aloysia triphylla* leaves (L'Herit.) from Algeria *in vitro*. Phytother. 14:181-187.

Choupani M., Arabshahi Delouee S. and Alami M. 2014. Antioxidant properties of various solvent extracts of Lemon verbena (*Lippia citriodora*) leaves. Inter. J. Adv. Biol. Biomed. Res. 2:1340-1346.

Cook N.C. and Samman S. 1996. Flavonoids- chemistry, metabolism, cardio protective effects and dietary sources. J. Nutr. Biochem. **7**:66-76.

Crabas N., Marongiu B., Piras A., Pivetta T. and Porcedda S. 2003. Extraction, separation and isolation of volatiles and dyes from *Calendula officinalis* L. and *Aloysia triphylla* (L'Her.) britton by supercritical CO_2. J. Essent. Oil Res. 15:350-355.

Delaquis P.J., Stanich K., Girard B. and Mazza G. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. Inter. J. Food Microbiol. 74:101-109.

Dewanto V., Wu X., Adom K. and Liu R.H. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50:3010-3014.

Di Leo Lira P., Van Baren C.M., López S., Molina A., Heit C., Viturro C. and De Lampasona M.P. 2013. Northwestern Argentina: A center of genetic diversity of lemon verbena (*Aloysia citriodora* PALÁU, Verbenaceae). Chem. Biodiv. 10:251-261.

Dixon R.A. and Paiva N.L. 1995. Stress-induced phenylpropanoid metabolism. The Plant Cell 7:1085-1097.

Domínguez-Avila J.A., González-Aguilar G.A., Alvarez-Parrilla E. and De la Rosa L.A. 2016. Modulation of PPAR expression and activity in response to polyphenolic compounds in high fat diets. Inter. J. Mol. Sci. 17 (7):1002.

Duarte M.C.T., Figueira G.M., Sartoratto A., Rehder V.L.G. and Delarmelina C. 2005. Anti-candida activity of Brazilian medicinal plants. J. Ethnopharmacol. 97:305-311.

El Babili F., Babili M.E.L., Souchard J.P., Chatelain C. 2013. Culinary decoctions: spectrophotometric determination of various polyphenols coupled with their antioxidant activities. Pharm. Crops, 4, 15-20.

El Hawary S., Yousif M., Abdel Motaal A. and Abd-El Hameed L. 2011. Composition and bioactivities of the essential oil from leaves of *Lippia Citriodora* Kunth cultivated in Egypt. J. Biol. Active Prod. Nat. 1:112-119.

El-Hawary S.S., Yousif M.F., Abdel Motaal A. and Abd-Hameed LM. 2012. Bioactivities, phenolic compounds and *in-vitro* propagation of *Lippia citriodora* Kunth cultivated in Egypt. Bull. Fac. Pharm. Cairo Univ. 50:1-6.

European Pharmacopoeia (Ph. Eur.) 9th ed. 2017. https://www.edqm.eu/en/european-pharmacopoeia-9th-edition.

Hanato T., Kagawa H., Yasuhara T. and Okuda T. 1988. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effect. Chem. Pharm. Bul.l 36:1090-1097.

Kim N.S. and Lee D.S. 2004. Headspace solid-phase microextraction for characterization of fragrances of lemon verbena (*Aloysia triphylla*) by gas chromatography mass spectrometry. J. Sep. Sci. 27:96-100.

Koleva I.I., Teris A.B., Jozef P.H., Linssen A.G., Lyuba N.E. 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13, 8–17.

Kulisic T., Radonic A., Katalinic V. and Milos M. 2004. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85:633-640.

Kurita N., Miyaji M., Kurane R. and Takahara Y. 1981. Antifungal activity of components of essential oils. Agric. Biol. Chem. 45:945-952.

Magwa M.L., Gundidza M., Gweru N. and Humphrey G. 2006. Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. J. Ethnopharmacol. 103:85-89.

Mau J.L., Chang C.N., Huang S.J. and Chen C.C. 2004. Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitoymyces albuminosus mycelia*. Food Chem. 87:111-118.

Moein M.M., Zarshenas M. and Etemadfard H. 2014. Essential oil composition and total flavonoid content of *Aloysia Citriodora* Palau under different cultivation systems. Inter. J. Plant Anim. Envir. Sci. 4:353-358.

Msaada K., Hosni K., Ben Taarit M., Chahed T., Kchouk M.E. and Marzouk B. 2007. Changes on essential oil composition of coriander (*Coriandrum sativum* L.) fruits during three stages of maturity. Food Chem. 102:1131-1134.

Oyaizu M. 1986. Studies on products of browning reaction: antioxidative activity of products of browning reaction. Jap. J. Nutr. 44:307-315.

Özek T., Kirimer N., Baser K.H.C. and Tümen G. 1996. Composition of the essential oil of *Aloysia triphylla* (L'Herit.) Britton Grown in Turkey. J. Essent. Oil Res. 8:581-583.

Pascual M.E., Slowing K., Carretero E., Šanchez Mata D. and Villar A. 2001. *Lippia*: traditional uses, chemistry and pharmacology: a review. J. Ethnopharmacol. 76:201-214.

Portmann E., López Nigro M.M., Reides C.G., Llesuy S., Ricco R.A. and Wagner M.L. 2012. Aqueous extracts of *Lippia turbinata* and *Aloysia citriodora* (Verbenaceae): assessment of antioxidant. Inter. J. Toxicol. 31:192-202.

Proestos C., Boziaris I.S., Nychas G.J.E. and Komaitis M. 2006. Analysis of flavonoids and phenolic acids in Greek aromatic plants: investigation of their antioxidant capacity and antimicrobial activity. Food Chem. 95:664-671.

Quirantes-Piné R., Funes L., Micol V., Segura-Carretero A. and Fernández-Gutiérrez A. 2009. High-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight and ion-trap tandem mass spectrometry to identify phenolic compounds from a *Lemon verbena* extract. J. Chromatogr. A 1216:5391-5397.

Ríos J.L. and Recio M.C. 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100:80-84.

Sacchetti G., Medici A., Maietti S., Radice M., Muzzoli M. and Manfredini S. 2004. Composition of functional properties of the essential oil of amazonian basil, *Ocimum micranthum* Willd. Labiatae in comparison with commercial essential oils. J. Agric. Food Chem. 52:3486-3491.

Santos-Gomes P.C., Fernandes-Ferreira M. and Vicente A.M.S. 2005. Composition of the essential oils from flowers and leaves of vervain (*Aloysia triphylla* (L'Herit.) Britton) grown in Portugal. J. Essent. Oil Res. 17:73-78.

Sharma A., Flores-Vallejo R.D.C. Cardoso-Taketa A. and Villarreal M.L. 2016. Antibacterial Activities of Medicinal Plants Used in Mexican Traditional Medicine. J. Ethnopharmacol. 208:264-329.

Statsoft. 1998. *STATISTICA for Windows (Computer Program Electronic Manual)*; Statsoft Inc.: Tulsa, OK.

Sun B., Ricardo-da-Silva J.M. and Spranger I. 1998. Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food Chem. 46:4267-4274.

Taghi Ebadi M., Azizi M., Sefidkom F. and Ahmadi N. 2015. Influence of different drying methods on drying period, essential oil content and composition of *Lippia citriodora* Kunth. J. App. Res. Med. Arom. Plants. 2:182-187.

Teixeira Duarte M.C., Leme E.E., Delarmelina C., Soares AA., Figueira G.M. and Sartoratto A. 2007. Activity of essential oils from Brazilian medicinal plants on *Escherichia coli*. J. Ethnopharmacol. 111:197-201.

Valentão P., Andrade P.B., Areias F., Ferreres F. and Seabra R.M. 1999. Analysis of vervain flavonoids by HPLC/diode array detector method. Its application to quality control. J. Agric. Food Chem. 47:4579-4582.

Vasco C., Ruales J. and Kamal-Eldin A. 2008. Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. Food Chem. 111:816-823.

Vinha A.F., Soares M.O., Castro A., Santos A., Oliveira M.B.P.P. and Machado M. 2012. Phytochemical characterization and radical scavenging activity of aqueous extracts of medicinal plants from Portugal. Eur. J. Med. Plants 2:335-347.

Zhang W. and Wang S.Y. 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49:5165-5170.

Paper Received October 10, 2018 Accepted February 1, 2019

ERYTHORBIC ACID AND SODIUM ERYTHORBATE EFFECTIVELY PREVENT PULP BROWNING OF MINIMALLY PROCESSED 'ROYAL GALA' APPLES

J.A. RIBEIRO*[a], M. SEIFERT[a], J. VINHOLES[b], C.V. ROMBALDI[a], L. NORA[a] and R.F.F. CANTILLANO[b]

[a]Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Universidade Federal de Pelotas, Campus Capão do Leão, s/n, Caixa Postal 354, CEP 96010-900, Pelotas, RS, Brazil

[b]Embrapa Clima Temperado, Rodovia BR 392, km 78, 9º Distrito, Monte Bonito, Caixa Postal 403, CEP 96010-971, Pelotas, RS, Brazil

*Corresponding author: Tel.: +555332758105; Fax: +555332758221

E-mail address: jardelaraujoribeiro@gmail.com

ABSTRACT

This study aimed to evaluate the effect of erythorbic acid (EA), sodium erythorbate (SE) and kojic acid (KA) to control the pulp browning of minimally processed (MP) 'Royal Gala' apples. Physicochemical and sensorial properties of MP apples were evaluated during a shelf life testing. SE and EA resulted in the highest levels of phenolic compounds, antioxidant activity and PPO and POD inhibition. Sensorial analysis results revealed that SE and EA treatments preserved the flavor, odor, color, succulence, firmness and overall quality of MP apples for up to 9 days. In conclusion, both SE and EA are suitable antibrowning agents for MP 'Royal Gala' apples.

Keywords: antioxidant activity, enzymes, minimal processing, phenolic compounds, sensorial analyses

1. INTRODUCTION

Apple (*Malus domestica* Borkh) is a widely consumed fruit in many Westernized diets due to its year-round market availability (VIEIRA *et al*., 2011). There is also a large retail demand for minimally processed (MP) apples due to practicality during consumption, transportation, and health benefits for those who consume. Nevertheless, apples are very sensitive to enzymatic browning, either by the high content of phenolic compounds and/or the activity of oxidative enzymes such as polyphenoloxidase (HOLDERBAUM *et al*., 2010) and peroxidase (ROJAS-GRAUE *et al*., 2008). Enzymatic browning in MP apple is a major problem for the food processing industry, and must be controlled to prevent undesirable changes in color, flavor and nutritional value (TOIVONEN and BRUMMELL, 2008; IOANNOU and GHOUL, 2013). An alternative to avoid the browning effect on minimally processed fruit pulps is the use of antioxidants (GALGANO *et al*., 2015). L-cysteine, erythorbic acid, sodium erythorbate, and kojic acid, used in combination with calcium chloride, are examples of antioxidants agents to preserve MP products quality. L-cysteine, commonly used to control/avoid pulp browning in MP products (ALI *et al*., 2016; CABEZAS-SERRANO *et al*., 2013; GHIDELLI *et al*., 2014), has been reported to have different mechanisms of action: a) inhibition of PPO (NI EIDHIN *et al*., 2006); b) adduct formation with *o*-quinones resulting in non-colored compounds during oxidation (RICHARD-FORGET *et al*., 1992); and c) reduction of *o*-quinones to their polyphenols precursors (CILLIERS and SINGLETON, 1990). Erythorbic acid (D-isoascorbic acid) (KALL and ANDERSEN, 1999), has also been used in MP products such as potatoes (CACACE *et al*., 2002), canned applesauce and beer (ANDERSEN, 1999). This antioxidant has been reported with similar antioxidants properties of its stereoisomer (ascorbic acid), however, it does not have the vitamin C activity, but it is at least five times cheaper (MARTIN-BELLOSO and SOLIVA-FORTUNY, 2011). Sodium erythorbate is a reducing agent (BUTA *et al*., 1999) commonly used in the food industry to reduce the superficial oxidation of MP fruits (GROSS *et al*., 2016; SAPERS and MILLER, 1998) and meat products (SOMMERS *et al*., 2002). Kojic acid has been used in Japan as food additive due to its inhibitory properties over different oxidase enzymes. This antioxidant has been reported in several food matrices such as syrup, flour, meet, flavoring and vegetables (SCPP, 2008). In addition, it has been reported that kojic acid significantly reduced the browning in Liberty apple slices (SON *et al*., 2001), Amasya apple juice (IYIDOGAN and BAYINDIRLI, 2004) and litchi pericarp (SHAH *et al*., 2017). Calcium chloride, when used in association with antioxidants, is responsible for the reduction of physiological imbalances and maintenance of color. In addition, it is recommended due to its firming action on a wide range of fruits (TECHAKANON and BARRETT, 2017). Despite the use of different antioxidants in MP apples, erythorbic acid, sodium erythorbate and kojic acid have never been tested as antibrowning agents in fresh-cut 'Royal Gala' apple, as far as we are concerned. In this context, the objective of this study was to evaluate the sensorial characteristics and physicochemical variables of MP 'Royal Gala' apples treated with erythorbic acid, sodium erythorbate and kojic acid associated with calcium chloride, under cold storage for 9 d, to simulate shelf-life conditions.

2. MATERIAL AND METHODS

2.1. Harvesting and storage of fruits

Royal Gala apples (*Malus domestica* Borkh), at commercial maturity stage, were harvested from a commercial orchard located in the Vacaria city (28º 30' 44" S, 50º 56' 02" W), Rio

Grande do Sul state, Brazil. The apples had the following average characteristics: starch content of 4.80, measured according to STREIF (1984); pulp firmness of 12.84 N; concentration of total soluble solids of 12.51ºBrix and titratable acidity of 0.23 g of malic acid 100 g^{-1} FW. The apples were selected by size (approximately 110 g) and for the absence of visible mechanical damage and rot and stored at 1.0°C ± 0.5°C and relative humidity of 90.0 % ± 5.0 %, at the Postharvest Physiology Laboratory from Embrapa Clima Temperado.

2.2. Treatments

Fruits were sanitized by dipping into a sodium hypochlorite solution (100 ppm, pH 6.5, at 6.5±1.5ºC) for 10 min, cut into eight wedge shape longitudinal slices with approximately the same size, with the removal of the central core and seeds, preserving the epidermis. The experimental treatments consisted of dipping the apple slices into different solutions for 1 min, as described below:

Treatments*	distilled water (W)	L-cysteine chloride 0.60 % (LC)	sodium erythorbate 5.00 % (SE)	erythorbic acid 3.00 % (EA)	kojic acid 0.07 % (KA)	$CaCl_2$ 1.00 % (CC)
W+CC (negative control)	X					X
LC+CC (positive control)		X				X
SE+CC			X			X
EA+CC				X		X
KA+CC					X	X

*The treatments W+CC (negative control) and LC+CC (positive control) were designed to be ineffective and highly effective against browning, respectively.

After dipping, the apple slices were drained for 5 min and placed onto expanded polystyrene trays, covered with PVC film (9 μm thickness) and stored for four different periods (0 d, 3 d, 6 d and 9 d), at 4±1.0°C and RH of 90.0±5.0 %, to simulate the shelf life of MP apples. The samples analyzed at 0 d had been exposed to each treatment for 8±1 h.

2.3. Reagents

Reagents were purchased from different suppliers: calcium chloride (≥ 99 %) from Synth (Diadema, SP, Brazil), L-cysteine (≥ 98 %) from Vetec (Duque de Caxias, RJ, Brazil), sodium erythorbate (≥ 98 %) and erythorbic acid (≥ 99 %) from Daxia Doce Aroma Ind. Com. Ltd. (São Paulo, SP, Brazil) and kojic acid (≥ 99 %) from Chengdu Jinkay Biology Engineering Co., Ltd. (São Paulo, SP, Brazil).

2.4. Physical and chemical analysis

2.4.1 Total soluble solids (TSS)

TSS was determined using a portable refractometer (ATAGO, model PAL-1) at 20ºC and the results were expressed in ºBrix.

2.4.2 Titratable acidity (TA)

TA was determined on apple pulp juice. The titration endpoint was determined with a pH meter (Quimis model Q400A). Briefly, 10 mL of pulp juice (diluted in 90 mL distilled water) was titrated with 0.1 M NaOH to a pH 8.1 endpoint. The results were expressed as grams (g) of malic acid per 100 g^{-1} of fresh weight (FW).

2.4.3 TSS/TA ratio

TSS/TA ratio was calculated by dividing the TSS values by TA values.

2.4.4 Loss of mass

Mass loss was calculated by the equation 1.

$$Loss\ of\ mass\ (\%) = \frac{Initial\ mass - Final\ mass}{Final\ mass}\ x\ 100 \qquad \text{Equation 1}$$

2.4.5 Pulp firmness

Measured according to MELO *et al*. (2009) using a Texture Analyser (TA-XT plus 40855, Stable Microsystems, England) with a 2 mm diameter probe, penetration depth of 5 mm, pre-test velocity of 1.0 mm s^{-1}; 2.0 mm s^{-1} test; post-test of 10.0 mm s^{-1} and force of 5 kg. The readings were performed in the middle portion of the pieces and the results were expressed in Newton (N).

2.4.6 Color

Apple color of equatorial region of slices was measured using a Minolta CR-400 colorimeter with a CIE L*a*b* reading system, proposed by the *Comission Internacionale de I'Eclairage* (CIE). The Browning Index was calculated from L*, a* and b* values according to PALOU *et al.* (1999) and hue or chromatic hue, represented by the Hue (h°) angle, was calculated as the tangent arc of b*/a* quotient. The result was expressed in degrees.

2.4.7 Total phenolic compounds

Measured according to the Folin-Ciocalteu method adapted from SWAIN and HILLIS (1959). Briefly, 250 μL aliquot of the extracts (the same used for DPPH· analysis) was combined with 250 μL of 0.25 M Folin-Ciocalteu reagent and 4000 μL ultrapure water. After 3 min of reaction, 500 μL of 0.5 M Na_2CO_3 was added, following incubation for 2 h at room temperature and absorbance reading at 725 nm. The results were expressed as grams of chlorogenic acid equivalents (CAE) per 100 g^{-1} of FW. Chlorogenic acid standard curve (0.0 mg mL^{-1} to 0.5 mg mL^{-1}) was used.

2.4.8 Antioxidant activity (DPPH)

The antioxidant activity was evaluated using the method described by BRAND-WILLIAMS *et al.* (1995) with some modifications. First, 10 mL of methanol was added to 2.5 g of fresh apple and homogenized during 1 min (ultra-turrax homogenizer, IKA, Artur Nogueira, SP). Extracts were centrifuged (Eppendorf - Centrifuge 5810 R) at 3050 g for 30 min at 1.0ºC. The supernatant was collected and stored at –18ºC until analysis. Apple extracts (100 μL) were added to 3900 μL DPPH solution (in methanol), and the reaction

mixture was kept in the dark for 24 h. After this period, the absorbances were spectrophotometrically read at 515 nm, and results expressed as mg of Trolox equivalent per 100 g^{-1} of FW.

2.4.9 Peroxidase (POD) enzyme activity

The POD enzyme activity was determined according to the methodology described by COELHO and SALAS-MELLADO (2014), adapted for micro quantities. Five grams (5.0 g) of apple pulp were homogenized (ultra-turrax homogenizer, IKA, Artur Nogueira, SP) with 0.2 g of polyvinylpyrrolidone (diluted in 20 mL of 0.2 M phosphate buffer pH 6.0) for 3 min. The homogenate was centrifuged (Eppendorf - Centrifuge 5810 R) at 3050 g, for 30 min at 1ºC. The supernatant (apple extract) was collected and maintained at ±4ºC. An aliquot of 45 μL of apple extract was mixed with 230 μL of a mixture of phosphate buffer (1.5 mL, pH 6.5, 0.05 M), distilled water (2.0 mL), hydrogen peroxide (1.0 mL, 0.08 %, v/v) and guaiacol (0.5 mL, 1 %). Samples were incubated at 37°C with readings (Molecular Devices - Spectramax 190 spectrophotometer) at 470 nm at time zero and after 15 minutes of reaction. The enzymatic activity was calculated based on the amount of protein, where a unit of enzyme activity was defined as the amount of enzyme causing the increase in absorbance of 0.01per min^{-1}. The results were expressed in units of enzyme/μg of protein.

2.4.10 Polyphenoloxidase (PPO) enzyme activity

PPO enzyme activity was measured by following the methodology described by RAI *et al.* (2011), with some adaptation. Briefly, 55 μL of the above apple pulp extract was added to 220 μL of the mixture consisting of phosphate buffer (1.5 mL, pH 6.5, 0.05 M), catechol (0.5 mL, 0.05 M) and water (2.0 mL). The reaction occurred at 37°C with reading (Molecular Devices - Spectramax 190 spectrophotometer) at 425 nm at time zero and 30 min. The enzymatic activity was calculated based on the amount of protein where a unit of enzyme activity was defined as the amount of enzyme, which causes an increase in absorbance of 0.01 per min^{-1}. The results were expressed in units of enzyme / μg of protein.

2.4.11 Protein

Protein concentration was determined according to BRADFORD (1976), using the reagents from Sigma-Aldrich® (Bradford Reagent, #B6916 and BSA Protein Standard, #P0834) and the corresponding protocol to assay the protein samples on 96 well plate. The absorbance was read at 595 nm in a spectrophotometer (Molecular Devices - Spectramax 190), and the results were expressed in μg of protein per μL.

2.5. Respiratory gas analysis

The O_2 and CO_2 concentration in the headspace of the hermetically sealed packages were determined every 3 d using Oxybaby 6.0 - Witt-Gasetechnik gauge analyzer. The results were expressed as a percentage (%).

2.6. Sensory analysis of minimally processed apple slices

A set of possible sensory judges, comprising male and female participants (between 20 and 40 years old) who consumed apple frequently, were trained for the evaluation of apple attributes under analysis during 30 days (2 weekly meetings of 15 minutes). The top 17 judges, those who showed better results, were selected. For each period of storage (0 d,

3 d, 6 d and 9 d), five samples (12.0±1.0 g each) were identified with three-digit code and randomly provided to the judges. A glass of water was provided for cleaning the palate between one sample and another. Flavor, odor, darkening, succulence, firmness and overall quality acceptability were the parameters evaluated using structured scales of 9 cm labelled "bad" to "excellent". This information was converted into scores from 0 to 9, respectively. The evaluation was performed at room temperature and in uniform laboratory conditions. The analysis was submitted to the evaluation by the Research Ethics Committee of the Faculty of Medicine of the Federal University of Pelotas and approved for being in accordance with Resolution 196/96 of the National Health Council (CAAE - 48625015.1.0000.5317).

2.7. Statistical analysis

The experimental design was completely randomized, in a 5 x 4 two-factorial scheme (five treatments x four storage periods) with three replicates. Data were analyzed for variance ($p \leq 0.05$) using the Statistica 7.0 program. For significant results, the means were compared by the LSD - Least Significant Difference ($p \leq 0.05$) test. The multivariate factorial analysis technique was also applied, using the Varimax rotation to improve the interpretation of the factors, using Statgraphics Centurion XVII software.

3. RESULTS AND DISCUSSION

It is important to notice that the samples analyzed at 0 d had been exposed to the corresponding treatment for about eight hours before analysis, causing the treatments to affect the results even at 0 d. Changes in the *ratio* (TSS/TA), commonly used to evaluate the fruit quality, can impact apple flavor (PIAGENTINI and PIROVANI, 2017). Treatments with W+CC, LC+CC, SE+CC and KA+CC resulted in a *ratio* increase (Fig. 1a) over the 9 days of storage. This *ratio* increase can be explained by the conversion of organic acids in non-acid molecules during the respiratory metabolism (PECH *et al.*, 2008), or due to the sugar accumulation by loss of humidity. Apples treated with EA+CC showed a decrease on *ratio*, probably due to the higher acidity (1.18 g of citric acid/100 mL, pH 2.39) of this antioxidant agent.

Mass loss can compromise the shelf-life of MP products (SANCHIS *et al.*, 2016). Minimal processing exposes fruits and vegetables tissues to a lower water pressure environment and can cause substantial loss of mass. W+CC and LC+CC were the treatments with higher mass loss (Fig. 1b). However, these values were lower than 1 %, not enough to change the fruit quality. In a similar study, PIZATO *et al.* (2013) and QI *et al.* (2011) obtained higher values for mass loss. Significant mass loss has physiological effect and can compromise fruits and vegetables appearance, texture and nutritional quality (SANCHIS *et al.*, 2016).

Results for pulp firmness (Fig. 1c) were not affected by the loss of mass, although slight variations occurred over the 9 days of storage. According to GANG *et al.* (2015), the use of $CaCl_2$ has an essential role in the maintenance of pulp integrity and firmness due to its action as bio-membrane stabilizer, supporting the integrity of the membrane cell wall. Besides, appropriated processing and storage conditions and use of antioxidants contribute to prevent pulp softening.

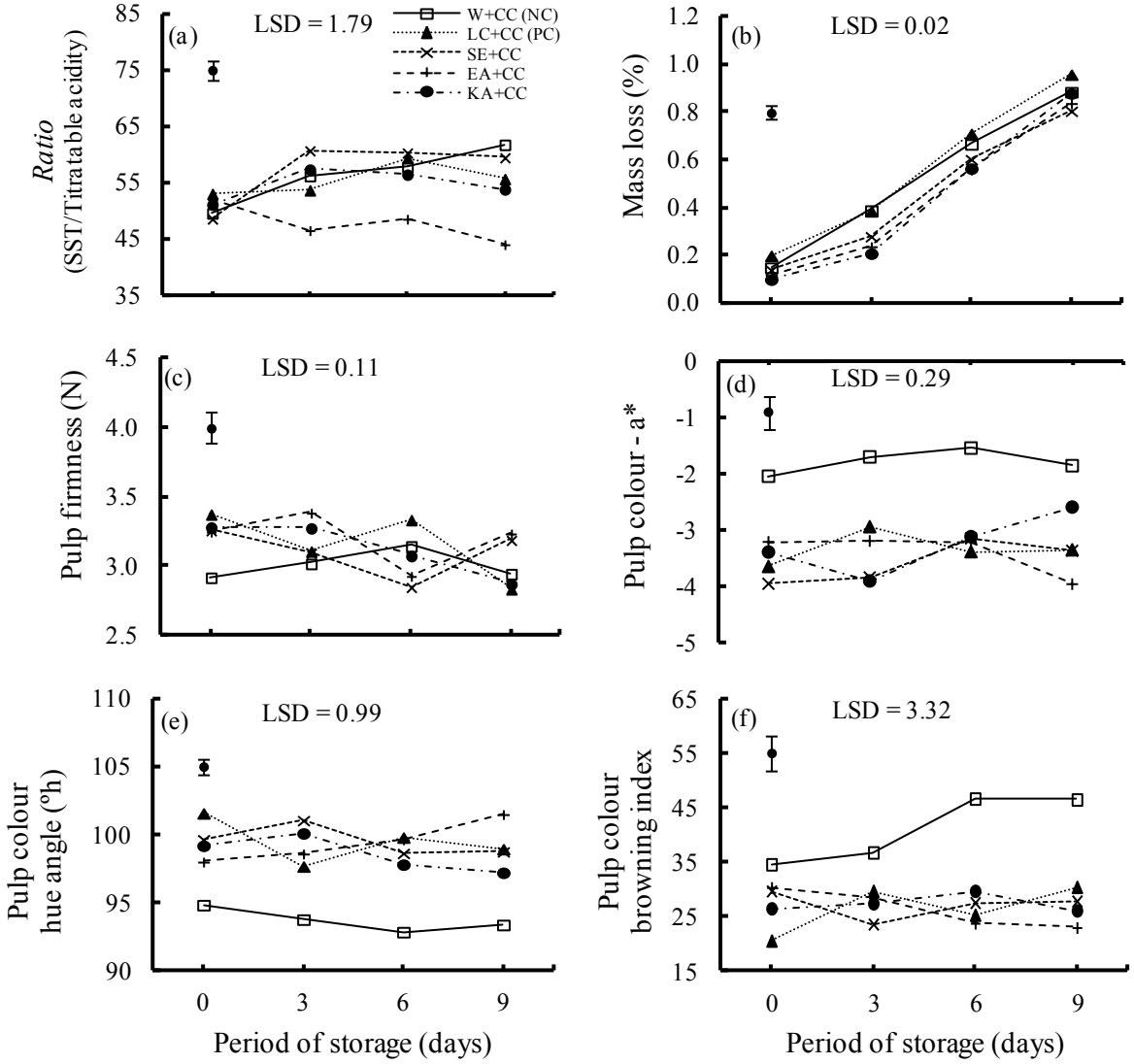


Figure 1. Effect of sodium erythorbate 5.0 % (SE) + calcium chloride 1.0 % (CC), erythorbic acid 3.0 % (EA) + CC and kojic acid 0.07 % (KA) + CC on physicochemical variables: mean values for *Ratio* (**a**), mass loss (**b**), pulp firmness (**c**) and pulp color variables: a* (**d**), hue angle (**e**) and browning index (**f**) for minimally processed 'Royal Gala' apple recorded at 0 d, 3 d, 6 d and 9 d, at 4.0ºC. Negative control (NC) consisted of distilled water (W) + CC, and positive control (PC) consisted of L-cysteine chloride 0.6 % (LC) + CC. The vertical bar indicates the Least Significant Difference (LSD) with ($p \leq 0.05$).

Color change in the pulp of the MP apples is associated with the oxidative processes due to the rupture of the cell membranes during product preparation. Green-red chromatic coordinate (a*) is an important parameter for the study of browning since brown color represents a combination between green (-a *) and red (+a *) (KIM and LEE, 2008). The green-red chromatic coordinate (a*) values were high for W+CC treatment in all periods of storage (Fig. 1d), corresponding to a more reddish apple pulp. Lower values of a* coordinate (yellow to green fruits) were observed for treatments LC+CC, SE+CC, EA+CC and KA+CC until day 6. However, the result of a* coordinate value for KA+CC treatment increased between day 6 and day 9. This increase on reddish color of apples treated with KA+CC can be due to the reversible linkage of kojic acid and PPO enzyme (CHEN *et al.*, 1991). The process of oxidative coloration is triggered by the cell membrane rupture and

the resulting mixture of polyphenol substrates with PPO (TOIVONEN and BRUMMELL, 2008). PPO enzyme when in contact with oxygen catalyzes two reactions: (1) hydroxylation of monophenols in diphenols and (2) oxidation of diphenols in quinones. The first is relatively slow and results in colorless products. The second one is relatively fast, and the resulting quinones are colored. Subsequent reactions of the quinones lead to the accumulation of melanoidin, which is the brown pigment (CORTELLINO *et al.*, 2015).
Fig. 1e displays the hue angle values obtained for the different antioxidant treatments. The treatments LC+CC, SE+CC, EA+CC and KA+CC resulted in a yellow-green hue throughout the nine days of storage. On the other hand, apples treated only with water and calcium chloride (W+CC), showed a tendency to brown, from the beginning of storage period, intensifying slightly over the 9 days of storage. These antagonistic results point to the importance of the antioxidant to inhibit the darkening of the pulp. Calcium chloride, besides maintaining pulp firmness, can also be responsible for the maintenance of color. This effect is related to its action on the prevention of the cellular membrane degradation, with consequent reduction in substrates release for PPO activity (TOIVONEN and BRUMMELL, 2008), protecting, therefore, the color of MP products (PEREZ-CABRERA *et al.*, 2011). Regarding the browning index (Fig. 1f), the treatments LC+CC, SE+CC, EA+CC and KA+CC maintained the pulp color, without statistical differences on the 6-day and 9-day of evaluation. Overall results for the color measurements revealed that all treatments maintained the apple pulp color satisfactorily, despite the differences between the mechanism of action of the antioxidants. L-cysteine has the ability to inhibit the enzymatic browning caused by PPO, through a competitive mechanism that captures *o*-quinones through the formation of cysteinyl adducts (RICHARD-FORGET *et al.*, 1992). The subgroup erythorbates comprise two compounds, erythorbic acid and sodium erythorbate. Erythorbic acid is a stereoisomer of ascorbic acid, with strong reducing properties, differing only from the relative position of the hydroxyl groups and of the hydrogen in the fifth carbon atom in the molecule (CAROCHO *et al.*, 2018). This acid reacts with oxygen and can thus remove it from a closed system (LEE *et al.*, 2012). Sodium erythorbate antioxidant activity is due to the quenching of singlet oxygen, hydrogen donation and as a reducing agent (CAROCHO *et al.*, 2018). Erythorbates have essentially the same function (FIDLER *et al.*, 2004) and are widely used as alternatives to ascorbic acid and its salts in products that the action of vitamin C is not necessary (MARTIN-BELLOSO and SOLIVA-FORTUNY, 2011). The antioxidant properties of kojic acid can be attributed to its ability to inhibit the thyroxinase and polyphenoloxidase enzymes, and its chelating activity (MITANI *et al.*, 2001). The mechanisms of action of kojic acid are similar to those of L-cysteine and ascorbic acid since they occur through interference with the absorption of O_2 required for the enzymatic reaction, reduction of the quinones to diphenols, or the combination of both (CHEN *et al.*, 1991).
The antioxidant activity (DPPH scavenging) of SE+CC treatment was higher than all the other treatments during the entire period of storage (Fig. 2a), with significant decrease (24.3 %) from day 0 to day 9. This result can be attributed to sodium erythorbate ability to prevent oxidation (FIGUEIREDO *et al.*, 2014; REISCHE *et al.*, 2002); or capture of the oxygen present in the medium through stable chemical reactions, preventing this oxygen of acting as propagator of auto-oxidation or as synergist in the regeneration of primary antioxidants (RAMALHO and JORGE, 2006). On the other hand, the EA+CC treatment showed a very similar activity to SE+CC treatment during the first six days of storage. However, a marked drop of DPPH activity (35.8 %) was observed between day 6 and day 9. This decrease can be attributed to the non-enzymatic degradation of erythorbic acid in aerobic conditions (CORZO-MARTÍNEZ *et al.*, 2012; CROPOTOVA *et al.*, 2016). Other treatments, W+CC, LC+CC and KA+CC, although oscillating and presenting statistical

difference between them, maintained a mean DPPH activity of 91.9 mg Trolox per 100 g of FW.

'Royal Gala' apple treated with SE+CC and EA+CC showed the highest values of total phenolic content (Fig. 2b), which was maintained throughout the storage period. Other treatments (W+CC, LC+CC and KA+CC) showed total phenolic compounds contents within the values reported in the literature for apples, 50 mg per 100 g FW to 380 mg per 100 g FW (CEYMANN *et al.*, 2012). Sodium erythorbate was reported to preserve the phenolic compounds in MP oyster mushroom (VENTURA-AGUILAR *et al.*, 2017). Ascorbic acid is a well-known antioxidant that prevents the polyphenol degradation due to its reducing action (GIL *et al.*, 1998). Thus, it can be inferred that its stereoisomer, erythorbic acid, has a similar capacity, effectively preserving the polyphenols content as observed in this experiment. Results of phenolic compounds and antioxidant activity in apples were correlated. Treatments that preserved the largest amount of phenolics were the same ones that maintained the highest antioxidant activity. Besides the antioxidant capacity, phenolic compounds are also responsible for apples flavor (ZHANG *et al.*, 2017). In addition, they have biological functions such as immunomodulatory and anti-inflammatory properties in humans and animals (ZHANG *et al.*, 2017). Therefore, the preservation of these properties is important to maintain the quality of the fruit and, consequently, the health benefit for consumers.

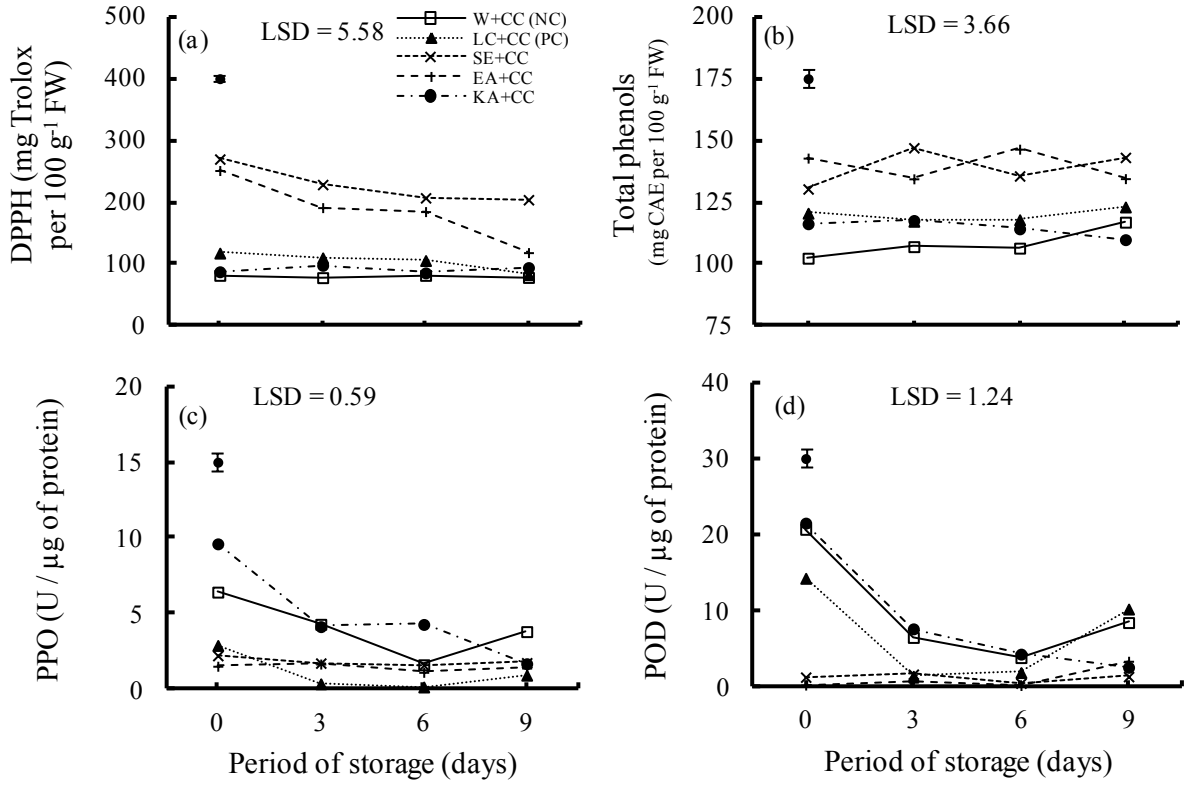


Figure 2. Effect of sodium erythorbate 5.0 % (SE) + calcium chloride 1.0 % (CC), erythorbic acid 3.0 % (EA) + CC and kojic acid 0.07 % (KA) + CC on DPPH activity (**a**), total phenolic content (**b**), PPO (**c**) and POD (**d**) activities for minimally processed 'Royal Gala' apple recorded at 0 d, 3 d, 6 d and 9 d, at 4.0ºC. Negative control (NC) consisted of distilled water (W) + CC, and positive control (PC) consisted of L-cysteine chloride 0.6 % (LC) + CC. The vertical bar indicates the Least Significant Difference (LSD) with ($p \leq 0.05$).

Regarding the enzymatic activity, changes in PPO activity (Fig. 2c) were treatment-dependent. Apples treated with SE+CC, EA+CC and LC+CC showed the lowest enzymatic activity. This result was reflected in the preservation of total phenolics, antioxidant activity, and maintenance of MP fruit color. Inactivation of PPO by EA+CC and LC+CC can also be attributed to the low pH of these solutions (2.22 and 1.62 respectively). As the ideal pH for PPO activity is 7.0, by reducing the pH its activity decreases rapidly, suggesting that the effect of pH is very important for most solutions that aim to inhibit the browning of the MP pulp. Similar results were obtained by TSOUVALTZIS and BRECHT (2017) studying "Russet Burbank" potatoes where the immersion of MP tubers in H_2SO_4 (< 0,04 %), pH 2.39, reduced the PPO activity in comparison to control samples. They attributed the reduction of PPO activity to the deviation from the ideal pH (5 to 7). Another mechanism for browning inhibition of L-cysteine is attributed to its conjugation with *o*-quinones, forming colorless compounds, or by reducing *o*-quinones to the precursor phenolic compounds (CILLIERS and SINGLETON, 1990). According to RICHARD-FORGET *et al.* (1992), these conjugated compounds may act as competitive inhibitors of PPO. However, they advert that in the presence of an excess of quinones, after all cysteine is consumed, the quinones can react with the cysteine-quinone addition compounds, forming violet pigments (RICHARD-FORGET *et al.*, 1992), which were not observed in our experiment. Nevertheless, SE+CC (pH 7.0) inactivated almost completely the enzymatic activity regardless of the pH value of the antioxidant solution. This antioxidant agent has no direct effect on PPO activity, but rather on browning since this compound acts as a free radical scavenger and changes the redox potential, reducing *o*-quinones to diphenols (MOSNEAGUTA *et al.*, 2012). KA+CC treatment did not completely inhibit the enzymatic activity, probably because this acid could not act satisfactorily in one of these mechanisms: prevent the absorption of the O_2 required for the enzymatic reaction; to reduce the quinones to diphenols; or to the combination of the two previously mentioned (CHEN *et al.*, 1991). Besides the positive effect provided by antioxidants, calcium chloride, which has similar properties to sodium chloride, generates chlorine dioxide under acidic conditions, which is involved in the PPO inhibition (GOMES *et al.*, 2014). This observation suggests that the enzymatic inactivation results from synergism between antioxidants agents and $CaCl_2$.

Although PPO is recognized as the main enzyme related to enzymatic browning in apples, it is also necessary to study the changes in POD enzymes activity, since these enzymes may also contribute to change the color of this fruit (JANG and MOON, 2011). Results obtained for POD activity (Fig. 2d) and PPO activity (Fig. 2c) were similar. Apples treated with LC+CC showed the highest enzymatic activity at day zero, which decreased at day 3 and day 6, increasing at day 9. POD activity was close to zero for SE+CC and EA+CC treatments. Since these antioxidants are structurally similar to ascorbic acid (AA), this effect is in accordance with those of JANG and MOON (2011), who reported that the presence of AA effectively reduced POD activity in MP apples. Reduction of POD activity in fruits treated with AA could be the result of lower oxidative stress on their surface due to the antioxidant nature of AA or the POD-enzyme-hydrogen-donor complex formation (SABA and SOGVAR, 2016). These results suggest that treatments containing sodium erythorbate and erythorbic acid may be an efficient way to maintain the quality of MP apples without pulp browning during storage.

Concerning the respiratory gases, oxygen (O_2) and carbon dioxide (CO_2) in the headspace of the packages, all the treatments showed a similar pattern, with a decrease in O_2 (Fig. 3a) and increase in CO_2 (Fig. 3b) over the 9 d of storage. The O_2 concentration for all the treatments (LC+CC, SE+CC, EA+CC, KA+CC) remained lower than the control treatment (W+CC), possibly due to the higher metabolism of apples not treated with antioxidants.

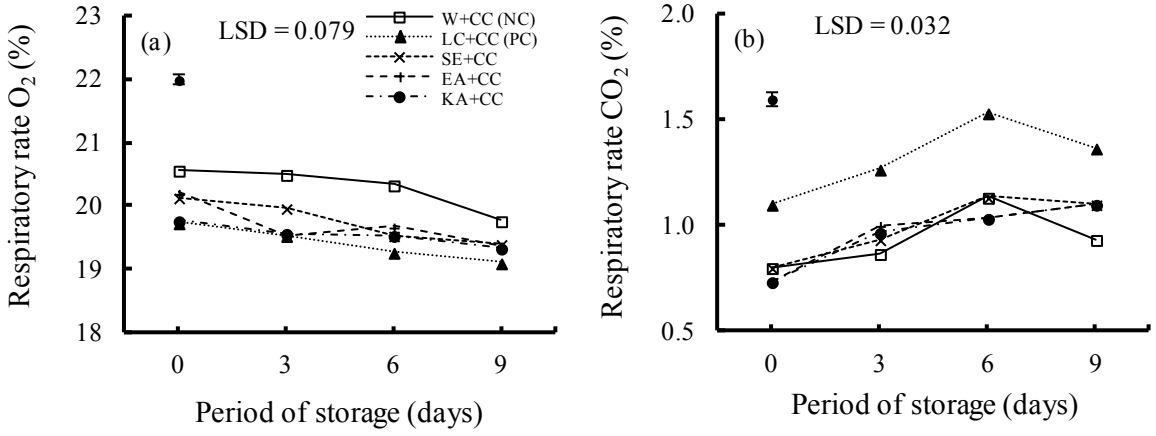


Figure 3. Respiratory rate of minimally processed 'Royal Gala' apples treated with sodium erythorbate 5.0 % (SE) + calcium chloride 1.0 % (CC), erythorbic acid 3.0 % (EA) + CC and kojic acid 0.07 % (KA) + CC based on concentration of O_2 (**a**) and concentration of CO_2 (**b**) in the headspace of the pack, recorded at 0 d, 3 d, 6 d and 9 d, at 4.0ºC. Negative control (NC) consisted of distilled water (W) + CC and positive control (PC) consisted of L-cysteine chloride 0.6 % (LC) + CC. The vertical bar indicates the Least Significant Difference (LSD) with ($p \leq 0.05$).

The respiratory process consumes O_2 and releases CO_2 because of the oxidation of carbohydrates, lipids and proteins, which are present in the product. In addition, higher respiration rates indicate faster overall metabolism and deterioration (VENTURA-AGUILAR *et al.*, 2017). According to MANURAKCHINAKORN *et al.* (2012), the reduction of O_2 and the increase of CO_2, a consequence of the respiratory process, is detectable in MP fruits even if stored in closed containers permeable for gas exchange. Interestingly, for unknown reasons, apples treated with LC+CC accumulated in the packs an average of 26.9 % more CO_2 than the apples treated with other antioxidants.

Sensory analysis showed that apples treated with the antioxidants LC+CC, SE+CC and EA+CC had no noticeable darkening in the pulp (Fig. 4a), being classified as very good and excellent. On the other hand, apples treated with W+CC presented a considerable increase in browning during the nine days of evaluation, reaching the marketing limit from the sixth day. The KA+CC also showed increased browning, however with lower intensity. Flavor parameter (Fig. 4b) was only affected for apples treated with LC+CC. Acceptability of apples treated with LC+CC was lower than for other treatments, already on the first day of evaluation. This behavior was intensified along the storage periods to the point of being between the marketing and inedible limits. The presence of calcium chloride did not significantly modify the flavor of the samples, which corroborates that this salt does not modify the flavor of the fruits. Due to the high acidity of the EA+CC treatment, it was necessary to carefully reduce the droplets of this antioxidant on the epidermis of the apple slices, since when this happened, the evaluators noticed and reported the unpleasant sensation caused by this antioxidant solution. This peculiarity makes this treatment a secondary option of using this product. Considering the odor attribute, apples treated with LC+CC also showed low acceptability (Fig. 4c). Evaluators were able to perceive the sulfur-containing compounds in the samples containing L-cysteine, which agrees with the results obtained by PEREZ-GAGO *et al.* (2006). Other treatments, although with lower intensity, also had a decrease in the odor attribute along the storage. This decrease can be attributed to the volatilization of compounds responsible for the aroma of the fruit due to damages caused in the pulp during the minimal processing. The attributes succulence and firmness (data not presented) did not present

statistical difference between the treatments, being in the range between very good and excellent. When including all evaluated attributes and score the overall quality, apples treated with SE+CC, EA+CC and KA+CC presented high acceptability since all evaluated attributes were satisfactory (Fig. 4d).

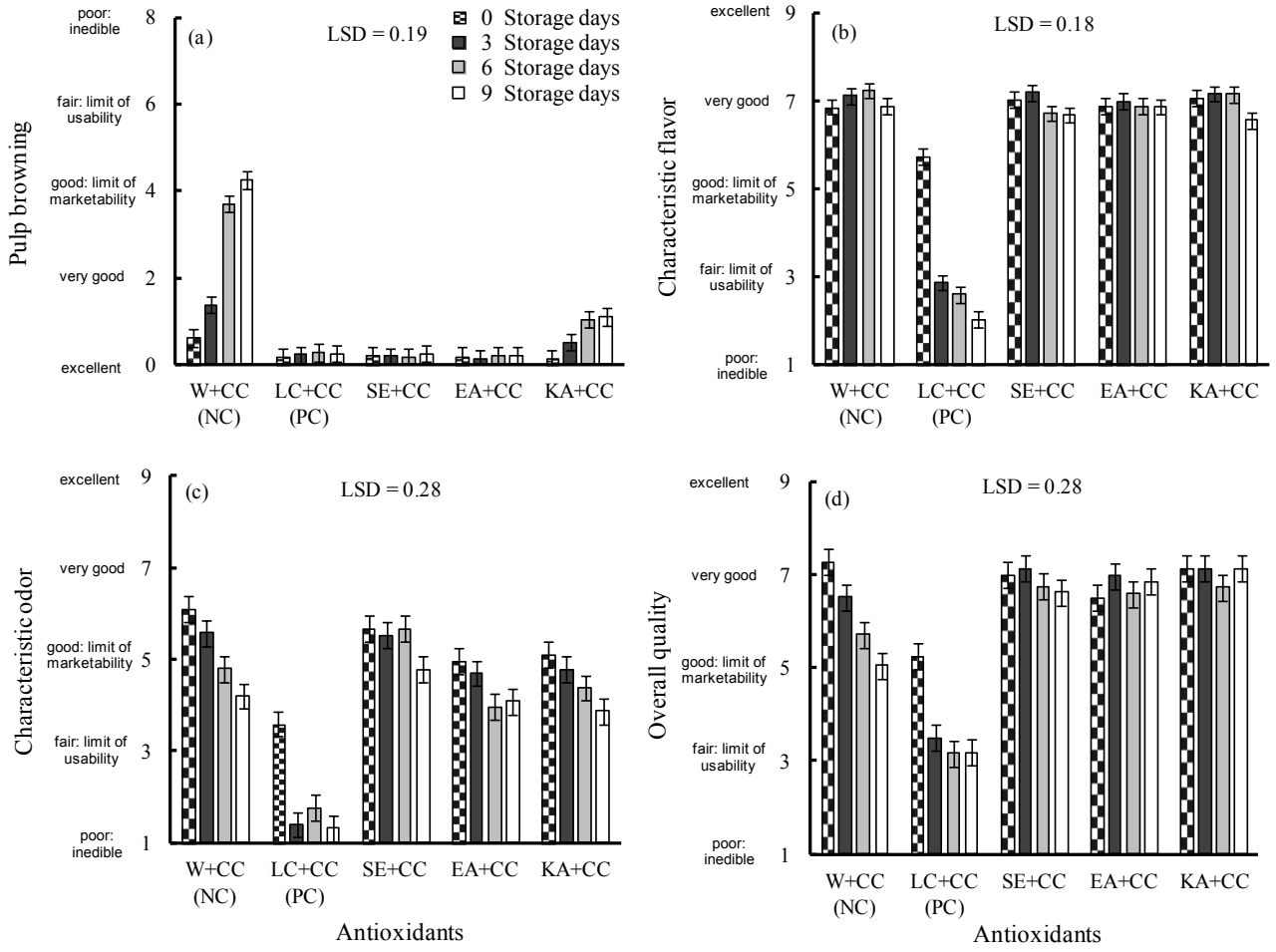


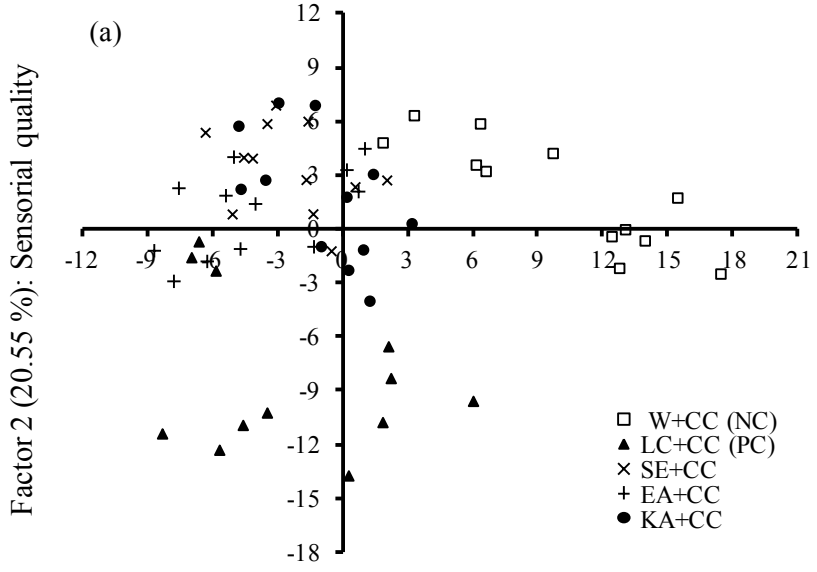
Figure 4. Sensorial characteristics of minimally processed 'Royal Gala' apple treated with sodium erythorbate 5.0 % (SE) + calcium chloride 1.0 % (CC), erythorbic acid 3.0 % (EA) + CC and kojic acid 0.07 % (KA) + CC, and recorded at 0 d, 3 d, 6 d and 9 d; at 4.0ºC: pulp browning (**a**); characteristic flavor (**b**), characteristic odor (**c**) and overall quality (**d**). Negative control (NC) consisted of distilled water (W) + CC, and positive control (PC) consisted of L-cysteine chloride 0.6 % (LC) + CC. The vertical bar indicates the Least Significant Difference (LSD) with ($p \leq 0.05$).

Opposite situation occurred with W+CC treatment, mainly due to the appearance of undesirable brown pigments on the surface of the slices due to the oxidation processes. LC+CC treatment was able to inhibit pulp browning completely, however, the apple taste and odor significantly changed, leading to low acceptability of apples treated with this antioxidant. The changes caused by L-cysteine in the odor are attributed to enzymatic reactions, mainly the degradation of methionine, cysteine and derivatives. These enzymatic degradations lead to the formation of volatile compounds containing aliphatic sulfur, such as thiols and polysulfides (VARLET and FERNANDEZ, 2010). In view of these results, the application of sodium erythorbate and erythorbic acid proved to be viable alternatives for the maintenance of the sensorial and physical-chemical attributes of

apples evaluated in this study. In addition, erythorbic acid has a relatively low cost because calcium 2-ketogluconate is easily converted during glucose fermentation (LEE *et al.*, 2012). This antioxidant is also reported to potentiate the non-heme iron uptake (FIDLER *et al.*, 2004), helping to provide the nutritional needs of iron (SINGH *et al.*, 2016). According to HALAGARDA and SUWALA (2018), the importance of sensory analysis at every stage of the product development or improvement process is due to significant cost and failure reduction after product launch. Moreover, the fulfilment of expectations, especially the sensorial ones, leads to consumer satisfaction, increasing the likelihood of commercialization of the product (GRUNERT, 2002).

The factorial analysis allowed us to verify the most important factors for fruit quality. In this analysis, five factors explained 70.86 % of the total variability of the experiment (data not shown). The Factor 1 (termed pulp color) explained 26.25 % of total variability (Fig. 5a), and showed higher correlation coefficients with the variables index of darkening (0.963), b* (0.946), chroma (0.942) and ºHue (-0.875). On the positive axis of the abscissa are the variables with the higher positive correlation coefficients values (index of darkening, b*, sensorial darkening and chroma) and on the negative axis is the variable with the higher negative correlation coefficient value (Hue).

It appears that these variables evolved together, being classified in the same factor, which indicates that the differences were detected by the sensory panel of judges. Regarding the distribution of the treatments in the factors, it can be observed that the treatment W+CC (negative control), as expected, resulted in slices of apples with higher levels of darkening, being grouped in the positive axis of the abscissa on the graph. The other treatments, with a low level of darkening, were grouped predominantly on the negative axis of the abscissa. The Factor 2 (termed sensorial quality), which explains 20.55 % of total variability, showed higher correlation coefficients with the variables odor (0.909), taste characteristics (0.882) and overall quality (0.865). All of them were located on the positive axis of the ordinate. It can be observed that the values of sensory quality in apples treated with SE+CC, EA+CC and KA+CC, resulted in a good level of acceptability by the sensory panel of judges. On the other hand, the application of LC+CC presented lower sensory quality. In relation to the storage periods (0 d, 3 d, 6 d and 9 d) it can be observed (Fig. 5b) that periods in which the MP fruit presented the best quality are located on the positive axis of the ordinate axis, and MP fruits of inferior quality are located on the negative axis of the ordinate.



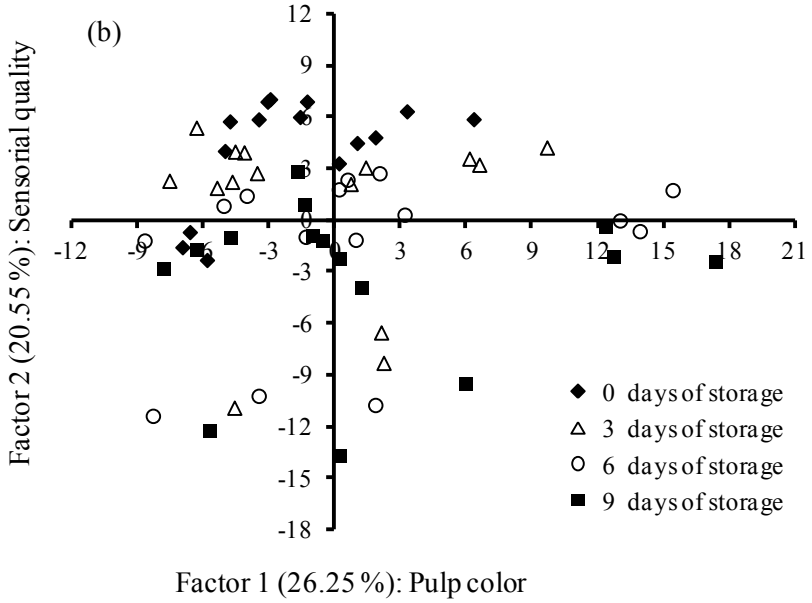


Figure 5. Factorial analysis of minimally processed 'Royal Gala' apples treated with sodium erythorbate 5.0 % (SE) + calcium chloride 1.0 % (CC), erythorbic acid 3.0 % (EA) + CC and kojic acid 0.07 % (KA) + CC (a) recorded at 0 d, 3 d, 6 d and 9 d (b); at 4.0ºC. Factor 1: color and Factor 2: sensorial quality after Varimax orthogonal rotation. Negative control (NC) consisted of distilled water (W) + CC, and positive control (PC) consisted of L-cysteine chloride 0.6 % (LC) + CC. The vertical bar indicates the Least Significant Difference (LSD) with ($p \leq 0.05$).

This result indicates that MP apples slices lose quality as the refrigerated storage period increased. However, this reduction in quality is a normal process, as many compounds are metabolized, resulting in senescence and cell death.

4. CONCLUSIONS

Apple browning is of high concern for the industry of minimally processed fruits since it has a high impact on color, flavor and nutritional value. Our results showed that treatment with either 5 % sodium erythorbate or 3 % erythorbic acid, associated with 1 % calcium chloride, controlled the apple pulp browning and preserved the phenolic compounds and the antioxidant activity for up to 9 days under cold storage. Besides, both antioxidants significantly inhibited the peroxidase and polyphenoloxidase enzymes and preserved the sensorial quality, which was found to be equivalent to the fresh fruit. Thus, the treatment of MP 'Royal Gala' apples with 5 % sodium erythorbate or 3 % erythorbic acid, associated with 1 % calcium chloride, can be suitable to extend the product shelf-life.

ACKNOWLEDGEMENTS

The authors acknowledge the Brazilian Government (Embrapa Clima Temperado, CAPES and CNPq) for funding this study, including a Ph.D. scholarship, and Rasip Agro Pastoril S/A for providing logistic support and experimental material.

REFERENCES

Ali S., Khan A.S. and Malik A.U. 2016. Postharvest l-cysteine application delayed pericarp browning, suppressed lipid peroxidation and maintained antioxidative activities of litchi fruit. Postharvest. Biol. Technol. 121:135 DOI: doi.org/10.1016/j.postharvbio.2016.07.015

Andersen F.A. 1999. Final report on the safety assessment of ascorbyl palmitate, ascorbyl dipalmitate, ascorbyl stearate, erythorbic acid, and sodium erythorbate. Int. J. Toxicol. 18(3_suppl):1. DOI: doi.org/10.1177/109158189901800303

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1-2):248. DOI: doi.org/10.1016/0003-2697(76)90527-3

Brand-Williams W., Cuvelier M.E. and Berset C. 1995. Use of a free-radical method to evaluate antioxidant activity. LWT - Food Sci. Technol. 28(1):25. DOI: doi.org/10.1016/S0023-6438(95)80008-5

Buta J.G., Moline H.E., Spaulding D.W. and Wang C.Y. 1999. Extending storage life of fresh-cut apples using natural products and their derivatives. J. Agric. Food Chem. 47(1):1. DOI: doi.org/10.1021/jf980712x

Cabezas-Serrano A.B., Amodio M.L. and Colelli G. 2013. Effect of solution ph of cysteine-based pre-treatments to prevent browning of fresh-cut artichokes. Postharvest. Biol. Technol. 75:17. DOI: doi.org/10.1016/j.postharvbio.2012.07.006

Cacace J.E., Delaquis P.J. and Mazza G. 2002. Effect of chemical inhibitors and storage temperature on the quality of fresh-cut potatoes. J. Food Qual. 25(3):181. DOI: doi.org/10.1111/j.1745-4557.2002.tb01018.x

Carocho M., Morales P. and Ferreira I.C.F.R. 2018. Antioxidants: Reviewing the chemistry, food applications, legislation and role as preservatives. Trends Food Sci. Technol. 71:107. DOI: doi.org/10.1016/j.tifs.2017.11.008

Ceymann M., Arrigoni E., Schaerer H., Nising A.B. and Hurrell R.F. 2012. Identification of apples rich in health-promoting flavan-3-ols and phenolic acids by measuring the polyphenol profile. J. Food Compost. Anal. 26(1-2):128. DOI: doi.org/10.1016/j.jfca.2011.12.002

Chen J.S., Wei C.I. and Marshall M.R. 1991. Inhibition-mechanism of kojic acid on polyphenol oxidase. J. Agric. Food Chem. 39(11):1897. DOI: doi.org/10.1021/jf00011a001

Cilliers J.J.L. and Singleton V.L. 1990. Caffeic acid autoxidation and the effects of thiols. J. Agric. Food Chem. 38(9):1789. DOI: doi.org/10.1021/jf00099a002

Coelho M.S. and Salas-Mellado M.d.l.M. 2014. Chemical characterization of chia (*Salvia hispanica* L.) for use in food products. J. Food Nut. Res. 2(5):263. DOI: doi.org/10.12691/jfnr-2-5-9

Cortellino G., Gobbi S., Bianchi G. and Rizzolo A. 2015. Modified atmosphere packaging for shelf life extension of fresh-cut apples. Trends Food Sci. Technol. 46(2):320. DOI: doi.org/10.1016/j.tifs.2015.06.002

Corzo-Martínez M., Corzo N., Villamiel M. and Castillo M.D.d. 2012. Browning reactions. In: Simpson B.K. (Ed.). Food biochemistry and food processing. 2nd ed: Oxford: Blackwell. pp. 39-83.

Cropotova J., Tylewicz U., Cocci E., Romani S. and Dalla Rosa M. 2016. A novel fluorescence microscopy approach to estimate quality loss of stored fruit fillings as a result of browning. Food Chem. 194:175. DOI: doi.org/10.1016/j.foodchem.2015.07.146

Fidler M.C., Davidsson L., Zeder C. and Hurrell R.F. 2004. Erythorbic acid is a potent enhancer of nonheme-iron absorption. Am. J. Clin. Nutr. 79(1):99. DOI: doi.org/10.1093/ajcn/79.1.99

Figueiredo B.C., Trad I.J., Mariutti L.R.B. and Bragagnolo N. 2014. Effect of annatto powder and sodium erythorbate on lipid oxidation in pork loin during frozen storage. Food Res. Int. 65:137. DOI: doi.org/10.1016/j.foodres.2014.07.016

Galgano F., Condeli N., Favati F., Di Bianco V., Peretti G. and Caruso M.C. 2015. Biodegradable packaging and edible coating for fresh-cut fruits and vegetables. Ital. J. Food Sci. 27(1):1. DOI: doi.org/10.14674/1120-1770/ijfs.v70

Gang C.C., Li J.L., Chen Y.Z., Wang Y.J., Li H., Pan B. and Odeh I. 2015. Synergistic effect of chemical treatments on storage quality and chilling injury of honey peaches. J. Food Process. Preserv. 39(6):1108. DOI: doi.org/10.1111/jfpp.12325

Ghidelli C., Mateos M., Rojas-Argudo C. and Perez-Gago M.B. 2014. Effect of antioxidants on enzymatic browning of eggplant extract and fresh-cut tissue. J. Food Process. Preserv. 38(4):1501. DOI: doi.org/10.1111/jfpp.12109

Giacalone G., Chiabrando V. and Bardi L. 2010. Changes in nutritional properties of minimally processed fresh fruit during storage. Ital. J. Food Sci. 22(3):305

Gil M.I., Gorny J.R. and Kader A.A. 1998. Responses of `fuji' apple slices to ascorbic acid treatments and low-oxygen atmospheres. HortScience 33(2):305

Gomes M.H., Vieira T., Fundo J.F. and Almeida D.P.F. 2014. Polyphenoloxidase activity and browning in fresh-cut 'rocha' pear as affected by ph, phenolic substrates, and antibrowning additives. Postharvest. Biol. Technol. 91:32. DOI: doi.org/10.1016/j.postharvbio.2013.12.013

Gross K.C., Wang C.Y. and Saltveit M.E. 2016. The commercial storage of fruits, vegetables, and florist and nursery stocks. Agriculture handbook 66. Washington, D.C.: U.S. Department of Agriculture.

Grunert K.G. 2002. Current issues in the understanding of consumer food choice. Trends Food Sci. Technol. 13(8):275. DOI: doi.org/10.1016/s0924-2244(02)00137-1

Halagarda M. and Suwala G. 2018. Sensory optimisation in new food product development: A case study of polish apple juice. Ital. J. Food Sci. 30(2):317. DOI: doi.org/https://doi.org/10.14674/IJFS-960

Holderbaum D.F., Kon T., Kudo T., and Guerra M.P. 2010. Enzymatic browning, polyphenol oxidase activity, and polyphenols in four apple cultivars: Dynamics during fruit development. HortScience 45(8):1150.

Ioannou I. and Ghoul M. 2013. Prevention of enzymatic browning in fruit and vegetables. Eur. Sci. J. 9(30):310. DOI: doi.org/10.19044/esj.2013.v9n30p%25p

Iyidogan N.F. and Bayindirli A. 2004. Effect of l-cysteine, kojic acid and 4-hexylresorcinol combination on inhibition of enzymatic browning in amasya apple juice. J. Food Eng. 62(3):299. DOI: doi.org/10.1016/s0260-8774(03)00243-7

Jang J.H. and Moon K.D. 2011. Inhibition of polyphenol oxidase and peroxidase activities on fresh-cut apple by simultaneous treatment of ultrasound and ascorbic acid. Food Chem. 124(2):444. DOI: doi.org/10.1016/j.foodchem.2010.06.052

Kall M.A. and Andersen C. 1999. Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples. J. Chromatogr. B 730(1):101. DOI: doi.org/10.1016/s0378-4347(99)00193-0

Kim J.-S. and Lee Y.-S. 2008. The influence of ph on the color development of melanoidins formed from fructose/amino acid enantiomer model systems. Journal of Food Science and Nutrition 13:306. DOI: doi.org/10.3746/jfn.2008.13.4.306

Lee D.E., Park K.M., Choi S.J. and Chang P.S. 2012. Optimal production and structural characterization of erythorbyl laurate obtained through lipase-catalyzed esterification. Food Sci. Biotechnol. 21(4):1209. DOI: doi.org/10.1007/s10068-012-0158-2

Manurakchinakorn S., Chamnan U. and Mahakarnchanakul W. 2012. Appearance and texture changes of modified atmosphere-packed fresh-cut mangosteen after preservative dips. J. Food Process. Preserv. 36(6):504. DOI: doi.org/10.1111/j.1745-4549.2011.00617.x

Martin-Belloso O. and Soliva-Fortuny R. 2011. Advances in fresh-cut fruits and vegetables processing. Boca Raton, FL: CRC Press.

Melo A.M.M., Vilas Boas E.V.B. and Justo C.F. 2009. Uso de aditivos químicos para a conservação pós-colheita de Banana 'Maçã' minimamente processada. Ciênc. Agrotec., Lavras. 33(1):228.

Mitani H., Koshiishi I., Sumita T. and Imanari T. 2001. Prevention of the photodamage in the hairless mouse dorsal skin by kojic acid as an iron chelator. Eur. J. Pharmacol. 411(1-2):169. DOI: doi.org/10.1016/s0014-2999(00)00873-6

Mosneaguta R., Alvarez V. and Barringer S.A. 2012. The effect of antibrowning agents on inhibition of potato browning, volatile organic compound profile, and microbial inhibition. J. Food Sci. 77(11):C1234. DOI: doi.org/10.1111/j.1750-3841.2012.02957.x

Ni Eidhin D.M., Murphy E. and O'Beirne D. 2006. Polyphenol oxidase from apple (malus domestica borkh. Cv bramley's seedling): Purification strategies and characterization. J. Food Sci. 71(1):C51. DOI: doi.org/10.1111/j.1365-2621.2006.tb12388.x

Palou E., Lopez-Malo A., Barbosa-Canovas G.V., Welti-Chanes J. and Swanson B.G. 1999. Polyphenoloxidase activity and color of blanched and high hydrostatic pressure treated banana puree. J. Food Sci. 64(1):42. DOI: doi.org/10.1111/j.1365-2621.1999.tb09857.x

Pech J.C., Bouzayen M. and Latché A. 2008. Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. Plant Sci. 175(1-2):114. DOI: doi.org/10.1016/j.plantsci.2008.01.003

Perez-Cabrera L., Chafer M., Chiralt A. and Gonzalez-Martinez C. 2011. Effectiveness of antibrowning agents applied by vacuum impregnation on minimally processed pear. LWT - Food Sci. Technol. 44(10):2273. DOI: doi.org/10.1016/j.lwt.2011.04.007

Perez-Gago M.B., Serra M. and del Rio M.A. 2006. Color change of fresh-cut apples coated with whey protein concentrate-based edible coatings. Postharvest. Biol. Technol. 39(1):84. DOI: doi.org/10.1016/j.postharvbio.2005.08.002

Piagentini A.M. and Pirovani M.E. 2017. Total phenolics content, antioxidant capacity, physicochemical attributes, and browning susceptibility of different apple cultivars for minimal processing. Int. J. Fruit Sci. 17(1):102. DOI: doi.org/10.1080/15538362.2016.1262304

Pizato S., Cortez-Vega W.R., Costa T.S., da, Oliveira L.M. and de, Prentice C. 2013. Improvement quality and shelf life assessment of minimally processed apples by use of protein coatings. J. Food Res. 2(5):24. DOI: doi.org/10.5539/jfr.v2n5p24

Qi H., Hu W., Jiang A., Tian M. and Li Y. 2011. Extending shelf-life of fresh-cut 'fuji' apples with chitosan-coatings. Innov. Food Sci. Emerg. Technol. 12(1):62. DOI: doi.org/10.1016/j.ifset.2010.11.001

Rai G.K., Kumar R., Singh J., Rai P.K. and Rai S.K. 2011. Peroxidase, polyphenol oxidase activity, protein profile and phenolic content in tomato cultivars tolerant and susceptible to Fusarium oxysporum f.sp. lycopersici. Pak. J. Bot. 43(6):2987.

Ramalho V.C. and Jorge N. 2006. Antioxidants used in oils, fats and fatty foods. Quim. Nova 29(4):755. DOI: doi.org/10.1590/s0100-40422006000400023

Reische D.W., Lillard D.A. and Eitenmiller R.R. 2002. Antioxidants. In: Akoh C.C., Min D.B. (Eds). Food lipids: Chemistry, nutrition, and biotechnology. 2nd ed. New York, NY: CRC Press. pp. 489-516.

Richard-Forget F.C., Goupy P.M. and Nicolas J.J. 1992. Cysteine as an inhibitor of enzymatic browning. 2. Kinetic-studies. J. Agric. Food Chem. 40(11):2108. DOI: doi.org/10.1021/jf00023a014

Rojas-Graue M.A., Soliva-Fortuny R. and Martin-Belloso O. 2008. Effect of natural antibrowning agents on color and related enzymes in fresh-cut fuji apples as an alternative to the use of ascorbic acid. J. Food Sci. 73(6):S267. DOI: doi.org/10.1111/j.1750-3841.2008.00794.x

Saba M.K. and Sogvar O.B. 2016. Combination of carboxymethyl cellulose-based coatings with calcium and ascorbic acid impacts in browning and quality of fresh-cut apples. LWT - Food Sci. Technol. 66:165. DOI: doi.org/10.1016/j.lwt.2015.10.022

Sanchis E., Mateos M. and Perez-Gago M.B. 2016. Physicochemical, sensory, and nutritional quality of fresh-cut "rojo brillante" persimmon affected by maturity stage and antibrowning agents. Food Sci. Technol. Int. 22(7):574. DOI: doi.org/10.1177/1082013216629262

Sapers G.M. and Miller R.L. 1998. Browning inhibition in fresh-cut pears. J. Food Sci. 63(2):342. DOI: doi.org/10.1111/j.1365-2621.1998.tb15738.x

Shah H.M.S., Khan A.S. and Ali S. 2017. Pre-storage kojic acid application delays pericarp browning and maintains antioxidant activities of litchi fruit. Postharvest. Biol. Technol. 132:154. DOI: doi.org/10.1016/j.postharvbio.2017.06.004

Singh A., Bains K. and Kaur H. 2016. Relationship of dietary factors with dialyzable iron and in vitro iron bioavailability in the meals of farm women. J. Food Sci. Technol. (Mysore) 53(4):2001. DOI: doi.org/10.1007/s13197-015-2153-0

Sommers C.H., Handel A.P. and Niemira B.A. 2002. Radiation resistance of listeria monocytogenes in the presence or absence of sodium erythorbate. J. Food Sci. 67(6):2266. DOI: doi.org/10.1111/j.1365-2621.2002.tb09538.x

Son S.M., Moon K.D. and Lee C.Y. 2001. Inhibitory effects of various antibrowning agents on apple slices. Food Chem. 73(1):23. DOI: doi.org/10.1016/s0308-8146(00)00274-0

Streif J. 1984. Jod-stärke-test zur beurteilung der fruchtreife bei äpfeln. Obst und Garten, Stuttgart 8:382

Swain T. and Hillis W.E. 1959. The phenolic constituents of *prunus domestica*. I. the quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10(1):63. DOI: doi.org/10.1002/jsfa.2740100110

Techakanon C. and Barrett D.M. 2017. The effect of calcium chloride and calcium lactate pretreatment concentration on peach cell integrity after high-pressure processing. Int. J. Food Sci. Technol. 52(3):635. DOI: doi.org/10.1111/ijfs.13316

Toivonen P.M.A. and Brummell D.A. 2008. Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. Postharvest. Biol. Technol. 48(1):1. DOI: doi.org/10.1016/j.postharvbio.2007.09.004

Tsouvaltzis P. and Brecht J.K. 2017. Inhibition of enzymatic browning of fresh-cut potato by immersion in citric acid is not solely due to ph reduction of the solution. J. Food Process. Preserv. 41(2):e12829. DOI: doi.org/doi:10.1111/jfpp.12829

Varlet V. and Fernandez X. 2010. Review. Sulfur-containing volatile compounds in seafood: Occurrence, odorant properties and mechanisms of formation. Food Sci. Technol. Int. 16(6):463. DOI: doi.org/10.1177/1082013210379688

Ventura-Aguilar R.I., Colinas-Leon M.T. and Bautista-Banos S. 2017. Combination of sodium erythorbate and citric acid with map, extended storage life of sliced oyster mushrooms. LWT - Food Sci. Technol. 79:437.
DOI: doi.org/10.1016/j.Iwt.2017.01.053

Vieira F.G.K., Borges G.D.C., Copetti C., Di Pietro P.F., Nunes E.D. and Fett R. 2011. Phenolic compounds and antioxidant activity of the apple flesh and peel of eleven cultivars grown in Brazil. Sci. Hortic. 128(3):261.
DOI: doi.org/10.1016/j.scienta.2011.01.032

Zhang J., Zhao P., Liu B. and Meng X. 2017. Use of oligochitosan as an inhibiting agent of apple juice enzymatic browning. J. Food Process. Preserv. 41(4):e13062. DOI: doi.org/10.1111/jfpp.13062

Paper Received November 29, 2018 Accepted March 3, 2019

PAPER

EFFECT OF ULTRASOUND TREATMENT ON PHYSICOCHEMICAL, FUNCTIONAL AND NUTRITIONAL PROPERTIES OF A SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) PROTEIN ISOLATE

M.R. ZUÑIGA-SALCEDO[a], J.A. ULLOA*[a,b], P.U. BAUTISTA-ROSALES[a,b], P. ROSAS-ULLOA[b], J.C. RAMÍREZ-RAMÍREZ[c], Y. SILVA-CARRILLO[d], R. GUTIÉRREZ-LEYVA[c] and C. HERNÁNDEZ[e]

[a]Programa de Maestría en Ciencias Biológico Agropecuarias. Unidad Académica de Agricultura, Universidad Autónoma de Nayarit, Carretera Tepic-Compostela Km 9, 63780 Xalisco, Nayarit, México

[b]Centro de Tecnología de Alimentos, Universidad Autónoma de Nayarit. Ciudad de la Cultura Amado Nervo, 63155 Tepic, Nayarit. México

[c]Unidad Académica de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Nayarit, Carretera Compostela-Chapalilla Km 3.5, 63700 Compostela, Nayarit, México

[d]Unidad Académica de Agricultura, Universidad Autónoma de Nayarit, Carretera Tepic-Compostela Km 9, 63780 Xalisco, Nayarit, México

[e]Centro de Investigación en Alimentación y Desarrollo Unidad Mazatlán, Av. Sábalo Cerritos S/N, 82100 Mazatlán, Sinaloa, México

*Corresponding author: Tel.: +523112118851; Fax +523112118861
E-mail address: arulloa5@gmail.com

ABSTRACT

The effect of ultrasound exposure time (15 and 30 min) at 130 W and 40 kHz on the physicochemical, functional, and nutritional properties of a safflower protein isolate (SPI) was evaluated. The moisture content, bulk density, and a_w of the SPI were significantly ($P < 0.05$) decreased by ultrasound compared to the untreated control. In contrast to the control, the SPI exposed to ultrasound for 30 min had increased protein solubility (by 9.7% and 3.7% at pH 6 and 7, respectively), least gelation concentration (by 2.0% at pH 6), and oil absorption capacity (by 3.0%). No significant differences ($P < 0.05$) were observed in amino acid composition, chemical score, or predicted protein efficiency ratio of the control and the SPI exposed to ultrasound. Ultrasound treatment would benefit the application of SPI in the food industry for ground meat formulations, meat substitutes and extenders, doughnuts, baked goods, and soups.

Keywords: ultrasound treatment, safflower protein isolate, physicochemical properties, functional properties, nutritional properties

1. INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is one of the oldest cultivated crops and usually is grown for flowers that are used for coloring, flavoring foods, dyes, medicinal properties, and livestock feed (PEIRETTI, 2017). Safflower is gaining attention due to its oil yield potential and the ability to grow under high temperatures, drought, and high salinity (HUSSAIN *et al*., 2016). Safflower is produced in over 20 countries, but in 2016, the Russian Federation, Kazakhstan, Mexico, and the USA were the main producers, producing about 71% of the 948,516 t of safflower produced worldwide (FAO, 2018).

Oil and meal are the two main products that come from safflower production. Oil is the primary product and has both food and industrial uses. The seed oil content of safflower ranges from 30 to 45% (LIU *et al*., 2016). Safflower meal, a by-product of the safflower-oil industry, contains approximately 20-25% protein, and is currently marketed as animal feed (TIRIL and KERIM, 2015). However, according to some studies, a protein isolate from safflower meal could represent an opportunity to recover proteins for human consumption (ULLOA *et al*., 2011; PAREDES-LÓPEZ and ORDORICA-FALOMIR, 1986).

On the other hand, the application of ultrasonic technology in the food industry is currently attracting much attention (HU *et al*., 2013). Ultrasound is an acoustic wave with a frequency >20 kHz (O'SULLIVAN *et al*., 2016a). High intensity ultrasound (HIUS, 20-100kHz) might have a wide variety of applications in the food industry (ZHANG *et al*., 2014) because it can alter the physicochemical properties and/or structure and functional properties due to cavitation effects on vegetable proteins such as soy protein isolate (JAMBRAK *et al*., 2009), black bean protein isolate (JIANG *et al*., 2014), and jackfruit seed protein isolate (RESENDIZ-VAZQUEZ *et al*., 2017).

A recent study by XIONG *et al*. (2018) showed that ultrasonic treatment caused partial unfolding of the proteins of pea protein isolate, which improved foam ability and stability. According to MALIK *et al*. (2017), solubility, emulsifying capacity, emulsion stability, foaming capacity, foam stability, and oil binding capacity of a sunflower protein isolate were improved significantly after application of HIUS. ZHANG *et al*. (2018) found that the HIUS treatment modified the protein structure and significantly enhanced the solubility of rice proteins, as well as emulsifying properties and foaming properties. However, the results of the application of ultrasound treatment on protein properties depend on some conditions such as frequency, amplitude, time, temperature, ionic strength, pH, and concentration, as well as intrinsic characteristics of the protein source (HIGUERA-BARRAZA *et al*., 2016). To our knowledge, no studies have focused on the potential effects of HIUS application on safflower protein isolate (SPI). Therefore, the objective of this study was to evaluate the effect of HIUS on the physicochemical, functional, and nutritive properties of a protein isolate obtained from safflower meal.

2. MATERIAL AND METHODS

2.1. Materials and chemicals

The safflower meal (23% protein, moisture 8%, 5.4% ash, 1.4% fat, and total carbohydrates 62.2%) used in this study was purchased from Aceitera La Junta, S.A. de C.V. (Limited Company of Variable Capital) (Guadalajara, Jalisco, Mexico). All chemical reagents were J.T. Baker analytical grade and purchased from Diseño Tecnológico en Laboratorios, S. A. de C.V. (Guadalajara, Jalisco, México).

2.2. Preparation of protein isolate

The SPI was prepared according to the method reported by ULLOA *et al*. (2011). Briefly, the protein was recovered in batches of safflower meal suspension (120 L) by adding one part safflower meal to 30 parts filtered tap water, which was then mixed for 45 min at room temperature (25°C). The pH of the suspension was adjusted to 9.0 with diluted NaOH during mixing. The insoluble residue of the protein suspension was separated by continuous centrifugation. The protein extract was concentrated using a pilot-scale ultrafiltration unit (Osmonic Inc., Minnetonka, USA) equipped with a polysulfonate membrane cartridge with a molecular weight cut-off of 100 kDa. Extracts were concentrated to one-fifth of their original volume, then diluted with filtered tap water and concentrated by diafiltration for further purification of protein retentate. Dilution consisted of adding an amount of water equal to four times the retentate volume after the first concentration. Three cycles of diafiltration were required to produce a protein isolate with a protein content ≥90.0% dry weight. Finally, the diafiltrated protein extract was spray dried in a Model Tower No. 1 drier (Niro Atomizer, SA, Monterrey, Nuevo León, Mexico) to obtain the powder of protein isolate.

2.3. Ultrasound treatment

Aqueous suspensions of SPI containing 10% of protein (w/v) were prepared in 100-mL beakers by magnetic stirring for 15 min. Ultrasound treatment was performed in an ultrasound bath Branson Model MTH-3510 (130 W and 40 kHz; a tank capacity of 5 L; internal dimensions of 290 × 150 × 150 mm; an acoustic energy density of 0.026 W cm^{-3}). Beakers containing SPI suspension were placed directly into the ultrasound bath to receive the treatment for 15 or 30 min, while the control SPI suspension was placed into a water bath at 25°C; the temperature during ultrasound treatment increased by <2°C. The ultrasonic intensity introduced in the system measured by calorimetry according to JAMBRAK *et al*. (2014) was 1 Wcm^{-2}. Afterward the suspensions of SPI exposed to ultrasound and the control were lyophilized in a FreeZone Freeze-Dry System (Labconco, Kansas City, MO, USA) and stored at room temperature in sealed containers for further analysis.

2.4. Physicochemical and microstructure characteristics

Moisture, protein (N × 6.25), and ash contents were determined according to AOAC methods (1995). Water activity (a_w) was measured at 25°C using an AquaLab 4TEV (Decagon Devices Inc., Pullman, Washington, USA), on coarse powder samples (3 g). Prior to testing samples, the water activity meter was turned on and allowed to warm up for 30 min and calibrated by filling a plastic disposable cup half filled with a saturated sodium chloride solution. The accuracy of water activity values was ±0.003. The color was determined with a Minolta CR-400 color meter (Konica Minolta Sensing Ltd, Inc., Tokyo, Japan). The measured values were expressed according to the CIELAB color scale L^* (lightness), a^* (redness-greenness), and b^* (yellowness–blueness). The L_s^*, a_s^*, and b_s^* values of the white standard tile used as reference were 94.44, -0.23 and 3.89, respectively. Total color difference (ΔE) was calculated as:

$$\Delta E^* = [(L_s^* - L^*)^2 + (a_s^* - a^*)^2 + (b_s^* - b^*)^2]^{1/2} \quad (1)$$

The bulk density was determined in triplicate using the method described by PIORNOS *et al*. (2015) and expressed as g mL^{-1}.

The microstructure of the freeze-dried SPI samples was observed with a scanning electron microscope (SEM; SEC, Mini-SEM SNE-3200M, South Korea) at an accelerating voltage of 20 kV. Before using the SEM, the samples were coated with gold using an ion sputter coater (MCM-100, SEC).

2.5. Functional properties

Protein solubility profile of the SPI as a function of pH was determined according to the method reported by PIORNOS *et al*. (2015). The water absorption capacity (WAC) and oil absorption capacity (OAC) were measured according to the method described by ULLOA *et al*. (2011) and expressed as g water or oil absorbed per g protein. Soy oil (Fábrica de Aceites la Central, S. A. de C.V., Guadalajara, Jalisco, México) was used in the determination of OAC. The emulsifying activity (EA) and emulsion stability (ES) were determined according to the method reported by DENG *et al*. (2011) using soy oil. The least gelation concentration (LGC) at pH 2, 4, 6, 8, and 10 was determined according to the method reported by BENELHADJ *et al*. (2016). Foaming capacity (FC) and foam stability (FS) were measured using the method described by STONE *et al*. (2015).

2.6. Amino acid composition and protein nutritive quality

Hydrolysis and quantification of amino acids were performed according to the methods described by VÁZQUEZ-ORTÍZ *et al*. (1995) using a Pro Star-210 Varian high-performance liquid chromatographic system (Varian Associates, Inc. USA). Amino acids were expressed on a protein basis, equivalent to g per 16 g of protein. The tryptophan content was not determined.
The nutritive quality of proteins was estimated by determination of chemical score (CS) and predicted protein efficiency ratio (PER) according to the procedure described previously by ULLOA *et al*. (2015).

2.7. Statistical analysis

Analyses of samples were done in triplicate and data were analyzed using one-way ANOVA. Significant differences ($P < 0.05$) between samples were determined from a Tukey's test using SPSS Statistics Version 20 (IBM Corporation, New York, USA).

3. RESULTS AND DISCUSSION

3.1. Physicochemical and microstructure characteristics

The effect of ultrasound exposure time on the physicochemical characteristics of SPI is shown in Table 1. The protein and ash contents of the SPI exposed to ultrasound for 15 and 30 min were not significantly ($P > 0.05$) different from the control (Table 1). However, the moisture content of SPI was significantly ($P < 0.05$) reduced from 5.08% (control) to 4.01% and 4.53% when samples were exposed to ultrasound for 15 and 30 min, respectively. The lower moisture content of SPI treated with ultrasound was due to a higher effect of compressions and expansions induced by the sound waves passing through the food medium, which make moisture removal easier (AWAD *et al*., 2012).
Bulk density is a property used to characterize powder products. It is of great importance for economical and functional reasons, for example, for reducing packaging costs, which depends on the combined effects of interrelated factors, like particle size, number of

contact points, and intensity of attractive inter-particle forces (PIORNOS *et al*., 2015). As shown in Table 1, the exposure to ultrasound of SPI significantly decreased the bulk density 4.9–6.5% as compared to the control. Such a decrease in the bulk density was due to the samples being treated with ultrasound and freeze drying, as these samples had larger and more heterogeneous structures in protein isolates in comparison with the control (HU *et al*., 2013; RESENDIZ-VAZQUEZ *et al*., 2017).

The a_w of a food system is a thermodynamic property, which is defined as the ratio of water vapor pressure of food to the saturated water vapor pressure at a given temperature. It is considered to be a good quality indicator for the safety and stability of foods with respect to microbial growth and biochemical reactions (TADAPANENI *et al*., 2017). The a_w of SPI exposed for 15 and 30 min to ultrasound was lower than that of the control (Table 1). As discussed previously, sound waves passing through the food medium make moisture removal easier, which also influenced the reduction in a_w of SPI as was observed in this study. However, both the control and the SPI exposed to ultrasound had values of a_w below a limiting level to ensure microbial stability, because it is generally accepted that no microbial growth will occur at $a_w < 0.66$ (ULLOA *et al*., 2015).

Table 1. Effect of ultrasound exposure time on the physicochemical properties of safflower protein isolate**.**

Property	Ultrasound exposure time (min)		
	0 (control)	15	30
Protein content (%)	87.54±0.52	88.17±0.13	87.03±1.01
Ash content (%)	6.92±0.11	6.88±0.12	6.81±0.13
Moisture content (%)	5.08^{a}±0.13	4.01^{c}±0.12	4.53^{b}±0.11
Bulk density (g mL^{-1})	0.61^{a}±0.1	0.57^{b}±0.1	0.58^{b}±0.1
Water activity	0.341^{a}±0.002	0.286^{c}±0.007	0.300^{b}±0.004
Color			
*L** (lightness)	60.62±0.11	60.35±0.19	60.19±0.21
*a** (redness-greenness)	4.21±0.06	4.23±0.03	4.18±0.08
*b** (yellowness-blueness)	17.45±0.29	17.41±0.17	17.77±0.25
Δ*E* (color difference)	36.70±0.17	36.94±0.21	37.22±0.27

Values are mean±standard deviation of three determinations. Values followed by different superscript letters in the same line are significantly different ($P < 0.05$).

The color characteristics of SPI exposed to ultrasound for 15 and 30 min in comparison with the control treatment are shown in Table 1. There were not significant ($P > 0.05$) differences in L^*, a^*, b^*, and ΔE values among samples of control SPI and those exposed to ultrasound. The effect of ultrasound on color in food depends on intrinsic characteristics of food and ultrasound conditions (BI *et al*., 2015). ADEKUNTE *et al*. (2010) reported a decrease in L^*, a^* and b^* values of tomato juice after sonication due to the degradation of lycopene. CHENG *et al*. (2007) found that guava juice treated by ultrasound for 30 min showed a significant change in the ΔE value due to a decrease in L^* value and an increase in a^* and b^* values. VALERO *et al*. (2007) found that ultrasound treatments had no significant effect on color scoring in orange juice. The degradation of color might be due to the effect of cavitation of ultrasound that could induce both chemical (e.g., by generation of radicals) and mechanical degradation of biomolecules (ADEKUNTE *et al*., 2010); however, such a phenomenon was not observed in the SPI exposed to ultrasound in this study. In general, a similar behavior on the effect of ultrasound exposure time on the

physicochemical properties of safflower protein isolate of this study was reported by FLORES-JIMÉNEZ *et al*. (2019) for canola protein isolate**.**
Fig. 1 shows a set of SEM images of control SPI (A) and SPI exposed to ultrasound for 15 (B) and 30 min (C). It was observed that samples B and C obtained after ultrasonic treatments and freeze-drying had larger and more heterogeneous structures than sample A (untreated SPI). These results might have been caused by ultrasound treatment inducing the unfolding of proteins and increasing the surface hydrophobic groups of the SPI molecules (HU *et al*., 2013; JIANG *et al*., 2014).

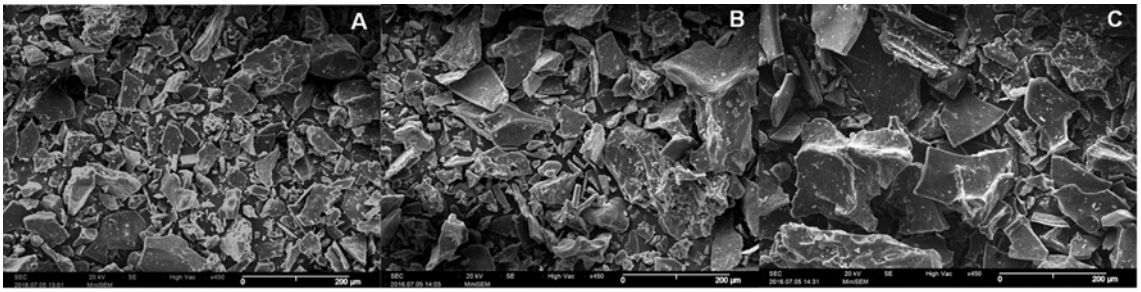


Figure 1. Effect of ultrasound exposure time (control = A, 15 min = B and 30 min = C) on the microstructure of safflower protein isolate.

3.2. Functional properties

Solubility is one of the most important functional attributes of proteins because it affects the texture, color, and the sensory properties of products containing them. It is closely associated with water retention capacity and other physicochemical and functional properties. Solubility of proteins depends on the composition, molecular weight, and surface characteristics of constituent amino acids and environmental factors such as pH, temperature, and ionic strength, and it can be affected by some chemical and physical treatments (TIMILSENA *et al*., 2016).
Table 2 shows the effect ultrasound exposure time on the protein solubility of SPI at different pH values. Only at pH 6 and pH 7, did the SPI exposed to ultrasound for 30 min have a beneficial effect on protein solubility, increasing 9.7% and 3.7%, respectively, in comparison to the control. This increase in protein solubility may be due to the conformational change during ultrasonic treatment and formation of soluble protein aggregates from insoluble protein (HU *et al*., 2013). However, such an increase in protein solubility depends on ultrasonic conditions (JIANG *et al*., 2014), as well as the pH at which the proteins are solubilized, as was observed in this study. Besides, for safflower proteins, the solubility values were minimum at the pH value of 5.0, which is the isoelectric point of safflower proteins, for ultrasound treatments and control treatment.
The results of WAC and OAC by effect of ultrasound exposure time of SPI are shown in Table 3. The WAC decreased from an initial value of 2.14 g H_2O g^{-1} protein to 1.94 g H_2O g^{-1} protein after ultrasound treatment for 30 min. According to RESENDIZ-VAZQUEZ *et al*. (2017), the ultrasound treatment might denature the molecular structure of proteins and cause an increase in the hydrophobic surface, which can lead to low values of WAC, as was observed in proteins from jackfruit seeds. In contrast, the OAC of samples of SPI exposed to ultrasound for 15 and 30 min were significantly ($P < 0.05$) higher compared to the control SPI. The OAC increased from an initial value of 0.99 g oil g^{-1} protein to 1.19 g oil g^{-1} protein and 1.30 g oil g^{-1} after exposure to ultrasound for 15 and 30 min, respectively. The

increase of OAC in proteins might be attributed to the exposure of hydrophobic groups after ultrasound treatment (HIGUERA-BARRAZA *et al*., 2016; ZHOU *et al*., 2016), which allowed the physical entrapment of oil (MEINLSCHMIDT *et al*., 2016).

Table 2. Effect of ultrasound exposure time at different pH on protein solubility (%) of safflower protein isolate.

pH	Ultrasound exposure time (min)		
	0 (control)	15	30
2	90.95±0.68	87.22±1.43	88.10±0.06
3	81.06^{a}±0.68	65.32^{b}±0.68	67.16^{b}±0.69
4	17.98^{a}±0.68	12.59^{b}±0.01	13.73^{b}±0.01
5	14.57^{a}±0.01	12.11^{b}±0.66	13.20^{ab}±0.69
6	49.61^{b}±1.37	50.32^{b}±0.01	54.41^{a}±0.61
7	61.16^{b}±0.01	57.94^{c}±1.28	63.44^{a}±0.04
8	89.75^{a}±0.74	71.88^{b}±0.68	73.49^{b}±1.43
9	92.32^{a}±0.12	86.34^{b}±1.31	88.32^{ab}±1.38
10	95.72^{a}±0.68	90.26^{b}±1.37	93.00^{ab}±0.06

Values are mean±standard deviation of three determinations. Values followed by different superscript letters in the same line are significantly different ($P < 0.05$).

Table 3. Effect of ultrasound exposure time on water and oil absorption capacity of safflower protein isolate.

Property	Ultrasound exposure time (min)		
	0 (control)	15	30
Water absorption capacity (g H_2O g^{-1} protein)	2.14^{a}±0.03	2.15^{a}±0.03	1.94^{b}±0.02
Oil absorption capacity (g oil g^{-1} protein)	0.99^{c}±0.01	1.19^{b}±0.02	1.30^{a}±0.01

Values are mean±standard deviation of three determinations. Values followed by different superscript letters in the same line are significantly different ($P < 0.05$).

Proteins are of particular interest as emulsifying agents in food formulations such as frozen desserts, salad dressings, comminuted meats, mayonnaise, cake batters, milks, and coffee whiteners, due to their ability to adsorb and form viscoelastic films at oil-water interfaces (O'SULLIVAN *et al*., 2016b). According to the results presented in Fig. 2, ultrasound treatment had no effect on EA and ES of SPI. YANJUN *et al*. (2014) reported that ultrasound pretreatment increased the emulsifying activity index (EAI) and ES index of milk proteins. ZHOU *et al*. (2016) found that the ultrasound treatment increased or decreased EAI of soybean glycinin depending on the ionic strength. In another study, the EA of defatted rice bran protein concentrate was higher ($P < 0.05$) than that of the ultrasound treatment, but the ES was not significantly different ($P > 0.05$) from the control (CHITTAPALO and NOOMHORM, 2009). The emulsifying properties of food proteins depend upon the solubility, molecular flexibility, surface hydrophobicity, and stability of the protein structure (ZHOU *et al*., 2016), which can be modified or not when are exposed to different ultrasound conditions as was observed in this study.

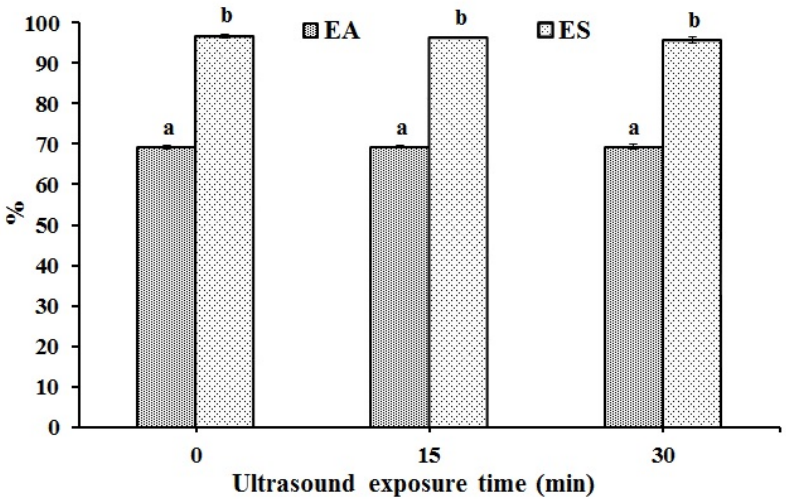


Figure 2. Effect of ultrasound exposure time on the emulsifying activity (EA) and stability (ES) of safflower protein isolate.

Gel properties are important functional characteristics of protein isolates, which are widely used as gelling agents to improve the texture and water holding capacity of meat products. Gelation is often an aggregation of denatured proteins, which involves the formation of a network, retaining significant amounts of water and transforming liquid sample to solid, which exhibits a certain degree of order where both covalent and non-covalent interactions are involved (CHEN *et al*., 2016). LGC indicates the gelling capacity of protein. Fig. 3 shows that the SPI exposed to ultrasound for 15 and 30 min at pH 2-10 had no effect on LGC, except for the ultrasound treatment of 30 min at pH 6, where the LGC increased from 6.0% (w/w) to 8.0% (w/w), which implies a reduction of the gelling capacity. The behavior of the LGC of SPI by effect of pH in this study was similar to that showed for lupin (PIORNOS *et al*., 2015), jackfruit (RESENDIZ-VAZQUEZ *et al*., 2017), and cashew nut shell (YULIANA *et al*., 2014) protein isolates.

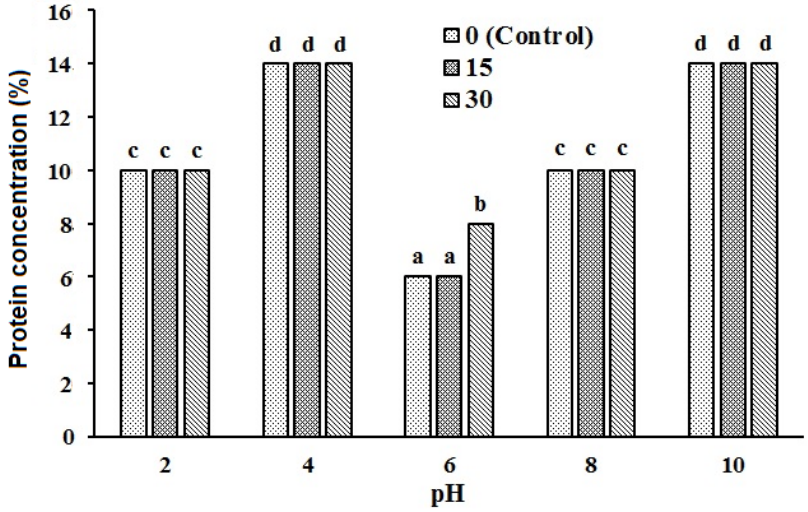


Figure 3. Effect of ultrasound exposure time on the last gelation concentration (LGC) of safflower protein isolate.

The FC and FS of protein isolates are functional properties that determine their application in food systems, where aeration and overrun is required (e.g. baked foods, whipped toppings and ice cream mixes) (SHEVKANI *et al.*, 2015). Because of surface-active properties, the proteins form foam when they are whipped (MALIK *et al.*, 2017). According to the results obtained in this study, the exposure to ultrasound for 15 and 30 min did not modify the FC and FS of SPI with respect to the control (Fig. 4).

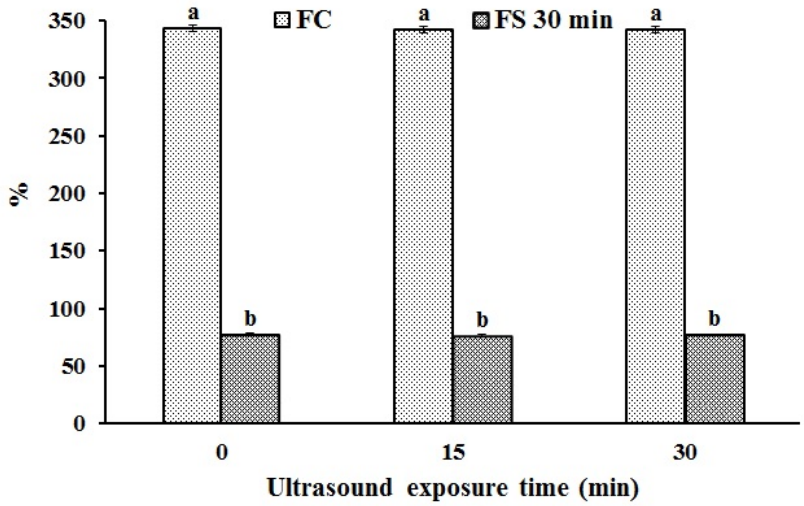


Figure 4. Effect of ultrasound exposure time on the foaming capacity (FC) and foam stability (FS) of safflower protein isolate.

Some reports showed that FC and FE of protein isolates from whey (JAMBRAK *et al.*, 2008) and soy (ZHANG *et al.*, 2014) were improved after ultrasound treatment for both 20 kHz and 40 kHz treatments, but no effect in foaming at 500 kHz treatment was observed, as occurred for SPI in this study. The improvement of the foaming properties in protein suspensions by ultrasound may be due to protein partial denaturation, which causes a higher air-water diffusion interface due to an increase in cohesiveness and flexibility of the foams (HIGUERA-BARRAZA *et al.*, 2016).

3.3. Amino acid composition and protein nutritive quality

Amino acid composition and nutritive quality of proteins in terms of CS and PER of SPI exposed to the ultrasound treatments in comparison with the control (Table 4) were not significantly different ($P < 0.05$). Of all the amino acids present in SPI, half corresponded to essential amino acids. On the other hand, glutamic acid was the amino acid with higher concentration in the SPI, as well as in the hempseed, soy (WANG *et al.*, 2008), and pennycress protein isolates (HOJILLA-EVANGELISTA *et al.*, 2014). According to amino acid requirements for adults (FAO/WHO, 1991), the first limiting amino acid of SPI was lysine. Therefore lysine was considered for calculating CS values for the SPI exposed to ultrasound for 15 min, 30 min, and the control, which were 48.4, 48.2, and 48.9 respectively, and were not significantly different ($P > 0.05$) from one another (Table 4).

Table 4. Effect of ultrasound exposure time on the composition of amino acid and protein quality of safflower protein isolate.

Parameter	Amino acid composition (g/16 g N) Ultrasound exposure time (min)			Reference for adults (FAO/WHO, 1991)
	0 (Control)	15	30	
Essential amino acid				
Lysine	2.69±0.08	2.66±0.15	2.65±0.05	5.5
Threonine	5.08±0.25	4.78±0.21	4.31±0.57	4.0
Valine	2.93±0.09	3.01±0.44	2.70±0.14	5.0
Methionine+cysteine	2.13±0.01	1.93±0.37	2.21±0.14	3.5
Cysteine				
Isoleucine	2.25±0.46	2.36±0.10	2.20±0.20	4.0
Leucine	6.39±0.94	7.06±0.36	6.57±0.14	7.0
Phenylalanine+tyrosine	4.10±0.94	4.16±1.12	3.56±0.60	6.0
Tyrosine	2.58±0.10	2.97±0.73	2.40±0.20	
Tryptophan	ND	ND	ND	
Total essential amino acids	25.58±0.70	25.96±1.10	24.19±0.80	
No essential amino acid				
Histidine	3.84±0.05	3.42±0.32	4.09±0.46	
Arginine	18.65±1.08	18.57±0.74	19.08±0.56	
Aspartic acid	6.78±0.57	6.73±0.41	6.60±0.48	
Serine	8.25±0.58	7.20±0.51	7.99±0.60	
Glutamic acid	20.96±0.86	22.28±0.73	21.17±0.83	
Glycine	8.83±0.52	9.09±0.58	9.61±0.26	
Alanine	7.11±0.37	6.75±0.55	7.27±0.86	
Total non-essential amino acid	74.42±0.81	74.04±0.75	75.81±0.90	
Nutritive quality				
Chemical score	48.9±1.53	48.4±2.7	48.2±0.83	
PER	2.16±0.22	2.42±0.20	2.26±0.04	

Values are mean±standard deviation of three determinations. Values followed by different superscript letters in the same line are significantly different ($P < 0.05$). ND = Not determined. PER = Predicted protein efficiency ratio.

With respect to the PER, the values obtained for the SPI exposed to ultrasound for 15 min, 30 min, and the control were not significantly different ($P < 0.05$) from one another and had values of 2.42, 2.26, and 2.16, respectively, which were higher than the values 2.14 and 2.04 for desi chickpea and soy protein isolates, respectively (WANG *et al*., 2010).

4. CONCLUSIONS

Application of ultrasound at 130 W and 40 kHz for 30 min to SPI increased the protein solubility at pH range of 6-7 and the OAC. It was demonstrated that the ultrasound did not affect the color parameters of L^*, a^*, b^* and ΔE, or the amino acid composition and nutritional quality of proteins of SPI. The improvement of the OAC of SPI could benefit its

application in the food industry for ground meat formulations, meat substitutes and extenders, doughnuts, baked goods, and soups.

ACKNOWLEDGMENTS

The authors wish to thank CONACYT-Mexico for the scholarship granted to QFB Minerva Marisol Zúñiga-Salcedo during her master's studies.

REFERENCES

Adekunte A.O., Tiwari B.K., Cullen P.J., Scannell A.G.M. and O'Donnell C. 2010. Effect of sonication on colour, ascorbic acid and yeast inactivation in tomato juice. Food Chem. 122:500-507.

AOAC.1995. Official Methods of Analysis. Association of Official Analytical Chemists, Arlington, USA.

Awad T.S., Moharram H.A., Shaltout O.E., Asker D. and Youssef M.M. 2012. Applications of ultrasound in analysis, processing and quality control of food: A review. Food Res. Int. 48:410-427.

Benelhadj S., Gharsallaoui A., Degraeve P., Attia H. and Ghorbel D. 2016. Effect of pH on the functional properties of *Arthrospira* (Spirulina) *platensis* protein isolate. Food Chem. 194:1056-1063.

Bi X., Hemar Y., Balaban M.O. and Liao X. 2015. The effect of ultrasound on particle size, color, viscosity and polyphenol oxidase activity of diluted avocado puree. Ultrason. Sonochem. 27:567-575.

Chen Z., Shi X., Xu J., Du Y., Yao M. and Guo S. 2016. Gel properties of SPI modified by enzymatic cross-linking during frozen storage. Food Hydrocoll. 56:445-452.

Cheng L.H., Soh C.Y., Liew S.C. and Teh F.F. 2007. Effects of sonication and carbonation on guava juice quality. Food Chem. 104:1396-1401.

Chittapalo T. and Noomhorm A. 2009. Ultrasonic assisted alkali extraction of protein from defatted rice bran and properties of the protein concentrates. Int. J. Food Sci. Technol. 44, 1843-1849.

Deng Q., Wang L., Wei F., Xie B., Huang F., Huang W., Shi J., Huang Q., Tian B. and Xue S. 2011. Functional properties of protein isolates, globulin and albumin extracted from *Ginkgo biloba* seeds. Food Chem. 124:1458-1465.

FAO. 2018. FAO Statistical Database. Food and Agriculture Organization. www.fao.org/faostat/es/#data/QC (accessed 13.06.18).

FAO/WHO. 1991. Protein Quality Evaluation. Rome, Food and Agriculture Organization.

Flores-Jiménez, N. T., Ulloa, J.A., Urías Silvas, J. E., Ramírez Ramírez, J. C., Rosas Ulloa, P., Bautista Rosales, P. U., Silva Carrillo, Y. and Gutiérrez Leyva, R. 2019. Effect of high-intensity ultrasound on the compositional, physicochemical, biochemical, functional and structural properties of canola (*Brassica napus* L.) protein isolate. Food Res. Int. In press. DOI: doi.org/10.1016/j.foodres.2019.01.025

Higuera-Barraza O.A., Del Toro-Sanchez C.L., Ruiz-Cruz S. and Márquez-Ríos, E. 2016. Effects of high-energy ultrasound on the functional properties of proteins. Ultrason. Sonochem. 31:558-562.

Hojilla-Evangelista M.P., Selling G.W., Berhow M.A. and Evangelista R.L. 2014. Preparation, composition and functional properties of pennycress (*Thlaspi arvense* L.) seed protein isolates. Ind. Crops Prod. 55:173-179.

Hu H., Wu J., Li-Chan E.C., Zhu, L. Zhang, F., Xu X., Fan G., Wang L., Huang X. and Pan, S. 2013. Effects of ultrasound on structural and physical properties of soy protein isolate (SPI) dispersions. Food Hydrocoll. 30:647-655.

Hussain M.I., Dionyssia-Angeliki L., Farooq M., Nikoloudakis N. and Khalid N. 2016. Salt and drought stresses in safflower:a review. Agron. Sustain. Dev. 36:1-31.

Jambrak A.R., Lelas V., Mason T.J., Kresic G. and Badanjak M. 2009. Physical properties of ultrasound treated soy proteins. J. Food Eng. 93:386-393.

Jambrak A.R., Mason T.J., Lelas V., Herceg Z. and Herceg L. 2008. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. J. Food Eng. 86:281-287.

Jambrak A. R., Mason T. J., Lelas V., Paniwnyk L. and Herceg, Z. 2014. Effect of ultrasound treatment on particle size and molecular weight of whey proteins. J. Food Eng. 121:15-23.

Jiang L., Wang J., Jiang Y.L., Wang J., Li Y., Wang Z., Chen Y., Ma W., Qi B., and Zhang, M. 2014. Effects of ultrasound on the structure and physical properties of black bean protein isolates. Food Res. Int. 62:595-601.

Liu L., Guan LL., Wu W., and Wang L. 2016. A review of fatty acids and genetic characterization of safflower (*Carthamus tinctorius* L.) seed oil. Organic Chem. Curr. Res. 5:160.

Malik M. A., Sharma H. K., and Saini C. S. 2017. High intensity ultrasound treatment of protein isolate extracted from dephenolized sunflower meal:Effect on physicochemical and functional properties. Ultrason. Sonochem. 39:511-519.

Meinlschmidt P., Sussmann D., Schweiggert-Weisz U. and Eisner P. 2016. Enzymatic treatment of soy protein isolates:effects on the potential allergenicity, technofunctionality, and sensory properties. Food Sci. Nutr. 4:11-23.

O'Sullivan J., Murray B., Flynn C. and Norton I. 2016a. The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. Food Hydrocoll. 53:141-154.

O'Sullivan J., Park M. and Beevers J. 2016b. The effect of ultrasound upon the physicochemical and emulsifying properties of wheat and soy protein isolates. J. Cereal Sci. 69:77-84.

Paredes-López O. and Ordorica-Falomir C. 1986. Production of safflower protein isolates:composition, yield and protein quality. J. Sci. Food Agric. 37:1097-1103.

Peiretti P.G. 2017. Nutritional aspects and potential uses of safflower (*Carthamus tinctorius* L.) in livestock. Ch. 1. In: "Agricultural Research Updates". Vol. 19. P. Gorawala and S. Mandhatri S. (Ed.), pp. 3-22. Nova Science Publishers, Inc., NY.

Piornos J.A., Burgos-Díaz C., Ogura T., Morales E., Rubilar M., Maureira-Butler I. and Salvo-Garrido, H. 2015. Functional and physicochemical properties of a protein isolate from *Alu*prot-CGNA:A novel protein-rich lupin variety (*Lupinus luteus*). Food Res. Int. 76:719-724.

Resendiz-Vazquez J.A., Ulloa J.A., Urías-Silvas J.E., Bautista-Rosales P.U., Ramírez-Ramírez J.C., Rosas-Ulloa P. and González-Torres L. 2017. Effect of high-intensity ultrasound on the technofunctional properties and structure of jackfruit (*Artocarpus heterophyllus*) seed protein isolate. Ultrason. Sonochem. 37:436-444.

Shevkani K., Singh N., Kaur A. and Rana, J.C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates:A comparative study. Food Hydrocoll. 43:679-689.

Stone A.K., Karalash A., Tyler R.T., Warkentin T.D. and Nickerson M.T. 2015. Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. Food Res. Int. 76:31-38.

Tadapaneni R.K., Syamaladevi R.M., Villa-Rojas R. and Tang J. 2017. Design of a novel test cell to study the influence of water activity on the thermal resistance of *Salmonella* in low-moisture foods. J. Food Eng. 208:48-56.

Timilsena Y.P., Adhikaria R., Barrow C.J. and Adhikaria B. 2016. Physicochemical and functional properties of protein isolate produced from Australian chia seeds. Food Chem. 212:648-656.

Tiril S.U. and Kerim M. 2015. Evaluation of safflower meal as a protein source in diets of rainbow trout [*Oncorhynchus mykiss*, Walbaum, 1792)]. J. Appl. Ichthyol. 31:895-899.

Ulloa J.A., Ibarra-Zavala S.J., Ramírez-Salas S.P., Rosas-Ulloa P., Ramírez-Ramírez J.C. and Ulloa-Rangel B.E. 2015. Chemical, physicochemical, nutritional, microbiological, sensory and rehydration characteristics of instant whole beans (*Phaseolus vulgaris*). Food Technol. Biotech. 53:48-56.

Ulloa J.A., Rosas-Ulloa P. and Ulloa-Rangel B.E. 2011. Physicochemical and functional properties of a protein isolate produced from safflower (*Carthamus tinctorius L.*) meal by ultrafiltration. J. Sci. Food Agric. 91:572-577.

Valero M., Recrosio N., Saura D., Muñoz N., Martí N. and Lizama, V. 2007. Effects of ultrasonic treatments in orange juice processing. J. Food Eng. 80:509-516.

Vázquez-Ortiz F.A., Caire G., Higuera-Ciapara I. and Hernández G. 1995. High performance liquid chromatographic determination of free amino acids in shrimp. J. Liq. Chromatogr. R T 18:2059-2068.

Wang X., Gao W., Zhang J., Zhang H., Li J., He X. and Ma H. 2010. Subunit, amino acid composition and in vitro digestibility of protein isolates from Chinese kabuli and desi chickpea (*Cicer arietinum* L.) cultivars. Food Res. Int. 43:567-572.

Wang X.S., Tang C.H., Yang X.Q. and Gao W.R. 2008. Characterization, amino acid composition and in vitro digestibility of hemp (*Cannabis sativa* L.) proteins. Food Chem. 107:11-18.

Xiong T., Xiong W., Ge M., Xia, J., Li, B. and Chen, Y. 2018. Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. Food Res. Int. 109:260-267.

Yanjun S., Jianhang C., Shuwen Z., Hongjuan L., Jing L., Lu L., Uluko H., Yanling S., Wenming C., Wupeng G. and Jiaping L. 2014. Effect of power ultrasound pre-treatment on the physical and functional properties of reconstituted milk protein concentrate. J. Food Eng. 124:11-18.

Yuliana M., Truong C.T., Huynh L.H., Ho Q.P. and Ju, Y. 2014. Isolation and characterization of protein isolated from defatted cashew nut shell: Influence of pH and NaCl on solubility and functional properties. LWT-Food Sci. Technol. 55:621-626.

Zhang L., Jin Y., Xie Y., Wu X. and Wu T. 2014. Releasing polysaccharide and protein from yeast cells by ultrasound: Selectivity and effects of processing parameters. Ultrason. Sonochem. 21:576-581.

Zhang L., Zheng P., Shen, K., Cai X., Zheng B. and Miao, S. 2018. Influence of ultrasound-assisted alkali treatment on the structural properties and functionalities of rice protein. J. Cereal Sci. 79:204-209.

Zhou M., Liu J., ZhouY., Huang J., Liu F., Pan S. and Hu H. 2016. Effect of high intensity ultrasound on physicochemical and functional properties of soybean glycinin at different ionic strengths. Innov. Food Sci. Emerg. Technol. 34:205-213.

Paper Received November 13, 2018 Accepted March 12, 2019

PAPER

THE IMPACT OF LEMON JUICE ON THE MARINATION OF ANCHOVY (*ENGRAULIS ENCRASICOLUS*): CHEMICAL, MICROBIOLOGICAL AND SENSORY CHANGES

V. ŠIMAT*[1], A. MIĆUNOVIĆ[1], T. BOGDANOVIĆ[2], I. LISTEŠ[2], I. GENERALIĆ MEKINIĆ[3], I. HAMED[1] and D. SKROZA[3]

[1]University Department of Marine Studies, University of Split, R. Boškovića 37, 21000 Split, Croatia

[2]Regional Veterinary Institute Split, Croatian Veterinary Institute, Poljička cesta 33, 21000 Split, Croatia

[3]Department of Food Technology and Biotechnology Faculty of Chemistry and Technology, University of Split, R. Boškovića 35, 21000 Split, Croatia

*Corresponding author: Tel.: +38521510192

E-mail address: vida@unist.hr

ABSTRACT

This paper investigates the qualitative changes (physical, biochemical, microbiological and sensory) in anchovy fillets during the maturation, in a marinade made from fresh lemon juice and olive oil with the addition of acetic acid. Marination was carried out at 4°C and it required 7 days until sensory acceptability of the product was achieved. At marination end point, a pH value of 4.2, a_w of 0.85 and NaCl content of 4% were measured for the products. Volatile and non-volatile amines were extracted from the marinated anchovy fillets, and the detected concentration implies that the accumulation of these components during processing was weak (<14 mg TVB-N/100 g, <0.5 mg TMA/100 g, <30 mg biogenic amines/kg). The lemon juice showed good preservative effect, it reduced the bacterial count in the products, while the addition of 0.5% acetic acid improved the sensory attributes of the product.

Keywords: marination, anchovies, quality control, TVB-N, TMA, biogenic amines, lemon juice

1. INTRODUCTION

Marinating is one of the oldest chemical preservation methods used for fish. During the process, the preservative effect is achieved by different concentrations of organic acids and salt. This effect is based on reducing the product's pH value and water activity, and increasing the salt content, resulting in a retardation of bacterial activity and enzymes. Marination promotes the structural characteristics of fish flesh, giving the product a characteristic flavour and aroma, and tender texture, but a limited shelf life of 1 to 6 months in refrigerated storage. Cold marinades achieve fish ripening in acetic acid and salt solution without any heat treatment. After maturation has been completed, the product is packaged in oil, sauces or a new salt/acid solution with the addition of spices (MCLAY, 1972). The quality of the final product is directly influenced by the initial quality of the raw material. In promoting the Mediterranean diet as a healthy way of living, minimally processed seafood products have become extremely popular. Thus, there is a growing interest in marinated fish products, and especially traditional carpaccio-like products (cold-marinated fish in fruit juice citric acid and salt). So far, the time required for the maturation of fish fillets in citric juice has been estimated empirically. Depending on the thickness of the raw material used for marination, it is generally considered that thin slices of bigger fishes (such as tuna) are ready for consumption after two hours, while the fillets of smaller fish (anchovy or sardine) need at least two days of maturation in a lemon juice-oil mixture. There is a real lack of information on how the properties of fillets change during maturation in citric juice, which would be useful for the industrial production of carpaccio-like products. In food preparation processes, citric acid has shown a positive effect on the texture and colour of raw materials (TOPUZ *et al*., 2016). Fresh lemon juice appears to be a good natural choice for marinating processes. It contains about 3% sugars, ascorbic acid, minerals such as potassium and phenolics, all of which have health-promoting properties and antioxidant effects (GONZÁLEZ-MOLINA *et al*., 2009).

The only European representative of the Engraulidae family, the anchovy (*Engraulis encrasicolus*, L.), belongs to the group of small pelagic fish caught along the Mediterranean shores and widely distributed in the Adriatic Sea. They are characterized by a gentler texture and lower fat content than sardines (ŠIMAT and BOGDANOVIĆ, 2012), which makes them a good raw material for minimally processed fish products. On the other hand, anchovies present a risk of infestation by *Anisakis* spp. (MLADINEO *et al*., 2012; ŠIMAT *et al*., 2015); thus, they must be frozen prior to processing. The marinating of thawed fish presents a challenge with respect to fillet texture, juiciness and colour, since the freezing process and storage in a freezer result in denaturation of muscle proteins. Anchovy carpaccio is a minimally processed, natural fish product traditionally prepared from fish fillets, marinated in lemon or lime juice and salt, with the addition of oil and spices. During maturation, fillets become glossy white, juicy and tender with a gentle acidic taste. The blood stains on the belly part of the fillet appear on the fillet as a result of the freezing process and ruin the appearance and overall acceptability of the product; therefore, the quality of the raw material is of key importance for the production of high quality products. Since lightly preserved fish products, in general, have a low salt content (<6 % NaCl w/w) and their pH value usually exceeds 5.0, carpaccio-like products are on the very edge of that definition; they have a low salt content and a pH value below 5. After processing, these products are stored at refrigerated temperatures (4-6°C) and consumed without prior heating.

Consequently, this research was conducted in order to investigate the optimal duration of maturation as well as qualitative changes (physical, chemical, microbiological and sensory) in anchovy fillets during the maturation process in a marinade made from fresh lemon juice and olive oil. Additionally, the effect of the addition of acetic acid (final

concentration of 0.5% and 1% in the marinating bath) to the lemon juice and olive oil marinade was investigated. The aim was to determine the time required for completion of the maturation process, and the physical, chemical and microbiological changes underlying the maturation process. The objectives of the maturation process were to obtain sensory acceptability of the product, glassy-white colour and soft fillet texture, characteristic flavour, satisfactory salt content and decrease in the pH and water activity values, and to investigate the preservative effect of lemon juice.

2. MATERIAL AND METHODS

2.1. Raw material preparation and marinating

Anchovies used for marinating were caught in southern part of the Adriatic Sea (FAO fishing area 37.2.1.), placed on ice onboard, transported to a local factory where they were frozen in a brine-air blast freezer and stored at -20°C for four months.
The traditional method of preparing carpaccio marinade was somewhat modified to attain a better quality product. The fish were thawed, beheaded, gutted and filleted, and the fillets transferred to a NaCl (200 g/L) solution (ratio 1:1) for 22 minutes to remove excess blood, intestines or scales. The fillets were then washed in icy water and drained. Marination was carried out in round polypropylene containers, in three lots. The first lot, 140 g of brined filets, were packed with 70 mL fresh lemon juice and 70 mL olive oil per container. Lots two and three were processed in the same way, with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath, respectively. The containers were closed, stored at 4±2°C and shaken several times before the first sampling for analyses. Preliminary investigation results were used for preparation of the sampling plan; thus, five randomly chosen containers were sampled on days 2 and 7 in order to investigate physical, chemical, microbiological and sensory qualitative changes.
Before the analyses, the liquid was drained from all the containers and the fillets were homogenized using a laboratory homogenizer (KINEMATICA Microtron MB 550, Switzerland). The homogenized mass of three containers was used for physical and chemical analyses. The remaining two containers were used for microbiological analysis and sensory assessment.

2.2. Chemical composition analyses

The chemical composition of anchovy fillets was determined as moisture content by gravimetric method, crude protein (AOAC, 2000), crude lipid (BLIGH and DYER, 1959) and crude ash (AOAC, 2000). All analyses were repeated six times and the results were presented on a wet weight basis (percentage).

2.3. Physical and chemical analysis

For the pH measurements, 10 g of fillets were homogenized with 10 mL distilled water. The pH value of the fish homogenate was measured using a digital Iskra pH-meter MA 5705 with a combined glass electrode (Iskra Model 0101, Slovenia). Water activity (a_w) was determined at room temperature using an a_w meter (Rotronic AG, HygroPalm AW1-set 40, RotronicInstrument Corp., Basserdorf, Germany). Total volatile basic nitrogen (TVB-N, mg/100 g) and trimethylamine (TMA, mg/100 g) were analysed according to ŠIMAT *et al.* (2009) using a Kjeldahl distillation unit (model B-324, Büchi, Switzerland) and automatic titration (Methrom 702 SET / MET titrino). The 2-thiobarbituric acid (TBA, mg

malonaldehyde/kg) assay, as an index of lipid oxidation, was performed according to the procedure of VYNCKE (1970) and LEMON (1975).
The total of eight biogenic amines, namely β-phenyletylamine, cadaverine, histamine, putrescine, spermidine, spermine, tryptamine and tyramine, were determined in samples using high-performance liquid chromatography (HPLC), according to the method described by EEROLA *et al*. (1993) and modified for smaller particle size column (ŠIMAT and DALGAARD, 2011). The standards of biogenic amines that were used were obtained from Sigma-Aldrich (St. Louis, MO, USA). The HPLC analyses were carried out using a classical Agilent 1200 Series LC system (Agilent Technologies Inc., Waldbronn, Germany) and the separation was performed on a C18 Agilent column (Zorbax Eclipse XDB 50 × 4.6 mm ID, 1.8 μm particle size). All analyses were done in triplicate and the results expressed as mg/kg of the sample.

2.4. Microbiological analysis

Samples and decimal dilutions were prepared according to the method described by ÖZOGUL *et al*. (2006). For all microbial counts, 25 g of anchovy fillet was taken and homogenized with 225 mL of buffered peptone water (BPW, Biolife Italiana). A series of decimal dilutions were obtained and inoculated on appropriate media. Total viable count was enumerated on Plate Count Agar (PCA, Biolife Italiana) at 30°C for 24-48 h using the pour plate method. Psychrotrophic microorganisms were also enumerated using PCA as the medium. Plates were incubated at 7°C for 10 days. The lactic acid bacteria count was determined using the pour plate method on De Man, Rogosa and Sharpe Agar (Biolife, Italiana) after incubation at 30°C for 72 hours. *Enterobacteriaceae* were enumerated from Violet Red Bile Glucose Agar (VRBGA, Biolife Italiana) after incubation at 37°C for 48 hours. The number of colony forming units (CFU) per gram of sample was calculated from the number of colonies obtained on selected plates retained from three successive dilutions and the results were presented as log CFU/g. Each dilution was poured in triplicate.

2.5. Sensory analysis

The sensory attributes of the marinated fish fillets were evaluated by a panel of 8 trained panellists according to the method described by SALLAM *et al*. (2007). The panellists, composed of MSc students, University staff and fish industry technologists, were educated on specific positive and negative sensory aspects of cold marinated fish. Every day, during seven evaluation days, fillet samples of two different products were served to each panellist in covered porcelain dishes coded with 3-digit randomly chosen numbers. An eight-point hedonic scoring scale was used for evaluation of the appearance (8 = extremely acceptable to 1 = extremely unacceptable), colour (8 = faint pink to 1 = deep brown), texture (8 = firm and consistent to 1 = extremely soft or extremely hard), odour (8 = characteristic saury odour to 1 = extreme off-odour), rancidity (8 = no rancidity to 1 = extremely rancid), juiciness and tenderness (8 = extremely juicy/tender to 1 = extremely dry/tough), sour and salty taste (8 = not sour/salty to 1 = extremely sour/salty), as well as flavour and aftertaste (8 = characteristic saury flavour to 1 = extreme off-flavour). A nine-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely) was used for evaluation of overall acceptability. The maturation process was performed at refrigerated temperature (4±2°C) and it was considered complete when sensory acceptability of the product was satisfactory (glassy-white colour, soft fillet texture, favourable effect in terms of odour and flavour). This was

achieved after seven days of maturation (preliminary study, data not shown). Thus, the sensory analysis 2-day data (generally accepted end of maturation) and 7-day data (end of maturation based on preliminary study) are presented in this paper.

2.6. Statistical analyses

In order to establish significant differences between data obtained for different marinating baths (with and without acetic acid), one-way variance analysis (ANOVA) was performed using the Statgraphics Centurion XVI 16.1 (StatPoint technologies, Inc.) statistical package, and Fisher's least significant difference (LSD) procedure at p value of <0.05.

3. RESULTS AND DISCUSSION

Taking into account the economic value of anchovy and increasing production of lightly preserved and uncooked fish products, knowledge on quality changes during processing will ensure standardization of the production processes as well as general safety requirements for consumers. In marinated products, a synergic effect of salt and acid content ensures specific product characteristics, such as taste, appearance or texture, but also environmental conditions that delay the growth of microorganisms causing spoilage. The addition of 0.3% acetic acid is considered to have a bactericidal effect, especially against Gram-negative bacteria (RAY and BHUNIA, 2008). Carpaccio products are prepared from raw fish fillets, thinly sliced and marinated in lemon juice and olive oil. Anchovy is a small pelagic fish whose fillets are thin and suitable for this type of product; however, the European anchovy in the Adriatic Sea represents a moderately highly-infected paratenic host of *Anisakis pegreffii* (MLADINEO *et al*., 2012; ŠIMAT *et al*., 2015) and, therefore, freezing of the fish before the cold marinade processing is obligatory. During freezing and storage, fish changes such as protein denaturation and lipid oxidation may influence the marinating process, which means that the overall acceptability of such products is usually lower, the colour and texture being less appealing to consumers.
Table 1 shows the moisture, protein, lipid and ash content of thawed, brined and marinated anchovy fillets after two and seven days of maturation at 4±2°C. The moisture content in thawed fillet was lower than in raw fish (0.78%, data not shown) due to the combined brine/air blast system used for freezing the fish. It is an individually quick freezing technology that uses a brine solution chilled at -21°C for freezing and air blast freezer for glaze fixation and final freezing of the product. Small pelagic fish such as anchovy reach a temperature of -18°C in 8-9 minutes but this results in lower moisture and higher NaCl content (0.23% in raw material) than in fresh samples. The lipid content of anchovies used in this study was lower than in previous reports for this species (DUYAR and EKE, 2009; KOCATEPE and TURAN, 2012; ÖZOGUL *et al*., 2017). Atlantic or Black Sea representatives of this species, in general, have different proximate composition and higher lipid content than anchovies caught in the Adriatic Sea (ŠIMAT and BOGDANOVIĆ, 2012). The brining of the fillets also decreased moisture content and this remained relatively constant during the marinating process. The statistical analysis attempted to determine the interaction of the investigated parameters, i.e. whether the chemical composition was dependent on the particular stage of production as well as the acetic acid addition at the beginning (day 2) and at the end of the maturation process (day 7). The brining of the fish before the marinating process had a statistically significant effect ($p<0.05$) on the chemical composition of the fillets (Table 1). Both brining and marinating significantly affected the lipid content (Table 1) when compared to thawed fillets. According to the statistical analysis, the difference between three groups of marinated

products was found to be significant ($p < 0.05$). The protein and ash content was higher in marinated samples, but did not change significantly during the marinating process. For fillet brining, strong brine (200 g/L) was used to avoid swelling of the flesh (weak brine) or surface crystallisation of filets (if saturated brines are used).
The marinating process also significantly influenced the changes of all the investigated chemical parameters (Table 2).
Table 2 shows changes in the physical and chemical parameters of anchovy fillets during marination in lemon juice, and lemon juice with added acetic acid (0.5 and 1%). The results allow concluding that the brining step ensured satisfactory content of NaCl in the fillets (4.12%) while lemon juice ensured pH values of 4.2 in all marinated products. When the brining step (dipping the fillets in NaCl solution for 22 minutes) was applied to raw fresh fillets, the salt content was higher than 6% (data not shown), which makes the fillets too salty in sensory terms. The brining step made the thawed fillets whiter, hardened the consistency of the fillets by drawing some water out of them and achieved appropriate salinity of the product (Tables 1, 2 and 4). The addition of acetic acid did not affect the NaCl content at the end of maturation (7 days). In the literature, significantly higher salt contents were found in marinated fish products. KILINC and CAKLI (2005) marinated sardine (*Sardina pilchardus*) in a solution containing 7% acetic acid and 14% salt. Marination was preceded by brining of the fish in 10% aqueous salt solution. The authors reported a statistically significant change in the salt content during marination and storage of marinated sardines. On the first day of maturation, the salt content was 4.93% and after 22 days 7.12%. The marinating process used for *Engraulis anchoitae* (brined in 10% NaCl, marinated in a solution containing 3% acetic acid and 10% salt) resulted in 4.97% NaCl in the fish meat on the first day of storage (YEANNES and CASALES, 2008).
Citric acid has shown to be an excellent choice of natural additive for fish maturation and preservation as it controls the acidity of the product, softens the consistency of muscle, gives it a lighter colour and sour taste, and enhances the impact of conservation. The pH value of the prepared lemon juice marinade was 2.35±0.15. The addition of acetic acid in the proposed concentrations did not have any effect on the pH of the marinade or the fillets during maturation. The combined effect of aw (< 0.9) and pH (4.2) in marinated anchovies places these products in the inhibition zone of growth of pathogenic bacteria and many of the deteriorant microorganisms (CABRER *et al*. 2002). Low pH values of fish marinated in acetic acid and salt solutions are recorded in the scientific literature. The pH values of 3.9 were recorded in anchovies marinated with acetic acid (concentration of 50-100 g/L) in a study by POLIGNE and COLLIGAN (2000). On the 20th day of storage, the pH value reached its maximum (4.21) and thereafter remained constant for 60 days. GÖKOĞLU *et al*. (2009) recorded decreasing pH in fresh anchovy, from 6.3 to 4.57, during marination in 30 g acetic acid/L and 150 g NaCl/L. KILNIC and CAKLI (2004) marinated sardine (pH = 6.72) in a solution with a pH value of 3.86, while in the marinated fillet they achieved a pH value of 4.23 at the beginning and 4.11 at the end of maturation (22 days). During 7 days of maturation in a bath prepared from 30 g acetic acid/L, FUSELLI *et al*. (1994) obtained a pH value of 4.2 for *E. anchoita* fillets.
Freshness, deterioration of freshness, and spoilage of seafood products can be monitored over time through the dynamics and speed of changes in biochemical parameters (ÓLAFSDÓTTIR *et al*., 1997) by numerous biochemical tests that have been developed. TBA has been used as an indicator of lipid oxidation of fish through the determination of secondary lipid oxidation products generated by hydroperoxide decomposition, while TVB-N and TMA values are widely used as criteria of freshness and degree of spoilage of fish muscle (ÓLAFSDÓTTIR *et al*., 1997).

Table 1. Changes of proximate composition of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=6).

	Thawed fillet	Brined fillet	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
% Moisture	74.88±0.26 [a]	70.05±0.34 [b]	70.07±0.34 [b]	69.68±0.24 [c]	70.75±0.18 [b]	69.19±0.36 [c]	70.75±0.11 [b]	69.42±0.14 [c]
% Protein	20.11±0.11 [a]	20.89±0.12 [a]	23.32±0.06 [b]	23.14±0.10 [b]	23.47±0.12 [b]	23.41±0.21 [b]	21.47±0.12 [c]	21.49±0.18 [c]
% Lipid	2.79±0.14 [a]	2.32±0.12 [b]	1.68±0.10 [c]	1.69±0.06 [c]	1.54±0.05 [d]	1.52±0.06 [d]	1.74±0.05 [e]	1.71±0.06 [e]
% Ash	2.31±0.01 [a]	4.62±0.06 [b]	4.07±0.03 [c]	4.24±0.02 [d]	3.94±0.03 [c]	4.18±0.02 [d]	3.35 ±0.03 [e]	4.47±0.02 [b]

[a-e]mean value±standard deviation in the same raw followed by different superscript are significantly different (p<0.05).

Table 2. Changes in the physical and chemical parameters of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=6).

Chemical analyses	Thawed fillet	Brined fillet	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
pH	6.22±0.02 [a]	5.67±0.03 [b]	4.23±0.04 [c]	4.22±0.11 [c]	4.24±0.04 [c]	4.21±0.06 [c]	4.21±0.04 [c]	4.23±0.08 [c]
a_w	0.88±0.01 [a]	0.77±0.01 [b]	0.85±0.04 [c]	0.85±0.07 [c]	0.85±0.08 [c]	0.86±0.06 [c]	0.84±0.04 [c]	0.86±0.02 [c]
NaCl (%)	1.34±0.12 [a]	4.12±0.21 [b]	4.02±0.2 [c]	4.10±0.14 [b]	3.87±0.12 [d]	4.02±0.14 [c]	3.61±0.12 [e]	4.27±0.26 [f]
TBA (mg malonaldehyde/kg)	2.04±0.14 [a]	2.31±0.18 [b]	3.67±0.21 [c]	3.37±0.17 [d]	4.06±0.21 [e]	3.65±0.12 [c]	2.94±0.14 [e]	2.10±0.19 [a]
TVB-N (mg/100 g)	12.41±0.21 [a]	14.04±0.36 [b]	10.35±0.32 [c]	10.47±0.24 [c]	8.05±0.26 [d]	9.59±0.32 [e]	8.24±0.16 [d]	8.75±0.12 [d]
TMA(mg/100 g)	0.36±0.04 [a]	0.32±0.04 [a]	0.29±0.02 [b]	0.27±0.04 [b]	0.25±0.08 [c]	0.22±0.05 [d]	0.24±0.08 [c]	0.21±0.05 [d]

[a-f] mean value±standard deviation in the same raw followed by different superscript are significantly different (p<0.05).

Changes in biochemical parameters of anchovy fillet during marination in lemon juice, and lemon juice with the addition of 0.5 and 1% acetic acid are shown in Table 2. Regarding the TBA test, quality fish products are limited to 5 mg malonaldehyde (MDA)/kg, while the fish is considered safe for consummation up to a maximum level of 8 mg MDA/kg (SALLAM *et al*., 2007).

The TBA values of thawed and brined fillets were low, <2.5 mg MDA/kg indicating good quality of the raw material. Both brining and marination processes significantly (p<0.05) increased TBA (>3 mg MDA/kg) values, while over 7 days of maturation these values were slightly lower. The marination bath was prepared using fresh lemon juice and olive oil, which might influence the TBA values in marinated fillets. The data suggest that the TBA values of marinated fish are within the good quality limits after 7 days of maturation.

The TVB-N value is related to species, catching season and region, age and sex of the fish (KILINC and CAKLI, 2004). According to HUSS (1988), the TVB-N value in fresh fish is typically between 5 and 20 mg/100 g of flesh, whereas 30 to 35 mg/100 g is considered a limit of acceptability. In our study, the TVB-N value of thawed anchovies was 12.41 mg/100 g, while data reported in the literature ranged from 7.32 to 12.77 mg/100 g (PONS-SÁNCHEZ-CASCADO *et al*., 2005b; OLGUNOĞLU *et al*., 2009; ÖZOGUL *et al*., 2010; TOPUZ *et al*., 2016). After the brining step, the TVB-N value increased to 14.04 mg/100 g; however, two days in the lemon juice/olive oil mixture resulted in a significant reduction (p<0.05) of TVB-N values. Reduction of TVB-N through the marinating process has been reported in the literature and can be attributed to the action of acid and salt whereby the TVB-N components are extracted from marinated filets. KILINC and CAKLI (2004) reported a decrease in the amount of TVB, from 10.24 to 6.53 mg/100 g, during marination of sardine (*S. pilchardus*) in 7% acetic acid and 14% salt bath. AKSU *et al*. (1997) report the same for anchovy; and the value of TVB decreased from 8.7 in the raw material to 7.41 mg/100 g in the final product (6% oacetic acid, 14% NaCl). PONS-SÁNCHEZ-CASCADO *et al*. (2005a) detected a decrease of TVB-N, from 7.32 to 6.04 mg/100 g, during marination of anchovy in a wine vinegar marinade. In this study, lemon juice appeared to have a strong extractive effect on TVB-N components, which can be attributed to the presence of citric acid. During a shrimp marination process, a marination bath containing citric acid (2%) and salt (4%) resulted in a lower TVB-N value, from the initial 27.5 to as low as 7 mg/100 g shrimp flesh (CADUN *et al*., 2008). A similar lowering effect of lemon juice was observed for TMA, a non-protein fraction found in marine fish produced from the reduction of trimethylamine oxide by bacterial activity. The TMA values observed in thawed and brined fillet (0.36 and 0.32 mg/100 g) were significantly reduced during maturation (Table 2). Since TMA values in fresh fish were around 1 mg/100 g, and the limit of acceptability was found to be 5 mg/100 g (HUSS, 1988), the quality of the investigated fillets, in terms of TMA, was good and remained good during the entire maturation period. The range of TMA for small pelagic fish ranges from 0.49 to 3.21 mg/100 g (PONS-SÁNCHEZ-CASCADO *et al*., 2005b; KILINC and CAKLI, 2005; GÖKOĞLU *et al*., 2009). The content of TVB-N and TMA were not analysed in the marinade bath during maturation, but based on previous research conducted by PONS-SÁNCHEZ-CASCADO *et al*. (2005a). It can be assumed that lemon juice marinade has a strong extraction effect on the TVB-N and TMA components of anchovy fillets and, considering the low initial values, high levels should not be expected in such products. During the storage of marinated sardines in marinating baths with the addition of 2 and 4% acetic acid and 10% NaCl at 4°C, GÖKOĞLU *et al*. (2004) reported a statistically significant increase in the amount of TMA in the flesh of sardines; however, in both types of baths, this amount did not exceed 1.7 mg/100 g after 150 days of storage. Similarly, TMA did not exceed 2 mg/100 g in marinated anchovies (30 g/L acetic acid and 150 g/L salt) after several months of storage of the marinated anchovies in oil and tomato sauce at

a temperature of 4°C (GÖKOĞLU *et al*., 2009). KILINC and CAKLI (2004) recorded values lower than 2 mg TMA/100 g in marinated sardine flesh (7% acetic acid and 14% salt) during the marinating process (22 days). PONS-SÁNCHEZ-CASCADO *et al*. (2005a) recorded a slight increase in the amount of TMA after 3 months of storage of cold marinated anchovy in wine vinegar, but these quantities did not exceed 1 mg TMA/100 g of flesh.

The presence of biogenic amines (BAs) in seafood is involved in various toxicological reactions and can constitute a risk to consumer health. The accumulation of high levels of BAs is associated with a relevant growth (>7 log CFU/g) of decarboxylating microorganisms (GARDINI *et al*., 2016) and indicates a need for better hygiene and process control during production. The decarboxylation process can be catalyzed by naturally occurring endogenous amino acid decarboxylases present in animal or plant cells and by exogenous enzymes produced by various microorganisms under favourable conditions (HALÁSZ *et al*., 1994). There is no legal regulation on the BA content of foods (besides histamine); however, a profile of BA in foods provides a better overview of the quality of certain products. The accumulation of BAs during brining and marination of anchovy fillets is shown in Fig. 1. Out of eight BAs (β-phenyletylamine, cadaverine, histamine, putrescine, spermidine, spermine, tryptamine and tyramine) determined in this work, only cadaverine, spermine and tyramine were detected in thawed and marinated anchovy fillets. A total of 20.62 mg BA/kg was observed in thawed fillets, which corresponds to the quantities of BAs found in fresh/thawed anchovy reported in the literature (VECIANA-NOGUÉS *et al*., 1996; Pons-SÁNCHEZ-CASCADO *et al*., 2005a, ÖZOGUL *et al*., 2017). After the brining process, the cadaverine and spermine values remained unchanged while tyramine concentration increased from 4.32 to 14.86 mg/kg. This resulted in significantly higher (p<0.05) BA content (30.58 mg/kg). The lowest content of total BA during the marinating process was observed for the lemon juice/olive oil mixture, with 0.5% acetic acid, and was 15.22 mg/kg. Spermine was detected in small amounts (4-6 mg/kg) and its concentration remained unchanged during maturation. Together with spermidine (undetected in marinated filets), spermine is not related to bacterial spoilage but rather a physiological BA needed for cell growth (VECIANA-NOGUÉS *et al*., 1996). The increased levels of tyramine in anchovies during the marination process suggest that some enzymatic processes occur during lemon juice/olive oil marination that supports BA formation. In marinades, amino acid decarboxylation by bacterial enzymes is limited by the pH value, NaCl content and a_w value, which directly affect the growth of bacteria (PONS-SNÁCHEZ-CASCADO *et al*., 2005b). Furthermore, acidic conditions increase the activity of cathepsin, which results in an increase of free amino acids, the precursors for BA formation (MEYER, 1965; PONS-SÁNCHEZ-CASCADO *et al*., 2005b). Tyramine is often reported as dominant BA in anchovy products, both marinated and salt-ripened (VECIANA-NOGUÉS *et al*., 1996; PONS-SÁNCHEZ-CASCADO *et al*., 2005a) but also in fresh anchovies during storage (ÖZOGUL *et al*., 2017). Tyramine production is generally related to lactic acid bacteria and orally administered tyramine (concentrations over 125 mg/kg) can be potentially toxic (LADERO *et al*., 2010). No other BAs were detected in marinated fillets; however, the BA content associated with a lemon juice/olive oil marinating mixture was not analysed. During production of vinegar-marinated anchovies, PONS-SÁNCHEZ-CASCADO *et al*. (2005a) recorded a total of 9.80 mg/kg of BAs per kilogram of raw material (not including agmatine), and 35.44 mg/kg after 3 months of storage. The authors did not find any β-phenylethylamine or tryptamine in the raw material or marinated product. They concluded that vinegar acts as an extractive solvent of biogenic amines from fish, and that the levels of some BAs (cadaverine and putrescine) decreased in fish flesh during the marinating process in tandem with their increased levels in vinegar. According to VECIANA-NOGUÉS *et al*.

(1996), β-phenylethylamine and tryptamine are present only in fish with a high content of other BAs. In general, *Enterobacteriaceae* and lactic acid bacteria are associated with the accumulation of the main biogenic amines: histamine, cadaverine, putrescine and tyramine in seafood products (ÖZOGUL *et al*., 2017). The low TMA values and BA accumulation data obtained through microbiological analyses are given in Table 3. The lemon juice/olive oil mixture had an inhibitory effect on total viable bacteria count, psychrophilic bacteria count and lactic acid bacteria count. The addition of acetic acid inhibited the growth of total viable count and psychrophilic bacteria at first two days of maturation, but by the end of maturation the investigated bacteria adapted to the new conditions and their count increased. No *Enterobacteriaceae*, which are primarily responsible for cadaverine production (MARINO *et al*., 2000; ÖZOGUL *et al*., 2017), were detected in the carpaccio products, which corresponds to low BA production in marinated anchovy fillets. Total or partial inhibition of microorganisms by the application of a marinating process has been recorded previously by FUSELLI *et al*. (1994) in marinated anchovy (*E. anchoita*), with 3% acetic acid and 10% salt; AKSU *et al*. (1997) in marinated anchovy *E. encrasicolus* (6% acetic acid and 16% salt); KILNIC and CAKLI (2004) in sardine marinade (7% acetic acid and 14% salt); OLGUNOĞLU *et al*. (2009) in anchovy *E. encrasicolus* (4.5% alcoholic vinegar, 10% salt and 0.2% citric acid).

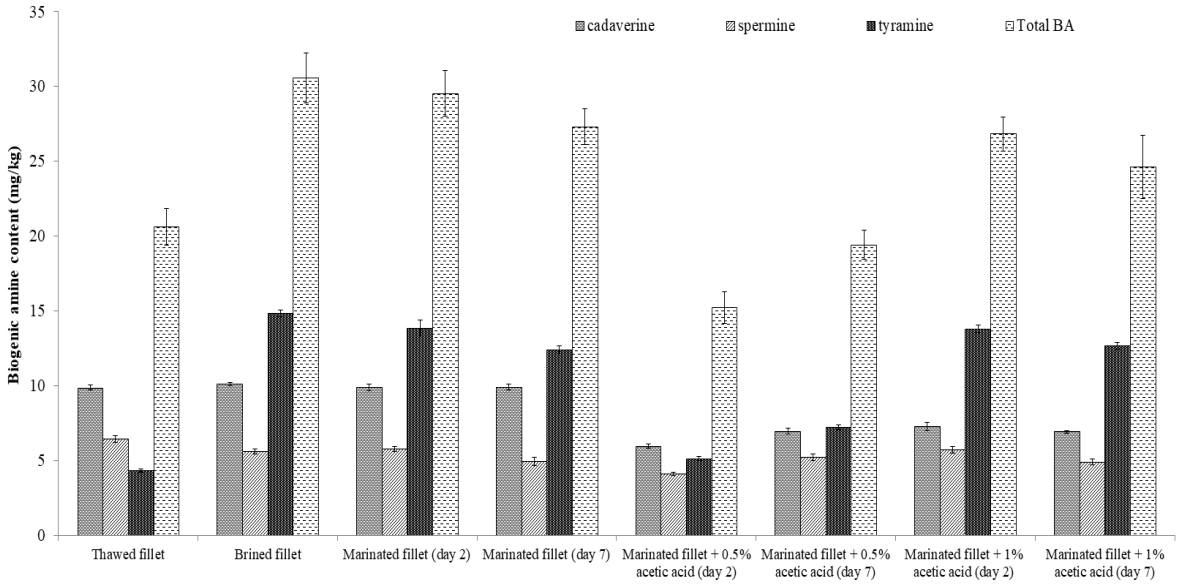


Figure 1. Biogenic amine formation in anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=3).

In general, the marinating process does not completely inactivate bacteria. They are able to continue to grow, faster or slower depending on their ability to adapt to the new media surrounding them (FUSELLI *et al*., 1994). Resistance of microorganisms to the acidic environment is variable and depends on their ability to adapt to new conditions. According to Ray and BHUNIA (2008), brief exposure of microorganisms under suboptimal conditions triggers their cellular mechanisms and allows them to be more resistant and better adapted to harsh environments. The authors state that acid tolerant bacteria may, by exposure to slightly acidic conditions (pH 5.0-5.8), exhibit resistance to survival at pH 2.4-4.0.

The results of sensory evaluation of marinated anchovies are given in Table 4.

Table 3. Changes in the microbiological count of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=3).

Microbiological analyses (log CFU/g)	Thawed fillet	Brined fillet	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
Total viable count	1.13±0.62	ND	0.91±0.24	0.26±0.14	0.34±0.11	0.42±0.18	$<10^{-1}$	0.28±0.09
Psychrophilic bacteria	1.16±0.14	ND	0.56±0.11	$<10^{-1}$	$<10^{-1}$	0.94±0.09	$<10^{-1}$	0.56±0.12
Lactic acid bacteria	0.56±0.02	ND	0.65±0.28	$<10^{-1}$	0.62±0.12	$<10^{-1}$	0.73±0.12	$<10^{-1}$
Enterobacteriaceae	$<10^{-1}$	ND	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$

mean value±standard deviation, ND – not determined.

Table 4. Results of the sensory analysis of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of 0.5 and 1% acetic acid (% on a wet weight basis) (n=6).

Sensory attribute	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
Appearance	7.87±0.92 ^{a}	7.90±0.46 ^{a}	7.90±0.62 ^{a}	7.98±0.08 ^{b}	7.76±0.91 ^{c}	7.90±0.85 ^{a}
Colour	6.90±1.08 ^{a}	7.47±0.88 ^{b}	6.73±0.81 ^{c}	7.93±0.17 ^{d}	6.87±0.74 ^{a}	7.83±0.65 ^{d}
Texture	7.92±0.94 ^{a}	7.93±0.68 ^{a}	7.53±0.99 ^{b}	8.00±0.08 ^{c}	8.00±0.02 ^{c}	7.47±0.62 ^{b}
Odour	7.41±0.94 ^{a}	7.94±0.68 ^{b}	7.62±0.74 ^{c}	7.81±0.56 ^{d}	7.96±0.99 ^{b}	7.38±0.87 ^{a}
Rancidity	6.97±1.12 ^{a}	7.42±0.74 ^{b}	7.24±0.42 ^{b}	7.45±0.99 ^{b}	7.79±1.21 ^{c}	7.45±0.91 ^{b}
Juiciness	7.92±0.81 ^{a}	7.93±1.18 ^{a}	7.53±0.60 ^{b}	7.98±0.22 ^{c}	8.00±0.69 ^{c}	7.47±0.88 ^{b}
Sour and salty taste	6.85±0.99 ^{a}	7.38±1.14 ^{b}	6.91±0.62 ^{a}	7.63±0.48 ^{c}	7.85±0.87 ^{d}	6.35±0.63 ^{e}
Flavour and aftertaste	5.61±0.88 ^{a}	6.27±0.97 ^{b}	5.45±0.71 ^{c}	7.81±0.87 ^{d}	7.72±0.49 ^{d}	6.48±0.47 ^{a}
Overall acceptability (scale from 1-9)	7.31±1.12 ^{a}	8.54±1.12 ^{b}	7.24±0.94 ^{a}	8.94±0.66 ^{c}	7.90±0.78 ^{d}	7.35±0.49 ^{a}

$^{a-e}$mean value±standard deviation in the same raw followed by different superscript are significantly different ($p<0.05$).

Based on the results of sensory analysis, the time required for maturation of anchovy fillets in the lemon/olive oil mixture was 7 days at 4°C. Depending on the acid and NaCl content, maturation temperature, bath:fish ratio etc., time needed for complete maturation of anchovy fillets ranges from 22 days at 4°C (PONS-SÁNCHEZ-CASCADO *et al*. (2005a) to 9 days at 20°C (CABRER *et al*., 2002). In general, better quality of fish is achieved when maturation takes place at lower temperatures over a longer period. Results for texture, juiciness and odour obtained on the second day of maturation in the lemon/olive oil mixture were high (7.9) and did not change during 7 days. However, products containing acetic acid showed improvement of these attributes over maturation. On day 2, all samples had a fishy (raw) flavour and a strong aftertaste and thus obtained lower grades from the panellists. Also, the colour was not glossy white, but yellowish and so colour grades were lower as well. After 7 days, the sensory acceptability of the product was satisfactory and grades were high for all investigated attributes. Glassy-white opaque colour, soft fillet texture, favourable effect in terms of odour and flavour were obtained. At this point, all assessed attributes (with the exception of odour) and overall acceptability of the product were the highest for the product in the lemon/olive oil mixture, with 0.5% acetic acid. The addition of 1% acetic acid resulted in an acidic aftertaste that covered the flavour and good aroma, thus changing the odour of the product and resulting in lower grades.
To the best to our knowledge, this paper presents the first results on the quality changes (physical, chemical, microbiological and sensory) of anchovy fillets during maturation in a lemon juice and olive oil marinade. The described process, consisting in the brining of fillets in a 200 g/L NaCl solution and marination of fillets in a lemon juice and olive oil mixture (50:50) over 7 days, achieved glassy-white colour and soft fillet texture, characteristic flavour and satisfactory salt content. The process resulted in a decrease of pH and water activity and good chemical characteristics of the final product. Low amine accumulation led us to assume that lemon juice has an extracting effect on volatile and non-volatile amines from anchovy fillets. Therefore, their role as an index of freshness/quality for carpaccio-like products is questionable. Further studies should include an analysis of the marination bath. Moreover, marination in lemon juice showed good preservative effects (total viable bacteria, psychrophilic bacteria and lactic acid bacteria count were reduced). The addition of 0.5% acetic acid improved the overall acceptability of the product. The results of this study could be of interest to the fish industry and contribute and enrich the offer of minimally processed seafood on the market.

ACKNOWLEDGMENTS

This work was fully supported by the Croatian Science Foundation, under project IP-2014-09-6897. The authors wish to thank Canicula d.o.o., Gizdavac, Croatia for their support during this investigation.

REFERENCES

Aksu H. Erkan N. Colak H. Varlik C. Gokoglu N. and Ugur M. 1997. Some changes in anchovy marinades during prodution in different acid – salt concentration and determination of shelf life. Yüzüncüyıl Üniversitesi Veteriner Fakültesi Dergisi. 8:86-90.

AOAC. Official Methods of Analysis. 2000. 17. ed. Association of Official Analytical Chemists. Washington, D.C.

Bligh E.G. and Dyer W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Cabrer A.I. Casales M.R. and Yeannes M.I. 2002. Physical and chemical changes in anchovy (*Engraulis anchoita*) flesh during marination. J. Aquat. Food Prod. Technol. 11:19-31.

Cadun A. Kişla D. and Çakli S. 2008. Marination of deep-water pink shrimp with rosemary extract and the determination of its shelf-life. Food Chem. 109:81-87.

Duyar H.A. and Eke E. 2009. Production and quality determination of marinade from different fish species. J. Anim. Vet. Adv. 8:270-275.

Eerola S. Hinkkanen R. Lindfors E. and Hirvi T. 1993. Liquid chromatographic determination of biogenic amines in dry sausages. J. AOAC Int. 76:575-577.

Fuselli S.R. Casales M.R. Fritz R. and Yeannes M.I. 1994. Microbiology of the marination process used in anchovy (*Engraulita anchoita*) production. LWT-Food Sci. Technol. 27:214-218.

Gardini F. Özogul Y. Suzzi G. Tabanelli G. and Özogul F. 2016. Technological factors affecting biogenic amine content in foods: a review. Front. Microbiol. 7:12-18.

Gökoğlu N. Cengiz E. and Yerlikaya P. 2004. Determination of the shelf life of marinated sardine (Sardina pilchardus) stored at 4°C. Food Control. 15:1-4.

Gökoğlu N. Osman K.T. and Yerlikaya P. 2009. Effect of pomegrante sauce on quality of marinated anchovy during refrigerated storage. LWT--Food Sci. Technol. 42:113-118.

González-Molina E. Domínguez-Perles R. Moreno D.A. and García-Viguera C. 2010. Natural bioactive compounds of Citrus limon for food and health. J. Pharm. Biomed. Anal. 51:327-45.

Halász A. Barath A. Simons-Sarkadi L. and Holzapfel W. 1994. Biogenic amines and their production by microorganisms in food. Trends Food Sci. Technol. 5:42-48.

Huss H. 1988. Fresh fish -quality and quality changes, Training Programme on Fish Technology and Quality Control, Rome: FAO Fisheries Series, FAO. pp. 132

Kilinc B. and Cakli S. 2004. Chemical, microbiological and sensory changes in thawed frozen fillets of sardine (*Sardina pilchardus*) during marination. Food Chem. 88:275-280.

Kilinc B. and Cakali S. 2005. The determination of the shelf life of sardine (*Sardina pilchardus*) marinades in tomato sauce stored at 4°C. Food Control. 16:639-644.

Kocatepe D. and Turan H. 2012. Proximate and fatty acid composition of some commercially important fish species from the Sinop region of the Black Sea. Lipids. 47:635-641.

Ladero V. Calles-Enriquez M. Fernandez M.A. and Alvarez M. 2010. Toxicological effects of dietary biogenic amines. Curr. Nutr. Food Sci. 6:145-56.

Lemon D.W. 1975. An improved TBA test for rancidity. New Series Circular No. 51. Halifax Laboratory, Nova Scotia. 1-4.

Marino M. Maifreni M. Moret S. and Rondinini G. 2000. The capacity of Enterobacteriaceae species to produce biogenic amines in cheese. Lett. Appl. Microbio. 31:169-73.

McLay B.R. 1972. Marinades. Ministry of Agriculture Fisheries and Food. Torry Advisory Note No. 56.

Mladineo I. Šimat V. Miletić J. Beck R. and Poljak V. 2012. Molecular identification and population dynamic of Anisakis pegreffii (Nematoda: Anisakidae Dujardin, 1845) isolated from the European anchovy (*Engraulis encrasicolus* L.) in the Adriatic Sea. Int. J. Food Microbiol. 157:224-229.

Ólafsdóttir G. Martinsdóttir E. Oehlenschläger J. Dalgaard P. Jensen B. Undeland I. Mackie I.M. Henehan G. Nielsen J. and Nilsen H. 1997. Methods to evaluate fish freshness in research and industry. Trends Food Sci. Technol. 8:258-265.

Olgunoğlu I.A. Özogul F. Özogul Y. and Kuley E. 2009. Chemical, sensory and microbiological assessment of marinated anchovy (*Engraulis engrasicholus* L., 1758) fillets stored at 1±1°C. Adv. Food Sci. 31:1-8.

Özogul Y. Özogul F. Kuley E. Özkutuk A.S. Gökbulut C. and Köse S. 2006. Biochemical, sensory and microbiological attributes of wild turbot (*Scophthalmus maximus*), from the Black Sea, during chilled storage. Food Chem. 99:752-8.

Özogul Y. Özogul F. and Kuley E. 2010. Effects of combining of smoking and marinating on the shelf life of anchovy stored at 4°C. Food Sci. Biotechnol. 19:69-75.

Özogul F. Öztekin R. and Kulawik P. 2017. Biogenic amine formation and microbiological quality of anchovy (*Engraulis encrasicolus*) treated with lavander and lemon balm ethanol extracts. J. Food Sci. 85:1278-1284.

Poligne I. and Collignan A. 2000. Quick marination of anchovies (*Engraulis encrasicolus*) using acetic and gluconic acids. Quality and stability of the end product. LWT-Food Sci. Technol. 33:202-209.

Pons-Sanchez-Cascado S., Veciana-Nogues M.T., Bover-Cid S., Mariné-Font A. and Vidal-Carou M.C. 2005a. Volatile and biogenic amines, microbiological counts, and bacterial amino acid decarboxylase activity throughout the salt-ripening process of anchovies (*Engraulis encrasicholus*). J. Food Prot. 68:1683-1689.

Pons-Sanchez-Cascado S. Vidal-Carou M.C. Mariné-Font A. and Veciana-Nogues M. T. 2005b. Influence of the freshness grade of raw fish on the formation of volatile and biogenic amines during the manufacture and storage of vinegar-marinated anchovies. J. Agric. Food Chem. 53:8586-8592.

Ray B. 2008. Bhunia A. Fundamental food microbiology. (4th ed.) Boca Raton: CRC Press, (Chapter 16).

Sallam Kh.I. Ahmed A.M. Elgazzar M.M. and Eldaly E.A. 2007. Chemical quality and sensory attributes of marinated Pacific saury (*Cololabis saira*) during vacuum packaged storage at 4°C. Food Chem. 102:1061-1070.

Šimat V. Maršić-Lučić J. Tudor M. and Mladineo I. 2009. Long-term storage influence on volatile amines (TVB-N and TMA-N) in sardines and herring utilized as food for tuna fattening. J. Appl. Ichthyol. 25:766-770.

Šimat V. and Dalgaard P. 2011. Use of small diameter column particles to enhance HPLC determination of histamine and other biogenic amines in seafood. LWT--Food Sci. Technol. 44:399-406.

Šimat V. Bogdanović T. Krželj M. Soldo A. and Maršić Lučić J. 2012. Differences in chemical, physical and sensory properties during shelf life assessment of wild and farmed gilthead sea bream (*Sparus aurata*, L.). J. Appl. Ichthyol. 28:95-101.

Šimat V. and Bogdanović T. 2012. Seasonal changes in proximate composition of anchovy (*Engraulis encrasicolus*, L.) from the central Adriatic. Acta Adriat. 53:125-132.

Šimat V. Miletić J. Bogdanović T. Poljak V. and Mladineo I. 2015. Role of biogenic amines in the post-mortem migration of Anisakis pegreffii (Nematoda: Anisakidae Dujardin, 1845) larvae into fish fillets. Int. J. Food Microbiol. 214:179-186.

Topuz O.K. Gökoğlu N. Yerlikaya P. and Uçak İ. 2016. Quality changes in marinated anchovy (*Engraulis encrasicolus*) sauced with olive oil-lemon juice emulsions. J. Aquat. Food Prod. Technol. 25:905-915.

Veciana-Nogués M.T. Albalá-Hurtado S. Mariné-Font A. and Vidal-Carou M.C. 1996. Changes in biogenic amines during the manufacture and storage of semipreserved anchovies. J. Food Prot. 59:1218-1222.

Vyncke W. 1970. Direct determination of the thiobarbituric acid value in trichloroacetic acid extracts of fish as a measure of oxidative rancidity. Fette-Seinfen Anstrichmittel. 72:1084-1087.

Yeannes M.I. and Casales M.R. 2008. Modifications in chemical compounds and sensorial attributes of *Engraulis anchoita* fillet during marinaion process. Cienc. Tecnol. Aliment. 28:798-80.

Paper Received July 23, 2018 Accepted February 14, 2019

PAPER

COAGULASE POSITIVE STAPHYLOCOCCI ENUMERATION AND ENTEROTOXINS DETECTION IN MILK AND DAIRY PRODUCTS FROM CENTRAL ITALY

L. IANNETTI[1], P. VISCIANO[2], C. MARFOGLIA[1], G. IANNITTO[1], G. PARISCIANI[1], M. SERICOLA[1], D. PETRONE[1], M.S. MANGIERI[2], F. POMILIO[1] and M. SCHIRONE*[2]

[1]Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via Campo Boario, 64100 Teramo, Italy

[2]Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Via R. Balzarini 1, 64100 Teramo, Italy

*Corresponding author: Tel.: +390861266911

E-mail address: mschirone@unite.it

ABSTRACT

This study aims at enumerating coagulase positive staphylococci (CPS) in 404 samples of milk and dairy products collected in own-checks or during the official controls from different dairy industries located in Central Italy. These microorganisms were enumerated using ISO 6888-2:1999/Amd. 1:2003 and only when they exceeded 10^5 CFU/g, the presence of any of the seven more common staphylococcal enterotoxins (SEA, SEB, SEC_1, SEC_2, SEC_3, SED and SEE) was also investigated. Own-checks samples resulted always below the detection limit, whereas among those collected by the competent authorities in the framework of official controls, *provola* (100%) and *mozzarella* (22.9%) samples were positive to CPS, with mean values of $1.8x10^2$ and $2.8x10^5$ CFU/g respectively. Such values exceeded the maximum limits set by Commission Regulation (EC) No. 2073/2005, resulting in a request of hygiene improvements in the first case; in the second case, the presence of staphylococcal enterotoxins in 8 (2.7%) *mozzarella* samples out of 298 investigated cheese products was also observed, resulting in their withdrawn from the market. Therefore, this study aims at highlighting that monitoring of CPS incidence in dairy products and subsequent testing of cheeses for enterotoxins when appropriate represent an important tool for public health in order to avoid the occurrence of foodborne outbreaks.

Keywords: Coagulase positive staphylococci, milk, dairy products, process-hygiene criteria, staphylococcal enterotoxins

1. INTRODUCTION

Italian cheese production boasts a great variety of dairy products ranging from fresh to ripened cheeses such as *mozzarella*, *pecorino* and *Parmigiano Reggiano*. Their quality is closely associated with parameters such as the territory of production, pedoclimatic characteristics, and anthropic components (SCHIRONE *et al*., 2012). Abruzzo and Molise regions located in Central Italy have no cheeses with protected designation of origin (PDO) or protected geographical indication (PGI), except for *caciocavallo silano* and *mozzarella di bufala campana* for Molise region, as reported in the publicly-accessible updated register of PDO and PGI of Regulation (EU) No. 1151/2012 (EU, 2012). In contrast, according to the Italian Decree No. 350 dated September 8, 1999 (MIPAAF Decree, 1999), 14 and 12 traditional cheeses are reported as traditional food products made in Abruzzo and Molise regions respectively. As par as such traditional cheeses are concerned, raw milk microbiota rather than microorganisms deriving from the dairy environment or equipment can affect the safety of the end product (SÁNCHEZ-GAMBOA *et al*., 2018; SCHIRONE *et al*., 2018).

Staphylococci are widespread in nature, being found on the skin and mucous membranes of animals and humans, but also in the environment (RUIZ *et al*., 2016). Coagulase positive staphylococci (CPS) are the most common causative agents of subclinical mastitis in dairy cows (RAJIC-SAVIC *et al*., 2015). Several studies (JORGERSEN *et al*., 2005; NEDER *et al*., 2011) reported that some strains isolated from bulk milk can produce enterotoxins and, as such, they are considered biological hazards for the dairy industry. Among them, *Staphylococcus aureus* is one of the most important disease-causing agents of foodborne intoxication in humans caused by the consumption of dairy products but, since it is also found in 30-80% of human population, the personnel working in a food industry can contaminate food with unhygienic food handling and manufacturing practices (SOLTAN DALLAL *et al*., 2016).

According to Commission Regulation (EC) No. 2073/2005 (EC, 2005) and further amendments, CPS should be investigated in different categories of cheese, both ripened an unripened, made from raw or pasteurized milk, or after a stronger heat treatment, for process control purpose. The process-hygiene criteria established by the above quoted Regulation differ on the basis of cheese categories and can be applied at the end of the manufacturing process or at the time when the number of staphylococci is expected to be the highest. The current legislation on microbiological criteria requires that only when such values exceed 10^5 CFU/g the cheese batch must be analyzed for staphylococcal enterotoxins, even if some authors reported that levels of *S. aureus* ranging from not detectable to $1.5x10^{10}$ CFU/g, with a median value of $3x10^7$ CFU/g, could cause foodborne outbreaks (WALLIN-CARLQUIST *et al*., 2010). Their formation is influenced by some parameters, such as temperature, pH, water activity, redox potential and bacteria antagonism (SCHELIN *et al*., 2011). The most common detected staphylococcal enterotoxins named as classical (SEA, SEB, SEC, SED and SEE) can cause several symptoms at rapid onset (2-8 h), including hypersalivation, nausea, vomiting, abdominal cramping and diarrhoea, after the ingestion of contaminated food (MARTINS *et al*., 2014; MEHLI *et al*., 2017). Staphylococcal food poisoning usually resolves within 24-48 h with no specific treatment; but it can be more severe or even fatal when occurring in infants, elderly or immune-compromised people (ROLA *et al*., 2016).

The aim of the present investigation was the enumeration of CPS in milk and dairy products produced in dairy industries located in Abruzzo and Molise regions, Central Italy, in order to check the process-hygiene criteria established by Regulation (EC) No. 2073/2005 and to detect staphylococcal enterotoxins only in not compliant samples.

2. MATERIAL AND METHODS

A total of 404 samples of milk and dairy products were obtained from different dairy industries located in Abruzzo and Molise regions (Central Italy) in the framework of own-checks (n=118) and official controls (n=286).
The milk samples (n=91) were differentiated into raw milk (n=14), bulk milk (n=28), whole milk (n=21), low-fat milk (n=19) and skimmed pasteurized milk (n=9). Among the dairy products, butter (n=9) and cream (n=6) were sampled. All the other remaining dairy products belonged to different categories of cheese (n=298) and could be grouped according to the process-hygiene criteria of Commission Regulation (EC) No. 2073/2005 – namely raw milk cheese (n=18), ripened cheese from pasteurized milk (n=36) and unripened cheese from pasteurized milk (n=244). Among the latter, only *caciotta* samples were produced from raw cow' or sheep's milk; while most of cheese samples were represented by unripened cheeses, made from pasteurized cow' or water buffaloes' milk, usually "pasta filata" fresh cheeses (i.e. *burrata*, *mozzarella*, *provola*, *scamorza* and *fiordilatte*), semi-soft cheeses and ricotta. Ripened cheeses were also from pasteurized cow' or sheep's milk and included semi-hard cheeses and grated cheese.
All samples were analyzed at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise – that is, the laboratory in charge for official controls in those regions, which also tested samples taken in the framework of own-checks from some local dairy industries. Samples collected over a 18-month period, including the whole year 2017 and the first 6 months of the year 2018, were involved in the present study. They were investigated for CPS enumeration by the analytical reference method ISO 6888-2:1999/Amd. 1:2003. Such a method is based on the use of Rabbit Plasma Fibrinogen Agar that allows the easy differentiation of coagulase positive and negative colonies with no need of confirmatory steps (VIÇOSA *et al.*, 2009). Briefly speaking, duplicate poured plates of the quoted medium were prepared and inoculated using decimal dilutions of the test sample (or initial suspension, if solid) and then incubated at 37°C for 18-24 h or more, if needed. The calculation of CPS per g of a sample was made counting typical colonies on petri dishes.
According to prescriptions from Commission Regulation (EC) No. 2073/2005, samples with CPS counts exceeding 10^5 CFU/g were also analyzed for the detection of staphylococcal enterotoxins using the VIDAS® Staph enterotoxin II (SET2) kit (Biomerieux, France) according to the FDA Bacteriological Analytical Manual (BAM), 8th Edition, chapter 13A (revision March 2011). Such a method allows the detection of any of the seven more common staphylococcal enterotoxins (SEA, SEB, SEC_1, SEC_2, SEC_3, SED, SEE). In a few words, toxins were extracted from the test samples (25 g or mL) adding extraction buffer, blending and centrifuging according to the manufacturer's instructions. The supernatant was pumped through moistened absorbent cotton placed in a syringe, using the plunger; then 500 µL of the filtrate was placed in the sample well of the VIDAS SET2 reagent strip and the assay was finally performed with a MINI VIDAS® instrument (Biomerieux, France).

3. RESULTS AND DISCUSSION

Most dairy products, including semi-hard and grated cheese, resulted below the detection limit of CPS (<1 CFU/mL or <10 CFU/g). Table 1 shows the results reported in the different cheese types, in relation to the presence of unsatisfactory results of CPS enumeration according to the Regulation (EC) No. 2073/2005.

Table 1. Number of cheese samples (n) and unsatisfactory CPS enumeration for each cheese type according to the category of the Regulation (EC) No. 2073/2005. Limits are expressed in parentheses; the lowest is allowed only for 2 samples out of 5, the highest is never allowed.

Samples	Raw milk cheeses (10^4-10^5CFU/g)		Ripened cheeses* (10^2-10^3 CFU/g)		Unripened cheeses* (10-10^2 CFU/g)	
	n	Unsatisfactory	n	Unsatisfactory	n	Unsatisfactory
Caciotta	18	0**				
Ricotta					10	0
Soft cheese					10	0
Semi-soft cheese					5	0
Semi-hard cheese			27	0		
Grated cheese			8	0		
Burrata					8	0
Mozzarella					35	8
Provola					5	5
Scamorza					73	0
Other "Pasta filata" cheeses					99	0

*made from milk or whey that has undergone pasteurization or a stronger heat treatment;
**CPS detected below regulatory limits

Coagulase positive staphylococci were only found in samples from official controls, while own-checks were always below the detection limit.
The results of CPS in milk samples were below the detection limit (<1 CFU/mL) except for 5 samples of raw milk collected in the framework of official controls from a small dairy industry in Molise region, that showed values ranging from $2.9x10^3$ to $3.5x10^3$ CFU/mL with a mean value of 3.5 Log CFU/mL (Fig. 1). The microbial contamination of raw milk may originate from animals, due to endogenous or udder infection, or from faeces and skin, and/or from the environment during milking or storage (VERRAES *et al*., 2014).
Only some categories of unripened cheese resulted positive to CPS – namely *caciotta*, *provola* and *mozzarella* samples. In particular, the values of CPS ranged from $4.8x10^3$ to $5.4x10^3$ CFU/g in 5 (33.3%) *caciotta* samples with a mean value of 3.7 Log CFU/g. However, they did not exceed the limits (10^4-10^5 CFU/g) of process-hygiene criteria established by Commission Regulation (EC) No. 2073/2005 for cheeses made from raw milk. All (100%) *provola* samples resulted positive to CPS, ranging from $1.2x10^2$ to $2.2x10^2$ CFU/g with a mean value of 2.3 Log CFU/g (Fig. 1). These values exceeded the limits (10-10^2 CFU/g) for unripened cheeses made from pasteurized milk and therefore process hygiene improvements were required to the involved dairy industry. In addition, 8 (22.9%) samples of mozzarella out of 35 exceeded the regulatory limits up to a maximum value of $3.5x10^5$ CFU/g, with a mean value of 5.4 Log CFU/g (Fig. 2). In such last case, as values over 10^5 CFU/g of CPS were detected, staphylococcal enterotoxins were also investigated according to Commission Regulation (EC) No 2073/2005 and their presence was always confirmed in 25 g (100%).

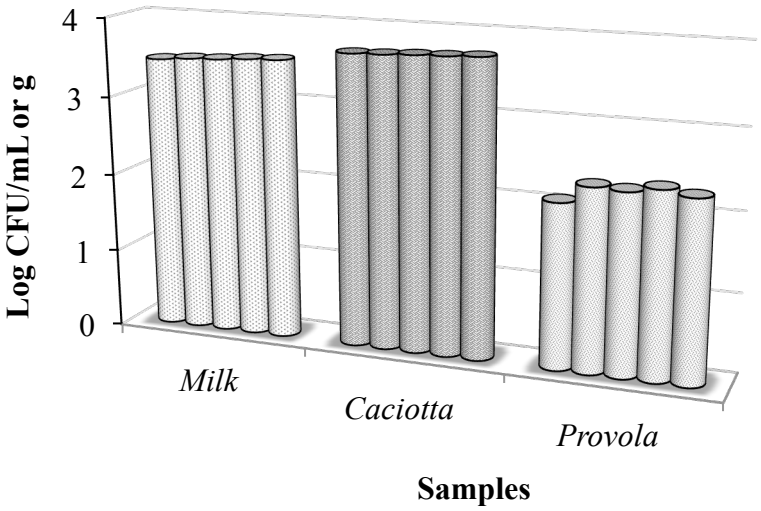


Figure 1. Coagulase positive staphylococci counts (CFU/mL or g) in milk and cheese samples.

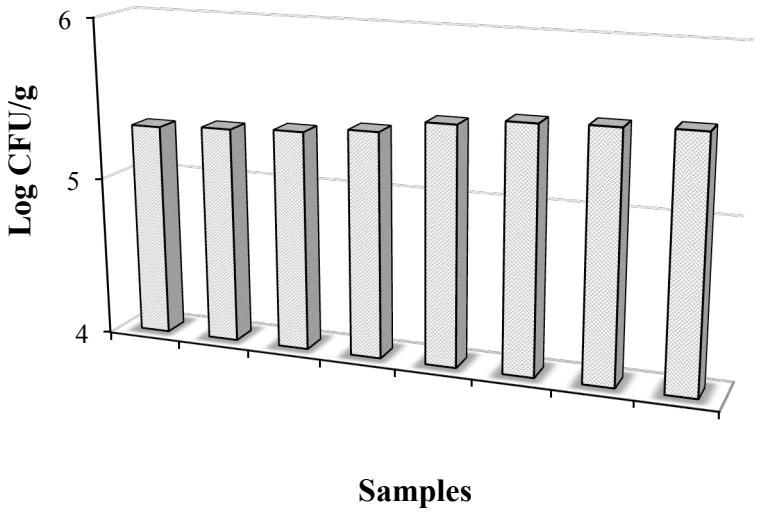


Figure 2. Coagulase positive staphylococci counts (CFU/g) in *mozzarella* samples exceeding the regulatory limit.

The results always below the detection limit for samples made in own-checks could be due to sampling made not at random, at least by some dairy industries – rather they aimed at selecting less manipulated products or those perceived to be safer, in order to reduce the possibility of encountering samples positive to CPS. On the contrary, some samples taken in the framework of official controls showed high values of CPS, stressing the importance of controls directly carried out by competent authorities. However, they usually did not exceed the process hygiene criteria, except for *provola* and *mozzarella* samples. These results could suggest the importance of good hygiene and manufacturing practices during cheese production in the first case, whereas the presence of staphylococcal enterotoxins in *mozzarella* samples with high values of CPS (> 10^5 CFU/g) confirmed a potential serious risk for consumers. All positive samples belonged to the same batch produced in a small

dairy industry in Molise region and were withdrawn or recalled in accordance with Article 19 of Regulation (EC) No. 178/2002 (EC, 2002). That batch was probably contaminated, even though it remains unknown if such contamination could be due to raw materials, lack of good hygiene practices or even to the handlers themselves, representing healthy carriers of CPS. However, some studies reported that pasteurized milk cheese caused outbreaks of foodborne diseases also at a higher incidence rate than those linked to raw milk cheese consumption (YOON *et al*., 2016). Moreover, failure of thermal processing, contamination of the products after pasteurization or abuse of storage temperature could allow the proliferation of CPS in dairy products as well, after heat treatment. Some bad hygiene habits such as coughing or sneezing and not washing hands when handling cheese production equipment could be a source of contamination of the end products (ROSENGREN *et al*., 2010).
Other authors (DELBES *et al*., 2006) reported lower CPS values than our results in raw cow's milk collected from France, ranging from undetectable to about $1.1x10^3$ CFU/mL. As par as cheeses are concerned, ROSENGREN *et al*. (2010) detected CPS in 69% of raw milk cheeses and 6% of cheeses made from pasteurized milk, even if staphylococcal enterotoxins were never detected. According to the results of our investigation, mean values of CPS ($2.6x10^3$ CFU/g) were detected in raw milk cheese produced in a South Tyrolean malga and the presence of staphylococcal enterotoxins was also confirmed, causing symptoms referable to staphylococcal food poisoning (ARMANI *et al*., 2016). According to the European Union summary report on trends and sources of zoonoses in 2016 (EFSA and ECDC, 2017), a monitoring study on milk samples from different animal species carried out in Italy reported that 110 and 55 out of 165 samples were positive for *S. aureus* and *Staphylococcus* spp. respectively. Another Italian survey on 6,482 milk samples described in the above quoted report showed that 451 were positive for *S. aureus*, 54 for *Staphylococcus intermedius* and 628 for *Staphylococcus* spp. JOHLER *et al*. (2018) found 80% of artisan raw milk cheese samples positive to *S. aureus* from 25 dairies located in Italy. Some isolates exhibited at least one gene encoding some of staphylococcal enterotoxins. Another Italian study (CREMONESI *et al*., 2007) on raw milk cheese samples (fresh, soft, semi-hard and hard cheeses) reported that no enterotoxins were detected in samples positive to high concentrations of *S. aureus*.

4. CONCLUSIONS

The results of the present study have highlighted the presence of CPS in some raw milk and unripened cheese, as well as the detection of staphylococcal enterotoxins in eight samples of *mozzarella*. Therefore, the monitoring of CPS incidence in milk and dairy products represents an important tool for public health, especially when high values are detected. The ubiquity of such microorganisms and the lack of good manufacturing practices in milk and cheese production represent a risk factor to consumers, so that the improvement of hygiene procedures such as regular checks of animal health, testing of milk and monitoring of critical points in the production process should be expected.

ACKNOWLEDGEMENTS

The authors thank Prof. Francesca Rosati (Sworn Translator) for her support in checking the manuscript for English form.

REFERENCES

Armani M., Macori G., Gallina S., Tavella A., Giusti M., Paolazzi G., Trentini L., Rabini M., Decastelli L. and Lombardo D. 2016. Coagulase positive staphylococci and food poisoning toxins – A case study of an outbreak investigation occurred in a sheperd hut. 17th International Congress on Infectious Diseases. Int. J. Infect. Dis. 45S:464.

Cremonesi P., Perez G., Pisoni G., Moroni P., Morandi S., Luzzana M., Brasca M. and Castiglioni B. 2007. Detection of enterotoxigenic *Staphylococcus aureus* isolates in raw milk cheese. Lett. Appl. Microbiol. 45(6):586-591.

Delbes C., Alomar J., Chougui N., Martin J.F. and Montel M.C. 2006. *Staphylococcus aureus* growth and enterotoxin production during the manufacture of uncooked, semihard cheese from cows' raw milk. J. Food Prot. 69(9):2161-2167.

EC 2002. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Off. J. Eur. Union L31:1-44.

EC 2005. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L338:1-26.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA J. 15(12):5077:1-228.

EU 2012. Regulation (EU) No 1151/2012 of the European Parliament and of the Council of 21/11/2012 on quality schemes for agricultural products and foodstuffs. Off. J. Eur. Union L343:1-29.

Johler S., Macori G., Bellio A., Acutis P.L., Gallina S. and Decastelli L. 2018. Short communication: Characterization of *Staphylococcus aureus* isolated along the raw milk cheese production process in artisan dairies in Italy. J. Dairy Sci. 101(4):2915-2920.

Jorgensen H.J., Mork T., Hogasen H.R. and Rorvik L.M. 2005. Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. J. Appl. Microbiol. 99(1):158-166.

Martins I.M., Kabuki D.Y., Miya N.T.N. and Pereira J.L. 2014. Occurrence and characterization of enterotoxigenic potential *Staphylococcus* isolated from dairy products. J. Food Safety 34(3):185-192.

Mehli L., Hoel S., Thomassen G.M.B., Jakobsen A.N. and Karlsen H. 2017. The prevalence, genetic diversity and antibiotic resistance of *Staphylococcus aureus* in milk, whey, and cheese from artisan farm dairies. Int. Dairy J. 65:20-27.

MIPAAF (Ministero delle Politiche Agricole, Alimentari, Forestali e del Turismo). Decreto 8 settembre 1999, n. 350. Regolamento recante norme per l'individuazione dei prodotti tradizionali di cui all'articolo 8, comma 1, del decreto legislativo 30 aprile 1998, n. 173.

Neder V.E., Canavesio V.R. and Calvinho L.F. 2011. Presence of enterotoxigenic *Staphylococcus aureus* in bulk tank milk from Argentine dairy farms. Rev. Argent. Microbiol. 43:104-106.

Rajic-Savic N., Katic V., Velebit B. and Colovic S. 2015. Characteristics of enterotoxigenic coagulase positive staphylococci isolated from bovine milk in cases of subclinical mastitis. Procedia Food Sci. 5:250-253.

Rola J.G., Czubkowska A., Korpysa-Dzirba W. and Osek J. 2016. Occurrence of *Staphylococcus aureus* on farms with small scale production of raw milk cheeses in Poland. Toxins 8(62):1-9.

Rosengren Å., Fabricius A., Guss B., Sylvén S., Lindqvist R. 2010. Occurrence of foodborne pathogens and characterization of *Staphylococcus aureus* in cheese produced on farm-dairies. Int. J. Food Microbiol. 144:263-269.

Ruiz P., Barragán I., Seseña S. and Palop M.L. 2016. Is staphylococci population from milk of healthy goats safe? Int. J. Food Microbiol. 238:146-152.

Sánchez-Gamboa C., Hicks-Pérez L., Gutiérrez-Méndez N., Heredia N., García S. and Nevárez-Moorillón G.V. 2018. Microbiological changes during ripening of Chihuahua cheese manufactured with raw milk and its seasonal variations. Foods 7(153):1-12.

Schelin J., Wallin-Carlquist N., Thorup Cohn M., Lindqvist R., Barker G.C. and Rådström P. 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Virulence 2(6):580-592.

Schirone M., Tofalo R., Perpetuini G., Manetta A.C., Di Gianvito P., Tittarelli F., Battistelli N., Corsetti A., Suzzi G. and Martino G. 2018. Influence of iodine feeding on microbiological and physico-chemical characteristics and biogenic amines content in a raw ewes' milk cheese. Foods 7(108):1-11.

Schirone M., Tofalo R., Visciano P., Corsetti A. and Suzzi G. 2012. Biogenic amines in Italian Pecorino cheese. Front. Microbiol. 3(171):1-9.

Soltan Dallal M.M., Reza Khoramizadeh M., Agha Amiri S., Saboor Yaraghi A.A. and Nezhad Fard R.M. 2016. Coagulase gene polymorphism of *Staphylococcus aureus* isolates. A study on dairy food products and other foods in Tehran, Iran. Food Sci. Human Wellness 5:186-190.

Verraes C., Claeys W., Cardoen S., Daube G., De Zutter L., Imberechts H., Dierick k. and Herman L. 2014. A review of the microbiological hazards of raw milk from animal species other than cows. Int. Dairy J. 39:121-130.

Viçosa G.N., Moraes P.M., Yamazi A.K. and Nero L.A. 2009. Enumeration of coagulase and thermonuclease-positive *Staphylococcus* spp. in raw milk and fresh soft cheese: an evaluation of Baird-Parker agar, Rabbit Plasma Fibrinogen agar and the Petrifilm™ Staph Express count system. Food Microbiol. 27:447-452.

Yoon Y., Lee S. and Choi K.H. 2016. Microbial benefits and risks of raw milk cheese. Food Control 63:201-215.

Wallin-Carlquist N., Márta D., Borch E. and Rådström P. 2010. Prolonged expression and production od *Staphylococcus aureus* enterotoxin A in processed pork meat. Int. J. Food Microbiol. 141:S69-S74.

Paper Received July 17, 2018 Accepted December 14, 2018

PAPER

CHARACTERIZATION OF PASTA WITH THE ADDITION OF *CICER ARIETINUM* AND *SALVIA HISPANICA* FLOURS ON QUALITY AND ANTIOXIDANT PARAMETERS

A.G. COTA-GASTÉLUM, M.G. SALAZAR-GARCÍA, A. ESPINOZA-LÓPEZ, L.M. PEREZ-PEREZ, F.J. CINCO-MOROYOQUI, O. MARTÍNEZ-CRUZ, F.J. WONG-CORRAL and C.L. DEL-TORO-SÁNCHEZ*

Department of Research and Postgraduate in Food (DIPA), University of Sonora. Blvd, Luis Encinas y Rosales S/N, Colonia Centro 83000, Hermosillo, Sonora, Mexico

*Corresponding author: Tel: +526622592208; Fax: +526622592208

E-mail address: carmen.deltoro@unison.mx

ABSTRACT

Quality parameters, antioxidant properties, *in vitro* digestion and consumer acceptance were determined in pasta prepared with chickpea and chia flours. Pastas fortified with chia and chickpea increased protein, fiber content, total phenols, and antioxidant capacity with respect to the control. More than 85% of the antioxidant capacity and over 90% of the phenolic compounds in cooked pasta were retained after *in vitro* digestion, which is considered high. Pasta prepared with 25% wheat semolina, 10% chia flour, and 65% chickpea flour has high quality parameters, phenol content, antioxidant capacity and consumer acceptance.

Keywords: *Cicer arietinum*, pasta, phenols, *Salvia hispanica*

1. INTRODUCTION

Pasta products are basic foodstuffs that are important in human food consumption (GELENCSÉR *et al*., 2008). The simplicity of pasta production (traditionally manufactured from durum wheat semolina), in addition to its ease-of-handling and storage stability has facilitated its popularity and widespread consumption around the world (CHILLO *et al*., 2008). Nevertheless, pasta is considered insufficient with regard to its nutritional value, due to a poor source of protein (unless supplemented) and its protein has low amounts of essential amino acids such as lysine. Hence, pasta is an excellent vehicle for supplementation with fiber, proteins, and many other valuable healthy components. It could be appropriately designed and would operate as a functional food if healthy components were to be incorporated into its formulation (BORNEO and AGUIRRE, 2008). Some of these ingredients could be chickpea (*Cicer arietinum*) and chia (*Salvia hispanica*) seeds.

Chickpea is an important grain legume because of its nutritional quality. It is a rich source of complex carbohydrates, protein, vitamins, and minerals (JUKANTI *et al*., 2012; HIRDYANI, 2014). Polyphenols are also present in chickpeas. Some researches indicate that one of the disadvantages of the presence of phenolic compounds (denominated anti-nutritionals) is that they bind to proteins through non-covalent interactions (electrostatic and hydrophobic interactions, and hydrogen bonding), which reduces their nutritional availability (MONDOR *et al*., 2009). However, these types of compounds are currently of great interest because they are bioactive compounds that can confer significant long-term health benefits (AGUILERA *et al*., 2011). Polyphenols have been recognized as the most abundant source of antioxidants in our diet due to their activities and beneficial properties such as antihypertensive and antibacterial actions as well as anticarcinogenic properties (YANG *et al*., 2001; THOMASSET *et al*., 2007; XU and CHANG, 2010). In majority of the cases, free radicals are responsible for degenerative diseases such as cancer and diabetes, among others (HAN *et al*., 2007), and polyphenols have the ability to scavenge free radicals, thereby preventing associated ailments.

The phenolic composition of the chickpea is represented by phenolic acids (*p*-hydroxybenzoic acid, vanillic acid, ferulic acid, and *p*-coumaric acid) and flavonols (quercetin, kaempferol, and myricetin), as well as isoflavones (daidzein and genistein) (MONDOR *et al*., 2009; SREERAMA *et al*., 2010; FARES and MENGA, 2012). One research was found that studied the effects of the toasting of chickpeas on the antioxidant properties of chickpea flour added to durum wheat pasta. In that research, it was concluded that toasting the chickpea increases its phenolic content and antioxidant activity (FARES and MENGA, 2012). However, to the best of our knowledge, there is no information on these compounds in fortified pastas after *in vitro* digestion. The majority of investigations focused on the health benefits accruing from the digestibility of starch, such as a lower glycemic index for persons with diabetes (GOÑI and VALENTÍN-GAMAZO, 2003; PETITOT *et al*., 2010; FLORES-SILVA *et al*., 2014) and for the prevention of cancer, as well as protection against cardiovascular diseases due to the dietary-fiber content of starch (CHILLO *et al*., 2008). The remainder of studies on chickpea-fortified pasta focused on improving its nutritional, cooking, and sensory qualities, in which the authors of these studies concluded that chickpea confers good quality and nutritional properties on pastas (ZHAO *et al*., 2005; SABANIS *et al*., 2006; WOOD, 2009; ABOU-ARAB *et al*., 2010; BASHIR *et al*., 2012; PADALINO *et al*., 2015).

Furthermore, chia (*Salvia hispanica*) which is native to southern Mexico and northern Guatemala has become an important raw material for obtaining functional foods due to its high content of fatty acids, protein, fiber, and secondary metabolites such as phenolic compounds (COATES and AYERZA, 1996; SANDOVAL-OLIVEROS and PAREDES-

LÓPEZ, 2013; MARTÍNEZ-CRUZ and PAREDES-LÓPEZ, 2014). Chia seeds are very rich in phenolic compounds and possess a high antioxidant capacity, suggesting that phenolic acids (gallic, caffeic, ferulic, chlorogenic, and rosmarinic), flavonols (quercetin and kaempferol), and isoflovonols (Genistein, Daidzin, and Glycitein) may decrease the invasiveness of cancer cells, remove ROS (Reactive Oxygen Species), and improve clinical outcome (SANDOVAL-OLIVEROS and PAREDES-LÓPEZ, 2013; MARTÍNEZ-CRUZ and PAREDES-LÓPEZ, 2014; REYES-CAUDILLO *et al*., 2008). According to MARINELI *et al*. (2014) and COELHO and SALAS-MELLADO (2014), the consumption of chia seed can therefore serve as an important alternative for improving consumer health, suggesting its use as a functional food in the human daily diet. Previous studies have shown that pasta made with chia flour (7.5% and 10%) had a higher nutritional value and superior technological characteristics (OLIVEIRA *et al*., 2015; MENGA *et al*., 2017).
Due to the many properties that chickpea and chia possess, they are good candidates for use in the fortification of pasta to improve the nutritional quality in pasta formulations. Thus, the purpose of this study was to characterize pasta fortified with chickpea flour and chia flour in terms of their quality parameters, antioxidant properties and *in vitro* digestion.

2. MATERIAL AND METHODS

2.1. Materials

Commercial wheat (*Triticum durum*) semolina, Kabuli-type chickpea (*Cicer arietinum*) and chia (*Salvia hispanica*) were purchased at a local store. Chickpea grains were manually cleaned and dried at 60°C for 6 h. To obtain chickpea flour, a Perten model LM3100 (PerkinElmer, USA) mill was used. The chia grains were ground in an analytical mill (Braun Aromatic Coffee grind KSM2, USA) to obtain chia flour. Both flours were then standardized to a particle size of 60 mesh (0.25 mm).
All chemical reagents were purchased from local laboratory suppliers and were of analytical grade.

2.2. Pasta elaboration

Each blend was made with different proportions of commercial wheat (*T. durum*) semolina, chickpea flour, and chia flour, as shown in Table 1. Chia flour was the same in all formulations (10%) because, in preliminary studies, this amount was optimal for the sensory quality of pasta (data not shown). For its preparation, 100 g of flour was utilized. Distilled water was added at 35% absorption (considering the moisture of each mixture, between 35 and 42 mL/100 g of the mixture) and were mixed at room temperature in a mixer machine (professional model 600 HD, USA) at low speed (set 1) for 10 min. Afterwards, the dough was allowed to rest for 10 min in a plastic bag at room temperature. First, the proofed dough was laminated in the pasta machine (IMPERIAL R 220 model RMN, Italy) at setting 3, and finally at setting 1. The pasta was hand-cut into strips approximately 20 cm in length (fresh pasta) using a scissors. The pastas were kept to air-dry for 2 days at ambient temperature according to CLEARY and BRENNAN (2006). The six pasta samples were placed individually in sealed containers to avoid moisture exchange and were then stored at 5°C.

Table 1. Formulations for pasta made with the wheat semolina, chia flour and chickpea flour.

	Formulations (g/100 g)					
Flour	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Wheat semolina	100	85	65	45	25	0
Chia	0	10	10	10	10	10
Chickpea	0	5	25	45	65	90

2.3. Chemical analysis

The testing was performed according to Association of Official Analytical Chemists (AOAC) Official Methods. Semolina, chia, and chickpea flours and each pasta sample were analyzed for crude protein (method 955.04) and ash (method 920.153) using the AOAC (2000) methods. Total dietary fiber and fat were determined by methods 985.29 and 920.85, respectively according to AOAC (1997). Carbohydrates were determined by difference.

2.4. Cooking quality determinations

The cooking quality of the pasta, such as optimal cooking time, water absorption, weight gain by pasta, and solid loss during cooking, were evaluated using method 66-50 of the American Association of Cereal Chemists (AACC) Official Methods (AACC, 2000).

2.5. Determination of color

Color was evaluated utilizing the CIELAB system (Hunter Lab MiniScan EZ 45/0-L Model, USA). The analyzed parameters included the following: L^* (which represents the percentage of brightness, where black is 0% and white is 100%); a^* (where $+a^*$ is red and $-a^*$ is green), and b^* (where $+b^*$ is yellow and $-b^*$ is blue). The readings were taken at room temperature on the surface of the pastas, with 10 repetitions for each evaluated sample (OLIVEIRA *et al*., 2015).

2.6. Antioxidant activity

In determining the antioxidant activity of the different pasta formulations, the extracts were obtained first. All samples (raw and cooked) were adjusted to 0.17 g/mL with 1% hydrochloric acid in methanol (REHMAN and SHAH, 2005; LI *et al*., 2007). Using an orbital shaker (Thermo Scientific, USA) for 3 h at room temperature, the super natants of the samples were separated by centrifugation (10,000 rpm, 15 min, and 4°C). DPPH [2,2-DiPhenyl-1-PicrylHydrazyl] and ABTS [2,2′-Azino-Bis-(3 ethylbenzoThiazoline-6-Sulfonic acid)] assays were employed to quantify the antioxidant activity. Additionally, quantification of total phenolic content was determined.

2.6.1 DPPH assay

The antioxidant properties of the pasta samples using the DPPH assay were measured by the method described by MOLYNEUX (2004). Briefly, a 0.1 mL aliquot of the sample solutions was mixed with 3.9 mL of a free radical DPPH solution (6×10^{-5} mol/L). The reaction mixtures were incubated for 30 min in darkness and their absorbance was measured at 515 nm. All analyses were carried out in triplicate and a Trolox standard

curve was utilized for quantification. The results were reported as micromole of Trolox Equivalent per gram of sample (μmol TE/g).

2.6.2 ABTS assay

The ABTS assay was performed using the procedure of RE *et al*. (1999). A 2.97 mL of the cation radical solution was combined with 0.03 mL of the extract. The samples were incubated for 30 min at room temperature, and later, absorbance was measured at 734 nm. A control was prepared containing the cation radical solution with no pasta extracts, and another, with the solvent used. All analyses were performed in triplicate and a Trolox standard curve was used for quantification. The results were reported as micromole of Trolox Equivalent per gram of sample (μmol TE/g).

2.6.3 Total phenolic content

Quantification of total phenols was assayed by a spectrophotometric method utilizing the Folin-Ciocalteau reagent (SINGLETON *et al*., 1999). Thereafter, 50 μL of each extract solution was combined with 3 mL of deionized water and 250 μL of 1 N Folin-Ciocalteau reagent. After 5 min of incubation at room temperature, 750 μL of a 20% sodium carbonate solution and 950 μL of deionized water were added, measuring absorbance at 760 nm. A gallic acid standard calibration curve (0–100 mg/L) was prepared and the results were expressed as mg of Gallic Acid Equivalents per gram of sample (mg GAE/g).

2.7. *In vitro* digestion

This assay was conducted according to GIL-IZQUIERDO *et al*. (2002), but with some modifications. The cooked pastas were the samples used in this analysis. A total of 20 g of pasta was cooked in 100 mL of distilled water for 7.5 min. After that, 1 g of cooked pasta was removed, cut into small pieces of approximately 2 mm, and dissolved in distilled water at a material-to-solvent ratio of 1:15. This preparation was mixed in a vortex mixer (Daigger, Vortex-Genie 2) and was adjusted to pH 2.0 with 1 M HCl. A total of 0.5 mL was removed and combined with 0.75 mL of pepsin (315 U/mL prepared in 0.2 M KCl buffer) and 1.75 mL of deionized water. The samples were neutralized with 1.25 M $NaHCO_3$ after incubation at 37°C in a shaking water bath (Wise Bath, DAIHAN Scientific, WSB-18) at 80 rpm for 2 h. A total of 0.375 mL of pancreatin solution (4 mg/mL, prepared in 0.1 M Phosphate Buffer Saline [PBS]) was added to the samples. Afterward, the samples were transferred onto dialysis membranes (12,000 Da), placed in an Erlenmeyer flask containing 35 mL of 0.1 M PBS solution, and incubated again in the shaking water bath (4 h, 37°C, 80 rpm). Antioxidant activity and total phenols were determined before (initial) and after digestion (inside and outside of the membrane).

2.8. Consumer acceptance test

To evaluate the acceptability of the pasta, a 5-point hedonic scale was used for acceptance testing, in which the upper and lower extremes, respectively, correspond to 5 (liked very much) and 1 (disliked very much). The pasta was placed in boiling water with 5 g of salt and 10 mL of oil, and was subsequently served to tasters. The sensory panel was composed of 100 untrained tasters who were recruited randomly (OLIVEIRA *et al*., 2015).

2.9. Statistical analysis

Statistical analysis of the data obtained in all experiments were performed by Analysis of variance (ANOVA) using the StatGraphics Centurion ver. 17.0 XV ver. 15.2.06 statistical software. Comparison of means was performed by the Tukey least significance test (p<0.05). The experiments were run in triplicate.

3. RESULTS AND DISCUSSION

The chemical composition of the samples is presented in Table 2. Chia and chickpea seeds are rich in protein (27.94 and 22.93%, respectively) and fiber (30.02 and 20.22%, respectively) when compared to other grains such as barley, rice, amaranth, corn, and wheat. Several authors found similar results in protein and fiber content in chia and chickpea (MONDOR et al., 2009; OLIVEIRA *et al*., 2015; SÁNCHEZ-VIOQUE *et al*., 1999; MUÑOZ *et al*., 2012; SARGI *et al*., 2013). Despite the fact that chia had the highest quantity of protein, it has not been marketed, to our knowledge, as a source of protein, due to the fact that the profile of amino acids is limiting for schoolchildren (OLIVEIRA *et al*., 2015). However, it can be mixed with other grains to improve protein balance in formulations for adults (AYERZA and COATES, 2011). This is one of the reasons for the addition of chickpea to the formulation in this study. An increase in protein and fiber content was observed in the pasta formulations by increasing the amount of chickpea, bearing in mind that the amount of chia was the same in all of the formulations. This result is significant because pasta is considered a low nutritional-value product. Hence, chickpea considerably fortified the pasta (from F3 to F5) with protein and fiber in comparison with semolina pasta (F1). However, the sample in which semolina was replaced with chia flour and chickpea flour (F6) had the highest amount of protein and fiber, indicating that both seeds are good options for elaborating pasta rich in this type of compound.

Table 2. Chemical properties of chia flour, chickpea flour, and wheat semolina and the different pasta formulations (% dry base).

	Protein (%)	Fat (%)	Ash (%)	Total Fiber (%)	Carbohydrates (%)
Chia	27.94±0.55^{a}	35.31±0.11^{a}	4.46±0.06^{a}	30.02±0.11^{b}	2.26±0.96^{e}
Chickpea	22.93±0.25^{b}	8.02±0.31^{c}	2.88±0.02^{b}	20.22±1.12^{a}	45.95±0.58^{c}
Semolina	14.9±0.37^{e}	1.06±0.03^{d}	0.93±0.03^{d}	4.72±1.41^{g}	78.39±0.34^{a}
F1	15.14±0.48^{e}	1.14±0.03^{d}	0.94±0.02^{d}	4.25±1.41^{g}	78.53±0.53^{a}
F2	15.01±0.02^{e}	11.31±1.27^{b}	1.06±0.01^{d}	11.42±0.56^{f}	72.62±1.31^{b}
F3	17.26±0.44^{d}	12.38±0.42^{b}	1.81±0.02^{c}	14.02±0.63^{e}	68.55±0.68^{c}
F4	17.96±0.35^{d}	11.07±0.32^{b}	2.08±0.01^{b}	17.66±1.32^{d}	68.89±0.74^{c}
F5	18.92±0.51^{d}	12.35±0.64^{b}	2.65±0.02^{b}	19.29±1.08^{d}	66.08±1.20^{c}
F6	24.12±0.38^{b}	10.90±1.42^{b}	3.03±0.10^{b}	21.04±0.99c	61.95±1.81^{d}

Different letters within the same column indicate statistical differences (p < 0.05). F1: pasta with 100% semolina; F2: pasta with 85% semolina, 10 % chia flour and 5% chickpea flour; F3: pasta with 65% semolina, 10% chia flour and 25% chickpea flour; F4: pasta with 45% semolina, 10% chia flour and 45% chickpea flour; F5: pasta with 25% semolina, 10% chia flour and 65% chickpea flour; F6: pasta with 10% chia flour and 90% chickpea flour. Carbohydrates were determined by difference.

Furthermore, it was observed that fat content for chia-seed is high (35.31%), while fat content (8.02%) for chickpea is low. In comparison with F1, an increase in fat content was observed, from F2 to F6; however, there were no significant differences (p>0.05) between the fat content in these latter samples, indicating that chia could be the principal seed responsible for this increase. Alpha-linolenic acid represents 46.72%-62.44% of the total fatty acids in chia seed (PEIRETTI, 2011). It is an omega-3 fatty acid and is popular for preventing and treating diseases of the heart and blood vessels, among other functions (MANTZIORIS *et al*., 1994; ZIA-UI-HAQ *et al*., 2007). Additionally, alpha-linolenic acid is denominated "essential" because it cannot be synthesized by humans, but can only be consumed through foods. Therefore, pasta formulations with chia comprise a good option for consuming this compound.

On the other hand, ash content in chia is high (4.46%) compared with chickpea (2.88%) and other cereals such as rice, wheat, and sorghum. Magnesium, calcium, iron, and phosphorus are the minerals found in abundance in chia seed and are essential for a healthy diet (OLIVEIRA *et al*., 2015; CAPITANI *et al*., 2012). The quality of many foods depend on the concentration and type of minerals they contain, including their taste, appearance, texture, and stability. In the pasta formulations, the major content of ash was in F5 and F6 (2.65 and 3.03%, respectively), without a significant difference between them (p>0.05). Generally, all pastas have a high content of carbohydrates (61.95–78.53%), especially F1, which had the highest content. From F2 to F6, a decrease in these amounts were observed due to the increment of fiber influencing the calculation of carbohydrates, which is determined by difference. Similar behavior was obtained by PADALINO *et al*. (2014) in pastas enriched with pea flour and by OLIVEIRA *et al*. (2015) with pastas enriched with chia flour.

3.1. Cooking quality and color parameters

Table 3 depicts the results of the cooking quality and color parameters of the pastas. Optimal cooking time was the same (7.5 min) for all pasta types. OLIVEIRA *et al*. (2015) reported from 15-16 min cooking time in pastas with chia flour (7.5, 15, and 30%) instead of wheat flour, which is longer than the time frame used in our study.

Table 3. Cooking quality and color parameters for the different pasta formulations.

	F1	F2	F3	F4	F5	F6
Optimum cooking time (min)	7.5^{a}	7.5^{a}	7.5^{a}	7.5^{a}	7.5^{a}	7.5^{a}
Water absorption (%)	64.1±1.6^{a}	64.5±2.1^{a}	64.7±1.8^{a}	61.1±1.1^{b}	59.0±1.0^{b}	59.6±0.7^{b}
Percentage weight increase (%)	118.2±4.1^{a}	116.7±7.3^{a}	113.5±5.3^{b}	110.3±9.2^{b}	96.2±1.9^{c}	69.6±3.0^{d}
Solid loss (%)	4.5±0.5^{b}	4.6±0.3^{b}	4.6±0.2^{b}	4.9±0.0^{b}	4.7±0.4^{b}	6.3±0.9^{a}
*L**	75.5±0.1^{a}	57.4±1.5^{b}	55.2±1.1^{b}	53.6±0.1^{bc}	51.3±0.7^{c}	48.9±0.6^{c}
a	2.9±0.2^{c}	5.0±0.3^{b}	5.4±0.2^{b}	5.9±0.1^{b}	6.5±0.1^{b}	7.1±0.2^{a}
b	29.5±0.1^{a}	28.6±0.3^{a}	28.1±0.1^{a}	28.1±0.4^{a}	27.0±2.3^{a}	21.5±0.1^{b}

Different letters in the same line differ statistically at the 5% level by the Tukey's test.
F1: pasta with 100% semolina; F2: pasta with 85% semolina, 10 % chia flour and 5% chickpea flour; F3: pasta with 65% semolina, 10% chia flour and 25% chickpea flour; F4: pasta with 45% semolina, 10% chia flour and 45% chickpea flour; F5: pasta with 25% semolina, 10% chia flour and 65% chickpea flour; F6: pasta with 10% chia flour and 90% chickpea flour.

Another study with pastas contained 30% of chickpea obtained the cooking time of 10.5 min (ZHAO *et al*., 2005). Spaghetti with 30% of chickpea flour exhibited an optimal cooking time of 5.75 min (WOOD, 2009), which is lower than that in our study. Thus, our results (7.5 min) are within the average optimal cooking time of pastas fortified with these types of seeds.

Moreover, water absorption, weight increase, and the loss of soluble solids demonstrated significant differences (p<0.05) across treatments. Water absorption was higher, from F1 to F3, without significant differences among them (p >0.05). In this case, the level of substitution did not affect water absorption. Several studies indicated that the presence of chia increased water absorption due to the high fiber content of chia flour. The fibers, when mixed with water, form three-Dimensional (3-D) networks by means of the mucilaginous compounds that this seed contains (OLIVEIRA *et al*., 2015; MUÑOZ *et al*., 2012; CAPITANI *et al*., 2012). However, in our study, water absorption was not affected by the presence of chia. This behavior could be due to the fact that the interactions, mostly with chickpea, possessed fewer free bonds for binding the water molecules, thereby limiting the formation of 3D networks. Within this context, the weight increase in the sample is related to the water absorption, demonstrating similar behavior. Highest weight increase was obtained for samples with the highest content of semolina (F1 and F2). This fact can be explained by the high interaction with the water of the protein gluten (gliadin and glutenin) and the starch derived from the semolina (FEILLET and DEXTER, 1996). Similar results were obtained by FLORES-SILVA *et al*. (2014) in spaghetti made with chickpea, unripe plantain, and maize flours.

In addition, when semolina was replaced with chia flour and chickpea flour (F6), it exhibited a greater loss of soluble solids, which differed statistically from the rest of the samples (p<0.05). However, there were no significant differences from F1 to F5 (p>0.05), showing that semolina and its interaction with chia and chickpea prevents the loss of soluble solids. HUMMEL (1966) classified pastas according to loss of solids: up to 6% is characteristic of very good quality, up to 8% average quality, and values equal to or greater than 10% are low-quality pastas. Hence, according to this classification, pasta enriched with chia and chickpea in the presence of semolina are very good quality pastas. This result can be attributed to the protein contained in the samples, which causes the retention of amylose during cooking (CHILLO *et al*., 2008). Although, F6 possesses the highest protein content, it is evident that the absence of the semolina proteins increases the loss of soluble solids. OLIVEIRA *et al*. (2015) observed that the addition of chia flour improved the quality of the pastas by reducing the loss of solids. On the contrary, ZHAO *et al*. (2005) studied pastas with flours of legumes such as beans and chickpeas, in which the authors observed an increase in loss of solids during cooking. This was attributed to losses in the structural changes of the protein network due to partial substitution of wheat protein by legume protein (TORRES *et al*., 2007). However, in our case, the interaction among semolina, chia, and chickpea conferred better quality pastas.

The addition of chia and chickpea flour to the pasta showed a reduction in brightness (lower L*), more red (higher a*), and less yellow (lower b*), when compared with semolina pasta (F1), principally when the proportion of chickpea flour was increased (Table 3). Results of redness (a*) values reached the maximal values (7.1). The greater intensity of the yellow color is a highly desirable feature in pasta products, because it is one of the most influential visual appeals in the acceptance of pastas (CHANG and FLORES, 2004). F1 pasta differed statistically from others, featuring greater intensity of the yellow color (b* value), thus favoring acceptability. These results were similar to those of several studies of pasta fortified with chia or chickpea (WOOD, 2009; ABOU-ARAB *et al*., 2010; OLIVEIRA *et al*., 2015).

3.2. Antioxidant activity and total phenols

DPPH, ABTS, and total phenol determinations of raw and cooked pasta samples are illustrated in Fig. 1. Antioxidant activity and total phenols content increased when the chickpea concentration increases. Similar results were observed by FARES and MENGA (2012) in durum wheat pasta enriched with chickpea flour and by KHAN *et al*. (2013) in durum wheat pasta enriched with sorghum. The samples that presented highest antioxidant capacity in both radicals were F5 (DPPH 698.54 μmol TE/g, ABTS 1029.16 μmol TE/g), and F6 (DPPH 756.12 μmol TE/g, ABTS 1063.8 μmol TE/g). However, ABTS exhibits more affinity (Fig. 1b) for the pasta compounds than DPPH (Fig. 1a). This could be explained by the ABTS reacting more specifically with an H-atom donor than DPPH, thereby accomplishing the ABTS reaction faster (PRIOR *et al*., 2005; ROGINSKY and LISSI, 2005; ROSA-ALCARAZ *et al*., 2017). Additionally, ABTS can measure hydro- and lipophilic compounds (KRISHNAIAH *et al*., 2011). On the other hand, SHARMA and BHAT (2009) indicated that the DPPH radical reacts to a greater degree with lipophilic compounds, but other researches mentioned that this radical does not react mainly with flavonoids, which contain no hydroxyl groups in the B-ring, as well as with aromatic acids containing only one OH-group (VON GADOW *et al*., 1997; YOKOZAWA *et al*., 1998). The phenolic compounds in chia (rosmarinic acid, protocatechuic acid, caffeic acid, and gallic acid, among others) and chickpea (*p*-hydroxybenzoic acid, vanillic acid, ferulic acid, and *p*-coumaric acid, among others) comprise hydrophilic compounds to a greater extent (FARES and MENGA, 2012; MARTÍNEZ-CRUZ and PAREDES-LÓPEZ, 2014; MENGA *et al*., 2017). Additionally, these compounds have more H-atom donor or aromatic acids containing only one OH-group and, according to what has been previously mentioned, this explains the behavior observed in high affinity for ABTS rather than for the DPPH radical.

In our study, antioxidant capacity is higher than in other uncooked pasta (maximum of 1,063.8 μmol TE/g); for example, in durum wheat pasta enriched with sorghum that has a maximum of 33.7 μmol TE/g (KHAN *et al*., 2013). In cooked pasta, the maximal amount obtained in our study was 800.67 μmol TE/g, a higher amount compared with pasta supplemented with germinated pigeon pea flour (5.8 μmol TE/g) (TORRES *et al*., 2007) and in pasta enriched with bean flour (1.26 μmol TE/g) (GALLEGOS-INFANTE *et al*., 2012). Temperature affects antioxidant activity, with a loss of approximately 50% in DPPH (Fig. 1a) and of 30% in ABTS (Fig. 1b). The same behavior was observed in the concentration of phenolic compounds, these decrease is approximately 30% in cooked pasta (Fig. 1c). Similar results were obtained by KHAN *et al*. (2013) in durum wheat pasta enriched with sorghum, where the authors demonstrated a decrease of 20-55% in antioxidant capacity and a decrease of 21-55% of phenolic compounds in cooked pasta.

There were no significant differences ($p > 0.05$) between 65% and 90% of chickpea in the formulations in each radical and in the amount of total phenols (14.76 and 16.34 mg GAE/g, respectively). These results were higher than those reported by GALLEGOS-INFANTE *et al*. (2012) in pasta enriched with bean flour (0.49 mg GAE/g), by TURCO *et al*. (2016) in pasta with faba bean flour (1.85 mg GAE/g), and by KHAN *et al*. (2013) in wheat pasta with sorghum (1.27-3.22 mg GAE/g). The correlation among antioxidant activities with phenols was carried out, and it was observed that this correlation was high ($R^2 = 0.921$). This indicates that phenols were the principal compounds responsible for antioxidant activity in the pasta, while pasta made only with semolina (F1) presented very low antioxidant activity and phenolic content, confirming the effect of the fortification accomplished with chia and chickpea.

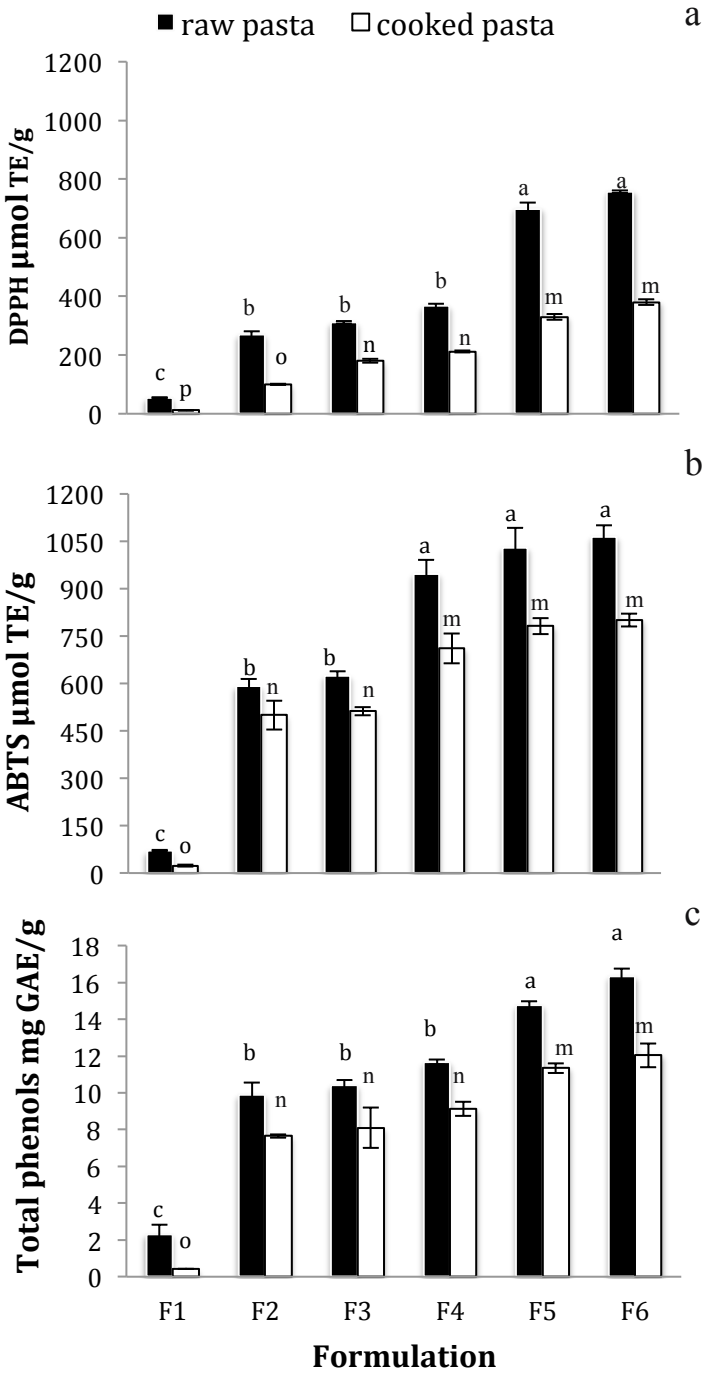


Figure 1. Antioxidant activity by DPPH (a), ABTS (b) and Total phenols (c) of raw and cooked pastas. Columns not sharing a common letter within the same treatment (raw or cooked) of pasta are significantly different ($P< 0.05$). F1: pasta with 100% semolina; F2: pasta with 85% semolina, 10 % chia flour and 5% chickpea flour; F3: pasta with 65% semolina, 10% chia flour and 25% chickpea flour; F4: pasta with 45% semolina, 10% chia flour and 45% chickpea flour; F5: pasta with 25% semolina, 10% chia flour and 65% chickpea flour; F6: pasta with 10% chia flour and 90% chickpea flour.

Cooked F1 pasta demonstrated a decrease of approximately 80% when compared with uncooked pasta, indicating that chia-chickpea-fortified pastas have lower cooking losses.

Some authors came to the same conclusion when chickpea-fortified spaghetti and apple by-product-fortified pasta were compared (WOOD, 2009; LONČARIĆ *et al*., 2014).

3.3. *In vitro* digestion

In vitro digestion was carried out only in cooked pastas using pepsin, pancreatin, and dialysis membranes to simulate the intestine. The most important finding in our study comprised the antioxidant capacity and the amount of phenolic compounds that pass through the membrane, where the outside of the membrane simulates the blood serum. The results revealed major antioxidant capacity in both radicals and the major amount of phenolic compounds outside of the membrane when the amount of semolina is decreased or absent in the formulations (Fig. 2). There were no significant differences (p>0.05) in antioxidant capacity between F5 and F6 (Figs. 2a and 2b). However, the highest antioxidant activity was detected in ABTS radical by F5 (655.1 ± 7.56 μmol ET/g) and F6 (703.4 ± 14.98 μmol ET/g). More than 85% of this antioxidant capacity was maintained outside of the membrane, which is considered high. Compared with the F5 and F6 undigested samples in ABTS radical (781.43 μmol ET/g and 800.67 μmol ET/g, respectively), there was an approximate decrease of 16% in F5 and 12% in F6 of antioxidant capacity.

Also, comparison with the F5 and F6 undigested samples (367 μmol ET/g and 388.21 μmol ET/g, respectively) in the DPPH radical revealed a major decrease of approximately 50% in F5 and 45.8% in F6 on the antioxidant capacity. As discussed previously, phenols were the principal compounds responsible for the antioxidant activity in the pasta; thus, if these compounds were affected by the digestive process, consequently, the antioxidant activity was affected. In our case, the phenolic compounds outside of the membrane in F5 (9.82 ± 0.1 mg GAE/g) and F6 (10.97 ± 0.3 mg GAE/g) exhibited the highest amounts, maintaining approximately more than 90% of these compounds compared with the initial amount (Fig. 2c). Hence, the differences between antioxidant activities in both radicals could be due to the affinity of the compounds in each sample and/or to their bioaccessibility (fraction of bioactive substance that is released from the food matrix) that consequently affects their bioavailability (amount of phenolic compounds reaching the blood circulation system), (RODRÍGUEZ-ROQUE *et al*., 2014). CATTANEO *et al*. (2015) found that after a simulated gastric condition, the antioxidant activity of puffed kernels was high compared to the undigested sample measured by ABTS and FRAP methods. RUFIÁN-HENARES and DELGADO-ANDRADE (2009) obtained similar behavior in flaked wheat-based breakfast cereals measured by ABTS, DPPH and FRAP methods.

In this context, some factors can affect the bioaccessibility and bioavailability of phenolic compounds. Studies indicated that in the cell wall of legume/seeds, these compounds could be present in insoluble-bond forms (covalent bonds, 20–60%) (SHAHIDI and YEO, 2016). These insoluble bonds affect the bioaccessibility and consequently, the bioavailability of the phenolic compounds because these bonds need to be cut by specific enzymes that generally are provided by intestinal microbiota (PEREZ-PEREZ *et al*., 2018). Most native polyphenols in foods are in glycoside form, which cannot be absorbed in the intestinal mucosa; therefore, the release of these performed by human and microbial enzymes is a necessary mechanism needed by them to break through the intestinal barrier (VALDÉS *et al*., 2015). In our study, we focused on the soluble forms (non-covalent bonds) of the phenolic compounds through *in vitro* digestion without the influence of microbiota. Thus, additional research should be carried out on the absorption of phenolics in the human colon. Also, hydrophobicity, the membrane-mediated transport, the stability of the compounds, environmental pH, degree of polymerization, interactions with other

compounds, molecular mass, and the complexity of the food matrix are some other factors that influence the absorption of phenolic compounds (TARKO *et al*., 2009; RODRÍGUEZ-ROQUE *et al*., 2014; SHAHIDI and YEO, 2016).
In our opinion, the majority of *in vitro* digestion studies of different fortified pastas have for long focused on starch digestibility. The principal interest of these studies is that sugars are progressively released from pasta during digestion, leading to a standard increase in postprandial blood glucose and insulin response (GOÑI and VALENTÍN-GAMAZO, 2003; PETITOT *et al*., 2010; FLORES-SILVA *et al*., 2014;PADALINO *et al*., 2015;GELENCSÉR *et al*., 2008; REYES-CAUDILLO *et al*., 2008; ZHENG *et al*., 2016). Our study focused on the bioaccessibility of phenolic compounds and antioxidant capacity after *in-vitro* digestion in order to provide additional and valuable information on the high effect of chia-chickpea-fortified pasta in the scavenging of free radicals. Thus, the antioxidant property of this fortified pasta could be involved in the defense mechanism of the organism against pathologies associated with the attack of free radicals, such as cancer, coronary heart disease, obesity, type 2 diabetes and hypertension, among others (PISOSCHI and NEGULESCU, 2011). With the results obtained in our research, pasta fortified with chia and chickpea can maintain high levels of phenolic compounds and antioxidant capacity after *in- vitro* digestion, therefore, making it capable of providing health benefits.

3.4. Consumer acceptance test

The results of consumer acceptability are presented in Fig. 3. Highest overall acceptability by tasters was the pasta with 100% semolina (F1) and samples containing wheat semolina (45% and 25%), chia (10%), and chickpea (45% and 65%) flours (F4 and F5, respectively), which revealed no significant differences (p >0.05). Similar results were obtained by ZHAO *et al*. (2005) in spaghetti fortified with chickpea. The pasta in which chia and chickpea are utilized instead of wheat semolina (F6) triggered the least acceptance. Therefore, the presence of wheat semolina is important for the sensory properties of fortified pastas. Additionally, PETITOT *et al*. (2010) noted that the addition of a high level of legume flour induced some minor structural changes in pasta. This explains the high acceptability of pastas with a high content of chickpea, containing semolina in their formulation. The addition of chia and chickpea causes inclusion of fibers, dilution of gluten proteins by albumins and globulins, and the largest amount of thin protein films, which may have favored the higher susceptibility of starch to digestive enzymes. Consequently, the acceptability of these types of fortified pastas is presently on the increase. If we were required to choose a pasta sample, it would of necessity be the sample that complies with all of the parameters (high quality parameters, phenol content, antioxidant capacity, and consumer acceptance). In accordance with all of the results in this work, this selected sample would be F5 (25% semolina, 10% chia flour, and 65% chickpea flour).

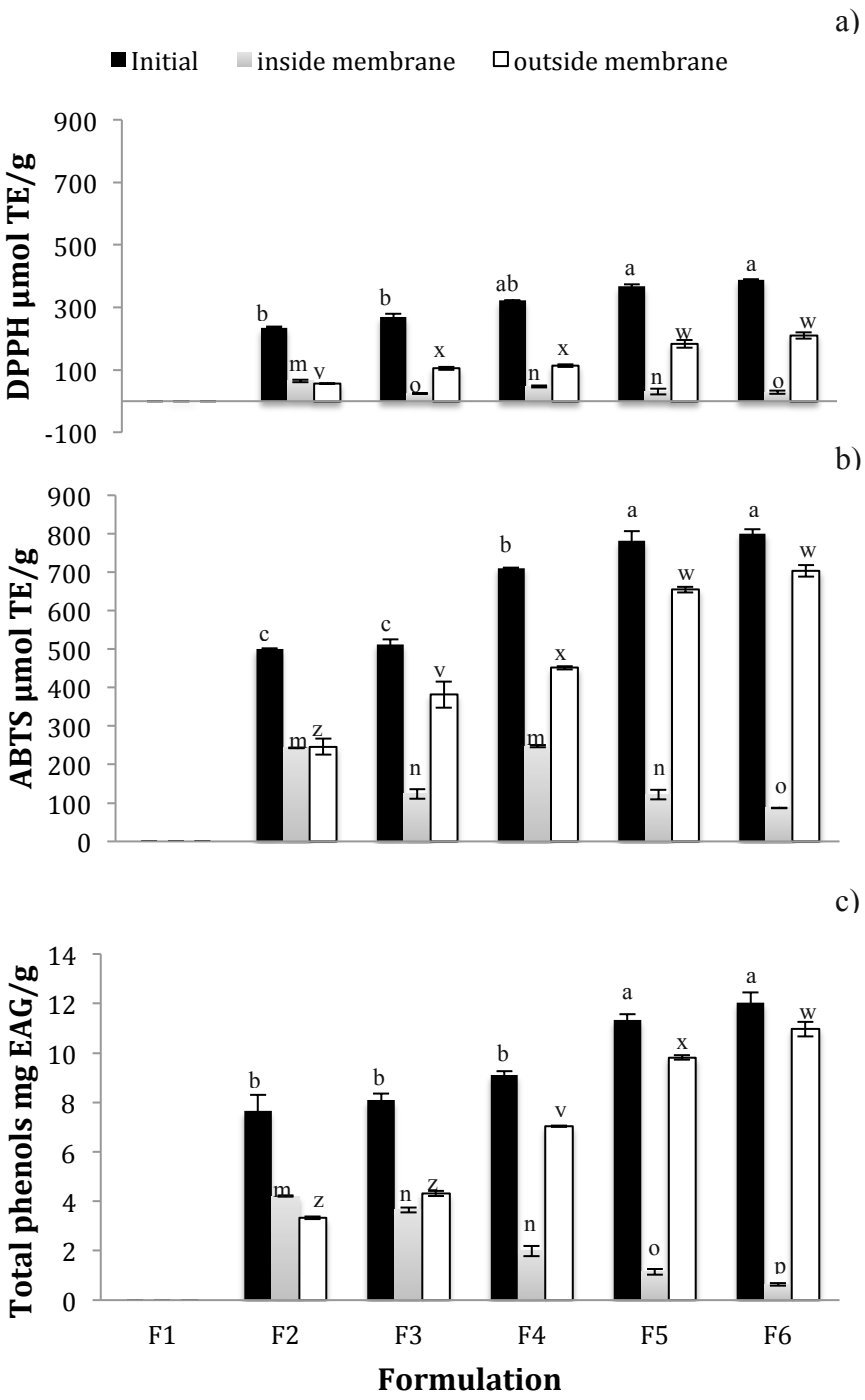


Figure 2. Antioxidant activity measured by DPPH (a) and ABTS (b) and total phenols (c) of digested and undigested (initial) cooked pasta.
Columns not sharing a common letter within the same status (initial, inside membrane and outside membrane) of pasta are significantly different ($P < 0.05$). F1: pasta with 100% semolina; F2: pasta with 85% semolina, 10 % chia flour and 5% chickpea flour; F3: pasta with 65% semolina, 10% chia flour and 25% chickpea flour; F4: pasta with 45% semolina, 10% chia flour and 45% chickpea flour; F5: pasta with 25% semolina, 10% chia flour and 65% chickpea flour; F6: pasta with 10% chia flour and 90% chickpea flour.

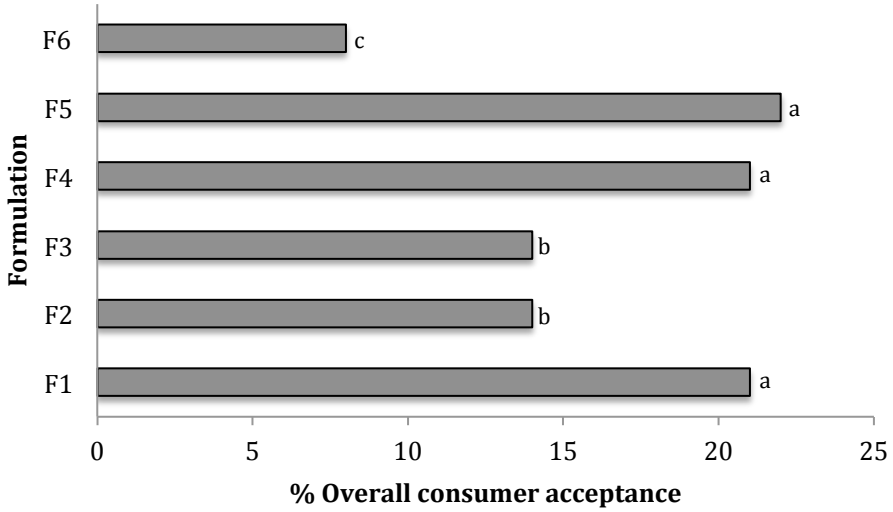


Figure 3. Percentage of consumer acceptance of the different pasta formulations.
The sensory panel was composed of 100 untrained tasters. Identical letters do not differ statistically at the 5% level by the Tukey's test. F1: pasta with 100% semolina; F2: pasta with 85% semolina, 10 % chia flour and 5% chickpea flour; F3: pasta with 65% semolina, 10% chia flour and 25% chickpea flour; F4: pasta with 45% semolina, 10% chia flour and 45% chickpea flour; F5: pasta with 25% semolina, 10% chia flour and 65% chickpea flour; F6: pasta with 10% chia flour and 90% chickpea flour.

4. CONCLUSIONS

In this work, we studied the effect of pasta with the addition of chickpea flour and chia flour in terms of quality parameters, antioxidant properties, and their *in vitro* digestion. The results revealed that the addition of these flours increases protein and fiber content, indicating that both seeds comprise good options for elaborating pasta rich in these types of compounds. Cooking parameters are important for consumers; in our results, the level of substitution of chia and chickpea did not affect water absorption; additionally, the presence of these seeds in the pasta avoided the loss of soluble solids. Thus, the interaction among semolina, chia, and chickpea yielded better quality pastas. Antioxidant activity and total phenols content increased in the fortified pastas. Although, cooking affected the amount of phenols and antioxidant capacity, *in vitro* digestion studies demonstrated that pasta fortified with chia and chickpea can maintain high levels of phenolic compounds and antioxidant capacity after *in-vitro* digestion, thus being able to provide health benefits. However, *in vivo* measurements are needed to confirm any biological effects. Chia-chickpea-fortified pasta would make an inexpensive, attractive, convenient and healthy food that is acceptable to consumers. Therefore, with the results obtained in our research, chia and chickpea can be utilized to develop more value-added products, thus rendering them more economical and affordable for developing countries.

ACKNOWLEDGEMENTS

This work was supported by Grant USO313002193 from the University of Sonora, Mexico.
We are grateful to CONACyT-México for supporting the PhD fellowship, number CVU 590932, granted to Liliana Maribel Perez-Perez.

REFERENCES

AACC. 2000. Official methods of the American Association of Cereal Chemists. 10th ed. St.Paul, MN, USA.

AOAC. 1997. Official methods of analysis. 17th ed. Gaithersburg, Md.: Assn. of Official Analytical Chemists.

AOAC. 2000. Association of Official Analytical Chemists. Official methods of analysis of AOAC. 19th ed, 2012. Arlington. USA.

Abou-Arab E.A., Helmy I.M.F. and Bareh G.F. 2010. Nutritional evaluation and functional properties of chickpea (*Cicer arietinum* L.) flour and the improvement of spaghetti produced from its. J. Am. Sci. 6(10):1055.

Aguilera Y., Dueñas M., Estrella I., Hernández T., Benitez V., Esteban R.M. and Martín-Cabrejas M.A. 2011. Phenolic profile and antioxidant capacity of chickpeas (*Cicer arietinum* L.) as affected by a dehydration process. Plant Foods Hum. Nutr. 66:187.

Ayerza R. and Coates W. 2011. Protein content, oil content and fatty acid profiles as potential criteria to determine the origin of commercially grown chia (*Salvia hispanica* L.). Ind. Crops Prod. 34(3):1366.

Bashir K., Aeri V. and Masoodi L. 2012. Physio-chemical and sensory characteristics of pasta fortified with chickpea flour and defatted soy flour. IOSR J. Environ. Sci. Toxicol. Food Technol. 1(5):34..

Borneo R. and Aguirre A. 2008. Chemical composition, cooking quality, and consumer acceptance of pasta made with dried amaranth leaves flour. LWT - Food Sci. Technol. 41:1748.

Capitani M.I., Spotorno V., Nolasco S.M. and Tomás MC. 2012. Physicochemical and functional characterization of by-products from chia (*Salvia hispanica* L.) seeds of Argentina. LWT. Food Sci. Technol. 45:94.

Cattaneo S., Hidalgo A., Masotti F., Stuknyte M., Brandolini A. and De Noni I. 2015. Heat damage and *in vitro* starch digestibility of puffed wheat kernels. Food Chem. 188: 286.

Chang Y. and Flores H. 2004. Qualidade tecnológica de massas alimentícias frescas elaboradas de semolina de trigo durum (*T. durum L*) e farinha de trigo (*T. aestivum L*). (Quality of fresh pastas obtained from blends of durum wheat semolina (*T. durum* L.) and wheat flour (*T. aestivum* L.).) Ciênc. Tecnol. Aliment. 24(4):487.

Chillo S., Laverse J., Falcone P. and Del Nobile M. 2008. Quality spaghetti in base amaranthus whole meal flour added with quinoa broad bean and chickpea. J. Food Eng. 84:101.

Cleary L. and Brennan C. 2006. The influence of a (1 fi 3)(1 fi 4)-b-D-glucan rich fraction from barley on the physico-chemical properties and *in vitro* reducing sugars release of durum wheat pasta. Int. J. Food Sci. Technol. 41:910.

Coates W. and Ayerza H.R. 1996. Production potential of chia in north-western Argentina. Ind. Crops Prod. 5(3):229.

Coelho M.S. and Salas-Mellado M.M. 2014. Chemical characterization of chia (*Salvia hispanica L.)* for use in food products. J. Food Nutr. Res. 2(5):263.

Fares C. and Menga V. 2012. Effects of toasting on the carbohydrate profile and antioxidant properties of chickpea (*Cicer arietinum* L.) flour added to durum wheat pasta. Food Chem. 131:1140.

Feillet P.Y. and Dexter J.E. 1996. Quality requirement of durum wheat of semolina milling and pasta production. Ch. 5. In "Pasta and noodle technology". Kruger, J.E., Matsuo, R.B. and Dick, J.W (Ed.), p. 95. St. Paul Minnesota. USA.

Flores-Silva P.C., Berrios De J.J., Pan J., Osorio-Díaz P. and Bello-Pérez L.A. 2014. Gluten-free spaghetti made with chickpea, unripe plantain and maize flours: functional and chemical properties and starch digestibility. Int. J. Food Sci. Technol. 49:1985.

Gallegos-Infante J.A., García R.M., Chang S., Manthey F., Yao R.F., Rynoso-Camacho R., Rocha-Guzmán N.E. and González-Laredo R.F. 2012. Effect of the addition of common bean flour on the cooking quality and antioxidant characteristics of spaguetti. J. Micriobiol. Biotechnol. Food Sci. 2(2):730.

Gelencsér T., Gál V., Hódsági M. and Salgó A. 2008. Evaluation of quality and digestibility characteristics of resistant starch-enriched pasta. Food Bioprocess. Tech. 1(2):171.

Gil-Izquierdo A., Zafrilla P. and Tomás-Barberán F.A. 2002. An *in vitro* method to simulate phenolic compound release from the food matrix in the gastrointestinal tract. Eur. Food Res. Technol. 214:155.

Goñi I. and Valentín-Gamazo C. 2003. Chickpea flour ingredient slows glycemic response to pasta in healthy volunteers. Food Chem. 81:511.

Han X., Shen T. and Lou H. 2007. Dietary polyphenols and their biological significance. Int. J. Mol. Sci. 8:950.

Hirdyani H. 2014. Nutritional composition of chickpea (*Cicer arietinum* L.) and value added products - a review. Indian J. Community Health. 26 (Suppl S2):102.

Hummel, C (Ed.). 1966. "Macaroni products**:** Manufacture, Processing and Packing" 2nd ed. Food Trade Press. London.

Jukanti A.K., Gaur P.M., Gowda C.L.L. and Chibbar R.N. 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br. J. Nutr. 108:S11.

Khan I., Yousif A., Johnson SK. and Gamlath S. 2013. Effect of sorghum flour addition on resistant starch content, phenolic profile and antioxidant capacity of durum wheat pasta. Food Res. Int. 54:578.

Krishnaiah D., Sarbatly R. and Nithyanandam R. 2011. A review of the antioxidant potential of medicinal plant species. Food Bioprod. Process. 89(3):217.

Li W., Pickard M.D. and Beta T. 2007. Evaluation of antioxidant activity and electronic taste and aroma properties of antho-beers from purple wheat grain. J. Agric. Food Chem. 55:8958.

Lončarić A., Kosović I., Jukić M., Ugarčić Z. and Piližota V. 2014. Effect of apple by-product as a supplement on antioxidant activity and quality parameters of pasta. Croat. J. Food Sci. Technol. 6(2):97.

Mantzioris E., James M.J., Gibson R.A. and Cleland LG. 1994. Dietary substitution with an alpha-linolenic acid-rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. Am. J. Clin. Nutr. 59(6):1304.

Marineli R.S., Aguiar M.E., Alves L.S., Teixeira G.A., Nogueira E.M. and Maróstica M.RJr. 2014. Chemical characterization and antioxidant potential of Chilean chia seeds and oil (*Salvia hispanica* L.). LWT - Food Sci. Technol. 59:1304.

Martínez-Cruz O. and Paredes-López O. 2014. Phytochemical profile and nutraceutical potential of chia seeds (*Salvia hispanica* L.) by ultra high performance liquid chromatography. J. Chromatogr. A. 1346:43.

Menga V., Amato M., Phillips T.D., Angelino D., Morreale F. and Fares C. 2017. Gluten-free pasta incorporating chia (*Salvia hispanica* L.) as thickening agent: An approach to naturally improve the nutritional profile and the *in vitro* carbohydrate digestibility. Food Chem. 221:1954.

Perez-Perez M., García-Borbón L., González-Vega R.I., Rodríguez-Figueroa J.C., Rosas-Burgos E.C., Huerta-Ocampo J.A., Ruiz-Cruz S., Wong-Corral F.J., Borboa-Flores J., Rueda-Puente E.O. and Del-Toro-Sánchez C.L. 2018. Release of linked phenolic compounds in chickpea (*Cicer arietinum*) using intestinal human microbiota. Biotecnia. 20(3):146.

Molyneux P. 2004.The use of the stable radical Diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26(2):211.

Mondor M., Aksay S., Drolet H., Roufik S., Farnworth E. and Boye J.I. 2009. Influence of processing on composition and antinutritional factors of chickpea protein concentrates produced by isoelectric precipitation and ultrafiltration. Innov. Food Sci. Emerg. Technol. 10:342.

Muñoz L.A., Cobos A., Diaz O. and Aguilera J.M. 2012. Chia seeds: microstructure, mucilage extraction and hydratation. J. Food Eng. 108:216.

Oliveira M.R., Novack M.E., Santos C.P., Kubota E. and Rosa R.Cda. 2015. Evaluation of replacing wheat flour with chia flour (*Salvia hispanica* L.) in pasta. Semina: Ciênc Agrár. 36(4): 2545.

Padalino L, Mastromatteo M., Lecce L., Spinelli S., Contò F. and Del Nobile M.A. 2014. Chemical composition, sensory and cooking quality evaluation of durum wheat spaghetti enriched with pea flour. Int. J. Food Sci. Technol. 49(6):1544.

Padalino L., Mastromatteo M., Lecce L., Spinelli S., Conte A. and Del Nobile M.A. 2015. Optimization and characterization of gluten-free spaghetti enriched with chickpea flour. Int. J. Food Sci. Nutr. 66(2):148.

Peiretti P.G. 2011. Fatty acid content and chemical composition of vegetative parts of perilla (*Perilla frutescens* L.) after different growth lengths. Res. J. Med Plants. 5:72.

Petitot M., Barron C., Morel M.H. and Micard V. 2010. Impact of legume flour addition on pasta structure: Consequences on its *in vitro* starch digestibility. Food Biophys. 5:284.

Pisoschi A.M. and Negulescu G.P. 2011. Methods for Total Antioxidant Activity Determination: A Review. Biochem. Anal. Biochem. 1:106.

Prior R.L., Wu X. and Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53(10):4290.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26(1):1231.

Rehman Z.U. and Shah W.H. 2005. Thermal heat processing effects on antinutrients, protein and starch digestibility of food legumes. Food Chem. 91:327.

Reyes-Caudillo E., Tecante A. and Valdivia-López M.A. 2008. Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican chia (*Salvia hispanica* L.) seeds. Food Chem. 107:656.

Rodríguez-Roque M.J., Rojas-Graü M.A., Elez-Martínez P. and Martín-Belloso O. 2014. *In vitro* bioaccessibility of health-related compounds as affected by the formulation of fruit juice- and milk-based beverages. Food Res. Int. 62:771.

Roginsky V. and Lissi E.A. 2005. Review of methods to determine chainbreaking antioxidant activity in food. Food Chem. 92(2):235.

Rosa-Alcaraz M.A., Torrescano-Urrutia G.R., Pérez-Álvarez J.A., Fernández-López J. and Sánchez-Escalante A. 2017. Evaluación de fitoquímicos y actividad antioxidante de suproductos de dátil (*Phoenix dactylifera* L.) producidos en el estado de Sonora. Biotecnia. 19(3):11.

Rufián-Henares J.A. and Delgado-Andrade C. 2009. Effect of digestive process on Maillard reaction indexes and antioxidant properties of breakfast cereals. Food Chem. 42:394.

Sabanis D., Makri E. and Doxastakis G. 2006. Effect of durum flour enrichment with chickpea flour on the characteristics of dough and lasagne. J. Sci. Food Agric. 86:1938.

Sánchez-Vioque R., Clemente A., Vioquea V., Bautista J. and Millán F. 1999. Protein isolates from chickpea (*Cicer arietinum* L.): chemical composition, functional properties and protein characterization. Food Chem. 64:237.

Sandoval-Oliveros M.R. and Paredes-López O. 2013. Isolation and characterization of proteins from chia seeds (*Salvia hispánica* L.). J. Agric. Food Chem. 61:193.

Sargi S.C., Silva B.C., Santos H.M.C., Montanher P.F., Boeing J.S., Santos Júnior O.O., Souza N.E. and Visentainer J.V. 2013. Antioxidant capacity and chemical composition in seeds rich in Omega 3: chia, flax, and perilla. Ciênc. Tecnol. Aliment. 33(3):541.

Shahidi R. and Yeo J.D. 2016. Insoluble-Bound Phenolics in Food. Molecules. 21(9):E1216.

Sharma O.P. and Bhat T.K. 2009. DPPH antioxidant assay revisited. Food Chem. 113:1202.

Singleton V.L., Orthofer R. and Lamuela-Raventós R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 299:152.

Sreerama Y.N., Shashikala V.B. and Pratape V.M. 2010. Variability in the distribution of phenolic compounds in milled fractions of chickpea and horse gram: Evaluation of their antioxidant properties. J. Agric. Food Chem. 58(14):8322.

Tarko, T., Duda-Chodak, A., Sroka, P., Satora, P. and Michalik, J. 2009. Transformations of phenolic compounds in an in vitro model simulating the human alimentary tract. Food Technol. Biotechnol. 47(4):456.

Thomasset S.C., Berry D.P., Garcea G., Marczylo T., Steward W.P. and Gescher A.J. 2007. Dietary polyphenolic phytochemicals promising cancer chemopreventive agents in humans? A review of their clinical properties. Int. J. Cancer. 120(3):451.

Torres A., Frias J., Granito M. and Vidal-Valverde C. 2007. Germinated *Cajanus cajan* seeds as ingredients in pasta products: Chemical, biological and sensory evaluation. Food Chem. 101:202.

Turco I., Bacchetti T., Bender C., Oboh G., Zimmermann B. and Ferretti G. 2016. Polyphenol content and glycemic load of pasta enriched with Faba bean flour. Funct. Food Health Dis. 6(5):291.

Valdés, L., Cuervo, A., Salazar, N., Ruas-Madiedo, P., Gueimonde, M. and González, S. 2015. The relationship between phenolic compounds from diet and microbiota: impact on human health. Food Funct. 6(8):2424.

von Gadow A., Joubert E. and Hansmann C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of Rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. J. Agric. Food Chem. 45:632.

Wood J.A. 2009. Texture, processing and organoleptic properties of chickpea-fortified spaghetti with insights to the underlying mechanisms of traditional durum pasta quality. J. Cereal Sci. 49:128.

Xu B.J. and Chang S.K.C. 2010. Phenolic substance characterization and chemical and cell-based antioxidant activities of 11 lentils grown in the northern United States. J. Agric. Food Chem. 58(3):1509.

Yang C.S., Landau J.M., Huang M.T. and Newmark H.L. 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr. 21:381.

Yokozawa T., Chen C.P., Dong E., Tanaka T., Nonaka G.I. and Nishioka I. 1998. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. Biochem. Pharmacol. 56(2):213.

Zhao J.H., Manthey F., Chang K., Hou H. and Yuan H. 2005. Quality characteristics of spaghetti as affected by green and yellow pea, lentil and chickpea, flours. J. Food Sci. 70(3):371.

Zheng Z., Stanley R., Gidley M.J. and Dhital S. 2016. Structural properties and digestion of green banana flour as a functional ingredient in pasta. Food Funct. 7(2):771.

Zia-UI-Haq M., Iqbal S., Imran M., Niaz A. and Bhanger M.I. 2007. Nutritional and compositional study of Desi chickpea (*Cicer arietinum* L.) cultivars grown in Punjab, Pakistan. Food Chem. 105(4):1357.

Paper Received August 11, 2018 Accepted December 10, 2018

PAPER

AN INVESTIGATION OF THE MECHANICAL, MICROSTRUCTURAL AND THERMO-MECHANICAL PROPERTIES OF COLLAGEN FILMS CROSS-LINKED WITH SMOKE CONDENSATE AND GLUTARALDEHYDE

S. BARBUT* and M. IOI
University of Guelph, Department of Food Science, Guelph, Ontario, Canada, N1G 2W1
*Corresponding author: Tel.: +15198244120
E-mail address: sbarbut@uoguelph.ca

ABSTRACT

Collagen films were produced from five commercial collagen dispersions used for in-line sausage co-extrusion production. Films were prepared by partially dehydrating in a salt solution (30%) and crossed linked with smoke condensate (15%) or glutaraldehyde (GA; 0-1%). Both treatments increased the tensile strength (0.32 to 0.91 MPa) and reduced % elongation while differences among the dispersions were observed. Overall, % elongation generally decreased with a higher degree of cross linking. Transmission electron micrographs revealed that collagen fibers were swollen to varying degrees, likely influencing the mechanical behaviours. Protein concentration affected the transparency of the films with a difference of 100% in light transmission between the clearest and most opaque film studied. Cross-linking with GA appeared to thermally stabilize films up to 80˚C. Aldehydes in the smoke condensate were identified by gas chromatography showing highest concentration of benzaldehyde, 4-hydroxy-3,5-dimethoxy.

Keywords: casings, coating, collagen, cross-linking, glutaraldehyde, smoke condensate

1. INTRODUCTION

Sausage products rely on casings to contain, form and bind the raw ground/emulsified meat placed inside them. After cooking (heat induced gelation) of the meat proteins, the casings can be removed from the product by the food processor (e.g., frankfurters produced in cellulose casings), by the consumer (salami produced in plastic casings) or consumed with the product (pepperettes produced in natural casings). Prior to the early 20th century almost all casings were derived from animal intestines. Since then, advancements in casing technology and manufacturing have led to several types of synthetic casings (e.g., cellulose, plastic) that can be tailored to a given process and product. With increasing sophistication, the production of manufactured casings was moved to designated plants, where collagen derived from hides is extracted, formed into tubes and then crosslinked with a strong crosslinking agent such as glutaraldehyde. When co-extrusion was more recently commercialized, on a large industrial scale (SAVIC and SAVIC, 2016), direct casing application was moved to the meat processing facilities. However, the issue of finding an acceptable mild crosslinking agent, that will also enable operating at a high speed production line, is still a challenge today (i.e., glutaraldehyde is not permitted to be used in food production areas, and in the off-premises casing manufacturing plants it is thoroughly washed away prior to shipping) and hence the need for the current investigation.

Co-extrusion is the process of extruding a cylindrical core of sausage meat, while simultaneously extruding an outer layer of casing material directly on to the meat's surface. Subsequent processes include: brining, smoking, drying and cooking. They are needed to stabilize and harden the casing material by osmotically removing water, and later chemically cross-linking the building blocks of the casing material (e.g., collagen, alginate). Stabilizing the casing is essential, as the casings require strength and elasticity to undergo linking, packaging, storage and reheating.

Since casings are formed directly on the meat's surface, co-extrusion systems must use a suitable brining (e.g., concentrated salt solution) and cross-linking agents that will set the casings fairly quickly (MORGAN *et al.*, 1998). Smoke condensates are commonly used as chemical cross-linking agents, as their aldehydes form covalent linkages between collagen molecules and fibers. Exposure to smoke condensates enhances the mechanical properties of the casing, and also provides the sausage with colour, flavour and some antimicrobial properties. In co-extrusion, smoke condensate is favoured over traditional smoking (burnt wood shavings) because of the ability to work with higher concentrations, product's uniformity, and the intense colour they can provide during a short exposure time (MORGAN *et al.*, 1998; BONTJER *et al.*, 2011). Smoke condensates are produced by combustion of hard woods (e.g., cherry, oak), while the smoke is moved through a shower of water droplets that collect the aldehydes, ketones, furans, phenols, and acids (TOLEDO, 2007).

When dry regenerated collagen casings are produced (in special plants), glutaraldehyde (GA) is used as a cross-linking agent, and is later thoroughly washed off the casings before the drying process (AVERY and BAILEY, 2008; MORGAN *et al.*, 1998; SAVIC and SAVIC, 2016). Glutaraldehyde is also used as a leather tanning agent, production of medical constructs made from collagen, and tissue fixative (COVINGTON, 1997; CHEUNG and NIMNI, 1982). It is able to form stable bonds among proteins and increase the mechanical properties of collagen fibers. The mechanism of glutaraldehyde's reactions has been studied quite a lot in order to help improve the modification of collagenous materials (CHEUNG and NIMNI, 1982; BARBUT, 2015). Overall, it has been shown that GA reacts with amine groups, specifically lysine, to form heterocyclic compounds. Subsequent oxidation reactions produce pyridine rings (ENGLERT *et al.*, 2007). Although GA is a

highly functional cross-linking agent, cytotoxic effects have limited its direct food applications, including in semi-liquid casing application as used in co-extrusion. However, as mentioned above in designated casing producing plants it is used as part of the manufacturing of regenerated collagen casings that subsequently go through an extensive washing process to remove all the GA residues.
Overall, there has been a substantial effort to understand the properties of collagenous materials and the effects of different cross-linking agents (O'SULLIVAN *et al*., 2006; WOLF *et al*., 2006; TOMIHATA *et al*., 1994; OLDE DAMIK *et al*., 1995). However, little has been published about the effectiveness of cross-linking agents on the performance of commercial co-extrusion collagen products. This study was designed to address this area in two steps. First, the effects of smoke condensate (currently used in meat processing plants for the wet co-extrusion process), and glutaraldehyde (used in special plants to make regenerated dry manufactured collagen casing) concentration and contact time were examined by evaluating the mechanical properties of collagen films. This was done to provide an insight into potential manipulation of the mechanical properties of co-extrusion collagen casings. The second study examined the mechanical, microstructural and thermal properties of cross-linked films to compare the similarities and differences in functionality of different commercial collagen dispersions.

2. MATERIAL AND METHODS

2.1. Study I. Manipulation of cross-linking conditions

2.1.1 Preparation of films

Five commercial collagen dispersions used for sausage extrusion processes were obtained from major manufacturers. The dispersions were labeled as: Collagen 1 through 5 (C1, C2, C3, C4, C5). Protein content was determined by the Dumas method (Leco FP528, St Joseph, MI, USA) using a nitrogen factor of 6.25. Moisture content was determined by drying triplicate samples (5 g) at 105°C for 24 h.
The method of film formation was adapted from the work of Harper *et al*. (2013) with alginate solutions and was performed at 4˚C to reduce adhesion during film formation. Briefly, the samples were degassed using a vacuum packager (Multivac Canada Inc., Woodbridge, ON, CAN) at 7.3 kPa for 25 s, then again at 7.3 kPa for 50 s and 75 s (settings 4, 6 and 8, respectively) to remove gas bubbles that were incorporated during processing, as they can create weak spots in the films. While still in the vacuum pack bags, the dispersions were mixed by rolling the dispersions 10 times in adjacent directions. Later, approximately 3 g portions were rolled on a stainless steel board between two layers of plastic film, with a stainless steel roller. The roller had a recess of 0.50 mm in order to achieve uniform film thickness. The plastic sheet on the roller side of the film was removed and the remaining plastic sheet with the collagen film on it was then placed in a 30 wt.% sodium chloride (prepared in deionized water) for 5 min, in order to dehydrate the film. The NaCl treatment mirrors the industrial practice of dehydrating the film (AVERY and BAILEY, 2008). After 5 min, the film was strong enough to hold together when removed from the plastic sheet. The plastic sheet was folded onto the formed film to prevent further dehydration of the film before it was moved to the cross-linking solution.
The goal of the first study was to determine the best exposure time and cross-linking agent concentration to obtain a good quality film/casing. For this part we used Collagen 2, which is one of the most common dispersions currently used by the meat industry. The partially dehydrated films (i.e., by the salt solution) were cross-linked with either liquid

smoke condensate (Charsol Select 24P Liquid Smoke, Red Arrow Products, Manitowoc, WI, USA) or glutaraldhyde (EM Grade, Canemco, Canton de Gore, QC, CAN). The application of liquid smoke is a standard treatment used by the meat industry, while glutaraldehyde was used here as a control treatment with a known aldehyde (cross-linker) concentration. The films were immersed in a 15 vol.% smoke condensate in deionized water, i.e., based on industry recommendation/procedure. Films were held in the diluted smoke condensate bath for 10, 20, 40 or 80 s. Following cross-linking, films were covered with a plastic sheet to avoid drying before testing. As indicated above, glutaraldehyde (GA) was used because its mechanism of cross-linking is better understood, thus its effects could be used as a reference/standard. Films were cross-linked in solutions of 0.1, 0.5 and 1.0 vol.% GA in 1M HEPES buffer at pH 7.4 (Fisher Scientific, Ottawa, ON, CAN). The partially dehydrated films were immersed in the GA solution for 5, 10 or 20 min intervals (note: longer times were used because of the low GA concentrations), followed by a 5 min rinse in water to remove GA residues. Similar to cross-linking with smoke condensate, the films were later covered with plastic to avoid dying before testing.

2.1.2 Mechanical properties

The standard tensile test (ASTM, 2010) was used, by employing a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY, USA) with a gripper distance set at 50 mm, trigger force at 5 g, test speed at 2 mm/s and test distance of 25 mm. Six measurements were made to calculate an average thickness, using a digital micrometer (Testing Machines Inc., Islandia, NY, USA), of each film in each of the three trials. Films were cut into 75 mm × 25 mm strips. The average thickness and width of the films were used for the tensile stress calculations. Tensile strength (maximum stress the film endured prior to breaking) and the percent elongation (maximum elongation the film reached prior to breaking) were determined from the generated stress–strain curve.
Puncture force was also examined using the texture analyzer. In this test, a 5 mm stainless steel ball probe was used to puncture films mounted in an extensibility fixture with 10 mm diameter round openings (TA-108S5, Texture Technologies, Corporation, Scarsdale, NY, USA). The test speed was 1 mm/s and the trigger force was 5 g. The distance to puncture and work of puncture were determined from the force–distance curve. A total of eighteen measurements were used for each of the treatments (six measurements per trial).

2.1.3 Experimental design and statistical analysis

The experiment was designed as a randomized complete block with 3 independent trials. Each trial consisted of evaluating six samples per treatment. The statistical analysis was performed using SAS Version 9.2 (SAS Inst., Cary, NC, USA). A General Linear Model was used for the analysis of variance (ANOVA). The film type means and interactions were compared by using Tukey's multiple comparison analysis with a P-value ≤ 0.05, which was used to detect statistical significance.

2.2. Study II. Evaluation of cross-linked collagen dispersions

2.2.1 Preparation of films

Collagen films were prepared from the five dispersions, as described before. In this part they were cross-linked with either 15 vol.% smoke condensate in deionized water, for 40 s, or with 0.5 vol.% GA in 1M HEPES buffer at pH 7.4 for 5 min, followed by a 5 min rinse.

2.2.2 Characterization of smoke condensate

The smoke condensate was diluted in methanol and injected directly to a gas chromatograph (GC-MS 1200, Varian, Palo Alto, CA, USA) to determine both volatile and semi-volatile compounds. Compounds were tentatively identified based on the NIST library provided with the instrument.

2.2.3 Transmission electron microscopy (TEM)

TEM was performed on collagen films, raw collagen and cellulose fibers as controls. The films were fixed in 2.0% glutaraldehyde, buffered with HEPES (pH 7.4) for 90 min. This was followed by fixing in osmium tetroxide and dehydration through a series of graded ethanol solutions (50%, 70%, 80%, 90%, and 100%), each for 10 min. Once the films were dehydrated, they were run through a series of propylene oxide and Spurr's resin solutions (1:0, 3:1, 1:1, 1:3 and 0:1) to ensure that the resin was thoroughly incorporated, prior to embedding. The resin was polymerized for 24 h at 60˚C. Samples were then cut into 70 to 90 nm sections on a microtome (Reichert Ultracut S, Leica Microsystems Inc., Concord, ON, CAN), fixed on grids, stained with saturated uranium acetate and lead citrate (Hayat, 2000).
Negative staining was used to prepare raw collagen and cellulose fibers as controls. The collagen control was prepared by hydrating dried bovine hide sample (provided by the Meat Laboratory at the University of Guelph) for 24 h at 23˚C. The pH was then lowered to 2 and blended to aid in dispersion and fiber swelling. The cellulose control was a 0.1 wt.% solution of powdered cellulose (Arbocel, JRS, Schoolcraft, MI, USA). One drop of the control solution was placed on a formvar-coated grid and the excess solution was removed with a filter paper. A drop of 2% uranium acetate was then applied to stain the sample. After 30 s, the excess uranium acetate was removed with a filter paper and the grids were dried, on the bench top.
Samples were examined by a TEM (Philips CM 10, Philips Scientifics, Eindhoven, NB, NL) and photographed (Olympus Morada camera, Olympus Soft Imaging System, Berlin, GER) using iTEM imaging software (Item Software, Whiteley, HPH, UK).

2.2.4 Optical property

The light transmission (380–780 nm) of the films was evaluated using a single beam spectrophotometer (USB 2000, Ocean Optics Inc., Dunedin, FL, USA). The following settings were used: integration time: 100 ms: scans to average 2; and boxcar width 4. The light transmission was measured on twelve films per collagen sample.

2.2.5 Mechanical properties of thermally treated films

Cross-linked films from Collagen 2 (C2) were mounted in the puncture fixture and placed in a plastic bag. The fixture and bag were lowered into a water bath at 40, 50, 60, 70 or 80˚C and held for 15 min. The puncture test was performed immediately after thermal treatment. The puncture test was performed as previously described. Once again, a total of eighteen measurements were used for each of the thermal treatments (six measurements per trial).

2.2.6 Experimental design and statistical analysis

The experiment was designed as a completely randomized block with 3 independent trials. Each trial consisted of evaluating six sub-samples per treatment. The statistical analysis was performed as indicated above.

3. RESULTS AND DISCUSSION

3.1. Study I. Manipulation of cross-linking conditions

3.1.1 Mechanical properties

The goal of the first study was to determine the best exposure time to obtain a good self-supporting collagen film, and for this we focused on Collagen 2, which is currently the most commonly used preparation. The results of the tensile test (Fig. 1) demonstrate that there were no significant differences ($p > 0.05$) in the tensile strength and % elongation when films were exposed to smoke condensate for 10 to 80 s. The results were obtained by using Collagen 2, which is one of the most commonly used collagen dispersions in the industry. The pH of the dispersion was 2.21 and is in line with the values of the other dispersions (C1=2.06; C3=2.01; C4=2.67; C5=2.04). According to manufacturers, the swelling agent for C3 was HCl, for C4 it was HCl/acetic acid; this information was not provided for C1, 2 and 5. Overall, the results reveal that after 10 s exposure the film gains a certain degree of cross-linking, and further exposure does not have a significant effect. It should be pointed out that after 10 s the film is removed from the solution but not rinsed so the amount of liquid smoke picked up by the film keeps on working. Overall, cross-linking, by intramolecular bonds, are formed between collagen molecules and fibers. The formation of covalent cross-links prevents collagen fibers from sliding past each other, thus reducing the film's ability to undergo deformation (RAULT *et al*., 1996; PAUL and BAILEY, 2003). From a practical point of view, the results indicate that the meat processor would not be able to significantly modify the tensile properties of their casings by increasing the contact time, within this range (10 to 80 s). As noted in the Method section, the concentration of smoke condensate used here was based on industry practices/recommendations.

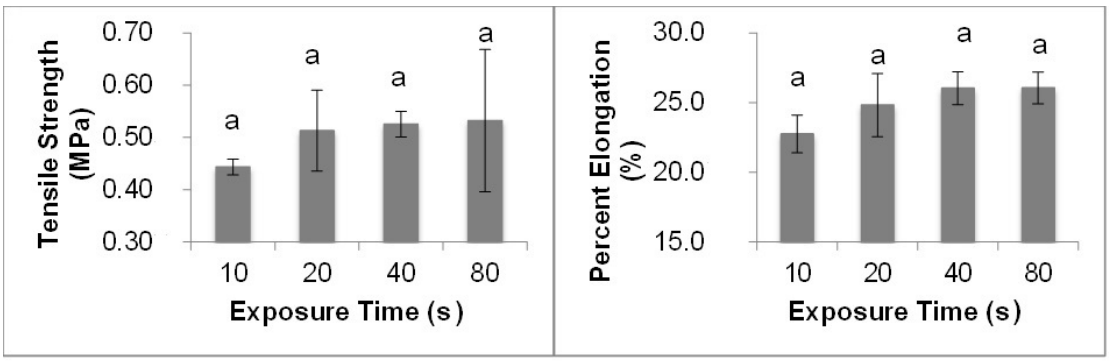


Figure 1. Mechanical properties of collagen films produced with increasing exposure times to smoke condensate (15 vol.% in deionized water). Samples made from Collagen 2. Means with the same letter are not significantly different ($p > 0.05$).

This study employed a commonly used liquid smoke condensate, currently used by various meat processors for co-extrusion applications. Overall, it is known that the composition of smoke condensates can vary, because of variations in the proportion of compounds such as aldehydes and phenols in the hard wood used to produce the condensates (GUILLÉN and MANZANOS, 1999; MONTAZERI *et al*., 2013). Since aldehyde compounds are linked to the smoke's cross-linking ability, variations in their concentration could potentially produce different results related to tensile strength. In order to address this point, laboratory grade glutaraldehyde (GA) with a known aldehyde concentration was also used in our study.

The mechanical properties of the glutaraldehyde-treated films (Table 1) show that the tensile strength and % elongation were basically in the same range as obtained by the smoke condensate. When using GA there were no significant interactions between the GA concentration and exposure time. Also, the results indicate that there was no significant difference ($p > 0.05$) in the percent elongation between 5 and 20 min exposure (Fig. 2).

Thus, exposure time appears to have a greater effect on tensile strength when cross-linking with GA. This difference might be attributed to the concentration of the cross-linking agent in the GA solution. The results also suggest that manipulating the concentration of GA will have significant differences ($p < 0.05$) on the tensile and puncture properties of the collagen films. The most notable effect of increasing GA concentration (0.1 to 1.0 vol.%) was the significant decrease in percent elongation and distance to break (Fig. 2). These observations are consistent with those discussed by AVERY and BAILEY (2008). Overall, it appears that over cross-linking with GA would make the films less stretchable.

Table 1. Mechanical properties of collagen films produced with increasing exposure time and concentration of glutaraldehyde; samples made from Collagen 2.

Concentration vol.%	Time min	Tensile Strength MPa	Percent Elongation %	Distance to Break mm	Work to Break N*mm
	5	0.564±0.14	22.475±2.11	3.012±0.26	1.537±0.56
0.1	10	0.765±0.09	23.213±1.72	2.911±0.31	1.590±0.28
	20	0.833±0.12	20.741±0.96	2.787±0.35	1.566±0.31
	5	0.502±0.06	20.137±1.21	2.517±0.24	1.068±0.05
0.5	10	0.664±0.02	20.381±1.43	2.338±0.44	1.058±0.37
	20	0.577±0.05	19.661±3.28	1.952±0.48	0.672±0.19
	5	0.708±0.08	20.921±2.96	2.071±0.63	1.630±0.90
1.0	10	0.680±0.15	15.877±2.80	1.784±0.72	1.276±0.61
	20	0.764±0.16	17.591±3.68	1.634±0.59	1.135±0.69

3.2. Study II. Evaluation of cross-linked collagen dispersions

3.2.1 Mechanical properties

The mechanical properties of casings play a crucial role in sausage production. They affect sausages' structural integrity, shape, volumetric changes, textural properties, and behavior during processing (SAVIC and SAVIC, 2016). For example, during traditional production, casings must withstand tensile stresses during stuffing, and hanging in the smokehouse. In addition, casings must provide compressive strength during meat protein gelation (heating) and exhibit elasticity during cooling the product (SAVIC and SAVIC, 2016).

Examining properties such as percent elongation, and tensile strength, can help to predict the success of a casing.

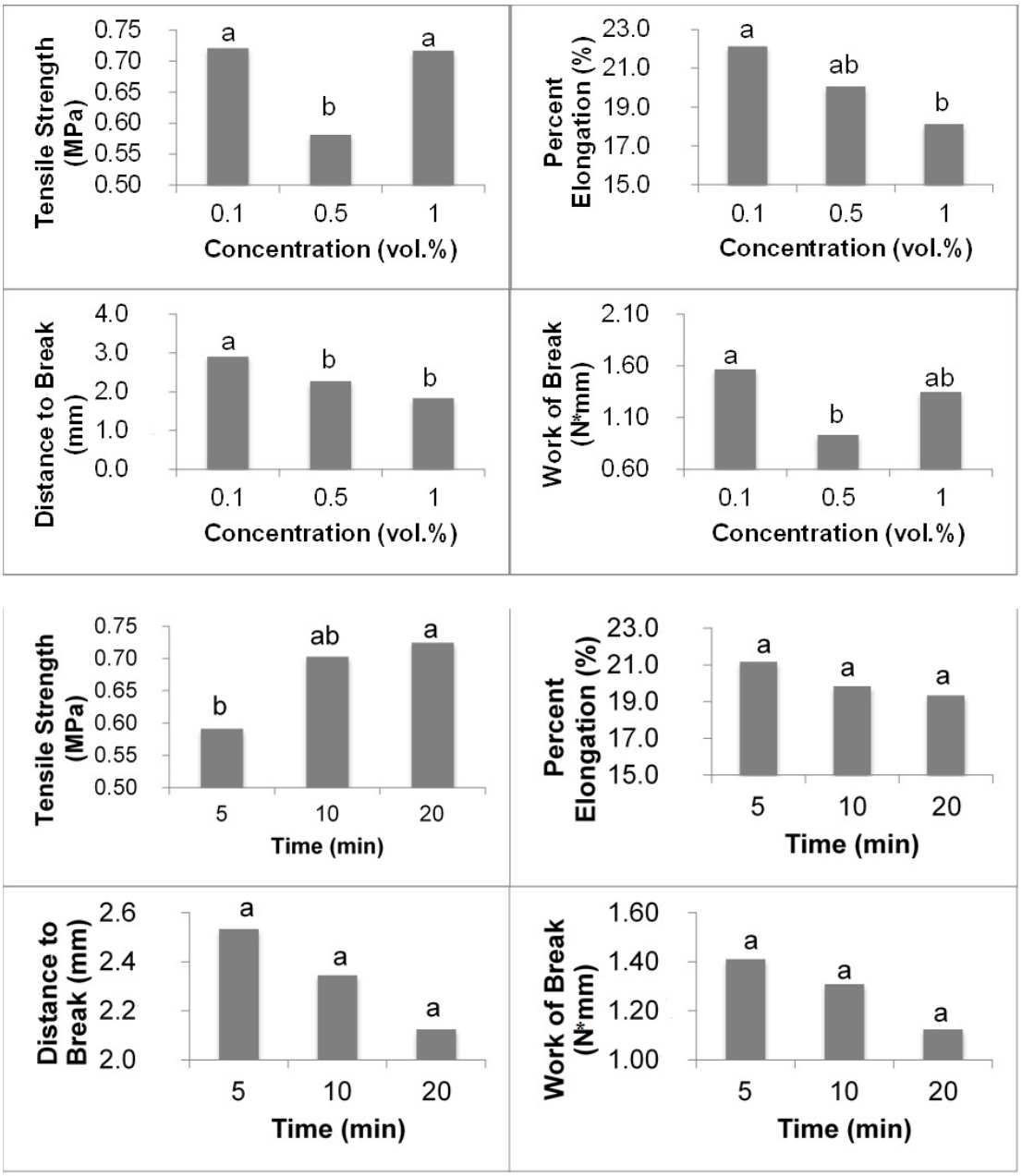


Figure 2. Mechanical properties of collagen films produced with increasing contact times to glutaraldehyde and concentration of glutaraldehyde. Samples made from Collagen 2. The contact time means were averaged across all concentration (0.1, 0.5, 1.0 vol.%) and the concentration means were averaged across all contact times (5, 10 and 20 min). Means in the same graph with same letter are not significantly different ($p > 0.05$).

In the second study, the five different collagen dispersions were compared. Overall, they all had a protein content or 4.3±0.8% (C1=5.1; C2=3.6; C3=3.7; C4=4.4; C5=4.8%) and moisture content of 94.5±1.0% (C1=93.5; C2=95.5; C3=93.5; C4=94.0; C5=93.7%). After partially drying the casings, by exposing them to a salt solution (30% salt as is also done by the industry), all the films showed a pretty similar moisture content (76.0±1.5%, where

C1=74.5; C2=77.5; C3=74.5; C4=75.0; C5=77.0%). Tensile strength and percent elongation results (Table 2) showed that there were some significant differences among the various collagen films. The film produced with Collagen 4 (C4) had the lowest tensile strength (0.32 MPa) when cross-linked with smoke condensate, and C3 (0.41 MPa) when cross-linked with GA. In any case, both C3 and C4 showed low tensile strength under both cross-linking treatments. SAVIC and SAVIC (2016) indicated that a greater degree of native and intact fibrillar structures produce higher strength and elasticity in collagen casings. The C3 and C4 collagen dispersions may have undergone further degradation during processing, resulting in their lower tensile properties. For instance, excessive mechanical or alkaline modification, during corium separation, can result in greater degradation of the native collagen fibers (SAVIC and SAVIC, 2016). The influence of protein concentration on the mechanical properties was also investigated (Table 3). When comparing adjusted mechanical properties (i.e., per % protein), again C3 and C4 were found to result in low tensile strength values when cross-linked with smoke condensate. When cross-linked with GA, C3 showed the lowest value (0.077 MPa/% protein). When looking at % elongation, the samples that showed the lowest values when tested as received (i.e., their original protein content) also showed the lowest values when compared on a % protein basis (sample C5 treated with smoke condensate, and sample C3 treated with GA; Tables 2 and 3). These results indicate that the mechanical properties of films may be more dependent on the quality of protein, rather than on protein content.

Another factor that may have led to differences in mechanical properties was the concentration of cellulose fibers within the partially dried films tested here. MATHEW *et al.* (2012) observed that interactions between cellulose nanofibers and collagen significantly increased the strength and break strain of fully dry collagen films. Note: see below additional discussion and micrographs concerning cellulose fibers.

It is important to note that cross-linking has a dramatic effect on the mechanical properties of partially dehydrated films (i.e., with salt solution). Cross-linking with smoke condensate or GA appeared to increase the tensile strength of collagen films by approximately 100% and 200% (respectively) over the values obtained after salt solution partial drying (BARBUT, unpublished data), which were in the range of 0.2 to 0.3 MPa. In addition, the distance to break was reduced by 30% and 60%, when cross-linked with smoke condensate and GA, respectively. As suggested by others (AVERY and BAILEY, 2008; COVINGTON, 1997; PAUL and BAILEY, 2003) cross-links increase the mechanical strength by forming bonds among the collagen molecules/fibers and preventing them from sliding past each other, creating a stiff but brittle network within the films.

Overall, there were some differences in the mechanical properties between films cross-linked with smoke condensate and GA. In general, films cross-linked with GA showed greater tensile strength than those cross-linked with liquid smoke (Table 2). Smoke condensates typically contain over 300 compounds (TOLEDO, 2007) therefore, the differences observed in tensile strength can likely be attributed to the purity of the cross-linking agent. The smoke condensate was analyzed to determine the type and concentration of its aldehyde compounds (Table 4). It was observed that the aldehyde compounds were all mono-functional aldehydes. The effect of various mono- and di-aldehydes (formaldehyde, gluteraldehyde, crotanaldehyde and glyoxal) on the thermal and conformational stability of type I collagen has been investigated by FATHIMA *et al.* (2004). Their work suggested that the aldehydes differ in their ability to improve the thermal stability of collagen. It was observed that the increase in thermal stability followed the order: formaldehyde > gluteraldehyde > glyoxal > crotanaldehyde, thus demonstrating that di-aldehydes are not necessarily more functional than mono-aldehydes.

Table 2. Mechanical properties of cross-linked films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

Collagen	Cross-linker[1]	Tensile Strength[2] MPa	Percent Elongation[2] %	Distance at Break[3] mm	Work to Break[3] Nmm	Thickness mm
C1	SC	0.67±0.04^{a}	24.80±1.23^{ab}	3.85±0.22^{a}	2.75±0.48^{a}	0.35±0.01^{ab}
C2	SC	0.53±0.02^{ab}	26.32±1.18^{a}	3.39±0.18^{a}	1.52±0.14^{a}	0.30±0.01^{b}
C3	SC	0.38±0.05^{b}	22.41±0.78^{abc}	3.21±0.30^{a}	1.24±0.34^{a}	0.34±0.01^{ab}
C4	SC	0.32±0.07^{b}	21.37±0.99^{bc}	3.27±0.35^{a}	1.23±0.31^{a}	0.36±0.02^{a}
C5	SC	0.39±0.16^{b}	18.81±2.08^{c}	2.59±0.04^{a}	0.85±0.10^{a}	0.38±0.03^{a}
C1	GA	0.91±0.17^{d}	26.26±4.65^{d}	2.77±0.52^{d}	1.79±0.57^{d}	0.36±0.03^{d}
C2	GA	0.66±0.02^{e}	20.38±1.43^{d}	2.34±0.44^{d}	1.06±0.37^{d}	0.45±0.01^{d}
C3	GA	0.41±0.13^{f}	18.95±2.56^{d}	2.66±0.39^{d}	1.35±0.27^{d}	0.38±0.05^{d}
C4	GA	0.61±0.20^{ef}	24.18±3.72^{d}	2.74±0.29^{d}	1.52±0.11^{d}	0.38±0.04^{d}
C5	GA	0.60±0.18^{ef}	22.04±2.60^{d}	2.87±0.60^{d}	1.91±0.76^{d}	0.39±0.05^{d}

[1]Smoke Condensate (SC), Glutaraldehyde (GA). [2]Tensile test. [3]Puncture test.
[4]Means in columns with same letter are not significantly different $p > 0.05$: letters [a-c]refer to Smoke Condensate treated films; [d-e]Glutaraldehyde treated films.

Table 3. Protein adjusted mechanical properties of cross-linked films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

Collagen	Cross-linker[1]	Tensile Strength[2] MPa / %protein	Percent Elongation[2] % / %protein	Distance at Break[3] Mm / %protein	Work to Break[3] N*mm / %protein
C1	SC	0.092±0.01^{ab}	3.36±0.17^{bc}	0.38±0.07^{a}	0.24±0.08^{a}
C2	SC	0.101±0.00^{a}	4.98±0.23^{a}	0.45±0.08^{a}	0.20±0.07^{a}
C3	SC	0.062±0.01^{b}	3.67±0.13^{b}	0.44±0.06^{a}	0.22±0.04^{a}
C4	SC	0.056±0.01^{b}	3.68±0.17^{b}	0.47±0.05^{a}	0.26±0.02^{a}
C5	SC	0.058±0.02^{b}	2.77±0.31^{c}	0.42±0.09^{a}	0.28±0.11^{a}
C1	GA	0.140±0.03^{d}	4.05±0.72^{d}	0.43±0.08^{d}	0.28±0.09^{d}
C2	GA	0.131±0.00^{d}	4.02±0.28^{d}	0.46±0.09^{d}	0.21±0.07^{d}
C3	GA	0.077±0.03^{e}	3.59±0.48^{d}	0.50±0.07^{d}	0.26±0.05^{d}
C4	GA	0.120±0.04^{de}	4.77±0.73^{d}	0.54±0.06^{d}	0.30±0.02^{d}
C5	GA	0.118±0.04^{de}	4.34±0.51^{d}	0.57±0.12^{d}	0.38±0.15^{d}

[1]Smoke Condensate (SC), Glutaraldehyde (GA). [2]Tensile test. [3]Puncture test.
[4]Means in columns with same letter are not significantly different $p > 0.05$: letters [a-c]refer to Smoke Condensate treated films; [d-e]Glutaraldehyde treated films.

It was suggested that the number of cross-links formed influence the stability of the cross-links, therefore the reactivity of the aldehyde is of greater importance. The reactivity of mono and di-aldehydes is thought to stem from sterochemical factors and pH (increase uptake at higher pH) as was indicated by BOWES and CATER (1968). Although according to our calculation the smoke condensate had a higher percentage of different aldehydes (0.95%; Table 4), compared to the 0.5% glutaraldehyde used in the experiment, it appears that glutaraldehyde is a more reactive cross-linker that overall provided higher tensile strength values to the films (Tables 2 and 3).
It should be mentioned that the pH of the cross-linking solution may have also affected the mechanical properties of the films. MORGAN et al. (1998) suggested that neutralizing the acidic collagen dispersion would result in water loss. Similar to dehydrating films with a salt brine, the removal of water will result in shorter distances between the collagen molecules and hence improve the stability of the collagen structure. The smoke condensate was used as is (i.e., not buffered; at pH 3.5) while the GA was buffered (pH 7.4). Thus the buffered solution could have increased the neutralization of the collagen gels, and in turn, improving stabilization.

Table 4. Tentative identification of aldehyde compounds found in the commercial smoke condensate, the relative concentration (%) and approximate concentration in the cross-linking bath.

Tentative ID	Relative %	Cross-linking Bath[1] %
2,4-Diethoxybenzaldehyde	0.47	0.07
4-Methyl 2,5-dimethoxybenzaldehyde	1.24	0.19
Benzaldehyde, 4-hydroxy-3,5-dimethoxy	3.22	0.48
2-Oxa-6-azatricyclo[3.3.1.1(3,7)]decane-6-carboxaldehyde	0.47	0.07
3,5-Dimethoxy-4-hydroxycinnamaldehyde	0.92	0.14
(Aldehyde in Smoke Condensate)	(6.32	0.95)

[1]The original Smoke Condensate received was diluted to 15 vol.% using deionized water.

3.2.2 Electron microscope imaging

The collagen fibers appear to have varying degrees of swelling or hydration (Fig. 3). Overall in natural tendon tissue, collagen fibrils are long, slender and show cylindrical structures, but can differ in length, diameter, uniformity, and telopeptide size, as a result of collagen type and interactions (WESS, 2008; CAMERON *et al.*, 2002). The alignment of collagen molecules in a staggered array conformation develops areas of overlap and gaps. The overlap and gap regions of the parallel arrays give collagen fibrils their distinctive striated pattern with a periodicity of 640-700 Å (WESS, 2008). The control collagen sample (obtained by us from beef hide) was imaged to compare the likeness of the banded fibers (Fig. 4A). It appeared that, at this magnification, the banded structures were indicative of collagen fibers. In addition, the bulk material, presented in this micrograph, shares similar characteristics to the collagen tissue studies by MEYER *et al.* (2005).
Although some collagen fibers display a banding pattern or cross-striations (Fig. 3), the majority of the fibers presented in the current work appear to be swollen. Since commercial collagen dispersions are extracted from hides that have varying degree of natural cross-linking, the degree of cross-links varies depending on animal age, growing conditions, overall muscle activity, etc. Overall, there is an increase in natural cross-links,

with increasing age and therefore the solubility also decreases (MEYER *et al*., 2005). In addition, the connective tissue extraction process would be expected to result in varying degree of fiber degradation.

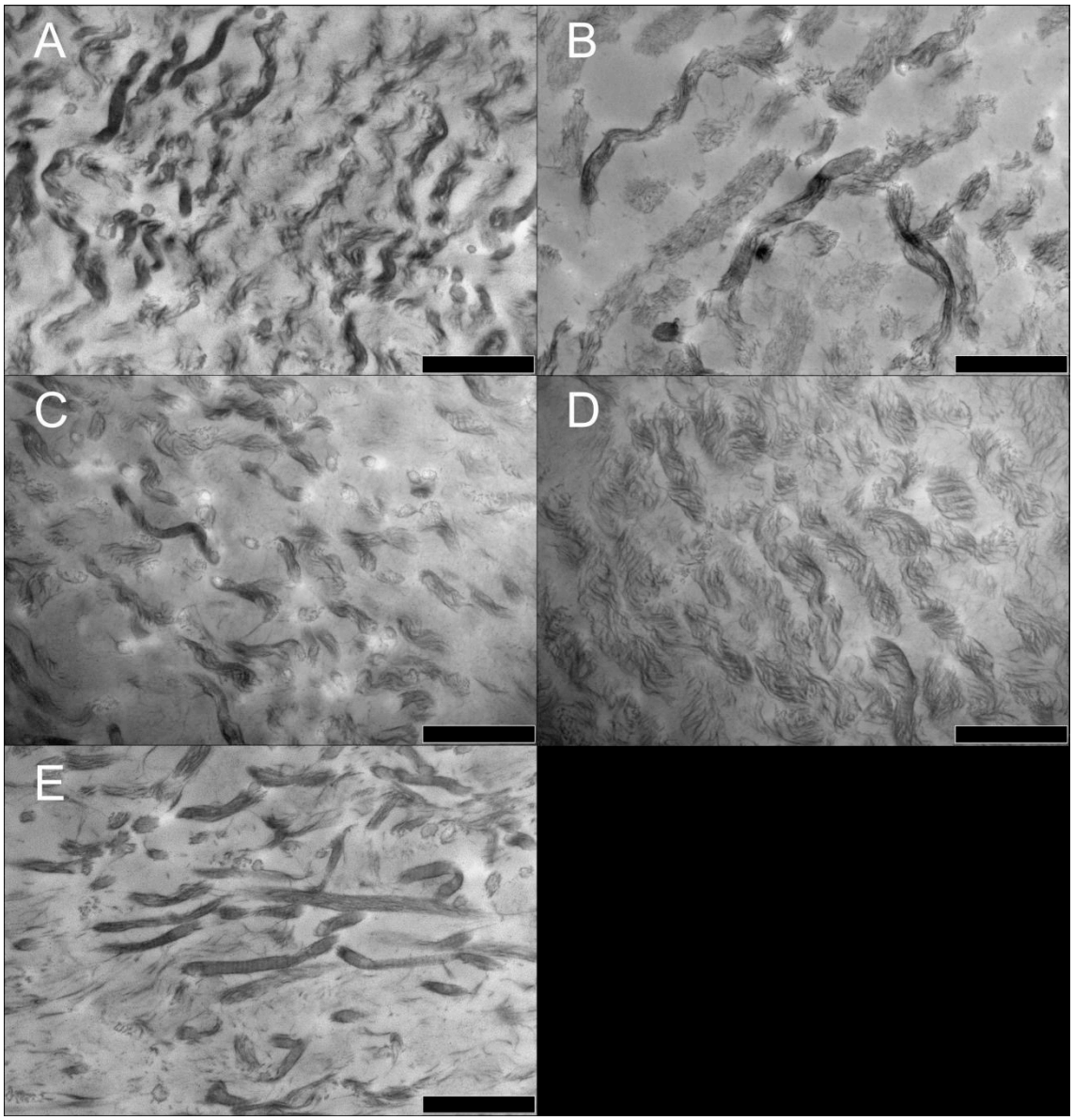


Figure 3. Transmission electron micrographs of collagen film: Collagen C1 (A), Collagen C2 (B), Collagen C3 (C), Collagen C4 (D), Collagen C5 (E). Black bar represents 1 μm.

The final collagen structure supports the mechanical properties of the film because a more intact fibrillar structure produces higher strength and elasticity in collagen casings (SAVIC and SAVIC, 2016). The C4 appeared to have the most swollen or hydrated fibers, with little visible banded fibrils remaining (Fig. 3D). Since C4 generally revealed lower tensile strength and percent elongation (Table 2), one may presume that greater swelling and hydration during collagen extraction, with HCl, and acetic acid resulted in lower mechanical properties. However, definite conclusions cannot be drawn as the orientation and plane at which the fibrils are viewed is not known with certainty.

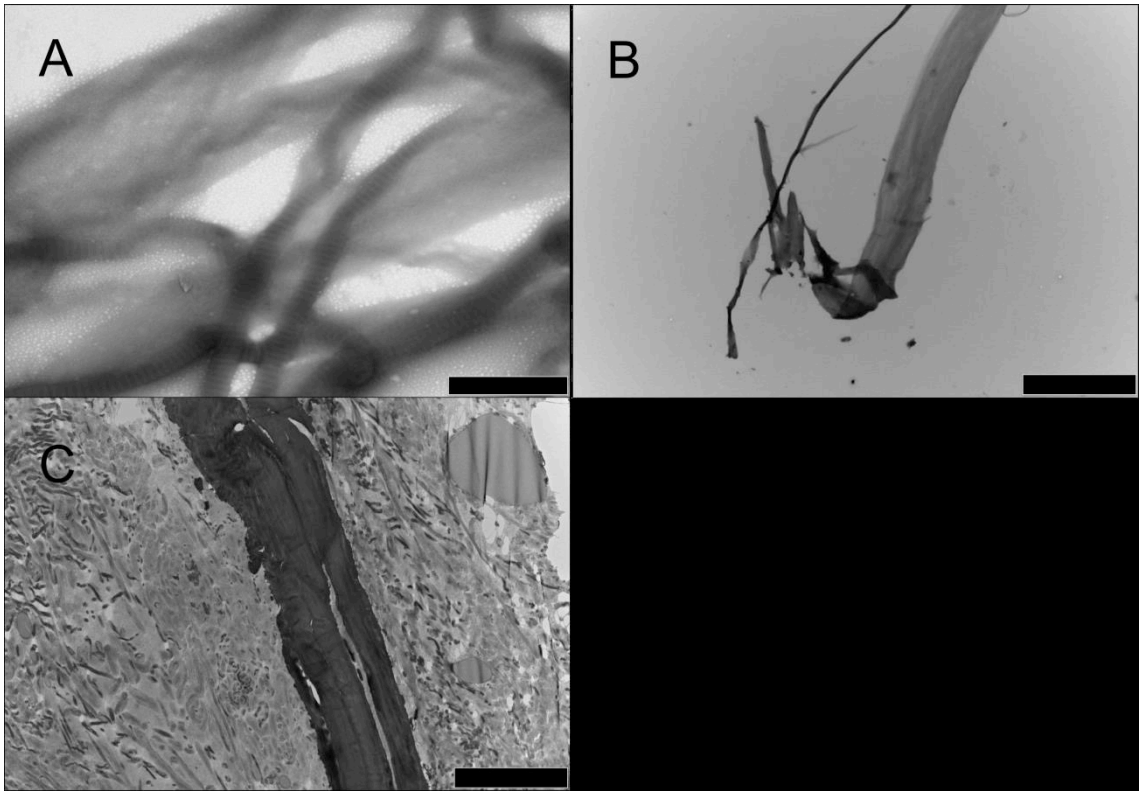


Figure 4. Transmission electron micrographs of a collagen control obtained from beef hide (A), a cellulose control (B) and a cellulose fiber embedded in a collagen film network (C). Black bar represents 1 μm in micrograph (A) and 5 μm in micrographs (B) and (C).

Another interesting observation was that there appears to be differences in the linearity of fibers. The fibers in C1 appear to be arranged in a less linear way compared to the other treatments. C1 also appears to have a higher concentration of more condensed fibers, which may have hindered alignment of the fibers during film preparation by rolling. It should be pointed out that during the formation of commercial co-extruded casings, a counter rotating extrusion head applies shear forces, which orientates and elongates the collagen fibers (HOOGENKAMP *et al*., 2015). Orienting the fibers improves the mechanical properties of casing by reducing splitting (BONTJER *et al*., 2011). Overall, it would be interesting to further investigate this topic to further assist the industry.
Microstructure imaging the films did not suggest significant differences in the concentration of cellulose in the five dispersions. As previously mentioned, cellulose fibers are commonly added to modify the mechanical properties and porosity of collagen casings (SAVIC and SAVIC, 2016; BARBUT, 2010). To verify that the larger fiber structures were derived from cellulose, a cellulose control (Fig. 4B) was imaged, demonstrating the difference in structure and magnitude. An example of a cellulose fiber embedded in the collagen network of one of the dispersions can be seen in Fig. 4C. Overall, cellulose fibers can be distinguished based on their larger size, lack of striation and electron density.

3.2.3 Optical properties of films

The degree of light transmission through casings affects consumer's ability to evaluate the meat product inside (SAVIC and SAVIC, 2016). Light transmission measurements were

taken to evaluate the transparency of cross-linked collagen films (Fig. 5). Among the collagen films, C5 was the least transparent and C2 was the most. Simple visual inspection also confirmed that C5 was more opaque than the other samples. The micrograph of the C5 films also showed high concentration of individual fibrils and sub-fibrils, which could result in higher light scattering. Light transmission is also affected by the composition of each casing (SAVIC and SAVIC, 2016). C2 was on the lower end of protein content (3.6%), which may have contributed to it being the most transparent.

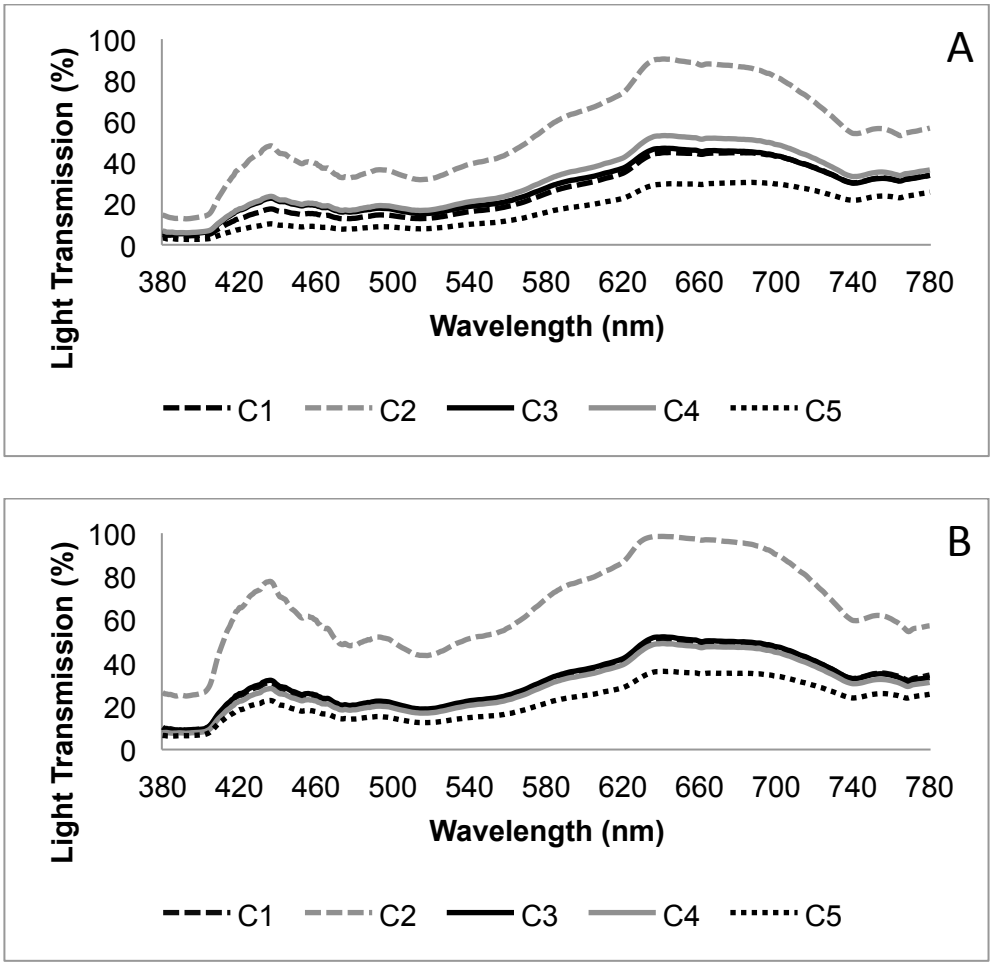


Figure 5. The light transmission (380 to 780 nm) through cross-linked collagen films; C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5). A - films cross-linked with liquid smoke; B - films cross-linked with glutaraldehyde.

Films treated with liquid smoke tended to have lower transmission than films treated with glutaraldehyde (Fig. 5). This observation is likely a result of some dark smoke components that became deposited on the films. There was no difference in the ranking of transparency between the liquid smoke and glutaraldehyde treated films. This observation is somewhat inconsistent with TANAKA *et al*. (2011), who observed that increased cross-linking results in lower transmission of wet collagen films cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodimide and N-hydroxysuccimide.

3.2.4 Mechanical properties of thermally treated films

Table 5 shows the puncture distance and work to puncture as an effect of heating temperature. In general, as heating increases, puncture distance significantly decreased and work to puncture showed a trend of decreasing. This is in line with the expectation that collagen tissue, in muscle foods, softens as temperature goes above the collagen denaturation temperature of 60˚C (MILES *et al.*, 2005). Overall, there were no measurements recorded from testing the films cross-linked with smoke condensate because the films became too fragile to be evaluated by the texture analyzer. As previously suggested, the collagen in films cross-linked with smoke condensate may have remained more swollen and had higher water content. The higher water content may have resulted in lower thermal stability of these films. MILES *et al.* (2005) suggested that the increased thermal stability is a result of the water content of the fibers. They observed that cross-linking reduced the axial separation between molecules, thus reducing the amount of entrapped water. AVERY and BAILEY (2008) observed that cross-linking agents, having different cross-link length, had no effect on denaturation temperature when hydration was done in the same way.

Table 5. Mechanical properties (puncture distance and work to puncture) of collagen films cross-linked with glutaraldehyde and thermally treated. Films produced with Collagen 2.

Thermal Treatment	Puncture Distance mm	Work of Puncture mJ
No Treatment	3.781±0.75[a]	2.576±0.82[a]
40°C	3.180±0.17[ab]	2.053±0.16[a]
50°C	3.151±0.57[ab]	2.584±0.45[a]
60°C	2.838±0.25[ab]	1.844±0.63[a]
70°C	2.423±0.13[b]	1.265±0.30[a]
80°C	2.578±0.23[b]	1.518±0.40[a]

[1]means in columns with a similar letter are not significantly different ($p > 0.05$).

In the present study, collagen may have not undergone full thermal denaturation by 80˚C when films were cross-linked with GA (AVERY and BAILEY, 2008). Cross-linking with GA has been observed to increase the denaturation temperature of collagen to approximately 100˚C (MILES *et al.*, 2005). Our results may suggest that the thermal stability of cross-linked casings depend more on moisture content than cross-links.

4. CONCLUSIONS

It was observed that manipulating the smoke condensate contact time (10, 20, 40, 80 s) did not result in significant differences ($p > 0.05$) in the mechanical properties of collagen films. Similarly, the GA contact time (5, 10, 20 min) did not result in significant differences in mechanical properties, when averaged across the GA concentrations employed. Although contact time had little effect, increasing the concentration of cross-linking with GA (0.1, 0.5 1.0%) significantly decreased the % elongation (22 to 17%) and distance to break (3.0 to 1.6 mm). This suggests that increasing the GA concentration increases cross-linking and brittleness of the film.

There were some significant differences between the mechanical properties of the different cross-linked films. It was suggested that fiber structure, cellulose content and pH of the cross-linking agent could influence the mechanical properties. Therefore manufacturers of regenerated collagen casings and producers of co-extruded sausages should consider these differences when selecting a source of collagen for their casings.
In addition, meat processors should monitor the concentration and pH of their cross-linking solution as solutions from a continuous liquid smoke drip/spray application are known to become diluted over time. As discussed in the paper, these changes may significantly affect the mechanical properties of the collagen films. Overall, proper understanding is required when selecting the raw collagen dispersion and the conditions for the co-extrusion process. Furthermore, if products are going to be cooked, the casings may require further heating and dehydration, which will also change their mechanical properties.

ACKNOWLEDGEMENTS

The authors would like to thank the Ontario Ministry of Agriculture, Food and Rural Affairs for financial support.

REFERENCES

ASTM. 2010. Standard test method for tensile properties of this plastic sheeting. Method D882-10, Philadelphia, PA.

Avery N. and Bailey A. 2008. Restraining cross-links responsible for the mechanical properties of collagen fibers: natural and artificial. In "Collagen Structure and Mechanics", P. Fratzl (Ed.), p. 81. Springer Science, New York, NY.

Barbut, S. 2015. Casings. In: "The Science of Poultry and Meat Processing", p.13-54 and 62. ISBN-978-088955-625-6. Free download: www.poultryandmeatprocessing.com.

Barbut S. 2010. Microstructure of natural, extruded and co-extruded collagen casings before and after heating. Ital. J. Food Sci. 22:126.

Bontjer M.B.H., Kuijpers M.W.J.T. and Van den Berg K.W. 2011. Method for preparing food products by co-extrusion, in particular sausage and food products obtained with this method. European patent WO 2006 135238.

Bowes J.H. and Cater C.W. 1968. The interaction of aldehydes with collagen. Biochim. Biophys. Acta 168: 41.

Cameron G.J., Alberts I.L., Laing J.H. and Wess T.J. 2002. Structure of type I and type III heterotypic collagen fibrils. An X-ray diffraction study. J. Struct. Biol. 137:15.

Cheung D.T. and Nimni M.E. 1982. Mechanism of crosslinking of proteins by glutaraldehyde II reaction with monomeric and polymeric collagen. Connect. Tissue Res. 10: 201.

Covington A.D. 1997. Modern tanning chemistry. Chem. Soc. Rev. 26:111.

Englert C., Blunk T., Muller R., von Glasser S.S., Baumer J., Fierlbeck J., Heid I.M., Nerlich M. and Hammer J. 2007. Bonding of articular cartilage using a combination of biochemical degradation and surface cross-linking. Arthritis Res. Therapy. 9(3):R47.

Fathima N.N., Madhan B., Rao J.R., Nair B.U. and Ramasami T. 2004. Interaction of aldehydes with collagen: effect on thermal, enzymatic and conformational stability. International J. Biol. Macromol. 34:241.

Guillén M.D. and Manzanos M.J. 1999. Smoke and light smoke study of an aqueous smoke flavouring from the aromatic plant *Thymus vulgaris L.* J. Sci. Food Agr. 79:1267.

Harper B.A., Barbut S., Lim L-T. and Marcone M.F. 2013. Characterization of 'wet' alginate and composite films containing gelatin, whey or soy protein. Food Res. Int. 52:452.

Hayat M.A. 2000. "Principles and techniques of electron microscopy biological applications" 4th ed. p. 120. Cambridge University Press, Cambridge, UK.

Hoogenkamp H.R., Bakker G.J., Wolf L., Suurs P., Dunnewind B., Barbut S., Friedl P., van Kuppevelt T.H. and Daamen W.F. 2015. Directing collagen fibers using counter-rotating cone extrusion. Acta Biomater. 12:113.

Mathew A.P., Oksman K., Pierron D. and Harmad M.F. 2012. Crosslinked fibrous composites based on cellulose nanofibers and collagen with in situ pH induced fibrillation. Cellulose 19:139.

Meyer M., Muhlbach R. and Harzer D. 2005. Solubilisation of cattle hide collagen by thermo-mechanical treatment. Polym. Degrad. Stabil. 87:137.

Miles C.A., Avery N.C., Rodin V., Bailey A.J. 2005. The increase in denaturation temperature following cross-linking is caused by dehydration of the fibres. J. Mol. Biol. 346:551.

Montazeri N., Oliveira A.C.M., Himelbloom B.H., Leigh M.B. and Crapo C.A. 2013. Chemical characterization of commercial liquid smoke products. Food Sci. Nutr. 1:102.

Morgan T.F., Frame G. and Kobussen P.J. 1998. Process for producing a linked co-extruded edible product. US Patent 5,795,605.

Olde Damik L.H.H., Dijkstra P.J., Van Luyn M.J.A., Van Wachem P.B., Nieuwenhuis P. and Feijen J. 1995. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. J. Material. Sci. - Material Med. 6:460.

O'Sullivan A.O., Shaw N.B., Murphy S.C., Van de Vis J.W., Van Pelt-Heerschap H. and Kerry J.P. 2006. Extraction of collagen from fish skins and its use in the manufacture of biopolymer films. J. Aquat. Food Prod. T. 15:21.

Paul R.G. and Bailey A.J. 2003. Chemical stabilization of collagen as a biomimetic. Sci. World J. 3:138.

Rault I., Frei V., Herbage D., Abdul-Malak N. and Huc A. 1996. Evaluation of different chemical methods for cross linking collagen gel films and sponges. J. Material. Sci. - Material Med. 7:215.

Savic Z. and Savic I. 2016. "Sausage Casings" 2nd ed. p. 75. Victus, Inc., Vienna, AUS.

Tanaka Y., Kubota A., Matsusaki M., Duncan T., Hatakeyama Y., Fukuyama K., Quantock A.J., Yamato M., Akashi M. and Nishida K. 2011. Anisotropic mechanical properties of collagen hydrogels induced by uniaxial-flow for ocular applications. J. Biomat. Sci. 22:1427.

Toledo R.T. 2007. Wood smoke components and functional properties. In: "International Smoke Seafood Conference Proceedings". D.E. Kramer and L. Brown. (Ed.), p. 55. Alaska Sea Grant College Program, Anchorage, USA.

Tomihata K., Burczak K., Shiraki K. and Ikada Y. 1994. Cross-linking and biodegradation of native and denatured collagen. In "Polymers of biological and biomedical significance". W. Shalaby, Y. Ikada, R. Langer and J. Williams. (Eds.), p. 275. American Chemical Society, Washington, USA.

Wess T.J. 2008. Collagen fibrillar structure and hierarchies. In: "Collagen Structure and Mechanics". P. Fratzl (Ed.), p.55. Springer Science, New York, NY.

Wolf K.L., Sobral, P.J.A. and Telis V.R.N. 2006. Characterization of collagen fibers for biodegradable films production. IUFoST 13:801.

Paper Received June 3, 2018 Accepted February 13, 2019

PAPER

POLYCYCLIC AROMATIC HYDROCARBONS IN SELECTED FOOD ITEMS COMING FROM THE CROATIAN MARKET

T. BOGDANOVIĆ[a], S. PETRIČEVIĆ[a], E. LISTEŠ[a] and J. PLEADIN*[b]

[a]Croatian Veterinary Institute, Regional Veterinary Institute Split, Poljička cesta 33, 21000 Split, Croatia

[b]Croatian Veterinary Institute Zagreb, Laboratory for Analytical Chemistry, Savska cesta 143, 10000 Zagreb, Croatia

*Corresponding author: Tel: +38516123626; Fax: +38516123670;
E-mail address: pleadin@veinst.hr

ABSTRACT

The study aims to evaluate the presence of 15 polycyclic aromatic hydrocarbons (PAHs) in fishery products, shellfish, meat products and spices (n = 140). Benzo[a]pyrene (BaP) was detected in the mean concentration of 0.11 μg/kg in mussels to 4.85 μg/kg in spices. However, none of the samples exceeded the maximal BaP and ∑PAH4 limit, set out under the European legislation, although high values determined in some food, especially dried herbs and spices, pointed towards a heavy contamination and the necessity for systematic controls. The study showed that processed food samples contained significantly higher ($p<0.05$) PAH levels in comparison to food coming from environmental sources.

Keywords: Croatian market, dried herbs and spices, fresh shellfish, meat products, polycyclic aromatic hydrocarbons, smoked fish

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are represented by roughly 660 different compounds and include highly hydrophobic and diverse organic compounds that have two or more fused aromatic rings (ZELINKOVA and WENZL, 2014; SINGH *et al.*, 2016). They pose as ubiquitous environmental pollutants that can be found in fresh water and marine sediments, the atmosphere and ice. The major reason for concern as regards human exposure to PAHs arises on the grounds of their carcinogenic, mutagenic and teratogenic effects (FALCÓ *et al.*, 2005; REINIK *et al.*, 2007; RENGARAJAN *et al.*, 2015). The United States Environmental Protection Agency (US-EPA) tagged 16 PAHs as priority pollutants based on the frequency of their occurrence and their carcinogenicity (EPA, 1994). The 16 PAHs include acenaphthene (ACE), acenaphthylene, anthracene (ANTHR), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (D[ah]A), fluoranthene (F), fluorene (FLR), indeno[1,2,3cd] pyrene (I[cd]P), naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR). Among them, those containing up to four fused benzene rings are known as light PAHs, while those containing more than four benzene rings are known as heavy PAHs and are more toxic and more stable as compared to their light counterparts.

Food consumption represents the main route of PAH exposure for non-smokers and non-occupationally exposed adults (ALOMIRAH *et al.*, 2011). The routes of PAH food contamination include direct contamination from natural and anthropogenic environmental sources present in air, water and soil, as well as contamination with PAHs formed throughout thermal food processing (e.g. drying, smoking, heating, baking, frying, roasting, grilling) (EFSA, 2008). PAHs most commonly found in food are BaP, BaA, CHR, D[a,h]H, PYR, ANTHR, F and BbF (YEBRA-PIMENTEL *et al.*, 2015). Available profile studies of both unprocessed (seafood, mussels) and processed food have shown the predominance of light over heavy PAHs. However, the presence of toxicologically important heavy (high molecular weight) PAHs that include CHR, BaA, BaP and BbF, has often been reported in certain whole smoked meat products (smoked pork speck, smoked chicken, smoked pork) and chopped meat products, such as various sausages (REINIK *et al.*, 2007; PURCARO *et al.*, 2009; KUBIAK *et al.*, 2015; ROZENTÄLE *et al.*, 2015; ROZENTÄLE *et al.*, 2018). Dominating PAHs of toxicological concern present in mussels are CHR, BbF and BkF (MERCOGLIANO *et al.*, 2016). It has been revealed that spices and herbs, which are important ingredients of many processed food items, are often contaminated with PAHs of the similar low molecular profile, with the prevalence of CHR similar to that in the above-mentioned foodstuffs (ROZENTÄLE *et al.*, 2018).

In order to protect public health and to keep food contaminants at toxicologically acceptable levels, food authorities of different countries have established the maximum levels (MLs) of PAHs for different food categories in which higher PAH levels are to be expected. The European Union legislation stipulates the limits for BaP and the sum of four PAHs - ∑PAH4 (BaA, CHR, BbF, BaP) in more than 10 groups of foodstuffs and is therefore the most comprehensive applicable regulation worldwide (EC REGULATION No. 835/2011, ZELINKOVA and WENZEL, 2016). Bivalve molluscs (fresh, chilled or frozen) in which environmental pollution might result in high contamination levels, are allowed MLs of 5μg/kg for BaP and 30 μg/kg for ∑PAH4. The MLs of PAHs in processed fish and meat products are set at 2 μg/kg for BaP and 12 μg/kg for ∑PAH4. However, there exists the list of EU countries that are allowed to continue using traditionally smoked fish and smoked meat products containing higher PAH levels (5 μg/kg for BaP and 30 μg/kg for ∑PAH4) (EC REGULATION No. 1327/2014). Despite the application of good smoking practices, lower PAH levels in these traditional foodstuffs have not been

achieved yet, since these products require smoking practices that significantly change their organoleptic characteristics. A three-year derogation from the obligation to observe and respect lower BaP and ∑PAH4 MLs expired in the autumn of 2017, but in half of the Member States the former MLs are still in force, pending the adoption of new, reassessment-based provisions. Recently, MLs of 10 μg/kg and 50 μg/kg were set out for BaP and ∑PAH4 in dried herbs and spices (EC REGULATION No. 1933/2015) in response to high PAH levels determined in the above in the recent years due to poor drying practices.

The main objective of this study was to evaluate the level of contamination of certain food items coming from the Croatian market with the ∑PAH15 referred to above (except for the non-fluorescent acenaphthylene), and to compare the levels of contamination from two major sources (environmental source *vs* food processing technique). In order to establish the impact of environmental sources, bivalve molluscs have been investigated, whereas the food processing impact was investigated in fishery products, meat products and spices. In order to establish the occurrence and toxicity of PAHs in food under study, low molecular PAH (ACE, ANTHR, BaA, CHR, F, FLR, NAP, PHEN, PYR), high molecular PAH (BbF, BAP, BkF, B[ghi]P, D[a,h]A, I[cd]P), ∑PAH4 (BaA, CHR, BbF, BaP) and ∑PAH8 (BaA, CHR, BbF, BaP, BkF, B[ghi]P, D[a,h]A, I[cd]P) contents were determined. Although the assessment of dietary exposure to PAHs does not fall within the scope of this study, for the sake of comparison the PAH contents and the respective sums are also expressed as benzo[a]pyrene equivalents (BaPE), so as to illustrate the toxicity of the investigated PAHs, as well as to simplify the interpretation of real-life risk for human health.

2. MATERIAL AND METHODS

2.1. Sampling and sample preparation

Food samples (n = 140) were obtained from the Croatian market during 2017 – 2018 and divided into four groups, as follows: fresh shellfish (n = 42), smoked fishery products (n = 8), meat products (n = 70) and dried herbs & spices (n = 20). Sample collection and storage were performed in accordance with the European legislation (EC REGULATION No. 333/2007, EC REGULATION No. 836/2011) so as to avoid PAH losses (ZELINKOVA and WENZEL, 2016).

Five fresh bivalve species (n = 42), including mussels (*M. galloprovincialis*, n = 29), oysters (*O. edulis*, n = 2*)*, variegated scallops (*C. varia*, n = 4), warty venus shells (*V. verruscosa*, n = 3) and smooth clam (*C. chione,* n = 4) were collected from local markets along the Croatian coastline. Samples containing approximately 4 kg of shellfish were transported to the Laboratory in cooled dim containers within 24 hours post sampling. Twenty five pieces of variegated scallops, mussels and warty venus shells and fifteen pieces of oysters and smooth clams of similar shell lengths were randomly selected from each sample and put together for the analysis; shells were than discarded, while soft tissues were homogenized (Grindomix, GM 200, Retsch, Haan, Germany) and stored at -20°C pending analysis.

Smoked fishery products (n = 8), including hot-smoked sea bass (n = 3) and sea bream (n = 1) fillets, cold smoked trout (n = 1) and tuna (n = 1) fillet, smoked sardine in sunflower oil (n = 1) and smoked salmon pate (n = 1), were obtained from the local Croatian market. In order to ensure a representative sample of fishery products, the whole package content was homogenized (Grindomix, GM 200, Retsch, Haan, Germany) and stored at -20°C pending analysis.

The meat sample pool (n = 70) consisted of 10 samples of fermented sausages (Istrian Rožica, dry homemade sausage, Kulen, tea sausage and other salami and sausages), 10 samples of semi-dry smoked sausages (Bodulska, ham, homemade sausage, Kranj sausage, Kvarner sausage, grill sausage and peasant sausage), 35 dry-cured meat samples (buđola, dry bacon, dry ham, dry sirloin and other products) and 15 semi-dry-cured meat samples (dry ham, smoked chops, smoked dry porcine shank, smoked rack, smoked ribs, smoked rolled shoulder with skin and other products). The samples were homogenized (Grindomix GM 200, Retsch, Haan, Germany) at different speeds for a different length of time depending on the type of meat product, and then stored at -20°C pending analysis.
The selected dried herb and spice samples (n = 20) included clove (*Caryophyllus aromaticus)* (n = 1), grounded garlic (*Allium sativum*) (n = 2), grounded ginger (*Zingiber officinale*) (n = 1), laurel (*Laurus nobilis*) (n = 1), grounded red paprika (*Capsicum spp*) (n = 2), grounded smoked red paprika (*Capsicum spp*) (n = 4), grounded black pepper (n = 4), mixed pepper (*Piper nigrum*) (consisting of black, green, white and red pepper, n=1), mixed dried spices (consisting of tomato, rosemary, basil and oregano) (n = 1), parsley (*Petroselinum crispum*) (n=1) and rosemary (*Rosmarinus officinalis*) (n = 2). Ungrounded samples were cut or crushed and then sieved through a 1.5-mm sieve.

2.2. Standards and reagents

All chemicals used (e.g. dichloromethane, acetonitrile) were of a HPLC grade. Ultrapure water was produced by a Millipore, Direct-Q 3 UV system (Millipore, Molsheim, France). The glassware was washed with a detergent and water, rinsed with acetone and dichloromethane, and dried at 50°C for an hour before use (European Commission, 2007).
The certified standard mix solution 16 Priority PAH, Cocktail 3, 16 comp.(10 μg of each/mL in acetonitrile) containing ACE, acenaphthylene, (ANTHR), (BaA), (BbF), (BkF), (B[ghi]P), (BaP), (CHR), (D[ah]A), (F), (FLR), I[cd]P, (NAP), (PHEN) and (PYR), was obtained from the Chiron, Trondheim, Norway. Standard mix working solutions (concentration ranges 0.15 μg/kg to 8.50 μg/kg) containing PAH16 were prepared by virtue of diluting the stock solution with acetonitrile and then stored at + 4°C in darkness. Benzo[b]chrysene (BbC) was supplied by Interchim (Montlucon, France).
The reference materials of (frozen) bivalve molluscs (ILC1060, ID025), smoked meat (ILC 424, ID109) and smoked black pepper (ILC 334, ID087), were supplied by the European Commission Joint Research Centre European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons (Institute for Reference Materials and Measurements, Geel, Belgium), while the smoked fish reference material (T0672QC) was purchased from FAPAS (Sand Hutton, York, UK).

2.3. Extraction and clean-up

PAHs were determined using the slightly modified method described by WEGRZYN and co-authors (2006) PAH isolation involves a preparative size-exclusion chromatography allowing for the efficient single-step lipid removal without saponification; within this frame, benzo[b]chrysene is used as an internal quantification standard. A homogenized sample (1 g) was spiked with BbC (50 μg/L, 100 μL) and diluted with dichloromethane to the final 4-mL volume. A sample was homogenized and vortexed for 10 min. The obtained mixture was centrifuged for 10 min at 3 500 rpm and 4°C. The supernatant was decanted and filtered through a 0.22-μm PTFE syringe on-line filter (Phenomenex, Torrance, USA) and transferred into a glass HPLC vial. Sample extracts were injected into a HPLC Agilent 1200 Series system (Agilent, Singapore, Singapore) for size-exclusion chromatography (SEC) with a fraction collector (Gilson, FC203B, Middleton, USA). The preparative SEC

was performed under isocratic conditions (100% dichloromethane) at the flow rate of 1 mL/min and room temperature using 2 size-exclusion columns connected in series (packed with PL gel based on PS/DVB, 300 × 7.8 mm i.d., 5 μm particle size and 50 Å) provided by Phenomenex (Phenomenex, Torrance, USA). The injection volume was 400 μL. Chromatograms were monitored at 254 nm and fractions were collected within 18 - 24 min timeframe. Aliquots were evaporated to dryness in a rotational vacuum concentrator (RCV2-18HCL; Christ, Osterode am Harz, Germany) at the speed of 1,300 rounds per minute; this stage took 40 min and went on at 20°C. The residue was dissolved in 100 μL of acetonitrile, so as to undergo chromatographic analysis (UPLC-FLD).

2.4. UPLC-FLD analysis

The UPLC PAH analysis was performed using an Ultra Pressure Liquid Chromatograph (Agilent 1290 Infinity UHPLC, Agilent, Singapore) equipped with a binary gradient pump (G4220A) and an auto-sampler having a thermostated sample compartment (G4226A), a thermostated column compartment (G1316C) and a fluorescence detector (G1321B). The separation of compounds was done in a Hypersil Green C18 PAH analytical column (150 mm x 3.0 mm i.d., 3.0 μm particle size) with a C18 guard Hypersil Green PAH column (10 mm x 3.0 mm i.d., 3.0 μm particle size) supplied by Thermo Scientific (Thermo-Scientific, Germany), the maintained temperature thereby being 30°C and the injection volume being 15 μL. The mobile phase consisted of a mixture of acetonitrile and acetonitrile/water (1/1) and was operated in the gradient mode at the flow rate of 0.8 mL/min (WEGRZYN *et al*., 2006). The initial composition of 100 % of acetonitrile/water (1/1) increased to 100% of acetonitrile in 30 min. The initial conditions were reached in 5 min. The total run time was 35 min. The excitation and emission wavelength pairs (excitation-Ex, emission-Em) used with fluorescence detection were as follows: Minute 2: Ex = 270 nm, Em = 340 nm for NAP; Minute 6.5: Ex = 250 nm, Em = 310 nm for FLR, ACE; Minute 8.5: Ex = 250 nm, Em = 380 nm for PHEN, ANTHR; Minute 11.0: Ex = 250 nm, Em = 460 nm for F, Minute12.1: Ex = 270 nm, Em = 385 nm for PYR; Minute: 15.5 Ex = 256 nm, Em = 395 nm for BaA, CHR; Minute 19.0: Ex = 295 nm, Em = 466 nm for BbF; Minute 21.5: Ex = 250 nm, Em = 410 nm for BkF BaP, D[ah]A, B[ghi]P; Minute 27.4: Ex = 295 nm, Em = 500 nm for I[cd]P; Minute 28.3: Ex = 460 nm, Em = 250 nm for BbC. The compounds were quantified using internal calibrations curves plotted for each of the 15 PAHs at seven concentration levels ranging from 0.15 to 8.5 μg/kg. Standard mix working solutions containing PAHs in different concentrations and a fixed amount of internal standard (5 μg/kg) were prepared in acetonitrile and injected in duplicates (15 μL per injection) so as to be able to come up with the linear regression lines.
Each sample of food products under study was analyzed in duplicate; the final PAH content was calculated as the mean of two parallel runs and expressed in μg/kg (of wet weight). The PAH content calculation also included the toxic equivalency factors (TEF) approach, so that PAH concentrations are also expressed as benzo[a]pyrene toxic equivalents; to that effect, the converting factors referred to by LAW *et al.* (2002) were used. The benzo(a)pyrene equivalent concentration (BaPE), expressed in μg/kg of food, is calculated as follows:

$$BaPE = \sum(BaPE) = \sum(C_{PAHi} \times TEF_{PAHi}),$$

where C_{PAHi} represents the concentration of the PAH congener i in food (μg/kg, while TEF_{PAHi} represents the toxic equivalency factor of the PAH congener i.

In order to illustrate the toxic potency of the investigated PAHs, the ∑PAH15 concentration, ∑PAH8 concentration and ∑PAH4 concentration are expressed as BaPE ($BaPE_{\sum PAH4}$, $BaPE_{\sum PAH8}$, $BaPE_{\sum PAH15}$).

2.5. Validation of the method and analytical quality assurance

In-house validated method for the respective matrices, i.e. dried herbs and spices, fishery products, shellfish and meat products, was applied. The performance assessment criteria included applicability, the limit of detection (LOD), the limit of quantification (LOQ), precision ($HORRAT_r$, $HORRAT_R$), specificity, linearity and recovery. The limit of detection (LOD) was calculated from the average of ten PAH15-negative samples (consisting of shellfish, smoked fish, smoked meat or spices), earlier analysed for PAH presence and used for validation as the blank material; to the above average, the tripled standard deviation was added (LOD = mean ± 3SD) (EC REGULATION No 582/2016). In order to determine the limit of quantification (LOQ), the mean concentration determined in ten PAH-negative samples of each matrix was summed up with the six-fold standard deviation (LOQ = mean ± 6SD). The precision of the method was assessed using the Horwitz equation as the Horrat value descriptive of each PAH at three concentration levels under repeatable (HORRATr) and reproducible ($HORRAT_R$) conditions. Food samples were spiked at the concentrations of 0.5, 1 and 1.5 of the MLs defined for 4 PAHs in triplicate. For each PAH, the mean recovery of a 36-sample set was calculated and used for the accuracy assessment, evaluated based on the intra-laboratory coefficient of variation. The specificity was checked by analyzing 15 PAHs in each of the ten blank samples per tested matrix and verifying the presence of interferences in the region of interest where single PAHs were expected to elute. The linearity was checked through the regression coefficients of determination (r^2) of the analytical curves using the standard mix working solution containing 15 PAHs at seven concentration levels ranging from 0.15 to 8.5 μg/kg. In this study, the presence or absence of matrix effects was identified using calibration curves and a fixed amount of internal standard (5 μg/kg) obtained with matrix-matched calibration standards (all matrices of food groups investigated in the study) and calibration solutions in the solvent. In the first step a three-point calibration curve (1, 2 and 4 μg/kg for meat and fish products; 2.5, 5 and 10 μg/kg for dried herbs and spices and shellfish products) was plotted using linear regression with the calibration standards in the solvent solution. In the next step, another three-point calibration curve of the same concentrations per food group and a fixed amount of internal standard (5 μg/kg) was plotted based on the measurement data of the matrix-matched calibration standards. The slopes of the regression curves representative of the two sets of calibration solutions were evaluated statistically. Internal quality control was pursued with each analytical batch using the available reference material (frozen mussels, ILC1060, ID025; T0672QC smoked fish, smoked meat, ILC 424, ID109 and ILC 334 and ID087 smoked black pepper), and was carried out by virtue of spiking the food samples so as to obtain the concentration of 2 μg/kg. Within each analytical series, the reference materials and the spiked food samples were analysed in duplicate and checked for recovery. The interpretation of validation and quality assurance results was performed as proposed by the EC REGULATION No 836/2011.

2.6. Statistical analysis

Data analysis was carried out using the XLSTAT 2018.3.51141 Software package (Addinsoft, New York, USA). The Kruskal-Wallis one-way analysis of variance was

performed for each data set so as to detect differences among food groups, considered to be statistically significant if estimated at the level of probability of $p = 0.05$.

3. RESULTS AND DISCUSSION

3.1. Validation of the method and quality assurance results

The results concerning linearity, LOQ, recovery and $HORRAT_R$ are presented in Table 1 and Fig. 1 shows a chromatogram descriptive of the target PAHs in a standard ($c = 1.06$ $\mu g/kg$) and a smoked ham sample spiked at the level of 1.96 $\mu g/kg$. The LOD established for the studied food groups ranged from 0.02 to 1.00 $\mu g/kg$, while the LOQ varied from 0.15 to 1.84 $\mu g/kg$.
The recoveries obtained within the frame of internal quality control spanned from 50 to 120 %, which is in accordance with the criteria established by the EC REGULATION No. 836/2011. The mean slopes of the regression curves for the standard and matrix sets of calibration solutions were not significantly different. Therefore, matrix-matched standard calibrations were not used. The applied analytical method fulfils all methodological requirements set out by the EC REGULATION No. 836/2011 and can therefore be considered as suitable for the determination of 15 PAHs in food categories under this study.

3.2. Shellfish products

Bivalve molluscs, posing in this study as unprocessed food representatives, are exposed to PAHs ubiquitously present in the marine environment due to polluted sediments, spill residues, shipping activities (de-ballasting waters), industrial and urban runoff, and atmospheric pollution (SORIANO *et al*., 2006). Furthermore, they are widely used in coastal monitoring programmes and pollution assessment studies as filter-feeders having a slow rate of detoxification and the ability to accumulate many toxic contaminants.
In this research, shellfish products were divided into two groups: mussels and other shellfish. The mussels were produced on farms, while other shellfish came from natural habitats in the Adriatic Sea. In cultured mussels, the predominance of PHEN, FLR, F and PYR was established (Table 2).
Other shellfish showed the abundance of PHEN, ACE and F, with the highest PHEN and ACE levels found in warty venus and the F levels in oysters. Statistically significant percent-shares of low molecular (78.9 % in mussels and 69.6 % in other shellfish) (Fig. 2) as compared to those of high molecular PAHs, obtained in this study (Fig. 3), were also reported in the studies by BIHARI *et al.* (2007), PERUGGINI *et al.* (2007), SERPE *et al.* (2011), and MERCOGLIANO *et al.* (2016) for shellfish coming from other Adriatic and Mediterranean areas. The majority of bivalve species collected from the Croatian market contained the investigated ∑PAH15 in concentrations ranging from 0.71 $\mu g/kg$ to 14.49 $\mu g/kg$ in mussels, and from 6.07 $\mu g/kg$ to 27.45 $\mu g/kg$ in other shellfish products (Table 3).
As for toxicologically important PAH markers, significantly higher ∑PAH4 and ∑PAH8 were found in other bivalve species, in particular in oysters, with a predominance of I[cd]P (6.99 $\mu g/kg$), BbF (4.02 $\mu g/kg$) and CHR (3.16 $\mu g/kg$). High- and medium-molecular PAHs in marine ecosystems are mostly of pyrolytic origins (MERCOGLIANO *et al*., 2016), so that their occurrence in other bivalve species under this study may also come from pyrolytic sources (antropogenic pollution coming from the mainland). A statistically significant difference in BaP content was found amongst the investigated bivalve

molluscs, above all when it comes to the smooth clam (Table 3). This study confirmed that farmed shellfish species (mussels) are characterized with lower levels of toxicologically important PAHs as compared to native shellfish species, which is in line with the results obtained in the studies by MERCOGLIANO *et al.* (2016) and ZELINKOVA *et al.* (2015). The majority of BaP and ∑PAH4 levels found in shellfish harvested along the Croatian coast were far below the MLs of 5 μg/kg and 30 μg/kg, respectively EC REGULATION No. 835/2011, as can be seen in Table 3, and are in accordance with the findings in mussels harvested along the Adriatic, the Campanian and the Ionian coasts of Italy (STORELLI and MARCOTRIGIANO, 2011; SERPE *et al*., 2010). The highest ∑PAH15 (27.5 μg/kg and 26.5 μg/kg) and ∑PAH8 (14.3 μg/kg) were determined in oysters and warty venus, whereas mussels showed the highest BaP content. Significant differences in $BaPE_{\Sigma PAH4}$, $BaPE_{\Sigma PAH8}$ and $BaPE_{\Sigma PAH15}$ values were observed, with the highest values established in oysters. Literature sources have reported $BaPE_{\Sigma PAH15}$ values of 1.56 μg/kg ww determined in Mediterranean mussels coming from the Adriatic Sea (PERUGINI *et al.,* 2007) and ranges of 0.1 to 4.5 μg/kg dry wt in shellfish coming from the Red Sea (EL NEMR *et al.,* 2016). PAH uptake depends on the physiology of the up-taking organisms and cyclic annual variations. In accordance with the EU legislation, monitoring of aqua-cultured shellfish is carried out along the Croatian coast, so as to ensure that PAH levels are within the consumer safety limits (BOGDANOVIĆ *et al.,* 2014). The study results revealed that sampling techniques developed for PAH monitoring in cultivated and wild shellfish found along the Croatian coast, minimize any risk for human health coming from the seafood consumption which has generally been tagged as one of the main sources of human exposure to severe pollutants.

3.3. Smoked fish

The majority of PAHs present in smoked food originate from wood smoke. While the amounts of PAHs in raw fish are very low due to the fish ability to oxidize and further metabolise PAHs absorbed from the environment, cold and hot-smoked fish are generally characterised with higher PAH contents that depend on fish properties, methods and parameters of fish smoking, composition of the smoke and the level of exposure of edible fish parts to the smoke released (DUEDAHL-OLESEN *et al.,* 2010; STOLYHWO *et al.,* 2005; ZELINKOVA *et al*., 2015). Furthermore, if a fishery product is canned in oil, the contamination may arise due to vegetable oil. Similar to meat products, the skin acts as a barrier against smoke particles, hence preventing any significant PAH penetration into fish muscles.

The mean PAH contents determined in smoked fish samples in this study are reported in Table 2. PAH profiling of fishery products coming from the Croatian market revealed the predominance of four light PAHs (ACE, PHEN, FLR and ANTHR) (Table 2, Fig. 2), which is in accordance with the results reported for commercial smoked fish (VARLET *et al.,* 2007; ZELINKOVA *et al*., 2015; DUEDAHL-OLESEN *et al*., 2018). PHEN, ANTHR, PYR and F were detected in all samples, the highest concentration of PHEN thereby being detected in smoked salmon pate (5.76 μg/kg). I[1cd]P (6.63 μg/kg) was determined in only one smoked sea bass sample. The analysis of the selected fishery products revealed the highest ∑PAH15 values in smoked salmon pate (118.05 μg/kg), followed by smoked sea bream (35.29 μg/kg) and smoked trout (32.13 μg/kg), while the lowest value was established in smoked tuna (1.17 μg/kg). The PAH amounts found in fish samples positively correlate with the lipid content of the same (SINGH *et al.,* 2016), which may explain the highest ∑PAH15 values obtained in smoked salmon pate within our study frame. As stated above, the amount of PAHs transferred by smoke particles into the final fishery product depends on several processing parameters including smoking technology,

combustion temperature, smoke composition, type of wood used, and the level of exposure of edible fish parts to the smoke released (STOLYHWO *et al*., 2005, DUEDAHL-OLESEN *et al*., 2010). Among the mutagenic/carcinogenic PAHs analysed, the highest BaP (0.76 μg/kg), ∑PAH4 (1.70 μg/kg) and ∑PAH8 (11.56 μg/kg) (Table 3) were detected in smoked sea bass fillets, while the lowest BaP (< LOD μg/kg), ∑PAH4 (0.18 μg/kg) and ∑PAH8 (0.22 μg/kg) were determined in smoked tuna. The aggregated average ∑PAH8 content decreased in the following order: I[cd]P, B[bghi]P, CHR, D[ah]A, B[b]F, B[a]P, B[a]A and B[k]F (Tables 2 and 3). The investigated fishery products complied to the PAH MLs of 2 μg/kg for B[a]P and 12 μg/kg for ∑PAH4, laid down under the EU legislation. BaP-based toxic equivalency factors calculated for ∑PAH4, ∑PAH8 and PAH15 showed a similar decreasing pattern starting with the highest values found in smoked sea bass that declined over smoked sardine in sunflower oil and smoked salmon pate down to the lowest values in smoked trout (Table 3). Smoked salmon pate, most heavily contaminated with PAH15, was characterized with low BaPE levels, while smoked sea bass fillets, moderately contaminated with PAH15, showed the highest BaPE levels, which can be attributed to the presence of heavy PAHs that have higher TEF values. A similar $BaPE_{PAHtotal}$ trend has been observed in different food items, for instance in the study by PERUGINI *et al.* (2007) in fish and shellfish, in the study by SANTOS *et al.* (2011) in meat products, and in the study by GOMES *et al.* (2013) in traditional meat sausages.

3.4. Meat products

Smoked meat products represent the principal source of PAHs that generate during an incomplete wood combustion (ALVES *et al.* 2018). As already well known, the amounts and types of PAHs present in contaminated smoke meat products depend on a number of factors, such as the fuel used, the smoking technique, the temperature at which the pyrolysis takes place, the air flow through the smoke generator, the distance between the meat sample and the heat source, the smoking chamber design, the smoked meat fat content, the duration of smoking, and the cleanness and maintenance of the equipment used (CODEX ALIMENTARIUS COMMISSION, 2009). Data on PAHs in smoked meat products are highly variable, this discrepancy being explained by differences in food smoking procedures and meat product characteristics (whole meat *versus* chopped meat products). Spices used in smoked meat production can also be contaminated with PAHs, therefore further increasing the levels of those hazardous contaminants.
Smoked meat products investigated in this study were divided into four groups, consisting of either whole meat samples in terms of dry-cured and semi-dry-cured meat products, or chopped meat products in terms of dry-fermented and semi-dry sausages. ∑PAH4 and ∑PAH8 composition pattern was dominated by light, harmless PAHs (93.0 % in semi-dry meat products to 98.3 % in dry-fermented sausages). Amongst them, the four PAHs most abundantly present in dry-cured meat products were (in descending order) PHEN, FLR, ACE and ANTHR. Similarly, dry-fermented sausages were also characterized by light PAHs supremacy, their representation thereby following virtually the same PHEN-ACE-FLR-ANTHR decreasing pattern described above, while both semi-dry-cured meat products and semi-dry sausages showed a little bit different PAH presence pattern dominated by PHEN, F, ACE and FLR in decreasing order for the former meat products and by PHEN, FLR, F and PYR in decreasing order of the latter meat products (Table 2, Fig. 2).

Table 1. Selected performance indicators of the method in use: linearity, limit of quantification (LOQ), recovery and precision (HORRAT$_R$).

PAHs [a]	Linearity (r^2)[b]	Dried herbs and spices			Shellfish			Smoked fish			Smoked meat		
		LOQ[c] (μg/kg)	Recovery[d]	HORRAT$_R$[e]	LOQ (μg/kg)	Recovery[d]	HORRAT$_R$[e]	LOQ (μg/kg)	Recovery[d]	HORRAT$_R$[e]	LOQ (μg/kg)	Recovery[d]	HORRAT$_R$[e]
NAP	0.998	3.14	71.22	1.88	1.85	76.18	1.66	2.90	77.72	0.99	2.97	119.2	1.08
FLR	0.999	3.30	103.5	0.99	3.23	113.9	0.76	2.94	76.88	0.76	3.30	120.0	1.33
ACE	0.999	3.27	91.03	1.15	0.63	102.2	1.02	2.61	71.08	0.98	3.00	88.20	1.25
PHEN	1.000	0.30	69.77	0.79	0.79	117.0	1.32	0.40	82.95	1.44	0.79	98.25	1.06
ANTHR	1.000	0.43	86.61	1.36	0.50	112.9	0.85	0.69	64.21	0.85	0.50	100.0	0.85
F	0.997	0.50	89.61	2.00	0.40	116.5	1.65	0.59	92.05	1.77	0.30	100.0	0.86
PYR	0.999	0.59	92.06	0.86	0.86	93.87	1.14	0.89	80.34	1.14	0.86	100.0	1.14
B(a)A	0.999	0.26	75.56	1.28	0.07	116.0	1.89	0.23	68.56	1.89	0.07	90.40	1.89
CHR	1.000	0.17	94.54	1.66	0.07	117.2	1.18	0.13	76.82	1.18	0.07	88.80	1.18
B(b)F	1.096	0.23	99.99	0.89	0.26	106.4	0.87	0.17	71.86	0.87	0.26	94.71	0.08
B(k)F	1.000	0.23	83.66	1.33	0.20	93.55	1.11	0.17	78.73	1.11	0.20	100.0	1.11
B(a)P	1.000	0.26	76.99	1.65	0.10	94.15	0.71	0.40	75.84	0.71	0.10	82.00	0.71
D[ah]A	0.998	0.63	88.77	1.18	0.66	88.48	0.84	0.69	81.05	0.84	0.76	77.00	0.84
B[ghi]P	0.972	0.56	70.66	1.44	0.56	86.49	1.13	0.53	81.99	1.13	0.50	100.0	1.13
I[cd]P	0.973	0.26	69.05	0.88	0.30	78.32	0.14	0.36	85.24	1.14	0.30	68.75	0.14

[a]Polycyclic aromatic hydrocarbons (PAHs): acenaphthene (ACE), acenaphthylene, anthracene (ANTHR), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (D[ah]A), fluoranthene (F), fluorene (FLR), indeno[1,2,3cd] pyrene I[cd]P, naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR); [b]Determination coefficients (r^2) of the analytical seven-point curves constructed for standard solutions (0.25-8.50 μg/kg);[c]Limit of quantification; [d]Mean recovery at three concentrations used in the precision assessment (selected food categories were spiked at the concentrations of 0.5, 1 and 1.5 of MLs); [e]Horrat coefficient (EC REGULATION No. 836/2011) for each PAH at three concentrations (selected food categories were spiked at the concentrations of 0.5, 1 and 1.5 of MLs value) in reproducibility (R) conditions used for the method precision assessment.

Table 2. Determination of PAHs levels in selected food from Croatian market.

	Fresh shellfish						Meat products									
	Mussels (n^a = 29)		Other shellfish (n^a = 13)		Smoked fish (n^a = 8)		Dry-cured meat products (n^a = 35)		Semi-dry cured meat products (n^a = 15)		Dry-fermented sausages (n^a = 10)		Semi-dry sausasges (n^a = 10)		Dried herbs and spices (n^a = 20)	
PAHs	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
NAP	$<0.56^{C}$	<0.56-6.62	$<0.56^{C}$	<0.56	<0.95C	<0.88-0.79	1.58^{B}	<0.55-27.60	$<0.95^{C}$	<0.55-4.09	2.55^{A}	<0.55-11.12	$<0.55^{C}$	<0.55-0.71	1.93^{B}	<0.95-27.68
ACE	0.24^{E}	<0.19-6.85	2.00^{D}	<0.19-8.11	22.30^{B}	<0.79-99.67	2.75^{D}	<0.91-25.97	1.56^{D}	<0.91-16.81	11.25^{C}	<0.91-45.69	$<0.91^{E}$	<0.91-1.01	76.34^{A}	<0.99-807.68
FLR	1.21	<0.98-4.01	<1.00	<0.98-1.27	2.79^{BC}	<0.89-9.86	4.15^{B}	<1.00-51.13	1.04	<1.00-2.33	11.14^{A}	<1.00-42.95	1.26^{C}	<1.00-4.48	6.56^{AB}	<1.00-31.74
PHEN	1.56^{E}	<0.24-4.17	2.16^{DE}	<0.24-6.29	3.14^{CDE}	<0.12-5.76	8.88^{BC}	<0.91-54.86	3.95^{CD}	<0.91-12.93	14.31^{B}	<0.91-63.54	3.19^{D}	<0.91-9.10	55.44^{A}	<0.09-473.05
ANTHR	0.16^{F}	<0.15-3.20	0.28^{EF}	<0.15-1.88	0.87^{DE}	<0.21-2.00	2.10^{CD}	<0.24-13.88	0.78^{E}	<0.24-3.38	5.38^{B}	<0.24-21.95	0.71^{E}	<0.24-2.78	13.24^{A}	<0.13-74.55
F	0.58^{CD}	<0.12-2.21	1.89^{B}	<0.12-6.96	0.56^{D}	<0.18-1.08	1.54^{BC}	<0.15-6.39	2.05^{B}	<0.15-10.22	1.80^{B}	<0.15-5.39	1.12^{BC}	<0.15-3.45	22.87^{A}	<0.15-124.63
PYR	0.49^{D}	<0.26-1.21	0.84^{CD}	0.30-1.94	0.78^{D}	<0.27-1.40	1.13^{C}	<0.26-10.88	1.02^{CD}	<0.26-4.27	1.94^{B}	<0.26-7.07	0.89^{C}	<0.26-2.71	26.31^{A}	<0.18-163.86
B[a]A	0.10^{C}	<0.02-0.62	0.22^{B}	<0.02-0.75	0.10^{C}	<0.07-0.19	0.15^{C}	<0.02-1.41	0.14^{C}	<0.02-0.66	0.09^{C}	<0.02-0.28	0.07^{C}	<0.02-0.21	5.76^{A}	<0.08-25.39
CHR	0.45^{B}	<0.02-0.98	0.53^{B}	<0.02-3.16	0.27^{CD}	<0.04-0.61	0.30^{CD}	<0.02-1.9	0.20^{CD}	<0.02-0.65	0.27^{CD}	<0.02-0.57	0.18^{D}	<0.02-0.38	10.15^{A}	<0.05-45.68
B[b]F	0.40^{BC}	<0.08-1.27	0.76^{B}	<0.08-4.02	0.19^{DE}	<0.05-0.52	0.26^{DE}	<0.08-1.78	0.13^{DE}	<0.08-0.49	0.14^{DE}	<0.08-0.30	0.08^{E}	<0.08-0.25	4.83^{A}	<0.07-20.86
B[k]F	0.23^{B}	<0.05-0.71	0.40^{B}	<0.05-2.14	$<0.05^{C}$	<0.05-0.09	0.15^{BC}	<0.06-1.55	0.10^{C}	<0.06-0.58	0.09^{C}	<0.06-0.17	$<0.06^{C}$	<0.06-0.19	1.55^{A}	<0.07-11.02
B[a]P	0.11^{C}	<0.12-0.69	0.21^{B}	<0.12-0.46	0.16^{BC}	<0.12-0.76	0.19^{B}	<0.03-1.47	0.11^{C}	<0.03-0.40	0.12^{C}	<0.03-0.30	0.15^{BC}	<0.03-0.37	4.85^{A}	0.15-21.88
D[ah]A	0.11^{D}	<0.20-1.39	0.48^{B}	<0.20-1.67	0.26^{C}	<0.21-1.59	$<0.23^{D}$	<0.23-1.72	$<0.23^{D}$	<0.23-0.52	$<0.23^{D}$	<0.23-0.78	$<0.23^{D}$	<0.23-0.25	8.68^{A}	<0.19-39.74
B[ghi]P	0.29^{C}	<0.17-1.32	0.69^{B}	<0.17-1.81	0.40^{C}	<0.16-1.63	0.31^{C}	<0.15-1.54	0.37^{C}	<0.15-0.70	0.30^{C}	<0.15-0.66	0.44^{C}	<0.15-1.45	3.70^{A}	<0.17-14.29
I[cd]P	0.24^{C}	<0.09-2.66	1.14^{B}	<0.09-6.99	0.83^{B}	<0.11-6.63	0.11^{C}	<0.09-1.29	$<0.09^{C}$	<0.09-0.59	$<0.09^{C}$	<0.09-0.53	0.29^{C}	<0.09-1.67	3.80^{A}	<0.08-22.10

[a]n – number of samples; [b]PAHs - polycyclic aromatic hydrocarbons, acenaphthene (ACE), acenaphthylene, anthracene (ANTHR), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (D[ah]A), fluoranthene (F), fluorene (FLR), indeno[1,2,3cd] pyrene I[cd]P, naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR); Values expressed as < (less than) denote values lower than the detection limit. Superscript uppercase letters A, B, C, D denote statistically significant difference ($p < 0.05$) in the investigated polycyclic aromatic hydrocarbons.

Table 3. Minimum, maximum, median and mean concentrations (μg/kg) of BaP[a], ∑PAH4[b], ∑PAH8[c] and PAH15[d] with benzo(a)pyrene toxic equivalents (BaPE[e], μg/kg) detected in different food from Croatian market

	Concentration μg/kg						
	BaP[a]	**∑PAH4[b]**	**∑PAH8[c]**	**PAH15[d]**	**[e]BaPE$_{PAH4}$**	**[e]BaPE$_{PAH8}$**	**[e]BaPE$_{PAH15}$**
			Mussels (n = 29)				
Minimum	<0.03	<0.02	0.18	0.71	<0.03	<0.03	0.03
Maximum	0.69	3.56	7.70	14.49	0.63	7.96	8.12
Median	0.06	0.95	1.38	5.28	0.12	0.15	0.21
Mean	0.11^{D}	1.06^{BC}	1.93^{CD}	6.55^{C}	0.17^{BC}	0.75^{DE}	0.82^{D}
			Other shellfish (n=13)				
Minimum	0.09	0.58	1.60	6.07	0.12	0.13	0.20
Maximum	0.46	7.94	14.33	27.45	0.83	9.31	9.44
Median	0.17	1.34	2.75	9.98	0.32	1.67	1.72
Mean	0.21^{AB}	1.72^{AB}	4.42^{AB}	12.08^{C}	0.31^{A}	2.84^{AB}	2.96^{AB}
			Smoked fish (n=8)				
Minimum	<0.12	0.18	0.22	1.17	0.05	0.05	0.14
Maximum	0.76	1.70	11.56	118.05	0.80	9.44	9.64
Median	<0.12	0.59	0.86	18.91	0.11	0.14	0.28
Mean	0.16^{BC}	0.72^{CD}	2.23^{BC}	29.64^{B}	0.19^{B}	1.58^{BC}	1.72^{B}
			Dry-cured meat products (n = 35)				
Minimum	<0.03	0.07	0.09	4.50	<0.03	<0.03	<0.03
Maximum	1.47	6.28	12.13	125.17	1.80	10.54	10.81
Median	0.08	0.55	0.92	13.21	0.12	0.19	0.32
Mean	0.19^{CD}	0.90^{DE}	1.59^{E}	22.04^{C}	0.24^{BC}	0.85^{E}	1.05^{D}
			Semi-dry cured meat products (n=15)				
Minimum	<0.03	<0.02	0.31	3.09	<0.03	<0.03	0.18
Maximum	0.40	2.15	3.68	30.15	0.48	3.15	3.28
Median	0.04	0.36	0.86	9.21	<0.08	<0.08	0.14
Mean	0.11^{D}	0.58^{E}	1.18^{E}	12.10^{C}	0.14^{C}	0.44^{DE}	0.59^{CD}

Dry-fermented sausages (n=10)							
Minimum	<0.03	0.09	0.31	9.26	<0.03	<0.03	0.18
Maximum	0.30	0.97	2.01	171.48	0.33	4.24	4.51
Median	0.09	0.67	1.20	28.04	0.13	0.25	0.63
Mean	0.12^{D}	0.61^{CDE}	1.21^{DE}	44.59^{AB}	0.14^{BC}	0.90^{CD}	1.26^{BC}
Semi-dry sausasges (n=20)							
Minimum	<0.03	0.16	0.17	0.28	<0.03	<0.03	<0.03
Maximum	0.37	1.10	4.32	23.02	0.39	1.84	2.24
Median	0.14	0.35	1.03	6.48	0.15	0.29	0.38
Mean	0.15^{BCD}	0.49^{E}	1.29^{CDE}	8.76^{C}	0.17^{BC}	0.36^{DE}	0.51^{D}
Dried herbs and spices (n=20)							
Minimum	0.15	1.24	3.63	7.22	0.21	0.31	0.48
Maximum	21.88	113.12	207.46	1009.53	27.27	278.36	300.76
Median	0.81	4.54	8.38	49.00	1.16	8.02	10.75
Mean	4.85^{A}	25.60^{A}	43.34^{A}	246.03^{A}	6.09^{A}	49.93^{A}	53.64^{A}

[a]BaP: Benzo(a)pyrene; [b]∑PAH4: The sum of benzo(a)anthracene - BaA, chrysene - CHR, benzo(a)pyrene - BaP and benzo(b)fluoranthene - BbF; [c]∑PAH8: The sum of benzo(a)anthracene - BaA, chrysene - CHR, benzo(a)pyrene - BaP and benzo(b)fluoranthene – BbF, benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P) dibenz[a,h]anthracene (D[ah]A) and indeno[1,2,3cd] pyrene I[cd]P; d∑PAH15: The sum of acenaphthene (ACE), anthracene (ANTHR), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (D[ah]A), fluoranthene (F), fluorene (FLR), indeno[1,2,3cd] pyrene I[cd]P, naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR) and [e]BaPE: The benzo(a)pyrene based toxic equivalency factors expressed to ∑PAH4, ∑PAH8 and ∑PAH15. Values expressed as < (less than) denote values lower than the detection limit. Different superscript uppercase letters A, B, C, D, E denote statistically significant difference ($p < 0.05$) in the investigated polycyclic aromatic hydrocarbons and benzo(a)pyrene based toxic equivalency factors of selected food groups (marked in columns).

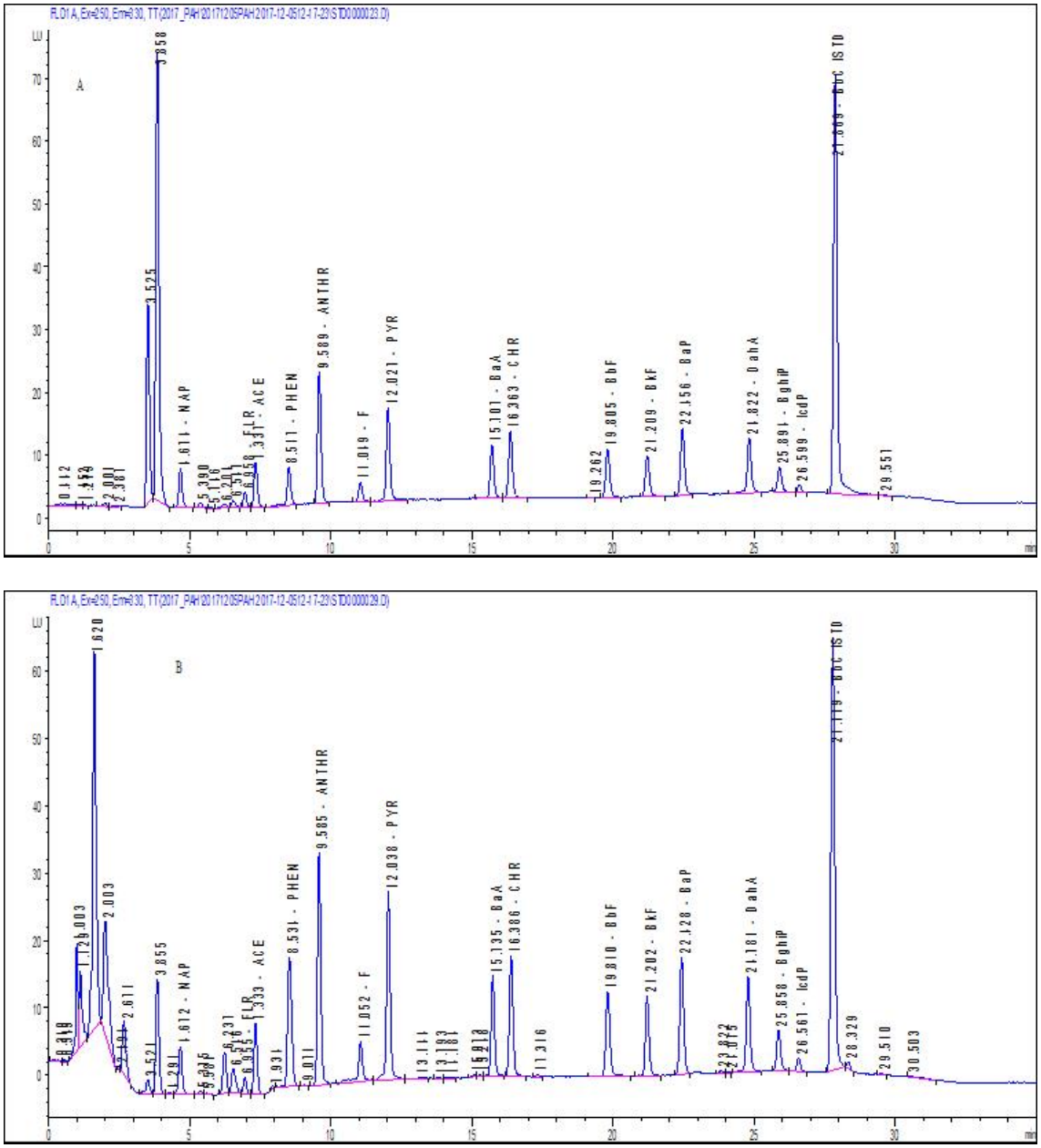


Figure 1. Chromatogram of the target PAHs (acenaphthene (ACE), anthracene (ANTHR), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), benzo[a]pyrene (BaP), chrysene (CHR), dibenz[a,h]anthracene (D[ah]A), fluoranthene (F), fluorene (FLR), indeno[1,2,3cd] pyrene I[cd]P, naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR) in standard sample at the level of 1.06 μg/kg (16 Priority PAH, Cocktail 3, Chiron) (A) and smoked ham sample spiked at the level 1.96 μg/kg (B)

A similar PAH profile was reported in the studies by SANTOS *et al.* (2011) and ROSEIRO *et al.* (2012), in which the prevalence of light PAHs was attributed to the unique sensorial properties of Portuguese traditional meat sausages. ALVES *et al.* (2017) also confirmed the similar pattern of low molecular PAHs' domination in fermented sausages of distinctive Portuguese and Serbian origin. Regarding the carcinogenic/mutagenic PAHs, BaP, ∑PAH4 and ∑PAH8 levels determined in this study were very low, with the highest values in sirloin (1.47 μg/kg for BaP, 6.28 μg/kg for ∑PAH4 and 12.13 μg/kg for ∑PAH8).

Significantly higher amounts were reported for BaP in smoked meat products produced in Latvia (from <0.05 μg/kg to 6.03 μg/kg) (ROZENTĀLE *et al.*, 2015) and traditional smoked meat products from the Baltic States (from 0.05 μg/kg to 166 μg/kg) (ROZENTĀLE *et al.*, 2018), with ∑PAH4 in ranges from 0.15-34.65 μg/kg and 0.42-628 μg/kg, respectively. None of the samples investigated in our study exceeded the MLs for BaP and ∑PAH4 stipulated by the pertaining legislation (2 μg/kg and 12 μg/kg, respectively) (EC REGULATION No. 835/2011). When comparing the investigated meat products based on their PAH contents expressed as benzo(a)pyrene toxic equivalencies (BaPE) (Table 3), a slightly higher $BaPE_{\sum PAH15}$ and $BaPE_{\sum PAH8}$ were established in dry-fermented sausages, while a slightly higher $BaPE_{\sum PAH4}$ was seen in dry-cured meat products. It should also be mentioned that literature sources have revealed significantly higher BaPE concentrations than those measured in whole meat and chopped meat products analysed in our study; for instance, SANTOS *et al.* (2011) and GOMES *et al.* (2013) reported that ∑PAH16 BaPE in Portuguese traditional meat/blood products range from 2.74 μg/kg to 52.38 μg/kg, while ∑PAH8 spans from 0.59 to 55.33 μg/kg and ∑PAH4 from 1.43 μg/kg to 20.18 μg/kg. As for the mean total 15 PAHs content in the studied meat products, statistically significant differences were observed. The highest average 15 PAHs sum of 171.48 μg/kg established in dry-fermented sausages comes as the consequence of the highest low molecular PAH content. When it comes to the total PAH levels, dry-cured, semi-dry-cured meat products and semi-dry sausages differed significantly from dry-fermented sausages ($p<0.05$). Statistically significant differences in toxicologically important BaP, ∑PAH4 and ∑PAH8 ($p>0.05$) failed to be found within the whole meat products' groups. Therefore, data on Croatian meat products examined within the frame of this study argue against the need for exceptions stated under EC REGULATION No. 1327/2014 under which the MLs for BaP and ∑PAH4 in traditional smoked meat and fish products is set at 5 μg/kg and 30 μg/kg, respectively. However, determination of PAHs in processed meat is a permanent process. A true assessment of risk resulting from the constant presence of PAHs in food chain requires a versatile and precise analytical method capable of measuring the level of a number of toxic PAH compounds, with the possibility of extension to additional compounds in accordance with the recommendation of the Scientific Committee on Food. Furthermore, according to the recommendation of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), benzo(c)fluorene as a compound usually, however inappropriately omitted from PAH food analyses, should be included due to its carcinogenic effects and scarce data on its occurrence in food.

3.5. Dried herbs and spices

Dried herbs and spices are defined as vegetable products, or mixtures thereof, which are free from any extraneous matter whatsoever, and are used for flavouring, seasoning and imparting the food aroma; therefore, they are classified as "all natural" (IS0, 1995; TORRE TORRES *et al.*, 2015). Since all spices come from plants, they have generally been recognised as safe (GRAS). However, even though used in small amounts, spices have also been recognized as a potential source of chemical hazards (ROZENTĀLE *et al.*, 2017). Very high levels of PAHs detected in herbs and spices present in foodstuffs (DG SANCO, 2004; EFSA, 2008) have recently resulted in new legislation requirements for BaP content, which should not exceed 10 μg/kg, and the sum of BaP, BaA, BbF and BaP, which should not exceed 50 μg/kg EC REGULATION No. 1933/2015).

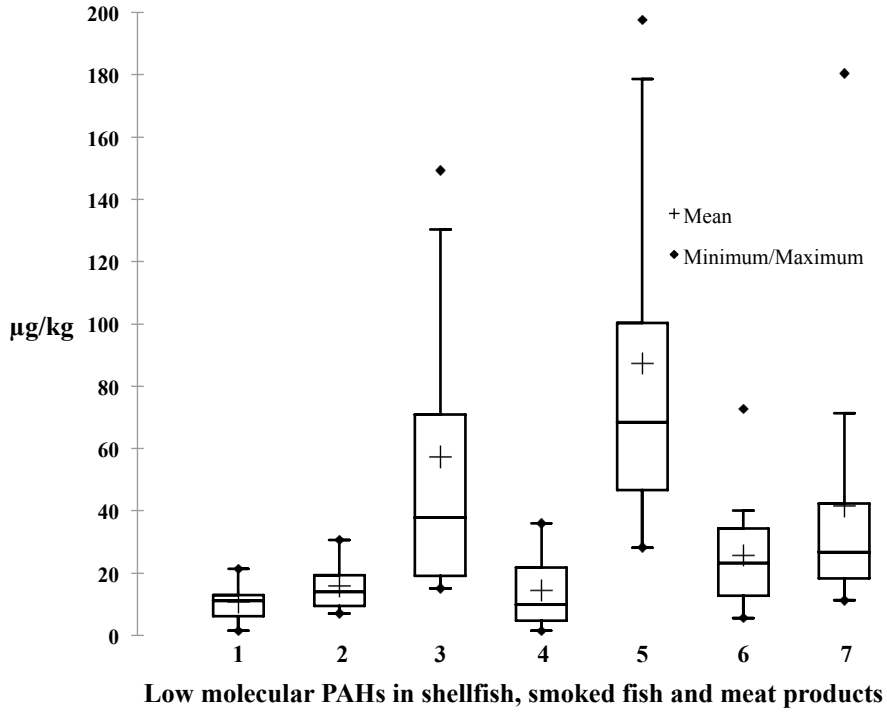


Figure 2. Contents (µg/kg fresh weight) of low molecular PAHs (acenaphthene (ACE), anthracene (ANTHR), benz[a]anthracene (BaA), fluoranthene (F), fluorene (FLR), naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR) in selected food from Croatian market (Food – 1: mussels; 2: other shellfish; 3:smoked fish; 4: semi dry sausages; 5: semi-dry cured meat products; 6: dry-fermented sausages; 7: dry-cured meat products).

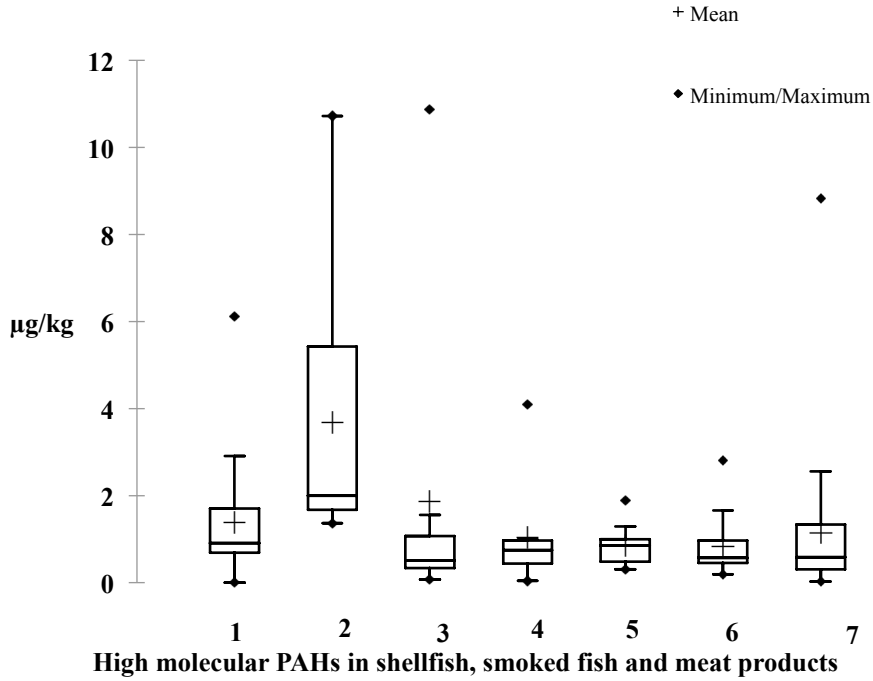


Figure 3. Contents (µg/kg fresh weight) of high molecular PAHs (benzo[b]fluoranthene (BbF), benzo[a]pyrene (BaP), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), dibenz[a,h]anthracene (D[ah]A), indeno[1,2,3cd] pyrene I[cd]P) in selected food from Croatian market (Food – 1: mussels; 2: other shellfish; 3:smoked fish; 4: semi dry sausages; 5: semi-dry cured meat products; 6: dry-fermented sausages; 7: dry-cured meat products).

Mean PAH concentrations obtained in this study in certain food categories are shown in Table 2. The majority of PAHs found in randomly sampled spices and herbs circulating on the Croatian market were low molecular ACE, PHEN and PYR, present in the total PAH15 content in the percent-share of 31.02 %, 22.53%, and 10.69%, respectively. As for the heavy ∑PAH4 and ∑PAH8, they were detected in all investigated herbs and spices (Fig. 4).

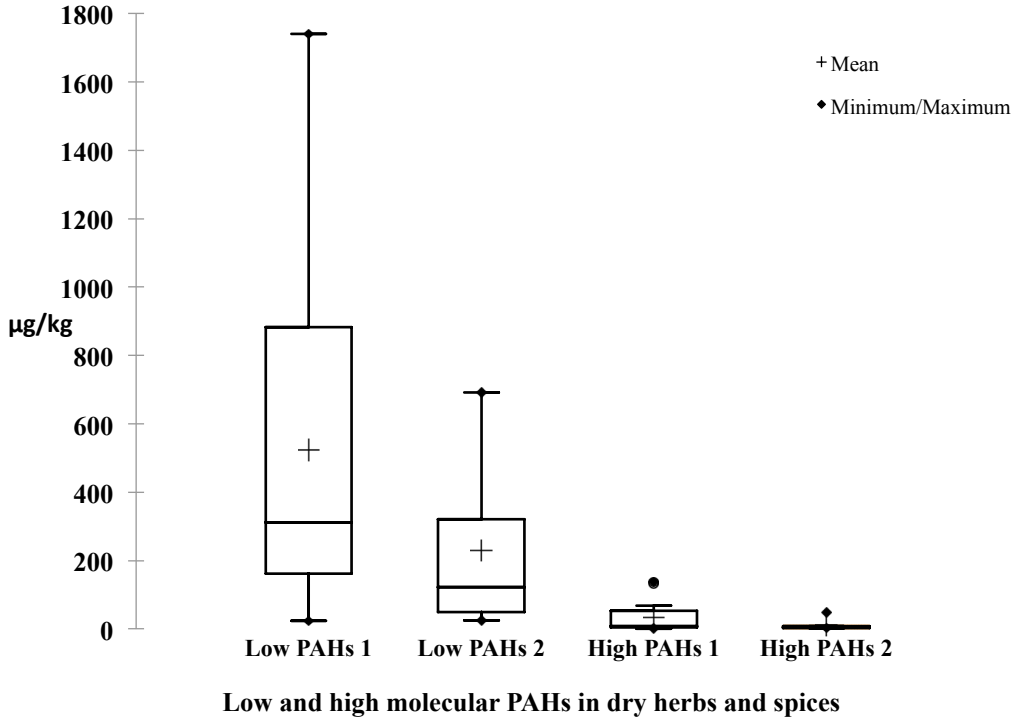


Figure 4. Contents (µg/kg fresh weight) of low (acenaphthene (ACE), anthracene (ANTHR), benz[a]anthracene (BaA), fluoranthene (F), fluorene (FLR), naphthalene (NAP), phenanthrene (PHEN) and pyrene (PYR and high molecular PAHs (benzo[b]fluoranthene (BbF), benzo[a]pyrene (BaP), benzo[k]fluoranthene (BkF), benzo[ghi]perylene (B[ghi]P), dibenz[a,h]anthracene (D[ah]A), indeno[1,2,3cd] pyrene I[cd]P) in selected dry herbs and spices from Croatian market.

The highest levels of the above were established in smoked paprika (∑PAH4 113.12 µg/kg, ∑PAH8 207.46 µg/kg) and the lowest in rosemary (∑PAH4 1.24 µg/kg, ∑PAH8 3.63 µg/kg) and garlic (∑PAH4 2.27 µg/kg, ∑PAH8 5.64 µg/kg). BaP concentration ranged from 0.15 µg/kg in garlic and 0.21 µg/kg in rosemary to 21.88 µg/kg in smoked paprika (Table 3). ∑PAH8 most abundantly present in the examined herbs and spices were CHR, D[ah]A, B[a]A, B[b]F and B[a]P (in decreasing order) (Table 2). The highest CHR, D[ah]A and B[a]A values (Table 2) were witnessed in smoked paprika, followed by mixed pepper (5.02 µg/kg, 6.09 µg/kg and 2.67 µg/kg, respectively) and rosemary (4.81 µg/kg, 37.92 µg/kg and 0.67 µg/kg, respectively).

In the study by ROZENTĀLE *et al.* (2017), the occurrence of four EU-regulated PAHs (B[a]P, B[a]A, CHR and B[b]F) was checked in 3 groups of herbs and 3 groups of spices. According to the authors, PAH concentration found to be the highest in almost all analysed seasonings, with the mean values ranging from 1.73 µg/kg (nutmeg) to 8.68 µg/kg (thyme), was that of CHR, its mean values established in red paprika and black pepper thereby being 3.18 µg/kg and 4.63 µg/kg, respectively. Similar to our study, the investigated seasonings showed variations in PAH levels. Contrary to the results of our

study, in the investigation by ROZENTÄLE *et al.* (2017) the highest BaP contamination was detected in black pepper at the level of 6.60 μg/kg, while in thyme the ∑PAH4 contamination of 37.39 μg/kg was determined. When it comes to $BaPE_{\Sigma PAH4}$, $BaPE_{\Sigma PAH8}$ and $BaPE_{\Sigma PAH15}$ calculated within this study frame, the obtained results exhibited a similar descending pattern with the highest results of 27.27 μg/kg, 278.36 μg/kg and 300.76 μg/kg, respectively, for smoked paprika, to the lowest BaPE values of 0.21 μg/kg, 0.31 μg/kg and 0.48 μg/kg, respectively, detected in garlic (Table 3). Traditional smoking and processing methods applied in the production of smoked paprika and smoked cardamom resulted in high PAH levels. However, given that the consumption of these spices is low, and to enable these smoked products to remain on the market, they were exempted from the maximal levels set out by the EC (2015). In summary, our results showed statistically significant ($p < 0.05$) shares of low molecular as compared to high molecular PAHs (Figure 2). However, the investigated herbs and spices were contaminated with PAHs at levels lower than the maximum levels established by the EU.

4. CONCLUSIONS

The results of this study confirmed that certain food items circulating on the Croatian market are contaminated with PAHs at levels below the established maximal limits set out under the pertaining legislation. A statistically significant difference ($p<0.05$) in toxicologically important PAH markers were found across the investigated food categories. With regard to BaP and ∑PAH4 contents, only certain spices showed significantly higher levels of the latter. Other shellfish, smoked fishery products and spices were characterized with higher ∑PAH8 marker values as compared to mussels and meat products. The highest total PAH amounts were found in dried herbs and spices, followed by meat sausages and smoked fish. The present study showed that processed foodstuffs are more severely contaminated with PAHs in comparison with food contaminated from environmental sources. Independent of food category, PAH composition pattern was dominated by low molecular PAHs. Being aware of the fact that the scope of the food-governing legislation is limited mostly due to the difficulty to define safe levels for complex PAH mixtures, future analyses should be extended to additional PAH compounds. A special attention should be paid to benzo[c]fluorene (BcF) as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), since data on its occurrence in food are still scarce, but the levels of benzo[c]fluorene-derived adducts are much higher than those of benzo[a]pyrene-derived adducts. Also, the margin of exposure (MOE) approach would be of interest for our further studies intended to evaluate certain food consumption patterns pursued in Croatia.

REFERENCES

Alomirah H., Al-Zenki S., Al-Hooti S., Zaghloul S., Sawaya W., Ahmed N. and Kannan K. 2011. Concentrations and dietary exposure to polycyclic aromatic hydrocarbons (PAHs) from grilled and smoked foods. Food Control 22:2035.

Alves S.P., Alfaia C.M., Škrbić B.D., Živančev J.R., Fernandes M.J., Bessa R.J.B. and Fraqueza, M.J. 2017. Screening chemical hazards of dry fermented sausages from distinct origins: biogenic amines, polycyclic aromatic hydrocarbons and heavy elements. J. Food Compos. Anal. 59:131.

Bihari N., Fafanđel M. and Piškur V. 2007. Polycyclic aromatic hydrocarbons and ecotoxicological characterization of seawater, sediment, and mussel *Mytilus galloprovincialis* from the gulf of Rijeka, the Adriatic Sea, Croatia. Arch. Environ. Contam. Toxicol. 52:387.

Bogdanović T., Ujević I., Sedak M., Listeš E., Šimat V., Petričević S. and Poljak V. 2014. As, Cd, Hg and Pb in four edible shellfish species from breeding and harvesting areas along the eastern Adriatic Coast, Croatia. Food Chem. 146:203.

Codex Alimentarius Commission (CAC) 2009. 'Code of Practice for the Reduction of Contamination of Food with PAH from Smoking and direct drying processes' with the objective of lowering PAH in smoke meat products and other foods.

Duedahl-Olesen, L., Christensen, J.H., Højgård A., Granby K. and Timm-Heinrich M. 2010. Influence of smoking parameters on the concentration of polycyclic aromatic hydrocarbons (PAHs) in Danish smoked fish. Food Addit. Contam. A, 27:1305.

EFSA 2008. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Polycyclic Aromatic Hydrocarbons in Food. EFSA J 724:1-114.

El Nemr A., Moneer A.A., Ragab S. and El Sikaily A. 2016. Distribution and sources of n-alkanes and polycyclic aromatic hydrocarbons in shellfish of the Egyptian Red Sea coast. Egypt J. Animal Sci. 31:172.

Environmental Protection Agency (EPA) 1994. Office of Air Quality Planning and Standards, Research Triangle Park, NC.

European Commission 2007. Commission Regulation No 333/2007 of 28 March 2007 laying down methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. Official Journal of the European Union L88:29-38.

European Commission 2011. Commission Regulation No 835/2011 of 19 August 2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs. Official Journal of the European Union L215:4-8.

European Commission 2011. Commission Regulation No 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. Official Journal of the European Union L215:9-16.

European Commission 2014. Commission Regulation No 1327/2014 of 12 December 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of polycyclic aromatic hydrocarbons (PAHs) in traditionally smoked meat and meat products and traditionally smoked fish and fishery products. Official Journal of the European Union L358:13-14.

European Commission 2015. Commission Regulation No 1933/2015 of 27 October 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in cocoa fibre, banana chips, food supplements, dried herbs and dried spices. Official Journal of the European Union L282:11-13.

European Commission 2016. Commission Regulation No 2016/582 of 15 April 2016 amending Regulation (EC) No 333/2007 as regards the analysis of inorganic arsenic, lead and polycyclic aromatic hydrocarbons and certain performance criteria for analysis.

Falcó, G., Bocio, A., Llobet, J.M. and Domingo, J.L. 2005. Health risks of dietary intake of environmental pollutants by elite sportsmen and sportswomen. Food Chem. Toxicol. 43:1721.

Gomes A., Santos C., Almeida J., Elias M. and Roseiro, L.C. 2013. Effect of fat content, casing type and smoking procedures on PAHs contents of Portuguese traditional dry fermented sausages. Food Chem. Toxicol. 58:374.

Kubiak M.S. and Polak-Śliwińska, M. 2015. The Level of Chosen Polycyclic Aromatic Hydrocarbons (PAHs) in Meat Products Smoked by Using an Industrial and a Traditional Method. Pol. J. Natur. Sc. 30:147.

Law R.J., Kelly C., Baker K., Jones J., McIntosh A.D. and Moffat, C.F. 2002. Toxic equivalency factors for PAH and their applicability in shellfish pollution monitoring studies. J. Environ. Monitor. 4:388.

Mercogliano R., Santonicola S., De Felice A., Anastasio A., Murru N., Ferrante M.C. and Cortesi M.L. 2016. Occurrence and distribution of polycyclic aromatic hydrocarbons in mussels from the gulf of Naples, Tyrrhenian Sea, Italy. Mar. Pollut. Bull. 104:390.

Perugini M., Visciano P., Giammarino A., Manera M., Di Nardo W. and Amorena M. 2007. Polycyclic aromatic hydrocarbons in marine organisms from the Adriatic Sea, Italy. Chemosphere 66:1904-1910.

Purcaro G., Moret S. and Conte L.S. 2009. Optimisation of microwave assisted extraction (MAE) for polycyclic aromatic hydrocarbon (PAH) determination in smoked meat. Meat Sci. 81:280.

Reinik M., Tamme T., Roasto M., Juhkam K., Tenno T and Kiis, A. 2007. Polycyclic aromatic hydrocarbons (PAHs) in meat products and estimated PAH intake by children and the general population in Estonia. Food Addit. Contam. 24:437.

Rengarajan T., Rajendran P., Nandakumar N., Lokeshkumar B., Rajendran P. and Nishigaki, I. 2015. Exposure to polycyclic aromatic hydrocarbons with special focus on cancer. Asia-Pac. J. Trop. Biomed. 5:189.

Roseiro L. C., Gomes A., Patarata L. and Santos C. 2012. Comparative survey of PAHs incidence in Portuguese traditional meat and blood sausages. Food Chem. Toxicol. 50:1896.

Rozentale I., Lun A.Y., Zacs D. and Bartkevics V. 2018. The occurrence of polycyclic aromatic hydrocarbons in dried herbs and spices. Food Control. 83:53.

Rozentāle I., Stumpe-Vīksna I., Začs D., Siksna I., Melngaile A. and Bartkevičs V. 2015. Assessment of dietary exposure to polycyclic aromatic hydrocarbons from smoked meat products produced in Latvia. Food Control. 54:22.

Rozentāle I., Zacs D., Bartkiene E. and Bartkevics V. 2018. Polycyclic aromatic hydrocarbons in traditionally smoked meat products from the Baltic states. Food Addit. Contam. B 11:145.

Santos C., Gomes A. and Roseiro L.C. 2011. Polycyclic aromatic hydrocarbons incidence in Portuguese traditional smoked meat products Food Chemic. Toxicol. 49:2347.

Serpe F.P., Esposito M., Gallo P. and Serpe L. 2010. Optimisation and validation of an HPLC method for determination of polycyclic aromatic hydrocarbons (PAHs) in mussels. Food Chem. 122:925.

Singh L., Varshney J.G and Agarwal T. 2016. Polycyclic aromatic hydrocarbons' formation and occurrence in processed food. A Review. Food Chem. 199:781.

Soriano J.A., Viñas L., Franco M.A., González J.J., Ortiz L., Bayona J.M. and Albaigés J. 2006. Spatial and temporal trends of petroleum hydrocarbons in wild mussels from the Galician coast (NW Spain) affected by the Prestige oil spill. Sci. Total Environ. 370:90.

Stołyhwo A. and Sikorski, Z.E. 2005. Polycyclic aromatic hydrocarbons in smoked fish -A critical review. Food Chem. 91:303-311.

Storelli, M.M. and Marcotrigiano G.O. 2001. Polycyclic aromatic hydrocarbons in mussels (*Mytilus galloprovincialis*) from the Ionian Sea, Italy. J Food Protect 64:406.

Torre Torres F.G., Kouassi A.P., Brar S.K. and Belkacemi K. 2015. Spice use in food: properties and benefits. Crit. Rev. Food Sci. 57:1078-1088.

Varlet V., Serot T., Monteau F., Le Bizec B. and Prost C. 2007. Determination of PAH profiles by GC-MS/MS in salmon processed by four cold-smoking techniques. Food Addit. Contam. 24:757.

Wegrzyn E., Grzeskiewicz S., Poplawska W. and Glod, B.K. 2006. Modified analytical method for polycyclic aromatic hydrocarbons, using SEC for simple preparation and RP-HPLC with fluoroesence detection, Aplication to different food samples. Acta Chromatogr. 17:249.

Yebra-Pimentel I., Fernández-González R., Martínez-Carballo E. and Simal-Gándara J. 2015. A critical review about the health risk assessment of PAHs and their metabolites in foods. Crit. Rev. Food Sci. 55:405.

Zelinkova Z. and Wenzl T. 2015. The occurrence of 16EPA PAHs in food - A review. Polycycl Aromat Compd. 35:284.

Paper Received November 16, 2018 Accepted February 8, 2019

PAPER

DRYING KINETICS, HEATING UNIFORMITY AND QUALITY CHANGES DURING THE MICROWAVE VACUUM DRYING OF ARTICHOKES (*CYNARA SCOLYMUS* L.)

Ç. MUŞTU and İ. EREN*
Manisa Celal Bayar University, Engineering Faculty, Food Engineering Department, 45140 Manisa, Turkey
*E-mail address: ismail.eren@cbu.edu.tr

ABSTRACT

In this study, our aim was to investigate the effect of process parameters such as the microwave power (450-800 W), system pressure (12.40 – 31.20 kPa) and drying time (0-25 min) on the drying kinetics, quality characteristics and heating uniformity indices during the microwave vacuum (MV) drying of artichokes. The microwave power was found to be more influential than the system pressure, and variations resulted in different drying characteristics. The effective diffusion coefficients were found to range from 2.279×10^{-6} - 3.454×10^{-6} m^2/s and $3.600\times10^{-}$ - $6.297\times10^{-}$ m^2/s for the first and second falling rate periods, respectively. Reducing microwave power and system pressure resulted in higher uniformity in temperature distributions. However, the average temperatures deviated slightly from the targeted drying temperatures at high microwave power. The highest shrinkage coefficient (0.3705±0.0233) and rehydration ratio (3.82±0.091) values were obtained at 800 W and 12.4 kPa. MV drying preserved the fresh colour characteristics of artichokes, and it could be used as an alternative to conventional drying.

Keywords: artichoke (*Cynara scolymus* L.), Microwave Vacuum Drying, Heating Uniformity, shrinkage, rehydration, Browning Index

1. INTRODUCTION

The globe artichoke (*Cynara scolymus* L.), which belongs to the Asteraceae family, is an important crop cultivated primarily in the Mediterranean area. The contribution of artichoke cultivation to the agricultural economy is often accompanied by the fresh consumption of the immature flower heads as a vegetable and the leaf extracts by the pharmaceutical industry (LATTANZIO *et al.*, 2009; LOMBARDO *et al.*, 2010). Several *in vitro* and *in vivo* experiments have shown the biological activity of artichokes, including their anticarcinogenic, anti-cholestatic, anti-inflammatory, and anti-genotoxic properties, which are due to its phytochemical composition, including its phenolic compounds, namely, its caffeoylquinic acids, apigenin, luteolin glycosides and tannins (ABU-REIDAH *et al.*, 2013; BEN SALEM *et al.*, 2017; FRATIANNI *et al.*, 2014). The demand for artichokes is steadily increasing because their associated nutritional characteristics are well suited to the increasing expectations of consumers regarding healthy foods. However, there is a limited season for fresh artichoke consumption, and this crop is highly susceptible to discolouration and nutritional losses due to enzymatic activity, even under cold storage. The canning and freezing of artichokes are the most common methods used in industrial production, but the resulting products do not fully meet consumer expectations due to nutritional losses and concerns about cost effectiveness (GUIDA *et al.*, 2013). Drying artichokes at low temperatures using methods such as freeze-drying and microwave vacuum (MV) drying would be advantageous for existing postharvest techniques in terms of preserving the nutraceutical properties for long periods as well as reducing storage, packaging and transportation costs.

The use of MV drying technology for food dehydration has emerged as an alternative drying method that has attracted the interest of researchers and the food industry in recent years (CLARY *et al.*, 2007; LOMBRAÑA *et al.*, 2010; MEDA *et al.*, 2016). The penetration of the product with microwaves results in rapid volumetric heating, increasing the vapour pressure at the interior and creating a driving force for moisture transfer to the surface. Applying a vacuum creates an additional pressure gradient for moisture transport and allows the user to adjust the temperature to the desired level. These combined effects cause rapid drying and prevent both the shrinkage and case hardening problems often seen in conventional drying methods (TSURUTA *et al.*, 2015; WRAY and RAMASWAMY, 2015).

The MV drying of potato cubes created a porous microstructure from the dried material, and there was low shrinkage of the dried potato particles as well as high rehydration capacity (MARKOWSKI *et al.*,2009). MV-dried green peas were characterized by minimal structural changes in terms of microstructure, shrinkage (43.3±0.1%) and colour changes compared to atmospheric freeze-drying with a heat pump fluidized bed (ZIELINSKA *et al.*,2013). In addition, the aroma, colour, and nutrients or other biologically active ingredients that are sensitive to thermal or oxidative degradation are better protected by MV drying than conventional hot air drying methods (CHAUHAN and SRIVASTAVA, 2009; MICHALSKA *et al.*, 2017; TEIN M. LIN, 1998; WOJDYŁO *et al.*, 2013). A lower degradation of polyphenols and anthocyanins has been reported for MV-dried raspberries compared to hot air-dried samples (SI *et al.*, 2016). LEUSINK *et al.* (2010) found that MV drying and freeze-drying methods had similar effects in preserving the anthocyanin content and antioxidant activity of cranberries, while lower drying times were needed during MV drying. Despite the numerous studies that have demonstrated the superiority of MV drying in terms of product quality, the drying of artichokes using a microwave vacuum has not been studied yet.

Designing an MV drying process is a complicated engineering problem because it is controlled by a large number of variables associated with both the system design (i.e., the cavity size, number and position of magnetrons, power density, duty cycle, and vacuum intensity) and the product properties (shape, size, geometry, dielectric and thermophysical properties) (CHEN *et al.*, 2015; PITCHAI *et al.*, 2012; ROGELIO *et al.*, 2015). Since microwaves are sinusoidal propagating electromagnetic waves and they are reflected from metal surfaces, a complex time-dependent field pattern generally forms in the drying cavity. This pattern results in the uneven heating of the product and the formation of hot spots. Therefore, the prediction of the temperature distributions in addition to the moisture transport in the product is critical to the product quality and process development (FENG *et al.*, 2012). From a practical point of view, empirical models have been used by several authors to simulate the drying curve of various products during MV drying. KIRANOUDIS *et al.* (1997) studied the MV drying kinetics of fruits by using the exponential model and reported that the drying constant is dependent on the microwave power and vacuum pressure. DROUZAS *et al.* (1999) used the Lewis model to predict the MV drying kinetics of model fruit gels and proposed an empirical correlation to estimate the drying rate constant as a function of the microwave power and the absolute pressure of the system. CUI *et al.* (2005) modelled the drying kinetics of carrot slices with a theoretical model and found a linear relationship between the drying rate and the microwave power level. In the same study, the temperature changes were also evaluated but the heating uniformity issues were not. It is obvious that the mathematical form of the proposed model and the drying times significantly changed for each food item in the above mentioned studies. Therefore, determining the heat and mass transfer behaviour simultaneously with the heating uniformity issues and quality characteristics would enhance our knowledge regarding the adoption of this relatively new technology for drying artichokes.

In this study, the use of microwave vacuum drying on fresh artichokes was investigated by determining the drying characteristics and temperature profiles under different drying conditions. For this purpose, the effects of the microwave power, system pressure and drying time on the drying rates, heating uniformity indices, colour changes, shrinkage and rehydration behaviour of artichokes were investigated. In addition, simple mathematical modelling approaches, including Fick's law of diffusion, were tested to predict the moisture transport.

2. MATERIAL AND METHODS

2.1. Materials

Fresh artichokes (*Cynara scolymus* L. cv. Sakız) were purchased from a manufacturer located in the Urla/İzmir area (Aegean coast of Turkey). Samples at the same commercial maturity level (as defined by the compactness of the fully developed buds) were carefully selected. All the globe artichokes were hand-trimmed on the same day using sharp stainless-steel knives to remove the external bracts and leaves and to create a uniform shape and size (10 ±1 cm diameter and 1±0.2 cm thickness). The average moisture content of the fresh artichoke hearts was found to be 6.1466±0.2909 kg H_2O/kg dry matter. Finally, the artichoke hearts were soaked in a 1% citric acid solution and stored at 4°C until the drying experiments.

2.2. Microwave Vacuum Drying System

A complete diagram of the laboratory-scale microwave vacuum dryer is presented in Fig. 1. The primary components of the microwave vacuum dryer consist of a vacuum chamber (7), microwave applicator set (17), heating plates (15), control panel (1-5), condenser (11), vacuum pump (12), pressure metre (13) and solenoidal valve (14). The microwave applicator (drying chamber) has the rectangular shape (30 cm wide, 21 cm high and 25 cm deep) and stainless-steel walls typical of many industrial and microwave multimode cavities. A magnetron emitting at a frequency of 2.45 GHz with a maximum microwave power of 1000 W was placed at the top of the drying chamber. The microwave power was transmitted through a waveguide (WR340), which is characterized by its TE10 mode pattern of field distribution. Heating plates were used to maintain the temperature of the cabinet walls at the targeted drying temperature (±1°C) to prevent condensation within the drying chamber. The MV drying system was completed with a condenser (water cooling system) to prevent water accumulation in the vacuum pump, which could damage the pump. The pressure control was performed using a pressure sensor (Autonics, Korea, model PSA-V01-Rc1/8) and its associated solenoid valve (Parker, USA) through an on-off control with a hysteresis value of±1.33 kPa (Fig. 1).

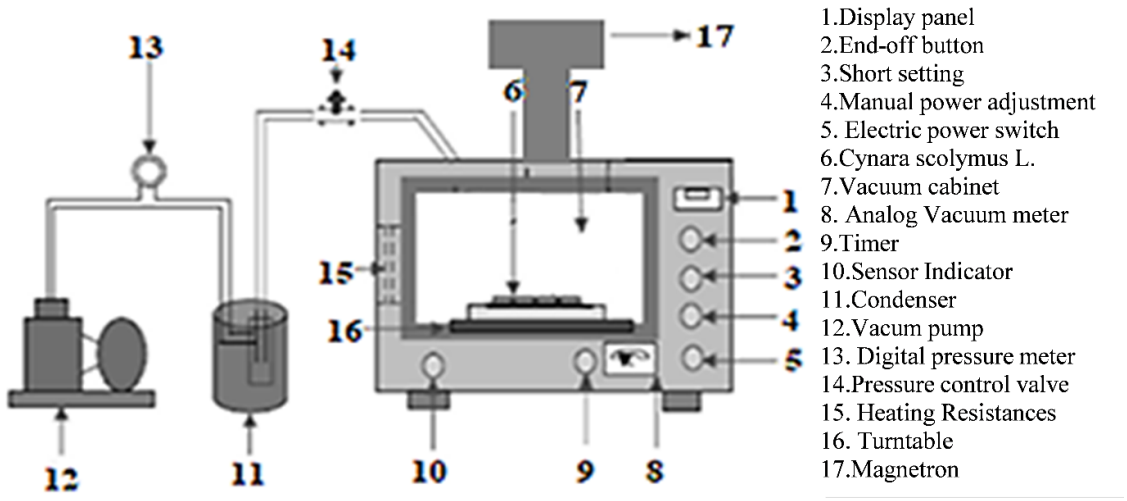


Figure 1. Microwave vacuum drying system.

2.3. Drying experiments

The artichoke hearts were removed from the 1% citric acid solution and drained. They were then gently blotted with filter paper to remove the adhering solution and weighed before being dried. The average moisture content of the artichokes prior to MV drying was found to be 8.9472±0.7152 kg H_2O/kg dry matter. Since the MV drying equipment was operated in batch mode due to the vacuum, new samples were used in each experiment. A single artichoke with an average weight of 52.2312±5.1249 g was used for each drying experiment. The artichokes were vertically positioned 15 cm below the top of the vacuum chamber and horizontally relative to the centre of the base. Microwave energy was supplied to the drying chamber as soon as the desired vacuum was attained. The rotation speed of the turntable was set to 5 rpm to avoid the uneven heating of the product by nonuniform microwave radiations. The artichokes were removed from the drying

chamber at specified sampling times after the vacuum was released. The dried samples were kept in airtight glass jars until the analysis.
MV drying experiments were performed at two levels of microwave power (450 W and 800 W) and two levels of absolute pressure (31.20 kPa and 12.40 kPa) until the final moisture content of the artichokes fell below 0.1 kg H_2O/kg dry matter. The microwave power levels were determined after preliminary trial runs with consideration for the calorimetric effect of the microwave system (4-6 W/g of sample). The drying time of the artichokes was recorded to be approximately 25 min at 450 W and 15 min at 800 W, regardless of the pressure level. Therefore, the quality characteristics (i.e., the shrinkage coefficient, rehydration ratio, and colour values) were determined at 5 min intervals up to 15 min for 800 W and 25 min for 450 W at all pressure levels.
The MV drying experiments were repeated separately at the specified combinations of microwave power and pressure levels to determine the drying kinetics and temperature profiles. The temperature distributions and weight loss of the samples were monitored at 1 min intervals. The weights of the samples were recorded with a digital balance that had an accuracy of 0.001 g (Sartorius, Germany). The surface temperature was measured from the top layer with an infrared camera (Testo 880-3, Germany). All the weight and thermal imaging recordings were completed in 10-15 sec. Two replicates were used for each drying experiment to determine the drying kinetics, temperature profiles and quality control parameters. The average values were used in the following calculations.

2.4. Drying characteristics and modelling the drying curves

2.4.1 Determination of drying kinetics

A graphical representation of the drying rates as a function of the drying time and free moisture content (M_t) was used to describe the drying behaviour of the artichokes during MV drying. The rate of drying (R) is expressed as the amount of water (kg) removed from the artichokes per unit area (h) per unit time (m^2) and is calculated by the following equation:

$$\boldsymbol{R} = -\frac{\boldsymbol{L_s}}{\boldsymbol{A(t)}} * \frac{\boldsymbol{dM}}{\boldsymbol{dt}} \quad (1)$$

where L_s is the dry solid weight (kg), $A(t)$ is the surface area (m^2) of an artichoke at any drying time (t), dM is the change in moisture content (kg water/kg dry solids) and dt is the time (h) between measurements (GEANKOPLIS, 2003). The moisture contents of the samples were determined by drying them in a vacuum oven at 70°C under 57.6 kPa gauge pressure (AOAC, 2005). The moisture content determinations were performed in triplicate and the average values were used in the calculations.

2.4.2 Models for drying curves

The moisture transport during the microwave vacuum drying of the artichokes was characterized by the model based on Fick's second law of diffusion, which is given in Eq. (2).

$$\frac{\partial M}{\partial t} = \nabla(D_{eff} \cdot \nabla M) \quad (2)$$

In this equation, $M(t, x)$ is the moisture content (kg water/kg dry matter.); t is time (s), x is the distance from centre (m), and D_{eff} is the effective diffusion coefficient (m^2/s).

Equation (2) can be solved analytically by using the separation of variables method for an infinite slab using the following initial (IC) and boundary conditions (BC1 and BC2) with the assumptions that the shrinkage, constant temperature and diffusion coefficients would be neglected. Equation (3) is the initial condition stating the uniform distribution of moisture within the samples. Equation (4) is the symmetry condition in the centre of the slab and Eq. (5) is the boundary condition at the surface, and it establishes that at this point, the equilibrium moisture content is reached immediately due to the effect of the vacuum (CRANK,1975).

$$\text{IC: } M(0,x) = M_0 \quad \forall x \tag{3}$$

$$\text{BC 1: } \left.\frac{\partial M}{\partial x}\right|_{x=0} = 0 \quad t > 0 \tag{4}$$

$$\text{BC 2: } M(t, L/2) = M_S = M_e \quad \text{t} > 0 \tag{5}$$

where M_0 is the initial moisture content (kg H_2O/kg dry matter.); M_s is the moisture content at the surface (kg H_2O/kg dry matter); M_e is the equilibrium moisture content (kg H_2O/kg dry matter); and L is the thickness of the slab.

$$\frac{M-M_e}{M_0-M_e} = \frac{8}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{(2n+1)^2} exp\left(-(2n+1)^2\frac{\pi^2 D_{eff}}{L^2}t\right) \tag{6}$$

Equation (6) gives the moisture concentration at a given time and a given location in an infinite slab. Since the experimental data were obtained for the total moisture loss from the entire product, Eq. (6) should be integrated throughout the entire volume to obtain the average moisture content of the sample as a function of time. In taking the average moisture contents and only the first term of the infinite series solution in case Fourier Number ($\tau = D_{eff}t/(L/2)^2$) > 0.2, Eq. (6) can be simplified to Eq. (7) as follows:

$$MR = \frac{\bar{M}-M_e}{M_0-M_e} = \frac{8}{\pi^2} exp\left(-\frac{D_{eff}\pi^2 t}{L^2}\right) \tag{7}$$

where *MR* is the dimensionless moisture ratio based on the average moisture contents.
In addition to the theoretical models, several researchers have proposed simple models to simulate the drying curves of foods that can provide adequate representations of experimental data, although the parameters of these models lack physical sense. Among the semi-empirical drying models, namely, the Lewis or exponential model (Eq. (9)), the Henderson and Pabis model (Eq. (8)) and the Page model (Eq. (10)) are used widely (EREN *et al.*, 2008; SUTAR and PRASAD, 2007)

$$MR = \frac{\bar{M}-M_e}{M_0-M_e} = \text{a exp } (-\text{kt}) \tag{8}$$

$$MR = \frac{\bar{M}-M_e}{M_0-M_e} = \text{exp } (-\text{kt}) \tag{9}$$

$$MR = \frac{\bar{M}-M_e}{M_0-M_e} = exp(-kt^n) \tag{10}$$

where k is the drying rate constant ($1/s$) or ($1/s^n$) and a and n are parameters in the models.

2.4.3 Temperature profiles and heating uniformity calculations

A heating uniformity evaluation of artichoke samples was conducted by using temperature uniformity indices, which are based on the average experimental temperatures (T_{av}) and the target temperature (T_{tg}) of drying. The target temperatures were 50 and 70°C because they correspond to the boiling temperatures of pure water at the applied chamber pressure levels. The indices given in Eq. (11) and Eq. (12) describe the temperature deviation of any point at the surface of the artichoke from the target and average temperatures at certain drying times as follows:

$$HUI_{av} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(T_i - T_{av})^2} \quad (11)$$

$$HUI_{tg} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(T_i - T_{tg})^2} \quad (12)$$

where T_i is the local temperature of any point at the surface of the product (°C) and N is the number of data points. If the temperatures deviate from the reference values, the HUI will be higher and represents worse heating uniformity. Image processing software (IRSoft PC v4, Germany) was used to analyse the surface temperature data from the dried artichokes. From each thermal image, at least 8619 individual temperature data points were used for the calculations.

2.4.4 Shrinkage coefficient calculations

The shrinkage is defined as the ratio of the volume (V) of an artichoke sample at any dryness level to that of the fresh sample (V_0). The volumetric shrinkage coefficient (S) is calculated using the following equation:

$$S = \frac{V}{V_0} \quad (13)$$

Volume measurements of the artichoke samples were conducted by volume displacement method as described by (SEGNINI *et al.*, 2004). Rapeseeds (*Brassica napus*) with a typical diameter of (~2 mm) were used as the granular material. At the first stage, a 500 mL beaker was filled with seeds and compressed manually to determine the calibrated amount of seeds. The seeds were then transferred carefully to a larger beaker containing an artichoke sample, and the excess seeds were swept away with a metal slab. The excess seeds were measured with a graduated cylinder (100±1 mL) to determine the apparent volumes of the samples. The volume measurement procedure was repeated three times for each artichoke sample and the average values were evaluated with a shrinkage coefficient calculation.

2.4.5 Rehydration ratio

The rehydration ratio (RR) is defined as the ratio of the weight of the rehydrated sample (WR) to the weight of the dried sample (WD). For this purpose, dried artichoke samples were immersed in 200 mL of distilled water at a temperature of 30±0.5°C. Samples were withdrawn from the solution at 20-min intervals and blotted with tissue paper to remove excess moisture. The weights of the rehydrated samples were then recorded for up to 80 min. The RR was calculated using the following equation:

$$R = \frac{WR}{WD} \quad (14)$$

The rehydration ratio determinations were performed in duplicate, and the average values were evaluated using calculations.

2.4.6 Determination of Colour Changes

The colours of fresh and dried artichoke samples were determined using a colourimeter (Minolta CR-410, USA) based on the CIELAB colour space. L^* represents the lightness of the luminance component (0 for black and 100 for white) and the coordinates a^* and b^* are the two chromatic components that represent the greenness to redness (-60 to +60) and the blueness to yellowness (-60 to +60), respectively. In addition, three derived colour parameters, the total colour differences (ΔE), hue angle ($h°$) and browning index (BI), were calculated using the following equations:

$$\Delta E = [(L_{ref} - L^*)^2 + (a_{ref} - a^*)^2 + (b_{ref} - b^*)^2]^{1/2} \quad (15)$$

$$h° = arctan(b^*/a^*) \quad (16)$$

$$BI = \frac{100(x-0.31)}{0.17} \quad (17)$$

$$x = \frac{(a^*+1.75L^*)}{(5.645L^*+a^*-3.012b^*)} \quad (18)$$

The colour parameters were also written in normalized form as $\boldsymbol{\Delta a}$ (a-a_{ref}), $\boldsymbol{\Delta b}$ (b-b_{ref}), $\boldsymbol{\Delta L}$ (L-L_{ref}), $\boldsymbol{\Delta L}$ (L-L_{ref}) and $\boldsymbol{\Delta BI}$ (BI-BI_{ref}). The subscript "*ref*" in calculations of the colour parameters denotes the reference values obtained from pre-treated artichokes prior to MV drying. The larger the ΔE values, the greater the colour change from the fresh sample (DONG *et al.*, 2018). The hue angle (h°) expresses the colour change (an angle of 0 or 360° represents a red hue, while angles of 90, 180, and 270° indicate yellow, green and blue hues, respectively). A decrease in the hue angle values is an indication of more browning colour and a shift away from yellowness (HAWLADER *et al.*, 2006). The browning index represents the purity of the brown colour and is an important parameter in processes in which enzymatic or nonenzymatic browning take place (PALOU *et al.*, 1999). The colour measurements were performed in duplicate, and six measurements were taken over the entire artichoke surface through rotation.

2.5. Statistical analysis

An analysis of variance was used to determine the effects of the microwave power, system pressure and drying time on the heating uniformity indices (HUI_{av} and HUI_{tg}), shrinkage coefficient, rehydration ratio and change in colour parameters of artichokes at the 95% level of significance. The experimental data for dimensionless moisture contents were fitted to the diffusion model, the Lewis model (exponential model), the Henderson-Pabis model and the Page model using a nonlinear regression analysis. All the statistical and regression analyses were performed using a statistical package program (SPSS v22.0, IBM Inc., 2013, USA). The coefficient of determination (R^2) and root mean square error ($RMSE$) were used to evaluate the goodness of the fit for the tested models. The best model

describing the drying behaviour was chosen as the one with the highest R^2 and the lowest *RMSE* (EREN *et al.*, 2008).
The RMSE values were calculated using the following equation:

$$RMSE = \left[\frac{1}{N}\sum_{i=1}^{N}(Y_{exp_i} - Y_{pre_i})^2\right]^{1/2} \quad (19)$$

where Y_{exp_i} is the experimental dimensionless moisture value, Y_{pre_i} is the predicted dimensionless moisture contents from the model, and *N* is the number of observations.

3. RESULTS AND DISCUSSION

The drying kinetics of artichokes were studied for average moisture contents ranging from 8.9472±0.7152 to 0.0643±0.0004 kg H_2O/kg dry matter by applying two different microwave powers (450 W and 800 W) and pressure levels (31.20 kPa and 12.40 kPa). The experimental moisture contents on a dry basis over the course of MV drying are given in Fig. 2-a. A non-linear relationship between the moisture contents and the drying times was observed under all the processing conditions. Similar MV drying curves were also obtained by JAYA and DURANCE (2007) for alginate-starch gels and by SONG *et al.* (2009) for potato slices. Microwave power significantly affected the drying curves and decreased the drying time of the samples from 25 to 15 min.
Fig. 2-b shows that the drying rates increased at the initial stages of drying (2-3 min) when the absorbed microwave energy was converted into sensible heat within moist artichokes, and the average temperature of the product increased with time. The average temperatures were increased very sharply with the corresponding boiling temperatures of pure water (Fig. 2-b). The saturation temperature is 50°C at 12.40 kPa and 70°C at 31.20 kPa. However, slight deviations were recorded in the average temperatures during the MV drying of the artichokes from the effects of the process parameters (Fig. 2-b). An increase in microwave power from 450 W to 800 W at 31.20 kPa caused an increase in the maximum average temperatures from 69°C to 75.9°C during MV drying. Similar behaviour in the average temperatures was also observed at lower vacuum levels (12.40 kPa), but the maximum average temperatures were reduced to 56.8°C and 60.4°C for 450 W to 800 W, respectively.
After this initial heating period, the vapour pressure of the product exceeded that of the drying chamber, and the thermal energy converted from microwave energy was used to evaporate moisture. At the low microwave power level (450 W), a constant drying rate region was observed regardless of the applied vacuum intensity. The occurrence of this constant rate period primarily resulted from the wet surface of the artichokes during the initial drying stages. A constant rate of drying was continued as long as water was supplied from the inside of the product to the surface at the rate of surface moisture removal. As the moisture content and the dielectric loss factor decreased, the rate of conversion of the electromagnetic waves to heat slowed and the internal mass transfer resistances became more pronounced. Therefore, a falling drying rate region was observed at moisture contents below 3-4 kg H_2O/kg dry matter at the low microwave power level (Fig. 2-d). CUI *et al.* (2004) reported lower critical moisture content values of 2-3 kg H_2O/kg dry matter after the MV drying of carrots.

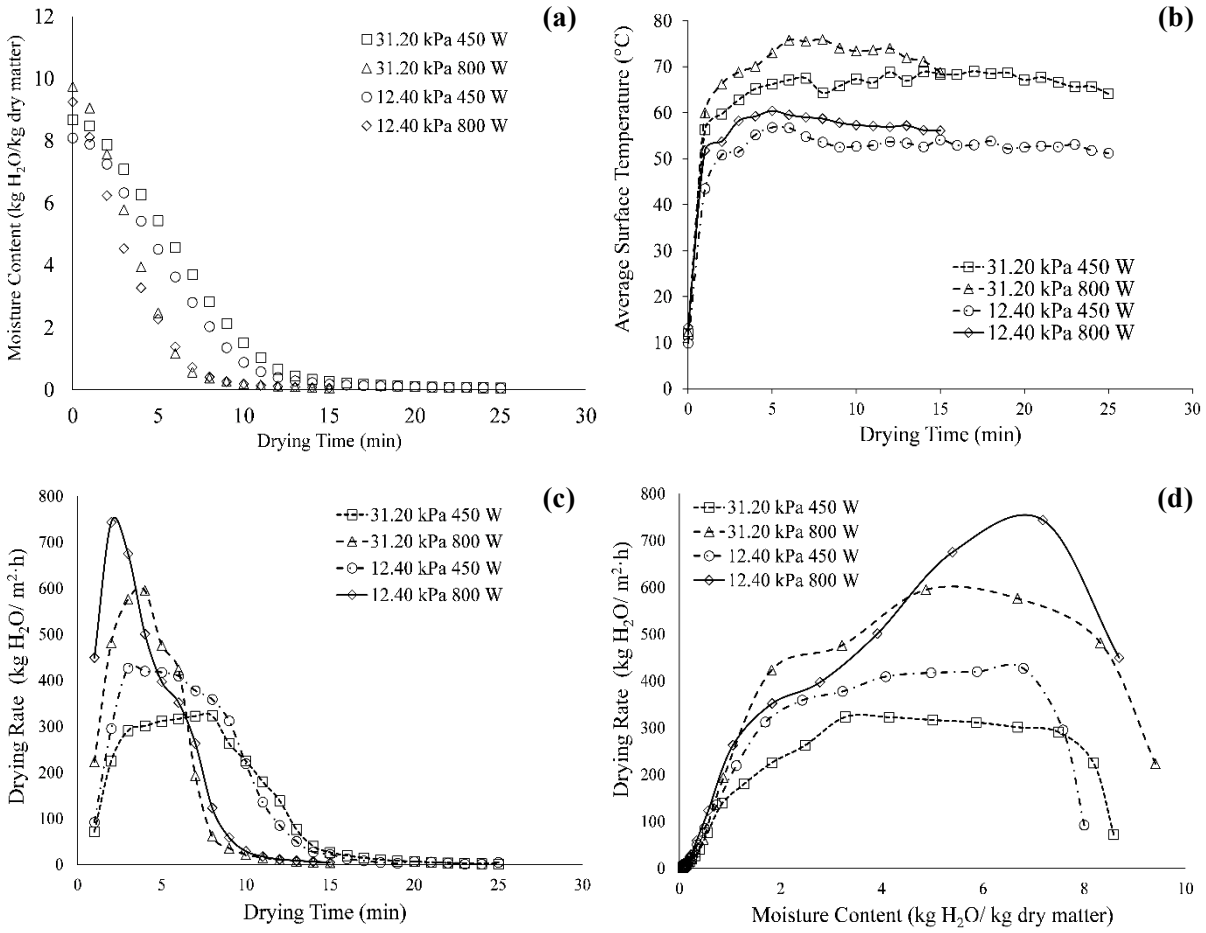


Figure 2. Effects of the process parameters on the drying characteristics and average surface temperatures of artichokes during MVD. (a) Moisture ratio vs. drying time; (b) drying rate vs. drying time; (c) drying rate vs. moisture content; and (d) surface temperature vs. drying time.

However, at the high microwave power level (800 W), the drying rate increased within 2-3 min, and then it decreased exponentially after reaching peak values of 595 and 744 kg $H_2O/m^2 \cdot h$ under 31.20 and 12.40 kPa vacuum pressure, respectively (Fig. 2-d). The high microwave power (800 W), which generated higher pressure inside the product, resulted in an additional pressure gradient that was responsible for the higher rates of moisture transport. Thus, the surface moisture was removed very quickly during the initial stage of drying, and drying took place directly during a falling rate period, which is an indication of an internal resistance-controlled drying mechanism. This result was expected because as the surface moisture is removed, the surface layer becomes dry and hard due to tissue shrinkage and creates resistance against the inside moisture moving towards the surface. Compared with microwave power, the effect of system pressure on the drying rates appears to have decreased towards the end while being more pronounced in the initial stages of drying. This result also indicated that the moisture transport during the MV drying of artichokes was primarily controlled by internal resistance mass transport.

The slopes of the drying rate curves in Fig. 2-d show two distinct falling rate periods for all the MV drying conditions. The first and second falling-rate periods were determined by using the locations of the critical moisture contents, which were calculated from the plot of the drying rate gradient vs. the moisture content (LAW *et al.*, 2003). At low microwave power (450 W), the critical moisture contents (x_{c1}) for the first falling-rate period were

found to be 3.277 and 4.075 kg H_2O/kg dry matter for 31.20 kPa and 12.40 kPa, respectively. Under high microwave power (800 W), the first critical moisture contents were increased to 4.867 kg H_2O/kg dry matter for 31.20 kPa and 7.184 kg H_2O/kg dry matter for 12.40 kPa. By contrast, as the microwave power increased to 800 W, the second critical moisture contents (x_{c2}) decreased from 0.399 to 0.263 kg H_2O/kg dry matter for 31.20 kPa and from 0.474 to 0.339 kg H_2O/kg dry matter for 12.40 kPa. Under all the MV drying conditions, the most relevant variable that affected the location of the critical points was the microwave power.

3.3. Evaluation of the mathematical models

Since different drying periods were observed during the MV drying of artichokes, the semi-empirical and theoretical models were fitted to the experimental data for both the first falling rate (*1st FRP*) and second falling (*2nd FRP*) rate periods. Table 1 shows the parameters of the models and the goodness of fit criteria for the semi-empirical models; namely, the Lewis model (Eq. (9)), the Henderson and Pabis model (Eq. (8)), the Page model (Eq. (10)) and the model based on Fick's second law of diffusion (Eq. (7)).

From the values for the coefficient of determination (R^2) and the root mean square error (*RMSE*), it is obvious that the Page model and the Henderson and Pabis model gave satisfactorily good fits for predicting the moisture contents of artichokes for all the MV drying periods. The R^2 values for these two semi-empirical models were greater than 0.982 and the *RMSE* values were less than 1.457×10^{-3}. However, the Page model had better R^2 and *RMSE* values for the *2nd FRP* than the Handerson and Pabis model. Therefore, the Page is the best model for all the processing conditions. The success of the Page model at characterizing MV drying was also reported by other researchers on the drying of bananas, potatoes and carrot slices using microwave vacuum technology (CUI *et al.*, 2004; MOUSA and FARID, 2002; SONG *et al.*, 2009).

The drying rate constants (k) of the semi-empirical models showed an increasing tendency with increased microwave power and vacuum for both falling rate periods (Table 1). This increase resulted from the increase in the drying rates by more thermal energy generation and consequently more internal vapour pressure inside the product. The drying rate constants (k) of the Page model were recorded over ranges of 3.906×10^{-6} - 3.610×10^{-4} (1/s) and 9.325×10^{-3} - 7.078×10^{-2} (1/s) for the 1st FRP and 2nd FRP, respectively. A graphical representation of the predicted moisture ratios by the Page model in comparison with the experimental moisture ratios under all the processing conditions is shown in Fig. 3. It is clear that the Page model gave good predictions at all stages of MV drying in artichokes.

During the MV drying of the artichokes, the shrinkage effects were ignored and the geometry of the disc-shaped artichokes was assumed to be an infinite slab due to the high diameter/height ratio (*10:1*). Therefore, the diffusional model was solved for an infinite slab assuming negligible shrinkage. When external resistance to mass transfer is considered negligible, the effective diffusion coefficient (D_{eff}) can be identified using Eq. (7) by minimizing the differences between the experimental and estimated MR. The low R^2 (<0.82) and high *RMES* (> 6.817×10^{-2}) values indicated that one or more combinations of different moisture transport mechanisms took place and thus reduced the accuracy of the diffusion model in the 1st FRP. The diffusion model simulated the drying curves more accurately (R2 > 0.82 and RMES < 4.327×10^{-3} in the 2nd FRP (Table 1). The effective diffusion coefficients were found to range from 2.279×10^{-6} - 3.454×10^{-6} m^2/s and 3.600×10^{-6}-6.297×10^{-6} m^2/s for the 1st FRP and 2nd FRP, respectively (Table 1).

Table 1. Parameters of the semi-empirical and diffusion models for the drying kinetics of artichokes.

Model		Model parameters/ goodness of fit	MV Drying Process Condition			
			31.20 kPa 450 W	31.20 kPa 800 W	12.40 kPa 450 W	12.40 kPa 800 W
Lewis Model	*1st FRP*	*k*	2.616×10^{-3}	4.087×10^{-3}	2.662×10^{-3}	4.578×10^{-3}
		R^2	0.760	0.778	0.942	0.973
		RMSE	5.919×10^{-2}	8.310×10^{-2}	7.700×10^{-2}	6.559×10^{-2}
	2nd FRP	*k*	3.783×10^{-3}	6.605×10^{-3}	4.087×10^{-3}	6.425×10^{-3}
		R^2	0.970	0.958	0.647	0.945
		RMSE	2.335×10^{-3}	3.310×10^{-3}	4.764×10^{-3}	2.776×10^{-3}
Page Model	*1st FRP*	*k*	3.906×10^{-6}	1.736×10^{-5}	9.944×10^{-6}	3.610×10^{-4}
		n	2.034	1.983	1.920	1.456
		R^2	0.999	0.998	0.999	0.999
		RMSE	1.849×10^{-3}	8.377×10^{-3}	5.899×10^{-3}	9.266×10^{-3}
	2nd FRP	*k*	9.325×10^{-3}	2.223×10^{-2}	2.135×10^{-2}	7.078×10^{-2}
		n	0.868	0.805	0.810	0.582
		R^2	0.985	0.992	0.968	0.980
		RMSE	1.629×10^{-3}	1.455×10^{-3}	1.457×10^{-3}	1.714×10^{-3}
Henderson and Pabis Model	*1st FRP*	*k*	5.202×10^{-3}	7.663×10^{-3}	5.050×10^{-3}	5.473×10^{-3}
		a	3.880	2.403	2,649	1.254
		R^2	0.993	0.985	0.986	0.990
		RMSE	9.777×10^{-3}	2.167×10^{-2}	2.049×10^{-2}	2.659×10^{-3}
	2nd FRP	*k*	3.284×10^{-3}	5.280×10^{-3}	2.267×10^{-3}	5.166×10^{-3}
		a	0.637	0.515	0.192	0.504
		R^2	0.982	0.989	0.955	0.975
		RMSE	1.754×10^{-3}	1.727×10^{-3}	1.695×10^{-3}	1.843×10^{-3}
Diffusion Model	*1st FRP*	D_{eff}	2.279×10^{-6}	3.367×10^{-6}	2.679×10^{-6}	3.454×10^{-6}
		R^2	0.682	0.669	0.727	0.819
		RMSE	6.817×10^{-2}	1.014×10^{-1}	9.224×10^{-2}	1.140×10^{-1}
	2nd FRP	D_{eff}	3.600×10^{-6}	6.297×10^{-6}	3.898×10^{-6}	6.120×10^{-6}
		R^2	0.979	0.973	0.942	0.960
		RMSE	1.754×10^{-3}	2.628×10^{-3}	4.327×10^{-3}	2.383×10^{-3}

The increasing trend in the D_{eff} with the microwave power was observed during both falling rate periods since a higher microwave power level would enhance the drying rate by generating more heat energy inside the product. As shown in Table 1, the D_{eff} values increased by 1.10 times for the *1st FRP* and remained almost constant for the *2nd FRP* as the system pressure decreased from 31.20 kPa to 12,40 kPa. The internal mass transfer resistance and the dry surface of the samples during the falling rate periods can be understood as the reason for the limited effect of the pressure on the D_{eff}. SUTAR and PRASAD (2007) also reported that the effect of the pressure on the D_{eff} of carrot slices during MV drying was non-significant over a range of 6.66-33.30 kPa at a 5% level of significance. The effect of microwave power on the D_{eff} was much more pronounced in the *2nd FRP*. This finding could be the result of the increased microwave power intensities in terms of watts per gram of product load due to the lower moisture contents in the *2nd FRP* of MV drying. Additionally, a higher moisture diffusivity in the *2nd FRP* during the

microwave drying of kiwifruits was associated with an increased moisture permeability caused by structural changes such as surface cracking, the destruction of cell walls and the formation of open-pore structures (MASKAN,2001).

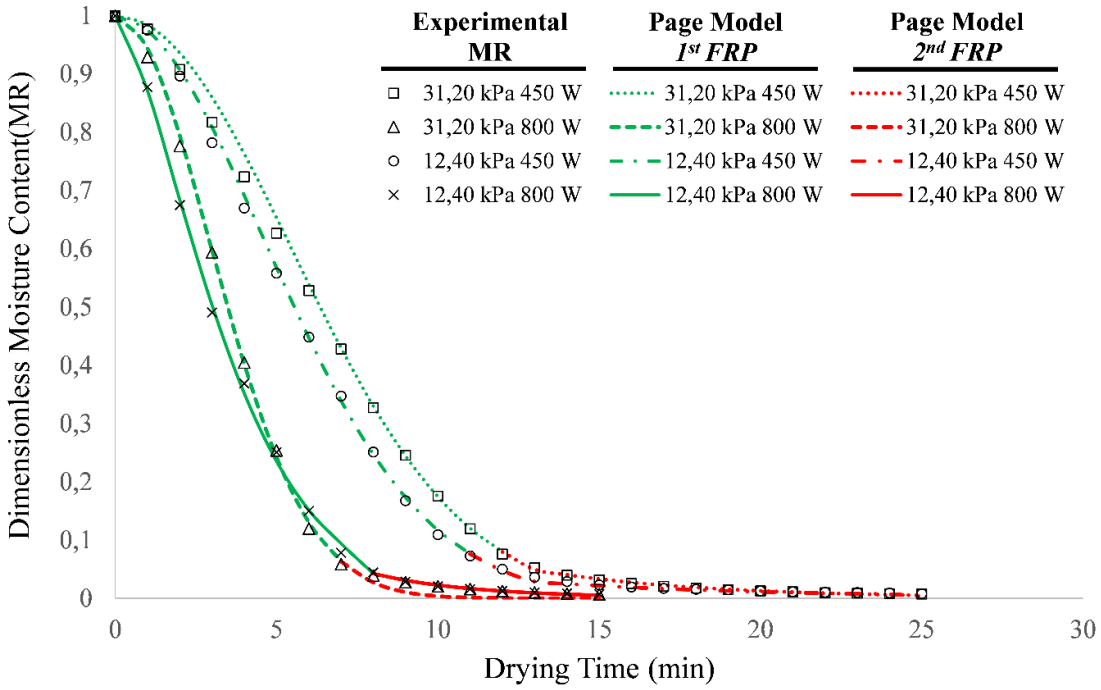


Figure 3. Experimental and predicted drying curves for artichokes using the Page model.

The experimental versus predicted dimensionless moisture ratios (*MR*) obtained by using the Page and the diffusion models at different stages of MV drying are plotted in Fig. 4. While the predicted MR values obtained by the Page model correlates well with the experimental data, clear deviations from the 45° line for the diffusion model can be seen in Fig. 4 for the *1st FRP*. Inaccurate predictions of the MR values by the diffusion model could have resulted from the fact that internal moisture transport took place in the presence of one or more combinations of different moisture transport mechanisms such as a hydrodynamically driven flow of vapour and liquid, liquid transport with capillary action, and evaporation-condensation. Conversely, the diffusion model eliminated the discrepancies between the experimental and predicted MR values for the *2nd FRP* (Fig. 4). This finding implied that liquid and vapour diffusion were the primary mechanisms of moisture transport during the MV drying of artichokes.

3.3. Temperature profiles and heating uniformity

The temperature profiles obtained at 5 min intervals with a thermal imager are represented in the form of a colour image (Fig. 5). It is clear that the temperature profiles varied significantly during the MV drying process depending on the process parameters. The formation of an annular hot zone, as represented by red coloured regions, was observed for its drying at high microwave power (800 W). The maximum temperatures reached up to 107°C and 89.2°C at a 31.20 kPa pressure level for 800 W and 450 W between 7 and 12 min of drying for a short time. As the absolute pressure decreased to 12.40 kPa, the maximum temperatures also decreased to 90.1°C and 77.9°C for 800 W and 450 W

microwave power levels. High product temperatures (greater than 100°C) were also reported by SUTAR and PRASAD (2007) for a short period during the MV of drying carrot slices.

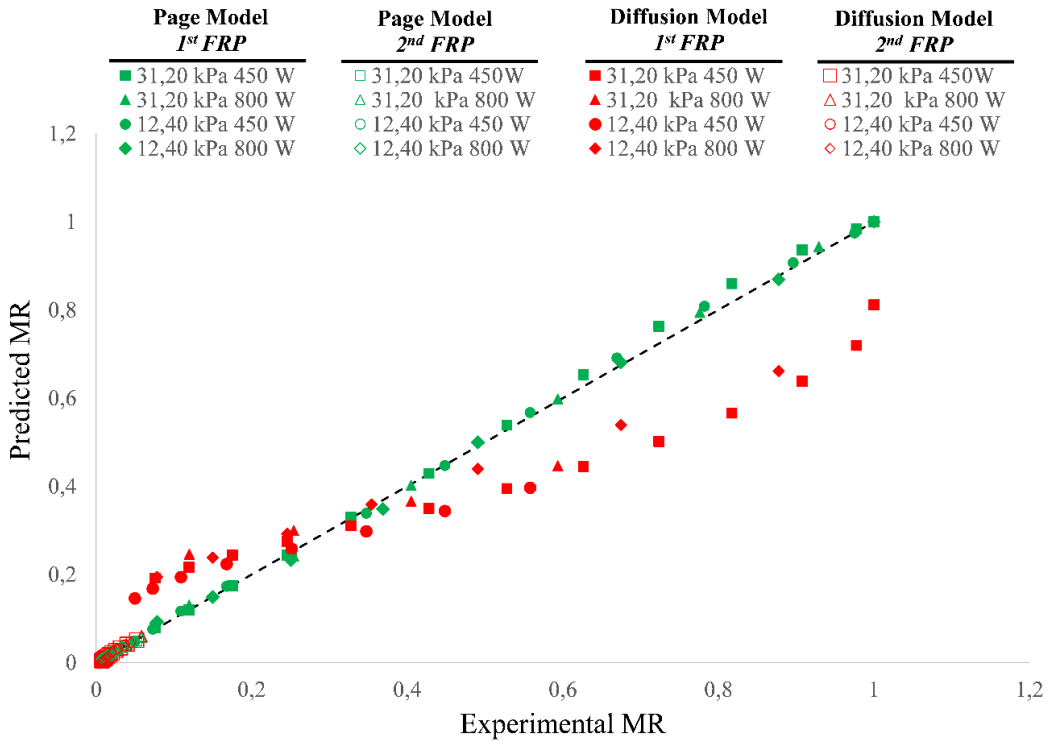


Figure 4. Comparison of the experimental and predicted dimensionless moisture contents simulated using the Page and diffusion models.

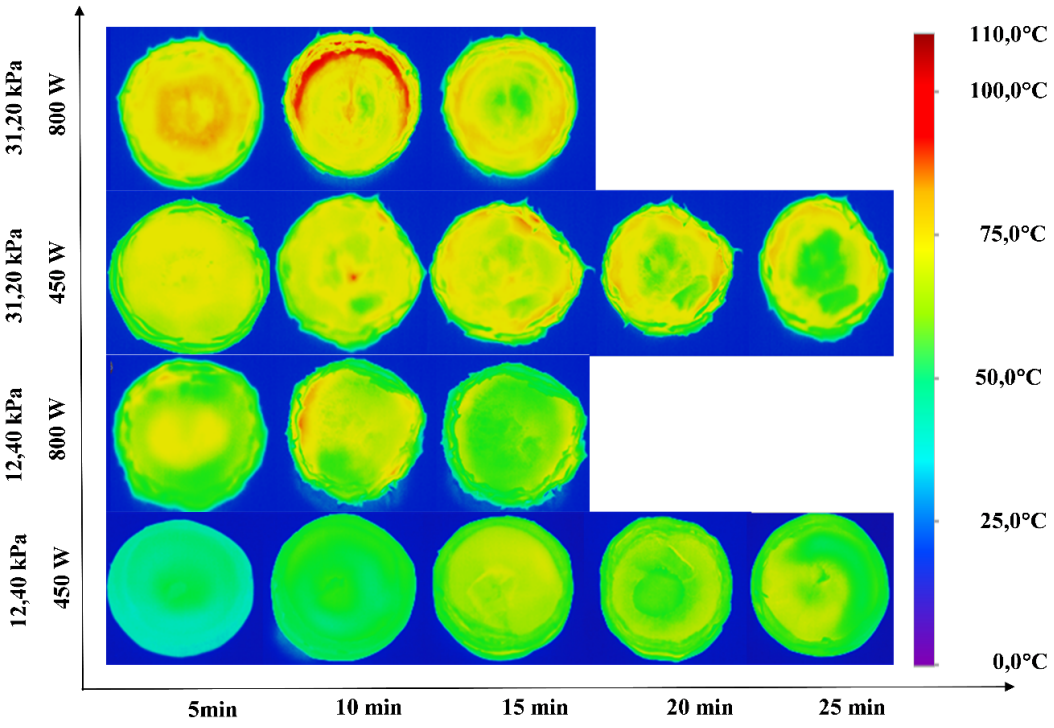


Figure 5. Surface temperature distribution of artichokes during MV drying.

Two different heating uniformity indices based on the average (HUI_{av}) and target (HUI_{tg}) temperatures were calculated to analyse the variation in the temperature distribution as MV drying proceeds (Table 2). The HUI_{av} values were found to range from 2.77±0.40-6.79±0.16. The HUI_{tg} values varied over a wider range between 3.47±0.32 and 15.17±0.07. The effects of process parameters such as the drying time, pressure and microwave power on the heating uniformity indices (HUI_{av} and HUI_{tg}) were statistically evaluated at a 95% confidence level using an analysis of variance (Table 3). The results showed that the variation in both heating uniformity indices could be explained by using the developed general linear models ($p<0.001$) with reasonable high R^2 values of 0.99 and 0.95 for the HUI_{av} and HUI_{tg}, respectively.

Microwave power ($p<0.001$) was the only MV drying parameter that significantly affected the HUI_{tg} values. Although the evaporation rate increase depends on the microwave power, the moisture removal rate has always been lower due to internal resistance because the MV drying takes place primarily during the falling rate period. Therefore, the increase in internal pressure due to vapour accumulation and the superheating of the entrapped vapour resulted in increased product temperatures and hence higher HUI_{tg} values. The effect of the system pressure ($p>0.01$) on the HUI_{tg} values were found to be non-significant at a 95% confidence level even though the average drying temperatures were decreased (Table 3). This result can be explained by the limited effect of the pressure on the MV drying characteristics during falling rate periods.

Table 2. Heating uniformity indices for MV drying of artichokes as a function of the drying time.

Drying Time (min)	MV Drying Process Conditions							
	31.20 kPa 450 W		31.20 kPa 800 W		12.40 kPa 450 W		12.40 kPa 800 W	
	HUI_{av}	HUI_{tg}	HUI_{av}	HUI_{tg}	HUI_{av}	HUI_{tg}	HUI_{av}	HUI_{tg}
5	4.76±0.22	6.19±0.17	5.61±0.20	14.03±0.09	3.53±0.32	9.69±0.11	5.09±0.19	12.58±0.07
10	3.85±0.26	4.91±0.20	6.79±0.16	15.01±0.08	3.47±0.32	3.47±0.32	6.03±0.16	15.17±0.07
15	4.80±0.21	5.08±0.20	5.32±0.19	10.17±0.11	3.55±0.30	11.83±0.09	4.35±0.25	9.12±0.14
20	5.37±0.21	5.46±0.20			2.77±0.40	10.00±0.10		
25	6.67±0.19	8.97±0.14			5.26±0.20	9.69±0.12		

Table 3. ANOVA table showing the effects of the process parameters on the heating uniformity indices.

Source	DF	HUI_{av}			HUI_{tg}		
		Sum of Squares	Mean Square	*p*-value	Sum of Squares	Mean Square	*p*-value
Model	7	389.326 [a]	55.617	<0.001	1554.157 [b]	222.022 [b]	<0.001
Pressure	1	5.221	5.221	.008	8.586	8.586	.354
MW Power	1	7.106	7.106	.003	101.475	101.475	.008
Time	4	6.669	1.667	.048	14.306	3.576	.806
Error	9	4.047	0.450		80.984	8.998	

Notably, the HUI_{av} values were significantly affected by all three process parameters at a 95% confidence level. It was observed that microwave power ($p<0.01$) increased the HUI_{av} values because it supported the formation of hot spots. The continuous supply of electromagnetic energy and the sinusoidal propagation characteristics of microwaves were

the primary reasons for the occurrence of annular hot zones during MV drying, especially at the 15th min for 450 W and the 10th min for 800 W. The effect of the pressure ($p<0.01$) seemed to be more effective due to evaporative cooling in the regions where the temperature was high, rather than lowering the average temperature. Table 3 also shows that the effect of the drying time ($p<0.05$) on the HUIav values is not as pronounced as the microwave power and pressure. A slight increase in the HUI_{av} values was observed through the end of the MV drying due to selective heating with a decrease in moisture content.

3.4. Shrinkage coefficient

The shrinkage coefficients of artichokes as a function of the moisture content under different microwave power and vacuum intensity conditions are given in Fig. 6. As the moisture contents decreased up to 60% (w/w), the shrinkage coefficients decreased sharply due to the high moisture removal rates during the initial stages of MV drying. The reduced volume of the artichoke samples for all the MV drying conditions were found to range from 63% to 78%, with the lowest shrinkage effect occurring when high microwave power (800 W) and low pressure (12.40 kPa) were applied. The standard deviations for each experimental shrinkage coefficient ranged between 0.01 and 0.33, which corresponds to experimental uncertainty limits between ±1.37% and ±9.21%.

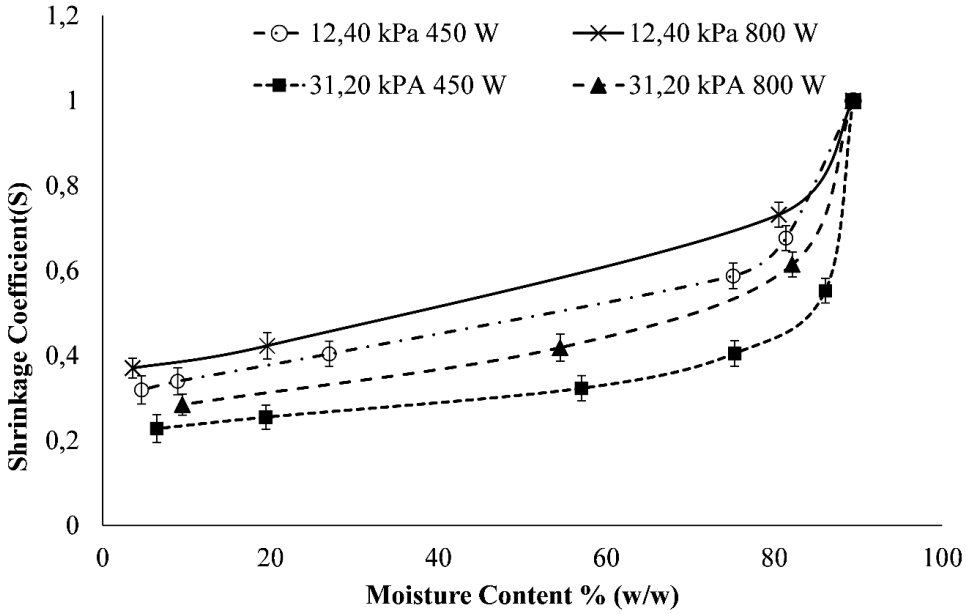


Figure 6. Shrinkage coefficient of artichokes as a function of moisture.

A statistical analysis using an ANOVA showed that the moisture content ($p<0.001$) and system pressure ($p<0.001$) have more pronounced effects on the shrinkage coefficients than microwave power ($p<0.05$). Removing water from plant structures reduces the turgor pressure created by intracellular fluids and a pressure imbalance between the inner and outer parts of the tissue occurs (MAHIUDDIN *et al.*,2018). Therefore, decreasing the system pressure from 31.20 kPa to 12.40 kPa minimized the pressure imbalances and resulted in higher shrinkage coefficients for a given microwave power level. The volumetric heating effect of the microwaves caused an additional increase in internal pressure due to vaporization, and the shrinkage-reducing effect of microwave power was

also found to be statistically significant. However, the decrease in the heat generation rate due to the reduced moisture contents at the falling rate period of drying caused this effect to be more limited. Similar results have also been reported by GIRI and PRASAD (2006) for the MV drying of button mushrooms.

3.5. Rehydration ratio

The rehydration ratios of artichoke samples that were dried at different system pressures and microwave power levels are depicted in Fig. 7. Initially high rates of moisture uptake were followed by a slower absorption rate in the final stages of rehydration. This typical rehydration behaviour has also been observed for MV-dried button mushrooms, potato cubes and green beans (GIRI and PRASAD, 2007; MARKOWSKI *et al.*, 2009; ZIELINSKA *et al.*, 2013). The equilibrium conditions for the rehydration process were reached within 80 min at 30°C. At that point, the rehydration ratio of the artichokes was determined to be within a range from 1,7326±0,1673 to 3,82±0,091 for various drying conditions. Similar to that of the shrinkage coefficients, the system pressure ($p<0.001$) and microwave power level ($p<0.05$) were found to be the statistically significant MV drying factors that affected the rehydration ratio at a 95% confidence level.

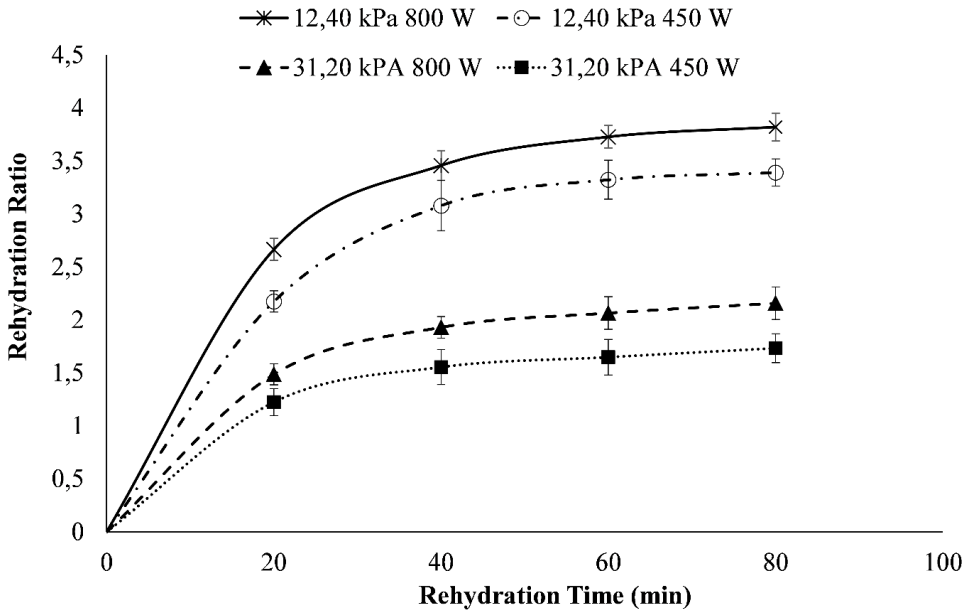


Figure 7. Rehydration Ratio of MV-dried artichokes as a function of rehydration time.

As indicated by the higher shrinkage coefficients, drying at a lower system pressure and higher microwave power enhanced the porous structure of the dried artichokes. Therefore, the capillaries and cavities with a larger volume absorbed much more water, leading higher rehydration ratios by decreasing the system pressure and increasing the microwave power.

3.6. Colour changes during MV drying of artichokes

Artichokes have been known to be very prone to enzymatic and non-enzymatic browning during storage and post-harvest processes due to their high enzymatic activity and

phenolic contents, in combination with temperature-dependent phase changes in the cellular membrane (DOĞAN *et al.*, 2005; LATTANZIO and LINSALATA, 1989). The mean values of the L^*, a^* and b^* for the artichoke samples prior to MV drying were found to be 73.32±6.97, -4.31±1.22 and 27.31±2.46, respectively. These values indicate that the artichoke hearts used in the experiments were light, yellowish and greenish, but there was considerable variability among the samples. It was observed that the changes in the L^*, a^* and b^* values of the samples during MV drying were lower than the variation in the initial colour values. A statistical analysis also showed that the MV drying parameters did not affect the L^*, a^* and b^* values of the dried artichokes significantly at the 95% confidence level. Therefore, the deviations in the colour values of the artichokes prior to MV drying were eliminated by normalizing (Δ) the dry product colour values relative to the colour values of the artichokes used in the experiments (Table 4).

Table 4. Normalized colour parameters for MV-dried artichoke hearts.

Drying Conditions	Drying Time (min)	ΔL	Δa	Δb	Δh°	ΔE	ΔBI
31.20 kPA 450 W	5	2.06	-1.05	6.59	-0.20	6.99	9.74
	10	2.64	-0.90	6.00	-0.38	6.62	11.17
	15	3.74	0.69	6.09	-2.75	7.18	11.28
	20	3.21	0.82	7.29	-2.70	8.01	16.08
	25	2.30	0.90	7.98	-3.01	8.35	16.56
31.20 kPa 800 W	5	3.17	1.06	7.43	-3.71	8.14	15.37
	10	3.51	1.00	7.18	-2.89	8.05	15.57
	15	3.45	1.11	8.58	-2.96	8.99	23.53
12.40 kPA 450 W	5	3.15	-1.36	5.32	0.86	7.52	5.25
	10	3.90	-1.07	5.44	0.28	6.78	6.32
	15	2.51	0.87	7.54	-4.14	7.99	16.41
	20	2.47	1.68	8.12	-5.26	8.65	19.22
	25	3.17	1.00	10.94	-4.09	10.99	24.14
12.40 kPa 800 W	5	3.46	-1.09	10.76	-1.84	11.36	17.50
	10	2.94	-0.06	9.56	-1.60	10.00	18.02
	15	1.88	0.67	12.97	-3.12	13.12	29.76

The ANOVA results revealed that the normalized colour parameters except for ΔL were affected by at least one MV drying parameter, i.e., the microwave power, system pressure and drying time at a 95% confidence level. The ***ΔBI***, ***ΔE*** and ***Δh°*** were found to be more explanatory at describing the colour changes during the Mv drying of the artichokes. The microwave power (p<0.01) and drying time (p <0.01) were the most influential MV drying parameters, since they affected all the normalized colour parameters for all the drying conditions. As the microwave power and drying time increased, the ***ΔE*** and ***ΔBI*** values increased and the ***Δh°*** values decreased. The negative correlation of the ***Δh°*** values to both ***ΔE*** and ***ΔBI*** were found to be statistically significant (p<0.05) with high Pearson's correlation coefficients (r>0.71) under all the drying conditions. The lower ***Δh°*** values revealed that the redness of the dried artichokes increased more than the yellowness, indicating more browning. HAWLADER *et al*. (2006) also stated that decreases in the hue angle values could be interpreted as an indication of a more browning colour and a shift away from yellowness.

Another important point regarding the colour changes during the MV drying of artichokes is that the effect of the system pressure on the $\Delta h°$ and ΔBI values was statistically insignificant (p >0,1). This finding may be because the Maillard reactions remained at a limited level and enzymes such as polyphenol oxidase (PPO) largely retained their activity at the studied temperature ranges (56.8-60.4°C at 12.40 kPa and 69-75.9°C at 31.20 kPa). A residual PPO activity of 32% was also reported by (JAISWAL *et al.*,2010) for the hot air drying of pomegranate arils in which the temperature was gradually reduced from 90°C to 50°C. Therefore, enzymatic browning reactions are thought to be more determinant of discolouration in artichoke samples. The highest ΔE and ΔBI values were recorded at the lowest system pressure (12.40 kPa) and the highest microwave power (800 W) as 13.12 and 29.76, respectively. PARIN (2004) reported higher ΔBI (38.35) and ΔE (42.80) values for artichokes dried with hot air at 60°C despite the pretreatments, such as blanching for 15 min followed by immersion in 1% (w/v) citric acid solution for 30 min.

4. CONCLUSIONS

Microwave power was found to be the most influential parameter on the drying characteristics of artichokes compared to system pressure. Both the constant and falling rate periods of drying were observed under low microwave power levels, whereas drying took place primarily during the falling rate period at high microwave power. Two distinct falling rate periods were evident from the drying characteristic curves. The low accuracy of the diffusion model in the *1st FRP* indicated that one or more combinations of different moisture transport mechanisms took place. The diffusion model simulated the drying curves more accurately in the *2nd FRP*. The effective diffusion coefficients were found to range from 2.279×10^{-6}- 3.454×10^{-6} m^2/s and $3.600x10^{-6}$ - $6.297x\ 10^{-6}$ m^2/s for the *1st FRP* and *2nd FRP*, respectively. An increasing trend in the D_{eff} with microwave power was observed during both falling rate periods since higher microwave power levels would enhance the drying rate by generating more heat energy inside the product. In addition, empirical models accurately simulated the drying curves during the MV drying of artichokes, even though the estimated parameters do not provide mechanistic information and lack physical meaning. Reducing the microwave power and system pressure resulted in lower HUI_{av} values, indicating a higher uniformity in the temperature distribution. However, the average temperatures deviated slightly from the targeted drying temperature at high microwave power and the HUI_{tg} values increased. An intermittent (pulsed) supply of electromagnetic energy would be an effective approach not for only eliminating the deviations from target temperatures but also for increasing the energy efficiency. The MV drying at lower system pressure and higher microwave power resulted in increased shrinkage coefficients and rehydration ratio values. High ΔBI and low $\Delta h°$ values indicated slight discolouration in the samples due to browning reactions induced by microwave power. Enzymatic browning is thought to be the primary discolouration mechanism during the MV drying of artichokes due to the relatively low average drying temperatures, but further investigations on the enzymatic activity are necessary for confirmation. It can be concluded that MV technology has great potential for drying artichokes in terms of drying times and dried product characteristics.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of this project from Manisa Celal Bayar University, Council of Scientific Research Projects (Project no: 2017-207).

REFERENCES

Abu-Reidah, I. M., Arraez-Roman, D., Segura-Carretero, A. and Fernandez-Gutierrez, A. 2013. Extensive characterisation of bioactive phenolic constituents from globe artichoke (Cynara scolymus L.) by HPLC-DAD-ESI-QTOF-MS. Food Chem. 141(3):2269-2277.

Ben Salem, M., Affes, H., Athmouni, K., Ksouda, K., Dhouibi, R., Sahnoun, Z., Hammami, S. and Zeghal, K. M. 2017. Chemicals Compositions, Antioxidant and Anti-Inflammatory Activity of Cynara scolymus Leaves Extracts, and Analysis of Major Bioactive Polyphenols by HPLC. Evid Based Complement Alternat Med, 2017:4951937.

Chauhan, A. K. S. and Srivastava, A. K. 2009. Optimizing Drying Conditions for Vacuum-Assisted Microwave Drying of Green Peas (Pisum sativumL.). Drying Technology, 27(6):761-769.

Chen, J., Pitchai, K., Jones, D. and Subbiah, J. 2015. Effect of decoupling electromagnetics from heat transfer analysis on prediction accuracy and computation time in modeling microwave heating of frozen and fresh mashed potato. Journal of Food Engineering, 144:45-57.

Clary, C. D., Mejia-Meza, E., Wang, S. and Petrucci, V. E. 2007. Improving grape quality using microwave vacuum drying associated with temperature control. J Food Sci, 72(1):E023-028.

Crank, J. 1975. The Mathematics of Diffusion,Second Edition ed., UK, Oxford University Press.

Cui, Z.-W., Xu, S.-Y. and Sun, D.-W. 2004. Microwave–vacuum drying kinetics of carrot slices. Journal of Food Engineering, 65(2):157-164.

Cui, Z.-W., Xu, S.-Y., Sun, D.-W. and Chen, W. 2005. Temperature Changes during Microwave-Vacuum Drying of Sliced Carrots. Drying Technology, 23(5):1057-1074.

Doğan, S., Turan, Y., Ertürk, H. and Arslan, O. 2005. Characterization and Purification of Polyphenol Oxidase from Artichoke (Cynara scolymus L.). J. Agric. Food Chem., 53(3): 76-785.

Dong, W., Cheng, K., Hu, R., Chu, Z., Zhao, J. and Long, Y. 2018. Effect of Microwave Vacuum Drying on the Drying Characteristics, Color, Microstructure, and Antioxidant Activity of Green Coffee Beans. Molecules, 23(5).

Drouzas, A. E., Tsami, E. and Saravacos, G. D. 1999. Microwave/vacuum drying of model fruit gels. Journal of Food Engineering, 39:117-122.

Eren, İ., Yıldız-Turp, G., Kaymak-Ertekin, F. and Serdaroğlu, M. 2008. The Effect of External Mass Transfer Resistance during Drying of Fermented Sausage. Drying Technology, 26(12):1543-1551.

Feng, H., Yin, Y. and Tang, J. 2012. Microwave Drying of Food and Agricultural Materials: Basics and Heat and Mass Transfer Modeling. Food Engineering Reviews, 4(2):89-106.

Fratianni, F., Pepe, R. and Nazzaro, F. 2014. Polyphenol Composition, Antioxidant, Antimicrobial and Quorum Quenching Activity of the "Carciofo di Montoro" (*Cynara cardunculus* var. *scolymus*) Global Artichoke of the Campania Region, Southern Italy. Food and Nutrition Sciences, 05(21): 2053-2062.

Geankoplis, C. J. 2003. Transport Processes and Separation Process Principles,4th ed., New Jersey, Prentice-Hall International Inc.

Giri, S. K. and Prasad, S. 2006. Modeling Shrinkage and Density Changes During Microwave-Vacuum Drying of Button Mushroom. International Journal of Food Properties, 9(3):409-419.

Giri, S. K. and Prasad, S. 2007. Optimization of Microwave-Vacuum Drying of Button Mushrooms Using Response-Surface Methodology. Drying Technology, 25(5):901-911.

Guida, V., Ferrari, G., Pataro, G., Chambery, A., Di Maro, A. and Parente, A. 2013. The effects of ohmic and conventional blanching on the nutritional, bioactive compounds and quality parameters of artichoke heads. LWT - Food Science and Technology, 53(2):569-579.

Hawlader, M. N. A., Perera, C. O. and Tian, M. 2006. Properties of modified atmosphere heat pump dried foods. Journal of Food Engineering, 74(3):392-401.

Jaiswal, V., Dermarderosian, A. and Porter, J. R. 2010. Anthocyanins and polyphenol oxidase from dried arils of pomegranate (*Punica granatum* L.). Food Chemistry, 118(1):11-16.

Jaya, S. and Durance, T. D. 2007. Effect of Microwave Energy on Vacuum Drying Kinetics of Alginate-Starch Gel. Drying Technology, 25(12):2005-2009.

Kiranoudis, C. T., Tsami, E. and Maroulis, Z. B. 1997. Microwave Vacuum Drying Kinetics of Some Fruits. Drying Technology, 15(10):2421-2440.

Lattanzio, V., Kroon, P. A., Linsalata, V. and Cardinali, A. 2009. Globe artichoke: A functional food and source of nutraceutical ingredients. Journal of Functional Foods, 1(2):131-144.

Lattanzio, V. and Linsalata, V. 1989. The Beneficial Effect of Citric and Ascorbic Acid on the Phenolic Browning Reaction in Stored Artichoke (Cynara scolymus L.) Heads Food Chemistry, 33:93-106.

Law, C. L., Tasirin, S. M. and Wan Daud, W. R. 2003. A New Variable Diffusion Drying Model for the Second Falling Rate Period of Paddy Dried in a Rapid Bin Dryer. Drying Technology, 21(9):1699-1718.

Leusink, G. J., Kitts, D. D., Yaghmaee, P. and Durance, T. 2010. Retention of antioxidant capacity of vacuum microwave dried cranberry. J Food Sci, 75(3):C311-316.

Lombardo, S., Pandino, G., Mauromicale, G., Knödler, M., Carle, R. and Schieber, A. 2010. Influence of genotype, harvest time and plant part on polyphenolic composition of globe artichoke [Cynara cardunculus L. var. scolymus (L.) Fiori]. Food Chemistry, 119(3):1175-1181.

Lombraña, J. I., Rodríguez, R. and Ruiz, U. 2010. Microwave-drying of sliced mushroom. Analysis of temperature control and pressure. Innovative Food Science & Emerging Technologies, 11(4):652-660.

Mahiuddin, M., Khan, M. I. H., Kumar, C., Rahman, M. M. and Karim, M. A. 2018. Shrinkage of Food Materials During Drying: Current Status and Challenges. Comprehensive Reviews in Food Science and Food Safety, 17(5):1113-1126.

Markowski, M., Bondaruk, J. and Błaszczak, W. 2009. Rehydration Behavior of Vacuum-Microwave-Dried Potato Cubes. Drying Technology, 27(2):296-305.

Maskan, M. 2001. Drying, shrinkage and rehydration characteristics of kiwifruits during hot air and microwave drying. Journal of Food Engineering, 48(2):177-182.

Meda, V., Mitra, P., Lee, J. H. and Chang, K. S. 2016. Optimization of microwave-vacuum drying processing parameters on the physical properties of dried Saskatoon berries. Open Agriculture, 1(1).

Michalska, A., Wojdyło, A., Lech, K., Łysiak, G. P. and Figiel, A. 2017. Effect of different drying techniques on physical properties, total polyphenols and antioxidant capacity of blackcurrant pomace powders. LWT - Food Science and Technology, 78:114-121.

Mousa, N. and Farid, M. 2002. Microwave Vacuum Drying of Banana Slices. Drying Technology, 20(10):2055-2066.

Palou, E., López-Malo, A., Barbosa-Cánovas, G. V., Welti-Chanes, J. and Swanson, B. G. 1999. Polyphenoloxidase Activity and Color of Blanched and High Hydrostatic Pressure Treated Banana. Journal of Food Science, 64(1):42-46.

Parin, H. 2004. Effect Of Pretreatment And Air Temperature On The Drying Rate, Rehydration Capacity And Color Of Artichoke. *MSc Thesis.* Middle East Technical University, Ankara, Turkey.

Pitchai, K., Birla, S. L., Subbiah, J., Jones, D. and Thippareddi, H. 2012. Coupled electromagnetic and heat transfer model for microwave heating in domestic ovens. Journal of Food Engineering, 112(1-2):100-111.

Rogelio, J. P., Liza, F. P., Abulencia, G. L., Soriano, J. K. T., Baldovino, R. G., Tolentino, T. a. U. and Jr., V. Y. M. 2015 An Optimized Design of a PLC-Based Controlled Microwave Vacuum Dryer for Preliminary Drying Studies on Rice Bran. *Proceedings of the World Congress on Engineering and Computer Science.* San Francisco, USA.

Segnini, S., Pedreschi, F. and Dejmek, P. 2004. Volume Measurement Method of Potato Chips. International Journal of Food Properties, 7(1):37-44.

Si, X., Chen, Q., Bi, J., Wu, X., Yi, J., Zhou, L. and Li, Z. 2016. Comparison of different drying methods on the physical properties, bioactive compounds and antioxidant activity of raspberry powders. J Sci Food Agric, 96(6):2055-2062.

Song, X.-J., Zhang, M., Mujumdar, A. S. and Fan, L. 2009. Drying Characteristics and Kinetics of Vacuum Microwave–Dried Potato Slices. Drying Technology, 27(9):969-974.

Sutar, P. P. and Prasad, S. 2007. Modeling Microwave Vacuum Drying Kinetics and Moisture Diffusivity of Carrot Slices. Drying Technology, 25(10):1695-1702.

Tein M. Lin, T. D. D. C. H. S. 1998. Characterization of vacuum microwave, air and freeze dried carrot slices. Food Research International, 31(2).

Tsuruta, T., Tanigawa, H. and Sashi, H. 2015. Study on Shrinkage Deformation of Food in Microwave–Vacuum Drying. Drying Technology, 33(15-16):1830-1836.

Wojdyło, A., Figiel, A., Lech, K., Nowicka, P. and Oszmiański, J. 2013. Effect of Convective and Vacuum–Microwave Drying on the Bioactive Compounds, Color, and Antioxidant Capacity of Sour Cherries. Food and Bioprocess Technology, 7(3):829-841.

Wray, D. and Ramaswamy, H. S. 2015. Novel Concepts in Microwave Drying of Foods. Drying Technology, 33(7):769-783.

Zielinska, M., Zapotoczny, P., Alves-Filho, O., Eikevik, T. M. and Blaszczak, W. 2013. A multi-stage combined heat pump and microwave vacuum drying of green peas. Journal of Food Engineering, 115(3):347-356.

Paper Received November 13, 2018 Accepted February 13, 2019

GUIDE FOR AUTHORS

Editor-in-Chief: **Paolo Fantozzi**
Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università di Perugia, Via S. Costanzo, I-06126 Perugia, Italy - Tel. +39 075 5857910 - Telefax +39 075 5857939-5857943 - e-mail: paolo.fantozzi@ijfs.eu

IJFS has a publication charge of € 350.00 each article

Publication Ethics and Publication Malpractice

Italian Journal of Food Science is committed to upholding the highest standards of publication ethics and takes all possible measures against any publication malpractices. All Authors submitting their works to Italian Journal of Food Science for publication as original articles attest that the submitted works represent their Authors' contributions and have not been copied or plagiarized in whole or in part from other works. The Authors acknowledge that they have disclosed all and any actual or potential conflicts of interest with their work or partial benefits associated with it. In the same manner, Italian Journal of Food Science is committed to objective and fair Editor(s) review of the submitted for publication works and to prevent any actual or potential conflict of interests between the editorial personnel and the reviewed material. Any departures from the above-defined rules should be reported directly to the Editor-in-Chief, who is unequivocally committed to providing swift resolutions to any of such a type of problems.

1. Manuscript Submission

Manuscripts must submitted as an electronic version to
http://www.chiriottieditori.it/ojs/index.php/IJFS/index
All the Authors must be registered with initals, surname and e-mail. The word processor used to generate the file should be indicated and the files should be saved in format "Text only"; **graphs, pictures and diagrams must be saved in JPG format**. Manuscripts must be typed single-space, using Times New Roman 12 pt as the advised font; employs italics, rather than underlining (except with URL addresses); **lines must be numbered**. A single file must contain the manuscript and all the tables and figures inserted at the appropriate points.

English is the official language. The Editor-in-Chief and/or Co-Editors reserve the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be previously revised for English by the authors**. If English is not the mother tongue of authors, they **must seek** help from one of the following agencies (or other similar official agencies):
WWW.JOURNALEXPERTS.COM
WWW.SCIENCEDOCS.COM
WWW.INTERNATIONALSCIENCEEDITING.COM
WWW.WRITESCIENCERIGHT.COM

WWW.GENEDITS.COM
WWW.MANUSCRIPT-PROOFREADING.COM

When the paper will be accepted, copy of the English Translator Certificate of the revised version must be uploaded.

2. Manuscript Preparation

(1) The paper should be divided under the following headings in this order:
Title. Informative of the content of the article (<50 characters + spaces).
Author(s). Initials and Surname, omit professional and official titles. The institute and address where the research was carried out and the current address of each author should be given on the title page.
Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. **Do not exceed 100 words**.
Key words. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.
Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.
Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.
Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.
Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, article number or thesis fulfilment may be included.
Units. A list of units particular to the paper may be included.
References. References in the Reference list should be arranged alphabetically (initials of first name, only), and, for the same author, should be arranged consecutively by year, typed double-spaced. Each individual reference should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - I1linois - USA). Literature citations in the text should be referred to by Surname and year in parentheses. If there are more than two authors, give the surname of the first author and add et al. and the year in parentheses. Examples: (SMITH, 2007), (SMITH and JONES, 2008) (SMITH et al., 2008).

(2) Tables should be as few and as simple as possible and include only essential data. Each table must be saved within the text at the appropriate points, and have an Arabic number, e.g. Table 4 NOT Tab. 4. Legends must be self-explanatory. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(3) Figures must be prepared and saved in **JPEG format,** and inserted in the manuscript at the appropriate points. They should be prepared so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. All figures must be given Arabic numbers, e.g. Fig. 3. Legends for figures must be self-explanatory.

(4) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in c1early defined units.
Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:
Reviews –They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.
Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.
Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**
(1) Manuscripts will be processed in the order received. The Editor-in-Chief will select papers to enter into the reviewing system based on originality and innovation. A letter will be sent to the authors acknowledging receipt of the manuscript along with a Declaration form stating that it has NOT been previously published, accepted or submitted for publication elsewhere and agreeing to the page charges upon acceptance of the paper. On receipt of the signed Declaration form, the Editor-in-Chief will send the manuscript to a Co-Editor and/or referees for evaluation.
(2) Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief and/or Co-Editor as soon as possible, usually within three weeks. The identity and report of the referees are made know to the Editor-in-Chief, but only the anonymous referee report is sent to the author(s). If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief and/or Co-Editors have the freedom to decide upon acceptance or rejection of the paper.
(3) The results of the refereeing process, accompanied by a letter from the Editor-in-Chief or the Co-Editor, will be sent to the author(s). **Papers needing revision must be returned to the Co-Editor within four weeks, otherwise the paper will be considered as withdrawn**. A letter announcing acceptance of the manuscript will be sent to the author(s) upon acceptance by the referees.
(4) The authors **will receive galley proofs** of the manuscript along **with the invoice** for the article charge which **must be paid in order to allow for publication**. The

proofs will be sent to the corresponding author as a PDF file by e-mail, only.
Once the page charge is paid, the DOI will be sent to the Author.

REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

(Anonymous)
Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)
AOAC. 1980. "Official Methods of Analysis" Association of Official Analytical Chemists, Washington, DC.
Weast, R.C. (Ed.). 1981 "Handbook of Chemistry" 62nd ed. The Chemical Rubber Co. Cleveland, OH.

(Bulletin, circular)
Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)
Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)
Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.
IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.
IFT Sensory Evaluation Div. 1981b. Guidelines for the prepaation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)
Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)
Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)
Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)
Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)
Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).

(Thesis)
Gejl-Hansen F. 1977. Microstructure and stability of Freeze dried solute containing oil-in-water emulsions Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)
Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.
Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2019.

ASSOCIATIONS and COMPANIES

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.)
Parma
Fax +39-0521-230507
www.aita-nazionale.it

Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Perugia
Fax +39-075-5857939
www.sistal.org

RESEARCH INSTITUTES

Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali (DI.VA.P.R.A.), Sezione Microbiologia ed Industrie Agrarie, Università di Torino
Grugliasco
Fax +39-011-6708549



ITALIAN JOURNAL OF FOOD SCIENCE
Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Alberto Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
del Tribunale di Perugia

ISSN 1120-1770 © 2019

CHIRIOTTI EDITORI srl - 10064 Pinerolo - Italy

publishes the technical magazines:

















VOLUME XXXI IJFS No. 3, 2019

CONTENTS

SURVEY

Household Characteristics Infuencing Fish Consumption in Van Province, Turkey
M. Terin ***416***

PAPERS

Effect of Storage Method: Aerobic Refrigerated, Vacuum Refrigerated and Frozen Storage, on the Quality of Churra Suckling Lamb Meat
A.R. Callejas-Cárdenas, S. Soto, I. Caro, D.E. Carballo, E.J. Quinto and J. Mateo ***427***

Effects of Probiotic Fermentation on Selected Milk and Whey Protein Preparations on Bioactive and Technological Properties
K. Skrzypczak, E. Fornal, A. Waśko and W. Gustaw ***437***

The Effect of an Increase in Paste Temperature Between Malaxation and Centrifugation on Olive Oil Quality and Yield: Preliminary Results
L. Guerrini, P. Masella, G. Angeloni, B. Zanoni, C. Breschi, L. Calamai and A. Parenti ***451***

Factors Affecting the Spectrophotometric Quantification of Flavonoids in Wine
O. Corona, M. Squadrito and A. Tirelli ***459***

Development of Fruit Leather From *Actinidia Arguta* By-Product: Quality Assessment and Shelf life Studies
G. Giacalone, T.M. Da Silva, C. Peano and N.R. Giuggioli ***470***

Drying of Lime Slices by Microwave and Combined Microwave-Convective Method
N. Izli, O. Taskin and G. Izli ***487***

Sensory Quality of Molecular Dishes and Consumers' Attitudes Towards Them
A. Głuchowski, E. Czarniecka-Skubina, M. Pielak and E. Ołubiec-Opatowska ***501***

Freeze Drying and Moisture Adsorption Kinetics of Kefir Powder
H. Isleroglu ***514***

Quality of Broiler Chicken Meat During Frozen Storage
A. Augustyńska-Prejsnar, M. Ormian and R. Tobiasz-Salach ***531***

The Effects of Locust Bean Gum and Rhubarb on the Physical and Functional Properties of the Gluten-Free Biscuits
H. Doğan and R. Meral ***542***

Chemical Composition, Antioxidant and Antimicrobial Activities of *Aloysia Triphylla* L. Essential Oils and Methanolic Extract
L. Rezig, M. Saada, N. Trabelsi, S. Tammar, H. Limam, I. Bettaieb Rebey, A. Smaoui, G. Sghaier, G. Del Re, R. Ksouri and K. Msaada ***556***

Erythorbic Acid and Sodium Erythorbate Effectively Prevent Pulp Browning of Minimally Processed 'Royal Gala' Apples
J.A. Ribeiro, M. Seifert, J. Vinholes, C.V. Rombaldi, L. Nora and R.F.F. Cantillano ***573***

Effect of Ultrasound Treatment on Physicochemical, Functional and Nutritional Properties of a Safflower (*Carthamus Tinctorius* L.) Protein Isolate
M.R. Zuñiga-Salcedo, J.A. Ulloa, P.U. Bautista-Rosales, P. Rosas-Ulloa, J.C. Ramirez-Ramirez, Y. Silva-Carrillo, R. Gutiérrez-Leyva and C. Hernández ***591***

The Impact of Lemon Juice on the Marination of Anchovy (*Engraulis Encrasicolus*): Chemical, Microbiological and Sensory Changes
V. Šimat, A. Mićunović, T. Bogdanović, I. Listeš, I. Generalić Mekinić, I. Hamed and D. Skroza ***604***

Coagulase Positive Staphylococci Enumeration and Enterotoxins Detection in Milk and Dairy Products from Central Italy
L. Iannetti, P. Visciano, C. Marfoglia, G. Iannitto, G. Parisciani, M. Sericola, D. Petrone, M.S. Mamgieri, F. Pomilio and M. Schirone ***618***

Characterization of Pasta with the Addition of *Cicer Arietinum* and *Salvia Hispanica* Flours on Quality and Antioxidant Parameters
A.G. Cota-Gastélum, M.G. Salazar-García, A. Espinoza-López, L.M. Perez-Perez, F.J. Cinco-Moroyoqui, O. Martínez-Cruz, F.J. Wong-Corral and C.L. Del-Toro-Sánchez ***626***

An Investigation of the Mechanical, Microstructural and Thermo-Mechanical Properties of Collagen Films Cross-Linked with Smoke Condensate and Glutaraldehyde
S. Barbut and M. Ioi ***644***

Polycyclic Aromatic Hydrocarbons in Selected Food Items Coming From the Croatian Market
T. Bogdanović, S. Petričević, E. Listeš and J. Pleadin ***661***

Drying Kinetics, Heating Uniformity and Quality Changes During the Microwave Vacuum Drying of Artichokes (*Cynara Scolymus* L.)
Ç. Muştu and İ. Eren ***681***