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PAPER

ASSESSMENT OF DURABILITY AND CHARACTERISTICS OF CHANGES IN KEFIR MADE FROM COW'S AND GOAT'S MILK

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ABSTRACT

Present study was aimed to determine the changes and the best consumption time of kefir samples made from cow and goat milk using starter culture during the storage at the refrigerator (+4°C for 35 days). It was found that goat kefir (GK) samples had higher pH and lipolysis values during storage while cow kefir (CK) samples had higher values of viscosity. The counts of lactococci, lactobacilli, leuconostoc, total mesophilic aerobic bacteria, acetic acid bacteria and yeast decreased during the storage. Amounts of lactic, acetic, citric, pyruvic and oxalic acids were higher in GK samples. Acetaldehyde, diacetyl and acetoin contents were higher in CK samples. The highest sensory scores were obtained for CK samples on 14th day and for GC sample on 21th day of the storage.

Keywords: cow milk, goat milk, kefir, shelf-life, storage

1. INTRODUCTION

Kefir is a fermented dairy product obtained by lactic acid and alcohol fermentation (WSZOLEK *et al*., 2001). It is originated from Caucasia region and believed that the word "kefir" is derived from the Turkish word "keyf", "keyif", "kefi" or "kef" (YAYGIN, 1994). In the Great Turkish Dictionary of the Turkish Language Institution (2019), it is reported that the word "kef" means "pleasure" and "foam", the word "keyif" means "body well-being", "health and vitality", the word "keyf" means "comfortable" and the word "kefi" means "foam". It is also known called kefyr, kephir, kefer, kiaphur, knapon, kepi and kippi in different regions (RATTRAY and O'CONNELL, 2011). However, it is called "kefir" in many languages now.

Kefir is defined as "the yogurt of the 21st century" (WSZOLEK *et al*., 2006). Kefir is traditionally produced with kefir grains. Nowadays, commercial freeze-dried kefir starter culture and the product that remains after the removal of kefir grains in addition to kefir grains are used in the production of commercial kefir (BENSMİRA and JIANG, 2012). But, kefir produced with kefir grains due to the different microflora of each kefir grain shows significant variation in taste, aroma and texture. Moreover, there is a risk of microbial contamination due to the use of kefir grains many times and necessity to separate the kefir grains from the kefir produced after fermentation in traditional kefir production. Furthermore, the kefir produced by the method has a short shelf life. Therefore, kefir grain is not suitable for industrial kefir production (GÜZEL-SEYDİM *et al*., 2010; KİM *et al*., 2018). Commercial kefir is produced using pure starter cultures that contain a mixture of limited number of bacteria and yeasts, which give a similar flavor to the traditional kefir, but do not cause bulge or leakage (O'BRIEN *et al*., 2016). The most common method in the production of commercial kefir is the use of commercial concentrated lyophilized kefir starter cultures (YAMAN, 2011).

Because of the health benefits of kefir, people's interest onto kefir has been increasing day by day. In the dairy industry, kefir is produced mostly from cow's milk (PURNOMO and MUSLİMİN, 2012). However, the researchers used goat milk (PURNOMO and MUSLİMİN, 2012; KACZYŃSKİ *et al*., 2018), sheep milk (WSZOLEK *et al*., 2001; CAİS-SOKOLİŃSKA *et al*., 2008), camel milk (KAVAS, 2015), buffalo milk (GUL *et al*., 2015), whey (MAGALHÃES *et al*., 2011), coconut milk (ISMAİEL *et al*., 2011), rice milk (NURLİYANİ *et al*., 2015), soy milk (KESENKAŞ *et al*., 2011; ISMAİEL *et al*., 2011), oat milk (DİNKÇİ *et al*., 2015) or peanut milk (BENSMİRA and JİANG, 2012) in addition to cow's milk in the production of kefir.

It has been interested in kefir produced from goat milk since the αs_1-casein, which has an important responsibility in cow's milk allergy is very low in goat milk. The studies on goat kefir produced with commercial culture mostly focused on physical, chemical and microbiological properties. However, it has been determined that there are not enough studies on the biochemical, organic acid and aroma profile of kefir produced by commercial culture.

Present study was aimed to determine the changes in chemical, physical, biochemical, microbiological, sensorial properties and also, organic acids and flavor components in kefir samples made from cow and goat milk fermented by kefir starter culture during storage at refrigerated condition. Also, the best consumption time of kefir during refrigerated storage was determined in this study.

2. MATERIALS AND METHODS

2.1. Materials

Cow and goat milk were used in production of the experimental kefir samples. Raw cows' milk was purchased from a factory and raw goats' milk from a farmer in Bolu. Kefir starter culture which is consisted of *Lactococcus lactis* ssp., *Leuconostoc* ssp., *Streptococcus thermophilus*, *Lactobacillus* ssp., kefir yeast, and kefir grain microflora according to the product information were obtained from Danisco Biolacta (Kefir DC1, Olsztyn, Poland). Sterilized bottles made of high-density polyethylene (HDPE) material were used for the preservation of the kefir samples.

2.2. Kefir Manufacture

The milk was transported to the research and development laboratory of Department of Food Engineering, Bolu Abant İzzet Baysal University. While the milk was transferred to the pasteurizer, it was filtered through cloth and steel strainer. Then, the temperature of the milk was increased to 55°C in the pasteurizer and a portion of the milk was passed through the cream separator to obtain skim milk. The resulting skimmed milk was used to adjust the fat content of the kefir milk to 3.1 %. Fat standardized milks were pasteurized at 90°C for 10 min and cooled to 28°C and starter culture was added to both the milks at a level of 0.0065 g L^{-1}. Afterwards, inoculated milk was filled into the sterilized HDPE bottles (1 L). All procedures were carried out aseptically as much as possible. After inoculation, kefir milk samples were incubated at 28°C until the pH reached 4.60. After incubation, kefir curd was broken by shaking down 10 times. The stirred kefir samples were kept at a refrigerator (4°C) for 35 days. The chemical, physical, biochemical, microbiological, sensorial properties and also organic acids and flavor components in kefir were determined on 1st, 7th, 14th, 21th, 28th and 35th day of storage period. Cow and goat milk kefir were coded as CK and GK, respectively.

2.3. Determination of chemical properties

Analysis of pH, titratable acidity, dry matter, fat and protein contents of kefir samples were done as described by KURT *et al*. (1993). The pH of milk and kefir samples was directly measured using a calibrated pH meter (WTW 720, Germany).

2.4. Determination of physical properties

Apparent viscosity of kefir samples was determined at 15°C, using a sine-wave vibro-viscometer SV-10 (A&D Company, Japan). The syneresis of kefir samples was determined by a procedure modified from Sodini, Montella, & Tong (2005). A sample of about 25 g of kefir (A) was centrifuged for 10 min at 1250*g* at 4°C. The expelled whey (B) was weighed. The syneresis (%) was calculated as: Syneresis (%) = (B/A)x100. A color measurement device (Konica Minolta CR400, Japan) was used to measure color values as CIE L*, a* and b* of the milk and kefir samples.

2.5. Determination of the lipolytic and proteolytic activity

The lipolysis as acid degree value (ADV) of kefir samples was determined by titrimetric method according to CASE *et al*. (1985). The proteolysis values in the milk and kefir samples were determined according to HULL (1947).

2.6. Enumeration of microorganisms

The kefir samples were dispersed and further diluents were made in Ringer solution (¼ strength, Merck, Germany). The Lactobacilli counts were determined on MRS medium under microaerophilic condition with Anaerocult C (Merck, Germany) at 30°C for 3 days (IRIGOYEN *et al*., 2005). The Lactococci counts were determined on M 17 medium under microaerophilic condition with Anaerocult C at 30°C for 2 days (IRIGOYEN *et al*., 2005). The Leuconostoc counts were determined on MSE agar, a selective medium for Leuconostoc, under aerobic condition at 25°C for 5 days (GARCÍA FONTÁN *et al*., 2006). The total mesophilic aerobic bacteria (TMAB) counts were determined on PCA agar under aerobic condition at 30°C for 2 days (MAINVILLE *et al*., 2001). The acetic acid bacteria counts were determined on APM agar under aerobic condition at 25°C for 5 days (WITTHUHN *et al*., 2005). The yeast counts were determined on YGC agar under aerobic condition at 25°C for 5 days (WITTHUHN *et al*., 2005).

2.7. Determination of organic acids

Lactic, citric, acetic, pyruvic, orotic and oxalic acid in milk and kefir samples were determined by HPLC (Perkin Elmer Flexar, USA) according to GÜZEL-SEYDİM (2000b) with some modifications. Four grams of the milk was diluted into 25 mL of 0.013N H_2SO_4 while four grams of kefir samples were diluted into 25 mL of 0.010N H_2SO_4. The mixture was vortexed for 1 min and centrifuged at 7000 *g* at 4°C for 7 min. The supernatant was passed through filter having a pore diameter of 0.45 μm (Millipore) and put into vial. The organic acids were separated in an isocratic system using a C 18 column (Perkin Elmer, 5μm, 250mm x 4.6mm i.d) at a column oven temperature of 50±1°C. The wavelengths of the UV detection were performed at 210 nm using Photodiode Array Detector. The mobile phase was carried out with 10 mM KH_2PO_4 adjusted to pH 2.5 with phosphoric acid. The flow rate of the mobile phase was adjusted at 0.5 mL min^{-1}. The amount of organic acids was determined on standard curves of individual organic acids.

2.8. Determination of volatile flavor components

The acetaldehyde, diacetyl, acetoin and ethanol contents of the kefir samples were analyzed at the research center of YENİGIDAM of Bolu Abant İzzet Baysal University, according to the method given by GÜZEL-SEYDİM *et al*. (2000b). The volatile flavor components in the product were determined by GC (Shimadzu GC 2010, Japan) with Flame Ionization Detector. The kefir samples (5 g) were weighed into 20 mL headspace vials. The prepared vials were heated in a dry block heater at 85°C for 5 min. At the end of the heating period, the air in the headspace of the vial using a gas-tight syringe (Supelco) was injected into the capillary column (Agilent, DB-23, 0.25 id 0.25mm x 60m). The column oven temperature was maintained at 40°C for 1 min and then, increased to 250°C and kept at this temperature for a further 3 minutes. The temperature rise time was 35 minutes. The

carrier gas was helium and the flow rate was 0.6 mL min^{-1}. The amount of the volatile components was determined on standard curves of individual volatile components.

2.9. Determination of sensory properties

Sensory evaluation of the samples was determined by modifying the hedonic scale system (DRAKE, 2009) and was performed by 11 trained panelists. The scoring was based on 5 points in three categories: 1) structure, consistence and texture, 2) taste and smell and 3) general appreciation.

2.10. Statistical Analysis

The CK and GK samples were manufactured two times and all the analyses were performed in two parallels. The differences between the characteristics of cow and goat milk kefir samples were determined by t-test and Mann Whitney U test according to the availability of the dependent variable in each group showing normal distribution. The changes occurred in the kefir samples during storage were analysed by one-way ANOVA based on the Tukey HSD test (DEVORE and PECK, 1993). Statistical analysis of all obtained data was performed with SPSS 20.0 package program (SPSS Inc., Chicago, IL) at the significance level 0.05.

3. RESULTS AND DISCUSSION

Some properties of both cow and goat milk used in kefir production were presented in Table 1. As seen from the table, dry matter, fat and protein contents of them were close to each other. The pH, viscosity and lipolysis values of goat milk were higher than that of cow milk. Moreover, L* value (lightness) of goat milk was higher than the value of cow milk. Goats convert carotene from green fodder completely into vitamin A, and therefore color of goat milk is whiter than cow milk (GÜRSOY, 2007). The tyrosine value was higher in cow milk.

Table 1. Some properties of standardized milk used in the study (n=2).

Analyses	Cow milk ($\bar{x}$)	Goat milk ($\bar{x}$)
Dry matter (%)	11.04	11.09
Fat (%)	3.00	3.06
Protein (%)	2.96	3.02
pH	6.60	6.64
Acidity (LA, %)	0.16	0.15
Viscosity (mPa.s)	2.032	2.064
Color L*	79.378	79.727
Color a*	-3.212	-3.121
Color b*	5.482	5.911
Lipolysis (meq KOH/100 g fat)	0.359	0.935
Proteolysis (mg tyrosine/5 mL milk)	0.171	0.143

$\bar{x}$: Mean of two repetitions, n: number of repetitions L*: lightness (0= black, 100= white), a*: green (-) or red (+), b*: blue (-) or yellow (+).

3.2. Chemical changes

Some chemical changes in the kefir samples made from cow and goat milk during 35-day storage were given in Table 2. The kefir samples made from goat milk (GK) had higher general mean dry matter content than the kefir samples made from cow milk (CK) ($P<0.05$). Similar results were observed in cow kefir (KAVAS, 2015) and goat kefir samples (KACZYŃSKİ *et al*., 2018). During storage, dry matter contents in kefir samples did not change ($P>0.05$). A similar result was obtained by ERTEKIN and GÜZEL-SEYDiM (2010).
There was no difference ($P>0.05$) between the general mean fat values of CK and GK samples. This was thought to be related with the standardization of the fat content of the milk used in the production of both samples. During storage, fat contents in kefir samples did not change ($P>0.05$).
The general mean protein contents of CK and GK samples were close to each other ($P>0.05$). During the storage, the change in protein values of kefir samples was found to be significant ($P<0.05$) in GK samples, but not significant ($P>0.05$) in CK samples.
The acidity as lactic acid (%) was higher in CK than GK samples in general ($P<0.05$). The reason for the slower development of acidity in GK samples compared to the CK sample might be related to the fact that the non-protein nitrogen content of goat milk and its buffering capacity are higher than cow milk (TRATNIK *et al*., 2006). In general, the acidity increased during storage and the change was significant ($P<0.05$) in both samples.
It was observed that the pH values of the CK samples were lower than that of the GK samples ($P<0.05$). The pH values of CK samples were like those determined by ERTEKIN and GÜZEL-SEYDİM (2010) while the pH value of GK samples were similar to those reported by KACZYŃSKI *et al*. (2018). This is related to the high buffering capacity of goat milk (TRATNIK *et al*., 2006). The pH value of the samples on the first day of storage was lower than 4.60. This decline was due to the lactic acid bacteria in the sample continued to produce lactic acid from lactose while the internal temperature of the samples was reaching from incubation temperature (28°C) to 4°C. The pH value of CK samples decreased until the 14th day of storage, there was a slight increase on the 21st day of storage, and then decreased again until the 35th day of storage. In the case of GK samples, the pH decreased until the 14th day of storage and then increased slightly up to 35th day of storage. In both kefir samples, the difference between day 1 and day 35 of storage was significant ($P<0.05$). The pH value of kefir did not change very fast during storage. This is related to yeasts found in kefir (O'BRIEN *et al*., 2016). Some yeast species assimilate lactic acid and cause to rise pH (RATTRAY and O'CONNELL, 2011).

3.3. Physical properties

The changes in some physical properties of the kefir samples during storage and statistical analysis results of these changes were given in Table 3. The general mean viscosity value of CK samples (64.80 mPa.s) was higher than GK samples (9.46 mPa.s) and the difference was significant ($P<0.05$). The viscosity value of CK samples was like those determined by YILDIZ-AKGÜL *et al*. (2018) while the viscosity value of GK samples was similar to those reported by GÜNEŞER and KARAGÜL-YÜCEER (2010). Similarly, TRATNIK *et al*. (2006) and GÜNEŞER and KARAGÜL-YÜCEER (2010) reported that the viscosity values of kefir samples made from goat milk were lower than the viscosity values of kefir samples made from cow milk. The reason for this is that the main fraction of goat milk casein is β-casein while the main fraction of cow milk casein is α_{s1}- and β-casein (HUMA *et al*., 2018). Goat milk protein micelles form softer and more brittle gel. As a result, weak texture occurs in

goat fermented dairy products (GÜNEŞER and KARAGÜL-YÜCEER, 2010). In addition, it has been thought that the difference between the acidity values of the samples affect viscosity of the samples. In general, viscosity values of both CK samples ($P<0.05$) and GK samples ($P>0.05$) increased during storage. Variability in viscosity during storage might be related to the production of exopolysaccharides and degradation of exopolysaccharides into monomers by microorganisms and enzymes in kefir.
The general mean syneresis value of GK samples was higher than that of CK samples ($P<0.05$). Compositional differences of goat milk such as low α_{s1}-casein concentration and smaller diameter fat globule lead to softer structure in fermented dairy products made from goat milk and more syneresis in these products (MILANI and WENDORFF, 2011). The changes in syneresis values during storage were found to be nonsignificant ($P>0.05$) in GK samples and significant ($P<0.05$) in CK samples. There was an inverse relationship between syneresis and viscosity values of the kefir samples during storage.
The general mean L*, a* (negative) and b* values of CK samples were higher than that of GK samples and the difference was significant ($P<0.05$). L* values of the kefir samples were higher than L* values of milk used in the production of the samples. In general, the L* values of both kefir samples tended to decrease during storage. The changes in L* and b* values of CK and GK samples was found to be significant ($P<0.05$) during storage. During storage, a* values of CK and GK samples showed fluctuant. However, this change was not significant ($P>0.05$). GUL *et al*. (2018) reported that milk variety has an effect on a* and b* values while no effect L* value.

3.4. Lipolysis and proteolysis

The changes in lipolysis and proteolysis of the kefir samples during storage and statistical analysis results of these changes were shown in Table 4. The lipolysis values of GK samples were higher than CK samples ($P<0.05$) and this may be related with some post-milking issues, such as milking time and storage type of goat milk. Because, when examining Fig. 1, it was seen that the amount of lactic and acetic acids in goat milk was higher than cow milk. This suggested that goat milk had been exhibited high lipolytic activity before reaching to the laboratory. WSZOLEK *et al*. (2001) reported that milk type used in the production of kefir influences the amount of free fatty acids. The lipolysis values of CK and GK samples increased during storage ($P<0.05$). CAIS-SOKOLIŃSKA *et al*. (2008) reported similar results.
The general mean values of proteolysis were found to be 0.597 mg tyrosine $5g^{-1}$ kefir for CK samples and 0.397 mg tyrosine $5g^{-1}$ kefir for GK samples ($P<0.05$) and this might be because of the number of microorganisms in kefir, especially the number of lactic acid bacteria. Microorganisms can obtain the amino acids needed from proteins and peptides through proteolytic systems (DİNKÇİ *et al*., 2015). Proteolysis values of CK and GK samples increased during storage and the increase was significant ($P<0.05$). DİNKÇİ *et al*. (2015) reported that proteolytic activity increased as long as storage time increased, and storage time affected proteolytic activity.

Table 2. Chemical changes in cow and goat kefir samples during storage.

Properties	Kefir	Storage time (Days) ($\bar{x}$±SD) (n=2)						
		1	7	14	21	28	35	General mean
Drymatter (%)	CK	10.78±0.006^{a*}	10.76±0.041^{a}	10.73±0.027^{a}	10.73±0.065^{a}	10.74±0.057^{a}	10.72±0.011^{a}	**10.74±0.038^{B*}**
	GK	11.13±0.029^{a}	11.14±0.011^{a}	11.19±0.062^{a}	11.15±0.044^{a}	11.09±0.092^{a}	11.13±0.008^{a}	**11.14±0.048^{A}**
Fat (%)	CK	3.10±0.071^{a}	3.14±0.159^{a}	3.09±0.059^{a}	3.12±0.057^{a}	2.94±0.035^{a}	3.01±0.124^{a}	**3.07±0.100^{A}**
	GK	3.18±0.035^{a}	3.15±0.005^{a}	3.15±0.071^{a}	3.13±0.009^{a}	3.13±0.053^{a}	3.04±0.018^{a}	**3.13±0.054^{A}**
Protein (%)	CK	3.10±0.013^{a}	3.10±0.020^{a}	3.09±0.000^{a}	3.06±0.047^{a}	3.07±0.008^{a}	3.06±0.002^{a}	**3.08±0.026^{A}**
	GK	3.06±0.003^{c}	3.10±0.003^{a}	3.10±0.009^{a}	3.10±0.004^{a}	3.09±0.012^{ab}	3.07±0.002^{bc}	**3.09±0.018^{A}**
Acidity (%)	CK	0.87±0.006^{c}	0.87±0.006^{bc}	0.90±0.003^{b}	0.94±0.001^{a}	0.94±0.000^{a}	0.95±0.013^{a}	**0.91±0.034^{A}**
	GK	0.80±0.003^{b}	0.82±0.003^{ab}	0.84±0.017^{a}	0.84±0.004^{a}	0.84±0.003^{a}	0.82±0.066^{ab}	**0.82±0.019^{B}**
pH	CK	4.31±0.011^{a}	4.27±0.020^{ab}	4.26±0.001^{bc}	4.27±0.009^{ab}	4.23±0.007^{bc}	4.21±0.014^{c}	**4.26±0.033^{B}**
	GK	4.47±0.002^{a}	4.44±0.009^{ab}	4.38±0.014^{c}	4.40±0.014^{bc}	4.41±0.007^{bc}	4.42±0.012^{bc}	**4.42±0.030^{A}**

CK: Cow kefir, GK: Goat kefir, $\bar{x}$: Mean, n: Number of repetitions, SD: Standard deviation, ^{a,b}: Means in each row show statistically difference (P>0.05 or P<0.05) among storage days for each property of the samples. ^{A,B}: Means in the same column show statistically difference between kefir samples in terms of related property (P<0.05).

Table 3. Physical changes in cow and goat kefir samples during storage.

Properties	Kefir	Storage time (Days) ($\bar{x}$±SD) (n=2)						
		1	7	14	21	28	35	General mean
Viscosity (mPa.s)	CK	42.17±2.165^{c*}	52.12±1.539^{c}	48.43±0.798^{c}	64.62±9.117^{bc}	93.23±10.438^{a}	88.24±3.906^{ab}	**64.80±20.916^{A*}**
	GK	8.44±0.188^{a}	8.58±0.119^{a}	10.37±0.268^{a}	9.66±0.285^{a}	10.13±0.502^{a}	9.61±1.493^{a}	**9.46±0.904^{B}**
Syneresis (%)	CK	46.26±0.837^{a}	42.42±0.338^{cd}	45.30±0.414^{ab}	43.83±0.219^{bc}	41.49±0.417^{d}	43.68±0.605^{bc}	**43.83±1.723^{B}**
	GK	62.04±0.250^{a}	61.58±0.799^{a}	55.61±1.848^{a}	59.19±0.609^{a}	58.80±0.219^{a}	60.80±4.270^{a}	**59.67±2.676^{A}**
Color L*	CK	82.810±0.057^{a}	82.723±0.025^{ab}	82.680±0.042^{ab}	82.583±0.081^{b}	82.530±0.050^{b}	82.685±0.057^{ab}	**82.668±0.104^{A}**
	GK	81.849±0.014^{a}	81.498±0.122^{b}	81.606±0.049^{ab}	81.562±0.064^{ab}	81.549±0.110^{ab}	81.447±0.035^{b}	**81.585±0.145^{B}**
Color a*	CK	-3.298±0.032^{a}	-3.338±0.032^{a}	-3.258±0.004^{a}	-3.318±0.110^{a}	-3.228±0.025^{a}	-3.358±0.025^{a}	**-3.299±0.060^{B}**
	GK	-3.103±0.040^{a}	3.047±0.013^{a}	-3.189±0.014^{a}	-3.189±0.023^{a}	-3.152±0.030^{a}	-3.174±0.077^{a}	**-3.142±0.062^{A}**
Color b*	CK	6.535±0.000^{c}	6.640±0.028^{bc}	6.743±0.025^{ab}	6.815±0.028^{a}	6.63±0.046^{bc}	6.830±0.035^{a}	**6.700±0.112^{A}**
	GK	5.741±0.191^{ab}	5.535±0.052^{b}	5.878±0.032^{ab}	5.960±0.042^{a}	5.891±0.044^{a}	5.804±0.043^{ab}	**5.801±0.157^{B}**

CK: Cow kefir, GK: Goat kefir, $\bar{x}$: Mean, n: number of repetitions, SD: Standard deviation, ^{a,b}: Means in each row show statistically difference (P>0.05 or P<0.05) among storage days for each property of the samples. ^{A,B}: Means in the same column show statistically difference between kefir samples in terms of related property (P<0.05). L*: lightness (0= black, 100= white), a*: green (-) or red (+), b*: blue (-) or yellow (+)

Table 4. Biochemical changes in cow and goat kefir samples during storage.

Properties	Kefir	Storage time (Days) ($\bar{x}$SD) (n=2)						
		1	7	14	21	28	35	General mean
Lipolysis (meqKOH 100g fat^{-1})	CK	0.45±0.016^{b*}	0.46±0.012^{b}	0.73±0.045^{ab}	1.03±0.018^{ab}	1.16±0.209^{a}	1.23±0.310^{a}	**0.84±0.349^{B*}**
	GK	0.99±0.002^{c}	1.12±0.050^{c}	1.33±0.001^{b}	1.63±0.000^{a}	1.43±0.086^{b}	1.42±0.060^{b}	**1.32±0.224^{A}**
Proteolysis (mg tyrosine 5g kefir^{-1})	CK	0.440±0.019^{d}	0.489±0.045^{cd}	0.582±0.031^{bc}	0.624±0.009^{b}	0.645±0.013^{b}	0.804±0.006^{a}	**0.597±0.124^{A}**
	GK	0.353±0.018^{b}	0.388±0.012^{ab}	0.412±0.014^{a}	0.407±0.009^{a}	0.404±0.009^{a}	0.422±0.006^{a}	**0.397±0.025^{B}**

CK: Cow kefir, GK: Goat kefir, $\bar{x}$: Mean, n: number of repetitions, SD: Standard deviation, ^{a,b}: Means in each row show statistically difference (P>0.05 or P<0.05) among storage days for each property of the samples. ^{A,B}: Means in the same column show statistically difference between kefir samples in terms of related property (P<0.05).

3.5. Microbial Profile

The changes in some microbial properties of the kefir samples during storage and statistical analysis were shown in Table 5. As seen from the table, there was no significant ($P>0.05$) difference in the counts of lactococci between the samples of CK and GK. Lactococci counts of the samples were consistent with the results obtained by WSZOLEK *et al*. (2001). The highest number of lactococci (~9 log cfu g^{-1}) was determined on the first day of storage in both kefir samples. The change in lactococci count between the 1st and 7th days of storage was significant in CK samples ($P<0.05$). In GK samples, the change in the lactococci count was statistically significant between the first and last day of storage ($P<0.05$). The number of lactococci was above 8 log units for both samples during storage. TEMIZ and KEZER (2015) reported that the decrease in the number of lactococci was less than about 1.25 log units after 28-day storage.

The general mean count of lactobacilli in CK samples was higher (6.74 log cfu g^{-1}) than that of GK samples (6.45 log cfu g^{-1}) and the difference was significant ($P<0.05$). WSZOLEK *et al*. (2001) reported the similar results. Lactobacilli counts of CK and GK samples decreased during storage ($P<0.05$). It was determined that 2.7 log-decrease in CK samples and 2.4 log-decrease in GK samples occurred on the 7th day of storage ($P<0.05$). IRIGOYEN *et al*. (2005) and GRØNNEVIK *et al*. (2011) reported that there was an approximately 2 log decrease in the number of lactobacilli in kefir samples on day 28 of storage when compared with the first day of storage.

The general mean count of leuconostocs of CK samples (6.74 log cfu g^{-1}) was higher than that of GK samples (6.50 log cfu g^{-1}), but not significant ($P>0.05$). WSZOLEK *et al*. (2001) reported the similar findings. The leuconostoc counts of the kefir samples decreased during storage and the decrease was significant ($P<0.05$). A statistically significant decrease was found in the leuconostoc count of CK samples on the 7th day of storage. On the other hand, a statistically significant decrease was found on the 1st, 7th and 14th days of storage of GK samples. At the end of storage, the number of leuconostoc of both kefir samples was over 6 log units. Some researchers reported that the number of leuconostoc in the kefir samples decreased below 6 log units at the end of storage (GRØNNEVIK *et al*., 2011; GUL *et al*., 2015).

The general mean counts of TMAB of each sample were found close to each other ($P>0.05$). TMAB counts of both samples decreased throughout storage time and the decrease was significant ($P<0.05$) for CK samples but not significant ($P>0.05$) for GK samples. In CK samples, the decrease was statistically significant between day 1st and day 14th of storage ($P<0.05$). TEMIZ and KEZER (2015) reported that storage time had effect on TMAB counts.

It was determined that the general mean counts of AAB of CK samples was higher than that of GK samples and this was significant ($P<0.05$). The similar findings were reported by IRIGOYEN *et al*. (2005). During storage, the number of AAB decreased in both samples ($P<0.05$). Approximately 1.2 log reduction ($P<0.05$) occurred between the 1st and 14th days of storage in GK samples. However, the counts of AAB in the GK samples remained almost constant ($P>0.05$) from the 14th day of storage to the end of storage. LEITE *et al*. (2013) reported a 0.6 log unit reduction in the counts of AAB during 28-day storage.

The general mean counts of yeast of CK and GK samples were 1.95 and 1.10 log cfu g^{-1}, respectively and the difference between them was significant ($P<0.05$). In general, kefir contains yeasts between 3-6 log cfu g^{-1} (ERTEKİN and GÜZEL-SEYDİM, 2010; DİNKÇİ *et al*., 2015).

Table 5. Microbial changes in cow and goat kefir samples during storage.

Properties	Kefir	Storage time (Days) ($\bar{x}$SD) (n=2)						
		1	7	14	21	28	35	General mean
Lactococci (log cfu g^{-1})	CK	9.23±0.057^{a*}	8.64±0.201^{b}	8.54±0.062^{b}	8.60±0.108^{b}	8.49±0.070^{b}	8.55±0.037^{b}	**8.67±0.275^{A*}**
	GK	8.98±0.008^{a}	8.65±0.054^{ab}	8.78±0.038^{ab}	8.68±0.154^{ab}	8.77±0.116^{ab}	8.49±0.043^{b}	**8.72±0.168^{A}**
Lactobacilli (log cfu g^{-1})	CK	9.04±0.163^{a}	6.33±0.341^{b}	6.28±0.031^{b}	6.22±0.047^{b}	6.26±0.003^{b}	6.28±0.151^{b}	**6.74±1.082^{A}**
	GK	8.62±0.054^{a}	6.20±0.237^{b}	5.96±0.054^{b}	5.96±0.136^{b}	5.96±0.170^{b}	6.01±0.143^{b}	**6.45±1.022^{B}**
Leuconostoc (log cfu g^{-1})	CK	8.33±0.231^{a}	6.53±0.112^{b}	6.41±0.082^{b}	6.39±0.011^{b}	6.42±0.023^{b}	6.35±0.364^{b}	**6.74±0.759^{A}**
	GK	8.44±0.001^{a}	6.61±0.103^{b}	5.92±0.108^{c}	6.01±0.067^{c}	5.94±0.126^{c}	6.11±0.052^{c}	**6.50±0.938^{A}**
TMAB (log cfu g^{-1})	CK	9.24±0.107^{a}	8.66±0.229^{ab}	8.64±0.137^{b}	8.57±0.021^{b}	8.36±0.144^{b}	8.26±0.173^{b}	**8.62±0.345^{A}**
	GK	9.35±0.100^{a}	8.55±0.013^{a}	8.71±0.078^{a}	8.20±0.730^{a}	8.64±0.161^{a}	8.20±0.067^{a}	**8.61±0.466^{A}**
AAB (log cfu g^{-1})	CK	7.37±0.012^{a}	6.36±0.146^{b}	6.11±0.040^{bc}	6.02±0.010^{bc}	6.03±0.001^{bc}	5.92±0.155^{c}	**6.30±0.525^{A}**
	GK	6.98±0.378^{a}	6.12±0.308^{ab}	5.77±0.128^{b}	5.81±0.083^{b}	5.76±0.161^{b}	5.72±0.218^{b}	**6.03±0.498^{B}**
Yeast (log cfu g^{-1})	CK	2.39±0.017^{a}	2.12±0.232^{a}	1.76±0.086^{a}	1.39±0.144^{a}	1.67±0.048^{a}	2.35±0.657^{a}	**1.95±0.443^{A}**
	GK	1.81±0.096^{a}	1.47±0.172^{a}	0.94±0.060^{a}	0.67±0.180^{a}	0.78±0.042^{a}	0.93±0.690^{a}	**1.10±0.477^{B}**

CK: Cow kefir, GK: Goat kefir, $\bar{x}$: Mean, n: number of repetitions, SD: Standard deviation, TMAB: Total mesophilic aerobic bacteria, AAB: Acetic acid bacteria, ^{a,b}: Means in each row show statistically difference ($P>0.05$ or $P<0.05$) among storage days for each property of the samples. ^{A,B}: Means in the same column show statistically difference between kefir samples in terms of related property ($P<0.05$).

However, some researchers reported yeast count between 0.5-3 log cfu g^{-1} during storage in kefir samples produced from cow and goat milk by using different commercial starter cultures (WSZOLEK *et al*., 2001; GARCIA FONTÁN *et al*., 2006; KESENKAŞ *et al*., 2011). In Codex standard for fermented milks (CODEX STAN 243-2003), it is stated that the number of yeasts in kefir should be at least 10^4 cfu g^{-1}. Yeasts produce ethyl alcohol and CO_2. The high number of yeasts in commercial kefir has caused packaging problems. In addition, due to consumer demand, low yeast counts have been generally preferred in kefir production in some countries. The difference of yeast number in kefir is varied depending on the type of culture used, the inoculation rate of the culture and the number of yeasts of the culture used. The number of yeasts in both CK and GK samples decreased until the 21st day of storage period. Then, the yeast count of the samples increased towards the end of the storage. During storage, the number of yeasts in both CK and GK samples was less than 2.5 log. The yeast count of CK samples was higher than that of GK samples during storage. Decrease in yeast count of both samples was nonsignificant ($P>0.05$) during storage DİNKÇİ *et al*. (2015) reported that storage time had no effect on yeast number.

3.6. Organic acid content

The changes in some organic acid contents of the kefir samples during storage were shown in Fig. 1.

General average lactic acid content in GK samples was greater than that of CK samples (Fig. 1a) and this was significant ($P<0.05$). TÜRKER *et al*. (2014) found that the lactic acid content of kefir samples from goat milk was higher than that of kefir samples from cow milk. The changes of lactic acid content in CK and GK samples were statistically significant ($P<0.05$) during storage. It was believed that fluctuations in the amount of lactic acid during storage were related to the amount of lactic acid produced by lactic acid bacteria and the assimilation of lactic acid by some yeast species (RATTRAY and O'CONNELL, 2011).

The average acetic acid content of GK samples was approximately 4 times higher than the values of CK samples (Fig. 1a) ($P<0.05$). TÜRKER *et al*. (2014) and GUL *et al*. (2015) reported that milk variety affected the amount of acetic acid in kefir. The acetic acid content of CK and GK samples were similar with values determined by MUIR *et al*. (1999) and GRØNNEVIK *et al*. (2011). The acetic acid content of CK samples was highest on day 1st of storage. The difference in the amount of acetic acid between the 1st and 7th days of storage was significant ($P<0.05$). GRØNNEVIK *et al*. (2011) reported that the change in the amount of acetic acid of kefir samples during storage was not significant. This was thought to be related to the fact that acetic acid is an intermediate product (LEITE *et al*., 2013).

The cow and goat milk used for kefir production in this study showed citric acid content of 1175 $\mu g\ g^{-1}$ and 433 $\mu g\ g^{-1}$, respectively. While the average amount of citric acid was 108.34 $\mu g\ g^{-1}$ in the GK samples, CK samples contained no citric acid (Fig. 1b). Citrate is a preferred substrate for the formation of acetoin and diacetyl by some lactic acid bacteria (GÜZEL-SEYDiM *et al*., 2000a). GRØNNEVIK *et al*. (2011) reported that there was more than 90 % reduction in the amount of citrate in kefir milk during fermentation and it was converted to other volatile components. ISMAIEL *et al*., (2011) could not detect citric acid in kefir produced under different fermentation conditions. The amount of citric acid in the GK samples increased up to the 14th day of storage ($P<0.05$), then suddenly decreased on the 21st day ($P<0.05$) and reached 35 $\mu g\ g^{-1}$. Although there was an increase in the amount of citric acid from the 21st day of storage to the end of storage, this increase was not

significant (P>0.05). KESENKAŞ *et al*. (2011) reported that the storage time affected the amount of citric acid in kefir.

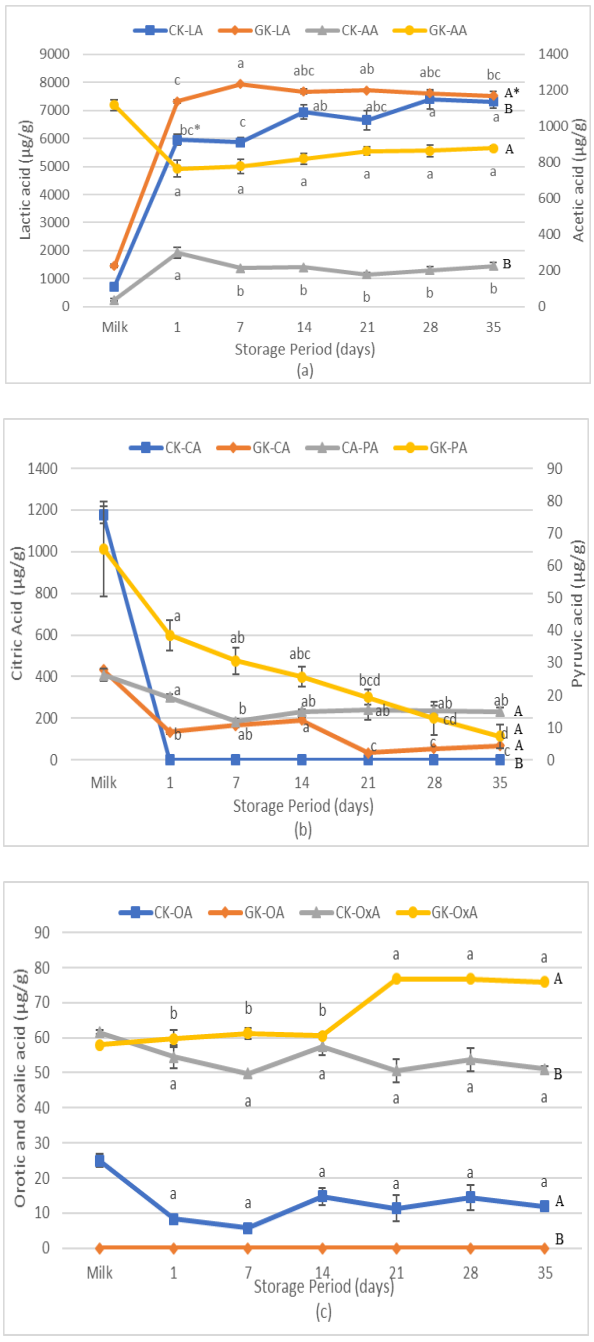

Figure 1. Lactic and acetic acid (a), citric and pyruvic acid (b), and orotic and oxalic acid (c) content in cow and goat kefir samples during storage (CK: Cow kefir, GK: Goat kefir, LA: Lactic acid, AA: Acetic acid, CA: Citric acid, PA: Pyruvic acid, OA: Orotic acid, OxA: Oxalic acid, ^{a,b,c,d}: show statistically difference (P>0.05 or P<0.05) among storage days for each property of the samples. ^{A,B}: show statistically difference between kefir samples in terms of related property (P<0.05).)

General mean pyruvic acid content of GK samples (22.39 $\mu g\ g^{-1}$) was higher than that of CK samples (15.31 $\mu g\ g^{-1}$) (P>0.05) (Fig. 1b). Pyruvic acid levels of both CK and GK samples were lower than the milk used in the production. MUIR *et al*. (1999) reported higher amounts in traditional and commercial kefir (47 and 60 $\mu g\ g^{-1}$). GÜZEL-SEYDİM *et al*. (2000b) and BESHKOVA *et al*. (2003) reported that pyruvic acid was completely consumed during storage. The amount of pyruvic acid significantly (P<0.05) decreased in both kefir samples during storage. Reduction in pyruvic acid might be due to the conversion to other organic compounds during storage (GÜZEL-SEYDİM *et al*., 2000b).
Orotic acid content was determined as 25 $\mu g\ g^{-1}$ in cow milk used in the production of CK samples and as 5.71-14-74 $\mu g\ g^{-1}$ in CK samples during storage (Fig. 1c). The values of CK samples were lower than findings of MUIR *et al*. (1999). On the other hand, no orotic acid was detected in both goat milk used in the production of the GK samples and GK samples during storage. In a study, while the amount of orotic acid decreased during fermentation of kefir, it increased during storage (GÜZEL-SEYDİM *et al*., 2000a,b). During storage time, no significant changes were observed in CK samples (P>0.05).
Overall mean content of oxalic acid of the GK samples was higher (P<0.05) than that of the CK samples (Fig. 1c). ISMAIEL *et al*. (2011) could not detect oxalic acid in kefir produced under different fermentation conditions. TÜRKER *et al*. (2014) reported that milk variety affects the amount of oxalic acid in kefir. oxalic acid might be found up to 169.15 mg L^{-1} in cow kefir and to 119.37 mg L^{-1} in goat kefir. During storage, fluctuations in the amount of oxalic acid in the kefir samples were determined. The changes in CK samples were not significant (P>0.05) during storage. In GK samples, the increase in the amount of oxalic acid on day 21st of storage was significant (P<0.05).

3.7. Aroma content

The changes in acetaldehyde, diacetyl, acetoin and ethanol content in the kefir samples during storage were shown in Fig. 2. The general mean value of acetaldehyde of CK samples was relatively higher, but insignificant (P>0.05). WSZOLEK *et al*. (2001) reported similar results. During storage, the amount of acetaldehyde decreased in both samples and the values were between 1.52-2.92 $\mu g\ g^{-1}$ in CK samples and 1.83-2.44 $\mu g\ g^{-1}$ in GK samples. These values were in agreement with values obtained by GRØNNEVIK *et al*. (2011). Differences in the amount of acetaldehyde in kefir may vary depending on milk fat ratio, starter culture type, the microbial diversity of the culture, the rate of use of culture, temperature and duration of incubation and storage (ERTEKIN and GÜZEL-SEYDIM, 2010; YILDIZ-AKGÜL *et al*., 2018). The changes in acetaldehyde values of kefir samples during storage were found to be significant only in the CK samples (P<0.05). BESHKOVA *et al*. (2003) reported a similar trend in amount of acetaldehyde during storage. The decrease in the amount of acetaldehyde is related to the conversion of acetaldehyde to ethyl alcohol by the enzyme, which called alcohol dehydrogenase.
Diacetyl content of the CK samples was higher than that of GK samples (Fig. 2a) (P<0.05). Diacetyl values obtained in this study were in accordance with the values determined by WSZOLEK *et al*. (2001) and BESHKOVA *et al*. (2003). In some studies, diacetyl was not detected during fermentation and storage of kefir (GÜZEL-SEYDİM *et al*., 2000a,b). During storage, the amount of diacetyl in CK samples increased up to the 14th day of storage and thereafter declined until the end of storage. In the GK samples, it increased until the 21st day of storage and decreased in the following days. The change in diacetyl values of kefir samples during storage was found to be significant (P<0.05) only in the CK samples. It has been reported that the optimum flavour balance for kefir is to be achieved when the ratio

of diacetyl to acetaldehyde is 3:1 (GRØNNEVIK *et al*., 2011). The highest ratio of diacetyl to acetaldehyde during storage was determined to be on the 14th day in the CK samples and on the 21st day in the GK samples. However, these rates were below 3. Actually, some of researchers found this ratio between 0-2 in their studies (WSZOLEK *et al*., 2001; GRØNNEVIK *et al*., 2011; YILDIZ-AKGÜL *et al*., 2018).

Acetoin content was determined to be between 13.86-17.26 $\mu g\ g^{-1}$ in the CK samples and 7.81-9.00 $\mu g\ g^{-1}$ in the GK samples during storage (Fig. 2b). General mean value of acetoin of CK samples was higher than that of GK samples ($P<0.05$). The values of the CK samples were similar with the values obtained (16-25 $\mu g\ g^{-1}$) by GÜZEL-SEYDİM *et al*. (2000b). BESHKOVA *et al*. (2003) were unable to detect acetoin in kefir samples in their study. Acetoin value of the samples tended to decrease during storage. The change in the amount of acetoin was significant ($P<0.05$) only in CK samples especially on day 21 during storage. GRØNNEVIK *et al*. (2011) reported that the amount of acetoin formed in kefir samples decreased during storage. The decrease in the amount of acetoin may be associated with further degradation, which results in 2.3-butanediol (GRØNNEVIK *et al*., 2011).

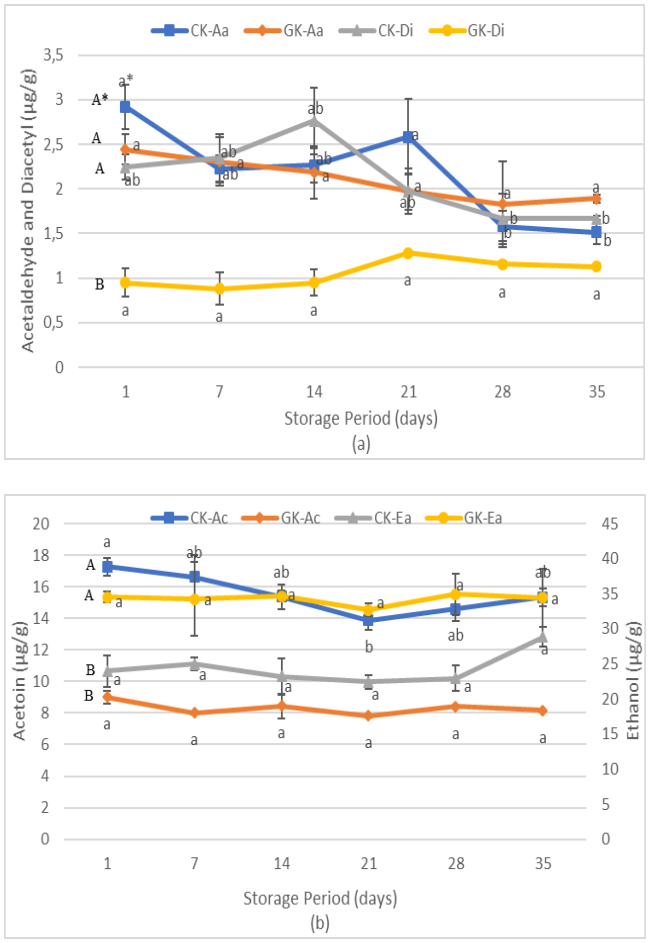

Figure 2. Acetaldehyde - diacetyl (a), acetoin - ethanol (b) contents in cow and goat kefir samples during storage (CK: Cow kefir, GK: Goat kefir, Aa: Acetaldehyde, Di: Diacetyl, Ac: Acetoin, Ea: Ethanol, ^{a,b,c,d}: show statistically difference ($P>0.05$ or $P<0.05$) among storage days for each property of the samples. ^{A,B}: show statistically difference between kefir samples in terms of related property ($P<0.05$).)

General mean value of ethanol was higher in the GK samples and this difference was significant (P<0.05) (Fig. 2b). The values obtained in this study were consistent with values reported by GRØNNEVIK *et al*. (2011) and GUL *et al*. (2015). Yeasts are primarily responsible for alcohol production in kefir, and some heterofermentative lactobacilli, such as *Lactobacillus kefir*, can also produce ethanol (GÜZEL-SEYDİM *et al*., 2000a; RATTRAY and O'CONNELL, 2011). In some studies, ethanol content in kefir was determined to vary between 0-9700 $\mu g\ g^{-1}$ (GÜZEL-SEYDİM *et al*., 2000b; WSZOLEK *et al*., 2001; GARCÍA FONTÁN *et al*., 2006; PURNOMO and MUSLIMIN, 2012; TEMIZ and KEZER, 2015; YILDIZ *et al*., 2018). The amount of ethanol in kefir varies greatly depending on the type of culture used, the inoculation rate of the culture and the microbial diversity of the culture. While a considerable change did not occur in the amount of ethanol in the CK samples until the 28th day of storage period, a sudden increase happened on the last day. The amount of ethanol in the GK samples did not change much during storage. Changes in the amount of ethanol in both samples were not significant (P>0.05). The increase in ethanol content of the CK samples on the last day of storage can be attributed to the high number of yeasts on the same day. WSZOLEK *et al*. (2001) found that storage time did not affect the amount of ethyl alcohol in kefir samples, like our results.

3.8. Sensory properties

The overall average scores of structure, consistence and texture, taste and smell, and general appreciation of CK samples was higher than the average values of GK samples (Fig. 3). While the difference between the structure, consistence and texture, and general appreciation scores of both samples were significant (P<0.05), the difference between taste and smell scores were nonsignificant (P>0.05). In terms of all sensory characteristics, the highest sensory scores were obtained on the 14th day of storage in the CK samples while the lowest sensory scores were on the 35th day of storage. The highest sensory scores were obtained on the 21st day of storage in the GK samples and lowest sensory scores were obtained on the 7th day of storage. In both samples, the change in sensory properties during storage period was significant (P<0.05) except for taste and smell characteristic of GC sample. Panelists reported that the GK samples had low consistence, did not give full sensation in the mouth and showed distinct the goat smell.

Figure 3. Sensory changes in cow and goat kefir samples during storage.

4. CONCLUSIONS

Based on the results of this study, it can be said that kefir samples produced from both cow and goat milk can be stored up to 35 days at refrigerator temperature. All samples had sensory scores above 3 during storage. However, the highest scores by sensory analyses and the highest diacetyl/acetaldehyde ratio in the kefir samples were obtained on the 14th day of storage in CK samples and the 21st day of storage in GC samples.

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REVIEW

ISOTOPE ANALYSIS AS A MEANS OF TRACING AQUATIC PRODUCTS AUTHENTICITY, SOURCE AND GEOGRAPHIC ORIGINS

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ABSTRACT

Aquatic products provide good sources of high-quality protein for humans. Tracing the origin of aquatic products is of critical importance both for consumers and suppliers. In recent years, isotope analysis is becoming a key instrument in food products authentication. This work reviews the use of isotope analysis to trace the production sources (wild or farmed) and geographic origins of aquatic products. Extensive research has studied the isotope values of freshwater fish to Atlantic salmon, sea bass, rainbow trout, and other commercial fish and shellfish. Generally, the ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes were successfully investigated in aquatic products in order to identify the production methods or geographic origins. However, the predictable confidence of isotope analysis can be enhanced in combination with other analytical techniques, such as fatty acids and multi-element profiling. Moreover, future research to combine isotope analysis with data fusion and multivariate data evaluation is recommended.

Keywords: food fraud, provenance, fish, seafood, isotopic abundance

1. INTRODUCTION

Aquatic products provide good sources of high-quality protein for humans (SAPKOTA *et al.*, 2008). According to the Food and Agriculture Organization's annual State of World Fisheries, total world fish production (capture and aquaculture, excluding aquatic plants) peaked at about 171 million tons in 2016, and is expected to reach 201 million tons in 2030 (FAO, 2018). However, the growing number of seafood production and economic globalization have exerted serious pressure on the variety of food products, resulting in food fraud and adulteration (DANEZIS *et al.*, 2016a). In addition, consumers are interested in knowing the origin of aquatic products. Numerous cases have been reported with fish products labelled falsely to increase the chances of marketing and sales (FOX *et al.*, 2018).

In recent years, tracing the origin of fish has become significantly important both for consumers, producers and regulators (POSUDIN *et al.*, 2015). In particular, verifying fish origin and its label description are in compliance is given high importance (DANEZIS *et al.*, 2016b). Traditionally, food authentication has been verified using several methods including genomics and proteomics techniques (CERUSO *et al.*, 2019, ORTEA *et al.*, 2016), chromatographic techniques (GRANATO *et al.*, 2018), isotopic and elemental techniques (GOPI *et al.*, 2019b), vibrational and fluorescence spectroscopy (COZZOLINO, 2015, DANKOWSKA, 2016), nuclear magnetic resonance spectroscopy (STANDAL *et al.*, 2010), sensory analysis (KIANI *et al.*, 2016), immunological techniques (CARRERA *et al.*, 2014) and others (DANEZIS *et al.*, 2016a, DANEZIS *et al.*, 2016b, GOPI *et al.*, 2019a). Amongst them, isotope analysis is one of the prominent analytical techniques (DANEZIS *et al.*, 2016b), that has not been commonly used previously but gaining momentum. In particular, isotope analysis could trace the production methods (wild or farmed) and geographic locations of various species; and it is relatively cost-effective (GOPI *et al.*, 2019a).

During the last several decades, research on food authentication has focused on wine, fruit, vegetables, cereals, meat, dairy products, oils, honey, and eggs (DANKOWSKA, 2016). However, there had been little interest in fishery products, but number of studies on fishery authentication are on the rise since 2007 (DANEZIS *et al.*, 2016a). Moreover, the investigations on isotope analysis of seafood products have been propelled to the forefront due to its advantages compared with other relevant methods, such as DNA, fatty acid and elemental profiling. For instance, the results of DNA profiling can be affected by the removal or degradation of DNA into small fragments in various treatments (NOVAK *et al.*, 2007, ŞAKALAR *et al.*, 2012). Fatty acid compositions depend on variability of seasons and diets (GRIGORAKIS, 2007), and it is difficult to distinguish the fatty acids profiles of wild and cultured samples (OSTERMEYER *et al.*, 2014), or between wild and organic samples (MOLKENTIN *et al.*, 2015). On the other hand elemental profiling needs more time to prepare samples and large database to discriminate the provenance of each species (GOPI *et al.*, 2019a).

In this paper, we review isotope analysis used in aquatic products authentication, particularly focusing on the production source (wild or farmed) and geographic origins. Our emphasis here is placed on applications, methods, accuracy and productivity of current studies attempting to elucidate the traceability of aquatic food products through isotope analysis.

2. ISOTOPE RATIO ANALYSIS

Isotopes are the atoms of the same element, which have equal numbers of electrons (and protons) but different numbers of neutrons (COPLEN, 2011, KELLY *et al.*, 2005). Different isotopes of the same element possess different masses. Isotopes have two specific types: stable and unstable (radioactive isotopes). Stable isotopes do not decay into other elements. In contrast, radioactive isotopes are unstable and decay into other elements. Stable isotopes can be grouped into light and heavy elements isotopes, depending on atomic mass (DANEZIS *et al.*, 2016b).
The less abundant stable isotope(s) of an element have one or two additional neutrons than protons, and thus are heavier than the more common stable isotope. The stable isotope abundance of an element is presented in ratio form as the ratio of the heavy-to-light isotopes (e.g. $^{13}C/^{12}C$ or $^{15}N/^{14}N$). Since this ratio is small, isotope ratio analysis is normally expressed by the ratio of the heavier and the lighter isotopes to a reference compound of normal isotope ratio, which is reported in standard delta (δ) as parts per thousand (per mil, ‰) (COPLEN, 2011, KELLY *et al.*, 2005) as Eq (1):

$$\delta_{ref} = \left(\frac{R_{samp} - R_{ref}}{R_{ref}}\right) \quad (1)$$

Where δ_{ref} is the isotope ratio of the sample expressed in delta units relative to the reference material.
R_{samp} and R_{ref} are the isotope ratios of the sample and reference material, respectively.
For bio-elements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$) isotope measurements, isotopic ratios are generally investigated by isotope-ratio mass spectrometry (IRMS) (DRIVELOS and GEORGIOU, 2012). For Sr, Pb and other heavy isotopes, thermal ionization mass spectrometry (TIMS), multi-collector-inductively couple plasma mass spectrometry (MC-ICP-MS), and dynamic reaction cell- inductively couple plasma mass spectrometry (MC-ICP-MS) are used (DRIVELOS and GEORGIOU, 2012).

3. ISOTOPE ANALYSIS IN FOOD AUTHENTICATION

The increasing global trade has challenged the guarantee of food safety, transparency and protection of human health (DANEZIS *et al.*, 2016b, KELLY *et al.*, 2005). In addition, aquatic food products are highly perishable commodities and traded worldwide, which give certain difficulties for characterizing its provenance (SCHRÖDER, 2008). Therefore, it is important to verify the authenticity of the aquatic products before entering markets.
Isotopic ratios have been known to be of extreme use in food authentication because food ingredients have variety of isotopes abundance that can reflect its trophic position, food sources, geographic origin, pedology and archaeological sites (DANEZIS *et al.*, 2016b, FULLER *et al.*, 2012). Stable isotopic ratios of $\delta^{13}C$ and $\delta^{15}N$ are natural biomarkers to evaluate the effects of different preservation methods on isotopic signatures of fish tissues (ARRINGTON and WINEMILLER, 2002, KELLY *et al.*, 2006, SYVÄRANTA *et al.*, 2008), trophodynamics and food sources in time and space (FULLER *et al.*, 2012, WYATT *et al.*, 2012). Similarly, geographic origin can be recognized by hydrogen, oxygen, sulphur and strontium isotope ratios (KELLY *et al.*, 2005).
There are various issues concerning the traceability and authentication of fishery and aquatic products. Among them, species of origin (fish species), production sources (wild

or famed), and geographic origins (locations) are desirable in traceability of fishery and aquatic products (MORETTI *et al.*, 2003). Consequently, isotope ratios can be used in tracing the authenticity of production sources (wild or famed), and geographic origins of aquatic products.

3.1. Wild and farmed

To date, there are numerous studies focusing to distinguish farmed and wild seafood products, especially since the beginning of the 21st century, due to the increased concerns amongst consumers, who wants to know the origin of fishes (DANEZIS *et al.*, 2016b). The feasibility of using stable isotopes to distinguish recently escaped farmed Atlantic salmon (*Salmo salar*) to wild specimen was investigated by DEMPSON and POWER (2004). Their results showed that muscle tissue of wild salmon had significantly enriched nitrogen $\delta^{15}N$ but depleted lipid corrected carbon $\delta^{13}C'$ (the residual $\delta^{13}C$ values) than those of escaped farmed salmon. In addition, those authors assumed that the differences of isotope fractionation between farmed and wild fish could be retained depending upon the time of year that farmed fish was escaped relative to the June to August rapid growth period. Moreover, their study also supported the fact that adipose tissue can be used as non-invasive utility to determine isotope values in salmonid fishes, as the average $\delta^{13}C'$ and $\delta^{15}N$ of white muscle and adipose fin tissue varied in absolute amount by only 0.5%.

The combined measurement of $\delta^{13}C$ and $\delta^{15}N$ can be useful to differentiate the origin, farmed or wild, Brazilian fresh water fish cachara (*Pseudoplatystoma fasciatum*), but seasonal variations need to be concerned (SANT'ANA *et al.*, 2010). Farmed cachara was found to have significantly enriched $\delta^{15}N$ in rainy but not dry season, whereas $\delta^{13}C$ was found to be enhanced in both seasons. Therefore, the authors assumed that $\delta^{13}C$ is a better indicator for cachara traceability.

The basic of isotope analysis in discrimination of wild (England) and cultured (Scotland and Greece) sea bass (*Dicentrarchus labrax*) was provided by BELL *et al.* (2007). The isotopic data indicated that $\delta^{13}C$ of individual fatty acids 16:0, 18:0, 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-9, and 20:4n-6 were significantly lighter in cultivated sea bass than those of wild specimen. In addition, total flesh oil of farmed sea bass had the lighter $\delta^{13}C$ compared to that of the wild specimen, but not for the $\delta^{18}O$ of the flesh oil. It was explained by the commercial aquafeed formulations contained more terrestrial-derived raw materials such as wheat, soybean, sunflower, maize, peas and rapeseed meals. Although wild bass had a higher choline nitrogen content than cultivated bass, higher $\delta^{15}N$ of the flesh lipid total glycerol/choline fraction was observed in cultivated bass than that of wild counterpart. It may be due to the differences in growth rate and maturity of wild (1690 g) and cultivated (386 g) sea bass or seasonal variations of $\delta^{15}N$. Nevertheless, those authors stated that because their study only discriminated fish origins from three geographic locations, it warrants further studies combining isotope analysis with other analytical methodologies such as the flesh fatty acids profiles.

The study of FASOLATO *et al.* (2010) highlighted that the utility of $\delta^{13}C$ and $\delta^{15}N$ abundance to discriminate the farmed to wild European sea bass. Because $\delta^{13}C$ abundance can be affected by the variability of tissue lipid content or intramuscular fat (FOCKEN and BECKER, 1998), the $\delta^{13}C$ abundance in this study was analyzed from free-fat muscle. Similar to previous study of BELL *et al.* (2007), the $\delta^{13}C$ values of farmed sea bass were

found to be lower than wild specimens. The $\delta^{15}N$ abundance showed higher values in wild specimen owing to the higher trophic level of fish feed from the Mediterranean Sea.
In an attempt to extend more heterogeneous range of samples, sea bass (*Dicentrarchus labrax*) from 18 different Italian and southern European sources was analyzed for its production sources, wild and cultivated (intensively, semi-intensively, and extensively) by determination of $\delta^{13}C$ and $\delta^{15}N$ isotopic compositions (FARABEGOLI *et al.*, 2018). However, the results of isotopic abundance were less satisfying as there were merely slight differences in isotopic abundance of $\delta^{13}C$ and $\delta^{15}N$ between cultivated and wild fish, as well as Italian and foreign intensively reared fish. According to the authors, $\delta^{13}C$ can be affected by dietary nutrients and habitat shifts; in addition, $\delta^{15}N$ can be influenced by the trophic level of fish feed formulations. These may affect results of isotopic abundance to distinguish between wild and cultivated sea bass.
It must be noted that the specific choice of isotopic abundance element in particular fatted/defatted samples is important. For example, the sole $\delta^{13}C$ abundance in defatted dry matter could not differentiate organic from wild salmon (MOLKENTIN *et al.*, 2015). In this case, the combination of $\delta^{13}C$ in lipid samples and $\delta^{15}N$ in defatted dry matter were needed in the differentiation of organic, conventional and wild fish (MOLKENTIN *et al.*, 2015).
To search for more authenticated method, WANG *et al.* (2018) suggested to use compound-specific amino acid $\delta^{13}C$ fingerprints ($\delta^{13}C_{AA}$) on large-numbered of salmon samples, to (1) discriminate organically, conventionally aquaculture to wild fish from Pacific to Atlantic regions; and (2) detect subtle diet changes by macroalgae or insects with controlled feeding experiments. The bulk isotope values of $\delta^{13}C$ and $\delta^{15}N$ could be used to trace the salmon origins. Those authors found that bulk isotope values resulted in poor discrimination between wild and organic salmon. However, the multivariate analysis of $\delta^{13}C_{AA}$ data could separate the wild, organic and conventional salmon with high certainty, as well as distinguish diets changes among lab-cultured experimental groups, even between the green (*Ulva rigida*) and red (*Palmaria*) macroalgae inclusion groups. In addition, $\delta^{13}C$ of essential amino acids (His, Phe, Val, Ile and Leu) in salmon tissue can reflect the dietary sources, therefore they can satisfactorily differentiate fish origins.
The research of VASCONI *et al.* (2019) used protein carbon and nitrogen isotope analysis to differentiate the wild and famed European eel (*Anguilla anguilla*) from Netherland, Denmark and Italy; and different farming modes (pond, recirculating aquaculture system, lagoon and wild). Multivariate data were performed by principal component analysis and sparse partial least squares discriminant analysis. The results showed that $\delta^{13}C$ and $\delta^{15}N$ abundance can predictively differentiate lagoon and wild eels, but cannot discriminate male and female eels from Netherland and Denmark. In addition, the stable isotope analysis results can ratify only the partial of what has been demonstrated by using the fatty acids profile in this study.

3.2. Geographic origin

In recent years, there has been an increasing interest in isotope analysis to authenticate the geographic origin of fish and shellfish. In particular, the study of ORTEA and GALLARDO (2015), using stable isotope ratio and/or multi-element (Pb, Cd, As, P, S) analyses, has shed some light on not only geographic origin, but also production method, and species authentication of commercially relevant shrimps. The shrimp samples were constituted by 45 individuals of seven different species in nine different geographical

origins. Multivariate analysis were used for data classification, including principal component analysis, cluster analysis, κ-means hierarchical classification and discriminant analysis (DA). The results showed that both stable isotope ratio and multi-element analyses can enhance the prediction capabilities of chemometric technique to discriminate shrimp samples into wild/ farmed, different geographical origins or even biological species, whilst cluster analysis was not appropriate to discern the farm origins. On the contrary, it is of interest to note that KIM *et al.* (2015) stated that isotope analysis is a reliable tool to trace the origin of commercial fish (mackerel, yellow croaker and pollock). Biplot of $\delta^{13}C$ and $\delta^{15}N$ values showed that Australian and Norwegian mackerel had different spatial and trophic position than those of Chinese and Korean counterparts (KIM *et al.*, 2015). The $\delta^{13}C$ of pollock from Japan and Russia, as well as the $\delta^{15}N$ between yellow croaker from Korea and China were likely similar because the two areas are close in their geographical distance (KIM *et al.*, 2015). Those authors also reported that $\delta^{13}C$ signature can be more effective in discrimination of geographic origin due to the distinct values of $\delta^{13}C$ of the three commercial fish.

The geographic origins of commercial hake species (n=60) were evaluated by the isotopic abundance of $\delta^{13}C$ and $\delta^{15}N$ using bivariate scatter plot, principal component analysis and Euclidean hierarchical clustering analysis (CARRERA and GALLARDO, 2017). The results facilitated a clear classification of hakes from six geographic coasts: Europe, North Africa, South Africa, North America, South America, and Australia. Most importantly, the $\delta^{13}C$ signature can corroborate the clear discrimination hake species according to latitude. For example, North African and South American hakes were in the adjacent range of $\delta^{13}C$ (-14 to -16), whilst Australian and North American hakes were in the range of -18 to -20 values of $\delta^{13}C$.

To study the carbon cycle at the molecular level, compound-specific isotope analysis is used as the combination of gas chromatography and isotope-ratio mass spectrometry (LIU *et al.*, 2017, RIELEY *et al.*, 1991). Compared to the conventional isotope analysis of bulk organic carbon, this technique can reflect the material source more accurately (LIU *et al.*, 2017) and understand the carbon fluxes within bio-geochemical systems (RIELEY *et al.*, 1991). Previous studies have applied this technique concerning with source of nutrients in aquatic and terrestrial food webs (LARSEN *et al.*, 2013) and discrimination of organically, conventionally aquaculture to wild fish (WANG *et al.*, 2018). However, few studies have demonstrated the traceability of compound-specific isotope analysis on geographic origins in seafood. One of the first attempts to trace the geographic origin of seafood by this technique was investigated on sea cucumber (*Apostichopus japonicas*) in the coastal area of China (LIU *et al.*, 2017). In this study, principal component analysis (PCA) and discriminant analysis (DA) were used to support the discrimination. Although a total of 28 fatty acids was detected in the fatty acid profiles, but stable carbon isotope compositions were only obtained from 26 fatty acids. The $\delta^{13}C$ values of fatty acids in both November 2015 and April 2016 were relatively enriched in Rushan, Wafangdian and Pikou, and depleted in the Danzi Island, the Changhai Island and Muping. Principal component analysis (PCA) and discriminant analysis (DA) allowed researchers to discriminate between different geographic locations of sea cucumber; except for the Changhai and Zhangzi Island in April 2016, because the two islands are both located in the Dalian sea and have similar environmental conditions. This study was followed up with the amino acids carbon stable isotope analysis of sea cucumber in an attempt to distinguish the sub-regions that are close together (ZHAO *et al.*, 2018). Because the $\delta^{13}C_{AA}$ fingerprint can supply

the information of biosynthetic origin and carbon acquisition (SCOTT *et al.*, 2006), as well as the environmental conditions and food sources (GANNES *et al.*, 1998, MCMAHON *et al.*, 2010); this method resulted in 100% of overall correct classification rate and cross-validation rate to discriminate 8 locations of wild samples and 3 locations of cultured sea cucumber. Especially, the sole $\delta^{13}C$ values of Gly and Ser could similarly discriminate the production method (wild versus cultured) and geographic provinces of sea cucumber, respectively.

Conservation efforts aimed at tracing the seafood geographic origins also involved the multi-element isotope analysis. For tracing the geographic origins (two northern Italian regions and other Italian regions) and type of feed (high or low fish content), the relationships between $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$ values of proteins and fat fractions of rainbow trout (*Oncorhynchus mykiss*) fillet and those of feed and tank water were evaluated (CAMIN *et al.*, 2018). Compared to the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ of feed, those isotopic values of fillet proteins were enriched; and $\delta^{13}C$ values of fish fat were depleted. The partial square regression showed that the C, N and S isotopic values of fillet and fish feed were positively correlated within and between material matrixes, and negatively correlated with isotopic values of H and O of feed and H of fillet. Whereas, the isotopic signature of environment water $\delta^{18}O$ was positively correlated with $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$ of fillet. Besides that, $\delta^{18}O_{fat}$ of fillet was less significant correlated with other isotopic ratios. In addition, the partial least squares-Discriminant analysis was applied to check the traceability of two geographic origins and feed type. Fish from Friuli Venezia Giulia region was predictably traced by $\delta^{15}N_{protein}$ and $\delta^{18}O_{protein}$; the Trentino fish was marked out by $\delta^{2}H_{protein}$ and $\delta^{18}O_{protein}$; whilst fish fed with high and low fat-feed were discriminated by $\delta^{34}S_{protein}$. The discriminant multiclass model reached the average accuracy of 94%. Furthermore, the authors suggested that geographical signature is extensively influenced by the local forage of diets. Previous studies have also shown that isotope analysis is informative variable to distinguish fish products between different farms (KIM *et al.*, 2015, TURCHINI *et al.*, 2008). To be more precise, however, isotope analysis would be a perfect complement to combine with other analytical techniques (CARTER *et al.*, 2015, GOPI *et al.*, 2019b, ORTEA and GALLARDO, 2015, TURCHINI *et al.*, 2008). One possibility is the combination with fatty acids profile. In an attempt to distinguishing the geographic traceability of sea cucumber (*Apostichopus japonicas*) in seven locations of northern China sea, the stable isotopes of carbon and nitrogen compositions partially overlapped in some areas, whilst fatty acids profile alone could not discriminate all of the origins (ZHANG *et al.*, 2017). However, the combination of $\delta^{13}C$ and 14:1n-5, or $\delta^{15}N$ and 16:0 content could be used to surmount the overlap areas. Additionally, the thorough separation of seven sampling locations were achieved when stable isotopes and fatty acid compositions combined with discriminant analysis and the recognition ability was 89.1%. ZHANG *et al.* (2019) extended the isotopic analysis of scallops (*Patinopecten yessoensis, Chlamys farreri,* and *Argopecten irradians*) of fatty acid $\delta^{13}C$ fingerprinting with fatty acid profile in seven sites of China. The results showed that all scallops of 75 samples were discriminated the geographic origins in combination of principal component analysis with the accuracy rate of 100%.

To trace the geographic origins of seafood, isotope analysis can be associated with trace metal compositions or elemental profiling (CARTER *et al.*, 2015, GOPI *et al.*, 2019b). For instance, CARTER *et al.* (2015) identified that the utility of $\delta^{2}H$ and $\delta^{13}C$ values in meat component of prawns could distinguish between Australian prawns and those imported from neighboring Asian countries. In addition, the data of potassium, zinc and arsenic

concentrations in prawn meat resembled the results obtained in isotope analysis with minor overlapped areas. Therefore, those authors surmised that the association of stable isotope and trace metal analysis would improve the accuracy of classification; however, the discriminant analysis for this combination had not been investigated.

To determine the geographic origins and production method (wild or farmed) of Asian seabass (*Lates calcarifer*), stable carbon and nitrogen isotope analyses and elemental profiling (31 different elements) were conducted in 38 samples from two Australian and one Malaysia regions (GOPI *et al.*, 2019b). Three statistical and ordination methods were used, including univariate (ANOVA) and multivariate (principal component analysis), linear discriminant analysis, and random Forest (R package). The accuracy of stable isotope, elemental profiling and the combination of two methods were 84, 72 and 81%, respectively. The incorrect predictions were two, one and none, respectively for three models. It was suggested that the combination of stable isotope and elemental profiling can be accommodated for seafood authentication. However, this study did not cover the seasonal variations of $\delta^{15}N$ and had limited sample size and species. To extend the provenance of geographic origins (17 different European areas located in Mediterranean Sea basin), 144 wild and farmed specimens of European sea bass were analyzed for the carbon and nitrogen isotope and rare earth elements (lanthanum, europium, holmium, erbium, lutetium, and terbium) (VARRÀ *et al.*, 2019). Data were anatomized by principal component analysis and orthogonal partial last square discriminant analysis (OPLS-DA). The results showed that the satisfactory classification can be achieved in tracing both for geographical origin and production method by OPLS- DA analysis.

In general, isotope analysis has been limited by the absence of reference database on a large scale of different species so that it can be officially applied. While establishing the reliable database, we need to pay attention to some drawbacks of isotope analysis. Particularly, isotope fractionations can be influenced by the environmental factors, e.g. growth conditions (LIU *et al.*, 2017, ZHAO *et al.*, 2018), and diet quality, e.g. high versus low dietary protein contents (FARABEGOLI *et al.*, 2018, WANG *et al.*, 2018). In addition, the results of isotope analysis can be overlapped due to the seasonal variability (SANT'ANA *et al.*, 2010), as well as the inappropriate utility of multivariate statistics and chemometrics methods (VARRÀ *et al.*, 2019).

4. CONCLUSIONS

Despite the limited studies, this review demonstrated that isotope analysis has been very promising in tracing aquatic food products provenance, especially in production sources and geographic origins. However, considerably more work will need to be done to authenticate the provenance of aquatic products. For example, the studies of isotopic abundance should be extended to various types of fish, seafood and aquatic products. The accuracy in analytical instrumentation and methods need to be firmly established and characterized. In addition, seasonal and environmental effects should be considered in isotopic values of food samples. More broadly, multidisciplinary approach and other analytical techniques, such as chemical characterization, fatty acids profile and multi-element profiling, combined with multivariate data evaluation and chemometrics can be further associated with isotopic analysis to improve the level of predictable confidence.

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PAPER

ENGINEERING PROPERTIES OF TWO HAZELNUTS VARIETIES AND ITS KERNEL RELATION TO HARVEST AND THRESHING

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ABSTRACT

In this research, several engineering properties of two hazelnut cultivars (Palaz and Çakıldak varieties) were determined and compared in terms of linear dimensions, mass, sphericity, surface area, projected area, true and bulk densities, porosity, repose angle, shell ratio, terminal velocity, rupture force, energy, deformation and drag coefficient. These properties are necessary for the design of much equipment for harvesting, processing, and transportation, sorting, separating and packing. Also, rupture force and deformations were determined which are the most discriminant parameters that can be used to describe the behavior under compression. In both cultivars, these values were also determined within the kernels.

Keywords: hazelnut, pomological properties, strength properties

1. INTRODUCTION

Hazelnut is the nut of the hazel and therefore includes any of the nuts deriving from species of the genus Corylus, especially the nuts of the species Corylus avellana. It is also known as cobnut or filbert nut according to species (MARTIN *et al*., 2014). A cob is roughly spherical to oval, about 15-25 millimeters (0.59-0.98 in) long and 10-15 millimeters (0.39-0.59 in) in diameter, with an outer fibrous husk surrounding a smooth shell (ANONYMOUS, 2016a)

It has been mentioned in historical documents that hazelnut was produced 2300 years ago in the Black Sea coast on the north of Turkey and it is known that hazelnut has been exported from Turkey to other countries for the last 6 centuries. Turkey, which is one of the few countries in the world with favorable weather conditions for hazelnut production, accounts for 75% of the global production and 70-75% of the exportation (ANONYMOUS, 2016b). The main hazelnut producing countries in the world are Turkey, Italy, Spain, USA and Greece. Although hazelnut is also produced in the Former Soviet Union, Iran, Romania and France, these countries do not have a major input in the world hazelnut trade. Turkey has an average production of 550,000 tons of shelled nuts in recent years. On the other hand, the production of Italy and Georgia, Additional important producing countries, varies between 100,000-125,000 tons and 40,000-80,000 tons respectively (ANONYMOUS, 2018).

The following hazelnut varieties are cultivated in Turkey: Tombul, Palaz, Cakildak, Kara, Fosa, Min-cane, Uzunmusa, Kan, Kargalak, Cavcava, Sivri, Aci, Kus, Yuvarlak Badem and Yassi Badem. They differ from each other in some properties (OZDEMIR and AKINCI, 2004). A specific know-ledge of some hazelnut engineering properties such as linear dimensions, shapes, porosity, volume, density, terminal velocity, rupture force, etc. and the variation between the hazelnut varieties is required to design of hazelnut processing instrument. The running of many types of machines is influenced accurately by the size and shape of the fruit enterer, and so in order to study a given process should be described accurately. For example, sphericity is one of the most important characteristics as it greatly affects the processability of hazelnuts for the food industry. For this reason, hazelnut varieties with better sphericity need to be grown more (MOHSENIN, 1980). The volume and density of agro-food products play an important role in applications such as design of silos, drying, mechanical compaction, stability of pellets and wafers, seperation and grading, evaluation of maturity, or quality evaluation (GLINSKI *et al*., 2011). Terminal velocity and drag coefficient plays also a significant assignment for the separation, the pneumatic conveying off goods and the cleaning foreign materials (GUNER, 2007). Similarly, the rupture force is also important and indicates failure over a significant. In mechanical processing of the fruits, most of the damage occurs in the harvesting and threshing as well as mechanical conveying and other equipment (OZDEMIR and AKINCI, 2004). For example; dynamic forces during fruit transport and handling cause by far the most bruise damage (ZEEBROECK *et al*., 2007). The evaluation of mechanical properties of hazelnuts (whole fruit, shell and kernel) has been developed over the past years with the objectives to obtain industrial processes and improve the use of hazelnuts as food ingredient. The experimental characterization of shells and kernels is a challenging topic to improve the quality of the final product. Many literature papers describe procedures to find the mechanical properties of raw and roasted kernels (BRAGA *et al*., 1999; AYDIN, 2002; ALASALVAR *et al*., 2003; DEMIR and CRONIN, 2004; ÖZDEMIR and AKINCI 2004; GHIRARDELLO *et al*., 2009; DELPRETE and SESANA 2014) and the experimental testing on hazelnuts generally relies on compressive testing of kernel and shell by means of

universal testing machines. In addition to these studies, some papers (e.g. GÜNER *et al*., 2003; KOYUNCU *et al*., 2004; VURSAVUŞ and ÖZGÜVEN, 2005; VALENTINI *et al*., 2006,) have experimental values of the compressive load needed to crack the shell of hazelnuts, walnuts and pine nuts.
Compressive force-crosshead displacement curves are widely used to measure textural properties in food products (CARCEL *et al*., 2012): initial slope, maximum force, energy until failure and other curve-related parameters have been described and correlated with textural parameters of hazelnuts.
The physical characteristics of the hazelnut kernel have an important role on the crispness and crunchiness sensory parameters especially on the roasted nuts (SAKLAR *et al*., 1999) and the water activities have direct effects on mechanical characteristic (BORGES and PELEG, 1997). In a test campaign (DEMIR and CRONIN, 2004), a small rectangular prismatic specimen, including the inner cavity present in the core of each hazelnut, was cut from the whole kernel to simplify the calculation of stress and elastic modulus when a compressive axial force loads the specimen section. Again the specimen geometry affected the results and it did not allow obtaining material properties. DI MATTEO *et al*., (2012) evaluated also some mechanical properties of chemical-peeled hazelnut kernels, such as firmness and rigidity, to study an original industrial process to improve the kernel pellicle removal. A mechanical characterization of whole nut, kernel and shell was conducted (DELPRETE and SESANA, 2014) in order to aid the design and construction of selecting machines. These mechanical properties are affected by numerous factors, such as the moisture content and loading direction (CHENGMAO *et al*., 2017). Also, Nut shell characteristics, such as hardness and thickness, were measured and correlated to the biological cycle of the nut weevil of *Curculio nucum* (Coleoptera: Curculionidae) pest and to the damage by its larvae (GUIDONE *et al*., 2007) stress the importance of physical properties evaluation.
There were three aims of this study. The first was to investigate the some pomological (physical) properties of two hazel nut varieties and its kernel widely grown in the Turkey. The second aim was to determine of strength properties of nuts and kernels and last aim was to determine of some frictional and aerodynamic properties of nuts and its kernel.

2. MATERIALS AND METHODS

2.1. Sample preparation and material testing system

In this study, two hazelnut varieties (Palaz and Çakıldak) that chosen randomly were used for all the experiments. The 30 nuts and kernels in four replicates of each variety were tested. Samples were supplied from the different hazelnut growers (2015 harvest season; Samsun, Turkey). The experiments performed as soon as possible after hazelnuts purchased. Samples were kept in a refrigerator until analyses were performed. The hazelnuts were cleaned manually to remove all foreign matter, immature, broken or spoilt nuts. These experiments were carried out in the Laboratory of the Department of Agricultural Engineering, Ondokuz Mayıs Univesity, Samsun. The mechanical properties of hazelnuts under compression load were measured by a Lloyd Instrument Universal Testing Machine (Lloyd Instrument LRX Plus, Lloyd Instruments Ltd, An AMATEK Company). The device has three main parts: moving head, driving unit and data acquisition system (load cell, computer and connections and NEXYGEN Plus software) (Fig. 1).

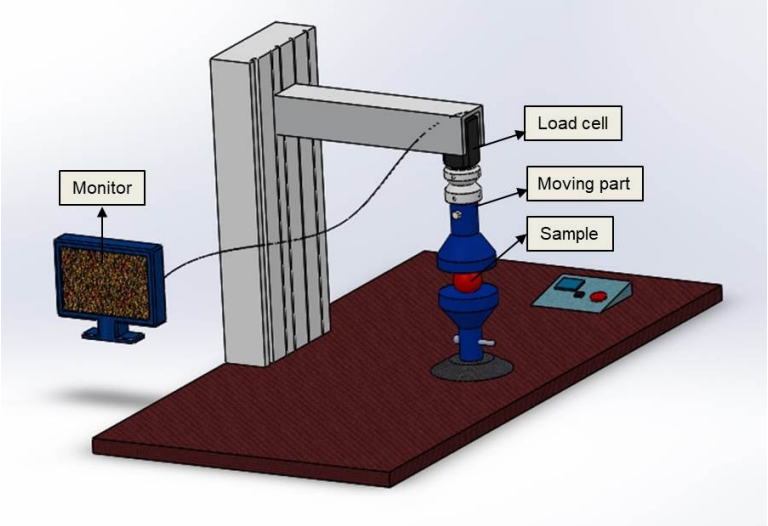

Figure 1. Lloyd instrument universal testing machine.

2.2. Determination of some pomological (physical) properties of nuts and kernels

The initial moisture content of hazelnut varieties (Palaz and Çakıldak) were determined by using a standard method and were found to vary between 6.38-7.38% and 6.52-7.71% db (db = dry basis) respectively (USDA, 1970).
To arrange the average linear dimensions (length (L), width (W) and thickness (T)) of the hazelnuts cultivars, a sample of 30 hazelnuts were randomly selected and the dimensions and mass of each hazel nuts used were determined. The dimensions of the hazelnuts were measured with a digital caliper, which had an accuracy of 0.01 mm. The geometric mean diameter, surface area and the sphericity of the hazelnuts were calculated by using the following relationships (MOHSENIN, 1980):

$$Dg = (LWT)^{1/3} \quad (1)$$

$$\phi = Dg / L * 100 \quad (2)$$

$$S = \pi . Dg^2 \quad (3)$$

where, Dg is the geometric mean diameter in mm; ϕ is the sphericity in %; S is surface area in mm^2 and L is the length (mm), W is the width (mm) and T is the thickness (mm) (Fig. 2).

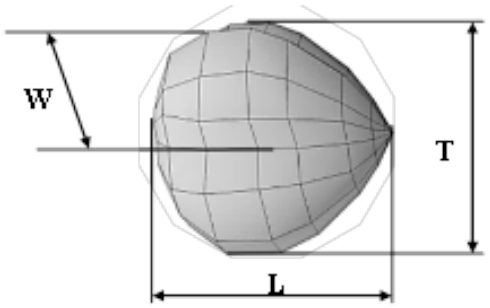

Figure 2. Three dimensions of nut.

Sample mass (M) and thousand mass (M1000) were measured by using a digital balance with a sensitivity of 0.001 g. The fruit mass was determined on 30 randomly selected hazelnuts and kernels and converted to a thousand mass. Also, shell ratio (Rs) was calculated by the measurement of nut mass (M) and shell mass (Ms) (ÖZDEMIR and AKINCI, 2004).
Projected area (P) (y axes) was determined from the pictures of hazelnuts and kernels which were taken by a digital camera (Canon 600 D), in comparison with the reference area to the sample area by using the Sigma Scan Pro 5 program.
The true density (ϱk), were determined using the liquid displacement method and The bulk density (ϱb) was determined with a weight per hectoliters tester which has calibrated in kilogram per hectoliters (DESHPANDE and OJHA, 1993; AYDIN, 2002; DEMIR *et al.*, 2002).
The porosity was determined by the following equation

$$\varepsilon=1-(\varrho b-\varrho k) \quad (4)$$

where (ϱb) is bulk density and (ϱk), is true density in kgm^{-3} (MOHSENIN, 1980; SITKEI, 1986).

2.3. Determination of strength properties of nuts and kernels

To determine the strength a property of hazelnuts and kernels, biological material test device (Lloyd Instrument Universal Testing Machine) was used (Fig. 1). In this study, hazelnuts and kernels were compressed between two parallel plates at a constant rate of 10 $mmmin^{-1}$ based on the preliminary tests.
Rupture force and deformation were determined from the force-deformation curve, where there is a sudden drop in force. To arrange the effect of loading positions on strength properties a coordinate system describing the three main compression positions of hazelnut and kernels is shown in Fig. 3. The energy absorbed in rupture point for was determined from the diagram by measuring the area under the force-deformation curves.

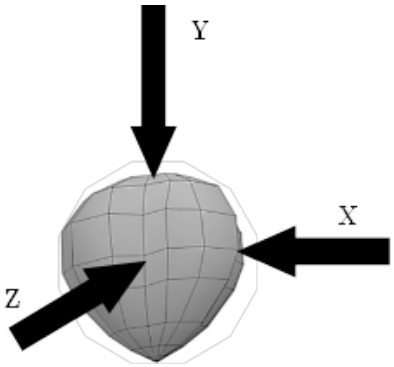

Figure 3. Applied force axis of nuts.

2.4. Determination of some frictional and aerodynamic properties nuts and its kernels

The determination of the angle of repose (ϕ) of nuts and kernels was used a funnel tube (smallest diameter 50 mm, biggest diameter 150 mm and height 300 mm) and a bow with discharge gate at the bottom. After filling the box with sample, the gate was quickly removed.
The height of fruit pile above the floor (h) and the diameter of the heap of sample (r) was measured and used to determine the angle of repose. The angle of repose was calculated with the measurement of the height (h) of conical shape at the center and radius (r) of the free samples over the surface (ERTEKIN *et al.*, 2006)

$$\phi = \tan^{-1}(h/r) \quad (5)$$

Terminal velocity was determined using a wind tunnel. For each test, a sample (nut, kernel) was dropped into the air stream from the top of the wind tunnel, and air was blown up the column to suspend the material in the air stream. The air velocity near the location of the sample suspension was measured by a digital hot-wire anemometer with an accuracy of 0.1 ms^{-1}.
In addition, the drag coefficient was calculated as following equation (Mohsenin 1980)

$$C_d = \frac{2mg(\rho_p - \rho_f)}{\rho_p \rho_f A_p V_t^2} \quad (6)$$

where: Ap is projected area of the particle (m^2), Cd is drag coefficient (-), g is acceleration due to gravity (9.81 ms^{-2}), m is mass of samples (kg), Vt is terminal velocity (ms^{-1}), ϱp is density of samples (kgm^{-3}) and ϱf is density of air (1.206 kgm^{-3})

3. RESULTS AND DISCUSSION

Frequency distributions of the dimensional properties of two hazelnut cultivars are given in Table 1.

Table 1. Frequency distributions of the dimensional properties of two hazelnut cultivars.

		Palaz		Çakıldak	
Dimensions	**Frequency**	**Nuts**	**Kernels**	**Nuts**	**Kernels**
Length (mm)		16.60-18.27	12.37-16.26	17.25-19.64	13.36-17.59
Width (mm)	%86.7	19.14-20.94	13.12-16.78	15.42-18.66	11.09-14.78
Thickness (mm)		16.35-17.96	11.98-15.27	14.38-16.40	9.89-13.49

According to the results of frequency distributions of the dimensional properties of two hazelnut and kernels cultivars; about 86.7% of the hazelnut were between 16.60 and 19.64 mm in length, 15.42 and 20.94 mm in width and 14.38 and 17.96 mm in thickness for both cultivars. Some physical properties of the Palaz and Çakıldak nuts and kernels are given in Tables 2 and 3.

The moisture contents were 6.63% and 6.96% for Palaz and Çakıldak nuts and 4.56% 4.50% for kernel respectively. While mean nut length was 17.73 mm, nut width was 19.89 mm and thickness was 17.08 mm and kernel length was 14.21 mm, kernel width was 14.94 mm and thickness was 13.36 mm for Palaz. These values were 18.44 mm, 16.85 mm, 15.20 mm and 14.70 mm, 12.75 mm, 11.82 mm for nuts and kernel of Çakıldak, respectively. All sizes of Palaz except length are bigger than those of Çakıldak.

Shell ratio (46.72 %) and thickness (1.21 mm) of the Palaz were higher than the Çakıldak (45.90 %) and (1.10 mm). For the Palaz, mean mass of nut and 1000 nuts mass was 1.58 g and 1591.98 g for nuts and 0.79 g and 793.61 g for kernels. The same values of Çakıldak were 1.59 g and 1612.18 g for nuts and 0.80 g and 792.23 g for kernels. When the nut mass in this study was compared with previous studies, the mean mass of the fruit was with in normal limits (GÜNER *et al*., 2003). The average value of the geometric mean diameter was calculated as 18.05 mm for Palaz and 16.76 mm for Çakıldak nuts, respectively. The same values were 14.12 mm and 13.00 mm for Palaz and Çakıldak kernels. Sphericity is an expression of a shape of a solid relative to that of a sphere of the same volume while the aspect ratio relates the width to the length of the fruit which is an indicative of its tendency toward being oblong in shape (ERTEKIN *et al*., 2006). These values were 1.042 and 0.91 for nuts and 1.37 and 0.89 for Palaz and Çakıldak, respectively.

Bulk densities for nuts and kernels of Palaz were between 392 kg/m^3 and 450 kg/m^3 and 482 kg/m^3 and 578 kg/m^3. The same values were between 406 kg/m^3 and 468 kg/m^3 and 483 kg/m^3 and 522 kg/m^3 for Çakıldak. The porosity ranged between 53.50% and 61.30% for Palaz nuts and 54.70% and 62.70% for Çakıldak nuts. The same values were between 58.70% and 64.30% for Palaz and 54.70% and 62.70% for Çakıldak for kernels. Bulk densities and porosities values were similar with the literature values (OZDEMIR AND AKINCI, 2004).

The mean surface and the projected area of the Palaz nut were found 889.17 mm^2 and 221.762 mm^2. For Çakıldak variety the same values were calculated as an 883.92 mm^2 and 211.740 mm^2 respectively. When the same properties were examined for the kernel of the Palaz and Çakıldak, the surface and the projected area were found 663.21 mm^2 and 167.23 mm^2, 533.03 mm^2 and 132.20 mm^2, respectively.

The mean and SD values of some mechanical properties of the two nut cultivars obtained from the measurements and calculations at moisture contents of 6.63% and 6.96% (w.b.) are shown in Table 4.

Table 2. Some physical properties of nuts.

	Cultivar							
	Palaz				Çakıldak			
	Min	Max	Mean	S.D	Min	Max	Mean	S.D
Length (mm)	15.53	18.43	17.33	0.024	15.06	20.01	18.44	0.045
Width (mm)	18.71	21.40	19.89	0.025	15.10	18.74	16.85	0.035
Thickness (mm)	16.21	18.21	17.08	0.021	13.92	16.42	15.20	0.024
Shell ratio (%)	44.33	48.71	46.72	0.230	44.69	46.78	45.90	0.215
Shell thickness (mm)	1.02	1.66	1.213	0.005	0.90	1.39	1.10	0.004
Geo. mean dia. (mm)	16.82	18.72	18.05	0.017	15.73	17.99	16.76	0.022
Sphericity	0.99	1.109	1.042	0.001	0.86	1.062	0.91	0.002
Surface area (mm^2)	889.17	1100.34	1024.65	1.957	777.66	1016.50	883.92	2.315
Projected area (mm^2)	221.76	298.84	257.23	0.785	201.73	247.13	211.74	0.598
True Density (kgm^{-3})	714.23	801.42	748.65	1.235	705.39	753.54	721.98	1.383
Bulk Density(kgm^{-3})	392.64	450.32	412.42	0.983	406.43	468.24	436.71	0.871
Porosity	0.53	0.613	0.587	0.005	0.54	0.62	0.59	0.004
Mass (g)	1.58	1.753	1.627	0.010	1.57	1.63	1.58	0.011
1000 Mass (g)	1591.98	1739.86	1669.25	12.26	1568.42	1634.83	1612.18	10.54

Table 3. Some physical properties of kernels.

	Cultivar							
	Palaz				Çakıldak			
	Min	Max	Mean	S.D	Min	Max	Mean	S.D
Lenght (mm)	12.08	17.010	14.21	0.057	10.16	18.87	14.70	0.060
Width (mm)	12.57	17.22	14.94	0.046	9.31	15.00	12.75	0.046
Thickness (mm)	11.26	15.79	13.36	0.037	8.82	13.87	11.82	0.046
Geo. mean dia. (mm)	12.88	15.52	14.12	0.025	10.83	14.78	13.00	0.033
Sphericity	0.84	1.15	1.37	0.004	0.69	1.18	0.89	0.004
Surface area (mm^2)	521.30	756.30	663.21	2.213	368.77	686.45	533.03	2.676
Projected area (mm^2)	140.79	198.50	167.23	0.898	112.64	178.06	132.20	0.785
True Density (kgm^{-3})	817.12	1020.12	984.42	3.452	824.56	978.76	845.31	4.349
Bulk Density (kgm^{-3})	482.17	578.67	523.23	1.924	483.33	522.35	489.60	1.345
Porosity	0.58	0.64	0.61	0.003	0.54	0.62	0.59	0.005
Mass (g)	0.78	0.92	0.842	0.012	0.72	0.84	0.79	0.015
1000 Mass (g)	793.61	934.51	869.73	10.25	735.25	865.79	792.23	10.743

The force values required to initiate nut rupture were obtained from the same experiments. From Table 4, it is seen that the rupture force was higher along the y-axis compared with the x- and z-axes for both cultivars. This could be because the area of contact between the nut pit and compression plates was larger along the y-axis than those along the x- and z-axes. This is in agreement with the finding of AKTAŞ *et al*. (2007).

Table 4. Some mechanical properties of nuts.

		Palaz				Çakıldak			
		Min	**Max**	**Mean**	**S.D**	**Min**	**Max**	**Mean**	**S.D**
Z axes	Rupture Force (N)	186.94	266.79	217.96	2.742	105.61	289.50	211.24	6.733
	Deformation (mm)	0.70	1.19	1.01	0.018	0.59	1.45	0.97	0.029
	Energy (J)	0.07	0.16	0.12	0.013	0.03	0.21	0.11	0.006
	Hardness (N/mm)	166.39	265.12	220.02	3.52	175.59	265.08	216.96	3.24
X axes	Rupture Force (N)	191.53	312.91	251.04	3.86	129.77	313.34	222.68	6.449
	Deformation(mm)	0.57	0.96	0.78	0.013	0.53	1.22	0.88	0.023
	Energy (J)	0.055	0.160	0.100	0.004	0.034	0.189	0.10	0.005
	Hardness (N/mm)	286.54	381.08	324.43	2.89	184.27	328.93	257.28	4.988
Y axes	Rupture Force (N)	191.81	339.39	274.88	5.341	155.61	396.46	239.57	7.248
	Deformation(mm)	0.56	1.32	0.97	0.023	0.45	2.77	1.02	0.067
	Energy (J)	0.051	0.23	0.13	0.005	0.04	0.27	0.10	0.007
	Hardness (N/mm)	244.47	342.78	288.25	3.442	95.32	408.73	273.16	8.272

In addition to rupture force, energy values, deformation and hardness were calculated as some mechanical properties. Maximum and minimum energy values except min energy for Çakıldak cultivar (0.034 J-x axes) were found along the y axes as 0.23 J for the Palaz and 0.27 J for the Çakıldak and 0.05 J for the Palaz. Depending on rupture force, maximum deformation was obtained on the y axes as a 1.32 mm and 2.77 mm for the Palaz and Çakıldak, respectively. The response of hardness to loading position in the x axes was higher than the hardness in the z and y axes loading position for the Palaz, while hardness value in the y axes was max for Çakıldak. This difference among the two type nut cultivars may be due to the shell properties of the varieties.
Terminal velocity, drag coefficient, repose angel and static and dynamic coefficient of the two hazelnut varieties and their kernels were given in Table 5.

Table 5. Terminal velocity, drag coefficient and repose angel of the Palaz and the Çakıldak.

		Cultivar							
		Palaz				Çakıldak			
		Min	Max	**Mean**	**S.D**	**Min**	**Max**	**Mean**	**S.D**
Nut	Terminal velocity (m/s)	11.26	13.89	13.15	0.744	11.84	13.67	13.30	0.544
	Drag coefficient	0.351	0.389	0.362	0.012	0.358	0.391	0.367	0.011
	Repose angel (°)	22.615	23.12	22.814	0.175	22.578	22.845	22.751	0.090
Kernel	Terminal velocity (m/s)	11.77	14.05	13.66	0.672	12.73	13.84	13.70	0.309
	Drag coefficient	0.363	0.377	0.369	0.005	0.370	0.388	0.377	0.005
	Repose angel (°)	24.231	25.813	24.934	0.464	23.887	24.867	24.53	0.389

The terminal velocity of hazelnuts and kernels, the values ranged from 13.26 to 13.89 m/s and from 11.77 to 14.05 m/s, respectively. The terminal velocity of kernels is higher than

that of hazelnuts. These differences in results can be attributed to the increase in mass of the individual nut or the kernel per unit when their frontal areas were presented to the air stream to suspend the material.
The repose angle with hazelnuts and kernels were varying from 22.58° to 23.12° and from 23.89° to 25.81° respectively. This is due to the higher sphericity of hazelnuts and kernels which results from allowing them to slide and roll on each other.

4. CONCLUSION

Several physical and mechanical properties of the two (The Palaz and the Çakıldak) hazelnut varieties were described in order to design a specific machine for harvesting, threshing, conveying, cleaning, separating, storing, etc. according to these results. Some remarkable points for the study can be summarised as follows.
1. Moisture content was 6.63% for hazelnut and 4.56% for kernel of the Palaz cultivar and 6.96% for hazelnut and 4.50% for kernel of the Çakıldak cultivar. The average hazelnut length, width, thickness and geometric diameter were 17.34 mm, 19.90 mm, 17.09 mm and 18.06 mm for hazelnut and 14.21 mm, 14.94 mm, 13.36 mm and 14.13 mm for kernels of the Palaz, respectively. The average hazelnut length, width, thickness and geometric diameter were 18.45 mm, 19.85 mm, 15.21 mm and 16.77 mm for hazelnut and 14.71 mm, 12.75 mm, 11.82 mm and 13.00 mm for kernels of the Çakıldak, respectively. The average 1000 mass for hazelnut and kernels were 1669.25 g and 869.74 g for the Palaz and 1612.18 g, 792.24 g for the Çakıldak, respectively.
2. The mean true and bulk density, angle of repose and terminal velocity of the Palaz hazelnut and kernel were 748 kg/m^3 - 984 kg/m3, 412 kg/m^3 - 523 kg/m^3, 22.81° - 24.93° and 13.15 m/s - 13.66 m/s, respectively. The same values were 721 kg/m^3 - 845 kg/m^3, 436 kg/m^3 - 489 kg/m^3, 22.75° - 24.53°and 13.30 m/s - 13.70 m/s, respectively.
3. The sphericity, surface area, projected area and porosity of the hazelnut and kernels were 1.04 % - 1.37 %, 1025 mm^2 - 663 mm^2, 257 mm^2 - 167 mm^2 and 0.59 - 0.61 for the Palaz cultivar, respectively. The same values were 0.91 % - 0.89 %, 883 mm^2 - 533 mm^2, 211 mm^2 - 132 mm^2 and 0.59 - 0.59 for the Çakıldak cultivar, respectively.
4. The rupture force was higher along the y-axis compared with the x- and z-axes for both cultivars. The biggest mean deformation value was 1.01 mm on z axes for the Palaz and 1.023 mm on y axes for the Çakıldak. The energy and hardness values of the Palaz cultivar were higher than the Çakıldak values within all axes.

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PAPER

QUALITY AND MYCOBIOTA COMPOSITION OF STORED EGGS

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ABSTRACT

The aim of this study was to monitor the quality and mycobiota composition of table eggs during storage period. The most significant changes in the egg weight and water activity were observed on Day 7. To identify the mycobiota present on the eggshell by PCR method, a newly designed procedure for the extraction of fungal DNA based on a combination of commercial isolation kit, proteinase K and ultrasound was implemented. Identified mold genera included *Penicillium* spp., *Cladosporium* spp., *Fusarium* spp. and *Alternaria alternata* group. Their ratio varied considerably during storage with the dominance of *Penicillium* spp. on Day 14.

Keywords: egg quality, Haugh units, micromycetes, PCR, storage, table eggs

1. INTRODUCTION

Egg is one of the most nutritious low-energy foods possessing all the proteins, vitamins and minerals needed for human health (TOLIK *et al*., 2014). Because of its beneficial composition, it may also be used as nutraceuticals and protein ingredients for food applications (ZAMBROWICZ *et al*., 2015). However, during egg collection, there is significant risk of contamination of the shell surface with microscopic filamentous fungi. By laying out, an egg gets to the external environment, which becomes its source of contamination. The majority of egg microbiological contamination studies deal primarily with microorganisms of bacterial origin (BAHOBAIL *et al*., 2012; JONES *et al*., 2015; ERKMEN and BOZOGLU, 2016; KARIMIAZAR *et al*., 2019), secondarily with fungal contamination (TOMCZYK *et al*., 2019). Micromycetes are able to grow under conditions which are unsuitable for bacteria (extreme pH, low a_w, wide range of enzymatic activity). Spores of various mold genera are regularly found on the shell surface and can furher penetrate into the egg contents (TOMCZYK *et al*., 2018). Contamination of the eggshell may be initiated by ambient conditions during egg collection and storage, which may be as a result of the quality of litter and feed, the presence of dust, air temperature and humidity. Therefore, the presence of numerous fungal genera (*Aspergillus*, *Cladosporium*, *Drechslera*, *Penicillium*, *Stemphylium* and *Fusarium*) on the eggshell has been reported in many studies (KOKKONEN *et al*., 2010; ROHWEDER *et al*., 2011; TOMCZYK *et al*., 2019).
Fungi not only degrade the substrate on which they occur, they also cause numerous diseases due to the presence of spores in the air (SKÓRA *et al*., 2015). Mycotoxins, which are secondary metabolites produced by some fungal strains, are of the greatest risk for consumers (SYPECKA *et al*., 2004).
Freshness, the characteristic most commonly related to egg quality, declines with time and temperature after laying (HIDALGO *et al*., 2006). Several chemical and physical changes occur during egg storage such as increase in albumen pH, thinning of the thick albumen, water evaporation through the shell (LUCISANO *et al*., 1996), enlargement of air cell, development of Maillard reaction (HIDALGO *et al*., 2006). The most common indices used to evaluated egg freshness are air cell height (EU, 2007) and thick albumen height, expressed as Haugh units (USDA, 1995).
Storage conditions (especially the temperature) also have a great impact on mycological contamination and the production of fungal exoproducts on the shell surface. Temperatures below 5°C slow the aging process, but primarily reduce the development of microorganisms. If the refrigerator temperature decreases below –2°C, the egg contents would start to freeze. According to the European legislation (EC, 2008), shelleggs must not be exposed to refrigeration at temperatures lower than 5°C. Another significant parameter in egg storage is the relative air humidity which is adjusted to reduce as much as possible the losses caused by water evaporation. This results in a weight decrease and changes in individual egg quality indicators. In general, the lower the storage temperature, the higher the relative humidity (STEINHAUSEROVÁ *et al*., 2003). Within the storage period, diversified mycoflora, including potentially toxinogenic and toxinogenic species of micromycetes, is found in the eggs. Therefore, the identification of micromycetes is necessary (THRANE *et al*., 2004). Current routine methods used for the detection and identification of microscopic filamentous fungi often require culture isolation and further morphological and physiological characterisation (SIMMONS, 2007). However, it does not provide sufficient distinction among species. The limitation of these techniques is unstable micromycete cell morphology, which may vary within the species and also dependent on ambient conditions (RAINIERI *et al*., 2003). The development of molecular techniques has

enabled the identification of not only the genus, but also the individual species of micromycetes. Currently, numerous identification methods are generally in use. DNA reassociation techniques are used to detect DNA complementarity. The disavantage of these methods is inability to distinguish closely related species. Gene sequence comparison no longer has this limitation. However, detection of rRNA genes, such as the internal transcriptional region (ITS), is the preferred method (KURTZMAN *et al*., 2011). For proper identification of micromycetes by PCR methods, sufficient amount and high purity of the DNA are necessary.
The aim of this study was to detect changes in quality of table eggs during the storage period of 28 days and to identify mold genera present on the eggshell using the PCR method. For correct and accurate identification it is necessary to obtain DNA which is often very complex in micromycetes. To extract the fungal DNA, the cell wall, cytoplasm, and nuclear membrane must be first effectively lysed. However, the structure of fungal cell wall itself prevents lysis and sufficient nucleic acid isolation (Čmoková *et al*., 2014). Therefore, a new effective procedure for lysing fungal cells and further DNA extraction has been designed and implemented in this study.

2. MATERIALS AND METHODS

Micromycetes were isolated from 120 brown-shell table eggs, laid by the *Lohmann Brown* crossbreed laying hens in cages, weight category M (EC, 2008). The eggs were graded, labeled and stored in the breeding stock at an average temperature of 14.3°C and a relative humidity of 61%. Sets of 30 sample units were taken every 7 days. Among them, water activity (a_w) of the eggshell was determined in 10 eggs, another 10 eggs were checked for selected egg quality indicators and the enumeration of microscopic filamentous fungi according to STN ISO 21527-1 (2010) and STN ISO 21527-2 (2010) was carried out using the last 10 eggs. Since molds are aerobic organisms and colonize mainly the shell, enumeration of molds was focused on the eggshell surface.

2.1. Determination of water activity (a_w) of eggshell and egg quality indicators

Water activity of the eggshell was determined by the LabMASTER-a_w (Novasina AG, Lachen, Switzerland) at a constant temperature (25°C) for 20 min±2.48 min. Measurements were repeated three times for individual egg. Eggshell breaking force was measured in accordance with the manufacturer's instructions by the Egg Force Reader (Orka Food Technology Ltd., Ramat HaSharon, Israel) - a compact system for automatic measuring of eggshell rupture point. The unit of strength measurement (kilogram-force, kgf) is calculated as gently applied force on the eggshell until it cracks.
Egg Analyzer™ (Orka Food Technology Ltd., Ramat HaSharon, Israel) was used to determine the egg weight, Haugh units (HU), quality grade and yolk color. The device is able to measure egg weight (g), height of the thick albumen (mm), and color of the yolk. The first two measurements were used for calculation of Haugh units that indicate egg quality. The equation for working out the rating is shown below:

$$HU = 100 \log (h - 1.7w^{0.37} + 7.6),$$

where:
HU = egg quality in Haugh units
w = egg weight in grams
h = height of the thick albumen in mm (NAGY *et al*., 2011)

Yolk color intensity was compared to the Roche Yolk Color Fan (RCF) ranging from 1 to 15 (1 means pale yellow and 15 dark yellow).
After evaluation, the egg contents were transferred to large Petri dishes with diameter of 200 mm each, they were then placed on a dark flat surface and both the height and the width of the egg yolk as well as those of the thick albumen were measured using a micrometer gauger and a ruler. Subsequently, the yolk index (YI) and albumen index (AI) were calculated according to NAGY *et al*. (2011) as follows:

$$\text{Albumen index} = \text{Height of the thick albumen (mm)} \times 100 / [\text{Length of the thick albumen (mm)} + \text{Width of the thick albumen (mm)}/2]$$

$$\text{Yolk index} = \text{Height of the yolk (mm)} / \text{Yolk diameter (mm)} \times 100$$

2.2. Fungal strains and culture condition

Microscopic filamentous fungi were removed from the eggshell surface as described by CUPÁKOVÁ *et al*. (2010). For this purpose, ten eggs of each experimental group were tested every week. Individual egg was transferred aseptically into a sterile plastic bag and sterile peptone water (0.1 wt%) in a volume of 100 mL was added. The sample was then shaken for 15 minutes using the Orbi-ShakerTMJR (Benchmark Scientific Inc., Sayreville, USA). Further decimal dilutions were prepared in accordance with STN EN ISO 6887-1 (2017). As described by the standard procedure, the first two decimal dilutions were spread in parallel on the surface of Dichloran Glycerol agar medium (DG-18; OXOID, Basingstoke, UK) and Dichloran-Rose Bengal Chloramphenicol agar medium (DRBC; OXOID, Basingstoke, UK) in a volume of 0.1 mL and incubated for 5 days at 25°C (STN ISO 21527-1 and STN ISO 21527-2, 2010). Colonies were further subcultured individually on the surface of Czapek agar medium (OXOID, Basingstoke, UK) and incubated at 25°C for another 7 days. After incubation, 1 to 5 colonies were subjected to macroscopic and microscopic identification.
Macroscopic evaluation included the growth rate, color, texture and topography of fungal colonies. Microscopic study of different mold genera was carried out by preparing slides stained with lacto phenol cotton blue and observed under light microscope.

2.3. DNA isolation

Because the cell wall of micromycetes is difficult to break, there was a challenge to obtain pure and concentrated fungal DNA. Therefore, the following three pre-isolation techniques were designed:
1. Mycelium in a quantity of 10-50 mg was taken with a sterile scalpel from the surface of Czapek agar medium, transferred into 1.5 mL eppendorf, exposed to liquid nitrogen for 5 min and then heated to 95°C for 5 min. Freezing and heating was repeated three times. The DNA isolation was then performed with the help of commercially available E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, USA).

2. Proteinase K (Macherey-Nagel, Düren, Germany) in a volume of 10 μL was added to eppendorfs with 10-50 mg mycelium and incubated for 30 min at 37°C. After that, the DNA isolation procedure was carried out in accordance with instructions of the commercially available E.Z.N.A.® Fungal DNA Mini Kit manufacturer (Omega Bio-Tek, Norcross, USA).
3. Both zircon and glass beads (1:1) in a volume of 0.2 mL and Proteinase K (Macherey-Nagel, Düren, Germany) in a volume of 10 μL were added to fungal mycelium (10-50 mg) in the eppendorf. Samples were incubated at 37°C for 30 min. After that, 800 μL of lysing solution FG1, which was a part of the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, USA) was added. Samples were then incubated at 65°C for 10 min with ultrasound waves of 500 Hz. Further DNA isolation was performed according to recommended procedure for the commercially available E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, USA, Norcross, USA).
The purity and concentration of DNA was measured using spectrophotometer (SHIMADZU, Wien, Austria). The obtained supernatant was used as a source of DNA in PCR reactions.

2.4. Primer design

The forward primer ITS 212d and the reverse primer ITS 549 amplified a specific DNA fragment for *Penicillium* spp. (336 bp; Table 1).

Table 1. DNA sequences of primers used in this study.

Primer name	Primer sequence (5′-3′)	Annealing temperature (°C)	PCR product	GenBank-EMBL accession number	Reference
	***Penicillium* spp.**				
ITS 212d	AAATATAAATTATTTAAAACTTTC	46	336 bp	LC 317718.1	Pedersen *et al.*, 1997
ITS 549	CTGGATAAAAATTTGGGTTG				
	***A. alternata* group**				
18S-F	AGGATCCATTGGAGGGCAAGT	61	99 bp	AY 563301	Pavón *et al.*, 2010
18S-R	TCCAACTACGAGCTTTTTAACTGCA				
Altsp-F	GAGAACAGCTTCATGGACTTCTCTTT	61	195 bp		
Altsp-R	CGCGGCAGTAGTTGGGAA				
Alt A–F	CGCATCCTGCCCTGTCA	60	118 bp		
Alt A–R	GTTGGTAGCCTTGATGTTGAAGC				
	***Cladosporium* spp.**				
MS1	CAGCAGTCAAGAATATTAGTCAATG	51	370 bp	AY 291273	Zeng *et al.*, 2006
MS2	GCGGATTATCGAATTAAATAAC				
Clado-PF	TACTCCAATGGTTCTAATATTTTCCTCTC	51	87 bp		
Clado-PR	GGGTACCTAGACAGTATTTCTAGCCT				
	***Fusarium* spp.**				
ITS-Fu-f	CAACTCCCAAACCCCTGTGA	55	410	MK 156681.1	Abd-Elsalam *et al.*, 2003
ITS-Fu-r	GCGACGATTACCAGTAACGA				

These primers were designed based on ITS region and *5.8S* rRNA sequences from *Penicillium* spp. available in the GenBank-European Molecular Biology Laboratory database (GenBank-EMBL; PEDERSEN *et al*., 1997).
Alternaria-specific and *Alternaria alternata*-group-specific primer pairs were designed based on *Alt a 1* gene sequences from *Alternaria* spp. available in the GenBank-EMBL database (Table 1; PAVÓN *et al*., 2010).
Both the forward primer AltspF and the reverse primer AltspR amplified a DNA fragment of 195 bp. in all *Alternaria* spp. The primer pair AltAF and AltAR amplified a specific DNA fragment (118 bp) for *Alternaria alternata* group. Finaly, the primer pair 18SF and 18SR, based on conserved *18S* rRNA gene, was used as positive amplification control of the assay (Table 1; MARTÍN *et al*., 2009; PAVÓN *et al*., 2010).
Mitochondrial (mt) small subunit rRNA (SSU rRNA) of *Cladosporium* was amplified by PCR using the universal fungal mitochondrial primers MS1 and MS2. Two specific primers Clado–PF and Clado–PR were designed for multiplex PCR assay (Table 1). The expected amplicon size for primer pair Clado-PF/R was 87 bp (ZENG *et al*., 2006).
Two primers were designed based on sequence information obtained to amplify specific fragments within the *ITS* regions of *Fusarium* spp. The initial tests for specificity revealed that the primer pair ITS-Fu-f and ITS-Fu-r were highly specific for *Fusarium* genus (Table 1; ABD-ELSALAM *et al*., 2003). All the primers were synthesized by the same commercial company (Ecoli s.r.o., Bratislava, Slovakia).

2.5. PCR amplifcation

The amplification was done in a volume of 20 μL containing 1 ng to 10 ng of DNA, 0.5 μL of each primer (concentration 10 pmol/μL), 4.0 μL HOT Firepol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) in the thermal cycler (TC-512, Techne UK, Staffordshire, United Kingdom) using the same cycling conditions for each primer sets, with an initial cycling step at 95°C for 12 min, followed by DNA denaturation at 95°C for 20 s, annealing at diferent temperatures depending on the type of primer used (Table 1) for 60 s, and elongation at 72°C for 2 min. The amplification was terminated by cooling to 6°C.
The following reference strains were used as positive controls in this study: *Penicillium chrysogenum* CCM F-362, *Fusarium sporotrichioides* CCM F-352, *Alternaria alternata* CCM F-397, *Cladosporium cladosporioides* CCM F-348 (Czech Collection of Microorganisms, Brno, Czech Republic).

2.6. Sensitivity of specific detection assays

To determine the minimum detectable amount of fungal DNA in three established PCR assays, variable quantities of fungal genomic DNA ranging from 10 ng to 100 ng were used as DNA template. The PCR products were size fractionated in agarose gels (1.5%) and visualized using GelRed™Nucleic Acid gel stain (Biotium INC., Fremont, USA). Amplicons were visualized by UV transillumination using the Mini Bis Pro® (DNR Bio-Imaging Systems Ltd., Neve Yamin, Izrael).
The identity of PCR products with the selected primers was confirmed by a commercial company (GATC Biotech AG, Cologne, Germany). The DNA sequences obtained from fungal strains were searched for homology to those available at the GenBank-EMBL database using the BLAST program (NCBI software package).

2.7. Statisical analysis

Two-way analysis of variance (ANOVA) and Tukey test for multiple comparison of means with a confidence interval set at 95% was conducted with R - statistics software (R CORE TEAM, 2018). The storage period was set as the main factor. Multiple factor analysis was conducted in R - statistics software (R CORE TEAM, 2018) with "FactomineR" (SEBASTIEN *et al*., 2008) and "factoextra" package (KASSAMBARA and MUNDT, 2007) according to SEMJON *et al*. (2018).

3. RESULTS AND DISCUSSION

Pre-market table eggs must be sorted, packed and stored under appropriate conditions to maintain temporary shelf life. According to Commission Regulation (EC) No. 589/2008, the minimum shelf life of fresh eggs is 28 days from the day it is laid. Storage conditions are limited by storage temperature where the lower limit is 5°C. However, the upper limit is not defined, it only requires maintaining the optimum egg quality.

3.1. Changes in egg quality indicators

Quality parameters of table eggs change during storage. Within the entire storage period, the average values of egg weight ranged from 60.47±2.838 to 57.83±3.368. The most significant egg weight loss (2.64 g±0.87) in this study was recorded on Day 7.
The decrease in egg weight was accompanied by a decrease in water activity (a_w) in the eggshell. Similarly, the most significant decrease in water activity was recorded on Day 7. On the same day, the average shell firmness achieved its maximum value. Shell firmness is related to water evaporation and subsequent drying of the eggs. The rate of water evaporation is influenced by the permeability of the shell and the number of pores in the shell (SIMEONOVOVÁ and MÍKOVÁ, 2003).
As already reported, the above-mentioned three egg indicators are interrelated. Due to egg drying, the increased water evaporation has a negative effect on egg weight (MATUŠOVIČOVÁ *et al*., 1986), which has also been confirmed in this study. Similar results were published by DE LEO *et al*. (2018). The authors reported an average weight loss of 1.67% after 35 days of egg torage.
According to SIMEONOVOVÁ *et al*. (1999), evaporation of water from egg contents depends on the environment, in particular temperature and humidity, as well as on the storage period. ALLEONI and ANTUNES (2004) also reported a reduction in egg weight during storage.
The HU indicates egg quality and the calculation is based on both the height of the thick albumen and the egg weight (CANER and YÜCEER, 2015). The initial value of HU represents the main marker for evaluating egg quality, and its expression provides an indication of the egg shelf-life as well as the storage conditions (FIGUEIREDO *et al*., 2014). As seen in Table 2, mean Haugh unit values ranged between 53.74 and 59.80 during egg storage in this study. DE LEO *et al*. (2018) also confirmed a HU decrease during the storage period of 42 days, which corresponds to similar earlier studies (CANER, 2005; CANER and YÜCEER, 2015; MORSY *et al*., 2015).
In general, Haugh units decrease during egg storage. This can be explained by changes in protein structure resulting in albumen thinning (SIMEONOVOVÁ *et al*., 1999). Results of JIN *et al*. (2011) were consistent with those obtained in this study. In contrast, lower values

within the entire storage period were reported by SAMLI *et al*. (2005) and AKYUREK and OKUR (2009). All of the cited authors stated that Haugh units were decreasing over time. As also reported by TOMCZYK *et al*. (2019), longer egg storage period resulted in a more noticeable decline in Haugh unit values.
A similar decrease was observed in the albumen index, which is based on both the width and height of the thick albumen. The values ranged from 1.370 to 10.500, depending on the storage period. Albumen index was decreasing with prolonged egg storage. The most significant decline occurred on Day 7, when the maximum weight loss and shell firmness, as well as minimum eggshell breaking point, water activity and Haugh units were all determined. Similarly, LAZAR (1990) also reported a decrease in albumen index value with increase in storage time and MÍKOVÁ and DAVÍDEK (2000) ranked the albumen index among the main indicators of egg freshness.

Table 2. Changes in physico-chemical parameters during egg storage.

Storage period (days)	1	7	14	21	28
Water activity (a_w)	0.91±0.00^{a}	0.91±0.00^{a}	0.91±0.01^{a}	0.91±0.00^{a}	0.91±0.00^{a}
Weight (g)	60.47±2.84^{a}	58.20±3.71^{a}	58.23±2.60^{a}	59.00±2.14^{a}	58.23±2.26^{a}
Color	8.33±1.63^{a}	9.17±2.23^{a}	9.50±1.64^{a}	9.33±0.52^{a}	9.00±1.26^{a}
Haugh units	59.80±23.03^{a}	57.85±11.91^{a}	58.33±14.28^{a}	56.15±13.83^{a}	54.57±9.76^{a}
Albumen index (%)	10.50±1.30^{a}	6.86±3.49^{b}	2.25±1.44^{c}	1.23±0.26^{c}	1.42±0.77^{c}
Yolk index (%)	36.70±2.90^{a}	35.51±2.59^{a}	32.92±2.63^{ab}	34.85±1.67^{ab}	31.02±2.99^{b}
Eggshell breaking force (kgf)	3.55±1.06^{a}	4.97±1.08^{a}	4.69±0.73^{a}	4.21±1.25^{a}	3.75±1.53^{a}
Egg quality grade	AA-B^{a}	AA-B^{a}	AA-B^{a}	AA-B^{a}	B^{a}

$^{a-c}$ Different superscripts in each row indicate significant differences between the mean values (Tukey´s, $p<0.05$).

The yolk index is also an excellent indicator of egg freshness, which is based on the measurements of the yolk height and width (YÜCEER and CANER, 2014). Yolk index ranged from 30.74% to 36.90% depending on the length of storage. Yolk width increased during storage as the vitelline membrane lost its elasticity due to aging. Similarly, in a study by DE LEO *et al*. (2018), the yolk index ranged from 30.99% to 39.96% during 42 days of egg storage.
As reported by NEDOMOVÁ (2012), the yolk index decreases with prolonged egg storage. During storage, the structure of vitelline membrane is changed, its strenght decreases, which may, in a combination with water gained from the egg albumen, lead to the enlargement and deformation of the yolk (SIMEONOVOVÁ *et al*.,1999). Similar decrease in yolk index during egg storage was also observed in other studies (SAMLI *et al*., 2005; AKYUREK and OKURK, 2009; NEDOMOVÁ and SIMEONOVOVÁ, 2010), where the storage period was identified as the limiting factor.
Another indicator of egg quality is the yolk color. The yolk color is a consumer factor without any nutritional significance. In this study, the yolk color ranged from 8 to 10 La Roche scale and became more intense as the egg aged. Similar to other studies (MÍKOVÁ and DAVÍDEK, 2000; JINN *et al*., 2011), it becomes more intense during egg storage.

Changes in egg quality parameters during storage may also be accompanied by egg contamination with microscopic filamentous fungi. Therefore, not only the temperature, but also the relative humidity is of great importance during egg storage. Microscopic filamentous fungi need sufficient moisture to grow. Thus, eggs are more frequently contaminated in high relative humidity environments. DE REU *et al*. (2006) and MESSENS *et al*. (2007) reported that a higher level of eggshell contamination led to a better possibility for penetration of micromycetes into the egg contents. Shell pores allow a potential route of entry for fungi and this can lead to both health hazards and a foul smell and taste of an egg. Therefore, detecting egg contamination is an important aspect of public health concern. In addition, volatile organic compounds (VOCs) are produced by micromycetes as they proliferate.These chemicals are emitted back into the environment through the eggshell. Currently, 69 fungi are known as volatile emitters (LEMFACK *et al*., 2014).

The fact that table eggs are often contaminated with fungal spores was also confirmed by TOMCZYK *et al*. (2019) during three weeks of egg storage under various storage conditions. NEAMATALLAH *et al*. (2009) also reported the presence of microscopic filamentous fungi in 38% of eggs in their study with an average count of 3.53 log CFU/mL. The authors have identified the following mold genera: *Aspergillus* (14%), *Penicillium* (9%), *Fusarium* (1%), *Mucor* (6%), *Rhizopus* (4%), and *Cladosporium* (5%).

In this study, micromycetes were isolated from the shell surface using DG–18 and DRBC selective culture media. According to STN ISO 21527-1, DRBC culture medium is used for enumeration of yeasts and molds in food products with water activity greater than 0,95 (e. i. egg contents). However, the measurements in this study gave a value of $a_w \leq 0.95$ in the eggshell which requires the use of DG–18 medium (STN ISO 21527-2). Some mold genera and species are xerophilic and need lower a_w to grow. Therefore, mycological examination of the eggshell was performed using both the above-mentioned culture media in this study. For genus identification, macroscopic evaluation of colony size and characteristics on special culture media is of great importance. Czapek agar medium often provides useful information with regards to growth rates on low water activity media. However, experience has shown that the brand of agar medium used may affect the appearance of colonies that grow on it (SAMSON and PITT, 2000). Therefore, microscopic evaluation of fungal structure is used for more accurate genus identification. Phenotypic key identification is difficult, because these characteristics are unstable and micromycetes sometimes appear to be atypical with slow spore formation and aberrant production of conidiophores (CHEŁKOWSKI and VISCONTI, 1992).

As seen in Table 3, fungal isolates in this study belonged to *Penicilium* spp., *Fusarium* spp., *Alternanria* spp. and *Cladosporium* spp. After inclusion of mold isolates into individual genera, the ability of both culture media to capture these genera was evaluated (Tables 2 and 3).

Despite the fact that STN ISO 21527 recommends the use of two selective culture media depending on water activity of particular food samples, different capture ability has been confirmed for both media. DG-18 medium was more suitable for isolation of *Cladosporium* spp. and *Alternaria* spp. On the contrary, capture of *Fusarium* spp. and to a lesser extent *Penicillium* spp., was significantly higher on DRBC medium. Similar results have been published by WEIDENBORNER *et al*. (1995) and PEREIRA *et al*. (2010). The authors reported that DRBC medium permited the isolation of a wider range of fungal genera/species, regardless of the type of food under study.

In this study, the counts and composition of mycoflora on the eggshell varied during the storage period. Similar to changes in egg quality indicators, changes in numbers of micromycetes were also confirmed between 7 and 28 days of egg storage. However, these

changes did not show any statistical significance (Table 2). With the use of DRBC medium, significant changes in the composition of mycoflora have been noticed on Day 21, when *Cladosporium* spp. was no more detected and a significant increase in *Fusarium* isolates (57%) has been observed. On Day 28, *Cladosporium* accounted for only 16% of the total mycological representation. Regarding the DG-18 medium, changes in the genus composition were first noticed on Day 14 with the dominance of *Penicillium* spp. (Table 3). The presence of *Alternaria* spp. (14%) was confirmed on Day 21 of egg storage.

Table 3. Mycological contamination of eggshells during the entire storage period.

Day	log CFU/mL	Percentage of isolates
		DRBC
1	1.95±1.98	*Cladosporium* spp. (50%) *Penicillium* spp. (25%) *Fusarium* spp. (25%)
7	2.23±2.88	*Cladosporium* spp. (50%) *Penicillium* spp. (33%) *Fusarium* spp. (17%)
14	1.85±1.65	*Cladosporium* spp. (42%) *Penicillium* spp. (16%) *Fusarium* spp. (42%)
21	1.85±1.76	*Penicillium* spp. (43%) *Fusarium* spp. (57%)
28	2.15±1.73	*Cladosporium* spp. (16%) *Penicillium* spp. (42%) *Fusarium* spp. (42%)
		DG-18
1	1.95±1.87	*Cladosporium* spp. (50%) *Penicillium* spp. (40%) *Fusarium* spp. (10%)
7	1.90±1.96	*Cladosporium* spp. (86%) *Penicillium* spp. (14%)
14	1.90±1.88	*Cladosporium* spp. (20%) *Penicillium* spp. (60%) *Fusarium* spp. (20%)
21	1.48±1.43	*Cladosporium* spp. (58%) *Penicillium* spp. (14%) *Fusarium* spp. (14%) *Alternaria* spp. (14%)
28	1.95±1.85	*Cladosporium* spp. (56%) *Penicillium* spp. (44%)

The results are expressed as the (average±standard deviation) of six independent measurements.

All the physico-chemical and mycological parameters of table eggs during the storage period of 28 days were further analyzed statistically using the method of multiple factor analysis (MFA) where the storage period was the main qualitative factor.

The results of MFA showed four selected components that explained 63.39% of the total variation in the results of experimental eggs. The first dimension (Dim1) explained 18.66% of variation, dimension 2 (Dim2) 17.22%, dimension 3 (Dim3) 15.20%, and dimension 4 (Dim4) 12.92%.

Contribution of the analyzed data in Dim1 was mainly related to storage period (51.36%, r=0.95) and physicochemical variables (46.70%, r=0.94). The first two dimensions explained 35.88% of variance (Fig. 1). The highest contribution in Dim 1 included albumen index (r=0.92), yolk index (r=0.62) and egg weight (r=0.41). Dim2 was characterized by the contribution of the effect of storage period (48.78%, r=0.89) and microbial parameters (37.25%, r=0.80) on analyzed variables. Microbiota count analyzed on DRBC agar (r=0.78) with water activity (r=-0.49) were correlated in Dim2. In the first two dimensions, these variables were correlated on statistical significant level of alfa<0.05 (Fig. 2).

Dim3 was mostly related with the storage period, as well as with combination of physicochemical and microbial parameters of experimental eggs. Correlation coefficients for microbial variables in Dim3 were determined as follows: CFU analyzed on DG–18 agar (r=0.57), water activity (r=0.45), yolk index (r=-0.39) and eggshell breaking force (r=-0.47).

The highest contribution on Dim4 was related with physicochemical variables (43.38%). However in Dim4 only the correlated CFU analysed on DG–18 agar (r=0.39) was statistically significant.

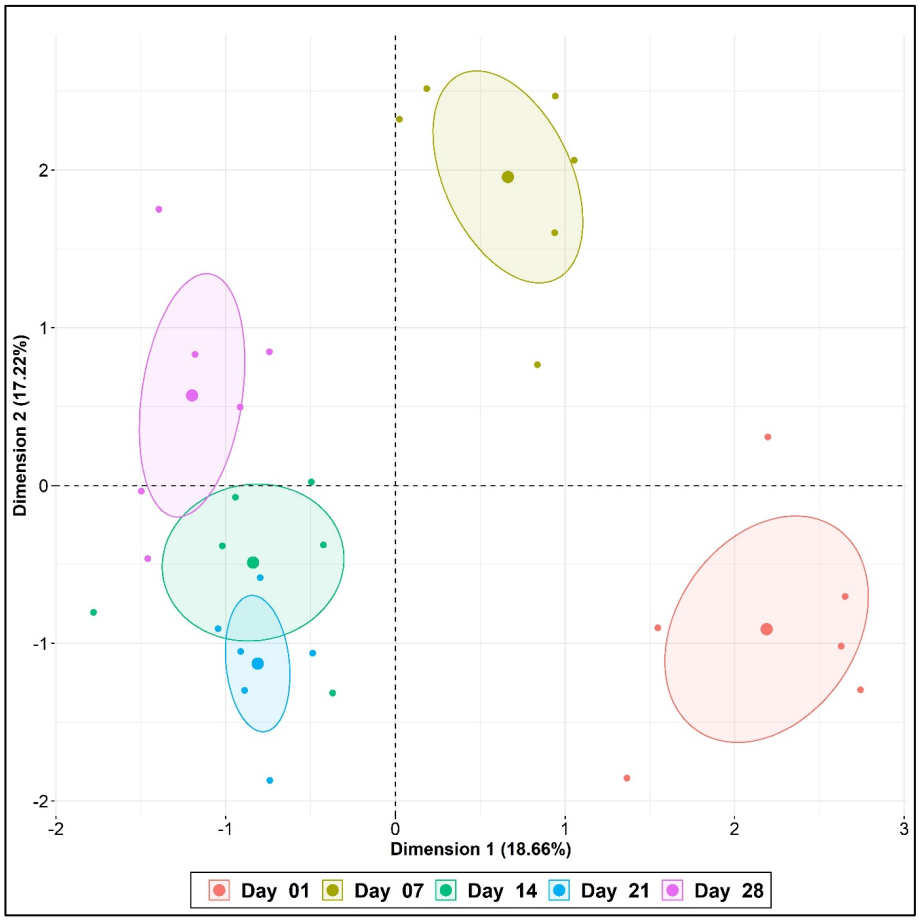

Figure 1. Plot of individuals in the first and second extracted dimensions during egg storage period.

From the results obtained by the MFA method, it can be concluded that the effect of storage conditions on experimental eggs was significant. The MFA method showed differences between experimental egg groups during the storage period. Analyzed variables of experimental eggs were more similar to each other on Days 14, 21 and 28 than on other days during the storage period. On experimental days 1 and 7, the eggs were significantly different from each other as well as from experimental eggs analyzed on Days 14, 21 and 28 in all monitored variables.

3.2. Isolation of fungal DNA

To overcome the poor diagnostic sensitivities and long turnaround times associated with the detection and identification of fungal pathogens in samples by cultivation, non-cultivation methods including the polymerase chain reaction (PCR) are increasingly being used for exact confirmation and more accurate identification of micromycetes. The

ultimate sensitivity of any PCR assay for the detection of fungal pathogens depends on the efficient lysis of fungal cells in the tissue sample and the purification of DNA that is free of PCR inhibitors. Fungi have cell walls that impede lysis and the recovery of nucleic acids. Furthermore, highly sensitive and specific nucleic acid-based methods for the detection of fungi necessitate the use of DNA extraction reagents that are free of contaminating fungal nucleic acids (FREDRICKS *et al.*, 2005).

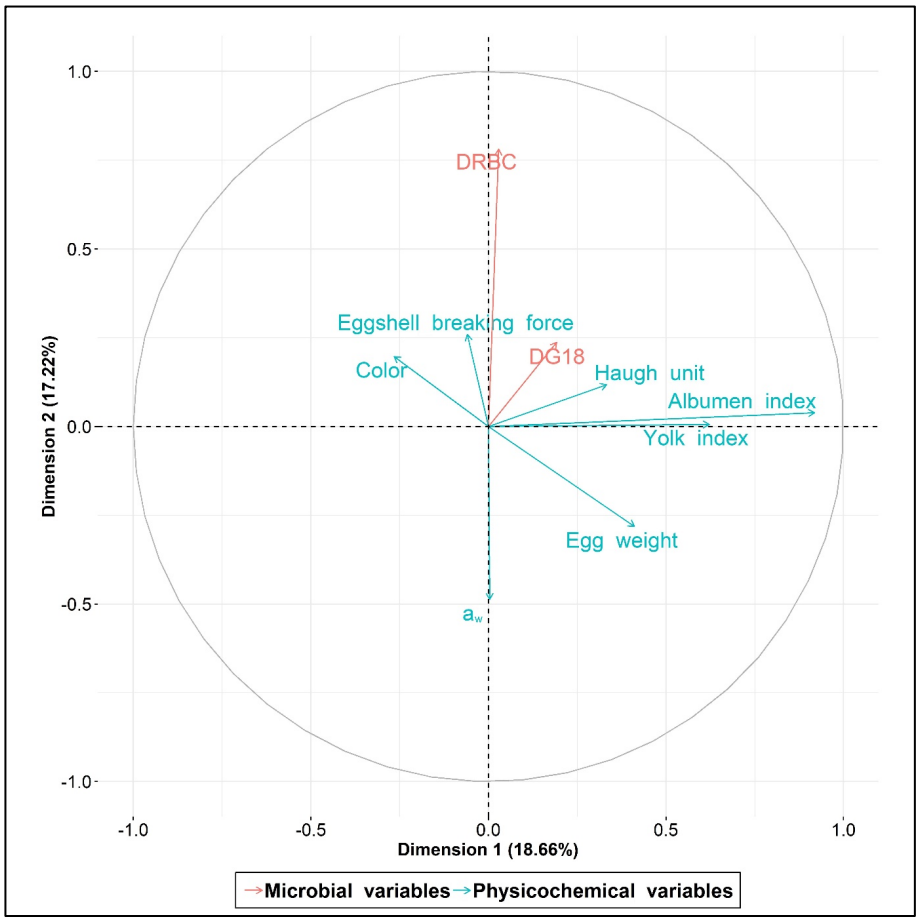

Figure 2. Correlation plot of variables in the first and second extracted dimensions during egg storage period.

In the case of the direct detection of molds in food samples (especially eggshell), DNA yield and purity depends primarily on the quantity and quality of the material taken. To isolate fungal DNA, it is first necessary to effectively disrupt the cell wall, lyse the cytoplasm and nucleus membrane, precipitate the proteins and remove the DNA from a number of inhibitors that can reduce the effectiveness of PCR. The individual steps can be executed using chemicals by known methodological procedures, or using commercial kits to facilitate isolation. In this case, however, it may be a problem to optimize the methodological process, because some chemicals in diagnostic kits are subject to corporate secrecy (ČMOKOVÁ *et al.*, 2014). The cell walls can be disrupted by homogenising of frozen sample in mortar using liquid nitrogen (GARG *et al.*, 2009; UCHIDA *et al.*, 2009), by

vortexing with glass or zircon beads (EBIHARA *et al*., 2009; SATO *et al*., 2010), or by repeated freezing (LITZ and CAVAGNOLO, 2010).
Membrane lysis is made with detergents (e.g. Triton X-100, sodium laurysulfate) followed by nucleic acids release into a buffered solution which contains chelators, most commonly EDTA and bonding calcium cations serving as a nucleases cofactor to prevent cleavage of the released DNA. To increase the nucleic acid purity, proteinase K is sometimes added into the lysis solution to cleave proteins including DNA-bound histones. A commonly used method is the incubation with proteinase K and subsequent completion of isolation using a commercial kit (BERGMANS *et al*., 2010; ALEXANDER *et al*., 2011; BEIFUSS *et al*., 2011; WISSELINK *et al*., 2011). In this study, three isolation procedures of fungal DNA were used and compared: 1. Combination of liquid nitrogen and heat 95°C; 2. Proteinase K and FG1 lysis solution; and 3. Zircon and glass bead isolation with simultaneous effects of proteinase K and ultrasonication of 50 Hz. Measurements of DNA concentration (Table 4) provided useful information on which of the three test procedures is the most effective for cell wall lysis and DNA extraction. DNA samples with the highest concentration and purity were used for further analysis. After PCR identification, the effectiveness of isolation procedures in relation to individual mold genera was re-evaluated.

Table 4. Comparison of DNA concentrations (ng/μL) yielded by three extraction procedures (average±SD).

	Procedure 1	Procedure 2	Procedure 3	One-Way ANOVA *P* value
Penicillium spp.	36.372±17.272^{C}	49.146±26.657^{B}	97.669±12.225^{A}	<0.001
Cladosporium spp.	12.462±4.807^{B}	35.585±11.433^{A}	38.175±20.410^{A}	<0.001
Fusarium spp.	22.335±9.134^{C}	64.222±18.859^{B}	71.554±17.674^{A}	<0.001
Alternaria alternata group	10.867±0.103^{C}	29.017±0.618^{B}	49.650±0.812^{A}	<0.001

$^{A-C}$ Different superscripts in each row indicate significant differences between the mean values (Tukey´s, $p<0.05$).

As can be seen in Table 4, the lowest average values for all identified genes were obtained by isolation procedure using a combination of thermal shock and liquid nitrogen. Among four mold genera, this procedure was most effective for *Penicilium* spp. isolates, where the average DNA concentration achieved a value of (36.372±17.272 ng/μL). The first procedure proved to be the worst for isolates of the genus *Cladosporium*. The second isolation procedure involving the use of proteinase K and a lysing solution FG1 appeared to be sufficient for isolates of the genus *Fusarium* (average DNA concentration was 64.222±18.859 ng/μL). The third method of isolation with a combination of zirconium and glass beads, proteinase K and ultrasonic waves, appeared to be the most effective for all four mold genera tested (Figs. 3-5). However, the highest average DNA concentration was recorded in *Penicillium* spp. (97.669±12.225 ng/μL). The last procedure was also sufficient for *Cladosporium* spp. (38.175±20.410 ng/μL) where the other isolation methods did not yield satisfactory results. In general, the purity of DNA obtained by all the three isolation procedures was very high (1.75-1.91).

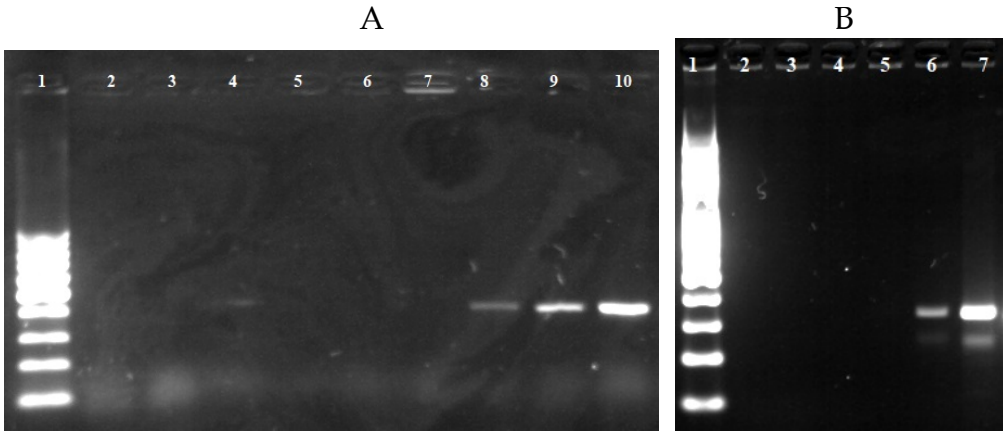

Figure 3. Identification of *Penicillium* spp. (A) and *Fusarium* spp. (B) - comparison of three DNA isolation procedures (1 - 3).
Figure A: Line 1 - 100 bp ladder; Lines 4,7,10 - reference strain *Penicillium chrysogenum* CCM F-362; Lines 2,3,5,6,8,9 - isolates of *Penicillium* spp. (336 bp). Lines 2,3,4 - Procedure 1; Lines 5,6,7 - Procedure 2; Lines 8,9,10 - Procedure 3.
Figure B: Line 1 - 100 bp ladder; Lines 2,4,6 - isolates of *Fusarium* spp.; Lines 3,5,7 - reference strain *Fusarium sporotrichioides* CCM F-352 (410 bp). Lines 2,3 - Procedure 1; Lines 4,5 - Procedure 2; Lines 6,7 - Procedure 3.

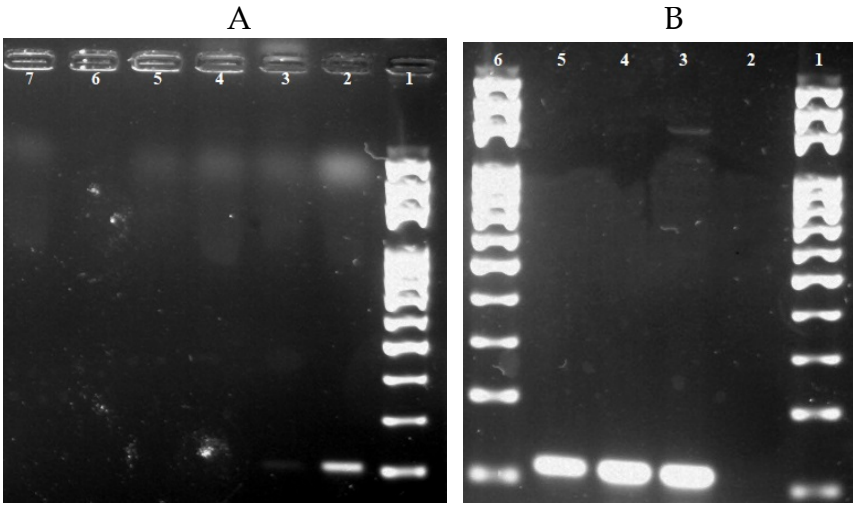

Figure 4. Identification of *Alternaria alternata* - comparison of three DNA isolation procedures (1 - 3).
Figure A: Line 1 - 100 bp ladder; Lines 2,5 - reference strain *Alternaria alternata* CCM F-397; Lines 3,4,6,7 - isolates of *Alternaria alternata* (118 bp). Lines 2,3,4 - Procedure 2; Lines 5,6,7 - Procedure 1.
Figure B: Line 1 - 100 bp ladder; Line 2 - negative control; Lines 3,4,5 - isolates of *Alternaria alternata*; Line 5 - reference strain *Alternaria alternata* CCM F-397 (118 bp). Lines 3,4,5 -Procedure 3.

Statistically significant differences (p<0.001) in the DNA concentrations obtained by three isolation procedures were observed in *Penicillium* spp., *Fusarium* spp. and *Alternaria alternata* group (Table 4). In *Cladosporium* spp., statistically significant difference was only recorded for the first isolation procedure where the minimum average concentration of DNA was obtained. The effectiveness of fungal DNA isolation using a combination of mechanical and chemical actions was also confirmed by LIU *et al*. (2000). However, other authors have reported various methods of cell wall destruction. In the most common

method, fungal mycelium is milled with liquid nitrogen or glass beads (LEE *et al*., 1988, WU *et al*., 2001). Some researchers also used dry ice (GRIFFIN *et al*., 2002), glass or magnetic beads (FAGGI *et al*., 2005), enzymatic cleavage (LI *et al*., 2002), or benzyl chloride (XUE *et al*., 2006). Although these techniques generally provide DNA of satisfactory quantity and quality, the greater problem arises with the *Cladosporium* DNA.
Melanized cell walls contain complex of polysaccharides and various secondary metabolites, including complex phenolic compounds, which hamper successful isolation of DNA (ADAMS *et al*., 1994). Due to the complexity of the fungal cell wall, conventional methods are often not appropriate for DNA extraction (KARAKOUSIS *et al*., 2006) and, compared to mammalian cells (WONG *et al*., 2007), the use of rigorous techniques is required (YANO *et al*., 2003).

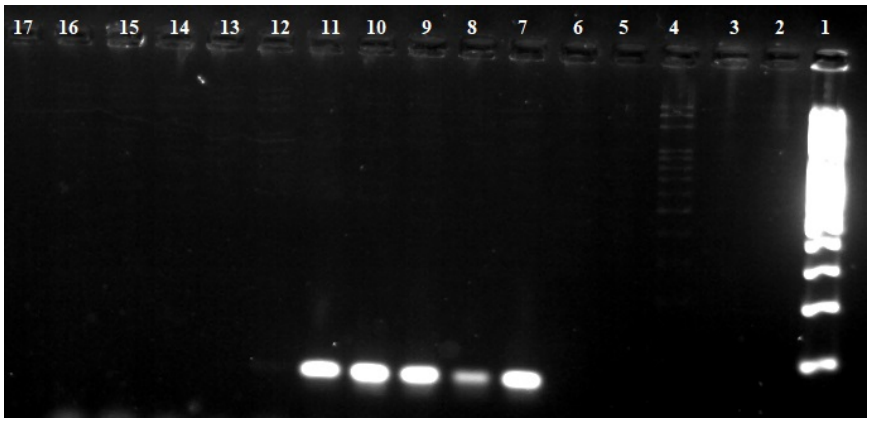

Figure 5. Isolation of *Cladosporium* spp. - comparison of three DNA isolation procedures (1 - 3).
Line 1 - 100 bp ladder; Lines 2,3,4,5,7,8,9,10,12,13,14,15,16 - isolates of *Cladosporium* spp.; Lines 6,11,17 - reference strain *Cladosporium cladosporioides* CCM F-348 (87 bp). Lines 2,3,4,5,6 - Procedure 1; Lines 7,8,9,10,11 - Procedure 3; Lines 12,13,14,15,16 - Procedure 2.

In this study, the combination of glass and zircon beads, proteinase K, ultrasound, and a commercially distributed insulating kit, yielded DNA in sufficient concentration and quality to identify the genus *Cladosporium* by PCR method. The PCR products obtained in this study were further separated by agarose gel electrophoresis. This procedure verified the DNA integrity required for reliable identification of fungal isolates at the genus level by the PCR method.

3.3. Identification of micromycetes

Identification of fungal isolates from table eggs was performed with the help of PCR method. The main advantage of this method lies in its high sensitivity and detection rate (MEHLIG *et al*., 2014) ranging from several hours to 2-3 days. Methods that allow the detection of genus without futher specification (pan-dermatophyte PCR) are focused on conservative DNA segments (KANO *et al*., 2003). Most current methods used to identify major fungal species are targeted at the ribosomal DNA domains (including 18S, ITS1, 5,8S, ITS2 and 28S). Specifically, the ITS region provides sufficient differentation among micromycete species. Methods based on classical PCR are most frequently used to detect fungal genus as a whole without more precise determination (BRILLOWSKA *et al*., 2007,

2010a,b; KONDORI *et al*., 2010; KIM *et al*., 2011). By this method, 30 isolates of *Fusarium* spp. (Fig. 6A) and 27 isolates of *Penicillium* spp. (Fig. 6B) were identified in this study, where the ITS region and 5.8S rRNA were used as the target site.

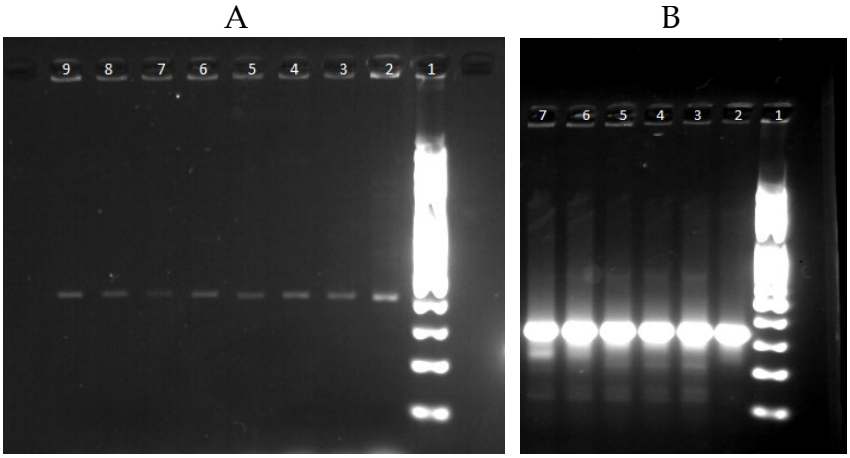

Figure 6. Identification of *Fusarium* spp. (A) and *Penicilium* spp. (B) by PCR method.
Figure A: Line 1 - 100 bp ladder; Lines 2,3,4,5,6,7,8,9 - isolates of *Fusarium* spp. (410 bp). Figure B: Line 1 - 100 bp ladder; Lines 2,3,4,5,6,7 - isolates of *Penicillium* spp. (336 bp).

Unlike classical PCR, multiplex PCR offers identification of major mold species (LI *et al*., 2002). Multiplex PCR was used to identify the prevalent species within *Alternaria alternata* group and *Cladosporium* spp. (Figs. 7A and 7B). *Alt a 1* gene sequence was selected as the target site for identification of *Alternaria alternata* group. Strains of *Cladosporium* spp. (35 isolates) were identified by specific sequence in mitochondrial (mt) small subunit rRNA (ZENG *et al*., 2006).

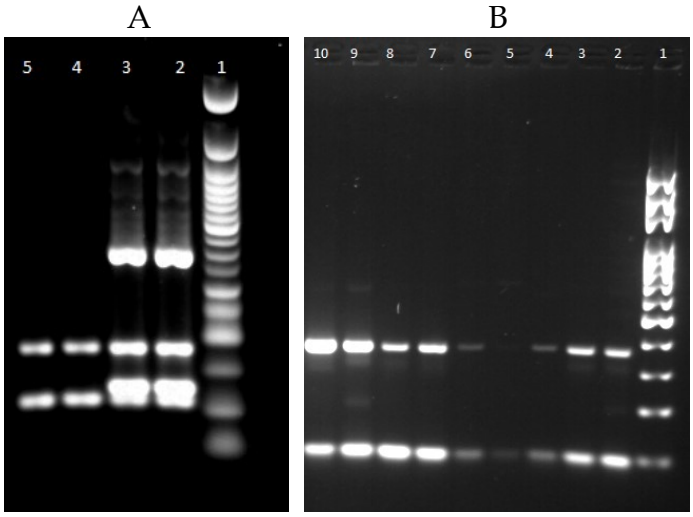

Figure 7. Identification of *Alternaria alternata* group (A) and *Cladosporium* spp. (B) by mutliplex PCR method.
Figure A: Line 1 - 50 bp ladder; Lines 2,3 - isolates of *Alternaria alternata* group (400 bp, 195 bp, 118 bp), Lines 4,5 - unidentified isolates.
Figure B: Line 1 - 100 bp ladder; Lines 2,3,4,5,6,7,8,9,10 - isolates of *Cladosporium* spp. (370 bp, 87 bp).

The PCR products obtained in this study were further sequenced and the homologues of the amplified DNA sequences were searched for in the GenBank-EMBL database. PCR genus identification correlated perfectly with the results of both macroscopic and microscopic identifications. Based on these results, fungal isolates from the eggshells were identified as *Cladosporium* spp. (35 isolates), *Fusarium* spp. (30 isolates), *Penicilium* spp. (27 isolates), and *Alternaria alternata* group (1 isolate). With the exception of *Cladosporium* spp., the remaining three fungal genera are potentially toxinogenic common pathogens of food crops. Their increasing occurrence in table eggs was reported by NEAMATALLAH *et. al*. (2009). The authors found that 38% of the examined eggs were contaminated by potentially toxinogenic micromycetes of the genus *Aspergillus* (14%), *Penicillium* (9%), *Fusarium* (1%), *Mucor* (6%), *Rhizopus* (4%) and *Cladosporium* (5%). GILARDI *et al*. (2015) confirmed the presence of the same species on the eggshell as found in this study (*Alternaria alternata* group), which was manifested as black spots on the inner shell membrane. Based on these findings, there is a high probability that the contaminating toxinogenic micromycetes on the eggshell may serve as a potential source of mycotoxins found in the egg contents. This theory has already been confirmed by EL MALT (2015).
Mycological analysis of stored eggs also indicated the presence of micromycetes on the shell surface in other studies. In Nigeria, micromycetes of the genus *Penicillium* were found in 82.5% of the examined egg samples (OBI and IGBOKWE, 2009). GRECO *et al*. (2014) reported that *Penicillium* is the second most common genus of microscopic filamentous fungi that contributes to the contamination of eggs in Buenos Aires, La Pampa and the province of Rio Negro. This genus was also placed at the forefront by EL MALT (2015). The prevalence of *Penicillium* spp. has also been demonstrated in this study.
Problems with the occurrence of microscopic filamentous fungi in stored eggs concern the entire world, but especially African countries which account for around 5% of the world egg production (MOTTET and TEMPIO, 2017). In these countries, environmental conditions are combined with very poor hygiene, resulting in the survival and proliferation of microorganisms (SALIHU, 2015).

4. CONCLUSION

The results of this study confirmed that storage period has significant impact on egg quality. Therefore, commercial freshness of table eggs can be extended by maintaining appropriate storage period and adequate storage conditions. In this study, changes in egg quality and the counts of micromycetes were observed during egg storage at an average temperature of 14.3°C and a relative humidity of 61%. The composition of eggshell mycoflora indicates a risk araising from the presence of potentially toxinogenic micromycetes belonging to *Fusarium* spp., *Penicillium* spp. and *Alternaria alternata* group. Rapid and reliable identification of mold genera by specific PCR methods requires high quality and purity of fungal DNA. For that purpose, a new effective method of DNA extraction from fungal cells based on the combination of a commercial isolation kit, proteinase K and ultrasound was designed and implemented in this study.

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PAPER

EVALUATION OF THE CHEMICAL AND NUTRITIONAL PROPERTIES OF TUNISIAN ALMOND CULTIVARS

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ABSTRACT

The aim of this research was to evaluate for the first time protein, oil content, fatty acid profile and sugar composition for the main commercial almond cultivars in Tunisia in comparison to foreigners. Thus, fruits from twelve locals and five introduced cultivars from France, Italy and Spain were analyzed over two years. In fact, total oil content varied from 52.28% ('Blanco') to 60.95% ('Lsen Asfour') in the first year and from 47.75% ('Zahaaf') to 56.15% ('Mahsouna') in the second. However, the highest oleic acid content was noted in 'Francoli' (76.2%) for both years. It was followed by 'Sahnoun' (75.11%) firstly and 'Abiodh' (73.02%) secondly. Likewise, the highest linoleic acid content was observed in 'Porto' for both studied years (22.87% and 23.67%). The highest palmitic acid content was detected in 'Porto' (7.02%) and in 'Tuono' for the consecutive years. Sugars profile was quite distinctive among cultivars. The cultivar 'Porto' presented the highest total sugars (5.8 g/100g DW) and sucrose contents (4.96 g/100g DW). Nevertheless, protein content doesn't show extreme values. For both years, the local cultivar 'Zahaaf' presented the highest protein content (27 g/100g DW) while introduced French cultivar 'Fournat de Breznaud' presented the lowest protein content (17 g/100g DW). All the analyzed components were different significantly according to cultivar and year effects. Results evidenced that the local Tunisian cultivars are highly rich in oil and fatty acids particularly oleic and linoleic acids, confirm the almond kernel as a high nutritional dietetic source and underline the high adaptability of some introduction.

Keywords: fatty acid composition, local cultivars, oil quality, *Prunus dulcis* L., sugar content

1. INTRODUCTION

The cultivated almond [*Prunus dulcis* (Miller) Webb] is a tree species whose domestication and spread has closely paralleled the rise of Eurasian civilizations. This tree-crop species is mainly planted for its edible seeds (kernels). Today, almonds are cultivated in more than 50 countries (http://faostat.fao.org), with approximately 95% produced in California, Australia and the Mediterranean Basin. In Tunisia, almond cultivation is present around the country mainly under rainfed conditions (GOUTA *et al.,* 2019). Moreover, the almond kernel represents the main nutrient source for many rural populations in the central and southern parts of the country. The high nutritive value of the almond kernel comes mainly from its high lipid content. In fact, it contains 52% of lipids, 20% of proteins and 20% of carbohydrates including 5% of water and 3% of soluble sugars (KADER, 1996). Almond quality was formerly related to the kernel flavor in addition to its physical parameters such as kernel size, percentage of double kernel and kernel rate without any attention to its nutraceutical composition (ROMOJARO *et al.,* 1988; NANOS *et al.,* 2002). At this moment, however, the nutritive value of almond kernel related to lipid, sugar, protein and mineral richness are being evaluated as main component of the almond kernel quality.
Different studies have reported that almonds consumption can significantly lower total and low-density-lipoprotein (LDL) cholesterol in plasma, reduce risk for heart disease and prevent several forms of cancer and inflammation (JENKINS *et al.,* 2008). The beneficial health effect of almond was attributed to its high content of mono and poly-unsaturated fatty acids (ROS and MATAIX, 2006). Moreover, the high (oleic acid/linoleic acid) ratio is used in determining the kernel quality due to its preventive effect on lipid oxidation and oil stability (KODAD *et al.,* 2010). In addition, negative cholesterol effects can be treated by an equilibrate lipid diet based on nut consumption including almonds (MUSA-VELASCO *et al.,* 2016). Furthermore, almond oil contains antioxidants and fat-soluble bioactive compounds that make it oil with interesting nutritional and cosmetic properties (RONCERO *et al.,* 2016). In this context several studies have been published about total oil and fatty acid profile of some almond cultivars (ÖZCAN et al., 2010; YILDIRIM *et al.,* 2016; ČOLIĆ *et al.,* 2017; SOCIAS I COMPANY *et al.,* 2018). In addition, sugars composition in the almond kernel has been reported in many studies (KAZANTZIS *et al.,* 2003; BALTA *et al.,* 2009). However, as far as we know, very few researches were carried out to characterize the nutraceutical values together with these chemical compositions (SOCIAS I COMPANY *et al.,* 2010; KODAD, 2017). This information is null regarding the rich almond germplasm from Tunisia considered as an almond diversification center.
The objective of this study was to determine for the first time the chemical and nutritional composition (including total oil, protein contents, fatty acid and sugar composition) of most important Tunisian almond cultivars. Moreover, the interaction of genotype x environment would be deeply discussed. Findings of the present work will be important for selecting cultivars with more stable macronutrients composition from year to year and consequently less subject to climate changes.

2. MATERIALS AND METHODS

2.1. Plant material

Plant material assayed included twelve Tunisian almond cultivars ('Dillou', 'Khoukhi', 'Blanco', 'Abiodh', 'Lsen Asfour', 'Achaak', 'Zahaaf', 'Fekhfekh', 'Ksontini', 'Sahnoun',

'Porto', and 'Mahsouna') and five almond cultivars originating from Italy ('Mazetto' and 'Supernova'), Spain ('Francoli'), France ('Lauranne' and 'Fournat de Breznaud') assayed as reference. The local cultivars used in this work (Fig. 1) are early flowering, auto-incompatible and their pomological and agronomical characteristics were previously well described (GOUTA *et al.*, 2011; GOUTA *et al.*, 2019). All studied almonds were collected from the national collection in Sidi Bouzid in central-western part of Tunisia during two consecutive years 2009 and 2010.

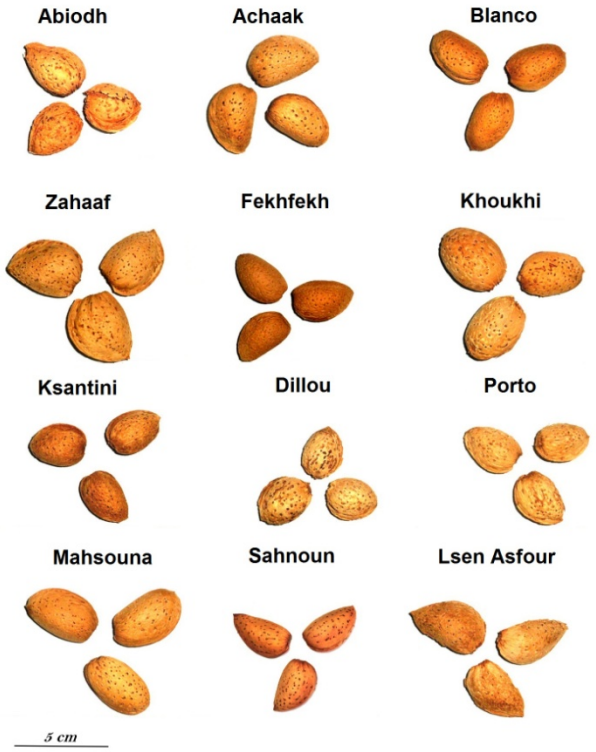

Figure 1. Pomological characteristics among the native Tunisian almond cultivars.

2.2. Oil and fatty acid determination

Kernels were preliminary blanched for 3 min in boiling water eliminating seed coat. The kernels were dried at 25°C until constant weight and then ground. Oil was extracted using about 5g of ground almond in a Soxtec Avanti 2055 fat extractor (Foss Tecator, Höganäs, Sweden) for 2 h using 70 ml of petroleum ether as solvent and keeping temperature at 135°C. To remove any residual ether, the extract was subject successively to vacuum evaporation for 15 min in a vacuum desiccator. Ten microliters of Butylated hydroxyl toluene methanol solution (BHT) as an antioxidant agent was added to each oil sample which was kept in an amber vial at -20°C until analysis. The percentage of the different fatty acids in oil samples was determined by capillarity gas chromatography of the fatty acid methyl esters (FAMEs). Methyl esters of the corresponding fatty acids were obtained by trans-esterification with KOH of each almond oil sample according to the official method UNE-EN (ISAO 5509, 2000). They were separated using a flame ionizing detector

(FID) gas chromatograph HP-6890 equipped with HP-Innowax column (30 m × 0.25 mm i.d.) and 0.25 μm film thinness (Agilent Technologies, Waldron, Germany). The FAMES identification was realized by comparison with relative chromatographic retention times of standard methyl esters mixture (Sigma-Aldrich, Madrid, Spain).

2.3. Sugar determination

Free sugar profiles were determined by a high performance liquid chromatography (HPLC, Agilent 1100, Germany) during the two consecutive years 2009 and 2010. In first step kernels samples were dried in an oven at 25°C until weight stabilized, ground in a mortar and then defatted using a soxhlet and ether petroleum as a solvent. Once defatted, a sample of 0.7 to 1.3 g of the remaining powder was moved to a falcon tube and mixed with of 9 ml MilliQ water. For protein denaturation, 0.5 ml of Carrez I (potassium ferrocyanide 15% w/v) and 0.5 ml of Carrez II (zinc acetate 30% w/v) solutions were added and kept under agitation in an agitator (Reax, Madrid, Spain) for 10 min. The resulting suspension was centrifuged at 8000 rpm for 20 min. The supernatant was recuperated and passed throw a nylon filter 0.45 μm before injection in a HPLC apparatus. A volume of 20 μl of the filtrate was injected in an interchange cationic column (Pb) CHO-682 (Transgenomic, Madrid, Spain). Sugar detection was performed according to the detection time of reference samples (Sigma, Madrid, Spain) of raffinose, sucrose, glucose and fructose.

2.4. Total protein determination

Protein fraction was obtained by the following formula:

$$\text{Protein percentage} = \text{total nitrogen percentage} \times Kc$$

with Kc presenting a conversion factor equal to 6.25 for almond. The total nitrogen content was obtained by the Dumas method (DUMAS, 1826). Almond kernels for each genotype were defatted as already mentioned (using soxhlet and ether petroleum solvent) and then analyzed by a LECO FP-528 Protein/Nitrogen Analyzer (LECO cooperation, Saint Joseph, MI, USA). A sample of 0.2 g of the resulting powder was incinerated at 850°C and the gases generated were passed through hot copper to remove oxygen. Nitrogen molecules with helium were measured in a cell differential thermo-conductivity. Then, data were read and interpreted with CPU-CAR-02 software. Results were expressed as percentage of nitrogen by kernel powder weight.

2.5. Statistical analysis

Three replicates of 20 kernels from each genotype were evaluated. The significance of cultivar, year and cultivar × year interaction effects for all studied components were tested on the 17 cultivars by ANOVA using SPSS 20.0. Differences between means were evaluated by using Duncan multiple range test. Correlations between traits were calculated from raw data of the two years using Pearson correlation coefficient. Trait mean values were used to perform a Principal Component Analysis (PCA).

3. RESULTS

3.1. Effect of the year and its interaction with the cultivar

The analysis of variance showed significant effect of cultivar and year for the fatty acids and sugars compositions and oil and protein contents in the seventeen almond cultivars assayed during two consecutive years. In addition, the interaction cultivar × year exhibited considerable variation for all analyzed parameters (Table 1). Besides the significant effect of the cultivar, a clear and significant environmental effect was noted in the oil content for all studied cultivars due to the specific climatic conditions of years tested. Some almond cultivars have shown high year to year stability in their fatty acids content compared to other cultivars. These results indicate that the year effect on the fatty acids composition in almond mainly depends on genotype. Stable values for some fatty acids were observed in cultivars such as 'Dillou', 'Sahnoun', 'Mazetto' and 'Mahsouna' for arachidic acid; 'Dillou', 'Khoukhi', 'Lsen Asfour', 'Sahnoun', 'Super Nova', 'Lauranne' and 'Mahsouna' for linolenic acid; 'Porto', 'Abiodh' and 'Mahsouna' for palmitic acid; 'Lsen Asfour' and 'Mahsouna' for palmitoleic acid; 'Lauranne' and 'Mahsouna' for stearic acid (Table 2).
The year effect was significant for different sugar amounts except raffinose percentage (Table 1). Moreover, studied cultivars show stable and similar year to year sugar percentage excepting the glucose percentage, confirming that the year to year stability depends on the specific characteristics of the genotype.

3.2. Oil content

The mean value of oil content over the 2 years varied from 47.75% for 'Zahaaf' to 60.95% for 'Lsen Asfour' (Table 2). In 2009, the mean value of total lipid was 56.23%, ranged for the local cultivars from 52.28% for Blanco to 60.95% for 'Lsen Asfour' and for the foreign cultivars from 53.36% for 'Francoli' to 55.93 % for 'Breznaud'. In 2010, the mean value of total oil was 51.39%, ranged for the local cultivars from 47.75% for 'Zahaaf' to 56.15% for 'Mahsouna' and for the foreign cultivars total lipid content ranged from 48.37% for 'Francoli' to 54.45% for 'Lauranne'. The values of total lipid content were found to be low for the European cultivars compared to the values registered in the Tunisian local cultivars. In fact, it was found in the range of 47-56% for 'Francoli' (Spain), 'Super Nova' and 'Mazetto' (Italy) and 'Laurane' and 'Fournat de Breznaud' (France).

3.3. Protein content

The mean value of the protein content was for almost studied cultivars higher in 2010 than in 2009, contrarily to the oil content which was higher in 2009 (Table 2). For the year 2009, the lowest contents were showed by the local cultivars 'Lsen Asfour' (14.49%) and 'Mahsouna' (17.34%) and the French cultivar 'Fournat de Breznaud' (17.84%) while the highest values ranged between 23 and 21.2% for 'Francoli', 'Zahaaf', 'Ksontini', 'Super nova' and 'Mazetto', respectively. In 2010 these same cultivars showed the highest protein content with a greater range of variation (27.15-23.35%). Likewise 'Mahsouna', 'Fournat de Breznaud' and 'Lsen Asfour' showed the lowest protein content (17.14-18.11%) the second year of study. However, the cultivars 'Dillou', 'Mahsouna' and 'Fournat de Breznaud' showed stable mean value of the protein content over the two year.

Table 1. Analysis of variance of fatty acid (Palmitic, Palmitoleic, Stearic, Oleic and Linoleic) content, total lipid content, sugar composition (Raffinose, Sucrose, Glucose, Fructose), total sugar content and protein content in the 17 assayed almond cultivars.

Source of variation	Df[1]	Mean squares											
		Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Total lipid	Raffinose	Sucrose	Glucose	Fructose	Total Sugar	Protein
Genotype (G)	16	**0.636**	**0.027**	**1.722**	**32.18**	**28.40**	**25.25**	**0.541**	**2.908**	**0.028**	**0.021**	**4.33**	**37.55**
Year (Y)	1	**1.547**	**0.048**	**1.498**	**189.36**	**172.58**	**596.627**	0.065	**15.514**	**0.036**	**0.003**	**19.651**	**50.28**
G × Y	16	**0.141**	**0.006**	**0.238**	**8.055**	**5.56**	**11.365**	**0.054**	**1.144**	**0.005**	**0.003**	**1.511**	**8.723**
Error	68	0.000	0.000	0.013	0.587	0.788	0.328	0.012	0.042	0.000	0.000	0.065	0.159

Mean squares in bold case present a level of significance of $P<0.001$.
[1]Df: Degree of freedom.

Table 2. Fatty acid (palmitic, palmitoleic, stearic, oleic, linoleic, arachidic, α-Linolenic), protein content and total lipid for each almond cultivar assayed during two consecutive years (2009 and 2010).

	Miristic		Palmitic		Palmitoleic		Margaric		Margaroleic		Stearic		Oleic	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
	Tunisian almond cultivars													
Dillou	0,04^{a}	0,05^{b}	6,19^{a}	6,46^{b}	0,52^{a}	0,56^{b}	0,04^{a}	0,05^{b}	0,09^{a}	0,10^{b}	1,36^{a}	1,53^{b}	72,10^{a}	69,49^{b}
Khoukhi	0,05^{a}	0,06^{b}	6,32^{a}	7,23^{b}	0,51^{a}	0,48^{b}	0,04^{a}	0,06^{b}	0,09^{a}	0,10^{b}	1,31^{a}	1,70^{b}	73,59^{a}	66,70^{b}
Blanco	0,04^{a}	0,04^{b}	6,14^{a}	6,29^{b}	0,41^{a}	0,43^{b}	0,04^{a}	0,05^{b}	0,09^{a}	0,09^{b}	1,40^{a}	1,35^{b}	70,65^{a}	70,42^{b}
Abiodh	0,03^{a}	0,06^{b}	6,88^{a}	6,85^{a}	0,59^{a}	0,54^{b}	0,05^{a}	0,02^{b}	0,09^{a}	0,05^{b}	1,75^{a}	1,62^{b}	73,56^{a}	73,02^{b}
Lsen Asfour	0,03^{a}	0,04^{b}	6,56^{a}	6,85^{b}	0,54^{a}	0,52^{a}	0,04^{a}	0,05^{b}	0,07^{a}	0,09^{b}	2,84^{a}	1,82^{b}	71,22^{a}	67,62^{b}
Achaak	0,02^{a}	0,05^{b}	6,62^{a}	7,25^{b}	0,53^{a}	0,40^{b}	0,05^{a}	0,03^{b}	0,08^{a}	0,06^{b}	2,69^{a}	2,40^{b}	73,06^{a}	67,95^{b}
Zahaaf	0,04^{a}	0,11^{b}	6,61^{a}	6,44^{b}	0,55^{a}	0,43^{b}	0,04^{a}	0,02^{b}	0,09^{a}	0,05^{b}	1,46^{a}	1,72^{b}	75,06^{a}	71,91^{b}
Fekhfekh	0,01^{a}	0,05^{b}	5,86^{a}	6,12^{b}	0,35^{a}	0,31^{b}	0,05^{a}	0,04^{b}	0,06^{a}	0,06^{a}	2,82^{a}	2,53^{b}	72,95^{a}	69,65^{b}
Ksontini	0,03^{a}	0,05^{b}	7,01^{a}	6,91^{b}	0,34^{a}	0,39^{b}	0,06^{a}	0,02^{b}	0,08^{a}	0,07^{b}	3,75^{a}	2,66^{b}	67,50^{a}	67,90^{b}
Sahnoun	0,03^{a}	0,03^{a}	6,28^{a}	6,42^{b}	0,53^{a}	0,46^{b}	0,05^{a}	0,05^{b}	0,09^{a}	0,10^{b}	2,00^{a}	1,92^{b}	75,11^{a}	71,05^{b}
Porto	0,03^{a}	0,03^{b}	7,02^{a}	7,04^{a}	0,52^{a}	0,43^{b}	0,05^{a}	0,06^{b}	0,08^{a}	0,10^{b}	2,38^{a}	2,13^{b}	66,70^{a}	66,28^{b}
Mahsouna	0,02^{a}	0,02^{a}	6,77^{a}	6,85^{a}	0,50^{a}	0,43^{a}	0,04^{a}	0,05^{b}	0,07^{a}	0,09^{b}	2,58^{a}	2,43^{a}	73,14^{a}	70,38^{a}

International reference almond cultivars														
Mazetto	0,02[a]	0,03[b]	6,77[a]	7,53[b]	0,47[a]	0,30[b]	0,05[a]	0,07[b]	0,08[a]	0,09[b]	2,63[a]	2,52[b]	72,56[a]	65,29[b]
Francoli	0,02[a]	0,04[b]	6,25[a]	6,40[b]	0,50[a]	0,49[b]	0,05[a]	0,06[b]	0,08[a]	0,10[b]	3,05[a]	2,50[b]	76,21[a]	76,15[b]
SuperNova	0,02[a]	0,02[b]	6,61[a]	7,24[b]	0,46[a]	0,48[b]	0,05[a]	0,06[b]	0,08[a]	0,10[b]	2,72[a]	2,17[b]	73,15[a]	69,89[b]
Lauranne	0,02[a]	0,02[b]	6,63[a]	6,79[b]	0,62[a]	0,55[b]	0,05[a]	0,05[b]	0,10[a]	0,10[a]	1,79[a]	1,79[a]	73,77[a]	70,67[b]
F. Breznaud	0,03[a]	0,03[b]	6,76[a]	6,84[b]	0,52[a]	0,51[b]	0,05[a]	0,05[b]	0,08[a]	0,09[b]	2,32[a]	1,94[b]	69,94[a]	69,56[b]
Min	0,01	0,02	5,86	6,12	0,34	0,30	0,04	0,02	0,06	0,05	1,31	1,35	66,70	65,29
Max	0,05	0,11	7,02	7,53	0,62	0,56	0,06	0,07	0,10	0,10	3,75	2,66	76,21	76,15
Mean	0,03	0,04	6,55	6,79	0,50	0,45	0,05	0,05	0,08	0,08	2,29	2,04	72,37	69,64
SD	0,01	0,02	0,33	0,39	0,07	0,08	0,01	0,01	0,01	0,02	0,70	0,40	2,53	2,65
CV	31,64	48,45	4,97	5,76	14,65	16,82	11,36	31,47	12,09	20,70	30,58	19,81	3,50	3,80

Table 2. Continues.

	Linoleic		**Arachidic**		**α-Linolenic**		**Gadoleic**		**Protein**		**Total Lipid**		**O/L ratio**		**USFA**	
	2009	**2010**	**2009**	**2010**	**2009**	**2010**	**2009**	**2010**	**2009**	**2010**	**2009**	**2010**	**2009**	**2010**	**2009**	**2010**
Tunisian almond cultivars																
Dillou	19,40[a]	21,37[b]	0,05[a]	0,03[a]	0,02[a]	0,02[a]	0,06[a]	0,07[b]	19,75[a]	19,49[a]	55,22[a]	52,81[b]	3,72[a]	3,25[b]	91,50[a]	90,86[b]
Khoukhi	17,86[a]	23,27[a]	0,05[a]	0,06[b]	0,01[a]	0,01[b]	0,06[a]	0,06[a]	20,48[a]	21,65[b]	55,80[a]	50,60[b]	4,12[a]	2,87[b]	91,45[a]	89,96[b]
Blanco	21,01[a]	20,84[b]	0,06[a]	0,06[b]	0,02[a]	0,02[b]	0,06[a]	0,07[b]	19,67[a]	18,39[b]	52,28[a]	53,83[b]	3,36[a]	3,38[b]	91,65[a]	91,26[b]
Abiodh	16,85[a]	17,54[b]	0,07[a]	0,07[b]	0,01[a]	0,07[b]	0,06[a]	0,03[b]	19,94[a]	18,48[b]	58,66[a]	48,61[b]	4,36[a]	4,16[b]	90,41[a]	90,55[b]
Lsen Asfour	18,40[a]	22,27[b]	0,09[a]	0,06[b]	0,02[a]	0,02[a]	0,07[a]	0,07[a]	14,49[a]	18,12[b]	60,95[a]	52,51[b]	3,90[a]	3,04[b]	89,62[a]	89,89[a]
Achaak	16,60[a]	21,51[b]	0,09[a]	0,09[b]	0,02[a]	0,06[b]	0,07[a]	0,03[b]	17,91[a]	18,37[b]	58,26[a]	55,53[b]	4,40[a]	3,16[b]	89,66[a]	89,46[b]
Zahaaf	15,91[a]	19,03[b]	0,06[a]	0,07[b]	0,02[a]	0,08[b]	0,06[a]	0,00[b]	22,89[a]	24,83[b]	54,01[a]	47,75[b]	4,712[a]	3,78[b]	90,97[a]	90,94[a]
Fekhfekh	17,60[a]	20,53[b]	0,10[a]	0,09[b]	0,02[a]	0,07[b]	0,07[a]	0,00[b]	17,74[a]	22,84[b]	57,76[a]	52,57[b]	4,14[a]	3,39[b]	90,55[a]	90,17[b]
Ksontini	20,89[a]	21,68[b]	0,12[a]	0,10[b]	0,02[a]	0,06[a]	0,08[a]	0,04[b]	21,95[a]	24,28[b]	54,33[a]	50,77[b]	3,23[a]	3,13[b]	88,38[a]	89,57[b]
Sahnoun	15,67[a]	19,34[b]	0,08[a]	0,08[a]	0,02[a]	0,02[a]	0,06[a]	0,07[a]	18,84[a]	20,17[b]	56,69[a]	49,96[b]	4,80[a]	3,67[b]	90,78[a]	90,39[b]
Porto	22,87[a]	23,67[a]	0,08[a]	0,08[b]	0,02[a]	0,00[b]	0,07[a]	0,07[a]	19,92[a]	22,71[b]	56,99[a]	49,44[b]	2,92[a]	2,80[b]	89,57[a]	89,95[b]
Mahsouna	16,54[a]	18,93[a]	0,10[a]	0,10[a]	0,02[a]	0,02[a]	0,08[a]	0,07[a]	17,35[a]	17,15[a]	60,37[a]	56,15[b]	4,70[a]	3,72[a]	89,67[a]	89,31[a]

International reference almond cultivars																
Mazetto	17,06[a]	23,67[b]	0,12[a]	0,12[a]	0,02[a]	0,03[b]	0,07[a]	0,06[a]	21,27[a]	27,15[b]	54,30[a]	48,82[b]	4,25[a]	2,76[b]	89,62[a]	88,96[b]
Francoli	13,45[a]	13,92[b]	0,11[a]	0,10[b]	0,02[a]	0,03[b]	0,08[a]	0,07[b]	23,02[a]	23,35[b]	53,36[a]	48,37[b]	5,66[a]	5,47[b]	89,66[a]	90,07[b]
SuperNova	16,58[a]	19,69[b]	0,11[a]	0,11[b]	0,03[a]	0,03[a]	0,08[a]	0,08[a]	21,63[a]	26,33[b]	55,48[a]	50,60[b]	4,41[a]	3,55[b]	89,73[a]	89,58[b]
Lauranne	16,79[a]	19,75[b]	0,08[a]	0,08[b]	0,03[a]	0,01[a]	0,06[a]	0,08[b]	20,55[a]	18,02[b]	55,52[a]	54,45[b]	4,39[a]	3,58[b]	90,55[a]	90,42[b]
F. Breznaud	20,08[a]	20,77[b]	0,08[a]	0,07[b]	0,02[a]	0,00[b]	0,06[a]	0,07[a]	17,84[a]	17,79[a]	55,93[a]	50,91[b]	3,43[a]	3,35[b]	90,02[a]	90,33[b]
Min	13,45	13,92	0,05	0,03	0,01	0,00	0,06	0,00	14,49	17,15	52,28	47,75	2,92	2,76	88,38	88,96
Max	22,87	23,67	0,12	0,12	0,03	0,08	0,08	0,08	23,02	27,15	60,95	56,15	5,67	5,47	91,65	91,26
Mean	17,86	20,46	0,08	0,08	0,02	0,03	0,07	0,06	19,72	21,12	56,23	51,39	4,15	3,47	90,22	90,10
SD	2,34	2,42	0,02	0,02	0,00	0,02	0,01	0,03	2,20	3,25	2,39	2,55	0,67	0,64	0,87	0,61
CV	13,11	11,81	28,85	27,81	22,02	79,53	12,56	46,97	11,18	15,39	4,24	4,97	16,24	18,33	0,96	0,68

Mean values of each parameter in each genotype in different years followed by a different lower-case letter are significantly different at P=0.01 by the Duncan test.

3.4. Fatty acid composition

The fatty acid profile of almond oil consisting of mystiric (C14:0), palmitic (C16:0), palmitoleic (C16:1), margaric (C17:0), margaroleic (C17:1 n-8), stearic (C18:0), oleic (C18:1 n-9), linoleic (C18:2 n-6), α-linolenic (C18:3 n-3), arachidic (C20:0), and gadoleic (C20:1 n-11) (Table 2). Fatty acid composition of studied almond kernel oil has shown three predominant fatty acids regardless of cultivar or year. The oleic acid is the main monounsaturated fatty acid, followed by linoleic acid the main polyunsaturated fatty acid and the palmitic acid the main saturated fatty acid. The ranges of variation of these three fatty acids were 65-76%, 13-23% and 5.8-7.5%, respectively. The contents of stearic and palmitoleic acids were <4%, and ranged between 1.3-3.7% and 0.3-0.6%, respectively.
In both years, the oleic, linoleic and palmitic acids varied among cultivars. In 2009, the highest values of oleic acid content were determined in 'Francoli' (76.21%), followed by cultivars 'Sahnoun' (75.11%) and 'Zahaaf' (75.06%). However, the lowest values were found in 'Porto' (66.70%) and 'Breznaud' (69.94%). For linoleic acid, 'Porto' represented the highest value (22.87%) and 'Francoli', 'Sahnoun' and 'Zahaaf' showed the lowest value (13.45-15.91%). For Palmitic acid, 'Porto' and 'Ksontini' demonstrated the highest palmitic content (7%) while 'Fekhfekh' showed the lowest value (5.86%). In 2010, the cultivar 'Francoli' showed the highest value of oleic acid content (76.15%), followed by 'Abiodh', 'Zahaaf' and 'Sahnoun' while 'Mazetto' recorded the lowest value (65.29%). For linoleic acid, the highest value was obtained for 'Porto' (23.67%) whereas the lowest value was obtained for 'Francoli' (13.92%). 'Mazetto' represented the highest palmitic acid content (7.53%) and 'Fekhfekh' represented the lowest palmitic content (6.12%). Thus the varieties 'Sahnoun', 'Zahaaf' and 'Francoli', are superior in marketing quality with high oleic acid content and low linleic and palmitic contents.
The oleic/linoleic (O/L) ratio showed a large variability among cultivars because of the high variability in oleic and linoleic acids contents. This ratio was generally higher in 2009 than in 2010 (Table 2). The cultivars 'Francoli' showed the higher (oleic/linoleic) ratio (5.6-5.4) during the two consecutive years followed by the cultivars 'Sahnoun', 'Zahaaf' and 'Mahsouna' (Table 2). Owing to their highest (O/L) ratio, these cultivars represented the greatest stability of almond kernels and oil. However, 'Porto' cultivar showed the lowest (oleic/linoleic) ratio (2.8-2.9) followed by the varieties 'Ksontini' and 'Mazetto' in 2009 and 2010, respectively. For the local cultivars it was noted that oleic and linoleic acids together accounted from 88.38 to 91.65% of the total extracted almond oil.

3.5. Sugar composition

Total sugar content varied from 2.3 to 6.5 g 100 g^{-1} of dry weight (DW), with an average content of 3.98 g 100 g^{-1}DW (Table 3). Sucrose, raffinose, glucose and fructose contents were analyzed separately. Sucrose was the sugar present at the highest concentration in all studied cultivars (1.9 to 5.8 g 100 g^{-1}DW) followed by raffinose (0.04 to 1.36 g 100 g^{-1}DW), glucose (0.019 to 0.43 g 100 g^{-1}DW) and fructose (0.007 to 0.322 g 100 g^{-1}DW).
The year effect was significant on the total sugar content (Table 1). The mean value of total sugar was higher in 2009 than in 2010 for all studied cultivars excepting 'Blanco' (Table 3).

Table 3. Sugar composition (Raffinose, sucrose, glucose, fructose), total sugar content and percentage of each type of sugars for each almond cultivar in two consecutive years (2009 and 2010).

	Raffinose		Sucrose		Glucose		Fructose		Total sugar		%raffinose		%sucrose		%glucose		%fructose	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
Tunisian almond cultivars																		
Dillou	1,049^{a}	0,958^{b}	3,592^{a}	3,558^{a}	0,061^{a}	0,062^{a}	0,010^{a}	0,010^{a}	4,712^{a}	4,589^{b}	0,223^{a}	0,209^{a}	0,762^{a}	0,775^{a}	0,013^{a}	0,014^{a}	0,002^{a}	0,002^{b}
Khoukhi	0,720^{a}	0,724^{a}	3,447^{a}	3,456^{a}	0,120^{a}	0,114^{a}	0,010^{a}	0,010^{b}	4,297^{a}	4,304^{a}	0,168^{a}	0,168^{a}	0,802^{a}	0,803^{a}	0,028^{a}	0,026^{a}	0,002^{a}	0,002^{a}
Blanco	1,046^{a}	1,365^{a}	3,131^{a}	4,139^{b}	0,067^{a}	0,053^{a}	0,010^{a}	0,010^{b}	4,254^{a}	5,567^{a}	0,246^{a}	0,245^{a}	0,736^{a}	0,743^{a}	0,016^{a}	0,010^{b}	0,002^{a}	0,002^{b}
Abiodh	0,612^{a}	0,301^{b}	5,807^{a}	2,741^{b}	0,076^{a}	0,069^{a}	0,010^{a}	0,040^{a}	6,505^{a}	3,151^{b}	0,094^{a}	0,095^{a}	0,893^{a}	0,870^{a}	0,012^{a}	0,022^{b}	0,002^{a}	0,013^{a}
Lsen Asfour	0,151^{a}	0,369^{b}	3,737^{a}	3,265^{b}	0,050^{a}	0,041^{a}	0,010^{a}	0,010^{b}	3,949^{a}	3,684^{b}	0,038^{a}	0,100^{b}	0,946^{a}	0,886^{b}	0,013^{a}	0,011^{a}	0,003^{a}	0,003^{b}
Achaak	0,516^{a}	0,390^{b}	3,256^{a}	2,722^{b}	0,058^{a}	0,031^{b}	0,007^{a}	0,010^{b}	3,837^{a}	3,154^{b}	0,134^{a}	0,124^{b}	0,849^{a}	0,863^{b}	0,015^{a}	0,010^{b}	0,002^{a}	0,003^{b}
Zahaaf	0,318^{a}	0,145^{b}	2,846^{a}	2,202^{a}	0,034^{a}	0,028^{a}	0,010^{a}	0,010^{b}	3,207^{a}	2,385^{a}	0,099^{a}	0,061^{b}	0,887^{a}	0,923^{b}	0,010^{a}	0,012^{a}	0,003^{a}	0,004^{a}
Fekhfekh	0,369^{a}	0,182^{b}	3,977^{a}	2,760^{b}	0,092^{a}	0,026^{b}	0,010^{a}	0,010^{b}	4,449^{a}	2,978^{b}	0,083^{a}	0,061^{b}	0,894^{a}	0,927^{b}	0,021^{a}	0,009^{b}	0,002^{a}	0,003^{b}
Ksontini	0,134^{a}	0,472^{b}	3,569^{a}	2,576^{b}	0,042^{a}	0,046^{a}	0,010^{a}	0,010^{b}	3,755^{a}	3,104^{b}	0,036^{a}	0,152^{b}	0,950^{a}	0,830^{b}	0,011^{a}	0,015^{b}	0,003^{a}	0,003^{b}
Sahnoun	0,279^{a}	0,227^{a}	4,657^{a}	2,790^{b}	0,063^{a}	0,023^{b}	0,010^{a}	0,040^{b}	5,009^{a}	3,080^{b}	0,056^{a}	0,074^{a}	0,930^{a}	0,906^{b}	0,013^{a}	0,007^{b}	0,002^{a}	0,013^{b}
Porto	0,714^{a}	0,789^{a}	5,074^{a}	4,845^{a}	0,060^{a}	0,063^{a}	0,010^{a}	0,050^{a}	5,858^{a}	5,747^{b}	0,122^{a}	0,137^{b}	0,866^{a}	0,843^{b}	0,010^{a}	0,011^{a}	0,002^{a}	0,009^{b}
Mahsouna	0,450^{a}	0,318^{a}	2,411^{a}	1,976^{b}	0,065^{a}	0,033^{b}	0,010^{a}	0,010^{b}	2,936^{a}	2,338^{b}	0,153^{a}	0,136^{a}	0,821^{a}	0,845^{a}	0,022^{a}	0,014^{b}	0,003^{a}	0,004^{b}
International reference almond cultivars																		
Mazetto	0,413^{a}	0,225^{b}	3,286^{a}	2,428^{b}	0,027^{a}	0,019^{a}	0,026^{a}	0,024^{b}	3,752^{a}	2,695^{b}	0,110^{a}	0,083^{b}	0,876^{a}	0,901^{b}	0,007^{a}	0,007^{a}	0,007^{a}	0,009^{b}
Francoli	0,416^{a}	0,214^{b}	3,990^{a}	2,536^{b}	0,141^{a}	0,032^{b}	0,116^{a}	0,036^{b}	4,663^{a}	2,818^{b}	0,089^{a}	0,076^{b}	0,856^{a}	0,900^{b}	0,030^{a}	0,011^{b}	0,025^{a}	0,013^{b}
Super Nova	0,326^{a}	0,152^{b}	3,238^{a}	2,611^{b}	0,056^{a}	0,052^{a}	0,038^{a}	0,038^{a}	3,658^{a}	2,854^{b}	0,089^{a}	0,053^{b}	0,885^{a}	0,915^{b}	0,015^{a}	0,018^{a}	0,010^{a}	0,013^{b}
Lauranne	0,234^{a}	0,042^{b}	3,903^{a}	3,128^{b}	0,434^{a}	0,212^{b}	0,322^{a}	0,174^{b}	4,893^{a}	3,556^{b}	0,048^{a}	0,012^{b}	0,798^{a}	0,880^{b}	0,089^{a}	0,060^{b}	0,066^{a}	0,049^{b}
F. Breznaud	0,230^{a}	0,244^{a}	5,083^{a}	4,025^{b}	0,161^{a}	0,077^{b}	0,104^{a}	0,067^{a}	5,578^{a}	4,413^{b}	0,041^{a}	0,055^{b}	0,911^{a}	0,912^{a}	0,029^{a}	0,017^{b}	0,019^{a}	0,015^{a}
Min	0,134	0,042	2,411	1,976	0,027	0,019	0,007	0,010	2,936	2,338	0,036	0,012	0,736	0,743	0,007	0,007	0,002	0,002
Max	1,049	1,365	5,807	4,845	0,434	0,212	0,322	0,174	6,505	5,747	0,246	0,245	0,950	0,927	0,089	0,060	0,066	0,049
Mean	0,469	0,419	3,824	3,045	0,095	0,058	0,042	0,033	4,430	3,554	0,108	0,108	0,863	0,866	0,021	0,016	0,009	0,009

Mean values of each parameter in each genotype in different years followed by a different lower-case letter are significantly different at P=0.01 by the Duncan test.

Taking into account both years of study, the Tunisian variety 'Porto' and French variety 'Fournat de Breznaud' represented the higher sugar (5.8 and 4.9 g 100 g^{-1}DW) and sucrose content (4.9 and 4.5 g 100 g^{-1}DW), while the varieties 'Zahaaf' and 'Mahsouna' showed the lowest contents. 'Blanco', 'Dillou', 'Porto' and 'Khoukhi' have the highest raffinose levels, in decreasing order, for both years. Concerning the fructose and glucose percentages, the French varieties 'Lauranne' and 'Fournat de Breznaud' demonstrated the highest mean values for the two years of study (Table 3). However, the Italian variety 'Mazetto' represented the lowest glucose content (0.02 g 100g^{-1}DW).
The two local varieties 'Achaak' and 'Porto' are the two most appreciated almond kernel by consumers. 'Porto' seems to be sweeter than 'Achaak' and showed two times more total sugar and four times more fructose percentage.

3.6. Correlation among nutraceutical properties

The correlation among oil content and fatty acids of the studied almond kernel cultivars is reported in Table 4. High significant negative correlation was found between linoleic and oleic acid contents (r= -0.969). Significant positive correlations were also found between stearic and arachidic acid contents (r= 0.848) and in margaric versus margaroleic and gadoleic (r= 0.686 and r= 0.705, respectively). A significant negative correlation was found between gadoleic versus myristic and linolenic (r= -0.704 and r= -0.810, respectively). For the sugar composition, total sugar content was positively and highly correlated with sucrose (r= 0.974) and raffinose (r= 0.539). Also, a significant and high correlation was found between glucose and fructose contents (r= 0.933).
Moreover, significant and negative correlations were observed between total sugar content and arachidic acid (r= -0.537) and linolenic acid (r= -0.547). Similarly, a significant and negative correlation was also found between raffinose and stearic acid (r= -0.527) and arachidic acid (r= -0.589). Significant positive correlation was found between glucose and palmitoleic acid (r= 0.511). These relationships between different biochemical traits of almond suggest that the selection for one of these fatty acids or sugars could negatively or positively modify the amount of the other. Finally, a significant negative correlation was found between the oil and protein contents (r= -0.647).

3.7. Chemical diversity analysis

A principal component analysis (PCA) was performed on biochemical data (fatty acid, total oil and protein contents and sugar composition) for screening and describing the similarities among the 17 studied almond cultivars (Fig. 2). The PCA yielded six significant components with eigenvalues ≥ 1 and accounting for 91% of the total variance in the dataset (Table 5). The first two PCs (PC1 and PC2) accounted for 48.24% of the total of variance. PC-1 and PC-2 represented 27.41% and 20.83% of the variance, respectively. Eigen analysis of the correlation matrix revealed that PC-1 was mainly contributed by total sugar, sucrose and raffinose contents. PC-2 was correlated to arachidic, gadoleic, margaric and stearic acids. The third and fourth PC accounted for 16.83% and 11.35%, respectively. PC-3 was represented by oleic, linoleic, fructose and glucose contents while PC-4 was highly correlated to oil and protein contents.

Table 4. Correlations between fatty acid and oil content composition, protein content, total lipid, sugar composition and total sugar content.

	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Arachidic	α-Linolenic	Protein	Total Lipid	Raffinose	Sucrose	Glucose	Fructose	Total sugar
Palmitic	1													
Palmitoleic	-0,047	1												
Stearic	0,165	**-0,400**	1											
Oleic	**-0,607**	**0,458**	-0,047	1										
Linoleic	**0,474**	**-0,405**	-0,175	**-0,968**	1									
Arachidic	**0,237**	**-0,436**	**0,848**	0,026	**-0,232**	1								
α-Linolenic	0,006	**-0,322**	0,055	-0,010	0,001	0,143	1							
Protein	**0,252**	**-0,373**	0,094	-0,119	0,080	**0,285**	**0,297**	1						
Total Lipid	-0,192	**0,294**	**0,223**	**0,260**	**-0,294**	0,055	**-0,322**	**-0,647**	1					
Raffinose	**-0,217**	0,060	**-0,458**	-0,117	**0,250**	**-0,521**	**-0,264**	-0,128	0,065	1				
Sucrose	-0,080	**0,346**	-0,066	0,005	0,032	**-0,238**	**-0,485**	**-0,271**	**0,362**	**0,305**	1			
Glucose	-0,067	**0,455**	-0,164	**0,205**	-0,170	-0,131	-0,166	-0,136	0,175	-0,103	**0,287**	1		
Fructose	0,010	**0,374**	-0,045	0,178	-0,181	0,069	-0,109	-0,002	0,014	**-0,269**	0,166	**0,896**	1	
Total sugar	-0,136	**0,363**	**-0,206**	-0,005	0,078	**-0,361**	**-0,503**	**-0,277**	**0,336**	**0,530**	**0,961**	**0,333**	0,181	1

Correlations shown in bold case are significant at $P<0.05$.

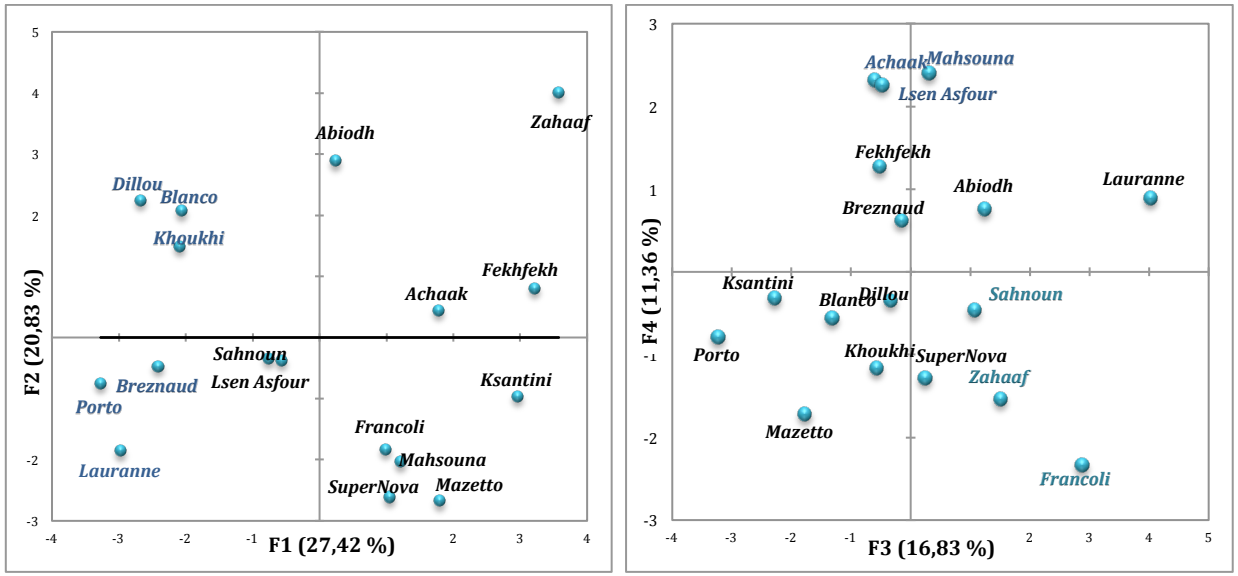

Figure 2. Score plot showed the Principal component analysis (PCA) based on nutraceutical data (fatty acid, total oil and protein contents and sugar composition) describing the similarities among the 17 studied almond cultivars.

Table 5. Eigenvectors of the four principal components axes from PCA analysis of the 17 almond cultivars for fatty acid and oil content composition, protein content, total lipid, sugar composition) and total sugar content. Eigenvalues and their contribution to total variation are listed at the bottom of columns.

Variable	F1	F2	F3	F4
Palmitic	-0,010	-0,400	-0,310	0,042
Palmitoleic	-0,588	0,161	0,581	0,114
Stearic	0,570	-0,663	-0,230	0,121
Oleic	0,228	0,160	0,860	-0,158
Linoleic	-0,375	0,062	-0,823	0,115
Arachidic	0,581	-0,777	-0,056	-0,050
α-Linolenic	0,856	0,312	0,154	0,063
Protein	0,419	-0,133	-0,056	-0,842
Total Lipid	-0,084	-0,133	-0,027	0,935
Raffinose	-0,485	0,440	-0,435	-0,156
Sucrose	-0,682	0,152	-0,203	-0,028
Glucose	-0,504	-0,183	0,586	0,114
Fructose	-0,391	-0,367	0,643	0,021
Total sugar	-0,798	0,240	-0,228	-0,067
Eigenvalue	4,935	3,749	3,030	2,045
Variance (%)	27,415	20,827	16,831	11,359
Cumulative (%)	27,415	48,243	65,073	76,433

Based on the PCA results (Fig. 2), same studied almond cultivars could be described by similarities in chemical characteristics considering oil and sugar composition while others had different chemical profile. PC-1 allowed the separation of 'Porto', 'Fournat de Breznaud', 'Blanco', 'Dillou', 'Khoukhi' and 'Lauranne' which are rich in total sugar, sucrose and raffinose. The cultivars 'Sahnoun', 'Zahaaf', 'Francoli' and 'Lauranne', separated along the positive direction of PC-3, were characterized by high oleic, fructose and glucose contents and low linoleic content. 'Mahsouna', 'Achaak', 'Lsen Asfour', 'Fekhfekh' and 'Lauranne' were situated in the positive side of PC-4 owing their high oil content opposing to 'Mazetto', 'Supernova', 'Zahaaf', 'Francoli' and 'Ksontini' on the negative direction with the highest protein content. This data suggests that almond kernels of 'Lauranne' cultivar offer unique nutritional potential, with high oil content, oleic acid and oleic to linoleic acids ratio and with superior total sugar content, especially fructose content. Moreover, the cultivars 'Lsen Asfour', 'Achaak' and 'Mahsouna' were associated together and represented some similarities in their composition.

4. DISCUSSION

The results showed that the main cultivated almonds in Tunisia are a potentially rich source of protein, unsaturated fatty acids and sugars. However, their contents on nutritional compound was affected by both genotype and harvest year. The year-to-year variation in fruit quality parameters may be explained by the differences in annual temperatures and precipitation over the two years of study (data not shown). The hard climatic conditions prevailing (dry and hot season) during 2010 were believed to be a contributing factor to the reported variation in sugar and oil content.

Significant genotypic and environmental effects were noted in the oil content for studied cultivars in the present study. The discrepancies in the possible year effect on oil content could be the result of the specific climatic conditions of the years tested (SOCIAS I COMPANY *et al.*, 2008). The variation between years indicated that climatic conditions had an effect on almond fruit development and thus severe deficiencies influenced lipid content (ZHU *et al.*, 2015). Therefore, the oil content trait appears to be under polygenic control (FONT I FORCADA *et al.*, 2011), with a clear environmental effect (ABDALLAH *et al.*, 1998; SATHE *et al.*, 2008; KODAD *et al.*, 2010). Moreover, the effect of harvest year on almond kernel oil content has been widely reported in the literature to be significant (BARBERA *et al.*, 1994; ABDALLAH *et al.*, 1998; SATHE *et al.*, 2008). YILDIRIM *et al*. (2016) reported that the total oil content changed significantly by year in fifteen commercial almond cultivars with the exception of cultivar 'Sonora'. However, no significant year effect was found by KODAD *et al.* (2011) in extensive two-year studies, although the interaction of genotype × year was significant. The magnitude of the effect of the external factors such as the climatic condition of the year probably depends on the genetic background of each cultivar, explaining the significant effect of the interaction genotype × year (KODAD *et al.*, 2011).

The variability range in total oil content in the present study was similar to the range of variability reported in previous studies. SATHE *et al.* (2008) have reported that oil content for eight almond Californian cultivars varied from 49.10% to 66.38%. ASKIN *et al.* (2007) reported that kernel oil content of 26 almond genotypes from eastern Anatolia (Turkey) varied from 25.19% to 60.77%. ČOLIĆ *et al.* (2017) reported that the range in total oil content for twenty almond spontaneous selections varied between 36.3 and 62.8%. Oil content of local almond genotype from Argentine varied from 48% to 57.5% (MAESTRI *et*

al., 2015). KODAK *et al.* (2008) found that total lipid contents ranged from 54 to 64.5% for European cultivars. They reported also that total lipid contents ranged from 35 to 53% for Australian cultivars and from 35 to 61% for Californian cultivars. Similarly, YADA *et al.* (2011) reported the variation range of kernel lipid contents of the most important commercial and local almond cultivars growing in USA-California (35-66%), Greece (56-61%), Italy (42-57%), Portugal (48-59%), Spain (40-67%), Turkey (25-61%), Afghanistan (43-63%), Egypt (55-59%), India (44-56%) and Iran (55-62%).

The heritability described for oil content is high (0.57) indicating an additive gene action, being a trait less influenced by environmental effects (FONT I FORCADA *et al.,* 2011). Consequently, selection for this trait will be more effective because it is less influenced by the environment (KODAD *et al.,* 2013). The local Tunisian cultivars with high and stable oil content could be incorporated into the almond breeding program in order to increase the oil content. In addition, the lipid portion, followed by the protein fraction, is the main component of the almond kernel, and is a major determinant of kernel flavor particularly following roasting (SOCIAS *et al.,* 2008). However, kernels with a relatively low percentage of oil such as 'Blanco'; 'Francoli', 'Ksantini' and ' Zahaaf' are required to produce almond milk, a dietetic product; because it's caloric level must be similar to that of cow's milk. Low lipid contents ('Lsen Asfour', 'Fourna de Breznaud') are also suitable for production of almond flour because of their correlation with high protein content (LONGHI, 1952).

For protein content, stability from year to year was observed for the cultivars 'Dillou' 'Mahsouna' and 'Fournat de Breznaud'. DROGOUDI *et al.* (2012), studying protein and mineral nutrient contents in kernels of 72 sweet almond cultivars and accessions grown in France, Greece and Italy, reported that the higher temperatures may have favored growth and nutrient utilization, resulting in greater nutrient contents in warmer year. Protein content in the seventeen studied almond cultivars ranged from 14 to 27%, which presented an interested range of variability compared with previous studies. In fact, protein contents ranged from 18.5 to 24.0 g $100g^{-1}$ of almond among all samples for the top ten almond-producing varieties in California and presently account for about 80% of the total commercial almond acreage (YADA *et al.,* 2013). KODAD *et al.* (2013) reported that the protein content ranging between 14.1 and 35.1% for 41 native almond genotypes grown in different geographical regions in Morocco. ÖZCAN *et al.* (2011) noted that crude protein content of five Turkish almonds varied from 12.7% to 16.3%. ASKIN *et al.* (2007) reported a wider range of protein content variability (16-31%) in 26 native genotypes from Turkey. All these results indicate the high range of variability of protein content depending on the genotype and the environmental conditions of the growing region (KODAD *et al.,* 2006). FONT I FORCADA *et al.* (2011) reported that the heritability estimate of protein content in almond is very low (h^2= 12.1%), confirming the strong effect of environmental conditions on its expression.

Almond oil has been reported to be very rich in monounsaturated fatty acids (MUFAs), especially in oleic and linoleic acids, whereas saturated fatty acids, especially palmitic, palmitoleic and stearic, are very low (YADA *et al.,* 2011). In commercial almond cultivars grown in various regions of the world, oleic and linoleic acids together accounts for about 90% of the total lipids, whereas, other fatty acids, including saturated fatty acids accounts for less than 10% (YADA *et al.,* 2011). This was consistent for the cultivars originate from the north of Tunisia that are 'Dillou', 'Khoukhi', 'Blanco' and 'Abiodh'. But overall the fatty acid composition, in the present paper, was in agreement with previous studies on almond grown around the world (SATHE *et al.,* 2008; MAESTRI *et al.,* 2015; ZHU *et al.,* 2015; ČOLIĆ *et al.,* 2017).

The variety 'Mahsouna' appears to present the most stable oil composition. Moreover, it presented stable value for oleic and linoleic acid contents. However, the oil composition of the varieties 'Blanco', 'Achaak', and 'Francoli' was more affected by the climatic conditions of the year studied. This confirmed that the year-on-year stability of each fatty acid depended on the specific characteristics of the genotype (ABDALLAH *et al.*, 1998; SATHE *et al.*, 2008; KODAD *et al.*, 2008, 2010; YADA *et al.*, 2011). KODAD *et al.* (2010) reported stable values for some fatty acids in some genotypes such as 'Marcona', 'Del Cid', and 'Castilla' for palmitic acid; 'Marcona' and 'Khoukhi' for palmitoleic acid; Desmayo Largueta and 'Del Cid' for stearic acid; 'Brézenaud' and 'Vivot' for oleic acid; and 'Desmayo Largueta', 'Khoukhi', 'Marcona', 'Retsou', and 'Vivot' for linoleic acid.

ABDALLAH *et al.* (1998) reported that the year effect was significant for all fatty acids except palmitoleic acid in twenty one Californian cultivars growing at four different sites. KODAD *et al.* (2010), after studying seventeen almond cultivars, reported that the year effect was significant for all fatty acids, except palmitic acid. Similarly, KODAD *et al.* (2011) noted that the year effect was not significant for palmitic and stearic acids. Furthermore, KODAD *et al.* (2010) reported that the genotype× year interaction was significant for all fatty acids except oleic acid, showing that the magnitude of the values changed each year. YILDIRIM *et al.* (2016) reported also that the effect of the cultivar, year and the interaction cultivar×year were significant for all fatty acids except heptadecanoic acid in fifteen commercial Turkish almond cultivars. Finally, SATHE *et al.* (2008) reported that the year effect was significant for all fatty acids in Californian cultivars growing at different sites, but stated that the year-to-year variability in fatty acid composition depended on the specific climatic conditions in that year.

Comparing linoleic acid levels in Spanish, Mediterranean, Californian and Australian almonds, ZHU *et al.* (2015) noticed that the regions producing almonds with lower linoleic acid were not irrigated, whereas Californian and Australian regions routinely apply irrigation to their orchards. NANOS *et al.* (2002), based on oil composition data, noted that irrigation resulted in almonds with superior oil quality as the oil had higher oleic acid content and oleic/linoleic acid ratio than almonds from non-irrigated trees. Consequently, Irrigation can affect almond kernel oil composition. For the others fatty acids, NANOS *et al.* (2002) reported that irrigation decreased the amounts of palmitic and palmitoleic acids, but did not affect the amount of stearic acid in 'Ferragnès' and 'Texas'.

The high content of unsaturated fatty acids, mainly of oleic acid, increases the phytonutrient value of the almond because this type of fatty acids does not contribute to the formation of cholesterol (KODAD *et al.*, 2011). Moreover, High levels of oleic acid and low levels of linoleic acid have been associated with prolonged shelf-life of almonds and are often advocated (ZHU *et al.*, 2015). Thus the varieties 'Sahnoun', 'Zahaaf' and 'Francoli', are superior in marketing quality with high oleic acid content and low linoleic and palmitic contents.

Furthermore, the higher oleic/linoleic (O/L) ratio was reported on 'Francoli' followed by 'Sahnoun', 'Zahaaf' and 'Mahsouna' cultivars. This ratio is considered a significant quality criterion of the oil kernel due to its preventive effect on lipid oxidation especially where almonds will be stored for long periods (KODAD *et al.*, 2010). In fact, a high O/L ratio is considered as an important factor providing stability in oils as well as a higher nutritional value and healthiest almond lipids (KODAD *et al.*, 2013; YILDIRIM *et al.*, 2016). For this, all oils of 'Abiodh', 'Francoli', 'Mahsouna', 'Sahnoun' and 'Zahaaf' can be considered of highly perfromant (Oleic/linoleic ratio > 4.2).

The two cultivars 'Achaak' and 'Francoli' were proved to be highly affected by the climatic conditions for sugars composition while 'Dillou' and 'Khoukhi' presented the most stable

sugar composition regarding harvest year. Sugar composition of almond kernel has vital value for good flavor and taste (NANOS *et al.,* 2002). It depends on the cultivar as well as the maturity stage but some sugar composition changes during maturation are cultivar-specific (NANOS *et al.,* 2002; KAZANTZIS *et al.,* 2003). In fact, KAZANTZIS *et al.* (2003) indicated that early harvested 'Ferragnes' almonds had higher raffinose content than late harvested almonds (due to sucrose accumulation with maturation and the preferential production of sucrose from raffinose and the other sugars) while the opposite held true for 'Texas' almonds. However, the effect of the year was reported to be non-significant on the expression of the sucrose content (YADA *et al.,* 2013). SÁNCHEZ-BEL *et al.* (2008) reported that sucrose and glucose contents in kernels of 'Guara' grown under drip-irrigated orchards were higher than those from non-irrigated orchards. The effect of year was reported to be significant on the total sugar content of the kernel of 'Ferragnes' and 'Mazeratto' varieties (BARBERA *et al.,* 1994).

Soluble sugars, while present in relatively low amounts, are sufficient to make kernels sweet-tasting (SCHIRRA, 1997). Free sugars are important nutritional components that affect the kernel flavor of almond (BALTA *et al.,* 2009). 'Porto' and 'Fournat de Breznaud' represented the higher sugar and sucrose content. Data regarding sucrose contents of this study were similar to those by KAZANKAYA *et al.* (2008) and BALTA *et al.* (2009). The prevalence of sucrose as the main sugar in almond is in agreement with previous works (FOURIE and BASSON, 1990; KADER *et al.,* 1996; NANOS *et al.,* 2002; KAZANKAYA *et al.,* 2008; BARREIRA *et al.,* 2010). They found, also, that sucrose was the main sugar constituent in almond followed by raffinose, glucose and fructose. FOURIE and BASSON (1990) obtained individual sugar contents of five almond cultivars ranging between 3.10 to 4.68 g 100 g^{-1}DW, 0.02 to 0.07 g 100 g^{-1}DW and 0.05 to 0.13 g 100 g^{-1}DW for sucrose, glucose and fructose, respectively. YADA *et al.* (2011) reported that the range variation of almond sugar contents (percentage of total weights) of commercially and locally almond cultivars growing in California is from 2.1 to 7%, in Greece from 2.6 to 4%, in India from 3.6 to 12%, in Italy from 2.1 to 5.5%, in Portugal from 2.5 to 7.1%, in Spain from 1.8 to 7.6%, and in Turkey from 2.5 to 13%.

Regarding relationships among the different nutraceutical almond parameters some interesting correlations were demonstrated in this work. Oleic and linoleic acids presented a conversely relationship. In the literature it has been reported that the proportion of oleic acid among total fatty acids is highly and negatively correlated with the linoleic acid levels with similar correlation coefficient (r= -0.9) (ABDALLAH *et al.,* 1998; ASKIN *et al.,* 2007; SATHE *et al.,* 2008; KODAD *et al.,* 2011; ZHU *et al.,* 2015). This high correlation between the two predominant fatty acids of almond kernels would allow accurate future predictions of total fatty acid composition by analyzing only linoleic acid level (ABDALLAH *et al.,* 1998). This higher correlation, could be considered as an index in any almond breeding program to improve almond quality (WANG *et al.,* 2019). In addition, the proportion of oleic acid was negatively correlated with palmitic level (r= -0.607). Similar results were reported in other almond cultivars (ASKIN *et al.,* 2007; SATHE *et al.,* 2008; KODAD *et al.,* 2011). Moreover, the correlation between stearic and arachidic was also approved by other authors (SATHE *et al.,* 2008).

Correlation coefficients, greater than 0.71 or smaller than -0.71, have been suggested to be biologically meaningful showing that this correlation is not influenced by climatic and environmental conditions and is genotype-dependent (KODAD *et al.,* 2011). No correlations were observed between the oil content and the percentages of the different fatty acids, even with the major fatty acid, which was also consistent with previous studies (SATHE *et al.,* 2008; KODAD *et al.,* 2011).

The negative correlation between protein and oil contents observed was previously reported by KODAD *et al.* (2013). On the other hand, BALTA *et al.* (2009) reported a positive correlation between maltose, glucose and fructose in sweet almond while this relationship was negative in bitter almond. Accordingly, these correlation findings indicate that inter-relationship among sugar contents vary according to kernel taste.

5. CONCLUSIONS

This work represents one of the most complete chemical and nutritional studies in almond characterizing the main almond cultivars grown in Tunisia. Results evidenced that oil, sugar and protein contents in almond depend of a polygenic background with a clear environment effect. Local Tunisian cultivars are highly rich in oil and fatty acids particularly oleic and linoleic acids with percentages between 88.4 and 91.6% of the extracted oil. In addition, the local cultivar 'Mahsouna' identified after a prospecting effort in the region of Sfax (South Tunisia) presented the most stable characteristics over the years regarding oil composition and protein content. The cultivar 'Porto' from the north of the country was performing in terms of sucrose and total sugar contents. This information would be essential to increase our knowledge on the local Tunisian almond diversity and their biochemical performance regarding traits to select adequate parents for future breeding programs. These results also support the importance of the characterization and preservation of genetic diversity being the granary for selecting in the coming future cultivars with high quality in a context of global warming offering valuable information for breeders about limits and capacities of the Tunisian almond cultivars to be used for breeding programs.

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PAPER

A COMPARATIVE STUDY ON THE EFFECT OF HIGH HYDROSTATIC PRESSURE ON RIPENING OF TURKISH WHITE CHEESE FROM DIFFERENT MILK SPECIES

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ABSTRACT

High pressure treatment has diverse effect on cheeses depending on their characteristics. In this study, pressure application on Turkish White cheese (300 and 450 MPa/5 min) and the changes during ripening were investigated. The 450 MPa pressure process showed an enhanced effect on proteolytic and lipolytic activity of cheeses. Besides, 450 MPa pressure treatment was very effective on the microbiological profile, but the other treatment condition exhibited a more moderate antimicrobial effect. Although, the total mold-yeast were detected after high-pressure treatment, their existence to a considerable degree was seen at the end of storage.

Keywords: high pressure, cow cheese, goat cheese, ripening, Turkish White cheese

1. INTRODUCTION

The ripening of cheese is an expensive process because of the high storage cost, so the reduction in storage cost without affecting the quality of the product would provide significant savings to the cheese industry (EL SODA AND AWAD, 2011). A reduction in the financial cost of a large quantity of cheese during storage, providing sufficient space for a new product by a fast cycle of stock can be ensured by accelerating ripening. Cheese ripening increases by elevated storage temperature, addition of exogenous enzyme or cheese slurries and modified microorganism usage in the dairy industry. Recently, the high-pressure treatment on cheese to accelerate ripening has been scientific and of commercial interest to numerous researchers (SALDO *et al*., 2002; O'REILLY *et al*., 2000). It has been reported in previous studies that high hydrostatic pressure (HHP) has been applied to cheese for destroying pathogenic and spoilage bacteria, reducing salting time, allowing the release of starter enzymes and changes in enzyme activity to reduce ripening time without changing cheese quality (SALDO *et al*., 2002). In the event of pressurization process, the breakdown of proteins structure affects textural development, aromatic compound formation and proteolysis by releasing amino acids. In the previous studies, the accelerating effect of HHP treatments on cheese ripening was reported as follows: alteration in enzyme configuration, structural changes in the protein matrix by increasing activity of proteases, as well as bacterial lysis which aid the release of microbial enzymes.
There have been various studies on the effect of HHP on cheese such as Mozzarella (SALDO *et al*., 2002; O'REILLY *et al*., 2000), Gouda (MESSENS *et al*., 2000), cheddar (RYNNE *et al*., 2008), sheep milk cheese (JUAN *et al*., 2007) and goat milk cheese (CAPELLAS *et al*., 2001). These researches showed that HHP affects cheese quality and ripening depends on cheese variety, chemical and physicochemical properties of cheeses, pressure level, processing time, and temperature conditions. Certain pressure parameters may lead to variable changes in composition, quality properties and texture of different cheeses (O'REILLY *et al*., 2001).
Although, a good number of researches were published on the cheese ripening properties after applying high hydrostatic pressure, no information has been found on ripening properties of Turkish White cheese under pressurization effect. The studies on Turkish White cheese have focused on the pressurization effect on microbial inactivation of pathogenic bacteria (*L. monocytogenes*), total mesophilic aerobic bacteria (TMAB), total molds and yeasts, Lactococcus, Lactobacillus and coliform bacteria (*Enterobacteriaceae)* in cheese (EVRENDİLEK *et al*., 2008), salt distribution of cheese during ripening (KOCA *et al*., 2018), as well as textural and microstructural changes after pressure treatment (KOCA *et al*., 2011). This study focused on the effect of pressure on ripening properties and hence microbiological changes because the diverse effects of HHP on cheese depend on milk species, composition, structure, and quality. There has been insufficient information on the effect of high pressure on the proteolysis and lipolysis properties of Turkish White cheese during ripening. In accordance, it was aimed to determine the effects of moderate level pressure treatment on the chemical, physicochemical, and biochemical changes of Turkish White cheese made from goat and cow milk during the ripening period.

2. MATERIALS AND METHODS

2.1. Cheese production

Full-fat cow and goat milk were obtained from two local dairy farms (İkizler Süt and Bolana, Bolu, Turkey) and cheeses were produced from 100 liters of pasteurized milk in two different vats. After pasteurizing of raw milk at 65°C for 30 min, the milk was cooled to 32°C. Mesophilic starter culture (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*) (DVS-R-704, Chr. Hansen) at a rate of 0.2% and $CaCl_2$ about 0.02% were added into pasteurized milk. Then liquid rennet (10g/100L cheese milk (CHY-MAX, Chr. Hansen) was supplemented as a coagulant when pH reached6.40, and coagulation was completed within 90 min. The coagulum was cut (1 cm cubes) and rested in the whey for 5 min. The curds were transferred into 7x7 cm molds, and the whey was drained spontaneously at room temperature for 8 hours until whey drops were stopped. The cheese blocks were taken in brine (14 g/100g NaCl) for 12 h at room temperature. Both goat and cow cheese samples were then packaged into airtight and watertight plastic boxes and were separated into three groups depending on the pressure treatment (i) control, (ii) 300 MPa for 5 min and (iii) 450 MPa for 5min for HHP application. After the HHP process, they were stored at +4°C for two months and examined at 0, 30, 60 days of storage.

2.2. High-pressure treatment

Pilot-scale high-pressure food processor unit (Avure Technologies, OH, USA) with one-liter pressure chamber was used. Vacuum packaged samples were processed with 300 and 450 MPa pressures for 5 min, while process temperature was kept below 6 ºC during the HHP application. Control samples without pressure application were packaged the same way as the HHP samples.

2.3. Microbiological analyses

TMAB (ISO, 2013), mold and yeast (ISO, 2004a), coliform (ISO, 2006), mesophilic lactic acid bacteria (ISO, 1998) and psychrotrophic bacteria (ISO, 2005) were determined in duplicate according to ISO (International Organization for Standardization) standards.

2.4. Physicochemical analyses

Chemical properties of cheese samples were determined with respect to International Dairy Federation (IDF) standards for total solid (ISO, 2004b), fat content (IDF, 2009), protein content (IDF, 2014), salt content (IDF, 2006), and titratable acidity (IDF, 2012). A pH meter (pH-720, Inolab, Germany) was used to measure pH value of the homogenate (10 g cheese sample+10 ml distilled water). Minolta CR-400 (Konica Minolta Sensing, Inc., Osaka, Japan) equipment was used for color measurements of cheese in D65 mode as L*, a*and b*.

2.5. Nitrogen fractionation determination

Water-soluble nitrogen (WSN) and Trichloroacetic acid-soluble nitrogen (TCA-SN 12%) fractions were prepared by KUCHROO and FOX (1982) and POLYCHRONIADOU *et al*.

(1999), respectively. The nitrogen contents of the soluble extracts were carried out using the Kjeldahl method (IDF 2014) and the ripening index (RI) was presented as a percentage of WSN to total cheese nitrogen. The acid degree value (ADV) of the samples was found in meq KOH/100g fat according to CASE *et al*. (1985).

2.6. Statistical analyses

The changes for 2 months-storage were determined by General Linear Model Repeated Measures. Analysis of Variance (ANOVA) and Tukey's multiple comparison tests were used to determine differences between cheese samples in different treatment groups by SPSS version 23 (SPSS Inc., Chicago, USA) package program. SIMCA (Soft independent modeling of class analogy) was used for the grouping of cheese samples based on properties under various pressure. The classes were observed to be significantly different from each other when the interclass distances were above 3.

3. RESULTS AND DISCUSSION

3.1. Microbiological properties of cheeses

The effect of high hydrostatic pressure processing on the microbial properties of cheese samples was shown in Table 1. Coliform bacteria were not found in both (control and pressurized) cow and goat cheese samples at 1/10 dilution. TMAB, lactococci and lactobacilli counts showed a significant decrease in bacterial growth at high pressure treated cheese samples (P<0.05). While aerobic mesophilic bacteria count in cow and goat control cheeses were around 9.13 and 9.6 log CFU/g respectively, average 2-3 log decreasing by 300 MPa and 4-6 log decreasing for 450 MPa pressure were observed in HHP-treated cow and goat cheese samples. The effect of high pressure on Lactococci and Lactobacilli counts were higher due to elimination of the all starter culture at 450 MPa pressure, processing nearly above the mesophilic bacteria counts. Besides, 300 MPa HHP-treated cheese samples showed significant decrease in microbial counts of lactic acid bacteria (P<0.05). Lactobacilli were affected by HHP more than lactococci in the study.
The lactobacilli were the most affected bacteria group from pressurization processing both in cow and goat cheeses. Although, total mold/yeast could not be determined for both control and pressure treated samples, their presence increased at the end of the storage and reached about 5.5 log levels at 60 days of storage. Growth of yeasts and molds in HHP treated samples compared to the control samples was delayed in goat cheese, but these microorganisms reached about 5 log levels during the storage period. EVRENDİLEK *et al*. (2008) noticed that total yeast and mold after 300-600 MPa high-pressure treatments were below the detection limit, so they could not be detected. Similarly, psychrotrophic bacteria in cow cheese were significantly detected at the end of the storage period (P<0.05). However, psychrotrophic bacteria was not found in all goat cheese samples during the storage period DARYAEI *et al*. (2008) reported the growth of yeast at 6 weeks of cheese samples treated under similar high-pressure parameters. Sublethal injury experiments regarding high pressure applications indicated that high pressure damaged the microorganism population more severely and it took longer to recover (O'REILLY *et al*., 2000). This fact may explain the existence of molds and yeasts counts in cheeses treated at 300 and 450 MPa at 60 days. Pressurization processing showed higher microbial reduction

effect in goat cheese as compared to cow cheese, this shows that the type of cheese affects the microbial inactivation (O'REILLY *et al.*, 2001).

Table 1. Microbiological changes during ripening (at 4ºC) of the cheeses after high pressure treatment at 300 and 450 MPa for 5 min (log cfu/g).

Cheese type	Microbial Group	Day	Treatment		
			Control	300 MPa	450 MPa
Cow cheese	TMAB	1	9.13±0.03[a]	7.24±0.02[b]	5.72±0.04[c]
		30	8.60±0.11[a]	7.07±0.06[b]	4.00±0.10[d]
		60	8.78±0.15[a]	7.41±0.61[b]	4.32±0.62[d]
	Lactobacilli	1	8.61±0.170[a]	N.D.	N.D.
		30	4.96±0.08[b]	3.51±0.11[d]	N.D.
		60	4.64±0.11[c]	3.06±0.44[e]	N.D.
	Lactococci	1	9.00±0.09[a]	6.82±0.27[b]	N.D.
		30	5.00±0.11[c]	3.79±0.09[e]	N.D.
		60	4.78±0.14[c]	4.28±0.73[d]	N.D.
	Mold/Yeast	1	N.D.	N.D.	N.D.
		30	N.D.	N.D.	N.D.
		60	5.32±0.10[a]	5.58±0.300[b]	5.41±0.10[c]
	Psychrotrophic bacteria	1	N.D.	N.D.	N.D.
		30	N.D.	N.D.	N.D.
		60	5.66±0.16[a]	5.75±0.13[a]	8.69±0.19[b]
Goat cheese	TMAB	1	9.6±0.07[a]	5.78±0.16[d]	3.67±0.09[g]
		30	7.68±0.16[b]	6.66±0.73[c]	4.29±0.66[f]
		60	6.04±0.05[d]	4.84±0.34[e]	3.30±0.25[g]
	Lactobacilli	1	9.03±0.11[a]	N.D.	N.D.
		30	4.91±0.10[b]	3.20±0.48[c]	N.D.
		60	0.50±1.00[d]	1.00±1.16[d]	N.D.
	Lactococci	1	9.69±0.17[a]	3.35±0.24[d]	1.19±1.38[e]
		30	5.67±0.09[b]	4.64±0.11[b]	N.D.
		60	4.27±0.17[d]	3.47±0.29[d]	N.D.
	Mold/Yeast	1	N.D.	N.D.	N.D.
		30	5.34±0.21[a]	1.46±1.695[b]	N.D.
		60	6.00±0.09[a]	5.67±0.11[a]	5.28±0.89[a]
	Psychrotrophic bacteria	1	N.D.	N.D.	N.D.
		30	N.D.	N.D.	N.D.
		60	N.D.	N.D.	N.D.

Values represented by mean ± standard deviation.

[a,b,c]Different superscript in the same microbial group indicates significant differences ($p<0.05$). N.D. = not determined in 1/10 dilution.

3.2. Physicochemical properties of cheeses

Pressure application did not significantly affect the total solids, protein, and fat contents of both cow and goat cheese samples (Fig. 1). The total solid content of the cheeses was found lower than HHP-treated Turkish White cheese pressurized up to 400 MPa for 5, 10 and 15 min reported by KOCA *et al*. (2011) and EVRENDİLEK *et al*. (2008) due to probably applying different pressing time and load in production. Moreover, a significant increase in total solid, protein, and fat contents was found in cow's milk cheeses on day 60 of ripening ($P<0.05$), while there were significant decreases in all parameters for goat cheeses ($P<0.05$). The high beta casein content of goat milk produces a firm and hard structure when processed in Turkish White cheese due to the long period of time and spontaneously straining of whey. The differences in structural properties were influenced by high hydrostatic pressure at different levels.

The placement of brine into cheese causes an increase in moisture content, in contrast, changes in para casein network due to HHP causes a decrease in moisture content. Moreover, SALDO *et al*. (2001) reported the existence of higher amount of bound water in HHP-treated cheese despite presence of same moisture content in control cheese. This phenomenon causes high moisture content in HHP-treated cheese during ripening. This fact also explains the reason for decrease in protein and fat content of goat cheese samples during ripening period. Also, the decrease in protein and fat contents may be as a result of the diffusion of proteolysis products and fat from the cheese into brine (HAYALOĞLU *et al*., 2002).

The goat cheese samples were higher dry solids content as compared to cow cheeses; therefore, goat cheeses resulted in higher moisture content by diffusion of brine into cheese with pressurization. On the other hand, cow cheese was not as firm as goat cheese, and pressure process was affected by the protein networks via breaking down. As a result, the HHP treatment might cause slight water expulsion and result to a decrease in moisture content in the pressure treated cheeses (HUPPERTZ *et al*., 2006). Compared to control samples, less water content was reported in La Serene cheese treated under 300-400 MPa HHP at 50 days (GARDE *et al*., 2007). While the higher water holding capacity was identified in high pressure treated ewes milk cheeses during ripening. None of changes were found for moisture content of goat cheeses (CAPELLAS *et al*., 2001). After the HPP treatment, structure of paracasein matrix of cheese plays an important role in the composition of cheese via acidity development and salt distribution (MOSCHOPOULOU *et al*., 2010). The quantity of salt in cheese samples increased both in control and pressurized samples during ripening due to salt diffusion from brine into cheese during storage. KOCA *et al*. (2011) reported an increase in the amount of salt in pressurized and unpressurized Turkish White cheeses at various pressure levels until the 14th day of ripening.

The titratable acidity as lactic acid basis showed a slight reduction by increasing the pressure level, whereas, pH values of the cheese samples increased by pressuring process (Table 2). These results were in agreement with results reported by KOCA *et al*. (2011) in 50-400 MPa high pressure treated Turkish White cheese. The pH-enhancing effect of high pressure on various cheeses was reported by some authors (MESSENS *et al*., 1998; MOSCHOPOULOU *et al*., 2010). The pH value in both cow and goat cheese samples showed a tendency to decrease at the ripening period. The carriage of colloidal calcium phosphate in two- sides may create these temporary changes in pH values (MOSCHOPOULOU *et al*., 2010). Besides, the usage of lactic acid, the formation of alkaline

compounds by degradation of big molecules and proteins may be the result of reducing effect (MESSENS *et al*., 1998).

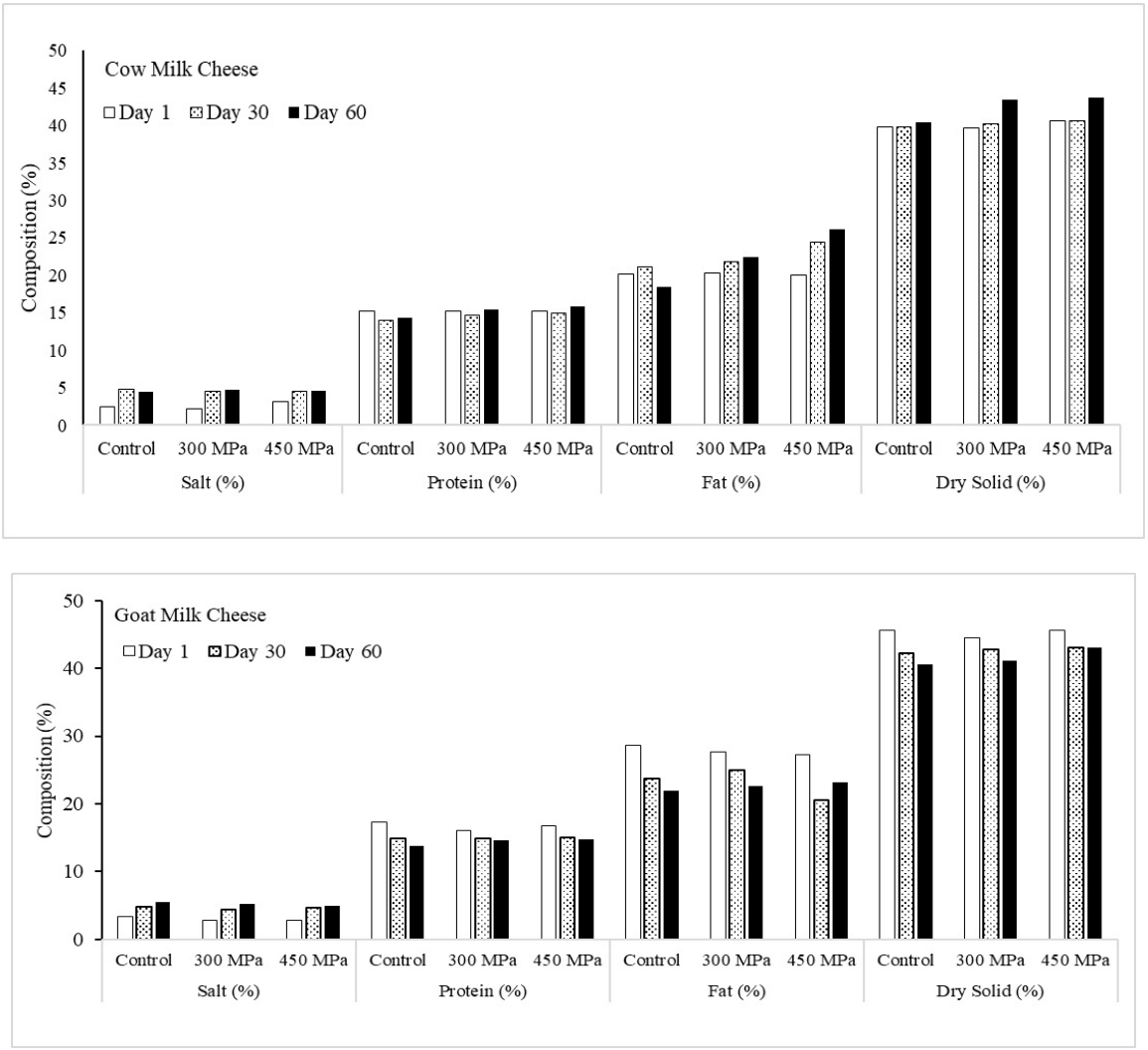

Figure 1. Effect of HHP treatment (300 and 450 MPa for 5 min) on compositional changes in cow and goat cheese samples during ripening.

The color properties (L*, a* b* value) of high hydrostatic pressure treated cheese samples is given in Table 2. The high-pressure process did not affect the L* (lightness) and b* (blue-yellow) values of the cow cheese samples significantly (P>0.05), whereas, there was a small change in goat cheese (P<0.05). Besides, a* (green-red) color parameter tended to increase the greenish color due to release of the whey. The color variations of cheeses are mainly affected by cheese manufacturing techniques and quality properties of the fat phase. As a result of the biochemical reactions during the ripening period, the compositional and structural changes occur and can affect the color changes of various cheeses. The cheese structure is the result of hydrophobic interactions between caseins and the effects of high hydrostatic pressure on the non-covalent bond by their breakdown. Therefore, the cheese components produce a new structure of different rheological and color characteristics (SALDO *et al*., 2002).

Table 2. The physicochemical changes in control and in the pressurized cheeses during ripening.

Cheese type		Day	Control	300 Mpa	450 Mpa
Cow cheese	Titratable acidity (% LA)	1	0.58±0.02^{a}	0.57±0.00^{a}	0.56±0.03^{a}
		30	0.35±0.03^{b}	0.41±0.04^{c}	0.36±0.02^{b}
		60	0.38±0.02^{bc}	0.37±0.0^{bc}	0.37±0.02^{bc}
	pH	1	4.54±0.02^{ab}	4.61±0.00^{a}	4.65±0.01^{b}
		30	4.57±0.04^{ab}	4.61±0.04^{ab}	4.59±0.01^{ab}
		60	4.52±0.08^{a}	4.56±0.06^{ab}	4.58±0.08^{ab}
	L*	1	92.38±0.40^{a}	92.26±0.01^{a}	92.79±0.01^{a}
		30	92.90±0.14^{a}	92.62±0.15^{b}	93.11±0.81^{a}
		60	92.07±0.82^{a}	92.90±0.16^{c}	91.45±1.85^{a}
	a*	1	-2.46±0.08^{a}	-2.38±0.00^{a}	-2.89±0.01^{a}
		30	-2.37±0.24^{a}	-2.51±0.05^{b}	-2.81±0.21^{ab}
		60	-2.27±0.20^{a}	-2.24±0.03^{c}	-2.51±0.18^{b}
	b*	1	16.38±0.64^{a}	16.69±0.04^{ab}	17.65±0.01^{a}
		30	16.34±2.18^{a}	17.54±0.00^{a}	16.68±0.26^{ab}
		60	16.40±1.57^{a}	16.33±0.44^{b}	16.52±0.74^{b}
Goat cheese	Titratable acidity (% LA)	1	0.61±0.03^{a}	0.58±0.07^{a}	0.50±0.05^{b}
		30	0.38±0.02^{c}	0.35±0.04^{d}	0.38±0.03^{cd}
		60	0.34±0.02^{d}	0.39±0.00^{cd}	0.42±0.02^{c}
	pH	1	4.75±0.10^{a}	4.83±0.08^{ab}	4.92±0.04^{c}
		30	4.81±0.07^{ab}	4.87±0.05^{bc}	4.88±0.03^{bc}
		60	4.84±0.02^{b}	4.83±0.01^{ab}	4.79±0.01^{ab}
	L	1	94.27±0.27^{a}	92.82±0.13^{bc}	91.89±0.40^{d}
		30	93.16±0.41^{b}	92.37±0.50^{cd}	92.76±0.33^{bc}
		60	92.66±0.15^{bc}	94.22±0.40^{a}	93.29±0.37^{b}
	a*	1	-2.42±0.11^{ef}	-3.20±0.21^{b}	-3.49±0.14^{a}
		30	-2.34±0.05^{fg}	-2.54±0.15^{de}	-2.68±0.08^{d}
		60	-2.22±0.07^{g}	-2.70±0.02^{d}	-2.96±0.08^{c}
	b*	1	9.23±0.32^{a}	10.71±0.87^{b}	11.28±0.74^{b}
		30	9.55±0.28^{a}	9.04±0.12^{a}	9.09±0.23^{a}
		60	9.51±0.27^{a}	9.47±0.16^{a}	9.20±0.19^{a}

Values represented by mean ± standard deviation.
^{a,b,c} Different superscript in the same parameter indicates significant differences ($p<0.05$).

3.3. The changes in nitrogen fractionations

The WSN, TCA-SN value and RI were determined for the monitoring of proteolysis progress, and acid degree value (ADV) for evaluation of lipolysis and the results were given in Table 3. No significant difference ($P>0.05$) was found in WSN or TCA-SN levels between HHP-treated cheeses and control cheese for 60 days ripening period in cow cheese. In contrast, both WSN and TCA-SN value of goat cheeses were determined by application of 300 MPa pressure compared to 450 MPa in cheeses samples ($P<0.05$). Higher proteolysis was observed in 300 MPa HP treated cheeses compared to control and

450 MPa treated samples. The chymosin activity is important for cheese ripening, however, chymosin enzyme has been affected by high hydrostatic pressure depending on pressure force. While most of the peptides were produced rapidly under HP treatment at <300 MPa pressure. The liberation of other peptides was inhibited by pressurization at >300 MPa pressures (SCOLLARD *et al*., 2000).

Table 3. The effect of high-pressure treatment (300 and 450 MPa for 5 min) on proteolysis and lipolysis of the cheeses during ripening.

Cheese type			Control	300 Mpa	450 Mpa
Cow cheese	WSN (%)	1	0.024±0.00^{a}	0.029±0.00^{ab}	0.027±0.00^{ab}
		30	0.028±0.00^{ab}	0.032±0.00^{bc}	0.034±0.00^{cd}
		60	0.037±0.00^{de}	0.040±0.00^{e}	0.039±0.00^{e}
	Ripening Index	1	1.016±0.01^{a}	1.189±0.03^{abc}	1.147±0.00^{ab}
		30	1.291±0.09^{abc}	1.376±0.13^{cd}	1.441±0.09^{de}
		60	1.670±0.227^{e}	1.662±0.29^{de}	1.588±0.17^{de}
	TCA-SN (%)	1	0.006±0.00^{a}	0.008±0.00^{ab}	0.009±0.01^{abc}
		30	0.010±0.00^{bcd}	0.011±0.00^{bcde}	0.009±0.00^{bc}
		60	0.013±0.00^{de}	0.014±0.00^{e}	0.012±0.00^{cde}
	Acid degree Value	1	0.86±0.08^{ab}	0.56±0.040^{a}	0.67±0.02^{a}
		30	1.21±0.39^{c}	1.08±0.09^{bc}	1.02±0.01^{bc}
		60	1.16±0.21^{bc}	1.18±0.20^{bc}	1.26±0.22^{c}
Goat cheese	WSN (%)	1	0.025±0.00^{a}	0.030±0.00^{b}	0.027±0.00^{a}
		30	0.026±0.00^{a}	0.033±0.00^{c}	0.036±0.00^{cd}
		60	0.028±0.00^{a}	0.034±0.00^{c}	0.038±0.00^{d}
	Ripening Index	1	0.938±0.02^{a}	1.204±0.06^{c}	1.036±0.11^{ab}
		30	1.118±0.09^{bc}	1.421±0.06^{de}	1.512±0.08^{e}
		60	1.199±0.06^{c}	1.351±0.09^{d}	1.429±0.03^{de}
	TCA SN (%)	1	0.009±0.00^{a}	0.011±0.00^{a}	0.010±0.00^{a}
		30	0.010±0.00^{a}	0.012±0.00^{a}	0.011±0.00^{a}
		60	0.461±0.02^{c}	0.345±0.19^{bc}	0.268±0.12^{b}
	Acid degree Value	1	0.95±0.01^{ab}	0.99±0.04^{ab}	1.09±0.05^{b}
		30	0.80±0.12^{a}	1.15±0.18^{b}	1.09±0.03^{b}
		60	1.77±0.29^{c}	1.92±0.14^{c}	1.89±0.03^{c}

Values represented by mean ± standard deviation.

^{a,b,c} Different superscript in the same parameter indicates significant differences ($p < 0.05$).

The ripening index value of goat cheese samples was higher than cow cheese samples. The destabilization of casein micelles after high hydrostatic pressure processing may increase as a result of residual coagulant or indigenous milk proteinases activity (HUPPERT *et al*., 2004) and can cause enhancement effect on their sensibility to proteolytic enzymes. It means that plasmin can play an important role in proteolysis after pressurization. The enhancer effect of high hydrostatic pressure on proteolysis was reported in other cheeses under different pressures (O'REILLY *et al*., 2000; RYNNE *et al*., 2008; VOIGT *et al*., 2010).

Similar to proteolytic changes, ADV values were affected by high-pressure treatment in goat cheese samples (P<0.05), whereas, there was no significant effect found in cow cheese samples (P>0.05). ADV values of all cheese samples showed an increase during storage time. It is obvious that high-pressure treatment influences biochemical reactions during the ripening period of cheese. This phenomenon may occur via the direct effect of pressure on enzyme or reaction and may affect the release of enzymes of lactic acid bacteria. Besides the changes during ripening via biochemical reaction on compositional and structural properties of cheeses, HHP produces conformational changes in proteins and may affect enzyme modulation site or active site directly (ROVERE, 1995). These changes affect proteolysis or lipolysis and show enhancing and reducing effect on the ripening of various cheeses.

3.4. Overall composition comparison of cheeses

SIMCA of the overall properties of the control and high pressure treated cheeses are presented in Fig. 2. It showed the distinctive pattern and 6 well-defined cheese groups. SIMCA is a supervised classification method and provides a model based on the principal component analysis and uses the distance of the mean in each group for discrimination. The distance of mean in each cluster or group are known as interclass distance and if this value is higher than 3, it is significant for identification (KVALHEIM and KARSTANG, 1992). The interclass distance for all control and pressurized cheeses ranged between 3.74-17.04 (Table 4).

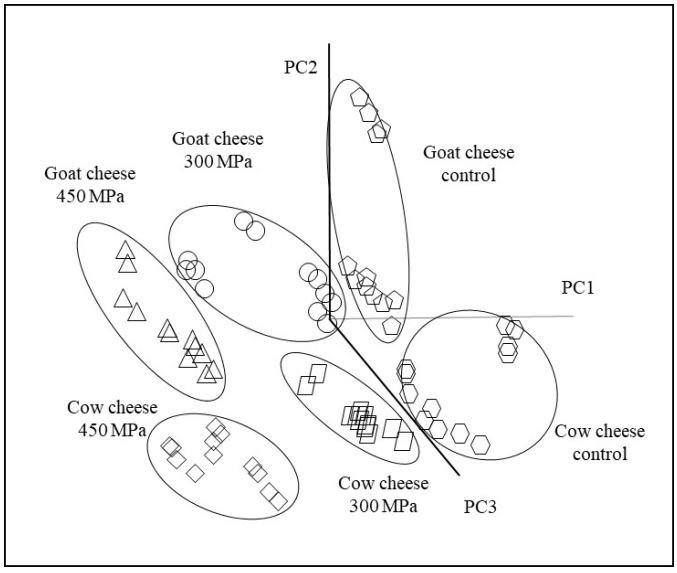

Figure 2. Soft independent modeling of class analogy (SIMCA) classification plot of cheese samples treated with high pressure at 300 and 450 MPa for 5 min. The all data were transformed into their centered and normalized mean prior to multiple analysis.

Table 4. Interclass distances between the goat and cow cheese treated with high pressure at 300 and 450 MPa for 5 min based on the SIMCA class projections.

Groups	Cow cheese control	Cow cheese 300 MPa	Cow cheese 450 MPa	Goat cheese control	Goat cheese 300 MPa	Goat cheese 450 MPa
Cow cheese control	0.00					
Cow cheese 300 MPa	3.74	0.00				
Cow cheese 450 MPa	7.38	6.89	0.00			
Goat cheese control	6.73	14.33	17.04	0.00		
Goat cheese 300 MPa	7.35	9.45	10.65	7.48	0.00	
Goat cheese 450 MPa	8.82	10.04	8.26	11.35	4.68	0.00

Interestingly, cow and goat cheese results showed good discrimination, although, both cheese types had similar results. The difference between goat and cow cheeses probably arises from results of the proteolysis and lipolysis test. When WSN and TCA-SN values of the cow cheese were not affected by high pressure application, pressurization showed enhancer effect on goat cheese samples, proteolysis and lipolysis properties. The SIMCA pattern showed that control, 300 MPa and 450 MPa pressurized samples of both cow and goat cheeses grouped in the same order and implying increase in pressure created a similar effect on the two cheese species.

4. CONCLUSION

The evaluation of the overall results obtained from the study showed that, two different levels of the high-pressure application on white cheeses samples produced from cow and goat milk had varying effects. This method shows the possibility of applying high-pressure treatment in the white cheese besides the predetermined reliability. Also, the microbial load of lactic acid bacteria is decreased by high-pressure practices. The HPP delayed the growth of yeasts/molds in goat cheese samples compared to the control groups at the end of the ripening period. Consequently, increase in the level of HPP provided significant decreasing effect on TMAB, Lactobacilli and Lactococci counts, and increasing effect on proteolysis and lipolysis. The fact that high-pressure has no significant difference in the chemical composition, but have positive results visually indicates the applicability of this technology.

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PAPER

BROMATOLOGICAL COMPOSITION AND EFFECT OF TEMPERATURE ON THE RHEOLOGY OF EGGPLANT PULP

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ABSTRACT

This study aimed to determine the bromatological composition and the behavior of rheological parameters on the pulp eggplant (*Solanum melongena)*. Bromatological analyzes were performed according to the reference methods, in which a percentage of moisture 90.98%, total carbohydrates 6.86%, crude fiber 1.94%, crude protein 1.19%, Fat 0.31% and 0.49% of ash has been obtained. Viscous flow curves were calculated in the steady state over a temperature range of 10-80ºC, and the rheological properties of the pulp were evaluated as a function of temperature. The pulp showed pseudoplastic behavior (shear thinning type) at all temperatures, and the relationship between viscosity and the Carreau-Yasuda model (R^2>0.99). The Arrhenius equation was fitted to the data for the apparent viscosity of the pulp with respect to temperature, with activation energy E_a=1081.61 J/mol. The results provide information on bromatological composition and the rheological behavior of eggplant pulp and may have applications in the design of processes using this raw material.

Keywords: Carreau-Yasuda model, eggplant (*Solanum melongena*), bromatological analysis, pulp, rheological behavior, shear thinning

1. INTRODUCTION

Eggplant or aubergine (*Solanum melongena*) is an economically important crop that is widely cultivated in tropical and subtropical areas of the world, and its cultivars produce a wide variety of fruits with different shapes, sizes and colors (SALUNKHE and DESAI, 1984; SAN JOSÉ *et al.*, 2013; NIÑO-MEDINA *et al.*, 2017). The global consumption of eggplant has increased in recent years, due to the numerous benefits of this crop, such as the presence of metabolites that contribute significantly to a healthy diet (SUN *et al.*, 2015). It has also been found that extracts of eggplant can successfully suppress the development and growth of tumors, metastasis, inflammation and heart disease (NISHA *et al.*, 2009). Eggplant is one of the vegetables on which great importance is placed in the Colombian Caribbean, due to its strong suitability for export, the aforementioned health benefits, its high content of phenols and antioxidant activity, and its contribution to the diet in terms of low calorie content, very low sodium content and high fiber content (ARAMENDIZ-TATIS *et al.*, 2010).

According to Food and Agriculture Organization - FAO (2015) data from 2015, eggplants used for consumption were fresh (more common), or in industrial processes, mainly frozen. In industrial processes, this vegetable can be used as cubes or dice, such as in slices, jams and jellies. The products of fruits and vegetables contain pulps as basic raw materials, which in most cases are transported via pipes and tanks and are agitated and mixed with other raw materials, pasteurized and evaporated in heat exchangers and continuous evaporators. In addition, other operations are applied such as sieving, snubbing, mixing and various thermal treatments. In order for these operations to be technically and economically feasible, it is important to have a knowledge of the properties of the pulp (IBARZ *et al.*, 1996; ORTEGA-QUINTANA *et al.*, 2015).

The rheological behavior is one of the most important properties in the development of new products, and is very useful in the design of unit operations, guarantees of high-quality food and beverages, and process optimization. In addition, rheological approaches are essential tools for food engineering, since rheology is linked to the processing and stability of food as well as its sensory qualities. Physical properties such as density, specific heat and thermal conductivity are of great importance for food and beverages, as they are closely related to their sensory and rheological characteristics (AUGUSTO *et al.*, 2012; DE CASTILHOS *et al.*, 2017).

Several factors affect the rheological behavior of fruit and vegetable pulps, and temperature is the most likely to affect the viscosity of the pulp (HOLDSWORTH, 1971). An understanding of the influence of temperature on viscosity is therefore fundamental in obtaining better knowledge of the rheological behavior of fruit and vegetable derivatives during processing at high temperatures (IBARZ *et al.*, 1996).

Various studies have been carried out on the rheological behavior of pulps from different botanical sources; most of these do not comply with Newton's law of viscosity, and it is said that they behave like non-Newtonian fluids. Their behavior can be described by a power law (HOLDSWORTH, 1971) or by the Herschel-Bulkley model in the case where they have a non-zero yield stress (STEFFE, 1996). The main objective of this research is to determine the effect of temperature on the behavior of the rheological parameters of eggplant pulp.

2. MATERIALS AND METHODS

The eggplants used were acquired from a food supply center in Cartagena, Bolivar-Colombia, and were in a state of commercial maturity and free from mechanical damage.

2.1. Pulp extraction

The selected fruits were weighed, washed and blanched at 80ºC for five minutes. Pulp was obtained using a refining 1.5 mm opening mesh, giving a pulp to facilitate rheological measurements. This was then packed in hermetic bags and stored in refrigeration at 4ºC for 24 hours for further rheological analysis.

2.2. Bromatological evaluation

Bromatological analyses were performed on the eggplant pulp. Moisture content, ash, protein, fat, total carbohydrates and raw fiber were determined using the methods described by the Association of Official Analytical Chemists (AOAC) (2000): moisture: Dehydration in an oven at 105ºC AOAC 33.7.03 Method 926.08; ash: y combustion at 450ºC for 12 hours AOAC 33.7.07 Method 935.42; crude protein: Using the method of Macro Kjeldahl, AOAC 33.7.12 Method 926.123; fat: AOAC 972.28 ethereal extraction method; total carbohydrates: AOAC 923.03; Crude fiber: A.O.A.C. 985.29.

2.4. Rheological evaluation

Steady flow tests were carried out to give viscosity curves at temperatures of 10ºC, 20ºC, 25ºC, 40ºC, 60ºC and 80ºC for samples without a previous history of shear, using a controlled-stress rehometer (Modular Advanced Rheometer System Mars 60, Haake, Thermo-Scientific, Germany), equipped with Peltier temperature control and measuring system, using a stainless steel plate-plate geometry (rough surface Ø 35 mm) with 1 mm gap, over a range of shear rates between 0.001 and 1000 s^{-1} (Franco *et al*., 1998). Prior to measurement, all samples were left at rest for 600 s to allow for relaxation, and the temperature of the samples was kept constant at 20±0.1°C using a Peltier system, following the methodology used by QUINTANA *et al.* (2017).

2.4. Statistical analysis

The data were analyzed with a unidirectional ANOVA using SPSS software (version 17.0 for Windows) in order to determine statistically significant differences ($p < 0.05$) between the samples. All tests were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Bromatological analysis

Table 1 shows the average values for moisture, total carbohydrates, total fiber, protein, fat and ash, obtained in triplicate. The results show that the eggplants used in this study mostly contained water at a percentage of 90.98±0.016%, a carbohydrate content of

6.86±0.17% and a fiber content of 1.94±0.07%, fairly close to the values reported by GARCÍA *et al*. (2003), MOREIRAS *et al*. (2013) and ICBF (2015).

Table 1. Bromatological analysis.

Sample (%)	Moisture	Total carbohydrates	Crude fiber	Crude protein	Fat	Ash
Eggplant	90.98±0.16	6.86±0.17	1.94±0.07	1.19 ±0.04	0.31±0.02	0.49±0.02

Eggplant represents an important source of dietary fiber, and therefore has health benefits in terms of its effects on digestive regularity and the prevention of diseases such as constipation, hypercholesterolemia, hyperglycemia and obesity, which are partly related to intake of fiber (ALVES DOS SANTOS *et al*., 2002). Its beneficial physiological effects lie in its texture and consistency, since it acts as a sponge that binds to foods rich in cholesterol, releasing accumulated wastes from the intestinal wall, which would otherwise be difficult to expel. It also increases the fecal mass, which results in a reduction in carcinogenic risk and faster elimination from the body (CAÑAS-ÁNGEL *et al* 2011.)

3.2. Rheological evaluation

The variation of the viscosity with the deformation speed was observed using steady flow tests at different temperatures. Fig. 1 shows the viscous flow curves for the eggplant pulp as a function of the deformation speed, at temperatures of 10°C, 20°C, 25°C, 40°C, 60°C and 80°C. These curves show the characteristics of a non-Newtonian fluid of rheofluidifying (shear thinning) type, since they combine the characteristic properties of elastic and both solid-like and liquid-like properties, characterized by a potential decrease in viscosity with respect to the shear rate (MULLER, 1973). On the other hand, a constant viscosity value η_0 and at high deformation η_∞ velocities are observed at low deformation speeds.

This behavior can be explained by the breaking of the reticular structure of polysaccharide molecules during shearing, as explained by BHANDARI *et al.* (2002). In a reticular system, the speed at which existing molecular interactions break becomes higher than the speed at which they reform, with increasing shear rate. The result is a lower intermolecular resistance to the flow, and hence a lower viscosity (DÍAZ-OCAMPO *et al.*, 2012). Similar behaviors have been observed in several fruit pulps, such as squash pulp (*Cucurbita moschata*) (QUINTANA[a] *et al.*, 2018); papaya pulp (*Carica papaya*) (QUINTANA *et al*., 2017); borojó pulp (*Borojoa patinoi cuatrec*) (DÍAZ-OCAMPO *et al.*, 2012); nispero pulp (*Achras sapota L.*) (ANDRADE *et al.*, 2009) and mango, papaya and peach purees (GUERRERO and ALZAMORA, 1998).

Among the models most often used to describe the rheological behavior of fruit pulps is the Ostwald de Waele power law (TORALLES *et al.*, 2006). Numerous authors have successfully described the flow behavior of various pulps using this model, for example, peach pulp (MUÑOZ *et al.*, 2012) and mango pulp of different varieties (VIDAL *et al.*,2004; ORTEGA-QUINTANA *et al.*, 2015; FIGUEROA-FLÓREZ *et al*., 2017). In this case, the experimental data for the viscosity and shear rate were fitted to the Carreau-Yasuda model (CARREAU, 1972), which gave us the best statistical parameters including a minimum correlation coefficient R^2>0.99282:

$$\eta = \eta_\infty + (\eta_0 - \eta_\infty)[1 + (\lambda_c \dot{\gamma})^a]^{\frac{n-1}{a}} \quad (1)$$

This model represents a fluid that follows Newton's law of viscosity at low deformation speeds and obeys a power law at high shear rates (MÉNDEZ-SÁNCHEZ *et al*., 2010). It uses five parameters: η_0 corresponds to the Newtonian viscosity at low shear rates; η_∞ is the Newtonian viscosity at high deformation speeds; λ_c is the Carreau time constant; a is the transition control factor, which is a dimensionless constant; and n is the parameter of the power law model. In the case where n=1, the model is reduced to the linear Newtonian model, for example, the Navier-Stokes equations. For fluidifying liquids (n<1), the viscosity decreases with an increase in the shear rate.

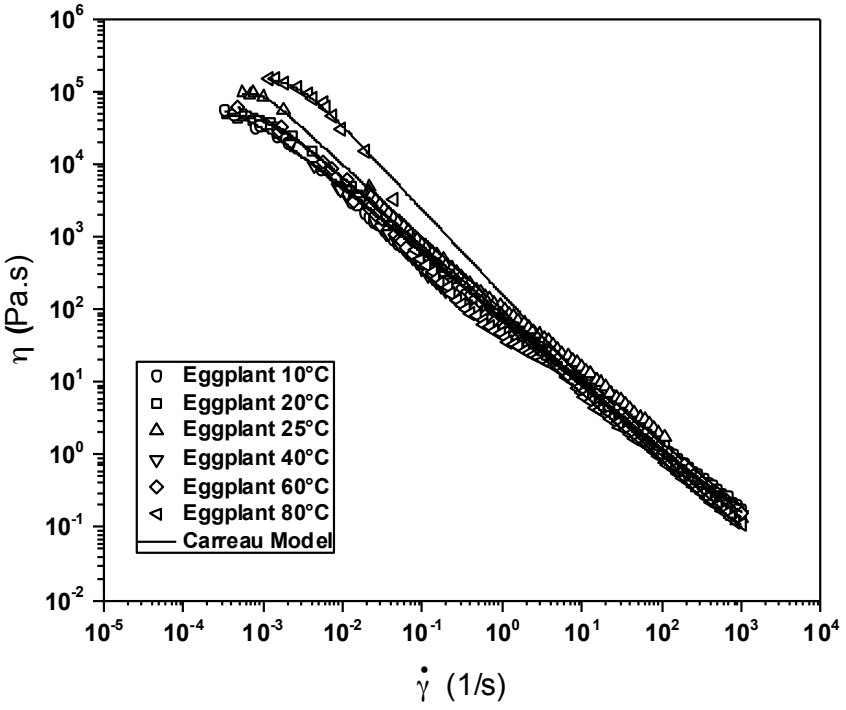

Figure 1. Viscous flow curves for *S. melongena* pulp at different temperatures (10ºC, 20ºC, 25ºC, 40ºC, 60ºC, 80ºC) fitted to the Carreau-Yasuda model.

Fig. 1 shows the experimental data (viscosity vs. shear rate) fitted to the Carreau-Yasuda model. At the initial points, the apparent viscosity tends to be constant, and is represented as the null viscosity η_o. It subsequently begins to decrease, and the viscosity curve enters a logarithmic drop phase, where at high deformation speeds it tends to behave like a Newtonian fluid, following the behavior of the Carreau model. This decrease in viscosity with an increase in shear rate is called shear thinning, and similar results have been obtained in rheological studies of papaya pulp (QUINTANA *et al*., 2017).

The fitting parameters of the Carreau-Yasuda model are shown in Table 2. The results demonstrate rheofluidifying (shear-thinning) behavior, with values for the flow behavior index of less than unity (n<1) for the different conditions of temperature. The viscosity decreases with the shear rate, and hence it is not considered an increase or decrease in the

fluidifying character of the pulps. However, changes in temperature affect the viscosity, since an increase in temperature produces greater intermolecular interaction in the aqueous phase of the pulp, causing repulsion between the suspended particles, lower resistance to flow, and consequently a decrease in the apparent viscosity (FIGUEROA-FLÓREZ *et al.*, 2017). This effect was also found by QUINTANA (2016), who showed that the results depended strongly on the heat treatment applied to the pulp before rheological analyses were carried out.

Table 2. Rheological parameters of the Carreau-Yasuda model for the viscosity of eggplant pulp with variation in shear rate, at temperatures of 10-80°C.

Temp.	η_0	η_∞	λ_c	a	n	R^2
10°C	70390.99±1495.38	4.31E-47±0.01	2129.66±136.20	1.50±0	0.10±0.02	0.99
20°C	48987.00±751.80	0.02±0.01	882.40±23.87	2.15±0.14	0.06±0.01	0.99
25°C	96193.78±583.63	5.14E-37±0	980.64±0	5.00±0.33	0.02±0.01	0.99
40°C	23964.83±654.76	0.08±0	643.23±10.68	17.61±5.43	0.08±0.01	0.99
60°C	83051.70±5478.35	0.13±0.3	1222.66±48.54	1.25±0.13	0.05±0.02	0.99
80°C	164922.94±5552.34	0.05±0.01	39.73±8.43	2.09±0.21	0.16±0.01	0.99

The effect of temperature on the apparent viscosity of fluid foods (at constant shear rate) can be explained by the Arrhenius equation (DAK *et al.*, 2007; RAO and TATTIYAKUL, 1999), expressed as:

$$\eta = Aexp\left(\frac{E_a}{RT}\right) \qquad (2)$$

where A is the pre-exponential factor; Ea is the activation energy, a parameter used to evaluate the thermal dependence (J/mol); R is the gas constant (8.314 J/mol K); and T is the temperature absolute (K). In this model, the apparent viscosity decreases in an exponential way with temperature. A shear rate of 15 s^{-1} was selected, since operations such as flow in pipes, mixing and agitation involve a range of shear speed of 10–1000 s^{-1} (STEFFE, 1996).

The experimental data for η can be well modeled by the Arrhenius equation, as shown in Fig. 2, and the values for R^2 are found to be greater than 0.9387. The effect of temperature on apparent viscosity (at a constant shear rate) can also be observed in Fig. 2.

In this case, the activation energy E_a=1081.61894 J/mol was lower than the values reported for squash pulp (E_a= 1229.46 J/mol) (QUINTANA *et al.*, 2018); acerola pulp (E_a=12637.89-14292.45 J/mol) (PEREIRA *et al.*, 2014); sapodilla pulp (E_a=12637.89 J/mol) (ANDRADE-PIZARRO *et al.*, 2010) and tomato paste (E_a=8600-13000 J/mol) (DAK *et al.*, 2008), indicating that eggplant pulp has a higher sensitivity to changes in temperature in a food transpiring process; in other words, the internal structure of eggplant pulp is more affected by temperature than the other pulps or food products mentioned above.

The activation energy is a very important parameter in terms of the movement of the molecules, as it influences when the temperature increases in the liquids, allowing them flow more easily, due to a high activation energy at high temperatures (MEMNUNE *et al.*, 2005). In this case, an increase in temperature causes a decrease in the viscosity of the

liquid phase, thus increasing the movement of the suspended particles and causing a decrease in the viscosity of the pulp (PELEGRINE *et al.*, 2002).

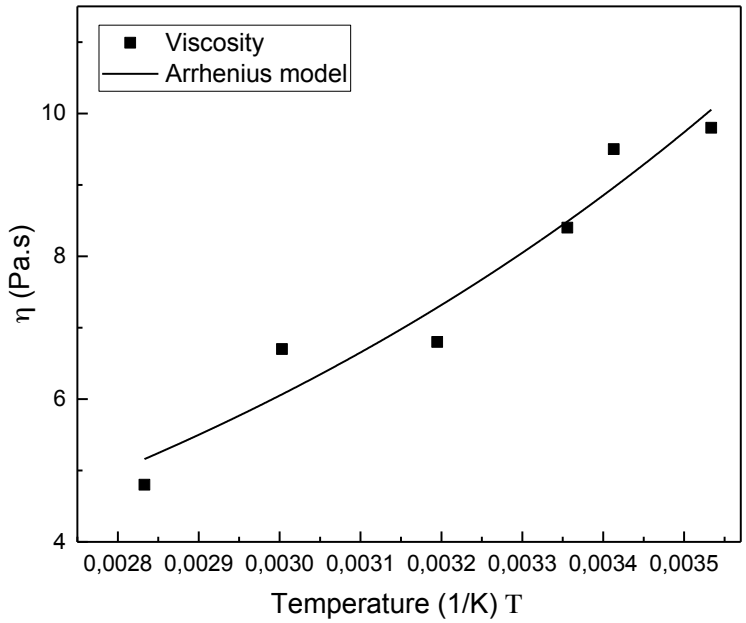

Figure 2. Variation in the values of η obtained at 15 s^{-1} as a function of temperature and fit to the Arrhenius model.

4. CONCLUSIONS

Based on the results obtained in this investigation, the eggplant pulp have a the mayor content expressed in percentage for moisture and carbohydrates, 90.98 and 6.86±0.17 respectively. The rheological properties of the pulp behave like a non-Newtonian fluid of rheofluidifying type (shear thinning). All samples showed a decrease in viscosity with shear rate, which was fitted to the Carreau-Yasuda model with R^2>0.99. The influence of temperature on the behavior of the pulp was observed, and the eggplant pulp was found to lose pseudoplasticity and become less consistent as the temperature increases and its rheological parameters are affected. The relationship between the temperature and the apparent viscosity of the pulp can be represented by the Arrhenius equation, where an increase in temperature causes a decrease in viscosity. Finally, by examining the rheological and flow properties of eggplant pulp, this study may encourage new applications and may help in the design of new food products from this raw material of national interest.

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PAPER

QUALITY OF ISTRIAN AND SLAVONIAN DRY-FERMENTED SAUSAGES

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ABSTRACT

The aim of this study was to investigate nutritive and sensorial quality of Istrian and Slavonian pork-meat dry-fermented sausages. Sensorial analysis resulted in statistically significant differences ($p<0.05$) between these products in 11 sensorial parameters analysed. Significantly higher protein content was determined in Istrian in comparison with Slavonian dry-fermented sausage, although the latter content significantly varied across the producing households. No significant differences in particular fatty acid esters and saturated, monounsaturated and polyunsaturated fatty acids were obtained. Although some of the nutritional quality indices failed to meet health recommendations, the obtained values are consistent with data published for pork meat products.

Keywords: Croatian households, dry-fermented sausages, nutritive properties, sensory properties, traditional pork meat sausages

1. INTRODUCTION

A long tradition of dry-cured meat production in rural households exists all over the world, especially in the Mediterranean countries. A very important group of such products are dry-fermented sausages, whose properties widely vary due to various processing procedures often lacking uniformity (TALON *et al.*, 2007; GARCÍA-GONZÁLEZ *et al*., 2013). Suitable chemical and sensory markers enable better linkage between raw matter and processing parameters, and thus result in higher uniformity and consistency of the products (CHIZZOLINI *et al*., 1996; VIRGILI and SCHIVAZAPPA, 2002; ZANARDI *et al*., 2010). A wide variety of dry-fermented sausages is characterized by specific flavour investigated into by a number of studies focused on clarifying the control mechanisms affecting the flavour development (TOLDRÁ, 1998; GARCÍA-GONZÁLEZ *et al*., 2013).

The highest impact on sensory properties of these products is that of smoking and ripening operations (JERKOVIĆ *et al*., 2010; KOVAČEVIĆ, 2014). Of particular significance are the processes of fat lipolysis, free fatty acids' formation, and degradation and oxidation of short-chain fatty acids, since these key reactions, taking place during ripening, affect the formation of specific odour and taste of the final product (KOZAČINSKI *et al*., 2006; MILIČEVIĆ *et al*., 2014; MARUŠIĆ *et al*., 2014). Raw material quality is influenced by farm animals' genotype, manners of their keeping and feeding, procedures applied before slaughtering, and post-slaughtering conditions. Technological processes, such as conditioning, fermentation, drying, smoking and ripening, as well as different technological parameters, such as temperature, relative humidity and air/smoke velocity, influence the properties of the final product, too. Due to the application of various technological processes, the activity of technological microflora, especially during the fermentation process and long-term maturation of sausage stuffing during production, complex microbiological, physicochemical and biochemical changes take place in fundamental building materials (fats, proteins and carbohydrates), resulting in water loss and increase in dry matter weight (KOVAČEVIĆ, 2014).

During the recent decades, studies devoted to fermented meat products have mainly focused on evaluation of physicochemical, microbiological and sensory properties (COMI *et al*., 2005, RANTSIOU *et al*., 2005, DI CAGNO *et al*., 2008) in order to contribute to the better characterization of final products, the definition of unique quality markers and the improvement of product specification protocols essential when dealing with products of the protected designation of origin (PDO) or geographical indication (PGI) (BOGDANOVIĆ *et al*., 2016). However, it is known that significant differences exist among traditional dry-fermented sausages produced in different countries or even in the same country (KOS *et al*., 2009; ZANARDI *et al*., 2010). As for traditional dry-fermented sausages produced in Croatia, Slavonian sausage is produced in the eastern Croatia and represents a very important Croatian PGI food brand. The same applies to Istrian sausage produced in the western Croatia, which is in the process of becoming a protected product. Even though both sausages are produced from pork meat, Istrian sausages contain less fat tissue and are spiced with pepper and garlic. The ingredient that makes these sausages specific is the local wine (Malvasia). Unlike these, Slavonian sausages are spiced with garlic and red pepper and contain in general a larger amount of fat tissue.

The production of Slavonian sausage calls for the minimum of 70% of the second- and the third-category pork meat and the minimum of 30% of solid fat tissue, while the production of Istrian sausage makes use of the meat of the same categories, but of not more than 6% of bacon. Mincing (6-8 mm hole for Slavonian and at least 10 mm for Istrian

sausage) is followed by the addition of salt and spices; in case of Slavonian sausage, sweet and hot red pepper and garlic are added, while in case of Istrian sausage pepper and Malvasia enriched with garlic extract and prepared according to a specific procedure, are added. In both cases, a thoroughly mixed mixture of the above-detailed ingredients is stuffed in pork small intestine used as casing, which should be at least 70 cm long when it comes to Slavonian, and not longer than 50 cm when it comes to Istrian sausage. In the further course of Slavonian sausage production, the product is conditioned for a day, cold-smoked for 14 days tops, and ripened and dried in chambers for at least 45 days at temperatures not higher than 16°C, relative humidity thereby being kept at 70-85%. Since Istrian sausages should not be smoked, the subsequent course of their production upon stuffing involves ripening in chambers at temperatures of 9-16°C and with relative humidity of 65 to 85%. Istrian sausages can be released to the market 30 days after the production commencement, while the entire production of Slavonian sausages takes 60 days at least. As for their physicochemical properties, Slavonian sausage is allowed to contain no more than 40% of fat, while water activity should be kept below 0.90; on the other hand, Istrian sausage should contain 16% of proteins at the minimum and 40% of water at the maximum.

According to specification, Slavonian sausage has an elongated cylinder shape; one sausage pair should be at least 35 cm long and measure 2-3 cm in cross-section (MA, 2019). The casing is reddish/brown and should be free of smudges, folders, cracklings and surface moulds. The texture is expected to be solid and elastic, but not rubberish; the sausage should be easy-to-cut, not prone to crumbling, and easy-to-chew. The stuffing should be dark red in its cross-section, except for the fat tissue, which is coloured either white or orange; the stuffing resembles a mosaic, should be well entangled and free of holes, lacking a dark margin under the casing. Prior to cutting, Slavonian sausage has a smoky smell of an ash tree, hornbeam or beech; once the sausage is cut, the smell of fermented meat and garlic is released. Istrian sausage assumes the shape of an elongated cylinder, measuring at least 50 cm in length and having a rounded diameter. The casing should not be damaged and should tightly adhere to the stuffing. In its cross-section, the sausage is solid, mosaic-like, the meat thereby being coloured red and the fat tissue being coloured white. The sausage should be compact and holes-free, while its stuffing should not get separated during cutting.

Given the fact that among complex factors that drive consumer dry-fermented sausages' choice, nutritive and sensory properties are the major criteria, the aim of this study was to investigate into these qualities of the most important types of traditional Croatian dry-fermented sausages. The study was performed on Istrian and Slavonian sausages produced in different households situated in two Croatian main fermented meat production regions. Based on fatty acid content, nutritional quality indices were also assessed for both types of products.

2. MATERIALS AND METHODS

2.1. Sampling

The study involved 40 samples of traditional Croatian dry-fermented sausages named Istrian (n=20) and Slavonian sausage (n=20). Sausages were collected randomly during a two-year period (2018-2019) from markets, fairs and producing households. The products originated from two Croatian regions: the western region (Istra and Primorje) and the

eastern region (Slavonia and Baranja). Pursuant to the Ordinance on Meat Products (OG 62/18) adopted by the Republic of Croatia, these sausages belong to the group of dry-cured sausages falling into the category of non-thermally processed meat products. The sausages were produced according to traditional recipes using traditional technologies observed by the producing rural household. To the end of their production, pork meat of the first, the second and the third category was used, together with fat and various amounts of salt and spices, depending on the sausage type (KOVAČEVIĆ, 2017).

2.2. Sensory characteristics

Sensory analysis was carried out by a trained panel (of 9 assessors). The assessors were selected and generically trained according to the ISO standard (ISO 11132:2012). Sensory analysis was carried out in the Sensory Laboratory of the Faculty of Food Technology and Biotechnology according to ISO 8589:2007. Sensory assessment made use of a quantitative descriptive analysis (QDA) and a unipolar numerical intensity scale was developed in collaboration with the Centro Studi Assaggiatori (Brescia, Italy). The intensity of each sensorial property was assessed using an ascending left-to-right numerical scale, "zero" thereby representing the absence of a given sensorial property and "nine" its highest intensity. At each panel session, a repeated sample of a given product group was available; for each assessor, a number of statistical parameters descriptive of his/her efficacy was calculated.
Plates containing single-coded samples were served into sensory compartments, together with all materials required for sensory assessment. Each assessor carried out a sensory assessment of the intensity of objective visual food item properties (colour of the minced meat, colour uniformity, fat content, cohesiveness), smell-related properties (favourable smell, unfavourable smell, smoky smell), taste-related properties (tenderness, juiciness, saltiness, sweetness, sourness, bitterness, spiciness) and aroma (coming from aromatic herbs, spice herbs, ripe meat, biochemical properties, fresh pork meat, moulds). The above was followed by the assessment of subjective properties (cross-section attractiveness, smell attractiveness, consistency attractiveness, maturity, richness of appealing aromas, steadiness of appealing aromas, overall attractiveness), which made use of the same numerical, intensity-measuring scale.

2.3. Physicochemical analysis

Samples were cut into small pieces and homogenized for 15 sec at 6,000 rpm using a Grindomix GM 200 (Retsch, Haam, Germany). Sample preparation was completed in full line with ISO 3100-1:1991. All samples were analysed for physicochemical parameters within the next 48 hours upon arrival into the Laboratory. The extracted fat was stored in a refrigerator at -18°C pending fatty acid composition analysis carried out within the next 48 hours. All chemicals used for analyses were of an analytical grade.
The pH value was determined in a homogenate diluted with distilled water (1:10, p/v) using a pH/Ion 510 – Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd/ Oakton Instruments, USA) according to the pH/Ion 510 Instruction Manual. Water activity (a_w) was measured at the room temperature (20±2ºC) using a Rotronic Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland). The pH-value and water activity given for each sample represent the mean value of two independent measurements. The water content was determined gravimetrically (ISO 1442:1997) at 103°C in an oven (UF75 Plus, Memmert, Schwabach, Germany), while the ash content was established according to ISO 936:1998 by

virtue of burning the samples in a furnace at 550°C (LV9/11/P320 Nobertherm, Lilienthal, Germany). The total fat content was determined using the Soxhlet method (ISO 1443:1973), which involves digestion of a sample in an acidic environment followed by fat extraction with petroleum ether using a Soxtherm 2000 automated device (Gerhardt, Munich, Germany). The total protein content was determined using the Kjeldahl method (ISO 937:1978) that employed a Unit 8 Basic digestion block (Foss, Höganäs, Sweden) and an automated distillation & titration device (Vapodest 50s, Gerhardt, Munich, Germany). The salt content determination made use of the multiple standard addition potentiometric technique that employs an ion-selective electrode and a Na EasyPlusTM analyser (Mettler Toledo, Germany). Based on the established sodium content, the representation of sodium chloride (salt) was determined stoichiometrically. The carbohydrate content was calculated based on the water, ash, total protein and fat content.
Each sausage sample was analysed in duplicate and the results were expressed in form of weight percentage (%) with the accuracy of 0.01%. Quality control of analytical methods used was performed using the Reference Material (RM) TET003RM (Fapas, York, England).

2.4. Fatty acid profile

Sample preparation for the analysis of fatty acid methyl esters was described earlier by PLEADIN *et al.* (2019). Fatty acid methyl esters were analysed using gas chromatography (GC) according to ISO 12966-4:2015 and EN ISO 12966-4:2015. To the above effect, a 7890BA gas chromatographer equipped with flame ionization detector (FID), a 60-m DB-23 capillary column having an internal capillary diameter of 0.25 mm and the stationary phase thickness of 0.25 μm (Agilent Technologies, Santa Clara, USA) was used. The components were detected by FID at the temperature of 280°C, hydrogen flow rate of 40 mL/min, air flow rate of 450 mL/min and nitrogen flow rate of 25 mL/min. The initial column temperature was 130°C; after a minute, it was increased by 6.5°C/min until the temperature of 170°C was reached. The temperature was further increased by 2.75°C/min until the temperature of 215°C was attained. The latter temperature was maintained for 12 min and then further increased rate by 40°C/min until the final column temperature of 230°C was reached, the latter being maintained for 3 min. One mL of a sample was injected into a split-splitless injector at the temperature of 270°C and with the partition coefficient of 1:50. The carrier gas was helium (99.9999%), flowing at the constant rate of 43 cm/sec. Fatty acid methyl esters were identified by comparing their retention times with those of fatty acid methyl esters contained by the standard mixture.
The results were expressed as a percent-share (%) of an individual fatty acid in total fatty acids, the accuracy thereby being 0.01%. Each sausage sample was analysed in duplicate. The material used for quality control was CRM BCR 163 (Institute for Reference Materials and Measurements, Geel, Belgium) that has a specified content of seven individual fatty acids.

2.5. Nutritional quality of lipids

Data on fatty acid composition in terms of the mean values obtained by the analysis of two replicates, were used for the calculation of the following lipid quality indices: the atherogenic index (AI), the thrombogenic index (TI) and the hypocholesterolaemic/hypercholesterolaemic ratio (HH). The atherogenic index (AI)

indicates the relationship between the sum of the main saturates and the sum of the main non-saturates. This parameter was calculated as follows:

$$AI = [(C12:0 + (4 \times C14:0) + C16:0)] / [\sum MUFA + PUFA\ n\text{-}6 + PUFA\ n\text{-}3]$$

(ULBRITCTH and SOUTHGATE, 1991)

The thrombogenic index (TI) is defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic FAs (MUFA, PUFA n-6 & PUFA n-3). The index was calculated as follows:

$$TI = (C14:0 + C16:0 + C18:0) / [0.5 \times \sum MUFA + 0.5 \times PUFA\ n\text{-}6 + 3 \times PUFA\ n\text{-}3) + (PUFA\ n\text{-}3 / PUFA\ n\text{-}6)].$$

The ratio of hypocholesterolaemic over hypercholesterolaemic fatty acids (HH) takes into account well-known effects of certain fatty acids on cholesterol metabolism (SANTOS-SILVA *et al*., 2002). It was calculated as follows:

$$HH = (C18:1n\text{-}9 + C18:2n\text{-}6 + C20:4n\text{-}6 + C18:3n\text{-}3 + C20:5n\text{-}3 + C22:5n\text{-}3 + C22:6n\text{-}3) / (C14:0 + C16:0)$$

(ULBRITCTH and SOUTHGATE, 1991).

2.6. Data analysis

Statistical analysis was performed using the SPSS Statistics Software 22.0 (SPSS Statistics, NY IBM, 2013) and the Big Sensory Soft (Centro Studi Assaggiatori, Brescia, Italy, 2005). In order to determine the differences between Istrian and Slavonian dry-fermented sausage in terms of physicochemical properties, fatty acid composition and sensory parameters, the independent sample t-test was used. The decisions on statistical significance were made at the significance level of $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Sensory characteristics

Sensory qualities of fermented meat products are adjudicated based on their aroma, appearance, flavour, texture, aftertaste and sound properties (FLORES, 2011). It is important to point out that contrary to some foods, sensory assessment of fermented meat products, including fermented sausages, is not standardized, since no consensus on sensory attributes that should be evaluated hasn't been reached yet. In several papers, these attributes have been selected and assessed through complex procedures involving sensory vocabulary generation so as to be able to compile a lexicon to be used to describe a sensory profile of a given fermented meat product, as well as through quantitative-descriptive analysis (RUIZ-PÉREZ-CACHO *et al*., 2005; GARCÍA-GONZÁLEZ *et al*., 2006; GARCÍA-GONZÁLEZ *et al*., 2008; BENEDINI *et al*., 2012). As for Croatian household-produced dry-fermented sausages, it has already been concluded that variations in their overall quality, especially sensory characteristics, represent a huge problem (FRECE *et al*., 2014).

The results of sensory evaluation of both Istrian and Slavonian dry-fermented sausage using quantitative descriptive analysis, carried out within this study frame, are shown in Fig. 1.

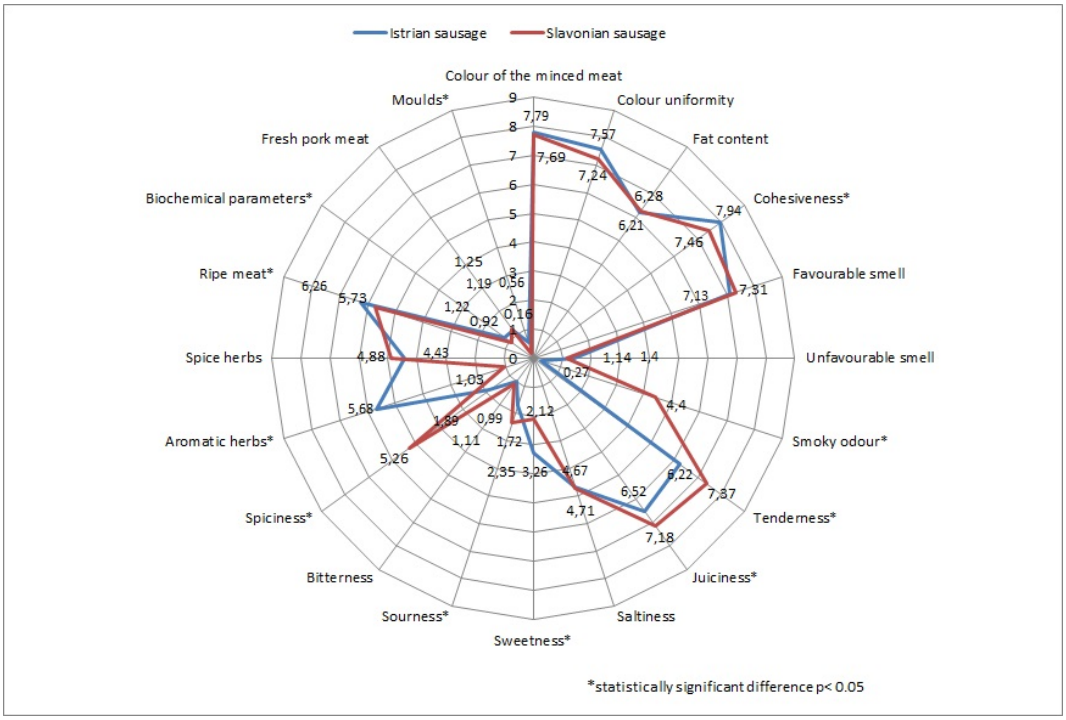

Figure 1. Descriptive sensory profile of objective characteristics (appearance, odour, texture, taste and aroma) of Istrian and Slavonian dry-fermented sausage.

Sensorial analysis resulted in statistically significant differences ($p<0.05$) between these products in 11 out of the total of 20 sensory characteristics analysed, including cohesiveness ($p=0.001$), smoky odour ($p<0.001$), tenderness ($p<0.001$), juiciness ($p=0.008$), sweetness ($p<0.001$), sourness ($p<0.001$), spiciness ($p<0.001$), aroma generated by the presence of aromatic herbs ($p<0.001$), ripe meat quality ($p=0.011$), biochemical parameters ($p=0.016$) and mould presence ($p<0.001$). These significant differences can be attributed to the differences in processing and to the use of different ingredients and spices during the production of the two.

The cohesiveness is stronger in Istrian in comparison with Slavonian sausage. Slavonian sausage scored higher for its smoky odour than did Istrian sausage, whose score in this regard was around 0. The latter was to be expected given that Slavonian sausage is smoked with cold smoke, while Istrian sausage should not be smoked at all. Slavonian sausage scored higher for tenderness and juiciness. It is known that these parameters are in correlation with moisture and fat content. Istrian sausage was shown to be sweeter, as oppose to Slavonian sausage, which was proven sourer, but they turned out to be equally salty. Istrian sausage must have a delicate taste (never sour) and should be moderately salty, while the taste of Slavonian dry-fermented sausage, according to its specifications (MA, 2019), should be mildly hot but never bitter, coming as a result of the combination of fermented meat, garlic, salt and red pepper used in its production. Slavonian dry-

fermented sausage is spicier as compared to Istrian one due to the presence of red pepper spiking, while Istrian sausage scores higher when it comes to aromatic herbs, which can also be attributed to spices added, i.e. garlic and local wine Malvasia.
The principal intensity scales applied in sensory profiling of similar dry-fermented sausages varied in terms of the different point intensity scales as well as the unstructured line scale (FLORES, 2011). The lack of consensus regarding the application of sensory scales sometimes makes the comparison and discussion of the results of similar studies quite difficult. However, the results pertaining to the texture descriptors (tenderness, juiciness), obtained in this study, are in agreement with those reported by KOS *et al.* (2015) for dry sausages made from domestic pig and wild boar meat. In general, studies that employed sensorial analysis in order to compare dry-ripened sausages with similar fat levels reported higher sensory scores for texture attributes (FONSECA *et al.*, 2015). Moreover, the amount of fat affects the colour of smoked sausages and is responsible for a high score obtained on the sensory attributes' assessment scale (AHMAD and AMER, 2012), as was the case in this study, too. The smell and the taste of smoked sausages come as the result of decomposition of carbohydrates, lipids and proteins mediated by enzymes, as well as the due to the spices used in the production and the production process itself (KABAN and KAYA, 2009). The results descriptive of smell- and taste-related attributes of Istrian and Slavonian sausages, obtained in this study, can also be compared to those obtained by KOS *et al.* (2015), who also claimed that dry sausages made from domestic pig and wild boar meat have an intense aroma, higher salinity, more pronounced spiciness, pronounced herbal and ripe meat aroma, and more stable flavour.
The intensity of subjective characteristics of Istrian and Slavonian dry-fermented sausage, assessed as a part of descriptive sensory analysis, is shown in Fig. 2.

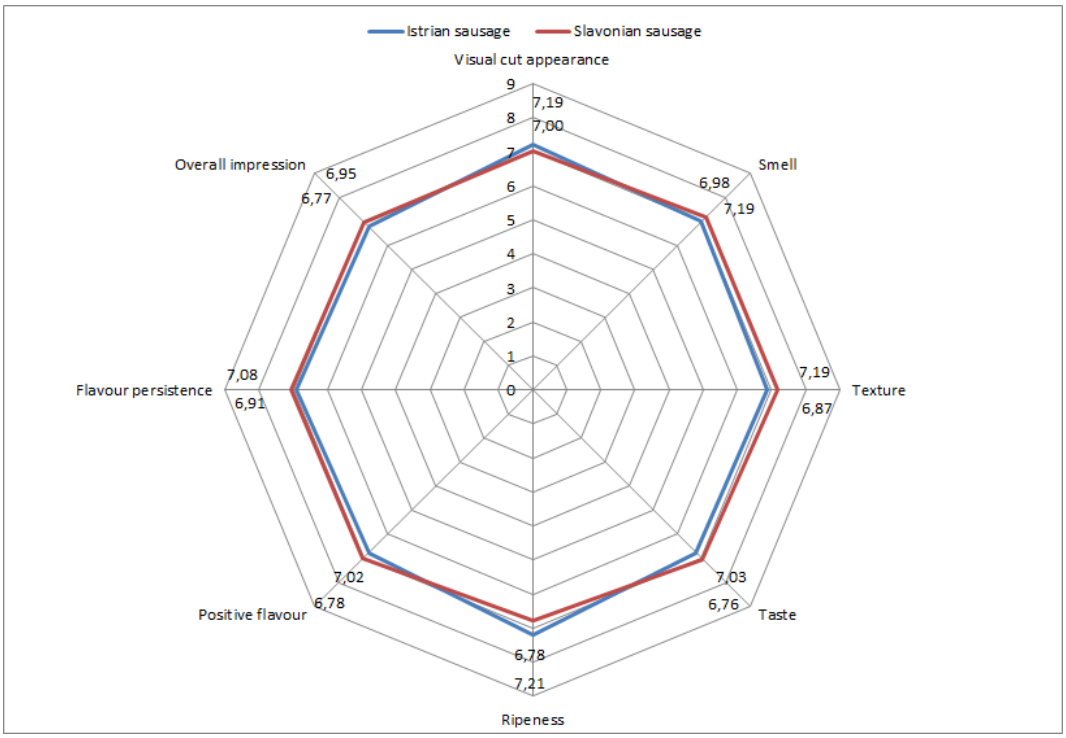

Figure 2. Descriptive sensory profile of subjective characteristics of Istrian and Slavonian dry-fermented sausage

No significant differences ($p>0.05$) in any of the assessed attributes were established, showing a good acceptability of both types of dry-fermented sausages.

3.2. Physicochemical properties

Unlike industrial production, household technologies applied during the production of fermented sausages are not regulated, so that a number of production-related factors, such as uneven weight and quality of raw materials and differences in production technologies, result in the diversity of composition of the finished products. Therefore, physicochemical properties of dry-fermented sausages show huge variability across individual producers and production periods (KOZAČINSKI *et al*., 2008). Industrial production of traditional dry sausages leans on traditional recipes also used in rural households, but, as opposed to seasonal household production, is carried out under controlled processing conditions and is not seasonal in nature, thus allowing for a continuous market supply throughout a year. Recent studies have shown that, due to healthy trends in meat products' consumption in terms of low-fat and low-salt products' preference, meat producers are facing a new challenge that comes down to attaining fat and salt reduction without any loss in sensory qualities (JIMÉNEZ-COLMENERO *et al*., 2009, FLORES *et al*., 2013).
Physicochemical parameters of Istrian and Slavonian dry-fermented sausage determined in this study are shown in Table 1. The results pertinent to chemical parameters indicate a similar nutritional composition of these two products, with statistically significant differences ($p<0.05$) in pH value, protein and ash content.

Table 1. Physicochemical parameters of Istrian and Slavonian dry-fermented sausages.

Parameter	pH	Water (% w/w)	Protein (% w/w)	Fat (% w/w)	Ash (% w/w)	Salt (% w/w)	CH (% w/w)
Istrian dry-fermented Sausage	5.72±0.59	24.99±5.79	30.66±5.05	38.76±8.44	5.00±0.72	5.51±0.96	0.59±0.43
Slavonian dry-fermented Sausage	5.12±0.27	28.10±4.28	26.68±3.57	39.48±8.58	4.54±0.59	4.16±0.67	1.20±0.92
p value	0.000	0.085	0.013	0.801	0.049	0.234	0.490

CH - carbohydrates.
Results are expressed as the mean value (%, mean±SD); one sausage sample was taken and analysed in duplicate.

The pH value is an indicator of fermentation and ripening of a meat product (SALGADO *et al*., 2005) and is commonly used for the assessment of their shelf-life. In dry-fermented sausages, the pH value spans from 4.7 to 6.3 (DELLAGLIO *et al*., 1996; ZANARDI *et al*., 2010; DEMEYER *et al*., 2000; MORETTI *et al*., 2012; PLEADIN *et al*., 2014). In this study, the mean pH values equalled to 5.11 for Slavonian sausage and 5.72 for Istrian sausage, i.e. were within the acceptable range specified above.
Literature data suggest that, due to the longer drying and ripening of dry-fermented sausages and a high share of lean meat used in the preparation of their stuffing, water and protein content in finished ripened sausages are on an equal level (about 30-40% w/w),

suggesting a high nutritional value of the finished product (PLEADIN *et al*., 2014). In dry-fermented sausages, the water content mostly raises up to 40% w/w (VIGNOLO *et al*., 2010). The ratio of water over proteins of 1.2 to 1.3 is typical of dry sausages (INCZE, 2007). In this study, a higher protein as compared to water content was determined in Istrian dry-fermented sausage, while in Slavonian dry-fermented sausage the ratio of water over proteins was determined to be 1.05. When analysing dry sausages produced in Croatian households, water over protein ratio ranged from 1.0 to 2.1, showing that these products are of a high nutritional value (KOZAČINSKI *et al*., 2008).
The amount of total fat present in dry-fermented sausages generally varies widely (21.70 to 55.40% w/w) depending on the recipe and the producing household, but also on the origin of raw materials. Such variations can be attributed to the differences in the amount of added fatback and the choice of more or less fatty meat made by individual manufacturers (PLEADIN *et al*., 2014). The ash content of meat products generally ranges from 3.52% to 6.06% w/w (OCKERMAN and BASU, 2007; JIMÉNEZ-COLMENERO *et al*., 2010; KAROLYI and ČURIĆ, 2012).
Salt (sodium chloride) is an essential ingredient of fermented sausages, so that meat products are one of the richest sodium chloride food sources contributing to the increased water and fat binding capacity, the formation of colour, taste and texture of the product and its microbiological safety. The salinity of a product depends on the amount of added salt and the duration of drying and ripening of the product (WIRTH, 1986), and has a significant impact on hardness and elasticity of meat products and their resistance to chewing (KOVAČEVIĆ *et al*., 2010). In fermented sausages' stuffing, an average share of salt ranges from 2.0% to 2.6%, whereas during the drying process the value keeps growing up to its final level found in the finished product (OCKERMAN and BASU, 2007; STAHNKE and TJENER, 2007). JIMÉNEZ-COLMENERO *et al.* (2001) demonstrated that larger amounts of salt exceeding the value of 4-6% have been established in numerous fermented meats studies, which was also the case in both sausages under this study (4.16-5.51%).
Since the glucose content in meat is too low or much too variable, different carbohydrates like glucose, sucrose, lactose, maltodextrin, corn syrups, starches and sorbitol are added into fermented sausages' stuffing (MASTANJEVIĆ *et al*., 2017) so as to enhance the growth of technological microflora (LUCKE, 1994). Sugars, mostly glucose, facilitate dry sausage fermentation process, since they pose as a substrate for the lactic acid production and contribute to the specific aroma development. Sugars added into fermented sausage stuffing in the maximal percent-share of 2% (usually 0.3-0.8% w/w) ensure the pH decrease from the initial 5.8-6.0 down to 4.8-5.4 (LUCKE, 2000). The carbohydrate content established in sausages under this study (0.59% in Istrian and 1.20% in Slavonian dry-fermented sausage) can be explained in view of the above.

3.3. Fatty acid profile

Table 2 shows the fatty acid composition and fat quality indices of Istrian and Slavonian dry-fermented sausage. Statistical analysis of the fatty acid composition-related data did not show any significant differences between these two types of sausages ($p>0.05$).
No significant differences in fatty acid esters and saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were obtained, revealing these sausages to have the fatty acid profile of a typical pork meat product, with small composition variations attributable to the differences in the amount of added fatback and the stuffing fatness. The proportions of fatty acid groups found in both analysed sausages were as

follows (in descending order): MUFA (46.66-46.83%) > SFA (42.91-43.00%) > PUFA (10.26-10.33%). This trend has also been confirmed in earlier studies of fatty acid profile of meat and meat products (MARUŠIĆ RADOVČIĆ *et al*., 2014; WOODS and FEARON, 2009; PLEADIN *et al*., 2014).

Table 2. Fatty acid composition (% of total fatty acids) of Istrian and Slavonian dry-fermented sausage.

Fatty acid	Istrian dry-fermented sausage	Slavonian dry-fermented sausage	p value
C10:0	0.09±0.01	0.09±0.01	0.565
C12:0	0.08±0.01	0.08±0.01	0.968
C14:0	1.41±0.11	1.43±0.15	0.714
C16:0	25.39±0.30	25.92±1.22	0.274
C17:0	0.39±0.09	0.40±0.12	0.779
C18:0	14.31±1.12	14.21±1.13	0.817
C20:0	0.43±0.23	0.43±0.19	0.961
C21:0	0.46±0.18	0.44±0.10	0.805
SFA	**42.91±1.98**	**43.00±2.22**	**0.921**
C16:1	2.31±0.34	2.56±0.29	0.087
C18:1*t*	0.19±0.04	0.20±0.03	0.749
C18:1*c*	44.18±2.50	43.74 ±2.99	0.689
C22:1	0.14±0.09	0.17±0.07	0.386
MUFA	**46.83±2.65**	**46.66±3.13**	**0.885**
C18:2n-6	8.97±3.17	9.13±2.64	0.586
C18:3n-6	0.23±0.02	0.24±0.03	0.469
C18:3n-3	1.01±0.16	0.95±0.20	0.365
PUFA	**10.26±3.25**	**10.33±2.59**	**0.805**
n-6	9.22±3.19	9.37±2.64	0.902
n-3	1.04±0.15	0.96±0.20	0.246

Results are expressed as the mean value (%, mean±SD); one sausage sample was taken and analysed in duplicate.
LOD (limit of detection)=0.05%.
SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

As proven earlier, fatty acids most represented in different pork meat sausages are oleic (18:1n-9c), palmitic (16:0), stearic (18:0) and linoleic (C18:2n-6c) acid (LEŠIĆ *et al*., 2017). The same was established in the study of fatty acid composition of industrial Slavonian Kulen (PLEADIN *et al*., 2014). Oleic acid, as the predominant fatty acid in meat and meat products, ranged from 43.74±2.99% in Slavonian sausage to the similar 44.18±2.50% in Istrian dry-fermented sausage, and accounts for roughly 94% of all MUFAs in both products. Among SFAs, palmitic and stearic acid were shown to be dominating and accounted for roughly 93% of all SFAs in both types of sausages. Linoleic acid, as the dominating PUFA, accounted for roughly 87-88% of all PUFAs in both sausages.

3.4. Nutritional quality indices

PUFA/SFA and n-6/n-3 ratios are the most common parameters used to evaluate the nutritional quality of fat (JIMENEZ-COLMENERO *et al*., 2010). Additional indices, which take into account different effects that a single fatty acid might have on the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation, i.e. the atherogenic (AI) and the thrombogenic index (TI), were also calculated. In order to gain insight into the effect of fatty acids on blood cholesterol, the ratio of hypocholesterolaemic over hypercholesterolaemic fatty acids (H/H) was determined, too.
Nutritional fat quality indices determined in this study for Istrian and Slavonian dry-fermented sausage are shown in Table 3. No statistically significant differences ($p>0.05$) in any of the indices were determined between the two.

Table 3. Nutritional fat quality indices calculated for Istrian and Slavonian dry-fermented sausage.

Nutritional quality indices	Recommended value	Istrian dry-fermented sausage	Slavonian dry-fermented sausage	p value
n-6/n-3	< 4	8.90±3.07	10.28±4.18	0.340
PUFA/SFA	< 0.4	0.24±0.08	0.24±0.06	0.971
AI	< 1	0.55±0.05	0.56±0.05	0.791
TI	< 1	1.45±0.13	1.46±0.13	0.879
H/H	as higher	2.01±0.17	1.98±0.17	0.676

H/H - hypo-/hypercholesterolaemic fatty acids ratio; AI - atherogenic index; TI - thrombogenic index.
Results are expressed as the mean value (%, mean±SD); one sausage sample was taken and analysed in duplicate.

Literature has revealed that consumption of animal fats is related to an excessive intake of SFAs and an increased proportion of n-6 PUFAs (n-6/n-3 ratio). It has been shown that in the current diet of consumers from western countries, the n-6/n-3 ratio is roughly 15-20+, while according to health recommendations it should be less than 4 if the incidence of chronic diet-related diseases is to be reduced (SIMOPOULOS, 2002; CORDAIN *et al*., 2005). In order to meet health recommendations or reduce the risk of cardiovascular, autoimmune and other chronic diseases, the PUFA/SFA ratio should be higher than 0.4 (SIMOPOULOS, 2002). In this study, the PUFA/SFA ratio was in line with health recommendations, whereas n-6/n-3 ratio was approximately two to three times higher than the maximal recommended value. The mean n-6/n-3 ratios obtained in this study for Istrian and Slavonian sausage are not extremely high (about 10), however, still not in accordance with health recommendations; at least, they are consistent with the results of previous studies of similar pork meat products (LEŠIĆ *et al*., 2017).
The AI is considered to be a particularly useful index because, in addition to describing MUFA content, it places the emphasis on myristic acid (C14: 0), which is believed to have the most harmful cardiovascular effects (HIGGS, 2002). The AI index takes into account the fact that some saturates, primarily myristic and palmitic acid, are considered to be pro-atherogenic (since they facilitate the adhesion of lipids onto the cells the immune and the circulatory system are composed of), while non-saturates are considered to be anti-atherogenic (since they inhibit the formation of plaques and diminish the levels of

esterified fatty acids, cholesterol and phospholipids, therefore preventing micro- and macro-coronary disease) (ULBRITCTH and SOUTHGATE, 1991). It is assumed that AIs below 1 are beneficial to human health (PLEADIN *et al*., 2019). For both types of sausages under this study, the AIs having the mean values of about 0.55 were in line with the recommendation, and similar to those found in other studies of different types of sausages (DEL NOBILE *et al*., 2009; STAJIĆ *et al*., 2011; RAZMAITE and ŠVIRMICKAS, 2012; ROMERO *et al*., 2013).

The TI indicates the risk of blood clotting and represents the ratio of pro-thrombogenic (certain saturated) and anti-thrombogenic (unsaturated) fatty acids. For both sausages analysed within this study, the TIs were about 1.5 times higher than the recommended values, which is consistent with literature data on similar types of sausages (DEL NOBILE *et al*., 2009; ROMERO *et al*., 2013). Recent studies show that fatty acids with an even number of C atoms (lauric, myristic and palmitic acid) increase the concentration of total and LDL cholesterol, as well as promote not only coagulation, but also inflammatory processes and insulin resistance (CALDER, 2015).

The H/H index takes into account known effects of certain fatty acids (especially oleic and linoleic acid) involved in cholesterol metabolism. The higher value of this index shows better effects for human health (SANTOS-SILVA *et al*., 2002). Oleic acid, cis-MUFA fatty acids in general and linoleic acid can reduce both total and LDL cholesterol, thereby reducing the risk of cardiovascular disease (CALDER, 2015). Due to the wide range of potential beneficial biological effects (cell membrane functionality, gene expression and lipid metabolism), n-3 PUFAs play a role in the prevention and treatment of inflammatory processes, thus reducing the risk of cardiovascular disease and some cancers (ARTERBURN *et al*., 2006). In this study, the H/H values approximated to 2 for both types of sausages; such a value is typical of meat, especially pork meat (SANTOS-SILVA *et al*., 2002; RAZMAITE and ŠVIRMICKAS, 2012).

4. CONCLUSIONS

Despite of the fact that Istrian and Slavonian dry-fermented sausages are produced in two climatically different regions, they are nutritionally related. Significant differences exist in their sensory properties due to the differences in traditional recipes used in their production. However, from the sensory evaluation standpoint, the existing sensory characteristics are highly accepted by consumers. Since this research showed that certain nutritional indices, especially n-6 over n-3 ratio, are not within the desirable limits and in line with health recommendations, modifications to the fatty acid composition of these sausages, to be attained primarily through changes in animal feeding practices, are needed in order to improve their nutritional properties and ultimately their beneficial effects on consumer health.

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DEVELOPMENT OF A DYNAMIC MODEL TO PREDICT THE FATE OF PATHOGENIC *ESCHERICHIA COLI* IN DICED CUCUMBER UNDER CHANGING TEMPERATURES

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ABSTRACT

Escherichia coli has been detected in a variety of foods, particularly in salad vegetables, such as diced cucumbers. However, it is difficult to control this pathogen in salad vegetables, because they are consumed without additional preparation or cooking. Thus, the objective of this study was to develop dynamic models to describe the kinetic behavior of *E. coli* in diced cucumber. The diced cucumber was inoculated with *E. coli*, and stored at 10°C, 20°C, 25°C, and 30°C; cells counts were then performed using Petrifilm™ plates. The Baranyi model was used to calculate lag phase duration (*LPD*; h) and maximum specific growth rate (μ_{max}.; log CFU/g/h). These parameters were then fitted to a polynomial model, as a function of temperature, and a subsequent dynamic model was developed in accordance with these primary and secondary models. The performance of the model was evaluated by comparing predicted data with observed data to calculate the root mean square error (*RMSE*). As temperature increased, *LPD* decreased, but μ_{max} increased. The secondary model effectively described the temperature effect on *LPD* and μ_{max}, where R^2 equaled 0.972-0.983. In the validation stage, *RMSE* value of 0.272 suggested that model performance was appropriate to predict cell counts in diced cucumber, and these predictions remained appropriate under changing temperatures. These results indicate that *E. coli* can grow rapidly in diced cucumber at high storage temperatures, and present a useful dynamic model for describing the kinetic behavior of *E. coli* in this vegetable.

Keywords: *Escherichia coli,* cucumber, mathematical model, dynamic model

1. INTRODUCTION

Interest in health and diet has led to an increase in the production and consumption of fresh vegetables in recent years (WIRSENIUS *et al*., 2010; VEREECKEN *et al*., 2015). A survey conducted by NGUYEN *et al*. (2015) reported that 26% of foodborne illness is due to consumption of contaminated fruits and vegetables. Although consumers usually think that fruit and vegetable salads are microbiologically safe, foodborne pathogens can survive and replicate in fresh vegetables (CALLEJÓN *et al*., 2015; BENNETT *et al*., 2018). In addition, fresh vegetables are eaten raw and can, therefore, be more dangerous than other food products, following exposure to foodborne bacteria. Specifically, there have been many cases of food poisoning due to contamination of sliced cucumber (DECRAENE *et al*., 2012; ANGELO *et al*., 2015).

Escherichia coli is a facultative anaerobic, Gram-negative bacillus that belongs to the Enterobacteriaceae family (PATERSON, 2006; STĘPIEŃ-PYSNIAK, 2010). *E. coli* is considered an indicator organism for contamination (CHOI *et al*., 2018) and major cause of foodborne illness, particularly via contamination of fresh vegetables by enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC) (NATARO and KAPER 1998; OLSEN *et al*., 2000; JANG *et al*., 2017). Among these pathotypes, ETEC is the most frequent cause of food poisoning and outbreaks caused by EHEC have been emerging in recent years (GOULD *et al*., 2013; CATFORD *et al*., 2014).

Predictive microbiology is a strategy that employs mathematical models to estimate the kinetic parameters of foodborne pathogens, and is aimed at securing food safety via the prevention of potential risks or hazards (WHITING and BUCHANAN, 1997; YOON, 2010). Most predictive models are developed using a constant temperature (HA *et al*., 2019; LEE *et al*., 2019); however, a number of variables, such as temperature and humidity, change during food storage and distribution. For this reason, a dynamic model should be used to describe the fate of foodborne pathogens under these changing conditions (HA *et al*., 2015; CHOI *et al*., 2016).

Therefore, the objective of this study was to develop a dynamic model to describe the kinetic behavior of *E. coli* in diced cucumber at a range of temperatures.

2. MATERIALS AND METHODS

2.1. *E. coli* prevalence in cucumbers

To evaluate *E. coli* contamination levels, 24 cucumbers were purchased from conventional markets or grocery stores in Korea. Twenty five-gram portions of cucumber were placed into sterile filter bags (3M, St. Paul, MN, USA), and 225 mL 0.1% buffered peptone water (BPW; Becton Dickinson and Company, BD, Franklin Lakes, NJ, USA) was added prior to homogenization for 60 sec. For quantitative analysis of *E. coli*, 1 mL of the homogenate was dispensed into a Petrifilm™ *E. coli*/Coliform Count Plate (3M, USA), which was then incubated at 37°C for 24 h. Any blue colonies with associated gas bubbles were identified and counted. For qualitative analysis of *E. coli*, 1 mL of the homogenate was added to *E. coli* (EC) broth (BD, USA) containing a durham tube and cultured at 44.5°C for 24-48 h. The aliquot in the gas-producing tube was then streaked on eosin methylene blue (EMB) agar (BD, USA) and incubated at 37°C for 24-48 h. The typical colonies representing *E. coli*, with a green color and metallic sheen, were counted. Finally, the 16S rRNA gene was

amplified using the following primers: 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') and sequenced for identification. To determine if the *E. coli* isolates were pathogenic, genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and amplified by PCR, using a Powerchek™ Diarrheal *E. coli* 8-plex Detection Kit (Kogene Biotech, Seoul, Korea), according to the manufacturers' protocols. The following PCR conditions were used: initial denaturation at 95°C for 12 min, followed by 32 cycles of 95°C for 30 sec, 60°C for 45 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. The characteristics of the genes used in PCR are shown in Table 1. To confirm amplification of the target gene, the PCR product was resolved on a 1.5% agarose gel in 1X TAE buffer (Biosesang, Seongnam-si, Korea).

2.2. Preparation of inocula

E. coli (NCCP14038, NCCP14039, NCCP15661, and NCCP11142) were cultured in 10 mL tryptic soy broth (TSB; BD, USA) at 37°C for 24 h. One milliliter of culture was transferred into 10 mL fresh TSB and subcultures were incubated at 37°C for 24 h. The subcultured strains were mixed into a tube, and then centrifuged at 1,912 ×*g* at 4°C for 15 min. The supernatants were discarded, and the cell pellets were washed twice with phosphate-buffered saline (PBS: pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4{\cdot}7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L distilled water). The cell pellets were then resuspended in PBS, and further diluted with PBS to a final concentration of 5 log CFU/mL for inoculation.

2.3. Development of predictive models

Cucumbers were diced into 25-g portions, then dipped into the pathogenic *E. coli* inocula for 3 min, drained for 10 min, and then placed in a sterile bag. The samples were stored at 10°C, 20°C, 25°C, and 30°C up to 96 h, depending on storage temperature. To enumerate *E. coli* in the cucumbers, the samples were aseptically transferred to sample bags (3M, USA) containing 225 mL BPW and homogenized using a pummeler (BagMixer; Interscience, St. Nom, France). The homogenates were serially diluted in BPW and 1 mL of each dilution was transferred to a Petrifilm™ Plate. The plates were incubated at 37°C for 24 h and the colonies were then manually counted. The experiment was repeated three times for each temperature. The primary model was developed by fitting the cell count data to the Baranyi model, using DMFit curve fitting software (Institute of Food Research, Norwich, UK) to calculate lag phase duration (*LPD*; h) and maximum specific growth rate (μ_{max}; log CFU/g/h). The equation was as follows:

$$N_t = N_0 + \mu_{max} \times A_t - \ln\left[1 + \frac{\exp(\mu_{max} \times A_t) - 1}{\exp(N_{max} - N_0)}\right] \quad \text{Eq. 1}$$

where N_t is the bacterial cell count at time t, and N_0 and N_{max} are the initial and final bacterial cell counts in a growth curve, respectively. A_t is the adjustment function, which denotes the physiological status of bacterial cells when defining the *LPD* (BARANYI and ROBERTS 1994). *LPD* and μ_{max} values were further-analyzed using a polynomial model as a function of temperature to develop a secondary model as follows:

$$LPD \text{ or } \mu_{max} = a_0 + a_1 T + a_2 T^2 \quad \text{Eq. 2}$$

where a_i are the coefficient values and T is the storage temperature (°C).
Also h0 values were calculated for describing the initial physiological status of bacterial cells.

2.4. Validation

To evaluate model performance, additional experiments were performed at 15°C and 23°C. During storage, "observed data" for *E. coli* cell counts were obtained as described above. These observed data were then compared to the predicted data, calculated using the model.

Table 1. Target genes for *Escherichia coli* pathogen type with PCR.

Pathogen	Target gene	Size (bp)
EAEC[1)]	*aggR*	757
EHEC[2)]	*VT1 (stx1)*	637
ETEC[3)]	*LT*	530
EPEC[4)]	*bfpA*	400
EHEC	*VT2 (stx2)*	297
EPEC	*eaeA*	231
ETEC	*ST (STh/STp)*	167
EIEC[5)]	*ipaH*	141

[1)]Enteroaggregative *E. coli.* [2)] Enterohemorrhagic *E. coli.* [3)]Enterotoxigenic *E. coli.* [4)]Enteropathogenic *E. coli.* [5)]Enteroinvasive *E. coli.*

The differences between the observed and predicted data were quantified by calculating the root mean square error (*RMSE*), bias factor (*B* factor) and accuracy factor (*A* factor) as follows:

$$RMSE = \sqrt{1/\mathrm{n} \times \sum(\text{observed data} - \text{predicticed data})^2} \quad \text{Eq. 3}$$

$$B \text{ factor} = 10^{[\sum \log((predictive\ values/observed\ values)/n]} \quad \text{Eq. 4}$$

$$A \text{ factor} = 10^{[\sum |\log(predictive\ values/observed\ values)|/n]} \quad \text{Eq. 5}$$

where n represents the number of data points.

2.5. Development of a dynamic model

To describe the *E. coli* growth in cucumbers at changing temperatures, a dynamic model was developed with the equation suggested by BARANYI and ROBERTS (1994), in accordance with primary and secondary models detailed above. To evaluate the performance of the dynamic model, *E. coli*-inoculated cucumber samples were stored at fluctuating temperatures (10°C-28°C), and cell counts were performed as described above.

These cell counts were then compared with the predicted cell counts generated using the dynamic model.

2.6. Statistical analysis

LPD and μ_{max} data were analyzed with a general linear model using SAS® software version 9.4 (SAS Institute, Inc., Cary, NC, USA). The mean comparisons among storage temperature were performed using a pairwise *t*-test at $\alpha = 0.05$.

3. RESULTS AND CONCLUSIONS

E. coli and coliform bacteria were not detected in the quantitative analysis of cucumber samples, although *E. coli* was detected in one sample by qualitative analysis. This organism was identified as non-pathogenic *E. coli* via 16S rRNA gene sequencing (Fig. 1). *E. coli* has been detected in salad vegetables in previous studies (VISWANATHAN and KAUR, 2001; RAHMAN and NOOR, 2012), and specifically, food poisoning associated with cucumbers has been reported in Canada and Sweden (DECRAENE *et al*., 2012; KOZAK *et al*., 2013). Thus, *E. coli* can be considered as an important risk in cucumbers and the behavior of this bacteria in cucumbers should be investigated.

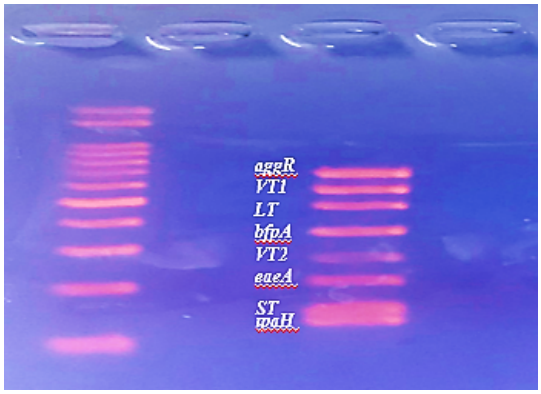

Figure 1. Multiplex PCR results for *Escherichia coli* pathogen type isolated from cucumbers using primers targeting *aggR, VT1, LT, bfpA, VT2, eaeA, ST*, and *ipaH* genes. Lane 1: 100 bp-marker; Lane 2: negative control; Lane 3: positive control; Lane 4: *E. coli* isolated from cucumbers.

Cell counts increased gradually when cucumber samples were stored at 10°C; however the counts increased rapidly in cucumbers stored at 20°C-30°C, reaching stationary phase within 12-24 h, depending on storage temperature (Fig. 2). In addition, *LPD* (1.73-5.00 h) was very short at this temperature range (1.73-5.00 h) (Table 2). Similarly, μ_{max} was measured as 0.01 log CFU/g/h in samples stored at 10°C, and increased values (0.29-0.42 log CFU/g/h) were recorded from samples stored at 20°C-30°C (Table 2). These results indicate that if cucumbers are contaminated with *E. coli*, the bacteria can replicate and cross contamination can occur during cutting. *E. coli* can grow very quickly in diced cucumber during preparation, indicated by low *LPD* values of 3.10 and 1.73 h for storage at 25°C and 30°C, respectively.

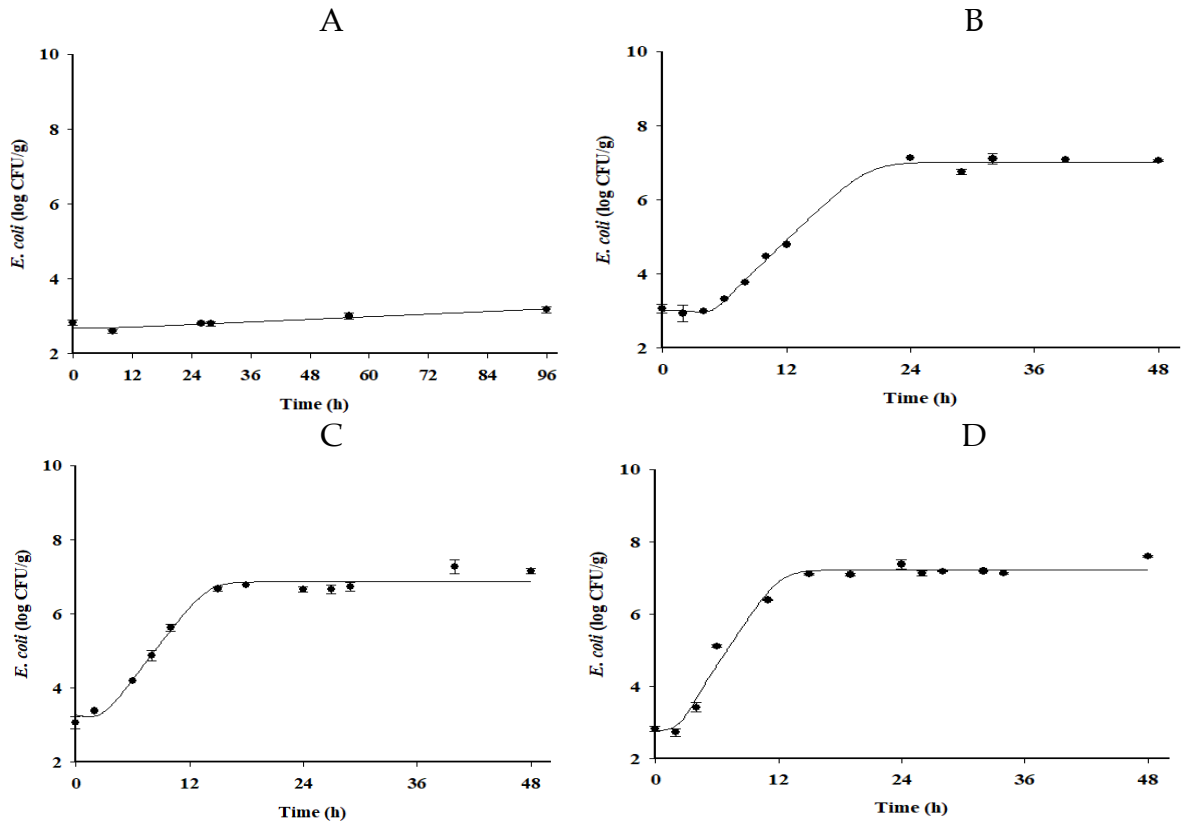

Figure 2. Bacterial populations of pathogenic *Escherichia coli* in cucumbers during storage at 10°C (A), 20°C (B), 25°C (C), and 30°C (D) for 96, 48, 48, and 48 h respectively; • observed value, - fitted line.

Table 2. The parameters calculated by the Baranyi model for pathogenic *Escherichia coli* growth in cucumber.

Storage temperature (°C)	*LPD*[1)] (h)	μ_{max}[2)] (log CFU/g/h)	h0[3)]	N_0[4)] (log CFU/g)	N_{max}[5)] (log CFU/g)	R^2
10	11.15±1.35^{A}	0.01±0.00^{D}	0.10	2.85±0.16	3.47±0.24	0.648
20	5.00±0.64^{B}	0.29±0.03^{C}	1.45	2.98±0.17	6.96±0.21	0.989
25	3.10±0.03^{C}	0.37±0.03^{B}	1.15	3.06±0.20	7.44±0.49	0.983
30	1.73±0.12^{C}	0.42±0.02^{A}	0.73	3.01±0.20	7.48±0.52	0.975

[1)]Lag phase duration. [2)]Maximum specific growth rate. [3)]Parameter specifying the initial physiological state of cells. [4)]Initial cell concentration. [5)]Maximum cell concentration.

[A-D]Means within the same column with different superscript letters are significantly different (p<0.05).

These results are consistent with those observed in a study by ABDUL-RAOUF *et al*. (1993). The h0 (which is a value multiplied by *LPD* and μ_{max}) is the value obtained by quantifying the initial physiological status (BARANYI and ROBERTS, 1994; GRIJSPEERDT and VANROLLEGHEM, 1999; MCKELLAR, 2001); this measure was higher at temperatures above 20°C (0.73-1.45) (Table 2). This indicates that cells grown at temperatures over 20°C can adapt to the actual environment more quickly, and therefore, storing contaminated cucumbers above 20°C may increase the risk.

To evaluate the effect of the temperature on the kinetic parameters (*LPD* and μ_{max}), a secondary model was developed in which R^2 was calculated to be 0.972-0.983 (Fig. 3), indicating that this model was appropriate to describe the effect of temperature on kinetic parameters. The *RMSE* value was calculated to evaluate model performance, where a value close to zero indicates that the predicted values are the same as the observed values (KIM *et al.*, 2018). In this study, *RMSE* was calculated as 0.272, indicating that the developed models were appropriate for describing the kinetic behavior of *E. coli* in cucumbers. Also, *B* factor and *A* factor were 0.98 and 1.04, respectively. ROSE (1999) showed that the developed model was suitable if the *B* factor was 0.9 to 1.05 and the *A* factor was below 1.15. Thus, the developed models in our study were appropriate.
Using the model, *E. coli* cell counts were predicted at changing temperatures (10°C-28°C), simulating storage, distribution, and preparation, and these predicted values were similar to the observed cell counts (Fig. 4).

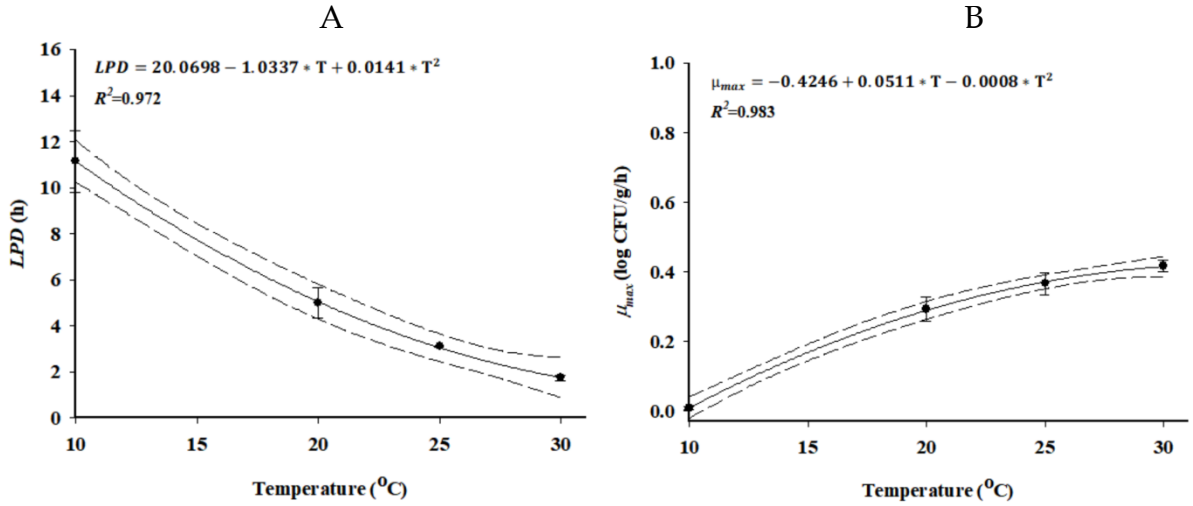

Figure 3. Secondary predictive model for lag phase duration (*LPD*) (A) and maximum specific growth rate (μ_{max}) (B) of *Escherichia coli* in cucumbers, as a function of temperature.

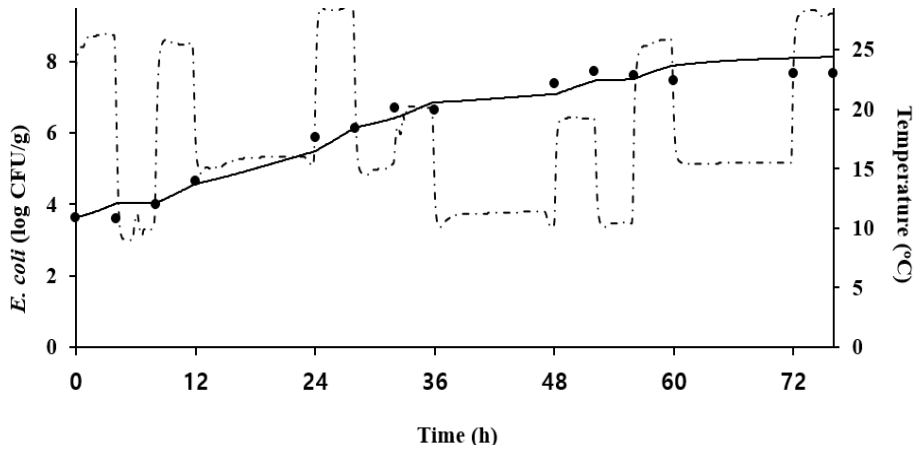

Figure 4. Dynamic model for *Escherichia coli* in cucumbers (Symbol: observed cell counts, line: predicted cell counts).

This result indicates that this dynamic model is appropriate for the description of *E. coli* growth in cucumbers even at changing temperature.
In conclusion, *E. coli* can grow rapidly in cucumbers if they exposed to temperatures above 10°C, which can occur during preparation and short-term storage at room temperature. Furthermore, the model developed here should be appropriate to predict *E. coli* growth in cucumbers, particularly under changing temperatures.

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PAPER

KRACHAI DAM (*KAEMPFERIA PARVIFLORA*) DRINKS: PHYSICOCHEMICAL PROPERTIES, CONSUMER ACCEPTANCE, PURCHASE INTENT, AND EMOTIONAL AND WELLNESS RESPONSES

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ABSTRACT

The rhizomes of Krachai Dam (*Kaempferia parviflora*) and their main effective methoxyflavones (5,7-dimethoxyflavone and 5,7,4′-trimethoxyflavone) have been reported to have beneficial medicinal effects. Additionally, emotions and wellness can play leading roles in product development in addition to liking. This study aims to formulate the Krachai Dam drink and to assess the effects of health benefit information on consumer acceptance, purchase intent, and emotional and wellness responses. The optimal formulation of Krachai Dam drink was 165 mg/100 ml dried Krachai Dam, 16% sugar and 0.32% citric acid, which had a high total phenolic content (TPC), total flavonoid content (TFC), and overall liking score. After consumers received the health benefit information of the product, increases were recorded in consumer acceptance, purchase intent, the highest price that consumers were willing to pay, and positive emotional and wellness responses.

Keywords: Krachai Dam drinks, *Kaempferia parviflora*, emotion, wellness, purchase intent

1. INTRODUCTION

Herbs have been recognized as having health benefits (FARZANEH and CARVALHO, 2015). Herbs and their extracts contain different bioactive compounds that can provide therapeutic effects. *Kaempferia parviflora* or Krachai Dam (KD) is a Thai herb that belongs to the Zingiberaceae family. Its rhizomes have been reputed for their beneficial medicinal effects. Pharmacological studies have claimed various benefits of KD and its main effective methoxyflavones, including cellular metabolism regulating activity, anticancer activity, vascular relaxation and cardioprotective activity, sexual enhancing activity, neuroprotective activity, antiallergic, anti-inflammatory, and antioxidative activity, antiosteoarthritis activity, antimicrobial activity, and transdermal permeation activity (CHEN *et al*., 2018). Normally, KD is used as a food supplement or traditional medicine in the form of capsules and tablets (CHEN *et al*., 2018; KANG *et al*., 2016; MEKJARUSKUL and SRIPANIDKULCHAI, 2019). However, using KD in food products is limited, such as wine (VICHITPHAN *et al*., 2007), yogurt (KANG *et al*., 2016), and fermented juice (CHAIYASUT *et al*., 2018). In Thailand, the use of KD in food supplements is approved by the Thai FDA (Thai Food and Drug Administration), which recommends using KD water extracts of no more than 200 mg of KD per day (Thai FDA, 2017). At higher doses, food products consisting of KD need more research and development to support preventive health care, improve new product options for consumers, and add value to agricultural products.

Classical sensory evaluations, such as consumer preference tests, seem to be insufficiently accurate at predicting whether a newly developed product will be successful in the market. Food-elicited emotions are introduced as a useful tool for new product development. Because emotions influence the eating behavior of people, including food choice, motivation to eat, and amount of food intake (JIANG *et al*., 2014), Jiang and others (2014) proposed that it is necessary for food industries to understand the emotional denotation of their products and use this connotation in the packaging, branding, and commercials for the marketing of their products. In recent years, several methods for measuring emotions associated with foods have been developed and reported, such as the EsSense Profile® (KING *et al*., 2010), consumer defined check-all-that-apply (CD-CATA) (NG *et al*., 2013), EmoSemio (SPINELLI *et al*., 2014) and the EmoSensory® Wheel (SCHOUTETEN *et al*., 2015). In addition, the health and wellness of food products is an interesting issue for consumers. Thus, King and others (2015) developed the WellSense Profile™, which is a questionnaire for measuring wellness associated with foods from a consumer perspective (KING *et al*., 2015). Accordingly, the emotional and wellness responses of consumers should be considered in food product development.

There are many kinds of herbal drinks in Thailand. Some herbal drinks are very popular, but some are not, even though they have health-promoting abilities. One reason is that there are many factors that influence buying decisions. An important factor is the emotional and wellness responses of consumers. This research aimed to formulate the Krachai Dam drink and to assess the effects of health benefit information on consumer acceptance, purchase intent, and emotional and wellness responses.

2. MATERIALS AND METHODS

2.1. Materials

Dried Krachai Dam rhizomes were purchased from Naikrajok Farm, Khao Kho, Phetchabun Province, Thailand. The Krachai Dam used in this research was cultivated in March 2018 and harvested in January 2019. Sugar was Mitr Phol brand from United Farmers & Industry Co., Ltd., Thailand. Citric acid (food grade) was purchased from Union Science Co., Ltd., Thailand.

2.2. Formulation and optimization of Krachai Dam drinks

Optimization of the herbal drink formulation was conducted using a central composite design (CCD), and the data were analyzed by using response surface methodologies (RSMs).
The three factors, including dried Krachai Dam (100-200 mg/100 ml), sugar (10-16%), and citric acid (0.2-0.4%), varied in 3 levels as low, middle, and high, which were coded as -1, 0, and 1, respectively (Table 1). A central composite design (face-centered design; alpha=1) consisted of a total of 18 runs (Table 2). Four replicates (runs 15, 16, 17, 18) at the center of the design were used to allow for estimation of the pure error as the sum of the squares.
The samples of 18 runs were prepared by using the process as follows. Dried Krachai Dam (KD) was extracted in water at 90°C for 10 min and filtered through two layers of white cloth. Sugar and citric acid were added to the KD extract, and the solution was adjusted to a specified volume by adding water and then pasteurized at 85°C for 10 min. A sample of the boiled KD drink was poured into a glass bottle while hot, closed with a bottle cap, and immediately cooled in cold water. The samples were stored at 4°C and subjected to analysis within 48 hours.

2.3. Determination of the physicochemical qualities of Krachai Dam drinks

To obtain the physicochemical properties, three replicate analyses were carried out for each sample.
The color of the Krachai Dam drink was measured at room temperature using a colorimeter (MiniScan EZ, Hunter Association Laboratory Inc., USA). CIE L*, a* and b* were determined and reported (LA PEÑA *et al*., 2010).
The pH of the Krachai Dam drink was determined using a pH meter (FiveEasy Plus, Mettler Toledo, Switzerland) (AYALA-ZAVALA *et al*., 2004).
The total acidity of Krachai Dam drinks was determined as the titratable acidity (AYALA-ZAVALA *et al*., 2004). Total acidity was determined by diluting each 5 ml aliquot of Krachai Dam drink with 95 ml of distilled water and then titrating to pH 8.2 using 0.1 mol/L NaOH.
The total soluble solids content of Krachai Dam drinks was determined at 20°C using a digital refractometer (0-85°brix, HI96801, Hanna Instrumants Inc., USA) (AYALA-ZAVALA *et al*., 2004).
The viscosity of approximately 500 ml of each herbal drink was measured using a rotary viscometer (Brookfield Ametek DV1, USA) at 60 rpm and 5°C (adapted from LA PEÑA *et al*., 2010).

Table 1. Factors and their levels for central composite design (face-centered design; alpha=1).

Level	Code level			True level		
	Krachai Dam	**Sugar**	**Citric acid**	**Krachai Dam** (mg/100 mL)	**Sugar** (%)	**Citric acid** (%)
Low	-1	-1	-1	100	10	0.2
Middle	0	0	0	150	13	0.3
High	1	1	1	200	16	0.4

Table 2. Central composite design arrangement (face-centered design; alpha=1).

Run	Code level			True level		
	Krachai Dam	**Sugar**	**Citric acid**	**Krachai Dam** (mg/100 mL)	**Sugar** (%)	**Citric acid** (%)
1	-1	-1	-1	100.00	10.00	0.20
2	-1	-1	1	100.00	10.00	0.40
3	-1	1	-1	100.00	16.00	0.20
4	-1	1	1	100.00	16.00	0.40
5	1	-1	-1	200.00	10.00	0.20
6	1	-1	1	200.00	10.00	0.40
7	1	1	-1	200.00	16.00	0.20
8	1	1	1	200.00	16.00	0.40
9	-1	0	0	100.00	13.00	0.30
10	1	0	0	200.00	13.00	0.30
11	0	-1	0	150.00	10.00	0.30
12	0	1	0	150.00	16.00	0.30
13	0	0	-1	150.00	13.00	0.20
14	0	0	1	150.00	13.00	0.40
15	0	0	0	150.00	13.00	0.30
16	0	0	0	150.00	13.00	0.30
17	0	0	0	150.00	13.00	0.30
18	0	0	0	150.00	10.00	0.30

The total flavonoid content of the Krachai Dam drinks was determined by using a modified colorimetric method, according to KAMTEKAR *et al*. (2014) with some modifications. Quercetin was used as a standard, and a series of standard solutions were prepared in the range of 100-1000 μg/mL. Briefly, 1 mL of sample or quercetin standard solution was mixed with 4 mL of distilled water, subsequently mixed with 0.3 mL of 5% sodium nitrite solution, and was allowed to react for 5 min. Then, 0.3 mL of 10% aluminum chloride was added and further reacted for 6 min before the addition of 2 mL of 1 M sodium hydroxide. Distilled water was added to bring the final volume of the mixture to 10 mL, and the solution was mixed well. The absorbance of the mixture was immediately measured at a wavelength of 415 nm against a prepared blank using a UV-VIS spectrophotometer (Libra S70, Biochrom Libra, UK). The flavonoid content was determined by a quercetin standard curve and expressed as the mean milligrams of quercetin equivalents per mL of sample±SD in triplicate.

The total phenolic content of Krachai Dam drinks was determined using the Folin-Ciocalteu method, according to RAHMAN *et al*. (2018) with some modifications. Gallic acid was used as a standard, and a series of standard solutions were prepared in the range of 0-125 μg/mL. A 1 ml sample of each Krachai Dam drink was transferred to a test tube and then mixed thoroughly with 2 ml of Folin-Ciocalteu reagent (10-fold diluted with distilled water). After mixing for 3 min, 10% (w/v) sodium carbonate (8 ml) was added. The mixture was mixed by using a vortex mixer and then allowed to stand for 30 min in the dark at room temperature. The absorbance of the mixture was measured at 760 nm using a UV-VIS spectrophotometer. The blank consisted of a solution with only Folin-Ciocalteu reagent (without sample). Determinations were carried out in triplicate and calculated from a calibration curve obtained from gallic acid. The total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE/mL sample). Data are expressed as the mean values±standard deviation (SD).

The antioxidant activity of Krachai Dam drinks was determined using the scavenging activity of the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) according to the method of IQBAL *et al*. (2015) with some modifications. Ascorbic acid was used as a standard, and a series of standard solutions were prepared in the range of 1-8 μg/ml. A series of sample concentrations with different ratios of samples to ethanol, i.e., 10:10, 8:10, 6:10, 4:10, 2:10, and 1:10 were prepared. Then, a solution of standard or sample (2 mL) was mixed with 2 ml of 0.1 mM DPPH-ethanol solution at room temperature. After incubation for 30 min in the dark, the absorbance of the mixture at 517 nm was determined using a UV-VIS spectrophotometer. The control was prepared without sample, while the blank contained DPPH solution. The percent DPPH radical scavenging activity of the standard solution and each sample were calculated as:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

The percent DPPH radical scavenging activity was plotted against the sample concentration to determine the amount of sample necessary to inhibit the DPPH radical concentration by 50% (IC_{50}). The assay was carried out in triplicate, and the data are expressed as the mean values±standard deviation (SD).

2.4. Assessment of the overall liking of Krachai Dam drinks

Sensory evaluation for the overall liking of 18 samples of Krachai Dam drinks was performed by 128 consumer panelists (51.6% male, 48.4% female, mean age=40.3). Panelists between the ages of 18 and 65 years were screened for potential food allergies, and they consumed herbal drinks at least once a week. Each sample of Krachai Dam drink (30 mL; Table 2) was put in a clear plastic cup, labeled with a three-digit code, and presented to all panelists in a random order. The samples were divided into three sets (6 samples per set) with a 10 min break between each set to prevent sensory fatigue. Panelists were asked to rate their overall liking of each sample using a 9-point hedonic scale (9=like extremely, 5=neither like nor dislike, 1=dislike extremely), and they were given drinking water to wash their palates before testing the next sample.

2.5. Consumer acceptance testing of the formulated Krachai Dam drink

The optimal formulation of Krachai Dam drink from the CCD was assessed for consumer acceptance by using a questionnaire that contained 3 sections, as shown in Table 3. A 30

mL sample of the optimally formulated Krachai Dam drink was put in a clear plastic cup and labeled with a three-digit code before being presented to all consumers (n=406; Table 8).

Table 3. Details of the questionnaire for assessing consumer acceptance of the optimally formulated Krachai Dam drink.

Section	Topic	Assessment
1	Before consumers received the details of the product and the benefits of Krachai Dam drinks. After consumers received the product details and health benefit information of Krachai Dam drinks as following: "The product that you are testing is Krachai Dam drink, which was produced from natural raw materials, no preservatives, no coloring agents, and no flavoring agents."	- Emotional and wellness responses - Consumer acceptance - Purchase intention
2	Krachai Dam (*Kaempferia parviflora*) or black ginger is a Thai herb which belongs to the Zingiberaceae family. Its rhizomes have been researched for beneficial health effects, such as cellular metabolism regulating activity, anticancer activity, vascular relaxation and cardioprotective activity, sexual enhancing activity, neuroprotective activity, antiallergic, anti-inflammatory, and antioxidative activity, antiosteoarthritis activity, antimicrobial activity, and transdermal permeation activity (CHEN *et al.*, 2018).	- Emotional and wellness responses - Consumer acceptance - Purchase intention
3	Demographic information of the participants.	- Gender - Age - Educational level - Occupation - Monthly income

Emotional and wellness responses were assessed by using the rate-all-that-apply (RATA) approach (adapted from JAEGER *et al*., 2018). Emotional terms from the EsSense profile® (KING *et al.*, 2010) and wellness terms from the WellSense Profile™ (KING *et al.*, 2015) were screened for relevance to the Krachai Dam drink using check-all-that-apply (CATA) (n=239; 46% male and 54% female). Emotional and wellness terms that were selected for at least 18% of participants were considered to have relevance to Krachai Dam drinks. The results of the term screening showed that 12 terms were selected, including seven positive emotion terms (active, energetic, good, happy, polite, satisfied, and warm) and five wellness terms (comforted, healthy, invigorated, relaxed, and refreshed). Additionally, three negative emotion terms (bored, disgusted, and worried) were added to the questionnaire to include both positive and negative emotions. Traditionally, at least 20% frequency of use was recommended for term screening by using the CATA questionnaire (KING *et al.*, 2010). However, some research has used lower than 20% frequency for screening relevant terms to products (SCHOUTETEN *et al.*, 2015). In addition, if the study objective was to assess the negative side of food experience, screening criteria in terms of usage frequency of negative emotions may be lower than that of positive emotions (JIANG *et al.*, 2014). Thus, a total of 15 emotional and wellness terms were listed randomly in the questionnaire. Consumers were asked to select the emotional and wellness terms that described how they felt after consuming the product and then rate the intensity of the

selected terms on a 5-point intensity scale from 1=slightly to 5=extremely (RATA approach).
Consumer acceptance was assessed by using a yes/no scale. Purchase intention was evaluated using a 3-point scale (1=purchase, 2=not sure, and 3=no purchase).

2.6. Statistical analysis

The physicochemical properties and overall liking data were analyzed by using ANOVA followed by DMRT to determine significant differences between treatments. Statistical analyses were performed by using IBM SPSS Statistics 19. The results were considered significant at a level of $p<0.05$ (95% confidence interval).
Response surface methodologies were conducted to determine the regression coefficients and statistical models for the experimental data, aiming at an overall optimal region for the response variables. After removing the nonsignificant coefficients from the initial model, three-dimensional surface plots were used to explain the effects of the independent variables (ingredients) on the response variables (product qualities). Graphical and numerical optimum formulations and predicted values for the response variables were based on the response optimizer.
For the consumer acceptance test, differences in the emotional and wellness responses before and after consumers had received the details and benefits of the Krachai Dam products were analyzed using a t-test. Consumer acceptance and purchase intent were analyzed using a frequency count, and the differences before and after the consumers had received the details and benefits regarding the Krachai Dam products were analyzed using the McNemar and McNemar-Bowker tests, respectively. Statistical analyses were performed using IBM SPSS Statistics 19. The results were considered significant at a level of $p<0.05$ (95% confidence interval).

3. RESULTS AND DISCUSSION

3.1. Formulation and optimization of the Krachai Dam drink

The physicochemical properties and overall liking of the KD drink products were evaluated. Table 4 shows that all physicochemical properties were significantly different between the 18 treatments, including L*, a*, b*, pH, % acidity, total soluble solids, viscosity, total phenolic content (TPC, μg GAE/ml), total flavonoid content (TFC, μg QU/ml) and antioxidant activity as DPPH scavenging activity (IC_{50}, ml/ml). Additionally, the overall liking showed significant differences between the 18 treatments.
The relationship between the responses and variables of CCD from response surface methodology are shown in Table 5. It should be noted that the adjusted R-square value of the b* value is very low, so this predicted equation is not appropriate to predict the b* value. The adjusted R-square values of % acidity, TSS, viscosity, TPC, and TFC are higher than 0.9, which means that their predicted equations provide confidence in the prediction of the values of the responses.
Then, the optimized formulation was predicted. The results show that the optimized formulation consisted of 165 mg/100 ml dried KD, 16% sugar and 0.32% citric acid (Table 6), which had a high predicted value of total phenolic content (TPC=40.10 μg/ml), total flavonoid content (TFC= 77.85 μg/ml), and overall liking (OA=7.1).

Table 4. Physicochemical properties and overall liking of Krachai Dam drinks from the central composite design treatments.

Run*	L*	a*	b*	TSS (°brix)	Viscosity (cP)	pH	% Acidity	TPC (μg GAE/ml)	TFC (μg QU/ml)	IC_{50} (ml/ml)	OA
1	70.69^{a}±0.81	5.63^{e}±0.34	-4.13^{cde}±0.11	10.23^{gh}±0.38	3.10^{fgh}±0.10	2.64^{a}±0.05	0.19^{l}±0.00	24.61^{i}±0.54	34.32^{m}±1.35	1.48^{bc}±0.01	5.42^{fg}±1.46
2	70.39^{a}±1.19	6.25^{de}±0.22	-4.09^{bcde}±0.13	10.73^{g}±0.47	3.13^{efgh}±0.15	2.43^{fg}±0.04	0.43^{b}±0.01	24.06^{i}±0.61	36.63^{l}±1.97	1.43^{d}±0.01	5.23^{g}±1.41
3	69.94^{ab}±0.97	6.17^{de}±0.22	-4.07^{bcde}±0.06	15.30^{bc}±0.95	3.43^{ab}±0.12	2.61^{ab}±0.04	0.20^{k}±0.00	26.90^{h}±0.07	40.60^{k}±1.11	1.56^{a}±0.01	5.90^{e}±1.11
4	68.31^{cd}±0.11	5.75^{e}±0.23	-3.86^{abc}±0.13	14.83^{c}±0.06	3.40^{abc}±0.17	2.39^{h}±0.01	0.44^{a}±0.00	26.15^{h}±1.52	43.81^{j}±2.14	1.52^{ab}±0.05	5.37^{fg}±1.16
5	65.21^{f}±0.44	10.28^{a}±0.77	-4.50^{f}±0.16	9.93^{hi}±0.06	3.07^{gh}±0.15	2.61^{ab}±0.03	0.21^{l}±0.01	37.72^{d}±0.42	76.24^{e}±1.18	1.22^{i}±0.05	5.27^{fg}±1.23
6	64.62^{fg}±0.57	9.70^{ab}±0.69	-4.00^{abcd}±0.29	9.30^{i}±0.70	2.97^{h}±0.21	2.41^{gh}±0.01	0.44^{a}±0.00	43.30^{c}±0.24	99.45^{a}±2.94	1.36^{ef}±0.02	5.13^{g}±1.55
7	66.07^{ef}±0.64	10.49^{a}±0.87	-4.02^{a-e}±0.37	15.97^{ab}±0.06	3.47^{a}±0.12	2.60^{b}±0.02	0.23^{i}±0.00	44.14^{bc}±0.57	82.14^{d}±1.55	1.37^{e}±0.02	6.37^{d}±1.28
8	65.88^{ef}±0.35	10.47^{a}±1.06	-4.03^{a-e}±0.19	15.43^{bc}±0.06	3.33^{a-e}±0.06	2.47^{def}±0.02	0.42^{c}±0.00	47.67^{a}±0.53	91.12^{fb}± 1.02	1.34^{efg}±0.04	6.61^{bcd}±1.38
9	69.54^{abc}±0.65	5.63^{e}±0.28	-3.75^{ab}±0.14	13.93^{d}±0.40	3.30^{a-f}±0.10	2.50^{cd}±0.01	0.31^{g}±0.00	26.86^{h}±0.26	45.09^{j}±0.59	1.50^{b}±0.04	5.64^{ef}±1.60
10	63.49^{g}±0.38	9.15^{bc}±0.67	-3.98^{abcd}±0.17	13.90^{d}±0.10	3.33^{a-e}±0.06	2.48^{de}±0.04	0.36^{d}±0.01	44.91^{b}± 0.53	87.40^{c}±1.94	1.22^{i}±0.01	6.43^{d}±1.71
11	69.48^{abc}±0.30	8.25^{c}±0.74	-4.08^{bcde}±0.17	10.40^{gh}±0.44	3.07^{gh}±0.15	2.54^{cd}±0.01	0.32^{f}±0.00	33.74^{g}±0.10	68.04^{f}±1.02	1.44^{cd}±0.01	6.57^{cd}±1.51
12	68.69^{bc}±0.59	8.82^{bc}±0.38	-4.32^{def}±0.06	16.20^{a}±0.10	3.37^{abcd}±0.06	2.43^{efg}±0.01	0.35^{e}±0.01	34.20^{fg}±0.19	66.24^{fg}±0.44	1.30^{gh}±0.01	7.03^{a}±1.39
13	63.60^{g}±0.66	7.02^{d}±0.34	-3.69^{a}±0.07	13.87^{d}±0.12	3.20^{c-g}±0.10	2.54^{c}±0.02	0.24^{h}±0.00	34.36^{fg}±0.45	54.71^{i}±0.22	1.31^{fg}±0.02	6.94^{abc}±1.46
14	67.25^{de}±0.42	9.65^{ab}±0.69	-4.37±0.07	13.03^{e}±0.31	3.17^{d-h}±0.06	2.43^{fgh}±0.01	0.42^{c}±0.00	37.64^{d}±0.37	56.12^{i}±0.38	1.22^{i}±0.02	6.91^{abc}±1.33
15	66.09^{ef}±2.42	8.64^{bc}±0.48	-4.50±0.45	12.30^{f}±0.20	3.20^{c-g}±0.10	2.45^{efg}±0.02	0.32^{f}±0.00	35.14^{f}±0.14	64.71^{g}±0.22	1.31^{fg}±0.02	7.25^{a}±1.53
16	64.70^{fg}±0.39	8.35^{c}±0.54	-4.13^{cde}±0.06	13.87^{d}±0.64	3.27^{a-g}±0.06	2.48^{de}±0.01	0.32^{f}±0.00	37.55^{d}±0.71	59.83^{h}±0.22	1.26^{hi}±0.04	7.10^{a}±1.73
17	64.82^{fg}±0.44	8.36^{c}±0.56	-4.29^{def}±0.03	13.07^{e}±0.15	3.23^{b-g}±0.15	2.45^{efg}±0.01	0.32^{f}±0.00	36.24^{e}±0.40	63.94^{g}±0.22	1.24^{i}±0.01	6.97^{ab}±1.97
18	64.73^{fg}±0.37	8.35^{c}±0.59	-4.25^{def}±0.17	13.00^{e}±0.26	3.23^{b-g}±0.06	2.46^{def}±0.01	0.32^{f}±0.00	37.01^{de}±0.43	65.60^{g}±0.80	1.25^{hi}±0.01	6.86^{abc}±1.93

*Corresponding to formulation runs listed in Table 2.
Values are means±standard deviations (SD).
Values in the same column with different letters are significantly different ($p<0.05$).
Abbreviations: TSS, total soluble solid; TPC, total phenolic content; TFC, total flavonoid content; IC_{50}, antioxidant activity as DPPH scavenging activity; OA, overall acceptability.

Table 5. The relationship between the responses and variables of central composite design.

Response	Predicted equation*	Adjusted R^2
L*	$Y=65.698-2.360K+0.205K^2-0.150S+2.775S^2+0.094C-0.885C^2$	0.70
a*	$Y=8.295+2.066K-0.775K^2+1.159S-0.370S^2+0.223C+0.170C^2$	0.84
pH	$Y=2.46+0.03K^2-0.009S+0.005S^2-0.087C+0.025C^2$	0.87
%acidity	$Y=0.328-0.009K-0.0002K^2+0.005S-0.0002S^2+0.108C-0.054C^2$	0.96
TSS (°brix)	$Y=13.409-0.049K+0.158K^2+2.714S-0.457S^2-0.198C-0.307C^2$	0.92
Viscosity (cP)	$Y=3.236-0.019K+0.075K^2+0.166S-0.190S^2-0.027C-0.054C^2-0.030KC$	0.96
TPC (μg/ml)	$Y=36.213-8.917K-0.057K^2+1.563S-1.967S^2+1.110C+0.061C^2+1.303KC$	0.97
TFC (μg/ml)	$Y=63.275-23.590K+3.213K2+0.923S+4.110S^2 + 3.910C-7.621C^2+3.333KC$	0.97
IC_{50}(ml/ml)	$Y=1.278-0.099K+0.075K^2+0.015S-0.081S^2-0.007C-0.020C^2$	0.65
Overall liking	$Y=7.046+0.224K-1.012K^2+0.367S-0.247S^2-0.064C-0.122C^2+0.244KS$	0.94

*Y=Response, K=Dried KD, S=Sugar, C=Citric acid.
Abbreviations: TSS, total soluble solid; TPC, total phenolic content; TFC, total flavonoid content; IC_{50}, antioxidant activity as DPPH scavenging activity.

In both adults and children, the WHO recommends reducing the intake of free sugars to less than 10% of total energy intake (WHO, 2015), which is equivalent to 50 g for a person of healthy body weight consuming approximately 2000 calories per day. In this research, the sugar level of the optimal formulation that consists of 16 g of sugar per serving (100 mL) is quite high but less than the sugar intake recommendation of the WHO. Moreover, 12 brands of Krachai Dam drinks from a market survey in Thailand consist of 8-20% sugar (data not shown). Additionally, the percent sugar in the optimal formulation is in the range of traditional Krachai Dam drinks. However, we recommend that future studies reduce the sugar or substitute the sugar using no calorie materials such as stevia.

Table 6. The optimal formulation of the Krachai Dam drink.

Ingredient	Code level	True level
Dried Krachai Dam	0.4	165 mg/100 ml
Sugar	1	16%
Citric acid	0.3	0.32%

Next, the formulated KD drink products were produced, and the physicochemical properties and overall liking were evaluated. The data were analyzed by using a t-test to compare the differences between the predicted values from the RSM and the actual value of the formulated product.
The results (Table 7) show that none of the physicochemical properties, except % acidity, were significantly different between the formulated KD drinks and their predicted values.

3.2. Consumer acceptance of the formulated Krachai Dam drink

A consumer acceptance test of KD drinks was administered to 406 consumers whose demographic information is shown in Table 8 using a Central Location Test in Khon Kaen

province. Fifty-four percent of the participants were female and 45% were male, and the participants were mostly over the age of 22 years. Their education was mostly at the Bachelor's degree level. Most were self-employed. Their monthly income was generally in the range of 10,000-20,000 baht.

Differences in emotional and wellness responses before and after the consumers received the details of the Krachai Dam product and the health benefit information were analyzed using a t-test, and the results are shown in Figure 1. After receiving the product details, all positive emotional and wellness responses were clearly higher than before the consumers had received product details ($p<0.05$). The participants did not have the "bored" emotion, while "disgusted" and "worried" responses had mean scores of nearly zero and no differences before and after receiving the product details (data not shown). Generally, most emotions elicited from foods are positive emotions, even when evaluating not well-liked products, and the intensity of negative emotions is relatively low (SPINELLI and JAEGER, 2019).

Consumer acceptance and purchase intent were analyzed using % frequency, and the differences before and after the consumers received the details of the product and its benefits were analyzed using the McNemar test and the McNemar-Bowker test, respectively. The highest price that consumers were willing to pay was reported as the mean value±standard deviation (SD). The differences between the highest price before and after consumers received the product details and health benefit information were compared using a t-test. The results (Table 9) show that most participants accepted the product (97.77% before and 100.00% after), and consumer acceptance before and after receiving the product details was not significantly different ($P>0.05$).

After consumers received the product details and health benefit information, the purchase intention (88.92% before and 96.31% after) and the highest price that consumers were willing to pay (17.58 baht/100 mL before and 29.02 baht/100 mL after) were significantly higher ($p<0.05$).

Table 7. Physicochemical properties and overall liking of formulated KD drinks.

Physicochemical properties	Predicted value	Actual value (mean±SD)	t-test (p-value)
L*	67.59	66.59±0.67	0.122
a*	9.63	9.18±0.67	0.364
b*	-4.25	-4.35±0.10	0.216
pH	2.45	2.43±0.01	0.130
% acidity	0.36	0.35±0.0015	0.006
TSS (°brix)	15.68	15.53±0.06	0.251
Viscosity (cP)	3.37	3.37±0.15	0.973
TPC (μg GAE/ml)	40.10	38.13±0.85	0.057
TFC (μg QU/ml)	77.85	76.12±1.02	0.098
IC_{50}(ml/ml)	1.34	1.32±0.04	0.516
Overall liking	7.17	7.07±1.19	0.499

Abbreviations: TSS, total soluble solid; TPC, total phenolic content; TFC, total flavonoid content; IC_{50}, antioxidant activity as DPPH scavenging activity.

Table 8. Demographic information of participants (n=406) in consumer tests of formulated KD drinks.

Demographic information	Frequency count	Percent
1. Gender		
Female	220	54.2
Male	186	45.8
2. Age		
18-21 years	23	5.7
22-40 years	134	33.0
41-59 years	128	31.5
≥60 years	121	29.8
3. Education level		
Secondary school	129	31.8
Bachelor's degree	227	55.9
Postgraduate	50	12.3
4. Occupation		
Student	30	7.4
Government employee	71	17.5
Self-employed/Merchant	118	29.0
Employee	60	14.8
Private company employee	33	8.1
Retiree	65	16.0
Unemployed	21	5.2
Other	8	2.0
5. Monthly income		
<10,000 baht	109	26.9
10,001-20,000 baht	137	33.7
20,001-30,000 baht	83	20.4
30,001-40,000 baht	50	12.3
40,001-50,000 baht	15	3.7
>50,000 baht	12	3.0

In summary, the product details and health benefit information of the KD drink increased the emotion and wellness responses, consumer acceptance, purchase intention, and the highest price that consumers were willing to pay. This result may be influenced by the relationship between emotions and food choice. Emotions can provide an internal stimulus or state that elicits a useful, corrective food choice. On the other hand, food may influence emotions through various factors, such as sensory and hedonic effects, social context, cognitive expectations, psychological distraction, appetite alteration, and brain function (GIBSON, 2006). Food companies must understand the emotional meaning of their food products and use these messages in the forms of packaging, branding, and advertisements to market their products. This also helps with finding target consumers, modifying emotional needs towards different consumers and discovering new markets (JIANG *et al.,* 2014). Therefore, the sensory-emotional optimization of products has been suggested as an alternative to sensory-hedonic product optimization based on the relation of a sensory property and an emotion (SPINELLI and JAEGER, 2019). In addition, a

combination of sensory intensities and emotional responses have been used to predict the consumer acceptance of commercial products such as vegetable juice (SAMANT and SEO, 2019).

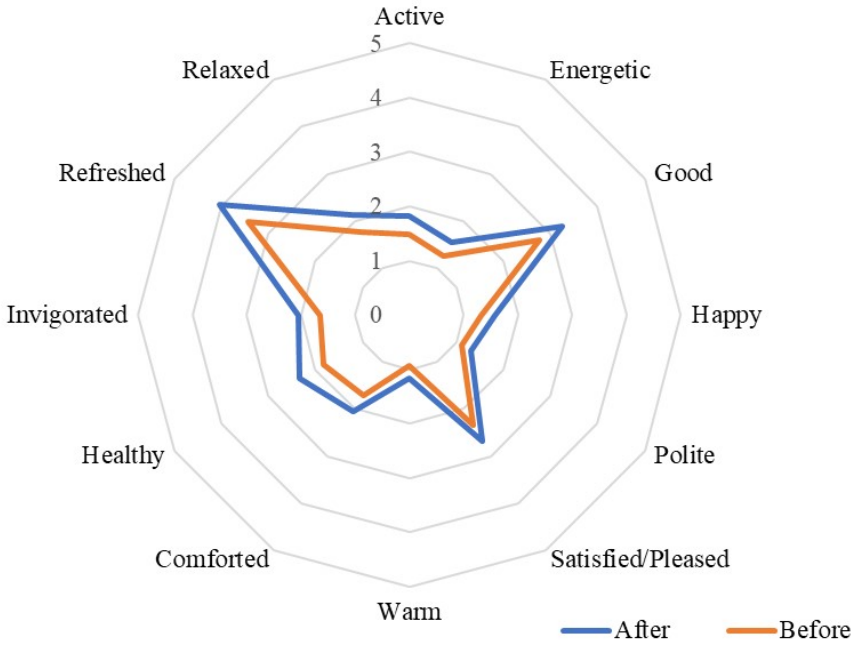

Figure 1. Positive emotional and wellness responses of the optimal formulated Krachai Dam drink before and after the consumers received the product details and benefits of KD (t-test, $p<0.05$).

Table 9. Consumer acceptance, purchase intention, and the highest price that consumers were willing to pay for the Krachai Dam drink before and after they received the product details and health benefit information (n=406).

Consumer test	Before	After	Statistical analysis
Consumer acceptance			
Accept	97.77%	100.00%	McNemar test,
Not accept	1.23%	0.00%	P-value=0.063
Purchase intention			
Purchase	88.92%	96.31%	McNemar-
Not sure	10.34%	3.45%	Bowker test,
No purchase	0.74%	0.24%	P-value=0.000
Highest price for 100 mL/bottle (baht)*	17.58±11.12	29.02±15.17	t-test, P-value=0.000

Values are means±standard deviations (SD).

4. CONCLUSIONS

The optimal formulation of the KD drink was 165 mg/100 ml dried KD, 16% sugar and 0.32% citric acid, which had high total phenolic content (TPC=38.13 μg/ml), total

flavonoid content (TFC= 76.12 μg/ml), and overall liking (OA=7.07). Most of the consumer panels (97.77%) were satisfied with the formulated product. After the consumers received the product benefit information, consumer acceptance, purchase intent, the highest price that consumers were willing to pay, and their positive emotional and wellness responses increased.

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PAPER

FATTY ACID PROFILE AND SENSORY PROPERTIES OF ROE DEER MEAT AFTER MODIFIED ATMOSPHERE STORAGE

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ABSTRACT

The aim of this study was to determine the effect of cold storage (2°C, 7 and 21 days) under vacuum and modified atmosphere (MA) conditions on the fatty acid (FA) profile and sensory properties of the *Longissimus thoracis et lumborum* from male roe deer. The total content of polyunsaturated FA tended to decrease during storage. Storage conditions had a limited ($P>0.05$) influence on the FA composition. Vacuum- and MA-packaged meat was characterized by high sensory quality during storage. However, samples packaged in MA composed of 40% CO_2+60% N_2 had a tendency to higher average scores for taste desirability after the third week of storage.

Keywords: game, cold storage, meat quality

1. INTRODUCTION

The consumption of game meat is relatively low despite its high nutritional and culinary value (SCHULP *et al.*, 2014). There are various reasons for the above, and one of them is a low variety of venison preservation techniques on the consumer market. In retail, game meat is available mostly in the form of vacuum-packaged and deep frozen products. Vacuum packaging inhibits the growth of aerobic microorganisms, limits lipid oxidation and prolongs the product's shelf life (STELLA *et al.*, 2018), but it also has several drawbacks, such as the dark color of meat and considerable drip loss, which compromises consumer acceptance (SAKOWSKA *et al.*, 2016).
The meat of wild animals is vacuum packaged on account of its unique quality attributes. Game meat is characterized by a high content of unsaturated phospholipids (VALENCAK *et al.*, 2015) and heme iron in myoglobin (WIKLUND *et al.*, 2006), and it is susceptible to auto-oxidation, which compromises its quality. To preserve the freshness of venison, oxygen is removed from the packaging during the vacuum packaging process. Freezing decreases water activity, slows down chemical and biochemical processes, inhibits microbial growth and extends the product's shelf life (ZHOU *et al.*, 2010). However, freezing, frozen storage and thawing lead to adverse changes in meat quality.
Modified atmosphere (MA) packaging preserves the attributes of fresh meat (DJENANE and RONCALÉS, 2018). The gas composition of MA is selected for a given type of meat to prevent undesirable changes in quality caused by microbial growth and oxidation or to preserve its attractive color (beef) (ZHANG *et al.*, 2015). Modified atmosphere packaging eliminates the drawbacks of vacuum packaging, such as considerable drip loss as well as packaging and product deformation, through the application of reduced pressure that increases consumer acceptance (ŠČETAR *et al.*, 2010). The effects of storage conditions (MA composition) and cold storage time on the quality of meat from farm animals, including poultry, and fish have been extensively researched (ZHANG *et al.*, 2015). However, their influence on the quality of venison, in particular meat from wild-living animals, remains insufficiently investigated. Therefore, the objective of this study was to determine the effect of vacuum packaging and MA packaging on the fatty acid profile and sensory properties of meat from male roe deer (*Capreolus capreolus* L.).

2. MATERIALS AND METHODS

2.1. Sampling, packaging and storage

The experimental materials comprised samples of the *Longissimus thoracis et lumborum* (LTL) muscle collected from the carcasses of 16 male roe deer aged 3 to 5 years, hunter-harvested in the forests of North-Eastern Poland (Sępopol Plain, Region of Warmia and Mazury) in June and July during one hunting season. During carcass dressing in a meat processing plant (within 48-54 h of harvest), right and left LTL muscles were cut out, placed in polyethylene bags on ice, and transported to the laboratory. Each muscle was divided into samples of similar weight, which were allocated to groups A, B, C and D. Samples A were immediately subjected to laboratory analyses, samples B were vacuum-packaged, samples C and D were packaged in MA containing 40% CO_2+60% N_2 and 60% CO_2+40% N_2, respectively. The samples were packaged in barrier bags made of ethylene-vinyl alcohol (EVOH) copolymer with the following gas permeability: O_2=1 ml/ m^2/24 h/bar/23°C, N_2<0.1 ml/m^2/24 h/bar/23°C, CO_2=1.6 ml/m^2/24 h/bar/23°C, H_2O=3

g/m²/24 h/23ºC using the PP-5MG (015) vacuum packaging machine (TEPRO S.A., Koszalin, Poland). The samples were stored in a cooling chamber without forced air-flow at a temperature of 2ºC for 7 and 21 days. Meat quality was determined based on an analysis of the fatty acid profile of intramuscular fat (IMF), an evaluation of the sensory properties of meat, and shear force measurements.

2.2. Analytical procedures

The sensory properties of meat were evaluated after cooking in 0.6% NaCl solution at 96ºC (±2°C) until internal temperature reached 80ºC, as described by DASZKIEWICZ *et al.* (2012). Five panelists on a 5-point scale (5 points - most desirable, 1 point - least desirable) evaluated meat quality. Prior to the evaluation, the panelists had been trained in the sensory properties of cooked venison based on cooked beef loin as the reference standard. The panelists assessed encoded samples composed of 1 cm x 1 cm x 1 cm meat cubes, cut from the center of each cooked sample, cooled to room temperature. Redistilled water was made available to the panelists for mouth cleansing between samples. The sensory properties (aroma, taste, juiciness, tenderness) of up to 5 meat samples were assessed per session.
The maximum shear force required to cut meat samples (5 cylinder-shaped samples, 1.27 cm in diameter, 2 cm in height) across the grain was measured using a Warner-Bratzler head (500 N, speed 100 mm/min) attached to the Instron 5542 universal testing machine (Instron, Canton, Massachusetts, USA). The samples were prepared as described by HONIKEL (1998).
Intramuscular fat (IMF) was extracted by Soxhlet extraction (AOAC, 1990) with diethyl ether as the solvent in the Soxtec™ 2050 Auto Fat Extraction System (FOSS Analytical, Hilleroed, Denmark). The fatty acid profile of IMF was determined by gas chromatography using a UNICAM PU-4600 gas chromatograph with a flame ionization detector (FID) on a glass capillary column (length: 2.10 m, inner diameter: 4.0 mm); detector temperature - 250ºC, injector temperature - 225ºC, column temperature - 200ºC, carrier gas - argon, carrier gas flow rate - 50 ml/min. Fatty acid methyl esters were prepared by the modified Peisker method with chloroform: methanol: sulphuric acid (100:100:1 v/v) (ŻEGARSKA *et al.*, 1991).

2.3. Statistical analysis

The results were processed statistically by one-way ANOVA with the use of STATISTICA ver. 13.3 software (TIBCO Software Inc.). The statistical significance of differences between mean values in groups was determined using the Bonferroni correction at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Sensory properties of meat

A sensory evaluation confirmed high quality of meat from male roe deer irrespective of storage conditions (Table 1). Stored meat samples were characterized by lower aroma intensity than non-stored samples, and the differences were higher ($P \leq 0.05$) in MA-packaged meat. Stored samples received similar scores for aroma intensity, only vacuum-packaged samples stored for 21 days scored higher ($P \leq 0.05$) than samples packaged in MA

containing 60% CO_2 and 40% N_2. Storage time had no influence (P>0.05) on aroma desirability but after 21 days, MA-packaged meat received higher (P≤0.05) scores for this attribute than vacuum-packaged samples.

Table 1. Sensory properties (points) and shear force values (N) of meat from male roe deer stored in a modified atmosphere (arithmetic means±SD).

Parameter	Storage (days)	Vacuum	Modified atmosphere	
			40% CO_2+60% N_2	60% CO_2+40% N_2
Aroma - intensity	0	4.13±0.85	4.13±0.85^{a}	4.13±0.85^{a}
	7	3.72±0.68	3.38±0.59^{b}	3.28±0.55^{b}
	21	3.75±0.86^{x}	3.34±0.54^{bxy}	3.22±0.45^{by}
Aroma - desirability	0	5.00±0.00	5.00±0.00	5.00±0.00
	7	4.88±0.22	4.94±0.17	4.97±0.13
	21	4.87±0.22^{x}	5.00±0.00^{y}	5.00±0.00^{y}
Taste - intensity	0	4.09±0.52	4.09±0.52^{a}	4.09±0.52^{a}
	7	3.91±0.55	3.78±0.45^{b}	3.75±0.37^{b}
	21	4.19±0.31^{x}	4.00±0.00^{aby}	4.06±0.17^{axy}
Taste - desirability	0	5.00±0.00^{a}	5.00±0.00^{a}	5.00±0.00^{a}
	7	4.84±0.24^{a}	4.84±0.24^{ab}	4.88±0.22^{a}
	21	4.28±0.82^{bx}	4.78±0.36^{by}	4.38±0.62^{bxy}
Juiciness	0	3.59±0.38	3.59±0.38	3.59±0.38^{ab}
	7	3.44±0.54	3.66±0.44	3.69±0.40^{a}
	21	3.38±0.43	3.38±0.43	3.38±0.47^{b}
Tenderness	0	4.44±0.48^{a}	4.44±0.48^{a}	4.44±0.48
	7	4.56±0.57^{a}	4.63±0.50^{ab}	4.72±0.41
	21	4.97±0.12^{bx}	4.88±0.29^{bxy}	4.75±0.37^{y}
Shear force	0	21.27±4.42^{a}	21.27±4.42^{a}	21.27±4.42^{a}
	7	17.98±3.39^{b}	16.94±2.76^{b}	18.86±4.46^{ab}
	21	16.24±2.20^{b}	17.14±2.48^{b}	16.72±2.68^{b}

Values within a row with different superscript letters ($^{x-y}$) are significantly different ($P \leq 0.05$). Values within a column with different superscript letters ($^{a-b}$) are significantly different (P≤0.05).

During storage, taste intensity decreased in samples stored for 7 days compared with non-stored samples and samples stored for 21 days. This trend was more pronounced (P≤0.05) in MA-packaged meat than in vacuum-packaged meat. An analysis of the effect of storage conditions on taste intensity revealed that after 21 days, vacuum-packaged samples scored higher (P≤0.05) than samples packaged in MA composed of 40% CO_2 and 60% N_2.

Roe deer meat stored for 21 days was characterized by lower (P≤0.05) taste desirability than non-stored meat and meat stored for 7 days. Taste deteriorated at the slowest rate in meat packaged in MA composed of 40% CO_2 and 60% N_2, and at the fastest rate in vacuum-packaged meat and meat packaged in MA containing 60% CO_2 and 40% N_2.

Similar results were reported by SEMAN *et al.* (1989) who observed a decrease in the flavor desirability of meat from farm-raised male red deer. In their study, vacuum-packaged samples and samples packaged in MA consisting of 100% CO_2 were stored for 6,

12 and 18 weeks at -1°C. The undesirable changes in palatability most likely resulted from enzymatic and chemical reactions, and the accumulation of microbial metabolites. When analyzing the effects of chemical processes on the sensory properties of meat, attention should be paid to peroxidation of lipids, in particular polyunsaturated fatty acids (PUFAs) (PAPUC *et al.*, 2017).

Meat juiciness was comparable (P>0.05) in non-stored samples and MA-packaged samples stored for 7 days. The value of this attribute decreased after 21 days of storage. Storage conditions had no effect (P>0.05) on meat juiciness. However, MA-packaged samples stored for 7 days received somewhat higher average scores for juiciness than vacuum-packaged samples.

No differences (P>0.05) in juiciness were reported by HUR *et al.* (2013) in beef, PIASKOWSKA *et al.* (2016) in fallow deer meat and by SEMAN *et al.* (1989) in red deer meat packaged in MA consisting of CO_2 and N_2 or 100% CO_2 vs. vacuum-packaged samples. ORKUSZ (2018) and CLAUSEN *et al.* (2009) observed a decrease in the juiciness of samples (goose meat and beef, respectively) stored in MA containing 50-80% O_2.

The analyzed meat was characterized by desirable tenderness, which further improved during storage, and differences (P≤0.05) were found for vacuum-packaged samples and samples packaged in MA consisting of 40% CO_2+60% N_2. After 21 days of storage, vacuum-packaged meat had the highest tenderness scores and meat packaged in MA composed of 60% CO_2+40% N_2 had the lowest tenderness scores (P≤0.05). The tenderness of samples packaged in MA consisting of 40% CO_2 and 60% N_2 was at an average level.

The results of tenderness evaluation were reflected in shear force values, which were lower (P≤0.05) in stored meat than in non-stored samples. No differences (P>0.05) were found between the average shear force values of meat stored for 7 and 21 days. Storage conditions had no effect (P>0.05) on shear force values.

The increase in tenderness of roe deer meat and the decrease in shear force values after cold storage, observed in our study, were related to the activity of endogenous and bacterial proteolytic enzymes. According to WIKLUND *et al.* (2014), meat from selected cervid species does not require aging due to its high tenderness, which probably would not show a further improvement during the process. PAULSEN *et al.* (2005) found no considerable differences in the average values of shear force between samples of roe deer meat stored in vacuum (3.5°C, 132 h) and samples collected from roe deer carcasses on day 5 *post mortem*. SEMAN *et al.* (1989) noted no differences (P>0.05) in the shear force values of meat from male red deer stored in vacuum and MA (100% CO_2) for 18 weeks.

3.2. Fatty acid composition of intramuscular fat

Storage time and conditions had no effect (P>0.05) on the content of saturated fatty acids (SFAs) in IMF (Table 2). Nevertheless, an increase in C 16:0 and C 18:0 content (in particular C 18:0) during storage contributed to an increase (P>0.05) in the total SFA pool.

Similarly to SFAs, storage time and conditions had a limited influence on the content of UFAs (Table 3). The differences between mean values in groups were small and statistically significant only for margoleic acid (C 17:1) and gadoleic acid (C 20:1). In vacuum-packaged meat, the content of C 20:1 was lower (P≤0.05) in non-stored samples and samples stored for 21 days compared with those stored for 7 days. After 7 days of storage, the content of C 17:1 and C 20:1 was higher (P≤0.05) in vacuum-packaged samples than in samples packaged in MA composed of 60% CO_2+40% N_2 and 40% CO_2+60% N_2, respectively.

Table 2. Percentage of saturated fatty acids in total fatty acids in the intramuscular fat of meat from male roe deer stored under vacuum and modified atmosphere conditions (arithmetic means ± SD).

Parameter	Storage (days)	Vacuum	Modified atmosphere	
			40% CO_2+60% N_2	60% CO_2+40% N_2
C 12:0	0	1.06±0.61	1.06±0.61	1.06±0.61
	7	1.02±0.49	1.09±0.71	0.92±0.35
	21	0.86±0.55	0.98±0.64	0.81±0.49
C 14:0	0	1.96±0.50	1.96±0.50	1.96±0.50
	7	1.90±0.27	2.05±0.66	1.81±0.34
	21	1.88±0.41	1.97±0.86	1.97±0.45
C 15:0	0	0.57±0.15	0.57±0.15	0.57±0.15
	7	0.56±0.18	0.59±0.19	0.53±0.11
	21	0.64±0.45	0.59±0.20	0.58±0.14
C 16:0	0	23.72±2.64	23.72±2.64	23.72±2.64
	7	23.59±1.47	23.86±1.84	23.58±2.16
	21	24.18±1.66	24.39±2.98	24.69±2.84
C 17:0	0	1.37±0.15	1.37±0.15	1.37±0.15
	7	1.37±0.15	1.40±0.23	1.35±0.19
	21	1.37±0.19	1.39±0.18	1.38±0.19
C 18:0	0	23.91±1.85	23.91±1.85	23.91±1.85
	7	24.22±3.14	24.56±2.18	24.03±1.85
	21	25.27±2.78	24.63±2.86	25.24±2.32
C 20:0	0	0.44±0.19	0.44±0.19	0.44±0.19
	7	0.45±0.17	0.49±0.30	0.39±0.10
	21	0.51±0.38	0.41±0.18	0.46±0.26
SFAs	0	53.04±4.13	53.04±4.13	53.04±4.13
	7	53.11±3.25	54.04±4.07	52.62±3.48
	21	54.72±4.09	54.35±3.78	55.13±3.70

SFAs - saturated fatty acids.

Storage time had no effect (P>0.05) on total content of UFAs, but the total content of PUFAs was lower in stored samples, particularly (P≤0.05) those vacuum-packaged and packaged in MA containing 40% CO_2 and 60% N_2, than in non-stored samples (Table 3). As a result, the PUFAs/SFAs ratio was slightly lower in stored meat (Table 4). The total content of monounsaturated fatty acids (MUFAs) increased (P>0.05) in meat stored for 7 days and decreased (P>0.05) in meat stored for 21 days (Table 3).

Increased PUFA content is desirable in view of the nutritional value and health-promoting properties of meat but they can also lead to undesirable changes in the product's quality during storage. Auto-oxidation of PUFAs contributes to undesirable changes in the aroma and taste of meat as well as to the formation of compounds that decrease the nutritional value of meat, and toxic compounds (PAPUC *et al.*, 2017).

Therefore, meat packaging must protect the product against the adverse effects of oxygen. This applies also to the meat of wild animals, which is rich in both PUFAs and heme iron that catalyzes auto-oxidation. In practice, vacuum and MA packaging are used to prevent

oxygen from entering the package - oxygen is replaced with appropriately selected gas mixtures.

Table 3. Percentage of unsaturated fatty acids in total fatty acids in the intramuscular fat of meat from male roe deer stored under vacuum and modified atmosphere conditions (arithmetic means ± SD).

Parameter	Storage (days)	Vacuum	Modified atmosphere	
			40% CO_2+60% N_2	60% CO_2+40% N_2
C 14:1	0	0.15±0.08	0.15±0.08	0.15±0.08
	7	0.17±0.07	1.12±0.06	0.15±0.07
	21	0.41±0.72	0.27±0.42	0.13±0.07
C 16:1	0	2.22±0.20	2.22±0.20	2.22±0.20
	7	2.38±0.43	2.50±0.45	2.36±0.38
	21	2.28±0.66	2.34±0.38	2.43±0.47
C 17:1	0	0.24±0.04	0.24±0.04	0.24±0.04
	7	0.29±0.04^{x}	0.26±0.05^{xy}	0.23±0.02^{y}
	21	0.29±0.12	0.27±0.05	0.27±0.10
C 18:1	0	26.28±4.46	26.28±4.46	26.28±4.46
	7	27.53±4.38	27.12±4.42	26.54±4.75
	21	26.79±3.92	26.02±4.89	25.88±2.99
C 18:2	0	12.07±2.41	12.07±2.41	12.07±2.41
	7	11.07±1.95	10.78±1.92	11.94±3.60
	21	10.75±2.46	11.39±2.81	11.22±3.25
C 18:3	0	2.19±0.47	2.19±0.47	2.19±0.47
	7	1.85±0.53	1.93±0.57	1.98±0.62
	21	1.80±0.60	1.92±0.71	1.75±0.59
C 20:1	0	0.39±0.13^{a}	0.39±0.13	0.39±0.13
	7	0.55±0.09^{bx}	0.44±0.10^{y}	0.48±0.10^{xy}
	21	0.40±0.05^{a}	0.41±0.12	0.42±0.15
C 20:4	0	4,80±4.73	4.80±4.73	4.80±4.73
	7	3.06±0.65	2.81±0.77	3.69±1.81
	21	2.57±1.25	3.03±1.00	2.77±1.07
MUFAs	0	29.28±4.31	29.28±4.31	29.28±4.31
	7	30.92±4.36	30.44±4.23	29.77±4.83
	21	30.16±3.46	29.31±4.98	29.13±2.97
PUFAs	0	19.07±4.89^{a}	19.07±4.89^{a}	19.07±4.89
	7	15.97±2.74^{b}	15.53±2.83^{b}	17.61±5.80
	21	15.12±3.62^{b}	16.35±4.14^{ab}	15.74±4.66
UFAs	0	46.96±4.13	46.96±4.13	46.96±4.13
	7	46.89±3.25	45.96±4.07	47.38±3.48
	21	45.28±4.09	45.65±3.78	44.87±3.70

MUFAs - monounsaturated fatty acids; PUFAs - polyunsaturated fatty acids; UFAs - unsaturated fatty acids (MUFAs + PUFAs).

Values within a row with different superscript letters ($^{x-y}$) are significantly different ($P \leq 0.05$). Values within a column with different superscript letters ($^{a-b}$) are significantly different ($P \leq 0.05$).

Table 4. The ratio of unsaturated fatty acids to saturated fatty acids in the intramuscular fat of meat from male roe deer stored under vacuum and modified atmosphere conditions (arithmetic means ± SD).

Parameter	Storage (days)	Vacuum	Modified atmosphere	
			40% CO_2+60% N_2	60% CO_2+40% N_2
UFAs/SFAs	0	0.90±0.14	0.90±0.14	0.90±0.14
	7	0.89±0.11	0.86±0.13	0.91±0.12
	21	0.84±0.13	0.85±0.13	0.82±0.12
MUFAs/SFAs	0	0.56±0.11	0.56±0.11	0.56±0.11
	7	0.59±0.11	0.57±0.11	0.57±0.11
	21	0.56±0.09	0.55±0.12	0.53±0.07
PUFAs/SFAs	0	0.36±0.11^{a}	0.36±0.11^{a}	0.36±0.11
	7	0.30±0.05^{ab}	0.29±0.06^{b}	0.34±0.12
	21	0.28±0.08^{b}	0.30±0.08^{ab}	0.29±0.10

MUFAs - monounsaturated fatty acids; PUFAs - polyunsaturated fatty acids; UFAs - unsaturated fatty acids (MUFAs + PUFAs).
Values within a column with different superscript letters ($^{a-b}$) are significantly different ($P \leq 0.05$).

The beneficial effect of oxygen-free packaging that limits lipid peroxidation in game meat was reported by FAROUK and FREKE (2008). They noted a lower content of malondialdehyde (an indicator of auto-oxidative changes) in samples of the *Semimembranosus* muscle of red deer, which were vacuum-packaged and stored for 9 months, in comparison with samples that were placed in bags that permitted free exchange of air between the inside and outside of the packaging.
Unfortunately, vacuum and MA packaging with oxygen-free gas mixtures cannot completely inhibit undesirable changes in the quality of cold-stored meat products. Under industrial conditions, O_2 cannot be entirely removed from the vacuum or MA packaging. According to BERRUGA *et al.* (2005), the presence of "residual oxygen", less than 2% is sufficient to promote lipid peroxidation. In the present study, this could be the reason for a decrease in PUFAs content in the IMF of roe deer after storage.

4. CONCLUSIONS

Due to the absence of significant changes in sensory properties and fatty acid profile between vacuum-packaged and MA-packaged samples of roe deer meat during storage, the suitability of those packaging methods and the composition of gas mixtures should be analyzed in view of the values of other attributes that are important from the consumer's perspective (color, drip loss, microbial counts).

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PAPER

VARIATIONS IN THE SUGARS AND ANTIOXIDANT COMPOUNDS RELATED TO ROOT COLOUR IN TUNISIAN CARROT (*DAUCUS CAROTA* SUBSP. *SATIVUS*) LANDRACES

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ABSTRACT

Carrot (*Daucus carota* L.) is the most widely consumed root vegetable since it is an important source of nutritional compounds, mainly antioxidants and sugars. In Tunisia, despite the genetic diversity observed in carrot germplasm, including landraces and wild relatives, no research has been conducted on the biochemical composition of carrot. Thus, this study aims to analyse carotenoids, soluble sugars, total phenols, total flavonoids and colour properties of 14 carrot landraces, in order to determine the diversity among them and evaluate the relationships among their biochemical contents. The main carotenoids identified were α-carotene, β-carotene and lutein. Orange carrots were richer in β-carotene and α-carotene than yellow carrots. The major sugars were sucrose, glucose, fructose and

galactose. Significant differences were observed among the Tunisian carrot landraces with respect to their biochemical composition and colour characteristics. Total carotenoids and total sugars ranged from 155.74 to 511.44 μg/g of dw and from 368.77 to 546.79 mg/g of dw, respectively. Total phenols and total flavonoids varied from 24.13 to 41.39 mg GAE/100 g of dw and from 16.51 to 24.85 μg CE/100 g of dw, respectively. Significant, positive and negative correlations were found among the measured parameters. A principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed to classify the Tunisian carrot landraces on the basis of colour properties and biochemical compounds. The PCA divided the landraces into four main groups and AHC classified them into two major clusters. The Tunisian carrot landraces were found to be rich in bioactive compounds; they could be good candidates for future breeding programs.

Keywords: antioxidant compounds, carotenoids, carrot, colour properties, sugars

1. INTRODUCTION

Carrot (*Daucus carota* L.) is one of the major vegetable crops grown worldwide (RUBATZKY *et al.*, 1999). This vegetable belongs to the *Apiaceae* family, being the most widely used member. The first carrots, with purple and yellow colours, originated in Central Asia, Asia Minor, Western Europe and England between the 11th and 15th centuries (BANGA, 1963). The orange carrot was domesticated in Europe between the 15th and 16th centuries (BANGA, 1957; STOLARCZYK and JANICK, 2011). Due to its nutritional value, moderate price and consumption modes, carrot is the most consumed root vegetable in the world (CHAUX and FOURY, 1994). Thus, its roots are eaten as crunchy salad; used to prepare juice, sweets and halwa; cooked with mixed vegetables; and preserved by salting, pickling, canning and drying (SINGH *et al.*, 2018).

Compared to other vegetables, carrot possesses the highest amount of carotenoids, defined as organic pigments that naturally occur in the chloroplasts and chromoplasts of plants (ZIELINSKA and MARKOWSKI, 2012). These bioactive compounds have an antioxidant activity, protecting plants against oxidative stress, and have been proven to be beneficial to human health, reducing oxidative stress by scavenging free radicals (TAN and NORHAIZAN, 2019; STAGOS, 2020).

Depending on its carotenoids content, the carrot root can be purple, orange, yellow or white (BARANSKI, 2012). β-carotene constitutes the major portion (60–80%) of the carotenoids, followed by α-carotene (10–40%) and lutein (1–5%), whereas the rest are other minor carotenoids (SUN and TEMELLI, 2006).

In general, carrot has great acceptability among consumers due to its sweetness, which is governed by its sugars content (SIMON *et al.*, 1980a). These compounds represent an important sensory indicator for consumers. Moreover, sweetness represents one of the most important factors in the acceptance of new commercial vegetable cultivars (NOOKARAJU *et al.*, 2010). The most abundant sugar found in carrot is sucrose, followed by glucose and fructose. The total sugars content in fresh carrot varies from 3% to 10%, whereas soluble sugars can represent up to 70% of the dry carrot (DOLORES *et al.*, 1999; CAZOR *et al.*, 2006).

Besides sugars and carotenoids, which determine carrot sweetness and colour, phenolic compounds also contribute to their organoleptic properties (RUBATZKY *et al.*, 1999). The major phenolic acids present in carrot are chlorogenic acid, caffeic acid, *p*-hydroxybenzoic acids, ferulic acid and other isomers of hydroxy cinnamic acid (ALASALVAR *et al.*, 2001). The content of these compounds varies depending on root colour. Chlorogenic acid is the main phenolic acid and represented 52.4, 57.1, 51.4, and 72.5% of the total phenolic compounds in orange, yellow, white, and purple carrots, respectively (SUN *et al.*, 2009). The different carrot tissues have similar composition, but the contents of individual phenolic compounds differ and they decrease from the exterior to the interior (GONÇALVES *et al.*, 2010). Phenolic compounds are involved in the resistance to physiological mechanism of plants, and their accumulation in carrots results from exposure to cold, injury or ethylene (RUBATZKY *et al.*, 1999). Flavonoids are represented mainly by anthocyanins, which are richer in purple carrot. They exert beneficial effects on human health, acting as vasodilators (CHENG et al., 1993) and platelet disaggregators (GRYGLEWSKI *et al.*, 1987), and also as efficient antioxidants with free radical scavenging abilities (BAHORUN *et al.*, 2003).

In Tunisia, carrots are widely cultivated with an annual production of 201.500 tons, representing 5% of the total vegetable production. They are produced on 5.700 ha of land (~94% as a winter crop and 6% as a summer crop) with an average yield of 35.35 tons/ha

(DGPA, 2019). Although these locally produced carrots can be yellow or orange, Tunisians prefer the latter. Tunisia is considered a center of biodiversity for *Daucus* and many other crops because of the diversity of ecosystems and climatic conditions (LE FLOC'H *et al.*, 2010). This diversity has been the subject of several studies in order to identify and enhance this genetic heritage. Thus, MEZGHANI *et al.* (2014; 2017; 2018) studied the genetic diversity of wild carrot, based on morphological and molecular data. This research revealed a great genetic variability among the accessions. Moreover, BEN AMOR *et al.* (2019) characterized Tunisian carrot (*Daucus carota* subsp. *sativus*) landraces collected from different regions by using agro-morphological descriptors related to roots and leaves. The study showed a high phenotypic variability, particularly in root colour, which could be reflected by the biochemical composition and content. Thus, the aim of this work was to determine the contents of individual carotenoids and sugars, as well as the total phenols and flavonoids, in different Tunisian carrot landraces, and to classify these landraces on the basis of their biochemical compounds and colour properties.

2. MATERIALS AND METHODS

2.1. Plant material

The study material consisted of 14 carrot (*Daucus carota* subsp. *sativus*) landraces, derived from a collection of 33 carrot landraces originating from the main regions of carrot cultivation in Tunisia and conserved at the National Gene Bank of Tunisia. The landraces were selected to maximize the phenotypic diversity, based on our previous study (BEN AMOR *et al.*, 2019). Seeds obtained from self-pollinated landraces were sown in the experimental site of the High Agronomic Institute of Chott Mariem in Tunisia (35.1182 N; 10.7297 E), in November 2016. At the maturity stage, the carrots were manually harvested. Uniform roots (five per landrace) were selected, and washed with tap water to remove soil and other dirt. Representative samples (500 g) of each set of five roots were taken for subsequent analysis.

2.2. Colour measurements

The colour measurements were performed on the skin of the carrot roots using a Minolta chromameter (CR-410). The measurements obtained are reported in the L*, a*, b* systems. The L* value varies from 0 to 100, representing the darkness or lightness of colour. The a* value ranges from green (-a*) to red (+a*). The b* value varies from blue (-b*) to yellow (+b*). The chroma C* and hue angle H^0 were also calculated, using the following equations: $C^* = (a^{*2} + b^{*2})^{1/2}$; $H^0 = \tan^{-1}(b^*/a^*)$. The C* value shows the saturation of colour and it is proportional to the colour intensity. The H^0 is the basic unit of colour and varies from 0^0 to 90^0, indicating red and yellow colour, respectively.
After the colour measurements, the roots were cut into slices and lyophilized. Dry samples were ground and the powders were stored in a refrigerator until analysis.

2.3. Extraction and analysis of carotenoids

Carotenoids were extracted from the lyophilized carrot (0.5 g) using 35 ml of methanol/tetrahydrofuran (1:1, v/v) containing 0.1% butylated hydroxytoluene. The mixture was blended for 5 min and then vacuum filtered through Whatman™ no.5 filter

paper (Whatman, England). The extraction was performed three times, leaving an uncoloured residue. The combined extracts were dried under vacuum at 37 °C in a rotary evaporator. The residue was re-dissolved in a methanol/*tert*-butyl methyl ether mixture (1:1, v/v) until the solution reached a final volume of 10 ml. The solution was centrifuged for 10 min at 14 000 rpm (at 4 °C) and then analyzed (BÖHM, 2001). The experiment was conducted under dark conditions in order to avoid carotenoids degradation. The quantification of carotenoids was carried out using high-performance liquid chromatography (HPLC) with diode array detection (DAD). The HPLC analysis was performed with methanol (solvent A) and methyl *tert*-butyl ether (solvent B), using a gradient procedure on a C_{30} column (250 mm x 4.6 mm, 5 μm, Trentec, Gerlingen, Germany) at 17 °C and a flow rate of 1.3 ml min^{-1}. Carotenoids were quantified at 450 nm and were identified on the basis of the retention time, as described by CHEN and TANG (1998), and according to the DAD spectra. Standard solutions of the main carotenoids were used to prepare calibration lines, in order to determine the concentrations corresponding to the different peaks of the chromatograms. The concentrations of individual carotenoids were expressed as μg/g of dry weight (μg/g of dw).

2.4. Extraction and analysis of soluble sugars

The measurement of the soluble sugars content was performed using HPLC, according to the method described by NOMURA *et al.* (1995). A 0.5 g sample of lyophilized carrot powder was extracted with 5 ml of 80% ethanol (v/v). The mixture was homogenized and then left in an ultrasound bath for 30 min at 60 °C, before centrifugation at 4 500 rpm for 15 min. The supernatant was recovered and the extraction was repeated twice more. The combined extracts were dried under vacuum at 80 °C in a rotary evaporator. The residue was re-dissolved in 2 ml of water, filtered and finally analyzed with HPLC. The chromatographic separation of the sugars was carried out with a Carbo Sep CHO-682 column (7.8 × 300 mm) held at 80 °C, with distilled water as the mobile phase at a flow rate of 0.4 ml min^{-1}. The temperature for the refractive index detector was set at 45 °C.
Standard solutions of sucrose, glucose, fructose and galactose were prepared and calibration lines were made for each one in order to determine the concentrations corresponding to the different peaks in the chromatograms. The concentrations of sugars were expressed as mg/g of dry weight (mg/g of dw).

2.5. Extraction and analysis of total phenols and flavonoids

2.5.1 Samples preparation

Total phenols and total flavonoids were extracted from 0.5 g of lyophilized carrots with 4 ml of an acidified methanolic solution (80% MetOH + 1% formic acid). The mixture was homogenized, left in an ultrasound bath for 5 min and finally centrifuged for 10 min at 5 000 rpm. The supernatant was collected for the analysis of total phenols and total flavonoids.

2.5.2 Analysis of total phenols

The quantification of total phenols was performed using a spectrophotometric technique based on the Folin-Ciocalteu method described by SINGLETON and ROSSI (1965). In microcuvettes, 1 ml of the sample, 500 μl of diluted Folin-Ciocalteu reagent (1:10, v/v) and

400 µl of Na_2CO_3 were mixed. The absorbance was measured at 750 nm after 2 h at room temperature. Gallic acid was used as the standard and the data were expressed as mg of gallic acid equivalents/100 g of dry weight (mg GAE/100 g of dw).

2.5.3 Analysis of total flavonoids

The quantification of total flavonoids was conducted spectrophotometric method described by DEWANTO *et al.* (2002). An aliquot of 100 µl of each methanolic sample was mixed with 625 µl of distilled water and 37.5 µl of $NaNO_2$ (5%). After 6 min, 37 µl of $AlCl_3$ were added and the mixture was left for 5 min. Finally, 250 µl of 1M NaOH and 162.5 µl of distilled water were added to the mixture. The absorbance was measured at 510 nm. Catechin was used as the standard and the data were expressed as µg of catechin equivalents/100 g of dry weight (µg CE/100 g of dw).

2.6. Statistical analysis

Statistical analyses were performed using appropriate packages in R software, available from the Comprehensive R Archive Network (CRAN) at http://CRAN.R-project.org/. For all parameters an analysis of variance (ANOVA) was carried out using the rcmdr package, to determine differences among cultivars. The data are expressed as the mean and standard deviation for each parameter. A Pearson correlation analysis was carried out to estimate the relationships among the studied parameters. Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed using the FactoMineR package, to determine relationships among the biochemical parameters and to group landraces into homogenous classes.

3. RESULTS

3.1. Colour parameters

The colour characteristics measured showed significant variability among the carrot landraces (Table 1). The a* value, indicating the intensity of red colour, ranged from 3.62 for NGBTUN560 to 27.73 for NGBTUN539. For yellowness b*, NGBTUN520 and NGBTUN556 showed the highest and lowest values, respectively. All carrot landraces had high values of C* (> 50), which could be due to the high intensity and saturation of the colour. Also, all landraces had high values of the lightness parameter L* and the hue angle H^0; in general, these values were higher in yellow landraces than in orange landraces.

3.2. Carotenoids content

The analysis of carotenoids with HPLC enabled the identification of three major carotenoids: α-carotene, β-carotene (the most abundant one in all samples) and lutein. In addition, the total carotenoids content was estimated as the sum of the individual carotenoids. The ANOVA showed that the individual and total contents of carotenoids depended to a significant extent on the carrot landrace (Table 2). The α-carotene content varied from 71.36 µg/g of dw for NGBTUN558 to 159.34 µg/g of dw for NGBTUN572. The content of β-carotene was highest for NGBTUN539 (418.58 µg/g of dw) and lowest for NGBTUN560 (71.74 µg/g of dw). For lutein, NGBTUN520 had the highest content

(68.99 μg/g of dw). The total carotenoids content ranged between 155.74 μg/g of dw (NGBTUN560) and 511.44 μg/g of dw (NGBTUN539), showing significant and marked differences among landraces.

Table 1. Colour measurements of 14 Tunisian carrot landraces[1].

Landrace	Skin colour	a*	b*	C*	H⁰	L*
NGBTUN512	Yellow	11.94±0.38 [f]	58.97±1.04 [e]	60.16±1.09 [ef]	78.55±0.15 [d]	81.38±0.04 [b]
NGBTUN514	Yellow	7.14±0.1 [gh]	54.98±0.32 [g]	55.44±0.33 [g]	82.59±0.06 [c]	74.32±0.59 [e]
NGBTUN520	Yellow	7.56±0.18 [g]	66.44±0.32 [a]	66.86±0.33 [a]	83.5±0.12 [b]	83.85±0.15 [a]
NGBTUN522	Yellow	6.15±0.03 [i]	60.74±0.17 [cd]	61.05±0.18 [e]	84.21±0.01 [b]	83.99±0.01 [a]
NGBTUN523	Yellow	6.95±0.5 [ghi]	62.37±1.44 [b]	62.75±1.48 [d]	83.64±0.31 [b]	80.51±1.04 [bc]
NGBTUN527	Orange	14.67±0.02 [e]	51.07±0.09 [h]	53.13±0.09 [h]	73.97±0 [g]	79.36±1.01 [c]
NGBTUN539	Orange	27.73±0.43 [a]	59.27±0.39 [e]	65.43±0.53 [b]	64.92±0.20 [j]	74.06±1.17 [e]
NGBTUN540	Orange	19±0.81 [c]	61.66±0.68 [bc]	64.52±0.40 [bc]	72.86±0.87 [h]	79.31±1.19 [c]
NGBTUN541	Orange	16.16±0.75 [d]	61.38±0.3 [bcd]	63.47±0.48 [cd]	75.25±0.58 [f]	79.52±0.63 [c]
NGBTUN556	Orange	18.6±1.09 [c]	50.81±0.66 [h]	54.1±1.00 [h]	69.9±0.84 [i]	76.78±1.15 [d]
NGBTUN558	Yellow	6.43±0.09 [hi]	60.48±0.1 [d]	60.82±0.90 [e]	83.93±0.09 [b]	83.73±0.61 [a]
NGBTUN560	Yellow	3.62±0.51 [j]	59.1±0.31 [e]	59.21±0.34 [f]	86.49±0.47 [a]	84.84±0.19 [a]
NGBTUN567	Yellow	14.73±0.09 [e]	59.19±0.2 [e]	60.99±0.17 [e]	76.02±0.13 [e]	75.87±0.28 [d]
NGBTUN572	Orange	21.34±0.28 [b]	56.66±0.4 [f]	60.54±0.47 [e]	69.36±0.12 [i]	76.72±1.22 [d]
p value	-	<0.0001	<0.0001	<0.0001	<0.0001	<0,0001
F value	-	613.53	157.14	122.88	844.81	62.86

[1]Data are expressed as the mean and standard deviation. [a-j]Different letters in the same column indicate significant differences for $p<0.05$.

3.3. Soluble sugars content

The main soluble sugars detected in the Tunisian carrot landraces were glucose, fructose, sucrose and galactose, with significant differences among the landraces (Table 3). Glucose and fructose were the most abundant soluble sugars, followed by sucrose and galactose. In general, the individual sugar contents were: for glucose >96.9 mg/g of dw, for fructose >119 mg/g of dw, for sucrose >46.2 mg/g of dw and for galactose >1.13 mg/g of dw, except for NGBTUN558 and NGBTUN560 in which galactose was not detected. The total sugars content, represented as the sum of the individual sugars, varied from 368.77 to 546.79 mg/g of dw for NGBTUN522 and NGBTUN540, respectively.

3.4. Total phenols and total flavonoids contents

The total phenols and total flavonoids were also analyzed and, as for the carotenoids and sugars, their contents varied significantly among the carrot landraces (Table 4). For total phenols, the highest content was recorded for NGBTUN520 (41.39 mg GAE/100 g of dw), whereas NGBTUN527 showed the lowest content (24.13 mg GAE/100 g of dw). The total flavonoids content ranged from 16.51 to 24.85 μg CE/100 g of dw for NGBTUN539 and

NGBTUN572, respectively. It is noteworthy that the flavonoids represented a small proportion of the total phenolic compounds in carrots.

Table 2. Content of individual and total carotenoids, expressed as μg/g of dry weight, in 14 Tunisian carrot landraces[1].

Landrace	α-carotene	β-carotene	Lutein	Total carotenoids
NGBTUN512	76.28±0.27 [ef]	116.77±1.46 [f]	61.36±1.93 [b]	254.41±0.19 [e]
NGBTUN514	79.00±1.48 [e]	113.31±1.66 [f]	50.10±2.66 [c]	242.41±2.83 [e]
NGBTUN520	77.12±0.19 [ef]	106.77±1.73 [fg]	68.99±2.94 [a]	252.88±4.86 [e]
NGBTUN522	73.64±0.06 [fg]	100.32±0.54 [g]	33.28±0.91 [de]	207.25±1.52 [f]
NGBTUN523	71.49±0.09 [g]	85.44±1.29 [h]	29.65±3.51 [e]	186.58±4.89 [g]
NGBTUN527	109.73±2.07 [d]	235.68±4.31 [c]	6.07±0.05 [i]	351.48±6.43 [c]
NGBTUN539	79.76±2.06 [e]	418.58±8.42 [a]	13.1±0.07 [h]	511.44±10.55 [a]
NGBTUN540	76.94±0.60 [ef]	210.98±7.33 [d]	13.17±2.38 [h]	301.09±10.31 [d]
NGBTUN541	122.72±3.21 [b]	203.29±13.91 [d]	22.81±8.29 [f]	348.82±18.99 [c]
NGBTUN556	121.72±0.86 [b]	168.73±5.8 [e]	5.17±0.84 [i]	295.62±7.5 [d]
NGBTUN558	71.36±0.1 [g]	74.99±0.39 [hi]	13.91±2.61 [gh]	160.26±3.11 [h]
NGBTUN560	72.11±0.19 [g]	71.74±0.44 [i]	11.89±1.69 [h]	155.74±2.32 [h]
NGBTUN567	115.38±3.78 [c]	201.31±2.06 [d]	35.79±1.76 [d]	352.49±4.08 [c]
NGBTUN572	159.34±4.99 [a]	292.09±17.21 [b]	18.77±3.67 [fg]	470.21±25.88 [b]
p value	<0.0001	<0.0001	<0.0001	<0.0001
F value	514.05	595.30	129.76	337.71

[1]Data are expressed as the mean and standard deviation. [a-i]Different letters in the same column indicate significant differences for $p<0.05$.

3.5. Correlation analysis

Pearson correlation coefficients (r) were calculated to determine the relationships among the studied parameters. A total of 61 features were correlated at the 0.05 or 0.01 significance level (Fig. 1). Total carotenoids were significantly and positively correlated with β-carotene (r=0.96) and the redness value a* (r=0.91), but were negatively correlated with the hue angle H^0 (r=-0.90) and the lightness parameter L* (r=-0.70). The hue angle H^0 was positively and significantly correlated with the lightness parameter L* (r=0.71), but negatively correlated with the redness value a* (r=-0.98), β-carotene (r=-0.90) and galactose (r=-0.71). Significant and positive correlations were also observed between β-carotene and the redness value a* (r=0.93); the yellowness value b* and the chroma C* (r=0.91). The total sugars showed a significant and positive correlation with fructose and glucose (r=0.79 and r=0.73, respectively) whereas glucose had a significant and negative correlation with galactose (r=-0.80).

Table 3. Content of individual and total sugars, expressed as mg/g of dry weight, in 14 Tunisian carrot landraces[1].

Landrace	Sucrose	Glucose	Fructose	Galactose	Total sugars
NGBTUN512	85.93±0.31 [d]	125.19±8.64 [ef]	200.15±36.94 [bc]	5.99±1.33 [d]	417.27±26.66 [def]
NGBTUN514	89.57±4.49 [d]	137.28±1.11 [de]	157.35±18.44 [cd]	4.88±0.56 [e]	388.90±13.40 [ef]
NGBTUN520	73.73±1.28 [e]	173.28±2.18 [c]	183.04±13.93 [bc]	1.43±0.08 [h]	431.49±17.48 [cde]
NGBTUN522	60.27±1.58 [f]	133.57±2.18 [e]	170.38±6.9 [bc]	4.54±0.18 [e]	368.77±10.85 [f]
NGBTUN523	46.20±3.18 [g]	188.18±13.96 [b]	194.43±4.93 [bc]	1.13±0.10 [h]	429.95±12.31 [cde]
NGBTUN527	137.7±14.29 [a]	124.02±1.22 [ef]	119.07±9.00 [d]	8.04±0.51 [c]	388.84±18.39 [ef]
NGBTUN539	123.95±1.27 [b]	118.18±5.19 [f]	156.79±20.15 [cd]	10.61±0.10 [b]	409.54±23.98 [def]
NGBTUN540	120.66±3.30 [b]	171.17±1.02 [c]	252.03±75.64 [a]	2.92±0.09 [g]	546.79±73.27 [a]
NGBTUN541	85.33±4.15 [d]	147.07±11.16 [d]	190.80±17.55 [bc]	3.47±0.72 [fg]	426.67±2.96 [cde]
NGBTUN556	100.22±8.05 [c]	96.90±5.84 [g]	159.55±14.61 [cd]	14.98±1.47 [a]	371.65±27.03 [f]
NGBTUN558	68.37±3.23 [ef]	245.47±5.84 [a]	219.52±17.68 [ab]	0.00 [i]	533.36±26.75 [a]
NGBTUN560	64.91±0.63 [ef]	240.00±0.37 [a]	193.28±22.68 [bc]	0.00 [i]	498.19±22.42 [ab]
NGBTUN567	121.13±2.25 [b]	171.63±5.02 [c]	171.80±18.42 [bc]	7.47±0.25 [c]	472.04±15.90 [bc]
NGBTUN572	50.49±2.19 [g]	188.56±15.47 [b]	206.12±11.71 [abc]	4.18±0.04 [ef]	449.35±29.41 [bcd]
p value	<0.0001	<0.0001	0.0005	<0.0001	<0.0001
F value	101.53	106.69	4.35	145.43	12.33

[1]Data are expressed as the mean and standard deviation. [a-i]Different letters in the same column indicate significant differences for $p<0.05$.

Table 4. Contents of total phenols and total flavonoids, expressed as mg of gallic acid equivalents/100 g of dry weight and μg of catechin equivalents/100 g of dry weight, respectively, in 14 Tunisian carrot landraces[1].

Landrace	Total phenols	Total flavonoids
NGBTUN512	33.29±0.24 [cd]	20.95±2.19 [c]
NGBTUN514	32.38±0.98 [d]	23.06±0.34 [b]
NGBTUN520	41.39±0.65 [a]	24.57±0.35 [a]
NGBTUN522	33.10±0.26 [cd]	20.56±0.78 [cd]
NGBTUN523	27.19±0.64 [gh]	17.56±0.01 [fg]
NGBTUN527	24.13±0.34 [i]	19.36±1.39 [cde]
NGBTUN539	36.70±1.55 [b]	16.51±0.08 [g]
NGBTUN540	27.01±0.17 [gh]	20.79±0.26 [c]
NGBTUN541	28.43±0.37 [f]	19.53±1.55 [cde]
NGBTUN556	33.98±0.17 [c]	22.91±0.30 [b]
NGBTUN558	29.70±0.17 [e]	19.06±0.34 [def]
NGBTUN560	24.60±0.83 [i]	18.41±0.88 [ef]
NGBTUN567	26.36±0.30 [h]	19.64±0.20 [cde]
NGBTUN572	28.07±0.08 [fg]	24.85±0.04 [a]
p value	<0.0001	<0.0001
F value	184.39	23.86

[1]Data are expressed as the mean and standard deviation. [a-i]Different letters in the same column indicate significant differences for $p<0.05$.

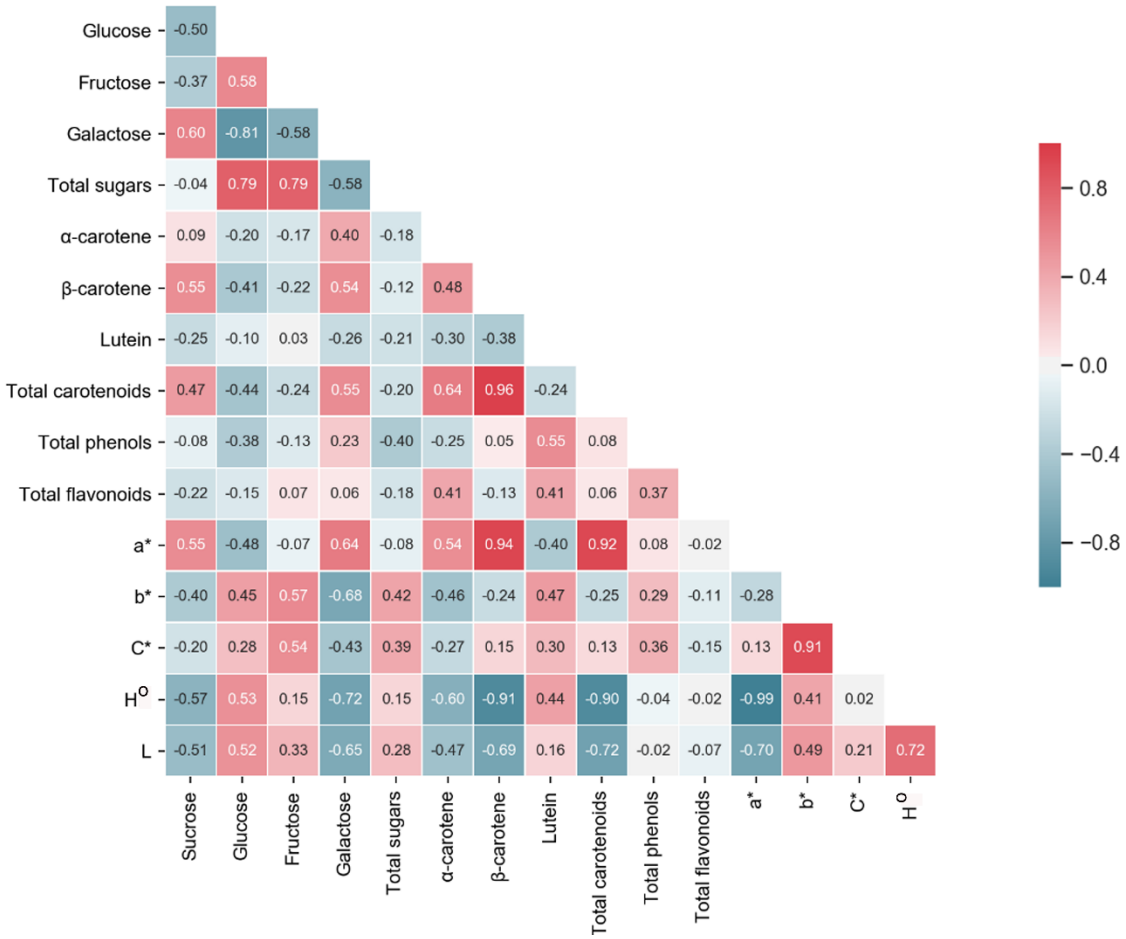

Figure 1. Pearson correlation coefficients among biochemical and colour parameters of 14 Tunisian carrot landraces. The intensity of the pink and blue colours indicates the significance of the correlation existing between each pair of studied parameters. The more the colour turns towards dark pink, the more significant is the positive correlation. The more the colour turns toward dark blue, the more significant is the negative correlation.

3.6. Multivariate analysis

A principal component analysis (PCA) was conducted to determine the differences in the studied parameters among Tunisian carrot landraces. This analysis generated 10 axes with distinct percentage contributions to the total variance (Fig. 2). The contribution of each parameter to the first five principal components is shown in Fig. 3. The first three principal components accounted for 76.1% of the total variance. The first axis explained 42.8% and was associated with the hue angle H^0, galactose, the redness value a* and total carotenoids. The second axis explained 18.2% and was associated with total sugars, the chroma C* and fructose. The third axis represented 15.1% of the total variance and was correlated with total phenols. The PCA scatter plot defined by the two principal components (Fig. 4) separated the carrot landraces into four distinct groups. The first group (G1) included NGBTUN539, 541, 567 and 572, originated from Kairouan, Sfax, Slimane and Siliana, respectively, and having similar contents of α-carotene, β-carotene, total carotenoids and sucrose and similar redness values a*, which were positively

correlated among themselves (Fig. 1). NGBTUN540, cultivated in Sfax, diverged from all the other landraces and formed the second group (G2) due to its high contents of total sugars and fructose (Table 3). The third group (G3) was formed by NGBTUN527 and 556, from Sidi-Bouzid and Gabes, respectively, which had higher contents of galactose than the other landraces (Table 3). NGBTUN512, 514, 520, 522 and 523, from Monastir (Moknine, Teboulba), and NGBTUN558 and 560, from Nabeul (Manzel-Temime), formed the fourth group (G4). These landraces are characterized by a yellow colour, related to the presence of lutein.

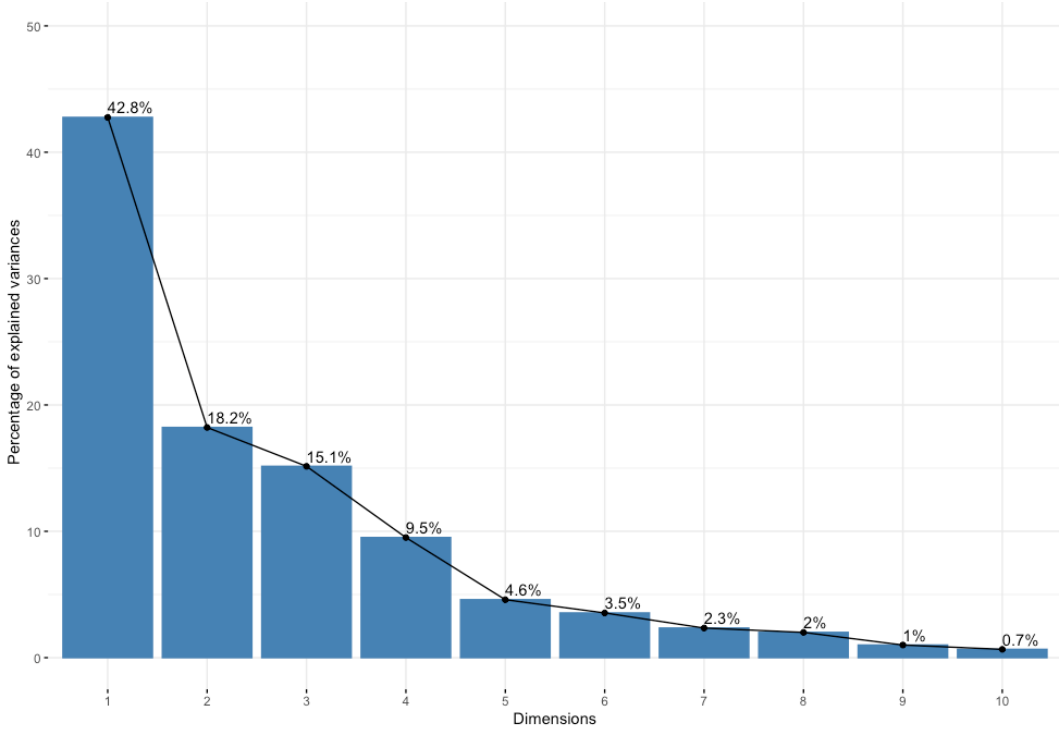

Figure 2. Decomposition of the total variation among the components of the PCA based on biochemical parameters of Tunisian carrot landraces.

Hierarchical clustering (AHC) was also performed and permitted the grouping of landraces on the basis of similarities (Fig. 5). Total carotenoids, β-carotene, sucrose and *α*-carotene were correlated and allowed the grouping of NGBTUN527, 539, 541, 556, 567 and 572 into cluster I. These landraces have similar contents of these compounds. Total sugars and glucose were strongly correlated, as were fructose and the lightness parameter L*. Landraces NGBTUN512, 514, 520, 522, 523, 540, 558 and 560 with similar values for these parameters were grouped together into cluster II. This grouping is in accordance with the PCA results.

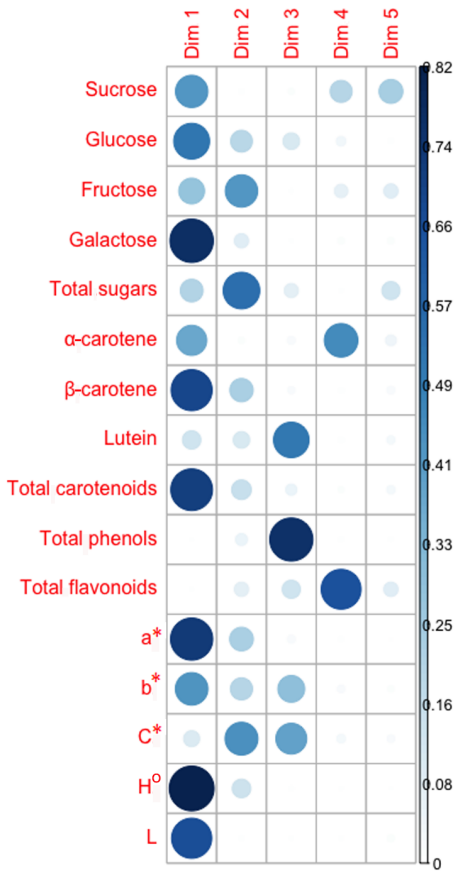

Figure 3. Contribution of biochemical parameters to the variability on the first five components of the PCA. The most significant correlations are indicated by a big, dark-blue circle.

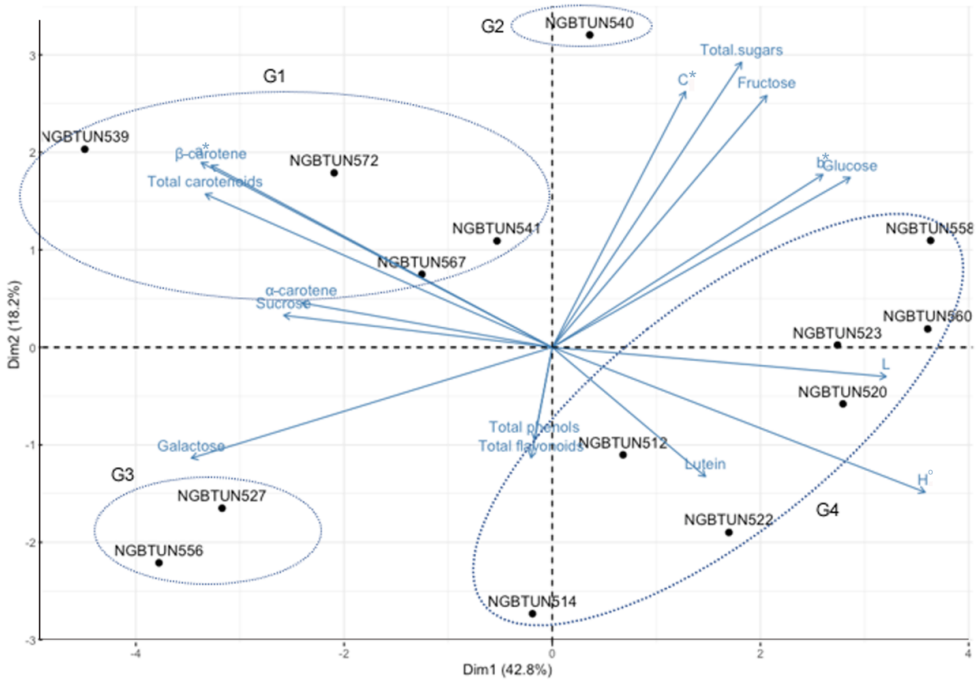

Figure 4. Scatter plot grouping of 14 Tunisian carrot landraces based on the first two principal components of the PCA.

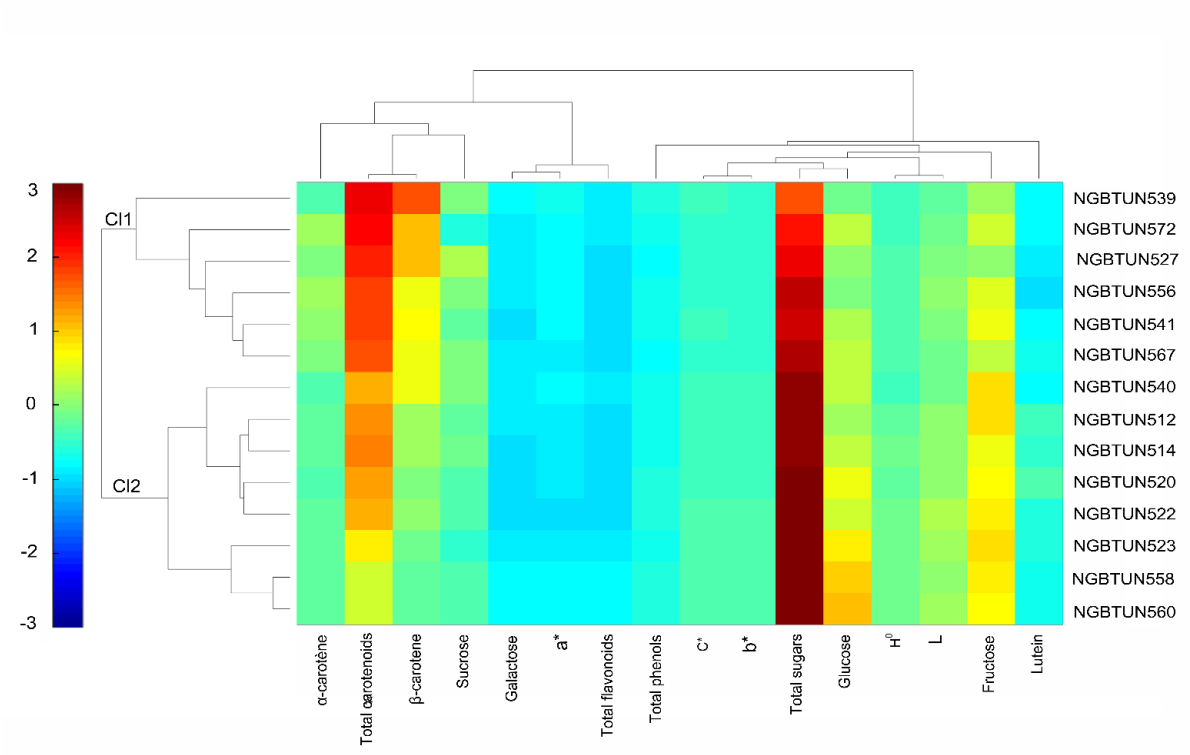

Figure 5. Dendrogram obtained from the cluster analysis of 14 Tunisian carrot landraces. The intensity and variation of colour (from dark red to blue) indicate the correlation existing between the parameters that allowed the discrimination of landraces.

4. DISCUSSION

In the present study, colour parameters and components of the biochemical composition such as carotenoids, sugars and phenolic compounds were identified in various carrot landraces in order to determine their genetic variability. The results prove that there is a large degree of variation among these landraces with respect to their biochemical composition and its relationship with root colour.

Food colour is a very important quality attribute influencing the consumer's choice and preferences (MELENDEZ-MARTINEZ *et al.*, 2005). It is widely used, together with measurements of pigments contents and flavor, because of its correlation with biochemical properties in vegetables. It is also used to assess food quality in post-harvest and processing conditions (PATHARE, 2013). Our correlation results highlight the association between colour and carotenoids accumulation. Thus, the redness value a* was highly correlated with β-carotene, α-carotene and total carotenoids, which give the orange colour to carrot landraces. The yellowness value b* was correlated with lutein and the hue angle H^0, which had greater values for yellow carrots than for orange ones. The high correlation between the hue angle H^0 and the lightness parameter L* was accompanied by carotenoids accumulation. The purity of carrot colour for all the landraces was indicated by the high chroma value C*. These results confirm that carotenoids contents can be determined by using a chroma meter (RUIZ *et al.*, 2005). The variation of colour in carrot is due mainly to the genotype, development of the plant, temperature and fertilization (BAJAJ *et al.*, 1980). The most suitable temperature for a better colour, accompanied by the highest content of carotenoids, is considered to be around 16 °C (BANGA *et al.*, 1955). The correlation results

are consistent with those of a previous study on pumpkin and squash conducted by ITLE and KABELKA (2009), who reported a positive correlation between a* and total carotenoids (r=0.91) and β-carotene (r=0.77); and a negative correlation between total carotenoids and H^0 (r=-0.83) and L* (r=-0.66), and between H^0 and β-carotene (r=-0.69). According to REEVES (1987), the best parameter to predict the total carotenoid content in pepper is a negative correlation with L*. Unlike previous studies (ALASALVAR *et al.*, 2001), we noted significant correlations between sugars and colour parameters. Sucrose was positively correlated with colour parameters, while glucose and fructose were negatively correlated with them. This could be explained by the fact that fructose and glucose accumulation is inversely proportional to sucrose accumulation (SUOJALA, 2000; SEKOLI *et al.*, 2016). KOROLEV *et al.* (2000 a, b) confirmed that the fructose and glucose contents increased in carrot root until 50 days after germination, while sucrose increased beyond this date until harvest (90 days). Similar to our results, correlations (positive or negative) between individual and total sugars contents have been reported in watermelon genotypes (YOO *et al.*, 2012). Glucose and fructose were negatively correlated with the total sugars, while sucrose had a positive correlation. These results show that each sugar contributes to the total sugars content to a different degree and that an increased sucrose content is accompanied by decreased fructose and glucose contents.

In the current work, carotenoids were quantified due to their contribution to carrot colour and the general quality of the roots. The type and amount of carotenoids differ among distinct parts of the plant (SAINI *et al.*, 2015). In carrot, the cortex (flesh) is richer in carotenoids than the core (KARABACAK and KARABACAK, 2019). Our experiment showed that the carotenoids profile was constituted mainly by β-carotene, α-carotene and lutein, whose contents varied distinctly among the carrot landraces, β-carotene and α-carotene being more highly abundant than lutein in orange coloured carrot .

These findings are in conformity with previous studies showing that α-carotene and β-carotene are the major carotenoids in orange carrots, unlike in yellow carrots (NICOLLE *et al.*, 2004; SURLES *et al.* 2004; BARANSKI *et al.*, 2010; JOURDAN *et al.*, 2015). Carrots with orange skin colour have been shown to possess higher amounts of total carotenoids than yellow carrots (ALASALVAR *et al.*, 2001; GRASSMANN, 2007; SUN *et al.*, 2009; SINGH *et al.*, 2018).

These new results indicate that Tunisian landraces are richer in carotenoids than ''Nante'' hybrids: namely, Nante/Berlikum (60.21 mg/100 g); Nante/Maestro (76.47 mg/100 g); Nante/Forto (72.45 mg/100 g); Nante/Bolero (72.93 mg/100 g) and Nante/Champion (79.47 mg/100 g) (RAKCEJEVA *et al.*, 2012).

Carotenoids biosynthesis depends on the cultivar (NICOLLE *et al.*, 2004) and is related to genetic factors (ROSENFELD *et al.*, 1997). Also, these compounds can be affected by the season (HORVITZ *et al.*, 2004), environmental conditions (SIMON, 2000), plant maturity (PHAN and HSU, 1973) and soil (HART and SCOTT, 1995). In fact, high temperatures and dry weather promote an increase in carotenoids contents (FIKSELOVÁ *et al.* 2010). Carotenoids have been found to be more abundant in carrots grown in clay soil than in carrots grown in sandy soil (MARTÍN-DIANA *et al.*, 2007; RICO *et al.*, 2007).

Cultivation practices can affect the content of biochemical compounds. Carotenoids accumulation was stimulated by fertilization based on NPK (SMOLEN and SADY, 2009), while it was inhibited by regular irrigation (FIKSELOVÁ *et al.*, 2010).

It has been noted that the post-harvest and storage conditions affect the carotenoids concentration (SAINI *et al.*, 2015). A temperature of 2-3 °C and 90% relative humidity reduced the carotenoids content by an average of 11% (MATĚJKOVÁ and PETŘÍKOVÁ, 2010). Hence, lyophilization is the most adequate method for better preservation of the nutritional quality of vegetables (SAINI *et al.*, 2014). The carotenoids and provitamin A

contents of carrots are important contributors to human health and the variability of carotenoids (determined using reliable HPLC methodology) could be considered as a parameter of selection in breeding programs (SIMPSON, 1983).
Sugars were also quantified due to their contribution to carrot taste and sweetness. Like carotenoids, the sugars content varies among the different parts of the carrot root. For example, total sugars are higher in the crown section (at the shoulders) than in the tip section (SEKOLI *et al.*, 2016).
Significant differences were observed in individual sugars as well as total sugars. Fructose and glucose were more abundant than sucrose and galactose, the latter being detected in Tunisian carrot landraces in lower amounts. Galactose has not been determined in similar research related to carrots and other crops. The results seem to be in direct contrast to the findings of DOLORES *et al.* (1999), ALASALVAR *et al.* (2001) and SIMON (1985), who showed sucrose to be the major soluble sugar, followed by glucose and fructose. The sugar content in plants, which contributes to sweetness, is controlled by the genotype and the environment (SIMON *et al.*, 1980.a, b; 1982; 1985). It is a polygenic control with a heritability estimated at 0.45 (BARANSKI *et al.*, 2012). The variation in sugars has been the subject of many studies, but sometimes the results are contradictory. SUOJALA *et al.* (2000) found low sugar levels in warm seasons, whereas NILSSON (1987), HOGSTAD *et al.* (1997) and ROSENFELD *et al.* (1998) noted the highest sugar levels at high temperatures. Fertilization can affect the variation in sugars. In this context, SEKOLI *et al.* (2016) found that the sucrose content increased proportionally with the increase in fertilization.
According to MELO *et al.* (2006), carrot belongs to the group of vegetables characterized by low phenolic contents since its mean content is <100 mg catechin equivalents/100 g of fresh weight. In our study, the phenolic compounds varied greatly among the carrot landraces. NICOLLE *et al.* (2004) found sizable differences in the content of total phenols among different carrot varieties (white, yellow, orange, dark-orange and purple), the values ranging between 4.3 and 4.4 mg GAE/g of dry weight for yellow carrot and between 3.3 and 6.0 mg of GAE/g of dry weight for orange carrots. However, in our study the carrots showed a lower content of total phenols, varying from 24.6 to 41.4 mg GAE/100 g of dry weight for yellow carrots and from 24.1 to 36.7 mg GAE/100 g of dry weight for orange carrots. Consequently, Tunisian carrot landraces appear to have lower contents of total phenols compared with other data of the scientific literature. This difference could be explained by the influence of the genetic factors, environmental conditions and variety (BRAVO, 1998). In addition, the phenolic compounds concentration depends on the fertilization method (SMOLEN and SADY, 2007). For example, the provision of nitrogen permits high concentrations of phenolic compounds (SMOLEN and SADY, 2009). Also, ROZEK *et al.* (2000) noted that the content of phenolic compounds are influenced more by the soil (light, medium or heavy texture) and climatic conditions than by fertilization.
The flavonoids are one of the most important groups of phenolic compounds since they exhibit important biochemical and pharmacological effects. Indeed, they contribute to the protection against reactive oxygen species (ROS) and the inhibition of platelet aggregation. They also have anti-inflammation, anti-atherosclerotic, antitumor, antimicrobial and antiallergic effects (KOLEY *et al.*, 2014). Quercetin, luteolin, kaempferol and myricetin are the main types of flavonoids found in carrot (BAHORUN *et al.*, 2004). However, the information related to the flavonoids content in carrot genotypes is limited. The low values of flavonoids in Tunisian carrot landraces are explained by the absence of anthocyanins, which are the most abundant type of flavonoids in the root and are responsible for purple and black colours (SINGH *et al.*, 2018). Different authors (LEJA *et*

al., 2013; KOLEY *et al.*, 2014; SINGH *et al.*, 2018) have reported lower amounts of flavonoids in orange and yellow carrots in comparison to purple, rainbow and black carrots.
Principal component analysis (PCA) and clustering (AHC) were performed to classify landraces on the basis of their similarities. Information obtained by the PCA can help breeders to distinguish between highly differentiated landraces to be used for plant breeding programs.

5. CONCLUSION

The contents of sugars and phenolic compounds in the roots varied significantly among 14 Tunisian carrot landraces due to agronomic and genetic factors. The colour, an important organoleptic characteristic in carrot, was significantly correlated with the content of carotenoids, oranges landraces having higher contents of α-carotene and β-carotene, whereas yellow landraces had more lutein. Considering the high variability of the biochemical parameters and taking into account their role in the nutritional value and health benefits of carrots, these results will be helpful in research and breeding programs to improve the overall quality of this plant food.

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PAPER

OPTIMIZATION OF EXTRACTION PROCESS OF PUMPKIN SEED OIL CHARACTERIZED WITH HIGH PUFA CONTENT VIA DOUBLE RESPONSE SURFACE IN COMBINATION WITH MATLAB METHODOLOGY

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ABSTRACT

Box-behnken-double response surface optimization combined with Matlab analysis was used to optimize the extraction of Inner Mongolia pumpkin seed crude oil based on the relative content of polyunsaturated fatty acids (PUFA) (Y_1) and crude oil yield (Y_2). The influence of three single factors (solid-liquid ratio, extraction temperature, extraction time) of pumpkin seed was investigated. By the optimization of dual response surface analysis, the optimal extraction process parameter was as follows: solid-liquid ratio of 1:26.8 g/mL, extraction temperature of 30°C, extraction time of 2.5 h. On this condition, the relative content of PUFA was 43.54±0.14%, and the crude oil yield was 14.84±0.10 %, which showed no significant difference from theoretical value (p >0.05). In addition, by Matlab analysis, when the extraction time was taken as the lower level (C=2.5 h), Y_1 was able to obtain larger theoretical values (45.8485%), however, when the temperature was taken as the higher level (C=3.5 h), Y_2 can obtain larger theoretical values (17.0563%). When the extraction time was fixed as 2.5 h, and the solid-liquid ratio was within the range of 1:24 ~ 1:27 g/mL, extraction temperature was within the range of 30～32.5°C, Y_1 and Y_2 both can get the theoretical maximum value. The results of dual response surface optimization and matlab optimization are consistent. The combination of the two methods can not only accurately obtain the best extraction scheme, but also more intuitively find out the reasonable process parameter interval of Pumpkin seed oil.

Keywords: pumpkin seed oil, polyunsaturated fatty acid (PUFA), crude oil yield, double response surface, matlab methodology

1. INTRODUCTION

The pumpkin, which is of relatively high economic importance, belongs to the family Cucurbitaceae (GEMROT *et al*., 2006; DURANTE *et al*., 2014). In the naked seed, there are about 22～64% lipids (YOUNIS *et al*., 2000; JIAO *et al*., 2014), which contains abundant oleic, linoleic and linolenic acids (DURANTE *et al*., 2014). The pumpkin seed oil contains various bioactive compounds, such as phytosterols (NISHIMURA *et al*., 2014), phenolic (REZIG *et al*., 2012), antioxidants (REZIG *et al*., 2012), tocopherols (RABRENOVIĆ *et al*., 2014; WANG *et al*., 2017) and small levels of carotenoids (WANG *et al*., 2017; PROCIDA *et al*., 2013), which has been widely used not only in the food industry as an edible oil, but also in different regions of the world as the commercial oil (CAN-CAUICH *et al*., 2019). In addition, the FAO indicates that unrefined pumpkin oil is considered to be of high quality for its taste, aroma and color, the characteristics of which have defined the use of this oil for salads and cold dishes (OJEDA-AMADOR *et al*., 2018).
As is known to all, the two essential polyunsaturated fatty acids (PUFAs) namely linoleic acid (omega-6 fatty acids) and alpha-linolenic acid (omega-3 fatty acids) are considered very important for human body to survive. Studies have shown that essential fatty acids can treat certain diseases such as cancers and diabetes (ORSAVOVA *et al*., 2015). Particularly, linoleic acid is recognized as the essential nutrient for humans. Moreover, the pumpkin seed oil is a good source of linoleic acid (C18:2,cis-9,12) (ORSAVOVA *et al*., 2015; LIANG *et al*., 2018). In the literature, a number of reports can be found describing the different methods for the extraction of oil from the seeds of pumpkins, such as organic solvent extraction (REZIG *et al*., 2018), mechanical pressing (RABRENOVIĆ *et al*., 2014), and supercritical fluid (DURANTE *et al*., 2017) etc. However, almost all the research optimized the extraction process based on the oil extraction rate. No research has been carried out to determine the influence of extraction method and condition on the content of bioactive compounds of PUFA portion in pumpkin oil. Thus, the aim of this study was to investigate the optimization of extraction process based on the PUFA content of pumpkin seed oil, by the methodology of double response surface in combination with Matlab analysis, in order to prepare the superior pumpkin seed oil characterized with high PUFA proportion, as well as the higher extraction rate.

2. MATERIALS AND METHODS

2.1. Samples of pumpkin seed

The pumpkin seed (Gold No. 2) were acquired in the locality of Hetao plain, Inner Mongolia (North latitude of 40°, East longitude of 107°), China, in September 2017. The complete seeds were selected manually, removing broken or cracked seeds. The selected seeds were dried (up to 4% humidity) in a convection oven at 30±1.0°C for 48 h and subsequently stored at 4°C in polyethylene bags until use. Each sample was analyzed as the whole nut, with the shell.

2.2. Oil extraction

The extraction of oil by organic solvent, was carried out according to the method described by MOO-HUCHIN *et al*. (2013), with a few modifications. 10 g of seeds were crushed in grinder (YB-2500A, Yongkang Fastfront Industrial and Trade Co. LTD.) until

the powder passed through No. 40 (about 0.43 mm) mesh. The oils from pumpkin seeds were extracted with petroleum ether (350 mL) in a Soxhlet apparatus by thermal cycles at 45°C for 4 h. In order to obtain the oil, the solvent was removed using a rotary vacuum evaporator at 40°C (Buchi, Flawil, Switzerland). The yield of extracted oil was calculated gravimetrically after collection.

2.3. The analysis of PUFAs in pumpkin seed oil

The pumpkin seed oil was methylated with boron fluoride-methanol (Sigma Aldrich) according to XUE (2016). The fatty acid methyl esters were analyzed by gas chromatograph by the method of XUE (2016). The fatty acids methyl esters were analyzed by a QP-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a split injector. One microliter of FA methyl esters was injected in split mode (5:1) onto a Rtx-Wax capillary column (Restek, Bellefonte, PA, USA; 30 m length × 0.25 mm id × 0.25 μm film thickness). The temperature of the column was programmed as follows: 1 min at 140°C, increments of 8°C/min to 180°C and held at 180°C for 2 min, increments of 3°C/min to 210°C then increments of 5°C /min to 230°C and held at 230°C for 10 min. The temperature of the injector and the detector both were 250°C. The flow rate of the carrier gas (N_2) was 1.5 mL/min. Identification of fatty acids was performed by comparison of the retention times with those of standards (Sigma-aldrich, Shanghai, China). The results were expressed as percent of the total fatty acids methyl esters present.

2.4. Single factor test

The effects of solid-liquid ratio (1:15, 1:25, 1:35, 1:45 and 1:55 g/mL), extraction temperature (25, 35, 45, 55, 65°C), and the extraction time (2, 3, 4, 5, 6 h) on the relative content of PUFA in pumpkin seed oil were investigated, respectively.

2.5. Response surface test

The box-behnken design (BBD) of double response surface methodology was employed using the Design Expert 7.0 software, based on the result of single factor test. The double response values are the relative content of PUFA (Y_1) and the crude oil yield (Y_2), in order to prepare the superior pumpkin seed oil characterized with not only higher PUFA proportion, but also the higher extraction rate. Three variables were optimized such as solid-liquid ratio, extraction temperature and extraction time. The range and level employed in the experiment are shown in Table 1.

Table 1. Experimental range and levels of independent variables in optimization study.

Level	Factors		
	A Solid-liquid ratio (mL/g)	B Extraction temperature (°C)	C Extraction time (h)
-1	1:20	30	2.5
0	1:25	35	3
1	1:30	40	3.5

2.6. Verification experiment of optimal combination

According to the results of box-behnken double response surface and matlab methodology, the optimal process parameters were obtained and verified by experiments.

2.7. Optimization experiment of matlab

The matlab methodology was based on the optimization calculation method and graphic processing function of algorithm language. By programming M (the program code), calculate the four-dimensional and three-dimensional interaction results, which can explain the effect of three factors (solid to liquid ratio (A), extraction temperature (B), extraction time (C)) on the relative content of PUFA (Y_1) and crude oil yield (Y_2) of pumpkin seed oil. The levels or range of values of the variables followed here were based on the results of the parametric study previously conducted.

2.8. Statistical analysis

All analysis was performed in duplicate and the data collected were subjected to ANOVA using Excel® 2010 software and Tukey tests (with a 95% confidence interval), to evaluate differences between the results.

3. RESULTS AND DISCUSSION

3.1. GC analysis of pumpkin seed oil

Fig. 1 shows the GC chromatogram of fatty acid methyl esters extracted from pumpkin seed oil by petroleum ether. The extracts of the seeds showed the presence of saturated fatty acids (such as palmitic acid (C16:0) and stearic acid (C18:0)), and unsaturated fatty acids (such as oleic acid (C18:1,cis-9), linoleic acid (C18:2,cis-9,12), and α-linolenic acid (C18:3,cis-9,12,15). Similar results for the fatty acid composition of pumpkin seed oil have been reported by MITRA *et al*. (2009), SALGIN and KORKMAZ (2011), DURANTE *et al*. (2014) and CUCO *et al*. (2019).

As shown in Table 2, abundant PUFA was observed (34.61%), especially the α-Linoleic acid (34.02%), which was higher than the amount extracted by pressurized CO_2 (CUCO *et al*, 2019). According to CUCO (2019), the extracts obtained using organic solvent, such as petroleum ether and *n*-hexane had higher concentrations of linoleic and stearic acids in relation to the extracts obtained with pressurized CO_2, which was consistent with the research results in this paper. Besides, the eicosanoic acid ($C_{20:0}$) portion was not detected, since the extraction of fatty acids may be related to the polarity of the solvents used (MEZZOMO *et al*., 2010). Differences observed in fatty acid composition of pumpkin seed oil may be attributed to the variation in climatic conditions, cultivation locations, agronomical practices, and genetic backgrounds of the seeds, as well as esterification and analysis methods (ÖZCAN *et al*., 2019).

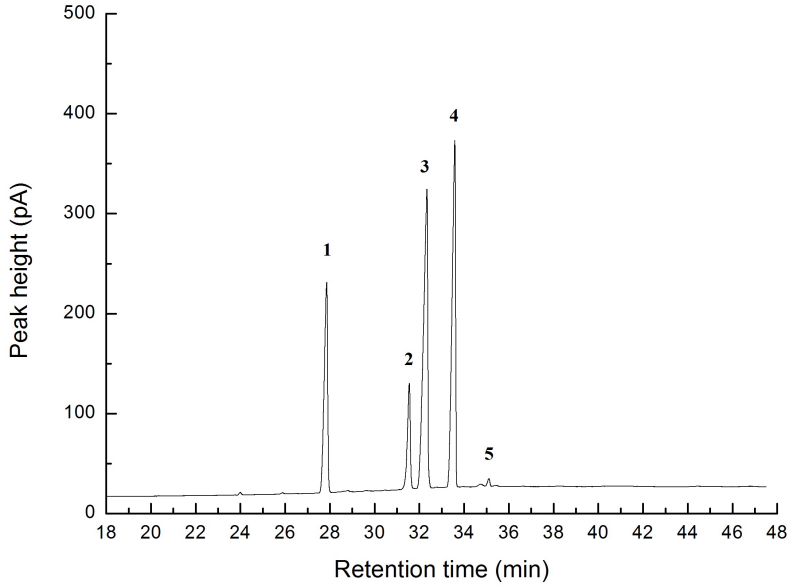

Figure 1. Gas chromatogram of fatty acid methyl ester extracted from pumpkin seed oil by petroleum ether.

Table 2. Composition of fatty acids of pumpkin seed oil.

No.	fatty acid	The relative content (%)
1	C16:0 Palmitic acid	20.75±0.23
2	C18:0 Stearic acid	8.79±0.01
3	C18:1,cis-9 Oleic acid	35.85±0.16
4	C18:2,cis-9,12 Linoleic acid	34.02±0.15
5	C18:3,cis-9,12,15 α-Linolenic acid	0.59±0.09
	Saturated fatty acid, SFA	29.54±0.22
	Monounsaturated fatty acid, MUFA	35.85±0.16
	Polyunsaturated fatty acid, PUFA	34.61±0.05
	N-3 PUFA	0.59±0.09
	N-6 PUFA	34.02±0.15

In recent years, the seed oil from vegetable, such as olive oil, sunflower seed oil, and algerian peanut landraces oil had been obtained for food use and biodiesel production (GIUFFRÈ *et al.*, 2017a; GIUFFRÈ *et al.*, 2017b; GIUFFRÈ *et al.*, 2016). The balance of fatty acids in the diet is important for health, and the fatty acids are among the most important parameters to establish the edibility of a vegetable oil (DENG, 2014). As for the monounsaturated fatty acids (MUFA), the content of oleic acid in the pumpkin seed oil (35.15%) was higher than that in the sunflower seed oil (32.47%) (GIUFFRÈ *et al.*, 2017b) and soybeans oil extracted by supercritical carbon dioxide (21.191%) (JOKIĆ *et al.*, 2013). The pumpkin seed oil was characterized with higher content of linoleic acid (34.02%), compared to that in olive oil (18.45%) (GIUFFRÈ *et al.*, 2017a), algerian peanut landraces oil (29.92%) (GIUFFRÈ *et al.*, 2016) and rapeseed (*Brassica napus ssp. oleifera* L.) oil (25.02%)

(BEYZI *et al.*, 2019). In addition, the α-linolenic acid portion in this pumpkin seed oil (0.59%) was significantly higher than that in the sunflower seed oil (0.06%) (GIUFFRÈ *et al.*, 2017b) and algerian peanut landraces oil (0.09%) (GIUFFRÈ *et al.*, 2016). Thus, pumpkin seed oil was a nutrient and biologically active vegetable oil, which had abundant PUFA playing the important beneficial role.

3.2. Single factor test results

The effect of solid-liquid ratio, extraction temperature, and extraction time on the relative content of PUFA in pumpkin seed oil was represented in Figs. 2-4, respectively. As shown in Fig. 2, with the increase of solid-liquid ratio, the relative content of PUFA in pumpkin seed crude oil first increased (p >0.05) and then decreased significantly (p <0.05). When at 1:25 g/mL, the content of PUFA reached the highest. From Fig. 3, at the range of 25～65°C, the relative PUFA content first increased from 25 to 35°C, while decreased with the rising of extraction temperature. The max content of PUFA was observed in 35°C. Similarly, when the extraction time was at 3 h, the PUFA have the maximum, and decreased significantly with extension extraction time (Fig. 4). Since heat treatment may cause lipid oxidation, and the temperature and time are key factors (BYRNEA *et al.*, 2002).

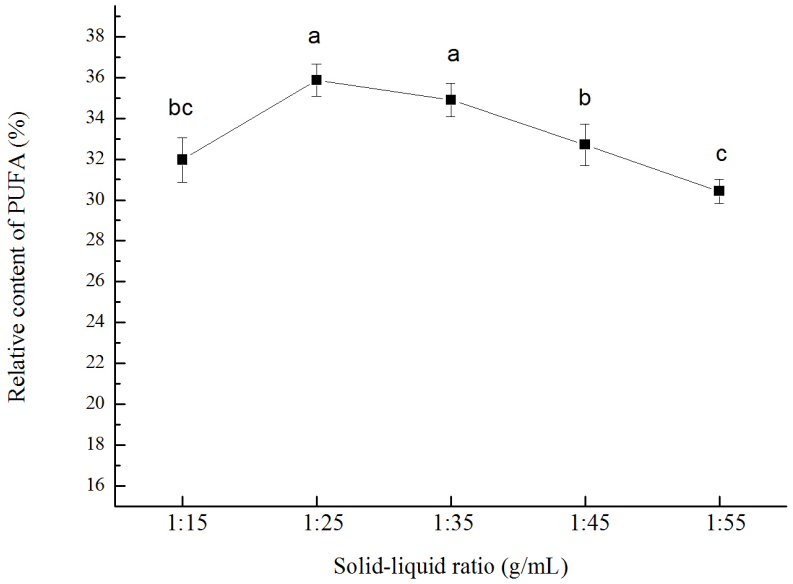

Figure 2. Effect of solid-liquid ratio on relative content of PUFA from pumpkin seed oil.

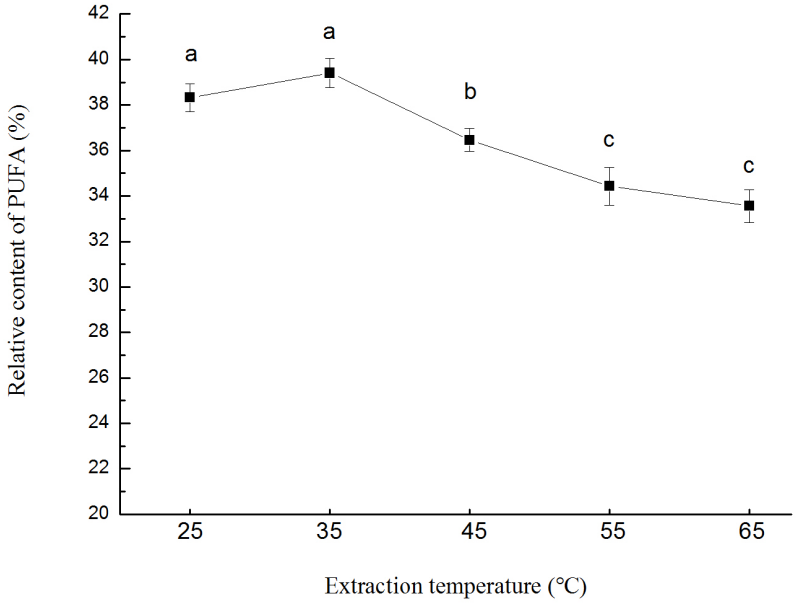

Figure 3. Effect of extraction temperature on relative content of PUFA from pumpkin seed oil.

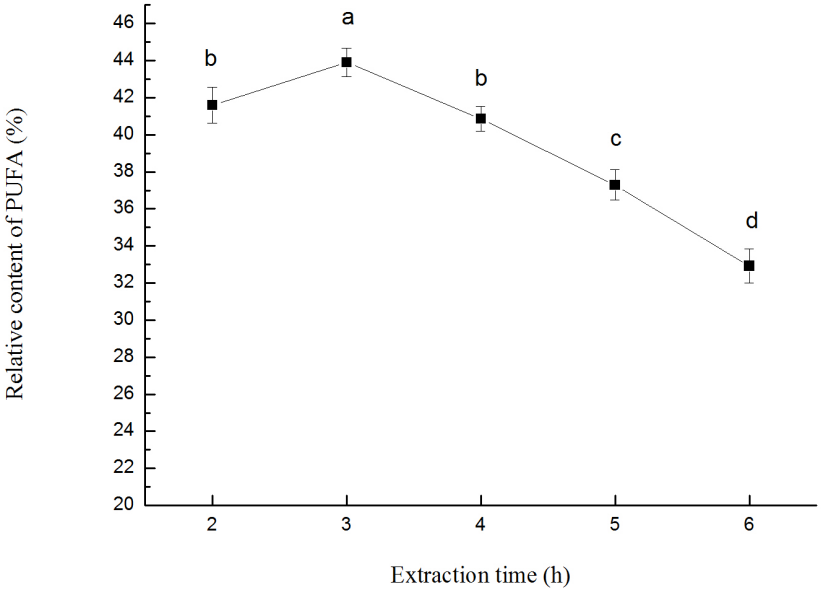

Figure 4. Effect of extraction time on relative content of PUFA from pumpkin seed oil.

3.3. Double response surface experiment results

BBD is an independent, rotatable or nearly rotatable second-order design based on three-level incomplete factorial designs. BBD is more efficient compared to other response surface designs, such as central composite designs. In addition, BBD can provide sufficient information for testing the lack of fit, and therefore is one of the best quadratic models for response surface methodology and has been widely used in analytical fields (SONG *et al*., 2009). The quadratic rotary combination design and test results can be seen in Table 3. In this study, coefficients of the models were calculated and the predicted models were as follows, respectively:

$$Y_1=38.29-1.31*A-1.74*B-2.59*C-0.84*AB-2.45*AC-0.025*BC+0.70*A^2+0.66*B^2-0.091*C^2 \quad (1)$$

$$Y_2=14.88+1.15*A+0.45*B+0.33*C+1.13*AB+0.38*AC+1.25*BC-0.38*A^2-0.18*B^2-0.26*C^2 \quad (2)$$

The results of ANOVA for the quadratic model were shown in Table 4 (Y_1) and Table 5 (Y_2), and the significance of each coefficient was determined by F-value and p-value. Generally, the larger magnitude of F-value and the smaller *p*-value had more significant effect on the corresponding coefficient. The model F-value of Y_1 and Y_2 implied that the model were significant. There was only a 0.0006 and 0.0071 chance that a model F-value of this size could occur due to statistical noise, respectively. The coefficient of determination (R^2) meant the proportion of the total variation in the response expected by the model. Higher coefficient of determination (R^2) of model implied that the response surface model was reasonable (HU *et al*., 2017).

Table 3. Experimental design and results of double response surface optimization.

Test No.	A：Solid-liquid ratio (mL/g)	B：Extraction temperature（°C）	C：Extraction time (min)	Y1: Relative content of PUFA (%)	Y2: Crude oil yield（%）
1	-1	-1	0	42.33	13.96
2	1	-1	0	40.08	13.8
3	-1	1	0	40.91	12.6
4	1	1	0	35.3	16.95
5	-1	0	-1	39.62	12.82
6	1	0	-1	43.22	14.57
7	-1	0	1	39.48	13.17
8	1	0	1	33.28	16.43
9	0	-1	-1	43.42	15.14
10	0	1	-1	39.61	13.55
11	0	-1	1	38.16	12.84
12	0	1	1	34.25	16.25
13	0	0	0	37.71	14.56
14	0	0	0	38.18	15.65
15	0	0	0	37.93	13.76
16	0	0	0	38.2	15.15
17	0	0	0	39.42	15.29

All data are mean values of duplicate measurements.

Table 4. The variance analysis of Y_1.

Source	Sum of squares	df	Mean square	F-value	*p*-value (Prob>F)	Significant
Model	122.4503	9	13.60559	17.17016	0.0006	**
A- Solid-liquid ratio	13.67645	1	13.67645	17.25959	0.0043	**
B- Extraction temperature	24.2208	1	24.2208	30.56649	0.0009	**
C- Extraction time	53.56125	1	53.56125	67.59395	<0.0001	**
AB	2.8224	1	2.8224	3.56185	0.1011	
AC	24.01	1	24.01	30.30046	0.0009	**
BC	0.0025	1	0.0025	0.003155	0.9568	
A^2	2.083841	1	2.083841	2.629794	0.1489	
B^2	1.853609	1	1.853609	2.339243	0.1700	
C^2	0.035252	1	0.035252	0.044487	0.8390	
Residual	5.54678	7	0.792397			
Lack of fit	3.7837	3	1.261233	2.861432	0.1680	
Pure error	1.76308	4	0.44077			
Cor Total	127.997	16				
Adj-R^2	0.9009					
Pred-R^2	0.5055					
C. V. (%)	2.29					

Note: *: $p < 0.05$; **: $p < 0.01$.

As for Y_1, the graphic plot of predicted values by the model vs. observed experimental values showed a linear distribution (R^2=0.9567) for the response, indicating that the predicted values obtained from the model had a linear relationship with the observed values.

As for Y_2, the R^2 for the predictive model was 0.9067, which also indicated that the experimental data well fitted the second-order polynomial equation. In addition, the ANOVA analysis presented in Table 4 (Y_1) and Table 5 (Y_2) indicated that the model was highly appropriate for the prediction. Overall, R^2, the fraction of the variation of the response by the model, adjusted R^2 and regression *p*-value and lack of fit values > 0.05 all indicated that model well fitted the experimental data points (BILGIC *et al*., 2012; BILGIÇ-KELEŞ *et al*., 2019).

The conditions for the maximum Y_1 and Y_2 were both generated by the optimizer function of the Design Expert 7.0 software. Based on the mathematical model established, the optimal experimental conditions were as follows: solid-liquid ratio (1:27 g/mL), extraction temperature (30°C) and extraction time (2.5 h), respectively. The Y_1 was obtained as 43.54%, and Y_2 obtained as 14.84%, which was satisfactorily close to the predicted value 43.44%, 14.78% at this point, respectively.

Table 5. The variance analysis of Y_2.

Source	Sum of squares	df	Mean square	F-value	*p*-value (Prob>F)	Significant
Model	26.08134	9	2.897927	7.556931	0.0071	**
A- Solid-liquid ratio	10.58	1	10.58	27.5895	0.0012	**
B- Extraction temperature	1.629013	1	1.629013	4.24798	0.0782	
C- Extraction time	0.851513	1	0.851513	2.220492	0.1798	
AB	5.085025	1	5.085025	13.26023	0.0083	**
AC	0.570025	1	0.570025	1.486456	0.2623	
BC	6.25	1	6.25	16.29814	0.0050	**
A^2	0.595267	1	0.595267	1.55228	0.2529	
B^2	0.134157	1	0.134157	0.349841	0.5728	
C^2	0.281357	1	0.281357	0.733695	0.4200	*
Residual	2.684355	7	0.383479			
Lack of fit	0.493675	3	0.164558	0.30047	0.8246	
Pure error	2.19068	4	0.54767			
Cor Total	28.76569	16				
Adj-R^2	0.7867					
Pred-R^2	0.6064					
C. V. (%)	4.27					

Note: *: $p < 0.05$; **: $p < 0.01$.

3.4. Analysis of the response surface

Three-dimensional response surfaces were applied to test the mutual effects of different variables, so as to better understand the independent variables' effect on relative content of PUFA, as well as crude oil yields. Contour plot and response surface plot of the interaction of two parameters can be seen in Fig. 5 (Y_1) (5a-5c) and Fig. 6 (Y_2) (6a-6c). Fig. 5a shows the mutual effects between solid- liquid ratio and extraction temperature extracted at fixed extraction time (3 h). It can be found that content of PUFA obviously decreased with growth of solid liquid ratio at a designated extraction temperature (Figure 5a), however, the growth of crude oil yield was increasingly slow when solid liquid ratio increased (Fig. 6a). The obviously reduction of PUFA content was found with growth of solid liquid ratio at a designated extraction time (Fig. 5b), whereas the growth of crude oil yield was increasingly when solid liquid ratio increased (Fig. 6b), similar with Fig. 6a. The PUFA content obviously decreased with growth of extraction temperature at a designated extraction time (Fig. 5c), while crude oil yield firstly decreased and then increased with the growth of extraction temperature, which had dual influences on crude oil yields (Fig. 6c).

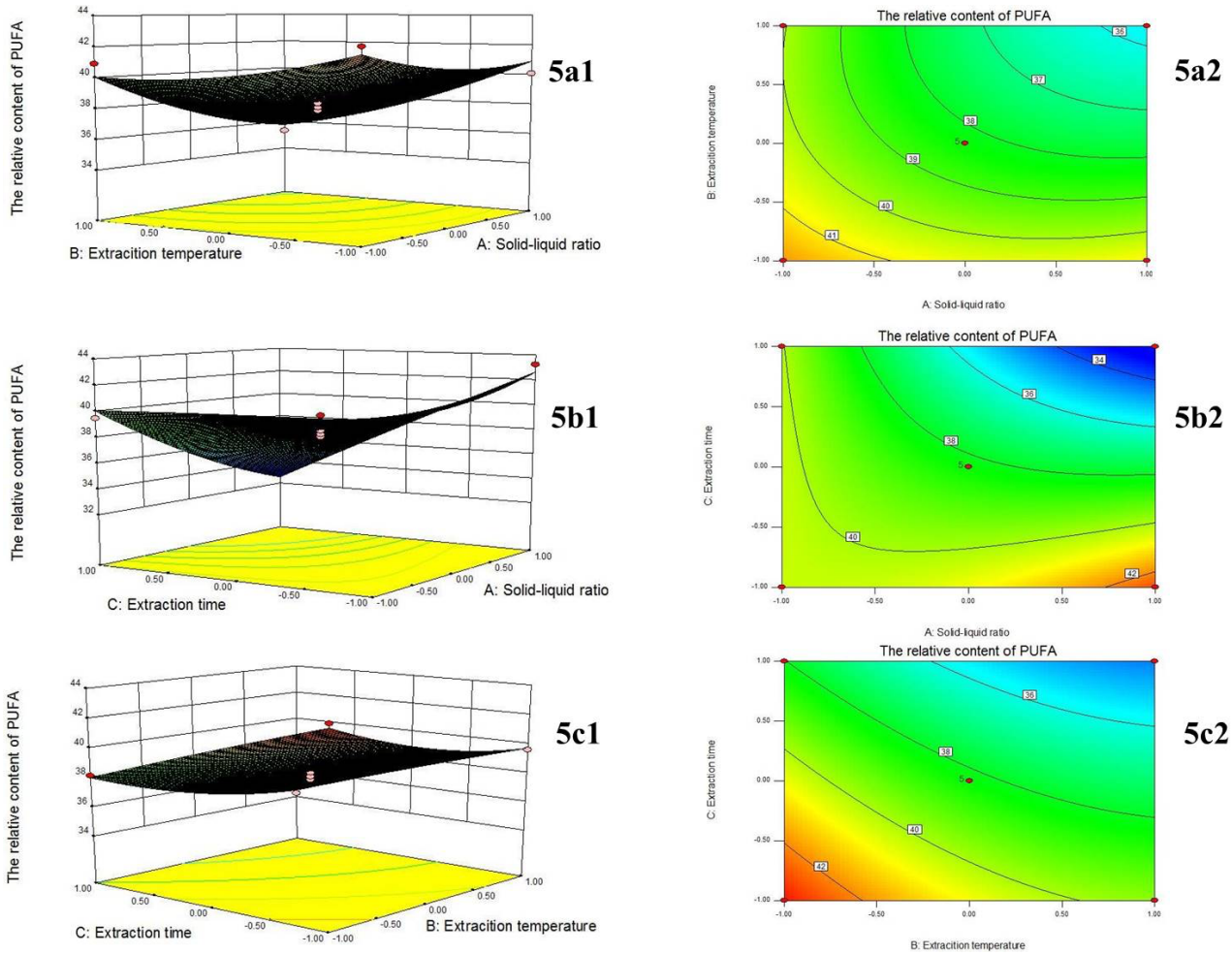

Figure 5. Contour plot and response surface plot of the influence of the interaction of various factors on the relative content of PUFA.

3.5. Matlab experimental results

Through programming, the 4-dimensional diagram of PUFA content and crude oil yield of pumpkin seed effected by solid-liquid ratio (g/mL), extraction temperature (°C) and extraction time (min) was presented (Figs. 7, 8). When the theoretical maximum value of Y_1 (45.8485%) was obtained, the conditional parameters were calculated by the matrix as bellow: the solid-liquid ratio of 1: 30 mL /g, the extraction temperature of 30°C, and the extraction time of 2.5 h. Similarly, when Y_2 reached the theoretical maximum value (18.7515%), the solid-liquid ratio was 1:30 g/mL, the extraction temperature was 40°C, and the extraction time was 3.5 h.

In order to better describe and analyze the interaction effects between data, three-dimensional response surface and contour projection diagrams of the interaction effects of Y_1 and Y_2 were drawn respectively (Fig. 9), when the extraction time was set short (2.5 h), medium (3 h) and long (3.5 h).

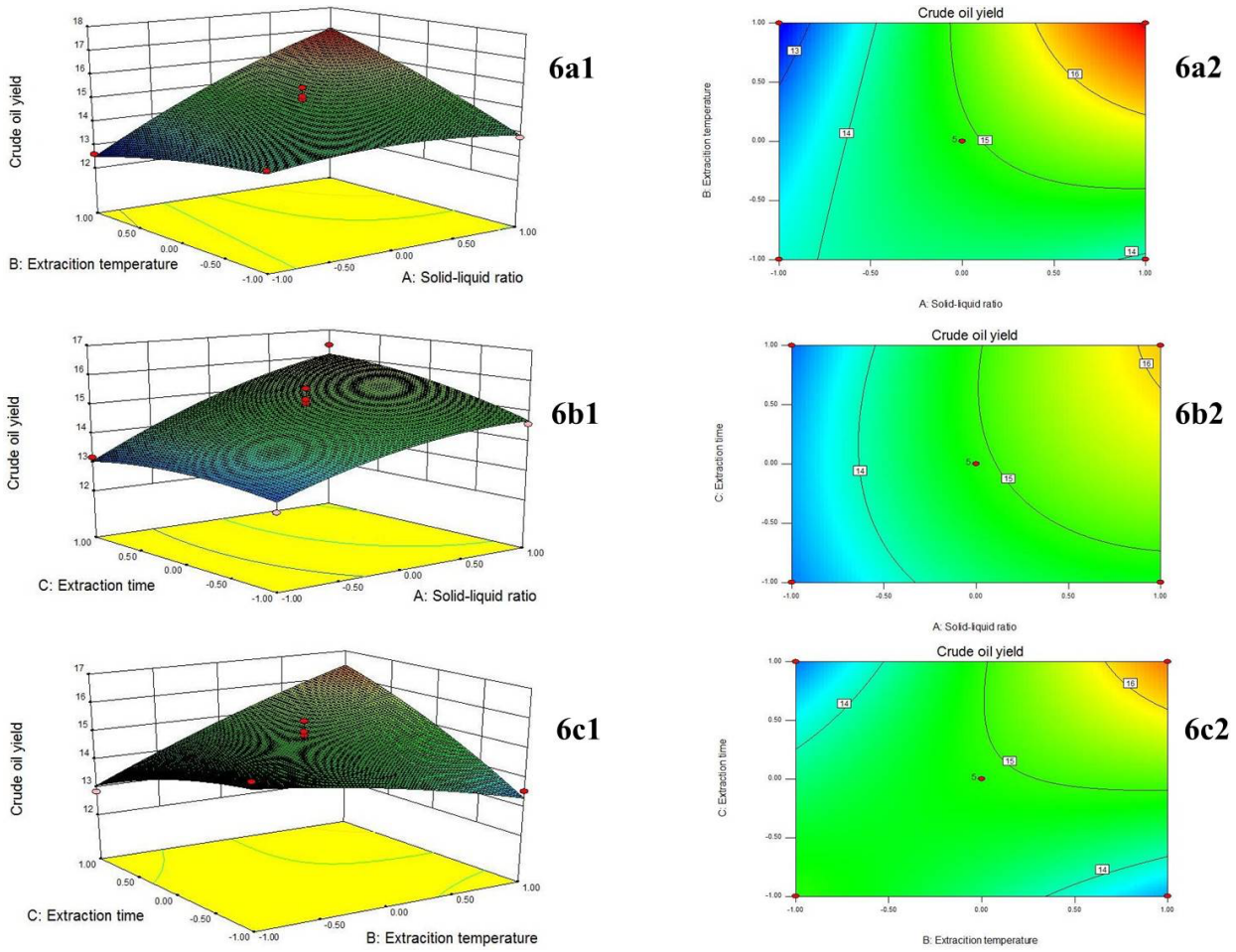

Figure 6. Contour plot and response surface plot of the influence of the interaction of various factors on the crude oil yield.

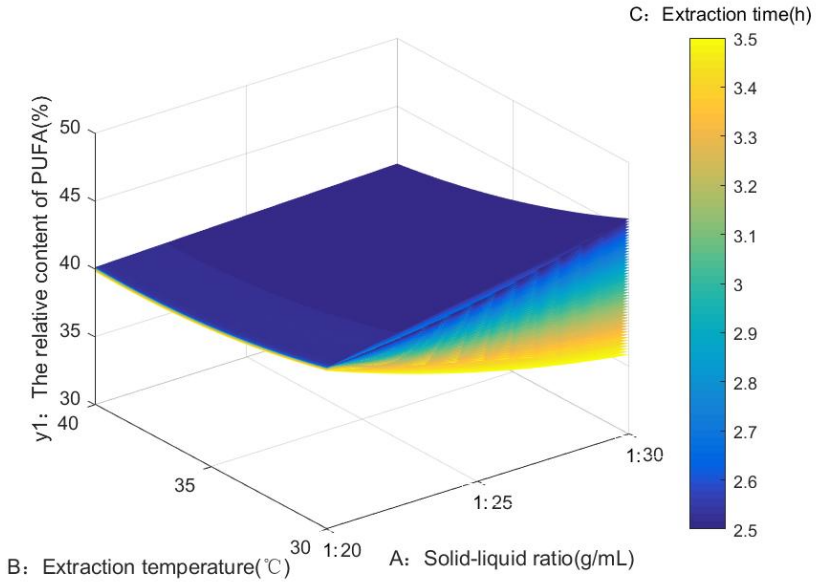

Figure 7. The 4-D interactive surface based on the optimizing of Y1.

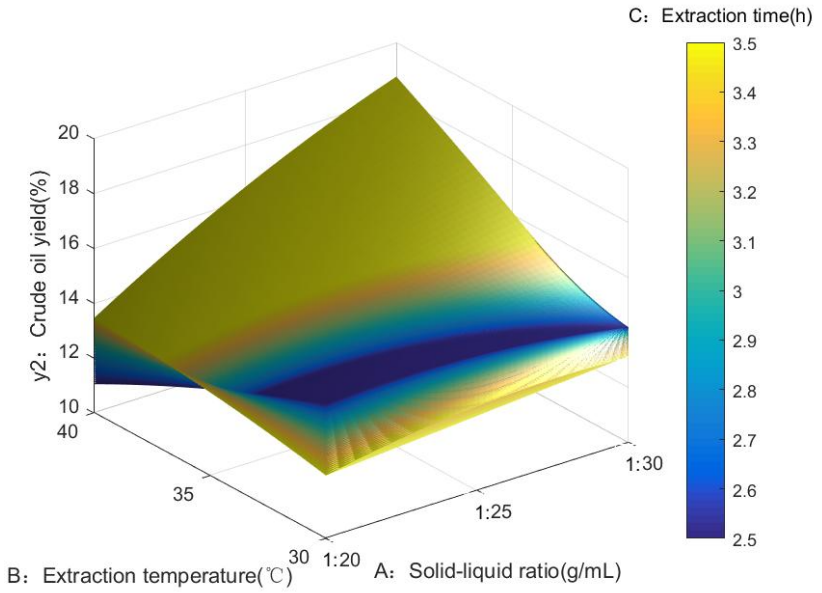

Figure 8. The 4-D interactive surface based on the optimizing of Y_2.

When the extraction time (C) set as lower level (C=2.5 h), the extraction temperature (B) was fixed, the value of Y_1 and Y_2 gradually increased with the increasing of solid-liquid ratio (A). When A value is fixed, both Y_1 and Y_2 decrease continuously with the increase of B. When the A value ranged from 1:24 to 1:27 g/mL, and the B value ranged from 30 to 32.5°C (the larger the value of A, the smaller the value of B), the Y_1 and Y_2 can approach the maximum value simultaneously. On this condition, the value range of Y_1 was 39.7~ 45.8485%, and the value range of Y_2 was 11.0440 ~ 15.0013%.

When the middle value (C) of extraction time (C=3 h) was set, and B value is fixed, with the increase of A, Y_1 continued to decrease, while Y_2 gradually increased. When A value was fixed, with the increasing of B value, Y_1 decreased and Y_2 increased. As A and B get closer to the minimum, Y_1 get closer to the maximum, and as A and B get closer to the maximum, Y_2 will get closer to the maximum. On this condition, the value range of Y_1 was 35.7675 ~ 41.8625%, and the value range of Y_2 was 12.5013 ~ 17.0563%, thus Y_1 and Y_2 cannot reach the maximum value simultaneously.

When the higher level of extraction time (C) (C=3.5h) was set, and B value was fixed, with the increasing of A, Y_1 continued to decline, while Y_2 showed a gradual increase trend. When fixed A value, as B increased, Y_1 continued to decline, while Y_2 gradually increased. The change rule was similar to what happened when C was set as the medium level (C=3 h). On this condition, the value range of Y_1 was 30.6135 ~ 41.6585%, and the value range of Y_2 was 12.2940 ~ 18.7515%. Only when A and B took a specific middle interval, can we ensure that both Y_1 and Y_2 get larger value simultaneously.

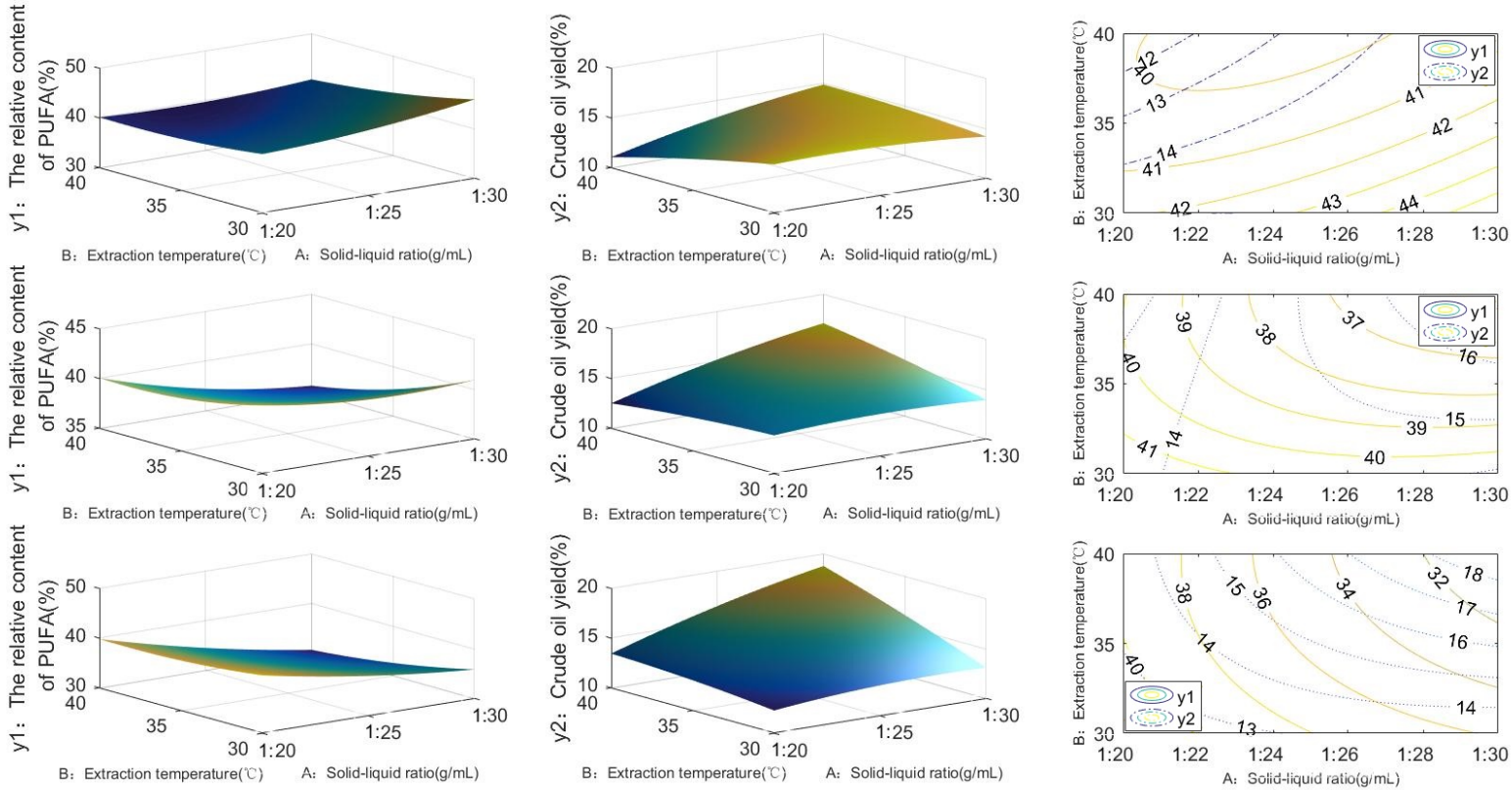

Figure 9. Contour plots and response surface plots of the effects of the interaction of various factors on relative content of PUFA and crude oil yield.

In conclusion, according to the software calculation, when the extraction time was set as the lower level (C=2.5 h), Y_1 can obtain the maximum theoretical value (45.8485%); when the extraction time was set as the higher level (C= 3.5 h), Y_2 can obtain the maximum theoretical value (17.0563%). When the value of C was 2.5 h, and the value of A was 1:24 ~ 1:27 g/mL, the value of B was 30 ~ 32.5°C, both Y_1 and Y_2 can obtain the larger theoretical value simultaneously, which is consistent with the above analysis conclusion.
Matlab, an acronym for MATrix LABoratory, is a product of the MathWorks, Inc.of Natick, MA (GILAT, 2011). The matlab is a high-performance and powerful graphics system for presenting and visualizing data. It integrates computation, visualization, and programming in an easy-to-use environment where problems and solutions are expressed in familiar mathematical notation (JALALVAND *et al*., 2019). Nowadays, the matlab is widely used in various research fields, such as optimization of oil extraction in chemical industry (HØRSHOLT *et al*., 2018), design and manufacturing of food machinery (CAO *et al*., 2014; ZHANG and DENG, 2018), and optimization of nutritional catering (TIAN and YU, 2009). At present, there is hardly any research on the optimization of pumpkin seed crude oil extraction process using matlab analysis method, which may be the development trend of food industry in the future.

4. CONCLUSIONS

The box - behnken dual response surface optimization method combined with matlab analysis method was used to optimize the extraction process of crude oil from Inner Mongolia pumpkin seed, based on the relative contents of PUFA, as well as the crude oil yield. The optimal process parameters were as bellow: solid-liquid ratio of 1:26.8 g/mL, extraction temperature 30°C, extraction time 2.5 h. At this point, the relative contents of PUFA pumpkin seeds oil was 43.54±0.14%, and the yield was 14.84±0.10%, which was not significantly different from the theoretical value (p >0.05). In addition, by the analysis of matlab, when the C value was set as the lower level (C=2.5 h), the solid-liquid ratio was within the range from 1:24 to 1:27 g/mL, the extraction temperature was within the range from 30 ~ 32.5°C, both Y_1 and Y_2 can obtain the larger theoretical value simultaneously, which was consistent with the conclusion of double response surface analysis. According to results, the optimization method combined dual response surface with matlab can not only accurately obtain the best extraction scheme, but also more intuitively find out the reasonable process parameter range, so that can provide theoretical basis and innovative basis for the research on industrial processing and utilization of pumpkin seed oil.

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PAPER

EVALUATION OF ENZYMATIC HYDROLYSIS APPLIED TO FISH BY-PRODUCT OIL THROUGH CHEMICAL PARAMETERS

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ABSTRACT

Large amounts of fish waste are produced by the fish processing plants. This waste could be used to obtain high value-added products, such as long chain polyunsaturated fatty acids. Thus, the aim of this work was to evaluate the enzymatic hydrolysis of low commercial value crude fish oil through chemical parameters. Crude fish oil was obtained from mixed fishmeal production and was chemically refined. *Candida rugosa* lipase AY "Amano" 30 was utilized to catalyze the enzymatic hydrolysis. The acidity index, iodine value, saponification index and peroxide value were used to characterize the samples studied. The chemical refining process yielded 62.75% and improved the quality of crude fish oil by reducing the acidity index around 91%. The best results of hydrolysis degree (23.45%) and iodine value (120 g I_2 g^{-1}) were obtained at 45ºC after 6 h of lipase action. The iodine value of crude oil was not affected by processing, indicating that the nutritional quality was preserved in the refined oil. Despite the hydrolysis process showed good results, it was not sufficient to concentrate the unsaturated fatty acids of refined oil, as indicated by the iodine value.

Keywords: acidity index, *Candida rugosa*, iodine value, lipase, refined fish oil

1. INTRODUCTION

The fish processing plants produce around 50% of waste from the total processed fish (ARRUDA *et al*., 2006). This waste is rich in organic and inorganic compounds, and its improper disposal causes negative environmental impacts, particularly on the margins of water bodies or in unlicensed landfills (FELTES *et al*., 2010). Fish waste could be utilized to obtain high value-added products, since fish oil can be produced from whole fish, roe and by-products from the processing of fish. Fish oil is widely used in aquafeeds as supply of long chain omega-3s fatty acids for aquaculture, especially for carnivorous fish *e.g.* salmonids. The direct use of fish oil in human foods and capsules is an increasingly significant outlet - the so-called, "nutraceuticals", which use has been increasing even more rapidly than that in aquaculture, at around 15% per annum (PIKE and JACKSON, 2010).

Fish oil is an important source of long chain polyunsaturated fatty acids (LC-PUFA), mainly the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (MONROIG *et al*., 2018). Recent studies revealed that LC-PUFAs prevented short and long term memory impairment induced by chronic sleep deprivation (AZOULBI *et al.,* 2019). There is also abundant evidence that increasing the intake of omega-3s fatty acids can soften the symptoms of neurodegenerative and neurological diseases, improving memory and cognitive function (CUTULI *et al*., 2014; ZHOU *et al.,* 2018). However, crude fish oil presents impurities *e.g.* free fatty acids, mono- and diglycerides, phosphatides, steroids, vitamins, hydrocarbons, pigments, carbohydrates, proteins and their degradation products and colloidal materials (MENEGAZZO *et al*., 2014). The production of high quality oils requires the largest possible removal of non-triglyceride components (PRIOR *et al*., 1991). The refining process aims precisely to remove these non-triglyceride components, conferring the oil best features.

One of the most promising techniques to fish oil processing is the use of lipase-catalyze enzymatic hydrolysis. This process favors the release of lipids from a protein matrix while preserving the nutritional value of fats for their application in food industry. The high specificity of lipases in relation to triacylglycerol substrate has suggested a large number of applications in the pharmaceutical and food areas, being used mainly for the production of particular fatty acids with low energetic consumption (PADILHA and AUGUSTO-RUIZ, 2007; FERREIRA-DIAS *et al*., 2013).

Concentrates of EPA and DHA may be prepared by selective hydrolysis of fish oils using lipases or by selective esterification of DHA and other free fatty acids (RANJAN-MOHARANA *et al*., 2016). The modification of lipids from oils usually involves a process catalyzed by lipase for fat hydrolysis, modification of triacylglycerol, and synthesis of esters. The process is attractive, since the lipase shows a high efficiency, using little amounts, especially in immobilization process (FERREIRA-DIAS *et al*., 2013; RAJENDRAN *et al*., 2009). Thus, this work aimed at evaluating the effect of enzymatic hydrolysis of low commercial value crude fish oil submitted to chemical refining. To this aim, samples submitted to chemical refining for increasing time and at different temperatures were characterized for chemical parameters.

2. MATERIALS AND METHODS

2.1. Crude fish oil and enzyme

Crude fish oil was obtained as a by-product of the mixed fishmeal production from the Torquato Pontes Fisheries Industry, located in Rio Grande, RS, Brazil. The crude oil was kept under refrigeration. The enzyme utilized was *Candida rugosa* lipase AY "Amano" 30, supplied by Amano Enzymes (USA).

2.2. Chemical refining of crude fish oil

The chemical refining of crude fish oil was realized according to the methodology described elsewhere (MORAIS *et al*., 2001; MENEGAZZO *et al*., 2014). Fig. 1A shows all the process steps.

2.3. Enzyme application in the crude fish oil

The enzyme application in crude fish oil was adapted from SUN *et al*. (2002) and is shown in Fig. 1B. The chemically refined fish oil was hydrolyzed with *Candida rugosa* lipase AY "Amano" 30 by adding phosphate buffer solution (pH 7.0) with the enzyme, and 100 μmol of $CaCl_2$ solution.

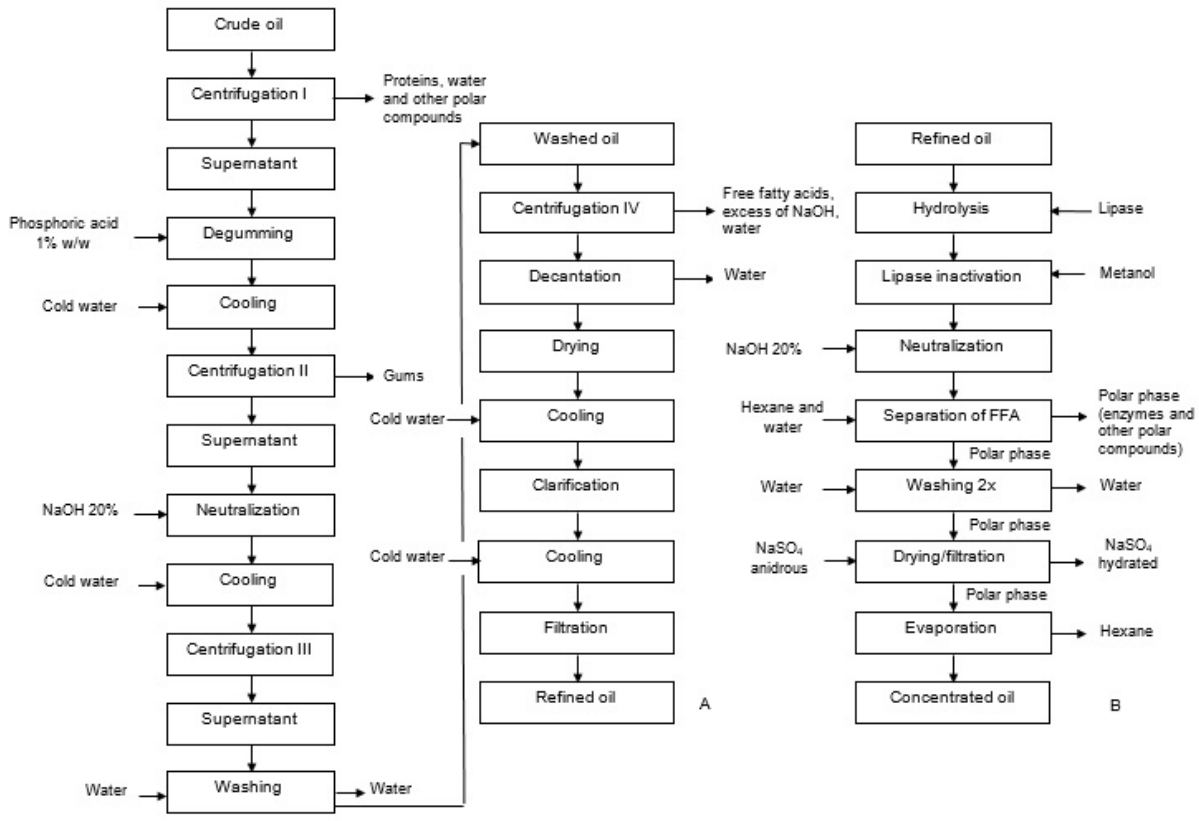

Figure 1. Chemical refining (A) and concentration (B) of fish oil.

The samples were placed in a reactor with stirrer and temperature-controlled bath. Aliquots were collected at defined intervals to determine the acidity index and the degree of hydrolysis (Enzymatic hydrolysis). After the reaction, lipases were inactivated with methanol (Lipase inactivation) and the free acids were neutralized with a NaOH solution 20% (Neutralization). The separation of glycerides from the fatty acids was performed by using a separator funnel. The oily mixture was added of 100 mL of hexane and 50 mL of distilled water (Separation of glycerides). The lower aqueous layer was separated and discarded, while the upper layer containing glycerides was washed twice with 50 mL of distilled water (Washing) and the remaining water was removed by a layer of anhydrous sodium sulfate (Drying/filtration). The glycerides were recovered after removal of solvent on a rotary evaporator (Evaporation). Then, unsaturated fatty acid was obtained as final product.

2.4. Chemical characterization of samples

The characterization of the crude, refined fish oils and the unsaturated fatty acids was carried out according to AOCS (2004) methods: acidity index (AI) (Cd 3d-63), iodine value (IV) (Cd 1b-87), peroxide value (PV) (Cd 8-53), and saponification index (SI) (Cd 3-25). All analyses were performed in triplicate.

2.5. Determination of lipase activity

The lipase activity was performed using the method described by SUGIHARA *et al*. (1990) An olive oil emulsion was prepared and added of 50 mM acetate buffer (pH 5.6) and 100mM $CaCl_2$. The enzyme solution added varied from 5 to 50 μL. The reaction was maintained at 37°C under stirring of 500 rpm for 30 min. After that, the reaction was inactivated with ethanol, then titrated with KOH 50mM, to determine the amount of fatty acids released by enzymatic reaction.[19] The lipase activity was calculated using Equation 1:

$$\text{Activity} = \frac{N\left(\frac{\text{mol}}{L}\right)\times 10^6\left(\frac{\mu\text{mol}}{\text{mol}}\right)\times\left(\frac{L}{1000\text{mL}}\right)\times\Delta\text{volume}_{\text{NaOH}}(\text{mL})}{\text{time(min)}} \quad (1)$$

2.6. Hydrolysis degree

The hydrolysis degree of oils after the enzymic treatment was calculated according to Equation 2:

$$\text{Hydrolysis}(\%) = \frac{\text{AI(hydrolyzedoil}-\text{AI(non}-\text{hydrolyzedoil)}}{\text{SI(non}-\text{hydrolyzedoil)}-\text{AI(non}-\text{hydrolyzedoil)}}x100 \quad (2)$$

where: SI is the saponification index, and AI is the acidity index.

2.7. Statistical analysis

A factorial experimental design 2^2, with three central points, was applied to obtain statistical models for the parameters studied (hydrolysis time and temperature) in function of the considered responses (hydrolysis degree and iodine value). The studied factors with the respective real and coded levels are shown in Table 1.

Table 1. Variable levels and limits for the 2^2 experimental factorial design.

Independent variable	Level		
	-1	0	1
Hydrolysis time (h)	2	4	6
Hydrolysis temperature (ºC)	45	50	55

All statistical analysis was performed using the Statistica 6.0 software and the validity of quadratic model was performed by variance analysis (ANOVA) at 5% probability by Fisher test.

3. RESULTS AND DISCUSSION

3.1. Characterization of fish oils

Table 2 shows the yield of each step of chemical refining. The refined fish oil yielded 62.75%, which is acceptable, since refining stages causes many losses during process, as occur in neutralization step with NaOH. This alkali was more effective in bleaching, even if it caused saponification of a small part of neutral oil in the same time it promotes the neutralization of free fatty acids. The washing was essential to remove all NaOH residue. Notwithstanding, the importance of neutral oil drying lies in that the moisture, during the oil storage, can cause hydrolysis and increase the acidity, as well as oxidation of the heated oil. At the filtering stage, the yield dropped from 67.74% to 62.75%. This loss could be associated to the use of adsorbents in higher amounts than the minimum required, resulting in a greater loss of oil.

Table 2. Yield of chemical refining process.

Stage of the process	Yield (%)
Crude fish oil	100.00
Degumming	91.85
Neutralization	81.65
Washing	74.82
Decantation	70.12
Drying	67.74
Filtration	62.75
Refined fish oil	62.75

The chemical characterization of crude fish oil and refined fish oil is shown in Table 3.

Table 3. Chemical characterization of crude fish oil and refined fish oil.

Parameter	Crude fish oil	Refined fish oil
Acidity index (AI) (mg NaOH g-1)	5.05±0.02a	0.45±0.04b
Iodine value (IV) (g I2 g-1)	121±0.13a	121±0.11a
Peroxide value (PV) (mEq. Kg-1)	n.d*	n.d*
Saponification index (AI) (mg KOH g-1)	182±0.25a	182±0.19a

*Not detectable.
**Different letters in the same line indicated a significant difference ($p<0.05$) by the Tukey test.

A significant difference ($p<0.05$) was observed between the AI of crude fish oil and refined fish oil, showing that the chemical refining was effective in reducing that value. Free fatty acids contents are usually associated with undesirable flavour and textural changes (VISENTAINER *et al*., 2015). The IV, which is associated with the degree of unsaturated fatty acids of oil (CREXI *et al*., 2010), showed no significant difference ($p>0.05$) between the samples studied. The SI also presented no significant difference ($p>0.05$) between results (Table 3). However, they are in accordance with the values reported for oils from marine animals (160-196 mg KOH g^{-1}) (ARAÚJO, 2001). The peroxide value was not detected due to the addition of antioxidant, which inhibited the oxidation-reduction reactions used to determine this parameter. In general, the recommendation of peroxide value for human consumption is of 8 meq Kg^{-1} oil (BORAN *et al*., 2006).
OLIVEIRA *et al*. (2016) also observed similar results for AI of crude and refined tuna by-product oils. These authors observed a reduction of 85% in AI, whereas a higher reduction of 91% was obtained in the present study (Table 2). Moreover, the AI of refined fish oil is in accordance to other fish oils, *e.g.* Nile tilapia (0.08 mg NaOH g^{-1}) and hybrid sorubim (0.03 mg NaOH g^{-1}) (MENEGAZZO *et al*., 2014). Regarding IV results, they indicated that the unsaturated compounds were preserved during the process, which was also observed in other study with Nile tilapia and hybrid sorubim oils (MENEGAZZO *et al*., 2014). In general, IV of both samples are in accordance to the value reported by ACKMAN (1966) for oils from marine animals, varying from 110 to 193 g I_2 g^{-1}. MENEGAZZO *et al*. (2014)

3.2. Determination of the lipase activity

The lipase activity for the enzyme *Candida rugosa* Amano AY was 1.78 U mL^{-1} (35.7 U g^{-1}) according to SUGIHARA *et al*. (1990). Two experiments were carried out to verify the lipolytic activity. The relation among the enzyme concentration and the lipolytic activity was similar to the value supplied by the manufacturer, which is 30,000 U g^{-1} at the enzyme concentration of 2.5×10^{-4} g mL^{-1}.

3.3. Enzymatic hydrolysis

The results of enzymatic hydrolysis according to the experimental design are showed in Table 4. A decrease in the IV was observed with the temperature increased from 50 to 55ºC. This behavior is associated to the oxidation of unsaturated fatty acids present in the sample, reducing the IV.

Table 4. Factorial experimental design matrix and results for hydrolysis degree and iodine value for the hydrolyzed oil.

Experiment	Temperature (°C)	Time (h)	Hydrolysis degree (%)	Iodine value (g I_2 g^{-1})
1	-1 (45)	-1 (2)	22.79±0.12 [b]	121±0.19 [b]
2	-1 (45)	+1 (6)	23.45±0.15 [a]	120±0.20 [c]
3	+1 (55)	-1 (2)	14.65±0.10 [f]	179±0.13 [a]
4	+1 (55)	+1 (6)	20.4±0.09 [c]	107±0.17 [g]
5	0 (50)	0 (4)	22.63±0.18 [b]	117±0.12 [d]
6	0 (50)	0 (4)	18.90±0.11 [e]	113±0.22 [f]
7	0 (50)	0 (4)	21.00±0.12 [d]	114±0.15 [e]

*Different letters in the same line indicated a significant difference ($p<0.05$) by the Tukey test.

The experiment 2 showed the best results with hydrolysis degree (23.45%) and IV (120 g I_2 g^{-1}), whereas the other experiments showed minor values. According to those results, it was noted that the optimal temperature and hydrolysis time with *Candida rugosa* AmanoAY was at 45ºC and 6 h. Therefore, another experiment was realized to correlate hydrolysis degree and acidity index. Fig. 2 shows the hydrolysis results obtained with the optimal conditions (45°C and pH 7.0) for the enzyme *Candida rugosa* AY "Amano" 30 after 12 h, according to SUGIHARA *et al*. (1990).

According to Fig. 2, a sharp increase was observed in hydrolysis at the first 0.5 h (up to 14.1%). The maximal hydrolysis degree was obtained at 8 h (27.8%). In this meantime, the hydrolysis degree augmented at a relatively constant rate. After that, the values oscillated without great variability, showing that was not necessary to prolong the period of hydrolysis for a long time and that acidity index showed similar behavior, as expected.

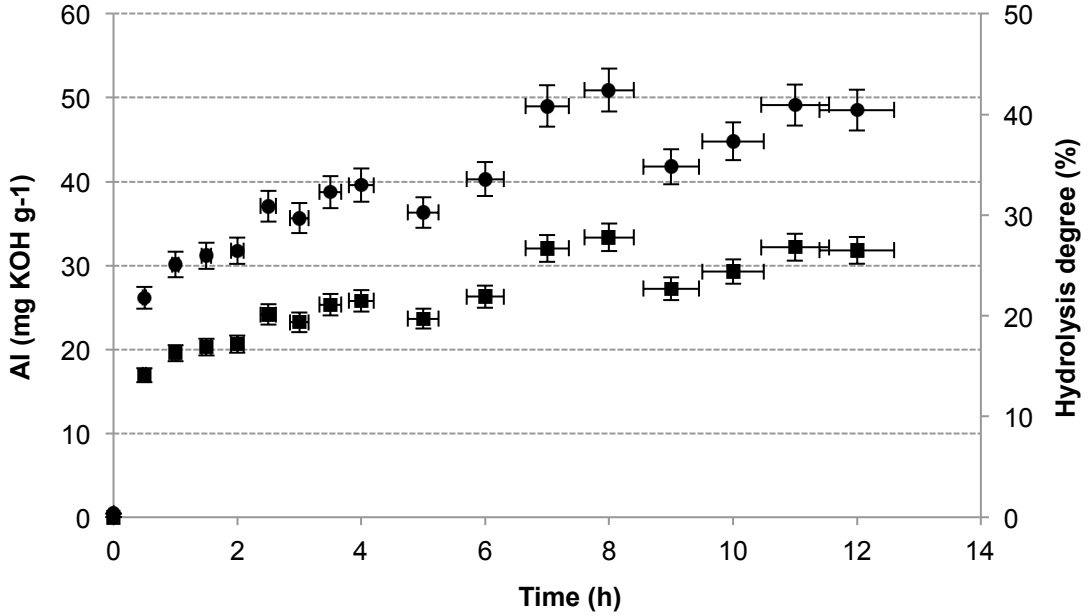

Figure 2. Kinetics of hydrolysis degree (■) (%) and acidity index (●) (mg KOH g^{-1} oil) of refined fish oil.

Other researchers also studied the enzymatic hydrolysis for extraction of unsaturated fatty acids from fish oil (IBERAHIM *et al*., 2018; BABAJAFARI *et al*., 2017). BABAJAFARI *et al*. (2017) compared enzymatic hydrolysis and chemical methods of oil extraction from rainbow trout. The chemical methods showed higher yield (16.58%) values than enzymatic hydrolysis (13.65% - 150ppm concentrated protease). However, the fatty acids composition showed that enzymatic hydrolysis concentrated the LC-PUFA (EPA and DHA), whereas the chemical methods concentrated MUFA and PUFA (linoleic acid and α-linolenic acid). Moreover, enzymatic hydrolysis is a safety method about food quality and environment-friendly extraction.

IBERAHIM *et al*. (2018) observed that catfish oil submitted to enzymatic hydrolysis showed no significant difference ($p<0.05$) in their fatty acids content, it means, MUFA content before hydrolysis was 48.41% and after 47.99%; PUFA content decreased from 20.32% to 19.32% after enzymatic hydrolysis by lipase. This behavior could be associated to oil auto-oxidation and photo-oxidation, since PUFA are more likely to undergo oxidation. PV and AI increased during the enzymatic hydrolysis, indicating the oil oxidation. Those results are similar to obtained in this study (Tables 3 and 4), since AI increased during the enzymatic hydrolysis process and IV indicated no change in fatty acids chains.

Despite results showed that hydrolysis of refine oil was affected by temperature and time (Table 4), no significant difference was observed between the IV of refined oil (Table 3) and hydrolyzed oil (Table 4). An increase in IV was expected with the hydrolysis process, since the enzyme has the ability to concentrate the unsaturated fatty acids. So, those results indicated that the hydrolysis process was not efficient. Thus, other process such as winterization (AMORIM *et al*., 2015) and ultrafiltration processes (KORIS *et al*., 2006) might be also evaluated to this kind of raw material to improve its nutritional quality, increasing the unsaturated fatty acids content.

4. CONCLUSIONS

The chemical refining yielded 62.75% and was efficient at removing undesirable compounds from crude fish oil, since the acidity index decreased by around 91% during the processing. The iodine value and saponification number of refined oil were not affected, ensuring its nutritional quality as a food ingredient. The best results of hydrolysis degree (23.45%) and iodine value (120 g I_2 g^{-1}) were obtained at 45ºC after 6 h of lipase action. However, this hydrolysis process was not efficient in concentrating the unsaturated fatty acids of the refined oil since no significant difference ($p>0.05$) was observed between the iodine value of the refined oil and the hydrolyzed oil. Thus, further research using this kind of residue is necessary in order to obtain isolated fatty acids (omega-3) or increasing the content of health beneficial fatty acids.

ACKNOWLEDGMENTS

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PAPER

THE EFFECT OF PEELING ON ANTIOXIDANT CAPACITY OF BLACK RADISH ROOT

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ABSTRACT

In this study, freeze-dried peeled and unpeeled root, as well as the juice from peeled and unpeeled root of black radish (*Raphanus sativus* L. var *niger*) cultivated in Mongolia were characterized for their DPPH· and $ABTS^{\cdot+}$ scavenging activity, reducing power, total phenolics, and flavonoids in order to evaluate the effect of the peel. The juice from the peeled root showed strong antioxidant potential may due to its high phenolic content. However, the ability of the dried unpeeled root extract to quench free radicals and reduce Fe^{3+} was higher than that of the dried peeled root extract.

Keywords: antioxidant capacity, black radish, peel, phenolic compounds, root

1. INTRODUCTION

Fruits and vegetables play a vital role in the prevention of degenerative diseases caused by oxidative stress and the improvement of general health as these contain vitamins, minerals, amino acids, dietary fibers, and phenolic compounds. For instance, the prevention of cancer and cardiovascular diseases has been strongly related to the intake of fresh fruits and vegetables rich in natural antioxidants. This suggests that a higher intake of such compounds will lower the risk of mortality from these diseases (WILLCOX *et al*., 2004).

Radish (*Raphanus sativus* Linn.) is an edible root vegetable of the *Brassicaceae* (*Cruciferae*) family with some other popular vegetables including white and red cabbage, broccoli, brussel sprouts, cauliflower, kohlrabi, rape, and mustard. Radish is originally from Europe and Asia. It grows in temperate climates at altitudes between 190 and 1240 m. It is 30-90 cm high and its roots are thick and of various sizes, forms, and colors (PEREZ GUTIERREZ and PEREZ, 2004). Radishes have different skin colors such as red, purple, through pink, black, yellow, and white, while its flesh is typically white (BANIHANI, 2017). The most popular varieties of the radish are red (*Raphanus sativus* L.), white (*Raphanus sativus* L. var *white*), and black (*Raphanus sativus* L. var *niger*). Among the people of Mongolia, black radish is less familiar than red and white radish. A few years ago, some Mongolians with diabetes firstly obtained the black radish root from Russia due to its positive effect on diabetic conditions. Furthermore, local farmers have been cultivating the black radish. It has a rough, black skin with hot-flavored. There are round and elongated varieties. Radishes are grown all over the world and mostly eaten raw as a crunchy vegetable, mainly in salads. These can be also brined, fermented (pickled), dried, and cooked in soups or stews. Recently, some people prefer to drink its juice due to its certain health benefits such as antioxidant, anti-microbial, anti-viral, anti-inflammatory, anti-tumorigenic, anti-mutagenic, anti-diabetic, anti-proliferative, hypocholesterolemic, antilithiasic, diuretic, nephroprotective, gastroprotective, and hepatoprotective. It is also very well known for its use in the treatment of bronchitis, diarrhea, gynecological disorders, and jaundice (JANJUA *et al*., 2013, AGARWAL and VARMA, 2014, BANIHANI, 2017, KUMAR and PATWA, 2018). In general, radish contains carbohydrates, sugars, dietary fibers, protein, various water-soluble vitamins (B_1, B_2, B_3, B_5, B_6, B_9, and C), and minerals (calcium, iron, magnesium, manganese, zinc, potassium, phosphorus, and fluoride). In addition to flavonoids, alkaloids, tannins, and phenolic compounds, the radish was found to have unique bioactive phytochemicals including glucosinolates and isothiocyanates (WANG *et al*., 2010, LUGASI and HOVARI, 2000, BANIHANI, 2017). Isothiocyanates are breakdown products resulting from the enzymatic hydrolysis of glucosinolates by the myrosinase. For instance, the most abundant glucosinolates in black radish and its juices are glucoraphasatin and glucoraphanin. These two glucosinolates and their degradation products (raphasatin and sulforaphane) are known for their antioxidant properties, which have been related to cancer and cholesterol gallstones prevention (CASTRO-TORRES *et al*., 2014, SARIKAMIS *et al*., 2015). In addition, radishes have a specific flavor and strong taste due to glucosinolates and their breakdown products.

BORS *et al*. (2015) investigated the influence of the variety and vegetative stage on the total phenolic content and antioxidant capacity of radish. Significant differences were found in total phenolic content between radish varieties; the highest level was noticed in black radish, followed by red and white radish. Black and red radish had similar and significantly higher antioxidant capacity than white radish. Regarding the vegetative stage, the highest total phenolic content and antioxidant capacity were found in sprouts,

followed by seeds and roots. A positive and highly significant correlation (r^2=0.939) was determined between total phenolic content and antioxidant capacity of the radish varieties. LUGASI *et al*. (1998) reported that squeezed juice from black radish root exhibited strong hydrogen-donating ability, reducing power, copper (II)-chelating property and showed marked free radical (H_2O_2/·OH) scavenging activity. In their study, only one glucosinolate, namely glucotropaeolin, was detected in the juice by the HPLC analysis. In addition, a significant amount of polyphenols was detected in the juice. Therefore, the authors supposed that the degradation products of glucosinolates and the polyphenolic compounds are the main biologically active components of the sample. Several physiologically active compounds such as isothiocyanates, thiocyanates, indoles, nitriles, epithionitriles, cyano-epithioalkanes, oxazolidine–2–thiones, are released from hydrolysis of glucosinolates by the enzyme myrosinase (LUGASI *et al*., 1998, CASTRO-TORRES *et al*., 2014, BANIHANI, 2017).

In a study by NIKOLIC *et al*. (2012), the phenolic compounds content and DPPH radical scavenging capacity of black radish depended on root size in such a way that bigger root had a higher content of phenolic compounds and higher scavenging capacity. They also found a positive correlation between the phenolic compound content and radical scavenging capacity. Therefore, black radish roots with a weight higher than 100 g are recommended for human nutrition and health benefits. JANJUA *et al*. (2013) analyzed five different extracts of black radish root peel for important 14 phytochemicals, namely tannins, saponins, flavonoids, phlobatannins, anthraquinones, carbohydrates, reducing sugars, steroids, phytosterol, alkaloids, amino acids, terpenoids, cardiac glycosides, and chalcones. According to the proximate analysis, the black radish root peel contained 7% moisture and 93% dry matter which was composed of crude protein (28.57%), fats (27.76%) and carbohydrates (39.82%), while fibers were only 1.4% and ash content was 2.43%.

The objective of this study was to evaluate antioxidant activity and content of polyphenolic compounds in the black radish root cultivated in Mongolia. The antioxidant activities of the black radish root samples were investigated by using three different assays, namely DPPH, ABTS, and FRAP. Total phenolic content and total flavonoid content were also determined by spectrophotometrically.

2. MATERIALS AND METHODS

2.1. Chemicals and apparatus

Folin-Ciocalteu reagent, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,4,6-tripyridyl-*S*-triazine (TPTZ), gallic acid, quercetin, l-ascorbic acid, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (MO USA). All other chemicals and reagents were of analytical grade from local suppliers in Mongolia. The water used was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Spectrophotometric determinations were carried out using a Shimadzu UV mini 1240 spectrophotometer.

2.2. Sample collection

Fresh black radish roots were purchased from a local produce market in Ulaanbaatar, Mongolia during the period September-October 2018. The radish roots were washed thoroughly under cold running tap water to remove surface impurities as well as to lower microbial load, spread on filter paper, cut-off their crown and tail. Afterward, the roots were halved with a stainless steel knife and separated into two parts. One part was peeled off with the knife. The other was processed with peel.

2.3. Sample preparation

Black radish root juice was obtained with the help of a laboratory-scale juice processor and then filtered using a sterilized muslin cloth. The obtained juice was stored at refrigeration temperature (4°C) and analyzed within 2 days. To obtain dried samples, the peeled and unpeeled black radish roots were sliced and freeze-dried for avoiding the loss of their juice rich in biologically active ingredients. The thoroughly dried samples were ground separately to a fine powder in a laboratory mill and then sifted through a mesh 0.5 mm in size. The powdered samples were stored in air-tight containers at 4°C until further use. To assay for antioxidant activity the powdered samples (1 g) were extracted with 50% (v/v) aqueous ethanol (50 mL) on a magnetic stirrer for 120 min at room temperature and centrifuged at 5000 rpm for 10 min at 4°C. The extracts were filtered with a Whatman No.5 filter paper and kept in dark at 4°C for further analysis.

2.4. DPPH free radical scavenging assay

$DPPH^{\cdot}$ scavenging ability of the samples was determined as described by ADEDAPO *et al*. (2009) with a minor modification. Briefly, 2 mL of 0.135 mM DPPH in ethanol (99.7%) were mixed with 100 μL of the sample solution. After 30 min in the dark, absorbance was measured at 517 nm against a blank (ethanol was used instead of the sample solution). Lower absorbance indicates higher scavenging activity. Percentage inhibition was calculated by comparing the absorbance of the test sample and the blank. Results were also expressed as Trolox equivalents (TE) by using a calibration curve (r^2=0.9973) of Trolox (0-300 μM).

2.5. ABTS radical cation ($ABTS^{\cdot+}$) scavenging assay

To evaluate $ABTS^{\cdot+}$ scavenging ability of the samples, the method of RE *et al*. (1999) was adopted. Firstly, to produce a stable $ABTS^{\cdot+}$ an aqueous solution of 7 mM ABTS was oxidized with 2.45 mM potassium persulfate for 12-16 h in the dark at room temperature. Before analysis, the $ABTS^{\cdot+}$ solution was diluted with distilled water to an absorbance of 0.75±0.05 at 734 nm. A 2 mL of $ABTS^{\cdot+}$ solution was mixed with 20 μL of the sample solution and absorbance was read at 734 nm exactly after 7 min. The percentage of $ABTS^{\cdot+}$ decolorization was calculated by comparing the absorbance of the test sample and the blank. The blank was prepared by replacing the sample solution with distilled water. The TE against $ABTS^{\cdot+}$ was also calculated using the calibration curve (r^2=0.9993) constructed with the standard Trolox (0.1-1.5 mM) under the same experimental conditions.

2.6. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay, a method for measuring total reducing power of electron-donating substances, was performed as previously described (BENZEI and STRAIN, 1996) with a slight modification. FRAP reagent containing 5 mL of 2,4,6-tripyridyl-*S*-triazine (TPTZ, 10 mM) in 40 mM HCl, 5 mL of ferric chloride hexahydrate (20 mM), and 50 mL of acetate buffer (300 mM, pH 3.6) was freshly prepared and warmed at 37°C before use. Sample solutions (100 μL) were allowed to react with 3 mL of the FRAP reagent for 30 min in the dark. Absorbance of the colored product was measured at 593 nm. A higher absorbance of the reaction mixture indicates greater reducing power. Aqueous solutions of ascorbic acid (0-0.1 mg/mL) were used for calibration (r^2=0.9998). The values were expressed as the concentration of ascorbic acid (vitamin C) that is most effective natural antioxidant having the ferric reducing ability.

2.7. Determination of total phenolic content

The total phenolic content of the black radish root samples was evaluated using the Folin-Ciocalteu colorimetric method (SINGLETON *et al*., 1999). In a test tube, each 20 μL of sample solution was mixed with 1.58 mL of distilled water and 100 μL of 1.8 N Folin-Ciocalteu phenol reagent and allowed to stand for 5 min. Then 300 μL of 20% aqueous sodium carbonate solution was added and shaken vigorously by a vortex. After incubation at room temperature for 2 h in the dark, the absorbance of the green-blue complex was measured at 765 nm. The results of total phenolic compounds were expressed as gallic acid equivalents (GAE), based on a calibration curve (r^2 = 0.9996) of gallic acid in the concentration range of 0.1 to 1.0 mg/mL.

2.8. Determination of total flavonoid content

Estimation of the total flavonoids in the black radish root samples was carried out using the procedure reported by ADEDAPO *et al*. (2009). Equal volume of sample solution and 2% anhydrous aluminium chloride in 50% (v/v) ethanol were mixed well and after 1 h at room temperature absorbance was measured at 420 nm. A yellow color indicates the presence of flavonoids. The interference background of the sample solution was corrected by preparing the test without aluminium chloride. The total flavonoid content was estimated from a calibration curve (r^2=0.9986) plotted at 420 nm with quercetin (10-50 μg/mL) and expressed as quercetin equivalents (QE).

2.9. Statistical analysis

All the measurements were taken five times and expressed as mean value ± standard deviation. The data were analyzed using one-way ANOVA for mean differences. Statistical significance was declared at $p < 0.05$.

3. RESULTS AND DISCUSSION

BORS *et al*. (2015) investigated the influence of the variety and vegetative stage on the total phenolic content and antioxidant capacity of radish. NIKOLIC *et al*. (2012) examined the

influence of black radish root size on the content and radical scavenging capacity of phenolic compounds. In this study, we evaluated the effect of the peel on the antioxidant activity of the juice and the ethanol extract prepared from black radish root cultivated in Mongolia. The juice obtained from black radish root and the ethanol extract of the freeze-dried root showed potent antioxidant activity in different test systems and contained a significant amount of polyphenols. Peeling increased the antioxidant capacity of black radish root juice significantly ($p<0.05$). On the other hand, the ethanol extract of the dried peeled and unpeeled root exerted statistically similar antioxidant potential.

3.1. DPPH· scavenging activity

It is well known that the DPPH· scavenging ability is attributed to the hydrogen donating ability of phytocompounds. A comparison of DPPH· scavenging abilities of the black radish root samples is shown in Fig. 1. Trolox, a synthetic water-soluble antioxidant analogue of vitamin E, was used as a reference compound, and DPPH· scavenging potentials expressed as TE of the studied samples were listed in Table 1. The black radish root juice were examined for their DPPH· scavenging ability after dilution by 10 times with distilled water. The strong DPPH· scavenging capacity of 60.9% was detected in the juice from peeled root, while the juice from unpeeled root exerted weak DPPH· scavenging capacity of 21.9% (Fig. 1), which reflects approximately 2.8-fold difference. However, DPPH· scavenging activities of the peeled and unpeeled root extracts were comparable to each other. At a concentration of 50 mg/mL, the percentage scavenging of the peeled root extract was 61.8% whereas that of the unpeeled root extract was 65.7%. The black radish root extracts quenched DPPH· with a mean of 414 μmol TE/100 g dry weight (Table 1).

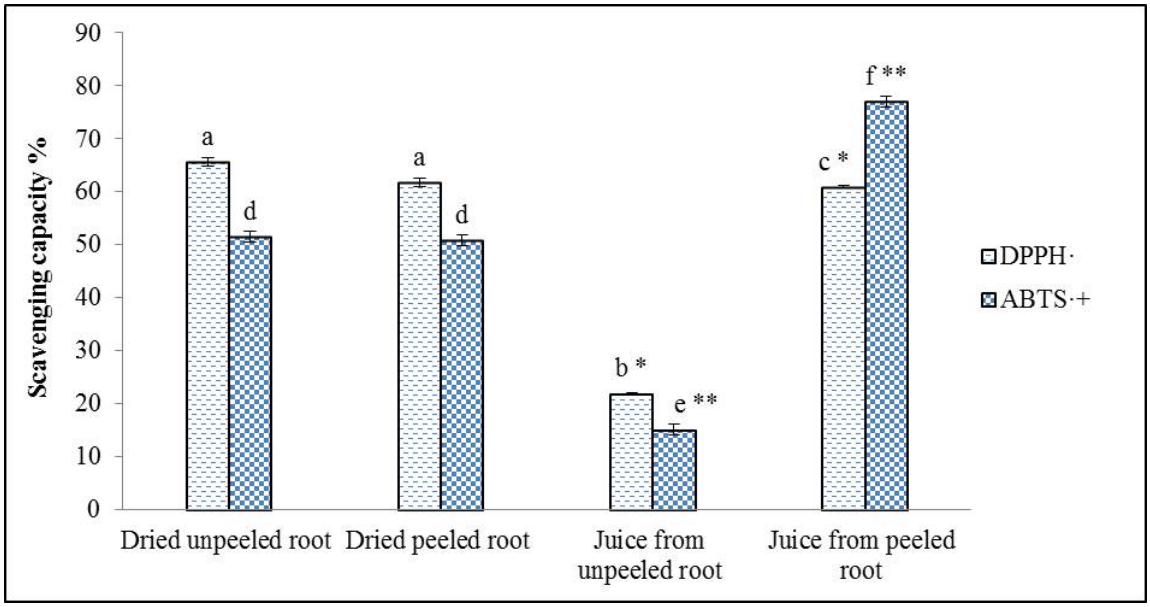

Figure 1. DPPH· and ABTS·· scavenging activity of the black radish root samples (%). Values are mean ± standard deviations of five parallel determinations. The vertical bars represent the standard deviations for each data point. Values with different superscript letters are significantly different ($p < 0.05$). *- diluted 10 times; **- diluted 5 times.

There are some reports describing DPPH· scavenging activity of black radish root and its juice, but the results varies depending on the experimental condition. For instance, DPPH· scavenging capacity of 80% (v/v) ethanol extract from black radish root ranged from 88.3% to 55.6% at a concentration of 5.5 mg/ml depending on the root size and the appropriate IC_{50} values were 1.59 and 3.54 mg/mL, respectively (NIKOLIC *et al*., 2012). According to the BORS *et al*. (2015), black radish root showed weak activity (12.23%) to scavenge DPPH· at the relatively high concentration (the dry extract prepared from 500 mg freeze-dried powder was recovered in 3 mL methanol). The squeezed juice from black radish has a significant scavenging activity to quench DPPH· with an IC_{50} value of 0.54 mL (LUGASI *et al*., 1998).

3.2. $ABTS^{\cdot+}$ scavenging activity

The antioxidant capacity of the samples was evaluated by $ABTS^{\cdot+}$ assay because proton radical scavenging is an important attribute of antioxidants. $ABTS^{\cdot+}$, a protonated radical, is reduced in the presence of hydrogen-donating antioxidants. Besides being one of the fastest, $ABTS^{\cdot+}$ method also provides good solubility, which allows the analyses of both lipophilic and hydrophilic compounds (RE *et al*., 1999). Fig. 1 showed the percentage inhibition of $ABTS^{\cdot+}$ by the studied samples, whereas Table 1 presented the scavenging abilities expressed as TE. The black radish root juice diluted previously 5 times with distilled water was analyzed by $ABTS^{\cdot+}$ assay. The juice from the peeled black radish root quenched 76.9% of $ABTS^{\cdot+}$ in the reaction mixture (Fig. 1), which is about 5.2-fold greater than that found in the juice obtained from the unpeeled root (14.9%). The same as the DPPH· scavenging activity, peeled and unpeeled root extracts showed similar ability to scavenge $ABTS^{\cdot+}$ with a mean of 1510.7 μmol TE per 100g dry weight (Table 1). At 50 mg/mL, the scavenging percentages were 50.7% and 51.4% for the peeled and unpeeled black radish root extracts, respectively (Fig. 1). To our best knowledge, there is almost no data on $ABTS^{\cdot+}$ scavenging potential of black radish root samples.

3.3. Ferric reducing antioxidant power

The reducing power of the black radish root samples was analyzed using the FRAP assay by comparing with reducing power of ascorbic acid (Table 1). The formation of blue-colored TPTZ–Fe^{2+} complex from colorless oxidized TPTZ–Fe^{3+} by the action of the electron-donating antioxidants under acidic condition (pH 3.6) was recorded at 593 nm. The reducing ability of the plant samples could be attributed to the number of hydroxyl groups in the phenolic and flavonoid compounds. The studied samples from black radish root intensively reduced Fe^{3+} to Fe^{2+}. It was found that 1 g (dry weight) of black radish root and 1 mL of its juice showed reducing power equal to that estimated in 3.5-3.7 and 0.8-1.0 mg of pure ascorbic acid, respectively. In a study by LUGASI *et al*. (1998), the reducing effect of 1 mL of black radish root juice was the same as that of 0.73 μmol ascorbic acid. It is equal to 0.13 mg ascorbic acid per 1 mL of the juice. According to HANLON and BARNES, the reducing power of the freeze-dried unpeeled root of black radish (Nero Tondo variety) was 23.7 μmol ascorbic acid/g which is equal to 4.17 mg ascorbic acid/g. It was comparable to our result.

Similarly to DPPH· and ABTS·+ scavenging ability, the dried unpeeled root extract and the juice obtained from peeled root showed higher reducing power. However, the margin of difference between reducing power of the juice obtained from the peeled and unpeeled root was narrow. The results indicated that phytoconstituents in black radish are capable of donating electrons that can react with free radicals to convert them into more stable compounds and reduce the oxidized intermediates of lipid peroxidation process.

Table 1. Antioxidant potential of the black radish root samples.

Sample	DPPH· scavenging activity	ABTS·+ scavenging activity	Fe^{3+} reducing power
Dried peeled root	401.47±5.54[1]	1500.45±21.24[1]	349.59±4.24[3]
Dried unpeeled root	426.52±4.91[1]	1520.95±109.58[1]	372.23±6.53[3]
Juice from peeled root	198.05±0.83[2]	569.66±4.41[2]	100.49±4.95[4]
Juice from unpeeled root	71.98±0.43[2]	109.36±1.16[2]	83.90±1.46[4]

[1]Expressed as μmol Trolox equivalents/100g.
[2]Expressed as μmol Trolox equivalents/100mL.
[3]Expressed as mg ascorbic acid/100g.
[4]Expressed as mg ascorbic acid/100mL.
Values are mean ± standard deviations of five parallel determinations.

3.4. Total phenolic content

Plant polyphenols are the significant group of compounds acting as free radical scavenging or primary antioxidants; therefore, it is justifiable to determine phenolic content in the plant extract. These can also chelate metal ions that could catalyze the formation of reactive oxygen species, which promotes lipid peroxidation (IQBAL *et al*., 2015). In many studies, phenolic compounds have demonstrated higher antioxidant activity than antioxidant vitamins and carotenoids (VELIOGLU *et al*., 1998). Total phenolic contents (expressed as GAE) in the black radish root samples are given in Table 2. The amount of total phenolics in the juice ranged from 103 to 146 mg/100mL. LUGASI *et al*. (1998) detected by the Folin-Denis method relatively low polyphenol content (25.5 mg/100 mL juice) in black radish root juice. In the case of the dried black radish root, total phenolic content was in the range of 750-791 mg/100 g (7.5-7.9 mg/g) dry weight. The lower level of total phenolic content was obtained by BORS *et al*. (2015) in black radish root (4.75 mg of GAE/g dry weight) and by HANLON and BARNES (2011) in the freeze-dried unpeeled root of the Nero Tondo black radish (2.4 μg of GAE/g). Total phenolic content in the unpeeled root was higher than those of the peeled root. These results revealed that black radish root skin contained a considerable amount of phenolic ingredients. However, the juice obtained from the unpeeled root contained lower amount of total phenolics compared to the peeled root juice. It indicated that the phenolic constituents present in the peel could not be extracted into the juice or these substances may be degraded during processing and storage by the action of any factors such as an enzyme. JANJUA *et al*. (2013) reported that phytochemicals of black radish root peel were not well dissolved in water as well as methanol. Moreover, the lowest yield was of water extract.

3.5. Total flavonoid content

Flavonoids exert potent antioxidant activity by several different mechanisms, such as scavenging of free radicals, chelation of metal ions, and inhibition of enzymes responsible for free radical generation. Depending on their structure, flavonoids are able to scavenge practically all known reactive oxygen species (PANDA, 2012). LUGASI and HOVARI (2000) demonstrated that radish contains a significant amount of flavonoids such as kaempferol, quercetin, myricetin, apigenin, and luteolin. Before that, some researchers observed 7 mg/kg (0.7 mg/100g) flavonoids including quercetin and myricetin in the radish root (LUGASI *et al*., 1998).
The amount of total flavonoid content in the black radish root samples was assessed by the aluminium chloride assay. Total flavonoid content in the juice prepared from the peeled root was 0.81 mg QE/100 mL, while total flavonoids were not detected in the juice from unpeeled root (Table 2). Dried peeled and unpeeled root of black radish contained comparable amounts of total flavonoids with a mean of 15.8 mg QE per 100 g of dry weight.

Table 2. Total phenolics and flavonoids of the black radish root samples.

Sample	Total phenolic content	Total flavonoid content
Dried peeled root	749.60±14.59[1]	16.00±0.04[3]
Dried unpeeled root	791.20±46.71[1]	15.69±0.09[3]
Juice from peeled root	146.00±3.42[2]	0.81±0.00[4]
Juice from unpeeled root	103.10±2.28[2]	–

[1]Expressed as mg gallic acid equivalents/100g.
[2]Expressed as mg gallic acid equivalents/100mL.
[3]Expressed as mg quercetin equivalents/100g.
[4]Expressed as mg quercetin equivalents/100mL.
Values are mean ± standard deviations of five parallel determinations.

4. CONCLUSIONS

The juice obtained from the peeled and unpeeled root showed considerable differences in antioxidant activity when it was calculated by the three different methods used in this study. The juice prepared from the peeled root showed strong antioxidant potential may due to its high phenolic content. However, the juice from the unpeeled root contained a relatively low amount of total phenolics and had no flavonoid content. It was supposed that the phenolic constituents including flavonoids present in the peel could not be extracted into the juice or these substances may be degraded. Although dried black radish root samples contained significantly different amounts of total phenolics, they showed statistically similar free radical scavenging activities and reducing power. Thus, the antioxidant activities of the dried root samples are probably from the combined action of phenolics and other constituents. The results of this study show that the root of black radish cultivated in Mongolia contained a significant amount of biologically active phenolics with antioxidant properties. Thus, the black radish root may serve as a potential source in functional food development.

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SHORT COMMUNICATION

OCCURRENCE OF DEOXYNIVALENOL IN BEERS COMMERCIALISED IN ITALY

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ABSTRACT

Deoxynivalenol (DON) is the most frequently detected mycotoxin in beer. This study represents a comprehensive assessment of DON occurrence in beers from the Italian market. Seventy-two craft and industrial beer samples were tested using the RIDASCREEN DON® ELISA method. DON was found in all samples. The average DON contamination was 34.3 μg/L (range: 6.1 - 111.2 μg/L). The highest contamination level was found in a wheat-based sample. Our study determined that wheat-based beers have a higher DON contamination than barley-based beers. Further studies are needed to verify the role of single ingredients on the risk of DON accumulation in beers.

Keywords: consumption level, ELISA, fermented beverage, food safety, mycotoxins, wheat

1. INTRODUCTION

Deoxynivalenol (DON) is one of the most frequently detected mycotoxins in cereals and cereal-based products (PEDROSO PEREIRA *et al.*, 2019). DON belongs to type-B trichothecenes mycotoxins and it is mainly produced by *Fusarium graminearum* and *Fusarium culmorum* (PASQUALI *et al.*, 2016). Type-B trichothecenes involve DON, its acetylated derivatives (3-Acetyl-DON and 15-Acetyl-DON) nivalenol, and deoxynivalenol-3-glucoside (D3G). D3G might be the result of plant metabolism of DON or of some food processing operations (BERTHILLER *et al.*, 2013). Although DON and its derivatives are not classified as being carcinogenic to humans, an exposure to this type of mycotoxins may be associated with a wide range of adverse health complications (EFSA CONTAM PANEL, 2017). The main effect of deoxynivalenol is the inhibition of protein synthesis. This leads to acute gastro-intestinal symptoms (e.g. emesis and diarrhoea) as well as, in case of long-term exposure, to immune system diseases or disorders (PEDROSO PEREIRA *et al.*, 2019). Considering its potential to cause serious health issues, the European Commission established a range of maximum limits for DON in cereals and cereal-based foods (FERRIGO et al., 2016). In 2006, the European Commission proposed the maximum level for DON in cereals intended for direct human consumption of 750 μg/kg (EFSA CONTAM PANEL, 2017). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has also established a provisional maximum tolerable daily intake (PMTDI) of 1 μg/kg body weight for the sum of DON and its derivatives (PEDROSO PEREIRA *et al.*, 2019). Since DON is largely detected in cereals and malt, which represent the key ingredients of beer, a DON contamination of this type of alcoholic beverages seems unavoidable (RODRÍGUEZ-CARRASCO *et al.*, 2015). It is worth specifying that maximum levels for DON in beer have not been set so far. Monitoring DON in beer is important considering that this widely popular fermented beverage may significantly contribute to the intake of mycotoxins and even exceed the safety levels when following a regular diet (PAPADOPOULOU-BOURAOUI *et al.*, 2004; RODRÍGUEZ-CARRASCO *et al.*, 2015). Therefore, exposure of consumers to DON and its derivatives through beer consumption should not be underestimated, especially in case of "heavy drinkers" (PASCARI *et al.*, 2018).

The occurrence of deoxynivalenol in beer has been studied in various surveys all around the world: in Belgium (PAPADOPOULOU-BOURAOUI *et al.*, 2004), in Poland (KUZDRALIŃSKI *et al.*, 2013), in Austria, Hungary, Croatia and Serbia (VARGA *et al.*, 2013), in Spain (RODRÍGUEZ-CARRASCO *et al.*, 2015), in Brazil (PIACENTINI *et al.*, 2015), in Germany (BAUER *et al.*, 2016), in Paraguay (ARRÚA *et al.*, 2019) and in Mexico (WALL-MARTÍNEZ *et al.*, 2019 b). Occurrence of DON in beers from the Italian market was assessed in two surveys with limited number of samples: by PIETRI *et al*. (2003) and by PETERS *et al.*, (2017). The surveys showed that the small number of Italian samples analysed, compared with samples produced in other countries, had the lowest DON contamination levels (below than 10 μg/L).

In 2018 the Italian beer consumption increased by 3.2%. Therefore, per capita consumption reached its historical peak of 33.6 L a year (ASSOBIRRA, 2018).

Considering both the limited number of surveys focusing on the occurrence of DON in beers purchased from the Italian market and the significant raise of Italian craft breweries the aims of this work were:

1. to assess the level of deoxynivalenol in beer samples sold on the Italian market from May 2018 to December 2018 in order to update and enrich the available data;

2. to compare the contamination of industrial beers to craft beers, taking into account the findings of PETERS *et al.*, 2017 who identified higher DON incidence in craft beers collected all over Europe;
3. to define whether wheat-based beers had higher DON contamination compared to barley based beers.

2. MATERIALS AND METHODS

Seventy-two beer samples were purchased from May to December 2018 in pubs, supermarkets and beer shops located in the North of Italy. Some of them were home brewed from semi-processed products. Most samples (53) were produced by Italian companies whereas nineteen samples came from different European countries. The foreign beers were produced in Germany (8), Belgium (5), Austria (1), Czech Republic (1), France (1), Netherlands (1), Sweden (1) and United Kingdom (1). None of the beers exceeded their expiration date. 1.5 mL of each sample were placed in separate test tubes and stored at -80°C for at least 24 hours, in order to reach a complete degassed condition. 50 μL of CO_2-free samples were subjected to the analysis. The commercial competitive ELISA RIDASCREEN DON® (R-Biopharm AG, Germany) was used. The declared detection limit of RIDASCREEN DON® is 3.7 ppb for beer samples, with a cross-reactivity to DON (100%), 3-Acetyldeoxynivalenol (>100%), 15-Acetyldeoxynivalenol (approximatively 19%), Nivalenol (approximatively 4%), Fusarenon-X (<1%) and T-2 Toxin (<1%). All reagents required for the analysis – including standards – were contained in the kit. The PBS-Tween washing buffer was prepared by dissolving the provided salt in Milli-Q® ultra-pure water. The test procedure issued by the producer was strictly followed.
Results were obtained by reading sample or standard absorbances at 450 nm using a Synergy (H1) microplate reader (BioTek®, US) spectrophotometer. The absorbance was inversely proportional to the DON concentration in the samples. Absorbance was expressed as a percentage value with respect to the zero standard (100 × (absorbance sample or standard)/(absorbance zero standard)). The values calculated for the standards were entered in a system of coordinates on a semilogarithmic graph against the DON concentrations (expressed in μg/L) using the online editor Line of Best Fit Generator "plot.ly" (PLOTLY TECHNOLOGIES, 2015). This allowed obtaining a calibration curve from which the DON concentration, actually contained in all the samples, was defined. All samples were measured at least twice in each analysis. Samples resulted off the chart were diluted using ultra-pure water, in ratio 1:2 and 1:5 and then reanalysed.
The pH of each sample was also measured using an XS Instruments® benchtop pHmeter supplied with an automatic temperature compensation and a microelectrode that was fit for the low-volume samples.
The statistical analysis was carried out using the statistical software JASP Version 0.11.1 (JASP TEAM, 2019). A linear regression was performed to assess relationship among DON, pH and alcohol content values. Furthermore, analysis of variance (ANOVA) followed by Bonferroni post hoc test was performed in order to determine the significance of fixed factors "wheat", "type of brewing process" and "type of fermentation" on the DON concentration. All tests were executed at a significance level of $P<0.05$.

3. RESULTS

With reference to the whole collection of beer samples, 46 samples were classified as "craft beer" whereas 26 samples were classified as "industrial produced beer". Wheat was one of the ingredients in 21 samples. Three of the selected samples were "gluten free" beers. The results of the analysis are summarised in Table 1.

Table 1. Samples description and results of the analysis.

Sample	Country of origin	[1]%ABV	Type of fermentation	Type of brewing process	Wheat	Special features	pH	[2]DON (*μg/L*)
B1	Germany	7.5	bottom	industrial			4.66	46.9±7.2
B2	Germany	5.3	top	industrial			4.36	71.5±15.7
B3	Germany	5.4	top	industrial			4.40	35.6±3.3
B4	Germany	5	bottom	industrial			4.48	25.7±8.4
B5	Germany	4.9	bottom	industrial			4.49	43.3±9.6
B6	Germany	5	top	industrial	yes		4.47	32.1±7.7
B7	Germany	5	bottom	industrial			4.50	57.2±19.5
B8	Italy	4.7	top	industrial			4.58	17.2±0.5
B9	Italy	6.5	top	craft	yes		4.51	45.0±2.9
B10	Italy	5.5	top	craft			4.57	55.9±14.6
B11	Italy	4.5	top	craft	yes		4.31	54.3±13.5
B12	Italy	5	top	craft	yes		3.82	61.4±5.4
B13	Italy	6.9	top	craft	yes		4.50	49.3±12.9
B14	Italy	5.2	top	craft	yes		4.64	25.8±0.8
B15	Italy	7.8	top	craft			4.96	54.8±7.1
B16	Italy	5.6	top	craft			4.58	50.5±15.6
B17	Italy	3.9	top	craft			4.17	18.1±2.7
B18	Italy	5	bottom	industrial			4.93	18.6±10.2
B19	Italy	4.5	bottom	craft			4.43	19.9±7.3
B20	Italy	5	bottom	craft	yes		4.64	44.5±17.8
B21	Italy	6	top	craft			4.64	18.4±2.1
B22	Italy	4.5	top	craft		gluten free	4.50	17.8±8.8
B23	Italy	5.6	top	craft	yes		4.46	20.7±0.6
B24	Italy	5.6	top	craft			4.36	13.1±2.1
B25	Italy	4.7	bottom	industrial			4.39	10.8±2.0
B26	Italy	4.7	bottom	industrial		gluten free	4.66	6.1±0.1
B27	Italy	5.5	bottom	industrial			4.60	20.0±0.8
B28	Italy	4.5	bottom	industrial			4.59	16.1±1.2
B29	Italy	4.7	bottom	industrial			4.44	15.7±1.4
B30	Italy	5	bottom	industrial			4.72	22.7±0.7
B31	Italy	5.5	top	craft		gluten free	4.53	13.1±1.7
B32	Italy	7	top	craft			4.31	95.8±5.7
B33	Italy	5	top	craft			4.41	31.9±1.0
B34	Italy	0.49	n.d.	industrial		alcohol-free	4.83	9.5±0.4

B35	Italy	5	top	craft	yes	4.05	65.9±12.2
B36	Belgium	4.9	top	industrial	yes	4.46	60.8±6.8
B37	Italy	5	top	industrial	yes	4.19	76.3±9.6
B38	Italy	5.1	bottom	industrial		4.22	12.0±0.8
B39	Belgium	8	top	craft	yes	4.40	27.8±4.0
B40	Italy	5.2	bottom	craft		4.80	10.7±0.5
B41	Italy	4.6	bottom	craft		4.55	22.3±3.2
B42	Belgium	7.5	top	industrial	yes	4.77	50.7±7.1
B43	Germany	12	top	industrial	yes	4.73	56.3±9.6
B44	Italy	8	top	industrial		4.62	36.4±7.9
B45	Italy	8	bottom	craft		4.70	80.3±2.1
B46	Italy	7.5	bottom	industrial		4.92	19.1±0.8
B47	Nether-lands	6.5	top	craft	yes	4.35	37.6±15.3
B48	United Kingdom	4.6	bottom	craft		4.55	9.3±0.6
B49	Italy	5.4	bottom	craft		4.56	54.5±9.8
B50	Italy	5	bottom	craft		4.73	18.9±1.8
B51	Belgium	9.5	top	craft		4.36	24.3±8.4
B52	Italy	8	top	craft		4.80	24.1±14.1
B53	France	5.5	bottom	industrial		4.43	12.7±3.1
B54	Italy	9	top	craft		4.67	10.1±0.2
B55	Czech Republic	4.4	bottom	industrial		4.69	15.0±0.7
B56	Belgium	6.5	spontaneous	craft	yes	3.45	111.2±14.1
B57	Italy	6	top	craft		4.66	10.3±1.3
B58	Sweden	5	bottom	craft	yes	4.55	23.3±3.2
B59	Italy	9	top	craft		4.53	45.0±6.2
B60	Italy	4.9	top	craft		4.56	50.5±15.7
B61	Italy	4.9	bottom	craft		4.79	48.5±8.2
B62	Italy	9.7	top	craft		4.80	82.6±13.8
B63	Italy	8.7	top	craft		4.60	35.2±5.1
B64	Italy	4.7	top	craft		4.78	7.5±1.2
B65	Italy	5.2	top	craft	yes	4.38	17.2±1.0
B66	Italy	4.5	bottom	craft		4.57	37.8±1.0
B67	Italy	8.5	top	craft		4.49	46.4±4.8
B68	Italy	4.6	top	craft	yes	4.31	25.5±2.2
B69	Italy	4.6	top	craft		4.39	28.9±2.8
B70	Italy	6.5	top	craft		3.98	35.0±6.9
B71	Italy	6.5	top	craft		4.77	19.1±4.9
B72	Italy	5.5	bottom	craft		4.51	20.5±5.6

[1]percentage alcohol by volume; [2]mean value±standard deviation.

DON contamination levels obtained by RIDASCREEN® DON ELISA

DON was found in all samples with a contamination incidence of 100%. The contamination ranged from 6.1 μg/L to 111.2 μg/L. The average contamination level was 34.3 μg/L, with a median of 25.8 μg/L. Only in 43.06% of samples (31) the DON contamination was greater than the average contamination level (34.3 μg/L). The highest contamination level (111.2 μg/L) was found in a sample that included wheat among the ingredients (Table 1).
pH values ranged from 3.45 to 4.96 with an average value of 4.51 and a median of 4.53.
The percentage alcohol by volume (%ABV) ranged from a minimum value below 0.5% (non-alcoholic beer sample) to a maximum of 12%. The average alcohol content was 5.8% with a median of 5.2%.
In order to assess the influence of the two variables pH and %ABV on DON contamination levels, a linear regression model was computed. Both %ABV and pH were partially correlated with samples DON content ($P>0.001$) but R^2 were negligible ($R^2 = 0.109$, $R^2 = 0.123$).
Analysis of Variance (ANOVA) was performed in order to determine the potential impact of both wheat (as ingredient) and the type of brewing process (industrial or craft beer) on DON content. The ANOVA revealed a positive effect of wheat as ingredient on DON contamination of the samples (*P* value = 0.001), with an average DON contamination of wheat-based beers of 46.6 μg/L (±23.1) and an average DON contamination of beers without wheat of 29.9 μg/L (±20.8). On the contrary, there was no difference between the types of brewing process on the final DON content (*P* value = 0.966).

4. CONCLUSIONS

This study represents the first comprehensive assessment of the DON level in beers sold on the Italian market. Moreover, it identifies wheat-based beers as potentially contributing to higher level of DON accumulation in consumers.
The screening results showed a weak correlation between the alcohol content (%ABV) and the DON contamination levels. Higher alcohol levels were related to significantly higher DON levels in beers by other researchers such as PAPADOPOULOU-BOURAOUI *et al.*, 2004; KOSTELANSKA *et al.*, 2009; PETERS *et al.*, 2017; KSIENIEWICZ-WOŹNIAK *et al.*, 2019; WALL-MARTÍNEZ *et al.*, 2019 b. The requirement of a higher input of fermentable sugars in malt wort, in order to reach higher alcohol levels, seems to be a possible explanation. Indeed, the further supplement of grains may be associated with a higher risk of mycotoxins contamination (KOSTELANSKA *et al.*, 2009; PETERS *et al.*, 2017; PASCARI *et al.*, 2018; WALL-MARTÍNEZ *et al.*, 2019 b).
A previous study (WALL-MARTÍNEZ *et al.*, 2019 a) did not find any correlation between pH and DON contamination values. However, in our study we observed a slightly negative correlation that suggests that higher beer pH is correlated to lower DON content. Further studies are needed to decipher this phenomenon. Given the minor pH diversity it is not possible to postulate that alkaline pH are the cause of decreased DON stability as it was found for baking products (YOUNG *et al.*, 1984). As it is known that the brewing process can include a pH correction before selling, our observations may not be associated to any specific processing of the beer.
According to our surveillance, wheat-based beers represent higher risk for consumers. As stated by a recent report of the U.S. Department of Agriculture Economic Research

Service, wheat may represent 5-10% of the whole malts used by U.S. breweries. The increased use of wheat may be related to the significant growth of craft beer production along with the increased popularity of several wheat beers produced by leading international brewers (JIN *et al.*, 2018). The scientific report of the European Food Safety Authority (EFSA, 2013) states that wheat has an average DON contamination about three times higher (434.4 μg/kg) than barley (176.1 μg/kg). That might explain the greater DON contamination values of wheat-based beers when compared to other beer types. Similar results were reported by KSIENIEWICZ-WOŹNIAK *et al.* (2019), who found a very high percentage of wheat-based beer samples positive to DON.

Previously published studies (PETERS *et al.*, 2017; WALL-MARTÍNEZ *et al.*, 2019 b) found a higher risk of mycotoxin contamination in craft beers. Diversely, our analysis did not reveal any significant differences between industrial and craft beers for what concerns DON contamination, which is in accordance with the study conducted by ARRÚA *et al.*, 2019.

EFSA estimates that the contribution of DON deriving from beer, in adult population, is approximatively 0.5-5.3% (EFSA, 2013). The average contamination value obtained in this study (34.3 μg/L) is three times higher than the average value (13.5 μg/L) taken into consideration by EFSA, 2013. Based on the average DON value found in our study, the consumption of a heavy drinker of 70 kg of body weight, consuming 0.5 L of beer per day, will determine a DON daily intake of 24.5 %. Considering the 2018 beer per capita consumption of 33.6 L (0.092 L of beer per day) of the italian consumers 4.5% of the TDI will be reached. These numbers are substantially higher than those found by PIETRI *et al.* (2003). Annual climate and weather variability may contribute to modify levels of mycotoxins in field crops (BEYER *et al.*, 2014), which will eventually lead to modified levels of DON in beer. For this reason, annual monitoring of grains for DON contamination would be essential to investigate the variability of malts contamination.

For the year 2018, our data suggests that through the consumption of beer, the Italian population received a higher percentage of DON from beer consumption compared to the average estimation from EFSA. Hence, our study suggests that the intake of DON through beer consumption should be updated for each nation, possibly on a yearly based manner. To verify if this can be simply attributed to a "year effect" or to the changes in the ingredients used for beer production further monitoring is needed.

Future studies should also focus on the impact of other grains on final DON levels in beer. Specifically, since maize is used in beer production and it has an average DON contamination (1041.9 μg/kg) significantly higher than barley (176.1 μg/kg) (EFSA, 2013), an investigation of maize impact on DON contamination would be of interest. Moreover experimental studies focused on the effects of different technological processes on DON accumulation will contribute to have a complete assessment of the factors that determine DON accumulation in beers.

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2. Manuscript Preparation

(1) The paper should be divided under the following headings in this order:
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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**.
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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.
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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.
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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.
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