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PAPER

EFFECTS OF ULTRASOUND TREATMENT ON STRUCTURAL, CHEMICAL AND FUNCTIONAL PROPERTIES OF PROTEIN HYDROLYSATE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) BY-PRODUCTS

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ABSTRACT

In this study, the effects of ultrasound treatment on biochemical, physical, structural and functional properties of fish protein hydrolysate of rainbow trout (*Oncorhynchus mykiss*) by-products were investigated. Enzymatic hydrolysis was conducted by Alcalase 2.4 L, pH 8, 1 h at 60°C, and enzyme/substrate ratio at 0.5%. A probe-type ultrasound was used for ultrasound assisted hydrolysis (UH) process. Higher protein recovery was obtained in UH than in the conventional enzymatic hydrolysis (CH). The highest foaming capacities of CH and UH were measured as 137.5% and 152.5%, respectively ($p<0.05$). Overall, our data suggest that ultrasound treatment helps to improve the functional properties such as foaming capacity and stability.

Keywords: by-products, fish protein hydrolysate, *Oncorhynchus mykiss*, rainbow trout, ultrasound hydrolysis

1. INTRODUCTION

Seafood processing industry generates high amounts of by-products during the processing steps. These by-products include backbone, head, tail, viscera, blood and cut-offs. Pertaining to species and the process applied, the volumes of these by-products vary from 20 to 70% of the whole raw material. Such amount of by-products brings about pollution and create severe problems at disposal points.

Based on the present industrial practice, most of the by-products are either discarded or used for various feed applications by processing them into fish silage, fishmeal and oil (HSU, 2010). The frame has a high-value biochemical composition with potential for higher-value food applications (ARASON *et al*., 2009; KLOMKLAO and BENJAKUL, 2018). An increasing trend in industrial applications for the utilization of fishery by-products, is the manufacture of water-soluble fish protein hydrolysates (FPH). This will give an increased yield of solubilized proteins due to reduced molecular weight and an increase in the number of ionizable groups (KRISTINSSON and RASCO, 2000).

Besides utilization in the replacement of animal meals from other sources (LI *et al*., 2018), protein hydrolysates can be used as functional additives in the food processing industry with many functional properties, such as water holding, gelling and foaming capacities, fat absorption, emulsifying and also antioxidant and antimicrobial activities. However, alternative methodologies have become necessary for improving yield, functional properties and bioactivity in protein hydrolysates. The quality and functional characteristics of the obtained products vary with the usage of different enzymes and production conditions. It is therefore, necessary to test alternative innovative technologies that will enhance product quality. These technologies need to be safe, cheap and easy to apply. It should also have no toxic and side effects.

Power ultrasound is an emerging and promising technology that has been applied in a variety of fields (ARVANITOYANNIS *et al*., 2015). Recently, enhancement in peptide production by ultrasound has become a focus of research in the food industry. With ultrasonic pretreatment of substrates, the enzymatic hydrolysis of wheat germ protein can be significantly stimulated (ZANG *et al*., 2015). In addition, hydrolysis of food protein can also be enhanced using sonicated enzymes (KADAM *et al*., 2015). The ultrasound treatment seems to be useful in accelerating the release of peptides. Ultrasound treatment is regarded as safe, non-toxic and environmentally friendly. It is also considered to be more advantageous to other technologies and is covered by "green technologies" (KENTISH and ASHAKKUMAR, 2011). Food technologists focus on protein production using high-intensity ultrasound application to support enzymatic hydrolysis, and produce high-throughput peptides.

In this study, it was aimed to determine the effects of ultrasound treatment on biochemical, physical, structural, antioxidant and functional properties of the fish protein hydrolysate that was produced from rainbow trout by-products.

2. MATERIAL AND METHODS

2.1. Materials

A total of 45 (weighing 12 kg) individual fresh rainbow trout (with average length and weight being 29.36±1.72 cm and 264.83±53.42 g, respectively), obtained from a local fish farming company, were transferred to the laboratory in styrofoam box with ice. After evisceration, the by-products (head, backbone, fins, tail and skin) were separated by hand and used as raw material; total weights of by-products are presented in Table 1. To

minimize microbial contamination and internal enzyme activity, viscera was excluded. The food-grade Alcalase 2.4 L (AU/kg Sigma Aldrich, Novozymes, Bagswaerd, Denmark) was used. All chemical reagents used for the experimental analysis were of analytic grade. The hydrolysation process was done on the same day the raw material reached the laboratory.

Table 1. Total weights of by-products of rainbow trout.

Type	Total weight (g)
Head	2074,10
Tail and backbone	1226,11
Fins and skin	1298,93
Total By-products used as raw material	4599,14
Viscera (not used)	2194,51

2.2. Methods

2.2.1 Preparation of raw material

By-products were chopped with mincing machine (Super meat grinder, 5 mm; pore size), mixed with distilled water (1:1 w/w) and homogenized (200 rpm for 2 minutes) using WiseTis‹HG-15D (Daihan, Seoul, Korea).

2.2.2 Preparation of protein hydrolysis

Protein hydrolysates were prepared using the pH-stat method according to SATHIVEL *et al*. (2005) and KANGSANANT *et al*. (2004), with slight modifications. For maximum activity and stability of the enzyme, all reactions were conducted at pH 8 (adjusted with 1N NaOH) and a temperature of 60°C. The prepared homogenate was used as raw material, divided into two equal aliquots and then placed in glass examination vessels. Experiments were carried out in the shaking water bath (Wisebath, Wertheim, Germany) agitating at 200 rpm for CH (Conventional Enzymatic Hydrolysis) and UH (Ultrasonic-Assisted Enzymatic Hydrolysis) processes, using Alkalase (0.5% by weight of raw material). For ultrasound assisted system, a probe type ultrasound equipment (Sonics vibra cell, USA, tapered micro tip, 142 x 6 mm) was used and the probe was immersed into the experimental vessel with 40% ultrasonic amplitude, pulse duration of 10 s on- time; 20 s off-time. In both vessels, the temperature was increased to 60°C for enzyme activation and kept constant during the experiment. Hydrolysis was initiated by addition of enzyme and terminated after 60 min, the enzyme was inactivated by increasing the temperature to 90 ºC for 10 min. Coarse filtration was applied to heated suspensions using glass cotton and filter paper. Thereafter, filtrates (6000 g) were centrifuged in a refrigerated centrifuge (Universal 320 R, Hettich, Germany) at 4°C for 35 min. After centrifugation, 3 separate phases occurred in the separation funnel; bottom phase: insoluble protein, middle phase: soluble protein heavily liquid, and upper phase: lipid fraction light liquid. The middle layer was collected. The supernatants were stored in a freezer at -80ºC and dried in a freeze-dryer (Labconco Freezone 2.5 Benchtop Freeze Dryer, USA) for 48 hours. The resulting powdery hydrolysates were vacuum packed and stored in a freezer at -80°C until analysis. The hydrolysis process of CH and UH groups were done in duplicate. All the analysis for the CH and UH groups were performed in three parallels.

2.2.2.1 Yield of FPH

FPH yield was calculated following the method used by ILHAN and GÜLYAVUZ, 2003.
Yield of FPH (%) = [Weight of FPH (g)/Weight of by-products (g)] x100 (1)
Yield of protein (%) = [(wf × Pf)/ (wi × Pi)] x100 (2)
Where wf is the weight in grams of FPH, Pf is the protein content (%) of FPH, wi is the weight of by-products in grams and Pi is the protein content (%) of by-products (PIRES *et al*., 2012).

2.2.3 Determination of the degree of hydrolysis (DH)

DH was analyzed with pH-stat method described by WROLSTAD *et al*., 2005. About 10 g of freeze-dried sample was weighted; hydrolysis conditions of fish by-products were applied. The solution was stirred with the magnetic stirrer (Ika, RCT Basic, Germany) and pH was adjusted to 8.0 with 0,1 N NaOH for 60 min. NaOH consumption was reported every 5 min. Results were given as a percentage. The equation used in the calculation is given below;

DH (%) = B × Nb × 1/α × 1/Mp × 1/htot × 100 (3)
B: Amount of alkali consumed (ml)
Nb: Normality of the alkali; 0.5 N (= 0.5 mmol/ml)
Mp: The mass of substrate (protein (g), %N ×6.25)
1/α: The calibration factors for pH-stat
htot: The content of peptide bonds.

ADLER-NISSEN (1986) assumed htot as 8.6 mmolg^{-1} of protein and α as 1 for fish.

2.2.4 Determination of biochemical composition

Total crude lipid content was determined by Soxhlet extraction method and crude protein content was analyzed by Kjeldahl method. The total protein content was calculated as %N using the standard conversion factor of 6.25. (AOAC, 1990, method 2.507); moisture and ash contents were determined using AOAC 1990 method 985.14 and method 7.009, respectively.

2.2.5 Amino acid analysis

Total amino acid analyzes were carried out in Kazlıçeşme R and D Test Laboratory (AB-0513-T), an accredited laboratory in Istanbul, Turkey. After pre-column derivatization with HPLC (Agilent 1260 Infinity), Agilent Eclipse AAA method was modified using FLD/DAD detectors and determined by an in-house laboratory method. A 0.2 g sample was weighed and mixed with 5 ml of 6 N HCl and stored in the condenser for 24 h. Depending on the amount of amino acid, 0.6 g to 2 g of sample was transferred to 100 ml balloon flask, after addition of 5 ml norvaline standard, the flask volume was completed to 100 ml. Thereafter, 0.5 µl of the filtered sample was injected into the device and analyzed. OPA (Ortho Phthalaldehyde), FMOC (Fluorenylmethoxy Chloroformate) and Borate was used as the derivatizing agent.

2.2.5.1 HPLC conditions

Mobile phase A; 40 mN Na_2HPO_4 (pH 7.8) and Mobile Phase B; Asentonitrile/ Methanol/Water (45/45/10), a flow rate of 2 ml/min. ZORBAX Eclipse-AAA 4.6 * 150 mm (3.5 μm) was used as the column. The column temperature was set at 40°C. The injection volume of sample was 0.5 μl. DAD detector wave lengths were 338nm, 10nm bw; Ref: 390 nm, 20 nm bw (for OPA-amino acid) and 262 nm, 16 nm bw; Ref: 324 nm, 8 nm bw (for FMOC-amino acid).

2.2.6 Measurement of the color

The color was measured using a color meter (Konica Minolta (Specktropen CR10 Japan)). Three measurements were taken from the samples of CH and UH.
L * (brightness), a * (redness), b * (jaundice), W (whiteness), chroma and h hue angle /saturation degree;

$$W = 100 - [(100-L^*)^2 + a^{*2} + b^{*2})]^{1/2} \quad (5)$$

$$Chroma = (a^{*2} + b^{*2})^{1/2} \quad (6)$$

$$h = b^*/ a^* \quad (7)$$

(PIRES *et al*. (2012)

2.2.7 Determination of functional properties

2.2.7.1 Protein solubility

Protein solubilities of CH and UH were determined as reported by American Oil Chemists Society (AOCS) (1989). FPH's were dispersed in the water (10 g/l); pH of solutions were adjusted to 3, 5, 7 and 9 with 0.5 N NaOH or 0.5 N HCl for 45 min with constant stirring. The solutions were then centrifuged for 30 min at 2.800 *g*. N contents in 15 ml of supernatants were determined according to the Kjeldahl method;
Protein solubility (%) = protein content of the supernatant/total protein content.

2.2.7.2 Foaming capacity and foaming stability

Foaming capacity (FC) and foaming stability (FS) were performed according to WILDE and CLARK, 1996 and SHAHIDI *et al*., 1995, with slight modifications. Three g of FPH was mixed with 100 ml of distilled water, then transferred into a 250ml graduated cylinder. The mixture was homogenized at 11000 rpm for 1 min at room temperature. The total volume was measured at 0, 1st, 5th, 10th, 40th and 60th min. FC was expressed as foam expansion at 0 min, while FS was expressed as foam expansion at 60 min.

2.2.7.3 Oil binding capacity and water holding capacity

Oil binding capacity was determined by the protocol of SHAHIDI *et al*., 1995. Five hundred mg hydrolysate was put in a centrifuge tube and 10 ml of sunflower oil was added. After being thoroughly vortexed for 1 minute, it was centrifuged (Hettich Universal 320 R Refrigerated Centrifuge) at 4500 g for 30 min at a temperature of 4°C, thereafter the unconnected oil was discharged. The oil binding capacity was expressed as weight of fat (g) absorbed per gram of sample.
Water holding capacity was analyzed following the centrifuge method described by COBB and HYDER (1972), with slight modifications. Five hundred mg of FPH was weighted into a centrifuge tube and 20 ml distilled water was added. The mixture was vortexed for 30 s,

then put in a dark place at room temperature for 6 h. Thereafter, the tube at 2800 g was placed into the centrifuge for 30 min. Obtained supernatant was filtered from Whatman Paper No: 1 and the volume of liquid was weighted. The water holding capacity calculation was done by dividing the volume of the filtrate obtained from the initially used water volume by the amount of sample. The results are expressed as ml/g.

2.2.8 Scanning electron microscopy (SEM)

The surface morphology of CH and UH thin films was investigated using a JSM-6610 (JEOL) scanning electron microscope (SEM) equipped with an energy dispersive X-ray (EDX) analyzer operated at 20 kV acceleration voltages. Prior to the observation, the investigated specimens were coated with about 250 angstroms of gold by QUORUM-SC7620 sputter coater.

2.2.9 Antioxidant activity assay

To observe antioxidant capacities of the groups, copper (II) ion reducing antioxidant capacity (CUPRAC) and Fe (III) ion reducing antioxidant power methods were used. Radical scavenging activity was determined by ABTS·2,2'-azinobis-(3-etilbenzotiazolin-6-sülphonic acid) radical scavenging method.

2.2.9.1 Copper (II) ion reducing antioxidant capacity assay (CUPRAC)

The method is based on the reduction of copper (II)-neokuproine to copper (I)-neokuproine after addition of antioxidant solution to the medium (APAK *et al*., 2004; MENTESE *et al*., 2015). A total of 10 mM Cu(II) chlorure (Sigma Chemical Co, USA), 7.5 mMneokuproine (Sigma Chemical Co, USA), and 1 M ammonium acetate tampon solution at pH 7.0 (one mL each) were pipetted into the test tubes. About 20 μL sample solutions were added to the medium and vortexed. Final volume was completed to 4.1 ml and 1080 μl distilled water was added and again vortexed. The same procedure was applied for Trolox® standard. After incubation at room temperature for 50 min, absorbance was read at 450 nm (1601UV-Shimadzu, Australia). Using Trolox® curve (8 - 4 - 2 - 1 - 0.5 - 0.25 - 0.125 - 0.0625 mM Trolox®, (r^2=0.999)), Trolox® equivalent antioxidant capacity (mg TEAC/mg substance) per mg substance was calculated for each substance.

2.2.9.2 Iron (III) ion reducing antioxidant capacity assay (FRAP)

The method is based on the measurement of the absorbance of the complex, Fe^{2+} - TPTZ complex at 593 nm (BENZIE and STRAIN, 1999; CAN and BALTAS, 2016). Firstly, 300 mM acetate buffer at pH 3.6 was dissolved in 40 mM HCl, and 10 mM TPTZ (2,4,6-tris (2-pyridyl)-s-triazine and 20 mM of $FeCl_3.6H_2O$ solution were prepared. Freshly prepared solutions were mixed in a ratio of 10: 1: 1 and FRAP reactive was obtained. A total of 100 ml aliquots of samples and 3000 μl FRAP reactive were transferred to each sample tube and vortexed. The reaction mixture was incubated for 5 min at room temperature, and absorbance was read at 593 nm. The same treatments were carried out for $FeSO_4.7H_2O$ standard (r2 = 0.999) prepared at concentrations of 15.63 - 31.25 - 62.50 - 125 - 250 - 500 - 1000 µM, respectively. The absorbance of the test tubes, which were allowed to incubate for 5 min at room temperature, was measured at 593 nm (1601UV-Shimadzu, Australia) and the standard $FeSO_4.7H_2O$ curve was used to calculate the equivalent antioxidant capacity (mM $FeSO_4.7H_2O$/mg substances).

2.2.9.3 ABTS$^{\cdot+}$ cationic radical scavenging method

The radical scavenging activity of the ABTS$^{\cdot+}$ [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] groups were studied according to RE *et al*., 1999; YILMAZ *et al*., 2017. A 7 mM solution of ABTS in water was prepared and 10 ml of this solution was mixed with 2.45 mM and 5 ml potassium persulphate solution and allowed to incubate at room temperature for 18 hours to enable the formation of ABTS$^{\cdot+}$ cationic radical. The resulting radical solution was diluted with phosphate buffer (PBS) at pH 7.4, to give an absorbance of 0.700 ± 0.020 at 734 nm. 200 µL of the test compound (dissolved in DMSO) was added to 1800 µl of the radical solution, vortexed, and after 5 min, the absorbance was read on the UV-Visible spectrophotometer (1601 UV-Shimadzu, Australia) at a wavelength of 734 nm. The radical scavenge value of the groups was calculated from the following formula. The study consisted of three replications for each substance and standard.

$$\text{Radical scavenging } (\%) = [(OD_{control}-OD_{test})/(OD_{control})x100]$$

2.2.10 Statistical analysis

The obtained data were analyzed by analysis of variance (one way ANOVA) and when significant differences were found, comparisons among means were carried out using the Tukey and Mann Whitney U test (data not provided in the normality of assumptions) under the program called JMP 5.0.1 (SAS Institute. Inc. USA) and SPSS 18.0 (SPSS Inc., Chicago, IL) (SOKAL and ROHLF, 1987). A significance level of 95% ($p<0.05$) was used throughout the analysis.

3. RESULTS AND DISCUSSION

3.1. Yields of by-products, FPH's (CH and UH) and the protein of FPH

The yield of by-product was calculated as 38.32 % using the data shown in Table 1. The yields of CH and UH were calculated as 9.82% and 10.54%, respectively. Ultrasound application may have increased the yield because ultrasound have a positive effect on alcalase activity due to the ability to break down the molecular aggregates, giving enzymes the opportunity to yield higher accessibility for reaction and increasing activity, (MCCLEMENTS, 1995). MA *et al*. (2011) studied the mechanism of ultrasonic impact on protease activity and their results showed that ultrasound had an effect on the activity of alcalase. Protein yields of FPH's were also calculated as 57.13% for CH and 61.76% for UH. LIASET *et. al*. (2000) produced protein hydrolysate from Atlantic salmon frames without heads. They used alcalase for conventional enzymatic hydrolysis and FPH protein recovery was observed to be 61.8%. The higher protein recovery of the present study may be affected by different hydrolysis conditions.

3.2. Biochemical composition of by-products from rainbow trout

Biochemical composition of raw material (trout by-products), CH and UH, are shown in Table 2.

Table 2. Proximate composition of by-products, fish protein hydrolysates (CH) and (UH)

	Protein(%)	Lipid(%)	Moisture(%)	Ash(%)
By-products	14.82±0.18[a]	6.45±0.48[a]	72.19±1.38[a]	3.54±0.24[a]
(CH)	86.40±0.32[b]	0.05±0.01[b]	1.36±0.08[b]	6.25±0.40[b]
(UH)	86.75±0.28[b]	0.05±0.01[b]	2.10±0.18[c]	5.95±0.32[b]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b) represent statistical differences amongst the groups ($p<0.05$).

Protein content in by-products was very low (14.82%). The CH and UH samples have high and similar protein contents. The high protein content of FPH is due to the characteristics of the hydrolysis process. During this process, proteins are solubilized and insoluble materials are removed by centrifugation (CHALAMAIAH *et al*., 2010). Lipid contents were the same for CH and UH and are very low (0.05%). This also may be as a result of characteristics of the hydrolysis process; the membranes tended to round up and form insoluble bubbles, which could cause the removal of membrane structural lipids. During the centrifugation stage after hydrolysis, these lipids separated into different layers and were removed from the medium. It is a desired feature for protein hydrolysates to have low lipid content (SHAHIDI *et al*., 1995). As estimated, the moisture content of by-products was high. Whereas, it was very low in CH and UH because FPHs were freeze-dried at the end of the hydrolysis process. The difference between CH and UH was significant ($p<0.05$). Since by-products have skin and bone parts, ash content was higher than trout flesh (average 1.21%) (TURCOMP, 2014), but it was higher in CH and UH than the by-products; this may be due to the increased salts by addition of alkali into the medium to adjust the pH 8 during the hydrolysis process (BENJAKUL and MORRISSEY, 1997). There was no significant difference in the ash contents of CH and UH.

3.3. Amino acids analysis

The functional differences in hydrolysates are closely related to the amino acid groups present in the structures. Table 3 shows the total amino acid contents in by-products, CH and UH, respectively.

Accordingly, values for by-products of amino acids were lower than all amino acid values of FPH ($p<0.05$). Amino acid contents of CH and UH were higher and close to each other. Total amino acid contents were calculated as 2.01g/100g for by-products, 80.54g/100g for CH and 82.65g/100g for UH. Generally, ultrasound application helps to open the surface of the substrate and increases the enzyme activity. As a result, it supports hydrolysis process. Glycine was the highest in all groups. Among the hydrophobic amino acids, (valine, methionine, leucine, isoleucine, alanine, tryptophan, phenylalanine and tyrosine), the value of leucine was the maximum and methionine was the minimum. Indicating the increased antioxidant activity, the sum of the hydrophobic amino acids was calculated as 22.98g/100g for CH and 23.72g/100g for UH. Differences for all amino acids of CH and UH were not significant, except valine *($p<0.05$)*. RAJAPAKSE *et al*., 2005 stated that hydrophobic amino acids, such as phenylalanine and glycine are highly soluble in lipids. Soluble amino acids have more capability to gain closer access to the radicals than neutral or hydrophilic amino acids.

Table 3. Total amino acid contents of by-products, CH and UH(g/100 g).

Amino Acid	By-product	CH	UH
Cysteine	0.00±0.00[a]	1.81±0.25[b]	1.94±0.01[b]
Aspartate	0.12±0.01[a]	6.80±0.06[b]	7.10±0.13[b]
Glutamate	0.09±0.01[a]	10.98±0.11[b]	11.45±0.28[b]
Aspargine	ND	ND	ND
Serine	0.11±0.01[a]	3.19±0.01[b]	3.24±0.17[b]
Glutamine	ND	ND	ND
Histidine	0.06±0.01[a]	1.93±0.00[b]	1.90±0.04[b]
Glycine	0.36±0.01[a]	12.05±0.16[b]	12.06±0.38[b]
Threonine	0.11±0.01[a]	3.00±0.01[b]	3.08±0.15[b]
Arginine	0.09±0.01[a]	5.59±0.06[b]	5.75±0.20[b]
Alanine	0.14±0.01[a]	5.70±0.06[b]	5.88±0.17[b]
Tyrosine	0.12±0.01[a]	1.95±0.05[b]	2.08±0.06[b]
Valine	0.14±0.01[a]	3.06±0.02[b]	3.21±0.03[c]
Methionine	0.11±0.01[a]	1.65±0.04[b]	1.74±0.02[b]
Norvaline	0.02±0.00[a]	0.01±0.01[a]	0.02±0.01[a]
Tryptophane	0.06±0.01[a]	0.33±0.06[b]	0.34±0.06[b]
Phenylalanine	0.08±0.00[a]	3.84±0.05[b]	3.99±0.11[b]
Isoleucine	0.07±0.01[a]	1.91±0.04[b]	2.05±0.08[b]
Leucine	0.07±0.01[a]	4.84±0.04[b]	5.13±0.11[b]
Lysine	0.09±0.01[a]	5.83±0.13[b]	5.89±0.08[b]
Hydroxyproline	0.13±0.01[a]	2.28±0.08[b]	2.07±0.16[b]
Sarcosine	ND	ND	ND
Proline	0.11±0.01[a]	3.89±0.05[b]	3.77±0.08[b]
Total	**2,01±0,04[a]**	**80,54±0,75[b]**	**82,65±0,60[b]**

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

3.4. Measurement of the color

At the end of the hydrolysis, yellowish brown liquid mixtures were obtained in both vessels (CH and UH). The liquid mixtures had three layers; the bottom, including bones brown in color, the middle; a dark yellowish brown clear liquid, and the top dense brown liquid. After centrifugation, collected liquids (CH and UH) were bright dark yellow. The colors of freeze-dried powders of CH and UH were creamy yellow and L*, a*, and b* values are presented in Table 4.

Table 4. L*, a* and b* and W, c, h values of CH and UH.

	L*	a*	b*	W	c	h
CH	85.80±0.84[a]	2.90±0.51[a]	22.60±1.35[a]	73.15±0.78[a]	22.79±0.48[a]	7.80±0.46[a]
UH	83.90±0.60[a]	3.50±0.30[a]	24.30±0.30[a]	71.10±0.56[a]	24.10±0.30[a]	6.80±0.34[a]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 10. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

L*, a*, and b* values observed in CH were similar with UH. Using L*, a*, b* values whiteness, chroma and h values were calculated (Table 4).

3.5. DH

Fig. 1 illustrates the variation in the DH of CH and UH under experimental conditions, which showed a rapid increase in both groups due to many peptide bonds cleaved up to around 20th min regardless of ultrasound application. After this time, as fewer peptide bonds were available for cleavage, the reaction rate reduced for CH and UH. After about 35-40 min, a small decrease occurred in the DH of UH, which was significantly lower than DH of CH ($p<0.05$). This observation was not in accordance with the theory that ultrasound application would yield a higher DH than the conventional hydrolysis. KANGSANANT *et al*. (2014) produced enzymatic hydrolysate from Nile tilapia assisted by continuous ultrasound with 40W. The researchers observed that ultrasound assisted hydrolysis provoked a decrease in DH, but this decrease was not significant compared to other researches which focused on different food items. This may be due to the low intensity of ultrasound applied in the present study (HUANG *et. al*., 2015; ZHANG *et. al*., 2015).

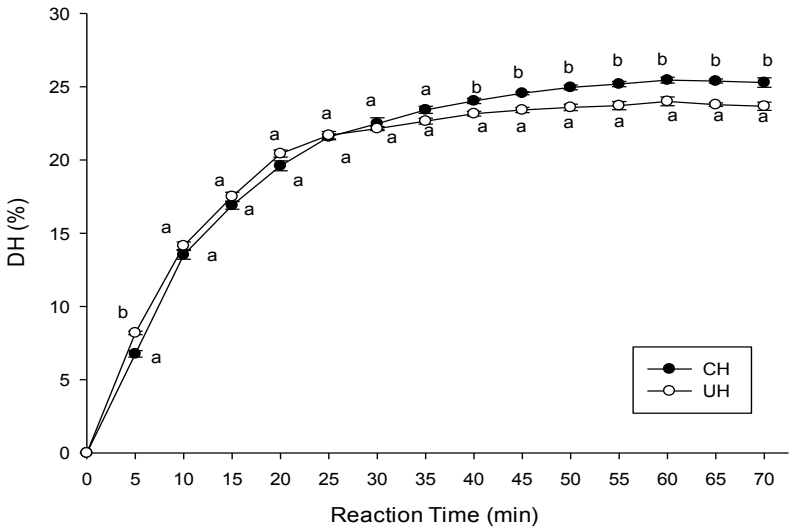

Figure 1. Evolution of DH during the hydrolysis of CH and UH.
CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different letters (a,b) represent statistical differences amongst the groups (p<0.05).

3.6. SEM analysis

To find out the structural effect of ultrasonic treatment on FPH, the microstructure of lyophilized CH and UH were observed by SEM under different magnifications. Fig. 2 illustrates the SEM images of CH and UH. As shown in Fig. 2, different microstructures were obtained in CH and UH. UH had larger aggregates plate-shaped morphology and a smooth surface structure, whereas CH had smaller aggregates both in the form of round structures and plate-shaped morphology. The differences might be due to the changes in application of ultrasound that led to the unfolding of UH molecules. As a result, higher hydrophobic groups might occur at the surface of the molecules and interaction of these

groups with each other formed larger structures. HU *et al*. (2013) found that treatments of different frequencies and times of ultrasound were effected on the structure of soy protein isolate dispersions. In their study, after ultrasound treatment, samples had larger and more heterogeneous structures. Also, they observed that longer ultrasound application might result in larger structure size. ZHOU *et al*. (2016) investigated the effects of heat, ultrasound and combinations of heat/ultrasound and ultrasound/heat on corn gluten meal hydrolysate. Researchers used 40 kHz frequency, on-time 10 s and off time 3 s, 40 min duration at 20°C. They observed that the control was in the form of massive texture, but the surface became incompact and porous after ultrasound pretreatment.

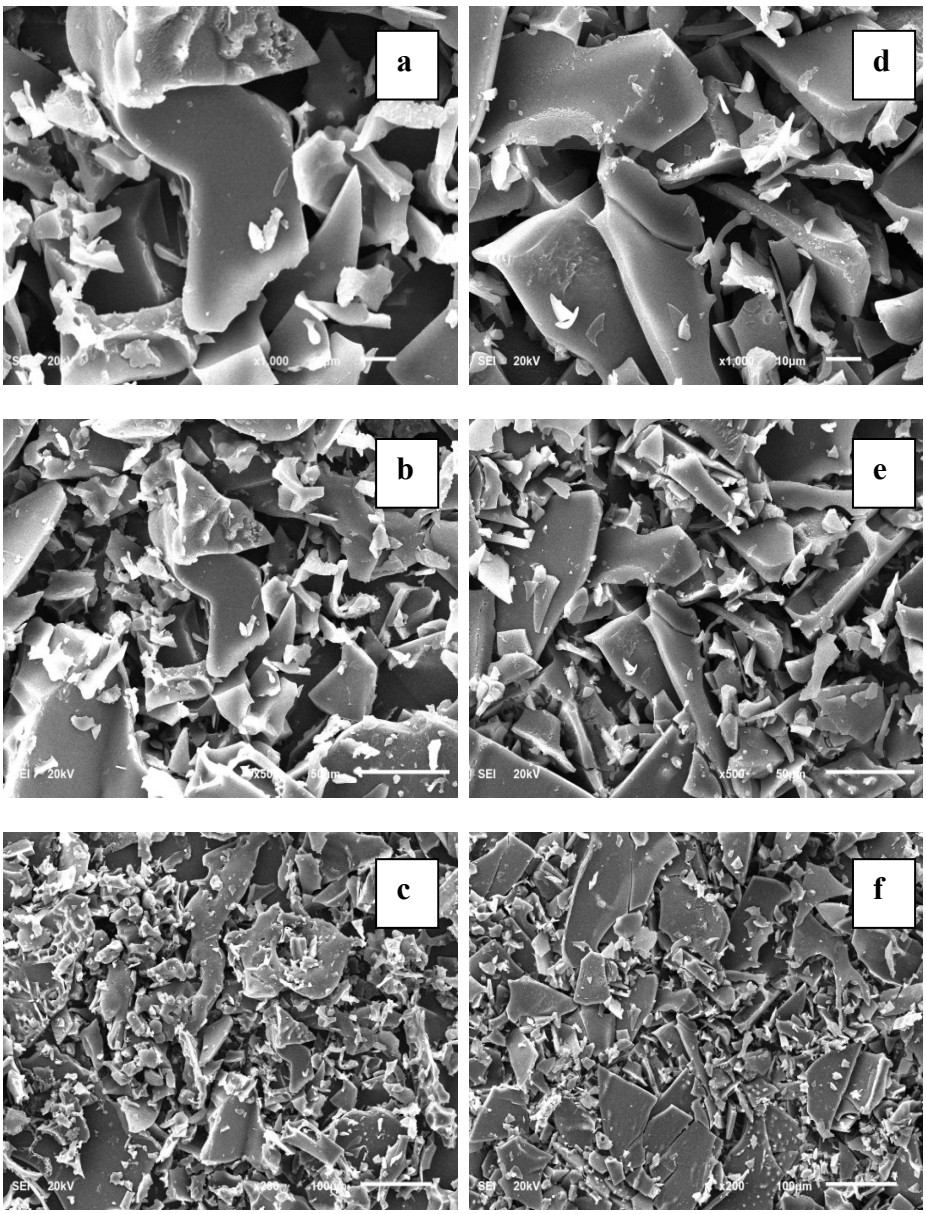

Figure 2. SEM analysis of CH (a,b,c) and UH (d,e,f).

In the present study, results are inconsistent with those emphasized studies. Different shapes may be due to the different ultrasonic conditions (pretreatment, ultrasonic-assisted hydrolysis and time, temperature, etc.) and the raw material used in the study.

3.7. Functional properties

In the food industry, proteins have a special attribution in food products due to their several significant functional characteristics. Among the functional properties, emulsifying, foaming, thickening and gelling capacities are often affected by their solubility (DAMODARAN, 1997). Soluble peptides obtained from enzymatic hydrolysis of proteins, can contribute to improving the emulsion and the foaming characteristics (RAYMUNDO *et al*., 2000). Ultrasound applications led to an improvement in the functional properties of different food items (BRYANT and MCCLEMENTS, 1999). But ultrasonic treatment conditions and variation in the rheological and thermos-physical properties of protein sources are considered effective on the functional properties of protein hydrolysates (AVAD *et al*., 2012).

3.7.1 Protein solubility

Protein solubility of CH and UH are shown in Fig. 3. It was low at acidic pH and gradually increased with increasing the pH, after neutral pH, it decreased again to pH 9. Both groups have the highest solubility at pH 7, and the lowest at pH 3. The differences between groups were significant, except pH 3 ($p<0.05$). As shown in Fig. 3, a parallel trend in CH and UH, ultrasound treatment was shown to improve the protein solubility.

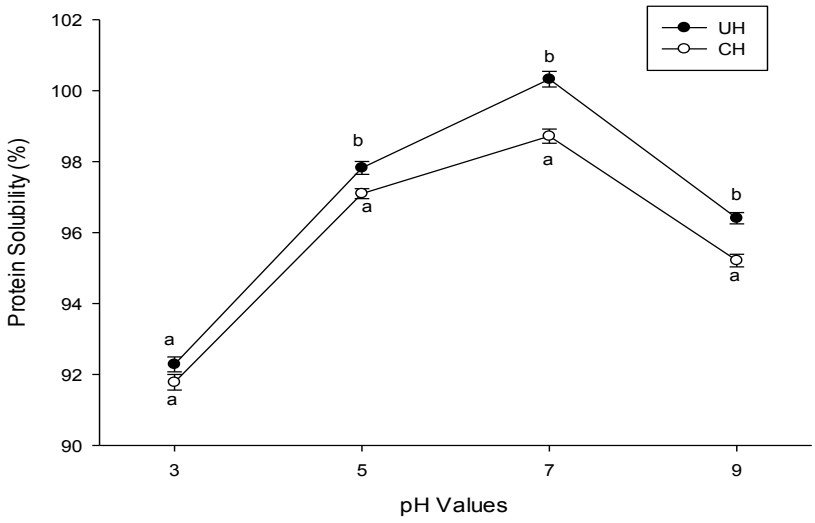

Figure 3. Protein solubility of CH and UH.

Ultrasound changes the conformation and structure of protein and hydrophilic amino acid residues directed towards water (ARZENI *et al*., 2011). This situation explains the case of higher solubility of UH than CH. Protein solubility is one of the most important representative factors in protein functionality. In the food industry, improvement in solubility led to a potential improvement in the functional properties of proteins (PELEGRINE and GASPARETTO, 2005).

3.7.2 Foaming capacity (FC) and foaming stability (FS)

FC and FS of CH and UH are shown in Fig. 4 a, b. The FC of UH in each duration was significantly higher than that of CH. The highest values for FC of CH and UH were measured accordingly as 137.5% and 152.5%, respectively. At the end of 60 min, it decreased to 11.0% in CH and 20.0% in UH. Diffusion of soluble proteins, rapid conformational change and reorganization of molecules at air-water interface are needed in the protein-based foam formation (NALINANON *et al*., 2011). Parallel to FC, FS of UH was also significantly higher than CH, this difference was significant ($p<0.05$) after the 5th to 60th min (Fig. 4b).

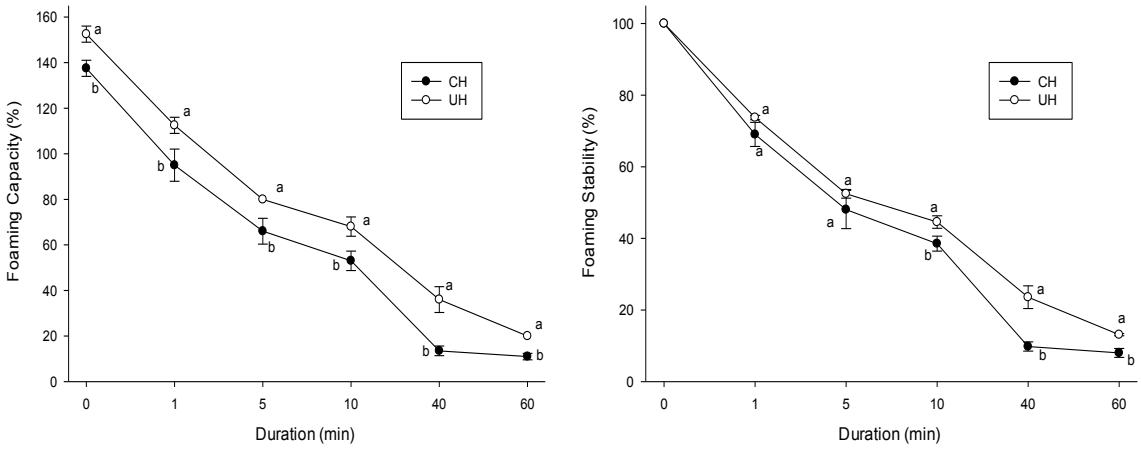

Figure 4. Foaming capacity (a) and stability (b) of CH and UH.
CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different letters (a,b) represent statistical differences amongst the groups (p<0.05).

It was reported that protein solubility has an important effect on functional properties of protein hydrolysates. In the present study, the results of FC and FS were in accordance with the solubility values. As the solubility increased with the ultrasound treatment, FC and FS increased. Researchers also reported that higher solubility results in higher foaming characteristics from different protein sources (SORIA-HERNÁNDEZ *et al*. 2015). JAMBRAK *et al*., (2009) illustrated that ultrasound application is an effective way to improve the physical properties of soy proteins. Anon, 2008 reported "The degree of hydrolyzation determines the functionality of the end products. Low degree of hydrolyzation results in highly functional foaming agents and high degree of hydrolyzation results in hydrolysed vegetable protein (HVP) which are used in soups and sauces as flavor enhancers".

3.7.3 Oil binding capacity (OBC) and water holding capacity (WHC)

OBC shows a major functionality of ingredients in the food industry. KRISTINSSON and RASCO (2000) stated oil binding capacity ranged from 2.86 to 7.07 mL of oil/g of protein for Atlantic salmon protein hydrolysates. The bulk density of the protein, the degree of hydrolysis and enzyme used in hydrolysis process affect this functionality. Water holding capacity is another important factor. It especially improves the textural properties of

foods. Different ingredients derived from proteins are used in muscle foods to improve water holding functions.
Data on OBC and WHC of CH and UH are presented in Table 5. UH has a better OBC than CH ($p<0.05$). On the contrary, WHC was lower in UH than CH, but this difference was not significant ($p>0.05$).

Table 5. Oil absorption and water holding capacity of CH and UH.

	CH	UH
Oil absorption capacity (g/g oil)	4.47±0.23[a]	6,36±0.40[b]
Water holding capacity (ml/g)	5.40±0.57[a]	4,70±0.14[a]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a, b, c..) represent statistical differences amongst the groups ($p<0.05$).

3.8. Antioxidant activity

Different measurement methods are used for antioxidant capacity determination. Since only one experiment cannot give reasonable results, it was observed that the item act as antioxidant. Accordingly, antioxidant activities of CH and UH were measured using the methods; CUPRAC, FRAP and $ABTS^{\cdot+}$ Radical Scavenging Activities. Many studies have shown that all protein hydrolysates consist of peptides or smaller protein fractions that are hydrogen donor and could react with radicals to convert them to more steady products, thereby finalizing the radical reaction (KITTIPHATTANABAWON *et al*., 2012).

3.8.1 CUPRAC and FRAP Antioxidant Activity

CUPRAC method is easily used to measure total antioxidant capacities of both hydrophilic and lipophilic antioxidants (YAVAŞER, 2011). The results of the antioxidant activity obtained using the CUPRAC and the FRAP methods of the UH and CH are given in Table 6. Trolox equivalent antioxidant capacity (TEAC) values of the groups (according to the CUPRAC method) were calculated on the Trolox® standard and $FeSO_4.7H_2O$ standard.

Table 6. Antioxidant activities of UH and CH, (CUPRAC (mM Trolox/mg compound) and FRAP (mM FeSO4.7H$_2$O/mg compound) methods).

Compounds	TEAC Values (μM Trolox®/mg mixture)	FRAP Values (μM $FeSO_4.7H_2O$/mg mixture)
UH	244.89±0.020[a]	13.175±0.009[a]
CH	230.23±0.017[b]	12.161±0.003[b]

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

TEAC method is based on electron transfer such as Trolox equivalent antioxidant capacity (SARMADI and ISMAIL, 2010). TEAC values for UH were significantly higher than CH ($p<0.05$). Reduction activities of UH and CH to iron (III) and iron (II) were calculated according to FRAP method. FRAP values of UH were higher than CH ($p<0.05$) (Table 6). In

both methods, higher antioxidant activities of UH samples might be due to change in the structures of fractions as the effect of ultrasound. JIANG *et al*. (2014) stated that ultrasonic treatment causes higher interactions of protein hydrophobic sites exposed to the surface of the molecules and buried inside the molecules.

3.8.2 $ABTS^{\cdot+}$ radical scavenging activity

The total radical scavenging capacities of CH and UH were determined using the $ABTS^{\cdot+}$ radical scavenging assay. $ABTS^{\cdot+}$ is generated by oxidation of ABTS with potassium per sulfate and is reduced in the presence of such as hydrogen or an electron donating antioxidant (BINSAN *et al*., 2008). The SC_{50} values for $ABTS^{\cdot+}$ radical scavenging activities of the CH and UH were presented in Table 6. The CH exhibited efficient radical scavenging activity when compared to UH, at the all final concentration (Table 7).
Increased compound concentrations caused an increase in radical scavenging ability. ABTS scavenging activity increased with increasing concentrations and it was stated that some amino acids like histidine, methionine, cysteine, phenylalanine and tyrosine might be effective in increasing the ABTS+ radicals scavenging activities (CHALAMAIAH *et al.* 2010). Aromatic amino acids in hydrolysates are capable of stabilizing free radicals by donating an electron. In the present study, total amounts of these amino acids were similar in CH and UH (11.18g/100g and 11.65g/100g, for CH and UH, respectively). Histidine shows capabilities of stabilizing free radicals by donating an electron and inhibiting lipid oxidation through chelating and lipid trapping of the imidazole ring. In the present study, histidine was higher in CH than UH. Lower SC_{50} values of CH display a higher radical scavenging effectiveness. The SC_{50} values for $ABTS^{\cdot+}$ method of CH and UH were found as 160.0 and 180.10 μg/ml, respectively (Table 7). In a study, ABTS scavenging activities were similar for control and ultrasound pretreated (91.2% and 92.7%) bighead carp hydrolysate, at a hydrolysate concentration of 30% (YANG *et al*., 2016).

Table 7. $ABTS^{\cdot+}$ radical scavenging activities at various final concentrations (%) and SC_{50} values of the UH and CH.

Compounds	ABTS$^{\cdot+}$ Method Radical Scavenging (%)						SC_{50} Values (μg/mL)
	1000 μg/mL	500 μg/mL	250 μg/mL	125 μg/mL	62.5 μg/mL	31.25 μg/mL	
UH	86.15±1.12^{a}	77.54±0.72^{a}	61.85±0.48^{a}	36.62±0.30^{a}	19.62±0.22^{a}	6.92±0.12^{a}	180.10±0.68^{a}
CH	87.08±0.90^{a}	79.08±0.50^{a}	64.31±0.56^{a}	42.58±0.42^{b}	24.46±0.28^{b}	9.85±0.08^{b}	160.00±0.45^{b}

CH: Conventional Enzymatic Hydrolysis, UH: Ultrasonic-Assisted Enzymatic Hydrolysis, ± SD: n: 3. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst the groups ($p<0.05$).

4. CONCLUSION

This research shows that FPH derived from trout by-products may have a potential utilization as a functional and nutritional ingredient in food systems with desirable properties. Ultrasound application improves protein solubility and it affects especially foaming capacity and stability, as well as oil absorption capacity of FPH. There were no significant differences observed in other functional properties. The SC_{50} value for $ABTS^{\bullet+}$ radical scavenging activity was gained by ultrasound treatment. Ultrasound assisted enzymatic hydrolysis of FPH can be used as a novel hydrolyzation process.

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PAPER

COMPARATIVE STUDY ON TOTAL POLYPHENOLS CONTENT OF TUNISIAN WILD *RHUS PENTAPHYLLA* FRUIT EXTRACTS AND THE EVALUATION OF THEIR BIOLOGICAL ACTIVITIES

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ABSTRACT

This study was focused on determining the total polyphenols content and biological activities from wild *Rhus pentaphylla* fruits at two different maturity stages, (stage 1: green-yellow fruits and stage 2: purple-red fruits Highly significant ($p \leq 0.01$) increase was observed in total phenolic contents of fruits ranging from 158.65±0.06 to 177.57±0.06 mg Eq.AG.100 mg^{-1}DW. In contrast, both total flavonoid (from 157.34±0.07 to 152.69±0.14 mg Eq.Cat.100 mg^{-1}DW) and condensed tannins contents (from 200.26±0.30 to 131.23±0.24 mg Eq.Cat.100 mg^{-1}DW) showed dramatic decreases ($p \leq 0.01$). Evaluation of biological activities has provided good effects. Our results show the importance of using this wild fruits in pharmaceutical industries.

Keywords: *Rhus pentaphylla,* ultrasonic extraction, total polyphenol content, antioxidant activity, antimicrobial potential

1. INTRODUCTION

The human body is constantly exposed to a multitude of microbes (bacteria, viruses, parasites, fungi) (TILLE, 2013). Although it has a complex defense system that allows it to meet or host these microbes without allowing them to invade its tissues, it is sometimes necessary to use antimicrobial agents, antivirals etc. (HAMBLIN and HASAN, 2004). But in some cases, these agents are unable to treat certain infectious diseases (ATANASOV *et al*.,2015). Faced with this incapacity, a trend has developed in recent years with the aim of finding new natural sources of plant-based bioactive molecules (CARINA, 2012).
The genus *Rhus pentaphylla* consists flowering species belonging to *Anacardiaceae* family, often grows in non-agricultural areas and widely used in foods (LEE *et al*., 2010). They are recognized in traditional medicine by their therapeutic interests (anticancer, anti-inflammatory, antidiarrhea), and it provides bioproducts that have desirable biological activities: antifungal, anti-inflammatory, antimalarial, antimicrobial, antitumorigenic, antioxidant, antiviral and hypoglycemic (ABED, 2013). The genus has several varieties in Spain, in the region of Malaga, as well, it is divided into northern Africa, which is common in the west of Algeria and Morocco (RACHED, 2009). In Tunisia, the genus *Rhus* is represented by two species: *Rhus tripartita* (Ucria) and *Rhus pentaphylla* which spreads in north and center of the country (ITIDEL *et al*., 2013). *R. pentaphylla* fruits, fresh or dried are slightly acidic (LAHSISSENE *et al*., 2010), and have a pleasant taste (RACHED, 2009), also it has been used in the treatment of diarrhea (LAHSISSENE *et al*., 2010). This genus has also been introduced as a medicinal vascular plant protection and cardiovascular diseases preventive in traditional medicine (SABZGHABAEE *et al*., 2014). Owing to its richness in tannins, flavonoids and coumarin, these natural molecules have anti-substantial activity of butyrylcholinesterase, and therefore can be used in the treatment of Alzheimer's disease (GHOUILA *et al*., 2014). This immense family of polyphenols has an important role in food quality through its antioxidant properties; contribute taste, astringency, flavor, color, long-term stability, and their antimicrobial activities (LARCHER *et al*., 2013). So in order to search for new drugs to contribute the bacteria and viruses, the aim of this study is to investigate the total polyphenols content of wild *R. pentaphylla* fruits collected at different maturity stages and to evaluate their biological activities.

2. MATERIAL AND METHODS

2.1. Fruit sampling

R. pentaphylla were collected from March to June 2015 in Kroumirie Mountains (Ain Draham Municipality, Governorate of Jendouba situated at an altitude of 602 m, at 36°44’57 North latitude and 08°41’12 34’’ East longitude) Tunisia (EL MAKNI and EL AOUNI, 2011). An experienced plant taxonomist presented during collection. At this point, 3 kg of fruits at two ripening maturities stages, green-yellow color fruits (un-ripe stage; S1) and purple-red color fruits (fully-ripe stage; S2) were collected at the same time to ensure subsequent maturity classes would develop on the clone. The collected fruits were transported in the same cooling and light conditions to chemistry department of Faculty of Sciences Bizerte. The different stage of fruits was immediately washed with distilled water and lyophilized at -80°C for 48 hours. Chemical analyses were conducted on the composite lyophilized fruit samples collected over time.

2.2. Chemicals, standards, and reagents

Gallic acid, (±)-catechin hydrate ($C_{15}H_{14}O_6$),and 2,2-diphenyl-1-Picrylhydrazyl (DPPH) were purchased from Univar and Sigma-Aldrich (France).Folin-Ciocalteu's reagent was purchased from Carlo Erba Reagents. 2,4,6-Tri (2-pyridyl)-s-triazine ($C_{18}H_{12}N_6$) was purchased from Acros Organics. Butylated hydroxytoluene (BHT, $C_{15}H_{24}O$), and Iodonitrotetrazolium chloride ($C_{19}H_{13}C_{11}N_5O_2$) were purchased from Fluka (Turkey). Aluminium chloride ($AlCl_3$), Vanillin, all other used chemicals and solvents (methanol, ethanol, acetone, hexane, HCl and DMSO) were purchased from Merck (Darmstadt, Germany).

2.3. Extract preparation

R. pentaphylla fruits at different maturity stages were processed separately. Soxhlet, maceration and ultrasonic extraction techniques were recommended in the literature (MOLLICA *et al*., 2018; GÜNAYDıN *et al*., 2017). Approximately, 6 g of freeze-dried fruits were stirred with 60 mL methanol: water (80:20,V/V) with vortex (IKA MS 3 basic) at 2300 rpm for 1 min. The mixtures were then extracted three times in an ultrasonic bath for 15 minutes (Elma Transsonic DIGITAL, T=25 °C, and F= 150W). The collected extracts were centrifuged at 4000 rpm (Eppendorf Centrifuge and Rotor Packages Model 5810), for 10 minutes, then filtered through a Whatman No. 4 paper and the organic phase (methanol) were evaporated under vacuum evaporator (Heidolph). The resulting aqueous phases were washed with water to dissolve the extracted material from the glassware. Afterwards the aqueous material was lyophilized and stored until use. The obtained lyophilized materials were dissolved in a known volume of methanol and DMSO for the quantification of polyphenols content, and the determination of antioxidant and microbial activities, respectively.

2.4. Determination of total phenolics content

The total phenolic contents of each extract were determined by the modified Folin Ciocalteu method described by Singleton (SINGLETON *et al*., 1999; BLAINSKI *et al*., 2013). Briefly, 1 ml of methanol extracts was added to 1 ml of Folin Ciocalteu reagent diluted with distilled water (1:9). The samples were left in room temperature for 5 minutes to develop color; the reaction was then stopped by adding 10 mL of Na_2CO_3 (7%). After homogenization and vortex, the mixture was heated for 10 minutes at 40°C, and then incubated in dark for 30 min. Measurement of the absorbance was carried out in UV spectrophotometer (Thermo scientific EVOLUTION 201) at 765 nm. Total phenol concentration was calculated from the regression equation of the calibration curve established using different concentrations from stock solution of gallic acid (12-152 $\mu g.mL^{-1}$). The results were expressed as mg of gallic acid equivalent per gram of dry weight (mg Eq. GA. 100 mg^{-1}DW).

2.5. Determination of flavonoids content

Flavonoids content of *R. pentaphylla* fruit extracts were carried out by following colorimetric assay using aluminum trichloride ($AlCl_3$) (ZHISHEN *et al*., 1999). 0.3 mL of $NaNO_2$ (5%) was added to 1 mL of methanol extract of each sample. After 6 min, 0.3 mL of 10% methanol solution of $AlCl_3.6H_2O$ was added to the extract samples. To the mixtures, 2 ml of NaOH (1M) and 10 mL of H_2O were added, followed by keeping them in dark for 2 hours. Absorbance was read at 520 nm after incubation in the dark at room temperature

for 10 min. The results were expressed in mg catechin equivalent 100 mg^{-1} dry weight (mg Eq.Cat.100 mg^{-1}DW).

2.6. Determination of condensed tannins content

Determination of condensed tannins is based on phenolic compounds condensation with vanillin under acidic conditions, which will provide a brown compound (PRICE *et al*., 1978). Briefly, 100 mg of lyophilized plant materials were dissolved in 1mL of methanol and added to 2 ml of 1% vanillin in 70% sulfuric acid H_2SO_4 (1 g vanillin in 77.77 mL of H_2SO_4 completed with distilled water to prepare 100 mL). The entire mixture was placed in a water bath for 15 min at 30°C protected from light. Finally the absorbance of the mixture was read at a wavelength λ = 500 nm using a Thermo scientific EVOLUTION 201 UV-Visible Spectrophotometer. The results were expressed in mg catechin equivalent 100 mg^{-1} dry weight (mg Eq.Cat.100 mg^{-1}DW).

2.7. Antioxidant activities

2.7.1 DPPH assay

Among the tests to determine the radical scavenging ability, 2,2-diphenyl-1-picrylhydrazyl (DPPH) analysis is one of the most widely used. Indeed, the DPPH assay is rapid, simple, stable, and economical to measure the antioxidant capacity of foods or plant extracts (JABBARI and JABBARI, 2016). Briefly for each extract, a 50 $mg.mL^{-1}$ stock solution had been prepared with methanol as solvent, and if necessary sequential dilutions were made (SOKMEN *et al*., 2004). Then, for all extracts solution, 50 μL was added to an equal volume of freshly DPPH radical solution (4.10^{-3}mM) and allowed to stand for 30 min in the dark at room temperature. The experiment was repeated for three times. The entire mixture was placed in the dark at room temperature for 30 min, and the absorbance of the mixture was read at λ = 517 nm using a UV-Visible Spectrophotometer. BHT was used as the standard control.

2.7.2 FRAP assay

Ferric reducing antioxidant power (FRAP) was performed according to the methods of BENZIE and STRAIN (1999) with slight modifications (PULIDO *et al*., 2000) were used as an evaluation of the reducing power of samples. The stock solution (50 $mg.mL^{-1}$) of each extract was prepared in methanol. A portion of 50µL solution was mixed with 1.5 mL of FRAP reagent in a test tube and vortexed. Blank samples were prepared with methanol and deionized water. Both samples and blank were incubated in a water bath for 30 minutes at 37°C and the absorbance of the samples was determined against blank at 593 nm. Series of stock solution at 62.5, 125, 250, 500 and 1000 µM were prepared using an aqueous solution of $FeSO_4.7H_2O$ as the standard curve. The values obtained were expressed as μM of ferrous equivalent Fe (II) per gram of freeze-dried sample. Increases in absorbance due to the formation of a colored TPTZ-Fe^{2+} complex were monitored at 593 nm. A Trolox per gram curve (0.1- 0.8 μM) was used as a standard.

2.8. Antimicrobial/Antifungal Tests

The same procedure given above was employed for the extraction. The evaluation of antimicrobial activities of *R. pentaphylla* extracts was carried out using three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcusfaecalis* ATCC 29212, *Listeria*

monocytogenes ATCC 19115) and two Gram-negative bacteria (*Escherichiacoli* ATCC 25922and *Pseudomonasaeruginosa* ATCC 27853) and against one fungal strain (*Candidaalbicans* ATCC 10231).

Broth dilution method

Micro-dilution technique was one of the most basic methods for antimicrobial susceptibility testing (BALOUIRI *el al*., 2016). The used pre-culture contain Muller-Hinton Broth medium (MHB), although a pre-culture McFarland 0.5~10^8 UFC.mL^{-1} (Colony Forming Unit) ~ 0.2 OD (optical density). The ad of 2 mL of sodium chloride (NaCl) or 0.9% phosphate buffered saline (PBS) to vials followed by the addition of bacteria with a cotton rod, and the optical density is measured at λ = 600 nm to obtain an absorbance 0.2 for all used bacterial strains. PBS is used as a blank. Each well of a sterile 96-well microtitre plate-shaped V was aliquoted with an equal volume of Mueller-Hinton broth and fruits extract initially dissolved in DMSO. We conducted a series of dilution, for the first eleven well, we added 100 μL of fruit extracts and we add then 100 μL of Muller Hinton Broth solution (Ca^{2+}, Mg^{2+}). Concentrations (5; 2.5; 1.25mg.mL^{-1}; and 4.88µg.mL^{-1}) are taken into a waterfall and the last well was used for the addition of control's bacteria. After 10 to 15 min, the microplate was incubated in the oven at 37°C for 24 hours. To each well 40μL of colored indicator iodonitrotetrazolium, $C_{19}H_{13}C_{11}N_5O_2$ chloride (200μg.mL^{-1}) was added.

3. Statistical analysis

All results are expressed as (±SD) of total phenolic, flavonoid and tannins contents. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a Tukey's HSD post hoc test. Correlation tests were used to determine relationships among the polyphenols content and antioxidant activities during ripening stages of *R. pentaphylla* fruits. Significance overall and within any correlation (confirmed by linear regression test) was accepted at $p<0.05$.

4. RESULTS AND DISCUSSION

4.1. Phenolic contents

R. pentaphylla fruits showed a highly significant ($p\leq0.01$) increase in total phenolic content during ripening stage, The order of total phenolic content (Table 1), of samples during ripening is [(S1)<(S2)].

The concentration increased from 158.65 for the unripe fruits to 177.57 mg Eq.AG.100 mg^{-1}DW for the fully-ripe fruits. From the obtained results, it can be seen that the phenolic contents of fruits could also be affected by experimental conditions and various factors such as variety, growing condition, maturity, season, amount of sunlight received. This result was in agreement with previous work (MARINOVA *et al*., 2005; NAVARRO *et al*., 2006), where phenolic compounds increase during the last stage especially for red colored varieties, and it's increasing with maturation.

Table 1. Total polyphenols content and antioxidant activities at different maturity stages of *R. pentaphylla* fruits extract.

		1st stage: un-ripe	2nd stage: fully-ripe
Total polyphenols content	TPC (mg Eq.AG. 100 mg^{-1}DW)	158.65±0.06**[a]	177.57±0.06**[a]
	TFC (mg Eq.Cat.100mg^{-1}DW)	157.34±0.07**[b]	152.69±0.14**[b]
	CTC (mg Eq.Cat.100mg^{-1}DW)	200.26±0.3**[c]	131.23±0.24**[c]
Antioxidant activities	DPPH (IC_{50} mg.mL^{-1})	0.42	< 0.5
	FRAP (µmol Fe^{2+}.g^{-1}DW)	64.36	81.59

Values are expressed as (±SD) (n=2), **highly significant ($p \leq 0.01$), [a]: statistic measurement between un-ripe and fully-ripe fruits for phenolic contents, [b]: statistic measurement between un-ripe and fully-ripe fruits for flavonoid contents, [c]: statistic measurement between un-ripe and fully-ripe fruits for tannins contents.

4.2. Flavonoid contents

Fruit ripening are related to important biochemical changes such as color, texture, taste and other quality traits modify. As seen, ripening stage caused a decrease in total flavonoids content; the results of the distribution of TFC in relation to fruit maturity/ripening stages are presented in Table 1. The concentration of flavonoid varies in the following order: [(S1)> (S2)], so as the fruit maturity progressed, there is a highly significant ($p \leq 0.01$) decrease from 157.34 to 152.69 mg Eq.Cat.100 mg^{-1} DW of FC in *R. pentaphylla* fruits. This decrease in TFC with advanced maturity is in agreement with the study of TLILI *et al*. (2014) who reported that the decrease of flavonoid content during ripening may be due to metabolic production of other phenolic compounds or degradation via enzyme action.

4.3. Condensed tannins content

As seen in Table 1, maturity stages of *R. pentaphylla* fruits had a marked effect on the amounts of condensed tannins. The variations of flavan-3-ol content during ripening follow this order [(S1) > (S2)] of ripening stages (a highly significant ($p \leq 0.01$) decrease). The higher concentrations of condensed tannins are found in S1 un-ripe stage (200.26mg Eq.Cat.100mg^{-1}DW) approximately two times more than those of fully-ripe (S2), Flavan-3-ol present a drastic declined during fruit ripening (KENNEDY *et al*., 2000). This change in tannins content in the later stages of ripening could be explained by the fact that different phenolic acids might have condensed to form complex phenolic compounds such as tannins and lignin (BEN AHMED *et al*., 2009).
A significant difference ($p < 0.05$) was demonstrated by ANOVA with a Tukey's HSD post hoc test (Table 1), which confirm that the variation of this polyphenols content (phenolic, flavonoid, and condensed tannins contents) was influenced by the stage of maturation and/or their interaction according to fruit shape.

4.4. DPPH assay

Methanol extracts of *R. pentaphylla* fruits crude were investigated through in vitro models radical scavenging activity using DPPH method. Inhibitory concentration IC_{50} value is the most critical value that reflects the antioxidant action of the tested species. If this value is low, the tested sample has a high antioxidant activity. The IC_{50} value of standard synthetic antioxidant BHT was ranging 0.17 $mg.mL^{-1}$. Table 1 shows that the 1st stage (un-ripe) of *R. pentaphylla* exhibited better antioxidant action about 0.42 $mg.mL^{-1}$, then the 2nd stage (fully-

ripe). The Antioxidant power of *R. pentaphylla* fruits is due to a wealth of flavonoid and tannins, which have been reported to have multiple biological effects, including antioxidant activity due to the redox properties of their phenolic hydroxy groups and the structural relationships between the different parts of the chemical structures (RICE-EVANS and MILLER, 1996).

4.5. FRAP assay

The ferric reducing antioxidant power (FRAP) was measured for each extract obtained at stages 1 and 2. Results were given in Table 1. The obtained values had been expressed as μM of ferrous equivalent Fe (II) per gram dry weight. From the results given in Table 1, the 2nd stage of *R. pentaphylla* exhibited better antioxidant principles then 1st stage (81.59 and 64.36 μmol $Fe^{2+}.g^{-1}$DWrespectively). The order of total activity is the same as total phenol content. A further investigation by PALAFOX-CARLOS *et al.* (2012), who suggest that the physiological and ripening process in fruit may affect directly the presence of phenolic compounds content and their antioxidant activity which increased during ripening.

4.6. Correlation between total polyphenols content and antioxidant activity

As result for this study, there was a positive correlation between the polyphenols content ($r = 0.876$), the two test (DPPH and FRAP) and the maturity stages, but this correlation is not significant (linear regression test $p = 0.0513$); the results of the regression is the same in the case of multiple regression (case of this test) and that when we made the regression one by one.

4.7. Antimicrobial potentials

Antimicrobial potential of *R. pentaphylla* extracts obtained from the two stages was tested separately. The results obtained from the first and the second stages using micro-dilution technique were given in Table 2.

Table 2. Antimicrobial activity of different crude extracts of *R. pentaphylla.*

	MIC($mg.mL^{-1}$) Methanol/Water	
Bacterial strains	**First stage**	**Second stage**
Escherichia coli	1.25	1.25
Staphylococcus aureus	-	-
Enterococcus faecalis	-	-
Pseudomonas aeruginosa	-	1.25
Listeria monocytogenes	-	-

The evaluation of in vitro antibacterial activity of methanol/water extract of *R. pentaphylla* fruits at different maturity stages against the in-use bacteria, showed that the two stages of fruits extract exhibited antibacterial activity against three positive Grams (*Enterococcusfaecalis*, *Listeria monocytogenes*, and *Staphylococcus aureus*) and two negative Grams (*Pseudomonas aeruginosa* and *Escherichia coli)* bacteria at different concentrations. MIC values of the two stages are ranged 1.25 $mg.mL^{-1}$ for the tested microorganisms. Methanol/water extracts of both stages of *R. pentaphylla* exhibited important antibacterial effects against *Escherichia coli* and *Pseudomonasaeruginosa*. From the obtained results

Escherichia coli was found to be the most sensitive organisms for the two stages. The results prove that there might be synergism between polyphenols content and antimicrobial (CUSHNI and LAMB, 2005). However, *R. pentaphylla* showed no antifungal effect against C*andida albicans* at its different maturity stages.

4. CONCLUSIONS

Quantitative analysis of widely *R. pentaphylla* fruit extracts revealed richness in secondary metabolites such as phenolic, flavonoids and condensed tannins. A highly significant difference was demonstrated which confirm that the variation of this polyphenols content was influenced by the stage of maturation and/or their interaction according to fruit shape. DPPH test showed that the first stage has the higher antioxidant activity, which is not the case for FRAP test. A good correlation between the polyphenols content, the two test (DPPH and FRAP) and the maturity stages. As a result of this study, the validation of bioactivity of this fruit as agro alimentary in operating pharmacy was confirmed. In fact, fruit extracts at different maturity stages present a good potential as antibacterial compounds against the tested microorganisms comparing with the commercial antibiotics. So we could suggest that this wild fruits could be used in the treatment of infectious diseases caused by resistant microbes.

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PAPER

INFLUENCE OF EXOPOLYSACCHARIDE ON THE GROWTH OF LACTIC ACID BACTERIA

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ABSTRACT

The highest production of exopolysaccharides (EPSs) by *Lactobacillus buchneri* GM3701 and *Lb. plantarum* RB-3 was 216 and 79.0 mg/L, when incubated in 10% glucose media at 25°C for 6 d and 5% glucose media at 25°C for 4 d, respectively. The EPSs consisted of mainly glucose. Bacterial growth in the media supplemented with the EPSs was investigated using various bacteria, including *Lactobacillus, Staphylococcus* and *Escherichia* strains. The EPS enhanced the growth of *Lb. farciminis* HM2001. This result suggests that the growth of some lactic acid bacteria can be enhanced by the supplementation with an EPS produced by *Lactobacillus* strains.

Keywords: exopolysaccharide, growth enhancement, Lactobacillus, yeast extract

1. INTRODUCTION

Exopolysaccharides (EPSs) are long-chain carbohydrate polymers that are released by a wide range of microorganisms, including fungi and bacteria (DONOT *et al*., 2012). EPSs are present outside of the cell wall, and they exhibit great diversity, not only in their sugar composition but also in their linkage, branching, and substitution (CHAPOT-CHARTIER and KULAKAUSKAS, 2014). EPSs can be bound or unbound to the cell wall, and cell-bound EPSs are distinguished into capsular polysaccharides (CPS) (CAGGIANIELLO *et al*., 2016). The physiological role of bacterial EPSs is not yet completely understood. EPSs may be associated with cell protection against unfavourable environmental conditions, like desiccation, the presence of oxygen or toxic compounds, low temperatures, high osmotic pressures, and bacteriophage attack, and they may contribute to the uptake of metal ions, biofilm formation, and cell adhesion mechanisms (CAGGIANIELLO *et al*., 2016; CERNING, 1990; SANCHEZ *et al*., 2006). On the other hand, LIU *et al*. (2017) reported that EPSs produced by *Lactobacillus plantarum* inhibited the biofilm formation of *Pseudomonas, Escherichia, Salmonella*, and *Staphylococcus*.

Generally, it is thought to be very unlikely that bacteria can use EPSs as an energy source; however, there are some studies that have reported that EPSs are degraded by lactic acid bacteria (LAB). PHAM *et al*. (2000) reported that EPSs produced by *Lactobacillus rhamnosus* were degraded by the enzymes of this strain and that some reducing sugars were liberated. Additionally, some *Bifidobacterium* strains can breakdown plant cell wall polysaccharides (VAN DEN BROEK *et al*., 2008). Furthermore, the growth of LAB and *Bifidobacterium* strains were enhanced by supplementing the cultures with the EPS produced by lactobacilli (HONGPATTARAKERE *et al*., 2012; KORAKLI *et al*., 2002; RUIJSSENAARS *et al*., 2000; TSUDA and MIYAMOTO, 2010). It is unclear if this enhancement was because of the utilization of monosaccharides degraded from the EPSs. To begin with, there are only a few reports about the influence of EPSs on the growth of LAB, and more studies are necessary to better understand the influence of EPSs on the growth of LAB. We should point out that crudely purified EPSs may contain mannan from the yeast extract in media, and the mannan may be used by some bacteria.

In this study, EPSs, produced by a *Lactobacillus* strain, were investigated for their yields and monosaccharide components. Furthermore, the influence of EPSs on growth was evaluated using *Lactobacillus, Streptococcus, Staphylococcus, Aerococcus,* and *Escherichia* strains.

2. MATERIAL AND METHODS

2.1. Bacterial strains

In the present study, 22 bacterial strains, including *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Staphylococcus, Aerococcus, Paenibacillus,* and *Escherichia coli*, were used (Table 1). The strains were isolated from Wagyu milk, Japanese pickles and fermented sushi at our laboratory unless otherwise stated (TSUDA *et al*., 2012; TSUDA, 2015). The lactic acid bacteria were incubated in TYG broth (10 g/L tryptone, 5.0 g/L yeast extract, 5.0 g/L glucose, 1.0 g/L Tween 80, and 0.1 g/L L-cysteine HCl monohydrate, pH 6.8 ± 0.2). The other strains were also incubated in TYG broth to compare the growth rate. All strains were stored in 10% reconstituted skim milk at -20°C. An inoculum of 1% was used for all tests.

Table 1. Strains used in the present study.

Species	Strain No.	Source
Lactobacillus reuteri	PUHM1004	Wagyu milk
Lb. coryniformis	SAB01	Japanese pickles
Lb. sakei subsp. *sakei*	SAB04	Japanese pickles
Lb. delbruckii subsp. *bulgaricus*	NBRC 13953	*
Lb. alimentarius	EM2001	Fermented sushi
Lb. casei	HM3701	Fermented sushi
Lb. buchneri	GM3701	Fermented sushi
Lb. farciminis	HM2001	Fermented sushi
Lb. acidipiscis	JAM3706	Fermented sushi
Lb. plantarum	JAB2001	Fermented sushi
Lb. plantarum	RB3	Japanese pickles
Lb. plantarum	PUHM1023	Wagyu milk
Enterococcus faecalis	PUHM1006	Wagyu milk
Lactococcus lactis	PUHM1014	Wagyu milk
Streptococcus thermophilus	NBRC 13957	*
Str. salivarius	AB3002	Fermented sushi
Str. pluranimalium	PUHM1022	Wagyu milk
Aerococcus viridans	PUHM5301	Wagyu milk
Staphylococcus auricularis	PUHM5201	Wagyu milk
Sta. aurigulas	PUHM5203	Wagyu milk
Paenibacillus turicensis	PUHM5101	Wagyu milk
Escherichia coli	NBRC 102203	*

*: NBRC: NITE Biological Resource Center.

2.2. Effects of the incubation conditions on exopolysaccharide (EPS) production

Lb. buchneri GM3701 and *Lb. plantarum* RB-3 were used as EPS-producing LAB. The EPS productivities were tested using the 22 strains in Table 1, and the cultures of the two strains showed ropiness. Therefore, strains GM3701 and RB-3 were selected as EPS producers. The ropiness was confirmed by inserting a sterile wire loop and pulling ropes from the media. A clear zone area using the Indian ink method was used as a simplified indicator of the EPS yield. Twenty microliter of LAB culture was put on a glass slide, and a few drops of Indian ink were added and mixed. A cover slip was placed over the mixture, and then, the prepared slide was observed microscopically with immersion oil. A clear zone area for each individual cell was obtained as follows: the cell area was subtracted from the clear zone area, and then, the obtained figures were divided by the cell numbers. This assay was performed with at least 10 clumps.
The effect of the incubation temperature on EPS production was investigated at 25, 30, and 37°C. Glucose, fructose, sucrose, and lactose were supplemented in TY broth (10 g/L tryptone, 5.0 g/L yeast extract, 1.0 g/L Tween 80, and 0.1 g/L L-cysteine HCl monohydrate, pH 6.8 ± 0.2) as carbon sources at 25, 50, and 100 g/L.
All assays were performed at least three times.

2.3. Preparation of the EPSs

A modified version of the method from LINDSAY *et al*. (2003) was used to prepare EPSs from bacterial culture. EPSs in the bacterial culture were precipitated with two volumes of cold ethanol, followed by stirring for 1 h at 4°C. The precipitated EPSs were collected by suction filtration, and the collected EPSs were dissolved in deionized water. The EPSs were again precipitated with two volumes of cold ethanol and subsequently lyophilized. Furthermore, EPSs from the TYG broth were prepared using the same method.
The lyophilized EPSs were analysed for their carbohydrate and protein content. The total amount of carbohydrates in the lyophilized EPSs was determined with the phenol-sulphuric acid method using glucose as the standard (DUBOIS *et al*., 1956). The protein content was determined using the protein-dye binding method with bovine serum albumin as the standard (BRADFORD, 1976).
All assays were performed at least three times.

2.4. Monosaccharide analysis of the EPSs

The lyophilized EPSs were hydrolysed in 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. After hydrolysis, the water and TFA were removed with a centrifugal concentrator (CC-181, TOMY, Tokyo, Japan), and the dry sample was dissolved in water. The hydrolysed EPS solution was spotted onto silica gel thin layer chromatography plate (Merck, Tokyo, Japan), along with standard solutions for glucose, galactose, mannose, arabinose, xylose, and rhamnose. The plate was developed in 1-butanol:2-propanol:H_2O (3:12:4), dried, sprayed with a phenol-sulfuric solution, and then heated at 110°C for 15 min to visualize brown spots (Adachi, 1965; Huebner *et al*., 2007). The monosaccharides of the EPSs were further analysed as follows. The sugar composition was determined by high pressure liquid chromatography with refractive-index detection (column: Sugar-D (Nacalai tesque, Kyoto, Japan); mobile phase: 85% acetonitrile; flow rate: 1.0 mL/min; temperature: 30°C). Glucose, mannose, N-acetylglucosamine, arabinose, xylose, and rhamnose were used as the standards.

2.5. Influence of the EPSs on bacterial growth

Growth in media with EPSs as the sole carbon source was tested with the 22 strains. Glucose medium was used as a control. The growth rate was determined using a modified version of the method from Tsuda and Miyamoto (2010). Briefly, the tested strains were inoculated into TY broth (10 g/L tryptone, 5.0 g/L yeast extract, 1.0 g/L Tween 80, and 0.1 g/L L-cysteine HCl monohydrate, pH 6.8 ± 0.2) containing 0.2% (w/v) glucose and the lyophilized EPSs, and the cultures were incubated for 24 h at 37°C. The optical density at 660 nm (OD_{660}) of the culture was measured at 0 and 24 h. All assays were performed at least three times. The growth rate against glucose was determined using the following equation:

$$\text{Growth rate} = (\text{Log } OD_{660} \text{ of TYE at 24 h} - \text{Log } OD_{660} \text{ of TYE at 0 h}) / (\text{Log } OD_{660} \text{ of TYG at 24 h} - \text{Log } OD_{660} \text{ of TYG at 0 h})$$

TYE: TY broth containing the EPSs produced by strains GM3701 or RB-3
TYG: TY broth containing glucose

2.6. Statistical analysis

To identify differences, a one-way analysis of variance (ANOVA) was applied to the means, and the Student-Newman-Keuls test ($P<0.05$) was applied using Statview 5.0 software (SAS Institute, Cary, NC, USA).

3. RESULTS AND CONCLUSIONS

3.1. Effects of the incubation conditions on EPS production

Lb. buchneri GM3701 and *Lb. plantarum* RB-3 are EPS-producing LAB strains. Ropiness was confirmed with a loop, and the clear zone surrounding the cell was confirmed by the Indian ink method. The effects of the incubation temperature and carbon source, which included glucose, fructose, sucrose, and lactose (100 g/L), on EPS production were investigated with *Lb. buchneri* GM3701 and *Lb. plantarum* RB-3 (Fig. 1). Concerning strain GM3701, the EPS yield was higher at 25 and 30°C than at 37°C for all of the sugars ($P<0.05$). There were no differences among the four tested sugars at 25°C, and the EPS yield in the glucose media was higher at 30°C than in the sucrose or lactose media ($P<0.05$). Concerning strain RB-3, the EPS yield was higher at 25 and 30°C than at 37°C for all of the sugars ($P<0.05$). The EPS yield in the glucose media was higher than in the sucrose media, and there were no differences among the four tested sugars at 30°C ($P<0.05$). From these results, it was presumed that a suitable incubation temperature and sugar for EPS production by strains GM3701 and RB-3 were 25°C and glucose, respectively. Therefore, these incubation conditions were applied in the series of tests.
Subsequently, the effect of the carbon concentration (25, 50, or 100 g/L) on EPS production was investigated with glucose at 25°C (Fig. 2). EPS production by GM3701 was higher at 100 g/L glucose after 5, 6, and 7 days than at 25 or 50 g/L ($P<0.05$), and the production by RB-3 was higher at 50 and 100 g/L glucose after 4 days than at 25 g/L ($P<0.05$).
The EPS yields are likely to decrease after reaching a maximum, as many studies have reported, and this is caused by enzymes, such as glycohydrolase, that are produced by bacteria (Pham *et al*., 2000). However, it is unclear whether the degraded EPSs were used for growth in that paper.

3.2. Characteristics of the EPSs

The EPS-producing strain was incubated at the above condition, and then, the EPSs were purified by ethanol precipitation and lyophilized. Similarly, the EPSs from the TYG broth were lyophilized. The polysaccharide (PS) yield from the TYG broth was 50.8 mg/L, and the EPS yields from strains GM3701 and RB-3 were 340 and 146 mg/L, respectively (Table 2). The carbohydrate and protein contents in these lyophilized EPSs are shown in Table 2. All of the EPSs contained more than 76% carbohydrates and less than 9.6% protein. These results confirmed that the lyophilized EPSs were not proteinaceous slime. The monosaccharide analysis of the EPSs was done using seven monosaccharides that are known as constituents of EPSs (glucose, galactose, mannose, N-acetylglucosamine, arabinose, xylose, and rhamnose) as standards. The PSs from the TYG broth consisted mainly of mannose.

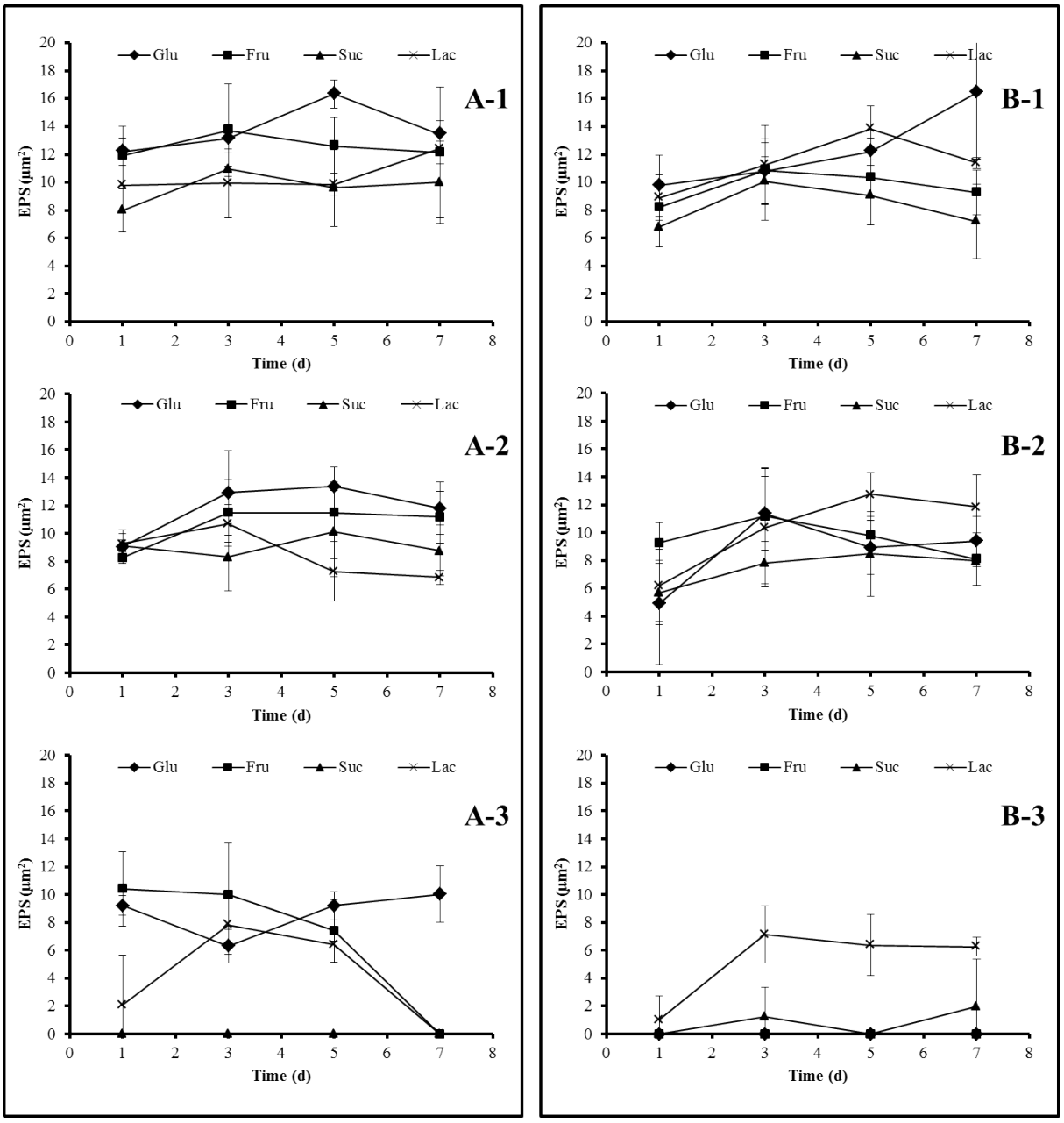

Figure 1. Effects of the incubation temperature (1: 25°C, 2: 30°C, 3: 37°C) and carbon source on EPS production by *Lb. buchneri* GM3701 (A) and *Lb. plantarum* RB-3 (B). Bars represent the standard deviation from the mean (n=3). ♦: glucose, ■: fructose, ▲: sucrose, ×: lactose.

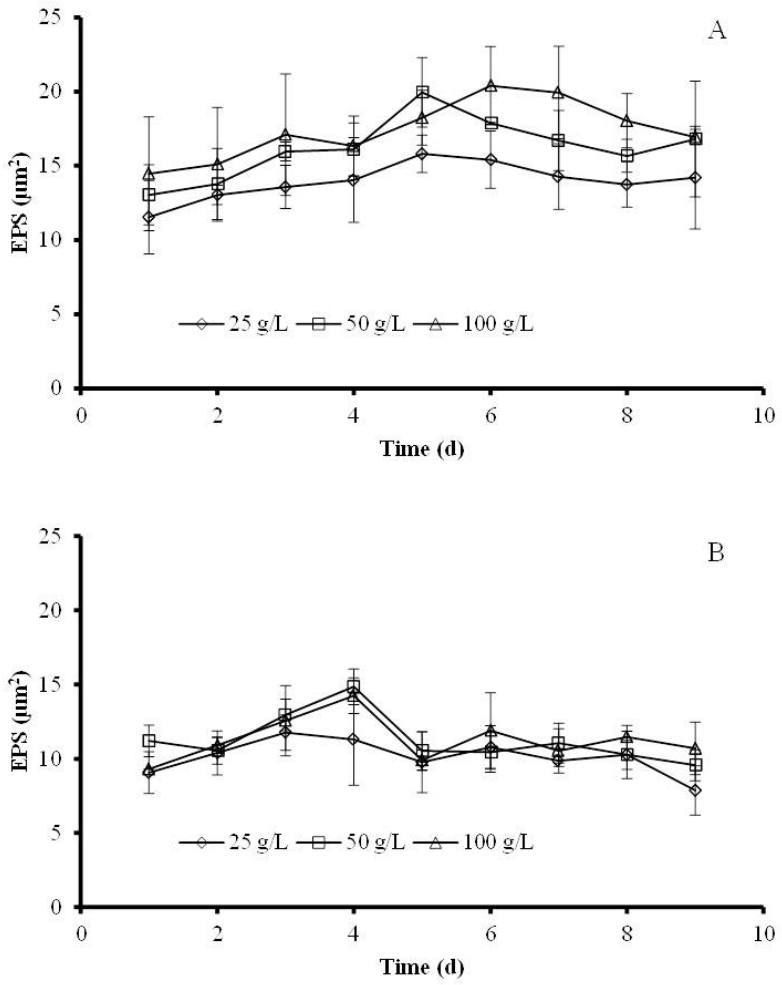

Figure 2. Effect of the glucose concentration on EPS production by *L. buchneri* GM3701 (A) and *L. plantarum* RB-3 (B). Bars represent the standard deviation from the mean (n=3). ◊: 25 g/L, □: 50 g/L, Δ: 100 g/L.

Table 2. EPS yields and the carbohydrate and protein concentrations in the lyophilized EPSs.

Strain	EPS yield (mg/L)	Carbohydrate (%)	Protein (%)	Composition of the EPS (%)			
				Glucose	Galactose	Mannose	Rhamnose
GM3701	340	76.0	9.6	73.2	-	23.7	trace*
RB-3	146	83.2	3.5	27.2	-	58.6	14.1
TYG broth	50.8	83.0	2.8	12.0	-	82.8	-

*: trace means less than 10%.

The correct yields and monosaccharide components of the EPSs produced by the LAB strains were estimated by subtracting the PS values, while taking the carbohydrate concentration into consideration (Table 3). The calculated EPS yields for the strains GM3701 and RB-3 were 216 and 79.0 mg/L, respectively. Glucose was found to be a major component of the EPS produced by strain GM3701, and glucose, mannose, and rhamnose

were found to be the predominant sugar residues in the EPS produced by the strain RB-3 (Table 3). Glucose and rhamnose are typical components of many EPSs produced by LAB. The quantities of the hetero-EPSs produced by LAB vary greatly. The production of EPS is 50-350 mg/L for *Str. thermophilus*, 80-600 mg/L for *Lc. lactis* subsp. *cremoris*, 60-150 mg/L for *Lb. delbrueckii* subsp. *bulgaricus*, 50-60 mg/L for *Lb. casei* (CERNING, 1995), and approximately 140 mg/L for *Lb. plantarum* (STAAF *et al*., 2000; TSUDA and MIYAMOTO, 2010). The highest recorded yields for hetero-EPSs are 2775 mg/L for *Lb. rhamnosus* RW-9595M (MACEDO *et al*., 2002) and 2500 mg/L for *Lb. kefiranofaciens* WT-2B (MAEDA *et al*., 2004). The EPS yields from *Lb. buchneri* GM3701 and *Lb. plantarum* RB-3 were 216 and 79.0 mg/L, respectively, and these values were thought to be a normal value for *Lactobacillus* EPSs.

Table 3. EPS yields and monosaccharide components of lyophilized EPSs, obtained by subtracting the values for the EPSs from the TYG broth.

Strain	EPS yield	Composition of the EPS (%)		
	(mg/L)	Glucose	Mannose	Rhamnose
GM3701	216	70.9	trace*	trace
RB-3	79.0	41.7	29.0	28.3

*: trace means less than 10%.

The PS from the TYG broth was thought to be mannan. It is well known that purified EPSs are contaminated with the mannan from the yeast cells in yeast extract. Glucose and rhamnose are the usual components of many EPSs produced by LAB (CAGGIANIELLO *et al*., 2016; DONOT *et al*., 2012; SANCHEZ *et al*., 2006), and the mechanisms of glucose incorporation into the polysaccharide chain are well known (DE VUYST *et al*., 2001). There are some reports about EPSs composed of glucose and mannose that are produced by *Lactobacillus* (HASHIGUCHI *et al*., 2011; SANCHEZ *et al*., 2006).

3.3. Growth enhancement by the EPSs

The growth rates of the EPSs against glucose for the 22 strains are shown in Fig. 3. The highest growth rate was observed with *Lb. farciminis* HM2001 ($P<0.05$). All of the 22 tested strains showed an OD_{660} of more than 0.3 when they were incubated in TYG broth for 24 h. The growth rates of the EPSs against glucose were calculated, and all of the tested strains showed a growth rate of less than 0.1, except for strain HM2001. The EPSs produced by strains GM3701 and RB-3 showed growth rates of 0.146 and 0.113 with strain HM2001, respectively.
Although the monosaccharide composition of the EPSs was different between the two EPSs (Table 3), the growth of *Lb. farciminis* HM2001 was enhanced by supplementation with either of the EPS produced by the LAB. The growth enhancement of this strain did not occur following supplementation with the PS from the TYG broth (data not shown). This suggested that the EPSs produced by *Lb. buchneri* GM3701 and *Lb. plantarum* RB-3 enhanced the growth of strain HM2001. On the other hand, the EPSs produced by *Lb. plantarum* RB-3 did not enhance the growth of the three tested *Lb. plantarum* strains.

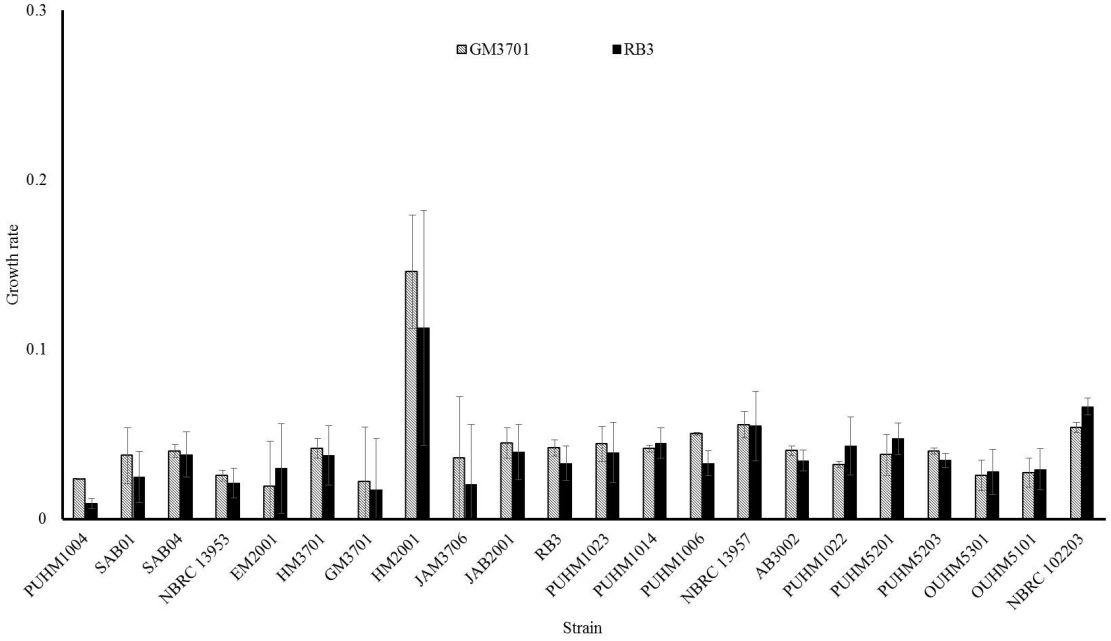

Figure 3. Growth rates of the EPSs against glucose for the 22 strains.

Therefore, no species specificity was shown for growth enhancement by EPSs in this study. We think that strain HM2001 may have the enzymes that degrade the EPSs produced by LAB, and the degraded sugars may be utilized by strain HM2001; the EPS or degraded carbohydrate chains may stimulate the growth by working like an extracellular signalling molecule. Another factor, such as the charge and linkage types of the EPS and the combinations of the enzymes that degrade EPSs, may be involved in the utilization of EPSs. RUSSO (2012) reported that β-D-glucan in EPSs enhanced the growth of *Lb. plantarum* and *Lb. acidophilus*. Therefore, a lot of β-D-glucan may exist in the EPSs used in this study. Further work is needed for a better understanding of the physiological importance of EPSs.

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PAPER

BIOACTIVE POLYPHENOL PROFILES AND ANTIOXIDANT ACTIVITY IN ITALIAN APPLES VARIETIES

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ABSTRACT

In this study ten organic apple varieties grown in Italy: Renetta Osiris, Gold Rush, Braeburn, Celato Cola, Limoncella, Cerina, Rosada, Topaz, Jonagored, Florina were analysed in order to evaluate some quality parameters. Individual phenolics compounds, total phenolics, glucose, fructose and antioxidant activity was determined in pulp and peel extracts. Results show that the chlorogenic acid was a predominant component in pulp and peel extracts, with highest value in Limoncella and Jonagored respectively. A significant correlation between phenolic composition and antioxidant activity was observed. Apple varieties Renetta Osiris and Gold Rush presented significant values on glucose and fructose in pulp.

Keywords: apple fruit, DPPH, HPLC, phytochemicals

1. INTRODUCTION

Apple (*Malus domestica*) is one of the most ancient fruits. It was born in Minor Asia, south of Black Sea and arrived in Greece from Egypt, where it was grown along Nyle valleys (XIII century B.C.). Afterwards, it quickly spread all over Europe and during the XVI century it appeared in North America (FORSLINE *et al*., 2003; COART *et al*., 2006). It is currently the second fruit produced and consumed all over the world (70 million tons). Italy produces 1.9 millions tons of apples a year and is the fifth largest producer worldwide and the third largest exporter in the world after China and Poland. Italy, along with India, is one of the major consumers of apples per capita, each person eats an average of 15 pounds of apples per year (two a week). In Germany 10, in France 8, in the United States only 5 (FAOSTAT, 2013). Apples are grown all over the Italian territory, but production is traditionally concentrated in the mountain and foothill regions, particularly in Valle d'Aosta, Piemonte, Veneto and Trentino-Alto Adige regions. In the past, the old varieties of fruits locally grown were quickly abandoned accordingly to a higher rationality of production with the choice of more productive varieties. In 1920, about 50 varieties of apples were marketed but they were reduced to 10 today with a loss of 80%. During the twentieth century the development of the sector has grown in connection with the increase of production, using particular attention to the territory and natural cultivation techniques with a low environmental impact. Currently, a particular attention is dedicated to the improvement of biodiversity and to the organic production (BERTSHINGER *et al*., 2004,; RAGANOLD *et al*., 2001). The quality of apple fruits depends on the bioactive compounds: polyphenols, organic acids and carbohydrates. The concentration of the compounds differs with varieties, maturity stage and environmental conditions (EISELE and DRAKE, 2005). Phenolic substances are considered important in nutrition because of the positive effect on human health as a result of their high antioxidant capacity (SERRA *et al*. 2012, EBERHARD *et al*., 2000). Epidemiologic studies associate the biological activity of these substances to the prevention of cancer and cardiovascular diseases, neuropathies and diabetes (RIBEIRO *et al*., 2014; BOYER *et al*., 2004; BALASURIYA *et al*., 2012; SCALBERT *et al*., 2000). The aim of this study is to evaluate the composition of different apples varieties, produced by organic methods representative of Italian biodiversity. The variability on the composition of bioactive compounds: individual phenolic compounds, total phenolics, fructose and glucose in pulp and peel tissue was determined. The influence of the total phenolic compounds on the antioxidant activity was evaluated.

2. MATERIAL AND METHODS

2.1. Standard and reagents

The standard compounds gallic acid, (+) catechin, (-) epicatechin, chlorogenic acid, phloretin, rutin, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), Formic Acid, Folin-Ciocalteau reagent, sodium carbonate, Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich (MO USA). Methanol of HPLC grade and hydrochloric acid were purchased from Merck (Germany). The water used was purified in a Milli-Q system (Millipore, Bedford, MA, USA). The extracts samples and standard solution were filtered through membrane 0.45 μm (Millipore Bedford, MA, USA).

2.2. Apple fruit samples

Apples samples from the varieties: Renetta Osiris (Trentino Alto Adige), Gold Rush (Toscana), Braeburn (Toscana), Celato Cola (Sicilia), Limoncella (Abruzzo), Cerina (Lazio), Rosada (Lazio), Topaz (Trentino Alto Adige), Jonagored (Trentino Alto Adige), Florina (Trentino Alto Adige), were purchased from local organic producers in different Italian regions during the period September-October 2015. The analysis was conducted within two days.

2.3. Samples preparation and procedure

The apple samples of peel and pulp have been separated and the pulp (5 g) and the peel (5 g) fractions were immediately homogenized with methanol containing 1% hydrochloric acid. The samples have been extracted with 10 ml of solvent for 30 min and 10 ml for 30 min using a Ultrasonic sonicator at room temperature (25°C). The extract, pulp and peel separately, were combined and filtered through a membrane 0,45 μm pore size prior to injection in HPLC for determining the individual phenolic compounds, antioxidant activity and total phenolics. Extracts were stored at -4°C for 1 day. Later at -20°C for 1 week.
The recovery efficiency of phenolic compounds was determined by spiking amounts of standards compounds considered to the fraction pulp and peel of Braeburn apple prior to extraction. The recovery study was performed in triplicate.

2.4. Apparatus

HPLC analyses were performed on a Shimadzu HPLC system, a LC-10AT liquid chromatograph equipped with four pumps FCV-10AL, a degasser DGU-14A, a Rheodyne 7725i injector with a 20 μl sample loop (Rheodyne, Berkeley, CA, USA) and a photodiode array detector SPD-M20A. The eluted compounds were monitored at 280 nm and the adsorption spectra between 250 and 350 nm. Spectrophotometric determinations were performed with a Shimadzu UV-Vis 1800 spectrophotometer.

2.5. Identification and quantification of individual phenolic compounds

The column used was a C18 Supelcosil LC (150 mm x 4.6 mm, 3 μm particle size), and a guard column. The mobile phase was a mixture: formic acid in water (2%, pH 3, solvent B) and formic acid in methanol (2%, pH 3, solvent A). The gradient program was time 0.01 95% B, 15 min 50% B, 20 min 30% B, 25 min 20% B, 30 min 95% B. 5 min of equilibration was required before the next injection. The flow rate was 0.6 mL min and the analyses were conducted at room temperature (25°C). The injected volume was 20 μL. Peak identification was performed by comparing the retention times and diode array spectral characteristics with external standards. Quantification was performed using calibration curve of standards. Data were processed using Shimadzu LC solution software. The determination of sugar content, glucose and fructose were determined according method previously described (KARKACIER *et al*., 2003).

2.6. Total phenolic content assay (TPC)

The total phenolics content in the apple extracts was determined using the Folin-Ciocalteau procedure according to procedure previously described (SINGLETON *et al*., 1999) with some slight modifications: 50 μl extract solution was added to 2.5 ml of Folin-

Ciocalteu reagent, 2 ml of 20% % Na_2CO_3 solution. The mixture was incubated for 30 min at 40°C. Absorbance increase was measured at 765 nm. Chlorogenic acid was used as standard (0-100 mg l^{-1}), and a calibrate equation was obtained (R^2 = 0.9961). Total phenolic content is expressed as mg chlorogenic acid g^{-1}.

2.7. Antioxidant activity assay

The antioxidant activity was estimated using the 1,1-diphenyl-2-picryldydrazyl (DPPH). Free Radical Scavenging Method according to method previously described with minor adaptation (SHARMA and BHAT, 2009). The DPPH reagent 25 mg were dissolved in 1000 ml of methanol(A), and the solution have been diluted 1:10 (B). Two ml of solution B were mixed with 100 μl of the extract methanolic solution and kept at 25°C for 30 min in a dark room. A decrease absorbance was determined at 515 nm using spectrophotometer. Radical Scavenging activity was calculated by the following formula:

$$\text{Scavenging } \% = [\text{Abs}_{t0min} - \text{Abs}_{t30min} \ / \ \text{Abs}_{t0min}] \times 100$$

where A_0 was the absorbance of DPPH blank solution, and A was the final absorbance of the tested sample after 30 min of incubation. Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) 0.05 mM in methanol was used as a standard to convert the inhibition capacity of the apple extract solution to the Trolox equivalent antioxidant activity (r^2= 0,9980).

2.8. Statistical data analysis and graphic program

All results were analyzed by Pearson correlation and R^2coefficient was determined. These data were elaborated with Graph Pad Prism 5.0 software and statistical significance was estabilished at $p<0.05$.

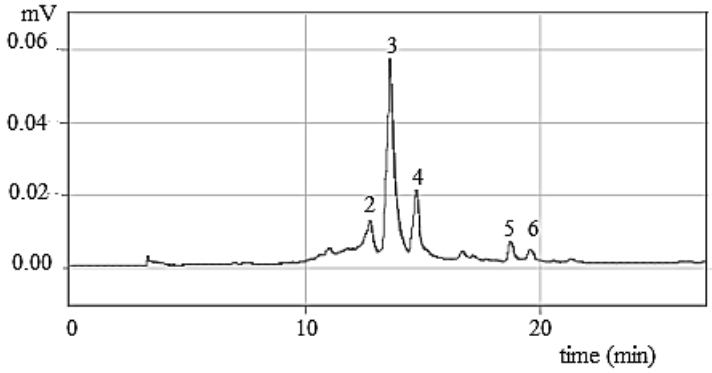
3. RESULTS AND DISCUSSION

In this study ten apple varieties, by organic production, were selected in order to represent the new orientation of the Italian quality organic production aiming to evaluate quality parameters. The apples types considered in the study are usually consumed as fresh fruits in Italy: Renetta Osiris, Gold Rush, Braeburn, Celato Cola, Limoncella, Cerina,Rosada, Topaz, Jonagored, Florina coming from the organic production of different Italian regions: Trentino Alto Adige, Toscana, Lazio, Abruzzo, Sicilia. Among them: Limoncella (Abruzzo), Cerina (Lazio), Gelato Cola (Sicilia) have been produced in small quantities and purchased from selected producers that aim to appraise enhances biodiversity of production territories.

3.1. Quantification of individual phenolic compounds

The determination of individual phenolic compounds was performed by RP-HPLC using the gradient elution method reported in the experimental section. Fig. 1 shows the chromatograms of the extracts from the pulp and peel apple of Braeburn varieties, obtained at λ 280 nm.

pulp



peel

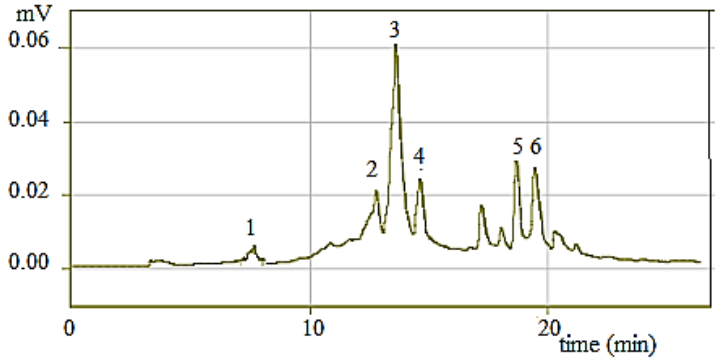


Figure 1. Chromatogram of apple Braeburn varieties, pulp and peel extracts using RP-HPLC, as 280 nm. Peaks: 1 gallic acid, 2 (+) catechin, 3 chlorogenic acid, 4 epicatechin, 5 rutin, 6 phloretin.

Table 1 shows the calibration parameters of the analytical method. Concentration of the linear range were determined, for all phenolic compounds, by triplicate analysis of four different standard concentrations. Recoveries were determined in both pulp and peel apple extract by spiking apple samples with a solution containing known amounts of all standard mixture compounds prior to the extraction procedure. The study was conducted in triplicate. The obtained values ranged from 92%±2.5 SD - 98%±2.2 SD for all analytes.
Compositions of the individual phenolic compounds: gallic acid, catechin, epicatechin, chlorogenic acid, rutin and phloretin in apples varieties, pulp and peel, are shown in Table 2 and Table 3. The results on apples pulp show that chlorogenic acid is the major compound present in all examined apples varieties and ranged, in the pulp, between 800.3 $\mu g g^{-1}$ Limoncella to 52.7 in Braeburn, in the peel between 766.6 $\mu g g^{-1}$ in Jonagored to 187.1 in Renetta Osiris.

Table 1. Retention time (T_r) and calibration parameters of apple phenolics.

Compounds	$T_{r\ min}$±SD	Linear range ($\mu g\ ml^{-1}$)	R^2	Detection limit LOD ($\mu g\ ml^{-1}$)
Gallic acid	7.5±0.04	2-50	0.9958	1.6
(+) Catechin	12.4±0.03	2.5-110	0.9902	1.5
Chlorogenic acid	13.6±0.06	1.5-200	0.9925	0.7
(-) Epicatechin	14.7±0.03	3.0-100	0.9928	0.6
Rutin	18.9±0.02	2.5-120	0.9958	0.5
Phloretin	19.8±0.03	08-80	0.9991	0.7

Mean value (n=3) $\mu g\ ml^{-1}$±SD, l 280 nm.

Table 2. Amounts of phenolics compounds in apples varieties, pulp methanolic extract.

Apples varieties	Gallic acid	Catechin	Chlorogenic acid	Epicatechin	Rutin	Phloretin
Renetta Osiris	4.7±0.4	34.1±2.2	224.1±2.2	30.3±0.8	35.5±1.3	1.0±1.2
Gold Rush	<1.6	14.9±0.8	243.6±2.0	70.6±1.3	20.1±0.8	3.4±0.8
Braeburn	<1.6	11.1±2.3	52.7±1.5	170.0±2.1	16.4±0.7	<0.7
Gelato Cola	4.4±1.7	60.7±2.5	400.1±3.1	89.4±1.7	20.1±0.6	1.7±0.8
Limoncella	6.3±1.8	21.2±1.8	800.3±3.5	72.6±1.5	47.2±0.8	<0.7
Cerina	5.0±1.0	69.3±1.6	328.5±2.8	79.3±2.1	46.8±1.1	<0.7
Rosada	8.3±1.1	110.5±2.8	247.7±1.6	30.0±1.6	13.6±1.1	2.5±1.1
Topaz	<1.6	44.6±1.6	230.1±2.7	60.5±1.4	18.0±1.2	3.1±1.2
Jonagored	7.7±2.1	36.3±1.5	400.2±1.6	18.3±0.8	13.4±0.8	3.2±1.0
Florina	<1.6	39.2±1.7	180.70±1.5	34.1±1.1	18.8±1.6	3.1±0.8

Data are expressed as the mean value ($\mu g\ g\text{-}1$)±SD (n=3).

Table 3. Amounts of phenolics compounds in apples varieties, peel methanolic extract.

Apples varieties	Gallic acid	Catechin	Chlorogenic acid	Epicatechin	Rutin	Phloretin
Renetta Osiris	4.4±1.2	45.9±0.6	187.1±1.8	65.6±1.0	102.4±2.3	<0.7
Gold Rush	9.8±1.8	35.9±1.6	400.2±3.2	131.8±0.8	79.4±2.4	4.3±1.3
Braeburn	26.7±1.4	77.8±2.8	315.4±3.1	71.2±0.6	131.3±1.8	1.1±1.0
Gelato Cola	7.2±1.5	100.6±3.1	466.4±3.3	80.1±1.3	128.2±1.6	<0.7
Limoncella	12.3±1.6	110.4±3.1	700.2±3.6	100.3±1.7	42.7±2.2	1.2±0.7
Cerina	6.3±1.1	120.6±3.3	366.6±2.6	166.8±1.3	71.8±2.1	2.2±0.6
Rosada	13.9±1.2	84.7±2.2	500.1±2.7	92.6±0.8	27.7±2.5	<0.7
Topaz	2.3±1.6	141.5±2.8	400.3±2.5	140.1±2.1	233.5±1.8	<0.7
Jonagored	13.9±1.2	127.2±1.8	766.6±3.1	89.3±2.0	186.4±1.6	10.6±0.5
Florina	2.2±2.1	194.3±1.8	203.0±1.8	227.5±2.2	145.8±1.3	19.8±0.7

Data are expressed as the mean value ($\mu g\ g1$)±SD (n=3).

Furthermore (−)epicatechin, (+) catechin, rutin, phloretin and gallic acid were minor phenolic constituents. In particular (+) catechin ranged between 11.1 $\mu g g^{-1}$ in Braeburn

apple to 110.5 in Rosada in pulp extracts, while on the peel between 35.9 $\mu g g^{-1}$ Gold Rush to 141.5 in Topaz. Epicatechin ranged from 18.3 $\mu g g^{-1}$ Jonagored to 170.0 $\mu g g^{-1}$ Braeburn in the pulp extracts, while in peel in the range between 65.6-227.5 $\mu g g^{-1}$ corresponding to Renetta Osiris and Florina, respectively. Rutin content on pulp, ranged from 13.4 $\mu g g^{-1}$ Jonagored to 47.2 Limoncella, on the peel 27.7 $\mu g g^{-1}$ Rosada to 233.5 in Topaz. Phloretin content ranged from, 0.6 $\mu g g^{-1}$ on Cerina and 3.4 $\mu g g^{-1}$ in Gold Rush on pulp, while on peel 0.6 $\mu g g^{-1}$ Renetta Osiris and 19.8 Florina. A poorly significant difference was observed in gallic acid in pulp and peel of all the examined cultivars. However, an higher content of all the examined phenols in peel extracts compared to the one in the pulp one was found with the exception of phloretin in Renetta osiris, Gelato cola, Rosada and Topaz.

3.2. Total Phenolics and antioxidant activity

Total phenol compounds (TPC) of apple peel extracts are always higher than those in pulp. This is in accordance with the fact that phenolic compounds have the tendency to accumulate in the peel tissues of the plant because of their potential roles in protection against UV radiation and pathogens. In addition, it is clear that total phenol compounds (TPC) of apple peel extracts differed significantly among ten cultivars: TPC was the highest in Florina followed, in decreasing order, by Jonagored, Topaz, Rosada, Braeburn, Limoncella, Cerina, Gelato Cola, Rush, Renetta Osiris. Florina was the variety with the highest TPC (9.60±0.8 μg chlorogenic acid g^{-1}) in the peel among all cultivars under study, whereas the lowest value 1.70±0.1 μg chlorogenic acid g^{-1} was observed in Renetta Osiris. In pulp, the values ranged between 0.9±0.1 to 4.9±0.5 μg g^{-1} chlorogenic acid; Rosada had the lowest values and Limoncella, Gelato Cola and Jonagored the highest value. It means that, also in the pulp, TPC is highly related to the specific cultivar. Evaluation of antioxidant activity was conducted by DPPH method which reveals the colorimetric decrease in absorbance of the radical DPPH due to the chemical trapping of the unpaired electron. Antioxidants interact with DPPH neutralizing its free radical character. Table 4 shows the results related to Total Phenolics and the DPPH activity of extracts obtained by using methanol acidified with 1% of HCl.

Table 4. Total phenolics and antioxidant activity in apple varieties, pulp and peel methanolic extract.

Apples varieties	Total phenolics*		Antioxidant activity** (μmol g $^{-1}$)	
	pulp	peel	pulp	peel
Renetta Osiris	1.0±0.3	1.7±0.1	243.9±0.2	297.2±0.2
Gold rush	1.0±0.2	2.3±0.3	236.2±0.2	293.2±0.2
Braeburn	1.4±0.2	3.9±0.4	294.8±0.2	363.2±0.3
Gelato cola	2.0±0.4	3.0±0.3	319.5±0.6	360.8±0.4
Limoncella	2.9±0.5	3.4±0.5	349.4±0.5	307.0±0.4
Cerina	1.9±0.2	3.3±0.7	290.7±0.3	348.2±0.4
Rosada	0.9±0.1	3.9±0.6	216.6±0.5	351.8±0.3
Topaz	4.9±0.5	1.5±0.6	311.1±0.7	397.4±0.4
Jonagored	2.0±0.4	6.6±0.6	325.4±0.7	370.9±0.5
Florina	1.5±0.2	9.6±0.8	319.8±0.7	377.3±0.6

Data espressed* (chlorogenic acid μg g^{-1})±SD mean value (n=3).
** data expressed (μmol g^{-1} Trolox)±SD mean value (n=3).

In the pulp, the value ranged between 216.6±0.5 to 325.4±0.7 (μmol g^{-1}Trolox) respectively for Jonagored and Rosada varieties, in the peel 293.2±0.2 to 377.3±0.6 (μmol g^{-1} Trolox) for Gold rush and Florina varieties. The highest values in peel are also evidenced for antioxidant activities. This is may be due to the different distribution of polyphenolics compounds: the peel, in addition to the compounds present in pulp, have additional phenolic compounds not found in the pulp such as anthocyanins and a high amount of quercetin glycosylate (VIERA *et al*. 2009). This appears more evident when we compared antioxidant activity in peel extracts between red and yellow apples. The relation between antioxidant activities and polyphenolic concentration is previously reported (VAN der SURS *et al*, 2001; D'ANGELO *et al*., 2007). In this study total phenolic compounds content was positively correlated with antioxidant activity in apple pulp (r^2= 0.870) and peel (r^2=0.699) respectively. The results suggest that, in apple, phenolic compounds have a significant contribution to the antioxidant capacity according to literature data (LEE *et al*., 2003).

3.3. Glucose and fructose content

In order to evaluate the sugar content, glucose and fructose have been quantified in apple pulp and peel extracts (Figure 2 and Figure 3). The fructose content ranged between 1.32 $g100g^{-1}$ (Florina) and 4.45 (Gold Rush); glucose from 1.25 $g100g^{-1}$ (Florina) to 3.0 (Rosada) in the pulp, while in the peel fructose ranged between 0.80 $g100g^{-1}$ (Florina) and 3.40 (Renetta Osiris), glucose 0.50 $g100g^{-1}$ (Florina) to 2.35 (Rosada). The results show the lowest amount in apple Topaz, Jonagored and Florina, whereas these cultivars present significant value in total phenolic content. On the contrary, apple varieties Renetta Osiris and Gold Rush, present significant values on glucose and fructose and low content of total phenolic compounds.

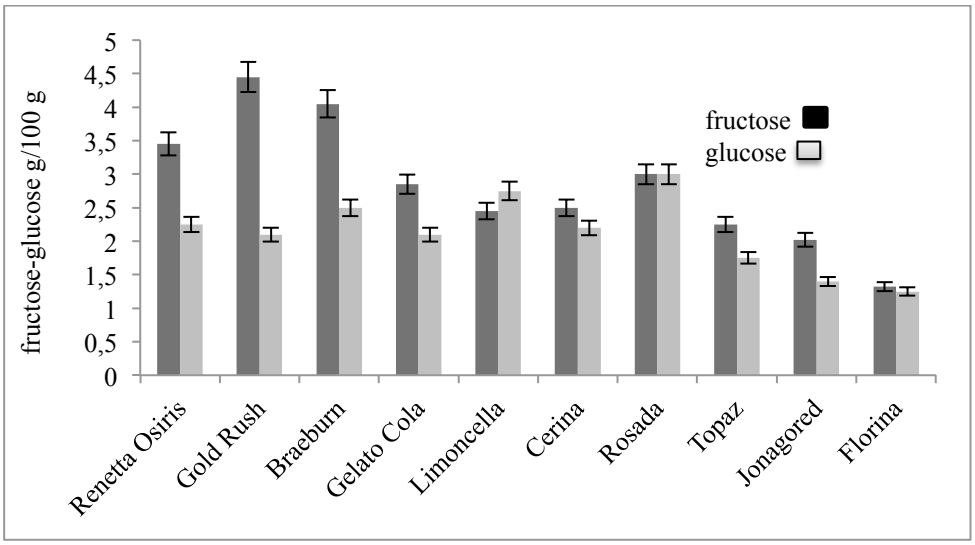


Figure 2. Fructose and Glucose content in pulp of apples varieties ($g\ 100g^{-1}$ ± SD, n=3).

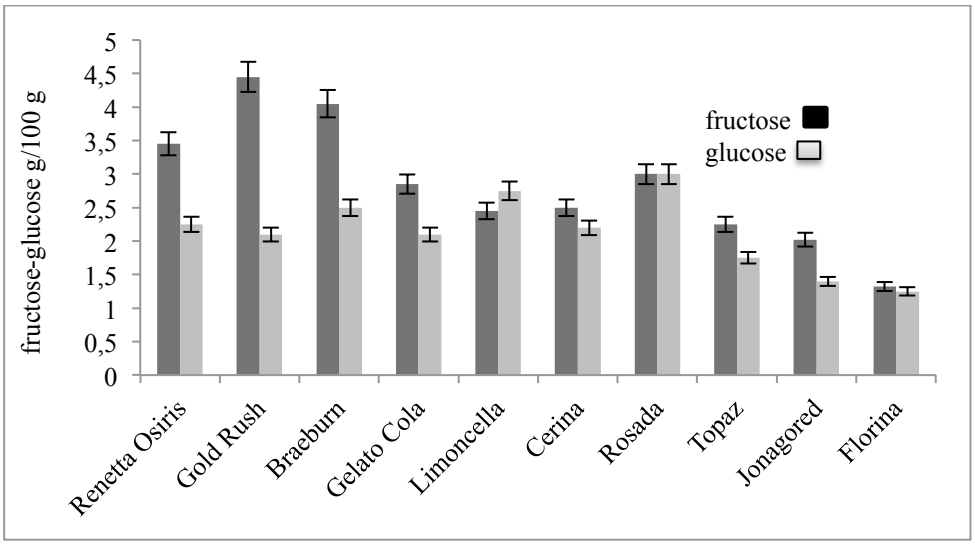


Figure 3. Fructose and Glucose content in peel of apples varieties (g $100g^{-1}$ ± SD, n=3).

4. CONCLUSIONS

In conclusion, phenolic compounds, secondary metabolites naturally present in plant material, are important to determine quality characteristics of apple. The bioactive substances quantified show that the commodity is a good source of nutraceutical compounds and denote significant differences in the apples varieties considered. Moreover, the apple peel, generally considered as waste, of the examined commodities presents significant contents of individual phenolic compounds, glucose and fructose and, consequently a high antioxidant capacity.

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PAPER

CHANGES IN THE ANTIOXIDANT CAPACITY AND POLYPHENOLS CONTENT OF RYE-BUCKWHEAT CAKES FORTIFIED WITH SPICES DURING THEIR LONG-TERM STORAGE

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ABSTRACT

The long-term storage of cakes may affect their functional and physicochemical properties. In this study, we investigated changes in the antioxidant capacity and polyphenols content of rye-buckwheat cakes with selected spices, e.g. cloves, nutmeg, cinnamon, vanilla, allspice and a commercial blend of spices, during their 18-month storage at 23°C, 60% humidity. Measurements were performed after each 6 months. During storage, chemical markers decreased considerably. The observed decrease in the antioxidant capacity was highly correlated with contents of total phenols, flavonoids and rutin. The use of cloves, cinnamon and a spice mix in rye-buckwheat recipe offered a higher functionality of cakes.

Keywords: antioxidant capacity, flavonoids, rye-buckwheat cakes, storage, total phenolics, spices

1. INTRODUCTION

According to the report of the Polish Agriculture Market Agency, the consumption of cookies and cakes had been increasing till 2008 and became stable for many years (AGRICULTURE MARKET AGENCY, 2013). Producers develop new recipes to attract consumers with new flavors and enrich confectionery products with healthy ingredients. Spices are well-known sources of natural antioxidants, which might be used as preservatives or flavor enhancers in cookies and biscuits (EMBUSCADO, 2015). A wide range of compounds with different molecular structures has been suggested to be responsible for the high antioxidant status of spices (YASHIN *et al.*, 2017). Therefore, they might significantly influence the quality and sensory parameters of confectionery products, but also increase their functional properties like e.g. their antioxidant activity (NANDITHA and PRABHASANKAR, 2009). PASQUALONE *et al*. (2014) described the positive influence of grape marc (by-product with high antioxidant potential) on biscuits' flavor and sensorial parameters, e.g. by more intense color and fruity odor formation. Powders are the main form in which spices are incorporated into confectionery products. Therefore, BAJEJ *et al.* (2006) demonstrated that better bakery parameters as well as consumer acceptability were achieved for biscuits incorporating mint powder than for biscuits with either mint extract or menthol. In some cases, however, essential oils of spices – which are generally acceptable by consumers – are added to provide the antioxidant capacity of confectionary goods (BASUNY *et al.*, 2012). Cinnamon, mint, nutmeg and cloves were indicated as the most commonly used spices and herbs in confectionary (NANDITHA and PRABHASANKAR, 2009). Garlic, ginger, basil, turmeric or coriander powders were also used in some studies with wheat bread (DZIKI *et al*., 2014). The addition of spices, as the main sources of antioxidants, is expected to prolong storage stability of confectionery products, ensure consumer acceptability and provide healthy properties.

According to many reports, consumer acceptability may be affected by the formation of oxidative compounds, which might create an unpleasant aroma and/or rancid and bitter taste in stored products. Oxidation may be effectively inhibited by antioxidants, therefore recipes of confectionery products were modified to increase antioxidants content in many ways. For example, WATANABE *et al.* (2014) reported that the addition of quinoa flour to wheat cookies increased their antioxidant capacity and then improved their oxidative stability during 50-day storage period (lower peroxide value of cookies incorporating 7.5% of quinoa flour). ZIELIŃSKI *et al.* (2012) demonstrated a greater increase in the antioxidant capacity of ginger cakes made of rye flour, instead of wheat flour, after long-time storage. They speculated this might also be due to the formation of some of the Maillard reaction products, e.g. melanoidins, which was indicated by the higher values of the browning index. In turn, JENSEN *et al.* (2011) reported no significant changes in the oxidative status during storage of wheat bread incorporated with a rosemary extract or an α-tocopherol solution. However, enhanced hydroperoxide formation was determined in the samples prepared with α-tocopherol, which means that the rosemary extract protected food from oxidation. In studies of NING *et al.* (2017) the addition of green tea powder to wheat bread significantly increased its overall antioxidant capacity and inhibited the formation of peroxides during 8-day storage (which was described as lower peroxide values, even at only 1% of green tea addition) as compared to wheat bread. Also interesting results were obtained by DAR *et al.* (2016), who developed rice snacks with a combination of cereal brans. According to their results, the antioxidant capacity of these snacks decreased significantly during 6 months of their storage, i.e. about 25 up to 28%. They also noticed the negative effect of storage on total phenolic contents, which might be due to low-stability of molecules in the matrix and advanced degradation process of phenolic

compounds. The reason of these changes was explained as "dilution of antioxidant components by increased moisture" or oxidation of antioxidant molecules during long-term storage, but these are only speculations as causes these changes are still unknown. Little literature focused on changes in the antioxidant capacity and phenolic content in cereal products is available today.
Therefore, the aim of this study was to determine changes in total phenolic and flavonoid contents during 18-month storage of rye-buckwheat cakes fortified with selected spices. The spices used in rye-buckwheat cakes formula were: cloves, nutmeg, cinnamon, vanilla, allspice and a commercial blend of spices for cakes. The content of rutin, as the main polyphenol occurring in buckwheat flour and honey, was determined to control its changes during storage period. Furthermore, antioxidant capacity was evaluated by measurements of the scavenging ability against $DPPH^{\cdot}$ and $ABTS^{\cdot\cdot}$ radicals. This study focused also on the antioxidant stability of rye-buckwheat cakes during storage. Moreover, spices with the best impact on storage stability of the analyzed cakes were selected. The measurements were performed every 6 month till the 18^{th} month of storage period and compared with results obtained immediately after baking.

2. MATERIAL AND METHODS

2.1. Chemicals

Standards of rutin (quercetin-3-rutinoside), gallic acid and catechin as well as chemicals used for antioxidant capacity determination: 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), sodium nitrite, aluminum chloride, and sodium carbonate were purchased from Sigma (USA). Ethanol, acetonitrile and formic acid were obtained from Merck (Germany). Folin-Ciocalteu reagent was provided by POCh (Poland). For HPLC analysis, water was purified with the Mili-Q-system (Milipore, USA).
The spices used in rye-buckwheat cakes formula as: cloves (*Syzygium aromaticum* L.), nutmeg (*Myristica fragrans* H.), cinnamon (*Cinnamomum verum* J.), vanilla (*Vanilla* Mill.), allspice (*Pimenta dioica* L.) and a commercial blend of spices for cakes, were provided from local market (Kotányi, Poland). According to the producer's declaration, commercial spice mix contains cinnamon, pepper, clove, anise, coriander, fennel and nutmeg. TPC values of each spice were previously calculated in our studies (PRZYGODZKA *et al.*, 2014): clove 156.67±3.52 (mg GAE g^{-1}), nutmeg 11.80±0.55, cinnamon 180.59±14.23, vanilla 8.08±0.49, allspice 183.92±1.26, and spice mix 101.33±5.66. Also TF of selected spices was measured (mg rutin g^{-1}): clove 19.78±0.54, nutmeg 10.21±0.17, cinnamon 96.81±1.95, vanilla 3.97±0.37, allspice 16.82±0.07, and spice mix 64.21±1.82.

2.2. Preparation of rye-buckwheat cakes and their storage conditions

Preparation of rye-buckwheat cakes with selected spices was described in detail in our previous work (PRZYGODZKA *et al.*, 2016).The composition of ingredients used is listed in Table 1. The rye-buckwheat cakes fortified with cloves, nutmeg, cinnamon, vanilla, allspice, and a spice mix were prepared while rye-buckwheat cakes without spices served as a control sample. After baking, the cakes were cooled down and part of them were freeze-dried, ground into powder and then used directly for analyses ("0"), whereas the others were packed into plastics bags with clips, stored at room temperature, without light

for 6, 12 and 18 months, and afterwards defrosted and ground into powder to prepare cake extracts.

Table 1. Composition of rye-buckwheat cakes with selected spices.

Ingredients (g)	C	Cakes with spices
rye flour	70	70
light buckwheat flour	30	30
buckwheat honey	50	50
sugar	20	20
baking powder	3	3
butter	25	25
cloves/ nutmeg/ cinnamon/ vanilla/ allspice or spice mix	0	2

2.3. Rye-buckwheat cake extracts preparation

About 100 mg of powdered rye-buckwheat cakes were placed in 2-mL Eppendorf flasks, to which 1 mL of an extraction solvent (ethanol/water 1:1, v/v, the solvent was selected according to our previous study (PRZYGODZKA *et al*., 2014) was added. The mixture was vortexed for 1 min and then extracted with ultrasonic waves for 30 sec. Then, it was centrifuged at the maximum speed and a temperature of 4ºC for 15 min. The supernatant was then transferred to a 5-mL volumetric flask and the residues were re-suspended in another 1-mL portion of the ethanol/water solution. This step was repeated until 5 mL of the extract had been obtained. These extracts were used for further analyses of rutin, total phenols and flavonoids contents, and for antioxidant capacity determination with ABTS and DPPH assays.

2.4. Determination of rutin, total phenolics and flavonoids contents

The content of rutin in rye-buckwheat cakes was determined with the HPLC system (Shimadzu, Japan) with a UV detector (SPD-10A) set up at 330 nm according to the procedure presented by ZIELIŃSKA *et al*. (2010). Five concentrations of a rutin standard were prepared in triplicate in the range of 1.0-40 μM, then solutions were filtered through nylon filter membranes, with pore size of 0.45 μm, before injection. The results were expressed in μg g^{-1} of dry weight. The TPC and TF contents were determined as previously described by PRZYGODZKA *et al.* (2014). TPC content was expressed in terms of mg gallic acid equivalents (GAE) g^{-1} of dry weight, whereas TF content was expressed as mg of catechin (CA) g^{-1} of dry weight.

2.5. Determination of antioxidant capacity (AC) by ABTS and DPPH assays

The antioxidant capacity of rye-buckwheat cakes enriched with spices was determined by ABTS assay as described by PRZYGODZKA *et al.* (2014). Also scavenging ability against DPPH radicals was measured according to PRZYGODZKA *et al.* (2015). The results provided by both ABTS and DPPH assays were expressed as μmol of Trolox equivalents (TE) $g^{-1}$of dry weight.

2.6. Statistical analysis

The results of the analyses are given as the means and the standard deviation of three independent extractions (mean±SD). Statistical one-way analysis of variance (ANOVA) using Fischer test was performed. The significance level was set at $p< 0.01$. Pearson correlation coefficients were calculated to enable correlations analysis of the results of rutin, total phenolic and flavonoids contents, and results of ABTS and DPPH assays. The correlations were calculated for each type of rye-buckwheat cakes fortified with selected spices over 18-month storage period.

3. RESULTS AND DISCUSSION

3.1. Changes of rutin, total phenols and flavonoids contents of rye-buckwheat cakes during storage

Changes in rutin content in cakes made of rye and buckwheat flour mixtures with selected spices during 18-month storage are illustrated in Fig. 1. Generally, a progressive reduction of rutin content was observed in all samples. It the control samples, it decreased significantly after 6 months of storage (almost 45%), to reach finally 86% decrease after 18 months of storage. In the case of cakes with spices addition, after 6 months the lowest decrease was noticed in cakes with allspice and cloves, from 173 to 89 $\mu g\ g^{-1}$ and 320 to 190 $\mu g\ g^{-1}$, respectively.

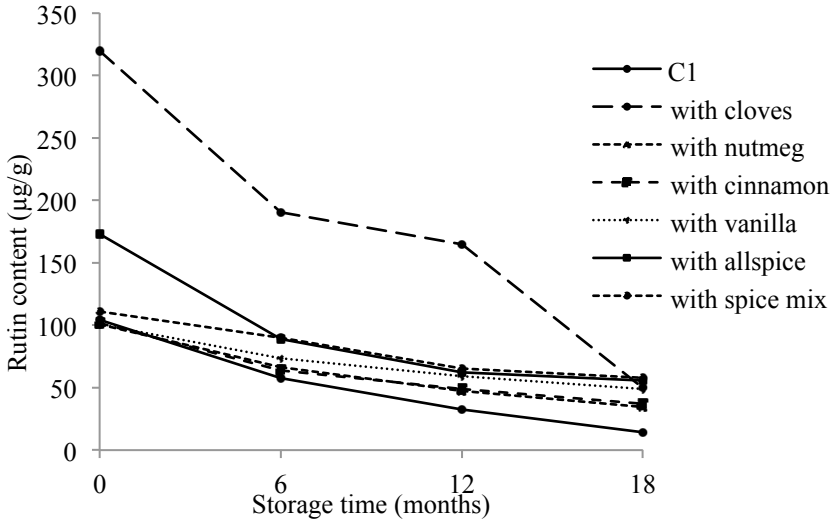

Figure 1. The influence of storage time on rutin content in rye-buckwheat cakes fortified with selected spices. SD were not higher than 10%.

A further decrease in rutin content was observed after 12 months, and after 18 months the lowest rutin content was determined in the cakes with cinnamon and the highest one - in the cakes with the spice mix and allspice. The final rutin content was the highest in the cakes fortified with the spice mix, cloves and vanilla. Our observations are in an agreement with findings reported by SAKAČ *et al.* (2016), who also observed a decrease of rutin content in rice-buckwheat cookies (80:20, v/v) during 16-month storage. They noticed the difference between rutin content in unpacked and packed cookies after the

first 6 months of storage, while afterwards no difference was demonstrated. In this case, it might be hypothesized that the lower rutin content was associated with the formation of new molecules with another compound like lysine, as it was described by ZHANG *et al.* (2016), or that rutin might be degraded to quercetin during the baking process (LUŠKIČ *et al*, 2016). Moreover, DADÁKOVÁ *et al.* (2011) noticed that 12- month storage of black elderflowers at 22ºC impacted a decrease of rutin amount, even much more than of chlorogenic acid. However, storage of asparagus at 4ºC or as freeze-dried flour had no significant influence on rutin content (STOFFYN *et al*., 2012). Moreover, rutin content decrease could be ascribed to the oxidation processes. In model studies of BUCHNER *et al.*(2006), a decrease in rutin concentration was observed in solutions with rutin kept under air perfusion. This effect might be reduced with vacuum packaging of cakes. Not many studies have been focused on changes in contents of individual polyphenols in bakery and/or confectionery products during storage, whereas a high number of research works is available on the monitoring of changes of total phenolic compounds.

Therefore, the total phenolic compounds (TPC) content was monitored every 6 months in rye-buckwheat cakes fortified with selected spices (Table 2). Its decrease was observed in all cakes over the entire period of storage. After 6-month storage it decreased by 6% in the control cakes and by 2% and 24% in the samples with spice mix and nutmeg, respectively. After another 6 months of storage, a significant decrease of TPC content was noticed in almost all cakes samples except for control and with allspice. Then, after 18 months the greatest loss of TPC was determined in the cakes with nutmeg (33%) and the lowest one in the cakes with the spice mix (9%). A significant decrease in TPC content, by 73 and 58%, was also observed in the cakes with cloves and spice mix. Despite the fact that the TPC content decreased by more than a half, still a very high TPC content was noted in the cakes with cloves. Moreover, the highest TPC content was reached in the cakes made of light buckwheat flour and fortified with spice mix, cloves, cinnamon and then allspice. The changes in TPC contents were higher compared to these presented previously by ZIELIŃSKI *et al.* (2012), which might be a consequence of rapid rutin degradation in cakes made of buckwheat flours. Therefore, the higher TPC content in rye-buckwheat cakes before the storage might be due to higher amounts of honey and ginger in their recipe. Our results are in accordance with findings reported by SAKAČ *et al.* (2016), who observed that TPC decreased along the prolonged storage period. After 6-month storage, the TPC content in rice-buckwheat cakes decreased by about 25%, then after 12 months by up to 55%. Moreover, these authors found no difference between TPC contents in the samples stored at 23 and 40°C, whereas slightly higher TPC values were observed in unpacked than in packed cakes.

Table 2. Content of total phenolic compounds (TPC) and flavonoids (TF) in rye-buckwheat cakes fortified with selected spices: cloves, nutmeg, cinnamon, vanilla, allspice and spice mix.

	TPC [mg GAE g^{-1}]						
	Storage time						
spices	0 m	6 m	0-6 m	12 m	0-12 m	18 m	0-18 m
C (without spices)	1.12 ± 0.03^{gA}	1.05 ± 0.07^{eAB}	↓ 6%	1.04 ± 0.01^{fB}	↓ 7%	0.85 ± 0.03^{eC}	↓ 24%
cloves	2.11 ± 0.04^{cA}	2.04 ± 0.06^{bA}	↓ 3%	1.73 ± 0.06^{bcB}	↓ 18%	1.68 ± 0.03^{bB}	↓ 20%
nutmeg	1.56 ± 0.10^{eA}	1.18 ± 0.04^{dB}	↓ 24%	1.09 ± 0.01^{eC}	↓ 30%	1.04 ± 0.01^{dD}	↓ 33%
cinnamon	2.28 ± 0.05^{bA}	2.01 ± 0.06^{bB}	↓ 12%	1.80 ± 0.06^{bC}	↓ 21%	1.66 ± 0.02^{bD}	↓ 27%
vanilla	1.32 ± 0.08^{fA}	1.25 ± 0.07^{dB}	↓ 5%	1.14 ± 0.01^{dC}	↓ 14%	1.01 ± 0.04^{dD}	↓ 23%
allspice	1.84 ± 0.07^{dA}	1.63 ± 0.05^{cB}	↓ 11%	1.62 ± 0.05^{cB}	↓ 12%	1.57 ± 0.02^{cB}	↓ 15%
spice mix	2.70 ± 0.09^{aA}	2.64 ± 0.03^{aA}	↓ 2%	2.53 ± 0.05^{aB}	↓ 6%	2.46 ± 0.03^{aB}	↓ 9%

	TF [mg CA g^{-1}]						
	Storage time						
spices	0 m	6 m	0-6 m	12 m	0-12 m	18 m	0-18 m
C (without spices)	2.02 ± 0.01^{fA}	1.59 ± 0.02^{eB}	↓ 21%	1.37 ± 0.07^{gC}	↓ 32%	1.24 ± 0.01^{eD}	↓ 38%
cloves	3.45 ± 0.01^{aA}	3.11 ± 0.06^{aB}	↓ 10%	3.01 ± 0.07^{aB}	↓ 13%	2.86 ± 0.05^{aC}	↓ 17%
nutmeg	2.20 ± 0.01^{eA}	2.13 ± 0.02^{dB}	↓ 3%	1.98 ± 0.01^{fC}	↓ 10%	1.63 ± 0.02^{dC}	↓ 26%
cinnamon	2.95 ± 0.05^{bA}	2.81 ± 0.05^{bB}	↓ 5%	2.46 ± 0.05^{bC}	↓ 16%	2.00 ± 0.02^{bD}	↓ 32%
vanilla	2.47 ± 0.02^{dcA}	2.35 ± 0.02^{cB}	↓ 5%	2.18 ± 0.01^{dC}	↓ 12%	1.65 ± 0.03^{dD}	↓ 33%
allspice	2.21 ± 0.02^{eA}	2.15 ± 0.05^{dAB}	↓ 3%	2.10 ± 0.02^{eB}	↓ 5%	1.83 ± 0.04^{cC}	↓ 17%
spice mix	2.96 ± 0.03^{bA}	2.79 ± 0.08^{bA}	↓ 6%	2.54 ± 0.04^{cB}	↓ 14%	1.94 ± 0.05^{bC}	↓ 34%

a, b- means in the same column with different letters as superscripts are significantly different ($p < 0.001$).
A, B- means in the same row with different letters as superscripts are significantly different ($p < 0.001$).

In our case, however, the changes in TPC contents were smaller. DAR, SHARMA and NAYIK (2016) also found a smaller decrease of TPC content (at about 17%) in snacks prepared from rice flour and a blend of three brans: wheat, oat and rice (2:1.5:1.5), during storage at room temperature. Also LIANG and WERE (2018) reported on the loss of phenols in butter cookies with different addition of sweeteners. Furthermore, the greatest decrease in TPC contents after 24-hour storage was observed in the cookies with honey and the lowest one in these with xylitol. This might be due to interactions between sugars (glucose, fructose etc.) and formation of Maillard reaction compounds. The loss of TPC was also explained by the formation of favorable polyphenol-sugar adducts which were further rearranged to brown pigments (melanoidins) (ZHANG, CHEN and WANG, 2013). The Pearson coefficient of correlation between TPC and rutin content (Table 4) was very high for almost all samples of cakes fortified with cinnamon (0.987), allspice (0.985), spice mix (0.982), nutmeg (0.974), and vanilla (0.937).

The main flavonoids of common buckwheat kernels are flavonol glycosides: rutin, quercetin, and kaempferol-3-rutinoside (TIAN, LI and PATIL, 2002). Total flavonoid (TF) content was also monitored during rye-buckwheat cakes storage and its significant changes were determined. In the control samples, TF values decreased from 2.02 for "0" time, through 1.59 after 6 months and 1.37 after 12 months and finally to 1.24 mg g^{-1} after 18 months. Furthermore, after 6 months no significant decrease was observed in the cakes with addition of nutmeg, and allspice, whereas 5% decrease was noticed in cinnamon and vanilla, and 6% decrease of TF was noticed in spice mix. Then, 12-month storage caused a further decrease in TF content in the range from 5 to 16% in the cakes with allspice and cinnamon, respectively. After 18 months, the least changes of TF contents were observed in the cakes fortified with cloves and allspice. Therefore, the highest TF content was determined in the cakes with cloves (2.86 mg g^{-1}), cinnamon (2.00 mg g^{-1}), and spice mix (1.94 mg g^{-1}). The decrease in TPC and TF contents in all samples of cakes was observed, however their values in the samples fortified with spices were still higher than in the control sample. According to results of TPC and TF content determination, after long-time storage of the spices-fortified cakes, the cloves, spice mix, and cinnamon might be recommended as good additives for confectionery goods. According to values of correlation coefficients (Table 4), it can be concluded that in the control samples and in the cakes with cloves and spice mix, rutin might have the highest contribution to TF levels in these samples. Whereas, a correlation between TPC and TF contents was the highest in the samples with vanilla (0.970), spice mix (0.950), and cinnamon (0.936).

3.2. Changes of the antioxidant capacity of rye-buckwheat cakes during storage

In the next step of our study, antioxidant capacity was monitored in rye-buckwheat cakes for 18 months of storage based on the scavenging ability of ABTS and DPPH radicals. Results of these determinations are presented in Table 3.
In control cakes, the ABTS values decreased only by 1 and 4% after 6 and 12 months of storage, respectively. Then, a high decrease (27%) was noticed after 18 months. In rye-buckwheat cakes fortified with spices and stored for 6, 12 and 18 months, the least changes in the antioxidant capacity were observed in the cakes with the spice mix (from 1 to 12%) and vanilla (from 4 to 14%). In turn, the greatest decrease in the antioxidant capacity was noticed in the cakes with cinnamon (17%) and allspice (14%) after 6 months, and in the cakes fortified with cinnamon (25- 27%) after 12 and 18 months. In the samples with nutmeg no change was observed after 6 months, then after 12 months the antioxidant capacity dropped drastically to 21% and finally after 18 months to 25%. A decrease of the antioxidant capacity of snacks enriched with bran was also described by DAR, SHARMA and NAYIK (2016). They reported that after 6 months of storage, ABTS values decreased by about 17-18% (conventional and microwave-assisted extraction, respectively). In turn, SAKAČ *et al.* (2016) demonstrated that in packed samples of cookies formulated from 80% of rice and 20% of buckwheat, the antioxidant capacity increased in the first months of storage but after 10 months started to decrease. Whereas from the beginning of storage period, a decreasing tendency was noticed in the packed samples, which were stored at higher than room temperature.

Table 3. Antioxidant capacity determined with ABTS and DPPH assays in rye-buckwheat cakes fortified with selected spices: cloves, nutmeg, cinnamon, vanilla, allspice and spice mix.

	ABTS [µmol TE g^{-1}]						
	Storage time						
spices	**0 m***	**6 m**	**0-6 m**	**12 m**	**0-12 m**	**18 m**	**0-18 m**
C (without spices)	21.13 ± 0.88^{fA}	20.94 ± 0.32^{fAB}	<1%	20.36 ± 0.94^{fB}	↓ 4%	15.36 ± 0.15^{gC}	↓ 27%
cloves	55.52 ± 2.73^{bA}	47.91 ± 0.40^{bB}	↓ 14%	45.62 ± 1.16^{bB}	↓ 18%	43.18 ± 0.40^{bC}	↓ 22%
nutmeg	30.49 ± 0.84^{eA}	30.47 ± 0.28^{eA}	<1%	24.16 ± 0.17^{eB}	↓ 21%	22.98 ± 0.36^{eC}	↓ 25%
cinnamon	49.38 ± 0.19^{cA}	41.10 ± 0.14^{cB}	↓ 17%	37.24 ± 0.41^{cC}	↓ 25%	36.04 ± 0.29^{cD}	↓ 27%
vanilla	21.87 ± 1.21^{fA}	20.91 ± 0.78^{fAB}	↓ 4%	19.83 ± 0.76^{gB}	↓ 9%	17.12 ± 0.25^{fD}	↓ 14%
allspice	40.86 ± 2.28^{dA}	35.98 ± 0.75^{dB}	↓ 12%	34.09 ± 0.63^{dB}	↓ 17%	32.55 ± 0.87^{dC}	↓ 20%
spice mix	63.24 ± 1.31^{aA}	62.61 ± 0.57^{aA}	<1%	57.03 ± 0.13^{aB}	↓ 10%	55.23 ± 0.72^{aC}	↓ 12%
	DPPH [µmol TE g^{-1}]						
	Storage time						
spices	**0 m***	**6 m**	**0-6 m**	**12 m**	**0-12 m**	**18 m**	**0-18 m**
C (without spices)	17.48 ± 0.57^{cA}	16.94 ± 0.67^{cAB}	↓ 3%	16.71 ± 0.10^{bB}	↓ 4%	16.30 ± 0.80^{bB}	↓ 7%
cloves	22.35 ± 0.70^{aA}	21.96 ± 0.88^{aA}	↓ 2%	21.99 ± 0.68^{aA}	↓ 2%	21.56 ± 0.04^{aA}	↓ 4%
nutmeg	9.99 ± 0.16^{gA}	9.96 ± 0.49^{gA}	↓ <1%	9.57 ± 0.35^{eA}	↓ 4%	9.08 ± 0.06^{fB}	↓ 10%
cinnamon	21.39 ± 0.61^{bA}	19.62 ± 0.16^{bB}	↓ 8%	16.38 ± 0.27^{bC}	↓ 23%	16.76 ± 0.65^{bC}	↓ 22%
vanilla	12.26 ± 0.72^{fA}	12.29 ± 0.53^{fA}	↑ <1%	12.11 ± 0.09^{dA}	↓ 2%	11.08 ± 0.02^{eB}	↓ 10%
allspice	15.96 ± 0.60^{dA}	13.78 ± 0.26^{eB}	↓ 12%	13.88 ± 0.08^{cB}	↓ 13%	12.07 ± 0.26^{dC}	↓ 24%
spice mix	15.12 ± 0.19^{eA}	15.23 ± 0.54^{dA}	↑ <1%	13.83 ± 0.25^{cC}	↓ 9%	14.71 ± 0.04^{cB}	↓ 3%

a, b- means in the same column with different letters as superscripts are significantly different ($p < 0.001$).
A, B- means in the same row with different letters as superscripts are significantly different ($p < 0.001$).

Table 4. Coefficients of correlations between rutin, TP and TF contents and antioxidant capacity provided by ABTS and DPPH assays for 18-month stored of rye-buckwheat cakes fortified with selected spices.

Type of cakes	rutin *vs* TPC	rutin *vs* TF	TPC *vs* TF	rutin *vs* ABTS	TPC *vs* ABTS	TF *vs* ABTS	rutin *vs* DPPH	TPC *vs* DPPH	TF *vs* DPPH	ABTS *vs* DPPH
C (without spices)	0.842	**0.999**	0.821	0.734	**0.983**	0.707	**0.987**	0.713	0.833	0.831
cloves	0.863	**0.981**	0.881	**0.966**	0.875	**0.998**	**0.989**	0.751	0.742	0.926
nutmeg	0.974	0.859	0.735	0.872	0.754	0.881	0.855	0.914	0.975	0.932
cinnamon	**0.987**	0.877	**0.936**	**0.995**	0.978	0.842	**0.931**	**0.958**	0.964	**0.948**
vanilla	0.937	0.848	**0.970**	0.890	**0.986**	**0.996**	0.703	**0.971**	**0.997**	**0.950**
allspice	**0.985**	0.695	0.717	**0.988**	0.977	0.798	0.908	0.946	**1.000**	**0.950**
spice mix	**0.982**	**0.885**	**0.950**	0.955	**0.982**	**0.916**	0.666	**0.947**	**0.987**	0.698

The antioxidant capacity was also measured using the DPPH method (Table 3) and a decreasing tendency was observed in DPPH values of the rye-buckwheat cakes. This result is in agreement with study of RINALDI *et al.* (2014), who observed DPPH decrease in chestnut- wheat bread even after 3 days of storage. The ability of control cake to scavenge DPPH· radicals mildly decreased from 3 to 7% during 18-month storage period. The 6-month storage had no significant influence on DPPH value decrease of ginger cakes with nutmeg, spice mix (both around 1%) and cloves (2%), whereas in the cakes with vanilla addition a slightly increase of DPPH value was observed (less than 1%). With time of storage, successive reduction was observed in the scavenging ability against DPPH radicals. In the cakes fortified with cinnamon, the DPPH value decreased even by 23%, whereas in the samples with cloves addition no significant change was noted. After 18 months, the highest reduction in DPPH values was determined in the cakes with cinnamon (22%) and allspice (24%) addition. In contrast, the least changes in DPPH values were observed in the cakes with spice mix (3%) and cloves (4%). Our observations are in accordance with findings reported by SAKAČ *et al.* (2016), and by DAR, SHARMA and NAYIK (2016), who observed that DPPH values increased by about 20% and 17% during storage of cereal products, respectively. It may be suggested that not only storage conditions but also the type and amount of flours used in formulae of cookies can influence the antioxidant capacity during storage. However more studies are needed in this respect because in other studies a decrease of the antioxidant capacity was also observed after 1 and 2 days of storage of barley bread, but it was not recognized as a significant change (HOLTEKJØLEN *et al*., 2008). According to ABTS and DPPH methods, the highest values of antioxidant activity were found in the buckwheat cakes with cloves, cinnamon and spice mix. Moreover, other methods could be used to evaluate the antioxidant activity to ensure better verification of tendencies in antioxidant activity changes. The highest coefficients of correlation (Table 4) between rutin content and ABTS values were calculated for the samples with cinnamon (0.995), allspice (0.988), cloves (0.966), and spice mix (0.955). Furthermore, high values of TPC *vs*. ABTS correlation were noted for the samples with vanilla (0.986), spice mix (0.982), cinnamon (0.978), and allspice (0.977). The high coefficients of correlation between TF and ABTS were calculated for the samples with cloves (0.998), vanilla (0.996), and spice mix (0.916). Whereas the DPPH radical method demonstrated a high correlation between rutin content and DPPH value in the buckwheat cakes with cloves (0.989), cinnamon (0.931), and allspice (0.908); as well as between TPC and DPPH value in the samples with addition of vanilla (0.971), cinnamon (0.958), spice mix (0.947), allspice (0.946), and nutmeg (0.914). Similar Pearson coefficients were calculated for the correlation between TF content DPPH value. It can be concluded

that the antioxidant capacity of rye-buckwheat cakes fortified with spices was highly related to bioactive compounds composition. The neo-formed antioxidants during baking or storage period have no significant contribution to the final antioxidant potential.

4. CONCLUSIONS

In this study, polyphenols, flavonoids and rutin contents, and antioxidant capacity of rye-buckwheat cakes enriched with cloves, nutmeg, cinnamon, spice mix, allspice or vanilla were monitored during 18-month storage. The loss of rutin, flavonoids and polyphenols, and antioxidant capacity was observed in both non-enriched and spices-fortified cakes. However, in the rye-buckwheat cakes fortified with selected spices all parameters were still higher as compared to the non-fortified ones. It was shown that the rye-buckwheat cakes fortified with cloves, cinnamon and spice mix maintained the highest functional parameters as TPC, TF, and rutin contents and antioxidant capacity after 18-month storage. Moreover, the loss of antioxidant capacity of the cakes fortified with spices was highly correlated with phenolics content. Therefore, the cloves, cinnamon and spice mix may be recommended to improve the quality of rye-buckwheat cakes. In future studies, it would also be advisable to develop cake formulas with other types of flour originating from tartary buckwheat or tartary buckwheat sprouts (being reach sources of antioxidants), whereas amounts of these ingredients need to be carefully studied to achieve also high consumer acceptance and mask their bitter taste and potential a stringent after taste in the finished product. Therefore, except for polyphenol-rich ingredients, a vacuum packaging or more appropriate bags should be used to prevent rapid degradation of polyphenols and/or formation of oxidation products.

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PAPER

COMPARISON OF BIOACTIVE COMPOUNDS AND SENSORY EVALUATION ON EDIBLE FLOWERS TEA INFUSION

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ABSTRACT

France Rose Buds, Jasmine Flower, and Osmanthus Flower are three edible flowers commonly available in Malaysia market. Composition of these 3 edible flowers is not widely studied. Hence, the caffeine, total phenolic content (TPC), volatile compounds, and overall acceptability of tea infusion from France Rose Buds, Jasmine Flower, and Osmanthus Flower were compared. Tea infusion from the edible flowers was prepared by boiling it with distilled water. None solvent extraction was carried out to determine the bioactive compounds. Tea infusion of Osmanthus Flower contains the highest caffeine (4.96±1.94 μg/ml), total phenolic content (4.33±0.03 mg GAE/g) and overall acceptability (6.16±2.05) compared to France Rose Buds and Jasmine Flower. The Jasmine Flower was found to have the highest number of volatile compounds (13) compared to France Rose Buds and Osmanthus Flower. This study indicates that the edible flowers have the potential for application as food ingredient.

Keywords: France Rose Buds, Jasmine Flower, Osmanthus Flower, caffeine, volatile compounds, sensory evaluation

1. INTRODUCTION

Edible flowers commonly seen include cauliflower, broccoli, and artichokes (CARTER *et al*., 2007). The edible part of cauliflower and broccoli is made of fleshy flower stalks and clusters of flower buds (CARTER *et al*., 2007). Edible flower petals and flower buds can be eaten raw in salads. Refreshing tea infusion can also be made from the flower petals (Lim, 2014). In terms of nutritional value of edible flowers; petal of the flowers is a source of vitamins, minerals, and antioxidants thus contribute to an increase interests for consumption (MLCEK and ROP, 2011). Other than as a decoration and culinary purposes, the nutritive and chemoprotective properties of certain edible flowers are well-documented or under study. Edible flowers can also be classified as nutraceutical food (MLCEK and ROP, 2011). Besides the well-recognized wholesome effects of green and black teas prepared from young leaves of *Camellia sinensis*, hot water infusions (teas) of many other plants also may have health benefits.

Flavonoids and phenolic acids have been used as antioxidants to prevent oxidative damage and control of diseases caused by oxidative stress (NA *et al*., 2014). Flower, seeds, and root of Osmanthus flower (*Osmanthus fragrans*) is also used as a acesodyne and as a folk medicine for the treatment of liver, stomachache and for other therapeutic purposes such as aerodontalgia, halistosis, rheumatism and physical pain (PENG and JI, 2004). The main bioactive components in the extracts of *Osmanthus fragrans* flowers are flavonoids and phenolic acids (XIONG *et al*., 2014), carotenoids, carotenoid-derivatives and volatile constituents (LEFFINGWELL, 2002).

Jasmine Flower (*Trachelospermum jasminoides*) is used in the perfume industry, and the scent has been included in at least 55 commercially sold perfumes (Basenotes Fragrance Search, 2014). The inner bark yields a strong fiber that is utilized for making rope, sacks and paper and the stem is used for treating rheumatism and injury in traditional Chinese medicine (Ill CHAN NOH, 2011; SHEU *et al*., 2009). Most publications on Jasmine Flower are rather concerned about the content of the plant (JING *et al*., 2012) than about the flowers (JOULAIN, 1987).

France Rose Buds (*Rosa* sp. var. Rosa de Castillo) are known as edible flowers and have been used for centuries as food components, either in the fresh form or in processed products, such as confectionary and beverages (GIRARD-LAGORCE *et al*., 2001). The combination of health benefits with recognized applicability in cuisine raises the possibility of using rose flowers in functional food products. Although the health benefits of some well-known edible flowers are commonly studied, the caffeine content, total phenolic content and volatiles compounds in tea infusion from France Rose Buds, Jasmine Flower, and Osmanthus Flower (*Osmanthus fragrans*) still remain unknown.

Research on sensory evaluation had been conducted by CHEN *et al*. (2010) on the attributes of taste, flavor, and overall acceptance on tea infusion from Pu-erh teas. ZHU *et al*. (2016) also conducted evaluation of volatile compounds in infusion of oolong tea (fully fermented *Camellia sinensis L.*). Instead of using hedonic scale to rate upon answering the questionnaire, 5 aroma terms were used to define the aroma by well-trained panel of ten members: 2-methylpyrazine for ''roast'' note, maltol for ''sweet'' note, hexanal for ''green and grassy'' note, dipropyl disulfide for ''sulphur'' note, phenylethyl alcohol for ''floral'' note. For the sensory analysis by BENVENUTI *et al*. (2016), 5 different organoleptic characteristics of which spiciness, sweetness, softness, scent, and bitterness were included in the evaluation scheme and were expressed in a scale of 1 to 100. In addition, sensory profile of tea infusion of France Rose buds, Jasmine Flower, and Osmanthus Flower corresponding to the volatile compositions is still insufficient. Therefore, this study was presented to compare the caffeine, total phenolic, volatile compounds, and overall

acceptability of the 3 types of edible flowers by untrained panelists. The flowers may be further exploit as a source of natural antioxidant for food and nutraceutical applications.

2. MATERIAL AND METHODS

2.1. Sample preparation

France Rose Buds, Jasmine Flower, and Osmanthus Flower were purchased from Yin Onn Shd. Bhd. (Mid Valley City) at Lower Ground Floor 035 & 036, Mid Valley City Megamall, Mid Valley City, Lingkaran Syed Putra,Kuala Lumpur, Malaysia. Extraction was prepared using boiled distilled water by 1: 8.82 ratio (ratio of dry mass sample to boil distilled water). The ratio used was modified from BISPO *et al*. (2002).

2.2. Chemicals

Methanols and acetic acid from Fisher Scientific, UK and caffeine standard from Sigma-Aldrich, China were used for High Performance Liquid Chromatography (HPLC) analysis. Folin-Ciocalteu and gallic acid from Merck, Germany and sodium carbonate from Fisher Scientific, UK were also used for Total Phenolic Content analysis.

2.3. Determination of caffeine content using HPLC

HPLC (Shimadzu Corporation, Japan) was carried out to determine and compare the caffeine content of France Rose Buds, Jasmine Flower, and Osmanthus Flower. The experimental procedure was adopted from BISPO *et al*. (2002) with some modifications. The modifications were on the amount of France Rose Buds, Jasmine Flower, and Osmanthus Flower applied in this study. About 17 g of dried edible flowers were used, instead of 4 g as referred to the method BISPO *et al*. (2002). The extraction time was also changed from 3 min to 10 min. Aqueous extract was obtained using 150 ml boiled distilled water with 17 g of dried edible flower for 10 min with continuous stirring. The extracts were filtered using syringe filter (Chemolab Supplies, Malaysia) of 0.45 μm twice (double filter) prior injected into HPLC. About 20 μL of each sample was injected into HPLC.

2.4. Total Phenolic Content (TPC) analysis

TPC of France Rose Buds, Jasmine Flower, and Osmanthus Flower were determined and compared using Folin-Ciocalteu method. The assay was conducted as described by BHEBHE *et al*. (2015). Total phenolic content was expressed as gallic acid equivalents (GAE). The results were obtained from representative samples prepared from each concentration. Calibration and linear curves were determined.

2.5. Analysis of volatile compounds using automated Gas Chromatography-Mass Spectrometry (GC-MS)

The volatile flavor constituents of each sample were analyzed using Trace GC Ultra Gas Chromatography system coupled with TSQ Quantum XLS Mass Spectrometer System (Serial No: TQU03227) from Thermo Fisher Scientific (USA) and capillary column of 30 m × 0.25 mm, 0.25 μm film thickness Model TG-5MS (Thermo Scientific, USA). The detail of experimental procedure was adopted from LI *et al*. (2013).

2.6. Sensory evaluation

Sensory analysis of France Rose Buds, Jasmine Flower, and Osmanthus Flower were conducted at room temperature (25±2°C) by 50 untrained panelists who need to complete the questionnaire containing 9-point hedonic scales. The evaluation covered the attributes of taste, color, aroma, and overall acceptability where scale 9 represented like extremely and 1 dislike extremely (HAJMOHAMMADI *et al*., 2016; LIM, 2011; ZHENG *et al*., 2014).

2.7. Statistical analysis

The experimental design was completely randomized. Statistical analysis and comparisons among means were carried out using the statistical package Minitab 17. The data collected was analyzed by one-way analysis of variance. The Tukey's post hoc test was applied for comparison of means, and differences are considered significant at the level of $p<0.05$. Linear regression for correlation analysis was performed using Microsoft Office Excel (2007).

3. RESULTS AND DISCUSSION

3.1. Caffeine content

Caffeine content of France Rose Buds, Jasmine Flower, and Osmanthus Flower were determined as shown in Table 1 with 17 g of each samples were brewed using boiled distilled water. From the data collected, it shows that Osmanthus Flower contain significantly ($p<0.05$) the highest amount of caffeine (4.96±1.94 μg/ml) than France Rose Buds and Jasmine Flower, at 0.17±0.00 μg/ml and 0.67±0.03 μg/ml respectively.
EL-SHAHAWI *et al*. (2012) reported that caffeine in tea infusion from tea (*Camellia sinensis* L.) is well-known as natural powerful antioxidant and help in the prevention of cardiovascular diseases and cancers. EL-SHAHAWI *et al*. (2012) also revealed that every consumption of 100 ml of brewed tea infusion (*Camellia sinensis* L.), about 1.04 to 212 mg of total catechins and 0.194 to 5.04 mg of caffeine were found in the tea infusion from 29 commercial green tea samples (*Camellia* sinensis L.) in Saudi Arabia. Coffee, tea, and fruits were the most important food sources of total polyphenols. A total of 437 different individual polyphenols were consumed, including 94 of them consumed at a level 1 mg/day (ZAMORA-ROS *et al*., 2015). To date, no specific dietary intake of caffeine in any food product including edible flowers tea infusion related to antioxidant activity has been reported.
EL-SHAHAWI *et al*. (2012) reported that twenty-nine green tea samples (*Camellia sinensis L.*) of different origins (China, Japan, Indonesia, Sri Lanka and Taiwan) from Saudi Arabian local market have caffeine content ranged from 0.09 to 2.23 mg/g. D'ARCHIVIO *et al*. (2016) also reported that average of 2.5±0.2 mg/g of caffeine was detected in tea infusion of oolong tea (semi-fermented *Camellia sinensis L.*). Caffeine content in tea infusion of France Rose Buds, Jasmine Flower, and Osmanthus Flower were relatively much lower than both of the green tea and oolong tea infusion as discussed above.

Table 1. Caffeine Content of France Rose Buds, Jasmine Flower, and Osmanthus Flower using HPLC.

Type of flower	Caffeine (μg/ml)
France Rose Buds	0.17±0.00[b]
Jasmine Flower	0.67±0.03[ab]
Osmanthus Flower	4.96±1.94[a]

Values expressed as mean±standard deviation (n=2). Means with different letters within the same column are significantly different at the level of $p<0.05$.

3.2. Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined by Folin-Ciocalteu assay and the result was shown in Table 2.The result was expressed as gallic acid equivalent (GAE). Table 2 shows that Osmanthus Flower had significantly the highest TPC value (4.33±0.03 mg GAE/g) among the three edible flowers in this study ($p<0.05$). In addition, the highest caffeine content was also detected in Osmanthus Flower at 4.96±1.94 μg/ml (Table 1) and this could also be related to the highest total phenolic content of Osmanthus Flower. Similarly, NA *et al*. (2014) reported that the total phenolic contents of *Osmanthus fragrans* was higher 16.00±0.57mg GAE/g than the other 50 edible types in a range of 0.63±0.03 to 35.84±1.67 mg GAE/g wet weight.
The TPC value of tea infusion of Osmanthus Flower in this study was lower than Na *et al*. (2014). The different results obtained may be due to the different parts of plant used, where leaves had higher content of TPC than fruit, stem, or brunches. Species type, age, maturity and or environmental stress may also contribute to differences in phenolic content of a plant (Watson, 2014). Generally, amount of polyphenols in plants is affected by genetics and environment. External environment also triggers the presence and content of polyphenols for the protection of the plant (WATSON, 2014). The most efficient solvent for polyphenols extraction was 50% DMF for black tea (fully fermented *Camellia sinensis L.*) and 50% acetone for mate tea. Higher phenolic content often linked with higher antioxidant activity where it is health benefiting in terms of medicinal properties such as antibiotic, anti-inflammation, anti-cancer and anti-allergic (BHEBHE *et al*., 2015).

Table 2. Total phenolic content of France Rose Buds, Jasmine Flower, and Osmanthus Flower.

Type of flower	Total Phenolic Content (mg GAE/g)
France Rose Buds	3.75±0.03[b]
Jasmine Flower	4.27±0.02[a]
Osmanthus Flower	4.33±0.03[a]

Results expressed in Gallic Acid Equivalent. Values are expressed as mean±standard deviation (n=5). Means with different letters within the same column are significantly different at the level of $p<0.05$

3.3. Volatile compounds

Volatile compounds of France Rose Buds, Jasmine Flower, and Osmanthus Flower were determined by automated headspace GCMS with the total retention time of 18.30 min. The

chromatography of volatile compounds detected in tea infusion of edible flowers and their relative content were summarized in Table 3. About 5 volatile compounds were detected in France Rose Buds by automated headspace GCMS. Phenylethyl alcohol was the major volatile compound identified from tea infusion of France Rose Buds at 57.12%, and this agrees with DUDAREVA and PICHERSKY (2006) that phenylethyl alcohol can be detected in France Rose Buds. Phenylethyl alcohol, also known as benzyl carbinol, 2-phenylethanol, and β-phenylethyl alcohol, is a colorless and viscous liquid, rose-like odor, initially a slightly bitter taste then sweet and reminiscent of peach (FENAROLI *et al*., 2000). Phenylethyl alcohol has been used as an antimicrobial, antiseptic, disinfectant, fragrance in perfumes and preservatives (BURDOCK, 1997).

Compound 1-Iodo-2-methylundecane (15.75%), tridecane (3.22%), cis-2-Methyl-7-octadecene (3.18%), and 1-Iodo-2-methylnonane (2.67%), classified as hydrocarbon group were detected in tea infusion of France Rose Buds. Antonelli *et al*. (1997) showed similar findings where the main component in 24 different rose varieties was phenylethanol, but some roses showed unusually high levels of benzyl alcohol. Antonelli *et al*. (1997) also detected 4-Vinylphenol, also known as 4-ethenylphenol, p-Vinylphenol, p-Hydroxystyrene, and 4-VP, in two samples of Rosa gallica out of 24 different rose varieties, but those volatile compounds was not found as analyzed in this study. Tridecane detected in this study was similar to Lin *et al*. (2013) where tridecane was extracted by HS-SPME in a total of 75 oolong tea (fully fermented *Camellia sinensis L.*) at the ranged of 0.30 to 2.50%.

Benzyl benzoate (6.81%) and 1,6-Octadien-3-ol,3,7-dimethyl- (3.81%), also known as linalool, were detected in both tea infusion of Jasmine Flower and Osmanthus Flower, similarly as Lim (2014). The (R)- (-)-linalool was found to be the key odorants of Jasmine tea flavor. Linalool was identified in a high proportion using different solid-phase micro extraction fibres. A total of 13 constituents were identified in the headspace of Jasmine Flower, with compound linalool (25.01%) was the highest in proportion, followed by benzyl acetate (23.71%) and 3-hexenyl acetate (13.80%) (LIM, 2014). Pregna-5, 14-diene-3, 20-diol-18-carboxylic acid, 3-acetate-, lactone were the major volatile compound detected in tea infusion of Jasmine Flower at 31.98%. However, this volatile compound has never been reported in the similar research as reported by LIM (2014). This might be due to the different part of the plants used in the experiments as different part consists of different volatile compounds. Volatile compositions vary according to genetics, soil, climate, and agricultural practices (TOCI and FARAH, 2008). Jasmine Flower can be further extracted and incorporated as new food ingredient due to its high volatile compounds.

3.4. Sensory evaluation

Sample preparation for sensory evaluation was conducted under the same preparation method for all the above analysis where the tea infusion was prepared by brewing it with boiling water without addition of sugar or honey. The prepared sample was kept at room temperature 25±2°C. About 50 untrained panelists were required to complete the questionnaire to score the attribute of taste, aroma, color, and overall acceptability. The result was recorded in Table 4. Although Jasmine Flower and Osmanthus Flower are originated from the same order and family, France Rose Buds and Osmanthus Flowers show significant different ($p > 0.05$) in sensory attributes, in terms of taste, aroma, color, and overall acceptability.

Table 3. Volatile compounds and their relative contents detected in France Rose Buds, Jasmine Flower, and Osmanthus Flower using HS GCMS.

No	Retention time (min)	Compound name	Molecular weight (g/mol)	France Rose Buds (%)	Jasmine Flower (%)	Osmanthus Flower (%)
•	4.48	Cyclohexene,methyl-5-(1-methylethenyl)-, (R)-		-	5.90	2.79
•	4.93	Ethyl2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-ylcarbonate		-	-	13.38
•	5.21	1,6-Octadien-3-ol,3,7-dimethyl- (Linalool)	154.25	-	3.81	13.04
•	5.49	Phenylethyl Alcohol	122.16	57.12	-	-
•	6.07	2H-Pyran-3-ol,6-ethenyltetrahydro-2,2,6-trimethyl-		-	-	5.42
•	6.18	Heptanediamide,N,N'-di-benzoyloxy-		-	2.07	-
•	6.79	1,6-Octadien-3-ol,3,7-dimethyl-,2-aminobenzoate		-	1.68	8.55
•	7.88	Megastigma-4,6(E),8(Z)-triene		-	-	8.05
•	8.44	1H-3a,7-Methanoazulene,2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-,		-	1.97	-
•	8.95	3-Buten-2-one,4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-		-	-	3.53
•	9.04	1,3,6,10-Dodecatetraene,3,7,11-trimethyl-, (Z,E)-		-	2.21	-
•	9.68	3-Hexen-1-ol benzoate			1.92	-
•	10.62	Tridecane	184.37	3.22	-	-
•	11.19	1-[2-O-benzoyl-3,5-O-dibenzyl-alpha-D-ribosyl]-5,6-dimethylbenzimide		-	1.07	-
•	11.44	Benzyl Benzoate	212.25	-	6.81	4.74
•	11.77	Phenylmalonic acid monobenzyl ester		-	9.60	6.21
•	12.06	cis-2-Methyl-7-octadecene		3.18	-	-
•	12.23	1-Iodo-2-methylundecane		15.75	-	-
•	12.31	Ethanone,2,2-dimethoxy-1,2-diphenyl-		-	6.52	3.55
•	13.51	1-Iodo-2-methylnonane		2.67	-	-
•	13.56	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde		-	3.96	-
•	13.85	Cholest-1-eno[2,1-a]naphthalene, 3',4'-dihydro-		-	-	2.38
•	14.23	Pregna-5,14-diene-3,20-diol-18-carboxylic acid,3-acetate-, lactone		-	31.98	

–, not found. Four constituents selected for analysis are indicated in bold type.

Table 4. Sensory evaluation on attributes including taste, aroma, color, overall acceptability of France Rose Buds, Jasmine Flower, and Osmanthus Flower.

Type of Flower	Taste	Aroma	Color	Overall acceptability
France Rose Buds	5.48±2.06[a]	6.24±1.84[a]	6.56±1.47[a]	5.90±1.62[a]
Jasmine Flower	4.34±2.05[b]	5.24±1.80[b]	5.60±1.63[b]	4.96±1.65[b]
Osmanthus Flower	5.76±2.36[a]	6.42±2.07[a]	6.42±1.66[a]	6.16±2.05[a]

Mean±standard deviation (n = 50). Values that are followed by different letters within each column are significantly different ($p< 0.05$).
1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely.

Research on sensory evaluation had been conducted by CHEN *et al*. (2010) on the attributes of taste, flavor, and overall acceptance on tea infusion from Pu-erh teas. ZHU *et al*. (2016) also conducted evaluation of volatile compounds in infusion of oolong tea (fully fermented *Camellia sinensis* L.). Instead of using hedonic scale to rate upon answering the questionnaire, 5 aroma terms were used to define the aroma by well-trained panel of ten members: 2-methylpyrazine for ''roast'' note, maltol for ''sweet'' note, hexanal for ''green and grassy'' note, dipropyl disulfide for ''sulphur'' note, phenylethyl alcohol for ''floral'' note. For the sensory analysis by BENVENUTI *et al*. (2016), 5 different organoleptic characteristics of which spiciness, sweetness, softness, scent, and bitterness were included in the evaluation scheme and were expressed in a scale of 1 to 100.
Table 4 displays a trend where France Rose Buds and Osmanthus Flower were scored significantly ($p<0.05$) higher than Jasmine Flower in terms of taste, aroma, color, and overall acceptability. Osmanthus Flower had the most acceptable sensory attributes with the highest score in terms of taste, aroma, and overall acceptability. Despite Jasmine Flower being scored as the lowest in all sensory attributes, it contains the highest number of volatile compounds among the 3 edible flowers. It is believed that the edible flowers serve more than just as decoration and ornamental plants.

4. CONCLUSIONS

This study compared the bioactive components contained in tea infusion of France Rose Buds, Jasmine Flower, and Osmanthus Flower. It was found that tea infusion of Osmanthus Flower has the highest caffeine (4.96±1.94 μg/ml), total phenolic content (4.33±0.03 mg GAE/g) and overall acceptability (6.16±2.05) compared to tea infusion of France Rose Buds and Jasmine Flower. Meanwhile, Jasmine Flower contains the highest number of volatile compounds among the 3 edible flowers. In addition, edible flowers contain the natural bioactive components and may be used in food preparation to reduce or replace the use of synthetic bioactive components. Thus, Osmanthus Flower can be further explored and developed into functional foods, nutraceutical, and pharmaceuticals to prevent and treat diseases caused by oxidative stress.

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PAPER

HOUSEHOLD FOOD WASTE IN MONTENEGRO

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ABSTRACT

Approximately one third of all food produced for human consumption is lost or wasted. This study provides a general overview about household food waste in Montenegro. An online survey was carried out in 2015 with a random sample of 371 adult Montenegrins (70.1% female; 91.1% less than 44 years old; 81.2% high-educated). Most of the respondents (90.8%) had high concerns related to food waste. Bakery products are the most wasted foods. Monthly economic value of food waste is 5-25 Euro. Raising Montenegrins' awareness about environmental, ethical and economic implications of household food wastage is crucial to address this issue.

Keywords: behavior change, food labelling, food waste cost, household food waste, Montenegro, online survey

1. INTRODUCTION

Food is lost or wasted throughout the supply chain, from initial agricultural production down to final household consumption (FAO, 2011; CZARNIAWSKA and LÖFGREN, 2013; FAO, 2017; HLPE, 2014). The causes of food losses and waste are mainly connected to financial, managerial and technical limitations in harvesting techniques, storage and cooling facilities, infrastructure, packaging and marketing systems (FAO, 2011; HLPE, 2014; OECD and FAO, 2015). Food wastage represents a missed opportunity not only to improve global food security (HLPE, 2014; OECD and FAO, 2015; KADER, 2005), but also to mitigate environmental impacts and resources use from food chains (HLPE, 2014; OECD and FAO, 2015; PARFITT *et al*., 2010; JEREME *et al*., 2013; FAO, 2013; CHAPAGAIN and JAMES, 2013; HODGES *et al*., 2010; SMIL, 2004; GRIZETTI *et al*., 2013; KUMMU *et al*., 2012).

Montenegro is a service-based economy; in 2016, the tertiary sector accounted for 71.9% of total gross domestic product (GDP), while the primary production – agriculture, forestry and fishing – accounted for 9.0% (EC, 2018). The green economy concept has a prominent place in the revised National Strategy for Sustainable Development of Montenegro for the period 2014–2020; however, Montenegro does not have a strategic document that would explicitly state the country's commitment to green economy (RADOVIC-MARKOVIC *et al*., 2015; MINISTRY OF TOURISM AND ENVIRONMENTAL PROTECTION-MONTENEGRO, 2007). Several laws are dealing with waste policies, and Montenegro has made notable efforts to harmonize its legislation with the *acquis* of the European Union (EU). The 2011 Law on Waste Management (GOVERNMENT OF THE REPUBLIC OF MONTENEGRO, 2011, 2016) requires the waste producer to make all efforts to prevent and reduce waste generation. The Law sets up ambitious targets in several areas of waste management and for biodegradable waste by 2020 – the aim is to reduce biodegradables by 50%. Other laws - such as the Law on Environment, a key legal act on the management and protection of the environment - aim to align with obligations resulting from Montenegro's international commitments and relevant EU's directives dealing with waste policy issues. Nevertheless, these laws cover waste in general and are not specifically addressing food waste. Government strategies and actions – e.g. Waste Management Plan 2014–2020, National Policy on Waste Management from 2004 and the 2005 Strategic Master Plan for Solid Waste Management of Montenegro – do not recognize food waste as an important subject (RADOVIC-MARKOVIC *et al*., 2015). Although several Non-Governmental Organizations (NGOs) deal with environmental protection and waste, they have rarely initiatives related to food waste (e.g. in 2014, Ecological Movement OZON promoted action "Stop food waste" during the European Week for Waste Reduction), with the exception of the NGO "Food Bank" dealing with decreasing food waste and fight against hunger. In several occasions (e.g. primary milk producers in 2013 and watermelon producers in 2011, who demonstratively destroyed their products due to poor government management policies), lack of effective market management resulted in significant food losses. In general, there is a lack of data about food waste and losses in Montenegro, as responsible authorities and relevant strategies do not deal with these issues and data on industrial and municipal solid waste do not seem to realistically reflect food waste generation. Thus, attention should be focused on waste generated by households and at consumer level, as these may be more reactive and likely to yield results faster (GRETHE *et al*., 2011).

In medium- and high-income countries, such as Montenegro, food is to a significant extent wasted at the consumption stage, meaning that it is discarded even if it is still suitable for human consumption (HLPE, 2014; LUNDQVIST, 2010). There is a growing body of literature dealing with household food waste in different countries and regions (e.g.

BYGRAVE *et al*., 2017; EVANS, 2011; GRAHAM-ROWE *et al*., 2014; JEREME *et al*., 2013; LEBERSORGER and SCHNEIDER, 2011; MONDÉJAR-JIMÉNEZ *et al*., 2016; NEFF *et al*., 2015; PRINCIPATO, 2018; PRINCIPATO *et al*., 2015; QUESTED *et al*., 2013; SECONDI *et al*. 2015; STENMARCK *et al*., 2016; WILLIAMS *et al*., 2012; WRAP, 2011), but the Balkan region in general and Montenegro in particular are largely underserved. Therefore, in order to address this literature gap, this exploratory study aims to provide a general overview about household food waste in Montenegro.

2. MATERIAL AND METHODS

The paper is based on the results of a voluntary self-administered online survey that was adapted to Montenegrin context and designed through elaboration of questionnaires previously used for similar research purposes e.g. Office of Environment and Heritage in the State of New South Wales (NSW), Australia (NSW-EPA, 2012), and the University of Bologna (LAST MINUTE MARKET, 2014). The questionnaire on food waste (FW) was available in Montenegrin online through Survio website (www.survio.com) from January until April 2015 (87 days in total) and the participation was entirely on a voluntary basis. Potential adult respondents were contacted using direct emails and social networks (Facebook, Twitter).

The online questionnaire included 25 one-option and multiple-choice questions dealing with: (i) food purchase behaviour and household food expenditure estimation; (ii) knowledge of food labelling information; (iii) attitudes towards FW; (iv) extent of household FW; (v) economic value of household FW; and (vi) willingness and information needs to reduce FW. The concept of FW was briefly presented in the introductory part of the online questionnaire to inform the respondents more about the topic and the research purpose (the following statement was included in the questionnaire: "*For the purpose of the present survey, food waste is considered food that was purchased by the household for human consumption but was thrown away i.e. was not consumed*"), as well as about approximate time needed to complete the survey (10-15 minutes).

The response rate (AAPOR, 2017) was used to provide essential information about the quality of the survey. Received unfinished questionnaires, contradictory or bad quality data, were excluded from further data processing (Table 1). Answering questionnaire on FW required time so that might be the reason why many of those who received the questionnaire did not answer (41.9%).

Table 1. Online survey visits and response rate.

Detail	No	%
Total visits	825	100
Just seen by respondents but not answered	346	41.9
Total questionnaires answered (completed and unfinished)	479	58.1
Total questionnaires completed by respondents	371	45.0
Total unfinished questionnaires	108	13.1
Responses included in final analysis	371*	45.0*

Source: Authors' elaboration based on survey results.
* The number of complete questionnaires divided by the number of total visits (those eligible in the sample).

The questionnaire was disseminated all over Montenegro but most of those who completed the questionnaire (69.5%) were from the capital of Montenegro - Podgorica.
Quantitative data collected through the questionnaire survey were analyzed using descriptive statistics (e.g. means, max, min, percentages), in order to get a general picture of frequencies of variables, using Microsoft Excel spreadsheets. Besides descriptive analysis, Chi-Square test was used to analyze the association between different variables in SPSS 16. The null hypothesis was that there is no relation between tested variables (gender, age, level of education, occupation, frequency of food purchasing, estimated amount of food waste, use of shopping list, knowledge about labelling, habits of respondents in terms of food preparation and use) as well as relation between the amount of uneaten food and frequency of throwing away food by households.
The major constraint faced during the research was the shortage and/or difficult access to secondary data on FW in Montenegro. A further limitation of the study is the non-probabilistic sampling design used for data collection as respondents were recruited on a voluntary basis. This also implies the non-representativeness of the recruited sample for the adult population in Montenegro. Moreover, while household food waste surveys are methodologically simple, they are mainly useful to provide qualitative information, because quantification of food wastage (cf. weight of food purchased and discarded, so not consumed) is prone to error as consumers often tend to underestimate their waste (and food waste) when self-reporting (e.g. BERETTA *et al*., 2013; NEFF *et al*., 2015; SIMUNEK *et al*., 2015; VENTOUR, 2008). Moreover, it should be highlighted that the questionnaire was prepared in English then translated into Montenegrin and this may have affected the understanding of respondents of issues regarding food wastage and, consequently, their answers.

3. RESULTS AND DISCUSSION

3.1. Main characteristics of respondents

Most of the interviewees were females (70.1%), quite young (77.4% less than 44 years old) and had high education level (more than 80% were holders of bachelor, master or PhD degrees). Taking into consideration obtained results, it is clear that mainly younger respondents use social media; the main tool utilized in the online questionnaire survey dissemination. Around 70% of respondents declared they have full-time or part-time paid work, 16.7% are unemployed persons (including housewives), 12.1% are students, while 1.1% is retired. More than a third of the respondents are married with children (37.2%). A significant share of the respondents still lives with parents (36.9%). As for household composition, they mainly consist of four or more members (47.7%).

3.2. Food purchase behavior and household food expenditure estimation

This part refers to respondents' food behavior and an estimation of their food expenditures in order to understand their attitudes towards food. It was found that about 77.4% of the respondents purchase their food from supermarkets and hypermarkets, 12.1% purchase from mini/small markets and 9.2% from small markets. The majority is buying food once a day (36.9%) and once a week (20.5%), while those who buy twice a week and every second day represent 15.4% and 17%, respectively. Food wastage could be attributed to poor planning when purchasing food as only 32.3% of the respondents use list prior to purchasing food. About 70.1% of the respondents spend monthly over 150 EUR for food.

Meanwhile, more than a half of the respondents (53.4%) are attracted to special food offers, which normally take place at super- and hypermarkets.

3.3. Knowledge of food labelling information and attitudes towards food waste

The results show respondents' knowledge about food labels, which might eventually affect food wastage among consumers and the respondents' attitudes towards food waste and food habits. It was indicated that 86.5% of the respondents understand and have knowledge about "use by" label as food must be eaten or thrown away by this date. This result could be attributed to the high educational level of the respondents. Whereas only 11.1% regarded the "best before" label as food is still safe to eat after this date.
It was evident that 90.8% of the respondents do worry about food waste and they try to avoid it, while 6.5% are aware about food waste problems but have no intention to change their current habits. Moreover, 82.3% of the respondents indicated that they dispose of "very little", or "reasonable amount" of uneaten food (Fig. 1).

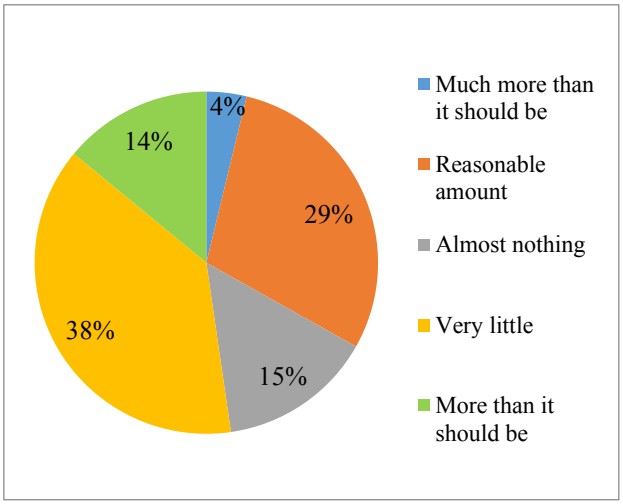

Figure 1. Quantity of uneaten food thrown away by Montenegrin households.

Regarding food waste management, 49.9% of the respondents give the remained food to animals and 44.7% said they dispose of it in the garbage. Meanwhile, when they were asked about how often they throw away leftovers or food, 56.1% of the respondents declared that they waste food at least once a week (Fig. 2).
Tracking consumers' food habits could explain their attitudes towards food waste and its quantity. In that regard, the survey results showed that 60.9% of the respondents cook a main meal from raw ingredients from 3 to 6 times/week. Furthermore, 73% of the respondents eat a meal left over from a previous day (less than twice/week), and 56.9% eat out of home less than two times a week.

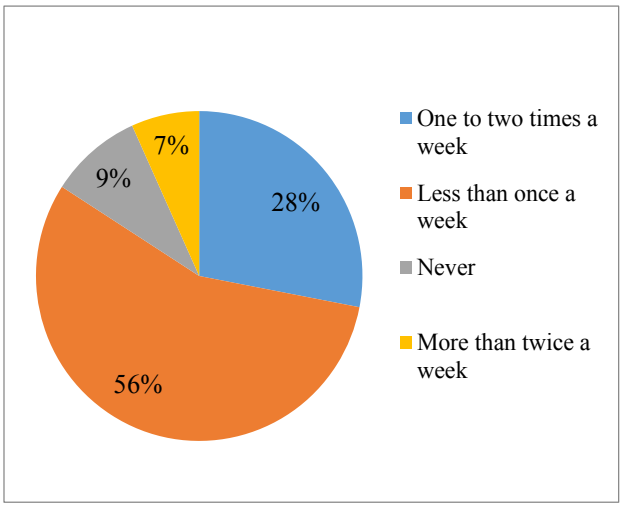

Figure 2. Frequency of throwing away food.

3.4. Quantity and value of food wasted and extent of household food waste

The analysis regarding the main reasons that contribute to throwing food showed that 45.6% of the respondents throw food because of 'expiration date' (without any differentiation between best-before and use-by dates); 46.6% of the respondents throw food leftovers; and 43.7% states that the main reason of throwing food is its long storage in the refrigerator.
In addition, this part of the results deals with the amount, extent and value of food waste. In Table 2 are presented the results of purchased food, which is thrown in households. The most wasted food group is bakery products while pulses and oilseeds, roots and tubers as well as fish and seafood are the least wasted food products by Montenegrin households.

Table 2. Respondents' estimation for food groups wastage (in percentage).

Items	Less than 2%	3 to 5%	6 to 10%	11 to 20%	Over 20%
Cereals and bakery products (bread, rice, pasta, etc.)	146 (39.4%)	96 (25.9%)	50 (13.5%)	33 (8.9%)	46 (12.4%)
Vegetables	227 (61.2%)	81 (21.8%)	41 (11.1%)	14 (3.8%)	8 (2.2%)
Milk and dairy products	231 (62.3%)	80 (21.6%)	36 (9.7%)	16 (4.3%)	8 (2.2%)
Fruits	240 (64.7%)	75 (20.2%)	31 (8.4%)	14 (3.8%)	11 (3.0%)
Meat and meat products	236 (63.6%)	76 (20.5%)	39 (10.5%)	11 (3.0%)	9 (2.4%)
Roots and tubers (potatoes, etc.)	258 (69.5%)	57 (15.4%)	36 (9.7%)	16 (4.3%)	4 (1.1%)
Pulses and oil seeds (e.g. peas, chickpeas, olives, sunflowers)	286 (77.1%)	48 (12.9%)	28 (7.5%)	4 (1.1%)	5 (1.3%)
Fish and seafood	311 (83.8%)	39 (10.5%)	12 (3.2%)	6 (1.6%)	3 (0.8%)

Source: Authors' survey.

As for the extent of food waste, 48.8% of the respondents do not throw away food that is still consumable, 23.5% throw less than 250 gr per week, and 18.1% throw between 250 and 500 gr per week.
Regarding the economic value of wasted food, it was revealed that for about 52.8% of the respondents the value of wasted food is between 5 and 25 EUR per month.

3.5. Willingness to behavioral change to reduce household food wastage

This part deals with the notion of consumers' willingness to change their behavior regarding food waste. Thus, the first step was to explore the respondents' perception of food waste reasons. It was evident from the results that most of the respondents are familiar with such reasons; for instance, 43.7% mentioned food is left in the fridge for too long time, followed by 45.6% of them that said 'food expired', 30.2% indicated food does not look eatable/good, and 46.6% referred to leftovers. While many respondents mentioned "food is left in the fridge for too long time" or "food has expired", it is important to consider the true reasons or root causes that led to this result and, consequently, to food wastage. These reasons are mainly related to inappropriate meals planning and inadequate food preseveration; which surprisingly were less mentioned by the respondents.
Eventually, since the respondents have clear vision about food waste causes or reasons then they could be willing to reduce such waste. Though, willingness is affected by information availability and other factors; so, 39.4% of the respondents mentioned that they will reduce food waste if the packaging was more suitable, followed by if they were better informed about the negative impacts of food waste on the environment (39.6%) or on the economy (21.3%).
Finally, as for the information needed to reduce food waste, 28.8% of the respondents said that they need recipes with leftovers, 48% need tips on how to conserve food properly, 33.7% need information about organizations and initiatives that deal with food waste prevention and reduction, and, finally, 36.7% need information on how to assess the freshness of products.

3.6 Relations between tested variables

The independence of variables was analysed by using Chi–Square Test. In particular, relations between the following variables were tested: gender, age, level of education, occupation, frequency of food purchasing, estimated amount of food waste, use of shopping list, knowledge about labelling, habits of respondents in terms of food preparation and use. All the tested relations were not statistically significant.
According to the conducted online survey, the following results were obtained when it comes to the quantity of uneaten food and frequency of throwing away food by households in Montenegro (Table 3). Regarding the frequency of throwing away food, regardless of the quantity of food, most respondents throw food less than once a week or one to two times a week. Also, regardless of the frequency of throwing away food, most respondents answered that they throw very little quantity of food.
Furthermore, the relation between two key questions was statistically tested by *Pearson's Chi-Square Test*:

(1) In general, how much of uneaten food your household usually throws away?
(2) How often your household throws away leftovers and food that you consider not usable?

The value of *Pearson's Chi-Square Test* of independence was 45.525, which is statistically significant at $p<0.01$ (Table 4). Therefore, there is a significant relation between the quantity of uneaten food and the frequency of throwing away food (Table 4). In other words, with increasing quantity of uneaten food, also the frequency of throwing away food increases. Therefore, it is crucial to pay particular attention to meals planning in order to reduce the quality of uneaten food and leftovers. Moreover, another strategy consists in providing households with more information (especially recipes) about the use of leftovers.

Table 3. Cross tabulation – The relation between quantity of uneaten food and frequency of throwing away food.

		How often your household throws away leftovers and food that you consider not usable? (choose one answer)				
		Never	Less than once a week	One to two times a week	More than twice a week	Total
In general, how much of uneaten food your household usually throws away? (choose one answer)	Very little	28	125	38	5	196
	Reasonable amount	4	59	35	11	109
	More than it should be	2	24	31	9	66
	Total	34	208	104	25	371

Source: Authors' survey.

Table 4. Chi-Square Test of independence.

	Value	**DF**	**Asymp. Sig. (2-sided)**
Pearson Chi-Square	44.525	6	.000**
Likelihood Ratio	45.744	6	.000**
No of Valid Cases	371		

Asymp. Sig.: Asymptotic Significance.
** - Statistically significant at $P< 0.01$.

3.7. Food wastage in Montenegro: urgent action is needed

Results show that most of the respondents have high concerns related to food waste. According to the respondents, food waste is prevalent in Montenegro and the most wasted foods are bakery products. More than half of Montenegrin respondents declared that the economic value of food waste generated each month is 5–25 EUR. Meanwhile, almost half of the interviewees declared that they throw food that is still edible/consumable.
Food waste is a serious issue that undermines food security and food system sustainability in the Mediterranean region (BERJAN *et al.*, 2018; CAPONE *et al.*, 2016; EL BILALI, 2018). The results of the present survey are in line with those obtained in similar studies on household food waste in the Mediterranean. These include surveys carried out in countries such as Algeria (ALI AROUS *et al.*, 2017), Egypt (ELMENOFI *et al.*, 2015; ABDELRADI *et al.*, 2018), Lebanon (CHARBEL *et al.*, 2016), Morocco (ABOUABDILLAH *et al.*, 2015), Tunisia (SASSI *et al.*, 2016) and Turkey (YILDIRIM *et al.*, 2016). In fact, all these

studies made the case for addressing urgently household food wastage given its negative environmental (FAO, 2013; CHAPAGAIN and JAMES, 2013; QUESTED *et al*., 2013; WRAP 2011), economic (HLPE, 2014; PRINCIPATO, 2018; RUTTEN, 2013) as well as ethical (STUART, 2009) implications.

Despite that, food waste is still not covered by overarching waste strategic documents in Montenegro. In fact, the *National Strategy for Sustainable Development of Montenegro by 2030* defines the strategic goals and measures for introducing green economy by, among others, improving waste management towards *circular economy*. Using the instruments of *circular economy* is possible to connect the activities and initiatives of producers, retailers, consumers and recyclers. Those strategic goals and measures are related to management of all kinds of waste, but there are no specific strategic goals and measures that address food waste management within circular economy. The new document entitled "A Comprehensive Assessment of the Current Waste Management Situation in South East Europe and Future Perspectives for the Sector Including Options for Regional Co-operation in Recycling of Electric and Electronic Waste" (EUNOMIA RESEARCH & CONSLUTING LTD, 2017) deals with national waste assessment and roadmap for improving of waste management in Montenegro. Furthermore, according to the Ministry of Sustainable Development and Tourism of Montenegro (MSDT), there are no existing initiatives on food waste reduction. The only novelty, and this per se is a complex task, is waste separation (on wet and dry fraction), as well as composting possibility. This task is covered with *Waste Management Strategy by 2030*, developed by MSDT. In addition to this should be mentioned that the administration of capital city, Podgorica, announced recently the start of a pilot project related to primary waste selection in households, in cooperation with NGOs and international organizations in Montenegro. The initiative should promote waste selection in households through informing citizens about existing recycling yards in Podgorica (total 6) as well as about possibility of selecting waste at these locations. In addition, big investments are expected in construction of sanitary waste baths, facilities for purification of water, production of energy from landfill biogas.

Local self-government authorities in charge of waste management are poorly staffed and trained, and are in need of stronger capacities. Very often, consumer health and food safety are at the center of regulators' attention and responsibilities for managing waste are broadly separated between government bodies leading to lack of coordination in implementation of policy on food waste reduction (Box 1). Focusing attention on the reduction of food waste generated by households is likely to yield results faster. Therefore, communication campaigns should target consumers with the objective to raise public awareness on the issue of food waste in order to change the behaviour of consumers towards food wastage.

Some potential causes of food waste result from business practices and private standards sometimes set at much higher levels than those set by the government. For instance, the "best before" date displayed on food products is not set by law but rather the result of industry practice that seeks to adapt to business liability constraints (NRDC, 2013). Likewise, marketing and sale strategies influence negatively the waste behaviour of individuals, especially youths (MONDÉJAR-JIMÉNEZ *et al*., 2016), so that retailers can play an important role in preventing food waste generation. Therefore, the private sector is engaged to reduce food waste throughout the food supply chain through various initiatives such as innovation (e.g. technologies, packages, production processes), corporate initiatives and consumer education via social media and other platforms (BIAC, 2013; BYGRAVE *et al*., 2017; DI TERLIZZI *et al*., 2016). Some supermarkets in Montenegro (e.g. VOLI, AROMA/CONTO, IDEA) have introduced the practice of promotional discount due to expiry date or sale of two products for the price of one. These initiatives are directed into improving of products sale as well as reduction of food wastage.

Box 1. Institutions dealing with environmental protection and waste management in Montenegro.

The main governmental authority responsible for policymaking on environment and sustainable development is the Ministry of Sustainable Development and Tourism within which operates the Environmental Protection Agency (EPA) that is responsible for implementation of environmental legislation. While the framework legislation related to food waste is under the responsibility of the Ministry, waste management is the responsibility of local governments and municipalities (*MINISTRY OF TOURISM AND ENVIRONMENTAL PROTECTION-MONTENEGRO, 2007*). In general, lack of investment and poor capacities of local self-government authorities and public enterprises responsible for waste management have been commonly recognized as restricting factors for implementation of the waste management policy. The Ministry of Agriculture and Rural Development (MARD) has responsibilities for food safety and supervises the authorities responsible for policy implementation, in particular, the Phytosanitary Administration, responsible for food safety. "Project – Consulting" Ltd (PROCON) was founded by the Government in 2008 to provide expert support in implementation of projects on environmental protection and communal services, adopted by the Government and/or local self-government authorities and supported by international financial institutions. In early 2014, the Centre for Sustainable Development was established as a programme, jointly implemented by the Montenegrin Government and United Nations Development Programme (UNDP) (PROCON, 2008).

Besides the state institutions responsible for environmental protection as well as for waste management, also NGOs should have a more active role in food waste reduction initiatives. There are three leading NGOs in Montenegro - i.e. Green Home (www.greenhome.co.me), Ozon (www.ozon.org.me), Zero Waste Montenegro (www.zerowastemontenegro.me/me) - that have undertaken activities related to waste management in the form of implementing projects and organizing roundtables. *Ozon* currently implements "Establishing of system for waste management in Budva municipality" project whose activities encompass waste collection, selective disposal and recycling of waste. The activities of *Zero Waste Management* include raising awareness on the concept of circular economy, promoting Zero Waste practices, lobbying against waste incineration, providing technical expertise to recycling facilities to increase their recycling rate, establishing a Zero Waste community, supporting establishment of First Zero Waste Municipality. In previous years, these NGOs organized roundtables on waste management within the projects funded by the European Union, but never exclusively on food waste management.

NGOs – in cooperation with public institutions and the private sector – can play an important role in initiatives such as educational campaigns directed to consumers and industry and food recovery as well as research and knowledge dissemination activities. National campaigns, such as consumer education campaigns on reading "use by" or "best before" date labels, can help change consumer behaviour (NRDC, 2013) thus contributing to the prevention and/or reduction of household food wastage. Such campaigns should focus on youths, who proved to be the population segment most inclined to waste food (MONDÉJAR-JIMÉNEZ *et al*., 2016; PRINCIPATO *et al*., 2015), and focus on concrete practices such as waste sorting, which was found to be positively associated with food waste reduction (SECONDI *et al*., 2015). However, actions against food wastage, especially educational campaigns, should also target social marketers, retailers and policy makers (PRINCIPATO *et al*., 2015). One possible way for rising the awareness of the new generations regarding the issues of waste in general and food waste in particular is the reform of the education system in Montenegro, with the aim of introducing more environment-related disciplines into educational programs at schools and universities (NORTH COUNTRY NGO, 2016).

4. CONCLUSIONS

Food losses can take place along the whole food chain from production, handling and storage, processing and packing, distribution and marketing, to final consumption. The purpose of this study was to assess food losses at consumer level (i.e. food waste) in Montenegro. Results show that household food wastage is high in Montenegro and that the most wasted foods are cereals and bakery products. Similarly, economic value of food waste is rather high (generally 5–25 EUR monthly). Despite the evident negative environmental and economic impacts of food waste, it is still not covered by waste management strategic documents in Montenegro. The paper highlights that focusing attention on waste generated at consumer level is likely to yield good results in food waste prevention and reduction strategies. Therefore, awareness raising initiatives should target consumers with the objective to change their attitude and behaviour towards food wastage. However, strategies for food wastage reduction can be effective only if there is a better coordination among all actors of the food chain i.e. public institutions, civil society (cf. NGOs) and the private sector (e.g. retailers). Meanwhile, responsibilities of local self-government authorities should be optimized in order to ensure exchange of information. In fact, data are rarely reported on a regular basis through national statistical databases in Montenegro. In order to establish a reliable food waste dataset in the country, an important first step is to develop a regular inventory for food waste estimation. For that, a more stringent enforcement of separate collection of waste in all Montenegrin municipalities is crucial. Involving broader range of stakeholders in food waste reduction will result in moving from the concept of 'managing food waste' to improving overall sustainability of the food chain in Montenegro.

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PAPER

NUTRITIONAL FEATURES OF LEEK CULTIVARS AND EFFECT OF SELENIUM-ENRICHED LEAVES FROM GOLIATH VARIETY ON BREAD PHYSICAL, QUALITY AND ANTIOXIDANT ATTRIBUTES

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ABSTRACT

Despite high nutritional value, *A. porrum* leaves are usually wasted. To evaluate the use efficiency of leek selenium fortified leaves in bread production, cultivar Goliath was selected among nine leek varieties based on quality and antioxidant characteristics. Fortified plants attained higher leaf content of Se and polyphenols compared to pseudo-stems. The addition of Se leaves increased bread polyphenol stability during baking, but it resulted in bread porosity decrease compared to both traditionally made bread and bread containing leaf powder of non-fortified plants. About 31 % of the daily required Se is provided by 100 g of bread enriched with Se.

Keywords: *A. porrum* cultivars, bread, polyphenols, porosity, Se-biofortification

1. INTRODUCTION

Among the several vegetables cultivated, the edible parts of *Allium* species are the most widely consumed around the world. The plants belonging to this genus are healthy not only for their nutritional features, but also for their wide biological activity. The latter is greatly attributed to the presence of some substances which *Allium* plants are able to accumulate, such as flavonoids, organosulfur compounds and methylated forms of selenium-containing aminoacids, i.e. selenomethyl selenocystein and γ-gluthamyl selenomethyl selenocystein, showing high antioxidant, cardioprotective and anticarcinogenic properties (IP *et al*., 2000; ADHIKARI, 2012). Notably, *Allium* plants fortified with Se reportedly display higher pharmacological activity than non-fortified ones (IP *et al*., 2000; ADHIKARI, 2012). Moreover, all *Allium* species belong to secondary accumulators of Se, even in the seeds (GOLUBKINA *et al*., 2015), with remarkable tolerance to high concentration of this element; accordingly, consumption of Se fortified *Allium* parts may give additional benefits to human health, taking also into account that Se deficiency has been detected in many countries worldwide (GOLUBKINA and PAPAZYAN, 2006).

Much attention is being paid for the last decade to waste valorization technologies of vegetable processing and to search for new natural sources of biologically active compounds. In this respect, the high content of antioxidants and organic sulfur compounds in *A. porrum* plant parts confer them high protective functions, such as antimicrobial, cardioprotective, hypocholesteremic, hypoglycemic and anticancer activity (RADOVANOVIĆ *et al*., 2015).

However, leek has not been deeply investigated as a potential source of pharmacologically beneficial compounds and, in most cases, pseudo-stems are just consumed whereas the leaf blades are discarded. Moreover, though this species is a natural source of methylated Se-containing amino acids with high anticancer activity, so far only one study concerning soil Se biofortification of leek has been carried out (LAVU *et al*., 2012) and no data on leaf Se accumulation have been reported.

The uneven distribution of elements in the earth crust is the cause of widespread Mg, Ca, Fe, Zn, Cu, I and Se deficiency in quite a few populations (WHITE and BROADLEY, 2009), which leads to numerous attempts to include these elements in bakery products (WIMALAWANSA, 2013; ROSELL *et al*. 2015). Among the latter, bread is largely consumed in most world countries and this justifies the relevant fortification with different nutrients, in order to produce functional food enriched with natural antioxidants, macro and trace elements (DE VALENÇA *et al*., 2017; ALLEN *et al*., 2006). Indeed, the interest in Se is due to its powerful antioxidant, immune-modulating, cardio-protective and anti-carcinogenic properties. It is estimated that the mean levels of wheat Se are 10-550 μg/kg, reaching 3-7 μg/kg in regions with Se deficient soils (several provinces of China) and 70 mg/kg in selenosis areas (India) (TAMAS *et al*., 2010). With the aim to solve the Se-deficiency issue in human organism, the use of Se biofortified cereals (LAZO-VÉLEZ *et al*., 2016; BRYSZEWSKA *et al*., 2007), Se enriched yeast (SCIENTIFIC OPINION, 2008) and bread supplement with Se fortified seedlings (BRYSZEWSKA *et al*., 2005) have been proposed. The main Se chemical form present in the latter products is selenomethionine (TAMAS *et al*., 2010), whereas *Allium* plants may provide more powerful anticarcinogen compounds, such as methylated forms of Se containing aminoacids (IP *et al*., 2000; ADHIKARI, 2012).

The higher dry matter in leek leaves compared with pseudo-stems simplifies the leaves dehydration process and gives wide chances to leaves powder utilization both as a spice and a supplement in functional food production with remarkable level of Se natural antioxidants.

Indeed, in recent years research has been frequently focused on the use of leaves and peel to produce powders with high antioxidants content and to the utilization of such powders in functional food preparation (FERREIRA *et al*., 2015; SONIA *et al*., 2016; ODUNLADE *et al*., 2017; LAKSHMI *et al*., 2017). However, so far leaves powder from Se enriched plants has never been used for these purposes, and in the case of *Allium* species this approach can also improve human organism protection against numerous diseases in addition to the increase of Se and other antioxidants consumption (GONZÁLEZ-MORALES *et al*., 2017).
The aims of the present research were: comparison of leek cultivars in terms of antioxidant and element composition in leaves and pseudo-stems; assessment of the effects of Se biofortification on quality characteristics of the most antioxidant-containing cultivar; evaluation of the efficiency of leek leaves powder use in the production of bread fortified with selenium-enriched and non-enriched leek leaves.

2. MATERIAL AND METHODS

2.1. Crop trials

Research was carried out on leek (*A. porrum* L.) at the experimental fields of Federal Scientific Center of Vegetable Production, in Odintsovo (Moscow, Russia, 55°39.51'N, 37°12.23'E) in 2015 and 2016 on a clay-loam soil, with pH 6.8, 2.1 % organic matter, 108 mg kg^{-1} N, 450 mg kg^{-1} P_2O_5, 357 mg kg^{-1} K_2O, exchangeable bases sum as much as 95.2%. Mean temperature and relative humidity values from May to October were 13.0, 16.1, 19.8, 18.6, 12.3, 6.4°C and 59.1, 63.8, 69.7, 72.4, 79.1, 81.0 % respectively. The experimental protocol was based on the comparison between nine cultivars (Goliath, Summer breath, Premier, Casimir, Kalambus, Campus, Vesta, Giraffe, Bandit), using a randomized complete block design with three replicates.
The sowing was performed on 5 December in 8 x 8 cm trays and the plantlets were transplanted in open field on 14 May, spaced 15 cm along the rows, the latter being 40 cm apart. Prior to planting, ploughing at 30 cm depth, hoeing at 15 cm and fertilization with 180 kg ha^{-1} N, 80 P_2O_5 and 120 K_2O were practiced; during the crops, 40 kg ha^{-1} N were supplied in three times at two-week intervals and, just in the last N application 7 kg ha^{-1} of P_2O_5 and of K_2O were also provided.
A further experimental trial was carried out in 2016 and 2017, assessing the effects of Se biofortification on leek pseudo-stem and leaf quality, by spraying the plants from June to August, once a week, with a total sodium selenate dose of 75 mg m^{-2}; cultivar Goliath was used, as it had showed the best quality indicators among the nine varieties compared in the previous year.
In either research, commercially ripe plants were harvested at mid-October and on samples taken in each plot the total, pseudo-stem and leaf blade weights were determined. Further plant samples were collected, gently washed with water to remove surface contaminants and dried with filter paper. Pseudo-stems and leaves were separated, cut with plastic knife, dried to constant weight and homogenized. The resulting powders were subjected to laboratory analysis and further used for bread production.

2.2. Bread production trial

Production of bread was achieved in 2016 and 2017 with three different processing procedures: bread obtained upon addition of leaves powder from leek selenium fortified plants to dough; bread obtained upon addition of leaves powder from leek non-fortified plants; traditionally-made bread.

Two hundred g of wheat flour with 90 μg selenium /kg d.w., 1.5 g salt and 8 g leek leaves powder were placed in a Kenwood dough mixer (Model A 907 D) set at highest speed and mixed for 1 min; control samples were not supplemented with leaves powder. Then, a suspension of 5 g yeast in 120 mL of water was added and the mixture was further run at high speed for 2 minutes. The dough was later kneaded on the kneading table, rounded into balls by hand and placed in lightly greased fermentation bowl in fermentation cabinet. The dough was then proofed for thirty minutes, baking was done at 250°C for 15 minutes and, next, the baked bread was allowed to cool at room temperature before performing determinations.
Baking was achieved in triplicate.

2.3. Volume, weight and specific volume of loaves produced

Loaf volume was measured by small seeds displacement method described by Khalil et al. (2000). Loaf was placed in a container of known volume where onion seeds were run until the container was full. The volume of seeds displaced by the loaf was considered as the loaf volumes, which were measured in a graduated cylinder. The weight of the loaf was determined using a sensitive weighing balance and the specific volume of the loaf was assessed by averaging the loaf volume with loaf weight. The specific volume was calculated according to the equation:

$$\text{specific volume } (cm^3/g) = \text{loaf volume}/\text{loaf weight}$$

2.4. Bread porosity

Bread porosity was determined according to GOST procedure (2001). Four cylindrical grooves from fresh bread were made with a volume of 27 (±0.5) cm each and weighed simultaneously. The porosity (%) was calculated by the following formula:

$$\text{porosity } (\%) = [(V\text{-}m/1.31):V] \times 100$$

where V is the total volume of bread grooves in cm^3; m is the mass of bread grooves in g; 1.31 is the density of non-porous mass of breadcrumb.

2.5. Dry matter content

The dry matter content in leaves and pseudo-stems of *A. porrum* as well as in bread samples was assessed after dehydration of the fresh samples in oven at 70°C, until they reached constant weight.

2.6. Selenium content

Se content in leek leaves and pseudo-stems as well as in bread samples was analyzed using the fluorimetric method previously described for tissues and biological fluids (ALFTHAN, 1984). The method includes digestion of dried homogenized samples via heating with a mixture of nitric-chloral acids, subsequent reduction of Se^{+6} to Se^{+4} with a solution of 6 N HCl, and formation of a complex between Se^{+4} and 2,3-diaminonaphtalene. Se concentration was assessed in triplicate by recording piazoselenol fluorescence value in hexane at 519 nm λ emission and 376 nm λ excitation. The results precision was checked

using a reference standard-lyophilized cabbage at each determination with 150 μg/Kg Se concentration (Institute of Nutrition, Russia).

2.7. Potassium content

Potassium content in leek pseudo-stems was assessed using AAS technique on Shimatsu 7000 spectrophotometer (Japan) after dry ashing of 2 g leek pseudo-stems and leaves at 420°C, and dissolution of the residues in 15 ml of 3% nitric acid (ANALYTICAL METHODS, 1996).

2.8. Total soluble solids (TSS) and sugars

Determination of total soluble solids was carried out in water extracts of leek leaves and pseudo-stems using TDS-3 conductometer (Russia).
Monosaccharides were determined using ferricyanide colorimetric method, based on the reaction of monosaccharides with potassium ferrycianide (SWAMY, 2008). Total sugars were analogically determined after acidic hydrolysis of water extracts with 20% hydrochloric acid. Fructose was used as an external standard.

2.9. Polyphenols

The concentrations of total phenolics in each sample of leaves, pseudo-stems and bread were determined in 70 % ethanol extract (1 hour at 80 °C) using the Folin-Ciocalteu colorimetric method, according to GOLUBKINA *et al.* (2017) by Unico 2804 UV (USA) spectrophotometer. The phenolic contents were calculated by using a calibration curve of gallic acid constructed with five concentrations of this compound (0-90 µg/mL). Phenolic contents were expressed as milligrams of gallic acid equivalents per 100 gram of dry weight (mg GAE/100 g d.w.).

2.10. Ascorbic acid

Ascorbic acid content in leek leaves and pseudo-stems was assessed by visual titration of fresh plant extracts in 6% trichloracetic acid with Tillmans reagent (AOAC, 2012). Five grams of fresh leek leaves were homogenized in porcelain mortar with 5 ml of 6% trichloracetic acid and quantitatively transferred to measuring cylinder. The volume was brought to 80 ml using trichloracetic acid, and the mixture was filtered through filter paper 15 min later. The ascorbic acid concentration was determined from the amount of Tillmans reagent, which went into titration of the sample.

2.11. Antioxidant activity

The antioxidant activity of leek leaves and pseudo-stems as well as bread samples was assessed using redox titration method (MAXIMOVA *et al*., 2001), via titration of 0.01 N $KMnO_4$ solution with ethanolic extracts of leaves, pseudo-stems and bread samples. Reduction of $KMnO_4$ to colorless Mn^{+2} in this process reflects the amount of antioxidants dissolvable in 70 % ethanol. The values were expressed in mg GAE/100 g d.w. The use of $KMnO_4$ acidic solution is known to be successfully used for the determination of *Ocimum basilicum* antioxidant potential (SRIVASTAVA *et al*., 2015) and antioxidant capacity of serum (ZHAN *et al*., 2014).

2.12. Statistical analysis

Data were processed by analysis of variance and mean separations were performed through the Duncan multiple range test, with reference to 0.05 probability level, using SPSS software version 21. The data expressed as a percentage were subjected to angular transformation before processing.

3. RESULTS AND DISCUSSION

3.1. Leek quality parameters

From the evaluation of nutritional indicators of nine leek cultivars, it arose that cultivar Goliath had significantly higher content of antioxidants, monosaccharides and potassium compared to the other varieties (Table 2). Notably, Goliath pseudo-stems attained 1.9 to 3.8 fold higher ascorbic acid, 1.6-2.6 fold polyphenols and 1.3-1.8 fold Se; leaf polyphenols concentration was 1.3-1.6 times higher. These results suggest that among the nine cultivars examined Goliath contains the highest content of polyphenols not only in pseudo-stems but also in leaves which are usually discarded, though their high potential benefits. Moreover, *A. porrum* leaves proved to be better sources of polyphenols than pseudo-stems in all cultivars. Conversely, selenium distribution between leaves and pseudo-stems in unfortified plants is less nutritionally important due to the low concentrations of this element.

A distinctive feature of cultivar Goliath was the high proportion of monosaccharides, accounting for 60.3 % of the total sugar amount in pseudo-stems compared to 18.2-41.9 % in the other varieties (Table 1). Moreover, Goliath pseudo-stems accumulated 2.2 to 11 fold more potassium (Fig. 1). Interestingly, positive correlations relevant to pseudo-stems were detected between polyphenols and K, Se and polyphenols, Se and K ($r = +0.96$; $r = +0.97$ and $r = +0.97$ respectively, at $P \leq 0.01$) and a negative correlation between leaves and pseudo-stems Se content ($r = - 0.88$ at $P \leq 0.01$). Due to the high nutritional value of cultivar Goliath, this variety was chosen in order to assess the effect of Se biofortification on leek quality and antioxidant features.

Table 1. Quality and antioxidant indicators of nine leek cultivars.

	Dry matter %	Ascorbic acid mg/100 g	Polyphenols mg GAE/100 g d.w.		Se µg/kg d.w.		Sugars %	
							monosaccharides	total
	plant	pseudo-stems	pseudo-stems	leaves	pseudo-stems	leaves	pseudo-stems	pseudo-stems
Goliath	12.35±0.4^{e}	13.0±0.7^{a}	683±56^{a}	964±72^{a}	107±7^{a}	14±1^{d}	3.8±0.3^{b}	6.3±0.4^{e}
Premier	15.20±0.5^{d}	8.9±0.5^{b}	432±34^{b}	650±21^{c}	80±5^{b}	65±3^{b}	4.4±0.3^{a}	10.5±0.7^{c}
Bandit	15.32±0.6^{d}	6.2±0.4^{c}	394±26^{bc}	647±26^{c}	75±5^{b}	48±2^{c}	3.5±0.2^{c}	8.6±0.5^{d}
Kalambus	17.64±0.6^{c}	4.5±0.4^{e}	319±19de	731±46^{b}	72±4^{b}	74±3^{b}	3.9±0.2^{b}	10.3±0.7^{c}
Cazimir	18.86±0.6^{c}	5.7±0.5^{cd}	284±20^{e}	728±53^{b}	60±3^{c}	76±4^{ab}	2.8±0.2^{d}	10.7±0.7^{c}
Giraffe	20.51±0.8^{b}	5.1±0.4^{d}	331±19^{d}	665±41^{bc}	73±4^{b}	49±2^{c}	3.4±0.2^{c}	11.0±0.7^{c}
Camus	21.36±0.7^{b}	4.5±0.3^{e}	347±21^{cd}	684±43^{bc}	64±3^{c}	81±5^{ab}	2.5±0.2^{e}	12.2±0.8^{b}
Vesta	23.39±0.8^{a}	4.5±0.3^{e}	301±19de	740±55^{b}	69±3^{bc}	84±5^{a}	2.6±0.2^{de}	14.3±0.8^{a}
Summer breath	24.28±0.9^{a}	5.4±0.4^{cd}	329±20^{d}	616±42^{c}	72±3^{b}	72±4^{b}	2.8±0.2^{d}	15.1±0.9^{a}
M	18.77	6.86	317	686	74,7	62,5	3.3	11.1
SD	3.24	2.69	71	37	8,4	17	0.6	2.1
CV (%)	17.3	39.3	22,4	5,4	11,2	27,2	18.2	18.9
Concentration range		4.5-16.9	284-683	616-964	60-107	14-84	2.5-4.4	6.3-15.1

Within each column, means followed by different letters are significantly different according to Duncan test at $P<0.05$.

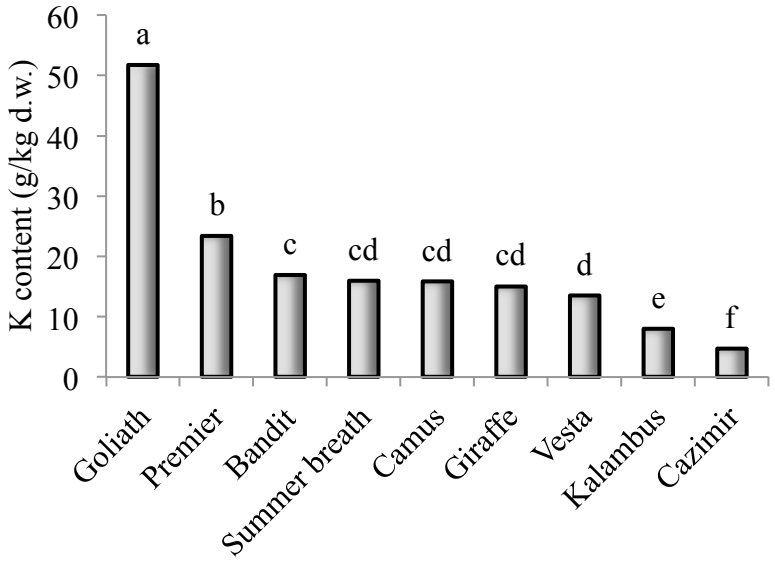

Figure 1. Intervarietal differences in K accumulation by leek pseudo-stems. Means followed by different letters are significantly different according to Duncan test at P<0.05.

3.2. Fortification of leek cultivar Goliath with Se

The data reported in Table 2 suggest that the enrichment of leek cultivar Goliath with selenium decreased plant weight due to leaf reduction, and significantly increased pseudo-stem occurrence to the total plant weight (+ 49.5%). Moreover, the leaves of selenium fortified *A. porrum* plants contained significantly higher contents of both dry matter and antioxidants compared to pseudo-stems. Notably, the contents of all the antioxidants detected were significantly higher in leaves than in pseudo-stems, i.e. 3.86 fold for ascorbic acid, 1.35 fold for polyphenols and 3.2 fold for Se. Total soluble solids value was also higher in leaves than in pseudo-stems (1.2 fold).

Table 2. Effect of Se application on yield, quality and antioxidant indicators of *A. porrum* cultivar Goliath.

	Se fortified plants		Ratio between fortified plants and non-treated control	
	pseudo-stems	leaves	pseudo-stems	leaves
Mean weight (g per plant)	196±15^{a}	200±9^{a}	1.1$^{n.s.}$	0.68*
Dry matter (%)	11.3±0.3^{b}	13.5±0.3^{a}	1.2*	1.38*
Ascorbic acid (mg/100 g f.w.)	15.0±0.6^{b}	57.9±0.8^{a}	1.15$^{n.s.}$	1.04$^{n.s.}$
Polyphenols (mg GAE/100 g d.w.)	1106±88^{b}	1494±76^{a}	1.62*	1.55*
Total sugars (%)	6.9±0.4	-	1.10$^{n.s.}$	-
Monosaccharides (%)	5.9±0.4	-	1.55*	-
Total soluble solids (mg/g)	70.0±2.7^{b}	83.2±3.6^{a}	1.27*	0.72*
Se content (μg/kg d.w.)	1451±50^{b}	4645±40^{a}	16.1*	332*
Potassium content (g/kg d.w.)	52.8±5.1^{a}	28.5±2.5^{b}	1.02$^{n.s.}$	1.04$^{n.s.}$

n.s. means no statistically significant differences between fortified and control plants;
*statistically significant at P≤0.05; within Se fortified plants, values along the rows followed by different letters are significantly different at P≤0.05.

According to the described data, leek foliar biofortification with Se performed on cultivar Goliath is beneficial to leek production, as it led to the increase of pseudo-stem yield as well as polyphenols and selenium concentration.
The investigations of LAVU (2013) on leek upon soil Se supply showed that the lower initial Se concentration in non-fortified pseudo-stems the higher the fortification level. The same phenomenon was observed in the present work: the fortification value reached 16.1 in pseudo- stems with high initial Se content (107 μg/kg), whereas it was as much as 332 in leaves with low initial Se concentration (14 μg/kg).
The uneven distribution of biologically active compounds in *A. porrum* plants was characterized by higher content of dry matter, ascorbic acid, polyphenols and total soluble solids in leaves, compared to pseudo-stems of selenium enriched plants. Similar distribution of these compounds was recorded in control plants, suggesting the nutritional importance of leek leaves, which are unfortunately discarded in the common farming practice. The antioxidant content increase in leaves and pseudo- stems as a result of selenium fortification is in agreement with the previously reported stimulating effect of selenium absorption on plant antioxidant defense (GOLUBKINA, 2016).

3.3. Supplementation of bread with leaves powder from Se fortified leek plants

Recent investigations have revealed that 4% supplementation of vegetable leaves power to wheat flour is optimal for producing functional bread (ODUNLADE *et al*., 2017).
Physical and biochemical characteristics of bread enriched with *A. porrum* leaves powder recorded in our research are reported in Table 4. The results suggest peculiar changes in bread quality upon supplementation of unfortified or Se fortified leaves to flour (Table 3).

Table 3. Physical, quality and antioxidant characteristics of bread enriched with *A. porrum* leaves powder.

Parameter	Additives		
	No additive control	*A. porrum* leaves powder	Se-enriched *A. porrum* leaves powder
Dry matter (%)	62.4±1.0^{ab}	60.4±1.0^{b}	64.5±1.1^{a}
Se content (μg/kg d.w.)	90±1^{b}	90±1^{b}	266±18^{a}
Total soluble solids (mg/g d.w.)	18.5±0.3^{c}	19.7±0.3^{b}	20.5±0.4^{a}
AOA (mg GAE/100g d.w.)	-	3.3±0.2^{b}	5.8±0.2^{a}
Polyphenols content (mg GAE/100g d.w.)	-	3.2±0.1^{b}	5.6±0.2^{a}
Specific volume (cm^3/g)	2.23±0.08^{a}	2.04±0.07^{b}	1.86±0.08^{c}
Bread porosity (%)	67.5±0.8^{a}	64.3±0.9^{b}	61.8±0.8^{c}
Colour	White	Light green	Light green

Along each row, values followed by different letters are statistically different according to Duncan test at $P \leq 0.05$.

As far as bread sensory attributes are concerned, the three products did not differ in terms of odor and flavor, whereas the bread supplemented with leek leaves powder showed a light green color, which did not vary between the samples treated with Se-fortified or non-fortified leaves. From a practical point of view, this unusual color may be preferred by consumers.

Notably, bread supplementation with selenium enriched leek leaves powder greatly differs from use of inorganic forms of selenium in bread production. Indeed, selenium salts (selenates and selenites) are known to be highly toxic and particularly dangerous upon overdosing. On the other hand, plant treatment with selenium inorganic salts allows to convert the latter to organic selenium derivatives of amino-acids and proteins, and in fact this process is named biofortification. As far as *Allium* species are concerned, such biofortification results in production of methylated forms of selenium-containing aminoacids showing remarkable anticancer activity (IP *et al*., 2000; ADHIKARI, 2012) which is significantly higher than that associated to the selenomethionine present in selenium enriched yeast (GOLUBKINA and PAPAZYAN, 2006)

Notably, the incorporation of leek leaves powder with high Se concentration into dough resulted in the highest values of Se content, total soluble solids, polyphenols and antioxidant activity of the final product. One hundred grams of such bread contain about 17 μg Se, which accounts for 31% of the required selenium consumption (Dietary Reference Intakes, 2000).

Se losses during bread baking were low and did not exceed 3%, which is consistent with previous investigation results (LYONS *et al*., 2005; ROSELL *et al*., 2015; GARVIN *et al*., 2011) about the stability of Se compounds during baking of bread from Se enriched wheat flour. Moreover, leek polyphenol stability during baking is higher in Se enriched product than under non fortified leaves use (93.7% vs 84.2% respectively). This phenomenon may reflect the well-known antioxidant protective effect of Se (GOLUBKINA and PAPAZYAN, 2006).

The results of the present work also prove the effect of Se supplementation on bread porosity and specific volume, the latter parameters decreasing according to the following sequence: control > bread with leaves powder from non fortified leek > bread supplemented with Se enriched leek leaves powder. The decrease in bread porosity and specific volume was reported in previous studies carried out on bakery products fortified with leaves powder from different plants by ODUNLADE *et al*. (2017). These authors explained that this phenomenon is the consequence of gluten concentration decrease due to the replacement of the flour portion containing leaves powder. Unfortunately, such a statement is not exhaustive for describing the phenomenon relevant to the effect of Se enriched leaves on bread porosity and specific volume; it is just indicative that using wheat with high Se content in bread production leads to 10% decrease of final product porosity (GARVIN *et al*., 2011), which is consistent with the 8.9% decrease relevant to Se fortified leek utilization detected in the present study. This peculiarity is presumably associated with Se, as the porosity decrease in the case of ordinary leek powder addition results in smaller changes of this parameter (4.7 %).

One of the reasons connected to dough rheological properties decrease in case of Se-enriched leaves powder use, compared to dough with non-fortified leaves and control dough, may be the increased concentration of plant polyphenols under selenium fortification. Indeed, despite their high nutritional value, polyphenols are known to cause changes in dough rheology via interaction with proteins, resulting in the decrease of enzymes and yeast activity and thus worsening bread porosity (WANG *et al*., 2007). In particular, gallate and hydroxylate benzol groups of polyphenols form noncovalent bonds with amino-, hydroxyl- and carboxyl groups of proteins (HUANG *et al*., 2004; ROSELL *et al*., 2015).

Another factor possibly affecting dough porosity is the high level of total soluble solids (ROSELL *et al*., 2015), which is increased in bread supplemented with *A. porrum* leaves powder, especially with high selenium content.

4. CONCLUSIONS

The experimental investigation carried out on leek in northern Europe allows to draw interesting remarks regarding both plant Se fortification and bread production using Se-enriched leaves. In this respect, Goliath was identified as the best responsive cultivar to Se application for obtaining nutrient-added pseudo-stems and its leaves powder was successfully mixed with dough during the bread making process. The mentioned practice is aimed to valorize both leek crop waste such as leaf blades and a widely consumed daily food such as bread. As arisen from this research, Se supply to *A. porrum* plants entails beneficial effects to human organism as a consequence of the use of either a fresh vegetable or a functional food.

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PAPER

UTILIZATION OF JERUSALEM ARTICHOKE POWDER IN PRODUCTION OF LOW-FAT AND FAT-FREE FERMENTED SAUSAGE

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ABSTRACT

The effects of Jerusalem artichoke powder (JAP) on the quality characteristics and storage stability of fermented sausage were investigated. The replacement of added beef fat with JAP were carried out during sausage manufacturing. Replacement of beef fat with JAP resulted in a significant decrease in TBARS and pH values and an increase in moisture and protein content in sausages during fermentation and storage ($p<0.05$). L* values decreased and a* values increased by adding JAP ($p<0.05$). JAP addition decreased hardness values and increased adhesiveness ($p<0.05$). The use of JAP encourages the development of lactic acid bacteria and positively affected their counts during the fermentation ($p<0.05$).

Keywords: sausage, Jerusalem artichoke powder, functional food, storage stability

1. INTRODUCTION

In recent years, functional meat products have attracted the attention of the consumer and their demands are rapidly increasing. Researchers and producers have tried to meet these demands with different strategies they have developed such as low fat, cholesterol, sodium chloride and nitrite and improved composition of meat products with incorporated health enhancing specific natural food ingredients (DEMEYER and ASTIASARÁN, 2007).
Natural additives have been more preferred than synthetic additives in food products because of their low toxicity, safety and cost efficiency (SIROCCHI *et al*., 2017; VAN HAUTE *et al*., 2016). Several natural and functional components mainly from plant origins have been introduced to meet consumer preferences and technological requirements. Therefore, improvement of meat products with some natural vegetable source compounds has been studied extensively over the past decades (HYGREEVA *et al*., 2014; OLMEDILLA-ALONSO *et al*., 2013). Vegetable sources with functional properties can be allowing many strategies to be carried out together in meat products and so many effects can be shown together such as reduction of beef fat, cholesterol and energy, enrichment with antioxidants, plant protein and dietary fiber and some technological improvements (GRASSO *et al*., 2014; OLMEDILLA-ALONSO *et al*., 2013). Jerusalem artichoke (*Helianthus tuberosus L.*) powder also may be a versatile alternative functional additive in dry fermented sausage to both reduction of beef fat and a consequent reduction of cholesterol, saturated fatty acid and energy value and increasing nutritional value and textural properties without adversely affecting quality characteristics.
Jerusalem artichoke contains high amounts of dietary fiber and most of it is inulin. Additionally, it contains some antioxidant compounds such as polyacetylenic derivatives, sesquiterpenes and coumarins (FURLAN *et al*., 2014). There are many studies about using of inulin and Jerusalem artichoke (GEDROVICA and KARKLINA, 2013) in many food products such as sausages, frankfurters, cookies, pasta and some dairy products and also successful results about technological and nutritional improvements have been reported in the literature (AFOAKWAH *et al*., 2015, FURLAN *et al*., 2014; PRAZNIK *et al*., 2002). However, study about the effects of Jerusalem artichoke powder in meat fermentation is limited in the literature. Therefore, the present study is aimed to assess the effect of Jerusalem artichoke powder as beef fat replacers on the quality characteristics and storage stability of dry fermented sausage, including physicochemical composition, lipid oxidation, color, textural and microbiological properties.

2. MATERIAL AND METHODS

2.1. Ingredients

A 24 h post-mortem *Longissimus thoracis et lumborum* cuts and beef fat (beef back fat) was purchased from a local butcher (Birlik Market, Nevsehir, Turkey) for each of 3 replications on separate production days. All meat cuts were trimmed from all subcutaneous and intermuscular fats. The lean beef and fat sources were separately ground in a 2 mm and 3 mm plate meat grinder, respectively (Arı Makine A.Ş, İstanbul). The freeze-dried culture mix, which contains *Lactobacillus curvatus, Staphylococcus xylosus, Staphylococcus carnosus* and *Pediococcus pentosaceus*, was used as starter culture mix.
Jerusalem artichoke (*Helianthus tuberosus L.)* tubers were obtained from a local producer (Nevsehir, Turkey) during harvest season. Jerusalem artichoke was washed, peeled and then dried using freeze dryer at −80°C and 0.01 mbar (Operon, OPR-FDU-8612, Korea).

Dried samples were ground to a fine powder using laboratory grinder (Yucebas Makine, Izmir, Turkey). Physicochemical properties, which are moisture, fat, ash, protein, carbohydrate and dietary fiber contents of Jerusalem artichoke powder was determined.

2.2. Sausage Preparation

The production and ripening of sausages were carried out as described in OZER and KILIC (2014). Five batches were prepared from ground beef. The control group incorporated with %72 lean beef and 20% beef fat was prepared without Jerusalem artichoke powder. The other experiment groups were given codes as JAP25, JAP50, JAP75 and JAP 100 in relation to the ratio of JAP powder used replacement of beef fat. JAP25, JAP50, JAP75 and JAP100 groups were produced with 15% beef fat and 5% JAP, 10% beef fat and 10% JAP, 5% beef fat and 15% JAP and 0% beef fat and 20% JAP, respectively. The other ingredients were added into sausages as follows: 2.5 % NaCl, 1.5 % garlic, 1.5 % red pepper, 0.8 % cumin, 0.5 % sucrose, 0.5 % black pepper, 0.5 % allspice and 150 ppm NaNO2. During the mixing, the starter culture mixture was added at a dose of 4-5 log cfu/g of sausage dough. After the filling up to cases of sausages samples, sausages were ripened at 95-70 % relative humidity (RH) at 25-18 ºC during 7 days; 24 h at 95 % RH at 24 ºC; 24 h at 90 % RH at 22 ºC; 12 h at 85 % RH at 20 ºC; 12 h at 80 % RH at 20 ºC; 48 h at 75 % RH at 18 ºC; and 48 h at 70 % RH at 18 ºC. Sausages were vacuum-packaged and then stored at 4 ºC for 30 days. The entire experiment was replicated three times on separate processing days.

2.3. Physicochemical composition

Fat, protein, ash and moisture content of sausages were measured at manufacturing and after fermentation day (AOAC 2005). pH of sausages were measured at manufacturing day, after fermentation and during storage period (7, 15, 30d) (AOAC 2005). Also, moisture, fat, ash, protein and total dietary fiber content of JAP according to AOAC (2005). Additionally, the total carbohydrate content of JAP was calculated by difference. Furthermore, total phenolic compounds were determined as described by TAKEUCHI and NAGASHIMA (2011) and total phenol concentration was quantified against a gallic acid (Sigma-Aldrich, St. Louis MO) standard curve. Finally, the water holding capacity of JAP was determined and expressed as g water per g of JAP (TAKEUCHI and NAGASHIMA 2011).

2.4. TBARS analysis

Thiobarbituric acid reactive substances (TBARS) values of samples were determined as described by KILIC and RICHARDS (2003) to evaluate of oxidation stability during fermentation and storage period (7, 15, 30 d) and TBARS values were expressed as μmol TBARS per kg of meat.

2.5. Color measurement

Color measurement was conducted by a Minolta Chroma Meter CR-200 (Minolta, Osaka, Japan) colorimeter using D65 as a standard daylight illuminant and a standard observer position of 10°. 8-mm-diameter circle and the specular component included (SCI) mode was used to measure. The colorimeter was standardized against a white calibration plate (D65, CIE L*=97.79, a*=−0.11, b*=2.69). Three readings were taken and averaged for each

of the three replications. Color values were determined at manufacturing day, after fermentation and during storage period (7, 15, 30 d).

2.6. Texture profile analysis

Samples were cut into slices (10±0.5 mm thick), wrapped with plastic film, and then held for equilibration to room temperature (20 ºC) for texture profile analysis (TPA). TPA tests and conditions were carried out as described in KıLıÇ and ÖZER (2017), using a texture analyzer (TA-XT2Í, Stable Micro Systems, UK). Test conditions were briefly as follows: rectangular probe (5 cm · 4 cm); pre-test speed 2 mm/s, test speed 5 mm/s, post-test speed 2 mm/s, 70 % compression and 50 kg load cell. Hardness (N), adhesiveness (Ns), springiness, cohesiveness, chewiness index, and resilience value of sausages were determined using 6 sausage slices per treatment.

2.7. Microbiological analysis

Sausage samples (10 g) were homogenized with sterile buffered peptone water (90 ml) in a stomacher at room temperature. Decimal dilutions in buffered peptone water were prepared and duplicate 0.1 ml samples of appropriate dilutions were spread. The following groups were investigated: total viable aerobic (TVAC) on Plate Count Agar (Merck, Darmstadt, Germany), incubated at 30°C for 48 h; lactic acid bacteria on De Man, Rogosa and Sharpe Agar (Merck, Darmstadt, Germany), incubated in anaerobic jar at 37°C for 48 h; yeast and molds on Potato Dextrose Agar (Merck, Darmstadt, Germany), incubated at 25°C for 72 h; coliform on Violet Red Bile Agar (Merck, Darmstadt, Germany), incubated at 37°C for 24 h.

2.8. Statistical analysis

The results were expressed as mean values with standard errors from the three replications. The statistical evaluation of the results was performed using the SPSS 22.0.0 (SPSS Inc., Chicago, USA). Data collected for physicochemical properties of sausages were analyzed by one-way analysis of variance (ANOVA). A completely randomized design was used with 5 treatment groups and 3 replications on separate production days. The treatments were one control group and four groups, which were assigned, and the data were analyzed using general linear model (GLM) procedure, in which treatment groups and storage time were assigned as fixed effects and replications as a random effect. Duncan multiple comparison test was used to compare means values and differences among mean values were considered significant when $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Microbiological analysis

Replacement of beef fat by JAP showed no significant effect on mold and yeast, total viable and coliform bacteria count during fermentation and storage periods (data is not presented) ($p<0.05$). The microbiological counts of sausage groups at the end of the fermentation ranged from 8.71 to 9.37 log cfu/g for total viable, from 5.61 to 5.82 log cfu/g for mold and yeast, and from 0.42 to 1.28 log cfu/g for total coliform bacteria, respectively. However, results of lactic acid bacteria count showed that the use of 50% and a higher rate

of JAP significantly increased lactic acid bacteria count during the fermentation period ($p<0.05$) (Fig. 1).

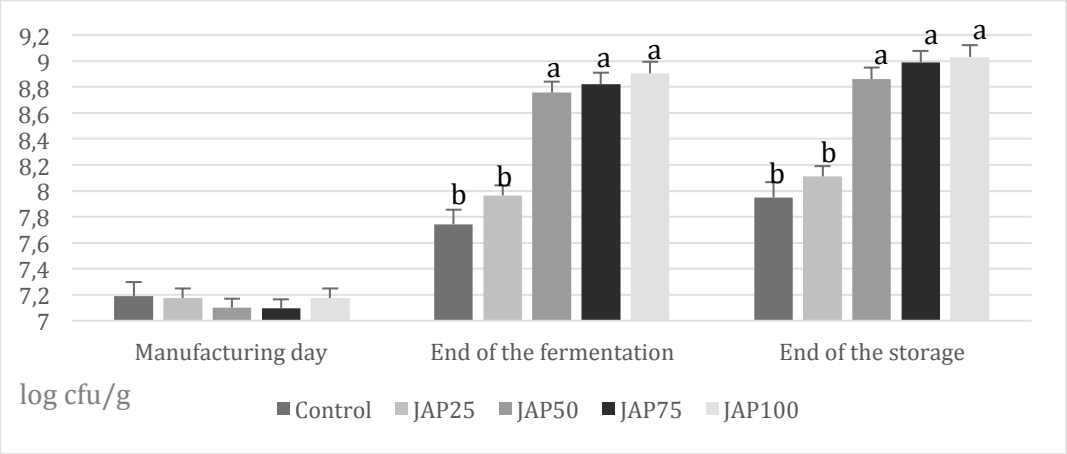

Figure 1. Lactic acid bacteria count in sucuk during the fermentation and storage periods.
a, b; Different letters in same day are significantly different ($P<0.05$).

This may be associated with the prebiotic effect of dietary fiber especially inulin and nutritional value of Jerusalem artichoke for lactic acid bacteria. It has reported that certain species of lactobacilli can ferment fructo-oligosaccharides and degrade even long-chain inulin-type fructans (CHOI *et al*., 2012). Additionally, CHOI *et al.* (2012) indicated that some *L. paracasei* strains could convert carbohydrates in Jerusalem artichoke to lactic acid without any pretreatment. There is numerous study about the prebiotic effect of added inulin to food products in literature and they indicated that inulin promotes the growth and activities of probiotic microorganisms in some dairy products such as yogurt, ice-cream and cheese (CARDARELLI *et al*., 2008).

3.2. Physicochemical composition analysis

Physicochemical properties of used JAP were determined and results showed that it contained 70.43%±0.68 carbohydrates, 8.18%±0.21 moisture, 9.46%±0.11 protein, 4.19%±0.18 ash and 7.13%±0.14 total dietary fiber. The determined mean proximate compositions for JAP are consistent with the literature (AFOAKWAH *et al*., 2015; PRAZNIK *et al*., 2002). Furthermore, JAP contained 103.41±21.6 mg GAE/kg total phenolic compounds. TAKEUCHI and NAGASHIMA (2011) have reported that peels of Jerusalem artichoke tuber were the main source of polyphenols and peels contains 10 times more polyphenols than peeled tubers and our result is similar to theirs. JAP revealed having the capacity to absorb at least 4.57±0.11 g of water/g at 20°C.
The proximate compositions of sausages formulated with different levels of JAP are given in Table 1. The use of JAP had shown significant differences in protein, ash, moisture and fat content of sausage dough. In addition, replacement of beef fat by JAP had shown significant differences on all components except ash values after fermentation. JAP75 and JAP100 had the highest protein content ($p<0.05$). Additionally, as expected, there were significant differences in the fat content of sausage dough ($p<0.05$) due to variation in the amount of beef fat used in sausage manufacture. Similar differences were also determined after fermentation ($p<0.05$) and control samples no containing JAP had the highest fat content after fermentation and storage ($p<0.05$). The moisture content for sausages after

fermentation was lower in the control group than other treatment groups ($p<0.05$). These results may be related to protein and carbohydrate content and water holding ability of JAP. It was also previously reported that compounds having water holding ability decelerate water loss and the drying rate during the ripening period in dry fermented meat products (CHOI *et al*., 2009). Additionally, Garcia *et al*. have reported that dietary fibers or additives containing rich carbohydrate and protein may cause a lower degree of water loss during ripening of fermented meat products such as dry sausage (GARCIA *et al*., 2002). As a result, the protein content proportionally increased by approximately 13% due to the drying process during the fermentation period, and fat content decreased by approximately 36% due to replacing beef fat with JAP in the final product.
pH of all sausage samples decreased during fermentation period ($p<0.05$) and were found to be at the same level for all sausage groups during the storage period (Table 2).

Table 1. Chemical composition* of sausage treatment groups.

Groups	Manufacturing Day	End of the Fermentation	Manufacturing Day	End of the Fermentation
	Protein (%)		Fat (%)	
Control	18.64±0.24 ^{cB}	25.91±0.24 ^{cA}	21.51±0.10 ^{aB}	31.35±0.18 ^{aA}
JAP25	18.98±0.10 ^{cB}	26.64±0.39 ^{cA}	19.39±0.27 ^{bB}	29.88±0.26 ^{bA}
JAP50	19.25±0.13 ^{bB}	27.83±0.14 ^{bA}	16.11±0.16 ^{cB}	25.71±0.13 ^{cA}
JAP75	19.70±0.16 ^{aB}	28.77±0.28 ^{aA}	12.91±0.20 ^{dB}	23.12±0.27 ^{dA}
JAP100	20.11±0.25 ^{aB}	29.41±0.43 ^{aA}	10.52±0.19 ^{eB}	19.91±0.19 ^{eA}
Groups	**Ash (%)**		**Moisture (%)**	
Control	1.91±0.04 ^{dB}	4.06±0.09 ^{bA}	56.94±0.28 ^{eA}	36.68±0.61 ^{eB}
JAP25	2.08±0.04 ^{cB}	4.28±0.14 ^{abA}	59.54±0.67 ^{dA}	39.20±0.39 ^{dB}
JAP50	2.19±0.16 ^{bcB}	4.52±0.21 ^{aA}	62.45±0.41 ^{cA}	41.94±0.27 ^{cB}
JAP75	2.40±0.13 ^{bB}	4.12±0.19 ^{abA}	64.99±0. 87 ^{bA}	43.99±0.74 ^{bB}
JAP100	2.76±0.08 ^{aB}	4.50±0.24 ^{aA}	66.60±0.48 ^{aA}	46.18±0.62 ^{aB}

*All values are the mean ± standard error of three replicates.
a, b, c, d, e (↓) Different letters within a column are significantly different ($p<0.05$).
A,B (→) Different letters within a row are significantly different ($p<0.05$).

Table 2. pH values* of sausage during the manufacturing, fermentation and storage periods.

Groups	Manufacturing Day	End of the Fermentation	Storage Days (d)		
			7d	15d	30d
Control	5.84±0.00 ^{bA}	5.11±0.00 ^{aB}	5.08±0.00 ^{aB}	5.07±0.00 ^{aB}	5.06±0.01 ^{aB}
JAP25	5.85±0.00 ^{abA}	5.06±0.02 ^{bB}	5.04±0.02 ^{bB}	5.02±0.03 ^{bB}	5.01±0.02 ^{bB}
JAP50	5.86±0.01 ^{abA}	5.02±0.01 ^{cB}	4.99±0.01 ^{cB}	4.98±0.02 ^{bB}	4.96±0.01 ^{cB}
JAP75	5.85±0.00 ^{abA}	4.94±0.00 ^{dB}	4.94±0.00 ^{dB}	4.93±0.00 ^{cB}	4.92±0.01 ^{dB}
JAP100	5.87±0.00 ^{aA}	4.90±0.00 ^{eB}	4.88±0.00 ^{eB}	4.84±0.00 ^{dB}	4.84±0.00 ^{eB}

*All values are the mean ± standard error of three replicates.
a, b, c, d, e (↓) Different letters within a column are significantly different ($p<0.05$).
A, B, C, D, E (→) Different letters within a row are significantly different ($p<0.05$).

Replacement of beef fat by JAP had shown no significant differences in sausage dough at manufacturing day. However, replacement of beef fat by JAP in sausage production affected the pH of sausages during the fermentation period. pH values of sausages decreased depending on the amount of replacement of beef fat by JAP in the sausage formulation and significant differences were determined among the treatment groups at the end of fermentation day and storage period ($p<0.05$).
MENDOZA *et al.* (2001) have reported a conflicting result with us that the use of inulin as a fat substitute in low fat-dry fermented sausages did not affect the pH during the fermentation period. However, the reason for decreases in pH of sausages in the present study is may be related to protein, carbohydrates and dietary fiber content of JAP. These components in JAP could be used as a nutrient by lactic acid bacteria and produced more lactic acid. Additionally, JAP may have created a suitable environment with high moisture content for development of starter culture by decelerating water loss and the drying rate during the fermentation period. Despite the effects of replacement of beef fat by JAP on the physicochemical properties of sausages produced in the present study were in accordance with the values reported by previous studies and Turkish standards for sausage (BOZKURT and BAYRAM, 2006; TSE, 2002).

3.3. TBARS analysis

TBARS values of sausages were measured throughout fermentation and the storage period shown in Table 3. The TBARS values of all sausage samples increased during fermentation and storage periods ($p<0.05$).

Table 3. TBARS values* of sausage (μmol/kg) during the manufacturing, fermentation and storage periods.

Groups	Manufacturing Day	End of the Fermentation	Storage Days (d)		
			7d	15d	30d
Control	0.74±0.02 ^{bE}	2.19±0.04 ^{aD}	2.82±0.02 ^{aC}	3.49±0.02 ^{aB}	4.88±0.02 ^{aA}
JAP25	0.78±0.07 ^{abE}	1.81±0.01 ^{bD}	2.46±0.10 ^{bC}	3.12±0.02 ^{bB}	4.26±0.10 ^{bA}
JAP50	0.78±0.02 ^{abE}	1.59±0.01 ^{cD}	2.22±0.02 ^{cC}	2.73±0.05 ^{cB}	3.82±0.06 ^{cA}
JAP75	0.82±0.03 ^{abE}	1.47±0.02 ^{dD}	2.01±0.09 ^{dC}	2.43±0.06 ^{dB}	3.47±0.03 ^{dA}
JAP100	0.85±0.01 ^{aE}	1.35±0.02 ^{eD}	1.89±0.02 ^{dC}	2.24±0.02 ^{eB}	2.92±0.02 ^{eA}

*All values are the mean ± standard error of three replicates.
a, b, c, d, e (↓) Different letters within a column are significantly different ($p<0.05$).
A, B, C, D, E (→) Different letters within a row are significantly different ($p<0.05$).

There were no differences among TBARS values among all treatment groups for sausage dough. However, highest TBARS values in control group and lowest TBARS values in JAP100 group were determined compared with other treatments at the end of fermentation and storage period ($p<0.05$). According to TBARS results, it can be said that the replacement of beef fat by JAP in sausage production significantly decreased TBARS values ($p<0.05$). The decrease in TBARS levels may be a result of both reducing the beef fat and increasing the JAP content. Additionally, it is conceivable that bioactive components such as flavonoids and phenolic compounds such as polyacetylenic derivatives, sesquiterpenes and coumarins in Jerusalem artichoke exhibiting antioxidative activities and contributing to decrease in TBARS values (FURLAN *et al.* 2014).

3.4. Color analysis

The color properties of used JAP were determined and results showed that L* value is 81.18±0.51, a* value are 2.32±0.11 and b* value is 8.71±0.23. Replacement of beef fat by JAP influenced CIE L* and CIE a* values in sausage dough, at the end of fermentation and during storage period ($p<0.05$) (Table 4). However, non-significant differences on CIE b* values were determined during fermentation and storage period ($p<0.05$).

Table 4. Color values* of sausages during the manufacturing fermentation and storage periods.

	Groups	Manufacturing Day	End of the Fermentation	Storage Days (d) 7d	15d	30d
L* values	Control	58.02±2.70 ^{aA}	50.07±2.46 ^{aB}	49.35±2.42 ^{aB}	49.91±2.45 ^{aB}	50.40±2.48 ^{aAB}
	JAP25	46.89±1.42 ^{bA}	42.67±1.30 ^{bcB}	42.06±1.28 ^{bcB}	42.53±1.30 ^{bcB}	42.96±1.30 ^{bcB}
	JAP50	47.44±1.44 ^{bA}	43.99±0.42 ^{bB}	43.36±1.40 ^{bB}	43.85±1.42 ^{bB}	44.29±1.43 ^{bB}
	JAP75	43.50±0.67 ^{cA}	40.09±0.66 ^{cB}	39.51±0.65 ^{cB}	39.96±0.65 ^{cB}	40.36±0.66 ^{cB}
	JAP100	41.21±1.06 ^{cA}	37.08±1.20 ^{dB}	36.55±2.17 ^{dB}	36.96±2.19 ^{dB}	37.33±2.22 ^{dB}
a* values	Control	12.33±1.37 ^{cA}	11.22±1.24 ^{cB}	11.11±1.23 ^{cB}	11.38±1.26 ^{cB}	11.50±1.28 ^{cAB}
	JAP25	14.85±0.49 ^{bA}	13.51±0.44 ^{bB}	13.37±0.44 ^{bB}	13.70±0.45 ^{bB}	13.84±0.46 ^{bB}
	JAP50	15.72±0.15 ^{bA}	14.31±0.13 ^{bB}	14.16±0.13 ^{bB}	14.51±0.13 ^{bAB}	14.65±0.14 ^{bAB}
	JAP75	15.91±0.30 ^{bA}	14.48±0.28 ^{bB}	14.33±0.36 ^{bB}	14.68±0.32 ^{bB}	14.83±0.01 ^{bAB}
	JAP100	17.34±0.22 ^{aA}	16.78±0.20 ^{aAB}	15.62±0.20 ^{aB}	16.00±0.20 ^{aAB}	16.16±0.80 ^{aAB}
b* values	Control	10.65±0.71 ^{aA}	10.86±0.72 ^{aA}	10.80±0.72 ^{aA}	10.75±0.72 ^{aA}	10.86±0.73 ^{aA}
	JAP25	12.32±2.76 ^{aA}	12.56±2.82 ^{aA}	12.50±2.80 ^{aA}	12.43±2.79 ^{aA}	12.56±2.82 ^{aA}
	JAP50	12.95±1.04 ^{aA}	13.21±1.06 ^{aA}	13.14±1.06 ^{aA}	13.08±1.05 ^{aA}	13.21±1.06 ^{aA}
	JAP75	11.63±1.04 ^{aA}	11.86±1.06 ^{aA}	11.80±1.05 ^{aA}	11.74±1.05 ^{aA}	11.86±1.06 ^{aA}
	JAP100	10.89±1.49 ^{aA}	11.11±1.57 ^{aA}	11.05±1.55 ^{aA}	11.00±1.53 ^{aA}	11.11±1.57 ^{aA}

*All values are the mean ± standard error of three replicates.
a. b. c (↓) Different letters within a column are significantly different ($p<0.05$).
A. B. C. D (→) Different letters within a row are significantly different ($p<0.05$).

When the replaced of beef fat by JAP, CIE L* values decreased and CIE a* values increased in sausage samples. It was determined that the control group had the highest CIE L* and lowest CIE a* values at all manufacturing period ($p<0.05$). Additionally, CIE L* values for all treatment groups decreased during fermentation period ($p<0.05$). Some researchers have reported that non-meat ingredients may affect the color properties of minced meat products because of the dilution of meat pigments rather than the color of the additives (SARTESHNIZI *et al*., 2015; TRESPALACIOS and PLA, 2007). These results in the present study were similar to some previous studies about the use of some vegetable source ingredient in meat products (ALAKALI *et al*., 2010; ERGEZER *et al*., 2014).

3.5. Texture profile analysis

Table 5 shows the TPA results of the sausages. Replacement of beef fat by JAP in sausage had shown a significant effect on hardness and adhesiveness. However, there were no significant changes in resilience, cohesiveness, chewiness index and springiness index among all sausage treatment groups. Results revealed that replacement of beef fat by JAP

resulted in an increase in adhesiveness values of sausages and a decreased in the hardness values compared control group ($p<0.05$). It is reported in many studies that fat reduction in comminuted meat products results in significant changes in textural properties and generally, hardness values of products increased (COLMENERO, 1996; KEETON, 1994; STEHLE, 2009). However, results of the present study showed that 50%, 75% and 100% replacement of beef fat by JAP in sausage resulted in a decrease in hardness values of sausages. JAP25 group, which contains 25% JAP and 75% beef fat, and the control group had the same level of hardness values.

Table 5. Texture profile analysis* of sausage at the end of the fermentation period.

Groups	Hardness (N)	Adhesiveness (mj)	Resilience	Cohesiveness	Springiness Index	Chewiness Index (N)
Control	61.69±2.39 ^{a}	0.43±0.05 ^{c}	0.02±0.00 ^{a}	0.40±0.07 ^{a}	0.60±0.09 ^{c}	0.90±0.25 ^{a}
JAP25	60.92±1.07 ^{a}	0.64±0.07 ^{b}	0.03±0.00 ^{a}	0.36±0.02 ^{a}	0.75±0.03 ^{bc}	0.88±0.26 ^{a}
JAP50	52.40±0.88 ^{b}	0.76±0.04 ^{a}	0.03±0.01 ^{a}	0.44±0.02 ^{a}	0.81±0.02 ^{b}	1.28±0.42 ^{a}
JAP75	35.20±0.63 ^{c}	0.80±0.00 ^{a}	0.05±0.01 ^{a}	0.44±0.07 ^{a}	0.88±0.02 ^{b}	1.35±0.33 ^{a}
JAP100	27.94±1.13 ^{d}	0.83±0.03 ^{a}	0.04±0.02 ^{a}	0.36±0.07 ^{a}	1.07±1.16 ^{a}	1.22±0.24 ^{a}

*All values are the mean ± standard error of three replicates
a, b, c (↓) Different letters within a column are significantly different ($p<0.05$)

The decrease in hardness values may be a result of increasing the proteins, carbohydrates and dietary fiber that have functional properties such as water binding and retention properties. Researchers have reported that fat replacer containing proteins, carbohydrates and dietary fiber may interact with water and fat of meat products and therefore lead to a change in textural properties (ERGEZER *et al*., 2014). The effects of using JAP on the moisture content of sausage also support this idea.

4. CONCLUSIONS

Regarding to results of lipid oxidation analysis, this study proved that improved lipid oxidation stability in fermented sausages during fermentation and storage can be achieved by replacing beef fat with Jerusalem artichoke powder. However, some textural differences, which can be undesirable by the consumer, can occur. Nevertheless, it can be concluded that meat products manufacturers should consider replacing beef fat with up to 25% Jerusalem artichoke powder in low-fat fermented sausage production to enhance positive nutritional effects such as lower beef fat and energy value and rich dietary fiber and improve the shelf life of sausage.

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PAPER

TURKISH-COFFEE ENRICHED WITH ROSE: A PROMISING COMBINATION

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ABSTRACT

The purpose of this study was to develop a new & healthy version of Turkish-coffee enriched with rose. Conventionally roasted *Coffee Arabica L.* beans for Turkish-coffee & dried-*Rosa Damascene Mill.*[7/0,7/0.5,7/1.5,7/2g(w/w)] was grinded. Total-Phenolic Contents (TPC), Total-Antioxidant (TAS) & Oxidant-Status (TOS) were measured and Oxidatif Stress Index (OSI) was calculated. Consumer panel testing was done. TPC of the coffee samples with 1.5 & 2g rose was different according to plain coffee ($p<0.05$). TAS value (mmol/L) of plain-coffee was 2.2±0.11 while the values of the coffees including 0.5g,1.5g,2g rose were 2.4±0.09,2.4±0.05,2.7±0.02, respectively. TOS value (μmol/L) of plain-coffee was 17.6±0.24, while the values of the coffees including 0.5 g, 1.5 g and 2 g rose were 13.0±1.00, 9.4±1.30, 9.4±0.31, respectively ($p<0.05$). OSIs of coffee samples including 0, 0.5, 1.5, 2 g rose were found to be 7.7, 5.2, 3.6, 2.7 respectively ($p<0.05$). The coffees including 1.5 & 2 g rose had the highest sensory-scores. Turkish-coffee including rose will strengthen already existing phenolic & antioxidant features of coffee, and thus contribute the improvement of health & taste.

Keywords: antioxidants, coffee, oxidants, polyphenols, rose, Turkish coffee

1. INTRODUCTION

Recent studies in nutrition & health have focused on more detailed research on the effects of foods on health (KRİS-ETHERTON *et al*., 2002; KRİS-ETHERTON *et al*., 2004; MRC, 2017). These studies are mostly conducted on the components of foods, processing techniques and alternative development (BIER *et al*., 2015; POTİ *et al*., 2015). The food industry has embarked on a consumer-oriented mission with new product development or product modifications matching with scientific nutrition and health recommendations (BIER *et al*., 2015). In particular, many herbal foods have become important raw materials for many fields due to their bioactive compounds, especially phenolic compounds (EL 2008). The bioactive compounds found and defined in foods vary according to their numbers, chemical structures and functions (FİLHO *et al*., 2007; KRİS-ETHERTON *et al*., 2002; KRİS-ETHERTON *et al*., 2004).

Coffee is a globally consumed beverage and recent studies showed that consuming coffee in acceptable amounts have potential health benefits (LİANG *et al*., 2016). It's known to be a natural antioxidant and recent studies comment on antioxidant effects of coffee along with other benefits and linking them to prevent various common diseases (AGUİAR *et al*., 2016). Turkish Coffee, a traditional delicacy for the Turks with its unique flavor and aroma is becoming a popular beverage globally (ÖZGÜR, 2012). *Coffee Arabica L*., which is the most used bean type for Turkish coffee, is widely used in pharmacology, homeopathy, therapeutics and gastronomy due to its health benefits (CAPEK *et al*., 2014).

Rosa damascena is a herb with economical value. Turkey is a leading manufacturer of rose and rose products especially around the city of Isparta (ANON 2003). In addition to its economical value, incorporation of "*Rosa damascena*" into pharmacology, homeopathy, therapeutics and gastronomy demonstrates a broad range of its uses and health benefits (BOSKABADY *et al*., 2011; MAHBOUBİ, 2016). Researchers indicate that rose products are also natural antioxidants, and thus use of rose as nutraceutical foods is useful for both health aspects and adding aesthetic value and taste to make it appealing to consumers (MLCEK AND ROP 2011; KOVATCHEVA-APOSTOLOVA *et al*., 2008). Additionally, there are studies regarding its anelgesic, antimicrobial, antioxidant, anti-inflammatory, antidiabetic and antidepressant features fields (BOSKABADY *et al*., 2011; MAHBOUBİ, 2016). It is considered that antioxidant and antimicrobial effects of *Rosa damascena* originate from its phenolic content and essential fat composition (ÖZKAN *et al*., 2004). Citronellol and geraniol are the two main compounds found in the essential oil of *Rosa damascena* and responsible for its pharmacological activities (MAHBOUBİ, 2016). Moreover, quarcetin, kaempferol and their glycosides are the flavonol glycosides, which are responsible for the high antioxidant activity of *Rosa damascena* flowers, petals and extract (BOSKABADY *et al*., 2011). However, it is reported that further research is required on the use of *Rosa damascena* plant in preclinical and clinical investigations (MAHBOUBİ, 2016).

The purpose of this study is to develop a new and healthy version Turkish coffee with rose through preserving its traditional and nutritional value while investigating consumers' liking and preferences. Besides contributing the efforts for improvement of health and strengthening already existing phenolic content and antioxidant features of coffee which is a widely consumed and traditional beverage in Turkey, this study will ensure that a Turkish-origin healthy beverage will be introduced as an innovative design to the world.

2. MATERIAL AND METHODS

This study was conducted on two stages. The first stage consists of the provision of sample, preparing the samples for chemical analysis and the measurements of phenolic content and antioxidant capacity. The second stage consists of the preparation of Turkish coffees and tasting the coffee samples prepared by using coffee and spent rose, which are mixed in certain amounts [7/0, 7/0.5, 7/1.5, 7/2 g (w/w)].

2.1. Preparation of Rose and Coffee Samples

Fresh (unfaded) petals of unprocessed *Rosa Damascena,* which were harvested in Isparta, were dried at room temperature. Before having been ground, rose petals and coffee beans were stored within closed dark glass jars at room temperature until analysis and/or tasting panel day so that they preserved their compound and freshness.
The most consumed coffee in the world (85-90%), *Coffee Arabica L.* beans were selected for the study. Raw coffee beans were roasted in the coffee shop for 4 minutes at 220 °C. The samples were prepared according to the traditional Turkish coffee standard published by The Turkish Coffee Culture and Research Association (ÖZGÜR, 2012).
Coffee beans and rose petals were fine ground with a grinding machine (Krupps F203 Electric Grinder®) on the analysis day. Fine ground 7 g coffee was prepared with 70 mL water (traditional coffee cup measure). To ensure the volume of coffee cup, the samples of Turkish coffee cups were also collected from coffee manufacturers and coffee shops, and their average service volumes were calculated.
Instead of steel/copper coffee pot, which is used for the preparation of traditional Turkish coffee, an electricity coffee pot (Arçelik K® 3300) was used to prepare multiple samples and ensure standardization. Coffee samples were prepared in avarege 1.15 minutes.
Fine ground coffee and rose petals were taken in certain amounts [7/0, 7/0.5, 7/1.5, 7/2 g (w/w)] to prepare four different samples as described above. Samples were taken from only the drinkable part of the coffee to eppendorf tubes one minute after the rose petal coffee was ready. Three samples of each experimental coffee were prepared and all samples were analyzed for three times.

2.2. Measurement of Total Phenolic Content (TPC)

The amount of phenolic compounds in the spent rose (*Rosa damascene Mill.*), Turkish coffee beans and all coffee samples with spent roses were determined by Folin-Ciocalteu colorimetric method (SİNGLETON *et al*., 1999). For sample extraction, spent rose and coffee beans were dissolved and homogenized in 80% ethanol and heated for 5 minutes. Extracts filtered with Whatman filter paper (number 4). Then, 80% ethanol was added to residue after filtration and heated for 10 minutes and filtered again up to ensure extraction. Extracts of dry coffee-rose samples and drinkable part of the coffee samples with rose in eppendorf tubes dilueted and analysed using a Folin-Ciocalteu reagent. TPCs were expressed as mg/L gallic acid equivalents (GAE) extract.

2.3. Measurement of Total Antioxidant Status (TAS)

TAS levels were measured using commercially available kits (Relassay, Turkey). The novel automated method is based on the bleaching of characteristic color of a more stable ABTS (2,2′ - Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The assay has excellent precision values, which are lower than 3%. The results were expressed as mmol Trolox equivalent/L (EREL, 2004).

2.4. Measurement of Total Oxidant Status (TOS)

TOS levels were measured using commercially available kits (Relassay, Turkey). In the new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L) (YUMRU *et al*., 2009).

2.5. Calculation of the Oxidative Stress Index (OSI)

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). For calculation, the resulting unit of TAS was converted to μmol/L, and the OSI value was calculated according to the following Formula (YUMRU *et al*., 2009):

$$\text{OSI (arbitrary unit)} = \text{TOS (}\mu\text{mol } H_2O_2 \text{ equivalent/L)} / \text{TAC (}\mu\text{mol Trolox equivalent/L)}$$

2.6. Sensory evaluation

The samples prepared in the same order as the coffees prepared for chemical measurements. Sugar was not added to coffee samples. Fine ground coffee and rose petals were taken in certain amounts [7/0, 7/0.5, 7/1.5, 7/2 g (w/w)] to prepare traditional Turkish coffee samples as described above.
Panelists were chosen among the people (20-64 years) who consume Turkish coffee regularly, accepted participating in the study voluntarily, do not smoke and do not have any mouth and dental (tooth decay etc.) and chronic disease that may affect their palate. Each group is expected to consist of 10-15 individuals in such studies considering the fact that there is not any data in the literature with regard to the appropriate number of panelists and qualitative research aspect of the study (ÇOKLUK *et al*., 2011). Therefore, 15 panelists participated in the study.
Panelists were chosen in "Food Preparation Laboratory" and they were seated in a way that they could not communicate with each other during the tasting stage. Each panelist was also given a number randomly. The panelists were informed for approximately fifteen minutes about the procedures of evaluation and the points to be taken into account before coffee presentation. The coffees prepared by the researchers with rose petals in four different amounts were presented in Turkish coffee cups, which had the same volume and design (70 mL). In order that panelists could differentiate the tastes of coffee samples, they were presented every ten minutes and participants were asked to eat a fat-free salt cracker and gargle with water at the intervals. After each coffee taste, the panelists wrote down their rating points form for each coffee sample. The evaluation was made from the lowest point (0 points) towards the highest point (10 points).
Panelist evaluation scores were assessed separately for each sample under the following criteria:

- Smell (the harmony of coffee and rose smell, dominant one etc.)
- Aroma (harmony and range of aroma component)
- Taste (the taste and aroma combination experienced when the coffee sample was tasted)

- Aftertaste (the duration of feeling the taste at the back of palate after swallowing the sample)
- Acceptability
- Preference (consumption preference)
- General impression (panelist's own remarks)

This study was approved by Baskent University Institutional Review Board (Project no: KA17/83) and supported by Baskent University Research Fund. Clear explanations were provided for the individuals with regard to the purpose of the study, after which written informed consent was obtained from all participants in accordance with the Declaration of Helsinki (World Medical Association).

2.7. Statistical analysis

All statistical analyses were performed using SPSS (The Statistical Package for Social Sciences) Version 20.0 (SPSS Inc., Chicago, IL, USA). Percentage, mean ± standard deviation (SD) values were taken for the evaluation of the data. In addition, a Kolmogorov-Smirnov test was used to determine whether the panelist evaluation ratings had a normal distribution. Panelists' evaluation ratings for the coffee samples were shown as mean ± SD and median (minimum-maximum). Kruskal Wallis and Mann-Whitney-U tests were used to compare the means of TPC, TAS, TOS and OSIs of coffee samples prepared with rose in four different amounts. Differences among means with $p < 0.05$ were accepted as representing statistically significant differences.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the Total Phenolic Content and Antioxidant/Oxidant Status

Table 1 shows the TPC (mg/L GAE), TAS (mmol/L), TOS (μmol/L) and OSIs of coffee samples prepared with raw and spent rose and coffee samples in different amounts. Accordingly, the TPC of raw dry coffee and rose was 611.3±1.19 mg/L GAE and 605.2±0.64 mg/L GAE respectively. There was no statistically difference between the TPC of plain coffee sample (445.7±6.66 mg/L GAE) and that of the samples prepared with 0.5g rose (458.4±11.75) ($p>0.05$). However, TPC of the coffee samples prepared with 1.5 and 2g rose (463.1±6.42 and 479.3±12.56 mg/L GAE) was statistically different compared to that of plain coffee sample. In addition, TPC of the coffee samples with 1.5 and 2g rose was not different from that of the sample with 0.5g rose ($p>0.05$). TPC of the coffee samples with 1.5 and 2 g rose was similar to each other ($p>0.05$).
The evaluation of TAS of the samples showed that the TAS of raw/dry coffee and rose samples was 3.1±0.00 mmol/L and 2.3±0.02 μmol/L respectively. The evaluation of TAS of the coffees prepared with rose in different amounts and without any rose showed that the TAS value of plain coffee was 2.2±0.011 mmol/L, while the values of the coffees including 0.5g, 1.5g and 2g rose were 2.4±0.09, 2.4±0.05 and 2.7±0.02 mmol/L respectively, and thus all TAS values of coffee samples with rose were different from each other ($p<0.05$).
The evaluation of TOS of the samples showed that the TOS values of raw/dry coffee and the spent rose were 9.5±0.02 μmol/L and 16.8±0.06 μmol/L respectively (Table 1). In addition, the evaluation of TOS of coffee samples showed that the TOS value of plain coffee was 17.6±0.24, while the values of the coffees including 0.5 g, 1.5g and 2g rose were 13.0±1.00 μmol/L, 9.4±1.30 μmol/L and 7.5±0.31 μmol/L respectively. The difference between the TOS values of all coffee samples with rose was important ($p<0.05$).

The evaluation of OSIs of all samples showed that the OSIs of raw/dry coffee and rose samples were 3.0 and 7.2 respectively. OSIs of coffee samples including 0, 0.5, 1.5, 2 g rose were found to be 7.7, 5.2, 3.6 and 2.7 respectively ($p<0.05$) (Table 1).

Table 1. The total phenolic content (GAE/g), total antioxidant status (mmol/L), total oxidant status (μmol/L) and oxidative stress indexes of coffee samples including rose in different amounts*.

Sample	TPC (mg/L GAE)	TAS (mmol/L)	TOS (μmol/L)	OSI
Powder and raw				
Dry coffee	611.3±1.19	3.1±0.00	9.5±0.02	3.0
Spent rose	605.2±0.64	2.3±0.02	16.8±0.06	7.2
Boiled (per Turkish coffee cup) (w/w)				
7 g coffee	445.7±6.66 [a]	2.2±0.11[a]	17.6±0.24[a]	7.7[a]
7 g coffee + 0.5 g rose	458.4±11.75[a]	2.4±0.09[a]	13.0±1.00[b]	5.2[b]
7 g coffee + 1.5 g rose	463.1±6.42 [b]	2.6±0.05[b]	9.4±1.30[c]	3.6 [c]
7 g coffee + 2 g rose	479.3±12.56 [b]	2.7±0.02[c]	7.5±0.31[d]	2.7[d]

*TPC: Total Phenolic Content, TAS: Total Antioxidant Status, TOS: Total Oxidant Status, OSI: Oxidative Stress Index.

[a-d]Values are the mean ± SD of three replicates. Values with different letters in the same column are statistically different ($p<0.05$).

3.2. Sensory evaluation

In this experiment, plain and coffee with rose (with different concentrations) were tasted in the panel and 66.7% of the panelists were female, while 33.3% of them were male. The mean age of the panelists was 43.8 ±7.5 years. 46.7% of the panelists were drinking Turkish coffee every day, 46.7% several times a week and 6.6% once a week. 66.7% of the participants were drinking sugar-free Turkish coffee, 26.7% of them preferred coffee with little sugar and 6.6% consumed coffee with sugar.

Table 2 shows the evaluation scores (smell, aroma, taste, aftertaste, acceptability, preference and general impression) of the panelists about the coffee samples with rose in different amounts. The sensory evaluation scores given to coffee samples with rose in different amounts show that panelists gave more points to the coffee samples containing 1.5 g and 2 g rose than plain coffee sample (6.2±2.2 vs. 6.1±2.3, respectively) (Table 2). In addition to this, the sensory evaluation scores of the coffee samples containing 0.5g rose are similar to or less than that of plain coffee (Table 2).

When general impression scores of the coffee samples as a result of the panel test and total antioxidant and total oxidant status of coffee samples are evaluated, the interpretation of consumer preference and both TAS and TOS of coffee samples showed that the coffee sample containing 2 g rose both had relatively the highest TAS and low TOS and got the highest score from the panelists. Additionally, the coffee sample containing 1.5 g rose content had relatively higher TAS and lower TOS than plain coffee and received higher general impression score from the panelists.

Table 2. Panelist evaluation scores of coffee samples with rose in different concentrations*.

Sample	Smell	Aroma	Taste	Aftertaste	Acceptability	Preference	General Impression
7 g coffee	5.7±2.8 5 (1-9)	5.8±1.7 6 (3-8)	6.3±2.4 7 (3-9)	6.8±1.6 7 (4-9)	6.1±2.3 6 (3-9)	5.8±2.3 6 (3-9)	5.8±1.77 6(4-9)
7 g coffee+0.5 g rose	6.6±1.5 6 (4-8)	5.7±1.8 6 (3-8)	6.4±1.7 7 (4-8)	6.8±0.7 7 (6-8)	5.3±1.34 6 (3-7)	5.6±1.9 6 (3-9)	5.4±2.09 6(3-9)
7 g coffee+1.5 g rose	7.3±1.4 8 (5-9)	7.0±1.7 8 (4-9)	6.5±1.8 6(4-9)	7.9±0.7 8(7-9)	6.4±1.7 6 (4-9)	6.1±1.7 6 (4-9)	6.2±2.2 6(4-9)
7 g coffee+2 g rose	7.0±1.6 8 (5-9)	6.3±1.9 6 (3-9)	6.4±1.9 6 (4-9)	7.4±1.8 8 (5-10)	6.0±2.3 5 (3-9)	5.7±2.2 5 (3-8)	6.1±2.3 5(3-9)

*Scores were shown as mean ± SD and median (minimum-maximum points).

4. DISCUSSION

Coffee is consumed worldwide and one of the most popular beverages. A number of epidemiologic and clinic studies proved that coffee consumption may prevent several chronic and degenerative diseases, such as cancer, cardiovascular disorders, diabetes, and Parkinson's disease (LUDWIG *et al*., 2014). Within this scope, this study was conducted to strenghten potential health effects and antioxidant feature of Turkish coffee and improve health condition by adding rose petals to the traditional beverage Turkish coffee which is widely consumed in Turkey.

It was found out in the study that TPC of the raw and dry coffee beans of *Coffee Arabica L* roasted for 4 minutes at 220° C was 611.3±1.19 mg/L GAE, while the value decreased to 451.4±1.20 mg/L GAE after the coffee was boiled. Moreover, TAS value decreased while TOS values and OSIs increased. An important family of phenolic compounds, chlorogenic acids are green coffee compounds which are formed by the esterification of caffeic, ferulic, and p-coumaric trans-cinnamic acids with (−)-quinic acid and associated with hepatoprotective, hypoglycemic and antiviral activities because of their antioxidant effects (FARAH AND DONANGELO, 2006). Chlorogenic acids (CGA) and related compounds (caffeoylquinic acids, dicaffeoylquinic acids, feruloylquinic acids, *p*-coumaroylquinic acids and mixed diesters of caffeic and ferulic acids with quinic acid and their isomers) constitutes the most important phenolic fraction of green coffee bean (CLIFFORD, 2000) and its content reaches up to 14% in dry substance (FARAH AND DONANGELO, 2006). The chlorogenic acid content of a 200 mL (7-oz) cup of coffee has been reported to range from 70-350 mg (CLİFFORD, 1999).

During the roasting, CGA can turn into isomerized, hydrolyzed or degraded lower molecular weighted materials. Roasted beans of *Coffee Arabica L.* contain 1.9-2.5 g/100g CGA in average, while the roasted beans of *Coffee Canephora* contain 3.3-3.8 g/100 g in average and these average values change depending on the type of coffee beans (FARAH, 2012). Especially roasting coffee beans at high temperatures (230°C) turns a part of CGA into quinolactons and melanoidins, and thus CGA content decreases after roasting (Farah 2006; FARAH, 2012). A study showed that roasting coffee beans causes 23% CGA loss because of degredation and constitutes condensed form (42-62 mmol/100 g) and ester-linked melanoiding forms (1.1-1.6 mmol/100 g) (COELHO *et al*., 2014). It was considered after this study that the decrease in the TPC after the roasted dry coffee was boiled in water at high temperature was the result of the transformation and condensation of the free forms of CGA into quinolactons and melanoidins through various mechanisms due to degredation. Moreover, the decrease in TPC also caused decrease in total antioxidant capacity and increase in oxidant capacity and OSI value.

The determination of TPC, TAS, TOS and OSI values of a coffee prepared in Turkish coffee preparation method (7 g/70 mL) for the first time was one of the important findings of this study. Coffees are prepared in different methods such as drip or filter, plunger or cafetière, espresso, cappuccino, moka-napoletana, percolator, soluble or instant and flavored (KARABUDAK *et al*., 2015). Preparation method of Turkish coffee is different from other coffee types (ÖZGÜR, 2012). For the traditional preparation of Turkish coffee, fine ground powder coffee is added to cold water and the coffee is boiled. This produces a strong coffee with a layer of foam on the surface and sediment (not meant for drinking) that settles on the bottom of the cup. LUDWİG *et al*. (2012) indicated that brewing time and preparation method affected the antioxidant amount of coffee (LUDWİG *et al*., 2012). It is generally reported that the total antioxidant capacity of 7-10 g of coffee is 150-300 mg/g (YASHİN *et al*., 2013). In a comprehensive evaluation made by using different methods, ferric reducing antioxidant power (FRAP) values of espresso, instant coffee, decaffeinated espresso coffee were 129.4, 108.6, 93.0 mol Fe^{+2}/L respectively (PELLEGRINI *et al*., 2003). Additionally, it was found out that total radical trapping antioxidant power (TRAP) of espresso was 66 mol Trolox/L, that of instant coffee 52.4 Trolox/L and that of decaffeinated espresso 45.8 mol Trolox/L. Trolox equivalent antioxidant capacity (TEAC) values for espresso, instant coffee and decaffeinated espresso were found to be 36.5, 32.5 and 27.0 mol Trolox/L respectively (PELLEGRİNİ *et al*., 2003). Therefore, the presence and amount of bioactive compounds have important roles in health effects depending on the preparation method of coffee (PETERS, 1991). However, the unstandardized methods and amounts (water and coffee amounts) and different unit of measurement (w/w, w/v, w/dose, and w/cup) make it difficult to compare the results of studies with other studies and the coffees prepared with different methods (CAPRİOLİ *et al*., 2015). This is one of the most important limitations in the literature.

Rosa damascena Mill's antimicrobial, anti-inflammatory, anticancer effects and protective effects against neurological, cardiovascular and liver diseases were proved in a number of *in vitro* and animal studies (NAYEBİ *et al*., 2017). The flowers, petals and hips of *Rosa Damascena* contain terpenes, glycosides, flavonoids and anthocyanins. In addition, this plant contains carboxylic acids, myrcene, vitamin C, kaempferol, quarcetin, tannin and essential oils and organic acids (BOSKABADY *et al*., 2011). A study showed that three flavonol glycosides, specifically quercetin-3-O-glucoside, kaempferol-3-O rhamnoside and kaempferol-3-O-arabinoside, contained in *Rosa Damascena*, are responsible for antioxidant activity. In our study, the TPC and TAS of spent rose leaf powder were found to be quite high (605.2±0.64 mg/L GAE and 2.3±0.02 mmol/L, respectively). In another study conducted by using *Rosa Damascena* extracts grown in the same region of Turkey, TPC of fresh leaf extract of *Rosa Damascena* was 276.0±2.93 mg/L GAE, while that of spent flower was found to be 248.9±2.96 mg/L GAE. Antiradical activities of fresh and spent leaf were determined, through α-diphenyl- α-picrylhydrazyl (DPPH), to be %74.51±1.65 and %75.94±1.72 at 100 ppm respectively. Additionally, determined by the method depending on phosphomolybdenum complex formation, the antioxidant activity of fresh leaf extract (372.2±0.96 mg/g) was higher than that of spent leaf extract 351.3±0.84 mg/g (ÖZKAN *et al*., 2004). The reason of the difference between the results of our study and above mentioned study may be a number of factors such as seasons and the difference in analysis techniques, land and improvement methods although *Rosa Damascenas* of the same region were used.

The aim of this study was to increase existing TPC and TAS value of Turkish coffee by adding spent rose flower grown in Isparta region of Turkey. As a result, TPC and TAS values increased and TOS and total OSI values decreased after 0.5 g, 1.5 g and 2.0 g rose aroma were added to plain coffee. The coffee sample containing 2g rose (the highest amount) had the highest TPC and TAS values and lowest oxidant content and OSI values.

Some studies suggest that *Rosa damascena* plant may be used as a medical source in the prevention and treatment of many diseases caused by free radicals (BOSKABADY *et al*., 2011). It is considered that it would be beneficial to carry out preclinical and clinical evaluations of above mentioned rose coffee samples in future studies.

Oxidative stress is associated with the excessive increase in oxidant levels and/or antioxidant capacity. The atoms or molecules, which contain one or more unpaired electron(s), are called oxidants or free radicals in biological system. Oxidants deteriorate cell structure and extracellular matrix and cause damages in genetic structure by distorting DNAs. Therefore, free radicals have a role in the pathogenesis of various diseases such as atherosclerosis, neurodegenerative diseases, cancer, allergies, diabetes and cataract (YUMRU *et al*., 2009). This study evaluated the OSIs of the coffee samples containing rose in different amounts and revealed that OSI values of coffee samples decreased as the amount of rose increased. In another study, rats were given 50, 75, 100 and 200 mg/kg/day of ethanol extract produced from *Rosa Damascena* petals for 10 days orally and it was found out that all doses of *Rosa Damascena* prevented lipid peroxidation and the highest antioxidant activity was observed after the consumption of 200 mg/kg (SHAHRİARİ *et al*., 2007). Therefore, coffee with rose consumption may be considered to support the antioxidant defense system in order to prevent free radical formation and prevent biological damage.

Coffee is one of the most widely consumed beverages throughout the world because of its unique sensory properties (KREUML *ET AL*., 2013). So, aromatic components are very important in coffee beverages, because they are the principal constituents of sensory experience for coffee consumers (JAIMES *et al*., 2015). The aroma of the coffee comes from caffeine and trigonelline alkoloid, chlorogenic acid, kahweol and cafestol and melanoidin, which is a Maillard reaction products (LUDWIG *et al*., 2014). One of the way for strenghten aroma profile of coffee is inserting a herb with rich source of aroma such as rose. Aroma compounds of rose vary according to parts of herb and its recovering periods (FENG *et al*., 2008; ZHAO *et al*., 2016). At the full opening stage of rose, β-citronellol citronellol acetate, phenethyl alcohol, geranyl acetate, geraniol, phenethyl acetate, nerol, *n*-hexyl acetate and *α*-myrcene, and alcohols are the major constituents of aroma (ZHAO *et al*., 2016).

Consumer appreciation/evaluation plays an important role in the innovation studies of food and various drinks. Therefore, this study includes panelist evaluation tests conducted on the coffee samples containing different amounts of rose aroma. In these evaluations, panelists ranked coffees in terms of their smell, aroma, taste, acceptability, preference and general impression and the coffee samples containing 1.5 g and 2 g rose aroma received relatively highest scores generally. These coffee samples had the highest phenolic content and antioxidant status, but lowest oxidant status and OSI values. Coffee consumers prefer products, not only good flavour and taste, but also good for health (JAIMES *et al*., 2015). So, through this study, it was to develop a new and relatively healthy version Turkish coffee with rose through preserving its traditional and nutritional value while investigating consumers' liking and preferences.

5. CONCLUSIONS

It is considered to strenghten existing phenolic content and antioxidant features of Turkish coffee especially with Turkish coffees containing 1.5 and 2 g rose as an innovative design. Moreover, these coffee samples are considered as the antioxidant & healthy products enriched with a different aroma and appreciated by consumers. Nonetheless, it is necessary to conduct a further study for more detailed investigation of the consumption

doses, possible potential short and long term health effects and risks of these promising combinations.

As far as is known, this is the first study that a promising combination of coffee and *Rosa Damascena* is shown as an innovative design. This study has a number of limitations. First of all, total antioxidant and oxidant profile were focused in this study. So, each phenolic composition of rose and coffee samples related to the antioxidant and oxidant activity were not determined. Nevertheless, it is considered that this study will light the way for other studies. Furthermore, this study focused on Turkish coffee, which was a particular kind of brewed coffee. So, the results could not be generalized to the effects of all coffee types. Finally, when comparing to other studies, our number of panelists might be relatively less or not because of rigid inclusion criteria. Further sensory evaluation may conduct with a larger sample size, including different age groups and populations. It is believed that taking into consideration these situations would be useful in future studies.

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PAPER

LIPID AUTOXIDATION OF FISH, LARD, CORN AND LINSEED OILS BY ISOTHERMAL CALORIMETRY

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ABSTRACT

This work proposes an approach to characterize lipid oxidation of oils based on the measurement of isothermal calorimetry data, including information on the duration of the monomolecular, bimolecular and termination periods. The approach has been exploited with fish and lard oil samples at temperatures from 40 to 80°C and with corn and linseed oils at 80°C. The length of the monomolecular period was the most sensitive to the variation of temperature. Accordingly, the monomolecular period was used as index of oxidative stability of oils. Thus, the highest oxidative stability was observed for corn oil samples at 80°C (t_{mono} = 2060 min), followed by linseed (t_{mono} = 390 min), fish (t_{mono} = 40 min) and lard oils (t_{mono} = 30 min). The different stability of the samples was discussed with their fatty acids profile and antioxidant activity. The results confirmed that the content in natural antioxidants is the key responsible for the final oxidative stability of the samples.

Keywords: autoxidation periods, fish oil, edible oils, isothermal calorimetry, oxidative stability

1. INTRODUCTION

Lipid autoxidation is a radical chain mechanism that involves a reaction between unsaturated fatty acids and oxygen (JOHNSON and DECKER, 2015; TUORILA and CARDELLO, 2002). This reaction is of great importance for the food industry, as its occurrence is often associated with quality defects, like rancidity or nutritional loss. The mechanism of reaction is generally described in three main stages: an "*initial pahse*", comprising the monomolecular phase of hydroperoxide formation. This is followed by the "*propagation phase*", where radicals are formed with autocatalytic, monomolecular and bimolecular or branching reactions, resulting in the exponential increase of the rate of lipid autoxidation. Then, the process ends with a "*termination phase*", where the rate of lipid reaction slow down and increase the formation of secondary oxidation products (GHNIMI, 2017). Between the first and second phase, the abrupt change in the rate of lipid autooxidation is often defined as the induction period (IP). This value is widespread used by food industry as index to characterize the oxidative stability of an oil sample (FRANKEL, 2014).

The measurement of the induction period is generally obtained by differential scan calorimetry (DSC) (SHAHIDI and ZHONG, 2015; LITWINIENKO *et al.*, 1999) or differential thermal analysis (DTA) (BACHA *et al.*, 2015). However, the IP value obtained with these techniques is often difficult to interpret when the samples have a complex composition. One drawback of DSC is that, at low temperatures, the exothermic peak of oxidation is flat, and the IP value cannot be determined unambiguously. Moreover, when DSC is run at high temperatures, the oxidative behavior of foods become dependent on the temperature gradient used and also on the geometry of the samples (FOX and STACHOWIAK, 2007; KOUSKSOU *et al.*, 2011). Furthermore, the analysis of oil samples that contain antioxidants at high temperatures may lead to misleading results when the antioxidants are volatile and can leave the sample before they can exert their protective action (HAMAMA and NAWAR, 1991).

An alternative approach for the analysis of lipid autoxidation is offered by isothermal calorimetry (HAMAN *et al.*, 2017a). This technique has some advantages respect DSC and DTA, as it can be used to monitor in real-time the rate of autoxidation of unsaturated fatty acids under mild temperatures. Its theoretical background is relatively simple and well-described by Willson (WILLSON *et al.*, 1995) and Hansen (HANSEN *et al.*, 1990). Recent applications of isothermal calorimetry include the study of oxidation of lipid oxidation (DRIDI *et al.*, 2016), the study of the radical scavenging activity of lipophilic antioxidants (HAMAN *et al.*, 2017b), antioxidant and pro-oxidant activity of spent coffee extracts (HAMAN *et al.*, 2018) and the study of shelf-life of foods (RIVA *et al.*, 2001). However, in these studies, the calorimetric trace was mainly used to provide the cumulative enthalpy evolution of the sample, or the induction time. In such way, only a small fraction of the information contained in the calorimetric trace is used.

For this reason, this work aims to propose a methodological approach that can extend the information normally achievable with calorimetric data, providing information about the duration of the monomolecular, bimolecular and termination phase of the lipid autoxidation process. For this purpose, fish and lard oil samples were analyzed by isothermal calorimetry at different temperatures (from 40 to 80°C), and the results compared with those obtained with linseed and corn oils at 80°C. The relevance and utility of this approach was discussed considering the fatty acid composition and the antioxidant capacity of the oils.

2. MATERIAL AND METHODS

2.1. Materials

Refined corn oil, fish oil, lard oil, cyclohexane (purity ≥ 99%), sodium thiosulphate (purity ≥ 99%) and potassium iodide (purity ≥ 99%) were purchased from Sigma-Aldrich (Milan, Italy). Refined linseed oil (cold pressed) was from Sabo Italia s.r.l. (Firenze, Italy). Chloroform (stabilized with ethanol) was purchased from PanReac AppliChem (Milan, Italy). Acetic acid (purity ≥ 99.7%) was purchased from Fluka (Milan, Italy).

2.2. Fatty acid profile by near infrared spectroscopy

Fatty acids were determined by near infrared (NIR) spectrometer with a Multipurpose Analyzer (MPA) (Bruker Optik GmbH, Germany). Acquisition software was OPUS 7.5 (Bruker Optik GmbH, Germany). The background spectrum consisted of empty cell at the same temperature of analysis. OPUS/QUANT package was used to quantify the composition of fatty acids (palmitic, stearic, oleic, linoleic and linolenic acid) in fish, lard, linseed and corn oils. The analysis was performed at the same conditions of the calibration kit (calibrations based on 8084 spectra) in the range of 12500-4000 cm^{-1} (resolution, 16 cm^{-1}; sample, 64 scans) at 40°C.

2.3. Isothermal calorimetry

A micro-calorimeter (Thermal Activity Monitor, Model 421 TAM III, TA Instruments), equipped with 24 channels, was used to measure the heat rate. Each channel of the instrument is a twin calorimeter where the two units are positioned above each other. In isothermal mode, the oil in the thermostat was maintained at a constant temperature with an absolute accuracy of ±0.0005 °C (manufacturer's data). The micro-calorimeters were equipped with built-in metal reference specimens having a heat capacity approximately equal to that of a vial.
The heat generated or absorbed was continuously measured over time. Following the manufacturer's instructions, each channel was calibrated prior to measurement using a gain calibration procedure with electric impulses. Ampoules were first lowered into the thermal equilibration position and left there for 15 min. Then, the ampoules were lowered into the measurement position and the heat flow rates recorded for up to 5 and 8 days at 10-second intervals. The auto-oxidation reaction was monitored by calorimetry with close glass ampoules containing 100±3 mg of oil in isothermal conditions in the presence of air in the head space.

2.4. DPPH assays for the determination of the antioxidant activity

The antioxidant activity of the oils was evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) as described by Brand Williams (BRAND-WILLIAMS *et al.*, 1995) with small modifications. DPPH is a free radical that can react directly with most of the antioxidants and be captured by them (KOLEVA *et al.*, 2002). The reduction of DPPH was measured by the decrease in absorbance at a characteristic wavelength (517 nm) and a determined time during the reaction (minimum 60 min). In the radical form (DPPH·) presented a violet color at 517 nm. With the addition of a reduced antioxidant (AH), its color changed from violet to yellow and consequently the absorption decreased as a result of the decrease of the DPPH concentration. Briefly, 10 mg of DPPH were dissolved in 250 mL of ethanol and sonicated for 1 min. A defined amount of oil was dissolved in 1 mL ethanol and sonicated

(DU-06, China) for 5 min. To perform the measurements, 1.9 mL of DPPH solution were transferred into the cuvettes and 100 μL of the extracts solutions were added. The samples were kept in dark for 1 h at room temperature (ca. 25 °C) and subsequently the absorbance was recorded at 517 nm with a spectrophotometer (Cary 100 Series UV-Vis Spectrophotometer, Agilent Technologies, Italy). The antioxidant capacity of the oils was determined as the IC_{50} - the concentration of antioxidant, which reduces the radical form (DPPH•) of 50%. A triplicate assay was performed for each sample.

2.5. Determination of peroxide value

The peroxide value (PV) of the samples was measured according to the AOAC Official Method 965.33 (HORWITZ, 2002). The method is based on an iodometric titration, which measures the iodine produced from the potassium iodide by the peroxides present in the oil. The measurements were performed in triplicate for each sample and PV values were expressed as meqO2 per Kg of oil.

2.6. Statistical analysis

Calorimetric results were reported as the mean of six independent experiments. All other measurements (NIR and DPPH) were performed in triplicate (n = 3). All the results are reported as mean, together with the relative standard deviation. To compare the means values, data were subjected to the analysis of variance (ANOVA) using Fisher's least significance difference method with a 95 % confidence level.

3. RESULTS AND DISCUSSION

3.1 Oxidative stability based on monomolecular and bimolecular periods

Figure 1 shows the heat-flow signal of a fish oil sample recorded at 40°C. At any point, the intensity of the heat-flow signal reflects the rate of the autoxidation process that is occurring to the sample, accordingly to the following general relationship (eq. 1) (WILLSON *et al.*, 1995):

$$\text{heat flow} = \text{rate x enthalpy} \qquad (1)$$

At the beginning of the experiment, the signal is negligible (from the beginning to the point (a), Fig. 1). This corresponds to a slow autoxidation process. At about 25 h (point (a) of Fig. 1), the rate of heat production suddenly increases, at point (b), reaching a maximum heat flow value at point (c). After that, the signal fades down to a minimum steady state, at point (d). The final heat-flow signal has an intensity close to that recorded at the beginning of the experiment.

Figure 1 also shows the changes in the apparent reaction order as a function of time. The apparent reaction order was determined at any time following an approach previously described by Haman (HAMAN *et al.*, 2017a). Briefly, for a reaction A → B, where A represents unsaturated fatty acids and B is their oxidation products, the heat-flow signal (dq/dt) is equal to $k \cdot H \cdot x^n$, where k is the rate constant, H is the reaction enthalpy, x is the number of moles of product formed at time t and n is the reaction order. Since x can be obtained, at any time, as the ratio between the heat (q) and the enthalpy (H), then this equation becomes (eq. 2):

$$\frac{dq}{dt} = k \cdot H \cdot \left(\frac{q}{H}\right)^n = k \cdot H^{1-n} \cdot q^n$$

The log-log plot of the heat flow with the heat leads a slope, which corresponds to the reaction order (n). Thus, by following the changes of n as a function of time, it is possible to identify three distinct phases of the autoxidation process. For fish oil, the first phase is characterized by a reaction order equals to ~ 0.5. This order corresponds to the *monomolecular period*, during which the reaction is catalyzed by monomolecular decomposition of the hydroperoxides (LOOH + LH → LO* + L* + H_2O) (LABUZA *et al.*, 1969; GHIMNI *et al.*, 2017). The duration of such phase lasts in about 25±0.3 h. Interestingly, this phase ends with the onset point of the calorimetric trace (also called induction point). This point is very informative since can be interpreted as the moment when a food become unacceptable for human consumption (LABUZA and DUGAN Jr, 1971). After this point, the reaction order becomes close to 1. It is during this period that the rate of lipid autoxidation rises exponentially. In this exponential stage, the bimolecular decomposition of hydroperoxides is the dominant mechanism (LOOH + LOOH → LO* + LOO* + H_2O). This process is known to follow the *bimolecular* period (LABUZA and DUGAN Jr, 1971). The length of such phase lasts in about 25±0.3 h (from point (a) to point (b) of Fig. 1).

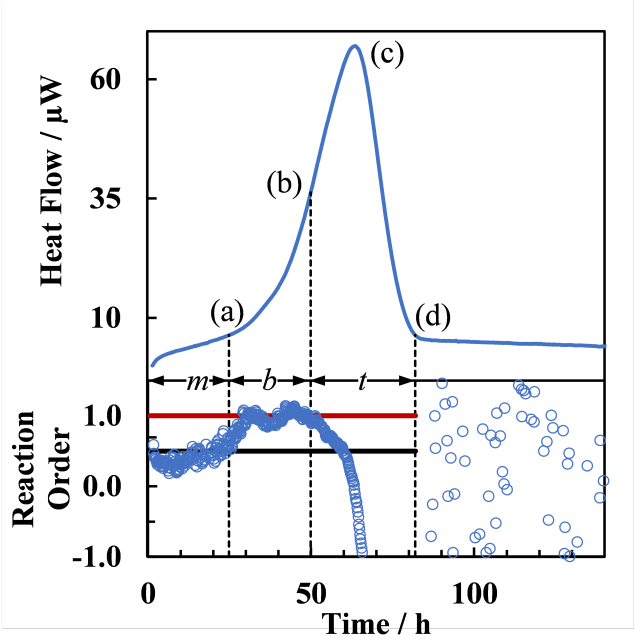

Figure 1. Calorimetric trace of fish oil in isothermal conditions at 40°C. Legend: (a) end of the monomolecular phase; (b) end of the bimolecular phase; (c) the maximum heat flow and (d) the end of the termination phase; Legend: *m* stands for monomolecular phase; *b* stands for bimolecular phase and *t* stands for termination phase.

After this point, the reaction order, suddenly, declines towards negative values. Negative reaction orders correspond to the so-called "*termination phase*", where the rate of the reaction is either inhibited by the high concentration of the reactants (i.e. radicals species) or by the absence of oxygen in the ampoule. In this stage, radical-radical combinations occur, and hydroperoxides start to exponentially decompose to secondary oxidation products, including aldehydes and ketones (GHINMI *at al.*, 2017). In the experimental

setup used here, the termination phase occurred when the concentration of oxygen in the ampoule was limiting. This was verified in a control experiment, where, successively to the analysis of a fish oil sample, the ampoule was opened without removing the sample, fresh air was left to enter into the headspace of the ampoule, and the recording of the calorimetric signal re-started. In such way, we observed that a new exothermic peak could be observed. This result confirms that the termination phase was limited by oxygen and that further monomolecular and bimolecular process could occur provided that oxygen is available. Accordingly, the termination period of the observed autoxidation process in the ampoule was defined as the period occurring from point (b), where the reaction order starts decreasing, to point (d), where the reaction is likely terminated because of oxygen depletion in the headspace of the ampoule. At times above 80 h, the reaction was completed. The measure of the reaction order become unpredictable because of the heat flow values were nearly constant, with slopes equals nearly to zero.

3.2. Oxidative stability of corn and linseed oil

The same approach used before with fish oil was next applied to characterize the oxidative stability of different oil samples, including fish, lard, linseed and corn oils. All the sample showed similar initial peroxide values. The resulting heat flows were plotted as a function of time (Fig. 2).

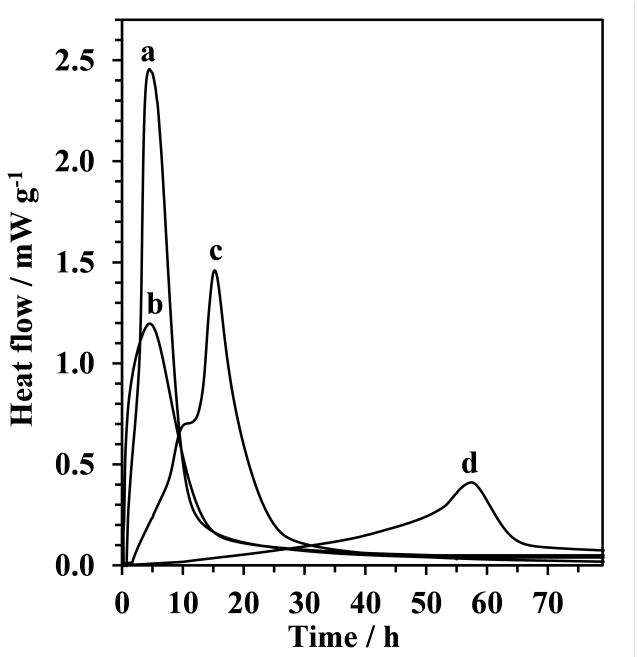

Figure 2. Heat flow *vs*. time curve plot of a) fish oil, b) lard oil c) linseed oil and d) corn oil, placed isothermally at 80°C into closed glass ampoule.

Table 1 reports the duration of the monomolecular, bimolecular and termination step. By comparing the duration of the monomolecular period at 80°C, the highest oxidative stability was observed for the corn oil sample (2060 min), followed by linseed (390 min), fish (50 min) and lard (30 min) oil samples.

Table 1. Kinetic and thermodynamic parameters of fish, lard, corn and linseed oil.

Oil	Temperature (°C)	Phase duration			ΔH
		Monomolecular (min)	Bimolecular (min)	Termination (min)	(kJ/mol O_2)
Fish	40	2220±20	802±10	1780±35	43.1±0.1
Fish	50	600±8	653±11	1102±24	59.3±0.4
Fish	60	220±2	450±4	802±8	71.9±0.5
Fish	70	90±4	311±2	648±6	84.0±0.6
Fish	80	40±3	224±4	470±9	97.8±0.3
Lard	40	2490±35	1519±25	5161±71	36.4±0.3
Lard	50	550±2	190±3	3120±59	47.8±0.7
Lard	60	200±3	121±3	2402±53	66.2±0.9
Lard	70	600±3	60±2	1570±31	74.7±0.8
Lard	80	30±4	104±18	1143±21	67.7±0.5
Linseed	80	390±7	1102±18	1681±31	77.3±0.2
Corn	80	2060±25	1121±11	1160±13	46.2±0.1

Furthermore, Table 1 shows the changes of the monomolecular, bimolecular and termination durations as a function of temperature. For fish and lard oil samples, higher temperatures corresponded with faster reaction rates and, consequently, shorter duration of these periods. Moreover, the relationship between the duration of such phases follows the so-called Arrhenius function, where the logarithm of the duration of each phase leads a linear relationship when plotted as a function of the inverse of temperature (ln(t) vs 1/T). For fish oil samples, the slopes of such linear relationships were $1.1 \cdot 10^4$ K, $3.6 \cdot 10^3$ K and $3.5 \cdot 10^3$ K, respectively, for the monomolecular, bimolecular and termination phase. Similar results were obtained for lard oil samples. The slopes were in this case equal to $1.2 \cdot 10^4$ K, $3.7 \cdot 10^3$ K and $4.1 \cdot 10^3$ K, respectively, for the monomolecular, bimolecular and termination phase of lard oil samples. These values suggest that the most sensitive phase to the changes in temperature is the monomolecular phase. This information is important for food manufacturers, since it provides an evidence that the antioxidants needed to stabilize such oils are those with the highest capacity to retard or stabilize the monomolecular phase.

Last experiments aimed to understand the reasons of the different oxidative stability observed between the oil samples. The ranking observed cold be explained considering either the quality of fatty acids composition (i.e. presence of mono- or poly-unsaturated fatty acids) or the antioxidant content of the samples. Table 2 reports the fatty acids profile and the antioxidant content of the oil samples. What is striking is that the ranking of oxidative stability of the oil samples obtained according to the duration of the monomolecular phase cannot be predicted simply by looking at the polyunsaturated fatty acid composition of the samples. Instead, the oxidative stability of the samples matches perfectly with the antioxidant capacity determined by the DPPH assay on the methanolic extracts of the samples. In practice, the oil samples with the highest oxidative stability (i.e. longer duration of the monomolecular phase, as determined by calorimetry) are also those with the highest antioxidant capacity (i.e. shorter value of the DPPH assay).

Table 2. Fatty acid composition, peroxide value and antioxidant capacity of fish, lard, corn and linseed oil.

	Corn oil	Linseed oil	Fish oil	Lard oil
Fatty acids (%, w/w)				
Palmitic acid (16:0)	14.7±0.6^{b}	6.3±0.5^{c}	15.6±1.4^{b}	26.6±1.6^{a}
Stearic acid (18:0)	2.9±0.3^{b}	4.2±0.6^{b}	3.5±0.8^{b}	10.7±0.9^{a}
Oleic acid (18:1)	30.8±1.8^{b}	18.8±1.6^{c}	11.1±0.9^{d}	46.8±2.8^{a}
Linoleic acid (18:2)	48.4±1.7^{a}	17.9±1.3^{b}	2.5±0.5^{d}	9.3±0.7^{c}
Linolenic acid (18:3)	1.1±0.2^{b}	50.4±3.1^{a}	1.5±0.3^{b}	1.8±0.2^{b}
Total SFA	17.6±0.5^{b}	11.1±0.7^{b}	23.1±1.1^{b}	37.3±1.5^{a}
Total PUFA	49.5±2.3^{b}	68.3±3.9^{a}	51.7±2.3^{b}	11.1±0.6^{c}
Total MUFA	30.8±1.4^{b}	19.2±1.8^{d}	22.6±2.1^{c}	49.6±2.4^{a}
Others	2.1±0.2^{a}	1.4±0.4^{a}	2.6±0.6^{a}	1.9±0.2^{a}
Peroxide value ($m_{eq}O_2$/kg oil)	2.9±1.2^{b}	1.6±0.5^{c}	4.2±0.3^{a}	5.4±1.7^{a}
DPPH (IC_{50})	70.3±1.5^{d}	83.9±2.7^{c}	251.8±6.4^{a}	98.9±3.4^{b}

Legend: SFA is saturated fatty acids; PUFA is polyunsaturated fatty acids; MUFA is monounsaturated fatty acids.
Values with different letters in the same raw are significantly different ($p < 0.05$).
Values are presented as mean±standard deviation ($n = 3$).

4. CONCLUSIONS

The results of the present study show the potential of isothermal calorimetry to monitor the oxidation processes of edible oils and exemplify a procedure to determine the monomolecular, bimolecular and termination periods according to the changes in the apparent reaction order of the overall process. In addition, we observed that the monomolecular phase was the most sensitive to the changes in temperature. This implied that the duration of the monomolecular phase is very informative to explain the oxidative stability of an oil sample. According to corn, linseed, lard and fish oil samples, their oxidative stability was not correlated with their qualitative composition in fatty acids. Instead, their oxidative stability was explained with their antioxidant capacity. On a more general perspective, this work demonstrated the potential of isothermal calorimetry to predict the oxidative stability of oil samples and characterizing the duration of the monomolecular, bimolecular and termination phase of the autoxidation process.

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PAPER

ANTIOXIDANT PROPERTIES, PROXIMATE ANALYSIS, PHENOLIC COMPOUNDS, ANTHELMINTIC AND CYTOTOXIC SCREENING OF *TEUCRIUM SANDRASICUM*; AN ENDEMIC PLANT FOR TURKEY

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ABSTRACT

This work was designed to evaluate the phenolic compounds and biological activities (antioxidant, cytotoxic and anthelmintic) of *Teucrium sandrasicum* extracts (ethanol and acetone) as well as to determine proximate parameters (such as proteins, carbohydrates, fat). The phenolic contents were identified using HPLC. The **e**thanol extracts exhibited higher free radical scavenging and antioxidant activities than acetone extracts. The reducing power, metal chelating and radical cation activities were found to be statistically different between the acetone and ethanol extracts. *T. sandrasicum* exhibited cytotoxic, anthelmintic activities with rich nutrient contents. Based on these results, this plant may be considered as a potentially useful source for the food and pharmaceutical industry.

Keywords: biological activities, medicinal plants, proximate, *Teucrium sandrasicum*

1. INTRODUCTION

Plants are good sources of natural functional compounds that are medically and biologically important and can be used in various fields, such as food ingredients, medicinal and pharmacological applications. For this reason, there has recently been an increase in the volume of research on the isolation and identification of these compounds. Many of these investigations especially relate to the designation of the biological activities of these compounds, such as antioxidant and cytotoxic activities (NICKAVAR and ESBATI, 2012; AL-DABBAS, 2017). The beneficial effect of medicinal plants on diseases have been revealed by a considerable number of researchers (CAKILCIOGLU and TURKOGLU, 2010). Medicinal plant species of the genus *Teucrium* have a wealth of phenolic compounds with powerful biological activities and many plants of the *Teucrium* genus have been used in the food industry, as natural preservatives and pharmaceutical applications (CANADANOVIC-BRUNET *et al*., 2006; SAROGLU *et al*., 2007; BAGCI *et al*., 2010). These plants are used to reduce inflammation and relieve indigestion and are also used as herbal medicines for coughs, asthma and stomach pain (AMIRI, 2010). In addition, *Teucrium* plants are well known for their hypoglycemic, antiseptic, antispasmodic and anthelmintic activities (GHARAIBEH *et al*., 1989; SAROGLU *et al*., 2007; REHMAN *et al*., 2016). The *Teucrium* genus is a member of the *Lamiaceae family,* of which there are more than 340 species widespread throughout the world (MAHMOUDI and NOSRATPOUR, 2013). Turkish flora includes 34 *Teucrium* species (DIRMENCI, 2012), eight of which are endemic (DAVIS, 1982). *Teucrium sandrasicum* is one of the endemic species of the *Teucrium* genus and the aerial parts of this plant are widely used in the daily diet (AKSOY-SAGIRLI *et al*., 2015). In previous limited research, several *T. sandrasicum* extracts (water, methanol, ethyl acetate, hydro-methanolic) have been evaluated for phenolic compounds, antioxidant activities and antiproliferative effects on various cell lines (AKSOY-SAGIRLI *et al*., 2015; KARAGOZ *et al*., 2015; TARHAN *et al*., 2016). According to the literature, the biological activities of plant materials are strongly based on the nature of extracting solvents, such as polarities. Therefore, the separate examination of plant extracts, obtained from different solvents, will make a significant contribution to medicinal plant studies and their pharmaceutical applications (CANADANOVIC-BRUNET *et al*., 2006; STANKOVIC *et al*., 2011). Consequently, more research is required on the biological activities of this aromatic and medicinal plant. Within this scope, we therefore consider that *T. sandrasicum* is a plant worthy of additional investigation. Furthermore a thorough investigation of the current literature indicates that no scientific reports have been published to date concerning the antioxidant capacities, and cytotoxic or anthelmintic properties of the ethanol and acetone extracts of *T. sandrasicum*. With these points in mind, the objectives of the present study are to evaluate the antioxidant capacities, the cytotoxic, anthelmintic activities and the total phenolic and flavonoid contents of the ethanol and acetone extracts of *T. sandrasicum,* as well as the chemical composition of the ethanol extracts. In addition, the other objective of this study was to determine the proximate content of this medicinal plant.

2. MATERIAL AND METHODS

2.1. Plant materials

T. sandrasicum was collected at an altitude of 1600 m from Sandras Mountain (between Denizli-Muğla, Turkey), in July 2017. The plant material was identified by Dr. Mehmet Çiçek from Department of Biology, Faculty of Arts and Sciences, Pamukkale University,

Denizli, Turkey. A voucher specimen (*T. sandrasicum*; Herbarium No: 2017-145) has been deposited in the private herbarium of Dr. M. Çiçek (PAU) at Pamukkale University, Denizli, Turkey.

2.2. Preparation of the plant extracts

The air dried aerial parts of *T. sandrasicum* were ground to a fine powder and extracted with ethanol and acetone. Each powdered sample (30 g) were mixed with 300 mL of solvents. Extraction was carried out by shaking at 50°C for 6 h in a temperature controlled shaker and the mixture was filtered using filter paper (Whatman No.1). This procedure was repeated twice. The solvent was evaporated using a rotary evaporator (IKA RV10D, Staufen, Germany) under vacuum at 40-50°C. Samples were lyophilized (Labconco FreeZone, Kansas City, MO) and stored at -20°C until tested. All experiments were carried out in triplicates.

2.3. Chemicals

β-carotene, Linoleic acid, 2,2-Diphenyl-1-picryl hydrazyl radical (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Phosphate buffer, Iron (III) Chloride, Quercetin, Sodium phosphate, Sodium carbonate, Potassium ferrocyanide, Gallic acid, methanol, chloroform, ethanol, and acetone were purchased from Sigma-Aldrich. Butylated hydroxy toluene (BHT), Folin-Ciocalteu reagent, Tween 20 was purchased from Merck (Darmstadt, Germany). Other chemicals and solvents were of analytical grade.

2.4. Determination of phenolic compounds

2.4.1 Total phenolic content

The total phenolic content of the *T. sandrasicum* extracts was evaluated using the Folin-Ciocalteu method (SLINKARD and SINGLETON, 1977). In this method, the extract solution (1 mg/mL) was mixed with Folin-Ciocalteu reagent (1 mL) and distilled water (46 mL). After resting at room temperature for 3 min, 3mL of 2% sodium carbonate solution was added to the mixture and mixed gently. The mixture was incubated at room temperature for 2 h. Following this procedure the absorbance was confirmed as 760 nm and the outcomes were shown as mg of the Gallic acid equivalents (GAE) per gram of extract.

2.4.2 Total flavonoid content

The total flavonoid content was evaluated using to method of ARVOUET-GRAND *et al.* (1994). One milliliter solution of $AlCl_3$ in methanol (2%) was combined with the equivalent quantity of extract solution. After about 10 min the absorbance of the reaction mixtures were determined as 415 nm.. The flavonoid content was calculated from a quercetin standard curve and expressed as milligram of quercetin equivalents (QE) per gram of extract.

2.5. Determination of antioxidant activity

2.5.1 β-carotene/linoleic acid method

In this method, antioxidant capacity was determined using the method of AMIN and TAN (2002). β-carotene stock solution was prepared as follows: 2 mg β-carotene was dissolved in 10 mL chloroform. Linoleic acid (20 μL) and 200 μL of 100% Tween 20 was added for one milliliter of the solution. A rotary evaporator was used to remove the chloroform. Then the remaining residue was added to 100 mL of distilled water and the 1 mL extracts were combined with this emulsion (24 mL). A spectrophotometer was immediately used to measure the initial absorbances at 470 nm. The reaction mixture was incubated for 2 hours at 50° C. Following this, the measurement of the absorbance of this mixture was repeated, and a synthetic antioxidant (BHT) was applied as the positive control. The total antioxidant activity (AA) was calculated in following way:

$$AA=[1- (A_{samp}-A_{co})/(A^{\circ}_{samp}-A^{\circ}_{co})] \times 100$$

(A_{samp} and $A_{co:}$ absorbance at the initial time of the incubation of samples and control, respectively and A°_{samp} and A°_{co}: absorbance in the samples and control at 120 min)

2.5.2 Phosphomolybdenum method

The total antioxidant property of *T. sandrasicum* extracts was determined using the phosphomolybdenum method according to PRIETO *et al*. (1999). Various concentrations of the extracts (0.1-1.0 mg/mL) were mixed reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture (3 mL) and extracts (0.3 mL) were dispersed into test tubes and the tubes were placed at 95° C for 90 min. The absorbances of the mixtures were measured at 695 nm using a spectrophotometer. The antioxidant capacity of the extracts was expressed as μg ascorbic acid equivalents (AA) per milligram of extract.

2.6. Evaluation of radical scavenging

2.6.1 Free radical scavenging activity (DPPH)

The radical scavenging activity of the *T. sandrasicum* extracts was determined using the DPPH, as described by MERIGA *et al*. (2012) with slight modifications. Extracts of different concentrations (1 mL) were combined with 4 mL of methanolic DPPH (0.004%) solution. After vortexing the reaction mixture, the decrease in absorbance of each extract and/or control (BHT) were measured at 517 nm after 30 minutes. Results were expressed as IC_{50} (the concentration of the sample that is required to scavenge 50% of DPPH radicals).

2.6.2 ABTS radical cation scavenging activity

Experiments were performed in accordance with the method used by SHALABY and SHANAB (2013) with slight modifications. ABTS (7mM) and potassium persulphate (2.45 mM) solutions were combined and stored in a dark room for 12-16 h prior to use. Before the analysis, the ABTS solution was diluted with ethanol to an absorbance of 0.700±0.05 at 734 nm. After the addition of 4.5 mL of the ABTS reaction mixture to the various concentrations (50-400μg/mL) of the extracts (1 mg/mL), the mixture was kept at room temperature for 15 min. There was a reading of 734 nm for the absorbances of the samples.

The radical-scavenging activity of the extracts was estimated based on the ABTS color reduction, by calculating the IC_{50} (concentration in μg/mL that cause 50% inhibition of ABTS radicals).

2.7. Ferric ion reducing power activities

The method described by OYAIZU (1986) with slight modifications was used to conduct the reducing power capacity of the extracts. Different concentrations of the samples (1 mL) were combined with 0.2M phosphate buffer (1 mL) and 1% potassium ferricyanide (1 mL). The mixture was kept at 50° C for 20 min. Trichloroacetic acid (1 mL, 10%) was added to reaction mixture. The aliquot of the upper layer (1.5 mL) was combined with distilled water (1.5 mL) and ferric chloride (0.1%). After 10 min the absorbance was read, at 700 nm. The activity was expressed as mg of ascorbic acid equivalents (AA) per milliliter of extract.

2.8. Metal chelating activity

The metal chelating power of the *T. sandrasicum* extracts was determined using the method of KARPAGASUNDARI and KULOTHUNGAN (2014) with slight modifications. One milliliter of the extract and 3.2 mL of deionized water were mixed with 2 mM $FeCl_2$ (0.1 mL) solution. After 30 s, 5 mM of ferrozine (0.2 mL) was added. The reaction was activated by adding ferrozine and then the mixture was left to stand for 10 min after which the absorbances of the solutions were measured at 562 nm. The metal chelating activity was calculated in following way:

$$\text{Chelating ability } (\%) = [(A_{co} - A_{samp}) / A_{co}] \times 100,$$

(A_{co} : absorbance of the control and A_{samp} : absorbance of the extract)

2.9. Proximate analysis

The *T. sandrasicum* plant samples were analyzed to determine proteins, fat, carbohydrates, ash and energy, according to the protocols mentioned in AOAC (1995). The macro-Kjeldahl method was applied to evaluate the crude protein content of the samples. The crude fat was evaluated with a Soxhlet apparatus for which a known weight of the powdered sample was extracted with petroleum ether. The volume of ash was established by burning at 650±15°C and total carbohydrates were calculated by difference. Energy was calculated based on the following equation:

$$\text{Energy(kilocalorie)} = 4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g fat}).$$

All parameters were made in triplicate.

2.10. Quantification of phenolic compounds by HPLC

Reversed-phase high performance liquid chromatography (RP-HPLC, Shimadzu Scientific Instruments) was used for the determination of the phenolic compound. Detection and quantification was made using a diode array detector (SPD-M20A), a LC-20AT pump, a CTO-10ASVp column heater, a SIL-10ACHT auto sampler, a SCL-10Avp system controller and a DGU-14A degasser. Separations were carried out using a C-18 reversed-phase column (Agilent ZORBAX Eclipse, 250 x 4.6 mm length, 5μm particle size). The

chromatograms were examined at 278 nm. The mobile phases were A: 3.0 % formic acid in dH_2O and B: methanol. The samples were dissolved in methanol and this solution (20 μL) was injected into the column. The phenolic composition of the *T. sandrasicum* ethanolic extract was determined according to the method of CAPONIO *et al*. (1999) with slight modifications. Gallic, 3,4 dihydroxybenzoic, 4-hidroxybenzoic, 2,5 dihydroxybenzoic, chlorogenic , vanillic, caffeic, p-coumaric, ferulic, cinnamic acid and quercetin epicatechin, rutin were used as standards. The amount of individual phenolic compound was determined, based on peaks. The quantity of each phenolic compound was expressed as μg/g of the extract.

2.11. Cytotoxic activity

The potential cytotoxic capacity of the *T. sandrasicum* extracts was evaluated using the brine shrimp lethality test. (MEYER *et al*., 1982). *Artemia salina* eggs (10 mg) were incubated in 500 mL artificial seawater and under artificial light for 48 h at 28ºC. After incubation, ten nauplii were collected with a Pasteur pipette and placed into test tubes containing brine solution. In the experiments, 0.5mL of plant extract (1000, 500, 100, 50 and 10 ppm) was mixed with 4.5 mL of brine solution. The number of survivors was counted in each concentration of the extracts and the control after about 24 h. The larvae were considered dead if no movement of the appendage was observed within 10 sec. To determine the LC_{50} values, the data was analyzed using the EPA Probit Analysis Program (version 1.5) (FINNEY, 1971).

2.12. Anthelmintic activity

The anthelmintic activity of the *T. sandrasicum* extracts was determined using the methods of DASH *et al*. (2002) with slight modifications. *Tubifex tubifex* (Annelida) was used in the experiments. The average size of *Tubifex tubifex* was 1-2 cm and 6 worms were placed in a petri dish containing 20 mL test solutions of ethanol and acetone extracts. Test samples of the extracts were prepared at different concentrations (2.5, 5, 7.5, 10 mg/mL) in distilled water. Albendazole (2.5, 5, 7.5, 10 mg/mL) was used as a reference standard, while distilled water was the negative control. The worms were observed, and the time taken for paralysis and the time taken death was noted in minutes. The mean time for paralysis was logged when movement was lost, or no movement could be perceived apart from when the worm was forcefully shaken. The time of death was recorded of each worm after ascertaining that the worm failed to move when shaken or when given external stimuli.

2.13. Statistical analysis

All analyses were performed in triplicate and the results presented as mean±SE (Standard Error) and the results analyzed using the MINITAB Statistical Package program. To see how the groups differed from each other, the variations between the different extracts were tested with Analysis of Variance (ANOVA) and Tukey (P<0.05), and the different groups were shown with different letters in the same column. If there were only two groups then a t-test was used.

3. RESULTS AND DISCUSSION

Antioxidant activity determination methods depend upon various parameters such as the concentration and the structure of the compound to be analyzed. For this reason, there is

no standard method for determining the antioxidant activity of a compound and one single method cannot fully describe the antioxidant activity (DU *et al*., 2009; JABRI-KAROUI *et al*., 2012). Consequently, we used six complementary antioxidant methods (radical scavenging (DPPH and ABTS), total antioxidant (β-carotene /Linoleic acid and phosphomolybdenum), metal chelating and reducing power activities) to evaluate the true antioxidant potential of the extracts.

3.1. Total phenolic and flavonoid content

The total phenolic and flavonoid contents in the ethanol and acetone extracts from *T. sandrasicum* have been determined in the present study. Our results revealed that in the ethanol extract, the total phenolic content with 107.94±0.59 mgGAE/g was higher than that of the acetone extracts with 78.2±1.5 mgGAE/g and this was found to be statistically different ($t=18.21$, $df=10$, $p<0.001$). The phenolic content for the acetone extract determined in the present study was lower than that determined by STANKOVIC *et al.* (2010) (acetone extract of *T. chamaedrys*). In addition, the variable amounts of total phenolic content in the different extracts may be due to solvent polarity (MARINOVA and YANISHLIEVA, 1997).
The total flavonoid content was found 65.96±0.19 and 51.61±0.56 mgQEs/g in acetone and ethanol extracts respectively and these were statistically different ($t=24.30$, $df=9$, $p<0.001$). These results obtained are in line with those of TARHAN *et al.* (2016) who found total flavonoid content varied from 30.23-95.12 mg/g in ethyl acetate, water and hydromethanolic extracts from *T. sandrasicum*. In addition, similar to our study, BAKARI *et al*. (2015) found acetone extract to have a higher total flavonoid content than the ethanol extract in *T. polium*.

3.2. Antioxidant activities

3.2.1 Total antioxidant activity (β-Carotene-linoleic acid and Phosphomolybdenum methods)

β-carotene/linoleic acid is used to measure antioxidant activity. Antioxidants minimize the oxidation of lipid components in cell membranes, or inhibit the conjugated diene hydroperoxides, known to be carcinogenic, generating from linoleic acid oxidation (TEPE *et al*., 2007). In this study, the antioxidant activity of the ethanol extract from *T. sandrasicum* (80.18±1.34%) was better than the acetone (73.61±0.95 %) extract (Fig. 1).
These results are in line with those of BAKARI *et al.* (2015) who found ethanol extract exhibited higher antioxidant properties than the acetone extract in *T. polium*. In addition, total antioxidant activity for the ethanol and acetone extracts determined in this study were higher than those reported by BAKARI *et al*. (2015) (*T. polium*). In this study, there were statistically differences among the antioxidant contents of the different extracts of T. *sandrasicum* and BHT ($F_{2,24}= 109.76$, $p<0.001$) (Fig. 1). Although the synthetic antioxidant (BHT) showed the highest antioxidant activity (over 90 %), the ethanol and acetone extracts were as effective as standard and they seemed to reduce the oxidation of linoleic acid, a key concern for the food industry.
The antioxidant activity of the samples was also evaluated using the phosphomolybdenum assay, according to the method of PRIETO *et al.* (1999). Similar to the β-Carotene-linoleic acid test system, in this method the ethanol extract from *T. sandrasicum* showed stronger antioxidant capacities than the acetone extract (Table 1).

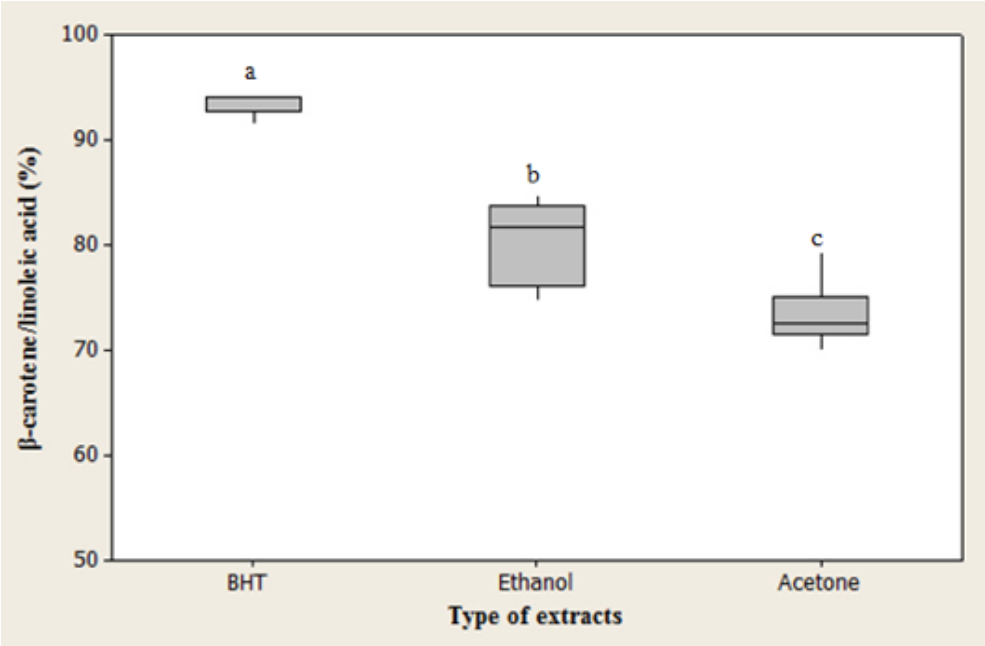

Figure 1. Antioxidant activity of *T. sandrasicum extracts.*
Ethanol extract of *T. sandrasicum*; Acetone extract of *T. sandrasicum,* BHT: Standard antioxidant.
(different groups were shown with different letters on each boxplot)

Table 1. Antioxidant properties of *T sandrasicum* extracts.

Sample	DPPH (IC_{50}, $\mu g\ ml^{-1}$)*	ABTS (IC_{50}, $\mu g\ ml^{-1}$)*	Phosphomolybdenum ($\mu g/mg$)*	Power reducing (mg/mL)*
Ethanol	122.60±1.35 b	174.86±1.52 a	104.03±3.3 a	0.32±0.01 a
Acetone	184.76±6.07 a	119.11±6.22 b	74.7±6 b	0.24±0.03 b
BHT	31.64±1.52 c	12.89±1.20 c	nt	nt

BHT: Standard antioxidant, nt: not tested.
*Values are mean of three replicate determinations (n=3)±standard error. Mean values followed by different letters in a column are significantly different ($p<0.05$).

The antioxidant activities were found to be statistically different between the ethanol and acetone extracts ($t=4.31$, $df=12$, $p<0.001$). As previously reported by NICKAVAR and ESBATI (2012) and CAKIR *et al.* (2003), our results also showed that the high antioxidant capacities of the ethanol extract of *T. sandrasicum* is due to the presence of high phenolic content.

3.3. Radical scavenging activity (DPPH and ABTS)

A stable free radical of a deep shade of purple on scavenging DPPH becomes yellow. The level of yellowing indicates the scavenging potential of the extracts, in terms of hydrogen donating ability. Consequently, DPPH is generally used as a substrate to ascertain antioxidant capacity (DUH *et al.*, 1999). The results of the DPPH in present study are given in Table 1. The higher DPPH radical scavenging activities were associated with the lower IC_{50} values. The ethanol extract exhibited higher scavenging activity than the acetone extract and there were statistically differences among the radical scavenging activities of

the different extracts of *T. sandrasicum* and BHT ($F_{2,24}$= 434.32, p<0.001). The present study has demonstrated that the ethanol and acetone extracts from *T. sandrasicum* have a radical scavenging capacity and the key role of the phenolic content as scavengers of free radicals has been emphasized in several other reports (KOMALI *et al*., 1999). When the acetone extract of *T. sandrasicum* was compared with *T. montanum* (STANKOVIC et al., 2011) and *T. polium* (BAKARI et al., 2015) of which the DPPH free radical scavenging activities were found to be 108.10 μg/mL and 13 μg/mL respectively, the DPPH free radical scavenging activity of the acetone extract of *T. sandrasicum* was lower than those of these species.
The ABTS scavenging capacity of plant extracts from *T. sandrasicum* were determined and the results are presented in Table 1. The values of IC_{50} were in the following order: BHT < acetone extracts< ethanol extracts. AKSOY-SAGIRLI *et al*., (2015) used ABTS for the determination of radical scavenging activity in methanol extracts from *T. sandrasicum.* In the present study we also used ABTS to investigate scavenging activity in ethanol and acetone extracts from *T. sandrasicum* and found that the ethanol and acetone extracts of *T. sandrasicum* have radical scavenging activity. Free radicals, which are produced in the human body by chemicals or metabolic processes are capable of oxidizing biomolecules (HALLIWELL and GUTTERIDGE, 1989) and exposure to free radicals causes cell damage, which may increase the risk of various diseases, such as cancer, heart diseases and diabetes. As with antioxidants, by inhibiting the formation of free radicals, free radical scavengers naturally protect cells from the damage caused by harmful molecules (PERCIVAL, 1998). The present study reveals that *T. sandrasicum* extracts could serve as free radical scavengers and due to these properties, they may be used as an ingredient in food.

3.4. Ferric ion reducing power activities

The reducing ability describes how easily one substance can give electrons to another. A powerful reducing agent is inclined to give electrons. The reducing power method measures the ability of components that act as antioxidants to reduce ferric ion (SINGH *et al*., 2012). In the present study, the reducing ability of ethanol and acetone extracts from *T. sandrasicum* were measured, and the results of this activity demonstrated that the ethanol extract showed a higher reduction ability than those of the acetone extracts (Table 1). The reducing power activities were found to be statistically different between the ethanol and acetone extracts (t=2.34, df=9, p<0.05). According to these results, the ethanol and acetone extracts of *T. sandrasicum* possess antioxidant capacity. This is because the reducing capacity of a compound serves as potential antioxidant activity (SINGH *et al*., 2012). In addition, the reducing power of ethanol extract may be due to the high level of phenolic content, which acts as an electron donor. Similarly, numerous studies advocate an association between the reducing power and the total phenolic content (GONCALVES *et al*., 2013).

3.5. Metal chelating properties

The metal chelating ability of the studied *T. sandrasicum* extracts were determined by measuring the iron-ferrozine complex. The metal chelating property of the ethanol and acetone extracts from *T. sandrasicum* were evaluated and these results showed that the ethanol extract (55.85±4.22 %) exhibited better metal chelating activity when compared with the acetone extract (26.67±1.48 %). Although the synthetic metal chelator (EDTA) exhibited the highest chelating activity (over 80 %), the ethanol and acetone extracts inhibited complex of ferrous, ferrozine and this revealed that they exhibit chelating activity (Fig. 2). The metal chelating capacity is important, because this activity reduces the

amount of catalyzing transition metal in lipid peroxidation (DUH *et al.*, 1999). For this reason, the presence of the chelating properties of the extracts contribute directly to their antioxidant properties.

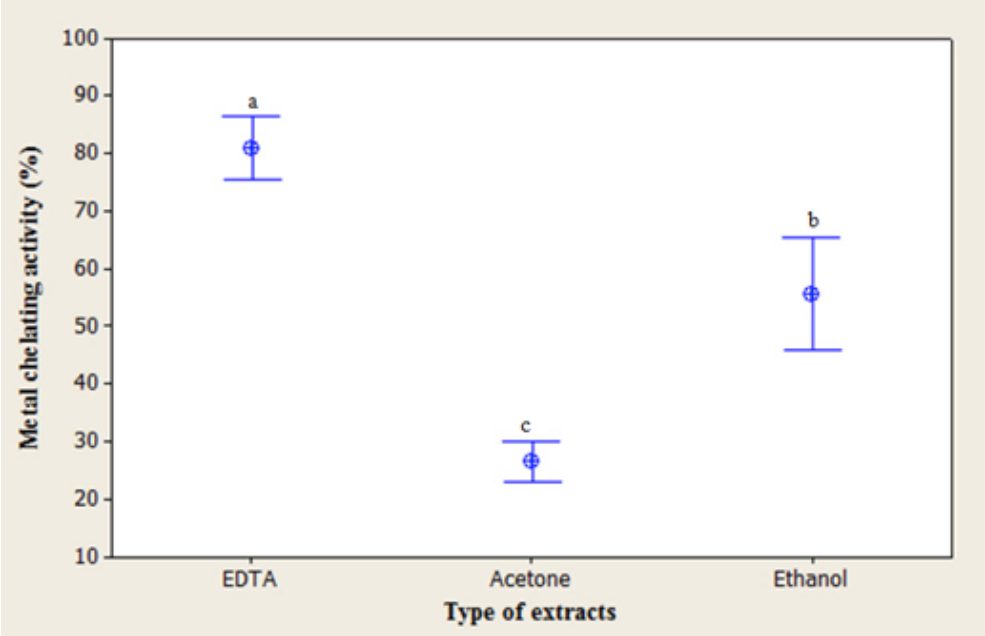

Figure 2. Metal chelating activity of *T. sandrasicum extracts.*
Ethanol extract of *T. sandrasicum*; Acetone extract of *T. sandrasicum*, EDTA: Standard antioxidant.
(different groups were shown with different letters on each bar)

3.6. Proximate analysis

The evaluation, determining the moisture, crude protein, crude fat, ash, carbohydrate, fibre and energy, as a proximate analysis of the aerial parts of *T. sandrasicum*, is presented in Table 2. When compared with earlier studies, the protein content of *T. sandrasicum* was found to be lower than those of *T. muscatense* (REHMAN *et al.*, 2016) and *T. polium* (HUSSAIN *et al.*, 2013). In contrast with the study of REHMAN *et al.* (2016), the carbohydrate content of *T. sandrasicum* was found to be lower than *T. muscatense*. The fat content and the energy value of the *T. sandrasicum* were lower than the fat content and the energy value of the *T. polium* (HUSSAIN *et al.*, 2013).

Table 2. Proximate analysis of *T. sandrasicum.*

Constituents	Aerial parts
Ash (g/100 g dw)	4.76±0.52
Carbohydrate (g/100 g dw)	17.06±1.28
Proteins (g/100 g dw)	2.43±0.10
Fat (g/100 g dw)	1.10±0.12
Moisture (g/100 g fw)	42.17±0.9
Fibre (g/100 g dw)	28.48±0.1
Energy (kcal/100 g dw)	87.86

The plants species, especially medicinal plants are also used as food or a food supplement and evaluating their nutritional contents can help to understand the significance of these plant species as a dietary supplement and for pharmaceutical applications. Proximate analysis of this plant plays a decisive role in assessing its nutritional significance and revealed that this species is good source of nutrients as well as can contribute towards nutritional requirements (PANDEY *et al.*, 2006; ADNAN *et al.*, 2010).

3.7. Phenolic composition

It has been established that the Lamiaceae species comprise a range of secondary metabolites, including phenolic acids and flavonoids. In present study, phenolic compositions of ethanol extract of *T. sandrasicum* were identified using HPLC method. Phenolic compound that were determined in ethanol extract are listed in Table 3 and the main phenolics were identified as caffeic acid and rutin (Fig. 3).

Table 3. Phenolic components in the ethanol extract of *T. sandrasicum*.

No	Phenolic component	Approximate Rt (min)	μg/g*
1	Gallic acid	6.8	917.35±0.08
2	3,4 dihydroxybenzoic acid	10.7	92.47±0.01
3	4-hydroxybenzoic acid	15.7	1066.40±0.08
4	2,5 dihydroxybenzoic acid	17.2	61.06±0.02
5	Chlorogenic acid	18.2	462.02±0.05
6	Vanilic acid	19.2	563.29±0.09
7	Epicatechin	21.3	1648.12±1.02
8	Caffeic acid	22.7	22727.28±5.06
9	p-Coumaric acid	26.1	1.47±0.05
10	Ferulic acid	30.1	72.06±0.01
11	Rutin	45.6	3392.28±1.06
12	Cinnamic acid	71.1	315.40±0.03
13	Quercetin	70.4	2893.08±0.02

*based on dry weights

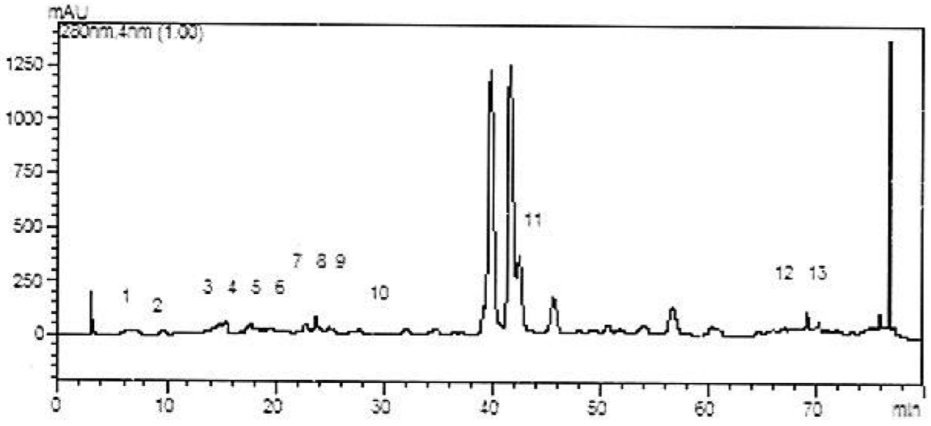

Figure 3. HPLC chromatograms of phenolic components in the ethanol extracts of *T. sandrasicum*.

Some phenolic compounds determined in present study, such as ferulic, gallic, caffeic, vanillic, chlorogenic acids and rutin, in previous studies were obtained from Lamiaceae plants (CANADANOVICH-BRUNET *et al*., 2006; ROBY *et al*., 2013; KASKA *et al.*, 2018). In addition, previously studies have shown that, from these phenolics, caffeic acid exhibits anticarcinogenic properties that act as a carcinogenic inhibitor (MAGNANI *et al*., 2014). In brief, nowadays the identification and measurement of plants phenolic compounds are considered to be effective mechanisms for ascertaining the importance of plants for human health (AMAROWICZ *et al*., 2010). This is because phenolic content can directly contribute to the antioxidant capacity of the plants (DUH *et al.*, 1999).

3.8. Cytotoxic activity

The brine shrimp cytotoxicity test is a practical and economic method for the investigation and assessment of toxicity, antifungal and pesticidal effects of plants. LC_{50} values of less than 1000 μg/mL are regarded as bioactive when using the brine shrimp lethality test to calculate the toxicity of plant extracts (MEYER *et al*., 1982). The lethality of ethanol and acetone extracts were 389.661 and 658.032 μg/mL respectively, and the extracts possessed high cytotoxic activities against brine shrimp. The lethality of these extracts from *T. sandrasicum* indicates the presence in this species of potent cytotoxic components, which require further investigation. The present study suggests the need for further investigations of this plant, in order to ascertain the potential cytotoxic compound.

3.9. Anthelmintic activity

In the present study, an investigation was made of the anthelmintic activities of *T. sandrasicum* extracts (ethanol and acetone). The results presented in Table 4 show that the ethanol and acetone extracts obtained from *T. sandrasicum* is active against *Tubifex tubifex*.

Table 4. *In vitro* anthelmintic activity of *T. sandrasicum*.

Type of extract	Concentration used (mg/mL)	Time (min) taken for paralysis (X±S.E.)*	Time (min) taken for death (X±S.E.)*
Control (Distilled water)	-	-	-
Ethanol	2.5	37.33±1.58 a	48.5±1.63 a
	5	21±1.93 b	31.67±1.38 b
	7.5	15.67±1.20 bc	21.67±0.62 c
	10	10.17±0.75 c	14±0.82 d
Acetone	2.5	22.33±1.52 a	31.5±1.43 a
	5	17.83±0.54 b	21±0.48 b
	7.5	12±0.45 c	16±0.45 c
	10	9±0.37 c	12.65±0.56 d
Albendazole (Standard)	2.5	52.33±2.73 a	61±3.33 a
	5	33±1.48 b	48.5±3.48 b
	7.5	21.17±2.01 c	35.5±3.21 c
	10	13±0.68 d	20.5±1.57 d

Values are mean±S.E. of six worms.
*Mean values followed by different letters in a column are significantly different ($p<0.05$).

All doses of the *T. sandrasicum* extracts (ethanol and acetone) showed better anthelmintic activity, in terms of promoting paralysis and causing death, than the standard. The strong anthelmintic activity of *T. sandrasicum* extracts may be due to the existence of rich polyphenolic compounds. Both humans and animals have benefitted from a broad range of medicinal plants in the treatment of parasitic infections in. Anthelmintics, derived from plant sources, present some advantages, such as pharmacological effectiveness and lower toxicity for animals and humans (PEIXOTO *et al*., 2013). Nowadays, there is an increasing interest in studies on the screening of new and effective medicinal plants that have anthelmintic properties. *T. sandrasicum* extracts possess wormicidal activity and could be effective against the parasitic infection of humans and animals. Hence, the lethality of the potent anthelmintic components in this species requires further investigation.

4. CONCLUSIONS

The results revealed in the present study have shown that the acetone and ethanol extracts from this plant have strong antioxidant properties. They have also shown that the plant possesses rich phenolic and flavonoid compounds, making it a good source of nutrients. Furthermore, *T. sandrasicum* extracts have been shown to have cytotoxic and anthelmintic activities. The present study therefore suggests that this plant could be considered as a source of natural agents in the food industry and can be used as a new anthelmintic and cytotoxic agent for pharmacological applications. Further investigation is required to isolate and identify the antioxidant, anthelmintic and cytotoxic components found in this plant. This will in turn increase information on the usability of the plant for the food industry and pharmacological applications.

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PAPER

EFFECT OF ANTIOXIDANTS AND PACKING CONDITIONS ON STORAGE STABILITY OF CEREAL BAR FORTIFIED WITH HYDROLYZED COLLAGEN FROM SEABASS SKIN

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ABSTRACT

Effect of antioxidants (green tea powder (GT) and citric acid (CA)) and packing conditions on storage stability of cereal bar fortified with hydrolyzed collagen (HC) from seabass skin was studied up to 6 months of storage at 25°C in dark. Up to 3 months of storage, the addition of antioxidants impeded lipid oxidation, especially those cereal bars packed in polypropylene with normal heat seal (PP). Changes in moisture content, water activity, color, texture, PV, TBARS and formation of volatiles were effectively retarded when samples were packed under N_2 gas in laminated polyethylene/aluminium foil bag (LF) for 6 months of storage.

Keywords: hydrolyzed collagen, cereal bar, antioxidants, packing condition, physicochemical parameters

1. INTRODUCTION

The market for functional foods has been continuously growing (VICENTINI *et al*., 2016). Fish hydrolyzed collagen (HC), has been demonstrated to contain low-molecular-weight peptides with a wide range of bioactivities (GÓMEZ-GUILLÉN *et al*., 2011). Bioactivity and health enhancing potentials have led to the use of HC, especially from marine animal, for developing functional foods, cosmetics and pharmaceutical products (RUSTAD *et al*., 2011; ZHUANG *et al*., 2009). With increasing consumer demand for health promoting food, fish HC is of increasing interest as a bioactive ingredient because of its associated nutraceutical aspects (THIANSILAKUL *et al*., 2007). It can be fortified in drinks to enhance bioactivity such as antioxidant activity (CHUAYCHAN *et al*., 2016). However, fish HC addition can potentially bring about a fishy odor in the finished product, leading to rejection by consumers. Recently, bioactive HC powder without fishy odor was developed using enzymatic hydrolysis of seabass skin (BENJAKUL *et al*., 2017). Thus, it could be fortified at higher level imparting increased bioactivities.
Cereal bars are eaten regularly due to their nutritive values and ease of consumption. Several kinds of cereal bars have been produced to serve the growing functional food market (DEAN *et al*., 2007; TALENS *et al*., 2012). From our preliminary study, the relationship between health concerns of the consumers and purchase intention for cereal bar fortified with HC was investigated. The results indicated that HC powder can be used as supplement for the development of functional cereal bar. Focus group results suggested that production of cereal bars consisting of many grains with high levels of HC powder (5%) was feasible. In general, cereal bars have a high oil content, mainly from nuts and some grains, which are susceptible to oxidation during storage. Furthermore, HC powder is hygroscopic and absorbs water easily. These changes directly affect physical, chemical and sensory properties of the cereal bars fortified with HC powder during the extended storage.
Antioxidants have been employed in foods to prevent lipid oxidation, which can result in off-odor and toxicity in foods (ROSTAMZAD *et al*., 2011). Due to safety concern, synthetic antioxidants have been commonly replaced by natural antioxidants. Antioxidative compounds such as citric acid and green tea powder were reported to retard lipid oxidation in food products (ROSTAMZAD *et al*., 2011; LORENZO and MUNEKATA, 2016). Moreover, packaging materials and storage conditions are important factors governing the shelf-life of foods via lowering water migration as well as oxygen permeability (NILSUWAN *et al*., 2016; BAKKALBAŞı *et al*., 2012). Lipid oxidation can be inhibited by using a packaging material with good protection and barrier properties or by storing the products in atmospheres containing a low oxygen content (NILSUWAN *et al*., 2016; BAKKALBAŞı *et al*., 2012). The application of antioxidants along with appropriate packaging condition could be an effective means for the shelf-life extension of cereal bars fortified with HC powder. The objective of this study was to investigate the effect of some antioxidants and packaging conditions on quality changes of cereal bars fortified with HC powder during storage at 25°C.

2. MATERIALS AND METHODS

2.1. Enzymes/materials/chemicals

Alcalase (EC 3.4.21.62) (food grade) from *Bacillus licheniformis* and papain (E.C. 3.4.22.2) from papaya (*Carica papaya*) latex were gifted from Siam Victory Chemicals Co, Ltd. (Bangkok, Thailand). Citric acid was procured from Chemipan Corporation Co., Ltd.

(Bangkok, Thailand). Rolled oat meal (McGarrett, PK Trading (Thailand) Co., Ltd, Bangkok, Thailand), all dried fruits, nuts and grains (My choice, Central Food Retail Company Ltd, Nonthaburi, Thailand), green tea powder (Changtong Factory, Hat Yai, Songkhla, Thailand), strawberry flavour, corn syrup, honey and ingredients for preparing cereal bar were purchased from a local market in Hat Yai, Songkhla, Thailand. Ammonium thiocyanate, 2-thiobarbituric acid and 1,1,3,3-tetramethoxypropane were obtained from Sigma (St. Louis, MO, USA).

2.2. Production of hydrolyzed collagen (HC) from seabass skin

HC from seabass (*Lates calcarifer*) skin was prepared using two-step enzymatic hydrolysis process as described by BENJAKUL *et al*. (2017). Briefly, skins of seabass (*Lates calcarifer*) were washed and cut into small pieces (3.0 × 3.0 cm^2). The skins were then pretreated to remove non-collagenous proteins by soaking in 0.10 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) for 3 h. Alkali-treated skins were washed until neutral or faintly basic pH of wash water was obtained. The pretreated skins were subsequently immersed in 0.1 M citric acid with a skin/solution ratio of 1:10 (w/v) for 2 h. The swollen skins were washed until wash water became neutral or faintly acidic. The prepared skins were subjected to hydrolysis.

To the pretreated skins, deionized (DI) water was added at a ratio of 1:5 (w/v). The pH of mixture was adjusted to 7.0 using 1.0 M NaOH and 1.0 M HCl and incubated at 40°C for 15 min. Papain (3% of solid content) was added and the hydrolysis was conducted at 40°C for 3 h with continuous stirring. The reaction was terminated by heating at 90°C for 15 min. For the second step of hydrolysis, Alcalase (2% solid content of fish skin) was added into the mixture, in which pH was readjusted to 8. Hydrolysis was performed at 50°C for 2 h. After inactivation at 90°C for 15 min, the resulting HC was filtered through 2 layers of cheesecloth and 2 layers of fiber grass filter to remove the debris. The filtrate was then fed to a filter unit, which consisted of 4 filter cartridges (CRC-20-BP-5, C.C.K, Taiwan) and 2 carbon capsules (Block carbon treatton 20, Treatton, Taiwan) using a diaphragm pump (R/O-450, Treatton, Taiwan). Feed flow rate was 2 L/min.

The resulting filtrate was then concentrated to obtain 10% solid using an alcohol recovery evaporator (Euro Best Technology Co., Ltd, Pathumthani, Thailand) at 50°C. HC concentrate was subjected to drying using a spray-dryer (LabPlant SD-06 Basic, North Yorkshire, England) equipped with a spry-drying chamber and a two-liquid-nozzle spray nozzle (0.5mm in size). The air inlet temperature was set at 200°C and outlet temperature was controlled at 90°C. The obtained powder was packed in laminated polyethylene/aluminium foil bag and sealed under vacuum. HC powder was kept at 4°C until used.

2.3. Preparation of cereal bar fortified with hydrolyzed collagen (HC)

The formulation and preparation of cereal bar fortified with HC are shown in Fig.1. Briefly, all dry ingredients were mixed together in a dough mixer (KitchenAid casserole multifunctional 5k, KitchenAid, Benton Harbor, MC, USA) at a low speed for 3 min. Liquid ingredients were separately mixed and preheated at 75°C for 5 min. Liquid mixture was then added to the dry mixture. The mixture was subsequently stirred at a low speed for 3 min. The resulting sticky cereal mixture (35 g) was placed on wax paper, transferred and pressed into aluminium baking tray (2.0×5.0 cm^2) with the height of 1.5 cm. The cereal bar in aluminium tray was baked in an electric oven (Mamaru MR-1214, Mamaru (Thailand) Co., Ltd., Bangkok, Thailand) at 180°C for 10 min. After baking, the cereal bars were allowed to cool at room temperature for 1 h. The resulting cereal bars were referred

to as 'Con' cereal bar. For another portion of cereal bar mixture, green tea powder (1.0% of total weight) and citric acid (0.01% of total weight) were mixed with cereal bar mixture in the same manner. Bars were formed and baked as previously described. The resulting cereal bars were named as 'GT+CA' cereal bar.

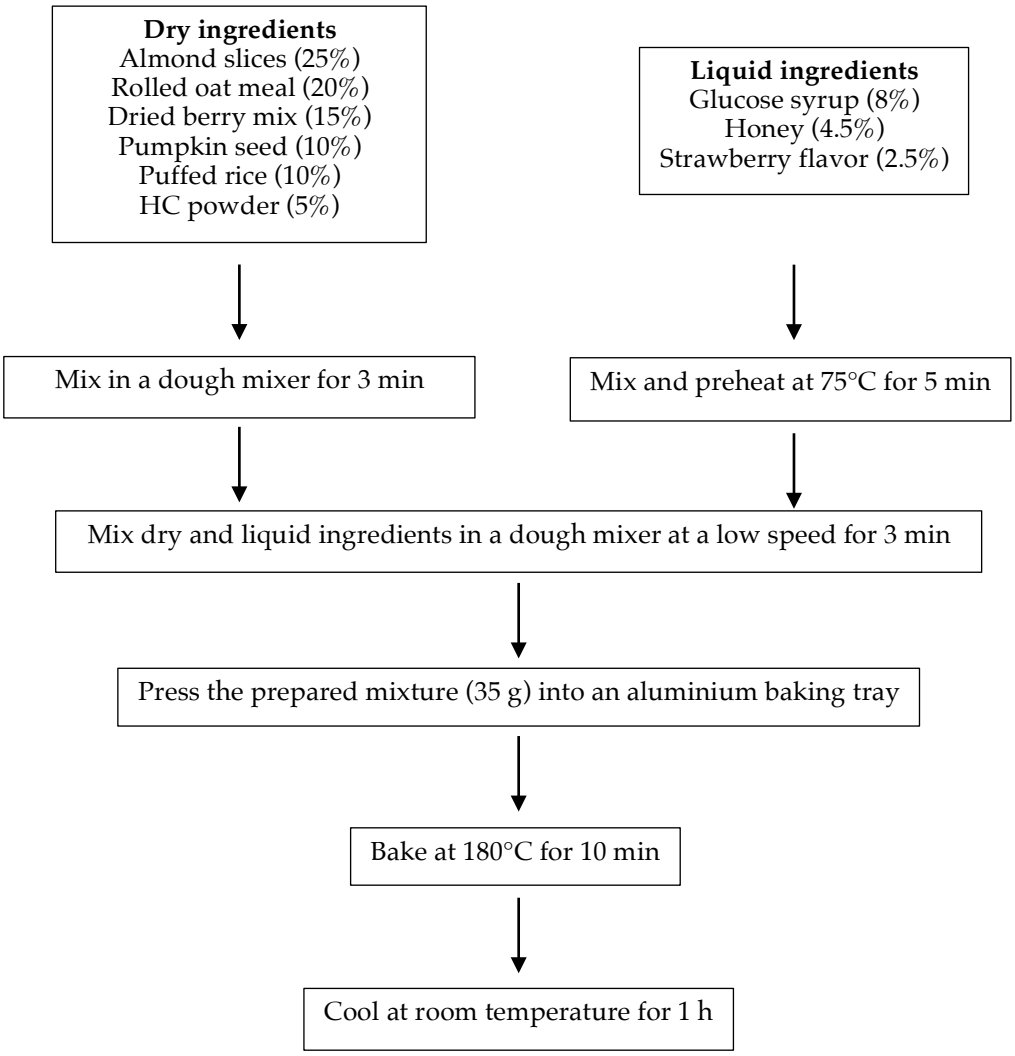

Figure 1. Diagram of preparation of cereal bar fortified with hydrolyzed collagen (HC).

2.4. Packing of cereal bars

Both 'Con' and 'GT+CA' cereal bars were packed under two different packing conditions. For the first group, the samples were placed in polypropylene (PP) pouch and heat sealed using an impulse sealer with a magnet Model ME-300HIM (S.N. MARK Ltd., Park, Nonthaburi, Thailand). The second group was placed in laminated polyethylene/aluminium foil bag with nitrogen gas flush (LF) (2.8-4.0% O_2) before being sealed using a Vacuum Sealer V-300 (FNB Machinery & Solutions, Bangkok, Thailand). PP bag (3.0×7.0 cm^2) had 89 μm thickness with a water vapor and oxygen permeabilities of 6.09 g.mm/day.m^2.mmHg (25°C) and 2.770 (mLO_2/day.pack), respectively. LF bag (3.0×7.0 cm^2) had 85 μm thickness with a water vapor and oxygen permeabilities of 5.76 g.mm/day.m^2.mmHg (25°C) and 0.002 (mL O_2/day.pack), respectively. The thickness of

packaging material was measured using a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mitutoyo Corp., Kawasaki-shi, Japan). Water vapor (WVP) and oxygen permeabilities were determined using ASTM method (SHIKU *et al*., 2004) and ambient oxygen ingress rate (AOIR) method (LARSEN *et al*., 2000), respectively. Oxygen content in packaging was investigated using Map-Pak Combi Gas Analyzer (AGC Instruments, Co Clare, Ireland).
The packaged samples were stored in the dark at the controlled temperature (25°C) for 6 months. At 0, 1, 2, 3, 4, 5 and 6 months of storage, the samples were taken for analyses.

2.5. Physicochemical analyses

2.5.1 Determination of moisture content and water activity

The samples were analyzed for moisture content using an oven method (AOAC, 2002). Water activity (a_w) was measured using a water activity meter (4TEV, Aqualab, Pullman, WA, USA).

2.5.2 Measurement of color parameters

The cereal bar was ground using a blender (Model MX-898N, Panasonic, Panasonic Sdn. Bhd., Kuala Lumpur, Malaysia) for 3 min. The color parameters of samples were then determined using a colorimeter (ColorFlex, Hunter Lab Reston, VA, USA). The color values were reported in the CIE system, including L^*, a^* and b^*, representing lightness, redness/greenness and yellowness/blueness, respectively. Total difference of color (ΔE^*) was calculated as described by TAKEUNGWONGTRAKUL *et al*. (2015).

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

where ΔL^*, $\Delta a^*$and $\Delta b^*$are the differences between the corresponding color parameter of the sample and that of day 0.

2.5.3 Measurement of textural properties

Hardness and crispiness of cereal bar were determined by a texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) using a test speed of 1.0 mm/s with a load cell of 50 kg. A special pasta blade and plate (probe TA 47, 60 mm x 20 mm) were used to imitate the biting action of a tooth. The maximum force required to break cereal bar and the distance at break were calculated for each sample. Ten measurements were made for each sample.

2.5.4 Determination of lipid oxidation

2.5.4.1 Peroxide value (PV)

PV was determined in oil extracted from the cereal bar using the Bligh and Dyer method (Bligh and Dyer 1959). PV was determined using the ferric thiocyanate method (TAKEUNGWONGTRAKUL *et al*., 2015). A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm. PV was expressed as μg cumene hydroperoxide/g sample.

2.5.4.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined using a distillation TBA method as described by KARNJANAPRATUM and BENJAKUL (2015b). Ten grams of sample, 97.5 mL of deionized water and 2.5 mL of 6 N HCl were transferred to a Kjeldahl flask. The mixture was heated and the distillate (200 mL) was collected. To determine TBARS, the distillate (0.2 mL) was added to 1 mL of TBAR solution (0.375% thiobarbituric acid, 15% TCA and 0.25M HCl) and heated in boiling water for 10 min. After cooling with running water and centrifugation at 5000xg for 10 min at room temperature, the absorbance of the pink solution was read at 532 nm. TBARS value was calculated from a standard curve of malondialdehyde (MDA) (0-10 mg/L) and expressed as μg MDA/g sample.

2.5.5 Volatile compounds

The volatile compounds in the cereal bar samples were determined at 0, 3 and 6 months of storage using solid-phase microextraction gas chromatography mass spectrometry (SPME GC–MS) following the method of TAKEUNGWONGTRAKUL and BENJAKUL (2017). Volatiles were expressed as the abundance (peak area).

2.6. Sensory evaluation

The packaged samples were taken for sensory evaluation at 0, 3 and 6 months. Sensory evaluation was performed by 50 untrained panelists. They were asked to evaluate for appearance, color, odor, flavor, taste, texture and overall likeness using a nine-point hedonic scale, in which a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely. The samples were labelled with random three-digit codes. Panelists were instructed to rinse their mouth with drinking water after each sample evaluation and the order of presentation of the samples was randomized (MEILGAARD *et al*., 2006). The samples with likeness score less than 7 (moderately like) were not used for further analyses.

2.7. Microbiological analysis

Total viable microbial count was enumerated by pour plating on Plate Count Agar (PCA, Difco Laboratories Inc., Detroit, MI, USA) at 37°C for 48 h. Yeast and mold counts were determined by pour plating using Potato Dextrose Agar (PDA, Laboratories Inc. Detroit, MI, USA) at 30°C for 72 h.

2.8. Statistical analysis

Completely randomized design was used. Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range tests (STEEL and TORRIE, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Changes in moisture content and water activity during storage

Moisture content and water activity of HC fortified cereal bars without (Con) and with (GT+CA) addition of antioxidants, packed under different conditions during storage are shown in Fig. 1.

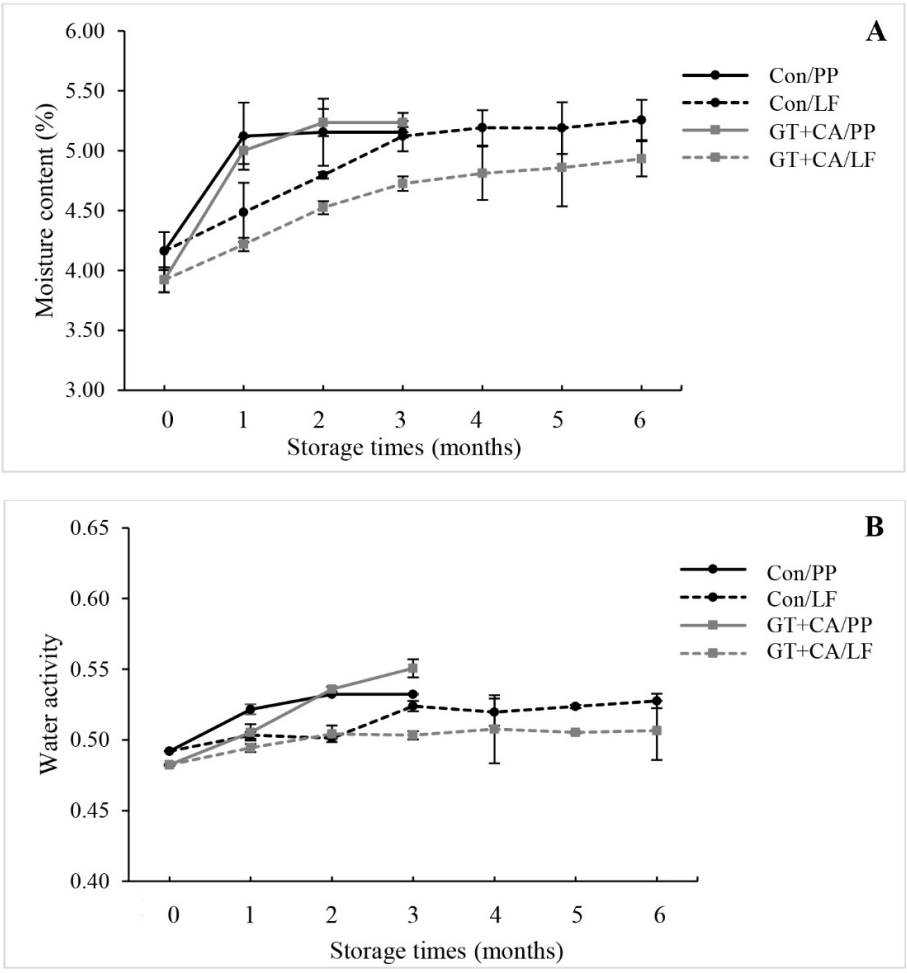

Figure 1. Moisture content (A) and water activity (B) of HC fortified cereal bars packed under different conditions during storage. Con; Cereal bar without antioxidants. GT+CA; Cereal bar with added antioxidants (1.0% Green tea powder, 0.01% Citric acid). PP; Polypropylene bag with normal heat seal. LF; Laminated polyethylene and aluminium foil bag with modified atmosphere pack (N_2 flush) before sealing. Bars represent the standard deviation (n=3).

Moisture contents of all samples during storage up to 6 months were in the range of 3.92-5.26% (w/w) (Fig. 1A). At Day 0, moisture contents of Con sample (4.16%) was significantly higher than that of GT+CA sample (3.92%) ($P<0.05$). Antioxidants (GT+CA) were added in the powder form, thereby lowering the moisture content of cereal bars. Sharp increase in moisture content was found for the samples packed in PP within the first month of storage ($P<0.05$), regardless of incorporation of antioxidants. Thereafter, the Con and GT+CA samples packed in PP bag had constant moisture content up to 3 months of storage ($P>0.05$). For the samples packed in LF bag, the moisture contents increased

linearly up to 3 months ($P<0.05$), irrespective of incorporation of antioxidant. However, no changes were found between 3-6 months of storage ($P>0.05$). It was noted that the Con sample had higher moisture content than GT+CA sample throughout the storage of 6 months. These results suggested that packaging played a profound role in preventing the migration of water into the cereal bars. LF bag showed the higher water vapor barrier property with a water vapor permeability of 5.67 g.mm/day.m^2.mmHg (25°C), compared with PP bag (6.09 g.mm/day.m^2.mmHg).

A similar result was observed for the change in water activity of cereal bars packed in different packing conditions during storage (Fig. 1B). At Day 0, the Con sample had higher water activity than GT+CA sample ($P<0.05$). Water activity of all samples continuously increased, especially those packed in PP bag, which showed the higher increasing rates, compared with those packed in LF bag, regardless of antioxidants used. Water activity of all samples during 6 months of storage was in the range of 0.48-0.53. Cereal bars with water activity lower than 0.6 are microbiologically safe under storage condition tested. FREITAS and MORETTI (2005) studied the stability of cereal bar with high protein using different packaging materials at room temperature. Increase in moisture content was observed during storage period tested and the best packaging with respect to water vapor permeability was the one containing aluminium (FREITAS and MORETTI, 2005). SENHOFA *et al*. (2015) found that the water activity of muesli stored in different packaging varied with packing materials and moisture permeability. In the present study, the packing condition had higher impact than the addition of antioxidants on moisture content and water activity change of cereal bars during storage.

3.2. Changes in color during storage

Color of HC fortified cereal bars without (Con) and with addition of antioxidants (GT+CA) packed under different packing conditions during 6 months of storage is presented in Table 1. For day 0, Con sample showed the lighter color as indicated by higher L^* values, compared to that of GT+CA sample ($P<0.05$). GT+CA sample was more greenish but less yellowish in color as indicated by the lower a^* and b^* values, respectively, compared to the Con sample ($P<0.05$). This could be related to green color of green tea powder used as antioxidant in the formulation, which contributed to the green color of product. Overall, the color of cereal bars was changed during storage, especially those packed in PP bag. The darker color with less yellowness as indicated by the decreased L^* and b^* values was observed for the Con sample packed in PP bag after storage for 3 months ($P<0.05$), compared to those found at Day 0. A similar result was obtained for the GT+CA sample packed in PP bag, in which less yellowness and redness indicated by lower a^* and b^* values were noted after 3 months of storage ($P<0.05$). On the other hand, a lower rate of change in color of samples during storage was observed when LF bag was used, particularly in combination of GT+CA addition. These results were in accordance with ΔE^* value. Increase in ΔE^* value at the higher rate was observed from the sample packed in PP bag, especially those of Con sample, during the storage. The addition of antioxidants (green tea and citric acid) could retard the discoloration of cereal bar during extended storage as evidenced by the retarded changes in lightness (L^* value) and yellowness (b^* value), compared with those of the Con sample. Moreover, lower O_2 permeability of LF bag with better water vapor barrier property could reduce chemical reactions, particularly lipid oxidation and Maillard reaction. Maillard reaction is predominant at room temperature in low-moisture products (a_w 0.5-0.7) with high protein content (BAPTISMA and CARVALHO, 2004). Lipid oxidation also resulted in propagation of the Maillard reaction (BECKER *et al*., 2009). Lipid oxidation occurred to a lower extent

when oxygen content in packaging was lowered and packaging material with low water vapor permeability was used (BAKKALBAŞı *et al*., 2012; NILSUWAN *et al*., 2016).

Table 1. Changes in color (L^*, a^*, b^* and ΔE^* values) of HC fortified cereal bars packed under different conditions during the storage.

Color	Storage time (month)	Con		GT+CA	
		PP	LF	PP	LF
L^*	0	67.94±0.96a		62.60±0.86a	
	1	65.56±0.70a	68.14±1.00a	61.83±1.54ab	61.82±1.43ab
	2	64.70±2.86a	66.91±1.03a	61.53±2.13ab	61.80±0.92ab
	3	61.23±2.65b	65.46±1.42a	61.33±3.50ab	60.52±2.21ab
	4	nd.	65.30±2.00a	nd.	60.10±3.72ab
	5	nd.	60.63±1.31b	nd.	60.10±1.39ab
	6	nd.	56.81±2.42c	nd.	58.28±0.89b
a^*	0	5.11±0.28abc		2.99±0.66a	
	1	5.73±0.68ab	6.28±1.34a	2.18±0.14bc	2.65±0.44ab
	2	4.28±1.33bc	5.42±1.14ab	2.16±0.64bc	2.52±0.21ab
	3	3.66±0.98c	5.17±0.23abc	1.46±0.55c	1.99±0.32bc
	4	nd.	5.13±0.56abc	nd.	1.68±0.21c
	5	nd.	4.31±0.40bc	nd.	1.60±0.33c
	6	nd.	4.31±0.39bc	nd.	1.55±0.47c
b^*	0	26.22±0.78a		22.29±0.46a	
	1	23.34±1.62bcd	24.97±0.37ab	22.57±0.74a	22.28±1.06a
	2	23.02±1.02bcd	24.84±0.41ab	20.67±0.29bc	22.14±0.61ab
	3	22.19±1.02cd	24.49±0.39ab	20.69±0.57bc	21.89±0.99ab
	4	nd.	23.43±1.98bc	nd.	21.33±0.64abc
	5	nd.	21.57±0.89cd	nd.	20.20±1.04cd
	6	nd.	21.22±1.54d	nd.	19.22±1.01d
ΔE^*	0				
	1	3.79±0.97a	1.72±1.14a	1.15±0.90a	0.85±0.86a
	2	4.63±2.18b	1.75±0.94a	2.11±1.28b	0.94±0.48b
	3	7.96±1.84c	3.02±0.61b	2.55±2.64b	2.34±1.49c
	4	nd.	3.84±1.61b	nd.	2.98±2.90c
	5	nd.	8.70±0.39c	nd.	3.54±0.85d
	6	nd.	12.23±1.65d	nd.	5.49±0.58e

Values and mean ± SD (n = 3). nd.; Not detected. Con; Cereal bar without antioxidants. GT+CA; Cereal bar with added antioxidants (1.0% Green tea powder, 0.01% Citric acid). PP; Polypropylene plastic with normal heat seal. LF; Laminated polyethylene and aluminium foil bag with modified atmosphere pack (N_2 flush) before sealing. Different lowercase letters for the same color attribute within the same column indicate significant difference ($P < 0.05$).

The generated lipid oxidation products, especially aldehydes and ketones, could be a carbonyl source for condensation with amines. As a consequence, browning discoloration via Maillard reaction could take place. Additionally, with increasing storage time,

Maillard reaction proceeded to higher extent. Thus, browning occurred to a higher degree, particularly for the Con sample packed in PP bag. Therefore, the addition of antioxidants and packing condition used played an important role in maintaining color of cereal bar during storage. The result indicated that LF bag showed higher preventive effect on color changes of cereal bar fortified with HC than PP bag during the extended storage.

3.3. Changes in hardness and crispiness during storage

Hardness and crispiness of cereal bars without (Con) and with addition of antioxidants (GT+CA) stored under different packing conditions during 6 months of storage are shown in Fig. 2.

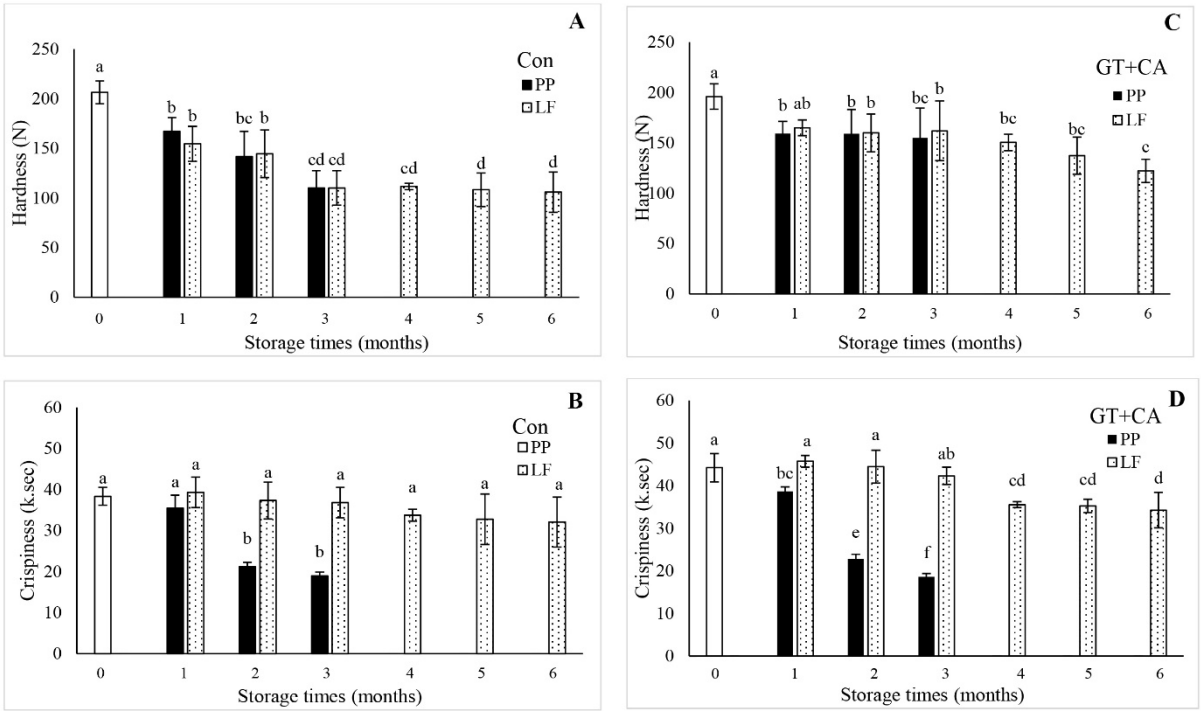

Figure 2. Hardness (A and C) and crispiness (B and D) of HC fortified cereal bars packed under different conditions during storage. Con; Cereal bar without antioxidants. GT+CA; Cereal bar with added antioxidants (1.0% Green tea powder, 0.01% Citric acid). PP; Polypropylene bag with normal heat seal. LF; Laminated polyethylene and aluminium foil bag with modified atmosphere pack (N_2 flush) before sealing. Bars represent the standard deviation (n=3).

The highest hardness (195.90-206.39 N) was observed for both Con (Fig. 2A) and GT+CA samples (Fig. 2C) at Day 0 of storage ($P<0.05$). Within the first 3 months of storage, hardness decreased for all samples packed in both PP bag and LF bag. There was no difference between cereal bars packed in PP bag and LF bag at the same storage time ($P>0.05$). After 3 months of storage, hardness of all samples packed in LF bag remained unchanged ($P>0.05$), excepted that of GT+CA samples after 6 months of storage, which had decreased hardness ($P<0.05$). This phenomenon was related to the slight increase in moisture content during storage (Fig. 1A). Increased moisture content more likely negatively affected the textural property of cereal bar. LF bag with better moisture barrier property packaging material prevented and reduced water vapor migration from environment to product, compared with PP bag. This was related with the lower crispiness of cereal bars packed in PP bag, compared to those packed in LF bag,

particularly within the first 3 months of storage (Fig. 2B and 2D). With extended storage, the samples packed in PP bag had marked decrease in crispiness ($P<0.05$). There was no change in crispiness for samples packed in LF bag throughout 6 months of storage ($P<0.05$), except those containing GT+CA, which had a slight decrease after 3 months of storage ($P<0.05$). Similar results were also reported for biscuits fortified with micro-encapsulated oil, in which high moisture content was related with lowered hardness (TAKEUNGWONGTRAKUL and BENJAKUL, 2017). The results indicated that packing condition had higher impact than added antioxidants on textural properties of cereal bars fortified with HC during storage of 6 months. Therefore, LF bag could be used for packing the cereal bars to maintain their textural property during storage.

3.4. Changes in peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) during storage

PV and TBARS of cereal bars without (Con) and with addition of antioxidants (GT+CA) stored under different packing conditions during 6 months of storage are depicted in Fig. 3. At day 0 of storage, GT+CA sample had lower PV than that of Con (Fig. 3A) ($P<0.05$). This result suggested that lipid oxidation could occur during cereal bar preparation and the addition of antioxidants, green tea powder and citric acid, could prevent lipid oxidation to some degree as indicated by the lower PV of GT+CA sample. PV of all samples notably increased within the first 2 months of storage ($P<0.05$) and remained unchanged during 2-3 months ($P>0.05$). After 3 months of storage, the highest PV was found for the Con packed in PP bag, compared with others ($P<0.05$). In general, PV of samples packed in PP bag was higher than that of samples packed in LF bag ($P<0.05$). Furthermore, the addition of antioxidants could suppress the formation of hydroperoxide. Nitrogen gas was purged into the sample packed in LF bag. This could also prevent oxidation in the sample in conjunction with antioxidants. In the low oxygen atmosphere, the oxidation of lipid occurs at negligible level. This result was in agreement with BAKKALBAŞı *et al*. (2012) who found that higher content of oxygen present in packaging increased lipid oxidation of walnuts during storage, especially at higher storage temperature. After 3 months of storage, sample packed in LF bag was further stored. It was found that the sharp decreases in PV were observed for both samples, Con and GT+CA samples. This was more likely caused by the decomposition of hydroperoxides to the secondary products (KARNJANAPRATUM and BENJAKUL, 2015a). The lower PV was observed for GT+CA sample, compared with those of Con sample ($P<0.05$). Addition of antioxidants in cereal bars had a preventive effect on lipid oxidation by retarding the radical chain reaction. Along with the nitrogen atmosphere, the oxidation of lipids in nuts or other ingredients used in formulation could be impeded more effectively.

Changes in TBARS of Con and GT+CA samples kept under different packing conditions during storage of 6 months are shown in Fig. 3B. The increase in TBARS indicated formation of secondary lipid oxidation products. Marked increases in TBARS were observed for all samples within the first month of storage ($P<0.05$). TBARS of Con sample packed in PP bag sharply decreased during 1-3 months, while GT+CA sample packed in PP bag showed gradual decrease after 2 months of storage ($P<0.05$). The decrease in TBARS with extended storage was probably due to the loss in those volatile secondary products (YARNPAKDEE *et al*., 2012). Green tea and citric acid added in cereal bars could prevent the formation of TBARS during storage of sample packed in PP bag, where oxygen was present to some extent. However, lower increase in TBARS value was observed for those packed in LF bag within the first month of storage ($P<0.05$), compared to those packed in PP bag. TBARS of samples packed in LF bag were slightly changed during 1-2 months of storage ($P<0.05$). During 2-3 months of storage, TBARS sharply

decreased in all samples (P<0.05). After 3 months of storage, drastic decrease in TBARS in sample packed in LF bag was observed. However, lower TBARS value was noticeable in the GT+CA sample, compared to Con sample. During 4-6 months of storage, TBARS value remained constant for both samples. Thus, the addition of antioxidants could prevent lipid oxidation of cereal bar during storage to some degree. Moreover, LF bag was able to effectively retard the early stages as well as the advanced stage of oxidation. As a result, the quality of cereal bar could be maintained during storage.

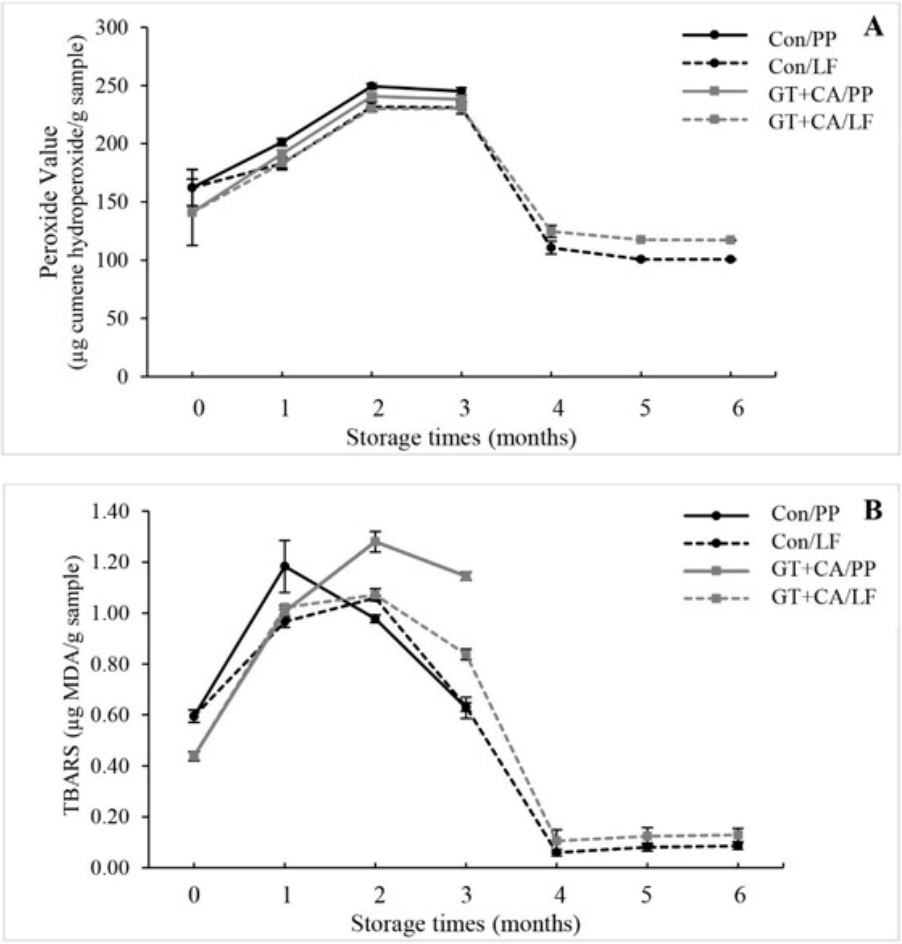

Figure 3. PV (A) and TBARS (B) values of HC fortified cereal bars packed under different conditions during storage. Con; Cereal bar without antioxidants. GT+CA; Cereal bar with added antioxidants (1.0% Green tea powder, 0.01% Citric acid). PP; Polypropylene bag with normal heat seal. LF; Laminated polyethylene and aluminium foil bag with modified atmosphere pack (N_2 flush) before sealing. Bars represent the standard deviation (n=3).

3.5. Volatile compounds

Volatile compounds in Con and GT+CA samples stored under different packing conditions at 0, 3 and 6 months of storage are displayed in Table 2. The types and abundance of volatile compounds detected in samples varied with antioxidants and packing conditions used as well as storage times. At day 0 of storage, 21 volatile compounds were identified for both samples, including 7 alcohols, 2 aldehydes, 3 ketones, 5 acids and 4 esters. It was noted that the abundance of total volatile compounds in GT+CA sample (308.83×10^6) was lower than that of Con sample (501.83×10^6). Some

volatiles were not detected in GT+CA sample, while they were found in Con sample. 1-Penten-3-ol was detected in GT+CA sample, indicating the presence of green odor/aroma in green tea (LEE *et al*., 2013). Most acids and ester volatile compounds were reported as fruity aroma (MOHAMED El HADI *et al*., 2013), which might be related to the mixed dried fruit and strawberry flavor used for preparing cereal bars. Several derivatives of volatiles can be formed by the oxidation of lipids. 1-Butanol 3-methyl-, 3-hexen-1-ol, 1,2-propanediol, 3-furanmethanol and benzyl alcohol were found in the Con sample with higher abundance, compared with those of GT+CA sample. Aldehydes and ketones are among the main contributors to flavor and their concentration was related to lipid oxidation (SAE-LEAW *et al*., 2016; TAKEUNGWONGTRAKUL and BENJAKUL, 2017). 2-Furan-carboxaldehyde, 5-hydroxymethylfurfural, 2-butanone, 3-hydroxy-, 2-propanone, 1-hydroxy-, ethanone, 1-(2-furanyl)-, butanoic acid ethyl ester, dipropylene glycol diacetate, 1,2-propanediol 1-acetate, 1,2-Propanediol 2-acetate and some acids were detected in the Con sample with higher abundance, compared with those of GT+CA sample. This result was in accordance with higher PV and TBARS of Con sample, compared to those of GT+CA sample at day 0 (Fig. 3). The results suggest that green tea and citric acid used in GT+CA sample could prevent lipid oxidation during cereal bar manufacture.

After 3 months of storage, marked increase in volatile compounds was observed for all samples packed in both PP and LF bags indicating that lipid oxidation took place in the cereal bars within 3 months of storage. Auto-oxidation could occur, especially in cereal, nuts and grains. Decomposition of lipid hydroperoxides is a complicated process and produces a multitude of constituents that may have biological effects and cause flavor deterioration in fat-containing foods (El-MAGOLI *et al*., 1982). This decomposition proceeds by homolytic cleavage of LO-OH to form alkoxy radicals LO·. These radicals undergo carbon-carbon cleavage to form breakdown products including aldehydes, ketones, alcohols, esters and furans (El-MAGOLI *et al*., 1982). New volatiles including 10 alcohols, 2 aldehydes, 1 ketone, 2 acids and 10 esters were identified for all samples tested. Notably, cereal bars packed in PP bag showed higher volatile abundance (706-921×10^6), compared to those packed in LF bag (419-505× 10^6). 1,2-propanediol, 3-hexen-1-ol and 1-hexanol were found as the major alcohols in all cereal bars. Hexanal and 2-furan-carboxaldehyde were dominant aldehydes, whereas 2-propanone, 1-hydroxy- was the predominant ketone. Aldehydes and ketones are known as the major contributors to the development of lipid oxidation off-odor and off-flavor (TAKEUNGWONGTRAKUL and BENJAKUL, 2017). Aliphatic alcohols such as 1-pentene-3-ol and 1-octen-3-ol contribute to off-flavor and they are produced by oxidative deterioration of food lipid (SAE-LEAW *et al*., 2016). Acetic acid was the major acid and butanoic acid ethyl ester was found as the high abundant ester in all samples, indicating the fruity flavor of cereal bars (MOHAMED EL HADI *et al*., 2013). Less volatile compounds were found for GT+CA sample, compared with the Con sample, regardless of packing condition used. This might be due to the influence of antioxidants used in GT+CA sample, which could prevent lipid oxidation. In addition, some new volatiles were detected only for those packed in PP bag such as 2-butanol and 2-penten-1-ol. This correlated well with the higher PV, compared with those packed in LF bag. The lowest abundance together with less types of volatiles was generally found for GT+CA sample packed in LF bag. Ethyl maltol, propanoic acid, propanoic acid ethyl ester, 2-hydroxypropyl propionate and 3-furancarboxylic acid methyl ester were found in all samples, excepted for GT+CA sample packed in LF bag. This was more likely owing to the higher barrier properties with lower oxygen content in LF bag as well as antioxidants added, which could prevent lipid oxidation during storage.

After 6 months of storage, some compounds were not found and new volatiles were generated. This was possibly due to the volatilization or decomposition of those aforementioned compounds. Simultaneously, new compounds were formed. The further

oxidation of lipids or some other reactions could change the abundance of volatile compounds during storage (ANDRÉS *et al*., 2004). Among alcohols, 2-butanol, 1-propanol,2-methyl-, 2-propanol, 1-methoxy-, 1-pentanol, 1-octanol, 2-propanol, 1,1'-oxybis-, benzene ethanol and maltol were not detected, while cyclohexanol was found as new volatile alcohol for both of Con and GT+CA samples packed in LF bag. 2-Propanone was generated as new ketone with disappearance of 2-butanone, 3-hydroxy- and 2(3H)-furanone, dihydro-. Marked increase in volatile compounds was observed for both Con and GT+CA samples with extended storage (6 month), compared with those of samples stored for 3 months. It was noted that GT+CA sample had lower abundance with less types of volatile compounds, compared to those of Con sample. The result indicated that lipid oxidation of GT+CA sample packed in LF bag could be reduced to some extent. Therefore, cereal bar fortified with HC with and without addition of antioxidants could be packed with LF bag to improve oxidative stability during 6 months of storage.

3.6. Sensory properties

Sensory properties of HC fortified cereal bar in the absence (Con) and the presence of antioxidants (GT+CA) stored under different packing conditions are shown in Fig. 4. At day 0, the likeness scores of all attributes tested, including appearance, color, odor, flavor, taste, texture and overall likeness for the Con and GT+CA samples were in the range of 7.5-8.2. Significant decrease in likeness score of both Con (Fig. 4A) and GT+CA samples (Fig. 4C) was observed for those packed in PP bag, especially after 3 months of storage ($P<0.05$). Likeness scores for all attributes of both samples stored in PP bag were lower than 7 (6.0-6.8) after 3 months of storage. There were no differences in likeness score of all attributes tested between both Con (Fig. 4B) and GT+CA samples (Fig. 4D) packed in LF bag during storage of 6 months ($P>0.05$). This result suggested that the packing condition had more impact than antioxidants on sensory properties during storage. The addition of antioxidants (GT+CA) had no marked influence on sensory qualities during storage. Con and GT+CA samples packed in PP were discarded at month 3 since likeness scores for all attributes tested were less than 7. Lower water vapor barrier property (6.09 g.mm/day.m^2.mmHg) with higher content of O_2 (18.0-18.8%) of PP bag could favor physical and chemical changes of cereal bar, whereas LF bag (2.8-4.0% O_2 content with water vapor permeability of 5.76 g.mm/day.m^2) showed superior property in keeping cereal bars. Water vapor from environment could migrate through packaging material and consequently increased the moisture content of product. This affected the appearance, color and texture of cereal bar. In addition, the high content of O_2 induced chemical change, especially lipid oxidation, during storage. Volatile compounds from lipid oxidation products were related to flavor deterioration known as rancidity (YARNPAKDEE *et al*., 2012). Moreover, aldehyde and ketone compounds from lipid oxidation were more likely involved in a yellowish discoloration via the Maillard reaction (YARNPAKDEE *et al*., 2012). Those reactions decreased the likeness score for appearance, odor, flavor and taste of cereal bars packed in PP bag. Similar results were reported for sensory property of stored muesli in different packaging (SENHOFA *et al*., 2015). Paper bag with low barrier property caused the greatest decrease of sensory quality during storage, compared with laminated low density polyethylene/aluminium foil container (SENHOFA *et al*., 2015). Also, LF bag was flushed with N_2 to replace air before sealing. This could help in retardation of lipid oxidation. Therefore, the packing conditions including packaging material and oxygen content, had direct impact on sensory properties of cereal bar fortified with HC during the storage. LF bag with nitrogen gas could improve the sensory quality of cereal bars during 6-month storage, regardless of antioxidant addition.

Table 2. Volatile compounds of HC fortified cereal bars packed under different conditions during storage.

Volatile compounds	Abundance (×10^6)							
	Month 0		Month 3				Month 6	
			PP		LF		LF	
	Con	GT+CA	Con	GT+CA	Con	GT+CA	Con	GT+CA
Alcohols								
2-Butanol	nd.	nd.	0.86	0.46	nd.	nd.	nd.	nd.
1-Propanol, 2-methyl-	nd.	nd.	1.99	1.69	1.53	1.31	nd.	nd.
2-Propanol, 1-methoxy-	nd.	nd.	0.42	0.36	0.35	0.28	nd.	nd.
1-Butanol	nd.	nd.	1.53	1.4	1.26	1.29	nd.	nd.
1-Penten-3-ol	1.29	nd.	2.71	2.69	nd.	nd.	nd.	nd.
1-Butanol, 3-methyl-	1.7	nd.	7.01	4.57	6.7	5.32	8.26	7.34
1-Pentanol	nd.	nd.	11.86	10.62	11.85	10.81	nd.	nd.
2-Penten-1-ol	nd.	nd.	1.59	1.4	nd.	nd.	5.88	6.14
1-Hexanol	3.98	3.1	31.97	31.09	29.49	23.48	207.7	117.33
3-Hexen-1-ol	90.1	41.21	89.01	65.77	64.47	72.34	303.91	295.12
Cyclohexanol	nd.	nd.	nd.	nd.	nd.	nd.	9.17	6.61
2,3-Butanediol	nd.	nd.	4.31	2.97	4.01	3.43	7.47	9.02
1-Octanol	nd.	nd.	0.32	0.69	0.31	nd.	nd.	nd.
1,2-Propanediol	258.83	167.47	342.48	255.17	175.83	111.76	71.61	94.19
3-Furanmethanol	2.8	2.28	4.98	4.56	3.23	5.87	46.86	55.63
2-Propanol, 1,1'-oxybis-	nd.	nd.	0.3	nd.	0.29	nd.	nd.	nd.
Benzyl alcohol	1.71	1.14	1.63	1.55	1.39	0.94	26.18	18.18
Benzeneethanol	nd.	nd.	0.21	nd.	0.2	nd.	nd.	nd.
Maltol	nd.	nd.	0.85	1.07	0.62	0.69	nd.	nd.
Ethyl maltol	nd.	nd.	1.19	1.16	1.09	nd.	21.73	21.79
Aldehydes								
2-Cyclopentenylethanal	nd.	nd.	nd.	nd.	nd.	nd.	234.1	51.68
Hexanal	nd.	nd.	9.61	9.3	6.66	2.87	23.07	9.74
2-furan-carboxaldehyde	16.17	5.37	6.72	5.82	4.83	4.32	65.28	62.25
Benzaldehyde	nd.	nd.	1.12	0.83	0.82	0.8	9.21	0.94

5-Hydroxymethylfurfural	2.52	nd.	0.19	nd.	nd.	nd.	nd.	nd.
Ketones								
2-Propanone	nd.	nd.	nd.	nd.	nd.	nd.	17.8	10.42
2-Butanone, 3-hydroxy-	3.89	nd.	1.9	1.9	1.88	1.37	nd.	nd.
2-Propanone, 1-hydroxy-	7.6	7.53	14.94	12.05	12.75	12.58	57.82	53.37
Ethanone, 1-(2-furanyl)-	0.84	nd.	1.61	1.32	1.25	1.22	17.16	9.45
2(3H)-Furanone, dihydro-	nd.	nd.	0.76	0.72	0.93	0.76	nd.	nd.
2-Butanone, 4-phenyl-	nd.	nd.	nd.	nd.	nd.	nd.	9.49	6.42
4H-Pyran-4-one, 3-hydroxy-2-methyl-	nd.	nd.	nd.	nd.	nd.	nd.	24.24	19.88
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	nd.	nd.	nd.	nd.	nd.	nd.	10.43	12.99
Acids								
Acetic acid	66.98	48.95	33.92	29.5	26.33	26.14	170.36	143.56
Propanoic acid	5.51	5.01	0.99	1.21	1.4	nd.	7.5	5.92
Butanoic acid	nd.	nd.	0.86	0.75	0.71	0.52	nd.	nd.
Pentanoic acid	0.41	nd.	0.56	0.55	0.41	0.36	7.19	5.58
Hexanoic acid	14.87	8.86	1.87	1.79	1.75	1.7	29.73	15.26
Hexanoic acid, 2-ethyl-	nd.	nd.	0.61	0.6	0.53	0.5	nd.	nd.
Butanoic acid, 2-methyl-	10.71	9.91	3.79	3.79	3.65	3.55	17.36	13.38
Esters								
Acetic acid ethyl ester	nd.	nd.	51.44	35.61	13.23	7.67	76.07	50.32
Propanoic acid, ethyl ester	nd.	nd.	1.48	0.75	0.46	nd.	nd.	nd.
Butanoic acid, ethyl ester	3.48	0.95	237.54	176.75	103.38	100.49	495.47	467.95
Butanoic acid, 2-methyl-, ethyl ester	nd.	nd.	12.01	7.81	5.15	4.77	48.12	47.71
Butanoic acid, 3-methyl-, ethyl ester	nd.	nd.	4.04	1.92	1.41	1.35	9.79	5.84
Hexanoic acid, ethyl ester	nd.	nd.	2.55	1.48	0.93	0.91	14.61	13.02
Butanoic acid, 2-ethyl-, methyl ester	nd.	nd.	nd.	nd.	nd.	nd.	25.15	22.09
Benzyl acetate	nd.	nd.	nd.	nd.	nd.	nd.	4.09	6.17
Methyl isobutyrate	nd.	nd.	2.09	1.72	1.64	1.28	nd.	nd.
Formic acid, octyl ester	nd.	nd.	nd.	nd.	nd.	nd.	7.82	5.43
Hexanoic acid, ethyl ester	nd.	nd.	2.55	1.48	0.93	0.91	13.02	14.61
Dipropylene glycol, diacetate	0.32	nd.	9.07	8.75	nd.	nd.	nd.	nd.
1,2-Propanediol, 1-acetate	4.57	3.78	5.8	5.55	5.15	5.04	33.97	nd.

2-Hydroxypropyl propionate	nd.	nd.	4.14	4.11	3.46	nd.	34.89	32.69
1,2-Propanediol, 2-acetate	3.55	3.27	2.79	2.64	2.54	2.27	11.31	11.25
Acetic acid, phenylmethyl ester	nd.	nd.	0.34	0.32	0.29	0.28	nd.	nd.
Ethane-1,1-diol dibutanoate	nd.	nd.	nd.	nd.	nd.	nd.	11.88	9.6
3-Furancarboxylic acid, methyl ester	nd.	nd.	0.51	0.44	0.22	nd.	9.64	7.4

nd.; Not detected. Con; Cereal bar without antioxidants. GT+CA; Cereal bar with added antioxidants (1.0% Green tea powder, 0.01% Citric acid). PP: Polypropylene plastic bag with normal heat seal. LF: Laminated polyethylene and aluminium foil bag with modified atmosphere pack (N_2 flush).

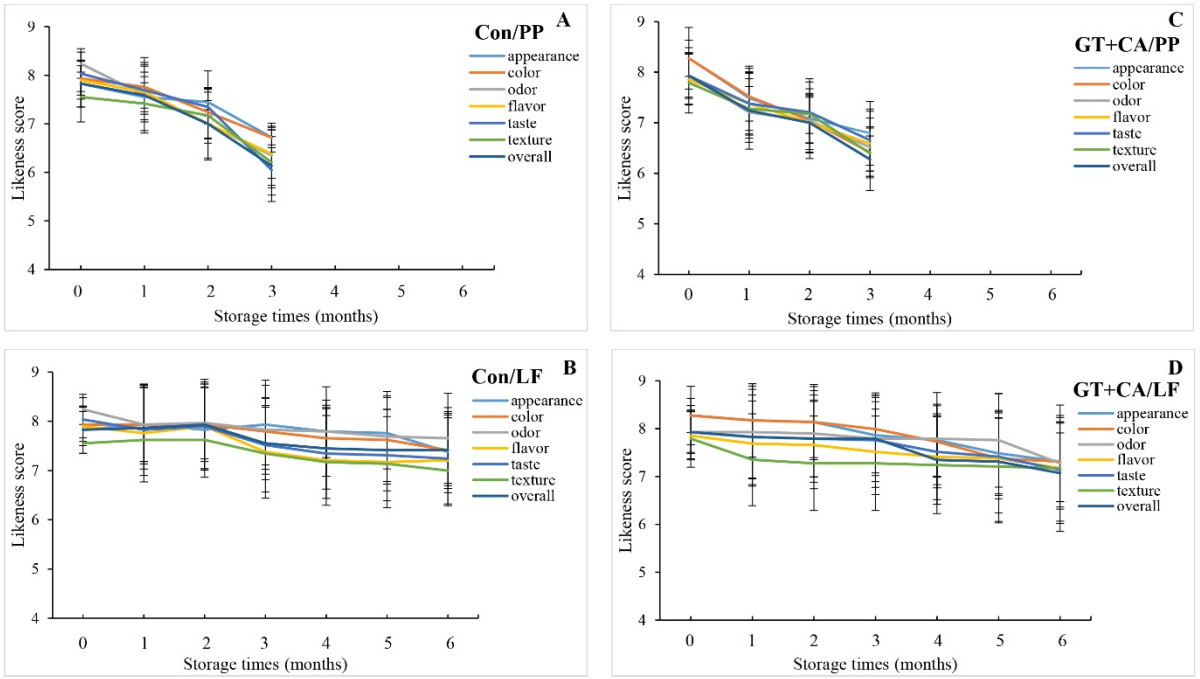

Figure 4. Sensory characteristics of HC fortified cereal bars packed under different conditions during storage. Con; Cereal bar without antioxidants. GT+CA; Cereal bar with added antioxidants (1% Green tea powder, 0.01% Citric acid). PP; Polypropylene plastic with normal heat seal. LF; Laminated polyethylene and aluminium foil bag with modified atmosphere pack (N_2 flush) before sealing. Bars represent the standard deviation (n=3).

3.7. Microbiological properties

Total viable count and yeast/mold counts of cereal bars stored under different packaging conditions were determined every month during storage of 6 months. Total viable counts for mesophilic aerobic microorganisms were less than 10^3 CFU/g sample for all samples tested during storage up to 6 months. On the other hand, yeast and mold counts were less than 10 CFU/g sample. The number of microorganisms was in accordance with the expected microbiological quality of processed foods, particularly cereal and cereal products (FDA, 2013). The result was in line with low water activity of the cereal bars, which was less than 0.6 during storage (Fig. 1B). This low water activity limits the microbial growth. SENHOFA *et al*. (2015) studied the storage stability of muesli in different types of packaging materials. Mesophilic aerobic bacteria, yeast and mold growth in muesli samples during storage was influenced by the presence of air and its diffusion through packaging material (SENHOFA *et al*., 2015). These results indicated that cereal bars with and without addition of antioxidants in all packaging conditions used were handled properly with appropriate storage condition, thus providing the product with microbiological safety and quality for consumers throughout the storage of 6 months.

4. CONCLUSIONS

Addition of antioxidants and packing conditions had profound impact on changes in quality and sensory properties of cereal bars fortified with HC during 6 months of storage at 25°C. Addition of green tea powder and citric acid as antioxidants could prevent some physicochemical changes and retard lipid oxidation to some degree, especially those cereal bars packed in PP bag. Based on sensory acceptance, cereal bars packed in LF bag in the presence of N_2 provided the better stability with lower lipid oxidation, compared with those packed in PP bag, regardless of antioxidant addition. Therefore, LF bag along with N_2 gas can be effectively used for packing cereal bars with improved storage stability.

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PAPER

EXTRACTION YIELD AND CHARACTERIZATION OF BURUNDIAN AVOCADO OIL OBTAINED BY MEANS OF MALAXATION WITH AND WITHOUT ENZYMATIC AID

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ABSTRACT

The mechanical extraction on the yield and quality of avocado oil extracted from different fruit varieties were investigated in this study. Batches of various varieties of ripen avocado in Burundi were processed in an oil mill located in the Gitega Region. Avocado oil was extracted considering the malaxation step carried out with and without the enzymes addition. Avocado pulp achieved by malaxation at 30°C for 90 min presented the highest yield and get the lessen acidity and peroxide values. Under the conditions applied in this study (dilution ratio between avocado paste and purified water at 1: 0.5 and malaxation temperature below 36°C), the addition of pectolitic and amylolytic enzymes did not reveal to increase the yield to such extent as to justify the cost of the treatment. According to what stated for classification of olive oils in EVO and VO categories, the results of chemical and sensorial indices allowed classifying most of the avocado oils obtained from malaxation without enzyme addition in the EVO category.

Keywords: avocado oil, oil mill, malaxation, enzymatic extraction, Burundi

1. INTRODUCTION

Avocado (*Persea americana* Mill.) is a drupe-shaped fruit, more or less elongated, with a thin, pale olive-green glossy shell. It is widely consumed today as an important and energetic fruit with high nutritional value and health benefits (DUARTE *et al*., 2016) due to the compounds of the lipid fraction that varies from 13.5 to 24%, in addition to significant levels of folic acid, minerals, sulphur, silicon, vitamins E, B1, B2, and D (DEMBITSKY *et al*., 2011). The fleshy pulp is a source of high quality oil with large levels of oleic and palmitic acids, and with physicochemical properties resembling those of olive oil (DUARTE *et al*., 2016). Besides, avocado oil can be considered as functional oil (TANGO *et al*., 2004), used in pharmaceutical and cosmetic industries, and for obtaining commercial oils for human consumption.

In this regard, the avocado pulp processing can contribute to the best use of the final oil (ROCHA, 2008). The most suitable varieties for oil extraction are Hass, Fuerte and Glória (TANGO *et al*., 2004). Depending on the location of the orchard, the oil content of these fruit flesh can range from 16-17% in September to 25-30% in April depending on the fruit ripening stage (REQUEJO-TAPIA, 1999).

In the oil-bearing cells, the major part of the oil is located in the vacuoles, where it is free, and the remaining part is bound or dispersed in the cytoplasm and is, therefore, not directly accessible in the extraction process and lost in the waste. The rupturing of the cell walls and of the structure of the finely-dispersed emulsion needs the extraction being performed in different ways (LEWIS *et al*., 1978). For cost reasons, most producers started to extract oil from dried fruits by means of solvents (MARTINEZ NIETO *et al*., 1988). In order to cut energy costs and minimise the air pollution caused by organic solvents, the avocado oil can be also separated from fruits by centrifugal or pressing forces, then oil cells are submitted to mechanical and/or enzymatic destruction (MARTINEZ NIETO *et al*., 1988; BIZIMANA *et al*., 1993). In addition, Moreno *et al.* (2003) have investigated the effect of different oil extraction methods on the physical and chemical properties of avocado oil. Extraction by heating the pulp up to 95°C using microwaves, followed by either Soxhlet extraction with hexane or pressing, was studied. Moreover, a method of fruit drying on the extractability of avocado oil with hexane and supercritical CO_2 has been studied (MOSTERT *et al*., 2007), as well as the extraction yield of Fortuna avocados oil as a function of the freeze-drying was evaluated (DOS SANTOS *et al*., 2013).

On the other hand, cold pressed avocado oil, greenish in colour, is relatively new oil in the commercial culinary oil field (WOOLF *et al*., 2009). It is defined as oil extracted using mechanical or physical means at temperatures below 50°C and it is extracted using methods similar to that used for extra virgin olive oil (KIRITSAKIS *et al*., 1998; WOOLF *et al*., 2009). Several studies have been conducted to find effective methods for the recovery of the oil enclosed in the cell and the need to destroy the cell walls through the use of specific enzyme to the breakdown of the individual types of polysaccharides in the cell wall structure has often been emphasized as a workable solution (HADJ-TAIEB *et al*., 2012; VIERHUIS *et al.*, 2001). The enzyme-assisted aqueous extraction has emerged as an alternative and environmentally friendly extraction process both for olive (ALIAKBARIAN *et al.*, 2008; HADJ-TAIEB *et al.*, 2012; NAJAFIAN *et al.*, 2009; VIERHUIS *et al.*, 2001) and avocado oil (FREITAS *et al*., 1993; MORENO *et al*., 2003). This process involves addition of selected enzymes into a mixture of oleaginous material with pre-determined amount of water at a given pH value, followed by incubation of the mixture at a pre-set temperature, time, and shaking speed (MAT YUSOFF *et al*., 2017). Hydrolytic enzymes, including cellulase and pectinase, are commonly used to hydrolyse and degrade cell wall constituents and improve the release of intracellular contents (HADJ-TAIEB *et al*., 2012).

The present study was focused to obtain extra virgin avocado oil in Burundian region both for local consumption and for export to foreign markets. Firstly, different time/temperature malaxation conditions, yield of oil extraction, and oil quality parameters, were investigated. Secondly, to the avocado batch getting the best oil yield, enzymatic trials were applied. Finally, a complete characterization of the avocado oil was carried out.

2. MATERIAL AND METHODS

2.1. Batches of avocado fruits used in oil extraction trials

Five avocado (*Persea Americana* Mill.) varieties (Fuerte = FU, Hass = HA, Local Rouge = LR, Local Vert = LV, and Washington = WA), were collected from a orchard located in the Murayi area (Gitega, Burundi). The varieties of Fuerte and Hass were already demonstrated to be among of the best for the oil content (GÓMEZ-LÓPEZ, 2002; OZDEMIR and TOPUZ, 2004), while the others three varieties, named Local Rouge, Local Vert, and Washington, were autochthonous of the Burundian region of Gitega.
The Table 1 reports the percentage of fruit varieties for each batch of production used in the extraction trials. Each batch has been prepared on real scale according to a fruit composition that couldn't be either standardised or replicated because of the cultivation of individual varieties of avocado trees in the context of Burundian agricultural system. This depends on the huge number of small-scale farmers, which generally practice an avocado production with limited know-how on the choice of cultivars, on inputs, and production techniques (JACQUES and JACQUES, 2012).
Since the avocado is classified as a climacteric fruit (BARMORE, 1976), avocados used for this study were collected unripe and were allowed ripening at a temperature of 24±2°C until visible changes in peel colour (from bright green to purplish) and pulp softening occurred. The degree of ripeness determined by measuring the firmness of the fruit (WONG *et al.*, 2010) to finger pressure, as like as the days required for fruits to soften (BARMORE, 1976) were the parameters used as general guide to avocado maturity. Hence to ensure the oil content in the avocados is at the maximum for processing, the fruit should ideally be mature (WONG *et al*., 2010).

2.2. Plant and process used for oil extraction trials

According to the Table 1, the nine batches of different avocado cultivar were processed according to the process depicted in Fig. 1. The plant was a semi-continuous system (Nuova M.A.I.P. Macchine Agricole Industriali Pieralisi, Jesi, Ancona, Italy) located in Murayi (Gitega, Burundi), and its technical scheme is shown in the Fig. 2. Before processing avocados were sorted and sanitized with a 100 ppm chlorine solution. After washing, the avocado fruits were manually cut in a half by sanitized knife to eliminate the kernel. The destoned avocado fruits were transported by means of a cochlea elevator (2.25 m), complete with hopper and kickstand in stainless steel, to one hammer crusher (15 HP) at low rotation speed (1.400 rpm) with double grid of 60 cm diameter along with grid holes of 6 mm. Besides to crush the destoned avocado fruit and enabling pigment extraction from skins, the hammer crusher is preferred to minimise the emulsion in order to optimise oil extraction (DI GIOVACCHINO *et al.*, 2002). These first process steps were very close to what reported in literature with the exception of leaving skins that are normally removed by fruits (COSTAGLI and BETTI, 2015; WONG *et al.* 2013).

The homogeneous paste mass with a creamy consistency was then pumped into the section equipped with malaxers (kneading machines). The kneading machine was composed of two tanks (350 + 350 kg) along with a mono-screw pump (P 50). Each kneading machine consisted of a stainless-steel tank with a central screw stirring the paste slowly and continuously at a monitored temperature. The effect of the kneading machine on the avocado paste (COSTAGLI and BETTI, 2015) was very similar to the one already described for the olive paste (DI GIOVACCHINO *et al.*, 2002): due to the coalescence phenomena (TRAPANI *et al.*, 2017) the small oil drops released during crushing of the destoned fruits merge into large drops that can be easily separated by centrifugal extraction. The separation of oil from solid and liquid phases was done using a centrifuge system, composed firstly by a three-phases centrifugal decanter (rotation speed 5.500 rpm), and then by a vertical centrifuge (rotation speed 6.900 rpm with a drum diameter of 30.5 cm). The paste inside the three-phases centrifugal decanter was separated into oil, vegetation water and solids i.e., pomace (exhausted pulp and residual skin). This device exploits the centripetal acceleration to separate continuously a mixture of particulate solids and liquids with phases having different densities (DI GIOVACCHNO *et al.*, 2002). Into the vertical centrifuge, the avocado oil was separated from vegetation water; then it was clarified through a bag filter in order to minimize the microbial contamination of the oil (GUERRINI *et al.*, 2015). Finally, the avocado oil was stored at 20°C into stainless steel tanks.

2.3. Extraction trials

Experiments applied a scalar approach and were aimed firstly to verify whether the avocado fruits cultivated in Burundian area behave similarly to olives during kneading. Since literature outcomes on cold avocado oil extraction techniques (WONG *et al.*, 2013; COSTAGLI and BETTI, 2015) stated temperature levels lower than 50°C, two main sets of experiments were performed at malaxing temperature of 30°C and of 36°C each coupled with specific malaxation time. Further, the addition of enzymes was also tested.

Table 1. Variety composition and maturity degree of the batches of avocado fruits used in the study.

Production batches	Maturity degree	Batch variety composition (%)*
A	Medium	50% WA + 25% LV + 25% LR
B	High	50% FU + 25% LR + 25% HA
C	High	50% FU + 25% LR + 25% HA
D	Medium	50% WA + 25% LV + 25% LR
E	High	50% HA + 25% LR + 25% FU
F	High	50% HA + 25% LR + 25% FU
G	Low	75% FU + 25% HA
H	Medium	50% HA + 25% LR + 25% LV
I	Low	75% HA + 25% FU

*where: FU = Fuerte; HA = Hass; LV = Local Vert; LR = Local Rouge; WA = Washington.

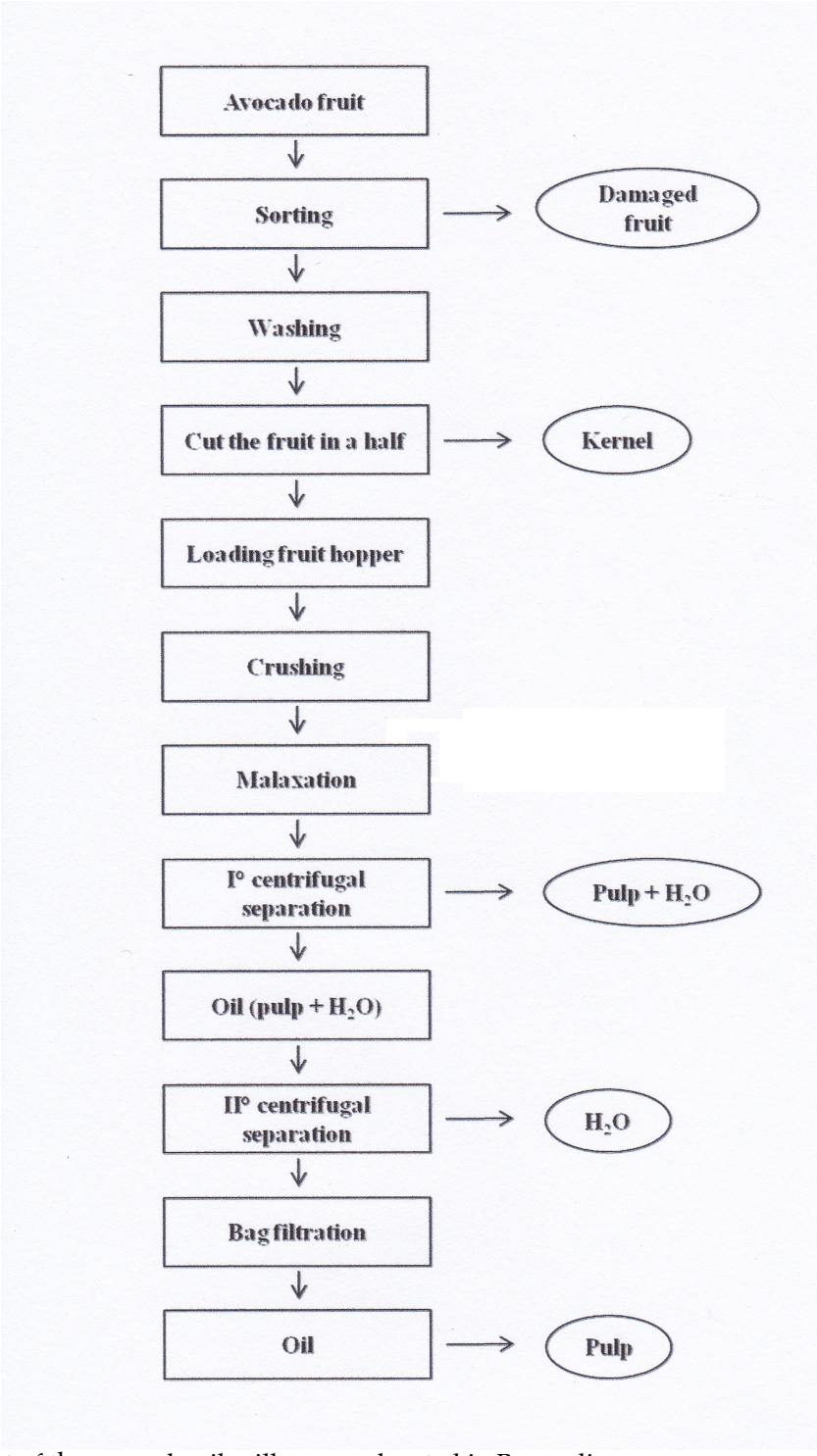

Figure 1. Flow sheet of the avocado oil mill process located in Burundi.

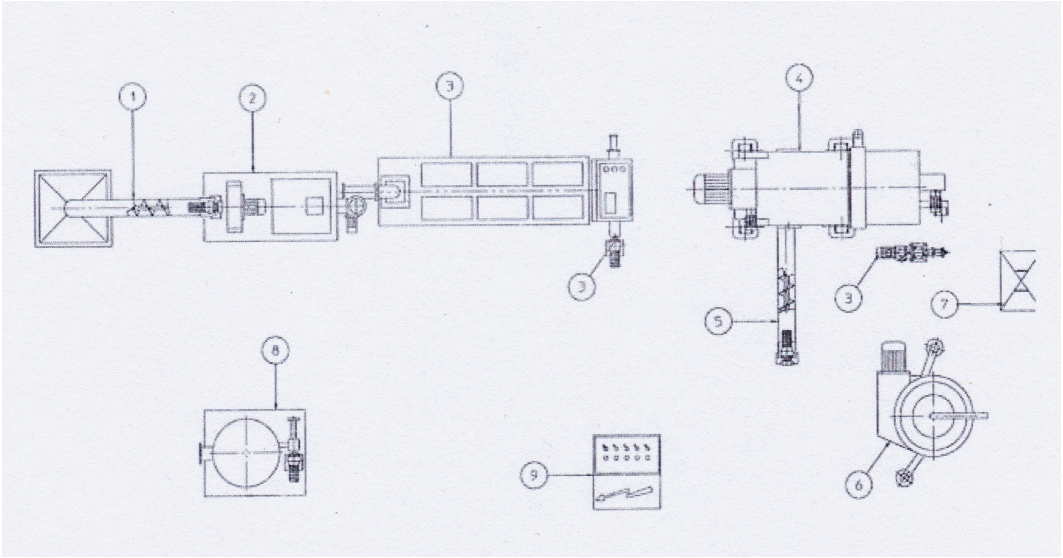

Figure 2. Technical scheme of the avocado oil mill process located in Burundi. 1. Cochlea; 2. Hammer crusher; 3. Pumps; 4. Decanter; 5. Unloading cochlea; 6. Centrifuge; 7. Decantation tank; 8. Boiler; 9. Control panel.

2.3.1 Malaxation trials

In the Table 2 the different operating conditions applied for each batch of production are shown. Noted that the crusher loading was not always constant, neither had reached the maximum level, due to the unfavourable seasonal trend which had not allowed the regular transfers/contributions of the avocado fruits in workable quantities. Moreover, as in these trials no dilution with water of the avocado paste was performed, the centrifugal decanter was used under the two-phases modality.

Table 2. Operating conditions applied to the malaxation trials.

Production batches	Pulp loading (kg)	T (°C)	t_{total} (min)
A	324	30±2	30
E	126	30±2	40
H	198	30±2	60
G	115	30±2	90
B	353	30±2	120
F	272	36±2	90
D	226	36±2	90
C	235	36±2	120
I	120	36±2	120

2.3.2 Enzymatic trials

With reference to the batch (Table 1) getting the best oil content, enzymatic extraction trials were performed by testing Maxoliva (pectinase, obtained from strains of *Aspergillus niger* and *Trichoderma longibrachiatum* belonging to the GRAS class, was supplied by DSM),

and Megazyme (α-amylase, $\geq$ 3.70 U/mg, from *Bacillus subtilis*, was supplied by Sigma-Aldrich Co., St. Louis, Mo. USA). Technical sheets outlined that: the Maxoliva enzyme is isolated specifically for the extraction of olive oil, with a balanced ratio of carbohydrate and pectinase activities, it is active between 20 and 55°C and between 3.0 and 5.0 pH. It has activity not less than 2000 units/mL. The Megazyme used in this study was an α-amylase enzyme that catalyses the hydrolysis of internal α-1.4-glucan links in polysaccharides containing 3 or more α-1.4-linked D-glucose units, yielding a mixture of maltose and glucose (TAKESHITA and HEHRE, 1975). It is active between 35 and 40°C, between pH 3.0 and 5.5, and has activity of 2000 units/mL.
The avocado paste obtained after crushing (Fig. 1) was diluted at a ratio 1:0.5 with purified water before being subjected to malaxation phase, as a consequence the centrifugal decanter was used in these trials under the three-phases modality. The temperature and time of the enzymatic hydrolysis were suggested by the previous malaxation trials (Table 2), by the condition of use of the enzymes, and considering the working pH (about 5.0). The enzymes were added at 1% w/w; the dosage was referred to each enzyme either when added individually, or in a mixture (Table 3). One unit of activity is defined as the amount of enzyme preparation that liberates 1 μmoL of reducing sugars per minute from the galacturonic acid of olive pectins (RANALLI *et al.*, 2003).

Table 3. Operating conditions applied to G batch for the enzymatic trials.

Trial	Pulp loading (kg)	T (°C)	t_{total} (min)	Enzyme
G1	191.5	30±2	90	Maxoliva
G2	190.5	30±2	120	Maxoliva
G3	230.0	30±2	90	Megazyme
G4	200.0	30±2	120	Megazyme
G5	215.0	30±2	90	Maxoliva + Megazyme
G6	393.0	30±2	120	Maxoliva + Megazyme

2.4. Oil extraction yield

For the oil content determination, the protocol was carried out following the procedure described by the EC Regulation (EEC 2568/91). Oil content in avocados was extracted from avocado paste with hexane using a Soxhlet apparatus. A cellulose thimble containing 5 g dried sample was placed in the Soxhlet device and extracted with 250 mL hexane for 6 h. The flask was removed and solvent evaporated using a rotary evaporator (Büchi Rotavapor R-3, 1000184809, Büchi Labortechnik AG, Switzerland). The oil in fruit pulp is calculated as the grams of the oil contained in 100 g of fresh fruit pulp.
The extraction yield was expressed as Business Yield (By) and as Process Yield (Py) in order to compare the different extraction steps and conditions. The By was obtained by the ratio between the weight of the oil extracted at the end of the process and the weight of the fruit pulp subjected to extraction.

$$Business\ yield\ (wt.\%) = \frac{\text{W oil extracted}}{\text{W fruit pulp}} * 100$$

The Py was calculated as the weight of oil extracted at the end of the process and the weight of the oil in the fruit pulp.

$$Process\ yield\ (wt.\%) = \frac{\text{W oil extracted}}{\text{W oil in fruit pulp}} * 100$$

2.5. Oil analysis

Filtered avocado oil was characterized for acidity value (% oleic acid/100 g avocado oil), peroxide value (mEq O_2/kg oil) and UV determinations according to the European Commission (EEC 2568/91) standard methods. Acidity value indicated the free fatty acids present in fats and oils. High degree acidity value can be related with the degree of triglyceride hydrolysis during preparation or storage. Free fatty acids are then oxidised to hydroperoxide that are measured by the Peroxide Value (PV). During the early stages of oxidation, the increase in UV absorption due to the formation of Conjugated Dienes (CDs) and Conjugated Trienes (CTs) is proportional to the uptake of oxygen and to the production of peroxides. Therefore, the content of CDs and CTs obtained measuring the oil absorbance at 232 and 270 nm (K232 and K270) also can serve as a relative measurement of oxidation. Spectrophotometric determinations were obtained using a Shimadzu UV-1601 spectrophotometer (Shimadzu Europe, Duisburg, Germany).

2.6. Determination of the phenolic fraction

Aliquots of oil (5 g) were added to 10 ml of a methanol/ water solution (80:20, v/v) in a 50-mL centrifuge tube, according to MONTEDORO *et al*. (1992). The mixture was blended (Ultraturrax, IKA, Staufen, Germany) for 5 min and then centrifuge for 5 min at 2500 *g*. The hydro-alcoholic extract was collected, and the oil phase was re-extracted with 2 x 10 mL methanol/ water solution. Finally, the hydroalcoholic fractions were combined and washed with n-hexane to remove the residual oil and then dried under vacuum at 30°C. The dry extracts were re-suspended in 1 mL methanol and the solutions were filtered through 0.2 μm regenerated cellulose filters. The absorbance of the filtered solutions was recorded at 765 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu Europe, Duisburg, Germany). The results were reported as gallic acid equivalents (mg/kg oil) based on the calibration curve (r^2 = 0.999). Folin-Ciocalteu reagent and gallic acid were obtained from Merck & Co. Inc. (Darmstadt, Germany).

2.7. Fatty acid and sterol composition

The fatty acid composition of the fatty acid methyl esters (FAME) was determined using a Shimadzu 2025 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an auto-sampler (model AOC-20s, Shimadzu), an auto-injector (model AOC-20i, Shimadzu), a flame ionization detector, and a CP-Select CB capillary column for FAME (100 m x 0.25 mm i.d.; 0.25 μm film thickness; Chrompack, Varian, Inc., CA). The injection volume was 1 μL in split mode (split ratio 30:1) and the carrier gas was hydrogen with a constant flow of 1.5 mL/min. The injector and detector temperatures were kept at 250°C. The column oven temperature was programmed following the procedure of Prandini *et al*. (2007) with minor modification: 60°C for 2 min, from 60 to 170°C at 10°C/min for 35 min, and from 170 to 240°C at 4°C/min for 9.5 min. Peak identification was possible with the aid of reference standards (Supelco 37 component FAME mix; conjugated octadecadienoic acid; Sigma Chemical Co, St. Louis, MO). Data were expressed as a percentage of total fatty acids, calculated with peak areas corrected by factors according to AOAC 963.22

method (2000). The content of β-sitosterol was determined according to EC Regulation (EEC 2568/91).

2.8. Sensory analysis

The sensory analysis was performed on the avocado oil samples from malaxation trials without enzyme addition by a panel trained according to the International Olive Council (IOC) requirements. Indeed, some researches have already been established both the resemblance of avocado oil to olive oil (SALGADO *et al*., 2008) and the recommended standards for avocado oil tasting used to ensure its quality in terms of sensory properties (KOCHHAR and HENRY, 2009; WONG *et al.*, 2010; WOOLF *et al.*, 2009). The panel test was established using a standard profile sheet IOC method (EN ISO/IEC 17025/2005) even leaving free choice to panelists for new descriptors. Each taster analysed all samples during three different sessions. The values of the median sensory data were calculated, and the test supervisor chose a significance level of 5%.

2.9. Statistical analysis

All analytical measurements were carried out in triplicate and the results were expressed as the mean value±standard deviation of three determinations. Comparisons of mean values were performed using one-way ANOVA with a Duncan post-hoc test and p-values of < 0.05 were considered significant. The IBM SPSS Statistics21 package (IBM Corporation, New York, USA) was used.

3. RESULTS

3.1. Malaxation trials

Under the conditions reported in the Table 2, the Py from malaxation trials ranged between 48 and 95% (Table 4). The highest By and Py were achieved in longer time (up to 90 min), both at 30°C and at 36°C, with some differences. Considering the By, malaxing for 90 min at 30°C allowed to gain the 9% w/w of oil, whilst time-temperature of 120 min-36°C and 120 min-30°C achieved lower values. On the other hand, malaxing for 120 min at 30°C lead to highest Py of 95% w/w.

As for the quality parameters (Table 4) of the avocado oil obtained under malaxation trials, the acidity levels were low and ranged between 0.32 and 1.02% of oleic acid. Oil from batch F, malaxed at 30°C for 90 min, showed the lowest acidity value. The PVs were also very low: from 2.50 to 1.48 mEq O_2/kg, when all the batches are considered. The values for the CDs and CTs ranged as 1.43÷2.04 and 0.09÷0.27 absorbance units for K232 and K270, respectively. For PVs, CDs and CTs the lowest values were registered for batches A and C. The results from sensory analysis performed on avocado oils obtained in the present study are reported in Table 4: the median of defects was > 0 only for the oils C, D, and E, while the median of the positive attributes was always > 0 for the remaining trials (A, B, F, G, H, and I). The positive attributes were identified as fruity, bitter, leaves, and almonds.

Table 4. Oil extraction yields and quality parameters of avocado oil obtained in the malaxation trials.

Production batches	Fruit oil (% w/w)	Business yield (% w/w)	Process yield (% w/w)	Acidity value (% oleic acid)	Peroxide value (mEq O_2/kg)	K232	K270	Panel test		Commercial class
								Negative attributes	Positive attributes	
	Limit value* for EVO			≤ 0.80	≤ 20	≤ 2.50	≤ 0.22	0	> 0	
A	6.30±0.32 c	3.00	48.00	0.66±0.03 c	1.48±0.07 c	1,43±0.09 e	0,19±0.01 b	0	3±0.8	EVO**
E	8.30±0.42 b	5.00	61.00	1.22±0.06 a	2.49±0.12 a	1,52±0.09 d	0,12±0.01 c	3±0.2	3±0.5	VO***
H	6.90±0.35 c	5.10	74.00	0.42±0.02 d	2.50±0.13 a	1,58±0.09 d	0,12±0.01 c	0	4±0.2	EVO
G	10.20±0.51 a	9.00	88.00	0.49±0.03 d	1.94±0.10 b	1,69±0.10 cd	0,17±0.02 b	0	2±0.7	EVO
B	8.70±0.44 b	8.20	95.00	0.39±0.05 d	1.99±0.11 b	1,80±0.11 b	0,12±0.02 ab	0	5±0.2	EVO
F	8.30±0.42 b	5.00	61.00	0.32±0.02 e	1.94±0.10 b	1,68±0.10 c	0,09±0.01 d	0	4±0.3	EVO
D	6.30±0.32 c	4.60	73.00	0.83±0.04 b	2.36±0.12 a	1,86±0.11 b	0,13±0.01 c	2±0.6	2±0.9	VO
C	8.70±0.44 b	7.80	90.00	1.15±0.07 a	1.50±0.08 c	1,69±0.10 c	0,09±0.01 d	1±0.5	1±0.2	VO
I	9.30±0.47 ab	8.40	90.00	0.44±0.03 d	2.00±0.10 ab	2,04±0.10 a	0,21±0.02 a	0	3±0.3	EVO

* Reg. CEE 1531/2001. Data in column with different letters mean significantly different values according to post-hoc Duncan test at $p<0.05$. **EVO, extra virgin oil; ***VO, virgin oil.

3.2. Enzymatic trials

In the Table 5, the results of the avocado oil extraction with enzymes (Table 3) were reported. Under the experimental conditions, the single enzyme Megazyme (G3 and G4) turned out to be the more efficient both at 90 min and 120 min than Maxoliva (G1 and G2). However, the joint of the two enzymes proved to be the best solution to increase the avocado oil yield (G5 and G6), both for the By and Py. Exactly, 4.7 and 9.7% w/w By, and 46 and 88% w/w Py were achieved at 90 and 120 min malaxation time, respectively.
Considering the avocado oil quality parameters, the acidity values ranged between 0.22 and 0.79%, with a mean value of 0.41±0.21% whilst the peroxides reached values between 3.4 and 4.4 of mEq O_2/kg. The major levels both of acidity and PVs were observed in G5 and G6 where the enzyme combination was employed.

Table 5. Oil extraction yields and quality parameters obtained in the malaxation trial G with enzymes addition.

Production batches	Business yield (% w/w)	Process yield (% w/w)	Acidity value (% oleic acid)	Peroxide value (mEq O_2/kg)
G1	2.60	26.00	0.31±0.02 cd	4.40±0.27 a
G2	3.20	31.00	0.50±0.04 b	3.70±0.23 b
G3	3.00	29.00	0.22±0.01 e	3.40±0.19 b
G4	7.00	68.00	0.29±0.02 d	3.40±0.24 b
G5	4.70	46.00	0.38±0.03 c	4.10±0.26 a
G6	9.70	88.00	0.79±0.05 a	4.40±0.29 a

Data in column with different letters mean significantly different values according to post-hoc Duncan test at $p<0.05$.

3.3. Chemical profile of the final avocado oil

The Table 6 showed the most significant parameters of the final avocado oil obtained through the malaxation under the best conditions identified in this study.

Table 6. Characterization of the EV avocado oil obtained from the malaxation experiment G.

Parameters	Value
Acidity value (% of oleic acid)	0.74±0.06
Peroxide value (mEq O_2/kg)	2.02±0.16
K232	1.70±0.14
K270	0.16±0.01
Total phenols (mg/L)	40.00±3.20
Oleic acid C18:1 (% of total fatty acids)	63.30±5.06
Linoleic acid C18:2 (% of total fatty acids)	10.50±0.84
Palmitic acid C16:0 (% of total fatty acids)	18.00±1.44
Palmitoleic acid C16:1 (% of total fatty acids)	5.30±0.42
β-sitosterol (% of total sterols)	76.40±6.11
Negative attributes (panel test)	0
Positive attributes (panel test)	4±0.90

The quality parameters (acidity value, peroxide value and K232 and K270) at very low levels (EEC 2568/91), together with the absence of negative sensory attributes, bearing out the quality grade of the avocado oil obtained under this study. Considering the lipid components, oleic acid (C18:1) was the major fatty acid oil, followed by palmitic (C16:0), palmitoleic (C16:1), and linoleic (C18:2) acids (Table 6). Finally, the percentage of β-sitosterol exceeded the value of 76% of the total sterols and 40 mg/L of the total phenols were detected.

4. DISCUSSION

A number of reports have indicated that the oil content in avocado fruits and the oil composition vary according to the location of the orchard, the variety, the number of days between flowering and harvest, the dry matter contents, and even to the part of the fruit measured (REQUEJO-TAPIA, 1999; OZDEMIR and TOPUZ, 2004). Considering all the varieties taken into consideration (Table 1), the average oil content was 8.11±1.35% w/w of avocado fruit (Table 4). The G batch registered an oil content greater than 10% w/w, followed by I (9.3% w/w), B and C, both with 8.7% w/w. As expected, FU and HA avocado varieties allowed achieving the highest oil content, confirming the study of Yanty *et al*. (2011).

4.1. Malaxation trials

In the EVOO production it should be desirable to strike a balance between oil yield and oil quality characteristics, but this requires studies to check whether a time-temperature could be applied in order to predict the potential effect of malaxation on extraction yield (TRAPANI *et al.*, 2017). Under the conditions applied this study, the malaxation (Table 2) was performed at a temperature either of 30 or 36±2°C, values that are close to the environmental temperature in Burundi and which were selected in order to reduce the energy consumption and processing variable costs. The optimal malaxing time and temperature conditions to reach the best compromise between quality and quantity of extracted avocado oil have been investigated, and the results have been reported in the Table 4. Data showed that at both 30 and 36°C longer times for malaxing improved the avocado oil yield. Malaxing at 30°C by increasing time from 30 to 40, 60, 90, till 120 min (respectively for batches A, E, H, G, and B), allowed at achieving an increase in Py by several percentage points. The same was observed for malaxing at 36°C, where the Py was maximized at a time of 120 min (batch I). According to literature data, an increase in time and temperature during malaxation causes a positive influence on olive oil extraction yield (TRAPANI *et al.*, 2017), albeit the state of advancement in the Italian olive oil production pointed out that the optimal setting of the malaxation parameters should be targeted for each individual cultivar (SELVAGGINI *et al.*, 2014).
Our results (Table 4) corroborated the fact that between heat and time, the period for kneading and mixing the avocado paste into the malaxer overcomes the only thermal energy needed to activate natural degrading enzymes, diffusion, and coalescence phenomena. Since the avocado oil comes in a finely dispersed emulsion inside the cells of the fruit pulp, the extraction process requires rupturing not only the cell walls, but also the structure of the emulsion (LEWIS *et al*., 1978) in order to favour the coalescence phenomena (TRAPANI *et al.*, 2017). The emulsions are surrounded by the lipoproteic membranes or the lipophilic solids of the paste, which can absorb part of the oil itself (COSTAGLI and BETTI, 2015) and thus the malaxing time is generally longer and the temperature is higher for avocados than olives (ANGEROSA *et al*., 2001). Indeed,

experimental trials (data not reported) highlighted that malaxation with times and temperatures lower than 20 min and 30°C, respectively, did not allow the oil separation in the malaxer and, consequently, in the centrifugal decanter. This was supported by WONG *et al*. (2011) who obtained the avocado oil by malaxing the mixture for 60 min at 45-48°C. On the other hand, the experience of COSTAGLI and BETTI (2015) showed that avocado mash malaxing time should not exceed 90 min with temperature below than 50°C.
Contrary to what expected when either temperature or time are increased, the chemical and sensory parameters of the avocado oil extracted under these malaxing conditions were maintained amply below the legal requirements (EEC 2568/91) to classify the products in the "virgin" category. Extra virgin oil (EVO), as well as virgin oil (VO), is a food product for which not only chemical parameters but also sensory characteristics must comply with values established by the EU regulation (EEC 2568/91; EN ISO/IEC 17025/2005). According to what stated for classification of olive oils in EVO and VO categories, the results of chemical and sensorial indices allowed classifying most of the avocado oils (A, B, F, G, H, and I) obtained from malaxation without enzyme addition in the EVO category, whilst the oils from batches C, D, and E did not result to comply with the quality level requested for EVOs by the current legislation because their median of defect was higher than 0. Considering both the yield and the oil quality, batch G composed of the varieties richer in oil (Table 4) and more widespread in the agronomic supply chain i.e., Fuerte and Hass (TANGO *et al*., 2004), and with a low degree of fruit maturity (Table 1) was deemed to be suitable to be tested with enzymatic preparations (Table 3).

4.2. Enzymatic trials

Advances in enzyme biotechnology applications have led to economically viable processes and improved extraction yield especially for oil pastes with more tenacious emulsions as avocado gets (BUENROSTRO and LOPEZ-MUNGUIA, 1986; COSTAGLI and BETTI, 2015). Many papers have been published on the effects of enzymes on the extraction and characteristics of olive oil (ALIAKBARIAN *et al.*, 2008; HADJ-TAIEB *et al.*, 2012; NAJAFIAN *et al.*, 2009; VIERHUIS *et al.*, 2001), while fewer are the reports on the assisted-enzymatic extraction of avocado oil (Freitas *et al*., 1993; MORENO *et al*., 2003; WONG *et al.*, 2013). In the present study, the enzyme addition was applied under the optimal operating conditions (either for 90 and 120 min at 30°C) for malaxing the G batch that got extra virgin oil with a high yield (Table 4). In such case, due to the batch composition made of Hass and Fuerte varieties (Table 1) greater oil content in the avocado paste was shown (Table 4). Thus considered, together with the harder texture of the fruits because of their unripen state (Table 1), corroborated the idea to evaluate the effect of enzyme addition both on oil extraction and quality. Many researchers confirmed that the enzyme addition hydrolyzes and breaks the cotyledon cell walls (MAT YUSOFF *et al*., 2017), degrading the walls of the oil-bearing cells, making the structure more permeable and further expose the oil component (ALIAKBARIAN *et al.*, 2008; HADJ-TAIEB *et al.*, 2012; NAJAFIAN *et al.*, 2009; VIERHUIS *et al.*, 2001). The most effective enzymes used in oil extraction technology are, cellulases, xylanases, and proteases, or enzyme mixture consisting mainly of pectinases, cellulases, hemicellulases (HADJ-TAIEB *et al.*, 2012; NAJAFIAN *et al.*, 2009).
The pectolitic Maxoliva enzyme used in this study (Table 3), which is commonly applied for the olive oil extraction at the industrial level (ALIAKBARIAN *et al.*, 2008; RANALLI *et al.*, 2003; NAJAFIAN *et al.*, 2009), provided a lower yield (Table 5) than the amylolytic Megazyme enzyme (Trials G1 vs G3, and G2 vs G4). The result was in line with other studies, which added α-amilase enzymes or a mixture of α-amilase and protease during mechanical extraction of avocado paste, getting a positive effect of the treatment

(COSTAGLI and BETTI, 2015). BUENROSTRO and LOPEZ-MUNGUIA (1986) obtained better extraction yields of avocado by using α-amylase alone which resulted in an extraction of 75% of the original oil content compared to 65% with the triple enzymatic mixture of polygalacturonase, α-amylase, and protease.
Data from this study (Table 5) obtained the higher Py when the enzymes were simultaneously added (G5 and G6), in accordance with Freitas *et al*. (1993) who improved the avocado oil yield by using mixtures of commercial preparations. With few exceptions (NAJAFIAN *et al*., 2009), the enzyme mixtures with combined activity give better results than individual enzymes to improve the rate of extracted oil (ALIAKBARIAN *et al*., 2008). This positive effect was obtained also without affecting the final oil quality (Table 5) as already demonstrated by Buenrostro and Lopez-Munguia (1986). Despite a negligible increase in the PVs (Table 5) if compared with the only malaxation without enzymes (Table 4), the use of the enzymatic preparation applied in this study allowed achieving acidity values for obtaining the extra virgin denomination (Table 5). By comparing G sample from malaxation trials (Table 4) to G samples from enzymatic trials (Table 5), it was surprising to observe generally lower extraction yields with enzymatic addition than without its use. This result might be rebutted from literature where the increasing in the oil extraction yield from malaxation with enzyme addition has been widely demonstrated (BUENROSTRO and LOPEZ-MUNGUIA, 1986; COSTAGLI and BETTI, 2015; FREITAS *et al*., 1996; MORENO *et al*., 2003; WONG *et al*., 2013). Even considering variability among fruit maturity and oil content in the within of batches of the same varietal composition, the lesser yield measured in oil G when aqueous enzymes were added (Table 5 *vs* Table 4) might depend on the altered kinetics of coalescence occurring during malaxation as negatively affected by paste dilution. Under the slight dilution conditions applied in this study, avocado paste could require higher temperature to reduce its pulp-water viscosity and an increase in oil yield (FREITAS *et al*., 1996; WONG *et al*., 2013). Further, during malaxation it is common to observe that part of the oil begins to physically separate and rise towards the surface of the olive paste. Since the speed of the oil's movement towards the surface of the paste depends on the oil viscosity, TRAPANI *et al*. (2017) led to the consideration that the condition to increase the oil process yield does not only include oil droplet coalescence but also the separation and rising to the surface of the oil. Such a phenomenon could be slowed in our enzymatic trials due to the increased shear stress from water dilution combined with an inadequate heat content of the avocado paste. Further, the slight dilution ratio 1:0.5 of the paste to purified water during malaxation could affect also the separation of the phases at centrifugal decanter with a decreased oil yield.

4.3. Chemical and sensory profile of the final avocado oil

Considering all the trials of avocado oil extraction performed on real scale in the Murayi oil mill, with and without the use of the enzymes; comparing yield, quality, process costs, and operation management, the most suitable extraction technique also taking into account the oil mill location could be considered as the malaxation extraction without the addition of the enzymes. In this regard, the final EV avocado oil obtained with the batch G was completely characterized (Table 6).
Even according to Moreno *et al*. (2003), who reported acidity value between 0.65 and 1.23 mg/KOH/g, the avocado oil achieved (Table 6) could be classified as an extra virgin (EEC 2568/91). Peroxide values were very far from those reported in the range of 5.1-12.3 mEq/kg (QUIÑONES-ISLAS *et al*., 2013). This is particularly interesting if data are compared to the study of INDRIYANI *et al*. (2016), where the peroxide values of the Indonesian avocado cultivars ranged from 14.9 to 166.1 mEq/kg oil i.e., at a clearly

oxidation state (Salgado *et al*., 2008). The contents of CDs and CTs, expressed with the specific absorptivity values reported in Table 6, were significantly low compared to the values of Indonesian avocado oil varied from 2.6-3.7 (INDRIYANI *et al*., 2016).
As for fatty acid profile (Table 6), avocado oil is characterized by having high levels of monounsaturated fatty acids (oleic and palmitoleic acids), low polyunsaturated fatty acids (linoleic acid), and relatively high levels of saturated fatty acid (palmitic and stearic acids). Likewise, ROCHA (2008) has reported that avocado oil from the varieties Wagner, Fortuna, Hass and Fuerte had higher levels of monounsaturated fatty acid ranging from 59 to 72% of total fatty acids, followed by saturated fatty acids, from 17 to 23%, and polyunsaturated fatty acids to a lesser extent with levels ranging between 10 and 14%. Regarding the avocado oils from the varieties Northrop, Duke, Wagner, Quintal, and Fuerte, they are characterized by having more than 63% oleic acid, while the oils from the varieties Rincon, Barker, Waldin, Prince and Panchoy showed less than 50% of this fatty acid. Palmitic acid content ranged between 15.38 and 32.37% in oils from different varieties. Therefore, the avocado variety affects the levels of palmitic acid and oleic acid, once varieties with high oleic acid levels had low palmitic acid levels and vice versa (DUARTE *et al*., 2016). The fatty acid composition is influenced by the cultivars, maturity stage, anatomical region of the fruit, and geographic location for plant growth (TANGO *et al*., 2004). According to this, the fatty acid composition of avocado oil from this study (Table 6) was in line with the literature (KOCHHAR and HENRY, 2009), achieving a higher percentage of oleic acid than values reported by YANTY *et al*. (2011) for the local Malaysian cultivars (43.65-51.22%) and by RAMIREZ-ANAYA *et al.* (2018) for Hass cv. malaxed at higher temperature than in our study.
Avocado oil contains substantial amounts of bioactive compounds such as phytosterols, especially in the lipid fraction, and the main representative is the β-sitosterol (DOS SANTOS *et al*., 2014). As shown in the Table 6, the avocado oil achieved more than 76% of β-sitosterol, higher than the Margarida avocado oil variety, in which β-sitosterol represents 71.8% of the total sterols (DOS SANTOS *et al*., 2014), but lower compared to Fortuna avocado (87.6%) of the MOIGRADEAN *et al*.'s (2012) study. With regard to polyphenols, avocado oil showed a lower proportion (Table 6) than most of olive oils (ALIAKBARIAN *et al.,* 2008; VIERHUIS *et al*., 2001). FORERO-DORIA *et al.* (2017) reported avocado oil with a phenolic concentration of 99.8±15 mg GAE/L of oil with similar antioxidant capacity as olive oils. The low values measured in the sample G (Table 6) could be due to the no addition of enzymes (VIERHUIS *et al.,* 2001) together with long malaxation time (ALIAKBARIAN *et al.,* 2008; STEFANOUDAKI *et al.,* 2011), while the temperature applied (Table 2) should have been improved the phenolic content (SELVAGGINI *et al.,* 2014). As for sensory, the avocado oil as characterized in Table 6 showed a greenish yellow color which corroborated the use to common Burundian use to process fruits with skins (WONG *et al.,* 2011) with a pleasant distinctive flavor of avocado as like as already reported for cold pressed avocado oil recovered by mechanical extraction at temperature less than 50°C with or without the use of enzymes (KOCHHAR and HENRY, 2009; WONG *et al.,* 2009; WOOLF *et al.,* 2010).

5. CONCLUSIONS

The extraction process realized in the Murayi oil mill allowed achieving high quality grade of avocado oil mechanically extracted by means of malaxation of the avocado pulp at 30°C for 90 or 120 min. On the other hand, the study confirmed that the addition of enzymes to avocado paste during malaxation requires training and deepened knowledge on the shear-stress occurring in the paste especially when a low water dilution is applied.

Further, the use of three-phases centrifugal decanter should be optimized for improving EV avocado oil extraction yields.

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PAPER

EFFECTS OF INNOVATIVE GLUTEN-FREE COATINGS ON QUALITY, SENSORY AND MICROBIAL PROPERTIES OF CHICKEN NUGGETS

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ABSTRACT

In parallel to the increased incidences of celiac diseases, the demand to produce a significant array of gluten-free products is rapidly growing. A total of 28 dry and liquid coatings of chicken nuggets were formulated using seven blend ratios of rice-to-corn flours (0:100, 100:0, 50:50, 60:40, 70:30, 40:60 and 30:70), two levels of corn starch (5 and 15%), 0.3% methyl cellulose (MC), and/or 0.5% carboxy methyl cellulose (CMC), and other ingredients such as 2% xanthan gum, 2% sucrose, 2.5% salt and 1% spice. Based on visual evaluations 16 groups were eliminated at first stage. After sensory analyses, six groups out of remaining 12 were selected and gluten analyses were performed. Although all had the gluten level below the legal limit of 20 mg/kg, three formulations (30:70, 40:60 and 60:40) with the lowest amounts of gluten out of six were selected, and the rest of the experiments were continued with these final three formulas. The CMC-containing formulations had higher sensory scores than the MC-containing ones. The addition of more than 40% rice flour adversely affected sensory and textural properties. The use of 15% cornstarch, instead of 5%, enhanced the textural parameters.

Keywords: celiac disease, poultry products, gluten-free batter; functional foods

1. INTRODUCTION

Celiac disease – a genetically based autoimmune condition – is characterized by human intolerance to gluten, a component of wheat, barley, and rye (LOEWE, 1993). The incidence of celiac disease has increased dramatically in recent years, with the projection that over 1% of Americans and 0.3% of Europeans are estimated to suffer from it (FASANA *et al*., 2003). The global incidences of food allergies were estimated at 8% for children and 2% for adults (JACKSON *et al*., 2006). Globally, there is a very narrow range of commercially-available gluten-free products such as biscuit, cakes, breads, and confectionary products, with a rapidly growing need for gluten-free poultry products.
Chicken nuggets are commercially produced using a coating of wheat flour, deep-frying, and quick-freezing (OWENS, 2001). Since wheat flour is rich in gluten (over 60%) (GALLAGHER *et al*., 2004), different replacements for wheat flour are being sought actively for chicken nuggets. One of such replacements is rice flour, that has been utilized in batters for frying owing to its high availability and nutritious and low-calorie nature (SHIH and DAIGLE 1999, 2006 MUKPRASIRT *et al*., 2000). The use of amaranth flour in batter, chicken paste, and chicken nuggets was also explored using different blends (TAMSEN *et al*., 2018). TASBAS *et al*. (2016) studied the effects of gluten-free chicken nuggets formulated using cellulose, egg powder, whey powder, pectin, and gluten-free wheat flour on physicochemical characteristics such as color, texture, and shelf-life.
Given the limited number of studies in available in literature, there is an urgent need to develop alternatives to wheat flour in the coating of chicken nuggets as well as to test them in terms of food quality, safety and stability. The objectives of this study were to (1) develop gluten-free coatings for chicken nuggets using corn and rice flours at seven blend levels, and (2) test their quality, safety and sensory properties.

2. MATERIALS AND METHODS

2.1. Preparation of ingredients

To be used in gluten-free coating formulations, chicken steaks, and skins of chicken breasts and legs were obtained from a local poultry firm (Erpiliç, Bolu, Turkey). AF V2 gluten-free binding agent (a mixture of green bean, bamboo, potato, and rice fiber), gluten-free spice mix tasty AG (a mixture of onion, garlic, and cumin powders), liquid casing used to cover nuggets (rice flour, corn flour, corn starch, methyl cellulose (MC), carboxy methyl cellulose (CMC), xhanthan gum, sucrose, and salt), and powder forms of sauces (rice flour, corn flour, yeast, sugar, salt, sunflower oil, emulsifier, and coloring agent) were acquired from DPS (Dutch Protein and Services Ingredients for the Food Industry, Holland).

2.2. Preparation of chicken nuggets

Chicken steaks were stored for 6 h at 1 to 4°C after slaughtering. Meat samples were grinded with a grinding machine (Hachoir 1500 watt) using a 5-mm blade to obtain minced meat. The meat temperature was kept under 4°C during the grinding process. Similarly, chicken skins were also grinded with the same machine under the same conditions but with a 1.3-mm blade. Minced meat samples were mixed with spice, binding agent, and water, kneaded to yield a typical nugget texture, shaped using a nugget-specific mold and rested for 6 h at a refrigeration temperature. The ingredients and preparation of chicken nuggets for the experiments are presented in Table 1 and Fig. 1.

Table 1. Ingredients of gluten-free chicken nuggets.

Ingredients	Percentage (%)
Chicken steak	74.5
Chicken skin	6.2
Water	12.4
Spice mix	1.6
Binding	5.3

Figure 1. (a) Chicken breast steak, (b) minced chicken breast, (c) chicken skin, (d) mixture of minced meat and skin, (e) minced meat, skin, and spice mix, (f) mixture of minced meat, skin, binging agent, and spice, (g) gluten-free nugget blend, (h) shaping, and (i) gluten-free nuggets before coating.

2.3. Formulations of liquid and dry coatings

Dry and liquid sauce formulations were based on the following seven blend ratios of rice to corn flours in percentages: 100% rice flour, 100% corn flour, 40:60, 30:70, 50:50, 60:40, and 70:30. Two levels of corn starch (5 and 15%), 0.3% MC, and/or 0.5% CMC, 2% xanthan gum, 2% sucrose, 2.5% salt, and 1% spice were also mixed in. All the mixture ingredients were in the powder form and used to yield a total of 28 liquid coating formulations whose codes are provided in Fig. 2

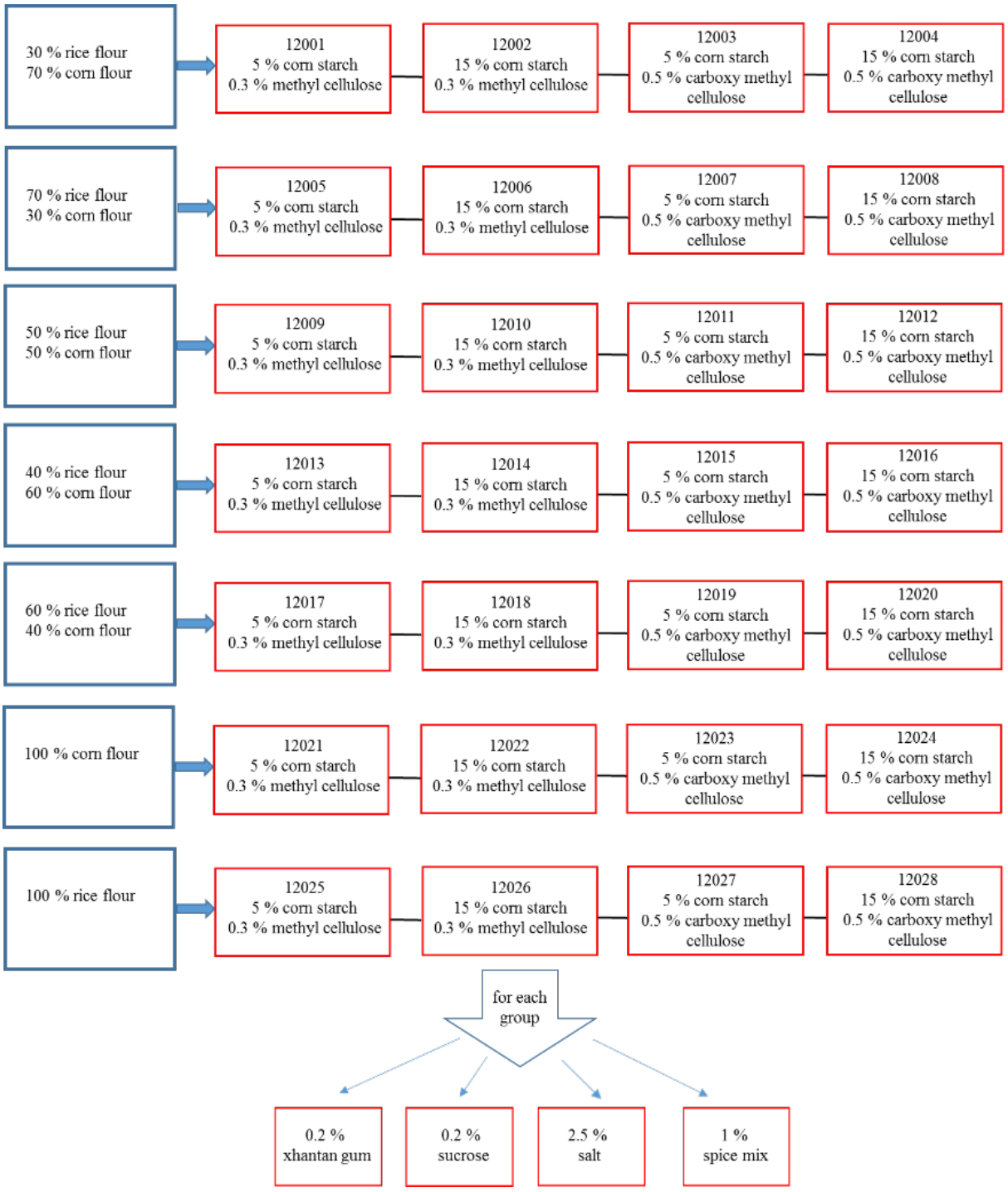

Figure 2. Formulations and codes of coatings used in this study.

Figure 3. Coating of gluten-free chicken nuggets with (a) batter, (b) batter preparation and mixing, (c) manual coating with batter, (d) manual coating with breader, and (e) coated gluten-free nuggets.

Once coated with the 28 liquid coating formulations (Fig. 3), the nuggets were manually coated with a dry sauce (Table 2) made up of the rice-to-corn flour blend of 50:50. After each formula was prepared and shaped, the liquid coating (batter) at the liquid coating-to-water ratio of 1:2, and then the dry coating (breader) were applied (Fig. 3).

Table 2. Formulations of gluten-free dry coatings.

Ingredients	Dry coating (%)
Rice flour	40
Corn flour	40
Yeast	3-5
Sugar	5-10
Salt	2-5
Sunflower oil	2-5
Emulsifier	< 2
Coloring agent	< 2

2.4. Frying, cooking, freezing and packaging of coated gluten-free chicken nuggets

Once coated, gluten-free chicken nuggets were fried (Bosch TFB3201 model fryer, Germany) at 180°C for 30 s using sunflower oil. Then, they were cooked in a steamed oven (CPS Cook Star, Holland) under the processing conditions of 4 min conveyor speed, 70% steam, 150°C oven temperature in order to reach 74°C at their coldest point. They were frozen inside a spiral freezer (CPS Tempo Frost, Holland) at -40°C and then packaged with 10 nuggets per package using Styrofoam plates and stretch films (Fig. 4).

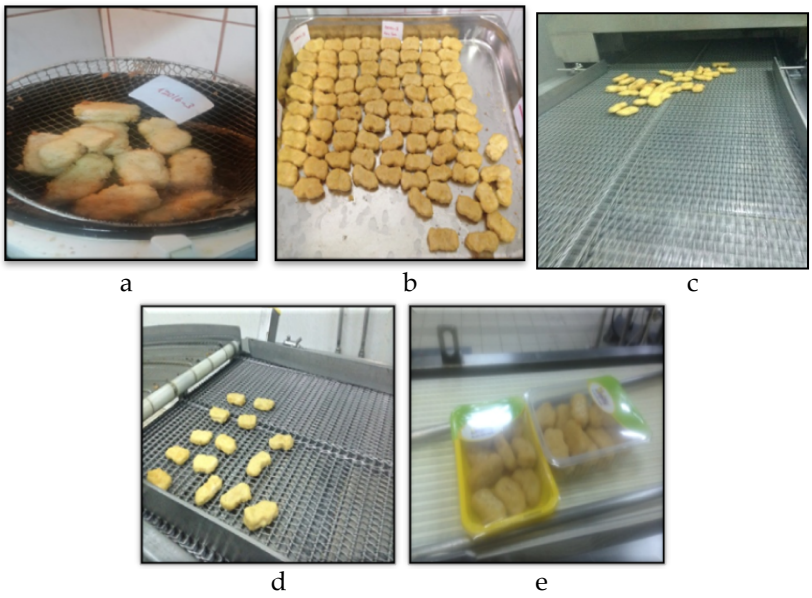

Figure 4. (a) Frying of gluten-free nuggets, (b) fried gluten-free nuggets, (c) feeding gluten-free nuggets to a steam oven, (d) feeding gluten free nuggets to a spiral freezer, and (e) packaging.

2.5. Selection of best coatings

Once produced, the coated gluten-free chicken nuggets were rested for a week to observe the integrity and homogeneity of the coatings. They were heated to detect whether or not nuggets retained their shape as well as there existed breakages for the coating layer. Based on the visual evaluations, 16 groups were eliminated, while the experiments were continued with the remaining 12 groups. Upon their sensory analyses, six groups out of 12 were selected. After the re-production of the six groups, the gluten analyses were performed. Although all had the gluten level below the legal limit of 20 mg/kg, three formulations with the lowest amounts of gluten out of six were selected.

2.6. Analyses of chemical composition and food quality

pH was measured after the homogenization of the mixture of 10 g samples with 100 mL distilled water by ultra turrax for 1 min (VURAL and OZTAN, 1996). Five g of the homogenous samples were taken into uniform containers and dried at 105°C until constant levels of weight and moisture (%) were reached. Their protein content was determined using the Kjeldahl method as a result of which N content (%) determined was multiplied by 6.25 to estimate protein fraction in % (AOAC, 2005).
The amount of fat in % was determined using the Soxhelet extraction by a gravimetric method. Three g of the homogenous samples were taken into a crucible, dried at 105°C for 4 h and then taken into an ash oven at 550°C until completely turning into grey-white ash. Ash (%) content was based on differences in weight before and after burning (AOAC, 2005). Gluten-free chicken nuggets were weighed before and after cooking to estimate product yield (%) (VERMA *et al.*, 2012).
After the nuggets were cut into two pieces, the three color values of L^*, a^*, and b^* were measured from both their surface and inside. The measurements were performed using a colorimeter (CR 300, Minolta, Japan) with calibrated standards (reference no: 1353123, Y = 92.7, x = 0.3133, y = 0.3193). Gluten-free chicken nuggets were weighed before and after coating to estimate surface coating (%) (ALTUNAKAR, 2003).

Both raw and cooked gluten-free chicken nuggets were dried at 105ºC for 24 h, and their moisture retention (%) was estimated according to HUANG *et al.* (1999). Dried nugget samples at 105ºC for 24 h were taken to determine oil retention (%). First, the fat content of the nuggets was determined, and their oil retention was calculated according to HUANG *et al.* (1999).

Thiobarbituric acid reactive substances (TBARS) were determined after the centrifugation (Hermle Z326K, Germany) at 10000 rpm for 5 min of the homogenized mixture of 10 g samples and 30 mL tricloracetic acid (7.5%). The centrifuged samples were filtered, and 5 mL filtrated samples were mixed with 5 mL 0.02 mol/L TBA solution prepared with 0.1 N HCl. After being subjected to vortexing, the samples were placed into a water bath at 100 °C for 35 min. The tubes were cooled down immediately and read at 538 nm (Perkin Elmer UV/VIS Spectrophotometer Lambda 35, USA) against blank. Results were calculated using a tetraethoxypropane (TEP) standard curve expressed in malonaldehyde/kg (MIELNIK *et al*., 2006).

The amount of hexanal was determined using a gas chromatography mass spectroscopy (GC-MS), with the samples being transferred into headspace vials and held at 40°C for 10 min. After the insertion of carboxen/polidimetilsiloxano (CARB-PDMS) fiber of 85 μm in thickness as an adsorbent into vials for 40 min to adsorb volatile compounds, the fiber was injected into GC-MS (Hewlett Packard 7890 GC with HP 5975 MS detector) equipped with FID detector and kept waiting for 10 min to desorb all its compounds to GC-MS column (DB- 624, 30 m in length; 0.25 mm in internal diameter, and 1.4 μm in internal thickness). The GC-MS conditions consisted of 250°C injection block and detector temperature, 1 mL/min flow rate, 230°C MS source temperature, and 150°C MS quadrupole temperature. Helium was used as the carrier gas at a flow rate of 1 mL/min.

Oven temperature program was set initially at 40 °C for 5 min with the increases from 40 to 110°C, 110 to 150°C, and 150 to 210°C at rates of 3°C/min, 4°C/min, and 10°C/min, respectively, and held for 12 min at 210°C. The mass spectrum ranged from 41 to 400 m/z with 70 eV electron energy. Similarity of the compounds was determined using the Wiley and NIST libraries (KIRALAN, 2010).

The amount of gluten was determined using the ELISA method. After five g of the samples were grinded, 0.25 g were mixed with cocktail solution and incubated for 40 min at 50°C water bath. After cooling, 7.5 mL of 80% ethanol was added and mixed thoroughly. After a 10-min centrifugation, the samples were diluted at the ratio of 1:12.5 with the dilution solution and transferred into vials. After the addition of washing solution three times, 100 µL of conjugate were added. The samples were incubated for 30 min at room temperature, and after the addition of washing solution again, 50 mL of substrate and chromogen were added. Upon the subsequent incubation at room temperature for 30 min, 100 µL of stop solution were added, and results were read at 450 nm (AOAC, 2010).

The apparent viscosity of the liquid coating was measured under a constant shear rate of 120/s at 25°C using a CP4/40 SCO 111 SS probe (diameter: 40 mm; slit: 0.15 mm) (Malvern Instruments, Kinexus Pro). The temperature of the batter samples was maintained during measurements using a circulating water bath. The texture of the gluten-free chicken nuggets was measured using a conical 45° probe with a 50-kg loading cell (TA plus, LLOYD Instruments, A trademark of Ametek Inc). Speed was adjusted to 100 mm/min to press 50% of the 15-mm sample thickness. Hardness (N), cohesiveness, springiness (mm), gumminess (N mm), chewiness (N mm), stickiness (g), and force of shear (N) were measured. Using a Craft knife adaptor (50 mm x 0.6 mm) at 1.5 mm/s, the speed of cutting force was controlled (ULU, 2006).

2.7. Microbial analyses

Ten g of the samples were diluted with 0.1% peptone (Merck, Germany) water to prepare serial dilutions. 0.1 mL of appropriate dilutions were surface-plated onto plate count agar (PCA, Merck, Germany) to count both total mesophilic aerobic bacteria (TMAB) and total psychrotroph aerobic bacteria (TPAB). TMAB and TPAB plates were incubated at 37°C for 24 h and at ±2°C for 10 days, respectively (HALKMAN and SAGDAS, 2011).
One mL of the appropriate three consecutive dilutions was transferred into Fluorocult-Lauryl Sulfate Tryptose (FLST) broth test tubes (Merck, Germany) containing Durham tubes. The tubes were incubated at 37°C for 24 to 48 h for the count of coliform. Cloudy and gas-formed tubes were evaluated as coliform positive. Positive tubes were inspected at dark under the UV lamb at 366 nm and 1 mL of Kovac's reagent (Merck, Germany) was added to the tubes emitting fluorescence light. The tubes whose color turned dark red were evaluated as *Escherichia coli* positive. By using the MPN tables, the numbers of both coliform and *E. coli* were expressed in MPN/g (HALKMAN and SAGDAS, 2011).
Twenty-five g of samples were diluted with 225 mL of *Listeria* enrichment broth (Merck, Germany). After their incubation at 33°C for 4 h, a selective enrichment (Merck, Germany) was added, and their incubation was continued for an additional 44 h. The enriched culture was streaked onto Oxford agar plates (Merck, Germany) incubated at 37°C for 24 to 48 h. Suspected colonies were transferred to Tryptone Soya Yeast Extract (TSYE) agar (Merck, Germany). After their incubation at 37°C for 24 h, developed colonies were transferred to 1% rhamnose containing Phenol Red Broth Base (PRBB) agar (Merck, Germany) and blood agar (Merck, Germany) for the further identification of *Listeria* spp. (HALKMAN and SAGDAS, 2011).
For the determination of *Salmonella* spp, 25 g of the samples were mixed with 225 mL peptone water (1% w/v) and incubated at 37ºC for 24 h. After their incubation, 0.1 mL of the pre-enriched culture was transferred to Rappaport Vassiliadis Soya (RVS) broth (Merck, Germany) and incubated at 41.5ºC for 24 h. The selective enriched culture grown was streaked onto Xylose Lysine Deoxycholate (XLD) agar plates (Merck, Germany) incubated at 37ºC for 24 h. Typical colonies were identified on Triple Sugar Iron (TSI) agar (Merck, Germany).

2.8. Sensory analyses

Sensory analyses of the frozen gluten-free chicken nuggets coated were conducted in the Sensory Analyses Lab of the Department of Food Engineering of Ankara University, Turkey. Sensory panel was formed using eight panelists professionally trained from the faculties and graduate students of the Department of Food Engineering. The samples were assigned to 3-digit numbers randomly and served to panelists. Each sample was evaluated for appearance, color, taste, aroma, texture, and overall acceptability on a 9-point hedonic scale with 1 being extremely bad and 9 extremely good (JACKSON *et al*., 2006).

2.9. Statistical data analyses

Significant differences among group means were determined using Tukey's multiple comparison tests following one-way analysis of variance (ANOVA) at $p < 0.05$. Data analyses were performed using MINITAB 17 (Minitab Inc. State College, PA, USA).

3. RESULTS AND DISCUSSION

3.1. Selection of the best batch of coatings

The selection of the best coating formulations for the gluten-free chicken nuggets was based on appearance and sensory properties, since some coatings lacked good adhesion properties after freezing, and reheating in microwave oven. In particular, the coatings with 100% rice or corn flour exhibited big cracks as well as uncovered or unevenly covered surfaces. The microwave heating resulted in burns and dissimilar color developments on some surfaces. Shrinkage and hardening were also observed for some coatings (Figs. 5a-d).

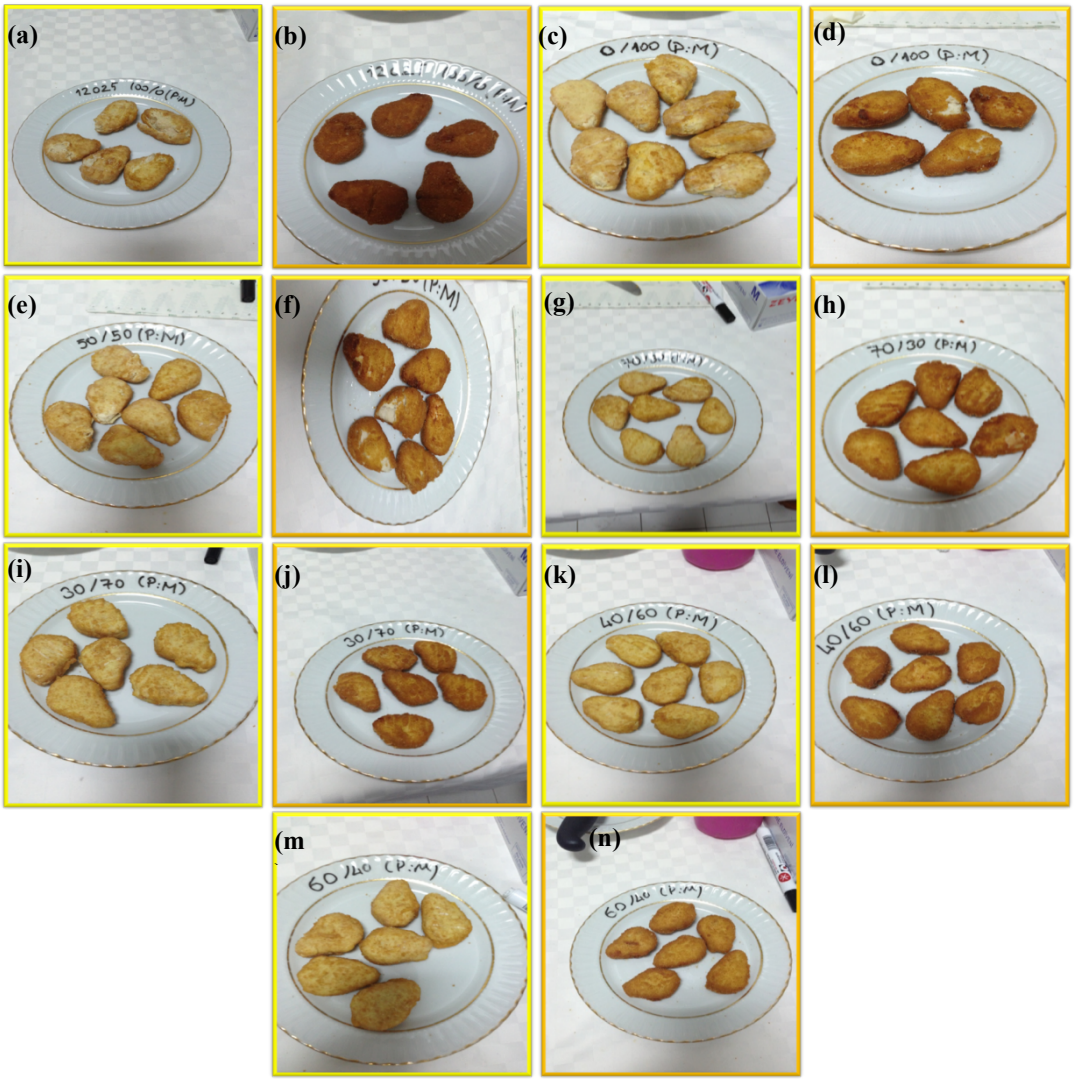

Figure 5. Frozen and fried gluten-free chicken nuggets coated with (a and b) 100% rice flour (R), (c and d) 100% corn flour (C), and R-to-C blend ratios (%) of (e and f) 50:50, (g and h) 70:30, (i and j) 30:70, (k and l) 40:60, and (m and n) 60:40.

The 50:50 blend ratio suffered, to a lesser extent, from non-homogenous coatings, cracking, the disintegration of coating, outpouring, and local burning due to heating (Figs. 5e-f). The 70:30 blend ratio led to a more homogenous coating, but the nuggets became very hard in

terms of their texture and lost their elasticity (Figs. 5g-h). The other three rice-corn blend ratios (30:70, 40:60, and 60:40) induced a more homogenous coating after freezing, with no disintegration and outpouring (Figs. 5i-n). These blend ratios were used in the remaining experiments with the 12 groups.

3.2. Microbial properties

Prior to the sensory analyses on a 9-point hedonic scale, the 12 coating formulations of the gluten-free chicken nuggets (coded as 12001 to 12004, and 12013 to 12020) were tested for the presence of TMAB, TPAB, and the three pathogen bacteria (coliform, *E. coli*, *L. monocytogenes* and *Samonellae* spp). The numbers of TMAB and TPAB were all below < 1 cfu/g, with no detection of the pathogen bacteria.

3.3. Sensory properties

Based on the sensory analyses, the scores ranged from 5.80 to 7.80 for appearance, 5.40 to 7.80 for color, 5.80 to 7.80 for taste, 5.8 to 7.6 for aroma, 4.40 to 7.20 for texture, and 5.60 to 7.40 for overall acceptability. The CMC-containing formulations (12004, 12015, 12016, 12019 and 12020) were rated higher than the MC-containing groups in terms of the sensory scores, in particular, for the textural properties (Fig. 6). Therefore, the MC-containing formulations were further eliminated after the sensory analyses.

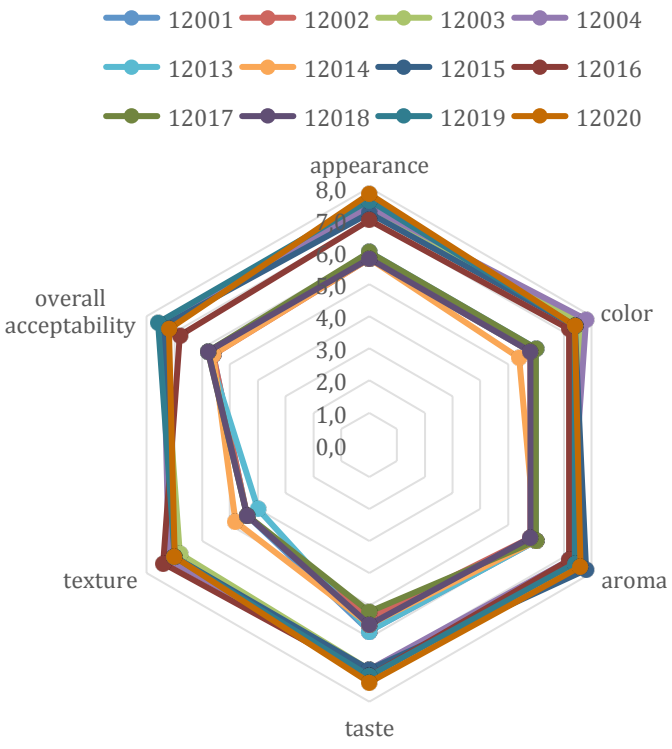

Figure 6. Sensory analyses of gluten-free chicken nuggets coated with three blend ratios of rice (R) to corn (C) flour (12001 = 30:70, 5% CS, 0.3% MC; 12002 = 30:70, 15% CS, 0.3% MC; 12003 = 30:70, 5% CS, 0.5% CMC; 12004 = 30:70, 15% CS, 0.5% CMC; 12013 = 40:60, 5% CS, 0.3% MC; 12014 = 40:60, 15% CS, 0.3% MC; 12015 = 40:60, 5% CS, 0.5% CMC; 12016 = 40:60, 15% CS, 0.5% CMC; 12017 = 60:40, 5% CS, 0.3% MC; 12018 = 60:40, 15% CS, 0.3% MC; 12019 = 60:40, 5% CS, 0.5% CMC; and 12020 = 60:40, 15% CS, 0.5% CMC).

3.4. Quality properties

The gluten contents of the six CMC-containing groups varied between < 3 and 27.78 mg/kg (Table 3). According to The Commission of the European Communities Regulation No 41/2009 issued on January 20, 2009, the final food products labeled with "very low gluten content" should have the gluten level of below 100 mg/kg, while foods labeled "gluten-free" should contain no more than 20 mg/kg (THOMPSON, 2018). Thus, the three formulas (12004, 12015 and 12016 with 0.5% CMC) with the lowest amounts of gluten out of six were selected for the further studies.

Table 3. Gluten contents of six coatings with carboxy methyl cellulose (CMC) for chicken nuggets.

Sample codes	Gluten (mg/kg)
12003	16.18
12004	< 3
12015	7.9
12016	< 3
12019	27.78
12020	18.33

12003 = 30:70 R/C flour, 5% CS; 12004 = 30:70 R/C flour, 15% CS; 12015 = 40:60 R/C flour, 5% CS; 12016 = 40:60 R/C flour, 15% CS; 12019 = 60:40 R/C flour, 5% CS; and 12020 = 60:40 R/C flour, 15% CS.

The chemical composition analyses of the final three products revealed similar values of pH, moisture, fat, ash, protein, and viscosity ($p > 0.05$) (Table 4). DEMIROK (2014) estimated the ranges of the moisture, fat, ash and protein contents, and pH of the commercially-available chicken nuggets in the Turkish market at 38.37 to 57.79%, 13.16 to 22.60%, 2.01 to 2.59%, 14.94 to 18.75%, and 6.03 to 6.17, respectively. The gluten-free chicken nugget formulations in the present study had lower fat and ash contents than the commercial products. The lower fat content may be attributed to less oil absorbance by the corn-and-rice-based gluten-free coating than the wheat-based coating during frying. The lower ash content may be the result of differences between our and commercial formulations.

Table 4. Chemical compositions of coated gluten-free chicken nuggets ($n = 3$).

Sample codes	Moisture (%)	Fat (%)	Ash (%)	Protein (%)	pH	Viscosity (Pa.s)
12004	59.75±1.45	9.07±0.03	1.37±0.01	14.76±0.32	6.21±0.02	3.13±0.02
12015	60.70±0.83	9.20±0.06	1.38±0.01	14.80±0.68	6.19±0.01	2.73±0.03
12016	60.73±1.22	9.16±0.05	1.37±0.01	14.85±0.55	6.21±0.03	3.36±0.04

12004 = 30:70 R/C flour, 15 % CS; 12015 = 40:60 R/C flour, 5 % CS; and 12016 = 40:60 R/C flour, 15 % CS.

XUE and NGADI (2007) estimated the viscosity of liquid coating formulated with 100% wheat, corn and rice flours at 5.44, 3.25 and 3.49 Pa.s, respectively. The viscosity values of liquid coatings formulated with soy and rice flours were reported as 4.43 and 3.52 Pa.s, respectively (DOGAN *et al.*, 2005). The viscosity values of the final three formulations

(12004, 12015, and 12016) in the present study were similar to those of the corn and rice flour-based formulations but lower than those of the wheat flour-based formulations by DOGAN *et al*. (2005), and XUE and NGADI (2007).

The surface coatings (%) of the final three formulations were between 26.29±2.46 and 25.17±3.30% ($p > 0.05$). The coating ingredients are directly related to viscosity, adhesion, and oil absorption during frying (ERGEZER *et al*., 2008; CHEN *et al*., 2008). The surface coating of the commercially-available formulations changed from 25.27 to 36.13% similar to that of our gluten-free formulations. The surface coatings based on the wheat, corn, rye and soy flours were found to range from 11.53 to 14.28%, with the rye flour with the highest value (GOKCE *et al*., 2016). DOGAN *et al*. (2005) found that the 5% soy flour led to the highest surface coating when compared to the wheat and 5% rice flours.

Gums were observed to enhance the surface coatings of the fish nuggets with the wheat and corn flours (Chen *et al*., 2009). According to Albert *et al*. (2009), the use of oxidized starch, xanthan gum, and hydroxyl methyl cellulose instead of flour did not increase the coating ability of the fish nuggets. The gluten-free coatings developed in the present study appeared to have a better surface coating ability than the other formulas reported in the related literature. Also, the addition of both CMC and xanthan gum in this study increased the coating ability and viscosity.

The product yield is a good indicator of the coating ability and rises with the increased economic value of a given product (ALTUNAR *et al*., 2004). The product yields of the final three formulations (12004, 12015, and 12016) were determined as 97.83±3.44, 96.78±4.12, and 98.82±3.26%, respectively ($p > 0.05$). ALTUNAR *et al*. (2004) stated that pre-jelatinized tapioca starch relative to amylo corn and waxy ones had the highest product yield for deep-fat fried chicken nuggets. DEVATKAL *et al*. (2011) pointed out that the risen amount of sorghum flour elevated the product yield (94.99%) of the gluten-free chicken nuggets. JEN *et al*. (1999) found that the sorghum flour enabled the formation of a three dimensional structure between starch and plant-based protein, thus boosting the emulsion stability and product yield.

The 5 and 10% oat flours significantly grew the product yields of the low-fat chicken nuggets (SANTHI and KALAIKANNAN, 2014). The product yield of the gluten-free chicken nuggets in the present study was higher than that of the sorghum-added ones and close to that of the oat-added ones. GOKCE *et al*. (2016) found that the corn and soy flours had the highest and lowest product yields of the chicken nuggets (82.64 and 78.10%), respectively, among the wheat, corn, rye and soy flours. These findings were attributed to the formation of a film layer on the coated surface by the high content of gelatinized starch without which the soy flour had the low product yield (GOKCE *et al*., 2016).

The moisture and oil retentions are the important quality parameters of plant-based flours as affecting the sensory and textural properties (KUMAR and SHARMA 2004). Depending on the rise of temperature inside a given product during frying, water that fills the pores is evaporated whose places are replaced by oil. The moisture retentions of the final coatings (12004, 12015, and 12016) were estimated at 69.22±4.22%, 67.25±3.12%, and 71.28±5.47%, while their oil retentions were 59.57±3.06%, 57.21±4.01%, and 59.12±4.46%, respectively ($p > 0.05$). The higher moisture than oil retention values appeared to stem from hydrocolloids and xanthan gum immersing less oil than did the wheat flour, as SANZ *et al*. (2004) also indicated. The oil retention of meat balls was estimated at 53.69, 52.39 and 50.21% when produced with the 2, 4 and 6% sorghum flours, respectively (Huang *et al*., 1999). DEMIROK (2014) estimated the fat contents of chicken nuggets at 11.62, 16.35 and 16.71% in response to cooking in steam-assisted oven, frying, and frying after microwave-assisted defrosting, respectively. Similarly, SHIH and DAIGLE (1999) reported that the rice-based coatings absorbed less oil during frying due to its chemical structure.

The high concentrations of TBARS indicate the presence of lipid oxidation compounds such as aldehyde, carbonyl, and hydrocarbones responsible for the spoilage of meat and meat products (AL-KUTBY, 2012; CAGDAS and KUMCUOGLU, 2015). In general, the products with below 3 mg MDA/kg TBARS are considered to be in good quality in terms of lipid oxidation (CADUN *et al*., 2008). The TBARS values in the present study were 0.17±0.07, 0.17±0.06, and 0.08±0.02 mg MDA/kg for 12004, 12015, and 12016, respectively ($p < 0.05$).

The hexanal concentration can be used as a quality indicator, in particular, for the stored products (MIELNIK *et al*., 2006). PIGNOLI *et al*. (2009) reported that the hexanal concentration changed during storage due to its reduced volatility as a result of its reactions with proteins. The hexanal concentrations were estimated at 161±23, 330±49, and 213±38 (x 10^6 AU) for 12004, 12015, and 12016, respectively ($p < 0.05$).

Although no significant difference among the three formulas was found in the inner or outer color values, the L^*, a^* and b^* values of the inner surface were significantly higher those of the outer surface ($p < 0.05$). The gluten-free chicken nuggets in the present study had slightly high L^* but similar a^* and b^* values than did the commercial chicken nuggets (64.38 to 68.41, 1.41 to 3.51, and 16.46 to 19.35, respectively) (LUKMAN *et al*., 2009). Turkey nuggets coated with corn flour had higher L^* (56.6) and b^* (42.3) values due to their carotenoid coloring pigments, while those coated with rice flour had lower L^* (38.5) and b^* (31.0) values than did those coated with wheat or soy flour (JUKIC *et al*., 2011). The deep-fat frying of the chicken nuggets with the wheat, corn, rye and soy flours was reported to generate the L^* values in the range of 28.79 to 39.16 with the lightest and darkest values from wheat and soy flours, respectively (GOKCE *et al*., 2016). JACKSON *et al*. (2009) revealed that the L^* value of nuggets were not affected by the wheat versus rice flour but by the cooking type. The lower L^* value of the fried nuggets in the present study was consistent with the finding by JACKSON *et al*. (2006) that the color of the fried nuggets grew darker. SOSA *et al*. (2006) also observed a darker color with the deep-fat frying of nuggets, tofus, and doughnuts. The L^* and a^* values of chicken nuggets with 5% and 10% sorghum flours were reported to decrease (DEVATKAL *et al*., 2011). DYKES and ROONEY (2006) found that the sorghum flour-based coatings resulted in darker nuggets than did the wheat flour ones due to their anthocyanin and tannin contents. The a^* value of the sorghum flour coating was lower than that of the regular coating for beef meatballs (VAN ZYL and SETSER, 2001).

3.5. Textural properties

No significant difference in the textural properties was found among the best three gluten-free coatings of the chicken nuggets (Table 5).

Table 5. Textural properties of the best three gluten-free coatings of chicken nuggets.

Textural properties	12004	12015	12016
Hardness (N)	51.23±6.50	43.00±12.10	53.24±6.45
Cohesiveness	0.55±0.06	0.65±0.00	0.61±0.01
Springiness (mm)	0.74±0.02	0.67±0.12	0.76±0,02
Gumminess (N mm)	27.17±6.99	26.54±9.37	33.09±4.45
Chewiness (N mm)	20.57±5.93	20.87±8.04	21.54±4.11
Stickiness (g)	-0.004±0.002	-0.009±0.005	-0.005±0.001
Force of shear (N)	12.42±0.77	11.01±1.37	13.18±0.42

The textural properties in this study were similar to those of the commercial chicken nuggets (LUKMAN *et al*., 2009). DEMIROK (2014) quantified the hardness, springiness, gumminess, chewiness, and stickiness values of the commercial chicken nuggets in the ranges of 2.59 to 5.05 N, 4.59 to 6.14 mm, 0.91 to 2.46 N mm, 5.56 to 11.53 N mm, and 0.01 to 0.08 g respectively.
The substitution of the wheat flour with the corn, soy, rice, soy/corn or rice/corn flours in coating turkey nuggets showed that the corn/soy flour had the highest hardness value (JUKIC *et al*., 2011). The reformulated nuggets had the higher hardness value with the rice than wheat flour (JACKSON *et al*., 2006). The chicken nuggets coated with the wheat and 5% and 10% sorghum flours did not differ in terms of hardness, with higher chewiness and gumminess for the 10% sorghum than wheat and 5% sorghum flours, and lower springiness for the 10% sorghum flour (DEVATKAL *et al*., 2011). The rye flour-coated chicken nuggets were found to be softest, while the soy and corn flour-coated ones had a similar hardness value (GOKCE *et al*., 2016).

4. CONCLUSIONS

The development of the gluten-free coatings for the chicken nuggets is of great importance to people with gluten intolerance. The replacement of the wheat flour by the gluten-free coatings developed in this study for the chicken nuggets enhanced their physical, sensory and textural properties. Given the similarity between the reformulated and commercial products, the gluten-free chicken nuggets performed well even after freezing and thawing. Further studies are needed to compare their shelf-life stability to the commercial products' one

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PAPER

IMAGING, SENSORY PROPERTIES AND FATTY ACID COMPOSITION OF PARMA HAM AND "NERO DI PARMA HAM"

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ABSTRACT

This paper provides preliminary results on "Nero di Parma" ham analysing fatty acid composition from fat and muscle tissues in three locations. Computerized Tomography (CT) and sensory characteristics were also performed. The same measurements were investigated in Parma ham too. Significant differences in terms of saturated and monounsaturated fatty acids composition were observed between hams resulting lower and higher in "Nero di Parma" ham than Parma ham respectively. Results detected by CT showed an inverse ratio of fat and muscle between the hams. "Nero di Parma" ham highlighted some descriptors such as oiliness, brightness, redness significantly different from Parma useful to define the sensory profile of a typical product, which had never been tested.

Keywords: fatty acids, "Nero di Parma ham", Parma ham, sensory profile

1. INTRODUCTION

Dry curing is a common way to preserve pork in some Mediterranean countries. In Italy, Parma ham is the main Consortium for the production of dry cured ham labeled with Protected Designation of Origin (PDO), processing over 8 million thighs (data referred to 2017).
Parma ham represents a product of great economic value covering about half of the trading value of carcasses from Italian heavy pigs. In addition, new interest is now addressed to the health promoting characteristics of Parma ham and therefore, the fatty acid composition of its lipids is important to relate the profile to medical guidelines.
Alongside the intensive pig farming conducted according to criteria of industrialization, an attractive production related to tradition is emerging. It concerns native breeds that are characterised by peculiar traits low input rearing conditions and suitable high quality food products. In the present work "Nero di Parma" breed has been considered. It is a modern recreation of the ancient Nera Parmigiana breed, which significantly declined almost to the point of extinction. However, selection programs are being designed to reintroduce the "Nero di Parma" pig breeding tradition; with the DM 11781 of 20 May 2016 the "Nero di Parma" pig obtained the recognition of the breed. Animals are fed fresh grass, corn, barley and wheat, as well as broad beans, berries, roots and acorns. The characteristics of the meat of "Nero di Parma" are linked to the presence of intramuscular fat due to the ability to accumulate subcutaneous fat between the muscle fibers compared to the white breeds such as the Large White and Landrace and their crossings. Fat plays an important role in the development of the chemical and sensorial characteristics of cured meat products (VENTANAS *et al*., 2007; JIMENEZ-COLMENERO *et al*., 2010). Moreover, much attention is being paid to fatty acid profile in animal products because of its effects on consumers' health (WOOD and ENSER, 1997). Scientific evidence (WHO, 2003) and nutritional guidelines recommend a reduction in total fat intake, particularly of saturated fatty acids (SFA), which are associated with an increased risk of obesity, hypercholesterolaemia and some cancers (WOOD *et al*., 2004), while a high intake of monounsaturated and n-3 polyunsaturated fatty acids has been shown to have an inverse effect (GANJI *et al*., 2003). However, there are few data on "Nero di Parma" pigs in terms of meat quality and those related to seasoned ham to the best of our knowledge are missing. The aim of paper is to provide preliminary results on some qualitative parameters of the "Nero di Parma" ham by evaluating fatty acid composition in fat and muscle in three locations (initial part, central and the final part of the ham), the structure of the ham through the computerized tomography (CT), sensory characteristics and measuring the same parameters in Parma ham.

2. MATERIAL AND METHODS

2.1. Sampling

A total of 6 raw cured hams, 3 "Nero di Parma" hams and 3 Parma (PDO) were provided by a local producer, within the same factory in order to ensure the same production technology. The two types of hams had different ripening maturation times: 30 and 24 months respectively. The choice depended on the high fat (either fat coverage either as intermuscular fat) of "Nero di Parma" that requires longer times of ripening. Therefore "Nero di Parma" ham is marketed at 30 months, and Parma ham at 24 months. Before starting chemical analysis, on the whole ham the spiral multi slice CT was carried out; subsequently the hams were sliced and used for sensory analysis. During the sensory

analysis whole slices (including muscle and external fat) at three different locations (initial, central and final part) were sampled according to Fig. 1. Samples were vacuum-packaged and stored at -20°C for 2 weeks until subsequent chemical analysis.

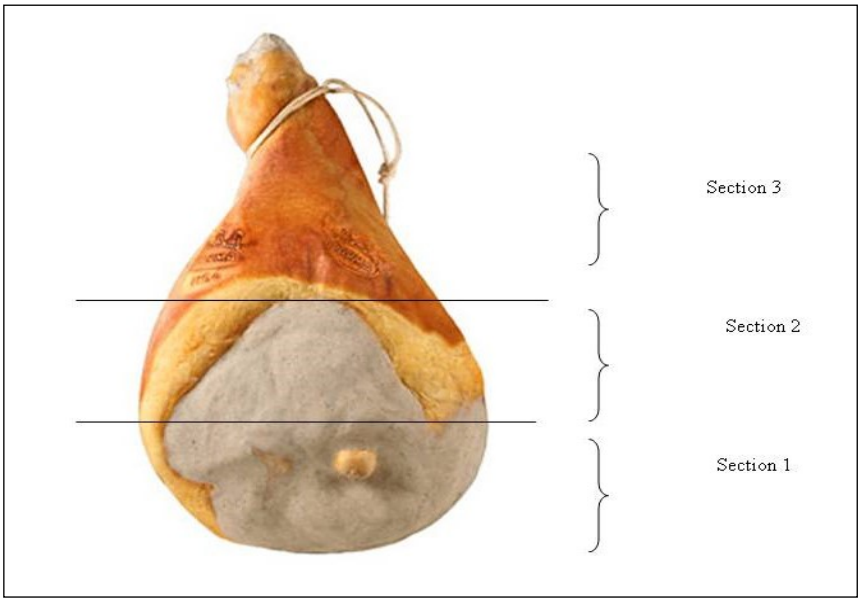

Figure 1. Sampling location. Section 1 corresponds to initial part; Section 2 corresponds to central part; Section 3: corresponds to final part

2.2. Fatty acid analysis

Lipid extraction was conducted using chloroform: methanol (2:1) according to the method of FOLCH *et al*. (1957). Lipids were extracted from each sample of the muscle, and from each sample of trimmed adipose tissue. The extracts were dried under vacuum on a rotary evaporator (Laborota 4000, Heidolph Instruments, Milan, Italy). Fatty acid composition was measured after methylation of the samples. Fatty acid methyl esters were prepared with boron trifluoride methanol according to procedures developed by MORRISON and SMITH (1964). The methyl esters were separated on a Carlo Erba Instruments chromatograph (GC 6000 Vega series 2) equipped with fused silica gel capillary column (0.25 mm i.d.25 m) having a 0.2-mm internal coating of cyanopropyl siloxane (CP-Sil 88, Chrompack). The furnace temperature was 180°C, and injector and detector temperatures were 240°C. For all samples, retention times and peak areas were determined by chromatography (Nelson Analytical, Manchester, NH). The identities of the peaks were verified by comparison with the retention times of standard fatty acid methyl esters. The results were expressed as the percentage of the total fatty acid composition. All the analyses were carried out in duplicate.
Atherogenicity (AI) and thrombogenicity (TI) indices (ULBRICHT and SOUTHGATE 1991), which characterize the potential proneness to atherosclerosis and thrombosis in humans were used to assess dietetic values of ham.
In detail IA indicates the relationship between the sum of the main saturated fatty acids and that of the main classes of unsaturated; the former being considered pro-atherogenic (favoring the adhesion of lipids to cells of the immunological and circulatory system), and the latter anti atherogenic (inhibiting the aggregation of plaque and diminishing the levels

of esterified fatty acid, cholesterol, and phospholipids, by preventing the appearance of micro and macro coronary diseases).
Index of thrombogenicity is defined as the relationship between the pro-thrombogenetic (saturated) and the anti-thrombogenetic fatty acids (MUFAs, PUFAs – n6 and PUFAs – n3).

2.3. Sensory analysis

Eleven trained assessors belonging to ONAS (National Organization Salumi Taster) undertook the sensory analysis on 2-mm slices of ham. The choice of descriptors was decided by open discussion in two preliminary sessions. Twenty sensory descriptors covering appearance (redness, whiteness, marbling, oiliness, brightness), odor (aged, fresh pork meat, rancid, mold), taste (sweetness, saltiness, bitter), flavor (aged, fresh pork meat, rancid, butter, fresh fat), texture (fibrousness, dryness, firmness) were chosen. A 9-point scale was used, where 0 meant absence and 9 meant maximum intensity of the descriptor. The sensory evaluation was repeated in three sessions carried out on three different days (EN ISO 13299, 2010). Each member of the panel assessed two types of hams in each session. Slices of ham samples were coded with three random numbers and were presented to the members according to MACFIE *et al*. (1989).

2.4. Computed axial tomography

Hams were submitted to 16-slices CT scanner (Brightspeed® - GE Medical Systems Italia S.p.A., Milan, Italy) applying the following protocol: Tube Voltage 110 kVp, X-Ray Tube Current 200 mA, Revolution Time 1, Slice Thickness 1.25 mm, Spiral Pitch Factors 0.9375, Convolution Kernel Standard, Rows and Columns 512x512.
All images were transferred to the picture archiving and communication system (MyPACS – MD Saronno - Italy), processed with a certified medical software (OsirixPRO® 64 Bit, Aycan Medical Systems) and reconstructed using smoothing and edge enhancement algorithms in all the three spatial transverse planes.
The program can differentiate the fat from the lean as a consequence of the significant difference in Hounsfield Units (HU) existing between the two tissues (negative values for fat and positive for lean). Hounsfield Units (also named CT numbers) express the X-ray attenuation of the tissues, which is a measure of its density in a given Region of Interest (ROI).
In each slice, a ROI based on the density of the tissues examined (fat or lean) was selected in order to obtain the HU surface area values. Therefore, the "Compute Volume" function of the software was used to calculate the volume.

2.5. Statistical analysis

The ham was the experimental unit for statistical analysis. Data were analyzed by means of variance analysis including typology (Parma and "Nero di Parma"), tissue (muscle and fat) and location (initial, central and distal part), as main effect. Newman-Keuls test was used to assess differences between means.
The sensory data for each attribute were submitted to three-way ANOVA with typology, judges, replicates and their interaction as effects. The significance of these effects was tested with the F-test and the comparison between mean values was tested with T Student. Differences with probability levels of $P<0.05$ were considered significant. Effects were deemed significant at $P<0.05$, and a trend was noted when $P<0.10$. Statistical analyses of the data were performed using SPSS software (SPSS Inc., Chicago, IL)

3. RESULTS AND DISCUSSION

Pig farming plays a key role within animal production, particularly in North Italy where about 70% of the Italian pig production is located. The main strength factor of the Italian pig industry is represented by the top quality of its production labeled with the PDO (Protected Designation of Origin) or PGI (Protected Geographical Indication) marks. They represent an important market value and must continuously be monitored from a qualitative point of view.

Fat and fatty acids, whether in adipose tissue or muscle, contribute importantly to various aspects of meat quality and are central to meat nutritional value (WOOD *et al*., 2008). It is generally assumed that intramuscular fat (IMF) content positively influences sensory quality traits, including flavor, juiciness and tenderness of meat, whereas a low amount of fat results in a less tasty meat (TOUS *et al*., 2013).

It is well known that traditional breeds are fattier than industrial ones. Generally, they have more adipose tissue thickness and more intramuscular fat (GANDEMER, 2009). Dry-cured ham from conventionally reared modern breeds of pigs compared to dry-cured ham from Iberian pigs had lower levels of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) (JIMÉNEZ-COLMENERO *et al*., 2010).

The FA composition of dry-cured hams is due to many factors, such as genetic features and differences in the rearing and feeding systems.

Results of FERNÁNDEZ *et al*. (2015) for fatty acid composition of analyzed samples of Spanish dry cured ham showed approximate mean percentages of SFA and monounsaturated fatty acid (MUFA) of about 43% of the total FA, and 13.87% of PUFA. In a study conducted by BERMÚDEZ *et al*. (2012) on the effect of the inclusion of chestnut in the finishing diet (from three different diets: concentrate, mixed and chestnut) on fatty acid profile of dry-cured Celta the percentage of SFA (35 %) PUFA (13%) and MUFA (51%) in mixed diet were similar to those found herein. In the present study the most abundant fatty acids in both types were monounsaturated fatty acids (MUFA) (mainly C18:1 c-n9) followed by SFA and finally polyunsaturated fatty acids. The different typology caused some changes in the fatty acid composition of dry-cured ham (Table 1).

In particular, "Nero di Parma" ham showed a significant lower SFA content and higher MUFA than Parma ham as a direct consequence of the high oleic acid content of acorns as reported in Iberian pigs (RUIZ *et al*., 1998; RUIZ-CARRASCAL *et al*., 2000). Numerically, the values of oleic acid in "Nero di Parma" agree with those found in Spanish ham (PEREZ-PALACIOS, 2010). Moreover, CAMPO and SIERRA (2011) monitored different varieties of Spanish dry cured ham produced from the same company in two years (1995-2007). The oleic acid (C18: 1n9) levels found overlap those found in "Nero di Parma" ham in the present work. The levels found in this study are lower but not too far from those of FERNÁNDEZ *et al*. (2007) who found approximately 49% of C18:1 n-9, considered a healthy product in a diet. Moreover, the oleic acid content of "Nero di Parma" ham agrees with values coming from pigs of Parma DOP circuit fed with 6% sunflower oil in which a significant increase of oleic acid in the treated group was found (BOSI *et al*., 2000). "Nero di Parma" ham compared to Parma showed a significantly lower content of both stearic acid (C18: 0) and myristic acid (C14: 0).

Moreover, the content of total n-3 PUFA and particularly α-linolenic acid (ALA, C18:3n-3) was greater ($P<0.05$) in "Nero di Parma" than Parma dry cured ham. This is likely due to the different rearing system, which characterize the two typologies. According to MURIEL *et al*. (2002), free-range rearing and feeding pigs on pasture increase the levels of long chain n 3 fatty acids in porcine muscles which is in agreement with the present results.

Table 1. Effects of typology (Parma ham and "Nero di Parma ham") on fatty acid composition of muscle and adipose tissue of dry-cured ham.

	Muscle tissue		Fat tissue		P value[1]		
	Parma	"Nero di Parma	Parma	"Nero di Parma"	T[2]	Typ[3]	Tex x Typ
C14:0	1.07±0.17	0.87±0.2	1.17±0.36	0.92±0.3	ns	*	ns
C16:0	24.00±0.87	23.40±0.86	24.01±0.85	24.03±2.4	ns	ns	ns
C16:1n7	2.42±0.81	2.37±0.77	2.00±0.81	2.24±0.59	ns	ns	ns
C17:0	0.16±0.53	0.15±0.48	0.15±0.44	0.17±0.39	ns	ns	ns
C17:1	0.12±0.04	0.15±0.03	0.13±0.04	0.15±0.02	ns	ns	ns
C18:0	11.00±0.95	9.15±1.26	11.23±1.40	9.53±1.37	ns	***	ns
C18:1n9	44.40±2.01	47.40±2.87	44.96±2.43	46.5±4.02	ns	***	ns
C18:2n6	10.38±0.83	9.90±2.27	11.11±1.12	10.74±1.40	ns	ns	ns
C20:0	0.08±0.01	0. 08±0.03	0.07±0.04	0.10±0.02	ns	ns	ns
C18:3n3	0.32±0.10	0.42±0.10	0.38±0.08	0.48±0.06	**	***	ns
C20:1n9	0.64±0.10	0.71±0.15	0.60±0.12	0.68±0.20	ns	ns	ns
C20:2n6	0.37±0.06	0.34±0.06	0.39±0.10	0.35±0.10	ns	ns	ns
C20:3n3	0.02±0.02	0.03±0.01	0.03±0.03	0.04±0.03	ns	ns	ns
C20:3n6	0.07±0.06	0.06±0.03	0.05±0.08	0.03±0.02	ns	ns	ns
C20:4n6	0.48±0.25	0.58±0.44	0.10±0.17	0.11±0.07	***	ns	ns
C23:0	0.23±0.21	0.15±0.14	0.04±0.05	0.13±0.30	ns	ns	ns
SFA	36.58±1.34	33.82±1.78	36.74±1.52	34.89±3.74	ns	***	ns
MUFA	51.77±1.56	54.85±2.94	51.18±2.03	53.83±3.75	**	***	ns
PUFA	11.65±0.95	11.32±2.77	12.07±1.39	11.77±1.55	ns	ns	ns
n-6 PUFA	10.94±0.93	10.53±2.65	11.27±1.30	10.89±1.47	ns	ns	ns
n-3 PUFA	0.35±0.11	0.44±0.11	0.41±0.10	0.52±0.07	†	***	ns

[2]T= tissue; [3] Typ: Tipology;
Probability [n]Statistical significance at $P<0.05$, [(*)] Statistical significance at $P<0.01$, [(**)] Statistical significance at $P<0.001$, (†) = $P<0.10$

Previous studies conducted in heavy pigs (PASTORELLI *et al*., 2003) (160±10 kg of BW) and in light pigs (SANTOS *et al*., 2008) showed that the fatty acid composition of dry cured ham depends on the dietary fatty acid composition. Both studies observed an increase in ALA content in dry-cured ham obtained from pigs fed diets enriched in n-3 PUFA.
The increase of linolenic acid and monounsaturated fatty acids content, in "Nero di Parma ham" is certainly positive considering the indications provided by human diet for the important role of monounsaturated fatty acids and fatty acids of the omega 3 series. A trend was noted for n-3 PUFA in the two tissues analysed (Table 1).
As far as linolenic acid is concerned, the content is higher in adipose tissue than in muscle (0.43 *vs* 0.37), according to MUSELLA *et al*. (2009) and as expected.
In contrast, eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) are preferentially stored in the organs or muscle rather than in the adipose tissue. In the present work, these fatty acids have not been quantified. No significant effect has been found for tissue x typology. Typology did not affect (P>0.05) linoleic acid (C18:2n6) in any of analysed tissue. Regarding the curing process of meat and in order to obtain adequate fat quality, adipose tissue should contain no more than 12% of C18:2n-6 and exceed 12% of C18:0 (MOUROT and HERMIER, 2001). Adipose tissues studied in the present research were within those ranges.

The nature and proportion of individual fatty acids, especially SFA to PUFA, is important in assessing the quality and nutritional value of fats. The proportions of these fatty acid groups have been used to calculate the very important risk factors for atherogenic index (AI) and thrombogenic index (TI). A lower AI value indicates a lower proportion of saturated to unsaturated acids, which reduces ability of endothelium of blood vessels to attach lipids and plaque formation. The TI at its lower index indicates a lower risk of occurrence of disturbance of the blood coagulation process and blood clotting. Both these indices in the present study were at an appropriate low level in both typologies of ham, close to that observed in wild boar meat (RAZMAITE *et al*., 2012). Both AI (0.42 *vs* 0.45) and TI (1.00 *vs* 1.20) indices were significantly lower in "Nero di Parma" ham than Parma ham. Our results are consistent with those obtained by CEBULSKA *et al*. (2018), who reported an average value of 0.40 and 1.07 for AI and TI respectively in pork meat originating from pigs of polish native pure breeds. A significant reduction of the nutritional indexes has been reported in cow's milk (CAROPRESE *et al*., 2010), ewe's milk (CIESLAK *et al.*, 2010; CAROPRESE *et al*., 2011), and rabbit meat (PEIRETTI and MEINERI, 2010) after linseed feeding.

CIESLAK *et al.* (2010) showed reduction of the atherogenic index from 1.4 to 0.5 and thrombogenic index from 0.8 to 0.4. OKROUHLÁ *et al.* (2013) reported a thrombogenic index (1.33 and 1.28 vs. 0.80 and 0.85) in meat of barrows and gilts.

According to previous studies (LORENZO *et al*., 2012, FRANCO *et al.*, 2006) that found the effect of carcass location on the fatty acid profile, we investigated the effect of location in three different locations of the ham. Fatty acid composition differences in relation to location were found. In particular, they were identified for the following fatty acids: C18:0, C18: 1n9, C20: 0, C20: 3n3, C20: 4n6 with consequent effect on SFA ($P = 0.007$), MUFA ($P<0.001$) and n-6 PUFA that showed just a trend ($P = 0.075$). In particular, it is noted that SFA exhibit a higher concentration in the initial part (36.73), the MUFAs in the final part (54.99) and the PUFA in the central part (12.49) of the sampled hams (Fig. 2).

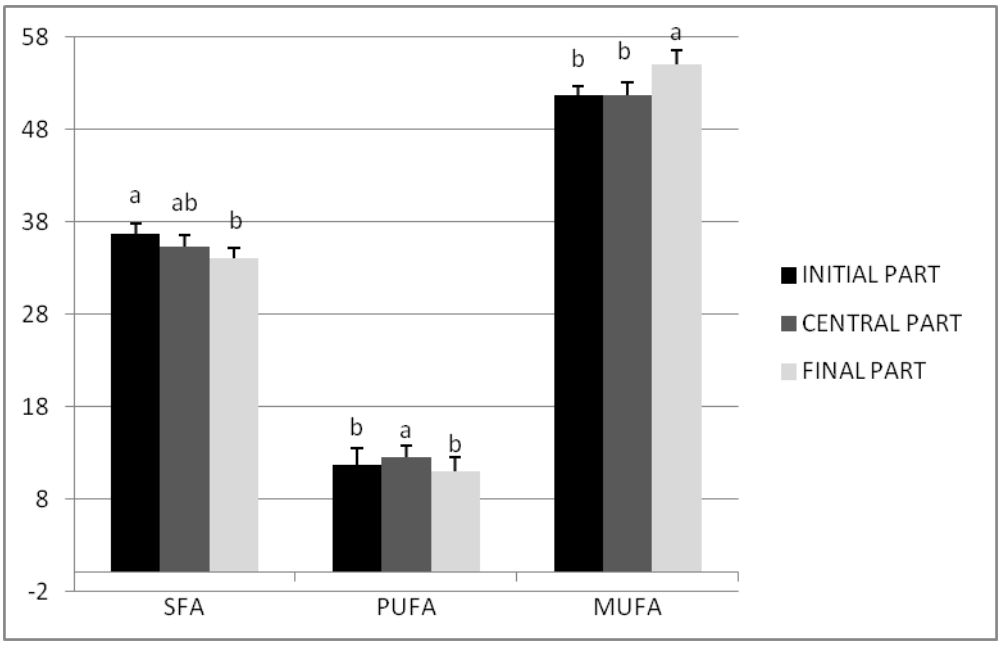

Figure 2. Effect of sampling location on Saturated fatty acids (SFA), Polyunsaturated fatty acids (PUFA) and Monounsaturated fatty acids (MUFA) content.

The highest content of MUFA in the final part agrees with results of CAVA *et al*. (2000) who found higher percentages of oleic acid and MUFA in a deeper muscle than in superficial one.
SFA are mainly located in the initial part corresponding to a location with higher adipose accumulation.
The nutritional value of adipose tissues is related to high values of PUFA/SFA, MUFA/ SFA ratios and low values for n-6/n-3 ratio. In the present study all cited ratios were significantly improved in "Nero di Parma" ham.

3.1. Sensorial analysis

There are various kinds of dry-cured hams in Mediterranean countries, distinguished by the origin and the quality of raw material. Dry-cured hams can be categorized into two types: (1) originating from traditional breeds, usually accompanied by extensive outdoor rearing systems and (2) originating from modern lean breeds, raised under intensive systems (ČANDEK-POTOKAR and ŠKRLEP, 2012). Consumer demand regarding the sensory quality of dry cured ham varies according to the region and local habits. Many highly regarded traditional products are based on the exploitation of natural resources like acorn pastures in the current case or in the case of the Iberian pig.
Taste is the key factor affecting consumer satisfaction with dry-cured ham (RESANO *et al*., 2011). However, familiarity with a product affects quality expectations and perceptions of consumers, which explains why different dry-cured ham characteristics are appreciated in different countries.
The least square means of the different attributes for the samples of dry cured ham are reported in the spider plot (Fig. 3). The panelist effect was significant ($P<0.01$) for all descriptors; this statement is very common in sensory evaluation because of a different use of scores and disagreement within the assessment of sample (LEA *et al*., 1997). No effect for replicates and no interaction between panelists × replicates and samples × replicates were found except brightness ($P=0.018$) and sweetness taste ($P = 0.024$). These results underline the excellent reproducibility of scores given by the panelists and excellent homogeneity of samples during repetitions.
Typology x replication interaction was not significant; this result would therefore seem to indicate that the mean scores for each sample given by the panelists for each descriptor are satisfactory estimates of the sensory profile of Parma and "Nero di Parma" ham. ANOVA of the sensory data showed a significant effect in the following descriptors: redness, marbling, oily, brightness, aged odor, rancid both in odor and flavor, sweetness, bitter and dryness. Numerical values of the considered descriptors of Parma ham are comparable with literature (LAUREATI *et al*., 2014).
Redness descriptor was higher in "Nero di Parma" (7.4 *vs* 5.88) than Parma ham. This result is consistent with those previously reported by (ESTÉVEZ *et al*., 2003, 2006) who described a higher a* value, chroma and iron content in muscles from rustic pig breeds than in those from selected ones. In addition to the breed effect, the characteristics of the Iberian pigs' livestock farming could have influenced since the pigments and iron concentrations and the red colour of the muscles increase with the animal age and the physical exercise (LAWRIE, 1998).
This descriptor is linked to the sensory perception of meat color: consumers consider bright red meat as fresh and are adverse to brown meat.
In addition, the highest oiliness appearance of "Nero di Parma" agrees with values of Montanera ham (JURADO *et al*., 2007) due to a high fat content of "ham as underlined by CT. The highest value of oiliness produced the highest brightness value in "Nero di Parma" (5.27 *vs* 4,22).

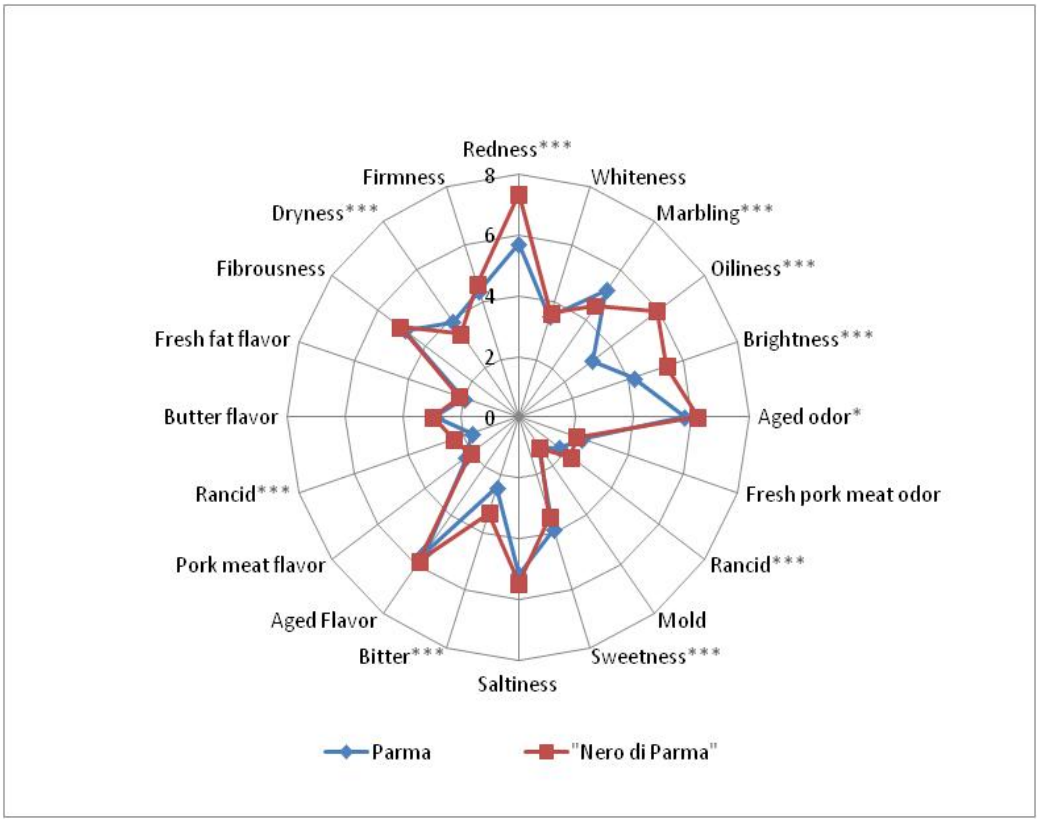

Figure 3. Sensory analysis results: mean values for each sensory descriptor by Parma and "Nero di Parma" ham. For each descriptor the relevant significance is reported.
(***significant at P< 0.001, **significant at P<0.01, *significant at P<0.05).

In addition, the fat unsaturation affects dryness of ham meat resulting higher in Parma ham according to results of RUIZ *et al*. (2002) obtained in meat.
However, an high fat content is associated with the phenomena of oxidation; the panel, both aroma and odor evaluation, found a greater value of rancid in the "Nero di Parma" ham (P <0.001 and P <0.003) than Parma ham. This attribute is often present in long-matured products, such as ham, and it is also reported in the Iberian typology (GARCIA-GONZALEZ *et al*., 2006; RUIZ *et al*., 2002). Rancidity is considered as a defective note of the product if present in high density. However, in contrast to the negative effect of lipid oxidation in pork, the formation of volatile lipid oxidation components during ripening/fermentation is important for the oxidative flavour note of Parma ham. It is well known that the typical ham aroma is related to intramuscular lipid composition and to the extent of lipolysis and oxidation of lipids during processing (BERDAGUÈ, *et al.,* 1993; BUSCAILHON *et al*., 1994).
In fact, muscle and adipose tissue lipids are subject to intense lipolysis by the action of lipases, generating free fatty acids that, at a second stage, are transformed to volatiles as a result of oxidation. Sensory profiles of dry cured ham are strongly affected by these enzymatic reactions (TOLDRA *et al.,* 1997).
"Nero di Parma" also showed the most intense aged odor according to the prolonged seasoning period (30 *vs* 24 months). As for marbling, a significant difference was obtained between the two types, higher in Parma than in "Nero di Parma" ham (5.13 *vs* 4.53). It must be emphasized that marbling score is not only affected by the total fat area of fat

flecks, but also by the distribution of fat (CHENG *et al*., 2015). The total lipid content of muscle was equal to an average value of 7.1% (s.e. 0.88) and 10.2% (s.e. 1.26) (P = 0.65) in Parma ham and "Nero di Parma ham" respectively. This result agrees with CT analysis as described below.

Taste descriptor related to sweetness, a typical characteristic of Parma ham, highlighted in Parma ham higher values (3.93 *vs* 3.43) than "Nero di Parma ham". In contrast, "Nero di Parma", characterized by a lower sweetness, resulted more bitter (3.18 *vs* 2.28); this attribute could be caused by a greater presence of myoglobin. Higher myoglobin levels in bull meat have been indicated as leading to greater sensations of metallic, liver, and bitter flavors. As recently reported, native pig breeds are characterized by the highest redness and the higher content of heme pigments (CEBULSKA *et al*., 2018).

In order to satisfy the requirements of the modern meat industry, it is important to develop some non-destructive, accurate, and rapid techniques for assessing meat quality and safety as recently reviewed (XIONG *et al*., 2017). Computerized tomography has been used mostly in medicine for diagnostic purposes. Nevertheless, it has also been proven to be useful in other fields and its application has been extended to palaeontology, geology and also to food technology and the meat science field. Application of computed tomography (CT) in meat science is based on the different X-ray attenuations that different tissues produce.

It has been demonstrated to be useful in the estimation of body composition in animals and to measuring lean percentage in pig carcasses (PICOUET *et al*., 2010).

In the present paper Hounsfield Units (HU), transformed by the software as cm^3, were then expressed as a percentage; the different distribution of lean and fat can be appreciated from the images reported in Fig. 4.

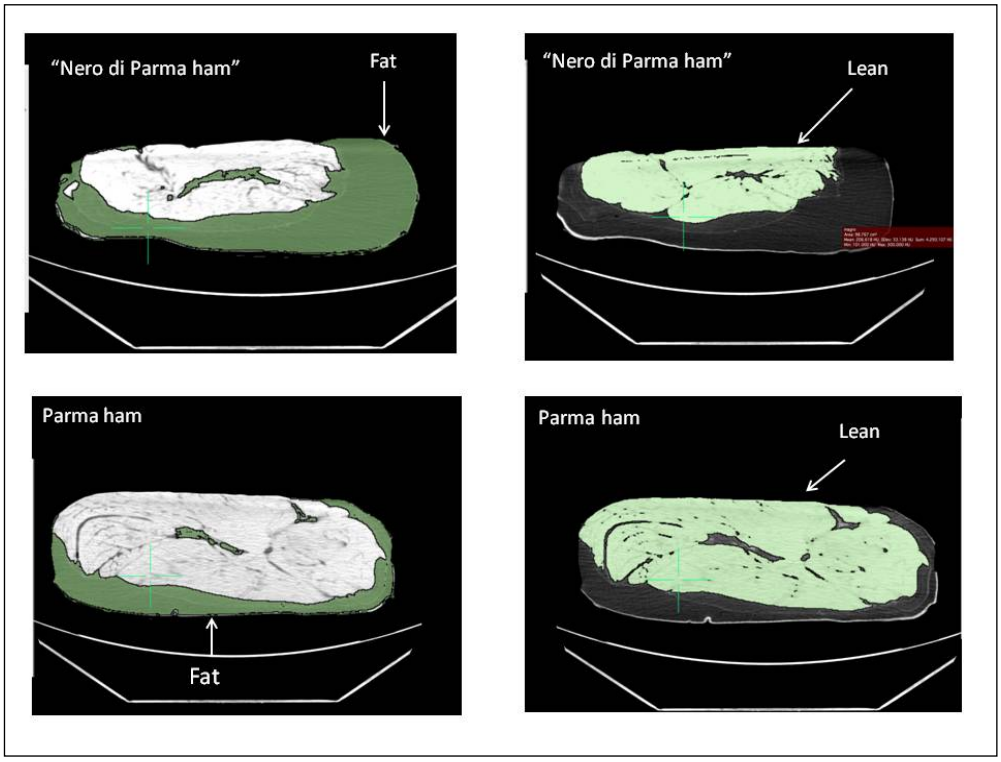

Figure 4. Distribution of fat and lean tissue in Parma ham and "Nero di Parma ham".

As graphically presented in Fig. 5 the opposite content of lean and fat component in the two types of analyzed hams ("Nero di Parma" 39% and 55%, Parma: 59% and 34%) could be noted respectively. Surprisingly, connective content is similar between the two types.
MONZIOLS *et al*. (2006) evaluating by magnetic resonance imaging the proportion of different carcass cuts reported a value of 62% (st. dev. 7.3) and 20.2% (st. dev. 6.3) in muscle and fat of ham respectively. The percentage of muscle overlaps the values found in Parma ham.
The highest fat content of "Nero di Parma ham" is due to an adaptive mechanism to the environment, which is known as thrifty genotype and which was firstly described in humans (NEEL, 1962) and also in Iberian pigs (GARCIA-CONTRERAS *et al*., 2018).
The thrifty genotype facilitates accommodation to seasonal cycles of feasting and famine because the ability to store fat in excess during food abundance enables survival during periods of scarcity.
An higher fat content implicates a longer ripening process from which depends all chemical and physical changes affecting volatile compounds during processing.

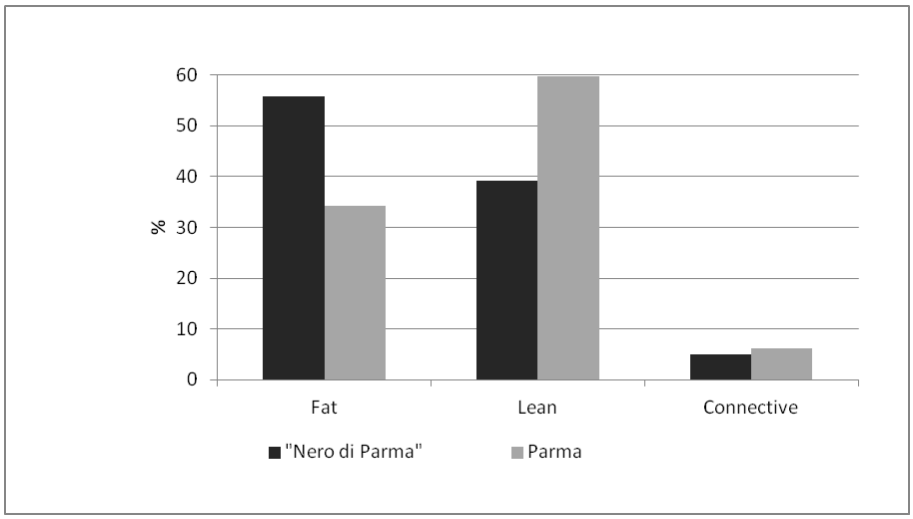

Figure 5. The lean, fat, and connective tissue surface ratio in "Nero di Parma" ham and Parma ham.

The connective tissue content of meat varies with species, chronological age, state of nutrition of the animal and muscle fiber characteristics (KLONT *et al.,* 1998). Meat texture is directly related to the size of muscle fiber and the amount of connective tissue (JOO *et al*., 2013). Moreover, connective tissue also undergoes morphological changes during meat-aging (BAILEY and LIGHT 1989; NISHIMURA 2015). The results of CT are consistent with the evaluation of the sensory analysis carried out on the ham; in this study no difference was found in texture also in accordance with the study of NISHIMURA (2010).

4. CONCLUSIONS

In conclusion, the results of the quality tests conducted on raw cured ham from the "Nero di Parma" pig emphasize high processing value and nutritional value of the meat of these animals. The profile of fatty acids and the proportions of individual fatty acids expressed in the form of disease risk indices (AI, TI) allow us to characterize the "Nero di Parma"

ham as a product with health value. “Nero di Parma” ham with the high monounsaturated and omega-3 fatty acids content, could play an important role for human health; moreover, the omega6/omega3 ratio was improved in “Nero di Parma” ham more than in Parma ham.
The present study provides additional data on Parma PDO ham with regard to the fatty acid composition, and sensory analysis, confirming literature and the positive evaluation of this product.
The sensorial profile of a typical product is the starting point for a strategy to enhance the typicality. The results of this preliminary study indicated that “Nero di Parma” ham, for which sensory investigations are not known, highlighted some descriptors such as oiliness, brightness, redness significantly different from Parma ham, and useful to define the sensorial profile of a typical product.
This study is only a first approach from which further investigations can emerge. The numbers will have to be expanded in order to reach a definitive sensorial profile and to characterize the “Nero di Parma” ham for the entire nutritional label.

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