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EXTRACTION AND PURIFICATION OF FERULIC ACID AS AN ANTIOXIDANT FROM SUGAR BEET PULP BY ALKALINE HYDROLYSIS

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ABSTRACT

Extraction of ferulic acid from sugar beet pulp was carried out using alkaline hydrolysis (NaOH) method and the effects of parameters on extraction were assessed. HPLC method and FT-IR spectrum of precipitate in purification method performed to prove that the isolated compound was ferulic acid. Finally the antioxidant activity of isolated ferulic acid was evaluated by ABTS⁺ method. Time, temperature and NaOH concentration were the most significant factors influencing ferulic acid extraction and severe alkali concentration has a negative dissociation effect on the ferulic acid content for all temperature/time conditions. The optimal conditions for extraction time, temperature and NaOH concentration were (12 hours, 41°C, and 2 M) respectively. Antioxidant capacity for isolated and pure ferulic acid was 0.39 ± 0.01 and 0.55 ± 0.01 respectively. It showed that sugar beet pulp is a potent source of ferulic acid that can be extracted and used as an antioxidant.

Keywords: antioxidant activity, ferulic acid, HPLC, response surface method, sugar beet pulp

1. INTRODUCTION

Extraction of major phenolic compounds from agricultural crop residues is important for the development of value-added products from renewable by-products. One of the functional compounds that may be extracted from agricultural biomass is ferulic acid (FA), which is the most abundant hydroxycinnamic acid found in plant cell walls that are covalently linked to polysaccharides and lignin (TILAY *et al.*, 2008). This phenolic compound is widely used in food, pharmaceutical and cosmetic industries because of different technologically beneficial functions as an antioxidant, anti-microbial and cross-linking agent (GRAF, 1992, MICARD *et al.*, 1999; OOSTERVELD *et al.*, 2001; ZHAO *et al.*, 2008) and also because of its therapeutic effects against cancer, diabetes, cardiovascular diseases (GHATAK *et al.*, 2010, DI DOMENICO *et al.*, 2009). Ferulic acid has been commercially produced from γ -oryzanol in rice bran oil because of easier process. However, the main part of this valuable phenolic acid is presented in plant cell walls cross linked with polysaccharides which may be released by enzymatic or chemical processes (OU *et al.*, 2007).

One of these potential sources is sugar beet pulp (SBP), a main by- product of sugar beet industries. It is a valuable by- product, but at present it is only used as animal feed. JANKOVSKA *et al.* (2001) set-up a method to determine the ferulic acid content of sugar beet pulp by high-pressure liquid chromatography. A few researches have been previously accomplished to extract ferulic acid from sugar beet pulp by alkaline and enzymatic methods (COUTEAU *et al.*, 1997; KROON *et al.*, 1996; JANKOVSKA *et al.*, 2005; DONKOH *et al.*, 2012), However, optimization of this process is necessary to understand the interactions between different parameters during extraction, and to minimize the negative effects of chemical processes. The main objective of this research was to compare total phenolic contents of methanol and alkaline sugar beet pulp extracts and optimize alkaline hydrolysis of sugar beet pulp for ferulic acid extraction through response surface methodology via central composite design. Finally antioxidant capacity of isolated ferulic acid from sugar beet pulp extract was evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Sugar beet pulp (SBP) was provided by Isfahan Sugar Factory (Isfahan, Iran). *Trans*-ferulic acid as external standard, ABTS (2,2'-Azino-Bis(3- ethylbenzthiazoline-6-Sulphonic acid)) and trolox was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), Sodium Hydroxide, ethanol, methanol, KBr, Gallic acid, ethyl acetate, potassium persulfate and Folin- Ciocalteu reagent were obtained from Merck Chemical Company.

2.2. Instrumentation

Mill type laboratory (Panasonic MX -J120-P made in Japan). HPLC (Agilent Technologies), equipped with a Zorbax C₁₈ column (length 150 mm×4.6mm dpi. 5 μ m particle size, 300 Å pore size, Agilent Technologies 1200 series, USA) and coupled online with a UV/Vis Agilent Technologies detector. Perkin Elmer spectrum 65 FT-IR spectrometer (USA). Perkin Elmer spectrophotometer, PTFE syringe-driven filters (0.22 μ m pore size) were provided by Biofil (Germany). Rotary evaporator (Heidolph co., Germany).

2.3. Sample preparation

Sugar beet pulp (SBP) was soaked in water for 3 hours to extract sugar residues and then it was dried in vacuum oven at 40°C for 12 h and ground in a laboratory mill (Panasonic MX-J120-P made in Japan). The powdered sample was passed through a sieve with mesh size 1 mm was taken for further investigations.

3. PREPARATION OF PLANT EXTRACTS

3.1. Extraction with methanol

Methanol was the most commonly used extraction solvent in the assay of phenolic compounds herbs in literatures (DAR *et al.*, 2011). In this study, 5 g SBP was mixed in methanol solution (99% v /v) and the extraction of phenolic compounds was performed by reflux for 6 hours at 60°C temperatures. Then, the pH of metalonic extracts was adjusted to 2.0, with HCl 6M for lignin precipitation. The mixture was filtered off, and subsequently the filtrate was centrifuged at 9000 rpm for 2 min. The supernatant was filtered and, evaporated to remove excess methanol followed by using a rotary evaporator (Heildolph Co., Germany) under reduced pressure at 40°C.

3.2. Extraction with sodium hydroxide

In this method, 5 g SBP was placed in an Erlenmeyer flask attached to a condenser, and mixed with a 100 mL NaOH (1M) solution. It was heated up to 60°C for 6 h and then cooled down to 20° C. Once the extraction process was completed, pH was reduced to 2.0, so that the hemicellulose would precipitate. The final mixtures were filtered off, and subsequently 150mL ethyl acetate was added to the filtrates in a 250mL baffled Erlenmeyer flask and was shaken in magnet stirrer (300 rpm) at room temperature for 15 min to carry out a liquid-liquid extraction (MAX *et al.*, 2009). The supernatant was vacuum evaporated to remove excess solvent. Then the concentrated extract, which contained the phenolic compounds was analyzed for total phenolic compounds.

3.3. Determination of total phenolics content

The total phenolics content of extracts was determined in accordance with a protocol described by TURKMEN and VELIOGLU (2005)[15]. 1 mL aliquot of each sample was mixed with 5 ml of Folin-Ciocalteu reagent (10% in distilled water) in a test tube. After 5 min, 4 ml of sodium carbonate (7.5% in distilled water) were added to each tube, the test tubes were cap-screwed and vortexed. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance was measured at 765 nm with a UV-vis spectrophotometer. Gallic acid was used as standard and the analyses were done in triplicate. The results were expressed as mg gallic acid (GAE)/g sugar beet pulp extract.

3.4. Experimental design

Response surface methodology (RSM) was used to determine optimum conditions for extraction of ferulic acid from sugar beet pulp. The experiments were designed according to the central composite design (CCD), the most widely used form of RSM. Three factors including time (x_1), temperature (x_2) and sodium hydroxide concentration (x_3) were chosen, and ferulic acid concentration (y) were determined using optimization method (Table 1).

Table 1: Independent variables and their coded and actual values.

Parameters	Symbols	Coded levels of variables				
		-1.6817(-α)	-1	0	1	1.6817(+α)
Time (hour)	X ₁	2	4.0	7	9.9	12
Temperature(°C)	X ₂	30	36.0	45	53.9	60
Concentration of NaOH(Molar)	X ₃	0.5	0.8	1.25	1.69	2

Each factor was studied at five different levels (-1.68, -1, 0, 1, 1.68). All variables were taken at a central coded value considered as zero. Equation (1) represents the three-variable mathematical model:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (\text{Eq.1})$$

where y is ferulic acid concentration, β_0 is the intercept term, β_1 , β_2 and β_3 are the linear terms, β_{11} , β_{22} and β_{33} are the quadratic terms and β_{12} , β_{13} and β_{23} are the interaction terms between three independent variables. The design contains a total of 20 experimental trials.

3.5. Determination of ferulic acid concentration

The prepared methanolic extracts were passed through 0.22 µm PTFE filters and 10 µl of the filterates were injected into a HPLC system, Agilent Technologies, equipped with a Zorbax C₁₈ column (length 150 mm×4.6mm dpi. 5 µm particle size, 300 Å pore size, Agilent Technologies 1200 series, USA) and coupled online with a UV/Vis Agilent Technologies detector. The flow rate was 1.0 mL/min and the oven temperature was 35°C. The mobile phase consisted of methanol and water (1% HAc)(65:35, v/v) and the detector was set at 320 nm. All quantitative analyses were made by the external standard method using ferulic acid as an analytical standard.

3.6. Conformity tests to declare the mathematical model validity

Conformity tests were carried out with the same experimental conditions to examine the accuracy of the mathematical model correlations. The error percentage was then calculated according to equation 2 (Madadi *et al.*, 2012):

$$\text{Error(\%)} = (\text{actual values} - \text{predicted values})/(\text{actual values}) \quad (\text{Eq.2})$$

3.7. Ferulic acid purification

Ethanol 95% was added to the brownish extracts to obtain a final concentration of 30% ferulic acid. Then, the murky solution was centrifuged at 11,000 g for 20 min (BURANOV *et al.*, 2009). The supernatant was vacuum evaporated to purify ferulic acid. For further purification of the ferulic acid, it was dissolved in a 6 ml anhydrous ethanol, resulting in a less intense murky solution, and again centrifuged for 20 min at 11,000 g. The supernatant was vacuum evaporated and precipitate was analyzed using Fourier transform infra-red (FT-IR) and the methanolic solution of precipitate was injected to high performance liquid chromatography (HPLC) (BURANOV *et al.*, 2009).

3.8. FT-IR spectroscopy

Isolated ferulic acid spectra was recorded on a FT-IR spectrometer in the range of 400-4000 cm^{-1} using a KBr disc containing 1% finely ground samples (KUNST *et al.*, 2003).

3.9. Measurement of antioxidant capacity of isolated ferulic acid

Antioxidant capacity of isolated ferulic acid from sugar beet pulp and pure ferulic acid were performed immediately by ABTS method. The method used was the ABTS $^{\bullet+}$ (radical cation) decolourisation assay (Alanon *et al.*, 2011). This assay was based on the ability of different substance to scavenge ABTS cation radical (ABTS $^{\bullet+}$ +(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid))). The radical cation was prepared by mixing 7mM of ABTS stock solution with 2.45mM potassium persulfate(1/1,v/v) and leaving the mixture for 4-16h until the reaction was complete and the absorbance was stable. The ABTS $^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734nm for measurement. The photometric assay was conducted on 0.9 ml of ABTS $^{\bullet+}$ solution and 0.1 ml of tested sample and mixed for 45 sec; measurements were taken immediately at 734nm after 15min. The antioxidant activity of the tested sample was calculated by determining the decrease in absorbance by using the following equation:

$$E = ((A_c - A_t) / A) \times 100$$

Where A_t and A_c are the respective absorbance of tested sample and ABTS $^{\bullet+}$ was expressed as μmol . Trolox chosen as standard antioxidant and the standard reference curve was constructed by plotting % inhibition value against Trolox concentration between 10 and 600 μM . Antioxidant activity measurement, expressed as Trolox equivalent antioxidant capacity (TEAC). Each sample was measured in triplicate. Mean and standard deviation ($n = 3$) were calculated.

3.10. Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's test was carried out to test for differences between extractants (methanol and alkaline hydrolysis) in the statistical program SPSS ver. 15.0. Significance of differences was defined at the 5% level ($p < 0.05$). The experimental design and statistical analysis were performed using MiniTab software (version 16).

4. RESULTS AND DISCUSSION

4.1 Total phenolic content

Significant differences were found in total phenolic contents between two extracts. alkaline extracts contained higher amounts of polyphenols than methanolic extract. The total phenolics content of methanolic and alkaline extract were 121.45 ± 1.32 and 758.638 ± 3.27 mg GAE/100 g db, respectively. The results showed that alkaline treatment led to retained higher phenolics, which might be due to an alkaline hydrolysis breaks the ester bond linking phenolic acids to the cell wall and thus is an effective way to release phenolic compounds from polysaccharides. It is clear that chemical processes are more efficient to extract phenolic compounds by hydrolyzing the covalent esteric bonds.

In structure of sugar beet pulp cell wall, phenolic compounds such as ferulic and cumaric acids were etherified to lignin and arabinoxylans and forms an alkali-labile cross-link between these two cell wall polymers (TORRE *et al.*, 2008). Such relatively higher content of alkali-labile cross-linkages within the lignin network or between lignin and polysaccharides might explain the fast and easy solubilisation of both phenolic acids by alkaline treatments (NOOR HASYIERAH *et al.*, 2011). In other researches, alkaline treatments are commonly used to extract bound phenolic acids and other related compounds from cereal grains, and alkaline hydrolysis totally releases the bound phenolics in a short time at high alkali concentration and temperature (OUFNAC *et al.*, 2007). Phenolic acids such as benzoic and cinnamic acids could not be effectively extracted with pure organic solvents, so mixtures of alcohol-water or alcohol-alkali are recommended. Our results are in agreement with the published results (STALIKAS, 2007; OUFNAC *et al.*, 2007).

4.2. Identification of ferulic acid

The intense peak at $R_t = 3.39$ min in the chromatogram of alkaline extracts revealed the presence of ferulic acid in the sugar beet pulp extract. (Fig. 1 a and b).

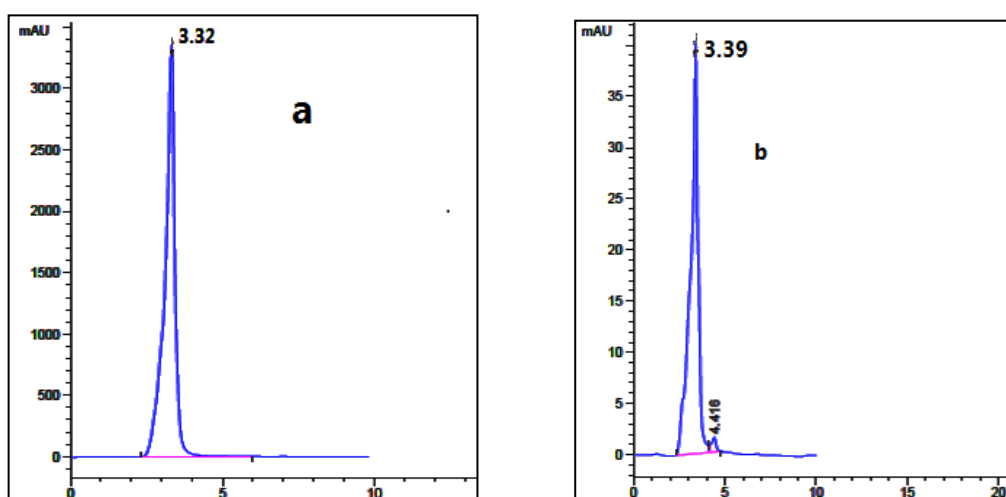


Figure 1: HPLC chromatogram of ferulic acid in C18 reverse phase chromatography at 320 nm. a) standard, b) alkali treated sugar beet pulp extract (peak of ferulic acid = 3.39 min).

Concentration of ferulic acid were determined according to calibration curve. The concentration of FA in the alkaline hydrolysates obtained in different extraction conditions designed by RSM are presented in Table 2.

Based on the RSM method, result of experimental analysis was presented in Table 3. In these results, those effects with calculated P-values less than 0.05 would be significant in the studied range of parameters.

Table 2: Experimental extraction conditions and ferulic acid concentration.

Run Order	Time (h) X_1	T(°C) X_2	NaOH (M) X_3	Concentration of ferulic acid (g/100g)
1	7.0	45.0	1.25	0.800
2	7.0	45.0	1.25	0.789
3	9.9	53.9	1.69	1.000
4	4.0	53.9	0.80	0.342
5	7.0	45.0	0.50	0.230
6	4.0	53.9	1.69	0.600
7	7.0	45.0	1.25	0.700
8	2.0	45.0	1.25	0.341
9	7.0	60.0	1.25	0.800
10	7.0	45.0	2.00	1.000
11	12.0	45.0	1.25	0.946
12	4.0	36.0	0.80	0.241
13	9.9	53.9	0.80	0.754
14	7.0	45.0	1.25	0.633
15	7.0	45.0	1.25	0.700
16	7.0	45.0	1.25	0.620
17	4.0	36.0	1.69	0.565
18	7.0	30.0	1.25	0.476
19	9.9	36.0	0.80	0.415
20	9.9	36.0	1.69	1.100

Based on the results shown in Table 3, the extraction of ferulic acid was significantly affected by time (x_1), temperature (x_2), NaOH concentration (x_3) and the coupling terms between x_1 and x_3 , while the interaction between terms x_1x_2 and x_1x_3 , and the second order effect of term x_1x_2 and x_3 on FA concentration were insignificant ($P > 0.05$).

Therefore, after eliminating the statistically insignificant values, the final model is given in Eq. (3) as follows:

$$y = -2.10380 + 0.035 x_1 + 0.051 x_2 + 1.355 x_3 - 0.0158 x_1 x_3 \quad (\text{Eq.3})$$

The positive and negative signs in front of the terms indicate the synergistic and antagonistic effect of each term on the model response, i.e. FA concentration. The coefficient of determination (R^2) for empirical equation from Eq. (3) was 0.952.

Table 3: ANOVA analysis for Central Composite Design.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Linear	3	1.11115	1.11115	0.370385	62.14	0.000	
A -Time	1	0.47184	0.47184	0.471844	79.16	0.000	Sign ≤ 0.05
B-Temp	1	0.06196	0.06196	0.061963	10.40	0.009	Sign ≤ 0.05
C-Concentration	1	0.57735	0.57735	0.577348	96.86	0.000	Sign ≤ 0.05
time*time	1	0.00444	0.00733	0.007332	1.23	0.293	
temp*temp	1	0.00658	0.00865	0.008651	1.45	0.256	
con*con	1	0.01535	0.01535	0.015347	2.57	0.140	
Interaction							
time*temp	1	0.00133	0.00133	0.001326	0.22	0.647	
time*con	1	0.01523	0.01523	0.015225	2.55	0.141	
temp*con	1	0.03188	0.03188	0.031878	5.35	0.043	Sign ≤ 0.05
Lack-of-Fit	5	0.03109	0.03109	0.006218	1.09	0.463	
Pure Error	5	0.02852	0.02852	0.005703			

$R^2 = 95.21$ & $\alpha = 0.05$ - Significant parameters ($P\text{-value} < 0.05$).

In order to determine the effect of the three independent variables on extraction yield, extraction parameter graph and response surface were generated as a function of two variables, while the third one was held constant at its middle level. The optimum region was determined in terms of maximum concentration of ferulic acid (Fig. 2).

To assess the quality of the model and to measure how well the suggested model fit the experimental data, the parameters, F-value, lack of fit and R^2 were used (Montgomery, 2005). In our research F-values were 82.85 and lack of fit of the model was not significant, which implied that the models were significant. The determination coefficients ($R^2 > 0.95$) were obtained from ANOVA of the quadratic regression models, indicating that less than 4.8% of the total variations was not explained by the suggested model.

4.3. Effect of the each process parameter on ferulic acid extraction

4.3.1. Temperature

As shown in Fig. 3, increasing the extraction reaction temperature may cause higher concentration of ferulic acid released, while the other two factors (i.e. time & alkali concentration) were constant. It is well known that sugar beet root contains significant quantities of ferulic acid which is etherified to lignin and /or arabinoxylans and through alkali-labile cross-linkages (SCALBERT, 1986). Therefore, cleavage of these bonds at the same time may be possible at higher temperatures. An increase in temperature from 30 to 60°C and time from 2 to 12 hr resulted in an increment of FA from 0.3 to 0.9g/100g when alkali concentration was fixed at the middle level (Fig. 2a).

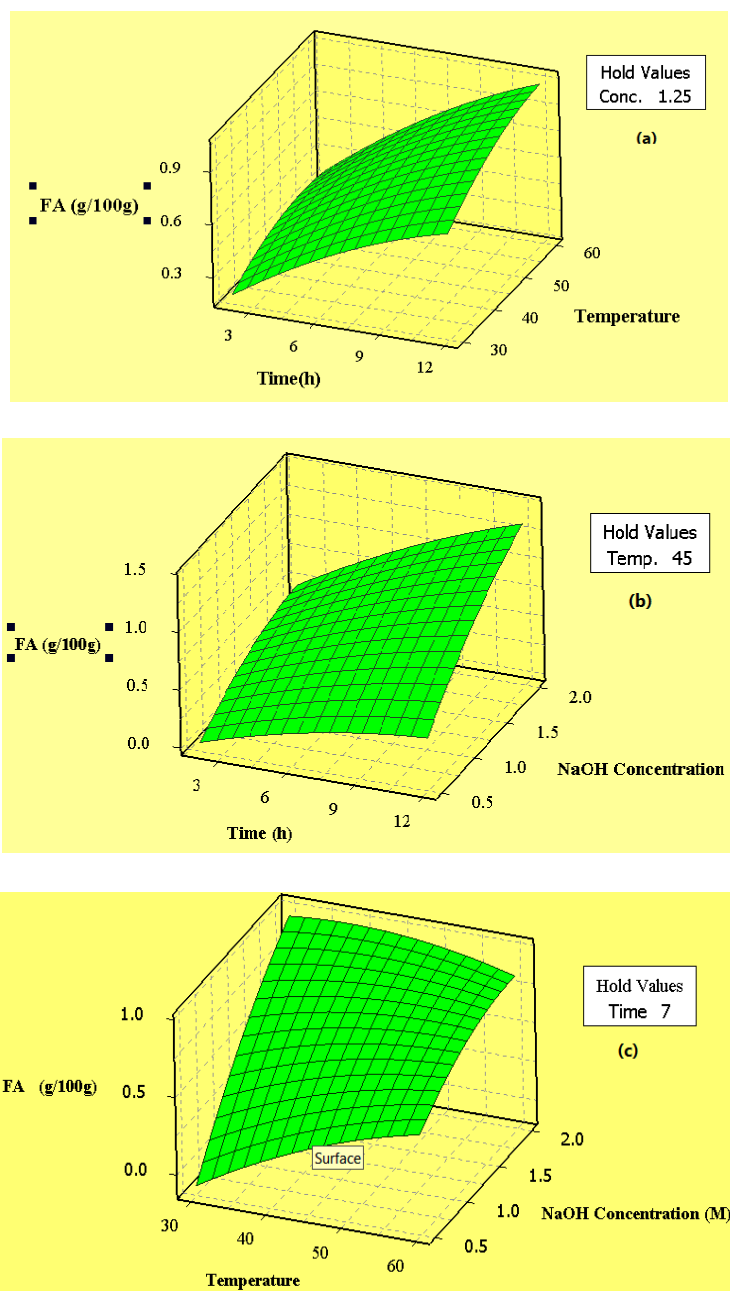


Figure 2: 3D response surface plot of the interactions. (a) time and concentration when temperature was fixed at 45°C, (b) temperature and time when concentration was fixed at 1.25 M, (c) concentration and temperature when time was fixed at 7 h

XU investigations (2005) showed that an increase in alkaline treatment temperature had an important effect on the release of ester-linked p-coumaric and ferulic acids from the cell walls of various cereal straws.

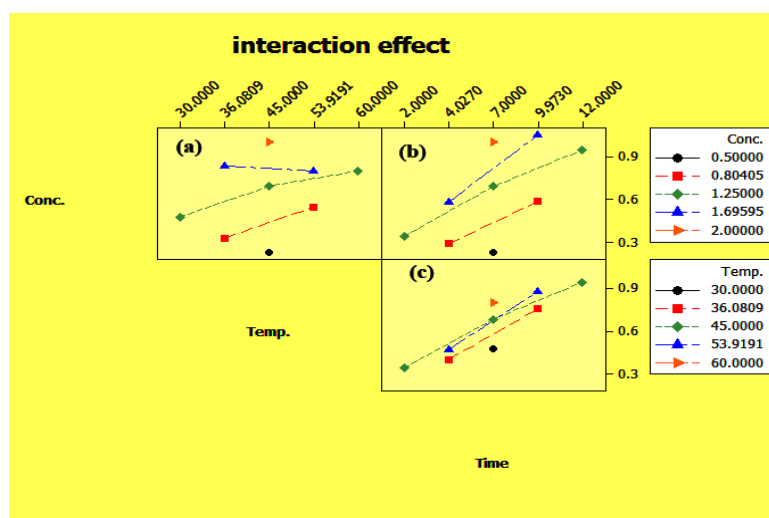


Figure 3: Interactions between extraction parameters NaOH concentration and temperature(a), NaOH concentration and time(b), time and temperature (c).

4.3.2. NaOH concentration

As presented in Fig. 2b, the ferulic acid concentration significantly increased with NaOH concentration. This is because raising the alkaline treatments may dissolve lignin by cleavage of ester linkages in lignin–polysaccharide complexes, which lead to the release and solubilization phenolic acids. Different alkaline compounds such as NaOH, Ca(OH)₂, NH₄OH, and H₂O₂ have been previously utilized as catalysts for hydrolysis treatments (RODRIGUEZ-VAZQUE *et al.*, 1994). In the present study, NaOH was selected because of its more selective action, compared to the other agents, in releasing phenolic compounds such as ferulic acids (TORRE *et al.*, 2008). It may be obvious that using a too mild alkaline condition may not act effectively for ferulic acid extraction. These results have also been confirmed in previous studies (MUSSATTO *et al.*, 2007; TORRE *et al.*, 2008).

The effect of time in alkaline hydrolysis on the extracted FA concentration is shown in Fig. 2b. By increasing reaction at constant temperature, alkali has more time to release ferulic acid. A positive interaction between the time and NaOH concentration and the time and temperature process on the extracted ferulic acid has been observed (Figs. 3 b and c). However for higher concentrations of NaOH (1.69M), the slope of graph was increased as compared to the low concentrations (0.8 M). At higher concentration of NaOH, accelerated solubilisation of ferulic acid has been observed. It may be emphasized that ferulic acid solubilization exhibited a time- dependent behaviour and reached a maximum concentration after 12 h of hydrolysis with 2.0N NaOH (Fig. 3 b).

But the main point that should be considered is that severe alkali concentration has a negative dissociation effect on the FA for all temperature / time conditions (Fig. 2c and Figs. 3 a and b).

According to the obtained results, the main key factors affecting the releasing rate of phenolic compounds are alkali concentration and hydrolysis duration. The same result has previously been reported for the effect of these factors on the yield of extraction from the other sources such as paddy straw, sugar cane baggass and agricultural wastes (NOOR HASYIERAH *et al.*, 2011; XU *et al.*, 2005; TILAY *et al.*, 2008).

According to the results in Table 3, interaction between temperature and NaOH concentration (x_2x_3) had a statistically significant effect on the extraction yield while the others (i.e: x_1x_2 and x_1x_3) were insignificant. Increasing the temperature from 36°C to 53°C at a constant time (7 h) and low concentration of alkali (0.8 M) improved the ferulic acid extraction (from 0.32 to 0.54 g/100g FA), but at the same temperature range, using high concentration of NaOH caused a reduction of FA concentration (Fig. 3a). This is because of the opposite effect of temperature and concentration of NaOH on FA extraction, which is shown in Eq. (3) by negative sign of the term, x_2x_3 .

A clear decrease in ferulic acid concentration took place after the threshold was exceeded and confirmed this negative interaction, so that when temperature was increased from 45 to 60 °C and alkali concentration was raised from 0.5 to 2.0M, an oxidative degradation might happen (Fig. 2c). No significant degradation was detected at the lowest alkali level (0.5M). It has also been previously shown that ferulic acid, in its monomeric form, is more resistant to oxidation than its dimeric form during alkaline hydrolysis (BAUER *et al.*, 2012).

Finally, it may be emphasized that the amount of ferulic acid obtained through CCD in the present research was 1.29 g/100 g, which is higher than the maximum amount reported by ZHAO (2008) in sugar beet pulp (0.800 g/100 g); and this improvement may be mainly due to the optimum condition selected for the extraction process.

Normal probability plot of residuals produced an approximately straight line which indicated a normal distribution of residuals (the deviation between predicted and actual values) and confirmed the accuracy of the model (NOOR HASYIERAH *et al.*, 2011).

4.4. Validating the optimal conditions

The optimum conditions, as suggested by the software, were 41°C, 2 M NaOH, 12 h corresponding to 1.33 g/100 g (predicted value). For verification of these conditions, four replicates were carried out to determine the highest experimental value of ferulic acid. The highest ferulic acid production obtained at these conditions was 1.29 g/100 g.

The results of the conformity test on the basis of determining the error percentage demonstrated that process optimization in CCD reliably predicted the FA with acceptable accuracy(3.1%).

4.5. FT-IR spectra and validation of precipitate

In the purification stage, the precipitate that obtained from alkaline extract of 5 g sugar beet pulp was analysed by FT-IR and HPLC method. The FT-IR spectrum of the precipitate was compared with the spectrum of the pure ferulic acid to confirm it (Fig. 4).

The FT-IR spectrum of sample clearly showed the existence of main functional groups in the ferulic acid structure, and the strong and broad band at 3,331.08cm⁻¹ is characteristic of the OH group in phenolic compound. A part from this, C-H stretching of the aromatic ring was at the 2925.82 cm⁻¹ band. The band at 1,649.02 cm⁻¹ corresponds to that of the carbonyl group (C = O). Stretching band at 1,328.87 cm⁻¹ is characteristic of C-H vibration on the methyl group, While the vibration for C = C on the aromatic ring is at 692.77 cm⁻¹ band (ROBERT, 1998).

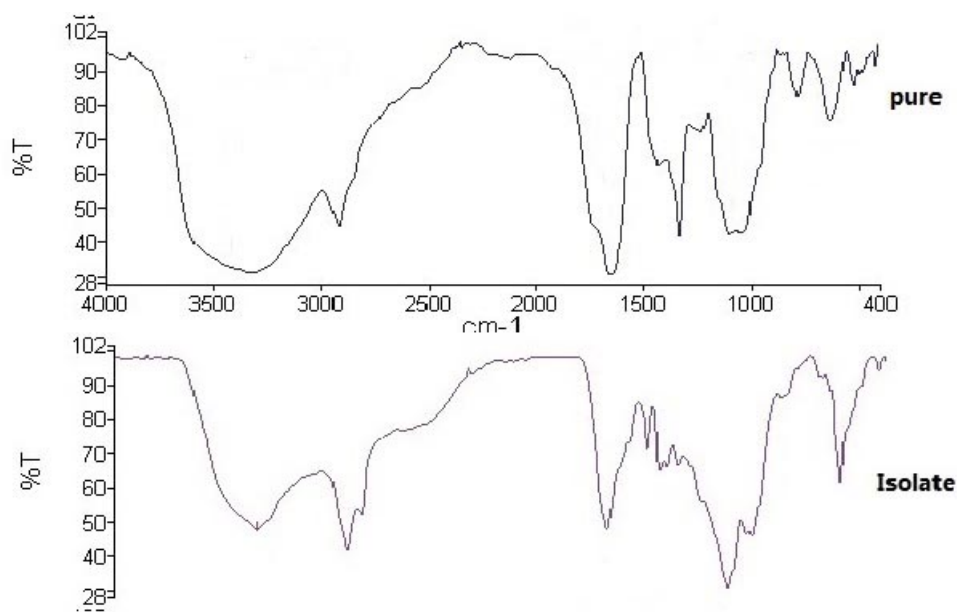


Figure 4: FT-IR spectra of ferulic acid (isolate and pure).

The precipitate was then analyzed using high performance liquid chromatography (HPLC) to reaffirm the results as characterized by the IR analysis. 0.15 mg of precipitate was collected and dissolved in 10 ml of methanol solution acting as the solvent before the analysis. Finally, the 10 μ l of concentrated sample was injected into a HPLC machine. The validation was performed with sample of precipitate based on the relative retention times (RRTs) and relative peak areas (RPAs). The percent of recovery was 64.88% and precision was assessed by analyzing three replicate samples and the relative standard deviation (RSD) was below 2.02% and 4.47% for RRTs and RPAs, respectively.

4.6. Measurement of antioxidant capacity of isolated ferulic acid

Antioxidant capacity results expressed as μ mol of Trolox equivalents per milligram of samples. The ABTS cation radical (ABTS \bullet^+) (Pisoschi and Negulescu2011) which absorbs at 734 nm (giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization. Antioxidant capacity for isolated and pure ferulic acid was 0.39 ± 0.01 and 0.55 ± 0.01 respectively. Significant differences were found at a significance level of $p < 0.05$ between isolated and pure samples in the antioxidant capacity values which indicates that precipitates need more purification stage to made pure. Result showed that sugar beet pulp is potent source of ferulic acid that can be extracted and use as an antioxidant.

5. CONCLUSIONS

The present study demonstrated that alkaline treatment led to release higher phenolic compounds than methanolic method and the results showed that temperature, time and NaOH concentration had significant effects on the ferulic acid solubilization in alkaline media for extraction. The coefficient of determinations (R^2) for predicted ferulic acid

content showed good correlation with the experimental data at 95% confidence level. The amount of extracted ferulic acid at optimized conditions obtained from the model (i.e: 12 h, 41°C and 2 M) was 1.29 g/100 g. The FT-IR spectrum of isolated sediment clearly showed the existence of main functional groups in the ferulic acid structure. Significant differences were found at a significance level of $p < 0.05$ between isolated and pure ferulic acid in the antioxidant capacity values which indicates that precipitates need more purification stage to made pure. In conclusion suggest that sugar beet pulp is potent source of ferulic acid that can be extracted and use as an antioxidant.

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QUALITY INDICATORS FOR MODIFIED ATMOSPHERE PACKAGING (MAP) STORAGE OF HIGH-QUALITY EUROPEAN PLUM (*PRUNUS DOMESTICA* L.) CULTIVARS

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ABSTRACT

The use of quality indicators is crucial in selling plums in more distant markets and the evaluation of freshness through multiple index is fundamental to evaluate the goodness of the storage technique. In this study we evaluated the quality of two european plums cultivars ('Ramasin' and 'Arido di Core' with purple and yellow flesh colour respectively) after modified atmosphere packaging (MAP) storage, through the selection of the most appropriate indicators. The headspace gas composition, the flesh fruit firmness (FFF), the soluble solid content (SSC), the titratable acidity (TA), the colour and the chlorophyll content of plums wrapped with 5 different films (F1, F2, F3, F4 and F5) were evaluated for up 21 days of storage (at $1\pm1^{\circ}\text{C}$ and 90-95% relative humidity). For both cultivars, the multilayered films (F1 and F2, 90 and 65 μm respectively) offered better effectiveness over other films. The total chlorophyll concentration, showing a good correlation with the colorimetric parameters of luminance (L^*) and chroma (respectively $R^2=0.92$ and $R^2=0.96$) confirmed, in the case of the Arido di Core cultivar, the results obtained by monitoring other parameters thus highlighting the usefulness of integrating multiple indexes in evaluating the performance of the storage methods used.

Keywords: Plum, film, quality index, chlorophyll, passive atmosphere

1. INTRODUCTION

Thanks to its adaptive behaviour in different climatic conditions, plums represent one of the most versatile of fruit trees species and its production is considered valuable for the future development of fruit trees sector (BLAZEK, 2007; SOTTILE *et al.*, 2010a). In recent years the recovery and development of high quality local germplasm cultivars, such as the Ramasin in Piedmont and Ariddo di Core in Sicily, have evidenced a high level of diversity along the Italian production of the European plum (*Prunus domestica* L.). These cultivars have been important for local market since years; more recently they are also being coveted by wider markets for the high nutraceutical characteristics of fruits (SOTTILE *et al.*, 2010b). The wide ripening period for such cultivars, combined with the different areas of cultivation, support an extension to the commercial calendar and represent a good opportunity for expanding the market for these fruits. The availability of numerous varieties, together with quality and price indicate the efficiency of the supply chain and distribution system which is today one of the most important sales channels in the area of horticultural fresh products (DEAN, 2011). The post-harvest management of these fruits appears problematic because plums evidence a cultivar-dependent high perishability, and require specific cares in terms of handling along all the supply chain. If not combined with other storage techniques, low refrigeration temperatures are not sufficient to maintain fruit quality up to the consumer; in fact, prolonged exposure to low temperatures would be responsible for enzymatic browning of the internal tissues and of the formation of skin damages (TAYLOR *et al.*, 1993; ABDI *et al.*, 1997). Among the different storage techniques such the use of absorbers (SHARMA *et al.*, 2012) or antagonists of ethylene (SINGH and SINGH 2012), the modified and the controlled atmospheres (ELZAYAT and MOLINE, 1995; PRANGE and DELONG 2006; GIUGGIOLI *et al.*, 2008; GIRGENTI *et al.*, 2010; DIAZ-MULA *et al.*, 2011 a, DIAZ-MULA *et al.*, 2011 b, SOTTILE *et al.*, 2013) are well known to have positive effect to improve the shelf life of plums. Active MAP on Sanacore and Ariddo di Core plums was performed with wrapped bulk preserving the quality more than 40 days for local consumption (PEANO *et al.*, 2010) and different MAP box liners were used to maintain the shelf life and the quality of 'Friar' plums (CANTÍN *et al.*, 2008). A key issue to success is also represented by high uniformity of the fruits as regards quality parameters (CRISOSTO *et al.*, 2004). For this reason the selection and the monitoring of quality indicators is important not only for defining the time for harvesting but also for the maintainance of the commercial value of the product. There are several studies on the evolution of quality parameters during plum post-harvesting fruit management (USENIK *et al.*, 2008; PÉREZ-MARÍN *et al.*, 2010; SOTTILE *et al.*, 2013;) but the identification of indicators useful to monitor quality along the distribution is still difficult. The pulp firmness and its relative evolution during storage is closely cultivar-dependent and specifically related to the stage of maturity at harvest time (SHARMA *et al.*, 2012); anyway, if not integrated with other quality indicators, this parameter would be impractical for the fresh fruit market due to the absence of reference classes especially for European plums (VALERO *et al.*, 2007; USENIK *et al.*, 2008). In case of deeply pigmented cultivars, due to an usual early change of the skin colour, the pulp firmness is usually adopted as a ripening indicator (CRISOSTO and Kader 2000; SOTTILE *et al.*, 2010 a). Generally, for stone fruits, the skin colour is one of the most important harvesting markers; the quantitative and qualitative development of the pigments in the skin is able to characterise the epidermis (chlorophyll, anthocyanins and carotenoids); the development of these pigments is closely related to the biological and physiological stress during the storage of the fruits (MERZLYAK *et al.*, 1997; ABBOTT 1999). According to previous studies (SHARMA *et al.*, 2012; VALERO *et al.*, 2013) in some case the skin colour of purple-flesh plums is not a parameter useful to assess the effectiveness of differing

storage treatments. All these aspects are very important in affecting the aesthetic appearance of the product (ABBOTT 1999), while they have several limits and they are not always positively correlated to a correct stage of fruit ripeness (USENIK *et al.*, 2008). It has been reported that a total soluble solids content (SSC) ranging from 14 to 16% (WESTWOOD 1978) or from 10 to 15% (DIAZ-MULA *et al.*, 2009) determines fruit ready for consumption. However, the aromatic profile of plums, as of most stone fruit species, is even more affected by the total titratable acidity (TA) value than to the sugar content (CRISOSTO *et al.*, 2004; CRISOSTO *et al.*, 2007). Many studies (ZIOSI *et al.*, 2008; INFANTE *et al.*, 2011) have revealed a close correlation between chlorophyll content within the pulp tissues of the stone fruit and the ripening degree of the fruit; this evidence demonstrates that visible UV spectroscopy is a non-destructive technique which could be considered useful for monitoring and characterising the different stages of fruit ripening (ZUDE *et al.*, 2003; CECCARELLI *et al.*, 2008). It is therefore evident that the evaluation of the effectiveness of any post-harvest treatment should consider the uniformity of the fruit by including multiple quality indices; this aspect should be more valuable for those cultivars that are often considered minor for the lower diffusion but with a high commercial capacities if new post-harvesting techniques, such as modified atmosphere packaging (MAP), are developed.

On the basis of these considerations, the aim of this work was to evaluate the influence of the different packaging films used for MAP storage up to 21 days at $1 \pm 0.5^\circ\text{C}$ of two European plum cultivars characterized by high tasting excellence and differing pigmentation (yellow and purple) also in order to assess the most important quality indices for fresh consumption.

2. MATERIALS AND METHODS

2.1. Fruit samples

Two European plum cultivars (*Prunus domestica* L.) were used, both belonging to local Italian germplasm and with different pigmentations: the cv. 'Ramasin' with a purple-coloured flesh is from the Piedmont territory and was harvested in mid-July; the cv. 'Ariddo di Core' is a yellow-coloured flesh variety from Sicily and is harvested in August. Both cultivars are characterised by fruits of small size and limited shelf life but with a high tasting quality well recognized by the local consumers. Fruits were picked by hand, and selected based on size uniformity and absence of damage. After a refrigeration (2 hours) they were placed in polyethylene terephthalate (PET) trays and transported within 24 hours to the fruit and vegetable warehouse (Agrifrutta Soc. Coop. S.R.L. - Piedmont, Italy) for storage-testing.

2.2. Packaging and storage conditions

The fruit samples were unwashed previously to be packaged. For both cultivars the sampling unit considered was the flowpack. The PET 0.250 kg trays (L14 x w9.5 x h5) were heat sealed with different films using a Taurus 700 model horizontal machine (Delphin, Italy). The materials used for the different packages were:

F1: multilayer produced by co-extrusion of PET, EVOH and PE of $90\mu\text{m}$, (Corapack, Italy);
F2: multilayer produced by co-extrusion of PET, EVOH and PE of $65\mu\text{m}$, (Corapack, Italy);
F3: polypropylene (PP) film, $25\mu\text{m}$, (Trepack, Italy);

F4: low density monolayer polyethylene (PE) film, 25 μm , (Trepack, Italy);
 F5: non commercial biodegradable film, 25 μm , (Novamont, Italy);

For each package, the control sample (Control) is represented by fruits preserved using a polypropylene (PP) macroperforated (6 mm diameter holes) film of 25 μm (Trepack, Italy). The O_2 and CO_2 transmission rate properties of the films were measured at 23°C and 50% of relative humidity in accordance with ASTM F 2622-08 and ASTM F 2476-05 standards (Table 1). With the exception of the biodegradable film (F5) whose water permeability value was supplied directly by the manufacturer (147 $\text{cm}^3 \text{ m}^{-2} 24\text{h}$), tested films resulted within the high water barrier film classification (VAN TUIL, 2000). All fruits were packed under normal atmospheric conditions (0.2 CO_2 kPa / 21.2 O_2 kPa). This was performed in order to create passive modified atmosphere packaging (MAP) during storage conditions through to the synergistic action of the fruit respiratory metabolism and the selectivity of the film to the gases. Due the macro hole (6-mm-diameter) the PP film (control) has no changed the atmosphere inside the packages along all the storage time. The fruits were stored for all the period under constant refrigeration conditions based on $1\pm0.5^\circ\text{C}$ with a relative humidity (RH) level of 90-95% and in the dark. Qualitative evaluations were performed at the picking time (0) and after 7, 14 and 21 days of storage.

Table 1: Characteristics of film used for MAP storage of plum fruits.

Film gas transmission rate at 23°C and 50% UR $\text{cm}^3/(\text{m}^2 24\text{h bar})$		
Film	O_2 (ASTM F2622-08)	CO_2 (ASTM F2476-05)
F1	1572	6111
F2	1572	6111
F3	1456	4616
F4	10990	55360
F5	2276	44494

2.3. Headspace Composition and Qualitative Parameters

Sampling of the gases (O_2 and CO_2) within the headspace of the packaging was performed with a Check Point II portable gas analyser (PBI Dansensor, Italy). Three random trays were used for each measurement for a total of 0.750 kg of fruit. In order to avoid any alteration to its internal atmosphere, the air sampled for analysis was fed back into the container using a porous septum (15 mm diameter PBI Dansensor, Italy) positioned on the surface of the film. Instrument calibration was performed after each measurement using a vacuum sample in normal atmosphere (ADAY and CANER, 2011). The value is recorded as kPa and it is the average of three measurements.

The weight of each container was measured using an electronic balance (SE622, WVR Science Education, USA) with an accuracy of 0.01 grams, at the harvest and at the end of each storage period. The relative weight loss was expressed as a percentage (%). The fruit flesh firmness (FFF) (kg/cm^2) was measured by a manual penetrometer (Facchini, Alfonsine, Italy) using a pipette tip with a 7.9-9 mm diameter in accordance with species standards. The skin of the fruit was not removed. Each value is the average of two measurements taken from opposite sides of each fruit. The data recorded is the average of

30 measurements (three random trays for a total of 0.750 kg of fruit). Soluble solids content (SSC) were determined in the juice (from three trays randomly chosen for each treatment) with a digital refractometer Atago PR-101 (Atago, Japan) at 20°C. Two readings (30 fruits) were taken on each fruit and averaged; results were expressed as °Brix.

The titratable acidity (TA) (meq/L) was measured with an automatic titrator (Titritino plus 484, Metrohm, Switzerland); 5 mL of pulp juice were used for each sample (shaken, centrifuged and filtered), diluted in 15 mL of distilled water which was neutralised with sodium hydroxide (NaOH) 0.1N. The value is the average of 3 measurements (three random plastic containers for a total of 0.750 kg of fruit).

2.4. Colour

In this study the colour evolution and total chlorophyll content were monitored only for the yellow-flesh cultivar cv. 'Ariddo di Core'. Colour was measured on the first 15 non-mouldy fruits from each tray (three trays were randomly chosen for each package). The mean of the 30 fruit measurements was used for data analysis. CIELAB or $L^*a^*b^*$ space was used to describe the color. This color space is device-independent and able to create consistent colors regardless of the device used to acquire the image. L^* is the luminance or lightness component, which ranges from 0 to 100, while a^* (green to red) and b^* (blue to yellow) are two chromatic components, with values varying from -120 to +120 (YAM and PAKADIS, 2004). These values were used to calculate chroma, which indicates the intensity or color saturation, using the following equation:

$$C^* = [a^{*2} + b^{*2}]^{1/2} \quad (2)$$

hue angle was calculated as follows:

$$h^\circ = \arctangent[b^* / a^*] \quad (3)$$

where 0° = red-purple, 90° = yellow, 180° = bluish-green, and 270° = blue (MCGUIRE, 1992).

2.5. Chlorophyll

Chlorophyll monitoring was carried out using UV-Vis spectrophotometry, a non-destructive analytical, qualitative and quantitative technique that makes use of a spectrophotometer to allow molecule recognition and quantification as a function of spectrum absorption. The UV-Vis analyses were performed using a Varian Cary 500 double beam spectrophotometer equipped with a Varian DRA-2500 integrating sphere. The background noise was subtracted using the Spectralon® as a reference. The spectra were recorded in a range between 350 and 800 nm at a resolution of 3 nm. Each UV-Vis measurement was made in diffuse reflection (DR) mode positioning the equatorial part of the surface of each fruit (95mm² area) in line with the reflection sphere. For each sample the chlorophyll concentration was calculated by processing the spectra acquired from each fruit (average of two fruit / 60 fruits / acquisitions) as per the Kubelka Munk (1931) F(R) function. It was first necessary to establish a calibration equation by means of the direct extraction of chlorophyll from plums exhibiting different degrees of maturity as per the official extraction methodology (AOAC, 2006).

2.6. Statistical analysis

All statistics were performed using SPSS for Windows version 20.0. The data obtained were treated with one-way analysis of variance (ANOVA) and the means were separated using the Duncan test ($p \leq 0.05$). As the sample sizes were identical, it was possible to perform a parametric test for the percentages.

3. RESULTS AND DISCUSSIONS

3.1. Headspace composition and qualitative parameters

Changes of O_2 and CO_2 (kPa) gases within the package headspaces are shown in Figs. 1 and 2 respectively for cv. 'Ramasin' and cv. 'Ariddo di Core'. For both cultivars each packaging film succeeded in changing the initial atmospheric conditions, equal to 0.2 kPa of CO_2 and 21.2 kPa of O_2 maintaining different MAP conditions up to the end of the storage period (21 days).

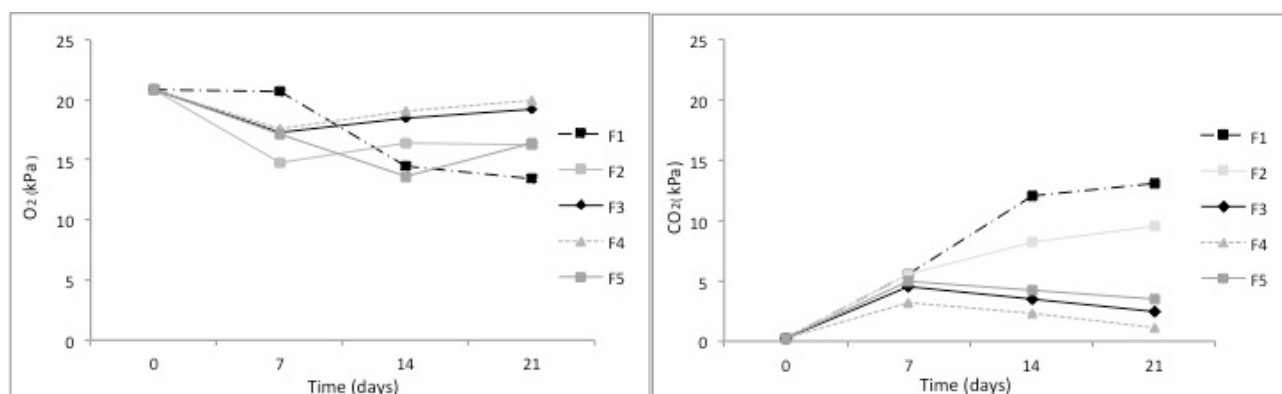


Figure 1: O_2 and CO_2 headspace gas composition of cv. 'Ramasin' plums stored in MAP at $1 \pm 0.5^\circ C$.

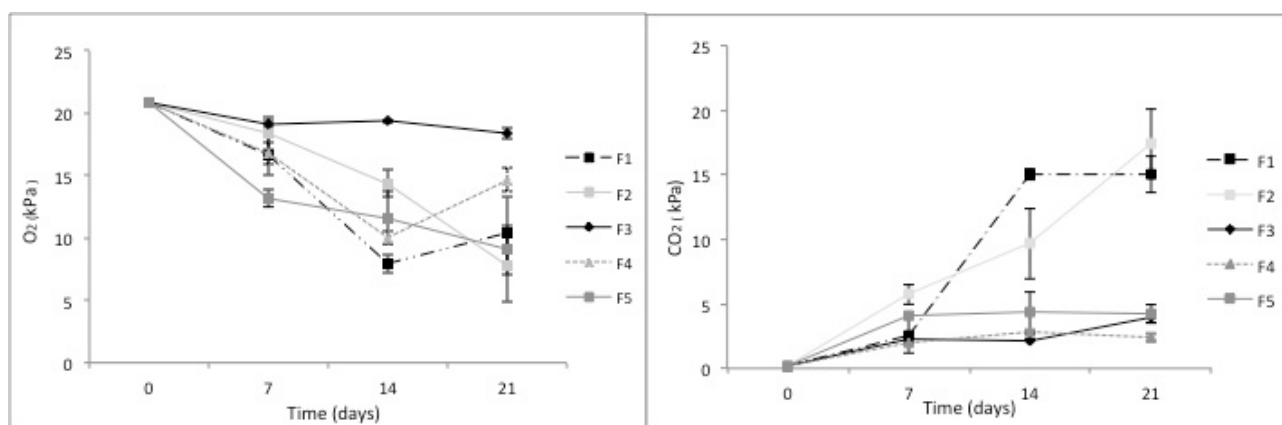


Figure 2: O_2 and CO_2 headspace gas composition of cv. 'Ariddo di Core' plums stored in MAP at $1 \pm 0.5^\circ C$.

In general a decreasing trend in O₂ content corresponds to an increase in the internal concentration of CO₂, product of the respiration of the plums; in this case, the CO₂ accumulation, at a constant temperature (1±0.5°C), is determined by the interaction of two factors: the permeability of the film and the storage period (EXAMA *et al.*, 1993; VAROQUAUX *et al.*, 2002). During the first 7 days O₂ and CO₂ contents did not differ significantly in both varieties. As the storage goes on, the values increase their differences evidencing the active performance of the different packaging films used. According to what reported in previous MAP studies on stone fruits (DIAZ-MULA *et al.*, 2011 a, GIRGENTI *et al.*, 2014) the multilayer films (F1 and F2) are able to maintain higher concentrations of CO₂ within the flow pack when compared with other films (F3, F4, F5) due to higher gas barrier properties (Table 1).

This condition is maintained by both the cultivars for all the storage time. In particular, after 21 days of storage, CO₂ ranged between 9.5 and 13.1 kPa for cv. 'Ramasin' and between 15.0 kPa and 17.4 kPa of CO₂ for cv. 'Ariddo di Core'. With the 'Ramasin' cultivar, the highest value is registered with the F1 film, while for the cv. 'Ariddo di Core' with the F2 film. All other films, from the 7th day of storage evidenced CO₂ values lower than 5 kPa. For both cultivars the lowest values were obtained with the F4 film (respectively 1.2 kPa for the cv. 'Ramasin' and 2.4 kPa for the cv. 'Ariddo di Core'). Throughout the storage period, the cv. 'Ariddo di Core', under all MAP conditions, presented higher values of CO₂ than the cv. 'Ramasin', suggesting a greater respiratory metabolism for the fruit of this cultivar. For the cv. 'Ramasin', the equilibrium point (13.4 kPa O₂ and 13.1 kPa CO₂) was only reached at the end of the storage period (21 days) with the F1 film, while in the case of the cv. 'Ariddo di Core' it is reached between the 14th and 21st day with both of the multilayer films (F1 and F2) with values ranging between 10 and 15kPa; this condition is however immediately lost. The weight losses observed (data not shown) increase with the storage time, but the rate for both cultivars is a function of the specific film employed. The control showed the greatest weight loss (3 % of the fresh weight after 21 days) confirming what was observed in previous MAP studies for stone fruits (SOTTILE *et al.*, 2013; GIRGENTI *et al.*, 2014). All MAP packages ensure controlled weight loss within a similar range of values (0.5-0.7% for cv. 'Ramasin' and 0.6-0.9 % for 'Ariddo di Core' after 21 days of storage). Based on these results, it is not possible to use the weight loss as quality parameter to identify the MAP film with the best performances.

The cv. 'Ramasin' (Table 2) and cv. 'Ariddo di Core' (Table 3) present very different FFF values at harvest (1.1 kg/cm² and 3.5 kg/cm² respectively). However both cultivars exhibit a similar evolution for this parameter. In fact, the FFF values decreased with time, reaching their lowest values after 21 days of storage. This result is associated with a decrease of pectin polymerisation within the cell tissues and although this trend is common to all packages, fruit stored under normal atmospheric conditions (control) showed a stronger decreasing trend and evidenced values significantly lower at the end of the storage period respect to MAP storage (0.5 kg/cm² for cv. 'Ramasin' and 1.1kg/cm² for 'Ariddo di Core').

As reported in previous studies (SOTTILE *et al.*, 2013) plums stored under MAP conditions with the highest levels of CO₂ use to evidence higher pulp firmness; for both cultivars the multilayer films (F1 and F2), ensuring higher values of CO₂ (Figs. 1 and 2), are able to better control pulp firmness decay as compared to other films.

In particular, for cv. 'Ramasin', the F1 film is significantly different compared to the F2 film, while in the case of the cv. 'Ariddo di Core', these two films do not exhibit significant differences in terms of performance.

Table 2: Evolution of qualitative characteristics of plums cv. 'Ramasin' stored in MAP at $1\pm0.5^{\circ}\text{C}$.

		Time (days)		
Film		7	14	21
		Harvest 1.10 ± 0.2^1		
FFF (kg/cm^2)	F1	0.86 ± 0.2^a	0.85 ± 0.1^a	0.80 ± 0.2^a
	F2	0.89 ± 0.1^a	0.84 ± 0.1^a	0.80 ± 0.1^b
	F3	0.77 ± 0.1^a	0.70 ± 0.1^a	0.78 ± 0.2^b
	F4	0.78 ± 0.1^b	0.70 ± 0.1^a	0.71 ± 0.1^b
	F5	0.76 ± 0.1^b	0.72 ± 0.1^a	0.73 ± 0.1^b
	Control	0.62 ± 0.1^c	0.53 ± 0.1^b	0.49 ± 0.2^c
		Harvest 16.0 ± 0.7		
SSC ($^{\circ}\text{Brix}$)	F1	$17.0\pm0.3^{n.s}$	17.5 ± 0.3^d	17.9 ± 0.5^d
	F2	$17.0\pm0.4^{n.s}$	17.7 ± 0.3^d	17.9 ± 0.7^d
	F3	$16.9\pm0.6^{n.s}$	18.2 ± 0.8^{bc}	18.6 ± 0.5^{bc}
	F4	$17.1\pm0.8^{n.s}$	18.0 ± 0.5^c	18.5 ± 0.6^c
	F5	$17.1\pm0.3^{n.s}$	18.3 ± 0.6^b	18.8 ± 0.3^b
	Control	$17.0\pm0.6^{n.s}$	18.8 ± 0.3^a	20.1 ± 0.5^a
		Harvest 5.1 ± 0.0		
TA (meq/L)	F1	$4.9\pm0.0^{n.s}$	4.8 ± 0.2^a	4.5 ± 0.0^a
	F2	$4.9\pm0.2^{n.s}$	4.8 ± 0.1^a	4.5 ± 0.1^a
	F3	$5.1\pm0.1^{n.s}$	4.6 ± 0.3^a	4.5 ± 0.0^{ab}
	F4	$5.0\pm0.1^{n.s}$	4.6 ± 0.1^a	4.4 ± 0.0^{ab}
	F5	$5.0\pm0.1^{n.s}$	4.5 ± 0.1^a	4.4 ± 0.0^b
	Control	$5.0\pm0.1^{n.s}$	4.2 ± 0.2^b	3.7 ± 0.1^c

Results were expressed as means \pm standard deviation.

Values in the column followed by different letters are significantly ($P<0.05$) different according to Duncan's test.

At harvest, the two cultivars present different SSC (16.0° Brix and 18.5° Brix respectively for cv. 'Ramasin' and cv. 'Ariddo di Core'). After the first 7 days of storage, in spite of the low refrigeration temperatures ($1\pm0.5^{\circ}\text{C}$), all packages presented an increase in the soluble solids content values in accordance with the observations of GUERRA and CASQUERO (2008), with the Green Gage variety and SOTTILE *et al.*, (2013) yellow-fleshcultivars; this trend is evident with continued storage. The increase is related to the concentration of sugars resulting from weight loss of the fruit (loss of water) and also due to the increasing extractability (sucrose inversion) during the increasing of the ripening degree. For both cultivars, over the whole storage period, the control presented a higher soluble solid content compared to the MAP packaged ones thus confirming the CO_2 effect during storage and its ability to delay the ripening processes in accordance with DÍAZ-MULA *et al.* (2009).

The F1 and F2 films showed statistically significant differences compared to the other MAP films (F3, F4 and F5) after 14 days of storage in the case of the cv. 'Ramasin' (Table 2) and after only 7 days of storage for the cv. 'Ariddo di Core' (Table 3) evidencing a better control of the soluble solids content dynamics and maintaining these values close to those measured at harvest.

Table 3: Evolution of qualitative characteristics of plums cv. 'Ariddo di Core' stored in MAP at 1±0.5°C.

		Time (days)		
	Film	7	14	21
FFF (kg/cm ²)		Harvest 3.55±0.11		
	F1	3.25±0.1 ^a	3.25±0.3 ^a	2.39±0.1 ^a
	F2	3.25±0.1 ^a	3.04±0.1 ^a	2.49±0.2 ^a
	F3	2.85±0.2 ^b	2.50±0.1 ^b	1.32±0.2 ^c
	F4	2.79±0.1 ^b	2.47±0.1 ^b	1.35±0.2 ^c
	F5	2.87±0.1 ^b	2.50±0.1 ^b	1.53±0.2 ^b
	Control	2.53±0.1 ^c	2.24±0.4 ^c	1.04±0.1 ^d
SSC (°Brix)		Harvest 18.5±0.1		
	F1	18.6±0.1 ^{bc}	18.7±0.1 ^c	19.1±0.2 ^c
	F2	18.6±0.1 ^{bc}	18.7±0.1 ^c	19.0±0.1 ^c
	F3	18.7±0.1 ^b	19.1±0.0 ^b	19.5±0.1 ^b
	F4	18.7±0.1 ^b	19.0±0.1 ^b	19.5±0.1 ^b
	F5	18.7±0.1 ^b	19.0±0.1 ^b	19.5±0.1 ^b
	Control	18.9±0.1 ^a	19.6±0.1 ^a	20.0±0.1 ^a
TA (meq/L)		Harvest 8.6±0.7		
	F1	5.0±0.1 ^{n.s}	4.3±0.0 ^{n.s}	4.1±0.1 ^a
	F2	5.0±0.1 ^{n.s}	4.4±0.1 ^{n.s}	4.1±0.0 ^a
	F3	4.5±0.0 ^{n.s}	4.3±0.0 ^{n.s}	3.6±0.0 ^b
	F4	4.5±0.1 ^{n.s}	4.3±0.0 ^{n.s}	3.5±0.0 ^b
	F5	4.5±0.0 ^{n.s}	4.3±0.1 ^{n.s}	3.5±0.0 ^b
	Control	4.5±0.0 ^{n.s}	4.1±0.1 ^{n.s}	3.9±0.0 ^c

Results were expressed as means ± standard deviation.

Values in the column followed by different letters are significantly ($P<0.05$) different according to Duncan's test.

Over the entire storage period, compared to the values measured at harvest (5.1 meq/L for cv. 'Ramasin' and 10.2 meq/L for cv. 'Ariddo di Core'), the TA diminished for all packages for both cultivars; both for Ramasin and Ariddo di Core cvs., MAP determined higher TA values compared to the control. The maturity index obtained from the SSC/TA ratio (data not shown) indicates a progressively increase during the whole storage period for both cultivars; for all MAP packages this value is lower respect to the control, thus confirming the observations reported from previous studies (DÍAZ-MULA *et al.*, 2009).

The colour variables a^* and b^* are not independent and their difficult perception to the human eye force to their correlation in order to calculate Chroma (C) and Hue angle parameters (ALCOBENDAS *et al.*, 2012). Table 4 reports the colour components L, Chroma and Hue angle as measured for the yellow flesh fruit cv. 'Ariddo di Core'. Just after 7 days of storage each film packaging influenced a change in skin colour thus indicating the presence of a maturation process even at low storage temperatures (1±0.5°C).

3.2. Colour and chlorophyll monitoring

The luminance values (L^*) decrease compared to those measured at harvest (46.9) thus indicating a darkening of the tissues as a result of the ripening (Table 4). Each of the MAP

packages presented no statistically significant differences compared to control as the fruit maintained higher L* values. The multilayer films (F1 and F2), to which correspond higher concentrations of CO₂ within the flow pack headspace (Fig. 2), are more differentiated than the other films presenting significantly higher values of L* over the entire storage period and reaching after 21 days values equal to 42.4. The chroma values follow in a similar way the decreasing trend of L* values. For each film the Hue angle values decreased at increasing storage time in accordance with findings reported by DÍAZ-MULA *et al.* (2011 a), but statistically significant lower values (redder fruit) were observed throughout the storage period in respect to control packed fruit.

Table 4: Colorimetric parameters of plums cv. 'Aridio di Core' stored in MAP at 1±0.5°C.

Time (days)	Film	L*	Chroma	h angle
7	Harvest	46.9±0.5 ^a	21.1±0.4 ^a	-1.31±0.01 ^d
	F1	43.7±0.2 ^b	17.0±0.6 ^{bc}	-1.39±0.02 ^c
	F2	43.7±0.3 ^b	17.1±0.9 ^{bc}	1.39±0.02 ^c
	F3	43.6±0.2 ^b	16.9±0.6 ^c	-1.42±0.01 ^b
	F4	43.3±0.3 ^c	16.9±0.8 ^b	-1.40±0.01 ^b
	F5	43.4±0.3 ^c	16.9±0.9 ^c	-1.42±0.01 ^b
	Control	42.5±0.3 ^d	15.8±0.9 ^d	-1.48±0.03 ^a
14	Harvest	46.9±0.5 ^a	21.1±0.4 ^a	-1.31±0.01 ^c
	F1	43.5±0.2 ^b	16.3±0.5 ^b	-1.36±0.54 ^{bc}
	F2	43.6±0.3 ^b	16.3±0.6 ^b	-1.45±0.04 ^{ab}
	F3	42.4±0.2 ^d	15.6±0.7 ^c	-1.47±0.02 ^a
	F4	42.5±0.2 ^{cd}	15.7±0.4 ^c	-1.48±0.03 ^a
	F5	42.5±0.3 ^c	15.6±0.3 ^c	-1.48±0.01 ^a
	Control	41.6±0.4 ^e	13.2±0.7 ^d	1.53±0.00 ^a
21	Harvest	46.9±0.5 ^a	21.1±0.4 ^a	-1.31±0.01 ^c
	F1	42.4±0.3 ^b	15.6±0.8 ^b	-1.47±0.04 ^b
	F1	42.4±0.5 ^b	15.3±0.8 ^b	-1.47±0.02 ^b
	F1	41.6±0.2 ^c	14.3±0.8 ^c	-1.47±0.02 ^b
	F1	41.7±0.2 ^c	14.6±0.7 ^c	-1.48±0.02 ^b
	F1	41.6±0.4 ^c	14.6±0.6 ^c	-1.48±0.02 ^b
	Control	39.5±0.3 ^d	12.6±0.8 ^d	-1.55±0.03 ^a

Results were expressed as means ± standard deviation.

Values in the column followed by different letters are significantly (P<0.05) different according to Duncan's test.

The skin changes from green to yellow color are closely correlated to the chlorophyll degradation process during ripening (ABDI *et al.*, 1997; CRISOSTO *et al.*, 2004) as shown in Fig. 3. The total pigment content, measured using UV-Vis spectrophotometry, decreases progressively throughout the storage period thus confirming evolution of the ripening processes even at low temperatures (ZUDE-SASSE *et al.*, 2002; SOLOVCHENKO *et al.*, 2005). For each quality control all MAP film tested evidenced to be able to manage the loss of total chlorophyll content maintaining higher values than the control. The high barrier capacity films (F1 and F2) were differentiated already within 7 days of storage thanks to the fruits have maintained the main quality values near to the time of harvest.

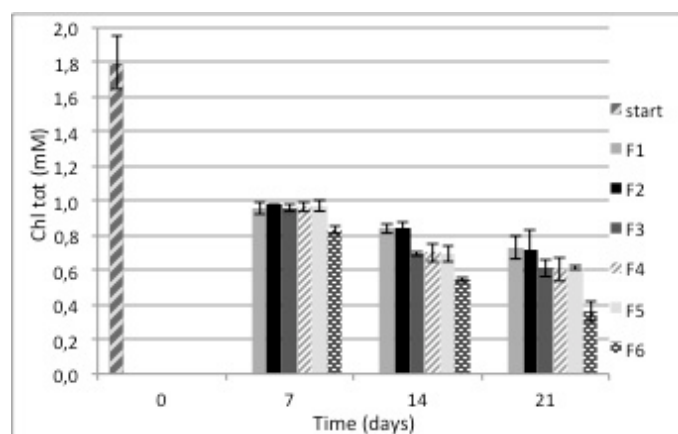


Figure 3: Chlorophyll total content (mM) in cv. 'Arido di Core' plums stored in MAP at $1\pm0.5^{\circ}\text{C}$.

The high CO_2 levels, corresponding to F1 and F2 films, (Fig. 2) would be responsible for the proper maintenance of the integrity and fluidity of cell membranes which are indispensable conditions for the stabilisation of the photosynthetic pigment content (PONGPRASERT *et al.*, 2011). The total chlorophyll content showed a good correlation to the colour components L^* (Fig. 4) and Chroma (Fig. 5) according to a linear regression index equal to $R^2 = 0.92$ and $R^2 = 0.96$, respectively. In particular, decreasing values in chlorophyll correspond to a loss of brightness and chromaticity (Table 4) associated with the concentration of carotenoids that are responsible for the change in colour from green to yellow (increasing value of a^*) of the fruit skin.

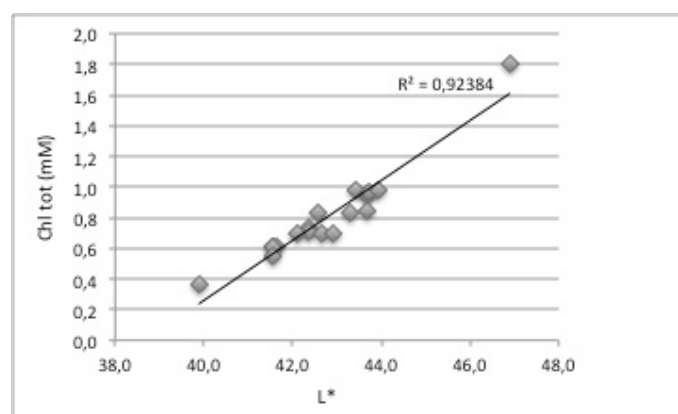


Figure 4: Linear regression of total chlorophyll content and the L^* colour parameter in cv. 'Arido di Core' plums stored in MAP at $1\pm0.5^{\circ}\text{C}$.

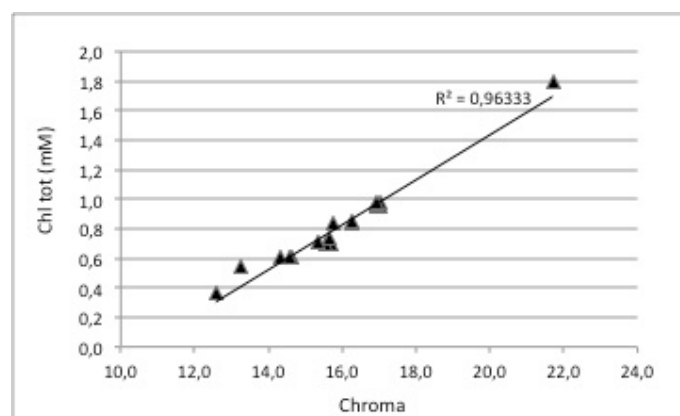


Figure 5: Linear regression of total chlorophyll content and the Chroma colour parameter in cv. 'Arido di Core' plums stored in MAP at $1\pm0.5^{\circ}\text{C}$.

4. CONCLUSIONS

Different and multiples indexes of quality have been considered to evaluate the goodness of the MAP technique for the storage of 'Ramasin' and 'Arido di Core' plum cultivars. The headspace composition showed how high CO_2 increments associated with higher barrier capacity films (multilayer) are usually related to a higher fruit quality at the end of the storage period. Among the qualitative index considered, for both cultivars, the flesh fruit firmness allowed a better evaluation of the effectiveness of different packaging since the beginning of the storage test. The colour parameter (lightness and chromaticity), linked to the chlorophyll content of the plums, is well known to be particularly appreciated by the consumers and for this it can be suggested to further evaluation of the performance of the tested films for the management of a MAP storage protocol. Results of this research confirm the correlation that exists between colour and the pigments content in organic matrices and more generally within food systems (RAMAKRISHNAN and FRANCIS, 1973; FRANCIS, 1985; WATADA and ABBOTT, 1975; TAKAHATA *et al.*, 1993; AMENY and WILSON, 1997; CHEN and TANG, 1998; ARIAS *et al.*, 2000; BRON *et al.*, 2005; CECCARELLI *et al.*, 2008). According to McGuire (1992) the monitoring of the total total chlorophyll content is highly correlated with the colour parameters, then it is important to consider also that it derives from a non-destructive technique such as UV-Vis whose numerous advantages are even more interesting when they can be applied to cultivars of excellence and high perishability such as those considered within this study.

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GENDER DIFFERENCES IN THE CHEMICAL COMPOSITION AND SELECTED PROPERTIES OF AFRICAN CATFISH (*CLARIAS GARIEPINUS* Burchell, 1822) MEAT

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ABSTRACT

The objective of this study was to analyze gender differences in the chemical composition and selected physicochemical properties of African catfish meat. The experimental material comprised fish younger than 1 year, with estimated body weight of 1 kg, cultured in an intensive fish farm. Fillet acidity (pH_{24}) was determined 24 h *post mortem*, and the color of external and internal fillet surfaces was described based on L^* , a^* , b^* , C^* and h° values. Samples of ground meat were analyzed to determine their proximate chemical composition, energy value and TBARS levels. The meat of male and female fish was characterized by high levels of total protein and fat, a low calorific value and optimal acidity. In males, internal and external fillet surfaces were darker and characterized by higher redness (a^*) and lower yellowness (b^*) values. In male fillets, significant ($p \leq 0.01$) correlations were noted between water content and fat content and between protein content and crude ash concentrations. The coefficients of correlation between protein content and L^* values and between ash content vs. L^* and b^* values were also statistically significant ($p \leq 0.01$). In meat samples collected from male fish, the values of color components a^* and b^* were also highly correlated. High negative correlations were observed between protein concentrations and crude fat levels, and between pH_{24} vs. TBARS values in female fillets. Color lightness was also significantly ($p \leq 0.01$) positively correlated with oxidative stability.

Keywords: African catfish, meat and fillets, chemical composition, energy value, TBARS, pH, color

1. INTRODUCTION

The geographic range of the African catfish (*Clarias gariepinus*) extends from Africa to south-east Asia. In the course of evolution, the species has adapted to unsupportive environmental conditions. In its natural habitat, the catfish is an omnivorous predator that feeds on zooplankton, arthropods, mollusks, fish, reptiles and amphibians (VITULE *et al.*, 2006; AMISAH *et al.*, 2009). In fish farms, the African catfish easily adapts to pond conditions and has relatively low requirements regarding water quality. The main goal of intensive cultures is to produce catfish with the highest body weight within a short period of time, which can only be achieved under optimal conditions. In commercial farms, fish weighing 800-1000 g are produced in 6-8 months. According to ADAMEK (2011), fish heavier than 1200 g are in highest demand on the Polish market. The meat of the African catfish is a valuable and cheap source of easily digestible protein of high quality. Catfish meat is pink, almost boneless and tasty, which contributes to its popularity. The chemical composition of fish meat (in particular its protein and fat content), its energy value and sensory properties such as color, texture, taste and flavor are determined by feeding intensity, type and quality of feed, as well as natural feed intake (JANKOWSKA *et al.*, 2007; PUCHAŁA and PILARCZYK, 2007).

There is a general scarcity of data relating to differences in the performance traits of *C. gariepinus* females and males raised in pond cultures. Therefore, the aim of the study was to analyze gender differences in the chemical composition and selected physicochemical properties of African catfish (*C. gariepinus*) meat.

2. MATERIALS AND METHODS

2.1. Experimental fish, diets and origin

The experimental material comprised 60 African catfish (*C. gariepinus*) younger than 1 year, with estimated body weight of 1 kg, and an equal number of males and females. Fish were harvested in autumn-winter of 2013 from a freshwater fish farm in northern Poland. Catfish were cultured in a 9000 L concrete pond (intensive system) with a closed circuit system and water temperature of 25±1°C. Fish were manually fed (every 3 h) pelleted feed prepared at the farm. Feed composition was as follows (per 100 kg): 17.8 kg of fish meal, 44.6 kg of extracted soybean meal, 14.9 kg of wheat grain, 7.4 kg of corn grain, 11.9 kg of rapeseed cake, 2.4 L of fish oil, and 1 kg of a vitamin-mineral premix. The nutrient content of feed was determined at the Laboratory of the Department of Animal Nutrition and Feed Science of the University of Warmia and Mazury in Olsztyn (Poland) according to AOAC guidelines (2005). The pelleted feed mixture contained: 33.57% of total protein, 5.82% of crude fat, 6.45% of crude ash and of 3.80% crude fiber. Its energy value was determined at 17.229 MJ/kg.

2.2. Preparation of skinned fillet samples

Fish were harvested 48 hours before slaughter, they were transferred to a separate pond at the farm, cleansed, stunned and slaughtered according to standard procedures (DIRECTIVE 2009/1099/EC). Catfish were manually gutted (body cavity was slit open, viscera and blood clots were removed), decapitated (cut behind epicranium outgrowths), fins were removed (caudal, dorsal, abdominal and pectoral fins were cut off approximately 0.5 cm from the base), and fish were filleted (the entire muscle was removed and skinned). The research material comprised 60 raw fillets without skin (left

side carcass), cooled for 24 h to a temperature of $4\pm1^{\circ}\text{C}$ in a Frost Co. chilling chamber with relative air humidity of 85%. All qualitative analyses were performed at the Laboratory for Meat Quality Assessment of the Department of Commodity Sciences and Animal Raw Material Processing of the University of Warmia and Mazury in Olsztyn (Poland).

2.3. Chemical composition, energy and TBARS values in meat

The fillets of male ($n=30$) and female ($n=30$) fish were ground in a laboratory grinder (three times) equipped with a 2 mm mesh, and they were thoroughly mixed to prepare samples for chemical analysis. The proximate chemical analysis involved determinations of: water content (PN-ISO, 2000a), total protein content – by the Kjeldahl method (PN-A, 2002) in Foss Tecator Kieltec 2200 System I (Höganäs, Sweden), crude fat content according- by Soxhlet extraction (PN-ISO, 2000b) in the Foss Tecator Soxtec™ Avanti 2050 extractor (Höganäs, Sweden), and crude ash content (PN-ISO, 2000c). Oxidative changes in intramuscular lipids were analyzed by measuring the content of thiobarbituric acid-reacting substances (TBARS) in accordance with the method proposed by RAK and MORZYK (2002). Absorbance was measured with the Analytik Jena AG Specord 40 spectrophotometer (Jena, Germany) and expressed in mg of malondialdehyde per 1 kg of meat. The energy value of meat was calculated using conversion factors of 4.00 kcal (16.78 kJ/g) for protein and 9.00 kcal (37.62 kJ/g) for fat (JESZKA, 2010).

2.4. Acidity and color parameters of fillets

Muscle acidity was measured 24 h *post mortem*, immediately after carcass cooling (left side carcass) ($n=60$), with the 340i pH-meter and WTW TFK 150/E temperature sensor (Weilheim, Germany) equipped with a Hamilton Double Pore combination glass electrode (Bonaduz, Switzerland). The pH-meter was calibrated against buffers with known pH before measurements (PN-ISO, 2002).

The color of fillets ($n=60$) was described based on the values of L^* (lightness), a^* (redness) and b^* (yellowness) in the CIELAB system (CIE, 1978) by measuring reflectance on the internal surface (abdominal side) and external surface of skinned fillets (Fig. 1) at the same points relative to the surface.

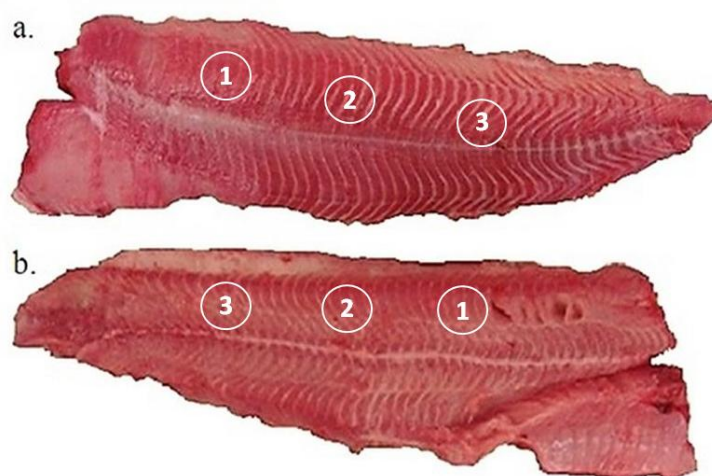


Figure 1: Color measurements on the external (a) and internal (b) surfaces of *C. gariepinus* fillets. Measurement points - 1, 2, 3 (own study).

Measurements were performed in three replications in the HunterLab MiniScan XE Plus spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA, USA). Chroma (C^*) and hue (h°) were calculated with the use of the respective formulas (HUNT *et al.*, 1991):

$$C^* = \sqrt{a^2 + b^2} \text{ and } h^\circ = \tan^{-1}(b/a).$$

Color analyses were performed with D65 light source, 10° standard observer and aperture diameter of 2.54 cm. Measurements were carried out in fillets chilled at $4 \pm 1^\circ\text{C}$ for 30 minutes. The spectrophotometer was calibrated against white and black standards before every measurement.

2.5. Statistical analysis

The results were processed statistically by one-way analysis of variance in the Statistica v. 10.0 program (2011). They were presented in tables as mean values, standard deviation and standard error of the mean (SEM). The significance of differences ($p \leq 0.05$ and $p \leq 0.01$) between the means of chemical composition, TBARS values and physicochemical properties in male ($n=30$) and female ($n=30$) catfish was analyzed by the Student's t-test. The coefficients of correlation between the analyzed groups of properties were determined separately for male and female fish. Data were checked for normal distribution (Shapiro-Wilk test) and equality of variances (Levene's test) before statistical analysis.

3. RESULTS AND DISCUSSIONS

The average protein content of fish is determined at 16-20%, but it can be as high as 25% in tuna fish. The African catfish is an interesting species in view of its processing suitability and chemical composition. According to KLASA and TRZEBIATOWSKI (1992), the muscle tissue of catfish is characterized by a low fat content (3.5%) and a high total protein content (17.9%). In a study by KAPELIŃSKI (2003), the fat content of farmed African catfish was estimated at 3-4%, which indicates that the species has a low calorific value and is particularly suited for cold smoking. ŁUCZYŃSKA *et al.* (2011) observed the lowest fat content (2.81%) in the dorsal muscle of carp, followed by trout (4.39%), whereas the highest fat levels were noted in salmon (11.57%). In this study, female fillets contained 17.42% of total protein and 5.76% of crude fat on a fresh weight basis. The above values were slightly higher than in males, but the observed differences were not statistically significant. Crude ash content, which is the total amount of mineral compounds remaining after incineration, is also an important indicator of meat quality. According to SKIBNIEWSKA *et al.*, (2012), the composition and content of those nutrients in the meat of livestock animals, including fish, is determined mainly by their availability in feed as well as by the species, physiological status and age of animals. The conducted statistical analysis also confirmed that gender had a significant influence on the ash content of African catfish meat. Females were characterized by a higher (by 0.07%) crude ash content than males. In addition to higher concentrations of total protein, fat and crude ash, female fillets were also characterized by a lower water content in comparison with male fillets (75.15% in females vs. 76.03% in males). Gender differences in the chemical composition of African catfish fillets have never been explored in the literature. The only relevant information contributed by other authors (POLAK-JUSZCZAK, 2007; SKAŁECKI *et al.*, 2013) was that the water content of muscle tissue in various fish species is inversely proportional to the combined content of total protein, fat and crude ash.

Table 1: Chemical composition, energy and TBARS values of meat and pH of *C. gariepinus* fillets.

Specification	Meat samples		SEM
	Male (n=30)	Female (n=30)	
Water content (%)	76.03±1.17	75.15±0.65	0.253
Total protein (%)	17.32±0.69	17.42±0.26	0.248
Crude fat (%)	5.15±1.35	5.76±0.65	0.298
Crude ash (%)	1.06 ^b ±0.09	1.13 ^a ±0.02	0.017
Energy value (kJ/100 g)	484.32±60.12	508.87±22.63	10.283
TBARS (mg MDA/kg meat)	0.24±0.10	0.36±0.12	0.026
pH ₂₄ (left-side carcass)	6.32±0.08	6.27±0.09	0.021

The results are expressed as means±SD.

The means denoted by different letters in rows differ significantly at ^a($p \leq 0.05$); SEM – standard error of the mean.

POLAK-JUSZCZAK (2007) determined the total protein content of African catfish fillets at 17.90%, free fat content at 5.30%, crude ash content at 0.98% and water content at 75.53%, and similar results were noted in our study. In comparison with the data presented in Table 1, the fresh meat of *C. gariepinus* evaluated by YANAR (2007) was characterized by significantly lower levels of fat (3.64%) and crude ash (0.68%), but somewhat higher content of protein (17.85%) and water (77.89%). In the work of GODA *et al.* (2007), the muscle tissue of African catfish administered standard feed contained 75.73% of water and 15.96% of protein. The cited authors also reported a significantly higher crude ash content (3.71%) and lower fat concentrations (4.60%). In studies of freshwater fish, the protein content of carp meat was determined at 17.21% by SKAŁECKI *et al.* (2013) and at 11.85-17.74% by PUCHAŁA and PILARCZYK (2007). Fat concentrations in carp muscles were determined at 4.44% by SKAŁECKI *et al.* (2013) and at 6.80-11.77% by PUCHAŁA and PILARCZYK (2007). Significant variations in the fat content of meat from carp and other fish species can be attributed mainly to the type of administered feed which also influences the final body weight and total length of fish (GODA *et al.*, 2007; PUCHAŁA, PILARCZYK, 2007); SKAŁECKI *et al.*, 2013).

The energy value of meat is determined by its carbohydrate, protein and fat content. The calorific value of an average serving of fish (100 g) ranges from less than 400 kJ to approximately 1225 kJ. Despite having higher gross energy value, fatty fish are still less calorific than other products of animal origin (JESZKA, 2010). In our study (Table 1), no statistical differences were determined between male and female fish, but a trend towards higher energy values (by 24.55 kJ) was noted in the meat of female catfish. As a species with 7% fat content, the African catfish can be classified into a group of medium-fat fish in line with the Polish Standards (PN-A, 1999). The calorific value of meat from *C. gariepinus* males and females was relatively low at 496.59 kJ/100 g on average. According to ROSA *et al.* (2007), the energy value of 100 g of fresh muscle tissue of catfish reached 457.90 kJ and was lower than the values presented in Table 1 (484.32 kJ/100 g for males and 508.87 kJ/100 g for females). Secondary products of lipid oxidation, whose presence is determined based on malondialdehyde (MDA) concentrations, are a direct symptom of autoxidation processes that adversely influence meat quality (KAMKAR *et al.*, 2014). In our study, chilled muscles of both male and female African catfish were characterized by low TBARS values (0.24 and 0.36 mg MDA/kg of meat, respectively), which was indicative of high oxidative stability of lipids. In the experiment conducted by YANAR

(2007), the average TBA levels of fresh *C. gariepinus* muscles reached 0.45 mg MDA/kg. The initial MDA content of fresh silver carp fillets was determined at 0.54 mg MDA/kg by KAMKAR *et al.* (2014).

pH value is a critical determinant of microbial growth and food spoilage. The pH of fish ranges from 6.7 to 7.0, and it fluctuates subject to season, feed, exposure to stress and activity levels (MERKIN *et al.*, 2010; YANAR, 2007). The data presented in Table 1 indicate that African catfish males and females were characterized by similar pH 24 h *post mortem* (6.32 in males, 6.27 in females). According to MARX *et al.* (1997), the boundary value of pH₂₄ for fresh fish meat is 6.5. In a study by SKAŁECKI *et al.* (2008), cod meat was characterized by significantly higher pH₂₄ (6.89) and pH₄₈ (6.74) values than herring meat (6.67 and 6.49, respectively).

The analysis of color on the internal surface of African catfish fillets (Table 2) revealed that gender was significantly correlated with lightness (L*), the contribution of the red component (a*) and the yellow component (b*), and hue (h°).

Table 2: Color parameters on the surface of *C. gariepinus* fillets.

Specification	Meat samples		SEM
	Male (n=30)	Female (n=30)	
Internal surface			
L*	46.65 ^B ±1.68	49.31 ^A ±0.85	0.422
a*	11.80 ^A ±1.34	8.69 ^B ±0.97	0.440
b*	15.15 ^B ±0.92	16.55 ^A ±0.41	0.220
C*	19.20±1.48	18.69±0.63	0.270
h°	52.08 ^B ±1.95	62.29 ^A ±2.10	1.268
External surface			
L*	41.81±1.69	43.07±1.43	0.376
a*	15.62±1.71	14.63±1.75	0.400
b*	11.20 ^b ±1.39	12.71 ^a ±1.52	0.365
C*	19.22±1.98	19.37±1.64	0.423
h°	35.64 ^B ±1.71	40.98 ^A ±2.17	0.810

The results are expressed as means±SD.

The means denoted by different letters in rows differ significantly at ^{ab} ($p \leq 0.05$) and ^{AB} ($p \leq 0.01$); SEM - standard error of the mean.

Female muscles were characterized by higher values of L* (49.31) and b* (16.55) in comparison with male fillets, and the observed differences were statistically significant ($p \leq 0.01$). JANKOWSKA *et al.* (2007) evaluated the quality of the meat of the European catfish (*Silurus glanis*) administered natural and formulated feed and did not find any differences in lightness or yellowness values. The L* values of the European catfish (47.98) were similar to those noted in the African catfish in our study (48.11). The contribution of yellow pigment in *C. gariepinus* fillets was determined at 15.85 on average in both sexes, and it was 5.81 higher than in *S. glanis* muscles. The color of fish meat is a species-specific trait which is determined by the number of red muscle fibers and pigment concentrations, including myoglobin, hemoglobin and carotenoids. WEDEKIND (1995) reported

significant sexual dimorphism in the quality of *C. gariepinus* fillets. The meat of males was characterized by intense red coloration, a lower fat content and higher cohesiveness (toughness) in comparison with female fillets. In our study, the value of the a^* coordinate measured on the internal surface of fillets was significantly higher ($p \leq 0.01$) in males (11.80) than in females (8.69), which is consistent with the results reported by WEDEKIND (1995). The results presented in Table 2 indicate that total chromaticity (C^*) measured on the ventral side of the fillets was similar in males and females at 18.95 on average. Despite an absence of statistically significant differences, chromaticity was lower (by 0.51) in female fillets. Color hue differed significantly (at $p \leq 0.01$) between genders, and the value of h° measured on the internal surface of fillets was higher in females (62.29).

The color profile of the external surfaces of *C. gariepinus* fillets is presented in Table 2. The values of L^* and a^* were not influenced by gender. Despite the above, male fillets were darker ($L^*=41.81$) and more saturated with the red component ($a^*=15.62$) than female fillets (43.07 and 14.63, respectively). The contribution of yellow, measured on the skinned side, was significantly higher ($p \leq 0.05$) in females at 12.71. Chromaticity (C^*) on the external surface of fillets was similar in both genders at 19.22 in males and 19.37 in females. Statistical calculations revealed that color hue on the external side of the fillets was significantly higher in females (40.98) than in males (where it was up to 5.34 lower).

The data presented in Table 3 point to a highly significant ($p \leq 0.01$) negative correlation between water content and fat content ($r = -0.88$) in the meat of male African catfish. In male fillets, protein content was significantly ($p \leq 0.01$) correlated with crude ash levels ($r = 0.84$). In female meat samples, a high ($p \leq 0.01$) negative correlation was observed between protein concentrations and fat content ($r = -0.85$).

Table 3: Coefficients of correlation between the chemical components of *C. gariepinus* fillets.

Parameter	Total protein		Crude fat		Crude ash	
	Male	Female	Male	Female	Male	Female
Water content	0.09	0.55	-0.88**	-0.57	-0.13	0.26
Total protein	-	-	-0.50	-0.85**	0.84**	0.50
Crude fat			-	-	-0.23	-0.10

Explanatory notes: *correlation coefficients are statistically significant at $*(p \leq 0.05)$ and $** (p \leq 0.01)$.

The proximate chemical composition of fish meat was not significantly correlated with pH_{24} values in males or females (Table 4).

Table 4: Coefficients of correlation between chemical composition vs. pH_{24} and color parameters on the internal surface of *C. gariepinus* fillets.

Parameter	pH_{24}		L^*		a^*		b^*	
	Male	Female	Male	Female	Male	Female	Male	Female
Water content	0.20	0.32	0.27	-0.16	0.29	-0.08	0.28	0.72*
Total protein	-0.12	0.44	-0.81**	-0.34	0.17	0.44	-0.45	0.31
Crude fat	-0.06	-0.60	0.06	0.38	-0.27	-0.48	-0.09	-0.13
Crude ash	-0.39	0.20	-0.76**	0.07	-0.17	-0.30	-0.77**	0.29

Explanatory notes: *correlation coefficients are statistically significant at $(p \leq 0.05)$ and $(p \leq 0.01)$.

A statistical analysis revealed a significant ($p \leq 0.01$) negative correlation between the protein and crude ash content of male fillets vs. color lightness measured on the internal surface of the fillets ($r = -0.81$ and $r = -0.76$, respectively). A significant ($p \leq 0.01$) negative correlation between crude ash content and the contribution of yellowness was also noted in male fillets ($r = -0.77$). Water content was significantly ($p \leq 0.05$) correlated with the yellow component on the external surface of female fillets (Table 5). In the meat of male catfish, a positive correlation ($p \leq 0.05$) was noted between fat content and the value of L^* ($r = 0.75$). Protein and crude ash content were not correlated with any color parameters on the external surface of fillets (Table 5).

Table 5: Coefficients of correlation between chemical composition vs. color parameters on the external surface of *C. gariepinus* fillets.

Parameter	L^*		a^*		b^*	
	Male	Female	Male	Female	Male	Female
Water content	-0.62	-0.35	0.44	-0.09	0.45	0.71*
Total protein	-0.48	-0.50	-0.00	0.60	-0.11	0.27
Crude fat	0.75*	0.34	-0.36	-0.43	-0.32	-0.22
Crude ash	-0.30	-0.41	-0.11	0.34	-0.16	0.35

Explanatory notes: *correlation coefficients are statistically significant at $(p \leq 0.05)$ and $(p \leq 0.01)$.

A significant ($p \leq 0.05$) negative correlation ($r = -0.68$) was observed between the lightness (L^*) and redness (a^*) on the internal surface of female fillets (Table 6). In male meat samples, parameter L^* was positively correlated ($p \leq 0.05$) with the contribution of yellowness ($r = 0.68$). No significant correlations between the values of a^* and b^* were noted on the internal surface of male and female fillets. In the meat of female catfish, a positive correlation ($p \leq 0.01$) was observed between the values of L^* and TBARS ($r = 0.78$), whereas a negative correlation ($r = -0.78$) was noted between pH_{24} and TBARS values (Table 6). In male fillets, significant correlations ($p \leq 0.05$) were noted between the values of b^* and acidity ($r = 0.65$) and between pH_{24} and oxidative stability ($r = -0.67$). In an analysis of parameters measured on the external surface of catfish fillets (Table 7), an increase in color lightness was accompanied by a decrease in the contribution of redness and yellowness ($r = -0.67$ and $r = -0.70$, respectively; $p \leq 0.05$), and an increase in TBARS values ($r = 0.70$, $p \leq 0.05$). In male fillets, a significant positive correlation was also noted between color parameters a^* and b^* ($r = 0.86$).

Table 6: Coefficients of correlation between color parameters on the internal surface of fillets vs. pH_{24} and TBARS values.

Parameter	a^*		b^*		pH_{24}		TBARS	
	Male	Female	Male	Female	Male	Female	Male	Female
L^*	-0.20	-0.68*	0.68*	-0.02	0.28	-0.49	-0.19	0.78**
a^*	-	-	0.21	-0.01	0.50	0.05	-0.59	-0.29
b^*			-	-	0.65*	-0.21	-0.60	0.01
pH_{24}					-	-	-0.67*	-0.78**

Explanatory notes: *correlation coefficients are statistically significant at $*(p \leq 0.05)$ and $** (p \leq 0.01)$.

Table 7: Coefficients of correlation between color parameters on the external surface of fillets vs. pH₂₄ and TBARS values.

Parameter	a*		b*		pH ₂₄		TBARS	
	Male	Female	Male	Female	Male	Female	Male	Female
L*	-0.58	-0.67*	-0.57	-0.70*	-0.10	-0.58	0.59	0.70*
a*	-	-	0.86**	0.01	0.17	0.34	-0.53	-0.52
b*			-	-	0.04	0.53	-0.29	-0.53

Explanatory notes: *correlation coefficients are statistically significant at $*(p \leq 0.05)$ and $** (p \leq 0.01)$.

4. CONCLUSIONS

A statistical analysis revealed that the chemical composition, energy and TBARS values in the meat of African catfish were not significantly influenced by gender. The analyzed fillets of male and female fish were characterized by a relatively high content of total protein, optimal concentrations of crude fat and a low calorific value. The muscle tissue of *C. gariepinus* was characterized by optimal acidity 24 h *post mortem*, and significant differences in this parameter were not observed between the genders. An analysis of color parameters revealed that the internal surface of male fillets was darker, more saturated with the red pigment (*a), less saturated with the yellow component (b*) and characterized by lower hue values (h°) in comparison with female meat samples. In female fillets, protein content was significantly correlated with fat concentrations, whereas in meat samples collected from male fish, significant correlations were noted between water content and fat content, and between total protein content and crude ash content. The value of parameter L* on the external surface of male fillets decreased with a rise in total protein and crude ash content. The contribution of yellowness (b*) was significantly correlated ($p \leq 0.05$) with water content in female fillets and with crude ash levels in male fillets ($p \leq 0.01$). In measurements performed on the external surface of male and female fillets, significant correlations were noted between fat content and color lightness, and between water content and yellowness (b*), respectively. The correlation coefficients indicate that an increase in TBARS values led to a significant increase in color lightness on the internal surface of fillets and a decrease in the pH₂₄ values of female muscles. A significant ($p \leq 0.01$) positive correlation was observed between redness and yellowness on the external surface of male fillets. In conclusion, the results of this study indicate that sexual dimorphism in the African catfish significantly differentiates the color parameters of fillets, but it has no influence on the chemical composition, pH, energy values and lipid stability of meat from males and females.

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FATTY ACID COMPOSITION IN WILD *BOLETUS EDULIS* FROM POLAND

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ABSTRACT

The aim of this study was to determine the content of fat and fatty acids profile in wild *Boletus edulis*. The research material consisted of 33 samples of wild *Boletus edulis* in the form of caps and stems, collected from selected regions of Poland. Methyl esters of fatty acids were prepared by Peisker's method. Separation of the examined compounds was performed by gas chromatography (FID). The dominant fatty acids in all samples under study were: C18:2, C18:1 and C16:0. The profile of fatty acids in *Boletus edulis* varied between the regions where the mushrooms were collected as well as the morphological parts of the fruiting body.

Keywords: *Boletus edulis*, edible mushrooms, fatty acids, human nutrition, Poland

1. INTRODUCTION

Edible wild mushrooms are a raw material consumed in many countries of the world as a delicacy (RIBEIRO *et al.*, 2009). They are most appreciated by gourmets as well as enthusiasts of mushroom picking which, apart from being a piece of cultural heritage, has recently become a highly valued recreational activity (KALAČ, 2009; 2013). Widespread consumption of mushrooms is related primarily to their taste and smell properties, which give a sophisticated flavor to dishes. In our previous studies we examined the toxicological aspects of edible mushrooms (chlorinated hydrocarbons residues) due to the fact they are considered to be bioindicators of the level of environment contamination (GAŁGOWSKA *et al.*, 2012). Nevertheless, in recent years, researchers have begun to focus on to their significant role in human nutrition. The growing awareness of consumers of food quality has made mushrooms a subject of scientific interest. The attention of researchers has been focused on their chemical composition and content of necessary nutrients essential for basic human diet supplementation. Since the last decade, a synoptic knowledge of the composition and nutritional value of the most important species of edible mushrooms has been available.

Bano suggested that the food value of mushrooms lies between vegetables and meat. Mushrooms are source of beneficial bioactive compounds (BANO, 1976). They are quite rich in protein, providing all the essential amino acids and contain relatively high amounts of carbohydrates and fiber (KALAČ, 2009). Due to the fact that mushrooms have a low fat content, they are considered low-energy functional foods, which could significantly contribute to the design of healthy dietary patterns (ALOBO, 2003; BARROS *et al.*, 2007; KAVISHREE *et al.*, 2008; LEE *et al.*, 2011). They contain significant amounts of vitamins and vitamin precursors, minerals and trace elements (KALAČ, 2009; 2013). Mushrooms also include sterols, with the predominance of ergosterol, the precursor of vitamin D (KALOGEROPOULOS *et al.*, 2013). Apart from rich composition, mushrooms have therapeutic properties, including prevention of such health problems as: atherosclerosis, diabetes mellitus, chronic inflammation, cancer and aging (YILMAZ *et al.*, 2006).

Physical and psychological development and health maintenance involves supplying the human body with proper nutrients, including animal and vegetable fat (BARROS *et al.* 2008, MOIOLI *et al.*, 2007). Lipids are a basic component of a diet and play many varied roles in the organism, primarily as the richest and the most concentrated source of energy (GAWEŃKI and HRYNIEWIECKI, 2000). Lipid consumption provides the body with the proper amounts of fatty acids, including saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Although each member of the group of fatty acids is important in the human diet, it is also necessary to preserve the relative proportions of their consumption. Interest in MUFAs is related to their function in preventing cardiovascular diseases such as atherosclerosis (HUNTER *et al.*, 2010; KANU *et al.*, 2007; MOTARD-BELANGER *et al.*, 2008; VON SCHACKY and HARRIS, 2007). The dietetic value of fat is to the highest extent, determined by the presence of PUFAs in its content, particularly of linoleic (C18:2 n-6) and α -linoleic acid (C18:3 n-3), which form a family of so-called "essential" fatty acids (INNIS, 2005; PRZYSŁAWSKI and BOLESŁAWSKA, 2006). They are converted to tissue hormones, which affect the functions of numerous tissues and organs and reinforce or weaken the regulatory effect of the hormonal and nervous systems. EFAs (essential fatty acids) prevent blood clotting and hypertension. Additionally, they increase the blood supply to the heart and contribute to the proper distribution of cholesterol in the body (KRIS-ETHERTON and ETHERTON, 2003; WILLIAMS, 2000).

Polish literature contains only few references concerning the profile of fatty acids in edible mushrooms. Based on international reports, mushrooms contain significant amounts of

unsaturated acids and low amounts of saturated acids (RIBEIRO *et al.*, 2009; KALAČ, 2009; 2013; BARROS *et al.*, 2007; KAVISHREE *et al.*, 2008; YILMAZ *et al.*, 2006; BARROS *et al.*, 2008; PEDNEAULT *et al.*, 2006). This is an important reason to recommend the raw material in various diets. The growing interest in the consumption of edible mushrooms provides an incentive to carry out a broad scope of analytical research concerning the composition of fatty acids in these raw materials.

In Poland, the picking and consumption of mushrooms is very popular due to the occurrence of large areas of forests. Of the many species of mushrooms, *Boletus edulis*, (a member of the *Boletaceae* family), is highly valued by consumers due to its unique flavor characteristics.

Taking the above into account, the aim of this study was to determine the content of the fat and fatty acid profile in wild *Boletus edulis*, indicate potential differences in the composition of fatty acids between the various morphological parts and examine the impact of the mushrooms' vegetative sites on the fatty acid profile.

2. MATERIALS AND METHODS

The research material consisted of 33 samples of whole fruiting bodies of wild *Boletus edulis* from four selected regions of Poland (Fig. 1) and 29 averaged samples in the form of caps and stems, collected in 2010 during the period from July to September. The samples were prepared (selected, cleaned, dried in a fruit and vegetable drier) according to PN-68/A-78508-1968 (PN-68/A-78508-1968). The dried and finely cut-up material was subjected to extraction in a Soxhlet's apparatus in order to obtain lipid substances PN-A-78509:2007 (PN-A-78509:2007). Methyl esters of fatty acids were prepared by Peisker's method, using a mixture of methyl alcohol, sulphurous acid and chloroform (PEISKER, 1964).



Figure 1: Location of sampling of *Boletus edulis*.

Separation of the examined compounds was performed by gas chromatography. The conditions of chromatographic separation involved a gas chromatographer (7890A Agilent Technologies) and a flame ionization detector (FID); capillary column Supelcowax 10: length - 30 m, inside diameter - 0.32 mm, liquid phase – Supelcowax 10, film thickness 0.25 µm; temperature: detector – 250°C, dispenser – 230°C and column – 195°C; carrier gas - helium, flow rate - 1.5 ml/min (51 cm/s); split 50:1.

The identification of fatty acids was carried out on the basis of their retention time in relation to the standard retention time of fatty acid methyl esters. For this purpose, a mixture of 37 standards of Supelco 37 Component FAME Mix (10 mg/ml in methylene chloride (varied)) was applied. For the calculation of the percentage share of fatty acids, a Chemstation computer program was used. In the experiment, there were 17 fatty acids identified from C12:0 to C24:1, which were divided into three groups: saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA).

Statistical analyses were conducted with Microsoft Excel software, which included calculation of the mean values as well as the standard deviations. The significance of difference of the mean values between the samples was determined using Statistica 10 software (Duncan's test, analysis at the level of significance of $p=0.05$).

3. RESULTS AND DISCUSSIONS

The fatty acid profiles of *Boletus edulis* are shown in Tables 1 and 2 and in Figs. 3 and 4. Statistical differences were found in the fat content, depending on the morphological parts of a mushroom. The average fat content in caps and stems of *Boletus edulis* was 4.38% and 1.75%, respectively (Fig. 2).

In *Boletus edulis* there 17 different fatty acids from C 12:0 to C24:1 were determined. Table 1 presents the fatty acids composition in whole fruiting bodies of wild *Boletus edulis* depending on research regions.

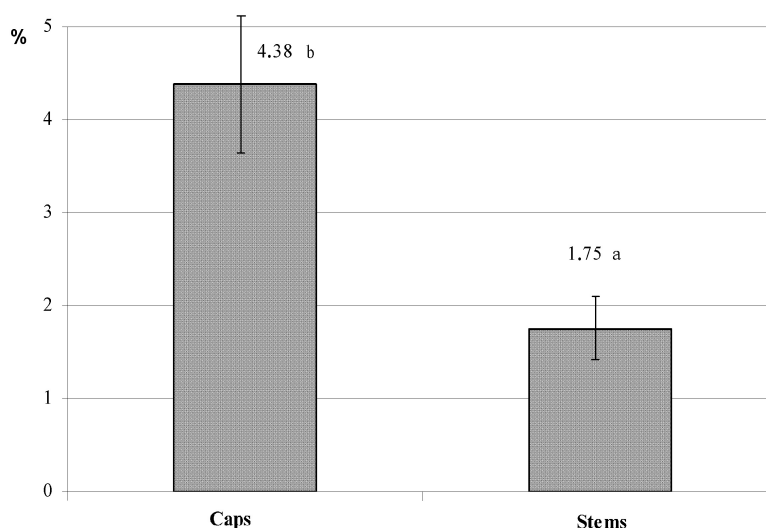


Figure 2: The average content of fat in *Boletus edulis* depending on the morphological parts of fruiting body.

The content of every fatty acid was varied depending on the region. Analyzing the dominant acids (C16, C18: 1 and 18: 2), the highest percentage of palmitic acid was found in the region B, while the lowest - statistically different from the rest - in the region A. However, for the region A, was the highest percentage share of C18:1 acid reported. The lowest contribution of this acid was observed for the region B. In the case of C18:2 acid no statistically significant differences were found between regions A and B. The highest content of this acid was indicated for the region D, and the lowest for region C. Determinated differences between regions can be related with different composition of the soil, weather conditions, *et al.*

Table 1: The fatty acids profile in *Boletus edulis* originating from different regions of Poland (n= 33).

Fatty acids	Region			
	A (n = 4)	B (n = 9)	C (n = 10)	D (n = 10)
C12:0	0.10±0.00 ^a	0.41±0.06 ^c	0.22±0.04 ^b	0.77±0.09 ^d
C14:0	0.13±0.01 ^a	1.11±0.18 ^c	0.65±0.11 ^b	0.76±0.13 ^b
C15:0	0.10±0.00 ^a	0.89±0.14 ^c	1.00±0.13 ^c	0.50±0.09 ^b
C16:0	8.39±0.42 ^a	28.84±3.35 ^c	25.10±1.03 ^c	16.82±1.21 ^b
C16:1	0.75±0.06 ^a	1.69±0.14 ^c	1.50±0.18 ^c	1.11±0.19 ^b
C17:0	0.10±0.00 ^a	0.24±0.04 ^c	0.32±0.07 ^c	0.19±0.03 ^b
C18:0	3.12±0.04 ^b	2.87±0.27 ^{ab}	2.78±0.38 ^{ab}	2.49±0.30 ^a
C18:1	44.75±0.70 ^d	19.56±3.11 ^a	31.71±2.56 ^c	24.33±0.95 ^b
C18:2	40.71±0.51 ^b	40.07±4.02 ^b	32.78±2.60 ^a	50.62±2.00 ^c
C18:3	0.31±0.02 ^a	1.30±0.22 ^d	0.60±0.12 ^c	0.42±0.06 ^b
C20:0	0.40±0.01 ^b	0.23±0.02 ^a	0.27±0.06 ^{ac}	0.40±0.07 ^{bc}
C20:1	0.74±0.05 ^b	0.35±0.06 ^a	0.73±0.11 ^b	0.35±0.05 ^a
C20:2	0.20±0.01 ^a	0.92±0.16 ^c	0.29±0.04 ^b	0.18±0.02 ^a
C22:0	0.26±0.08 ^a	0.68±0.09 ^d	0.54±0.06 ^{cd}	0.44±0.06 ^{bc}
C22:1	0.16±0.04 ^a	0.23±0.04 ^a	0.53±0.08 ^b	0.43±0.07 ^b
C24:0	0.10±0.00 ^a	0.51±0.10 ^b	0.77±0.11 ^c	0.10±0.02 ^a
C24:1	0.11±0.01 ^a	0.10±0.00 ^a	0.22±0.05 ^b	0.10±0.01 ^a

^{a-d} The significance of difference of the mean values between the samples; p = 0.05.

A similar relation in the case of content of predominant fatty acids (linoleic acid (C18:2), oleic (C18:1) and palmitic (C16:0)) in this species was also observed by other authors (KAVISHREE *et al.*, 2008, BARROS *et al.* 2008). In presented studies, linoleic acid content ranged from 32.78-50.62%. A similar amount of C18:2 was found by the following authors: BARROS *et al.* (2008) - 44.32% (Portugal), KAVISHREE *et al.* (2008) - 33.80% (India), PEDNEAULT *et al.* (2006) - 42.20% (Canada), YILMAZ *et al.* (2006) - 33.60% (Turkey). The content of C18:1 acid in the *Boletus edulis* under study ranged from 19.56 to 44.75%. In their research BARROS *et al.* (2008) found 39.72%, PEDNEAULT *et al.* (2006) - 36.01%, KAVISHREE *et al.* (2008) - 31.10%, YILMAZ *et al.* (2006) - 30.20%. Palmitic acid (C16:0) was

found in samples in the range of 8.39 - 28.84%. The content of the acid in the studies of PEDNEAULT *et al.* (2006) was that of 9.80%, of KAVISHREE *et al.* (2008) - 21.60% and of BARROS *et al.* (2008) - 10.03%.

In none of the samples were short-chained fatty acids detected, while BARROS *et al.* (2008) and PEDNEAULT *et al.* (2006) observed the presence of trace amounts of C6:0, C8:0 and C10:0 in *Boletus edulis*. The presence of fatty acids in the *trans* configuration was also not stated.

The obtained results varied depending on the morphological parts of the mushroom, what is presented in Table 2.

Table 2: The fatty acids profile in *Boletus edulis* depending on morphological parts of the mushroom.

Fatty acids	Caps (n = 15)	Stems (n = 14)
C12:0	0.57±0.16 ^b	0.30±0.07 ^a
C14:0	0.69±0.03 ^a	0.61±0.07 ^a
C15:0	0.46±0.11 ^a	1.45±0.20 ^b
C16:0	15.96±4.77 ^a	35.95±0.95 ^b
C16:1	1.11±0.07 ^a	1.94±0.11 ^b
C17:0	0.16±0.02 ^a	0.26±0.06 ^b
C18:0	2.69±0.48 ^a	2.40±0.14 ^a
C18:1	34.51±0.80 ^b	20.69±1.12 ^a
C18:2	40.71±1.41 ^b	32.68±1.18 ^a
C18:3	0.90±0.20 ^b	0.48±0.04 ^a
C20:0	0.36±0.08 ^a	0.33±0.02 ^a
C20:1	0.61±0.09 ^b	0.35±0.05 ^a
C20:2	0.26±0.06 ^a	0.18±0.03 ^a
C22:0	0.36±0.03 ^a	0.85±0.11 ^b
C22:1	0.31±0.08 ^a	0.55±0.07 ^b
C24:0	0.23±0.02 ^a	0.86±0.12 ^b
C24:1	0.10±0.01 ^a	0.11±0.02 ^a

^{a-d} The significance of difference of the mean values between the samples; p = 0.05.

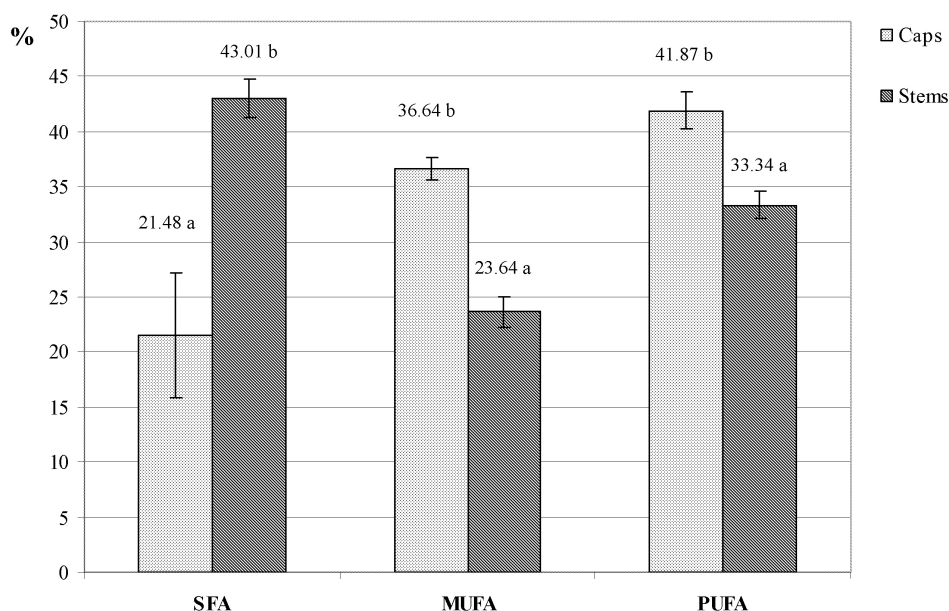
The predominant fatty acids in caps were C18:2, C18:1 and C16:0 (40.71%, 34.51%, 15.96%, respectively). In the case of three predominant fatty acids in stems, the biggest share had C16:0 (35.95 %), next – C18:2 and C18:1 (32.68%, 20.69%, respectively).

In the examined morphological parts of the mushrooms statistically significant differences in the content of C18:2 acid were found, where in the caps 40.71% was determined and 32.68% in the stems. The stems of the same species of mushroom from Turkey had 28.40% C18:2 acid content (YILMAZ *et al.*, 2006). A statistically significant difference in the content of oleic acid (C18:1) in the caps (34.51%) and stems (20.69%) was found. The content of this acid in the stems at level of 8.30% was determined by YILMAZ *et al.* (2006). The stems of

Boletus edulis (35.95%) were characterized by a significantly higher content of C16:0 acid compared to caps (15.96%).

The content of other fatty acids ranged from 0.10 - 2.69% and was dependent on the morphological parts of the mushroom.

It was found that the percentage share of each group of fatty acids (SFA, MUFA, PUFA) in the studied morphological parts of *Boletus edulis* was statistically different (Fig. 3).



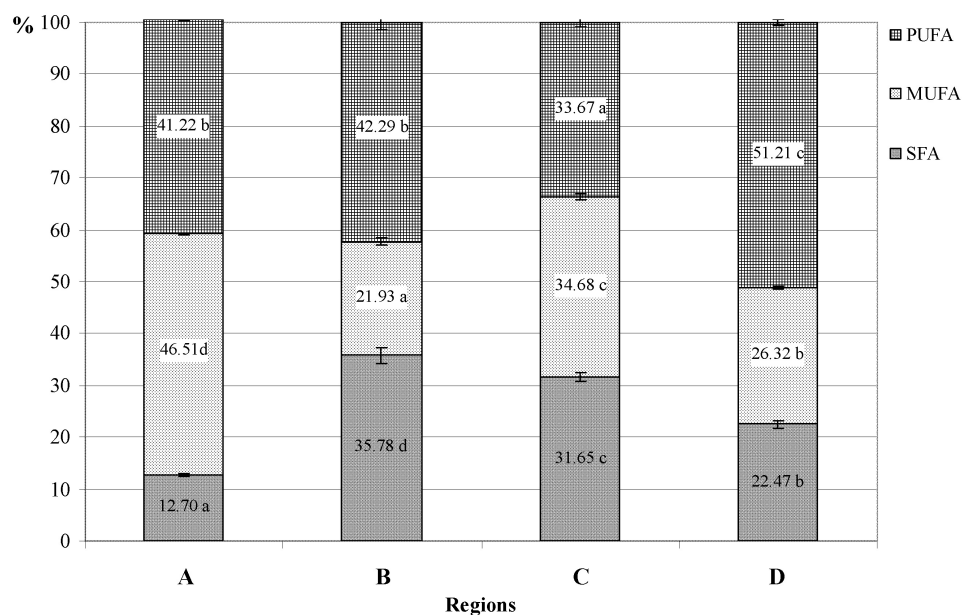
^{a-d}The significance of difference of the mean values between the samples; $p = 0.05$.

Figure 3: Percentage contribution of SFA, MUFA, PUFA in *Boletus edulis* depending on morphological parts of the mushroom.

In the case of saturated fatty acids the share was about twice as high in caps (43.01%) than in the stems (21.48%). The caps were characterized by a higher contribution of monounsaturated (36.64%) and polyunsaturated fatty acids (41.87%) compared to the stems (23.64% and 33.34%, respectively).

The main groups of fatty acids present in the mushrooms under study were unsaturated fatty acids (from 64.22 to 87.73 %). PEDNEAULT *et al.* (2006) also obtained similar amounts 84.50%. There were significant statistical differences found in the contents of particular groups of fatty acids (SFA, MUFA, PUFA) in *Boletus edulis* from different regions (Fig. 4). The analyzed samples had the largest share of polyunsaturated (33.67 - 51.21%) and the lowest contribution of saturated fatty acids 12.70 - 35.78%.

The biggest share of unsaturated fatty acids (41.22% of PUFA and 46.51% of MUFA) was observed in *Boletus edulis* from region A (87.73%). In the regions B and C there were similar contributions of unsaturated fatty acids (64.22% and 68.35%, respectively) determined. Nevertheless the region B characterized the higher content of PUFA (42.29%) and only 21.92% of MUFA, while in the region C there was indicated 33.67% of PUFA and 34.68% of MUFA. The mushrooms from the region D were the richest source of PUFA (51.21%) (Fig. 4).



^{a-d} The significance of difference of the mean values between the samples; $p = 0.05$.

Figure 4: Percentage contribution of SFA, MUFA, PUFA in *Boletus edulis* originating from different regions of Poland.

4. CONCLUSIONS

The profile of fatty acids in *Boletus edulis* varied between the regions where the mushrooms were collected as well as the morphological parts of the fruiting body. This confirms view of SANMEE *et al.* (2003) and DIEZ and ALVAREZ (2001), that a number of factors usually influence the nutritional composition of mushrooms. These factors include growing site, type of substrates, mushroom type, developmental stages and part of the fungal samples analyzed (DIEZ and ALVAREZ, 2001; SANMEE *et al.*, 2003). The observed differences in the fatty acids content in mushrooms under study may result, among others, from the different chemical composition of the substrate in particular regions. However, this thesis requires confirmation in further studies.

In terms of human nutrition, caps of *Boletus edulis* are a more valuable raw material than stems due to the significantly higher content of MUFA and PUFA and lower SFA. Among polyunsaturated fatty acids, the most noteworthy is linoleic acid C18:2 (Ω -6), which is a precursor of 1-acetic-3-ol the major component of mushrooms giving them a specific aroma (RIBEIRO *et al.*, 2009; BARROS *et al.*, 2007). The high content of this acid in Polish *Boletus edulis* provides that the mushroom may be recommended in different types of diets for people with high blood cholesterol (KAVISHREE *et al.*, 2008; VAZ *et al.*, 2011).

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SEA-BUCKTHORN OIL IN VEGETABLE OILS STABILISATION

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ABSTRACT

The paper proposes the development of blends of vegetable oils with a high content of easily oxidizable unsaturated fatty acids with sea-buckthorn fruit oil as a natural method of their preservation. The predominant lipophilic compounds in sea-buckthorn oil included β -carotene, α -tocopherol, and β -sitosterol. Strong correlations were found between oxidative stability of the blends and α - and β -tocopherols, lutein and β -carotene concentration (r ranging from 0.96 to 0.99). The observed effect may result from both the particularly huge increase in the carotenoid content (from 64- to 171-fold) in the obtained blends, and the synergistic interaction between tocopherol mixtures and carotenoids.

Keywords: oils stabilisation, sea-buckthorn fruit oil, natural antioxidants

1. INTRODUCTION

Consumption of vegetable oils provides the body with energy and ingredients with structural, regulatory and protective functions (AYALA *et al.*, 2014). Lipid components form, *inter alia*, the structure of cell membranes; moreover, they are involved in the formation and functioning of nerve cells, regulation of intrasystemic metabolism, and maintaining the oxidation/reduction balance in the cells (MURRAY *et al.*, 1995). The composition of fatty acids and the profile of low-molecular lipophilic compounds depend on the botanical source of origin, and the method of oil production. Most oils contain predominantly unsaturated fatty acids which allow them to maintain the liquid state at room temperature. However, the presence of unsaturated fatty acids promotes the initiation of oxidation processes, which are particularly rapid for polyunsaturated acids. With an increase in the number of unsaturated bonds in the cell, the number of carbon atoms separating them also increases. The carbon-hydrogen bond near such a carbon atom is characterized by lower dissociation energy, which leads to easy formation of free radicals. According to COSGROVE *et al.* (1987) the oxidation of oleic acid is 50 times slower than that of linoleic acid, and 100 times slower than that of linolenic acid. The slightly lower rate of the oxidation of oleic acid as compared to linoleic acid (10-40 times lower rate) was specified in the study by MCCLEMENT and DECKER (2008).

Oxidation of an oil begins at the moment of the extraction thereof from a plant matrix. Due to the destruction of natural cell structures, they become susceptible to the action of enzymes, oxygen, light, and other free radical generators (SZUKALSKA, 2003). The basic and primary indicator of the oxidation of an oil is an increase in the value of peroxide value (PICURIC-JOVANOVIC *et al.*, 1999; BROADBENT and PIKE, 2003). For cold-pressed oils, the value of peroxide value as permitted by Codex Alimentarius (2005) is 15 mEqO₂/kg. The value of peroxide value, however, does not allow clear determination of freshness of an oil. Only the performance of additional analyzes on the secondary products of oil oxidation e.g. by the TBA test, determination of the anisidine value, or the measurement of absorbance of conjugated dienes and trienes allows a more precise determination of the degree of oxidation (JERZEWSKA, 1991).

Despite the presence of oxidizable unsaturated fatty acids, vegetable oils contain numerous stabilizing compounds exhibiting antioxidant action (CZAPLICKI *et al.*, 2011; OGRODOWSKA *et al.*, 2014; ROSZKOWSKA *et al.*, 2015). They get to the oil from the plant matrix, or are added at the packaging stage. The compounds of particular significance are terpenoid compounds such as tocopherols, sterols, carotenoids, and squalene. Most often, however, the content of natural antioxidants is not sufficiently high to fully protect the oil against oxidation. A study by CZAPLICKI *et al.* (2011) on nine popular bio-oils found that the content of unsaponifiable fraction ranged from only 0.48% (poppseed oil) to 7.12% (amaranth oil). It therefore seems that vegetable oils valued for their unique compositions of fatty acids, such as linseed, borage, and evening primrose oils, should be enriched with either natural or synthetic antioxidants. Literature describes attempts to increase the oxidative stability of oils through the addition of compounds such as e.g.: tocopherols, tocotrienols, sesamol, butylated hydroxyl toluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), tertiarybutylhydroquinone (TBHQ) and ascorbyl palmitate (AP) (HAMDO *et al.*, 2014; HWANG and WINKLER-MOSER, 2013; ÖNAL-ULUSOY and ERGIN, 2002). Similarly, a positive effect on the oxidative stability of oils was observed in tests using grape seed extract, green tea extract, microalgae *Scenedesmus almeriensis* extracts, and extracts of herb such as rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), marcela (*Achyrocline satureioides*), and carqueja (*Baccharis trimera*) (POIANA, 2012; CHEN *et al.*, 2013; VIEITEZ *et al.*, 2013; LIMÓN *et al.*, 2015). However, the introduction of pure substances or lyophilized extracts into an oil results in the need for standardization

of their concentrations in the oil, which may be limited by the solubility of the compound or extract being added. It was demonstrated, *inter alia*, that in corn oil at a temperature of 25°C, only 3% sterols can be dissolved (VAIKOUSI *et al.*, 2007). The practice of introducing into oils substances which do not occur in them naturally gives rise to controversy associated with the loss of the “natural” characteristic of a product.

On the other hand, a natural manner of increasing the content of antioxidants in oils may be the development of their blends with oils being particularly rich in natural antioxidants. An oil which is characterized by an exceptionally high content of phytosterols, tocopherols, and carotenoids, is the oil extracted from sea-buckthorn fruits. This study attempted to determine the effects of the addition of sea-buckthorn oil as a stabilizer of linseed, borage, and evening primrose oils which are characterized by different compositions of fatty acids and contents of endogenous antioxidants. The following were assessed: the content of natural terpenoid antioxidants in the obtained blends, the composition of fatty acids, and their oxidative stability.

2. MATERIALS AND METHODS

2.1. Chemicals

Chromatography-grade solvents: methanol, methyl tert-butyl ether (MTBE), iso-propanol, hexane, pyridine, N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich (St. Louis, MO, United States, supplier Poznań, Poland). Analytical-grade reagents: methanol, dichloromethane, chloroform, sulphuric acid, potassium hydroxide, sodium sulphate, powdered zinc (POCH, Gliwice, Poland) were used. Analytical standards: 5 α -cholestane (97%), β -Apo-8'-carotenal (>96%) and fatty acids mixture were purchased from Sigma-Aldrich and tocopherols mixture (95%) from Merck (Darmstadt, Germany). Deionized water was obtained from HLP 5s deionizer (Hydrolab, Gdańsk, Poland).

2.2. Materials

The linseed oil, borage seed oil, evening primrose seed oil and sea-buckthorn fruits oil were used in this study.

Oils were obtained by pressing the raw material on a IBG Monforts & Reiners, Komet CA59G (Germany) laboratory expeller. The oils were purified by centrifugation at 8000 x g on a Eppendorf centrifuge (type 5810R). The oils blends were prepared by 15% of sea buckthorn oil addition to analysed linseed, borage seed and evening primrose seed oils.

In oils and prepared mixtures fatty acids compositions and bioactive compounds (carotenoids, sterols and tocopherols) concentrations were analysed as well as oxidative stability was also examined.

2.3. Determination of fatty acid composition

Ten micrograms of sample was dissolved in 1.5 mL of chloroform-methanol-sulphuric acid (100:100:1, v/v/v), transferred into 2 mL-pharmaceutical vials and sealed hermetically over a gas burner (ZADERNOWSKI and SOSULSKI, 1978). The fatty acids methylation was carried out by heating the vials at 70°C for 2 hours. After cooling, the vials were opened and the powdered zinc was added to decompose remained sulphuric acid. Obtained methyl esters were dried in a stream of nitrogen, purified in a hexane extraction and analysed by gas chromatography with a GC-MS QP2010 PLUS (Shimadzu,

Japan) system. Separation was performed on a BPX70 (25 m x 0.22 mm x 0.25 μ m) capillary column (SGE Analytical Science, Victoria, Australia) with helium as the carrier gas at a flow rate of 0.9 mL/min. The column temperature was programmed as follows: a subsequent increase from 150°C to 180°C at the rate of 10°C/min, to 185°C at the rate of 1.5°C/min, to 250°C at the rate of 30°C/min, and then 10 min hold. The interface temperature of GC-MS was set at 240°C. The temperature of the ion source was 240°C and the electron energy 70 eV. The total ion current (TIC) mode was used in 50-500 m/z range. According to the shares of individual fatty acids oils oxidation index (U) was calculated using the formula given by COSGROVE *et al.* (1987):

$$U = (0.02 \cdot (C_{16:1} + C_{18:1}) + 1 \cdot C_{18:2} + 2 \cdot C_{18:3}) / 100.$$

2.4. Determination of carotenoids

Carotenoids in oils were analysed with a reversed phase high performance liquid chromatography (RP-HPLC) technique. The sample of oils was diluted in hexane contained β -Apo-8'-carotenal as an internal standard and saponified with 6 mL of 40% methanolic KOH solution in a shaker at room temperature in the dark for 16 h. Next, 30 mL of hexane to the sample was added and then the tube was filled up to 50 mL with 10% Na₂SO₄. The lower phase was separated, triple-rinsed with 10 mL of hexane and collected with the upper organic phase. The organic solvent was evaporated at 40°C under a nitrogen stream and dissolved in 2 mL of a methanol: dichloromethane (45:55 v/v) solution. The chromatographic analysis of carotenoids was conducted according to modified EMENHISER *et al.* (1995) method. Briefly, the analysis was carried out using a 1200 series liquid chromatograph manufactured by Agilent Technologies (Palo Alto, CA, USA), equipped with a diode array detector (DAD) from the same manufacturer. Separation was performed at 30°C on a YMC-C₃₀ 250 x 4.6 mm, 5 μ m column and YMC-C₃₀ 10 x 4.6 mm, 3 μ m precolumn (YMC-Europe GmbH, Germany). A methanol- methyl tert-butyl ether (MTBE) gradient was programmed as it is presented in Table 1.

The absorbance was measured at the wavelength of 450 nm. Carotenoids were identified, based on retention times of available standards (Sigma-Aldrich, USA), and by comparing the UV-Visible absorption spectra.

Table 1: HPLC gradient conditions established for analysis of carotenoids.

Time [min]	Methanol [%]	MTBE [%]	Flow rate [mL/min]
0-5	95	5	1
25	72	28	1.25
33	5	95	1.25
40	95	5	1
60	95	5	1

2.5. Determination of phytosterols

The content of sterols in oils was determined by gas chromatography coupled with mass spectrometry (GC-MS QP2010 PLUS, Shimadzu, Japan) according to the method described

by VLAHAKIS and HAZEBOEK (2000). The sample was saponified by adding a 0.5 mL 2M NaOH methanolic solution at ambient temperature for 2 hours. Unsaponifiables were extracted with diethyl ether which was evaporated under nitrogen conditions. The dry residues were re-dissolved in 1.5 mL of n-hexane and a 0.2 mL of 5 α -cholestane internal standard solution was added (0.4 mg/mL). After evaporation, the residues were re-dissolved in 100 μ L of pyridine and 100 μ L BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) and left in 60°C for 60 minutes to complete derivatization. One mL of hexane was then added to the sample and 1 μ L of the obtained mixture was analysed. A ZB-5MSi capillary column was used for the separations of sterols with helium as a carrier gas at a flow rate of 0.9 mL/min. The injector temperature was set at 230°C and the column temperature was programmed as follows: 70°C for 2 min, a subsequent increase to 230°C at the rate of 15°C/min, to 310°C at the rate of 3°C/min, and then 10 min hold. The interface temperature of GC-MS was set at 240°C. The temperature of the ion source was 220°C and the electron energy 70 eV. The total ion current (TIC) mode for quantification (100-600 m/z range) was used. The quantifications using the internal standard method were done.

2.6. Determination of tocopherols

The tocopherols analysis was carried out by high performance liquid chromatography (HPLC), according to the method described by CZAPLICKI *et al.* (2011). Briefly, 0.1 g of oil (\pm 0.001 g) was diluted in hexane in a 10 mL measuring flask. After subsequent centrifugation (10 min. at 25000 x g) in a 5417R-type Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany), the sample was transferred to a chromatographic vial and 20 μ L was injected into the chromatographic system. The analysis was performed using a 1200 series liquid chromatograph manufactured by Agilent Technologies (Palo Alto, CA, USA), equipped with a fluorescence detector from the same manufacturer. The separation was done on a Merck LiChrospher Si 60 column, 250 mm x 4 mm, 5 μ m. A 0.7% isopropanol solution in hexane at a 1 mL/min flow rate was used as the mobile phase. The fluorescence detector was set at 296 nm for excitation and 330 nm for emission. Peaks were identified on the basis of retention times determined for α -, β -, γ - and δ -tocopherol standards (Merck, Darmstadt, Germany) separately, and their content was calculated using external calibration curves.

2.7. Determination of induction time

Induction time of oils was tested on a Rancimat apparatus 743 (Metrohm, Herisau, Switzerland). The analysis was performed according to method described by FARHOOSH (2007). Briefly, 2.5 g of oil in a reaction vessel was weighed and after capping the vessel was placed in a thermostated electric heating block at temperature 110°C. An air flow rate of 20 L/h was given. Determination of the induction time was based on the conductometric detection of volatile oxidation products. The time that elapsed until these oxidation products appeared was saved as the induction time.

2.8. Determination of initial state of oils rancidity

The acid (AV), peroxide (PV), and p-anisidine (p-AV) values were determined in accordance with procedures of CEN ISO 660:2009, CEN ISO 3960:2010, and CEN ISO 6885:2008, respectively.

2.9. Statistical analysis

The obtained results of all analysis (performed in triplicate) were statistically analysed using Statistica 12.0 PL software (StatSoft Inc., Kraków, Poland). In order to indicate the significance of differences between oil samples, unvaried analysis of variance (ANOVA) with a Duncan test at $p \leq 0.05$ significance level was used. The intra-sample quality variation of fresh oils and their blends with the sea buckthorn oil was assayed using principle component analysis (PCA) at $p \leq 0.05$ significance level.

3. RESULTS AND DISCUSSIONS

The initial rancidity of three used in this study highly unsaturated oils (linseed, borage seed, and evening primrose seed) was relatively low, with an AV value from 1.37 to 3.15 mg KOH/g, and a PV value from 0.93 to 3.76 mEq O₂/kg (Table 2). According to requirements of the Codex Alimentarius Commission these results met the standard for cold-pressed and virgin oils, determined as 4 mg KOH/g, and 15 mEq O₂/kg of oil, respectively. Values of a p-AV of these oils, which reflect the content of secondary products of lipid oxidation, varied from 2.00 to 7.85 (Table 2), and were similar for example to results for raspberry seed oil (OOMAH *et al.*, 2000). Determined AV, PV, and p-AV values showed that used oils were of food-grade quality typical for other cold pressed and virgin oils.

Table 2: Rancidity indices of linseed, evening primrose, and borage oils before enrichment with sea-buckthorn oil.

	Acid value [mg KOH/g oil]		Peroxide value [mEq O ₂ /kg oil]		Anisidine value [-]	
	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD
Linseed	1.37	0.02	0.93	0.05	2.00	0.26
Evening primrose	1.57	0.01	3.76	0.11	7.85	0.61
Borage	3.15	0.22	1.45	0.10	4.61	0.52

The fatty acid compositions of the studied linseed, borage seed, and evening primrose seed oils, and of their blends with sea-buckthorn oil, are presented in Table 3. It was found that linseed, borage seed, and evening primrose seed oils were characterized by a high percentage of polyunsaturated fatty acids (PUFA). The oils being richest in these acids included linseed oil (73%) and evening primrose oil (82%), and the total share of acids containing at least one unsaturated bond in these oils amounted to nearly 90%. In turn, borage oil was characterized by the highest share of γ -linolenic acid (7%) of all the studied oils, and unsaturated fatty acids were also represented by oleic (26%) and linoleic acid (33%). The oils used in the experiment are valued for their composition of fatty acids; however, both the great number of unsaturated bonds and the degree of their unsaturation have an adverse effect on the oxidative stability of an oil. The susceptibility of the oils under study to oxidation is expressed as an oxidizability index calculated according to the formula proposed by COSGROVE *et al.* (1987). Borage oil turned out to be the oil being least susceptible to oxidative changes; the oxidizability index for this oil amounted to 0.61. In turn, linseed oil, which contained almost 60% of α -linolenic acid, exhibited the highest value of the oxidizability index (1.33). As compared with e.g.

rapeseed oil (ROSZKOWSKA *et al.*, 2015), the oxidizability indices of the oils under study had values higher by 65, 143, and 259% for, respectively, borage oil, evening primrose oil, and linseed oil.

Table 3: Fatty acids composition (%), the main bioactive compounds content in oils [mg/100 g of oil], and their oxidation index [-] and induction time [h].

Compound	Linseed oil		Borage seed oil		Evening primrose seed oil		Sea buckthorn fruit oil	
	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD
palmitic (C _{16:0})	7.33 ^a	1.13	19.60 ^b	1.25	7.98 ^a	0.11	36.31 ^c	0.01
palmitoleic (C _{16:1})	nd ^a		nd ^a		nd ^a		40.97 ^b	0.04
stearic (C _{18:0})	3.28 ^a	0.75	7.46 ^b	0.44	2.28 ^c	0.05	0.55 ^d	0.01
oleic (C _{18:1})	15.88 ^a	1.8	26.40 ^b	1.44	7.12 ^c	1.34	8.77 ^c	0.11
linoleic (C _{18:2})	14.49 ^a	0.32	32.64 ^b	1.41	75.47 ^c	1.88	12.12 ^d	0.13
α-linolenic (C _{18:3})	59.02 ^a	3.36	nd ^b		nd ^b		1.28 ^c	0.04
γ-linolenic (C _{18:3})	nd ^a		13.91 ^b	1.73	7.17 ^c	0.39	nd ^a	
Σ PUFA	73.51 ^a	3.68	46.55 ^b	3.14	82.64 ^c	2.27	13.40 ^d	0.17
Oxidizability index	1.33^a	0.07	0.61^b	0.05	0.90^c	0.03	0.16^d	0.00
lutein	0.22 ^a	0.01	0.09 ^a	0.01	0.07 ^a	0.01	3.24 ^b	0.49
all-trans β-carotene	0.22 ^a	0.05	0.07 ^a	0.05	0.14 ^a	0.04	118.36 ^b	9.58
other carotenoids	0.04 ^a	0.01	0.01 ^a	0.01	0.09 ^a	0.08	74.82 ^b	6.25
total carotenoids	0.48^a	0.06	0.18^a	0.07	0.30^a	0.12	206.04^b	15.63
α-tocopherol	6.21 ^a	0.30	nd ^b		26.42 ^c	0.17	144.14 ^d	4.10
β-tocopherol	nd ^a		nd ^a		nd ^a		3.98 ^b	0.23
γ-tocopherol	37.02 ^a	1.01	11.95 ^b	0.21	40.41 ^c	0.86	4.63 ^d	0.32
δ-tocopherol	nd ^a		100.25 ^b	0.50	nd ^a		0.75 ^a	0.00
total tocopherols	43.23^a	1.32	112.20^b	0.71	66.83^c	0.69	153.50^d	4.10
campesterol	40.44 ^a	0.63	38.30 ^a	3.04	57.35 ^b	3.08	7.87 ^c	0.45
Δ5-avenasterol	13.37 ^a	0.53	43.42 ^b	0.77	62.92 ^c	5.95	20.46 ^d	1.54
β-sitosterol	110.21 ^a	0.29	37.96 ^b	2.05	595.94 ^c	6.46	536.30 ^d	1.25
Δ7-stigmastenol	nd ^a		nd ^a		5.95 ^b	0.28	nd ^a	
Δ7-stigmasterol	14.75 ^a	0.35	nd ^b		nd ^b		nd ^b	
cycloartenol	162.38 ^a	0.53	51.02 ^b	4.74	nd ^c		28.50 ^a	0.63
other sterols	45.44 ^a	1.64	37.13 ^a	1.22	8.58 ^b	0.19	262.81 ^c	16.61
total sterols	386.58^a	0.83	207.81^b	7.49	730.74^c	4.06	855.94^d	8.95
Induction time	2.44^a	0.23	3.91^b	0.25	3.97^b	0.20	>48^c	

nd – not detected

Values within a row with different letters are significantly different ($p \leq 0.05$).

Sea-buckthorn oil, used as a stabilizer, owed its resistance to oxidation to, *inter alia*, the relatively low share of polyunsaturated fatty acids. The oxidizability index calculated for this oil only amounted to 0.16, and the induction time in the Rancimat test was longer than 48 h (in a temperature of 110°C), which demonstrates its extraordinary resistance to oxidative changes. However, this resistance is owed not only to the characteristics of fatty acids but also to the abundance of natural antioxidants. The average content of carotenoids in sea-buckthorn oil amounted to 206 mg/100 g, and the predominant one was β -carotene (65%). In addition to β -carotene, sea-buckthorn oil also contained, *inter alia*, α -carotene, lutein, zeaxanthin, and β -cryptoxanthin. Sea-buckthorn oil also contained tocopherols at an amount of 144 mg/100 g, with the predominant α homologue (94%), and phytosterols at an amount of 856 mg/100 g, with the predominant β -sitosterol (63%).

Linseed, evening primrose, and borage oils were characterized by significantly lower contents of these antioxidants. The carotenoid content did not exceed the value of 0.48 mg/100 g and, in the extreme case, was ca. 1100 times lower than that in sea-buckthorn oil. Sea-buckthorn oil was also significantly richer in tocopherols, as it contained, respectively, 3.6-, 1.4-, and 2.3-times more of these compounds than, in turn, linseed, borage seed, and evening primrose seed oils. In the group of these compounds, γ -tocopherol (linseed and evening primrose oils), and δ -tocopherol (borage seed oil) were predominant. As regards phytosterols, the total content thereof being similar to that of sea-buckthorn oil was found in evening primrose seed oil, while linseed oil and borage seed oil contained 2.21- and 4.12-times less of those compounds, respectively. The predominant phytosterols in the enriched oils included cycloartenol (linseed oil and borage seed oil) and β -sitosterol (evening primrose seed oil).

The addition of sea-buckthorn oil resulted in an over 64-, 171-, and 103-fold increase in the carotenoid content of linseed oil, borage seed oil, and evening primrose seed oil, respectively, with a particularly apparent increase in the share of all-trans β -carotene (Table 4).

The enrichment with sea-buckthorn oil resulted in an increase in the content of this compound to a level ranging from approx. 3 mg/100 g in an evening primrose seed oil blend to almost 5 mg/100 g in a linseed oil blend. The enrichment with sea-buckthorn oil also contributed to an increase in tocopherol content. This change was biggest for linseed oil (45%), and the content of α -tocopherol in this oil increased over three-fold. Borage seed oil, which was the only one containing no α -tocopherol, was enriched with this component to an amount of almost 29 mg/100 g. At the same time, the addition of sea-buckthorn oil caused a significant increase in the content of β -sitosterol in linseed oil (58%) and borage seed oil (197%). Evening primrose seed oil was the only oil in which no significant change to the concentration of this sterol was noted, with the total increase in the share of sterols by 2.57%.

At the same time, the enriched oils were characterized by significantly lower oxidizability index (a decrease by 12-30%), and the induction time being increased by approx. 21-32% (Table 4). The noted relative increase in the stability of oil was statistically significant, and reached the highest value for linseed oil. However, the actual induction time of this oil only increased to a value of 3.21 h (from the initial value of 2.44 h), which may be explained by the particularly high content of PUFA. This phenomenon may be explained not only by the change to fatty acid concentration but also by the more than 3-fold increase in the concentration of α -tocopherol, and over 60-fold increase in the concentration of carotenoids.

Table 4: Fatty acids composition (%), the main bioactive compounds content [mg/100 g of oil] in oils blends with sea buckthorn oil and their oxidation index [-] and induction time [h].

Compound	Linseed oil		Borage seed oil		Evening primrose seed oil	
	\bar{x}	SD	\bar{x}	SD	\bar{x}	SD
palmitic (C _{16:0})	10.87 ^a	0.83	27.15 ^b	1.97	13.69 ^c	1.99
palmitoleic (C _{16:1})	5.68 ^a	0.01	7.10 ^b	0.53	7.17 ^b	0.69
stearic (C _{18:0})	2.96 ^a	0.37	6.74 ^b	0.47	1.87 ^c	0.19
oleic (C _{18:1})	15.19 ^a	0.17	25.22 ^b	0.75	8.19 ^c	0.29
linoleic (C _{18:2})	14.39 ^a	0.21	24.94 ^b	1.11	63.32 ^c	2.09
α-linolenic (C _{18:3})	50.91 ^a	1.18	nd ^b		nd ^b	
γ-linolenic (C _{18:3})	nd ^a		8.87 ^b	0.05	5.77 ^c	5.77 ^c
Σ PUFA	65.30 ^a	1.39	33.81 ^b	1.16	69.09 ^a	3.15
Oxidizability index	1.17 ^a	0.03	0.43 ^b	0.01	0.75 ^c	0.04
lutein	0.67 ^a	0.04	0.56 ^a	0.10	0.55 ^a	0.13
all-trans β-carotene	17.94 ^a	0.20	17.81 ^a	0.77	17.87 ^a	0.47
other carotenoids	11.26 ^a	0.16	11.23 ^a	0.80	11.30 ^a	0.46
total carotenoids	31.31 ^a	0.29	31.06 ^a	1.9	31.16 ^a	0.46
α-tocopherol	26.90 ^a	2.18	21.62 ^b	1.26	44.08 ^c	0.60
β-tocopherol	0.60 ^a	0.43	0.60 ^a	0.44	0.60 ^a	0.06
γ-tocopherol	32.16 ^a	1.05	10.85 ^b	0.54	35.04 ^c	0.19
δ-tocopherol	0.11 ^a	0.00	85.33 ^b	1.64	0.11 ^a	0.00
total tocopherols	59.77 ^a	1.65	118.40 ^b	2.87	79.83 ^c	0.85
campesterol	35.55 ^a	1.81	33.74 ^a	2.12	49.93 ^b	3.94
Δ5-avenasterol	14.43 ^a	3.25	39.98 ^b	2.15	56.55 ^c	3.52
β-sitosterol	174.12 ^a	6.16	112.71 ^b	8.99	586.99 ^c	29.61
Δ7-stigmastenol	nd ^a		nd ^a		5.06 ^b	0.44
Δ7-stigmasterol	12.54 ^a	0.66	nd ^b		nd ^b	
cycloartenol	142.30 ^a	6.05	47.64 ^b	3.51	4.28 ^c	0.39
other sterols	78.05 ^a	7.55	70.98 ^a	5.30	46.71 ^b	2.45
total sterols	456.98 ^a	11.25	305.03 ^b	10.15	749.52 ^c	14.28
Induction time	3.21 ^a	0.23	4.80 ^b	0.25	4.81 ^b	0.18

nd – not detected

Values within a row with different letters are significantly different ($p \leq 0.05$).

A PCA analysis confirmed strong correlation between the induction time for the oil and the shares of palmitoleic acid (0.98) and palmitic acid (0.77) being typical of sea-buckthorn oil (SHAFI *et al.*, 2008) (Fig. 1a), and between the induction time and the content of α- and β-tocopherol as well as lutein and β-carotene – correlations within a range of 0.96–0.99 (Fig. 2a). Stability of the oils was affected, to a much smaller extent, by the content of β-sitosterol, with the correlation coefficient being only equal to 0.41.

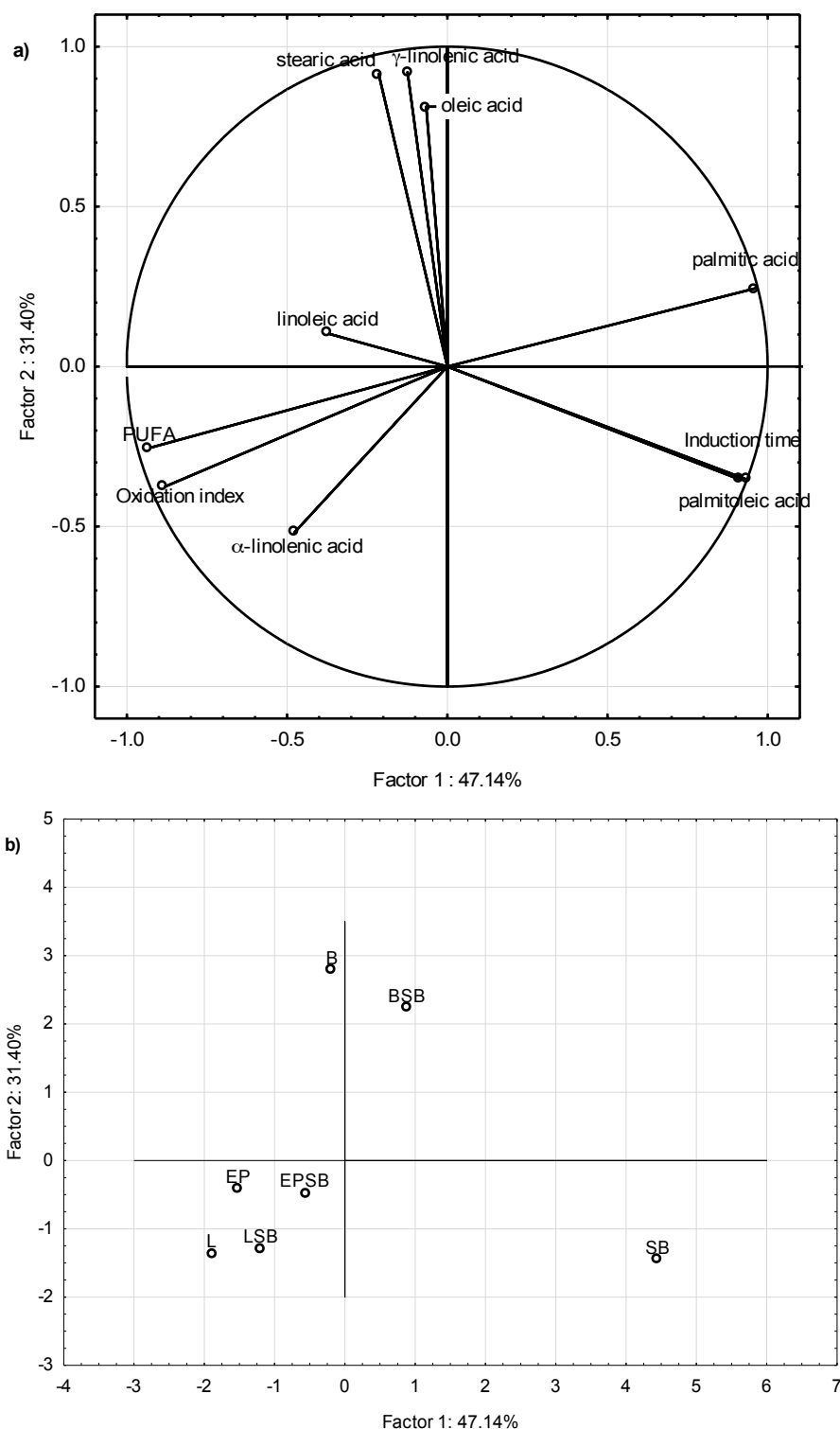


Figure 1: a) PCA loading plot of tested variables; b) Score plot of the two first principal components after PCA analysis of fatty acid composition, oxidation index and induction time of fresh oils and their blends with sea buckthorn fruit oil.

B – borage seed oil, BSB – borage seed oil enriched with sea buckthorn fruit oil, EP – evening primrose seed oil, EPSB - evening primrose seed oil enriched with sea buckthorn fruit oil, L – linseed oil, LSB - linseed oil enriched with sea buckthorn fruit oil, SB - sea buckthorn fruit oil.

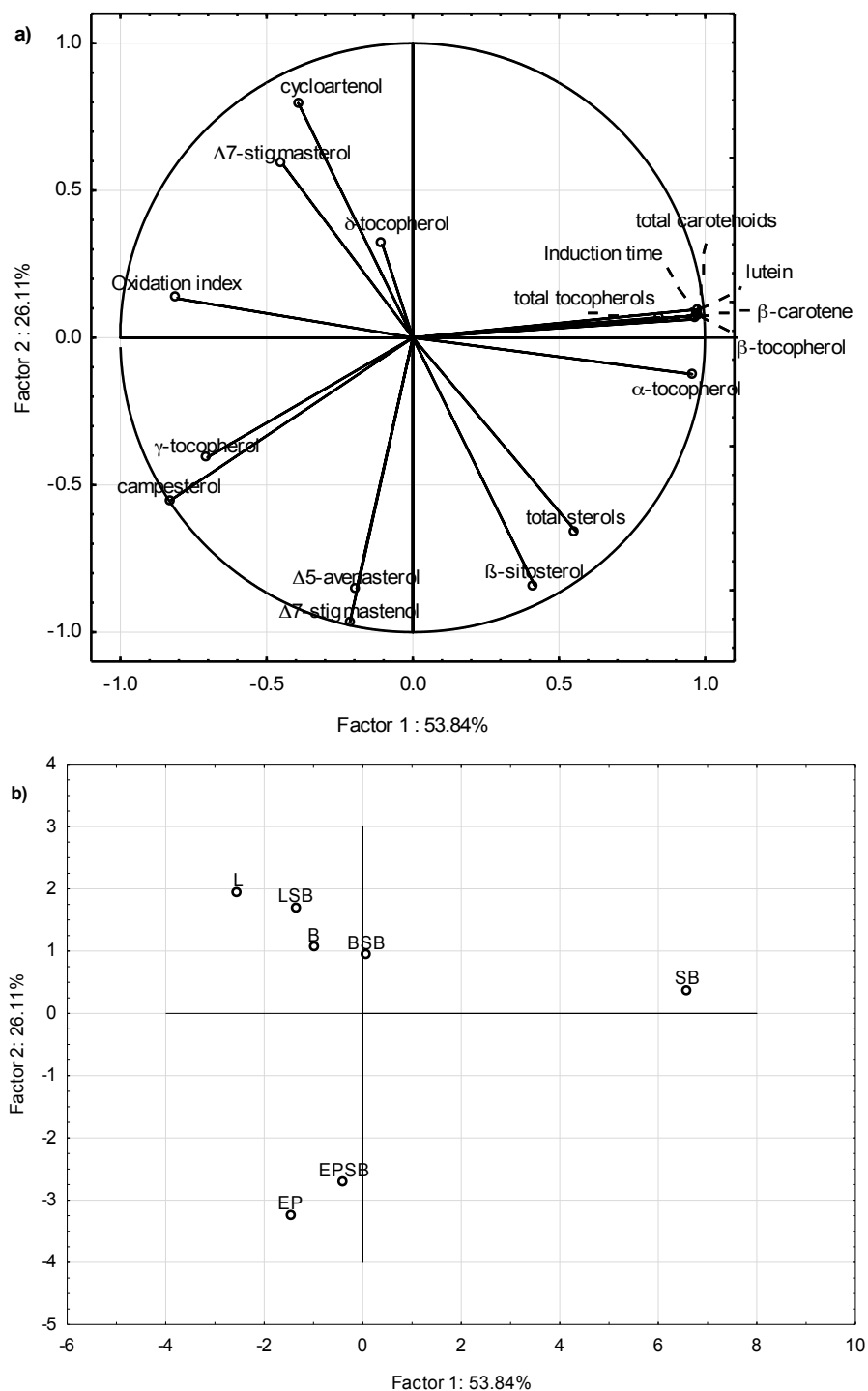


Figure 2: a) PCA loading plot of tested variables; b) Score plot of the two first principal components after PCA analysis of bioactive compounds content, oxidation index and induction time of fresh oils and their blends with sea buckthorn fruit oil.

B – borage seed oil, BSB – borage seed oil enriched with sea buckthorn fruit oil, EP – evening primrose seed oil, EPSB – evening primrose seed oil enriched with sea buckthorn fruit oil, L – linseed oil, LSB – linseed oil enriched with sea buckthorn fruit oil, SB – sea buckthorn fruit oil.

The relationship between the induction time for an oil and its potential oxidizability is rarely noted, since an oil is a complex mixture of compounds. Strong correlations could

possibly be noted for a pure phase of proper lipids. However, BHATNAGAR *et al.*, (2009) demonstrated that the addition of coconut oil to refined sunflower oil and rice bran oil had a positive effect on the stability of blends due to a change to the proportions of fatty acids. Correlations between the induction time (stability of an oil) and the antioxidants content were found significantly more often (MATEOS *et al.*, 2005). However, in the case of mixture of antioxidants the resultant activity depends not only on their content and composition, but also on their synergistic or antagonistic activity, as well as lipophilic or hydrophilic properties (KMIECIK *et al.*, 2011). A relationship between the stability of an oil and the content of β -carotene, being similar to that noted in this study, was found earlier by GOULSON and WARTHESEN (1999) in relation to high oleic rapeseed oil. They demonstrated that β -carotene significantly inhibited the oxidation of this oil in the dark at a concentration of approx. 5 mg/100 g, while with the simultaneous exposure, the antioxidant effect was already observed at a concentration of approx. 2.75 mg/100 g. However, the impact of particular antioxidant components is not only dependent on the concentrations at which they occur. Synergistic interactions which were demonstrated, *inter alia*, between α -tocopherol and β -carotene, are also important (SCHROEDER *et al.*, 2006). Tocopherols are considered to be the main lipid antioxidants. CHOE and MIN (2006), referring to numerous studies, report that the tocopherols' capacity to quench free radicals depends on their structure and the concentration in the oil. According to the cited authors, the highest activity as regards quenching free radicals is exhibited by δ -tocopherol, and the value for this activity decreases for γ -, β - and α -tocopherol, respectively. The addition of α -tocopherol at an amount of 10 mg/100 g of oil may, in certain cases, even accelerate the oxidation (CHOE and MIN, 2006). In turn, with concentrations of tocopherols exceeding $4 \cdot 10^{-3}$ M, the activity of particular homologues does not differ significantly (JUNG *et al.*, 1991).

Sterols were the predominant group of compounds in the unsaponifiable fraction of all oils under study. The noted low coefficient of correlation between their concentrations and the induction time indicates a weaker resultant effect of the action of these compounds on the oxidation mechanism. Earlier studies indicate that these compounds may exhibit both pro- and antioxidative action. A weak pro-oxidative action was demonstrated for, *inter alia*, β -sitosterol (LAMPI *et al.*, 1999), while ergosterol, lanosterol, stigmasterol and cholesterol exhibit no antioxidative properties in relation to thermally oxidized safflower oil (SIMS *et al.*, 1972). On the other hand, antioxidative activity is exhibited by sterols containing an ethylidene group within their structure (LAMPI *et al.*, 1999). This group is found in, *inter alia*, Δ^5 -avenasterol which, in the oils tested as part of this study, occurred at an amount ranging from approx. 13 mg/100 g (linseed oil) to 63 mg/100 g (evening primrose oil). The noted increase in the content of Δ^5 -avenasterol by approx. 8% in enriched linseed oil could have affected the increase in the stability of this oil in relation to a non-enriched oil. The mechanism of antioxidative action of sterols having an ethylidene group involves their capacity to easily split off a proton, and form stable tertiary radicals. The resulting compounds are so durable that they do not initiate autooxidation (MAŁECKA, 1995). Our recent study showed that the thermal degradation of rapeseed oil sterols was faster than tocopherols and carotenoids (ROSZKOWSKA *et al.*, 2015).

Analysis of plot score (Fig. 1b) confirmed a close similarity of linseed oil and evening primrose seed oil, and blends of these oils with sea-buckthorn oil. Then, a separate group comprised both borage seed oils (enriched and natural). Both groups of oils were clearly distinguished from sea-buckthorn oil in terms of the composition of fatty acids. On the other hand, other chemical components analysis (Fig. 2b) showed a close similarity of linseed oil and borage seed oil, and their blends with sea-buckthorn oil. In this case, a separate group comprised natural and enriched evening primrose seed oils.

4. CONCLUSIONS

Results of the study suggest that the development of blends of oils containing valuable and unique polyunsaturated fatty acids with sea-buckthorn oil being rich in antioxidant compounds increases the oxidative stability of these oils. In addition, the enrichment with sea-buckthorn oil contributes to both significant increase in the content of carotenoids (mainly β -carotene), α -tocopherol and β -sitosterol, and relative decrease in the share of polyunsaturated acids. Enriched oils gain new sensory qualities (the color derived from carotenoids) and health-promoting properties (the inhibition of radical formation, and the presence of numerous antioxidants); moreover, they have an extended shelf life.

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FUNCTIONAL SAUSAGE MADE FROM MECHANICALLY SEPARATED TILAPIA MEAT

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ABSTRACT

Physicochemical, bacteriological and sensory parameters of sausages made from waste of Nile tilapia with the prebiotic inulin added and reduced sodium were investigated. Mechanically separated carcass meat and mechanically separated head meat were processed into sausages with or without inulin and salt replacer. T1 and T3 showed greatest lipid, but lowest carbohydrate levels, a^* and b^* values ($P < 0.05$). In general, the inulin formulations showed higher acceptability and purchase intent ($P < 0.05$). The addition of inulin to low-sodium tilapia sausages is a promising technological strategy to minimize negative effects on the taste and texture from KCl increment.

Keywords: fish waste, healthy products, inulin, *Oreochromis niloticus*, potassium chloride, salt replacer

1. INTRODUCTION

The Nile tilapia is the most important species for aquaculture in the world. Tilapia is marketed either as whole fish or as fresh or frozen fillets, although consumers prefer fillets. Reported fillet yields of Nile tilapia vary widely (30-40%), generating a large amount of wastes that are commonly underutilized, used as animal feed, or discarded (MONTEIRO *et al.*, 2012; MONTANHINI NETO and OSTRENSKY, 2013).

The utilization of fish-processing waste to develop new products provides an important opportunity for the food industry to produce sustainable and value-added products as a source of omega-3 polyunsaturated fatty acids (DECKELBAUM and TORREJON, 2012) and high-value protein. Further studies are needed to develop ways to use fish waste to produce functional products with lower costs (MONTEIRO *et al.*, 2014a; PALMEIRA *et al.*, 2014a; PALMEIRA *et al.*, 2014b).

Some investigators have reported the beneficial effects of including inulin as a fat replacer in chicken and pork sausages, with desirable effects such as improved texture. In addition, inulin has a prebiotic physiological effect, attributed to fructan, which stimulates the growth of beneficial bacteria in the intestine of the host (MENDOZA *et al.*, 2001; MENEGAS *et al.*, 2013).

The reduction of sodium in processed products has constituted a challenge for the food industry, particularly in view of the contemporary consumer's awareness of the relationship between food and health. The need to reduce salt contents is further reinforced by agreements between the industrial sector and the World Health Organization (WHO). The main challenge is to maintain the acceptability as well as physicochemical parameters of the food (DESMOND, 2006; DOTSCHE *et al.*, 2009; MONTEIRO *et al.*, 2014b), considering microbiological quality standards. However, inulin and sodium replacers in fish products have not been studied.

This study evaluated the effect of sodium reduction and inulin addition on physicochemical, bacteriological and sensory parameters of sausages manufactured from mechanically separated meat of Nile tilapia.

2. MATERIALS AND METHODS

2.1. Obtaining mechanically separated tilapia meat

The wastes from heads and carcasses of tilapia were obtained from a fish-farming cooperative in Rio de Janeiro, Brazil. The fish heads and carcasses were passed through a deboning machine (KME, São Paulo) to remove the muscle from the bones, and were then washed.

The mechanically separated carcass meat (MSCM) and mechanically separated head meat (MSHM) were frozen and packed in 1kg polyethylene packages at -18°C. The samples were transported in cold boxes to keep them frozen during transport to the pilot plant of the Centro de Tecnologia SENAI Alimentos e Bebidas, Rio de Janeiro, Brazil, where they were stored in a freezer (-18°C) until the MSHM and MSCM were used to produce sausages. Four different sausage formulations were prepared: 100% NaCl without inulin (T1), 100% NaCl + 6% inulin (T2), 50% NaCl and 50% KCl + without inulin (T3), and 50% NaCl and 50% KCl + 6% inulin (T4). In formulations without inulin, MSHM was used as a replacer, and potassium chloride (KCl) was used to reduce the sodium content. All formulations are described in Table 1.

Table 1: Formulations of sausages manufactured from mechanically separated tilapia meat.

Ingredients (%)	Treatments*			
	T1	T2	T3	T4
MSCM**	81.91	81.91	81.91	81.91
Sodium chloride	1.3	1.3	0.65	0.65
Potassium chloride	-	-	0.65	0.65
Soybean protein	2.0	2.0	2.0	2.0
MSHM**	10.0	4.0	10.0	4.0
Inulin	-	6.0	-	6.0
Polyphosphate	0.5	0.5	0.5	0.5
Manioc starch	2.0	2.0	2.0	2.0
Sodium erythorbate	0.3	0.3	0.3	0.3
Carmine	0.05	0.05	0.05	0.05
Collagen	1.0	1.0	1.0	1.0
Nitrite	0.25	0.25	0.25	0.25
Onion	0.3	0.3	0.3	0.3
Garlic	0.2	0.2	0.2	0.2
White pepper	0.1	0.1	0.1	0.1
Cilantro	0.05	0.05	0.05	0.05
Ginger	0.04	0.04	0.04	0.04

*T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin), T3 (50% NaCl and 50% KCl + without inulin), T4 (50% NaCl and 50% KCl + 6% inulin).

** MSCM - mechanically separated carcass meat; and MSHM - mechanically separated head meat.

2.2. Experimental design

Ten kg and thirty-kg of MSHM and MSCM were obtained, respectively. For each experimental replication ($n = 2$), the samples were divided into four groups each with 3.5 kg of MSHM (T1 and T3), 1.5 kg of MSHM (T2 and T4), and 7.5 kg of MSCM. Each group was divided at random into 40 g portions, resulting a total of 40 samples per group which were separated in the proposed formulations and analyzed on days 0 and 34 of refrigerated storage, totaling 80 samples units.

2.3. Sausage manufacturing

To compose the sausage, the ingredients (Table 1) were mixed in a cutter. After the emulsion was formed in the cutter, the sausage mass was placed in a manual sausage maker using sausage collagen casings of 21 mm diameter, and separated into sections. The sausage was heat-treated, starting with pre-cooking in a drying oven (Incomaf Indústria Ltda., São Paulo, Brazil) with circulating hot air at 50°C for 15 min with an open chimney, then raised to 60°C for 30 min with a closed chimney and increased by 5°C every 5 min (steam drying) until the sausage reached 72°C internally, as measured with the aid of a thermocouple. The sausages were then cooled in a water bath to an internal temperature

of 40°C. The sausages were stored in a cooling chamber at 5°C until the next day, when the membranes were delaminated and the products were vacuum-packed in polythene bags and stored under refrigeration (5°C). The products were placed in isothermal boxes and transported to the laboratory in Rio de Janeiro, Brazil, where they were kept under refrigeration (4°C) for 34 days until analytical procedures. The analyses on day 0 were performed immediately after the arrival of the samples.

2.4. Physicochemical analyses

Water, ash, protein and lipid contents were evaluated in MSHM and MSCM on day 0 (AOAC, 2012). In the sausages, pH values were determined with a digital pH meter (Digimed®DM-22) equipped with a DME-R12 electrode (Digimed®) according to CONTE-JÚNIOR *et al.* (2008). Water, ash, protein, lipid and carbohydrate contents were determined by standard methods (AOAC, 2012). The carbohydrate content was estimated taking into account the ingredient composition. The energy content (kcal/100g) was estimated according to TRIKI *et al.* (2013). These analyses were performed immediately after manufacture and repeated after 34 days of storage, to assess the maintenance of quality and identity of the product.

In addition, on day 0 the cooking yield was calculated as the cooked weight of the sausages divided by the weight of the pre-cooked sausage, multiplied by 100 (HORITA *et al.*, 2011). All analyses were performed in quadruplicate for each treatment.

The instrumental color parameters were determined using a Konica Minolta CR-400 colorimeter (Konica Minolta Sensing, Osaka, Japan) previously calibrated with CIE Standard Illuminant D65, a 8 mm-diameter aperture, and a 2° standard observer (AMSA, 2012). Samples were macerated to a thickness of 3 cm in a beaker with a diameter of 15 cm. Results are expressed in CIELAB L^* (lightness), a^* (redness) and b^* (yellowness) values. Measurements were performed in triplicate for each treatment.

2.5. Bacteriological analysis

Bacteriological analyses of the MSHM, MSCM and sausages were performed on day 0. Total aerobic mesophilic bacteria (TAMB), Coagulase-positive *Staphylococcus* bacterial counts, and most probable number of thermotolerant coliforms were evaluated. The presence of *Salmonella* spp. was evaluated and the results were expressed as presence or absence in 25 g of sample (APHA, 2001).

2.6. Sensory evaluation

The panelists were recruited from the students, faculty, and staff of the Universidade Federal Fluminense, Brazil. Sausage samples were analyzed at room temperature (20°C) and were coded with three-digit random numbers. The samples were presented to 100 untrained panelists (38 men and 62 women, 18 to 47 years old) who were instructed to evaluate their overall liking for each sausage sample, using the 9-point hedonic scale (1=dislike extremely, 9=like extremely). The panelists also recorded their purchase intent (5-point scale: 1 = “definitely would not buy”, 5 = “definitely would buy”) (STONE and SIDEL, 1998). These consumer tests were conducted in two stages: with no information (blind – first stage), and with the information “Mechanically separated tilapia meat was used to make the sausages, and its use reduces environmental pollution and encourages sustainability” (informed – second stage) (GARCIA *et al.*, 2009).

In the first stage, color, bitter taste, salty taste, succulence, elasticity and softness were also evaluated using a five-point Just About Right (JAR) scale (1 = much too weak, 2 =

somewhat weak, 3 = just about right, 4 = somewhat strong, and 5 = much too strong) according to CERVANTES *et al.* (2010).

An unsalted cracker and a glass of water at 25°C were offered to cleanse the palate between samples. The sensory evaluation was performed two days after the sausage was manufactured, to ensure adequate bacteriological quality.

2.7. Statistical analysis

Data for chemical composition, energy value, pH, overall liking and purchase intent were evaluated separately for treatment and time (days 0 and 34) or stages (blind and informed) by ANOVA. Cooking yield, instrumental color parameters, JAR data and bacteriological results were analyzed using one-way ANOVA. These data were further analyzed using a Tukey test when the means were considered different ($P < 0.05$). Chemical composition of MSHM and MSCM was evaluated by one-way ANOVA at a 95% confidence interval.

In addition, a principal components analysis (PCA) was performed to assess the parameters that were influenced by addition of inulin and potassium chloride. Partial least squares regression (PLSR) was performed to assess if the determinant parameters contributed positively or negatively to the overall liking of the samples. Penalty analysis (PA) was used to analyze the JAR data in order to identify possible alternatives for product improvement. Pearson's correlation at a 5% significance level ($P < 0.05$) was performed to correlate the physicochemical and sensory data (color and texture parameters). All statistical analyses were performed using the software XLSTAT version 2012.6.08 (Addinsoft, Paris, France).

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical and bacteriological analyses of MSHM and MSCM

MSCM and MSHM exhibited 0.43% (± 0.0006) and 0.73% (± 0.0008) ash, 86.14% (± 0.001) and 69.41% (± 0.0069) water, 2.34% (± 0.0013) and 18.39% (± 0.0112) lipid, and 4.78% (± 0.0056) and 12.62% (± 0.0044) protein, respectively. The MSHM had a higher content of lipids, ash and proteins and lower water content than the MSCM. MONTEIRO *et al.* (2012) also observed higher lipid and ash, but lower water content in MSHM than in MSCM.

Regarding bacterial quality, *Salmonella* spp. and Coagulase-positive *Staphylococcus* were not detected in the samples. The most probable number of thermo-tolerant coliform and TAMB counts were within the official limits (ICMSF, 1986) which was also observed by MONTEIRO *et al.* (2012) in mechanically separated tilapia meat.

Our results suggest that the process of obtaining mechanically separated meat was conducted appropriately, and that the use of tilapia wastes can be a viable alternative for the development of value-added products.

3.2. Physicochemical parameters of tilapia sausage

Water, ash and protein contents did not differ significantly among the treatments ($P > 0.05$) on both days 0 and 34 (Table 2). T1 and T3 showed higher lipid and lower carbohydrate contents than T2 and T4 ($P < 0.05$). This can be explained by inulin addition, replacing the MSHM in T2 and T4. In agreement with our findings, MENEGAS *et al.* (2013) reported that foods with added carbohydrate (inulin) as a fat replacer showed lower lipid and higher carbohydrate contents.

Although the lipid content of T1 and T3 differed from T2 and T4, no difference ($P > 0.05$) was observed in energy value among all treatments, regardless of storage period. Thus, in the sausages with lower fat content, the loss of energy value was offset by the increased carbohydrate levels. However, sausages made with mechanically separated tilapia meat residue still have a very low caloric value, similar to low-fat sodium reduced fresh merguez sausage (TRIKI *et al.*, 2013). This is a desirable factor for both fish processors and consumers.

The pH values of the sausages did not differ ($P > 0.05$) among the treatments on days 0 and 34 of refrigerated storage. MONTEIRO *et al.* (2014c) also observed no difference among pH values of restructured tilapia steaks.

The samples were stored under refrigeration, and after 34 days of storage at $4 \pm 1^\circ\text{C}$, the analyses were repeated. No significant differences ($P > 0.05$) were observed in water, ash, protein, lipid, carbohydrate, energy value and pH values (Table 2), which indicates that the identity of the product was maintained after refrigerated storage.

Table 2: Chemical composition, energy value and pH of sausages made from mechanically separated tilapia meat.

Parameters	Storage time (days)	Treatments*			
		T1	T2	T3	T4
Water	0	74.05 ^{ax} ±1.76	70.28 ^{ax} ±2.09	73.33 ^{ax} ±3.89	70.22 ^{ax} ±1.50
	34	72.86 ^{ax} ±2.69	69.30 ^{ax} ±3.07	73.43 ^{ax} ±2.66	70.02 ^{ax} ±2.53
Ash	0	2.99 ^{ax} ±0.51	2.92 ^{ax} ±0.52	3.06 ^{ax} ±0.52	3.02 ^{ax} ±0.38
	34	3.05 ^{ax} ±0.51	2.94 ^{ax} ±0.46	3.03 ^{ax} ±0.36	2.93 ^{ax} ±0.42
Protein	0	14.26 ^{ax} ±1.51	13.77 ^{ax} ±1.36	14.56 ^{ax} ±1.38	13.71 ^{ax} ±1.00
	34	12.61 ^{ax} ±1.37	12.51 ^{ax} ±2.09	14.04 ^{ax} ±0.70	14.33 ^{ax} ±1.29
Lipid	0	2.64 ^{ax} ±0.06	0.86 ^{bx} ±0.15	3.16 ^{ax} ±0.09	1.38 ^{bx} ±0.49
	34	3.82 ^{ax} ±1.24	1.72 ^{bx} ±1.05	3.02 ^{ax} ±0.39	2.02 ^{bx} ±0.82
Carbohydrate	0	6.06 ^{bx} ±2.45	12.18 ^{ax} ±2.70	5.90 ^{bx} ±2.09	11.68 ^{ax} ±0.82
	34	7.66 ^{bx} ±2.33	13.54 ^{ax} ±1.84	6.48 ^{bx} ±2.18	10.71 ^{ax} ±0.82
Energy value	0	105.05 ^{ax} ±5.21	111.52 ^{ax} ±6.17	110.24 ^{ax} ±13.14	113.12 ^{ax} ±6.63
	34	115.44 ^{ax} ±14.84	119.68 ^{ax} ±15.48	109.28 ^{ax} ±10.80	116.55 ^{ax} ±6.86
pH	0	6.72 ^{ax} ±0.02	6.71 ^{ax} ±0.08	6.75 ^{ax} ±0.04	6.76 ^{ax} ±0.01
	34	6.68 ^{ax} ±0.10	6.73 ^{ax} ±0.01	6.72 ^{ax} ±0.06	6.71 ^{ax} ±0.06

* T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin), T3 (50% NaCl and 50% KCl + without inulin), T4 (50% NaCl and 50% KCl + 6% inulin).

Water, ash, protein, lipid and carbohydrate expressed in g/100g of sample.

Energy value expressed in kcal/100g.

Values are means±SD.

↔ Means in a row without common superscripts are different ($P < 0.05$); $n = 2$.

· Means in a column with common superscripts did not exhibit difference ($P > 0.05$); $n = 2$.

With regard to cooking yield, no differences ($P > 0.05$) were observed among the treatments (Table 3). Similarly, HORITA *et al.* (2011) and MONTEIRO *et al.* (2014b) found no significant differences in cooking yield when NaCl was replaced with KCl in low-fat

bologna and restructured tilapia steaks, respectively. However, TRIKI *et al.* (2013) reported that replacing sodium chloride with a salt mixture (potassium chloride, calcium chloride and magnesium chloride) increased cooking losses.

The direct relationship between fat content and cooking yield is due to the emulsion stability provided by fat. Inulin has the ability to form a gel and acts similarly to fat (FRANCK, 2002). In this study, there was no relationship between fat content and cooking yield. Similarly, BRENNAN *et al.* (2004) found no differences in cooking loss (fluid loss) with the inclusion of inulin in spaghetti pasta. However, ÁLVAREZ and BARBUT (2013) suggested that inulin powder produces a higher yield compared to inulin gel. FELISBERTO *et al.* (2015) suggested that significant fluid losses were observed in formulations containing inulin or polydextrose. Our results suggest that inulin and KCl addition at the levels of the present study did not affect the product yield.

Color from tilapia muscle can be strongly affected by meat processing, and consequently influence consumer acceptability. Therefore, it is important to study color behavior in fish waste destined for human consumption, to predict changes that may occur in the final product (RAWDKUEN *et al.*, 2009; MONTEIRO *et al.*, 2014a). T4 exhibited a higher L^* value ($P < 0.05$) than the other treatments (Table 3). MENEGAS *et al.* (2013) observed that the inclusion of inulin increased the lightness of dry-fermented chicken sausage. In addition, MONTEIRO *et al.* (2014b) observed that the L^* value was intensified in restructured tilapia steaks manufactured with 50% NaCl and 50% KCl. T2 and T4 presented higher a^* values ($P < 0.05$). These formulations had a lower lipid content (inulin added to replace MSHM). CANDOGAN and KOLSARICI (2003) noted that products with reduced lipid tend to be redder, due to concentration of lean meat, which in this study, was represented by the MSCM. T3 exhibited lower ($P < 0.05$) a^* and b^* values than the other treatments (Table 3), suggesting that replacing 50% NaCl with KCl can affect color parameters, as also observed by MONTEIRO *et al.* (2014b) in tilapia products. In contrast to our findings, HORITA *et al.* (2011) and CANTO *et al.* (2014) found no difference ($P > 0.05$) in the a^* and b^* values in reduced-fat mortadella and restructured caiman steaks after replacing 50% NaCl with KCl, respectively. Nevertheless, it is important to evaluate if the differences detected with instrumental analyses will be perceived by consumers.

Table 3: Physicochemical parameters of sausages manufactured from mechanically separated tilapia meat.

Physicochemical parameters	Treatments*			
	T1	T2	T3	T4
Cooking yield (%)	78.48 ^a ±0.08	82.67 ^a ±0.04	77.75 ^a ±0.04	77.07 ^a ±0.03
L^*	63.31 ^b ±3.96	63.80 ^b ±3.45	63.97 ^b ±2.41	67.20 ^a ±4.21
a^*	16.57 ^b ±0.67	18.15 ^a ±0.60	16.13 ^c ±0.42	18.53 ^a ±0.14
b^*	6.85 ^{ab} ±0.15	7.27 ^a ±0.70	6.44 ^b ±0.08	7.35 ^a ±1.23

*T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin), T3 (50% NaCl and 50% KCl + without inulin), T4 (50% NaCl and 50% KCl + 6% inulin).

L^* ranges from 0 (black) to 100 (white); a^* ranges from red ($+a^*$) to green ($-a^*$); and b^* ranges from yellow ($+b^*$) to blue ($-b^*$).

Values are means±SD.

-- Means in a row without common superscripts are different ($P < 0.05$); $n = 2$.

3.3. Bacteriological quality of tilapia sausages

No difference ($P > 0.05$) was observed among treatments. TAMC counts were below the limit of 7.0 log CFU/g (ICMSF, 1986) in all treatments, ranging from 3.06 to 3.38 log

CFU/g. *Salmonella* sp., Coagulase-positive *Staphylococcus* and thermo-tolerant coliforms were not detected in any sausage formulation.

In the present study, the partial replacement of salts and inulin addition did not compromise the bacteriological quality of the product. DESMOND (2006) and MENEGAS *et al.* (2013) reported similar results. Nevertheless, the adoption of hygienic procedures before, during, and after processing is essential to obtain a product with optimum quality. Our findings suggest that the sausage-making process was conducted appropriately.

3.4. Sensory evaluation of tilapia sausages

With regard to overall liking, all treatments exhibited mean scores between “like slightly” and “like moderately” (Table 4). OLIVEIRA FILHO *et al.* (2010) found lower overall liking for sausages with different percentages of inclusion of minced tilapia. In this study, differences were observed among the blind samples, where T2 showed higher ($P < 0.05$) overall liking than T3. After the scorers received information about the products, T3 and T4 exhibited lower ($P < 0.05$) overall liking than T1 and T2. All formulations received mean scores between “maybe/maybe not” and “probably would buy” (Table 4), which indicates the consumer acceptability of this new product. In general, T1 and T2 showed higher scores ($P < 0.05$) for purchase intent than T3 and T4. The sentence “Mechanically separated tilapia meat was used to make the sausages, and its use reduces environmental pollution and encourages sustainability” submitted to the panelists did not affect ($P > 0.05$) the overall liking and purchase intent of all treatments. However, our results indicate that the inulin formulations had better potential, regardless of any information provided. MENEGAS *et al.* (2013) reported that acceptability of dry-fermented chicken sausages was not affected by inulin addition.

According to FRANCK (2002), when thoroughly mixed with water or another aqueous liquid, inulin forms a particle-gel network, resulting in a white creamy structure with a spreadable texture. This formulation can easily be incorporated into foods to replace up to 100% of the fat.

On the other hand, our findings for overall liking and purchase intent indicated that sodium reduction can be still a major challenge for the industry. Nevertheless, all formulations exhibited color, taste (bitter and salty), and texture (succulence, elasticity, and softness) attributes close to ideal (2.51 – 3.55) (Table 4). Color, salty taste, succulence and elasticity did not differ ($P > 0.05$) among treatments, suggesting that the consumers were unable to differentiate the low-sodium and inulin added in the formulations by these attributes. T3 showed a stronger ($P < 0.05$) bitter taste than T2, whereas softness was lower ($P < 0.05$) in T1 compared to the T4 formulation.

ARMENTEROS *et al.* (2012) noted that reduction of the salt content by more than 40–50% negatively affected the sensory quality of ham, especially taste, with some bitter and metallic after tastes perceived by consumers.

In agreement with our results, PALMEIRA *et al.* (2014a) observed taste (spicy and bitter) and texture attributes close to ideal in trout meatballs with salt replacement; however, the consumers perceived difference among formulations by these attributes. MONTEIRO *et al.* (2014b) found close to ideal taste (bitter and salty) and texture attributes in restructured tilapia steaks manufactured with 50% salt replacer (KCl). CANTO *et al.* (2014) found a salty taste and texture attributes close to ideal in restructured caiman steaks with 50% KCl. Both authors reported that consumers were not able to differentiate among treatments with respect to attributes evaluated.

Table 4: Sensory evaluation of sausages made from mechanically separated tilapia meat.

Overall liking	Treatments*			
	T1	T2	T3	T4
Blind	6.61 ^{abx} ±1.41	6.79 ^{ax} ±1.51	6.17 ^{bx} ±1.52	6.33 ^{abx} ±1.63
Informed	6.93 ^{ax} ±1.35	6.80 ^{ax} ±1.58	6.08 ^{bx} ±1.72	6.20 ^{bx} ±1.69
Purchase intent	T1	T2	T3	T4
Blind	3.51 ^{abx} ±0.93	3.57 ^{ax} ±1.15	3.16 ^{bx} ±1.01	3.17 ^{bx} ±1.04
Informed	3.68 ^{abx} ±1.00	3.76 ^{ax} ±1.10	3.17 ^{cx} ±1.26	3.28 ^{bcx} ±1.15
JAR attributes	T1	T2	T3	T4
Color	2.74 ^a ±0.79	2.55 ^a ±0.76	2.77 ^a ±0.96	2.51 ^a ±0.75
Bitter taste	3.10 ^{ab} ±0.54	2.91 ^b ±0.60	3.18 ^a ±0.66	3.07 ^{ab} ±0.70
Salty taste	3.00 ^a ±0.55	3.00 ^a ±0.65	2.88 ^a ±0.73	2.85 ^a ±0.69
Succulence	2.90 ^a ±0.75	2.96 ^a ±0.67	2.89 ^a ±0.84	2.92 ^a ±0.88
Elasticity	2.79 ^a ±0.74	2.73 ^a ±0.83	2.68 ^a ±0.91	2.69 ^a ±0.90
Softness	3.17 ^b ±0.83	3.38 ^{ab} ±0.84	3.40 ^{ab} ±0.91	3.55 ^a ±0.85

*T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin), T3 (50% NaCl and 50% KCl + without inulin), T4 (50% NaCl and 50% KCl + 6% inulin).

Blind - with no information; and informed - "Mechanically separated tilapia meat was used to make the sausages, and its use reduces environmental pollution and encourages sustainability".

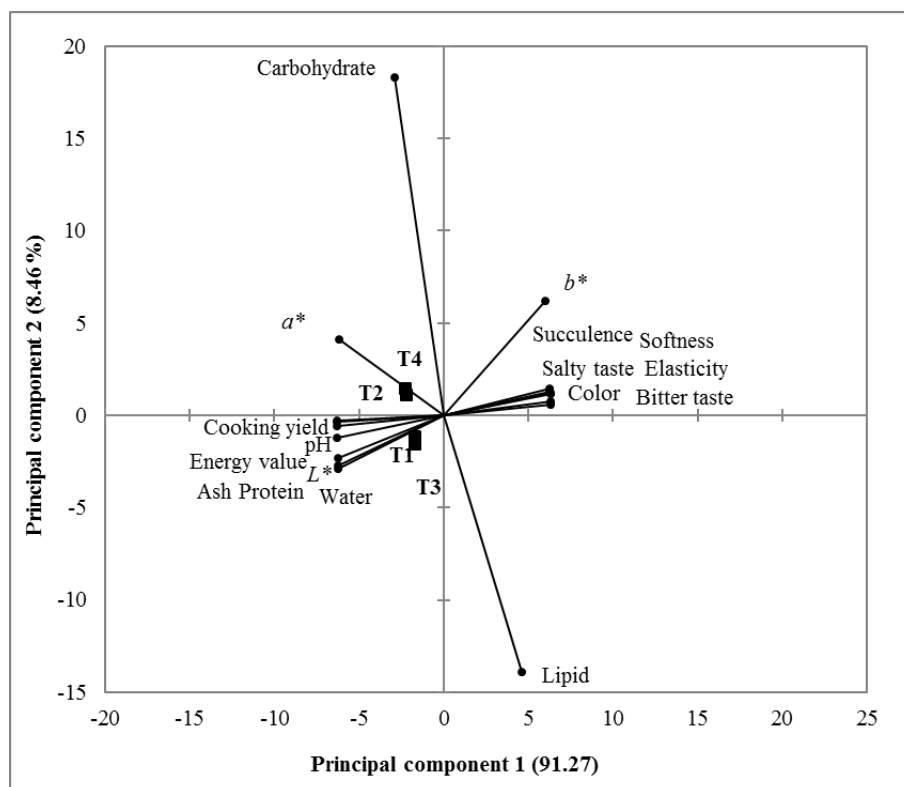
Values are means±SD.

* Means in a row without common superscripts are different ($P < 0.05$).

* Means in a column with common superscripts did not exhibit difference ($P > 0.05$).

Two principal components (PC1 and PC2) explained 99.73% of total data variance (Fig. 1) and separated two groups (T1 and T3; T2 and T4), based mainly on lipid and carbohydrate contents, bitter taste, texture parameters, and a^* and b^* values. The inulin treatments (T2 and T4) were characterized by greater softness, succulence, carbohydrate, a^* and b^* values, with a less bitter taste and lower lipid content than T1 and T3. Taste and texture attributes were the most important for the salt-replacer formulations. T3 showed a stronger bitter taste and less softness than T4. MONTEIRO *et al.* (2014b) found that replacing 50% of NaCl with KCl slightly increased the bitter taste and negatively influenced the succulence and softness. MENDOZA *et al.* (2001) found a softer texture in cooked meat products when the inulin was added. Our results suggest that adding inulin can minimize the negative effects of KCl on the sensory parameters of food products.

The most important correlations were between softness and succulence ($r = 1.00$), between softness and elasticity ($r = 1.00$), between cooking yield and bitter taste ($r = -0.99$), between cooking yield and salty taste ($r = -0.99$), between lipid and carbohydrate ($r = -0.92$) and between lipid and a^* values ($r = -0.85$). These correlations may explain the stronger bitter taste perceived in the formulations with salt replacer (T3 and T4), which was probably influenced by cooking yield. The direct relationship between softness and succulence as well as the inverse relationship between lipid, carbohydrate and a^* values may clarify the positive effect of inulin addition on the chemical composition, color and texture parameters observed.

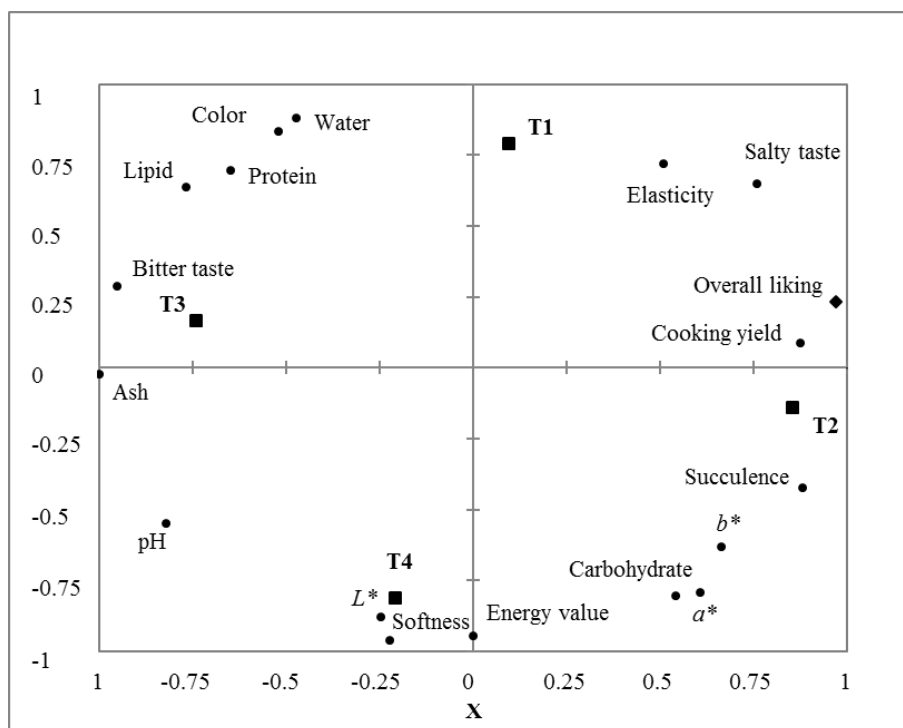


T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin),
T3 (50% NaCl and 50% KCl + without inulin), T4 (50% NaCl and 50% KCl + 6% inulin).

Figure 1: Physicochemical and sensory data of sausages made from mechanically separated tilapia meat defined by two principal components.

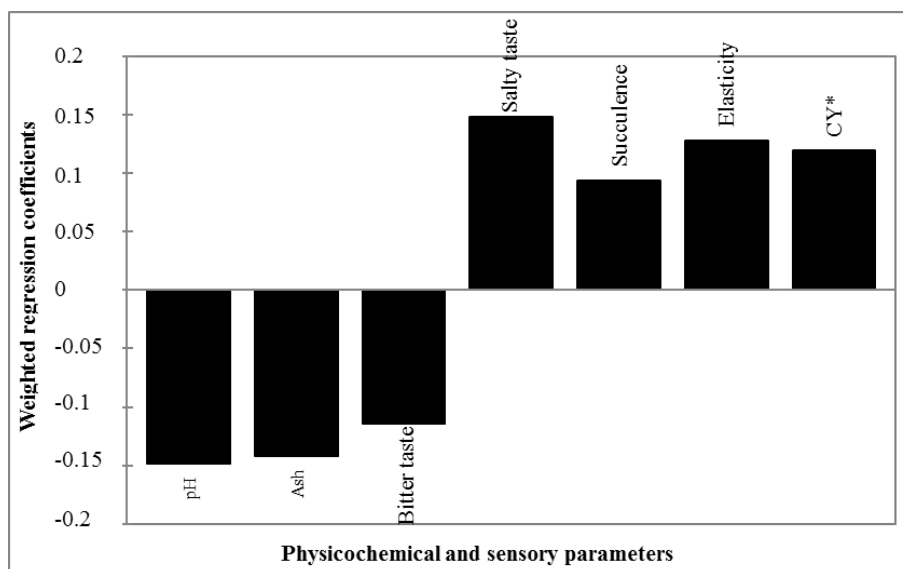
The PLSR model (Fig. 2) explained 99.4% of consumer acceptance (Y-axis) and 93.1% of the untrained-panel sensory scores and physicochemical parameters (X-axis), with a cumulative Q^2 of 0.925. The X-axis parameters were considered important when their respective 'Variable Important to the Projection' was > 1.0 (WOLD *et al.*, 2001). Ash, pH and bitter taste were detrimental to overall liking whereas cooking yield, salty taste, succulence and elasticity positively contributed to overall liking (Fig. 3). With regard to cooking yield, T3 and T4 showed similar results, as did T1 and T2. This can be explained by the greater ionic strength of potassium (1.33) than sodium (0.95), which decreases the electrostatic repulsion between the peptide chains and the space between the myofibrils, retaining less water between the myofibrillar spaces (MARCHESE and BEVERIDGE, 1984; DAMODARAN *et al.*, 2007).

Regarding to Penalty Analysis, major detrimental attributes were those with a > 0.5 penalty score and more that 20% occurrence. T1, T2 and T3 were penalized as having too-weak color (Table 5). Color intensity (a^* and b^*) was lower in T1 and T3 than T2 and T4, suggesting that the panelists were unable to differentiate color among treatments. Moreover, color was not determinant for acceptability. Only formulation T3 was penalized for having a too-bitter taste. The consumers penalized the T3 and T4 formulations as having a too-weak salty taste. This results suggest that use of 50% KCl can decrease the salt perception, which was important for product acceptability. Regarding the texture attributes, T1, T3 and T4 were penalized as having too little succulence, and T1 and T3 as having too little elasticity. Moreover, only formulation T1 was penalized for being too soft.



X axis = physicochemical parameters; Y axis = overall liking.
 T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin), T3 (50% NaCl and 50% KCl + without inulin),
 T4 (50% NaCl and 50% KCl + 6% inulin).

Figure 2: Partial Least Square regression model for sensory attributes and physicochemical parameters of sausages made from mechanically separated tilapia meat.



*CY – Cooking yield.

Figure 3: Weighted regression coefficients of physicochemical and sensory parameters detrimental to acceptability by partial least squares regression.

Apparently, KCl affected texture parameters that were determinant for acceptability, whereas inulin addition to foods together with sodium reduction may be an alternative to improve the acceptability of these products, taking into account the enhancement of taste and texture.

The effect of fiber addition in products differs, depending on the type and the level of the fiber added, as well as by the presence of other ingredients (JIMENEZ-COLMENERO *et al.*, 2005).

Table 5: Consumer penalty analysis of the JAR diagnostic attributes (percentage of consumers and mean decreases).

Treatments	Color		Bitter taste		Salty taste	
	Too weak	Too strong	Too weak	Too strong	Too weak	Too strong
T1	35.0 [*] (0.90) [#]	-	-	-	-	-
T2	45.0 (1.01)	-	-	-	-	-
T3	36.0 (0.53)	-	-	29.0 (0.83)	27.0 (0.36)	-
T4	-	-	-	-	29.0 (0.77)	-

Treatments	Succulence		Elasticity		Softness	
	Too weak	Too strong	Too weak	Too strong	Too weak	Too strong
T1	27.0 (1.22)	-	33.0 (0.75)	-	-	31.0 (0.57)
T2	-	-	-	-	-	-
T3	32.0 (1.04)	-	42.0 (0.73)	-	-	-
T4	34.0 (1.44)	-	-	-	-	-

T1 (100% NaCl without inulin), T2 (100% NaCl + 6% inulin), T3 (50% NaCl and 50% KCl + without inulin), T4 (50% NaCl and 50% KCl + 6% inulin).

* The percentage of consumers who found treatments to be too weak or too strong for JAR color, bitter taste, salty taste, succulence, elasticity, and softness.

The number in parentheses is the change in mean compared to the consumer response score to overall liking.

(-) indicates that less than 20% of consumers choose that JAR category.

Our findings indicate that the level of inulin used was sufficient to maintain the physicochemical and sensory parameters of the tilapia sausages. On the other hand, 50% KCl as a sodium-chloride replacer negatively influenced the sensory attributes. Further studies should be performed to evaluate lower levels of KCl and/or the use of other ingredients in this product since herbs and spices is a promising alternative to suppress or decrease the sensory effects caused by the use of KCl (AHN *et al.*, 2004).

4. CONCLUSIONS

Sausages manufactured with mechanically separated tilapia meat represent a potential alternative for sustainable use of this waste, with high consumer acceptance. The inclusion of inulin is an option to produce a low-fat food, improve the emulsion stability, and ensure the prebiotic effect of the sausage. Replacing sodium with 50% KCl decreased the

acceptance and purchase intent of the tilapia sausages; however, the inclusion of inulin in fish products with sodium reduction is a promising technological strategy to solve possible problems with taste and texture due to KCl addition.

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PEST DETECTED IN PACKED FOOD: TEN YEARS OF ANALYSIS

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ABSTRACT

More than one hundred food complaints, coming from food industries, food stores, and customers, were analyzed over a ten-year period (2004-2013). In the samples of plant products and animal products, the prevalent pests were insects and rodents while in animal products, mites were also found. The highest percentages of stored products' pests in plant products were represented by Coleoptera (62.1) and Lepidoptera (48.2), while Diptera were mainly crop pests (12.5) or species of hygienic concern (33.3). In animal products, the highest number of complaints concerned milk and dairy products, and the contaminations were caused by insects, mites, and mice.

Keywords: pests, infestation, products contamination

1. INTRODUCTION

Different types of foreign matter are reported in food, and insects are considered one of the most important foreign matter problems (LEWIS, 1993; EDWARDS and STRINGER, 2007).

As stated by the FDA Defect Levels Handbook (2014), an infestation is: “The presence of any live or dead life cycle stages of insects in a host product, ...; or evidence of their presence ...; or the establishment of an active breeding population ...”. An inaccurate use of Integrated Pest Management and of Hazard Analysis and Critical Control Points in food processing and retailing can facilitate the occurrence of pests; the detection of extraneous materials in processed food causes the significant loss of revenue and image to the companies involved.

Animal contaminations can derive not only from crops, food industries, and stores but also from dwellings when food is improperly conserved (TREMATERRA and FLEURAT-LESSARD, 2015). The presence of insect in food repulses customers, and moreover, the presence of pests can cause hygienic problems, e.g. cockroaches, domestic flies and rodents can contaminate food with pathogens (GORHAM, 1991; MACOVEI *et al.*, 2008; SULAIMAN *et al.*, 2011; PAVA-RIPOLL *et al.*, 2012; WASALA *et al.*, 2013).

Complaints about cereal products were considered in a previous paper, and the pests most frequently associated with contamination were flying insects. The moth *Plodia interpunctella* (Hbn.) (Lepidoptera, Pyralidae) and the beetle *Sitophilus oryzae* (L.) (Coleoptera, Curculionidae) were the pests that were most commonly responsible for food contamination, and rodent droppings were found in a few cases (SÜSS *et al.*, 2014). Pasta was more commonly infested by insects because the strip of cardboard packaging is not always well-glued, and one or two series of aeration holes in flexible packaging allow insect entry (GORHAM, 1991; LOCATELLI and GAMBARO, 1999; SÜSS *et al.*, 2014; TREMATERRA and SAVOLDELLI, 2014). *P. interpunctella* contaminated confectionery products, made with ingredients that are susceptible to attack, namely, flour, cocoa, nuts, and dried fruits (SÜSS *et al.*, 2014).

In the present study complaints about extraneous materials, such as insects, rodent droppings, and hairs, visible to the naked eye, in plant and animal food source were analyzed.

2. MATERIALS AND METHODS

Samples, coming from food industries, food stores, and customers, were analyzed in the entomological laboratory of University of Milan from 2004 to 2013. In the present study, we analyzed food related complaints of samples belonging to plant products (88), and animal products (16), in a total of 104 samples.

Samples were delivered in original packages, unwrapped packages, or without packages. In some cases, the samples were delivered frozen or cooled. Samples were stored at the temperature of the retail store and analysed within 48 hours.

When the sample was delivered in the original package, the first step in the analysis was a visual inspection to ensure the integrity of the package and the presence of any obvious sign of infestation. The presence of mechanical-related holes, holes due to the activity of insects, or sealing defects, was verified before opening packages. Airtight packages were then verified by immersion in water. Holes were scrutinized under a stereo-microscope to verify if they were of mechanical origin or due to the insect activity (RIUDAVETS *et al.*, 2007). Where insects were found, developmental stage and larval age were noted, and we also noted if the insects were dead or alive (SÜSS *et al.*, 2014).

For each category, we reported the number and/or the percentage of samples contaminated with insects, rodents or other animals. Insects were classified and divided into two different categories: crop pests and stored product pests.

3. RESULTS

In the samples of plant products and animal products, the most represented pests were insects and rodents (Table 1), while mites were also found in animal products.

Among insects (Table 2), Coleoptera (40.3%) and Lepidoptera (37.5%) were the most represented in plant products, while Diptera (33.3%) were in animal products. Only 15.2% of plant product complaints concerned food in opened packages, while animal product complaints mainly concerned unwrapped packages.

Table 1: Percentages of pests in samples of plant products (88) and animal products (16).

Pests	Plant products	Animal products
Insects	81.8	70.5
Mites	-	11.8
Anellida	1.1	-
Rodents	11.4	11.8
Other vertebrates	1.1	-
Other contaminants*	4.6	5.9

*plastic fragments, feather, seed and vegetable debris.

Table 2: Percentages of insect Orders present in samples of plant products and animal products.

Insect	Plant products	Animal products
Coleoptera	40.3	25.0
Lepidoptera	37.5	16.8
Diptera	12.5	33.3
Hymenoptera	-	8.3
Thysanura	1.4	-
Orthoptera	1.4	-
Dermaptera	1.4	8.3
Dictyoptera	1.4	-
Hemiptera	1.4	8.3
Psocoptera	2.7	-

3.1. Plant products

In complaints about plant products, pests of stored products represented the highest percentages in Coleoptera (62.1) and Lepidoptera (48.2), while Diptera were mainly crop pests or species of hygienic concern (Table 3).

Fruits and vegetables (21.6%), canned vegetables (18.2%), and cocoa (15.9%) were the foods most susceptible to complaints (Table 4) followed by frozen vegetables (11.4%), mushrooms (9.1%), ready to eat fresh vegetables (7.9%), and fresh vegetables (4.5%).

Table 3: Complaints about plant products: relative values of Lepidoptera, Diptera and Coleoptera (each Order was considered 100) distributed according to origin of pests (in the case of "other", Lepidoptera pests were unidentified, in the case of Diptera pests were of hygienic relevance).

Origin of pests	Lepidoptera	Diptera	Coleoptera
Crop	37.0	66.7	37.9
Stored products	48.2	-	62.1
Other	14.8	33.3	-

Table 4: Number and percentage of complaints about different food plant products.

Food	Complaints	
	No.	%
Canned vegetables	16	18.2
Cocoa	14	15.9
Dried fruits and vegetables	19	21.6
Frozen vegetables	10	11.4
Fruit juice	2	2.3
Grinded coffee	1	1.1
Mushrooms	8	9.1
Olive oil	2	2.3
Ready meals	1	1.1
Ready to eat fresh vegetables	7	7.9
Sugar	2	2.3
Vegetable stock cube	2	2.3
Vegetables	4	4.5

Among crop pests, noctuid moths larvae or locusts were found in salads and spinach, *Acanthoscelides obtectus* Say, *Callosobruchus maculatus* (F.) and *Zabrotes subfasciatus* (Bohemann) (Coleoptera, Chrysomelidae) infested dried and canned pulses, *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae) and elaterid larvae were detected in tomatoes, Noctuid larvae were found also in canned artichokes and tomatoes, and elaterids in canned jam. *Ostrinia nubilalis* (Hübner) (Lepidoptera, Crambidae) contaminated grilled

peppers, while Carabidae, ground beetles, were detected in spinach and also in chamomile.

Alive stored product pests such as *Lasioderma serricorne* (F.) (Coleoptera, Anobiidae) developed in chamomile, spices and herbal tea, *Plodia interpunctella* infested cocoa products, and nuts and dead larvae of *P. interpunctella* were found in coffee, vegetable stock cube, and instant mashed potatoes. In cocoa products, *Ephestia* spp. (Lepidoptera, Pyralidae) were also detected, and accidental contamination by blow flies, *Forficula auricularia* L. (Dermaptera, Forficulidae), and *Attagenus* sp. (Coleoptera, Dermestidae) was observed; the presence of *Ahasverus advena* (Waltl) (Coleoptera, Silvanidae) and *Carpophilus* sp. (Coleoptera, Nitidulidae) in cocoa beans revealed the presence of molds.

One adult psocid (Psocoptera), probably present in the cupboard, contaminated an open sugar bag while another was detected in a bottle of olive oil.

Sometimes, species not directly linked to the food were found, but their presence seriously increases the risk of contamination, particularly with regard to insects of hygienic relevance. Examples were one larva of *Musca domestica* L. (Diptera, Muscidae) and one adult of *Muscina* sp. (Diptera, Muscidae) in tomato sauce and a *Periplaneta americana* (L.) (Dictyoptera, Blattidae) nymph in dried mushrooms.

Occasional infestations caused by larvae of *Attagenus* sp. (Coleoptera, Dermestidae) in an orange soft drink and in fruit juice, blow fly in cocoa, and by parts of other animals, such as one lizard tail, one earthworm, and one feather, were also detected.

Mus domesticus Schwartz & Schwartz (Rodentia, Muridae) contaminated different foods: dead mice were found in the cocoa bean, in frozen vegetables, in tomatoes and potatoes. Legs of mice and one *Apodemus* sp. (Rodentia, Muridae) were also present in spinach. Also, droppings of mouse were detected in sugar, oil, and sesame seeds, while mouse hairs were found in dates.

3.2. Animal products

In the case of food complaints in animal products the packages were already unwrapped, except for eggs. The highest percentage of complaints concerned milk and dairy products (62.5%) and the contaminations were caused by insects, mites, and mice. In detail, one adult of Diptera Sciaridae contaminated a milk bottle, while different insects were detected in dry milk: larvae of dermestid and anobid beetles, and the thorax of an adult stink bug, *Forficula auricularia* (Dermaptera, Forficulidae), was found in fruit yoghurt. Cheese was contaminated with *Tyrophagous putrescentiae* (Schrank) (Acarididae, Acaridae) mites, but also with plastic debris. In dry milk, an adult of *M. domesticus* was found, while droppings were detected in cheese.

Diptera were also found in salami, one blowfly larva, and one muscid pupa in an egg package.

Only in one case was a larva of *P. interpunctella* recorded in a meat product: this involved a breadcrumb chicken breast and the infestation derived from the breadcrumb.

As far as fish products are concerned, one larva of *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) was in canned tuna, one larva of *M. domestica* was in canned octopus, and one larva of an unidentified moth was in fish baby food.

4. COMMENTS AND CONCLUSION

Food infestation mainly concerned insects and rodents to a lesser extent. Crop insects were frequently detected in frozen and ready to eat vegetables, as the pest hides in the vegetable and leaves are sometimes difficult to wash due to their conformation.

Vegetable food samples included wrapped and unwrapped packages while complaints about animal products were always about already unwrapped packages. This case makes it difficult to exactly state the origin of contamination, but it should frequently be ascribed to improper conservation after purchase.

Plodia interpunctella was the most frequent pest; it infested cocoa products, dried and dehydrated vegetables, dried beans, chamomile, spices and herbal tea, nuts, coffee, vegetable stock cubes, instant mashed potatoes, and was also recorded in breadcrumbed meat.

Ready to eat products with dried mushrooms were infested by dead larvae of Diptera Mycetophilidae. Fungus gnats avoid already senescent or decaying mushroom, usually spoiled by Diptera Phoridae, Sciaridae, and Calliphoridae (LOCATELLI *et al.*, 2006). Therefore, the presence of Mycetophilidae indicated that the mushroom was fresh.

Ahasverus advena and *Carpophilus* sp., which infest nuts and dried fruits that are incorrectly stored (WOODROFFE, 1962), were detected in cocoa beans; their presence reveals the development of molds (SINHA, 1974; PIERCE *et al.*, 1991) that can produce mycotoxins (DAVID *et al.*, 1974). Also, cockroaches and flies in food cause concern as they are linked to the transmission of pathogens (SASAKI *et al.*, 2000; DE JESU'S *et al.*, 2004; TALLEY *et al.*, 2009).

In a few cases, the infestation was caused by different live insect stages; often, only one dead insect was detected in the samples. In a previous paper on cereal products (SÜSS *et al.*, 2014), live insects in different stages were detected. This time, few live insects were in dried food such as chamomile, cocoa, nuts and herbal tea.

Rodent droppings and hair were found both in plant and animal food. Rodent contamination is unacceptable for health reasons (MEERBURG and KIJLSTRA, 2007) and indicates negligence and laxity in applying prevention measures during production and storage in warehouses and dwellings. Sometimes, customers confused soil and pieces of plastic with droppings.

Some pests not typically associated with the products were also found. Larvae of *P. interpunctella* often nestled under lids and in jars. In these cases, the insects used the packaging as a shelter, but the effect on the customer was nevertheless extremely negative. In other cases, the insect was embedded in the multilayer film, as sometimes packaging industries overlook insect prevention in processing departments (RIUDAVETS *et al.*, 2007). An integrated approach of controlling food safety throughout the entire food production chain has become an important issue in attaining a greater food safety level (VALEEVA *et al.*, 2004; TREMATERRA, 2013; TREMATERRA and FLEURAT-LESSARD, 2015).

Quality assurance of food industries asks entomologists about the identification of pests' species, and information on biology and ethology of pests in order to establish the weak point of production processes. In the majority of our cases, it was not possible to trace the origin of infestation. Consumers frequently reported contamination several days or weeks after purchase, i.e. packages have already been opened (sometimes a part of the package was missing) and part of the food has been consumed. As TURNER and ALI (1996) reported, often products which are left, partially used and open, in a cupboard will absorb water vapor and possibly become attractive to psocids in the kitchen. In many cases, no information was available about storage conditions after the packages were opened.

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VENDING MACHINE FOODS: EVALUATION OF NUTRITIONAL COMPOSITION

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ABSTRACT

The nutritional quality of vending machine foods may be a factor that contributes to significantly increase obesity and associated diseases, and the vending industry is significantly growing worldwide. This study aims to evaluate the nutritional composition of vending machine foods and to compare it with the consumption of the Gran Canaria population.

Food products from 74 snack and 71 refrigerated vending machines located in Las Palmas (Gran Canaria), and on university campuses, were nutritionally assessed. The percentages of sales per food type were accessed during a 12 month-period to verify user preferences. Significant differences ($p < 0.05$) were found in the content of nutrients compared with the Kruskal-Wallis test with all the food groups. Sandwiches (wholemeal and white bread) had the lowest energy levels, while croissants had the highest. We highlight the increased sodium content in baguettes compared to the other foods.

The findings suggest that vending machine foods contain more fat/saturated fat, calories and sodium than recommended. Further studies on the nutritional assessment of vending machine foods, governments' awareness and policies that promote the intake of healthy foods are essential to increase the amount of foods with an appropriate nutritional profile according to recommendations in vending machines.

Keywords: consumer, food choices, nutritional assessment, vending machines

1. INTRODUCTION

Recent decades have witnessed a significant increase in industrial vending machine development. Japan is the world leader in this sector, the USA makes 30 billion American dollars per year (LIN *et al.*, 2011), and the UK makes approximately 1,700 million pounds sterling (MINTEL, 2009).

Spain is a European power in using vending machines, with a consolidated industry and highly integrated use (RAPOSO *et al.*, 2015). There are 560,000 vending machines across Spain; that is, one machine for every 80 inhabitants. Japan, the industry leader, has 5.5 million vending machines, which is one for every 23 people (MTV, 2008).

For decades, the nutritional value of foods present in vending machines has been consistently documented (CHENEY, 1974; EZELL *et al.*, 1985; HRUBAN, 1977; KOEHLER *et al.*, 1977; SHEARER *et al.*, 1980). In general, authors have voiced concern about the relatively low value of nutrients, and the high sugar and fat contents in many of the most frequently chosen items (HUNTER, 1992; KUBIK *et al.*, 2013; PASCH *et al.*, 2011). Nowadays, such concern has led to some governments, as in Spain, to legislate the products sold in vending machines in schools and colleges (Spanish Law 17/2011, of July 5, of Food Safety and Nutrition, especially Article 41), and also because of the high childhood obesity rates and unhealthy food habits detected among consumers (CAVALIERE *et al.*, 2014; EBENEGGER *et al.*, 2010; MACKAY, 2011). Although the vending machine policy has been effective in changing snack behaviour in a wide variety of settings, no published research has investigated vending snack behaviour in a university campus community (CARUSO *et al.* 2014).

Despite the potential risks or health benefits, very little is known about the variety of food and beverages sold in vending machines on university campuses. A study carried out by BYRD-BREDBENNER *et al.* (2012), which aimed to assess the drinks and snacks sold in vending machines in different universities, reported that snacks and drinks offered poor nutrient quality. Most snacks were low in fibre and had high calorie and fat contents, and almost half contained too much sugar. Most drinks also contained high levels of sugar and calories. The findings of this study suggested that vending machines offer limited healthy options. Other studies have demonstrated that the sale and consumption of healthy foods can be influenced by increased availability of healthy foods (FRENCH *et al.*, 2004; LYTLE *et al.*, 2006; MUCKELBAUER *et al.*, 2009; PERRY *et al.*, 2004). Similarly, purchase behaviour can be positively influenced by driving customers to healthier choices through strategies such as labelling, and by providing information, reminders and reinforcement (GEREND, 2009; HARNACK *et al.*, 2008; HOLDSWORTH *et al.*, 2004). As the effect of labelling itself is slight, it is likely to be more effective if combined with other methods (FRENCH *et al.*, 2001; GRUNERT *et al.*, 2010; SACKS *et al.*, 2009); e.g. strategies that influence purchasing behaviour by reducing low-calorie food prices (FRENCH *et al.*, 2001). The results of another study (KOCKEN *et al.*, 2012) indicated that when the availability of low-calorie foods increased and was combined with labelling that highlighted nutritional properties, and prices lowered at the same time, students made healthier choices without buying more or fewer products from vending machines.

Vending machines reflect the good availability and suitability of food and beverages in western society, and also in many environments, including schools (RIDEOUT *et al.*, 2007; VAN DER HORST *et al.*, 2008), workplaces (FRENCH *et al.*, 2010) and health centres (LAWRENCE *et al.*, 2009). In some places, availability of alternative foods and beverages from vending machines may be limited (FARLEY *et al.*, 2010). Based on these data, providing healthy varied foods in such machines is extremely important to counteract the facts that some studies have reported (FINKELSTEIN *et al.*, 2008; FRENCH *et al.*, 2003;

LYTLE *et al.*, 2006), which have documented that most vending machines are typically stocked with food and beverages that are rich in energy, but low in nutrients.

Some studies have shown the relationship between students who use vending machines regularly and excessive consumption of sugary drinks (WIECHA *et al.*, 2006). However, not only sugary drinks from vending machines contribute to a significant increase in obesity and associated diseases, but snack vending machines form part of an environment that may lead to obesity, which encourages easy access to energy-dense and nutrient-poor food (KUBIK *et al.*, 2015; PARK and PAPADAKI, 2016). Typically vending machines offer few healthy options (LAWRENCE *et al.*, 2009). We must take into account that vending machines increasingly grow in number, become more prevalent and available, thus they supply daily energy intake to more and more individuals. So it is important to accurately assess and monitor the nutritive value of vending machine products (MATTHEWS and HORACEK, 2015).

For all these reasons, and taking into account the implementation of Law 17/2011 in Spain on Food Safety and Nutrition, we believe that it is necessary to determine the nutritional composition of the food dispensed in vending machines to compare not only the nutritional profiles of various foods, but also the consumption of such foods in the Gran Canaria population (Canary Islands, Spain).

2. MATERIALS AND METHODS

For this work, we comprehensively considered the Gran Canaria island (Spain) and placed particular emphasis on the ULPGC (*Universidad de Las Palmas de Gran Canaria*) university campuses located on it. The ULPGC has 25,172 students enrolled in various degree programmes distributed over four campuses on Gran Canaria (ULPGC, 2015).

This study was carried out with 108 vending machines located in the town of Las Palmas de Gran Canaria (50 snack vending machines and 58 refrigerated vending machines that dispensed solid food products), and all 37 vending machines of snacks/refrigerated solid food products in ULPGC campus buildings (24 snack vending machines and 13 refrigerated vending machines that dispensed solid food products).

Only the vending machines (145) of a single company in the sector were considered, which has 65% of the volume of business in Las Palmas de Gran Canaria, Spain. Regarding the 37 machines located at the ULPGC, the company has been awarded the exclusive installation of these machines throughout the campus.

2.1. Nutritional Assessment

Over 3 months we accessed the cuisine of the vending company involved, which collaborated in this study and calculated all the products it made. While food handlers were preparing food, ten samples of each product were taken to be weighed on a balance (Model 2200c, Precisa, Dietikon, Switzerland).

Finally the mean weight of each ingredient was used to calculate nutrients (proteins, total and saturated fats, carbohydrates and fibre), the total energy value and sodium present in each product item. It should be noted that the nutritional information of 61% of the ingredients used in all the products was supplied by the vending company's providers. For the remaining 39% for which it was not possible to obtain such information, we used the food table of the DIAL program (2011), developed by a group of professors from the Department of Nutrition and Bromatology I in the Faculty of Pharmacy at the *Universidad Complutense de Madrid* (Spain).

Among the processed products, the following categories appeared: 9 different types of wholemeal baguettes (85 g); 12 different types of croissants (100 g); 11 different types of white bread baguettes (85 g); 14 different types of white bread sandwiches (50 g); 5 different kinds of wholemeal sandwiches (50 g). All these products were composed only of the ingredients described in Tables 1-5, except those prepared with tuna, chicken, crab and watercress, which also contained mayonnaise.

We randomly took ten different products marketed in vending machine foods: five chocolate bars and five industrial pastry products. The purpose of this procedure was to establish the nutritional, total energy and sodium values of the above food categories by collecting the information provided by these foods.

Finally from the vending company, we requested the percentage of sales for each food type during a 12-month period to verify what user preferences were.

Table 1: Nutritional assessment - wholemeal baguettes.

Wholemeal baguette (85 g)	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
Salami and cheese	547.6	23.8	33.6	13.4	34.1	6.5	1732.5
Chicken and cheese	537.2	52.0	20.7	12.3	32.3	6.4	516.8
Salami	437.3	14.9	25.4	9.1	33.9	6.6	1559.5
Tuna and corn vegetable	432.9	14.5	20.4	3.1	42.9	9.9	723.3
Tuna and corn	427.9	13.9	23.6	3.6	36.5	7.0	737.5
Pork loin and cheese	424.9	41.2	12.8	5.9	33.0	6.4	1450.9
Ham and cheese	396.7	32.4	13.1	6.8	33.5	6.4	1198.3
Goat cheese	349.4	15.9	16.1	8.6	32.3	6.4	792.4
Tortilla	291.5	10.4	8.6	1.8	39.5	7.5	885.7
\bar{x}	427.3	24.3	19.4	7.2	35.3	7.0	1066.3
σ	80.7	14.5	7.7	4.0	3.7	1.2	431.2

\bar{x} : mean of all types of wholemeal baguette.

σ : standard deviation of all types of wholemeal baguette.

2.2. Statistical analysis

The data analysis of this work was carried out with the statistical software package SPSS 20.0 (SPSS, Chicago, IL, USA) for MAC OS X (Apple Computers, Cupertino, CA, USA).

For the nutritional evaluation, different products were grouped by category, wholemeal baguette; croissant; white bread baguette; white bread sandwich; wholemeal sandwich, and the means and standard deviations of the nutrients (markers), sodium and total energy values per category were calculated.

Variables (markers) were summarised as medians and interquartile ranges (IQR). They were compared by the Kruskal-Wallis test. Multiple comparisons were made by the Wilcoxon test. Statistical significance was set at $p < 0.05$.

Table 2: Nutritional assessment - croissants.

Croissant (100 g)	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
Tomato, salad, turkey, tuna and corn	758.8	40.4	34.5	10.9	68.3	6.0	2676.3
Tuna and corn	686.8	18.9	42.5	12.2	55.2	3.2	763.3
Tuna, corn and vegetables	675.4	19.1	36.7	11.3	63.4	7.0	696.5
Tuna and peppers	671.2	21.5	40.3	11.8	54.0	4.4	690.2
Corn, crab and pineapple	668.6	13.3	40.8	11.8	59.1	4.9	685.8
Crab	658.5	13.2	43.1	12.3	52.6	2.5	658.3
Salami and cheese	647.7	37.1	35.8	19.1	50.3	2.3	998.5
Chicken	622.4	14.7	39.6	11.7	50.2	2.5	595.6
Watercress	597.3	8.4	39.4	11.6	50.5	2.6	580.1
Tuna	571.9	18.3	32.6	10.6	49.8	2.4	612.7
Ham and cheese	570.3	32.8	25.8	15.8	50.0	2.3	721.2
Tortilla	474.0	13.1	20.4	9.2	57.7	3.5	870.6
\bar{x}	633.6	20.9	36.0	12.4	55.1	3.6	879.1
σ	72.9	10.3	6.9	2.6	6.0	1.6	578.3

\bar{x} : mean of all types of croissant.

σ : standard deviation of all types of croissant.

Table 3: Nutritional assessment – white bread baguettes.

White bread baguette (85 g)	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
Sobrasada and cheese	570.4	20.0	34.5	12.6	43.0	3.0	947.9
Salami and cheese	555.4	25.6	29.6	10.3	44.5	3.0	1622.4
Pork loin and cheese	468.3	45.2	11.7	5.8	43.7	3.0	1519.4
Tuna and corn	404.4	14.3	17.1	2.6	46.2	3.5	692.7
Chicken and cheese	403.5	40.3	7.2	3.6	43.0	3.0	552.8
Ham and cheese	396.3	28.7	11.1	6.8	43.5	3.0	766.2
Tuna, corn, tomato and vegetables	393.3	14.2	13.8	2.1	50.3	5.5	666.0
Salami	383.5	14.1	16.1	5.8	44.0	3.0	1203.5
Goat cheese	372.5	17.6	13.8	7.9	43.0	3.0	792.6
Tortilla	304.8	11.8	5.9	1.2	49.0	3.9	829.9
Serrano ham and tomato	285.1	16.9	3.1	0.9	45.2	3.9	1381.7
\bar{x}	412.6	22.6	15.0	5.4	45.0	3.4	997.7
σ	89.2	11.2	9.7	3.8	2.5	0.8	371.4

\bar{x} : mean of all types of white bread baguette.

σ : standard deviation of all types of white bread baguette.

Table 4: Nutritional assessment – white bread sandwiches.

White bread sandwich (50 g)	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
Tortilla, ham and cheese	363.3	23.7	15.9	5.4	31.6	3.5	1272.8
Sobrasada and cheese	337.5	17.0	21.0	8.9	21.2	2.1	490.1
Salami and cheese	336.9	19.6	19.5	9.0	21.9	2.1	785.6
Chicken	326.4	8.9	23.1	3.6	21.8	2.3	464.1
Tuna, corn, tomato and vegetables	319.9	10.7	17.4	2.7	29.8	4.9	515.9
Corn, crab and pineapple	313.5	7.0	19.7	3.0	27.3	3.7	489.1
Two cheeses	312.6	18.0	17.3	10.2	22.1	2.1	652.8
Tuna	310.6	12.1	20.1	3.1	21.4	2.2	498.0
Watercress	308.6	4.0	23.2	3.6	22.0	2.4	458.5
Tuna and peppers	290.1	11.1	17.3	2.7	23.9	3.3	467.7
Turkey and cheese	283.0	26.9	8.5	4.5	25.6	2.1	1446.6
Ham and cheese	276.1	25.1	10.1	4.9	22.4	2.1	996.6
Crab	266.8	6.2	17.3	2.7	22.9	2.2	436.0
Tuna and corn	257.6	9.8	14.4	2.2	23.5	2.4	467.1
\bar{x}	307.4	14.3	17.5	4.8	24.1	2.7	674.4
σ	29.7	7.4	4.3	2.7	3.3	0.9	331.5

\bar{x} : mean of all types of white bread sandwich.

σ : standard deviation of all types of white bread sandwich.

Table 5: Nutritional assessment – wholemeal sandwiches.

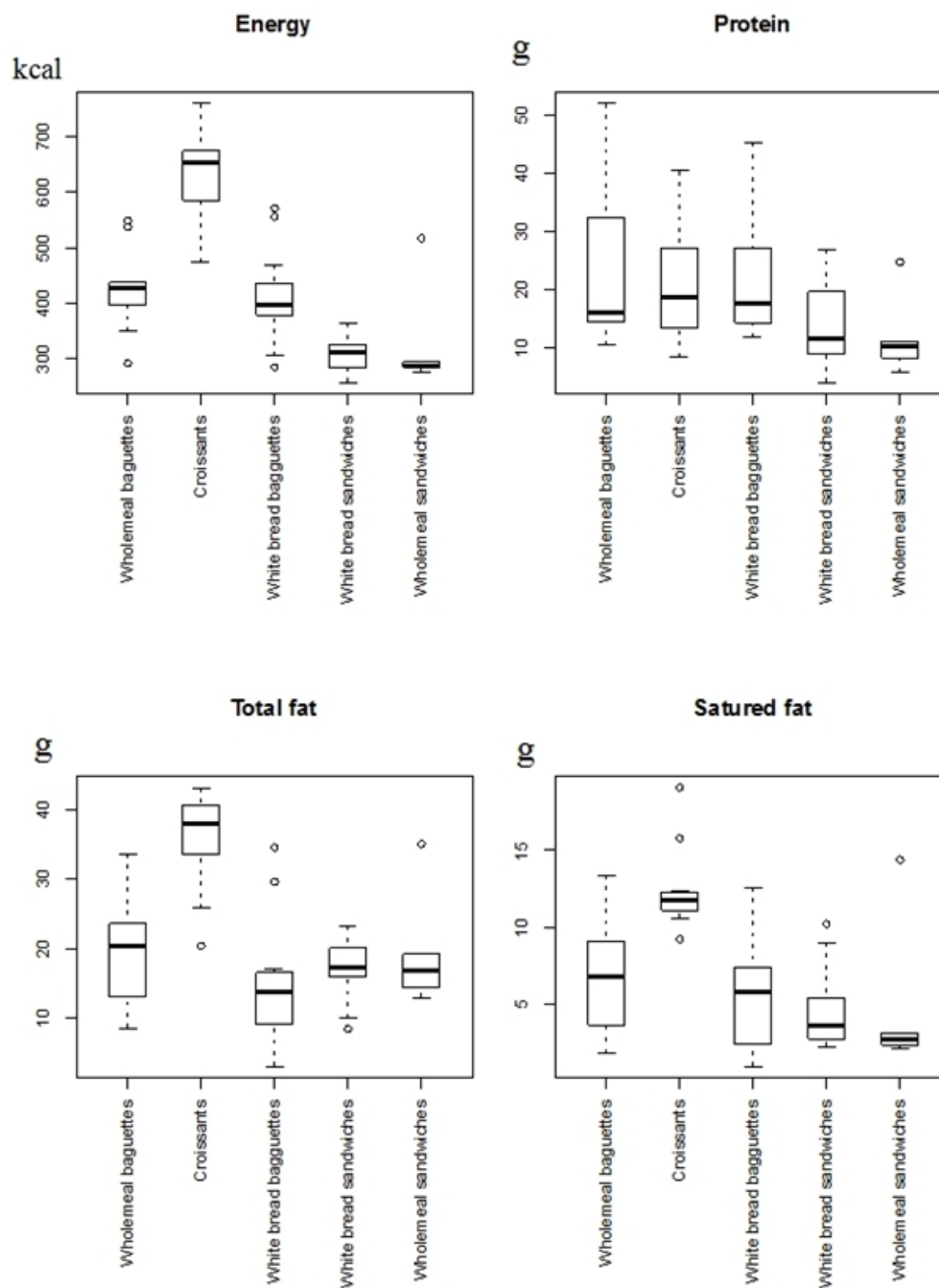
Wholemeal sandwich (50 g)	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
Salami and cheese	516.6	24.7	35.0	14.4	24.0	3.1	1623.8
Watercress	294.4	5.8	19.2	3.1	22.7	3.2	394.3
Tuna, corn and vegetables	286.3	11.0	13.0	2.1	28.7	5.4	428.0
Crab	285.4	8.2	16.8	2.7	23.7	3.1	402.8
Tuna and corn	275.5	10.3	14.4	2.3	24.5	3.4	429.3
\bar{x}	331.6	12.0	19.7	4.9	24.7	3.6	655.6
σ	103.6	7.4	8.9	5.3	2.3	1.0	541.4

\bar{x} : mean of all types of wholemeal sandwich.

σ : standard deviation of all types of wholemeal sandwich.

3. RESULTS AND DISCUSSION

By means of box plots, Fig. 1 shows the seven categories of processed food products against markers by their quartiles, where data are medians and IQR.



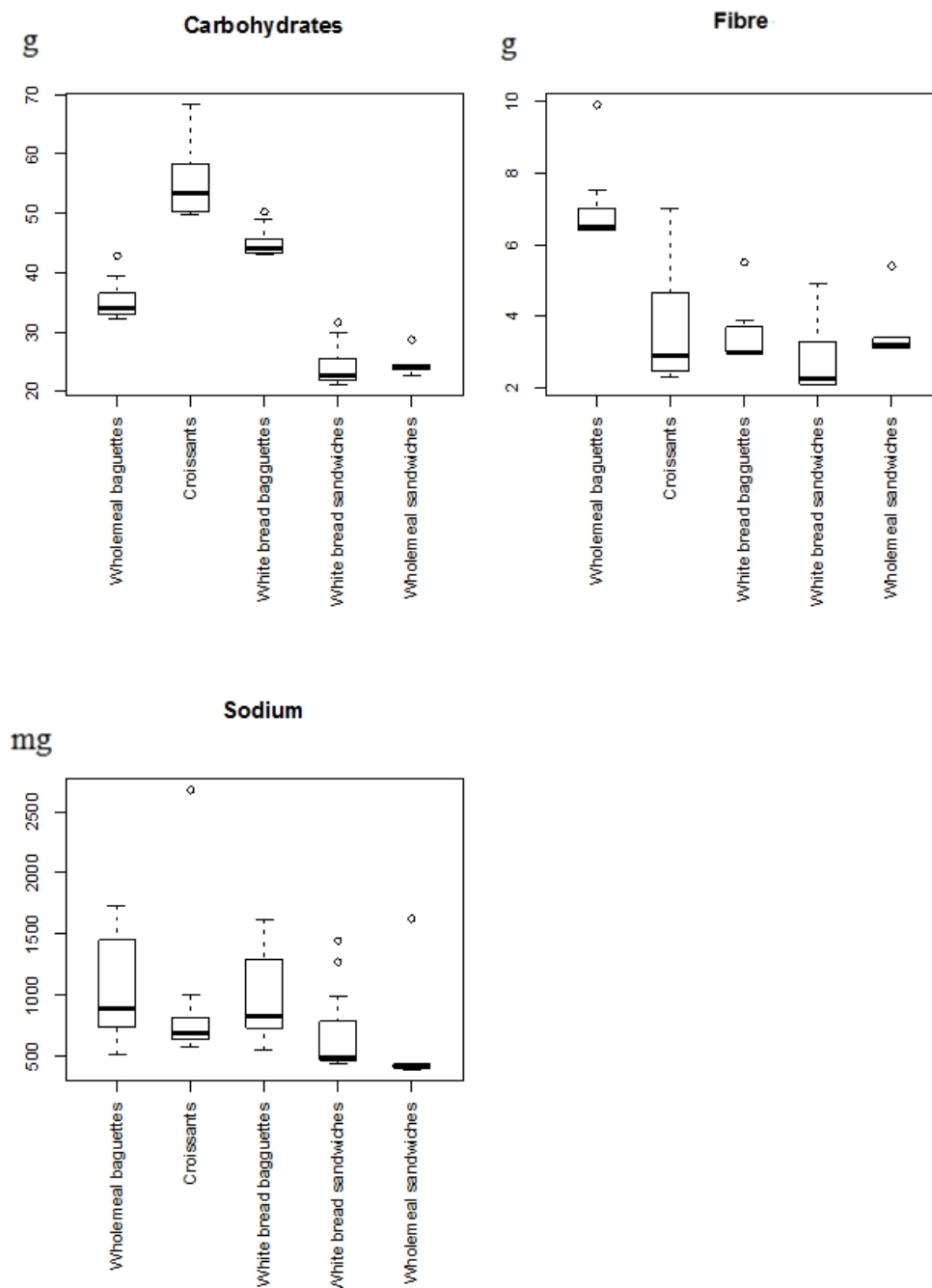


Figure 1: Categories of processed food products against markers by box plots graphics.

3.1. Processed foods in the vending company

Tables 1-5 show the nutritional results calculated from the foods processed in the vending company, which were subsequently sold on the market in vending machines. Note that the products in these tables prepared with tuna, chicken, crab and watercress also contained mayonnaise.

Table 6 shows the nutritional value of each bread/croissant type (with no added ingredients) used to make various types of processed products.

Table 6: Nutritional assessment – types of bread / croissant.

Product	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
Croissant (100 g)	355.0	8.0	13.4	7.7	49.4	2.3	390.0
White bread baguette (85 g)	219.3	8.2	0.9	0.2	43.0	3.0	484.5
Wholemeal baguette (85 g)	187.9	6.0	2.5	0.5	32.3	6.4	467.5
Wholemeal sandwich (50 g)	129.5	5.5	1.5	0.4	22.0	3.0	265.0
White bread sandwich (50 g)	113.0	3.6	2.2	0.4	21.2	2.1	300.0
\bar{x}	200.9	6.3	6.8	1.8	33.6	3.4	381.4
σ	96.3	1.9	6.8	3.3	12.5	1.7	97.8

\bar{x} : mean of all types of bread / croissant.

σ : standard deviation of all types of bread / croissant.

To analyse the results presented in Tables 1-6, it was necessary to compare them to the reference Daily Value (DV) values obtained from the Food and Drug Administration (FDA) (FDA, 2013), which are provided in Table 7.

For sodium, it should be noted that on January 31, 2013, the World Health Organization (WHO) considered that its reference value for daily intake for adults should be < 2000 mg (2013).

Table 7: Reference daily intake values (FDA, 2013).

Caloric Intake (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total Carbohydrate (g)	Dietary Fibre (g)	Sodium (mg)
2000	50	65	20	300	25	2400

In the analysis of the results shown in Table 6, the high calorie intake (355 kcal) in croissants was checked and compared to the other products. Their energy value more than tripled that of white bread sandwiches. A large quantity of total and saturated fats in croissants was also noted compared with other products. A croissant had about 20 times more saturated fat than a white bread sandwich. Sodium levels were generally high: a white bread baguette contained 484.5 mg of sodium, which means that according to the WHO (2013), the consumers who ate this product with no added ingredients had already eaten 24.2% of their daily sodium needs.

When interpreting the results of the nutritional calculations made with all the bread/croissant types with added ingredients, those foods with a high sodium content and lots of calories also presented high levels of saturated fat.

When we focused on the different wholemeal baguette types (Table 1), we saw that the average sodium levels surpassed almost half the daily WHO recommendations (2013) (\bar{x} =

1,066.3 mg), while the baguettes that contained salami contained more than one third of the sodium level according to the same recommendations. Another point was that two baguette types (salami and cheese / chicken and cheese) contained more than 50% of the DV (FDA, 2013) for saturated fat. Two other types of wholemeal baguettes had a high protein content: the chicken and cheese baguette (52 g) and the pork loin and cheese baguette (41.2 g). When choosing a wholemeal baguette type, e.g. for a mid-morning snack, tortilla was the best option since it contained the least calories (291.5 kcal), less saturated fat (1.8 g) and its sodium content (885.7 mg) was below the average value (1,066.3 mg) of other types of wholemeal baguettes.

The sodium levels of the white bread baguettes (Table 3) were high (\bar{x} = 997.7 mg), which also occurred with the wholemeal baguettes. Two types of white bread baguettes (sobrasada and cheese / salami and cheese) surpassed 50% of the DV (FDA, 2013) for saturated fat. Protein content was high in the pork loin and cheese (45.2 g) and the chicken and cheese (40.3 g) sandwiches. When it comes to choosing a white bread baguette, it is important to avoid the sobrasada and cheese and salami and the cheese kinds because, apart from having a high saturated fat content, they contained a large amount of sodium which, for the salami and cheese baguette, represented 81.12% of the WHO's daily recommendations (2013).

No major differences sandwiches were found between wholemeal and white bread and their average nutritional values were similar (Tables 4 and 5). Perhaps wholemeal sandwiches make consumers feel fuller given their high fibre content. The high energy value of the wholemeal salami and cheese sandwich was noteworthy (516.6 kcal), as was its sodium content (1,623.8 mg), compared to the other wholemeal and non-wholemeal sandwiches. Sandwiches that were not made with wholemeal bread stood out for their high sodium content: turkey and cheese (1,446.6 mg) and tortilla with ham and cheese (1,272.8 mg).

Croissants (Table 2) had very high values of sodium, saturated fat and calories. Only one (tortilla) of the twelve available croissant types contained less than 50% of the DV (FDA, 2013) for saturated fat. Its average energy intake value was 633.6 kcal. The croissant that contained tomato, salad, turkey, tuna and corn should be noted as its sodium content was 2,676.3 mg, which surpassed the WHO's 676.3 mg daily recommendation (2013), and its calorie content was 758.8 kcal, which represented 37.94% of the recommended daily intake (FDA, 2013).

Regarding the statistical relationship among the different products grouped by category, Table 8 summarises the seven markers analysed as medians and IQR in each food group. All the markers (energy, proteins, total fat, saturated fat, carbohydrates, fibre and sodium) showed significant differences among the five food groups ($p < 0.05$).

Sandwiches (wholemeal and white bread) had the lowest energy levels, while croissants had the highest. The croissants showed a significantly larger amount of total and saturated fats and carbohydrates than the other studied food groups, and also protein when compared with both sandwich types (white bread and wholemeal) (Table 8).

The carbohydrate values in both sandwich types were significantly lower (Table 8).

The larger amount of fibre content detected in the wholemeal baguettes stood out from the other foods, while the sodium content levels in the white bread and wholemeal sandwiches were significantly lower compared to the other food groups analysed herein (Table 8). We highlight the high sodium content in the baguettes compared to the other foods (Table 8).

Table 8: Comparison of nutritional markers between groups of processed foods.

	Wholemeal baguettes	Croissants	White bread baguettes	White bread sandwiches	Wholemeal sandwiches	P*
Energy	427.9 ^a (369 ; 437)	653.1 ^b (591 ; 672)	396.3 ^a (378 ; 436)	311.6 ^c (285 ; 325)	286.3 ^c (285 ; 294)	< .001
Protein	15.9 ^{a,b} (14.5 ; 32.4)	18.6 ^{a,b} (13.3 ; 24.3)	17.6 ^a (14.2 ; 27.1)	11.6 ^{b,c} (9.1 ; 19.2)	10.3 ^c (8.2 ; 11.0)	.044
Total fat	20.4 ^a (13.1 ; 23.6)	38.0 ^b (34.0 ; 40.4)	13.8 ^a (9.1 ; 16.6)	17.4 ^a (16.2 ; 20.0)	16.8 ^a (14.4 ; 19.2)	< .001
Saturated fat	6.8 ^a (3.6 ; 9.1)	11.8 ^b (11.2 ; 12.2)	5.8 ^a (2.3 ; 7.3)	3.6 ^a (2.8 ; 5.3)	2.7 ^a (2.3 ; 3.1)	< .001
Carbohydrates	33.9 ^a (33.0 ; 36.5)	53.3 ^b (50.3 ; 58.1)	44.0 ^c (43.2 ; 45.7)	22.6 ^d (21.9 ; 25.2)	24.0 ^d (23.7 ; 24.5)	< .001
Fibre	6.5 ^a (6.4 ; 7.0)	2.9 ^b (2.5 ; 4.5)	3.0 ^b (3.0 ; 3.7)	2.2 ^c (2.1 ; 3.1)	3.2 ^{b,c} (3.1 ; 3.4)	< .001
Sodium	885.7 ^a (737 ; 1451)	693.4 ^a (647 ; 790)	829.9 ^{a,c} (729 ; 1293)	494.1 ^b (467 ; 752)	428.0 ^c (402 ; 429)	.004

Data are medians (IQR).

Different superscript letters ^{a,b,c,d} indicate significant differences (p < 0.05).

(*) Kruskal-Wallis test.

Based on all these findings, we once again stress the very high content in sodium and saturated fat of many processed foods, and their high calorie intake. Croissants are the least suitable choice because they contain high saturated fat compared to other foods, and they also contain many calories and a lot of sodium. Sandwiches are the most suitable snack choice. Choosing a food type by considering the ingredients that it contains is important to help avoid eating the foods that contain sausage meat because its sodium, saturated fat and energy values are very high.

3.2. Chocolate and industrial pastry products

The nutritional values that correspond to chocolate and industrial pastry products on sale in vending machines are included in Tables 9 and 10.

Table 9. Nutritional assessment – chocolates.

Chocolate	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
1	280.0	4.0	14.0	5.0	35.0	1.0	140.0
2	245.0	4.9	12.7	4.9	29.4	8.6	24.5
3	233.0	3.0	12.0	0.0	29.0	0.0	0.0
4	213.0	3.0	11.0	4.1	26.0	0.8	118.0
5	134.0	1.1	6.8	0.0	18.2	0.0	0.0
\bar{x}	221.0	3.2	11.3	2.8	27.5	2.1	56.5
σ	54.4	1.4	2.7	2.6	6.1	3.7	67.4

\bar{x} : mean of all samples of chocolate.

σ : standard deviation of all samples of chocolate.

Table 10. Nutritional assessment – industrial pastry products.

Industrial pastry product	Energy value (kcal)	Protein (g)	Total fat (g)	Saturated fat (g)	Total carbohydrate (g)	Dietary fibre (g)	Sodium (mg)
1	240.0	3.5	16.0	11.5	19.0	3.0	105.0
2	237.0	5.0	13.8	7.8	25.2	2.3	383.0
3	223.0	4.8	8.4	1.7	31.5	1.3	0.1
4	175.0	2.1	7.2	1.3	26.3	1.4	172.0
5	132.0	1.5	7.0	1.1	15.4	0.3	0.1
\bar{x}	201.4	3.4	10.5	4.7	23.5	1.7	132.0
σ	46.7	1.6	4.1	4.7	6.3	1.0	158.2

\bar{x} : mean of all samples of industrial pastry products.

σ : standard deviation of all samples of industrial pastry products.

All these foods obtained similar nutritional composition values. It should be noted that most of the carbohydrates in these foods were sugars. Back in the 1990s, HUNTER (1992) voiced concern about the relatively low value of the nutrients and the high sugar content of the foods most frequently chosen from vending machines, including snacks like those cited in this section of our study.

Table 11 shows the relationship of the consumption percentages for all the foods sold in vending machines over a 12-month period. These results reflect a consumer preference for snacks, i.e. chocolate (29.04%) and industrial pastry products (33.84%), and a very low consumption of baguettes (4.18%).

A recent study (BYRD-BREDBENNER *et al.*, 2012) detected snacks in vending machines with a high content of fat and sugar, lots of calories and very little fibre. These results coincide with the products studied herein. In fact since the 1970s, the reduced nutritional value of most products sold in vending machines (CHENEY, 1974; EZELL *et al.*, 1985; FRENCH *et al.*, 2010; KUBIK *et al.*, 2011; LAWRENCE *et al.*, 2009), their high sugar content (EZELL *et al.*, 1985; HRUBAN, 1977; KOEHLER *et al.*, 1977; SHEARER *et al.*, 1980) and their high energy contribution (FINKELSTEIN *et al.*, 2008; FRENCH *et al.*, 2010; KIBBLEWHITE *et al.*, 2010; NAYLOR *et al.*, 2010; PASCH *et al.*, 2011) have been known.

Table 11: Percentage of consumption from vending.

Consumption during a 12-month period	
Category	Total
Baguettes	4.18%
Wholemeal baguettes	22.83%*
White bread baguettes	77.17%*
Chocolate	29.04%
Croissants	10.05%
Industrial pastry products	33.84%
Sandwiches (white bread and wholemeal)	13.96%

These values correspond to the breakdown in the category Baguettes. So the total % of Baguettes consumption was 4.18%, and 22.83% for baguettes made with wholemeal bread and 77.17% for white bread baguettes.

3.3. Future perspectives and recommended strategies

Based on the similarity between the results of the above-cited recent studies and those obtained in this paper, the implementation of strategies to promote the sale and consumption of healthier foods is believed necessary. To go about this, it is advisable to adopt measures like those proposed by KOCKEN *et al.* (2012). Their study increased the availability of foods in vending machines with a lower calorie intake, included labelling that highlighted nutritional properties, and also lowered the price of these foods. Another measure to adopt could be to lower the prices of foods with a low fat content, as demonstrated in the study conducted by FRENCH *et al.* (2001).

The impact of implementing these strategies, along with policies to encourage eating healthier foods, and the possibilities of improving the diet of a high percentage of the population would be strong, and the costs associated with treating a group of diseases caused by inadequate diet could lower. Law 17/2011, of July 5, on Food Safety and Nutrition already imposes new legislative terms to this problem by preventing the sale of foods with high contents of saturated fat, sodium and sugar in schools. This has been the first step to ensure that the general population becomes more aware of healthier food choices when choosing food from vending machines.

4. CONCLUSIONS

Plenty of additional work is needed to enhance the nutritional quality and appropriate portion size of the foods offered in vending machines. To improve vending machine selections on university campuses, benchmark data like those reported herein can help stakeholders set priorities and work with decision makers on campuses to advocate healthy campus food environments, which include all food retail outlets. The ultimate goal of studies like the present one is to compel environmental changes that make healthy choices possible and easier. Future research should also investigate the behavioural and financial impact of eliminating unhealthy snacks from vending machines.

Thus we propose that vending companies take steps to change the processed foods sold in their machines. It would be much more appropriate to provide these machines with foods that contain fewer calories, especially with much lower levels of saturated fat and sodium, compared to those currently available to vending users. In this way, the intake of these foods would be more in line with WHO recommendations (2013) and with the DV set by the FDA (2013). We believe that awareness of governments and the adoption of policies to promote consumer intake of healthy food choices are essential measures to increase the amount of foods with an appropriate nutritional profile that meets these recommendations (WHO, 2013; FDA, 2013). Therefore, conducting further studies would help support and implement public health policies and environmental changes, which could improve healthy food access and availability in vending machines.

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ANTHOCYANIN PROFILE AND ANTIOXIDANT ACTIVITY OF FRESHLY SQUEEZED POMEGRANATE (*PUNICA GRANATUM* L.) JUICES OF SICILIAN AND SPANISH PROVENANCES

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ABSTRACT

Pomegranate (*Punica granatum* L., *Punicaceae*) fruit is traditionally consumed in several countries, especially in Middle East, and has gained increasing popularity all over the world due to its assumed health benefits. Juices derived from the arils of the seeds were shown to be rich in anthocyanin glucosides, typically composed of cyanidin, delphinidin, and pelargonidin. The aim of the present study was the characterization of diverse Sicilian and Spanish pomegranate accessions regarding their anthocyanin and total polyphenol contents using the Folin-Ciocalteu reagent. The anthocyanin profiles were determined by HPLC-DAD and LC-MS, and color parameters were characterized using the CIELAB coordinates. Antioxidant activities were measured using a fluorimetric assay, and expressed as ORAC values. The anthocyanin and polyphenol contents were correlated with their antioxidant activities. Results obtained were correlated and evaluated for the identification of the most suitable accessions to be selected for cultivation, juice processing, and further breeding.

Keywords: pomegranate, juice, ORAC, anthocyanins, antioxidant, polyphenols, cultivars

1. INTRODUCTION

Pomegranate is a wide-spread fruit crop in the entire Mediterranean area. The fruit is highly appreciated due to the sweet taste of its juice made from the edible part of the fruit, the arils. The species native to Iran and the Himalayas in northern India has a deeply rooted history dating back to ancient times. As reported by MELGAREJO *et al.* (2012), the world production is probably exceeding 3,000,000 mt, and over 80% thereof is originating from Iran, India and China (HOLLAND and BAR YAAKOV, 2008). The growing interest in pomegranate cultivation stems from a number of advantages ranging from agronomic performance in marginal environments to the broad variety of products obtained therefrom, and the health properties of the juice traditionally used for the treatment of diabetes, osteoporosis, cataracts, cardiovascular and neoplastic diseases (ASGARY *et al.*, 2014; KUMAGAI *et al.*, 2015; MALIK and MUKHTAR, 2006; VIUDA-MARTOS *et al.*, 2010). Many research dealt with the anthocyanins profile and the antioxidant activity of pomegranate juices originating from Iran, Israel, Morocco, Spain, Turkey, USA including their benefits for human health (LEGUA *et al.*, 2012; MOUSAVI DERAZMAHALLEH *et al.* 2013; QU *et al.*, 2011; RINALDI *et al.*, 2013; SCHWARTZ *et al.*, 2009); however, little is known about the different characteristics of the varieties despite recent findings indicating a broad genetic and geographic diversity (CRISTOFORI *et al.*, 2011; FERRARA *et al.*, 2011; FISCHER *et al.*, 2011a). Sicilian pomegranate germplasm has been characterized so far (BARONE *et al.*, 2001; DOMINA *et al.*, 2007), and a new promising accession named "Primosole" has been described (LA MALFA *et al.*, 2009). Different authors also considered the germplasm of other Italian regions. Recently, CALANI *et al.* (2013) evaluated the phenolic profiles of some ancient Italian accession.

In this paper, the phenolic contents of 12 Sicilian accessions should be studied comparing them with four Spanish varieties for the first time. In order to shed light on the diversity of anthocyanin profiles and antioxidant activities and other basic quality traits such as total soluble solids, total acidity, pH, and ascorbic acid contents of 16 accessions originating from Sicily (Italy) and Spain should be determined. This study is aimed to identify high-yielding varieties with potential health benefits suitable for growing in marginal areas.

2. MATERIALS AND METHODS

2.1. Sample preparation

Sixteen accessions, 12 belonging to the Sicilian germplasm, and four originating from Spain, (Table 1) were grown applying standard horticultural practices in the experimental farm of the Catania University (Italy) located near the eastern coast of Sicily (lat. 37°24'37" N; long. 15°03'16" E); 20 kg of fruits from four different trees of each accession were harvested at marketing ripeness stage (i.e. October 10 for Violetto and Valenciana, November 10 for PG-CT5, PG-CT6 and Valenti, October 25 for the others) transported to the laboratory, and stored at 4°C for 24 h. Fruits were sanitized with chlorinated tap water; seeds (arils) were isolated using a commercial pomegranate aril separator (Pomeke, Netanya, Israel), and juice was freshly squeezed with a juice extractor (Termozeta, model 405003, Milan, Italy). Juices obtained were analyzed for their pH, total acidities (TA), soluble solids (TSS), maturity index (MI), total polyphenol contents, anthocyanin contents, ellagic acid, tannin contents, and antioxidant activities. All analyses were performed in triplicate, and the data collected were treated statistically.

Table 1: Composition of juices made from the arils of 16 pomegranate accessions.

Accession	pH	TA (g/100 ml of citric acid)	TSS (°brix)	M Index
Dente di cavallo acc.1	4.00±0.02 ^{abc}	0.18±0.01 ^c	15.49±0.89 ^e	86.04±1.95 ^f
Dente di cavallo acc.2	3.88±0.01 ^{de}	0.25±0.01 ^a	14.88±0.39 ^g	59.55±0.94 ^h
Noto	4.04±0.03 ^a	0.15±0.00 ^d	14.88±0.68 ^g	99.22±2.45 ^e
PG-CT1	4.03±0.04 ^{ab}	0.25±0.02 ^a	16.09±0.72 ^c	64.36±1.12 ^h
PG-CT5	3.90±0.03 ^{de}	0.14±0.00 ^{de}	17.59±0.95 ^a	125.62±3.11 ^a
PG-CT6	3.85±0.01 ^e	0.14±0.00 ^{de}	15.49±0.69 ^e	110.64±3.43 ^{bc}
PG-SR1	3.96±0.02 ^{abcd}	0.20±0.01 ^b	14.58±0.56 ^h	70.92±2.25 ^g
PG-SR3	3.95±0.01 ^{bcd}	0.14±0.00 ^{de}	14.58±0.56 ^h	104.16±3.21 ^{de}
Primosole	3.67±0.02 ^g	0.15±0.01 ^d	15.78±0.74 ^d	105.27±3.74 ^{cde}
Rosolini	3.89±0.03 ^{de}	0.14±0.01 ^{de}	15.49±0.81 ^e	110.64±2.96 ^{bc}
Valenti	3.94±0.02 ^{cd}	0.14±0.01 ^{de}	15.49±0.79 ^e	110.64±2.58 ^{bc}
Violetto	3.96±0.01 ^{bcd}	0.18±0.01 ^c	13.36±0.52 ⁱ	74.24±1.96 ^g
Mollar de Elche	3.84±0.02 ^{ef}	0.14±0.01 ^{de}	17.59±0.89 ^a	125.64±4.79 ^a
Piñon tierno	3.53±0.03 ^h	0.13±0.00 ^e	15.18±0.67 ^f	116.80±3.41 ^b
Piñonenca	3.75±0.01 ^{fg}	0.21±0.01 ^b	16.69±0.93 ^b	80.82±1.85 ^f
Valenciana	3.71±0.02 ^g	0.14±0.01 ^{de}	14.88±0.74 ^g	106.29±3.63 ^{cd}

Values in the same column not followed by the same letter are significantly different ($p < 0.01$). pH value, TA total acidity expressed as citric acid, TSS total soluble solids, Mindex maturity index TSS/TA.

2.2. Chemicals

All reagents and solvents were of analytical or HPLC grade. Trolox [(+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid], ABTS [2,20-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] and Folin-Ciocalteu reagent were supplied by Sigma (St. Louis, MO, USA). Cyanidin 3-glucoside (CY3), delphinidin 3-glucoside (DP3) pelargonidin 3-glucoside (PL3), cyanidin 3-rutinoside (CY3R), gallic acid and ellagic acid were from Extrasynthèse (Genay, France). Delphinidin-3-rutinoside was from Apin Chemicals (Oxfordshire, UK). All other reagents and solvents were from Carlo Erba (Milan, Italy).

2.3. Determination of ascorbic acid

The ascorbic acid concentration was determined according to RAPISARDA and INTELISANO (1996) using an HPLC (Shimadzu, Japan) equipped with two pumps (LC-10A), a control system (SCL-10A), an injector (Rheodyne with 20 μ L loop), a photodiode detector (SPD-M10A), a C18 Alltima ODS Hypersil column 250 mm x 4.6 mm I.D. (Milan, Italy), and a similarly packed pre-column. The elution was performed using a buffer consisting of $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ at pH 2.3, at a flow rate of 1 mL/min, and detection wavelength was set at 260 nm.

2.4. Determination of anthocyanins and ellagic acid

Total anthocyanins were determined spectrophotometrically as described by RAPISARDA *et al.* (2000). An aliquot of juice (2 mL) was diluted up to 25 mL with a pH 1 solution (125 mL of 0.2 M KCl and 375 mL of 0.2 M HCl). A second aliquot (2 mL) was diluted to 25 mL with a pH 4.5 buffered solution (400 mL of 1 M sodium acetate, 240 mL of 1 M HCl, and 360 mL of H₂O). Absorbance of the solutions was measured at 510 nm. Anthocyanin concentrations were calculated by Equation (1):

$$C_{mg/L} = (Abs_{pH1} - Abs_{pH4.5}) * 484.82 * 1000 / 24825 * DF \quad (1)$$

where the term in parentheses is the difference of absorbance at 510 nm measured at pH 1 and pH 4.5, 484.82 is the molecular mass of cyanidin-3-glucoside chloride, 24825 is its molar absorptivity (ϵ) at 510 nm in the pH 1 solution, and DF is the dilution factor. Anthocyanins and ellagic acid were also quantitated by HPLC-DAD, and assigned by HPLC-MS according to Fischer *et al.* (2011b). Anthocyanin analyses were conducted using a Merck Hitachi La-Chrom Elite HPLC system (Merck, Darmstadt, Germany) equipped with an L-2200 auto sampler, an L-2130 pump, a jetstream column oven, and a L-2450 diode array detector. The separation was carried out on an analytical Phenomenex (Torrance, CA, USA) C 18 Synergi 4 μ m Hydro-RP 80 Å pore size (150 x 3.0 mm) column fitted with a Phenomenex security guard column (4 x 3.0 mm) operated at 30°C. The acquisition range of the diode array detector was set at 200-600 nm. The mobile phase consisted of 5% (v/v) formic acid in water (eluent A), and of water, formic acid and methanol (10/10/80, v/v/v; eluent B). The rate flow was 0.4 mL/min, and the gradient program was optimized as follows: 10-14% B (5 min), 14-23% B (11 min), 23-35% B (5 min), 35-40% B (14 min), 40-100% B (3 min), 100% B isocratic (3 min), 100-10% B (3 min), 10% B isocratic (4 min). Total run time was 48 min, the injection volume of all samples was 10 μ L, and monitoring was performed at 520 nm and 280 nm for ellagic acid.

In order to remove undesirable sugars, acids, amino acids, and proteins that could interfere with anthocyanin separation, solid-phase extraction (SPE) was conducted prior to instrumental analysis employing C18 ODS SPE cartridges (Sep-Pak Waters Milford, MA, USA). Aliquots of anthocyanin extracts (10 mL) were passed through SPE preconditioned cartridges with methanol (5 mL) and water (10 mL). Cartridges were washed with water (15 mL), and anthocyanins eluted drop wise with methanol containing formic acid 0.4% (10 mL). The methanolic extract was concentrated under reduced pressure using a Büchi rotavapor (Flavil, Switzerland) at 30°C, and the residue diluted with 5 mL of deionized water containing 7% (v/v) of formic acid. LC-MS analyses were carried out using an Agilent HPLC 1100 system (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1379A degasser, a model G1312A binary gradient pump, a model G1313A thermo-auto sampler, a model G1316A column oven, and a model G1315A diode array detection system. The HPLC system was connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source. The LC-MS program was the same used for LC-DAD. Data acquisition and processing were done using Esquire Control software. Positive ion mass spectra of the column eluate were in the range of m/z 50-1500 at a scan speed of 13000 $m/z/s$. Nitrogen was used both as drying gas at flow rates of 10.0 L/min and nebulizing gas at pressures of 50.0 psi. The nebulizer temperature was 365°C. Helium was used as collision gas at a pressure of 4×10^{-6} mbar.

2.5. Determination of total polyphenols and total tannins

The total polyphenol (TP) and tannin (TT) contents of the samples were estimated by a colorimetric assay based on the procedures described by Glasl (1983) with slight modifications. For the determination of total tannins, a sample size of 10 g was used. Briefly, the samples were dissolved in 250 mL water to obtain the mother liquor (ML). A 5 mL aliquot of ML was diluted with water to 25 mL, and 2 mL of this solution were transferred into a 25 mL vial containing 1 mL of Folin-Ciocalteu reagent and 10 mL of bi-distilled water, and subsequently made up to volume of 25 mL with a 10.0% sodium carbonate solution. After 15 minutes, the absorbance was read at 730 nm. Water was used as the blank. To determine the non-adsorbed polyphenols (NAP), 10 mL of ML was mixed with 100 mg of hide powder (Merck) and shaken for 90 min. A 2 mL aliquot of this solution was assayed for polyphenolics as above. The absorbance wavelength (730 nm) was previously selected by spectrophotometric scanning of samples of extract and gallic acid. The percentage of total phenolics and tannins were determined as follows (Equation 2):

$$TP\% = \frac{15625 \cdot Abs}{1000 \cdot m} \quad NAP\% = \frac{15625 \cdot Abs}{1000 \cdot m} \quad TT\% = TP - NAP \quad (2)$$

where TP are the total polyphenolics (%); NAP the non-adsorbed phenolics (%); TT the total tannins (%); Abs the absorbance; and m the mass (g).

2.6. Determination of antioxidant activity

In addition to the Folin-Ciocalteu assay, which does not only measure the total content of phenolics but also that of the total reducing capacity of the samples, the ORAC assay was performed as described by CAO *et al.* (1993) with some modifications. The measurements were carried out on a Wallac 1420 Victor III 96 well plate reader (EG and Wallac, Turku, Finland) with a fluorescence filter (excitation 485 nm, emission 535 nm). Fluorescein (116 nM) was the target molecule for free radical attack by AAPH (153 mM) used as the peroxy radical generator. The reaction was performed at 37°C, pH 7.0, and Trolox (1 µM) was taken as the control standard, while phosphate buffer was used as blank. All solutions were freshly prepared prior to analysis. All samples were diluted with phosphate buffer (1:25-100, v/v) prior to analysis, and results were expressed as micromoles (µMol) of Trolox equivalents per 100 mL of juice.

2.7. Color analysis

The CIELAB coordinates of pomegranate juice were determined without previous sample preparation by reading the L^* a^* b^* -values on a Cary 1E spectrophotometer (Varian, Mulgrave-Victoria, Australia), and estimating the color intensity by Cary Color Calculation software. Color index (C*index) was calculated according to equation (3):

$$(180 - \text{Hue}) / (L^* + \text{Chroma}) \quad (3)$$

2.8. Statistical analysis

Experimental data were processed by statistical analyses using the Statistica software (StatSoft, Tulsa, OK, USA). The descriptive analysis was followed by an analysis of variance method applying the Tukey's HSD test. Principal component analysis (PCA) and cluster analysis (CA) were also performed.

3. RESULTS AND DISCUSSIONS

In this study, juices were obtained from the arils of four Spanish and 12 Italian pomegranate accessions whose pomological characteristics were previously described (LA MALFA *et al.*, 2009) illustrated by Fig 1. Their main chemical characteristics are compiled in Table 1. The lowest pH value was determined for “Piñon tierno” (pH 3.5), while “Noto” and “PG-CT1” were characterized by higher pH values (pH 4.0). “Dente di cavallo acc. 2” had the highest acidity (TA = 0.25 g/100mL), whereas the remaining cultivars (Piñon tierno”, 0.14 “PG-SR3”, “Rosolini”, “PG-CT5”, “Valenti”) had considerably lower total acidities around 0.13-0.14. To provide information about the sensory quality of the fruits, the maturity index (MI) being the ratio of TSS and TA measured in the juices was also determined. The highest maturity index (MI) was found for “Mollar de Elche” (125.14), and the lowest in “Dente di cavallo acc.2” (60.73). All accessions were classified as sweet according to the classification of Martínez *et al.* (2006). The contents of the main bioactives are listed in Table 2. In accordance with Chace *et al.* (1981), all accessions are suitable both for fresh consumption and juice production.



Figure 1: Pomegranate accessions investigated: from top to bottom and from left to right: Valenciana, Mollar de Elche, Primosole, Piñonenca, Piñon tierno, Valenti, Violetto, Rosolini, Noto, Dente di cavallo acc. 1, PG-SR3, PG-SR1, PG-CT1, PG-CT5, Dente di Cavallo acc. 2, PG-CT6.

Ascorbic acid content; however, without considering the dehydroascorbic acid contents, ranged between 80.2 and 417.4 mg/L with statistical significance between all accessions. In particular, maximum values were found for the “Violetto”, “Rosolini”, “PG-CT1” and “PG-SR1” accessions that are well separated among others accessions. We detected very high values of ascorbic acid much higher than the values reported in the literature (FERRARA *et al.*, 2011; DROGOUDI *et al.*, 2005; TEHRANIFAR *et al.*, 2010).

Table 2: Contents of bioactive compounds and antioxidant potential of 16 pomegranate accessions.

	Ascorbic Acid (mg/L)	Anthocyanins (mg/L)	Tannins (mg/L)	Ellagic Acids (mg/L)	Total polyphenols (mg/L)	ORAC-value (μ mol TE/100mL)
Dente di cavallo acc. 1	179.8 \pm 9.3 ^{de}	76.9 \pm 2.2 ^{ef}	3.3 \pm 0.2 ^{fgh}	37.3 \pm 1.7 ^f	948.6 \pm 42.4 ^{gh}	7272 \pm 369 ^{gh}
Dente di cavallo acc. 2	100.6 \pm 5.7 ^{hi}	154.1 \pm 6.1 ^b	4.2 \pm 0.1 ^{cde}	47.5 \pm 2.7 ^d	1186.9 \pm 58.3 ^{efg}	7269 \pm 362 ^{gh}
Noto	80.2 \pm 4.4 ⁱ	56.6 \pm 1.5 ^g	5.2 \pm 0.24 ^b	34.8 \pm 1.7 ^f	1338.8 \pm 65.2 ^e	7050 \pm 353 ^h
PG-CT1	358.8 \pm 14.5 ^b	104.3 \pm 9.8 ^{cd}	7.4 \pm 0.3 ^a	83.2 \pm 3.6 ^b	2118.7 \pm 101.8 ^{cd}	10540 \pm 536 ^{bc}
PG-CT5	124.5 \pm 6.9 ^{fghi}	160.2 \pm 8.0 ^b	4.4 \pm 0.2 ^{bcde}	33.4 \pm 1.9 ^f	2497.0 \pm 122.8 ^b	8408 \pm 410 ^{defg}
PG-CT6	106.6 \pm 5.1 ^{ghi}	156.3 \pm 8.5 ^b	4.8 \pm 0.1 ^{bc}	70.5 \pm 3.0 ^c	1100.9 \pm 55.0 ^{efgh}	8536 \pm 427 ^{def}
PG-SR1	417.4 \pm 20.2 ^a	97.5 \pm 5.2 ^{cd}	2.7 \pm 0.2 ^h	49.0 \pm 2.6 ^d	857.0 \pm 42.8 ^h	8317 \pm 280 ^{defg}
PG-SR3	139.4 \pm 7.7 ^{fg}	65.0 \pm 3.3 ^{fg}	3.6 \pm 0.2 ^{efg}	26.0 \pm 1.2 ^g	2252.8 \pm 112.6 ^{bc}	7721 \pm 368 ^{fgh}
Primosole	175.9 \pm 9.2 ^{de}	196.6 \pm 10.3 ^a	3.9 \pm 0.2 ^{defg}	41.7 \pm 2.0 ^e	3055.1 \pm 151.1 ^a	11835 \pm 585 ^a
Rosolini	252.0 \pm 11.5 ^c	168.1 \pm 4.1 ^{ab}	4.1 \pm 0.2 ^{cdef}	24.7 \pm 1.0 ^g	1011.2 \pm 60.2 ^{fgh}	8285 \pm 415 ^{defg}
Valenti	164.7 \pm 8.2 ^{ef}	92.9 \pm 7.4 ^{de}	4.7 \pm 0.2 ^{bcd}	19.0 \pm 0.9 ^h	1205.6 \pm 97.0 ^{efg}	8548 \pm 417 ^{def}
Violetto	209.0 \pm 10.2 ^d	53.0 \pm 2.0 ^g	7.1 \pm 0.1 ^a	41.8 \pm 2.1 ^e	1899.5 \pm 96.0 ^d	9400 \pm 469 ^{cd}
Mollar de Elche	101.5 \pm 5.3 ^{hi}	102.8 \pm 3.2 ^{cd}	4.6 \pm 0.2 ^{bcd}	36.5 \pm 1.4 ^f	1281.3 \pm 66.6 ^{ef}	9064 \pm 458 ^{de}
Piñon tierno	115.9 \pm 6.5 ^{gh}	64.5 \pm 1.1 ^{fg}	2.7 \pm 0.1 ^h	16.1 \pm 0.4 ^h	1982.0 \pm 96.1 ^{cd}	11411 \pm 568 ^{ab}
Piñonenca	184.8 \pm 10.6 ^{de}	114.6 \pm 4.9 ^c	3.7 \pm 0.2 ^{fgh}	104.3 \pm 4.6 ^a	3097.7 \pm 154.4 ^a	5593 \pm 269 ⁱ
Valenciana	118.2 \pm 6.0 ^{gh}	97.3 \pm 2.8 ^{cd}	3.2 \pm 0.1 ^{gh}	34.3 \pm 1.2 ^f	2398.1 \pm 119.3 ^b	8010 \pm 406 ^{efgh}

Values in the same column not followed by the same letter are significantly different ($p < 0.01$).

Total anthocyanins content as determined by spectrophotometry (Table 2) ranged between 53.0 and 196.6 mg/L with a mean value of 110.0, and a standard deviation of 44.3, thus revealing a high variability among the accessions; in particular “Rosolini” and “Primosole” showed the highest anthocyanins contents. This was in according with previous reports providing evidence of high variability in other accessions investigated (SCHWARTZ *et al.*, 2009; DAFNY-YALIN *et al.* 2010). Analysis of the anthocyanin profiles (Table 3) revealed statistically significant differences between the accessions. In particular, cyanidin 3-O-glucoside (CY3) was the predominant anthocyanin followed by cyanidin 3,5-O-diglucoside (CY3.5), and delphinidin 3,5-O-diglucoside (DP3.5). In six out of 16 accessions, among them two of the Spanish accessions, anthocyanins diglucoside (DI) contents exceeded those of the monoglucosides (MO), and seven out of total accessions were richer in monoglucosidic (MO) than diglucosidic anthocyanins (DI). In particular, the cumulated relative amounts of DI accounted for 23.7% and 78.3% of total anthocyanins,

with “Violetto” and “Piñonenca” showing the lowest and highest proportions, respectively (Table 3).

Table 3: Individual and total anthocyanin contents of 16 pomegranate accessions including the relative amount of mono (MO) - and diglycosidic (DI) anthocyanins of total anthocyanin contents.

Accession	Anthocyanins (mg/L)							Total	% MO	% DI
	CY3	PL3	CY3R	DP3.5	CY3.5	PL3.5	DP3			
Dente di cavallo acc. 1	19.2	4.0	0.4	4.9	17.8	2.3	2.1	50.7	50.5	49.5
Dente di cavallo acc. 2	40.5	4.8	0.8	23.5	41.3	2.6	14.2	127.7	47.2	52.8
Noto	15.8	2.0	0.4	3.8	5.8	0.8	1.8	30.4	65.9	34.1
PG-CT1	78.7	5.8	1.7	47.3	46.7	1.9	44.7	226.8	57.7	42.3
PG-CT5	44.9	5.3	0.8	43.8	55.3	3.4	23.1	176.6	41.9	58.1
PG-CT6	58.7	6.2	1.1	38.5	48.1	2.8	33.1	188.5	52.6	47.4
PG-SR1	53.6	6.1	0.8	18.0	41.1	2.5	12.7	134.8	54.3	45.7
PG-SR3	24.5	4.2	0.4	4.0	28.6	2.7	1.7	66.1	46.6	53.4
Primosole	52.7	5.6	0.9	59.1	57.4	3.2	33.6	212.5	43.7	56.3
Rosolini	49.5	8.1	0.7	4.1	32.4	2.7	3.6	101.1	61.2	38.8
Valenti	67.1	8.5	1.0	21.8	47.9	3.1	16.0	165.4	56.0	44.0
Violetto	21.1	2.2	0.5	0.0	9.0	0.7	7.4	40.9	76.3	23.7
Mollar de Elche	38.4	9.8	0.5	3.9	48.8	5.9	3.0	110.3	46.9	53.1
Piñon tierno	24.3	3.6	0.5	8.7	23.6	2.1	4.8	67.6	49.2	50.8
Piñonenca	12.6	4.6	0.2	17.2	52.1	7.8	4.0	98.5	21.7	78.3
Valenciana	19.7	4.5	0.4	5.7	19.1	2.7	2.7	54.8	49.7	50.3

CY3 cyanidin 3-O-glucoside; PL3 pelargonidin 3-O-glucoside; CY3R cyanidin 3-O-rutinoside; DP3.5 delphinidin 3,5 diglucoside; PL3.5 pelargonidin 3,5 diglucoside; DP3 delphinidin 3-O-glucoside; %MO per cent of monoglucoside anthocyanins; %DI per cent of diglucoside anthocyanins.

To the best of our knowledge, this approach has so far solely been applied by FISCHER *et al.* (2011a), and we found the same results for accessions of identical provenance, being in agreement with the data reported by FISCHER *et al.* (2011a), the Spanish accession showed the same proportion of anthocyanin diglucoside as “Piñonenca” accounting for 77% of the total amount, and the glycosidic relations of the Italian cultivars IT-1 and IT-2 were quite similar to the ratios found for “Dente di cavallo acc1” and “Rosolini”, both exhibiting 35% of diglucosidic anthocyanins. LEGUA *et al.* (2012) characterized the profiles of six Spanish cultivars, while we found a relative amount of anthocyanin diglucosides ranging between 36 and 51%. However, the latter group did not determine total phenolic contents, thus precluding the comparison of anthocyanin and total phenolic ratios. ZHAO *et al.* (2013) studied the anthocyanin profiles of three Chinese cultivars, reporting the predominance of monoglucosidic anthocyanins accounting for 92% of their total amount. BOROCHOV-NEORI *et al.* (2011) characterized four pomegranate accession for anthocyanins

composition showing a different anthocyanins glucosylation dependent on the harvest date and climate. GOMEZ-CARAVACA *et al.* (2013) found same results on 17 cultivars from different provenances. We evaluated the proportion of total anthocyanins to total phenolics, finding consistent differences to earlier published data. In particular, in our samples, anthocyanin share in total polyphenols ranged between 2.9 and 16.6%, being in agreement with the results published by GIL *et al.* (2000) for commercial juices and juices made from fresh arils, whereas according to FISCHER *et al.*, (2011a) the ratio of total anthocyanins to total phenolics was even higher (17-93%) in juices produced from the aril. In an investigation of 20 Iranian cultivars, TEHRANIFAR *et al.* (2010) found total anthocyanin ratios to total phenolics ranging between 0.88 and 3.29. Such differing traits may be used as a discriminant factor of diverse accessions. Fig. 2 shows the anthocyanin patters of juices made from the arils of “Primosole” pomegranate cultivar analyzed by HPLC–DAD. Identification and anthocyanins peak assignment were based on the comparison of their UV–Vis spectra and retention times (RT) with those of standards and references (FISCHER *et al.*, 2011b; WU and PRIOR, 2005), eight peaks were found, of which six were identified according to FISCHER *et al.* (2011b). In addition, we were able to tentatively identify a cyanidin-pentoside (CYPENT) and a cyanidin-rutinoside (CY3RUT) for the first time in Sicilian accessions (MS data not show). In particular, all accessions contained cyanidin pentoside (CYpent), while small amounts of cyanidin rutinoside were found in “Primosole”, “PG-CT1”, “PG-CT5”, “PG-CT6”, and “Mollar de Elche”, respectively.

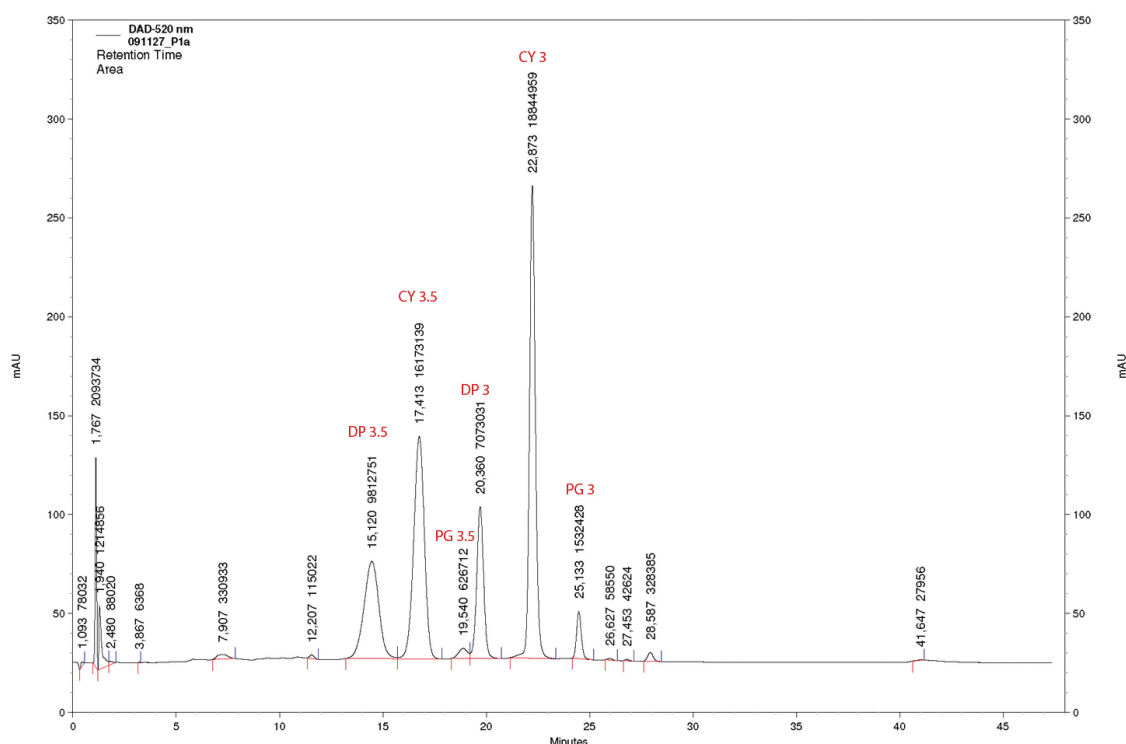


Figure 2: HPLC anthocyanin profile of a pomegranate juice of the “Primosole” accession.

Colorless phenolic compounds appear to be mostly responsible for the health-promoting properties of pomegranate fruits and juices obtained therefrom. Total polyphenol contents ranged between 948.6 and 3097.6 mg/L being in agreement with the literature data (GIL *et al.*, 2000; EL CAR *et al.*, 2011; OZGEN *et al.*, 2008). While “Primosole” accession showed the highest value, each of the accessions exhibited a high level of antioxidant activity ranging from 5,593 to 11,835 ORAC value compared to strawberry, plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear, and honeydew melon (WANG *et al.*, 1996; KALT *et al.*, 1999; WU *et al.*, 2004). Among the Sicilian accessions, “Primosole” had a slightly higher antioxidant activity (11,835 ORAC units) than “Piñon tierno” (11,411 ORAC units) exhibiting the maximum value among the Spanish accessions. Hydrolyzable tannins comprising gallotannins and ellagitannins together with gallagyl esters, were the predominant phenolic compounds in pomegranate fruits and juices prepared from the entire fruits, accounting for an average of 99 to 100 % of total phenolics in the pericarps, 87 % in pomegranate juices from entire fruits, and only 33 % in pomegranate juices made from isolated arils⁴². In the present study, tannin contents were rather uniform within the different accessions, with “Violetto” showing the highest value. However, in contrast to FISCHER *et al.* (2011a; 2011b; 2013) the method applied did not allow the distinction of gallotannins, ellagitannins and gallagyl esters, respectively. “Valenti” and “Piñon tierno” were characterized by low ellagic acid contents in contrast to “PG-CT1” and “Piñonenca” displaying the maximum values. Variability of ellagic acid contents was found to be high, which was confirmed by statistical analysis allowing to divide into 8 separate groups of the 16 accessions considered. Based on these findings, selection of high-yielding cultivars is recommended.

Anthocyanin amounts varied markedly which went along with visually noticeable differences in the red color of the juices. Therefore, monitoring of total color variations using the *CIE-L*a*b** color space, was instrumental. Higher *a** values were characteristic of a more reddish overall impression of the juices, whereas *b** values reflected blue (*-b**) to yellow (*+b**) tonalities of the juices.

Color analysis revealed significant differences among the cultivars as can be seen from the differing values of color parameters (Table 4) (SCHWARTZ *et al.*, 2009; FISCHER *et al.*, 2011a; DAFNY-YALIN *et al.*, 2010; FISCHER *et al.*, 2013).

In particular, it has been possible to distinguish deeply pigmented pomegranate juices by their high anthocyanin content which well corresponded with their high *a** values. This may allow rapid analysis and identification of adulterated commercial pomegranate juice because the pattern of anthocyanins is strictly related to the *a** value in accordance with DAFNY-YALIN *et al.* (2010) who found a significant correspondence ($p < 0.05$) between *a** values and anthocyanin contents. Calculating the ΔE values, and taking “Primosole” as a reference, i.e. the index for the differences perceived by the human eye, differences between “Primosole”, “PG-CT1”, “Valenti”, “PG-CT5” and “Valenciana” were insignificant; however, we found significant differences between “Primosole” and “Noto”, “Dente di Cavallo acc.1”, “PG-SR3”, “Violetto” among Italian accessions and significant differences between “Primosole” and “Piñon tierno”. We have calculated the ΔE values taking “Piñonenca” as a reference of the Spanish accessions, and we did not find perceivable differences by the human eye.

From Table 5, a significant correlation ($P < 0.05$) between ORAC values, anthocyanin contents and total polyphenol contents is obvious. The same holds true for the anthocyanin contents and the *C** values.

Our findings are in accordance with previously published data (LEGUA *et al.* 2012; TZULKER *et al.*, 2007; CUI *et al.*, 2013), thus confirming the high nutritional relevance of pomegranate juice. PCA analysis allowed identifying different clusters within the accessions. The total variability was explained by the 24 principal components, 9 of which

representing 95.6% of quality. Using Kaiser's rule to identify the principal components, only the eigenvalues greater than 1 have been considered (Table 6).

Table 4: Color characteristics of 16 pomegranate accessions.

	<i>L</i> [*]	<i>a</i> [*]	<i>b</i> [*]	<i>C</i> [*]	<i>h</i> [°]	Color index	Δ <i>E</i>
Dente di cavallo acc.1	92.7±2.5 ^{abc}	4.8±0.4 ^g	4.0±0.2 ^d	6.2±0.3 ^g	40.2±0.5 ^c	1.4±0.0 ^{gh}	9.8±0.1
Dente di cavallo acc.2	89.0±2.0 ^f	5.0±0.2 ^g	4.1±0.2 ^{cd}	6.5±0.4 ^{fg}	38.9±0.5 ^c	1.5±0.0 ^{de}	8.4±0.1
Noto	93.1±2.6 ^a	4.3±0.4 ^h	3.2±0.1 ^f	5.3±0.3 ^h	36.8±0.4 ^d	1.5±0.0 ^{ef}	10.5±0.1
PG-CT1	89.2±2.2 ^{fg}	11.6±0.6 ^b	4.5±0.2 ^b	12.4±0.7 ^b	21.3±0.3 ^{hi}	1.6±0.0 ^{ab}	2.3±0.0
PG-CT5	85.8±2.1 ⁱ	12.9±0.5 ^a	5.5±0.4 ^a	14.0±0.8 ^a	23.0±0.4 ^h	1.6±0.0 ^a	1.8±0.0
PG-CT6	91.6±2.1 ^{de}	6.0±0.3 ^e	3.5±0.1 ^{ef}	7.0±0.5 ^{de}	30.1±0.4 ^f	1.5±0.0 ^c	8.2±0.1
PG-SR1	91.8±2.3 ^{cde}	6.4±0.4 ^d	4.8±0.4 ^b	8.0±0.6 ^c	36.7±0.5 ^d	1.4±0.0 ^{fg}	7.9±0.1
PG-SR3	93.4±2.3 ^a	3.1±0.3 ⁱ	3.4±0.1 ^f	4.6±0.2 ⁱ	47.7±0.6 ^{ab}	1.3±0.0 ⁱ	11.7±0.1
Primosole	87.5±2.3 ^h	13.0±0.6 ^a	4.7±0.2 ^b	13.9±0.6 ^a	20.0±0.3 ⁱ	1.6±0.0 ^a	*
Rosolini	92.0±2.1 ^{bcd}	5.6±0.4 ^f	3.8±0.2 ^{de}	6.8±0.4 ^{ef}	34.4±0.5 ^e	1.5±0.0 ^{de}	8.7±0.0
Valenti	87.4±2.1 ^h	11.2±0.6 ^c	5.4±0.3 ^a	12.4±0.6 ^b	26.0±0.4 ^g	1.5±0.0 ^{bc}	2.0±0.0
Violetto	92.8±2.3 ^{ab}	4.3±0.3 ^h	4.7±0.3 ^b	6.3±0.5 ^g	47.6±0.5 ^{ab}	1.3±0.0 ⁱ	10.2±0.1
Mollar de Elche	91.0±2.4 ^e	5.9±0.3 ^e	4.0±0.4 ^d	7.2±0.3 ^d	34.0±0.4 ^e	1.5±0.0 ^d	7.9±0.0
Piñon tierno	93.2±2.5 ^a	2.8±0.1 ⁱ	3.2±0.2 ^f	4.3±0.2 ⁱ	49.1±0.5 ^a	1.3±0.0 ⁱ	11.8±0.2
Piñonenca	88.7±2.1 ^g	4.2±0.2 ^h	4.5±0.4 ^{bc}	6.2±0.4 ^g	46.5±0.6 ^b	1.4±0.0 ^h	8.9±0.1
Valenciana	88.7±2.2 ^g	13.0±0.6 ^a	4.7±0.4 ^b	13.9±0.7 ^a	20.0±0.3 ⁱ	1.6±0.0 ^{ab}	1.2±0.0

Values in the same column not followed by the same letter are significantly different ($p < 0.01$).

*Primosole value is the reference for Δ*E* calculation. Δ*E* indicates the values that have a just noticeable difference.

Table 5: Correlation matrix of characteristics of different pomegranate juices.

	MI	ASC	PT	TAN	ELL	ORAC	<i>L</i> [*]	CI	ANT
MI		0.1908	0.0904	-0.2902	-0.5223	0.2081	-0.1434	0.2392	-0.0463
ASC	n.s.		0.0275	-0.1068	-0.0540	0.0280	-0.1249	0.2793	-0.1420
PT	n.s.	*		0.0218	0.2999	0.1921	-0.4858	0.1278	0.1753
TAN	*	n.s.	n.s.		0.2869	0.2010	-0.0624	0.1529	0.2482
ELL	***	n.s.	*	*		-0.2606	-0.2026	0.1090	0.3998
ORAC	*	n.s.	*	*	*		-0.0820	0.1980	0.3538
<i>L</i>[*]	n.s.	n.s.	***	n.s.	*	n.s.		-0.7755	-0.6710
CI	*	*	n.s.	n.s.	n.s.	*	***		0.7221
ANT	n.s.	n.s.	*	*	**	**	***	***	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s.: not significant.

MI maturity index; ASC ascorbic acid content; PT total polyphenols; TAN tannins content; ELL ellagic acid; ORAC ORAC value; *L*^{*} lightness parameter; CI color index; ANT anthocyanins content.

Table 6: Eigenvalues and proportion of total variability among pomegranate accessions as explained by the first 24 principal components (PC).

PC	Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative%
1	7.384571	30.76905	7.38457	30.7690
2	4.426785	18.44494	11.81136	49.2140
3	3.448635	14.36931	15.25999	63.5833
4	2.490409	10.37670	17.75040	73.9600
5	1.770037	7.37515	19.52044	81.3351
6	1.303753	5.43230	20.82419	86.7675
7	0.949781	3.95742	21.77397	90.7249
8	0.710033	2.95847	22.48400	93.6833
9	0.448733	1.86972	22.93274	95.5531
10	0.356859	1.48691	23.28959	97.0400
11	0.230059	0.95858	23.51965	97.9986
12	0.216912	0.90380	23.73656	98.9024
13	0.129161	0.53817	23.86573	99.4405
14	0.096041	0.40017	23.96177	99.8407
15	0.019683	0.08201	23.98145	99.9227
16	0.010061	0.04192	23.99151	99.9646
17	0.004536	0.01890	23.99605	99.9835
18	0.002681	0.01117	23.99873	99.9947
19	0.000949	0.00395	23.99968	99.9987
20	0.000139	0.00058	23.99982	99.9992
21	0.000095	0.00040	23.99991	99.9996
22	0.000062	0.00026	23.99998	99.9999
23	0.000020	0.00008	24.00000	100.0000
24	0.000005	0.00002	24.00000	100.0000

The projections of the observations on the first two principal component axes are shown in Fig. 3. The accessions are distributed on the factor plane. These two coordinates represent 49.2 % of the total variance. The major contribute on factor 1 is due to anthocyanin contents, whereas b^* values contribute to the factor 2. “Violetto” has a positive contribution to factor 1, being well distributed along the factor 2 direction. On the other hand, both the “Valenciana” and “PG-CT5” have negative contributions on factor 1 and factor 2. “PG-CT1” positively contributes to factor 1, but opposite sign contributions on factor 2. Cultivars “Piñonenca” as well as “Dente di cavallo acc.2” and PG-SR1” were neither discriminated by the principal factor 1 x factor 2 not by any of the others. PCA is a good starting point to understand the component issues relevant for the discrimination of the different pomegranate accession samples. In fact, we found significant differences among the accessions, and this information may be useful for the authenticity control of pomegranate juices from different origins.

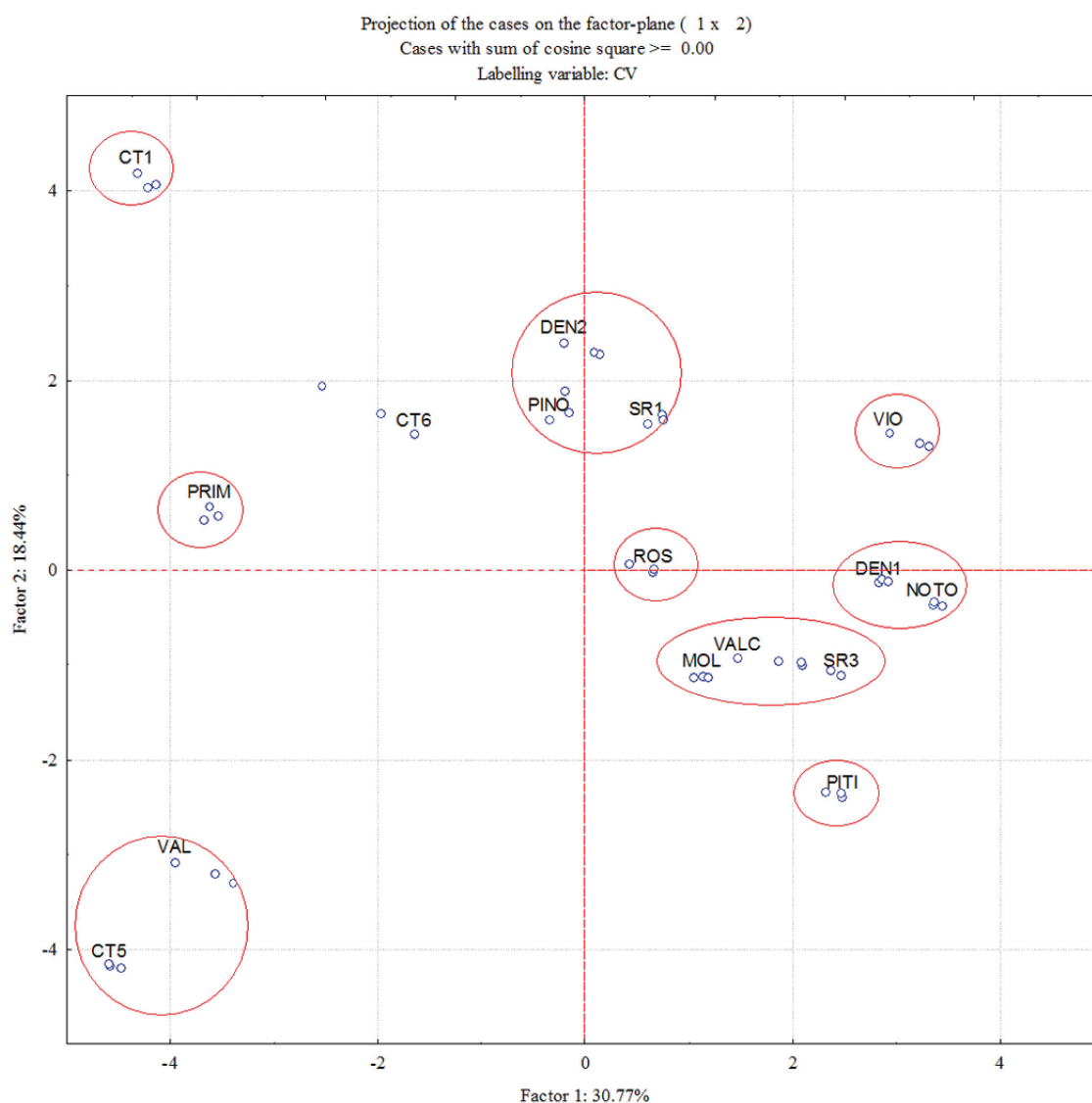


Figure 3: Projections of sample replicates of the five groups on the factor 1 vs. factor 2 plane.

Fig. 4 shows the hierarchical cluster analysis with the linkage method. We found seven main groups with the first main group made of “Dente di Cavallo acc.1” “Dente di cavallo acc. 2” and “Noto”, the second one was made of “PG-CT5” “Valenti” “PG-SR1” and “Rosolini”. High dissimilarity levels were found for accessions “Primosole”, “Mollar del Elche”, and “Violetto”, being highly heterogeneous among the studied accessions. Among all cultivars investigated, “Primosole” has interesting properties making this cultivar suitable for juice production and further breeding. In further investigations, anthocyanin pattern and contents in pomegranate of different provenances should be investigated in more detail.

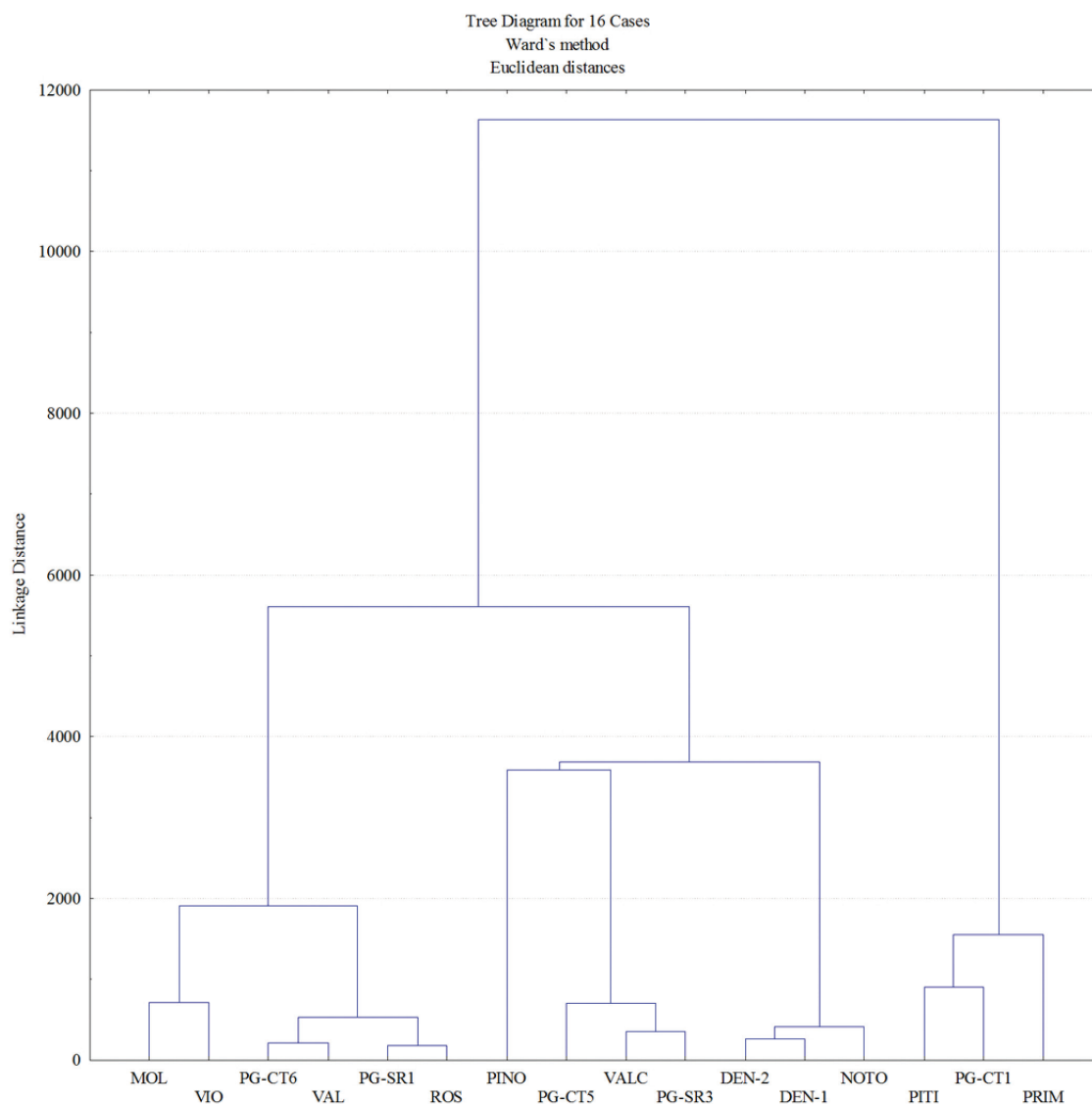


Figure 4: Cluster dendrogram of the pomegranate accessions under investigation.

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EFFECT OF SOME AMINO ACIDS ON YIELD AND CHARACTERISTICS OF PACIFIC WHITE SHRIMP TREATED WITH ALKALINE SOAKING SOLUTION

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ABSTRACT

Effects of alkaline soaking solution (ASS), 0.75% NaOH containing 2.5% NaCl, pH 11.5, containing different amino acids at various concentrations on yield and characteristics of Pacific white shrimp (*Litopenaeus vannamei*) were studied. The lowest cooking loss with the highest cooking yield was obtained for the sample treated with ASS containing 3% glutamic acid ($P < 0.05$). When shrimp were treated with ASS having 3% monosodium glutamate (MSG) with the same mole equivalent to glutamic acid, the higher weight gain but slightly lower cooking yield were obtained ($P < 0.05$). ASS containing 3% MSG had no effect on color and shear force of cooked shrimp.

Keywords: glutamic acid, monosodium glutamate, amino acids, alkaline, treatment, Pacific white shrimp

1. INTRODUCTION

Nowadays, Pacific white shrimp (*Litopenaeus vannamei*) is one of important species having a high market demand worldwide due to its appealed appearance, taste, flavor and texture (MANHEEM, 2013). Thailand is well known as the world largest shrimp producer, manufacturer and exporter. In 2013, shrimp was exported from Thailand with the value of 28,617 million bahts (THE CUSTOMS DEPARTMENT, 2013). Shrimp processing, especially freezing, can lead to the denaturation or aggregation of proteins (CARNETRO *et al.*, 2013). These changes can result in drip loss and sensorial changes in the product (GONÇALVES and RIBEIRO, 2009). Furthermore, cooking also causes the changes in quality attributes, mainly by affecting textural and physicochemical properties and inducing weight loss (CARNETRO *et al.*, 2013). The addition of water binding agent is therefore required in order to retain the quality of shrimp during processing. Phosphate and bicarbonate have been widely used as water binding agents, which can increase water uptake and lower cooking loss (RATTANASATHERIN *et al.*, 2008; CARNETRO *et al.*, 2013 and CHATARASUWAN *et al.*, 2011). Bicarbonate treatment could increase the weight gain of shrimp, mainly due to the repulsion between protein molecules mediated by its alkaline pH (CHATARASUWAN *et al.*, 2011). Due to the strict regulation of the uses of phosphate and bicarbonate in shrimp and shrimp products, an alternative additive with the property equivalent to both phosphate and bicarbonate are currently gaining attention for shrimp processing industry.

Some amino acids were reported to retard protein denaturation and retain protein functionality of frozen fish muscle (CAMPO-DEANO *et al.*, 2009; ZHOU *et al.*, 2006). Owing to the hydrophilic nature of some amino acids, their uses along with alkaline treatment could show the synergism in water uptake or water binding of shrimp muscle. Amino acid with different side chains, pI and molecular properties might exhibit varying efficacy as processing aid for shrimp processing. Monosodium glutamate (MSG) is the sodium salt of glutamic acid. It has been used widely as a flavor enhancer with umami taste. Since it is readily soluble in water, it can be used with ease for shrimp treatment and yield the product with improved properties. However, no information regarding the uses of amino acids and their salts as the processing aid for quality improvement of shrimp exists. The objective of this study was to investigate the impact of amino acids in conjunction with alkaline treatment on yield and characteristics of Pacific white shrimp.

2. MATERIALS AND METHODS

2.1. Collection and preparation of shrimp

Pacific white shrimp (*Litopenaeus vannamei*) (55-60 shrimp/kg) were purchased from a local market in Hat Yai, Songkhla province, Thailand. Shrimp with storage time less than 6 h after capture were stored in the insulated box containing ice using a shrimp/ice ratio of 1:2 (w/w). The samples were transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, shrimp were cleaned using tap water. Shrimp were peeled and deveined manually. Prepared shrimp were placed in polyethylene bag and stored in ice until use.

2.2 Effects of amino acids at various concentrations in alkaline soaking solution on weight gain, cooking loss and cooking yield of shrimp

2.2.1 Preparation of treated shrimp

Shrimp (peeled and deveined) samples were mixed with alkaline soaking solution (ASS; 0.75% NaOH containing 2.5% NaCl, pH 11.5) in the presence of glycine, glutamic acid or arginine at levels of 1, 2 and 3% (w/v) using the shrimp/solution ratio of 1:2 (w/v). The mixtures were stirred gently for 30 min at 4 °C and allowed to stand at 4 °C for 30 min. After treatment, the shrimp samples were placed on the plastic screen for 5 min (4 °C) to drain off solution. Sample soaked in 2.5% NaCl containing 3% mixed phosphates (sodium tripolyphosphate+tetrasodium pyrophosphate; 1:2, w/w) (MANHEEM, 2013) and those treated with 2.5% NaCl containing 0.75% NaOH (pH 11.5) were used as the positive controls. Those without soaking were used as the control.

All shrimp samples without and with different treatments were divided into two portions. The first portion was used as raw shrimp and another portion was subjected to cooking. To prepare cooked shrimp, the samples were subjected to steaming until the core temperature of the second segment of shrimp reached 85°C. The samples were cooled rapidly in iced water for 1 min, and drained on a screen for 5 min at 4 °C. Both raw and cooked shrimp samples were weighed and the weight gain, cooking yield and cooking loss were calculated.

2.2.2. Analyses

2.2.2.1 Determination of weight gain

Weight gain was determined by weighing the shrimps before and after soaking in the solutions. Weight gain was calculated as follows:

$$\text{Weight gain (\%)} = [(B-A)/A] \times 100$$

where: A = initial weight (before soaking)

B = weight after soaking, followed by draining

2.2.2.2 Determination of cooking loss and cooking yield

Cooking loss and cooking yield were measured by weighing the shrimps before and after heating according to method of MANHEEM *et al.* (2012). Cooking yield and cooking loss were calculated by the following equations:

$$\text{Cooking loss (\%)} = [(B-C)/B] \times 100$$

$$\text{Cooking yield (\%)} = (C/A) \times 100$$

where: A = initial weight (without soaking and steaming)

B = weight after soaking, followed by draining

C = weight after steaming, followed by cooling in iced water

2.3 Effect of glutamic acid at various concentrations in ASS adjusted to different pHs on shrimp treatment

Shrimp (peeled and deveined) samples were mixed with ASS (0.75% NaOH containing 2.5% NaCl) in the presence of glutamic acid at various concentrations (1, 2, and 3%) having pH 7 and 11.5. To adjust the pH of ASS, 6 M and 2 M HCl was used. Shrimp samples were treated with solutions and analysed for weight gain, cooking loss and cooking yield as described previously.

2.4 Comparative study of glutamic acid and monosodium glutamate in ASS on shrimp treatment

2.4.1. Preparation of treated shrimp

Shrimp (peeled and deveined) samples were mixed with ASS (pH 11.5) in the presence of glutamic acid or monosodium glutamate (MSG) at various concentrations (1, 2, and 3%) with pH of 11.5. MSG was used at the same mole equivalent to glutamic acid. Samples were treated as mentioned above. Both raw and cooked shrimp were analysed for weight gain, cooking loss and cooking yield. Additional analyses were performed as follows:

2.4.2. Determination of color

Color of raw and cooked shrimp were determined and expressed as L^* (lightness), a^* (greenness/ redness) and b^* (yellowness/ blueness). The second segment of shrimp was subjected for measurement using a Hunterlab colorimeter (Hunter Associates Laboratory, Inc., Reston, Virginia, USA) using a CIE Lab scale (YOUNG and WHITTLE, 1985).

2.4.3. Determination of shear force

Shear force of raw and cooked shrimp was measured using the TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a Warner-Bratzler shear apparatus (BRAUER *et al.*, 2003). The operating parameters consisted of a cross head speed of 10 mm/s and a 25 kg load cell. The shear force, perpendicular to the axis of the second segment muscle fibers, was measured.

2.4.4. Determination of protein pattern of soaking solutions

After being soaked with shrimp, the resulting soaking solutions were subjected to SDS-PAGE to determine the patterns of proteins leached out into solutions. SDS-PAGE was performed using 10% running and 4% stacking gels as described by LEAMMLI (1970). Soaking solution (20 ml) was mixed with 10 ml of 10% (w/v) SDS solution. The mixture was then homogenized at 11,000 rpm for 1 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The sample was then centrifuged at 7,500 $\times g$ for 15 min to remove undissolved debris using a microcentrifuge (MIK-RO20, Hettich Zentrifugan, Tuttlingen, Germany). Protein concentration of the supernatant was determined by the Biuret method (ROBINSON and HOGDEN, 1940). Sample (10 μg protein) was loaded onto the gel consisting of 4% stacking gel and 10% separating gel. Separation was performed by electrophoresis apparatus (Mini-Protein III, Bio-Rad, USA) using 30 mA. Protein was fixed and stained for 3 h in 1.25% Coomassie Brilliant Blue R-250 in 40% methanol and 10% glacial acetic acid. Gels were destained for 15 min with destaining

solution I (50% methanol and 7.5% glacial acetic acid) and with the destaining solution II (5% methanol and 7.5% glacial acetic acid) for 3 h. Wide range molecular weight standards were used and the molecular weight of protein was estimated.

2.4.5. Sensory evaluation

Cooked samples were subjected to sensory analysis. The samples were evaluated by 30 panelists from the Department of Food Technology with the age of 25-35, using the 9-point hedonic scale, where 9 = like extremely; 7 = like moderately; 5 = neither like or not dislike; 3 = dislike moderately; 1 = dislike extremely (MEILGAARD *et al.*, 1990). Panelists were acquainted with shrimp consumption and had no allergies to shrimp. All panelists were asked to evaluate for color, appearance, flavor, taste, texture and overall likeness. Samples were presented in the plates coded with three-digit random numbers.

2.5. Statistical analyses

A completely randomized design (CRD) was used for the whole experiments. Experiments were run in triplicate using different lots of shrimp samples. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test. Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for window, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1 Weight gain, cooking loss and cooking yield of Pacific white shrimp treated with ASS containing amino acids at various concentrations

Weight gain of Pacific white shrimp treated with ASS (pH 11.5) in the presence of amino acids at different concentrations (1, 2 and 3%) is shown in Fig. 1a. Weight gain of the treated shrimp increased, when the concentrations of amino acids in ASS increased ($P < 0.05$), except those treated with ASS containing glutamic acid, in which weight gain decreased with increasing concentration ($P < 0.05$). Amongst all amino acids, glutamic acid showed the higher increasing effect on weight gain at low level (1%). Under the alkaline condition, carboxyl groups, both at α -carbon and γ -carbon, were deprotonated and COO^- became dominant. Those negatively charged residues might penetrate into the swollen muscle along with ASS. Some COO^- groups of glutamic acid could interact with positively charged domain of proteins via ionic interaction, while the rest of COO^- groups were able to bind with water. As a result, the water could be retained in the muscle after treatment. Nevertheless, in the presence of an excessive glutamic acid, those COO^- groups in the solution (aqueous phase) more likely competed with muscle proteins in binding with water. As a consequence, the less water was retained in the shrimp muscle as indicated by the lowered weight gain, when the level of glutamic acid in ASS was higher than 1%. Glycine has been known as the smallest amino acid and has H atom as the side chain. Due to its small molecule, it could migrate easily into shrimp muscle and subsequently interact with water by hydrogen bonding via side chains within muscle. Additionally, carboxyl groups at α -carbon of glycine, which was deprotonated under the alkaline condition, could interact with water via ionic interaction. Consequently, water could be more imbibed, particularly when the level of glycine increased. This was reflected by the increased weight gain of shrimp after being treated with glycine at higher concentrations.

For shrimp treated with ASS containing arginine, weight gain increased as the levels of arginine increased ($P < 0.05$). At pH 11.5, some carboxyl groups of arginine ($pI = 10.76$) became deprotonated. Those groups could interact with muscle proteins and simultaneously bound with water. It was noteworthy that pH of solution (11.5) was close to pI of arginine. Therefore, COO^- group of arginine in ASS was present at low intensity. Owing to the lower abundance of COO^- group in ASS containing arginine, water was less bound with proteins, in comparison with that containing glutamic acid. WOLFENDEN *et al.* (1981) found that hydration potential of arginine was high at pH 7. At neutral pH, NH_2 group mostly became protonated, in which NH_3^+ was formed. Those positively charged groups effectively bound with water. However, in the present study, the pH of solution was 11.5, which was above pI (10.76). As a result, NH_3^+ was not present in ASS, and NH_2 group at the side chain became abundant. Arginine is also reported frequently to form hydrogen bonds with other side chains and water molecule (BORDERS *et al.*, 1994). Thus, charge of amino acid under the alkaline condition and the way those amino acids interacted with water and proteins in muscle more likely affected weight gain of treated shrimp.

Differences in weight gain among all treatments were plausibly governed by the differences in water binding or water holding capacity of different amino acids under the alkaline conditions. It was found that weight gain of shrimp treated with ASS containing 3% arginine was higher than those treated with mixed phosphates and other samples ($P < 0.05$). It is noted that treatment of shrimp with ASS containing 1% glycine, 1% arginine or 3% glutamic acid led to the lower weight gain, compared with ASS without amino acids. Thus, amino acids in alkaline solution had varying impact on weight gain of shrimp after treatments.

Cooking loss and cooking yield of shrimp samples treated with ASS containing different amino acids at various concentrations are shown in Figs. 1b and 1c, respectively. Cooking loss of shrimp decreased when the concentration of glutamic acid in ASS increased ($P < 0.05$). However, no differences in cooking yield were noticeable, when glycine at different levels was used ($P \geq 0.05$). Overall, when shrimp samples were treated with ASS in the presence of all amino acids, the decrease in cooking loss was obtained, in comparison with those treated with only ASS ($P < 0.05$). The weight loss was in the descending order in samples treated with ASS containing glycine, arginine and glutamic acid, respectively. Shrimp treated using ASS comprising 3% glutamic acid had the lower cooking loss than that treated with M-P ($P < 0.05$). Generally, the lowest cooking loss was found with the sample treated with ASS containing 3% glutamic acid ($P < 0.05$). When heat was applied, denaturation and coagulation of proteins were augmented, which in turn lowered water-holding capacity. Moreover, the increased protein-protein interaction was obtained (NIAMNUY *et al.*, 2008), leading to the enhanced release of water from shrimp muscle.

After cooking, the cooking yield of shrimp with different treatments varied (Fig. 1c), in which water molecules might be bound with amino acids or proteins in different fashions. The highest cooking yield was obtained with shrimp treated with ASS containing glutamic acid, compared with other treatments ($P < 0.05$). It was suggested that glutamic acid had the potential to bind water in shrimp muscle, when heat was applied. The efficacy in water holding during heating was dependent on concentrations used ($P < 0.05$). However, the concentration of glycine had no effect on cooking yield of shrimp ($P > 0.05$). Efficiency in increasing cooking yield was in the descending order: glutamic acid, arginine and glycine. The negatively charged residues, especially carboxyl group at α -carbon and γ -carbon of glutamic acid are strongly hydrated (COLLINS *et al.*, 2007). The two amino acids that have the highest water-binding ability are aspartic acid and glutamic acid (LOW *et al.*, 1978). An

ionic side chain of aspartic acid, glutamic acid and lysine has been claimed to bind 4-7 water molecules (ZAYAS, 1997).

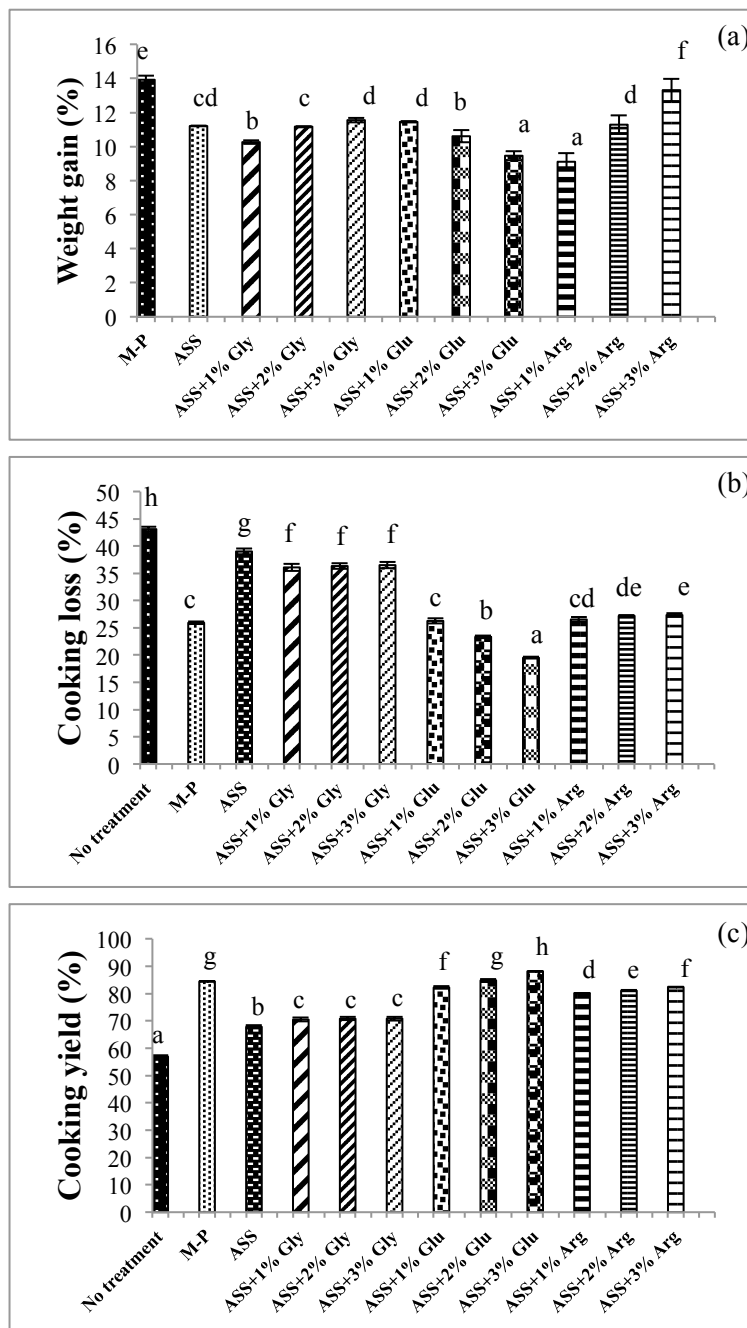


Figure 1: Weight gain (a), cooking loss (b) and cooking yield (c) of Pacific white shrimp treated with 0.75% NaOH containing 2.5% NaCl (pH 11.5) in the presence of amino acids at different concentrations. Note: M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1, (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), Gly: glycine Glu: glutamic acid, Arg: arginine. Different lowercase letters on the bars indicate significant differences (P<0.05). Bars represent the standard deviation (n=3).

When comparing with the sample treated with ASS alone, all samples treated with amino acids showed the increased cooking yield ($P < 0.05$). Only sample treated with ASS containing 3% glutamic acid had the higher cooking yield than that treated with M-P ($P < 0.05$). The sample treated with ASS having 2% glutamic acid had similar cooking yield to those treated with mixed phosphate ($P \geq 0.05$). Therefore, glutamic acid in ASS was shown to play a vital role in increasing the cooking yield by holding water in muscle during heating.

3.2 Weight gain, cooking loss, cooking yield and physical properties of Pacific white shrimp treated with ASS containing glutamic acid at various concentrations and pHs

Weight gain, cooking loss and cooking yield of Pacific white shrimp samples after soaking in ASS in the presence of glutamic acid at various concentrations with different pHs (7.0 and 11.5) are shown in Fig. 2. Weight gain and cooking yield of shrimp samples soaked in ASS, pH 11.5 were higher than those treated with ASS at pH 7.0 ($P < 0.05$), for all concentrations of glutamic acid used. At pH above pI or very alkaline pH, proteins had more negative charge, in which protein molecules repulsed each other, resulting in the swollen muscle structure (CHANTARASUWAN *et al.*, 2011b). As a consequence, water could be more uptaken into shrimp (CHANTARASUWAN *et al.*, 2011b). Simultaneously, glutamic acid could be taken into the muscle along with NaCl. Glutamic acid might favor the water binding via its COO⁻ group. Weight gain was slightly decreased as the concentrations of glutamic acid used increased ($P < 0.05$), regardless of pH. Generally, weight gain of all samples was lower than that of samples treated with ASS and with M-P ($P < 0.05$), except that treated with ASS containing 1% glutamic acid (pH 11.5) that showed the similar weight gain ($P > 0.05$). Among Pacific white shrimp treated with sodium carbonate and sodium bicarbonate at various pHs (5.5-11.5), the highest weight gain and cooking yield were observed in those treated at pH 11.5 (CHANTARASUWAN *et al.*, 2011b). Therefore, pH of soaking solution was the prime factor determining weight gain of shrimp.

Cooking loss (Fig. 2b) of shrimp decreased, when the concentration of glutamic acid in ASS increased ($P < 0.05$), irrespective of pH used. Lower cooking loss was found as ASS had pH of 11.5, compared with pH 7.0 ($P < 0.05$). The decrease in cooking loss was in accordance with the increase in cooking yield. The lowest cooking loss was observed in shrimp soaked with ASS containing 3% glutamic acid (pH 11.5) ($P < 0.05$). Glutamic acid could provide negative charge to muscle, thereby enhancing the water binding capacity of muscle proteins. Bound water was held tightly during heating as indicated by the lowered cooking loss and increased cooking yield. AASLYNG *et al.* (2003) suggested that a higher cooking loss was found in the sample with the low pH, whereas high water holding capacity was achieved at medium and high pH. When comparing the cooking loss of shrimp treated with mixed phosphates (M-P), the sample treated with ASS containing glutamic acid at levels of 2 or 3% (pH 11.5) had the lower cooking loss ($P < 0.05$). This reflected the high efficiency of ASS containing glutamic acid at a sufficient amount in lowering the cooking loss. The augmented repulsion of muscle compartment also allowed glutamic acid with the high negative charge to penetrate into muscle and bind more water, thus resulting in the lowered cooking loss.

In the presence of glutamic acid at 2 and 3%, no differences in cooking yield were obtained, compared with the sample treated with ASS alone ($P < 0.05$), when pH of soaking solution was 7.0 (Fig. 2c). On the other hand, cooking yield of samples treated with ASS containing glutamic acid at all levels were higher than that of sample treated with ASS alone, when pH was 11.5 ($P < 0.05$). The result indicated the paramount role of pH in water holding capacity of muscle when heating was implemented. As the shrimp were treated

with ASS (pH 11.5) containing glutamic acid at levels higher than 1%, similar cooking yield was obtained, compared to that found in the sample treated with mixed phosphates ($P<0.05$). Therefore, ASS containing glutamic acid showed the potential in improving the cooking yield, and its efficacy was comparable to mixed phosphates.

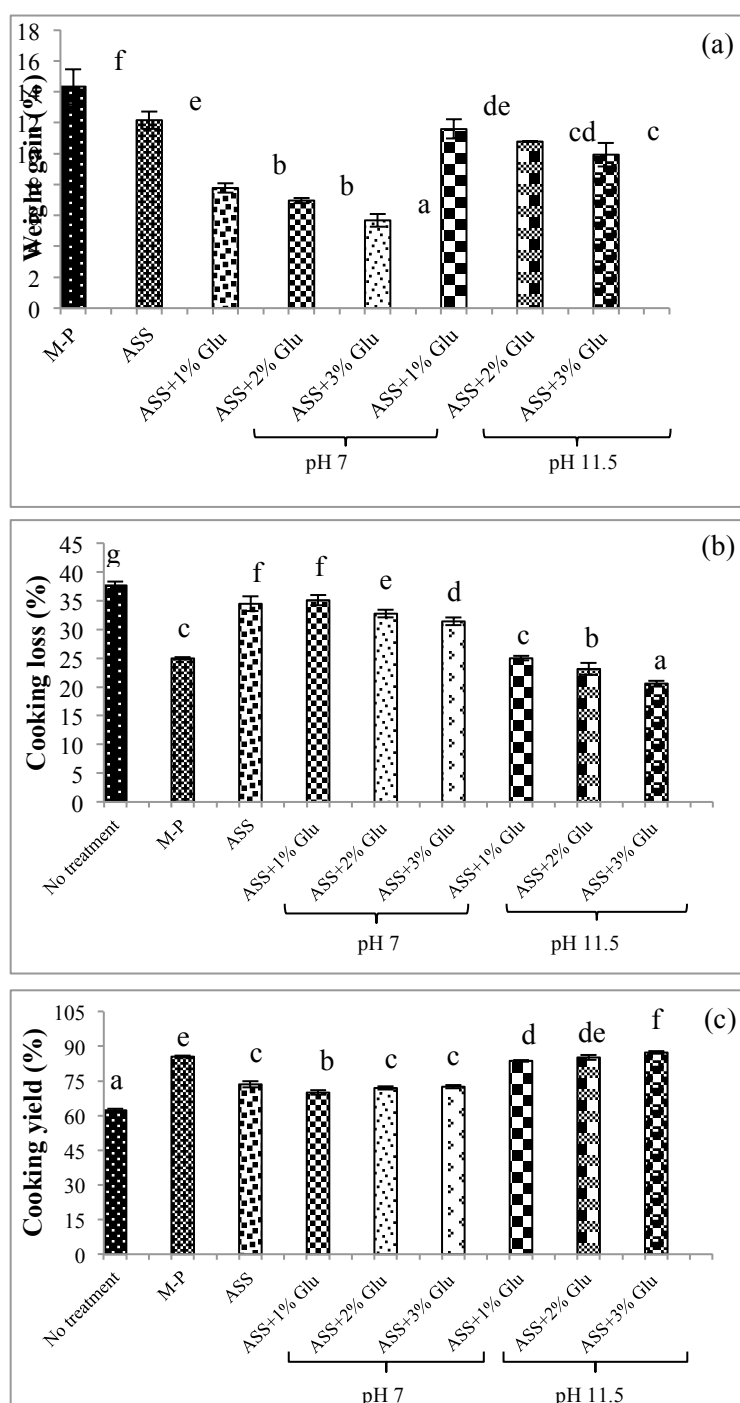


Figure 2: Weight gain (a), cooking loss (b) and cooking yield (c) of Pacific white shrimp treated with 0.75% NaOH containing 2.5% NaCl with pHs 7.0 and 11.5 in the presence of glutamic acid at various concentrations. Note: M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1, (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), Glu: glutamic acid. Different lowercase letters on the bars indicate significant differences ($P<0.05$). Bars represent the standard deviation ($n=3$).

3.3 Comparative study of ASS containing glutamic acid and MSG on shrimp treatment

Glutamic acid with high efficacy in increasing cooking yield was selected as the potential additive in ASS. However, glutamic acid had low solubility. Conversely, MSG, a salt form, was cheaper and soluble with ease in water. MSG was used at the same mole equivalent to glutamic acid for shrimp treatment.

3.3.1 Weight gain, cooking loss and cooking yield

Weight gain of shrimp treated with ASS containing glutamic acid at the levels of 1%, 2% and 3% or MSG at the levels of 1%, 2% and 3% mole equivalent of glutamic acid is shown in Fig. 3a. Sample treated with ASS containing 1 or 2% MSG had similar weight gain to that treated with mixed phosphate (positive control) ($P \geq 0.05$). It was postulated that the negatively charged glutamic acids from MSG were able to bind tightly with water molecule via ionic interaction within protein compartment, leading to the increased water holding in shrimp muscle. It was found that MSG showed higher ability in water holding than glutamic acid as indicated by higher weight gain.

For cooking loss (Fig. 3b), shrimp treated with ASS containing MSG had the higher cooking loss than those treated with glutamic acid counterpart, particularly at levels of 2 and 3% ($P < 0.05$). The cooking loss was lower in samples treated with ASS comprising both glutamic acid and MSG at higher concentrations ($P < 0.05$). For samples treated with mixed phosphate and ASS alone, the cooking losses of 19.19% and 31.07%, respectively were observed. The cooking loss of 13.76-19.62% was obtained for sample treated with ASS having glutamic acid, while cooking loss of 17.51-20.41% was found for the sample treated with ASS containing MSG. During cooking, muscle proteins underwent denaturation to a higher extent, while the amount of water retained in shrimp meat decreased with coincidental increase in fat and protein contents (MANHEEM *et al.*, 2012; BENJAKUL *et al.*, 2008). The result suggested that shrimp muscle could retain more water when glutamic acid and MSG were incorporated in ASS. However, glutamic acid showed the slightly higher ability in lowering cooking loss of shrimp, compared with MSG, especially at level of 2-3%.

For cooking yield (Fig. 3c), the opposite results were observed, in comparison with cooking loss. The lowest cooking yield was observed in the control (without treatment). Cooking yield of treated shrimp increased, when the concentrations of both glutamic acid and MSG increased ($P < 0.05$). Similar cooking yield was found in shrimp treated with ASS containing 2% glutamic acid or 3% MSG ($P > 0.05$). It was found that shrimp treated with ASS containing 2 or 3% glutamic acid or 3% MSG had the higher cooking yield than that of sample treated with mixed phosphates ($P < 0.05$). Since MSG was more soluble and cheaper than glutamic acid, it was selected for treatment of shrimp. The appropriate concentration of MSG in ASS was 3%.

3.3.2 Shear force

Shear force of raw and cooked Pacific white shrimp treated with ASS (pH 11.5) in the presence of glutamic acid and MSG at different levels (1%, 2% and 3%) is presented in Table 1. For raw shrimp, all treatments had no impact on shear force ($P > 0.05$). It was noted that shrimp treated with ASS containing glutamic acid and MSG at all levels (1-3%) had similar shear force ($P > 0.05$). All samples treated with ASS containing either glutamic acid or MSG had the similar shear force to those treated with mixed phosphates ($P > 0.05$), except those treated with ASS containing 3% glutamic acid, which showed the lower shear force ($P < 0.05$). Generally, shrimp tended to have the non-significant decrease in shear

force after treatment, particularly with increasing concentrations of glutamic acid and MSG in ASS used for treatment. When proteins imbibed water within their structure, the muscle had the lower resistance to the force applied.

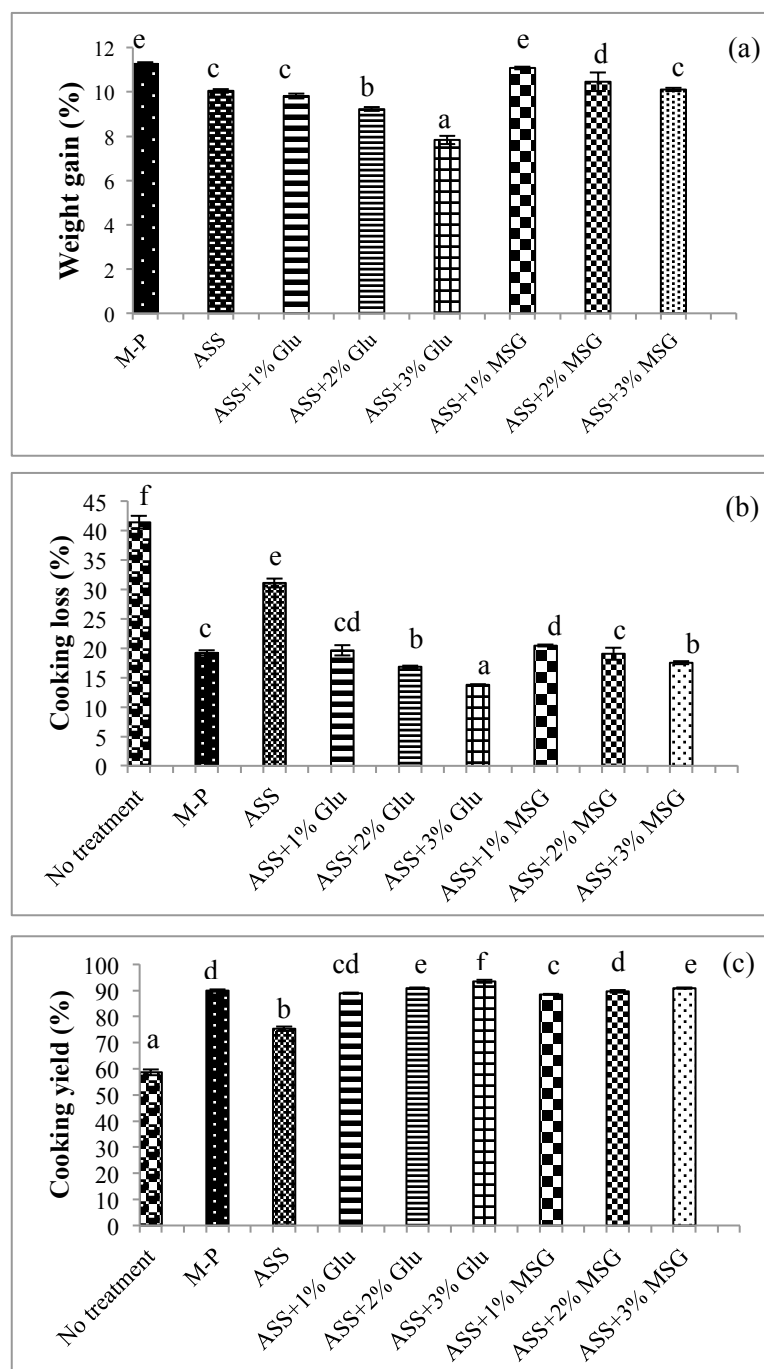


Figure 3: Weight gain (a), cooking loss (b) and cooking yield (c) of Pacific white shrimp treated with 0.75% NaOH containing 2.5% NaCl (pH 11.5) in the presence of glutamic acid and MSG at various concentrations. Note: M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1, (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), Glu: glutamic acid. MSG: monosodium glutamate. Different lowercase letters on the bars indicate significant differences (P<0.05). Bars represent the standard deviation (n=3).

Table 1: Shear force of raw and cooked of Pacific white shrimp treated with 0.75% NaOH (pH 11.5) containing 2.5% NaCl in the presence of glutamic acid and MSG at various concentrations.

Treatments	Shear force (g)	
	Raw	Cooked
No treatment	1970±177 ^{†,abc}	2194±326 ^b
M-P	2057±212 ^{bc}	1578±341 ^a
ASS	1890±416 ^{abc}	2031±345 ^b
ASS+1% MSG	2127±219 ^c	1549±130 ^a
ASS+2% MSG	1995±284 ^{abc}	1525±334 ^a
ASS+3% MSG	1794±275 ^{abc}	1472±83 ^a
ASS+1% glu	1972±329 ^{abc}	1555±205 ^a
ASS+2% glu	1665±253 ^{ab}	1515±126 ^a
ASS+3% glu	1618±243 ^a	1449±118 ^a

†Mean±SD (n=3).

Note: M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1, (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), Glu: glutamic acid. MSG: monosodium glutamate. Different lowercase superscripts in the same column indicate significant differences (P<0.05).

For cooked shrimp, lower shear force was observed in all treated samples, in comparison with those without treatment and those treated with ASS alone (P<0.05). Nevertheless, all samples had the similar shear force, compared to those treated with mixed phosphates (P>0.05). When heat was applied, protein denaturation and aggregation took place. Those phenomena resulted in the toughness as well as high resistance to force. No differences in shear force were found among shrimp treated with ASS containing glutamic acid and MSG at different concentrations used (P>0.05). Myofibrillar proteins with increased negative charge favored the repulsion of polypeptide chains, which resulted in the swelling of muscle and became less resistant to shear force applied. When the concentration of glutamic acid or MSG in ASS increased, non-significant decrease in shear force was obtained (P≥0.05). Therefore, treatment of shrimp using ASS containing glutamic acid or MSG did not had the negative impact on textural property and their shear force was comparable to that of shrimp treated with mixed phosphates.

3.3.3 Color

Color parameters (L^* , a^* and b^*) of raw and cooked shrimp treated with ASS (pH 11.5) in the presence of glutamic acid or MSG at different concentrations are shown in Table 2. For raw shrimp, L^* value increased when concentrations of MSG in ASS increased (3%) (P<0.05). The a^* value generally increased as the concentrations of both glutamic acid and MSG in ASS increased (P<0.05). Basically, a^* value indicates the reddish color. Conversely, no changes in b^* value were observed as the level of glutamic acid or MSG in ASS increased (P<0.05). Thus, raw shrimp turned to be slightly reddish as the levels of glutamic acid or MSG in ASS increased. Appearance of the product plays a significant role in maintaining high consumer acceptance (BONO *et al.*, 2012). Glutamic acid or MSG in ASS might induce the change of proteins associated with carotenoid, named carotenoprotein. This led to the enhanced exposure of free carotenoids, especially astaxanthin, as evidenced by more reddish color. Astaxanthin was reported as the major

pigment in shrimp meat (NIAMNUY *et al.* 2008). After cooking, no difference in color was observed among shrimp treated with ASS containing glutamic acid or MSG at all levels ($P>0.05$). It was obvious that L^* , a^* and b^* -values of cooked shrimp increased, in comparison with raw counterparts. The increase in intensity of red color after cooking is caused by muscle protein denaturation and the release of carotenoid pigment bound to the protein (carotenoproteins) (LATSCHA 1989; NIAMNUY *et al.* 2008). The a^* - and b^* - values of samples treated with ASS containing 2 or 3% glutamic acid were lower, compared with the control (no treatment). During soaking, some proteins including carotenoproteins were partially solubilized or leached out. As the results, less pigments were retained in the meat. Coincidentally, the soaking solution was more reddish in color as the glutamic acid or MSG concentrations in ASS increased (data not shown). There was no difference in all color parameters between cooked shrimp treated with ASS containing glutamic acid or MSG at all levels and those treated with mixed phosphates.

Table 2: Color of raw and cooked of Pacific white shrimp treated with 0.75% NaOH (pH 11.5) containing 2.5% NaCl in the presence of glutamic acid and MSG at various concentrations.

Samples	Treatments	L^*	a^*	b^*
Raw	No treatment	46.46±1.58 ^{ab}	-1.64±1.05 ^a	0.12±1.91 ^{cd}
	M-P	46.33±3.43 ^{ab}	-1.69±0.36 ^a	-3.23±1.79 ^a
	ASS	44.96±2.10 ^a	-1.66±0.34 ^a	-2.01±1.61 ^{ab}
	ASS+1% MSG	45.42±2.58 ^a	0.27±0.96 ^b	-1.13±0.48 ^{bc}
	ASS+2% MSG	46.81±2.10 ^{ab}	1.28±1.13 ^{bc}	-0.88±1.28 ^{bc}
	ASS+3% MSG	48.87±2.51 ^b	2.30±1.15 ^d	0.16±1.96 ^{cd}
	ASS+1% glu	45.22±2.39 ^a	0.89±1.51 ^b	-0.02±1.63 ^{cd}
	ASS+2% glu	46.09±0.88 ^a	2.01±0.74 ^{cd}	0.08±1.95 ^{cd}
	ASS+3% glu	46.35±1.22 ^{ab}	2.77±0.87 ^d	1.19±2.71 ^d
Cooked	No treatment	70.02±4.04 ^c	13.18±4.40 ^d	16.34±3.98 ^b
	M-P	65.50±4.30 ^{ab}	8.55±2.45 ^{ab}	12.37±4.63 ^a
	ASS	69.35±5.37 ^{bc}	11.85±4.02 ^{cd}	13.39±3.20 ^{ab}
	ASS+1% MSG	65.09±2.40 ^a	11.17±2.77 ^{abcd}	14.34±3.86 ^{ab}
	ASS+2% MSG	64.77±3.95 ^a	8.86±1.31 ^{ab}	11.28±3.63 ^a
	ASS+3% MSG	63.17±4.55 ^a	8.27±1.94 ^a	10.92±3.18 ^a
	ASS+1% glu	65.50±3.75 ^{ab}	11.32±1.91 ^{bcd}	15.64±3.06 ^{ab}
	ASS+2% glu	64.94±2.38 ^a	9.69±1.60 ^{abc}	14.40±3.13 ^{ab}
	ASS+3% glu	64.44±2.05 ^a	9.04±1.30 ^{abc}	11.78±2.18 ^a

†Mean±SD (n=3).

Note: M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1, (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), Glu: glutamic acid. MSG: monosodium glutamate. Different lowercase superscripts in the same column under the same state of sample indicate significant differences ($P<0.05$).

3.3.4 Protein patterns of soaking solutions

Protein patterns of soaking solutions after shrimp treatments are shown in Fig. 4. Band intensity of myosin heavy chain (MHC) and actin slightly increased as concentration of both glutamic acid and MSG in ASS increased. The result suggested that more MHC and actin were solubilized and leached out to solution to a higher extent when glutamic acid and MSG levels in ASS increased. The increase in MHC band intensity in soaking

solutions was in agreement with the higher cooking yields (Fig. 3c). Protein extraction and dissociation of myofibrillar proteins were mainly due to the ionic effect and pH alteration (BENDALL, 1954). Apart from MHC and actin, protein with MW of 25.6 and 18.2 kDa also increased with increasing levels of glutamic acid and MSG in ASS. CHANTARASUWAN *et al.* (2011) reported that Pacific white shrimp soaked in sodium bicarbonate and sodium carbonate solution had the increase in band intensity of MHC as pH of soaking solution increased. Protein patterns of ASS and ASS with mixed phosphates after shrimp soaking were similar, in which actin and protein with MW of 18.2 kDa were dominant. After those proteins were leached out from shrimp muscle, the soaking solution could be more penetrated to looser muscle compartment and retained inside the muscle as indicated by higher weight gain and cooking yield.

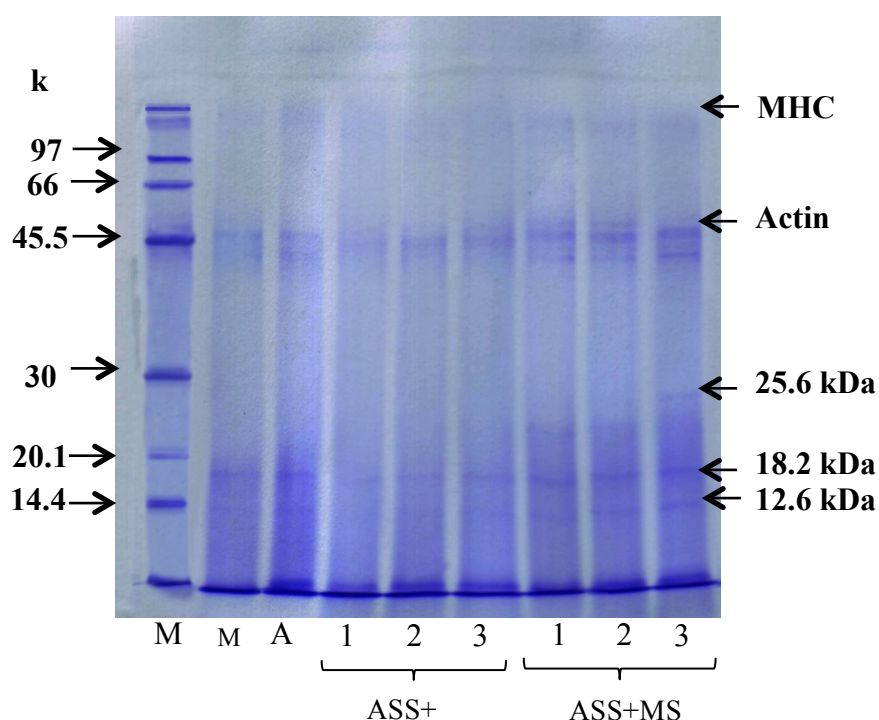


Figure 4: Protein patterns of soaking solution (0.75% NaOH and 2.5% NaCl, pH 11.5) in the presence of glutamic acid and MSG solutions at different concentrations after soaking with shrimp. M, Standard marker; M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1 (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), MSG: monosodium glutamate, MHC: myosin heavy chain. Numbers indicate the concentration of Glu or MSG (%).

3.4 Sensory properties of cooked shrimp treated with alkaline soaking solution containing MSG

Sensory properties of cooked shrimp without and with different treatments are shown in Table 3. The lowest likeness score for all attributes tested except flavor was found in the control sample (without treatment) ($P < 0.05$). During heating, the shrinkage of muscle of shrimp occurred, resulting in more compact in microstructure of shrimp with less juiciness. Among all samples, ASS +3%MSG sample showed the highest score for all attributes except for flavor that showed the lower score than the control and M-P sample

($P < 0.05$). This might be associated with fishy odor/flavor developed in of ASS +3%MSG sample. MSG is sodium salt of glutamic acid and provides a flavoring function similar to naturally occurring free glutamate in foods (YAMAGUCHI and NINOMIYA, 2000). Monosodium L-glutamate (MSG) has been used as a flavor enhancer since 1908, when it was identified as the source of umami taste (pleasant savory taste) (IMADA *et al.*, 2014). Since ASS +3%MSG samples had the high water holding capacity, more water was retained in shrimp meat as shown by a high cooking yield (Fig. 3). Decreased toughness with high juiciness contributed to the higher likeness score for texture and appearance. For color likeness, the highest score was observed for ASS +3%MSG sample ($P < 0.05$). Treatment of shrimp using 0.75% NaOH containing 2.5% NaCl and 3% MSG (ASS+3%MSG) rendered the resulting cooked shrimp with the highest overall likeness score ($P < 0.05$). However, the score was similar to that of shrimp treated with mixed phosphates ($P > 0.05$).

Table 3: Likeness score of cooked Pacific white shrimp with different treatments.

Attributes	No treatment	M-P	ASS	ASS+3% MSG
Appearance	5.50±1.25 ^{†,a}	7.76±0.66 ^c	7.00±0.98 ^b	8.00±0.74 ^c
Color	6.03±1.56 ^a	7.76±0.66 ^c	7.07±1.01 ^b	7.87±0.82 ^c
Flavor	7.10±1.03 ^b	7.31±1.13 ^b	6.80±1.19 ^{ab}	6.40±1.57 ^a
Texture	5.57±2.10 ^a	7.55±0.81 ^{bc}	7.00±1.74 ^b	7.80±0.85 ^c
Taste	5.80±1.85 ^a	7.83±0.73 ^b	7.73±0.78 ^b	8.13±0.73 ^b
Overall	5.77±1.45 ^a	7.76±0.85 ^{bc}	7.40±0.81 ^b	8.07±0.69 ^c

†Mean±SD (n=3).

Note: M-P: solution containing 2.5% NaCl and 3% mixed phosphates (tetrasodium pyrophosphate and sodium tripolyphosphate, 2:1 (w/w)), ASS: 0.75% NaOH containing 2.5% NaCl (pH 11.5), ASS+3% MSG: 0.75% NaOH containing 2.5% NaCl (pH 11.5) in the presence of 3% monosodium glutamate. Different lowercase superscripts in the same row indicate significant differences ($P < 0.05$).

4. CONCLUSION

Amino acids in ASS had the pronounced impact on quality improvement of Pacific white shrimp. Glutamic acid showed the marked effect on increasing cooking yield, while arginine could increase weight gain effectively. pH of ASS played a paramount role in water uptake and lowering the cooking loss of treated shrimp. MSG, the water soluble salt, showed the comparable impact on quality improvement to glutamic acid. Glutamic acid or MSG at higher level in ASS enhanced the solubility of MHC and actin, facilitating the migration of soaking solution and water holding capacity of shrimp muscle. Cooked shrimp treated with ASS containing MSG had the increased overall likeness score, but showed less score in flavor associated with the slightly fishy odor. The use of ASS containing 3% MSG was recommended as the promising soaking solution for shrimp treatment.

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QUALITY ASSESSMENT OF MEDITERRANEAN SHRIMPS DURING FROZEN STORAGE

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ABSTRACT

Aim of the research was to evaluate the effects of frozen storage on the quality of two Mediterranean wild shrimps, namely *Parapenaeus longirostris* (deepwater pink shrimp) and *Parapandalus narval* (narwal shrimp) in order to promote the marketing of these little-known shrimp species as frozen products, strengthening and enhancing their economic value. Quality changes were determined by sensory evaluation combined with chemical and chemical/physical analyses, including determination of volatile aroma constituents. In particular, raw and cooked shrimp samples were evaluated at various frozen storage intervals up to sixteen months. The variation observed for the chemical and chemical-physical indices did not diminish the sensory quality of both shrimp species. The results confirmed that freezing allows maintaining a good sensory quality of the considered shrimp species.

Keywords: Deepwater pink shrimp, frozen storage, narwal shrimp, sensory evaluation, volatile aroma compounds

1. INTRODUCTION

The Mediterranean shrimp species include, among others, the deepwater pink shrimp (*Parapenaeus longirostris*, Lucas 1846) and the narwal shrimp (*Parapandalus narval*, Fabricius 1787). Both species live in deep waters on muddy or muddy-sandy bottoms. *Parapenaeus longirostris* has a wide geographical distribution, being found both in the Eastern and Western Atlantic (OLASO, 1990), as well as in the Mediterranean and its adjacent seas (MASSUTTI, 1963); Italy is the country with the largest catches (HOLTHUIS, 1980) especially in the channel of Sicily and in the Ionian Sea. *Parapandalus narval* has an Eastern Atlantic-Mediterranean distribution (THESSALOU-LEGAKI, 1992) and is very common in the sea of Ustica Island.

Shrimps presumably represent the most important market for seafoods. In 2014 the global shrimp production was around 7×10^6 metric tons, of which almost 4×10^6 from aquaculture (LARKIN *et al.*, 2015). The international trade is dominated by USA, Japan and European countries as importers, whereas developing nations, especially South East Asian countries, act as the main shrimp suppliers of the world.

Italy imports most of its demand mainly as farmed shrimp whereas the two Mediterranean wild shrimps, namely *Parapenaeus longirostris* and *Parapandalus narval*, are of little commercial importance, sold only as fresh locally and close to the fishing grounds since their limited shelf life.

Although freezing is an effective method for preserving foods, some deterioration in frozen food quality can occur during storage, such as colour fading (CHANDRASEKARAN, 1994; OKPALA and BONO, 2016), lipid oxidation (RIAZ and QADRI, 1990), denaturation of protein (BHOBE and PAI, 1986), sublimation and recrystallization of ice (LONDAHL, 1997). These can result in off-flavours, rancidity, dehydration, loss of juiciness, textural changes (BHOBE and PAI 1986; YAMAGATA and LOW 1995; LONDAHL, 1997) and increase in volatile basic nitrogen (RIAZ and QADRI 1990; YAMAGATA and LOW, 1995).

Some papers are present in literature on the quality changes of frozen shrimps during storage (YAMAGATA and LOW 1995; BAK *et al.*, 1999; BOONSUMREJ *et al.*, 2007; TSIRONI *et al.*, 2009; BONO *et al.*, 2016). Only few of these evaluated the sensory quality or related the sensory evaluation to the volatile aroma compounds (ROCHAT *et al.*, 2009; ALAM and SOLBERG, 2009), but according to our knowledge, no informations are reported on *Parapenaeus longirostris* and *Parapandalus narval*.

In view of the fact that frozen shrimp is a product of high commercial value and increasing demand due to its competitive price and extended shelf life (TSIRONI *et al.*, 2009), the aim of the research was to evaluate the effects of frozen storage time on the quality of the deepwater pink and narwal shrimps in order to promote the marketing of these little-known shrimp species as frozen products, strengthening and enhancing their economic value. Since the consumer is the ultimate judge of quality, chemical and instrumental methods were matched with the sensory evaluation: chemical and chemical-physical indices, volatile aroma constituents and sensory properties were determined at different times during frozen storage both on raw and cooked samples.

2. MATERIALS AND METHODS

2.1. Sampling

Shrimp specimens of *Parapenaeus longirostris*, Lucas 1846 (FAO name: deepwater pink shrimp) and *Parapandalus narval*, Fabricius 1787 (FAO name: narwal shrimp) were caught

off the southeast coast of Sicily (Porto Palo, Siracusa, Italy - FAO 37: Mediterranean, Black sea; Subarea 37.2: Central Mediterranean; Division 37.2.2: Ionian) in March 2013. Samples of frozen shrimps (size: *Parapenaeus longirostris*, 10-15 cm; *Parapandalus narval*, 7 cm) were provided by a Sicilian company that owns and operates both the fishing boats and the packing and storage facilities. After catch, shrimps were put into ice, then quick-frozen at -40°C, stored at -18°C under vacuum (-0.8 bar) and transported frozen (at constant -18°C) to the laboratory. All shrimp samples came from the same frozen batch; for each shrimp species eight packages in total were purchased and stored at constant -18°C for sixteen months. The chemical and sensory analyses were carried out immediately after arriving at the lab and at specific intervals during storage. At fixed time, one package was thawed at room temperature and sufficient quantities were used for analyses. Unpeeled raw and cooked shrimp samples were analysed immediately after thawing. Cooked shrimps were obtained steaming unpeeled specimens for 15 min. All determinations were made in triplicate.

2.2. pH Measurement

The pH values of the raw samples were determined on homogenates of samples in distilled water (1:2 w/w) by using a pHmeter MP220 (Mettler Toledo, Milan, Italy) at 25°C.

2.3. Determination of the total volatile basic nitrogen (TVB-N)

For the determination of the total volatile basic nitrogen (TVB-N) of the raw samples, steam distillation of an extract deproteinised by trichloroacetic acid extraction was used according to official method (EU, 1995). Results were expressed as mg TVB-N/100 g of wet sample.

2.4. Colour measurement

Quantification of the colour change was based on measurement of CIELab values (L^* -value: lightness; a^* -value: redness and greenness; b^* -value: yellowness and blueness), using a NR-3000 Colourimeter (Nippon Denshoku Ind. Co. Ltd, Tokyo, Japan). The instrument was standardized under "C" illuminant condition according to the CIE (Commission International de l'Eclairage) using a standard white reference tile. At predetermined times of storage, according to the design, measurements were conducted for raw and cooked shrimp at five points. All measurements were carried out on three different shrimp samples.

The average values were reported and values of ΔE were determined:

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

where L_0^* , a_0^* , and b_0^* are the values of L^* , a^* and b^* colour parameters at storage time zero.

2.5. HS-SPME sampling

The method of headspace solid phase microextraction (HS-SPME/GC-MS) was used for the isolation and concentration of volatiles. The analyses were conducted on peeled raw and cooked shrimp samples. To 5 g of each shrimp sample, placed in a 40 ml vial, 14 ml of a NaCl saturated aqueous solution were added. Extraction was performed in the headspace vial kept at 35°C using a DVB/CAR/PDMS fibre of 50/30 µm film thickness (Supelco, Bellefonte, PA, USA) housed in its manual holder (Supelco, Bellefonte, PA,

USA). The sample was equilibrated for 30 min and then extracted for 30 min. During the extraction, the sample was continuously stirred. After the sampling, the SPME fibre was introduced onto the splitless injector of the GC/MS maintained at 260°C for 3 min for the thermal desorption of the analytes. No artefacts were observed after a SPME analysis of the saturated saline solution performed as blank analysis.

2.6. GC-MS analysis

A Varian 3800 gas chromatograph directly interfaced with a Varian 2000 ion trap mass spectrometer (Varian Spa, Turin, Italy) was used. The conditions were as follows: injector temperature, 260 °C; injection mode, splitless; capillary column, CP-Wax 52 CB, 60 m, 0.25 mm i.d., 0.25 µm film thickness (Chrompack Italy s.r.l., Turin, Italy); oven temperature, 45°C held for 5 min, then increased to 200°C at a rate of 5°C/min and to 240°C at 3°C/min; 240°C held for 20 min; carrier gas, helium at a constant pressure of 10 psi; transfer line temperature, 250°C; acquisition range, 40–250 *m/z*; scan rate, 1 scan/s. Each volatile component was identified using mass spectral data, NIST 11 library (NIST/EPA/NIH Mass Spectra Library, version 2.0 g, USA), linear retention indices, literature data and injection of standards where available. The linear retention indices (LRI) were calculated according to VAN DEN DOOL and KRATZ (1963) equation.

2.7. Sensory analysis

The sensory profiles of the shrimp samples were evaluated following the UNI 10957, 2003 method. Twenty-five judges were submitted to preliminary tests to determine their sensory performance on basic tastes and the aromas associated with shrimps. The sensory profile (UNI 10957, 2003) was defined by using a selected panel of twelve judges trained over four sessions. Panelists were asked to score appearance and odour of raw peeled shrimp and appearance, odour, texture, and taste of cooked shrimp. A list of descriptors was selected based on the frequency (60 %) of the terms used by the judges in several sessions. Reference standards were available to define descriptors. The descriptors were quantified using a nine-point intensity scale, where 1 = “not perceptible” and 9 = “strongly perceptible”. The shrimp samples were tested in triplicate. Each judge evaluated the shrimp samples in two sessions. All evaluations were conducted from 10.00 to 12.00 AM in individual booths (ISO 8589, 2007) illuminated with white light. The order of presentation was randomized among judges and sessions. Water was provided for rinsing between shrimp samples. All data were acquired by a direct computerized registration system (FIZZ Biosystemes. ver. 2.00 M, Couternon, France).

2.8. Statistical analysis

Chemical and sensory data were subjected to analysis of variance (ANOVA), using Statgraphics Plus software (ver. 5.1). Duncan’s multiple range test was applied to the data to identify any significant differences between the analysed samples. The model was statistically significant with $P < 0.05$.

3. RESULTS AND DISCUSSIONS

Figure 1 shows the variation of the pH values during frozen storage in raw deepwater pink and narwal shrimps. For both shrimp species the pH values ranged between 7.3–7.8 in agreement with CADUN *et al.* (2005) who reported pH values of 7.64 for frozen

deepwater pink shrimp; no statistically significant ($P>0.05$) increase resulted till four month. The pH determination is one of the most frequently used physical methods for the quality control of seafood products since they are affected by the changes in the concentrations of free hydrogen and hydroxyl ions because of the shifts in the oxidation–reduction balance of the food by the activity of micro-organisms or enzymes (VARLIK *et al.*, 2000). Generally, the pH value of crustaceans is higher than that of fish and mammal species because of their higher content of nonprotein nitrogenous compounds (SHAHIDI, 1994). From our results, the pH values of analysed shrimp samples during storage resulted always below 8, the critical acceptability limit for most shellfish products (SCHORMULLER, 1968).

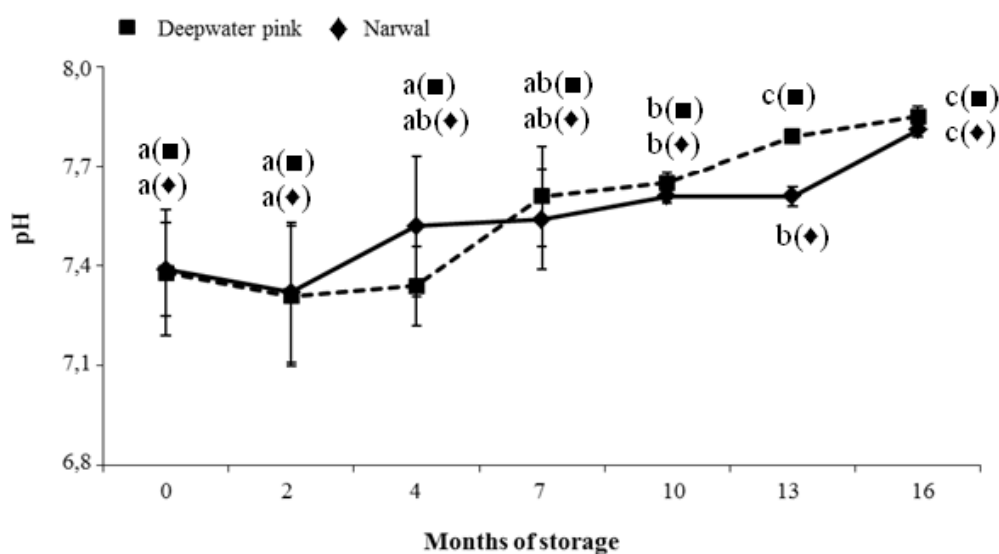


Figure 1: pH variations of raw shrimp samples during frozen storage (Error bars indicate standard error of measurements).

For each species different letters indicate statistically significant differences among mean values at $P \leq 0.05$ by Duncan's multiple range test.

Figure 2 shows the total volatile basic nitrogen (TVB-N) values in raw deepwater pink and narwal shrimps during storage. The TVB-N content resulted around 29 mg/100 g of wet weight in the shrimp samples of the two species at the beginning of storage, and increased reaching the values of 35.09 mg/100 g and 35.85 mg/100 g at the end of storage for deepwater pink and narwal shrimps, respectively. Statistically significant increases were observed after four month of storage for both shrimp species. The increase in TVB-N values is consistent with the results of the pH values: the TVB-N increase during the storage turns the medium alkaline and as a result pH increases. The level of TVB-N is considered a useful index of microbial spoilage in different fresh and lightly preserved seafood (OZOĞUL and OZOĞUL, 2000). The limit of TVB-N values for seafood products of good sensory quality has been reported to be 30 mg/100 g. However, this limit may be questionable for shrimp species since the average TVB-N values for fresh crustaceans are often higher (OEHLENSCHLÄGER, 1997); in a study by COBB *et al.* (1973) the initial TVB-N content of fresh shrimp tails from different batches of shrimp stored on ice, ranged from 13.5 to 38.2 mg N/100 g; however a high sensory quality of shrimp samples was

perceived. As a consequence, TVB-N content cannot be considered as an indicator of freshness by itself but it must be always enhanced by the sensory tests (CHEN *et al.*, 1995). Table 1 illustrates the changes of L^* , a^* and b^* in raw and cooked deepwater pink and narwal shrimps during frozen storage. L^* values increased whereas a^* and b^* values decreased when storage time increased; the colour of shrimp changed from red and yellow to dull lighter colour. In Table 1 ΔE values for raw and cooked shrimps are also reported. ΔE values, indicating the total colour changes, increased with an increase in storage time. For raw deepwater pink shrimp statistically significant variations resulted at the beginning and at the end of storage, whereas for cooked samples a significant increase was observed only after thirteen months. Regarding the narwal shrimps, ΔE values significantly increased until ten months both in raw and cooked samples. The rate of increase was higher for narwal shrimp samples, both raw and cooked, than for deepwater pink shrimps probably due to the deeper colour of the former as indicated by the higher scores of the colour sensory descriptor (Table 3). Shrimp colour is linked to the content of astaxanthin and its esters, that are the major pigments in shrimps; drying and storage conditions affect shrimp colour due to the astaxanthin oxidation and isomerisation reactions that lead to colourless compounds and, thus, to the loss of the typical redness and yellowness (CHEN *et al.*, 1995; NIAMNUY *et al.*, 2008).

Table 2 shows the amount of the volatile compounds detected in raw and cooked deepwater and narwal shrimp samples at different storage time. A total of thirty compounds have been observed, such as aldehydes, ketones, alcohols, esters, acids, nitrogen- and sulfur- containing compounds.

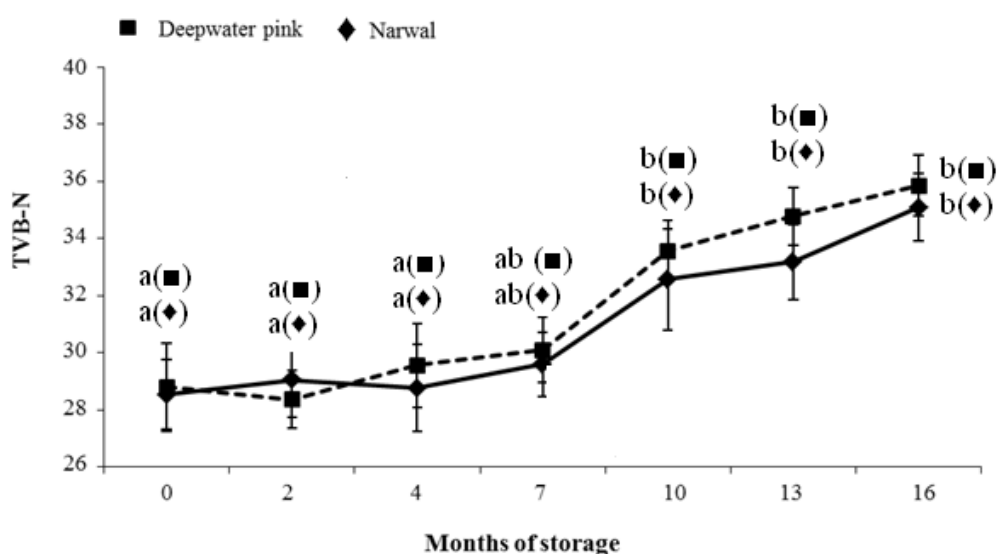


Figure 2: Total volatile basic nitrogen values (mg N/100 g shrimp flesh) of raw shrimp samples during frozen storage (Error bars indicate standard error of measurements).

Figure legend: For each species different letters indicate statistically significant differences among mean values at $P \leq 0.05$ by Duncan's multiple range test.

Slight differences in the qualitative and quantitative volatile composition of the two shrimp species and during the storage period, as well as between raw and cooked samples, resulted. In raw shrimps, hexanal, 2-ethyl-1-hexanol and dimethylsulphide were

the most abundant compounds, followed by 6-methyl-5-hepten-2-one and nonanal; 1-penten-3-ol, octanal (only in deepwater pink shrimp samples), 1-octen-3-one and γ -butyrolactone were also detected in a good amount. Some of these volatiles, namely dimethyl sulphide, 1-penten-3-ol, 1-octen-3-one, 2-ethyl-1-hexanol and γ -butyrolactone, decreased during the storage period but they remained among the most abundant components of volatile fraction up to sixteen months. Aldheydes, ketones and alcohols containing 6, 8 and 9 carbon atoms are derived from long-chain polyunsaturated fatty acid via 12- or 15-lipoxygenases and hydroperoxide lyase (BAEK and CADWALLADER, 1997) and are responsible of the pleasant planty, green and melony aromas and flavours of fresh seafoods. In particular hexanal contributes to the distinct green plant-like, grassy and apple-like aromas of fresh shrimp, whereas 2-ethyl-1-hexanol, together with short-chain alcohols, contributes to the typical sweet aroma of shrimps (ALASALVAR *et al.*, 1997). Also dimethyl sulphide provides a pleasant seashore-like smell in fresh seafoods (IIDA, 1988); in fact, although sulphur compounds are usually associated with deteriorated seafoods, there is evidence that they can be present even in fresh ones and are considered important volatile aroma components in marine crustaceans (ALASALVAR *et al.*, 1997). γ -Butyrolactone has faintly sweet odour reminiscent of rancid butter; lactones have been identified in roasted shrimp and boiled scallops; they derived from aliphatic saturated and unsaturated γ -hydroxycarboxylic and δ -hydroxycarboxylic acids (PAN and KUO, 1994). Dimethylamine, trimethylamine, acetic acid, and phenol were detected in low amount in fresh samples and slightly increased during frozen storage, except for trimethylamine, whose variations during storage were not statistically significant both in narwal and in deepwater shrimp samples. The formation of trimethylamine in frozen seafoods is prevented by the inhibition of microbial growth, yet dimethylamine is produced enzymatically: CASTELL *et al.* (1970) reported the increase of dimethylamine content in frozen fish muscle due to enzymatic breakdown of trimethylamine oxide. Also acetic acid could be formed by enzymatic decomposition of either lipid autoxidation or secondary hydroperoxides of fatty acids (ALASALVAR *et al.*, 1997), whereas phenol formation occurs via decarboxylation of phenolic carboxylic acids (SPURVEY, 1998).

In all the samples, 6-methyl-5-hepten-3-one and geranylacetone (spicy and flowery notes) were present; these compounds, deriving from carotenoid degradation, showed a statistically significant increase during storage. Their amount and the rate of increase during storage are higher in narwal than in deepwater pink shrimps. 2,4-Decadienal, (*E,Z*)-2,4-heptadienal, (*E,Z*)-3,5-octadien-2-one and (*Z*)-4-heptenal, deriving from lipid autoxidation during storage, were not detected. The absence of these compounds, responsible of stale and oxidized aromas and of fish cold-stored off-flavours (ALASALVAR *et al.*, 1997), was probably due to the very low fat content of deepwater pink and narwal shrimps.

As expected, the cooked shrimps showed some differences in volatile aroma profile compared to the raw ones. After cooking, hexanal and dimethylsulphide remained the main constituents of the volatile fraction but their amount decreased when freezing time increased. 2-Methylbutanal, 3-methylbutanal, 2,6-dimethylpyrazine, dimethylsulfoxide, 1-dodecanol were detected only in cooked shrimps samples and their amount did not vary among the cooked samples. Among these compounds, 1-dodecanol, with a flower-like odour, has been already recognized as an important volatile of cooked shrimps (MANDEVILLE *et al.*, 1992). 2-Methylbutanal and 3-methylbutanal are well known as amino acid degradation products and may be generated either thermally (Strecker degradation) or enzymatically from isoleucin and leucin.

Table 1: ΔE , L^* , a^* and b^* values of shrimp samples during frozen storage.

Months of storage	Deepwater pink shrimp								Narwal shrimp							
	Raw				Cooked				Raw				Cooked			
	ΔE	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE	L^*	a^*	b^*
0		31.31a	10.87d	-2.03d		45.47a	3.07d	-1.46d		43.99a	17.66c	10.84b		23.06a	12.73c	14.63c
2	0.54a ^(a)	33.86a	9.93c	-4.12c	1.13a	58.76b	1.46c	-2.59c	0.92a	44.86a	11.73c	9.92b	0.18a	28.88a	12.17c	13.52c
4	0.93b	38.22b	6.41b	-4.51c	1.15a	58.69b	-1.44b	-3.12b	1.18b	45.47a	11.56c	9.04b	0.59b	36.75b	11.38b	11.34b
7	0.99b	39.16b	6.29b	-5.41b	1.16a	58.31b	-3.22a	-3.78b	1.30c	48.72a	8.07b	2.53a	0.78c	38.33b	11.33b	11.13b
10	1.04b	39.79b	4.72a	-5.67b	1.17a	58.39b	-3.92a	-4.12b	1.40d	53.57b	6.82a	1.37a	1.16d	43.52c	10.89b	8.34a
13	1.13c	42.47c	4.41a	-6.32b	1.22b	60.13c	-4.07a	-4.88b	1.41d	58.84b	6.06a	1.11a	1.25d	43.53c	6.61a	6.73a
16	1.19c	48.43c	3.40a	-8.29a	1.28b	62.11c	-4.97a	-6.73a	1.45d	59.98b	5.12a	1.02a	1.29d	46.07c	4.83a	6.68a

^(a) Different letters in the same column indicate statistically significant differences among mean values at $P \leq 0.05$ by Duncan's multiple range test.

Table 2: Volatile fraction composition^a of shrimp samples during frozen storage.

Months of storage	Deepwater pink								Narwal							
	Raw				Cooked				Raw				Cooked			
	0	4	10	16	0	4	10	16	0	4	10	16	0	4	10	16
Volatiles^(b)																
Dimethylamine	84b ^(d)	90b	114a	149a	60b	68b	88a	99a	50b	67b	107a	113a	90b	92b	109a	111a
Trimethylamine	48	46	40	43	52	56	57	54	56	51	51	54	64	67	62	65
Dimethyl sulphide	920a	875a	585b	332c	2246a	2352a	1391b	596b	704a	743a	304b	298b	2656a	2021a	1231b	698c
3-Hydroxy-2-butanone	25	28	38	43	-	-	-	-	-	-	-	-	-	-	-	-
2-Butanone	65	60	53	48	-	-	-	-	-	-	-	-	-	-	-	-
2-Methyl butanal	- ^(c)	-	-	-	68	71	75	69	-	-	-	-	82	79	86	89
3-Methyl butanal	-	-	-	-	52	63	58	57	-	-	-	-	60	71	68	75
Hexanal	1120	1155	1051	1197	1855a	1693a	1076b	1038b	1157	1136	1156	1098	1931a	1832a	1123b	1311b
1-Penten-3-ol	232a	160b	115c	48d	96a	41b	38b	16c	104a	88b	41c	48c	29a	17b	10b	9b
Heptanal	-	-	-	-	32	42	32	28	-	-	-	-	23	22	28	11
2-Methyl-1-butanol	33	24	35	29	-	-	-	-	78	59	60	65	29	25	11	12
1-Pentanol	-	-	-	-	39	31	25	17	-	-	-	-	31	28	14	21
Octanal	100	116	115	126	127	143	143	121	21	36	24	43	59	60	89	34
6-Methyl-5-hepten-2-one	321b	358b	397b	498a	119b	130b	168b	262a	413b	452b	479b	681a	121b	164b	279a	325a
2,6-Dimethylpyrazine	-	-	-	-	43	37	38	46	-	-	-	-	46	56	53	47
Nonanal	260	287	242	225	350	327	321	312	330	341	294	288	380	372	222	287
Ethyl octanoate	-	-	-	-	-	-	-	-	-	-	85	93	-	-	-	-
1-Octen-3-one	186a	148a	96b	82b	41a	49a	18b	11b	216a	228a	182a	106b	-	-	-	-
Acetic acid	99b	108b	120b	160a	236b	246b	254b	294a	89b	99b	111b	142a	47b	54b	116a	135a
2-Ethyl-1-hexanol	913a	605b	465c	428c	-	-	-	-	1589a	1295b	861c	973c	-	-	-	-
Dimethyl sulfoxide	-	-	-	-	59b	68b	125a	130a	-	-	-	-	19b	31b	49a	66a
1-Nonanol	43	39	53	32	36	42	59	48	45	40	28	32	7	7	5	3
γ-Butyrolactone	191a	70b	68b	74b	143a	89b	66b	64b	122a	56b	21c	25c	111a	53b	22c	20c
4-Butoxy-1-butanol	48	34	39	30	30	25	26	28	58	48	51	63	50	49	47	53
Dodecanal	-	-	-	-	58	63	83	64	-	-	-	-	91	86	93	89
Methyl dodecanoate	8	4	5	5	-	-	-	-	10	8	15	13	-	-	-	-
Hexanoic acid	23	28	32	35	31	28	36	38	8	7	10	6	31	28	34	36
1-Dodecanol	-	-	-	-	158	174	164	169	-	-	-	-	201	198	231	243
Geranylacetone	11b	15b	42a	50a	166b	164b	236a	242a	62b	45b	125a	175a	185a	179a	265b	271b
Phenol	54b	46b	71a	86a	-	-	-	-	26b	23b	42a	53a	-	-	-	-

^(a)Expressed as peak areas, arbitrary scale. ^(b)Volatile compounds have been reported according to the order of elution on the column CP-WAX 52 CB column. ^(c)Not detected. ^(d) Different letters in the same row indicate statistically significant differences among mean values at P < 0.05 by Duncan's multiple range test.

Table 3: Sensory scores of shrimp samples during frozen storage.

Months of storage	Deepwater pink								Narwal							
	Raw				Cooked				Raw				Cooked			
	0	4	10	16	0	4	10	16	0	4	10	16	0	4	10	16
Volatiles																
Flesh colour	3.5b ^(a)	2.9a	2.8a	2.1a	3.5b	3.7b	2.3a	2.6a	7.5c	6.2b	2.7a	2.8a	7.4d	6.8c	6.0b	4.8a
Sheen	4.5b	4.4b	4.1a	3.9a	-	-	-	-	6.5c	5.8b	6.0b	4.0a	-	-	-	-
Compactness	6.5	6.2	6.0	6.2	-	-	-	-	6.5	5.9	6.3	6.4	-	-	-	-
Sea aroma	5.1	4.8	4.7	5.0	4.2	4.7	4.2	4.3	5.3	4.8	4.8	5.1	5.8	4.4	4.5	4.1
Algae aroma	4.3	4.9	4.5	4.7	-	-	-	-	4.9	4.9	4.7	4.3	-	-	-	-
Shrimp aroma	5.0	5.9	5.4	5.4	6.9	6.3	6.6	6.3	6.2	5.9	5.4	6.4	7.1	6.3	6.5	6.2
Off-odour	2.5	2.7	3.3	3.3	2.2	2.9	3.3	3.4	2.6	2.6	3.7	3.8	2.0	2.8	3.4	3.8
Flesh firmness	5.3	4.2	4.5	4.8	4.6	3.3	3.8	3.8	5.1	4.2	5.0	5.5	4.8	3.6	3.3	3.6
Bitter	-	-	-	-	2.5	3.3	2.6	3.0	-	-	-	-	3.0	3.1	2.8	2.8
Salty	-	-	-	-	3.4	3.0	3.1	2.8	-	-	-	-	3.8	3.7	4.5	3.7
Sour	-	-	-	-	2.3	2.1	2.0	1.9	-	-	-	-	2.3	2.1	2.5	1.7
Sweet	-	-	-	-	4.6	3.8	4.0	3.9	-	-	-	-	5.7	4.6	4.0	4.5
Juicy	-	-	-	-	5.1	4.8	4.1	4.2	-	-	-	-	5.5	4.4	4.5	4.7
Chewy	-	-	-	-	3.9	4.0	4.2	4.5	-	-	-	-	3.4	3.6	4.0	4.0
Sea flavour	-	-	-	-	4.8	3.8	3.7	4.6	-	-	-	-	4.4	4.0	3.9	4.0
Shrimp flavour	-	-	-	-	6.8	5.9	5.9	6.8	-	-	-	-	7.0	6.2	6.6	6.4
Off-flavour	-	-	-	-	2.2	2.8	3.0	3.0	-	-	-	-	2.3	3.3	3.5	3.0
Overall	7.4	7.1	7.4	7.2	7.6	7.6	7.4	7.5	8.0	8.1	7.8	7.9	8.3	8.2	8.2	8.1

^{a)} Different letters in the same row indicate statistically significant differences among mean values at $P < 0.05$ by Duncan's multiple range test.

Since these branched aldehydes were not present in uncooked samples, their thermal generation could be presumed. Also 2,6-dimethylpyrazine has a thermal origin: in fact alkylpyrazines may be formed by the involvement of lipid oxidation products in Maillard reaction (HUANG *et al.*, 1987) by heating of food at or above 100 °C. Alkylpyrazines, including methylpyrazine, 2,5- and 2,6-dimethylpyrazine have been detected in several cooked crustaceans, identified as having a roasted, nutty/meaty aromas in boiled crayfish and considered to contribute more to boiled rather than roasted odours in proteinaceous food (SPURVEY, 1998).

Other volatiles such as the 2-ethyl-1-hexanol were not identified in the cooked shrimps. Otherwise, as below reported 2-ethyl-1-hexanol, together with short-chain alcohols, contributes to the typical sweet aroma of raw shrimps (ALASALVAR *et al.*, 1997).

As happened for the raw shrimps, both deepwater pink and narwal, the volatile profile of the cooked shrimps considered as a whole, remained almost stable at least until ten months of freezing storage.

Table 3 reports the results of sensory evaluation of raw and cooked deepwater pink and narwal shrimps. In particular, for the raw samples the descriptors were three for the appearance (colour of flesh, sheen, compactness) four for the aroma (shrimp, sea, algae, off odour) and one for the rheological properties (flesh firmness). Regarding the cooked shrimps four descriptors for the aroma (shrimp, sea, algae odour, off odour), three for the flavour (shrimp, sea, off flavour), one for the rheological properties (flesh firmness), four for the taste (bitter, salty, sour, sweet), one for the oral perception (juicy) and finally, one for the texture (chewy) were selected.

The analysis of variance applied to the sensory data showed statistically significant differences only for sensory scores linked to colour and sheen descriptors both for raw and cooked deepwater pink and narwal shrimps. This was in accordance with the colour analysis that showed colour changes during storage and with the volatile analysis that evidenced an increase in the amount of carotenoid degradation products. No statistically significant variation was observed for the "overall" descriptor up to sixteen month of frozen storage. The differences observed in chemical indices and volatile aroma constituents little influenced the sensory attributes of raw shrimp samples during the freezing storage. The same occurred for the sensory attributes of the samples cooked after different periods of freezing.

4. CONCLUSIONS

The main goal of this research was to evaluate the effect of freezing storage on the quality of deepwater pink shrimps and narwal shrimps. To this end, pH, TVB-N, colour analysis, volatile fraction analysis and sensory evaluation were carried out. The assessment of shrimp quality mainly considered the impact of the preservation method on the sensorial characteristics since they are major concerns of consumers. The chemical and chemical-physical data and the volatile profile, considered the most important parameter for shellfish flavour quality, evidenced slight variations during the freezing storage but were unable to affect the sensory quality as confirmed by the maintenance of high scores for the "overall quality" descriptor throughout the entire storage period. Our results demonstrate the maintenance of a good sensory quality during the considered period, therefore these two shrimp species could be of great economic interest if marketed as frozen products.

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GRAPE SEED RIPENING EVALUATION BY *ORTHO*-DIPHENOL QUANTIFICATION

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ABSTRACT

Two millennia of viticulture recognize the seed browning importance on tannin ripening and, thus, on grape and wine quality. This color change was recently attributed to phenolic oxidations. However an objective chemical index able to quantify the oxidation status of seed tannins was missing, probably due to the heterogeneous oxidation polymerizations. This work suggests the adoption of the *ortho*-diphenol quantification as indication of the tannin ripening process, because *ortho*-dihydroxylated substitutions are highly susceptible to oxidation. The method proposed is based on the *ortho*-diphenol characteristic complexation with molybdenum. Different cultivars (Merlot, Pinot noir, Croatina, Aladasturi, Alexandrouli, Odjaleshi and Tavkveri) were studied during three vintages in Oltrepò pavese, Italy. The color darkening correlated with the *ortho*-diphenol decrease. We believe this index could find useful applications in viticulture, supporting harvesting time decisions.

Keywords: *Vitis vinifera*, tannins, seed browning, viticulture, wine quality

1. INTRODUCTION

"... *Naturalis autem maturitas est, si cum expresseris vinacea, quae acinis celantur, iam infuscata et nonnulla praeter modum nigra fuerint. Nam colorem nulla res vinaceis potest adferre nisi naturae maturitas ...*". In his treatise "*De Re Rustica*", Columella (4-70 A.D.) suggested to use the seed darkening as the best grape ripening index. In 2000, Kennedy, Matthews and Waterhouse described the seed color change during berry development, and, in 2005, RISTIC and ILLAND, published a color chart to define the grape seed ripening. Two millennia of grapevines cultivation confirmed the importance of grape seed color. Also winegrowers are aware of the importance of this character: traditionally they check the seed color to evaluate the grape ripening status. Despite the increased knowledge in grape chemistry and physiology, the visual observation of the seed color is still one of the best available methods to evaluate the grape phenolic ripening. This method has some disadvantages: i) it is a subjective evaluation; ii) seeds have not an homogeneous color; iii) considering the color chart published by RISTIC and ILLAND (2005), it is not easy to discriminate the different brown tonality, especially in the last phenological steps (the most important for ripening estimation). For decades, a number of researchers focused their attention on phenolic content and polymeric subunit composition (KENNEDY *et al.*, 2000) or extractability during winemaking (ROLLE *et al.*, 2013). They payed attention to the phenolic concentration, overlooking this evident physiological color change. It should be noted that ROLLE *et al.* (2013) proposed an interesting acoustic method based on the physiological seed hardening during ripening, but the instruments required for this analysis are not widely diffused among grape and wine analytical laboratories.

Traditionally, winemakers consider seed lignification as the main responsible of this color variation. In their belief, lignin deposition would act as a barrier against tannins extraction, resulting also in a decrease in the detectable total phenolics (RIBEREAU-GAYON *et al.*, 1998). However, the grape seed hardening is due to the lignification of the inner layers of the outer integument (RISTIC and ILLAND, 2005), whereas nearly all of the soluble seed phenolics are localized in the thin-walled cells between the epidermis and the inner lignified layers (ADAMS, 2006). Adams (2006) also suggests that the seed browning characteristic of fruit ripening is the result of tannins and flavan-3-ols oxidation. Also KENNEDY *et al.* (2000) proposed seed polyphenol oxidation as the best explanation for their ripening study results. PILATI *et al.* (2007) described a rapid accumulation of H₂O₂ and an activation of the ROS scavenging enzymes at veraison. Thus, the hypothesis of a characteristic oxidative burst during ripening seems to be agreed among researchers.

In wine industries, tannins play a fundamental role, affecting the product quality in terms of astringency, body and bitterness. Their organoleptic feature change during ripening. At the moment, winegrowers usually assess the seed tannin ripening status by: i) a subjective visual color evaluation; and/or ii) a subjective organoleptic estimation by tasting; and/or iii) an approximate relationship between the anthocyanin accumulation or total phenolic content and the seed tannins evolution. Thus, an objective and accessible method to describe seed tannin ripening is still missing.

The aim of this work is to develop an index representative of the oxidative status of the seed phenolic compounds.

2. MATERIALS AND METHODS

Grapevines were all cultivated in the same germplasm collection located in Oltrepò Pavese (Lombardy region, northern Italy) already described in RUSTIONI *et al.* (2013). Plant material was collected during 3 growing seasons: 2009, 2010 and 2011. In the first

experimental year, Merlot and Pinot noir grapes were studied. In 2010, also Croatina (a local cultivar) was analyzed, together with Merlot and Pinot noir. In 2011, other 4 cultivars were included in the study: Aladasturi, Alexandrouli, Odjaleshi and Tavkveri (all of them are Georgian varieties). The list of cultivars and sampling dates is reported in SI 1. All samples were collected in 3 biological replications and the fresh seeds were extracted. A number of berries per replication were analyzed (depending on the seed number) as shown in SI 1. Berries were weighed and seeds were separated, counted, weighed and the color class was attributed following RISTIC and ILLAND (2005). Seeds were then extracted in 25 ml of methanol for 20 hours, and, then, in other 25 ml of methanol for 4 hours. All the extractions were performed in dark conditions and under continuous shaking. Finally the two extracts were mixed and kept at -20°C until analysis.

Within 3 months samples were analyzed following the method proposed by Maestro DURÁN *et al.* (1991). Two solutions were prepared: sol. A (water:ethanol 50:50) and sol. B (5% sodium molybdate in sol. A). 10 ml of diluted sample were added by 2 ml of sol. A (blank) and with sol. B (reacted) for 15 minutes. The reacted sample was then read against the blank at 370 nm by a JASCO 7800 spectrophotometer (JASCO, Mary's Court, Easton, Maryland). Dilutions were set up to optimize the absorbance range. A calibration curve was obtained by using standard caffeic acid solutions. Samples were also reacted with the Folin Ciocalteu solution to quantify the total phenolic content following Di Stefano, CRAVERO and GENTILINI (1989).

Data were statistically analyzed using the SPSS® statistical software (Version PASW Statistics 19, SPSS Inc, Chicago, Illinois).

3. RESULTS AND CONCLUSIONS

The list of samples analyzed, together with some general information, are reported in SI1. The number of berries considered depended on the expected number of seeds of each cultivar. Generally, in 50 ml of methanol, about 20-50 seeds were extracted. However, taking into account the seeds and berries number and weights, it was possible to elaborate the data concerning total phenolic content and *ortho*-diphenols concentrations in relation to the sampled grapes (e.g.: mg/seed; mg/kg of grapes).

Information concerning the number of seeds per berry as well as their phenolic content could be a useful support for winemaking technique optimization (maceration timing, seeds separation, aging expectations ...) (Table 1).

These data could also be interesting for phenotypic characterization (RUSTIONI *et al.*, 2014). However the important environmental effect on these parameters should not be forgotten. As an example, in our experimental conditions, Pinot noir generally had a high number of seeds/berry, and these seeds had a high phenolic concentration. In the opposite situation we found Odjaleshi. Nevertheless, intermediate characteristics were also recorded (e.g.: Aladasturi had a lot of seeds but they were not particularly concentrated in phenolic compounds).

Seed browning was studied following the method proposed by RISTIC and ILLAND (2005).

Fig. 1 reports the obtained data. In Fig. 1a, each point represents the average value of the three biological replications: the seed color level generally increased during berry development. In Fig. 1b each dataset (cultivar and year) is reported in a different color. It appears that the trend was consistent in all the varieties. Nevertheless, an important variability among the samples was recorded. The explanation could be found in the vintage and cultivar diversity (phenological shifts) as well as in the ripening degree variability among seeds.

Table 1: Cultivar characteristics: seed phenolic content and number of seeds per berry.

	Cultivar	Average	Standard deviation	Minimum	Maximum
Seed phenolic content mg/seed	Pinot noir	1.520	0.698	0.373	2.922
	Merlot	1.022	0.519	0.220	2.311
	Croatina	1.002	0.583	0.287	2.617
	Tavkveri	0.746	0.402	0.206	1.606
	Alexandrouli	0.596	0.144	0.347	0.844
	Aladasturi	0.505	0.319	0.226	1.358
	Odjaleshi	0.422	0.233	0.179	1.139
Number of seeds per berry	Aladasturi	3.2	0.2	2.9	3.5
	Pinot noir	2.3	0.4	1.6	3.3
	Tavkveri	2.2	0.6	1.3	3.4
	Alexandrouli	1.8	0.2	1.4	2.2
	Merlot	1.7	0.3	1.1	2.8
	Croatina	1.6	0.3	1.1	2.9
	Odjaleshi	1.5	0.2	1.1	2.1

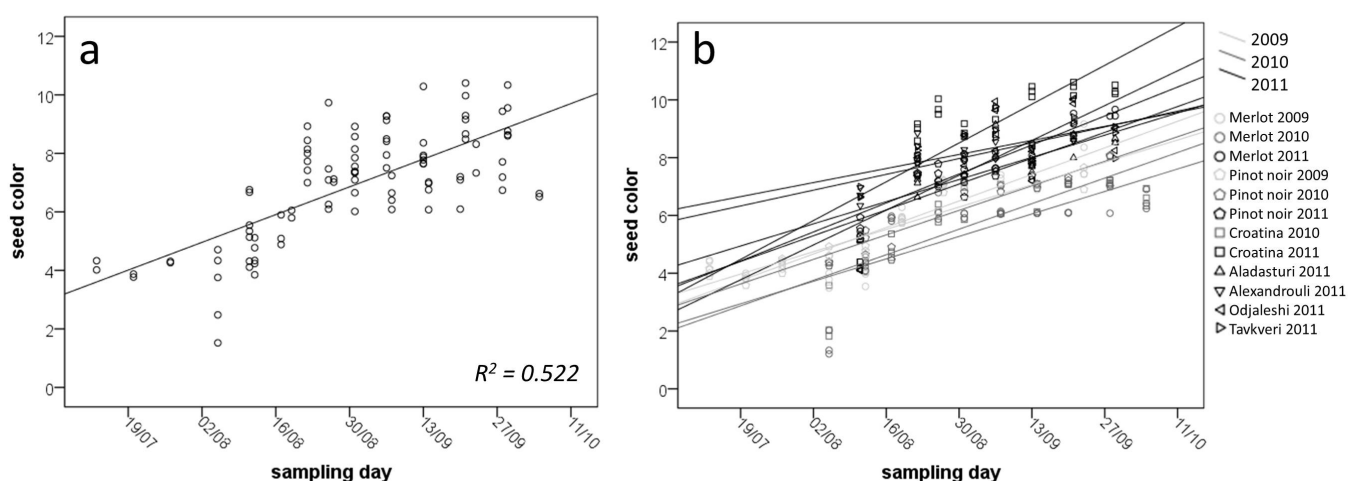


Figure 1: Seed color change during the growing season. In figure 1a each point represents the average value of the three biological replications. In fig. 1b each dataset (cultivar and year) is labeled by a different symbol. The obtained regression lines are classified depending on the sampling year (2009: light grey; 2010: dark grey; 2011: black).

A similar variability was found in the *ortho*-diphenol content decreasing trends during berry development (Fig. 2). Also in this case we attribute this heterogeneity to vintage, cultivar and seed variability. However, we observed a consistent decrease in the *ortho*-diphenol concentration in seeds (Fig. 2b), and we propose to adopt this value as an indicator of the grape phenolic ripening status. Recently, the central role of phenolic

oxidation in seed browning process has been underlined (KENNEDY *et al.*, 2000; RISTIC and ILAND, 2005; ADAMS, 2006; PILATI *et al.*, 2007).

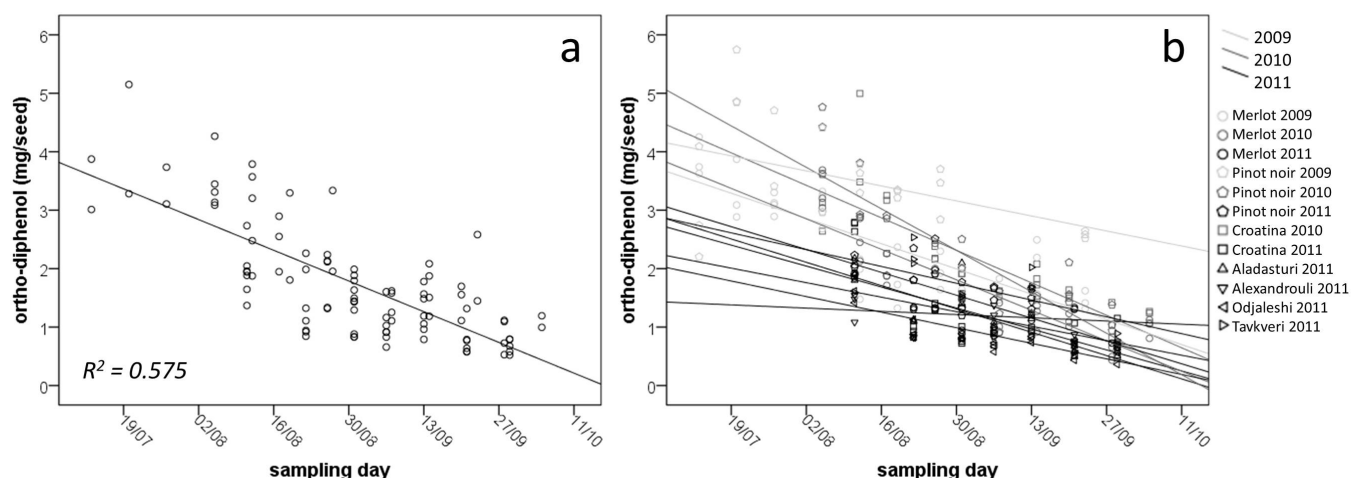


Figure 2: *Ortho*-diphenol concentration decrease during the growing season. In fig. 2a: each point represents the average value of the three biological replications. In fig. 2b each dataset (cultivar and year) is labeled by a different symbol. The obtained regression lines are classified depending on the sampling year (2009: light grey; 2010: dark grey; 2011: black).

However, in our knowledge, an “in deep” assay able to quantify tannin oxidation does not exist: via-radical polymerizations produce heterogeneous products due to the high reactivity of the instable reaction intermediates. *Ortho*-diphenol could be easily oxidized because the resulting phenoxyl semi quinone radical is stabilized by a second oxygen atom, while *meta*-diphenol are less susceptible to oxidation (WATERHOUSE and LAURIE, 2006). Thus, the catechol (*ortho*-diphenol) moieties are probably the most oxidizable groups in flavan-3-ols and proanthocyanidins. For that reason, it is possible to correlate the decrease in *ortho*-diphenol concentration to the oxidation processes characteristic of seed ripening. A number of studies have been carried out to clarify the role of anthocyanins-metal complexes on fruits and foods colors, and the importance of the *ortho*-diphenol substitutions are well known (KONDO *et al.*, 1992; BOULTON 2001). Of course, complex solutions such as grape and wine could encourage further studies considering multiway interactions (RUSTIONI, 2015). Nevertheless, in our knowledge, considering non pigmented phenolics, any paper reports interferences in the *ortho*-dihydroxylated quantification through Molybdenum complexation by copigments.

Fig. 3 reports the correlation between the *ortho*-diphenol quantification and the seed color level (RISTIC and ILAND, 2005). The data dispersion should be attributed to the fundamentally different approaches of the tested methods. Nevertheless, the trend clearly appears: a decrease in *ortho*-diphenol content corresponds to the browning process characteristic of seed ripening.

The majority of the tannin synthesis occur before veraison. However, winegrowers are aware of the quality impact produced by the phenolic evolution during fruit ripening. Traditionally they use a visual inspection of the seed browning as signal of the phenolic ripening status, which results from proanthocyanidin oxidation. Thus, the method proposed by RISTIC *et al.* (2005) improved this qualitative evaluation. However, this technique is limited by the color inhomogeneity and by the subjectivity of the records.

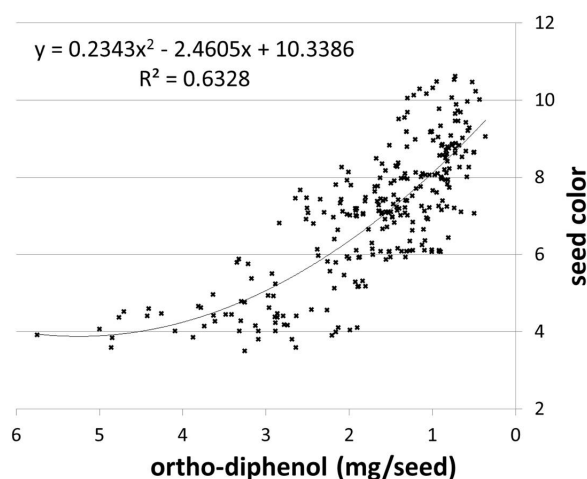


Figure 3: Correlation between *ortho*-diphenol concentration and seed color.

Thus, the aim of the present work is the production of an objective chemical index able to describe the tannin seed ripening. It does not require exclusive equipment and it is easy and fast to achieve. For these reasons we hope it will be a useful and practical support for the grape and wine industry. Moreover, this index could be adopted by researchers to characterize fruit quality in relation to treatments or vineyard managements.

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THE COMBINED EFFECT OF *THYMUS VULGARIS* EXTRACT AND PROBIOTIC BACTERIA (*LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM BIFIDUM*) ON AFLATOXIN M₁ CONCENTRATION IN KEFIR BEVERAGE

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ABSTRACT

The current study was conducted to evaluate the effect of different doses of *Thymus* extracts (0, 2, 4 and 6) gr/L and various values of *Lactobacillus acidophilus* and *bifidobacterium bifidum* on decreasing of fixed amount of aflatoxin in kefir beverage. The results showed that *Thyme* extracts reduced the amount of Aflatoxin M₁. *Thyme* extract in combination with probiotic bacteria decreased Aflatoxin M₁ more than *Thymus* alone. The most reduction of Aflatoxin M₁ levels was detected by using 4 gr/L *Thymus* extract and 1×10^8 *Lactobacillus acidophilus*. Moreover, results revealed that *Thymus* extracts and probiotic bacteria could reduce AFM₁ in kefir drink.

Keywords: aflatoxin M₁ reduction, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Thymus* extracts, Kefir

1. INTRODUCTION

Thymus is a small aromatic perennial herbaceous plant that cultivated in frequency due to their wide use in the food, cosmetic, and pharmaceutical industries (NABAVI *et al.*, 2015). The genus *Thymus* is a taxonomically complex group of aromatic plants traditionally used for medicinal purposes because of their antiseptic, antispasmodic and antitussive properties (PINA-VAZ *et al.*, 2004, NABAVI *et al.*, 2015). Several researchers demonstrated that extracts and essential oils of some *Thymus* spp. have antiviral, antibacterial and antifungal activities. (FAZELI, *et al.*, 2007, BEHNIA, 2008, SHARAFZADEH and ALIZADEH, 2012).

Probiotics are used in dairy products as well as in food supplements and in agriculture as feed additives because of their beneficial health effects (NAGPAL *et al.*, 2012). According to the currently international FAO/WHO definition (2001), probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Probiotics improve the health of both animals and humans through the improvement of its intestinal health by the regulation of microflora, stimulation and development of the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance and reducing the risk of certain other diseases (KUMAR *et al.*, 2011; NAGPAL *et al.*, 2012).

At present, probiotics are introduced as suitable replacing of antibiotics in order to confront with pathogens in animals and human being, and consumption of probiotic food products and medicines have considerable vogue. Several investigations suggested that using probiotic is associated with reducing the risk of antibiotic-associated diarrhea (32). The foods which contain probiotic bacteria are put in the group of special products, and according to the dairy products International Federation (IDF) recommendation, these probiotic products should have a minimum concentration of 10⁶ CFU/g probiotic bacteria and consumer a total of some 10⁸ to 10⁹ probiotic microorganisms should be consumed daily for the suitable probiotic effects (15).

The most commonly important probiotics belong to *Lactobacillus* and *Bifidobacterium* genus. There are many well-characterized strains of *Lactobacilli* and *Bifidobacteria* available for human use (HUSSAIN *et al.*, 2009; KECHAGIA *et al.*, 2012). Some of the beneficial properties of probiotics are anticarcinogenic, immunologic enhancement, antiatherogenic, cholesterol-lowering, anti-obesity and antidiabetic characteristics (NAGPAL *et al.*, 2012).

Kephir (kefir) is a viscous, highly acidic beverage produced from cow, goat, sheep or mare milks. The fermentation is initiated by “kefir grains” (clusters of yeast and bacteria), which are added to raw, pasteurized or UHT-treated milk (SARKAR, 2007; RIBEIRO and RIBEIRO, 2010). Kefir contains high contents of thiamine, riboflavin, pantothenic acid, vitamin C, protein and minerals; hence, kefir has both therapeutic and nutritional attributes (SARKAR, 2007). Kefir also has greater amounts of threonine, serine, alanine and lysine than milk (SARKAR, 2007). Kefir also has medical effects in order to treat hypertension, body skin fineness, stress and depression, cholesterolemia and arthritis (XIAO *et al.*, 2003; NINANE *et al.*, 2005).

Aflatoxins are naturally occurring mycotoxins that are produced by some species of fungi like *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin M₁ (AFM₁) is one of the metabolites of Aflatoxin B₁ that is excreted into milk when lactating animals are given feed with aflatoxin contaminated food (10). Contamination of milk or milk products with AFM₁ is considered as a potential risk for public health (11, IARC, 2002).

International Agency for Research on Cancer (IARC) classified AFM₁ as a group 2B agent (possibly carcinogenic to humans) and exposing to chronic aflatoxin causes acute liver problems, mutation and liver cancer (BEHFAR *et al.*, 2012). Aflatoxin M₁ is relatively stable during pasteurization, sterilization and preparation of dairy products (FALLAH,

2010). Therefore, industries sustain irretrievable economic losses, if it is not controlled, and its potential risks for human health especially for children (ELKHOURY *et al.*, 2011). The aim of this research was to produce kefir and reviewing *Thymus* extract effects in combined with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on the AFM₁ concentration in kefir drink.

2. MATERIALS AND METHODS

2.1. Chemicals and instrumentation

Lyophilized *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (CHR Hansen Company, Denmark) were used as probiotic bacteria. The pasteurized low fat milk (1.5% fat) and kefir grain (Iran) were used to produce kefir drink. *Thymus* extracts (Zard band Company, Iran) (were prepared at 3 different concentrations including 2, 4, 6 g/L. AFM₁ was procured from Merk Company, Iran) and 200 ppb concentration was used to add the kefir drink as follow explanation.

2.2. Producing kefir drink

In order to produce kefir drink, 1 gram kefir grain was added to 1 liter pasteurized low fat milk (1.5% fat), and this was incubated at 38°C and acidity measurements were performed at different times until reaching 42°C (Standard and industrial search of Iran). At least this product was kept in refrigerator at 4°C, subsequently (MOATTER and SHAMS KASHANI, 1378; Iran standard and industry research institute, 1385).

2.3. Adding aflatoxin M₁ and *Thymus* extracts to produced kefir

Ten mL of produced kefir was poured equally in 4 tubes and 10 mL of contaminated milk with 200 ppb of AFM₁ was added to each tube. Finally, different doses of *Thymus* extract (2, 4, 6 g/L) were added to the samples (OTLES and CAGINDI, 2003; MARHAMATIZADEH *et al.*, 2011; MARHAMATIZADEH *et al.*, 2012).

2.4. Adding aflatoxin M₁, *Thymus* extract and *Lactobacillus acidophilus* or *Bifidobacterium bifidum* to kefir

Four g/L fixed *Thymus* with 1×10^8 , 3×10^8 and 6×10^8 *Bifidobacterium bifidum* or *Lactobacillus acidophilus* were poured in 4 tubes and 10 mL of contaminated milk by 200 ppb of AFM₁ was added to them, subsequently (OTLES and CAGINDI, 2005; MARHAMATIZADEH *et al.*, 2011; MARHAMATIZADEH *et al.*, 2012). Finally, all samples were kept into the incubator for 24 hours at 24°C. After 24 hours, the coagulation was separated from liquid by using a cloth filter, and remained liquid was incubated at 14°C for 24 hours. It was kept for 48 hours in refrigerator at 4°C and all samples were analyzed with ELISA reader (Europroxima Company), subsequently.

2.5. ELISA test

Samples were centrifuged at 2,000 rpm for 10 min at 7°C and the upper oil layer was removed. Then, 100 mL of these samples were used for ELISA test. ELISA reader device in three repetitions and distinguished three optical densities for every sample was done. And every of three optical densities were put in Excel program (collected by Euro- Proxima

Company) and three concentrations were got that the average of these three shows AFM_i reminder in every samples.

2.6. Statistical analyses

The data was analyzed by using Kruskal-Wallis test in a meaningful surface at $P < 0.05$ by SPSS software (SPSS for Windows, version 20, SPSS Inc, Chicago, IL, USA).

3. RESULTS

3.1. Evaluating primary milk contamination with AFM_i

The results showed that primary milk contains 32 ppb AFM_i and then 200 ppb of AFM_i were added to milk that totally AFM_i becomes 232 ppb. Table 1 shows the changes of aflatoxin level in the present of *Thymus* extracts, *Bifidobacterium bifidum* and *Lactobacillus acidophilus* bacteria in various groups.

Table 1: Comparison of AFM_i concentration in the presence of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* bacteria and *Thyme* extracts.

Sample tubes	Added Aflatoxin (ppb) AFM _i value added in the form of (ppb)	Initial Aflatoxin (ppb) AFM _i value primary	The remaining amount of aflatoxin (ppb) AFM _i value	Reduced aflatoxin (ppb) AFM _i value Decreased (ppb)	Reduced aflatoxin (%) AFM _i value decreased (%)
40 g kefir	200	232	174.3	57.7	24.87
2 g/l <i>Thyme</i> extract	200	232	154.6	77.4	33.36
4 g/l <i>Thyme</i> extract	200	232	155.9	76.1	32.80
6 g/l <i>Thyme</i> extract	200	232	162	69.8	30
4 g <i>Thyme</i> extract and 1×10^8 <i>Bifidobacterium bifidum</i>	200	232	169.5	62.5	26.93
4 g/l <i>Thyme</i> extract and 3×10^8 <i>Bifidobacterium bifidum</i>	200	232	175.3	56.7	24.43
4 g/l <i>Thyme</i> extract and 6×10^8 <i>Bifidobacterium bifidum</i>	200	232	148.3	83.7	36.07
4 g/l <i>Thymus</i> extract and 1×10^8 <i>Lactobacillus acidophilus</i>	200	232	125.8	106.2	45.77
4 g/l <i>thymus</i> extract and 3×10^8 <i>Lactobacillus acidophilus</i>	200	232	186.1	45.9	19.78
4 g/l <i>thymus</i> extracts and 6×10^8 <i>Lactobacillus acidophilus</i>	200	232	169.1	63	27.15

3.2. Evaluating kefir containing *Thymus* in detecting AFM_i

The results indicated in Table 1 showed that *Thymus* extracts in kefir caused AFM_i reduction largely. The reduction percent of AFM_i in the kefir samples containing different concentrations of *Thymus* extracts 2, 4 and 6 gr/L were 33.36%, 32.80% and 30%,

respectively. The most reduction of AFM₁ was detected by 2 gr of *Thymus* and *Thymus* extract with 6 g/L concentration showed the least percent of AFM₁ reduction.

3.3. Evaluating probiotic kefir containing *Thymus* and *Lactobacillus acidophilus* in AFM₁ reduction

The data in Table 1 shows that fixed quantity of *Thymus* (4 gr/L) with different amount of *Lactobacillus acidophilus* declined Aflatoxin. Reduction of AFM₁ in the presence of *Thymus* extract and different value of *Lactobacillus acidophilus* 1×10^8 , 3×10^8 and 6×10^8 were 45.77%, 19.78% and 27.15, respectively. The most decrease of Aflatoxin was detected with 1×10^8 level of *Lactobacillus acidophilus* and the amount 3×10^8 of bacteria has the least reduction of aflatoxin.

3.4. Evaluating probiotic kefir containing *Thymus* and *Bifidobacterium bifidum* in deleting AFM₁

The results showed that *Bifidobacterium bifidum* bacteria decrease the amount of AFM₁ in the samples. The most decline percent of AFM₁ in the presence of 6×10^8 of *Bifidobacterium bifidum* occurred with 36.07%; and 1×10^8 and 3×10^8 of bacteria with 26.93% and 24.43%, respectively, had the least AFM₁ reduction.

4. CONCLUSIONS

The aim of the present study was the evaluation of the effect of *Thymus* extract and *Lactobacillus acidophilus* and *Bifidobacterium bifidum* bacteria on the reduction of Aflatoxin amount in kefir beverage. Furthermore, the possibility of producing a new probiotic food based on kefir and *Thymus* was assessed.

The results of present study indicated that *Thymus* extract has anti-aflatoxin activity and capability for aflatoxin reduction. The *Thymus* extract in combination with probiotic bacteria has the more ability for aflatoxin decline. The results of the current research showed that *Thymus* extract with 2 gr/L concentration in combination with 6×10^8 *Bifidobacterium bifidum* have the most effect in reduction of AFM₁ and the strongest anti-aflatoxin activity was shown by the *Thymus* extract with 4 gr/L concentration in combination with 1×10^8 *Lactobacillus acidophilus*. Our results are agreed with other studies (OTLES and CAGINDI, 2003; LEE *et al.*, 2003; TRATNIK *et al.*, 2006).

Probiotic foods have been produced in order to treat intestinal infections as well as genital diseases. In three decades ago, commercial probiotic products have been supplied to the world market grew. Kefir is a fermented dairy product which originates from the Caucasus Mountains in Eastern Europe (TRATNIK *et al.*, 2006). Kefir is the most popular probiotic product in Europe. Kefir has beneficial effects in healing and homeostasis due to its vitamins, minerals and essential amino acids (OTLES and CAGINDI, 2003). The vitamin content of kefir affects both type of milk and microbiological flora (SARKAR, 2007, ARSLAN 2014).

Aflatoxins are a group of fungal secondary metabolites which are recognized as being of economic and health importance (10). AFB₁ is currently of great interest due to their toxic, carcinogenic and mutagenic nature on human and animal health (11). A number of studies reported that many micro-organisms, including bacteria, yeasts, moulds, actinomycetes and algae are able to remove or degrade small amounts of aflatoxin in foods and feeds (LEE *et al.*, 2003).

Several lactic acid bacteria strains have shown different capabilities for binding AFM₁ in solutions and in milk (HASKARD, *et al.*, 2001). The various studies have reported that certain *Lactobacilli* and *Bifidobacteria* are capable of removing AFB₁ from liquid solutions by binding the toxin (PELTONEN *et al.*, 2001; HASKARD *et al.*, 2001). Some researchers have suggested that aflatoxin binds predominantly to polysaccharides and peptidoglycans of the bacterial cell wall (LAHTINEN *et al.*, 2004). LOPEZ *et al.*, (2003) showed that *Saccharomyces* yeast reduced 90% AFM₁ in the milk.

Powders and extracts of many herbs, plants and spices have been reported to inhibit the production of aflatoxin (PARANAGAMA *et al.*, 2003). A recent study showed that the essential oils of *T. daenensis* and *Thymus* spp. (Elam ecotype) flowers exhibited antibacterial activities against *L. monocytogenes* from chicken meat (GHASEMI PIRBALOUTI *et al.*, 2009). LIXANDRU *et al.*, (2010) evaluated antimicrobial activity of some plant essential oils against bacterial and fungal species. The results showed *Thymus*, *Coriander* and *Basil* oils proved the best antibacterial activity, while *Thymus* and *Spearmint* oils better inhibited the fungal species. *Thymus vulgaris* extracts with 0.01 and 1% concentration decreased the AFB₁ production by 83 and 91 % (FOUAD *et al.*, 2013). HAMZAWY *et al.*, (2012) reported that ethanolic and aqueous extracts of *Thymus vulgaris* has potential hepatorenoprotective effects against aflatoxins because of antioxidant properties and radical scavenging activity.

In conclusion, the results of the current study revealed that *Thymus* extracts and probiotic bacteria can reduce AFM₁ in kefir drink. Our results are agreed with other researches that showed probiotic bacteria and *Thymus* extract have anti-aflatoxin activity. Moreover; it may be stated that aflatoxins are not only a big problem at crop production level, but also it has become a global health topic due to the consequences following their consumption in animals and human being. So it is important to develop the useful protocols to eliminate aflatoxins from contaminated food.

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EFFECTS OF TOMATO POMACE SUPPLEMENTATION ON CHEMICAL AND NUTRITIONAL PROPERTIES OF CRACKERS

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ABSTRACT

Tomato paste waste materials are rich in bioactive food components, but have a low economic value. In this study, the potential use of tomato pomace in crackers was studied. Wheat flour was partially (4%, 8%, 12%) substituted with dried tomato pomace meal. Tomato pomace addition caused a significant ($p < 0.05$) increase in protein, ash, dietary fiber (soluble, insoluble, total), minerals (Mg, Ca, K, P, Mn, Zn, Fe), total phenolics, antioxidant capacity, Hunter *a* and *b* color values, but decrease in *L* value. Although the samples having 12% tomato pomace had lower scores, the crackers were liked statistically equally by the panelists.

Keywords: Cracker, tomato pomace, total phenolics, dietary fiber, minerals, sensory

1. INTRODUCTION

Tomato (*Lycopersicon esculentum* L) is the world's second largest vegetable crop (VALENCIA *et al.*, 2002). Worldwide, about 37 million tons of tomatoes are processed in the industry (KESKIN, 2012). A large part of the world tomato crop is processed into tomato paste, which is used as an ingredient in many products such as soups, sauces, and ketchup (SANCHEZ *et al.*, 2003). The problems of industrial wastes are becoming harder to solve, and much effort will be needed to develop the nutritional and industrial potential of by-products, waste and under-utilized agricultural products (AJAYI *et al.*, 2006). Tomato industry generates large amounts of by-products, and these by-products representing 10-30% of total processed tomatoes contain tomato seeds, peels, pulp and cores (RAHMATNEJAD *et al.*, 2009). Seeds and peels present in tomato pomace (TP) consist of the substances that are rich in nutritional value. It is underlined in some studies that they are rich in biologically active compounds, such as dietary fiber, protein, oil, mineral matters, phenolic compounds and carotenoids (EL-ADAWY and TAHA, 2001; SCHIEBER *et al.*, 2001; SOGI *et al.*, 2002; KNOBLICH *et al.*, 2005; CALVO *et al.*, 2008).

Crackers are popular snack foods in human diet (SEDEJ *et al.*, 2011). They are dry, thin and crisp bakery products and the low level of moisture, decreased even further with baking, left no medium for mold growth (HAN *et al.*, 2010). There are different types of crackers such as; saltiness cracker, soda cracker, sprayed cracker, cream cracker, savory cracker, matzos cracker, water cracker, Graham cracker, etc. (YONEYA and NIP, 2006). The basic ingredients in cracker production are wheat flour, fat (or shortening), salt, leavening agents (yeast, chemical leaveners, or combination), whey powder, sugar and/or glyucose syrup (YONEYA and NIP, 2006; GUNDOGDU SERTAKAN, 2006). Crackers are usually produced with soft white flour (KWEON *et al.*, 2014). But, the contents of some components in white flour, like amino acids (lysine, tryptophan) and dietary fiber that play an important role in nutrition, are low (ELGUN and ERTUGAY, 1995). Crackers could be an alternative food for the consumption of tomato pomace that is rich in biologically active compounds.

In the study, white wheat flour used in soda cracker production was substituted with tomato pomace meal to improve the nutritional and functional properties of crackers and it was aimed to determine the potential use of tomato paste waste material, usually utilized in animal feeding and rich in biologically active components, in human diet. The use of waste materials in human diet could also decrease environmental pollution problem

2. MATERIALS AND METHODS

2.1. Materials

Commercial wheat flour (Type 650), wheat starch, corn oil, sugar, salt and baking powder (sodium bicarbonate, sodium acid pyrophosphate) were purchased from local markets in Denizli, Turkey. Waste materials of tomato paste production were obtained from Honaz Paste Plant (Honaz, Denizli, Turkey).

2.2. Methods

2.2.1. Preparation of tomato pomace meals

Tomato pomace was dried in a cabinet dryer (Yücebaş Machine Analytical Equipment Industry, Izmir, Turkey). The dryer was consisted of a centrifugal fan to supply the

airflow, an electric heater, and an electronic proportional controller (ENDA, EUC442, Istanbul, Turkey). The air velocity was kept constant at 0.2 m s⁻¹ during drying at 60°C. The relative humidity of ambient air changed between 19 and 21%. After drying, pomaces were ground with a grinder (Tope TKS-16S, Izmir, Turkey) to a particle size of < 1000 µm.

2.2.2. Production of crackers

Soda crackers were prepared according to the procedure of HAN *et al.* (2010) with some modifications. Formulations presented in Table 1 were used to produce crackers. Crackers containing tomato pomace meals were prepared by substituting 4, 8 or 12% of flour in the formulae with dried tomato pomace. The preparation of crackers included steps of mixing of dry and liquid ingredients for 3-4 minutes to form a dough (KitchenAid, Artisan Series, Model 5KSM150, USA), resting of dough for 10 minutes, passing of dough through a set of smooth stainless steel rotating drums 3 times (KitchenAid, Artisan Series, Model 5KSM150, USA) by sheeting and laminating the dough, and cutting square shaped crackers from the dough sheet. Crackers were baked in an electric oven (Özköseoglu, Istanbul, Turkey) at 200°C for 10 minutes. After baking, crackers were left in the oven for an additional 2 minutes with the heat off but with forced air circulation. This process simulated the drying and cooling stages of a tunnel-type commercial baking oven. Baked crackers were then removed from the oven and allowed to cool down to room temperature.

Table 1: Soda cracker formulations.

Ingredients (g)	Control	TP4 ^a	TP8 ^b	TP12 ^c
Wheat flour	450.0	430.0	410.0	390.0
Dried tomato pomace meal	-	20.0	40.0	60.0
Wheat starch	50.0	50.0	50.0	50.0
Water	200.0	250.0	250.0	250.0
Corn oil	75.0	75.0	75.0	75.0
Sugar	17.5	17.5	17.5	17.5
Salt	5.5	5.5	5.5	5.5
Baking powder	5.0	5.0	5.0	5.0

^aTP4: 4% of wheat flour was substituted with dried tomato pomace powder, ^bTP8: 8% of wheat flour was substituted with dried tomato pomace powder, ^cTP12: 12% of wheat flour was substituted with dried tomato pomace powder.

2.2.3. Analytical measurements

Total solids, ash, and oil contents of the samples were determined according to the methods of AOAC (1990). Crackers were analyzed for their total protein contents by a Dumatherm nitrogen-determination system run under the combustion method (Gerhardt Analytical Systems, Dumatherm, Germany) (ANONYMOUS, 2011).

Dietary fiber contents were determined with the fiber assay kit (Megazyme K-TDFR, Wicklow, Ireland) according to the Mes-Tris AOAC method 991.43 (1995) and AACC method 32-07 (1995). Samples were first suspended in the Mes-Tris buffer and then,

digested by heat-stable α -amylase, protease, and amyloglucosidase to remove starch and protein. Insoluble dietary fiber was recovered from the enzyme digestate after filtration. Soluble dietary fiber in the filtrate was precipitated with ethanol and filtered. All dietary fiber fractions collected were dried at $103\pm 2^\circ\text{C}$ for a night. Total dietary fiber content was calculated as the sum of insoluble and soluble dietary fiber contents. All dietary fiber contents were corrected for residual protein, ash, and blank.

Inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer, Optima 8000, Massachusetts, USA) was used to determine the mineral elements (Mg, Ca, K, P, Mn, Zn and Fe) of wheat flour, tomato pomace and crackers. In the pretreatment stage, digestion of 1g sample was performed using a mixture of $\text{HNO}_3:\text{H}_2\text{O}_2$ (8:4) in a microwave oven (Milestone Start D, Sorisole, Italy) (GOPALANI *et al.*, 2007; Anonymous, 2016). In the first step of digestion, the samples were digested for 15 min until reaching 110°C , and in the second step they were kept at this temperature for 15 min. The optimal operation conditions for ICP-OES analysis of mineral matters were as follows: RF power, 1450 W; plasma gas (Ar) flow rate, 15 L/min; auxiliary gas (Ar) flow rate, 0.2 L/min; nebulizer flow rate, 0.7 L/min; sample flow rate, 1.5 mL/min; delay time, 15s. Sensitive wavelengths for mineral identification were obtained from the tables provided by the manufacturer (BOSS and FREDEEN, 2004).

For the extraction of phenolics, crackers were mixed with 70% (v/v) methanol with a 1:10 (w/v) ratio and homogenized (IKA-Ultra Turrax, Staufen, Germany). Then the mixture was treated in an ultrasonic water bath (Elma E 60 H, New Jersey, USA) for 10 minutes and shaken in a mechanical orbital shaker (WiseShake SHO-1D, Wertheim, Germany) for 15 minutes. Finally, the liquid extract was separated from solids by centrifugation (26,000g for 20 minutes at 4°C , Hettich, Universal 30 RF, Massachusetts, USA). The supernatant was recovered and the extraction step was duplicated for the precipitate. The supernatants of the two steps were mixed and stored at -24°C until total phenolic content and antioxidant activity analyses.

Folin-Ciocalteu method (SINGLETON *et al.*, 1999) was used to determine total phenolic content. 1 mL of extract was mixed with 5 mL of 1:10 (v/v) Folin-Ciocalteu reagent:water mixture for 5 minutes and 4 mL of 75g/L sodium carbonate (Na_2CO_3) was then added. After incubation at room temperature for 2 hours, the absorbance of the reaction mixture was measured by a spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments Ltd., Leicestershire, United Kingdom) at 760 nm. Gallic acid (0–100mg/L) was used as a standard to produce the calibration curve and the total phenolic content of extracts was expressed in mg of gallic acid equivalents (GAE)/100 g dry matter of wheat flour, tomato pomace meal and crackers.

DPPH assay was used to determine the antioxidant activity of extracts (THAIPONG *et al.*, 2006). The stock solution was prepared by dissolving 24 mg DPPH with 100mL methanol and then stored at -20°C until needed. The working solution was obtained by mixing 10mL stock solution with 45mL methanol to obtain an absorbance of 1.10 ± 0.02 units at 515 nm using the spectrophotometer. Cracker extracts (150 μL) were mixed with 2850 μL of the DPPH solution and allowed to react for 24 h in the dark. Then the absorbance was taken at 515 nm. The standard curve was linear between 25 and 800 μM Trolox. Results were expressed in μM TE/100 g dry mass. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.

2.2.4. Physical and sensory properties of crackers

Color values (Hunter L, a, b) of crackers were determined by a Hunter LabMini Scan XE model colorimeter (Reston, VA, USA) (ANONYMOUS, 1995).

In sensory evaluation, a panel of 48 subjects in the Department of Food Engineering (Pamukkale University, Denizli, Turkey) evaluated the sensory properties of soda crackers and assigned scores for color, smell, flavor, crispiness, and overall acceptability on a hedonic scale from 1 (dislike extremely) to 7 (like extremely). The panel consisted of students, staff and faculty members (30 females, 18 males), and 60% of the subjects were between 18 and 25, 27% between 26 to 40 years old, and 13% older than 40 years. The samples were labeled randomly with three-digit numerical codes. During the panel, subjects were instructed to rinse their mouths with water, and eat unsalted crackers before tasting each sample. The panel was performed in partitioned booths equipped with daylight.

2.2.5. Statistical analysis

“Minitab 13 Statistical Software” was used for the statistical analysis of data. ANOVA (one-way analysis of variance) with Tukey’s multiple comparison test was performed to determine significant differences at $\alpha=0.05$.

3. RESULTS AND DISCUSSION

Crude protein, crude oil, crude ash, soluble, insoluble and total dietary fiber contents, mineral matters (Mg, Ca, K, P, Mn, Zn, Fe) and total phenolic compounds contents, antioxidant activity values and color values of wheat flour and tomato pomace meal are given in Table 2. Results indicated that tomato pomace used in this study is a good source of crude protein, crude oil, dietary fiber, and minerals, and has higher total phenolic compounds and antioxidant capacity values than wheat flour. In the present work, soda cracker was supplemented with tomato pomace meal in order to enrich the nutritional status of the final product.

In previous studies, tomato pomace was reported to have a protein content of 16.27-19.65%, oil content of 5.85-10.75%, dietary fiber content of 54.79-59.03% and ash content of 3.472-4.046% (ALVARADO *et al.*, 2001; DEL VALLE *et al.*, 2006; ISIK, 2013). Our results were mostly similar to those reported in the literature. The small differences can be due to several factors including climate, geography, geochemistry, agricultural practices like fertilization and genetic composition (TOLEDO and BURLINGAME, 2006).

Tomato pomace powder had higher *a* and *b* values in color than wheat flour, which can be explained by higher carotenoid contents of tomato pomace, especially lycopene (SHARMA and LE MAGUER, 1996; SCHIEBER *et al.*, 2001; KNOBLICH *ET AL.*, 2005; SIKORA *et al.*, 2008). Lycopene is an important carotenoid which gives red color to tomato, and lycopene content of tomato peel is about 3025 $\mu\text{g}/100\text{ g}$ (SHARMA and LE MAGUER, 1996; SIKORA *et al.*, 2008).

3.1. Effect of tomato pomace addition on the proximate chemical composition of crackers

Ash, crude protein, soluble, insoluble and total dietary fiber, and mineral contents of soda crackers increased significantly ($p < 0.05$) with tomato pomace powder addition (Table 3). The reason is most likely that tomato pomace meal, which substituted wheat flour in cracker production, had higher crude protein, dietary fiber, crude ash and mineral contents than wheat flour (Table 2).

Table 2: Chemical and nutritional properties of wheat flour and tomato pomace meal.

Parameter	Wheat flour ^a	Tomato pomace meal
Crude protein (%)	10.87	16.31
Crude oil (%)	1.66	5.38
Total dietary fiber (%)	2.89	59.94
Soluble dietary fiber (%)	1.39	4.91
Insoluble dietary fiber (%)	1.50	55.03
Crude ash (%)	0.480	3.492
Mg (ppm)	398.3	2850.6
Ca (ppm)	380.4	3625.5
K (ppm)	1950.4	24500.3
P (ppm)	1403.2	4625.1
Mn (ppm)	9.5	40.1
Zn (ppm)	12.5	41.5
Fe (ppm)	19.1	130.5
Total phenolic compounds (mg GAE/100g)	104.12	427.81
Total antioxidant activity (μ mol TE/100g)	2.364	80.34
Hunter color values		
L	94.43	54.95
a	0.44	16.12
b	9.20	19.65

^aAll values are in dry basis.

A generous intake of dietary fiber may help to reduce risk for developing diseases such as coronary heart disease, stroke, hypertension, diabetes, obesity and certain gastrointestinal disorders such as constipation, diverticulitis and large bowel cancers (ANDERSON *et al.*, 2016; MUDGIL and BARAK, 2013). Dietary fiber intake recommendations for adults generally fall in the range of 20 to 35 g/day or 10 to 13 g per 1,000 kcal energy intake (MARLETT *et al.*, 2002). In this case, by the consumption of 100g of control, TP4, TP8 and TP12 crackers, an adult can take about 6.2%, 17.7%, 22.3% and 28.5% of his daily recommended dietary fiber intake, respectively. Crackers containing tomato pomace powder at different ratios provide significantly higher dietary fiber intakes than control cracker for the consumers.

Minerals are essential for a wide variety of metabolic and physiologic processes in human body. They are useful for many actions in the body like muscle contraction, normal heart rhythm, nerve impulse conduction, oxygen transport, oxidative phosphorylation, enzyme activation, immune functions, antioxidant activity, bone health, and acid base balance of the blood (WILLIAMS, 2005; SALDAMLı and SAĞLAM, 2007; LAKSHMI, 2014). An adequate daily amount of minerals is necessary for optimal functioning.

Table 3: Chemical and nutritional properties of crackers supplemented with various amounts of tomato pomace powder.

Parameter	C ^{a,b}	TP4	TP8	TP12
Crude protein (Nx5.7) (%)	7.35±0.16c	7.63±0.08b	7.80±0.01ab	7.82±0.04a
Crude oil (%)	16.50±1.15a	16.75±0.29a	17.62±0.88a	18.06±0.97a
Total dietary fiber (%)	1.86±0.29d	5.30±0.55c	6.68±0.43b	8.54±0.58a
Soluble dietary fiber (%)	1.02±0.49b	1.82±0.19ab	2.01±0.22a	2.50±0.55a
Insoluble dietary fiber (%)	0.84±0.21d	3.48±0.41c	4.67±0.30b	6.04±0.42a
Crude ash (%)	1.269±0.192b	1.520±0.151ab	1.554±0.109ab	1.693±0.120a
Mg (ppm)	185.0±39.6b	283.9±54.6ab	306.1±21.4ab	400.7±114.7a
Ca (ppm)	213.9±15.9c	356.2±15.0b	493.9±8.3a	569.9±89.4a
K (ppm)	1491.1±109.2d	2108.9±41.9c	2738.7±140.5b	3090.0±132.7a
P (ppm)	1871.8±164.6b	2123.1±157.1ab	2250.2±252.8ab	2314.2±147.2a
Mn (ppm)	9.7±1.7b	12.3±2.4b	14.4±1.6ab	23.0±8.5a
Zn (ppm)	8.8±2.6b	11.0±3.1b	17.8±2.5a	22.6±2.9a
Fe (ppm)	8.9±2.4c	12.6±2.5bc	15.8±1.0b	22.1±4.3a
Total phenolic compounds (mg GAE/ 100g)	52.52±6.50d	68.59±3.67c	104.402±6.69b	127.585±9.26a
Total antioxidant activity (μmol TE/ 100g)	7.20±0.97c	7.67±2.12c	13.23±2.11b	18.11±1.59a
Hunter color values				
L	68.55±1.82a	63.04±0.26b	57.17±0.40c	53.06±1.14d
a	4.15±1.06d	7.28±0.27c	9.67±0.20b	10.87±0.20a
b	21.10±0.90b	22.39±0.15a	23.08±0.12a	22.82±0.45a

^aAll values are in dry basis;

^b Different letters within the row across the table show significant differences at $\alpha=0.05$.

In this study, addition of tomato pomace meal to cracker formulation caused increases ($p<0.05$) in mineral contents (Mg, Ca, K, P, Mn, Zn and Fe). Substitution of wheat flour by tomato pomace powder in crackers at a level of 12% increased mineral levels in crackers between a ratio of 23.6% (for P) and 166.4% (for Ca). According to our calculations, an adult can take about 15.45% of K, 5.70% of Ca, 10.83% of Mg, 28.93% of P, all of Mn, 22.60% of Zn and 24.55% of Fe daily requirements by the consumption of 100g of TP12 cracker. On the other hand, he or she can take about 7.45% of K, 2.14% of Ca, 5.00% of Mg, 23.39% of P, 44.09% of Mn, 8.80% of Zn and 9.88% of Fe daily requirements by the consumption of 100g of control cracker (BAYSAL, 2007; SALDAMLI and SAĞLAM, 2007).

3.2. Effect of tomato pomace addition on total phenolic compounds and total antioxidant activity values in crackers

The results in Table 3 show that the substitution of wheat flour by tomato pomace meal powder increased the total phenolics contents and antioxidant activity values of crackers. Cracker samples having all levels of tomato pomace powder had significantly ($p<0.05$)

higher total phenolics contents than control crackers. Crackers having 8 and 12% of tomato pomace powder had significantly ($p<0.05$) higher antioxidant activity values than control crackers. The reason for these results is most likely that the tomato pomace meal had a higher total phenolics content and antioxidant activity value than wheat flour (Table 2). Indeed, tomato skins and seeds, which are the main portion of tomato pomace, include polyphenolic compounds primarily the quercetin, rutin, chlorogenic acid, naringenin and kaempferol (VERHOEYEN *et al.*, 2002; SIKORA *et al.*, 2008; NAVARRO-GONZALEZ *et al.*, 2011; KAMILOGLU *et al.*, 2013). Additionally, tomato skin, which presents the important portion of tomato pomace, is a rich source of lycopene (SHARMA and LE MAGUER, 1996; SCHIEBER *et al.*, 2001; KNOBLICH *et al.*, 2005) and lycopene is a carotenoid which has the highest antioxidant activity in common carotenoids (ASICIOGLU, 2005; KAMILOGLU *et al.*, 2013). Lycopene is the most abundant carotenoid in tomatoes, accounting about 83% of the total pigments present, and is responsible for the bright red color of tomatoes (KAMILOGLU *et al.*, 2014). Tomato skin and seeds also contain other components which have high antioxidant activity primarily β -carotene as a carotenoid and vitamin C (KNOBLICH *et al.*, 2005; SIKORA *et al.*, 2008; STRATI and OREOPOULOU, 2011).

3.3. Effect of tomato pomace addition on physical and sensory properties of crackers

Addition of tomato pomace powder had a decreasing effect on L color value, and increasing effect on a and b color values (Table 2). These are mostly due to their higher a and b, and lower L values of tomato pomace than wheat flour (Table 2). These higher a and b color values of TP4 and TP8 were also liked more by the panelists in sensory evaluation (Table 4).

Sensory evaluation results of soda crackers are presented in Table 4. Control crackers and crackers substituted with tomato pomace received similar ($p>0.05$) scores in color, smell, flavor, crispiness and overall acceptability. Although increasing the substitution level of tomato pomace powder to 12% caused some reductions in the scores, this decrease was statistically insignificant.

In the sensory evaluation, a number of panelists reported that TP12 crackers had a little bitterness taste (data not shown). It's thought that this was most likely from a bitter component, named TFI, presented in tomato seeds. TFI is a furostanol saponin and it's chemical structure was established as 5 α -furostane-3 β ,22,26-triol-3-[O- β -D-glucopyranosyl (1 \rightarrow 2)- β -D-glucopyranosyl (1 \rightarrow 4)- β -D-galactopyranoside] 26-O- β -D-glucopyranoside by SATO and SAKAMURA (1973).

Table 4: Results of sensory evaluation.

Cracker sample	Color	Smell	Flavor	Crispiness	Overall Acceptability
Control	4.48 \pm 0.60	4.88 \pm 0.48	4.71 \pm 0.25	4.81 \pm 0.24	4.88 \pm 0.24
TP4	5.17 \pm 0.23	4.92 \pm 0.29	4.71 \pm 0.48	4.65 \pm 0.30	4.92 \pm 0.16
TP8	4.79 \pm 0.91	4.77 \pm 0.58	4.50 \pm 0.57	4.73 \pm 0.27	4.69 \pm 0.24
TP12	4.15 \pm 0.59	4.77 \pm 0.31	4.38 \pm 0.25	4.56 \pm 0.12	4.54 \pm 0.53

4. CONCLUSIONS

Byproducts of tomato processing industries have been known as a good source of biologically active food components, but have a low economical value. In this research, potential use of tomato pomace powder in human diet was studied and successful results were obtained. Tomato pomace powder addition increased the crude protein, soluble, insoluble and total dietary fibers, mineral, total phenolic contents and total antioxidant capacity of crackers. Crackers having tomato pomace powder had higher a and b color values than control, and colors of TP4 and TP8 had higher scores in sensory analysis in spite of the difference with others was insignificant ($p>0.05$). Panelists liked crackers equally in terms of color, smell, flavor, crispiness, and overall acceptability. But results of sensory evaluation indicated that substitution of wheat flour higher than 12% by tomato pomace powder in the production of soda crackers is not recommended.

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STATUS OF BENZOIC ACID AMOUNT DURING PROCESSING FROM YOGHURT TO ITS BY-PRODUCT DRINK (DOOGH)

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ABSTRACT

The addition of benzoic acid is forbidden in by-product drink of yoghurt "Doogh" in Iran. However, this preservative can be naturally found in milk and its products. A total of 24 and 48 samples of yoghurt and Doogh were analyzed by HPLC method to assess the natural and permitted amount of benzoic acid. All samples of yoghurt and Doogh contained benzoic acid in mean amount of 1.5-5.0 mg/kg and 0.8-4.7 mg/L, respectively. These findings showed that the amount of 6 mg/L can be defined for benzoic acid as admissible limit for Doogh in Iran.

Keywords: benzoic acid, yoghurt, Doogh, admissible limit, HPLC, Iran

1. INTRODUCTION

Doogh is a traditional fermented drink which is widely used in Asia and produced from salt, set yoghurt and water (ZAMANI MAZDEH *et al.*, 2014). This product is known with different names like "Ayran" in Turkey and "Lassi" in India (YILDIZ *et al.*, 2012; HINGMIRE *et al.*, 2009). Similar to other acidified drinks, one of the main problems with Doogh is microbial contamination leading to the spoilage and safety reduction of this product (ELVIRA *et al.*, 2014; SOSPEDRA *et al.*, 2012). For this purpose, the antimicrobial preservatives such as benzoic acid and/or its salts are usually added to Doogh to impart many advantages including increasing their shelf life, but the unpleasant effects of these additives have been reported in some surveys (ZENGIN *et al.*, 2011; KAMANKESH *et al.*, 2013; ZAMANI MAZDEH *et al.*, 2014). The use of chemical additives in different countries is restricted by the specific policies adopted. In Iran, the scientific panel on the food additives in "Food and Drug Administration: FDA" and "Institute of Standards and Industrial Research of Iran: ISIRI" has prohibited the use of benzoic acid in the dairy products. The existence of benzoic acid in those products leads to fine payment by the producers and suspension of the production licenses (ISIRI, 2008). Recent evidences suggest the natural occurring of benzoic acid in yoghurt and its transfer to diluted and salted formulation prepared thereof (Doogh) (ESFANDIARI *et al.*, 2013; ZAMANI MAZDEH *et al.*, 2014; AMIRPOUR *et al.*, 2015). This can cause the misinterpretation of the inspected results regarding the existence of the natural or added benzoic acid to Doogh. In fact, the use of benzoic acid is not allowed in the dairy products in Iran even though the presence of the naturally-occurring benzoic acid in the fermented dairy products has been reported by many workers (SIEBER *et al.*, 1995; IAMMARINO *et al.*, 2011; HORNICKOVA *et al.*, 2014). The source of benzoic acid can be hippuric acid as the natural compound in milk that changes to benzoic acid through the fermentation of the lactic acid bacteria in yoghurt (SIEBER *et al.*, 1999). To author's knowledge, there have been no controlled study which determines the natural amount of benzoic acid in Doogh. Therefore, this project was undertaken to assess the level of benzoic acid as the natural and permitted value in Doogh. These findings could help FDA and ISIRI to reassess the status of the enacted rules for benzoic acid in Doogh and resolve the legislative issue in Iran.

2. MATERIALS AND METHODS

2.1. Samples

Set yoghurt samples (n=24) were taken from three commercial brands of the dairy processing plants (A, B and C; for each 4 samples) and small scale brands (D, E and F; for each 4 samples) with high sale in Isfahan, Iran. A total of 12 Doogh samples were manually prepared by mixing 0.7, 40 and 59.3 g of salt (NaCl), yoghurt sampled in the previous stage (for each brand 2 samples) and water, respectively. A sum of 36 Doogh samples from three aforementioned brands (A, B and C; for each 12 samples) were purchased from the local food stores located in Isfahan, Iran. All samples were kept at 4° C before the examination would be begun. The analysis was performed in two replicates to determine the mean of the measurements for benzoic acid.

2.2. Instrumentation and quantification

A Hewlett–Packard 1090-II liquid chromatograph (now Agilent, Waldbronn, Germany) equipped with a diode array detector was used. The system was equipped with a

Rheodyne 7725i injector with a 20- μ L loop. The separations were carried out at the room temperature on a 3- μ m, 150 \times 3 mm I.D. Hecor-A C18 reversed-phase column (RStech Co., South Korea) was preceded by a guard column (4 \times 4 mm, 5 μ m) of the same packing material from Merck (Darmstadt, Germany). The separation was performed isocratically using a mobile phase consisting of HPLC grade methanol (40%, v/v) and 0.25 mM ammonium acetate aqueous solution (60%, v/v at pH = 4.5). The mobile phase flow rate was 0.4 mL/min and the detection was performed at 230 nm.

The stock standard solution of benzoic acid (99%, Merck) was prepared in methanol at 1000 mg/L concentration level. More diluted standard solutions were prepared in the pure water at the concentrations ranging from 1 to 50 mg/L. Pure water was prepared by Overseas Equipment & Services water purification system (OK, USA).

Estimation of benzoic acid amount was performed according to a previous reported method (GUARINO *et al.*, 2011). A 2-g yoghurt sample was mixed with 5 mL methanol/water (35/65, v/v). For Doogh analysis, 3 mL of the sample was mixed with 2 mL methanol. The mixture was ultrasonicated for 5 min. It was then heated at 50 °C for 5 min and centrifuged in 2000 rpm (5 min). The clear supernatant solution was injected into HPLC.

3. RESULTS AND DISCUSSIONS

The results corresponding to benzoic acid level in the industrial (A, B and C) and small scale brands (D, E and F) yoghurt samples are listed in Table 1. The mean values of benzoic acid were in the range of 1.5-2.9 and 3.6-5.0 mg/kg in the samples collected from three yoghurt processing plants and small scale brands, respectively. Our findings imply to the presence of benzoic acid in all samples of yoghurt. The surveys on the presence of benzoic acid in all yoghurt samples in various studies, performed by MIHYAR *et al.* (1999), EL ZEINY (2009), YILDIZ *et al.* (2012), CAKIR and CAGRI-MEHMETOGLU (2013) and AMIRPOUR *et al.* (2015), revealed that the ranges found were higher than those described in the current study.

MIHYAR *et al.* (1999) showed that the content of benzoic acid was in the range of 10.6 to 1998.8 mg/Kg of Labaneh yoghurt in Amman. EL-ZEINY (2009) also pointed out high concentration level of benzoic acid in the amount of 921 mg/Kg in yoghurt ice dressing in Saudi Arabia. Furthermore, the amount of benzoic acid is stated from 9.36 to 26.21 in all 25 yoghurt samples gathered from five cities in Turkey (YILDIZ *et al.*, 2012). In another study in Turkey, a mean of 35.2 mg/kg of benzoic acid found in all 21 yoghurt (CAKIR and CAGRI-MEHMETOGLU, 2013). In a recent experiment by AMIRPOUR *et al.*, (2015) in Iran, the mean amount of 29.3 \pm 8.6 mg/kg of benzoic acid detected in all yoghurt samples collected from four different brands of the dairy processing plants.

In contrast, the detection of benzoic acid was reported in 80% of yoghurt samples in Turkey (KOYUNCU and UYLASER, 2009). Additionally, Benzoic acid was not observed in yoghurt samples examined in Spain, Brazil and China (GONZALEZ *et al.*, 1998; TFOUNI and TOLEDO, 2002; WANG *et al.*, 2006).

Table 1 illustrates the benzoic acid value in the manually manufactured Doogh. Benzoic acid was found in the range of 0.8-2.2 and 2.6-4.0 mg/kg in Doogh made from the yoghurt collected from the dairy processing plants and small scale brands, respectively. Since Doogh is a diluted and salted formulation prepared from yoghurt, processing yoghurt to Doogh leads to a decrease in the benzoic acid content with amount of around 60-75%

As table 1 shows the amount of benzoic acid was higher in yoghurt and Doogh samples collected from the small scale brands. A probable explanation could be related to the lack

of controlling the quality criteria in the laboratory and proper hygiene practices as observed by the researchers.

Table 1: Mean of benzoic acid concentration (mg/kg) in yoghurt and manually manufactured Doogh (mg/L).

Sample Brand	Yoghurt ^a (Mean±SD)	Manually manufactured Doogh ^b (Mean±SD)
A	2.9±0.13	2.2±0.34
B	1.7±0.14	1.2±0.02
C	1.5±0.10	0.80±0.10
D	4.0±0.39	2.6±0.34
E	3.6±0.12	2.6±0.21
F	5.0±0.21	4.0±0.25

^aStandard deviation calculated for 4 samples of yoghurt.

^bStandard deviation calculated for 2 samples of manually manufactured Doogh.

Benzoic acid concentrations quantified in the industrially made Doogh are revealed in Table 2. The results demonstrated all samples presented benzoic acid with the levels ranging between 3 and 5.6 mg/L. The presence of benzoic acid in our study was near agreement with (YILDIZ *et al.*, 2012 and ESFANDIARI *et al.*, 2013) that reported the content of benzoic acid in Ayran and Doogh samples in Turkey and Iran ranged from 1.54 to 16.57 and 0.94 to 9.77 mg/ L, respectively. AKBARI-ADERGANI *et al.* (2013) and ZAMANI MAZDEH *et al.*, (2014) pointed out the high concentration of benzoic acid with the mean of 195.9 and 21.3 in 27 and 130 Doogh samples in Iran. In similar study in Iran, benzoic acid content in Doogh samples purchased from four dairy brands including A, B, C and D were 22.2, 20.0, 19.4 and 21.5 mg/kg, respectively (AMIRPOUR *et al.*, 2015).

Table 2: Benzoic acid (mg/ L) level in the investigated Doogh.

Sample brand	Concentration range	Mean±SD (n=12)
A	3.9-4.2	4.0±0.1
B	3.0-5.2	4.3±0.7
C	4.0-5.6	4.7±0.5

The amount of benzoic acid in the industrially manufactured Doogh was higher than manually ones. Whereas manually manufactured Doogh was prepared under the laboratory conditions and the industrially manufactured Doogh was purchased from the local stores, it seems that the storage, handling and hygiene conditions in the dairy processing plants is plausible justification for this status. Therefore, this part of the food chain requires continuous monitoring for good hygienic practice in the milk processing (Smith, 2003).

Totally, the reason for the difference in the amount of benzoic acid in yoghurt and Doogh of the present study and others is not clear but it may be attributed to the several factors including the feed of the milk- producing animal, the season of milking, the breeding

conditions, the content of hippuric acid in the raw milk, samples size, type of commercial lactic acid bacteria starter, processing technique, storage condition and type of yoghurt analyzed as mentioned in other studies (SIEBER *et al.*, 1999; GARMIENE *et al.*, 2008; QI *et al.*, 2009; HORNICKOVA *et al.*, 2014; JAVANMARDI *et al.*, 2015).

The low level of benzoic acid found in the manually and industrially manufactured Doogh indicates that this compound is as the indigenous constituent transferring from yoghurt to Doogh. Regarding with the codex standard act, the permitted amounts of benzoic acid as preservative used in the fermented milk drink is 300 mg/kg (Codex, 2003). Therefore the amount of benzoic acid detected in present study should not affect the public health. To sum up, the permissible amount of benzoic acid could define less than 6 mg/L in Doogh. Below this value, samples can be considered "acceptable" in Iran because such a low concentration could originate from the natural endogenous formation of benzoic acid and not from the fraudulent addition.

4. CONCLUSIONS

An investigation of 72 yoghurt and Doogh samples was carried out to define the natural occurring amount of benzoic acid. In the current study, it was found that all yoghurt and Doogh analyzed contained benzoic acid in low level at less than 6 mg/L. This amount can be a permitted amount without having any harmful effect on the human health and considered as the admissible level for benzoic acid in Doogh in the national act by Iranian supervision authorities. Moreover, it is suggested further studies on the continuous monitoring and the measuring of benzoic acid content in milk and its products to declare the amount of this compound on the labels of packaging. The inserted data on the labels of packaging can be useful for the exposure estimation of the consumers with this compound.

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