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OPINION PAPER

INSOLUBLE TOMATO-FIBER EFFECT ON WHEAT DOUGH RHEOLOGY AND COOKIES' QUALITY

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ABSTRACT

Commercial insoluble tomato fiber (ITF) was incorporated in wheat-flour dough to prepare cookies at amounts of 2.50, 5, 7.50, and 10 %. It was demonstrated that all wheat dough samples exhibited non-Newtonian-thixotropic behaviors at shear rates from 0.001 to 1000 s-1. Besides, the oscillatory rheology analysis confirmed that the storage modulus predominated the loss one in the whole frequency range and increased significantly with the increase in the ITF concentration. Actually, the latter's incorporation from 0 to 10 % increased Farinograph water absorption, pasting temperature and peak consistency, and decreased dough stability, amylograph pasting viscosities and fermentation parameters of all tested wheat flours. Furthermore, the ITF addition was proven to affect the formed cookies, indicating a significant increase in the samples' breaking strength and decrease in their spread ratio. The total polyphenol contents of the formed cookies ranged from 86.98mgGAE/ 100 g to 376.02 GAE/g cookies. The ITF incorporation increased the antioxidant activities as measured by DPPH, ABTS and FRAP scavenging activities. Correlations between the analyzed parameters of the cookies' color and IC50 are statistically significant ($p< 0.01$), suggesting the possible use of ITF as an alternative source of bioactive compounds to improve the cookies' quality.

Keywords: Insoluble tomato fiber, wheat flour, rheological properties, cookies, antioxidant activities, quality characteristics

1. INTRODUCTION

Every year, millions of tons of tomatoes are processed with a residue waste being considered as a good source for food supplements, such as insoluble dietary fibers (IDF), fats, proteins and bioactive compounds, namely lycopene and polyphenols (KAUR *et al*., 2008). IDF from by-products, whose chemical constituents are chiefly non-starch polysaccharides, namely cellulose, arabinoxylans and β-glucan, is not commonly incorporated in food products due to its adverse effects on food quality, like sensory effects and functionality (AHMED *et al*., 2013).

Nowadays, many research works have revealed that IDF is beneficial to human health. Indeed, the ingestion of insoluble fiber from fruits and vegetables could significantly reduce the plasmatic concentration of cholesterol, implying a decrease in the risk of cardiovascular disease, colon cancer, diabetes and obesity (SLAVIN, 2013). The European Prospective Investigation into Cancer and Nutrition (EPIC) has shown 40% reduction of colorectal cancer risk when consuming more than 30 g of fiber per day (SUMCZYNSKI *et al*., 2015). Actually, the Food and Nutrition Board recommends 38 g/day for dietary fiber intake (SUMCZYNSKI *et al*., 2015).

Tomato fiber is a by-product of tomato processing industry with high content of IDF (71.82 %), soluble dietary fiber (14.33 %), protein (13.30 %), lipid (6.01 %) and ash (3.01) (NAVARRO-GONZALEZ *et al*., 2011). Some sugars, as glucose, xylose and galactose are present in tomato residue fiber (GARCIA-HERRERA *et al*., 2010). The latter is reported to comprise mainly cellulose (75%), hemicelluloses (15%) and pectin (10%) (HUA *et al*., 2017). Since it is rich in bioactive compounds, namely polyphenols and lycopene, it could be used in the development of functional food formulations (NAVARRO-GONZALEZ *et al*., 2011).

Cookies and biscuits can be supplemented with dietary fibers from various sources, wheat bran, inulin carob fiber and many other biopolymers such as galactomannans, pectins and β-glucan (MILDNER-SZKUDLARZ *et al*., 2013). The incorporation of dietary fiber into wheat flour interacts directly with the structural elements of three dimensional gluten networks, disrupts the starch gluten matrix and finally changes the mechanical properties of blended dough during mixing, fermentation and baking (LIU *et al*., 2017; MARTINEZ *et al*., 2014; AHMED *et al*., 2013).

Since there is no published data on cookies formulations containing ITF, to our knowledge, this study is the first to examine the effect of ITF incorporation on both physicochemical and rheological wheat dough, and the quality characteristics of the formulated cookies. The obtained results would contribute to better valorize the ITF in the cereal-based foods and support product authenticity.

2. MATERIALS AND METHODS

2.1. Materials

The samples of commercial wheat flour produced by "Minoterie Soukra de Tunis", Tunisia" were studied for proximate composition. ITF was supplied by "Conservas Vegetales de Extremadura" (CONESA), Extremadura, Spain, and packaged in vacuum bags until the samples were opened for analysis. The ITF proximate composition: water content 6.22 g/100 g, protein 0.93 g/100 g, dietary fiber 91.27 g/100 g, lipid 1.01 g/100 g, ash 0.57 g/100 g), ITF 79.82 g/100g and soluble dietary fiber 11.45 g/100g. Wheat dough samples were packed in low-density polyethylene bags, and then stored for analysis at different ITF concentrations.

2.2. Proximate composition analyses

Water, protein, lipid and ash contents were determined according to the approved AOAC method (AOAC, 1990). Dietary soluble and insoluble fiber contents were identified according to the method of PROSKY *et al*. (1998). Total carbohydrate content was estimated by mean-value difference: 100− (% water +% protein+% ash + % lipid+% total fibres).
The assessment of wet and dry gluten contents, and gluten index was performed by Glutomatic (Perten Instruments, Hägersten, Sweden) according to the AACC method (AACC, 2000). Wheat flour was substituted by ITF at different concentrations (g/100 g) to make a dough blend. Both wheat flour and ITF was premixed in dry condition using a mixer with a spiral blade, typically used for dough mixing. The wheat dough samples were prepared by mixing different blends in Farinograph at a consistency of 500 UB at 30°C. The wheat flour sample without TIF was considered as control.

2.3. Rheological properties of wheat dough

2.3.1 Rheological measurements

The rheological properties of prepared dough samples were determined using a strain/stress controlled Rheometer (Thermo-Haake, Rheostress 1, Germany) equipped with a temperature-control unit (Thermo-Haake, Karlsruhe K15 Germany). The Rheometer had a cone-plate configuration with a 35-mm cone radius and a 0.14-mm gap between the cone and plate. Measurements were conducted in the shear rate range of 0.001 to 1000 s^{-1} at constant temperature (20°C). Twenty-five data points were recorded at 10 s intervals during the shearing. Each measurement was replicated seven times on the same sample with two repetitions. Experimental data were fitted to Herschel-Bulkley and power-law (Ostwald) equations (1) and (2), respectively:

$$\sigma = \sigma_0 + K\dot{\gamma}^n \quad (1)$$

$$\sigma = K\dot{\gamma}^n \quad (2)$$

Where σ is the shear stress (Pa), K is the consistency coefficient (Pa.s^n), $\dot{\gamma}$ is the shear rate (s^{-1}), σ_0 is the yield stress and n is the flow behavior index (dimensionless).
The thixotropic hysteresis loop area was computed as the difference between the area under the up-flow and the down-flow curve using RheoWin v.2.93 (Haake, Germany) software.

2.3.2 Dough viscoelasticity

Using a parallel plate system (4 cm dia.) at a 500-mm gap, the dynamic rheological measurements were conducted with a Rheometer (AR 1000, TA Instruments, New Castle, DE, USA). Dynamic shear data were obtained from frequency sweeps over the range of 0.1-100 rad/s. The strain value was obtained in the linear viscoelastic region at 1.5 % strain, and the frequency-sweep tests were performed at 20°C. To obtain the experimental data and to calculate the elastic (G') and viscous moduli (G''), data analysis software (version VI. 1.76) was used. Aiming to relax the samples before taking the dynamic shear rheological measurements, all samples were allowed a5-min rest at the initial temperatures. These rheological measurements were performed in triplicate.

2.3.3 Farinograph, alveograph and visco-amylograph tests

The ITF effect on dough rheology was determined by Farinograph alveograph and visco-amylograph tests according to AACC method (AACC, 2000).

2.4. Fermentation parameter determination

Fermentation parameters were assessed by the Rheofermentometer F3 (Tripette and Renaud, France) according to the supplier's specifications. The fermentation parameters of dough development were determined as follows: maximum dough fermentation height (H_m) and the time at which dough reaches maximum height (T_1). The measured gas parameters were the volume of gas produced throughout fermentation (V_T), the gas retained in the dough at the end of the assay (V_R), and the loss volume of gas (V_L) in both milliliters (mL). All assays were performed in triplicate, and the average values were adopted.

2.5. Formulation of cookies

2.5.1 Cookie preparation

Cookies were prepared from the wheat flour (WF) and other ingredients such as shortenings, sugar, salt and sodium bicarbonate. Composite flours, ITF and other dry ingredients (sodium bicarbonate, sugar and salt) were mingled together in a bowl and shortenings were added, then mixed in a Hobart mixer for 6 min to obtain creamy dough. The specified amount of water was added gradually during continuous mixing until slightly firm dough was obtained. A hundred of baked cookies each weighing approximately 13.4 g were obtained for each recipe. Kneaded dough was manually rolled into sheets of required thickness and cut into round shapes, using a 5-cm diameter and 1-cm high biscuit cutter. Cookies were baked in batches at 195±2.0°C for 20 min. Baked cookies were cooled to room temperature (22±1.0°C) and stored in polyethylene bags until analysis.

2.5.2 Color determination

The color parameters (L*, a* and b*) of the prepared wheat flours and formulated cookies were assessed using a CR-300 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan). Color intensity was measured and expressed using the CIE L* a∗ b* coordinate system, where L* represents color lightness, a∗ characterizes red (positive value) and green (negative value) colors. The parameter b∗ indicates yellow (positive value) and blue (negative value) colors. The above analysis was realized in 20 replicates.

2.5.3 Physical parameters

The cookies' diameter was measured by laying six cookies edge to edge with the help of a scale, rotating them through 90, remeasuring them and then taking the average value. The cookies' thickness was measured by stacking five samples one on top of the other and taking the average value. The spread ratio was estimated as diameter/thickness.

2.5.4 Texture analysis of cookies

The cookies texture was assessed using breaking test and whose parameters are breaking strength and fracturability, was determined using a TA.TX. Plus Texture Analyzer (Stable Micro Systems, Godalming, Surrey, U.K.). The test speed is of 1 mm/s using a knife probe. The peak force from the resulting curve was measured as the breaking strength or breaking force of the cookies. Twenty cookies from each formulation were analyzed.

2.5.5 Determination of total polyphenol content and antioxidant activities of cookies

Total polyphenol content and antioxidant activities by DPPH and FRAP of the formulated cookies were determined using the method described by Ismail *et al*. (2014). The total phenol content is expressed as the Gallic acid equivalent (mg GAE/100 g) of the sample. The cookies antioxidant activities by ABTS assay were assessed using the method of PASSOS *et al*. (2017). The antioxidant activities of the formulated cookies are expressed as inhibitory concentration at 50 % (IC_{50}), which in turn, represents the amount of cookies (mg) necessary for 50 % reduction of $ABTS^{\cdot+}$, $DPPH^{\cdot}$ and FRAP. IC_{50} values were calculated according to the non-linear regression algorithm of the plotted inhibition graph percentage compared with the cookie sample concentration.

2.5.6 Sensory evaluation of cookies

For sensory evaluation, 50 panelists were chosen from the Food Technology and Science Department of Nutraceutical Institute of Foods at Laval University (25 males and 25 females). The tests were performed under daylight room conditions. Sensory analyses were conducted on ITF-enriched cookies samples due to their higher physical properties. The order of samples presentation to the panel was randomized. The color, texture, flavor, crispness and overall acceptability of cookies were rated on a 1-9 scale: 1 - dislike extremely; 2 - dislike very much; 3 - dislike moderately; 4 - dislike slightly; 5 - neither like nor dislike; 6 - like slightly; 7 - like moderately; 8 - like very much; 9 - like extremely.

2.6. Statistical analysis

Except for the sensory evaluation that was evaluated in duplicate, all the others were realized in triplicate. The results were statistically analyzed using SPSS (SPSS Inc., Chicago, USA), version 18. While the analysis of variance (ANOVA) was used to identify the significant difference between the results, Duncan's test was used to separate the mean with a significance level of 5%.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

The physicochemical properties of the wheat flour incorporated with ITF were examined (Table 1), revealing that the ITF incorporation levels into wheat flour decreased the water and protein contents from 14.05 to 12.38 % and from 9.58 to 4.33 %, respectively, (p<0.05). The ash content is also known to be another parameter used for the determination of the wheat flour purity. In this study, the ash and fat contents ranged from 0.42 to 0.94 and 0.26 to 1.41 %, respectively, hence the significant variations (p<0.05) between ITF-enriched samples and control flour. Additionally, the total fiber increased and the carbohydrate

contents decreased substantially with the increase in ITF from 0 to 10 % (p<0.05) depending on the ITF's concentration.
Likewise, wet and dry gluten contents decreased from 28.20 to 22.56 % and from 12.48 to 9.98 %, respectively, with ITF incorporation (p>0.05), which is the same with the gluten index values (p<0.05). Furthermore, the results demonstrated that dry gluten content decreased significantly with the incorporation of 2.5 % ITF, due to reduction of protein content in the tested wheat flours. The ITF incorporation will compete for water during the dough making process, thus making the dough thicker, which in turn may 'work' the gluten network more to enable an enhanced water uptake. Further ITF incorporation would out-compete gluten for the available water and make the gluten network less able to take up water, hence reducing the water binding capacity beyond the ITF incorporation in wheat flour.

Table 1. Chemical composition, gluten analysis and color parameters of wheat flour containing insoluble tomato fibre.

	ITF concentration (g/100 g)				
	Control	**2.5**	**5**	**7.5**	**10**
Chemical composition					
Water	14.05±0.03^{a}	13.63±0.03^{b}	13.22±0.02^{c}	12.80±0.02^{d}	12.38±0.02^{e}
Protein	9.58±0.05^{a}	6.77±0.04^{b}	5.95±0.05^{c}	5.14±0.03^{d}	4.33±0.02^{e}
Ash	0.42±0.02^{a}	0.55±0.01^{b}	0.68±0.01^{c}	0.71±0.01^{d}	0.74±0.01^{e}
Lipids	0.26±0.01^{a}	0.55±0.01^{b}	0.84±0.01^{c}	0.92±0.01^{d}	0.94±0.01^{d}
Insoluble fiber	3.54±0.05^{a}	10.21±0.04^{b}	11.87±0.03^{c}	14.54±0.02^{d}	18.20±0.02^{e}
Soluble fiber	6.75±0.05^{a}	7.08±0.10^{ab}	7.41±0.14b^{c}	7.73±0.20^{cd}	8.06±0.24^{d}
Total fibers	10.20±0.01^{a}	17.29±0.05^{b}	19.28±0.11b^{c}	22.27±0.17d	26.26±0.23^{e}
Total carbohydrates	65.40±1.05^{a}	61.21±0.38^{ab}	60.03±0.80b^{c}	58.16±1.24^{cd}	55.35±1.67^{d}
Gluten analysis					
Wet gluten	28.20±0.03^{a}	26.79±0.02^{b}	25.38±0.02^{c}	23.97±0.02^{d}	22.56±0.03^{e}
Gluten Index	86.86±0.08^{a}	82.51±0.07^{b}	78.17±0.07^{c}	73.84±0.06^{d}	69.48±0.06^{e}
Dry gluten	12.48±0.02^{a}	11.86±0.02^{b}	11.23±0.01^{c}	10.61±0.03^{d}	9.98±0.02^{e}
Color parameters					
L*	95.34±0.04^{a}	93.69±0.03^{b}	92.04±0.03^{c}	90.40±0.04^{d}	88.75±0.03^{e}
a*	-0.74±0.02^{a}	-0.36±0.02^{b}	1.20±0.01^{c}	4.38±0.02^{d}	6.75±0.01^{e}
b*	12.78±0.04^{a}	16.27±0.03^{b}	19.67±0.02^{c}	23.27±0.03^{d}	26.76±0.03^{e}

Chemical composition and gluten analysis were expressed as %.
Values given are the means of three replicates ± standard deviation. Different letters within the same row indicate significant differences (one- way ANOVA and Duncan test, p< 0.05).

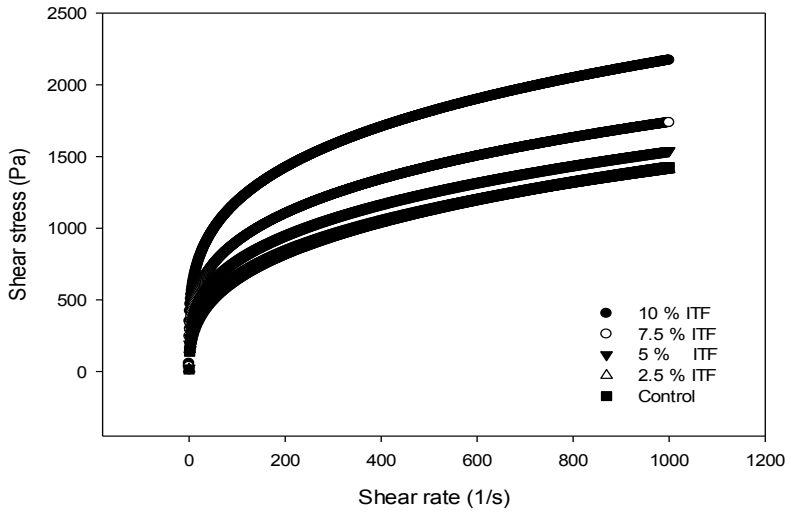
Table 1 lists CIELAB color parameters (L*, a*, and b*) for different wheat flour samples incorporated with ITF, indicating the considerable impact of ITF incorporation on these parameters. Briefly, the lightness value (L*) of the whole-wheat flour was 95.34, which drastically dropped to 88.75 for the highest ITF content (10 %). Nevertheless, a* value for the whole wheat flour sample was -0.74, which increased significantly, about 4 to 9 times after ITF incorporation with the highest value of 6.75 for the sample with the highest ITF amount. The yellowness parameter (+b*) increased significantly as ITF amount increased. The increase in color values a* and b* in wheat flour samples could be attributed to the

creation of more surface areas that possibly increase color intensity as reported by AHMED and AL-ATTAR (2015).

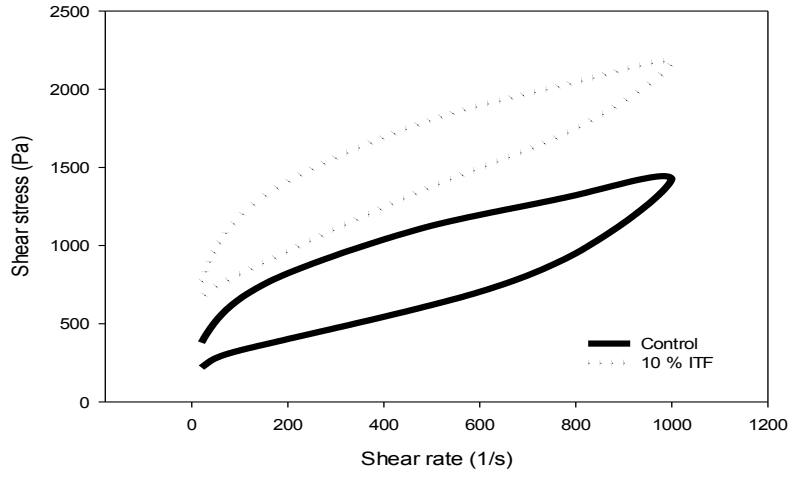
3.2. Rheological properties of formulated wheat dough

3.2.1 Flow behavior

All formulated dough samples enriched with ITF exhibited non-Newtonian behavior at shear rates from 0.001 to 1000 s^{-1} at 20°C (Fig. 1).



A



B

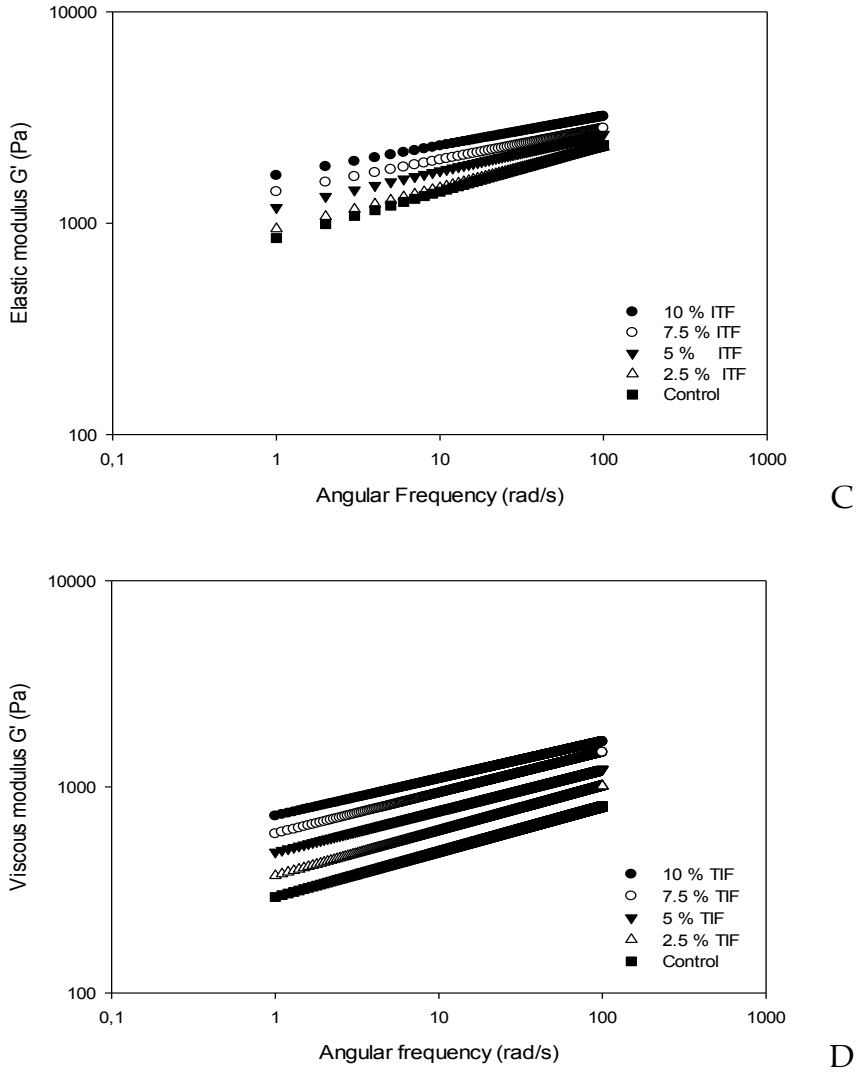


Figure 1. Shear stress versus shear rate (A). Flow curves with a controlled shear rate measured by increasing (forward measurements) and decreasing shear rate (backward measurements) (B). Variation of storage (C) and loss modulus (D) with angular frequency (ω) for insoluble tomato fibre-enriched wheat dough at 20°C.

There is a nonlinear relationship between shear stress (σ) and shear rate ($\dot{\gamma}$), which is in good agreement with the findings of GUADARAMA-LEZAM *et al*. (2016). The power-law and Herschel-Bulkley models were used to characterize the flow curves of the formulated dough samples. Based on the regression coefficient values (R^2), the power-law was found to be a better-fit model for flow curves ($R^2 > 0.99$), and only the rheological parameters of this model were determined in this study (Table 2). Furthermore, flow behavior index values (n), at different ITF amounts, were in the range of 0.339-0.264 and significant differences between all wheat dough samples ($p<0.05$) were noted. Indeed, the flow plot of the shear stress against shear rate of the investigated dough showed a flow index (n) less than 1 (thinning fluid), indicating that the flow behavior of the examined samples can be described by following a non- Newtonian profile. Consequently, all values are less than 1, which further confirms the pseudoplastic behavior of the whole ITF-enriched dough.

The obtained results also showed that the flow index (n) decreased with the increase in ITF concentration. Actually, the consistency coefficient (K) indicates the viscous nature of a matter. Yet, the consistency values (K) of the whole-wheat flour suspension within the studied concentration domain were in the range of 137.22-350.60 Pa·s^n. The higher consistency values of the formulated dough resulted from the antiplasticizing effect of sugars against water. Another reason for the non-Newtonian pseudoplastic flow behavior of samples emanates from the presence of high-molecular-weight macromolecules as fibers, gluten, sugars and starch. MARTINEZ *et al*. (2014) have confirmed that the addition of insoluble fibers increases dough consistency due to the insoluble fibers' effect on the internal structure of wheat doughs.
Figure 1B shows the flow curves of control and wheat dough at 10 % of ITF. Shear stress measurements at increasing and decreasing shear rates from 0.001 to 1000 s^{-1} gave hysteresis loops. The hysteresis area (A) was determined and illustrated in Table 2, confirming that its values decreased with the increase in ITF concentration. However, significant variations with ITF concentration exist in all tested samples ($p<0.05$). The hysteresis loops indicate a time dependency of the wheat dough rheological properties. This thixotropic behavior is observed with concentrated suspensions and macromolecular solutions due to structural breakdown happening in the specimen during the rheological test.

3.2.2 Viscoelastic behavior

The oscillatory spectra of the formulated dough samples displayed viscoelastic properties (Figs. C-D). The characteristic slopes of the double-logarithmic plots of the storage and loss modulus (G′ and G″) vs angular frequency were quite similar. In this case, the elastic modulus was significantly high and different from the viscous one throughout the covered frequency range.
All examined dough samples exhibited higher storage modulus (G′) values than those of G″, thus confirming the elastic behavior of the dough samples and indicating that both moduli values increased with the increase of the angular frequency and ITF concentration. These results are comparable with those found by AHMED *et al*. (2013), GUADARRAMA-LEZAMA *et al*. (2016), who have proven that wheat dough was characterized by a solid-like behavior. Indeed, they suggested that the increase of mechanical properties is due to limited plasticization effect and the presence of fiber nanoparticles.
The experimental data was fitted with power-law models, described as follows:

$$G'(\omega) = K'.\omega^{n'} \quad (3)$$

$$G"(\omega) = K".\omega^{n"} \quad (4)$$

Where G′ is an elastic modulus (Pa), G″ is a loss modulus (Pa), ω is an angular frequency (rad/s), and k′, k″, n′ and n″ are the experimental constants.
Table 2 presents the power-law parameters of the elastic and viscous moduli (k′ and k″) of the tested samples undergoing an increase with the increase of ITF concentration. In all cases, the *k′* values were higher than those of *k″*, and increased significantly with the rise in the ITF level in the wheat dough, (from 850.82 to 1680.92 and from 291.88 to 720.68 Pa, respectively). In the same vein, BENGTSSON *et al*. (2011) found that the ITF increase led to the increase of G′ and G″. Thus, the highest k′ and k′′ values were observed for dough sample with 10 % of ITF. The exponents n′ and n″ were then found to decrease from 0.22 to 0.12 and from 0.23 to 0.181, respectively. Besides, no significant variations in exponent values at high levels of ITF ($p>0.05$) were noticed.

3.2.3 Farinograph properties

The use of elevated ITF concentration in the wheat flour increased significantly the Farinograph water absorption (WA), dough consistency (DC) and dough development time (DDT) (p<0.05) (Table 2). While the increase in WA may be due to the presence of protein, fiber and resistant starch contents, the increase in the DDT showed a delay in the hydration and gluten development in the presence of these macromolecules. Obviously, variations in WA depend on the protein content, the chemical structure and porosity of ITF, the association between molecules and the particle sizes (THEBAUDIN *et al*., 1997).
Recently, WANG *et al*. (2002) have demonstrated that the variations in WA is mainly attributed to the greater number of hydroxyl group existing in the fiber structure, allowing more water interaction through hydrogen bonding. These results are in accordance with those found in the literature (MIS *et al*., 2012; AHMED *et al*., 2013).
Dough stability time is an indicator of the flour strength, with higher values, indicating stronger doughs, which shows that the different ITF amounts modify the DDT (p<0.05). The use of ITF improved significantly the dough stability compared with control flour (p< 0.05), which could be explained by higher interactions among ITF, water and gluten. Furthermore, mixing dough could release water to the dough matrix, causing a quick reduction in dough consistency, and therefore a reduction in DDT (MAJZOOBI *et al*., 2011).
The mixing tolerance index (MTI) of ITF-enriched dough decreased considerably, with the increase in concentration from 0 to 10 % of ITF. Table 3 shows that control and wheat dough enriched with 2.5 % of ITF has a better dough mixing tolerance with a lower value than the other samples. The increase in mixing tolerance index upon ITF addition is probably due to the dilution of gluten protein with the fibers, which may be explicated by the interaction between fiber and gluten influencing the dough mixing properties (AHMED *et al*., 2013). However, quality index (QI) insignificantly increased from 53.48 to 60.60 BU by the ITF addition as a function of increasing rate substitution (p>0.05). AHMED *et al*. (2013) suggested that the quality index increase emanates from the interaction between gluten and fiber.

3.2.4 Alveographic properties

Table 3 lists the alveographic parameters of dough with and without ITF, proving that its incorporation caused an increase in the tenacity (P) and decrease in the extensibility (L), and consequently in the swelling index (G) of the formulated wheat dough (p<0.05). After the ITF addition, the configuration curve ratio (P/L) increased and the alveograph strength decreased. This may be due, at least partly, to the reduction in the gluten content caused by the presence of other components, as proteins, fibers, lipids and non-gluten-forming proteins, which interfere with gluten formation. It can be concluded that the protein existing in the ITF might have instigated partial disruption of the gluten network, causing change in the equilibrium of elasticity and extensibility properties of the whole wheat flour dough. The obtained results also corroborated significant differences between control and ITF-enriched dough in term of the deformation energy (W) (p < 0.05). These results are clearly in conformity with those obtained by BOUBAKER *et al*. (2016). Yet, many studies, as WANG *et al*. (2002), reported that commercial fibers' incorporation greatly improved the development of wheat protein behavior. They also stated that the fibres 'incorporation induced a decrease of the proteolytic degradation, being practically neutralized by inulin.

3.2.5 Visco-amylograph properties

To determine the ITF incorporation effect on wheat flour-water interaction in cookies dough, a rapid viscoanaylzer (RVA) was used, revealing that ITF significantly diminished the pasting viscosities (peak, breakdown, final, and setback viscosities) (p<0.05). Similarly, the values of the peak paste viscosity of the formulated dough decreased significantly from 1285.56 to 450.44 cP, which was explained probably by their association with high protein amount. Thus, the tomato protein is estimated to form complexes with starch granule surface, preventing the release of exudates and lowering the peak viscosity. However, the pasting temperature increased from 86.20 to 90.75°C in flour blends (p<0.05), which, according to Martinez *et al*. (2014), might be related to delay or restricted starch swelling. Actually, the amylose leaching and lower pasting viscosities are indications of reduced starch available for gelatinization. Therefore, the ITF addition is proven to significantly diminish the degree of breakdown from 0.60 (control) to 0.25 (flour at 10 % of TIF), showing increased resistance of the swollen starch granule to rupture after cooking as reported by SOZER *et al*. (2014).

3.3. Fermentation parameters

The fermentation parameters of the ITF-enriched wheat dough were assessed by the rheofermentometer, Table 3. The maximum dough height (H_m) decreased dramatically from 69.17 to 30.84 mm, with the ITF addition, regardless of its concentration (p<0.05). However, no significant difference between samples containing 7.5 and 10 % of ITF (p> 0.05) was observed. Similar results were obtained by MARTINEZ *et al*. (2014) and LIU *et al*. (2017) who discovered that the addition of insoluble fibers led to the decrease of the maximum dough height. Nevertheless, the time required to reach the maximum dough height decreased from 2.95 to 1.30 h with the increase in ITF concentration (p<0.05), demonstrating substantial differences in the time of reaching the maximum height between wheat flour samples. Besides, the CO_2 production, retention and loss volumes were also determined. Indeed, although the ITF incorporation decreased the CO_2 production and retention volumes, it increased its loss volume (p<0.05). These results accord well with those found by MARTINEZ *et al*. (2014), who added different fibers to the wheat flour, proving that the influence differs with the type of fiber used. They suggested that insoluble fibers with larger particles can create points to split the structure, thus enabling gas to escape, affirming the assumption that ITF incorporation to wheat flour decreased gas holding capacity. However, CORREA *et al*. (2014) have hypothesized that the addition of hydrocolloids could form hydrophilic complexes with protein or starch, which further changed the viscoelasticity and influenced the fermentation character.

3.4. Properties of formulated cookies

3.4.1 Physical properties

Table 4 summarizes the physical parameters of the analyzed cookies by substituting wheat flour with ITF from 0 to 10% levels. As the ITF concentration increased, the diameter of cookies (D) decreased from 53.94 to 51.83 mm, but no significant variations were found between cookies with 5 and 7.5 % of ITF. The thickness of cookies (E) increased, confirming the dilution of gluten as reported by AJILA *et al*. (2008). The differences in diameter and thickness are revealed in the spread ratio (D/E), consistently decreasing from 5.17 to 4.02 with ITF levels increase (p<0.05).

Table 2. Power-law parameters of insoluble tomato fibre-enriched wheat dough enriched at 20°C.

ITF (%)	$\sigma = K\dot{\gamma}^n$				$G' = K'.\omega^{n'}$			$G'' = K''.\omega^{n''}$		
	n	K [Pa.s^n]	A [Pa.s^{-1}]	r^2	n'	k'[Pa.s$^{n'}$]	r^2	n''	k''[Pa.s$^{n'}$]	r^2
Control	0.339±0.003^a	137.22±9.98^a	4.09±0.03^a	0.986	0.220±0.002^a	850.82±12.00^a	0.989	0.230±0.002^a	291.88±15.03^a	0.994
2.5	0.321±0.002^b	153.40±11.80^a	3.83±0.02^b	0.991	0.194±0.003^{ab}	940.49±14.80^b	0.972	0.218±0.003^b	370.21±15.21^b	0.992
5	0.302±0.002^c	191.26±15.74^{ab}	3.51±0.03^c	0.978	0.173±0.02^{ab}	1187.66±19.84^c	0.988	0.201±0.001^c	482.46±12.30^c	0.980
7.5	0.284±0.003^d	244.19±17.81^b	3.33±0.04^d	0.983	0.153±0.003^{bc}	1409.37±24.83^d	0.991	0.198±0.002^c	591.10±12.95^d	0.979
10	0.264±0.002^e	350.60±19.40^c	3.18±0.03^e	0.995	0.120±0.003^c	1680.92±30.01^e	0.998	0.181±0.003^d	720.68±15.88^e	0.996

A: area of hysteresis loop; n: flow index; K: consistency coefficient; ITF: insoluble tomato fibre; r: Regression coefficient; ITF: Insoluble tomato fibre.
Values given are the means of three replicates ± standard deviation. Different letters within the same row indicate significant differences (one- way ANOVA and Duncan test, $p< 0.05$).

Table 3. Farinograph, alveograph, viscoamylograph and rheofermentation tests of insoluble tomato fibre-enriched wheat flour.

Test		Concentration (%, g/100g)				
	Parameters	**Control**	**2.5**	**5**	**7.5**	**10**
Farinograph	W (%)	58.80±0.20a	65.70±0.30b	71.42±0.22c	74.04±0.16d	78.68±0.32e
	DC (BU)	510±3.00a	525±3.00a	565±5.00b	590±10.00c	620±8.00d
	DDT (min)	2.00±0.05a	2.30±0.04b	4.02±0.10c	5.10±0.07d	6.30±0.10e
	DST (min)	7.40±0.10a	7.30±0.05a	7.30±0.08a	6.45±0.10b	5.67±0.13c
	DMI (BU)	43.20±0.10a	48.55±0.15b	60.40±0.25c	65.23±0.23d	67.78±0.41e
	QI (BU)	53.48±1.73a	55.00±2.50a	57.09±1.91a	60.20±2.00a	60.60±2.00a
Alveograph	P (mmH_2O)×10^{-4}	89.32±2.50a	123.75±0.50b	160.11±1.30c	163.69±2.30c	174.20±2.20d
	L (mm)	86.56±1.40a	65.44±1.03b	30.19±0.80c	22.77±0.50d	18.82±0.60e
	G (mm)	20.65±0.16a	17.96±0.14b	12.20±0.16c	10.59±0.12d	9.63±0.15e
	P/L ratio	1.03±0.01a	1.89±0.03b	5.30±0.10c	7.19±0.06d	9.26±0.18e
	W_L (J×104)	225.40±5.20a	200.36±3.30b	180.22±1.20c	140.43±2.55d	120.60±3.40e

Viscoamylograph	PV (cP)	1285.56±5.70a	1179.25±4.80a	813.60±4.81b	550.02±2.98c	450.44±5.56d
	HPV (cP)	771.34±3.66a	553.33±4.67b	341.71±2.80c	154.00±3.02d	112.61±1.80e
	FV (cP)	1421.63±7.70a	1149.11±9.89b	842.91±6.09c	605.81±5.90d	514.11±3.89e
	SV (cP)	650.30±4.04a	595.78±5.22b	500.20±3.29c	451.81±2.90d	401.50±5.69e
	PT (°C)	86.20±0.02a	87.36±0.06b	87.93±0.04c	88.46±0.06d	90.75±0.05e
	BD	0.60±0.00a	0.49±0.00b	0.42±0.00^{c}	0.28±0.00d	0.25±0.00e
Rheoermentator	Hm (mm)	69.17±2.33a	41.23±1.23b	36.89±0.50^{c}	32.76±1.01d	30.84±1.02d
	T_1 (h)	2.95±0.05a	2.52±0.03b	1.83±0.02c	1.47±0.03d	1.30±0.03e
	V_T (mL)	1720.27±19.73a	1680.60±14.40ab	1651.04±14.96bc	1644.3±11.93bc	1610.30±7.85c
	VR (mL)	1440.06±25.10^{a}	1372.82±18.00^{ab}	1314.10±24.95b	1234.28±15.89c	1144.45±13.70d
	VL (mL)	280.21±1.27a	307.78±1.40^{b}	336.94±4.95^{c}	410.09±3.97^{d}	465.70±5.85e

ITF: insoluble tomato fibre; W: water absorption; DC: Dough consistency; DDT: dough development time; DST: dough stability time; DS: degree of softening; QI: Quality index; P: tenacity (mmH_2O); L: dough extensibility; G: swelling index; P/L: configuration curve ratio; W_i: deformation energy; PV: pasting viscosity; HPV: hot pasting viscosity; FV: final viscosity; SV: setback viscosity; PT: pasting temperature; DB: degree of breakdown; V_t: total volume of dioxide carbon; V_r: retention volume of carbon dioxide; V_l: loss volume of carbon dioxide.
Values given are the means of three replicates ± standard deviation. Different letters within the same row indicate significant differences (one- way ANOVA and Duncan test, $p< 0.05$).

Table 4. Physical, colour, textural and biochemical parameters of insoluble tomato fibre-enriched cookies.

		ITF concentration (g/100 g)			
Parameters	**Control**	**2.5**	**5**	**7.5**	**10**
Physical parameters					
Diameter (mm)	53.94±0.06^{a}	53.17±0.03^{b}	52.20±0.10^{c}	52.06±0.06^{c}	51.83±0.03^{d}
Thickness (mm)	10.43±0.17^{a}	11.24±0.15^{b}	11.67±0.25^{c}	12.22±0.25^{c}	12.91±0.15^{d}
Spread ratio	5.17±0.08^{a}	4.73±0.06^{b}	4.47±0.10b^{c}	4.26±0.09^{cd}	4.02±0.04^{d}
Color parameters					
L*	75.79±0.09^{a}	70.51±0.06^{b}	66.17±0.03^{c}	63.64±0.05^{d}	62.36±0.04^{e}
a*	3.22±0.01^{a}	3.94±0.01^{b}	4.17±0.02^{c}	4.91±0.01^{d}	5.43±0.02^{e}
b*	27.72±0.03^{a}	31.45±0.05^{b}	35.58±0.02^{c}	38.76±0.03^{d}	39.68±0.01^{e}

Textural parameters					
Breaking strength (N)	19.88±0.11[a]	20.94±0.06[b]	24.47±0.04[c]	28.06±0.06[d]	33.47±0.05[e]
Fracturability (mm)	0.32±0.02[a]	0.45±0.02[b]	0.56±0.02[c]	0.59±0.02[cd]	0.63±0.01[d]
Biochemical parameters					
Total polyphenol content (mg GAE/100)	86.98±2.58[a]	189.50±7.50[b]	239.51±3.50[c]	305.00±10.03[d]	376.02±6.02[e]
ABTS (mg/mL)	2.40±0.03a	1.84±0.04[b]	1.34±0.01[c]	1.13±0.02[d]	0.89±0.03[e]
DPPH (mg/mL)	181.00±7.00[a]	91.03±8.04[b]	14.47±0.07[c]	9.10±0.10[c]	3.32±0.05[c]
FRAP (mg/mL)	35.89±2.34[a]	16.62±2.12[b]	6.62±0.03[c]	1.95±0.05[cd]	0.78±0.04[d]

ABTS, DPPH, and FRAP were expressed as inhibitory concentration at 50 % (IC_{50}).
Values given are the means of three replicates ± standard deviation. Different letters within the same row indicate significant differences (one- way ANOVA and Duncan test, $p< 0.05$).

However, the decrease in the spread ratio of ITF-fortified cookies is probably attributed both to the fact that the composite flour increased water absorption, and the absence of free water in the cookie dough. SHARMA *et al*. (2016) mentioned that the decrease in the spread ratio of germinated flour blend cookies may be due to the enzymatic degradation of the starch and protein into smaller sugars and peptides, resulting in the increase of hydrophilic nature within cookies. Table 4 shows that cookies became darker (lower L* values), more reddish (higher a* values) and more yellowish (higher *b** values) than control cookies with the increase in ITF amount. The lightness (L) also decreased proportionally with the increase of the ITF amount, reflecting the darkening of the cookies. This color change was confirmed by cookies photos (Fig. 2). The decrease of lightness is probably due to the high protein and free sugar contents may accelerate the Maillard reaction and therefore change the color of the products. As suggested by MILDNER-SZKUDLARZ *et al*. (2013), cookies color depends mainly on their ingredients because the temperature is not high enough for Maillard and caramelization reactions. The ITF's color values may affect the color of cookies' crust. The 10 % level of ITF presented more yellowish crust color than control cookies (Fig. 2).

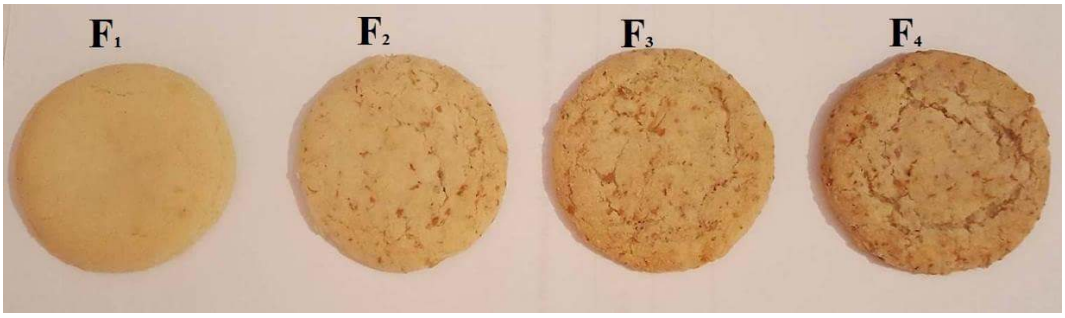


Figure 2. Photos of formulated cookies at different levels of insoluble tomato fibre.

The breaking strength values of formulated cookies with different ITF concentration are illustrated in Table 4, which reveals that the breaking force value increased with the increase in ITF concentration ranging from 19.88 to 34.47 N. Meanwhile, the results showed that there are significant variations in cookies' hardness among all cookies samples (p<0.05) (Table 4). The increase in breaking force may be due to the increase in cookies thickness, which gives physical strength to the cookies structure. Yet, higher positive correlation was found between ITF concentration and breaking strength (r = 0.965, p<0.05), which is in perfect accordance with the study of NASIR *et al*. (2010) who found a positive correlation between the concentration of vegetable flours and cereal foods. Besides, the results showed that the ITF incorporation decreased the cookies' fracturabilities (p<0.05), revealing a negative correlation between thickness and fracturability of the formulated cookies (r=-0.96, p<0.05).

3.4.2 Total polyphenols and antioxidant activities of cookies

The results of the total polyphenol content and antioxidant activities are shown in Table 4. The total polyphenol content of cookies, with 2.5 to 10 % ITF, increased significantly compared to the control cookies, i.e., it increased in parallel with ITF supplementation level. While highly negative correlation was found between lightness (L*) parameter and the total polyphenol content (r =-0.977, p< 0.01), positive correlations between a* and b*

parameters and total polyphenol content of cookies (r=0.994, p<0.01and r=0.982, p<0.01, respectively).
To characterize the antioxidant potential of the formulated ITF-enriched cookies, DPPH, FRAP and ABTS radical scavenging activities were determined, Table 4. Actually, all IC_{50}values of antioxidant tests decreased with the ITF increase, and significant differences between all cookies samples were found (p<0.05). Comparing the activities, the highest one was ABTS followed by those of FRAP and DPPH, whose relationship with the Maillard reaction products (e.g., color parameters) was identified by a multivariate analysis to find the possible correlations among measured variables. High correlations were found between the total polyphenol content and antioxidant activities (IC_{50}) (r =-0.982 (ABTS); r=-0.919 (DPPH); r=-0.940 (FRAP), p< 0.01). Correlations between antioxidant activities (IC_{50}) and lightness (L*) (r =-0.998 (ABTS); r=-0.977 (DPPH); r=-0.985 (FRAP), p< 0.01) were also detected. This implies that the amount and type of polyphenolic substances in cookies are variable, depending mainly on the ITF incorporation level. The baking can further increase the antioxidant activities, and the formation of dark color pigment due to Maillard browning during baking process can increase the antioxidant properties of the cookies. These findings were confirmed with the high correlations between the lightness and inhibitory concentrations (IC_{50}), which are similar to those obtained by MICHALSKA *et al*. (2008). The latter indicated that Maillard reaction products possess cookies' antioxidant activities at elevated temperature, which are in turn in good agreement with the studies of MISAN *et al*. (2011) and AGILA *et al*. (2008). At a 10 % ITF incorporation, the content of polyphenols were 4 times higher than control cookies.

3.4.3 Sensory evaluation

The score results of sensory evaluation of the tested cookies are illustrated in Fig. 3.

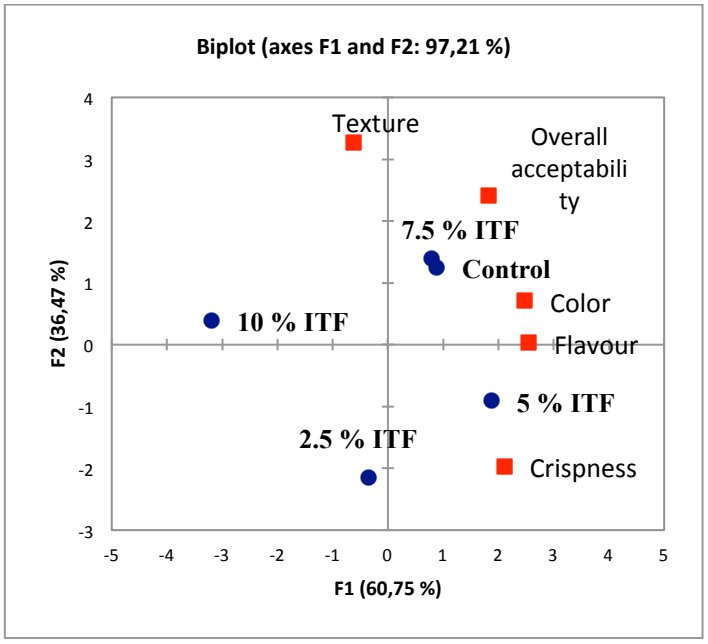


Figure 3. Principal component analysis plot of data from sensory evaluation of insoluble tomato fibre-enriched cookies.
● ITF concentration; ■ Sensory parameters

The first (PC1) and the second (PC2) principal components were 60.75 and 36.47 %, respectively, accounting for 97.21 % of the total variance. The results also revealed that all sensory parameters were positively correlated with both components. Indeed, while color, crispness and flavor were positively and highly correlated with PC1, texture and overall acceptability were positively correlated with PC2.
The score plot showed that control cookies and those enriched with 7.5 % of TIF had high scores of color and overall acceptability. However, compared to control, the sensory parameters of the formulated cookies were found to be different. According to the panels, there are no a significant difference between control and cookies with 7.5 % of ITF in term of overall acceptability (Fig. 3). It can be concluded that ITF can be incorporated into cookies at 7.5 % level.

4. CONCLUSIONS

In this study, the effect of ITF on the physicochemical, rheological and antioxidant properties of wheat flour and the formulated cookies were investigated. The results showed that the ITF incorporation improved the nutritional and rheological properties of wheat dough and diminished the rheofermentation parameters and pasting properties: higher water absorption, tenacity, stability and peak consistency and smaller extensibility. Moreover, ITF significantly increased the hardness and the total polyphenol content and enhanced the antioxidant activities of cookies. The cookies enriched with 7.5 % ITF had similar overall acceptability with the control cookies. However, compared to the latter, differences on the formulated cookies color and texture were found. Therefore, ITF might be used for the novel formulation of cookies as an alternative source of dietary fiber and bioactive compounds.

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REVIEW

EDIBLE *ALLIUM S*PECIES: CHEMICAL COMPOSITION, BIOLOGICAL ACTIVITY AND HEALTH EFFECTS

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ABSTRACT

Since ancient times edible *Alliums* play an important role in human diet and traditional medicine. The most commonly cultivated *Allium* species are onion (*Allium cepa* L.), garlic (*Allium sativum* L), leek (*Allium ampeloprasum* L.), chive (*Allium schoenoprasum* L.) and Welsh or Japanese bunching onion (*Allium fistulosum* L.). These species are rich sources of biologically active compounds such as flavonoids, organosulfur compounds and saponins. Numerous studies we reviewed in this paper, confirmed their significant antioxidant, antibacterial, anti-inflammatory, antiproliferative and anticancer activities, which makes them an important source of phytonutrients that can contribute to the protection and preservation of human health.

Keywords: *Alliums*, biological activity, flavonoids, organosulfur compounds

1. INTRODUCTION

Allium species have been used for centuries in human diet because of their pungent smell and specific taste, but also for medicinal purposes because of their remarkable medicinal properties. The best known and most cultivated species of the genus *Allium*, onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) are widely used as spices and medicinal plants and are therefore the subject of numerous studies. To date, their chemical structure has been thoroughly explored as well as their biological activity. *Allium* species are rich in phytonutrients, mostly flavonoids and organosulfur compounds which exhibit strong antioxidative, antimicrobial, anti-inflammatory and anticarcinogenic activity (AHIABOR *et al*., 2016; ALBISHI *et al*., 2013; ASHRAF *et al*., 2011; BENEKEBLIA, 2004; BOROWSKA *et al*., 2013; CHANG *et al*., 2013; COLINA-COCA *et al*., 2017; HERMAN-ANTOSIEWICZ and SINGH, 2004; JOHNSON *et al*., 2016; KAUR *et al*., 2016; KHAZAEI *et al*., 2017; KIM *et al*., 2013; KOCA *et al*., 2016; KUMARI and RANJAN, 2014; KWON *et al*., 2002; LANZOTTI *et al*., 2014; LANZOTTI, 2006; LI *et al*., 2016; LI *et al*., 2014; MIN KIM *et al*., 1997; NDOYE FOE *et al*., 2016; ORTIZ, 2015; PAN *et al*., 2018; QUINTERO-FABIÁN *et al*., 2013; SHIN *et al*., 2013; SULEIMAN and ABDALLAH, 2014; THOMAS *et al*., 2017; THOMSON and ALI, 2003; YANG *et al*., 2001b).
Many epidemiological studies showed that regular consumption of *Allium* vegetables can decrease the risk of various diseases such as cardiovascular, respiratory, gastrointestinal diseases and different types of cancer (CHEN *et al*., 2009; FLEISCHAUER *et al*., 2000; GONZÁLEZ *et al*., 2006; GUERCIO *et al*., 2014; KIM *et al*., 2018; O'GARA *et al*., 2000; POURZAND *et al*., 2016; TURATI *et al*., 2015, 2014; YOU *et al*., 1989; ZHOU *et al*., 2011).

2. CHEMICAL COMPOSITION

The chemical composition of these species is very complex. They contain a variety of different phytochemicals, and the most important constituents are organosulfur compounds. These compounds provide them with characteristic odor and flavor, as well as the majority of biological properties. Another important group of chemically active compounds are polyphenols, which include phenolic acids and flavonoids, responsible for the characteristic color of onion bulbs. Edible onion parts are rich in carbohydrates, mostly glucose and fructose, while the outer scales of onion bulbs contain significant content of galactose and arabinose. Essential amino acids (arginine and glutamic acid), which may be important nitrogen reserves, also contribute to the nutritional value of onion species. They also contain several other complex bioactive components such as saponins, vitamins (A, C, B6, and folate) and minerals (P, K, Ca, Mg, Zn, Mn, Na, Fe, Br, J, Se, and Cu).

2.1. Flavonoids

Flavonoids are the largest group of polyphenolic compounds present in fruit, vegetables, nuts, tea, wine and other food ingredients. Common onion (*A. cepa* L.), garlic (*A. sativum* L.), and other *Allium* species are the richest sources of dietary flavonoids.
The best described property of flavonoids is their antioxidant activity. Their structure is essential for their ability to act. The configuration, substitution and number of hydroxyl groups determine their antioxidant activity and ability to scavenge free reactive species. Flavonoids have a characteristic structure with two benzene rings (A and B rings, shown in Fig. 1) connected with a pyran ring (heterocyclic ring containing oxygen, the C ring, shown in Fig. 1).

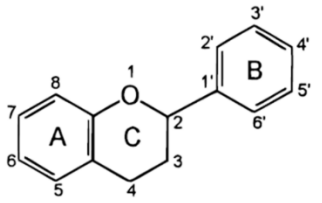


Figure 1. The basic structure of flavonoids (PIETTA, 2000).

So far, more than 4000 different kind of flavonoids have been identified. They are divided into groups (Fig. 2) by the number and position of hydroxyl groups, level of oxidation and pattern of substitution of the C ring: i) anthocyanins - glycosylated derivative of anthocyanidin, present in colorful flowers and fruits; ii) anthoxanthins - a group of colorless compounds divided in several categories, including flavones, flavonols, flavanones, flavanols, isoflavones and their glycosides (HAN *et al*., 2007). Two flavonoid classes are mainly found in onion, flavonols, responsible for yellow and brown skin and anthocyanins which give red to purple color to some onion varieties (RODRIGUES *et al*., 2017).

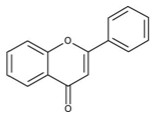
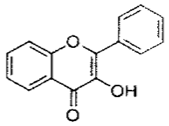
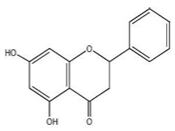
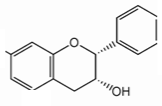
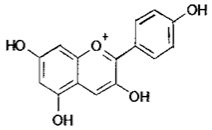
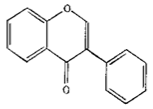
Flavonoid	Basic structure
Flavones	
Flavonols	
Flavanones	
Flavanols	
Anthocyanidins	
Isoflavones	

Figure 2. Chemical structure of the main classes of flavonoids (REIS GIADA, 2013).

Flavonoids are plant pigments, which participate in plant protection against different ecological and physiological stresses such as UV radiation, heat, herbivores and pathogens. Flavonoids have been shown to possess a diverse biological property such as antioxidant, anti-inflammatory, antiallergic, antimicrobial, antiviral, anticancer and neuroprotective activity. These properties are structure dependent which makes flavonoids one of the best antioxidants, scavengers of free radicals and a chelator of bivalent cations that cause DNA damage which is associated with the development of many diseases (HALLIWELL *et al*., 2005; PANCHE *et al*., 2016).
Anthocyanins and flavonols are the most common subgroups of flavonoids present in *Allium* species. The most abundant flavonols in common onions are two quercetin conjugates, namely quercetin 3,4'-diglucoside and quercetin 4'-monoglucoside (CARIDI *et al*., 2007; FREDOTOVIĆ *et al*., 2017; GRIFFITHS *et al*., 2002). These compounds together account for about 80% of total flavonol content with some differences among various *Allium* cultivars.
Quercetin, myricetin, kaempferol and isorhamnetin glycosides accounted for the remaining 15% total of flavonols in onions. Compared to yellow and white onions, red onions showed higher total flavonol content (PRAKASH *et al*., 2007). Also, total flavonol content is higher in the outer layers compared to the inner layers of onion bulb.
In addition to flavonoids, onions are rich source of anthocyanins. Anthocyanins are class of natural pigments responsible for the color of fruits, vegetables, flowers and grains. They give red or purple color to outer layers of onion bulbs. The most frequent anthocyanins in red onion are cyanidin derivatives (over 50% of all anthocyanins), cyanidin-3-(6''-malonylglucoside), cyanidin-3-(6''-malonyl-3''glucosyl-glucoside) and cyanidin-3-glucoside. There are also minor amounts of peonidin, petunidin and delphinidin derivatives (FERRERES and GIL, 1996; FOSSEN *et al*., 1996; GENNARO *et al*., 2002; RODRIGUES *et al*., 2017; SLIMESTAD *et al*., 2007).

2.2. Organosulfur compounds

Allium species are characterized by the rich content of sulfur compounds such as S-alk(en)yl cysteine sulfoxide (ACSO), sulfides, alkyl polysulfides and amino acids (WHO, 1999). First sulfur compounds were isolated from *Allium* species in the middle of the 19th century (SEMMLER, 1892; WERTHEIM, 1844). It has been discovered that volatile sulfur compounds are responsible for the pungent odor of onion species. Also, it became clear that these disulfide compounds are not present in intact bulbs, but they are formed by the enzymatic cleavage of precursors upon disruption of the bulb tissue. Alliin or (+)-S-allyl-cysteine sulfoxide, was the first odorless sulfur compound isolated from the cytoplasm of intact garlic cell (STOLL and SEEBECK, 1948).
After crushing the onion cell, the enzyme allinase is released from vacuole. The enzyme allinase belongs to the group of C-S lyases and plays a key role in the formation of volatile compounds of the genus *Allium* (KEUSGEN, 2011). ACSO is decomposed by the enzymatic reaction of allinase to pyruvate, ammonia and sulfenic acid. Sulfenic acid immediately produce thiosulfinates by a quick condensation reaction (BLOCK *et al*., 1992). Thiosulfinates are very unstable and break down to the mixture of compounds, responsible for specific onion taste: polysulfides, thiosulfinates, capaenes and zwiebelanes (Fig. 3). The main thiosulfinate in garlic is diallyl sulfide, derived from allicin, and dipropyl disulfide in common onion, derived from isoalliin (ROSE *et al*., 2005). NDOYE FOE *et al.* (2016) identified the main components of *A. sativum* and *A. cepa* essential oil. *A. sativum* essential oil was rich in diallyl trisulfide, diallyl disulfide, allyl methyl trisulfide, diallyl sulfide and diallyl tetrasulfide while those from *A. cepa* was rich in diallyl trisulfide, dipropyl trisulfide, 2-methyl-3,4-dithiaheptane, methyl propyl trisulfide, dipropyl

tetrasulfide and 2-propenyl propyl disulfide. These compounds are most likely responsible for their excellent antioxidant and anti-inflammatory activity.

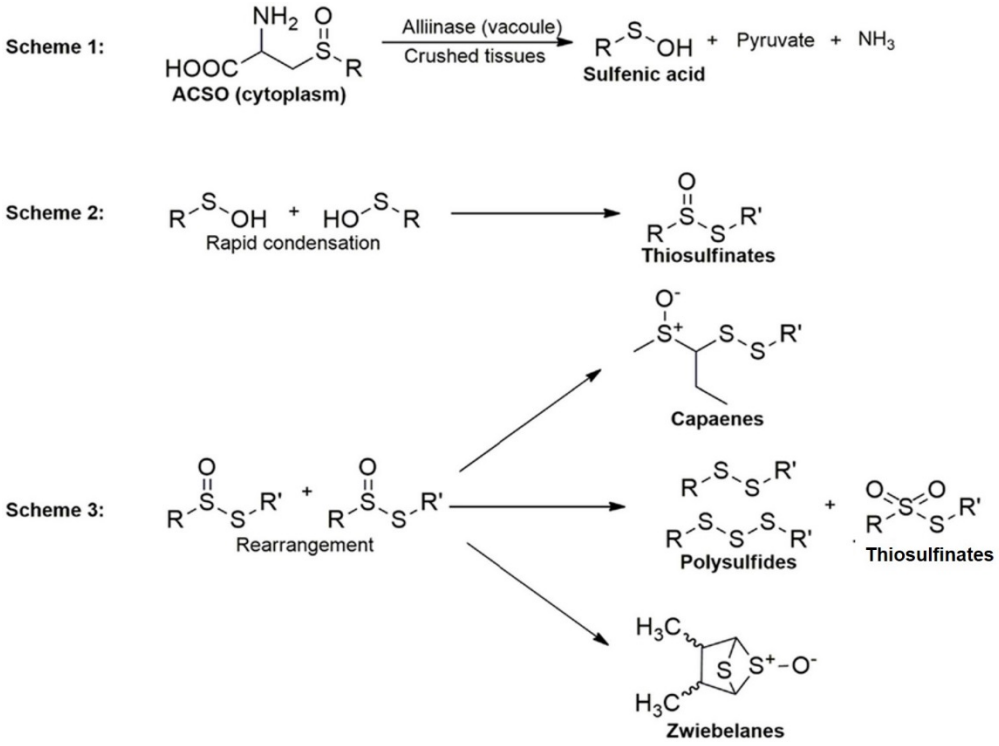


Figure 3. Overview of organosulfide formation from S-alk(en)yl-L-cysteine sulfoxide (ACSO) in *Allium* species (TOCMO *et al.*, 2015).

2.3. Other bioactive compounds

Recent scientific research found several interesting novel compounds isolated from onion such as saponins and peptides. They have been identified so far in over 40 different *Allium* species (SOBOLEWSKA *et al.*, 2016). Saponins are one of the largest classes of surface-active secondary metabolites widely distributed in plants, however, their biological functions are not completely understood. They are generally considered to have important roles in defense of plants against pathogens, pests and herbivores and several studies indicated that they can act as natural remedies in treatment of many diseases (AUGUSTI, 1990; LANZOTTI, 2006; MORRISSEY and OSBOURN, 1999; OSBOURN *et al.*, 2011; SOBOLEWSKA *et al.*, 2016; SPARG *et al.*, 2004). Recent study reported that saponins from *Allium* species possess high cytotoxic activity, which makes them potential candidates for future development as anticancer agents (LANZZOTI *et al.*, 2014).

3. BIOLOGICAL ACTIVITY AND HEALTH EFFECTS OF EDIBLE ONIONS

Onions are used since ancient times as vegetable and spice because of their special taste and smell. They were also used in folk medicine for treatment of bacterial infections such as dysentery, ulcers, wounds, scars, keloids, and asthma. They have also been used as an

adjuvant therapy for diabetes, for prevention of high blood pressure, and loss of appetite (WHO, 1999).
Over the last 50 years, an intensive research has been conducted on the evaluation of biological activity of *Allium* plants, their extracts and essential oil. Garlic and onion are the best known and two mostly tested *Allium* species. Garlic extracts have been reported to possess strong antibacterial (BENEKEBLIA, 2004), antidiabetic (ASHRAF *et al*., 2011), antiproliferative activity (THOMSON and ALI, 2003; YANG *et al*., 2001a), and ability to inhibit development of cardiovascular diseases (THOMSON *et al*., 2006). Similar but weaker effects have been proved for onion extracts.

3.1. Antioxidant activity

Allium plants are one of the main food antioxidants. Antioxidants prevent cell and DNA damage by chelating free oxygen or nitrogen radicals (ROS or RNS), inhibiting their production, or activating antioxidative enzymes (superoxide dismutase 2- SOD2, catalase-CAT and glutathione peroxidase- GP_x). Analysis of the antioxidant activity of *Allium* species is also important because of the proven link between oxidative stress and development of diseases such as atherosclerosis, various forms of cancer and even aging itself. The first study of antioxidant properties of *Allium* plants was performed with crude extracts. Investigators have concluded that the organosulfur compounds are primarily responsible for observed antioxidant effects (AUGUSTI, 1990; MIN KIM *et al*., 1997; SIEGERS *et al*., 1999a). YIN and CHENG (1998), and BENEKEBLIA (2004) concluded that antioxidant activity of onions is not just related with organosulfur compounds but also with phenolic compounds.
In vitro and *in vivo* data reported that onion extracts showed stronger ability to scavenge free radicals, compared to garlic extracts and red onion was more active than yellow onion (GORINSTEIN *et al*., 2008; NUUTILA *et al*., 2003). Red onion is the rich source of flavonoids especially quercetin, presented in conjugated form. The dry outer layers of onion, which are wasted during food preparation, contain huge amounts of quercetin and its derivatives (CORZO-MARTÍNEZ and CORZO, 2007). KAUR *et al.* (2016) proved that methanolic extract of *Allium cepa* have higher antioxidant activity compared to aqueous extract. Interestingly, it was observed that aqueous extract had higher phenolic content. KIM *et al*. (2005) investigated antioxidant activity of flavonols isolated from garlic leaves and shoots. They confirmed that quercetin and its compounds possess strong antioxidant activity. KUMARI and RANJAN (2014) study showed that methanolic extract of *Allium sativum* exhibited strong antioxidant activity, which is in correlation with rich phenolic content. FREDOTOVIĆ *et al*. (2017) determined antioxidant potential of two onion methanolic extracts, *Allium* × *cornutum* and *A. cepa*. Both onions showed strong antioxidant activity. However, *A.* × *cornutum* extract showed slightly higher antioxidant activity which was in correlation with his higher total phenolic content. Comparison of antioxidant activity of garlic and onion cultivars grown in Turkey showed that both species possesses good antioxidant properties which are significantly correlated with their total phenolic content (TPC). Among all samples of onion and garlic, red onions had the highest TPC and antioxidant activity (KOCA *et al*., 2016). KIM *et al*. (2018) performed a comparative study of bioactive organosulfur compounds and antioxidant activity in three *Allium* species, *A. cepa* (onion), *A. sativum* (garlic) and *A. ampheloprasum* (elephant garlic). Results showed that garlic possessed the strongest antioxidant activity followed by elephant garlic and onion. Their findings demonstrated a positive correlation between antioxidant activity and organosulfur compounds content. The study of COLINA-COCA *et al*. (2017) confirmed that feeding rats with high-cholesterol (HC) diet resulted in oxidative stress. HC diet enriched with onion ingredients significantly decreased oxidative

stress by activating antioxidant defense system. BOYLE *et al*. (2000) performed an *in vivo* experiment and confirmed the powerful antioxidant effect of onions. They showed that consumption of food rich in flavonoids (in this experiment it was fried red onion) is associated with an increased resistance of human lymphocyte DNA to DNA strand breakage.
Different *Allium* species, both cultivated (*A. nutans* L., *A. fistulosum* L., *A. vineale* L., *A. pskemense* B. Fedtsch, *A. schoenoprasum* L., *A. cepa* L. and *A. sativum* L.) and wild (*A. flavum* L., *A. sphaerocephalum* L., *A. atroviolaceum* Boiss, *A. vineale* L., *A. ursinum* L., *A. scorodoprasum* L., *A. roseum* L. and *A. subhirsutum* L.), were investigated in order to evaluate the antioxidant properties of their bulbs (ŠTAJNER *et al*., 2008). The results confirmed that the bulbs and leaves of cultivated *Allium* species possess better antioxidant ability in comparison with other wild species, which makes them promising sources of non-toxic natural antioxidants. Therefore, the bulbs and leaves of *Allium* species could be used not only in human diet but also as a source of natural antioxidants and for medical purposes.

3.2. Antimicrobial activity

In 1858. Louis Pasteur described the antibacterial effect of garlic for the first time. During World War II garlic was used as an antiseptic to prevent gangrene (PETROVSKA and CEKOVSKA, 2010). Recent studies confirmed antibacterial properties of garlic. Garlic is effective against gram-positive and gram-negative bacteria, although its extracts were more effective on gram-negative bacteria (DANKERT *et al*., 1979; ELNIMA *et al*., 1983; YOSHIDA *et al*., 1999a, 1999b). JOHNSON *et al*. (2016) showed significant antimicrobial potency of aqueous garlic extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Aqueous extracts of *Allium sativum* and *Allium tuberosum* were tested against penicillin-sensitive *S. aureus* (PSSA) and MRSA. Both extracts were able to reduce staphylococcal infection, although only *A. sativum* showed *in vitro* anti-staphylococcal activity, but neither of them wasn't effective against MRSA (VENÂNCIO *et al*., 2017). HAN *et al*. (1995) showed that biological activity of 1 mg allicin works the same as 15 IU of penicillin. This proved that allicin and other organosulfur compounds present in garlic essential oil are responsible for strong antibacterial activity (ANKRI and MIRELMAN, 1999; MATSUURA, 1997). WALLOCK-RICHARDS *et al*. (2014) reported the first evidence for antimicrobial activity of allicin containing garlic extract against BCC (*Burkholderia cepacia* Complex), the major bacterial phytopathogen for alliums and intrinsically multiresistant human pathogen.
Red onion is not so effective against bacteria compared to garlic (BAKHT *et al*., 2013; BENEKEBLIA, 2004; HUGHES and LAWSON, 1991; MIN KIM *et al*., 1997; SANTAS *et al*., 2010). Onion essential oil showed strong inhibitory effect on growth of gram-positive bacteria. Their water extracts showed strong *in vitro* inhibitory effect on growth of *Escherichia coli*, *Serratia marcescens*, *Streptococcus* sp., *Lactobacillus odontolyticus*, *Pseudomonas aeruginosa*, *Salmonella typhosa* and *Prevotella intermedia* (BAKRI and DOUGLAS, 2005). Raw onion extract showed good antimicrobial activity against *S. aureus* while boiled extract had no activity. In addition, the boiled onion extract showed no antimicrobial activity against both *S. aureus* and *E. coli* (ORTIZ, 2015). AHIABOR *et al*. (2016) confirmed antimicrobial activity of undiluted crude extracts of red and yellow onion (*A. cepa* L.) and shallot (*A. aescalonicum* L.) against *S. typhi*, *E. coli* and *S. aureus*. It is interesting that onion and garlic extracts can prevent growth and development of oral bacteria that cause caries (JAIN *et al*., 2015; KIM, 1997; MISHRA *et al*., 2016; THOMAS *et al*., 2017).
Beside antibacterial effect, onion and garlic are also effective against broad spectrum of fungi and yeasts. Garlic showed strong inhibitory effect against *Candida* sp., *Cryptococcus*

sp., *Trichophyton* sp., *Epidermophyton* sp. and *Microsporum* sp. (ANKRI and MIRELMAN, 1999; DAVIS *et al*., 1994; SHAMS-GHAHFAROKHI *et al*., 2006; YAMADA and AZUMA, 1977) as well as against fungi that produce mycotoxins such as *Aspergillus parasiticus*, *A. niger*, *A. flavus* and *A. fumigates* (BENEKEBLIA, 2004; YIN and TSAO, 1999). Recent research showed antifungal effect of garlic essential oil against three *Trichophyton* species (*Trichophyton rubrum*, *T. erinacei*, *T. soudanense*) responsible for severe mycoses in humans (PYUN and SHIN, 2006). Water extract of red onion was effective against *Malassezia furfur* and *Candida* sp. (SHAMS-GHAHFAROKHI *et al*., 2006). The newest studies are consistent with those published research and confirm strong antifungal activity of garlic aqueous and petroleum ether extracts as well as garlic oil tested on *Candida albicans* (LI *et al*., 2016), *Aspergillus*, *Curvularia* and some *Dermatophyte* species (SULEIMAN and ABDALLAH, 2014).

Allium species stimulate growth of probiotic bacteria (genus *Lactobacillus* and *Bifidobacterium*), which can ferment oligosaccharides, prebiotics that human organism cannot digest on its own. Ingestion of probiotic bacteria may reduce the severity and frequency of diarrheal diseases, and development of colon cancer as well as improve lactose digestibility among lactose-intolerant individuals (KAPLAN and HUTKINS, 2000). LI *et al*. (2016) investigated the effect of onion juice on milk fermentation by *Lactobacillus acidophilus*. The onion juice stimulated the growth of probiotic bacteria *L. acidophilus* and maintain their viability. The authors assume that whole constituents of onion juice including polyphenols, sulfur compounds, minerals and fructans together are responsible for stimulation of growth and viability of *L. acidophilus.*

3.3. Anti-inflammatory activity

It has been proved that most of the *Allium* members possesses anti-inflammatory effect. Mechanism of their anti-inflammatory action can be explained through the interaction of oxidative stress and inflammation. Overexpression of pro-inflammatory enzymes such as iNOS (inducible nitrogen oxide synthetase) and COX-II (cyclooxygenase) is noticed in some diseases such as atherosclerosis, some types of cancer and inflammatory diseases. Their overexpression leads to production of pro-inflammatory mediators such as NO (nitrogen oxide) and PG (prostaglandin) which play important role in maintenance of normal blood pressure, inflammatory processes, wound healing and regulation of body temperature (REUTER *et al*., 2010).

However, their overexpression can also lead to development of diseases such as colon cancer, atherosclerosis, inflammatory bowel diseases, multiple sclerosis and Alzheimer disease. iNOS and COX-II enzymes which activate mediators are under control of transcriptional factor NF-κB. NF-κB controls the expression of more than 150 different genes included in regulation of inflammatory processes. Recent research has been proved that antioxidants can inhibit activation of NF-κB, and thus reduce the symptoms, and development of mentioned diseases and conditions (DEVI *et al*., 2009)

Quercetin and apigenin are potent inhibitors of nitric oxide (NO) and prostaglandin E2 (PGE2) production induced by lipopolysaccharide (LPS) in the macrophage cell line J774A.1 (RASO *et al*., 2001). They modulate iNOS and COX-II enzyme expression. Reduced activity of both enzymes by the action of apigenin and quercetin affects the expression of NF-κB (WADSWORTH and KOOP, 1999). Modulation of iNOS and COX-II enzymes by these two flavonoids may be important in the prevention of inflammation and indicate that they might be used as potent anti-inflammatory agents. Three active garlic compounds, caffeic acid, S-allyl cysteine and uracil inhibited UVB-induced skin wrinkle formation in mice by decreasing oxidative stress as follows: i) caffeic acid and S-allyl cysteine decrease oxidative stress by direct affecting and modulating NF-κB or AP-1

(activator protein 1), ii) all three active compounds achieve anti-inflammatory effect through the suppression of COX-2 and iNOS (KIM *et al*., 2013). SAC, caffeic acid, uracil, diallyl trisulfide, diallyl sulfide and other garlic compounds can inhibit activity of NF-κB by inhibiting transcription of cytokine genes involved in proinflammatory response. Phenolic extract isolated from onion skin showed the potency of inhibition human LDL cholesterol oxidation and COX-2 expression even at concentrations as low as 5 μg/ml (ALBISHI *et al.,* 2013). QUINTERO-FABIÁN *et al*. (2013) examined the effects of alliin in lipopolysaccharide- (LPS-) stimulated 3T3-L1 adipocytes by RT-PCR, Western blot, and microarrays analysis of 22,000 genes. The phosphorylation of ERK1/2, which is involved in LPS-induced inflammation in adipocytes, was decreased following alliin treatment. Also, the gene expression profile by microarrays showed an upregulation of genes involved in immune response and downregulation of genes related with cancer. Their results have shown that alliin is able to suppress the LPS inflammatory signals by generating anti-inflammatory gene expression profile and by modifying adipocyte metabolic profile.

Garlic prevents oxidation of low density lipoprotein (LDL). The oxidation of LDL is under control of lipoxygenase (LOX) and inducible NO synthase (iNOS) whose activity is regulated by transcriptional factor NF-κB. Oxidized LDL promotes adhesion and platelet aggregation, which stimulates the inflammatory process, resulting in damage of cardiovascular system and development of diseases such as atherosclerosis. Garlic water extract and its main component, S-allyl cysteine (SAC) inhibits iNOS in human macrophages and thus reduce oxidation of LDL (GENG *et al*., 1997; IDE and LAU, 2001). Thanks to its strong antioxidative capacity, SAC can remove superoxide radical that reacts with NO and thus prevent its activity. Diallyl-disulfide (DADS) also can decrease NO production, expression of proinflammatory cytokines and protein expression in RAW264.7 murine macrophage cell line (SHIN *et al*., 2013) Garlic extract and its main compound SAC, may be useful for prevention of atherosclerosis (Kim *et al*., 2001).

KIM *et al*. (2005) isolated four flavonols from garlic leaves and shoots and checked their antioxidant activity by measuring inhibition of lipoxygenase (LO) and hyaluronidase (HYA). Quercetin showed the strongest antioxidant activity while its glycosides, isoquercitrin and reynoutrin showed slightly lower activity. Allicin, the active substance of garlic, inhibited degradation of IκB (inhibitor of transcriptional factor NF-κB). The degradation of IκB releases active NF-κB, which is then translocated to the nucleus and regulates gene expression. Inhibition of NF-κB reduce proinflammatory cytokine expression and synthesis of inflammatory enzymes COX/LOX (LANG *et al*., 2004). ALI *et al*. (2000) demonstrate that *A. cepa* and its thiosulfates can inhibit COX and LOX activity, as well as platelet aggregation in the blood. They also confirmed antiasthmatic activity of onion extract and ability to inhibit cancer development. JAISWAL and RIZVI (2014) explored the effect of onion extract in the regulation of PON1 (paraoxonase 1) expression in male Wistar rats subjected to mercuric chloride induced oxidative stress. PON1 is an important enzyme with capability of protection against low-density lipoprotein (LDL) oxidation. Onion extract significantly decreased mercuric chloride induced oxidative damage by up-regulating the activity of PON1 enzyme and protected against LDL-oxidation and lipid peroxidation.

3.4. Antiproliferative and anticancer activity

Antiproliferative effect of *Allium* species have been reported in several studies using different cell cultures. SEKI *et al*. (2000) reported about the ability of garlic and onion oil to inhibit proliferation of human promyelocytic leukemia cells. Interesting experiment of SIEGERS *et al*. (1999b) showed that garlic powder and extract alone are unable to inhibit

tumor cell growth, but when extract and garlic powder are supplemented simultaneously, there was a significant inhibition of cell proliferation. They suggested that antiproliferative effect of garlic is due to the catalytic break-down of alliin induced by alliinase enzyme. After catalytic breakage of alliin, allicin and polysulfides are synthesized, which are responsible for such a powerful antiproliferative effect.

It was also found that onionin A, a natural compound isolated from onion, strongly inhibited ovarian cancer cell proliferation, so it could be used for additional treatment of patients with ovarian cancer (TSUBOKI *et al*., 2016). FREDOTOVIĆ *et al*. (2017) demonstrated strong antiproliferative effect of methanolic extracts of two onion species, triploid onion *A.* × *cornutum* and *A. cepa*, on glioblastoma and breast cancer cell lines. The inhibition of glioblastoma cell growth was stronger than the inhibition of breast cancer lines in both onion extracts treatments.

As we have already mentioned, *Allium* species are rich sources of flavonoids and organosulfur compounds. The molecular mechanism of antiproliferative action is related with both type of compounds. Quercetin glucosides (Q 3,4'-diglucoside and Q 4'-monoglucoside), isolated form four *Allium* species, *A. chinese* (Chinese onion), *A. sativum* (garlic), *A. cepa* L. (onion) and *A. fistulosum* L. (Welsh onion) showed to be an effective inhibitor on cell growth of HepG2, PC-3 and HT-29 cells (PAN *et al*., 2018). These results suggest that combination of quercetin glucosides may be responsible for antiproliferative activity of onion extracts on cancer cells (LI *et al*., 2014). CHANG *et al*. (2013) demonstrated that among all isolated glucosides from onion extract, Q 4'-monoglucoside exhibited the highest antioxidant activity on cancer cells. They also suggested that quercetin glucosides may act as activators of apoptosis in different cancer cell lines. The antiproliferative action of flavonoids may involve the inhibition of the prooxidant process. Flavonoids are effective inhibitors of xanthine oxidase (CHANG *et al*., 1993), COX and LOX (MUTOH *et al*., 2000), and therefore they inhibit tumor cell proliferation. Also, they can inhibit polyamine biosynthesis. Ornithine decarboxylase is enzyme involved in polyamine biosynthesis and correlated with the rate of DNA synthesis and cell proliferation in different tissues. Different experiments showed that flavonoids are able to inhibit ornithine decarboxylase and decrease polyamine level leading to inhibition of DNA synthesis and cell proliferation (MAKITA *et al*., 1996; TANAKA *et al*., 1997a, 1997b). The antiproliferative effect of organosulfur compounds seems to be related with their ability to induce apoptosis. The study of SUNDARAM and MILNER (1996) showed that DADS (diallyl disulfide) can reduce the growth of colon, lung and skin tumor cells. DADS' antiproliferative activity depends on the presence of both diallyl and disulfide groups. The antiproliferative mechanism of DADS and DATS (diallyl trisulfide) is related with the increase of the intracellular free-calcium concentration which may activate calcium-dependent endonuclease leading to DNA fragmentation and apoptosis (SAKAMOTO *et al*., 1997; SUNDARAM and MILNER, 1996). DATS possesses anticancer activity both *in vitro* and *in vivo*. BOROWSKA *et al*. (2013) showed that DATS is more toxic to prostate cancer cells than to noncancerous epithelial cell line PNT1A. Cytotoxicity of DATS toward PNT1A cell line was reduced which means that PNT1A cells had higher resistance to DATS-induced cell death than PC-3 cells. DADS also induced apoptosis in HL60, HCT-15 and neuroblastoma cells by the production of ROS followed by the induction of p53 and activation of caspase-3 which leads to cell death (FILOMENI *et al*., 2003; HONG *et al*., 2000; KWON *et al*., 2002). DADS can also affect the cell cycle in human HCT-15 cells. It can induce G2/M phase arrest and inhibition of p^{34} kinase activity because of the decreased p^{34}/cyclin B1 complex formation and subsequent p^{34} hyperphosphorylation (KNOWLES and MILNER, 2000). YANG *et al*. (2009) demonstrated that DADS induced ROS formation and accumulation of Ca^{2+} ions, which induced the apoptosis by promoting caspase-3 activity in COLO 205 cells. Apoptosis is followed by the increased level of Fas,

phosphorylation of Ask1 and JNK, p53 and apoptotic genes Bak and Bax leading to the decrease of the antiapoptotic genes Bcl-2 and Bcl-X_L.
The flower extract of *A. atroviolaceum* induced antiproliferative effect against the Hella cell line. The mechanism of its action seems to involve the induction of apoptosis through the down regulation of the antiapoptotic *Bcl-2* gene expression and activation of caspase-9 and caspase-3 mitochondrial death pathway (KHAZAEI *et al*., 2017). SOUID *et al*. (2017) first demonstrated that dried aqueous extract (DAE) of *A. roseum* possesses an excellent antiproliferative effect on Chronic Myeloid Leukemia (CLM) K562 cells. The mechanism of DAE antiproliferative action was associated with the inhibition of both $ERK_{1/2}$ and PI3K/Akt signaling antiapoptotic pathway and induction of apoptotic caspase pathway. Furthermore, DAE wasn't toxic for normal mouse fibroblast cells. Chemical analysis of DAE identified a different organosulfur compounds and high amount of allicin, which are known as potent anticancer agents.
Based on these findings, we can conclude that *Allium* vegetables possess exceptional antiproliferative activity against different cell lines (BOIVIN *et al*., 2009). This activity is in correlation with their anticancer properties observed in many epidemiological and laboratory studies (FLEISCHAUER and ARAB, 2001; GALEONE *et al*., 2006; MILNER, 2001; TALALAY and FAHEY, 2001). These effects are related with both flavonoid and organosulfur compounds.
Many epidemiological studies have shown that regular consumption of *Allium* vegetables is associated with decreased risk of developing cancer, especially gastrointestinal cancer (BIANCHINI and VAINIO, 2001; GAO *et al*., 1999; LAWSON, 1998; WITTE *et al*., 1996). Two cohort studies and meta-analysis of 19 case-control studies showed that consumption of high levels of *Allium* vegetables reduced risk for gastric cancer development (ZHOU *et al*., 2011).The review with summarized findings from epidemiological studies based on *Allium* vegetables intake and gastric cancer risk once again confirmed the beneficial effect of this vegetables (GUERCIO *et al.,* 2014). They concluded that high intakes of alliums, mainly garlic and onion, can prevent gastric cancer development. New epidemiological studies are in line with those of GUERCIO *et al*. (2014). Results from case-control study and meta-analysis confirmed that high intake of alliums, garlic and onion, may reduce gastric cancer risk (TURATI *et al*., 2015).
A large cohort study carried out in 10 European countries: the European Prospective Investigation into Cancer and Nutrition (EPIC) confirmed the link between increased intake of fruits and vegetables (including *Allium* vegetables) and decreased risk of stomach cancer development (GONZÁLEZ *et al*., 2006). In a case-control study conducted in China, it was demonstrated that higher intake of red onion, garlic, Chinese onion, Welsh onion and leek was correlated with lower risk of esophagus and stomach cancer (GAO *et al*., 1999). The protective role of this vegetables on stomach, esophageal and duodenal cancer development is likely to be associated with their antibacterial activity against *Helicobacter pylori*, a bacterium that plays a key role in the development of these types of cancer (O'GARA *et al*., 2000; YOU *et al*., 1989). Similar investigation conducted in Shanghai confirmed the link between increased intake of food rich in garlic, red onion, chive and leek and decreased risk of prostate cancer development (HSING *et al*., 2002). Although these studies suggested that regular garlic and allium vegetables consumption reduce gastric cancer risk, KIM *et al.* (2018) found no statistically significant association between garlic intake and reduction of gastric cancer risk.
It was shown that consumption of red onion and garlic can inhibit colorectal cancer development. Six different studies showed that increased intake of raw or cooked garlic reduces the risk of colorectal cancer development from 10 to 50% (FLEISCHAUER *et al*., 2000). In contrast, meta-analysis of eight different cohort studies showed that large intake of *Allium* vegetables does not reduce risk for colorectal cancer (ZHU *et al*., 2014). TURATI

et al. (2014) found that high garlic intake is associated with a 15% reduction in colorectal cancer risk. These case-control studies showed a link between high intake of alliums and a reduction of colorectal adenomatous polyps.
YANG *et al*. (2009) found that the intake of raw onion and garlic can be protective against esophageal cancer in Taiwanese man. Other case-control studies reported that consumption of 7 or more portion of onions per week can be significantly protective against esophageal cell carcinoma (GALEONE *et al*., 2006).
It has also been confirmed the association between consumption of *Allium* vegetables and lower risk for lung (SANKARANARAYANAN *et al*., 1994) and brain cancer development (HU *et al.,* 1999). The case-control study was carried out among Iranian woman with newly diagnosed breast cancer to investigate the effect of onion, garlic and leek on the breast cancer. The results suggested that the consumption of garlic and leek significantly reduced a risk for breast cancer development, while high consumption of cooked onion may be related with higher risk of breast cancer development (POURZAND *et al.,* 2016).
Several studies have reported that organosulfur compounds as well as flavonoids, such as quercetin 3,4′-diglucoside and quercetin 4'-monoglucoside can protect from cancer development in different tissues. They can activate or inactivate a wide variety of mechanisms to prevent cancer propagation. There are several proposed mechanisms of chemopreventive action of biologically active substances from *Allium* vegetables: inhibition of oxidative damage thanks to their strong antioxidant activity (LAWSON *et al.,* 1991; NUUTILA *et al.,* 2003; PERCHELLET *et al*., 1990; SYED *et al*., 2013), inhibition of cell proliferation and induction of apoptosis (ADAMS-CAMPBELL, 2011; ALTUNDAL *et al*., 2016; ANTONY and SINGH, 2011; ATASHPOUR *et al*., 2015; AQUILANO *et al*., 2010; CHAN *et al*., 2013; CHEN *et al*., 2011; CHOU *et al*., 2010; DUO *et al*., 2012; HERMAN-ANTOSIEWICZ and SINGH, 2004; ICIEK *et al*., 2012; KELKEL *et al*., 2012; KIM *et al*, 2013; KNOWLES and MILNER, 2000; LEE YJ *et al*., 2015; LEE WJ *et al*., 2015; LEE *et al*., 2010; NAGARAJ *et al*., 2010; NIU *et al*., 2011; PERCHELLET *et al*., 1990; REN *et al*., 2015; RUSSO *et al*., 2014; VIDYA PRIYADARSINI *et al*., 2010; YI *et al*., 2010a,b), inhibiting of procarcinogens activation by the their effect on cytochrome P450 (CHEN *et al*., 2009; CHOI *et al*., 2011; KUMAR and PANDEY, 2013; WARGOVICH, 2006; YANG *et al*., 2001b), inhibiting DNA damage (anticlastogenic effect) (FREDOTOVIĆ *et al*., 2014; HAZA *et al*, 2011; KHANUM *et al*., 2004; LAU *et al*., 1990), inhibition of lipoxygenase and cyclooxygenase activity (anti-inflammatory effect) (ADÃO *et al*., 2011; ALI, 1995; BELMAN *et al*., 1989;BYUN *et al*., 2013; DIRSCH and VOLLMAR, 2001; CHANG *et al*., 2005; ELBERRY *et al*., 2014; PARK, 2011; PERCHELLET *et al*., 1990; PRASANNA and VENKATESH, 2015; ROSE *et al*., 2005; WANG *et al*., 2012). Additional studies need to be done to confirm the chemopreventive effect of this vegetables as well as the exact mechanism of their action.

4. CONCLUSIONS

For centuries, *Allium* vegetables have been very suitable ingredients in a wide variety of cuisines worldwide. They produce specific chemicals, mostly organosulfur compounds and flavonoids that give them the unique taste and smell but also are responsible for their biological activity. Beside these main active compounds, they possess small amounts of saponins which contribute to the health benefits of these vegetables. The antimicrobial activity of *Allium* species has been proved against a wide range of bacteria, fungi and yeasts. Numerous studies confirmed them as potent antioxidants capable to catch and inactivate free radicals and therefore prevent oxidative cell damage. Strong antioxidant activity was found to be related mainly with sulfur-compounds as well as flavonoids.

Different studies have indicated that *Alliums* possess anti-inflammatory properties via scavenging reactive oxygen species and through the inhibition of proinflammatory cytokines expression. *Alliums* possess remarkable antiproliferative activities against different cell lines which is directly related with their anticancer activities. There are proposed several potential mechanisms for their anticancer action, but it is necessary to perform more research to confirm them as effective anticancer agents.

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PAPER

LEMON VERBENA (*LIPPIA CITRIODORA* KUNTH) BEVERAGES: PHYSICOCHEMICAL PROPERTIES, CONTENTS OF TOTAL PHENOLICS AND MINERALS, AND BIOACCESSIBILITY OF ANTIOXIDANTS

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ABSTRACT

In this study, dried lemon verbena (*Lippia citriodora* Kunth) leaves were used for functional beverage production with addition of sucrose and/or sweeteners. Carbonated or mineral enriched versions of these beverages were also produced. The highest antioxidant bioaccessibility was obtained from sucrose added and natural mineral water enriched beverage both in FRAP (47.01 %) and CUPRAC (11.13 %) assays. In general, all beverages were rich in potassium and the mineral enriched beverages were high in magnesium and calcium. The ascorbic acid value was maximum in carbonated beverages. While energy reduced beverages were rich in total phenolics, sucrose added and natural mineral water enriched beverages showed the highest functionality in terms of bioaccessible antioxidants.

Keywords: antioxidant capacity, bioaccessibility, herbal tea, lemon verbena, total phenolics

1. INTRODUCTION

There has been a growing interest in functional food consumption as a result of an increment in the public awareness on healthy and balanced diet. Functional foods are recognized with their health benefits, which related to the high ratios of bioactive components like ascorbic acid, carotenoids, vitamin E and phenolic compounds (MIRON *et al*., 2013). These natural antioxidants are widely distributed in several parts of higher plants (bark, flowers, fruits, leaf pods, seeds, stems and wood) and have been investigated worldwide. Herbs and spices are among the most important sources of antioxidants and phenolics (YANISHLIEVA *et al*., 2006). Lemon verbena (*Lippia citriodora*), one of these herbal plants, grows spontaneously in South America and is cultivated in North Africa and Southern Europe. It is preferred for refreshing effect, which associated with its lemony flavor since ancient times. Fresh leaves are mainly used as a flavoring agent in fish and poultry dishes, vegetable marinades, salad dressings, jams, puddings, and beverages, while dried leaves are mostly used in herbal teas and sorbets (FUNES *et al*., 2009). Generally, the leaves of this plant are reported to possess digestive, antispasmodic, antipyretic, antioxidant, analgesic, anti-inflammatory, sedative and stomachic properties. In addition, it has been used in infusions for the treatment of asthma, cold, fever, flatulence, colic, diarrhoea and indigestion (RAGONE *et al*., 2007).

Previous studies on lemon verbena mainly concentrated on its chemical characterization and revealed the presence of several phenolic compounds like iridoids, flavonoids, phenolic acids and phenylpropanoids especially verbascoside (FUNES *et al*., 2009). It is well known that the health benefits of polyphenols are proportional with the amount of consumption. The bioaccessibility, the amount of an ingested antioxidative compounds that is available for absorption in the gut after digestion (PALAFOX-CARLOS *et al*., 2011), should be known, since the phytochemicals must be previously available to exert their biological activities (COSTA *et al*., 2014). Bioaccessibility of constituents might be changed according to physical properties and chemical composition of the food, its release from the food matrix, possible interactions with other food components, the presence of suppressors or co-factors and individual digestive capacity (PARADA and AGUILERA, 2007). There is very limited information about the bioaccessibility of antioxidant capacity of herbs or herbal drinks.

Today, herbal tea is traditionally prepared by brewing the fresh or dried leaves, stems, roots or seeds with boiled water or using ready to infuse commercial tea bags. However, brewing methods and parameters of plant species differ from one to another. Due to erroneous brewing practices, the expected health benefits may be minimized and even adverse effects might seen.

The main objective of this study is to produce a new alternative beverage to benefit from the nutritional and functional properties of lemon verbena in different forms. Together with physicochemical properties, total phenolic content of beverages, antioxidant capacity and bioaccessibility of antioxidants were investigated. Furthermore, the optimization of the process parameters with regard to prevent the mistakes applied in traditional techniques and so producing microbiologically safe, standard and value added beverages were aimed in this research.

2. MATERIALS AND METHODS

2.1. Chemicals

All the reagents were analytical grade. TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) and bile salts were purchased from Fluka (Switzerland). Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), neocuproine (2,9-dimethyl-1,10-phenanthroline), DPPH (2,2-diphenyl-2-picrylhydrazyl), methanol, sodium carbonate, gallic acid, oxalic acid, nitric acid, sodium bicarbonate and sodium hydroxide were purchased from Sigma Aldrich (Germany). Pepsin, pancreatin, iron (III) chloride hexahydrate, Folin-Ciocalteu reagent, 2,6 dichlorophenol indophenol, copper (II) chloride, ammonium acetate and hydrochloric acid were supplied from Merck (Germany).

2.2. Materials

Lemon verbena was purchased from Kurtsan Food Company (Bursa, Turkey) in the dried form for infusion in the production process of the beverages. Natural lemon flavor was obtained from Aromsa Company (Kocaeli, Turkey) and natural mineral water was acquired from Uludag Beverage Company (Bursa, Turkey).

2.3. Methods

2.3.1 Beverage production

The beverages were produced at a pilot scale. A synthetic cloth bag was used as infuser. The plant was infused (1 %) in a boiled water without additional heating. Then the extract was cooled down to room temperature and used as the main ingredient of the beverages. Afterwards the addition of other ingredients, mixtures were plate filtered (plate filter 60X60 CFP, Zambelli Enotech, Italy).
The brix values of the first group of beverages (sucrose added beverages-SB) were adjusted to 8°±0.5 by using sucrose, citric acid, ascorbic acid and natural lemon flavor. The second group (Energy reduced beverages-EB) was produced using aspartame and acesulfame-K along others. The amount of substituted aspartame and acesulfame–K in place of sugar were calculated according to the relative sweetness values of these sweeteners. Due to the replacement of some sucrose to sweeteners for energy reduction, the brix values of these group beverages were adjusted to 5°±0.5. Four different types of beverage were also produced by carbonation-C (ECB, SCB) and mineral enrichment-M (EMB, SMB). Eventually, six different beverages were formulated (Fig. 1).
The carbonation process was applied to improve the refreshing trait of produced beverages. The antimicrobial agents (Na-benzoate and K-sorbate) were used in these beverages, so they were not subjected to pasteurization process, since CO_2 and antimicrobial agents provided for preservation.
In the mineral enrichment process, the volume of the tap water was reduced by half and the remaining volume was replaced with natural mineral water.
The beverages produced according to these steps mentioned above, were filled into 200 mL glass bottles and pasteurized (except carbonated ones) after capping. The bottles were then cooled and stored at room temperature until analyzed (Fig. 1).

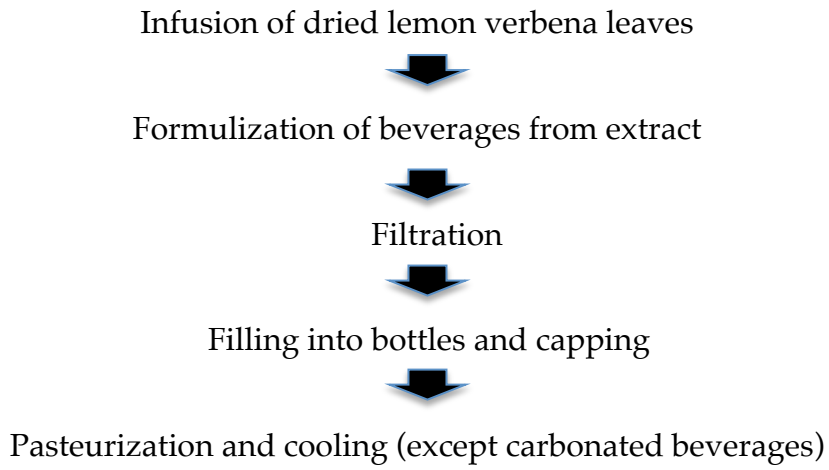

Figure 1. Flow diagram of beverage production.

2.3.2 Evaluation of some physicochemical properties of raw material and beverages

The brix (water soluble dry matter) (AOAC, 1990), titratable acidity and pH (AOAC, 2005), ascorbic acid (SIMONA *et al.*, 2011), color values (*L, a, b*) (BAKKER *et al.*, 1986) and turbidity (TAJCHAKAVİT *et al.*, 2001) analyses were performed on beverages. Moisture content, ascorbic acid, and color (*L, a, b*) were determined in dried lemon verbena leaves to show the physical and chemical properties of raw material. The brix contents of the beverages were analysed using a digital RA-500 model KEM refractometer. Sevencompact pH/Ion Mettler Toledo pH meter, Shimadzu UV 1208 model spectrophotometer, HunterLab Colour Analyzer (MSEZ4500L; HunterLab, Virginia, USA), Hach turbidimeter (Hach, 2100Q) instruments were used for other analyses. All analyses were repeated three times to ensure accuracy of the results.

2.3.3 Mineral content

To determine the quantity of Fe, Ca, Mg and K in raw material and beverages, NMKL (2007) method was applied using Agilent 7500 CX (Agilent Technologies, USA) model ICP-MS. Argon (99.9995 % pure, Linde, Turkey) was used as a carrier gas on ICP-MS and ultra-pure water (18 MΩ·cm at 25 °C resistivity) was generated by purifying distilled water with the New Human Power I (Scholar-UV-PF, 15L/Hr) water purification system. Standard stock solutions containing 1000 mg L^{-1} of each element (Merck, Darmstadt, Germany) were used to prepare the calibration standards. Standards were prepared in 1 % (v/v) HNO_3 on a daily basis.
Approximately 0.5 g of sample was weighed directly in polytetrafluoroethylene (PTFE) flasks after adding 4 mL HNO_3 (Merck Suprapur-65 %) and 1 mL H_2O_2 (Merck Ultrapur-35 %), then flasks were digested in Berghof MWS 3+ (Germany) microwave digestion system. After cooling down to room temperature, the digested sample was transferred to a 50 mL volumetric flask and diluted with distilled water. All samples were filtered with 0.45 μm filters (Hydrophilic PVDF Millipore Millex-HV) prior to the analysis. The instrumental operating conditions for ICP-MS were as follows; Plasma Parameters; RF-Power: 1550 W, Sample depth: 8 mm, Carrier Gas Flow: 0.95 L min^{-1}, Make up Gas Flow: 0.15 L min^{-1},

Nebuliser Pump 0.1 rps, Spray Chamber Temperature: 2 °C, Detector Parameters; Discriminator: 8.0 mV, Analog HV: 1710 V, Pulse HV: 1490 V.
For mineral determination of water used in process, HNO_3 was directly added to water sample. While K ve Ca were analysed by using Eppendorf Elex 6361 model flame photometer, Mg and Fe were analysed with Perkin Elmer Optima 2100 DV model ICP-OES (AYYILDIZ, 1983).

2.3.4 In Vitro Digestion Procedure

To evaluate the functional properties of the beverages, total phenolics, antioxidant capacity and bioaccessibility of antioxidants were investigated. The samples directly taken from each beverage was used for determining the total phenolics and antioxidant capacity. An in vitro digestion enzymatic extraction method, slightly modified version of the one described by VITALI *et al.* (2009) that mimics the conditions in the gastrointestinal tract was used to measure the bioaccessibility of antioxidants. The simulation of gastrointestinal conditions using commercial digestive enzymes (pepsin and pancreatin) is a widely used method for specifying the potential availability of bioactives. Briefly, 10 mL of distilled water and 0.5 mL of pepsin (20 g L^{-1} in 0.1 mol L^{-1} HCl) were added to 1 mL of sample, pH was adjusted to 2 by using 5 mol L^{-1} HCl and sample was incubated at 37 °C in a shaking water bath for 1 h. Simulation of gastric digestion was stopped by the addition of 1 M $NaHCO_3$ (to adjust pH to 7.2). 2.5 mL of bile/pancreatin solution (2 g L^{-1} of pancreatin and 12 g L^{-1} of bile salt in 0.1 M $NaHCO_3$) and 2.5 mL of NaCl/KCl (120 mmol L^{-1} NaCl and 5 mmol L^{-1} KCl) were added to the sample and simulation of intestinal digestion was conducted for the following 2 h. Samples were centrifuged at 3500 rpm for 10 min and the supernatant was used for the analysis. After gastric and intestinal digestion, digested samples were used to determine the bioaccesibility of antioxidants. Bioaccessibility was calculated as the percentage of antioxidant capacity.

2.3.5 Total phenolics

Folin-Ciocalteu reagent was used to determine total phenolics as described by SPANOS and WROLSTAD (1990). In brief, an aliquot (0.25 mL) of sample, 2.3 mL of deionised water and 0.15 mL of Folin-Ciocalteu reagent (FC/Water, 1:5 v/v) were mixed within 10 mL volumetric flask and vortexed for 15 s at room temperature. After 5 min, 0.3 mL of 35 % Na_2CO_3 was added and mixed thoroughly. The absorbance of the mixtures was measured at 725 nm, after incubation for 2 h at room temperature. Water was used as the blank, and gallic acid (GA) solution was used for the calibration of the standard curve (R^2=0.9835). The phenolic content was expressed as gallic acid equivalents (GAE).

2.3.6 Antioxidant capacity

Antioxidant capacity determination methods aim to measure capacity of the antioxidant substances reliably and quickly. So far, various methods were developed, yet only several of them are recommended to be used together to determine the in vitro available antioxidant capacity.

2.3.6.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Antioxidant capacity of the beverages and digested extracts by using DPPH free radical was measured using a modified version of the KATALINIC *et al.* (2006). In this method, the antioxidants were allowed to react with the stable radical in methanolic solution. The

discoloration of the DPPH radicals was monitored through the decrease in absorbance at a characteristic wavelength during the reaction. First, 0.1 mL sample was added to 3.9 mL of 6 x 10^{-5} M methanolic solution of DPPH radical and vortexed (Vortex Mixer Classic, Velp Scientifica, Italia) for 15-30 s. The reaction was allowed to occur in dark at room temperature for 30 mins. A trolox calibration curve (R^2=0.9951) was obtained by measuring the reduction in absorbance of the DPPH solution in 517 nm in the presence of different concentrations of trolox (10-100 μmol L^{-1}).

2.3.6.2 FRAP (ferric reducing antioxidant power) assay

According to BENZIE and STRAIN (1996), 3 mL of daily prepared FRAP reagent (incubated at 37 °C) was mixed with 300 μL of distilled water and 100 μL of the test sample (or extraction solvent for the reagent blank). The test samples, digested extracts and blank were incubated at 37 °C for 60 min. At the end of incubation, absorbance was measured immediately at 595 nm. The FRAP reagent was prepared by mixing 25 mL of 0.3 mol L^{-1} acetate buffer (pH 3.6), 2.5 mL of 20 mmol L^{-1} $FeCl_3$ x 6 H_2O and 2.5 mL 10 mmol L^{-1} TPTZ solution in 40 mmol L^{-1} HCl. The results were calculated from calibration curve as μmol trolox mL^{-1} for beverages (R^2=0.9975).

2.3.6.3 CUPRAC (cupric ion reducing antioxidant capacity) assay

Estimation of cupric ion reducing antioxidant capacity was achieved based on the method of APAK *et al.*, (2008). 1 mL 1 x 10^{-2} M copper (II) chloride + 1 mL 7.5 x 10^{-3} M neocuproine + 1 mL 1 M ammonium acetate were added to x mL 10^{-3} M antioxidant neutral solution + (1-x) H_2O:VT =4 mL; and the final absorbance was measured at 450 nm after 30 min (R^2=0.9947). Calculation of antioxidant capacity was done as trolox equivalents (TEAC values).

2.3.7 Sensory analysis

The beverages were evaluated based on color, odor, appearance and taste by a panel consisting of 10 trained members using a ranking test (ALTUĞ and ELMACI, 2011). According to this test, the panelists ranked the samples from their best favourite one to their least favorite by giving points between 1 and 6. As a result of this statistical test, samples within the range of 22 - 48 did not show any statistical difference while the samples that ranked below 22 (the mean of all of the panelist's point values) were preferred and samples ranked above 48 were rejected at the 95 % probability level ($p < 0.05$).

2.3.8 Statistical analysis

The experiment was conducted in a completely randomized design with three replications. The results were statistically evaluated by one-way analysis of variance (ANOVA) using the JMP software package version 6.0 (SAS Institute Inc. NC, 27513). When significant differences were found ($p < 0.05$), the Least Significant Difference (LSD) test was used to determine the differences among means.

3. RESULTS AND DISCUSSION

3.1. Physico-chemical properties

In this study, the physico-chemical properties of dried lemon verbena leaves and beverages were performed in addition to the bioactive content. The moisture content of dried lemon verbena leaves was measured as 7.24±0.04 g 100 g^{-1}. This result was regarded appropriate by the limits Turkish Standards Institute, which was defined as 10 g 100 g^{-1} max for dried herbs (ANONYMOUS, 2014). EBADI *et al.*, (2015) similarly determined 9 g 100 g^{-1} moisture content in *Lippia citriodora* Kunth leaves with different drying methods.
Physico-chemical properties of lemon verbena beverages are shown in Table 1. All data in Tables are expressed as means±standard deviations (n = 3).
The brix and titratable acidity values of the beverages were adjusted based on the results of market survey on similar beverages conducted prior to the production (ice tea, natural and flavored mineral water, lemonade). The differences between the water soluble dry matter contents of all beverages were found statistically significant ($p < 0.05$) (Table 1). Titratable acidity of the beverages was lower than the sum of citric acid and ascorbic acid added in the production. This decrease could be explained by neutralization of the acidity by the hardness of the water used or the buffer salts of the extract (CEMEROĞLU, 2007). The highest acidity value of ECB could be the result of dissolution of carbondioxide in aqueous medium as carbonic acid (ADEBAYO *et al.*, 2015). The pH values of all beverages were similar with the pH values of some commercial herbal tea beverages determined by PHELAN and REES (2003).

Table 1. Physico-chemical properties of lemon verbena beverages.

Analyses	SB	SCB	SMB	EB	ECB	EMB
Water soluble dry matter (g 100 g^{-1})	8.10±0.00^{a}	7.50±0.01^{c}	7.80±0.00^{b}	5.20±0.00^{e}	5.50±0.10^{d}	4.90±0.00^{f}
Titratable acidity (g 100 mL^{-1})*	0.18±0.00^{b}	0.17±0.01^{c}	0.15±0.00^{d}	0.18±0.00^{b}	0.19.±0.01^{a}	0.14±0.00^{e}
pH	3.32±0.01^{d}	3.62±0.00^{b}	3.79±0.01^{a}	3.33±0.00^{d}	3.54±0.00^{c}	3.79±0.02^{a}
Ascorbic acid (mg 100 mL^{-1})	20.48±0.27^{bc}	28.15±0..30^{a}	20.30±1.60^{bc}	19.36±2.06^{c}	29.57±0.47^{a}	22.19±0.36^{b}
Color						
*L***	11.63±0.15^{cd}	12.20±0.26^{ab}	12.53±0.31^{ab}	12.03±0.49^{bc}	11.47±0.31^{d}	12.70±0.17^{a}
*a***	-1.63±1.00^{a}	-3.73±0.49^{d}	-3.07±0.38^{bcd}	-2.37±0.46^{ab}	-2.60±0.20^{abc}	-3.37±0.49^{cd}
*b***	5.33±0.21b^{c}	6.07±0.11^{a}	4.90±0.40^{c}	5.70±0.20^{ab}	5.70±0.26^{ab}	5.40±0.17^{b}
NTU***	5.14±0.22^{b}	2.40±0.04^{f}	4.02±0.20^{c}	6.00±0.10^{a}	2.86±0.14^{e}	3.38±0.19^{d}

SB: sucrose added beverage; SCB: sucrose added and carbonated beverage; SMB: sucrose added and mineral enriched beverage.
EB: Energy reduced beverage; ECB: Energy reduced and carbonated beverage; EMB: Energy reduced and mineral enriched beverage.
*: Citric acid.
** *L* means lightness of the beverages, and ranges from black to white (0-100). A negative value of *a* indicates green, while *a* positive value indicates red-purple color. Positive *b* indicates yellow and negative blue color.
*** Nephelometric Turbidity Unit.
(Mean values within a column with unlike superscript letters were significantly different ($p < 0.05$))

The ascorbic acid content of beverages was contributed as both antioxidant source and acidity regulator with citric acid. The highest ascorbic acid contents of SCB and ECB samples were related with the production process in which samples were carbonated after addition of antimicrobial agents instead of pasteurization. Therefore, the thermal degredation of ascorbic acid was not occured in these beverages. Reduction of ascorbic acid in other samples was related with the heat treatment (LEŠKOVÁ *et al.*, 2006). The differences between all samples were found to be statistically significant ($p < 0.05$). The ascorbic acid content of lemon verbena leaves were 5.73±0.27 mg 100 g^{-1} and the higher ascorbic acid values of beverages compared to the raw-material in this study can be explained by the intentional addition of ascorbic acid during production. COSTA *et al.*, (2012) reported the ascorbic acid content as 7.20±0.20 mg 100 mL^{-1} in a beverage prepared with the 0.75 % infusion of rooibos red tea leaves (*Aspalathus linearis*) and 21.40±0.10 mg 100 mL^{-1} in a beverage produced with 1 % green tea (*Camellia sinensis*) infusion. SUNA *et al.*, (2016) determined the ascorbic acid content of a mineral enriched *Erica arborea* herbal tea beverage as 28.15±0.30 mg 100 mL^{-1}. The difference between the results might be due to the variety of herb used in the process or its concentration. Additionally, TAMER *et al.*, (2016) determined ascorbic acid content of linden enriched lemonade as 597.9 mg kg^{-1} which was found higher than our results as a consequence of high amount of ascorbic acid coming from lemonade.

According to the results of the statistical analysis, the overall color parameters for beverages are affected significantly by the different production processes ($p < 0.05$). As shown in Table 1, beverages were in green and yellow colour tones. While EMB had the highest *L* (brightness) value, ECB had the lowest value. The highest turbidity value (6.00±0.1) was measured to be the sample EB, which also had the highest total phenolic content (Table 3). It is stated that, chemical turbidity consisted of complexing of some organic compounds like starch, polyphenols, proteins, pectin and minerals like Cu and Fe (SIEBERT, 1999). Accordingly, polyphenols may increase the turbidity by complexing with metals and proteins over time (BEVERIDGE, 1997). The color and turbidity values of the products could not be compared due to the absence of similar beverage.

3.2. Mineral contents

Mineral content of raw-material and beverages are given in Table 2. It is clear that plants take minerals, which are essential for their life-cycle, from soil. The mineral composition of the plants is also affected from the physical and chemical characteristics of soil, usage of natural or artifical fertilizers, storage conditions, climate, region etc. Additionally, the mineral content of the infusions obtained from these plants vary with the mineral amount in leaves and extraction yield (COSTA *et al.*, 2002). The beverages were rich in K, Ca and Mg as the raw material used in their production. The same minerals were found in similar values in herbal tea of *Lippia multiflora* in various researches (TETTEY-LARBI, *et al.* 2015; CHRISTINE, *et al.* 2017). Fe, Ca, Mg and K content of water used in our process was 0.03 mg L^{-1}, 13.0 mg L^{-1}, 1.72 mg L^{-1} and 0.51 mg L^{-1}, respectively. The highest amount of Ca and Mg were determined in SMB and EMB whereas ECB had the highest amounts of Fe and K. While dried lemon verbena leaves and water used in process were rich in Ca, K was higher in beverages. It could be explained with different extraction ratios of minerals. Regarding this issue, PYTLAKOWSKA *et al.* (2012) studied efficiency of mineral extraction from tea leaves and classified the elements in herb infusions as highly-extractable (>55 %) as K; moderately-extractable (20-55 %) including Mg, Na, P, B, Zn and Cu and poorly-extractable (<20 %) comprising Al, Fe, Mn, Ba, Ca and Sr. The researchers also determined the content of some minerals in *Melissa officinalis* infusion (1 %, 10 min brewed) and reported values of 3.90±0.07 $\mu g\ g^{-1}$ (mg kg^{-1}) for Fe, 21.0±0.20 mg kg^{-1} for Ca, 198.00±5.00 mg

kg^{-1} for Mg, 1449.00±12.00 mg kg^{-1} for K, respectively. Similarly, ÖZCAN and AKBULUT (2008) analysed the mineral content of *Melissa officinalis* infusion (2 %) as 20.11 mg 100mL^{-1} for Ca. While our Fe and Ca values were higher, Mg and K contents were lower than these studies. It could be related to raw material and differences in processing conditions.

Table 2. Mineral content of dried lemon verbena leaves (mg kg^{-1}) and beverage samples (mg L^{-1}).

	Fe	Ca	Mg	K
SB	0.11±0.00c	89.72±2.93d	28.87±0.56d	184.35±4.58c
SCB	0.26±0.00b	103.58±1.16c	30.26±0.37c	193.48±2.81b
SMB	0.01±0.00d	122.48±5.17b	43.47±1.29b	121.38±3.37e
EB	0.12±0.05c	88.98±0.74d	28.57±0.25d	197.38±1.10b
ECB	0.37±0.00a	87.51±0.7d	27.98±0.22d	222.28±1.71a
EMB	0.01±0.00d	129.85±1.85a	46.21±0.75a	130.78±1.160d
Raw-material	82.61±1.30	24800±0.02	2814.78±20.00	15600±0.00

*Mean values within a column with unlike superscript letters were significantly different ($p < 0.05$).

3.3. Phenolic contents

Phenolic compounds play an important role regarding in antioxidant effects and defensive action in plants or the human body (BOO *et al.,* 2012). Total phenolic contents of the beverages were given in Table 3.

Table 3. Total phenolic contents of the beverages.

Sample	Total phenolics (mg GAE 100 mL^{-1})
SB	209.35±3.77d
SCB	239.33±14.64c
SMB	232.25±7.67c
EB	360.78±14.11a
ECB	302.55±11.84b
EMB	306.80±7.50b

Mean values within a column with unlike superscript letters were significantly different ($p < 0.05$).

In the average of 2.74-4.71 % of dried lemon verbena polyphenols (7653.46±36.62 mg GAE/100 g^{-1}) were transferred to beverages after extraction process. Energy reduced beverages had generally higher total phenolic contents than sucrose added beverages. Among these EB showed the highest total (360.78±14.11 mg GAE 100 mL^{-1}) phenolic value. Due to the lack of literature data dealing with phenolic content of lemon verbena beverages, our results were compared with polyphenol content of similar types of samples. For instance, DIAS *et al.* (2012) analysed total phenolic content of *Melissa officinalis* infusion (0.50 %) in lyophilized extracts of commercial bag and granulated forms as 959.54±10.02 mg GAE mL^{-1} and 657.06±0.80 mg GAE mL^{-1} respectively, whereas COSTA *et al.*, (2012) determined green tea infusions' (1 %) total phenolic content as 29.10±0.50 mg GAE 100 mL^{-1}. GUIMARÃES *et al.,* (2011) studied infusion and decoction of lemon verbena

and fennel mixed herbs and reported phenolics value of decoction and infusion respectively as 389.73±4.00 mg GAE g^{-1} and 438.08±0.19 mg GAE g^{-1}. ATOUI *et al.*, (2005) also determined total phenolic content of 1.25 % infusion of chinese green tea (*Camellia sinensis*) and greek mountain tea (*Sideritis syriaca*) approximately as 507 mg GA 100mL^{-1} and 37 mg GA 100 mL^{-1}, respectively. Our results were different from the literature data owing to the differences in extraction method, concentration and the material. According to VELIOGLU *et al.*, (1998) antioxidant activity and total phenolics were found to be positively and significantly correlated.

3.4. Antioxidant capacity and bioaccessibility

The antioxidant capacity and the bioaccessibilities of the antioxidant capacity of the beverages are given in Table 4. The differences between the antioxidant capacities of the beverages were significant ($p < 0.05$). The different values obtained from the three assays are due to the different reaction mechanisms or kinetics of the test materials (i.e. DPPH, Cu^{2+} and Fe^{3+}) quenched/reduced by beverages react according to different mechanism and kinetics (JESZKA-SKOWRON *et al.*, 2015). However, the extraction method or differences in concentration make difficult to compare the results. YOO *et al.*, (2008) reported DPPH antioxidant capacity of lemon verbena infusion as 86.90±2.20 %. SB, EB and ECB samples analysed with respectively FRAP, CUPRAC and DPPH assays showed higher antioxidant capacity values than those of others (Table 4).

Table 4. Bioaccessibility of antioxidant capacity of the beverages (µmol trolox mL^{-1}).

Sample	DPPH (µmol trolox mL^{-1*})	DPPH Bioaccessibility (%)	FRAP (µmol trolox mL^{-1*})	FRAP Bioaccessibility (%)	CUPRAC (µmol trolox mL^{-1*})	CUPRAC Bioaccessibility (%)
SB	27.17±0.09a	0.99	32.00±0.78a	19.63	73.35±1.27b	9.26
SCB	23.75±0.70b	0.93	19.97±0.25cd	27.84	62.65±6.89c	10.39
SMB	24.85±2.64b	0.85	17.55±2.35d	47.01	30.46±6.53e	11.13
EB	27.64±0.08a	0.58	26.60±1.82b	32.07	81.72±2.11a	7.13
ECB	27.78±0.30a	0.50	22.61±1.51c	43.74	69.24±0.84bc	7.47
EMB	27.03±0.27a	0.89	19.19±1.72d	37.99	40.83±1.97d	9.65

Mean values within a column with unlike superscript letters were significantly different ($p < 0.05$).

The bioaccesibility ratios were higher in order of FRAP, CUPRAC and DPPH assays. Especially SMB had the highest bioaccessibility obtained from FRAP (47.01 %) and CUPRAC (11.13 %) assays. A possible reason of varying bioaccessibility of antioxidant capacity values could be associated with several factors related to the process conditions, chemical interactions with other phytochemicals, biomolecules present in the food and also the protocols used for the measurements (PARADA & AGUILERA, 2007). The bioaccessibility of antioxidant capacity was decrased in this study. In agreement with our data, HENNING *et al.*, (2014) reported a 21.5 % and 8.1 % decrease of TEAC (trolox equivalent antioxidant capacity) in green tea and grape seed samples during in vitro simulated digestion. In another study, ŞAHAN *et al.*, (2017) reported the bioaccessibility of chicory cultivars with $TEAC_{CUPRAC}$ and $TEAC_{DPPH}$ assays between 62.12–73.48 % and 64.66-76.21 %, respectively. DEĞIRMENCIOĞLU *et al.*, (2016) concluded $TEAC_{CUPRAC}$ bioaccessibility of fermented vegetable juices between 16-36 %. Although the polyphenols supply major antioxidant potency of the samples, our results displayed that digestion may

alter antioxidant properties depending on the variations in polyphenol content (HENNING *et al.,* 2014). Besides, it is known that, structural changes after GI digestion affect both further polyphenol uptake and result in a significant loss of the antioxidant activity (RODRÍGUEZ-ROQUE *et al.,* 2013). Data of this study confirmed that different applications and methods may have an influence on the release of total phenols and their antioxidant capacity, therefore, on the bioaccessible fraction. Likewise, phenolic compounds which demonstrate antioxidant capacity would be bioavailable after digestion and might contribute to protection of humans from several diseases (PÉREZ-VICENTE *et al.,* 2002).

3.5. Sensory findings

Sensory properties of the beverages are shown in Fig. 2. For sensorial test, panelists were briefed about the properties of the beverages. The product should have light yellow color related with the extract and should not contain any particles, while the typical odor and taste of the extract should be appreciated. There were no significant differences in color, odor, appearance and taste values between samples and all of the beverages were generally accepted by the panelists ($p < 0.05$).

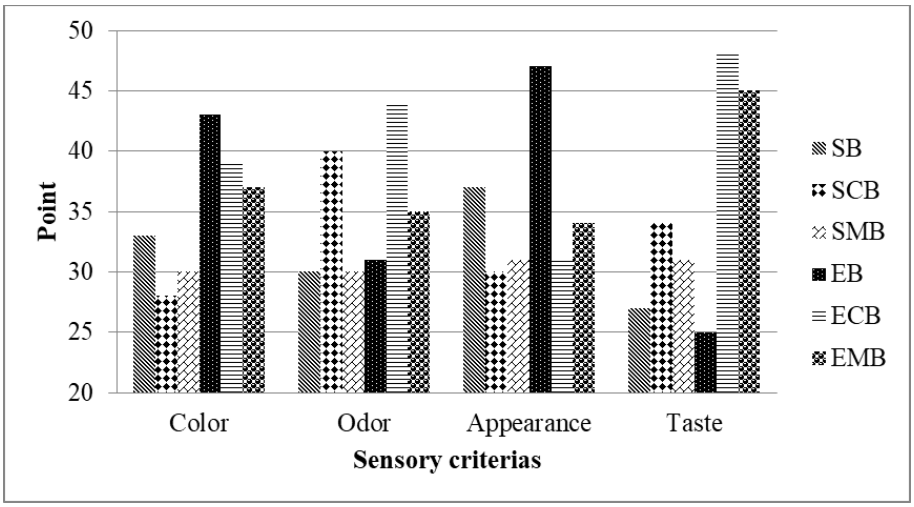

Figure 2. Sensory properties of lemon verbena beverages.

4. CONCLUSIONS

Overall, the sucrose added and also the sucrose added and mineral enriched beverages were found to be the most nutritive beverages among our products because of the highest bioaccessibility values of antioxidant capacity. In general, energy reduced beverages had higher phenolic content. All samples were preferred as sensorial. As a result of growing market interest in functional drinks, natural herbal extracts became popular due to their high bioactive components. In addition, they are easy to formulate and process. Nevertheless, bioaccessibility of a novel herbal tea beverage has not yet been reported in the literature data so far. From this perspective, the bioactive components and their bioaccessibilities in herbal infusions and herbal beverages are needed to be investigated in further studies.

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PAPER

EFFECTS OF CHIA (*SALVIA HISPANICA* L.) SEED ROASTING CONDITIONS ON QUALITY OF COOKIES

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ABSTRACT

Our aims were to analyze physical changes and antioxidant properties of chia seeds roasted under various conditions (160-200°C, 5-15 min) and to investigate the effects on quality characteristics of cookies. Weight loss and water-holding capacity rapidly changed after roasting at 180°C. Fatty acid composition showed no significant change, while antioxidant activity of roasted seeds increased. Cookies were prepared by replacing 3% of flour with roasted chia seeds (180°C, 0-15 min). Baking loss, hardness, and brightness were inversely proportional to roasting time. Roasting of chia seeds affected texture and sweetness scores in a consumer preference test.

Keywords: chia seed, cookie, cooking quality, roasting, sensory evaluation

1. INTRODUCTION

Cookies are low-moisture, tasty, and crispy baked products comprising three main ingredients: flour, sugar, and butter. Cookies are loved by all generations owing to the unique taste and long shelf life. The increase in the awareness about a healthy lifestyle and nutrition among consumers has encouraged many studies on the nutritional ingredients of cookies (JAN *et al*., 2016; PARK *et al*., 2015).
Seeds of chia (Salvia hispanica L.), an annual plant originating from Central America, were used as a staple food by ancient Aztecs in pre-Columbian times (VALDIVIA-LÓPEZ and TECANTE, 2015). Chia seeds are rich in protein (15-25 g per 100 g), fats (30-33 g per 100 g), dietary fiber (18-30 g per 100 g), and unsaturated fatty acids (17.83 g per 100 g) (ÁLVAREZ-CHÁVEZ *et al*., 2008; MARTÍNEZ-CRUZ and PAREDES-LÓPEZ, 2014). In addition, chia seeds exert a strong antioxidant effect, owing to the presence of phenol compounds such as quercetin, kaemferol, caffeic acid, and chlorogenic acid (REYES-CAUDILLO *et al*., 2008; TAGA *et al*., 1984). For culinary uses, chia seeds are processed into flour, seed oil, or whole seeds. Studies on the application of chia seeds to bread, ice-cream, pound cake, and sausage have been carried out (CAMPOS *et al*., 2016; LEE, 2013; PIZARRO *et al*., 2013; SCAPIN *et al*., 2015).
The ancient Aztec roasted chia seeds and used them in the preparation of chiapinolli, a type of flour used in tortillas, tamales, and beverages (CAHILL, 2003). Roasting is a food-processing method employed to impart a unique flavor and color to a food. Roasting is mainly used for the manufacture of coffee, cocoa, and barley tea. Roasting promotes extraction of seed oils and antioxidants owing to modification of the cellular structure of the seed (KIM *et al*., 2002). In addition, roasting is accompanied by a browning reaction, resulting in the production of brown pigments and aroma components. These amino-carbonyl reactants are known to have antioxidant properties and to improve the taste and flavor of the food (DEWANTO *et al*., 2002; LIN *et al*., 2016).
Given the changes in the characteristics of chia seeds after roasting, different qualities of cookies with chia seeds may be obtained by controlling roasting conditions. Few studies have shown the changes in roasted chia seeds and their applications in food industry. The aims of this study were to investigate the effects of roasting on physicochemical and antioxidant properties of chia seeds and to find the optimal roasting conditions by evaluation of the quality characteristics of cookies containing roasted chia seeds.

2. MATERIALS AND METHODS

2.1. Raw materials

Soft flour (CJ Cheiljedang Co., Ltd., Incheon, Korea), sugar (CJ Cheiljedang Co., Ltd.), butter (Seoul Dairy Co., Ltd., Seoul, Korea), and eggs were purchased at a retail market located in Seoul to prepare cookies. Chia seeds, produced in Paraguay in September 2014, were purchased from a supplier (Chowonherb, Seoul, Korea).

2.2. Roasting

The roasting temperature was set to 160°C, 180°C, or 200°C. Chia seeds (10 g) were roasted for 5, 10, or 15 min in an oven (Zippel DE68-04072D, Samsung, Seoul, Korea). Roasted chia seeds were sufficiently cooled at room temperature (25°C) and stored in the freezer (-20°C) until analysis.

2.3. Physical analysis of chia seeds

Changes in the mass of chia seeds during roasting were measured using a scale (Libror EB-2200HV, Shimadzu, Kyoto, Japan). The water-holding capacity (WHC) of the roasted chia seeds was measured by the modified method of ALFREDO *et al*. (2009). Briefly, 1 g of chia seeds was placed in a flask containing 10 mL of distilled water in a water bath (BS-20, Jeio Tech, Gimpo, Gyeonggi) for 24 h incubation at 25°C. The suspension was centrifuged (Universal 32 R, Hettich, Tuttlingen, Germany) at 3,000 rpm for 20 min, and the supernatant was weighed. WHC was expressed as the weight of water held per gram of the sample. The browning index (BI) was measured by the method of MASKAN (2001). Briefly, 10 g of roasted chia seeds was placed on a Petri dish (∅ 90 mm × 15 mm). Color values (CIE L*, a*, b*, and ΔE) of the chia seeds on the petri dish surface were measured with a colorimeter (CR-400, Konica Minolta, Osaka, Japan) in triplicate. Chromameter was calibrated with a standard whiteboard (L = 96.90, a = 0.45, b = 1.49). BI was calculated via the following formula:

$$\text{Browning Index (BI)} = \frac{[100(x - 0.31)]}{0.17}$$

$$x = \frac{(a + 1.75L)}{(5.645L + a - 3.012b)}$$

2.4. Analysis of fatty acids of chia seeds

The fatty acid composition of chia seeds was analyzed for fatty acid methyl esters (FAMEs) by gas chromatography with the methods of AOCS Ce 2-66 and Ce 1-62 (AOCS, 1998). An Agilent Technologies 7890N gas chromatograph with a flame ionization detector and a fused silica capillary column (SP™-2560, 100 m × 0.25 mm internal diameter [i.d.] × 0.2 μm film thickness, Supelco) was used for the analysis. The operating conditions were as follows: split ratio 200:1, flow rate 1.0 mL He/min, injector temperature 225°C, detector temperature 285°C, initial oven temperature 100°C for 4 min, and endpoint oven temperature 240°C for 17 min (an increase at a rate of 3°C/min). FAMEs were identified by comparing their retention times with those of the standards, and their relative concentrations were calculated as grams per 100 grams of a sample.

2.5. Content of total phenols and flavonoids

Total polyphenol content of roasted chia seeds was analyzed by the Folin-Denis method (SINGLETON and ROSSI, 1965). The results were expressed in terms of gallic acid equivalents (mg GAE/g). Total flavonoid content was measured by the method suggested by DAVIS (1947) and expressed as quercetin equivalents (mg QE/g).

2.6. Antioxidant activities of chia seeds

DPPH (1,1-diphenyl-2-picrylhydrazyl) antioxidant activity was measured by the method of MOLYNEUX (2004), and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical-scavenging activity was measured by the procedure of RE *et al*. (1999). Ascorbic acid (Sigma Aldrich, Darmstadt, Germany) was used as a reference. The percentage inhibition at various concentrations (100, 50, 33.3, 25, 20, and 16.66 mg/mL) of each

sample was calculated using the following formula to estimate the half-inhibitory concentration (IC_{50}; mg/mL) values of DPPH and ABTS:

$$Percentage\ inhibition = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

Where $A_{control}$ is the absorbance of 100 μl of ethanol, A_{sample} is the absorbance of a 100 μl sample.

2.7. Cookie preparation

Raw chia seeds (group RT0) and chia seeds roasted at 180°C for 5 min (group RT5), 10 min (RT10), and 15 min (RT15) were freeze-dried (FD8508, Ilshin Biobase Co., Ltd., Gyeonggi, Korea). The unroasted and roasted seeds were pulverized by a high-speed grinder (CRT-04, Hungchuan Machinery Enterprise, Taipei, Taiwan) and filtered through a 40-mesh sieve. Cookies were prepared by the AACC method 10-52 (AACC, 2000) from flour (300 g), butter (180 g), sugar (120 g), and eggs (60 g). Each chia seed powder (groups RT0, RT5, RT10, and RT15) was added to cookies via replacement of 3% (9 g) of the flour. Butter was creamed by means of a mixer (KM400, Kenwood, Havant, Britain) and mixed with sugar and eggs for 5 min. Sieved flour and chia seed powder were added to the mixture. The cookie dough was rolled out, cut into a cylindrical shape (∅ 40 mm × 5 mm), and baked for 20 min at 170°C in an oven (Zipel DE68-04072D, Samsung, Seoul, Korea). The cookies were cooled for 1 h at room temperature (25°C) and then subjected to analysis.

2.8. Cookie properties

2.8.1 Dough density, baking loss, the spread factor, and pH

Dough density was measured as an increase in the volume of water. Baking loss of cookies was calculated by the comparison between cookie mass and dough mass. The spread factor of cookies was calculated by the procedure of AACC (2000). Briefly, six randomly selected cookies were stacked in a line, and their diameter and thickness were measured. The spread factor was calculated by dividing the diameter of a cookie by its thickness. The pH level of dough was measured with a pH meter (SP-701, Suntex Instruments Co., Ltd., Taipei, Taiwan).

2.8.2 Quantification of the color of cookies

The photographs of cookies with roasted chia seeds were taken by a digital camera (Canon IXUS 500, Tokyo, Japan). Color values (CIE L*, a*, and b*) of six cookies randomly selected from each group were evaluated with a chromameter. The total color difference (ΔE) values were calculated as follows:

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

Where ΔL, Δa, and Δb are the difference of L, a, and b value between white board (L: 96.90, a: 0.45, b: 1.49) and sample, respectively.

2.8.3 Hardness of cookies

This parameter was measured repeatedly 15 times for each sample using a rheometer (Compac-100II rheometer Sun, Sun Scientific Co., Ltd., Tokyo, Japan) with a No. 5 probe (∅ 5 mm). The operating conditions were as follows: mastication test mode (mode 20), 5 mm distance, and 120 mm/min table speed.

2.9. Sensory evaluation

A consumer preference test of the cookies was conducted by a panel of 30 people (age 25-35 years, 15 males and 15 females). Samples were served on a white plate with water. Cookies were evaluated for appearance, flavor, texture, an oily taste, sweetness, savory taste, and aftertaste. A method with a 7-point scale, 1 = strongly dislike and 7 = strongly like, was employed to measure the seven parameters.

2.10. Statistical analysis

All results obtained by measurements were subjected to one-way analysis of variance (ANOVA) in the SPSS software ver. 23.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD). The significance of each experimental value was analyzed by Duncan's multiple-range test ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Physical analysis of chia seeds

The mass loss and WHC of roasted chia seeds are shown in Table 1. At all temperatures, the mass loss increased with roasting time. The mass of roasted chia seeds decreased with an increase in the temperature. In particular, the mass loss of chia seeds roasted at 180°C or 200°C was significantly higher than that of the unroasted samples. Wang and lim (2014) described weight loss as a general indicators for determining the roasting degree, and it was divided into two stages: the first stage mainly due to vaporize, and the another stage by formation of CO2 and volatiles compounds. Since the moisture content of the raw chia seed was 7.00% (data not shown), further mass reduction can be presumed to be due to several volatile compounds such as CO2, aldehydes, ketones, alcohols and pyrazines produced by the Maillard reaction between sugars and amino acids (XIAO *et al*., 2014). WHC of the roasted chia seeds significantly decreased with roasting time. Protein denaturation and extraction of seed surface oil during heat treatment may contribute to the rapid decrease in WHC. ÖZTÜRK *et al*. (2002) reported that WHC affects the hardness and spreadability of cookies. Thus, the process of roasting of chia seeds was expected to affect the quality of cookies. During the roasting process, the food color gradually darkened due to the formation of a brown pigment from the Maillard reaction and caramelization. This change is related to the roasting temperature and time, which are the major parameters that control roasting conditions and processes (KAHYAOGLU and KAYA, 2006). The BI measurement results on chia seeds roasted at 160°C, 180°C, and 200°C are presented in Fig. 1. An increase in the BI is an indicator of the nonenzymatic browning process such as the Maillard reaction and caramelization (HELOU *et al*., 2016). The BI showed no significant change at 160°C but increased at temperature >180°C as a function of roasting time. These results indicated that the Maillard reaction proceeded actively in chia seeds above 180°C.

Table 1. Mass loss and water-holding capacity (WHC) of chia seeds roasted under.

Roasting condition		Mass loss (g / 100 g)	WHC (g of water retained /g of sample)
Temperature (°C)	Time		
-	0	-	7.67±0.01[a]
	5	3.27±0.31[f]	7.16±0.01[b]
160	10	5.93±0.12[d]	6.71±0.00[c]
	15	6.87±0.50[c]	3.99±0.00[e]
	5	5.53±0.50[de]	7.69±0.01[a]
180	10	6.93±0.50[c]	4.59±0.00[d]
	15	7.93±0.31[b]	3.84±0.01[e]
	5	5.00±0.72[e]	7.65±0.01[a]
200	10	7.47±0.23[bc]	3.88±0.01[e]
	15	9.40±0.60[a]	3.95±0.01[e]

[a,b,c,d,e,f]Means with different superscript letters in each column are significantly different according to Duncan's multiple-range test ($p < 0.05$).

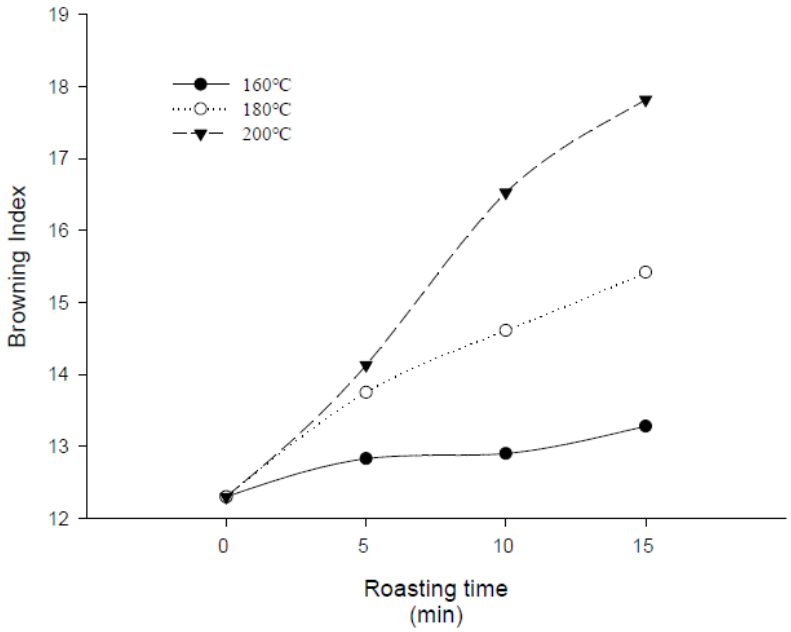

Figure 1. Changes in the browning index (BI) of roasted chia seeds with roasting time.

3.2. Fatty acid composition

It is known that the fatty acid composition of seed oil determines the physicochemical and nutritional characteristics of seed oil, and can be changed by roasting (HAMA, 2017). There was no significant difference in the fatty acid composition between raw chia seeds and those roasted under different conditions (Table 2). It is expected that there will be no significant change in the quality of seed fat in the temperature and time conditions that we

set. YOSHIDA and TAKAGI (1997) reported no significant difference in the quality of sesame oil roasted at temperatures below 200°C. Because the roasting conditions in our study were set within the range of normal baking temperatures (160-200°C), the mechanism underlying the change in the fatty acid composition after roasting at higher temperatures and for longer periods remains unclear. YEN (1990) reported that the fatty acid composition of sesame, which is similar to that of chia seeds, changed rapidly (to linoleic acid in particular) after roasting at temperatures above 240°C. Further studies are needed to evaluate the effect of roasting of chia seeds above 200°C.

Table 2. Fatty acids composition of chia seeds roasted under various temperature and time conditions.

Roasting condition		palmitic acid (g/100 g)	stearic acid (g/100 g)	oleic acid (g/100 g)	linoleic acid (g/100 g)	α-linoleic acid (g/100 g)
Temperature (°C)	Time					
-	0	6.28±0.04^{NS}	3.26±0.10^{NS}	6.94±0.22^{NS}	18.90±0.99^{NS}	65.22±0.06^{NS}
	5	6.31±0.19	3.24±0.02	6.86±0.09	18.40±0.00	64.76±0.31
160	10	6.25±0.03	3.19±0.01	6.74±0.07	18.33±0.16	65.06±0.19
	15	6.55±0.48	3.37±0.15	6.70±0.17	18.42±0.18	64.10±0.92
	5	6.26±0.19	3.23±0.04	6.80±0.10	18.44±0.07	64.85±0.15
180	10	6.27±0.07	3.26±0.01	6.83±0.07	18.47±0.05	64.90±0.05
	15	6.21±0.07	3.22±0.07	6.76±0.09	18.33±0.15	65.06±0.37
	5	6.33±0.00	3.32±0.03	6.85±0.00	18.78±0.02	64.34±0.00
200	10	6.18±0.05	3.21±0.01	6.76±0.07	18.43±0.27	64.99±0.36
	15	6.49±0.07	3.33±0.06	7.03±0.13	18.74±0.33	64.12±0.68

NS = not significant in each column according to Duncan's multiple-range test ($p < 0.05$).

3.3. Antioxidant activities of chia seeds

Polyphenol compounds act as antioxidants and can be obtained from fruits, vegetables, and plants. Table 3 shows that the total polyphenol and flavonoid content of chia seeds increased with roasting time. These results were similar to those observed for roasted almonds and sesame seeds (JEONG *et al*., 2004; LIN *et al*., 2016). The amino-carbonyl products formed by the Maillard reaction act as new antioxidants, thereby enhancing the antioxidant properties (DEWANTO *et al*., 2002; NICOLI *et al*., 1999). LEE *et al*. (2013) reported higher total polyphenol and flavonoid contents for green beans as compared to coffee extracts roasted at 190°C. Nonetheless, the reverse observation at a high temperature was reported (over 200°C). Under all temperature conditions, the IC_{50} value of DPPH and ABTS decreased with roasting time (Table 3). Although no significant difference was observed in the IC_{50} of DPPH at 180°C and 200°C, the antioxidant activity tended to increase with an increase in roasting temperature. JEONG *et al*. (2004) mentioned that roasting of sesame seeds at different temperatures and for various periods enhances the antioxidant activities, which positively correlate with the production of melanoidin. DURMAZ and ALPASLAN (2007) demonstrated an increase in the antioxidant activity after the Maillard reaction. Overall, the roasting process was able to enhance the antioxidant activity of chia seeds.

Table 3. Antioxidant activities of chia seeds roasted under various temperature and time conditions.

Roasting condition		Total Polyphenols	Total Flavonoids	DPPH IC_{50}	ABTS IC_{50}
Temperature (°C)	Time	(μg GAE/ g)	(μg QE/ g)	(mg/mL)	(mg/mL)
-	0	358.00±5.62^{f}	286.07±2.68^{e}	26.99±9.51^{a}	38.70±0.62^{a}
	5	369.70±3.12^{ef}	299.21±17.05^{e}	14.94±0.56^{b}	33.96±0.70^{b}
160	10	438.14±24.01^{d}	359.51±6.50^{c}	14.75±0.79^{b}	28.32±1.28^{d}
	15	512.87±1.56^{ab}	394.25±16.34^{b}	11.68±0.63^{b}	23.75±0.46^{f}
	5	383.21±6.79^{ef}	299.38±5.44^{e}	15.29±1.24^{b}	31.08±2.15^{c}
180	10	476.85±48.73^{c}	351.49±11.96^{c}	12.50±0.11^{b}	24.20±0.71^{ef}
	15	517.37±8.68^{ab}	380.41±19.84^{b}	10.32±0.19^{b}	21.36±1.01^{g}
	5	393.12±6.80^{e}	323.37±4.76^{d}	13.47±0.19^{b}	25.44±1.15^{ef}
200	10	493.96±5.63^{bc}	392.93±7.05^{b}	12.47±0.32^{b}	25.94±1.30^{e}
	15	538.09±8.11^{a}	421.58±5.20^{a}	11.67±0.29^{b}	21.36±1.01^{g}

^{a,b,c,d,e,f,g}Means with different superscript letters in each column are significantly different according to Duncan's multiple-range test ($p < 0.05$).

3.4. Cookie properties

3.4.1 Dough density, baking loss, the spread factor, and pH

On the basis of the above results, we roasted chia seeds at 180°C for 5, 10, or 15 min for further experiments. Table 4 shows the properties of cookies containing roasted chia seeds. The density and pH of the dough are major indicators of cookie quality, owing to their effects on the hardness, flavor, and color of a cookie (HADINEZHAD and BUTLER, 2009). No significant difference was observed in dough density (range 1.23-1.26) among the treatment groups. The duration of roasting of chia seeds had no significant effect on pH of the dough; however, pH of the control (6.63) was slightly higher as compared to that of other groups. This observation may be related to the pH difference between the chia seed powder (5.42) and wheat flour (6.82). The baking loss of cookies containing chia seeds, including unroasted seeds, was lower as compared with that of the control (15.82%). These results indicated that the amount of water released during the baking process was smaller because the moisture content (7.00%) of chia seed powder was lower than that of wheat flour. The spread factor determines cookie quality, and high spreadability is indicative of a better cookie (MILLER and HOSENEY, 1997). The spread factor was the lowest in group RT0; RT10 and RT15 had a higher spread factor than the control did. Cookie spreadability tends to decrease with an increase in the concentration of dietary fiber, owing to the increase in the WHC of cookies (MANCEBO *et al*., 2015). Studies have shown 34.4 g of dietary fiber per 100 g of chia seeds (MUÑOZ *et al*., 2013), explaining the lower spread factor for RT0 as compared with that of the control. Nevertheless, WHC significantly decreased after roasting of chia seeds, suggesting that spreadability increased with roasting time.

Table 4. Properties of cookies containing roasted chia seed powders.

Property	Density (g/mL)	pH	Baking loss (g/ 100 g)	Spread factor	*L*	*a*	*b*	*ΔE*	Hardness (N)
Control	1.23±0.02^{NS}	6.63±0.03^{a}	15.82±0.01^{a}	6.15±0.17^{ab}	69.97±1.06^{a}	0.10±1.12^{b}	28.44±0.16^{a}	38.03±0.83^{d}	31.28±3.18^{b}
RT0	1.26±0.02	6.41±0.01^{b}	13.03±0.22^{c}	5.65±0.13^{c}	64.57±1.24^{b}	0.60±0.91^{b}	23.33±0.17^{c}	38.98±0.97^{d}	36.19±0.36^{a}
RT5	1.24±0.02	6.31±0.02^{c}	13.61±0.00^{b}	5.99±0.07^{b}	62.36±1.23^{c}	1.22±0.25^{b}	23.35±0.37^{c}	40.85±0.86^{c}	32.00±1.14^{b}
RT10	1.26±0.02	6.32±0.01^{c}	13.68±0.00^{b}	6.32±0.20^{a}	60.98±0.29^{c}	2.96±0.52^{a}	23.96±0.47^{b}	42.43±0.03^{b}	31.18±1.35^{b}
RT15	1.26±0.02	6.33±0.03^{c}	13.63±0.00^{b}	6.22±0.01^{a}	57.74±0.18^{d}	4.11±0.14^{a}	22.64±0.14^{d}	44.66±0.18^{a}	31.54±1.05^{b}

Control: without added chia seeds, RT0: with chia seeds (raw), RT5: with roasted chia seeds (180°C, 5 min), RT10: with roasted chia seeds (180°C, 10 min), RT15: with roasted chia seeds (180°C, 15 min).

^{a,b,c,d}Means with different superscript letters in each row are significantly different according to Duncan's multiple-range test ($p < 0.05$).

NS = not significant.

3.4.2 Quantification of the color of cookies

A photograph of the cookies is presented in Fig. 2. Longer roasting time of chia seeds corresponded to darker and larger cookies. The *L* (lightness) value of the control sample was higher than that of the cookies with chia seeds and tended to decrease with roasting time. The *a* (redness) value was higher in groups RT10 and RT15 (2.96 and 4.11, respectively). The *b* (yellowness) value was significantly lower for cookies with chia seeds as compared with that of the control (28.44). ΔE (total color difference) was the lowest (38.03) in the control group and increased with roasting time. The dark color of cookies is attributed to the Maillard reaction or caramelization (WALKER *et al*., 2012). The dark color of chia seeds affected ΔE of cookies. Higher pH of cookies contributes to a better browning reaction (MARTINS *et al*., 2000).

Figure 2. Photographs of cookies containing roasted chia seed powders.
Control: without added chia seeds, RT0: with chia seeds (raw), RT5: with roasted chia seeds (180°C, 5 min), RT10: with roasted chia seeds (180°C, 10 min), RT15: with roasted chia seeds (180°C, 15 min).

3.4.3 Hardness of cookies

This parameter is known to be influenced by moisture content, pore development, and density of cookie dough (CHABOT, 1979). As illustrated in Table 4, the difference in hardness between groups control and RT0 was likely to be associated with the high concentration of dietary fiber (in chia seeds) that increases WHC. In comparison to RT0, groups RT5, RT10, and RT15 showed a decreasing trend of hardness; this phenomenon may be due to the inverse relation between hardness and moisture retention. WHC of roasted chia seeds decreased with roasting time. Sugar loss during the Maillard reaction (WONG *et al*., 2008) is reported to affect the hardness of cookies. Our results are in line with those reported by VETTER *et al*. (1986) who found a positive correlation between cookie hardness and the amount of added sugar.

3.5. The consumer preference test

Table 5 shows the results of the survey of consumer preferences regarding the cookies containing chia seeds powder. There were no significant differences in the appearance, flavor, oily taste, sweetness, savory taste, and aftertaste among all the groups. Nonetheless, groups RT5, RT10, and RT15 yielded higher texture scores than the control group did, whereas RT0 had the lowest score. Hardness was found to be the highest for RT10 (36.19 N). These results are similar to those reported in another study (on cookies containing oak mushroom powder), wherein an inverse relation was observed between mechanical strength and texture preference (JUNG and JOO, 2010). On the other hand, our

results contradict the observations reported by JOO and CHOI (2012). As a consequence, RT5 and RT15 showed a high score in overall preference.

Table 5. Sensory preference scores for cookies containing roasted chia seed powders.

	Appearance	Flavor	Texture	Overall preference
Control	5.25±1.25^{NS}	4.70±1.34^{NS}	4.60±1.35^{ab}	4.66±1.33^{b}
RT0	4.75±1.07	4.75±1.45	4.05±1.61^{b}	4.61±1.30^{b}
RT5	5.00±1.08	5.05±1.05	5.20±1.24^{a}	5.09±1.10^{a}
RT10	4.70±1.17	4.90±1.41	5.45±1.28^{a}	4.97±1.25^{ab}
RT15	4.90±1.29	4.95±1.43	5.35±1.27^{a}	5.16±1.27^{a}

Control: without added chia seeds, RT0: with chia seeds (raw), RT5: with roasted chia seeds (180°C, 5 min), RT10: with roasted chia seeds (180°C, 10 min), RT15: with roasted chia seeds (180°C, 15 min).
^{a,b}Means with different superscript letters in each column are significantly different according to Duncan's multiple-range test ($p < 0.05$).
NS = not significant.

4. CONCLUSIONS

In this study, the effects of roasting conditions on chia seeds and cooking quality of cookies containing chia seeds were investigated. This study confirmed that roasting at 160-200°C changes WHC, the BI, and color as well as increases the antioxidant activity of chia seeds. Cookies with roasted chia seeds, especially RT10 and RT15, were superior in spreadability, hardness and overall preference than control. In conclusion, roasting chia seeds for 10 min at 180°C is preferable for making cookies.

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CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM TRADITIONAL DAIRY PRODUCTS OF SMALL-SCALE ALPINE FARMS

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ABSTRACT

This study investigated the prevalence of *Staphylococcus aureus* in raw milk dairy products handcrafted in traditional alpine small-scale farms, and characterised the enterotoxigenicity and resistance to methicillin. Among the analysed samples, 69% exceeded the international microbiological recommendations. The highest counts were observed for cheese or fatty products (~10^6 cfu/g). Conversely, lower contamination levels concerned raw milk and whey cheese (~10^2 cfu/g). A total of 163 *S. aureus* isolates were collected, and the prevalence of MRSA was low (1.7%) but not negligible. The finding of enterotoxins genes in 67% of the isolates is of concern for the public health.

Keywords: alpine small-scale dairies, dairy products, molecular characterisation, staphylococcal enterotoxins, *Staphylococcus aureus*

1. INTRODUCTION

Staphylococcal Food Poisoning (SFP) is one of the most common foodborne diseases worldwide caused by the ingestion of food contaminated with preformed Staphylococcal Enterotoxins (SEs) produced by Staphylococcus aureus (HENNEKINNE and DRAGACCI, 2012). SFP is generally characterised by self-limiting gastrointestinal symptoms, but occasionally the disease can be more severe or even fatal (BENKERROUM, 2017). *S. aureus* is ubiquitous in the environment and it is also one of the major causes of bovine mastitis (Boss *et al*., 2016). Therefore, raw milk and raw milk dairy products may be contaminated with *S. aureus*, due to the shedding of large segments of the organism into milk (D'AMICO and DoNnelly, 2011; ROLA and OSEK, 2016). Moreover, cheese-makers may carry enterotoxin-producing *S. aureus* in their noses or on their hands, and the lack of proper hygienic measures during food processing increases the probability of contamination with *S. aureus*, especially in small-scale artisanal dairies (ANDRÉ *et al*., 2008). Indeed, dairy products are among the foods most commonly involved in SFP outbreaks (BENKERROUM, 2017; DE BUYSER and LAFARGE, 2001).

To date, 23 different SEs have been described and many *S. aureus* strains harbour more than one SEs gene. SEs can be divided into classic types (i.e. A to E) and new variants classified at present as SEs or SEs-like (SEls) based on their ability to cause emesis. SEs are synthesised when *S. aureus* cell density reaches 10^5-10^8 cfu g^{-1}. However, all of these toxins are heat-stable and can therefore be still present in the food even when the microorganism is inactivate or the contamination level is reduced by processing (BENKERROUM, 2017).

Among *S. aureus* strains, those that are Methicillin-resistant (MRSA) have spread in the last decades as hospital-acquired pathogens (HA-MRSA) throughout the world, causing serious life-threatening infections not responding to a lot of antimicrobial treatments. More recently, community-acquired (CA-MRSA) and livestock-associated (LA-MRSA) MRSA have also emerged (BARDIAU *et al*., 2013). MRSA have been identified in different foods worldwide, and several food-borne MRSA outbreaks have been reported demonstrating the zoonotic risk of transmission to humans (DOULGERAKI and NYCHAS, 2017). The screening of *S. aureus* isolates from food of animal origin is therefore essential to estimate the MRSA emergence and the related zoonotic hazard (Bardiau *et al*., 2013).

In alpine regions, in particular in the Lombardy Region, raw milk dairy products are handcrafted in small-scale artisanal dairies built in pastures. These products and practices are closely linked to environmental, economic and tourist aspects, important for the safeguard and development of alpine culture and society (DELLA TORRE, 2017). In this context, traditional cheeses represent appealing products to the new trends of searching for natural and authentic foods, and many of them have been awarded with the Protected Designation of Origin (POD) label (Lombardia, 2014). However, since the hygienic conditions of traditional plants are very diverse, a specific surveillance plan for the safety of cheese produced in pastures has been developed (Italian Ministry of Health, 2017). Data on *S. aureus* isolates recovered from small-scale alpine dairies are however scarce.

The aims of this study were to investigate the prevalence of *S. aureus* and to characterise isolates from the production chain of artisanal raw milk dairy products. In particular, we tested the isolates for the presence of enterotoxins genes and for resistance to methicillin.

2. MATERIAL AND METHODS

2.1. Retrospective database analysis

IZSLER database was asked to obtain data on the prevalence and level of contamination of *S. aureus* for all milk and dairy products samples referred to our laboratory throughout 2016. Samples with $\geq 10^2$ cfu g^{-1} *S. aureus* counts were considered as exceeding international microbiological recommendations *(Reg. CE n. 2073/2005)*.

2.2. Sample selection and S. aureus isolation and identification

S. aureus isolates were collected from products tested within the alpine pastures surveillance plan carried out in the Lombardy Region. The samples were collected in 2016 from a total of 40 small-scale dairies. For *S. aureus* isolation, serial dilution of each sample homogenate were plated on Baird Parker agar + rabbit plasma fibrinogen (RPF agar) (Biolife Italiana, Milano, Italy) and incubated at 37°C for 48 h. Up to 5 characteristic colonies for each sample were planted on blood agar to confirm *S. aureus* hemolytic property. The species identification was confirmed with PCR of the *nuc* gene as described by Brakstad *et al*. (BRAKSTAD and MAELAND, 1992). DNA was obtained by boiling a suspension of the isolates in 2 ml of demineralised water for 5 min at 99°C. The suspension was then centrifuged at 13 000 g for 5 min and supernatant was used for all the following PCR assays.

2.3. Detection of mecA and mecC (methicillin resistance)

The detection of *mecA* and *mecC* (*mecA* homologue) was carried out by means of two PCR protocols using specific primers as reported by Pichon *et al*. (PICHON *et al*., 2012). Briefly, for both *mecA* and *mecC* the PCR reaction mix (final volume 20 µL) contained 1X HotStarTaq Master Mix (Qiagen INC, Hilden, Germany), 0.5 µM of each primer, and 1 µL DNA. The thermic profile was 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 1 min. The final elongation step was performed at 72°C for 10 min. The amplified PCR products were distinguished by electrophoresis in a 2.5% agarose gel (Agarose Multi Purpose, Roche -120 V for 40 minutes), stained with Eurosafe Nucleic Acid Stain (Euroclone, 1X). 100 bp DNA ladder (Invitrogen, 0.5 µg/µL) was included.

2.4. Staphylococcal enterotoxins

Two multiplex PCR protocols were used as described in Bianchi *et al*. (BIANCHI *et al*., 2014) to detect *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selj*, *selp*, and *ser* SEs genes. The electrophoresis conditions were the same for detection of mecA and mecC.

3. RESULTS AND DISCUSSION

3.1. Retrospective database analysis

Among the 4177 samples of milk or milk-derived products of different origin analysed by IZSLER for the presence of coagulase positive staphylococci during 2016, 145 were from small alpine pastures dairies. While for the other dairy products those exceeding the international microbiological recommendations were 22% (867/4032), for the traditional alpine products the proportion increased to 69% (100/145). The level of contamination varied between the different products tested. It is interesting to note that, in general, raw

milk has lower contamination values than the final products (Fig. 1). This could be due to an exponential growth of *S. aureus* in the early phases of cheese-making, when the milk is heated to about 40°C, which is the optimum temperature range for *S. aureus* growth and enterotoxin production (HENNEKINNE *et al*., 2012). In addition, secondary events of contamination from the cheese-maker's skin may happen due to inappropriate hygienic procedures. The only product with low level of contamination is whey cheese; as for its production, the whey is heated above 85°C.

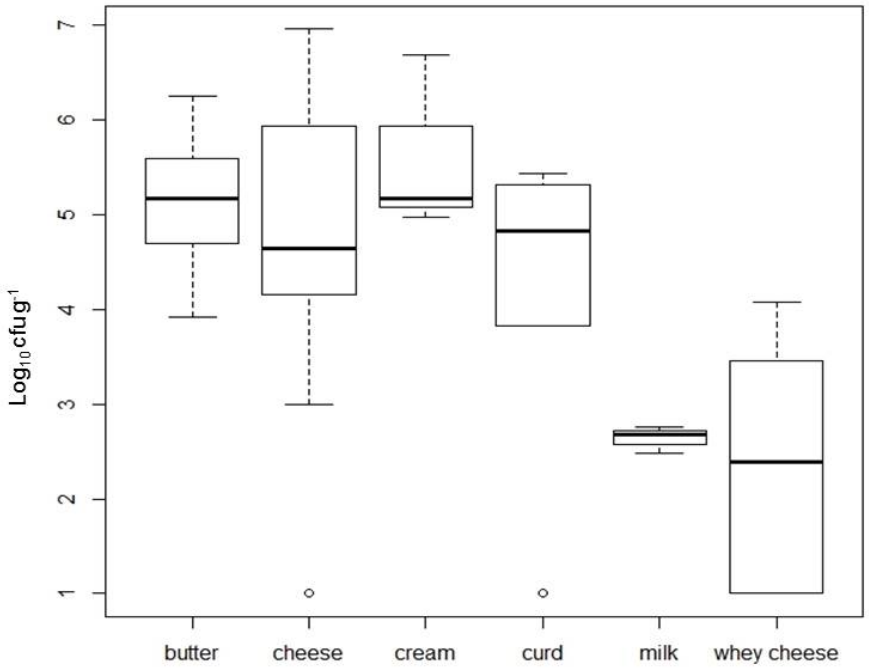

Figure 1. Distribution of the *S. aureus* counts in the different products analysed.

3.2. Isolates

Out of a total of 81 samples (n=23 from raw milk, n=7 from curd, n=39 goat or bovine cheese, n=11 butter, n=1 cream), 172 coagulase positive staphylococci isolates have been collected. A total of 163 (95%) isolates were confirmed as *S. aureus* by the *nuc* PCR, and used for further characterisation (S1).

3.3. MRSA isolates

Among the 163 isolates analysed, 3 (1.7%) were MRSA (*mecA*+; S1). None of the isolates was found to be *mecC* positive. The isolation frequency of MRSA raw milk and dairy products in the present study is consistent with the low prevalence estimates previously reported. Studies from Greece and Italy have revealed MRSA prevalence estimates of 3% (PAPADOPOULOS *et al*., 2018), 3.8% (CORTIMIGLIA *et al*., 2016) and 0.7% (GIACINTI *et al*., 2017). However, given the fact that traditional herding systems on alpine pastures should be extensive and characterized by low rates of antimicrobials administration, the results of this study raise some concern.

3.4. SEs genes detection

At least one SEs gene was found in 67% of the isolates (n=110) and 29 different SEs genes profiles were distinguished (Table 1; S1).

Table 1. Enterotoxins gene profiles. The number at the end of each line represents the number of isolates bearing a specific enterotoxins gene profile. The number at the bottom of each column represents the number of isolates bearing a specific enterotoxin gene.

Enterotoxins genes											
sea	**seb**	**sec**	**sed**	**see**	**ser**	**seg**	**seh**	**sei**	**selj**	**selp**	**no. of isolates**
•											21
•	•		•		•				•		1
•		•									4
•			•								4
•			•				•				2
•			•		•						5
•			•		•				•		13
•						•		•			2
•							•				1
•					•				•		4
•					•				•		1
	•										9
		•									4
		•				•		•			1
			•								9
			•			•		•			1
			•				•				1
			•						•		4
			•		•				•		10
			•		•				•	•	1
				•							1
						•					1
						•		•			2
							•				2
							•	•			1
								•			1
									•		1
					•						2
					•		•	•	•		1
58	10	9	51	1	38	7	8	9	36	1	

sea was detected in 53% (n=58) of the isolates, followed by *sed* (n=51; 46%), *ser* (n=38; 35%) and *selj* (n=36; 33%) genes. SEA and SED are the SEs most frequently associated with SFP, and they have caused outbreaks linked to the consumption of dairy products (HUMMERJOHANN and GRABER, 2014; JOHLER *et al*., 2015; SABIKE and EDRIS, 2014). Twenty-five isolates (23%) contained the SEs gene pattern *sed*, *sej*, *ser*, which are carried on

the same plasmid (BENKERROUM, 2017), with more than half of them (14/25) additionally carrying *sea*. These patterns have been correlated with genotype B *S. aureus* as identified by RS-PCR, which has been reported to be a particularly virulent bovine-associated type of *S. aureus*, and the one most widespread in Switzerland and central European countries (HUMMERJOHANN *et al*., 2014).
For SED and SER, the exhibition of emetic activity is well established (SCHUBERT and BANIA, 2017), while the situation for SElJ remains unclear (BENKERROUM, 2017). Nevertheless, all SEs and SEls belong to the family of superantigens, molecules able to stimulate T-cell proliferation (5000-fold more than in a conventional immune response), driving a massive release of cytokines that cause a life threatening systemic inflammation and toxic shock (TSS). However, to date it is not clear whether exposure to SEs/SEls via food can lead to TSS, and it has been suggested that it is the dose that makes the difference between evolution in TSS or SPF in case of SEs/SEls ingestion (BENKERROUM, 2017). *seh*, which also has been linked to milk-based SFP outbreaks (BIANCHI *et al*., 2014), was detected in 8 isolates (7%).
Improper handling and storage of raw milk and cheese in the early stages of processing contaminated with *S. aureus* can result in the production of SEs, which is also dependent on the initial dose of *S. aureus* contamination (SABIKE *et al*., 2014). Based on our data, the contamination of raw milk averaged 10^{2} cfu g^{-1}, while the average contamination of cheese was 10^{6} cfu g^{-1}, indicating that the alpine pasture process of cheese-making allows exponential growth of *S. aureus*, that reaches a concentration critical for the production of SEs (10^{5}-10^{8} cfu g^{-1};(BENKERROUM, 2017). Indeed, one of the samples included in our study was referred to our laboratory for the suspect involvement in a SFP episode. It was an aged cheese (isolate 20.1) which proved positive for SEA even if the count of *S. aureus* was 10^{3} cfu g^{-1} (data not shown) suggesting that the *S. aureus* population declined during aging. In the European Union, milk-derived products are examined for enterotoxin content only when the number of coagulase-positive staphylococci exceeds 10^{5} cfu g^{-1} (Reg. CE n. 2073/2005). In the light of our findings, this measure may not be appropriate with regard to aged cheese. Moreover commercial kits commonly used for SEs detection are only available for classical SEs (i.e. A to E), leading to an underestimation of the actual incidence of new SEs and SEls. Conversely, the production of SEs/SEls depends on the expression of the SEs/SEls genes, which is dependent on a complex regulatory system influenced by specific environmental conditions (i.e. temperature, pH, a_w, Eh, and salt concentration). It is therefore possible that even when the *S. aureus* contamination reaches critical levels, the SEs/SEls are not produced, highlighting again the modest value of *S. aureus* count as indicator of the presence or absence of SEs in food (BENKERROUM, 2017). Indeed, this situation is routinely observed in our laboratory (data not shown).

4. CONCLUSIONS

Despite the high overall *S. aureus* prevalence (69%) in dairy products manufactured in alpine small-scale farms, the estimated MRSA prevalence in our study was low (1.7%) but not negligible. It is therefore necessary to keep monitoring foods and apply control measures against *S. aureus* in herds to minimise the dissemination of MRSA in animals and subsequently in the community.
Milk and milk products are considered to be of particular significance as a staphylococcal enterotoxin (SE) source. Given the high levels of contamination found in many of the products analysed, the presence of enterotoxigenic strains of *S. aureus* should raise concern. Indeed, the technologies used in alpine pasture dairies are not effective in hindering and limiting the proliferation of *S. aureus* in the early phases of production, and

both human and animal sources can be responsible for contamination. Within this scope, in traditional dairies major benefits could derive from the application of basic good manufacturing practices, starting from control of the health status of cows and milking hygiene. Focused educational interventions and further studies aimed at assessing the routes of transmission of *S. aureus* in small-scale alpine farms could have a great impact on the quality and safety of these precious and peculiar productions.

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PAPER

MODELING AND OPTIMIZATION OF PROCESS PARAMETERS FOR IMPROVING OSMOTIC DEHYDRATION OF KIWIFRUIT

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ABSTRACT

Osmotic conditions for kiwifruit dehydration were optimized using central composite rotatable design and response surface methodology. The optimal conditions included osmotic time of 4.29 h, sucrose concentration of 70 %, and osmotic temperature of 50 °C. At these optimum values, water loss (WL) exhibited a response value of 45.64 %. The optimized condition was validated and found to be fitted with the experimental values. Quadratic regression equations describing the effects of these factors on WL were developed. The osmotic dehydration of kiwifruit was significantly influenced by osmotic temperature, osmotic time, and sucrose concentration. Moreover, osmotic process at relatively high temperatures caused a significantly depletion of V_c content in kiwifruit.

Keywords: kiwifruit, osmotic dehydration, optimization, response surface methodology, water loss rate

1. INTRODUCTION

Kiwifruit is one of the delicious fruits originating from China. Although with full of phytochemicals, vitamins, and minerals, it has a relatively short shelf-life due to its highly perishable nature (KAYA *et al*., 2010). The short shelf life of fresh kiwifruit after harvest is becoming one of the main factors that affect the rapid development of kiwifruit processing industry. Osmotic dehydration is a potential preservation technique to reduce postharvest losses of fruits and vegetables and produce high-quality intermediate-moisture products (AHMED *et al*., 2016). It is widely used for partial removal of water from food materials as a pretreatment before further processing to improve texture characteristics, sensory, functional and nutritional properties (CHIRALT *et al*., 2001; TORREGGIANI and BERTOLO, 2001; TALENS *et al*., 2002; RASTOGI and RAGHAVARAO, 2004). The use of osmotic dehydration can prolong the shelf life of the kiwifruit, as the water content reduction slows down deteriorative reactions.

In osmotic dehydration process, food materials are used to immerse in concentrated solution creating a concentration gradient between the osmotic solution and food materials, the simultaneous mass transfer phenomena mainly include flow of water from the product to the solution, transfer of solute into the product, and leaching of the components of the product. The water is mainly removed by capillary flow and diffusion; meanwhile, leaching and solute uptake occur through diffusion (SHI and XUE, 2009). The rate of mass transfer during osmotic dehydration can be influenced by many factors, such as type and concentration of osmotic agents, temperature, agitation/circulation of solution, food to solution ratio, food structure, shape and size, thickness of food material, and pre-treatment (DA CONCEICAO *et al*., 2012; AKBARIAN *et al*., 2013). Osmotic temperature and solution concentration are the important factor, which affects osmotic mass transfer (TORTOE, 2010). LOMBARD *et al*. (2008) investigated the influence of the process temperature, pressure and osmotic concentration on the mass transfer process during the osmotic dehydration of South African grown Cayenne type pineapple pieces, and the results showed water loss and solids gain increased with temperature and concentration. FALADE *et al*. (2007) studied the osmotic mass transfer phenomenon of water melon slabs using three different concentrations of sucrose solution (40, 50 and 60°Brix). The water loss and solid gain of the watermelon slabs treated with the higher osmotic solution concentration were found to be higher. CAO *et al*. (2006) found that the optimal conditions for osmotic dehydration of kiwifruit slice were 60% sucrose concentration, 30–40° C osmotic temperature, 150 min osmotic time, and 8 mm slice thickness. Meanwhile, the influence of each factor or interactions among the factors should be determined to understand the behavior involved in mass transfer during osmotic dehydration. Individual screening of these factors at a time is laborious and requires much experimental work (FERNANDES *et al*., 2006). Therefore, an optimization technique for osmotic dehydration parameters must be established.

Response surface methodology (RSM) is an effective mathematical and statistical tool. It not only defines the effect of independent variables but also their interaction effects (MYERS and MONTGOMERY, 1995). The present study aims to determine the optimal osmotic dehydration conditions of independent variables (osmotic temperature, osmotic time, and sucrose concentration) for kiwifruit and validate the optimized conditions based on water loss rate by using RSM coupled with central composite rotatable design. In addition, the effects of different sucrose concentrations on kiwifruit water loss (WL) and solid gain (SG) rates were analyzed.

2. MATERIALS AND METHODS

2.1. Sample preparation and osmotic treatment

Fresh kiwifruits of *xuxiang* cultivar were obtained directly from a producer from qinyuan orchard located at *Mei* county (*Shaanxi*, China). The average values of single weight, titratable acidity, and total soluble solid contents in the kiwifruits were 94.2±0.2g, 1.32± 0.34% and 16.2±0.65 Brix, respectively. The kiwifruits were washed and cut into cubes (1 cm × 1 cm × 1 cm) to prepare samples. Then the cube samples were subjected to osmotic dehydration under different osmotic temperatures, osmotic times, and sucrose concentrations based on the experimental design shown in Table 1. The ratio of the sample to the osmotic solution was 1:5 (wt/wt). In order to ensure concentration of the osmotic solution did not change significantly during the experiment, the osmotic system in a vessel was covered with a wrap to prevent evaporation without agitation. The temperature was controlled using a constant temperature water bath. After the osmotic treatment, the samples were removed from the osmotic solution, washed with distilled water, and blotted gently with a tissue paper to remove adhering water for the next analysis (ALI *et al.*, 2010; TYLEWICZ *et al.*, 2011).

2.2. Central composite rotatable design for optimizing process parameters during kiwifruit osmotic dehydration

A central composite rotatable design was used to optimize the conditions for osmotic dehydration of kiwifruit cubes. Osmotic temperature (23-57°C), osmotic time (2.3-5.7h), and sucrose concentration (43-77%, w/w) were taken as independent variables to optimize WL rate and determine the efficiency of osmotic dehydration. The experimental data were fitted using multiple linear regression in Equation (1) (BAŞ and BOYACI, 2007; PENG *et al.*, 2015):

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=(i+1)}^{3} b_{ij} X_i X_j, \quad (1)$$

where Y is the WL rate, *i and j* are the linear and quadratic coefficients, respectively, X_i and X_j represent the independent variables, and b_0, b_i, b_{ii}, and b_{ij} are the regression coefficients.

2.3. Mass transfer determination

The process kinetic variables of WL and SG rates of the samples were calculated as described by SINGH *et al.* (2007) and FALADE *et al.* (2007) by using Equations (2) and (3) with some minor adjustments:

$$WL\% = \frac{(M_0 - m_0) - (M_t - m_t)}{M_0} x100\% \quad (2)$$

$$SG\% = \frac{m_t - m_0}{M_0} x100\% \quad (3)$$

where M_0 and m_0 are the initial mass weights of the kiwifruit samples and the dry solid mass in the samples (g), respectively; M_t and m_t are the mass weights of the samples and the dry solids (g) in the samples after the osmotic dehydration time *t*.

2.4. Analytical determination

Moisture content was determined gravimetrically using a vacuum oven by drying to constant weight (AOAC, 1997).
Ascorbic acid (Vitamin C, V_c) in kiwifruit is the most important vitamin for human nutrition. A standard ascorbic acid solution method was used to determine V_c of kiwifruit based on the titration of ascorbic acid with 2,6-dichloroindophenol in acidic solution by the AOAC's official titrimetric method (AOAC, 1990). The analysis was done in triplicate, and the result for each sample was averaged.

2.5. Statistical analysis

All tests were run in triplicate. Analysis of variance (ANOVA; Origin software, OriginLab Corporation, Northampton, MA, USA) was used to indicate significant differences among tests. Differences were considered significant at the $p \leq 0.01$ level.

3. RESULTS

3.1. Model fitting

In this study, central composite rotatable design coupled with RSM was used to optimize osmotic dehydration for kiwifruit cubes. The response of WL rate was selected on the basis that the response directly influenced the following drying efficiency of the product. The three independent variables, namely, osmotic time, sucrose concentration, and osmotic temperature (coded *A*, *B*, and *C*, respectively) were used to optimize the response of WL rate coded *Y*. The experimental design and obtained values are shown in Table 1. Regression analysis of the response was conducted by fitting a suitable quadratic model in the case of the response variable to assess how well the model represented the data. The results of the analysis of variance (ANOVA) are shown in Tables 2 and 3. According to the estimated regression coefficients of the quadratic polynomial model in Table 2, non-significant factors were removed. The regression model equation in terms of coded value was obtained to express the relationship between the investigated factors and WL rate:

$$Y=37.32 + 2.54 \times A + 2.86 \times B + 8.24 \times C - 0.21 \times A \times B - 0.98 \times \times A \times C - 0.34 \times B \times C - 2.31 \times A^2 - 0.82 \times B^2 - 1.82 \times C^2 \quad (4)$$

The regression model was a function of changes in sucrose concentration, osmotic temperature and time. The *F*-value of 38.80 implied that the model was very significant ($p < 0.01$) and accurately predicted the WL rate of the samples. Moreover, the R^2 of 0.7884 for the model is in reasonable agreement with the adjusted R^2 of 0.9471, and the adequate precision of 21.532 indicates that the model has an adequate signal to noise ratio (Table 3).
As shown in Table 2, osmotic time, sucrose concentration, and osmotic temperature significantly affected the kiwifruit dehydration rate ($p < 0.01$); the model of $Prob > F$ and less than 0.01 indicated that the regression equation exhibited high significance and reliability. Meanwhile, the R^2 of the regression model was found to be 0.972, greater than 90%, indicating the significant relationship between the independent variable and the response value (Table 3).

Table 1. Experimental design and measured values of WL rate for osmotic dehydration of kiwifruit.

No.	*A*-Osmotic time /h	*B*-Sucrose concentration/%	*C*-Osmotic temperature/°C	*Y*-Water loss rate*/%
1	4.00(0)	76.82(+1.68)	40.00(0)	41.20±1.84
2	4.00(0)	60.00(0)	40.00(0)	37.31±1.53
3	5.00(+1)	50.00(-1)	50.00(+1)	40.44±1.97
4	3.00(-1)	50.00(-1)	50.00(+1)	38.41±1.25
5	4.00(0)	60.00(0)	23.18(-1.68)	20.65±1.13
6	5.00(+1)	50.00(-1)	30.00(-1)	23.23±0.89
7	2.32(-1.68)	60.00(0)	40.00(0)	24.64±1.54
8	4.00(0)	43.18(-1.68)	40.00(0)	29.09±1.32
9	4.00(0)	60.00(0)	56.82(+1.68)	44.02±1.28
10	4.00(0)	60.00(0)	40.00(0)	37.31±1.05
11	5.68(+1.68)	60.00(0)	40.00(0)	37.26±1.57
12	4.00(0)	60.00(0)	40.00(0)	37.31±1.44
13	5.00(+1)	70.00(+1)	50.00(+1)	43.81±1.58
14	3.00(-1)	70.00(+1)	30.00(-1)	23.23±1.88
15	3.00(-1)	50.00(-1)	30.00(-1)	17.67±1.05
16	4.00(0)	60.00(0)	40.00(0)	37.31±2.15
17	3.00(-1)	70.00(+1)	50.00(+1)	43.01±1.86
18	4.00(0)	60.00(0)	40.00(0)	37.31±1.43
19	5.00(+1)	70.00(+1)	30.00(-1)	28.34±1.62
20	4.00(0)	60.00(0)	40.00(0)	37.31±1.45

( ) Coded levels for actual values of different parameters during osmotic dehydration of kiwifruit.
*Each combination with triplicate and water loss rate expressed by average value ± standard deviation.

Table 2. ANOVA of WL rate regression model for osmotic dehydration of kiwifruit.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F* Value	*p*-value*
Model	1251.66	9	139.07	38.80	<0.0001
A	88.29	1	88.29	24.63	0.0006
B	111.41	1	111.41	31.08	0.0002
C	926.79	1	926.79	258.54	<0.0001
AB	0.35	1	0.35	0.098	0.7602
AC	7.68	1	7.68	2.14	0.1739
BC	0.91	1	0.91	0.25	0.6251
A^2	76.60	1	76.60	21.37	0.0009
B^2	9.75	1	9.75	2.72	0.1302
C^2	47.52	1	47.52	13.26	0.0045
Residual	35.85	10	3.58		
Lack of Fit	35.85	5	7.17		
Pure Error	0.000	5	0.000		
Total	1287.51	19			

A: Osmotic time (h); B: Sucrose concentration (%); *C*: Osmotic temperature (℃).
*p-Values less than 0.01 indicate model terms are significant, and values greater than 0.1 indicate the model terms are not significant.

Table 3. ANOVA for response surface quadratic model.

Terms	Values
Standard deviation	1.89
Mean	33.94
Coefficient of variation (%)	5.58
R^2	0.9722
Adjusted R^2	0.9471
Predicted R^2	0.7884
Adequate precision*	21.532

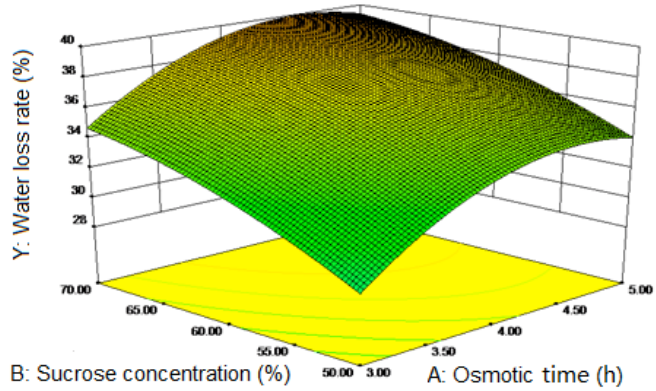
*Adequate precision measures the signal to noise ratio. A ration greater 4 is desirable.

3.2. Linear effect of osmotic variables on WL rate for kiwifruit dehydration

Osmotic time, sucrose concentration, and osmotic temperature significantly affected ($p < 0.01$) the WL rate of the samples at the linear level (Table 2). The coefficients of linear terms in the regression equation (Equation 4) indicated that the WL rate of the samples was mainly influenced by osmotic temperature ($p \leq 0.01$), followed by sucrose concentration ($p \leq 0.01$) and osmotic time ($p \leq 0.01$). In addition, the quadratic terms of osmotic temperature and time ($p < 0.05$) had significant effects, while the interaction of factors had no significant effect ($p > 0.05$) on WL rate within the investigated range (Table 2).

3.3. Interactive effect of osmotic variables on WL rate for kiwifruit dehydration

Considering the interactive effect of osmotic variables, Fig.1 shows the response surface plot and contour plot of kiwifruit WL rate under the effects of input parameters of osmotic time, sucrose concentration, and osmotic temperature. Some profiles for the quadratic response surface plot in the optimization of the two parameters were obtained by keeping the other parameter at zero levels for WL rate. As shown in Fig.1a, the WL rate first gradually increases with increasing osmotic time and sucrose concentration and subsequently maintains a steady state. This trend may be rationalized by considering that the intracellular free water movement speed in kiwifruit accelerates with increasing sucrose concentration. The WL rate will gradually decrease with decreasing amount of free water. When the osmotic pressure between the solution and the internal kiwifruit cells reach the equilibrium, the WL rate will not change. Fig. 1b and 1c demonstrate the same trends that the WL rate first increases and subsequently maintains a steady state under the interaction between two parameters. At lower sucrose concentration with increasing osmotic temperature, the WL rate increases gradually, but as the sucrose concentration increases, the WL rate increases rapidly with increasing osmotic temperature (Fig.1b). Similarly, the interaction between osmotic temperature and time showed similar positive correlation (Fig.1c).This might be due to higher temperature led to swelling and plasticizing cellular membrane and rapider release of moisture from the kiwifruit cells, and viscosity of the sucrose solution was lower at higher temperature, which improved water loss from common surface of kiwifruit and osmotic solution.



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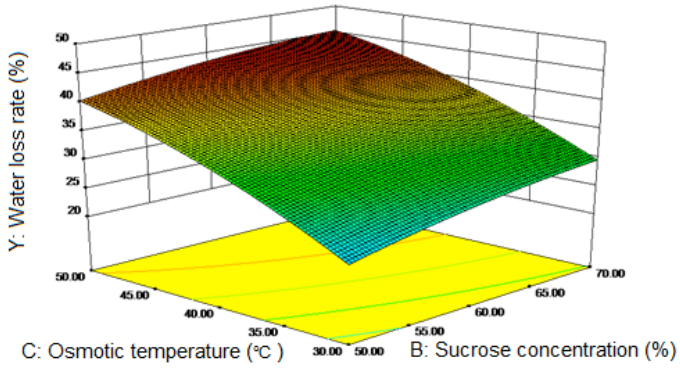

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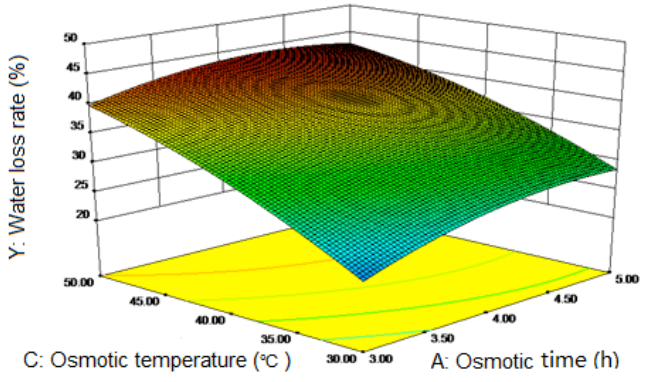

C

Figure 1. Response surface and contour plots for response of kiwifruit water loss rate during osmotic dehydration (**a**: the interaction between the osmotic time and sucrose concentration; **b**: the interaction between the sucrose concentration and the osmotic temperature; and **c**: the interaction between the osmotic time and temperature).

3.4. Determination and experimental validation of optimal conditions

Process parameters can be optimized by finding the stationary point of the model equation in the ranges of tested independent parameters (PENG *et al.*, 2015). The optimal conditions were determined by maximizing the desirability of the response using Design Expert

software (version 6.0.4 by Stat-Ease, Inc., MN, USA). The optimal conditions included osmotic time of 4.29 h, sucrose concentration of 70 % , and osmotic temperature of 50 ℃ with a predicted response value of 45.64 % for WL rate. A confirmation test was conducted using the optimum parameters identified by RSM to verify the adequacy of the regression models. The fitted values predicted by the models were compared with the experimental data. Under these optimal conditions, the experimental value of WL rate is consistent with the predicted value with 3.89 % standard deviation (Table 4). These values did not show any significant difference ($p > 0.05$). Response surface method is reasonable and effective for optimization of WL rate of kiwifruit.

Table 4. Optimal conditions and validation.

Osmotic time (h)	Osmotic temperature (°C)	Sucrose concentration (%)	Predicted water loss rate (%)	Experimental water loss rate (%)*	Standard deviation (%)
4.29	50	70	45.64	43.81	3.89

*Experimental water loss rate expressed by average value with triplicate to eliminate the errors.

3.5. Effect of sucrose concentration on WL and SG rates

Change in WL and SG rates for osmotic dehydration of kiwifruit under different sucrose concentrations was showed in Fig. 2. The results showed that the WL and SG rates have similar trends in 50%, 60%, and 70% sucrose concentrations. Osmotic time had a substantial effect on mass transfer kinetics. Increasing the time increased the percentage of water loss and solid gain. From Fig. 2a, WL rate rapidly increased in the first 5 h of osmosis, then increasing slowly. This phenomenon is due to the largest pressure difference between the kiwifruit cells and the surrounding hypertonic solution, thereby promoting the osmotic dehydration of kiwifruit in the initial stage of the penetration process and inducing rapid diffusion of the water molecules. As osmotic time continues, the pressure difference gradually decreases and the structural changes in kiwifruit tissues gradually occur, the mass transfer tends to reach the dynamic equilibrium state. The WL rate increases with increasing sucrose concentration, and higher concentrations of osmotic solution could facilitate the removal of moisture from the texture of food product and resulted in lower moisture contents and higher WL rate from the texture, consistent with some other reports. LENART (1992) reported that increasing the concentration of an osmotic solution led to high WL rate until the equilibrium level was achieved; by contrast, low-concentrated sucrose solution led to small WL and SG rates (TORTOE, 2010). Similarly, RAMASWAMY (2005) studied the effect of osmotic time on mass transfer, and the results showed that mass exchange occurred at a faster rate within the initial 2h followed by a reduction in drying rate during further processing time.

The kiwifruit SG rate showed similar trends in 40%, 60%, and 80% sucrose concentrations (Fig. 2b). The SG rate increased continuously throughout the osmotic time in the test range, and the increase in the sucrose concentration could raise the SG rate. High concentration promotes sucrose mass transfer from the solution to the kiwifruit cells. The concentration of an osmotic agent affects the mass transfer kinetics during osmotic dehydration (HERMAN-LARA *et al.*, 2013). The difference in osmotic potential between the solution and the fruit sample resulted in a high diffusion rate of the solute and water (AZOUBEL and MURR, 2004; PHISUT, 2012). Similarly, LAZARIDES (1994) reported that apple processed at a temperature of 30 and 50°C resulted in higher sugar gain (up to 55%)

compared to room temperature condition. It is due to the swelling of membrane and plasticizing effect, which enhances the permeability of the membrane.

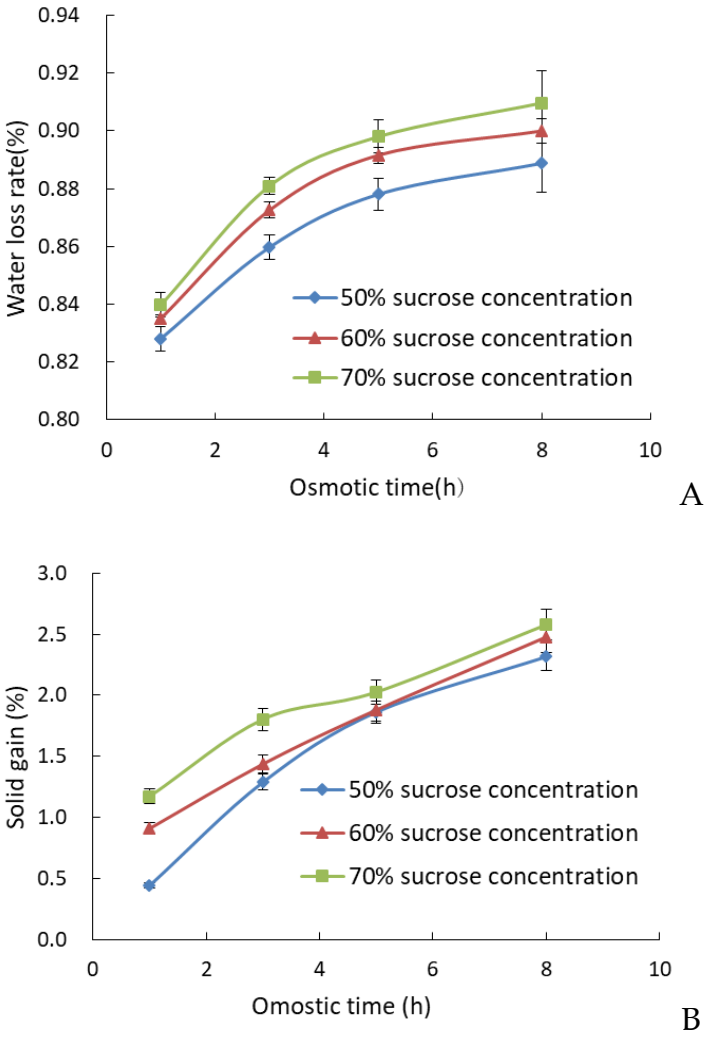

Figure 2. Variation of WL and SG rates with time during kiwifruit osmotic dehydration under different sucrose concentrations at 50℃.

3.6. Change of V_c content in untreated and osmotic treated kiwifruits

Comparison of ascorbic acid (V_c) content in untreated and osmotic treated kiwifruit samples at different osmotic temperatures was showed in Fig. 3. The V_c content of kiwifruit was significantly decreased by osmotic dehydration. It may be that V_c is transferred from the kiwifruit to the osmotic solution with the water molecule moving from the inside of the kiwifruit during the osmotic process. Moreover, the V_c content of osmotic treated kiwifruit was decreased significantly with increasing the osmotic temperature, this may be because the internal molecular movement in kiwifruit osmotic system at high temperature were much faster, which accelerated the loss of internal V_c molecules. This result is in agreement with the report by CAO *et al.*(2006), which osmotic temperature was the most significant factor affecting the ascorbic acid loss. However, CHAKRABORTY and SAMANTA (2016) found that the optimally dehydrated kiwifruit

demonstrated a significant increase in the ascorbic acid content by simultaneous osmotic dehydration using fructose as osmotic solution and vacuum drying under far - infrared radiation.

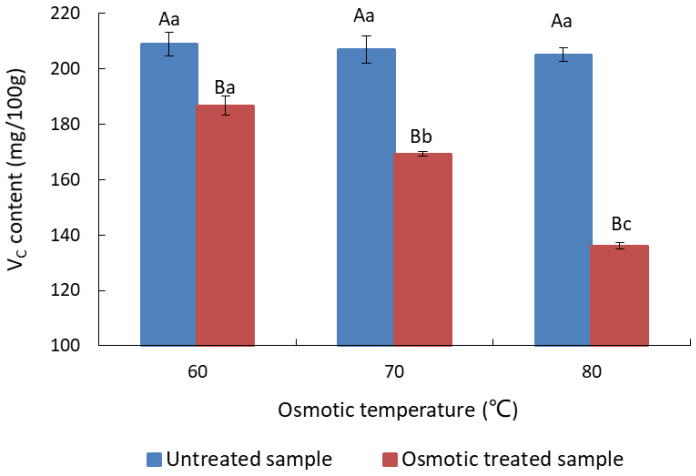

Figure 3. Comparison of V_c content in untreated and osmotic treated kiwifruit samples at different osmotic temperatures. Bars with different capital letters at each osmotic temperature are significantly different at $p< 0.05$. Bars with different small letters at treated samples are significantly different at $p< 0.05$.

4. CONCLUSIONS

The optimization of the osmotic conditions for kiwifruit dehydration was successfully examined using the RSM. The optimal conditions comprised osmotic time of 4.29 h, sucrose concentration of 70 %, and osmotic temperature of 50 ℃ with a response value of 45.64 % for the WL rate. The WL rate of the kiwifruit cubes was mainly influenced by osmotic temperature ($p \leq 0.01$), followed by sucrose concentration ($p \leq 0.01$) and osmotic time ($p \leq 0.01$). Moreover, the V_c content was decreased significantly with increasing the osmotic temperature. The optimized condition was validated and found to be fitted with the experimental values. Therefore, osmotic dehydration of kiwifruit highly depends on osmotic temperature, osmotic time, and solvent concentration. The predicted model for WL rate established by the response surface quadratic regression provided an adequate mathematical description of kiwifruit osmotic dehydration.

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PAPER

THE EFFECT OF INTRAMUSCULAR FAT CONTENT ON THE MEAT QUALITY OF PLW X PL PIGS

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ABSTRACT

This study aimed to determine the effect of intramuscular fat content (IMF) on the quality of meat from PLW x PL pigs. Meat was evaluated in terms of intramuscular fat content (n=80), technological properties, visual and tactile aspects, and colour measurements. It was demonstrated that meat containing more IMF was characterised by higher tenderness and marbling, but lower water content and a lower proportion of the yellow b* colour. Furthermore, it had a lower C* saturation, a lower h° hue, and lower muscle pigment content.

Keywords: pigs, intramuscular fat, meat quality

1. INTRODUCTION

There are numerous works indicating a significant role of intramuscular fat content (IMF) in determining the traits of pork (FERNANDEZ *et al.*, 1999a, b; FORTIN *et al.*, 2005). Its content in meat depends on numerous factors such as species, breed and sex (HOCQUETTE *et al.*, 2010; WOOD *et al.*, 2004; BOCIAN *et al.*, 2009; 2012; JANKOWIAK *et al.*, 2010; TYRA and ŻAK, 2012; feeding (WOOD *et al.*, 2004; ALONSO *et al.*, 2010), method of maintenance and age of slaughtered animals (ANDRÉS *et al.*, 2001), body weight at slaughter (LATORRE *et al.*, 2004), carcass muscling and subcutaneous fat thickness (PIETRUSZKA *et al.*, 2015). Irrespective of the above factors, intramuscular fat content is intimately related to some quality traits of meat.
Intramuscular fat (IMF) in pork is one of the main factors that influence the sensory quality parameters of meat, especially suitability for processing and cooking (FERNANDEZ *et al.*, 1999a; DASZKIEWICZ *et al.*, 2005; CZARNIECKA-SKUBINA *et al.*, 2010). The number and size of intramuscular adipocytes is related to variability in IMF content (HOCQUETTE *et al.*, 2010). The muscle fibre microstructure and fibre types -affect the accretion rate of intramuscular fat (FIEDLER *et al.*, 2003; WOJTYSIAK, 2014). According to DE VRIES *et al.* (2000), consumers mostly prefer pork with an IMF content of 2.5-3%. CZARNIECKA-SKUBINA *et al.* (2007) stated that pork with a higher (>2.51%) IMF content, despite having better processing value, tenderness, juiciness, palatability, and marbling, is less accepted by consumers. A minimum IMF content of 1.5% was considered necessary to ensure appropriate juiciness, tenderness, and palatability (FORTIN *et al.*, 2005). Reducing IMF content may have a negative impact on sensory and processing features of meat (CZARNIECKA-SKUBINA *et al.*, 2007; BOCIAN *et al.*, 2009). According to ELLIS (2006) an IMF content ranging from 1.8% to 2.6% is an indicator of good quality pork.
The most popular and common pig breeds in Poland are the Polish Large White (PLW), the Polish Landrace (PL), and their crossbreds (BLICHARSKI and SNOPKIEWICZ, 2017). The study aimed to determine the effect of IMF on some meat quality traits of Polish Large White x Polish Landrace (PLW x PL) pigs.

2. MATERIALS AND METHODS

2.1. Animals and sampling

The tested meat was obtained from 80 fattening pigs, F_1 crossbreds (Polish Large White x Polish Landrace), 50% gilts and 50% hogs. The crossbred PLW x PL fattening pigs came from and were kept on the same farm under the same environmental conditions, in accordance with welfare requirements. The animals were fed *ad libitum* with the same complete mixtures, according to standard requirements (GRELA and SKOMIAŁ, 2014). The composition and nutritional value of the complete mix are given in Table 1.
When fattening was complete, the animals were individually weighed and transported to a slaughterhouse about 100 km away. The slaughter was carried out in accordance with the applicable procedures after a 2-hour rest. The average live weight of slaughtered pigs was 106±9.57 kg.

Table 1. The nutritional value of feed mixtures.

Composition of feed mixture	Fattening period	
	30 to 70 kg	70 to 110 kg
Ground wheat (%)	20	15
Ground barley (%)	25	10
Ground triticale (%)	40	60
Protein concentrate[a] (%)	15	15
Metabolizable energy (MJ/kg)	13.20	13.34
Crude protein (g/kg)	156	158

[a]Composition: metabolizable energy, 13.30 MJ/kg; crude protein, 37.60%; crude fibre, 2.50%; crude ash, 17,80%; crude fat, 1.20%; Ca, 4.40%; P, 1.20%; Na, 1.0%; Lysine, 4.50%; Methionine, 0.56%; Tryptophan, 0.50%; Threonine, 1.60%; Methionine + cystine, 1.40%. Vitamin-micromineral per kilogram of complete diet: vitamin A (E 672), 60000 IU; vitamin D3 (E 671), 16600 IU; vitamin E alfa tocopheryl acetate, 716 IU; vitamin K3 as sodium sulfate, menadione 16 mg; thiamine, 12 mg; riboflavin, 24 mg; pyridoxine, 20 mg; cobalamin, 200 mcg; biotin, 400 mcg; niacin, 113 mg; Ca-D-pantothenate, 70 mg; betaine, 1080 mg; Cu, 160 mg as copper sulfate; Fe, 640 mg as iron sulfate monohydrate; Mn, 320 mg as manganese oxide; Zn, 640 mg as zinc oxi; Se, 3.0 mg as sodium selenate; I, 16 mg as anhydrous calcium iodate.

On the day following the slaughter, the carcass fat and meat content were determined according to RÓŻYCKI and TYRA (2010). The thickness of backfat was determined on the cold right half-carcass at points over the shoulder (at the thickest point), on the back (behind the last thoracic vertebra and the first lumbar vertebra), and at three locations over the loin (cross-section of the gluteal muscle): over the rostral edge of the gluteal muscle, in the middle of the gluteal muscle, and over the caudal edge of the gluteal muscle. The arithmetic mean was calculated from the five measurements of backfat thickness. On a cross-section of the *longissimus lumborum* muscle taken from the last thoracic vertebra and the first lumbar vertebra, the surface contour was measured (loin eye), and then the determined cross-sectional area was measured using the LUCIA system (Image for Image Processing and Analysis, version 4.82.2004). The research did not require the consent of the Local Ethical Committee.

2.2. Meat analysis

The acidification of muscle tissue at 45 minutes post slaughter (pH_{45}) and at 48 hours post slaughter (pH_{48h}) was determined using an Elmetron CP-401 pH-meter with a blade electrode. The equipment was calibrated using Elmetron pH 7.0 and pH 4.0 buffers. The meat quality was evaluated at 48 h post slaughter based on the *longissimus lumborum* muscle, which were stored at a temperature of 4-6°C. Water-holding capacity (WHC) was determined using the method developed by GRAU and HAMM (1952) and modified by POHJA and NIINIVAARA (1957). A 300 mg sample of minced meat was placed on a Whatman 1 filter paper and put between two glass plates; then an even load of 2 kg was applied to it for 5 minutes. The area of juice infiltration was used to calculate the percentage of free water content in the meat, assuming that 1 cm^2 of infiltration corresponds to 10 mg of water. The surface of meat juice infiltration was measured using a LUCIA computer analysis system (System for Image Processing and Analysis, version 4.82.2004).
Thermal drip was determined at 48 h post slaughter using the method developed by WALCZAK (1959). A 20 g sample of minced meat (20 g) was placed in a hygroscopic gauze and heated in a water bath at a temperature of 85°C for 10 min. After taking the

sample out of the water bath, the gauze was removed, then the sample was cooled to a temperature of 4°C and weighed. Based on the difference in weight before and after the heat treatment, the percentage weight loss was calculated.
Shear force was measured using the INSTRON 3342 strength testing equipment with a Warner-Bratzler attachment (WBSF), in accordance with the methodology provided by SZALATA *et al*. (1999). A 120 g meat sample was heated in a water bath until the sample reached a temperature of 70°C on the inside. The heat treatment was performed in a 0.85% NaCl solution. Then, 10 mm × 10 mm bars were cut along muscle fibres, which were subsequently cut perpendicularly to the muscle fibres. The results were read as maximum shear force expressed in N.
The chemical composition of the meat, i.e. water, dry mass, total protein, and intramuscular fat content, was determined in accordance with POLISH STANDARD PN-A-82109:2010 with near-infrared transmission spectroscopy (NIT) using calibration on artificial neural networks (ANN) with the FOSS FoodScan equipment.
Visual and tactile evaluation was determined 48 h after slaughter on a slice of raw meat weighing 120 g. Visual and tactile assessment of the meat was carried out by a trained 10-person team. All evaluators had 4 years of experience in assessing pork meat. Visual properties of the raw meat were assessed: visual colour intensity according to a 6-grade scale (POLISH STANDARD PN-ISO 4121:1998) on which 1=very light, 6=dark purple; marbling based on Canadian and American models on a 10-grade scale (CHENG *et al*., 2015; NPPC, 1999) where 1=no intramuscular fat content, 10=very high marbling. Tactile evaluation of firmness was on a 7-grade scale (PN-ISO 4121:1998) where 1=very firm, 7=very soft.
Meat colour was also measured on a slice of raw meat at 48 h post slaughter using a Minolta CR 310 photocolorimeter (Konica Minolta, Japan) with a measuring port diameter of 50 mm. The equipment was standardized using a CR310 white calibration plate with the following coordinates: Y=92.80, x=0.3175 i y=0.3333. Colour parameters were determined in the CIE system, L*a*b* (L* - lightness, a* - participation of red, b* - participation of yellow) (CIE, 1986) using illuminant D 65 and a standard 2° observer. Chroma (C*) and hue angle (h°) were calculated according to the formula provided by BEATTIE *et al*. (1999) and BREWER *et at*. (2001):

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

Muscle pigment was determined by colorimetry according to the method developed by HORNSEY (1956). A 40 ml mixture of acetone, water, and concentrated HCl in proportions 40:2:1 was poured over minced meat samples (10 g), which were then extracted for 1 hour. After filtering, the absorbency of the tested solutions was measured using a Marcel Media spectrophotometer at a wavelength of 640 nm. The optical density value (E) was multiplied by a factor of 680 in order to obtain the proper concentration of hematin expressed as micrograms of hematin per 1 g of meat.

2.3. Statistical analysis

The results were statistically analysed; the arithmetic mean and the standard deviation for carcass traits, and the standard error (SEM) for meat quality traits were calculated. Data were verified for homogeneity of variance with the Leven'e Test; in the absence of homogeneity of variance, statistical significance between groups was calculated using the nonparametric Kruskal-Wallis Test. A probability of $P < 0.05$ was considered statistically significant.

The obtained test results were compiled and analysed in three groups that were defined in terms of the intramuscular fat content (IMF) of the meat of the PLW x PL pigs according to the normal distribution of features (Gaussian curve): group I, <1% of IMF content; group II, 1-2.5% of IMF content; group III, >2.5% of IMF content. The meats were divided into IMF groups to verify the impact on meat quality of increased IMF in the meat of PLW x PL pigs.
Pearson's simple correlation coefficients between the IMF content and the meat slaughter traits and quality traits were calculated to numerically summarize the degree of association between any two variables. All calculations were conducted using Statistica PL.8.0 data analysis software (StatSoft Inc. STATISTICA, 2008).

3. RESULTS AND DISCUSSION

The data regarding the quantity and weight measurement of warm carcasses, average backfat thickness, and loin eye area are shown in Table 2. The obtained values of average backfat thickness indicate a higher fat content of pig carcass than reported in other studies (CZARNIECKA-SKUBINA *et al*., 2007; TYRA and ŻAK, 2012).

Table 2. Mean and standard deviation of carcass characteristics.

	Mean and standard deviation
Number (n)	80
Hot carcass weight (kg)	86.83±8.61
Average backfat thickness (mm)	23.23±5.27
Loin eye area (cm^2)	53.87±7.41

The characteristics of the technological properties of the tested pork are presented in Table 3 and analysed according to the intramuscular fat content. The 16 meat samples fell within the first group (lowest fat content), 48 meat samples within the II group and 16 meat samples within III group (highest fat content). In numerous tests, the highest percentage of meat samples had up to 2% of IMF (DASZKIEWICZ *et al.*, 2005; TYRA and ŻAK, 2012). The IMF content in the meat studied ranged from 0.79% to 3.20% and significantly differed between all the groups ($P<0.01$).
The acidity of muscle tissue is one of the parameters that determines meat quality and is used to determine the processing and cooking suitability of meat (KAJAK *et al*., 2007). Meat acidity is measured 45 minutes after slaughter and is a widely recognised criterion that reflects the intensity of post-slaughter changes that lead to meat quality defects such as PSE (HOFMANN, 1994). The ultimate pH is an indicator of meat quality and is associated with water-holding capacity, colour, and tenderness (KAJAK *et al*., 2007).
In this study, higher pH_{45} values were observed in group I than in group III ($P<0.05$); this can be explained by the fact that the group with the lowest IMF content contained carcasses with values from 6.21 to 6.84 pH, which had an impact on higher pH_{48} values. The meat pH value measured 48 h post slaughter was the highest in the meat with the lowest IMF content and differed significantly between group I and group II and III ($P<0.01$). The results of pH_{45} and pH_{48} presented in the paper are compatible with the results obtained by JAWORSKA *et al.* (2007), who showed that with an increase from 1.72% to 2.63% of IMF content in meat, the values of pH_{45} (6.40 to 6.36) and pH_{48} (5.52 to 5.50) decreased. CZARNIECKA-SKUBINA *et al.* (2007) demonstrated that pork with the

highest IMF content (>2.51%) was characterized by a significantly higher final pH (5.63), compared to meat with the lowest (<1.5%) and average (1.51-2.5%) IMF content (5.51 and 5.54) (P<0.05); moreover, it was characterized by a darker colour and lower protein content. DASZKIEWICZ *et al.* (2005) demonstrated that meat with lower IMF content (<1.0%) had lower pH_{45} (6.17) and similar values to obtained in this study at pH_{48} (5.44). In turn, KLONT (2005) indicated that a lower final pH causes the meat to have less water retention capacity and to be lighter in colour, while a higher final pH gives it a darker colour, less juice leakage during storage, and positively effects meat quality traits, i.e. succulence, tenderness and taste. The values obtained and presented in this study of pH_{45} as well as the final pH were typical for meat of good quality in accordance with the assumptions of HOFMANN (1994) and KLONT (2005).

Table 3. Characteristics of the technological properties of meat quality (mean value and standard error) in relation to IMF content.

	Group - IMF				
	I	**II**	**III**	**SEM**	***P***
	<1%	**1-2.5%**	**>2.5%**		
Number (n)	16	48	16		
Number (%)	20.00	60.00	20.00		
IMF (%)	0.79^{A}	1.59^{B}	3.20^{C}	0.10	0.001
pH_{45}	6.50^{a}	6.37	6.32^{b}	0.02	0.027
pH_{48h}	5.54^{A}	5.46^{B}	5.44^{B}	0.01	0.005
WHC (% of free water)	19.38^{a}	19.99	21.67^{b}	0.31	0.033
Thermal drip (%)	21.20	21.05	21.75	0.24	0.378
WBSF (N)	55.94^{A}	48.38^{A}	37.46^{B}	1.48	0.001
Chemical composition of meat					
Water content (%)	73.30^{A}	74.09^{B}	72.73^{C}	0.12	0.001
Total protein content (%)	23.09	23.40	23.11	0.07	0.343

$^{(A-C)}$Row means with different superscripts differ significantly at $P<0.01$.

$^{(a-b)}$ Row means with different superscripts differ significantly at $P<0.05$.

IMF - Intramuscular fat content; WHC - Water holding capacity; WBSF - Warner Bratzler shear force (N - Newton); pH_{45} - pH at 45 minutes post slaughter; pH_{48h} - pH at 48 hours post slaughter.

The meat with the lowest WBSF (shear force) contained the highest amount of IMF (group III) and was more tender than groups I and II ($P<0.01$). VAN LAACK *et al.* (2001) evaluated the impact of ultimate muscle tissue acidity (pH_u) and IMF content on tenderness and tenderization of pork. Similarly, RAMSEY *et al*. (1990) showed that increasing meat IMF content decreases its shear force.

The studies demonstrated that, along with increasing IMF content of the tested meat, the water content decreased and the dry mass content increased ($P<0.01$). DASZKIEWICZ *et al*. (2005) observed a similar relationship between the level of IMF and the chemical composition of pork. They showed that as the content of IMF and marbling increased, the content of dry matter increased and the content of total protein and ash decreased.

Table 4 contains the results of a visual and tactile evaluation, and an evaluation of colour and muscle pigment of meat. With the increase in IMF, marbling in meat increased (P <0.01).

Higher marbling of pork is associated with a higher IMF content (FERNANDEZ *et al*., 1999a; VAN DER WAL *et al*., 1992; VAN LAACK *et al*., 2001). CZARNIECKA-SKUBINA *et al*. (2007) showed that meat with the highest IMF content (≥2.51%) was characterized by a higher marbling (6.06 points) in relation to meat with the lowest (≤1.5%) IMF content (3.92 points). Also, PRZYBYLSKI *et al*. (2010) showed more marbling (4.28 points) in meat with 2.27% IMF content compared to 1.67 IMF meat (2.70 points) ($P < 0.05$).

Table 4. The results of visual and tactile evaluation of meat colour and muscle pigment content (mean value and standard error) in relation to IMF content.

	Group - IMF				
	I	**II**	**III**	**SEM**	***P***
	<1%	**1-2.5%**	**>2.5%**		
Visual and tactile evaluation					
Visual colour intensity (1-6 scale)	3.7	3.5	3.1	0.08	0.127
Marbling (1-10 scale)	1.0^{A}	2.3^{B}	3.9^{C}	0.13	0.001
Firmness (1-7 scale)	3.8^{a}	4.2	4.6^{b}	0.08	0.011
Colour measurements					
L^*_{48}	53.73	54.78	55.23	0.32	0.560
a^*_{48}	16.19	15.78	15.11	0.13	0.037
b^*_{48}	6.80^{A}	4.90^{Ba}	3.32^{Bb}	0.22	0.001
C^*_{48}	17.60^{A}	16.61^{A}	15.52^{B}	0.16	0.001
h^o_{48}	22.63^{A}	17.02^{Ba}	12.36^{Bb}	0.70	0.001
Muscle pigment (micrograms of hematin per 1 g of meat)	34.27^{Aa}	29.83^{b}	26.99^{B}	0.64	0.002

$^{(A-C)}$Row means with different superscripts differ significantly at $P<0.01$.
L* value represents lightness; a* proportion of red; b* proportion of yellow; C* saturation; h^o hue angle.

As evaluated tactilely, the highest hardness of meat was observed in samples in group I with a minimum content of IMF, compared to group III which was less hard ($P < 0.05$).
Meat colour constitutes an important quality indicator (POŁOM and BARYŁKO-PIKIELNA, 2004). Although lightness L* was highest for meat with the highest fat content, there were no statistically significant differences in terms of IMF content. The obtained results were typical of normal meat quality (WARRIS *et al*., 2006). There were no significant differences in the proportion of red a*, but there was a significant difference in the proportion of yellow b*, which was highest in the group with the lowest IMF ($P < 0.01$). Quality features of meat colour include colour saturation, colorimetric purity, and dominating light wavelength, referred to as hue. The highest colour saturation C* and h° hue were observed in meat with the lowest (<1%) IMF content ($P<0.01$). Similar values of meat colour parameters L*, a*, b*, and saturation C*, and higher values of h° hue than the ones obtained in this study were shown in previous studies (BOCIAN *et al*., 2015).
Muscle pigment content is one of the main factors that affect the evaluated meat colour. This study demonstrated significant differences in muscle pigment content. The highest content of muscle pigment was in meat from group I, which also had the lowest IMF content compared to group III, which had higher IMF content ($P<0.01$). These values are similar to the ones obtained previously by BOCIAN *et al*. (2015) for PLW x PL meat.
For more detailed interrelations between intramuscular fat content and the characteristics of the tested meat, the simple linear correlations between them were computed. The

coefficients of simple correlation between IMF and processing properties of meat, subjective visual and tactile evaluation, and its colour are shown in Table 5. The correlations between IMF and carcass weight, backfat thickness and loin eye area were also computed. The study confirmed a significant negative relationship between IMF and meat acidity pH_{45} (P<0.05) and pH_{48h}, WBSF, water content (P <0.01), visual colour intensity (P <0.05), proportion of red a* and yellow b* colour, its saturation C*, and h° hue, as well as muscle pigment content (P<0.01).

Table 5. Correlation coefficients between intramuscular fat content and other variables in meat.

	IMF
pH_{45}	-0.242*
pH_{48h}	-0.292**
WHC	0.140
Thermal drip	0.032
WBSF	-0.493**
Water content	-0.779**
Total protein content	-0.106
Visual colour intensity	-0.259*
Marbling	0.908**
Firmness	0.305**
L^*_{48} - value represents lightness	0.147
a^*_{48} - proportion of red	-0.302**
b^*_{48} - proportion of yellow	-0.584**
C^*_{48} - saturation	-0.473**
h^o_{48} - hue angle	-0.560**
Muscle pigment	-0.422**
Hot carcass weight	0.353**
Av. backfat thickness	0.195
Loin eye area	-0.006

(*)Statistical significance at P<0.05, (**) Statistical significance at P<0.01.

Intramuscular fat content was not related to carcass fat and meat content; only carcass weight was positively correlated with IMF (P<0.01), which may be explained by the impact of age on the higher animal body weight at slaughter. PIETRUSZKA *et al*. (2015) reported a significant positive correlation between IMF content and average backfat thickness (r=0.31, P<0.05), and a negative correlation with the loin eye area (r=-0.64, P<0.01); however, in contrast to this study, no significant relationship between IMF and pH_{45} was demonstrated. The high positive correlation between the IMF content and marbling (r=0.908) and the negative relationship with the shear force (r=-0.493) (P<0.01) obtained in this study are in line with the findings of LI *et al*. (2013), which also showed lower shear force of meat containing higher IMF content.

4. CONCLUSIONS

The meat obtained from the most popular Polish crossbred pigs contained more intramuscular fat and was characterized by greater shear force, lower pH_{45} and pH_{48h}, more marbling with lower water content, and a smaller proportion of yellow colour b*. In addition, it was characterized by a lower saturation of colour C*, its tone h°, and lower content of muscle pigment.

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PAPER

CHARACTERISTICS OF LIPIDS FROM IMMUNOCASTRATED MEDIUM-HEAVY PIGS FED EITHER A RESTRICTED DIET OR *AD LIBITUM*

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ABSTRACT

We studied the feeding level-related variations in lipid characteristics in the adipose tissues of pigs. The lipid content, fatty acid profile, oxidative stability, iodine value, thrombogenic and atherogenic indices were determined in individual samples from 24 immunocastrated males (Duroc x Large White), fed either restricted or *ad libitum*. In backfat, feed restriction increased the polyunsaturated fatty acid proportion and iodine value and lowered the thrombogenic and atherogenic indices. Intramuscular lipid content was reduced by restriction, which did not affect either the fatty acid composition or the oxidative stability in both raw and cooked muscle. Feed restriction improved the nutritional quality of lipids without impairing their technological attributes.

Keywords: adipose depots, fatty acid profile, feeding restriction, immunocastration, medium-heavy pigs

1. INTRODUCTION

The consumption of meat, a common constituent of western balanced diets, is associated with the development of serious cardiovascular diseases (KONTOGIANNI *et al.,* 2008; ABETE *et al.,* 2014; LARSSON and ORSINI, 2014) because of the amount and characteristics of the fat it provides (SIMOPOULOS, 1999; WILLIAMS, 2000).
In pig meat, several factors affect the deposition of lipids and their fatty acid (FA) composition. Of these, feeding strategies (WOOD *et al*., 1986; CAMERON *et al*., 2000; LO FIEGO *et al*., 2005a; LEBRET, 2008), genetic factors mainly related to the adipogenetic aptitude of breeds (CAMERON *et al*., 2000; PIEDRAFITA *et al*., 2001; WOOD *et al*., 2008), sex, age and live weight at slaughter (LEBRET and MOUROT, 1998; LO FIEGO *et al*., 2005b) have been demonstrated to play a role.
In Italy, pork for fresh consumption, obtained mainly from subjects slaughtered at 95-100 kg body weight (BW), is generally imported. National production is centered on the rearing of heavy pigs slaughtered when they reach 9/10 months of age and 160-170 kg BW, to be processed into typical, high quality, dry cured Protected Designation of Origin (PDO) products.
A production chain based on medium–heavy pigs, slaughtered at a final weight of approximately 140 kg at about 6-8 months of age and not subjected to the strict PDO rules, provides meat for both fresh consumption and processed products such as salami, sausages, seasoned and cooked hams. For this production, the same genetic types used for heavy pig production can be used. Compared to heavy pigs, medium–heavy pigs have a shorter fattening period and, thus, show a better feed conversion efficiency (LO FIEGO *et al*., 2010; DALLA BONA *et al*., 2016).
Male pigs destined to be slaughtered at such high BW and age are surgically castrated to limit aggressiveness and the development of boar taint, a potentially off-putting odor in the meat (DUNSHEA *et al*., 2001; XUE and DIAL, 1997). However, this technique is stressful for the animal, with negative effects on health and animal welfare (ZAMARATSKAIA *et al*., 2008). Hence, in view of possible EU legislative measures aimed at restricting this practice, the investigation of suitable alternatives has been encouraged.
One method of inhibiting sexual development and boar taint is immunization against gonadotropin releasing hormones (GnRHs) (DUNSHEA *et al*., 2001). The vaccine is administered in two doses. After the second vaccination, immunocastrated pigs perform similarly to surgically castrated subjects (FÀBREGA *et al*., 2010; GISPERT *et al*., 2010; PAULY *et al*., 2009). In fact, when fed *ad libitum*, immunocastrated subjects show a very high feed intake, which can lead to excessive fat deposition. This, in turn, can influence the fatty acid composition of lipid depots, which become more saturated. Conversely, decreasing energy intake brings about an increase in the degree of unsaturation in pig adipose tissue (LEBRET and MOUROT, 1998).
The effects of feed restriction in immunocastrated pigs after the second vaccination, aimed at improving feed efficiency and avoid excessive carcass fatness, have been poorly investigated, and studies have only been performed in lean light pigs. Moreover, according to a review by ČANDEK-POTOKAR *et al*. (2015), research has yielded conflicting results. Finally, the effect of this feeding practice on the fatty acid composition of subcutaneous adipose tissue and intramuscular fat has not been taken into account.
Thus, this research was carried out on finishing immunocastrated pigs slaughtered at about 140 kg BW, in order to verify the effects of feed restriction, on fatty acid composition of backfat and intramuscular fat.

2. MATERIALS AND METHODS

2.1. Animals, diet and sampling

A total of 24 crossbred (Italian Duroc x Italian Large White) pigs were involved in the trial, conducted at CREA (San Cesario S/P, Modena, Italy). All pigs were immunocastrated against GnRH using two doses of Improvac® vaccine (Zoetis, Belgium, S.A.), each dose consisting of 300 μg of GnRH-protein conjugate in 2 ml of aqueous solvent. Applications were performed by subcutaneous injection, at 90 and 162 days of age.
The animals were housed collectively until they reached 120 days of age (51.84±4.38 kg BW). Subsequently, the pigs were housed evenly in six adjacent pens, each with four subjects, balanced for weight, and were fed *ad libitum* until they were 162 days old (103.3±6.9 kg BW). Thereafter, until slaughtering, at 197 days of age (142.32±6.8 kg BW), three pens were fed restricted (R) at 7.5% $BW^{0.75}$ and the remaining three received the same farm concentrate *ad libitum* (AL). This dietary restriction level is commonly adopted in the finishing of traditional Italian heavy pigs. Feed composition is shown in Table 1. Water was always available through nipple drinkers.

Table 1. Determined and calculated diet analyses.

Determined analysis[(a)]	%
Crude protein	13.83
Crude fibre	5.05
Crude fat	2.50
Fatty acids (FAs)	(% total FAs)
C14:0 (myristic)	0.23
C16:0 (palmitic)	21.62
C16:1n-7 (palmitoleic)	0.33
C18:0 (stearic)	2.57
C18:1n-9 (oleic)	24.27
C18:2n-6 (linoleic)	48.27
C18:3n-3 (α-linolenic)	2.71
Σ SFA (saturated FAs)	24.42
Σ MUFA (monounsaturated FAs)	24.60
Σ PUFA (polyunsaturated FAs)	50.98
Calculated analysis[(b)]	
Starch, %	46.01
Digestible lysine, %	0.80
Net energy, Mcal/kg	2.29

The ingredients in the diet in % (as fed): corn meal 48.6, barley meal 21.0, beet pulp 6.0, soybean meal dehulled 13.5, wheat bran 7.5. Added amino acids 0.55 (l-lysine HCl 0.38; dl-methionine 0.05; l-threonine 0.07; l-tryptophan 0.05); added salts 2.45 (calcium carbonate 1.05; dicalcium phosphate 1.00; sodium chloride 0.40) vitamins and trace elements 0.40.

[(a)]According to the Association of Official Analytical Chemists (1995). [(b)]According to Sauvant *et al*. (2004).

All animals were slaughtered on the same day, following routine abattoir procedures. After carcass grading, 1 hour *post mortem*, individual samples of the *Longissimus lumborum* (LL) muscle and subcutaneous adipose tissue were collected at the last rib level. An

aliquot of the samples was vacuum-packed, in individual bags, and stored at -20°C, for subsequent laboratory analysis.

2.2. Analyses

2.2.1 Lipid content and oxidative stability

Each sample of LL muscle was analyzed in duplicate to determine:
- On fresh muscle (24 h *post mortem*) - lipid oxidation by the 2-thiobarbituric acid reactive substances (TBARS) measurement, according to SIU and DRAEPER (1978); results were expressed as mg of malondialdehyde (MDA)/kg muscle.
The same determination was carried out on vacuum-packed slices (approx. 100 g), put in a water-bath at 80°C, and left until the core temperature reached 70°C.
- After thawing - ether extract content using petroleum ether (Carlo Erba reagents, MI, Italy) and a Soxhlet apparatus (AOAC, 1995) with previous acid hydrolysis. Results were expressed as the percentage of wet matter.

2.2.2 Fatty acid profile

Fatty acid (FA) composition of lipids of subcutaneous adipose tissue and LL muscle was determined using a TRACE™GC Ultra (Thermo Electron Corporation, Rodano, Milan, Italy) equipped with a Flame Ionization Detector, a PTV injector, and a TR-FAME Column (Thermo Scientific, Rodano, Milan), 30 m long, 0.25 mm i.d., 0.2 μm film thickness. Total lipids were extracted from the samples of subcutaneous adipose tissue, separately for the outer and inner layers (IUPAC, 1979) and from LL muscle (FOLCH *et al*., 1957).
An aliquot of 25 mg was then subjected to methylation by means of a methanolic solution of potassium hydroxide (KOH 2N) according to FICARRA *et al*. (2010), using tridecanoic acid (C13:0) (Larodan Fine Chemicals AB, Malmö, Sweden) as internal standard. The injection of the fatty acid methyl ester sample (1 μl) was performed in split mode with a split flow of 10 mL/min, operating at a constant flow of 1 mL/min of helium as a carrier gas. The temperature of the injector and detector was kept at 240°C. The temperature program used for the analysis started from 140°C, was maintained for 2 min, then increased to 250°C, at a rate of 4°C/min, and kept at this temperature for 5 min.
The peaks of the fatty acids were recorded and integrated using Chrom-Card software (vers. 2.3.3, Thermo Electron Corporation, Rodano, Milan, Italy) and identified by comparison with the retention times of standard solutions with known quantities of various methyl esters (Supelco® 37 Component FAME mix, PUFA standard n.2, Animal Source, Supelco, Bellafonte, PA, USA). For quantification purposes, the response factor was calculated, and an internal standard was used. The amount of each FA in the sample was expressed as FA relative percentage with respect to the total amount of FAs. The iodine value (IV) of the outer and inner layers of backfat was calculated adopting the equations proposed by LO FIEGO *et al*. (2016):

$$IV = 85.703 + [C14:0] \times 2.740 - [C16:0] \times 1.085 - [C18:0] \times 0.710 + [C18:2n\text{-}6] \times 0.986$$

In addition, atherogenic and thrombogenic indices (AI and TI) were calculated after ULBRICHT and SOUTHGATE (1991):

$$AI = \frac{(C12:0 + 4xC14:0 + C18:0)}{(MUFA + PUFA)}; \quad TI = \frac{(C12:0 + C16:0 + C18:0)}{0.5x(MUFA + n-6\ PUFA) + 3x(n-3\ PUFA) + (n-3\ PUFA/n-6 PUFA)}$$

2.3. Statistical analysis

The data were statistically analyzed by a MIXED model (SAS Institute Inc., Cary, NC, USA) including dietary treatment as the fixed effect and pen as the random effect.

3. RESULTS AND DISCUSSION

As expected, and in agreement with QUINIOU *et al*. (2012) and BATOREK *et al*. (2012), R pigs gained less final BW (135.4 *vs* 149.3 kg), yielded lighter carcasses (113.4 *vs* 125.1 kg) and thinner backfat (22.7 *vs* 28.1 mm) compared to the AL group (P<0.01) (*data not reported*). Table 2 shows the data regarding the intramuscular fat (IMF) content and lipid oxidation (TBARS values) in LL muscle.

Table 2. Intramuscular fat (IMF) and TBARS (thiobarbituric acid reactive substances) content of *Longissimus lumborum* muscle of immunocastrated male pigs submitted to different feeding regimes (least squares means).

Items	Treatment		SE[$]
	***Ad libitum* (n=12)**	**Restricted (n=12)**	
IMF (%)	3.97[a]	2.43[b]	0.617
TBARS (mg MDA/kg raw muscle)	0.057	0.052	0.008
TBARS (mg MDA/kg cooked muscle)	1.159	1.298	0.139

[$]Standard error of the differences; [a,b]=$P<0.05$.

The IMF content was lower than that found by MINELLI *et al*. (2013) in pigs slaughtered at a heavier weight and similar to those observed by ROSSI *et al*. (2014) and by DALLA BONA *et al*. (2016) in medium-heavy pigs slaughtered at 135-140 kg body weight. Feeding restriction significantly lowered ($P<0.05$) the IMF content. BATOREK *et al*. (2012), instead, found no difference in this trait between *ad libitum* or restricted fed immunocastrated pigs.
The feeding regimen did not affect the oxidative stability of fresh and cooked LL muscle. The TBARS values fell within the range observed by ROSSI *et al*. (2014) and indicated a very low level of lipid oxidation in both fresh and cooked muscle.
Table 3 shows the data on the effect of the dietary treatment on the fatty acid composition of the outer layer of backfat.
Total saturated fatty acids (SFAs) were lower in restricted pigs ($P<0.05$); the difference being mainly represented by the variations in the proportions of palmitic acid (C16:0; $P<0.05$) and stearic acid (C18:0), although the latter did not decrease significantly. The percentage of monounsaturated fatty acids (MUFA), except for heptadecenoic acid (C17:1) that increased in the R pigs ($P<0.05$), was not affected by the feeding regimen, whereas the percentage of total polyunsaturated fatty acids (PUFAs) was significantly higher in the R group ($P<0.01$). This outcome is mainly ascribable to the variation in the proportion of linoleic acid (C18:2n-6; $P<0.05$), the single most represented PUFA in backfat, although all the individual PUFA percentages, except for eicosadienoic (C20:2n-6), eicosapentaenoic (C20:5n-3) and docosadienoic (C22:2n-6) acids, were significantly higher in the R group.
Accordingly, restricted pigs showed higher PUFA/SFA ratio values ($P<0.01$), total n-6 ($P<0.05$) and n-3 FAs, IV ($P<0.01$), and lower values for TI ($P<0.01$) and AI ($P<0.05$).

Table 3. Fatty acid (FA) composition (%) of the outer layer of backfat in immunocastrated male pigs submitted to different feeding regimes (least squares means).

Fatty acids	Treatment		SE[($)]
	***Ad libitum* (n=12)**	**Restricted (n=12)**	
C 10:0 (capric)	0.09	0.08	0.006
C 12:0 (lauric)	0.08	0.08	0.004
C 14:0 (myristic)	1.37	1.39	0.041
C 16:0 (palmitic)	25.48[a]	24.21[b]	0.471
C 17:0 (heptadecanoic)	0.37	0.41	0.050
C 18:0 (stearic)	12.91	12.03	0.504
C 20:0 (eicosanoic)	0.18	0.15	0.012
C 16:1n-7 (palmitoleic)	2.21	2.41	0.170
C 17:1n-7 (heptadecenoic)	0.32[b]	0.43[a]	0.041
C 18:1n-7 (vaccenic)	2.51	2.69	0.114
C 18:1n-9 (oleic)	39.31	38.47	0.612
C 20:1-n9 (eicosenoic)	0.79	0.75	0.052
C 18:2n-6 (linoleic)	12.50[b]	14.66[a]	0.776
C 18:3n-3 (α-linolenic)	0.65[B]	0.76[A]	0.034
C 18:3n-6 (γ-linolenic)	0.15[B]	0.19[A]	0.011
C 20:2n-6 (eicosadienoic)	0.53	0.60	0.043
C 20:3n-3 (eicosatrienoic)	0.10[b]	0.12[a]	0.007
C 20:4n-6 (arachidonic)	0.25[B]	0.32[A]	0.018
C 20:5n-3 (eicosapentaenoic)	0.01	0.01	0.001
C 22:2n-6 (docosadienoic)	0.01	0.01	0.001
C 22:4n-6 (docosatetraenoic)	0.11[B]	0.13[A]	0.006
C 22:5n-3 (docosapentaenoic)	0.06[B]	0.08[A]	0.005
C 22:6n-3 (docosaesaenoic)	0.03[B]	0.04[A]	0.003
Σ SFA (saturated FA)	40.47[a]	38.34[b]	0.755
Σ MUFA (monounsaturated FA)	45.15	44.74	0.226
Σ PUFA (polyunsaturated FA)	14.38[B]	16.91[A]	0.882
PUFA/SFA	0.36[B]	0.44[A]	0.026
Σ n-6 (ω-6 FA)	13.54[b]	15.91[a]	0.840
Σ n-3 (ω-3 FA)	0.85[B]	1.00[A]	0.044
n-6/n-3	15.98	15.85	0.313
IV (iodine value)	64.98[B]	69.17[A]	1.151
TI (thrombogenic index)	1.21[A]	1.09[B]	0.039
AI (atherogenic index)	0.52[a]	0.48[b]	0.014

[a,b]=$P<0.05$; [A,B]=$P<0.01$; [($)]standard error of differences;
IV=85.703 + [C14:0] x 2.740 - [C16:0] × 1.085 - [C18:0] × 0.710 + [C18:2n-6] × 0.986);

$$TI = \frac{(C12{:}0+C16{:}0+C18{:}0)}{0.5x(MUFA+n-6\ PUFA)+3x(n-3\ PUFA)+(n-3\ PUFA/n-6PUFA)}\ ;\quad AI = \frac{(C12{:}0+4xC14{:}0+C18{:}0)}{(MUFA+PUFA)}$$

Table 4 reports the data regarding the effects of feed restriction on the fatty acid composition of the backfat inner layer.

Table 4. Fatty acid (FA) composition (%) of the inner layer of backfat in immunocastrated male pigs submitted to different feeding regimes (least squares means).

Fatty acids	Treatment		SE[($)]
	Ad libitum (n=12)	Restricted (n=12)	
C 10:0 (capric)	0.07	0.07	0.006
C 12:0 (lauric)	0.07	0.07	0.003
C 14:0 (myristic)	1.30	1.28	0.045
C 16:0 (palmitic)	26.62[a]	25.87[b]	0.347
C 17:0 (heptadecanoic)	0.34[b]	0.47[a]	0.049
C 18:0 (stearic)	16.05	15.94	0.691
C 20:0 (eicosanoic)	0.20[a]	0.18[b]	0.009
C 16:1n-7 (palmitoleic)	1.71	1.71	0.166
C 17:1n-7 (heptadecenoic)	0.24[b]	0.32[a]	0.037
C 18:1n-7 (vaccenic)	2.02	2.08	0.114
C 18:1n-9 (oleic)	37.70	36.62	0.740
C 20:1-n9 (eicosenoic)	0.82	0.79	0.057
C 18:2n-6 (linoleic)	11.22[B]	12.70[A]	0.452
C 18:3n-3 (α-linolenic)	0.55[B]	0.63[A]	0.022
C 18:3n-6 (γ-linolenic)	0.12[B]	0.16[A]	0.012
C 20:2n-6 (eicosadienoic)	0.50	0.55	0.032
C 20:3n-3 (eicosatrienoic)	0.09[b]	0.10[a]	0.005
C 20:4n-6 (arachidonic)	0.20[B]	0.24[A]	0.012
C 20:5n-3 (eicosapentaenoic)	0.00	0.01	0.001
C 22:2n-6 (docosadienoic)	0.01	0.01	0.001
C 22:4n-6 (docosatetraenoic)	0.09[b]	0.10[a]	0.005
C 22:5n-3 (docosapentaenoic)	0.05[B]	0.06[A]	0.003
C 22:6n-3 (docosaesaenoic)	0.02[B]	0.03[A]	0.002
Σ SFA (saturated FA)	44.66	43.88	0.780
Σ MUFA (monounsaturated FA)	42.50	41.53	0.947
Σ PUFA (polyunsaturated FA)	12.84[B]	14.59[A]	0.504
PUFA/SFA	0.29[B]	0.33[A]	0.013
Σ n-6 (ω-6 FA)	12.12[B]	13.77[A]	0.479
Σ n-3 (ω-3 FA)	0.72[B]	0.82[A]	0.026
n-6/n-3	16.88	16.70	0.225
IV (iodine value)	60.04[b]	62.35[a]	0.821
TI (thrombogenic index)	1.45	1.39	0.045
AI (atherogenic index)	0.58	0.55	0.015

[a,b]=$P<0.05$; [A,B]=$P<0.01$; [($)]standard error of differences;
IV=85.703 + [C14:0] x 2.740 - [C16:0] × 1.085 - [C18:0] × 0.710 + [C18:2n-6] × 0.986);

$$TI = \frac{(C12{:}0+C16{:}0+C18{:}0)}{0.5x(MUFA+n-6\ PUFA)+3x(n-3\ PUFA)+(n-3\ PUFA/n-6PUFA)}\ ;\quad AI = \frac{(C12{:}0+4xC14{:}0+C18{:}0)}{(MUFA+PUFA)}$$

In this layer the decrease in total SFAs was less marked than in the outer layer. Again, the most significant variation was shown by C16:0, which decreased significantly ($P<0.05$) in R pigs. In this group, eicosanoic acid (C20:0) decreased ($P<0.05$) and heptadecanoic acid (C17:0) increased ($P<0.05$). The feeding regimen did not affect total MUFA percentage and, as already observed in the outer layer for this class of FA, only the heptadecenoic acid (C17:1) proportion was higher in R pigs ($P<0.05$).
The total PUFA percentage was significantly higher ($P<0.01$) in R pigs. As found in the outer layer, all the PUFAs except C20:2n-6, C20:5-n3 and C22:2n-6 increased with feed restriction, however the difference was mainly related to the increase in linoleic acid proportion ($P<0.01$). As in the outer layer, also in the inner layer PUFA/SFA ratio, the total n-3 and n-6 fatty acid percentage ($P<0.01$) and IV ($P<0.05$) were higher in restricted fed subjects. The TI and AI values did not differ between treatments. The inner layer was thus characterized by a higher percentage of saturated fatty acids and a lower PUFA/SFA ratio than the outer layer, as also observed by DUNKER *et al*. (2007), DAZA *et al*. (2017) and BEE *et al*. (2002). According to MONZIOLS *et al*. (2007), this could be explained by the larger *de novo* synthesis of SFAs (especially C16:0) exhibited in the inner layer, which leads to a dilution of dietary PUFAs (especially C18:2n-6). Concerning the effect of feeding restriction in the finishing phase on subcutaneous fatty acid composition, our data show that decreasing the feed allowance and thus the availability of energy for the *de novo* synthesis, which mainly yields SFAs, leads to a higher unsaturation of backfat lipids connected to a relative increase in PUFAs, which are of strict feed origin (DAZA *et al*., 2007).
Table 5 shows the fatty acid composition of IMF in LL.
The feeding level only marginally affected the fatty acid composition of intramuscular fat. Palmitic acid decreased by 0.9 percentage points in restricted pigs ($P<0.05$) as well as C20:0 which decreased by 0.01 percentage points ($P<0.05$), whereas C20:2n-6 and C20:3n-3 increased by 0.03 ($P<0.01$) and 0.01 ($P<0.05$) percentage points, respectively. The non-significant variations exhibited by total SFAs and PUFAs resembled those observed by DALLA BONA *et al*. (2016). It is well known that diet affects fatty acid composition in muscle less than in subcutaneous adipose tissue (CORINO *et al*., 2002).
Our data indicate that the nutritional quality of adipose depots is in general improved by a dietary restriction. This is evident especially in backfat, where the PUFA/SFA ratio and total omega-3 PUFA percentage were higher ($P<0.01$) in both layers, and TI and AI were lower, however the differences were statistically significant only in the outer layer. These nutritional parameters showed the same trend in the IMF, where the differences were not statistically significant. However, in the present research, the n-6/n-3 ratio of both layers of backfat and of LL muscle was not favorably modified by the feed restriction which increased the proportions of both n-6 and n-3 PUFA. This confirms the findings of WIECEK *et al*. (2011) in *Longissimus thoracis* from light pigs slaughtered after 64 and 83 days of treatment.
Regarding the technological attributes of lipids, in both layers of backfat, the iodine value was significantly higher in restricted pigs but lower than 70, which is the threshold indicated by most authors (BARTON-GADE, 1987; MADSEN *et al*., 1992) as a guarantee of good preservation aptitude. GIRARD *et al*. (1988) suggest that in order to obtain an adequately firm fat that is not too susceptible to oxidation, its content in stearic and linoleic acid should be higher than 12% and lower than 15%, respectively. These requisites were met in both the *ad libitum* and restricted groups, in both layers. Thus, feeding restriction did not impair the technological quality of subcutaneous adipose tissue.

Table 5. Fatty acid (FA) composition (%) of *Longissimus lumborum* muscle in immunocastrated male pigs submitted to different feeding regimes (least squares means).

Fatty acids	Treatment		
	***Ad libitum* (n=12)**	**Restricted (n=12)**	**SE[($)]**
C 10:0 (capric)	0.12	0.12	0.001
C 12:0 (lauric)	0.09	0.08	0.005
C 14:0 (myristic)	1.40	1.38	0.066
C 16:0 (palmitic)	27.99[a]	27.10[b]	0.413
C 17:0 (heptadecanoic)	0.23	0.27	0.028
C 18:0 (stearic)	14.43	14.63	0.745
C 20:0 (eicosanoic)	0.17[a]	0.16[b]	0.008
C 16:1n-7 (palmitoleic)	3.08	3.03	0.249
C 17:1n-7 (heptadecenoic)	0.24	0.25	0.026
C 18:1n-7 (vaccenic)	3.83	3.89	0.208
C 18:1n-9 (oleic)	42.53	41.95	0.731
C 20:1-n9 (eicosenoic)	0.80	0.80	0.040
C 18:2n-6 (linoleic)	4.18	5.11	0.743
C 18:3n-3 (α-linolenic)	0.19	0.20	0.025
C 18:3n-6 (γ-linolenic)	0.12	0.13	0.017
C 20:2n-6 (eicosadienoic)	0.15[B]	0.18[A]	0.011
C 20:3n-3 (eicosatrienoic)	0.02[b]	0.03[a]	0.003
C 20:4n-6 (arachidonic)	0.31	0.51	0.144
C 20:5n-3 (eicosapentaenoic)	0.00	0.01	0.002
C 22:2n-6 (docosadienoic)	0.00	0.00	0.000
C 22:4n-6 (docosatetraenoic)	0.07	0.11	0.026
C 22:5n-3 (docosapentaenoic)	0.03	0.04	0.010
C 22:6n-3 (docosaesaenoic)	0.01	0.01	0.002
Σ SFA (saturated FA)	44.44	43.75	0.990
Σ MUFA (monounsaturated FA)	50.48	49.91	0.880
Σ PUFA (polyunsaturated FA)	5.08	6.34	0.969
PUFA/SFA	0.11	0.15	0.026
Σ n-6 (ω-6 FA)	4.84	6.05	0.934
Σ n-3 (ω-3 FA)	0.25	0.29	0.038
n-6/n-3	19.52	20.62	2.020
TI (thrombogenic index)	1.50	1.45	0.061
AI (atherogenic index)	0.61	0.58	0.019

[a,b]=$P<0.05$; [A,B]=$P<0.01$; [($)]standard error of differences.

$$TI = \frac{(C12{:}0+C16{:}0+C18{:}0)}{0.5x(MUFA+n-6\ PUFA)+3x(n-3\ PUFA)+(n-3\ PUFA/n-6PUFA)}\ ;\quad AI = \frac{(C12{:}0+4xC14{:}0+C18{:}0)}{(MUFA+PUFA)}$$

The fatty acid composition of lipids is strongly influenced by the carcass fatness (LO FIEGO, 1996), therefore any factor that modifies carcass fat content, such as age or BW at slaughter (LEBRET and MOUROT, 1998; LO FIEGO *et al*., 2010), feeding strategies (CAMERON *et al*., 2000; BEE *et al*., 2002) and genetic type (PIEDRAFITA *et al*., 2001; LO FIEGO *et al*., 2005b), can also modify lipid composition, especially the PUFA content in backfat depots. In the present research, final BW and carcass backfat thickness were found to be significantly lower in R pigs, which also showed a markedly higher degree of lipid unsaturation in both layers of backfat. This thus confirms the effect of adiposity underlined in the quoted studies on light pigs and on Italian heavy pigs.

4. CONCLUSIONS

Our results indicate that, from a nutritional point of view, the fatty acid profile of the lipids of immunocastrated medium-heavy pigs can be improved by feeding restriction in the finishing period, without significantly impairing their technological attributes.
Rearing medium–heavy pigs is becoming increasingly common in Italy. This production chain uses the same genetic types as for PDO productions and, by adopting suitable feeding plans, yields pigs for either fresh pork or seasoned non-PDO salami. The optimal feeding strategy thus depends on carcass destination. *Ad libitum* feeding, which favors carcass fat covering and the deposition of less unsaturated intramuscular fat and, thus, is more appropriate for processing, is suitable for seasoned products. If the production goal is pork for fresh consumption, some degree of feeding restriction is advisable since it elicits leaner carcasses and meats with healthier lipids.
However, further studies are needed to define the most appropriate level of restriction to prevent excessively lengthening the rearing period.

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PAPER

EFFECT OF THE ADDITION OF SMOKED TROUT FILLET POWDER TO THE QUALITY PROPERTIES OF PASTA

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ABSTRACT

In this study, quality properties of six pasta samples, prepared by substituting semolina with smoked trout fillet powder (STFP), were investigated. The addition of STFP to the pasta formulation resulted in an increase in protein, fat and ash content, energy value, antioxidant activity, phenolic content, optimum cooking time, cooking loss, and a* and b* values, but a decrease in carbohydrate content, water absorption capacity, swelling index and L* value. Furthermore, SEM images revealed that addition of STFP leads to an increase in protein matrix around the starch molecules, and its addition in quantities up to 15% showed acceptable sensory scores.

Keywords: pasta enrichment, pasta quality, smoked trout fillet powder

1. INTRODUCTION

Pasta is the most commonly consumed food product after bread among cereal products, and is traditionally produced from wheat semolina and water. The popularity of pasta is a result of its low cost, long shelf life, sensory characteristics and nutritional properties (FRADIQUE *et al*., 2013; KAUR *et al*., 2013; GOES *et al*., 2016). The Food and Drug Administration and the World Health Organization both recognize pasta as a good vehicle for enrichment with nutrients (BELEGGIA *et al*., 2011), and it is also known to be a rich source of complex carbohydrates and B vitamins, but as a poor source of protein and essential amino acids. This has led to many studies being carried out to enrich pasta with protein-rich ingredients such as meat, seafood (such as fishes and shrimp), legumes (such as chickpea, split pea, lentil, cowpea, mung bean and faba bean flour) (GALLEGOS-INFANTE *et al*., 2010; KADAM and PRABHASANKAR, 2012; LAKSHMI DEVI *et al*., 2013; LIU *et al*., 2016; PETITOT *et al*., 2010; SAVITA *et al*., 2013; WOJTOWICZ and MOSCICKI, 2014; WOOD, 2009; ZHAO *et al*., 2005). Such enrichments can have an effect on certain qualities of pasta, such as color, sensory characteristics, texture and cooking parameters (MERCIER *et al*., 2011).

Fish provide several nutritional benefits, such as high quality proteins that contain essential amino acids, and are a good source of lipids, complex B vitamins and such minerals as phosphorus, magnesium, zinc and iron (GOES *et al*., 2016). Fish is usually consumed in its fresh form, but can also be consumed after being dried, salted or smoked. Smoking techniques have been used as a preservation method for centuries. Besides affecting the characteristic color and flavor of food, smoking processes also has antimicrobial and antioxidative effects that are known to extend the shelf life of foods (LINGBECK *et al*., 2014).

Before marketing, smoked fish fillet is packed in standard weights. The edges of smoked fillets must be cut to meet a standard weight for packaging. These pieces that showed the same nutritional characteristics as the fish fillets, are discarded as production waste or are processed into low value-added products. Such waste can be used in the manufacture of enriched, high value-added food products.

The objective of this study is to investigate the chance of utilization of such fillet pieces and to examine the possibility of pasta nutritional value enhancement. It was also aimed to determine the changes in the cooking characteristics, antioxidant activity, microstructural properties, sensory properties and color attributes of pasta samples with the addition of smoked trout (*Oncorhynchus mykiss*) fillet powder (STFP).

2. MATERIAL AND METHODS

2.1. Raw materials

Smoked trout fillet pieces were dried in a cabinet dryer (Yucebas Machine Analytical Equipment Industry, Izmir, Turkey) at 50°C until <10% moisture content was achieved. The airflow rate in the cabinet dryer was 0.2 m/s and the relative humidity of the air was in a range of 19–21 %. After drying, the samples were ground into powder of a <1000 μm particle size, and the STFP was then stored at -18 °C until use.

Wheat semolina (particle size <450 μm), deionized water and common salt were used in the preparation of each pasta sample.

2.2. Pasta production

The STFP was added to the pasta formulation in quantities of 5, 10, 15, 20 and 25. The formulations of the pasta samples are shown in Table 1. The salt content of the STFP was found to be 6.3 %, and so the salt content was adjusted to 1.5 % in the pasta formulations. The ingredients were mixed in the kneading vessel of the laboratory pasta machine (Dolly pasta machine, La Monferrina, Italy) at room temperature for 10 min to make the pasta dough, and the dough was then extruded using the pasta machine. A no. 28 die (6 mm wide, 0.85 mm thick, PTFE) was used to shape the dough, which was then cut into 10 cm lengths. The pasta samples were dried at room temperature until a <10% moisture content was reached (~20 h).

Table 1. Formulations of pasta samples.

Ingredients	STFP Inclusion Level					
	0%	5%	10%	15%	20%	25%
Wheat Semolina (g)	100	95	90	85	80	75
STFP (g)	0	5	10	15	20	25
Deionized Water (mL)	34	34	36	39	39	41
Salt (g)	1.5	1.2	0.9	0.5	0.2	0

The control sample (unenriched) and the enriched pasta samples were packaged in a moisture-proof material (PET+COEX PA) and stored at room temperature for further analyses.

2.3. Proximate composition analysis

The crude protein content of the samples was determined using the Dumas method (SHEA and WATTS, 1939) with a Dumatherm analyzer (Gerhardt GmbH & Co. KG, Königswinter, Germany), while fat content, moisture content and ash content were measured using AOAC (1990) methods. Carbohydrate content was estimated by subtracting the moisture, protein, fat and ash content from 100% Energy values were calculated using the following equation (SOUCI *et al*., 2000);

$$Energy\ value(kcal/g) = (Carbohydrates \times 4) + (Proteins \times 4) + (Lipids \times 9)$$

2.4. Total phenolic content and total antioxidant activity of pasta

For the extraction of phenolics, 10 mL of aqueous methanol (70:30 v/v) was added to 1 g of the samples. After 10 min of sonication in an ultrasonic bath (E 60 H Model, Elma Co., Germany), the mixture was shaken in a mechanical shaker (WiseShake SHO-1D, Wertheim, Germany) for 15 min at room temperature. At the end of the centrifugation (NF 1200 R, Nuve, Turkey) of mixture at 8500 g at 4°C for 20 min, supernatants were collected. The total phenolic content and antioxidant activity of the extracts were analyzed in duplicate.
Total phenolic content (TPC) analyses were carried out by the method of SINGLETON *et al*. (1998), in which 1 mL of extract is poured into a test tube, after which, 5 mL of 10-fold

diluted Folin-Ciocalteu and 4 mL of Na_2CO_3 (7.5%) solutions were added. After 2 h incubation in the dark, the absorbance of the mixtures was measured at 760 nm against a reagent blank and standards using a spectrophotometer (PG-80 UV-Vis Spectrometer, PG Instruments, United Kingdom). The results were expressed as milligrams gallic acid equivalent (GAE)/100 g wet basis.

Total antioxidant activity (TAA) was measured using the 2.2-Diphenyl-1-picrylhydrazyl (DPPH) method according to THAIPONG, *et al*. (2006). Twenty four mg of DPPH was dissolved in 100 mL of methanol for prepared as a stock solution and then stored at -20 °C until use. The working DPPH solution was prepared by mixing the stock solution with methanol to obtain an absorbance value of 1.1±0.02 at 515 nm. The extracts (150μL) were allowed to react with the working solution (2850 μL) for 24 h in the dark, after which the absorbance of the samples was measured at 515nm. The standard calibration curve was linear between 25 and 800 μM Trolox, and the results were expressed in μmol Trolox equivalent (TE)/100 g wet basis.

2.5. Pasta cooking tests

2.5.1 Optimum Cooking Time

The optimum cooking time (OCT) of the samples was determined according to AACC (2000). Briefly, 25 g of pasta was broken into 5 cm constant lengths and added into 300 mL of boiling distilled water. Every 30 seconds, a piece of pasta was taken out and squeezed between two glass plates. The OCT was defined as the time taken until the white center of the sample disappeared.

2.5.2 Cooking loss

For the determination of cooking loss, 10 g of pasta was cooked for the OCT, after which the sample was rinsed with distilled water in a Buhner funnel. Cooking loss was measured by evaporating the cooking water and rinsing with water in a hot air oven at 105 °C. The residue was weighed and reported as a percentage of the uncooked pasta sample (TUDORICA *et al*., 2002).

2.5.3 Water absorption capacity

After cooking the 10 g pasta samples at OCT, the water absorption capacity (WAC) was calculated using the following equation (MARTI *et al*., 2013):

$$WAC(\%) = \frac{Weight\ of\ cooked\ pasta - Weight\ of\ uncooked\ pasta}{Weight\ of\ uncooked\ pasta} \times 100$$

2.5.4 Swelling index

The swelling index (SI) of the samples was determined according to the CLEARY and BRENNAN (2006). Twenty-five g of pasta was cooked in 250 mL of boiling distilled water for OCT, and then dried at 105°C. The SI was expressed as:

$$SI = \frac{Weight\ of\ cooked\ pasta - Weight\ of\ dried\ pasta}{Weight\ of\ dried\ pasta}$$

2.6. Microstructure of raw pasta

The pasta samples were freeze-dried (Thermo Savant ModulyoD-230, USA) for 8 h, and the freeze-dried samples were coated with gold. The microstructure of the surface of the raw pasta samples was visualized with scanning electron microscopy (SEM) (FEI Quanta 250 FEG, USA).

2.7. Color measurement

The surface color values of the cooked pasta were measured using a Hunter Lab Miniscan XE Colorimeter (Hunter Associates Laboratory, Reston, VA). L*, a* and b* parameters were recorded, and the changes in color resulting from the addition STFP to the pasta formulation were determined according to a color differential index (ΔE) that calculated the following equation;

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

Where:
ΔL* was calculated as L* sample-L*control;
Δa was calculated as a* sample-a* control;
Δb was calculated as b*sample-b*control

According to the Handbook of Colour Science (YAMAUCHI, 1989), ΔE values describe visual color differences as follows: (0–0.5, trace difference); (0.5–1.5, slightly discernible; hard to detect with the human eye); (1.5–3.0, noticeable; detectable by trained people); (3.0-6.0, appreciable; detectable by ordinary people); (6.0–12.0, large; large difference in the same color group); (Larger than 12; extreme; another color group).

2.8. Sensory evaluation

The pasta samples were cooked (100 g of pasta in 1 L of boiling water with 10 g salt) for OCT and drained. Pasta samples were presented individually on plastic trays to each panelist. The sensory evaluation was made by 44 panelists from Pamukkale University, Department of Food Engineering (29 females, 15 males; age range 20–50). The panelists scored each sample for color, odor, taste, texture and overall acceptability on a hedonic scale ranging from 1 (dislike extremely) to 7 (like extremely). The samples were labeled with randomly selected three-digit numerical codes. Bread and water were given to the panelists for rinse their palates between samples. The final scores were calculated from the average of the 44 scores (AYDIN and GOCMEN, 2011; CARDENAS-HERNANDEZ *et al.*, 2016).

2.9. Statistical analysis

All experiments were performed in duplicate. Statistical analyses were made using SPSS 22.0 (IBM SPSS Inc., Chicago, IL, USA) software. A Duncan's multiple range test was used and the levels were considered significantly different at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Raw materials

The moisture, protein, fat, ash and carbohydrate content, and the energy value, TAA, TPC and color values of wheat semolina and STFP are shown in Table 2.

Table 2. Nutritional and Chemical Properties of wheat semolina and STFP.

	Wheat semolina	STFP
Moisture (%)	13.41±0.07	7.61±0.45
Protein (%)	10.91±0.02	72.49±0.82
Fat (%)	1.79±0.37	14.29±0.74
Ash (%)	0.90±0.03	5.03±0.22
Carbohydrate (%)	73.00±0.40	0.59±0.31
Energy Value (kcal/100g)	351.69±1.71	420.92±4.57
Total Antioxidant Activity (μmol TE/100g)	2.20±0.10	10.06±0.39
Total Phenolic Content (mg GAE/100g)	33.30±0.46	51.22±0.91
Hunter color values		
L*	85.61±0.08	53.33±1.06
a*	0.04±0.03	6.27±0.18
b*	18.45±0.08	27.40±0.52

STFP: Smoked Trout Fillet Powder.

The moisture value of semolina was determined under the maximum limit (14.5%) of the Turkish Food Codex (2002). In addition, the protein content of semolina was detected above the minimum protein content (10.5%) defined in the Turkish Food Codex (2002). The results reveal that while STFP is a remarkable source of protein and fat, semolina is a good source of carbohydrate. It was determined that the use of fish powder in pasta production enhances the nutritional quality of enriched pasta. Additionally, the energy values of semolina and STFP were determined as 351.69 kcal/100g and 420.92 kcal/100g, respectively. STFP has a higher energy value than semolina due to its high protein and fat content. Furthermore, STFP demonstrates higher total antioxidant activity and total phenolic content than wheat semolina. The high level of antioxidant activity in the STFP can be attributed to the applied smoking process, in that smoke contains various phenolic compounds (GOULAS and KONTOMINAS, 2005; KJALLSTRAND and PETERSSON, 2001). The color measurement indicated that STFP has higher a* and b* values and lower L* values than wheat semolina.

3.2. Proximate composition of samples

The proximate composition of the control sample and the enriched samples are presented in Table 3.
Moisture content was increased after the addition of STFP, although all results were found to be under the critical moisture value of 13% identified in Turkish Standard 1620 (2017). The STFP may have increased the moisture content of the samples due to the water holding capacity of STFP proteins during dough preparation (CHIN *et al*., 2012; ZAYAS, 1997).

Table 3. Proximate composition of samples.

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Carbohydrate (%)	Energy Value (kcal/100g)
Control	9.44±0.15^{b}	13.87±0.28^{d}	1.70±0.19^{d}	1.33±0.03^{b}	73.66±0.59^{a}	365.39±0.47^{c}
STFP 5%	10.12±0.06^{ab}	14.29±0.21^{d}	3.61±0.73^{cd}	1.39±0.17^{b}	70.59±0.41^{a}	371.96±4.08^{bc}
STFP 10%	10.32±0.26^{a}	17.97±0.18^{cd}	5.52±0.71^{bc}	1.45±0.20^{b}	64.74±0.96^{b}	380.51±3.27^{ab}
STFP 15%	10.06±0.55^{ab}	19.72±2.40^{bc}	5.79±1.54^{ab}	1.56±0.08^{b}	62.87±4.41^{bc}	382.48±5.84^{ab}
STFP 20%	10.15±0.18^{ab}	23.27±2.93^{ab}	7.22±0.42^{ab}	2.20±0.04^{a}	57.16±2.73^{cd}	386.69±3.00^{a}
STFP 25%	10.44±0.45^{a}	25.76±2.51^{a}	7.88±0.88^{a}	2.29±0.11^{a}	53.63±2.19^{d}	388.40±6.66^{a}

STFP: Smoked Trout Fillet Powder.
Different superscript letters in columns indicate statistical differences ($p<0.05$).

The protein, fat and ash content of the samples increased with the addition of STFP. In particular, the protein content of the sample with the addition of 25% STFP was detected to be 1.9 times higher than the control sample due to the high protein content (72.49%) of STFP. The fat content of the control sample and 25% STFP-added sample were detected as 1.70% and 7.88% respectively. The control sample was significantly lower than the 10, 15, 20 and, 25 % STFP-added samples ($p<0.05$). The ash content analysis revealed 1.33% ash content for the control sample and 2.29% for the 25% STFP-added sample. The ash content of the 25% and 20% STFP added samples were significantly higher than the rest of the groups ($p<0.05$). The statistical analysis revealed that the control and STFP 5% samples were similar in this respect ($p>0.05$).
The carbohydrate ratio decreased significantly with the addition of STFP, while the energy value increased ($p<0.05$). Although the energy value was higher, it was not regarded as a negative outcome due to the higher fat and protein contents of the enriched samples.
The results of the present study concur with those of LIU *et al*. (2016) and CHIN *et al*. (2012).

3.3. Total phenolic content and antioxidant activity

Traditionally, meat, fish and some other foods are subjected to smoking not only to give them a special taste, color and odor, but also to improve shelf life, based on the antioxidant and antibacterial properties of smoke (SEMANOVA *et al*., 2016; SOARES *et al*., 2016).
The TAA and TPC of the samples are shown in Table 4.

Table 4. Total antioxidant activity and phenolic content of samples

Sample	Total Antioxidant Activity (μM TE/100g)	Total Phenolic Content (mg GAE/100g)
Control	1.79±0.29^{b}	35.93±1.75^{c}
STFP 5%	4.75±0.88^{a}	36.36±1.90^{bc}
STFP 10%	4.76±0.92^{a}	38.77±2.24^{abc}
STFP 15%	4.99±0.76^{a}	38.83±0.99^{abc}
STFP 20%	5.10±1.11^{a}	40.92±2.43^{ab}
STFP 25%	5.12±0.41^{a}	42.21±0.30^{a}

STFP: Smoked Trout Fillet Powder.
Different superscript letters in columns indicate statistical differences ($p<0.05$).

TAA and TPC were both detected in higher values in the STFP than in the semolina (Table 2). So enriched samples had higher TAA and TPC values than the control samples. The TAA values of the samples ranged between 1.79 μM TE/100g and 5.12 μM TE/100g. The TAA of the control sample was significantly lower than STFP-added samples ($p<0.05$).
The TPC of the samples increased significantly with the addition of STFP to the pasta formulation ($p<0.05$), and it was determined that the TPC of the 25% STFP-added sample was 1.2 times higher than the control sample.
Previous studies have investigated the effects of smoking processes on certain foods, and similar results have been found. SHAIBAN *et al*. (2006) investigated the effect of different types of woods used for smoking cheese, and found that smoked cheese showed higher total phenolic content and total antioxidant activity than non-smoked cheese. SEROT *et al*. (2004) investigated 10 major phenolic compounds in herring fillets smoked using different methods, and found that the sum of the content of 10 phenolic compounds in smoked fillets was strongly affected by the method used.

3.4. Pasta cooking tests

Cooking characteristics are a strong indicator of pasta quality. The cooking characteristics of the pasta samples are shown in Table 5. OCT ranged from 9.50 to 11.00 minutes, and compared to the control sample, the OCT showed an increasing trend with the addition of STFP. Higher optimum cooking times have also been reported in meat-based pasta. KADAM and PRABHASANKAR (2012) reported that cooking time increased from 8.5 min (control sample) to 14.0 min with the addition of 30% shrimp meat. Cooking time is related to the starch gelatinization and water penetration rate. Water enter into the starch granule may be restricted by more complex protein networks, which may cause a delay in the start of the gelatinization process (LIU *et al*., 2016).
The swelling index of pasta is an indicator of the water absorbed by the proteins during cooking (GOPALAKRISHNAN *et al*., 2011). Increasing the STFP ratio in the formulation caused the water absorption capacity and swelling index to decrease significantly ($p<0.05$), which could be related to the lower water absorption capacity of the protein network in STFP than in the gluten network. Previous studies into bran-enriched and fish powder-enriched pasta have reported similar decreases in water absorption capacity and swelling index (ARAVIND *et al*., 2012; DESAI *et al*., 2018; GATTA *et al*., 2017). PETITOT *et al*. (2010) determined that pasta samples made with split pea flour and faba bean flour had lower water absorption capacities than the control samples. In contrast, BASKARAN *et al*. (2011) reported that pasta enriched with skimmed milk powder and whey protein concentrate had higher swelling index values than the control sample.

Table 5. Cooking characteristics of the control and STFP-enriched pastas.

Sample	Optimum Cooking Time (Min)	Water Absorption Capacity (%)	Cooking Loss (%)	Swelling Index
Control	9.50 ± 0.01^{d}	208.32 ± 1.62^{a}	5.87 ± 0.04^{b}	2.74 ± 0.03^{a}
STFP 5%	9.75 ± 0.35^{cd}	204.76 ± 3.21^{ab}	7.50 ± 0.01^{a}	2.72 ± 0.05^{a}
STFP 10%	10.00 ± 0.01^{bcd}	197.48 ± 5.08^{bc}	7.57 ± 0.23^{a}	2.62 ± 0.07^{ab}
STFP 15%	10.50 ± 0.71^{abc}	197.80 ± 2.84^{bc}	7.82 ± 0.69^{a}	2.63 ± 0.01^{ab}
STFP 20%	10.75 ± 0.35^{ab}	190.20 ± 1.65^{cd}	8.15 ± 0.50^{a}	2.55 ± 0.04^{b}
STFP 25%	11.00 ± 0.01^{a}	182.85 ± 2.53^{d}	8.42 ± 0.15^{a}	2.51 ± 0.08^{b}

STFP: Smoked Trout Fillet Powder.
Different superscript letters in columns indicate statistical differences ($p<0.05$).

Cooking loss is defined as the amount of solids lost into the cooking water of a sample cooked for OCT (SOZER *et al*., 2007). An analysis identified statistically similar levels of cooking loss in the enriched samples ($p>0.05$), while the control sample was significantly different ($p<0.05$). Increasing the STFP ratio in the pasta formulation increases the level of cooking loss of the samples. The cooking loss values of the samples ranged between 5.87% and 8.42% all of which fall under the acceptable limit of 9% (AACC, 2000). An increase in the proportion of STFP in the formulation results in a decrease in the amount of semolina-based components, such as gluten, in pasta, which leads to a weakening of the gluten network. Similarly, some studies on the enrichment of pasta with various ingredients have also shown an increase in the level of cooking loss (ARAVIND *et al*., 2012; GALLEGOS-INFANTE *et al*., 2010; ISLAS-RUBIO *et al*., 2014; SANT'ANNA *et al*., 2014). The supplementation of different non-gluten flours in pasta formulation has been reported to dilute the gluten strength and weaken the pasta structure, which may increase the level of dry matter lost into the cooking water (GALLEGOS-INFANTE *et al*., 2010). In addition, it was stated that pasta had low cooking loss when dried at high temperature. PASQUALONE *et al*. (2016) reported that when a high-temperature drying program was adopted, cooking losses were ranged from 3.2 to 4.8 % in control pasta and in pasta samples enriched of lyophilized tomato matrix or with durum wheat bran extracts produced by supercritical carbon dioxide or ultrasound.

3.5. Microstructure of raw samples

SEM images demonstrate the arrangement of the gluten network and starch in pasta (RAJESWARI *et al*., 2013). The surface microstructure of raw pasta samples is illustrated in Fig 1. Images of the control pasta sample show numerous starch granules that appear to vary in both size and shape (Fig. 1a). The outer surface of the pasta appears to be coated with a thin protein film (Fig. 1a), which has also been reported in previous studies (PASQUALONE *et al*., 2016; ALIREZA SADEGHI and BHAGYA, 2008; CUNIN *et al*., 1995).

As dried pasta has a very limited water system, the starch and protein compete strongly for water during cooking. The less protein surrounding the starch granules, causes the faster starch swelling and gelatinization. (DE NONI and PAGANI, 2010).With the addition of STFP to the pasta formulation, the protein matrix around the starch molecules enhances, and the starch molecules are coated with a thicker protein network by the increase of STFP addition ratio (Fig. 1b, 1c, 1d, 1e, 1f)., as also observed by ALIREZA SADEGHI and BHAGYA, (2008) and LIU *et al*., (2016).This phenomenon also explained the extension of cooking time by the increasing addition ratio of STFP.

3.6. Color measurement

The color of pasta is very important quality parameter in terms of consumer preferences (CARINI *et al*., 2009). The color parameters of the enriched and control samples are shown in Table 6. It can be noted that the L* value (lightness) decreases with the increasing supplementation levels of STFP. The L* values of the control and the 5% STFP-added sample were statically similar ($p>0.05$), and significantly higher than the other enriched samples ($p<0.05$). Pasta samples enriched with STFP had a significantly higher a*(redness) and b*(yellowness) values than the control sample ($p<0.05$). Overall, the color values indicate that enriched samples have more redness (a*) and more yellowness (b*), but less lightness (L*) values than the control sample. The changes in color were found to be related to the original color of the STFP and the semolina (Table 2).

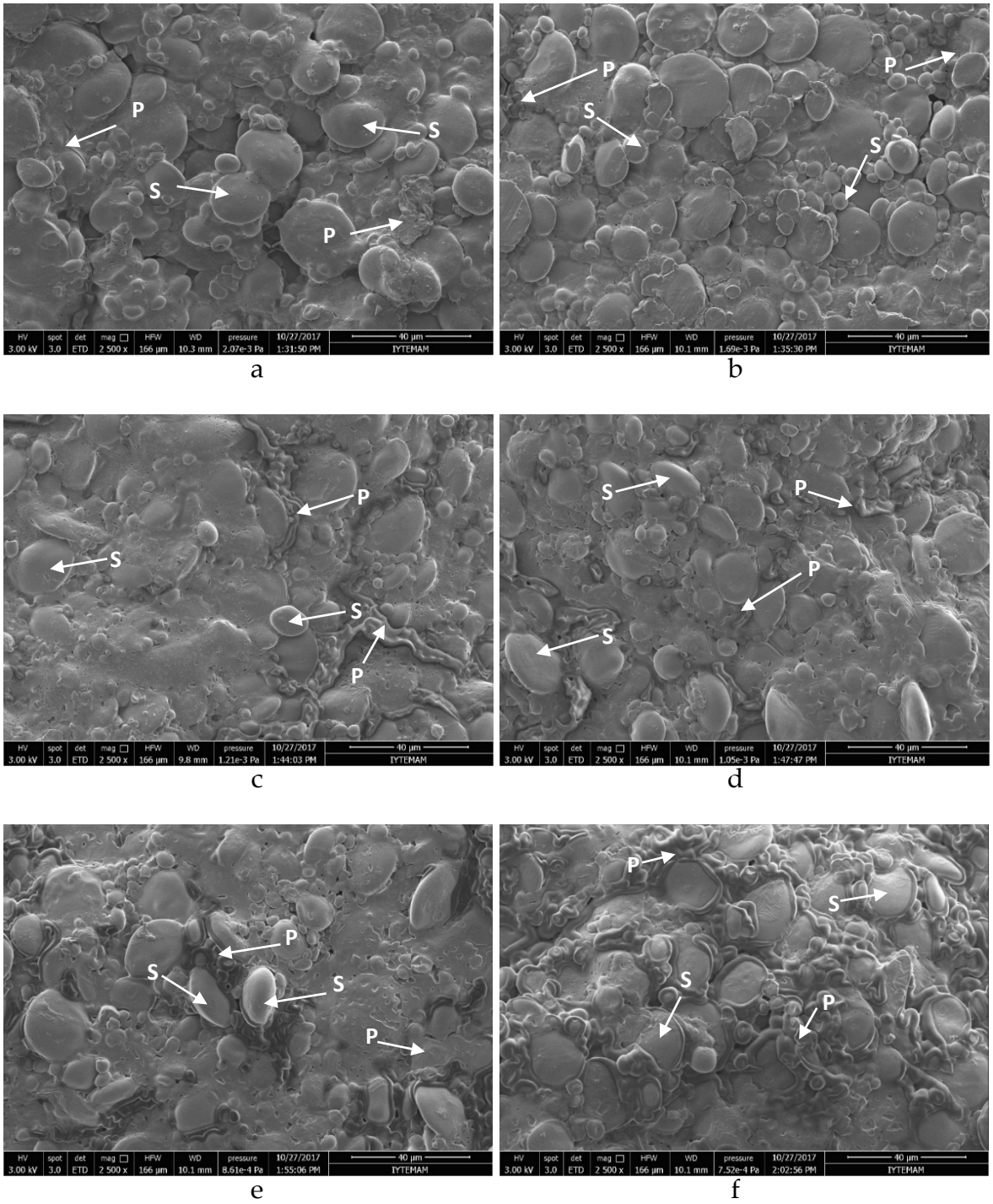

Figure 1. Surface SEM images of (a) control pasta, (b) 5% STFP pasta, (c) 10% STFP pasta, (d) 15% STFP pasta, (e) 20% STFP pasta, (f) 25 % STFP pasta. S: starch granules, P: protein network. Magnification 2500x.

These results were in close agreement with KADAM and PRABHASANKAR (2012), AYDIN and GOCMEN (2011), ALIREZA SADEGHI and BHAGYA (2008), who reported that samples supplemented with oat flour, shrimp meat and mustard protein isolate had lower L values and higher a and b values than the control sample due to the darker color of the enrichment materials than semolina.

The color differential index (ΔE) was indicated the color changes between the control sample and the enriched samples. The ΔE values of the samples was increased with the addition of STFP to the pasta formulation. According to the Handbook of Colour Science, 10%, 15% and, 20% STFP-added samples can be classified as "large difference in the same color group", while the classification of the 5% STFP-added sample and the 25% STFP-added sample were "appreciable; detectable by ordinary people" and "extreme, another color" respectively. DESAI *et al*., (2018) reported that the incorporation of fish powder into pasta increased ΔE values, while KHAN *et al*. (2014) investigated the color changes in sorghum flour-enriched pasta, stating that all the ΔE values of the cooked enriched samples were greater than 12, being classified as "extreme, another color" by YAMAUCHI (1989) in the Handbook of Colour Science.

Table 6. Color parameters of cooked pasta samples.

Sample	L*	a*	b*	ΔE
Control	68.01±0.45^{a}	-3.06±0.02^{f}	14.81±1.10^{e}	
STFP 5%	67.00±0,34^{a}	-2.30±0.10^{e}	18.63±0.01^{d}	4.04±1.05^{d}
STFP 10%	65.79±0.47^{b}	-1.55±0.10^{d}	20.62±0.33^{c}	6.40±0.66^{c}
STFP 15%	64.49±0.52^{c}	-0.42±0.27^{c}	22.76±0.64^{b}	9.09±0.30^{b}
STFP 20%	62.84±0.59^{d}	0.45±0.13^{b}	24.26±0.95^{ab}	11.32±0.01^{a}
STFP 25%	61.87±0.35^{d}	1.24±0.11^{a}	25.25±0.66^{a}	12.85±0.45^{a}

STFP: Smoked Trout Fillet Powder.
Different superscript letters in columns indicate statistical differences ($p<0.05$).

3.7. Sensory Evaluation

The sensory properties of the samples were evaluated based on color, odor, taste, texture and overall acceptability, and the results are shown in Table 7. The control sample and the 5% STFP-enriched samples were found to be statistically similar in all parameters ($p>0.05$).

Table 7. Sensory properties of control and smoked trout fillet powder enriched pastas.

Sample	Color	Odor	Taste	Texture	Overall Acceptability
Control	4.84±0.21^{a}	4.86±0.22^{a}	4.78±0.34^{a}	5.01±0.31^{a}	4.90±0.25^{a}
STFP 5%	4.98±0.03^{a}	4.88±0.23^{a}	4.77±0.27^{a}	5.00±0.11^{a}	4.86±0.26^{a}
STFP 10%	4.75±0.11^{a}	4.50±0.06^{b}	4.36±0.21^{ab}	4.34±0.23^{ab}	4.38±0.23^{ab}
STFP 15%	4.84±0.18^{a}	4.17±0.01^{c}	3.94±0.33^{bc}	4.25±0.41^{ab}	4.00±0.30^{bc}
STFP 20%	4.77±0.08^{a}	4.19±0.03^{c}	3.46±0.18^{c}	3.84±0.23^{b}	3.63±0.12^{c}
STFP 25%	4.48±0.44^{a}	3.90±0.03^{d}	3.48±0.08^{c}	3.73±0.57^{b}	3.56±0.33^{c}

STFP: Smoked Trout Fillet Powder.
Different superscript letters in columns indicate statistical differences ($p<0.05$).

No difference was identified between the color values of all samples ($p>0.05$), while odor and taste values decreased significantly with the addition of STFP ($p<0.05$). Some of the panelists reported an excessive fish odor and taste in the 20% and 25% STFP-added

samples, and a decrease in texture values was detected with STFP supplementation. During cooking, it was observed that especially the 20% and 25% STFP-added pastas had a tendency to rupture, and the panelists also pointed out that these pastas were very easily ruptured. The addition of STFP, which causes gluten reduction and a weakening of the gluten network, might reduce the resistance of the pasta. It was determined that the overall acceptability values of all the samples scored above 3.5, which the midpoint in a 7-point hedonic scale.

This study follows on from the research by CHIN *et al*. (2012), who reported that the color, taste and overall acceptability scores of noodles decreased with the addition of surimi powder.

On the other hand KADAM and PRABHASANKAR (2012), when supplementing pasta with shrimp meat, found from a sensory analyses that the addition of 20% shrimp meat had the best result in the overall score. LIU *et al*. (2016) enriched pasta with beef emulsion, and the overall preference scores showed that a 30% beef emulsion-added sample was statically similar to a commercial pasta sample.

4. CONCLUSIONS

The consumption of fish is known to have many benefits to human health. It can be added to various foods as a good source of enrichment, due mainly to its high protein content. Fish meat is considered to be a good source of enrichment for pasta. In the present study, it was determined that the addition of STFP increased the nutritional value of pasta, and also increased the antioxidant activity and phenolic content. The cooking analysis showed that the addition of STFP increased OCT and cooking loss, and decreased WAC and swelling index. As can be observed from the SEM images, the addition of STFP to the pasta formulation leads to an increased protein matrix around the starch molecules. An increased L* value, and decreased a* and b* values have been found in the enriched-STFP samples due to the darker color of STFP than semolina. The sensory analysis revealed overall acceptability scores of all samples above 3.5, although the 20% and 25% STFP-added samples were reported by the panelists to have an excessive taste and odor of fish. The 20% and 25% STFP-added samples were also noted to rupture during cooking. It was determined that the addition of 15% STFP enhanced the nutritional value of pasta, and also had acceptable cooking quality and sensory characteristics. Therefore, it can be concluded that fish pieces (production waste material) can be thought as pasta enrichment ingredients in case they are used in the mentioned concentration.

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ENCAPSULATION OF CAROTENOID-RICH PAPRIKA OLEORESIN THROUGH TRADITIONAL AND NANO SPRAY DRYING

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ABSTRACT

The objective of this research was to evaluate the physicochemical properties and stability during controlled storage of the particles of oleoresin paprika (*Capsicum annuum* L.) produced by Micro and Nano spray dryer. Micro and Nano spray dryer produced micro and submicron particles, respectively. Micro and submicron particles exhibited similar values of solubility, but showed significant differences in all parameters of colour and in the moisture content and water activity. At the end of spray dried process, both powders presented significant differences in the carotenes recovery. Micro- particles showed higher content of carotenes on the surface than the submicron particles. However, both particles showed similar protection to the carotenes at the end of storage. The best storage condition for both particles was around water activity (a_w) of 0.529 with about 50% carotenes retained at 30 days.

Keywords: carotenes, encapsulation, microcapsules, oleoresin, powder

1. INTRODUCTION

Oleoresin paprika (*Capsicum annuum* L.) is a lipophilic matrix obtained by pepper fruit processing and is mainly used as a natural food colourant due to its carotenoid pigments (RASCÓN *et al*., 2011). However, carotenes are highly susceptible to degradation during the processing and storage of foods (RODRÍGUEZ-AMAYA, 2016). The polyenic structure of carotenoid pigments is responsible for their colourant properties, antioxidant activities and biological functions. It also makes them very sensitive to heat, light and prooxidant conditions, promoting isomerisation and oxidation reactions, which diminishes their activities (RASCÓN *et al*., 2015). So, it is necessary to implement a strategy to prevent carotenoid degradation in paprika oleoresin and minimise their contact with oxygen. Nanoemulsions are used as vehicles for the encapsulation of various compounds (MEHRNIA *et al*., 2017). Encapsulation of the paprika emulsions is a technique may be used to protect the principal components of paprika and to improve their stability during processing and storage. The formation of micro- particles was the main focus of research and development efforts in the 1980s, whereas the formation of submicron and nano particles had been the focus of more recent efforts (JAFARI *et al*., 2017). Micro- and submicron particles have been incorporated into many types of food and beverage products, having a wide range of food applications (MIHINDUKULASURIYA and Lim, 2013). There are several methods for producing micro- and submicron particles, such as the spray drying is a widely available and low-cost technique that has been used extensively to transform liquids into solid powders, facilitating the handling of sensitive food ingredients and providing high storage stability powders (LI *et al*., 2010). The speed of the process and consequently, the short drying time, enables the drying of temperature-sensitive products without degradation (SCHUCK *et al*., 2008). Several spray dryers have been used in the production of microcapsules. In the typical spray drying process a liquid feedstock is atomized into a spray of fine droplets and then brought into contact with the hot drying gas at sufficient temperature for the moisture evaporation to take place (LEE *et al*., 2011) and it regularly encompasses four fundamental steps: atomization of feed into a spray, spray–air contact, drying of spray, and separation of dried product from the drying air (MASTER, 1976). However, traditional atomizers do not allow the generation of solid particles in the submicron range (ARPAGAUS *et al*., 2018). In addition, Buchi® has introduced a laboratory scale spray dryer that is able to generate submicron particles in the size range of 300 nm to 5 μm for milligram sample quantities at high yields (ARPAGAUS *et al*., 2018; BUCHI, 2018; SCHMID *et al*., 2010). This equipment had a vibrating mesh technology for fine droplets generation and a piezoelectric crystal driven spray head is incorporated with a small spray cap that contains a thin perforated membrane (spray mesh) having an array of precise micron-sized holes. However, it is important to know the properties and advantages of the particles developed by Micro and Nano spray drying to select the most appropriate equipment for a specific application. Therefore, the objective of this work was to evaluate the physicochemical properties and stability during the storage of particles of paprika oleoresin rich in carotenes obtained by the Büchi Micro and Nano Spray Dryer.

2. MATERIAL S AND METHODS

2.1. Material, chemicals and reagents

Paprika oleoresin with carotenoid content of 67560 $\mu g/g$ was obtained from AMCO (Xalapa, Veracruz, Mexico city, Mexico). Maltodextrin (10 dextrose equivalents, DE) was purchased from INALMALT (Mexico), Acetone and distillated water was HPLC grade.

2.2. Preparation of micro- and submicron particles by spray drying

To preparation of particles, nanoemulsion was prepared by combining paprika oleoresin (1 g), canola oil (9 g), surfactant (6 g; Tween 20, hydrophilic-lipophilic balance, HLB = 16.7), distilled water (54 g) and the wall material, maltodextrin 10 DE (30 g). The mixture was allowed to stand for 24 h and then 50 g of sample were ultrasonicated (Digital Sonifier 250D, Branson, USA) at 35% amplitude with pulsations of 5 x 5 s during 5 min, in an ice bath to keep the temperature constant (<40°C). Finally, the mixture was microfluidized at 30 MPa (microfluidizer M110PII, Microfluidics, USA) for 11 cycles, to achieve a particle size less than 100 nm.

Micro- particles were prepared from the selected emulsion using a mini Büchi 290 Spray Dryer (Flawil, Switzerland). The operating conditions for the dryer were: inlet air temperature of 150 °C and outlet air temperature of 90 °C. The spray flow rate of the feed solution was 6.66 x 10^{-4} L/s and the atomization pressure of the air gas was 543.80 kg/m^2. A 4 x 10^{-4} m diameter nozzle was used, the aspirator was set at 70% of the maximum capacity and the volumetric flow of the drying air was about 28 m^3/h. The feed rate was 2.5 mL/min. The micro- particles were recovered from the collecting chamber, weighed into amber bottles and maintained under a nitrogen atmosphere until analysis.

Submicron particles were prepared from the selected diluted nano-emulsion (1:20 v/v) using a B-90 Nano Spray Dryer (Flawil, Switzerland). The input temperature was 110 °C, the feed rate ranged from 5–35 mL/min, the volumetric flow of the drying air was 6 m^3/h and a spray mesh of 5.5 μm pore diameter was used. The fine droplets were dried into solid particles, which were collected by electrostatic charging and deflected to the collecting electrode (LI *et al*., 2010).

2.3. Retention and encapsulation efficiency of carotenes after spray drying

The retention of the carotenes after spray drying was evaluated by yield, non-encapsulated (superficial) carotenes content and encapsulation efficiency for both processes (micro- and submicron particles). The yield encapsulation percentage was defined as the ratio of total carotenoid in the final dried micro- particles to that in the emulsion. Carotenes concentration in the emulsion and particles was determined spectrophotometrically, as proposed by HORNERO and MÍNGUEZ (2001) using following equations:

$$C^R = \frac{A_{508} x\ 2144.0 - A_{472}\, x\ 403.3}{270.9}\ (\mu g/mL)$$

$$C^Y = \frac{A_{472}\, x\ 1724.3 - A_{508}\, x\ 2450.1}{270.9}\ (\mu g/mL)$$

$$C^T = C^R + C^Y (\mu g/mL)$$

Where A represents the absorbance at specific wavelength, C_R represents the red isochromatic fraction content, C_Y represents the yellow isochromatic fraction content, and C_T represents total carotenoid content.
Quantification of carotenes in the emulsion (control experiment) prior to encapsulation was necessary for determination of yield and encapsulation efficiency. Both particles (0.025 g) were dissolved in a volumetric flask containing 100 mL of acetone, and then filtered and the absorbance measured in a diode array spectrophotometer (Agilent model 8453, USA) at 454 nm. Surface carotene in both powders was assessed using the method described by WAGNER and WARTHESEN (1995). Briefly, triplicate samples (50 mg) of powder were weighed into test tubes and extracted with 25 mL hexane. After shaking (100 rpm) for 15 s, the powder particles were centrifuged at 1000 × *g* for 1 min and the carotenes concentration in the supernatant measured at 454 nm. The percentage of surface carotenes was determined by dividing the surface concentration by the total carotenes concentration in the powder. Encapsulation efficiency was calculated considering the total carotenes in the capsules, including carotenes on the surface of the capsules and non-encapsulated (superficial) carotenes.

2.4. Physicochemical properties of the particles

The moisture content was determined gravimetrically using vacuum oven-drying at 60 °C to constant weight. The a_w of the spray-dried powders was measured using an a_w meter (AquaLab, 3TE, Decagon, USA). The solubility of the particles was determined by a gravimetric method, which involved adding 0.5 g of the sample to an Erlenmeyer flask containing 50 mL of distilled water and homogenising at 6 *x g* at room temperature for 30 min. Then, the solution was centrifuged at 3000 × *g* for 5 min before a 25-mL aliquot of the supernatant was transferred to a previously weighed Petri dish and maintained in an oven at 105 °C until complete evaporation of the water. The dishes were weighed and the solubility was calculated from the weight difference (CANO-CHAUCA *et al*., 2005). The colour was analysed using a ColorFlex V1-72 colourimeter (Hunter Lab, Reston VA, USA) by measuring the L*, a* and b* parameters and subsequently calculating the secondary hue angle (H°) and Chroma (C*). The range of diameter of the particles was determined using image analysis with the image j 7.50i software.

2.5. Physical properties of the particles

Bulk density, tapped bulk density, particle density, compressibility, and the angle of repose were determined. The particle density was measured by a pycnometric method using toluene. Bulk density was determined in 2 g of powder, which was loosely weighed into a 10-mL graduated cylinder. The final volume was recorded and the bulk density was calculated by dividing the sample weight by the volume. The compact density was determined by the method of "Tappin" in which 2–5 g of particles was placed in a 10-mL test tube. The probe was hit on a flat surface until constant volume. The percentage compressibility was determined as the ratio of the compression volume to the initial volume. For the angle of repose test, 5 g of sample was weighed and added to a dropping funnel which was placed at 10 cm above a flat surface. The height of the cone formed and its radius was measured.

2.6. Antioxidant activity by the linoleic acid method

The total antioxidant activity in the extract of the microcapsules was performed by the linoleic acid method. First a solution of β-carotene in chloroform (3.34 mg/mL) was added

to a flask containing 40 mg of linoleic acid and 400 mg of Tween 20 and mixed. Then, the chloroform was then removed by rotary evaporation at 40 °C for 5 min before 100 mL of distilled water was slowly added to the residue, with vigorous stirring, to form an emulsion.
By other hand, both particles (0.025 g) were dissolved in a volumetric flask containing 100 mL of acetone, and then filtered and prepared to tubes containing 0.2 mL at 200 mg/mL of the antioxidant solution (sample). Then, aliquots of the emulsion (5mL) were transferred into different test tubes containing 0.2 mL of samples to different concentration. The tubes were placed in a water bath at 40 °C and absorbance at 470 nm was recorded at initial time (t=0) and each 15 min intervals for 120 min. A blank consisting of an emulsion without β-carotene and 0.2 mL of ethanol in 5 mL of emulsion was used as the control. The antioxidant activity by using the following equation:

$$AA = \left(1\ \frac{A_0 - A_t}{A_0^0 - A_t^0}\right) x\ 100$$

Where, A_0 and A_0^0 are the absorbance values measured at initial time for sample and control, respectively, while, A_t and A_t^0 are the absorbance values measured in the samples and control, respectively at t=120 min.

2.7. The carotenes degradation kinetics

The spray-dried powders were placed in desiccators equilibrated to various a_w (0.108, 0.318, 0.515 and 0.743) using saturated salt solutions, and temperatures (25, 35 and 45 °C) during 30 days. The degradation kinetics of the total micro- and submicron particles carotenes was respectively evaluated according to HATEGEKIMANA *et al.* (2015). A first-order model was used to fit the thermal degradation data of the carotenes (CHEN *et al.*, 2009), using the following equations:

$$\ln\left(\frac{C_t}{C_0}\right) = kt$$

$$t_{1/2} = \frac{0.693}{k}$$

Where, C_0 and C_t are the quality parameters at time zero and time t (days), respectively, k is the first-order kinetic constant and $t_{1/2}$ is the time (days) required for the total carotenoid content to decrease by 50%.

2.8. Moisture sorption isotherms of the particles

Moisture sorption isotherm of the micro- and submicron particles were determined by the dynamic dew point isotherm (DDI) method in a vapour sorption analyser (AquaLab VSA, Decagon Devices Inc., Pullman, WA, USA) within the a_w range of 0.1–0.850 at 25 °C. The samples used in each analysis were first dried over phosphorus pentoxide (P_2O_5) until the weight variation at 25 °C was less than 0.01%. Then, one gram of each sample was placed in the equipment to make the isotherm. The equipment was programmed so that the sample was maintained in each relative humidity until reaching equilibrium, that is, the

weight variation was less than 0.01%. The moisture sorption isotherm data were correlated to the a_w (relative humidity) using the Guggenheim–Anderson–de Boer (GAB) equation according to WEISSER (1985).

$$M = \frac{M_0 C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)}$$

Where a_w is water activity; M is water content of the sample on dry basis; M_0 is the monolayer water content; C is the Guggenheim constant; and *K* is the constant correcting properties of multilayer molecules with respect to bulk liquid. The parameters values of GAB equation (M0, C and k) were estimated by fitting the mathematical model to the experimental data, using non-linear regression using the Kaleidagraph 4.0 package (Synergy Software, 2457 Perkiomen Avenue Reading, PA 19606-2049, USA).

2.9. Scanning Electron Microscopy

For visualising the outer topography of the micro- and submicron particles, the particles were equilibrated at 0.529 a_w using a supersaturated solution of magnesium nitrate and then the specimen was mounted on a holder with double-sided adhesive tape. The structure of the particles was observed after coating the specimens with gold and examining under a Quanta FEG 250 scanning electron microscope.

2.10. Statistical analysis

All experiments were performed on triplicate samples and were repeated at least twice. Differences between means were determined by Tukey's test ($P < 0.05$) using one-way analysis of variance (ANOVA) followed by the multiple comparison procedure (Hol-Sidak method) with Minitab release 12 software.

3. RESULTS AND DISCUSSION

3.1. Micro- and submicron particles properties

According with image analysis Micro and Nano spray dryer produce micro- particles and submicron particles with a range of diameters of 1-250 μm and 0.5-10 μm, respectively. The evaluation of the micro- and submicron particles immediately after spray drying revealed that the micro- particles had a higher ($P \leq 0.001$) moisture content (1.40 versus 0.50 g/100g) and a_w (0.465 versus 0.011) compared to the submicron particles (Table 1). Similarly, submicron particles resulted in higher carotenes retention (71.40 mg/mL) than micro- particles (46.37 mg/mL) at the end of the drying process, which was reflected in the submicron encapsulation yield (63.46%), based on the carotenes concentration. The submicron particles also showed higher encapsulation efficiency (98.50%) than the micro-particles (81.15%), indicating a higher concentration and better protection of carotenes within the submicron particles, which was reflected in the colour parameters. The micro-particles had colour parameter values (a* = 29.30, b* = 59.71, L* = 66.13) significantly different ($P \leq 0.05$) from the submicron particles (a* = 0.06, b* = 13.74, L* = 92.20), possibly due to the comparably lower concentration of carotenes on the surface of the submicron

particles. The difference in physicochemical properties between micro- and submicron particles may be due to the different drying temperature used by each of the spray dryers.

Table 1. Physicochemical properties of micro- and submicron particles the oleoresin of paprika obtained by Micro and Nano spray drying technology.

Property	Micro- particles	Submicron particles
Moisture content (g/100 g)	1.40±0.11^{b}	0.50±0.00^{a}
Solubility (g/100 g)	7.08±1.15^{a}	9.88±1.68^{a}
a_w	0.465±0.00^{b}	0.011±0.01^{a}
Total carotenes (mg/mL)	46.37±0.05^{a}	71.40±0.01^{b}
Antioxidant activity (%)	80.00±0.01^{a}	78.26±0.01^{a}
Encapsulation Yield (%)	41.21±5.20^{a}	63.46±4.50^{b}
Surface carotenes (%)	19.30±2.50^{b}	1.49±0.05^{a}
Encapsulation Efficiency (%)	81.15±2.20^{a}	98.50±2.10^{b}
Colour parameters		
L	66.13±0.12^{a}	92.20±0.17^{b}
a	29.30±0.28^{b}	0.06±0.16^{a}
b	59.71±0.12^{b}	13.74±0.27^{a}
Hue angle (°)	63.85±0.17^{a}	87.37±0.75^{b}
Chroma	66.51±0.24^{b}	13.75±0.26^{a}
Bulk density (kg/m^3)	350.25±0.00^{b}	240.50±0.00^{a}
Tapped density (kg/m^3)	480.30±10.50^{b}	330.40±20.00^{a}
Particle density (kg/m^3)	250.10±30.25^{a}	200.15±32.40^{a}
Angle of repose (°)	28.89±2.02^{a}	24.70±0.57^{a}
Compressibility (%)	27.36±3.23^{a}	29.56±0.58^{a}
Haussner index	1.37±0.06^{a}	1.34±0.12^{a}

Results are expressed as the mean (n=3)±SD. Means followed in same row are significantly different by Tukey's test (p<0.05).

Since, higher moisture content and some physicochemical properties of the micro particles is associated to lower outlet temperature (GOULA and ADAMOPOULOS, 2005) and the higher temperature used in this process of drying, which was slightly higher during micro- than submicron encapsulation (GOULA and ADAMOPOULOS, 2005). Drying outlet temperature used in the traditional Micro and Nano spray dryers was 90 and 110°C, respectively. The micro- particles had a high concentration (around 19%) of superficial carotenes, whereas the submicron particles presented only 1.49% non-encapsulated carotenes, which helps explain why the micro- particles had a relatively lower encapsulation efficiency. Similar results were reported when pure β-carotene was microencapsulated using traditional spray drying (DESOBRY *et al*., 1999). These results on surface oil coincide with those reported by JAFARI *et al*. (2007), who found that large particles contain more un-capsulated oil on their surface than the smaller particles. These differences in performance and encapsulation efficiency between the micro- and submicron particles have been explained by other authors, who mention that the performance of the encapsulation during the drying process depends of equipment design differences, while the retention rate of the core is associated with the physicochemical properties and characteristics of both the core and the wall material used in the

encapsulation (NUNES and MERCADANTE, 2007). The outlet temperature of the Nano Spray Dryer was higher than that exhibited by the Micro Spray Dryer, possibly resulting in faster crust formation (or solidification) of the particles which may be associated with lower levels of superficial carotenes (JAFARI *et al*., 2008). Despite the difference in carotene retention, both particles showed a similar antioxidant activity (about 80%), suggesting that in the micro- particles, components other than the carotenes present in paprika oleoresin, such as polyphenols, might confer certain antioxidant activity (MUDRIĆ *et al*., 2017). ABBEDDOU *et al*. (2013) explained that the degradation of carotenes did not imply a decrease in the antioxidant activity of the processed product.
On the other hand, the flow properties were evaluated to help characterize the particles obtained for both methods. Micro- and submicron particles showed significant differences ($P \leq 0.05$) in the bulk and tapped density. The bulk density was 350 and 240 kg/m^3 whilst tapped density was 480.30 and 330.40 kg/m^3 for the micro- and submicron particles, respectively. Instead, micro- and submicron particles no showed significant differences ($P > 0.05$) in the angle of repose, compressibility percentage and Haussner index. The mechanical properties of the micro- and submicron particles are influence their stability during transport, storage and packaging design (FERY and WEINKAMER, 2007) and differences in the values of bulk density between micro- and submicron particles suggest a high tendency to brittle fracture, which may influence the flow properties (KAGAMI *et al*., 2003). The bulk density values for both particles are in the range of reported for folic microcapsules (ASSADPOUR and JAFARI, 2017). There was no significant difference between the micro- and submicron particles in the angle of repose, compressibility and Haussner index, suggesting that both capsules have poor flow and strong cohesiveness of the powder (ABDULLAH and GELDART, 1999). These properties are possibly due to the high hygroscopicity of the maltodextrin used as the wall material. The particle density is an important parameter because it considers the volume occupied by the capsules, without considering the volume of the pores of the capsule. According to the results obtained, the micro- and submicron particles had similar values of particle density explaining their similar solubility.

3.2. Degradation of carotenes in controlled storage conditions

The use of mathematical models for the release of bioactive compounds provides information on the processes of mass transfer and the influence of parameters such as the morphology and distribution of encapsulated compounds (ASSADPOUR *et al*., 2017). Table 2 shows the degradation kinetics of carotenes degradation of micro- and submicron particles during storage at various temperatures and water activities. The micro-particles presented two stages of degradation. A first stage with a rapid degradation rate with k values ranging from 0.039 to 0.087 days^{-1} and a second stage with a lower degradation rate with k values of 0.009 to 0.030 days^{-1} derived from retention of the carotenes inside the capsule. In the first stage the highest percentage of retained carotenes is degraded. It can be observed that the half-life varied from 7.96 to 19.80 days, being smaller when the micro-particles were stored at higher water activities. The time corresponding to the change in the slope between two stages was longer in micro- particles stored at 25 °C and 0.529 a_w. The submicron particles presented a single stage of degradation with higher degradation of carotenes when the samples were stored at 0.729 of water activity and 45 °C. The carotenes degradation in submicron particles showed a good fit to a first-order reaction.
Micro- and submicron particles showed higher carotenes retention when the samples were stored at 0.529 a_w at 25 °C, and under these conditions, a 40 and 50% retention of the carotenes was estimated by micro- and submicron particles, respectively at the end of 30 days of storage (Fig. 1).

Table 2. Kinetic parameters of the degradation of carotene micro- and submicron particles under different water activity and temperature storage conditions.

T(°C)	a_w	Submicron particles (Single stage)		Micro- particles First stage		Second stage	
		k (days^{-1})	$t_{1/2}$ (days)	k (days^{-1})	$t_{1/2}$ (days)	k (days^{-1})	$t_{1/2}$ (days)
25	0.328	0.023	29.61	0.047	17.74	0.012	57.75
	0.529	0.021	33.00	0.035	19.80	0.009	77.00
	0.753	0.022	31.50	0.054	12.83	0.014	49.50
35	0.321	0.028	24.06	0.067	10.34	0.018	38.50
	0.515	0.024	28.87	0.039	17.76	0.021	33.00
	0.743	0.029	23.89	0.060	11.55	0.025	27.72
45	0.309	0.037	18.72	0.055	12.60	0.024	28.87
	0.496	0.032	21.65	0.075	9.24	0.020	34.65
	0.729	0.039	17.76	0.087	7.96	0.030	23.10

Coefficient of determination (R^2) of the degradation of carotene was higher than 0.80 was in all case. Data are mean of three determinations (n=3).

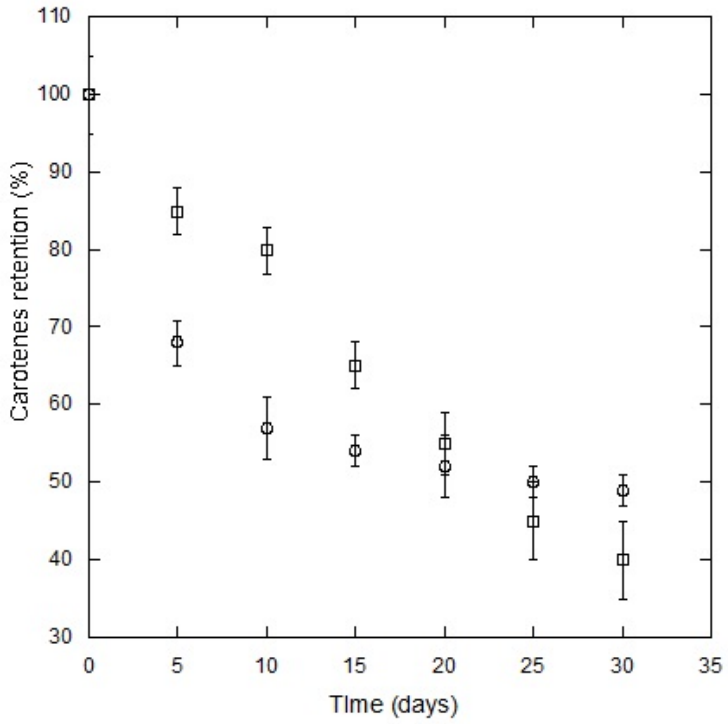

Figure 1. Carotenes retention (%) in micro- (○) submicron (□) particles obtained by spray drying and stored at 0.529 water activity at 25 °C during 30 days.

In both particles, the retention of carotenes depended on water activity and storage temperature, being higher in the range of water activities from 0.496 to 0.529 and at 25 °C. The differences in the kinetics of degradation between micro and submicron particles may be due to the fact that micro- particles have a higher concentration of carotenes on the surface compared to submicron particles, which is mostly exposed to environmental

conditions (light, oxygen and moisture) favouring the degradation of carotenes present on the surface. This same behaviour has previously been reported for the degradation of carotenes microencapsulated using maltodextrin as wall material (DESOBRY *et al*., 1999). Possibly, the lower retention of the micro- particles compared to the submicron particles at the end of the storage is due to the fact that the retention of the encapsulated carotenes depends on the particle size and the carotene content on the surface of the particle, as well as the state of the material of wall used in the encapsulation. It has been reported that low water activities maltodextrin had pores on the surface of the capsules favour carotene oxidation, whereas at higher water activities, the maltodextrin collapse, causing release and degradation of the carotenes (DESOBRY *et al*., 1999). So, an intermediate water activity would favor its stability. In turn, submicron particles have a larger surface area so water can be adsorbed onto the surface of carbohydrates at their polar sites; the dissolution of carbohydrates occurs favouring the degradation of encapsulated carotenes (AYRANCI *et al*., 1990).

The isotherm showed a type III form, according to the Brunauer-Emmet-Teller classification (VALENZUELA and AGUILERA, 2015). As shown in Fig. 2 there is no effect of the size of the particle on the water adsorption isotherm possibly this is due to other factors that affect the adsorption capacity such as pore volume, number of pores, pore diameters, total area of pores, distribution and shape of the particle, among others (ZOU and REZAEE, 2016, CHEN *et al*., 2017, ABDUL-MANAP *et al*., 2018). The water absorption isotherms and the GAB parameters of the micro and submicron particles were similar, indicating that particles exhibit similar behaviour in the equilibrium. In this work, the monolayer moisture content for the submicron (4.631 g H_2O/100 g) and micro- particles (4.531 g H_2O/100 g) was very similar and, in both instances and the value of the monolayer corresponded to the water activity about 0.500, which is close to that which provides the least carotenes degradation, corroborating that the best stability conditions for both particles is in the range 0.496- 0.529 a_w when the samples are stored at 25 °C.

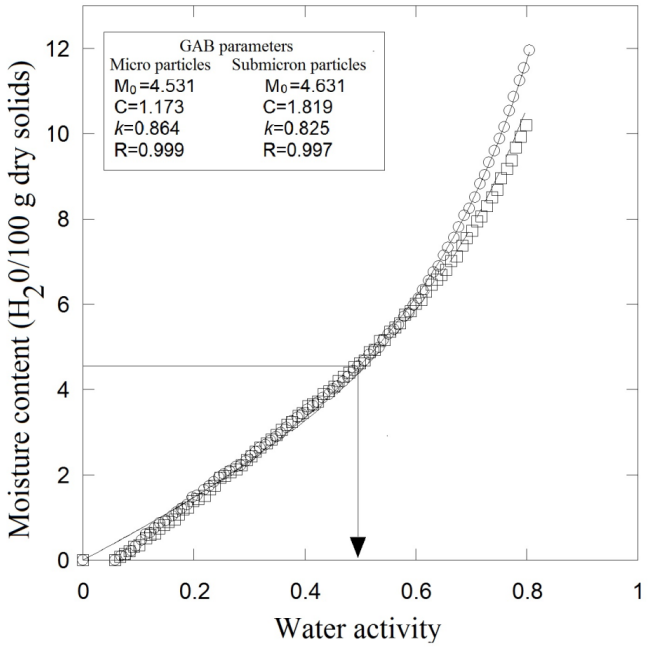

Figure 2. Water sorption isotherms of micro- (○) submicron (□) particles of paprika oleoresin with maltodextrin obtained at 25 °C.

The shape of the isotherm and the estimated GAB parameters are similar to those reported for juçara pulp microcapsules using maltodextrin as wall material (BIGETTI-GUERGOLETTO *et al*., 2017). It is well-known that the estimation of the monolayer moisture content and a_w, respectively, is important to define the conditions affording the highest stability and proper storage conditions (Kaya and Kahyaoglu, 2005). It has been reported that the monolayer moisture content corresponds to the minimum integral entropy zone, which the water molecules are best organized and less available to take part in reactions, because this humidity level, sufficient water was absorbed by the capsules to form a dough-like mass which acts as a shell opposed to oxygen diffusion into the capsule core, inasmuch as water is absorbed without initiating the wall dissolution process (BERISTAIN *et al*., 2002).

3.3. Particle morphology

The scanning electron micrographs of the particles (Fig. 3) and image analysis illustrated that the micro- particles stored at 0.529 a_w at 25 °C, were spherical with a smooth surface. In contrast, the submicron particles were spherical but much smaller with a rough.

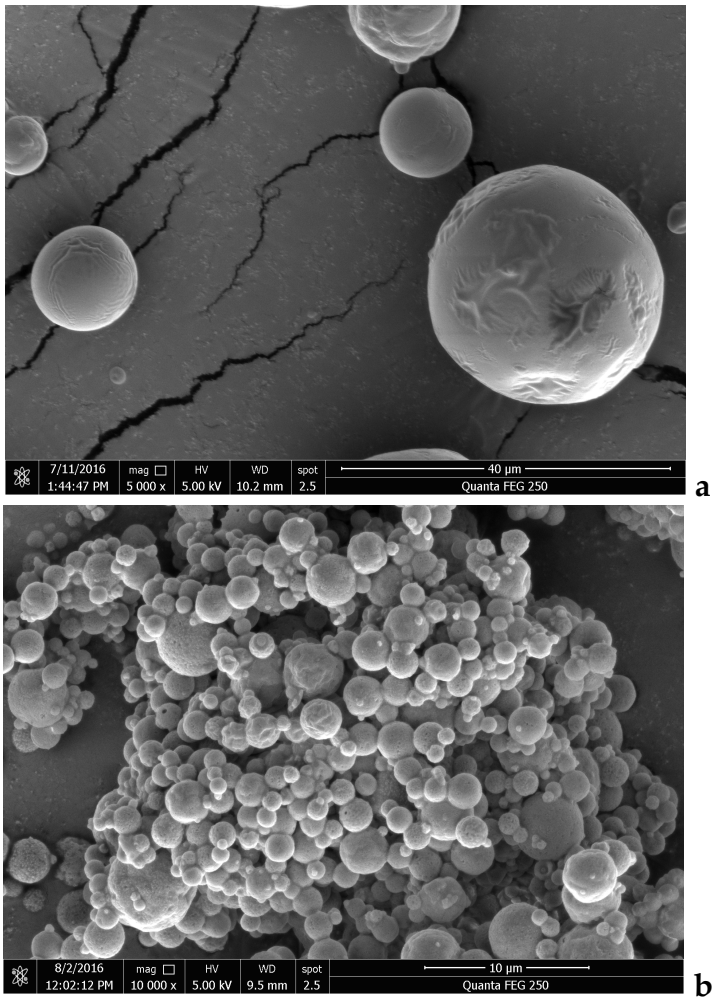

Figure 3. Scanning electron micrographs of the (a) micro- and (b) submicron particles, equilibrated at 0.529 water activity at 25 °C.

The differences in the morphology and particle size of the micro- and submicron particles are mainly due to the type of nozzle and formation of the drop during the drying process. Nano Spray Dryer uses vibration mesh technology, creating tiny droplets (before evaporation), making possible to produce powder with narrow distribution in the range of submicron particles (Li *et al*., 2010).
Particle size plays is also influenced by other factors, like shape, surface texture, and surface roughness. Similar morphology has been shown for starch capsules, displaying a smooth but depressed surface where smaller particles tended to agglomerate among themselves (HATEGEKIMANA *et al*., 2015). In addition, the powder particle size depends on the method used to obtain the capsules, the process parameters and the emulsion properties. The formation of indented surfaces of the micro- particles during spray drying is attributed to particle shrinkage during the drying process at low or high inlet temperatures. The particle shrinkage could be caused by the rapid evaporation and high pressure found inside the particles (ALAMILLA-BELTRÁN *et al*., 2005). These irregularities on the surface may favour a porous structure, in which there is a better arrangement of the water molecules and confers enhanced stability (VIVEROS-CONTRERAS *et al*., 2013).

4. CONCLUSIONS

This study showed that the carotenes present in the paprika oleoresin were successfully encapsulated through Micro and Nano spray dryer producing micro-particles and submicron particles, respectively. Submicron particles exhibited at higher yield, efficiency encapsulation and lower surface carotenes than the micro- particles. Submicron- and Micro- particles presented carotene degradation in one and two stages, respectively. However, both particles showed a similar stability to water activities of 0.529 at 25 °C after 30 days of storage. In general, the particles produced by each type of dryer exhibited differences in size, colour and encapsulation efficiency. However, they presented similarities in adsorption properties and stability. According to this, its application can be proposed in various food products, cosmetics or pharmaceutics.

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PAPER

FATTY ACIDS PROFILE IN *CARASSIUS SPP.* FROM LAKE GOPŁO, POLAND

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ABSTRACT

Effects of sex and age on fatty acids profile in the meat of *Carassius* spp. were evaluated. *Carassius auratus* fillets displayed a higher content of SFA and MUFA than *Carassius carassius*. In contrast, *Carassius carassius* had a higher content of PUFA (higher proportion of linoleic, arachidonic, eicosapentaenoic, docosatetraenoic and docosapentaenoic acids) and total n-3 and n-6 PUFA, compared with *Carassius auratus*. Moreover, γ-linolenic acid was higher in fillets from *Carassius auratus*. What is more, fillets from females had a greater content of eicosapentaenoic acid than males. Finally, 4-year-old fish had a lower content of ALA, higher MUFA content and better nutritional indexes (n-3/n-6, AI) than 3-year-old fish.

Keywords: age, *Carassius*, fatty acids, sex

1. INTRODUCTION

Fish meat is considered a high quality-food for human consumption. It is also considered as a functional food that can promote superior health (HOSOMI *et al*., 2012). Fish can be a great source of high amounts of protein, vitamins and minerals and most of all polyunsaturated fatty acids (PUFA) such as omega-3 (n-3) long-chain PUFA, including eicosapentaenoic acid (C20:5 n-3, EPA) or docosahexaenoic acid (C22:6 n-3, DHA), considered as 'essentials' (HUYNH and KITTS, 2009). In fact, these long-chain PUFA can be synthesized by humans from α-linolenic acid (C18:3 n-3, ALA), but due to low conversion efficiency, it is recommended to obtain EPA and DHA from additional sources, such as seafood. EPA and DHA are dietary fats with an array of health benefits. Indeed, a daily intake of 250–500 mg of EPA+DHA decreases the risk of mortality from coronary heart disease and sudden cardiac death (EFSA 2010). Moreover, EPA and DHA are also the precursors of several metabolites that are potent lipid mediators, considered by many investigators to be beneficial in the prevention or treatment of several diseases (SERHAN *et al*., 2008). In contrast, EPA in blood is an extremely potent antithrombotic factor.
There are various studies, which have examined the effects of exogenous (SHIRAI *et al*., 2002; LUZIA *et al*., 2003; GULER *et al*., 2008; KALYONCU *et al*., 2009; JABEEN and CHAUDHRY, 2011) and endogenous (AKPINAR *et al*., 2009; JABEEN and CHAUDHRY, 2011) factors on the fatty acids composition of fish meat. It should be noted that fish body composition is determined by different factors, such as sex (AKPINAR *et al*., 2009), age (ALEMU *et al*., 2013), feed availability, fishing season, location of reservoir, type of tissue and species (KALYONCU *et al*., 2009, 2010; JABEEN and CHAUDHRY, 2011).
To our best knowledge, no research has been found on the effects of age and sex on muscle fatty acid composition in two different species of *Carassius*. Thus, the aim of this study was to analyze the impact of sex and age on the fatty acids profile in the meat of *Carassius carassius* L. and *Carsassius auratus gibelio* Bloch collected in the same exogenous conditions, in the autumn, from the Lake Gopło.

2. MATERIALS AND METHODS

2.1. Study area

Lake Gopło is located in the southern part of Kuyavian-Pomeranian province. The western part of this lake is a strict nature reservoir. The main morphometric indicators of Lake Gopło are as follows; surface area: 22 km^2, maximum depth: 16 m, average depth ca 4.7 m and length of shoreline: 90 km (ŁUCZYŃSKA *et al*., 2008). Based on the limnological classification, it is an eutrophic reservoir, and, based on fishing classification, it is a zander type of the lake.

2.2. Biological material collecting

The study involved 56 individuals of *Carassius* (28 individuals of *Carassius carassius* L. and 28 *Carassius auratus gibelio* Bloch), selected according to the age (n= 28 for fishes > 3-year-old and n= 28 for fishes > 4-year-old) and sex (females: n= 28; males: n= 28). Fish were caught in natural conditions (Lake Gopło), in autumn (November 2015) and body weight and length were determined for each fish (Table 1).

Table 1. Body weight and length of *Carassius carassius* L. and *Carassius auratus gibelio* B. according to the age and sex.

	***Carassius carassius* L.**		***Carassius auratus gibelio* B.**	
	Body weight (g)	**Length (cm)**	**Body weight (g)**	**Length (cm)**
Age				
>3 years	176.9-206.4	16.3-17.4	176.5-209.0	16.0-17.5
>4 years	267.1-297.5	18.0-19.6	255.3-317.0	19.5-20.8
Sex				
Male	176.9-287.8	16.3-19.1	176.5-288.6	16.0-19.8
Female	178.6-297.5	16.6-19.6	175.1-317.0	16.4-20.8

The fillet samples for analyses were taken from the large side muscle of the fish body above the lateral line. The samples were vacuum packaged and stored frozen (-20°C) until analyzed for fatty acid composition.

2.3. Fatty acids analyses

Lipid extraction from muscle samples was performed by modification of the BLIGH and DYER (1959) and FOLCH *et al*. (1957) methods. Briefly, 100 mg of freeze-dried muscle was treated with chloroform/methanol/water (1:2:0.8) and stirred for 4 hours. Then, to obtain a better separation between the two phases, chloroform, 2N KCl/0.3 N HCl, and H_2O were added consecutively, and the samples were centrifuged for 5 min at 3000 g. The lower lipid-containing phase was separated from the upper phase, and retained for use. Subsequently, another extraction with chloroform was repeated. The extracted lipids were esterified and then analyzed by gas chromatography (GC Trace 2000 ThermoQuest EC Instruments) equipped with a flame ionization detector (260°C) and a fused silica capillary Column (Omegawax 320, Phenomenex, Torrance, CA, USA) 30 m x 0.32 mm x 0.25 μm film thickness. Helium was used as the carrier gas at a flow rate of 1.0 mL/min with constant flow compensation. GC inlets were held at a temperature of 240°C, and the detector was maintained at a temperature of 250°C. The oven temperature was programmed from 150°C, and was followed by a ramp-up at a rate of 5°C/min till 240°C with a final hold of 15 min (the total analysis time was 33.00 min). The individual fatty acids peaks were identified by comparison of retention times with those of known mixtures of standard fatty acids (PUFA-2, Supelco, Bellofonte, PA, USA) run under the same operating conditions. Results were expressed as percentage of the total fatty acids identified. An example of gas chromatography analysis (GC profile) is presented in Fig. 1. To assess the nutritional implications, the n-6 PUFA/n-3 PUFA and the PUFA/SFA ratios were calculated. The group of the fatty acids analyzed included saturated fatty acids (SFA) (C14:0, C16:0, C18:0), monounsaturated fatty acids (MUFA) (C16:1 n-7, C18:1 n-9, C18:1 n-7, C20:1 n-9,) and polyunsaturated fatty acids (PUFA) (C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:3 n-3, C20:4 n-6, C20:5 n-3, C22:4 n-6, C22:5 n-3, C22:6 n-3). In order to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, respectively, the atherogenic index (AI) and the thrombogenic index (TI) were calculated, according to the formulas suggested by ULBRICHT and SOUTHGATE (1991). AI=[12:0+(4×14:0)+16:0]/[n-6 PUFA+n-3PUFA+MUFA]; TI=[14:0+16:0+18:0]/[(0.5×MUFA)+(0.5×n-6 PUFA)+(3×n-3PUFA)+(n-3 PUFA/n-6 PUFA)].

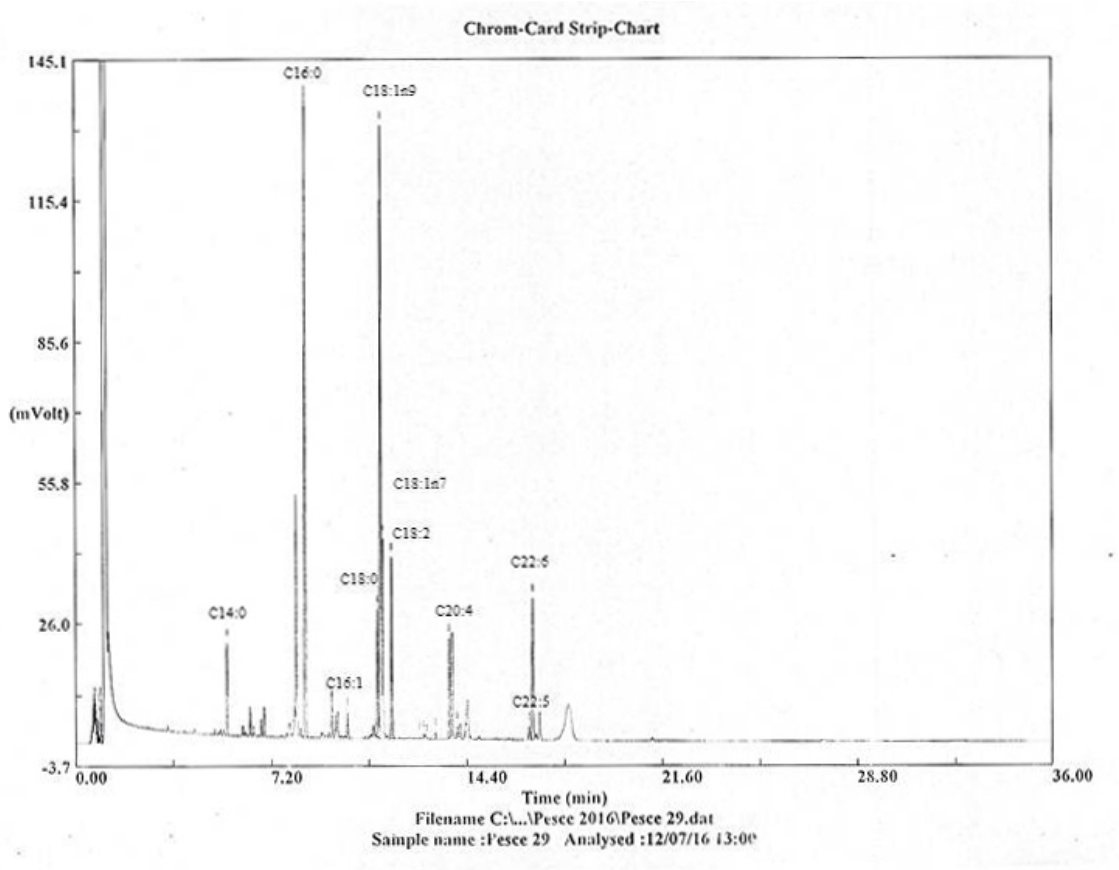

Figure 1. An example of gas chromatography profile.

2.4. Statistical analyses

Fatty acid composition and nutritional ratio data were analysed by 2×2×2 factorial ANOVA with interactions, where species, sex and age were the main factors. Each individual fish was considered as the experimental unit. Data analysis was performed using the SPSS package (SPSS, 2010). Data are presented as means±SEM, and a value of $p<0.05$ was used to indicate statistical significance.

3. RESULTS

The fillet fatty acids composition and nutritional ratios are presented in Table 2. Overall, the MUFA were the most abundant acids (39.86±0.42%), followed by SFA (31.79±0.39%) and PUFA (27.22±0.38%). The factor of greatest relevance affecting SFA content was fish species. *C. auratus* recorded significantly higher (+3%) SFA concentration compared to *C. carassius* ($p<0.001$), and particularly, higher proportions ($p<0.001$) of myristic (C14:0) and stearic (C18:0) acids, as well as palmitic acid (C16:0; $p>0.05$). As analyses indicated, sex did not significantly affect the total content of SFA or the content of the single SFA. The age factor affected only the palmitic acid content, which was higher ($p<0.05$) in fillets from 3-year-old fish compared to 4-year-old ones. Age did not influence the total content of SFA. In general, the palmitic acid was the most abundant (25.7±0.37%) among SFA, followed by the stearic (4.0±0.10%) and myristic acids (2.1±0.09%).

Table 2. Effects of species, sex and age on fatty acids composition (% of total fatty acids) and nutritional ratios of fish fillets.

Fatty acids‡	Species (SP)†		Sex (S)		Age (A)		SEM	Significance						
	CC	CA	Male	Female	>3 years	>4 years		SP	S	A	SPxS	SPxA	SxA	SPxSxA
C14:0	1.65	2.56	1.99	2.22	2.14	2.07	0.09	***	ns	ns	ns	*	ns	ns
C16:0	25.14	26.23	25.48	25.90	26.46	24.92	0.37	ns	ns	*	ns	ns	ns	ns
C18:0	3.50	4.50	4.13	3.87	3.91	4.08	0.10	***	ns	ns	ns	**	ns	ns
C16:1	3.66	3.02	3.41	3.27	3.49	3.19	0.05	***	ns	**	ns	ns	ns	ns
C18:1n-9	28.06	29.49	28.75	28.80	27.22	30.33	0.41	ns	ns	***	ns	ns	ns	ns
C18:1n-7	5.72	7.11	6.36	6.48	6.52	6.32	0.11	***	ns	ns	ns	ns	ns	ns
C20:1n-9	1.31	1.35	1.24	1.41	1.32	1.33	0.05	ns	ns	ns	ns	ns	ns	ns
C18:2n-6	10.72	8.23	9.57	9.38	9.84	9.11	0.19	***	ns	ns	*	ns	ns	ns
C18:3n-6	0.26	0.36	0.32	0.30	0.32	0.30	0.02	*	ns	ns	ns	ns	*	ns
C18:3n-3	0.18	0.24	0.23	0.19	0.26	0.16	0.02	ns	ns	*	ns	ns	**	ns
C20:3n-3	0.43	0.37	0.43	0.37	0.43	0.37	0.03	ns	ns	ns	ns	ns	ns	ns
C20:4n-6	4.95	4.41	4.60	4.76	4.85	4.51	0.10	*	ns	ns	ns	ns	ns	ns
C20:5n-3	1.67	0.57	0.95	1.29	1.15	1.09	0.06	***	**	ns	**	ns	ns	**
C22:4n-6	0.32	0.24	0.29	0.28	0.30	0.26	0.01	*	ns	ns	**	ns	ns	ns
C22:5n-3	1.12	0.72	0.97	0.86	0.90	0.94	0.05	***	ns	ns	*	ns	**	ns
C22:6n-3	10.13	9.52	9.80	9.85	9.62	10.02	0.17	ns	ns	ns	ns	ns	ns	ns
Partial sums														
SFA	30.28	33.29	31.59	31.99	32.51	31.07	0.39	***	ns	ns	ns	ns	ns	ns
MUFA	38.76	40.97	39.76	39.96	38.56	41.17	0.42	*	ns	**	ns	ns	ns	ns
PUFA	29.79	24.66	27.16	27.29	27.68	26.77	0.38	***	ns	ns	**	ns	ns	ns
Total n-6	16.25	13.25	14.78	14.72	15.31	14.19	0.25	***	ns	*	*	ns	ns	ns
Total n-3	13.53	11.41	12.38	12.57	12.36	12.58	0.22	***	ns	ns	**	ns	ns	ns
Nutritional ratios														
n-3/n-6	0.84	0.87	0.85	0.87	0.82	0.90	0.02	ns	ns	*	ns	ns	ns	**
P/S	1.00	0.75	0.88	0.87	0.87	0.88	0.02	***	ns	ns	**	ns	ns	ns
AI	0.46	0.56	0.50	0.52	0.53	0.49	0.01	***	ns	*	ns	ns	ns	ns
TI	0.44	0.54	0.49	0.50	0.51	0.47	0.01	***	ns	ns	*	ns	ns	ns

†CC = *Carassius carassius* L.; CA = *Carassius auratus gibelio* Bloch; ‡SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; P/S = PUFA/SFA ratio; AI = Atherogenic index; TI = Thrombogenic index. Significance: *** $p<0.001$; ** $p<0.01$; * $p<0.05$; ns: not significant.

Age effect was evident in the oldest *C. auratus* fish for both myristic and stearic acid contents, which were in lower ($p<0.05$ and $p<0.01$) amounts in 4-year-old fish.
The total MUFA content was higher ($p<0.05$) in *C. auratus* fillets compared to those of *C. carassius* (+2.21%), with higher ($p<0.001$) content of vaccenic acid (C18:1n-7) and slightly higher ($p>0.05$) oleic acid (C18:1n-9). In contrast, percentage content of palmitoleic acid (C16:1) was lower in *C. auratus* fillets compared to those of *C. carassius* ($p<0.001$). Sex did not significantly affect total MUFA and the proportions of the single MUFA. As opposed to *C. carassius*, age has been a relevant factor for the total MUFA content. In fact, with the increase of the age of the fish, total MUFA content increased (<0.01), as did oleic acid ($p<0.001$), whereas palmitoleic acid decreased ($p<0.01$).
C. carassius also had higher content of total PUFA (+5.13%), in comparison with fillets of *C. auratus*, and this difference was statistically significant at $p<0.001$. A higher percentage ($p<0.05$ and $p<0.001$) content of linoleic (LA, C18:2n-6), arachidonic (AA, C20:4n-6), eicosapentaenoic (EPA, C20:5n-3), docosatetraenoic (DTA, C22:4n-6) and docosapentaenoic (DPA, C22:5n-3) acids, and content of total n-3 and n-6 fatty acids was found in the fillets of *C. carassius*, while γ-linolenic acid (GLA, C18:3 n-6) was higher ($p<0.05$) in fillets from *C. auratus*. A high content of docosahexaenoic acid (DHA, C22:6 n-3) was also detected in both species, but no significant differences were found. Sex and age had a minimal influence on PUFA composition. Fillets from females had a higher ($p<0.01$) content of EPA than males. Comparison among ages showed that fillets of 3-year-old fish had a higher ($p<0.05$) content of α-linolenic (ALA, C18:3 n-3) and total n-6 fatty acids, compared to those of 4-year-old fish. Significant interactions were found between species and sex for total PUFA ($p<0.01$), LA ($p<0.05$), EPA ($p<0.01$), DTA ($p<0.01$), DPA ($p<0.05$), total n-6 ($p<0.05$) and n-3 ($p<0.01$), between sex and age for GLA ($p<0.05$), ALA ($p<0.01$) and DPA ($p<0.01$), as well as among species, sex and age for EPA ($p<0.01$). Analyses indicated that the n-3/n-6 ratio (ranging from 0.82 to 0.90) was affected only by the age, being higher ($p<0.01$) in 4-year-old fish than in 3-year-old. In addition, significant interaction was found among species, sex and age in n-3/n-6 ratio ($p<0.01$). The PUFA/SFA ratio was affected only by species, being higher ($p<0.001$) in *C. carassius* than in *C. auratus* fish. However, a significant interaction ($p<0.01$) was found among species and sex for PUFA/SFA ratio. In the present study, AI and TI were lower ($p<0.001$) in *C. carassius* than in *C. auratus* fish, and AI was lower ($p<0.05$) in 4-year-old fish than in younger individuals. In addition, a significant ($p<0.05$) interaction was found among species and sex for TI.

4. DISCUSSION

The results on the fillet fatty acids composition (31.79% of SFA, 39.86% of MUFA and 27.22% of PUFA) are in line with the values obtained by KOŁAKOWSKA *et al*. (2000) in roach (*Rutilus rutilus* L.), caught in the Odra and Regalica River (Poland), with higher amounts of total SFA (34.98%) and MUFA (46.83%) than total PUFA (18.19%). Different trends were reported by POLAK-JUSZCZAK and KOMAR-SZYMCZAK (2009), on roach caught from the Vistula Lagoon (25.10% of SFA, 32.49% of MUFA and 42.41% of PUFA), and by STANEK *et al*. (2008) on perch (*Perca fluviatilis* L.) from Włocławski Reservoir (46.02-47.53% of SFA, 23.83-32.24% of MUFA and 21.73-28.56% of PUFA).
Regarding the proportion of single SFA, palmitic acid was the most abundant, followed by stearic and myristic acids, respectively. The highest presence of palmitic acid (14.6-16.6%) was found in the meat of carp (*Cyprinus carpio* L.), caught in all four seasons (GULER *et al*., 2008). In the meat of roach originating from the Mazurian Great Lakes region (Poland), palmitic acid content was the dominant SFA (25.38%) and the total amount of SFA for this

species accounting for 35.38% (ŁUCZYŃSKA *et al*. 2008). The same results were confirmed by ÖZOGUL *et al*. (2007) for the fish called kutum (*Rutilus frisii*) caught in the Seyhan Dam Lake in Adana (Turkey).
It is reported that SFA with 12-16 carbon atoms increase serum concentrations of LDL cholesterol (MENSINK and KATAN, 1992). In particular, palmitic acid increases total serum cholesterol, but its effect is lower than that of the myristic acids (DALEY *et al*., 2010), which, in the present study, is approximately 2%. Stearic acid is considered a "neutral" fatty acid since it does not affect the plasmatic level of LDL or HDL cholesterol in humans (MENSINK and KATAN, 1992; WILLIAMSON *et al*., 2005). This effect of stearic acid has been attributed to its reduced digestibility and easy desaturation into oleic acid. From a nutritional point of view, the oleic acid, the most common MUFA present in considerable quantities in both animal and plant sources, has a relevant importance in the human diet because it acts on lipaemia, reducing both LDL cholesterol and the triglycerides (MOZAFFARIAN and CLARKE, 2009) and providing other health benefits (reviewed in SALES-CAMPOS *et al*., 2013).
In the present study, oleic acid ranged from 27.2% to 30.3 %, with the highest values in the fillet of 4-year-old fish. Regarding the composition of the single PUFA, the high amount of DHA in comparison with the low level of α-linolenic acid observed in *Carassius* is in agreement with the results obtained by MURZINA *et al*. (2016). DHA cannot be synthesized *de novo* in vertebrates, it is biosynthesized from its precursor α-linolenic acid during desaturation and elongation on biochemical pathways (STEFFENS and WIRTH, 2005; TOCHER, 2015). It is well known that the freshwater food system contains higher levels of linoleic and α-linolenic acids (TOCHER, 2003, 2010) leading to evident differences between freshwater and marine fish in terms of fatty acids distribution. In fact, the marine food chain is rich in EPA and DHA. However, some fish species from freshwater can also be a valuable source of EPA and DHA (STEFFENS and WIRTH, 2005; REITER and GRIMM, 2012), which derive from freshwater plankton (BRETT *et al*., 2009) and by endogenous metabolism (TOCHER, 2003). The dominant PUFA found in roach from Brda River (Poland) was linoleic acid (7.41-10.11%) (STANEK *et al*., 2012). Other studies in the meat of roach have found, among PUFA, the highest proportion of EPA, ranging from 7 to 12% (AHLGREN *et al*., 1994), 10% (GRAHL-NIELSEN *et al*., 2011) and 9% (UYSAL *et al*., 2008). However, variable values (ranging from 1.54 to 20.15%) were also found in 9 freshwater fish species from the Tigris River (CENGIZ *et al*., 2010). A diet rich in n-3 EPA is believed to shift the physiological state to one that is less inflammatory than that of a diet containing high amounts of n-6. In addition, marine-derived omega-3 fatty acids are recommended for the treatment and prevention of many chronic diseases, including cardiovascular disease and metabolic syndrome (HARRIS *et al*., 2010).
As analyses indicated, sex and age of *Carassius* had a minimal influence on PUFA composition. Analyses carried out by STANEK *et al.* (2012), concerning roach caught in fall and spring, indicated that the total PUFA content in the fish meat, ranged from 19.96 to 27.42%, and was not affected by sex.
Concerning the nutritional ratios, the n-3/n-6 ratio (ranging from 0.82 to 0.90) was affected only by age, being higher in 4-year-old fish. The value of n-3/n-6 ratio was found low (0.23) in the fillet of Nile tilapia (*Oreochromis niloticus*) (HERATH *et al*., 2016) and in common carp (0.47) reared in natural temperatures with water from Lake Trasimeno (GERI *et al*., 1995). GULER *et al*., (2008) found in fillets of carp caught from Lake Beysehir, values of n-3/n-6 ratio near 1 in winter, spring and summer and 0.5 in autumn. In contrast, in the meat of carp, caught in Ivriz Dam Lake, n-3/n-6 ratio were found to be 1.08, 1.43, 1.64 and 1.60 in spring, summer, autumn and winter, respectively (KALYONCU *et al*., 2010). The n-3/n-6 ratio has been suggested to be a useful indicator for comparing relative nutritional values of fish oils (PIGOTT and TUCKER, 1990). Furthermore, an

increase in the human dietary n-3/n-6 fatty acids ratio is essential to prevent cardiomyopathy (by reducing plasma lipids) and to reduce cancer risk. Until recently, the dietary balance between n-3 and n-6 PUFA has been evenly balanced with a n-3/n-6 ratio of approximately 1; the modern western diet is now dominated by a n-3/n-6 ratio of approximately 0.06 (SIMOPOULOS, 2002). The ratio of n-3/n-6 PUFA in lipids of freshwater fish varies mostly between 0.5 and 3.8, whereas it varies between 4.7 and 14.4 in marine fish (GULER *et al*., 2008).
The PUFA/SFA is used for the assessment of lipids on the basis of the proportions of the different fatty acid groups. In our study, the PUFA/SFA ratio was affected only by species, being higher in *C. carassius* than in *C. auratus* fish. The PUFA/SFA ratios obtained in the present study are similar to those reported in fillets of Nile tilapia (0.83–1.32; HERATH *et al*., 2016). However, the PUFA/SFA ratio value of the fillets of the present study, ranged from 0.75 to 1.00, is well above the minimum value of 0.45 recommended by the Department of Health of the UK (HMSO, 1994) and confirms that these freshwater fish species are suitable for human consumption.
The AI and TI represent the criteria for evaluating the level and interrelation through which some fatty acids may have atherogenic or thrombogenic properties, respectively. In particular, these indexes take into account the different effects that single fatty acid might have on human health, and, in particular, on the probability of increasing the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation (GARAFFO *et al*., 2011). In the present study, AI and TI were lower in *C. carassius* than in *C. auratus* fish, and AI was lower in 4-year-old fish than in younger. The AI (ranging from 0.46 to 0.56) and TI (ranging from 0.44 to 0.54) values found in the current study can be considered low according with literature reports (ULBRICHT and SOUTHGATE, 1991; JANKOWSKA *et al*., 2010; STANEK *et al.*, 2012), and not comparable with the values reported in the meat of ruminants (AI: 1.29±0.26, TI: 1.54±0.179, D'ALESSANDRO *et al.,* 2012) but is similar to poultry meat (AI: 0.56±0.13, TI: 0.55±0.14, LAUDADIO and TUFARELLI, 2010; AI: 0.43±0.02, HE *et al*., 2015; AI: 0.42±0.01, TAVANIELLO *et al.,* 2018).

5. CONCLUSIONS

In conclusion, the results of this study indicate that the meat from *C. auratus* and *C. carassius* is characterized by a favourable fatty acids profile from the human health point of view. Considering that fish were caught in the same season, sex had a negligible effect on fatty acids composition, while species and age had a marked effect on it. Furthermore, *C. auratus* fillets displayed significantly higher contents of SFA and MUFA, and a lower content of PUFA compared to those of *C. carassius*. Fillets of 4-year-old fish had a lower content of ALA, higher MUFA content and better nutritional indexes (n-3/n-6 and AI) compared to those of 3-year-old fish. Both species of *Carassius*s lightly differ in their feeding trait and trophic level. This may cause differences in the fatty acid composition. In light of the obtained results, further experimental investigations are needed to deepen knowledge on these freshwater fish species, considering the different place and different season in which they were caught, in view of their potential exploitation for human diet or aquaculture.

All procedures performed in studies were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The authors declare that they have no conflict of interest.

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PAPER

EFFECT OF THE PRODUCTION PROCESS ON THE CONTENT OF ANTHOCYANINS IN DRIED RED-FLESHED POTATO CUBES

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ABSTRACT

This study aimed at determining the effect of particular stages in the laboratory manufacture of dehydrated potato cubes on the stability of anthocyanin content in red-fleshed potato varieties. The raw material used in the study was potatoes of the following three red-fleshed varieties: Rosemarie, Herbie 26, and Rote Emma.

The analysed potato varieties differed in their respective content of anthocyanins and polyphenols. A higher content of these compounds was found in potatoes of Rote Emma cv. (216 mg/100 dm polyphenols and 37.3 mg/100 g anthocyanins).

The greatest losses of anthocyanins were noted after peeling and pre-drying and those of total polyphenols were noticed after blanching, pre-drying and drying. In comparison to the raw material, only ca. 25% of anthocyanins and ca. 31% of total polyphenols remained in the finished product. Among the analysed varieties, Rote Emma might be recommended for the production of dried potato cubes. This is because the highest content of biologically active compounds was present in potatoes of this variety after the production process.

Keywords: colour-fleshed potatoes, anthocyanins, dehydrated potato cubes

1. INTRODUCTION

In comparison to other plant materials (fruits or vegetables), potatoes are convenient study materials, owing to their wide applicability, high availability, high consumption across the world and very good adaptation capabilities. They are one of the few plant materials that produce high crop yields in different climatic zones and under various soil conditions. Although potatoes are materials that are well known to consumers, many scientists worldwide are still undertaking analyses of their chemical composition. Such a high interest in this raw material results from its varietal diversity and, therefore, from its rich chemical composition (BROWN *et al.*, 2008; LISIŃSKA *et al.*, 2009; RYTEL *et al.*, 2014). Recent studies have addressed biologically active compounds of the potatoes varieties that have intensively coloured flesh, containing anthocyanins, which are known for their antioxidative properties (LACHMAN and HAMOUZ, 2005; FURRER *et al.*, 2017; VALIÑAS *et al.*, 2017).

Anthocyanins constitute a large group of plant pigments included in the natural phytonutrients that are soluble in water and occur in almost all parts of a plant (BRIDLE and TIMBERLAKE, 1997; RODRIGUEZ-SAONA *et al.*, 1998; CASTAÑEDA-OVANDO *et al.*, 2009, PIĄTKOWSKA *et al.*, 2011). In cells, they occur in vacuoles in the form of granules of various sizes. A few hundred natural pigments and over 100 chemically synthesised ones are known today. Anthocyanins are widely applied in the food industry as colourants due to their intensive and attractive colour. In addition, their therapeutic properties have been used in folk medicine for years. Today, they are being increasingly used in the cosmetic and pharmaceutical industries (WROLSTAD, 2000; EICHORN and WINTERHALTER, 2005).

Anthocyanins are unstable compounds. They undergo various transformations in the water environment depending on pH value, which, in turn, might contribute to a change in the colour of the products that contain them. Few scientific reports are available on the effect of processing conditions on anthocyanins in red- or purple-fleshed potato varieties (PERLA *et al.*, 2012; LACHMAN *et al.*, 2013; KITA *et al.*, 2015). Anthocyanins of potatoes are acylated derivatives of cyanidin, and their colour differs depending on the medium pH. Potatoes contain anthocyanins, which are stable not only in an acidic medium, such as in the form of pigments isolated from fruits, but also in neutral and slightly basic media (EICHORN and WINTERHALTER, 2005; FRIEDMAN and LEVIN, 2009; CHUNG *et al.*, 2017).

A dynamic increase has recently been observed in the manufacture of potato products, the main ones including French fries, chips and dehydrated potato products. Production of the latter is successively increasing in response to the needs of the market. Today, consumers look for 'convenient' foods that not only enable the fast preparation of meals in households or catering facilities but which are also characterised by high organoleptic and nutritional values. The drying process facilitates the possibility of manufacturing a wide array of preserved semi-products or potato products that meet these criteria. A new and interesting solution is the use of red-fleshed and purple-fleshed potatoes to manufacture such products.

This study aimed at determining the effect of particular stages in the laboratory manufacture of dehydrated potato cubes on the stability of anthocyanin content in red-fleshed potato varieties.

2. MATERIAL AND METHODS

2.1. Material

The raw material used in the study was potatoes of the following three red-fleshed varieties: Rosemarie, Herbie 26 and Rote Emma, all of which were sourced from the plantations of the Czech University. The study was conducted in the growing season from 2015 to 2016 in three technological replications. The effect of particular stages in the laboratory manufacture of dehydrated potato products on changes in the content of anthocyanins in the raw material, semi-products and finished products that were made from colour-fleshed potatoes was investigated.
The method of dehydrated dice production in laboratory conditions was as follows:
The potatoes were washed, peeled (1.5 mm) using a laboratory carborundum peeler, diced into 10×10×10 mm cubes by a manual cutting device in the laboratory and rinsed with distilled water at a temperature of 20°C. Subsequently, the potato cubes were blanched in water at 75°C for 5 min and pre-dried in a laboratory oven at 120°C for 1 hour. Afterward, the temperature of drying was decreased between 55 and 60°C to obtain a final moisture content of about 12% (about 8 hours). A total of 1 kg samples of potato were taken during each stage of laboratory processing (LISIŃSKA and LESZCZYŃSKI, 1989; RYTEL, 2012; RYTEL *et al*., 2014; RYTEL *et al*., 2017).
The raw material (unpeeled potatoes) was determined for the following proximate chemical composition: dry matter, starch, total and reducing sugars. Wet samples, which included unpeeled potatoes, peeled potatoes, skins, potato after blanching and pre-drying, were frozen and lyophilised by using a freeze dryer (temperature -35°C, pressure 5 Pa, time 12 h) (Edwards, England). All raw materials, semi-products and finished products obtained during laboratory production were ground in a laboratory mill. The prepared samples were examined for determining the content of anthocyanins.

2.2. Extraction of anthocyanins

The samples were prepared according to the method described by NEMŚ *et al*. (2015). The freeze-dried raw materials, semi-products and finished products were extracted with 70% aqueous acetone (0.1% acetic acid) in a graduated tube. The mixture was homogenised using a vortex and allowed to stand for 2 h at room temperature. The acetone-water solution was partitioned with chloroform to remove lipophilic compounds. Next, the acetone-water fraction was collected and put into a Büchi rotary evaporator (Merck, Darmstadt, Germany) until all residual acetone evaporated. The remaining extract was brought to a known volume with 50% methanol and stored at 20°C until it was analysed. The samples were filtered with 0.45 μm and 0.22 μm filters before HPLC-PDA and UPLC-MS/MS analyses.

2.3. Quantification of anthocyanins by HPLC-PDA

The content of anthocyanins was determined according to KUCHARSKA *et al*. (2017) by using a Dionex (USA) HPLC system equipped with an Ultimate 3000 model of a diode array detector, an LPG-3400A quaternary pump, an EWPS-3000SI autosampler and a TCC-3000SD thermostated column compartment, all of which were controlled by the Chromeleon v. 6.8 software. The Cadenza Imtakt column C5-C18 (75 × 4.6 mm, 5 μm) (Portland, USA) was used. The following solvents constituted the mobile phase: 4.5% formic acid (Solvent A) and 100% acetonitrile (Solvent B). The following elution conditions were applied: 0-1 min 5% B in A; 1-20 min 25% B in A; 20-27 min 100% B in A; and 27-30

min 5% B in A. The flow rate was 1 mL/min, and the injection volume was 40 μL. The column was operated at 30°C. Anthocyanins were monitored at 520 nm, and their content was expressed in cyanidin 3-O-glucoside equivalents (CygE)/100 g dm.

2.4. Identification of anthocyanins by UPLC-qTOF-MS/MS

The method for anthocyanin identification was previously described by MIZGIER *et al*. (2016). Anthocyanins were identified on Acquity ultra-performance liquid chromatography (UPLC) system coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UPLC/Synapt Q-TOF MS, Waters Corp., Milford, MA, USA) with an electrospray ionisation (ESI) source. They were separated on an Acquity TM BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters) (Merck, Darmstadt, Germany). The detection wavelength was set at 520 nm. The mobile phase was a mixture of 4.5% formic acid (Solvent A) and 100% acetonitrile (Solvent B). The gradient program was as follows: initial conditions - 99% (A), 12 min - 75% (A), 12.5 min - 100% (B) and 13.5 min - 99% (A). The flow rate was 0.45 mL/min, and the injection volume was 5 μL. The column was operated at 30°C.
The major operating parameters for the Q-TOF MS were set as follows: capillary voltage, 2.0 kV; cone voltage, 40 V; cone gas flow, 11 L/h; collision energy, 28-30 eV; source temperature, 100°C; dissolution temperature, 250°C; collision gas, argon; dissolution gas, nitrogen; flow rate, 600 L/h; data acquisition range, m/z 100-2000 Da; and ionisation mode, positive. The data were collected by the Mass-Lynx TM V 4.1. software.

2.5. Analytical methods

The dry matter content of fresh potato samples and freeze-dried materials was determined by the reduced weight after drying at 105°C until a constant weight was achieved (HORWITZ and LATIMER, 2005). The contents of total and reducing sugars were determined by the colorimetric method with DNS (HORWITZ and LATIMER, 2005). The starch content was determined in raw potato tubers by measuring their specific gravity while the quantity of anthocyanins was analysed by HPLC-PDA (KUCHARSKA *et al*., 2017), and their profile by UPLC-qTOF-MS/MS (MIZGIER *et al*., 2016) of samples that were prepared as described in the work of NEMŚ *et al*. (2015). The polyphenol content was determined using the Folin-Ciocalteu colorimetric method, as described by SINGLETON *et al*. (1999) and KITA *et al*. (2015). All analyses were carried out in triplicate.

2.6. Statistical analysis

The study's results were subjected to statistical calculations by using the Statistica 13.1 software (StatSoft Polska Sp. Z o.o., Kraków, Poland). The significance of the differences between mean values was determined by conducting a multi-way analysis of variance and Duncan's test ($P \leq 0.05$). All experiments were performed in three technological replications within two years of investigation, and the present results show the mean values of all data in a combined way.

3. RESULTS AND DISCUSSION

Potatoes of red-fleshed and purple-fleshed varieties are rarely used to manufacture fried or dried food products. This is because of their lesser popularity among producers and consumers and, consequently, their lower availability in the market. In addition, potatoes

of colour-fleshed varieties usually have a higher content of total sugars and reducing sugars in comparison to those of the common yellow-fleshed or white-fleshed varieties. These compounds determine the colour of the finished product (KITA *et al.,* 2015). Dehydrated potato products should be manufactured from potatoes that have a high content of dry matter (from 21 to 25%) and starch (from 15 to 19%) and those in which the content of reducing sugars is below 0.5% (LISIŃSKA *et al*., 2009). In the potatoes of red-fleshed varieties that were analysed in our study, contents of dry matter, starch and reducing sugars met the above requirements in tubers of Herbie 26 and Rote Emma var. Potatoes of Rosemarie var. had a lower content of dry matter and starch and over 0.6% of reducing sugars (Table 1).

Table 1. Chemical composition of raw potatoes.

Chemical compounds (g/100 g fresh matter, fm)	Potato variety		
	Herbie 26	Rosemarie	Rote Emma
Dry matter	22.8±0.12^{c}	17.9±0.09^{a}	20.9±0.10^{b}
Starch	15.4±0.11^{b}	14.0±0.11^{a}	15.4±0.12^{b}
Total sugar	0.71±0.09^{a}	0.86±0.07^{b}	0.69±0.10^{a}
Reducing sugar	0.51±0.08^{a}	0.60±0.09^{b}	0.69±0.10^{a}

a, b, c - different letters indicate significant differences among the varieties following the LSD test ($p>0.05$), ± SD (standard deviation); n = 6.

Taking into consideration the attractive colour of the flesh and the higher content of biologically active compounds, potatoes of red- and purple-fleshed varieties might be an interesting alternative to traditional light-fleshed potato varieties and might be recommended for the manufacture of potato products. Tables 2 and 3 present the contents of anthocyanins and total polyphenols determined at particular stages in the laboratory manufacture of dehydrated potato cubes.

Table 2. Contents of pigments (mg/100 g dry matter, dm) and total polyphenols (mg/100 g dm) in red-fleshed potatoes and skins.

Pigments	Potato variety					
	Herbie 26		Rosemarie		Rote Emma	
	Unpeeled potatoes	Skins	Unpeeled potatoes	Skins	Unpeeled potatoes	Skins
Pelargonidin-3-rutinoside-5-glucoside	3.20±0.12^{b}	6.81±0.11^{a}	-	-	9.46±0.13^{b}	10.5±0.12^{a}
Pelargonidin-3-rutoside	2.04±0.11^{b}	4.35±0.10^{a}	2.90±0.12^{a}	1.36±0.07^{c}	2.33±0.10^{a}	6.32±0.10^{b}
Pelargonidin-3-caffeoylrutinoside-5-glucoside	4.85±0.20^{a}	0.74±0.08^{a}	4.94±0.09^{a}	2.98±0.08^{b}	8.48±0.11^{b}	15.5±0.14^{c}
Pelargonidin-3-pcoumaorylrutinoside-5-glucoside	2.95±0.10^{b}	0.40±0.06^{a}	1.81±0.11^{a}	2.20±0.10^{b}	10.5±0.12^{c}	-
Pelargonidin-3-feruloylrutinoside-5-glucoside	13.1±0.11^{c}	6.38±0.11^{b}	5.10±0.13^{a}	6.31±0.12^{b}	6.52±0.11^{b}	0.43±0.03^{a}
Sum of analysed anthocyanins	**26.1**±0.13^{b}	**18.7**±0.10^{b}	**14.7**±0.12^{a}	**12.8**±0.11^{a}	**37.3**±0.22^{c}	**32.8**±0.17^{c}
Total polyphenols	188±4.53^{a}	196±5.13^{b}	186±3.99^{a}	94±4.01^{a}	216±5.32^{b}	204±4.89^{c}

a, b, c - different letters indicate significant differences among varieties following the LSD test ($p>0.05$), ± SD; n = 6.

Table 3. Content of pigments (mg/100 g dm) and total polyphenols (mg/100 g dm) in potatoes after particular technological stages.

Potato variety	Technological stage	Pigments						
		Pelargonidin-3-rutinoside-5-glucoside	Pelargonidin-3-rutoside	Pelargonidin-3-caffeoyl-rutinoside-5-glucoside	Pelargonidin-3-p-coumarylrutinoside-5-glucoside	Pelargonidin-3-feruloylrutinoside-5-glucoside	Sum of analysed anthocyanins	Total polyphenols
Herbie 26	Potato after peeling	2.54±0.10^{c}	1.62±0.09^{b}	0.52±0.07^{b}	0.07±0.06^{c}	9.39±0.12^{d}	**14.1**±0.12^{d}	**185**±3.71^{c}
	Potato after blanching	2.22±0.11^{b}	0.15±0.04^{a}	0.54±0.05^{b}	0.04±0.008^{b}	5.51±0.10^{c}	**8.46**±0.11^{c}	**179**±2.55^{b}
	Potato after pre-drying	0.60±0.04^{a}	0.10±0.01^{a}	0.50±0.06^{b}	0.01±0.007^{a}	4.30±0.09^{b}	**5.51**±0.10^{b}	**172**±4.01^{b}
	Potato after drying	-	-	0.18±0.08^{a}	-	2.10±0.08^{a}	**2.28**±0.09^{a}	**141**±1.98^{a}
Rosemarie	Potato after peeling	-	1.03±0.09^{c}	3.51±0.10^{d}	1.22±0.09^{c}	2.49±0.11^{c}	**8.25**±0.12^{d}	**181**±4.02^{d}
	Potato after blanching	-	0.39±0.01^{b}	3.45±0.11^{c}	0.96±0.08^{b}	2.09±0.08^{b}	**6.89**±0.11^{c}	**168**±3.25^{c}
	Potato after pre-drying	-	0.29±0.03^{b}	2.79±0.12^{b}	0.41±0.06^{a}	1.81±0.10^{a}	**5.30**±0.12^{b}	**151**±3.44^{b}
	Potato after drying	-	0.20±0.02^{a}	2.47±0.10^{a}	-	1.80±0.11^{a}	**4.47**±0.09^{a}	**146**±2.56^{a}
Rote Emma	Potato after peeling	1.32±0.09^{c}	0.94±0.07^{b}	3.32±0.14^{c}	10.7±0.14^{d}	5.90±0.12^{b}	**22.2**±0.17^{c}	**194**±2.07^{d}
	Potato after blanching	1.02±0.08^{b}	0.72±0.05^{a}	2.41±0.10^{b}	7.18±0.12^{c}	6.02±0.12^{b}	**17.3**±0.18^{b}	**152**±2.67^{c}
	Potato after pre-drying	0.81±0.03^{a}	-	1.10±0.09^{a}	4.18±0.10^{a}	4.99±0.11^{a}	**11.1**±0.10^{a}	**128**±1.98^{b}
	Potato after drying	-	-	1.09±0.08^{a}	5.42±0.11^{cb}	4.88±0.10^{a}	**11.4**±0.11^{a}	**112**±1.91^{a}

a, b, c, d - different letters indicate significant differences among varieties following the LSD test ($p>0.05$), ± SD; n = 6.

The mean content of total polyphenols in potatoes of the analysed varieties was 197 mg/100 g dm. The highest content was found in both skins and whole tubers of Rote Emma potatoes, whereas the lowest was found in Rosemarie potatoes (Table 2). The content of anthocyanins in the analysed potatoes ranged from 14.7 mg/100 g dm (Rosemarie var.) to 37.3 mg/100 g dm (Rote Emma var.). Their contents were lower in skins and were on average 21.4 mg/100 g dm (Table 2). However, the lowest anthocyanin content was determined in skins of Rosemarie var. According to other authors (FOSSEN *et al*., 2003; FRIEDMAN and LEVIN, 2009; FURRER *et al*., 2017), the content of anthocyanins in potatoes might vary greatly from a few to a few dozen mg per 100 g dm. As reported by HAMOUZ *et al.* (2011), the anthocyanin content in potatoes of purple-fleshed varieties ranged from 6.88 mg/100 g^{-1} dm (Valfi var.) to 57.3 mg/100 g dm (Violette var.) and in potatoes of red-fleshed varieties from 13.5 mg/100 g dm (Rosalinde var.) to 21.2 mg/100 g dm (Highland Burgundy Red var.). In turn, according to KITA *et al.* (2013), purple-fleshed potatoes contain these compounds in a range from 40.2 to 184.7 mg/100 g dm. The quantitative and qualitative composition of anthocyanins in potatoes is highly diverse and depends, primarily, on the potatoes' variety, cultivation site and weather and climatic conditions (LACHMAN *et al*., 2009; HAMOUZ *et al*., 2011). According to SULC *et al*. (2017), the red-fleshed potato varieties might also contain other anthocyanin glucosides, e.g. peonidin, apart from pelargonidin.

In contrast, as claimed by other authors (LACHMAN *et al*., 2009; NEMŚ *et al*., 2015), both the flesh and skins of potatoes contain the same anthocyanins. However, potatoes of the red-fleshed varieties that were analysed in the present study differed in both the composition and content of acylated compounds (Table 2). In Rote Emma var. potatoes, the majorly identified anthocyanin glucoside was pelargonidin-3-*p*-cumaorylrutinoside-5-glucoside, whereas, in potatoes of Herbie 26 and Rosemarie varieties, it was pelargonidin-3 feruloylrutinos-5-glucoside (Table 2). Pelargonidin 3-rutinoside-5-glucoside was also found to be a predominating glucoside in skins of Herbie 26 and Rote Emma var. potatoes. In contrast, the skins of potatoes of Rote Emma var. differed in the composition of glucosides. They did not contain pelargonidin-3-*p*-cumaorylrutinoside-5-glucoside, and the majorly identified compound in it was pelargonidin-3-feruloylrutinoside-5-glucoside (Table 2). According to VALIÑAS *et al*. (2017), the flesh of potatoes differs in the composition and contents of individual anthocyanins. This is probably due to the migration and transport of metabolites between flesh and skin or vice versa. As of now, however, no research works have addressed this issue.

The first technological stage of processing potatoes into most dried products is peeling. In this study, the potatoes were peeled manually, so the depth of peeling might be greater and exceed 1.5 mm. There were no differences in the composition of anthocyanin glucosides in the peeled potatoes, but their losses were observed. After peeling, the total content of anthocyanins decreased by 43% on average (Fig. 1).

According to FURRER *et al.* (2017), peeled potatoes contain ca. 7% fewer anthocyanins in comparison to the non-peeled ones. The great differences in the loss of anthocyanins, which was observed after potato peeling, might have resulted from the manner and depth of skin removal. In the study conducted by FURRER *et al*. (2017), the potatoes were industrially peeled; therefore, their peeling depth could be significantly less than after manual peeling. During deeper manual peeling, the skin was removed along with the layer of flesh underneath. According to LACHMAN *et al*. (2013), manual peeling of potatoes up to a depth of ca. 1-2 mm does not affect anthocyanin loss; however, the lack of such an effect depends on the variety. In potatoes of the purple-fleshed variety, namely, Violette, the content of anthocyanins decreased by 41% after peeling, whereas it increased by 127 to 286% in potatoes of other colour-fleshed varieties (LACHMAN *et al.,* 2013). Anthocyanins occur in higher amounts in the flesh of potatoes rather than in the skin.

Therefore, during the shallower peeling of tubers, the percentage content of these compounds in dry matter increases.

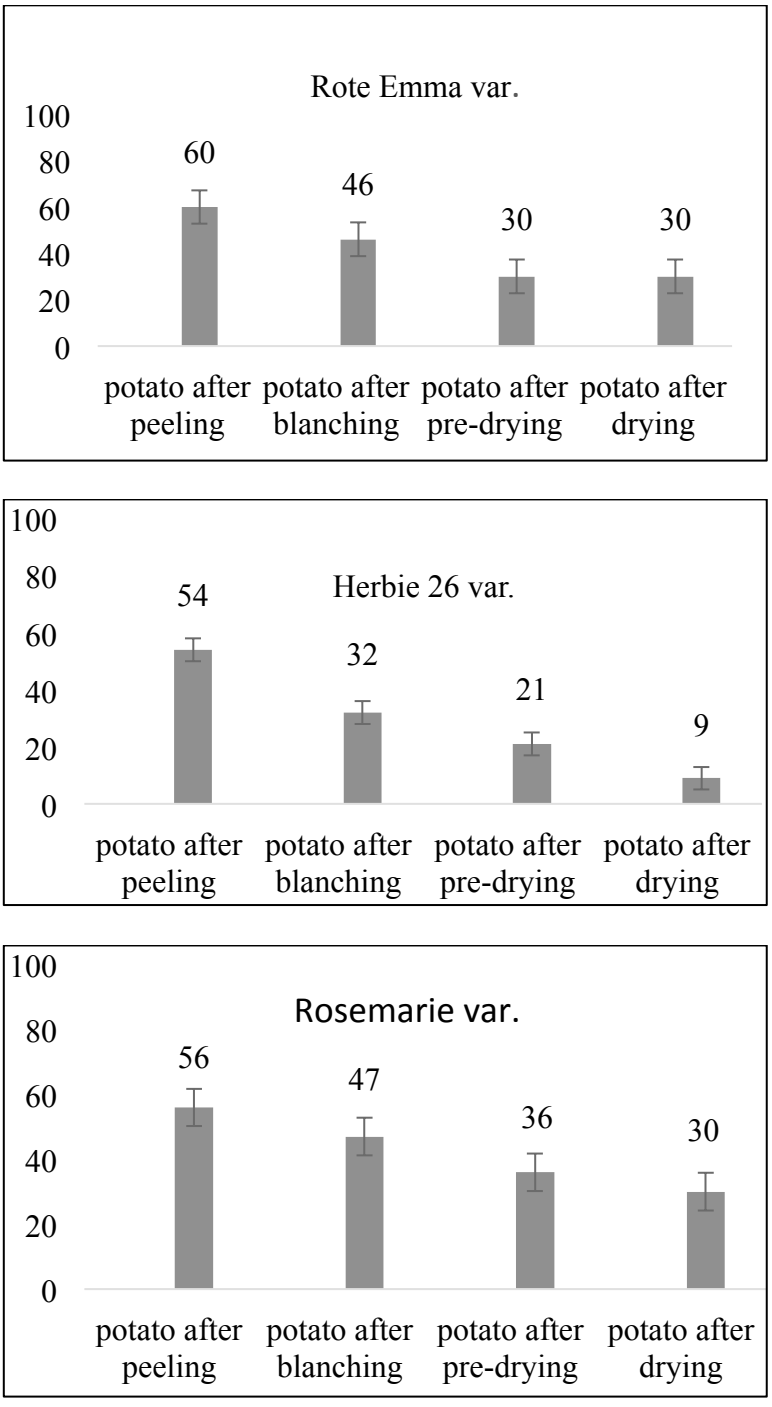

Figure 1. Anthocyanin residue (%) in potatoes after technological stages of dehydrated dice processing (mean of years).

Another stage of processing involves blanching potato cubes. The extensive disintegration of the raw material (cubes) and its exposure to a temperature of 75°C for 15 min caused successive loss of anthocyanins (Table 3, Fig. 1). After blanching, their total content of

anthocyanins decreased by 19% on average in potatoes of the Rote Emma and Rosemarie varieties, and by 40% in those of Herbie 26 var. compared to the peeled potatoes (Table 3). As reported by other authors (MULINNACI *et al.*, 2008; LACHMAN *et al.*, 2013), losses of anthocyanins after blanching might range from 16 to 29%. The extent of these losses can be determined by the pH value of the blanching bath, the degree of raw material disintegration, temperature and the time during which the product is exposed to it (MULINNACI *et al.*, 2008). According to FURRER *et al*. (2017), the stability of anthocyanins in potatoes depends on their variety and the type of heat treatment, specifically, after thermal processing (i.e. blanching, freezing, roasting and frying), the content of anthocyanins decreased by 3 to 29% on average in purple-fleshed potatoes. However, it increased by a few percentage points in red-fleshed potatoes. As demonstrated by LACHMAN *et al.* (2013), processes, such as roasting, microwaving and steaming, prevent anthocyanin losses. After such their processes were conducted, these authors reported a significant increase in total anthocyanins in purple-fleshed and red-fleshed potatoes, even though the above processes were applied to whole tubers with skins (non-peeled and non-disintegrated). Probably, this method of material preparation has a protective effect on the anthocyanin content in potato tubers. As reported by BROWN *et al*. (2008), microwaving and cooking cause smaller changes in anthocyanin content than frying or roasting. In our study, blanched potato cubes were pre-dried at 120°C for 1 hour. The impact of high temperature on the material contributed to the successive loss of anthocyanins. After this stage, their content decreased by 23% (Rosemarie var.) to 35% (Herbie 26 var.) compared to the blanched potatoes; however, no changes were found in the composition of the analysed anthocyanin glycosides (Table 3). Further drying of potato cubes at 50°C for 8 hours caused changes in the composition of the studied compounds. In the case of Herbie 26 var., the potato cubes that were dried to a moisture content of ca. 12% (finished product) did not contain pelargonidin-3-rutinoside-5-glucoside, pelargonidin-rutinoside-5-glucoside or pelargonidin-3-*p*-cumaorylrutinoside-5-glucoside and were characterised by the lowest total content of anthocyanins (2.28 mg/100 g) in comparison to the potatoes of the other varieties (Table 3). According to other authors (KITA *et al*., 2013; NEMŚ *et al*., 2015), high-temperature processes (over 100°C), such as frying or extrusion, not only cause greater losses of anthocyanins that range from 50 to 80% but also changes in the composition of anthocyanin glycosides. According to CASTANEDA-OVANDO *et al.* (2009), anthocyanins are highly unstable and susceptible to degradation. Their stability depends on multiple factors, e.g., pH, storage temperature, chemical structure, their content, exposure to light and oxygen, solvent and presence of enzymes, flavonoids, proteins or metal ions. In our study, the greatest losses of anthocyanins in the production process of dehydrated potato cubes were attributed to the processes of peeling (43% on average) and pre-drying (31% on average), whereas blanching caused their content to decrease by only 26% on average (Fig.1). This was despite the considerable disintegration of the material. Changes in the content of anthocyanins varied depending on the variety, and the greatest losses were found upon processing potatoes of Herbie 26 var.

The production process of dry potato cubes also caused losses in total polyphenols (Table 3). The greatest loss of these compounds was noted after the following thermal processes: blanching, pre-drying and drying. According to KITA *et al.* (2015), the heat processes used in potato production not only cause the degradation of phenolic compounds but also the transformation of different groups of polyphenols.

The content of anthocyanins in the finished product was ca. 23% on average (Fig. 1) and that of polyphenols was ca. 31% of their initial content in the raw material (Tables 2 and 3). Despite high losses of total polyphenols and anthocyanins during the manufacture of dehydrated products, potatoes of red-fleshed and purple-fleshed varieties might be an alternative to the potatoes with yellow or white flesh. Literature data support the

conclusion that smaller losses of anthocyanin compounds occur upon processing potatoes with skin, and, perhaps, the use of this type of material should be recommended.

4. CONCLUSIONS

The analysed potato varieties met the established requirements for tubers intended for the manufacture of dehydrated potato products. However, only potatoes of Rosemarie var. had lower than recommended content of dry matter and starch and over 0.6% of reducing sugars. Potatoes of Rote Emma variety might be recommended for the production of dried potato cubes, as they m*et al*l the requirements and had the highest content of total polyphenols and anthocyanins. In addition, dried potato cubes made of this variety preserved the highest content of biologically active compounds.
The process of laboratory production of dehydrated potato cubes caused losses of anthocyanins and total polyphenols in semi-products and finished products. The greatest losses of anthocyanins were noted after peeling, and pre-drying and those of total polyphenols were noticed after blanching, pre-drying and drying. In comparison to the raw material, only ca. 23% of anthocyanins and ca. 31% of total polyphenols remained in the finished product.

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PAPER

CORN GRAIN BRUSHING FOR DEOXYNIVALENOL REDUCTION

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ABSTRACT

For the purpose of deoxynivalenol (DON) reduction in corn samples, a laboratory brusher was developed. The brusher had two main parts, motionless screen and fast rotating brush. Corn kernels were placed on the motionless screen of the brusher, and fast rotating round shaped polypropylene bristle brush was put into the motionless screen in order to clean the surface of the kernels. The experiment consisted of 27 brushing trials with variations in process parameters (DON concentration, brushing time, and speed of rotating brush). Brushing of corn that is DON infected resulted in the reduction of DON concentration in all samples. The maximal limit of DON reduction at optimal conditions was 83.6%. Thus, the presented process can be considered as highly efficient. The proposed technology did not cause any changes in the physical appearance of kernel, nor were damages observed on kernel surface during the brushing process. Also, there were no whole grain losses detected in any of the process parameter combination.

Keywords: brushing, corn, decontamination, deoxynivalenol

1. INTRODUCTION

Well-known and frequently mentioned secondary metabolites of fungi, mycotoxins, commonly occurred in cereal grains and other food and feedstuffs. They belong to the most toxic contaminants among a wide range of food commodities (COUNCIL FOR AGRICULTURAL SCIENCE AND TECHNOLOGY, 2003; KOUROUSEKOS and THEODOSIADOU, 2018). These highly harmful compounds are extremely thermostabile; for instance, pure aflatoxin B1 can be destroyed only at temperatures above 160°C (KARLOVSKY *et al.*, 2016). During "field to fork" chain, contaminated food materials are not exposed to temperature, which can lead to mycotoxin decontamination, or if it is exposed, the process does not last long enough to be decontaminated. Moreover, conditions on the field as well as conditions during further manipulation with cereal products oftentimes promote fungal growth and mycotoxin production.

Exposure to the mycotoxins is somehow unavoidable (MAGAN and OLSEN, 2006). Chronic consumption of contaminated goods can adversely affect human and animal health and can be lethal to some animal species. Main toxic effects of mycotoxins are teratotoxicity, carcinogenicity, hepatotoxicity, nephrotoxicity, embryotoxicity and immunosuppression (PESTKA, 2010; ANFOSSI *et al.*, 2010).

One of the best ways to manage contamination is to apply pre-harvest and post-harvest preventive strategies, that is by avoiding the emergence of mycotoxins. However, when the material is already contaminated, mycotoxins should be inactivated, destroyed, or removed from the commodity. Besides, the nutritive value and acceptability of the products should be preserved, and technological properties of the product should be retained (AVANTAGGIATO, 2012). Nowadays, various procedures are applied for mycotoxin decontamination, which can be divided into three main groups: chemical, biological, and physical.

Chemicals, such as oxidizing agents, chlorinating agents, acids, and alkali, have been used for the deactivation of mycotoxins. Despite the fact that some of these agents were found to be effective, the chemical procedures are not widely used due to their negative effect on product palatability and nutritive value, potential toxicity, high operational costs, and long operational time (AMÉZQUETA *et al.* 2009; ZAKI *et al.*, 2012).

Biological detoxification, which is based on biotransformation and/or biodegradation principles, is widely used in the deactivation of mycotoxins contaminated feedstuffs, as well as for food and beverages. Enzymes, microorganisms or a specific organic compound derived from microorganisms interact with mycotoxins in the gastrointestinal tract of animals to form a non-toxic stable complex. To enable sufficient contact surface between contaminated substrate and biological additive, grain materials are usually milled before the addition of bio-binder. Therefore, when biological detoxification is performed, the grains could not be preserved in a whole kernel form, nor the effects of the binders observed before grains are consumed by animals (KOLOSOVA and STROKA, 2011; KARLOVSKY *et al.*, 2011).

Physical treatment of contaminated materials includes, sorting, screening, milling, washing, irradiation, thermal treatments, adsorbing, etc. Some of these procedures can be found in conventional grain storage and/or processing technologies, with the main purpose of improving the nutritional quality of grains, and not to remove mycotoxins (TRENHOLM *et al.*, 1991). Taking into account that most of these operations do not destroy mycotoxins, the effect of physical procedures is often weak to moderate (KABAK, 2009; MANAFI and KHOSRAVINIA, 2012; MARIN *et al.*, 2013). Generally, the selected method for mycotoxin decontamination should be efficient, relatively simple, inexpensive, and not time-consuming.

For the purpose of mycotoxin decontamination in grain, a team of scientists at the Institute of Food Technology, University of Novi Sad, Serbia, developed an intensive laboratory grain brusher. Preliminary trials have shown a significant effect of the brushing process on the aflatoxin (AF) removal (ČOLOVIĆ *et al.*, 2013). Although there is a considerable number of papers referring to the effects of the scouring process on mycotoxin reduction, the literature on the effects of cleaning grain surface without damage of pericarp is limited (SCHAARSCHMIDT and FAUHL-HASSEK, 2018).

Considering all the aforementioned facts, the main objective of this study was to present a new method of physical detoxification, primarily. Furthermore, we wanted to investigate the influence of processing parameters (processing time (t), and rotational speed (v)) on reduction rate of DON in maize samples naturally contaminated with this mycotoxin in three different concentrations and to determine the optimal conditions of brush procedure applied. Response Surface Methodology (RSM) was used since it was proven to be an effective tool for optimizing a wide variety of processes (LIAUDANSKAS *et al.*, 2018). Experimental results were subjected to analysis of variance (*ANOVA*) to show the relationship between applied assays.

2. MATERIALS AND METHODS

2.1. Material

Samples of mycotoxin contaminated corn were collected from commercial warehouses within the Serbian Northern Province of Vojvodina. Sampling of three corn samples was performed in accordance with Commission Regulation 401/2006 (European Commission, 2006). The total amount of aggregate sample (10 kg) was homogenized using Nauta mixer, model 19387 (Nauta patenten, Netherlands). After homogenization, the aggregate sample was quartered to get 3000 g of the representative sample. Obtained representative corn samples were again homogenized using rotation drum mixer (model SYTH0,05, Muyang Group, China) and quartered to get sub-samples of 100 g per contaminated sample (27 sub samples of each contaminated sample). Initial DON concentrations in naturally contaminated samples were 7.5 mg/kg (DON1), 10.6 mg/kg (DON2), and 14.8 mg/kg (DON3).

2.2. Chemicals and reagents

Acetonitrile used for HPLC analysis (all HPLC grade, purity ≥ 99.9%) was purchased from Merck (Darmstadt, Germany). Ultra-pure water was produced by Milli-Q purification system (Milli-Q from Millipore, USA). DON (concentration of 100 μg/mL) standard was purchased from Sigma-Aldrich (Steinhem, Germany). Standard solutions were prepared in acetonitrile and stored at –10 °C. Those solutions were used for solvent based calibration and for fortification of blank corn samples.

2.3. Sample preparation

Sub-samples of 100 g were ground to a 1 mm particle size using laboratory mill (KnifetecTM 1095 mill, Foss, Hoganas, Sweden), and additionally quartered to obtain sub-samples of 25 g. The sub-samples were further extracted with 100 ml of acetonitrile:water (84:16, v/v) and shaken vigorously for 30 minutes on a laboratory Griffin flask shaker (Griffin and George, Wembley, England). The extract was filtered through no. 4 filter paper (Whatman, Maidstone, UK). Three ml of extract was cleaned up on MycoSep® 225

Trich for DON determination (Romer Labs, Inc., Union, MO). The cleaned-up extract was evaporated to dryness (Reacti-Therm ™ manifold, Thermo Fisher Scientific, Inc., USA), and reconstituted in 300 ml of mobile phase.

2.4. HPLC analysis of DON and validation procedure

Agilent 1200 (Agilent Technologies Inc., USA) HPLC instrument system equipped with diode array detector (DAD) was used for determination of DON. The detection was performed at 220 nm. Agilent column Eclipse XDB-C18, 1.8 μm, 4.6 x 50 mm was used. The mobile phase consisted of an isocratic mixture of water-acetonitrile (80:20, v/v), with a flow rate of 0.25 ml/min. Each sample was analysed in duplicate.
Before application in the experiment shown, HPLC/DAD method was developed and validated for DON determination. The validation parameters were determined and calculated according to EU Commission Decision procedure (2002/657/EC) by analysing certified reference material as well as spiked corn samples at three different levels. CRM with certified DON content of 900±100 μg/kg (D-W-164) was supplied by Trilogy Analytical Laboratory (Trilogy® Reference Material, Washington, USA). The results of methodology validation are shown in Table 1. As can be seen, the obtained validation parameters were in compliance with the recommendations given in Regulation 2006/401/EC (EC, 2006).

Table 1. Validation parameters for DON determination.

Validation parameters	CRM	Spiked concentration (μg/kg)		
		1000	5000	10000
RSD_r	6.56	7.12	8.15	5.94
RSD_R	8.81	9.22	10.1	6.56
Recovery	96.7	94.5	92.5	95.1

CRM - certified reference material (naturally contaminated corn, D-W-164, Trilogy® Reference Material, Washington, USA).
RSDr- relative standard deviation calculated under repeatability conditions (%).
RSD_R- relative standard deviation calculated under reproducibility conditions (%).

2.5. Processing

Contaminated corn, in a kernel form, was subjected to the brushing process. For this purpose, laboratory brusher developed at the Institute of Food Technology, University of Novi Sad, Serbia, was used (Fig. 1). The brusher had two main parts, fast rotating brush (A) and motionless screen (B). Corn kernels were placed onto the motionless screen to cover the surface (approximately 100 g) of the brusher (laboratory test sieve was used for this purpose), and fast rotating round shaped polypropylene bristle brush was put down into the motionless screen. The purpose of the corn kernel brushing was to remove dust from the surface of whole kernels, and to brush it out together with broken kernels through the openings in the motionless screen. Aspiration of the dust is provided from the bottom side of the screen by connecting it to the central aspiration system, in order to facilitate separation, and to prevent excessive dusting, as well as inhalation of toxic substances. Brushing time was set at 30, 60, and 90 s, respectively, while speed of rotating brush was set at 400, 800, and 1200 rpm, respectively.

Figure 1. Laboratory grain brusher. A - fast rotating brush, B - motionless screen.

Extent of DON reduction (E_{DON} (%)) was calculated using the following equation:

$$E_{DON} = \left(1 - \frac{C_{DON\ \text{Red.1–3}}}{C_{DON\ 1–3}}\right) \cdot 100\% \qquad \text{(Equation 1)}$$

2.6. Experimental design and statistical analysis

The experimental data used for the study of experimental results were obtained using a 3^2 full factorial experimental design; each of the 3 specific DON contaminated samples were processed at 2 parameters and at 3 levels. Independent experimental factors for each of the samples are shown in Table 2.

Table 2. Independent experimental factors and their levels.

Experimental factor	Symbol	Coded factor's level		
		-1 (low)	0 (centre)	+1 (high)
t – Brushing time (s)	X_1	30	60	90
v – Speed of rotating brush (rpm)	X_2	400	800	1200

Descriptive statistical analyses of all the obtained results were expressed by means, for each treatment. Collected data were subjected to ANOVA to explore the effects of process variables. The evaluation of RSM and ANOVA of the obtained results was performed using Statistica software version 12 (StatSoft Inc. 2013, USA)®.

The experimental data used for the analysis were derived according to RSM. The main advantage of RSM is a reduced number of experimental runs that provide sufficient information for statistically valid results. The RSM equations describe the effects of the test variables on the observed responses, determine test variable interrelationships and represent the combined effect of all test variables in the observed responses, enabling the experimenter to make efficient exploration of the process (ČOLOVIĆ *et al.*, 2016, BRLEK *et al.*, 2013).
The following second order polynomial (SOP) model was fitted to the experimental data. Six models of the following form were developed to relate six responses (Y) and three process variables (X), for each of the different mixtures.

$$Y_k = \beta_{k0} + \sum_{i=1}^{2} \beta_{ki} \cdot X_i + \sum_{i=1}^{2} \beta_{kii} \cdot X_i^2 + \beta_{k12} \cdot X_1 \cdot X_2, \quad \text{(Equation 2)}$$

where: β_{k0}, β_{ki}, β_{kii}, β_{k12} are constant regression coefficients; Y_k, deoxynivalenol concentration after reduction (C_{DONRed}), for three initial concentrations of deoxynivalenol (DON_{1-3}), while $X1$ - brushing time (t); $X2$-speed of rotating brush (v).

3. RESULTS AND DISCUSSION

The results for the effects of brushing process on DON reduction have been shown in Fig. 2 from the least to the most contaminated samples, respectively. The brushing of infected corn resulted in the reduction of concentration of DON in all samples, matter on DON concentration, brushing time or speed of rotating brush.
The best results of brushing for DON concentration of 7.5 mg/kg (DON_1) and 14.8 mg/kg (DON_3) were recorded at the highest levels of rotating speed and the longest time of brushing, respectively. The samples before and after brushing for rotating speed of 1200 rpm and brushing time of 90 s, as well as tailings collected from the aspiration system has been shown in Fig. 3. However, samples contaminated with concentration of 10.6 mg/kg (DON_2) have shown the highest level of decontamination at rotating speed of 1200 rpm and a duration of 60 s. Looking at Fig. 2, it is clear that the results of decontamination level were unpredictable for rotation speed of 400 and 800 rpm. The reason for that is probably due to the insufficient rotating speed of brush, so decontamination level is rather accidental and not dependent on brushing time. At rotation speed of 1200 rpm, decontamination level increased with the increase in processing time. A similar conclusion can be made for DON_2 concentration. Yet, decontamination level for DON_2 samples differ insignificantly for rotation speed of 1200 rpm and processing time of 60 and 90 s (approx. 81% and 78% respectively). It seems that for this DON concentration, sufficient time of brushing for maximum decontamination is 60s at a brushing speed of 1200 rpm.
An average mycotoxin reduction of 27 samples was 50.9%, showing that the grain brushing process was very effective in the removal of DON. Also, only five of 27 samples had DON reduction less than 40%, while only two of those had DON reduction less than 20%. Generally, for a short brushing time and low speed of rotating brush, DON reduction was lower. VISCONTI *et al.* (2004) showed that concentration of DON is the highest in the outer layers of grains such as bran. This also explains why intensive cleaning of the outer layers of corn applied in our study reduced DON concentration.

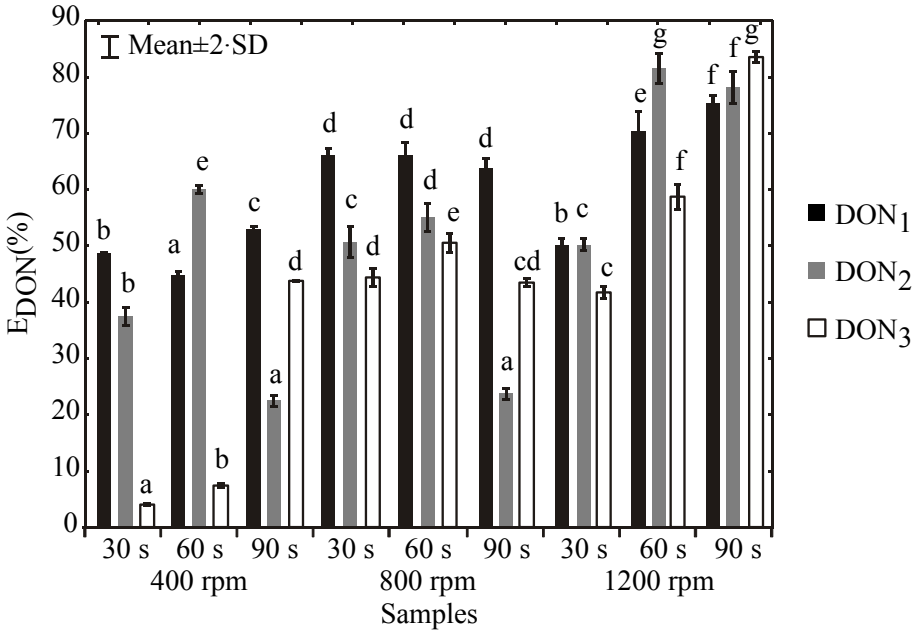

Figure 2. Extent of DON reduction (%) by application of grain brushing process.
a-g - Different letters within the same set of samples show significantly different means of observed data at $p<0.05$ level.

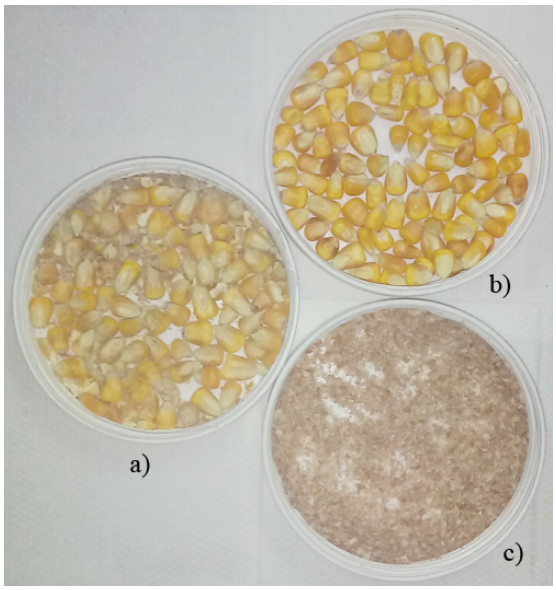

Figure 3. The infected sample, contaminated with heavy DON concentration (a), the sample after brushing treatment (for rotating speed of 1200 rpm and brushing time of 90 s) (b), and the brushed tailings collected from the aspiration system (c).

Physical dehulling of the outer layer of maize can reduce AF decontamination by 92% (SIWELA *et al*., 2005). However, this method removes natural protection of kernel and it does not preserve its integrity. For any combination of process parameters, changes in physical integrity of kernel or damages to the kernel surface were not observed during the brushing process. Since there is no damage of the grain, kernels could preserve its biological function and its natural protection from microbial contamination. Therefore, it is

reasonable to expect that kernels decontaminated by brushing have a longer shelf life than kernels decontaminated with similar mechanical treatments, which include surface breakage of kernels.

It cannot be neglected that, by this method, none of the total mass of treated kernels was removed and as a result, in that way, there were no material losses. As WU and MUNKVOLD (2008) stated in their paper that deals with the economic costs of mycotoxin's presence in feed, removing of screenings and broken kernels from maize after sieving reduced DON contamination by 73%, however, mass loses were extensively high and accounted for approx. 69%. Removal of contaminated maize can also be performed by flotation and density segregation (GRENIER *et al.*, 2014). Fungal damaged kernels are mostly, also, mycotoxin-contaminated. Since they have different physical properties than non-infected kernels, they can be separated by density segregation or by fractionation on so-called gravity tables. However, these methods are not mycotoxin specific, so kernels contaminated with fungi, but without presence of mycotoxins are also removed.

A group of Italian authors showed in their paper an electronic optical technique of sorting infected and healthy apricot kernels based on the discoloration of contaminated kernels (ZIVOLI *et al.*, 2016). Although, they succeeded in removing up to 59% of aflatoxin accumulated in naturally-contaminated samples, the obtained results were highly variated, such that the proposed method cannot be considered as reliable and need to be improved. It may be less effective in comparison with the manual method of sorting, such as removal of contaminated grains, which is, on the other hand, highly time demanding.KUSHIRO (2008) in his review paper showed that DON concentration could be decreased by 86% when infested wheat kernels are removed. On the other hand, PARK (2002) combined removal of extensive mould growth kernels with the cleaning of maize kernels for reduction of AF content, and obtained a reduction of 40% to 80%. That is similar to the maximal mycotoxin reduction in our study, where extensive mould growth kernels were not removed. However, the maize was not infected with the same mycotoxin. Same author also used dry milling process for fractionation of AF B1 content. Highest levels of mycotoxin were found in the germ and hull fractions. Grits, low-fat meal and low fat flour contained only 6 to 10% of AF. Since a prerequisite for fractionation is to comminute the kernel, authors of present study changed the physical structure of corn kernels. Also, fractionation resulted in the concentration of toxin in separate fractions, without removal of mycotoxins from the material.

Washing grains with tap water significantly reduced the mycotoxin level and this can be applied to food and feeds (FANDOHAN *et al.*, 2005). Yet, costs of drying grains are too high, so it is reasonable to use this method only prior to wet milling or ethanol fermentation. The same goes for rinsing and flotation techniques.

Most of the physical methods of decontamination can be generally considered as efficient, but the major problems occur with implementation of these processes on a commercial scale. As earlier mentioned, milling, combined with sieving causes significant mass losses, and most of other established methods are time consuming, which cannot be said for the presented method of kernel brushing. Laboratory grain brusher presented in this study has been shown to be generally efficient on a small scale level. Meanwhile, development of a continuous semi-industrial scale brusher is in progress with the accent on maintaining same efficacy as it was on a lab scale device.

ANOVA shows the significant effects of independent variables to the responses (Table 3). The SOP models for all variables were found to be statistically significant and the response surfaces were fitted to these models. The linear term of v was the most influential in the DON_1 reduction calculation (statistically significant, at $p < 0.10$ level). The prediction of DON reduction was influenced by a linear term of v and the quadratic term of t (both

statistically significant at $p < 0.10$ level). The linear term of v was very influential for DON reduction calculation ($p < 0.05$), for DON_3.
All SOP models had an insignificant lack of fit tests, which means that all the models represented the data satisfactorily (Table 3).
A high r^2 is indicative that the variation was accounted and that the data fitted satisfactorily to the proposed model (SOP in this case). The coefficients of determination for DON reduction prediction were very good and showed the good fit of the model to experimental results.

Table 3. ANOVA calculation for DON reduction during grain brushing process.

	dF	$C_{DONRed\ 1}$	$C_{DONRed\ 2}$	$C_{DONRed\ 3}$
t	1	124.519	32.491	1082.945
t^2	1	1.660	947.083**	42.844
v	1	407.825**	1350.000**	2764.210*
v^2	1	135.942	279.609	78.972
t × v	1	108.160	462.656	1.097
Error	3	161.312	442.659	757.681
r^2		0.828	0.874	0.840

Abbreviations: t - brushing time, v - speed of rotating brush, dF - degrees of freedom. + significant at $p<0.01$ level, *significant at $p<0.05$ level, **significant at $p<0.10$ level, error terms have been found to be statistically insignificant.

Using these models, the contour plots of the extent of DON reduction (E_{DON}) were plotted and superimposed to ascertain the optimum processing conditions (Fig. 4).

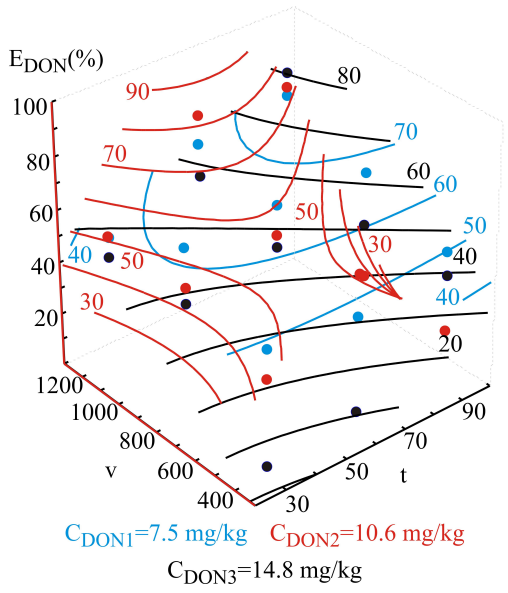

Figure 4. Optimum region obtained after superimposing the contour plots of the system response.

Optimization of the process is performed to ensure rapid processing conditions with high DON reduction. Optimum operating conditions were derived with a few iterative steps in finding processing parameters that gave the highest reduction of DON. Contour plots of the extent of DON reduction showed that maximum reduction was obtained at higher initial concentration and higher speed of rotating brush, as was expected. The optimal conditions for DON reduction were t = 90 s, v = 1200 rpm, which consistent with experimental results. The maximal extents of DON reduction at optimal conditions were: 75.3%, 78.2% and 83.6%, for the initial DON concentration of 7.5 mg/kg, 10.6 mg/kg and 14.8 mg/kg, respectively.

4. CONCLUSIONS

This study presented fast technological process which successfully detoxified corn without causing any changes in the physical integrity of the kernel, nor damages of kernel surface. Therefore, the presented laboratory brusher could easily grow into its industrial version, especially due to its simple construction. The higher reduction of DON was obtained for the higher initial concentration, longer polishing time, and higher speed of rotating brush. Further, brushing of corn infected with DON resulted in reduction of concentration in all processed samples. It is important to note that the proposed process did not lead to whole grain loss, but only fine particles, unlike other effective physical decontamination methods, such as sieving or fractionation.

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PAPER

GASTROINTESTINAL STABILITY OF CAROTENOIDS FROM RAW AND FREEZE-DRIED VEGETABLES

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ABSTRACT

In this study, the gastrointestinal stability of carotenoids (lycopene and *β*-carotene) extracted from raw and freeze-dried samples of tomato, carrot and red pepper was investigated. Extracted carotenoids fractions (lycopene and *β*-carotene) were submitted to a two-phase *in vitro* digestion process using human gastrointestinal enzymes. The use of freeze-drying has a strong effect on the enhancement of the gastrointestinal stability of carotenoid, especially after simulated intestinal phase. In addition, the effect of pH on carotenoid stability is much lower in freeze-dried plant material than in raw samples of tomato, carrot and red pepper. The food matrix also plays important role in carotenoids gastrointestinal stability rate, which was found to be the most stable in red pepper.

Keywords: carotenoids, *in vitro* digestion, lycopene, *β*-carotene

1. INTRODUCTION

Environmental influences such as contamination, ultraviolet (UV) radiation, smoking, stress and improper diet may result in cell damage caused by free radicals, believed to be the cause of many degenerative diseases, such as certain types of cancer, cardiovascular disease, type 2 diabetes, age-related macular degeneration (AMD) and cataracts, among others, and even mortality caused by some of these serious diseases (MAIANI *et al*., 2009; FIEDOR and BURDA, 2014; MeyErs *et al*., 2015; NOLAN *et al*., 2015; SLUIJS *et al*., 2015). In order to protect itself, the body uses antioxidants and neutralizers of the free radicals, which are commonly provided by the diet. Carotenoids, which are considered as the most widely distributed pigments in nature (SCHWARTZ *et al*., 2008), are known to be very efficient scavengers of singlet oxygen ($^1O_2$), as well as other reactive oxygen species (ROS). Carotenoids are responsible for the attractive, yellow to red colour of fruit and vegetables, which is the first attribute that consumers evaluate. Although more than 700 different carotenoids have been identified so far, just six of them are commonly analysed in foods and blood: three hydrocarbon compounds - carotenes (*β*-carotene, *α*-carotene, lycopene) and three oxygenated forms - xanthophylls (*β*-cryptoxanthin, lutein, zeaxanthin) (OLMEDILLA-ALONSO, 2017).

The composition and the content of carotenoids in foods are dependent on different factors e.g. variety and maturity of species, cultivation practices and methods of food processing. Several reviews and databases on food sources of carotenoids, intake, stability and bioavailability have been published (HEINONEN *et al*., 1989; HART and SCOTT, 1995; LETH *et al*., 2000; MURKOVIC *et al*., 2000; O'NEILL *et al*., 2001; KIM *et al*., 2007; FERNÁNDEZ-GARCÍA *et al*., 2012; NAGAO, 2014; MEYERS *et al*., 2015; OLMEDILLAALONSO, 2017). Therefore, in order to understand the relationship between nutrition and health in humans, it is important to know not only the amounts of consumed carotenoids but to what extent they are absorbed from the different dietary sources, their bioavailability, respectively (OLMEDILLA-ALONSO 2017). In general, stability under environmental conditions, gastrointestinal stability, the bioaccessibility as well as the bioavailability of functional food ingredients represent main factors affecting usefulness of the intake of certain foodstuffs (NAGAO, 2014). Carotenoids have very low bioavailability because they are quite susceptible to conditions found in the digestive tract (temperature, pH). Likewise, they are less bioavailable due to extreme hydrophobicity, and it also depends on other factors: release from the food matrix, solubilization in the digestive tract, absorption in intestinal epithelia, and metabolism (Nagao 2014). Also, it may be due to the fact that they can be bound in carotenoproteins; in green leafy vegetables carotenoids are found bound in chloroplasts and in carrot root, *α*- and *β*-carotene are largely in crystal forms. Therefore, the carotenoids are not easily solubilized out of these tissues by the digestive process (Institute of Medicine 2000), mostly due to rigid cell walls. They are more readily released in ripe fruit as well as processed vegetables then in fresh one, which substantially improves their bioavailability (Nagao 2014). It is known that the application of a thermal treatment and/or mechanical homogenization, as well as addition of fats and oils in diet, are all techniques that enhanced the bioavailability of dietary carotenoids (FERNÁNDEZ-GARCÍA *et al*., 2012), just because of increased bioaccessibility by dispersing them in digestive tract. On the other hand, dietary fibres have been thought to decrease bioaccessibility by binding bile acids (NAGAO, 2014). Following digestive release in stomach and upper intestine, the hydrophobic components aggregate in lipid emulsion droplets, which partitioned into mixed micelles in the small intestine. The formation of micelles allows carotenoids to be soluble in the hydrophobic interior and carotenoids, which are not in that form are not typically bioaccessible and remain unabsorbed (FERNÁNDEZ-GARCÍA *et al*., 2012; NEILSON *et al*., 2017).

In order to study the rate of gastrointestinal stability of biologically active components, the use of *in vitro* digestion models procedures represents effective tool due to its simplicity, low cost and putative production of many digesta fractions. A large number of *in vitro* studies have been carried out on the bioavailability of carotenoids and their assimilation during the digestive process and a number of models are suggested to mimic *in vivo* digestion over the years (FERRUZZI *et al*., 2006; GRANADO-LORENCIO *et al*., 2007; FAILLA *et al*., 2008; COURRAUD *et al*., 2013; KOPEC *et al*., 2017), but there is a lack of studies on the stability of carotenoids under gastrointestinal conditions using human gastrointestinal juices which comprise a complex mixture of enzymes present in multiple isoforms, enzyme inhibitors and bile salts that are important for the digestion process. The aim of this study was to determine carotenoid stability after simulated gastric and duodenal phases of simulated digestion process. Raw and freeze-dried samples of tomato, carrot and red pepper, vegetables rich in carotenoids, were used for their extraction. The stability rate of carotenoids (*β*-carotene and lycopene) in relation to digestion enzymes and to the effect of pH after each simulated digestion phase was determined spectrophotometrically.

2. MATERIAL AND METHODS

2.1. Chemicals

Lycopene, *β*-carotene and BHT (butylhydroxy toluene) were purchased from Sigma – Aldrich (Basel, Switzerland). All solvents (hexane, acetone, ethanol) were of pro analysis purity and were purchased from Kemika (Zagreb, Croatia).

2.2. Samples

Raw samples of carrot, tomato and red pepper were purchased from green local market. Raw samples were processed immediately after the purchase. Samples were firstly cut in small pieces and immediately homogenized in the blender to obtain a pulp. Then, the pulp was treated with argon in order to avoid rapid oxidation process. The pulp was not stored, because for repetition of experiments only fresh samples were used. Immediately after purchasing, one part of samples was freeze-dried using Freeze-dry system (Labconco, USA) at the temperature of –50°C and the pressure of 0.2 mb, during the period of 3 days. After freeze-drying, samples were grinding into a spice grinder and immediately used for further analyses. Second part of samples were fresh (raw) and were immediately prepared for extraction in the manner that samples were cut into approximately 2 to 4 cm cubes, and then homogenized using a hand blender (Bosch Maxomixx, Germany).

2.3. Extraction of carotenoids

The extraction of carotenoids from freeze-dried and raw samples of carrot, tomato and red pepper was done using two procedures described by ALDA *et al*. (2009) and FISH *et al*. (2002). For extraction procedure, the following solvents were used: acetone, hexane and ethanol (2:1:1). Sample from homogenized raw plant material or freeze-dried plant material (1 g) was mixed with 25 mL of solvent mixture, under subdued lighting at room temperature and the bottle was treated with argon in order to protect lycopene from degradation. After solvent addition, the samples were shacked during the period of 30 min (180 rpm, room temperature). After that, 1 mL of deionized water was added and the samples were left for approximately 5 min in order to obtain two separate layers. Upper

layer (the fraction with lycopene) was separate and stored in glass, dark flasks, treated with inert gas (argon) and stored at – 20°C until analysis.

2.4. Extraction of β-carotene

Extraction of β-carotene was done according to procedure described by DAVIS *et al*. (2008). For extraction procedure, the following solvents were used: BHT in acetone (0.05%), ethanol and hexane (1:1:2). Homogenized raw plant material or freeze-dried plant material (0.6 g) was mixed with 15 mL of solvent mixture under subdued lighting at room temperature and the bottle was treated with argon to protect β-carotene from degradation. After solvent addition, the samples were shacked during the period of 10 min (180 rpm, room temperature). After that, 3 mL of deionized water was added and the samples were left for approximately 5 min in order to obtain two separate layers. Upper layer (the fraction with β-carotene) was separate and stored in glass, dark flasks, treated with inert gas (argon) and stored at –20°C until analysis.

2.5. Spectrophotometric measurement of lycopene and β-carotene

Spectrophotometric measurements were performed on UV/Vis spectrophotometer Specord 200 spectrometer (Analytik Jena GmbH, Germany) and IRAffinity-1 Fourier Transform infrared (FTIR) spectrometer (Shimadzu, Japan). IR spectra were recorded by using KBr transmision cell, in the spectral area 4000-400 cm^{-1} and with resolution 4 cm-1. Abbreviations used are for streching (*n*), deformation (*d*).
For measurement of lycopene and β-carotene, the calibration curve was done using different concentrations of lycopene and β-carotene. A 0.01 g of β-carotene and lycopene was dissolved in hexane (100 mL) to obtain the concentration of 100 μg/mL (stock solution). After that, the working solution was done (20 μg/mL) and the following concentrations were prepared: 7, 6, 5, 4, 3, 2, 1 μg/mL.
The concentration of lycopene in prepared samples was determined by UV/Vis spectrophotometer at 503 nm. Hexane was used for detection of zero. Concentration of lycopene in raw and freeze-dried samples of carrot, tomato and red pepper was determined according to following formula (FISH *et al*., 2002):

$$(A_{503} \times 31.2) / \text{g of sample}$$

Absorbance of β-carotene was determined using UV/Vis spectrophotometer at 450 nm.

2.6. Isolation of human juices

Human gastric and duodenal juices were collected from four donors (two males and two female) without known gastrointestinal pathology, and who were not taking acid secretion inhibitors or antibiotics. Gastric and duodenal juices were aspirated through the endoscope. Eight hours before the procedure, all liquid or food intake was ceased. For each patient, 3 mL of initially aspired juice were discarded and the remaining amount was collected in a sterile tube, which was centrifuged to remove mucus and cell debris. In order to reduce inter-individual variations, batches of pooled gastric and intestinal juices were prepared and then stored at –20°C until use. The approval for the collection of digestive juices was obtained from the Ethics Committee of the University Hospital Centre Split, Croatia.

2.7. Enzymatic activity of juices

The procedure described by ALMAAS *et al*. (2006) was used to determine enzymatic activity of the prepared pooled human gastric juice samples. Pepsin activity was measured using 2.5% solution of bovine haemoglobin. The solution was prepared in 0.2 mol/L phosphate buffer (pH=7.6) and then acidified (to pH=3) using H_2SO_4. In order to determine the human duodenal juice activity, casein solution (1%) dissolved in 0.2 mol/L phosphate buffer (pH=7.6) was used. A volume of 500 μL of prepared protein solutions was incubated with 5, 20 or 50 μL of gastrointestinal juice. The digestion reactions were stopped with the addition of 1 mL of 10% trichloroacetic acid (TCA). Samples were measured spectrophotometrically at 280 nm. One unit of enzyme activity (U) is defined as the amount of enzyme that causes the absorbance change of 1 between the blank and the sample, after 20 min at 37°C.

2.8. *In vitro* digestion

A two-phase digestion procedure was performed according to the method described by FURLUND *et al*. (2013). Gastric and intestinal digestion phases were performed at 37°C, in horizontal shaking bath (180 rpm). The volume of digestive juice corresponding to 1 U of enzymatic activity was 20 μL of human gastric juice and 25 μL of human duodenal juice. The pH of the samples was adjusted to pH=2.5 using 1 mol/L HCl for gastric phase, and to pH=7.5 using 2 mol/L NaOH for intestinal phase. The concentration of human juices used for this assay was 20 U per g of plant material for gastric and 62.4 U per g of plant material for intestinal phase. A 0.6 g of both fresh and freeze-dried samples were used for digestion process. The incubation period of gastric phase was 30 min, while digested intestinal samples were collected after 120 min of intestinal phase. Before spectrophotometric analyses, the digested samples were centrifuged before spectrophtometrical analyses by microcentrifuges mySpin 12 (Thermo Scientific, USA) at room temperature, during 10 min at 9000 rpm. Enzymatic reactions were stopped on ice and the samples were stored at –20°C until analyses. All digestion processes were run in duplicate. Stability rate of lycopene and β-carotene represents the ratio of their concentrations before *in vitro* digestion and after gastric or intestinal digestion phases. Samples were dissolved in *n*-hexane and according to UDDIN *et al*. (2009) and POOJARI *et al*. (2009) digestive enzymes retained their stability in non-polar solvents such as *n*-hexane. The gastrointestinal stability rate (%) of lycopene and β-carotene was calculated according to the following formula:

(Sample concentration after digestion/sample concentration before digestion) x 100

2.9. Statistics

Statistical analysis was performed using GraphPad InStat3 software (GraphPad Software Inc., San Diego, CA, USA). The relationship between the obtained parameters was described using Pearson's correlation coefficient *r*. Differences at $p<0.05$ were considered to be statistically significant

3. RESULTS AND DISCUSSION

The influence of digestion process on the carotenoids stability is not completely explored. In this study we explored the stability rate of carotenoids (lycopene and *β*-carotene) from raw and freeze-dried red carrot (*Daucus carota*), tomato (*Solanum lycopersicum*) and red pepper (*Capsicum annuum*) after gastric and duodenal simulated digestive phase using human digestive juices.

The analysis of the UV/Vis spectrum of the obtained extracts showed strong peaks at 444, 471 and 502 nm. FTIR spectral peaks, in the range of 3082 - 2835 cm-1 indicate the presence only of C-H bonds, *ie.* 3082 - 3011 cm-1 which correspond to C(sp2)-H bonds and 2965 - 2835 cm-1 to C(sp3)-H bonds stretching. Peaks are observed at 1643 *n* (C=C, alkene), 1435 and 1375 *d* (CH2, CH3, bend). The quantities of carotenoids in the extracted fractions of raw freeze-dried samples were determined spectrophotometrically by previously described methods. Although not the absorbance peak of greatest magnitude in hexane, the absorbance peak at 503 nm was used for lycopene determination in order to minimize interference from other carotenoids. If generally accepted, nominal carotenoid contents of red-fleshed watermelon, fresh red tomato, and pink grapefruit are utilized (HOLDEN *et al*., 1999) together with molar extinction coefficients at 503 nm in hexane for those carotenoids (ZECHMEISTER *et al*., 1943; ZECHMEISTER *et al*., 1943a), the potential error can be estimated if absorbance contributions by other carotenoids are ignored. Such a calculation suggests that constituent carotenoids other than lycopene will contribute to the absorbance at 503 nm 0.2% for red-fleshed watermelon, 0.4% for fresh red tomatoes, and 0.6% for pink grapefruit (FISH *et al*., 2002). Previous reports of the major carotenoids detected in the investigated material showed that the carrot is a significant source of *β*-carotene (BYSTRICKA *et al*., 2015), *S. lycopersicum* of lycopene (BARANSKA *et al*., 2006), while the unique keto carotenoids of red pepper capsanthin, capsorubin and cryptocapsin impart brilliant red colour to ripen chilly pods, while the yellow orange colour is from *β*-carotene, zeaxanthin, violaxanthin and *β*-cryptoxanthin (ARIMBOOR *et al*. 2015). Results showed that the concentration of carotenoids (lycopene) in raw and freeze-dried samples ranged from 37.73 to 53.93 mg/kg and 61.09 to 61.92 mg/kg, respectively, with the highest one in red pepper extracts. On the other hand, the absorbance peak at 450 nm was used for *β*-carotene estimation in the investigated samples. The results showed that the carotenoid (*β*-carotene) concentration in raw and freeze-dried samples ranged from 15.14 to 27.92 mg/L, and 21.43 to 56.17 mg/L, respectively.

The difference in the stability of carotenoids (lycopene and β-carotene) between raw and freeze-dried plant material as well as the difference in their gastrointestinal stability in relation to different plant matrix were detected. The high stability of carotenoids from freeze dried food rich with carotenoids is already reported by several authors (CINAR, 2005; CHEN *et al*., 2007; VASQUE-CAICEDO *et al*., 2007). However, this is the first report on the gastrointestinal stability of carotenoids from fresh and dried samples. Gastrointestinal stability of β-carotene and lycopene was evaluated using gastric and duodenal human juices. Some authors reported that colonic microbiota can maximize the bio-accessibility of carotenoids by digestion of plant cell walls (DJURIC *et al*., 2017).

Results presented in Table 1 show that the stability of carotenoids (lycopene) were significantly higher in red pepper than in carrot and tomato after simulated gastric phase. Generally, lycopene stability after simulated gastric digestion was much higher in freezedried plant material than in raw plant material. The difference in lycopene gastric stability was lower in freeze-dried plant matrix (carrot, tomato and red pepper). Interestingly, the stability rate of lycopene after simulated gastric digestion was extremely high in freezedried red pepper and tomato (96.04 and 92.09%, respectively). Lycopene was not stable after simulated duodenal digestive phase in carrot and in tomato, or its stability

was very low (27.98% in raw red pepper). The use of freeze-drying significantly improved its duodenal stability. Generally, lycopene stability in raw and in freeze-dried plant material was much lower after simulated duodenal phase in comparison with its stability after simulated gastric phase as shown in Table 2. COURRAUD *et al*. (2013) also reported high stability of carotenoids after simulated gastric incubation (in their study they used commercial digestive enzymes).

Table 1. Stability rate of carotenoids (lycopene) from a) raw, and b) freeze-dried samples of carrot, tomato and red pepper after simulated gastric phase.

		Sample		
		Carrot *(Daucus carota* L.)	**Tomato *(Solanum lycopersicum* L.)**	**red pepper *(Capsicum annum* L.)**
a)	Concentration in raw sample [mg/kg]	37.73±0.12	45.48±0.20	53.93±0.77
	Concentration after gastric phase [mg/kg]	16.35±0.84	20.12±0.94	32.80±0.19
	Stability [%]	43.00±0.23	44.14±0.47	60.81±0.45
b)	Concentration in freeze-dried sample [mg/kg]	61.09±0.24	60.45±0.55	61.92±0.41
	Concentration after gastric phase [mg/kg]	52.26±0.12	52.54±0.90	59.47±0.34
	Stability [%]	86.91±0.87	92.09±0.55	96.04±0.63

Table 2. Stability rate of carotenoids (lycopene) from a) raw, and b) freeze-dried samples of carrot, tomato and red pepper after simulated duodenal phase.

		Sample		
		Carrot *(Daucus carota* L.)	**Tomato *(Solanum lycopersicum* L.)**	**Red pepper *(Capsicu annum* L.)**
a)	Concentration in raw sample [mg/kg]	37.73±0.12	45.48±0.20	53.93±0.77
	Concentration after duodenal phase [mg/kg]	/	/	15.09±0.56
	Stability [%]	/	/	27.98±0.36
b)	Concentration in freeze-dried sample [mg/kg]	61.09±0.24	60.45±0.55	61.92±0.41
	Concentration after duodenal phase [mg/kg]	38.08±0.78	36.12±0.24	42.16±0.20
	Stability [%]	62.33±0.11	59.75±0.22	68.08±0.14

According to results presented in Tables 3 and 4 the gastrointestinal stability of carotenoids (β-carotene) differs from that of lycopene. After duodenal digestive phase β-carotene was not the most stable in red pepper, as it was the case for lycopene. In comparison with lycopene, β-carotene showed moderate stability rate after duodenal phase in both, raw and freeze-dried plant material, while lycopene was not stable in raw carrot and tomato after duodenal phase. Interestingly, the stability rate of β-carotene significantly decreased after duodenal digestive phase in freeze-dried red pepper (Table 4).

Table 3. Stability rate of carotenoids (β-carotene) from a) raw, and b) freeze-dried samples of carrot, tomato and red pepper after simulated gastric phase.

		Sample		
		Carrot *(Daucus carota* L.)	**Tomato *(Solanum lycopersicum* L.)**	**Red pepper *(Capsicum annum* L.)**
a)	Concentration in raw sample [mg/kg]	15.14±0.35	21.54±0.40	27.92±0.24
	Concentration after gastric phase [mg/kg]	3.57±0.26	10.47±0.34	20.41±0.25
	Stability [%]	23.59±0.17	48.64±0.32	73.13±0.30
b)	Concentration in freeze-dried sample [mg/kg]	21.43±0.29	33.28±0.35	56.17±0.27
	Concentration after gastric phase [mg/kg]	6.42±0.45	18.89±0.29	51.84±0.18
	Stability [%]	30.00±0.28	56.77±0.38	92.30±0.45

Table 4. Stability rate of carotenoids (β-carotene) from a) raw, and b) freeze-dried samples of carrot, tomato and red pepper after simulated duodenal phase.

		Sample		
		Carrot *(Daucus carota* L.)	**Tomato *(Solanum lycopersicum* L.)**	**Red pepper *(Capsicum annum* L.)**
a)	Concentration in raw sample [mg/kg]	15.14±0.35	21.54±0.40	27.92±0.24
	Concentration after duodenal phase [mg/kg]	5.38±0.36	12.80±0.29	13.54±0.19
	Stability [%]	35.59±0.22	59.45±0.26	48.50±0.34
b)	Concentration in freeze-dried sample [mg/kg]	21.43±0.29	33.28±0.35	56.17±0.27
	Concentration after duodenal phase [mg/kg]	11.42±0.26	22.93±0.40	11.38±0.24
	Stability [%]	53.33±0.20	68.93±0.24	20.26±0.36

In this study the influence of pH on carotenoids (lycopene and β-carotene) stability was explored (Tables 5 and 6). As it is shown in Table 5 the influence of pH on lycopene stability was stronger in raw than in freeze-dried plant material. Generally, it can be seen that carotenoids (lycopene and β-carotene) were more stable at pH 2.5 than at pH 8.0. Correlations between the stability of carotenoids in acidic and alkaline conditions were found to be significant (r=0.6926, p=0.0125) and extremely significant (r=0.9170, p<0.0001) for Tables 5 and 6, respectively. Freeze-drying technique significantly improve lycopene stability at low pH. Interestingly, concerning the stability of β-carotene there is no significant difference between raw and freeze-dried plant material.

Table 5. The influence of pH (gastric and duodenal) on the stability rate of carotenoids (lycopene) from a) raw, and b) freeze-dried samples of carrot, tomato and red pepper.

		Sample		
		Carrot *(Daucus carota* L.)	**Tomato *(Solanum lycopersicum* L.)**	**Red pepper *(Capsicum annum* L.)**
a)	Concentration in raw sample [mg/kg]	37.73±0.12	45.48±0.20	53.93±0.77
	Concentration at pH 2.5 [mg/kg]	16.06±0.89	23.76±0.22	37.61±0.87
	Stability [%]	42.56±0.78	52.12±0.47	69.73±0.87
	Concentration at pH 8 [mg/kg]	10.70±0.78	8.51±0.32	31.32±0.56
	Stability [%]	27.21±0.51	18.67±0.23	58.07±0.45
b)	Concentration in freeze-dried sample [mg/kg]	61.09±0.24	60.45±0.55	61.92±0.41
	Concentration at pH 2.5 [mg/kg]	55.03±0.44	52.47±0.70	57.30±0.66
	Stability [%]	90.65±0.54	86.79±0.10	92.53±0.53
	Concentration at pH 8 [mg/kg]	8.44±0.20	39.30±0.24	38.52±0.30
	Stability [%]	13.81±0.12	65.01±0.17	62.20±0.78

Table 6. The influence of pH (gastric and duodenal) on the stability rate of carotenoids (β-carotene) from a) raw, and b) freeze-dried samples of carrot, tomato and red pepper.

		Sample		
		Carrot *(Daucus carota* L.)	**Tomato *(Solanum lycopersicum* L.)**	**Red pepper *(Capsicum annum* L.)**
a)	Concentration in raw sample [mg/kg]	15.14±0.35	21.54±0.40	27.92±0.24
	Concentration at pH 2.5 [mg/kg]	6.80±0.12	12.06±0.26	17.58±0.35
	Stability [%]	44.97±0.54	56.02±0.22	62.98±0.26
	Concentration at pH 8 [mg/kg]	4.41±0.29	4.54±0.30	16.75±0.28
	Stability [%]	29.13±0.18	21.12±0.22	60.02±0.56
b)	Concentration in freeze-dried sample [mg/kg]	21.43±0.29	33.28±0.35	56.17±0.27
	Concentration at pH 2.5 [mg/kg]	12.18±0.15	21.04±0.29	46.22±0.52
	Stability [%]	56.87±0.45	63.23±0.17	82.29±0.13
	Concentration at pH 8 [mg/kg]	5.38±0.18	7.74±0.42	33.93±0.25
	Stability [%]	25.12±0.20	23.27±0.09	60.42±0.15

4. CONCLUSIONS

Results of this study showed that the use of freeze-drying greatly improved gastrointestinal stability of carotenoids (lycopene and *β*-carotene) from carrot, red pepper and tomato in comparison with raw plant material, especially after intestinal digestive phase. In addition, the effect of pH on the stability of carotenoids is lower in freeze-dried plant material. Also, carotenoids stability depends on the food matrix (carotenoids were the most stable in red pepper).

This article contains a study with human digestive juices. The approval for the collection of digestive juices was obtained from the Ethics Committee of the University Hospital Centre Split (11/09/2014).

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PAPER

DETERMINATION OF SUNSET YELLOW IN DIFFERENT BRANDS OF ORANGE JELLIES OF BANGLADESH BY HPLC

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ABSTRACT

Synthetic colorants may have adverse health effects, therefore, it is necessary to ensure controlled use of these colorants in various alimentary products. In this study, 54 samples of six brands of orange jellies were analyzed for the determination of 'Sunset Yellow' color by High Performance Liquid Chromatography. The results showed that the value of Sunset Yellow in one brand of orange jelly exceeded the Bangladesh Standard and Testing Institute (BSTI) value, the values in four other brands were within the range of the BSTI value, and it was absent in the last one. One brand yielded more than twice the maximum BSTI value and four times the maximum value from the European Union. These results indicated that there is a need to monitor the amounts of synthetic colorants used in food products to protect the public health from serious adverse effects related to such chemicals and to create awareness to the consumers as well as policy makers.

Keywords: HPLC, orange jellies, sunset yellow-FCF, synthetic food color, BSTI

1. INTRODUCTION

Sunset yellow (SY) is a common synthetic food color, appearing reddish-orange upon application, that is added to food to improve the color, texture, and overall appearance (KUCHARSKA and GRABKA, 2010; ABBEY *et al.*, 2014). Dairy products, cereals, candies, jellies, ice-cream, soft drinks, yogurts, fillings, liqueurs, and powdered juices are the most common food items in which this dye is added (MEINICKE and JORGE, 2008; YUAN *et al.*, 2016). Overuse of synthetic colorants is a major source of food intoxication (KOUTSOGEORGOPOULOU *et al.*, 1998). There are various adverse health effects associated with ingesting excess amounts of synthetic colorants, such as cancer, genetic diseases, etc. (TSUDA et al., 2001; DAS and MUKHERJEE, 2004), asthma, abortions, weakened immune systems, and allergic reactions (GEOFFREY and FELIX, 1991; HINTON, 2000; BHATTACHARJEE, 2014). Additionally, synthetic colorants have been associated with behavioral effects in children, such as hyperactivity, decreased IQ scores, and boosted aggression (GEOFFREY and FELIX, 1991; HINTON, 2000; BHATTACHARJEE, 2014). Synthetic SY specifically has been linked with anaphylactic reactions and cardiovascular complications (angioedema, vasculitis, and thromboxane synthesis inhibition) in individuals who present a sensitivity to the compound (SARDI *et al.*, 2010). Studies have shown that most synthetic colorants bind directly to DNA and cause structural and numerical incongruities (HAMDY *et al.*, 2000; MPOUNTOUKAS *et al.*, 2010). In addition, it has been found that semi-toxic doses of sunset yellow leads to changes in total lipid storage of the body when exposed to animal models. As lipids have structural functions in biological membranes of the body, this might stimulate their metabolism and may cause potential liver injuries such as necrosis (MATHUR *et al.*, 2005).
Similar to most other developing countries, it is a common scenario in Bangladesh that industrial and non-industrial sectors are involved in food production and processing activities. Food industry uses various synthetic colorants, which are the most interesting groups of food additives as the colorful food products attract consumers (KUCHARSKA and GRABKA, 2010). However, their range of use and amounts are restricted across the world (SUN *et al.*, 2013). The non-industrial sector produces two- to three-times the amount of food items compared to the industrial sector. Unfortunately, quality control systems are lacking in the non-industrial sectors, leading to high production of sub-standard food products potentially compromising the health of consumers and thus, indirectly leading to a financial burden for the nation. Based on the results of the International Research and Recommendation of Codex Committee on Food Additives and Contaminants (CCFAC), the Acceptable Daily Intake (ADI) value of food colorants is set across the world (BESSONOV *et al.*, 2011; GANESAN *et al.*, 2011). The ADI of permitted food colorants varies from 0.1 mg/kg body weight (Erythrosine red) to 25mg/kg body weight (Fast Green FCF) (SWAROOP *et al.*, 2011). However, there is warranted concern over the amount of synthetic colorants in food products as certain companies exceed the upper limit recommended by the ADI. Therefore, it is necessary to monitor the total daily intake of all food colorants to ensure the ADI is not exceeded (JOINT, on FOOD ADDITIVES, ORGANIZATION and OTHERS, 1965, 1991).
Due to the wide industrial use of food dyes to color foods it is important to determine the amounts of synthetic colorants commonly added to foods in Bangladesh. Current analytical detection methods are costly and require substantial resources that are not available in Bangladesh. Therefore, the present study was conducted to determine the color range of SY in different brands of orange jellies collected from different shops of Tangail City, Bangladesh, using a simple and cost effective high-performance liquid chromatography (HPLC) method. The analytical determination of this particular color

may aid in establishing a method for detecting synthetic colorants and contribute to overall quality assurance and consumer safety.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

Sodium acetate 97%, HPLC-grade chloroform, n-hexane, and acetonitrile were obtained from Merck (Darmstadt, Germany). Glacial acetic acid was procured from Sigma Chemical Co. (Darmstadt, Germany). Diethyl acetate from RCI Lifescan Ltd. was used. Deionised water (18.2 MΩ) used for chromatography processing was procured from a Barnstead Nanopure water purification system (Barnstead, USA). Sunset Yellow FCF (E110) was purchased from Rayner, Co. Ltd. (London, England).

2.2. Samples

A total of 54 samples from six brands of orange jellies (research raw material) were purchased from three stores in Tangail city, Tangail, Bangladesh after getting verbal consent from the shopkeepers. All samples were considered valid based on their expiry dates. The weights of the samples were 200 gm to 500 gm. The collected samples were preserved in refrigerator at 4 °C in the laboratory of the Department of Food Technology and Nutritional Science, Mawlana Bhashani Science and Technology University (MBSTU). Ethical approval was taken from the research cell of MBSTU.

2.3. Mobile phase

The mobile phase was prepared using the modified method of PYLYPIW and GRETHER (2000). It consisted of a mixture of 3.0 mM acetate buffer (pH ~4.00) and HPLC-grade acetonitrile with a ratio of 17:3. Acetate buffer was prepared by mixing of 1.0 ml of glacial acetic acid and 1.0 g of sodium acetate trihydrate in 1.0 L de-ionized water and mixed well. The mixture was filtered with a filter membrane (Pore size 0.2 μm).

2.4. Preparation of standard solution

Approximately100 mg of anhydrous sunset yellow was taken in a 25 ml volumetric flask. Ten ml 85% aqueous acetonitrile was added to the volumetric flask and was shaken well. Finally, 85% aqueous acetonitrile was added up to mark. The solution was filtered with syringe filter. The standard stock solution-1 was labeled as 4 mg/ml. Approximately 5 ml of stock solution-1 was taken in 50 ml volumetric flask and mobile phase was added up to the mark. The standard solution-2 was labeled as 0.4 mg/ml standard solution. From the stock solutions, working standard solutions 0.0, 1.0, 5.0, 10.0, 20.0, 40.0 μg/ml were prepared by dilution of aliquots. The solution was filtered through sample filters (Pore size 0.2 μm) prior to inject into the column.

2.5. Preparation of sample solution

Approximately 1.0 gm of orange jelly was weighted accurately and placed in a conical flask and it was made to 10 ml by adding aqueous 85% acetonitrile solution and mixed well by vigorous shaking for 10 minutes. About 2.0 ml of the solution was filtered through sample filter (Pore size 0.2 μm) and the filtrate was then diluted 5 times and placed in an

eppendorf tube. Finally, 20 μl was injected into the HPLC column. The concentration of injected sample solution was 20 μg/ml.
Calculation of sample concentration:

$$\frac{1\ gm \times 200\ ml}{10\ ml \times 1000\ ml} = 0.02\ \text{mg/ml} = 20\mu\text{g/ml}$$

2.6. Sample solution preparation for Spiked/Recovery assay

Approximately 2.0 gm of orange jelly and 1.0 mg of SY was weighted accurately and placed in a conical flask and 85% aqueous acetonitrile solution was added to it to make 20 ml solution and mixed well by vigorous shaking for 10 minutes. About 2.0 ml of the solution was filtered through sample filter (Pore size 0.2 μm) and the filtrate was then diluted 5 times and placed in an eppendorf tube. Finally, 20 μl was injected into the HPLC column. The concentration of injected sample solution was 20 μg/ml.

2.7. Chromatographic analysis

The chromatographic system consisted of a Shimadzu isocratic pump, a degasser, column, oven, a UV-Vis detector, and a LC Workstation Class-VP for data acquisition and analysis. Each of orange jelly samples of 1.0 g was diluted 1:10 with mobile phase and then the sample was again diluted 1:5 with mobile phase. After that the solution was transferred into dry eppendorf tube. The clear aqueous solution was filtered through a PTFE syringe filter. Then the solution was transferred to the dry HPLC vials. 20 µl of the sample was injected into the injector. For the chromatographic analysis, a Luna 5µ C18 (2) 100A column (250 × 4.6 mm) was used and the column temperature was set at 33 °C. The sunset yellow analysis was performed with isocratic solvent system using sodium acetate and acetic acid buffer (pH ~4.0)/acetonitrile- 17:3 with a flow rate of 1.0 ml/min.

2.8. SY identification and quantification

Optimum absorption wavelength for SY color was evaluated before and using standard solutions with UV-spectrophotometer. The determined wavelength for the analysis of SY was 480 nm. Several runs were made to determine the retention time for the analysis. The retention time for this color was used for the identification of the color present in different brands of orange jellies.
Quantification of the studied colors was done by external standard calibration. Five level analytical curves (0.00, 1.0, 5.0, 10.0, 20.0, 40.0 μg/ml) were used and the mean of 3 injections of each standard was used to represent each calibration point. By plotting analytes (y) against the concentration (μg/ml) of the color the peak areas were measured. To determine the slope, y-intercept and the correlation coefficients of the standards plots, least square linear regression analysis was used. Limit of detection (DL) and limit of quantification (QL) were determined by considering 3 and 10 times the signal to noise ratios respectively estimated by the regression lines as mentioned in the previous report (MACDOUGALL *et al.* 1980).
For HPLC method validation the performance parameters, i.e., precision, linearity, limit of detection, limit of quantification, the expanded uncertainty were calculated. By spiking known amounts of the studied colors to the unprocessed sample and comparing the output with the same sample without spiking, recovery evaluations were carried out.

Recoveries were calculated by differences of concentrations and were expressed as percentages.

2.9. Statistical analysis

Each test was performed in triplicate. The descriptive analyses (means, median, standard errors coefficient of variation) were summarized. Data were expressed as mean±standard error (SE). One-way analysis of variance (ANOVA) was carried out using SPSS software version 20 at a significance level of 5%. The Least Significant Difference (LSD) test was used to detect differences in means.

3. RESULTS AND DISCUSSION

3.1. Analysis of chromatogram

Numerous analytical methods have been developed for analyzing synthetic colorants in response to the concern regarding their adverse effects. The most preferred technique for quantitative determination of the colorants is using HPLC, as it is relatively simple, cost effective, and has less environmental impact due to fewer hazardous chemicals used. In this study, an efficient and accurate HPLC analytical method was used for the determination of sunset yellow in different brands of orange jellies collected from local markets of Tangail city, Bangladesh. This method was simple to use, had good operational stability, and gave reliable and reproducible results.
The developed HPLC method was applied to analyze the SY color range in orange jellies. The retention time of the sunset yellow standard was 5.62±0.2 minutes and approximate time was set at 10 minutes. (Figs. 1 and 2).
The calibration curve (Fig. 3) for SY was obtained by plotting the peak areas of different concentrations of the working standard solutions (0.0, 1, 5, 10, 20 and 40 μg/ml). The recovery range was 96-131%.
Table 1 describes the analytical characteristics of the HPLC method. There was a strong linear relationship ($R^2 = 0.999$) obtained between the concentration of SY and the peak area within the HPLC chromatogram at 480 nm (Fig. 3). The detection and quantification limits were calculated as 1.14 mg/100 ml and 3.46 mg/100 ml, respectively.

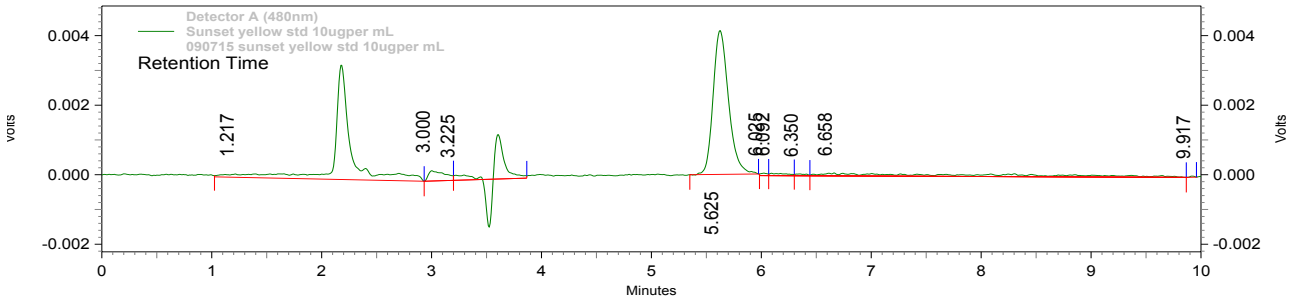

Figure 1. HPLC chromatogram of 10 μg/ml sunset yellow standard solution with a retention time of 5.625 min.

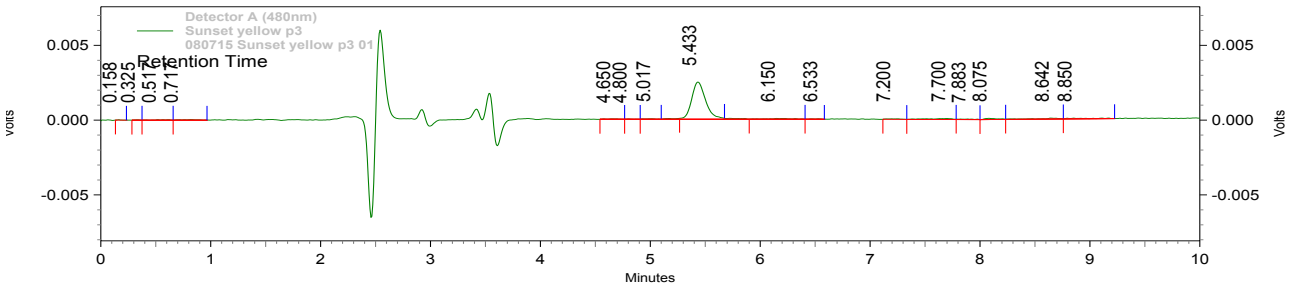

Figure 2. HPLC Chromatogram of sunset yellow present in orange jelly brand-1 (obtained from shop 1) with a retention time of 5.433 min.

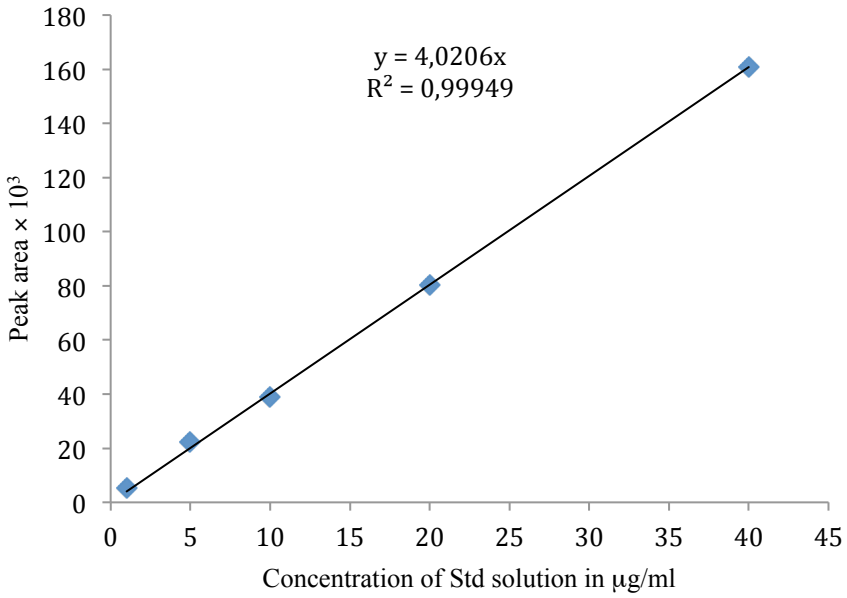

Figure 3. Calibration curve for the sunset yellow standard.

Table 1. Analytical characteristics of HPLC method.

Parameter	Value
Accuracy	107±14.3
Slope	4020
Intercept	0
Linearity range	1.32 µg/ml to 40 µg/ml
Correlation coefficient	0.999
STEYX	1390
LOD	1.14 mg/100 ml
LOQ	3.46 mg/100 ml

3.2. Calculation of % recovery of SY in spiked samples

Approximate 500 μg of SY was added to the 1.0 g of unspiked sample containing SY 4049.41 μg/g. The observed value of SY of the spiked sample was calculated 4560.60 μg/g. So the recovered added SY was 511.19 μg. Finally, the % recovery was 102.23%.

3.3. Determination of SY in samples

The results in Tables 2 and 3 show the levels of the SY from the six brands of orange jellies collected from three different shops with different batches. After HPLC analysis, majority of the orange jellies contained the same synthetic color as was mentioned on their respective product labels. Chromatogram's peak from five out of the six orange jelly brands, were identified as SY matched with the SY standard. No peak in the chromatogram of the samples of brand 6 was matched to the peak of SY standard.

Table 2. Level of SY (mg/100 g) in different brands of orange jellies.

Orange Jelly	Concentration of sunset yellow (mg/100 g)				Mean±SD (n = 3)	p-value
	Shop	Sample 1	Sample 2	Sample 3		
Brand 1	1	25.10	27.40	28.70	27.07±1.49	0.118
	2	17.30	15.20	25.80	19.43±4.58	
	3	25.70	28.20	17.90	23.93±4.39	
Brand 2	1	42.50	40.40	42.00	41.63±0.90	0.351
	2	37.33	44.30	39.20	40.28±2.95	
	3	39.20	40.50	37.90	39.20±1.06	
Brand 3	1	10.10	8.70	8.89	9.23±0.62	0.833
	2	8.89	10.79	7.49	9.06±1.35	
	3	9.02	9.25	8.07	8.78±0.51	
Brand 4	1	19.40	18.50	17.40	18.43±0.82	0.109
	2	13.8	17.00	18.70	16.50±2.03	
	3	18.00	20.3	19.70	19.33±0.97	
Brand 5	1	12.90	13.60	13.30	13.27±0.29	0.003
	2	13.10	11.30	12.10	12.17±0.74	
	3	9.95	11.20	10.10	10.42±0.59	
Brand 6	1	ND	ND	ND	ND	
	2	ND	ND	ND	ND	
	3	ND	ND	ND	ND	

ND = Not detected.

The concentrations of SY in the studied orange jellies varied between 8.78 to 41.63 mg/100 g, depending on the brand in Table 2. Among the collected samples of Brand 1, SY concentration (Mean ± SD) of two samples were higher than the BSTI value as a whole, but the difference was not statistically significant ($p = 0.118$). SY values in all samples of brand 1 exceeded the recommended value of EU. When compared to the SY concentration of brand 2, all values exceeded the BSTI and EU recommended values. No samples of brand 3 exceeded the EU and BSTI values. On the other hand, all samples of brand 4 exceeded

the EU recommended value, but did not exceed the BSTI value. The SY values in brand 5 sample were within the BSTI value, but only one was within the EU value. However, brand 6 showed no trace of SY. A discrepant finding compared to the product label from brand 6 was observed. Added synthetic color can be reduced during manufacturing process due to formation of inorganic salts as byproducts e.g. NaCl (KIRSCHBAUM *et al.*, 2003). Orange jellies from brand 2 contained the highest amount (41.63 mg/100 g) of SY in Table 3, which was 2 times more than the maximum value of BSTI and 4 times from the EU value (Fig. 4). Again, when comparing among different brands, SY showed significant difference among the brands ($p = 0.003$) in Table 3. Among the statistical parameters, the mean value, standard errors, and coefficient of variance were 19.5 mg/100 g, 13.0 and 76% respectively.

Table 3. Summary of concentration of SY (mg/100 g) in selected brands of orange jellies.

Sample	Concentration of sunset yellow (mg/100 g)			Mean ± SD (mg/100 g) (n = 9)	p-value
	Shop 1	Shop 2	Shop 3		
Brand 1	27.07	19.43	23.93	23.48±3.14	$p_B = 0.079$, $p_E < 0.001$
Brand 2	41.63	40.28	39.20	40.37±0.99	$p_B < 0.001$, $p_E < 0.001$
Brand 3	9.23	9.06	8.78	9.02±0.19	$p_B < 0.001$, $p_E = 0.018$
Brand 4	18.43	16.50	19.33	18.09±1.18	$p_B = 0.018$, $p_E < 0.001$
Brand 5	13.27	12.17	10.42	11.95±1.17	$p_B < 0.001$, $p_E = 0.003$
Brand 6	ND	ND	ND	ND	

*ND= not detected; p_B– value compared with BSTI; p_E– value compared with EU.

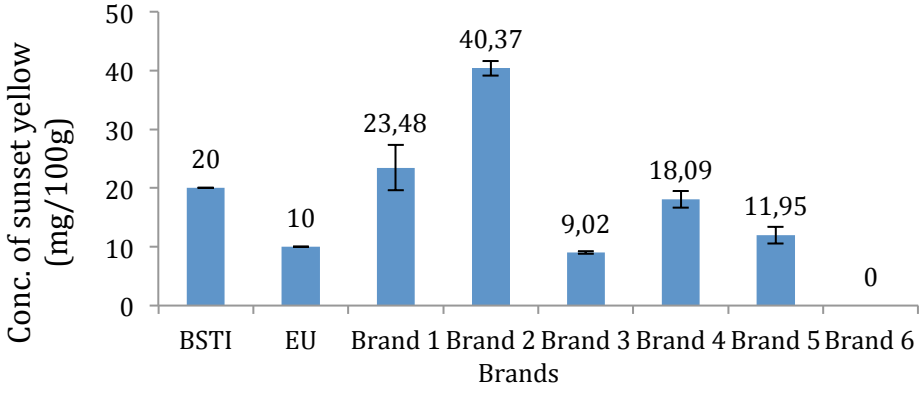

Figure 4. Comparison of SY concentration in different brands of orange jelly with BSTI and EU standard range.

ALVES *et al.* (2008) showed that the concentration of SY in one brand of mango juice powder was significantly higher compared to the maximum regulated value of 10 mg/100 g. The intakes of colors such as tartrazine, erythrocine and SY were higher in children due to the ingestion of foods containing high concentrations of colors (9.45 and 4.0 mg) (RAO and SUDERSHAN, 2008). Another study showed that the consumption of chewing gum

contains the greatest amount of tartrazine (E102), and jellies contain quinoline yellow (E104), ponceau 4R (E124) and allura red (E129) (MALCZYK *et al.,* 2015). On the other hand, the consumption of colored beverages significantly increases the adoption of SY (E110) and azorubine (E122) (MALCZYK *et al.,* 2015). The amount of tartrazine that is not secreted through urine, widely metabolized by intestinal microflora in which some metabolites are absorbed through the intestine (KHERA *et al.,* 1979; WATABE *et al.,* 1980; ELHKIM *et al.,* 2007).
BENTO *et al.* (2015) found the highest concentration containing 75.30±3.85 mgL^{-1} of INS102 colorant in one sample of milk drink among 15 samples of yogurt and milk drink. They also mentioned that INS122 was the most commonly used dye which was 33% of yogurt and milk drink samples ranging from 1.43 to 11.75 mgL^{-1}.

4. CONCLUSIONS

All but two of the samples tested in this experiment contained sunset yellow in accordance with the standard range accepted by the BSTI. Sunset yellow was absent in one brand, while the other brand substantially exceeded the amount of sunset yellow compared to the standard range accepted by the BSTI and EU. The differences in sunset yellow concentrations across the six brands of orange jellies highlight the need for improved product labeling. By providing this information to consumers and manufacturers would not only be aiding the health of consumers but they would also be assuaging the food adulteration reputation that currently taints the food product system. According to the Bangladesh Pure Food Ordinance (2005), there is prohibition of use of intoxicated food color in food. This is considered as a frightening issue which might be hazardous for public health. Therefore, further research is required to evaluate different category food products with this method to see the reproducibility of the results described here.

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S.H.K. designed the study, and A.I. and M.S. collected data from the experiments. A. I. and L. B. contributed to data analysis and manuscript preparation. Additional editing was performed by M.I.H. and M.Z.A. References were obtained by M.A.Z. All authors carefully read and approved the manuscript.

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PAPER

PRESENCE OF DESTRUXIN A AND BEAUVERICIN IN CEREALS

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ABSTRACT

A LC-MS/MS method for the detection of destruxin A (DTX A) and beauvericin (BEA) in cereals was developed, validated and applied to commercial products collected in Italian markets in the years 2015-2016. Results showed that BEA contaminated 59 % of the samples even if only 15 of them (34%) showed quantifiable residues (comprised between 0.11 and 7.51 ng/g). The sample of red rice contaminated with the highest BEA level was also contaminated with DTX A (0.28 ng/g). Finally, no significant differences were detected between contaminated samples based on the production year and the agronomic technology used (organic or conventional farming).

Keywords: LC-MS/MS, mycotoxins, organic, conventional

1. INTRODUCTION

Cereals supply and demand have been steadily increasing in recent years (USDA, 2017), however these products are exposed to pre-/post-harvest fungal infections potentially dangerous for humans and animals and responsible for economic losses (WHO-IARC, 2002; PERAICA *et al*., 1999; ZAIN, 2011).
Fungal toxicity is mainly due to the production of mycotoxins. These secondary metabolites, produced by molds as natural protection, are generally thermostable and resistant to food transformation processes (KARLOWSKY *et al*., 2016). For these reasons, mycotoxins are considered the main chronic dietary risk factors and therefore a correct evaluation of the real contamination and co-occurrence of these products is required. Among all mycotoxins, European Union has set a maximum level in food only for aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, T-2 toxin and HT-2 toxin (Commission REGULATION 1881/2006; COMMISSION RECOMMENDATION 2013/165/EU).
Recently, a particular attention was paid in EC to enniatins and beauvericin (BEA). EFSA's Panel on contaminants has reported the occurrence of enniatins and BEA in European foods and feeds in 2014 in the food chain (EFSA, 2014). Even if no concerns for human health have been related to the acute exposure to these mycotoxins, given the lack of relevant *in vivo* toxicity data, no reliable conclusions can be drawn on chronic exposure to these compounds (contam, 2014).
Many fungi, such as *Beauveria bassiana* and *Fusarium* spp., produce BEA and, in the last period, *B. bassiana* is widely used as entomopathogenic mycoinsecticide alone or in combination with *Metarhizium anisopliae* (WANG and XU, 2012).
The *Metarhizium* spp. and other ubiquitous soil fungi, produce a family of cyclic peptide toxins termed destruxins (DTXs). To date, a number of DTXs have been identified and placed in five major subgroups (A-E) with DTX A, B and E as the most predominant one (HSIAO and KO, 2001; WANG *et al*., 2009; ibraim and asker, 2012). They are known to possess cytotoxic and cytostatic effects on mammalian and insect cells with DTX A and E being the most toxic (SKROBECK and BUTT, 2015).
For some authors, mycotoxins from mycoinsecticides have limited ways to enter in environment and the risks of contaminating foods are negligible (HU and ZHANG, 2016). Differently from BEA, no analytical data are currently available on the occurrence of DTXs in food chain and the present work aimed to investigate of the real occurrence of DTX A, along with BEA, in cereals purchased from the Italian market in 2015-2016 period. Based on our previous experience with these two analytes and on the extraction solvents reviewed in literature (HSIAO and KO, 2001; WANG *et al*., 2009; CITO *et al*., 2014 and 2016; BUTT *et al*., 2009; TAIBON *et al*., 2015; BLESA *et al*., 2012; SØRENSEN *et al*., 2008), a validated LC-MS/MS method was optimized in order to determine simultaneously both analytes in commercial organic or conventional farming cereals samples.

2. MATERIALS AND METHODS

2.1. Chemicals

The standards of DTX A and BEA were obtained from Sigma-Aldrich S.r.l (Milano, Italia). All reagents were obtained by Sigma unless stated otherwise. Acetonitrile, dichloromethane and ethyl acetate, used for the mycotoxins extractions, were of analytical grade while acetonitrile used for chromatographic analysis was of HPLC grade. Milli-Q quality water (Millipore, Milford, MA, USA) was used.

2.2. Standard solutions

Standard solutions were prepared by dissolving each compound with methanol in a volumetric flask and then diluted with methanol to make the working solutions.

2.3. Sample extraction

The cereals samples (maize, barley, oat, rice, red rice, amaranth, millet, wheat and spelt) were purchased in local supermarkets. In the first step, a representative portion of the cereal samples (100 g) was mixed well with a food chopper. An accurately weighed portion of all the samples (10 g) was placed in a centrifuge tube and 25 mL of acetonitrile or dichloromethane: ethyl acetate (1:1, v/v) was added. The extractions were carried out using an IKA Labortecnhik homogenizer model T25 basic (IKA WERKE GmbH & Co., Staufen, Germany) for 5 min at 13500 rpm. The supernatant was transferred after centrifugation, and another aliquot of extraction solvent was added to the residue and homogenized. The organic fractions were pooled and evaporated to dryness under vacuum by rotary evaporation (temperature of the bath, 20°C), and the residue redissolved in 500 μL of acetonitrile. The sample was filtered with 0.45 μm Minisart SRP 4 (Sartorius: Goettingen, Germany) and used for the LC/MS-MS analysis.

2.4. Quantification and recovery

The quantitative analysis of BEA and DTX A was based on calibration curves obtained analysing spiked samples (10 g of equimolar mixture of barley, oat, maize, rice, wheat and spelt) at different concentrations ranged between 0.1 and 100 ng/g. For the equations, six points with different concentrations were used. Extraction recoveries were determined by spiking untreated powdered equimolar mixture of cereals with standard solutions to obtain tree different final concentrations (0.1, 10 and 100 ng/g for each investigated compound). After the solvent evaporated, the samples were extracted, as reported above. Recovery values were calculated as the ratio of the peak area obtained from the extraction of the fortified samples to the corresponding peak area determined by a single-point calibration standard.

2.5. LC–ESI-tandem MS analysis

Chromatography-mass spectrometry system consisted of a Varian apparatus (Varian Inc.) including a vacuum solvent degassing 20 unit, two pumps (212-LC), a Triple Quadrupole MSD (Mod. 320-LC) mass spectrometer with ESI interface and Varian MS Workstation System Control Ver. 6.9 software. The chromatographic separation was performed by using a Kinetex 2.6μm C18 100Å column (Phenomenex) (100 mm×4.6 mm). The sample was injected (5 μL) after filtration. Chromatographic analysis was carried out by using acetonitrile and aqueous solution of formic acid (0.05%) (3:97 v/v). The flow rate was 0.1 mL/min.

The instrument operated in positive mode and ESI parameters were: detector voltage 1250 V, drying gas pressure 18.0 psi, desolvation temperature 300.0°C, nebulizer gas 42.0 psi, needle voltage 6000 V and shield voltage 250 V. Nitrogen was used as nebulizer and drying gas. Collision induced dissociation was performed using argon as the collision gas at a pressure of 1.8 mTorr in the collision cell. The selected reaction monitoring (SRM) transitions as well as the capillary voltage and the collision energy are summarized in Table 1. Quantitative analysis was performed in SRM to maximize sensitivity. For each investigated compound the $[M+H]^+$ species were selected as precursor ions. Two SRM

transitions (Table 1), the first one for quantification and the second one for confirmation purpose, were acquired by using the experimental conditions described above.

Table 1. Chromatographic and selected reaction monitoring (SRM) parameters used in the analysis (retention time (t_R), quantification and confirmation transitions, collision energy and capillary voltage).

Compound	t_R (min)	Quantification transition (*m/z*)	Collision energy (eV)	Confirmatory transition (*m/z*)	Collision energy (eV)	Capillary voltage (V)
DTX A	3.52±0.08	578.1→465.1	-28.5	578.1→436.8	-22.5	86.29
BEA	4.32±0.06	784.2→244.0	-25.0	784.2→262.0	-24.5	140.00

2.6. Validation procedure and evaluation of the matrix effect

The specificity of the method was assessed by analysing blank samples (one sample for each analysed cereals) and blank samples spiked with the investigated compounds, according to the procedure reported above. Assay selectivity was defined by evidence of non-interference at retention times and ion channels identical to those of BEA and DTX A in the blank samples.

In order to determine the linearity of the method, calibration curves (obtained from five replicate experiments) were constructed by analysing spiked cereal samples (10 g of equimolar mixture of barley, oat, maize, rice, wheat and spelt fortified before extraction) ranged between 0.1 and 100 ng/g. The linearity was evaluated by linear least-squares regression analysis.

The detection limit (LOD) was defined as the concentration for which a signal-to-noise ratio equal to 3 was obtained. The quantification limit (LOQ) was defined as the lowest concentration for which an accuracy between 80% and 120% and a precision with a coefficient of variation of±20% or less was obtained over six measurements, with a signal-to-noise ratio superior or equal to 10.

Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying added blank cereal samples, six replicates set at the same concentration (0.1, 10 and 100 ng/g), during the same day. The between-day precision was studied by assaying added blank cereals samples, six replicates set at the same concentration (0.1, 10 and 100 ng/g), on different days (5 days). The accuracy of the method was also evaluated at the same concentration levels and expressed as relative error % (RE).

The recovery data were determined by spiking blank cereal samples with standard solutions (three concentrations analysed in triplicate). After spiking, the samples were extracted as previously described. Recovery values were calculated by comparing the analytical results of the samples through overall extraction procedure with those obtained from blank samples fortified after extraction.

In order to study the matrix effect (ME), blank samples were processed and spiked later to obtain three final concentration levels (set B: six samples with final concentrations of 0.1, 10 and 100 ng/g). The response (peak area) was compared with directly injected standard solutions (set A: six samples prepared in methanol at the same concentration levels). The matrix effect (ME) was evaluated by comparing the mean peak area of the spiked samples (post-extraction addition) with corresponding standard solutions at equivalent concentrations prepared in methanol. The ME values were then calculated as follows: ME (%) = A/B×100 (MATUSZEWSKI *et al.*, 2003).

3. RESULTS AND DISCUSSION

3.1. LC–ESI-tandem-MS optimization

The selected reaction monitoring (SRM) was performed to enhance sensitivity and specificity of the analysis. The MS/MS dissociation study was optimized, for each single standard compound, by varying the cone voltage and collision energy, using the flow injection analysis (FIA) of working standard solutions at a flow rate of 0.01 mL/min directly through the electrospray probe. $[M+H]^+$ ions were found to be the most abundant ones and selected as precursor ions for the target compounds.
The MS–MS breakdown for DTX A showed a fragmentation pattern similar to that reported by other authors (WANG *et al*., 2009; BUTT *et al*., 2009). As expected the DTX A ion $[M+H]^+$ at 578.1 *m/z* represented the most abundant ion without any adduct. The collision-induced dissociation (CID) experiments showed common losses of amino acids following ring opening.
As previously described by other authors, BEA tends to be readily ionized via ESI to form $[M+H]^+$ ion. As determined in this work, $[M+H]^+$ ion was found to be the most abundant one and selected for BEA analysis. The MS/MS tuning experiments displayed product ions scan spectra of BEA in accordance with those reported in literature (SØRENSEN *et al*., 2008; SONG *et al*., 2009). Most intense fragments were selected for DTX A and BEA quantification and confirmatory purposes (Table 1).

3.2. Method optimization and validation procedure

The method was validated for accuracy, precision, specificity, linearity and sensitivity. In order to control for variability in recovery from biological samples and factors that can affect the instrumental response, various cereals samples were assayed. Cereals used for calibration and for recovery studies were analysed to verify the absence of each investigated compound before performing the analysis. The analysis of blank samples showed the absence of interfering endogenous compound peaks at the same ion channel or retention time of DTX A or BEA.
Two extraction methods were tested in order to identify a unique system to quantify contemporarily both mycotoxins. Satisfactory mean recoveries for BEA (89 and 72 % respectively) and only with binary mixture for DTX A (56 %) were obtained by using both selected extraction solvents (acetonitrile and dichloromethane: ethyl acetate 1:1 v/v) (Table 2).

Table 2. Mean extraction recoveries obtained with the two extraction procedures (experiments conducted on equimolar mixture of barley, maize, oat, rice, spelt and wheat) (n=3).

	Spiked concentration (ng/g)	Extraction with Acetonitrile		Extraction with CH_2Cl_2:ethyl acetate (1:1 v/v)	
		Recovery (mean±SD)	RSD%[a]	Recovery (mean±SD)	RSD%[a]
DTX A	0.1	38.6±7.8	20.3	66.7±6.66	10.4
	10	43.1±6.4	14.8	68.2±5.52	8.10
	100	39.2±5.8	14.9	79.9±3.46	4.3
BEA	0.1	88.4±5.0	5.9	55.8±6.7	12.0
	10	88.7±3.1	3.4	54.9±6.2	11.2
	100	89.4±2.9	3.2	57.5±4.7	8.1

[a]Relative standard deviation.

The matrix-induced effects, such as signal enhancement or suppression, were also evaluated according to MATUSZEWSKI *et al*. (2003), and the results obtained in presence of an extract of equimolar mixture of barley, maize, oat, rice, spelt and wheat are summarized in Table 3. An enhancement of the absolute response was observed for both analytes with both the extraction systems. Very close results were obtained for all matrices when tested separately (results not shown), therefore, calibration curves were generated from blank constituted of an equimolar-mixed cereals sample (barley, oat, maize, rice, wheat and spelt) spiked before extraction to avoid and minimize any uncertainty related to the matrix-induced effects.

Table 3. Matrix effects obtained with the two extraction procedures (experiments conducted on equimolar mixture of barley, maize, oat, rice, spelt and wheat) (n=3).

	Spiked concentration (ng/g)	Matrix effect (mean±SD)	
		Acetonitrile	CH_2Cl_2:ethyl acetate (1:1 v/v)
DTX A	0.1	146.1±11.3	120.6±10.6
	10	137.8±9.9	130.6±9.6
	100	136.9±7.9	124.8±9.0
BEA	0.1	147.9±8.5	156.6±10.7
	10	148.7±4.7	137.2±8.7
	100	143.1±8.5	140.8±8.5

Based on the results obtained in these preliminary stages, the binary extraction mixture was selected for the continuation of the work, ensuring satisfactory recovery values for both analytes. Calibration curves (five replicate experiments) were constructed and the method was found to be linear within the range 0.1–100 ng/g with correlation coefficient above 0.9994. The equations of the curves, obtained by a least squares fit, are reported in Table 4.

Table 4. Regression plot parameters for DTX A and BEA quantification in mixed cereals (experiments conducted on equimolar mixture of barley, maize, oat, rice, spelt and wheat).

	Range (ng/g)	Regression plots parameters	R^2	LOQ[a] (ng/g)	LOD[b] (ng/g)
DTX A	0.1-100	y = 24423x+6127	0.9994	0.1	0.03
BEA	0.1-100	y = 46020x+7695	0.9997	0.1	0.03

[a]Quantification limit.
[b]Detection limit.

The selected method was validated in term of precision and accuracy (results are reported in Table 5). Intra-day and inter-day precision, expressed as the relative standard deviation (RSD %) values, were always less than 15 % (n = 6) for both the analytes. The relative errors (RE %) ranged from −12.00 % for BEA to +14.00 % for DTX A obtained at LOQ levels. The reported results indicated that the developed method is precise, accurate, reproducible and utilizable for determination of the two compounds in cereal-based foods. Compared to the LOQ present in literature our values are comparable for both analytes (TAIBON *et al*., 2015; BLESA *et al*., 2012; Sørensen *et al*., 2008; TOLOSA *et al*., 2017; MALACHOVÁ *et al*., 2014).

Table 5. The intra-day and inter-day precision and accuracy of the method (n=6).

Compound	Analysis type	Spiked concentration (ng/g)	Measured concentration (media±SD)	RSD%[a]	Accuracy (relative error %) [b]
DTX A	Intra-day	0.1	0.11±0.01	9.09	+8.20
		10	10.52±1.12	10.65	+5.20
		100	101.03±3.40	3.40	+1.00
	Inter-day	0.1	0.12±0.01	11.67	+14.00
		10	10.54±0.84	7.97	+5.40
		100	103.06±2.35	2.28	+3.06
BEA	Intra-day	0.1	0.09±0.01	14.44	-12.00
		10	9.90±1.24	12.53	-1.00
		100	101.20±4.23	4.18	+1.20
	Inter-day	0.1	0.09±0.01	13.33	-9.40
		10	9.95±0.90	9.35	-0.50
		100	109.20±3.68	3.37	+9.20

[a]Relative standard deviation.
[b]Accuracy = relative error % = (measured-spiked)/spiked x 100.

3.3. Analysis of DTX A and BEA content in cereals

The validated method (extraction accomplished with dichloromethane: ethyl acetate, 1:1 v/v) was successfully applied to quantify DTX A and BEA levels in 44 commercial products collected in the years 2015-2016 (Table 6).
Results showed that BEA contaminated 59 % of the sample even if in only 15 samples (34%) quantities were higher than LOQ (included in the range 0.11 and 7.51 ng/g). Contamination data are in accord with BEA occurrence reported by various authors and collected in the EFSA CONTAM Panel report (2014).
The high number of positive samples is due to the good LOD values obtained with our method (0.03 ng/g for both compounds). Either no significant differences were detected between percentages of contaminated samples based on the year of production or the agronomic technology used (organic or conventional farming).
Only one sample (red rice presenting the highest BEA levels) resulted contaminated by DTX A (0.28 ng/g).
Although the number of analyzed samples is limited, the low levels of BEA and the substantial absence of DTX A confirm that acute exposure to these toxins could do not indicate concern for human health. However, careful monitoring in foods is essential in order to provide a correct estimate of chronic exposure to these toxins.

Table 6. Occurrence and content of DTX A and BEA in commercial products (44 cereals samples) collected in the years 2015-2016 (each sample was analysed in triplicate).

Year	BEA content (ng/g±SD)									
	Amaranth	Barley	Oat	Oat flakes	Maize	**Millet**	**Red rice**	**Rice**	**Spelt**	**Wheat**
2015	---- [a]	---- [a]	0.18±0.01	0.17±0.02[a]	0.19±0.02[a]	0.58±0.05[a]	7.51±1.16[b]	1.28±0.16	0.53±0.07[a]	3.92±0.13
		0.27±0.03[a]	---- [a]	----	2.48±0.48		----	0.98±0.11[a]	----	----
				>LOD	----		>LOD	----		
2016	---- [a]	>LOD	>LOD	---- [a]	0.21±0.03	>LOD [a]	----	>LOD [a]	>LOD [a]	>LOD
			----	>LOD	1.54±0.18		>LOD	---- [a]	----	----
				----				0.11±0.01		5.12±0.59
								>LOD		
								----		

[a]Organic product.

[b]Red rice sample containing also DTX A (0.28±0.04 ng/g).

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(4) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in c1early defined units.
Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

3. Editorial and Review Policy

Scientific contributions in one of the following forms may be submitted:
Reviews –They can be submitted directly to the Editor-in-Chief or articles can be requested directly by the Editor-in-Chief.
Short Communications, Surveys and Opinions – They do not need to have the formal organization of a research paper; they will receive priority in publication; maximum of five pages allowed.
Papers – The paper must follow the guidelines as specified under the section Manuscript Preparation.

Reviews, Papers, Short Communications and Surveys will be **subjected to critical review by referees.**
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