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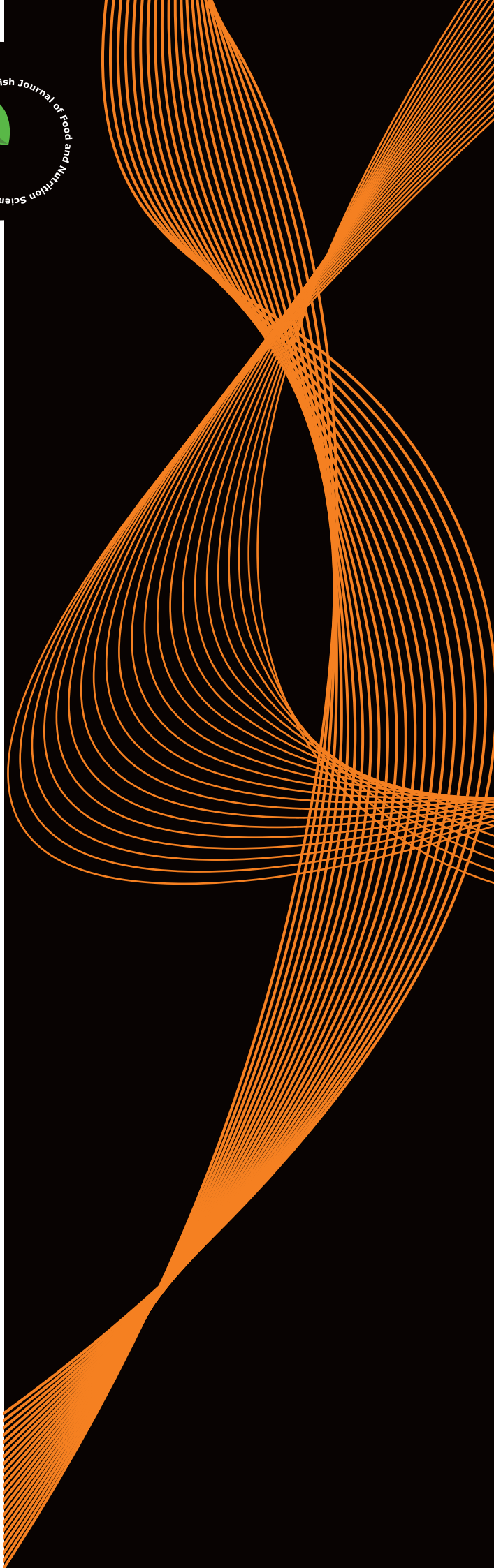

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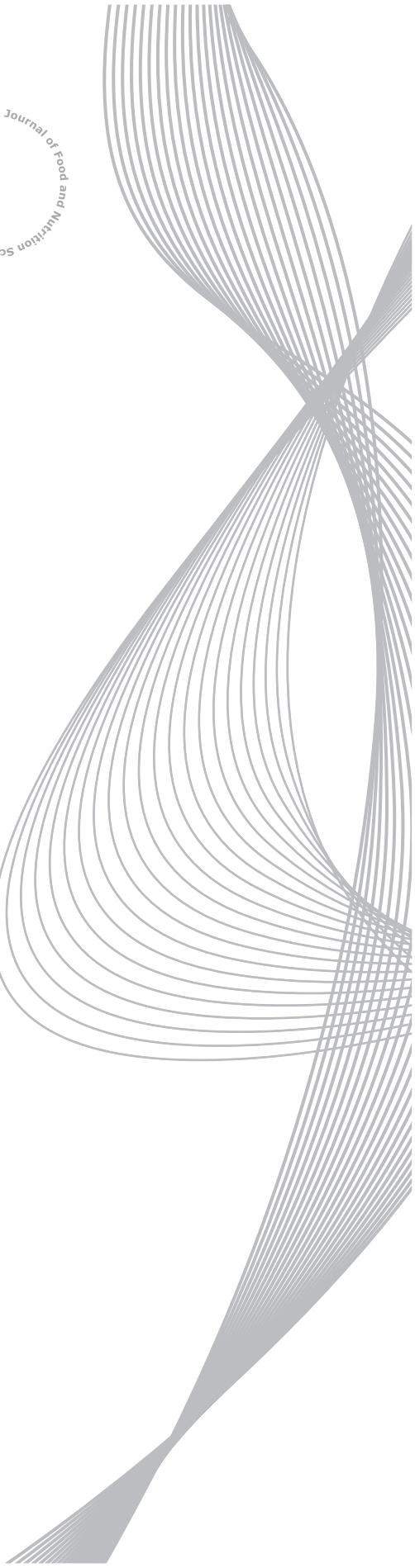

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Original research article
Section: Food Quality and Functionality

Functional Properties of Enzymatic Hydrolysate and Peptide Fractions from Perilla Seed Meal Protein

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Key words: perilla seed meal, enzymatic hydrolysate, peptide, ultrafiltration, agricultural by-products, physical property

The present study aimed to decompose perilla seed meal (PSM) proteins *via* enzymatic degradation and to evaluate the functional properties of the resulting enzymatic hydrolysate and peptide fractions for the utilization of PSM, a by-product in the production of perilla seed oil. PSM protein hydrolysate was fractionated based on molecular weight using an ultrafiltration system, and the physical properties required to utilize the hydrolysate and peptide fractions as functional food components were determined. The enzymatic hydrolysate and peptide fractions showed higher solubility, oil absorption capacity, and emulsifying and foaming properties than protein isolates. In particular, peptide fractions below the 5 kDa showed high solubility, emulsifying activity, and foaming capacity. Therefore, these peptide fractions are ideal as functional raw materials and substitutes that can be used to improve the quality of various processed food products and protein supplements.

INTRODUCTION

Perilla (*Perilla frutescens var. Japonica* HARA) is a seedling of a perennial plant belonging to the family Lamiaceae and is cultivated in some Asian countries such as Korea, China, and Japan [Meng *et al.*, 2009a]. Perilla leaves are used as a fresh vegetable and to process pickles due to their unique scent. Perilla seeds were also considered as an interesting oil source for non-food uses and this variety has become widely cultivated in China and Korea. Perilla is not only used as a food ingredient but also for skin creams, soaps, and medicinal preparations, because of its recognized bioactivities, such as antioxidant, anti-allergic, anti-inflammatory, and anti-HIV-1 activity [Meng *et al.*, 2009b]. In particular, perilla oil has excellent physiological functions such as lowering blood pressure, improving thrombosis, and inhibiting cancer cell proliferation [Mohammad, 2011], and perilla cultivation is increasing due to an increase of perilla oil consumption. Perilla seed meal (PSM), a byproduct of the production of perilla oil, is expected to be very useful as a functional food component because it contains high amounts of proteins and active ingredients [Di Bernardini *et al.*, 2011].

Enzymatic hydrolysates, such as sugars, proteins, and lipids, are incorporated in the diets of individuals or patients suffering from impaired metabolic conditions. Protein hydrolysates have long been used in various processing applications, including dietary supplements for athletes and elderly people who need protein. Furthermore, they are used in animal feed to replace expensive protein sources [Schmidl *et al.*, 1994; Córdova-Murueta & García-Carreño, 2002]. Hydrolysates containing low molecular weight peptides are easier to digest and absorb than long polymeric proteins and are widely available in various foods and processed products [Megias *et al.*, 2009]. Peptides produced *via* enzymatic hydrolysis of food proteins exhibit physicochemical properties that are different from those of the original proteins because of lower molecular weights and exposure of hydrophobic residues caused by changes in the molecular structure, while maintaining physiological activity. Peptides present in food products exert biological control functions and provide nutritional value by supplying amino acids. In addition, peptides have excellent emulsifying properties and desired physical properties, such as gel-forming ability, solubility, viscosity, and emulsion properties, and strong affinity fat, and are thus ideal components of food products, such as sports drinks, dietetic foods, and health supplements [Chalamaiah *et al.*, 2012; Benitez *et al.*, 2008]. Various bioactive peptides have been produced from proteins, and their potential use as a functional food material has been proposed.

Therefore, in the present study, PSM protein hydrolysate was prepared *via* enzymatic degradation, and the peptides from the hydrolysate were fractionated based on the molecular weight using an ultrafiltration system. The amino acid composition and physical properties, including solubility, emulsification, foaming, oil retention, and water absorption properties, of the protein hydrolysate and peptide fractions were evaluated to serve as the basis for the utilization of PSM as functional ingredients in food products.

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MATERIAL AND METHOD

Materials

The PSM was obtained from Queensbucket Co. (Seoul, Korea) and ground and stored in a deep freezer (MDF-435, Sanyo, Tokyo, Japan) at –42°C for use in subsequent experiments. The proximate composition of PSM was measured according to methods of Bhattacharjee *et al.* [2013] and Park & Yoon [2014] with some modifications. Moisture content was measured using an infrared moisture analyzer (FD-720, Kett, Tokyo, Japan). The contents of crude protein and crude fat were determined using a Micro Kjeldahl system (Distillation Unit B-323, Buchi, Flawil, Switzerland) and an auto fat extraction system (Soxtec 2050, Foss, Sweden), respectively. Crude fiber content was analyzed with a raw fiber extractor (Fiber test F-6, Raypa, Spain). Crude ash content was determined by weighing the remaining inorganic residue after being completely burned at a temperature of 550°C in a muffle furnace. The total carbohydrate content (%) in the PSM was determined by the method involving the summing up of the total values of crude protein, crude fat, crude fiber, and crude ash constituents of the sample and subtracting it from 100. The moisture content of PSM was 26.55%, and crude protein, crude fiber, crude fat, crude ash, and carbohydrate contents were 45.50, 30.74, 18.69, 4.81, and 0.27 % dry weight, respectively.

Preparation of perilla seed meal (PSM) protein isolate

The PSM protein isolate was prepared according to the alkaline extraction and acid precipitation method of Gnanasambandam & Heitiarachchy [1995] with slight modifications. The PSM was added with distilled water at a ratio of 1:10 (w/v) and stirred in a shaking water bath (BS-11, JeioTech, Seoul, Korea) at 25°C for 1 h; the pH of the mixture was adjusted to 10.0 with 1 N NaOH. The resulting slurry was incubated at 25°C for 1 h and centrifuged for 17,000 ×*g* for 30 min at 4°C to obtain the supernatant. The supernatant was adjusted to pH 4.0 with 1 N HCl and incubated at 25°C for 30 min to precipitate the proteins. The precipitated proteins were then collected by centrifugation at 16,000 ×*g* for 30 min, and the residue was homogenized with a homogenizer (AM-1, Nihonseiki Kaisha LDD, Nissei, Japan) by the addition of distilled water equivalent to twice the weight of the residue. The pH of the mixture was adjusted to 7.0, then the mixture was lyophilized using a freeze dryer (FD-1, Eyela, Tokyo, Japan), and used as the protein isolate for hydrolysate preparation.

Preparation of PSM protein hydrolysate and membrane fractions

The PSM protein hydrolysate was prepared following the method of our previous study to find optimal hydrolysis conditions [Park & Yoon, 2018]. As a result of previous experiments, optimal hydrolysis conditions of the PSM protein isolate were determined to be pH 7.0, hydrolysis temperature of 50°C, hydrolysis time of 4 h, and enzyme concentration of 10 U using Flavourzyme (Novo Nordisk Co., Bagsvaerd, Denmark). Thus, the following hydrolysis conditions were used in the present study: PSM protein isolates (5% w/v) were suspended in 25 mL of 0.1 M phosphate buffer (pH 7.0) in a reaction vessel equipped with a stirrer and hydrolyzed with 10 U of Flavourzyme at 50°C for 4 h. After enzymatic hydrolysis, the reaction mixture was boiled at 95°C for 10 min to inactivate the enzyme and then centrifuged at 18,000 ×*g* for 20 min. A part of the supernatant (hydrolysate solution) was passed through ultrafiltration membranes with varying molecular weight cut-offs (3, 5, and 10 kDa) using an Amicon Stirred Ultrafiltration Cell (8050, Millipore, Bedford, MA, USA). The permeate from each molecular weight cut-off membrane was collected as <3, 3–5, 5–10, and >10 kDa peptide fractions. The hydrolysate solution and all permeates were freeze-dried and stored at –42°C for subsequent determination of physical properties.

Amino acid analysis

Amino acid composition of protein isolate, hydrolysate, and peptide fractions was determined using an amino acid analyzer (Biochrom 30, Biochrom, Cambridge, UK). The sample (30 mg) was mixed with 3 mL of 6 N HCl and then hydrolyzed at 110°C for 24 h. The hydrolyzed mixture was filtered with a glass wool to remove the residue. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator (R-124, Buchi, Flawil, Swizerland) at 45°C, and then diluted to 100 mL with a sodium citrate buffer (pH 2.2). The filtrate filtered through a 0.45 μm membrane filter (Millipore, Billeria, MA, USA) was used as a sample for amino acid analysis. The amino acid content was expressed as a percentage of total amino acids in the sample.

Solubility measurements

The solubility of each sample was measured following the method of Nalinanon *et al.* [2011] with some modifications. The sample was mixed with distilled water at 1:100 (w/v) and stirred for 1 h. Each dispersion was adjusted to pH 2, 4, 6, 8, and 10 and subsequently centrifuged at 4,000 × g for 20 min. The protein content of the supernatant was measured by performing a bicinchoninic acid assay [Jang *et al.* 2016] and expressed as the percentage of the total protein content in the sample, using the following equation (1):

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \times 100 \quad (1)$$

Water and oil absorption capacity

Water and oil absorption capacities were measured according to the method of Beuchat [1977]. Each sample (1 g) was mixed with 10 mL of distilled water, vortexed, and incubated at room temperature for 30 min. After centrifugation at 3,000 ×*g* for 15 min, the volume of the supernatant was measured using a 10-mL graduated cylinder. Water absorption capacity (WAC) and oil absorption capacity (OAB) were expressed as g of distilled water or soybean oil adsorbed to 1 g of sample.

Emulsifying properties

The emulsifying activity was determined following the method of Yim & Lee [2000] with modifications. Each sample (1 g) was mixed with 100 mL of distilled water, dispersed by vortexing, and the pH of the dispersion was ad-

justed to 2, 4, 6, 8, and 10. Then, 20 mL of the dispersion was added to 20 mL of soybean oil and dispersed for 5 min to form an emulsion. The emulsion was centrifuged at 1,100 ×*g* for 5 min, and the height of the emulsified layer and the total height of the tube were determined. The emulsifying activity was measured according to the following equation (2):

$$\text{Emulsifying activity (\%)} = \frac{\text{Height of emulsified layer}}{\text{Height of total contents in the tube}} \times 100 \quad (2)$$

For the measurement of emulsion stability, the emulsion was prepared by the same method as that used for emulsifying activity. The emulsion was then heated in a hot water bath at 80°C for 30 min, cooled to 15°C and centrifuged at 1,100 ×*g* for 5 min, and the emulsion layer was measured. Emulsion stability was expressed using equation (3):

$$\text{Emulsion stability (\%)} = \frac{\text{Height of emulsified layer after heating}}{\text{Height of the initial emulsified layer}} \times 100 \quad (3)$$

Foaming properties

Determination of foaming capacity was determined by a modified method of Sathe & Salunkhe [1981]. The sample (1 g) was added to 99 mL of distilled water, and each solution was adjusted to pH 2, 4, 6, 8, and 10, respectively. The mixture was transferred to a graduated cylinder and bubbled at 10,000 rpm for 30 s using a homogenizer (AM-1, Nihonseiki Kaisha LDD, Nissei, Japan), and the corresponding increase in volume (mL) was measured.

Foam stability was measured by comparing the foam volume at constant time to the initial foam volume of samples. In other words the foam was formed in the same method as that of foaming capacity and the initial volume of the bubble was measured. The foam was allowed to stand for 30 and 60 min at room temperature, and its volume was measured again. Foam stability was calculated according to equation (4):

$$\text{Foam stability (\%)} = \frac{B - A}{B} \times 100 \quad (4)$$

where A represents the volume after standing (mL), and B represents volume before whipping (mL).

Statistical analysis

Results were expressed as the mean and standard deviation of triplicate experiments. Statistical analysis was performed using SPSS (Ver. 21, Chicago, IL, USA) statistical program. Statistical significance was considered at $p<0.05$. Significant differences between the mean values of the tests were verified by conducting Duncan's multiple range test.

RESULTS AND DISCUSSION

Amino acid composition

The amino acid composition of protein isolate, hydrolysate, and peptide fractions is presented in Table 1. All samples were characterized by high levels of aspartic acid (7.92±13.26–13.26±1.13%) and glutamic acid (19.61±27.03–28.61±0.24%), *i.e.* negatively charged amino acid, which would suggest that PSM protein hydrolysate possessed acidic characteristic. In addition, all samples contained high levels of lysine and arginine, and, especially, the lysine content of <3 kDa fraction was 14.02±4.52%, which was significantly the highest in the samples. It is important to note that lysine is nutritionally important as cases of growth retardation in children increase as a consequence of deficiency in dietary lysine [Tan *et al.*, 2014]. All samples also contained the high amount of the essential amino acids (EAA), and the EAA content of peptide fractions increased with decreasing molecular weight. For example, EAA contents of protein isolate, hydrolysate, >10 kDa, 5–10 kDa, 3–5 kDa, and <3 kDa peptide fractions were 29.26±0.11, 30.93±1.98, 30.34±0.51, 37.75±0.03, 38.18±0.17, and 44.91±0.76%, respectively. Hence, the results suggested that hydrolysate and peptide fractions could be used as an alternative source for cereal-based foods which are low in lysine. These results indicated also that peptide fractions obtained from PSM could be used as dietary protein supplements within food formulations to design protein products. Protein isolate and hydrolysate showed the higher levels of glutamic acid, arginine, and glycine contents compared to peptide fractions, while peptide fractions except for >10 kDa fraction had higher contents of lysine, leucine, valine, and phenylalanine than protein isolate and hydrolysate. As a result, the peptide fractions except for the >10 kDa fraction showed significantly higher contents of hydrophobic amino acids (25.9±0.91–30.79±3.79%) than the protein isolate (18.73±0.07%) and hydrolysate (22.57±1.06%). Amino acid composition has been reported to influence the structure, hydrophobicity, and functionality of peptides [Kimatu *et al.*, 2017]. Therefore, it could be expected that the physicochemical and functional properties of protein isolate and hydrolysate would be different from these of the peptide fractions.

Solubility

The solubilities of the hydrolysate and peptide fractions obtained from PSM proteins were measured to evaluate the potential use of PSM in various food applications. Results were compared relative to the protein isolates (Table 2). The solubility of protein isolate, hydrolysate, and peptide fractions obtained *via* ultrafiltration ranged from 22.81±0.61% to 39.21±1.27%, 38.84±1.39% to 57.35±1.97%, and 31.79±0.93% to 61.73±1.77%, respectively. The solubilities of the hydrolysate and peptide fractions were relatively higher than those of protein isolates, and fractions with smaller molecular weights showed higher solubilities. In particular, the <3 kDa peptides showed the highest solubilities among peptide fractions under all pH conditions, and the highest solubility (61.73±1.77%) was observed at pH 10.0. These results are consistent with the previous findings by Lee *et al.* [1995], in which proteins with smaller molecular sizes were found to exhibit higher solubility. The peptide permeates prepared *via* ultrafiltration and papain modification treatment of soy protein were almost completely soluble at all pH values, except for pH 5.0 [Wu *et al.*, 1998]. In general, the degradation of proteins into smaller peptides leads to the forma-

TABLE 1. Amino acid composition of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal.

Amino acid	Protein isolate	Hydrolysate	>10 kDa	5–10 kDa	3–5 kDa	<3 kDa
Aspartic acid	10.40±0.08^{b}	13.26±1.13^{a}	7.92±0.22^{d}	10.44±0.35^{b}	9.67±0.20^{c}	10.84±0.21^{b}
Threonine	2.85±0.19^{bc}	2.89±0.12^{b}	2.43±0.05^{d}	3.44±0.30^{a}	3.43±0.04^{a}	2.64±0.14^{c}
Serine	4.73±0.11^{cd}	4.85±0.01^{c}	5.07±0.03^{b}	5.73±0.11^{a}	5.82±0.13^{a}	4.62±0.19^{d}
Glutamic acid	27.03±0.07^{b}	25.59±0.01^{c}	28.61±0.24^{a}	22.33±0.33^{d}	20.5±0.53^{e}	19.61±0.04^{f}
Proline	n.d	n.d	n.d	n.d	n.d	n.d
Glycine	6.92±0.04^{a}	5.73±0.01^{b}	5.97±0.09^{b}	4.41±0.23^{d}	4.98±0.07^{c}	4.06±0.60^{e}
Alanine	3.70±0.07^{c}	4.73±0.07^{b}	3.57±0.19^{cd}	4.59±0.36^{b}	4.84±0.24^{b}	5.59±0.39^{a}
Cystine	n.d	n.d	n.d	n.d	n.d	n.d
Valine	3.62±0.09^{cd}	3.45±0.21^{c}	2.98±0.01^{d}	4.64±0.25^{b}	4.63±0.11^{b}	5.71±0.46^{a}
Tryptophan	n.d	n.d	n.d	n.d	n.d	n.d
Methionine	1.98±0.02^{bc}	1.75±0.15^{c}	3.40±0.11^{a}	2.01±0.11^{bc}	1.00±0.01^{d}	2.30±0.91^{b}
Isoleucine	1.42±0.05^{c}	1.95±0.60^{abc}	1.70±0.47^{bc}	2.04±0.34^{ab}	2.16±0.01^{ab}	2.47±0.71^{a}
Leucine	4.11±0.04^{e}	5.25±0.41^{d}	5.10±0.16^{d}	6.30±0.30^{c}	7.47±0.04^{b}	8.02±0.71^{a}
Tyrosine	2.86±0.05^{a}	2.38±0.28^{a}	2.91±0.13^{a}	2.52±0.04^{a}	3.13±0.11^{a}	2.38±1.33^{a}
Phenylalanine	3.90±0.10^{d}	5.45±0.07^{c}	3.57±0.04^{e}	6.32±0.24^{b}	6.66±0.10^{ab}	6.71±0.61^{a}
Histidine	3.59±0.08^{ab}	4.04±1.29^{a}	3.49±0.00^{ab}	3.15±0.09^{b}	3.63±0.01^{ab}	3.05±0.22^{b}
Lysine	7.80±0.21^{bc}	6.16±2.15^{c}	7.68±0.02^{bc}	9.84±0.36^{b}	9.19±0.04^{b}	14.02±4.52^{a}
Arginine	15.09±0.09^{a}	12.54±0.64^{bc}	15.59±0.02^{a}	12.23±0.27^{c}	12.87±0.35^{b}	7.99±0.65^{d}
HHA	18.73±0.07^{d}	22.57±1.06^{c}	20.32±0.39^{c}	25.90±0.91^{b}	26.77±0.07^{b}	30.79±3.79^{a}
PCAA	26.48±0.38^{b}	22.74±0.23^{a}	26.75±0.07^{b}	25.23±0.01^{b}	25.70±0.39^{b}	25.06±3.64^{b}
NCAA	37.43±0.01^{b}	38.85±1.14^{a}	36.53±0.01^{c}	32.77±0.68^{d}	30.16±0.33^{e}	30.45±0.25^{e}
TEAA	29.26±0.11^{d}	30.93±1.98^{c}	30.34±0.55^{cd}	37.75±0.03^{b}	38.18±0.17^{b}	44.91±0.76^{a}

The amino acid content was expressed as the mean ± SD (n = 3) as a percentage of the total amino acids. Values with different letters in the same row are significantly different at $p<0.05$. HHA, hydrophobic amino acids-Ala, Vla, Ile, Leu, Phe, Try, Pro, Met and Cys; PCAA, positively charged amino acids-Arg, His and Lys; NCAA, negatively charged amino acids- Asp and Glu; TEAA, total essential amino acids. n.d, not detected.

TABLE 2. Solubilities of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal at varying pH conditions.

Fraction	Solubility (%)				
	pH 2.0	pH 4.0	pH 6.0	pH 8.0	pH 10.0
Protein isolate	37.81±0.88^{cA}	22.81±0.61^{dB}	37.86±1.07^{cA}	38.43±0.57^{dA}	39.21±1.27^{eA}
Hydrolysate	54.98±1.54^{aA}	38.24±1.39^{aB}	55.39±1.66^{abA}	56.25±1.66^{bA}	57.35±1.97^{cA}
>10 kDa	50.27±1.58^{bA}	31.79±0.93^{cB}	48.51±1.45^{bA}	49.58±1.48^{cA}	51.92±1.59^{dA}
5–10 kDa	50.63±2.31^{bB}	32.22±0.94^{cC}	50.49±1.59^{bB}	51.41±1.54^{cB}	56.04±1.60^{cA}
3–5 kDa	55.05±1.84^{aA}	36.40±0.95^{bB}	55.52±1.74^{abA}	56.34±1.68^{abA}	58.16±1.66^{bA}
< 3 kDa	55.87±1.72^{aB}	39.82±1.07^{aC}	58.48±1.70^{aAB}	59.66±1.72^{aAB}	61.73±1.77^{aA}

Mean±SD (n=3). Values with different small letters in the same column are significantly different at $p<0.05$. Values with different capital letters in the same row are significantly different at $p<0.05$.

tion of more soluble products [Klompong *et al.*, 2017], since smaller peptides have increased the accessibility of hydrophilic groups, and facilitated the reaction of hydrophilic amino acid to the aqueous medium [Nguyen *et al.*, 2017; Sarabandi *et al.*, 2018]. The high solubility of the peptides obtained *via* ultrafiltration using molecular weight cut-off membranes is presumably caused by the exposure of all hydrophilic groups of small molecules to the aqueous environment [Wu

et al., 1998]. Regarding the solubilities of the samples under various pH conditions, all samples showed the lowest solubilities at pH 4.0, and solubility increased at pH 2.0 and above pH 6.0. The above results are consistent with those reported by Tan *et al.* [2014], who showed that the solubilities of soybean and pinto bean protein isolates were the lowest at the pH range from 4.0 to 5.0. The low solubilities of protein hydrolysates and peptides at pH 4.0 were attributed to the low solubilities of amino acids and proteins at isoelectric point, leading to the formation of precipitates [Singh *et al.*, 2005]. Several factors are known to influence protein solubility, including surface charges, pH, molecular size, ionic strength and type, and temperature of solvent. The pH is considered the most significant factor influencing protein solubility [Kinsella, 1979], and alkali treatment usually improves the solubility of proteins [Pearson, 1994]. Peptides obtained from the PSM protein hydrolysate showed higher solubilities than those of the protein isolates and thus could be used as components of various food products.

Water and oil absorption capacity

Water absorption capacities (WACs) of protein isolate, hydrolysate, and ultrafiltration fractions obtained from PSM are shown in Table 3. The highest WAC of protein isolates was 1.23±0.47 g/g, while the WAC of the hydrolysate was 0.68±0.05 g/g. The WACs of peptide fractions ranged from 0.72±0.07 to 0.76±0.16 g/g, with no significant differences among the peptide fractions. The interactions of protein with water are important in relation to dispersibility or wettability, water absorption and binding, swelling, viscosity, gelation, and surfactant properties [Lee, 2015]. The oil adsorption capacities (OACs) of the hydrolysate (2.45±0.21 g/g) and peptide fractions (2.43±0.25–2.79±0.08 g/g) were higher than those of the protein isolates (1.36±0.15 g/g), and the OAC of the peptide fraction tended to increase with decreasing molecular weight. Manak *et al.* [1980] reported that the OAC of soy protein isolates prepared *via* ultrafiltration was 2.52 mL/g, which is similar to those of the peptide fractions obtained *via* ultrafiltration. Hermansson [1975] reported that the OAC of proteins is influenced by pH and ion concentrations and is dependent on protein composition, amino acid ratios, and the presence of carbohydrates. In addition, oil/water holding capacity relies on surface properties, overall charge density, thickness and hydrophobic/hydrophilic nature of the food particle [Dehnad *et al.*, 2016]. From the above results, the peptide fractions from PSM showed lower WAC and higher OAC than protein isolate, which is considered to be related to the result that the peptide fraction with the smaller molecular weight had the higher content of hydrophobic amino acids. In other words, it is presumed that the hydrophobic peptides exposed by enzymatic hydrolysis are due to increased binding to the oil as compared to the interaction with water [Tan *et al.*, 2014].

Emulsifying properties

Protein emulsifying activity is the ability of the protein to participate in emulsion formation and to stabilize the newly created emulsion. Emulsifying properties are useful functional characteristics which play an important role in the development of new sources of plant protein products for uses as foods [Cabra *et al.*, 2008; Dehnad *et al.*, 2016]. The emulsifying activities of protein isolate, hydrolysate, and peptide fractions obtained from PSM are shown in Table 4. The emulsifying activity of protein isolate ranged from 40.18±0.58% to 52.46±0.28%. The emulsifying activity of the hydrolysates at various pH conditions ranged from 48.58±2.63% to 64.87±0.76%, while those

TABLE 3. Water and oil absorption capacities of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal.

Fraction	Water absorption capacity (g/g)	Oil absorption capacity (g/g)
Protein isolate	1.23±0.47^{a}	1.36±0.15^{c}
Hydrolysate	0.68±0.05^{b}	2.45±0.21^{b}
>10 kDa	0.76±0.16^{b}	2.43±0.25^{b}
5–10 kDa	0.72±0.07^{b}	2.48±0.23^{b}
3–5 kDa	0.76±0.01^{b}	2.57±0.12^{ab}
<3 kDa	0.75±0.20^{b}	2.79±0.08^{a}

Mean±SD (n=3). Values with different small letters in the same column are significantly different at $p<0.05$.

TABLE 4. Emulsifying activities of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal at varying pH conditions.

Fraction	Emulsifying activity (%)				
	pH 2.0	pH 4.0	pH 6.0	pH 8.0	pH 10.0
Protein isolate	52.46±0.28^{dA}	40.18±0.58^{cD}	45.79±0.45^{cC}	49.22±0.38^{cB}	49.65±0.78^{cB}
Hydrolysate	64.87±0.76^{aA}	48.58±2.63^{bC}	53.23±2.77^{abB}	54.93±3.55^{bB}	57.10±0.77^{abB}
>10 kDa	55.48±0.19^{cA}	47.77±2.74^{bB}	49.16±2.28^{bcB}	55.30±0.76^{bA}	55.33±0.27^{bA}
5–10 kDa	61.91±1.45^{bA}	49.23±3.02^{abB}	49.37±0.68^{bcB}	56.28±2.84^{abA}	56.97±5.65^{abA}
3–5 kDa	64.72±0.68^{aA}	50.67±2.74^{abC}	55.84±0.55^{aB}	56.05±0.50^{abB}	57.56±0.29^{abB}
< 3 kDa	65.74±1.12^{aA}	55.26±4.03^{aB}	56.67±5.33^{aB}	60.07±3.10^{aAB}	60.63±2.56^{aAB}

Mean±SD (n=3). Values with different small letters in the same column are significantly different at $p<0.05$. Values with different capital letters in the same row are significantly different at $p<0.05$.

TABLE 5. Emulsion stabilities of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal at varying pH conditions.

Fraction	Emulsion stability (%)				
	pH 2.0	pH 4.0	pH 6.0	pH 8.0	pH 10.0
Protein isolate	44.04 ± 0.82^{bA}	33.18 ± 1.64^{cC}	38.81 ± 2.52^{cB}	40.90 ± 3.21^{cAB}	42.40 ± 2.29^{cAB}
Hydrolysate	54.68 ± 2.48^{aA}	38.81 ± 3.22^{bcD}	46.82 ± 2.12^{bC}	48.64 ± 2.88^{abBC}	52.07 ± 2.48^{abAB}
>10 kDa	47.05 ± 3.01^{bA}	39.90 ± 2.92^{bcB}	45.09 ± 1.19^{bA}	47.99 ± 1.50^{bA}	48.81 ± 2.89^{bA}
5–10 kDa	53.50 ± 2.18^{aA}	41.60 ± 6.40^{abC}	43.51 ± 1.94^{bBC}	47.90 ± 1.45^{bABC}	49.22 ± 1.89^{bAB}
3–5 kDa	57.28 ± 0.83^{aA}	44.25 ± 2.92^{abC}	51.29 ± 1.94^{aB}	51.30 ± 2.35^{aB}	52.48 ± 1.59^{abB}
< 3 kDa	56.61 ± 2.36^{aA}	47.45 ± 3.59^{aC}	50.78 ± 2.26^{aBC}	53.13 ± 3.40^{aAB}	54.92 ± 3.55^{aAB}

Mean±SD (n=3). Values with different small letters in the same column are significantly different at $p<0.05$. Values with different capital letters in the same row are significantly different at $p<0.05$.

TABLE 6. Foaming capacities of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal at varying pH conditions.

Fraction	Foaming capacity (mL)				
	pH 2.0	pH 4.0	pH 6.0	pH 8.0	pH 10.0
Protein isolate	4.70 ± 0.26^{cAB}	3.70 ± 0.26^{cC}	4.20 ± 0.36^{cBC}	4.50 ± 0.35^{dAB}	4.80 ± 0.26^{cA}
Hydrolysate	6.10 ± 0.10^{bA}	5.00 ± 0.24^{bB}	5.73 ± 0.14^{bA}	5.93 ± 0.33^{cA}	6.10 ± 0.10^{bA}
>10 kDa	7.00 ± 1.00^{abAB}	5.23 ± 0.31^{bC}	5.53 ± 0.47^{bC}	5.93 ± 0.40^{cBC}	7.10 ± 0.56^{aA}
5–10 kDa	6.73 ± 0.35^{abA}	5.40 ± 0.20^{bB}	6.77 ± 0.61^{aA}	6.63 ± 0.564^{bA}	6.97 ± 0.25^{aA}
3–5 kDa	7.29 ± 0.30^{abA}	6.20 ± 0.26^{aB}	7.10 ± 0.35^{aA}	7.30 ± 0.17^{aA}	7.46 ± 0.42^{aA}
< 3 kDa	7.10 ± 0.53^{aA}	6.20 ± 0.17^{aB}	7.01 ± 0.32^{aAB}	7.29 ± 0.30^{aA}	7.40 ± 0.38^{aA}

Mean±SD (n=3). Values with different small letters in the same column are significantly different at $p<0.05$. Values with different capital letters in the same row are significantly different at $p<0.05$.

of the ultrafiltered fractions ranged from 47.77±2.74% to 65.74±1.12%. The hydrolysate and peptide fractions obtained *via* ultrafiltration showed higher emulsifying activities than those of the protein isolates. The emulsifying activity tended to increase with decreasing molecular weight of the peptides. All samples showed the highest emulsifying activity at pH 2.0 and the lowest emulsifying activity at pH 4.0. The emulsifying activity tended to increase again at pH values above 6.0. In general, the emulsifying activity of proteins increased with higher protein solubility and was found to be significantly influenced by the pH, temperature, and ionic strength conditions [Crenwelge *et al.*, 1974; Kinsella 1979]. Wu *et al.* [1998] reported that the emulsifying activities of soy protein peptides prepared *via* papain modification and ultrafiltration were significantly higher compared to those of the protein isolates, which is consistent with our current findings. The above results suggested that hydrolysates with higher solubilities and smaller molecular weights promote diffusion and spread at oil-water interfaces [Wu *et al.*, 1998]. In addition, the findings of Lui *et al.* [1989] suggested that ultrafiltration improves the emulsifying ability by increasing the number of peptides with emulsifying power, exposing hydrophobic residues of the proteins, and increasing the balance between hydrophilicity and hydrophobicity. Moreover, the findings of Wu *et al.* [1998] suggested that solubility and molecular size, rather than surface hydrophobicity, are the major factors responsible for the strong emulsifying activity of the small peptides.

The emulsion stability of the protein isolates ranged from 33.18±1.64% to 44.04±0.82%. The emulsion stability of the hydrolysate ranged from 38.81±3.22% to 54.68±2.48% and that of the peptide fractions ranged from 39.90±2.92% to 57.28±0.83% (Table 5). The hydrolysate and peptide fractions showed relatively higher emulsion stabilities than those of the protein isolates. These results are consistent with those reported by Lui *et al.* [1989], in which the fractions fractionated *via* ultrafiltration demonstrated higher emulsion stabilities than soy. Yim & Lee [2000] also reported that <1 kDa peptides obtained *via* fractionation of soybean protein hydrolysates obtained from proteolytic enzymes of Meju showed the highest emulsifying power and emulsion stability. Emulsions with small molecular oil droplet sizes were found to be more stable than those with larger droplets [Chove *et al.*, 2002]. The emulsion stability of each sample at various pH values showed a similar trend with that of emulsifying activity. The emulsion stability was highest at pH 2.0, except for the >10 kDa fraction, and lowest at pH 4.0. Chove *et al.* [2002] reported that soy protein isolates microfiltered using the 0.1-μm membrane improved the emulsion-stabilizing ability of the resulting retentate. In addition, fractions richer in species with isoelectric point values of around 4.6 showed poor emulsifying stability in-

TABLE 7. Foam stabilities of protein isolate, hydrolysate, and peptide fractions obtained from perilla seed meal at varying pH conditions.

Fraction	Foam stability (%)									
	Standing time (30 min)					Standing time (60 min)				
	pH 2.0	pH 4.0	pH 6.0	pH 8.0	pH 10.0	pH 2.0	pH 4.0	pH 6.0	pH 8.0	pH 10.0
Protein isolate	45.5 ± 2.8^{bB}	0.0 ± 0.0	35.7 ± 1.2^{bD}	51.1 ± 2.5^{cA}	47.5 ± 1.8^{dC}	32.0 ± 1.5^{aB}	0.0 ± 0.0	28.3 ± 1.6^{bC}	37.8 ± 2.8^{cA}	34.2 ± 0.7^{nsB}
Hydrolysate	49.2 ± 6.7^{bC}	0.0 ± 0.0	35.1 ± 3.6^{bD}	77.0 ± 2.4^{aA}	59.3 ± 2.9^{bB}	32.8 ± 0.7^{aB}	0.0 ± 0.0	33.3 ± 0.5^{aC}	49.2 ± 1.5^{aA}	33.9 ± 1.5^{B}
>10 kDa	46.7 ± 2.9^{bA}	0.0 ± 0.0	34.5 ± 2.5^{bC}	67.8 ± 5.7^{bcA}	60.6 ± 3.3^{bB}	20.0 ± 2.5^{cC}	0.0 ± 0.0	23.6 ± 2.2^{cC}	41.0 ± 6.5^{bA}	31.0 ± 2.5^{B}
5–10 kDa	30.8 ± 3.5^{cD}	0.0 ± 0.0	36.8 ± 3.3^{bC}	68.2 ± 4.2^{bA}	52.9 ± 4.2^{cB}	26.2 ± 1.8^{bB}	0.0 ± 0.0	17.6 ± 1.3^{dC}	34.8 ± 1.8^{cA}	32.9 ± 2.8^{A}
3–5 kDa	56.3 ± 2.0^{aB}	0.0 ± 0.0	40.8 ± 2.1^{aC}	71.2 ± 2.5^{bA}	69.3 ± 5.3^{aA}	32.4 ± 2.9^{aB}	0.0 ± 0.0	12.7 ± 1.1^{eC}	41.1 ± 2.6^{bA}	32.0 ± 4.3^{B}
< 3 kDa	48.2 ± 1.8^{bC}	3.6 ± 0.4^{E}	42.8 ± 0.8^{aD}	72.1 ± 1.5^{bA}	60.1 ± 2.8^{bB}	30.8 ± 5.3^{aB}	0.0 ± 0.0	28.3 ± 2.9^{bC}	36.4 ± 2.0^{cA}	35.8 ± 4.0^{AB}

Mean±SD (n=3). Values with different small letters in the same column are significantly different at $p<0.05$. Values with different capital letters in the row are significantly different at $p<0.05$.
ns, no significant difference

dices and emulsifying activity indices. In the present study, the lowest emulsifying activity and emulsion stability values of all samples at pH 4.0 were attributed to the isoelectric point of the PSM protein being pH 4.0.

Foaming properties

Foaming properties are physicochemical characteristics of proteins that facilitate the formation and stabilization of foams. The foaming capacity and foam stability of protein isolate, hydrolysate, and peptide fractions at different pH values are shown in Tables 6 and 7, respectively. The foaming capacities of the hydrolysate (5.00±0.24–6.10±0.10 mL) and peptide fractions (5.23±0.31–7.46±0.42 mL) were higher than those of protein isolates (3.70±0.26–4.80±0.26 mL). This result might be due to the fact that the small peptides diffuse more rapidly to the air-water interface and encapsulate air bubbles, thereby developing a foam [Wierenga & Gruppen, 2010]. The highest and lowest forming capacities of all samples were observed at pH 10.0 and pH 4.0, respectively, indicating that the foaming properties were significantly influenced by pH. Adebowale & Lawal [2003] reported that the foaming ability of a mucana bean protein concentrate was lowest at pH 4.0 and highest at pH 10, which was consistent with our result. Our current findings were also similar to these reported by Elderidge *et al.* [1963], in which the foaming capacity of soy protein was observed to be the lowest at the isoelectric point. The foaming capacity of protein improves the sensory properties of food, including the appearance and texture. Proteins with high foaming capabilities are used as foaming agents in the manufacture of food products and affect the texture and volume of bread [Kim & Ahn, 2007]. The ultrafiltration process induces structural changes in the proteins and reduces S-S interactions, which also influence bubble formation [Bang *et al.*, 1996]. In general, the foaming capacity of proteins was improved by increasing the structural flexibility *via* exposure of hydrophobic residues and by increasing the capacity to decrease surface tension [Mutilangi *et al.*, 1996]. In addition, Kinsella & Melachouris [1976] reported that protein hydrolysis exposes the hydrophobic groups of polypeptide and promotes the foam formation. Therefore, the strong foaming capacities of hydrolysate and peptide fractions from PSM protein were attributed to the exposure of hydrophobic residues to the enzymatic hydrolysis.

After standing for 30 min (Table 7), the foam stability was the highest in the 3–5 kDa fraction, and the lowest in protein isolates at various pH. The foam stability of each sample was high ranging from 51.1±2.5% to 77.0±2.4% at pH 8.0, but foam of all samples except for <3 kDa fraction was destroyed at pH 4.0. PSM protein hydrolysate showed the highest foam stability, while >10 kDa fraction showed the lowest foam stability at all pH values except pH 6.0 at standing for 60 min. In addition, the foam stability according to pH was the highest at pH 8.0 with the same tendency as at 30 min, and no foam remained at pH 4.0. These results are similar to those reported by Klompong *et al.* [2017], in which the protein hydrolysates obtained from yellow stripe trevally (*Selaroides leptolepis*) showed the lowest foam stability at pH 4, and foam stability of the hydrolysate was found to be higher at pH 6.0 or higher and eventually decreased again at pH 10.0. The low foam stability was concomitant with the low solubility at pH 4.0. Protein solubility and the pH of the dispersing medium are important factors that determine the foaming properties of proteins, especially foam stability. The reduced foam stability at very acidic or alkaline pH could be attributed to the repulsion of peptides *via* ionic repulsion [Townsend & Nakai, 1983; Klompong *et al.*, 2017].

CONCLUSIONS

The protein hydrolysate and peptide fractions produced from perilla seed meal (PSM) were found to have a very high content of essential amino acids including lysine. In addition, protein hydrolysate and peptide fractions showed superior functionality compared to PSM protein isolates. Especially <3 kDa and 3–5 kDa peptide fractions exhibited high solubility, oil absorption capacity, emulsifying activity, foam capacity, and foam stability. The results from this study indicate that peptides have the potential to be used as value-added food ingredients where functional properties

are sought after (*e.g*. protein beverage formulations, bread, emulsion *etc*.). Furthermore, it is also important to invest their physiological activity to improve utilization as functional materials in food industry.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Original research article
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Functional Properties of Protein Isolate and Acid Soluble Protein-Rich Ingredient Co-Produced from Ethanol-Treated Industrial Rapeseed Meal

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Rapeseed meal is produced in large quantities as a by-product of vegetable oil production. To enhance the utility and profitability of the rapeseed meal, it was treated with ethanol and used for concomitant preparation of two protein-rich ingredients, namely protein isolate (PI) and acid soluble protein (ASP). Their functional properties were evaluated in response to two boundary concentrations of NaCl (0.03 and 0.25 mol/L) in a wide pH range (2 to 10). The PI exhibited the lowest protein solubility at isolectric point (pH 4.5) which increased both at lower and higher pH. In contrast, ASP exhibited high protein solubility (>70%) which was negligibly influenced by pH. The addition of 0.03 mol/L NaCl increased its protein solubility to almost 100% at acidic pH. The water holding capacity of PI was positively influenced by the addition of 0.25 mol/L NaCl. The ASP did not exhibit any capacity to hold water but demonstrated higher ability to absorb oil compared to the PI. Both ingredients exhibited different thermal stability in response to salt addition at pH 7 and 8. PI and ASP exhibited completely different pattern of emulsion stability as influenced by pH. While the stability of PI emulsions was close to 100% and only negligibly affected by pH, the ASP emulsion stability significantly varied in response to pH variation. The concomitant production of PI and ASP resulted in products with distinctive techno-functional properties, which makes them suitable for different applications as additives in the formulation of new food products.

INTRODUCTION

Rapeseed meal is a by-product of vegetable oil production [Kreps *et al*., 2014]. In recent years, a steady increase in the generation of rapeseed meal worldwide is observed due to enhanced interest in rapeseed oil as a stock for biofuel production [Zentková & Cvengrošová, 2013]. Its quantity may reach up to 48% of the rape seeds used [Ivanova, 2012]. The most typical application of the rapeseed meal is as a protein-rich additive for the feed industry [Kreps *et al*., 2014]. However, high fiber and anti-nutrient availability limit its inclusion levels due to adverse physiological effect on animals. This is especially valid for rapeseed meal, which, compared to canola meal, contains higher amounts of phytates, allyl isothiocyanates, glucosinolates, and polyphenols [Das Purkayastha *et al*., 2014]. Alternatively, the rapeseed meal can serve as a raw material for preparation of protein-rich ingredients for the food industry [Ivanova *et al*., 2016; Wanasundara *et al*., 2016]. According to Das Purkayastha *et al*. [2014], antinutrients may remain as a part of the final product and negatively affect its nutritive quality and functional properties.

To reduce the antinutrient level in the rapeseed meal and increase the applicability of this by-product, various pre-treatment approaches have been studied [Ghodsvali *et al*., 2005; Gu *et al*., 2011; Das Purkayastha *et al*., 2013]. Among them, treatment with an aqueous ethanol solution is one of the most widely used due to its high reduction efficiency on phenols and glucosinolate and possible use in the food industry as a safe reagent [Chabanon *et al*., 2007]. However, this specific treatment may alter the nutritive quality or functional properties of the protein-rich derived products and, as a consequence, impact their application in the food industry.

The most common approach for preparation of protein isolates includes alkaline extraction of proteins with NaOH followed by isoelectric precipitation. Rapeseed meal protein isolates are characterized with relatively balanced amino acid composition and attractive techno-functional properties because of which they are considered alternatives to plant protein ingredients currently utilized in the food industry [Wanasundara *et al*., 2016]. However, regardless of the advantages of the rapeseed meal protein isolates as protein-rich ingredients, their commercial production is highly limited due to low protein yield recovery (approximately 20%) [Lqari *et al*., 2002; Chabanon *et al*., 2007] and as a consequence, low profitability [Li & Guo, 2017]. Therefore, a concordant production of multiple value-added products would result in enhanced efficiency of the rapeseed meal use, diversified application of rapeseed meal-derived products and overall better economical outcome [Li & Gui, 2017]. The aim of the current

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research was to evaluate functionality of two protein-rich products, a protein isolate (PI) and an acid soluble protein--containing ingredient (ASP), concomitantly produced from ethanol-treated industrial rapeseed meal. Due to the large influence of salts and pH on the functional properties of proteins, the impact of NaCl at two boundary concentrations (0.03 and 0.25 mol/L) in a wide pH range (2.0–10.0) was evaluated as well.

MATERIAL AND METHODS

Co-production of protein isolate (PI) and an acid soluble protein-rich ingredient (ASP)

Industrially manufactured rapeseed meal was provided by a local company. It was produced after thermal treatment of rape seeds at 110–115°C followed by extraction with hexane at 60–65°C for approximately 1 h. Proximate composition of the meal was previously analyzed in our laboratory by Ivanova *et al.* [2016]. The meal was ground and sieved to obtain samples with uniform size particles (≤0.315 mm). To enhance the practical application of the study and achieve more efficient utilization of the rapeseed meal as a by-product, it was not subjected to further modifications, except for treating with ethanol, which aimed at reducing phenol and glucosinolate levels. The procedure included a 4-step treatment with a 75% aqueous ethanol solution (v/v) at a meal to solvent ratio of 25% (w/v) for 30 min at room temperature as described by Chabanon *et al*. [2007]. The residue was collected by decanting, dried in air and stored in a closed container for further use as a source for preparation of the protein-rich ingredients. The PI was produced by extraction of 5% (w/v) meal dispersion with 1 mol/L NaOH (pH 12.0) at 40°C for 60 min under continuous agitation. Extracted proteins were precipitated with 1 mol/L HCl at pH 4.5 which corresponded to the lowest protein solubility. The resulting sediment (PI) was collected by centrifugation for 15 min at 1800×*g* (MPW-251, Med. Instruments, Poland) and dried by lyophilization (Lyovac GT2, Leybold-Heraeus, Germany). The supernatant, containing acid soluble protein, was collected and lyophilized (Lyovac GT2, Leybold-Heraeus, Germany) as well to obtain ASP. The crude protein content of both products was established by Kjeldahl method [AOAC, 1990] with a conversion coefficient of 6.25.

Protein solubility

Protein solubility of PI and ASP in water was determined as described by González-Pérez *et al*. [2007] with some modifications. Samples were dispersed in water to a final protein concentration of 4 mg/mL. The pH was varied from 2 to 8.5 with an increment of 0.5 by using either concentrated or diluted NaOH and HCl solutions as appropriate. NaCl was added to a final concentration of 0.03 or 0.25 mol/L when needed. After 2 h at room temperature, samples were centrifuged for 15 min at 1800×*g* (MPW-251, Med. Instruments, Poland) and supernatants were collected. Protein solubility was calculated as a ratio of the amount of the protein in a supernatant as determined by the biuret method [AACC, 1983] and the initial concentration of the protein used in the test system. The result was multiplied by 100 to express in percentage. Bovine serum albumin was used as a standard protein.

Determination of water- and oil absorption capacity

Water absorption capacity (WAC) was determined as described by Rodriguez-Ambriz *et al*. [2005]. Each protein sample (100 mg) was mixed with 1 mL distilled water. The resulting suspension was centrifuged for 20 min at 1800×*g* (23°C) (MPW-251, Med. Instruments, Poland) and the supernatant was decanted for 10 min at a 45° angle. WAC was calculated by dividing the weight of the absorbed water (g) by the weight of the protein sample (g).

Oil absorption capacity (OAC) was determined by the method of Lin & Zayas [1987]. Each protein sample (100 mg) was mixed with 1 mL sunflower oil and vortexed for 30 s. The emulsion was incubated at room temperature (23°C) for 30 min and subsequently centrifuged for 10 min at 13,600×*g* (MPW-251, Med. Instruments, Poland). The supernatant was decanted and drained for 20 min at a 45° angle. OAC was calculated by dividing the weight of the absorbed oil (g) by the weight of the protein sample (g).

Thermal stability

Thermal stability was determined as described by Tang *et al*. [2012]. Aliquots of 5 mL of protein solutions (2 mg protein/mL) were adjusted to either pH 7 or 8 and were heated for 20 min at temperatures varying from 50 to 90°C with increments of 10°C. After cooling to room temperature (23°C), the turbidity of the solutions was measured at λ=500 nm (Spekol 11, Carl Zeiss Jena, Jena, Germany). Distilled water was used as a control. Thermal stability was evaluated by changes in samples turbidity relative to turbidity of the sample at room temperature (23°C) and multiplied by 100 to express in percentage. Negative values indicate decrease in turbidity.

Emulsifying properties

Emulsifying activity and emulsion stability were determined as described by Neto *et al*. [2001]. Five milliliter protein solution (5 mg protein/mL) was homogenized with 5 mL sunflower oil. The emulsion was centrifuged at 1800×*g* for 5 min and the height of the emulsified layer was recorded. The emulsifying activity was calculated as a ratio of the height of the emulsified layer and the height of the total content of the tube and multiplied by 100 to express in percentage. Emulsion stability was established by heating protein emulsion at 80°C for 30 min followed by centrifugation at 1800×*g* for 5 min. Emulsion stability was calculated by the height of emulsified layer after heating divided by the height of the emulsified layer before heating and multiplied by 100 to express in percentage. NaCl was added to a test system to reach a final concentration of either 0.03 or 0.25 mol/L whenever appropriate. The influence of pH on emulsifying properties was tested by varying pH from 2 to 10 with an increment of 2.0 by using either concentrated or diluted NaOH and HCl solutions as appropriate.

Statistical analysis

Results are presented as means of at least three independent determinations ± standard deviation (SD). Statistical evaluation was performed by using one-way analysis of variance (ANOVA) of the IBM SPSS Statistics program (Somers, NY, USA). Mean differences were established by Fisher's

least significant difference test for paired comparison with a significance level of $\alpha=0.05$.

RESULTS AND DISCUSSION

Protein solubility

Solubility of protein is a key determinant of the other functional properties such as gelling, foaming and emulsification and, as a consequence, their application as food additives [Vioque *et al*., 2000]. Protein solubility is influenced by numerous factors including amino acid composition, protein conformation, interaction with other food compounds, and pH and NaCl, which are the parameters of the highest practical impact [Kinsella *et al*., 1985]. The pH was studied in a wide range of values (from 2 to 8.5), while the two supplementation levels of NaCl were chosen as the most typical boundary concentrations used in food processing [Antova *et al*., 2008; Dragoev *et al*., 2009].

PI and ASP exhibited different protein solubility behavior in response to NaCl supplementation and pH variation (Figures 1A and B). The PI expressed negligible protein solubility (2.80%) at isolectric point (pH 4.5). Improvement of the protein solubility was observed below or above the pI value, but more prominently in alkaline media (Figure 1A). This is a typical trend for solubility of protein isolates obtained from oilseed crops [Xu & Diosady, 1994; Vioque *et al*., 2000; Ogunwolu *et al*., 2009; Ivanova *et al*., 2013]. At pH in the range of 2.5–6, the protein solubility of PI did not exceed 30%. This feature limits the application of the PI as an additive in food processing, since numerous food products have a typical pH from 4.5 to 6.0 [FDA, 2018]. Our results agree with findings reported by Alashi *et al*. [2013] who implied low utilization of canola/rapeseed protein isolates in the food industry due to their poor solubility in neutral and low acidic media. The addition of the lower concentration NaCl (0.03 mol/L) did not change the protein solubility pattern. However, supplementation of the reaction mixture with 0.25 mol/L NaCl resulted in more than a 10-fold increase of protein solubility at the isoelectric point (pH 4.5). The protein solubility in the low acidic area was augmented and reached 51% at pH 6.

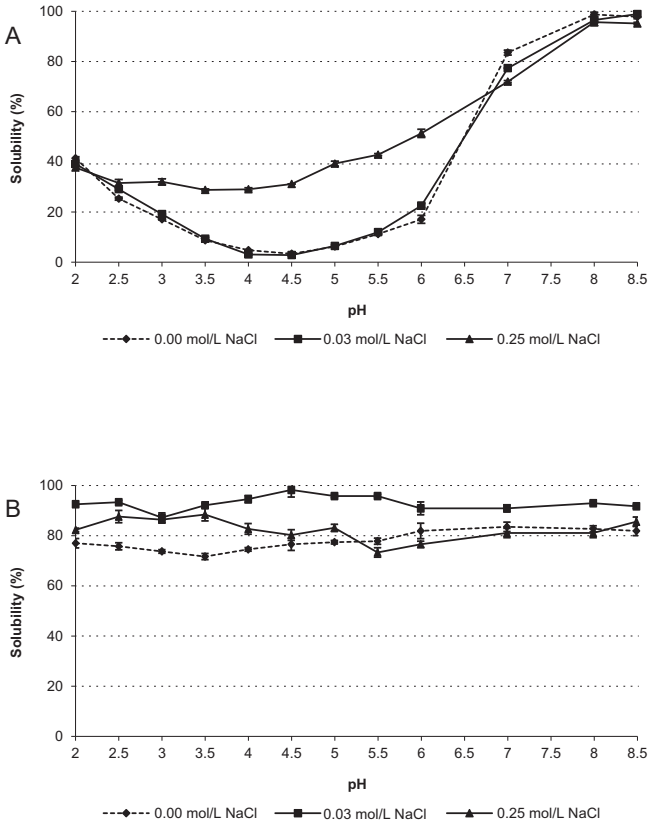

FIGURE 1. Solubility of protein isolate – PI (A) and acid soluble protein ingredient – ASP (B) at different pH values and NaCl concentrations.

In contrast, ASP exhibited protein solubility higher than 70% over the entire pH range studied (Figure 1B). It was slightly influenced by pH in the acidic range and insensitive to alkaline media. A similar influence of pH on solubility of Chinese rapeseed protein isolate, obtained by membrane processing, was observed by Xu & Diosady [1994]. A greater solubility of ultrafiltered protein isolate compared to a precipitated protein isolate was reported by Yoshie-Stark *et al*. [2008] as well. The better protein solubility of the ASP compared to PI is partially due to its protein profile. A previously performed SDS-PAGE study by Ivanova *et al*. [2017] revealed that ASP was composed of proteins with low molecular weights which did not exceed 33 kDa. The PI contained proteins with higher molecular weights which accounted for the higher susceptibility to agglomeration and coagulation near of pI. Our observations agreed with findings reported by Aider & Barbana [2011] who underlined the importance of low molecular proteins for acquiring good solubility. Improved solubility of rapeseed meal protein isolate was achieved by Vioque *et al*. [2000] after partial hydrolysis. Except at pH 3, the protein solubility of ASP increased to more than 90% for all pH values after supplementation with 0.03 mol/L NaCl. The higher ash content of ASP (20.5%) compared to PI (2.3%) [Ivanova *et al*., 2017] is most probably responsible for the higher impact of the low concentration NaCl (0.03 mol/L) (Figure 1B). The addition of 0.25 mol/L NaCl slightly enhanced the protein solubility at acidic pH but not in alkaline media. The higher impact of NaCl addition on protein solubility of both PI and ASP in acidic than in alkaline media may be due to the greater affinity of Cl^- to positively charged proteins in the acidic range than that of Na^+ to negatively charged proteins in alkaline media [Schnepf, 1992]. The influence of salts on protein solubility is complex because it is not only pH dependent but also affected by salt concentration and protein conformation.

Water and oil absorption capacity

The ability of protein-containing ingredients to absorb and retain water and oil is an important feature which influences texture and mouthfeel of food products [Okezie & Bello, 1988]. The PI and ASP, prepared in our study, exhibited significant differences in both WAC and OAC (Table 1). The WAC of the PI (2.00 g H_2O/g sample) was lower than that of commercial soybean protein isolates, such as Purina Protein 500E and 760 [Zayas, 1997], but higher than the WAC of an industrial sunflower meal protein isolate [Ivanova *et al*., 2014], cashew nut protein concentrate [Ogunwolu *et al*., 2009], and a rapeseed protein isolate [Vioque

TABLE 1. Water and oil absorption capacity of protein isolate and acid soluble protein ingredient at different concentrations of NaCl.

Sample	Water absorption capacity (g H_2O/g sample)			Oil absorption capacity (g oil/g sample)		
	NaCl concentration (mol/L)			NaCl concentration (mol/L)		
	0.00	0.03	0.25	0.00	0.03	0.25
Protein isolate	2.00±0.13^{b}	2.16±0.16^{b}	2.70±0.02^{a}	1.29±0.10^{a,B}	1.45±0.22^{a,B}	1.47±0.04^{a,B}
Acid soluble protein	–	–	–	2.77±0.12^{a,A}	2.79±0.00^{a,A}	3.03±0.09^{a,A}

$^{a-b}$Means in a row for a particular functional property with common lowercase superscripts do not differ significantly (p≥0.05). $^{A-B}$Means in a column with common uppercase superscripts do not differ significantly (p≥0.05).

et al., 2000]. The addition of the higher NaCl concentration (0.25 mol/L) significantly increased the WAC to 2.70 g H_2O/g sample, while the lower NaCl level (0.03 mol/L) did not alter the capacity of the PI to retain water. The ASP did not exhibit any capacity to hold water (Table 1). This may be due to the relatively lower protein content of ASP (28.8%) compared to PI (86.8%). Lower WAC of a sunflower protein isolate with a lower protein content compared to a similar protein isolate but with higher protein level was observed by Ivanova *et al*. [2014]. In contrast, the ASP showed higher OAC than PI (Table 1). This feature may be due to differences in the chemical composition and protein fractional profiles of the two protein-containing products as previously established in our laboratory by Ivanova *et al*. [2017]. The authors reported that the ASP was composed of proteins with low molecular weights up to 33 kDa with a prevailing 8 kDa fraction (approximately 35%). According to Vioque *et al*. [2000], small polypeptide chains provide better exposure of amino acid nonpolar sides to hydrocarbon chains, thus contributing to an increase of OAC. Both levels of NaCl supplementations did not alter OAC of either PI or ASP (Table 1).

Thermal stability

Thermal stability of functional protein ingredients is essential when a heat treatment is a part of the food processing scheme. Aiming at comparison between PI and ASP, the thermal stability was evaluated at pH 7 and 8 where the two ingredients exhibited similar protein solubility. PI and ASP showed completely different behavior in response to temperature increase, which was also highly influenced by pH (Table 2). The PI exhibited better thermal stability at pH 7 than at pH 8, regardless of the higher protein solubility of this ingredient at the higher pH (Figure 1A). In contrast, the ASP expressed higher thermal stability at pH 8 than at pH 7, as evidenced by the decrease of turbidity. Since the protein solubility of the ASP at both pH values is not significantly different,

TABLE 2. Thermal stability of protein isolate and acid soluble protein ingredient at pH 7 and 8.

Temperature (°C)	Turbidity alteration (%)					
	Protein isolate			Acid soluble protein		
	NaCl concentration (mol/L)			NaCl concentration (mol/L)		
	0.00	0.03	0.25	0.00	0.03	0.25
pH 7						
50	-0.15±0.1^{bB}	-3.58±0.28^{aC}	15.19±0.11^{eA}	0.57±0.1^{eA}	-3.46±0.52^{eB}	-7.19±0.43^{eC}
60	1.19±0.3^{aB}	-5.69±0.18^{cC}	9.91±0.16^{bA}	6.86±0.1^{dA}	-0.34±0.14^{dB}	-5.42±0.43^{dC}
70	-1.09±0.4^{cB}	-4.50±0.16^{bC}	41.67±0.48^{bA}	15.5±1.2^{cA}	5.99±0.15^{cC}	11.86±0.58^{cB}
80	-1.23±0.5^{cB}	-6.79±0.33^{dC}	12.51±0.21^{dA}	36.38±0.65^{bA}	19.83±0.53^{bC}	22.22±0.58^{bB}
90	1.43±0.4^{aB}	-4.92±0.26^{bC}	42.71±0.27^{aA}	55.56±0.4^{aA}	35.44±0.25^{aC}	26.98±0.16^{aB}
pH 8						
50	0.62±0.3^{cB}	-4.99±0.36^{cC}	20.62±0.36^{eA}	-13.27±0.5^{dA}	-18.82±0.24^{dB}	-19.34±0.15^{eC}
60	0.41 ±0.2^{cB}	-6.83±0.31^{dC}	33.96±0.69^{dA}	-13.61±0.4^{dA}	-19.06±0.71^{dC}	-17.76±0.55^{dB}
70	0.72±0.2^{cB}	-3.97±0.36^{bC}	66.44±0.55^{cA}	-8.98±0.4^{cA}	-12.16±0.49^{cC}	-11.46±0.80^{cB}
80	1.34±0.5^{bB}	-3.85±0.18^{bC}	114.07±0.43^{bA}	-3.81±0.1^{bA}	-6.82±0.23^{bC}	-5.16±0.30^{bB}
90	5.73±0.2^{aB}	-1.93±0.18^{aC}	167.35±0.75^{aA}	4.56±0.6^{aBA}	-2.04±0.36^{aC}	-0.70±0.15^{aB}

$^{a-e}$ Means in a column with same lowercase letter do not differ significantly (p≥0.05). $^{A-B}$ Means in a row for a specific ingredient with same capital letter do not differ significantly (p≥0.05). Negative values indicate decrease in turbidity.

TABLE 3. Emulsifying activity of protein isolate and acid soluble protein ingredient at different pH and NaCl concentrations.

Sample	NaCl concentration (mol/L)	Emulsifying activity (%)				
		pH				
		2	4	6	8	10
Protein isolate	0.00	$59.00 \pm 0.25^{c,A}$	$57.46 \pm 0.19^{d,B}$	$62.20 \pm 0.00^{a,A}$	$58.99 \pm 0.23^{c,A}$	$61.46 \pm 0.39^{b,B}$
	0.03	$57.83 \pm 0.71^{d,A}$	$59.28 \pm 1.34^{cd,B}$	$62.50 \pm 0.00^{a,A}$	$61.41 \pm 0.32^{ab,A}$	$60.48 \pm 0.33^{bc,C}$
	0.25	$60.97 \pm 1.74^{a,A}$	$63.29 \pm 0.00^{a,A}$	$63.81 \pm 1.85^{a,A}$	$62.11 \pm 1.68^{a,A}$	$63.04 \pm 0.13^{a,A}$
Acid soluble protein	0.00	$59.88 \pm 0.87^{b,A}$	$58.13 \pm 0.28^{c,B}$	$61.22 \pm 0.05^{a,A}$	$61.46 \pm 0.39^{a,A}$	$62.05 \pm 0.21^{a,A}$
	0.03	$59.75 \pm 0.36^{b,A}$	$60.12 \pm 0.17^{b,A}$	$60.06 \pm 0.76^{b,AB}$	$59.52 \pm 0.68^{b,B}$	$61.46 \pm 0.39^{a,A}$
	0.25	$59.52 \pm 0.68^{a,A}$	$59.35 \pm 0.75^{a,AB}$	$59.08 \pm 0.62^{a,B}$	$59.39 \pm 0.18^{a,AB}$	$60.59 \pm 0.83^{a,A}$

$^{a-d}$ Means in a row with same lowercase superscripts do not differ significantly ($p \geq 0.05$). $^{A-B}$ Means in a column for a specific sample with same letter do not differ significantly ($p \geq 0.05$).

it cannot account for the different pattern of thermal stability observed. Overall, PI had better thermal stability than ASP at pH 7 but worse at pH 8, regardless of the similar protein solubility (80%) at the higher pH (Figures 1A and B). According to Jiang *et al*. [2010], proteins with higher solubility are supposed to have a better thermal stability. However, our data imply that more factors might be involved in this process. The previously established differences in the chemical composition and protein fractional profile of PI and ASP [Ivanova *et al*., 2017] might affect the number and position of hydrogen bonds, protein density packing and the number and fractional volume of protein cavities formed, which are significant determinants of protein thermal stability [Vogt & Argos, 1997]. Internal van der Waals' packing and interplay of the hydrophobic and electrostatic interactions may also be important for stabilizing proteins against heat [Kaushik & Bhat, 1999]. In addition, variations in long-range protein contacts, influencing the formation of protein-stabilizing centers and the content of stabilization center elements under the studied conditions, may occur [Magyar *et al*., 2016]. According to the same authors, an increase in the latter and, therefore, an increase of thermal stability of thermolabile proteins may occur up to 80°C. This might explain the sharp decrease in thermal stability of both protein-containing ingredients at 90°C under all studied conditions (Table 2).

The addition of NaCl significantly influenced the thermal stability of both ingredients at the two pH values studied, but to a different extent. A decrease in turbidity and, therefore, an increase in thermal stability of PI and ASP, was observed at the lower concentration of NaCl (0.03 mol/L). Fontanari *et al*. [2012] implied that the stabilizing effect of low salt concentrations was due to improved hydration of protein molecules as a result of electrostatic induction and/or structural alteration of water molecules surrounding the protein. In contrast, supplementation with 0.25 mol/L NaCl reduced the thermal stability which was more pronounced for the PI. At a higher ionic strength, the reduced protein stability is rather attributed to nonspecific osmotic effects [Pegram *et al*., 2010] and protein conformational changes [Beauchamp & Khajehpour, 2012]. Different response of PI and ASP to salt addition is most probably due to differences in their protein composition and profile, which in turn leads to variations in electrical charge on the protein surface and interaction with water molecules. Arntfield *et al*. [1986] also observed alteration of the heat resistance of faba bean legumin and vicilin in response to different NaCl load.

Emulsifying properties

Emulsifying activities of PI and ASP are presented in Table 3. For both protein-containing ingredients, the emulsifying activity varied in a narrow range, from 57 to 63%, under all studied conditions. The lowest activity of PI was observed at pH 4, which is in the pH range with the lowest protein solubility for this ingredient (Figure 1A). According to Kinsella [1985], poor emulsifying properties of plant proteins at a pH close to their isoelectric point are due to weak electrostatic repulsive forces. Similar observations were reported by Ivanova *et al*. [2014] and Mao & Hua [2012] who studied the emulsifying properties of protein isolates obtained from sunflower meal and walnut, respectively. The ASP also exhibited the lowest emulsifying activity at pH 4. In contrast to PI, this ingredient was highly soluble over the entire pH range studied (Figure 1B) and, therefore weak electrostatic repulsive forces cannot be a probable explanation. Due to relatively high impurity of the ASP [Ivanova *et al*., 2017], other interactions in addition to the protein-protein ones may also influence the overall emulsifying capacity. The addition of 0.25 mol/L NaCl neutralized the influence of pH on the emulsifying activity of both PI and ASP (Table 3).

PI and ASP exhibited a completely different pattern of emulsion stability as influenced by pH. While the stability of PI emulsions was close to 100% and only negligibly affected by all pH conditions studied (Figure 2), the ASP emulsion stability highly varied depending on pH (Figure 3). The highest stability was observed at pH 4, 8, and 10 (Figure 3). Except at pH 10, the emulsion stability of ASP was lower than that of PI (Figures 2 and 3). Our results differ from these obtained by Xu & Diosady [1994] who reported higher emulsion stability of a soluble protein isolate (111.1%) than that of a protein isolate (98.4%) obtained from Chinese rapeseed meal. This may be due to differences in the methodologies used for their preparation, and, as a consequence, in their chemical composition. The influence of NaCl on

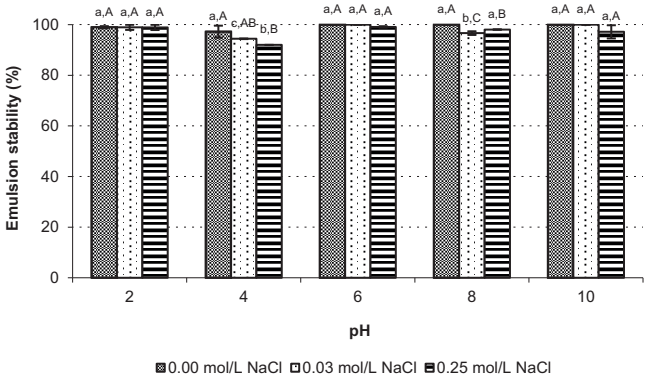

FIGURE 2. Emulsion stability of protein isolate (PI).

[a-c] Means with same lowercase letter for a particular NaCl concentration do not differ significantly (p≥0.05). [A-C] Means with same capital letter for a particular pH do not differ significantly (p≥0.05).

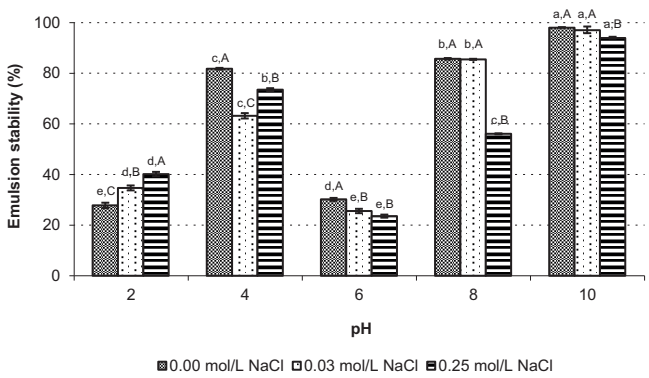

FIGURE 3. Emulsion stability of acid soluble protein ingredient (ASP).

[a-e] Means with same lowercase letter for a particular NaCl concentration do not differ significantly (p≥0.05). [A-C]Means with same capital letter for a particular pH do not differ significantly (p≥0.05).

the emulsion stability of ASP also differed from that of PI (Figures 2 and 3). The addition of 0.25 mol/L NaCl reduced the emulsion stability of ASP at all pH values except at pH 2 (Figure 3). A similar trend was observed by Cheung *et al*. [2015] who observed destabilization of napin protein isolate emulsions after NaCl addition.

The chosen methodology for the evaluation of emulsifying activity and stability of the PI and ASP is a quick approach which allows discrimination between emulsifying capacities of the two protein-containing products. Once the superiority of emulsifying properties of the PI to the ASP is established, additional experiments and analyses, including but not limited to hydrophobicity evaluation, particle size measurement, and determination of emulsion stability from turbidity measurements, are needed to provide a better insight and a more profound explanation of the phenomenon.

CONCLUSION

The study demonstrated the opportunity to concomitantly prepare two protein-containing ingredients from ethanol-treated industrial rapeseed meal with valuable functionality. While PI protein solubility was pH dependent, the one of the ASP was slightly sensitive to pH and remained higher than 70% for the entire pH range studied. Compared to the PI, the ASP demonstrated higher ability to absorb oil and higher thermal stability at pH 8 than at pH 7. The emulsifying activity of PI and ASP varied from 57.46% to 63.81% for all combinations of pH and NaCl. The emulsion stability of the ASP was lower than that of the PI. Distinctive techno-functional properties of the two ingredients define their wide and versatile application in the food industry as additives. The simplicity of the procedure and the lack of purification steps lead to a better and more complete use of the industrially produced rapeseed meal, with the potential for a higher profitability.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Original research article
Section: Food Quality and Functionality

Composition of Anthocyanins in Colored Grains and the Relationship of Their Non-Acylated and Acylated Derivatives

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Key words: acylated and non-acylated anthocyanins, blue and deep purple maize, black soybean, purple wheat

Colored grains are rich sources of anthocyanins that could play an important role in the prevention of various diseases associated with oxidative stress. Bearing in mind that cereals are widely grown crops, anthocyanins-rich colored grains could be used as a functional food ingredient that provides health benefits to a large part of human world's population. This study investigated composition and content of anthocyanins in the grain of blue popping maize, deep purple maize, purple wheat, and black soybean. The relationship of acylated and non-acylated forms before and after alkaline hydrolysis of anthocyanin extracts has been studied as well. Deep purple maize had the highest content of total anthocyanins reaching as much as 4988.90 mg CGE/kg d.m. Ten anthocyanins were identified in blue popping maize, of which two are isomers of cyanidin-3-(malonylglucoside) and three of cyanidin-3-(dimalonyl-β-glucoside). Seven, nine, and three anthocyanins have been identified in the deep purple maize, purple wheat, and black soybean, respectively. Cyanidin derivatives were predominant and their acylated forms accounted for about 98, 29, 71, and 0% of the total anthocyanins content in the grains, respectively. According to the study, acylated derivatives were completely degraded under the effect of highly alkaline pH. However, at the beginning of their degradation they were transformed to their non-acylated parents.

The results could be useful to better understanding of the nature of anthocyanin in colored grains and, in that regard, their use for the derivation of food products with functional potential, as well as of natural dyes and pharmaceutical ingredients.

INTRODUCTION

Anthocyanins are natural pigments responsible for red, purple, and blue colors in the plant kingdom. They are common components of the human diet as they are present in many foods, fruits, and vegetables, especially in berries and red wine. Anthocyanins add not only colors to the food, but also potential health benefits to consumers because of their free radical scavenging capacity [Kähkönen & Heinonen, 2003]. Health benefits associated with anthocyanins include enhancement of sight acuteness, treatment of various blood circulation disorders and neurodegenerative damages, anti-cancerous and anti-inflammatory properties, controlling diabetes, and possibly others due to their diverse action on various enzymes and metabolic processes [Giusti & Wrolstad, 2003]. These qualities make anthocyanins attractive alternatives to synthetic dyes. However, anthocyanins incorporation to food is a certain challenge due to their low stability regarding the factors such as light, oxygen, temperature, and pH. The same applies to their fate in the digestive tract and their bioavailability.

According to recent data, almost 1000 anthocyanins have been identified in nature [Trouillas *et al.,* 2016]. It is very rare that natural anthocyanins exist in aglycon form (as anthocyanidins). There were 31 anthocyanidins found in plants of which only six: cyanidin (Cy), delphinidin (Dp), pelargonidin (Pg), peonidin (Pn), malvidin (Mv), and petunidin (Pt), are widespread and usually found in fruits and vegetables [Andersen & Jordheim, 2010]. According to the study of Kong *et al.* [2003], their distribution is: Cy 50%, Dp 12%, Pg 12%, Pn 12%, Mv 7%, and Pt 7%. Mostly, natural anthocyanins are present as various glycosides of Cy, Dp, Pg, Pn, Mv, and Pt differing from each other by the successive addition of one more hydroxyl group at 3', 4', and at 5' that can be methylated. Among the 539 anthocyanins reported to be identified in a review article by Andersen & Jordheim [2010], 97% are glycosidated. In addition to the differences in the aglycone structure and glycosylation, anthocyanins differ in acylation moieties. The sugar residues can be acylated by aliphatic acids or by aromatic acids [Giusti & Wrolstad, 2003]. Generally, they are linked to sugar in its 6- or 3-position but other substitutions are also present counting di-, tri- or poly- acyl substitution [Cortez *et al.,* 2017]. Malonic acid and *p*-coumaric acid are the most frequent aliphatic and aromatic acyl groups, respectively. By 2010, out of the total

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number of the reported anthocyanins with properly identified structures, 65% of them were acylated, whereby acylation with phenolic acids was 1.5 more frequent than with aliphatic acids [Andersen & Jordheim, 2010]. However, there are dissimilar results of such modifications on anthocyanins stability. Giusti & Wrolstad [2003] reported that acylated anthocyanins (mostly di- or poly-acylated) possessed higher color stability to pH changes (from weakly acidic to slightly alkaline). Similar observations have been made by Torskangerpoll & Andersen [2005] at pH 1.1 to 10. Further, Zhao *et al.* [2017] provided an overview of numerous studies, which confirmed that anthocyanin glycosyl acylation typically increased *in vitro* and *in vivo* chemical stability of acylated anthocyanins. However, unlike the above-mentioned reports in the literature, Luna-Vital *et al.* [2017] reported that the malonyl glycoside forms of the anthocyanins were more susceptible to degradation caused by time and pH than the non-acylated forms. In addition, neither the malonoyl derivative in purple corn nor the coumaroyl derivative in grape pomace showed enhanced stability at pH 3 and 4 compared to their non-acylated parents [West & Mauer, 2013]. According to results of Mora-Rochín *et al*. [2016], nixtamalization processing increased the relative percentage of glycosylated anthocyanins at alkaline pH and decreased the acylated anthocyanins. It has been established that differences in hydroxylation, methoxylation, glycosylation, and acylation patterns of anthocyanins had a critical impact on its color and antioxidant capacity, and, in this connection, on their health-beneficial effects expression in human body. Thus, *in vitro* activity of anthocyanidins in preventing the oxidation of human LDL decreased in the order delphinidin = cyanidin > malvidin > peonidin > pelargonidin > petunidin, while glycosylation altered the activity order. Overall, glucosylated forms were less active than free forms in LDL suspensions [Kähkönen & Heinonen, 2003]. The same authors state that the quality of the sugar substituent affected the activity of anthocyanins as well.

The use of numerous potential food plants as commercial sources of anthocyanins is limited by the availability of raw material, as well as by economic considerations. Some colored grains, such as blue/purple maize, blue/purple wheat, blue barley, black rice, as well as black soybean, hold promise as nutraceutical foods. Cereal processing can generate a large amount of anthocyanins-rich by-products at low cost given that anthocyanins are located in the outer layers of the grain, which could be separated. Although poorly represented in the diet, at present, colored grains are used for making blue or pink tortillas [Mora-Rochín *et al.,* 2016], pink cookies [Žilić *et al.,* 2016], multigrain bread [Bartl *et al.,* 2015], and tea. Possibility of wide application of colored grains, especially in the bakery and confectionery industry, reflects the need to better understand their anthocyanins profile, as well as behavior of anthocyanins in different conditions of grain processing. Therefore, the aim of this study was to determine the composition of anthocyanins in the grain of blue popping maize, deep purple maize, purple wheat, and black soybean. In order to determine with certainty the presence of acylated derivatives in the colored grains, alkaline hydrolysis was used to separate aliphatic or/and aromatic acids from anthocyanins. Hence, the aim was to determine the effect of the applied alkaline hydrolysis method for the identification of phenolic compounds on the content of acylated, as well as non-acylated derivatives in anthocyanin extracts.

MATERIALS AND METHODS

Plant materials

The genotype of blue popping maize, deep purple maize, purple wheat, and black soybean used in this study was obtained from the Maize Research Institute Zemun Polje (MRIZP) gene bank (Serbia). The grains of blue aleurone popping maize genotype were collected in the vicinity of Kragujevac (Central part of Serbia). Deep purple standard-grain maize was bought recently at the market from local farmers from the vicinity of Santiago (Chile). The purple color of its grain, almost black, comes from a combination of anthocyanin genes for blue pericarp and blue aleurone. The used wheat cultivar Indigo, with light purple color of aleurone, was released in United Kingdom. Soybean variety Black Tokyo, with black seed coat and yellow cotyledons, originates from Japan. Although black soybean is native to tropical Asia and Southeast Asia where it is widely used as a basic ingredient of food, Black Tokyo variety has been used extensively for the past five years in the breeding process of the Maize Research Institute, Serbia.

Analytical procedures

Chemicals and reagents

All chemicals and solvents were of the HPLC or analytical grade. Potassium persulfate (dipotassium peroxodisulfate), 2,2'-azino-bis/3-ethylbenzothiazoline-6-sulfonic acid (ABTS), delfinidin-3-glucoside (De-3-Glu), cyanidin-3,5--diglucoside (Cy-3,5-diGlu), cyanidin-3-glucoside (Cy-3-Glu), petunidin-3-glucoside (Pt-3-Glu), pelargonidin-3-glucoside (Pg-3-Glu), malvidin-3-glucoside (Mv-3-Glu), and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile were purchased from J.T. Baker (Avantor, The Netherlands). Hydrochloric acid and potassium hydroxide were purchased from Merck (Darmstad, Germany). Ultrapure water was used throughout the experiments (LaboStar PRO TWF, Evaqua Water Technology, Germany).

Analysis of total anthocyanins

Anthocyanins were extracted from 150, 40, 400, and 150 mg of blue popping maize, deep purple maize, purple wheat, and black soybean, respectively, by mixing with 10 mL of methanol acidified with 1 M HCl (85:15, v/v) and shaking for 30 min at ambient temperature. The crude extract was centrifuged at 10,000 rpm for 5 min at 4°C. Absorbance was measured at 535 and 700 nm to detect anthocyanins. A UV/VIS spectrophotometer Agilent 8453 with Peltier Temperature Controller Agilent 89090A was used. Using the molar extinction coefficient of 25965 Abs/M×cm and a molecular weight of 449.2 g/mol the content of anthocyanins was calculated and expressed as mg of cyanidin 3-glucoside equivalent (CGE) per kg of dry matter (d.m.) [Lee *et al*., 2005].

Analysis of total antioxidant capacity

The antioxidant capacity of maize, wheat, and soybean flour were measured according to the QUENCHER method described by Serpen *et al.* [2008], using 7 mM aqueous solution of ABTS (2,2-azino-bis/3-ethil-benothiazoline-6-sulphonic acid) with 2.45 mM $K_2O_8S_2$ as the stock solution. The working solution of $ABTS^{\bullet+}$ was obtained by diluting the stock solution in water/ethanol (50:50, v/v). Depending on the sample, 6 to 9 mg of the flour were mixed with 20 mL of $ABTS^{\bullet+}$ working solution, and the mixture was rigorously shaken for 25 min. After centrifugation at 9200×g for 5 min at 4°C the absorbance was measured at 734 nm. A UV/VIS spectrophotometer Agilent 8453 with Peltier Temperature Controller Agilent 89090A was used. The total antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) in mmol of Trolox per kg of d.m.

Analysis of individual anthocyanins by HPLC

Individual anthocyanins were determined by HPLC analysis from the prepared extracts after their filtration through a nylon syringe filter of 0.45 μm. Pure anthocyanin compounds such as De-3-Glu, Cy-3,5-diGlu, Cy-3-Glu, Pt-3-Glu, Pg-3-Glu, and Mv-3-Glu were used as references for concentration, retention time, and UV characteristic. Identified non-acylated anthocyanin peaks were confirmed and quantified using the Thermo Scientific Dionex Chromeleon 7.2. chromatographic software and the results were expressed as μg per g of d.m. Stock standard solutions were prepared in methanol acidified with 1 M HCl (85:15, v/v) at a concentration of 1.0 mg/mL. The working solutions were prepared by diluting the stock solutions with acidified methanol to concentrations of 5.0, 10.0, 20.0, 40.0, 50.0, and 100.0 μg/mL. The HPLC analysis was carried out with the HPLC-DAD system (Thermo Scientific Ultimate 3000). Chromatograms were obtained at 530 nm after injection of 10 μL of the sample. Separation was performed on a Thermo Scientific Hypersil GOLD aQ C18 column (150 mm × 4.6 mm, i.d., 3 μm) using a linear gradient elution program with a mobile phase containing solvent A (formic acid/H_2O, 1:99, v/v), and solvent B (formic acid/acetonitrile, 1:99, v/v) at a flow rate of 0.7 mL/min and the column oven temperature of 30°C. The following gradient was established: linear gradient elution from 10% B to 30% B, 0–30 min; linear gradient elution from 30% B to 100% B, 30–35 min; isocratic elution of 100% B, 35–38 min; linear gradient elution from 100% B to 10% B, 38–40 min; isocratic elution of 10% B, 40–45 min.

In order to confirm the presence of acylated forms of anthocyanin, alkaline hydrolysis of the extracts was carried out according to the method described by Pedreschi & Cisneros-Zevallos [2007] with slight modifications. 150 μL of 10% KOH was necessary for the appearance of a blue color in 1 mL of extract of anthocyanins. All hydrolysates were kept in darkness for 10 min. Then, 6 M HCl was added to the solution until stable red color appeared (a total of about 50 μL). After centrifugation at 10,000 rpm for 3 min at 4°C and filtration through a nylon syringe filter of 0.45 μm, the supernatants were used for the analysis of anthocyanins according to the HPLC method described above. The content of acylated derivatives of cyanidin and pelargonidin was calculated after confirmation of their presence in extracts before and after alkaline hydrolysis by mass spectrometry. After comparing the data obtained with HPLC and HPLC-MS analysis, the acylated derivatives were quantified using HPLC peak area values (mAU*min) and external standard curves for Cy-3-Glu and Pg-3-Glu. The content of acylated derivatives of cyanidin and pelargonidin was calculated as equivalent to their glucoside forms and expressed as μg per g of d.m.

Analysis of individual anthocyanins by HPLC-MS

As indicated above, anthocyanin compounds were determined by comparison of the spectroscopic and chromatographic properties with those of authentic anthocyanin standards (De-3-Glu, Cy-3-Glu, Pt-3-Glu, Pg-3-Glu, and Mal-3-Glu). The remaining compounds were tentatively identified using a combination of the retention time, peak spectra, mass-to-charge ratio and pattern of fragmentation. Samples were injected into a Waters HPLC system consisting of 1525 binary pumps, a thermostat, and a 717+ autosampler connected to the Waters 2996 diode array and an EMD 1000 single quadrupole detector with an ESI probe (Waters, Milford, USA). Separation of anthocyanins was performed on a Symmetry C-18 RP column 125 × 4 mm packed with 5 μm diameter particles (Waters, Milford, MA, USA) and connected to an appropriate guard column. Two mobile phases, A (1% formic acid) and B (acetonitrile), were used at a flow rate of 1 mL/min with the following gradient profile: initial 10% B; in 30 min linear rise to 30% B; from 30–35 min to 50% B; in next 10 min return to 10% B with additional 5 min of equilibration time. A post column flow splitter (ASI, Richmond, CA, USA) with a 5/1 split ratio was used to obtain the optimal mobile phase inflow for the ESI probe. For LC/MS analysis, signals for each compound were detected in the positive ESI single ion recording (SIR) mode with the following parameters: capillary voltage of 2.5 kV, cone voltage of -30 V, extractor and RF lens voltages of 3.0 and 0.2 V, respectively. Source and desolvation temperatures were 130°C and 400°C, respectively, with N_2 gas flow of 500 L/h. Data acquisition and spectral evaluation for peak confirmation were carried out by the Waters Empower 2 Software (Waters, Milford, USA).

Statistical analyses

All analyses were done in duplicate per genotype and the results were statistically analyzed using the Statistica software version 5.0 (StatSoft Co., Tulsa, OK, USA). The analytical data are reported as the mean ± standard deviation. Significance of differences between genotypes means were analyzed by Tukey's (HSD) test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Total anthocyanins content and antioxidant capacity

The content of total anthocyanins in the grain of blue popping maize, deep purple maize, purple wheat, and black soybean, as well as the antioxidant capacity of the grains are shown in Table 1. According to our study, the content of total anthocyanins varied significantly between colored grains.

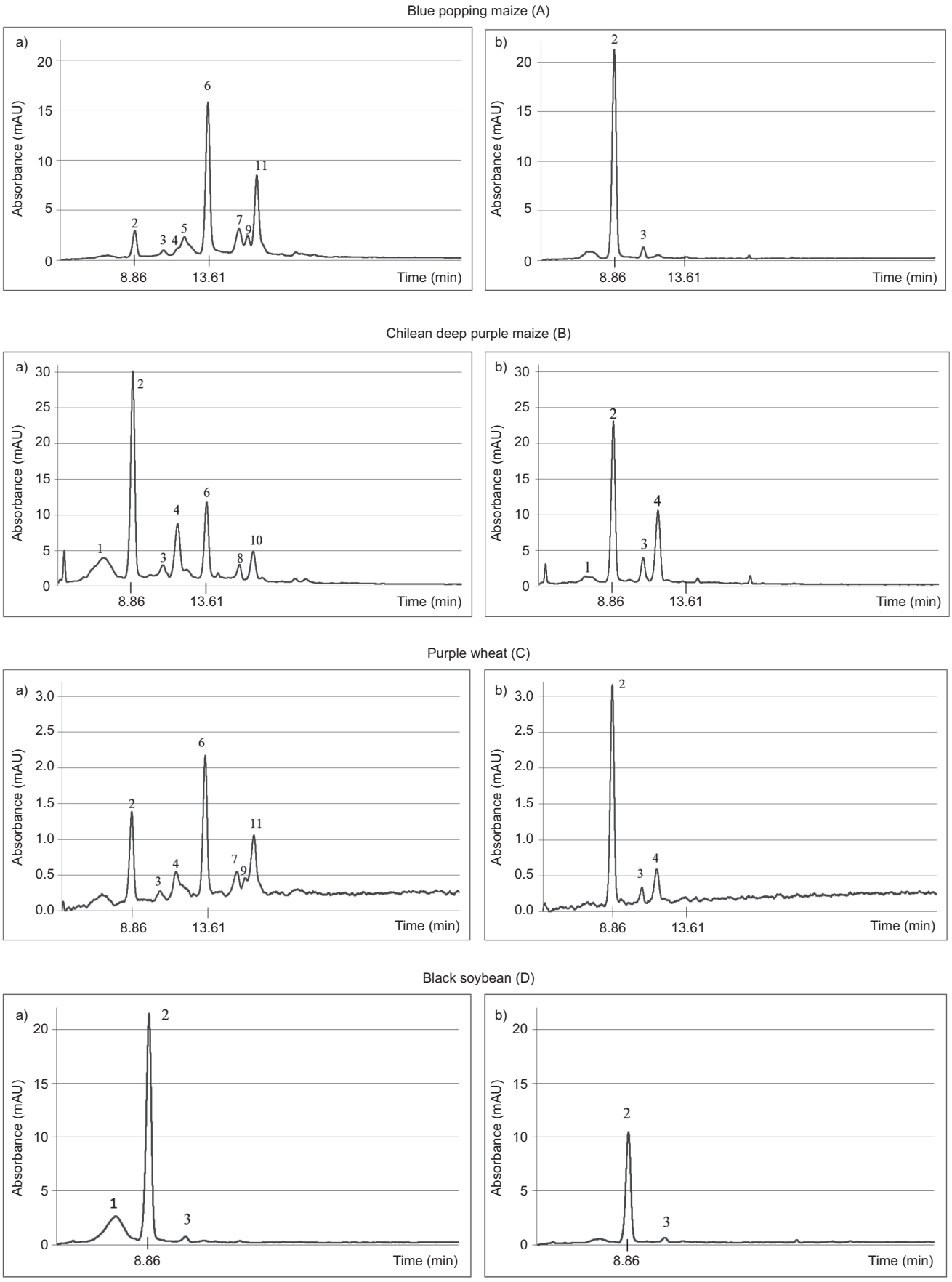

FIGURE 1. Chromatogram at 530 nm corresponding to A – blue popping maize, B – Chilean deep purple maize, C – purple maize, and D – black soybean extracts a) before and b) after alkaline hydrolysis. The peak numbers show the major anthocyanins that are identified in Table 2.

TABLE 1. Content of anthocyanins and antioxidant capacity of colored grains.

Sample	Total anthocyanins (mg CGE/kg d.m.)	Antioxidant capacity of flour (mmol Trolox Eq/kg d.m)
Blue popping maize	907.51±9.8^{b}	37.62±3.39^{d}
Deep purple maize	4988.90±450.1^{a}	230.88±1.83^{a}
Purple wheat	91.72±4.3^{d}	41.13±0.80^{cd}
Black soybean	583.42±26.3^{c}	92.74±1.47^{b}

Means followed by the same letter within the same column are not significantly different, according to Tukey's test (p=95%).

Chilean deep purple maize had the highest content of total anthocyanins reaching as much as 4988.90 mg CGE/kg d.m. It was higher than that in chokeberry (*Aronia melonocarpa* E.) (4341 mg CGE/kg d.m.) [Jakobek *et al.,* 2007] and significantly higher than in other berry fruits [Jakobek *et al.,* 2007]. In comparison with the blue popping maize, purple wheat and black soybean, Chilean maize had 5.5, 54.4, and 8.5 times higher content of total anthocyanins, respectively. However, the antioxidant capacity of Chilean deep purple maize was higher by only 5.5 and 2.5 times than that of purple wheat and black soybean, respectively. Having that in mind, it can be concluded that the anthocyanins contained in the grain--matrix of deep purple maize exhibited a lower antioxidant capacity compared to other bioactive compounds present in grains of wheat and soybean. The same applies to anthocyanins in the grain of blue popping maize. In the grain of this maize, the total anthocyanins content of 907.51 mg CGE/kg d.m. was higher by 10 and 1.5 times, while its antioxidant capacity of 37.62 mmol TEAC/kg d.m. was even lower by 1.1 and 2.5 times compared to the values reported in the grain of wheat and soybean, respectively. Among other things, this can be a consequence of a synergistic, additive or antagonistic interaction that arises from the coexistence of anthocyanins and numerous other antioxidant compounds in the grains. For instance, it has been established that catechin, which is relatively inefficient at inhibiting linoleic acid oxidation, regenerates the highly efficient antioxidant Mal-3--Glu and, at a lower extent, peonidin-3-glucoside (Pn-3-Glu). The Mal-3-Glu recycling by catechin strongly increased the antioxidant efficiency of these two antioxidants [Rossetto *et al.,* 2002]. Such a high antioxidant capacity of black soybean can be a consequence of this catechin/anthocyanin interaction. As previously established [Žilić *et al.,* 2013], the soybean variety Black Tokio, used in this study, had a high content of catechin in seed coat, accounting for over 2000 μg/g d.m. Contrary to the above, the study of Hidalgo *et al.* [2010] has shown that DPPH scavenging activities were reduced in combinations of individual anthocyanins by promoted antagonistic effects.

Composition of anthocyanins, their relative quantitative content, and relationship of acylated and non-acylated forms

It has been established that small differences in the chemical structure of anthocyanins may have an important impact on spectral characteristics and antioxidant properties of the pigments [Kähkönen & Heinonen, 2003]. Therefore, the composition of anthocyanins in grains was analyzed and HPLC chromatograms at 530 nm of crude extract are shown in Figures 1A-1D. The results of HPLC and HPLC--MS analysis for anthocyanins are summarized in Tables 2 and 3. The composition and content of anthocyanins in grains after alkaline hydrolysis are shown as well.

Anthocyanins profile of maize grain (blue popping and deep purple standard-grain maize)

Anthocyanin profiles observed in the grain of blue popping maize and in the grain of deep purple maize were different. Overall, the identified anthocyanins in maize grains were Dp, Cy, Pg, and Pn conjugated with glucose, and their acylated forms, including mono- and di-malonyl derivatives (Table 2, Figure 1Aa and 1Ba).

In the grain of blue popping maize, five major anthocyanins were identified, including cyanidin-3-glucoside (Cy-3--Glu; peak 1, m/z 449), pelargonidin-3-glucoside (Pg-3-Glu; peak 2, m/z 433), peonidin-3-glucoside (Pn-3-Glu; peak 3 m/z 463), cyanidin-3-(malonylglucoside) (Cy-3-Mal--Glu; peaks 4 and 5, m/z 535), and cyanidin-3-(dimalonyl--β-glucoside) (Cy-3-diMal-Glu; peaks 6, 7 and 8, m/z 621). Pelargonidin-3-(6'-malonylglucoside) (Pg-3–6Mal-Glu, m/z 519) and peonidin-3-(6'-malonylglucoside) (Pn-3–6Mal--Glu, m/z 549) were present in traces (Table 2, Figure 1Aa). Some derived compounds had similar spectroscopic properties and the same mass-to-charge ratio, but different retention times (Table 2). This could be explained by the presence of isomers of anthocyanins, hence two isomers of Cy-3-Mal--Glu and three isomers of Cy-3-diMal-Glu were identified. By comparing with published data [Wu & Prior, 2005], isomers of Cy-3-Mal-Glu, peaks 4 and 5, sharing the same MS spectra at different retention times of 12.08 and 13.61 min, were identified as cyanidin-3-(3'-malonylglucoside) (Cy-3--3Mal-Glu) and cyanidin-3-(6'-malonylglucoside) (Cy-3--6Mal-Glu), respectively. In the grain of two landraces of blue Mexican maize, Mora-Rochín *et al.* [2016] reported the presence of three isomers of Cy-3-Mal-Glu, two isomers of cyanidin-3-(succinylglucoside) (Cy-3-Suc-Glu), and two isomers of cyanidin-3-(disuccinylglucoside) (Cy-3-diSuc-Glu). Unlike these studies, the previous research of Žilić *et al.* [2012] showed the presence of two di-glucosides (Cy-3,5-diGlu and Pg-3,5-diGlu) in the total of 10 anthocyanins identified in the grain of blue popping maize. The most abundant anthocyanins in the grain of blue popping maize used in this study were Cy-3–6Mal-Glu (peak 5) and one of the isomers of Cy-3-diMal-Glu (peak 8) accounting for 41.01 and 23.06% of the total peaks area of anthocyanins, respectively. These results are consistent with the previous research of Salinas-Moreno *et al.* [2012] for blue/purple grain of Mexican maize. In our study, the peak area of Cy-3-Glu (peak 4) was 6-fold lower than that of Cy-3–6Mal-Glu. It is evident that acylated forms of anthocyanins were dominant in the grain of blue popping maize. Furthermore, they accounted for 85% of the total peaks area of all detected anthocyanins. According to the research of Urias-Lugo *et al.* [2015], acylation affects the reduction of cancer cell viability in mammary glands,

TABLE 2. Anthocyanins composition in grains of blue popping maize, deep purple maize, purple wheat, and black soybean. Chromatographic characteristics of peaks in Figure 1A-1D.

Peak	t_R (min)	Area (%) (530 nm)	$[M^+]$ (m/z)	MS/MS (m/z)	Identity
Blue popping maize					
Before alkaline hydrolysis					
2	8.87	6.68	449	287	Cyanidin 3-glucoside
3	10.73	1.40	433	271	Pelargonidin 3-glucoside
4	11.70	1.74	463	301	Peonidin 3-glucoside
5	12.08	6.14	535	287	Cyanidin 3-(3'-malonylglucoside)[1]
6	13.61	41.01	535	287	Cyanidin 3-(6'-malonylglucoside)[1]
7	15.63	9.73	621	438/287	Cyanidin 3-(dimalonyl-β-glucoside)[2]
	15.71	trace	519	303	Pelargonidin 3-(6'-malonylglucoside)
9	16.19	5.00	621	438/287	Cyanidin 3-(dimalonyl-β-glucoside)[2]
10	16.60	trace	549	301	Peonidin 3-(6'-malonylglucoside)
11	16.77	23.06	621	438/287	Cyanidin 3-(dimalonyl-β-glucoside)[2]
After alkaline hydrolysis					
2	8.87	84.95	449	287	Cyanidin 3-glucoside
3	10.73	3.72	433	271	Pelargonidin 3-glucoside
Deep purple maize					
Before alkaline hydrolysis					
1	6.99	13.79	465	303	Delphinidin 3-glucoside
2	8.86	35.22	449	287	Cyanidin 3-glucoside
3	10.79	3.90	433	271	Pelargonidin 3-glucoside
4	11.74	12.73	463	301	Peonidin 3-glucoside
6	13.61	14.38	535	287	Cyanidin 3-(6'-malonylglucoside)
8	15.71	3.17	519	303	Pelargonidin 3-(6'-malonylglucoside)
10	16.60	6.09	549	301	Peonidin 3-(6'-malonylglucoside)
After alkaline hydrolysis					
1	6.99	4.95	465	303	Delphinidin 3-glucoside
2	8.86	51.96	449	287	Cyanidin 3-glucoside
3	10.79	8.08	433	271	Pelargonidin 3-glucoside
4	11.74	27.43	463	301	Peonidin 3-glucoside
Purple wheat					
Before alkaline hydrolysis					
2	8.86	18.17	449	287	Cyanidin 3-glucoside
3	10.73	2.58	433	271	Pelargonidin 3-glucoside
4	11.71	8.76	463	301	Peonidin 3-glucoside
6	13.61	31.08	535	287	Cyanidin 3-(6'-malonylglucoside)
Before alkaline hydrolysis					
7	15.64	8.15	621	438/287	Cyanidin 3-(dimalonyl-β-glucoside)[2]
8	15.71	trace	519	303	Pelargonidin 3-(6'-malonylglucoside)
9	16.19	3.58	621	438/287	Cyanidin 3-(dimalonyl-β-glucoside)[2]
10	16.60	trace	549	301	Peonidin 3-(6'-malonylglucoside)
11	16.75	19.08	621	438/287	Cyanidin 3-(dimalonyl-β-glucoside)[2]
After alkaline hydrolysis					
2	8.86	82.03	449	287	Cyanidin 3-glucoside
3	10.79	4.92	433	271	Pelargonidin 3-glucoside
4	11.78	12.99	463	301	Peonidin 3-glucoside
Black soybean					
Before alkaline hydrolysis					
1	6.99	26.71	465	303	Delphinidin 3-glucoside
2	8.86	69.30	449	287	Cyanidin 3-glucoside
3	10.79	1.74	433	271	Pelargonidin 3-glucoside
After alkaline hydrolysis					
2	8.86	87.25	449	287	Cyanidin 3-glucoside
3	10.79	3.10	433	271	Pelargonidin 3-glucoside

[1–2]Compounds with identical m/z ratio within superscript.

liver, colon, and prostate. These authors point out that an appropriate extraction method of maize anthocyanins must be selected to obtain a high yield of Cy-3-Mal-Glu more than only Cy-3-Glu. Taking into account the high level of Cy-3--Mal-Gly in old blue popping maize from Balkan used in this study, and the poor adaptability of colored maize originating from South America to growing conditions in Europe, it can be used in breeding of modern hybrids for temperate areas as a source of genes that play a structural or regulatory role in anthocyanin biosynthesis [Vancetović *et al*., 2014].

In contrast to the blue popping maize, the glucosidic forms of anthocyanins were dominant in the grain of Chilean deep purple maize. Seven major anthocyanins were identified in the grain of this maize, while different isoforms of certain anthocyanins were not detected. Four of them were glucosides: De-3-Glu (peak 1, m/z 465), Cy-3-Glu (peak 2, m/z 449), Pg-3-Glu (peak 3, m/z 433), and Pn-3-Glu (peak 4, m/z 463), while three of them were acylated forms: Cy--3-6Mal-Glu (peak 5, m/z 535), Pg-3–6Mal-Glu (peak 6, m/z 519), and Pn-3–6Mal-Glu (peak 7, m/z 549) (Table 2, Figure 1Ba). Cy-3-Glu (peak 2), in terms of peak area, represented the major type of anthocyanin in the grain of Chilean deep purple maize comprising 35.22% of the total anthocyanins, followed by the acylated Cy-3-Glu (peak 5) that represented 14.38%. Similar composition and mutual relative relation of the present anthocyanins was found by Pedreschi & Cisneros-Zevallos [2007] in the grain of Andean purple maize, in which Cy-3-Glu and the corresponding acylated

form represented 44.37 and 26.87%, respectively. In our study, compared with blue popping maize, the relative peak area of Cy-3-Glu and Cy-3-6Mal-Glu presented in the grain of deep purple maize was about 5-fold higher, *i.e.* 3-fold lower. Taking into account the same comparison, Pn-3-Glu, as well as its corresponding acylated form (peaks 4 and 7) were found in relatively high proportions in the grain of deep purple maize (17.73 and 6.09% of the total peaks area of anthocyanins, respectively).

Anthocyanins profile of wheat grain

Purple wheat used in this study had an almost identical anthocyanin profile as the blue popping maize. Cy-3-Glu (peak 1, m/z 449), Pg-3-Glu (peak 2, m/z 433), Pn-3-Glu (peak 3 m/z 463), Cy-3–6Mal-Glu (peak 4, m/z 535), three isomers of Cy-3-diMal-Glu (peaks 5, 6 and 7, m/z 621), as well as Pg-3–6Mal-Glu and Pn-3–6Mal-Glu in trace were identified (Table 2, Figure 1Ca). In the same purple variety Indigo, Liu *et al.* [2010] detected only Cy-3-Glu. In turn, using newly developed HPLC-MS method, Bartl *et al.* [2015] managed to separate and define 19 anthocyanins in the blue wheat and 26 in the purple wheat. As in the grain of popping maize, acylated forms of anthocyanins were dominant in the grain of purple wheat. However, they accounted for about 62% of the total peak area of anthocyanins that was 23% lower than their proportion in the grain of blue popping maize. The acyl derivative of anthocyanins *viz* Cy-3–6Mal-Glu (peak 4) was the most abundant compound in purple wheat accounting for 31.08% of the total anthocyanins. Other predominant anthocyanins included isomer of Cy-3-diMal-Glu (peak 7), Cy--3-Glu (peak 1), and Pn-3-Glu that represented 19.08, 18.17, and 8.76% of the total peaks area of anthocyanins, respectively. Abdel-Aal *et al.* [2006] identified De-3-Glu and Cy-3--Glu as the major anthocyanins in Canadian blue and purple wheat, respectively. According to the results of these authors, eight of the 10 anthocyanins identified in the grain of purple wheat were acylated by malonic and succinic acid but no acylated anthocyanins were found in the blue wheat.

Anthocyanins profile of soybean

Three anthocyanins were detected in black soybean. They were De-3-Glu, Cy-3-Glu, and Pg-3-Glu (peaks 1, 2 and 3) (Table 2, Figure 1Da). No acylated anthocyanins were found in the soybean that is consistent with the findings reported by Zhang *et al.* [2011]. Cy-3-Glu was the major anthocyanin representing 69.30% of the total peaks area of anthocyanins, followed by De-3-Glu and Pg-3-Glu with 26.71 and 1.74%, respectively. Interesting, in addition to the three major anthocyanins, in the grain of two black soybean varieties Koh *et al.* [2014] identified cyanidin, as well as acylated Pg-3-Glu (Pg--3-6Mal-Glu) in trace amounts.

Quantification of anthocyanins

As shown in Table 3, cyanidin derivatives, in the total amount of 465.2, 1460.3, 80.1, and 254.0 μg/g d.m., were predominant anthocyanins in the grain of blue popping maize, Chilean deep purple maize, purple wheat, and black soybean, respectively. Acylated forms of cyanidin derivatives accounted for 431.99, 423.09, 61.88, and 0 μg/g d.m., *i.e.* for about 98, 29, 71, and 0% of the total content, respectively. According to results of Zhao *et al.* [2008], the total content of cyanidin derivatives in five Chinese purple maize hybrids ranged from 87.1 to 2248.3 μg/g d.m., while the acylated forms constituted between 59.3 to 86.1% of this content. Small amounts of pelargonidin derivatives were detected in the grain of popping maize, wheat, and soybean, while their total content in the grain of Chilean maize was high (330.5 μg/g d.m.). The content of De-3-Glu in this maize was about 4 times higher than that in the grain of soybean, where it was also identified.

If the main anthocyanins are compared with literature data, it can be concluded that the content of Cy-3-Glu and its malonyl derivatives (Cy-3-Mal-Glu) was significantly higher in the grain of blue popping and Chilean deep purple maize than in Mexican blue genotypes where it ranged from 2.6 to 19.8 μg/g and from 3.4 to 26.0 μg/g d.m., respectively [Mora-Rochín *et al.,* 2016], and similar to those in the grain of some Chinese purple maize genotypes (12.1 to 916.0 μg/g and 55.0 to 1100. 3 μg/g d.m., respectively) [Zhao *et al.,* 2008]. The study of Zhao *et al.* [2008] is one of the few in which the presence of Cy-3-diMal-Glu in maize grain was reported at a high concentration of 20.0 to 628.4 μg/g d.m. In our study, the content of the dominant isomer of Cy-3-di-Mal-Glu, identified in blue popping maize, was 113.50 μg/g d.m., while the content of its two isomers occurring at retention times of 15.63 and 16.19 min was 2.4 and 5.4 times lower, respectively. Although Bartl *et al.* [2015] identified a large number of anthocyanins in the grains of blue and purple wheat, they measured the low content of Cy-3--Glu in them, only at about 1.0 to 1.7 μg/g d.m. However, our results are comparable with the values obtained by Abdel-Aal *et al.* [2006] for the content of Cy-3-Glu amounting to 20.3 μg/g d.m. in the grain of blue wheat. In turn, two Canadian genotypes of purple wheat were found to contain Cy-3-Glu at 103.0 μg/g [Hosseinian *et al.,* 2008]. In these genotypes, the authors did not identify the presence of malonyl derivatives of cyanidin which, according to our investigations, were dominant in purple wheat with a total content of 50.1 μg/g d.m. Very low content of Cy-3-Mal-Glu at 1.2 μg/g d.m. was measured by Abdel-Aal *et al.* [2006] in purple Canadian wheat. In the grain of black soybean used in this study, the content of the dominant anthocyanins, Cy--3-Glu and De-3-Glu, can be compared with those in the seed coat of some Korean genotypes. Namely, the content of Cy--3-Glu and De-3-Glu in seed coat of 56 genotypes of black soybean ranged from 122 to 12073 and from 0 to 2164 μg/g d.m., respectively [Lee *et al.,* 2016]. Only one anthocyanin, Cy-3-Glu, was detectable in the whole kernel of black soybean by Xu & Chang [2008a], and its content amounted to 86.3 μg/g d.m. The same authors in one of their studies indicated the content of Cy-3-Glu in whole grain of black soybean at 356.8 μg/g d.m. [Xu & Chang, 2008b]. As Abdel-Aal *et al.* [2008] already observed, the variation in anthocyanin content and composition in colored maize, wheat and soybean may be due to the differences between genotypes. Furthermore, Zhao *et al.* [2008] confirmed that in addition to the variety, growing conditions of the purple maize also influence their anthocyanin content.

TABLE 3. Content of individual anthocyanins from colored grains extracts before and after alkaline hydrolysis.

Compounds*	t_R	Blue popping maize		Deep purple maize		Purple wheat		Black soybean	
		BAH	AAH	BAH	AAH	BAH	AAH	BAH	AAH
De-3-Glu	6.99	n.d.	n.d.	518.41±18.1^{a}	112.65±4.7^{b}	n.d.	n.d.	124.93±6.3^{b}	n.d.
Cy-3-Glu	8.86	33.21±1.1^{g}	299.53±11.9^{e}	1037.24±36.3^{a}	927.98±38.9^{b}	18.17±0.9^{g}	42.67±2.2^{fg}	254.04±13.9^{d}	87.25±2.6^{e}
Pg-3-Glu	10.73	11.06±0.4^{cd}	20.80±0.8^{c}	182.25±6.4^{b}	229.15±9.6^{a}	2.58±0.13^{d}	4.11±0.22^{d}	10.11±0.55^{cd}	3.10±0.09^{d}
Cy-3–3Mal-Glu	12.08	45.81±3.8^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cy-3–6Mal-Glu	13.61	203.36±9.2^{b}	n.d.	423.09±14.8^{a}	n.d.	31.08±1.6^{c}	n.d.	n.d.	n.d.
Cy-3-diMal-Glu	15.63	48.13±3.1^{a}	n.d.	n.d.	n.d.	8.15±0.41^{b}	n.d.	n.d.	n.d.
Pg-3–6Mal-Glc	15.71	trace	n.d.	148.21±5.2^{a}	n.d.	trace	n.d.	n.d.	n.d.
Cy-3-diMal-Glc	16.19	21.19±0.9^{a}	n.d.	n.d.	n.d.	3.58±0.19^{b}	n.d.	n.d.	n.d.
Cy-3-diMal-Glc	16.77	113.50±4.3^{a}	n.d.	n.d.	n.d.	19.07±1.0^{b}	n.d.	n.d.	n.d.
Total anthocyanins		476.26±21.4^{c}	320.33±8.2^{d}	2309.20±80.8^{a}	1269.78±53.3^{b}	82.63±4.3^{e}	46.78±2.5^{e}	389.08±21.4^{cd}	90.35±2.7^{e}
Total cyanidin derivatives									
Non-acylated		33.21±1.1^{g}	299.53±11.9^{e}	1037.24±36.3^{a}	927.98±38.9^{b}	18.17±0.9^{g}	42.67±2.2^{fg}	254.04±13.9^{d}	87.25±2.6^{e}
Acylated		431.99±19.6^{a}	n.d.	423.09±14.8^{a}	n.d.	61.88±3.2^{b}	n.d.	n.d.	n.d.

*- μg/g d.m.; BAH-before alkaline hydrolysis; AAH-after alkaline hydrolisys; n.d.-not detected. Means followed by the same letter within the same row are not significantly different, according to Tukey's test (p=95%).

Effects of the applied alkaline hydrolysis method for the identification of phenolic compounds on the content of acylated and non-acylated derivatives in anthocyanin extracts

According to our results, total degradation of all acylated forms of anthocyanins identified in the grain of blue popping maize, Chilean deep purple maize, and purple wheat occurred during alkaline hydrolysis (Tables 2 and 3, Figure 1Ab-1Cb). The extreme alkaline environment caused the breakdown of ester bonds and the separation of the acyl groups from the anthocyanins. Hence, acylated derivatives were transformed to non-acylated forms. It has already been indicated that acylated forms can transform to non-acylated forms at the beginning of the anthocyanins degradation [Riaz *et al*., 2016] under the influence of different conditions. The study of West & Mauer [2013] has shown that about 68% of the purified cyanidin 3–6Mal-Glu was lost during storage at 40°C and converted primarily to Cy-3-Glu. According to the latest research of Simić *et al*. [2018], the proportion of Cy-3--Glu and its acylated derivative in blue popping maize flour, also used in this study, as well as in crumb of bread baked at 230°C for 20 min and its crust amounted to 25 and 75%, 42, and 58% and finally 58 and 42% of the total cyanidin derivatives, respectively. This conversion of cyanidin derivatives probably contributed greatly to the preservation of attractive pinkish color of bread. According to our results, in blue popping maize and purple wheat samples, which had low content of Cy-3-Glu and high content of its malonyl derivatives in grains, about 62 and 40% of the acylated cyanidin derivatives were transformed into non-acylated ones, respectively. (Table 3). In the case of Chilean deep purple maize and black soybeans, it can be concluded that the applied method significantly reduced non-acylated forms of anthocyanins. The content of Cy-3-Glu was lower by 65% after alkaline hydrolysis of the black soybean extract. However, in the case of Chilean deep purple maize, Cy-3-Glu reduction was lower and amounted to about 36% (Table 3). It can be speculated that the 6M HCl, which was used after alkaline hydrolysis to achieve a stable red color of extracts, affected the separation of aglycons and degradation of anthocyanin glucosides. After alkaline hydrolysis of soybean and Chilean maize extracts, the content of De-3-Glu was reduced by 100 and 80%, respectively. On the other hand, although the content of the acylated form of pelargonidin was not high in maize and wheat samples, after alkaline hydrolysis of their extracts the content of Pg-3-Glu was increased (Table 3). Pedreschi & Cisneros--Zevallos [2007], whose modified alkaline hydrolysis method has been applied in this study, quantified anthocyanins only in the non-hydrolyzed samples.

Alkaline hydrolysis was performed under extreme conditions in this study. However, many other authors observed the behavior of anthocyanins under the influence of different pH conditions. According to results of McDougall *et al.* [2007], all of the anthocyanins were reduced after pancreatic digestion in the alkaline environment but the acylated forms were notably more stable than the non-acylated ones. There was also a relationship between the type of acylated hydroxycinnamic acid and resistance to pancreatic digestion. The study of Cabrita *et al*. [2000] revealed that, for some of the anthocyanin-3-glucosides (*e.g.* malvidin-3-glucoside), the bluish color was rather intense and its stability was relatively high in the alkaline region. As presented in the study of Torskangerpoll & Andersen [2005], aromatic acylated cyanidin derivatives showed higher color stability than the non-acylated forms at pH values vary-

ing from 1.1 to 10.5 during 98-day period at 10°C. Contrasting with many reports in the literature, some of them are listed above, the malonyl glycoside forms of the anthocyanins from purple maize pericarp were more susceptible to degradation caused by time (12 weeks) and pH (2.0 to 6.0) than the non-acylated forms [Luna-Vital *et al.,* 2017]. In turn, Howard *et al*. [2016] reported that acetylated derivatives in blueberry juice were more prone to losses during storage than glycosides, especially in acidified juices.

CONCLUSIONS

It can be concluded that the blue popping maize, deep purple maize, purple wheat, and black soybean used in this study differ significantly both by composition and by content of individual anthocyanins. The acylated forms of anthocyanins were dominant in the grain of blue popping maize, as well as purple wheat and represented 85 and 62% of the total peaks area of anthocyanins, respectively. In the grain of Chilean deep purple maize, that was the richest in anthocyanins, the glucosidic forms were dominant. Cy-3-Glu represented the major type of anthocyanin in the grain of deep purple maize constituting 35.22% of the total peaks area of anthocyanins. Furthermore, no acylated anthocyanins were found in the black soybean. Rich in anthocyanins, colored grains could be used as functional food ingredients that provide health benefits to a large part of human world's population.

The extreme alkaline environment caused the breakdown of the ester bonds, the separation of the acyl groups from the anthocyanins, complete degradation of acylated derivatives, and their partial transformation to its non-acylated parents. On the other hand, partial degradation of glycosides in all samples could be a consequence of the acid that is added to the base hydrolysate. These studies confirm that the applied alkaline hydrolysis method can be used only for the identification of acylated anthocyanins. Moreover, this study confirms the importance of plant sources with a high content of anthocyanins precisely because of their low stability.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Original research article
Section: Nutritional Research

Dietary Chicory Inulin-Rich Meal Exerts Greater Healing Effects than Fructooligosaccharide Preparation in Rats with Trinitrobenzenesulfonic Acid-Induced Necrotic Colitis

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Key words: TNBS-induced colitis, microbiota activity, short-chain fatty acids, chicory roots, Wistar rat

The aim of this study was to compare the effects of chicory root inulin-rich meal (containing a polyphenolic fraction as well) and pure fructooligosaccharides (FOS) on necrotic colitis induced with trinitrobenzenesulfonic acid (TNBS) in Wistar rats. Both chicory preparations significantly reduced the pH value of colonic digesta and favourably lowered the caecal activity of bacterial *β*-glucuronidase as well as the caecal concentration of putrefactive short-chain fatty acids in comparison to the control TNBS rats. In addition, they enhanced the production of total short-chain fatty acid (SCFA pool) and concentration of anti-inflammatory propionic acid in the caecal digesta. Nevertheless, only dietary chicory meal favourably increased the total SCFA concentration and thus decreased the pH value of caecal digesta. The increased caecal SCFA production may explain the observed greater reduction in mucosal necrosis and increased glandular mucosal regeneration in rats fed a diet with chicory root meal. Both chicory preparations beneficially regulated physiological parameters in the lower part of the rat intestinal tract after TNBS-induced colitis, however the dietary treatment with chicory meal showed stronger reduction of mucosal disturbances caused by colitis. Those beneficial effects might be related to the higher polymerization of inulin *vs*. FOS and to the presence of biologically active compounds in the meal, *i.e*., phenolic compounds, which had a strong impact on intestinal microbial activity and thus indirectly alleviated mucosal disturbances caused by colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract whose etiology has not yet been fully elucidated [Motavallian-Naeini *et al*., 2012]. IBD is a multifactorial intestinal disorder that involves interactions among the immune system, genetic susceptibility and environmental factors, especially the intestinal microbiota [Witaicenis *et al.*, 2010]. The incidence of IBD is continually increasing, and it has become a worldwide healthcare problem [Zhang & Li, 2014]. There is evidence suggesting that the appropriate modulation of intestinal microbial activity regulates intestinal physiology and immunological function, which may assist in the prevention and treatment of IBD [Lara-Villoslada *et al.*, 2006].

Well-known dietary ingredients that modulate the activity of the intestinal microbiota are nondigestible carbohydrates. Some studies have shown that a diet enriched in dietary fibre successfully maintains the remission of ulcerative colitis in humans and experimental animal models because of an associated increase in the luminal production of short-chain fatty acids (SCFA) [Zhong *et al.*, 2000; Rodríguez-Cabezas *et al.*, 2002]. It has also been reported that dietary fructooligosaccharides (FOS) have a beneficial effect on intestinal inflammation in a rat model of colitis induced by trinitrobenzenesulfonic acid (TNBS); the applied dietary constituents reduced the extent of the damage and promoted epithelial healing [Cherbut *et al.*, 2003]. These and other authors suggested that the main beneficial effects were associated with the end products of FOS fermentation, such as lactic acid and SCFA, as well as with the acidification of the luminal contents [Kosmala *et al*., 2015]. More complex nondigestible dietary carbohydrates that also modulate the activity of the microbiota in the gastrointestinal tract are inulin-type fructans [Juśkiewicz *et al*., 2011a]. Some studies have shown that dietary supplementation with these compounds beneficially stimulates the numbers of *Bifidobacteria* [Fotschki *et al.*, 2014] and *F. prausnitzii* [Ramirez-Farias *et al.*, 2009], as well as the production of SCFA [Fotschki *et al.*, 2014; Kosmala *et al*., 2014].

Chicory is a rich source of dietary fibres such as inulin and FOS, which have health-promoting properties on consum-

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ers [Żary-Sikorska *et al*., 2016]. From all parts of the chicory plant (peel, seed, leaf), the highest content of inulin was determined in root [Jurgoński *et al*., 2011]. Inulin consists of linear chains of *β*-2,1-linked fructosyl units terminating at the reducing end with a glucose residue attached *via* a sucrose-type linkage. In nature, inulin is the second most abundant storage carbohydrate after starch [Singh *et al.*, 2016]. Inulin is often used as a substrate to produce high purity FOS preparations [Ganaie *et al.*, 2014]. Despite the differences in the degree of polymerization between inulin and FOS (inulin>FOS), both nondigestible carbohydrates have been termed 'prebiotics' [Gibson *et al.*, 1995] and have been found to beneficially stimulate the growth and/or activity of the microbiota, thus affecting various physiological functions and ultimately having health-promoting impacts on consumers [Ganaie *et al.*, 2014]. Nevertheless, the different degrees of polymerization of nondigestible carbohydrates have considerable effects on the specific activity of the microbiota and intestinal immune functions [Żary-Sikorska & Juśkiewicz, 2008]. Moreover, unprocessed dietary fibre occurs as fibre–phenolic complexes, which present more health-promoting effects to consumers than the highly purified preparations of dietary fibre [Fotschki *et al.*, 2015]. The chicory root is also a source of phenolic compounds, such as caffeoylquinic acids (CQAs) more specifically mono- and di-CQAs isomers [Jurgoński *et al*., 2011]. Zorrilla *et al.* [2014] showed that a polyphenolic-enriched almond extract can effectively improve epithelial barrier function and ameliorate colonic inflammation in a rat model of TNBS-induced colitis. Therefore, the present study with Wistar rats tested the hypothesis that dietary supplementation with unprocessed inulin-rich chicory root meal is more effective in reducing intestinal inflammation in a rat model of TBNS-induced colitis than are equivalents of highly purified chicory FOS.

MATERIALS AND METHODS

Chicory preparations

In this study, two kinds of chicory preparations were used: meal from chicory roots obtained from industrial processing and a commercial preparation of fructooligosaccharide produced *via* the enzymatic hydrolysis of chicory inulin (Raftilose®P95, ORAFTI, Belgium). The raw material used to produce the meal was industrially produced chicory semolina (ZPC Cykoria S.A., Poland) dried with a convective dryer at a temperature below 70°C. The dried chicory material was disaggregated in a ball grinder and sieved through a screen with a mesh diameter of 0.8 mm. The commercial dietary FOS preparation consisted of 96.4% dry matter, 0.83% ash, 0.6% glucose, and 95% short-chain FOS. The chicory root meal consisted of 96.7% dry matter, 5.87% crude protein, 1.81% crude fat, 3.32% ash, 6.25% cellulose, 4.30% monosaccharides, 75% fructans (15% oligofructose and 60% inulin), and 0.13% phenolic compounds. The determination of the proximate chemical composition was performed according to the method described in the previous studies [Jurgoński *et al.,* 2011; Wang *et al.,* 2018]. Total phenolic content was determined with the Folin–Ciocalteu's phenol reagent [De Pascual-Teresa *et al*., 2000]. Absorbance was measured at 720 nm wavelength and chlorogenic acid was used as a standard (Sigma, Poznan, Poland).

Animals and experimental design

The rats were used in compliance with the European Guidelines for the Care and Use of Laboratory Animals [Directive 2010/63/EU], and the animal protocol was approved by the local institutional animal care and use committee (Permission No. 32/2012; Olsztyn, Poland). To determine the number of animals in group, the sample size estimation was done according to the approaches proposed by Dell *et al.* [2002]. The rats were housed individually. The selection of the animals and their maintenance over the experiment followed common regulations. The environment was controlled, with a 12-h light-dark cycle, a temperature of 21±1°C, a relative humidity of 50±5%, and 20 air changes/h. The experiment lasted 28 days and was conducted on 32 male Wistar rats aged 7 weeks and weighing 205.6±12.9 g. The rats were divided into 4 groups. Each group was fed a modified version of the semipurified diet recommended for laboratory rodents. All experimental diets were similar in terms of dietary ingredients with the exception of the phenolic content and fibre source (Table 1). Healthy control rats and control rats with induced colitis were fed diets with dietary cellulose (groups C and CC, respectively), and the rest of the rats with induced colitis were fed diets with either a dietary FOS preparation or chicory root meal (groups CCF and CCM, respectively). The rats had free access to tap water and the semipurified diets *ad libitum*, which were prepared and then stored at 4°C in hermetic containers until the end of the experiment.

Assessment of colonic condition

At the beginning of the experiment, all animals were fasted overnight and, except for the untreated control group, were rendered colitic by the method originally described by Morris

TABLE 1. Composition of experimental diets (%).

	Group[1]		
	C and CC	CCF	CCM
Main components	90	89.4	86.05
Fibre sources			
Cellulose	10.0	–	–
FOS preparation	–	10.6	–
Chicory root meal	–	–	13.5
Calculated content			
Protein	15.50	15.50	15.50
Fibre	10.00	10.07	10.12
Total phenolic compounds	–	–	0.018

[1]C, healthy control rats fed dietary cellulose; CC, control rats with induced colitis fed a diet with dietary cellulose; CCF; rats with induced colitis fed a diet with a dietary fructooligosaccharide preparation; CCM, rats with induced colitis fed a diet with chicory root meal; FOS, fructooligosaccharide preparation (Raftilose®P95, ORAFTI, Belgium).

et al. [1989]. Briefly, the rats were anesthetised with isoflurane and given 10 mg of TNBS (Sigma, Poznań, Poland) dissolved in 0.25 mL of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm into the anus. The cannula was left in place for 1 min to ensure that the solution was not immediately expelled by the rat. Rats in the noncolitic group were intracolonically administered 0.25 mL of phosphate-buffered saline (PBS) instead of TNBS. The rats were kept in a head-down position for an additional 30 s and returned to their cages.

At the termination of the experiment, the rats were anaesthetized with sodium pentobarbital according to the recommendations for euthanasia of experimental animals. After laparotomy, the caecum and colon were removed and weighed. Subsequently, the colon was flushed with PBS and dried, and the tissue was immersed for 7 days in a 10% solution of buffered formalin. Colonic segments were embedded in paraffin blocks. Paraffin sections (1–2 μm) were cut with a Reichert's microtome and tissue fragments were passed through increasing concentrations of alcohol solutions, acetone and xylene (de-waxed). The preparations were stained with hematoxylin and eosin (H&E; Merck, Darmstadt, Germany) according to the method described by Fischer *et al.* [2008]. The tissue sections were evaluated and images taken by standard light microscopy using a computer program for image analysis, B-cell, and Olympus BX50 microscope with a digital camera (Olympus Co., Tokyo, Japan).

A semiquantitative score was used to evaluate severity of histological lesions in the inflamed colon (Histopathological Colitis Score, HCS) [Engel *et al.,* 2008]. The HCS featured the parameters inflammation extent, crypt architecture, edema, and infiltration with inflammatory cells, with a maximum of 12 points (Table 2). The cross-sectioned segments from the colon were scored by investigators, blinded to the experimental groups tested.

TABLE 2. Histopathological Colitis Score (HCS).

Feature	Description	Score
Inflammation extent	none	0
	mucosa	1
	mucosa + submucosa	2
Damage in crypt architecture	none	0
	regeneration	1
	destruction	2
Hyperemia / Edema	without	0
	mild	1
	moderate	2
	severe	3
Infiltration with inflammatory cells	without	0
	mild	1
	moderate	2
	severe	3

(ulceration and/or crypt abscess respectively +1)

These features describe inflammation criteria to determine colitis severity [Engel *et al.,* 2008].

Analytical procedures

The individual feed consumption and final body weight of the rats were determined. Samples of the caecal and colonic digesta were collected, and their pH was immediately measured using a microelectrode and a pH/ION meter (model 301; Hanna Instruments, Vila do Conde, Portugal). In the fresh caecal digesta, the dry matter was determined by drying to constant weight at 105°C, whereas the ammonia concentration was determined by the microdiffusion method in Conway's dishes [Hofirek & Haas, 2001].

After storage of the caecal digesta at −70°C, SCFA concentrations were measured using a gas chromatography system (Shimadzu GC-2010, Kyoto, Japan) with a capillary column (SGE BP21, 30 m × 0.53 mm; SGE Europe Ltd., Milton Keynes, UK), as previously described [Jurgoński *et al*., 2015]. The concentrations of the caecal putrefactive SCFA (PSCFA) were calculated as the sum of isobutyric, isovaleric, and valeric acids. All SCFAs analyses were performed in duplicate. Standards of acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids were obtained from Sigma Co. (Poznań, Poland). They were used to plot calibration curves and calculate individual fatty acid concentration in the digesta.

Caecal fermentation processes were analysed based on the activities of selected bacterial enzymes (α- and β-glucosidase, α- and β-galactosidase, and β-glucuronidase), as measured by the rate of release of *p*-nitrophenol or *o*-nitrophenol from the respective nitrophenyl glucosides, according to a previously described method [Juśkiewicz *et al.*, 2011b]. The following substrates were used: *p*-nitrophenyl-α-D-glucopyranoside (for α-glucosidase), *p*-nitrophenyl-β-D-glucopyranoside (for β-glucosidase), *p*-nitrophenyl-α-D-galactopyranoside (for α-galactosidase), *o*-nitrophenyl-β-D-galactopyranoside (for β-galactosidase), and *p*-nitrophenyl-β-D-glucuronide (for β-glucuronidase). To measure the activities of enzymes secreted by bacterial cells into the caecal environment, a reaction mixture containing 0.3 mL of a substrate solution (5 mM) and 0.2 mL of a 1:10 (v/v) dilution of the caecal sample in 100 mM phosphate buffer (pH 7.0) after centrifugation at 4°C, 7211 × g (MPW-350R, MPW Med. Instruments, Warsaw, Poland) for 15 min was prepared. The samples were incubated (10-min, 37°C) and then mixed with 2.5 mL of 0.25 M cold sodium carbonate to stop the reaction. The absorbance was measured at 400 nm for *p*-nitrophenol and at 420 nm for *o*-nitrophenol (Spectrophotometer Unicam Helios α, Thermo Fisher Scientific Polska Sp. z o.o., Warsaw, Poland). The enzymatic activity was expressed as μmol product formed per hour per g of fresh digesta. All analyses were performed in duplicate.

Statistical analysis

The results were examined statistically using one-way analysis of variance, and significant differences between groups were determined with Duncan's multiple range test at a significance level of $P \leq 0.05$. Calculations were made using STATISTICA 12.0 software (StatSoft Corp., Kraków, Poland).

TABLE 3. Body weight, diet intake, and basic indices of the distal intestine in rats with TNBS-induced colitis.

	Group[1]			
	C	CC	CCF	CCM
Initial body weight (g)	206±4	205±3	205±4	206±3
Final body weight (g)	309±7[a]	264±4[b]	279±4[b]	279±8[b]
Diet intake (g/4 weeks)	557±15[a]	477±13[bc]	456±13[c]	500±9[b]
Feed conversion ratio (g/g)	5.50±0.26[b]	8.61±0.79[a]	6.53±0.57[b]	7.35±0.82[ab]
Caecum				
Tissue mass[2]	0.27±0.01[c]	0.32±0.02[c]	0.61±0.02[a]	0.43±0.02[b]
Digesta mass[2]	0.83±0.04[c]	0.92±0.04[bc]	1.83±0.18[a]	1.21±0.14[b]
pH of digesta	6.82±0.06[a]	6.81±0.06[a]	6.33±0.15[ab]	6.21±0.14[b]
Dry matter of digesta (%)	23.9±0.53[a]	24.3±0.53[a]	18.4±0.93[b]	16.4±0.40[c]
Ammonia in digesta (mg/g)	0.36±0.02	0.38±0.02	0.38±0.03	0.34±0.02
Colon				
Mass with digesta[2]	0.99±0.04[b]	3.54±0.49[a]	1.09±0.10[b]	1.01±0.10[b]
pH of digesta	6.80±0.13[ab]	7.06±0.06[a]	6.59±0.15[b]	6.44±0.06[b]

Values are expressed as the mean ± standard error of the mean; TNBS, trinitrobenzenesulfonic acid. [1]C, healthy control rats fed a diet with dietary cellulose; CC, control rats with induced colitis fed a diet with dietary cellulose; CCF, rats with induced colitis fed a diet with a dietary fructooligosaccharide preparation; CCM, rats with induced colitis fed a diet containing chicory root meal. [2]g/100 g body weight. [a-c]Values with different superscript letters within a row are significantly different at P≤0.05.

RESULTS AND DISCUSSION

In the present study, after 28 days of the experiment, the animals administered TNBS exhibited the overt typical clinical signs of colitis: lesser weight gain related to a decrease of diet intake and an increase in the feed conversion ratio (C *v*s. CC; P<0.05) (Table 3). Similar signs were observed by Gonçalves *et al.* [2013] in Wistar rats with TNBS-induced colitis. Compared to the untreated animals (C group), the induction of colitis in the CC group was manifested by a significant increase of the colon mass with digesta (Table 3) and activity of caecal bacterial *β*-glucosidase (P<0.05; Table 3). The results obtained in the present study showed that diet enrichment with chicory preparations, especially chicory root meal, partly reduced these typical clinical signs of the TNBS--induced inflammatory process.

The experimental groups of animals with TNBS-induced colitis (CCF and CCM) consumed less food and thus gained significantly less in weight than the rats from group C (Table 3). Among the rats treated with TNBS, the dietary intake differed between the CCF and CCM groups (CCF<CCM; P<0.05). Animals fed the diet with FOS utilised the diet significantly better than the control group animals with induced colitis (CC group; P<0.05). The highest relative caecal tissue mass was noted in the rats fed dietary FOS, and the values of that parameter decreased as follows among the groups: CCF>CCM>CC. Dietary inclusion of the FOS preparation resulted in a significant increase in the caecal digesta mass in comparison to other groups (P<0.05). The concentrations of caecal digesta dry matter in the CCF and CCM groups *vs*. those of the C and CC (P<0.05) groups, were respectively reduced.

The administration of experimental diets to the rats with TNBS-induced colitis caused positive effect by decreasing *β*-glucuronidase activity in the caecal digesta (CCF, CCM vs. CC; P<0.05) and compensating the increase of the *β*-glucosidase during the inflammation processes caused by TNBS (Table 4). Bacterial enzymes, such as *β*-glucuronidase, may exert toxic, carcinogenic or mutagenic effects in the colon [Klewicka *et al*., 2009]. Roberton *et al.* [1982] suggested that the most important factor in the modulation of *β*-glucuronidase activity in the rat large bowel is bile flow. Dietary fibre increases peristalsis, and thus greater quantities of bile are transported to distal segments of the gastrointestinal tract. Some studies reported that diet with dietary fibres and polyphenols may reduce the activity of such enzymes as *β*-glucuronidase and *β*-glucosidase [Lahouar *et al.*, 2012; Kosmala *et al.*, 2017]. Juśkiewicz *et al.* [2011b] also examined the effects of the chicory dietary polyphenolic fraction and found favourable changes in the activity of bacterial *β*-glucuronidase in the faeces and caecal digesta of rats.

In comparison to the dietary treatment with cellulose, dietary inclusion of the commercial FOS preparation as well as chicory root meal exerted a positive effect by lowering the pH value in the colonic digesta (Table 3). It is noteworthy that the acidification of the digesta promotes positive microbiota proliferation and decreases the growth of pathogenic bacterial species [Topping & Clifton, 2001]. In the CCM group, this dietary preparation lowered the percentage of dry matter and the pH value in the caecum (Table 3), indicating more intensive fermentation of indigestible components ahead

TABLE 4. Microbial enzyme activities and short-chain fatty acid (SCFA) concentrations, profile, and pool in the caecal digesta.

	Group[1]			
	C	CC	CCF	CCM
Enzyme activity (μmol/h/g digesta)				
α-Glucosidase	10.9±1.16[b]	11.6±3.11[b]	47.0±6.16[a]	30.2±5.59[a]
β-Glucosidase	5.29±0.82[c]	13.1±1.8[a]	7.57±0.80[bc]	9.48±1.21[ab]
α-Galactosidase	6.28±1.17[c]	10.9±1.80[bc]	55.6±8.50[a]	31.0±5.73[ab]
β-Galactosidase	24.0±1.80[c]	27.6±1.85[bc]	222±21.45[a]	128±11.33[ab]
β-Glucuronidase	18.8 ±2.8[ab]	30.4±4.7[a]	8.69±1.46[b]	8.63±2.43[b]
SCFA concentration (mmol/g digesta)	91.8±7.1[b]	88.9±6.6[b]	98.3±9.9[b]	141±9[a]
Acetic acid	66.4±5.94	63.0±5.1	53.1±5.7	71.5 ±5.1
Propionic acid	14.4±1.23[b]	14.9±1.2[b]	38.0±6.1[a]	61.3±8.0[a]
Butyric acid	8.89±0.74[a]	8.14±0.70[a]	5.59±0.42[b]	6.86±0.81[ab]
PSCFA[2]	2.16±0.20[ab]	2.79±0.29[a]	1.60±0.54[b]	1.16±0.29[b]
SCFA profile (%)				
Acetic acid	72±1.3[a]	71±1.2[a]	55±1.3[b]	51±3.4[b]
Propionic acid	16±0.6[b]	17±0.5[b]	37±2.1[a]	43±3.5[a]
Butyric acid	10±1.2[a]	9±0.8[a]	6±0.9[b]	5±0.7[b]
SCFA pool (mmol/100 g body weight)	77.1±8.4[b]	86.4±5.5[b]	169±11[a]	173±24[a]

Values are expressed as the mean±standard error of the mean. The colitis has been induced by trinitrobenzenesulfonic acid (TNBS). [1]C, healthy control rats fed a diet with dietary cellulose; CC, control rats with induced colitis fed a diet with dietary cellulose; CCF, rats with induced colitis fed a diet with a dietary fructooligosaccharide preparation; CCM, rats with induced colitis fed a diet containing chicory root meal. [2] Short-chain fatty acids of putrefactive origin (isobutyric acid + isovaleric acid + valeric acid). [a-c]Values with different superscript letters within a row are significantly different at P≤0.05.

of the colon. In the examined preparations, only the feeding with the diet with chicory root meal was able to significantly elevate the caecal concentration of SCFAs, as the main fermentation products of the abundant microbiota (Table 4). The caecal SCFAs pool was found to be significantly increased upon dietary application of both tested preparations (CCF and CCM *v*s. C and CC; P<0.05). The SCFAs are essential for the constant repair of the colon epithelium [Sturm & Dignass, 2008] and exert anti-inflammatory and anticarcinogenic effects [Van der Beek *et al.*, 2017]. The secretory response to SCFAs, in combination with the contractile response, seems to act as a lubricant for the movement of luminal content in the colon [Yajima *et al.,* 2011]. In addition, dietary application of chicory preparations resulted in the highest caecal propionic acid concentration (CCF and CCM *vs*. C and CC, respectively; P<0.05). As a result, in comparison to control animals the application of both experimental diets (CCF, CCM) significantly increased percentage of propionic acid in the SCFA profile at the expense of acetic and butyric acids (P<0.05) (Table 4). Acetic and propionic acids are known to reduce the production of anti-inflammatory mediators such as TNF-*a* by human neutrophils. Propionic acid is also able to inhibit the expression of proinflammatory mediators [Vinolo *et al.*, 2011]; in addition it stimulates chloride secretion and exerts a range of health-promoting functions [Yajima *et al.*, 2011; Louis & Flint, 2017]. Jurgoński *et al.* [2011] found no significant difference in the production of butyric acid in the caecum of rats fed a diet supplemented with chicory root extract. In the present study, the caecal concentration of butyric acid, which is a major nutritive factor for colonic enterocytes, was reduced when the rats were feed CCF and CCM diets. The butyric acid exhibits a wide spectrum of cellular effects on the mucosa, such as enhanced differentiation and apoptosis. It is presumed that butyric acid exerts local anti-inflammatory effects [Hamer *et al.,* 2008]. In the studies performed on the isolated perfused rat colon, it has been shown that butyric acid increased the secretion of mucin [Barcelo *et al.,* 2000; Shimotoyodome *et al.,* 2000]. Putrefactive SCFAs are products of bacterial fermentation of undigested proteins [Cardona *et al.,* 2005]. Both experimental groups, in comparison to the CC group, exhibited significantly reduced production of PSCFAs (Table 4), which may suggest the less intensive anaerobic bacterial fermentation of polypeptides and amino acids.

The histological examination of the colonic tissue in rats not treated with TBNS showed normal morphology of the mucosa. Numerous small lumps of lymphocytes were found in the mucosa (Figure 1). The number of lumps appearing in the lymph mucosa was higher, and they had the characteristics of proliferated lymphocytes with few eo-

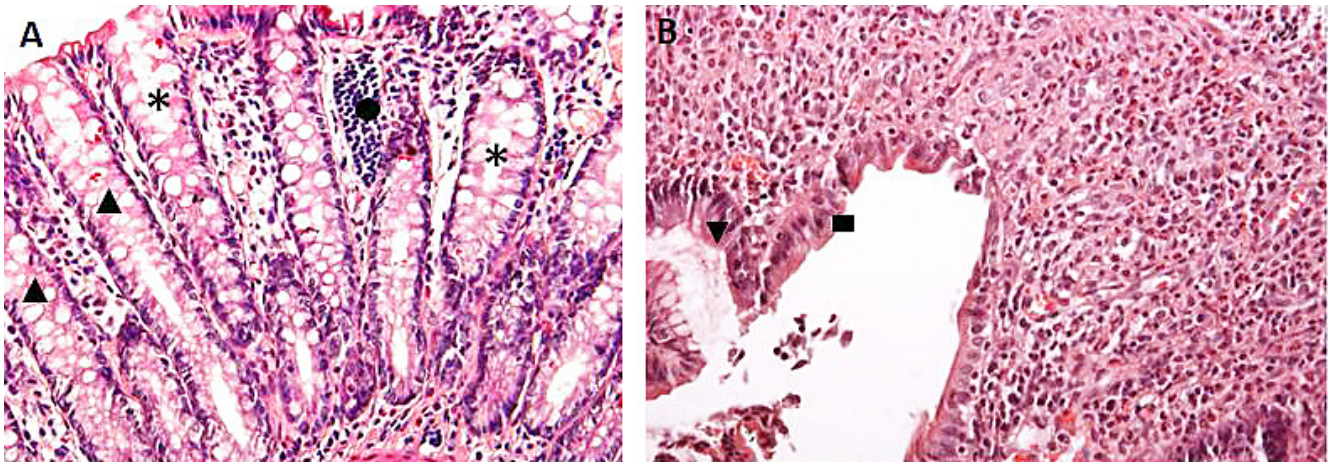

FIGURE 1. A – Example of colonic mucosa from the healthy control (C group) Wistar rat group; Goblet cells (black triangles); Intestinal glands in the crypts (black asterisks); Small clump of lymphocytes (black dot); B – Regeneration of mucosal glands in a rat from the colitic control group (CC group); Brush border (black square); Simple columnar epithelium (black arrowhead). The specimens were stained with hematoxylin and eosin. The original magnification was 40×.

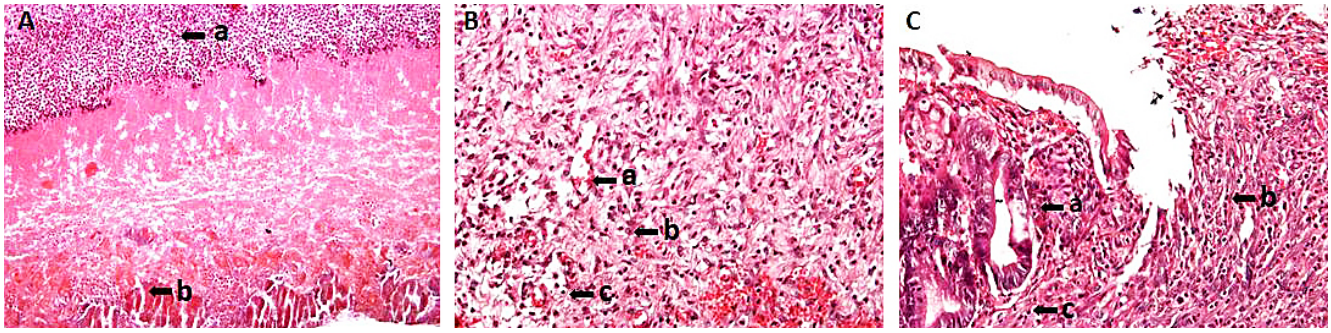

FIGURE 2. A – Necrosis of colonic mucosa and leukocyte infiltration in a rat from the colitic group fed a dietary fructo-oligosaccharide preparation (CCF group); a -leukocytes; b – necrotic changes; B – Thick layer of young connective tissue (granulation) with fibroblasts under the dead mucosa in a rat from the colitic group fed chicory root meal; a – erythrocytes, b – macrophages; c – lymphocytes (CCM group); C – Mucosal regeneration on the border of damage in a rat from the colitic group fed a diet containing chicory root meal (CCM group). Glandular cells are seen arising on young granulation tissue, in which eosinophils are present alongside fibroblasts; a – macrophages; b – eosinophils, c – lymphocytes. The specimens were stained with hematoxylin and eosin. The original magnification was 20× (A) and 40× (B, C).

sinophil cells. The administration of TNBS, which induces inflammation in rats, significantly increased the weight of the colon. This observed effect might be related to a higher level of edema and inflammatory processes. The administration of TNBS resulted in widespread mucosal necrotic inflammation in the entire colon in all animals. A similar effect was observed by Yue-Meng *et al.* [2011] in a nutritional study regarding the therapeutic effects of Peifeikang (a probiotic compound) on rats with experimental TNBS-induced colitis. The defensive response to the necrotic inflammation observed in all animals was leukocyte (neutrophil) infiltration. Neutrophil infiltration is one of the most prominent histological features in the inflamed colonic mucosa of colitis [Liu & Wang, 2011]. In the present study, the largest regenerative changes in the colon were found in the group fed chicory root meal. The lowest average thickness of tissue necrosis was observed in the animals from the CCM group, and this thickness increased among the groups as follows: CCM<CCF<CC ($P<0.05$) (Table 5). The observed beneficial effects might be related to the production of SCFA and to the regulation of leukocytes ability to migrate to loci of inflammation [Vinolo *et al.,* 2011]. The SCFAs regulate differentiation of mucosal T_{reg} cells, modulate Toll-like receptor 4 signalling, suppress the production of proinflammatory cytokines, and reduce the infiltration of colonic mucosa by leukocytes, thereby directly suppress the immune response and regulate colonic inflammatory processes [Van der Beek *et al.*, 2017]. Directly under the necrosis area in all groups treated with TNBS was a zone of leukocyte infiltration (Figure 2). Moreover, under the zone of leukocyte infiltration, the CCM group had a considerably smaller area of connective tissue with edema and infiltration of eosinophil cells and fibroblasts than the CC group had ($P<0.05$) (Figure 1 and 2, Table 5). The infiltration of cells with the ability to produce pro-inflammatory mediators or cytokines affects the formation and differentiation of the connective tissue [Debnath *et al.*, 2013]. Of great importance is the infiltration of eosinophil cells, which potentiates inflammatory processes such as the formation of young granulation tissue. In all animals treated with TNBS, there was an increase in new connective tissue, which was dominated by fibroblasts and numerous eosinophil cells as well as single plasmatic cells and mononuclear macrophages. In all

TABLE 5. Effects of chicory root preparations on the histopathology of colitis induced in rats by TNBS.

	Group[1]		
	CC	CCF	CCM
Mucosal necrosis (μm)	262±7.8[a]	211±19.2[b]	145±10.7[c]
Leukocyte infiltration under the necrosis zone (μm)	144±15.0	131±14.7	114±15.5
Connective tissue with edema and infiltration of eosinophils, plasmatic cells and fibroblasts (μm)	416±13.0[a]	–	65.5±19.1[b]
Granulation tissue with eosinophilic infiltration (μm)	1124±34.5[a]	843±57.2[b]	1096±24.3[a]
Signs of glandular mucosa regeneration (no. of rats out of 8)	5	3	7
Histopathological Colitis Score (HCS)	10±2[a]	6±2[b]	4±1[b]

Values are expressed as the mean±standard error of the mean; TNBS, trinitrobenzenesulfonic acid. [1]CC, control rats with induced colitis fed a diet with dietary cellulose; CCF, rats with induced colitis fed a diet with a dietary fructooligosaccharide preparation; CCM, rats with induced colitis fed a diet containing chicory root meal. [a-c]Values with different superscript letters within a row are significantly different at P≤0.05. The scoring method for HCS is described in Table 2.

animals treated with TNBS, the largest thickness of the new connective tissue in the colon was observed in the rats fed cellulose and chicory meal (P<0.05) (CC>CCM; Table 4). Furthermore, the infiltration of lymphocytic and eosinophil cells in the muscularis and under the colonic adventitia was observed in all animals. In both preparations examined, the addition of chicory meal to the diet most effectively stimulated the regeneration of the glandular mucosa in the form of small foci or the renewal of intestinal epithelial glands (Figure 2). Also the value of Histopathological Colitis Score used to evaluate histological lesions in the inflamed colon presented more favourable effect of chicory meal than of FOS (CCM<CCF<CC; Table 5).

CONCLUSIONS

The present study showed that the meal from chicory roots and the commercial chicory preparation of fructooligosaccharides exerted positive changes in rats with TNBS--induced colitis. Both examined preparations significantly reduced the pH value of colonic digesta and favourably lowered the activity of bacterial β-glucuronidase as well as the production of putrefactive SCFAs in rats treated with TNBS. Nevertheless, only the dietary chicory root meal treatment favourably increased the SCFAs concentration and decreased the pH value of caecal digesta. Moreover, the morphological characteristics of the colon showed that the feeding with the diet containing chicory root meal most effectively reduced TNBS-induced colitis by reducing the level of mucosal necrosis and the field of granulation tissue with eosinophilic infiltration, as well as by increasing the signs of glandular mucosal regeneration (7/8 animals). The observed beneficial effects of chicory root meal might be related to the higher polymerization of inulin (*vs*. FOS) and to the presence of biologically active compounds, *e.g.*, phenolic compounds, which have a strong impact on intestinal microbial activity and thus indirectly alleviate colonic mucosal disturbances caused by colitis. Based on these results, the examined chicory products, especially chicory root meal, might be considered a valuable source of dietary fibre that regulates the intestinal physiological fermentative processes and thus reduces disorders associated with colitis. Further *in vivo* studies are needed to better understand the potential clinical relevance of chicory products in processes related to the abnormalities in the gut immune system associated with inflammatory bowel disease.

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Original research article
Section: Food Quality and Functionality

Nutritional Quality, Potential Health Promoting Properties and Sensory Perception of an Improved Gluten-Free Bread Formulation Containing Inulin, Rice Protein and Bioactive Compounds Extracted from Coffee Byproducts

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Key words: α-glucosidase, sugars bioaccesibility, coffee by-products, inulin, antioxidant coffee fibre, gluten-free bread

The present study aimed to improve the formulation of a gluten-free commercial bread-making premix by adding inulin, rice protein and extracts of coffee by-products (silverskin and husk) to obtain healthier products enriched in protein and dietary fiber with the potential to reduce the risk of chronic diseases. The new formulation combines well-known and novel food ingredients. To validate the novel ingredients, we determined the physicochemical characterization, caffeine and chlorogenic acid contents, and food safety of coffee silverskin (CSE) and husk (CHE) extracts. Sensory and nutritional quality, bioactive compounds (chlorogenic acid, total phenolic compounds, melanoidins and browning), *in vitro* bioaccessibility, and health-promoting properties (overall antioxidant capacity and α-glucosidase activity) of the breads were evaluated as well.

Results support the feasibility of CSE and CHE as natural sustainable sources of antioxidants, α-glucosidase inhibitors and colorants. The observed health-promoting properties suggest that coffee by-product extracts could potentially be used as functional food ingredients or supplements to reduce the risk of chronic diseases associated with oxidative stress and to control postprandial glucose levels. Based on consumers' preferences, we obtained new bread formulations with a high nutritional and sensorial quality, suitable for celiacs and with the potential to reduce the risk of gastrointestinal disease related to oxidative stress. Data on *in vitro* digestion indicated a significant ($p<0.05$) decrease in the bioaccessibility of sugars and a significant increase ($p<0.05$) in antioxidants.

ABBREVIATIONS

CD, celiac disease; CGA, chlorogenic acid; CHE: coffee husk extract; CR, commercial recipe; CSE, coffee silverskin extract; GI, glycaemic index; IDF, insoluble dietary fibre; NF, new formulation; NFS, new formulation with 25 g CSE/kg dry matter CSE; NFH, new formulation with 25 g CHE/kg dry matter; OTA, Ochratoxin A; SDF, soluble dietary fibre; TAC, total antioxidant capacity; T2D, type 2 diabetes, TDF, total dietary fibre; TPC, total phenolic compounds.

INTRODUCTION

Gluten-free products present a major challenge for the food industry in terms of organoleptic, technological and nutritional characteristics [Conte *et al*., 2019]. Among gluten-free foods, bread is the most consumed in Europe [Padalino *et al*., 2016]. The absence of gluten in these products affects starch digestibility increasing the postprandial glycaemic response. Consequently, people on a gluten-free diet have a higher risk of suffering from chronic diseases like type 2 diabetes (T2D) [Giuberti *et al*., 2015]. However, the addition of dietary fibre and protein has been found to reduce the glycaemic response of gluten-free products such as bread [Scazzina *et al*., 2015].

Coffee husk is the outer skin and pulp obtained from the wet processing of coffee berries [del Castillo *et al*., 2019]. It has a high content of carbohydrates (35–85%), soluble fibers (30.8%), minerals (3–11%), and proteins (5–11%). It is also rich in insoluble dietary fiber and can be a source of phytochemicals such as tannins (5–9%) and cyanidins (20%) for the food industries [del Castillo *et al*., 2019].

Coffee silverskin is a thin tegument of the outer layer of the two beans forming the green coffee seed and represents 4.2% (w/w) of the coffee cherry. It is obtained when green coffee beans are roasting, being the unique byproduct of the roasting process. Coffee silverskin has high contents of dietary fiber (68–80%) and polysaccharides (60–70%), and presents a very high antioxidant activity [Pourfarzad *et al*., 2013; Bresciani *et al.,* 2014]. Prior studies have proposed the use of coffee silverskin as an innovative food ingredient [Ballesteros *et al*., 2014; Bresciani *et al.,* 2014].

The aqueous extracts of coffee silverskin (CSE) and coffee husk (CHE) have recently been proposed as a source of two safe food ingredients: aqueous extracts enriched in phyto-

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chemicals and antioxidant dietary fiber [Iriondo-DeHond *et al*., 2018]. CSE was used in the preparation of biscuits with no added sugar, obtaining a product with a good nutritional quality and improved texture and colour [Garcia-Serna *et al*., 2014]. Besides, CSE can be used to prevent or treat age-related chronic diseases caused by oxidation and inflammation, such as T2D. The bioactive compounds present in CSE affect several pathways involved in the pathogenesis of T2D, thereby reducing the risk of this disease [del Castillo *et al*., 2016].

This study aimed to evaluate the use of CSE and CHE as functional ingredients in the formulation of gluten-free breads with a high nutritional value and the potential to control postprandial glucose. To the best of our knowledge, the use of CHE for diabetes has not been previously reported.

MATERIALS AND METHODS

Reagents

The reagents used in this study were: intestinal acetone powders, 4-methylumbelliferyl α-D-glucopyranoside (4-MUG), acarbose, α-amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3200–4500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, cholestyramine resin, chlorogenic acid (CGA), caffeine, phloroglucinol, salicylic acid; 2,2'-azinobis [3-ethylbenzothiazoline-6-sulphonic acid] (ABTS), Folin-Ciocalteu reagent, Nα-Acetyl-L-lysine, ortho-phthalaldehyde (OPA), methanol HPLC-grade, phenol, concentrated sulphuric acid (93–98%), and ammonium chloride were from Sigma-Aldrich (St. Louis, MO, USA). Sucrose, D-fructose, D-glucose kit, and the dietary fibre kit were from Megazyme (International Ireland Ltd, Bray, Ireland).

Buffered peptone water (BPW) was from Biocult, BD™ Difco™ plate count agar (PCA) medium and brain BD™ Bacto™ heart infusion (BHI) agar from BD (New York, NJ, USA) and Sabouraud dextrose agar (SDA) with chloramphenicol from CONDA (Torrejón de Ardoz, Madrid, Spain). Water was purified using a Milli-Q and Elix system (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

Apparatus

Microplate spectrometers BioTek powerWaveTM XS (BioTek Instruments, Winooski, VT, USA) and FP-6200 (JASCO, Easton, MD, USA) were used for the spectrophotometric assays. Bloc-digest 12 (Selecta, Barcelona, Spain) was employed for the digestion process in the Kjeldahl method. Accela liquid chromatograph (Thermo Scientific, San Jose, CA, USA) equipped with a DAD and coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyser *via* an electrospray ionisation (ESI) interface was used to determine caffeine and CGA content. CertoCLAV A-4050 autoclave (CertoCLAV, Traun, Austria), Stomacher® 400 Circulator (Seward, UK), horizontal laminar flow bench Mini-H (Telstar, Madrid, Spain), Nüve EN120 incubator (Nüve, Ankara, Turkey) and SANYO Mir154 incubator (SANYO Electric Biomedical Co., Ltd., Osaka, Japan) were used for microbiological analysis. Bread was made using a PAN 850 bread machine (Fagor, San Sebastián, Spain).

Commercial food ingredients

Bread was prepared using a gluten-free commercial baking pre-mix. The composition of the pre-mix was: 753 g/kg of corn starch, 52 g/kg of dietary fibre, 20 g/kg of salt, 13 g/kg of fat, and 10 g/kg of protein. ORAFTI® inulin and Remypro N80+ rice protein from Beneo Ibérica (Barcelona, Spain) were used to fortify the breads.

Coffee by-products ingredients

Arabica coffee husk (*Coffea arabica*) was provided by Delikia (Pontevedra, Spain) while Robusta coffee silverskin (*Coffea canephora*) was provided by Fortaleza S.A. (Vitoria, Spain). Raw materials were stored at room temperature in a dry and dark place until the preparation of extracts.

Aqueous coffee byproduct extracts

Robusta CSE and Arabica CHE aqueous extracts were obtained with hot water (50 g/L) at 100°C for 10 min as described in the patent WO 2013/004873 [delCastillo *et al*., 2013]. Extracts were freeze-dried and stored at -20°C until use.

Microbiological analyses of CSE and CHE

The safety of coffee byproduct extracts as food ingredients was evaluated by assaying counts of: (1) total aerobic microorganisms, (2) aerobic microorganisms forming endospores, and (3) moulds and yeasts. All assays were performed in sterile conditions with previous solubilization of the samples (10 g) in BPW (90 mL) using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (1) pour plate method, PCA medium, incubation at 30°C 72 h; (2) pour plate, BHI agar medium, preincubation (80°C, 10 min) and incubation at 37°C 48 h; and and (3) spread method, SDA with chloramphenicol and incubation at 25°C 120 h. Results were expressed as colony-forming units per gram (CFU/g).

Physicochemical analyses of CSE and CHE

For the analyses, each freeze-dried extract (250 mg) was dissolved in Milli-Q water (10 mL) for 5 min using a vortex mixer at room temperature and then centrifuged at 4,000×*g* for 10 min. The supernatants were filtered through a 13 mm PTFE filter (pore size 0.45 μm). Samples were kept at -20°C until analysis. The following components of CSE and CHE were analyzed: browning, content of total carbohydrates, glycemic sugars, total proteins, total dietary fibre (TDF), total lipids, total phenolic compounds (TPC), CGA and caffeine content, melanoidins, total antioxidant capacity (TAC), and α-glucosidase activity.

Bread-making

The studied bread formulations are shown in Table 1. Control breads were prepared using a gluten-free baking premix (commercial recipe, CR) following the manufacturer's instructions. To reduce the bioaccessibility of sugars and increase the nutritional value of the commercial preparation, new bread formulations (NF) containing inulin and rice protein with no sugar were proposed. These basic formulations were supplemented with 25 g of CSE per kg of dry matter (d.m.) (NFS) or 25 g of CHE per kg d.m. (NFH). The breads were made with a domestic bread-maker using the gluten-free programme, obtaining 1000 g of the product.

TABLE 1. Bread formulations.

Ingredients (g)	CR	NF	NFS	NFH
Gluten-free baking pre-mix	410	385	372	372
Sourdough	20	13	13	13
Sunflower oil	90	19	19	19
Sugar	40	0	0	0
Rice protein	0	55	55	55
Inulin	0	40	40	40
CSE	0	0	13	0
CHE	0	0	0	13
Water	240	500	500	500
Estimated calories (kcal/100 g bread)	241	180	178	177
Estimated protein energy value (% on total kcal)	1.2	26.2‡	26.5‡	26.6‡
Estimated fibre content (g fibre/100 g bread)	3.8*	11.7ϕ	11.6ϕ	11.6ϕ

CR – commercial recipe; NF – new formulation; NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

*CR might be "source of fibre" (≥3 g fibre/100 g bread); ‡NF, NFS and NFH might be "high in protein" (≥20 % energy value of bread provided by protein); ϕ NF, NFS and NFH might be "high fibre content" (≥6 g fibre/100 g bread).

Homemade loaves were sliced (10 mm thick), and the slices were frozen and subsequently freeze-dried. The freeze-dried slices (approximately 50 g) were ground and sieved through a 40-mesh screen to obtain a powder. For the analyses, the bread powder (1 g) was dissolved in Milli-Q water (10 mL) for 5 min using a vortex mixer at room temperature and then centrifuged at 4,000×*g* for 10 min. The supernatants were filtered through a 13 mm PTFE filter (pore size 0.45 μm). Samples were kept at -20°C until analysis.

Sensory analysis

A preliminary sensory analysis of the NFS and NFH breads found that the maximum amount of CSE and CHE considered acceptable was 25 g/kg d.m. (data not shown). A sensory analysis of NF bread was carried out to evaluate its acceptance and of NFS and NFH breads to estimate the influence of extract supplementation on their sensory perception. Colour, texture, taste, and overall acceptance were evaluated in a hedonic sensory test involving 30 untrained panellists. Results of the verbal scale test were converted to a 7-point scale from 1 (lowest) to 7 (highest). Breads were considered acceptable if the average of the panellists' scores for all the parameters was equal to or above 4.

Physicochemical analyses

Total carbohydrate content

Total carbohydrate content was determined using the phenol–sulphuric method described by Masuko *et al*. [2005]. Samples (0.1 mL) were mixed with concentrated sulphuric acid (0.3 mL; 93–98% purity) and phenol (0.09 mL; 50 g/L) in a glass flask. The mixtures were incubated at 90°C for 5 min and cooled at room temperature. Absorbance was measured at 490 nm. A calibration curve was constructed using glucose (0.1–0.85 g/L). Reagent blank and sample blank were also analysed in each set of samples. All measurements were performed in triplicate, and results were expressed as g/100 g.

Glycaemic sugars

Glucose, fructose, and sucrose contents were determined with a K-SUFRG kit (Megazyme) adapted to a microplate format following the manufacturer's instructions. Analysis was carried out in triplicate. Results were expressed as g/100 g.

Dietary fiber

Insoluble (IDF), soluble (SDF), and TDF contents were determined using an enzymatic-gravimetric assay based on the AOAC-991.43 [1995] and AACC-32.07.01 [1995] methods. Analysis was carried out in duplicate for each sample. Results were expressed as g/100 g.

Total proteins

Total protein content was determined following the Kjeldahl method [AOAC-920.87, 32.1.22, 1995]. This procedure was carried out in triplicate. Results were expressed as g/100 g.

Free amino groups

An OPA assay was carried out to determine free amino groups content [Michalska *et al*., 2008] in the soluble fraction of digested bread. Quantitative analysis was performed using a calibration curve of Nα–acetyl–l–lysine (0.01–1 mmol/L). All measurements were performed in triplicate. Results were expressed as mg Lys eq./kg of digested bread.

Total lipids

Total lipid content was determined according to Toschi *et al*. [2014] with minor modifications. Briefly, the sample (1 g) was mixed with 50 mL of *n*-hexane, homogenized by Ultra-Turrax for 3 min and then subjected to ultrasound treatment for 20 min. The organic layer (containing the lipid matter) was separated by centrifugation (1,620×*g* for 20 min). The lipid fraction was collected and dried in a rotary evaporator. The fat content was determined gravimetrically. The analysis was carried out in triplicate. Results were expressed as g/100 g.

Total phenolic compounds

TPC content in the samples was analyzed using Folin–Ciocalteu adapted to a micromethod [Contini *et al*., 2008]. The reaction was initiated by mixing 10 μL of the sample with 150 μL of the Folin–Ciocalteu reagent. After incubation at room temperature for 3 min, 50 μL of sodium bicarbonate solution were added. The kinetics of the reaction at 37°C was followed for 120 min by measuring absorbance at 735 nm once each minute. Sample blank and reagent blank were also analyzed in each set of samples. The CGA calibration curve was used for quantification (0.01–0.6 g/L). All measurements were performed in triplicate, and results were expressed as mg CGA eq./g freeze-dried sample.

Caffeine and CGA content

Caffeine and CGA content were determined with UPLC--MS/MS as described by Fernandez-Gomez *et al*. [2016]. Samples were diluted with Milli-Q water. For CGA quantification, 50 μg/mL of phloroglucinol were added as an internal standard, and 50 μg/mL of salicylic acid as an internal standard for caffeine. All the analyses were performed in triplicate, and results were expressed as mg caffeine or CGA/g freeze-dried sample.

Browning

Browning was measured at 405 nm. All measurements were made in triplicate, and results were expressed as absorbance (Abs)/g freeze-dried sample.

Melanoidins

Melanoidins content was determined according to Silván *et al*. [2010]. Samples were subjected to ultrafiltration using a Microcon YM-10 regenerated cellulose 10 kDa (Millipore, Bedford, MA, USA) at 12,000×*g* for 10 min. Melanoidins were measured spectrophotometrically at 405 nm. The analysis was carried out in triplicate and results were expressed as absorbance (Abs)/g freeze-dried sample.

Health-promoting properties of bread

Antioxidant capacity

An indirect $ABTS^{\bullet+}$ decolourisation assay was performed according to Oki *et a*l. [2006]. An $ABTS^{\bullet+}$ stock solution was prepared by adding 44 μL of potassium persulfate (140 mmol/L) to a 2.5 mL $ABTS^{\bullet+}$ aqueous solution (7 mmol/L). The mixture was allowed to stand for 16 h at room temperature. The working solution of the radical $ABTS^{+}$ was prepared by diluting the stock solution 1:75 (v/v) in a sodium phosphate buffer (5 mmol/L, pH 7.4) to obtain an absorbance value of 0.7±0.02 at 734 nm. Samples (30 μL) were added to 270 μL of the working solution of $ABTS^{\bullet+}$ in a microplate. Absorbance was measured at 734 nm and 30ºC for 18 min, every 2 min. The reaction was complete after 5 min. CGA calibration (0.15–2 mmol/L) was used. All measurements were performed in triplicate. Results were expressed as mg CGA eq/g freeze-dried sample.

The antioxidant capacity of the insoluble fraction of the digested breads was determined by a direct $ABTS^{\bullet+}$ assay or QUENCHER assay [Açar *et al*., 2009]. Briefly, 10 mg of the sample were mixed with 70 mg of cellulose and stirred. After that, 10 mg of the mixture were mixed with 1.7 mL of the $ABTS^{\bullet+}$ solution in a thermomixer (600 rpm, 37ºC, 5 min) and centrifuged (4500×*g*, 25ºC, 2 min). Absorbance (734 nm) of the supernatant was measured in a microplate. A CGA calibration curve (0.01–0.25 g/L) was used. Measurements were performed in triplicate and results expressed as g CGA eq/ 100 g.

α-Glucosidase activity

Alpha-glucosidase activity was determined following Berthelot & Delmotte [1999] with slight modifications. Briefly, 0.1 g of rat intestine powder was dissolved in 3 mL of NaCl (90 g/L), sonicated in an ice bath for 6 min and then centrifuged at 10,000×*g* for 30 min to extract the enzyme contained in the supernatant. In a 96-well microplate, 100 μL of the sample dissolved in phosphate buffered saline (100 mmol/L, pH 6.9) were mixed with 100 μL of α-glucosidase (diluted 1/10) and 100 μL of 4-MUG (2 mmol/L). Fluorescence was then monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm at 37°C for 30 min. Blank of sample and negative control (buffer, enzyme and 4-MUG) were included. Acarbose was used as a positive control of the inhibition of enzymatic activity. Curves of samples and acarbose were assayed to cover the whole range of inhibition of the enzyme (~ 0.5–96 %). The percentage of α-glucosidase inhibition was calculated using the equation: % α-glucosidase= $[(F_{n.c.-} F_s) / F_{n.c}] \times 100$, where $F_{n.c.}$ is the fluorescence of the negative control (without inhibitor) and F_s is the fluorescence of the sample. All measurements were performed in triplicate, and results were expressed as sample concentration (g/L) causing 50% α-glucosidase inhibition (IC_{50}).

Release of nutrients and bioactive compounds during bread digestion

Bioaccessibility of the nutrients and bioactive compounds composing the breads was estimated applying an *in vitro* oral-gastrointestinal digestion system according to Hollebeeck *et al*. [2013] with minor modifications. One g of sample was digested as follows: salivary step (pH 6.9, 10 mL, 5 min, 3.9 U-amylase/mL, aerobic), gastric step (pH 2, 13 mL, 90 min, 71.2 U pepsin/mL, aerobic), and duodenal step (pH 7, 16 mL, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/mL, aerobic). The obtained digests were centrifuged (1700×*g*, 4°C, 20 min) and separated into soluble and insoluble fractions. The soluble fraction was treated to mimic human intestinal reabsorption of bile salts with cholestyramine resin (100 g/L) [Martinez-Saez *et al*., 2017]. The soluble and insoluble fractions were freeze-dried and stored in a dry cold place until analysis.

The soluble fractions of the digested breads were analyzed for contents of glycemic sugars, free amino acids, TPC, CGA and caffeine content, TAC, and α-glucosidase activity as described in the section of physicochemical analyses and health-promoting properties of bread, respectively. The insoluble fraction was recovered, and its antioxidant capacity was analyzed by direct $ABTS^{\bullet+}$ assay as described in the section of health-promoting properties of bread.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses were performed using Statistica 7.1 (Stat Software Inc., California, USA). Experimental data were analyzed using ANOVA and significant differences among means from triplicate analyses at $p \leq 0.05$ were determined using post hoc Duncan's test.

RESULTS AND DISCUSSION

Validation of CSE and CHE as food ingredients

Food safety

The microbiological analyses of CSE showed values of 3.25×10^5 CFU/g of endospores, 4.3×10^3 CFU/g of total aerobic microorganisms, and a yeast and mould con-

tent of less than 10^2 CFU/g. The analyses of CHE showed values below 10^2 CFU/g for all the microorganisms under study. The main safety hazard is the presence of ochratoxin A (OTA), a mycotoxin released by *Aspergillus* potentially nephrotoxic, carcinogenic, teratogenic and genotoxic. OTA limits have been defined for roasted coffee (5 μg/kg) and soluble coffee (10 μg/kg) [European Parlamient regulation (EC) N.123/2005, 2005]. Since CSE and CHE are extracts of two coffee by-products, there is no specific OTA regulation limit. However, the absence of moulds in these extracts reduces the risk of OTA contamination. Adding CSE and CHE to the bread formulation did not increase microorganism content, suggesting good microbiology quality of the food ingredients.

Physicochemical analyses

Data on the physicochemical characterization of CSE and CHE are shown in Table 2. Differences in the composition of CSE and CHE can be explained by their nature, as they come from different anatomic parts of the coffee fruit with different chemical components [del Castillo *et al*., 2019].

Total carbohydrate contents were 17.95 g/100 g and 24.18 g/100 g in CSE and CHE, respectively. Results obtained for CSE are in line with those reported by del Castillo *et al*. [2019]. As in the coffee silverskin raw material, CSE had a low content of simple sugars like glucose (0.25 g/100 g), fructose (0.83 g/100 g) and sucrose (1.93 g/100 g). CHE had a higher glucose content (8.88 g/100 g), while the contents of sucrose (0.12 g/100 g) and fructose (0.01 g/100 g) were lower than in CSE. Both extracts, especially CSE, presented low amounts of glycemic sugars.

The results of TDF content determination in CSE (36.06 g/100 g) agree with those reported by del Castillo *et al*. [2019]. CSE dietary fibre is mainly composed of SDF (27 g/100 g of TDF). CHE presented a high amount of TDF (68.43 g/100 g), mainly SDF (67.45 g/100 g of TDF). Both extracts can be considered good sources of SDF, especially CHE (Table 2).

The amount of soluble proteins present in CSE was 17.54 g/100 g (Table 2), which is similar to the soluble protein content reported by Narita & Inouye, 2014 for CSE obtained under subcritical water conditions. CHE had a lower soluble protein content of 5.07 g/100 g close to the 7 g/100 g described by Gouvea *et al*. [2009] for coffee husk raw material. Therefore, CSE may be a better natural source of protein than CHE.

Similarly, caffeine content was also higher in CSE (53.25 mg/g) than in CHE (13.93 mg/g) with values similar to those reported by Mesías *et al*. [2014] and Murthy & Naidu [2012]. Since the safety limits of caffeine intake fixed by the European Food Safety Authority [2015] are 400 mg per day for non-pregnant adults and 200 mg per day for pregnant women, the daily intake of CSE considered to be safe would be ~7.5 g for non-pregnant adults and ~4 g for pregnant women. Consequently, the safe daily intake of CHE would be 4 times higher than for CSE. Caffeine content should be considered when applying these extracts for human nutrition and, in particular, considering certain members of the population.

TABLE 2. Physicochemical characterization of coffee silverskin (CSE) and coffee husk (CHE) extracts.

Analysis	CSE	CHE
Total carbohydrates (g/100 g)	17.95±1.62^b	24.18±1.64^a
Glucose (g/100 g)	0.25±0.03^b	8.88±0.10^a
Fructose (g/100 g)	0.83±0.02^a	0.01±0.01^b
Sucrose (g/100 g)	1.93±0.21^a	0.12±0.01^b
TDF (g/100 g)#	36.06±1.67^b	68.43±4.50^a
SDF (g/100 g) #	27.90±1.14^b	67.45±4.65^a
IDF (g/100 g) #	8.17±0.53^a	0.99±0.15^b
Total proteins (g/100 g)	17.54±0.82^a	5.07±0.38^b
Lipids (g/100 g)	1.83±0.58^a	1.14±0.02^a
Caffeine (mg/g)	53.25±1.65^a	13.93±0.80^b
CGA (mg/g)	21.30±6.17^a	1.71±0.27^b
Browning (abs/g)	374.93±18.09^a	58.27±3.72^b
Melanoidins (abs/g)*	306.13±4.00^a	52.13±2.00^b
TPC (mg CGA eq./g)	81.03±5.56^a	19.67±1.24^b
TAC (mg CGA eq./g)	190.86±4.25^a	52.30±0.93^b
Estimated calories kcal/100 g extract	158.4	127.3
α-Glucosidase ($IC_{50,}$ mg/mL)	1.42±0.32^b	2.46±0.21^a

Data are expressed as mean (n=3) ± standard deviation. Values in each row having different letters indicate significant differences at $p<0.05$ (Duncan's test). CGA, chlorogenic acid; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; TAC, total antioxidant capacity; TDF, total dietary fibre; TPC, total phenolic compounds.

#Results are expressed as mean (n=2) ± standard deviation; *Analyses performed on aqueous extracts fraction >10 kDa.

CGA content was significantly higher in CSE (21.30 mg/g) than in CHE (1.71 mg/g). These values are lower than those described by other authors [Mesías *et al*., 2014; Murthy & Naidu, 2012] but higher than those reported by Bresciani *et al*. [2014]. These differences might be attributed to solvent extraction, set conditions, quantification method or origin of the by-products.

Differences in browning and melanoidin were also significant between the coffee by-product extracts (Table 2). The high browning and melanoidins values of CSE are associated with the Maillard reaction occurring during the roasting process. Data on browning and melanoidins suggest that CSE may be a good source of natural colorants to be applied in the food industry. The use of natural pigments is the current marketing trend because of consumers' concern about the safety of artificial food dyes, which is reinforced by the possible health benefits of natural pigments [Rodriguez-Amaya, 2016]. In this regard, coffee melanoidins exert several beneficial effects on human health due to their antioxidant, antimicrobial, anticarcinogenic, anti-inflammatory, antihypertensive, and antiglycative properties [Moreira *et al*., 2012].

A TPC content of 81.03 mg CGA eq/g was found in CSE (Table 2). This value was 4 times higher than in CHE

(19.67 mg CGA eq/g). This difference in TPC content can be explained the on basis of CGA content, one of the main phenolic compounds in coffee by-products [del Castillo *et al*., 2019]. Similarly, TAC was 3.6 times higher in CSE than in CHE (Table 2). This great difference may be associated with their different contents of melanoidins and phenolic compounds such as CGA which has been described as the main contributor to the overall antioxidant power of coffee by-products [Mesías *et al*., 2014].

Effects of CSE and CHE on α-glucosidase activity

As seen in Table 2, the IC_{50} values obtained for each extract were significantly different. An IC_{50} value of 1.42 mg/mL was obtained for CSE which is comparable to values previously described by our group [del Castillo *et al*., 2016]. Differences in the effect of the extracts on α-glucosidase activity might be associated with their CGA content. This bioactive compound previously showed an IC_{50} value of 0.25 mg/mL [del Castillo *et al*., 2016]. Other authors have proposed that CGA strongly inhibits α-glucosidase activity and reduces postprandial blood glucose [Oboh *et al*., 2015]. Data on the inhibitory effect on α-glucosidase activity pinpoint CSE and CHE as ingredients to be used for better control of postprandial glucose level.

Food application of CSE and CHE

Sensory analysis of innovative breads

Breads were considered acceptable if their average score was equal to or higher than 4.0 (Figure 1). The sensory analysis indicated that the quality of NF made with inulin and rice protein was good (all values >5.1), and no significant differences were observed between NF and NFS or NFH breads. Therefore, the addition of these two coffee by-products extracts (25 g/kg d.m.) did not affect the sensory quality of the product. Comparing NFS and NFH with NF, color was the most appreciated parameter, especially for NFH. The appearance (color) of the breads containing CSE and CHE supports its feasibility as a natural colorant, providing the typical color of wholemeal breads. These results highlight the potential application of these extracts as natural colorants for gluten-free bread formulations, since the darkening of the crumb color is desirable in the consumer choice of a bread [Matos & Rosell, 2012].

Nutritional analysis of innovative breads

Total carbohydrate content of the innovative breads (NF) and those containing CSE or CHE (25 g/kg d.m.) and inulin (NFS and NFH) were significantly different from the commercial pre-mix gluten-free bread (CR) (Table 3). Fructose, glucose, and sucrose contents were significantly lower in all new bread formulations (NF, NFS and NFH). As seen in Table 1, the reduction of starch and the substitution of sugar by inulin in the innovative breads decreased glycemic carbohydrates, as described by Rizzello *et al*. [2016].

TDF content of NF, NFS and NFS breads was significantly higher than CR. Adding inulin to the NF duplicated the TDF content compared to CR. CSE and CHE accounted for approximately 13% of TDF in the new bread formulations (NFS and NFH). Coffee silverskin has previously been proposed as an ingredient for reducing caloric density in biscuits [Garcia-Serna *et al*., 2014]. The new bakery products could be labelled as having a "high fibre content", as they contain at least 6 g of fibre per 100 g [European Parliament Regulation [EC] N. 1924/2006 [2006]. Therefore, the use of inulin as dietary fibre improved the nutritional value of the bread.

Total protein values were significantly higher in the innovative breads (NF, NFS and NFH) compared to CR due to the addition of rice protein (Table 3). CSE provided 7% extra protein in NFS breads compared to NF in accordance with the higher protein content of CSE (Table 2). Therefore, NF, NFS and NFH breads can be considered products that are "high in protein", containing at least 20% of the energy value of the food provided by protein [European Parliament Regulation [EC] N. 1924/2006]. Several studies have demonstrated that the consumption of rice protein can be associated with a lower risk of oxidative stress preventing the occurrence of several diseases such as hyperlipidemia [Wang *et al*., 2016].

Caffeine content of NFS and NFH breads was determined to evaluate the safety of consuming these products (Table 3). The daily bread intake recommended by WHO [2003] for European countries is 250–350 g. Considering the safety limits of the intake fixed by EFSA [2015], both products can be considered safe for non-pregnant adults. In the case of pregnant women, NFH consumption is safe if the daily consumption of NFS does not exceed 250 g.

CGA content in the NFS and NFH breads supplemented with coffee by-products extracts (25 g/kg d.m.) degraded by 68–70% (based on initial CGA concentration in CSE and CHE) (Table 2). Other studies have also found high thermal degradation of CGA during the baking process [Rupasinghe *et al*., 2008]. The low sugar content in NF bread produced the highest reduction in browning compared to CR bread (Table 3). Browning values of NFS and NFH breads were higher and similar to CR, respectively. As reported in Figure 2, the addition of the extracts provided a natural color and the typical appearance of wholemeal breads. As shown in Table 3, the inclusion of extracts as food ingredients in NFS and NFH breads significantly improved

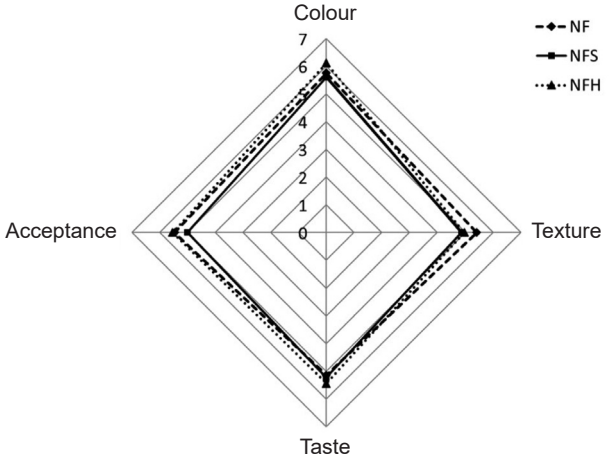

FIGURE 1. Mean scores (n=30) of 1–7 scale for overall acceptance of innovative gluten-free breads NF, NFS and NFH. NF – new formulation, NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

TABLE 3. Chemical characterization of CR, NF, NFS and NFH breads.

Analysis	CR	NF	NFS	NFH
Total carbohydrates(g/100 g)	27.74±2.08^{a}	18.76±2.78^{b}	18.21±1.26^{b}	18.38±1.40^{b}
Glucose (g/100 g)	2.70±0.06^{a}	0.26±0.01^{c}	0.28±0.01^{c}	0.61±0.02^{b}
Fructose (g/100 g)	5.10±0.06^{a}	1.52±0.10^{b}	1.53±0.02^{b}	1.47±0.12^{b}
Sucrose (g/100 g)	0.42±0.02^{a}	0.06±0.02^{c}	0.04±0.02^{c}	0.10±0.02^{b}
TDF (g/100 g)	5.73±0.91^{b}	11.28±0.37^{a}	12.72±0.52^{a}	12.82±1.23^{a}
SDF (g/100 g)	4.20±0.33^{b}	5.90±0.20^{ab}	6.20±0.20^{ab}	6.80±0.80^{a}
IDF (g/100 g)	1.53±0.58^{c}	5.30±0.10^{b}	6.50±0.30^{a}	6.00±0.40^{ab}
Total proteins (g/100 g)	1.21±0.25^{c}	8.36±0.21^{b}	9.03±0.40^{a}	7.36±0.98^{b}
Lipids (g/100 g)	14.89±0.47^{a}	2.14±0.38^{c}	3.22±0.22^{b}	3.30±0.31^{b}
Caffeine (mg/g)	nd	nd	0.72±0.05^{a}	0.36±0.02^{b}
CGA (mg/g)	nd	nd	0.25±0.01^{a}	0.02±0.002^{b}
Browning (Abs_{405}/g)	1.63±0.09^{b}	0.90±0.05^{c}	2.22±0.08^{a}	1.21±0.02^{b}
Melanoidins* (Abs_{405}/g)	1.05±0.05^{b}	0.64±0.05^{c}	1.54±0.03^{a}	1.11±0.13^{b}
TPC (mg CGA/g)	175.86±2.43^{b}	54.69±0.81^{d}	254.92±7.73^{a}	121.12±6.12^{c}
TAC (mg CGA/g)	87.24±1.82^{c}	76.10±1.28^{d}	288.27±3.57^{a}	129.39±1.80^{b}
Estimated calories (kcal/100 g bread)	250	128	138	133
α-Glucosidase (IC_{50} mg/mL)	38.10±2.10^{b}	108.60±1.76^{a}	27.00±1.95^{d}	32.70±2.56^{c}

CR – commercial recipe; NF – new formulation; NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

Data are expressed as mean (n=3) ± standard deviation. Values in each row having different letters indicate significant differences at $p<0.05$ (Duncan's test). CGA, chlorogenic acid; IDF, insoluble dietary fibre; nd, not detected; SDF, soluble dietary fibre; TAC, total antioxidant capacity; TDF, total dietary fibre; TPC, total phenolic compounds. *Analyses performed on aqueous extracts fraction >10 kDa.

antioxidant capacity (3.78 and 1.70-times higher compared to NF control bread for NFS and NFH breads, respectively). This higher antioxidant capacity could be related to phenolic compounds such as CGA and melanoidins. Antioxidants can play a double role as food preservatives and health-promoting compounds.

The highest TPC content was found in NFS bread (Table 3). Differences in TPC may be due to bread composition. CR bread had highest content of gluten-free baking pre-mix (Table 1) which had corn flour. Soong *et al*. [2014] have reported that baked muffins made with corn flour had a higher TPC content than those baked with rice flours. Consequently, NF had the lowest TPC content which increased to 79% and 55% with the addition of CSE and CHE, respectively. These results are in line with TPC values shown by CSE and CHE (Table 2).

Effect of bread components on α-glucosidase activity

As seen in Table 3, the addition of CSE and CHE to NF inhibited α-glucosidase activity in accordance with the results obtained for CSE and CHE ingredients (Table 2). The inhibitory activity of CSE was related to the presence of CGA [del Castillo *et al*., 2016]. IC_{50} values of NFS (27.00 mg/mL) and NFH (32.70 mg/mL) breads are comparable to those reported for similar products such as pasta enriched with faba bean flour [Turco *et al*., 2016].

Bioaccessibility of nutrients and bioactive compounds composing the innovative breads

As reported in Table 4, substituting the sugar and starch of the gluten-free baking pre-mix with inulin significantly reduced glycemic sugar content in digested NF, NFS and NFH compared to CR. Total glucose and fructose content was by at least 35% lower in NFH and NFS. A low content of free glucose available for absorption is an important factor in the prevention of diabetes.

CGA was not detected in NFS and NFH digested breads (Table 4). Previous studies have reported a significant decrease in CGA concentration during CSE digestion, especially after the hydrolysis reaction of the duodenal step [Fernandez-Gomez *et al*., 2016]. Part of the CGA could be linked to the maillardized dietary coffee fibre structure which could protect against oxidative damages in the large intestine. The inclusion of phenolic groups, especially CGA, in the coffee melanoidin skeleton has been reported [Moreira *et al*., 2012].

The antioxidant capacity of digested fractions of NFS and NFH breads was significantly higher than CR but was

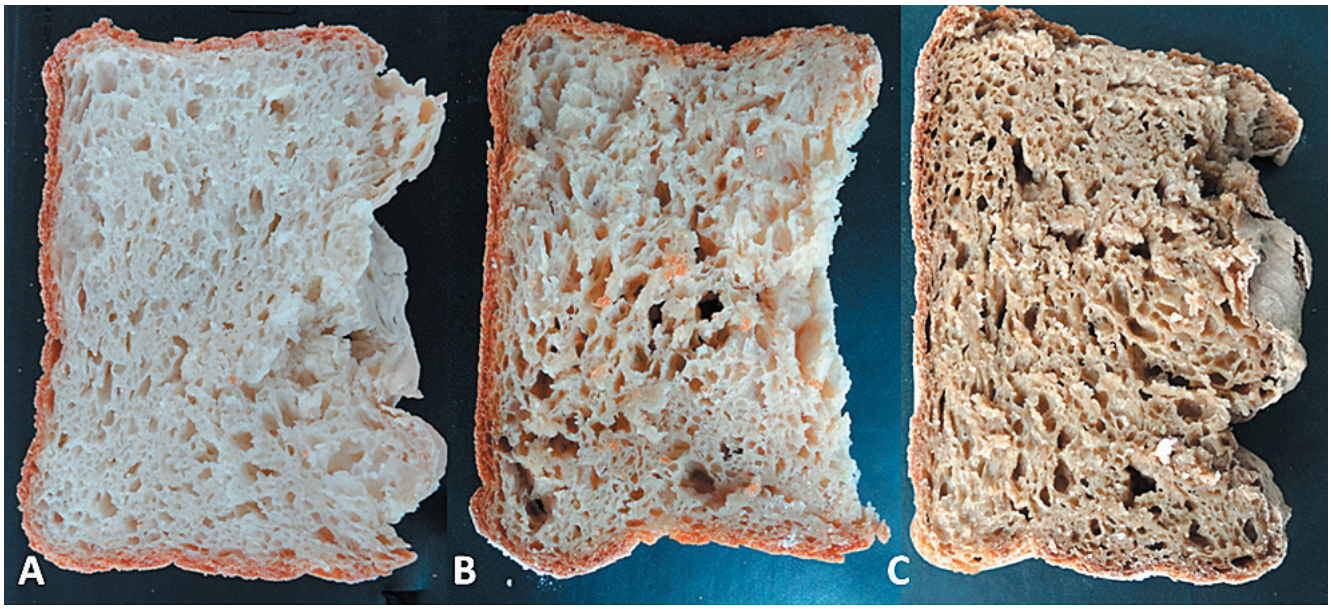

FIGURE 2. Cross section of innovative breads: (A) NF – new formulation, (B) NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; (C) NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

similar to NF. This might be related to the release of several peptides with antioxidant properties caused by the hydrolysis of the rice protein during the digestion process. The rice protein used in this study is enriched in sulfur-amino acids which exert an antioxidative effect by scavenging free radicals [Métayer *et al*., 2008].

As seen in Table 4, the antioxidant capacity of the insoluble fraction recovered from the digestion process of NFS and NFH breads was significantly higher than that found for NF samples. Maillardized dietary coffee fibre may be responsible for the higher antioxidant power of the non-bioaccessible fraction [Silván *et al*., 2010].

No significant differences were found among the IC_{50} values of the bioaccessible fractions of the digested breads (Table 4). The reduced inhibitory effect may be associated with the loss of free CGA during the digestion process. CGA inhibited α-glucosidase activity in a dose-dependent manner [Oboh *et al*., 2015].

TABLE 4. Chemical characterization of CR, NF, NFS and NFH digested breads.

Analysis	CR	NF	NFS	NFH
		Soluble fraction		
Glucose (g/100 g)	7.47±0.13^{a}	7.05±0.19^{b}	5.86±0.08^{c}	5.72±0.28^{c}
Fructose (g/100 g)	3.42±0.11^{a}	3.37±0.08^{a}	1.15±0.01^{c}	1.58±0.10^{b}
Sucrose (g/100 g)	0.42±0.02	nd	nd	nd
FAG (mg lys eq/1000 g)	0.20±0.01^{b}	0.26±0.07^{b}	0.33±0.12^{ab}	0.45±0.05^{a}
Caffeine (mg/g)	nd	nd	0.43±0.01^{a}	0.17±0.02^{b}
CGA (mg/g)	nd	nd	nd	nd
TPC (mg CGA/g)	134.86±6.05^{c}	227.92±17.56^{b}	265.70±18.02^{a}	222.18±7.89^{b}
TAC-I (mg CGA eq/g)	573.96±30.70^{b}	742.62±23.55^{a}	756.29±22.76^{a}	721.34±27.86^{a}
α-Glucosidase (IC_{50}, mg/mL)	5.40±0.85^{a}	5.20±1.20^{a}	5.20±0.87^{a}	5.90±1.45^{a}
		Insoluble fraction		
TAC-D (g CGA eq/100 g)	nd	22.88±2.80^{c}	35.01±0.97^{a}	30.68±0.94^{b}

CR – commercial recipe; NF – new formulation; NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

Data are expressed as mean (n=3) ± standard deviation. Values in each row having different letters indicate significant differences at $p < 0.05$ (Duncan's test). CGA, chlorogenic acid; nd, not detected; FAG, free amino groups; TAC-D, total antioxidant capacity direct method; TAC-I, total antioxidant capacity indirect method; TPC, total phenolic compounds.

CONCLUSIONS

CSE and CHE are sustainable natural sources of antioxidants and α-glucosidase inhibitors. Furthermore, the extracts can be employed as natural colorants in the high dietary content formulation to provide the typical appearance of wholemeal bread. The new gluten-free bread formulations developed during this study may provide additional health benefits in people with specific nutritional requirements. Proof-of-concept human intervention studies are needed to verify the positive effects of these breads in controlling postprandial glucose. In conclusion, the nutritional properties of the new gluten-free bread formulations were greatly improved compared to the gluten-free commercial breads.

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Original research article
Section: Food Technology

Effect of the Addition of Polysaccharide Hydrocolloids on Sensory Quality, Color Parameters, and Anthocyanin Stabilization in Cloudy Strawberry Beverages

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Key words: polysaccharide hydrocolloids, strawberry beverages, sensory evaluation, color, stability of anthocyanins, ultra-performance liquid chromatography–mass spectrometry

This manuscript presents results of the qualitative characteristics of strawberry cloudy juice and beverages with the addition of 0.2% and 0.3% of carboxymethylcellulose (CMC), guar gum (GG), locust bean gum (LBG), and xanthan gum (XG). Fresh products were evaluated with reference to their sensory quality (5-point scale). Changes in $L^*a^*b^*$ parameters and in the stability of anthocyanins (ultra-performance liquid chromatography--mass spectrometry) were monitored in the storage experiment (6 months, 4°C).

Most of the hydrocolloids have contributed to the improvement of the taste and the consistency of strawberry products. In overall taste evaluation, the highest scores were given to the samples with CMC, whereas in the consistency evaluation, to the samples with CMC, GG, and LBG addition at a dose of 0.2%. The study of color parameters of the products has indicated significant changes in their chromatic space during storage. After 6 months, beverages with CMC, GG, and LBG were darker in comparison to the control sample. The contribution of red color in beverages was higher, and of yellow color was lower than in the strawberry juice.

Strawberry juice was characterized by a high degree of anthocyanins degradation (*Dd*=84%), especially of pelargonidin-3-glucoside and cyanidin--3-malonylglucoside. The use of hydrocolloids has contributed to the partial reduction of this phenomenon.

In conclusion, the most beneficial protective effect on anthocyanins (*Dd*= 65%) and the impact on the sensory characteristics in strawberry beverages was provided by LBG application.

INTRODUCTION

Polysaccharide hydrocolloids are a large group of food additives with universal applications in the food industry. They are high-molecular-weight biopolymers and are obtained by extraction from terrestrial or sea plants, from plant exudates, or *via* the microbiological pathway. A number of derivatives of natural polysaccharide hydrocolloids, obtained by chemical or enzymatic treatment of raw materials, were identified as well [Dickinson, 2003]. Due to numerous functional properties, including stabilization, emulsification, thickening or gelling, they are used, among others, for the production of dairy products (including yogurts, desserts, beverages, cheeses) [Varela & Fiszman, 2013], ice creams, pastry and confectionery products, meat and convenience foods, soups, sauces, or salad dressings [Saha & Bhattacharya, 2010]. Fruit processing offers interesting application perspectives for the hydrocolloids. Texture and gelling properties of pectin are used in the production of bars and fruit jams [Raju & Bawa, 2006]. Alginates are used, among others, for the production of structured fruits [de Almeida Lins *et al*., 2014], whereas carboxymethylcellulose (CMC) is comprehensively used in concentrated fruit juices, as a filling in cakes, in juice dehydration, as an additive reducing syneresis and providing brighter appearance to the processed fruit products, and so on. Guar gum (GG) added to nectars acts as a thickener by increasing its viscosity [Somogyi, 2005]. Solutions of locust bean gum (LBG) are stable over a wide pH range, which makes them excellent stabilizers and thickeners in the production of beverages [Barak & Mudgil, 2014].

Functional properties of hydrocolloids, their mechanism of action, and their influence on the rheological properties of food are the well-known and well-described issues in previous studies. For example, some research have addressed the influence of these biopolymers on the stability and viscosity of cloudy apple juices [Genovese & Lozano, 2001] and peach nectars [Pastor *et al.*, 1996]. In turn, Chaikham & Apichartsrangkoon [2012] described the dynamic, viscoelastic, and physicochemical properties of longan juices, while Mirhosseini *et al*. [2008] investigated the physical stability, turbidity loss rate, and cloudiness of orange bev-

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erage emulsion. However, in the case of juices, nectars or beverages production technology, the role of hydrocolloids requires consideration in a wider context. The following issues can be considered particularly important for the quality of these products: 1) the impact of hydrocolloids on the development of sensory characteristics (especially concerning the perception of sour and tart tastes [Pangborn *et al*., 1978]), and 2) the effect they might exert on the stability of bioactive compounds, including anthocyanins [Heins *et al*., 2001]. This problem is particularly important in strawberry processing. Anthocyanins that can be found in fruits are characteristic of undergoing strong degradation. The process of their decomposition in juices, nectars, drinks, or purees occurs very rapidly. After few weeks or even days of storage, strawberry products can change color from red to brown [Gössinger *et al*., 2009]. Meanwhile, the scientifically proven beneficial effect of anthocyanins on human health [Tsuda, 2012; Gupta *et al*., 2009; Ellingsen *et al*., 2008] speaks for their preserving properties as best as possible in the fruit products. The protective effect of hydrocolloids on the stability of anthocyanins is possibly associated with intermolecular interactions or introduction of anthocyanins to the structure of hydrocolloids. They form a three-dimensional network in an aqueous phase, which leads to the compartmentation effect of colored compounds. Furthermore, there are known interactions between cationic molecules and certain hydrocolloids (*e.g.* pectin and sodium alginate). A similar mechanism could also apply to the flavylium cation, which is the parent structure of anthocyanidins [Heins *et al*., 2001]. From the point of view of sensory attributes of fruit products, the important property of polysaccharides is their ability to reduce taste sensations, like astringency [Troszyńska *et al*., 2010]. Because of their viscosity, hydrocolloids reduce the feeling of friction caused as a result of the reduction of oral cavity moistening during the consumption of astringent products. Another possible mechanism results from the interactions between polysaccharides and polyphenols [Taira *et al.*, 1997]. The formation of polysaccharide complexes with, *e.g.,* tannins prevents binding of the latter ones to salivary proteins.

Given the above premises, the objective of this study was to investigate the effect of selected polysaccharide hydrocolloids: carboxymethylcellulose (CMC), guar gum (GG), locust bean gum (LBG), and xanthan gum (XG) on sensory properties, color parameters, and stability of anthocyanin compounds in cloudy beverages made of strawberry of "Roxana" cultivar.

MATERIALS AND METHODS

Reagents and chemicals

Methanol and acetic acid (HPLC purity) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (UPLC; gradient grade) and *L*-ascorbic acid were from Merck (Darmstadt, Germany). Analytical standards of anthocyanins: pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, pelargonidin-3-*O* rutinoside, and pelargonidin-3-*O*-galactoside were purchased from Extrasynthese (Lyon, France). Carboxymethylcellulose sodium, guar gum, locust bean gum, and xanthan gum were from Brenntag (Kędzierzyn Koźle, Polska).

Strawberry cultivar for juice and beverages production

Fruits of strawberry ("Roxana" cultivar (cv); *Fragaria x ananassa* Duchesne) were collected at a commercial strawberry plantation (Smolna, Poland, 51º09'24"N, 17º25'56'E) in the 2016 season. Fully mature, expanded, free from stems, and undamaged fruits were hand washed and in this form used for juice and beverages production.

Production of cloudy strawberry juice (laboratory scale)

Strawberry fruits were homogenized and heated (10 s, 75ºC,) in a Thermomix device (Vorwerk, Wuppertal, Germany). The pulp was pressed in a hydraulic basket press (SSRE, Warsaw, Poland) for 5 min at a piston thrust of 5000 KG/ cm^2. Fresh juice was heated to 90ºC for 2 min (Thermomix), poured into 80-mL colorless jars, left for 10 min for pasteurization, and cooled in a water bath to 20ºC. Three replicates of cloudy strawberry juice preparation were carried out. Juices were analyzed twice: after processing and after 6 months of cold storage (4ºC, no light exposure).

Selection of hydrocolloids

The choice of hydrocolloids for the present experiment was based on the analysis of literature data on the functional properties of these biopolymers and consultations with an adviser from Brenntag Polska Sp. z o.o. company (use of selected substances for fruit and vegetable processing). First, the hydrocolloids were added to the model product in the concentration range from 0.1 to 0.5%. Taking into account the changes in the consistency of the analyzed samples (sensory evaluation, viscosity measurement), two concentrations of hydrocolloids were selected for further studies (0.2 and 0.3%).

Production of strawberry beverages with hydrocolloids

Fresh, unpasteurized cloudy strawberry juice was heated in the Thermomix to 40ºC. When this temperature had been reached, hydrocolloids (CMC, GG, LBG, XG) were added at the dose of 0.2% and 0.3%, mixed, and heated with juice in the Thermomix to 90ºC. Beverages were poured into 80 mL colorless jars, left for pasteurization (10 min), and cooled in a water bath to 20ºC. Three replicates of beverages preparation were carried out.

Sensory evaluation of juice and beverages

The sensory evaluation of fresh strawberry cloudy juice and beverages was carried out by a group of 12 trained panelists (10 women, 2 men) using a universal 5-point scale [ISO 13299:2003]. The evaluators had methodical (theoretical and practical) preparation in the field of sensory analysis (senses, sensory language, properties and techniques).

The evaluation included the following sensory attributes: color, overall taste, aroma, consistency (I part of evaluation), as well the intensity of sweet/ sour/ tart/ foreign taste (II part of evaluation). Accordingly, an average score of 1 was equivalent to non-detectable taste in the sample, whereas score 5 denoted the maximum intensity. The intensity scales for the descriptors were provided in Table 1 and developed by the panelists.

Coded samples (four-digit codes) were provided to the panelists for the evaluation at a temperature of *ca*. 20ºC

TABLE 1. Sensory parameters of strawberry juice and beverages evaluated in descriptive analysis.

Quality parameter	Score				
	1	2	3	4	5
Color	Strongly changed or atypical	Changed, not very intense or with a brown shade	Clearly darker or lighter than the color of strawberry flesh	Intense, slightly darker or lighter than the color of strawberry flesh	Very intense, slightly darker or lighter than the color of strawberry flesh
Aroma	Strongly foreign	Unperceptible or weak, foreign	Poorly perceptible, no foreign odour	Aromatic, harmonized	Very aromatic, fresh, harmonized
Overall taste	Foreign	Changed, strawberry taste- unperceptitble, bitter, empty	Low-intensity, non-harmonized (eg. too sweet, too sour)	Intense, strawberry, harmonized	Very intense, strawberry, harmonized
Consistency	Strongly changed (undrinkable, too thick) or atypical	Changed (too thick) or atypical (heterogeneous, clearly stratified)	Drinkable, changed (thick)	Semi-liquid, homogeneous	Smooth, delicate, homogeneous, semi-liquid

in uniform, transparent, 50-mL plastic containers in complete randomized order. The members of sensory panel were seated in individual booths in a light and temperature controlled room. Distilled water was used to clean the palate between samples. All of strawberry products were evaluated in one session (9 samples).

Analysis of anthocyanins content in juice and beverages by UPLC-PDA

The content of anthocyanins in strawberry products was analyzed directly after processing and after 6 months of storage (4°C, no light exposure). Juice and beverages (2 mL) were centrifuged for 10 min (4°C, 15,000 rpm). The MillexSamplicity® Filters System (Merck Millipore, Darmstadt, Germany) was used for samples filtration. The filtered samples were kept at 4°C, whereas the analytical column was thermostated at 30°C (column oven). The mobile phase consisted of 4.5% formic acid (solvent A) and acetonitrile (solvent B). The program parameters were as follows: 0–1 min – isocratic elution with 99% of 4.5% formic acid; 12 min – linear gradient, lowering of solvent A to 0%; 12.5–13.5 min – return to 99% of solvent A (the initial composition). The injection volume was 5 μL and the flow rate was 0.45 mL/min. The runs were monitored at 520 nm. Calibration curves of anthocyanins (concentrations ranging from 0.05 to 5 mg/mL; $r^2 \leq 0.9998$) were made from cyanidin-3-*O*-glucoside, pelargonidin-3-*O*--glucoside, pelargonidin-3-*O*-galactoside, and pelargonidin--3-*O*-rutinoside as standards. Pelargonidin-3-malonylglucoside and cyanidin-3-malonylglucoside were expressed as pelargonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, respectively. The samples were analyzed in triplicate. Results were expressed in mg/L of juice/ beverages.

Identification of anthocyanins by the ultra-performance liquid chromatography–mass spectrometry (LC–ESI-MS) method

According to the procedure previously described by Wojdyło *et al.* [2014], identification of anthocyanins in strawberry juice and beverages was carried out using an ACQUITY Ultra Performance LCTM system (UPLC™) with a binary solvent manager and a Waters Micromass Q-Tof Micro mass spectrometer (Manchester, U.K.) equipped with an ESI (electrospray ionization) source operating in the positive ion mode.

Color measurements

Color properties of juice and beverages (L^*, a^*, b^*) were determined with a Color Quest XE colorimeter (Hunter Lab, Reston, Virginia, USA). The samples were filled into a 1-cm cell, and $L^*a^*b^*$ parameters were determined using 10° observer angle and Illuminant D65. The color measurement was done in triplicate. Products were analyzed directly after processing and after 6 months of cold storage (4°C, no light exposure).

Statistical analysis

Statistica version 12.5 (StatSoft, Poland) was used for statistical analyses of the results of color measurements and anthocyanin content determinations. One-way analysis of variance (ANOVA) by Duncan's test was used to compare the means. Differences were found significant at $p<0.05$. Results were presented as mean ± standard deviation of three determinations.

RESULTS AND DISCUSSION

Sensory evaluation of strawberry juice and beverages with added hydrocolloids

Members of the sensory panel evaluated a total of nine strawberry products (one juice and eight beverages) with 0%, 0.2%, and 0.3% of hydrocolloids of plant origin, that is CMC, GG, LBG, and the microbiological XG. The first part of the study included the evaluation of color, aroma, taste, and consistency, whereas in the second part, the panelists were focused on the evaluation of the intensity of taste sensations (Figure 1a-1d).

The strawberry products were characterized by attractive red color, however, its intensity was significantly changing as a result of the addition of hydrocolloids. Hence, for the evaluators the color attributed to 100% strawberry juice was considered best (average score 4.88), whereas the color of other beverages was perceived as less desirable. This effect was observed in all the products containing hydrocolloids. At the same time,

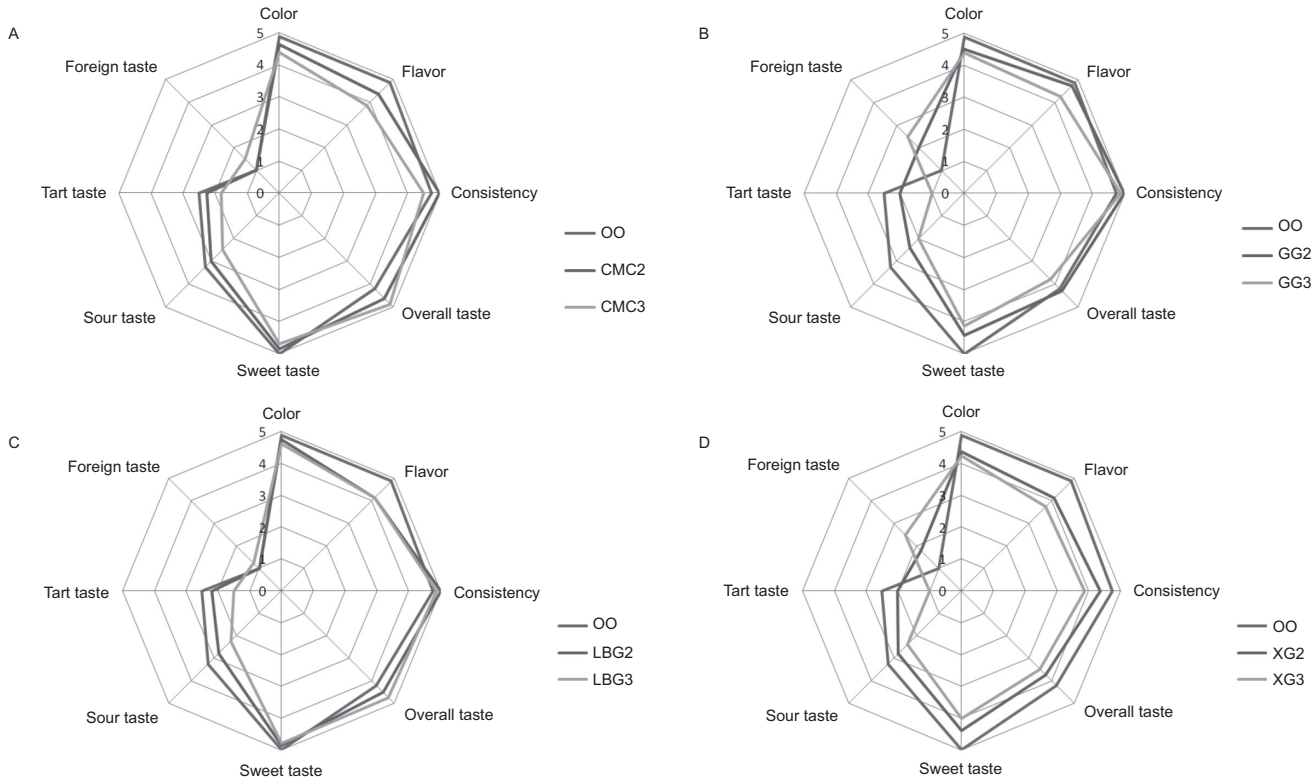

FIGURE 1. Sensory evaluation of strawberry juice and beverages with the addition of polysaccharide hydrocolloids.

OO – control sample (juice), CMC – beverages with carboxylmethylcellulose, GG – with guar gum, LBG – with locust bean gum, XG – with xanthan gum; 2 and 3 – 0.2% and 0.3% addition of hydrocolloids.

with an increase in their concentration, the scores in color evaluation were particularly lower. The least favorable effect on the color of beverages was reported in the case of addition of XG (average scores of 4.38 and 4.25 for products with 0.2% and 0.3% of XG, respectively).

In the sensory evaluation, higher scores were given to beverages containing plant hydrocolloids, especially LBG (average scores 4.75 and 4.63 for products with 0.2% and 0.3% of LBG, respectively). Products with CMC (average scores 4.63 and 4.38) and GG (average scores 4.50 and 4.38) were characterized by a less intense color.

In the concentration ranges studied, these substances also affected the assessment of aroma of the products. With the increase in the proportion of hydrocolloids in beverages, their aroma became less perceptible. However, it did not result from the emergence of foreign, undesirable odor in the tested samples, but only from masking the natural strawberry aroma. Sensory evaluation results indicated that such properties were attributed only to XG (at both concentrations) and among the substances of plant origin — to LBG and CMC at a dose of 0.2% and 0.3%, respectively. In the case of XG and LBG, their 0.2% addition to beverages caused a significant weakening of their aroma in relation to the control sample (in both cases, the score was 4.13). However, increasing the dose of both the hydrocolloids up to 0.3% appeared to be disadvantageous only in the case of XG (average score 3.75). The use of 0.2% additive of guar gum (GG2; average score 4.75) and 0.2% carboxymethylcellulose (CMC2; average score 4.38) affected the quality of strawberry beverages to the least extent. At their higher concentration, the effect of aroma masking was more prominent; however, the sample containing carboxymethylcellulose was given lower scores (CMC3; average score 3.88).

Interesting information was provided by the results of overall taste evaluation of strawberry beverages. Evaluators recognized samples containing plant hydrocolloids (except for guar gum at a concentration of 0.3%) as more tasty, in comparison to the control (100% cloudy juice made of strawberries; average score: 4.20). Simultaneously, the greater was the addition of hydrocolloids, the higher scores were given to the products by the evaluators. The highest scores were given to beverages made of CMC (average scores: 4.63 and 4.88 for CMC2 and CMC3, respectively). A beneficial effect on the overall taste was also reported upon the addition of LBG (average scores: 4.50 and 4.75 for LBG2 and LBG3, respectively). In the case of GG, a slight improvement in the flavor of the beverages was observed only at a dose of 0.2% (average score: 4.30). Unfavorable taste modification was caused by the addition of XG to the strawberry juice. In sensory evaluation, beverages with this additive were given the lowest scores (3.75 for XG2 and 3.50 for XG3). This proves that the impact of hydrocolloids on the food taste is not just a matter of the dose used, but above all of the specific, individual properties of these substances. The analysis of the intensity of taste sensations associated with the consumption of juice and strawberry beverages appeared to be helpful in the understanding of this phenomenon.

The "Roxana" cultivar of strawberries, used to produce juice in this experiment, is a dessert type cultivar. Hence, in all the products, sweet taste was predominant. Although the use of hydrocolloids significantly reduced the sweetness of strawberry drinks (proportionally to the dose used), it did not have a decisive influence on the results of previously described overall taste evaluation (Figure 1a-1d). It can be clearly observed in the case of the control sample containing 100% of cloudy juice. This product was the sweetest of all the products tested (average score: 5.00); however, in terms of overall taste perception, it cannot be equal to the beverages containing even CMC or LBG. It was more sour (average score: 3.25) and more tart (average score: 2.50; Figure 1a-1d), compared to the products with hydrocolloids (more balanced and milder taste).

The strength of sour taste masking by hydrocolloids was most evident in the beverages containing GG. With the addition of 0.2% GG, the detection of sour taste was expressed on a 5-degree scale by an average score of 2.40. By comparison, the control product obtained the score of 3.25 for the same property. Increasing the amount of GG up to 0.3%, the degree of reduction of sour taste was already so important that evaluators have identified its intensity as weakly perceptible (average score: 2.00). Guar gum effectively masked the tartness of strawberry products. This taste was not strongly constituted in the products tested (in the case of 100% cloudy juice, the average score was 2.50), although evaluators have clearly indicated the differences in the intensity of tartness among them. On the addition of 0.3% GG, the tart taste in the strawberry beverages was not identified at all (average score: 1.00). The use of XG provided equally strong effect; on addition to strawberry juice, it caused a decrease in the flavor intensity in the final product to a level equal to 2.0 (dosage: 0.2%) and then to 1.0 on a 5-point intensity scale (dosage: 0.3%). Despite these advantageous properties of both GG and XG, beverages with their addition were not as attractive in terms of taste as those containing CMC or LBG. It may probably be due to the perception of undesirable flavor in the products with GG and XG. However, all hydrocolloids used in the experiment provided a foreign flavor to the products, only for GG and XG was this effect not neutral among evaluators and had a negative impact on the overall assessment of the desirability of the taste of the beverages.

During the consumption of products containing hydrocolloids, a major role in the perception of taste and aroma is attributed to their rheological properties. The higher the values of viscosity (synonymous with increasing concentration of colloids), the lesser the ability of man to respond to sensory stimuli. This relationship was described by Pangborn *et al*. [1978], who studied the effect of the addition of xanthan gum and CMC on sensory properties of tomato juice, orange drink, and instant coffee. They have observed that increasing the concentration of hydrocolloids reduces the taste and favors the intensity of all products, regardless of the test temperature. The use of gums has significantly reduced the sourness and saltiness of tomato juice, sourness of orange drink, and bitterness of coffee. Baines & Morris [1987] found a similar effect of GG on sweetness and flavor of strawberry. In order to decrease the sensibility of this flavor by three fold, it was necessary to increase the viscosity by at least 2 orders of magnitude. Similar properties are also attributed to other hydrocolloids such as sodium alginate, pectins [Hayashi *et al*., 2005; Sun-Waterhouse & Wadhwa, 2013], and carrageenan [Calton & Wood, 2002]. Reduced perception of sour and tart taste in the strawberry products was probably due to the inhibition of hydrocolloids interactions with substances imparting the sensory properties to food in the cell membrane and taste receptors of the oral cavity [Sun-Waterhouse & Wadhwa, 2013].

Given the influence of hydrocolloids on the sensory quality of juices and fruit beverages, tartness masking appears to be a particularly important aspect. Although consumers occasionally consume products with intensively tart taste, for example, bitter chocolate, tea, or dry wine, in general, this taste is considered undesirable and largely contributes to the lack of acceptance of new products. The issue of tartness is the more problematic, since consumers are increasingly looking for products with reduced content of, for example, sugar, which naturally masks the undesirable tastes. In general, it is assumed that tartness is a sensory sensation derived from complexes of polyphenols with salivary proteins [Kallithraka *et al*., 2001]. Due to significant differences in the chemical structure of hydrocolloids, providing explanation for these interactions is difficult. For instance, due to the presence of functional groups in the molecule derived from acetic acid and pyruvic acid, XG is classified as an anionic compound [Sun *et al*., 2007; Garćia-Ochoa *et al.*, 2000]. In this case, it can be assumed that the suppression of astringency reflects interactions between the above mentioned groups and the ionic form of tannic acids. In addition, the spiral structure of XG allows "trapping" substance molecules, which are the carriers of tart taste. Another mechanism of action is attributed to nonionic hydrocolloids, for example, GG composed of polymannan chain randomly substituted with galactose molecules. The masking of astringency is based on the physical adsorption of substances with astringent properties to the polysaccharide surface [Troszyńska *et al*., 2010]. Hydrocolloids improve the sensory characteristics of food with no need of using *e.g.* sweetening agents. Consequently, they facilitate the processing of raw materials rich in bioactive compounds, but characterized with a specific and intense flavor profile.

Hydrocolloids are commonly used to concentrate fruit juices. This property leads to changes in the specific physical parameters (*i.e.* viscosity and stability of cloudiness), which can be visually observed as the change of consistency. Under the influence of addition of hydrocolloids, the tested strawberry products resembled more of smoothies than the cloudy juice. Nevertheless, the members of the sensory panel positively evaluated their texture, especially when these substances were used at a lower dose. Beverages containing 0.2% of CMC, GG, and LBG received the highest scores in the assessment of consistency among all samples analyzed (average value 5.00). At higher concentration of colloids (0.3%), this effect was not that positive. However, the consistency of the products with GG and LBG (average score: 4.88) was still perceived as better in comparison with the control product (average score: 4.75). The lower scores (XG2=4.38;

TABLE 2. Changes of color parameters of strawberry juice and beverages during storage.

Sample code	L^*		a^*		b^*	
	0 months	6 months, 4°C	0 months	6 months, 4°C	0 months	6 months, 4°C
OO	34.64±0.01^{g}	39.75±0.00^{c}	18.93±0.05^{h}	14.59±0.10^{i}	9.03±0.05^{g}	11.94±0.00^{b}
CMC2	34.65±0.01^{g}	38.12±1.50^{g}	19.36±0.10^{c}	18.21±0.30^{a}	8.97±0.00^{h}	11.02±0.10^{c}
CMC3	34.82±0.30^{e}	37.53±0.00^{i}	18.96±0.00^{g}	14.92±0.00^{f}	8.92±0.10^{i}	10.59±0.00^{g}
GG2	34.50±0.20^{h}	38.63±0.80^{f}	19.23±0.40^{d}	16.70±0.01^{c}	9.39±0.10^{c}	10.83±0.05^{f}
GG3	34.84±1.40^{d}	39.19±0.01^{d}	19.07±0.00^{f}	16.10±0.05^{d}	9.15±0.00^{e}	10.87±0.00^{e}
LBG2	34.70±0.10^{f}	38.95±0.20^{e}	19.13±0.01^{e}	15.23±0.50^{e}	9.26±0.06^{d}	10.91±0.00^{d}
LBG3	35.00±0.02^{c}	38.08±0.00^{h}	19.12±0.00^{e}	17.96±0.20^{b}	9.07±0.20^{f}	10.42±0.00^{h}
XG2	35.67±0.03^{b}	40.21±0.10^{b}	20.86±0.20^{b}	14.66±0.00^{h}	10.53±0.30^{b}	11.93±0.08^{b}
XG3	38.46±0.10^{a}	40.86±1.30^{a}	24.42±0.00^{a}	14.80±0.00^{g}	12.82±0.00^{a}	12.92±0.20^{a}

OO – control sample (juice), CMC – beverages with carboxylmethylcellulose, GG – with guar gum, LBG – with locust bean gum, XG – with xanthan gum; 2 and 3 – 0.2% and 0.3% addition of hydrocolloids.

Values were expressed as mean ± standard deviation (n=3); a.b.c… – statistically homogenous groups according to values in column (Duncan test. $p \leq 0.05$).

XG3=3.88) were given by the panelists to the beverages with XG,. Consistency of these products was strongly changed (to thick, undrinkable). Moreover, other sensory quality indicators, *i.e*., color, aroma, and taste, were not favorably perceived by evaluators, which potentially also affected the results of consistency assessment.

Color measurement in the CIE *L*a*b system**

Measurements of color parameters of juice and strawberry beverages were conducted using fresh products and these stored at 4°C for 6 months. Colorimetric analysis results are shown in Table 2 and Figure 2. In the control sample, the value of parameter L^* (lightness) was 34.64, whereas that of a^* parameter (proportion of red/green color) was 18.93 and that of b^* parameter (proportion of yellow/blue color) was 9.03. As a result of the addition of hydrocolloids, individual color components of beverages were shifted to a chromatic space, and the direction of these changes was dependent on both the dose and the type of the substance used. In the samples before storage, a positive and proportional relationship was observed between the dose of the hydrocolloid and the values of parameter L^* (Table 2).

Among products containing hydrocolloids of plant origin, an increase in the value of ΔL^*, that is brightness in relation to the standard (100% strawberry juice), was observed under the influence of CMC and LBG (Figure 2). However, in both the cases these values were close to zero, which indicates that in the range of concentrations tested, these hydrocolloids do not cause significant changes in the brightness of the products. A significant increase in the brightness was reported in the beverages containing XG, in which the values of ΔL^* were 1.03 and 3.82 (for doses of 0.2% and 0.3%, respectively). Considering the changes in the other parameters of the chromatic space (a^*, b^*), always the same relationship could be observed in these products always, that is, increasing the contribution of a particular color component in relation to the control sample, proportional to the hydrocolloid dose used (Figure 2).

Thus, both red and yellow color were more strongly constituted with XG in comparison to the control, and the values of Δa^* and Δb^* coefficients were 1.93 and 1.50 (0.2% XG) as well as 5.49 and 3.75 (0.3% XG), respectively. The addition of plant hydrocolloids (CMC, GG, LBG) to strawberry juice did not cause such dynamic color changes. Furthermore, the values of Δa^* and Δb^* estimated in the colorimetric test suggested that the mechanism of action of these substances differs from that observed in the case of XG. In the samples with added CMC, the contribution of red color was indeed higher than in the control sample; however, with an increase of CMC additive, this difference decreased (Figure 2). A similar regularity was found in the samples containing GG and LBG. The direction of changes in the values of parameter b^* was different. In beverages with added CMC, slightly smaller proportion of yellow color in relation to 100% strawberry juice was observed, wherein the greater the amount of hydrocolloid in the product, the greater was the color difference Δb^*. In the beverages with added GG and LBG, the contribution of b^* was higher than in the control sample, although with a decreasing trend in the value of color difference and increasing concentrations of hydrocolloids.

As a result of storage of strawberry products (6 months at 4°C), dynamic changes were observed in their chromatic space. All samples analyzed were lighter, less red, and more yellow in relation to the samples prior to storage (Table 2). In the control product, changes within the parameters L^* and b^* were more advanced in comparison to beverages containing hydrocolloids. This trend was partially confirmed by the results of red color intensity measurement. In beverages with added plant hydrocolloids (CMC, GG, LBG), differences between the values of parameter a^* before and after storage were lower than these observed in the control prod-

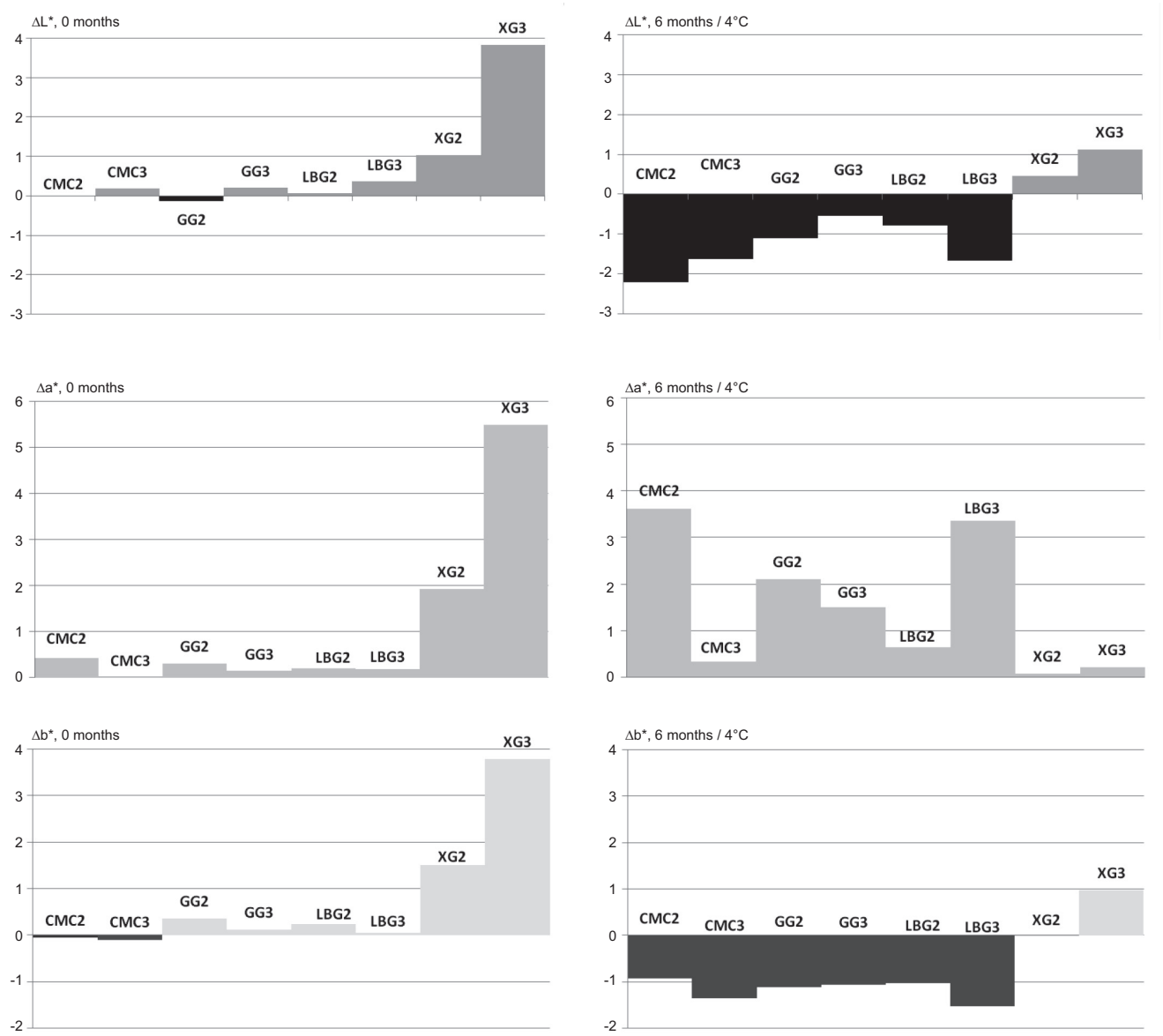

FIGURE 2. Effect of polysaccharide hydrocolloids addition on color parameters of strawberry beverages compared to control sample (100% juice) after and before storage (6 months, 4°C).

*ΔL** – changes of brightness; *Δa** – changes of redness/greenness; *Δb** – changes of yellowness/blueness.

uct. The proportion of red color was significantly reduced in the samples containing XG (Table 2).

Color conversion in the products containing hydrocolloids was also considered in relation to the parameters of the control sample, determining the values of *ΔL**, *Δa**, and *Δb** (Figure 2). After 6 months of storage, beverages containing CMC, GG, and LBG were darker than the control sample and (in case of CMC and GG addition) these differences were smaller at a higher dose of these substances. In all the other beverages, the contribution of red color was greater than that of 100% strawberry juice. Depending on the type and dose of hydrocolloids, the values of *Δa** ranged from 0.07 (XG2) to 3.62 (CMC2). In turn, the contribution of yellow color was observed to be lower in all the other beverages, except for those containing XG, in comparison with the control product. However, the influence of hydrocolloid dose on the direction of these changes appeared to be ambiguous. In drinks containing CMC and LBG, the values of *Δb** decreased with an increase in the proportion of hydrocolloids. In turn, in beverages with 0.2% and 0.3% addition of GG, the contribution of yellow color was similar, which resulted in slightly different values of *Δb**.

The ambiguous impact of hydrocolloids on the color of strawberry drinks could be related to the analytical technique used involving the measurement of radiation reflected from the surface. The control product contained mainly water in the free form, and the light was reflected from its surface differently than in the case of strawberry beverages characterized with a structure altered by the addition of hydrocolloids. Water can adsorb the radiation resulting in its lesser reflection from the surface [Pasławska *et al*., 2010]. Another factor affecting the measured values of color components could

also be an increase in samples turbidity caused by the addition of hydrocolloids and intensification of the phenomenon of oxygen incorporation into the products [Laaman, 2011].

Chaikham & Apichartsrangkoon [2012] have observed that pasteurized juice made of longan fruits (*Euphoria longana*) containing 0.15% of XG was characterized by lower brightness and a higher proportion of yellow and red color compared to the control sample. In the paper by Azoubel *et al*. [2011], concerning the restructuring of passion fruit (*Passiflora cincinnata*) pulp using hydrocolloids (gelatin, pectin, and alginate), changes were observed in the *L*a*b** color components. The addition of structure-forming substances caused an increase in the proportion of red color and a decrease in the proportion of yellow one, whereas the values of *L** parameter were changing ambiguously. Color changes observed in our study during the storage of strawberry products were probably associated with advanced polymerization of anthocyanins into brown compounds for which, among others, the degradation products of sugars and ascorbic acid are responsible for. The conversion of the monomeric forms of the anthocyanins into oligo- or polymeric pigments induces significant color changes toward the reddish brown color [Monagas *et al.*, 2006], which may result in an increase in $+a^*$ parameter value. This phenomenon is characteristic for long stored products [Piątkowska *et al.*, 2011; Krifi & Metche, 2000]. Polyphenol compounds are largely responsible for color conversion of juices, concentrates, beverages, and other fruit products. The final products of their decomposition can have different colors, which can be quantified using instrumental methods. As a result of the reaction between hydroxycinnamic acid and flavan-3-ols, colorless or slightly yellow caffeine, dehydrodicatechin A (yellow) and B (colorless), procyanidin A (colorless), or hetero dimers (some of them are red) are formed [Alonso-Salces *et al.*, 2005]. Changes in the pH value (from acidic to neutral) adversely affect the stability of the anthocyanins, causing even a complete loss of color [Giusti & Wrolstad, 2001]. The formation of *o*-quinones, as a result of the reaction of anthocyanins and polyphenoloxidase, leads to color change of food from red through blue to brown [Fang *et al.*, 2007]. All these processes affect color intensity and colloidal stability of the fruit products [Saucier *et al.,* 1997].

Content and storage stability of anthocyanin compounds

In the strawberry products, five anthocyanin compounds were identified including three pelargonidin glycosides (3-*O*--glucoside, 3-*O*-rutinoside, and 3-malonyl-glucoside) and two cyanidin glycosides (3-*O*-glucoside and 3-malonyl-glucoside) (Figure 3, Table 3). Their structure was confirmed *via* UPLC--MS/MS analysis, in which the retention times and mass spectra of anthocyanins present in the beverages were compared to the retention times and mass spectra of the standards (Table 3). In the case of acylated anthocyanins, their identification was based on a comparison of results obtained in our study to the data from previous studies. The total content of anthocyanins (TA) in a fresh 100% cloudy strawberry juice was 114 mg/L (Table 4). More than 90% of this value was mainly due to the two pelargonidin glycosides:

TABLE 3. Identification of anthocyanins in strawberry juice and beverages by UPLC-MS/MS method.

Peak number	λ_{max} (nm)	MS (m/z)	MS^2 (m/z)	Compound
1	516	449.109	287.056	Cyanidin-3-*O*-glucoside
2	518	433.113	271.061	Pelargonidin-3-*O*-glucoside
3	506	579.171	271.061	Pelargonidin-3-*O*-rutinoside
4	518	535.104	287.057	Cyanidin-3-malonylglucoside
5	520	519.113	271.069	Pelargonidin-3-malonylglucoside

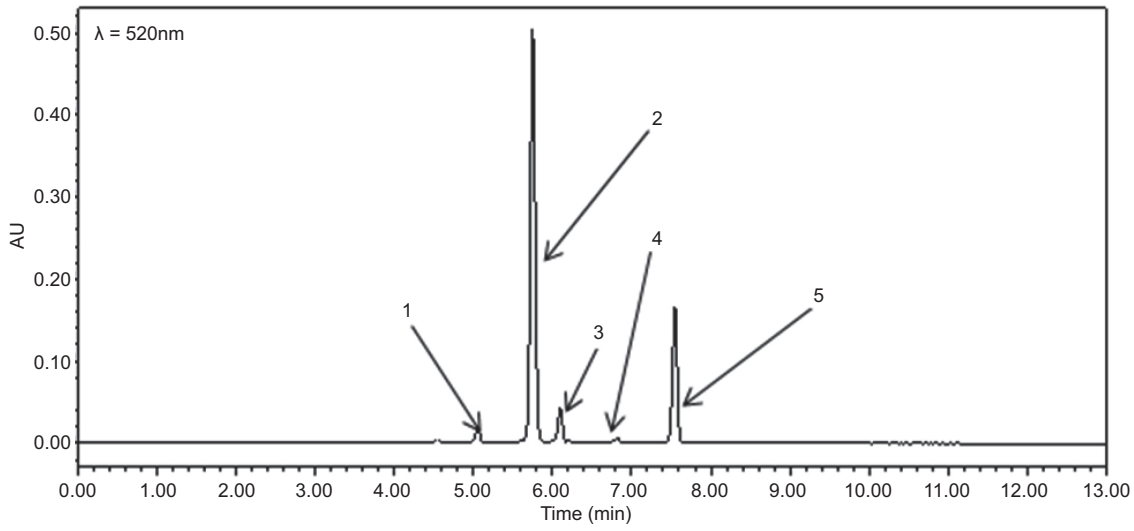

FIGURE 3. LC-PDA chromatogram of strawberry juice/beverages anthocyanins.

1 – cyanidin-3-*O*-glucoside, 2 – pelargonidin-3-*O*-glucoside, 3 – pelargonidin-3-*O*-rutinoside, 4 –cyanidin-3-malonylglucoside, 5 – pelargonidin-3-malonylglucoside.

TABLE 4. Changes in anthocyanins content (mg/L) in strawberry juice and beverages during storage

Sample code	Storage conditions	C-3-Glu	*Dd* (%)	P-3-Glu	*Dd* (%)	P-3-Rut	*Dd* (%)	C-3-mGlu	*Dd* (%)	P-3-mGlu	*Dd* (%)	TA	*Dd* (%)
OO	0 months	3.70^{a}		86.0^{a}		3.23^{a}		1.32^{b}		19.7^{a}		114 ^{a}	
	6 months, 4°C	0.652^{G}	82.4	14.1^{G}	83.5	0.00	100	0.00	100	3.41^{G}	82.7	18.2^{G}	84.0
CMC2	0 months	3.44^{d}		82.4^{d}		2.93^{f}		1.38^{a}		18.5^{d}		109 ^{c}	
	6 months, 4°C	1.04^{B}	69.7	23.4^{B}	71.6	0.00	100	0.00	100	4.70^{B}	74.4	29.2^{B}	73.2
CMC3	0 months	3.16^{h}		73.8^{i}		3.20^{b}		0.797^{g}		17.2^{i}		98.2^{i}	
	6 months, 4°C	1.25^{A}	60.5	16.6^{E}	77.5	0.00	100	0.00	100	4.69^{C}	72.7	22.5^{E}	77.0
GG2	0 months	3.57^{c}		82.5^{c}		2.83^{g}		0.917^{f}		18.8^{c}		108 ^{d}	
	6 months, 4°C	0.997^{C}	72.1	17.7^{D}	78.5	1.15^{C}	59.3	0.00	100	3.81^{E}	79.7	23.7^{D}	78.2
GG3	0 months	3.18^{g}		75.2^{h}		2.50^{i}		1.09^{e}		17.6^{h}		99.6^{h}	
	6 months, 4°C	0.719^{E}	77.4	18.5^{C}	75.4	1.20^{A}	52.1	0.00	100	3.95^{D}	77.5	24.4^{C}	75.5
LBG2	0 months	3.60^{b}		82.8^{b}		3.19^{c}		1.33^{b}		19.3^{b}		110^{b}	
	6 months, 4°C	0.725^{E}	79.9	15.9^{F}	80.8	0.250^{D}	92.2	0.00	100	3.45^{F}	82.2	20.3^{F}	81.5
LBG3	0 months	3.41^{e}		77.9^{f}		2.96^{e}		1.05		18.1^{e}		103 ^{f}	
	6 months, 4°C	0.862^{D}	74.7	26.3^{A}	66.3	1.18^{B}	60.2	0.00	100	7.80^{A}	56.8	36.1^{A}	65.1
XG2	0 months	3.00^{i}		80.8^{e}		3.07^{d}		1.18^{d}		17.9^{g}		106^{e}	
	6 months, 4°C	0.676^{F}	77.5	13.9^{H}	82.8	0.00	100	0.00	100	3.40^{G}	81.1	17.9^{H}	83.1
XG3	0 months	3.20^{f}		77.4^{g}		2.83^{h}		1.21^{c}		18.0^{f}		102 ^{g}	
	6 months, 4°C	0.592^{H}	81.5	13.5^{I}	82.6	0.00	100	0.00	100	3.13^{h}	82.6	17.2^{I}	83.2

C-3-Glu – cyanidin-3-glucoside, P-3-Glu – pelargonidin-3-glucoside, C-3-mGlu – cyanidin-3-malonylglucoside, P-3-mGlu – pelargonidin-3-malonyl-glucoside, TA – total anthocyanins, *Dd* – degradation degree

a.b.c… – statistically homogenous groups according to values in column for fresh samples (Duncan test. $p \leq 0.05$); A, B,C… – statistically homogenous groups according to values in column for samples stored for 6 months at 4°C (Duncan test. $p \leq 0.05$).

the dominant pelargonidin-3-*O*-glucoside (P-3-Glu; 74% TA) and pelargonidin-3-malonyl-glucoside (P-3-mGlu; 17% TA). Significantly lower concentrations were observed for cyanidin-3-*O*-glucoside (C-3-Glu; 3.25% TA). In strawberry beverages obtained by adding hydrocolloids to 100% cloudy juice, lesser amounts of anthocyanins were determined. As expected, in these products, the detectable concentration of the test compounds was inversely proportional to the dose of the hydrocolloids. Therefore, probably, these substances may trap the colored compounds, limiting the degree of their extraction to solutions that are subjected to chromatographic analysis. Of all the beverages tested, the most anthocyanins were determined in samples with LBG (LBG2=110 mg/L and LBG3=103 mg/L, respectively; $p<0.05$). In the samples with the addition of XG, their concentration was lower by 7% and 10% in relation to the control (for the XG2 and XG3, respectively). Beverages with CMC and GG were characterized by a similar content of TA (Table 4). In the concentration ranges of hydrocolloids under study, 5%–14% less anthocyanins were determined compared to strawberry juice. The key issue of the study was to determine how the addition of hydrocolloids can reduce the distribution of anthocyanins in beverages. As demonstrated by our previous study, the cloudy strawberry juice ("Roxana" cv.) obtained after the addition of hydrocolloids was characterized by a high degree of degradation of anthocyanin pigments [Teleszko *et al*., 2016]. At the same time, during the stage of fruit processing (laboratory scale), no addition of inactivators/PPO inhibitors was observed, which could exclude the potential impact of the additional factors or their interactions with hydrocolloids on the color stability of the products. The pasteurization process commonly used in fruit and vegetable industry was selected as a method of food preservation. The cloudy juice obtained and the beverages were stored at 4°C in the dark for 6 months. The storage experiment has shown that the addition of hydrocolloids to cloudy strawberry juice led to the partial degradation of anthocyanins. This effect, however, was directly dependent on the type of hydrocolloid.

After 6 months, only 16% of the initial content of the test compounds remained in 100% cloudy strawberry juice (Table 4). There was no presence of P-3-Rut and C-3-mGlu, and the degree of degradation (*Dd*) of other anthocyanin monomers was found in the range between 82.4% (C-3-Glu) and 83.5% (P-3-Glu). The addition of hydrocolloids did not prevent the degradation of C-3-mGlu, which is revealed due to the presence of a trace amount of this compound in the products tested (1% TA on average); however, this observation was not so relevant for the present experiment. Nev-

ertheless, the ability of hydrocolloids to stabilize compounds such as C-3-Glu or P-3-Rut differs significantly. It was also observed that increasing the concentration of hydrocolloids in the samples did not ensure an increase in the stability of active compounds during storage.

The lowest degree of degradation of anthocyanins was observed in the beverage containing 0.3% of LBG. After 6 months of storage, 36.1 mg TA/L was determined in this product, which corresponded to the amount of *Dd* at the level of 65.1%. Interestingly, when using a lower dose of LBG (0.2%), the protective effect of anthocyanins was negligible, and the degree of their decomposition was similar to that observed in the control sample (*Dd*=81.5%). Such strong diversity in the stability of TA depending on the concentration of hydrocolloid was only observed in the samples with LBG (Table 4). By comparison, in the beverages with GG, the values of *Dd* were 78.2% and 75.5% (for GG2 and GG3, respectively). In the products with CMC, the decomposition of anthocyanins was more advanced in the sample with 0.3% addition of this hydrocolloid, and *Dd* value was in the range of 73.1%–77.0% (for CMC2 and CMC3, respectively). There was, however, no significant effect of the use of XG on the inhibition of anthocyanins degradation in beverages.

In this case, the rate of TA degradation was not only the highest among samples containing the addition of hydrocolloids, but also the least diverse (XG2=83.1% and XG3=83.2%). The degree of degradation of anthocyanins was investigated not only in relation to their total content in the products, but also in terms of individual monomers. The average values of *Dd* coefficient of three major anthocyanins identified in beverages were comparable and were found between 74.1% (C-3-Glu) and 76.9% (P-3-Glu). Decomposition of C-3-Glu was reduced to the greatest extent by the addition of CMC, wherein the higher the concentration of this hydrocolloid in the product, the lower was the degree of degradation of C-3-Glu (CMC2=69.7%; CMC3=60.5%).

By comparison, in a control product stored under the same conditions, the value of *Dd* calculated for C-3-Glu was 82.4% (Table 4). P-3-Glu and P-3-mGlu were best preserved in the beverage containing 0.3% of LBG (*Dd* accounted for 66.3% and 56.8%, respectively). Moreover, the effect of hydrocolloids on the stability of P-3-Rut appeared to be interesting. It was observed that only the use of GG contributed to a satisfactory reduction in decomposition of this compound. By adding 0.2% GG, the degree of degradation of P-3-Rut in the product tested was reduced to 59.3%, while by adding 0.3% of GG, the value of *Dd* was reduced to 52.1% (Table 4). The protective effect against P-3-Rut was observed neither in beverages containing CMC, nor in those with XG. This effect was partially indicated in the beverage containing 0.3% LBG, for which the value of *Dd* was 60.2%.

Hubbermann *et al*. [2006] studied the effect of hydrocolloids (sodium alginate, citrus pectin, locust bean gum, carrageenan, and corn starch) on the stability of anthocyanins contained in elderberry concentrates and black currant. They demonstrated that the majority of hydrocolloids tested caused only slight color changes. Only sodium alginate significantly affected color stability of elderberry concentrate. In the case of longer storage, a trend for a^* value stabilization was also observed for pectin, corn starch, and sodium alginate in black currant concentrate. The molecular mechanism of anthocyanins binding with hydrocolloids is not well-understood. Fernandes *et al.* [2014] have described this process in relation to low-methylated pectin and pure anthocyanin preparations: cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside. Their studies have indicated that hydrogen bonds and hydrophobic interactions are involved in pectin–anthocyanins interactions.

CONCLUSION

The use of hydrocolloids in fruit processing allows obtaining new, sensory-attractive products with increased stability of anthocyanin compounds. The key issue is the selection of these substances, both in terms of quantity and quality. In the case of strawberry beverages, the best results were obtained using the addition of LBG. These products (at both concentration ranges of LBG) obtained higher scores in the taste and consistency assessment in comparison to 100% cloudy strawberry juice, which was the control sample. Above all, however, the use of LBG at a dose of 0.3% has contributed to the reduction of anthocyanins degradation up to 65%. Given their advanced decomposition in juice (*Dd* 84%), this effect can be considered satisfactory and important from the perspective of the quality of the analyzed products.

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Original research article
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Effect of Thermal Processing on Simultaneous Formation of Acrylamide and Hydroxymethylfurfural in Plum Purée

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Key words: acrylamide, hydroxymethylfurfural, plums purée, temperature, time, thermal treatment

The formation of acrylamide (ACR) and hydroxymethylfurfural (HMF) at different time and temperature combination in plum purée derived from two species was investigated. An optimized method for reducing ACR and HMF formation in thermally-treated plum purée was developed using a Central Composite Design model. Precursors of contaminants and their influence on the heating of plum purée were evaluated as well. The contaminants content was determined in thirteen running variants in the temperature range of 59.3–200.7°C, and heating time between 5.9 and 34.1 min. The model allowed establishing that the lowest ACR content was reached at 5.9-min exposure time and 130°C temperature, for both plum species (3.91 μg/kg and 8.73 μg/kg for *Prunus cerasifera* (P1) and *Prunus domestica* (P2), respectively). The lowest quantity of HMF was found at 20-min exposure time and 59.3°C temperature for both plum species (0.25 mg/kg and 0.18 mg/kg for P1 and P2, respectively). The results obtained allowed predicting the ACR/HMF levels in plum purée at different heating conditions.

INTRODUCTION

Plums are fruits rich in vitamins, minerals, antioxidants, and other bioactive components, with numerous health benefits [Birwal *et al.,* 2017]. Consumption of fresh thermally-treated plums (prunes, juice, compote, and jam) may prevent from anemia, constipation, obesity, and cardiovascular diseases [Sahamishirazi *et al.,* 2017]. During thermal treatment of plums, beside the desired sensorial properties, different compounds can be found such as advanced glycation end products (AGEs) and low-molecular-mass browning products such as acrylamide (ACR) and 5-hydroxymethylfurfural (HMF) [Nguyen *et al.,* 2016]. Their possible mutagenic, carcinogenic, and/or cytotoxic effects have been proved in previous research [Nursten, 2005]. In 2002, the Swedish National Food Administration added ACR to the list of food-borne toxic compounds, which have been found in high amounts in some heat-treated, carbohydrate-rich foods such as potato chips and crisps, coffee and bread [Swedish National Food Administration, 2002], and later in hazelnuts and almonds [Amrein *et al.,* 2007], dried fruits [Kukurová *et al.,* 2015], and vegetables [Constantin *et al.,* 2014]. Different pathways for ACR formation in foods have been reported such as Maillard reaction between free asparagine as the primary precursor and sugars [Zyzak *et al.,* 2003; Blank *et al.,* 2005]; formation from acrolein and acrylic acid [Yasuhara *et al.,* 2003]; formation from wheat gluten [Claus *et al.,* 2006], and from 3-aminopropionamide (3-APA) [Granvogl & Schieberle, 2006].

HMF is an organic compound included into the class of furans. It is formed as an intermediate in the Maillard reaction or through fructose dehydration under acidic conditions at elevated temperatures. HMF was found in bakery products, honey, malt, fruit products, coffee, vinegar, and dried fruit [Capuano & Fogliano, 2011]. In literature, a high variability in ACR and HMF contents has been reported between different studied products, which was mainly influenced by the difference in food composition including precursors' content (free asparagine and reducing sugars) present in raw materials and in process conditions applied (pH, water activity, temperature/time combination, presence of divalent cations) [Gökmen *et al.,* 2008]. These two neo-formed contaminants (NFC) are very interesting because of their high occurrence in food and toxicological potential such as mutagenic, carcinogenic and cytotoxic effects [Capuano & Fogliano, 2011]. The purpose of this study was to find out information on acrylamide and 5-hydroxymethylfurfural formation during

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thermal treatment of plum purée at different combinations of time and temperature. This study will help food processors to establish the optimal temperature/time combination to obtain lower amounts of ACR and HMF in the final product.

MATERIALS AND METHODS

Reagents and chemicals

Internal standards: 2,2,3-*d3*-2-propenamide (*d3*-ACR) and 2,4,4-*d3*-glutamic acid (*d3*-Glu), purity 97–98% were achieved from Cambridge Isotope Laboratories (Andover, Maryland, USA); standard of acrylamide (ACR), purity 99%, 21 L-Amino Acids Kit: L-alanine ≥98% (Ala), L-arginine monohydrochloride ≥98% (Arg), L-asparagine ≥98% (Asn), L-aspartic acid ≥98% (Asp), L-cysteine hydrochloride anhydrous ≥98% (Cys), L-glutamine ≥99% (Gln), L-glutamic acid ≥99% (Glu), L-glycine ≥99% (Gly), L-histidine monohydrochloride monohydrate ≥98% (His), *trans*-4-hydroxy-L--proline ≥98% (Hyp), L-isoleucine ≥98% (Ile), L-leucine ≥98% (Leu), L-lysine monohydrochloride ≥98% (Lys), L-methionine ≥98% (Met), L-phenylalanine ≥98% (Phe), L-proline ≥99% (Pro), L-serine ≥99% (Ser), L-threonine ≥98% (Thr), L-tryptophan ≥98% (Trp), L-tyrosine ≥98% (Tyr), L-valine ≥98% (Val), and L-ornithine ≥98% (Orn), hydroxymethylfurfural (HMF) 99% purity, perfluorooctanoic acid (PFOA) 96%, and acetonitrile HPLC gradient grade were purchased from Sigma-Aldrich (Steinheim, Germany); ethyl acetate and acetic acid glacial grade were purchased from Fischer Scientific (Loughborough, UK); methanol HPLC-grade, potassium hexacyanoferrate trihydrate, and zinc sulfate heptahydrate were achieved from Merck (Schuchardt, Germany). Nylon syringe filters (0.45 μm) were obtained from Waters (Milford, Milford, MA, USA).

Plant material

Fruits of two plum species: *Prunus cerasifera* – cherry plum (P1) and *Prunus domestica* Angeleno (P2), were used in this study. The selected plums represented the offseason species available on the Romanian market. Plums have been purchased on the local market and stored at 4°C before analysis. The dry matter content was determined with a classic thermogravimetric method (removing the water using an oven at 105°C temperature) and revealed the following values in plums: 10.18% for P1 and 9.03% for P2. Plums were washed, homogenized at 10,000 rpm for 15 s (Grindomix Retsch GM200), and heat treated according to the experimental model.

Extraction and analysis of plum purée amino acids

For the determination of amino acids, 2 g of plum purée were weighed into a 10-mL centrifuge tube. The extraction solution (20 mL, 0.1 % acetic acid (v/v)) was added to the sample and stirred for 30 min at 150 rpm (Heidolph Unimax 2010, Schwabach, Germany) and centrifuged at 19,621×*g* for 10 min (Sigma 2–16 KC, Germany). The samples were diluted using the following protocol: 100 μL of the clear supernatant of each sample were transferred to a 10-mL volumetric flask, 50 μL of *d3*-Glu (stock solution 10-fold diluted) were added, and the flask was filled up with 0.1% acetic acid (v/v). The extract was filtered through a 0.45 μm nylon syringe filter before the LC/MS analysis. The LC/ESI-MS-MS analyses for quantification of free amino acids profile were performed in the HPLC system 1200 series coupled to an Agilent 6410 Triple Quad detector equipped with ESI interface (Agilent Technologies, Santa Clara, California, USA). The analytical separation was performed on a Purospher STAR RP-8ec column (4.6×150 mm, 3μm particle size) (Merck, Darmstadt, Germany) using an isocratic elution with a mixture of 100 mL of acetonitrile and 900 mL of aqueous solution of PFOA (0.05 Mm) at a flow rate 0.5 mL/min at ambient temperature. All parameters of the electrospray ionization tandem mass spectrometry (ESI-MS-MS) system were based on in-source generation of the protonated molecular ions of the amino acids measured and the internal standard (*d3*-Glu) as well as collision-induced production of amino acid-specific fragment ions for Multiple Reaction Monitoring (MRM) experiments [Constantin *et al.,* 2014].

Reducing sugars content determination

Determination of the content of reducing sugars was performed using the 3,5-dinitrosalicylic acid (DNS) according to AOAC method [1995]. Briefly, 2 mL of the sample homogenized with 2 mL of 0.04 M DNS solution were maintained in a boiling-water bath for 10 min. After cooling, the contents of the tubes were brought to the volume of 10 mL with distilled water. The absorbance of the mixture was measured at 535 nm wavelength against a prepared blank using a Jenway 6506 UV-Vis Spectrophotometer (Cole-Parmer, Stone, United Kingdom). The results were expressed as mg glucose/g DW.

Thermal treatment and experimental design

Plum purée was subjected to heat treatment using a thermostat (Liebisch Labortechnik, Germany), in a range of temperatures between 59.3 and 200.7°C, and heating times between 5.9 and 34.1 min according to the experimental model parameters of which are presented in Table 1. Central Composite Design (CCD) and response surface modeling have been used to optimize the thermal treatment of plum purée to obtain ACR and HMF. CCD builds a quadratic model for response variables. The design involves three distinct sets of steps: a factorial design on the variables studied, a set of focal points and a set of points or samples axial stay. The design investigates five levels of each variable studied. Circumscribed data set was used, with a distance of ±1.4142 proven stars. The experiments were carried out in the order given by the software to determine the influence of external factors in the analysis. Two parameters were analyzed such as temperature and time of thermal treatment, and contents of ACR and HMF were selected as the answers Design-Expert® software (Stat-Ease, Inc.) was used for data analysis.

The experimental conditions can be described by equation (1).

$$R = b_0 + b_1A + b_2B + b_3AB + b_4A^2 + b_5B^2 \quad (1)$$

where: A, B are independent variables studied and b_0 – intercept, b_1-b_5 represent regression coefficients, constants for

TABLE 1. Matrix of experimental design (coded levels and real values) with responses in terms of acrylamide (ACR) and hydroxymethylfurfural (HMF) content in purée made of *Prunus cerasifera* (P1) and *Prunus domestica* (P2) plums.

Run	Coded levels		Actual levels		Plum purée P1		Plum purée P2	
	Temperature (°C) (A)	Time (min) (B)	Temperature (°C) (A)	Time (min) (B)	ACR (μg/kg DW)	HMF (mg/kg DW)	ACR (μg/kg DW)	HMF (mg/kg DW)
1	0	0	130	20	21.50	34.73	9.92	4.68
2	-1	+1	80	30	35.28	25.38	50.55	2.85
3	-1.41	0	59.3	20	8.49	0.25	31.72	0.18
4	-1	-1	80	10	5.48	0.34	15.99	0.51
5	0	0	130	20	30.18	34.69	12.30	4.53
6	0	0	130	20	27.37	34.53	9.85	5.48
7	0	0	130	20	22.74	32.75	11.57	3.94
8	0	-1.41	130	5.9	3.91	35.17	8.73	0.45
9	+1.41	0	200.7	20	1024.89	139.06	634.75	90.40
10	0	0	130	20	22.70	31.86	15.07	3.79
11	+1	0	180	30	876.68	100.72	596.89	92.88
12	0	+1.41	130	34.1	552.29	95.36	79.42	25.19
13	+1	-1	180	10	447.77	116.89	263.18	57.03

the effect of the general process, the linear and quadratic effects of each independent variable, as well as the interaction effects of the variables on the content of ACR and HMF.

ACR content determination

After heat processing of plum purée, ACR was extracted with 30 mL of 0.1% acetic acid (v/v) and further pre-extracted with ethyl acetate to avoid the negative impact of salts in the chromatographic system according to procedures published before by Constantin *et al.* [2014] and Ciesarová *et al.* [2009]. Acetic acid extraction step was repeated three times. The samples were shaken for 1 min and then clarified with 1 mL of Carrez solution I (15% potassium ferrocyanide) and 1 mL of Carrez solution II (30% zinc acetate). The acetic acid extracts were collected and brought to a total volume of 100 mL with 0.1% acetic acid (v/v). After mixing for 1 min and sonication for 10 min, the samples were shaken by a vortex mixer for 1 min, sonicated for 5 min, and centrifuged at -5°C for 10 min at 19,621×*g* (Sigma 2–16 KC, Germany). A volume of 5 mL of the clear supernatant was transferred to a test tube with the addition of 100 μL of internal standard *d3*-ACR solution (2 mg in 100 mL of water) and 5 mL of ethyl acetate, and mixed well for 1 min. The ethyl acetate top layer was removed to a clean test tube. The step of pre-extraction with ethyl acetate was repeated three times, and all the ethyl acetate layers were collected and evaporated under vacuum at 35°C to dryness. The dry residue was dissolved in 1 mL of 0.1% acetic acid (v/v) and filtered through a nylon syringe filter with 0.45 μm pore size to glass vials before LC--MS analysis.

The LC/ESI-MS-MS technique using the 1260 Infinity HPLC system coupled to 6410 Triple Quad LC/MS equipped with ESI interface (Agilent Technologies, Santa Clara, California, USA) was applied. The analytical separation was performed on Atlantis dC18 column (30×100 mm, 3 μm pore size, Waters, Milford, MA, USA) using isocratic elution of 1% methanol (v/v) and 0.2% acetic acid (v/v) in water as a mobile phase at 0.4 mL/min flow rate at 25°C. All parameters of the electrospray ionization tandem mass spectrometry (ESI-MS-MS) system were based on in-source generation of the protonated molecular ions of ACR and the internal standard (*d3*-ACR), as well as collision-induced production of specific fragment ions for MRM experiments (transition for ACR: 72 → 55, transition for *d3*-ACR: 75 → 58). The following instrumental parameters were used for ACR analysis in the ESI+ mode: drying gas (N_2) flow of 8 L/min, gas temperature of 350°C, nebulizer pressure of 345 kPa, capillary voltage of 2.5 kV, fragmentor of 80 V, collision energy of 5 eV, and dwell 50 ms. Calibration was performed by diluting the ACR stock solution (5 mg in 100 mL of water) in the range of 10 – 2000 ng/10 mL with 50 μL of the internal standard (*d3*-ACR).

HMF content determination

After heat processing of plum purée, HMF was extracted with 30 mL of a methanol: water mixture (80:20, v/v). The mixture was sonicated for 5 min, centrifuged for 10 min at 19,621×*g* (Sigma 2–16 KC, Germany) and filtered through 0.45 μm nylon membrane syringe filters. Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, California, USA) equipped with UV/VIS detector (DAD) was used for HMF analysis. The chromatographic separation was performed on C18 SB column (4.6×250mm, particle size 5 μm, Waters, Milford, MA, USA) using the gradient elution at a flow

TABLE 2. Contents of amino acids and reducing sugar in purée made of *Prunus cerasifera* (P1) and *Prunus domestica* (P2) plums.

Compound		Plum purée	
		P1	P2
Amino acids (mg/kg DW)	Hyp*	0.28±0.09	0.00±0.00
	Asp*	5.56±0.10	4.93±0.10
	Pro*	51.93±0.75	39.65±0.45
	Asn*	178.41±1.01	138.78±0.35
	Ser	5.95±0.02	5.88±0.19
	Gln*	34.08±0.36	91.51±0.01
	Thr*	3.32±0.05	3.40±0.07
	Glu*	5.64±0.10	8.59±0.23
	Gly*	0.73±0.08	0.72±0.11
	Ala*	10.22±0.22	13.15±0.18
	Val	3.06±0.02	5.05±0.06
	Met	0.00±0.00	0.00±0.00
	Tyr	0.00±0.00	0.00±0.00
	Ile*	2.38±0.02	6.08±0.03
	Leu*	2.40±0.02	6.07±0.03
	Phe*	1.40±0.05	3.58±0.02
	His*	2.71±0.23	1.34±0.05
	Orn*	0.27±0.03	0.38±0.01
	Lys*	0.54±0.00	0.97±0.00
	Arg*	0.32±0.05	0.79±0.08
	Trp	0.16±0.00	0.41±0.00
	Sum	14.75±0.11	15.77±0.00
Reducing sugars (RS) (mg/g DW)	RS*	11.12±1.03	9.18±0.34

Explanations: Hyp – 4-trans-hydroxyproline, Asp – aspartic acid, Pro – proline, Asn – asparagine, Ser –serine, Gln – glutamine, Thr –threonine, Glu – glutamic acid, Gly – glycine, Ala – alanine, Val – valine, Met – methionine, Tyr – tyrosine, Ile – isoleucine, Leu – leucine, Phe – phenylalanine, His – histidine, Orn – ornithine, Lys – lysine, Arg – arginine, and Trp – tryptophan.

* indicates significant difference between samples ($p<0.05$); DW – dry weight.

rate of 0.8 mL/min at 25°C. The mobile phase consisted of % of methanol (A), 0.01 M H_3PO_4 (B), and acetonitrile (C). Gradient composition for HMF determination was applied as following: 0–1.5 min, 0–2% A, 100–95% B, 0–3% C; 1.5–2.1 min, 2% A, 95% B, 3% C; 2.1–3.0 min, 2–8% A, 95–86% B, 3–6% C; 3.0–11.0 min, 8% A, 86% B, 6% C; 11.0––11.5 min, 8–94% A, 86–0% B, 6% C; 11.5–20.0 min, 94% A, 0% B, 6% C; 20.0–20.1 min, 94–2% A, 0–95% B, 6–3% C; 20.1–30.0 min, 2% A, 95% B, 3% C.

The HMF was detected at its absorption maximum of 280 nm and quantified using external calibration curve in the range from 0.05 to 1.0 μg/mL.

Statistical analysis

Data were analyzed using multivariate data analysis and Design Expert v. 10.1 software from Design-Expert® (Stat-Ease, Inc., Minnesota, USA) and by paired t-test using SPSS19.0 (IBM, New York, NY, USA).

RESULTS AND DISCUSSION

Contents of amino acids and reducing sugars in plum purée

The purées from fresh red plum were analyzed for ACR and HMF precursors, and the results of determinations of contents of amino acids and reducing sugars are presented in Table 2. The amino acids analysis showed a high asparagine content in both plums compared to other amino acids (178.41±1.01 mg/kg DW for P1 and 138.78±0.35 mg/kg DW for P2). It is known that asparagine is a crucial participant in the production of ACR in the Maillard reaction [Mottram *et al.,* 2002]. Its high content in P1 and P2 plum purée may be considered responsible for the ACR formation.

Reducing sugars content in P1 and P2 plums purée was 11.12±1.03 mg/g DW and 9.18±0.34 mg/g DW, respectively. Leong & Oey [2012] obtained similar results (9.70±0.98 – 16.54±0.04 mg/g DW) for plum from the Otago region (South Island, New Zealand). The presence of reducing sugars is essential in both ACR and HMF formation, as they form a Schiff base with asparagine, and then by decarboxylation ACR, while HMF is formed by their caramelization or thermal dehydration [Friedman, 1996; Abraham *et al.,* 2011].

Thermal treatment optimization

The Central Composite Design (CCD) and surface response modeling were used to determine the optimal parameters (temperature and time) of the thermal treatment of plum purée, and to achieve minimal contents of ACR and HMF. Table 1 presents the matrix of the complete CCD used in optimization with actual values of the main variables studied, and the corresponding values of the ACR and HMF content measured. The optimized coded model for contaminants was represented using ANOVA (Table 3 and Table 4).

Acrylamide formation

ACR is generally formed in thermally treated foods (>120°C), with a high level of carbohydrates [Tareke *et al.,* 2000, 2002; Gökmen, 2015]. As it can be seen in Table 4, the quantities of ACR in the thermally treated plum purée have considerably fluctuated. These differences could depend on plum species, their chemical composition (reducing sugar and amino acid content), and processing conditions (time and temperature) [Becalski *et al.,* 2011]. The model selected revealed the concentration of the ACR formed in the treated purée to highly depend on the selected parameters. A low ACR content was obtained for the eighth running variant (130 min/5.6°C) for both plum species (Table 1). Furthermore, the content of ACR determined in sample P1 was almost 2-fold higher when compared to the sample P2. This significant difference can be due to the higher content of asparagine and reducing sugars. ACR was suggested to be formed by the specific amino acid route due to the suf-

TABLE 3. ANOVA for the square surface of the acrylamide (ACR) formation during thermal treatment of purée made of *Prunus cerasifera* (P1) and *Prunus domestica* (P2) plums.

Statistical parameters	Sum of Squares	df	Mean Square	F Value	p-value
Plum purée P1					
Model	1.578E+006	5	3.156E+005	101.10	< 0.0001
A-Temperature	*9.255E+005*	*1*	*9.255E+005*	*296.54*	*< 0.0001*
B-Time	*1.904E+005*	*1*	*1.904E+005*	*61.01*	*0.0001*
AB	*39823.27*	*1*	*39823.27*	*12.76*	*0.0091*
A^2	*3.740E+005*	*1*	*3.740E+005*	*119.83*	*< 0.0001*
B^2	*88163.21*	*1*	*88163.21*	*28.25*	*0.0011*
Residual	21847.92	7	3121.13		
Lack of Fit	*21792.95*	*3*	*7264.32*	*528.60*	*< 0.0001*
Pure Error	*54.97*	*4*	*13.74*		
R-Squared			0.9863		
Adj R-Squared			0.9766		
Plum purée P2					
Model	*5.933E+005*	*5*	*1.187E+005*	*63.15*	*< 0.0001*
A-Temperature	*3.388E+005*	*1*	*3.388E+005*	*180.30*	*< 0.0001*
B-Time	*27406.26*	*1*	*27406.26*	*14.58*	*0.0066*
AB	*22371.76*	*1*	*22371.76*	*11.91*	*0.0107*
A^2	*2.046E+005*	*1*	*2.046E+005*	*108.88*	*< 0.0001*
B^2	*5039.41*	*1*	*5039.41*	*2.68*	*0.1455*
Residual	*13153.71*	*7*	*1879.10*		
Lack of Fit	*13135.39*	*3*	*4378.46*	*955.88*	*< 0.0001*
Pure Error	*18.32*	*4*	*4.58*		
R-Squared			0.9783		
Adj R-Squared			0.9628		

ficient contents of reducing sugars related to the content of asparagine. The CCD allowed estimating equations which enable predicting the most suitable models for the production of ACR for both samples (P1 and P2) as follows (Eq. 2 and 3):

$$\text{P1 ACR} = +24.90 + 340.14\,A + 154.28\,B + 99.78\,AB + 231.87\,A^2 + 112.58\,B^2 \quad (2)$$

$$\text{P2 ACR} = +11.74 + 205.79\,A + 58.53\,B + 74.79\,AB + 171.49\,A^2 + 26.91\,B^2 \quad (3)$$

Optimized coding models for ACR contents were represented by regression analysis and variance analysis (ANOVA), and the quadratic models were applied. From the ANOVA (Table 3), it can be seen that both models (P1 and P2) fitted well to optimization data (R^2=0.9863 and R^2 is 0.9783, respectively), and the F values (101.10 and 63.15) indicated that the pattern was significant. In this case, the significant model terms were: A, B, AB, A^2, B^2 for P1 and A, B, AB, A^2 for P2. The ACR content determined in samples P1 and P2 was positively correlated with all individual terms, with the greatest influence of the A^2 (square of temperature response) for P1, and A for P2 sample.

Figure 1 shows the correlative effect of temperature and time on ACR formation. The content of ACR increased as the processing temperature of the plum purée increased. As it can be seen from the response surface graph (Figure 1A and B), ACR formation was minimal at the shortest time of thermal treatment, between 10 and 20 min. For the plum purée P1, the lowest ACR formation was at the minimum exposure time, between 15 and 20 min. Moreover, an increase in ACR formation at high temperatures (≥180°C) was correlated with an extended thermal treatment interval (≥25 min) (Figure 1A). For the plum purée P2, the combined effect of time and temperature revealed an increased effect on ACR formation at temperatures above 180°C and up to 25 min (Fig-

TABLE 4. ANOVA for the square surface of the hydroxymethylfurfural (HMF) formation during thermal treatment of purée made of *Prunus cerasifera* (P1) and *Prunus domestica* (P2) plums.

Statistical parameters	Sum of Squares	df	Mean Square	F Value	p-value
Plum purée P1					
Model	23234.07	5	4646.81	39.46	< 0.0001
A-Temperature	*18836.10*	*1*	*18836.10*	*159.97*	*< 0.0001*
B-Time	*1104.44*	*1*	*1104.44*	*9.38*	*0.0183*
AB	*424.31*	*1*	*424.31*	*3.60*	*0.0995*
A^2	*1851.49*	*1*	*1851.49*	*15.72*	*0.0054*
B^2	*1386.95*	*1*	*1386.95*	*11.78*	*0.0110*
Residual	824.25	7	117.75		
Lack of Fit	*817.24*	*3*	*272.41*	*155.44*	*0.0001*
Pure Error	*7.01*	*4*	*1.75*		
R-Squared			0.9657		
Adj R-Squared			0.9413		
Plum purée P2					
Model	14022.45	5	2804.49	89.37	< 0.0001
A-Temperature	*9394.83*	*1*	*9394.83*	*299.38*	*< 0.0001*
B-Time	*669.31*	*1*	*669.31*	*21.33*	*0.0024*
AB	*280.75*	*1*	*280.75*	*8.95*	*0.0202*
A^2	*3589.86*	*1*	*3589.86*	*114.40*	*< 0.0001*
B^2	*292.43*	*1*	*292.43*	*9.32*	*0.0185*
Residual	219.66	7	31.38		
Lack of Fit	*217.86*	*3*	*72.62*	*161.41*	*0.0001*
Pure Error	*1.80*	*4*	*0.45*		
R-Squared	0.9846				
Adj R-Squared	0.9736				

ure 1B). Therefore, time is also an important parameter involved throughout the process, besides the temperature, with a crucial influence in ACR formation. ACR was also formed at the lower temperatures (59.3°C and 80°C), but in lower amount (8.49 and 5.48 μg/kg DW, respectively). In a study conducted by Roach *et al.* [2003], the formation of ACR in prune juice was highlighted at a temperature range below 120°C (98–116°C) at higher moisture conditions. Additionally, in a study conducted by Amrein *et al.* [2007], substantial amounts of ACR were found in plums dried at temperatures below 90°C. Furthermore, besides the temperature and time, the starting reactants present in the food matrices are also important in the Maillard reaction, such as sugar and amino acid type [Yaylayan & Stadler, 2005]. Although, the ACR formation involves the condensation of the amino group of asparagine (as the principal precursor) and the carbonyl groups of reducing sugars, when the samples are subjected to heat [Becalski *et al.,* 2003; Mottram *et al.,* 2002; Stadler *et al.,* 2002; Zyzak *et al.,* 2003], other amino acids may have a positive effect in obtaining small amounts of ACR in some model systems, such as proline, tryptophan, cysteine, glycine, lysine, *etc*. [Yu *et al.,* 2013; Koutsidis *et al.,* 2009]. The differences in ACR content between P1 and P2 plum purée at a temperature below 80°C, apart from the variations of heating duration, could also be caused by the higher content of proline in P2 sample, which could lead to a low ACR production. In a study reported by Koutsidis *et al.* [2009], proline and tryptophan (80%) were the most effective amino acids involved in decreasing the ACR levels followed by cysteine and glycine (45–55%). Thermal treatment (temperature above 120°C) can initiate the deamination and decarboxylation of asparagine, with a higher yield of ACR when a carbonyl source is present [Yaylayan *et al.,* 2003; Weisshaar *et al.,* 2002]. This fact can explain the ACR content in P1 and P2 plums, that are rich in reducing sugars and have a high level of asparagine (Table 2). According to Mottram *et al*. [2002], the ACR production is slightly influenced by the presence of glutamine and aspartic acid; the ACR quantities obtained were very low,

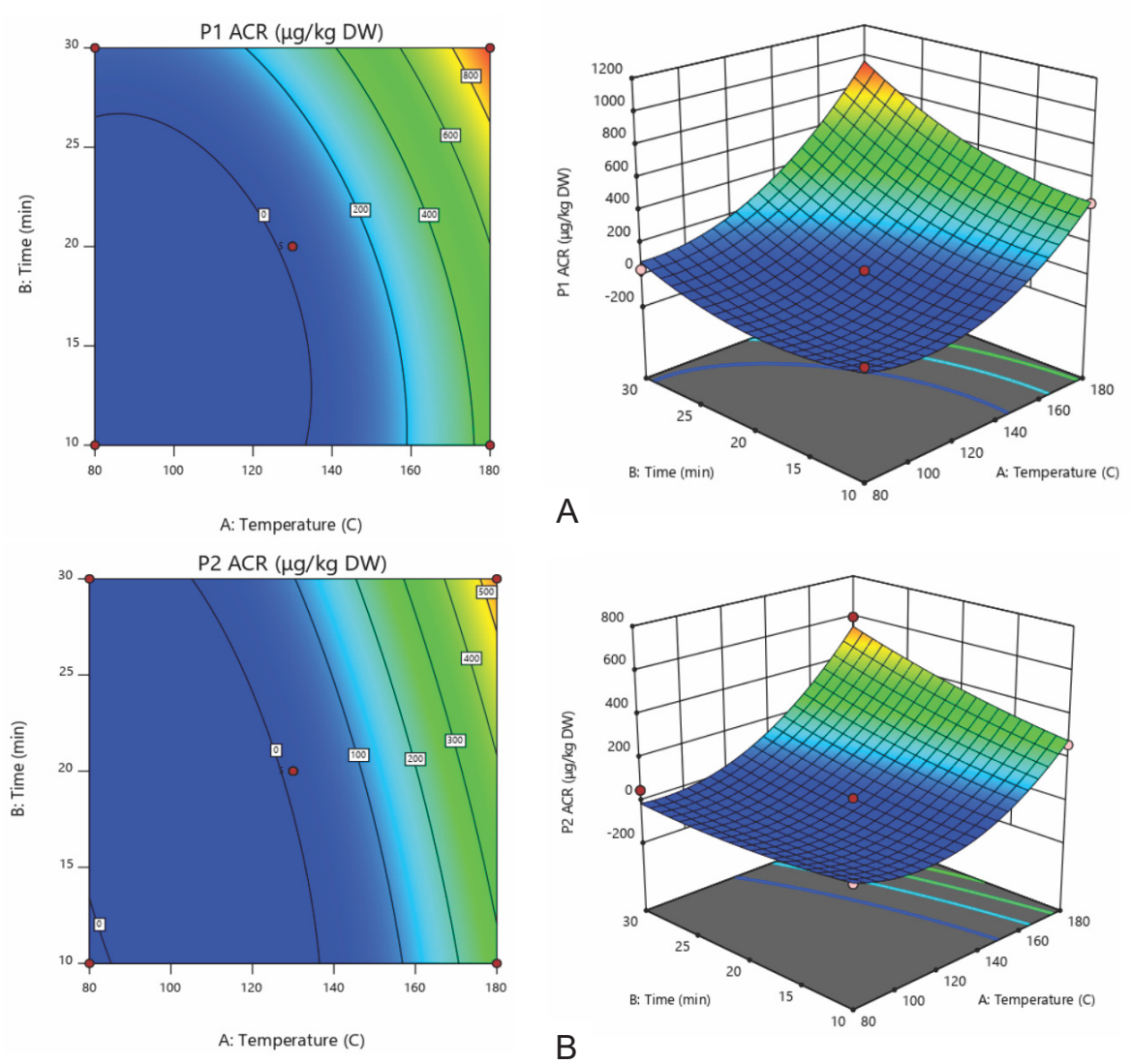

FIGURE 1. Contour graphs describing the quantitative correlative effects of time and temperature on the formation of acrylamide (ACR) in puree of plum *Prunus cerasifera* (P1) (A) and *Prunus domestica* (P2) (B).

in the range of 0.5–1.0 mg/mol. In another study, Zyzak *et al.* [2003] used a system model containing: amino acid, glucose, maltodextrin, emulsifier, potato starch, and water, to identify the ACR formation mechanism. The study revealed that a high quantity of ACR was formed (9270 µg/kg) in the model system with asparagine. Additionally, high quantities of ACR were obtained in the system with glutamine (156 µg/kg) and low quantities (<50 µg/kg) in the systems with: alanine, arginine, aspartic acid, cysteine, lysine, methionine, threonine, and valine. In our study, among the amino acids involved in the ACR formation, the highest content was found for asparagine, however limited quantities of alanine, arginine, aspartic acid, lysine, and valine were found as well (Table 2). There is a few data on the ACR levels in thermally obtained plum products such as jams. In a survey on ACR content in various thermally processed plum products from the Slovak market, the level of ACR varied between 15 µg/kg and 46 µg/kg [Kukurová *et al.,* 2015]. In a similar study regarding the ACR levels in foods from the Turkish market, Ölmez *et al.* [2008] found less than 10 µg/kg ACR in strawberry jam. However, different ACR contents in thermally treated fruits were reported, such as: 14.74–1680 µg/kg in dried plums [Amrein *et al.,* 2007; De Paola *et al.,* 2017], 1432–1502 µg/kg in dark pears, 0–19 µg/kg in pears, 173.43–879.92 mg/kg in Abu variety banana fritter, and 30.07–201.18 mg/kg in Awak variety banana fritter [Daniali *et al.,* 2013].

Regulatory bodies have not established so far the minimum and maximum content of ACR in thermally treated products. Meanwhile, the industrial environment has continuously been concerned to implement measures to reduce the amount of ACR in food based on the application of effective mitigation and quantification strategies. The European Commission recommends the selection of raw material with a reduced level of ACR precursors for the baby jar foods (low-acid and prune-based foods) [Commission Regulation (EU) 2017/2158].

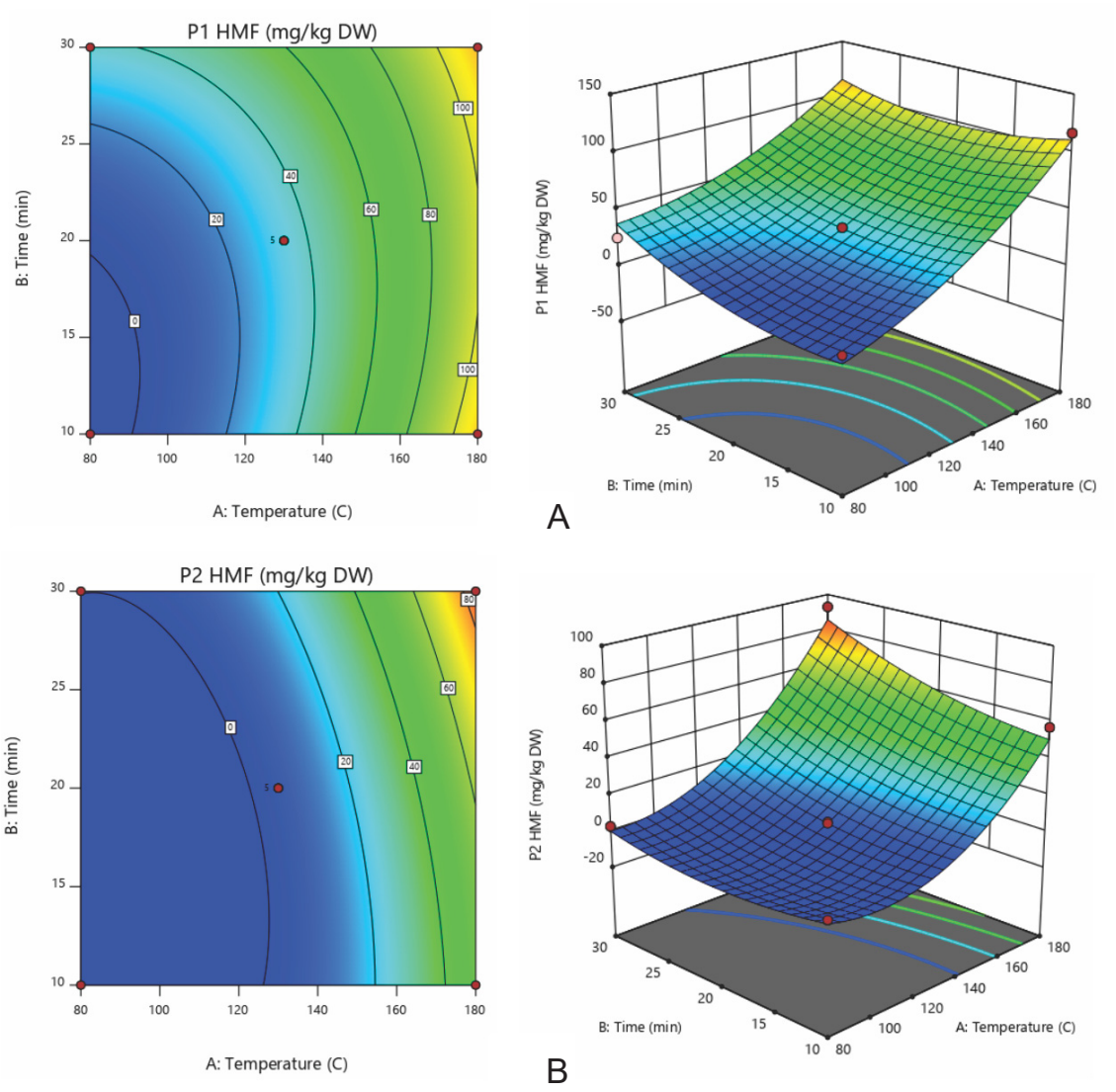

FIGURE 2. Contour graphs describing the quantitative correlative effects of time and temperature on the formation of hydroxymethylfurfural (HMF) in puree of plum *Prunus cerasifera* (P1) (A) and *Prunus domestica* (P2) (B).

Hydroxymethylfurfural formation

As it can be seen in Table 1, a low HMF content was found for variants 3 and 4 of P1 sample and for variants 3, 4 and 8 of P2 sample. By using low temperatures for heat treatment of plums, the amount of HMF formed was lower, even if the time treatment was longer. The CCD allowed estimating equations which enable predicting the most suitable models for the production of HMF for both samples (P1 and P2) as follows (Eq. 4 and 5):

$$P1\ HMF = +33.71 + 48.52\ A + 11.75\ B - 10.30\ AB + 16.31\ A^2 + 14.12\ B^2 \quad (4)$$

$$P2\ HMF = +4.49 + 34.27\ A + 9.15\ B + 8.38\ AB + 22.72\ A^2 + 6.48\ B^2 \quad (5)$$

From the ANOVA (Table 4) for the model chosen, the significant terms are A, B, A^2, and B^2 for plum purée P1 and A, B, AB, B^2 and A^2 for plum purée P2. The HMF content in samples P1 and P2 was positively correlated with all individual terms; the A term (temperature response) having the greatest influence on HMF formation. In the case of P1 sample, HMF content was negatively correlated with AB. Figure 2 (A, B) depicts the temperature-time effect on HMF production. HMF formation was minimal at exposure time between 10 and 25 min, and at exposure temperature of around 120°C. HMF is formed by heating, as an intermediate in the Maillard reaction, [Mauron, 1981; Glatt & Sommer, 2006]. Kavousi *et al.* [2015] suggested that the presence of the amino acids, glutamine, glutamic and aspartic acids led to an accelerated formation of HMF compared with the addition of basic amino acids in the model systems. Another mechanism for HMF formation implies direct thermal dehydration of fructose, sucrose, and glucose, without the presence of amino groups [Antal *et al.,* 1990].

By analyzing the 13 running variants, the highest level of HMF was obtained in the plum purée exposed to 200°C for 20 min for P1 plums (139.06 mg/kg DW) and to 180°C for 30 min for P2 plums (92.88 mg/kg DW) (Table 1). According

to Kocadagli *et al.* [2012], chlorogenic acid promotes the hydrolysis of sucrose to fructose, at a temperature above 180°C, that may contribute to HMF formation. Moreover, in a study conducted by Zhang *et al.* [2016] by heating fructose with or without aspartic acid (at 90°C /48 h), to simulate plums drying, it was found that chlorogenic acid increased HMF concentration. In this case, the tested temperature was below 100°C, but the time of exposure was longer.

The amino acids present in the food matrix may also contribute to the increase of HMF content, probably due to the sucrose hydrolysis which is catalyzed by the presence of amino acids [Lee & Nagy, 1990]. Moreover, a higher content of HMF was formed by heating fructose with aspartic acid at pH 7.0, compared to fructose alone [Zhang et al., 2016]. In a study conducted by Rada-Mendoza *et al.* [2002] where HMF was analyzed in various types of fruit jams (apple, apricot, banana, bilberry, fig, lemon, mulberry, orange, pineapple, plum, strawberry), the obtained results varied between5.5 and 37.7 mg/kg. In our study, the HMF content varied as a function of temperature/time coordinates between 0.25 and 139.06 mg/kg in P1, and between 0.18 and -92.88 mg/kg in P2, respectively.

The HMF levels in food are established only for honey by Codex Alimentarius, that allows a maximum concentration of 40 mg HMF /kg of productand also by the industry that set an upper limit for fruit juice at 20 mg/kg [Morales *et al.,* 2008]. For plum products and jams, the regulatory bodies have not set a limit for HMF content.

Global optimization

The "Desirability" approach is one of the most widely used methods in the industry for optimizing multiple response processes. The global desirability function is defined as the geometric mean of the partial functions. A non-zero value of desirability implies that all the selected criteria were in a good combination and a value closest to 1 shows the best of combinations. Therefore, for sample P1 a 0.895 value of Desirability was obtained, that implied value of 27.8 mg/kg DW for HMF and a value of 3.90 μg/kg DW for ACR. For sample P2, similar results were obtained, such as a 0.979 value of Desirability which correlates to a minimum of 3.95 mg/kg DW for HMF and 8.74 μg/kg DW for ACR.

CONCLUSIONS

Our study data suggest that ACR is formed in the heat-treated plum purée samples through the specific amino acid pathway and is due to the high contents of reducing sugars and asparagine. In general, the contents of ACR and HMF in the tested plum purées highly depended on the applied combination of time and temperature, with a minimum ACR content obtained between 10 and 20 min and an increase in contents of both tested toxicants at higher temperature.

Central Composite Design model was used to estimate equations predicting the most suitable models for the production of ACR and HMF in the treated samples. The global desirability of the both selected varieties were close to 1, showing the best of combinations. Therefore, the optimal parameters in terms of temperatures and time combination that should be applied are: 114.2°C/23.8 min for *Prunus cerasifera*, and 127.7°C/21 min for *Prunus domestica*.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Original research article
Section: Food Quality and Functionality

Predicting the Botanical Origin of Honeys with Chemometric Analysis According to Their Antioxidant and Physicochemical Properties

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Key words: honey, botanical origin, physicochemical analyses

The aim of this study was to develop models based on Linear Discriminant Analysis (LDA), Classification and Regression Trees (C&RT), and Artificial Neural Network (ANN) for the prediction of the botanical origin of honeys using their physicochemical parameters as well as their antioxidative and thermal properties. Also Principal Component Analysis (PCA) and Cluster Analysis (CA) were performed as initial steps of data mining. The datasets consisted of 72 honey samples (false acacia, rape, buckwheat, honeydew, linden, nectar-honeydew and multifloral) obtained from different regions of Poland and collected between April 2014 and November 2016. Ash content, pH, free acidity, colorimetric coordinates in the CIELAB space (L*, a*, b*, h*, C*), total phenolics content, antioxidant activity, and glass transition temperatures (T_g) of the honey samples were determined. The first four principal components accounted for about 85% of the total variance. PC1 was highly correlated with colour intensity, the hue angle (h*), and total phenolics content, whereas PC2 was dominated by chroma (C*) value and glass transition temperatures (T_g). The CA dendrogram displays two clusters: one with light coloured honey samples and second with dark coloured honey samples. On the basis of the LDA analysis, the colour parameters possessed the highest discrimination power according to the botanical origin of honey samples. The models based on ANN and C&RT algorithms were characterized by 100% accuracy. Study results demonstrate that the chemometric approach enables high-accuracy classification of honeys according to their botanical origin.

INTRODUCTION

Honey is the only food product produced by honeybees (*Apis mellifera*). Pursuant to the definition of the Council Directive [Council Directive, 2001/110/EC], honey is a sweet substance made by honeybees from the nectar of flowers, plant saps or excretions of plant-sucking insects. During honey production, bees transform the collected materials by combining them with specific substances of their own and leave the honey in honeycombs to ripen and mature. Thus, the unique composition and properties of honey stem from its origin [Bertelli *et al.,* 2010]. All the honey is composed mostly of carbohydrates and also contains minerals, amino acids, proteins, vitamins, enzymes (diastase and invertase), organic acids (gluconic acid, acetic acid), volatile compounds, and phenolic compounds [Gheldof *et al.,* 2002; Bogdanov *et al.,* 2008; De La Fuente *et al.,* 2011]. As it was shown in previous studies, honey exhibits various biological properties, including the antioxidant activity which is ensued from the content of such bioactive compounds as: antioxidant enzymes (catalase, peroxidase), ascorbic acid, carotenoids and phenolic compounds, including both the flavonoids and phenolic acids as well as products of Maillard reaction [Al-Mamary *et al.,* 2002; Gheldof *et al.,* 2002; Bertoncelj *et al.,* 2007; Estevinho *et al.,* 2008; Alvarez-Suarez *et al.,* 2010; Brudzynski & Miotto, 2011a,b]. The phenolic content and composition of honey depend strongly on nectar type [Meda *et al.,* 2005; Al *et al.,* 2009; Habib *et al.,* 2014], thus the phenolic profile could potentially be used to determine the botanical origin of the honey [Beretta *et al.,* 2005; Anjos *et al.,* 2015; Nayik *et al.,* 2016; Nayik & Nanda 2016]. The origin of honey also highly influences its sensory properties, like colour and flavour. The colour of honey is closely related to its composition such as the presence of pigments, mainly chlorophylls, carotenoids and some phenolic compounds [Lazaridou *et al.,* 2004]. Moreover, honey colour is influenced by storage conditions like time and temperature [Baltrušaitytė *et al.,* 2007]. The antioxidant activities of honeys have frequently been associated with their colour. Generally, the following tendency could be observed: the more darker the colour of the honey is, the most potent antioxidant properties it exhibits [Brudzynski & Miotto, 2011b, a]. Some studies have focused on the colour parameters to enable determination of the botanical origin of honey [Beretta *et al.,* 2005; Juszczak *et al*., 2009; Tuberoso *et al.,* 2014; Anjos *et al.,* 2015; Nayik & Nanda, 2016; Siddiqui *et al*., 2017]. The physicochemical properties of honey, like pH, conductivity, water and ash content, are also influenced by its composition and thereby by its botanical origin.

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Recently, instead of measurements of individual parameters, a global approach has been recommended in the determination of the botanical origin of honeys. Thus, thermal analysis can be applied alongside other parameters. Differential scanning calorimetry enables the analysis of the thermal behaviour of a honey sample under changing temperatures. Honey, as a supersaturated aqueous solution, can undergo a characteristic phenomenon, *i.e.* glass transition, which occurs as an effect of heat capacity variation. The glass transition temperature (T_g) can be used for the prediction of honey rheological behaviour [Lazaridou *et al*., 2004], for the assessment of its authenticity [Cordella *et al.,* 2002], or for the determination of possibility of its spray drying [Tonon *et al.,* 2009]. Glass transition temperature depends mainly on the moisture and honey type. Generally, the T_g increases with a decreasing water content and an increasing molecular weight of solutes [Tomaszewska-Gras *et al.,* 2015]. The main sugars of honey: fructose and glucose, also affect the T_g value. However, the final T_g is governed by the sugar ratio which depends on the botanical source and environmental conditions [Ahmed *et al.,* 2007]. Unifloral honeys exhibit differences in flavours, biological activity, colours and other properties and together with honeydew honey are the most highly-priced bee products. In recent years, as an effect of the increasing fertilization of plant cultivars, honey has become the product of limited supply. All these favour the practice of honey adulteration. To protect the high quality of honey and to ensure its authenticity, specific regulations has been set by the EU [Council Directive, 2001/110/EC]. Thus, the authentication of honey – which means it should agree with its botanical and/or geographical origin – has become an important issue nowadays.

In recent years, numerous studies have addressed determinations of the botanical origin of honeys. The most frequently used method for the determination of honey adulteration and authenticity is the pollen analysis (mellisopalnyology). Nevertheless, as this procedure requires highly specialised personnel and is very time-consuming, it loses its importance as a fast routine method for assessing honey quality [Benedetti *et al.,* 2004].

The botanical origin of honey can be assessed by combining physicochemical parameters (pH as content, total acidity *etc*.) with multidimensional data analysis. Such an approach has shown good approximation [Anjos *et al.,* 2015; Nayik & Nanda, 2016; Popek *et al.,* 2017].

To the best knowledge of authors, studies on the classification of Polish honeys are scarce [Madejczyk & Baralkiewicz, 2008; Chudzinska & Baralkiewicz, 2010; Popek *et al.,* 2017].

The aim of the present investigation was to describe and classify Polish honeys (from various regions of Poland) according to their botanical origin based on a combination of their antioxidant properties, results of their thermal analysis, and values of their physicochemical parameters. The novelty of this study is to use thermal analysis and antioxidant properties together with other physicochemical parameters for the determination of the botanical origin. The underlying interrelations between parameters were studied using chemometric methods including unsupervised and supervised pattern recognition techniques.

MATERIAL AND METHODS

Honey samples

The present study was carried out using eight different types of honey, namely: false acacia (*Robinia pseudoacacia*) n=12, rape (*Brassica* spp.) n=6, buckwheat (*Fagopyrum esculentum*) n=12, honeydew n=9, heather *(Calluna vulgaris)* n=6, linden (lime) n=12, nectar-honeydew n=3, and multifloral n=12. The honeys came from various regions of Poland, were collected between April 2014 and November 2016, and were obtained from local associations of beekeepers. Honey samples were stored in the dark at room temperature 20–25°C. Before analysis, they were heated in a bath (35°C) for 20 min and homogenised.

Ash content

Ash content was determined by incinerating honey samples in a muffle furnace (Thermo Scientific™ M110 Muffle Furnaces, Germany) at a temperature of 550°C [AOAC, 1995], and expressed in g/100 g.

Free acidity and pH

Free acidity was measured by a titrimetric method AOAC 962.19 [AOAC, 1996]. 0.1 M NaOH was added to a 10% w/v solution of honey. The solution of honey was prepared with CO_2 free distilled water, up to pH 8.3. The results were expressed as milliequivalents/kg (meq/kg).The pH values were measured with a pH-meter (Microcomputer pH Meter CP-551, Elmetron, Poland) in a 10% w/v solution of honey in distilled water, according to the AOAC Official Method 962.19 [AOAC, 1996].

Colour determinant values

Colour indices (L* and a*, b*) were measured by the CIELab system using a Minolta Chroma-meter (Spectrophotometer CM-5, Konica Minolta, Japan) with illuminant D65. It expresses colour as three numerical values, L* for the lightness and a* and b* for the green–red and blue–yellow colour components. Also the hue angle (h*) and chroma (C*) were calculated as:

$$h^* = tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (1)$$

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

Samples were illuminated at 45°. Measurements were made on homogenised honey samples.

Colour intensity

Honey sample colour intensity was measured using the method of Beretta *et al.* [2005]. Honey samples were diluted to 50% (w/v) with warm (45°C) deionised water and the solution was then filtered through a 0.45 μm pore size filter. The absorbance readings were taken at 450 and 720 nm using a spectrophotometer (Varian Cary 1E UV/Visible Spectrophotometer, USA) and the difference in absorbance (A_{450} $-A_{720}$) was expressed as mAU.

Total phenolics content

Total phenolics content was determined according to the method of Singleton & Rossi [1965] with some modifications [Muzolf-Panek *et al.,* 2016]. For total phenolics content determination, honey (10 g) was dissolved in 10 mL of deionised water. The solution was mixed in the dark at room temperature for 30 min, and filtered (0.45 μm pore size filter). Then, the filtrate was centrifuged at 4000 rpm for 5 min. The supernatant was removed and filtrated again. The sample was prepared each time on the day of the analysis.

An aliquot of 20 μL of honey extract was mixed with 100 μL of Folin-Ciocalteu reagent and left to stand in a dark place at room temperature for 3 min. Then, 300 μL of sodium carbonate (20% m/v) and 1580 μL of deionised water were added and mixed. After 2 h of incubation, the absorbance was read at 765 nm against a blank sample (prepared as previously described but deionised water was used instead of the extract). For each sample, three separate determinations were conducted. All results were presented as mg gallic acid equivalent (GAE) per 100 g of honey sample.

DPPH radical scavenging activity

The antioxidant activity of honeys was evaluated by the so-called DPPH method according to the procedure of Sánchez-Moreno *et al.* [1998] with some modifications [Muzolf-Panek *et al*., 2016]. In brief, an aliquot of 10 μL of honey extract (prepared as described above) was mixed with 990 μL of 0.1 mM DPPH in methanol. After 30 min of incubation at room temperature without the access of light, the absorbance was read at 515 nm using a spectrophotometer (Varian Cary 1E UV/Visible Spectrophotometer, USA). For each sample, three separate determinations were conducted. After the addition of the honey sample to the solution of DPPH$^{\bullet}$ in methanol, a decrease of absorbance was observed in comparison to the control sample. The higher was the concentration of the honey sample, the greater decrease of absorbance was obtained, and from the difference between the absorbance of the control sample and samples with honey the percentage of the scavenged DPPH$^{\bullet}$ was calculated. Then, the slope of the linear plot of the scavenged DPPH$^{\bullet}$ by the honey extracts *versus* honey concentration was determined. The antioxidant activity (AA) of honey sample was expressed as mmol Trolox equivalent (TE) per 100 g of honey. Additionally, IC_{50} values defined as the concentration of the sample which ensures 50% reduction of DPPH$^{\bullet}$ concentration were calculated.

Thermal analysis

Differential scanning calorimeter DSC 7 (Perkin Elmer, Norwalk, USA) was used to determine the glass transition temperature (T_g) of honey samples. The device equipped with an Intracooler II and running under Pyris software 10.0 was calibrated using the standards of indium (T_m=156.60 °C, ΔH= 28.45 J/g, Perkin Elmer) and n-dodecane (99.8 purity, Tm = -9.65 °C, Merck). The honey samples were weighed into aluminum pans (Perkin Elmer, 50 μL total volume, No. B016–9321) and hermetically sealed. The analysis of T_g involved the following three steps: (1) heating from 25°C to 100°C, with a scanning rate of 10°C/min; (2) cooling from 100°C to -65°C; and (3) heating from -65°C to 100°C, with a scanning rate of 10°C/min. The reference was an empty, hermetically sealed aluminum pan. All the samples were analysed by the DSC in at least three replications. The obtained glass transition temperatures (T_g) were calculated from the second heating scans as a midpoint temperature according to Tomaszewska-Gras *et al.* [2015].

Statistical analysis

Each honey sample was analysed in triplicate and the results are expressed as mean ± standard deviations (SD). All statistical tests were performed using Statistica 13.0 software (StatSoft, Tulsa, Oklahoma, USA). Prior to multivariate analysis, the entire data matrix was standardised (except C&RT analysis). Person's linear correlation coefficients (r) between selected parameters were calculated. Principal Component Analysis (PCA) and Cluster Analysis (CA) were used as the first step of multivariate data analysis to visualize information and to find patterns in data sets. Furthermore, linear discriminant analysis (LDA), classification and regression trees (C&RT), and artificial neural networks (ANN) were used to calculate classification rules for sample discrimination. The significance level was set at 5%.

RESULT AND DISCUSSION

Physicochemical characteristics

The results of the physicochemical measurements of all the types of honey samples are shown in Table 1. All the measured parameters are in accordance with the Polish Standards for honey [Polish Standard PN-A-77626:1998]. The highest ash content was recorded for honeydew honey (0.8 g/100 g), next for nectar-honeydew honey (0.46 g/100 g). This is consistent with the study of Popek *et al.* [2017] where ash content was 0.6 and 0.5 g/100 g, respectively. The lowest value in ash content was noted for multifloral honey (0.17 g/100 g). The colour parameters of the selected honey types showed a high variability. The L*, a* and b* values of honey samples varied from 26 to 50, -3 to 7 and 5.8 to 23, respectively (Table 1). The obtained values indicate that almost all of the honey samples were dark coloured. Only the acacia honey can be classified as light coloured with an L* value equaling 51 [Gonzalez-Miret *et al.,* 2005]. Acacia, rape and multifloral honey samples possessed a green component (negative value of index a*) whereas all the others possessed the red component (positive value of index a*). All the analysed honey samples had a yellow constituent in their colour. The colour intensity of a 50% (w/v) honey solution varied from 263 mAu for the pale acacia honey to 1506 mAu for the dark honeydew honey. This parameter is directly related to the occurrence and concentration of compounds containing conjugated double bonds such as terpenes, carotenoids, and flavonoids that absorb light in the visible range (400–700 nm) [Młodzińska, 2009]. As reported in Table 1, the total phenolic content was low in the pale honeys of monofloral origin, like in rape (5.5 mg GAE/100 g) and acacia (10.3 mg GAE/100 g) honeys, whereas the highest TPC value was noted for dark buckwheat honey (>60 mg GAE/100 g). A similar polyphenol content was recorded in the heather, arbutus, and locust pod-shrub honey collected from various regions of Portugal [Alves *et al.,* 2013]. The TPC level recorded in this study for Polish buckwheat honey was markedly lower than the results obtained

TABLE 1. Results of physicochemical parameters, antioxidant activity (IC_{50}, AA), total phenolic content (TPC), and glass transition temperature (T_g) of individual types of honey.

Type of honey/ Measurement	Acacia	Buckwheat	Linden	Nectar-honeydew	Rape	Honeydew	Multifloral	Heather
L*	51±5.6	33±8.7	40±3.6	31±0.1	43±0.5	26±1	42±1.9	26±0.4
a*	-3.41± 0.44	2.25±3.84	0.46±1.86	7.9±0.1	-3.03±0.36	6.32±1.22	-1.14±1.05	0.54±0.16
b*	18.6±4.59	8.39±3.48	21.53±10.61	15.57±0.06	16.32±0.25	7.89±1.39	23.7±8.99	5.8±0.21
h*	-0.19±0.05	0.22±0.35	-0.04±0.17	0.47±0.01	-0.18±0.02	0.67±0.04	0.05±0.04	0.09±0.03
C*	18.9±4.49	9.29±3.88	21.66±10.49	17.46±0.07	16.6±0.29	10.11±1.81	22.74±9	5.83±0.2
Colour intensity (mAu)	263±9.3	1424±449.8	532.8±191.6	852.7±17.2	229.1±28.1	1506.8±104.1	491.4±81.4	1199 ±24.5
pH	4.19±0.06	4.07±0.16	4.23±0.05	4.78±0.01	4.22±0.02	5.02±0.4	4.21±0.27	4.25±0.01
Free acidity (meq/kg)	12.8±0.82	34.25±10.67	31.09±11.26	22±0.5	10.5±1.05	32.67±1.49	34.04±25.33	32.33±1.03
Ash content (g/100 g)	0.2 ±0.02	0.22±0.12	0.19±0.09	0.46±0.1	0.22±0.1	0.8±0.04	0.17±0.04	0.41 ±0.02
IC_{50} (mg/mL)	69.4±9.2	28.1± 3.7	74.3±5.1	19.9±2.7	184.8±28.1	14.28±1.1	91.4±17.1	113.8±18.8
TPC (mg GAE/100 g)	10.3±3.51	62.33 ±9.64	20.48±5.16	42.85±5.83	7.53±2.21	48.75±9.98	19.03±4.6	31.72 ±6.23
AA (mmol TE/100 g)	56.3±6.4	95.56±5.42	30.50±1.28	101.24±3.75	11.29±2.12	142.27±9.86	27.63±5.97	35.97±8.04
T_g (°C)	-40.6±1.16	-42.21±1.90	-41.18±3.93	-35.19±0.48	-43.78±5.69	-41.55±3.56	-40.76±2.41	-47.11±0.95

by Mellen *et al.* [2015] (296.22 mg GAE/100 g) and Dżugan *et al.* [2018] (135 mg GAE/100 g). These authors reported also the highest TPC values for rape, linden, nectar-honeydew, and honeydew honeys accounting for 25.45, 40.91, 63.03, and 60.01 mg GAE/100 g, respectively [Dżugan *et al.,* 2018]. The results of the present study showed (Table 1) that the unifloral honeys, rape and heather, had the lowest antioxidant activity. Their IC_{50} values were 184.8 and 113.8 mg/mL, respectively. The highest radical scavenging activity was noted in honeydew and nectar-honeydew honey samples (14.28 and 19.9 mg/mL, respectively). It is difficult to compare directly results of the antioxidant activity and TPC of honeys with literature data. In previous researches, authors used various modifications of the DPPH assay and different units to express the results. However, the results concerning the antioxidant activity of acacia honey were similar to that obtained by Krpan *et al.* [2009] who showed that the mean antioxidant activity of acacia honey expressed as IC_{50} value was equal to 111 mg/mL. Also the antioxidant activity of acacia honey reported by Bertoncelji *et al.* [2007] was very similar. However, the TPC value obtained in this study for acacia honey (10.3 mg GAE/100 g) was 2.5 times higher compared to literature data [Krpan *et al.,* 2009; Bertoncelji *et al.,* 2007]. Nevertheless, the TPC value of multifloral honey presented in this study (19 mg GAE/100 g) was similar to the TPC values reported by Bertoncelji *et al.* [2007] (15.7 mg GAE/100 g). Other authors reported a higher content of polyphenols in multiflower honey. In the Mellen *et al.* [2015] study, TPC values determined for multiflower honeys from different areas of Poland ranged from 61.12 to 99 (mg GAE/100 g). Also Dżugan *et al*. [2018] obtained higher TPC values for multifloral honey(49 mg GAE/100 g). Such large differences may be due to a very different content of pollen derived from various plants in the final product.

Generally, the pH value of honey is between 3.5 and 5.5 due to the presence of inorganic ions, such as phosphate and chloride and organic acids especially gluconic acid [Bogdanov *et al.* 2004]. Active acidity (pH) measurements can also be included in the identification of the botanical origins of honey [Sanz *et al*., 2005]. The highest pH value was noted in the honeydew honey samples (5.02) while the lowest one in the buckwheat (4.07) honey samples. The lowest value of free acidity was noted for rape honey (10.5 meq/kg), whereas honey with the highest acidity turned out to be buckwheat honey (with the mean value of 34.25 meq/kg). These valeus are in accordance with the results obtained by Pasini *et al.* [2013] for buckwheat honey (19.2–50.3 meq/kg). It is known that honeydew honeys show a higher average acidity and pH value than blossom honey [Bentabol Manzanares *et al.,* 2011].

Thermal analysis

Glass transition, measured by the DSC technique, was manifested by a change in heat capacity which induces a step-like change in the baseline of the curve (heat flow *vs.* temperature). This transition occurs when the material changes upon cooling from a rubbery-like state into a hard, glassy state or conversely from a glassy solid to a rubbery state upon heating. Table 1 shows the values of the glass transition temperatures (T_g) which were determined from the heating DSC curve as the midpoint. The T_g values of the pure honey samples varied between -35.19°C and -47.11°C and were in strong agreement with literature data [Cordella *et al.,* 2002; Tomaszewska-Gras *et al.,* 2015]. The lowest temperature was determined for heather honey, which is known as one of those with the highest water content. According to Council Directive 2001/110/EC, water content in heather honey is allowed up to 23%, while

TABLE 2. Linear correlation coefficients between all measured parameters.

	L*	a*	b*	pH	Free acidity	Ash content	IC_{50}	TPC	AA	T_g	h	C	Colour intensity
L*	1.00												
a*	-0.74	1.00											
b*	0.57	-0.32	1.00										
pH	-0.42	0.47	*-0.18*	1.00									
Free acidity	-0.33	0.31	-0.30	*-0.06*	1.00								
Ash content	-0.68	0.67	-0.47	0.78	*0.18*	1.00							
IC_{50}	*0.19*	-0.50	*0.20*	-0.25	-0.35	-0.34	1.00						
TPC	-0.72	0.70	-0.55	0.24	*0.37*	0.44	-0.60	1.00					
AA	-0.46	0.63	-0.47	0.56	*0.19*	0.68	-0.76	0.71	1.00				
T_g	*0.18*	*0.14*	*0.11*	*0.16*	*0.03*	*-0.03*	-0.26	*-0.07*	*0.09*	1.00			
H*	-0.75	0.96	-0.34	0.55	0.24	0.76	-0.48	0.72	0.70	*0.08*	1.00		
C*	0.53	-0.23	0.99	*-0.14*	-0.31	-0.41	*0.17*	-0.50	-0.41	*0.12*	-0.26	1.00	
Colour intensity ABS 450	-0.85	0.74	-0.61	0.30	0.43	0.64	-0.45	0.89	0.68	*-0.21*	0.78	-0.56	1.00

In italic there are reported the correlation coefficient values statistically insignificant at $p>0.05$.

in the remaining types of honey it should not exceed 20%. It is well established that water acts as a plasticizer and this caused a decrease in the T_g point. In turn, the highest value of T_g (-35.19°C) was recorded for the nectar-honeydew honey which was one of the darker honeys (L*= 31) with a high ash content (0.46%).

Correlation, principal component and cluster analyses

In the preliminary investigation of the different types of honey, a correlation analysis as well as a principal component analysis (PCA) were performed. The correlation matrix (Table 2) showed a significant correlation between almost all parameters. The highest value of Pearson's correlation coefficient (r=0.89) was determined between TPC and colour intensity. The significant correlation between the TPC and parameter L* (measure of lightness) was negative, which indicated decreases in the TPC values with increases in light colour. This is proved by multivariate analysis (Figure 1). Principal component analysis was performed to explore dataset and to find any relationship between the variables. Using

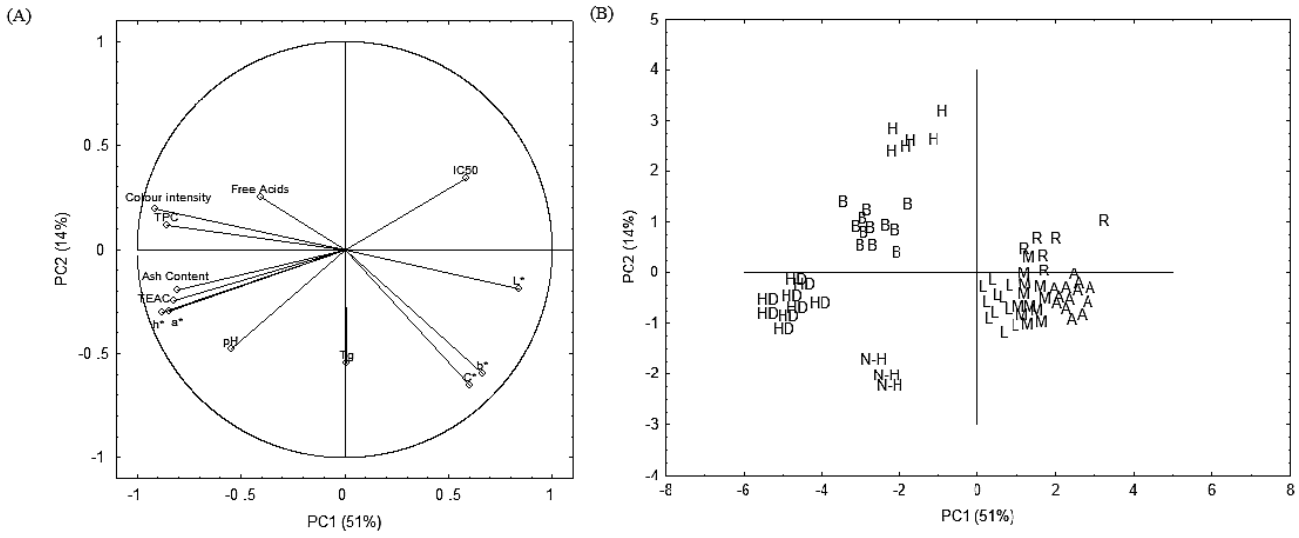

FIGURE 1. Principal component analysis results.

A) Projections of the variables on the facto plane. L*,a*,b*,h* and C* – colour indices, TPC – total phenolic content, IC50, AA – antioxidant activity, Tg – glass transition temperature. B) Projections of the scores on the factor plane. A – acacia honey, R – rape honey, L – linden honey, M – multifloral honey, N-H – nectar-honeydew honey, B – buckwheat honey, H – honeydew honey, H – heather honey.

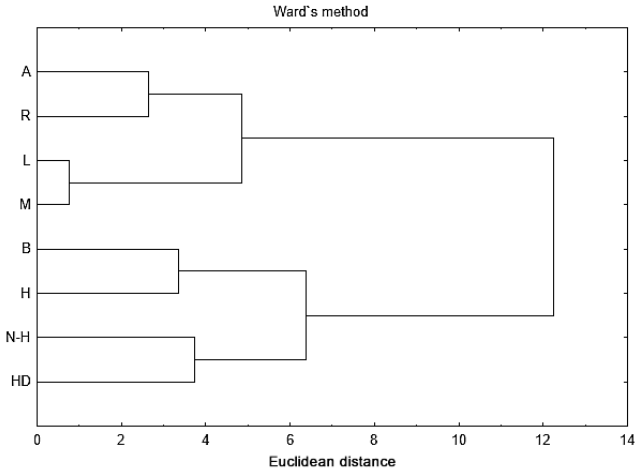

FIGURE 2. Dendogram of different honey types according to cluster analysis of similarity on the basis of antioxidant activity, thermal analysis, and physicochemical parameters.

(A – acacia honey, R – rape honey, L – linden honey, M – multifloral honey, N-H – nectar-honeydew honey, B – buckwheat honey, H – honeydew honey, H – heather honey).

the graphical criterion, the first four principal components, with Eigen values greater than 1, were derived. The first four principal components accounted for about 85% of the total variance. The highest and the lowest loading values indicate the highest importance of parameters in determining sample distribution along the first PC. The first (PC1), second (PC2), third (PC3), and fourth (PC4) principal component explained 51%, 14%, 10%, and 8% of the variance, respectively. The first component was predominated by colour intensity, h* value, and total phenolic content, while the highest loading values of C*, b* and T_g were noted in the second principal component. Figure 1B showed the score plot of PCA. All light coloured honey samples on the right side of PC1 are linked to L* colour parameter whereas dark coloured honey samples are located on the left side of PC1. Additionally, heather and buckwheat honeys are linked to free acidity and are located on the left side of PC2 while nectar-honeydew honey and honeydew honey are characterized by the higher ash content, the higher antioxidant activity, and the higher values of a* colour parameter.

Another unsupervised pattern recognition method is cluster analysis (CA). The Ward method, known as the minimum variance method with Euclidean distance between centroids, was applied. In accordance with the CA dendrogram (Figure 2), eight well-separated clusters were observed. As shown in Figure 3, all darker honeys (N-H – nectar-honeydew honey, B – buckwheat honey, H – honeydew honey, H – heather honey) were heaped together in one cluster, while light coloured honeys (A – acacia honey, R – rape honey, L – linden honey, M – multifloral honey) were heaped in the second separate cluster. The most similar, according to the measured parameters, were the linden and multifloral honeys. The smallest Euclidean distance was measured between these honeys. This similarity cloud disrupts the determination of linden and multifloral honeys. In addition, the distance determined between rape and acacia honeys was smaller than among other honeys.

Linear Discriminant Analysis

Linear Discrimination Analysis (LDA), is probably the most commonly used one among the supervised pattern recognition methods [Berrueta *et al*., 2007]. Standardised dis-

TABLE 3. Standardised canonical discriminant function coefficients.

Variable	Root 1	Root 2	Root 3	Root 4	Root 5	Root 6
L*	0.453	1.035	-0.119	-1.053	0.453	0.214
a*	5.288	-3.381	-2.504	-0.407	1.696	-0.469
b*	7.255	13.833	-10.880	6.093	6.679	2.110
h*	-5.675	4.720	0.845	1.043	-1.101	0.811
C*	-6.286	-15.385	11.407	-6.325	-7.234	-1.752
Colour intensity	-0.008	1.242	-0.196	-0.305	-0.999	-0.770
pH	-0.282	-0.937	-0.515	0.078	-0.631	-0.133
Free acidity	-0.127	-0.886	0.080	0.461	-0.774	0.612
Ash content	-0.651	-0.303	-0.015	-0.277	0.715	0.204
IC_{50}	1.488	-0.353	-0.177	-0.520	1.146	1.340
TPC	2.505	0.125	-0.309	-0.787	0.991	1.049
AA	0.131	-0.470	0.074	-0.508	0.352	0.353
T_g	0.631	-0.427	0.350	-0.781	-0.299	0.109
Discrimination (%)	48.48	24.28	18.98	5.41	2.06	0.69
Cumulative (%)	48.48	72.76	91.74	97.15	99.21	99.89

L*,a*,b*,h* and C* – colour indices, TPC – total phenolic content, IC_{50}, AA – antioxidant activity, T_g – glass transition temperature.

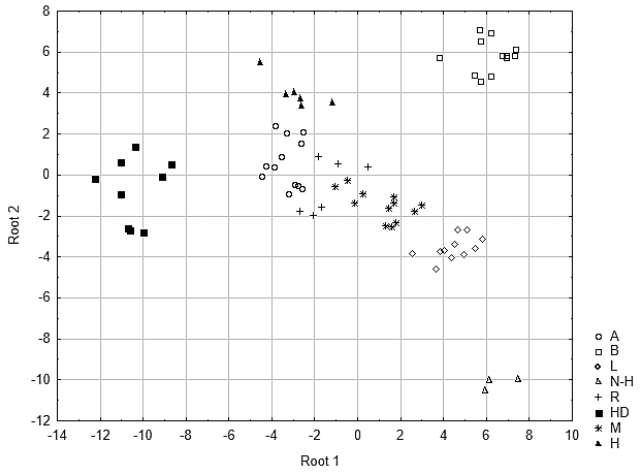

FIGURE 3. Linear discriminant analysis (LDA) sample classification of honeys.

(A – acacia honey, R – rape honey, L – linden honey, M – multifloral honey, N-H – nectar-honeydew honey, B – buckwheat honey, H – honeydew honey, H – heather honey).

criminant coefficients were used to compare the relative importance of the independent variables (Table 3). The higher the absolute value of the standardised coefficient, the more important was the related independent variable. The first six discriminant functions were statistically significant and accounted for 99.9% of the total variance. The first discriminant function accounted for 48.5% of the total variance and the second for 24.2%. Colour parameters (b*, a*, h*, and C*) contributed the most to the canonical variable (Table 3). This means that the colour of honey had the highest discrimination power according to the botanical origin. Colour parameter b* contributed the most to the first canonical variable (standardized coefficient = 7.255) accounting for most of the discrimination between honey classes. The second canonical variable was related to chroma (C*) parameter (standardized coefficient = -15.385) expounding the discrimination between the honeys according to their botanical origin. A scatter plot of canonical values (Figure 3) shows that the discrimination of honey types is noticeable. We can observe an excellent discrimination of dark coloured honeys (buckwheat, honeydew and nectar-honeydew honey) because the colour parameters possess the highest discriminating power. According to the classification matrix, almost all types of honey were classified correctly (98.61%). Only one sample of multifloral honey was incorrectly classified as a rape honey type. Linear discriminant analysis was successfully used to predict the group membership of acacia, pine honeydew, and multifloral honey based on their mineral content and antioxidant properties [Nayik *et al.,* 2016]. In that research, potassium contributed the most to the first canonical variable. Whereas, the second canonical variable was related to calcium. It was found that the mineral composition also helped in the determination of honey type. Similar results were obtained by Nayik & Nanda [2016]. The cherry, apple, saffron, and wild bush honeys were discriminated using LDA. Mineral content, colour parameters, and electrical conductivity contributed the most to the first, second, and third canonical variable, respectively. The classification matrix proved the very strong classification ability of the constructed model.

Classification and Regression Trees

Classification and regression trees (C&RT) are machine-learning methods for building prediction models from data. The tree develops by carefully searching for predictors in each branch for the best division in each node. The goal is that descendants are more homogeneous than their parents. When the split creates the positive change in prescient exactness, splits at every node will happen. V-fold cross-validation (v=10) was used to avoid model over-fitting. Figure 4 shows the graph of the Decision Tree model for the classification of honey types.

The TPC values play the main role in the differentiation between light and dark coloured honeys, whereas the colour parameters possess the highest discrimination power in distinguishing between multifloral and linden honey samples. The ash content and temperature of glass transition (T_g) were useful in the differentiation between different dark coloured types of honey. All honey samples were correctly classified. Therefore, the accuracy of the model is 100%. In other research, the decision tree (C&RT) was successfully used to determine honey type. Discrimination according to the botanical origin of honeys was based on their physicochemical properties. Clear rules that characterise the type of honey were obtained and in only one case was heather honey incorrectly classified as a multifloral one [Popek *et al.,* 2017]. But in conclusion more honey samples (types of honey, regions and years of collection) should be examined to generalise the developed model.

Artificial Neural Network

The determination of the tested honey types was tackled with a pattern recognizer based on artificial neural network (ANN) providing nonlinearity in the multivariate classification performance. The available set of 72 points has been divided into: learning (75%), training (15%), and validating (15%) sets. Multilayer feed-forward fully connected ANN has been trained with the Broyden-Fletcher-Goldfarb-Shanno learning algorithm (200 epoch). The search for an appropriate ANN model was performed using multilayer perceptron (MLP) and radial basis function (RBF) networks. The best seven networks of total 30 were chosen. The network structure developed for honey data included an input layer, one hidden layer and an output layer. The input layer made up of 13 neurons, 10–13 neurons in a hidden layer and eight neurons in the output layer (classification of honeys based on their botanical origin). The sums of squares and the cross-entropy error function were used during the network training process. The best seven ANN-MLP networks are presented in Table 4. In all the obtained networks, the hyperbolic tangent function was used in the hidden layer, whereas the sine linear transfer and Softmax functions were also used in the output layer. The success of the model to classify objects can be evaluated as: training performance as a percentage of the samples in the learning set correctly classified during the networks learning step; test performance as a percentage of the samples

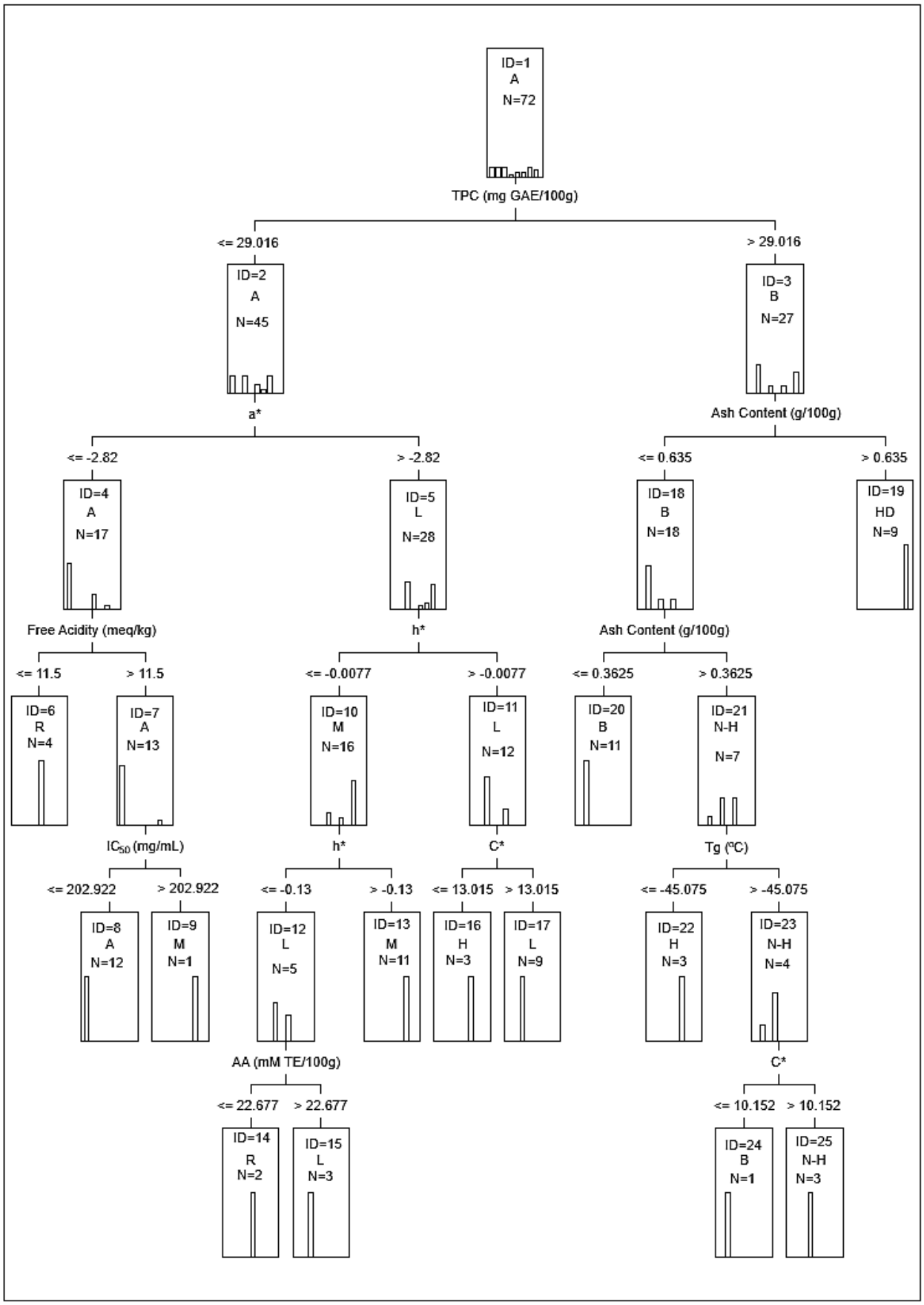

FIGURE 4. Classification and regression tree (C&RT) for honey types.

L*,a*,b*,h* and C* – colour indices, TPC – total phenolic content, IC_{50}, AA – antioxidant activity, Tg – glass transition temperature, A – acacia honey, R – rape honey, L – linden honey, M – multifloral honey, N-H – nectar-honeydew honey, B – buckwheat honey, H – honeydew honey, H – heather honey.

TABLE 4. Summary of Artificial Neural Network – Multilayer Perceptron (ANN-MLP) models.

MLP model	Training performance	Test performance	Validation performance	Training algorithm	Error function	Hidden activation	Output activation
13–13–8	100	100	100	BFGS 67	SOS	Tanh	Sinus
13–10–8	100	100	100	BFGS 67	SOS	Tanh	Tanh
13–10–8	100	100	100	BFGS 68	SOS	Tanh	Tanh
13–13–8	100	100	100	BFGS 71	SOS	Tanh	Tanh
13–10–8	100	100	100	BFGS 52	SOS	Tanh	Identity
13–10–8	100	100	100	BFGS 13	Entropy	Tanh	Softmax
13–10–8	100	100	100	BFGS 17	Entropy	Tanh	Softmax

SOS – sum of squares, BFGS – Broyden-Fletcher-Goldfarb-Shanno technique for training neural networks.

in the testing set correctly classified during the networks testing step; and validation performance as a percentage of the samples in the validation set (samples not used in the learning and testing steps) correctly classified by the models during the networks validation step. Performances of the obtained models are presented in Table 4. All the built networks were characterised by a perfect learning and testing performance (100%). Only the validation performance for the fourth model was lower than 100%.

CONCLUSIONS

In perspective of the outcomes achieved inside this investigation, it can be concluded that a discriminant approach based on the combination of physicochemical parameters, thermal analysis and antioxidant properties together with appropriate chemometric techniques is a promising and effective way for differentiating honeys conforming to their botanical origin. The colour parameters were important in discriminating honey samples using all multivariate technics. The rest of the analysed in this study physicochemical properties of honey were also important but their discriminating power depends on the statistical methods which were applied. In conclusion, this study demonstrated the high potential of the selected chemometric methods combined with the honeys selected properties in honey analysis for providing reliable results and models of simple application. The results presented in this paper jointly with the models could certainly be developed further to completely approve the adequacy of the method, using a greater number of samples from other honey types collected from other regions.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Original research article
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Sprouted and Non-Sprouted Chickpea Flours: Effects on Sensory Traits in Pasta and Antioxidant Capacity

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Chickpea flour, mainly from non-sprouted chickpeas, serves as an alternative to wheat flours. Sprouting legumes may improve antioxidant potential, but sensory effects of sprouting chickpeas for flour are largely unknown. This study evaluated sensory effects of up to 40% substitution of Sprouted Chickpea Flour (SCF) and Non-Sprouted Chickpea Flour (NSCF) in pasta. Total phenolics and antioxidant potential (as Trolox equivalency) of the flours were also assessed. Results showed phenolic contents and antioxidant potential were significantly higher in SCF than NSCF. By descriptive analysis, chickpea flour levels corresponded with decreases in chewiness and pasta flavor, and increases in mushiness, grittiness, bitterness, and earthiness. Effects on bitterness, earthiness, and pasta flavor were greater with SCF than NSCF. By consumer assessment, 20% SCF did not exhibit significantly lower overall hedonic measures than the other samples. With attention given to possible organoleptic challenges, SCF may warrant consideration as a more antioxidant-rich alternative to NSCF.

INTRODUCTION

Chickpea flour has been recently increasing in prominence as an alternative to wheat flour, with Future Market Insights [FMI] forecasting the global chickpea flour market to likely surpass a valuation of five billion USD by the end of 2026 [FMI, 2018]. The market for chickpea flour includes direct sales to consumers (*e.g.* as bags of flour for home use) and for use in product manufacturing (*e.g.* commercial breads and pastas made with chickpea flour). At present, non-sprouted chickpea flour (NSCF) is much more common than sprouted chickpea flour (SCF) in commercial production, but there is evidence that sprouting legumes such as chickpeas prior to flour production may improve antioxidant potential and other nutritional attributes [Devi *et al.*, 2015; Gunashree *et al.*, 2014]. As with other gluten-free flours, the direct substitution of chickpea flours for wheat flour can present sensory challenges, and the differences between SCF and NSCF in sensory properties are not well understood [Melini *et al.*, 2017; Zafar *et al.*, 2015]. Therefore, the nutritional and sensory properties of SCF, specifically regarding comparison with NSCF, is a matter in need of further investigation.

Chickpea flours may appeal to consumers for their perceived nutritional benefits and lack of gluten. Chickpeas are pulse legumes, which have received substantial attention for their apparent healthfulness [Świeca *et al.*, 2013]. Currently, many nutritionists and dietitians are recommending increased consumption of pulse legumes [Venn *et al.*, 2010]. Furthermore, the American Diabetes Association [Polak *et al.*, 2015] and the American Heart Association [Stone *et al.*, 2014] recommend pulses for better cardiovascular health and blood glucose control, as well as for a healthy source of protein and starch [Świeca *et al.*, 2013]. Chickpeas, specifically, are calculated to have 25.3–28.9 g 100 g protein content [Khattak *et al.*, 2007] and have been noted for their historic role in the Mediterranean diet [Gupta *et al.*, 2017]. Chickpeas are also known to be good sources of a large variety of vitamins, minerals, and polyphenolics [Bouchenak & Lamri-Senhadji, 2013; Segev *et al.*, 2011; Khattak *et al.*, 2007].

Multiple studies have shown improvements in the nutritional properties of legumes as an effect of sprouting [Ramesh & Swami, 2016; Devi *et al.*, 2015; Nakitto *et al.*, 2015; Masood *et al.*, 2014]. Noted effects have included increases in minerals, aspartic acid, folic acid, and vitamins, with decreases

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in crude fat, crude carbohydrate, and antinutrients such as trypsin inhibitors and α-amylase inhibitors [Devi *et al.*, 2015; Gao *et al.*, 2015; Gunashree *et al.*, 2014; Świeca *et al.*, 2013]. A recent study concluded that sprouting chickpeas increased the content of protein, minerals, and fiber, while decreasing fat content [Masood *et al.*, 2014].

It has been shown that sprouting can increase the total antioxidant activity or phenolic contents of legumes [Ramesh & Swami, 2016; Gharachorloo *et al.*, 2013; Świeca *et al.*, 2013]. A recent study focusing on chickpea flour showed SCF to positively influence brachial artery flow mediate dilation *in vivo* [Enrique *et al.*, 2018].

Wood [2009] studied consumer acceptability of spaghetti fortified with NSCF at levels up to 30%. Although pasta firmness decreased with increases in chickpea fortification, the study concluded that non-sprouted chickpea-fortified spaghetti was acceptable to consumers [Wood, 2009]. Another study found that adding small amounts of NSCF to wheat flour created a dough with higher strength and added elasticity [Sabanis *et al.*, 2006]. This improvement was noted at substitution levels ranging from 5–20%, but there was noted quality deterioration when more than 30% was added to the flour. An investigation of substituting chickpea flour in cracker production found that higher substitution levels were associated with increased leguminous odor and bitter taste [Kohajdová *et al.*, 2011]. Despite the sensory challenges of chickpea flour substitution, the practice has proved viable, as evidenced by the growing market for chickpea flour and chickpea-flour rich products [FMI, 2018]. Efforts to improve the sensory quality of chickpea-flour products has included optimization of substitution levels, and addition of hydrocolloids such as pectin and gums, to name a few [Padalino *et al.*, 2015]. How these sensory challenges would be different when using SCF rather than NSCF is currently unknown.

With the substantial rise in chickpea flour use, there has been an increasing need to further understand the differences between SCF and NSCF in regard to nutritional and sensory properties. The objectives of this study were to compare the total phenolics contents and antioxidant capacity between these two flours, and to determine the effects of both flour types on sensory properties and consumer acceptability when used in pasta production.

MATERIALS AND METHODS

Chickpea sprouting

Dry Goya chickpeas were sprouted in the Food Science Laboratory at Montclair State University (Montclair, NJ, USA) using a protocol described in a previous study [Khattak *et al.*, 2007]. In brief, the chickpeas were submerged in deionized water for 18 h, and then placed in a porous colander and rinsed with deionized water three times per day for six days. During this period, the chickpeas were placed in a 0.56°C refrigerator overnight to prevent bacterial growth [Kumar *et al.*, 2006]. At the conclusion of the incubation, chickpeas that had not visibly sprouted were discarded and the sprouted chickpeas were placed into an Excalibur Food Dehydrator (Excalibur, Sacramento, CA, USA) at 49°C for 15 h.

Sprouted and non-sprouted chickpea flour production

For both flour types, Goya chickpeas were ground into flour in a Vitamix Blender (Vitamix, Cleveland, Ohio, USA). SCF utilized chickpeas that had undergone the sprouting procedure described above, and NSCF utilized untreated dry chickpeas.

Total phenolics content

Total phenolics content (TPC) was evaluated in triplicate for each chickpea flour type in accordance with the methodology described by Singleton *et al.* [1999] with minor modifications. For each assessment, 5 g of dry flour sample was extracted twice at room temperature for 15 min using gentle shaking and sonication in 40 mL of 4:1 (v/v) acetone/water. The extracts were combined and the solvents were removed under reduced pressure by rotary evaporation at 40°C followed by high vacuum at room temperature. The resulting material was dissolved in methanol. Assessments were performed in cuvettes containing water, methanolic sample, commercial Folin-Ciocalteu reagent, and saturated Na_2CO_3, with a final volume of 1.0 mL (using solution without sample extract as a blank). Each cuvette was incubated at room temperature for 1 h before recording the absorbance at 750 nm *vs.* a blank containing no sample. The instrument used was a Cary 300 Bio UV/Visible Spectrophotometer (Agilent Technologies, Santa Clara, California, USA). Absorbance values were correlated to the best fit line of a standard curve constructed using 0.85–8.50 μg/mL gallic acid and reported as mg gallic acid equivalents (GAE)/g flour.

Trolox antioxidant activity

DPPH radical scavenging activity was determined in quadruplicate for NSCF and SCF by measurement of Trolox Antioxidant Activity (TAA). The protocol was as described in Brand-Williams *et al.* [1995] with minor modifications. Hydrophilic fractions of 1.25 g of flour were extracted with 4:1 (v/v) acetone/water solvents, with the solvents subsequently removed by evaporation under reduced pressure. The fractions were dissolved in 99.7:0.3 (v/v) water/formic acid to a final volume of 7.5 mL. 10 μL of sample solution were added to 290 μL of DPPH solution (0.10 mM prepared in 4:1 (v/v) methanol/H_2O) in wells of a VersaMax ELISA Microplate Reader (VersaMax, Sunnyvale, CA USA). Absorbance was recorded at 517 nm following 30-min incubation at 25°C. The values were plotted to a standard curve constructed using solutions of 0–5 mM Trolox in 1:1 (v/v) acetone/water. TAA values were reported as mmol Trolox equivalent/100 g flour.

Pasta preparation

Five different flour compositions were prepared for use in pasta sample preparation, representing incorporations of NSCF and SCF into semolina flour at a range of concentrations (100% semolina, 20% NSCF, 40% NSCF, 20% SCF, and 40% SCF). These substitution levels were chosen partially with consideration of a prior investigation by Wood [2009], but also with consideration of our own preliminary trials that determined substantial texture changes at substitution levels greater than 40%. The semolina flour was "Bob's Red Mill Semolina Flour" (Bob's Red Mill, Milwaukie, OR, USA).

The production of dough from flour was consistent for all sample types, accomplished by combining 400 g of the flour mixture with 118 mL of water. The flour mixture was mixed with water until it formed a solid dough. This dough was then kneaded and wrapped in plastic wrap and left to sit at room temperature for 10 min. The dough was then formed into smaller balls and placed into a Kitchen Aid Gourmet Pasta Press attachment of a Kitchen Aid machine (Benton Harbor, MI, USA) and used to make fusilli pasta. The pasta was refrigerated at 0.56°C for 24 h. Prior to the serving of samples, 173 g portions of pasta were placed in 710 mL of boiling water for 5 min and drained with a colander.

Descriptive analysis

A modification of the Spectrum™ Descriptive Analysis Method was used to determine textural attributes of pasta "chewiness", "mushiness" and "grittiness"; taste attributes of "saltiness", "sweetness", "bitterness"; and flavor attributes of "earthiness" and "pasta flavor". Panelists (n=8) marked assessments on 15 cm lines, where the leftmost side was labeled as "not perceptible" and the rightmost side was labeled as "high intensity" [Meilgaard *et al.*, 1999]. The scores were measured by a ruler and reported on a 0 to 1 scale of intensity.

Participants were recruited from the students and employee population of Montclair State University (Montclair, NJ, USA). The panelists received two training sessions, consisting of calibration to the intensity of listed sensory traits according to sensory standards [Meilgaard *et al.*, 1999].

Panelists assessed each of the five pasta types in triplicate. Assessments consisted of three separate sessions, with five samples evaluated during each testing session. Samples were pre-coded with 3-digit random numbers and evaluated in a counterbalanced order. Assessments took place under white light by panelists seated in individual booths. The uncooked pasta samples were always freshly prepared the day prior to assessment and then cooked immediately prior to assessment.

Consumer assessment

Consumer assessment followed ASTM methodology with minor modifications [ASTM, 2011]. The assessment was completed by 108 untrained panelists recruited from the students and employee population of Montclair State University (Montclair, NJ, USA). Each panelist evaluated all five pasta samples. Samples were pre-coded with 3-digit random numbers and prepared and presented as described above for the descriptive analysis.

Panelists were asked to rate each sample for their hedonic assessments of appearance, texture, flavor, and overall likability. The panelists were presented a 7-point hedonic scale ranging from "dislike extremely" to "like extremely". Following self-reporting by the panelists, investigators converted panelists' responses on the lingual 7-point scale into a numeric scale ranging from zero ("dislike extremely") to six ("like extremely").

Statistical analysis

All statistical analyses were performed with IBM SPSS Statistics for Windows (Version 24.0, IBM Corp, Armonk, NY, USA). TPC and TAA values were each evaluated for significant differences between samples by unpaired t-test ($\alpha=0.05$). Significant differences between samples for descriptive analysis and consumer assessment results were determined by one-way ANOVA with Tukey's Studentized Range test ($\alpha=0.05$).

Multivariable linear regression models were produced by modeling NSCF and SCF levels as independent variables *vs.* each of the assessed descriptive traits. The stepwise linear regression function in SPSS was implemented with an exclusion criteria of $\alpha=0.10$. If no variable exceeded a p-value of 0.10, no model was reported for that measure.

TABLE 1. Antioxidant assessments: total phenolics content and Trolox antioxidant activity of NSCF and SCF.

Assessment	NSCF[a]	SCF[a]	% Difference[b]
TPC (mg GAE/g flour; n=3)	7.3 ± 0.08^{B}	8.4 ± 0.22^{A}	+15.1%
TAA (mmol Trolox equivalent/100 g flour; n=4)	2.06 ± 0.04^{B}	2.36 ± 0.03^{A}	+14.6%

[a]Mean ± SD; NSCF = Non-Sprouted Chickpea Flour; SCF = Sprouted Chickpea Flour; [b]Percentage difference in SCF value *vs.* NSCF value. Values followed by the same superscript capital letter within a row were not significantly different from one another ($\alpha=0.05$) according to t-test *via* SAS software.

RESULTS AND DISCUSSION

Antioxidant assessments: total phenolics content and Trolox antioxidant activity

The results of the TPC and TAA evaluations are shown in Table 1. SCF (8.4 mg GAE/g flour) had significantly greater TPC values than NSCF (7.3 mg GAE/g flour; $p=0.0013$). SCF (2.36 mmol Trolox equivalent/100 g flour) also had significantly greater TAA values than NSCF (2.06 mmol Trolox equivalent/100 g flour; $p<0.0001$).

Notably, the observed increases associated with sprouting were extremely similar for both TPC and TAA (15.1% and 14.6%, respectively). These observations also correspond very closely to the 13.6% increase in the total antioxidant capacity (assessed by phosphomolybdenum method) following sprouting of chickpeas observed recently by Ramesh & Swami [2016].

A prior study has determined isoflavonoid content and diversity to increase dramatically (*i.e.* up to 500%) within chickpeas during germination [Wu *et al.*, 2012], so this may well be substantially contributing to our observed increases in phenolic contents and antioxidant activity. Although the specific *in vivo* effects of increased isoflavonoid consumption are a matter of continuing investigation and debate [Miadoková, 2009], it has received specific study for its role in cancer prevention. There are multiple proposed mechanisms other than antioxidant activity by which isoflavonoids may contribute directly to cancer prevention. These include induction of cell cycle arrest and apoptosis, induction of detoxification enzymes, regulation of host immune system, and changes in cellular signaling [Ito *et al.*, 2006; Birt *et al.*, 2001]. It is therefore feasible that our observed increases in phenolics within chickpeas during sprouting may be contributing health benefits other than

TABLE 2. Descriptive analysis of pasta samples with different levels of chickpea flour substitution[a].

	Attribute	100% Semolina	20% NSCF[b]	40% NSCF[b]	20% SCF[b]	40% SCF[b]
Texture	Chewiness	0.62^{A}	0.53^{AB}	0.26^{C}	0.45^{AB}	0.35^{BC}
	Mushiness	0.24^{B}	0.28^{AB}	0.48^{A}	0.29^{AB}	0.43^{A}
	Grittiness	0.08^{B}	0.21^{A}	0.19^{A}	0.21^{A}	0.26^{A}
Taste	Saltiness	0.06^{B}	0.09^{AB}	0.14^{A}	0.11^{AB}	0.13^{A}
	Sweetness	0.25^{A}	0.31^{A}	0.20^{A}	0.23^{A}	0.19^{A}
	Bitterness	0.06^{C}	0.12^{BC}	0.17^{AB}	0.28^{A}	0.28^{A}
Flavor	Earthiness	0.10^{C}	0.33^{B}	0.30^{B}	0.37^{B}	0.57^{A}
	Pasta flavor	0.61^{A}	0.45^{AB}	0.38^{B}	0.32^{BC}	0.22^{C}

[a]Determined by descriptive analysis by eight trained panelists. All panelists assessed each sample at three distinct evaluation sessions. Results reported on 0 (low) to 1 (high) scale. [b]Sample label refers to amount (by mass) of semolina flour in formula that was replaced with chickpea flour. NSCF = Non-Sprouted Chickpea Flour; SCF = Sprouted Chickpea Flour. Values followed by the same superscript capital letter within a row were not significantly different from one another (α=0.05) according to ANOVA and means separation with Tukey's Studentized Range *via* SAS software.

TABLE 3. Predictive modeling of descriptive analysis results according to chickpea flour substitution levels[a].

	Attribute	Intercept	Coefficients		R^2 (Adj.)
			NSCF (%)[b]	SCF (%)[b]	
Texture	Chewiness	0.636	-0.00857	-0.00757	82.5%
	Mushiness	0.204	0.00627	0.00537	77.3%
	Grittiness	0.0933	0.00275	0.00450	61.6%
Taste	Saltiness	0.0614	0.00186	0.00186	87.5%
	Sweetness		*no significant variables*[c]		
	Bitterness	0.127	0.00275	0.00459	81.2%
Flavor	Earthiness	0.147	0.00489	0.0107	79.8%
	Pasta flavor	0.570	-0.00500	-0.00950	84.6%

[a]Models based upon sensory scores determined by descriptive analysis by eight trained panelists. All panelists assessed each sample at three distinct evaluation sessions. Results were reported on 0 (low) to 1 (high) scale. [b]Percentage of chickpea flour used in substitution of semolina flour in pasta formula. NSCF = Non-Sprouted Chickpea Flour; SCF = Sprouted Chickpea Flour. [c]Variables with $p < 0.10$ were excluded from reported models. Linear regression models made with SAS software.

radical quenching. To this point, a recent *in vivo* investigation determined that consumption of sprouted chickpea pasta resulted in greater brachial artery flow mediated dilation than consumption of semolina flour pasta [Enrique *et al.*, 2018].

Our results suggest that sprouting chickpeas increases the concentration of antioxidant compounds and *in vitro* antioxidant potential, and provide evidence of a possible consistency in the approximate magnitude of these changes. The results also affirm that this improvement of antioxidant contents and potential is present within the samples exposed in this study to sensory evaluation. Further research into the composition differences (and the associated *in vivo* effects) between SCF and NSCF may be warranted to further elucidate the mechanism of the observed changes.

Descriptive analysis

The results of the descriptive analysis are shown in Table 2 and the predictive models for these attributes according to substitution are shown in Table 3. Figure 1 depicts descriptive characteristics of the 100% semolina sample alongside the average value for the two NSCF samples (20 and 40% substitution) and the average value for the two SCF samples (20 and 40% substitution). Regarding textural attributes, the data suggests that substitution of semolina with chickpea flour at levels of 40% results in reductions in chewiness and increases in mushiness and grittiness. For all three of the assessed textural attributes, both 40% chickpea flour substitutions were significantly different from the 100% semolina sample. Notably, though, in none of the textural assessments were significant differences found between the 40% NSCF and 40% SCF samples. Examination of the coefficients of the predictive models indicate decreases in chewiness and increases in mushiness and grittiness with greater levels of chickpea flour substitution. The coefficients of the models suggest greater magnitude of the effect associated with NSCF for chewiness and mushiness, and with SCF for grittiness.

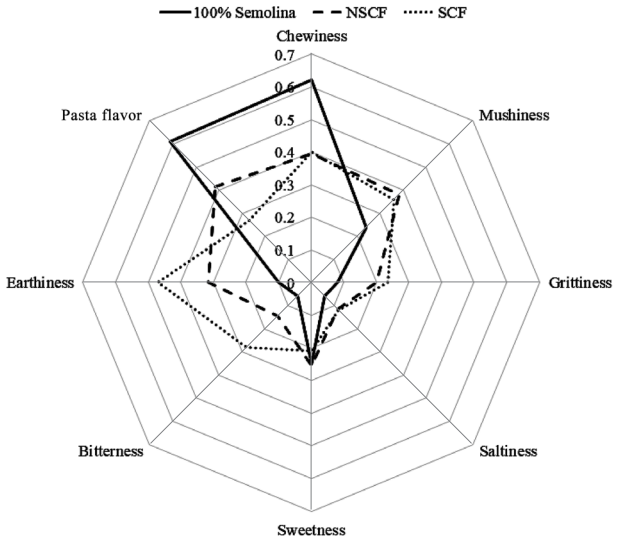

FIGURE 1. Descriptive analysis of pasta samples with non-sprouted and sprouted chickpea flour substitution[ab].

[a]Determined by descriptive analysis by eight trained panelists. All panelists assessed each sample at three distinct evaluation sessions. Results reported on 0 (low) to 1 (high) scale. [b]"100% Semolina" had no substitution; "NSCF" is average value for samples of 20% and 40% substitution non-sprouted chickpea flour; "SCF" is average value for samples of 20% and 40% substitution sprouted chickpea flour.

The loss of chewiness and increases in mushiness and grittiness would all likely suggest quality impairment, and these effects correspond well to documented challenges with the substitution of gluten-free flours [Kohajdová *et al.*, 2011; Wood, 2009]. Our data suggests, however, that these challenges may not be exacerbated by the use of SCF rather than NSCF.

Regarding tastes, the data shows no significant differences between any samples for sweetness, and neither chickpea flour was a significant variable for this output in the models. For bitterness, 40% NSCF, 20% SCF, and 40% SCF each had significantly higher values than the 100% semolina sample ($p=0.0054$, $p=0.0001$, and $p=0.0001$, respectively). None of these three samples, however, were significantly different from one another. NSCF and SCF were both significant positive predictors of bitterness in the models, with the coefficient of SCF 1.6 times greater than that of NSCF. Increases in bitterness associated with chickpea flour incorporation has been shown in a study previously [Kohajdová *et al.*, 2011], and our study indicates this effect may be more noticeable when using SCF rather than NSCF.

Interestingly, our data show significant increases in perceptions of saltiness at a substitution level of 40% for both NSCF and SCF, and both variables were determined to be positively associative in the predictive models. To the knowledge of the authors, this particular effect has not been previously documented, but this perception of the panelists may feasibly be due to increases in other tastes and flavors associated with the chickpea flour. This suggests that the use of chickpea flour in place of semolina may allow for salt reduction in formulation.

Regarding flavors, all samples with chickpea flour had significantly greater earthiness flavor than 100% semolina. 40% NSCF, 20% SCF, and 40% SCF each had significantly lower pasta flavor than 100% semolina. For both assessed flavor attributes, 40% SCF was significantly different from either NSCF sample, and the models for both attributes show a greater magnitude of coefficient for SCF than for NSCF. The data suggests that these flavor changes are more substantial when substituting SCF rather than NSCF for semolina flour in pasta.

A prior study [Rayas-Duarte *et al.*, 1996] that investigated buckwheat substitution in pasta (and examined two of the same sensory attributes as our own study) helps us to contextualize the magnitude of our observed effects. Specifically, our study found that 40% SCF resulted in a 325% increase in grittiness and 570% increase in earthiness *vs.* the control. The study on buckwheat flour determined that 30% dark buckwheat substitution resulted in 1,467% increase in grittiness and a 300% increase in earthiness *vs.* the control. So although our data indicates significant sensory effects are associated with the substitution of SCF, effects of similar and greater magnitude have been observed for other health-promoting flour substitutions in pasta.

Consumer assessment

The results of the consumer assessments are shown in Table 4. For the measure of appearance, 40% NSCF was the only sample that differed significantly from the 100% semolina sample ($p=0.0246$). The deterioration of appearance in pasta with NSCF substitution levels exceeding 20% has been shown once previously in lasagna noodles [Sabanis *et al.*, 2006]. Our results indicate that this organoleptic challenge may be diminished when substituting SCF rather than NSCF.

TABLE 4. Consumer assessments of pasta samples with different levels of chickpea flour substitution[a].

Hedonic measure	100% Semolina	20% NSCF[b]	40% NSCF[b]	20% SCF[b]	40% SCF[b]
Appearance	4.38^{A}	4.27^{A}	3.93^{B}	4.36^{A}	4.63^{A}
Texture	4.67^{A}	4.70^{A}	4.36^{A}	4.55^{A}	4.41^{A}
Flavor	5.05^{A}	4.57^{B}	4.36^{B}	4.56^{B}	3.90^{C}
Overall	4.86^{A}	4.48^{BC}	4.51^{ABC}	4.63^{AB}	4.25^{C}

[a]Determined by consumer assessment by 108 untrained panelists. Results reported on 0 (low) to 6 (high) scale. [b]Sample label refers to amount (by mass) of semolina flour in formula that was replaced with chickpea flour. NSCF = Non-Sprouted Chickpea Flour; SCF = Sprouted Chickpea Flour. Values followed by the same superscript capital letter within a row were not significantly different from one another ($\alpha = 0.05$) according to ANOVA and means separation with Tukey's Studentized Range *via* SAS software.

Despite the descriptive results indicating loss of chewiness and increases in mushiness and grittiness with chickpea flour substitution, the hedonic assessments showed no significant differences in texture between any of the samples. Wood [2009] previously found that substitution with NSCF reduced pasta firmness, but still resulted in acceptable product quality. Our results indicate this effect to not be different when using SCF rather than NSCF.

The most substantial observed changes in hedonic measures occurred with flavor, for which all chickpea flour samples performed significantly worse than the 100% semolina sample. Moreover, the 40% SCF samples were significantly worse than all other samples in this regard. This observation corresponds well with the descriptive analysis results which indicated SCF to more substantially influence earthiness (positive association) and pasta flavor (negative association) than NSCF. The positive associative effect of SCF on bitterness (although technically a taste) may also have contributed to the flavor assessment. The results suggest that SCF may present greater challenges to flavor quality than NSCF. It is worth noting, though, that at 20% substitution, the SCF sample was not significantly different from NSCF. Therefore, this separation in effect may only occur at relatively high substitution levels (exceeding 20%).

In the overall hedonic assessment, 40% SCF was the only sample to differ significantly from 100% semolina ($p=0.0007$). As with flavor, the results suggest that SCF substitution may present more organoleptic challenges than NSCF substitution, but that this distinction may only be present with substitution levels exceeding 20%.

CONCLUSIONS

Sprouting of chickpeas prior to flour production can increase phenolic contents and *in vitro* antioxidant potential. However, sensory evaluation indicates that the use of SCF rather than NSCF may present some challenges to product quality – particularly regarding bitterness and effects on flavor. Notably, this distinction between chickpea flour types may only occur at substitution levels in excess of 20%. Considering the observed increases in healthful components, SCF may merit consideration as an alternative to NSCF in bulk production and product formulation, but attention must be given to the effects on flavor and taste quality that may occur at high levels of substitution. Further studies investigating methods to mitigate these quality concerns may be warranted.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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