ISSN (1230-0322)
2024, Vol. 74, No. 2

Food

Polish Journal of Food and Nutrition Sciences

formerly Acta Alimentaria Polonica

Published
by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn

Published since 1957 as
Roczniki Chemii i Technologii Żywności and Acta Alimentaria Polonica (1975–1991)

SCOPE: The Journal covers fundamental and applied research in food area and nutrition sciences with a stress on interdisciplinary studies in the areas of food, nutrition and related subjects.

POLICY: Editors select submitted manuscripts in relation to their relevance to the scope. Reviewers are selected from the Advisory Board and from Polish and international scientific centres. Identity of reviewers is kept confidential.

AUTHORSHIP FORMS referring to Authorship Responsibility, Conflict of Interest and Financial Disclosure, Copyright Transfer and Acknowledgement, and Ethical Approval of Studies are required for all authors.

FREQUENCY: Quarterly – one volume in four issues (March, June, September, December).

COVERED by Web of Science, Current Contents/Agriculture, Biology & Environmental Sciences, Journal Citation Reports and Science Citation Index Expanded, BIOSIS (Biological Abstracts), SCOPUS, FSTA (formerly: Food Science and Technology Abstracts), CAS (Chemical Abstracts), AGRICOLA, AGRO-LIBREX data base, EBSCO, FOODLINE, Leatherhead FOOD RA data base FROSTI, AGRIS and Index Copernicus data bases, Biblioteka Nauki ICM, Biblioteka Narodowa – POLONA, and any www browser; ProQuest: The Summon, Bacteriology Abstracts, Immunology Abstracts.

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Polish Journal of Food and Nutrition Sciences

ADVISORY BOARD OF PJFNS 2023–2026

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W latach 2022–2024 kwartalnik naukowy ***Polish Journal of Food and Nutrition Sciences*** realizuje projekt nr RCN/SP/0520/2021/1 finansowany ze środków budżetu państwa – Ministerstwo Edukacji i Nauki w ramach programu „Rozwój czasopism naukowych". Dofinansowanie wynosi 120 000 zł. W ramach projektu podejmowane są działania zmierzające do podniesienia poziomu praktyk wydawniczych i edytorskich, zwiększenia wpływu czasopism na rozwój nauki oraz utrzymania się czasopism w międzynarodowym obiegu naukowym.

Ministry of Education and Science
Republic of Poland

In the years 2022–2024, the scientific quarterly ***Polish Journal of Food and Nutrition Sciences*** accomplishes a project no. RCN/SP/0520/2021/1 financed from the state budged – Ministry of Education and Science Republic of Poland in the framework of a program "Development of scientific journals". The financing amounts to 120,000 PLN. The program aims at improving the level of publishing and editing practices, increasing the impact of scientific journals on science development, and extending the international range of scientific journals.

Subscription
2024 – One volume, four issues per volume. Annual subscription rates are: Poland 150 PLN, all other countries 80 EUR. Prices are subject to exchange rate fluctuation. Subscription payments should be made by direct bank transfer to Bank Gospodarki Żywnościowej, Olsztyn, Poland, account No 17203000451110000000452110 SWIFT code: GOPZPLWOLA with corresponding banks preferably. Subscription and advertising offices at the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, ul. J. Tuwima 10, 10-747 Olsztyn, Poland, tel./fax (48 89) 5234670, fax (48 89) 5240124, e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

Zamówienia prenumeraty: Joanna Molga (e-mail: pjfns@pan.olsztyn.pl)

Wersja pierwotna (referencyjna) kwartalnika PJFNS: wersja papierowa (ISSN 1230–0322)
Nakład: 70 egz.; Ark. wyd. 14; Ark. druk. 14
Skład i druk: ITEM

ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 111–119
DOI: 10.31883/pjfns/186589
ISSN 1230-0322, e-ISSN 2083-6007

Classification of Slovenian Dry-Cured Ham – Kraški pršut – According to Texture Profile

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The effects of the supplier and the mass of pork thighs together with some production parameters including mass loss during ham production, mass of the final products and relative content of non-protein nitrogen (NPN) on the texture profile of Slovenian dry-cured ham – Kraški pršut were investigated to determine its optimal texture. The study included 32 dry-cured hams from two mass classes (heavy with thigh mass at salting of 11.7–12.1 kg and light with thigh mass at salting of 10.5–10.7 kg) and two different pork thigh suppliers from regular production. Slices cut transversally from the thigh at approximately 8 cm from the femur head were used for physicochemical parameter (pH, contents of moisture, total fat, protein, NaCl, and NPN) analysis, instrumental texture analysis profile analysis (TPA), and sensory analysis. Pork thigh mass had a significant effect on the TPA parameters; light samples were harder, chewier, springier, more cohesive and resilient compared to the heavy samples. NaCl content and sensory scores for saltiness were higher in the light Kraški pršut samples than in the heavy ones. Based on the median for hardness in the sensory analysis, the samples were classified into three ranks of texture using linear discriminant analysis (9 variables, 100% correct classification): optimal (median 4.0; 19% of samples), slightly too soft (median 3.5, 72% of samples), and soft (median 3.0; 9% of samples). Kraški pršut mass and hardness were significantly correlated to saltiness, pastiness, aroma, and all TPA parameters ($p \leq 0.05$). Positive correlation ($p \leq 0.05$) was also observed between NPN and pastiness. These findings indicate the importance of supplier, mass of pork thighs, and production parameters for optimizing the texture of Kraški pršut.

Keywords: meat products, pork thighs, proteolysis, texture parameters, sensory profile

INTRODUCTION

A general tendency to reduce the salt content in consumer diet, in agreement with the Global Strategy on Diet, Physical Activity and Health, has led producers to reduce the salt content in dry-cured hams [Desmond, 2006; Waxman, 2004]. However, this technological intervention resulted in a modified texture of the product; salt reduction during processing led to excessive activity of proteolytic enzymes, resulting in a soft, pasty texture, being the two main texture problems [Contreras *et al.*, 2020, 2021; Ruiz-Ramírez *et al.*, 2006; Virgili *et al.*, 1995]. Currently, the slightly tough texture, typical for Kraški pršut – Slovenian dry-cured ham, has been replaced by a soft, very soft, and even pasty texture, which represents a significant challenge for producers. The frequency and intensity of this shift in texture during industrial processing remain unknown, as the phenomenon of pasty texture of dry-cured ham is very complex [Morales *et al.*, 2007b]. Several process factors that affect the texture of dry-cured ham have been studied; for example, one of the most important is salting because when the salt content decreases, fibre swelling decreases, resulting in poor texture [Desmond, 2006; Gou *et al.*, 2008]. The production of high-quality ham requires the consideration, monitoring, and control of a variety of processing factors

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Submitted: 25 October 2023
Accepted: 28 March 2024
Published on-line: 18 April 2024

(ripening time and temperature, salting, mass loss, water activity and pH values) related to textural properties (*e.g.*, hardness, pastiness, adhesiveness) and proteolytic activity [Pérez-Santaescolástica *et al.*, 2018].

Information on the relationship between the extent of proteolysis and the texture of ham is somewhat contradictory. Hence, in this study, technological parameters (pork thighs supplier, thigh mass, salt content, and mass loss after production) were investigated in relation to quantitative parameters of proteolysis, including non-protein nitrogen (NPN) content and proteolysis index (PI), and texture parameters of the final product. Therefore, the initial aims were to determine the sensory acceptable texture of Kraški pršut, identify a deficient texture and link it to instrumental measurements of texture, degree of proteolysis, mass classes of pork thighs, suppliers and mass losses during production. Based on the assessments of the panel of sensory experts, we tried to find values for optimal texture parameters, as well as values for undesirable texture (too soft or too firm). In addition, the influence of mass losses during the production and the content of NPN on the texture parameters and sensory attributes of dry-cured ham was studied. All findings are expected to contribute to the improvement of the final product.

MATERIAL AND METHODS

Material and experimental design

Slovenian dry-cured ham – Kraški pršut (KP) – with protected geographical indication was used in the study. A total of 32 samples of Kraški pršut (410±33 days after salting) were included in the experiment. The manufacturer's data, ham mass before salting and at the end of the production process, and mass loss at the end of ripening were determined for the samples. The KP samples were divided according to mass of pork thighs at salting into two classes of 16 pieces each: light (L; 10.5–10.7 kg) and heavy (H; 11.7–12.1 kg), and within each mass class according to two suppliers of pork thighs (A and B); class L: 4 pieces from supplier A and 12 pieces from supplier B, class H: 8 pieces from supplier A and 8 pieces from supplier B. All KP samples were cut in half. The halves were weighed and sliced for various analyses (**Figure 1**). The pH and contents of water and intramuscular fat, NaCl, protein, and non-protein nitrogen (NPN) were measured on slice A; instrumental texture profile analysis (TPA) was carried out on slice B; and sensory analysis of the textural attributes of the samples was performed on slice C.

Physicochemical properties analysis

The superficial connective and adipose tissue were removed from the dry-cured ham slices. Then, approximately 100 g of a representative sample was homogenized for 20 s using a Grindomix homogenizer (GM 200; Retsch, Haan, Germany) at 5,000–6,000 rpm. After determination of pH and moisture content, the reminding homogenized samples were vacuum packed and stored at –20°C until further chemical analyses.

The pH was measured in an aqueous extract of homogenized ham. The extract was obtained by mixing 5 g of homogenate with 50 mL of distilled water, followed by filtration of the suspension after 30 min. The pH of the filtrate was measured using a combined glass-gel spear electrode (Type 03, Testo Pty Ltd, Croydon South, Victoria, Australia) with the accuracy of 0.01 units. Two buffers (pH 4.00 and pH 7.00) were used for the pH--meter calibration.

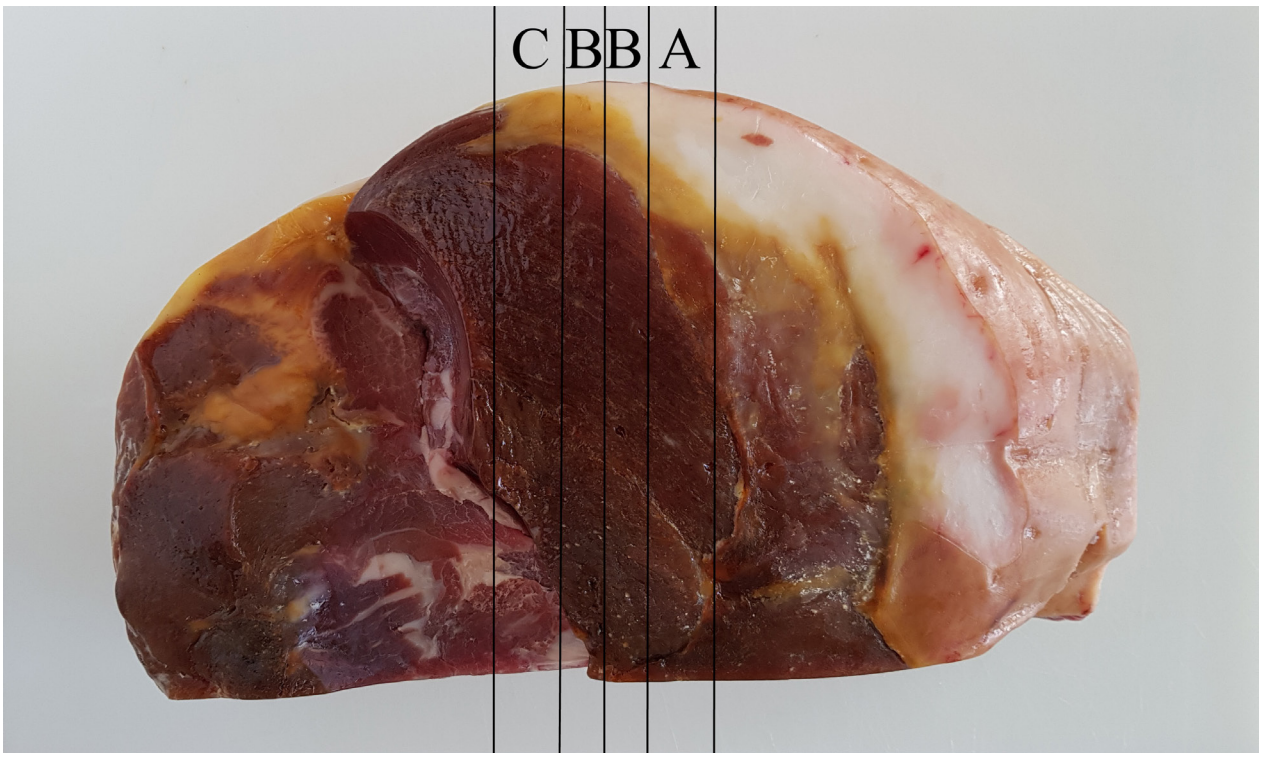

Figure 1. Place of cutting dry-cured ham slices for physicochemical properties analysis (**A**), instrumental texture profile analysis (**B**), and sensory analysis (**C**).

Moisture content was determined as the mass loss of 5 g of the homogenized sample dried to constant mass at 105°C by the official method 950.46 of AOAC International for moisture in meat [AOAC, 1997].

Total fat was determined by weight after Soxhlet extraction (Soxhlet™ 2050, Foss, Hillerød, Denmark) using petroleum ether as a solvent.

The Kjeldahl method was used to determine the total nitrogen content. The assay was carried out in accordance with the method 928.08 of AOAC International – Nitrogen in meat [AOAC, 1997]. The total nitrogen content was multiplied by 6.25 to calculate the protein content.

Non-protein nitrogen (NPN) content was determined by precipitating proteins with trichloroacetic acid and then determining the nitrogen content in the extract by the Kjeldahl method [AOAC, 1997]. Non-protein nitrogen was expressed as a percentage of non-protein nitrogen relative to total nitrogen. The proteolysis index (PI) was calculated as the ratio of NPN to total nitrogen (expressed as a percentage) [Careri *et al.,* 1993*;* Virgili & Schivazappa, 2002].

The salt content (NaCl) was measured using a sodium ion-selective electrode (DX223; Sodium Analyser AP214, Mettler Toledo GmbH, Zurich, Switzerland).

All parameters were determined in parallel and given as arithmetic means. The contents of moisture, total fat, protein, NPN and NaCl were expressed as a percentage of the initial mass.

Instrumental texture profile analysis

For instrumental measurement of textural properties, two 15 mm thick slices were taken from each KP sample. Using a scalpel, 12 squares measuring 20×20×15 mm were carefully cut from the muscles: – *semimembranosus* (SM) and *biceps femoris* (BF) (6 pieces for each muscle), covered with plastic wrap to prevent drying, and conditioned at a temperature of 4°C for 2 h. TPA was performed as described previously by Morales *et al.* [2007a] on three samples using the XT Plus texture analyser (Stable Micro Systems Ltd., Surrey, UK) for each muscle. The specimens were compressed twice to 50% vertical to the muscle fibres (with an interval of 5 s between these compression cycles) and at a crosshead speed of 5 mm/s. The force-time curves were recorded, and the following parameters were calculated: hardness (expressed as maximum force of the first compression), adhesiveness (expressed as the negative work between the two cycles), springiness (expressed as a ratio or percentage of the original downstroke compression), chewiness (expresses as the product of hardness, cohesiveness and springiness), and resilience (expressed by dividing the upstroke energy of the first compression by the downstroke energy of the first compression).

Sensory analysis

A panel of six qualified and experienced experts in the field of meat products was appointed to evaluate the sensory characteristics of dry-cured ham. The panel has been trained [ISO 8586:2014-05] and has participated in the sensory profiling of Kraški pršut on the Slovenian market for at least 10 years. The preparation of the descriptors was carried out during the last three sessions. The sensory evaluation of KP samples was carried out under defined, precisely prescribed, controlled and reproducible conditions which included arrangement of laboratory, samples, accessories and organization of assessment [ISO 8589:2007; ISO 8586:2012]. Assessment of the coded samples took place in a standard sensory laboratory. For each sample, two thin slices (0.5 mm) at room temperature (20°C) were randomly served to the panellists on a white plate. To neutralise the taste, the panel used the middle dough of white bread and water. The panel evaluated the samples separately in two sessions (days) with 16 samples each. The analytical descriptive test [Gašperlin *et al*., 2012] was performed by scoring the sensory attributes on a structured scale from 1 to 7 points, where a value of 1 means that the attribute is not expressed, and a value of 7 means a strongly expressed attribute. The panel could also use half values. The sensory profile of the KP samples was evaluated using seven descriptors divided into four blocks related to (I) visual attributes on the surface of the slice, namely marbling; (II) texture attributes,

Table 1. Definitions of descriptors used in the sensory evaluation of dry-cured hams.

Descriptor	Definition	Scale
Marbling	The proportion of intramuscular fat (visual assessment)	1 – absence of marbling 7 – extreme marbling
Hardness	Effort required to bite the sample thoroughly and bring it to a swallowable state	1 – very soft/tender 7 – very hard
Pastiness	Mouth-coating sensation during chewing	1 – absence of pastiness 7 – fully expressed pastiness
Solubility	Rate of disintegration of the slices during chewing before swallowing	1 – not soluble 7 – extremely soluble
Odour	Odour associated with dry cured ham	1 – absence of dry-cured ham odour 7 – fully expressed dry-cured ham odour
Aroma	Aroma associated with dry cured ham	1 – absence of dry-cured ham aroma 7 – fully expressed dry-cured ham aroma
Saltiness	Basic taste sensation elicited by NaCl	1 – not salty 4 – optimal saltiness 7 – extremely salty

namely overall hardness, pastiness, and solubility; (III) olfactory attributes, namely odour intensity; and (IV) aroma attributes, namely aroma intensity and saltiness. The definitions of the descriptors are listed in **Table 1.**

Statistical analysis

Data were analysed using the SPSS statistical program (version 23.0, SPSS Inc., Chicago, IL, USA). The normality of the distribution and the homogeneity of variance were tested using the Shapiro-Wilk test (α=0.05) and the Levene test. The experiment aimed to evaluate the physicochemical, TPA parameters and sensory attributes of KP samples was performed with a 2×2×8 mixed factorial experimental design (2 types of mass classes (H and L), 2 types of thighs suppliers (A and B), and 8 production replicates). As the interaction mass class × thigh supplier was not significant (p>0.05), it was removed from model 1. A statistical model 2 was used to analyse the differences between the KP samples, which were classified into three ranks according to the calculated median value (SPSS, Descriptive Statistics, Explore) of six ratings of mouth hardness (M) by the panellists for each KP sample (ranks: median 4.0 (optimal), median 3.5 (soft), median 3.0 (very soft)). Differences between the groups/ranks were considered significant at p≤0.05. Pearson correlation coefficients between the parameters were calculated using the Correlate procedure of SPSS software, and the interactions between the variables were analysed using the multivariate linear descriptive analysis (LDA) method.

RESULTS AND DISCUSSION

Physicochemical parameters and sensory attributes of Kraški pršut

As shown in **Table 2**, there were significant effects of mass classes and thighs suppliers on some physicochemical parameters of KP samples: dry-cured ham mass, pH value, moisture and NaCl contents. The mass of KP after sampling or before slicing ranged from 7.07 kg for light to 8.08 kg for heavy hams, where the masses from supplier A were significantly higher (p≤0.001) from those of supplier B. Values of pH were between 6.01 for light samples and 6.09 for heavy samples, with higher values noted for those from supplier A (6.11). NaCl and moisture contents were both observed higher in the products from supplier B, where light KP samples had higher content of NaCl (5.30 g/100 g) and lower content of moisture (42.17 g/100 g). Mass classes had no significant effect on mass loss during the production process, higher values were determined for the KP samples from supplier B than supplier A (33.53% vs 32.86%). Mass classes and thighs suppliers had no effect on protein content and proteolysis index. In contrast, total fat content was found to be significant higher (p≤0.01) in the KP samples from supplier A (23.06 g/100 g), whereas NPN content was higher in the KP samples from supplier B (7.04 g/kg).

For comparison, data from the Slovenian Nutrition Tables – Meat and Meat Products [Golob *et al.,* 2006] given for Kraški pršut before protected geographical indication were as follows: water content of 51.8 g/100 g (44.4–57.9 g/100 g), total protein 32.1 g/100 g (25.0–43.9 g/100 g), total fat 7.4 g/100 g (6.5–8.3 g/100 g), and salt 7.6 g/100 g (4.6–10.1 g/100 g). Žlender *et al.* [2013], in a study on KP, found average total mass loss due to dehydration and trimming during processing from 34.75% to 36.63%, moisture content 36.99±11.77 g/100 g, protein 29.12±4.84 g/100 g, fat 27.69±14.91 g/100 g, and NaCl 5.26±1.17 g/100 g. Andronikov *et al.* [2013] determined higher NPN in BF muscle (22.0–23.2%) than in SM muscle (14.1–15.2%) in KP samples. Toldrá [2006] explained these high NPN values in dried meat by the fact that proteolytic enzymes are still relatively active at water activity (a_w) values at the end of the production process (0.85–0.90), although a low a_w value reduces the activity of cathepsins and other muscle enzymes such as aminopeptidases. The average proteolysis index in our KP samples was lower (16.7%) than these in dry-cured Italian protected designation of origin (PDO) hams (28.6%, 24.6%, and 22.4% in Parma ham, San Daniele, and Toscano prosciutto, respectively) [Piasentier *et al.,* 2021]. In general, KP analysed in our study contained less moisture but had a comparable content of protein and salt to other better-known European dried meats, *e.g.*, Corsican, French (Bayonne), and Italian (Parma) prosciutto (53.3–60.8%, 26.5–32.5%, 5.3–9.2%, respectively) [Virgili & Schivazappa, 2002].

The instrumental texture parameters of the KP samples are shown in **Table 2**. Mass classes had a significant effect on all tested parameters, with the exception of adhesiveness. The light KP samples, regardless of thighs supplier, were significantly harder, chewier, more springy, cohesive and resilient compared to the heavy samples. The main differences in ham texture may be explained by moisture content [Monin *et al.,* 1997; Serra *et al.,* 2007]. The hypothesis that a lower salt content promotes proteolysis, resulting in a softer texture of the final product [Gou *et al.,* 2008], applies only to heavy hams in the present study.

Mass classes and thighs suppliers had no influence (p>0.05) on the sensory evaluated marbling of the KP samples (**Table 2**). Panellists estimated that the light KP samples were significantly harder (p≤0.01) than the heavy ones, which coincides with instrumental measurements. Changes in hardness during ripening of Bayonne ham were attributed by researchers to both moisture content and the extent of protein proteolysis [Monin *et al.*, 1997]. Some researchers explain the higher hardness (by mouth) of dry hams by lower pH than is typical of normal quality [Guerrero *et al.,* 1999]. In the present study, the differences in pH values between KP samples were too small to explain the variability in hardness (M). The panellists found no significant differences (p>0.05) in pastiness and solubility between KP samples. Surprisingly, the panellists could not detect any differences in odour intensity between the KP samples. Most likely, the differences in the intensity of this olfactory property were not sufficient enough for the panellists to detect. However, they noticed significant differences in the aroma of the KP samples, where the heavy samples were estimated with higher scores (5.42) than the light ones (5.19). The light KP samples were saltier compared to the heavy samples (5.49 *vs* 5.13). Saltiness was also affected by thighs suppliers, with saltier KP samples from supplier B. These results are in accordance with chemically analysed NaCl content. In general, all KP samples were too salty - estimated above the optimal value of 4.0 [Gašperlin *et al.,* 2012]. The NaCl content and sensory evaluated saltiness of KP samples were also higher compared

Table 2. Effects of mass classes (heavy, H and light, L) and thighs suppliers (A and B) on physicochemical and texture profile analysis (TPA) parameters, and sensory attributes of Kraški pršut hams.

Parameter/attribute	Mass class			Thigh supplier		
	H (*n*=16)	L (*n*=16)	p_M	A (*n*=12)	B (*n*=20)	p_S
Physicochemical parameter						
Mass loss (%)	33.6±2.8	33.0±0.7	Ns	32.9±3.0	33.5±1.0	*
Dry-cured ham mass (kg)	8.08±0.42	7.07±0.30	***	7.94±0.71	7.34±0.44	***
pH	6.09±0.10	6.01±0.07	***	6.11±0.09	6.02±0.08	***
NaCl (g/100 g)	4.51±0.56	5.30±0.50	***	4.34±0.46	5.24±0.52	***
Moisture (g/100 g)	44.2±2.1	42.2±3.3	***	42.5±3.9	43.5±2.3	**
Total fat (g/100 g)	20.8±3.4	21.7±6.6	Ns	23.1±4.8	20.3±5.4	**
Protein (g/100 g)	26.2±1.9	26.5±1.9	Ns	25.9±2.0	26.6±1.8	Ns
Proteolysis index (%)	16.9±1.6	16.6±1.2	Ns	16.4±0.8	16.9±1.7	Ns
Non-protein nitrogen (g/kg)	6.93±0.69	7.05±0.42	Ns	6.91±0.42	7.04±0.62	*
TPA parameter						
Hardness (TPA) (N)	69±37	89±26	**	74±39	83.3±30	Ns
Adhesiveness (N×mm)	–3.26±1.08	–3.04±0.87	Ns	–3.53±1.06	–2.93±0.86	**
Springiness (–)	0.62±0.07	0.66±0.05	**	0.64±0.06	0.64±0.07	Ns
Cohesiveness (–)	0.53±0.05	0.57±0.04	***	0.54±0.05	0.56±0.05	Ns
Chewiness (N)	24±148	34±12	***	27±16	30±13	Ns
Resilience (–)	0.13±0.03	0.15±0.02	**	0.14±0.04	0.14±0.02	Ns
Sensory attribute						
Marbling (1–7)	1.72±0.38	1.66±0.36	Ns	1.79±0.48	1.63±0.27	Ns
Hardness (M) (1–7)	3.35±0.43	3.73±0.77	**	3.48±0.67	3.59±0.65	Ns
Pastiness (1–7)	1.98±0.44	1.88±0.39	Ns	1.91±0.34	1.93±0.46	Ns
Solubility (1–7)	5.15±0.37	5.13±0.34	Ns	5.18±0.37	5.12±0.34	Ns
Odour (1–7)	5.56±0.48	5.43±0.54	Ns	5.52±0.61	5.48±0.46	Ns
Aroma (1–7)	5.42±0.32	5.19±0.42	**	5.38±0.40	5.26±0.39	Ns
Saltiness (1–4–7)	5.13±0.48	5.49±0.48	**	5.09±0.48	5.44±0.50	**

Results are shown as mean ± standard error; *n*, number of dry-cured hams; p_M, significance of mass classes effect; p_S, significance of thigh supplier effect; Ns, not significant ($p>0.05$); *, significant ($p\leq0.05$); **, highly significant ($p\leq0.01$); ***, very highly significant ($p\leq0.001$); M, hardness by mouth.

to some other European hams, such as Parma ham (*m. biceps femoris*, 4.0–5.5%) [Benedini *et al.,* 2012; Virgili *et al.,* 2007] and de Bayonne ham (*m. semimembranosus*, 4.9%; *m. biceps femoris*, 5.6%). The high saltiness was due to high dehydration and total mass loss (average 33.3%) and was higher than in Italian ham (di Parma, San Daniele) [Virgili *et al.,* 2007; Virgili & Schivazappa, 2002] and similar to Spanish Seranno [Ruiz-Ramírez *et al.*, 2006] and French de Bayonne ham [Monin *et al.*, 1997].

Classification of Kraški pršut according to sensory evaluated texture

To evaluate the texture profile, the KP samples were classified into ranks (**Table 3**) according to the median values obtained for hardness in sensory evaluation. Median of hardness (M) was evaluated by six panellists and used to categorise the KP samples into three ranks: median 4.0 (optimal), median 3.5 (soft), median 3.0 (very soft). The highest proportion of KP samples was categorised into rank soft (72%), followed by rank optimal (19%) and rank very soft (9%). The KP samples with optimal texture had a significantly higher NaCl content and a lower moisture content than the KP samples with soft and very soft texture. Significant differences were also observed for all TPA parameters, with the highest values noted for the KP samples with optimal texture. We did not find any similar classification of dry-cured hams in the available literature, although the statistical technique LDA is a widely applied sensory profiling method which identifies

Table 3. Physicochemical and instrumental texture profile analysis (TPA) parameters and sensory attributes of Kraški pršut hams categorised into three ranks (4.0, 3.5 and 3.0) formed according to the calculated median values for hardness evaluated by sensory panellists.

Parameter/attribute	4.0 (*n*=6)	3.5 (*n*=23)	3.0 (*n*=3)	p_R
Physicochemical parameter				
Mass loss (%)	32.92±0.31	33.48±0.47	32.20±1.87	Ns
Dry-cured ham mass (g)	7028±110	7713±126	7717±527	Ns
pH value	6.01±0.01^b	6.06±0.01^a	6.06±0.02^a	*
NaCl (g/100 g)	5.45±0.11^a	4.75±0.05^b	4.92±0.14^b	***
Moisture (g/100 g)	41.75±0.57^b	43.45±0.24^a	43.49±0.28^a	**
Total fat (g/100 g)	21.37±1.68	21.17±0.59	22.94±0.93	Ns
Protein (g/100 g)	27.04±0.34^a	26.25±0.16^a	24.55±0.32^b	***
Proteolysis index (%)	16.75±0.21^{ab}	16.64±0.13^b	17.37±0.28^a	Ns
Non-protein nitrogen (g/kg)	6.99±0.05^b	6.95±0.05^b	7.41±0.10^a	**
TPA parameter				
Hardness (TPA) (N)	105.8±5.7^a	84.2±2.8^b	61.1±4.7^c	***
Adhesiveness (N×mm)	−2.65±0.14^a	−3.13±0.08^b	−3.91±0.34^c	***
Springiness (–)	0.67±0.01^a	0.64±0.01^a	0.58±0.02^b	***
Cohesiveness (–)	0.58±0.01^a	0.55±0.00^b	0.52±0.01^c	***
Chewiness (N)	40.5±2.2^a	29.9±1.2^b	19.1±2.1^c	***
Resilience (–)	0.15±0.00^a	0.14±0.00^a	0.12±0.01^b	***
Sensory attribute				
Marbling (1–7)	1.61±0.01	1.70±0.00	1.70±0.10	Ns
Hardness (M) (1–7)	4.32±0.10^a	3.51±0.01^b	3.11±0.10^c	***
Pastiness (1–7)	1.70±0.10^c	1.90±0.01^b	2.31±0.10^a	***
Solubility (1–7)	5.30±0.10^a	5.20±0.00^{ab}	5.10±0.11^b	Ns
Odour (1–7)	5.42±0.10	5.60±0.02	5.61±0.11	Ns
Aroma (1–7)	5.20±0.10^b	5.41±0.00^a	5.41±0.10^a	**
Saltiness (1–4–7)	5.50±0.10^a	5.21±0.00^b	5.30±0.10^b	*

Results are shown as mean ± standard error; *n*, number of dry-cured hams; p_R, significance of rank effect; Ns, not significant ($p>0.05$); *, significant ($p\leq0.05$); **, highly significant ($p\leq0.01$); ***, very highly significant ($p\leq0.001$); M, hardness by mouth. Values with different letters (a–c) within parameter are significantly different ($p\leq0.05$).

patterns in features to distinguish between different classes [Liu *et al.*, 2018; Marques *et al.*, 2022].

Figure 2 shows the projection of LDA parameters and data onto different ranks in the area defined by the first two functions (F1 and F2, 100% of the total variance explained). For a 100% correct distribution of KP samples into defined three ranks, only nine parameters were needed, namely, TPA parameters and sensory attributes – hardness, pastiness and solubility. The plot shows the ability of the analyses used to discriminate KP samples according to their texture. On F1, the KP samples with optimal texture (median 4.0) grouped on the left side of the plot were clearly distinguished from the KP samples with soft to very soft texture (median 3.5 and 3.0) on the right side of plot. Along F1, the KP samples with a median of 4.0 were described by the sensory descriptor hardness and the TPA parameters chewiness and springiness. In addition, the KP samples with ranks 3.5 and 3.0 (samples appeared to be more scattered) were characterised by softness (the opposite of hardness TPA) and pastiness. Compared to the samples with rank 3.5, which are located in the upper right part of the plot, the samples with rank 3.0 are located in the lower right part of the plot and are clearly related to the sensory descriptor pastiness and less related to cohesiveness, which was measured instrumentally.

From the manufacturer's point of view, it is also important from which mass class the undesirable textures of KP samples were produced, such as soft and very soft textures (**Table 4**). KP samples with optimal texture were produced from light thighs (100%), soft KP samples were made from heavy (61%) and light

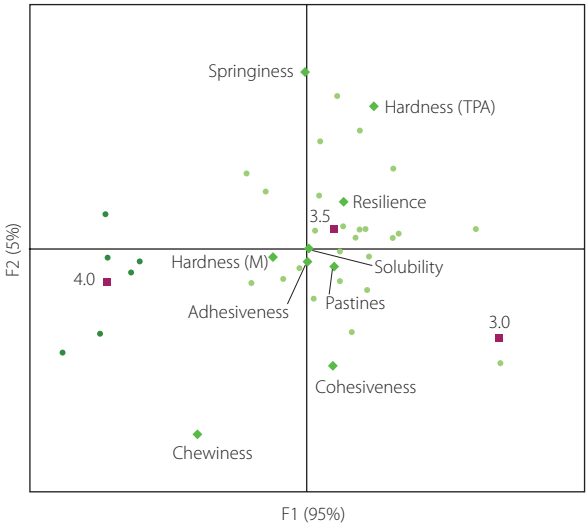

Figure 2. Linear discriminant analysis of the results of sensory and instrumental texture profile analyses of the 32 Kraški pršut samples, divided into three ranks according to the median values for hardness obtained in the sensory analysis.

Table 4. Composition of ranks of Kraški pršut (KP) hams according to sensory assessed texture.

KP ranks	Number of KP	Mass class (pcs)	Supplier (pcs)
Optimal	6	Light (6)	A (2), B (4)
Soft	23	Light (9), heavy (14)	A (9), B (14)
Very soft	3	Light (1), heavy (2)	A (1), B (2)

(39%) thighs, and very soft KP samples were mainly produced from heavy pork thighs (66%). No significant differences were found in the texture type distribution of KP samples among suppliers (χ^2=2.000, critical value χ^2=3.841, df=1, p=0.157). However, since the goal of manufacturers is to produce a larger quantity of the final product, they want to use heavy pork thighs as raw material. To achieve this goal and reduce the undesirable texture of dry-cured hams, certain technological steps must be adapted.

It was assumed that major mass losses in the production of KP samples as a result of drying, ripening and, last but not least, the quality of meat with dark, firm, dry (DFD) or pale, soft, exudative (PSE) properties were related to the texture parameters and sensory attributes of the final product. However, the results of the correlation analysis showed the opposite phenomenon; the losses were not strongly related to the mentioned parameters of KP samples (**Table 5**). A positive correlation coefficient was found between KP sample mass and aroma (p≤0.01; r=0.27) and pastiness (p≤0.05; r=0.16), a negative correlation coefficient between KP sample mass and hardness (M) (p≤0.01; r=–0.32) and saltiness (p≤0.01; r=–0.41) as well TPA parameters (hardness (TPA), springiness, cohesiveness, chewiness and resilience) (p≤0.01; r=–0.22 to –0.33) and a very strong correlation with NaCl content (p≤0.01; r=–0.80). The lighter hams were harder and saltier, more cohesive and chewier than the heavier hams. Similar relationships as for the KP sample mass were also found for the mass of pork thighs.

The NPN content is interesting because it is an indicator of the extent of ripening, tenderizing and aroma development

Table 5. Coefficients of Pearson correlations between production parameters, physicochemical parameters, instrumental texture profile analysis (TPA) parameters and sensory attributes of Kraški pršut (KP) hams (n=192).

Parameter	Pork thigh mass[a]	Mass loss[a]	KP mass[a]	NaCl	NPN	PI
Hardness (M)	–0.34**	–0.03	–0.32**	0.25**	0.00	–0.02
Pastiness	0.18*	–0.09	0.16*	–0.10	0.17*	0.17*
Solubility	–0.02	0.10	–0.05	0.00	0.05	–0.05
Odour	0.10	0.07	0.08	–0.16*	–0.03	–0.10
Aroma	0.27**	0.00	0.27**	0.35**	–0.06	–0.16*
Saltiness	–0.38**	0.17*	–0.41**	0.53**	0.06	0.04
Hardness (TPA)	–0.27**	0.01	–0.29**	0.31**	–0.08	–0.03
Adhesiveness	–0.13	0.00	–0.17*	0.20**	–0.06	–0.08
Springiness	–0.26**	0.18*	–0.22**	0.10	–0.03	–0.18*
Cohesiveness	–0.30**	0.21**	–0.30**	0.22**	–0.07	–0.20**
Chewiness	–0.33**	0.05	–0.33**	0.31**	–0.07	–0.10
Resilience	–0.26**	0.12	–0.24**	0.13	–0.09	–0.18*
NaCl	–0.62**	–0.15	–0.80**	1	–0.03	–0.06
NPN	–0.06	–0.06	–0.09	–0.03	1	0.60**
PI	–0.16*	–0.02	–0.02	–0.04	0.60**	1

[a]The batch average value was included in the calculation. n, Number of comparisons; *, significant (p≤0.05); **, highly significant (p≤0.01); NPN, non-protein nitrogen; PI, proteolysis index; M, texture by mouth.

of the final product. In the correlation analysis between the NPN content and the texture parameters, a positive coefficient and very weak correlation was found for pastiness ($p \leq 0.05$; $r=0.17$) (**Table 5**). For the PI, the same finding was noticed for pastiness and a negative correlation coefficient with aroma as well as TPA parameters (springiness, cohesiveness and resilience) ($p \leq 0.05$; $r=-0.20$ to -0.16).

CONCLUSIONS

Physicochemical properties, instrumental texture profile analysis parameters and sensory attributes have contributed to classifying Kraški pršut as optimal (19%), soft (72%) and very soft (19%). The correlation between the texture of KP samples, the degree of proteolysis and mass loss during production cannot be fully demonstrated; therefore, increasing the level of drying hams to a higher mass loss does not ensure a better texture of the product. However, in the present study, the mass of KP samples was negatively related to saltiness and hardness (determined by both sensory and instrumental analysis), and positively related to pastiness and aroma, with the light hams being harder and saltier but less pasty and aromatic than the heavy ones. Thus, to achieve the optimal texture of dry-cured ham Kraški pršut, certain technological steps must be adapted. The use of light thighs is one of the solutions, and it is better if producers adjust/divide the whole production scheme, starting from the separation of the thighs by weight, limiting dehydration and extending the ripening phase.

ACKNOWLEDGEMENTS

Authors wish to thank Monika Kočar and Nina Mihelj who participated in analyses. Results presented in the manuscript are part of their master's theses.

RESEARCH FUNDING

The work was financially supported by the research programs funded by the Slovenian Research Agency (P4-0234).

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 120–129
DOI: 10.31883/pjfns/186717
ISSN 1230-0322, e-ISSN 2083-6007

Antibacterial Effect of Sea Buckthorn (*Hippophae rhamnoides L.*) Fruit Extract on Radish Seeds Prior to Sprouting

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Sprouts consumption is increasing worldwide due to their nutritional and health benefits. However, they can be sources of foodborne bacterial diseases, including sprout-related outbreaks caused primarily by Shiga toxin-producing *Escherichia coli* and *Salmonella* spp. The aim of this study was to determine the efficacy of a dried sea buckthorn fruit extract in sanitization of contaminated radish seeds. The efficacy of 15-min seed treatment and 3-h soaking in the extract solution was evaluated in comparison to the treatment with 20,000 mg/L calcium hypochlorite, recommended by the U.S. Food and Drug Administration. The effect of the sea buckthorn fruit extract on radish seed germination capacity and the profile of phenolic compounds in the extract were also determined. Decontamination effect of the extract after a 15-min seed treatment ranged from 72.1% against *Staphylococcus aureus* to 93.0% against *Listeria monocytogenes* and was higher than that of active chlorine against *E. coli*, *S. aureus* and *Salmonella enterica*. Soaking seeds for 3 h in the extract increased the decontamination efficiency only against *S. aureus* (81.9%). Compared to water, after 4 days of sprouting, no significant differences were found in radish seed germination capacity and the length of roots and hypocotyls of sprouts. The sea buckthorn fruit extract had a high content of phenolic compounds, namely isorhamnetin 3-rhamnosylglucoside (12.99 mg/L), isorhamnetin 3-rutinoside (8.25 mg/L), protocatechuic acid (5.43 mg/L), isorhamnetin 3-glucoside (3.41 mg/L), and gallic acid (3.36 mg/L). The extract can be used as a substance limiting bacterial contamination of radish seeds and, as a result, sprouts, as well as a valuable source of phenolic compounds.

Keywords: sea buckthorn fruit, antibacterial activity, radish seed decontamination, seed germination, phenolic compounds

INTRODUCTION

Radish sprouts have gained popularity worldwide due to their nutritional value and a high content of bioactive compounds including flavonoids, phenolic acids, glucosinolates, isothiocyanates, and ascorbic acid [Gamba *et al.*, 2021]. Their ingestion may elicit a hypoglycemic effect by lowering the plasma levels of fructosamine and glucose followed by a decrease in the plasma level of insulin and improved insulin sensitivity. Radish sprouts are known to exhibit high antioxidant activity and to lower the risk of development of certain cancers and heart diseases [Abellán *et al.*, 2019]. Moreover, health-promoting phytochemicals are more concentrated in sprouts than in adult plant edible organs [Gamba *et al.*, 2021].

Although consumption of sprouts has been associated with health benefits, they have also been claimed to be one of the most common sources of bacterial foodborne illnesses. There have been documented multiple outbreaks of foodborne diseases induced by sprout consumption, with the causative agents being primarily Shiga toxin-producing *Escherichia coli* and various serotypes of *Salmonella* [Miyahira & Antunes, 2021; NACMCF, 1999]. According to the Centers for Disease Control and Prevention (CDC) and U.S. Food and Drug Administration

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Submitted: 19 January 2023
Accepted: 2 April 2024
Published on-line: 18 April 2024

(FDA) data [CDC, 2023; FDA, 2023], there have been ten sprouts-related outbreaks noted over the last 10 years, in which a total of 367 people were infected and 77 people required hospitalization. In a *Listeria monocytogenes* outbreak linked to mung bean sprouts in 2014, two of the five hospitalized people died, while no deaths were reported in other outbreaks.

The other pathogens most frequently isolated from sprouted seeds were *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus cereus* [Kang *et al.*, 2011; NACMCF, 1999; Viswanathan & Kaur, 2001]. Contaminated seeds have been recognized to be the main source of pathogenic bacteria introduced into sprouts [Miyahira & Antunes, 2021; NACMCF, 1999; Sikin *et al.*, 2013]. Seeds usually harbour native aerobic microbiota (10^2–10^7 CFU/g) and fecal coliforms (10^2–10^3 CFU/g) [Michalczyk & Kowalińska, 2009; Sikin *et al.*, 2013]. However, during sprouting the number of bacteria can increase significantly, reaching from >10^6 to even 10^{12} CFU/g [Lang *et al.*, 2016; Miyahira & Antunes, 2021; NACMCF, 1999; Sikin *et al.*, 2013].

In order to reduce the risk posed to the consumer by the consumption of contaminated sprouts, various methods of seeds disinfection have been applied. It is known that disinfection of seeds prior to sprouting is more effective than that of sprouts [Sikin *et al.*, 2013]. According to FDA recommendations [FDA, 2023], soaking seeds in 20,000 mg/L of a chlorine solution before sprouting should be applied as a preventive method. However, this method has been proved poorly effective in eliminating *Salmonella* spp. and *E. coli* O157:H7 [Montville & Schaffner, 2004]. Moreover, the use of chlorine as a disinfectant can result in the formation of carcinogenic compounds and the emergence of new, more tolerant pathogens [Allende *et al.*, 2008]. The use of 20,000 mg/L calcium hypochlorite may also pose a risk to the environment and worker safety. Therefore, new, safe and effective methods are being sought to disinfect seeds prior to sprouting and thus enhance consumer safety.

Sea buckthorn (*Hippophae rhamnoides* L.) has been used in traditional medicine by Tibetans and Mongolians for over 2,000 years [Chen *et al.*, 2023]. Currently, the most important area of its application, apart from the production of pharmaceuticals, is food and feed production. The plant, especially its berries, is used as a raw material to produce functional food or food supplements. It can also be successfully provided to consumers who promote a healthy lifestyle or those who are interested in preventing or alleviating diet-related diseases [Chen *et al.*, 2023; Jaśniewska & Diowksz, 2021]. Sea buckthorn berries are valued for their high content of various essential nutrients, such as essential amino acids, polyunsaturated fatty acids (especially omega-7 palmitoleic acid), vitamins (mainly vitamin C and E), minerals and a variety of bioactive compounds with flavonoids and phytosterols being the main medicinal active ingredients [Chen *et al.*, 2023]. The health benefits for the consumer are related to antimicrobial activity of the berries, their anti-inflammatory effects, support of tissue regeneration, protection against cancer, strengthening the immune system, as well as anti-myocardial, anti-hyperlipidemic, fatty liver-preventing, and wound-healing effects [Jaśniewska & Diowksz, 2021]. Sea buckthorn fruit, as a rich source of many active substances with health-promoting properties, seems to be a valuable material for use in a broad range of products, as available in some countries fruit vinegar, sea buckthorn yogurt, sea buckthorn fermented soy milk, sea buckthorn nutritional milk powder, sea buckthorn probiotic yogurt or probiotic drink, and others [Chen *et al.*, 2023].

This study was undertaken to determine the efficacy of the extract from dried sea buckthorn fruit in the disinfection of contaminated radish seeds in comparison to the treatment with 20,000 mg/L active chlorine. Previous studies have shown seed germination to be the critical moment from the microbiological viewpoint, and sprouting conditions to promote bacteria development [NACMCF, 1999; Sikin *et al.*, 2013]. Hence, the efficacy of 15-min treatment as well as 3-h soaking of the seeds in the extract was evaluated in this study.

MATERIALS AND METHODS

Preparation of sea buckthorn fruit extracts

The dried sea buckthorn fruit (Dary Podlasia Adam Nowicki, Bielsko Podlaskie, Poland) were ground to powder, and 10 g of the powder were suspended in 90 mL of water and in 90 mL of 5% (*v/v*) ethanol to obtain water and water-ethanol extract, respectively. Extraction was carried out for 40 min in an ultrasonication bath (Sonorex, Bandelin Electronic, Berlin, Germany) at 40°C [Rajkowska *et al.*, 2023]. After cooling, the samples were filtered through a Whatman filter paper (grade 1:11 µm) and centrifuged at 2,800×*g* for 10 min. In order to obtain sterile extracts, supernatants were filtered using Minisart syringe filters with a pore size of 0.45 µm (Sartorius Stedim Biotech, Goettingen, Germany). The extracts were stored at 4°C in the dark up to 1 month.

Assessment of antibacterial activity

Antibacterial activity of water and water-ethanol extracts of sea buckthorn fruit, and a water solution of calcium hypochlorite (Warchem Sp. z o.o., Zakręt, Poland) at a concentration of 20,000 mg/L was determined with the agar diffusion plate method using the following bacterial strains: *Escherichia coli* ATCC 8739, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 19111, and *Staphylococcus aureus* ATCC 6538 (purchased from Merck Life Science Sp z o.o., Poznań, Poland). One hundred microliters of a bacterial suspensions (10^7–10^8 cells/mL) in a sterile saline solution (sodium chloride 8.5 g/L) were spread on the surface of Tryptic Soy Agar (TSA) (pancreatic digest of casein 15 g/L, papain digest of soya 5 g/L, sodium chloride 5 g/L, agar 15 g/L; pH 7.2; Merck KGaA, Darmstadt, Germany). Then, blank antimicrobial susceptibility discs (Ø 6 mm, Thermo Fisher Scientific, Waltham, MA, USA) were placed centrally on the plates, and 20 µL of the tested substances were applied. In addition, the antibacterial effect of 5% (*v/v*) ethanol was tested as a control for the water-ethanol extract. The plates were incubated at 37°C for 24 h. The results were expressed as bacterial growth inhibition zones around the discs (mm) as an arithmetic mean from three independent experiments.

Inoculation and disinfection of contaminated radish seeds

The bacterial suspensions were prepared in a sterile saline solution from TSA slants cultures of the tested microorganisms to achieve a final inoculum containing 10^8 to 10^9 cells/mL, according to McFarland standards. Radish seeds for sprouts and microgreens were obtained from a commercial supplier (Zielone Witaminy Kamil Stankiewicz, Lidzbark, Poland) and stored at room temperature until use. Radish seeds (100 g) were added to the inoculum of the tested bacteria monocultures (150 mL) and gently mixed for 2 min. The seeds were drained, spread over double-layer of sterile Miracloth (Calbiochem, San Diego, CA, USA) on the top of a plastic rack, and air-dried for 48 h under a laminar flow hood (Airstream, Esco Lifesciences, Friedberg, Germany).

Ten grams of seeds contaminated with bacteria were submerged with 25 mL of sterile distilled water (control), 20,000 mg/L calcium hypochlorite solution or sea buckthorn fruit extracts for 15 min at room temperature with gentle agitation on a platform shaker (100 rpm). The seeds were drained and those treated with calcium hypochlorite were rinsed in 100 mL of distilled water.

The efficacy of disinfection after seed soaking, which is the standard procedure for seeds before germination, was also assessed. Seeds previously treated with calcium hypochlorite were soaked in sterile distilled water, and those treated with the sea buckthorn fruit extract were soaked in the same extract, for 3 h at room temperature. Seeds soaked in sterile distilled water served as a control.

Enumeration of microorganisms

The number of bacteria was determined before and after treatment with calcium hypochlorite, sea buckthorn fruit extract, and water as control, as well as after soaking. Ten grams of seeds were weighed and transferred to 90 mL of a peptone (1.0 g/L) water solution (after treatment with water and sea buckthorn fruit extract) or to 90 mL of a peptone (1.0 g/L) water solution with the addition of 37.0 g/L sodium thiosulfate as a neutralizer [Jeong *et al.*, 2010]. The samples were homogenized in a stomacher (Devimix, De Ville Biotechnology, Raszyn, Poland) for 2 min, and serial tenfold dilutions were prepared. Enumeration of bacteria was performed by the pour plate method using selective media, *i.e.*, Tryptone-Bile-X-Glucuronate (TBX) agar (bile salts 1.5 g/L, X-β-D-glucuronide 0.075 g/L, peptone 20.0 g/L, agar 15.0 g/L; pH 7.2; Merck KGaA, Darmstadt, Germany) for *E. coli*, Xylose Lysine Deoxycholate (XLD) agar (ferric ammonium citrate 0.8 g/L, lactose 7.5 g/L, L-lysine hydrochloride 5.0 g/L, phenol red 0.08 g/L, sodium chloride 5.0 g/L, sodium deoxycholate 1.0 g/L, sodium thiosulfate 6.8 g/L, sucrose 7.5 g/L, xylose 3.75 g/L, yeast extract 3.0 g/L, agar 15.0 g/L; pH 7.4; Merck KGaA, Darmstadt, Germany) for *S. enterica*, Listeria mono Differential Agar Base according to Ottaviani & Agosti (casein enzymic hydrolysate 6.0 g/L, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside 0.05 g/L, disodium hydrogen phosphate anhydrous 2.5 g/L, glucose 2.0 g/L, lithium chloride 10.0 g/L, magnesium glycerophosphate 1.0 g/L, magnesium sulfate 0.5 g/L, meat peptone 18.0 g/L, sodium chloride 5.0 g/L, sodium pyruvate 2.0 g/L, yeast extract 10.0 g/L, L-α-phosphatidylinositol 0.016 g/L, selective supplement 40 mL/L: nalidixic acid 0.02 g/L, ceftazidime 0.02 g/L, amphotericin B 0.01 g/L, and agar 15.0 g/L; pH 7.2; Merck KGaA, Darmstadt, Germany) for *L. monocytogenes*, and Baird-Parker agar (casein peptone 10.0 g/L, glycine 12.0 g/L, lithium chloride 5.0 g/L, meat extract 5.0 g/L, sodium pyruvate 10.0 g/L, yeast extract 1.0 g/L, egg yolk emulsion 50 mL/L, agar 15.0 g/L; pH 6.8; Merck KGaA, Darmstadt, Germany) for *S. aureus*. The plates were incubated at 37°C for 48 h. The number of colony forming units *per* 1 g of seeds (CFU/g) was counted.

On the basis of the number of bacteria after treatment in water as control ($N_{control}$) and after treatment with the tested substances (N_{test}), the decontamination effect was calculated as microbial reduction percentages, according to formula (1) [Mascarenhas *et al.*, 2022]:

$$\text{Microbial reduction (\%)} = ((N_{control} - N_{test})/N_{control}) \times 100 \quad (1)$$

Radish seed germination

Uninoculated radish seeds treated with water, calcium hypochlorite, and water sea buckthorn extract for 15 min as described above, were placed on sterile moistened filter pad (Whatman, grade 1:11 µm) in sterile Petri dishes (140×20 mm, Biospace, Poznań, Poland). The filter paper was moistened with 10 mL of distilled water once a day or more frequently if necessary [Charkowski *et al.*, 2001]. The percentage of seeds that germinated at room temperature (22–24°C) after 24, 48, 72 and 96 h was determined. The length of hypocotyls and roots of all sprouts was also measured after 96 h. A total of 100 radish seeds were evaluated in triplicate.

High-performance liquid chromatography analysis of sea buckthorn fruit extract

Chromatographic separation of phenolic compounds of the sea buckthorn fruit extract was carried out according to Czyżowska *et al.* [2020] on a Spherisorb ODS2 column (250×4.6 mm, 5 µm packing) (Waters, Milford, MA, USA). The mobile phase consisted of 5% formic acid (solvent A) and 95% acetonitrile (solvent B). The samples were eluted with the following gradient: 2 min, 97% (A); 13 min, 97–85% (A); 9 min, 85–82% (A); 31 min, 82–75% (A); 5 min, 75–70% (A). The flow rate was 0.8 mL/min, and the injection volume was 10 µL of the standard solution or extract. Analyses were performed using a Finnigan Surveyor high-performance liquid chromatography (HPLC) system equipped with an autosampler and a diode array detector (Thermo Scientific, Waltham, MA, USA). Phenolic compounds were quantified according to calibration curves established, at specific wavelengths, for gallic acid (280 nm), caffeic acid (320 nm), and quercetin-glucoside (360 nm). The content of compounds with λ_{max} closest to 280 nm was expressed as gallic acid equivalent, the content of compounds with λ_{max} closest to 320 nm was expressed as caffeic acid equivalent and flavonols as quercetin-glucoside equivalent. The results were expressed as mg/L of the extract.

Compounds were identified using available standards and based on the literature [Guo *et al*., 2017; Wang *et al*., 2022]. UV absorption spectra were scanned in the range of 210–500 nm. The peaks were identified by comparisons of the UV absorption spectra to those of the reference compounds. Most standards (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid) were purchased in Sigma-Aldrich/Merck (Poznań, Poland). Quercetin 3-rutinoside, isorhamnetin 3-rutinoside, isorhamnetin 3-glucoside, and kaempferol 3-rutinoside were purchased from Extrasynthese (Genay, France).

Statistical analysis

The results were expressed as the arithmetic mean and standard deviation from three independent experiments. The significance of differences between means was determined using analysis of variance (one-way ANOVA; OriginPro 8.1, OriginLab Corporation, Northampton, MA, USA) and Tukey's Honest Significant Difference (HSD) test, with $p<0.05$.

RESULTS AND DISCUSSION

Antibacterial activity of sea buckthorn fruit extracts

Initially, the antibacterial potential of water and water-ethanol extracts from dried sea buckthorn fruit was determined using the disc diffusion method, compared to the recommended calcium hypochlorite solution at a concentration of 20,000 mg/L. The assay was performed against selected strains of bacteria infecting sprouts and posing a potential health hazard to the consumer [Fleckenstein *et al*., 2021; Miyahira & Antunes, 2021; NACMCF, 1999; Viswanathan & Kaur, 2001], *i.e.*, *E. coli*, *S. enterica*, *L. monocytogenes*, and *S. aureus*. All tested substances showed antibacterial activity against these strains (**Figure 1**). Calcium hypochlorite elicited a significantly higher inhibitory effect on the growth of *E. coli*, *S. enterica*, and *L. monocytogenes*. However, sea buckthorn water extract was comparably effective against *S. aureus* and, concurrently, produced the largest growth inhibition zone (15.3 mm) compared to other tested bacteria (7.8 mm for *E. coli*, 8.8 mm for *S. enetrica*, and 8.9 mm for *L. monocytogenes*). Due to the significantly higher antibacterial activity of the water extract (growth inhibition zones of 7.8–15.3 mm) than the water-ethanol extract (6.8–11.0 mm), the water extract of sea buckthorn fruit was used in further studies on radish sprouts contaminated with bacteria.

The results obtained in this study are consistent with reports on the high antimicrobial activity of water and methanolic sea buckthorn extracts against *S. aureus*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*, and *Klebsiella pneumoniae* [Chaman *et al*., 2011]. Ivanišová *et al.* [2020] showed that also other products of *Hippophae rhamnoides*, *i.e.*, oil, juice, dried berries and tea, had strong antimicrobial activity against *E. coli*, *S. enterica*, *Bacillus thuringiensis*, *L. monocytogenes*, *S. aureus*,

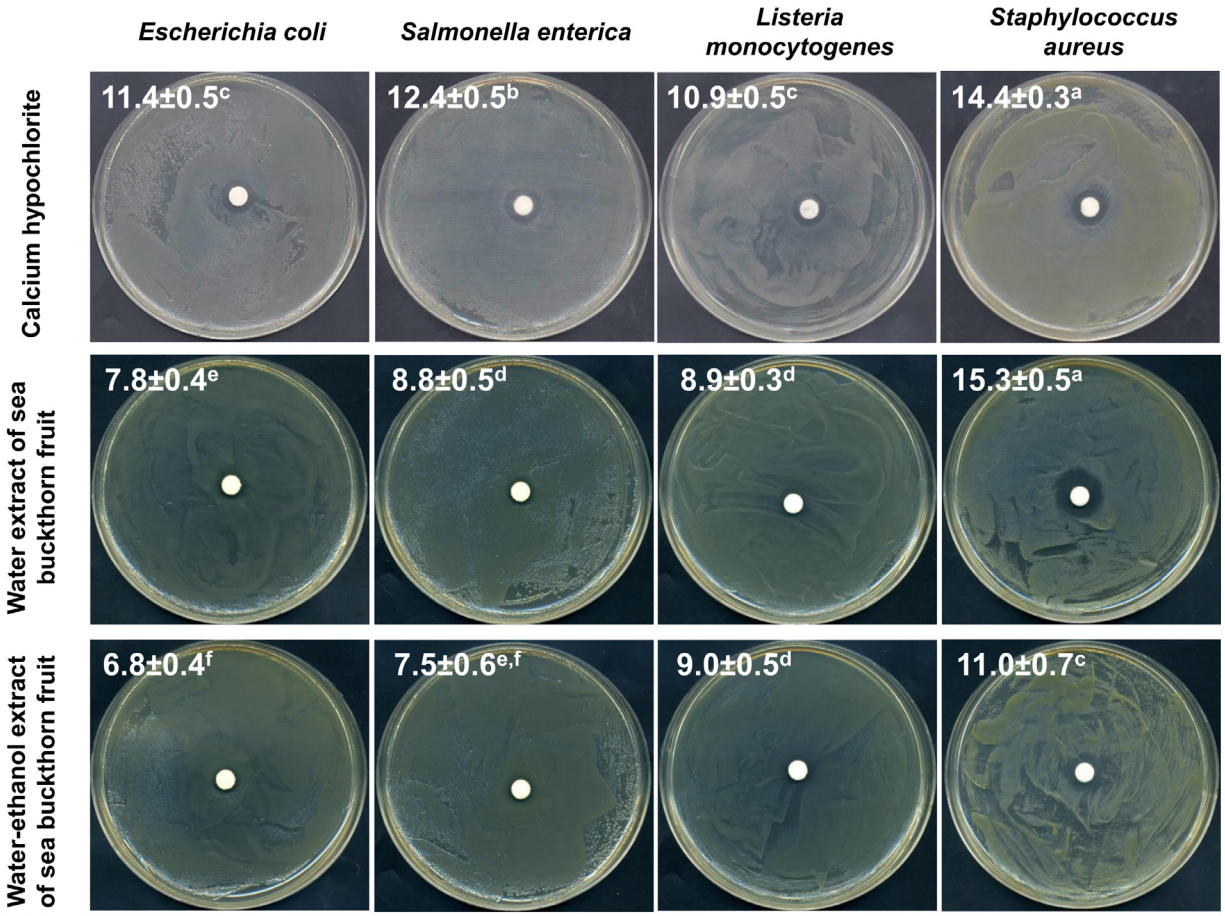

Figure 1. Photographic images of disc diffusion test results with values of zones of bacterial growth inhibition (mm) after treatment with calcium hypochlorite at a concentration of 20,000 mg/L, water extract and water-ethanol extract from sea buckthorn fruit. Means followed by different letters differ significantly ($p<0.05$).

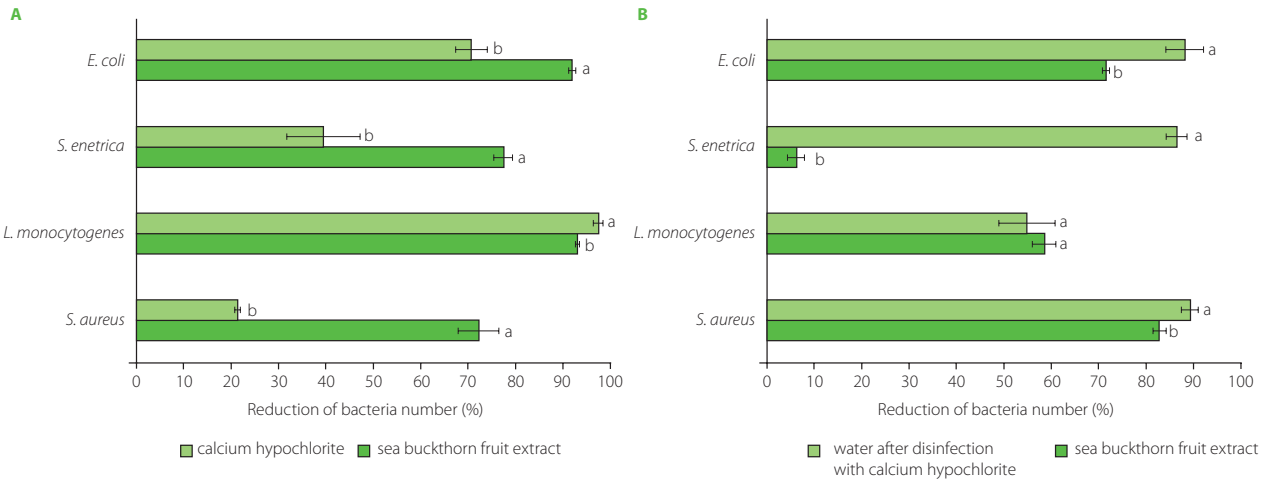

Figure 2. Effectiveness of radish seed decontamination after 15-min treatment with calcium hypochlorite and sea buckthorn fruit extract (**A**), and 3-h soaking of seeds in water or sea buckthorn extract (**B**). Means within individual strains followed by different letters differ significantly ($p<0.05$).

and especially *Yersinia enterocolitica*. Sea buckthorn leaves also seem to be a valuable source of compounds with antibacterial activity, and both aqueous and hydroalcoholic leaf extracts inhibited the growth of *Bacillus cereus*, *P. aeruginosa*, *S. aureus*, and *Enterococcus faecalis* [Upadhyay *et al.*, 2010]. The antimicrobial properties of *H. rhamnoides* extracts against microorganisms causing serious food poisoning and infections, namely *E. coli*, *Salmonella typhimurium*, *S. aureus*, *Candida albicans*, and *Pichia jadinii* were investigated in other studies which demonstrated their antifungal activity to be comparable with antifungal drugs – ketoconazole and mycostatin [Jeong *et al.*, 2010].

Radish seed decontamination

In the study, the disinfecting effect of sea buckthorn extract and calcium hypochlorite was determined on radish seeds for sprouts inoculated with bacteria at a level of 10^6–10^7 CFU/g. The seeds were treated for 15 min, and the decontamination efficacy was additionally determined after soaking the seeds for 3 h. A reduction in the number of bacteria was found after 15-min treatment with both sea buckthorn extract and active chlorine (from calcium hypochlorite) (**Figure 2A**), while treatment with water, as a control, resulted in an increase in the bacteria number by 0.1 log CFU/g (*E. coli*, *S. aureus*), 0.2 log CFU/g (*S. enterica*), and 1.0 log CFU/g (*L. monocytogenes*). Contrary to the results presented above from the disc diffusion method, on seeds, the extract exhibited significantly higher antibacterial activity against *E. coli*, *S. enterica* and *S. aureus* than calcium hypochlorite (**Figure 2A**). The disinfecting effect of the extract ranged from 72.1% for *S. aureus* to 92.0% for *E. coli*, while for active chlorine these values were 21.3% for *S. aureus* and 70.7% for *E. coli*. The number of *L. monocytogenes* was reduced the most, by 93% after the treatment with the sea buckthorn extract and by 97.5% in the presence of active chlorine.

According to published data, the treatment of seeds with 20,000 mg/L calcium hypochlorite resulted in a variable reduction in bacterial counts of 1.0 to 6.5 log CFU/g [Beuchat *et al.*, 2001; Montville & Schaffner, 2004]. Summarizing a broad review of the scientific literature, Montville & Schaffner [2004] demonstrated that 20,000 mg/L calcium hypochlorite treatment yielded a median reduction of 2.5 log CFU/g, although sometimes its efficacy was barely 0.5 log CFU/g or even less [Ding *et al.*, 2013]. Putative factors contributing to such variability in results include different properties of various seed types. Barak *et al.* [2002] reported differences in the attachment and growth of *S. enterica* serovars and *E. coli* O157:H7 to alfalfa sprouts, which directly determine the disinfecting effect. As it has been shown, the adhesive properties of bacteria depend, among others, on roughness, wettability and porosity of seed surface [Fransisca *et al.*, 2012; Zheng *et al.*, 2021]. Furthermore, wrinkled seeds were not only characterized by much higher microbiological contamination but were more difficult to sanitize than the smooth ones [Charkowski *et al.*, 2001].

As shown by Montville & Schaffner [2004], the efficacy of 20,000 mg/L calcium hypochlorite in reducing pathogen number depends on the duration of sanitization. Moreover, their analysis showed a linear correlation between inoculum size and microbial reduction. In the light of these findings, different sanitization efficacy can be expected depending on the type of seed, sanitization time and the level of seed contamination with bacteria, which also makes it difficult to compare and discuss published results. It should be noted that all the factors discussed above will also determine the activity of the sea buckthorn extract and the efficacy of disinfection conducted with its use.

In the study, the effect of decontamination of radish seeds varied significantly and differed after 3 h soaking process, with the seeds treated with calcium hypochlorite being soaked in water, and those treated with the sea buckthorn extract being further soaked in the same extract (**Figure 2B**). After soaking, the number of *E. coli* was reduced by 70.8% and 87.3% after the treatment with the extract and active chlorine, respectively. Interestingly, treatment and soaking in water resulted in a 0.7 log CFU/g reduction in *E. coli* counts, which may simply be due to the bacteria being washed off the seed surface.

In accordance with the recommendations of Codex Alimentarius Commission [2001] and European Sprouted Seed Association (ESSA) hygiene guideline for the production of sprouts and seeds for sprouting [ESSA, 2017], seeds should be thoroughly rinsed with water before the disinfection treatment to remove dirt and increase the efficiency of disinfection, and as appropriate, seeds should also be rinsed after the disinfection to eliminate its residues. The Commission also recommended a final water rinse of sprouts, which will remove the hulls, cool the product to slow down microbial growth and may reduce microbial contamination of the sprouts. Research carried out on germinated seeds washed in a stream of running water for 30 s showed that the number of microorganisms could be reduced in this way by a maximum of 1 log [Michalczyk & Kowalińska, 2009].

In the study, calcium hypochlorite was found to ensure a better disinfecting effect against *E. coli*, *S. enterica* and *S. aureus* after soaking the seeds than after a 15-min treatment, with a reduction of 87.3%, 85.5%, and 88.3%, respectively (**Figure 2B**). This may be due to the action of active chlorine, which remained on the seeds surface despite rinsing. Similar observations for hypochlorite used to disinfect rice seeds were reported by Miché & Balandreau [2001], who hypothesized that a chlorine coating was formed on the seeds, which could not be removed despite intensive rinsing. The possibility of removing bacteria from radish seeds during subsequent stages of rinsing, soaking and drying cannot also be excluded. However, in water as a control, the number of *S. aureus* increased by 0.5 log CFU/g and that of *L. monocytogenes* increased by 0.9 log CFU/g, while the number of *S. enterica* did not change after 3-h soaking in water.

In contrast to active chlorine, the seeds soaked in the sea buckthorn fruit extract resulted in lower decontamination efficiency than a 15-min treatment against *E. coli*, *S. enterica* and *L. monocytogenes*. A greater reduction after 3-h soaking in the extract was noted for *S. aureus*, and the number of bacteria was reduced by 81.9% (**Figure 2B**). After prolonged treatment, both substances also showed a much weaker sanitizing effect on the seeds contaminated with *L. monocytogenes*. The lower activity of the extract after 3-h soaking may result from bacterial cells being trapped in cracks or crevices of seed coat, which may additionally enlarge during the swelling and germination of seeds. According to Weissinger & Beuchat [2000], this phenomenon may limit direct exposure of pathogen cells to aqueous sanitizers and result in reduced efficacy of disinfectants.

Both *L. monocytogenes* and *S. enterica* are known to persist long-term in foods with low water activity, including seeds, in spite of the fact that their growth is not promoted in dehydrated products. However, during rehydration, bacteria can proliferate intensively, and their number may increase by 2.28 to 6.25 log CFU/g [Fay *et al.*, 2023]. Also, during early stages of sprouting, the number of bacteria may increase by as much as 7–8 log CFU/g [Fransisca *et al.*, 2012]. In this context, it seems that soaking seeds in sea buckthorn fruit extract may protect them against excessive and undesirable bacterial proliferation, even if its efficacy is lower than after 15-min seed treatment.

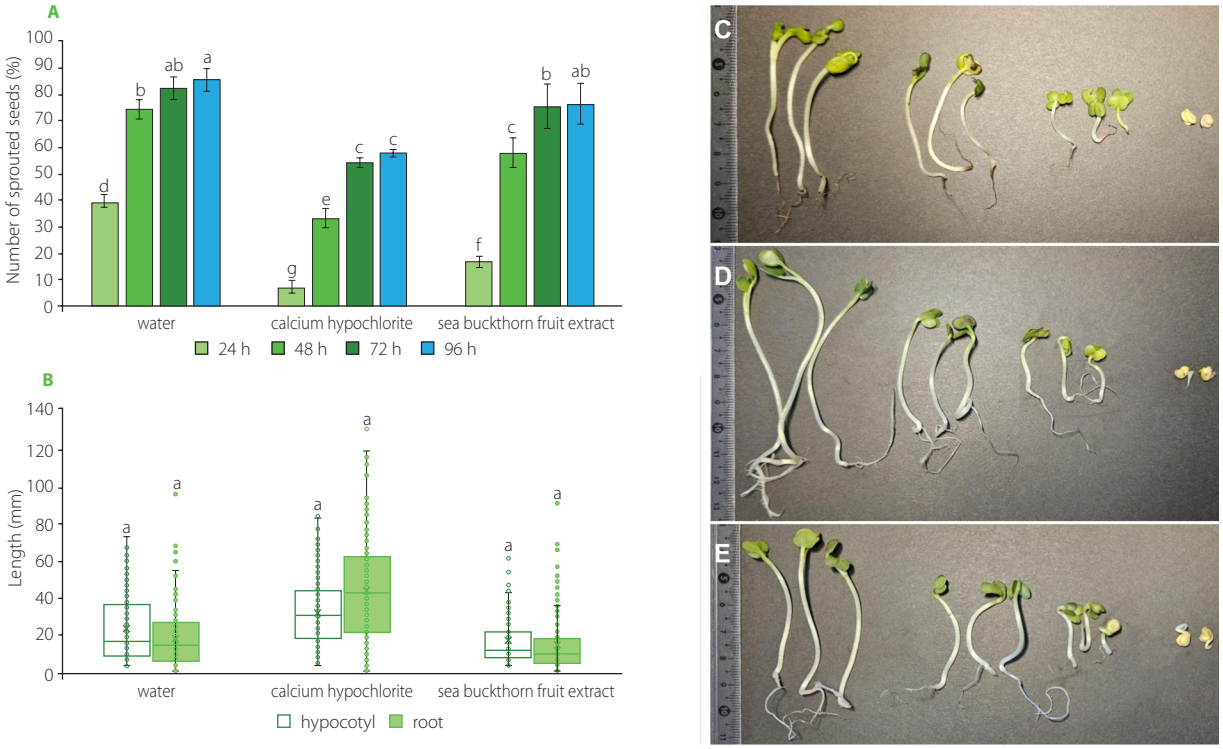

Figure 3. Graphs of germination capacity of radish seeds (**A**) and the length of hypocotyls and roots of sprouts on the 4th day of cultivation (**B**), and photographic images of sprouts after treatment with water (**C**), calcium hypochlorite (**D**) and sea buckthorn fruit extract (**E**). Means followed by the different letters differ significantly ($p<0.05$).

Influence of sea buckthorn extract and calcium hypochlorite on radish seed germination

For sprout producers, it is important not only to reduce the risk of development of pathogenic microorganisms, but also to identify the impact of the methods and substances used on seed germination ability and the related efficiency of the sprout breeding process. For this reason, the percentage of germinated radish seeds and the length of roots and hypocotyls of sprouts were also determined in the study. Compared to water treatment, the seeds treated with the sea buckthorn fruit extract showed significantly lower germination capacity after 24 and 48 h (**Figure 3A**). However, in the following days, the number of germinated seeds was comparable and amounted to 85.3% and 76.3% after 4 days, respectively. Whereas the number of sprouts obtained from the seeds treated with active chlorine was significantly lower than after the treatment with water or the extract. After 4 days, the number of germinated seeds was only 57.7%. A significant reduction in germination capacity after the treatment with calcium hypochlorite was also reported for alfalfa seeds [Beuchat *et al*., 2001; Kim *et al*., 2003].

On the other hand, regardless of the seed treatment method, no significant differences were found in the length of roots and hypocotyls of sprouts (**Figure 3B**). This seems to be due to the large variation in the length of sprouts in population and the resulting large dispersion of the results obtained. However, the average values indicate similar parameters for sprouts from the seeds previously treated with water and extract, as well as longer hypocotyls and roots after seed treatment with active chlorine (**Figure 3B-E**). In the context of sprout breeding, however, uniform seed germination and sprout growth should be considered favorable.

Profile of phenolic compounds of sea buckthorn fruit extract

The water extract of sea buckthorn fruits contained 6 phenolic acids and 10 flavonoids from the flavonol group (**Table 1**). Among the phenolic acids, protocatechuic acid dominated, with its content in the extract reaching 5.43 mg/L. The second major acid was gallic acid (3.36 mg/L). The remaining acids were present at levels of approximately 1 mg/L or less. The dominant group of phenolic compounds of the extract were flavonoids including mono- and diglycosides of flavonols. Depending on their content, these compounds occurred in the sea buckthorn fruit extract in the following order: isorhamnetin 3-rhamnosylglucoside > isorhamnetin 3-rutinoside > isorhamnetin 3-glucoside > isorhamnetin 3-sophoroside-7-rhamnoside > isorhamnetin 3,7-diglucoside. The contents of the first three compounds mentioned were in the range of 12.99 to 3.41 mg/L. Other compounds whose contents in the extract were significant (above 1 mg/L) were quercetin 3-glucoside-7-rhamnoside and quercetin 3-sophoroside-7-rhamnoside.

Research on the phytochemical profile and the antioxidant and antiproliferative activities of sea buckthorn berries conducted by Guo *et al.* [2017], like our study, protocatechuic and gallic acids to be the major phenolic acids in terms of content. Taking into account flavonols, their contents were among the highest in *Hippophae rhamnoides* fruits [Guo *et al.*, 2017; Wang *et al.*, 2022]. According to Yang *et al.* [2009], sea buckthorn berries subsp.

Table 1. Content of phenolic compounds in the sea buckthorn fruit extract (mg/L).

Retention time (min)	λ_{max} (nm)	Content (mg/L)	Compound
11.49	284	3.36±0.11	Gallic acid
16.76	255	1.02±0.02	*p*-Hydroxybenzoic acid
17.55	301, 307	0.57±0.03	Hydroxycinnamic acid derivative
20.40	260	0.21±0.01	Vanillic acid
25.65	355	0.45±0.01	Isorhamnetin 3,7-diglucoside
26.04	348	0.53±0.04	Isorhamnetin 3-sophoroside-7-rhamnoside
27.18	355	0.73±0.04	Quercetin glucoside-rhamnoside-7-rhamnoside
30.72	356	0.15±0.05	Quercetin 3-rutinoside
33.64	326	1.30±0.04	Ferulic acid
36.05	355	2.43±0.09	Quercetin 3-glucoside-7-rhamnoside
37.38	337	1.38±0.05	Quercetin 3-sophoroside-7-rhamnoside
38.09	354	8.25±0.68	Isorhamnetin 3-rutinoside
43.36	353	0.72±0.04	Kaempferol 3-rutinoside
46.30	354	12.99±0.87	Isorhamnetin 3-rhamnosylglucoside
47.69	354	3.41±0.08	Isorhamnetin 3-glucoside

λ_{max}, wavelength of absorption maximum of the UV spectrum.

mongolica contained several times higher amounts of flavonol glycosides than in the case of chokeberry, cranberry, blackcurrant or bilberry. The most numerous flavonols identified in the sea buckthorn fruit extracts were isorhamnetin derivatives with one, two or three glycoside residues in the structure [Teleszko *et al.*, 2015].

Previous investigations have indicated a discernible correlation between the antimicrobial activity of phenolic compounds and their structural attributes, notably pertaining to the configuration, quantity, and positioning of hydroxyl or methoxyl functional groups within the molecular framework [Gulcin, 2012; Wu *et al.*, 2013]. Various studies have found that phenolic compounds can interact with bacterial cell walls [Bhattacharya *et al.*, 2018; Tang *et al.*, 2016; Yi *et al.*, 2010; Zhang *et al.*, 2015]. Phenolic compounds exhibit an affinity for bacterial cell walls, leading to perturbations in membrane integrity and subsequent efflux of intracellular constituents. Notably, Gram-negative bacterial species manifest a heightened resilience to phenolic compounds compared to Gram-positive bacteria. This phenomenon is intricately linked to the structural composition of their outer membrane, characterized by an abundance of phospholipids, imparting a formidable barrier against macromolecular ingress. The augmented resistance of Gram-negative bacteria to phenolic compounds may stem from the activity of enzymes localized within the periplasmic space. These enzymes are conjectured to catalyze the degradation of exogenously introduced molecules, thereby attenuating their cytotoxic effects [Efenberger-Szmechtyk *et al*., 2020]. However, there are also studies which suggest that phenolic compounds can cause the breakdown of the outer membrane of Gram-negative bacteria, leading to an increase in its permeability [Yi *et al.*, 2010].

Research by Bhattacharya *et al.* [2018] showed that the polyphenol fraction of kombucha containing mainly catechin and isorhamnetin, as well as catechin and isorhamnetin themselves, have the ability to cause the permeability of the internal membrane of *Vibrio cholerae*. According to other researchers [Tang *et al.*, 2016; Zhang *et al.*, 2015], the damage to cell walls in *S. aureus* is probably caused by binding to the peptidoglycan layer, while in *E. coli* it is caused by oxidative stress. It is already known that phenolic compounds are able to induce endogenous oxidative stress in bacterial cells by inducing the formation of reactive oxygen species (ROS) [Tang *et al.*, 2016; Zhang *et al.*, 2016]. Damage to the cell wall may lead to the leakage of intracellular components. Plant extracts may cause an increase in intracellular protein leakage, depending on the concentration and incubation length [Tang *et al.*, 2016; Zhang *et al.*, 2015]. Furthermore, phenolic compounds have been empirically demonstrated to induce cytoplasmic leakage of various cellular constituents, encompassing nucleotides and ionic species, such as potassium and phosphate ions [Yi *et al.*, 2010]. Furthermore, Dadi *et al.* [2009] showed that phenolic compounds (resveratrol, piceatannol, quercetin and its derivatives) were able to inhibit the activity of ATPase and ATP synthase in *E. coli*.

CONCLUSIONS

Sea buckthorn fruit extract showed varying antibacterial activity, and was more effective than the recommended 20,000 mg/L calcium hypochlorite in decontaminating radish seeds inoculated with *E. coli*, *S. aureus* and *S. enterica*. Soaking seeds in the extract may protect them against excessive and undesirable bacterial multiplication, although its decontamination efficacy was lower than after 15-min seed treatment. Moreover, the extract did not affect the germination capacity of radish seeds or the length of roots and hypocotyls of sprouts.

Sea buckthorn fruit extract can be used to reduce bacterial contamination of radish seeds prior to sprouting, additionally constituting a valuable source of phenolic compounds. Phenolic compounds determined in the extract have well documented health-promoting properties. However, further research is needed to investigate the extract's impact on human health. Moreover, the use of the extract in seed sanitization requires microbiological quality control of both seeds and sprouts before they reach the consumer.

The demonstrated antibacterial effect of the sea buckthorn fruit extract allows recommending it as a natural product to be used to reduce bacterial contamination of seeds for sprouts without compromising their viability. The use of plant extracts as an effective means to increase the microbiological safety of sprouts fosters great potential in the food industry and represents a viable alternative to chemical antibacterial agents. However, further study is necessary to investigate strategies to prevent pathogen development during germination and sprout growth.

RESEARCH FUNDING

The research did not receive any external funds.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 130–136
DOI: 10.31883/pjfns/186806
ISSN 1230-0322, e-ISSN 2083-6007

Hyperbaric Storage at Subzero Temperature – the Effect on the Shelf-Life and Selected Quality Characteristics of Raw Pork Sausages

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The aim of this study was to determine the microbiological stability and changes occurring in selected physicochemical properties of raw pork sausages during hyperbaric storage at subzero temperature (HS-ST). It was shown that storage at moderate pressures (up to 111 MPa) led to a reduction in microbial populations. After 21 days of storage under these conditions, no microorganisms were detected in the samples. Additionally, the inhibition of thiobarbituric acid reactive substance (TBARS) formation was observed under HS-ST conditions. The negative effect of the storage at 111 MPa/−10°C was the sausage colour change from pink to grey-pink or grey, visible to the naked eye. Such changes did not occur in the samples stored at 60 MPa/−5°C for 35 days. The results showed that applying HS-ST at 60 MPa/−5°C allowed the shelf-life of raw pork sausages to be effectively extended by at least 35 days without visible quality deterioration.

Keywords: raw meat product, high pressure, TBARS, shelf-life, denaturation of proteins, drip loss

INTRODUCTION

Meat and meat products are nutrient-rich products usually exhibiting high water activity, making them susceptible to deterioration and spoilage. Their improper processing can lead to economic losses, food waste and loss of consumer confidence. The meat industry strives to meet growing consumer demand for high-quality raw meat and meat products with extended shelf-life. In this context, raw meat refers to meat that is stored fresh, uncooked, including cut or minced meat, with or without preservatives and not heat-treated.

High pressure processing (HPP) is a non-thermal preservation method most widely used in the food industry. Meat products account for nearly 25% of the market of foods produced using this technology [Bolumar *et al*., 2021]. The effect of pressure on the quality of meat and meat products has been described in numerous review papers [Bolumar *et al*., 2021; Cheftel & Culioli, 1997; Chuang & Sheen, 2022; Hygreeva & Pandey, 2016; Rajendran *et al*., 2022]. Raw meat cannot be pressurized under conditions that significantly reduce the microbial load (400–600 MPa), as this causes changes to meat colour [Bak *et al*., 2019; Jung *et al*., 2003]. An improvement in meat colour can be achieved at moderate pressures (130 MPa); however, processing in these conditions is not sufficient to reduce the number of meat microbiota [Jung *et al*., 2003].

Moderate pressures, from 20 to 200 MPa, are used in hyperbaric storage (HS). This innovative storage technique extends the shelf-life of raw meat without adverse changes in colour and may offer a promising alternative for the meat industry [Basso *et al*., 2022; Santos *et al*., 2020]. Santos *et al*. [2020] showed that the storage of beef and pork meat for 60 days at pressures of 50–100 MPa at room temperature had little effect on their colour. At pressures higher than 50 MPa, the growth of meat

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Submitted: 11 December 2023
Accepted: 4 April 2024
Published on-line: 23 April 2024

microbiota was also inhibited or inactivated. Unfortunately, storage at higher pressures (≤75 MPa) promoted the formation of secondary products of meat lipid oxidation which additionally occurred faster than during refrigerated storage at atmospheric pressure [Santos *et al*., 2021]. It is likely that lowering the HS temperature can lead to slower oxidation processes. Such conditions of pressure and subzero temperature, in which the water remains unfrozen, seem particularly interesting. To the best of our knowledge, there are very limited data on changes in food stored at moderate pressures and subzero temperatures. From an economic point of view, comparing HS-ST to conventional frozen storage, the former method allows for significant energy savings as storage can be carried out at a higher temperature than is possible at atmospheric pressure. Moreover, since water does not crystallise under HS-ST, the cold demand (energy required for cooling) will be much lower than in conventional freezing, where the cold demand to freeze a food product includes the amount of heat that must be removed from the product as a result of lowering the initial temperature to the cryoscopic temperature (cooling), the phase transformation of water into ice (freezing proper) and the lowering of the temperature of the frozen product from the cryoscopic temperature to the final freezing temperature. With lower energy requirements, the HS-ST represents a more environmentally friendly alternative to conventional frozen storage.

The aim of our study was to determine the selected quality characteristics and shelf-life of raw pork sausages during hyperbaric storage under moderate pressures (60 and 111 MPa) in subzero temperatures (without freezing water) for a long time (up to 35 days).

MATERIALS AND METHODS

Sample preparation

The raw sausages used in experiments were purchased from a local manufacturer and consisted of pork meat (82 g/100 g), water, flavours, salt, glucose, spices, spice extracts, acidity regulator: sodium citrate, yeast extract and garlic extract. Final products were portioned and packed in protective atmosphere by the producer and transported to company store. According to producer's declaration, their shelf-life from manufacture was 7 days under refrigeration. The raw material was transported from market to the laboratory with a cold chain continuity and then unpacked from collective packaging and divided for single portions (about 130 g) which were vacuum-packed in commercially available plastic bags characterized by high barrier properties to oxygen and to water vapor (polyamide/linear low-density polyethylene, Food Saver, Gdynia, Poland, cat. no. 72459) using vacuum packaging machine (Hendi, De Klomp, The Netherlands).

Hyperbaric storage

Hyperbaric storage was performed using equipment designed at the Department of Food Chemistry, Technology and Biotechnology, Gdańsk University of Technology (Poland) and built by DS-Technology Ltd. (Słupsk, Poland). Pressure was generated as described in our previous publication [Malinowska-Pańczyk *et al*., 2014]. The raw sausages were subsequently stored at 60 MPa/−5°C and 111 MPa/−10°C. The conditions of hyperbaric storage at subzero temperature (HS-ST) were selected based on previous research results in which no changes were found in the properties of raw pork meat after HPP at subzero temperature [Malinowska-Pańczyk *et al*., 2014]. Samples were placed in 620 mL pressure vessels that were filled with distilled water, next secured with a spring that kept the sample in the upper unfrozen zone and then sealed tightly. The pressure vessel was placed from the side of the closure in a cryogenic bath and gradually immersed to cool the system and generate pressure. The total time for this step was 90 min. After this time, the pressure vessels were transferred to a storage section and kept therein for up to 35 days. The magnitude of the pressure generated in such a system depends on the temperature [Bridgman, 1912; Rubinsky *et al*., 2005]. Simultaneously samples were kept at 4, −5°C and −10°C at 0.1 MPa and were used as controls (15 samples for each condition). Before analyses, the samples were thawed at 0–2°C.

Microbial analysis

Microbial enumerations were performed after 1, 3, 7, 10 and 14 days of storage at 4°C for the control samples. In the case of raw sausages treated with HS-ST and sausages stored at −5°C and −10°C at 0.1 MPa, analysis was carried out after 3, 7, 14, 21, and 35 days of storage. Minced samples (10 g) were transferred to a stomacher bag and homogenised in a stomacher (Masticator Basic Panoramic, IUL Instruments, Barcelona, Spain), with 90 mL of saline peptone water for 1 min. Decimal dilutions were also prepared in saline peptone water and then plated (in duplicate) onto an appropriate agar medium and incubated (**Table 1**). Cultures from dilutions that contained between 10 and 300 colony forming units (CFU) were selected for colony counting. The number of coagulase-positive staphylococci was

Table 1. Media and incubation conditions used during microbial analysis.

Group of microorganisms	Medium	Incubation conditions
Total bacteria count (TBC)	Plate count agar	30°C, 48 h
Psychrophilic bacteria count (PBC)	Plate count agar	4°C, 10 days
Coliforms	Chromocult® coliform agar	30°C, 48 h
Escherichia coli count	Chromocult® coliform agar	30°C, 48 h
Coagulase-positive staphylococci count	Baird-Parker agar	37°C, 48 h

determined in accordance with the standard established by the International Organization for Standardization (ISO) [PN-EN ISO 6888-1:2022]. The media were purchased from Merck KGaA (Darmstadt, Germany). Microbial counts were expressed as log CFU/g of stored raw sausages.

Determination of thiobarbituric acid reactive substance content

Thiobarbituric acid reactive substance (TBARS) content was determined as described by Salih *et al*. [1987] with a small modification where butylhydroxytoluene (BHT) was used instead of butylhydroxyanisole. To 10 g of minced sausages after storage, 34.25 mL of 4% (*v/v*) perchloric acid at 4°C and 0.75 mL of 0.01% BHT solution were added. The sample was homogenised at 10,000 rpm for 2 min using Silent Crusher M (Heidolph, Schwabach, Germany). The homogenate was filtered through Whatman paper No. 1. The filtrate was made up to 50 mL by washing the precipitate on the filter with 4% (*v/v*) perchloric acid. Five millilitres of a 0.02 M aqueous solution of 2-thiobarbituric acid (TBA) were added to 5 mL of the filtrate, which was then boiled for 1 h. Afterwards, the samples were cooled in a cold, tap water. Absorbance at 532 nm was then measured against a blank containing 5 mL of 4% (*v/v*) perchloric acid instead of filtrate. The TBARS content was calculated from the standard curve and expressed as mg of malondialdehyde (MDA) equivalent in 1 kg of sausage. Determinations of TBARS content in the control samples stored at 4°C were carried out on days 1, 10, 14 and 17, whereas in the samples stored at subzero temperature under atmospheric and moderate pressure on days 5, 14, 21 and 35.

Determination of protein solubility

The solubility of protein in sausages before storage and stored for 5, 7, 14, 21 and 35 days at 60 MPa/–5°C and 111 MPa/–10°C or at –5°C and –10°C at 0.1 MPa was assayed. The minced sample was homogenised in 0.9 M saline at pH 7.5 for 5 s at 7,000 rpm followed by 30 s at 12,000 rpm using Silent Crusher M (Heidolph) [Malinowska-Pańczyk *et al*., 2014]. The homogenate was centrifuged at 4,500×*g* for 30 min at 4°C. The biuret method was used to determine the concentration of proteins (mg) in 1 mL of the supernatant [Gornall *et al*., 1949]. Relative protein solubility (%) was calculated from the ratio of soluble protein determined in the control initial samples to the soluble protein content of the stored samples.

Drip loss determination

Drip loss was calculated as the percentage ratio of the weight of the sample after 5, 7, 14, 21 and 35 days of storage at atmospheric (0.1 MPa) or moderate pressure to the initial weight, according to formula (1):

$$DL = [(m_1 - m_2)/m_1)] \times 100 \quad (1)$$

where: DL, drip loss (%); m_1, the weight of the sample before storage (g); and m_2, the weight of the sample after storage (g).

Statistical analysis

The storage experiments were conducted on three different batches of raw sausage from a single manufacturer. Determinations of microbial and physicochemical parameters were performed in triplicate. The results in the figures are presented as mean value and standard deviation (SD). To test the statistical significance of the observed differences in selected qualitative traits (TBARS content, degree of protein denaturation, drip loss) and microbiota count between samples stored at different times and different temperatures under atmospheric and moderate pressure conditions, a two-way analysis of variance (ANOVA) was conducted. Differences were considered statistically significant at $p<0.05$. Differences between groups were determined with a Tukey test. The statistical analysis was conducted using Prism software, version 10.2.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

The effect of hyperbaric storage at subzero temperature on the microbial quality of sausages

Sausages sold in the raw form are the type of the meat products for which the European Communities (EC) Commission Regulation on Microbiological Criteria for Foodstuffs 2073/2005 established that the upper limit for *Escherichia coli* and *S. aureus* should not exceed 3.7 log CFU/g (5×10^3 CFU/g) [Commission Regulation (EC), 2005]. *E. coli* were not detected in the control sausages (data not shown), which indicates good hygienic conditions during production and distribution. The number of coagulase-positive staphylococci in raw sausages before storage was 3.0 log CFU/g (**Figure 1D**). It also did not exceed the upper limit established by the above-mentioned EC Commission Regulation. Because the total microbial load in meat determines its shelf-life and spoilage [Fougy *et al*., 2016; Vasilopoulos *et al*., 201], the total bacteria count (TBC), psychrophilic bacteria count (PBC) and coliform count were additionally determined in the study. The initial TBC in the unpressurised samples was 4.3 log CFU/g (**Figure 1A**). This level of microbial contamination indicated a good product quality. Psychrophilic bacteria were also present in the samples, at 3.9 log CFU/g (**Figure 1B**).

When the samples were stored under refrigeration, it was found that TBC and PBC increased in the control samples (**Figure 1A** and **1B**). TBC exceeded the upper acceptable limit (<6.7 log CFU/g) on the 7th day of storage. After 14 days, the TBC and PBC in the control samples were at *ca*. 8 and 7 log CFU/g, respectively. TBC in sausages stored for 35 days under 60 MPa/–5°C decreased only slightly (0.6 log cycle). However, at 111 MPa/–10°C, TBC lowered to an undetectable level after 21 days. In the samples stored at 0.1 MPa/–5°C and 0.1 MPa/–10°C at the same time, the TBC did not change significantly ($p\geq0.05$). Santos *et al*. [2020] showed that storage of pork and beef meat at 50 MPa at room temperature not only did not inhibit the growth of total aerobic mesophiles but even increased the number of microbial populations. Inactivation of the meat microbiota was achieved only during storage at higher pressures of 75 and 100 MPa [Santos *et al*., 2020]. PBC decreased to 2 log cycles in the samples stored at 60 MPa/–5°C for 35 days

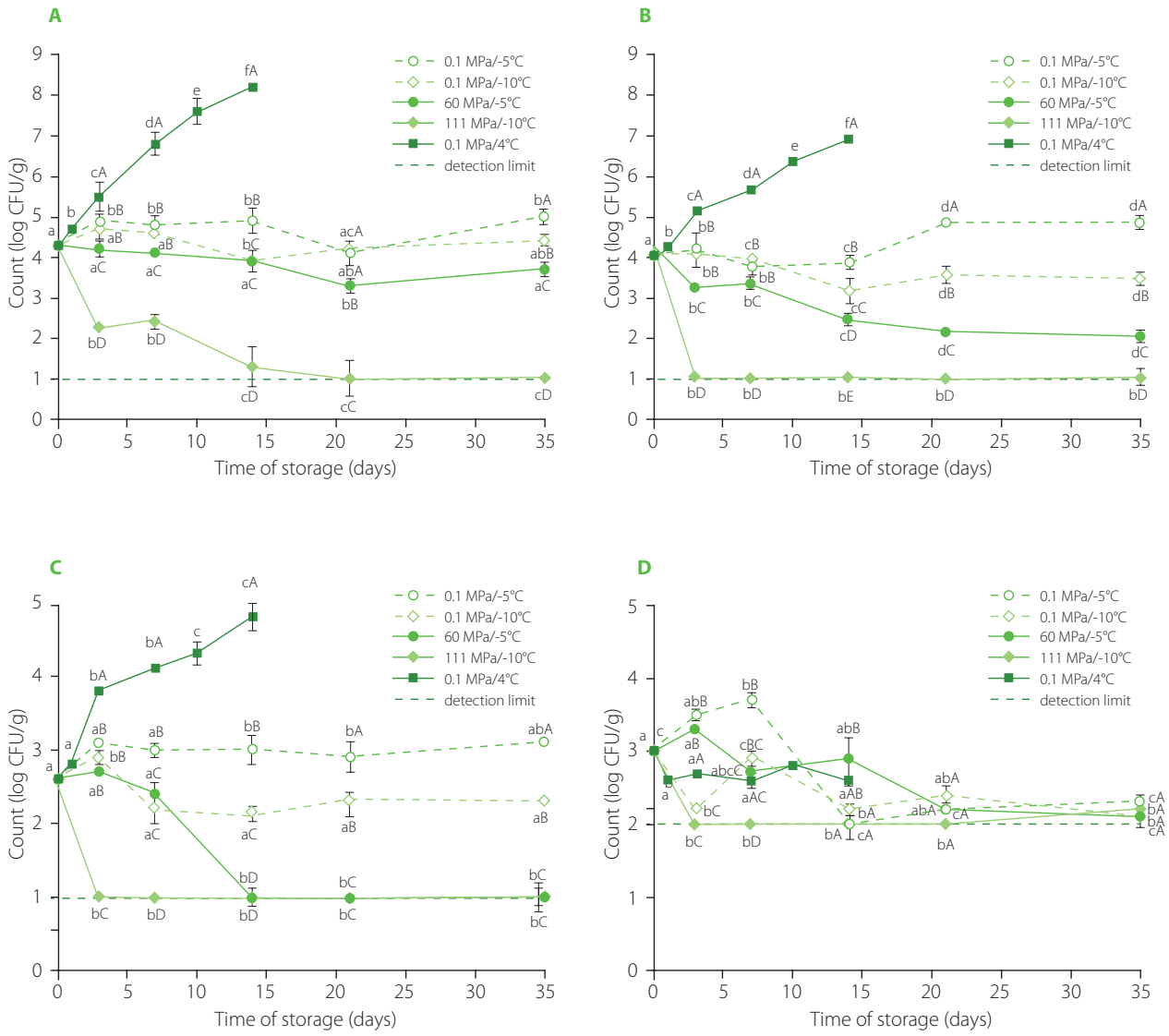


Figure 1. Total bacteria count (**A**), psychrophilic bacteria count (**B**), coliform count (**C**), and coagulase-positive staphylococci count (**D**) of raw pork sausages stored at different temperatures under atmospheric and moderate pressure conditions. The results are shown as mean and standard deviation. The different lowercase letters (a–f) indicate the significant differences among different storage times for the same storage conditions, and the different capital letters (A–E) indicate the significant differences among different storage conditions at the same time ($p<0.05$). The dashed line indicates the detection limit.

and was not detected after 3 days of storage at 111 MPa/–10°C (**Figure 1B**). A reduction of psychrophilic microbiota by about 1.25 log cycle in fresh salmon stored at 60 MPa/10°C for 50 days was also observed by Fidalgo *et al.* [2019].

During storage of control sausages for 14 days in chilled conditions, the number of coliforms increased by 2.3 log cycles (**Figure 1C**). HS-ST of sausages caused a successive decrease in the count of coliforms, which were not detected after the 3rd and 14th day of storage at 111 MPa/–10°C and 60 MPa/–5°C, respectively. In the case of the samples stored at 0.1 MPa/–5°C, the number of coliforms increased by 0.5 log cycle, while in those stored at 0.1 MPa/–10°C it decreased by 0.3 log cycle. However, these changes were not statistically significant ($p\geq0.05$).

The population of coagulase-positive staphylococci did not change ($p\geq0.05$) throughout the storage period of the control samples at 4°C (**Figure 1D**). In the case of the sausages stored under pressure at subzero temperature, they were not detected after 3 days of storage at 111 MPa/–10°C, whereas in these stored at 60 MPa/–5°C, their number decreased successively. A reduction in the number of coagulase-positive staphylococci was also observed in the samples stored at analogous temperatures at atmospheric pressure.

The effect of hyperbaric storage at subzero temperature on the content of thiobarbituric acid reactive substance in sausages

The main factor in non-bacterial spoilage of meat and meat products that leads to the loss of nutritional value is lipid oxidation. Secondary products formed during this reaction readily react with proteins, causing changes in the colour, aroma, and taste of raw meat [Fuentes *et al.*, 2010]. Lipid oxidation in meat can be initiated endogenously *via* metal ions, especially haeme iron, and by exogenous reactive oxygen species [Amaral *et al.*, 2018]. HPP can promote lipid oxidation reactions [Cheah & Ledward,

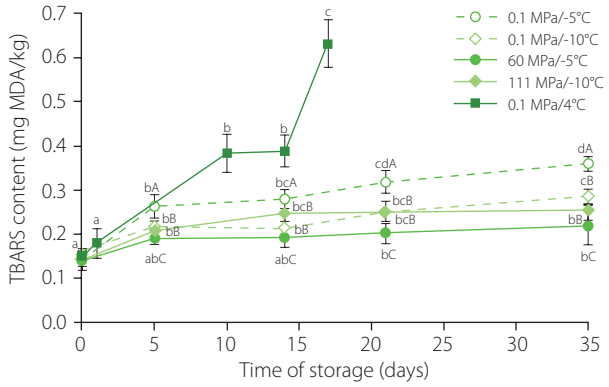


Figure 2. Content of thiobarbituric acid reactive substances (TBARS) in raw pork sausages stored at different temperatures under atmospheric and moderate pressure conditions. The results are shown as mean and standard deviation. The different lowercase letters (a–d) indicate the significant differences among different storage times for the same storage conditions, and the different capital letters (A–C) indicate the significant differences between different storage conditions at the same time (p<0.05).

1996]. Pressure causes conformational changes in haemoproteins and leads to the release of iron from haeme, which induces lipid oxidation [Beltran *et al.*, 2003; Rajendran *et al.*, 2022].

The initial level of secondary lipid oxidation products in the raw sausages was 0.15 mg MDA/kg (**Figure 2**), which indicates the good quality of the raw materials and the maintenance of appropriate conditions during sausage stuffing production. A successive increase in TBARS value was found in the control samples during refrigerated storage. After 17 days of storage, the TBARS content in these samples was 0.63 mg MDA/kg.

The content of secondary metabolites of lipid oxidation in the samples stored at subzero temperatures increased slightly (**Figure 2**). The TBARS content of the samples stored at 0.1 MPa/−5°C was higher on each day of storage compared to the samples stored at the same temperature under 60 MPa/−5°C (p<0.05). For sausages stored at −10°C, there were no differences between TBARS content in sausages stored at 0.1 and 111 MPa (p≥0.05). After 35 days of storage at 0.1 MPa/−5°C and 0.1 MPa/−10°C, it was 0.36 and 0.28 mg MDA/kg sausages, respectively. In the case of the samples stored at 60 MPa/−5°C and 111 MPa/−10°C, the TBARS values determined on the last day of storage were 0.23 and 0.26 mg MDA/kg sausages, respectively. The difference in the TBARS content between the samples at the beginning and end of HS-ST was significant (p<0.05). The TBARS content of sausages stored for 35 days at subzero temperatures both at moderate and atmospheric pressure was considerably lower than of the control sausages stored under refrigeration for 17 days. A rancid odour and taste of pork is detected by consumers even when the TBARS value is 0.5–1.0 mg MDA/kg [Wood *et al.*, 2008]. Only in the control sausages stored at 4°C did TBARS levels exceed the threshold value. The data showed that HS-ST effectively retarded lipid oxidation in raw meat products. Such a phenomenon was not observed in pork (in pieces) and beef (minced) stored for 60 days at 60 and 75 MPa at 10 and 25°C, respectively [Santos *et al.*, 2021]. The authors showed that under these conditions, the formation of secondary lipid oxidation products occurred more rapidly than in meat stored under refrigeration at atmospheric pressure. It seems that one of the main factors affecting the rate of meat lipid oxidation during hyperbaric storage is the temperature of the process. During storage at atmospheric pressure, oxidation occurs more slowly the lower the process temperature is [Shimizu & Iwamoto, 2022]. Probably, the oxidation rate is less dependent on the value of pressure, but this relationship needs to be confirmed.

The effect of hyperbaric storage at subzero temperature on denaturation of proteins and drip loss of sausages

Figure 3 shows changes in protein solubility and drip loss of sausages after HS-ST. It was noticed that protein solubility, which is an indicator of the degree of protein denaturation, depended on pressure value and time of exposure. A successive decrease in protein solubility was determined in the samples stored at

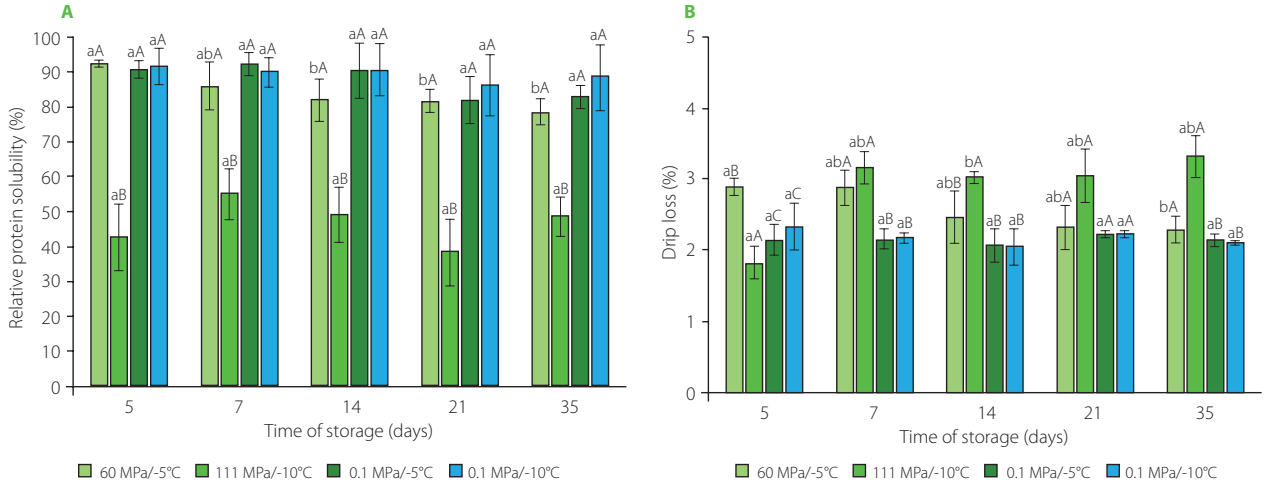
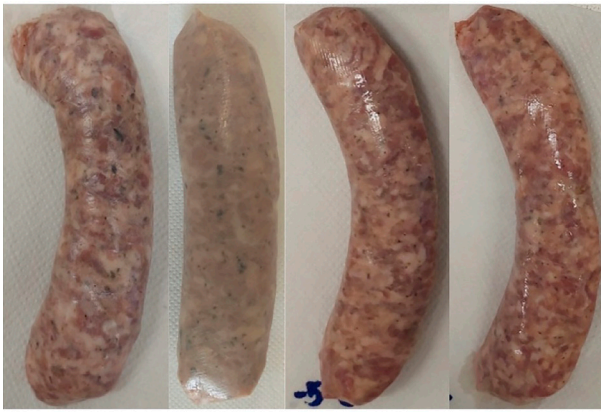


Figure 3. The relative solubility of proteins (**A**) and drip loss (**B**) in raw pork sausages stored at different temperatures under atmospheric and moderate pressure conditions. The results are shown as mean and standard deviation. The different lowercase letters (a–b) indicate the significant differences among different storage times for the same storage conditions, and the different capital letters (A–B) indicate the significant differences between different storage conditions at the same time (p<0.05).



60	111	0.1	0.1
-5	-10	-5	-10

Figure 4. The appearance of the sausage links after 35 days of storage at different temperatures under atmospheric and moderate pressure conditions.

60 MPa/−5°C, to 21.5% on the 35th day of storage compared to the samples before storage. Still lower protein solubility was observed in the samples stored at 111 MPa/−10°C. Already after 5 days of storage, the solubility decreased by 57.5% compared to that of raw sausages before storage and did not change ($p \geq 0.05$) during further storage. For the samples stored at −5°C and −10°C at atmospheric pressure, there were no significant changes ($p \geq 0.05$) in solubility over the storage period. Additionally, greater level of denaturation was correlated with a higher drip loss (**Figure 3B**). According to Fernández *et al*. [2007], water is less retained in pressure-treated meat due to the denaturation of myofibrillar proteins induced under these conditions. Drip loss increased even in the first five days of hyperbaric storage, and then remained at a constant level. The greatest changes were observed in the samples stored at 111 MPa/−10°C.

Changes were also observed after 35 days of storage in the appearance and colour of the samples (**Figure 4**), particularly in the samples stored at 111 MPa/−10°C with a shift in colour from pink to greyish-pink or grey. According to Ludikhuyze *et al*. [2001], a reduction in the colour intensity of meat subjected to 200–350 MPa treatment occurs due to the denaturation of globin, or the displacement or release of haeme from the myoglobin molecule.

CONCLUSIONS

The study showed HS-ST to be a useful technique to extend the shelf-life of raw sausages. Under these conditions, the growth of the microbiota was inhibited, or the population numbers decreased with the extension of the storage period. Sausage storage at 60 MPa/−10°C significantly reduced the formation of secondary lipid oxidation products. Such a phenomenon was not found during storage of the samples at 111 MPa/−10°C and at −5°C and −10°C at atmospheric pressure. A negative phenomenon observed during storage at 111 MPa/−10°C was a change in the colour of sausages that was noticeable to the naked eye. In addition, during HS-ST (111 MPa/−10°C), there was an intense drip loss, which means not only financial losses related to such meat, but also the loss of valuable ingredients such as vitamins, mineral salts or water. Our research shows that HS-ST can be an effective method for extending the shelf-life of raw meat without drastic changes in quality, but the applied pressure must be less than 100 MPa.

Future research will provide knowledge of the phenomena that occur during storage at moderate pressure and subzero temperature, which will allow the full potential of HS not only to extend the shelf-life of meat, but also to adapt certain properties of meat raw materials to develop new meat products. The latter aspect is so far completely unexplored but represents a very promising research topic. The ability to track the transformation of components during storage of muscle tissue at temperatures below 0°C without freezing the water will bring new elements to the existing knowledge of the phenomena occurring in meat at low temperature, including under moderate pressure, and additionally perhaps enable the verification of some hypotheses regarding the denaturation of muscle proteins induced by freezing.

RESEARCH FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 137–146
DOI: 10.31883/pjfns/187799
ISSN 1230-0322, e-ISSN 2083-6007

Chemical, Physical, and Sensory Properties of Bread with Popped Amaranth Flour

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This study investigated the effect of substitution of wheat flour with popped amaranth flour in bread formulation on the chemical, physical, and sensory characteristics of breads. The raw and popped amaranth grain flours of four Peruvian varieties: Oscar Blanco, Centenario, Taray, and Imperial, were characterised for chemical composition and pasting properties using Rapid Visco-Analyzer (RVA). Both types of amaranth flour had a high nutritional value, but the peak and final viscosity of popped amaranth flour were closer to the wheat flour. Breads were formulated with the popped amaranth flour, at four substitution levels of 0, 10, 20, and 30%. A significant increase in contents of protein (around 12%) and raw fiber (more than 100%), and a decrease in carbohydrate content (around 6%) in breads at the highest substitution level compared to wheat bread were observed. At this substitution level, the RVA profile parameters, specific volume, pore area, and colour coordinates (L^*, a^*, and b^*) differed significantly. In the sensory analysis using Flash profile technique, consumers identified that the Taray and Imperial bread varieties at 10 and 20% substitution level were similar to the wheat bread. Adding popped amaranth flour to bread improved the nutritional value, ensuring good physical and sensory properties. Popped amaranth flour can, thus, be an alternative to wheat flour in the development of healthy bakery products.

Keywords: *Amaranthus caudatus*, popped grains, pasting properties, bread physical properties, Flash profile

INTRODUCTION

The genus Amaranthus is a pseudocereal from the Amaranthaceae family with more than 60 species. Only three species are used in the production of edible grains: *Amaranthus hypochondriacus, Amaranthus cruentus,* and *Amaranthus caudatus* [Kaur *et al*., 2010]. The most important Andean species is *A. caudatus*, cultivated in the Andes of Peru, Bolivia, Ecuador, and Argentina [Repo-Carrasco-Valencia *et al*., 2009]. Its grains have recently attracted interest for their high protein content (12.5–16 g/100 g) [Bressani *et al*., 1987]. Additionally, the amino acid profile of proteins is well-balanced in the context of human nutritional requirements [Drzewiecki, 2001], with the content of lysine, an amino acid with a high biological value, being two to three times higher than in other cereals, and a high content of methionine, cysteine, tryptophan, threonine, leucine and phenylalanine, which are the limiting amino acids in the protein profile of other cereals

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Submitted: 16 December 2023
Accepted: 22 April 2024
Published on-line: 14 May 2024

such as wheat [Bresani *et al*., 1987; Cotovanu & Mironeasa, 2021; Palombini *et al*., 2013; Paucar-Menacho *et al*., 2018; Pavlik, 2012]. In addition, 100 g of amaranth grain contains between 7.7 to 12.8 g of lipids, which are rich in unsaturated fatty acids [Bressani *et al*., 1987]. Moreover squalene, beneficial for human health, has a significant contribution to the lipid profile of amaranth [He *et al*., 2002; Venskutonis & Kraujalis, 2013]. It is a natural biosynthetic precursor of cholesterol and has photoprotective properties. The amaranth grains also contain high amounts of dietary fiber and minerals such as calcium and magnesium [Bodroza-Solarov *et al*., 2008]. They are also a rich source of nutrients; however, the presence of phytic acid, has adverse effects on the bioavailability of their minerals [Sanz-Penella *et al*., 2013].

The amaranth grain can be consumed roasted, popped, extruded, in flour, or as an added ingredient for bread, cakes, muffins, pancakes, cookies, dumplings, crepes, and noodles [Sanz-Penella *et al*., 2013]. Researches have demonstrated that the partial substitution of wheat flour with amaranth flour (5 to 20%) improves nutritional value and final sensory acceptance of breads [Cotovanu & Mironeasa, 2021; Kamoto *et al*., 2018; Tömösközi *et al*., 2011]. Others studies have pointed out that a higher addition of amaranth flour to bread dough (greater than 40% substitution) not only improves the nutritional value of the product, but also allows the physical and sensory properties to be maintained at acceptable levels [Martínez *et al*., 2013; Miranda-Ramos *et al*., 2019; Rosell *et al*., 2009; Sanz-Penella *et al*., 2013]. Both raw and popped amaranth grain flour was used as a substitute for wheat flour in the bread preparation. Bodroza-Solarov *et al*. [2008] obtained a denser crumb structure, more uniform porosity, improved crust colour and great sensory acceptance of bread with popped *A. cruentus* grain flour at substitution level of 10–20% compared to wheat bread. Calderón de la Barca *et al*. [2010] produced gluten-free bread with up to 70% popped amaranth and 40% raw amaranth. They obtained acceptable physical characteristics of bread (loaves with homogeneous crumb and high specific volume) and rheological behaviour of the doughs, with a high nutritional value even without added hydrocolloids. Thus, popped amaranth could improve rheological and digestibility properties of breads since it is mainly composed of starches and proteins [Bodroza-Solarov *et al*., 2008]. Furthermore, the partial removal of the pericarp could reduce the content of antinutrients, such as phytic acid (IP6), since phytates are known to be concentrated in the bran of most cereals [Hama *et al*., 2011].

Several varieties of *A. caudatus* are cultivated in Peru, including Oscar Blanco, Centenario, INIA 414 Taray, and INIA 430 Imperial. This raised the interest in evaluating popped amaranth flour in various bread formulations. However, flours from popped grains of these varieties have not yet been studied in terms of addition to bread. Therefore, the objective of the present study was to compare the chemical and rheological properties of flour from raw and popped amaranth grains of four Peruvian varieties and evaluate the effect of the addition of popped amaranth flour on the chemical, physical, and sensory properties of bread.

MATERIALS AND METHODS

Materials

Grains of four Peruvian varieties of *Amaranthus caudatus* were used in study. The varieties Oscar Blanco (OB), INIA 414 Taray (T), and INIA 430 Imperial (I) were provided by Instituto Nacional de Innovación Agraria (INIA) station Andenes Cusco, Peru (harvest 2019). The Centenario (C) variety was acquired at UNALM, Lima, Peru (harvest 2019). The grain sizes of the four amaranth varieties used were as follows: Oscar Blanco – 1.1±0.18 mm, Centenario – 1.4±0.13 mm, Taray – 1.2 ±0.09 mm, Imperial – 1.3±0.12 mm. Wheat flour from "Los gallos mill" in Hermosillo, Sonora-México was used.

Preparation of popped grain flours

The amaranth grains were placed in a stainless-steel pot at 200°C for 15 s until complete popping was achieved [Amare *et al*., 2015; Bodroza-Solarov *et al*., 2008]. Subsequently, each variety of popped grains was pulverized in a disc mill, and the resulting flour was sieved through a 0.5 mm mesh, and identified as popped Oscar Blanco (POB), popped Taray (PT), popped Imperial (PI), and popped Centenario (PC). The flours were stored in polyethylene bags at 4°C until use.

Dough preparation and baking

The formulation for the control bread (with wheat flour) and the breads with 10, 20 and 30% substitution of wheat flour with popped amaranth flour are shown in **Table 1**. The solid ingredients were mixed and kneaded with water. Then the dough was placed in stainless steel molds, fermented at 40°C for 40 min, and baked at 150°C for 40 min. The loaves were cooled to room temperature (19°C), removed from the mold, and packed in high-density polyethylene containers.

Proximate analysis

The proximate composition of raw amaranth flours, popped amaranth flours and breads was analysed. The determinations were performed using the official methods of the AOAC International [AOAC, 2005]. The contents of proteins (method 984.13), lipids (method 2003.05), ash (method 942.05), moisture (method 950.46), and raw fiber (method 962.09) were determined. Carbohydrate content was determined by subtraction of the total content of other constituents (expressed in g/100 g) from 100 g. Moisture determination for pasting profile analysis was performed using AACC International method 44-19 [AACC, 1995].

Determination of pasting properties

The pasting properties of the flours were analysed using the AACC International method 76-21 [AACC, 2000]. Wheat flour (WF), raw and popped amaranth flours, and blends of WF with popped amaranth flour (in proportion as in bred formulations) were analyzed on 3.5±0.01 g of sample adjusted to 14% moisture. The amount of water incorporated was 25±0.01 g, which was obtained from the flour adjustment [Shittu *et al*., 2007]. Rapid Visco-Analyzer (RVA, Super 4, Newport Scientific, Sydney, Australia) and Thermocline software (Newport Scientific) were used to

TABLE 1. Formulations of breads without (control) and with the popped amaranth flour.

Ingredient	Control	Substitution level (% of total flour)		
		10	20	30
Wheat flour (g)	300	270	240	210
Flour of popped amaranth (g)	0	30	60	90
Instant yeast (g)	8	8	8	8
Brown sugar (g)	40	40	40	40
Vegetable shortening (g)	30	30	30	30
Sodium chloride (g)	2	2	2	2
Water (mL)	170	Variable	Variable	Variable

obtain pasting profiles. An initial temperature of 50°C and mixing at 960 rpm were applied, decreasing the speed to 160 rpm after 10 s. The temperature was maintained at 50°C for 1 min and then increased to 95°C at 4.42 min, remaining until 7.42 min. At 11 min the temperature dropped to 50°C and the test ended at 13 min. The parameters recorded were pasting temperature, peak and final viscosities.

Bread specific volume determination

Volume of bread loaves (mL) was measured using laser topographic equipment (BVM-6610, Perten Instruments, Sweden) and their weight (g) with analytical balance (Entris 224-IS, Sartorius Lab Instruments GmbH & Co.). The specific volume (mL/g) was calculated by dividing volume by weight [Vidaurre-Ruiz *et al*., 2019].

Bread porosity determination

Bread slices were photographed in colour using a camera. The images were scanned (Canon MG3610, Tokyo, Japan) at 600 dpi resolution, converted to gray colour, and processed with ImageJ software, version 1.51j8 (National Institutes of Health, Bethesda, MD, USA). The pixel values were converted into length units using dimensions of known lengths. The images were then binarized (pores in black and crumb in white), determining the number of pores *per* cm^2 and the area percentage of the pores [Vidaurre-Ruiz *et al*., 2019].

Instrumental bread colour measurement

The bread colour coordinates were measured in the CIELab space using a CSM7 portable colourimeter (PCE instruments, Deutschland GmbH, Meschede, Germany). L^* (0 – black, 100 – white), a^* (positive value – red, negative value – green), and b^* (positive value – yellow, negative value – blue) were recorded. Three points were measured for each bread slice [Yamsaengsung *et al*., 2010].

Flash profile sensory evaluation

For the sensory evaluation of bread, Flash profile (FP) technique was used, which is a descriptive sensory technique derived from the free-choice profile (each evaluator qualifies samples comparatively with their own words). Breads were evaluated in three sessions. In the first session, the samples were shown simultaneously and randomly, and the evaluators were asked to list sensory characteristics (attributes). The second session consisted of a consensus avoiding the repetition of two terms describing the same thing and then choosing their definitive list of attributes. In the third session, the samples were again presented simultaneously and randomly with a sensory evaluation of the chosen attributes. Finally, the evaluators were instructed to classify them in increasing order of intensities on an ordinal scale, allowing ties [Dairou & Sieffermann, 2002]. The information was analysed *via* a generalized Procrustes analysis (GPA). The evaluation was carried out with 24 evaluators (consumers) between men and women.

Statistical analysis

Data of nutritional composition and physical characteristics were expressed as mean and standard deviation and analyzed using the InfoStat free version 2017 software (InfoStat Group, Universidad Nacional de Córdoba, Argentina). The differences between treatments were established through the analysis of variance and Tukey multiple comparisons, considering statistically significant values of $p<0.05$. Flash profile sensory evaluation data were evaluated by PGA with XLSTAT 2014 trial version software (Addinsoft, New York, NY, USA).

RESULTS AND DISCUSSION

Raw and popped amaranth flours and bread proximate analysis

The proximate composition of the flours of raw and popped amaranth grains of four varieties is shown in **Table 2**. Significant differences ($p<0.05$) between varieties were found in contents of protein (highlighting varieties I and T), lipids (highlighting the OB and C varieties), and ash (the highest content in flour of I variety grains). No differences ($p\geq0.05$) were found in raw fiber and carbohydrate content in raw amaranth flours. The results were similar to those reported for *A. caudatus* [Alvarez-Jubete *et al*., 2009; Amare *et al*., 2016; Repo-Carrasco-Valencia *et al*., 2009] and *Amaranthus* spp. [USDA, 2019]. **Table 2** also shows an increase in protein, fat, ash and a decrease in carbohydrate and raw fiber content in the four popped amaranth varieties compared to the raw samples. The protein contents in PI and PT were different ($p<0.05$) from the rest of the varieties; ash content was significantly higher ($p<0.05$) in PC than in POB and PT. No differences ($p\geq0.05$) between varieties were found in lipid, raw fiber, and carbohydrate contents. These data were consistent with findings from other studies for popped *A. cruentus* (increasing content compared to raw grains of lipids from 5.88 to 7.27% and soluble fiber from 8.61 to 9.22% [Bodroza-Solarov *et al*., 2008] and popped *A. caudatus* var. Centenary (increasing content of total protein from 10.30 to 11.81 g/100 g dry weight and lipids from 7 to 8.17 g/100 g dry weight, decreasing content of carbohydrates from 80.3 to 70.15 g/100 g dry weight) [Paucar-Menacho *et al*., 2018].

TABLE 2. Proximate chemical composition (g/100 g dry matter) of flours of raw and popped amaranth grains of four Peruvian varieties, and breads without (control) and with popped amaranth flours

Flour/bread	Proteins (*N*×6.25)	Lipids	Raw fiber	Ash	Carbohydrates
Raw amaranth flour					
OB	13.45±0.29^{b}	6.90±0.21^{a}	3.89±0.12^{a}	1.97±0.09^{c}	73.79±0.47^{a}
C	13.16±0.40^{b}	6.64±0.00^{a}	3.98±0.23^{a}	2.35±0.04^{b}	73.87±0.13^{a}
T	14.9±0.20^{a}	6.42±0.16^{ab}	4.25±0.09^{a}	1.90±0.02^{c}	72.53±0.43^{a}
I	14.22±0.35^{ab}	5.96±0.16^{b}	3.76±0.19^{a}	2.64±0.05^{a}	73.42±0.74^{a}
Popped amaranth flour					
POB	14.59±0.07^{b}	8.03±0.04^{a}	3.58±0.17^{a}	2.10±0.12^{c}	71.7±0.16^{a}
PC	14.08±0.03^{c}	7.49±0.14^{a}	3.08±0.39^{a}	3.14±0.08^{a}	72.21±0.57^{a}
PT	15.25±0.15^{a}	7.72±0.43^{a}	2.87±0.10^{a}	2.37±0.05^{bc}	71.79±0.33^{a}
PI	15.54±0.15^{a}	7.23±0.09^{a}	3.24±0.08^{a}	2.80±0.28^{ab}	71.19±0.05^{a}
Bread					
Control	11.35±0.82^{b}	8.77±0.05^{g}	0.53±0.04^{d}	1.29 ±0.02^{de}	78.06±0.85^{a}
POB10	12.96±0.12^{a}	10.80±0.02^{ab}	0.73±0.14^{cd}	1.17 ±0.10^{e}	74.36±0.39^{cde}
POB20	12.64±0.12^{a}	9.07±0.07^{fg}	1.09±0.18^{abcd}	2.04±0.02^{abc}	75.16±0.35^{bc}
POB30	12.74±0.34^{a}	9.32±0.01^{efg}	1.27±0.03^{abc}	1.82±0.01^{abcd}	74.85±0.30^{bcd}
PC10	12.39±0.09^{ab}	10.15±0.36^{bcd}	0.78±0.02^{bcd}	1.48±0.25^{cde}	75.2±0.73^{bc}
PC20	12.41±0.06^{ab}	10.94±0.09^{a}	1.05±0.24^{abcd}	2.06±0.25^{abc}	73.54±0.16^{de}
PC30	12.54±0.00^{ab}	10.90±0.03^{a}	1.36±0.17^{ab}	2.04±0.13^{abc}	73.17±0.27^{e}
PT10	12.29±0.21^{ab}	10.60±0.04^{abc}	0.86±0.00^{bcd}	1.21±0.03^{e}	75.05±0.22^{bcd}
PT20	12.35±0.07^{ab}	10.32±0.09^{abc}	1.18±0.25^{abc}	1.26±0.14^{de}	74.98±0.54^{bcd}
PT30	12.36±0.06^{ab}	9.49±0.19^{def}	1.53±0.01^{a}	2.23±0.29^{a}	74.39±0.03^{cde}
PI10	12.26±0.10^{ab}	9.97±0.17^{cde}	0.97±0.18^{abcd}	2.01±0.06^{abc}	74.78±0.06^{bcd}
PI20	12.29±0.54^{ab}	8.69±0.08^{g}	1.11±0.10^{abcd}	1.53±0.20^{bcde}	76.38±0.16^{b}
PI30	12.26 ±0.01^{ab}	8.87±0.44^{fg}	1.32±0.25^{abc}	2.11±0.05^{ab}	75.44±0.22^{bc}

Results are shown as mean ± standard deviation (n=3). Different letters in superscript in each column, separately for raw amaranth flours, popped amaranth fours and breads, represent significant differences (p<0.05). OB, Oscar Blanco variety; C, Centenario variety; T, Taray variety; I, Imperial variety; POB, PC, PT and PI, popped OB, C, T and I, respectively; POB10, PC10, PT10 and PI10, breads with 10% substitution of wheat flour with POB, PC, PT and PI, respectively; POB20, PC20, PT20, PI20, breads with 20% substitution of wheat flour with POB, PC, PT and PI, respectively; POB30, PC30, PT30, PI30, breads with 30% substitution of wheat flour with POB, PC, PT and PI, respectively.

Finally, **Table 2** shows the proximate composition of breads with substitution in formula from 0 to 30% of wheat flour with amaranth popped flour of the four Peruvian varieties. As the percentage of WF substitution increased, the content of protein and raw fiber in bread showed an increasing tendency, while the content of carbohydrates – a decreasing one. The increase in the contents of protein and raw fiber and a decrease in carbohydrate content in breads with the highest substitution level compared to WF accounted for around 12%, more than 100% and around 6%, respectively. These trends were similar to those reported by Bodroza-Solarov *et al*. [2008], who made bread with popped *A. cruentus* at substitution levels of 10, 15, and 20%, resulting in higher contents of protein and crude fiber but a lower content of carbohydrates. Similar results were also reported for breads with raw flours of *A. cruentus* [Sanz-Penella *et al*., 2013], *A. spinosus* and *A. hypochondriacus* [Miranda-Ramos *et al*., 2019], *A. hypochondriacus* [Kamoto *et al*., 2018], and *Amaranthus* spp. [Cotovanu & Mironeasa, 2021] with substitution levels ranging from 5 to 50%. Moreover, a significant increase (p<0.05) in ash and lipid contents was observed in bread with popped amaranth flours compared to the control bread (with a few exceptions) (**Table 2**). These results agree with those reported in bread made from flours of popped *A. cruentus* [Bodroza-Solarov *et al*., 2008], raw *A. cruentus* [Sanz-Penella *et al*., 2013], and raw *Amaranthus* spp. [Cotovanu & Mironeasa, 2021]. The improved nutritional properties of breads with popped amaranth flour can result from the favorable chemical composition of amaranth flour compared to wheat flour (higher protein and fiber content). A larger share of amaranth

in bread is beneficial not only because of its protein content, but also because of a high nutritional value of these proteins. Amaranth albumins and globulins are rich in essential amino acids, including lysine, and are easily digestible [Venskutonis & Kraujalis, 2013]. Moreover, according to the literature, heat treatment in popping of amaranth grains increases the protein efficiency ratio and the gelatinisation of starch positively affects the stability, strength and freshness of the crumb [Bodroza-Solarov *et al.,* 2008]. Furthermore, it could affect protein digestibility due to the reduction of exogenous factors such as tannins, phytates, and trypsin inhibitors that reduce protein digestibility [Amare *et al*., 2015].

Pasting properties

Parameters of the pasting profile of raw and popped amaranth flours and WF, including pasting temperature, peak and final viscosities, determined using RVA are show in **Figure 1A**. Pasting profiles show the flour viscosity changes during heating in excess water under constant agitation. The flour behaviours of raw and popped amaranth grain flours and WF showed significant differences ($p<0.05$). A slightly higher pasting temperature was determined for raw amaranth flours than WF. However, the viscosity parameters of raw grain flours showed a considerably lower value. These results were similar, although slightly smaller differences were observed,

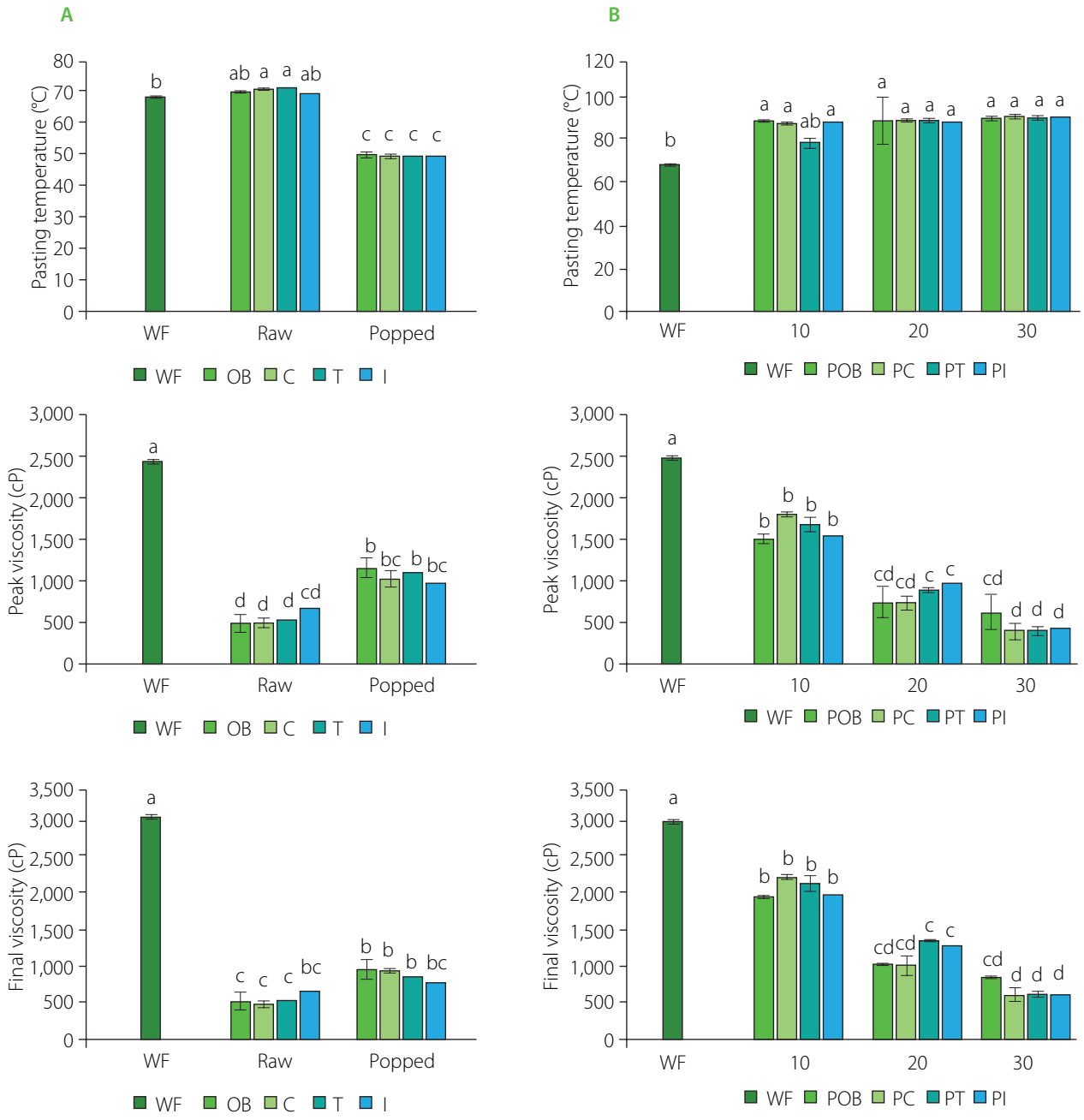

FIGURE 1. Pasting characteristics of wheat flour (WF) and flours of raw and popped amaranth grains of four Peruvian varieties (**A**), and blends of FW with 10, 20 and 30% popped amaranth flour (**B**). OB; Oscar Blanco; C, Centenario; T, Taray; I, Imperial; POB, PC, PT and PI, popped OB, C, T and I, respectively. Results are shown as mean and standard deviation ($n=3$). Different letters above bars indicate that the treatments are significantly different ($p<0.05$).

to those reported in 11 lines of *A. caudatus* [Kaur *et al*., 2010] and *A. hypochondriacus* [Sindhu & Khatkar, 2016]. The pasting temperature was the lowest for flours of popped amaranth grains (**Figure 1A**). The other RVA profile parameters were also lower for popped grain flours than WF but higher than for raw grain flours. Our analyses showed lower values of pasting profile parameters than those reported for flours of raw and popped *A. cruentus* and *A. hypochondriacus* grains by Muyonga *et al*. [2014]. Popped samples would have better viscosity properties than raw amaranth flour because starch granules disintegrate during heating, becoming more susceptible to hydration, which is associated with increased viscosity [Lai, 2001] and possibly due to the impact of extreme dehydration when raw grain bursts [Muyonga *et al*., 2014].

Figure 1B also shows the differences in every RVA profile parameter of blends of wheat flour with popped amaranth flour of the four varieties used in bread formulation. The pasting temperature was higher ($p<0.05$) for the blends compared to WF; however, the proportion of WF and amaranth flour in the blend did not change pasting temperature significantly ($p\geq0.05$). In the rest of the viscosity characteristics, considerably lower values were found for blends than in the control flour and as the substitution level of WF with popped amaranth flour in the blend increased. These results were similar to the findings from a study of low-gluten bread made with amaranth flour, with substitution level of 0 to 40% [Duda *et al*., 2019]. It was found that as the substitution level increased, the peak and final viscosity were considerably reduced, and a slight increase in the pasting temperature was observed. Another study described bread with raw quinoa flour addition. The peak and final viscosity of the flours decreased slightly, while the dough temperature did not show differences as the substitution level increased from 0 to 20% [Vásquez *et al*., 2016]. In another study on pastes, in which wheat was substituted with *Amaranthus mantegazzianus* flour, the viscosity values were reduced as the substitution level increased up to 50% [Martínez *et al*., 2013]. This is possibly due to the low starch and amylose contents of the whole meal amaranth flour with respect to bread wheat flour. The grains with a low starch content swell and release amylose, resulting in a lower viscosity [Martínez *et al*., 2013]. Amaranth starch is characterised by a low amylose content from 4.7 to 12.5% [Kong *et al*., 2009] and a higher amylopectin content from 20 to 25% [Cotovanu & Mironeasa, 2022] affecting its functional properties [Kong *et al*., 2009]. Starch gelatinisation is a key factor in starch behaviour, which occurs when the dough is heated to 60°C. Low consistency values reached at the starch gelatinisation stage can be explained by the increased interactions between the low amount of amylose and the large length of amylopectin chain of amaranth starch, which generates a synergistic effect on the final viscosity and these on starch retrogradation [Corke *et al*., 2016; Piga *et al*., 2021]. This would indicate that the addition of amaranth flour could limit starch retrogradation and increase the shelf life of bread [Cotovanu & Mironeasa, 2022], but it would also contribute to the weakening of gluten [Šárka & Dvořáček, 2017].

Physical characteristics

The physical characteristics of the breads with different levels of popped amaranth substitution from the four Peruvian varieties are shown in **Table 3**. Additionally, the appearance of bread cross-sections is shown in **Figure 2**. The breads POB10 and POB30 had lower weight compared to the others. The volume and specific volume decreased with the increase in the substitution of WF with popped amaranth flour in bread formulations. The WF bread presented the highest specific volume, although POB10, POB30 and PT10 did not differ significantly ($p\geq0.05$) from control in this respect. Our findings were consistent with the study by Bodroza-Solarov *et al*. [2008] who reported a reduction in the specific volume of bread by 33% when a 20% popped *A. cruentus* flour was used in formulation. Similar results were also obtained for amaranth flours of other species [Cotovanu & Mironeasa, 2021; Miranda-Ramos *et al*., 2019; Sanz-Penella *et al*., 2013; Tömösközi *et al*., 2011]. The reduction in the specific volume results from high-fiber ingredients [Iglesias-Puig *et al*., 2015]. This could also be explained by the dilution of gluten and decrease of α-amylase activity by globular proteins (11S and 9P) of amaranth, which reduces maltose availability for yeast during the bread-making process [Cotovanu & Mironeasa, 2021]. Whole-grain pseudocereal flours are high in dietary fiber but are gluten-free. However, proteins such as albumins and glutenins from WF can interact through disulfide bonds, maintaining the viscoelastic properties of gluten under acceptable conditions [Oszvald *et al*., 2009].

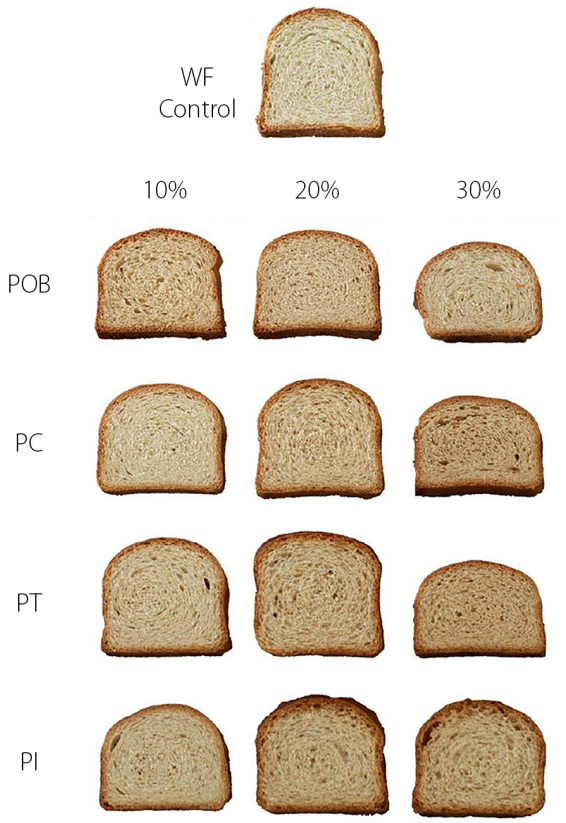

FIGURE 2. Appearance of wheat flour (WF) bread and breads with 10, 20 and 30% substitution in formulation of WF with popped amaranth flours from four varieties including Popped Oscar Blanco (POB), Popped Centenario (PC), Popped Taray (PT), Popped Imperial (PI).

TABLE 3. Physical characteristics and colour coordinates of breads without (control) and with popped amaranth flours.

Bread	Weight (g)	Volume (mL)	Specific volume (mL/g)	Number of pores *per* cm²	Pore area (%)	Colour coordinate		
						*L**	*a**	*b**
Control	129.2±1.1^{a}	487.00±16.97^{a}	3.76±74.96^{a}	60.00±2.20^{b}	46.95±2.26^{bcd}	74.96±1.23^{a}	3.99±0.48^{a}	16.46±0.88^{a}
POB10	120.85±0.35^{b}	448.50±0.7^{ab}	3.71±61.94^{ab}	23.28±0.24^{d}	63.85±1.66^{a}	71.74±2.28^{ab}	6.28±0.30^{bc}	20.25±0.44^{ab}
POB20	132.85±1.34^{a}	417.00±12.72^{bc}	3.14±69.89^{cd}	33.06±2.12^{d}	57.77±0.94^{ab}	69.89±1.08^{abc}	6.92±0.46^{bcd}	21.64±1.04^{bcd}
POB30	122.90±1.27^{b}	411.00±26.87^{bc}	3.34±71.74^{abc}	78.61±7.46^{a}	34.76±1.04^{def}	61.94±0.96^{def}	9.14±0.39^{e}	25.10±0.95^{cde}
PC10	132.70±0.28^{a}	429.50±7.77^{abc}	3.24±70.38^{bcd}	26.11±2.20^{d}	52.04±1.38^{abc}	70.38±1.47^{abc}	6.02±0.35^{bc}	20.18±0.33^{ab}
PC20	134.50±2.26^{a}	413.50±6.36^{bc}	3.07±66.91^{cd}	48.89±0.31^{c}	27.74±4.05^{ef}	66.91±4.78^{bcd}	6.75±1.71^{bc}	21.50±2.62^{bcd}
PC30	133.30±0.98^{a}	396.50±0.7^{bc}	2.97±61.25^{cd}	75.94±4.32^{a}	29.40±3.35^{ef}	61.25±0.35^{ef}	9.19±0.42^{e}	25.66±1.01^{e}
PT10	131.10±0.14^{a}	453.50±7.77^{ab}	3.46±69.49^{abc}	24.44±0.79^{d}	53.21±2.23^{abc}	69.49±1.11^{bc}	5.76±0.19^{ab}	20.20±0.44^{ab}
PT20	134.65±0.07^{a}	405.50±9.19^{bc}	3.01±61.91^{cd}	49.39±0.71^{bc}	44.43±0.80^{bcd}	61.91±0.71^{def}	6.93±0.96^{bcd}	21.11±2.30^{bc}
PT30	131.85±0.35^{a}	372.50±4.94^{c}	2.83±59.29^{d}	78.17±1.96^{a}	36.07±0.38^{def}	59.29±1.26^{f}	8.83±0.23^{de}	25.26±0.41^{de}
PI10	129.40±2.26^{a}	419.50±9.19^{bc}	3.24±65.97^{bcd}	47.33±1.26^{c}	27.51±2.96^{f}	65.97±0.78^{cde}	5.98±0.41^{abc}	20.77±1.19^{b}
PI20	133.10±2.69^{a}	418.00±12.73^{bc}	3.14±62.74^{cd}	53.61±0.39^{bc}	41.85±2.22^{cde}	62.74±0.62^{def}	7.53±0.85^{bcde}	22.66±2.20^{bcde}
PI30	132.05±1.62^{a}	371.50±43.13^{c}	2.82±58.84^{d}	74.78±2.36^{a}	39.86±10.48^{cdef}	58.84±0.84^{f}	7.85±0.40^{cde}	23.06±0.95^{bcde}

Results are shown as mean ± standard deviation (n=3). Different letters in superscript in each column, separately for raw amaranth flours, popped amaranth fours and breads, represent significant differences (p<0.05). POB, Popped Oscar Blanco; PC, Popped Centenario; PT, Popped Taray; PI; Popped Imperial; POB10, PC10, PT10 and IP10, breads with 10% substitution of wheat flour with POB, PC, PT and PI, respectively; POB20, PC20, PT20 and PI20, breads with 20% substitution of wheat flour with POB, PC, PT and PI, respectively; POB30, PC30, PT30 and PI30, breads with 30% substitution of wheat flour with POB, PC, PT and PI, respectively; *L**, lightness; *a**, redness–greenness; *b**, yellowness–blueness.

The number of pores *per* cm² and the pore area percentage in the bread crumbs are shown in **Table 3**. A higher substitution of WF with popped amaranth flour caused a higher number of pores/cm² and, for most amaranth varieties, a decrease in pore area (for flour from variety Imperial, an increase in pore area was determined with an increase in the share of flour in the bread formula). A bread substituted with *A. spinosus* and *A. hypochondriacus* showed a higher number of cells/cm² as the substitution percentage increased [Miranda-Ramos *et al*., 2019]. In bread fortified with germinated *Amaranthus sp* flour, the number of pores/cm² and the pore area also increased as the substitution increased [Guardianelli *et al*., 2021]. A bread formulation with 50% raw amaranth flour from *A. spinosus* and *A. hypochondriacus* showed a higher number of cells with a lower specific volume compared to control, but was found not significant [Miranda-Ramos *et al*., 2019]. It could be due to a loss of dough elasticity since low gluten availability. These results can be compared to those obtained in our research. On the other hand, the technological parameters significantly decrease compared to the control because of the weakening of the gluten network, which leads to a decrease in volume, porosity, and elasticity in formulations greater than 20% [Cotovanu & Mironeasa, 2021], since, when mixed with water, wheat proteins are hydrated, allowing the development of a dough with a balanced interrelation of cohesiveness, elasticity (glutenins) and viscosity, extensibility (gliadins), forming gluten network through S–S bonds and hydrogen bonding, retaining gas during fermentation and baking [Wieser *et al*., 2022]. The increased level of substitution results in a dilution of the protein fractions involved in gluten formation. Besides, the amaranth 11S proteins and globulin P are not enough to have a gluten network in the mixture, resulting in a softer dough [Cotovanu & Mironeasa, 2021]. Bread shape, crumb porosity, and other characteristics depend mainly on new grain ingredients, which generally cause quantitative and qualitative changes in the protein-proteinase and carbohydrate-amylase complex of the flour, modifying the sensory properties of the product [Derkanosova *et al*., 2020].

Finally, the results of the instrumental colour evaluation of breads are shown in **Table 3**. Lightness (*L**) had a progressive decrease (p<0.05) as popped amaranth substitution increased, resulting in darker bread. The values of *a** and *b** also significantly (p<0.05) increased. The samples changed to red as *a** increased and yellow as *b** increased. Popped amaranth caused hue changes, obtaining a darker and opaque crumb at the highest substitution level. Similar behaviour was observed in raw amaranth bread from *A. cruentus* substituted for up to 40% [Sanz-Penella *et al*., 2013], in crude *A. caudatus* var Centenario with substitution of 0 to 100% [Rosell *et al*., 2009], and crude *Amaranthus* spp. flour [Cotovanu & Mironeasa, 2021; Nasir *et al*., 2020].

Sensory evaluation

The bread samples of the 12 formulations were evaluated, including the control. Each consumer described between four to ten sensory attributes of their own, generating a total of 71 terms, subdivided into: general appearance (20), texture (29), aroma (05), colour (07), and taste (10). In addition, a consumer consensus index (Rc) value of 0.61 (61%) was obtained, resulting in a positive correlation.

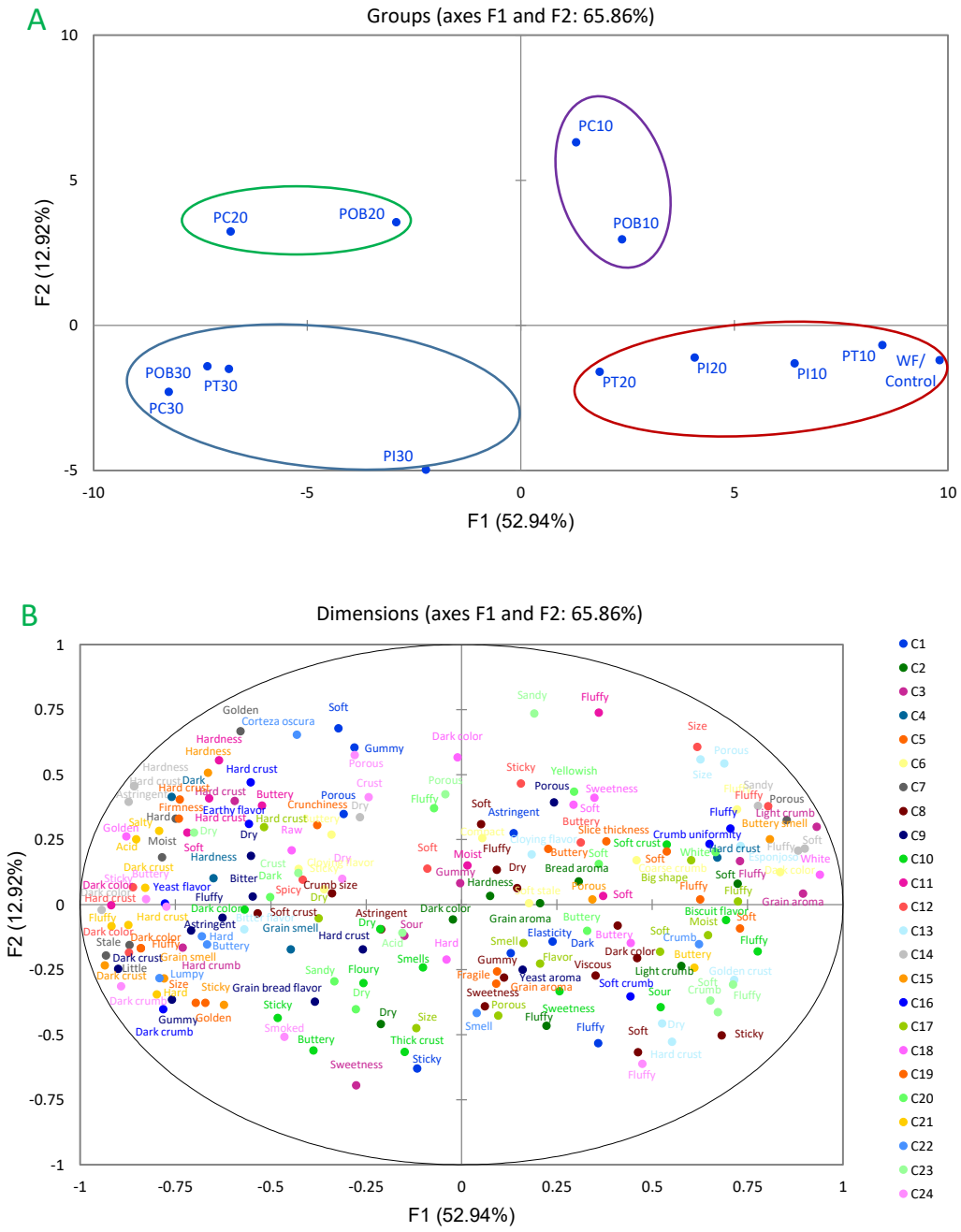

FIGURE 3. Generalized Procrustes analysis (GPA) plots of Flash profile data for wheat bread and breads with 10, 20 and 30% substitution in formulation of WF with popped amaranth flours. (**A**) Sensory space of samples – breads, (**B**) Sensory space of attributes or descriptors. POB, Popped Oscar Blanco; PC, Popped Centenario; PT, Popped Taray; PI, Popped Imperial; WF, wheat flour (control); C1–C24, individual consumers.

The results of the Flash profile analysis (sensory attributes submitted by the consumers) were subjected into the GPA, which results are shown in **Figure 3**. The first two factors of GPA explained 65.86% of data variability (F1=52.94% and F2=12.92%). This value was lower than that reported for GPA of data for breads with raw *A. hypochondriacus* (86% variability) [Kamoto *et al*., 2018] and with germinated basul flour (72.99%) [Vilcanqui-Pérez *et al*., 2022]. The sample location in the sensory space is shown in **Figure 3A**. Four groups were evident in dimensions F1 and F2. Consumers identified the same sensory attributes within groups. The first group included the control and PT10, PI10, PT20 and PI20 samples, located in the positive areas of dimension 1 and negative ones of dimension 2. The second group included POB10 and PC10 samples, located in the positive areas of the two dimensions. The third group included POB20 and PC20, located in the negative zones of dimension 1 and positive of dimension 2. Finally, the fourth group comprised the treatments with the highest level of addition (30), located in the negative zones of the two dimensions. These results coincide with those reported by Kamoto *et al*. [2018] for breads with raw amaranth flour,

which were divided in GPA into three groups (breads with substitution levels 0–5%, 10–15, and 20–25) and by Vilcanqui-Pérez *et al*. [2022]. showing similar attributes in three groups consisting of breads with 0%, 5-10%, and 15-20% of WF substitution with germinated basul flour.

Figures 3A and **3B** show the sensory space for samples and attributes, respectively. Sensory attribute differences are observed between groups. The first group (control, PT and PI, at 10 and 20% substitution) was characterised as fluffy, sticky, moist, light, and yeasty, with a soft crumb and sweetness. Groups with PC10 and POB10 were described as buttery, sticky, astringent, soft, smooth, porous, and smelling like bread. Third group (PC20 and POB20) had attributes such as yeasty flavour, hardness, dryness, and gold. Finally, all the samples at 30% substitution were characterised as lumpy, small, not very fluffy, and with a dark crumb. Thus, PT and PI samples at 10 and 20% substitution were similar in their sensory profile to the control and showed the best attributes in taste and appearance. Descriptors found for bread with raw amaranth were crusts browning, alveolus size and regularity, earthy aroma, yeasty aroma, saltiness, sweetness, chewiness, crisp crust, elasticity, coarseness, crumb graininess, and stickiness [Kamoto *et al*., 2018]. Attributes found in sprout basul breads were a sweet taste, sticky texture, and fluffy appearance [Vilcanqui-Pérez *et al*., 2022].

The higher addition of popped amaranth to bread affects the structure of the crumb, elasticity, and crumb colour. Thicker cell walls and the grayish colour of the crumb are also observed, but uniform cells with thicker walls ensure higher breadcrumb stability and strength [Bodroza-Solarov *et al*., 2008]. The lipid content of amaranth flour is six times higher than WF; hence, it can act as a surface-active agent and gas-stabilizing agent during baking, which could contribute to bread elasticity [Alvarez-Jubete *et al*., 2010].

CONCLUSIONS

Bread with 30% substitution of FW with popped amaranth flour had improved nutritional value with a lower carbohydrate content and higher protein and raw fiber contents compared to FW bread. The physical characteristics (peak and final viscosity, specific volume, pore percentage, and colour coordinates) showed reductions as substitution levels increased. According to the sensory evaluation, Taray and Imperial bread varieties at 10 and 20% substitution level were similar to the control with the best sensory attributes, specifically in taste and appearance. The results suggest that bread with popped amaranth flour could become a healthy alternative to wheat bread. However, further study on the phytate and mineral effect in bread is needed.

ACKNOWLEDGEMENTS

The authors thank Instituto Nacional de Innovación Agraria (INIA) station Andenes Cusco-Perú for the amaranth samples Oscar Blanco, INIA 414 Taray, and INIA 430 Imperial.

We also thank M. Carmen Granados N. from the CIAD Hermosillo cereal laboratory for technical support in rheology analysis and bread making.

RESEARCH FUNDING

The authors thank Vicerrectorado de investigación, Universidad Nacional Micaela Bastidas de Apurímac (UNAMBA) for funding the project from mining canon fees (Resolution 048-2019-CU-UNAMBA).

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 147–161
DOI: 10.31883/pjfns/187941
ISSN 1230-0322, e-ISSN 2083-6007

Effect of the Addition of Apple Pomace and Erythritol on the Antioxidant Capacity and Antidiabetic Properties of Shortbread Cookies

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Apple processing into juice generates vast amounts of a by-product, namely fruit pomace, which poses a serious problem for the processing industry. At the same time, fruit pomace features a high health potential. The aim of the present study was to develop recipes of 8 variants of cookies with wheat flour substituted by apple pomace (0, 10, 30 and 50% of flour weight), sweetened with sucrose and erythritol. The cookies were analyzed for their nutritional value; antioxidant capacity ($ABTS^{\bullet+}$ scavenging activity and oxygen radical absorption capacity – ORAC); the ability to inhibit α-amylase, α-glucosidase and pancreatic lipase; and consumer acceptability. In total, 13 phenolic compounds were identified in the cookies with pomace. Cookies with 50% addition of apple pomace had an approximately 8-fold higher content of dietary fibre than traditional products (without the apple pomace) and simultaneously reduced energy value (by 32.6 and 40.5 kcal/100 g of cookies sweetened with sucrose and erythritol, respectively). The antioxidant capacity of cookies was 0.032–0.316 mmol TE/100 g in the ABTS assay and 1.153–2.070 mmol TE/100 g when ORAC was determined. The IC_{50} enabling α-amylase and α-glucosidase inhibition ranged from 138.1 to 221.8 mg/mL and from 976.4 to 1374.9 mg/mL, respectively. The anti-lipase activity of cookies with the addition of 50% apple pomace and erythritol was the highest (IC_{50} of 7.3 mg/mL). Both antioxidant capacity and antidiabetic potential increased significantly with the increasing proportion of pomace in cookies. Replacing sucrose with erythritol favorably influenced the consumer assessment. The study results show that the proposed products can be a perfect alternative to traditional sweet snack products, especially for consumers with diet-related diseases. The feasibility of using waste raw materials, which are a challenge to the food industry, has been proven as well.

Keywords: apple pomace, erythritol, shortbread cookies, LC-MS, antidiabetic activity, antioxidant activity

INTRODUCTION

Apples are one of the most commonly grown and consumed kinds of fruit in different parts of the world. The fruit and vegetable industry focused on apple processing generates a significant mass of waste in the form of pomace – about 11 million tonnes worldwide/year [USDA, 2018]. This by-product is most often transported, stored, composted and destined for low-value animal feed. Such processes generate high costs and pose a threat to the environment.

Apple pomace contains, among others, large amounts of dietary fibre, which constitutes about 40 g/100 g of their dry matter (d.m.) [Alongi *et al.*, 2019] and phenolic compounds with anti-tumor [Nile *et al.*, 2021; Sudha *et al.*, 2016], anti-inflammatory [Barreira *et al.*, 2019; Zhang & Ying, 2011], antibacterial [Santos

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Submitted: 15 January 2024
Accepted: 25 April 2024
Published on-line: 17 May 2024

et al., 2023; Zhang *et al.*, 2016] and antiviral properties [Suárez *et al.*, 2010]. Particular attention should be paid to the antioxidant and antidiabetic properties of apple pomace. Their antioxidant capacity is associated with a high content of phloretin, chlorogenic acid and quercetin [Gorjanović *et al.*, 2020]. Research showed that including pomace in food formulations increased antioxidant properties of food products [Tańska *et al.*, 2016]. With the increase in the proportion of apple pomace, the total phenolic content and total flavonoid content was reported to increase significantly, causing an increase in antioxidant activity measured as the ability to scavenge the radicals [Mir *et al.*, 2017]. In addition, the high content of phenolic compounds in these by-products makes them able to improve the impaired oxidative state associated with type 2 diabetes [Grindel *et al.*, 2014]. The low glycemic index of products with apple pomace addition normalizes blood glucose levels and influences body weight control, contributing to the improvement of carbohydrate metabolism and preventing type 2 diabetes [Alongi *et al.*, 2019]. Studies have shown that phenolic compounds are found primarily in the skin of apples [Francini & Sebastiani, 2013]. Thus, the apple skin is a valuable but underused product. Currently, the food industry focuses on sustainable production and the use of health-promoting by-products of the fruit and vegetable industry [Kammerer *et al*., 2014]. Due to the relatively low cost of obtaining apple pomace and at the same time the multi-faceted health benefits that can be obtained from it, it is recognized as a valuable material for further processing in the food industry. Studies involving humans have shown that the consumption of fibre-rich foods can promote health and thus prevent a number of chronic diseases, especially those associated with inflammation (for example, type 2 diabetes and cardiovascular diseases) [He *et al.*, 2022]. In addition, fermentation of fibre by the intestinal microflora induces the production of short-chain fatty acids (SCFA), which play, among others, an immunoregulatory role [Yang *et al.*, 2020].

Taking into account the impact of dietary fibre and phenolic compounds on human health, current trends in food production and the growing consumer awareness and increased demand for so-called "clean label" foods, a study was conducted to evaluate the bioactive potential and nutritional properties of designed low-energy snacks (shortbread cookies) with a functional additive (apple pomace replacing 10, 30 and 50% of wheat flour by weight). The market success of a new food product, in addition to its high nutritional value, also requires acceptance by potential consumers. Therefore, two additional variants of cookies were developed – sweetened with sucrose and its substitute – erythritol. It was assumed that it is feasible to develop a recipe of shortbread cookies that will be characterized by a high bioactive potential and at the same time will be accepted by consumers. Literature data show the beneficial effect of apple pomace addition to bakery/confectionery products on their nutritional value, phenolic content and antioxidant properties [Alongi *et al.*, 2019; Cantero *et al.*, 2022; Ghadam *et al.*, 2023; Kruczek *et al.*, 2023; Sudha *et al.*, 2016; Usman *et al.*, 2020; Valková *et al.*, 2022]. The studies also assessed the organoleptic characteristics of the products obtained [Alongi *et al.*, 2019; Ghadam *et al.*, 2023; Lauková *et al.*, 2016; Rocha Parra *et al.*, 2019; Usman *et al.*, 2020; Valková *et al.*, 2022]. It should be noted, however, that in the studies conducted so far, the addition of apple pomace was most often at the level of 8–30% [Alongi *et al.*, 2019; Cantero *et al.,* 2022; Ghadam *et al.*, 2023; Lauková *et al.*, 2016; Rocha Parra *et al.*, 2019; Sudha *et al.*, 2016; Usman *et al.*, 2020; Valková *et al.*, 2022]. In the present study, the maximum proportion of pomace was as high as 50%. Additionally, studies conducted so far have most often assessed only the impact of adding apple pomace to specific food products. An innovative element of this research is the simultaneous use of various additions of apple pomace and erythritol. Erythritol has been shown to be an excellent substitute for sugar in sweet snacks due to its low energy value (0.2 kcal/g). [Regnat *et al.*, 2018]. It is well tolerated by consumers and has no significant effect on blood glucose and insulin levels, making it suitable for both healthy persons and diabetic patients. Moreover, it has been shown that the consumption of erythritol induces the secretion of intestinal hormones that modulate the feeling of satiety, which promotes weight loss [Mazi & Stanhope, 2023]. It is also considered a beneficial substitute for sucrose due to its anti-caries and endothelium-protective effects [Boesten *et al.*, 2015]. Therefore, composing a recipe for shortbread cookies based on apple pomace and erythritol seems to be an excellent alternative to sweet snacks, both for healthy consumers and for consumers with carbohydrate metabolism disorders.

MATERIALS AND METHODS

Reagents and chemicals

Chemicals for the determination of nutritional value (potassium dichromate, sulfuric acid (VI), sodium thiosulfate, sodium hydroxide, hydrochloric acid, diethyl ether) and dietary fiber (petroleum ether, phosphate buffer, α-amylase, proteases, hydrochloric acid,

Table 1. Recipe of individual variants of shortbread cookies.

Cookie variant (%)*	Composition (g/100 g)				
	Wheat flour type 450	Apple pomace	Butter (82% fat)	Egg yolks	Sugar/erythritol
0	45.4	0.0	30.3	9.1	15.2
10	40.9	4.5	30.3	9.1	15.2
30	31.8	13.6	30.3	9.1	15.2
50	22.7	22.7	30.3	9.1	15.2

*Percentage of replacing wheat flour with apple pomace (by weight).

amyloglucosidase, ethanol, acetone) were purchased from Idalia (Radom, Poland) and Sigma-Aldrich (Steinheim, Germany), respectively. Certified reference material (CRM; the validated reference material BCR-191 was used to confirm the accuracy of the method for determining the mineral content) was purchased from MS Spectrum (Warsaw, Poland). Acetonitrile for chromatography was purchased from Merck (Darmstadt, Germany). Cyanidin 3-*O*-glucoside, quercetin 3-*O*-glucoside, chlorogenic acid, (+)-catechin, and procyanidin B_2 were purchased from Extrasynthese (Lyon, France). 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), formic and acetic acids, phloroglucinol, methanol, 3,5-dinitrosalicylic acid, potassium sodium tartrate tetrahydrate, sodium phosphate monobasic, starch from potato, pancreatic α-amylase from porcine pancreas (type VI-8), dipotassium hydrogen orthophosphate dihydrogen, *p*-nitrophenyl-α-D-glucopyranoside, and intestinal α-glucosidase from *Saccharomyces cerevisiae* (type I) were purchased from Sigma-Aldrich. Ultra-performance liquid chromatography (UPLC) grade water was prepared in the HLP SMART 1000s system (Hydrolab, Gdańsk, Poland), and additionally filtered through a 0.22 µm membrane filter immediately before use.

Materials

Dried apple pomace was purchased from a company GreenHerb-Kuźniar Dariusz (Łańcut, Poland) certified by the Institute of Consumer Research (Poland). The producer dried wet pomace, a by-product of juice production, in the Agromech M829 tumble dryer (Rogoźno, Poland) at 70°C for 3 h, crushed it as needed on Scorpion slicers and grinders (Rozdrażew, Poland) and sieved it using sifters from the same manufacturer. Wheat flour type 450, sucrose, erythritol, butter (82% fat) and eggs, necessary to make the cookies, were purchased from retail outlets (Wroclaw, Poland).

Shortbread cookie preparation

The subject of the research were shortbread cookies with a varied addition of by-product of juicing in the form of dried and powdered apple pomace (10, 30 and 50% of flour weight). Sucrose and its substitute – erythritol were used as sweeteners. A total of 8 variants of shortbread cookies with apple pomace were prepared including 4 variants sweetened with sucrose (SA10, SA30 and SA50, respectively) and 4 sweetened with erythritol (EA10, EA30 and EA50, respectively). Cookies with only wheat flour (without pomace), sweetened with sugar (SA0) or erythritol (EA0) were prepared as control samples. The recipe composition of individual cookie variants is presented in **Table 1**.

The preparation of the shortbread cookies consisted of the following steps: mixing the dry ingredients – wheat flour with apple pomace and ground sucrose/erythritol; adding the butter and egg yolks and kneading the dough for 3 min (KitchenAid model 5KPM5 mixer; Springfield, OH, USA); forming a ball and chilling the dough for 1 h at 4°C; rolling out the dough and cutting out circles (thickness 0.5 cm, diameter 5 cm); and baking for 8 min at 180°C (convection-steam furnace Rational; Landsberg am Lech, Munich, Germany). Three batches of each cookie variant were baked (100 cookies of each type).

Proximate analysis

The methods of the Association of Official Analytical Chemists (AOAC) were used to determine contents of dry matter (AOAC 925.49-1925), ash (AOAC 940.26), proteins (AOAC 920.152), dietary fibre (AOAC 985.29), and fat (AOAC 996.06) [AOAC, 2005] in apple pomace and shortbread cookies. The amount of total carbohydrates was calculated by subtracting the content of dietary fibre, fat, protein and ash from the dry matter content. The energy value was determined using the Rosenthal method [Gronowska-Senger, 2018].

Determination of sugar content

Sugar content in apple pomace and shortbread cookies was determined using the high-performance liquid chromatography (HPLC) system (Merck-Hitachi L-7455; Merck KGaA, Darmstadt, Germany) with an evaporative light scattering detector (ELSD 1000, Polymer Laboratories Inc., Amherst, MA, USA) as of the procedure described by Wojdyło *et al.* [2018]. Weighed samples of the shortbread cookies or apple pomace (4-5 g) were suspended in distilled water (100 mL), vortexed and subjected to sonication for 15 min (Sonic 6D; Polsonic, Warsaw, Poland). Then, the temperature of the suspensions was raised to 90°C, and heating was continued for 30 min with occasional stirring. After this step, the mixtures were centrifuged for 10 min at 19,000×*g* (MPW-55 centrifuge; Warsaw, Poland), and supernatants were subjected to purification on Sep-Pak C-18 columns. Finally, before injection into the HPLC system, the samples were filtered using hydrophilic membrane filter type PTFE (0.20 mm; Millex Samplicity; Merck). Chromatography separation was carried out using the Prevail™ Carbohydrate ES HPLC column (250×4.6 mm, 5 µm; Imtakt, Kyoto, Japan) with an injection volume of 20 µL, a flow rate of 1 mL/min and temperature analysis of 30°C. Isocratic elution based on the mobile phase consisting of acetonitrile and water (75:25, *v/v*) was used. Flow of nitrogen gas was 1.2 mL/min and temperature of nebuliser and evaporation was 80°C. Quantification of sugars was carried out based on standard curves obtained after the injection of standard solutions of sucrose, glucose, fructose and erythritol with known concentrations ranging from 0.05 to 0.10 mg/mL (r^2=0.999–0.997). The results of quantitative analysis were reported as the mean (n=3) and expressed in g/100 g of the cookie variant or apple pomace.

Determination of mineral content

Determination of the content of selected minerals (Cu, Mg, Mn, Fe, Zn, Ca, Na, K) in apple pomace and shortbread cookies was carried out in the certified Laboratory of Food Research of the Department of Human Nutrition of the Wrocław University of Environmental and Life Sciences, Poland. The atomic emission spectrometry (FEAS) method – potassium, sodium, calcium – and the atomic absorption spectrometry (FAAS) method – copper, zinc, iron, manganese, magnesium – were

used to determine the content of minerals. The Varian AA240FS atomic absorption spectrometer (Mulgrave, VIC, Australia) was used for the analyses. For the determination of mineral content, approximately 0.5 g of each variant of cookies or apple pomace was weighed out. Subsequently, 5 mL of 65% nitric acid and 1 mL of hydrogen peroxide were added to each sample. The MARS 6 closed microwave system was used for sample mineralization (CEM, Matthews, NC, USA). Mineralization temperature and time were 210°C and 15 min, respectively. In the next step, samples were quantitatively transferred using double distilled water into 10-mL tubes. Validated reference material BCR-191 was used to confirm the accuracy of the method. The measurement uncertainty was 5% [CEN, 2009, Food and Nutrition Institute, 2013]. The results of the mineral content were reported as the mean (n=3) and expressed in mg/100 g of the specified cookie variant or apple pomace.

Chromatography analysis of phenolic compounds

The qualitative and quantitative analysis of phenolic compounds of apple pomace and shortbread cookies was carried out using a UPLC system with a photo diode array (PDA) detector connected to a quadrupole time-of-flight–mass spectrometer (Q/TOF-MS) controlled by Empower 3 software and MassLynx 4.0 ChromaLynx software (Waters Corporation, Milford, MA, USA), as previously described by Tkacz *et al.* [2019]. The sample was grounded in a laboratory mill for extraction, weighed (cookie: 2.0 g and apple pomace: 0.5 g), suspended in 5–6 mL of a mixture of methanol, water, ascorbic acid and acetic acid (3:7:2:1, *v/v/w/v*), placed in a sonicated water bath (Sonic-6D; Polsonic, Warsaw, Poland) at 20°C for 15 min and then extracted for 24 h at 4°C. Next, the mixture was centrifuged (10 min at 19,000×*g*; MPW-350; Warsaw, Poland), and supernatant was separated. Finally, before analysis, all obtained extracts were filtered through a hydrophilic membrane (PTFE, 0.20 µm; Millex Samplicity Filter; Merck). The same chromatographic parameters were used as in our previous study [Wojdyło *et al.,* 2018], *i.e.*, UPLC BEH C18 column (2.1×100 mm, 1.7 µm; Waters Corporation), injection volume 5 µL, column temperature 30°C, elution at a flow rate 0.42 mL/min in a gradient mobile phase system consisting of (A) 2% formic acid and (B) 100% acetonitrile. PDA spectra were reordered, and absorbance at 520 nm for anthocyanins, at 360 nm for flavonols, at 320 nm for phenolic acids, and at 280 nm for flavan-3-ols and dihydrochalcones was measured. Quantification of phenolic compounds was carried out by injecting solutions of chlorogenic acid, procyanidin B_1, quercetin 3-*O*-glucoside and quercetin 3-*O*-galactoside with known concentrations ranging between 0.05 and 0.50 mg/mL and preparing standard curves ($r^2 \leq 0.9998$). The optimized MS parameters included: dissolution and source temperatures of 300 and 100°C, respectively, dissolution and cone gas flow of 300 and 40 L/h, respectively, cone and capillary voltage of 30 and 2500 V, respectively. The mass range from 100 to 1,000 *m/z* in positive (for anthocyanins) and negative ionization (for phenolic acids, flavonols, flavanols, and dihydrochalcone) was used for MS analysis. The results were presented as the mean of 3 replicates (n=3) and expressed in mg *per* kg of apple pomace powder or shortbread cookies.

Determination of antioxidant capacity and ability to inhibit activity of α-amylase, α-glucosidase and pancreatic lipase

To determine bioactivities of shortbread cookies and apple pomaces, first the extracts were prepared. The products were grounded, then weighed (about 0.5 g of apple pomace and 2.5 g of cookies), suspended in 5 mL of a mixture of 80% (*v/v*) methanol with 1% (*v/v*) HCl in the proportion of 8:1 (*w/v*), and sonicated (Sonic 6D water bath; Polsonic) for 15 min. After extraction, suspensions were centrifuged (5 min; 1,000×*g*), and supernatants were collected.

Determination of antioxidant capacity with the $ABTS^{\bullet+}$ was performed according to Re *et al.* [1999]. The $ABTS^{\bullet+}$ was obtained by mixing a solution of 7 mM ABTS with 140 mM potassium persulfate for about 16–18 h prior to analysis in darkness at the room temperature. The working solution was made by mixing $ABTS^{\bullet+}$ diluted with distilled water until its absorbance was 0.70±0.02 at 732 nm. Then, 0.1 mL of the extract was mixing with 2 mL of the working $ABTS^{\bullet+}$ solution in a cuvette, and reaction absorbance was measured after 6 min. Trolox was used as a standard, and the results were expressed as mmol Trolox equivalent (TE) *per* 100 g of apple pomace powder or shortbread cookies.

Oxygen radical absorption capacity (ORAC) assay was performed according to Ou *et al.* [2001]. The mixture of fluorescin (40 nM) with the extract was placed in a cuvette, pre-incubated at 37°C for 15 min and then mixed with 2,2′-azo-bis(2-amidinopropane) dihydrochloride (18 mM). The fluorescence was reordered every 1 min at 493 and 515 nm for excitation and emission, respectively, during 45 min. The results were calculated based on a standard curve plotted for Trolox and presented as mmol Trolox equivalent (TE) *per* 100 g of apple pomace powder or shortbread cookies.

The ability of apple pomace and cookies to inhibit activity of α-amylase, α-glucosidase and pancreatic lipase was determined according the to the procedures described previously in detail by Podsędek *et al.* [2014]. The starches from potato, *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl acetate were used as substrates in α-amylase inhibition, α-glucosidase inhibition and pancreatic lipase inhibition assays. The solutions of substrates were mixed with phosphate buffer saline (pH 6.9) and apple pomace or cookie extracts with different concentrations. After pre-incubating the samples at 37°C in a water bath for 5 min, the reactions were started by adding the enzyme solutions. The reactions were carried out at 37°C for 10, 15, 10 min in α-amylase inhibition, α-glucosidase inhibition and pancreatic lipase inhibition assays, respectively, and the absorbance was recorded at 540, 405, and 400 nm, respectively, using a spectrophotometer type UV-2401 PC (Shimadzu, Kyoto, Japan). The half maximal inhibitory concentration (IC_{50}) defined as the amount of inhibitor that is able to reduce the activity of a given enzyme by 50% was calculated and expressed as mg of apple pomace or cookies *per* mL of the reaction mixture under assay conditions.

Consumer evaluation

Consumer evaluation of shortbread cookies was performed using a 9-point hedonic scale. Each cookie variant was evaluated for five characteristics: colour, taste, odor, crispness, and overall acceptability [Land & Shepherd, 1988]. The study involved 62 participants who were informed about the purpose of the study. Each of them then received a questionnaire to record their own sensory perception. The scoring description was as follows: 1–definitely dislike, 2–very dislike, 3–dislike, 4–slightly dislike, 5–neither like or dislike, 6–slightly like, 7–like, 8–very like, 9–definitely like. The tests were conducted in a sensory laboratory, free of foreign odors, disturbing light and sound. Each participant in the consumer evaluation has given written consent to participate in the study. The study was based on the guidelines of the Declaration of Helsinki [World Medical Association, 2013]. The personal data of the participants of the organoleptic evaluation were coded in accordance with the guidelines of the General Regulation of the European Parliament on the Protection of Personal Data (GDPR 679/2016). Participants gave informed consent *via* the statement: "I am aware that my responses are confidential, and I agree to participate in this survey" where an affirmative reply was required to enter the survey. They were able to withdraw from the survey at any time without giving a reason. The products tested were safe for consumption. The research was approved by the Research Ethics Committee of the Wrocław University of Environmental and Life Sciences, Poland (no. 28/2023).

Statistical analysis

The data obtained were statistically analyzed using STATISTICA version 13.3 (StatSoft®; Tulsa, OK, USA). A one-way analysis of variance (ANOVA) and Tukey's multiple range test were performed with the significance level set at $p \leq 0.05$. Principal component analysis (PCA) and Spearman's correlation were also used. Data from three replicates were presented as mean and standard deviation.

RESULTS AND DISCUSSION

Nutritional value

Table 2 presents the nutritional value of the apple pomace and shortbread cookies. The energy value of cookies was reduced with increasing pomace addition – for cookies sweetened with sucrose, the decrease was 32.6 kcal/100 g (SA0 *vs.* SA50); for cookies sweetened with erythritol, 40.5 kcal/100 g (EA0 *vs.* EA50). The erythritol-sweetened cookies had a significantly lower energy value than their sucrose-sweetened counterparts. This was due to the difference in the energy value of the sweeteners used – sucrose, 4.0 kcal/g and erythritol, 0.2 kcal/g [Regnat *et al.*, 2018]. According to literature data, the dry matter content of wheat flour and apple pomace ranges from 85.7 to 92.4 g/100 g [Czaja *et al.*, 2020] and 20.0–24.5 g/100 g, respectively [Hang & Woodams, 1987; Villas-Bôas & Esposito, 2000]. In our study, the dry matter content of apple pomace was significantly higher and amounted to 95.54 g/100 g (**Table 2**). The differences are related to the fact that the other authors analyzed the composition of wet pomace, while dry apple pomace powder was used in our study. The fat and protein contents of the apple pomace powder were also different compared to the literature results reported for the dry matter of apple pomace – the fat content was 0.9–4.3 g/100 g d.m., while the protein content was 1.6–4.6 g/100 g d.m. [Hosseini & Pazhouhandeh, 2023; Yadav & Gupta, 2015]. The share of dry matter in apple pomace powder was similar to that of wheat flour; hence, the differences between cookie variants were small (**Table 2**). For fat content, the only source was butter, the use of which was the same in all variants of the cookies (**Table 1**). The slight differences in protein content of the tested cookies were due to differences in protein content between pomace and wheat flour (6.98 g/100 g and 11.00 g/100 g, respectively). The largest differences were observed for dietary fibre and ash contents which increased significantly with the increasing proportion of pomace in the cookies. This applied to cookies with both sweeteners. The dietary fibre content increased an approximately 8-fold from 2.79 (SA0) to 22.54 g/100 g (SA50), while the ash content ranged from 0.44 (EA0) to 0.73 g/100 g (SA50). Studies involving the addition of apple pomace to shortbread cookies were also conducted by Alongi *et al.* [2019]. The share of dietary fibre in apple pomace was lower and amounted to almost 40%; additionally, pomace was only added at levels of 10 and 20%. Despite the lower addition levels of pomace, the glycemic index of the tested cookies decreased by 5 and 10%, respectively, compared with those of products without the addition of pomace.

Erythritol was included in the total carbohydrate content; thus, carbohydrate share in individual cookies variants was similar and ranged from 55.49 (EA10) to 57.73 g/100 g (EA50) (**Table 2**).

Analysis of the nutritional value of cookies with apple pomace showed that they were characterized by a favorable nutritional profile in terms of the increasing content of dietary fibre, as well as a decreasing energy value with an increasing proportion of pomace and replacement of sucrose to erythritol.

Sugar content

The sugar content of the various types of cookies and apple pomace is presented in **Table 2**. Four sugars/polyols were identified: glucose, fructose, sucrose and erythritol. The largest quantities were found for sucrose and erythritol, which was due to their use as sweeteners in the shortbread cookies. Apple pomace and SA50 cookies also contained glucose at 4.96 and 1.03 g/100 g, respectively. The content of fructose in pomace was lower than that of glucose, but fructose was also identified in the cookies with a pomace content of 30% and 50%, sweetened with both sucrose and erythritol. SA50 products, compared to the other cookie variants, were characterized by a significantly higher total sugar content (20.12 g/100 g). The difference in total sugars between SA50 and EA50 was 3.93 g/100 g. The results reported by other authors showed that the content of glucose in apple pomace ranged from 2.5 to 12.4, and that of fructose from 18.0 to 31.0 g/100 g [Queji *et al.*, 2010]. Antonic *et al.* [2020] found that such discrepancies may result from, among other factors, the production process, harvest time and variety of apples.

Table 2. Nutritional profile, contents of ash, dry matter, sugars and minerals of apple pomace and shortbread cookies containing apple pomace.

	SA0	SA10	SA30	SA50	EA0	EA10	EA30	EA50	AP
Nutritional profile, ash and dry matter (*per* 100 g)									
Energy value (kcal)	519.72±5.17^{a}	496.82±6.08^{ab}	491.67±1.96^{ab}	487.09±9.99^{b}	426.26±11.36^{c}	414.82±8.00^{cd}	405.84±5.68^{cd}	385.78±10.71^{d}	114.81±1.57
Fat (g)	31.16±0.07^{ab}	31.42±0.03^{ab}	32.24±0.08^{a}	32.28±0.13^{a}	29.51±1.68^{b}	30.46±0.10^{ab}	31.49±0.08^{ab}	31.86±0.36^{ab}	nd
Protein (g)	8.41±0.07^{a}	7.50±0.24^{b}	7.25±0.12^{b}	6.81±0.24^{b}	6.81±0.24^{b}	6.81±0.24^{b}	6.81±0.24^{b}	5.26±0.00^{c}	6.98±0.24
Total carbohydrates (g)	57.51±0.05^{a}	57.57±0.56^{a}	56.74±0.25^{a}	57.37±0.42^{a}	56.66±1.48^{a}	55.49±0.13^{a}	57.19±0.12^{a}	57.73±0.38^{a}	87.06±0.29
Dietary fiber (g)	2.79±0.14^{d}	6.83±0.05^{c}	15.23±0.27^{b}	22.54±0.30^{a}	3.17±0.03^{d}	7.03±0.04^{c}	15.40±0.23^{b}	22.73±0.00^{a}	59.04±0.26
Dry matter (g)	97.51±0.03^{a}	97.01±0.02^{c}	96.86±0.04^{d}	97.19±0.03^{b}	93.43±0.03^{g}	93.26±0.00^{h}	96.09±0.03^{e}	95.53±0.00^{f}	95.54±0.03
Ash (g)	0.45±0.02^{d}	0.51±0.01^{c}	0.63±0.00^{b}	0.73±0.01^{a}	0.44±0.01^{d}	0.48±0.03^{c}	0.61±0.01^{b}	0.70±0.03^{a}	1.65±0.07
Sugar content (g/100 g)									
Glucose	nd	nd	nd	1.03±0.19^{a}	nd	nd	nd	nd	4.96±0.81
Fructose	nd	nd	0.86±0.09^{bc}	1.43±0.42^{ab}	nd	nd	0.27±0.00^{c}	1.75±0.16^{a}	2.08±0.61
Sucrose	15.04±1.29^{b}	15.17±0.12^{b}	14.09±0.80^{b}	17.66±0.83^{a}	nd	nd	0.25±0.06^{c}	0.63±0.27^{c}	2.60±0.41
Erythritol	nd	nd	nd	nd	13.59±0.30^{a}	13.98±0.03^{a}	12.87±0.96^{a}	13.80±0.51^{a}	nd
Total sugars	15.04±1.29^{b}	15.17±0.17^{b}	14.95±0.71^{b}	20.12±1.44^{a}	13.59±0.30^{b}	13.98±0.03^{b}	13.40±1.02^{b}	16.19±0.40^{b}	9.97±1.90
Mineral content (mg/100 g)									
Cu	0.19±0.05^{d}	0.24±0.04^{cd}	0.31±0.04^{bc}	0.42±0.04^{a}	0.20±0.03^{d}	0.24±0.04^{cd}	0.39±0.02^{ab}	0.39±0.07^{ab}	0.96±0.02
Mg	19.81±0.28^{d}	22.99±0.21^{c}	30.59±0.36^{b}	36.11±0.84^{a}	20.21±0.05^{d}	23.10±0.38^{c}	31.05±0.7^{b}	36.96±1.86^{a}	92.62±3.89
Mn	0.22±0.01^{d}	0.25±0.01^{d}	0.35±0.02^{c}	0.49±0.07^{a}	0.13±0.02^{e}	0.17±0.01^{e}	0.31±0.00^{cd}	0.43±0.02^{b}	1.43±0.11
Fe	1.70±0.03^{b}	2.37±0.46^{b}	4.04±0.06^{b}	12.95±9.42^{a}	1.28±0.16^{b}	1.92±0.19^{b}	5.76±1.37^{ab}	6.93±3.02^{ab}	19.59±0.89
Zn	0.74±0.03^{abc}	0.68±0.02^{bcd}	0.72±0.01^{abcd}	0.76±0.05^{ab}	0.66±0.07^{cd}	0.63±0.01^{d}	0.77±0.02^{ab}	0.82±0.06^{a}	0.85±0.19
Ca	42.08±2.21^{d}	55.74±0.98^{c}	76.57±1.40^{b}	93.67±2.97^{a}	40.66±0.89^{d}	52.40±0.34^{cd}	74.01±2.40^{b}	105.16±0.12^{a}	170.36±15.01
Na	13.13±0.51^{b}	20.80±7.20^{a}	18.11±0.31^{ab}	18.20±0.68^{ab}	17.63±0.53^{ab}	17.37±0.26^{ab}	17.37±0.17^{ab}	17.57±1.30^{ab}	17.83±0.96
K	68.13±1.63^{d}	81.11±1.80^{c}	108.79±2.83^{b}	128.01±2.01^{a}	70.77±0.84^{d}	81.12±0.48^{c}	110.39±1.03^{b}	127.38±4.27^{a}	348.05±16.17

Values (mean of three replications) ± standard deviation followed by the same letter (a, b, c,…), within the same row, are not significantly different ($p>0.05$; Tukey's test); apple pomace (AP) was not subjected to statistical analysis. SA; sucrose-sweetened shortbread cookies; EA, erythritol-sweetened shortbread cookies; 0, 10, 30 and 50 after EA and SA, percentage (by weight) of replacing wheat flour with apple pomace in the cookie recipe; nd, not detected.

In turn, based on multivariate analysis of the spectroscopic profile of the sugar fraction of apple pomace, Gabriel *et al.* [2013] showed a significant impact of apple variety on the sugar profile of apple pomace. It has also been shown that the sugar content of apples and therefore of apple pomace can vary depending on the position of the fruit in the tree canopy, with south-facing fruit having a lower sugar content due to increased respiration and oxidation of carbohydrates [Lazar *et al.*, 2009]. In the case of Golden Delicious apples, the sugar content was also influenced by the harvest date – the percentage of sucrose increased with later harvest dates, while the glucose content decreased [Núñez-Gastélum *et al*., 2015].

Mineral content

The content of selected minerals in the various variants of shortbread cookies and apple pomace is shown in **Table 2**. With the increase in pomace proportion in the recipe, the mineral content of baked goods increased. This relationship applied to all minerals except sodium. The minerals found in the highest content were potassium (from 68.13 mg/100 g for SA0 to 128.01 mg/100 g for SA50), calcium (from 40.66 mg/100 g for EA0 to 105.16 mg/100 g for EA50), magnesium (from 19.81 mg/100 g for SA0 to 36.96 mg/100 g for EA50) and iron (from 1.28 mg/100 g for EA0 to 12.95 mg/100 g for SA50). In most cases, no significant effect of the sweetener on the content of individual minerals was found ($p>0.05$). Similar conclusions were reached by Mir *et al.* [2017], who analyzed brown rice-based apple pomace biscuits and showed that the mineral content of crackers increased with apple pomace ratio increase in the formula. Also, the study of Er & Özcan [2010] reported the high content of Ca, Mg and K in pomace from different apple varieties, which was 8,420.50, 4,707.83 and 929.85 mg/kg, respectively.

The mineral content in the cookies differed from what would be expected based on the mineral content in apple pomace and the share of pomace in the recipe of individual cookie variants (**Table 2**). This may be because the process of baking cookies can affect the mineral content. Bredaliol *et al.* [2020], who examined the impact of baking conditions on the content of minerals in wheat bread, showed that as the baking temperature increased, the content of macro minerals decreased. However, taking into account the longer baking time, the losses of macro minerals were smaller. Baking at a temperature of 220°C and simultaneously extending the baking time from 15 to 20 min resulted in an increase in the content of the macro minerals. The parameters used in the initial baking phase most likely resulted in a more intense reduction of phytate content, which favored greater availability of macro minerals. The optimal temperature for phytase activity is 55°C; therefore, the degradation of phytic acid probably still occurred during baking (especially in the initial stage) due to the activation of phytase contained in the flour. This process promoted the reduction of phytates and thus increased the content of macro minerals. It should be noted, however, that baking parameters had a significant impact on the content of macro minerals, but were not significantly related to the content of micro minerals.

Identification of phenolic compounds

Identification of phenolic compounds was carried out with the UPLC-PDA-Q/TOF-MS method. The results are shown in **Table 3**. Five groups of phenolic compounds were identified in the tested cookie variants (with the addition of pomace): anthocyanins, flavonols, phenolic acids, flavan-3-ols and dihydrochalcones. The first identified fraction in the resulting products was that of anthocyanins, represented by two compounds: cyanidin 3-*O*-glucoside (t_R=4.77 min) and cyanidin 3-*O*-glucoside (t_R=4.28 min), both of them with $[M–H]^+$ at *m/z*=449 with an MS/MS fragment at *m/z*=287. The anthocyanins were previously detected in apples of different varieties with red-colored skin [Wojdyło *et al.*, 2008]. The next group of phenolic compounds identified in the cookies was that of compounds representing flavonols (**Table 3**), including: quercetin 3-*O*-rutinoside ($[M–H]^-$ at *m/z*=609; MS/MS fragment at *m/z*=301, and t_R=6.52 min), quercetin 3-*O*-galactoside ($[M–H]^-$ at *m/z*=463; MS/MS fragment at m/z=301, and t_R=6.58 min), quercetin 3-*O*-glucoside ($[M–H]^-$ at *m/z*=463; MS/MS fragment at *m/z*=301, and t_R=6.73 min), quercetin 3-*O*-xyloside ($[M–H]^-$ at *m/z*=433; MS/MS fragment at *m/z*=301, and t_R=7.00 min), quercetin hexoside ($[M–H]^-$ at m/z=463; MS/MS fragment at *m/z*=301, and t_R=7.34 min), and quercetin 3-*O*-rhamnoside ($[M–H]^-$ at *m/z*=447; MS/MS fragment at m/z=301, and t_R=7.55 min). The flavonols identified in this study were consistent with the flavonol profiles reported by other authors studying apple pomace [Ćetković *et al.*, 2008; Gumul *et al.*, 2021]. Phenolic acids of cookies with apple pomace were represented by chlorogenic acid (t_R=3.96 min) (**Table 3**). Also, two dihydrochalcones – phloretin 2'-*O*-xyloglucose ($[M–H]^-$ at *m/z*=567; MS/MS fragment at *m/z*=273, and t_R=7.66 min) and phloretin 2'-*O*-glucose ($[M–H]^-$ at *m/z*=435; MS/MS fragment at *m/z*=273, and t_R=8.40 min) – and flavan-3-ols were determined. The latter fraction consisted of procyanidins B_1 and B_2 with $[M–H]^-$ at *m/z*=577; MS/MS fragment at *m/z*=289, and t_R=2.62 min, and t_R=1.81 min, respectively. Chlorogenic acid, phloretin glycosides and procyanidins were previously determined in apple pomace and bakery/confectionary products with apple pomace [Gorjanović *et al*., 2020; Gumul *et al.*, 2021; Kruczek *et al*., 2023]. Among the important bioactive compounds, procyanidin B_2 deserves special attention, as it exhibits a significant antioxidant activity and inhibits the oxidation of the low-density lipoprotein (LDL) cholesterol [Xiao *et al.,* 2020]. Chlorogenic acid is another compound worthy of note for its anti-cancer potential [Kasai *et al.*, 2000]. In turn, quercetin elicits a preventive effect in the development of hormone-dependent cancers and cardiovascular diseases [Gormaz *et al.*, 2015; Zand *et al.,* 2002].

Content of phenolic compounds

The content of phenolic compounds in individual variants of cookies and apple pomace is presented in **Table 4**. Flavonols (44.4%) and flavan-3-ols (37.8%) had the highest share of the total content of phenolic compounds of apple pomace. Considerably fewer compared to flavonols and flavan-3-ols were dihydrochalcones (10.0%), phenolic acids (4.8%) and anthocyanins (3.1%). The content of each phenolic compound in cookies increased

Table 3. Phenolic compounds identified with ultra-performance liquid chromatography with a photodiode detector-quadrupole/time-of-flight mass spectrometry (UPLC-PDA-Q/TOF-MS) in shortbread cookies containing apple pomace.

Group of phenolics	Compound	t_R (min)	λ_{max} (nm)	Parent ion* (*m/z*)	MS/MS ion (*m/z*)
Anthocyanin	Cyanidin 3-*O*-glucoside	4.77	520	449+	287+
	Cyanidin 3-*O*-galactoside	4.28	519	449+	287+
Flavonol	Quercetin 3-*O*-rutinoside	6.52	352	609	301
	Quercetin 3-*O*-galactoside	6.58	355	463	301
	Quercetin 3-*O*-glucoside	6.73	350	463	301
	Quercetin 3-*O*-xyloside	7.00	350	433	301
	Quercetin hexoside	7.34	357	463	301
	Quercetin 3-*O*-rhamnoside	7.55	345	447	301
Phenolic acid	Chlorogenic acid	3.96	320	353	191
Flavan-3-ol	Procyanidin B_2	1.81	280	577	289/245
	Procyanidin B_1	2.62	280	577	289/245
Dihydrochalcone	Phloretin 2′-*O*-xyloglucose	7.66	285	567	273
	Phloretin 2′-*O*-glucose	8.40	285	435	273

*$[M–H]^+$ for anthocyanins (positive-ion mode) and $[M–H]^-$ for other phenolic compounds (negative-ion mode). t_R, retention time; λ_{max}, absorption maximum of PDA UV spectrum.

with the increasing share of pomace in their recipe, in the products sweetened with either sucrose or erythritol. The total content of phenolic compounds determined in apple pomace was 2515.35 mg/kg, which was higher compared to the value of 89.4 mg gallic acid/100 g d.m. determined by Gumul *et al.* [2021]. Also, Leyva-Corral *et al.* [2016] reported a different content of these compounds, reaching 324.2 mg gallic acid/100 g d.m. Both literature values were obtained *via* the colorimetric method, which may explain the differences from the values in our studies. Analysis of apple pomace conducted by Oszmiański *et al.* [2011] showed a quite high content of polymeric procyanidins and flavan-3-ols, dihydrochalcones and flavonols. Polymeric procyanidins, dihydrochalcones and flavonols accounted for 57.0%, 5.5%, 3.0% of total phenolics in pomace, respectively.

The share of individual phenolic compounds from the group of flavonols in apple pomace and cookies with their addition was as follows: quercetin 3-*O*-galactoside > quercetin 3-*O*-rhamnoside > quercetin hexoside > quercetin 3-*O*-xyloside > quercetin 3-*O*--glucoside > quercetin 3-*O*-rutinoside (**Table 4**). The most abundant flavonol turned out to be quercetin 3-*O*-galactoside, with its content in cookies ranging from 17.35 (EA10) to 108.66 mg kg (SA50). The cookies with 50% pomace addition had about 6–8 times higher flavonol content compared to the variants with 10% pomace addition. The flavonol content of the apple pomace powder was 1313.84 mg/kg. This value was close to the result shown by Kruczek *et al.* [2023] (103.19 mg/100 g d.m.), although Ćetković *et al.* [2008] and Gumul *et al.* [2021] determined a lower content of quercetin glycosides (28.6–61.0 and 73.14 mg/100 g d.m., respectively). Nevertheless, as in our studies, literature data indicate quercetin 3-*O*-galactoside as the major flavonol of apple pomace [Gorjanović *et al.*, 2020; Gumul *et al.*, 2021; Kruczek *et al.*, 2023].

The most abundant flavan-3-ol, both in the pomace and in individual variants of cookies, was procyanidin B_2 (**Table 4**). Its content in cookies ranged from 52.45 (EA10) to 365.20 mg/kg (SA50) and in apple pomace it was 372.28 mg/kg. Procyanidin B_1 was present in smaller amounts – from 4.47 (EA10) to 38.04 mg/kg (SA50) in cookies and 231.09 mg/kg in apple pomace. According to earlier experimental studies, the content of procyanidin B_2 in apple pomace was lower and ranged from 2.61 to 16.00 mg/100 g d.m. [Gumul *et al.*, 2021; Schieber *et al.*, 2001].

The content of phloretin 2′-*O*-glucose ranged from 6.69 (SA10) to 43.48 mg/kg (SA50) and that of phloretin 2′-*O*-xyloglucose – from 3.46 (EA30) to 13.21 mg/kg (SA50) (**Table 4**). The presence of phloretin 2′-*O*-glucose in apple pomace has been previously reported by other authors within a broad content range from 0.7 to 18.0 mg/100 g d.m. [Ćetković *et al.*, 2008; Gumul *et al.*, 2021; Leyva-Corral *et al.*, 2016].

The phenolic compounds found in the lowest amounts were chlorogenic acid and anthocyanins (**Table 4**). The content of chlorogenic acid in cookies ranged from 3.60 (SA10) to 27.95 mg/100 g (SA50) and in apple pomace was 154.15 mg/100 g. Other authors determined chlorogenic acid in apple pomace as a major phenolic acid, with its content ranging from 8.2 to 41.6 mg/100 g d.m. [Ćetković *et al.*, 2008; Gorjanović *et al.*, 2020; Kruczek *et al.*, 2023; Leyva-Corral *et al.*, 2016].

The anthocyanin content of apple pomace was relatively low (**Table 4**). These polyphenolic compounds are found in red-colored apple varieties [Wojdyło *et al.*, 2008]. In our study, we used a commercially available pomace powder. According to the manufacturer's declaration, it was obtained from a by-product after squeezing juice from different varieties of apples with different fruit colors.

Table 4. Content of phenolic compounds (mg/kg) in apple pomace and shortbread cookies containing apple pomace.

Compound	SA0	SA10	SA30	SA50	EA0	EA10	EA30	EA50	AP
Cyanidin 3-*O*-glucoside	nd	1.85±0.09^{b}	5.39±0.29^{ab}	9.28±0.57^{a}	nd	2.35±0.11^{b}	8.17±2.56^{a}	9.43±0.35^{a}	73.82±6.20
Cyanidin 3-*O*-galactoside	nd	nd	2.99±0.22^{a}	4.49±1.52^{a}	nd	nd	nd	4.03±0.95^{a}	35.60±2.02
Quercetin 3-*O*-rutinoside	nd	nd	7.10±0.14^{c}	14.19±1.65^{a}	nd	2.25±0.33^{d}	10.68±0.22^{b}	12.24±1.23^{ab}	47.74±4.18
Quercetin 3-*O*-galactoside	nd	17.41±0.75^{c}	55.27±5.17^{b}	108.66±2.24^{a}	nd	17.35±1.28^{c}	93.40±13.87^{a}	93.98±1.43^{a}	501.55±6.04
Quercetin 3-*O*-glucoside	nd	nd	9.88±0.38^{b}	19.97±0.37^{a}	nd	3.18±0.39^{c}	16.93±2.57^{a}	18.09±0.32^{a}	97.85±0.07
Quercetin 3-*O*-xyloside	nd	nd	11.45±0.76^{b}	24.75±0.68^{a}	nd	2.98±0.23^{c}	21.61±3.46^{a}	22.42±0.23^{a}	135.10±9.03
Quercetin hexoside	nd	7.19±0.04^{c}	23.95±0.71^{b}	47.35±0.94^{a}	nd	6.15±0.36^{c}	41.15±5.56^{a}	41.28±0.87^{a}	261.69±15.88
Quercetin 3-*O*-rhamnoside	nd	8.11±0.05^{c}	27.09±1.65^{b}	54.56±1.64^{a}	nd	6.44±0.45^{c}	46.36±6.05^{a}	45.93±1.73^{a}	269.91±19.02
Chlorogenic acid	nd	3.60±0.35^{d}	13.17±1.16^{c}	27.95±0.28^{a}	nd	3.88±0.15^{d}	19.68±1.12^{b}	23.52±1.99^{b}	154.15±0.80
Procyanidin B_2	nd	69.55±1.05^{d}	208.79±9.99^{c}	365.20±0.04^{a}	nd	52.45±4.00^{d}	231.84±10.88^{c}	295.38±15.63^{b}	372.28±8.26
Procyanidin B_1	nd	5.90±0.62^{d}	16.42±3.21^{c}	38.04±2.77^{a}	nd	4.47±0.91^{d}	23.85±2.21^{bc}	26.51±1.52^{b}	231.09±26.90
Phloretin 2′-*O*-xyloglucose	nd	nd	5.91±0.82^{b}	13.21±0.96^{a}	nd	nd	3.46±0.00^{c}	3.48±0.22^{c}	17.72±0.47
Phloretin 2′-*O*-glucose	nd	6.69±0.10^{c}	22.11±2.93^{b}	43.48±1.16^{a}	nd	6.97±1.17^{c}	41.88±3.25^{a}	42.37±3.46^{a}	290.67±8.59
Total phenolic compounds	**nd**	**120.30±2.30^{d}**	**409.51±13.50^{c}**	**771.15±2.13^{a}**	**nd**	**108.46±7.93^{d}**	**560.56±26.47^{b}**	**637.09±8.85^{b}**	**2,515.35±34.56**

Values (mean of three replications) ± standard deviation followed by the same letter (a, b, c,…), in the same row, are not significantly different (p>0.05; Tukey's test); apple pomace (AP) was not subjected to statistical analysis. SA, sucrose-sweetened shortbread cookies; EA, erythritol-sweetened shortbread cookies; 0, 10, 30 and 50 after EA and SA, percentage (by weight) of replacing wheat flour with apple pomace in the cookie recipe; nd, not detected.

The sweetener used was found not to affect the content of phenolic compounds in the individual variants of the products.

Antioxidant capacity

Two methods were used to determine the antioxidant capacity of shortbread cookies and apple pomace – ABTS and ORAC assays. The results of both determinations showed that the antioxidant capacity of cookies increased significantly with the increase in pomace share in their recipe (**Table 5**). This applied to the cookies with both sweeteners. $ABTS^{\bullet+}$ scavenging activity ranged from 0.022 (EA0) to 0.363 mmol TE/100 g (SA50) and ORAC from 1.116 (EA0) to 2.253 mmol TE/100 g (SA50). In most cases, cookies sweetened with sucrose were characterized by significantly higher antioxidant capacity than those sweetened with erythritol. This was most likely due to the fact that sucrose, unlike erythritol, participates in Maillard reactions. The antioxidant properties of processed foods exposed to high temperatures also result from the formation of compounds during the Maillard reaction or heating of reducing sugars for example, caramelization products, Amadori compounds, reactive reductones, premelanoidins, and melanoidins [Billaud *et al.*, 2003]. Michalska *et al.* [2008] studied the influence of baking rye bread on the formation of Maillard reaction products and their antioxidant activity. They showed that the Maillard reaction products formed during baking, mainly melanoidins, were able to scavenge the peroxyl radicals and ABTS cation radicals and reduce the Folin-Ciocalteu reagent. Also, the study conducted by Patrignani *et al.* [2016] on cookies showed that the Maillard reaction products, apart from their key role in imparting color and flavor to baking products, had antioxidant properties. Therefore, the higher antioxidant capacity of cookies determined in our study and associated primarily with a higher proportion of pomace (and thus a higher content of bioactive compounds) could also result from the formation of Maillard reaction products upon high-temperature baking. Moreover, Žilić *et al.* [2016] showed that the highest antioxidant activity was determined in cookies with the addition of ammonium bicarbonate, which proves that the Maillard reaction proceeds at an increased rate under alkaline conditions, resulting in an increase in antioxidant activity.

In order to understand the relationship between antioxidant capacity of cookies and phenolic compound contents, a correlation analysis was carried. For each group of phenolic compounds, the correlations were positive and significant with the value of correlation coefficient (r) much higher than 0.6 (**Table S1** in Supplementary Materials). For $ABTS^{\bullet+}$ scavenging activity, the strongest correlation was shown with chlorogenic acid (r=0.9952) and for ORAC with phloretin 2′-*O*-glucose (r=0.9929).

Gumul *et al.* [2021] conducted studies aimed at evaluating the health-promoting properties of gluten-free bread prepared with the addition of apple pomace. They showed that the 5% pomace addition contributed to a 66-fold increase in antioxidant activity. In our research, the $ABTS^{\bullet+}$ scavenging activity of cookies with 10% pomace increased almost 4 times compared to the cookies without apple pomace (**Table 5**). This concerned cookies with both sucrose and erythritol. For ORAC, the increase was 1.5 times for the sucrose-sweetened cookies and 1.2 times for the erythritol-sweetened ones. These results were in agreement with the study of Zlatanović *et al.* [2019] who reported that the addition of apple pomace to cookies contributed to a 3–5.5-fold increase in antioxidant activity compared with the control sample. The lower antioxidant activity of products sweetened with erythritol than with sucrose was previously found by Nowicka & Wojdyło [2016], who checked, among others, the stability of polyphenols and antioxidant properties of cherry puree prepared with the addition of various sweeteners.

Table 5. Antioxidant capacity measured as $ABTS^{\bullet+}$ scavenging activity (ABTS assay) and oxygen radical absorption capacity (ORAC), and α-amylase, α-glucosidase and pancreatin lipase inhibitory activity of apple pomace and shortbread cookies containing apple pomace.

Cookie variant/apple pomace	ABTS assay (mmol TE/100 g)	ORAC (mmol TE/100 g)	α-Amylase inhibitory activity	α-Glucosidase inhibitory activity	Pancreatin lipase inhibitory activity
			IC_{50} (mg/mL)		
SA0	0.032±0.004^{f}	1.153±0.065^{e}	221.8±4.00^{a}	1374.9±7.9^{a}	16.28±0.88^{a}
SA10	0.116±0.008^{d}	1.697±0.041^{c}	20.3±2.8^{d}	1149.3±8.6^{d}	16.84±0.86^{a}
SA30	0.236±0.005^{c}	1.939±0.018^{b}	13.1±1.4^{de}	1106.8±7.9^{d}	11.18±1.11^{bc}
SA50	0.363±0.005^{a}	2.253±0.052^{a}	<0.5±0.0^{e}	931.3±9.5^{f}	7.97±0.32^{c}
EA0	0.022±0.001^{f}	1.116±0.058^{e}	202.3±4.9^{ab}	1202.6±3.9^{c}	16.76±1.22^{a}
EA10	0.082±0.002^{e}	1.353±0.037^{d}	198.7±1.4^{b}	1287.3±4.7^{b}	13.04±0.76^{ab}
EA30	0.260±0.013^{c}	1.985±0.012^{b}	186.4±5.8^{b}	1131.2±9.4^{d}	9.92±1.05^{bc}
EA50	0.316±0.009^{b}	2.070±0.038^{b}	138.1±3.6^{c}	976.4±7.4^{e}	7.26±0.56^{c}
AP	1.715±0.065	8.760±0.171	26.4±1.1	395.5±4.5	2.28±0.32

Values (mean of three replications) ± standard deviation followed by the same letter (a, b, c,…), within the same column, are not significantly different (p>0.05; Tukey's test); apple pomace (AP) was not subjected to statistical analysis. TE, Trolox equivalent; IC_{50}, half maximal inhibitory concentration; SA, sucrose-sweetened shortbread cookies; EA, erythritol-sweetened shortbread cookies; 0, 10, 30 and 50 after EA and SA, percentage (by weight) of replacing wheat flour with apple pomace in the cookie recipe.

Inhibition of α-amylase, α-glucosidase and pancreatic lipase activity

The next stage of the study aimed to determine the ability of apple pomace and individual variants of cookies to inhibit the activity of α-amylase, α-glucosidase and pancreatic lipase, and respective data are shown in **Table 5**. Extracts obtained from cookies with apple pomace addition at various levels were tested at different concentrations. This allowed calculating the IC_{50} value (mg/mL). Enzymes such as α-amylase and α-glucosidase play a key role in the digestion of carbohydrates; hence, the inhibition of their activity plays a significant role in diabetes control [Tundis *et al.*, 2010]. Pancreatic lipase catalyzes the conversion of fat molecules into vitamins A, D, E, K, fatty acids and salts of bile acids [Lunagariya *et al.*, 2014]. For each enzyme, it was shown that the increasing addition of pomace in the cookie recipe was associated with an increased inhibition of its activity (lower IC_{50} values) (**Table 5**). The exception was α-glucosidase inhibitory activity of EA10 cookies, for which significantly ($p \leq 0.05$) higher IC_{50} values were obtained compared to the EA0 variant. The difference was only 84.7 mg/mL. Perhaps the addition of 10% pomace was insufficient to induce an inhibitory effect on α-glucosidase. For α-amylase inhibitory activity, the IC_{50} ranged from <0.5 (SA50) to 221.8 mg/mL (SA0). The ability of SA50 cookies to inhibit α-amylase activity was more than 400 times higher than that of cookies without added pomace (SA0). For α-glucosidase inhibitory activity, IC_{50} values ranged from 931.3 (SA50) to 1,374.9 mg/mL (SA0), and for lipase from 7.26 (EA50) to 16.76 mg/mL (SA10). It was also observed that the higher the addition of apple pomace was, the higher was the α-amylase, α-glucosidase, and pancreatin lipase inhibitory activity of cookies. Additionally, it was observed that shortbread cookies prepared with erythritol elicited significantly greater effect on the activity of α-amylase and α-glucosidase than the sucrose-sweetened ones. From the data presented in **Table 5**, it appears that the main factor influencing the increase in enzyme inhibition was the increasing proportion of apple pomace in cookie formula

Results of correlation analysis (**Table S1** in Supplementary Materials) showed that certain phenolic compounds, such as phloretin 2′-*O*-xyloglucose (r=–0.6220) and procyanidin B_2 (r=–0.5934), significantly affected the α-amylase inhibitory activity of the cookies. Significant correlations were found between α-glucosidase inhibitory activity of the cookies and contents of selected phenolic compounds, including quercetin 3-*O*--rutinoside (r=–0.4385), phloretin 2′-*O*-xyloglucose (r=–0.4289), quercetin 3-*O*-glucoside (r=–0.4198), and quercetin 3-*O*-xyloside (r=–0.4119). Phenolic compounds that had the greatest effect on lipase inhibition were procyanidin B_2 (r=–0.8841), quercetin 3-*O*--rutinoside (r=–0.8608), phloretin 2′-*O*-xyloglucose (r=–0.8375), quercetin 3-*O*-galactoside (r=–0.8262), and quercetin 3-*O*-glucoside (r=–0.8250).

The phenolic compounds contained in apples play a key role in the treatment of diabetes. One of the mechanisms is the inhibition of the activity of α-amylase and α-glucosidase, which reduces the rate of carbohydrate digestion. In addition, flavonoids have been shown to exert the anti-diabetic effect, affecting obesity, diabetes, insulin resistance, hyperglycemia, and hyperlipidemia [Alkhalidy *et al.*, 2018]. However, there are no results in the available literature showing the extent of inhibition of the aforementioned enzymes by sweet snack foods with apple pomace added. Researchers have focused more often on the technological properties of these types of products and sensory sensations after their consumption [Lyu *et al.*, 2020].

Consumer evaluation

The results of the consumer evaluation of the individual variants of cookies are shown in **Figure 1**. Products were rated for color, taste, odor, crispness and overall acceptability. According to the participants, the highest scores were given to SA0 and EA0 (overall acceptability 7.85 and 7.98, respectively). This can be attributed to the known characteristics of commonly consumed shortbread cookies. It was shown that the higher the addition of pomace was, the lower was the acceptance by potential consumers, but even with the 50% pomace addition the overall acceptability was at 5.76 (SA50) and 6.24 (EA50). The high scores may be related to the fact that apple pomace is a potential flavoring ingredient for short-crust pastry, which certainly affects its acceptance [Sudha *et al.*, 2007]. Studies aimed to evaluate apple pomace cookies were also conducted by Sudha *et al.* [2007], but in this case the maximum pomace addition was 30%. Other authors also evaluated the effect of apple pomace addition to bakery and confectionery products in terms of consumer acceptance [Sudha *et al.*, 2007, Usman *et al.*, 2020]. Products with 20% pomace addition usually had a pleasant apple taste and were well accepted by the panelists [Sudha *et al.*, 2007]. However, with the increase in pomace share in the cookie formula, their sensory characteristics were more often decreased; as low to moderate acceptability of cookies with 30%–50% pomace was reported [Usman *et al.*, 2020].

Interestingly, for each variant of cookies and each distinguishing feature, the use of erythritol instead of sucrose had a positive impact on the panelists' assessment (the difference in assessment between variants of cookies sweetened with sucrose and erythritol, depending on the distinguishing feature, ranged from 0.13 for the cookies without added pomace for overall acceptability to 1.08 for the products with 30% added pomace for taste) (**Figure 1**). A study by Laguna *et al.* [2013] led to different results, showing that replacing 50% sucrose with erythritol in shortbread cookies negatively affected consumer acceptance. These differences may be due to the fact that product analyzed in the present study were made with fruit pomace. The use of erythritol could affect the achievement of a cooling effect, which eliminated the aftertaste of the pomace.

Principal component analysis

Principal component analysis (PCA) was used to summarize the relationship between the variables (nutritional, bioactivity and sensory properties) and show the effect of apple pomace and sweetener addition in the design of a new snack product (**Figure 2**). The determined probability of the occurring dependencies

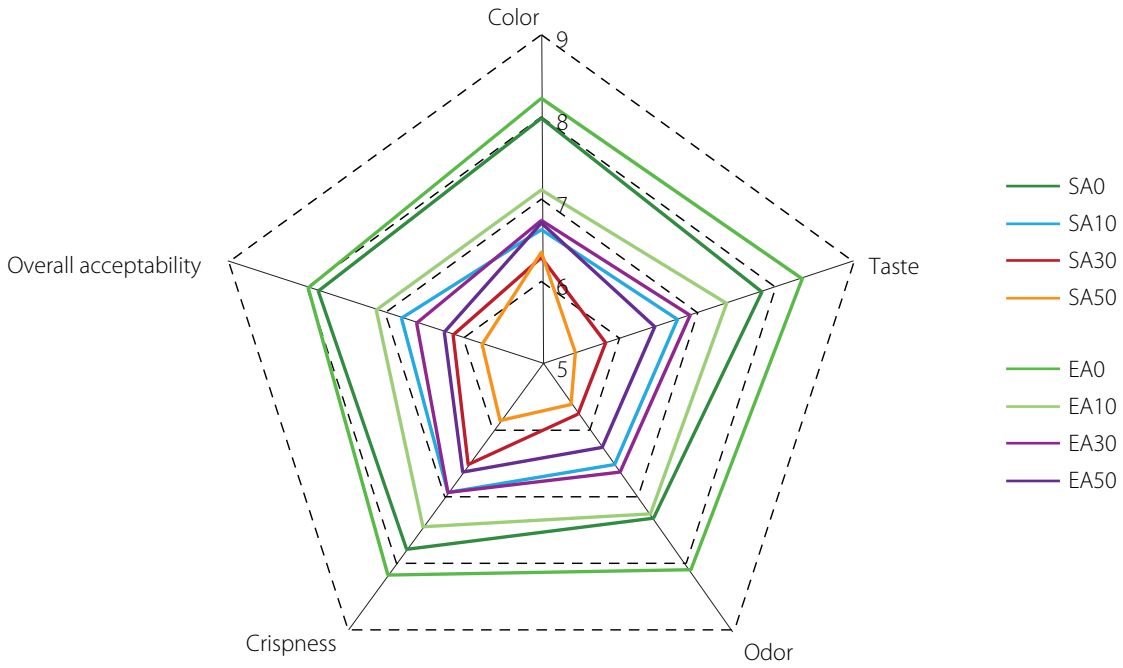

Figure 1. Consumer evaluation of shortbread cookies containing apple pomace. SA, sucrose-sweetened shortbread cookies with specific addition of apple pomace; EA, erythritol-sweetened shortbread cookies with specific addition of apple pomace; 0, 10, 30 and 50 after EA and SA, percentage (by weight) of replacing wheat flour with apple pomace in the cookie recipe.

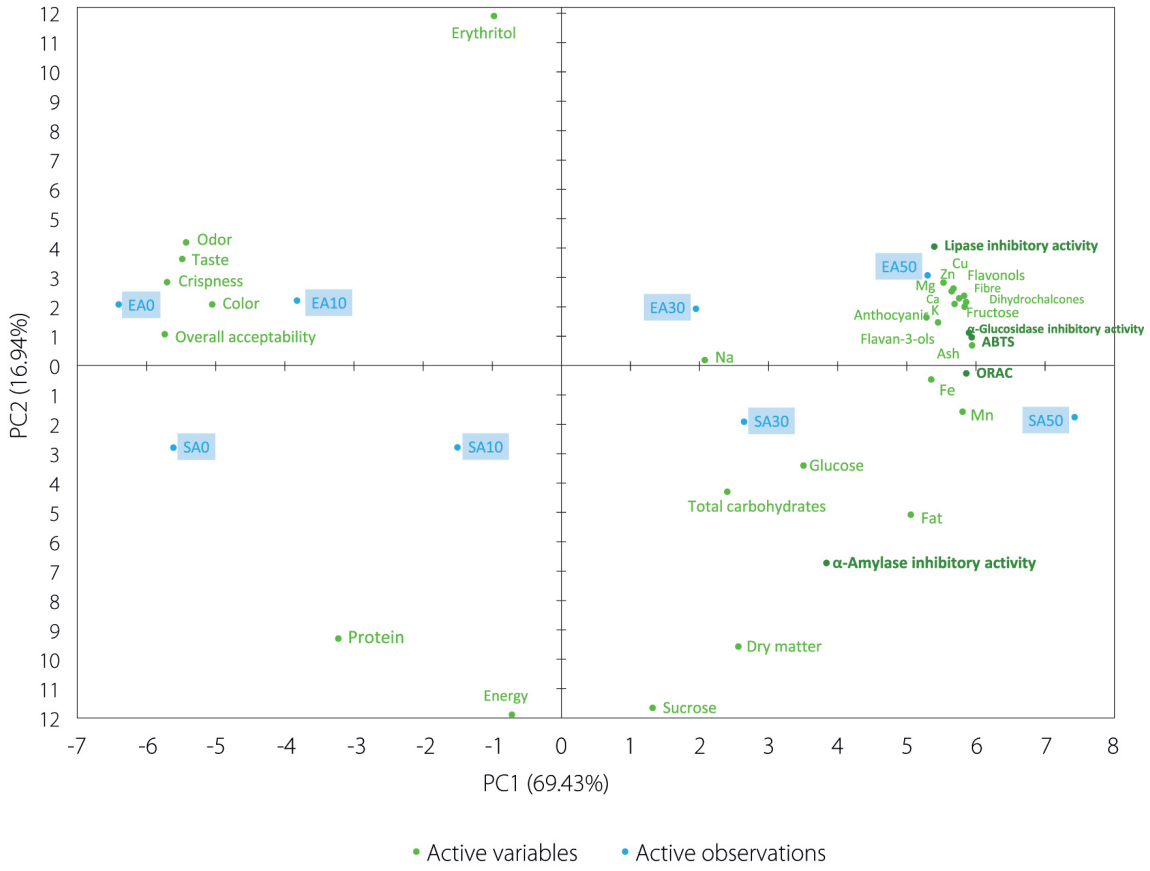

Figure 2. Principal component analysis (PCA) of the impact of erythritol/sucrose and apple pomace in cookies recipe on nutritional, bioactive and sensory parameters of cookies. SA, sucrose-sweetened shortbread cookies with specific addition of apple pomace; EA, erythritol-sweetened shortbread cookies with specific addition of apple pomace; 0, 10, 30 and 50 after EA and SA, percentage (by weight) of replacing wheat flour with apple pomace in the cookie recipe; d.m., dry matter.

(PC1 *vs.* PC2) was very high – 86.37%. Eigenvalues for PCA are shown in **Table S2** and **Figure S1** in Supplementary Materials. Both the addition of a sweetener (erythritol/sucrose) and the pomace had a significant effect on the active observation ranking of the cookies studied (**Figure 2**). The analyzed active observations were arranged opposite along the horizontal axis in the same order and distance. The acceptability of the analyzed sensory traits of products with erythritol and a low content of apple pomace (EA10) and without pomace (EA0) was higher compared to the other analyzed products. The highest addition of pomace was the least acceptable in this evaluation. In addition, a very strong relationship was observed between active observations (EA50 and SA50) and active variables, such as phenolic compounds (flavonols, dihydrochalcones, anthocyanins, flavan-3-ols), minerals (Zn, Cu, Mg, Ca, Na, Fe, Mn, K), antioxidant capacity (ABTS$^{\bullet+}$ scavenging activity and ORAC) and α-glucosidase inhibitory activity. Weaker impacts were measured between phenolic compounds or minerals and lipase inhibitory activity or α-amylase inhibitory activity. This was also confirmed by the correlation analysis, whose results are presented in **Table S1** in Supplementary Materials. In turn, PCA analysis showed that flavan-3-ols, anthocyanidins and dihydrochalcones exerted a stronger effect on α-glucosidase inhibitory activity and ABTS$^{\bullet+}$ scavenging activity than flavonols. By contrast, the activity of lipase inhibitory activity was influenced not only by flavonols but also by the presence of dietary fibers, and was unaffected by total carbohydrate or fat, and in the case of α-amylase inhibitory activity, the relationship was proportionally inverse. Protein content had no significant impact on the health-promoting potential of the analyzed products. It should be noted that the energy value was negatively correlated with the presence of erythritol and positively correlated with the content of sucrose.

CONCLUSIONS

The study compared 8 variants of shortbread cookies prepared with different addition levels of apple pomace and sucrose/erythritol. The most beneficial properties were obtained for the products with 50% added pomace. The content of dietary fiber increased about 8 times compared to the products without pomace addition, while the energy value decreased. Thirteen polyphenolic compounds, including quercetin glycosides, phloretin glycosides, cyanidin glycosides, procyanidins and chlorogenic acid, were identified in the cookies with added pomace. The antioxidant capacity and antidiabetic properties of cookies increased significantly as the share of pomace increased in cookie formula. The highest consumer acceptability was shown for the cookies without added pomace, but the cookies with 50% added pomace received relatively high scores. The highest scores given by panelists to SA0 and EA0 cookies result from the fact that the sensory values of traditional shortbread cookies (without the addition of other raw materials apart from the basic ones: wheat flour, butter, sweetener, egg yolks) are widely known and accepted by consumers. This is most likely due to a lack of a habit or an experience in consuming products with the addition of raw materials featuring functional properties. For each cookie variant, replacing sucrose with erythritol favorably influenced the consumer assessment. The study demonstrated that it was feasible to develop recipes of cookies that will offer high health benefits and at the same time will be accepted by consumers, which inscribes into a recent global trend that focuses on developing sustainable food formulas offering the mentioned values. Therefore, in the future, the possibilities of using fruit pomace should be expanded to minimize the problem of disposing its excess in an environmentally friendly way, while at the same time producing food of a high nutritional value and health quality, by incorporating this valuable functional ingredient into recipes.

ADDITIONAL INFORMATION

The publication was the result of the activity A.W. and P.N. as research group of "Plants4Food".

RESEARCH FUNDING

This research was funded by the National Science Centre, Poland, grant number DEC-2020/04/X/NZ9/00656 (MINIATURA 4).

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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SUPPLEMENTARY MATERIALS

The following are available online at **http://journal.pan.olsztyn.pl/Effect-of-the-Addition-of-Apple-Pomace-and-Erythritol-on-the-Antioxidant-Capacity,187941,0,2.html**; **Table S1**. Spearman correlation analysis (*r*).; **Table S2**. Eigenvalues for principal component analysis.; **Figure S1**. Plot of eigenvalues and cumulative variability of principal component analysis.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 162–168
DOI: 10.31883/pjfns/188110
ISSN 1230-0322, e-ISSN 2083-6007

High-Fiber Crackers Supplemented with Asparagus Hard-Stem: Impacts of Supplementation Ratios and Water Amounts in Cracker Recipe on the Product Quality

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Spears or cladophylls are edible parts of asparagus (*Asparagus officinalis* L.) implemented in a human diet while its hard-stem by-products have been used for animal feeding. In this study, the asparagus hard-stem was proved to be rich in dietary fiber and total phenolics with high antioxidant capacity. Wheat flour was partially replaced by asparagus hard-stem powder (AHP) in the cracker recipe and the AHP ratios were 0 (control), 5, 10, 15, 20% of the blend weight; the nutritional constituents, antioxidant capacities, physical attributes, and sensory overall acceptability of crackers were then evaluated. As the AHP ratio increased from 0 to 20%, the dietary fiber and total phenolic contents of the fortified crackers were improved by 5.0 times and 3.2 times, respectively, while their ferric reducing antioxidant power and DPPH scavenging capacity were enhanced by 6.1 and 1.4 times, respectively. Besides, the elevated ratio of AHP also increased the product hardness and reduced its overall acceptability. The impacts of water amount used in the dough kneading on the high-fiber cracker quality were then investigated. At 20% AHP level, the appropriate water amount was 55 g/100 g of the flour blend to reduce hardness and improve overall acceptability of the fortified crackers. The study results show that AHP is a potential dietary fiber and antioxidant ingredient for high-fiber cracker making.

Keywords: antioxidant capacity, *Asparagus officinalis* L., color parameters, consumer acceptability, dietary fiber, hardness

INTRODUCTION

Dietary fiber is generally known as edible parts of plants, including polysaccharides and lignin, that are resistant to enzymatic digestion in the human intestine [Prosky, 2000]. Its frequent intake exerts various positive effects on reducing cholesterol levels, blood pressure, incidence of cardiovascular diseases and some types of intestinal problems. Additionally, food antioxidants play an essential role in promoting human immune system and preventing free radical-related diseases [Brambilla *et al.*, 2008]. Dietary fiber and antioxidants from a variety of plant sources have been added to various food products such as bakery [Salehi & Aghajanzadeh, 2020] and pasta products [Bianchi *et al.*, 2021; Nguyen *et al.*, 2023; Ta *et al.*, 2023] to enhance their health-promoting properties.

Crackers are a common type of bakery products around the world due to low price, ease of consumption, and long shelf-life [Tiwari *et al.*, 2023]. Wheat crackers are rich in starch but poor in dietary fiber and antioxidants; as a result, their nutritional composition is not well balanced [Ujong *et al.*, 2023]. Recently, crackers have been supplemented with dietary fiber

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Submitted: 8 February 2024
Accepted: 29 April 2024
Published on-line: 17 May 2024

and antioxidants from by-product flours of the agri-food industry to improve their health effects [Chatziharalambous *et al.*, 2023].

Asparagus (*Asparagus officinalis* L.) is a widely known vegetable which contains flavonoids and other phenolic compounds with strong antioxidant activity [Nindo *et al.*, 2003]. In agricultural production, the harvested asparagus spear accounts for about 20-25% of the total weight of the trees, while the by-products including hard-stem leftovers and roots account for 70-75% [Chitrakar *et al.*, 2019]. High levels of dietary fibers and phenolics are reported in asparagus hard-stem [Nielsen, 2010], but the use of this by-product for food formulation has not been considered.

In the present study, various ratios of asparagus hard-stem powder (AHP) were added to the cracker recipe to improve dietary fiber content and antioxidant capacity of the fortified crackers. The aim of the research was to clarify the impacts of AHP addition on the nutritional composition, antioxidant capacities, physical attributes and sensory acceptability of crackers. The effects of water amounts used in the recipe of high-fiber crackers on their textural and sensory quality were examined as well.

MATERIALS AND METHODS

Materials and chemicals

Asparagus hard-stems were collected from an asparagus (*Asparagus officinalis* L.) field in Cu Chi district, Ho Chi Minh City. The collected asparagus hard-stems were washed with municipal water, sliced to 5-mm thick pieces, and dried at 50°C for 2–2.5 h to reach the moisture content of 10–12 g/100 g. The dried hard-stems were then ground and sieved through a 70-mesh screen (0.210 mm) to obtain AHP which was preserved in plastic bags at 4°C for further experimentation.

Ingredients for cracker making including wheat flour, cooking oil, refined saccharose, baking powder, sodium chloride, calcium dihydrogen phosphate, and lecithin were bought in a local supermarket.

All chemicals of analytical grade were from Sigma-Aldrich Inc. (Saint Louis, MO, USA); commercial enzymes, including Dextrozyme® GA, Termamyl® S, and Alcalase® 2.5 L, used for fiber determination were from Novozymes A/S (Bagsværd, Denmark).

Experimentation

The flour blend included wheat flour and AHP. In the making of dietary fiber-enriched crackers, the weight ratio of AHP to total flour blend (200 g) was 0% (control sample), 5%, 10%, 15%, and 20%. Other ingredients included: 28 g cooking oil, 10 g refined saccharose, 0.52 g lecithin, 0.58 g calcium dihydrogen phosphate, 4.08 g baking powder, 1.46 g salt, and 90 g water.

The cracker making process began with mixing the cooking oil, sugar, and lecithin within a mixer (5KSM7590, KitchenAid, Benton Harbor, MI, USA) at a speed of 200 rpm for 1 min. Calcium dihydrogen phosphate was dissolved in a required volume of water and then added to the mixture, which was mixed at 300 rpm for another 1 min. A mixture of wheat flour, AHP, baking powder, and sodium chloride was finally added and kneaded at 100 rpm for 11 min. The obtained dough was then incubated for 12 min in a convectional incubator (V222, Incucell, Munich, Germany) and flattened to 2 mm thickness by a rolling pin. The rolled dough was shaped using molds with a diameter of 42 mm and pricked with a fork. The crackers were baked at 230°C for 10 mins in an oven (VH-309N2D, Sanaky, Ho Chi Minh city, Vietnam), and the moisture of all cracker samples was less than 5 g/100 g. After baking, the crackers were naturally cooled down to 25°C and preserved in zip-lock bags for 1 day before analysis.

In order to enhance quality of the AHP-fortified crackers, the water amount used in the kneading of 20% AHP added sample was 90, 100, 110, 120 g/200 g flour blend. Other ingredients and the procedure of cracker making were fixed as mentioned above.

Analytical methods

Determination of chemical composition

Wheat flour, AHP, and crackers were analyzed to determine their chemical composition according to the methods of the Association of Official Analytical Chemists [AOAC International, 2023]. Moisture content was determined by AOAC 925.10 method. Protein was evaluated according to AOAC 984.13 method. Lipid was measured using AOAC 960.39 method. Ash was quantified by AOAC 930.30 method. Insoluble dietary fiber (IDF), soluble dietary fiber (SDF) and total dietary fiber (TDF) were measured by AOAC 985.29 method. Starch was estimated using AOAC 996.11 method. Results were expressed in g *per* 100 g on dry basis (db).

Determination of total phenolic content and antioxidant capacity

Wheat flour, AHP and crushed crackers were extracted with acidified methanol (the volume ratio of methanol/HCl/water was 80/1/20) at 30°C for 1 h. The material/solvent ratio was 1/15 (*w/v*). The extract was separated from the residue by centrifugation at 1,600×*g* for 20 min (3K30, Sigma Zentrifugen Ltd., Osterodeam Harz, Germany). The total phenolic content in the obtained extract was determined with the spectrophotometric method and Folin-Ciocalteau reagent [Agbor *et al.*, 2014]. Briefly, 0.2 mL of the extract and 1 mL of the Folin-Ciocalteu reagent were mixed by vortexing for 30 s. The reaction mixture was left at room temperature in the dark for 2 h, and the absorbance was recorded at 760 nm (UV 2600i spectrophotometer, Shimadzu Co., Kyoto, Japan). Total phenolic content was presented as mg gallic acid equivalent *per* g of the sample dry basis (mg GAE/g db). Antioxidant capacities were evaluated using ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays [Benzie & Strain, 1999; Brand-Williams *et al.*, 1995]. For DPPH assay, 0.1 mL of the diluted extract was added into 3.9 mL of a 60 µM DPPH radical solution in methanol. The reaction mixture was incubated in the dark at room temperature. The absorbance at 515 nm was monitored at 0 and after 30 min of incubation using methanol as the blank.

For FRAP assay, 3.8 mL of the FRAP working solution (25 mL of 0.3 M acetate buffer pH 3.6, 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine solution in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3 \times 6H_2O$) were mixed with 0.2 mL of the diluted extract. The reaction mixture was incubated in the dark at 37°C for 5 min, the absorbance at 593 nm was measured with an acidified methanol as the blank. For both assays, the antioxidant capacity was expressed as µmol Trolox equivalent *per* g of the sample dry basis (µmol TE/g db).

Physical analysis

Water holding capacity and oil holding capacity of AHP and wheat flour were measured following the procedure described by Fernández-López *et al.* [2009]. About 3 g of the sample were mixed with 30 mL of water or soybean oil (Tuong An Vegetable Oil Joint Stock Company, Ba Ria-Vung Tau Province, Vietnam) by vortexing for 30 s. After 2 h, the mixture was centrifuged at 1,000×*g* for 20 min. The supernatant was then decanted. The absorbed water or oil was determined by the difference between the sediment and the initial sample weight. Results were expressed as g of water or oil *per* g of the sample dry basis.

Hardness was evaluated using a texture profile analyzer (TA-XT Plus, Stable Micro System, Godalming, UK) and 3-point break measurement. Thickness and diameter of cracker samples were measured using a vernier caliper; the thickness was evaluated by measuring the height of a stack of six crackers and dividing by six, while the diameter was determined by measuring the width of six crackers placed edge to edge and dividing by six [Park *et al.*, 2015]. Instrumental color analysis was performed using a colorimeter (CM-3700A, Konica Minolta, Japan) in the CIELab color space; L^* (lightness), a^* (redness-greenness) and b^* (yellowness-blueness) values were recorded. Total color difference of the cracker samples (ΔE) was computed by the following formula (1):

$$\Delta E = \sqrt{(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2} \quad (1)$$

where: L_0^*, a_0^* and b_0^* are the color values of the crackers without AHP addition; L^*, a^*, and b^* are the color values of the AHP-supplemented crackers.

Sensory evaluation

Sensory overall acceptability of all cracker samples was evaluated with an acceptance test and a 9-point hedonic scale. The participants gave scores 1–9 to the cracker samples, ranging from "extremely dislike" to "extremely like" [Mai *et al.*, 2022]. Sixty-two untrained participants were chosen from academic staff and students of the Ho Chi Minh City University of Technology.

Statistical analysis

All cracker samples were done in triplicates to calculate the average result. Results were presented as mean and standard deviation. One-way analysis of variance was conducted using Statgraphics Centurion XV.I software, and the significance of differences was compared with Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Chemical composition, antioxidant capacities and physical characteristics of asparagus hard-stem powder and wheat flour

The chemical composition, antioxidant capacities and physical characteristics of AHP and wheat flour are illustrated in **Table 1**. The lipid and starch contents of AHP were 1.4 and 34.1 times, respectively, lower than those of the wheat flour, while the by-product contained 1.1 and 8.0 times more protein and ash, respectively. The amounts of IDF, SDF, and TDF of AHP were 27.9, 2.0, and 15.3 times greater, respectively, than those of the wheat flour. The dietary fiber contents of asparagus hard-stems in the present research were nearly similar to those reported by Iwassa *et al.* [2019] (57.18 g/100 g db), but they were higher than the findings of de Paula Laidens *et al.* [2021] (47.86 g/100 g db) probably due to the difference in asparagus varieties, cultivation and harvesting conditions.

The AHP contained 5.5 times more total phenolics than the wheat flour (**Table 1**). Additionally, the antioxidant capacities of AHP measured by FRAP and DPPH assays were 47.2 and 18.4 times, respectively, greater than those of wheat flour. Asparagus hard-stems are reported to contain rutin, quercetin, kaempferol

Table 1. Chemical composition, antioxidant capacities and physical characteristics of asparagus hard-stem powder and wheat flour.

Characteristic	Asparagus hard-stem powder	Wheat flour
Protein (g/100 g db)	14.3±0.1^a	12.7±0.2^b
Lipid (g/100 g db)	1.8±0.1^b	2.5±0.1^a
Ash (g/100 g db)	6.4±0.0^a	0.8±0.0^b
Starch (g/100 g db)	2.1±0.1^b	71.6±0.3^a
SDF (g/100 g db)	3.6±0.4^a	1.8±0.3^b
IDF (g/100 g db)	53.0±1.3^a	1.9±0.3^b
TDF (g/100 g db)	56.6±1.2^a	3.7±0.4^b
IDF/SDF	14.9±1.9^a	1.1±0.3^b
Total phenolic content (mg GAE/g db)	11.42±0.16^a	2.08±0.35^b
Ferric reducing antioxidant power (µmol TE/g db)	99.31±1.14^a	2.10±0.04^b
DPPH radical scavenging capacity (µmol TE/g db)	60.55±1.13^a	3.29±0.32^b
L^*	73.5±0.0^a	93.6±0.2^b
a^*	1.6±0.0^b	0.3±0.0^a
b^*	22.8±0.1^b	8.7±0.0^a
ΔE	24.6±0.2^b	0.0±0.0^a
Water holding capacity (g water/g db)	6.1±0.2^b	1.2±0.0^a
Oil holding capacity (g oil/g db)	3.0±0.0^b	1.0±0.0^a

Each value is expressed as mean ± standard deviation (n=3) and means having different letter superscripts within the same row are significantly different ($p<0.05$); TDF, total dietary fiber; SDF, soluble dietary fiber; IDF, insoluble dietary fiber; GAE, gallic acid equivalent; TE, Trolox equivalent; L^*, lightness; a^*, redness–greenness; b^*, yellowness–blueness; ΔE, total color difference; db, dry basis.

and other polyphenols and they exhibit high antioxidant activity [Guo *et al.*, 2020].

According to the instrumental color analysis, the AHP was darker than the wheat flour as indicated by a higher *L** value (**Table 1**). Additionally, the AHP had higher *a** and *b** values than the wheat flour, indicating that the yellowness and redness of AHP were more intense. Besides, the water holding capacity and oil holding capacity of wheat flour were approximately 5.1 and 3.0 times, respectively, lower than those of AHP. Water holding capacity may be related to SDF content since this fiber group has an ability to retain water. Oil holding capacity is associated with a chemical structure of plant polysaccharides and proteins; it is influenced by surface properties and hydrophobic nature of fiber particles [Fernández-López *et al.*, 2009].

Based on the chemical composition and physical characteristics, the supplementation of AHP to cracker recipe was expected to enhance dietary fiber and antioxidant contents of the fortified crackers; however, the use of AHP could affect their textural and sensory quality.

■ Impacts of various ratios of asparagus hard-stem powder on the cracker quality

■ Impacts on the nutritional composition and antioxidant capacities of crackers

Table 2 presents nutritional composition and antioxidant capacities of the cracker samples. The supplementation of AHP to cracker formula improved the contents of protein, ash, and dietary fiber whilst the lipid and starch contents of the fortified crackers were significantly reduced. This observation was attributable to the difference in the chemical composition between AHP and wheat flour. Specifically, the 20% AHP added crackers contained 5.0, 5.3 and 3.8 times more TDF, IDF and SDF, respectively, than the control crackers. A similar increase in dietary fiber content was previously reported when 5–15% chickpea husk was supplemented to the cracker formula [Bose & Shams-Ud-Din, 2010]. It can be noted that when the ratio of AHP was 15% or greater, the fortified crackers were considered as food with a high fiber content according to the Food and Agriculture Organization since the TDF content was higher than 6% [FAO, 1997]. Nevertheless, high AHP level in the recipe enhanced the IDF/SDF ratio of the fortified crackers. The appropriate ratio of IDF/SDF should be about 3/1 for food products since IDF and SDF exert different health effects on human [Rodríguez Galdón *et al.*, 2009].

Additionally, a crucial improvement was found in the total phenolic content and antioxidant capacities of the fortified crackers (**Table 2**) due to the greater amount of phenolics in AHP compared to that in wheat flour. At 20% AHP level, the total phenolic content of the fortified crackers was increased 3.1 by times while their FRAP and DPPH radical scavenging capacity were improved by 6.1 and 1.8 times, respectively as compared to those of the control crackers.

■ Impacts on the physical attributes and overall acceptability of crackers

The augmentation of the AHP ratio in the cracker formulation from 0 to 20% enhanced the product diameter by 9% while decreased its thickness by 27% (**Table 3**). This can be explained by the reduced gluten content of the cracker dough. Moreover, asparagus hard-stem proteins are poor in cysteine and methionine [Guan *et al.*, 2015], resulting in a weak gluten network [Gambuś *et al.*, 2011] when the AHP addition ratio was increased. A similar trend was recorded when the powder of old stalks of asparagus was supplemented to biscuit products [Liu *et al.*, 2016].

As the incorporation ratio of AHP was enhanced from 0 to 20%, the cracker hardness was doubly increased (**Table 2**). Lee *et al.* [2022] recently claimed an increase in cracker hardness when

Table 2. Nutritional composition, total phenolic content and antioxidant capacities of crackers supplemented with various ratios of asparagus hard-stem powder (0–20% of flour blend).

Characteristic	0	5%	10%	15%	20%
Protein (g/100 g db)	6.8±0.1^{e}	8.3±0.3^{d}	8.8±0.1^{c}	10.4±0.1^{b}	11.0±0.3^{a}
Lipid (g/100 g db)	15.1±0.2^{a}	14.4±0.7^{ab}	14.1±0.1^{ab}	13.7±0.2^{ab}	13.2±0.9^{b}
Ash (g/100 g db)	2.0±0.0^{e}	2.2±0.0^{d}	2.4±0.1^{c}	2.5±0.0^{b}	3.0±0.0^{a}
Starch (g/100 g db)	61.0±3.6^{a}	60.2±2.0^{a}	59.1±0.8^{a}	53.7±1.7^{b}	49.4±2.9^{b}
TDF (g/100 g db)	2.0±0.1^{e}	3.0±0.1^{d}	5.4±0.1^{c}	7.5±0.3^{b}	10.0±0.4^{a}
SDF (g/100 g db)	0.4±0.0^{b}	0.7±0.1^{ab}	1±0.2^{ab}	1.4±0.4^{a}	1.5±0.5^{a}
IDF (g/100 g db)	1.6±0.0^{e}	2.2±0.0^{d}	4.3±0.0^{c}	6.2±0.1^{b}	8.5±0.2^{a}
IDF/SDF	3.7±0.4^{ab}	3.1±0.6^{b}	4.2±0.7^{ab}	4.9±1.6^{ab}	6.1±2.6^{a}
Total phenolic content (mg GAE/g db)	1.45±0.06^{e}	2.09±0.11^{d}	2.98±0.17^{c}	3.81±0.26^{b}	4.57±0.24^{a}
Ferric reducing antioxidant power (μmol TE/g db)	4.01±0.03^{e}	8.33±0.07^{d}	13.44±0.30^{c}	17.07±0.29^{b}	24.45±0.03^{a}
DPPH scavenging capacity (μmol TE/g db)	18.09±0.69^{e}	21.84±0.12^{d}	25.46±0.46^{c}	28.47±0.64^{b}	32.05±0.86^{a}

Each value is expressed as mean ± standard deviation (n=3) and means having different letter superscripts within the same row are significantly different (p<0.05); TDF, total dietary fiber; SDF, soluble dietary fiber; IDF, insoluble dietary fiber; GAE, gallic acid equivalent; TE, Trolox equivalent; db, dry basis.

Table 3. Physical attributes and overall acceptability of crackers supplemented with different ratios of asparagus hard-stem powder (0–20% of flour blend).

Characteristic	0	5%	10%	15%	20%
Diameter (mm)	38.69±0.31^{e}	39.50±0.07^{d}	40.67±0.10^{c}	41.72±0.07^{b}	42.17±0.05^{a}
Thickness (mm)	3.82±0.10^{a}	3.49±0.15^{b}	3.22±0.03^{c}	2.89±0.04^{d}	2.79±0.02^{e}
Hardness (N)	21.04±1.32^{e}	25.87±0.36^{d}	32.84±1.14^{c}	37.98±0.54^{b}	42.18±2.75^{a}
*L**	83.2±0.3^{a}	74.9±0.4^{b}	68.4±0.2^{c}	65.8±0.2^{d}	65.5±0.2^{d}
*a**	3.1±0.1^{e}	4.9±0.0^{d}	6.4±0.2^{c}	6.7±0.2^{b}	7.2±0.0^{a}
*b**	22.5±0.1^{b}	25.7±0.3^{a}	25.7±0.7^{a}	26.1±0.7^{a}	26.2±0.2^{a}
Δ*E*	0.0±0.0^{d}	9.1±0.3^{c}	15.5±0.3^{b}	18.1±0.2^{a}	18.5±0.3^{a}
Sensory score	7.0±1.2^{a}	6.3±1.3^{b}	6.3±1.4^{b}	5.0±1.4^{c}	4.2±1.6^{d}

Each value is expressed as mean ± standard deviation (n=62 for sensory score and n=3 for other characteristics); means having different letter superscripts within the same row are significantly different (p<0.05); *L**, lightness; *a**, redness–greenness; *b**, yellowness–blueness; Δ*E*, total color difference.

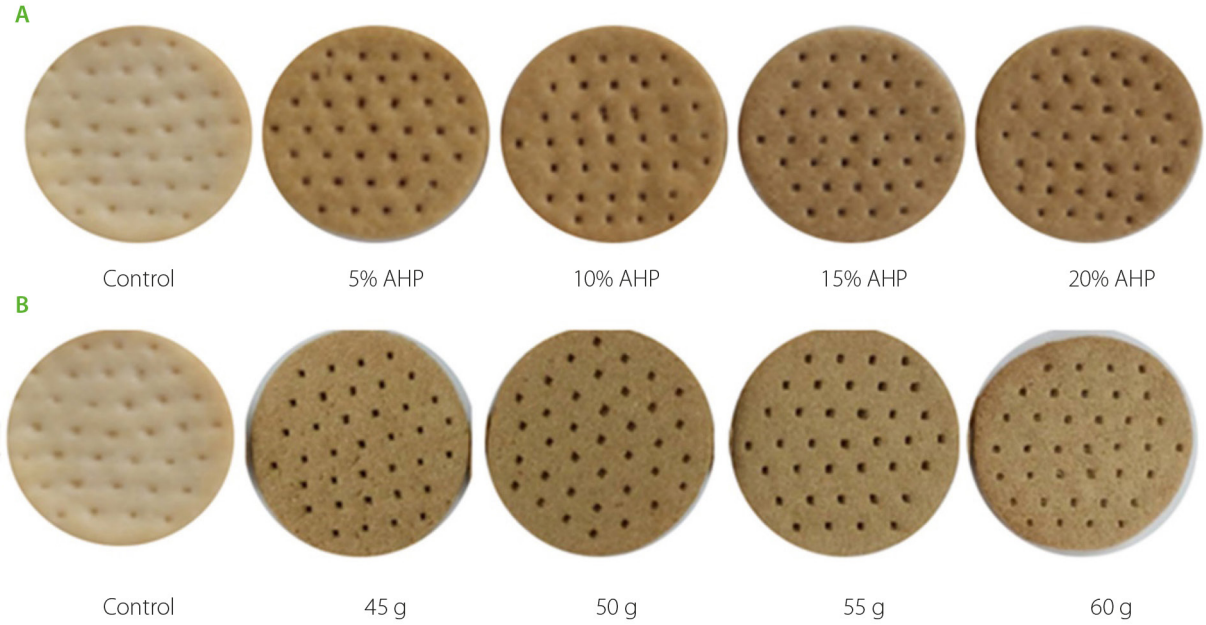

Figure 1. Pictures of crackers (**A**) fortified with 5, 10, 15 and 20% asparagus hard-stem powder (AHP), the water amount in cracker recipe was fixed at 45 g *per* 100 g the flour blend; and crackers (**B**) fortified with 20% AHP, the water amount was 45, 50, 55 and 60 g *per* 100 g the flour bend. The controls were crackers without AHP and the water amount of 45 g *per* 100 g wheat flour.

supplementing red ginseng marc from 0 to 20% to the product formulation and explained that it was due to the enhancement in dietary fiber content. The greater the dietary fiber content in food was, the greater was food hardness [Damat *et al.*, 2019].

The use of AHP also enhanced darkness of the cracker samples as well as their redness and yellowness (**Table 2**), since the color of AHP and wheat flour greatly varied. The Δ*E* values of all fortified crackers were higher than 5, indicating their color difference as compared to that of the control crackers. The appearance of the crackers, shown in **Figure 1A**, confirmed an increase in darkness of the crackers as the AHP ratio was enhanced in the product recipe. A similar increase in product darkness was also observed when asparagus spear powder was supplemented to the pasta formulation [Vital *et al.*, 2020].

The increased AHP ratio in the cracker formulation successively decreased the overall acceptability of the fortified crackers (**Table 2**) mainly due to their enhanced hardness. The control crackers received the highest sensory score. The sensory scores of the 5, 10 and 15% AHP-added crackers can be considered acceptable; however, the sensory score of the crackers with 20% AHP was lower than 5, indicating that this sample was not accepted by consumers.

Generally, a high AHP ratio in the cracker recipe improved dietary fiber and phenolic contents, as well as antioxidant capacity of the fortified crackers, but increased their hardness and decreased their sensory quality. The presence of AHP with high water holding capacity in the cracker dough resulted in a reduced amount of free water for gluten network development

Table 4. Physical attributes and sensory scores of crackers fortified with 20% asparagus hard-stem powder (AHP) and kneaded with different amounts of water.

Characteristic	Control cracker	Water amount used for cracker dough kneading (g water/100 g flour blend)			
		45	50	55	60
Diameter (mm)	39.12±0.74^{d}	42.64±0.14^{ab}	42.19±0.33^{bc}	41.72±0.13^{c}	41.65±0.15^{c}
Thickness (mm)	3.87±0.09^{a}	2.71±0.02^{cd}	2.83±0.12^{bc}	3.07±0.05^{b}	2.87±0.02^{c}
Hardness (N)	21.46±0.79^{d}	42.53±0.86^{a}	33.41±1.65^{b}	25.02±0.71^{c}	16.95±0.72^{e}
*L**	81.2±0.3^{a}	65.9±0.1^{b}	64.3±0.6^{c}	64.0±0.3^{c}	63.3±0.2^{d}
*a**	4.0±0.0^{d}	7.2±0.1^{c}	7.6±0.2^{b}	7.8±0.2^{ab}	7.9±0.1^{a}
*b**	23.8±0.1^{d}	26.2±0.3^{c}	27.0±0.3^{b}	27.1±0.5^{b}	28.0±0.3^{a}
Δ*E*	0.0±0.0^{d}	15.9±0.3^{c}	17.7±0.2^{b}	18.0±0.5^{b}	18.9±0.2^{a}
Sensory score	7.0±1.2^{a}	4.3±1.5^{d}	5.9±1.0^{c}	6.6±1.4^{b}	6.4±1.0^{bc}

Each value is expressed as mean ± standard deviation (n=62 for sensory score and n=3 for other characteristics); means having different letter superscripts within the same row are significantly different (p<0.05); Control crackers were made from wheat flour without AHP and the water amount in the recipe of 45 g/100 g wheat flour; *L**, lightness; *a**, redness–greenness; *b**, yellowness–blueness; Δ*E*, total color difference.

during the cracker kneading. The effects of water amounts used in the recipe of 20% AHP-added crackers needed to be clarified to improve their texture and sensory quality.

■ Effects of water amounts used in the high-fiber cracker recipe on the quality of 20% AHP-fortified crackers

When the water amount used in the high-fiber cracker recipe was enhanced from 45 to 60 g/100 g the flour blend, the product hardness was reduced by 2.5 times (**Table 4**). This reduction can be explained by a better development of a gluten network during the kneading of high-fiber dough when the water content was increased. The gluten network accounts for dough elasticity, resulting in crackers with low hardness [Beverly, 2014]. However, so high water level in dough interferes with the gluten structure and makes the dough difficult to hold its shape [HadiNezhad & Butler, 2009]. At water level of 60 g/100 g the flour blend, the hardness of high-fiber crackers was about 1.3 times lesser than that of the control crackers (**Table 4**).

The diameter of 20% AHP-fortified crackers was slightly reduced towards the value of the control crackers as the water amount in the product recipe changed from 45 to 60 g/100 g the flour blend (**Table 4**). For thickness, this attribute was improved and achieved the highest value at the water amount of 55 g/100 g of the flour blend. A higher water level decreased the product thickness since the dough became flabby [Hoseney & Rogers, 1990].

The increase in water amount used in the cracker formulation from 45 to 60 g/100 g the flour blend slightly reduced *L** value of the product color (p<0.05) (**Table 4**) probably due to improved Maillard reactions. However, all *L**, *a** and *b** values were changed in narrow ranges. **Figure 1B** shows that the change in the water level in the cracker recipe did not strongly affect the color of the fortified crackers.

It can be noted that the acceptance level of high-fiber crackers was successively increased from 4.3 to 6.6 points (out of 9) when the water content was increased from 45 to 55 g/100 g the flour blend (**Table 4**) due to a reduced hardness. At water level of 55 and 60 g/100 g the flour blend, the overall acceptability of the crackers did not differ significantly (p≥0.05). Generally, the recommended water level was 55 g/100 g the flour blend for the 20% AHP-added cracker recipe; and a change in the water level used in high-fiber cracker making was a potential technique to improve hardness and sensory quality of the product.

CONCLUSIONS

AHP was proved to be an ingredient with a high dietary fiber content and antioxidant capacities. The increment in the AHP ratio in the cracker formulation from 0 to 20% increased the total dietary fiber and phenolic content and antioxidant capacities of the fortified crackers. However, the increased ratio of AHP in the cracker recipe significantly enhanced the product hardness while reduced its thickness and sensory score. The appropriate AHP ratio was 15% since the fortified crackers were deemed high-fiber food and accepted by consumers. Change in the water level in the recipe of high-fiber crackers successfully reduced the hardness and improved the sensory score of high-fiber crackers. When the AHP addition ratio was increased to 20%, the recommended water level was 55 g/100 g the flour to improve its textural and sensory quality. AHP can therefore, be considered a potential ingredient of dietary fiber and antioxidants for fortification of cracker products.

ACKNOWLEDGEMENTS

We acknowledge the support of time and facilities from Ho Chi Minh City University of Technology (HCMUT), VNU-HCM for this study.

RESEARCH FUNDING

This research was funded by Vietnam National University Ho Chi Minh City (VNU-HCM) under grant number NCM2020-20-01.

CONFLICT OF INTERESTS

The authors have declared no conflicts of interest for this article.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 169–176
DOI: 10.31883/pjfns/188139
ISSN 1230-0322, e-ISSN 2083-6007

Fatty Acid Composition and Anticancer Activity of Neutral and Polar Lipids of Pacific Oyster (*Crassostrea gigas*) Cultured in Khanh Hoa Coast in Vietnam

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In this study, we investigated the fatty acid composition and anticancer activity of neutral and polar lipid fractions extracted from *Crassostrea gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast, harvested during the five months of January, April, May, September, and November. Analysis revealed that saturated fatty acids (SFAs) were the most abundant fatty acids in the neutral lipid fraction, followed by monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Conversely, the polar lipid fraction exhibited a different order, with PUFAs being the most abundant, followed by MUFAs and SFAs. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were identified as the most prevalent polyunsaturated fatty acids, while oleic acid and palmitic acid were the predominant monounsaturated and saturated fatty acids, respectively. Notably, the combined content of EPA and DHA in the polar lipid fraction consistently exceeded 30% throughout all five months of analysis. Thrombogenicity index (TI) values ranged from 0.13 to 0.29 for the polar lipid fraction and from 0.6 to 1.1 for the neutral lipid fraction. Moreover, the polar lipid fraction exhibited significantly higher *n*3/*n*6 ratios compared to the neutral lipid fraction. The polar lipid fraction exhibited stronger inhibitory effects on the growth of the three cancer cell lines (HepG2, MDA-MB-231, and RD) compared to the neutral lipid fraction. The findings of the present study show that lipids extracted from *C. gigas* oysters cultured in Khanh Hoa Coast have a weak anticancer activity but may still aid in prevention and treatment of certain cancer types.

Keywords: anticancer activity, fatty acid profile, lipid fraction, *n*3/*n*6 ratio, thrombogenicity index

IINTRODUCTION

The lipid content and composition of marine organisms, including fish, mollusks, and various other marine species, exhibit significant variability influenced by factors such as species, season, gender, and geographical location [Kandemir & Polat, 2007; Nguyen *et al.*, 2024]. The lipid content is also influenced by the dietary nutritional profile [Anjos *et al.*, 2017]. In our previous study, we demonstrated that Pacific oysters (*Crassostrea gigas*) cultured in Khanh Hoa Coast, Vietnam, were rich in exploitable lipids, particularly polyunsaturated fatty acids (PUFAs), and their composition varied with season and location [Nguyen *et al.*, 2024]. Oyster lipids, comprising a mixture of neutral and polar lipids, play essential roles in biological functions and exhibit bioactivities with potential health benefits [Tan *et al.*, 2022]. Neutral lipids primarily consist of triacylglycerols and wax esters, while polar lipids are composed of phospholipids and glycolipids. The structure

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Submitted: 17 March 2024
Accepted: 30 April 2024
Published on-line: 23 May 2024

and function of polar and neutral lipids vary among different species [Şen Özdemir *et al.*, 2019]. Understanding the specific fatty acid composition and potential health implications of these lipids is crucial for exploring their nutritional and pharmaceutical potential.

Studies have shown that extracts from a range of marine organisms, including mollusks, possess bioactivities with potential health benefits, including antiviral activity, antioxidant, anti-inflammatory, immunomodulatory, and anticancer properties [Hamed *et al.*, 2015; Khan & Liu, 2019]. The active compounds in these extracts include proteins and glycoproteins (active against viruses) [Dang *et al.*, 2015], and PUFAs with reported anti-inflammatory properties among others. Marine-derived lipids have been demonstrated to exhibit anticancer activity in various studies [Lauritano *et al.*, 2020; Li *et al.*, 2020; Martínez Andrade *et al.*, 2018]. Similarly, extracts from marine microorganisms and algae have shown potential as novel anticancer agents, highlighting the abundance of bioactive compounds present in marine ecosystems [Samarakoon *et al.*, 2014; Tommonaro *et al.*, 2020]. Kim *et al.* [2010] demonstrated that the hexane lipid extract from *C. gigas* exhibited growth-inhibitory activity against the prostate cancer cell line. This lipid fraction was comprised of palmitic, margaric, and stearic acids. In a study by Nappo *et al.* [2012] conducted with marine diatoms *Cocconeis scutellum* Ehrenberg (Bacillariophyceae), the diethyl ether extracts rich in EPA induced apoptosis and decreased viability in BT20 cells. Oyster lipids contain several glycerophospholipids with triacylglycerols containing *n*3 long chain-PUFAs that are presumed to have better tissue delivery capacity and bioavailability [Liu *et al.*, 2020].

It is important to find new sources of bioactive compounds which are useful in the development of therapeutic agents for cancer treatment. Challenges can be encountered in harvesting potential organisms and isolating and purifying the bioactive compounds, which call for more organisms to be explored. Investigation into the fatty acid composition and anticancer properties of oyster lipids, particularly those cultured in diverse geographical locations such as the Nha Phu Lagoon, Khanh Hoa Coast, is useful in this scenario.

In this study, we investigated the fatty acid composition and potential anticancer activity of neutral and polar lipids extracted from Pacific oysters cultured in the Nha Phu Lagoon, Khanh Hoa Coast, Vietnam harvested during January, April, May, September, and November. Specifically, our study aimed to assess the effects of oyster lipids on three human cancer cell lines: MDA-MB-231 (human breast cancer), HepG2 (liver cancer), and RD (muscle rhabdomyosarcoma-A) cell lines.

MATERIAL AND METHODS

Materials and reagents

Sample collection and preparation

Commercial-quality Pacific oysters (*Crassostrea gigas*) were obtained from Nha Phu Lagoon, located in Ninh Hoa District, Khanh Hoa Province, Vietnam, during multiple harvests conducted in January, April, May, September, and November 2021. The sample collection and preparation were carried out following the procedure described by Nguyen *et al.* [2024]. Briefly, the oyster muscle was separated from the shells, vacuum-sealed in polyamide bags, and promptly frozen at −35±2°C using an air-blast freezer (Seatecco Corporation, Da Nang, Vietnam). Frozen samples were stored at −80°C and thawed completely at 2°C before analysis.

Analytical materials and reagents

Analytical-grade materials and reagents including *n*-hexane, chloroform, methanol, L-ascorbic acid, thin-layer chromatography (TLC) plates (TLC silica gel 60 F254), silica gel 60 (0.040–0.063 mm), diethyl ether, potassium chloride, sodium sulfate, trypan blue, and ninhydrin were procured from Sigma-Aldrich (Burlington, MA, USA). Additionally, L-glutamine, penicillin-streptomycin, trypsin-EDTA, Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), and fetal bovine serum (FBS) were obtained from Gibco (Billings, MT, USA). Doxorubicin was sourced from EBEWE Pharma (Unterach am Attersee, Austria), while all other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany). Human cell lines, including MDA-MB-231 (human breast cancer), HepG2 (liver cancer), and RD (muscle rhabdomyosarcoma-A), were procured from ATCC (Manassas, VA, USA).

Lipid extraction and fractionation

Total lipids were extracted from oyster muscle following the method of Bligh & Dyer [1959]. Briefly, oyster muscle was homogenized using an Ultra-Turrax homogenizer (T25 basic, Ika Labortechnik, Staufen, Germany) in a mixture of chloroform, methanol, and 0.88% KCl (1/1/0.5, *v/v/v*). The homogenate was then subjected to centrifugation at 1,942×*g* (Hermle Z326K universal refrigerated centrifuge, Wehingen, Germany) for 20 min at 4°C. The lipid layer was collected, and the chloroform was completely evaporated at 40°C in a water bath using a liquid nitrogen stream. Subsequently, total lipids were separated into neutral and polar lipid fractions *via* column chromatography on silica gel, following the procedure described in our earlier work [Nguyen *et al.*, 2024]. A solution containing 0.8 g of the lipid sample in 1 mL of chloroform was loaded onto a column (180 mm in diameter and 330 mm in length) filled with silica gel 60 (0.040–0.060 mm). The neutral lipid fraction was obtained by eluting with a chloroform-methanol solvent. The polar lipid fraction was recovered by elution with methanol. The complete recovery of the neutral and polar lipid fractions was confirmed using thin-layer chromatography (TLC) [Deranieh *et al.*, 2013]. The larger volumes of the solvents of each fraction were removed under vacuum at 40°C (Yamato RE-801-AW2 rotary evaporator, Yamato, Japan). The residual solvent was evaporated under a stream of nitrogen at 35°C. The obtained lipid fractions were used for the analysis of fatty acid composition and anticancer activity.

Fatty acid composition analysis

Fatty acid methyl esters (FAMEs) of neutral and polar lipid fractions were prepared *via* base-catalyzed esterification according

to the American Oil Chemists' Society (AOCS) official method (Ce 1b-89, 2017) [AOCS, 2017]. Subsequently, the FAMEs were subjected to gas chromatography (GC) analysis using a Shimadzu GC 17A chromatograph (Shimadzu Corp., Kyoto, Japan) with flame-ionization detector. Separation was carried out on a Zebron ZB-wax column (0.25 mm × 30 m, 0.25 µm; Phenomenex, Torrance, CA, USA). The initial temperature of column oven and injection port was 170°C. This temperature was maintained for 2 min, then increased to 240°C at a rate of 5°C /min, from 240°C to 250°C at a rate of 1.6°C /min, and finally held at 250°C for 10 min. The inlet pressure of nitrogen, which was used as a carrier gas, was 2.0 kg/cm^2. Results were expressed as g *per* 100 g of the lipid fraction (g/100 g LF), and tricosanoic acid (C23:0) was used as an internal standard.

Thrombogenicity index calculation

The thrombogenicity index (TI) was computed according to the equation proposed by Ulbricht & Southgate [1991], which evaluates the relationship between pro-thrombogenic saturated fatty acids and anti-thrombogenic monounsaturated fatty acids (MUFAs) and PUFAs.

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{(0.5 \times PUFA_{n6}) + (3 \times PUFA_{n3}) + (0.5 \times MUFA) + \frac{PUFA_{n3}}{PUFA_{n6}}} \quad (1)$$

Determination of anticancer activity

The assessment of the anticancer properties of polar and neutral lipid fractions of *C. gigas* oysters harvested in January followed the protocol outlined by Tran *et al.* [2020], with modifications. MDA-MB-231, HepG2, and RD cell lines were cultured in EMEM and DMEM media. The lipid fractions were initially dissolved in dimethyl sulfoxide, DMSO (10 mM) and subsequently diluted in the culture medium to obtain various concentrations of 6.25, 12.50, 25.00, 50.00, and 100.00 µg/mL for the cell proliferation assays. Following treatment with lipid fractions at varying concentrations, cells were incubated for 72 h at 37°C and 5% CO_2. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide (MTT) assay as outlined by Denizot & Lang [1986]. Doxorubicin was employed as the reference compound, while DMSO served as the blank control. IC_{50} values, representing the concentrations of lipid fractions that inhibit 50% of cell viability, were determined based on the dose-response inhibition curves. The inhibition rates of the neutral and polar lipid fractions against the three tested cancer cell lines were calculated using the following equation (2):

$$\text{Inhibition rate (\%)} = (1 - OD_{Sample}/OD_{DMSO}) \times 100 \quad (2)$$

where: OD_{Sample} is optical density of the final assay mixture with lipid fraction, OD_{DMSO} is optical density of the final assay mixture with DMSO.

Statistical analysis

The experiments were performed in triplicate, and the data were expressed as mean values and standard deviations (SD). Statistical analyses were conducted using the SPSS software (version 26, SPSS Inc., Chicago, Il, USA). One-way analysis of variance (ANOVA) was employed to analyze the results. Significance of differences between the samples was determined using the Student-Newman-Keuls post-hoc test at a significance level of 5%.

RESULTS AND DISCUSSION

Fatty acid composition in the neutral lipid and polar lipid fractions

The changes in fatty acid composition of the neutral and polar lipid fractions in *C. gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast, Vietnam, for the months of January, April, May, September, and November are presented in **Table 1**. Neutral lipids extracted from *C. gigas* were abundant in SFAs, followed by PUFAs, and MUFAs. The SFAs content of the neutral lipid portion increased from January (46.94 g/100 g LF) to a maximum in September (53.35 g/100 g LF) and then decreased in November (47.17 g/100 g LF). The SFAs content was significantly different ($p<0.05$) for the five months. The MUFAs content of the neutral lipid fraction showed slight variation, with the highest content recorded in the month of May (22.91 g/100 g LF), while the lowest was in the month of September (19.66 g/100 g LF). The MUFA content of the neutral lipid fraction for the months of January, April, and May was not significantly ($p<0.05$) different from each other, but significantly different ($p<0.05$) from that obtained in September. The major MUFAs in neutral oyster lipids was myristoleic acid (C14:1*n*9), while palmitic acid (C16:0) was identified as the predominant saturated fatty acid (SFA). The PUFAs content was relatively stable in January, April, and November and only decreased ($p<0.05$) during May and September. The main PUFAs in the neutral lipids were the *n3* PUFAs with eicosapentaenoic acid (EPA, C20:5*n*3, 5.12–7.94 g/100 g LF) and docosahexaenoic acid (DHA, C22:6*n*3, 5.52–9.61 g/100 g LF). The EPA and DHA contents were significantly ($p<0.05$) lower in the months of May and September. In contrast to neutral lipids, polar lipids exhibited a higher abundance of PUFAs (36.12––47.22 g/100 g LF), followed by MUFAs (27.68–33.64 g/100 g LF), and then SFAs (20.74–30.52 g/100 g LF). The oysters harvested in May and September had lower ($p<0.05$) PUFA contents compared to those harvested in January, April, and November. These findings were consistent with the higher SFA contents determined in the oysters harvested in May and September. The PUFAs content of the polar lipids was over 40 g/100 g LF except in the months of May and September, where it was approximately 36 g/100 g LF. Similar to the neutral lipid fraction, EPA and DHA were the dominant PUFAs; however, their levels in the polar lipid fraction were significantly higher ($p<0.05$) compared to those found in the neutral lipids. In the polar lipid fraction, oleic acid (C18:1*n*9) was the predominant MUFA, whereas palmitic acid (C16:0) remained the most abundant SFA. The total DHA and EPA content was over 30 g/100 g LF for all the months. The PUFA content in the polar lipid fraction was significantly ($p<0.05$) higher than that of the neutral lipid fraction. Glycolipids and phospholipids are typically esterified with EPA [Da Costa *et al.*, 2021], and marine polar lipids play a crucial role as carriers

Table 1. Fatty acid composition (g/100 g lipid fraction) of neutral and polar lipid fractions of *Crassostrea gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast harvested during different months

Fatty acid	Neutral lipid fraction					Polar lipid fraction				
	January	April	May	September	November	January	April	May	September	November
C14:0	9.84±0.05^{b}	10.41±0.12^{b}	10.12±0.14^{b}	12.76±0.13^{a}	10.12±0.12^{b}	0.54±0.01^{d}	1.69±0.02^{c}	1.13±0.09^{cd}	1.84±0.03^{c}	0.94±0.05^{cd}
C15:0	8.87±0.11^{b}	8.67±0.09^{b}	10.06±0.13^{a}	10.59±0.14^{a}	8.89±0.11^{b}	0.47±0.04^{c}	0.65±0.04^{c}	0.65±0.04^{c}	0.86±0.10^{c}	0.59±0.02^{c}
C16:0	17.84±0.14^{c}	18.41±0.12^{c}	17.18±0.14^{cd}	17.76±0.11^{c}	16.48±0.11^{d}	16.74±0.15^{d}	18.22±0.12^{c}	21.25±0.11^{b}	22.64±0.11^{a}	16.51±0.11^{d}
C18:0	10.38±0.10^{c}	12.82±0.16^{b}	14.82±0.16^{a}	12.24±0.15^{b}	11.68±0.13^{bc}	2.98±0.11^{e}	3.73±0.09^{e}	4.93±0.10^{d}	5.18±0.12^{d}	2.88±0.10^{e}
SFA	**46.94±0.12^{d}**	**50.32±0.14^{c}**	**52.18±0.18^{b}**	**53.35±0.16^{a}**	**47.17±0.11^{d}**	**20.74±0.14^{h}**	**24.29±0.09^{g}**	**27.95±0.11^{f}**	**30.52±0.09^{e}**	**20.92±0.11^{h}**
C14:1*n*9	5.23±0.08^{a}	5.29±0.09^{a}	5.94±0.09^{a}	4.41±0.09^{b}	4.29±0.09^{b}	4.53±0.06^{b}	4.69±0.08^{b}	5.37±0.06^{a}	5.83±0.08^{a}	4.95±0.08^{ab}
C16:1*n*7	4.05±0.10^{b}	4.65±0.10^{b}	4.29±0.11^{b}	3.94±0.12^{b}	4.05±0.10^{b}	4.35±0.04^{b}	4.69±0.06^{b}	4.88±0.06^{ab}	5.73±0.06^{a}	5.29±0.11^{ab}
C17:1*n*7	3.93±0.11^{bc}	2.94±0.08^{c}	4.41±0.10^{b}	3.81±0.10^{bc}	4.13±0.09^{b}	3.53±0.08^{bc}	4.02±0.10^{b}	5.65±0.12^{a}	4.45±0.08^{b}	4.29±0.08^{b}
C18:1*n*9	3.53±0.05^{c}	3.79±0.07^{c}	3.79±0.07^{c}	3.79±0.90^{c}	3.53±0.09^{c}	9.35±0.11^{a}	7.41±0.11^{b}	9.33±0.10^{a}	10.39±0.10^{a}	9.86±0.05^{a}
C20:1*n*7	4.18±0.14^{c}	3.65±0.12^{cd}	4.47±0.11^{c}	3.71±0.12^{cd}	5.08±0.12^{bc}	6.12±0.07^{b}	6.86±0.06^{b}	8.43±0.06^{a}	2.84±0.07^{d}	4.94±0.12^{c}
MUFA	**20.92±0.17^{de}**	**20.33±0.10^{de}**	**22.91±0.14^{d}**	**19.66±0.11^{e}**	**21.08±0.10^{d}**	**27.88±0.18^{c}**	**27.68±0.14^{c}**	**33.64±0.16^{a}**	**29.24±0.12^{b}**	**29.33±0.15^{b}**
C18:3*n*6	6.18±0.09^{a}	6.48±0.10^{a}	6.68±0.11^{a}	6.76±0.09^{a}	5.86±0.10^{a}	1.62±0.06^{c}	2.17±0.06^{bc}	1.65±0.06^{c}	2.82±0.06^{b}	1.29±0.06^{c}
C20:4*n*6	5.07±0.12^{b}	5.73±0.10^{ab}	6.73±0.12^{a}	6.94±0.12^{a}	5.47±0.10^{b}	1.15±0.05^{d}	1.04±0.07^{d}	2.60±0.07^{c}	1.98±0.09^{cd}	1.59±0.10^{cd}
C20:5*n*3	7.94±0.12^{e}	6.91±0.14^{e}	5.41±0.14^{f}	5.12±0.11^{f}	7.24±0.11^{e}	16.74±0.07^{a}	15.59±0.09^{b}	13.58±0.10^{c}	11.96±0.10^{d}	16.17±0.11^{ab}
C22:6*n*3	9.61±0.15^{d}	8.06±0.10^{e}	5.59±0.12^{f}	5.52±0.12^{f}	9.06±0.09^{de}	27.71±0.11^{a}	24.63±0.13^{b}	18.53±0.11^{c}	19.36±0.14^{c}	26.80±0.09^{a}
PUFA	**28.80±0.16^{e}**	**27.18±0.02^{f}**	**24.41±0.08^{g}**	**24.35±0.09^{g}**	**27.64±0.07^{f}**	**47.22±0.18^{a}**	**43.43±0.12^{c}**	**36.35±0.07^{d}**	**36.12±0.17^{d}**	**45.85±0.15^{b}**
Other	3.35±0.11^{b}	2.18±0.10^{c}	0.51±0.07^{d}	2.64±0.11^{bc}	4.11±0.11^{ab}	4.16±0.13^{ab}	4.60±0.09^{a}	2.06±0.11^{c}	4.12±0.10^{ab}	3.90±0.10^{ab}

Results are expressed as mean ± standard deviation (n=3). Means with different lowercase letters in the same row show significant differences (p<0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

of *n*3 fatty acids, containing higher levels of *n*3 PUFAs compared to triglycerides [Lordan *et al.*, 2011; 2017]. The observed increase in the SFAs content of the neutral lipid fraction during the months of May and September (spawning season) may be attributed to the elevated energy demands necessary during this period. The increased levels of PUFAs in January and November align with phytoplankton blooms during the rainy seasons. Since PUFAs have a high tendency to be esterified to phospholipids rather than triacyclglycerols, their content increases in phospholipids during gonadal maturation. PUFAs serve a significant role in the composition of structural membrane lipids and are stored as lipovitellins in oocytes. These lipovitellins act as reserves for the cellular division process that occurs after fertilization [De La Parra *et al.*, 2005]. Palmitic acid, EPA, and DHA were identified as principal constituents of phospholipids, which are characteristic of marine animals [Pogoda *et al.*, 2013]. Previous studies have consistently demonstrated that oysters are rich sources of EPA and DHA [Liu *et al.*, 2020; Martino & Cruz, 2004]. High levels of EPA are indicative of the presence of EPA-rich phytoplankton. Previous studies have also revealed the energetic role of EPA [Qin *et al.*, 2021]. In mollusks, the fatty acid C20:4*n*6 acts as a precursor for prostaglandins, which play a crucial role in the regulation of reproductive processes [Soudant *et al.*, 1999]. It has been suggested that marine bivalves exhibit a greater need for accumulating *n*3 PUFAs compared to *n*6 PUFAs [Abad *et al.*, 1995]. This observation elucidates the higher levels of *n*3 PUFAs compared to *n*6 PUFAs discovered in our study. Pogoda *et al.* [2013] similarly reported that the fatty acid composition of *Ostrea edulis* and *C. gigas* was predominantly composed of C16:0, EPA, and DHA, consistent with the findings of our study. The fatty acid composition of oysters is influenced by both intrinsic factors such as sex, age, and size as well as extrinsic factors including temperature, salinity, and diet [Martino & Cruz, 2004].

Nutritional quality of neutral and polar lipid fractions

The changes in thrombogenicity index (TI) and an *n*3/*n*6 ratio of neutral and polar lipids of oyster harvested in different months are shown in **Figure 1**. A high ratio of *n*3/*n*6 is necessary and important in marine organisms for growth and survival [Soudant *et al.*, 1999]. Significantly higher *n*3/*n*6 ratios were observed in the polar lipid fraction compared to the neutral lipid fraction (**Figure 1A**). In the polar lipid fraction, the *n*3/*n*6 ratio exhibited a significant decline from January to September, followed by a significant increase in November. Notably, the *n*3/*n*6 ratios of polar lipids extracted from oysters harvested in May and September were significantly lower (p<0.05) compared to the ratios observed in other months. The observed reduction in *n*3/*n*6 ratios during May and September could be attributed to lipid accumulation and the conversion of glycogen into neutral lipids that occur during the spawning season. The pattern of changes

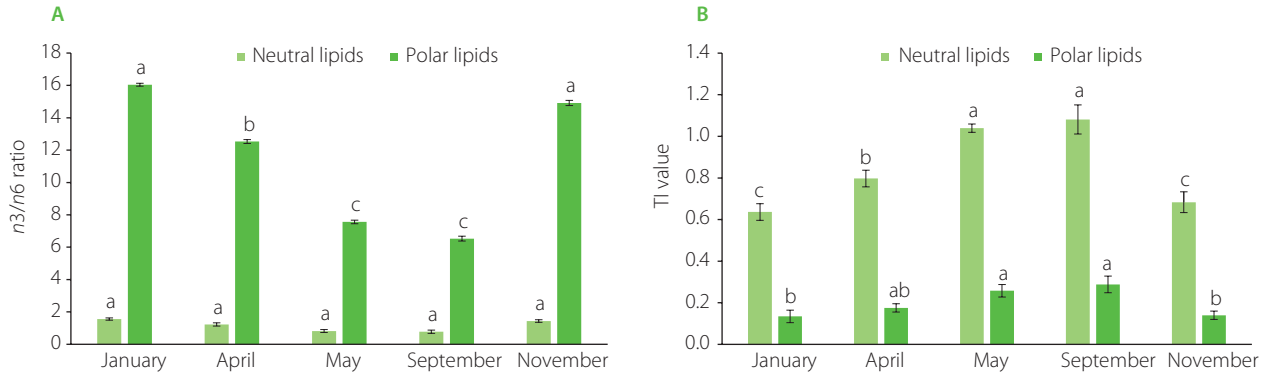

Figure 1. Variations in *n3/n6* ratio (**A**) and thrombogenicity index, TI (**B**) of neutral and polar lipids of *Crassostrea gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast harvested during different months. Different letters above bars separately for each lipid fraction indicate significant differences ($p<0.05$).

in the *n3/n6* ratio of the neutral lipid fraction was similar, but no significant ($p\geq0.05$) differences were found in the *n3/n6* ratio among oysters harvested in different months. Diets with low *n3/n6* ratios are associated with an increase in inflammatory diseases [Qin *et al.*, 2021]. Therefore, the consumption of polar lipids from oysters is crucial for maintaining good health, as they contain elevated levels of omega-3 fatty acids in comparison to neutral lipids.

The TI for both the neutral and polar lipid fractions gradually increased from January to September and then decreased in November (**Figure 1B**). The TI values for the neutral lipid fraction were significantly higher ($p<0.05$) than those of the polar lipid fraction. TI values for the polar lipid fraction ranged between 0.13 and 0.29, while those for the neutral fraction ranged between 0.6 and 1.1. The TI is associated with the risk of thrombosis, and its values exceeding 1.0 are considered hazardous to human health [Chakraborty *et al.*, 2016]. High TI values were observed for the neutral lipid fraction in May and September, reaching 1.0 and 1.1, respectively. These elevated TI values are attributed to the increased content of saturated fatty acids during the spawning season. As spawning requires significant energy, oysters utilize their lipid reserves to meet these demands. Since polar lipids contain a higher proportion of PUFAs, the TI values for the polar lipid fraction are significantly lower than those of the neutral lipid fraction.

Anticancer activity of neutral and polar lipid fractions

The anticancer activity of the polar and neutral lipid fractions extracted from *C. gigas* was evaluated against human breast cancer (MDA-MB-231), liver cancer (HepG2), and muscle rhabdomyosarcoma-A (RD) cell lines using the MTT assay. Both the polar and neutral lipid fractions of *C. gigas* exhibited weak inhibitory effects on the cell growth of the three tested cell lines. The extent of this effect was dependent on the concentration of the lipid fractions. After 72 h of treatment with the neutral and polar lipid fractions at a concentration of 100 µg/mL, the inhibition rates of the polar lipid fraction on the proliferation of HepG2 and RD cells were significantly ($p<0.05$) higher than those of the neutral lipid fraction. Inversely, the inhibition rate of the neutral lipid fraction on the proliferation of MDA-MB-231 cells was significantly ($p<0.05$) higher compared to that of the polar lipid fraction (**Figure 2**). For the polar lipid fraction, the inhibitory effect on cell proliferation decreased gradually in the following order: HepG2 cells>MDA-MB-231 cells>RD cells. In terms of the neutral lipid fraction, the inhibitory effect on the proliferation of the three tested cell lines decreased gradually in the following order: MDA-MB-231 cells>HepG2 cells>RD cells. After a 72-h treatment, the IC_{50} values of the polar and neutral lipid fractions against the three tested cell lines were both found to be above 100 µg/mL (**Table 2**). In comparison, the IC_{50} values of the doxorubicin positive control were 0.60 µg/mL, 1.35 µg/mL, and 1.40 µg/mL for MDA-MB-231, HepG2, and RD cells, respectively. The results obtained regarding the cell growth inhibitory effects of the polar and neutral lipid fractions against the three tested cell lines corresponded to the morphological changes observed in cancer cells treated with the studied samples, as

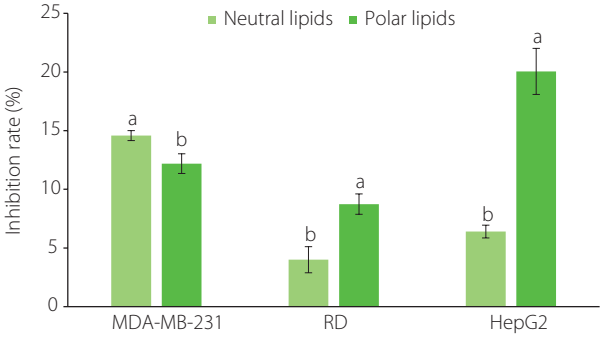

Figure 2. Inhibition rates against MDA-MB 231, RD and HepG2 cell lines treated at a concentration of 100 µg/mL for 72 h with neutral and polar lipids of *Crassostrea gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast. Different letters above bars separately for each cancer cell line indicate significant differences ($p<0.05$).

Table 2. Anticancer activity of neutral and polar lipid fractions of *Crassostrea gigas* oysters against cancer cell lines.

Lipid fraction	IC_{50} (µg/mL)		
	MDA-MB-231	RD	HepG2
Neutral lipids	>100	>100	>100
Polar lipids	>100	>100	>100
Doxorubicin*	0.60±0.04	1.40±0.10	1.35±0.04

*Doxorubicin was used as a positive control. Results are expressed as mean ± standard deviation ($n=3$).

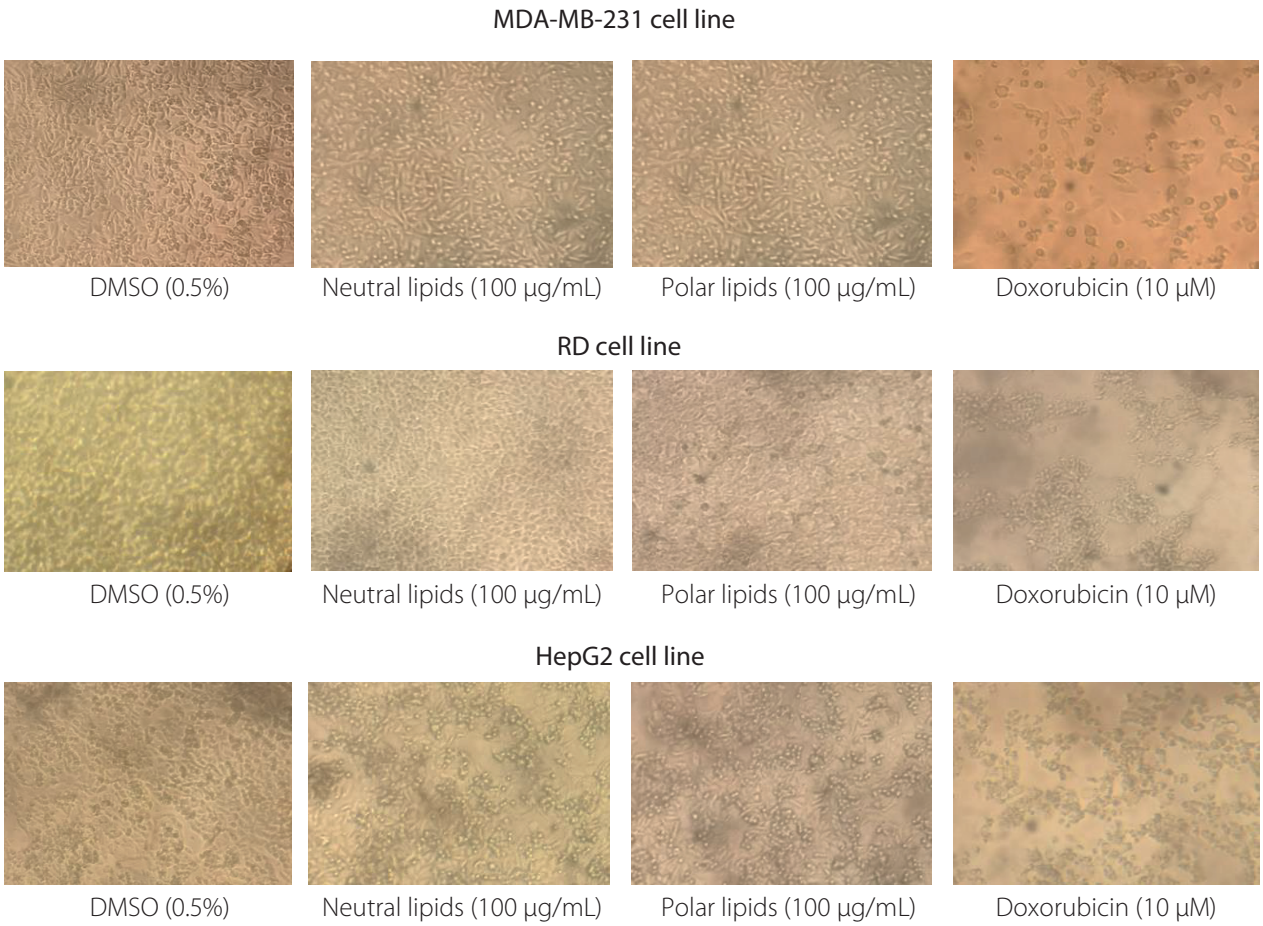

Figure 3. Morphological changes in MDA-MB 231, RD and HepG2 cell lines treated with neutral lipid and polar lipid fractions of *Crassostrea gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast.

examined through phase contrast microscopy (**Figure 3**). It is worth noting that a previous study conducted by Wang *et al.* [2017] reported similar findings. In their study, the IC_{50} value of the polar lipid fraction extracted from the brain of silver carp against MCF-7 cells was significantly lower compared to that of the total lipid and neutral lipid fractions. Additionally, consistent with our study, the IC_{50} values of all lipid fractions in their research exceeded 100 µg/mL. The IC_{50} values of the neutral lipid extracted from *Scylla paramamosain* against five cancer cell lines, including SK-LU-1 cells, HL-60 cells, HT-29 cells, HepG2 cells, and MCF7 cells, were found to be above 100 µg/mL, whereas the IC_{50} values of the polar lipid fraction ranged from 85.4 to 95.8 µg/mL [Nguyen *et al.*, 2020]. The difference in inhibitory activity of lipid fractions against various cell lines can be attributed to variations in the fatty acid composition of the lipid extract [Nappo *et al.*, 2012; Wang *et al.*, 2014].

Previous studies have demonstrated that PUFAs show cytotoxicity in cancer cells [De Gaudry *et al.*, 2014; Dekoj *et al.*, 2007; Kang *et al.*, 2010], and supplementation with DHA has been shown to significantly enhance apoptosis [Das & Das, 2016]. In this study, we observed that the cytotoxicity of the lipid fractions in the three cancer cell lines was relatively low. Nonetheless, the polar lipid fraction exhibited promising cytotoxic activity specifically against HepG2 cells. Similar findings have been reported by Nguyen *et al.* [2020], where polar lipids derived from crabs exhibited cytotoxic activity against five different cancer cell lines, including HepG2 cells. Wang *et al.* [2017] proposed that the cytotoxicity of the lipid fractions of oysters could potentially be attributed to apoptosis caused by the increased level of reactive oxygen species in cells and a consequent increase in mitochondrial membrane permeability. Apoptosis in breast cancer BT20 cells and three human pancreatic cancer cell lines induced by EPA was reported by Nappo *et al.* [2012] and Shirota *et al.* [2005], respectively. In our study, we observed that the polar lipid fraction had a higher content of EPA compared to the neutral lipid fraction. This observation could potentially explain why the growth inhibition effect of the polar lipid fraction in all three cell lines was higher than that of the neutral lipid fraction. It has been demonstrated that higher *n*3/*n*6 ratios play a beneficial role in preventing the development and progression of cancer [Aronson *et al.*, 2001].

CONCLUSIONS

In conclusion, the fatty acid composition of the neutral and polar lipid fractions obtained from *C. gigas* oysters cultured in Nha Phu Lagoon, Khanh Hoa Coast, Vietnam, exhibited variations throughout the year. The oysters harvested in May and September had a higher content of SFAs in the neutral lipid fraction and a lower content of PUFAs in the polar lipid fraction compared to oysters harvested in January, April, and November. The neutral lipid

fraction was predominantly composed of saturated fatty acids, while the polar lipid fraction contained abundant PUFAs. The polar lipid fraction, rich in DHA and EPA, displayed favorable lipid indices, such as the TI and the *n*3/*n*6 ratio, which are important in the context of the effects of lipids on human health. The polar lipid fraction extracted from oyster muscle demonstrated stronger inhibitory effects on the growth of the three cancer cell lines (HepG2, MDA-MB-231, and RD) compared to the neutral lipid fraction. This study highlights the potential of *C. gigas* oysters cultured in Khanh Hoa Coast as a valuable source of PUFA-rich lipids that are beneficial for human health. Although they exhibited weak anticancer activity, they may still hold potential in the prevention and treatment of certain cancer types.

ACKNOWLEDGEMENTS

The authors are grateful to the staff and students of the Faculty of Food Technology, Nha Trang University for their kind assistance.

RESEARCH FUNDING

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under Grant Number 106.05-2019.341.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest or competing interests.

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ORIGINAL ARTICLE
http://journal.pan.olsztyn.pl

Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 177–187
DOI: 10.31883/pjfns/188316
ISSN 1230-0322, e-ISSN 2083-6007

Extraction, Chemical Composition and Antidiabetic Potential of Crude Polysaccharides from *Centella asiatica* (L.) Urban

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The global prevalence of diabetes mellitus (DM) is escalating, posing a significant challenge to human healthcare systems. In the present study, mineral and monosaccharide compositions, and antidiabetic potential of polysaccharide fractions separated from *Centella asiatica* (L.) leaves (CAL) were assessed. Initial single-factor experiments identified key extraction parameters, further optimized through response surface methodology (RSM) with optimal conditions determined as a liquid-to-solid ratio of 24.43:1 (mL/g), extraction time of 60.76 min, and a temperature of 83.31°C. Alcohol insoluble residue (AIR) extraction yielded three crude polysaccharide fractions (P50, P70, and P90). P50 was obtained with the highest yield (14.31%) and exhibited the highest content of total sugars and uronic acids (68.01 and 24.28 g/100 g, respectively). Calcium, magnesium, and manganese were dominant minerals in P50 and P70. Monosaccharide composition analysis *via* high-performance liquid chromatography (HPLC) demonstrated enrichment of galacturonic acid, galactose, and arabinose in P50 and P70. Furthermore, both fractions (P50 and P70) exhibited dose-dependent inhibitory effects on α-amylase and α-glucosidase. At 5 mg/mL, P50 caused 68.3% α-amylase inhibition and 62.3% α-glucosidase inhibition, while P70 triggered 46.8% and 34.1% inhibition, respectively. In conclusion, this study is the first to provide valuable insights into optimizing CAL extraction conditions. Potential utilization of polysaccharide fractions obtained from CAL as components for formulating functional foods can be explored in the future.

Keywords: alcohol insoluble residue, antidiabetic, mineral elements, monosaccharide composition, response surface methodology

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder resulting from inadequate insulin production by the pancreas or ineffective utilization of the insulin produced. The International Diabetes Federation (IDF) reported a current global diabetic prevalence of 537 million individuals in 2021, with a projected increase to over 783 million by the year 2045 [Saeedi *et al.*, 2019; Sun *et al.*, 2022]. This trajectory of diabetic cases poses a substantial challenge to human health care systems worldwide. Pharmaceutical interventions like oral metformin, DPP-4 inhibitors, SGLT-2 inhibitors, GLP-1 agonists, and α-glucosidase inhibitors help to regulate blood glucose levels. However, prolonged usage

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Submitted: 18 February 2024
Accepted: 6 May 2024
Published on-line: 24 May 2024

of these drugs often leads to side effects and drug resistance [Tahrani *et al.*, 2016].

Numerous research groups are currently focusing their efforts on investigating herbs and functional foods in order to find antidiabetic molecules with reduced side effects compared to modern pharmaceutical drugs [Ahn *et al.*, 2011; Hussain *et al.*, 2022; Shahid *et al.*, 2023; Sharmin *et al.*, 2013]. Recent studies have highlighted the remarkable antidiabetic potential of polysaccharides derived from natural sources [Ji *et al.*, 2023]. Polysaccharides extracted from both traditional medicinal plants [Zheng *et al.*, 2019] and commonly consumed foods including pumpkin [Zhang *et al.*, 2013], legumes [Bai *et al.*, 2020], some fruits (*e.g.*, goji berry) [Zhu *et al.*, 2013], and green tea [Wang *et al.*, 2015] were used by researchers to manage the blood glucose in diabetic patients and animals or insulin secretion in cell lines. Polysaccharides, recognized as significant biological macromolecules, exhibit also other activities, including lipid reduction, induction of tumor cell apoptosis, immunomodulation, prebiotic effects and antioxidant properties [Kakar *et al.*, 2020; Wang Y. *et al.*, 2019; Zeng *et al.*, 2020].

Centella asiatica (L.) Urban, a member of the Apiaceae (Umbelliferae) tropical plant family, which was identified by Carl Linnaeus, is a globally distributed herb renowned for its extensive traditional and medicinal applications [Biswas *et al.*, 2021]. This botanical species is indigenous to South and Southeast Asian nations, including India, China, Sri Lanka, Indonesia, and Malaysia. It is also naturally abundant in South Africa and Madagascar [Hamid *et al.*, 2002]. Traditionally, *C. asiatica* is recognized for its culinary usage in salads and juices. It has gained prominence in the global herbal market, primarily due to its multifaceted medicinal potential [Biswas *et al.*, 2021]. Notably, previous research studies have explored the health-promoting properties of *C. asiatica* through extracts, polyherbal formulations, and purified compounds. However, no research study has investigated the potential bioactivity of polysaccharides derived from *C. asiatica* leaves. Therefore, the present study optimized the extraction process and investigated the composition, and antidiabetic potential of *C asiatica* leaves (CAL) crude polysaccharides, and explored the potential of *C. asiatica* polysaccharides as an antidiabetic functional food product to be used in the future.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals used were of the highest available grade. Sodium carbonate, monosaccharide standards (glucose, fructose, galactose, arabinose, rhamnose, xylose, mannose), glucuronic and galacturonic acids, α-glucosidase, α-amylase, and soluble starch were procured from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China). 4-Nitrophenyl-α-D-glucopyranoside (PNPG) and 3,5-dinitrosalicylic acid (DNS) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Acarbose and soluble starch were sourced from Beijing Biotopped Science & Technology Co., Ltd. (Beijing, China). Sodium hydroxide, trifluoroacetic acid (TFA), hydrochloric acid, 1-phenyl-3-methyl-5-pyrazolone (PMP), and phosphate buffered saline (PBS) were purchased from Nanjing Chemical Material Corp (Nanjing, China).

Sample preparation

Centella asiatica (L.) Urban leaves (CAL) were collected in October 2022 from the local farm in Semenyih, Selangor, Malaysia. The specimen of CAL was taxonomically identified, and its voucher was deposited at the herbarium of the Universiti Kebangsaan Malaysia, Bangi, Malaysia. The freshly collected leaves were dried (50°C), pulverized into a fine powder using a high-powered mill (Model SF-2000, Chinese Traditional Medicine Machine Works, Shanghai, China), and then sifted through a 40-mesh sieve. The materials were stored in a desiccator at ambient temperature until needed.

Optimization of extraction conditions

Single-factor experiment

For the extraction process of CAL powder, three critical factors were considered: liquid (water) to solid ratio (mL/g), extraction temperature (°C), and extraction time (min). Each factor was investigated at five distinct levels, precisely: extraction temperatures (A) at 60°C, 70°C, 80°C, 90°C and 100°C; extraction times (B) at 30 min, 40 min, 50 min, 60 min, and 70 min; and liquid-to-solid ratio (C) at 15:1, 20:1, 25:1, 30:1 and 35:1. The crude extract was separated from the residue by centrifugation (3,000×*g*, 20 min) and dried until a constant weight had been achieved. The extraction yield was calculated using the following formula (1):

$$\text{Extraction yield (\%)} = \frac{\text{Crude extract weight (g)}}{\text{CAL powder weight (g)}} \times 100\ \% \quad (1)$$

Response surface methodology

Based on the results of single-factor experiments, a three-level model was employed, comprising 17 experimental runs designed using the response surface methodology (RSM) with Box-Behnken design (BBD) to optimize extraction conditions. The experimental configurations were generated using the Design Expert 12 (State-Ease Inc., Minneapolis, MN, USA) software. Regression analyses of the experimental data were conducted and a non-linear quadratic model was fitted using the following formula (2) [Su *et al.*, 2022]:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where: Y is the estimated response, X_i and X_j are the independent variables, β_{ij} is the interaction term, β_{ii} is the quadratic coefficient, β_i is the linear coefficient, and β_0 is the intercept.

Aqueous extract preparation

The aqueous extract of CAL was prepared under the conditions used the in single-factor experiment, giving the highest extraction yield, and at the same time as close as possible to those optimized by RSM, *i.e.*, 50 g CAL powder was suspended in water at a ratio of 25:1 (*v/w*) and extracted for 60 min at 80°C. The mixture was than centrifuged, and supernatant was separated. The extraction process was conducted three times.

Preparation of polysaccharide fractions

The CAL aqueous extracts were treated with ethanol to obtain alcohol-insoluble residues (AIR). Solvent was sequentially added to the extract to achieve final concentrations of 50% (*v/v*), 70% (*v/v*) and then 90% (*v/v*). Mixtures were left to stand for 24 h at room temperature to precipitate AIRs from which, after deproteinization, polysaccharide fractions designated as P50, P70, and P90, respectively, were obtained. Protein was removed by the Sevag method [Yao *et al.*, 2020]. The resulting AIRs and Sevag reagent (chloroform and butyl alcohol mixture, 4:1, *v/v*) were thoroughly mixed in a shaker, vigorously shaken for 20 min, centrifuged at 1,500×*g* for 10 min, and the supernatants were collected. The procedure was repeated five times. The deproteinized solutions were re-precipitated in anhydrous ethanol at ten times the solution volume. The precipitates were collected by centrifugation (3,000×*g*, 20 min) and air-dried at 50°C until a constant weight had been achieved. The dried precipitates – polysaccharide fractions – were then stored in a dryer until use. The yield of polysaccharide fractions was calculated using the following formula (3):

$$\text{Fraction yield (\%)} = \frac{\text{Fraction weight (g)}}{\text{CAL powder weight (g)}} \times 100\% \quad (3)$$

Determination of contents of total sugars, uronic acids, and proteins

Total sugar content in the CAL polysaccharide fractions (P50, P70 and P90) was determined by the phenol-sulfuric acid method using D-glucose as a standard, as described by Yue *et al.* [2022]. The absorbance of the reaction mixture was read at 492 nm using a plate reader (Biochrom EZ Read 800 Microplate Reader, Cambridge, UK). The uronic acid content of P50, P70, and P90 was determined by the carbazole sulfuric acid method [Li *et al.*, 2007], using D-galacturonic acid as a reference substance. In addition, protein content was analyzed using the Bradford's method, with bovine serum albumin serving as the reference [Bradford, 1976]. The contents of total sugars, uronic acids, and proteins were expressed in g *per* 100 g of CAL polysaccharide fraction.

Mineral composition analysis

The mineral composition of CAL polysaccharide fractions (P50 and P70). was determined using a flame atomic absorption spectrometer (GGX-600, Beijing Haiguang Instrument Co., Ltd., Beijing, China) following the method outlined by Santos *et al.* [2014]. CAL polysaccharide fraction samples (0.20 g) were digested overnight with 10 mL of mixed acids (nitric acid and perchloric acid in a ratio of 4:1, *v/v*) and then, at 120°C for 3 to 5 h until the solution became transparent and colorless. The digested solution was transferred to a 25-mL volumetric flask, 2 mL of concentrated hydrochloric acid was added, and finally the flask was filled up with distilled water. Then, the atomic absorption spectrometry measurements were performed. The contents of magnesium, manganese, copper, zinc, calcium, iron, and selenium were determined, and respective results were expressed in mg *per* kg of the polysaccharide fraction.

Monosaccharide composition analysis

High-performance liquid chromatography (HPLC) was used to determine the monosaccharide composition of the P50 and P70 according to a previously described method [Chen *et al.*, 2018] with slight modifications. Initially, 20 mg of the fractions were hydrolyzed at 100°C for 6 h using 2 mL of a 4 M trifluoroacetic acid (TFA) solution. The hydrolysates were diluted with anhydrous ethanol, and the solvents were evaporated at 45°C under vacuum. A new portion of ethanol was added to the solids, and then evaporation was repeated. This procedure was repeated several times until the hydrolysates were neutral. After hydrolysis, the monosaccharides of the P50 and P70 were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP). Briefly, the hydrolysates were mixed with 1 mL of a 0.5 M PMP solution in methanol and 0.5 mL of a 0.4 M NaOH solution and reacted at 80°C for 60 min. After centrifugation, 0.4 M HCl (0.5 mL) and distilled water were added to the supernatant. Chloroform was then used to remove the excess PMP from the supernatant. The aqueous phase was filtered through a 0.45-µm membrane and taken for HPLC analysis.

The composition of monosaccharides was analyzed using the Shimadzu HPLC system (Model 2010AHT, Kyoto, Japan). A UV-Vis detector (SPD-20A, Shimadzu) was employed with the wavelength set at 250 nm. The chromatographic column used was an Athena-C18 (4.6×250 mm, 5 µm, ANPEL Laboratory Technologies, Shanghai, China). Separation was done with a solvent flow rate of 1 mL/min at room temperature. Mobile phase consisted of a 0.05 M ammonium acetate buffer (A), and a mixture of acetonitrile and water in a ratio of 80:20 (*v/v*) (B). The injection volume was 10 µL. The monosaccharides were identified by comparing the retention times of the analyte peaks in the sample to those of known standards analyzed under the same chromatographic conditions. The relative content (%) of individual monosaccharides of CAL polysaccharide fractions was calculated based on peak areas.

Analysis of antidiabetic potential

The antidiabetic potential of CAL polysaccharide fractions was assessed based on the ability of the fraction compounds to inhibit α-amylase and α-glucosidase activity. The α-amylase inhibitory activity of the CAL polysaccharide fractions was determined by using the iodine-starch method, following the protocol described by Wu *et al.* [2020]. Briefly, 100 µL of CAL polysaccharide fraction solutions with different concentrations (1 to 5 mg/mL) were combined with an α-amylase solution (100 µL, 0.5 mg/mL) prepared in 0.2 M phosphate buffered saline (PBS, pH 6.9). After incubation at 37°C for 10 min, 200 µL of a 1% soluble starch solution in 0.2 M PBS (pH 6.9) was added. Following another 10-min incubation at 37°C, a 3,5-dinitrosalicylic acid (DNS) solution (200 µL) was introduced. The mixture was then heated at 100°C for 10 min to deactivate α-amylase. Subsequently, the mixture was diluted with 4 mL of distilled water, and the absorbance was measured at 540 nm using a microplate reader (SpectraMax M3, Molecular Devices, San Jose. CA, USA). Acarbose served as the positive control in this assay.

The α-amylase activity inhibition (%) by CAL polysaccharide fractions was calculated using the formula (4):

$$\text{Inhibition (\%)} = \frac{[(A_{cl} - A_{bl\text{-}cl}) - (A_{spl} - A_{spl\text{-}cl})]}{(A_{cl} - A_{bl\text{-}cl})} \times 100 \quad (4)$$

where A_{cl} and $A_{bl\text{-}cl}$ are the absorbances of reaction mixtures giving 100% enzymatic activity and 0% enzymatic activity, respectively; A_{spl} and $A_{spl\text{-}cl}$ are the absorbances of the reaction mixture with the CAL polysaccharide fraction and the CAL polysaccharide fraction itself, respectively.

The inhibitory potential of CAL polysaccharide fractions against α-glucosidase was evaluated using a method described by Salahuddin *et al.* [2020] with adjustment. An aliquot of 200 µL of CAL polysaccharide fraction solutions (concentration in range of 1 to 5 mg/mL) was mixed with 1.2 mL of 0.1 M PBS (pH 6.8) and 200 µL of an α-glucosidase solution (0.24 U/mL in 0.1 M PBS, pH 6.8). The mixture was incubation at 37°C for 10 min, and then, 200 µL of a 2.5 mM 4-nitrophenyl-α-D-glucopyranoside (PNPG) solution in the same PBS was added. After incubation at 37°C for 10 min, 0.8 mL of a 0.2 M sodium carbonate solution, was pipetted in. The absorbance of the mixture was recorded at 405 nm. Acarbose served as the positive control. The α-glucosidase activity inhibition (%) by CAL polysaccharide fractions was calculated according to formula (4) given above.

Statistical analysis

Differences among values were assessed using one-way analysis of variance (ANOVA) in SPSS 23.0 (IBM SPSS, Armonk, NY: IBM Corp., USA), and graphing was performed using Origin2021 (OriginLab Corp., USA). Duncan's multiple range test was employed for multiple comparisons. The results were presented as mean and standard deviation (SD). Statistical significance was set at a p-value less than 0.05. All measurements were conducted in triplicate.

RESULTS AND DISCUSSION

Single-factor experiment

The results of single-factor extraction revealed that the liquid-to-solid ratio, extraction temperature, and extraction time significantly influenced the yield of CAL extraction. The optimal conditions were determined as a liquid-to-solid ratio of 25:1 (mL/g), an extraction temperature of 80°C, and an extraction time of 60 min (**Figure 1**). These moderate conditions of extraction time and temperature seem safe for preserving CAL polysaccharides. As previously documented, higher temperatures and extended extraction times may enhance polysaccharide yield; however, they may induce structural changes in polysaccharides [Su *et al.*, 2022; Zhang *et al.*, 2015].

Extraction optimization using response surface methodology

To optimize the extraction yield of CAL, an RSM optimization experiment was conducted with a three-factor, three-level design. Traditional approaches, such as orthogonal testing, are commonly used in similar studies; however, RSM mitigates random errors in experiments and enables continuous analysis of experimental levels, thereby generating a continuous predictive model [Cai *et al.*, 2019; Su *et al.*, 2022]. The experimental design of the present study, yielding a binary multiple equation (5) to model the relationship between the CAL extraction yield (Y) and the three influencing factors was as follows:

$$Y = 14.03 + 0.78A + 0.5613B - 0.3563C - 0.87AB + 0.55AC + \\ + 0.3525BC - 0.9048A^2 - 1.23B^2 - 1.24C^2 \quad (5)$$

where: Y is extraction yield of CAL, A signifies the liquid-to-solid ratio, B denotes the extraction temperature, and C represents the extraction time.

Optimal extraction parameters for CAL polysaccharides were identified as a liquid-to-solid ratio of 24.43:1 (mL/g), an extraction time of 60.76 minutes, and an extraction temperature of 83.31°C (**Table 1**). The optimization model was well fitted to experimental data, as indicated by an F-value of 66.84 (p<0.0001), a lack of fit value of 0.2973 (p>0.05), and a determination coefficient (R^2) of 0.9885 (**Table 2**). The coefficient of variation (cv) was found to be 1.83%. Furthermore, response surface diagrams and contour maps were generated using a multiple quadratic

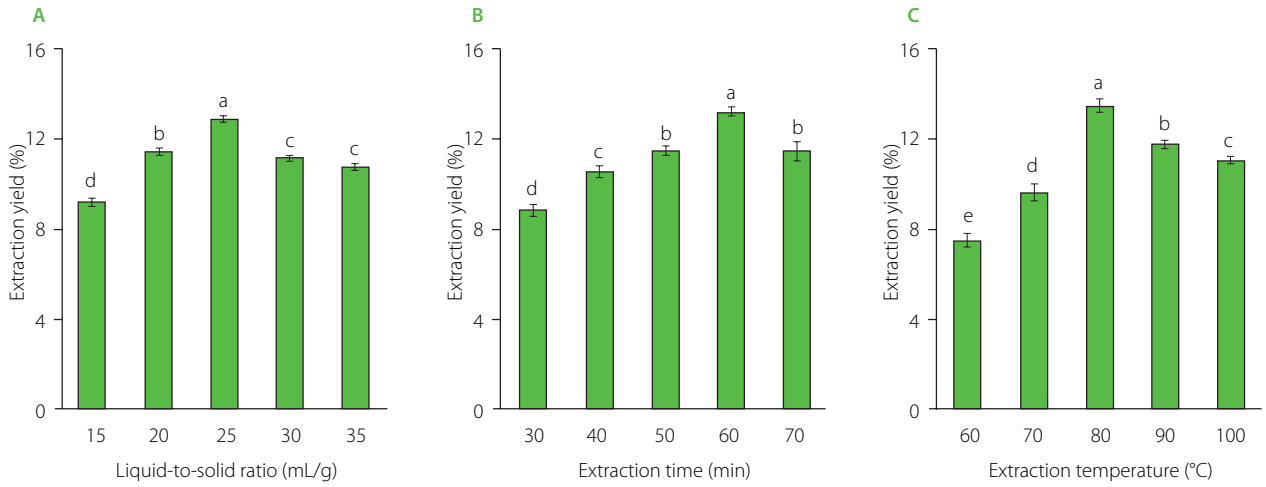

Figure 1. Extraction yield of *Centella asiatica* leaves (CAL) at different liquid-to-solid ratios (**A**), extraction times (**B**) and extraction temperatures (**C**). Different letters above the bars indicate significant differences (p<0.05).

Table 1. Box-Behnken design including factors (liquid-to-solid ratio, temperature and time), and response (extraction yield) used to optimize the *Centella asiatica* leaves (CAL) extraction.

Number	Liquid-to-solid (mL/g)	Temperature (°C)	Time (min)	Predicted extraction yield (%)	Actual extraction yield (%)
1	25	80	60	13.88	13.91
2	20	80	50	11.64	11.57
3	30	90	60	12.74	12.91
4	30	80	70	12.34	12.27
5	20	70	60	12.07	11.97
6	25	90	70	12.28	12.18
7	30	80	50	10.26	10.37
8	25	90	50	12.93	12.87
9	30	70	60	9.88	9.94
10	25	70	50	9.67	9.88
11	25	70	70	12.51	12.67
12	25	80	60	13.98	14.07
13	25	80	60	14.10	14.19
14	25	80	60	13.94	13.89
15	20	90	60	12.65	12.74
16	20	80	70	12.18	12.06
17	25	80	60	14.01	14.10

Table 2. Data of analysis of variance for a regression model used in response surface methodology (RSM) for *Centella asiatica* leaves (CAL) extraction.

Source	Sum of squares	df	Mean square	F-value	*p*-Value
Model	31.23	9	3.47	66.84	<0.0001
A	4.87	1	4.87	93.75	<0.0001
B	2.52	1	2.52	48.54	0.0002
C	1.02	1	1.02	19.56	0.0031
AB	3.03	1	3.03	58.32	0.0001
AC	1.21	1	1.21	23.31	0.0019
BC	0.497	1	0.497	9.57	0.0175
A^2	3.45	1	3.45	66.39	<0.0001
B^2	6.34	1	6.34	122.15	<0.0001
C^2	6.45	1	6.45	124.15	<0.0001
Residual	0.3634	7	0.0519		
Lack of fit	0.2973	3	0.0991	6	0.0581
Pure error	0.0661	4	0.0165		
Cor total	31.59	16			
R^2	0.9885				
Adjusted R^2	0.9737				
Predicted R^2	0.8462				
cv (%)	1.83				
Adeq. precision	24.8538				

A, extraction temperature; B, extraction time; C, liquid-to-solid ratio; R^2, determination coefficient; cv, coefficient of variation.

regression model (**Figure 2**). The steepness of the response surface directly correlated with response sensitivity, and contour maps revealed the strength of interactions [Wang L. *et al.*, 2019]. Notably, contour lines plotted for the liquid-to-solid ratio, extraction time, and temperature exhibited an oval pattern, indicating their significant interactions (**Figure 2**).

Fraction yield, and contents of total sugars, uronic acids, and proteins

Following the CAL aqueous extraction, polysaccharide precipitation from crude extract was carried out, resulting in three polysaccharide fractions termed P50, P70, and P90. The results demonstrated significantly highest P50 fraction yield (14.31%), followed

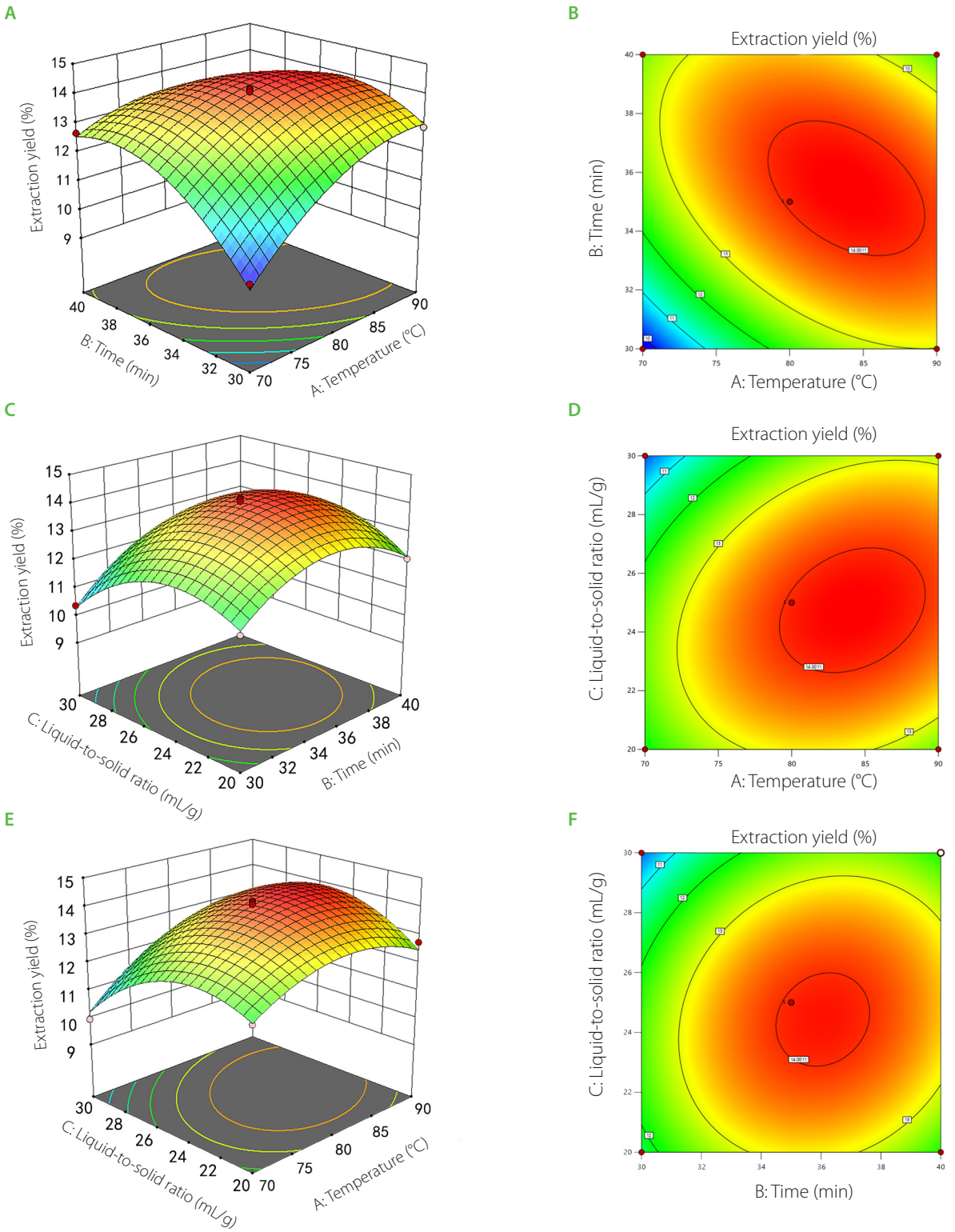

Figure 2. Response surface plots (**A**, **C** and **E**) and contour plots (**B**, **D** and **F**) showing the effects of extraction time, liquid-to-solid ratio, and extraction temperature on the extraction yield of *Centella asiatica* leaves.

Table 3. Fraction yield and content of total sugars, uronic acids, and proteins in *Centella asiatica leaves* (CAL) polysaccharide fractions (P50, P70 and P90).

Fraction	Yield (%)	Total sugar (g/100 g)	Uronic acid (g/100 g)	Protein (g/100 g)
P50	14.31±0.02^{a}	68.01±0.15^{a}	24.28±0.09^{a}	1.19±0.03^{a}
P70	4.75±0.04^{b}	37.38±0.09^{b}	11.23±0.13^{b}	1.24±0.01^{a}
P90	1.11±0.02^{c}	6.52±0.11^{c}	1.22±0.03^{c}	1.04±0.03^{b}

Each value is expressed as means ± standard deviation. Means with different letters within a column are significantly different (p<0.05).

by P70 and P90, respectively (**Table 3**). Moreover, the contents of total sugars and uronic acids (68.01 and 24.28 g/100 g, respectively) were also found to be higher in the P50 fraction of CAL, followed by P70 and P90. It was noted that the P90 fraction had the lowest yield and contents of total sugars, uronic acids, and proteins. Therefore, this fraction was excluded, and only the P50 and P70 fractions were subjected to downstream experiments.

Mineral and monosaccharide composition

The results of mineral content determination demonstrated that the CAL polysaccharide fractions (P50 and P70) had a high level of calcium, followed by magnesium and a low level of copper, and selenium (**Table 4**). Overall, the mineral content in both fractions (P50 and P70) was found to be in the order of calcium > magnesium > manganese > zinc > iron > copper > selenium. The analysis of the mineral composition of food and related products is essential for comprehending their nutritional significance, as these elements play a pivotal role in maintaining the health of both humans and animals [Jomova *et al.*, 2022]. Several minerals and trace elements have been recognized for their significance in antihyperglycemic effect of plants traditionally used in the treatment of diabetes. These include sodium, potassium, magnesium, calcium, iron, copper, zinc, and others [Gholamhoseinian *et al.*, 2020]. Moreover, essential trace elements like zinc, magnesium, manganese, and chromium play roles in insulin synthesis and secretion, and their levels are influenced by diabetes [Asif, 2017]. The results suggest that the CAL polysaccharide fractions hold a significant nutritional value as they are rich in essential mineral elements, particularly magnesium, calcium, and manganese. Moreover, these elements may be linked to their potential anti-hyperglycemic effects.

Table 4. Content of minerals (mg/kg) in *Centella asiatica* leaves (CAL) polysaccharide fractions (P50 and P70).

Element	P50	P70
Magnesium	12,846±37^{A}	12,408±30^{B}
Manganese	4,005±12^{A}	2,722±10^{B}
Copper	5.32±0.45^{A}	6.01±0.22^{A}
Zinc	397.0±5.2^{A}	192.3±3.3^{B}
Calcium	269,839±71^{B}	296,430±60^{A}
Iron	143.9±4.2^{A}	100.1±4.0^{B}
Selenium	0.28±0.04^{A}	0.24±0.04^{A}

Each value is expressed as means ± standard deviation. Means with different letters within a row are significantly different (p<0.05).

The HPLC separation of monosaccharides derived from polysaccharides of P50 and P70 fractions are shown in **Figure 3**. Eight compounds were identified: mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, and arabinose. **Table 5** provides the monosaccharide relative contents of P50 and P70 fractions. In P50, the predominant monosaccharides were galacturonic acid (24.55%) and galactose (20.55%). Similarly, in P70, a higher share of galactose (16.15%) and galacturonic acid (15.93%) was found in the composition of monosaccharides. The monosaccharide composition of various plant extracts has been observed to impact their inhibition potential against α-amylase and α-glucosidase. For instance, polysaccharide fractions derived from sesame seed hulls, particularly those rich in rhamnose, glucose, glucuronic acid, and galacturonic acid, have caused significant inhibition of both enzymes [Darwish *et al.*, 2023]. Deng *et al.* [2020] found that a polysaccharide from *Chaenomeles speciosa* seeds, composed of rhamnose, glucuronic acid, galacturonic acid, and arabinose, proved to be an effective inhibitor of α-amylase and α-glucosidase.

Antidiabetic potential

The key ones among the human enzymes responsible for the digestion of carbohydrates include glucosidase from the small intestine and pancreatic α-amylase. α-Amylase catalyzes the breakdown of long-chain carbohydrates, while α-glucosidase in the intestinal brush border is vital for oligosaccharide digestion [Hu *et al.*, 2018]. The inhibition of α-amylase and α-glucosidase activities significantly impedes glucose conversion into bloodstream glucose, attenuating postprandial blood glucose levels.

The results of the present study demonstrated concentration-dependent inhibition of α-amylase activity by CAL polysaccharide fractions (**Figure 4**). Acarbose, the positive control, exhibited the highest inhibitory potential (82.7% at 5 mg/mL). Notably, the P50 fraction demonstrated the most substantial inhibitory effect at 5 mg/mL, yielding 68.3% inhibition, while the P70 fraction displayed a 46.8% inhibitory potential at the same concentration against α-amylase. A previous study by Wu *et al.* [2020] investigated polysaccharides from *Rosa roxburghii* Tratt leaves and reported half inhibitory concentrations (IC_{50}) values of 10.16 mg/mL and 8.58 mg/mL for two polysaccharides, RLP-1.2 and RLP-2.1, respectively. Similarly, another relevant study by Fang *et al.* [2020] on *Mentha haplocalyx* polysaccharides reported an IC_{50} value of 11.65 mg/mL. Moreover, *Schizophyllum*

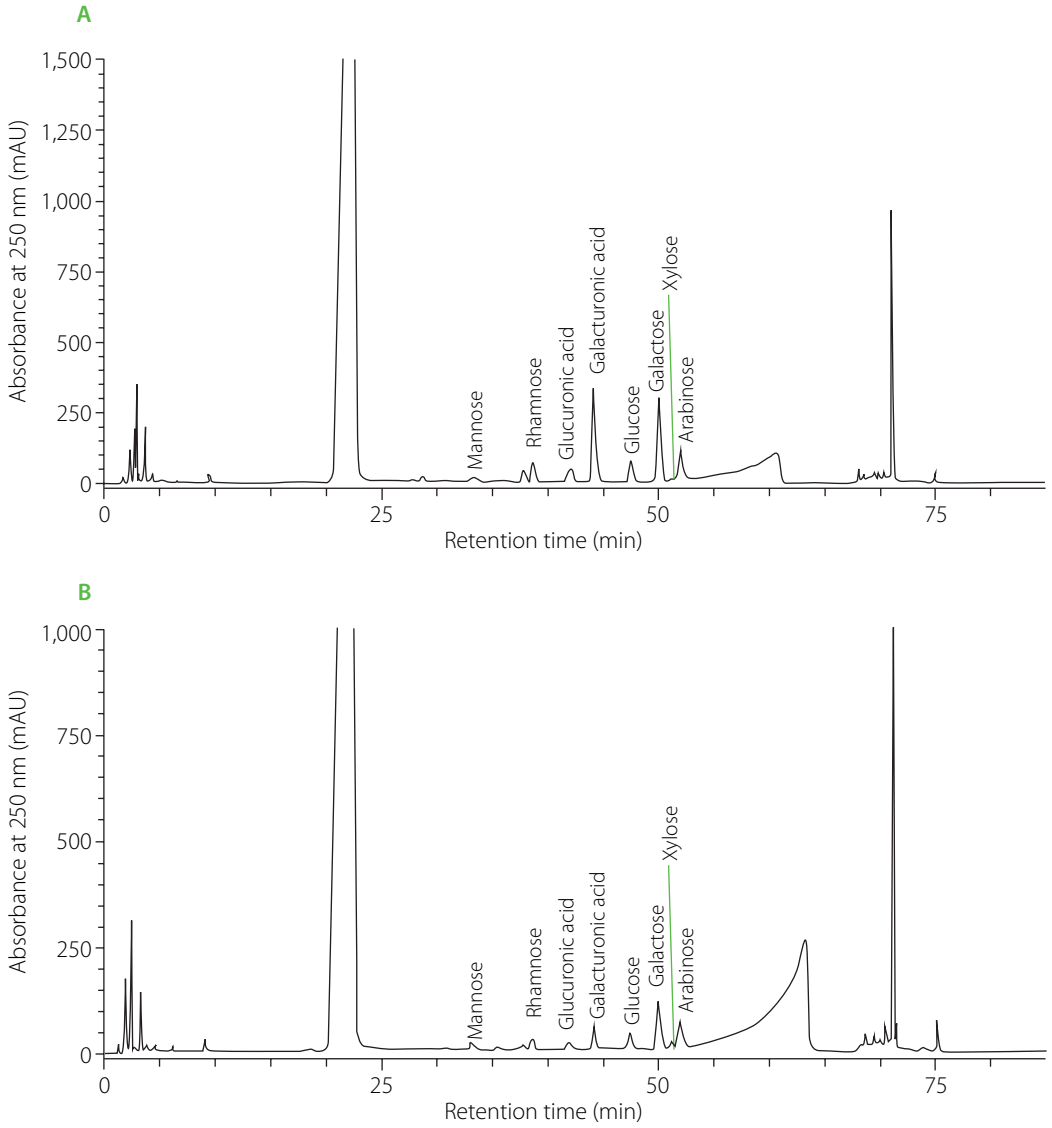

Figure 3. High-performance liquid chromatography (HPLC) separation of monosaccharides derived from polysaccharides of *Centella asiatica* leaf (CAL) fractions – P50 (**A**) and P70 (**B**).

Table 5. Relative content (%) of monosaccharides derived from polysaccharides of *Centella asiatica* leaves (CAL) fractions (P50 and P70).

Monosaccharide	P50	P70
Mannose	2.09 ± 0.09^{dB}	2.36 ± 0.27^{dA}
Rhamnose	9.71 ± 0.27^{cA}	3.75 ± 0.13^{dB}
Glucuronic acid	9.00 ± 0.11^{cA}	6.23 ± 0.21^{cB}
Galacturonic acid	24.55 ± 0.43^{aA}	15.93 ± 0.37^{aB}
Glucose	10.01 ± 0.12^{cA}	6.74 ± 0.19^{cB}
Galactose	20.55 ± 0.21^{aA}	16.15 ± 0.29^{aB}
Xylose	1.52 ± 0.13^{dB}	2.25 ± 0.09^{dA}
Arabinose	14.79 ± 0.23^{bA}	9.26 ± 0.19^{bB}

Each value is expressed as means ± standard deviation. Means with different letters a-d within a column and different letters A and B within a row are significantly different ($p<0.05$).

commune polysaccharide, when tested at 10.0 mg/mL, exhibited 55.89% inhibition against α-amylase [Chen Z. *et al.*, 2020]. Notably, our results surpassed these previous findings, indicating that P50 and P70 fractions may harbor active polysaccharides with potent α-amylase inhibitory properties.

Similar to the α-amylase inhibition potential, P50 and P70 also demonstrated concentration-dependent inhibition of α-glucosidase activity (**Figure 4**). Acarbose exhibited the highest inhibitory potential (81.7% at 5 mg/mL). Between CAL polysaccharide fractions, P50 showed a stronger inhibitory effect against α-glucosidase at 5 mg/mL (62.3%), than P70 (34.1%) (**Figure 4**). Wu *et al.* [2020] previously studied polysaccharides from *Rosa roxburghii* Tratt. leaves, reporting IC_{50} values for RLP-1.2 and RLP-2.1 against α-glucosidase at 6.29 mg/mL and 9.59 mg/mL, respectively. Another study on ginger stems and leaves reported an IC_{50} value of 7.66 mg/mL for polysaccharides obtained using hot water extraction [Chen X. *et al.*, 2020]. The α-glucosidase inhibition activity of polysaccharides extracted from CAL has not been previously investigated, the results of the current study surpass those achieved in the aforementioned previous studies.

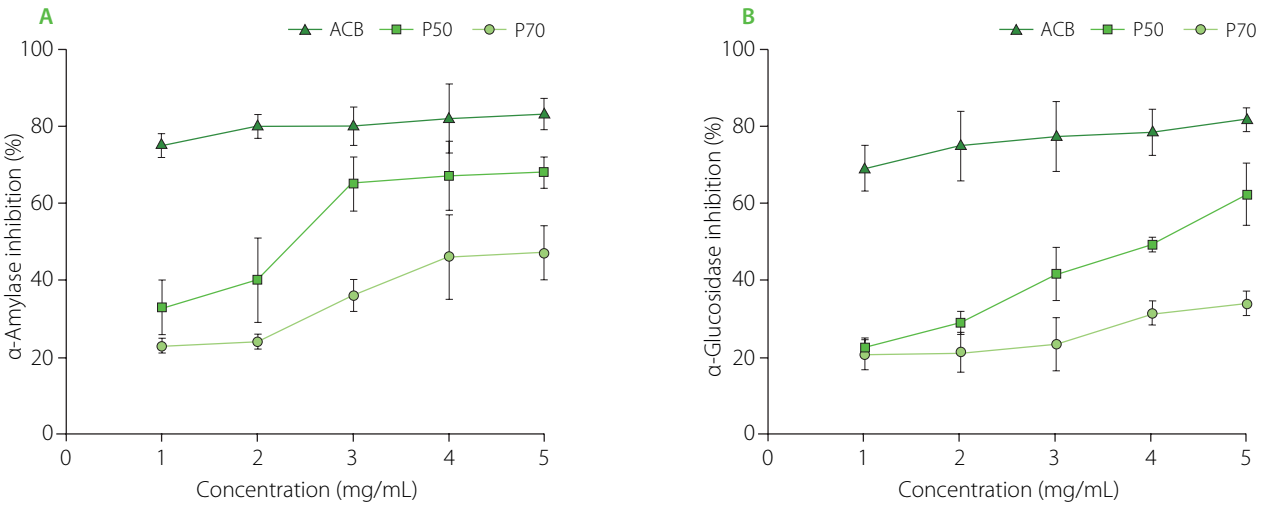

Figure 4. Inhibition of α-amylase (**A**) and α-glucosidase (**B**) activity by *Centella asiatica* leaf (CAL) polysaccharide fractions (P50 and P70). The results are presented as mean and standard deviation (*n*=3). ACB; acarbose.

At each concentration (ranging from 1 to 5 mg/mL), P50 exhibited significantly higher inhibition of α-amylase and α-glucosidase compared to P70 (**Figure 4**). Previous research demonstrated that polysaccharides extracted from *Hovenia dulcis* (HDPs) using the accelerated solvent extraction technique (ASE-HDPs) exhibited greater α-amylase inhibitory activity than those extracted using the hot water method (HWE-HDPs), which was potentially attributed to the lower molecular weight of ASE-HDPs compared to HWE-HDPs [Yang *et al.*, 2019]. Furthermore, the presence of hydroxyl and carboxyl groups on the branched chains of polysaccharides allows them to interact with amino acid residues of digestive enzymes, thereby reducing α-amylase activity [Nie *et al.*, 2017]. Moreover, inorganic trace elements, such as zinc and manganese, were shown to exhibit antidiabetic properties, contributing to the efficacy of medicinal herbs [Lachkar *et al.*, 2021]. Therefore, the elevated α-amylase and α-glucosidase inhibition activity observed in P50 could also be attributed to its higher content of elements such as zinc and manganese, as well as uronic acid.

Acarbose, used in the treatment of type 2 diabetes, may lead to side effects such as abdominal bloating due to its potent inhibitory activity on starch hydrolase, causing the accumulation of undigested carbohydrates [Deng *et al.*, 2015]. These undigested carbohydrates can undergo fermentation by bacteria in the colon, resulting in the production of gas. Therefore, identifying a new inhibitor of α-amylase and α-glucosidase with a lower inhibitory activity than acarbose would be preferable. This study is the first to reveal the significant α-amylase- and α-glucosidase-inhibiting potential of CAL polysaccharides, suggesting their applicability as nutraceutical food ingredients for diabetes management.

CONCLUSIONS

In this study, the parameters for optimizing the extraction of crude polysaccharides from *Centella asiatica* leaves (CAL) were determined using the response surface methodology (RSM). Subsequent analysis of the extracted polysaccharides revealed the presence of significant amounts of total sugars, with distinct variations in uronic acid and protein content among different fractions (P50, P70, and P90). The mineral composition demonstrated that CAL polysaccharide fractions had a high level of calcium, followed by magnesium and manganese. Among monosaccharides, galacturonic acid and galactose were dominant in the P50 and P70 fractions. Furthermore, both P50 and P70 fractions demonstrated concentration-dependent inhibition of α-amylase and α-glucosidase, suggesting their potential as nutraceutical food ingredients. Future studies could explore the underlying mechanisms of their antidiabetic effects and assess their safety and efficacy in *in vivo* models, paving the way for the development of novel and sustainable therapeutic interventions for diabetes.

RESEARCH FUNDING

This study was supported by Academic New Talent Training program, Xinyang Agriculture and Forestry University, Evaluation of characteristic germplasm resources and selection of good germplasm of wild oil tea in Dabie Mountain, Henan Province, China (242102110247), INTI International University Seed grant (INTI-FHLS-01-26-2023) and Fundamental Research Grant Scheme (FRGS), Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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Polish Journal of Food and Nutrition Sciences
2024, Vol. 74, No. 2, 188–196
DOI: 10.31883/pjfns/188615
ISSN 1230-0322, e-ISSN 2083-6007

Application of Flour Blends from Modified Cassava and Suweg Flours in Gluten-Free Steamed Brownies

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This study aimed to formulate flour blends from modified cassava flour (MCF) and suweg flour (SF) and use them to produce gluten-free steamed brownies (GFSB) with desirable qualities. The blends varied in the proportions of MCF and SF by weight, namely 80:20 (F1), 70:30 (F2), and 60:40 (F3). Wheat flour-based steamed brownies (WFSB) served as the control. The results showed that the MCF to SF ratio significantly influenced various parameters of flour blends, such as chemical compositions, color characteristics, pasting, and functional properties. Higher SF content in the blend decreased peak viscosity, breakdown, and setback. Water absorption capacity also decreased as the MCF to SF ratio in the flour blend increased. The color of flour blends with an increasing proportion of SF was getting darker and more different from the color of wheat flour. GFSB exhibited lower volume expansion than WFSB. Increasing SF content in the flour blend increased the texture parameters of GFSB including hardness, cohesiveness, gumminess, and chewiness. Values of color difference, representing color variation between gluten-free and control brownies, ranged from 2.30 to 6.32, where the GFSB-F1 was more similar in color to WFSB. Preference levels for color, aroma, and texture of GFSB did not significantly differ from WFSB. However, GFSB-F2 was preferred in taste and overall acceptance over WFSB. The utilization of modified cassava flour and suweg flour blends in gluten-free steamed brownies offers a promising avenue for diversifying gluten-free baking options.

Keywords: *Amorphophallus paeoniifolius*, flour blends, gluten-free steamed brownies, hedonic sensory test, *Manihot esculenta,* pasting properties, texture parameters

INTRODUCTION

Wheat flour, containing gluten, is commonly used in bakery and pasta items due to its unique properties. The presence of gluten within wheat flour contributes to the strength and elasticity of dough, resulting in a well-structured final product. Gluten is comprised of two main protein fractions: glutenins and gliadins [Kamal *et al.*, 2023]. Glutenins contain intra-molecular disulfide bonds in their structure, their structure tends to be linear, and this protein fraction is insoluble in alcohol. While gliadins, soluble in alcohol, have inter- and intra-molecular bonds and their structure tends to be spherical and dense [Li *et al.*, 2021]. Gliadins and glutenins bind and make the dough more elastic, thus facilitating the forming process and making the dough fluffier [Li *et al.*, 2020]. Apart from its advantages, gluten can trigger celiac or proximal enteropathic conditions associated with reversible immune reactions. Individuals with celiac disease experience inflammation in the small intestine due to an adverse response to gluten ingestion, damaging the intestinal lining [Zerbini *et al.*, 2024]. In Indonesia, wheat flour is extensively utilized in diverse food preparations, serving as a significant source of calories.

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Submitted: 14 March 2024
Accepted: 13 May 2024
Published on-line: 24 May 2024

However, individuals with autoimmune disorders, gluten ataxia, wheat allergy, non-celiac gluten sensitivity (NCGS), and dermatitis herpetiformis must also avoid gluten-containing foods [Singla *et al.*, 2024]. Hence, substituting wheat flour with gluten-free alternatives becomes imperative.

Certain gluten-free indigenous tubers include cassava and suweg. Cassava, scientifically known as *Manihot esculenta* Crantz., is a tropical plant species native to regions across Asia, Africa, and South America [Burns *et al.*, 2012]. Indonesia is the largest cassava producer in the world. Among its various uses, cassava can be processed into cassava flour. However, utilizing gluten-free flour as a replacement for wheat flour poses several challenges. Typically, products made from gluten-free flour lack the desirable characteristics found in wheat flour-based products. One strategy to broaden the application of gluten-free flours involves modifying the starch in the flour. Several studies into the chemical, physical and microbial modifications of starch structure aimed to improve its characteristics have been reported [Cahyana *et al.*, 2020; Li *et al.*, 2023; Liu *et al.*, 2022; Magallanes-Cruz *et al.*, 2023; Marta *et al.*, 2019a,c; Marta *et al.*, 2022; Xu *et al.*, 2021; Zhang *et al.*, 2021; Zheng *et al.*, 2020]. In the case of cassava flour, Liu *et al.* [2022] produced modified cassava flour (MCF) by fermentation. MCF had a neutral aroma and good nutritional values with carbohydrate content of 87.55 g/100 g, proteins at 2.03 g/100 g, lipids at 0.43 g/100 g, ash at 0.85 g/100 g, and crude fiber at 4.17 g/100 g.

Suweg (*Amorphophallus paeoniifolius* (Dennst.) Nicolson), a member of the *Araceae* family, represents a gluten-free tuber commodity indigenous to Indonesia. However, its utilization is currently restricted due to the limited range of products derived from suweg. Processing suweg tubers into flour offers a viable solution for expanding the potential applications of suweg tubers. Suweg flour is rich in carbohydrates (76.32–78.36 g/100 g) and proteins (10.14–11.37 g/100g), and its ash and fat content is 2.30–3.22 g/100 g and 0.49–0.55 g/100 g, respectively [Suriya *et al.*, 2016].

A flour blend is a mixture of different flours rich in starch, protein, and other nutrients with or without wheat flour [Fetriyuna *et al.*, 2021; Kılıç Keskin *et al.*, 2022; Shittu *et al.*, 2007]. Flour blending aims to improve the final product's characteristics such as sensory characteristics, nutritional content, functional properties, *etc*. by combining the benefits of each flour. In this study, modified cassava flour and suweg flour were used as a flour blend to replace wheat flour in steamed brownies. Modified cassava flour offers the advantage of being gluten-free, suitable for individuals with gluten intolerance or celiac disease, and its high fiber content [Liu *et al*., 2022]. However, it lacks the binding properties of gluten, which may affect the texture of the final product. On the other hand, our previous study showed that suweg flour is rich in fiber and nutrients, and provides a natural sweetness to baked goods [Marta *et al.*, 2023]. Additionally, the neutral taste of modified cassava flour complements the distinct flavor of suweg flour, resulting in a more harmonious flavor profile.

Several studies have been conducted on steamed brownies that used local commodities as a substitute for wheat flour. Previous studies have reported that steamed brownies from 100% modified cassava flour [Lubis *et al.*, 2021] and 100% pumpkin flour [Subaktilah *et al.*, 2021] had lower protein contents. Furthermore, Lubis *et al.* [2021] reported that steamed brownies with a flour blend from breadfruit flour, purple sweet potato flour, modified cassava flour, and saga seed flour with a ratio of 35:45:5:15 (*w/w/w/w*), had weaker aroma and good volume expansion compared to wheat flour steamed brownies. Meanwhile, steamed brownies with 100% suweg flour [Bela Monica & Setyaningrum, 2022] and 100% purple sweet potato flour [Lubis *et al.*, 2021] produced a dark color and dense texture. Information regarding the use of modified cassava and suweg flours in making gluten-free steamed brownies is still limited; hence, the aim of this study was to evaluate the physicochemical properties of modified cassava and suweg flour blends in various proportions and to determine textural and sensory characteristics of gluten-free steamed brownies produced from these blends.

MATERIALS AND METHODS

Materials

The main materials used in this study were commercial medium wheat flour (Segitiga Biru by Bogasari Flour Mills, Indofood Sukses Makmur, Jakarta, Indonesia), commercial cassava flour modified by fermentation (Ladang Lima by Agung Bumi Agro, Surabaya, Indonesia), and suweg tubers with a harvest age of 1 year (Madiun, East Java, Indonesia). Additional ingredients for making steamed brownies were: cocoa powder (Van Houten by PT Perusahaan Industri Ceres, Bekasi, Indonesia), salt (Cap Kapal by Susanti Megah, Surabaya, Indonesia), powdered sugar (Gulaku by Sugar Group Companies, Lampung Tengah, Indonesia), emulsifier and baking powder (Koepoe Koepoe by Gunacipta Multirasa, Tangerang, Indonesia), margarine (Blue Band by Unilever Indonesia Tbk, Bandung, Indonesia), chicken eggs (Eggspert, Bandung, Indonesia), and a dark chocolate compound (Collata by Gandum Mas Kencana, Tangerang, Indonesia).

Suweg flour preparation

The suweg flour (SF) was prepared according to the method provided in Marta *et al.* [2019a] with a slight modification. The suweg tubers were peeled manually using a knife to carefully remove the skin in thin layer, and sliced (±1 mm) using a food processor. The chips were soaked in water to inhibit the browning reaction. Then, they were drained and dried in a drying oven at 50°C overnight. The dried chips were then milled and sieved using a 100-mesh sieve.

Flour blend preparation

A flour blend was prepared by mixing two types of single flour, modified cassava flour (MCF) and suweg flour (SF), until homogeneous. The ratios of MCF and SF (by weight) used in flour blends were 80:20 (F1), 70:30 (F2), and 60:40 (F3).

Brownie preparation

The preparation method for steamed brownies followed that described by Lubis *et al.* [2021] with a slight modification. First,

the dry ingredients, such as flour blend or wheat flour (WF), powdered sugar, cocoa powder, baking powder, and salt, were mixed until homogeneous in a container (mixture 1). Then, the eggs were beaten and mixed with an emulsifier at the highest mixer speed in another container for 15 min or until stiff peaks were produced (mixture 2). Afterward, the dry ingredients were gradually stirred with mixture 2. The melted margarine and dark chocolate were added and stirred until homogeneous. The mixture was poured into the pan (21×9×7 cm^2) and then steamed for 45 min. Formulations of steamed brownies are presented in **Table 1**. Gluten-free steamed brownies prepared using F1, F2 and F3 were coded as GFSB-F1, GFSB-F2 and GFSB-3 respectively. Steamed brownie with wheat flour (WFSB) served as the control. All brownie variants were prepared in three repetitions.

Analysis of proximate composition and crude fiber content of flours

The proximate composition and crude fiber content were analyzed according to AOAC International methods [AOAC, 2005]. Moisture content was determined using an oven-drying method, drying at 135°C for 2 h (method no. 930.15). Ash content was determined by incinerating the flour sample in a furnace (method no. 930.05). Lipid content was determined using the Soxhlet extraction (method no. 930.09). Protein content was determined using the Kjeldahl method (method no. 978.04). Crude fiber content was determined using method no. 978.10. The contents of ash, lipids, proteins and crude fiber were expressed in g *per* 100 g of dry base (db) of flour blend or wheat flour.

Analysis of pasting properties of flours

A Rapid Visco Analyzer (RVA StarchMaster2, Parten Instruments, Warriewood, Australia) was used to determine the pasting properties of flours. The flour samples (2.8 g) were added to 25 mL of distilled water in an RVA canister. The temperature was initially held at 50°C for 1 min, then increased from 50 to 95°C at a rate of 6°C/min, kept at 95°C for 5 min, and then decreased to 50°C at a rate of 6°C/min. Finally, the gel was held at 50°C for 5 min. The visco-amylograms were recorded and several parameters were calculated, including pasting temperature (PT), peak viscosity (PV), holding viscosity (HV), breakdown (BD), final viscosity (FV), and setback (SB). PT was the temperature at which the viscosity increases during the heating process. PV was the highest viscosity reached during the heating phase of a pasting curve. HV was the minimum viscosity reached after the PV during the cooling phase. FV was the viscosity at the end of the pasting curve after the sample has cooled down. BD was the decrease in viscosity from PV to HV during the heating phase and was calculated by subtracting HV from PV. SB was the increase in viscosity after reaching PV and cooling the sample. SB was calculated by subtracting PV from FV.

Table 1. Formulation (g) of wheat flour-based steamed brownies (control) and gluten-free steamed brownies with modified cassava and suweg flour blends in different flour proportions (80:20, 70:30 and 60:40 by weight).

Ingredient	Control	F1 (80:20)	F2 (70:30)	F3 (60:40)
Wheat flour (WF)	85	–	–	–
Modified cassava flour (MCF)	–	68	59.5	51
Suweg flour (SF)	–	17	25.5	34
Sugar powder	150	150	150	150
Chocolate powder	35	35	35	35
Margarine	120	120	120	120
Dark chocolate compound	75	75	75	75
Eggs	200	200	200	200
Baking powder	5	5	5	5
Emulsifier	5	5	5	5
Salt	2.5	2.5	2.5	2.5

Color evaluation of flours and steamed brownies

The color parameters were measured using a CM-5 spectrophotometer (Konica Minolta Co., Osaka, Japan) with Spectra Magic software. The color measurement included lightness (L^*), redness-greenness (a^*), yellowness-blueness (b^*), and hue. The calibration was performed with a white calibration plate (CM-A120) and a zero-calibration plate (CM-A124) using a large target mask (CM-A203). The whiteness index and color difference (ΔE) for flours and steamed brownies were calculated according the Equations (1) and (2) previously used by Chaple *et al.* [2020] and Diaz *et al.* [2019], respectively.

$$\text{Whiteness index} = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (1)$$

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

where: the ΔL^*, Δa^*, and Δb^* are the differences between the color parameters of the sample and the control: wheat flour (for flour blends) and wheat flour-based steamed brownie (for gluten-free brownies).

Determination of functional properties of flours

Swelling volume (SV), solubility (SOL), and water absorption capacity (WAC) of flours were determined. The SV and SOL were assessed following the previous method by Marta *et al.* [2019b]. The flour (0.35 g on a dry basis) was placed into a centrifuge tube, followed by the addition of 12.5 mL of distilled water. The mixture was vortexed for 20 s, then heated in a water bath at 92.5°C with regular stirring for 30 min. Subsequently, the sample was swiftly cooled for 1 min in an ice water bath and then centrifuged at 2,050×g for 15 min. The resulting supernatant was carefully decanted, and its volume was recorded. To determine solubility, the supernatant was dried in an oven, and the solubility percentage was calculated. SV and SOL were calculated using the Equations (3) and (4):

$$\text{SV (mL/g)} = \frac{\text{Total volume} - \text{Supernatant volume}}{\text{Weight of flour (db)}} \quad (3)$$

$$\text{SOL (\%)} = \frac{\text{Weight of dried supernatant}}{\text{Weight of flour (db)}} \times 100\% \quad (4)$$

WAC was evaluated using the following procedure: 1 g of the flour was combined with 10 mL of distilled water in a centrifuge tube and mixed thoroughly using a vortex mixer. Afterward, the sample was allowed to condition at room temperature (26±2°C) for one hour before being centrifuged at 2,050×g for 30 min. The volume of the resulting supernatant was recorded, and the WAC was calculated using Equation (5):

$$\text{WAC (g/g)} = \frac{\text{Volume of water absorbed}}{\text{Weight of flour (db)}} \quad (5)$$

Volume expansion test of steamed brownies

The volume expansion test procedure referred to Gandikota & MacRitchie [2005] with a slight modification. The percentage of volume expansion was calculated based on the final volume of steamed brownies (after steaming) and the initial volume of the mixture (before steaming). Initial and final volumes were calculated by multiplying the length, width and height of the dough and brownies, respectively. Volume expansion was calculated using Equation (6):

$$\text{Volume expansion (\%)} = \frac{\text{Final volume} - \text{Initial volume (cm}^3\text{)}}{\text{Initial volume (cm}^3\text{)}} \quad (6)$$

Evaluation of texture parameters of steamed brownies

Texture parameters were evaluated using a texture analyzer (TA.XTExpress, Stable Micro System, Godalming, UK) and Exponent Lite Express software for data collection and calculation. The brownie samples (2×2×3 cm^3) were pressed using an aluminum cylinder probe P36R with a 2 kg load cell, distance of 10 mm, force of 5 g, strain of 50%, and 5 mm/s speed for 5 s. Their hardness, springiness, cohesiveness, gumminess, chewiness, and resilience were measured.

Hedonic sensory test of steamed brownies

A hedonic sensory test of the brownies was conducted with 60 semi-trained participants aged between 19 and 24, consisting of men and women. Steamed brownies were served in size (2×2×3 cm^3) from the center and placed on white plastic dishes coded with random three-digit numbers. Steamed brownies were evaluated based on the acceptability of their color, texture, aroma, taste, and overall acceptance using a 7-point hedonic scale. The scale ranged from "like very much" to "dislike very much," corresponding to the highest and lowest scores of "7" and "1", respectively. This test procedure refers to Lubis *et al.* [2021].

Statistical analysis

Data were expressed as mean and standard deviation (SD) of triplicates. The data were analyzed using the one-way analysis of variance (ANOVA), followed by the Duncan test to determine the significance of differences at $p<0.05$. The IBM SPSS 27.0 statistical software (Armonk, NY, USA) was used for statistical analyses.

RESULTS AND DISCUSSION

Proximate composition and crude fiber content of flours

The proximate composition and crude fiber content of flour blends and wheat flour are presented in **Table 2**. The ash content of flour blends was higher than that of wheat flour. Flour blends contained 1.64–2.38 g of ash in 100 g db, and showed the following dependency: the higher the proportion of suweg flour (SF) in the blend, the higher the ash content of the flour. In contrast, the lipid and protein contents of flour blends were lower than those of wheat flour. The protein content of wheat flour was up to 10 times higher than of flour blends. The flour blends with the increasing share of SF were characterized by increasing protein contents. A previous study has reported that SF had a higher protein content than MCF, where the protein content of SF and MCF are 44.4% db and 1.13% db, respectively [Marta *et al.*, 2023].

All flour blends had a higher crude fiber content than wheat flour (**Table 2**). This difference can be important when considering the baking properties of flour blends affecting the gluten-free steamed brownies prepared from them. A previous study has reported that the lower volume expansion of muffins might be due to the higher crude fiber content in wheat and flaxseed flour blend [Kaur & Kaur, 2018].

Pasting properties of flour blends

The visco-amylograms and pasting parameters of flours are presented in **Figure 1** and **Table 3**, respectively. The pasting temperature (PT), peak viscosity (PV), and breakdown (BD) of flour blends were higher than these of the wheat flour; conversely, the setback (SB) of flour blends was lower than that of the wheat flour. PT, PV, and SB of F1 significantly ($p<0.05$) differed from the other flour blends. It had a lower PT and higher PV and SB than the other flour blends. The higher the PT of blends with higher suweg flour proportion could be caused by the increasing content of non-starch components in the flour blends (**Table 2**).

Table 2. Proximate composition and crude fiber content of wheat flour and flour blends with different ratios of modified cassava flour (MCF) to suweg flour (SF).

Flour	Water (g/100 g db)	Ash (g/100 g db)	Lipid (g/100 g db)	Protein (g/100 g db)	Crude fiber (g/100 g db)
Wheat flour*	14.52±0.16	0.69±0.01	1.36±0.43	10.55±0.18	0.38±0.15
F1	11.37±0.15^a	1.64±0.02^c	0.55±0.03^c	1.34±0.06^c	0.86±0.18^c
F2	11.16±0.25^a	2.00±0.02^b	0.67±0.16^b	1.61±0.22^b	1.48±0.09^b
F3	10.86±0.53^a	2.38±0.04^a	0.78±0.06^a	1.88±0.01^a	1.91±0.04^a

*Values for wheat flour were previously published [Marta *et al.*, 2023]. Means within columns with different superscripts are significantly different ($p<0.05$). F1, MCF to SF ratio of 80:20 (*w/w*); F2, MCF to SF ratio of 70:30 (*w/w*); F3, MCF to SF ratio of 60:40 (*w/w*); db, dry base.

Non-starch components, such as protein, lipid, and fiber, can inhibit starch gelatinization which needs more energy to gelatinize, and increase the PT [Yang *et al.*, 2021]. On the other hand, the presence of the non-starch components decreased the PV, BD, and SB of flour blends.

The higher BD and the lower SB of all flour blends compared to the wheat flour indicated that these flours have lower thermal stability and do not retrograde easily, respectively [Charles *et al.*, 2016]. Flour with low SB is desirable for making steamed flour products because it is not easily retrogradable, so its texture quality is more stable during storage [Li *et al.*, 2024]. Retrogradation refers to the process where starch molecules in baked goods reorganize themselves into a more crystalline structure, leading to staling and changes in texture over time. Flour with low SB can maintain steamed brownies' soft and moist texture for longer, improving their quality during storage.

Color of flours

The color parameters of wheat flour and flour blends are shown in **Table 4**. The *L**, hue, and whiteness index (WI) of flour blends were lower than those of wheat flour, while *a** was higher. Significant ($p<0.05$) differences existed in the color parameters of flour

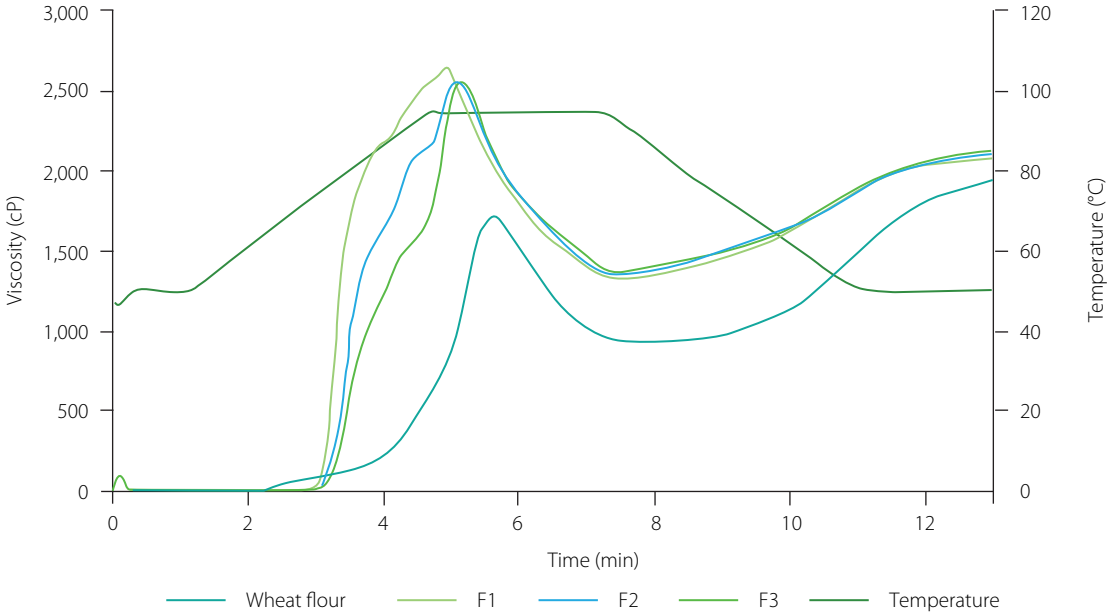

Figure 1. Visco-amylograms of wheat flour and flour blends (F1, F2 and F3) with different ratios of modified cassava flour to suweg flour (80:20, 70:30 and 60:40 by weight, respectively).

Table 3. Pasting properties of wheat flour and flour blends with different ratios of modified cassava flour (MCF) to suweg flour (SF).

Flour	Pasting temperature (°C)	Peak viscosity (cP)	Hold viscosity (cP)	Final viscosity (cP)	Breakdown (cP)	Setback (cP)
Wheat flour*	64.91±0.60	1,718±5	936±15	1,943±24	783±10	1,007±10
F1	73.11±0.82^{b}	2,656±11^{a}	1,327±43^{c}	2,088±26^{b}	1,329±52^{a}	761±30^{a}
F2	74.34±0.45^{a}	2,579±8^{b}	1,356±53^{b}	2,111±25^{ab}	1,222±61^{b}	755±33^{b}
F3	74.53±0.24^{a}	2,576±33^{b}	1,381±30^{a}	2,134±21^{a}	1,195±52^{c}	753±18^{b}

*Values for wheat flour were previously published [Marta *et al.*, 2023]. Means within columns with different superscripts are significantly different ($p<0.05$). F1, MCF to SF ratio of 80:20 (*w/w*); F2, MCF to SF ratio of 70:30 (*w/w*); F3, MCF to SF ratio of 60:40 (*w/w*).

Table 4. Color parameters of wheat flour and flour blends with different ratios of modified cassava flour (MCF) to suweg flour (SF).

Flour	*L**	*a**	*b**	Hue	Whiteness index	Δ*E*
Wheat flour*	93.09±0.21	0.45±0.01	9.81±0.12	1.53±0.01	87.99±0.03	–
F1	89.36±0.03^{a}	1.29±0.01^{c}	9.29±0.06^{b}	1.43±0.00^{a}	85.82±0.03^{a}	3.85±0.23^{c}
F2	87.94±0.03^{b}	1.52±0.01^{b}	9.60±0.08^{a}	1.42±0.01^{b}	84.51±0.06^{b}	5.26±0.19^{b}
F3	86.88±0.08^{c}	1.68±0.03^{a}	9.76±0.09^{a}	1.40±0.00^{c}	83.56±0.12^{c}	6.33±0.22^{a}

*Values for wheat flour were previously published [Marta *et al.*, 2023]. Means within columns with different superscripts are significantly different ($p<0.05$). F1, MCF to SF ratio of 80:20 (*w/w*), F2, MCF to SF ratio of 70:30 (*w/w*), F3, MCF to SF ratio of 60:40 (*w/w*); *L**, lightness; *a**, redness-greeness; *b**, yellowness-blueness; Δ*E*, color difference compared to wheat flour.

blends, except for the *b** value between F2 and F3. F1, consisting of 80% MCF and 20% SF, displayed higher *L**, hue, and WI compared to the other flour blends, whereas its *a**, *b**, and Δ*E* were lower. Increasing the proportion of MCF in the flour blend resulted in higher *L**, hue, and WI, as well as lower *a** and *b**. The Δ*E* represents the color variation between wheat and blend flours, ranging from 3.85 to 6.33 with the lowest value for F1, indicating closer color similarity to wheat flour.

Color is crucial in consumer acceptance of food products, as it is typically the first perceived sensory aspect. A higher proportion of SF in the flour blend led to a lower whiteness index (WI), which in turn negatively correlated with an ash content. Previous research has shown that higher ash content of chickpea, carob and rice flours determined their lower brightness [Ammar *et al.*, 2022]. In addition, the phenolics can cause color changes due to forming oxidation products, imparting a brown color to flour [Shete *et al.*, 2015].

Functional properties of flours

The functional properties of flours, including swelling volume, solubility, and water absorption capacity (WAC) are shown in **Table 5**. Flour blends exhibited higher swelling volume, solubility, and WAC compared to wheat flour. While the functional properties of all flour blends were generally similar, there was a notable difference in WAC. The WAC of F1 was significantly ($p<0.05$) higher than that of F2 and F3.

The lower swelling volume (SV) observed in wheat flour compared to flour blends could be attributed to its higher lipid and protein contents. Non-starch components such as lipids and proteins can inhibit water absorption and starch swelling [Cahyana *et al.*, 2021; Chao *et al.*, 2020; Marta *et al.*, 2019b]. Cahyana *et al.* [2021] reported that the lipids could interact with starch to form a starch-lipid complexes, where the presence of protein promoted the formation of these complexes. The formation of starch-lipid complexes hindered the starch swelling.

Table 5. Functional properties of wheat flour and flour blends with different ratios of modified cassava flour (MCF) to suweg flour (SF).

Flour	Swelling volume (mL/g)	Solubility (%)	WAC (g/g)
Wheat flour*	11.53±0.32	14.17±2.00	0.95±0.11
F1	19.70±0.71^{a}	14.27±0.79^{a}	1.98±0.06^{a}
F2	19.58±0.38^{a}	14.21±0.62^{a}	1.66±0.07^{b}
F3	19.06±0.37^{a}	13.81±0.45^{a}	1.65±0.05^{b}

*Values for wheat flour were previously published [Marta *et al.*, 2023]. Means within columns with different superscripts are significantly different ($p<0.05$). F1, MCF to SF ratio of 80:20 (*w/w*); F2, MCF to SF ratio of 70:30 (*w/w*); F3, MCF to SF ratio of 60:40 (*w/w*); WAC, water absorption capacity.

Volume expansion of steamed brownies

The volume expansion of wheat flour-based steamed brownies and gluten-free steamed brownies is presented in **Table 6**. All variants of gluten-free steamed brownies (GFSB-F1, GFSB-F2, and GFSB-F3) exhibited a lower volume expansion compared to wheat flour-based steamed brownies (WFSB). The presence of gluten in WFSB notably contributed to its higher volume expansion. Additionally, the volume expansion in steamed brownies can be enhanced by using baking powder and resulting production of CO_2 during the heating or steaming process [De Leyn, 2014]. Apart from incorporating baking powder, various factors such as mixing technique, heat distribution, egg beating, and the characteristics of the pan significantly influence the volume expansion of steamed brownies. Furthermore, in addition to the absence of gluten, the reduced volume expansion observed in gluten-free steamed brownies (GFSB) can also be attributed to their high fiber content [Rai *et al.*, 2018].

Texture parameters of steamed brownies

The texture analysis of steamed brownies in this study encompassed hardness, springiness, cohesiveness, gumminess, chewiness, and resilience (**Table 6**). In the case of all texture parameters, gluten-free steamed brownies showed lower values compared to the wheat flour-based steamed brownies, except for hardness, where GFSB-F2 and GFSB-F3 exhibited higher levels than WFSB. Among the GFSB, an increase in the proportion of SF in flour blends corresponded to higher values of texture parameters, except for springiness and resilience. It suggests that SF primarily influences the enhancement of texture parameters in GFSB.

The SF in flour blend causes an increase in the hardness of GFSB, which might be due to the high content of crude fiber in SF. Suweg flour contains 3.15 g of crude fiber in 100 g, which is higher than in MCF (1.18 g/100 g) [Marta *et al.*, 2023]. Furthermore, the higher the SF proportion in the blend, the higher the cohesiveness, gumminess, and chewiness of GFSB. The high

Table 6. Volume expansion and texture parameters of wheat flour-based steamed brownies (WFSB) and gluten-free steamed brownies (GFSB) prepared with modified cassava and suweg flour blends (F1, F2 and F3) in different flour proportions (80:20, 70:30 and 60:40 by weight, respectively).

Steamed brownie	Volume expansion (%)	Hardness (N)	Springiness	Cohesiveness	Gumminess	Chewiness	Resilience
WFSB	74.77±5.91^{a}	11.19±0.29^{b}	0.86±0.01^{a}	0.52±0.02^{a}	592±31^{a}	507±28^{a}	0.20±0.01^{a}
GFSB-F1	67.03±5.31^{b}	9.51±0.35^{c}	0.81±0.01^{b}	0.30±0.01^{c}	287±22^{c}	232±19^{c}	0.12±0.00^{b}
GFSB-F2	65.66±3.92^{b}	13.74±0.64^{a}	0.82±0.02^{b}	0.34±0.01^{b}	481±39^{b}	396±23^{b}	0.13±0.01^{b}
GFSB-F3	65.05±2.07^{b}	14.19±0.39^{a}	0.83±0.01^{b}	0.36±0.01^{b}	519±8^{b}	431±12^{b}	0.13±0.00^{b}

Means within columns with different superscripts are significantly different ($p<0.05$).

cohesiveness of the steamed brownies indicated that the product could retain more gas and volume. The texture attributes of GFSB-F2 were more similar to WFSB and did not exhibit significant differences ($p \geq 0.05$) compared to GFSB-F3. The increased gumminess and chewiness observed in GFSB with the higher SF content can be attributed to the crude fiber content of the flour. A previous study has indicated that incorporating more flour with a low fiber content led to brownies with reduced gumminess or excessive hardness [Lubis *et al.*, 2021].

Color of steamed brownies

The appearance and color parameters of steamed brownies are presented in **Figure 2** and **Table 7**, respectively. None of the color parameters of GFSB-F1 showed significant differences ($p \geq 0.05$) from WFSB. Moreover, an increase in the proportion of SF in the flour blend corresponded to higher values across all color parameters of GFSB, including L^*, a^*, b^*, hue, and ΔE.

The ΔE value signifies the color distinction between GFSB samples and WFSB (control). A ΔE value below 0.2 suggests no discernible difference in color between the two substances. A ΔE value ranging from 0.2 to 1.0 indicates a minor color variation, while a ΔE value between 1.0 and 3.0 suggests a slight color difference. A ΔE value of 3.0 to 6.0 denotes a moderate color difference, whereas a ΔE value exceeding 6.0 indicates a substantial color difference [Sharma, 2003]. The ΔE values of all GFSB ranged from 2.30 to 6.32 (**Table 7**). GFSB-F1 displayed the smallest ΔE value (2.30), indicating a slight color difference compared to WFSB. The ΔE value of GFSB corresponded with the ΔE value of flour blends, whereby an increase in SF proportion in the blend led to a higher color difference between the sample and the control.

The color of flour influences the color of steamed brownies. Steamed brownies typically exhibit a dark brown or blackish-brown hue, influenced by ingredients such as chocolate and sugar, as well as the baking process. Cocoa powder and chocolate bars are common chocolate sources in brownie recipes. Moreover, finer sugar particles absorb color more effectively, resulting in a darker brown shade [Richardson *et al.*, 2018].

Hedonic sensory evaluation of steamed brownies

The results of the participants' preference for steamed brownies are presented in **Table 8**. The hedonic attributes of GFSB did not significantly differ ($p \geq 0.05$) from WFSB, except for taste and overall acceptance preference of GFSB-F2. The taste and overall acceptance preference for GFSB-F2 were notably higher ($p < 0.05$) than for WFSB and did not significantly differ ($p \geq 0.05$) from the other GFSB samples. Overall, all GFSBs received hedonic scores between 5 and 6, indicating they fell within the "like moderately" category and were generally accepted by the participants. MCF typically has a neutral aroma [Triyono *et al.*, 2019]. As the proportion of SF increases, the distinct aroma of suweg tubers became more pronounced, enhancing the taste of GFSB. The aroma can also be intensified by the caramelization process occurring during baking, resulting in a distinctive caramel aroma. Moreover, the aroma can be further influenced by the Maillard reaction products and the quality and quantity of volatile compounds released [Capuano *et al.*, 2009].

The texture parameters assessed by instrumental analysis for GFSB differed significantly from those of WFSB. However, the texture preference for GFSB did not significantly differ from WFSB. The texture preference for all steamed brownies ranged from 5.25 to 5.60 (**Table 8**), indicating a preference for "like slightly". Interestingly, a parallel trend to ΔE was observed; although the ΔE value for all GFSB samples was significantly different, the results

Figure 2. The visual appearance of wheat flour-based steamed brownies (WFSB) and gluten-free steamed brownies (GFSB) prepared with modified cassava and suweg flour blends (F1, F2 and F3) in different flour proportions (80:20, 70:30 and 60:40 by weight, respectively).

Table 7. Color parameters of steamed wheat flour-based brownies (WFSB) and gluten-free brownies (GFSB) prepared with modified cassava and suweg flour blends (F1, F2 and F3) in different flour proportions (80:20, 70:30 and 60:40 by weight, respectively).

Steamed brownie	L^*	a^*	b^*	Hue	ΔE
WFSB	17.68 ± 1.46^{c}	4.22 ± 0.50^{b}	3.51 ± 0.39^{b}	0.69 ± 0.00^{b}	–
GFSB-F1	19.86 ± 0.60^{bc}	4.50 ± 0.24^{b}	3.81 ± 0.38^{b}	0.70 ± 0.03^{b}	2.30 ± 1.34^{b}
GFSB-F2	21.04 ± 1.45^{b}	3.91 ± 0.27^{b}	3.54 ± 0.14^{b}	0.74 ± 0.02^{ab}	3.50 ± 1.97^{ab}
GFSB-F3	23.61 ± 1.19^{a}	5.45 ± 0.26^{a}	5.29 ± 0.38^{a}	0.77 ± 0.03^{a}	6.32 ± 2.15^{a}

Means within columns with different superscripts are significantly different ($p < 0.05$). L^*, lightness; a^*, redness-greenness; b^*, yellowness-blueness; ΔE, color difference compared to WFSB.

Table 8. Results of hedonic sensory test of wheat flour-based steamed brownies (WFSB) and gluten-free brownies (GFSB) prepared with modified cassava and suweg flour blends (F1, F2 and F3) in different flour proportions (80:20, 70:30 and 60:40 by weight, respectively).

Steamed brownie	Color	Aroma	Texture	Taste	Overall acceptance
WFSB	5.85±1.14^{a}	5.65±0.93^{a}	5.25±1.52^{a}	5.65±1.14^{b}	5.45±1.05^{b}
GFSB-F1	6.15±0.99^{a}	5.60±0.94^{a}	5.55±1.10^{a}	5.85±0.99^{ab}	5.75±0.85^{ab}
GFSB-F2	6.20±1.00^{a}	5.45±1.28^{a}	5.60±1.23^{a}	6.35±0.81^{a}	6.10±1.07^{a}
GFSB-F3	6.05±1.94^{a}	5.30±0.92^{a}	5.35±1.14^{a}	5.60±1.00^{b}	5.85±0.75^{ab}

Means within columns with different superscripts are significantly different ($p<0.05$).

of the hedonic test showed that the color preferences for all GFSB and WFSB samples were not significantly different. The difference trend between the results of analysis using instruments (ΔE) and hedonic tests (color preferences) was also found in another study [Cahyana *et al.*, 2020]. The color preference for all steamed brownies samples ranged from 5.85 to 6.20, which means "like moderately" (**Table 8**).

Organoleptic tests still play a crucial role, especially in food and beverage industries where sensory perception is central to product quality. While instruments can provide quantitative data, organoleptic tests offer qualitative insights and can assess overall consumer acceptability, which instruments alone may not capture. So, while instruments may be more sensitive in certain aspects, organoleptic tests remain essential for comprehensive product evaluation.

CONCLUSIONS

The ratio of modified cassava and suweg flour significantly affected various properties of flour blends, including their chemical composition, color characteristics, pasting and functional properties. Suweg flour notably contributed to increased ash, lipids, proteins, and crude fiber contents in the flour blend. As the proportion of suweg flour increased, peak, breakdown, and setback viscosities decreased. Water absorption capacity of flour blends decreased, and the color difference (compared to wheat flour) increased with a higher suweg flour content. Incorporating composite flour into gluten-free steamed brownies significantly influenced their properties. Gluten-free steamed brownies exhibited a lower volume expansion compared to wheat flour-based steamed brownies. Brownies made from the flour blend with a higher content of suweg flour displayed higher texture parameters, except for springiness and resilience. The color of GFSB prepared with the blend with a lower proportion of suweg flour was more similar to color of WFSB. Preference levels for color, aroma, and texture of GFSB did not significantly differ from WFSB. However, GFSB prepared with the modified cassava and suweg flours in the ratio of 80:20 (*w/w*) was preferred in taste and overall acceptance over WFSB. Overall, the study suggests that the ratio of modified cassava and suweg flours plays a crucial role in determining the chemical, physical, and sensory properties of flour blends and their respective gluten-free steamed brownies. The findings provide valuable insights for the development of gluten-free products with improved sensory attributes and consumer acceptance.

RESEARCH FUNDING

This research was funded by Grant of Ministry of Education, Culture, Research, and Technology (MoECRT), Republic of Indonesia [grant number: 2393/UN6.3.1/PT.00/2022], and The APC was funded by Universitas Padjadjaran.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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