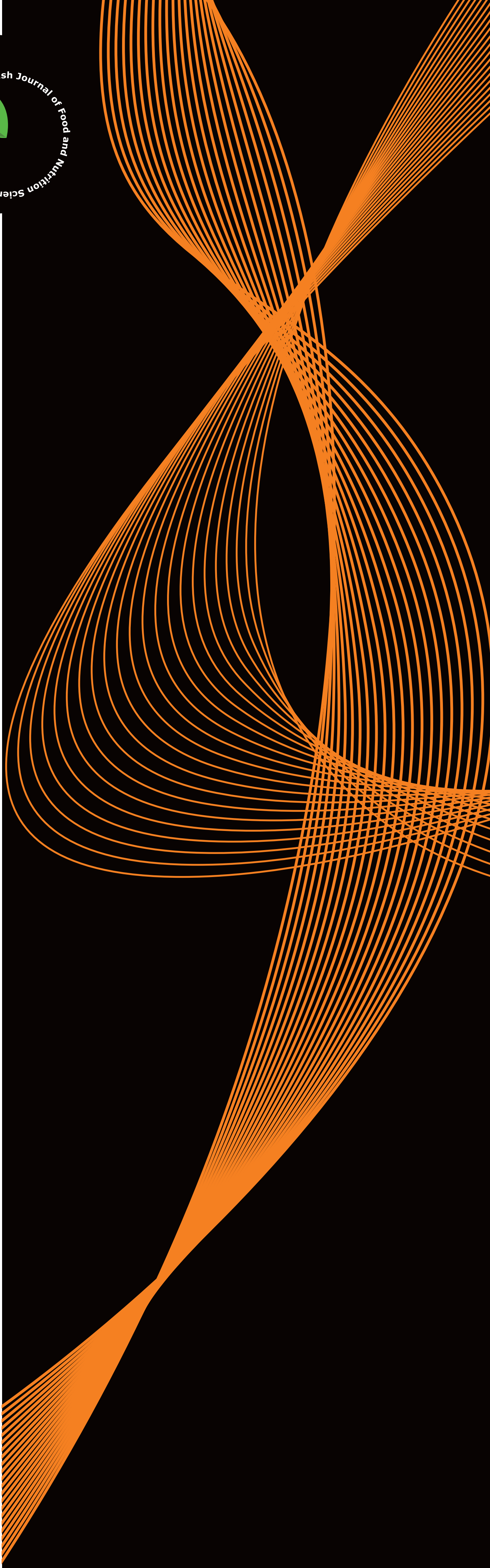
ISSN (1230-0322)

2020, Vol. 70, No. 4

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. Referees are selected from the Advisory Board and from Polish and international scientific centres. Identity of referees 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, Polish Scientific Journals Contents (PSJC) Life Science data base available at http://psjc.icm.edu.pl and any www browser; ProQuest: The Summon, Bacteriology Abstracts, Immunology Abstracts.

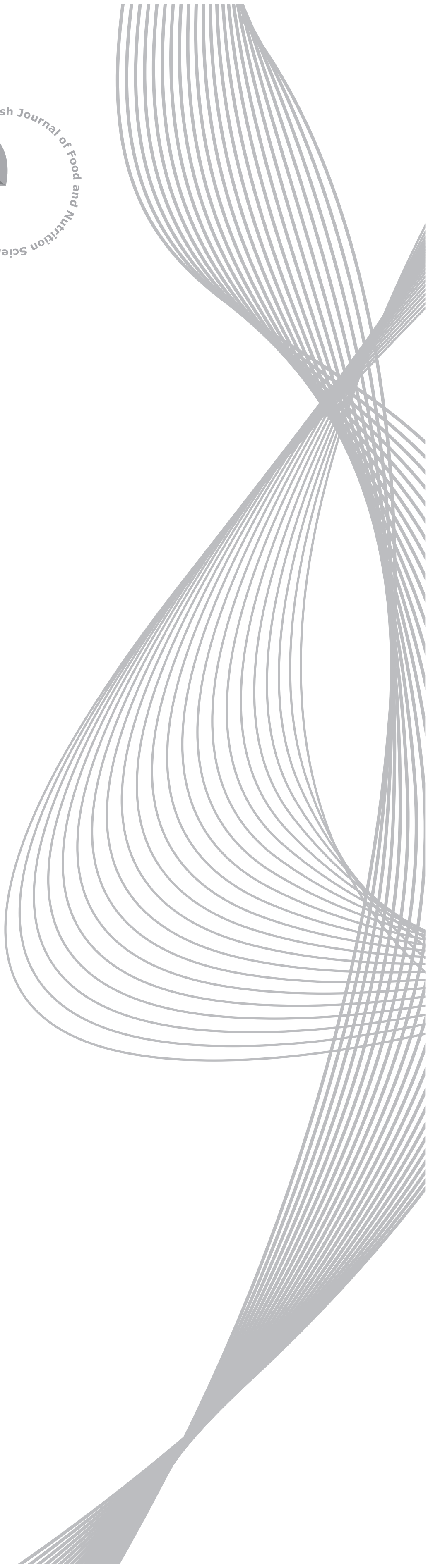
EDITORIAL AND BUSINESS CORRESPONDENCE: Submit contributions (see Instructions to Authors) and address all communications regarding subscriptions, changes of address, etc. to:

CORRESPONDENCE TO: Ms. Joanna Molga
Polish Journal of Food and Nutrition Sciences
Institute of Animal Reproduction and Food Research
of Polish Academy of Sciences
ul. Tuwima 10, 10-747 Olsztyn, Poland
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

ISSN (1230–0322)
2020, Vol. 70, No. 4

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

Pol. J. Food Nutr. Sci. 2020, Vol. 70, No. 4

Advisory Board of PJFNS 2019–2022

Marek Adamczak
University of Warmia and Mazury in Olsztyn, Poland
Huda Al-Kateb
Birmingham City University, Birmingham, UK
Wilfried Andlauer
University of Applied Sciences and Arts Western Switzerland
Valais, Sion, Switzerland
Anna Brzozowska
Warsaw University of Life Sciences, Poland
Zuzana Ciesarova
VUP Food Research Institute, Bratislava, Slovak Republic
Maria Dolores Del Castillo
CSIC-UAM, Madrid, Spain
Juana Frias
Institute of Food Science, Technology and Nutrition
(ICTAN), Madrid, Spain
Liwei Gu
University of Florida, Gainesville, USA
Henryk Jeleń
Poznań University of Life Sciences, Poland
Georgios Koutsidis
Northumbria University, Newcastle-upon-Tyne, UK
Andrzej Lenart
Warsaw University of Life Sciences, Poland
John Lodge
Northumbria University, Newcastle-upon-Tyne, UK
Adolfo J. Martinez-Rodriguez
CSIC-UAM, Madrid, Spain
Francisco J. Morales
CSIC, Madrid, Spain
Zhongli Pan
University of California, Davis, USA;
World Food Center, China
Ron B. Pegg
University of Georgia, Athens, USA
Mariusz K. Piskuła
Institute of Animal Reproduction and Food Research
of the Polish Academy of Sciences in Olsztyn, Poland
Da-Wen Sun
National University of Ireland, Dublin, Ireland
Lida Wądołowska
Warmia and Mazury University, Olsztyn, Poland
Zenon Zduńczyk
Institute of Animal Reproduction and Food Research
of the Polish Academy of Sciences in Olsztyn, Poland
Henryk Zieliński
Institute of Animal Reproduction and Food Research
of the Polish Academy of Sciences in Olsztyn, Poland

Pol. J. Food Nutr. Sci. 2020, Vol. 70, No. 4

POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

covered by CURRENT CONTENTS/AGRICULTURE, BIOLOGY & ENVIRONMENTAL SCIENCES,
JOURNAL CITATION REPORTS AND SCIENCE CITATION INDEX EXPANDED, BIOSIS, SCOPUS,
IFIS Publishing and CAS abstracts, and AGRO-Librex, FROSTI, PSJC, AGRIS and Index Copernicus data bases

2020, VOL. 70, NO. 4

REVIEW

ORIGINAL PAPERS

Ministerstwo Nauki
i Szkolnictwa Wyższego

KOMUNIKAT

W latach 2019–2020 kwartalnik naukowy ***Polish Journal of Food and Nutrition Sciences*** realizuje zadania z zakresu digitalizacji i upowszechniania publikacji naukowych, które ukazują się na jego łamach oraz utrzymania procedur zapewniających ich oryginalność, w ramach zadania nr 1 pt. ***Digitalizacja artykułów publikowanych w kwartalniku PJFNS w celu zapewnienia otwartego dostępu do nich przez sieć Internet*** oraz zadania nr 2 pt. ***Utrzymanie procedur zabezpieczających oryginalność artykułów naukowych publikowanych w kwartalniku PJFNS***. Oba zadania finansowane są na podstawie umowy nr 607/P-DUN/2019 ze środków Ministra Nauki i Szkolnictwa Wyższego przeznaczonych na działalność upowszechniającą naukę.

ANNOUNCEMENT

In the years 2019–2020, the scientific quarterly *Polish Journal of Food and Nutrition Sciences* accomplishes tasks in the scope of digitalization and dissemination of published articles and in the scope of ensuring originality of published works in the framework of task no. 1: ***Digitalization of scientific manuscripts published in PJFNS quarterly to enable open access to them in the Internet*** and task no. 2: ***Maintenance of procedures assuring originality of scientific manuscripts published in PJFNS quarterly***. Both tasks are financed under the agreement no. 607/P-DUN/2019 from funds of the Minister of Science and Higher Education intended for science dissemination activities.

Subscription

2020 – 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: 100 egz.; Ark. wyd. 19; Ark. druk. 19
Skład i druk: Mercurius, www.mercurius.com.pl

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 321–336
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/127635
http://journal.pan.olsztyn.pl

Review article
Food Quality and Functionality Section

Phenolic Compounds from Apples: Reviewing their Occurrence, Absorption, Bioavailability, Processing, and Antioxidant Activity – a Review

Małgorzata Starowicz[1], Bohdan Achrem–Achremowicz[2], Mariusz K. Piskuła[1], Henryk Zieliński[1]*

[1]Department of Chemistry and Biodynamics of Food, Division of Food Sciences, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, ul. Tuwima 10, 10-748 Olsztyn, Poland
[2]Department of Technology and Evaluation of Plant Products, Faculty of Biology and Agriculture, University of Rzeszów, ul. Zelwerowicza 4, 35-601 Rzeszów, Poland

Key words: apples, bioactive compounds, phenolic compounds, functional properties, geographic origin, cultivars, production influence

This review summarises the research on phenolic compounds in apples in relation to their geographical origin, cultivars, technological processes, and human health effects due to their antioxidant activity and bioavailability. Apples are popular among consumers and are known as a rich source of vitamins, minerals, and biologically active compounds. In this review article, we have focused on their phenolic compounds. Some epidemiological studies have confirmed the protective effects of apples against chronic diseases, which can be linked to the high content of phenolics in plant tissues and their bioavailability. However, according to the available literature, the geographical origin and variety of apples influence the content of these bioactive compounds and are highly related to their antioxidant activity fluctuation. The gathered studies have confirmed that the choice of polyphenol-rich raw material as well as proper processing are important to achieve high-quality fruit-based products with a high content of antioxidants after a few stages of production. It was proven that the processing of apples could significantly modify the content of phenolics in the finished products, including juices, concentrates, vinegars, and ciders. For instance, the use of high pressure or vacuum has been proposed as a highly potent solution in inhibiting flavonoid degradation during intensive processing. Moreover, several methods have been screened to monitor the phenolic content and antioxidant activity of apple samples, based on spectrophotometry, HPLC, LC-MS, and LC-MS/MS techniques for chemical compound separation and identification. The main assumptions of these techniques and results obtained are described in this review.

INTRODUCTION – THE PROTECTIVE EFFECTS OF BIOACTIVE COMPOUNDS FROM FRUITS AND VEGETABLES IN HUMANS

Data published by the World Health Organization (WHO) show that low consumption of fruits and vegetables might be the reason for the increased incidence of chronic diseases, and could be the cause of 14% of deaths from gastrointestinal cancers, 11% of those from coronary heart disease, and 9% of those from strokes [WHO, 2009; Arts & Hollman, 2005]. One of the hypotheses is that vegetables and fruits contain high amounts of biologically active compounds eliciting protective effects against lifestyle-related diseases [Arts & Hollman, 2005]. This effect might be due to the antioxidant properties of certain compounds. Antioxidants inhibit oxidation reactions, thus scavenging oxygen free radicals formed upon these reactions. Free radicals can bind proteins, damage lipids of cell membranes, or disrupt the structure of nucleic acids. They are naturally counteracted by the human organism; however, this natural balance might be upset by illnesses, stress, excessive consumption of highly-processed foods, and high environmental pollution. The consumption of fruits and vegetables is highly recommended to support the body in this respect. The main antioxidants found in fruits and vegetables are A, E, and C vitamins; β-carotene; lycopene; and a diverse group of compounds named phenolics. Therefore, it is hard to select one specific compound responsible for health benefits. That is why, Ohr [2004] stated that "scientists are currently studying how mixtures of antioxidants … work together. They are looking for synergistic effects among individual antioxidants."

The most important group of antioxidants is the phenolic compounds (Figure 1). They are organic chemical compounds with at least one phenol unit, represented by, *i.a.*, tannins (hydrolysable and condensed), flavonoids, and lignans. Phenolic compounds can be found in a variety of plant species. Their high content is correlated with antioxidant activity, which decreases the risk of coronary disease and protects against neurodegeneration [Giacalone *et al.,* 2015]. The positive effect of flavonoids on memory improvement was also studied [Whyte & Williams, 2011]. Of 12 epidemiological surveys about coronary heart disease risk assessment compared by Arts & Hollman [2005], seven demonstrated the protective effects of a diet enriched with polyphenols. An inverse relationship has also been determined between poly-

---

* Corresponding Author: Tel.: +48 895234639; Fax: +48 895234693;
E-mail: m.starowicz@pan.olsztyn.pl (Dr. M. Starowicz)

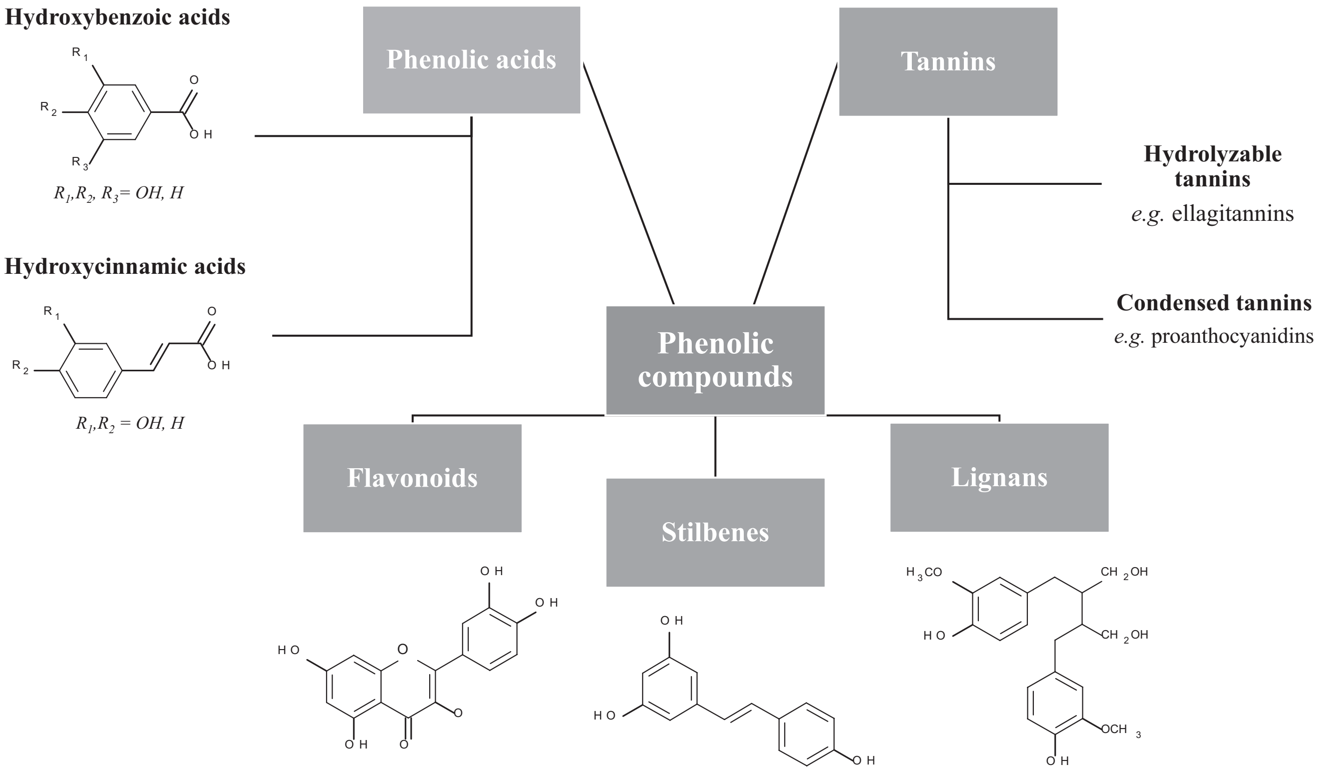


FIGURE 1. Representatives of phenolic compounds with their chemical structures.

phenol intake and stroke and breast cancer risk [Bosetti *et al*., 2005; Knekt *et al*., 2002]. In turn, Jiao *et al.* [2019] have demonstrated that phenolics affect obesity by modulating the gut microbiota and prebiotic agents. Moreover, the anti-inflammatory properties of flavonoids were discussed by Maleki *et al.* [2019], who suggested that the possible mechanism of their action is by inhibiting enzyme activity and transcription factors responsible for inflammation. In epidemiological studies conducted on the Finnish population (>10,000 people), it was proven that flavonoid intake might be crucial for decreasing the risk of development of chronic diseases [Knekt *et al*., 2002]. Also, in clinical studies, a positive correlation was observed between flavonoid administration and the progress of type 2 diabetes [Guo *et al.*, 2019]. Finally, a positive effect of nutritional supplementation with flavonoids was seen in the research on skeletal muscle atrophy to prevent oxidative stress-induced muscle waste [Salucci & Falcieri, 2020].

One of the most affordable fruit-based products on the market is juice. Juices have been proved to contain a high number of antioxidants, such as vitamin C, and to be rich in varied polyphenols [Jacob *et al*., 2008; Martí *et al.,* 2009]. Therefore, it has been experimentally confirmed that the polyphenols from grape juice prevent heart disease and that compounds with high antioxidant activity from berries or apple juice inhibit hypercholesterolemia [Habanova *et al.,* 2019]. In another example, juice from pineapples, oranges, and grapefruits was seen to be a rich source of folic acid derivatives, which help prevent nervous system diseases and malformations, such as spina bifida. In turn, cranberry juice has been shown to decrease urinary tract infections [Jepson *et al*., 2004].

Among the polyphenols, considerable attention has been paid to flavonoids, the intake of which varies from 60 to 600 mg per day among different dietary patterns. Ample studies have focused on determining their content in different food products using novel methodologies and advanced equipment. Today, their bioavailability in the human body is a topic related to phenolic compounds' influence on human health [Manach *et al.,* 2005].

APPLE PHENOLIC COMPOUNDS – PROFILE AND INTAKE

Apples are a rich source of flavonoids, including flavones, flavanols, anthocyanins, and dihydrochalcones (Figure 2). The major individual flavonoids in apples are quercetin and its glycosides, (–)-epicatechin, (+)-catechin, and phloridzin and its derivatives [Lee *et al.,* 2003; Francini & Sebastiani, 2013]. Cyanidin glycosides can be found only in apples of red varieties. Other apple polyphenols are procyanidins – the oligomers of (–)-epicatechin and (+)-catechin, with their content in apple fresh weight ranging from 38 to 154 mg [Hellstrom *et al.*, 2009]. Phenolic acids, *e.g*., chlorogenic acid, are also present in apple flash and skin. However, the quantitative and qualitative profile of phenolic compounds found in apples differs depending on their variety, country of origin, and special pre-treatment, which is summarised in Table 1.

Zhang *et al.* [2019] reported that the positive effect of phenolic content and composition might be due to the pollination of apples with quince pollen. A significant increase in (+)-catechin, (–)-epicatechin, chlorogenic acid, phlorizin, quercetin, and quercetin 3-*O*-galactoside contents was obtained by this modification.

Only a part of phenolic compounds is found in the free form in plant tissues, and most are bound to cell wall components. Important is that apples have the highest portion of free phenolics when compared to other fruits [Sun *et al.*, 2002]. The apples' bound phenolics are less bioavailable than free phenolics since they need to be released from the food matrix during digestion [Williamson *et al*., 2018]. Therefore, an important aspect of research is to study phenolics occurrence in natural food products and then to determine their intake by consumers. Apples, blackcurrants, strawberries, raspberries, chokeberries, cranberries, blackberries, plums, apricots, pomegranates, oranges, pineapples, and grapefruits are known to be the richest sources of polyphenols [Calderón-Oliver & Ponce-Alquicira, 2018]. Their consumption is estimated to range from 100 to 200 g per day. Apples provide approx. 22% of polyphenols in the diet [Scalbert & Williamson, 2000]. An apple portion contains 400 mg of polyphenols, whereas the average portion of pears or grapes provides 300 mg of polyphenols, and a portion of cranberries, cherries or blueberries provides 200–400 mg [Scalbert & Williamson, 2000]. In general, the intake of polyphenolic compounds varies widely among populations with the average approximating 1 g per day [Scalbert & Williamson, 2000]. For example, the main source of flavonoids in a diet of the Australian population is soy and its products, whereas 55.2% of total flavonoids come from non-alcoholic beverages in a diet of the French population [Del Bo *et al*., 2019]. In many countries (*e.g*., Poland), tea and fruits are also a common source of flavonoids in the diet. According to data selected by Murphy *et al.* [2019], flavonoid intake also varies by gender: women in Australia consume a higher amount of flavonoids than men: 659 *vs.* 566 mg per day. The interest in purchasing natural food products with a high flavonoid content by consumers might be driven by their positive effects on human health.

APPLE PHENOLIC COMPOUNDS – THE BIOLOGICAL EFFECTS

As mentioned in the Introduction section, dietary phenolics can play a positive role in reducing the risk of development of chronic diseases associated with oxidative stress. The biological effects of apple phenolic compounds are also of interest. Flavonoids extracted from apple peel were implicated in the suppression of breast cancer and the growth of carcinogenic cells in the bronchi [George & Rupasinghe, 2017; Loung *et al*., 2019]. In turn, Zielińska *et al.* [2019] proved the potential benefits of apple-derived polyphenols in modulating inflammation at the intestinal level. Apple extracts can also regulate carbohydrate metabolism through the inhibition of α-glucosidase activity, thereby potentially reducing the risk of type 2 diabetes development [Raphaelli *et al.*, 2019]. The newest research from Zou *et al.* [2020] showed that apple phenolics might be effective in the prevention of obesity *via* promoting brown adipogenesis. There is no clear evidence as to whether apple flavonoids protect lipids and proteins in plasma and decrease LDL cholesterol level [Zou *et al.,* 2020]. Zeng *et al*. [2020] explained, with the example of apple proanthocyanidins, that the hypocholesterolemic activity of polyphenols might be related to their ability to bind cholesterol. In their *in vitro* study, tannins were potent

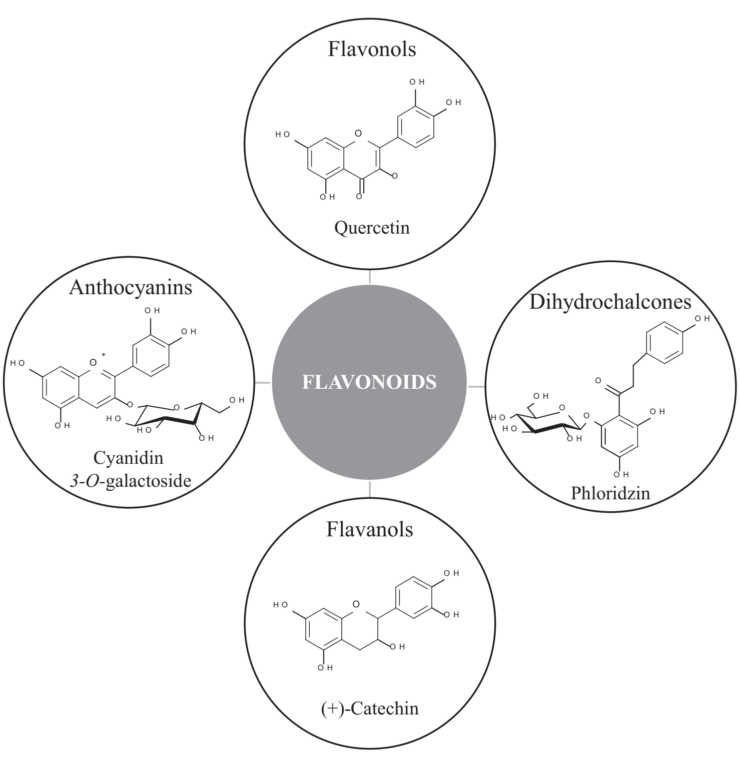


FIGURE 2. Flavonoid classes with examples of each class and their structure.

TABLE 1. The summary of latest studies about major phenolic compounds found in apples according to variety, country of origin, and special pre-treatment.

Material	Country of origin	Apple variety	Content of major phenolic compounds	Main remarks	Reference
Apple peel	Croatia	*‘Apistar’* *‘Bobovac’* *‘Božićnica’*	(–)-epicatechin: **119.5** mg/100g DW (–)-epicatechin: **131.8** mg/100g DW (–)-epicatechin: **50.4** mg/100g DW	The content of phenolics in peel differs among varieties	Loncarić *et al.* [2020]
Raw apple	Spain	‘*Golden Delicious’*	procyanidin B_2: **91.55** mg/100g DW (–)-epicatechin: **76.30** mg/100g DW quercetin 3-*O*-rhamnoside: **8.35** mg/100g DW	High-pressure releases flavonoids from the matrix	Fernández-Jalao *et al.* [2019]
High-pressure treated apple (400 MPa at 35°C for 5 min)			procyanidin B_2: **95.02** mg/100g DW (–)-epicatechin: **73.08** mg/100g DW quercetin 3-*O*-rhamnoside: **11.08** mg/100g DW		
Raw apple	Italy	‘*Golden Delicious’*	procyanidin B_2: **74.51** mg/100g DW (–)-epicatechin: **67.89** mg/100g DW quercetin 3-*O*-rhamnoside: **9.73** mg/100g DW		
High-pressure treated apple (600 MPa at 35°C for 5 min)			procyanidin B_2: **103.57** mg/100g DW (–)-epicatechin: **98.58** mg/100g DW quercetin 3-*O*-rhamnoside: **20.15** mg/100g DW		
Raw apple	Italy	‘*Golden Delicious’* organic	(–)-epicatechin: Before frozen storage: **19.3** mg/100g DW Dipped: **21.2** mg/100g DW Vacuumed with lemon juice: **18.1** mg/100g	Vacuum pre-treatment method in acid environment inhibits degradation of flavonoids during storage	Santarelli *et al.* [2020]
			After frozen storage: **19.4** mg/100g DW Dipped: **16.7** mg/100g DW Vacuumed with lemon juice: **18.0** mg/100g		
	Italy	‘*Golden Delicious’* conventional	(–)-epicatechin: Before frozen storage: **19.6** mg/100g DW Dipped: **20.0** mg/100g DW Vacuumed with lemon juice: **25.4** mg/100g		
			After frozen storage: **15.0** mg/100g DW Dipped: **16.9** mg/100g DW Vacuumed with lemon juice: **19.0** mg/100g		
Raw apple	Japan	‘*Golden Delicious’*	(–)-epicatechin: peel: **20.37–41.24** mg/100g DW pulp: **10.36–22.21** mg/100g DW	The profile of phenolics differs among apple parts and growing locations at different altitudes	Dhyani *et al.* [2018]
		‘*Red Delicious’*	(–)-epicatechin: peel: **37.74–52.97** mg/100g DW pulp: **13.21–22.45** mg/100g DW		
		‘*Royal Delicious’*	Total tannin content (TTC): peel: **16.03–33.29** TAE/g FW pulp: **11.64–25.83** TAE/g FW		
Crabapple	China	Malus ‘*Red splendor*’	procyanidin B_1: **103.57** mg/kg DW (–)-epicatechin: **36.34** mg/kg DW phloridzin: **24.57** mg/kg DW hyperin: **72.09** mg/kg DW isoquercitrin: **25.01** mg/kg DW	Total polyphenol content is the highest in apple cultivar ‘*Red splendor*’	Han *et al.* [2019]
		M. micromalus ‘*Haihongguo*’	procyanidin B_1: **847.88** mg/kg DW (+)–catechin: **430.96** mg/kg DW procyanidin B_2: **203.47** mg/kg DW (–)-epicatechin: **1220.76** mg/kg DW phloridzin: **94.42** mg/kg DW hyperin: **72.09** mg/kg DW isoquercitrin: **49.06** mg/kg DW		
		M. micromalus	procyanidin B_1: **1534.78** mg/kg DW (+)–catechin: **1326.33** mg/kg DW procyanidin B_2: **2151.86** mg/kg DW (–)-epicatechin: **2727.69** mg/kg DW cyanidin 3-*O*-galactoside: **190.17** mg/kg DW		

DW – dry weight; FW – fresh weight; TAE – tannic acid equivalent.

to precipitate cholesterol *via* interactions of ions and hydrophobic and intermolecular hydrogen bonds. Moreover, Hamauzu *et al.* [2005] demonstrated apple phenolic compounds to be significant inhibitors of the influenza viral activity. Then, in later research, Pastene *et al*. [2009] observed suppressed *Helicobacter pylori* urease activity after incubation with apple peel extracts, which suggests that apple phenolic compounds have a strong gastro-protective effect. It was also proved that apple extracts had an antibacterial effect against Gram-positive bacteria [Pires *et al*., 2018].

Flavonoids are metabolized *in vivo*, which suggests that they have a low impact on the antioxidant activity in humans. The significant importance of the higher overall antioxidant activity after the consumption of foods with a high flavonoid content (*e.g.*, fruits, vegetables, tea, wine, or chocolate) might be explained by the change in the concentration of antioxidant metabolites in human plasma (for example, uric acid) [Lotito & Frei, 2004a,b; 2006]. The addition of '*Red Delicious*' apple extract to human plasma *in vitro* increased ferric reducing antioxidant power (FRAP). In this case, FRAP values of plasma increased from 454 μM to 486 μM (+7%) and up to 532 μM (+17%), respectively, after the addition of 7 and 14 μg of apple polyphenols to 1 mL of plasma [Lotito & Frei, 2006]. Such an increase in plasma FRAP is comparable to the results reported after the consumption of other polyphenol-rich foods [Lotito & Frei, 2006]. Further *in vivo* study of Wruss *et al*. [2015] has shown that the antioxidant activity of human plasma increased after apple consumption. The highest oxygen radical absorbance capacity (ORAC) of plasma was determined 1 h (120% of change) and 6 h (120% of change) post intake.

Moreover, the addition of apple polyphenols to plasma *in vitro* significantly increases its antioxidant resistance. Although apple polyphenols do not inhibit the peroxidation of ascorbate in plasma, the endogenous half-life of urate and α-tocopherol and the delay in lipid oxidation were observed to be higher. Lotito & Frei [2004a] demonstrated a high content of polyphenols in extracts from '*Red Delicious*' apples, as reflected by a high radical scavenging activity *in vitro*. To prove this *in vitro* observation, in a further *in vivo* study by Lotito & Frei [2004a], six volunteers consumed five apples per day to deliver a total of 1825 mg of polyphenols. Plasma was collected 6 h after consumption, and antioxidant activity was measured. The results of an *ex vivo* study did not prove the *in vitro* measurements; no significant impact of apple polyphenols on plasma antioxidant status was found. Furthermore, the antioxidant activity of plasma measured by the FRAP method significantly increased 1 h after apple consumption, to 60 μM of TE [Lotito & Frei, 2004b]. In the next step, volunteers consumed 260 g of bagel, as a control without flavonoids and 750 mL of water; the amount was matched to the consumed amount of carbohydrates and weight of apples. Approx. 60 mg of vitamin C was delivered with apples, which led to a slightly higher amount of ascorbic acid in plasma. The removal of ascorbate from plasma by ascorbate oxidase did not increase the antioxidant capacity. The results indicated that vitamin C from apples did not significantly modulate the antioxidant activity. The bagel consumption resulted in a decreased level of FRAP in plasma, which suggested the effect of food consumption *per se* [Lotito & Frei, 2004a,b]. This hypothesis was also tested on the same six volunteers. They drank a solution of 64 g of fructose in 1000 mL of water, and the fructose amount was estimated according to the weight of five apples. Plasma activity, expressed as FRAP, significantly increased after about 1 h, up to 40 μM, since fructose administration. Moreover, the changes in antioxidant activity depending on time were comparable to those observed after apple consumption. The concentration of uric acid in plasma significantly increased in every patient after fructose administration; the same was observed after apple consumption, while the level of ascorbate in plasma remained unchanged [Lotito & Frei, 2004b]. This research is consistent with the findings reported by Wruss *et al.* [2015], who observed that an increase in the total phenolic content over time did not correlate with an observed, highly elevated antioxidant capacity in the blood plasma after apple juice consumption. In addition, they concluded that the antioxidant activity was rather due to a high fructose content of the apple juice.

ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS

The antioxidant activity of different food products has been broadly addressed in recent investigations [Shahidi & Ambigaipalan, 2015]. For example, such products as coffee, tea, chocolate, wine, and beer possess high antioxidant activities that are linked to the high content of polyphenols. The total polyphenol content, including flavonoids, in fruits and juices is also correlated with the antioxidant activity of these products *in vitro* [Murillo *et al.,* 2012].

Flavonoids are strong antioxidants *in vitro*, mainly because of their low redox potential and ability to donate electrons and hydrogen atoms; however, their activities vary For example, flavanols have a redox potential of +0.53––0.57 V. From a thermodynamic point of view, they can protect urates (+0.59 V) but not ascorbate (+0.28 V) against oxidation by peroxide radicals (+1.06 V). Therefore, they are more effective than ascorbate in the antioxidant protection of α-tocopherol in a micellar solution [Frank *et al*., 2006]. However, high flavonoid contents are not always strictly correlated with strong antioxidant activity in the studied samples. For example, the highest antioxidant activity (ORAC assay) of blueberries was noted in their '*Northland*' and '*North country'* varieties, *i.e.*, 6747 and 6265 μmol TE/100 g of fresh matter, respectively, whereas the highest total flavonoid content was determined in those of '*North country'* and '*Blomidom*' varieties [Wang *et al.,* 2017]. A weak correlation was also demonstrated between antioxidant activity and total phenolic compounds for different varieties of apple [Jelodarian *et al*., 2012]. Despite the high antioxidant activity of flavonoids *in vitro*, their effectiveness *in vivo* is limited. In *in vitro* studies, the antioxidant activity of biologically active compounds of apples and their extracts were determined using the FRAP and ORAC methods [Lotito & Frei, 2006]. Water extracts of the apple variety '*Red Delicious*' contained 176 mg of phenolic compounds per 100 g of apples. The FRAP and ORAC values were at

1421 and 1508 μmol TE/100 g apples, respectively. However, polyphenols contribute 14–18% of FRAP and ORAC values. This could mean that other biologically active compounds also make a high contribution to the overall antioxidant activity measured *in vitro*.

The high antioxidant activity of apples might also be related to their procyanidin contents. Procyanidins are found in fruit and cocoa products and are deemed good antioxidants due to their multiple hydroxyl groups [Hellstrom *et al.*, 2009]. Their antioxidant potential depends on the chain length of oligomers and the type of oxygen they react with [Spranger *et al.*, 2008; Zhou *et al.*, 2014]. This also applies to apple procyanidins [Lu & Foo, 2000]. Procyanidins and their monomer, possess comparable antioxidant efficiency against peroxide radicals, whereas procyanidins with different degrees of oligomerization different significantly in their lipid-protecting ability against oxidation induced by iron and ascorbate [Plumb *et al.*, 1998]. Procyanidins are stronger antioxidants than more absorbable (–)-epicatechin and (+)-catechin.

As it was mentioned above, the effectiveness of flavonoids *in vivo* is limited; however, some studies reported an increase in the overall antioxidant activity in plasma. This observation leads to the general conclusion that flavonoids play an important role as antioxidants in plasma and tissues [Arts & Hollman, 2005]. After the ingestion of food or a beverage, flavonoids in the ingested matrix must pass from the gut lumen into the circulatory system in order to be absorbed. Since *in planta* almost all flavonoids are in the form of glycosides, the attached sugar must be removed following consumption before absorption can take place [Williamson *et al.,* 2018]. Some studies have pointed out that flavonoids are poorly absorbed and that their plasma concentrations are low even after flavonoid-rich food consumption [Lotito & Frei, 2006].

PHENOLIC COMPOUNDS – BIOAVAILABILITY AND ABSORPTION

A crucial aspect of research dedicated to molecules with antioxidant properties is their bioavailability from food. The list of factors that influence the bioavailability of antioxidants is quite long [Porrini & Riso, 2008]. They can be divided into four main categories: 1) related to the antioxidant (structure, molecular linkage, amount introduced, *etc*.); 2) related to food and its preparation (matrix characteristics, technological processing, presence of positive/negative effectors, *etc*.); 3) related to the host (disorders and/or pathologies, enzyme activity, gender, *etc*.); and 4) external (exposure to different environment and food availability). Also, the complex relationship between factors was discussed. In the mucosa of the intestine and liver, flavonoids are being modified *via* glucuronidation, methylation, and sulfating. Biotransformation influences their physical properties; therefore, they are more soluble in water and have better antioxidant properties. Some metabolites of phenolics have the same antioxidant activity as their precursors; however, in general, metabolites are weaker antioxidants because of the modifications of their catechol and phenol groups. Moreover, phenolics are degradable by digestive tract bacteria and their metabolites exhibit biological activity *via* an antioxidant or non-antioxidant mechanism [Minatel *et al.,* 2017]. Phloretin was established as a urinary biomarker of apple intake by Saenger *et al.* [2017]. Phlorentin content determined in urine 12 h after apple consumption allows discriminating low- and high apple consumers. Therefore, the study of Yuste *et al.* [2018] proved that glucuronide derivatives of phloretin, catechol, and hydroxyphenyl-γ--valerolactone, and also sulfate derivatives of dihydroxyphenylpropionic acid and (methyl) catechol are the main apple phenolic metabolites detected in human blood and plasma. In a further study, Yuste *et al.* [2019] specified that phlorentin glucuronide, and galactosides of cyanidin and peonidin were the intake markers of red-fleshed apples.

Quercetin is one of the most studied phenolic compounds because of its wide distribution in food and high antioxidant potential *in vivo*. Its main sources include onions, apples, tea, and wine. Quercetin occurs in a conjugated form as a quercetin glycoside. It is transferred to enterocytes *via* a sodium-dependent transporter of glucose 1. Some other studies pointed out that, before absorption in the intestine, enzymatic or bacterial deglycosylation takes place. Quercetin glycosides can persist in the bloodstream for more than 10 h, which is longer than other flavonoids like anthocyanidins and flavanols. After the intake of foods rich in quercetin, its blood plasma level was at 0.7–7.6 μM [Lotito & Frei, 2006]. In contrast to quercetin, flavanols are the main compounds of tea and chocolate, but are also found in apples (the amounts of (+)-catechin in apples and apple products are presented in Tables 1 and 2). Usually, flavanols occur in plants as aglycones and can be directly absorbed into the bloodstream. However, the concentration of free flavanols in the plasma is low were they are found in glucoronated and methylated forms. Anthocyanidins from berries and wines are absorbed intact, as glycosides. It was shown that their ethylated and glucuronated conjugates can be found in blood plasma in the nanomolar ranges [Porrini & Riso, 2008]. Other important phenolics in apples, phloretin and phloridzin, were investigated in the plasma of rats by Crespy *et al*. [2001]. They showed that the highest concentration of phloretin, in both conjugated and unconjugated forms (conjugated forms of phloretin were dominant at 85–95% of the total amount), was found in plasma after 10 h and rapidly decreased after 24 h. In turn, the research conducted by Rios *et al*. [2003] has shown that procyanidins are degraded by bacterial intestinal microflora to more absorbable phenolic acids with low molecular weights. While the bioavailability of quercetin, flavanols, and anthocyanidins in organisms is limited, the absorption of non-degradable procyanidins seems to be even lower, or almost null [Serra *et al*., 2010].

The absorption of flavonoids in humans is rather low in comparison to other antioxidants, such as vitamins C and E. Their maximum concentration in human plasma is observed between 1 and 3 h after flavonoid-rich food consumption. Levels of flavonols, flavanols, and flavanones are established at 0.006–7.6 μM, while these of anthocyanidins are lower than 0.15 μM [Hollman *et al.,* 1997]. Moreover, the half-life of flavonoids in human plasma is short, reaching only a few hours. These limitations influence the higher activity of diet-derived flavonoids as antioxidants *in vivo*, in comparison to other active compounds present in higher amounts,

TABLE 2. Phenolic compounds determined in products from apples according to apples' variety, country of origin and special pre-treatment.

Material	Country of origin	Apple variety	Content of dominant phenolic compounds	Main remarks	Reference
Juice	Germany	*'Elstar Elshof'* (variety susceptible to scab)	procyanidin B$_2$: **74.6** mg/L (–)-epicatechin: **61.8** mg/L procyanidin C$_1$: **46.6** mg/L	Higher content of flavonoids was determined in varieties resistant to scab	Schempp *et al.* [2016]
		'Rubinola' (scab-resistant variety)	procyanidin B$_2$: **106.6** mg/L (–)-epicatechin: **89.9** mg/L procyanidin C$_1$: **53.3** mg/L		
Cloudy juice	Poland	*'Shampion'*	procyanidin B$_2$: **169.5** mg/L (–)-epicatechin: **95.1** mg/L	Addition of ascorbic acid preserved the content of flavonoids in juice during storage	Kolniak-Ostek *et al.* [2013]
Cloudy juice after 3-year storage			procyanidin B$_2$: **157.6** mg/L (–)-epicatechin: **95.1** mg/L		
Cloudy juice with ascorbic acid (AA) addition			procyanidin B$_2$: **202.1** mg/L (–)-epicatechin: **73.7** mg/L		
Cloudy juice with AA addition after 3-year storage			procyanidin B$_2$: **165.9** mg/L (–)-epicatechin: **82.3** mg/L		
Cider after ethanol fermentation	Turkey	*'Red Delicious'*	(+)–catechin: **1.46** mg/L (–)-epicatechin: **3.33** mg/L caffeic acid: **0.75** mg/L chlorogenic acid: **16.50** mg/L *p*–coumaric acid: **0.04** mg/L	Pulp maceration increased the content of phenolic compounds in cider	Budak *et al.* [2015]
Cider after maceration with pulp and ethanol fermentation			(+)–catechin: **2.13** mg/L (–)-epicatechin: **4.63** mg/L caffeic acid: **0.96** mg/L chlorogenic acid: **24.13** mg/L *p*–coumaric acid: **0.03** mg/L		
Vinegar (Zi Lin)	China	no information, commercial samples	protocatechuic acid: **0.82** μg/mL chlorogenic acid: **10.91** μg/mL *p*–coumaric acid: **0.17** μg/mL	The content of phenolic compounds differed among vinegars from various producers	Liu *et al.* [2019]
Vinegar (Heng Shun)			protocatechuic acid: **1.54** μg/mL chlorogenic acid: **2.99** μg/mL		
Cider vinegar (Heinz)			protocatechuic acid: **1.00** μg/mL chlorogenic acid: **0.23** μg/mL		
Cider vinegar (Xin He)			chlorogenic acid: **4.67** μg/mL		
Vinegar	Turkey	*'Malus domesticus'*	gallic acid: **0.8** mg/100 mL *p*–hydroxybenzoic acid: **0.2** mg/100 mL (+)–catechin: **2.4** mg/100 mL syringic acid: **0.12** mg/100 mL caffeic acid: **0.4** mg/100 mL *p*–coumaric acid: **0.08** mg/100 mL	Significant changes appeared in phenolic compounds during vinegar making	Bakir *et al.* [2016]
Pomace	Cameroon	*'Malus sylvestris'*	(–)-epicatechin, caffeic acid and quercetin Total amount: **141.30** mg/100 g DW	The content of phenolic compounds increased after blanching	Dibanda *et al.* [2020]
Pomace after microwave blanching; 50°C, 5 min			Gallic acid, (–)-epicatechin, caffeic acid, *p*–coumaric acid, ferulic acid, rosmarinic acid, quercetin. Total amount: **93.13** mg/100 g DW		

e.g., 30–150 μM of vitamin C, 160–450 μM of urinate, or 15–40 μM of vitamin E. Long-term consumption of flavonoid-rich food does not lead to the accumulation of their high amounts in plasma. Concentrations of quercetin in human plasma are lower than 1 μM [Loito & Frei, 2006]. Data about the concentration of flavonoids in human tissues are not available, but it is unlikely that flavonoids have a significant impact on the antioxidant activity in cells, where ascorbate and reduced glutathione can be found in mM concentrations [Loito & Frei, 2006]. Poorly absorbed phenolic compounds are metabolized in intestines and liver. Moreover, they are good substrates and indicators of phase II enzymes [Williamson *et al.*, 1996]. This suggests that they are recognized by organisms as xenobiotics, potentially toxic compounds, because of their polyhydroxy structure and strong redox potential [Dunnick & Hailey, 1992]. Therefore, by inducing the detoxification of enzymes, flavonoids protect the organism against mutagenic and carcinogenic factors, thus preventing cancer development [Knekt *et al.,* 2002]. Probably, the absorbed flavonoids (in low concentrations) and their metabolites exert other biological activities, including *e.g.*, changing cell signaling and gene expression, which contributes to the health benefits. Hence, flavonoids after intensive metabolic transformations and chemical forms of flavonoids present in fruits and vegetables (mainly glycosides, except for flavanols in aglycon forms), differ from the metabolites formed *in vivo*.

FACTORS INFLUENCING THE CONTENT OF PHENOLIC COMPOUNDS IN APPLES

There are a lot of factors influencing the phenolic content in apples. In the West Himalayan apple varieties, the phenolic content was significantly differentiated among cultivars and locations [Bahukhandi *et al*., 2018]. It was also confirmed that the variety and maturity of apples significantly affected their chemical composition, content of phenolics, and antioxidant activity [Oszmiański *et al*., 2008]. Oszmiański *et al*. [2008] compared 120 apple varieties and found they were highly diversified in terms of pehnolics content. According to this research, the highest phenolic content was found in apples of '*Ozark Gold'* variety (~2116.03 mg/kg), whereas the lowest one in these of '*Ligol*' variety (~814.17 mg/kg). The quality and quantity of phenolics in apples directly influences their antioxidant activity [Wojdyło *et al.,* 2008]. Orchard cultivation system has an impact on the chemical composition of apples as well. Apples from organic farming have a higher content of phenolics in comparison to those from conventional cultivation [Wojdyło *et al.*, 2010]. Sut *et al.* [2019] noted that the content of phloretin 2-*O*-xyloglucoside and quercetin 3-*O*-arabinoside was significantly higher in ancient apple varieties ('*Friuli Venezia Giulia'*, northern Italy) than in the commercial ones (*e.g*., '*Golden Delicious'*, '*Red Delicious'*, '*Granny Smith'*, and '*Royal Gala'*). Also, ancient Croatian apple varieties were observed to be an important source of phenolics, especially proanthocyanidins, phenolic acids, and dihydrochalcones, compared to the commercial ones [Jakobek *et al.*, 2013]. Also, Kschonsek *et al.* [2018] noticed the significant difference in phenolics content between old (29 mg/ 100 g) and new (13 mg/ 100 g) cultivars of apples from Germany. It might be said that even the ancient cultivars can be a good source of bioactive compounds in the formulation of novel products. However, the results are not unambiguous in comparison to other research data. For example, the peel, pomace, and juice made of the old Spanish cultivar '*Verde Doncella'* had lower contents of flavanols and quercetin derivatives compared to '*Red Delicious'* cultivar. However, this study pointed out that '*Verde Doncella'* had a higher content of *p*-coumaric acid and procyanidin B_2 in the peel [Krawitzky *et al*., 2014]. Interesting results were described by Skłodowska *et al*. [2018], who observed that apple leaves might be a rich source of bioactive compounds due to the variety and geographical origin, which significantly differentiated the apple leaf phenolics. The methods and conditions of apple processing also have a major impact on polyphenols.

FACTORS INFLUENCING THE CONTENT OF PHENOLIC COMPOUNDS IN APPLE PRODUCTS

In Poland, clarified beverages and wines are the most popular products made from apple juice concentrate. This type of manufacturing leads to large differences in the chemical composition between the raw material and the final product, such as an almost total lack of pectin and vitamin C, and 90% loss of phenolics. The lowest content of valuable procyanidin is found in apple juice. Procyanidins bind to the cell walls of apples and are removed during the clarification process of juice. This leads to a significant decrease in the polyphenols in juices [Kolniak-Ostek *et al.*, 2013]. Oszmiański & Wojdyło [2009] noted a 6-fold lower antioxidant activity of apple juice compared to blackcurrant juice. Cloudy and puree juices are good alternatives to clarified apple juices. The content of phenolic compounds depends on the apple variety and type of manufactured juice. Clarified juice made of '*Idared*' apples contained only 250.1 mg/L of phenolics, whereas the content in cloudy juice made from '*Szampion*' was 5-fold higher reaching 1044.4 mg/L of juice [Oszmiański *et al.*, 2007]. Cloudy juice made of '*Szampion'* apples had up to 31% higher antioxidant potential, measured by the DPPH method, and that made of '*Idared'* apples had about 45% higher antioxidant potential than the respective clarified juices [Oszmiański *et al.*, 2007]. In other research, cloudy juices produced without the enzymatic treatment, clarification, or filtration contained more biologically active compounds [Oszmiański & Wojdyło, 2009]. The juice clarification process removes brow products of phenolic compounds oxidation. Omitting this step in cloudy juice manufacture leads to their unattractive brown color. Cloudy juices should be produced from high-quality, fresh, and matured apples. Their cloudiness can range from 15% (var. '*Elstar'*) to 47% (var. '*Gostar'*) [Wojdyło *et al.,* 2008]. Schempp *et al*. [2016] noted that '*Elstar'* variety was a rich source of flavanols, *e.g*., (–)-epicatechin, and their oligomers, *e.g*., procyanidin B_2 and C_1, with their content approximating 240.0 mg/L. Considering the oxidation and browning of juices, they should be manufactured in an inert gas environment, after ascorbic acid addition to the pulp. The quality of cloudy juices can be improved by employing decanting instead of pressing. Juice production using decanters takes only a few seconds, which inhibits the oxidation of crushed fruits. The addition of ascorbic acid or rhubarb to apple pulp during fruit crushing reduces the effects of oxidation processes, including color change. The addition of ascorbic acid proved necessary in all types of juices except for those made of var. '*Szampion*' apples. Juices with ascorbic acid contain significantly more phenolic compounds (approx. 1.2-fold higher) and have higher antioxidant activity (3.5-fold higher) compared to juices without its addition [Kolniak-Ostek *et al.*, 2013]. Promising results in terms of decreasing the browning of juices were achieved by using high-pressure carbon dioxide treatment [Murtaza *et al*., 2020]. This processing of juices also increased (–)-epicatechin and (+)-catechin concentrations; however, more studies need to be done to establish the appropriate temperature and pressure conditions to achieve the highest content of phenolics. In another study, an attempt to increase the content of phenolic compounds and antioxidant activity in apple juices and beverages by the enzymatic treatment of apple pulp and the addition of apple leaves proved successful [Zielinski *et al.,* 2014]. The phenolic compound contents increased by 2 to 5 times, whereas the antioxidant potential by about 1.29 times. Moreover, the addition of apple puree produced from different enzymatic formulations increased the phenolic content in the studied juices from 441 mg/L to 612 mg/L, and antioxidant activity by 5–21% [Zielinski *et al.,* 2014]. The obtained data show that enriching natural cloudy juices with ascorbic acid, apple pulp or leaves is useful. If enriched with this technology, juices can be an attractive product, with antioxidants that will have a beneficial influence on human health. Oszmiański *et al.* [2008] demonstrated a high content of polyphenolic compounds and antioxidant activity in juices made of '*Idared*' apples. Furthermore, the addition of blackcurrants not only enriched apple juice with phenolic compounds and improved its color, but also protected it from enzymatic oxidation. Ascorbic acid from blackcurrants, used in this reaction, also effectively protected the polyphenols of '*Idared*' apples from oxidation. Antioxidant activity is measured as the ability to scavenge DPPH radicals; therefore, the antioxidant activity of blackcurrant juice was about 40-fold higher than that of apple juice. The addition of 20% blackcurrant juice to apple juice increased the antioxidant activity about 8-fold in comparison to apple juice without any additives [Oszmiański *et al.,* 2008]. However, Średnicka-Tober *et al.* [2017] demonstrated that the addition of chokeberry juice to apple juice led to a decrease in quercetin and kaempferol contents. Whereas green tea extract addition resulted in a significant enrichment of apple juice with phenolic acids (~2- times) and flavonoids (~10- times), with molecules characteristic for tea: (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, and (–)-epigallocatechin 3-*O*-gallate. Bat *et al.* [2018] successfully used phytochemicals as markers to prove the origin and variety of apple juices. Moreover, Adamenko *et al.* [2019] established a new product, *i.e*., fermented apple beverage with Cornelian cherry addition (10%) having a strong antioxidant properties (ABTS, DPPH, and FRAP tests) and enriched with phenolic compounds and iridoids. This treatment caused the highest content of 5-*O*-caffeoylquinic acid (from 70.0 to 96.8 mg/L). In turn, Verdu *et al.* [2014] presented that the apple progeny significantly influenced the phenolic compounds content of another type of apple beverage, namely cider. The content of 12 phenolics varied in apples as a genetic effect and between harvest years.

Kahle *et al.* [2005] pointed out that the phenolic compounds content was significantly different in juices made from dessert or cider apples. Moreover, there was a difference in phenolic contents between apple cultivars, while freshly squeezed juices were richer in phenolic compounds than commercially available juices. The influence of storage on phenolic stability in apple juices was also studied by Maragó *et al.* [2015]. They demonstrated that the contents of total phenolics and flavanols were almost 5-fold and 8-fold higher, respectively, in '*Panaia-red'* juice than in juice made of '*Golden Delicious'* apples. Higher stability of phenolics was achieved in '*Panaia-red'* juice. Pavun *et al.* [2018] highlighted that the use of fruit nectar definitely lowered the phenolic content in commercial juices, which was ultimately correlated with their antioxidant activity. It was also demonstrated that not pasteurization but pre-pasteurization had a significantly stronger impact on phenolics content during processing of not from concentrate (NFC) juices [Tian *et al*., 2018].

Another important aspect affecting contents of antioxidants is the technological preparation of raw fruits. The microwave-treatment of apple peels [Dibanda *et al.,* 2020] and their storage method affected phenolics content and antioxidant activity [Santarelli *et al.,* 2020]. According to Santarelli *et al.* [2020], there is a difference between the response of tissues of organicaly and conventionally farmed apples to freezing. The antioxidant activity of organic apples after freezing was reduced by only 13%, whereas in the conventional ones a decrease of up to 25% was noted. This was related to the higher resistance of organic apple tissue to thermal stress, which caused a decrease in free and conjugated phenolics related to antioxidant activity. Fernández-Jalao *et al.* [2019] increased extractability of flavonoids from apple pulp by up to 54% using high-pressure processing at 600 MPa. In another study, Ferrentino *et al*. [2018] successfully used supercritical fluid extraction to improve the recovery of extracted phenolics from apple pomace. The mechanism of these two methods might be related to the flavonoid compounds from cell walls being trapped in an insoluble matrix.

Also, the consumption of dried apples is quite popular among consumers nowadays. It is known that food product stability over storage period is an important aspect to be considered during its production process. In this context, the addition of a green tea extract to dried apples might be a perfect solution, providing a new type of product that would combine the preservation of apples and an increased phenolic content in the final product [Corey *et al.*, 2011]. Pająk *et al.* [2017] proposed biofilms made of white or green tea extracts to prolong the freshness of sliced apples. The proposed application of white tea allowed them to obtain a stored product with a similarly high content of total phenolics to that in the fresh material. Tappi *et al.* [2018] used cold atmospheric plasma to study the effect of phenolic behavior in fresh-sliced apples. They noticed that phenolic content significantly decreased in slices after 2 h (1.774 mM/kg fresh weight); however, it was not significantly lower after half an hour (from 2.142 to 2.092 774 mM/kg fresh weight). The high content of phenolic compounds and health-promoting properties of food products play an important role in consumer acceptance. From the sensory point of view, in the study of Wang *et al*. [2019a], the most preferred by the panelists were the dried apple slices pretreated with steam blanching and osmotic dehydration of the apple slices. Freeze-dried apple powders were also found to be good sources of antioxidants [Raudone *et al*., 2016], like chlorogenic acid and procyanidin C_1 which were responsible for their high antioxidant activity.

Apples are eagerly consumed mostly because of their pleasant taste and the fact that they are widely available worldwide. FAO statistics reported that, in 2016, the global production of apples exceeded 80 million tons, and over 3 million were converted into juice [Matsuoka, 2019]. According to these statistics, a large amount of waste is simultaneously produced. Therefore, growing attention has been paid to the reuse or limitation of the amount of waste produced. The benefits of apple pomace consumption were described in a publication prepared by Skinner *et al.* [2018]. The positive antioxidant effect on human health might be related to the phenolics in this apple waste. Therefore, quercetin (5.72 μg/kg), phloretin (2.01 μg/kg), and phloridzin (1.23 μg/kg) were determined to be the main phenolics in apple pomace [Rana *et al.*, 2015]. Apple peel is also a rich source of bioactive compounds [Raudone *et al.,* 2017]. Its main flavonoids are (–)-epicatechin and quercetin, at 0.28 and 0.19 mg/g, respectively [Bhagwat *et al.*, 2011]. This is in agreement with Escarpa & González [2001], who found high amounts of phenolic compounds (in the order: rutin, chlorogenic acid, and (+)-catechin) in "*Golden*" and "*Reinette*" apples (both peel and pulp). A possible innovation is the use of apple peels after microwaving, which is a good method for releasing phenolic compounds bound in the cell wall [Dibanda *et al*., 2020]. Furthermore, apple peels can be a good component to formulate functional foods, because of the high total phenolic content (1.1 g GAE/g of dry weight). Du *et al*. [2020] proposed "cold-pressing technology" as an effective method for peeling and deseeding apple fruits, with a positive effect on phenolic compounds retention in pomace. A 3- to 18-fold higher content of gallic and vanillic acids, (–)-epicatechin 3-*O*-gallate, and phloridzin was detected in apple pulp than in juice, cider, and/or vinegar. Xu *et al.* [2016] pointed out that seeds could also be a rich source of phenolic compounds. However, the content of phenolics differs in seeds from different apple cultivars. The dominant phenolics determined in seeds were phloridzin > hyperin > chlorogenic acid. Other examples of apple products and the main conclusions about phenolics variation during apple processing are summarized in Table 2.

METHODS OF PHENOLICS DETERMINATION AND ANTIOXIDANT ACTIVITY MEASUREMENT IN APPLES

A broad review of phenolic compounds' determination was prepared by Naczk & Shahidi [2004]. They gathered the main existing knowledge about phenolic compound extraction and analysis of their profile using spectrophotometric and chromatographic methods; however, since then, some new insights have been presented in this respect. The Folin–Ciocalteu's reagent is still commonly used to spectrophotometrically characterize the total content of phenolic compounds in apples and their products [Azlan *et al*., 2018; Bakir *et al*., 2016; Dhyani *et al*., 2018; Xu *et al*. 2016]. Total phenolic content is one of the most frequently analyzed factors, even after it was shown that the spectrophotometric method can lead to inaccurate results because of the strong interference of sugars and proteins in the assay results [Escarpa & Gonzalez, 2001]. In this context, HPLC-UV-VIS was used to determine the phenolic compound profiles in apple samples, preceded by an appropriate extraction procedure. Solvent extraction, sample fragmentation and its pretreatment, and compound-of-interest isolation are also important steps [Starowicz, 2019]. For example, Stan *et al.* [2017] proposed conventional extraction as a more efficient method to extract phenolics from dried apples than microwave- or ultrasound-assisted extraction. However, they noted that the content of compounds extracted can be different upon using different extraction procedures. Chlorogenic acid, (+)-catechin, and quercetin contents were higher after conventional than microwave-assisted extraction. The microstructure analysis conducted by Wang *et al.* [2019b] revealed that ultrasound treatment released phytochemicals from fruit cell walls, proving to represent a more efficient technique to increase phenolic compounds extractability. An interesting procedure was presented by Loncarić *et al.* [2020], who extracted flavonoids from apple peels using micro-matrix solid-phase dispersion (micro-MSPD) with sea sand and a solvent mixture of methanol:water:formic acid. Also, different instrumentation is used to increase the efficiency of compound determination. Both qualitative and quantitative methods are in matter of interest, and can be used with internal/external standards to determine phenolic compounds content. The HPLC with a diode array detector (DAD) is the most common method applied for phenolic compound determination in apples [Duda-Chodak *et al.,* 2010]. However, its modifications were proposed in further studies. Gomes *et al.* [2016] used the electrochemical properties of flavonoids to measure their content in fruit powders by RP-HPLC-EC. This method was appropriate to determine low content of selected polyphenols in either fresh or dried samples. The LC-MS/MS was also adapted to phenolic compound determination in samples of apples because of its low sensitivity and good repeatability [Loncarić *et al.,* 2020]. In the study of Jakobek *et al.* [2013], two different detectors were used to determine contents of specific phenolic compounds in apples, *i.e.,* UV-Vis and MS/MS. Moreover, using various HPLC columns and conditions in the inter-laboratory study, Hollands *et al.* [2017] developed, validated, and assessed analytical method for the separation, identification and quantification of procyanidins in an apple extract. Additionally, many researchers have tried to establish a relationship between bioactive compounds and antioxidant activity, to determine which phenolic compound has the greatest influence on antioxidant activity.

There are several mechanisms of the antioxidant action of phenolic compounds and therefore different methods must be used to determine their antioxidant activity. Alam *et al.* [2013] found that the most frequent methods used to determine antioxidant activity *in vitro* in plant material are: α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay, hydroxyl radical scavenging assay, and superoxide radical scavenging activity determination. According to these authors, the *in vivo* methods frequently used to determine antioxidant activity included lipid peroxidation assay (LPO) in tissues and determination of superoxide dismutase (SOD) and catalase (CAT) activities in erythrocytes of experimental animals. The antioxidant activity is often measured by using a few methods. The DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities of apple extracts were measured by Duda-Chodak *et al.* [2010]. A significant difference was found between the analyzed ABTS$^{\bullet+}$ scavenging activities of apple extracts, whereas no significant differences were noted in the DPPH method. Also, the FRAP method is usually introduced to evaluate antioxidant capacity. Pająk *et al.* [2017] found a high correlation between the antioxidant activities measured by FRAP, DPPH, and ABTS assays, and total phenolic content (with correlation coefficients of 0.914, 0.950, and 0.965, respectively). Furthermore, ethanol, water, and methanol are the solvents most commonly used to extract phenolic compounds before antioxidant activity determination usually conducted with spectrophotometric methods using cuvettes. As an innovation, Horszwald & Andlauer [2011] proposed 96-well plates (measurement done on a microplate reader) instead of cuvettes. The main advantages of this method are the low amounts of chemicals and extracts needed, and the very good repeatability of the results obtained. Gökmen *et al.* [2009] presented a novel approach, "QUENCHER," to the analysis of antioxidant activity. A direct QUENCHER procedure allows measuring the antioxidant activity in the samples in which "a great part of antioxidants is bound to insoluble matrices" [Gökmen *et al.,* 2009]. An interesting method was presented by Raudone *et al.* [2017], in which a combination of HPLC-DAD and FRAP assay was applied to measure the flavonoid content and antioxidant activity in apples and apple peels. Based on the HPLC-DAD–FRAP post-column analysis, it was established that among 12 flavanol compounds, flavanols and phenolic acids exhibited antioxidant activity, whereas no ferric ion reducing activity was observed for phloridzin. Therefore, it was observed that phloridzin cannot be used as a reference compound in antioxidant activity determination.

CONCLUSIONS

Apples are an important source of phenolics in the human diet, and their consumption has been implicated in the prevention of degenerative diseases. The polyphenols that can be found in apple peel and pulp are flavonols (mainly quercetin and its glycosides), flavanols ((–)-epicatechin and (+)-catechin), procyanidins, anthocyanidins, and dihydrochalcones (phloridzin and its derivatives). Their content was strictly related to the geographical origin, variety, and processing techniques of the apple products. Information about cultivar--typical apple polyphenol content and profile is important for bioactivity studies and, consequently, essential for the development of consumer-relevant products with particular nutritional functionalities. As to what the future holds, much work should be put into determining the bioavailability, transport, and bioactivity of dietary flavonoids. Moreover, a strong emphasis should be put in further studies on the reuse of apple by-products, *e.g.*, peels and seeds, as novel food components.

ORCID IDs

M. Starowicz https://orcid.org/0000-0001-6053-2250;
M.K. Piskuła https://orcid.org/0000-0001-7953-0909;
H. Zieliński https://orcid.org/0000-0002-4333-6677.

REFERENCES

1. Adamenko, K., Kawa-Rygielska, J., Kucharska, A.Z., Piórecki, N. (2019). Fruit low-alcoholic beverages with high contents of iridoids and phenolics from apple and Cornelian cherry (*Cornus mas* L.) fermented with *Saccharomyces bayanus*. *Polish Journal of Food and Nutrition Sciences, 69*(3), 307–317. https://doi.org/10.31883/pjfns/111405
2. Alam, Md.N., Bristi, N.J., Rafiquzzaman, Md. (2013). Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, *21*(2), 143–152. https://doi.org/10.1016/j.jsps.2012.05.002
3. Arts, I.C., Hollman, P.C. (2005). Polyphenols and disease risk in epidemiologic studies. *American Journal of Clinical Nutrition*, *81*(1), S317-S325. https://doi.org/10.1093/ajcn/81.1.317S
4. Azlan, A., Kok, Y.W., Khoo, H.E. (2018). Antioxidants content and activity of polyphenol-rich mixtures. *Journal of Engineering and Applied Sciences*, *13*(9), SI, 6973–6979. http://doi.org/10.36478/jeasci.2018.6973.6979
5. Bahukhandi, A., Dhyani, P., Bhatt, I.D., Rawal, R.S. (2018). Variation in polyphenolics and antioxidant activity of traditional apple cultivars from West Himalaya, Uttarakhand. *Horticultural Plant Journal*, *4*(4), 151–157. https://doi.org/10.1016/j.hpj.2018.05.001
6. Bakir, S., Toydemir, G., Boyacioglu, D., Beekwilder, J., Capanoglu, E. (2016). Fruit antioxidants during vinegar processing: changes in content and *in vitro* bio-accessibility. *International of Molecular Sciences, 17*(10), art. no. 1658. https://doi.org/10.3390/ijms17101658
7. Bat, K.B., Vodopivec, B.M., Eler, K., Ogrinc, N., Mulič, I., Masuero, D., Vrhovšek, U. (2018). Primary and secondary metabolites as a tool for differentiation of apple juice according to cultivar and geographical origin. *LWT – Food Science and Technology*, *90*, 238–245. https://doi.org/10.1016/j.lwt.2017.12.026
8. Bhagwat, S.B., Haytowitz, D.B., Holden, J.M. (2011). USDA database for the flavonoid content of selected foods. Release 3.1. Available on-line at [https://www.ars.usda.gov/ARSUserFiles/80400525/Data/Flav/Flav_R03–1.pdf]. Accessed 21 Janaury 2020.
9. Bosetti, C., Spertini, L., Parpinel, M., Gnagnarella, P., Lagiou, P., Negri, E., Franceschi, S. (2005). Flavonoids and breast cancer risk in Italy. *Cancer Epidemiological Biomarkers Prevention*, *14*, 805–808. https://doi.org/10.1158/1055-9965.EPI-04-0838
10. Budak, N.H., Ozçelik, F., Güzel-Seydim, Z.B. (2015). Antioxidant activity and phenolic content of apple cider. *Turkish Journal of Agriculture – Food Science and Technology*, 3(*6*), 356–360. https://doi.org/10.24925/turjaf.v3i6.356-360.265
11. Calderón-Oliver, M, Ponce-Alquicira, E. (2018). Fruits: a source of polyphenols and health benefits. Eds. A.M. Grumezescu, A.M. Holban, In *Handbook of Food Bioengineering, Natural and Artificial Flavoring Agents and Food Dyes*, Academic Press, London, United Kingdom, pp. 189–228. https://doi.org/10.1016/B978-0-12-811518-3.00007-7
12. Corey, M.E., Kerr, W.L., Mulligan, J.H., Lavelli, V. (2011). Phytochemical stability in dried apple and green tea functional products as related to moisture properties. *LWT – Food Science and Technology*, *44*(1), 67–74. https://doi.org/10.1016/j.lwt.2010.07.005
13. Crespy, V., Aprikian, O., Morand, Ch., Besson, C., Manach, C., Demigné, Ch., Rémésy, Ch. (2001). Bioavailability of phloretin and phloridzin in rats. *The Journal of Nutrition*, 131(12), 3227–3230. https://doi.org/10.1093/jn/131.12.3227
14. Del Bo, C., Bernardi, S., Marino, M., Porrini, M., Tucci, M., Guglielmetti, S., Cherubini, A., Carrieri, B., Kirkup, B., Kroon, P., Zamora-Ros, R., Liberona, N.H., Andres-Lacueva, C., Riso, P. (2019). Systematic review on polyphenol intake and health outcomes: is there sufficient evidence to define a health-promoting polyphenol-rich dietary pattern? *Nutrients*, *11*(6), art. no. e1355. https://doi.org/10.3390/nu11061355
15. Dhyani, P., Bahukhandi, A., Rawat, S., Bhatt, I.D., Rawal, R.S (2018). Diveristy of bioactive compounds and antioxidant activity in Delicious group of apple in Western Himalaya. *Journal of Food Science and Technology – Mysore*, *55*(7), 2587–2599. https://doi.org/10.1007/s13197-018-3179-x

16. Dibanda, R.F., Akdowa, E.P., Rani, P.A. Tongwa, Q.M., Mbofung C.M.F. (2020). Effect of microwave blanching on antioxidant activity, phenolic compounds and browning behaviour of some fruit peelings. *Food Chemistry*, *302*, art. no. 125308.
https://doi.org/10.1016/j.foodchem.2019.125308
17. Du, G.R., Zhu, Y.Y., Wang, X.Y., Zhang, J., Tian, C.R.., Liu, L., Meng, Y.H., Guo, Y.R. (2019). Phenolic composition of apple products and by-products based on cold-pressing technology. *Journal of Food Science and Technology – Mysore, 56*(3), 1389–1397.
https://doi.org/10.1007/s13197-019-03614-y
18. Duda-Chodak, A., Tarko, T., Satora, P., Sroka, P., Tuszyński, T. (2010). The profile of polyphenols and antioxidant properties of selected apple cultivars grown in Poland. *Journal of Fruit an Ornamental Plant Research*, *18*(2), 39–50.
19. Dunnick, J.K., Hailey, J.R. (1992). Toxicity and carcinogenicity studies of quercetin, a natural component of foods. *Toxicological Sciences*, *19*(3), 423–431.
https://doi.org/10.1093/toxsci/19.3.423
20. Escarpa, A., González, M.C. (2001). Approach to content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta*, *427*(1), 119–127.
https://doi.org/10.1016/S0003-2670(00)01188-0
21. Fernández-Jalao, I., Sánchez-Moreno, C., De Ancos, B. (2019). Effect of high-pressure processing on flavonoids, hydrocinnamic acids, hydrochalcones and antioxidant activity of apple 'Golden Delicious' from different geographical origin. *Innovative Food Science & Emerging Technologies*, *51*, SI, 20–31.
https://doi.org/10.1016/j.ifset.2018.06.002
22. Ferrentino, G., Morozova, K., Mosibo, O.K., Ramezani, M., Scampicchio, M. (2018). Biorecovery of antioxidants from apple pomace by supercritical fluid extraction. *Journal of Cleaner Production*, *186*, 253–261.
https://doi.org/10.1016/j.jclepro.2018.03.165
23. Francini, A., Sebastiani, L. (2013). Phenolic compounds in apple (*Malus x domestica* Borkh.): compounds characterization and stability during postharvest and after processing. *Antioxidants*, *2*(3), 181–193.
https://doi.org/10.3390/antiox2030181
24. Frank, J., Budek, A., Lundh, T., Parker, R.S., Swanson, J.E., Lourenço, C.F., Gago, B., Laranjinha, J., Vessby, B., Kamal-Eldin, A. (2006). Dietary flavonoids with a catechol structure increase α-tocopherol in rats and protect the vitamin from oxidation *in vitro*. *Journal of Lipid Research*, *47*(12), 2718–2725.
https://doi.org/10.1194/jlr.M600291-JLR200
25. George, V.C., Rupasinghe, H.P.V. (2017). Apple flavonoids suppress carcinogen-induced DNA damage in normal human bronchial epithelial cells. *Oxidative Medicine and Cellular Longevity*, *2017*, art. no. 1767198.
https://doi.org/10.1155/2017/1767198
26. Giacalone, M., Di Sacco, F., Traupe, I., Pagnucci, N., Forfori, F., Giunta, F. (2015). Blueberry polyphenols and neuroprotection. Eds. R.R. Watson, V.R. Preedy, In *Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease, Prevention and Therapy,* 1, Academic Press, Cambridge, USA, pp. 17–28.
https://doi.org/10.1016/B978-0-12-411462-3.00002-3
27. Gökmen, V., Serpen, A., Fogliano, V. (2009). Direct measurement of the total antioxidant capacity of foods: the 'QUENCHER' approach. *Trends in Food Science & Technology*, *20*(6–7), 278–288.
https://doi.org/10.1016/j.tifs.2009.03.010
28. Gomes, S.M.C., Ghica, M.-CE., Rodrigues, I.A., de Souza Gil, E., Oliveira-Brett, A.M. (2016). Flavonoids electrochemical detection in fruit extracts and total antioxidant capacity evaluation. *Talanta*, *154*, 284–291.
https://doi.org/10.1016/j.talanta.2016.03.083
29. Guo, X.F., Ruan, Y., Li, Z.H., Li, D. (2019). Flavonoid subclasses and type 2 diabetes mellitus risk: a meta-analysis of prospective cohort studies. *Critical Reviews in Food Science and Nutrition*, *59*(17), 2850–2862.
https://doi.org/10.1080/10408398.2018.1476964
30. Habanova, M., Saraiva, J.A., Holovicova, M., Moreira, S.A., Fidalgo, L.G., Haban, M., Gazo, J., Schwarzova, M., Chlebo, P., Bronkowska, M. (2019). Effect of berries/ apple mixed juice consumption on the positive modulation of human lipid profile. *Journal of Functional Foods*, *60*, art. no. 103417.
https://doi.org/10.1016/j.jff.2019.103417
31. Hamauzu, Y., Yasui, H., Inno, T., Kume, C., Omanyuda, M. (2005). Phenolic profile, antioxidant property, and anti-influenza viral activity of Chinese quince (*Pseudocydonia sinensis* Schneid.), quince (*Cydonia oblonga* Mill.), and apple (*Malus domestica* Mill.) fruits. *Journal of Agricultural and Food Chemistry, 53*(4), 928–934.
https://doi.org/10.1021/jf0494635
32. Han, M., Li, G., Liu, X., Li, A., Mao, P., Liu, P., Li, H. (2019). Phenolic profile, antioxidant activity and anti-proliferative activity of crabapple fruits. *Horticultural Plant Journal*, *5*(4), 155–163.
https://doi.org/10.1016/j.hpj.2019.01.003
33. Hellstrom, J.K., Torronen, A.R., Mattila, P.H. (2009). Proanthocyanidins in common food products of plant origin. *Journal of Agricultural and Food Chemistry*, *57*(17), 7899–7906.
https://doi.org/10.1021/jf901434d
34. Hollands, W.J., Voorspoels, S., Jacobs, G., Aaby, K., Meisland, A., Garcia-Villalba, R., Tomas-Barberan, F., Piskuła, M.K., Mawson, D., Vovk, I., Needs, P.W., Kroon, P. (2017). Development, validation and evaluation of an analytical method for the determination of monomeric and oligomeric procyanidins in apple extracts. *Journal of Chromatography A*, *1495*, 46–56.
https://doi.org/10.1016/j.chroma.2017.03.030
35. Hollman, P.C., van Trijp, J.M., Buysman, M.N., van der Gaag, M.S., Mengelers, M.J., de Vries, J.H., Katan, M.B. (1997). Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Letters*, *418*(1–2), 152–156.
https://doi.org/10.1016/S0014-5793(97)01367-7
36. Horszwald, A., Andlauer, W. (2011). Characterization of bioactive compounds in berry juices by traditional photometric and modern microplate methods. *Journal of Berry Research*, *1*(4), 189–199.
https://doi.org/10.3233/JBR-2011-020
37. Jacob, K., Periago, M.J., Böhm, V., Berruezo, G.R. (2008). Influence of lycopene and vitamin C from tomato juice on biomarkers of oxidative stress and inflammation. *British Journal of Nutrition*, *99*(1), 137–146.
https://doi.org/10.1017/S0007114507791894
38. Jakobek, L., García-Villalba, R., Tomás-Barberán, F.A. (2013). Polyphenolic characterization of old local apple varieties from Southeastern European region. *Journal of Food Composition and Analysis, 31*(2), 199–211.
https://doi.org/10.1016/j.jfca.2013.05.012
39. Jelodarian, S., Ebrahimabadi, A.H., Khalighi, A., Batooli, H. (2012). Evaluation of antioxidant activity of *Malus domestica*

fruit extract from Kashan area. *Avicenna Journal of Phytomedicine*, *2*(3), 139–145.

40. Jepson, R.G., Mihaljevic, L., Craig, J. (2004). Cranberries for preventing urinary tract infections. *Cochrane Database of Systematic Reviews*, *2*, art. no. CD001321.
https://doi.org/10.1002/14651858.CD001321.pub3
41. Jiao, X., Wang, Y., Li, Y., Lang, Y., Li, E., Zhang, X., Zhang, Q., Feng, Y., Meng, X., Li, B. (2019). Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6J mice by modulating the gut microbiota. *Journal of Nutritional Biochemistry*, *64,* 88–100.
https://doi.org/10.1016/j.jnutbio.2018.07.008
42. Kahle, K., Kraus, M., Richling, E. (2005). Polyphenols profiles of apple juices. *Molecular Nutrition & Food Research*, *49*(8), 797–806.
https://doi.org/10.1002/mnfr.200500064
43. Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A. (2002). Flavonoid intake and risk of chronic diseases. *American Journal of Clinical Nutrition*, *76*(3), 560–568.
https://doi.org/10.1093/ajcn/76.3.560
44. Kolniak-Ostek, J., Oszmiański, J., Wojdyło, A. (2013). Effect of L-ascorbic acid addition on quality, polyphenolic compounds and antioxidant capacity of cloudy apple juices. *European Food Research and Technology*, *236*(5), 777–798.
https://doi.org/10.1007/s00217-013-1931-z
45. Krawitzky, M., Arias, E., Peiro, J.M., Negueruela, A.J., Val, J., Oria, R. (2014). Determination of color, antioxidant activity, and phenolic profile of different fruit tissue of Spanish 'Verde Doncella' apple cultivar. *International Journal of Food Properties*, *17*(10), 2298–2311.
https://doi.org/10.1080/10942912.2013.792829
46. Kschonsek, J., Wolfram, T., Stöckl, A., Böhm, V. (2018). Polyphenolic compounds analysis of old and new apple cultivars and contribution of polyphenolic profile to the *in vitro* antioxidant capacity. *Antioxidants*, *7*(1), art. no. 20.
https://doi.org/10.3390/antiox7010020
47. Lee, K.W., Kim, Y.J., Kim, D.-O., Lee, H.J., Lee, Ch.Y. (2003). Major phenolics in apple and their contribution to the total antioxidant capacity. *Journal of Agricultural and Food Chemistry*, *51*(22), 6516–6520.
https://doi.org/10.1021/jf034475w
48. Liu, Q., Tang, G-Y., Zhao, C.-N., Gan, R.-Y., Li, H-B. (2019). Antioxidant activities, phenolic profiles, and organic acids contents of fruit vinegars. *Antioxidants*, *8*(4), art. no.78.
https://doi.org/10.3390/antiox8040078
49. Lončarić A., Matanović K., Ferrer P., Kovač, T., Šarkanj, B., Skendrović Babojelić, M., Lores, M. (2020). Peel of traditional apple varieties as a great source of bioactive compounds: extraction by micro-matrix solid-phase dispersion. *Foods*, *9*(1), art. no. 80.
https://doi.org/10.3390/foods9010080
50. Lotito, S.B., Frei, B. (2004a). Relevance of apple polyphenols as antioxidant in human plasma: contrasting *in vitro* and *in vivo* effects. *Free Radical Biology & Medicine*, *36*(2), 201–211.
https://doi.org/10.1016/j.freeradbiomed.2003.10.005
51. Lotito, S.B., Frei, B. (2004b). The increase in human plasma antioxidant capacity after apple consumption is due to the metabolomics effect of fructose on urate, not apple-derived antioxidant flavonoids. *Free Radical Biology & Medicine*, *37*(2), 251–258.
https://doi.org/10.1016/j.freeradbiomed.2004.04.019
52. Lotito, S.B., Frei, B. (2006). Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomena. *Free Radical Biology & Medicine*, *41*(12), 1727–1746.
https://doi.org/10.1016/j.freeradbiomed.2006.04.033
53. Loung, Ch.-Y., Fernando, W., Rupasingne, H.P.V., Hoskin, D.W. (2019). Apple peel flavonoid fraction 4 suppresses breast cancer cell growth by cytostatic and cytotoxic mechanisms. *Molecules*, *24*(18), art. no. 3335.
https://doi.org/10.3390/molecules24183335
54. Lu, Y.R., Foo, L.Y. (2000). Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chemistry*, *68*(1), 81–85.
https://doi.org/10.1016/S0308-8146(99)00167-3
55. Maleki, S.J., Crespo, J.F., Cabanillas, B. (2019). Anti-inflammatory effects of flavonoids. *Food Chemistry*, *299*, art. no. 125124.
https://doi.org/10.1016/j.foodchem.2019.125124
56. Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans: I. Review of bioavailability studies. *American Journal of Clinical Nutrition, 81*, 230–242.
https://doi.org/10.1093/ajcn/81.1.230S
57. Maragò, E., Iacopini, P., Camangi, F., Scattino, C., Ranieri, A., Stefani, A., Sebastiani, L. (2015). Phenolic profile and antioxidant activity in apple juice and pomace: effects of different storage conditions. *Fruits*, *70*(4), 213–223.
https://doi.org/10.1051/fruits/2015015
58. Martí, N., Mena, P., Cánovas, J.A., Micol, V., Saura, D. (2009). Vitamin C and the role of citrus juices as functional food. *Natural Product Communications*, *4*(5), 677–700.
https://doi.org/10.1177/1934578X0900400506
59. Matsuoka, K. (2019). Anthocyanins in apple fruit and their regulation for health benefits. In *Flavonoid – a Coloring Model for Cheering Up Life*, IntechOpen. Available on-line at: [cdn.intechopen.com/pdfs/66144.pdf] (accessed 11 February 2020).
https://doi.org/10.5772/intechopen.85257
60. Minatel, I.O., Borges, C.V., Ferreira, M.J, Gomez Gomez, H.A., Chen, Ch.-Y.O., Pereira Lima, G.P. (2017). Phenolic compounds: functional properties, impact of processing and bioavailability. Eds. M. Soto-Hernandez, M. Palma-Tenango, M. del Rosaria Garcia-Mateos, In *Phenolic Compounds. Biological Activity*, IntechOpen, London, UK.
https://doi.org/10.5772/66368
61. Murillo, E., Britton, G.B., Durant, A.A. (2012). Antioxidant activity and polyphenol content in cultivated and wild edible fruits grown in Panama. *Journal of Pharmacy & Bioallied Sciences*, *4*(4), 313–317.
https://doi.org/10.4103/0975-7406.103261
62. Murphy, K.J., Walker, K.M., Dyer, K.A. (2019). Estimation of daily intake of flavonoids and major food sources in middle-aged Australian men and women. *Nutrition Research*, *61*, 64–81.
https://doi.org/10.1016/j.nutres.2018.10.006
63. Murtaza, A., Iqbal, A., Marszałek, K., Iqbal, M.A., Waseem Ali Sh., Xu, X., Pan, Sh., Hu, W. (2020). Enzymatic, phyto-, and physicochemical evaluation of apple juice under high-pressure carbon dioxide and thermal processing. *Foods*, *9*(2), art. no. 243.
https://doi.org/10.3390/foods9020243
64. Naczk, M., Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, *1054*(1–2), 95–111.
https://doi.org/10.1016/S0021-9673(04)01409-8

65. Ohr, L.M. (2004). Dietary antioxidants. *Food Technology*, *58*(10), 67–74.
66. Oszmianski, J., Wolniak, M., Wojdylo, A., Wawer, I. (2007). Comparative study of polyphenolic content and antiradical activity of cloudy and clear apple juices. *Journal of the Science and Food Agriculture*, *87*(4), 573–579. https://doi.org/10.1002/jsfa.2707
67. Oszmiański, J., Wojdyło, A. (2009). Effects of blackcurrant and apple mash blending on the phenolics contents, antioxidant capacity, and color of juices. *Czech Journal of Food Sciences*, *27*(5), 338–351. https://doi.org/10.17221/20/2008-CJFS
68. Oszmiański, J., Wolniak, M., Wojdyło, A., Wawer, I. (2008). Influence of apple puree preparation and storage on polyphenols contents and antioxidant activity. *Food Chemistry*, *107*(4), 1473–1484. https://doi.org/10.1016/j.foodchem.2007.10.003
69. Pająk, P., Socha, R., Łakoma, P., Fortuna, T. (2017). Antioxidant properties of apple slices stored in starch-based films. *International Journal of Food Properties*, *20*(5), 1117–1128. https://doi.org/10.1080/10942912.2016.1203931
70. Pastene, E., Troncoso, M., Figueroa, G., Alarcon, J., Speisky, H. (2009). Association between polymerization degree of apple peel polyphenols and inhibition of *Helicobacter pylori* urease. *Journal of Agricultural and Food Chemistry*, *57*(2), 416–424. https://doi.org/10.1021/jf8025698
71. Pavun, L., Usković-Marković, S., Jelikić-Stankov, M., Đikanović, D., Đurđević, P. (2018). Determination of flavonoids and total polyphenol contents in commercial apple juices. *Czech Journal of Food Sciences*, *36*(3), 233–238. https://doi.org/10.17221/211/2017-CJFS
72. Pires, T.C.S.P., Dias, M.I., Barros, L., Alves, M.J., Oliveira, M.B.P.P., Santos-Buelga, C., Ferreira, I.C.F.R. (2018). Antioxidant and antimicrobial properties of dried Portuguese apple variety (*Malus domestica* Borkh. cv Bravo de Esmolfe). *Food Chemistry*, *240*, 701–706. https://doi.org/10.1016/j.foodchem.2017.08.010
73. Plumb, G.W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., Williamson, G. (1998). Antioxidant properties of catechins and proanthocyanidins: Effect of polymerisation, galloylation and glycosylation. *Free Radical Research*, *29*(4), 351–358. https://doi.org/10.1080/10715769800300391
74. Porrini, M., Riso, P. (2008). Factors influencing the bioavailability of antioxidants in foods: a critical appraisal. *Nutrition, Metabolism & Cardiovascular Diseases*, *18*(10), 647–650. https://doi.org/10.1016/j.numecd.2008.08.004
75. Rana, Sh., Gupta, S., Rana, A., Bushau, S. (2015). Functional properties, phenolic constituents and antioxidant potential of industrial apple pomace for utilization as active food ingredient. *Food Science and Human Wellness*, *4*(4), 180–187. https://doi.org/10.1016/j.fshw.2015.10.001
76. Raphaelli, C.D., Pereira, E.D., Camargo, T.M., Vinholes, J., Rombaldi, C.V., Vizzotto, M., Nora, L. (2019). Apple phenolic extracts strongly inhibit α-glucosidase activity. *Plant Foods for Human Nutrition*, *74*(3), 430–435. https://doi.org/10.1007/s11130-019-00757-3
77. Raudone, L., Raudonis, R., Liaudanskas, M., Janulis, V., Viskelis, P. (2017). Phenolic antioxidant profiles in the whole fruit, flesh and peel of apple cultivars grown in Lithuania. *Scientia Horticulturae*, *216*, 186–192. https://doi.org/10.1016/j.scienta.2017.01.005
78. Raudone, L., Raudonis, R., Liaudanskas, M., Viškelis, P., Pukalskas, A., Janulis, V. (2016). Phenolic profiles and contribution of individual compounds to antioxidant activity of apple powders. *Journal of Food Science*, *81*(5), C1055-C1061. https://doi.org/10.1111/1750-3841.13277
79. Rios, L.Y., Gonthier, M.P., Rémésy, C., Mila, I., Lapierre, C., Lazarus, S.A., Williamson, G., Scalbert, A. (2003). Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *American Journal of Clinical Nutrition*, *77*(4), 912–918. https://doi.org/10.1093/ajcn/77.4.912
80. Saenger, T., Hűbner F., Humpf, H.-U. (2017). Short-term biomarkers of apple consumption. *Molecular Nutrition & Food Research*, *61*(3), art. no. 1600629. https://doi.org/10.1002/mnfr.201600629
81. Salucci, S., Falcieri, E. (2020). Polyphenol and their potential role in preventing skeletal muscle atrophy. *Nutrition Research*, *74*, 10–22. https://doi.org/10.1016/j.nutres.2019.11.004
82. Santarelli, V., Neri, L., Sacchetti, G., Di Mattia, C.D., Mastrocola, D., Pittia, P. (2020). Response of organic and conventional apples to freezing and freezing pre-treatments: focus on polyphenols content and antioxidant activity. *Food Chemistry*, *308*, art. no. 125570. https://doi.org/10.1016/j.foodchem.2019.125570
83. Scalbert, A., Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition, 130*(8), 2073S-2085S. https://doi.org/10.1093/jn/130.8.2073S
84. Schempp, H., Christof, S., Mayr, U., Treutter, D. (2016). Phenolic compounds in juices of apple cultivars and their relation to antioxidant activity. *Journal of Applied Botany and Food Quality*, *89*, 11–20.
85. Serra, A., Macia, A., Romero, M.P., Valls, J., Blade, C., Arola, L., Motilva, M.J. (2010). Bioavailability of procyanidin dimers and trimers and matrix food effects in *in vitro* and *in vivo* models. *British Journal of Nutrition*, *103*, 944–952. https://doi.org/10.1017/S0007114509992741
86. Shahidi, F., Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects – a review. *Journal of Functional Foods*, *18*, Part B, 820–897. https://doi.org/10.1016/j.jff.2015.06.018
87. Skinner, R.Ch., Gigliotti, J.C., Ku, K.M., Tou, J.C. (2018). A comprehensive analysis of the composition, health benefits, and safety of apple pomace. *Nutrition Reviews*, *76*(12), 893–909. https://doi.org/10.1093/nutrit/nuy033
88. Skłodowska, M., Mikiciński, A., Wielanek, M., Kuźniak, E., Sobiczewski, P. (2018). Phenolic profiles in apple leaves and the efficiency of selected phenols against free blight (*Erwinia amylovera*). *European Journal of Plant Pathology*, *151*(1), 213–228. https://doi.org/10.1007/s10658-017-1368-5
89. Spranger, I., Sun, B., Mateus, A.M., de Freitas, V., Ricardo-Da-Silva, J.M. (2008). Chemical characterization and antioxidant activities of oligomeric and polymeric procyanidin fractions from grape seeds. *Food Chemistry*, *108*(2), 519–532. https://doi.org/10.1016/j.foodchem.2007.11.004
90. Stan, A., Bujor, O.-C., Badulescu, L. (2017). Extraction of phenolic compounds from organic dried apples: comparison between conventional, microwave- and ultrasound- assisted extrac-

tion methods. *Journal of Horticulture, Forestry and Biotechnology*, *21*(3), 8–14.

91. Starowicz, M. (2019). Food and Nutritional Analysis. In *Encyclopedia of Analytical Science* (third edition), Elsevier, London, UK, pp. 265–270.
92. Sun, J., Chu, Y.-F., Wu, X.Z., Liu, R.H. (2002). Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*, *50*(25), 7449–7454. https://doi.org/10.1021/jf0207530
93. Sut, S., Zengin, G., Maggi, F., Malagoli, M., Dall'Acqua, S. (2019). Triterpene acid and phenolics from ancient apples of Friuli Venezia Giulia as nutraceutical ingredients: LC-MS study and *in vitro* activities. *Molecules*, *24*(6), art. no. 1109. https://doi.org/10.3390/molecules24061109
94. Średnicka-Tober, D., Kazimierczak, R., Rembiałowska, E., Strok, T., Świąder, K., Hallmann, E. (2017). Bioactive compounds in organic apple juices enriched with chokeberry and green tea extract. *Journal of Research and Applications in Agricultural Engineering, 62*(4), 173–177.
95. Tappi, S., Ramazzina, I., Rizzi, F., Sacchetti, G., Ragni, L., Rocculi, P. (2018). Effect of plasma exposure time on the polyphenolic profile and antioxidant activity of fresh-cut apples. *Applied Sciences – Basel*, *8*(10), art. no. 1939. https://doi.org/10.3390/app8101939
96. Tian, Y., Sun, L., Yang, Y., Gou, X., Niu, P., Guo, Y. (2018). Changes in the physicochemical properties, aromas and polyphenols of not from concentrate (NFC) apple juice during production. *CyTA – Journal of Food*, *16*(1), 755–764. https://doi.org/10.1080/19476337.2018.1471102
97. Verdu, C.F., Childebrand, N., Marnet, N., Lebail, G., Dupuis, F., Laurens, F., Guilet, D., Guyot, S. (2014). Polyphenol variability in the fruits and juices of a cider apple progeny. *Journal of the Science of Food and Agriculture*, *94*(7), 1305–1314. https://doi.org/10.1002/jsfa.6411
98. Wang, H.L, Guo, X.B, Hu, X.D, Li, T., Fu, X., Liu, R.H. (2017). Comparison of phytochemical profiles, antioxidant and cellular antioxidant activities of different varieties of blueberry (*Vaccinium* spp.). *Food Chemistry*, *217*, 773–781. https://doi.org/10.1016/j.foodchem.2016.09.002
99. Wang, J., Wang, J., Ye, J.H., Vanga, S.K., Raghavan, V. (2019b). Influence of high-intensity ultrasound on bioactive compound of strawberry juice: profiles of ascorbic acid, phenolics, antioxidant activity and microstructure. *Food Control*, *96*, 128–136. https://doi.org/10.1016/j.foodcont.2018.09.007
100. Wang, Y., Zhao, H., Deng, H., Song, X., Zhang, W., Wu, S., Wang, J. (2019a), Influence of pretreatments on microwave vacuum drying kinetics, physicochemical properties and sensory quality of apple slices. *Polish Journal of Food and Nutrition Sciences*, *69*(3), 297–306. https://doi.org/10.31883/pjfns/110734
101. WHO (2009). Fruits, vegetables and NCD disease prevention. Available on-line at: [https://www.who.int/dietphysicalactivity/media/en/gsfs_fv.pdf] (accessed 9 September 2020).
102. Whyte, A.R., Williams, C.M. (2011). The effect on memory of an acute blueberry flavonoid intervention with 8–9-year-old children. *Appetite*, *57*(2), art. no. 567. https://doi.org/10.1016/j.appet.2011.05.099
103. Williamson, G., Kay, C.D., Crozier, A. (2018). The bioavailability, transport, and bioactivity of dietary flavonoids: a review from a historical perspective. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1054–1112. https://doi.org/10.1111/1541-4337.12351
104. Wojdyło, A., Oszmiański, J., Bielicki, P. (2010). Chemical composition, phenolic compounds and antioxidant activity of three varieties of apple from organic and conventional farming. *Journal of Research and Applications in Agricultural Engineering*, *55*(4), 173–177.
105. Wojdyło, A., Oszmiański, J., Laskowski, P. (2008). Polyphenolic compounds and antioxidant activity of new and old apple varieties. *Journal of Agricultural and Food Chemistry*, *56*(15), 6520–6530. https://doi.org/10.1021/jf800510j
106. Wruss, J., Lanzerstorfer, P., Huemer, S., Himmelsbach, M., Mangge, H., Höglingen, O., Weghuber, D., Weghuber, J. (2015). Differences in pharmokinetics of apple polyphenols after standardized oral consumption of unprocessed apple juice. *Nutritional Journal*, *14*, art. no. 32. https://doi.org/10.1186/s12937-015-0018-z
107. Xu, Y., Fan, M., Ran, J., Zhang, T., Sun, H., Dong, M., Zhang, Z., Zheng, H. (2016). Variation in phenolic compounds and antioxidant activity in apple seeds of seven cultivars. *Saudi Journal of Biological Sciences*, *23*(3), 379–388. https://doi.org/10.1016/j.sjbs.2015.04.002
108. Yuste, S., Ludwig, I.A., Rubio, L., Romero, M.P., Pedret, A., Valls, R.M., Sola, R., Motilva, M.J., Macia, A. (2019). *In vivo* biotransformation of (poly)phenols and anthocyanins of red-fleshed apple and identification of intake biomarkers. *Journal of Functional Foods*, *55*, 146–155. https://doi.org/10.1016/j.jff.2019.02.013
109. Yuste, S., Macia, A., Ludwig, I.A., Romero, M.P., Fernandez-Castillejo, S., Catalan, U., Motilva, M.J., Rubio, L. (2018). Validation of dried blood spot cards to determine apple phenolic metabolites in human blood and plasma after an acute intake of red-fleshed apple snack. *Molecular Nutrition & Food Research*, *62*(23), art. no. 1800623. https://doi.org/10.1002/mnfr.201800623
110. Zeng, X., Du, Z., Ding, X., Jiang, W. (2020). Characterization of the direct interaction between apple condensed tannins and cholesterol *in vitro*. *Food Chemistry*, *309*, art. no. 125762. https://doi.org/10.1016/j.foodchem.2019.125762
111. Zhang, M., Wang, Z., Mao, Y., Hu, Y., Yang, L. Wang, Y. (2019). Effects of quince pollen pollination on fruit qualities and phenolic substance contents of apples. *Scientia Horticulturae*, *256*, art. no. 108628. https://doi.org/10.1016/j.scienta.2019.108628
112. Zhou, H.C., Tam, N.F.Y., Lin, Y.M., Ding, Z.H., Chai, W.M., Wei, S.D. (2014). Relationships between degree of polymerization and antioxidant activities: a study on proanthocyanidins from the leaves of a medicinal mangrove plant *Ceriops tagal*. *PLoS ONE*, *9*(10), art. no. e107606. https://doi.org/10.1371/journal.pone.0107606
113. Zielinski, A.A.F., Alberti, A., Braga, C.M., da Silva, K.M., Canteri, M.H.G., Igarashi-Mafra, L., Granato, D., Nogueira, A., Wosiacki, G. (2014). Effect of mash maceration and ripening stage of apples on phenolic compounds and antioxidant power of cloudy juices: a study using chemometrics. *LWT – Food Science and Technology*, *57*(1), 223–229. https://doi.org/10.1016/j.lwt.2014.01.029

114. Zielińska, D., Laparra-Llopis, J.M., Zieliński, H., Szawara-Nowak, D., Giménez-Bastida, J.A. (2019). Role of apple phytochemicals, phloretin and phloridzin, in modulating processes related to intestinal inflammation. *Nutrients*, *11*(5), art. no. 1173. https://doi.org/10.3390/nu11051173
115. Zou, Y., Wang, T., Li, Sh., Liu, Y., You, J. (2020). Dietary apple polyphenols promote fat browning in high-fat diet-induced obese mice through activation of adenosine monophosphate-activated protein kinase α. *Journal of the Science of Food and Agriculture*, *100*(6), 2389–2398. https://doi.org/10.1002/jsfa.10248

Submitted: 15 April 2020. Revised: 12 July and 10 September 2020. Accepted: 18 September 2020. Published on-line: 15 October 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 337–346
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/126617
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Nutritive Parameters and Antioxidant Quality of Minimally Processed "Cime di Rapa" (*Brassica rapa* subsp. *sylvestris*) Vary as Influenced by Genotype and Storage Time

Donato Giannino[1*#], *Giulio Testone*[1#], *Chiara Nicolodi*[1], *Lucia Giorgetti*[2], *Lorenza Bellani*[2,3], *Maria Gonnella*[4], *Marco Ciardi*[2], *Paolo Cappuccio*[5], *Stefano Moscatello*[6], *Alberto Battistelli*[6], *Vincenzo Longo*[2]

[1]Institute for Biological Systems, National Research Council of Italy (CNR),
via Salaria km 29, 300 – Moterotondo, Rome, Italy
[2]Institute of Agricultural Biology and Biotechnology, National Research Council of Italy (CNR),
Unit of Pisa, via Moruzzi 2, Pisa, Italy
[3]Department of Life Sciences, University of Siena, via A. Moro, 2 – 53100 Siena, Italy
[4]Institute of Sciences of Food Production, National Research Council of Italy (CNR),
Via Amendola, 122/O – 70126 Bari, Italy
[5]San Lidano Soc. Coop. Agr. a.r.l., Via Migliara 46 – Sezze Scalo, Latina, Italy
[6]Research Institute on Terrestrial Ecosystems (IRET), National Research Council of Italy (CNR) – Porano, Terni, Italy

Key words: "cime di rapa" (broccoli-raab, rapini), dietary fibre, glycemic carbohydrates, antioxidant parameters, packaged product quality, hybrid and conventional genotypes

In order to assess the quality and performance of bagged broccoli-raab, a recently marketed product, several nutritive parameters were determined in novel hybrid and conventional cultivars at pre- and post-packaging stages in the industrial environment. The characterization of shoots and composing organs at post-cut stage included contents of dietary fibre (DF), glycaemic carbohydrates (GC), antioxidant compounds (ACC) and capacity (AOC), which were determined by chromatographic methods and spectrophotometric assays. ACC and AOC were analysed during shelf life of bagged products. Genotype and storage effects were addressed as variability factors at fixed packaging conditions. Contents of DF and GC (39.64–34.57; 7.56–2.21 g/100 g), glucosinolates (37.47–24.63 mg/g SIN), and ACC (total phenolics: 18.64–14.92 mg GAE/g; flavonoids: 34.74–30.96 mg/g CE; flavonols: 14.62–14.08 mg QE/g), and AOC (Oxygen Radical Absorbance Capacity: 354.62–293.25 μmol/g TE; DPPH$^\bullet$ scavenging activity: 59.35–46.14) were lower in shoots of the hybrid than marketed cultivar. In both genotypes, AOC was maximal in leaves, followed by florets and stems. The integrated analyses suggested that the hybrid genotype was better suited for fresh consumption and that increased ratio of florets/leaves *vs.* stem is expected to raise product antioxidant properties. The comparison of unprocessed and bagged products pointed at a value decay of most parameters except for glucosinolates and correlation analyses supported the necessity of performing multiple antioxidant assays to enhance product quality evaluation. As for shelf life, storage time was the major factor affecting antioxidant properties, while genotype and interaction effects were minimal.

ABBREVIATIONS

TPC – total polyphenol content; FID – flavonoids; FOL – flavonols; GSL – glucosinolates; AOC – antioxidant capacity; ACC – antioxidant compound content; DW – dry weight; FW – fresh weight; hpp, hours post packaging.

INTRODUCTION

Cruciferous vegetables encompass nutritionally and economically important crops worldwide and the "broccoletto" group (*Brassica rapa* L.) was proposed based on morphological traits [Bonnema *et al.*, 2011] and molecular phylogeny. It includes "cime di rapa" and "friariello" vegetables from south Italy [Elia & Santamaria, 2013], also known as broccoli-raab or rapini [PlantUse, 2020] and consumed as cooked or raw (salads or sauces).

Botanically, broccoli-raab falls into the subspecies *sylvestris* Janch var. *esculenta* Hort. [Romani *et al.*, 2006], and the edible products are shoots bearing leaves, stems, and corymb-inflorescences (florets). Local farmers usually select parental lines phenotypically, aiming at progenies with superior traits and minimal inbreeding depression. Seed companies exploit these local selections to develop synthetic varieties or to create inbred lines for F_1 hybrids with new traits such as regrowth attitude (for multiple cutting), product tenderness and antioxidant content. Landraces cultivation spans fall-winter cycles of 50–90 days in open field [Elia & Santa-

---

* Corresponding Author: Tel.: +39 0690672210;
E-mail: donato.giannino@cnr.it (Dr. D. Giannino)
[#] Authors have equally contributed to this work

maria, 2013] with minimal agronomic practices and pesticide treatments; greenhouse and hydroponic systems are also feasible [De Pascale *et al.*, 2007].

Nutritional quality of broccoli-raab relies on dietary fibre [U.S.D.A., 2019] and on bioactive phytochemicals, such as tocopherols, ascorbate (vitamin C) [Annunziata *et al.*, 2012; Cefola *et al.*, 2010; Conversa *et al.*, 2016], carotenoids [https://fdc.nal.usda.gov], phenolics [De Pascale *et al.*, 2007; Romani *et al.*, 2006], and glucosinolates (GSL) [Annunziata *et al.*, 2012; Cefola *et al.*, 2010; Conversa *et al.*, 2016]. These latter are typical of *Brassicaceae* and some specific classes could fingerprint “cime” ecotypes. The GSL breakdown products exert effects against different cancer types and their bioavailability is affected by product processing [Barbieri *et al.*, 2008]. As for phenolics (PP), broccoli-raab abound in phenolic acids (chlorogenic and ferulic) and flavonols (a flavonoid subclass) that are mainly quercetin, kaempferol, isorhamnetin, and respective conjugates [De Pascale *et al.*, 2007; Romani *et al.*, 2006]. Phenolics health-promoting action is mediated by interactions with gut microbiota and potential direct effects of *Brassica* phenolics are reported [Cartea *et al.*, 2010]. Chemical antioxidant capacity (AOC) of foods can be assessed by *in vitro* assays that detect various scavenging mechanisms. The AOC measurement is often unlinked to *in vivo* outcomes and feeble to provide health claims, though valid to assess potential effects in *B. rapa* vegetables [Soengas *et al.*, 2011].

Found that consumers associate local products to quality, tradition and health, industries were prompted to release bagged broccoli-raab into the ready-to-use market, favouring the *ex situ* consumption; with nutritional value preservation, undisclosed florets, stay-green leaves, typical taste without off-odours being the major quality parameters. Some factors (*e.g.,* seasonality, cultivation techniques) affect these characteristics during conservation [Barbieri *et al.*, 2009] and airflow modifications were effective to prolong floret storage [Cefola *et al.*, 2010]. Moreover, increased sulphur fertilization during cultivation and light exposure during storage enhanced the antioxidant quality of film-and-tray packaged product [Barbieri *et al.*, 2009]. Lab-scale results identified suitable gas equilibria and wrapping material for modified atmosphere packaging [Conte *et al.*, 2011; Torrieri *et al.*, 2010].

This work intended to gain novel information on dietary fibre and glycaemic carbohydrate content of two broccoli-raab genotypes, a novel F_1 hybrid and a marketed cultivar, and to provide information on the antioxidant quality of bagged products derived from an industrial process by monitoring ACC and AOC. Genotype and storage effects were the studied variability factors at fixed packaging conditions.

MATERIALS AND METHODS

Plant materials and growth conditions

Plants of ‘Broccoletto di rapa novantino riccio San Marzano’ (BSM90) were marketed by “Domenico Pagano e Figli” (Scafati, SA, IT); plants of the unpatented ‘F_1 Hybrid 39’ (H39) were provided by a seed company. They were grown under a 90-day cycle at the “Di Pastina and Meletti” farm (LT, Italy); Table 1 reports information on environment, cultivation, and farming practices. The parental lines of BSM90 and H39 derive from landraces of south Italy (Apulia and Campania regions) and the breeding schemes are undisclosed. Phenotypically, H39 showed less fringed margin leaves (Figure 1f), synchronous flower disclosure, and erect habitus with a primary axis prevailing over secondary branching (not shown) as compared to BSM90 (Figure 1e).

TABLE 1. Environment and cultivation parameters of two genotypes of *Brassica rapa* subsp. *Sylvestris.*

Murillo Sezze, Latina	
Coordinates	
Latitude	N 41° 27’ 7.382”
Longitude	E 13° 3’ 45.666”
Altitude (m asl)	5.0
Climate[1]	
Temp. min (°C)	8.7±3.0
Temp. max (°C)	17.2±3.0
Temp. mean (°C)	12.2±2.6
Relative humidity (%)	77.7±9.7
Total rain (mm)	127.0
Soil[2]	
Clay (<0.002 mm) (%)	440
Silt (0.05–0.002 mm) (%)	220
Sand (2–0.05 mm) (%)	340
Total nitrogen (%)	1.40
Organic matter (%)	24.9
P_2O_5 available (mg/kg)	462.6
K_2O exchangeable (mg/kg)	450.6
E.C. (mS/cm)	0.480
pH	7.84
Cation Ex. Cap. (meq/100 g)	30.3
Cultivation	dates
Di-ammonium phosphate (100 kg/ha) Panfertil, IT, 18 % P_20_5, 46 % NH_4^+	05/11/2015
Sowing – density 70 plants/m^2	07/11/2015
Harvest	12/02/2016
Farm delivery	15/02/2016
Processing	16/02/2016

[1]sowing-harvest; www.arsial.it/portalearsial/agrometeo; [2]USDA classification [U.S.D.A., 2006]

Product processing, sampling criteria, and sample treatment

Seventy plants were processed by “San Lidano” company (http://www.sanlidano.it/) according to quality management standard of standard for quality [ISO 9001, 2008] and traceability [ISO 22005, 2007]. Personnel performed manual selection and cutting to produce 10–20 cm long shoots; the average yield was *ca*. 45 g out of 100 g of starting material. Sanitary treatment (sodium hypochlorite 30 mg/L), water wash (both at 4–6°C), mechanized drying (automatic electric spin dryer extra compact – Turatti Group, Venice, Italy) at 4400/5000 round per minutes using a load of 5–8 kg. The bags were flushed (active modified atmosphere packaging) to reach a final concentration of 7.2% O_2, 8.8% CO_2, and 84% N_2, measured by Portable Gas Analyzer Checkpoint3 Dansensor (Mocon ® Europe, Milan, Italy). Filling was at calibrated weight of 500 g±2%. Weight parameters (g) were measured using ten bags (gros: 514.51±9.33, net: 503.8±10.33, **b**ag + **h**umidity: 10.83±1.27, **i**nflorescences: 28.67±4.49, **s**tems: 101.83±7.43, **l**eaves: 351.56±10.09; **i+s+l**: 482.06±11.85, **b+h+i+s+l**: 492.19±10.24). Automated vertical packaging (Olimpia 4000 Simotion, Miele, Italy) produced bags made of anti-fog polypropylene film (size 645 mm x 380 mm, thickness 35 mm; density 910 kg/m^3; model PP AFM035, Masterpack S.p.A., Milan, Italy). Film permeability parameters were 8.155 (standard test method ASTM D3985), 37.69 (ASTM D1434) and 22.93 (ASTM F1249) 10^{-12} mol/mm^2 s Pa respectively for O_2, CO_2, and H_2O. Filled-in bags were stored in cold rooms at 7±1°C in the dark.

As for sampling of unprocessed material (Figure 1 a–f), three bulks (each of 1 kg, biological replicate) of delivered shoots were used to generate four batches, each consisting of entire shoots (*ca*. 15 cm), flowers, stems, and leaves. Dietary fibre and glycaemic carbohydrates were determined in entire shoots, while antioxidant parameters were assessed in entire shoots and composing organs. As for the packaged material, the content of each bag represented a replicate batch (Figure 1 g–h). All samples were immediately frozen and crunched in liquid nitrogen, stored at -80°C. An aliquot of 100 g was weighed without thawing, lyophilized at -50°C for 72 h (laboratory freeze dryer with stoppering tray dryer, FreeZone®, Labconco Corp., Kansas City, MO, USA) and stored at -20°C. Three replicates were used in all assays and all the measurements were in triplicate. The Italian law imposes consumption within 2 days after bag opening (DM n° 3746–2014); factories have conventionally fixed the sell-by dates at 7 days post packaging (pp) because consumer's choice is affected by storage prolongation [Stranieri & Baldi, 2017]. In this work, the storage time analyses of antioxidant parameters included plant material before processing and bagged shoots at 1, 48, and 96 h pp (hpp), considering this lapse as the one during which the consumer most likely eats the product.

Contents of extractive, dietary fibre, glycaemic carbohydrate, and ashes

Entire shoots were sampled at the post-cut stage; quantification of cellulose, hemicellulose, and lignin was performed on ground lyophilized tissue (0.3 g) after separation of extractive; it was based on acid treatment to depolymerize polysaccharide and release Klason and soluble lignin fractions [Sluiter *et al.*, 2008b]. Monomeric sugars were analysed by HPAE chromatography, using pulsed amperometric detection (Thermo Scientific™ Dionex™ ICS-5000, Sunnyvale, CA U.S.A.) and CarboPac PA20 (4 mm x 250 mm)/dedicated guard columns[Dionex Technical note 40, 2016].

Runs were carried out at 30 °C, flow rate of 0.4 mL/min, and a sodium hydroxide/sodium acetate-stepped gradient that consisted of: 2.4/0 mM, 0–18 min; 100/0 mM,18––20 min; 100/0 mM, 20–30 min; 100/100 mM, 30–30.1 min; 100/100 mM, 30.1–46 min; 200/0 mM, 46–46.1 min; 200/0 mM, 46.1–50 min; 2.4/0 mM, 50–50.1 min. The eluents and the standard solutions were prepared using HPLC grade reagents (Sigma, Steinheim, Germany). Data were processed by Chromeleon software versions 6.8. Dietary fibre was calculated as the sum of total lignin, hemicellulose, pectin, and cellulose. Extractive separation and ashes determination were carried out exactly as described [Sluiter *et al.*, 2008a,c]. Starch and soluble sugars (glucose, fructose and sucrose) were determined as previously reported [Moscatello *et al.*, 2017] with minor modifications. Briefly, 10 mg of lyophilized tissue powder was added to 1.5 mL of an extraction ethanol-buffer mixture – 80% ethanol and 20% buffer (Hepes-KOH 100 mM, 10 mM $MgCl_2$, pH 7.1), gently shaken (80°C, 45 min) and centrifuged (14,000×*g*, 5 min). The supernatant was collected for soluble sugars analysis (5 min centrifugation at 16,000×*g*). The pellet containing starch was washed four times with 1 mL of acetate buffer (40 mM, pH 4.5), autoclaved (1 mL of washing buffer, 45 min, 120°C) to solubilize starch, and finally incubated with a-amylase and amyloglucosidase (4 and 40 U, respectively) for 1 h at 50°C up to complete hydrolysis into glucose. The quantification of glucose, fructose, and sucrose from the extraction supernatant

FIGURE 1. Industrial process chain: "cime di rapa" at San Lidano industry (a). Batches of flowers (b), leaves (c), and stems (d) that derived from shoots of BSM90 before processing (e). Shoots of H39 (f), arrows indicate leaf margin that is less fringed than BSM90. Packaged product (g) and inner content (h). Dietary fibre and glycaemic carbohydrates were determined in shoots (e and f), while antioxidant parameters were assessed in all samples.

and of glucose from starch hydrolysis (after centrifugation, 16,000×*g*, 5 min) was achieved by spectrophotometric coupled enzymes assays, using a Plate reader (Spectrotar Nano BMG, Labatech Gmbh, Ortenberg, Germany).

Extraction and determination of total contents of phenolics, flavonoids, flavonols, and glucosinolates

Extraction was carried out based on previous protocols [Li *et al.*, 2011]. Briefly, lyophilized material (0.2 g) was added to (final concentration of 10 mg/mL) a water-methanol mixture (20–80%, *v/v*). The suspension was shaken for 3 h in the dark at 4°C, and then centrifuged at 3,000×*g* for 10 min at 4°C. The supernatant was recovered, aliquoted at 4°C for the content determinations, and stored at –80°C. Phenolic compounds were measured spectrophotometrically at 760 nm according to the Folin–Ciocalteu method [Li *et al.*, 2011] and the total phenolic content was expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of dry weight (DW). Total flavonoid amount determination followed a method used for fruit [Kim *et al.*, 2003] using absorbance at 430 nm, and values were converted into milligrams of catechin equivalents per gram of DW (mg/g CE). Total flavonol quantification was at 360 nm [Castillo-Muñoz *et al.*, 2009] using quercetin as standard and expressed in milligram of quercetin equivalents per gram of DW (mg QE/g). Total glucosinolates were determined at 365 nm [Giorgetti *et al.*, 2018]. Briefly, aliquots (0.5 mL) from extracts were vacuum-dried, the pellet was re-dissolved in 0.2 mL distilled sterile water and treated with myrosinase (28 U/mL) for 1 h at 37°C to catalyse the glucosinolates conversion into isothiocyanates. Cyclo-condensation of these was achieved by adding a predissolved mixture of 80 mM 1,2-benzenedithiol (0.1 mL), methanol (0.9 mL), and 0.1 M potassium phosphate buffer (0.78 mL) in a final volume of 2 mL and incubated at 64°C for 1 h. The reaction generated 1,3-benzedithiol-2-thione from 1,2-benzenedithiol (ε=23,000 /M cm at 365 nm) and glucosinolates were quantified referring to the calibration curve of sinigrin and expressed as milligram of sinigrin equivalents per gram of DW (mg/g SIN).

Antioxidant activity by chemical assays

The oxygen radical absorbance capacity (ORAC) assay was performed by minor modifications [Ninfali *et al.*, 2005]. Briefly, 100 μL of the extract was diluted (1:10, 1:100: 1:1000, *v/v*) and added to a mixture of 1 mL final volume. The mixture contained 800 μL sodium phosphate buffer (75 mM, pH 7.0) with fluorescein sodium salt (0.05 μM) plus a 100 μL solution of 2,2'-azobis(2-amidinopropane) dihydrochloride (400 mM). The standard mixture consisted of 100 μL of 50 μM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); the control consisted of sodium phosphate buffer (75 mM, pH 7.0). The Perkin-Elmer Victor™ X^3 apparatus (Waltham, MA) measured fluorescence every 5 min at 37°C at 485 nm excitation, 520 nm emission for 60 cycles. The ORAC values were calculated by the formula (As-Ab/At-Ab)*KA, where: As, area subtended by the curve (AUC) of fluorescein in the sample (Perkin Elmer 2030 Work Station); At and Ab, Trolox and control AUCs, respectively. K, dilution factor; and A, Trolox concentration (μM). The ORAC unit was expressed in micromoles of Trolox equivalents per g of DW (μmol/g TE). The radical scavenging activity was also determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) assay [Boudjou *et al.*, 2013]. The extract (50 μL) was added to a solution of 60 μM methanol dissolved DPPH$^\bullet$ (final volume 2 mL), vortexed and incubated at 25°C for 60 m in the dark. Blank consisting of pure methanol and control was ethanol (50 μL) instead of extract; absorbance was measured at 517 nm. The antiradical activity (ARA) was expressed as percentage of DPPH inhibition relative to the control by the equation: ARA = [1-(As/Ac)] × 100, with As and Ac as absorbance values of sample and control, respectively.

Statistical analysis

All parameters were analysed according to a completely randomised design with three replications. ANOVA was carried out by a General Linear Model (GLM, SAS Software, Cary, NC, USA). Fibre and sugar content data were analysed in relation to genotypes by a one-way ANOVA, while two-way ANOVA was applied to the antioxidant data deriving from post-cut fresh product (genotype/organs) and from packaged product (genotype/storage). The Least Significant Difference test (α=0.05) was used to separate means. PROC CORR in SAS performed variable correlation analyses on samples of entire shoots and composing parts at the time 0 plus bagged products sampled at 1, 48, and 96 h of storage for both genotypes analysed.

RESULTS AND DISCUSSION

Dietary fibre and glycaemic carbohydrates of pre-packaged shoots

The H39 and BSM90 entire shoots were sampled after cutting during the industrial process (Figure 1) and assayed for the contents of sugars, dietary fibre, extractives, and ashes (Table 2). Overall, the extractives (polar and non-polar soluble fractions included sugars, organic acids, lipids, phenolics, soluble proteins, and pigments) were the most abundant category and the genotypes showed no significant difference. Sugars (Table 2) were grouped into dietary fibre (cell wall components and lignin, DF) and glycaemic carbohydrates (GC) following their nutritional function [EFSA Panel on Dietetic Products Nutrition and Allergies, 2010]. DF was over 5 times higher than GC and higher in BSM90 than H39 (39.64 *vs*. 34.57 gram per 100 grams of DW). As for total lignin, the genotypes shared similar contents (9.21–9.56 g/100 g DW), though the soluble component was higher in BSM90 (4.11 *vs.* 3.07 g/100 g DW), while the Klason fraction was higher in H39 (6.13 *vs.* 5.45 g/100 g DW) leading to a Klason/soluble ratio of 2.0 and 1.3 in H39 and BSM90, respectively. Galactose, arabinose, xylose, and mannose participated to the hemicellulose polymer, which had contents of 5.67 and 7.61 g/100 g DW in H39 and BMS90, respectively. The most abundant monomer was galactose followed by arabinose, xylose, and mannose, respectively representing the 35%, 29–34%, 19–24%, and 12–13% fractions of the total content. The BSM90 genotype was *ca*. 1.3-fold richer in total hemicellulose than H39 due to

TABLE 2. Fibre and sugar contents of entire shoots of “cime di rapa” after cutting.

Compounds[1]	BSM90 (g/100 g)	H39 (g/100 g)	*Signif.*
Lignin (Klason)	5.45±0.03^{b}	6.13±0.32^{a}	*
Soluble lignin	4.11±0.22^{a}	3.07±0.2^{b}	**
Total lignin	9.56±0.19	9.21±0.13	*n.s.*
Arabinose	2.58±0.01^{a}	1.63±0.11^{b}	***
Galactose	2.67±0.03^{a}	1.97±0.18^{b}	**
Xylose	1.48±0.04^{a}	1.34±0.07^{b}	*
Mannose	0.89±0.06^{a}	0.73±0.05^{b}	*
Total hemicellulose	7.61±0.15^{a}	5.67±0.4^{b}	**
Ramnose	0.79±0.05^{a}	0.70±0.04^{b}	*
Galacturonic acid	11.20±0.40^{a}	9.82±0.49^{b}	*
Total pectin	11.99±0.45^{a}	10.52±0.53^{b}	*
Cellulose	10.48±0.35^{a}	9.18±0.70^{b}	*
Dietary fibre	39.64±1.14^{a}	34.57±1.69^{b}	**
Glucose	3.48±0.16^{a}	0.80±0.08^{b}	***
Fructose	2.80±0.15^{a}	0.99±0.15^{b}	***
Sucrose	0.70±0.01^{a}	0.27±0.03^{b}	***
Soluble sugars	6.97±0.32^{a}	2.06±0.26^{b}	***
Starch	0.59±0.04^{a}	0.15±0.05^{b}	***
Glycaemic carbohydrates	7.56±0.28^{a}	2.21±0.30^{b}	***
Other parameters			
Residual humidity	3.58±0.05	3.27±0.07	*n.s.*
Ashes	10.84±0.02^{b}	13.15±0.17^{a}	**
Extractives	47.41±5.07	46.75±1.36	*n.s.*

[1] mean ± standard deviation; g, grams of dry weight. Significance letters refer to the genotype incidence; *n.s.*, non-significant; *, **, *** = significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Mean ratios (percentage) of dry (lyophilized) *vs.* fresh weight tissues were 11.75±1.03 and 10.58±0.43 with no significant difference for BSM90 and H39, respectively. Division factors of 8.5 and 9.5 were used to convert data into g/100 g fresh weight for BSM90 and H39, respectively.

the higher level of each monosaccharide, and particularly of arabinose and galactose. The pectin group consisted of galacturonic acid and rhamnose (93 *vs.* 7% of the total pectin, respectively) and total contents were higher in BSM90 (11.99 *vs.* 10.52 g/100 g DW). BSM90 also had a higher amount of cellulose (10.48 *vs.* 9.18 g/100 g DW) and, overall, the great DF content was due to the major contribution of hemicellulose. GC included glucose fructose, sucrose, and starch and showed abundance differences between the two genotypes. Looking at mean values of soluble sugars in H39 and BSM 90, glucose (0.80 *vs.* 3.48 g/100 g DW) and fructose (0.99 *vs.* 2.80 g/100 g DW) prevailed on sucrose contents (0.27 *vs.* 0.70 g/100 g DW). The amounts of soluble sugars and starch (0.15 *vs.* 0.59 g/100 g DW) were at least 2.5-fold higher in BSM90 than H39 shoots, accounting for the higher total carbohydrate content in the former (7.56 *vs.* 2.21 g/100 g DW). Finally, ashes were more abundant in H39 than BSM90 (13.15 *vs.* 10.84 g/100 g DW, respectively).

Rapini nutrient contents are expressed as g/100 g fresh weight (FW) in the USDA database [https://fdc.nal.usda.gov] and those of BSM90 and H39 were converted accordingly (see notes of Tables 2 and 3 for DW/FW ratios and conversion factors). The comparisons of BSM90-H39 *vs*. USDA showed that values of the former were higher as for ashes (1.3–1.4 vs 0.9) and fibre (4.7–3.6 *vs*. 2.7), lower as for total carbohydrates (0.9–0.2 *vs*. 2.9), and in comparable ranges as for soluble sub-fraction (0.8–0.2 *vs*. 0.4). Quantification of DF components was previously undescribed in broccoli-raab (*B. rapa* subsp. *sylvestris*) that showed higher levels than turnip greens of *B. rapa* subsp. *rapa* [Francisco *et al.*, 2011] and comparable to that of broccoli (*B. oleracea*), which have positive impact on human microbiota with a 200 g daily consumption [Kaczmarek *et al.*, 2019]. Given that a 25 g daily consumption of DF is optimal [EFSA Panel on Dietetic Products Nutrition and Allergies, 2010], 200 g serve of BSM90 and H39 would respectively provide 37% and 22% of the requirement. Content variations of lignin, pectin, and hemicellulose monomers were under genotype effects as also reported in turnip greens [Francisco *et al.*, 2011]. Galactose, arabinose, xylose, and mannose were here included as hemicellulose components, however the abundance of arabinose and galactose may also derive from arabinogalactans. The total content of pectin was underestimated because only galacturonic acid and rhamnose were quantified; however they were the most abundant fraction of DF. The GC higher content in BSM90 *vs.* H39 may confer a sweeter flavour (appreciated by consumers) that counterbalances the bitterness associated to glucosinolates [Schonhof *et al.*, 2004]. Finally, starch quantification enriched information on “cime” nutrient compared to the USDA database.

Antioxidant compounds and capacity of shoots and composing organs before packaging

Comparing BSM90 with H39 (Table 3), mean values from entire shoots were 18.64–14.92 mg GAE/g of total phenolic compounds (TPC) content, 34.74–30.96 mg/g CE of flavonoids (FID) content, 14.62–14.08 mg QE/g of flavonols (FOL) content, 37.47–24.63 mg/g SIN of glucosinolates (GSL) content, 354.62–293.25 μmol/g TE of ORAC, and 59.35–46.14 % ARA against DPPH$^{\bullet}$. The sum of values measured in each shoot component was not always consistent with the values of entire shoots (*e.g.*, +48–56% for FOL content, +12–20% for ORAC, +10–14% for ARA against DPPH$^{\bullet}$), likely because of the variability of organ frequency in the shoots. The leaves of both genotypes showed the highest values of all parameters, followed by those of florets and stems. Specifically, leaves had on average double ORAC and contents of FID, FOL, and GSL than florets, and at least five-fold higher levels of FID and GSL than stems. The ANOVA pointed at the genotype (G) effects on TPC, GSL, ARA against DPPH$^{\bullet}$ and ORAC levels ($P \leq 0.01$ and 0.001) that were recurrently higher in BSM90 than H39. The organ type (O) significantly

TABLE 3. Antioxidant compound and glucosinolate contents and antioxidant capacity in shoots and organs of H39 and BMS90 "cime di rapa" at post-cut phase[1].

Genotype	Organ	TPC (mg/g GAE)	FID (mg/g CE)	FOL (mg/g QE)	GSL (mg/g SIN)	ORAC (μmol/g TE)	ARA (%)
BSM90	Shoot	18.64±1.29	34.74±8.51	14.62±2.76	37.47±6.39^{a}	354.62±39.21	59.35±5.51^{a}
	Leaves	9.85±1.82	33.07±12.42	19.51±5.42	28.60±7.37^{b}	242.97±21.19	29.57±3.44^{c}
	Flowers	7.47±2.10	17.03±2.82	10.21±1.46	8.70±4.57^{cd}	125.22±4.26	26.17±0.11^{c}
	Stems	3.15±0.88	5.69±1.54	3.27±0.44	3.21±1.14^{d}	73.17±5.07	10.43±0.39^{e}
H39	Shoot	14.92±0.98	30.96±9.07	14.08±2.54	24.63±2.04^{b}	293.25±18.14	46.14±0.56^{b}
	Leaves	7.66±1.13	36.03±23.36	16.21±4.82	14.69±3.12^{c}	190.48±5.13	25.36±6.61^{c}
	Flowers	5.89±2.24	12.94±2.91	7.73±2.20	6.3±4.89^{d}	82.66±1.53	18.54±1.46^{d}
	Stems	2.35±0.40	4.30±0.61	2.93±1.15	3.18±1.16^{d}	61.25±2.80	9.86±0.89^{e}
Signif.	G	**	n.s.	n.s.	***	***	***
	O	***	***	***	***	***	***
	G x O	n.s.	n.s.	n.s.	*	n.s.	*

[1] mean ± standard deviation; g, grams of dry weight. G, genotype; O, organ. Significance letters refer to G×O interactions; n.s., non-significant; *, **, *** = significant at P≤0.05, 0.01 and 0.001, respectively. TPC, total phenolic compounds; FID, flavonoids; FOL, flavonols; GSL, glucosinolates; ORAC, oxygen radical absorbance capacity; ARA, DPPH$^{\bullet}$-based antiradical activity. Other abbreviations are in Materials and methods. See Table 2 for division factors to convert into mg/g fresh weight.

influenced all parameters (P≤0.001), whereas G × O interactions affected only GSL and ARA against DPPH$^{\bullet}$ variations (P≤0.05). The higher contents of TPC were likely to explain the 1.2-fold higher antioxidant activity (ARA against DPPH$^{\bullet}$, ORAC) of BSM90 *vs.* H39 shoots.

Hereafter we refer to literature results based on methods and units comparable to this work. As for the entire shoot, TPC ranges of "friariello" were 89–118 and 220–250 mg GAE/100 g FW [De Pascale *et al.*, 2009; Romani *et al.*, 2006] and are consistent with those of Table 3, converted into 157–-219 mg GAE/100 g FW. FID determination included values of 39–104 mg CE/g FW from *B. oleracea* vegetables [Reilly *et al.*, 2014], namely over four times lower than broccoli-raab shoots (325–408 mg CE/g FW). As for the shoot components, FOL content ranges of *B. oleracea* florets were 4.5–5.4 mg QE/g DW [Naguib *et al.*, 2012] and lower than those of "cime di rapa" (Table 3). These FOL content differences (higher contents in *B. rapa* "friariello" than *B. oleracea*) were also observed by chromatographic quantifications of quercetin, kaempferol, and isorhamnetin forms [Romani *et al.*, 2006]. As for GSL, total sums of different classes revealed similar contents between leaves (10.6–39.8 μmol/g DW) and entire shoots (20.9–31.4 μmol/g DW) in "friariello" [Barbieri *et al.*, 2008; De Pascale *et al.*, 2007], while in this study, leaf GSL content was 60–76% of the entire shoot. The discrepancies may be due to different type of sampling or genotypes. Moreover, GSL amounts of rapini florets (66.32–102.35 mg SIN/100 g FW) were comparable with those of broccoli [Fernández-León *et al.*, 2013]. Regarding ORAC values, those previously reported [Wu *et al.*, 2004] for broccoli-raab fell in the ranges of this work (30.8 *vs.* 30.8–41.7 μmol/g TE FW); ARA percentages of *B. rapa* var. *rapa* leaves [Iqbal *et al.*, 2013] were higher than stems, 25–35% *vs.* 20–25% similarly to "cime di rapa". The G, E, and G×E effects were reported for phenolic compound contents and antioxidant properties of "friariello" [Barbieri *et al.*, 2009; De Pascale *et al.*, 2007], while GSL contents were under control of these factors in several *Brassica* vegetables [Reilly *et al.*, 2014], consistently with results of this work. "Cime di rapa" contained higher TPC and AOC values in leaves than flowers similarly to other *Brassica* vegetables [Bhandari & Kwak, 2015]. Finally, leaves, stems, and florets represented the 72.95±2.08, 21.12±1.28 and 5.94±0.83% of the packaged product. Considering that florets have higher TPC/AOC values than stems and are much enjoyed by consumers, increasing the flower/stem ratio (by selective cutting or genetic improvement) can improve the final product antioxidant quality.

Antioxidant quality profile of bagged shoots during shelf life

The product at 48 hpp was assumed as the most probably consumed; the mean values for BMS90 and H39 were respectively: 16.07–14.54 mg GAE/g of TPC content, 12.17–9.39 mg/g CE of FID content, 2.96–4.29 mg QE/g of FOL content, 34.55–24.71 mg/g SIN of GSL content, 185.49–178.36 μmol/g TE of ORAC, and 86.80–90.44% of ARA against DPPH$^{\bullet}$ (Table 4). During the 1 to 96 hpp lapse, the storage time (ST) effects were strongly significant on FID, FOL, and ORAC variables (P≤0.001), less on TPC and ARA ones (P≤0.05), and unrevealed on GSL (Table 4). More precisely, ST effects were linear in ORAC (-25%) along the whole time lapse, and oscillatory for FID and FOL contents (*e.g.*, for FOL -50 % at 48 hpp and -10% at 96 *vs.* 1 hpp). The genotype had a modest influence on the variation of TPC and ORAC values and the G × ST interaction mildly affected FID variations, essentially due to an increase in FID content in H39 at 96 hpp, opposite to the general decreasing trend. Finally, looking at the compound con-

TABLE 4. Variation of compound contents and antioxidant capacity during storage of “cime di rapa” bagged products[1].

Genotype	Storage (hour pp)	TPC (mg/g GAE)	FID (mg/g CE)	FOL (mg/g QE)	GSL (mg/g SIN)	ORAC (μmol/g TE)	ARA (%)
BSM90	1	15.21±1.67	21.85±5.81^{a}	8.51±2.11	31.00±8.43	248.13±35.77	87.13±1.29
	48	16.07±1.00	12.17±2.12^{b}	2.96±0.31	34.55±6.02	185.49±16.17	86.80±2.57
	96	12.43±1.16	14.34±2.73^{b}	5.38±0.76	26.86±11.32	195.26±13.46	90.26±1.07
H39	1	12.71±1.27	20.52±4.91^{ab}	8.44±1.51	22.81±1.19	222.37±28.94	87.07±1.06
	48	14.54±0.13	9.39±1.52^{b}	4.29±1.01	24.71±1.19	178.36±3.56	90.44±0.99
	96	11.79±2.63	23.36±2.16^{a}	5.97±0.63	26.50±6.68	156.46±3.42	89.34±1.48
Signif.	G	*	n.s.	n.s.	n.s.	*	n.s.
	ST	*	***	***	n.s.	***	*
	G x ST	n.s.	*	n.s.	n.s.	n.s.	n.s.

[1] mean ± standard deviation; g, grams of dry weight. Mean ratios of dry *vs.* fresh weight (percentage) calculated for the 1–48–96 hpp timing were 9.15±0.39 and 7.86±0.21, with no significant difference for BSM90 and H39, respectively. Division factors of 10.9 and 12.7 were used to convert data into mg/g fresh weight for BSM90 and H39, respectively. Conversion factors specifically refer to packaged material. TPC, total phenolic compounds; FID, flavonoids; FOL, flavonols; GSL, glucosinolates; ORAC, oxygen radical absorbance capacity; ARA, DPPH$^{\bullet}$-based antiradical activity. G, genotype; ST, storage time. Significance letters refer to G × ST interactions; n.s., non-significant; *, **, *** = significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

tents and chemical antioxidant capacity, the ORAC positive correlation with TPC was very strong ($r \geq 0.80$) and strong ($0.60 \leq r < 0.80$) with GSL and FID. The ARA against DPPH$^{\bullet}$ antiradical activity showed strong positive correlation with TPC and GSL amounts, however a significant weak positive correlation ($r=0.45$) occurred between the two antioxidant assays (Table 5).

TABLE 5. Correlations between antioxidant activities and compound contents[1] in *B. rapa* subsp. *sylvestris*.

	TPC	FID	FOL	GSL	ARA	ORAC
TPC	1.00	0.37*	0.21n.s.	0.83***	0.77***	0.82***
FID		1.00	0.82***	0.34*	0.05n.s.	0.64***
FOL			1.00	0.25n.s.	-0.22n.s.	0.58***
GSL				1.00	0.71***	0.79***
DPPH					1.00	0.45**
ORAC						1.00

[1] Pearson’s correlation coefficients (r) and related significance (asterisks). The correlation was established using the whole data set of unprocessed and bagged materials (n=42). TPC, total phenolic compounds; FID, flavonoids; FOL, flavonols; GSL, glucosinolates; ORAC, oxygen radical absorbance capacity; ARA, DPPH$^{\bullet}$-based antiradical activity. n.s., not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Processing steps are known to alter nutritive contents of packaged vegetables and, consistently, the comparison of shoots at the post-cut phase *vs.* bagged products at 48 hpp (Tabs 3 and 4) pointed at level drops of most variables (3–14% for TPC, 65–70% for FID, 70–80% for FOL, 0–8% for GSL, and 39–48% for ORAC). Storage time behaves as an additive factor affecting ACC and AOC of *B. rapa* [Pouria & Seid Mahdi, 2019] and its effects on FID, FOL, and ORAC values were significant in this work. The FID fall-and-raise trend of packaged broccoli-raab recalled that of kale at similar modified atmosphere packaging (MAP) and storage conditions [Kobori *et al.*, 2011]; this behaviour may reflect time-regulated interactions between stress response and senescence pathways as described in lettuce leaves [Ripoll *et al.*, 2019]. Moreover, specific FID classes occur in distinct *Brassica* spp. tissues [Fernandes *et al.*, 2007], and fluctuations may also derive from organ-specific stress responses. Regarding GSL, the O_2/CO_2 equilibria (3–10% *vs.* 5–10%) in MAP are crucial for brassica product quality and specific conditions for broccoli-raab (8% O_2 and 2% CO_2) increased shelf-life, while higher CO_2 was envisaged to improve it [Conte *et al.*, 2011]. Here, the 7.2% O_2 and 8.8% CO_2 contributed to stabilize the GSL content until 96 hpp and, concurrently, off-odours (not quantified in this work) were unperceived by subjective sensorial analyses. The genotype effects on TPC and ORAC were non- or lowly-significant in pre-processed shoots *vs.* bagged products; the mitigation of differences between H39 and BSM90 might have been due to diversified responses to work steps before packaging. TPC levels showed a positive correlation with those form both AOC assays, in agreement with several studies on *Brassica* vegetables [Li *et al.*, 2018]. Focusing on GSL, their radical scavenging activity depends on the chemical assay and accounts for a small percentage of the total antiradical activity [Cabello-Hurtado *et al.*, 2012]; the GSL positive correlations *vs.* both AOC assays confirm their (minimal) contribution. In addition, the modest positive correlation between ORAC/ ARA against DPPH$^{\bullet}$ assays has been previously observed and rely in different sensitivity to diverse antioxidant compounds and/or their mixtures [Floegel *et al.*, 2011], some of which were untested in this work (*e.g.*, vitamin C, carotenoids *etc*.). Finally, the ORAC value drop and the moderate ARA against DPPH$^{\bullet}$ value raise during storage may reflect this differential sensitivity and confirm the necessity of multiple and combined assays to assess antioxidant quality criteria.

CONCLUSIONS

Genotype-dependent differences were observed in fresh unprocessed "cime di rapa" as for the contents of dietary fibre, glycaemic sugars, phenolics, flavonoids, flavonols, glucosinolates, and antioxidant capacity; most values were lower in the hybrid genotype, which may specifically target fresh consumption considering that low fibre levels enhance tenderness. Reducing the stem component in favour of leaves and florets will enhance the antioxidant properties of the product. Level decay of most parameters (except for glucosinolates) occurred in the minimally processed products compared to the pre-packaged ones. The antioxidant quality of packaged product was strongly affected by storage time while feebly by genotype (and factor interactions). Proper gas composition in MAP may have compensated genotype differences and stabilized glucosinolates content variation. Correlation analyses between antioxidant contents and antioxidant capacity support the necessity of performing multiple antioxidant assays to evaluate the product quality.

ACKNOWLEDGEMENTS

Dr. Tiziano Biancari, agronomist, is thanked for carrying out cultivation, and providing plant material according to the experimental design. We are grateful to the native English speaker Dr. Carla Ticconi for language editing (PhD Genetics, UC Davis, CA).

RESEARCH FUNDING

This research was supported by the project "Nutrizione, Alimentazione & Invecchiamento Attivo (NUTR-AGE)" of the National Research Council of Italy.

CONFLICT OF INTERESTS

The authors report no conflicts of interest and are responsible for the content of this article.

ORCID ID

D. Giannino https://orcid.org/0000-0001-5854-7912;
G. Testone https://orcid.org/0000-0002-6355-3871;
Ch. Nikolodi https://orcid.org/0000-0002-2953-4052;
L. Giorgetti https://orcid.org/0000-0003-3295-4549;
L. Bellani https://orcid.org/0000-0002-9135-7632;
M. Gonnella https://orcid.org/0000-0001-5465-880X;
M. Ciardi https://orcid.org/0000-0003-4121-8279;
P. Cappuccio https://orcid.org/0000-0002-2361-8319;
S. Moscatello https://orcid.org/0000-0001-7265-305X;
A. Battistelli https://orcid.org/0000-0002-8613-4688;
V. Longo https://orcid.org/0000-0001-8972-8564

REFERENCES

1. Annunziata, M.G., Attico, A., Woodrow, P., Oliva, M.A., Fuggi, A., Carillo, P. (2012). An improved fluorimetric HPLC method for quantifying tocopherols in *Brassica rapa* L. subsp *sylvestris* after harvest. *Journal of Food Composition and Analysis, 27*(2), 145–150. https://doi.org/10.1016/j.jfca.2012.05.006
2. Barbieri, G., Bottino, A., Orsini, F., De Pascale, S. (2009). Sulfur fertilization and light exposure during storage are critical determinants of the nutritional value of ready-to-eat friariello campano (*Brassica rapa* L. subsp *sylvestris*). *Journal of the Science of Food and Agriculture, 89*(13), 2261–2266. https://doi.org/10.1002/jsfa.3718
3. Barbieri, G., Pernice, R., Maggio, A., De Pascale, S., Fogliano, V. (2008). Glucosinolates profile of *Brassica rapa* L. subsp *sylvestris* L. Janch. var. *esculenta* Hort. *Food Chemistry, 107*(4), 1687–1691. https://doi.org/10.1016/j.foodchem.2007.09.054
4. Bhandari, S.R., Kwak, J.-H. (2015). Chemical composition and antioxidant activity in different tissues of brassica vegetables. *Molecules, 20*(1), 1228–1243. https://doi.org/10.3390/molecules20011228
5. Bonnema, G., Del Carpio, D.P., Zhao, J. (2011). Diversity analysis and molecular taxonomy of brassica vegetable crops. In Sadowski J. and Kole C. (Eds.), *Genetics, Genomics and Breeding of Vegetable Brassicas*, 1 ed, CRC Press, Boca Raton, Florida, USA, pp. 81–124.
6. Boudjou, S., Oomah, B.D., Zaidi, F., Hosseinian, F. (2013). Phenolics content and antioxidant and anti-inflammatory activities of legume fractions. *Food Chemistry, 138*(2–3), 1543–1550. https://doi.org/10.1016/j.foodchem.2012.11.108
7. Cabello-Hurtado, F., Gicquel, M., Esnault, M.-A. (2012). Evaluation of the antioxidant potential of cauliflower (*Brassica oleracea*) from a glucosinolate content perspective. *Food Chemistry, 132*(2), 1003–1009. https://doi.org/10.1016/j.foodchem.2011.11.086
8. Cartea, M.E., Francisco, M., Soengas, P., Velasco, P. (2010). Phenolic compounds in Brassica vegetables. *Molecules, 16*(1), 251–280. https://doi.org/10.3390/molecules16010251
9. Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., Gómez, M.V., Velders, A.H., Hermosín-Gutiérrez, I. (2009). Flavonol 3-*O*-glycosides series of *Vitis vinifera* Cv. Petit Verdot red wine grapes. *Journal of Agricultural and Food Chemistry, 57*(1), 209–219. https://doi.org/10.1021/jf802863g
10. Cefola, M., Amodio, M.L., Cornacchia, R., Rinaldi, R., Vanadia, S., Colelli, G. (2010). Effect of atmosphere composition on the quality of ready-to-use broccoli raab (*Brassica rapa* L.). *Journal of the Science of Food and Agriculture, 90*(5), 789–797. https://doi.org/10.1002/jsfa.3885
11. Conte, A., Scrocco, C., Brescia, I., Mastromatteo, M., Del Nobile, M.A. (2011). Shelf life of fresh-cut Cime di rapa (*Brassica rapa* L.) as affected by packaging. *LWT – Food Science and Technology, 44*(4), 1218–1225. https://doi.org/10.1016/j.lwt.2010.11.006
12. Conversa, G., Bonasia, A., Lazzizera, C., Elia, A. (2016). Bio-physical, physiological, and nutritional aspects of ready-to-use cima di rapa (*Brassica rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort.) as affected by conventional and organic growing systems and storage time. *Scientia Horticulturae, 213*, 76–86. https://doi.org/10.1016/j.scienta.2016.10.021
13. De Pascale, S., Maggio, A., Pernice, R., Fogliano, V., Barbieri, G. (2007). Sulphur fertilization may improve the nutritional value

of *Brassica rapa* L. subsp *sylvestris*. *European Journal of Agronomy, 26*(4), 418–424.
https://doi.org/10.1016/j.eja.2006.12.009

14. De Pascale, S., Orsini, F., Vallone, S., Barbieri, G. (2009). Crop season effects on yield and quality of hydroponically grown *Brassica rapa* var. *sylvestris*. *Acta Horticulturae*, *87*, 427–432.
https://doi.org/10.17660/ActaHortic.2009.807.60
15. Dionex Technical note 40 (2016). Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation. in Inc., T.F.S. Ed., Sunnyvale, CA, USA, p. 10.
16. EFSA Panel on Dietetic Products Nutrition and Allergies (2010). Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. *EFSA Journal, 8*(3), 1462–1539.
https://doi.org/10.2903/j.efsa.2010.1462
17. Elia, A., Santamaria, P. (2013). Biodiversity in vegetable crops, a heritage to save: the case of Puglia region. *Italian Journal of Agronomy, 8*(1), 21–34.
https://doi.org/10.4081/ija.2013.e4
18. Fernandes, F., Valentão, P., Sousa, C., Pereira, J.A., Seabra, R.M., Andrade, P.B. (2007). Chemical and antioxidative assessment of dietary turnip (*Brassica rapa* var. *rapa* L.). *Food Chemistry, 105*(3), 1003–1010.
https://doi.org/10.1016/j.foodchem.2007.04.063
19. Fernández-León, M.F., Fernández-León, A.M., Lozano, M., Ayuso, M.C., Amodio, M.L., Colelli, G., González-Gómez, D. (2013). Retention of quality and functional values of broccoli ‘Parthenon’ stored in modified atmosphere packaging. *Food Control, 31*(2), 302–313.
https://doi.org/10.1016/j.foodcont.2012.10.012
20. Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S.I., Chun, O.K. (2011). Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of Food Composition and Analysis, 24*(7), 1043–1048.
https://doi.org/10.1016/j.jfca.2011.01.008
21. Francisco, M., Velasco, P., Lema, M., Cartea, M.E. (2011). Genotypic and environmental effects on agronomic and nutritional value of *Brassica rapa*. *Agronomy Journal, 103*(3), 735–742.
https://doi.org/10.2134/agronj2010.0439
22. Giorgetti, L., Giorgi, G., Cherubini, E., Gervasi, P.G., Della Croce, C.M., Longo, V., Bellani, L. (2018). Screening and identification of major phytochemical compounds in seeds, sprouts and leaves of Tuscan black kale *Brassica oleracea* (L.) ssp *acephala* (DC) var. *sabellica* L. *Natural Product Research, 32*(14), 1617–1626.
https://doi.org/10.1080/14786419.2017.1392953
23. Iqbal, S., Younas, U., Chan, K.W., Saeed, Z., Shaheen, M.A., Akhtar, N., Majeed, A. (2013). Growth and antioxidant response of *Brassica rapa* var. *rapa* L. (turnip) irrigated with different compositions of paper and board mill (PBM) effluent. *Chemosphere, 91*(8), 1196–1202.
https://doi.org/10.1016/j.chemosphere.2013.01.035
24. ISO 9001 (2008). Quality management systems – Requirements. [https://www.iso.org/standard/46486.html], Last accessed 26/08/2020.
25. ISO 22005 (2007). Traceability in the feed and food chain – General principles and basic requirements for system design and implementation. [https://www.iso.org/standard/36297.html], Last accessed 26/08/2020.
26. Kaczmarek, J.L., Liu, X., Charron, C.S., Novotny, J.A., Jeffery, E.H., Seifried, H.E., Ross, S.A., Miller, M.J., Swanson, K.S., Holscher, H.D. (2019). Broccoli consumption affects the human gastrointestinal microbiota. *The Journal of Nutritional Biochemistry, 63*, 27–34.
https://doi.org/10.1016/j.jnutbio.2018.09.015
27. Kim, D.-O., Chun, O.K., Kim, Y.J., Moon, H.-Y., Lee, C.Y. (2003). Quantification of polyphenolics and their antioxidant capacity in fresh plums. *Journal of Agricultural and Food Chemistry, 51*(22), 6509–6515.
https://doi.org/10.1021/jf0343074
28. Kobori, C.N., Huber, L.S., Sarantópoulos, C.I.G.L., Rodriguez-Amaya, D.B. (2011). Behavior of flavonols and carotenoids of minimally processed kale leaves during storage in passive modified atmosphere packaging. *Journal of Food Science, 76*(2), H31-H37.
https://doi.org/10.1111/j.1750-3841.2010.01988.x
29. Li, H., Deng, Z., Liu, R., Young, J.C., Zhu, H., Loewen, S., Tsao, R. (2011). Characterization of phytochemicals and antioxidant activities of a purple tomato (*Solanum lycopersicum* L.). *Journal of Agricultural and Food Chemistry, 59*(21), 11803–11811.
https://doi.org/10.1021/jf202364v
30. Li, Z., Lee, W.H., Liang, X., Liang, D., Wang, Q., Huang, D., Ong, N.C. (2018). Profiling of phenolic compounds and antioxidant activity of 12 Cruciferous vegetables. *Molecules, 23*(5), art. no. 1139.
https://doi.org/10.3390/molecules23051139
31. Moscatello, S., Proietti, S., Buonaurio, R., Famiani, F., Raggi, V., Walker, R.P., Battistelli, A. (2017). Peach leaf curl disease shifts sugar metabolism in severely infected leaves from source to sink. *Plant Physiology and Biochemistry, 112*, 9–18.
https://doi.org/10.1016/j.plaphy.2016.12.001
32. Naguib, A.E.-M.M., El-Baz, F.K., Salama, Z.A., Abd El Baky Hanaa, H., Ali, H.F., Gaafar, A.A. (2012). Enhancement of phenolics, flavonoids and glucosinolates of Broccoli (*Brassica olaracea*, var. *italica*) as antioxidants in response to organic and bio-organic fertilizers. *Journal of the Saudi Society of Agricultural Sciences, 11*(2), 135–142.
https://doi.org/10.1016/j.jssas.2012.03.001
33. Ninfali, P., Mea, G., Giorgini, S., Rocchi, M., Bacchiocca, M. (2005). Antioxidant capacity of vegetables, spices and dressings relevant to nutrition. *British Journal of Nutrition, 93*(2), 257–266.
https://doi.org/10.1079/BJN20041327
34. Pouria, G., Seid Mahdi, J. (2019). Antioxidant components of brassica vegetables including turnip and the influence of processing and storage on their anti-oxidative properties. *Current Medicinal Chemistry, 26*(24),4559–4572.
http://doi.org/ 10.2174/0929867325666181115111040
35. Reilly, K., Valverde, J., Finn, L., Rai, D.K., Brunton, N., Sorensen, J.C., Sorensen, H., Gaffney, M. (2014). Potential of cultivar and crop management to affect phytochemical content in winter-grown sprouting broccoli (*Brassica oleracea* L. var. *italica*). *Journal of the Science of Food and Agriculture, 94*(2), 322–330.
https://doi.org/10.1002/jsfa.6263
36. Ripoll, J., Charles, F., Vidal, V., Laurent, S., Klopp, C., Lauri, F., Sallanon, H., Roux, D. (2019). Transcriptomic view of detached lettuce leaves during storage: a crosstalk between wounding, dehydration and senescence. *Postharvest Biology and Technology, 152*, 73–88.
https://doi.org/10.1016/j.postharvbio.2019.02.004

37. Romani, A., Vignolini, P., Isolani, L., Ieri, F., Heimler, D. (2006). HPLC-DAD/MS characterization of flavonoids and hydroxycinnamic derivatives in turnip tops (*Brassica rapa* L. subsp *sylvestris* L.). *Journal of Agricultural and Food Chemistry, 54*(4), 1342–1346. https://doi.org/10.1021/jf052629x
38. Schonhof, I., Krumbein, A., Brückner, B. (2004). Genotypic effects on glucosinolates and sensory properties of broccoli and cauliflower. *Nahrung/Food, 48*(1), 25–33. https://doi.org/10.1002/food.200300329
39. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. (2008a). Determination of ash in biomass. Technical Report NREL/TP-510–42622, in *Laboratory*, N.R.E. (Ed. *Laboratory Analytical Procedure (LAP)*, Cole Boulevard, Golden, Colorado.
40. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. (2008b). Determination of structural carbohydrates and lignin in biomass. NREL Laboratory Analytical Procedure NREL/TP-510–42618, in *Laboratory*, N.R.E. (Ed. *Laboratory Analytical Procedure (LAP)*, Cole Boulevard, Golden, Colorado.
41. Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. (2008c). Determination of extractives in biomass. Technical Report NREL/TP-510–42619, in *Laboratory*, N.R.E. (Ed. *Laboratory Analytical Procedure (LAP)*, Cole Boulevard, Golden, Colorado, p. 12.
42. Soengas, P., Sotelo, T., Velasco, P., Cartea, M.E. (2011). Antioxidant properties of Brassica vegetables. In Books, G.S. (Ed.) *Functional Plant Science and Biotechnology*, 1 ed, Isleworth, pp 43–55,
43. Stranieri, S., Baldi, L. (2017). Shelf life date extension of fresh-cut salad: a consumer perspective. *Journal of Food Products Marketing, 23*(8), 939–954. https://doi.org/10.1080/10454446.2017.1266545
44. Torrieri, E., Perone, N., Cavella, S., Masi, P. (2010). Modelling the respiration rate of minimally processed broccoli (*Brassica rapa* var. *sylvestris*) for modified atmosphere package design. *International Journal of Food Science and Technology, 45*(10), 2186–2193. https://doi.org/10.1111/j.1365-2621.2010.02387.x
45. U.S.D.A. (2006). Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Official Soil Series Descriptions. [https://www.nrcs.usda.gov], Last accessed 26/8/2020.
46. U.S.D.A. (2019). FoodData Central, 2019. United Staes Department of Agriculture, Agricultural Research Service. [https://fdc.nal.usda.gov/], Last accessed 26/8/2020.
47. Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E., Prior, R.L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry, 52*(12), 4026–4037. https://doi.org/10.1021/jf049696w

Submitted: 22 May 2020. Revised: 3 August 2020. Accepted: 21 August 2020. Published on-line: 4 September 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 347–360
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/127399
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Use of Principal Component Analysis and Cluster Analysis for Differentiation of Traditionally-Manufactured Vinegars Based on Phenolic and Volatile Profiles, and Antioxidant Activity

Ayse Karadag[1]*, *Fatih Bozkurt*[1,2], *Hatice Bekiroglu*[1], *Osman Sagdic*[1]

[1]*Food Engineering Department, Yıldız Technical University, 34210, Esenler-Istanbul, Turkey*
[2]*Food Engineering Department, Mus Alparslan University, 49100, Mus, Turkey*

Key words: vinegar, antioxidant, phenolics, volatiles, OXITEST, PCA

This study aimed to characterize twelve vinegar samples produced by the traditional method with the use of whole fruits and without any preservatives in terms of their physicochemical properties, total phenolic content (TPC), total flavonoid content (TFC), phenolic compound profiles, antioxidant activity (DPPH$^\bullet$ scavenging activity, FRAP, CUPRAC), and volatile compositions, as well as their abilities to delay oxidation in mayonnaise. Types of raw material significantly affected all of the above parameters ($p<0.05$). Gallic acid, protocatechuic acid, and caffeic acid were detected as the major phenolic acids in all vinegar samples. Among, flavonoids, rutin, and kaempferol were also identified. The major volatiles belonged to acetic acid esters and alcohol groups, and isoamyl acetate was determined in all vinegar samples at changing ratios. The high positive correlation coefficient ($r>0.70$) was determined between DPPH$^\bullet$ scavenging activity of vinegars and induction period of accelerating oxidation based on the OXITEST of mayonnaises produced with these vinegars. Vinegar types significantly affected the oxidative stability of mayonnaise ($p<0.05$). Furthermore, it was demonstrated that vinegar samples could be clearly discriminated by principal component and cluster analyses. This study suggests that fruit type should be considered as a crucial factor in the production of vinegars affecting not only sensory properties but also their physicochemical and bioactive properties.

INTRODUCTION

Vinegar is produced from fruits and vegetables containing sugar or starch through a two-stage fermentation process, namely alcohol and subsequently acetic acid fermentation. In the first stage, fermentable sugars are converted to ethanol and CO_2 under anaerobic conditions by yeast, and in the second stage where alcohol formed in the first stage, is converted to acetic acid by acetic acid bacteria [Ho *et al.*, 2017]. Vinegar is mainly used for pickling of fruits and vegetables and in the preparation of mayonnaise, salad dressings, mustard, and other food condiments due to its taste and aroma. Besides, it is one of the most famous folk medicines used to curb infections [Chen *et al.*, 2016]. The presence of various types of polyphenols and other bioactive compounds contribute to its therapeutic effects, among them antimicrobial, antidiabetic, antihypertensive, antiobesity, and lipid-lowering ones [Chou *et al.*, 2015; Samad *et al.*, 2016].

The chemical composition and physicochemical parameters of vinegar are affected by the manufacturing techniques and raw materials used. Traditional vinegar typically results from a long fermentation (up to a month) and uses natural vinegar as the starter culture, whereas industrial vinegar typically can be manufactured in approximately one day [Budak *et al.*, 2014]. Research on producing new types of vinegar has been ongoing to obtain not only different organoleptic and sensorial properties but also provide a varying phenolic composition, antioxidant activities, and volatile compounds. For example, in the study of De Leonardis *et al.* [2018], compared to apple, white wine, and balsamic vinegars, olive vinegars provided the highest amount of total phenolics (3600 mg GAE/L, almost three times higher than those of balsamic vinegar, 1227 mg GAE/L) and displayed a high presence of hydroxytyrosol (1019 mg/L) which is a potent antioxidant and its daily intake of 5 mg can prevent low-density lipoprotein (LDL) oxidation [Lopez-Huertas & Fonolla, 2017]. In other respect, the use of second-quality strawberry to produce vinegar rich in anthocyanins also resulted in the formation of furaneol, mesifurane, and γ-decalactone which are considered to be the major contributors of fruit flavor due to their low odor threshold and their high quantities [Ubeda *et al.*, 2013]. Among other different raw materials used for vinegar production, onion juice [Horiuchi et al., 1999], oat, buckwheat [Yu *et al.*, 2018], coconut, pineapple juice [Mohamad *et al.*, 2018], hawthorn, artichoke [Ozturk *et al*., 2015], and tomato [Lee *et al.*, 2013] can also be listed. In Turkey, apple, lemon, and grape are the most widely used raw materials for vinegar production, however, vinegar production with new sources and traditional methods has attracted growing interest lately due to the increase in consumer demand and the market value of vinegars.

Thus, the aim of the present study was to perform a comparative analysis of vinegar samples manufactured according

---

* Corresponding Author: +90 212 383 45 83; Fax: +90 212 383 45 71; E-mail: karadaga@yildiz.edu.tr (A. Karadag)

to the traditional method using different raw materials, *i.e.* rosehip, fig, lemon, jujube fruit, artichoke, blackberry, guelder-rose, walnut, pomegranate, red grape, apple, and hawthorn, concerning their physicochemical properties (acidity, pH, color, and °Brix), bioactive properties (phenolic compound profiles and antioxidant activities), and volatile composition. Also, mayonnaise samples were produced with the use of vinegars and subjected to accelerated lipid oxidation tests, and the correlation between the antioxidant activity values of vinegars and oxidative status of mayonnaise samples was evaluated.

MATERIALS AND METHODS

Materials

Twelve vinegar samples, namely rosehip, fig, lemon, jujube fruit, artichoke, blackberry, guelder-rose, walnut, pomegranate, red grape, apple, and hawthorn vinegar, at least three samplings for each vinegar, were supplied from the same manufacturer (Nahita, Icmeli Dogal Urunler Co) that produces according to traditional methods by using whole fruits and without adding any preservatives. The vinegar samples were stored in the laboratory at a constant temperature of 25±1°C before analysis. All chemicals and reagents used for the analyses were of analytical or high-performance liquid chromatography (HPLC) grade and obtained from Merck (Darmstadt, Germany) unless otherwise specified. 6-Hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox, 97%), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95%), 2,4,6-tripyridyl-s-triazine (TPTZ), neocuproine and phenolic standards used for HPLC analysis were obtained from Sigma-Aldrich Ltd. (Steinheim, Germany).

Physicochemical properties

The pH values of the samples were measured by using a pH meter (InoLab 720, WTW GmbH, Weilheim, Germany). The titration acidity of the samples was calculated as acetic acid equivalents. After titration of 5 mL of vinegar with 0.1 N NaOH, spent volume of NaOH was noted, and titration acidity as a percent was calculated:

$$\text{Titration acidity (\%)} = \frac{V \times E \times 100}{M} \tag{1}$$

where: V was the spent volume of NaOH, E was taken as 0.006005 g acetic acid (major acid for vinegar) equivalent to 1 mL of 0.1 N NaOH spent, and M was the sample weight [Bakir *et al.*, 2017].

°Brix values of the vinegars were measured using an Abbe refractometer (Reichert, Benchtop Refractometers AR 700, New York, NY, USA) calibrated with distilled water. The values were expressed as °Brix.

Color values of the vinegar were measured using a chromameter (Lovibond RT Series Reflectance Tintometer, Amesbury, UK). Color was expressed as L* (whiteness/darkness), a* (redness/greenness), and b* (yellowness/blueness).

Total phenolic and total flavonoid content

Vinegar samples were filtered using a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter and appropriately diluted with methanol for further analysis. Total phenolic content (TPC) of the vinegar was determined with the Folin-Ciocalteu (FC) reagent according to the method described by Singleton & Rossi [1965]. Gallic acid was chosen as a reference standard. An aliquot of 0.5 mL of the sample was added to 2.5 mL of FC reagent (0.2N) and 2 mL of Na_2CO_3 (2%). The final mixture was incubated for 30 min at room temperature in the dark, the absorbance was measured at 760 nm using a Shimadzu 150 UV-1800 spectrophotometer (Kyoto, Japan). The results were presented as mg gallic acid equivalent (GAE) per 100 mL of vinegar. The linear range of the standard curve was from 0.01 to 0.6 mg/mL (r^2=0.999).

Total flavonoid content (TFC) of the vinegar was determined according to the method described by Zhishen *et al.* [1999]. The sample (1 mL) was mixed with 4 mL of distilled water, 0.3 mL of $NaNO_2$ (5%), and 0.3 mL of $AlCl_3$ (10%) solution, and allowed to stand for 6 min. Then, 2 mL of NaOH (1 M) was added and the volume was completed to 10 mL with distilled water. Absorbance was measured at 510 nm using a Shimadzu 150 UV-1800 spectrophotometer. The results were presented as mg catechin equivalents (CE) per 100 mL of vinegar. The linear range of the standard curve was from 0.01 to 0.5 mg/mL (r^2=0.998)

Antioxidant activity assays

The DPPH assay was performed as described by Brand-Williams *et al.* [1995]. Volumes of 0.1 mL of each vinegar diluted with the same ratio (1:12, *v/v*) were added to 4.9 mL of $DPPH^\bullet$ solution (6×10^{-5} M in methanol). The mixture was incubated at room temperature for 20 min in the dark. The absorbance was measured at 517 nm by Shimadzu UV-1800 spectrophotometer, and the results were given as inhibition percentage (*I%*) according to the following equation.

$$I\ (\%) = \frac{Abs_C - Abs_S}{Abs_C} \times 100 \tag{2}$$

where: Abs_S and Abs_C were the absorbances of the sample and control ($DPPH^\bullet$ solution), respectively.

The CUPRAC assay (the cupric-reducing antioxidant capacity) was carried out according to the method of Apak *et al.* [2004] with slight modifications. The 1-mL portions of $CuCl_2$ solution (0.01 M), neocuproine (7.5 mM), and 1 M ammonium acetate buffer (pH 7.0) solutions were added to a test tube. After the addition of 0.1 mL of vinegar sample, the total volume was adjusted to 4.1 mL with distilled water. All samples were incubated at room temperature for 1 h in the dark. The absorbance was measured at 450 nm using a Shimadzu UV-1800 spectrophotometer. The results were expressed as mg Trolox equivalent (TE) per 100 mL of vinegar. The standard curve ranged from 25 to 400 μM (r^2=0.994).

The FRAP assay (ferric reducing antioxidant power) was performed according to Benzie & Strain [1996]. The FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3\times6H_2O$ in a 10:1:1 (*v/v/v*) ratio just before use. The TPTZ solution was prepared in 40 mM HCl. A 100 μL sample was mixed with 900 μL of H_2O and 2 mL of FRAP reagent and incubated at room temperature for 30 min in the dark. The absorbance was measured at 593 nm using a spectrophotometer. The results were expressed as mg Trolox equivalent (TE) per 100 mL of vinegar. The curve for the Trolox was linear in the concentration range of 10-100 μM ($r^2=0.999$).

HPLC analysis of phenolic compounds

Phenolic profiles of vinegar were evaluated using the HPLC system (LC-20AD pump, SIL-20A HT autosampler, CTO-10ASVP column oven, DGU-20A5R degasser, and CMB-20A communications bus module) coupled to a diode array detector – SPDM20A DAD (Shimadzu Corp., Kyoto, Japan). Standard calibration curves were prepared by using gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, syringic acid, *p*-coumaric acid, rutin, and kaempferol. The samples were filtered through a 0.45-μm membrane filter and 1 mL of the filtered samples was placed into vials and analyzed. Separations were conducted at 40°C on an Inertrsil® ODS C-18 reversed-phase column (250 ×4.6 mm, 5 μm particle size, GLSciences, Tokyo, Japan). The mobile phase included solvent A (distilled water with 0.1% (*v/v*) acetic acid) and solvent B (acetonitrile with 0.1% (*v/v*) acetic acid). A gradient elution as follows: 10% B (0 to 2 min), 10% to 30% B (2 to 27 min), 30% to 90% B (27 to 50 min) and 90% to 100% B (51 to 60 min), and at 63 min returns to initial conditions. The flow rate was 1 mL/min. Chromatograms were recorded at 278, 320, and 360 nm. Identification and quantitative analyses were done based on the retention times and external standard curves. HPLC-DAD results were presented as mg of individual phenolic per L of vinegar for all samples.

Volatile compound analysis

Volatile compounds of vinegars were identified using the GCMS-QP2010 system (Shimadzu, Milan, Italy) combined with a CTC-Combi-PAL-Autosampler (Bender and Holbein, Zurich, Switzerland).

The column used for chromatographic separation was Restec (Bellefonte, USA) Rtx-5MS fused silica capillary column (30 m×0.25 mm, 0.25 μm). Firstly, vinegar samples were transferred to 20 mL of headspace vials. The samples were heated to 70°C and agitated at 500 rpm for 15 min. The headspace parameters used were: incubation temperature, 70°C; incubation time, 15 min; syringe temperature, 70°C; agitation speed, 500 rpm; injection volume, 500 μL; fill speed 200 μL/s; pull up delay 500 ms; injection speed, 350 μL/s; pre-injection delay, 500 ms; and post-injection delay, 1500 ms. Volume of 0.5 mL of the headspace sample was injected into the column of GC-MS system. GC conditions were: injection temperature, 150°C; oven temperature, 40°C for 3 min, then programmed at 8.0°C/min to 176°C, finally 176°C for 20 min; interface temperature, 280°C; and ion source temperature, 230°C. The carrier gas was helium with a flow rate of 1.71 mL/min. The mass spectrometer was operated in the selected ion-monitoring mode with an electron impact ionization voltage of 70 eV, and data were collected over a range of *m/z* 35–550. Analyses were performed in duplicate for each sample. The identification of volatiles was performed by comparison of the mass spectra of detected volatile compounds with the commercial mass spectra libraries (NIST27 and WILEY7). Quantification was performed based on the relative peak areas that were used directly to give the percentage volatile composition of the vinegar by dividing the area of each peak by the total area under all of the peaks.

Analysis of lipid oxidation in mayonnaise samples

Preparation of mayonnaise samples

The recipe contained the following ingredients in a weight ratio (*w/w*): sunflower oil (70%), egg yolk (10%), vinegar (18%), sugar (0.82%), salt (0.82%), and xanthan gum (0.36%). A coarse emulsion was initially formed by dissolving egg yolk, sugar, salt, xanthan gum, and vinegar. Mayonnaise was prepared by adding the oil to the aqueous mixture at a steady rate and mixing the ingredients using an IKA T-25 Ultra-Turrax high-speed homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 7000 rpm for 5 min until a homogenous emulsion was obtained.

Oxidation tests

Oxidation of mayonnaise samples was monitored using an OXITEST–Oxidation stability Reactor (Velp Scientifica, Usmate, Milan, Italy), equipped with two separate oxidation chambers. After placing the sample in a chamber, it was hermetically sealed and heated to 90°C. Then, pressurized oxygen (99.9999% purity) was injected into the chamber. The analysis was initiated after the oxygen pressure reached 6 bar. The OXITEST reactor monitors the absolute pressure change inside the chambers calculating the oxygen uptake of the oxidizable compounds of the samples and automatically generates the induction period (IP) of oxidation. The higher the IP value, the higher the resistance of the sample to the oxidation.

The data obtained from the OXITEST reactor were set to first-order oxidation kinetics to estimate the oxidation rate constant (*k*). The change in the pressure by time was fitted to the first-order kinetic equation, kinetic parameters were calculated by using nonlinear regression analysis using Statistica software (StatSoft, Tulsa, USA). First-order kinetic equation followed:

$$C = C_0 \times \exp(-k \times t) \tag{3}$$

where: C_0 represents the initial pressure value (bar) in the sample vessel of the OXITEST device, *k* introduces the rate constant for oxidation kinetics, C represents the pressure that varies with time, and time is defined as *t* in hours.

Statistical analysis

All data were presented as a mean of at least three measurements, *i.e.* ± standard deviation for each vinegar. The differences among the vinegar samples were evaluated by one-way analysis of variance (ANOVA) combined with the Tukey comparison test at $p<0.05$ significance level. Principal component analysis (PCA) was performed to analyze all data. Multivariate data analysis was performed to discriminate vinegar samples by applying PCA and hierarchical clustering analysis (HCA). PCA data matrix consisted of TPC, TFC, color parameters, antioxidant activity, induction period, and phenolic content as variables. HCA data matrix consisted

TABLE 1. Physicochemical properties of vinegars.

Vinegar type	Color values			Total acidity (%)	pH	°Brix
	L*	a*	b*			
Rosehip	60.4 ± 0.426^{de}	7.62 ± 0.278^{c}	39.4 ± 0.241^{a}	3.74 ± 0.040^{d}	2.59 ± 0.006^{e}	4.01 ± 0.006^{c}
Fig	79.0 ± 0.620^{bc}	-0.227 ± 0.029^{g}	13.7 ± 1.54^{f}	4.01 ± 0.028^{b}	2.66 ± 0.006^{d}	4.87 ± 0.058^{a}
Lemon	82.1 ± 0.780^{a}	-1.54 ± 0.078^{h}	5.70 ± 0.530^{h}	3.04 ± 0.046^{g}	2.71 ± 0.010^{b}	4.17 ± 0.058^{c}
Jujube Fruit	77.3 ± 0.200^{c}	-1.36 ± 0.086^{h}	14.2 ± 0.355^{f}	2.55 ± 0.023^{h}	2.64 ± 0.000^{d}	4.53 ± 0.058^{b}
Artichoke	80.5 ± 1.06^{ab}	-1.41 ± 0.040^{h}	8.84 ± 1.47^{g}	2.19 ± 0.006^{j}	2.78 ± 0.006^{a}	3.67 ± 0.148^{d}
Blackberry	60.2 ± 0.772^{de}	6.41 ± 0.355^{d}	32.4 ± 0.311^{c}	3.80 ± 0.009^{d}	2.56 ± 0.006^{f}	4.13 ± 0.058^{c}
Guelder-rose	55.1 ± 1.07^{gh}	11.5 ± 0.354^{b}	36.7 ± 0.905^{ab}	5.04 ± 0.030^{a}	2.55 ± 0.010^{f}	4.03 ± 0.028^{c}
Walnut	57.2 ± 1.32^{fg}	2.70 ± 0.562^{f}	20.4 ± 1.79^{e}	3.78 ± 0.009^{d}	2.68 ± 0.006^{c}	4.43 ± 0.058^{b}
Pomegranate	41.5 ± 0.556^{i}	12.5 ± 0.146^{a}	22.4 ± 0.475^{e}	3.29 ± 0.015^{f}	2.50 ± 0.006^{g}	3.23 ± 0.058^{ef}
Red grape	58.0 ± 0.68^{ef}	11.8 ± 0.180^{ab}	28.1 ± 0.118^{d}	3.89 ± 0.009^{c}	2.35 ± 0.006^{h}	3.33 ± 0.058^{e}
Apple	53.7 ± 1.07^{h}	8.19 ± 0.375^{c}	34.5 ± 0.220^{bc}	3.50 ± 0.016^{e}	2.72 ± 0.006^{b}	4.17 ± 0.106^{c}
Hawthorn	61.3 ± 0.62^{d}	4.47 ± 0.343^{e}	33.1 ± 1.72^{c}	2.29 ± 0.017^{i}	2.76 ± 0.006^{a}	3.17 ± 0.058^{f}

Data represent the means ± standard deviations of three measurements. The comparison is between values in rows, means with the same letter are not significantly different ($p>0.05$).

of major volatiles observed in vinegar samples. Data analyses were conducted with Minitab® 17.3.1 (Minitab Inc., State College, USA) software. The Pearson correlation test was employed to determine the correlation coefficients between antioxidant assays and total phenolic and flavonoid contents.

RESULTS AND DISCUSSIONS

Physicochemical properties

The physicochemical properties of vinegar samples are given in Table 1. The pH levels of the vinegar samples were between 2.35 and 2.77, and the total acidity ranged between 2.19 and 5.04%, the guelder-rose vinegar had the highest, whereas artichoke and hawthorn vinegars had the lowest acidity. °Brix value represents the sugar equivalents in vinegar, and it is related to the fermentation since the level of soluble sugars decreases as a result of microorganism activity. The raw material, type of starter cultures, and the methods of production affect °Brix values [Nakamura *et al.*, 2010]. In our study, fig vinegar had the highest °Brix value. The color properties of vinegar are important regarding consumer perception. L*, a*, and b* parameters indicate the lightness-darkness, redness--greenness, and yellowness-blueness of the samples, respectively. L* values of the samples ranged from 41.5 (pomegranate vinegar) to 82.1 (lemon vinegar), a* values ranged from -0.227 (fig vinegar) to 12.5 (pomegranate vinegar), b* values were between 5.70 (lemon vinegar) and 39.4 (rosehip vinegar), and the color of the vinegar samples was mainly related to the raw material.

Total phenolic, total flavonoid content and antioxidant activities

Bioactive properties, namely total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, cupric-reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power (FRAP) of vinegar are given in Table 2. TPC of vinegar ranged from 25.0 to 88.1 mg GAE/100 mL, and TFC varied from 9.74 to 34.9 mg CE/100 mL of vinegar. ANOVA revealed significant differences between the vinegar samples ($p<0.05$) according to the type of raw material. The highest contents of both TPC and TFC were determined in blackberry, rosehip, and guelder-rose vinegars, whereas the lowest ones in lemon and artichoke vinegars. Except for artichoke vinegar, TPC and TFC of vinegars in our study were higher (between 17 and 90 mg GAE/100 mL for TPC, and 2.4 and 34 mg CE/100 mL for TFC) than those found for the similar vinegars studied by Bakir *et al.* [2017] who investigated the antioxidant activities of different types of vinegar in Turkey. The DPPH radical scavenging activity of vinegar in our study ranged from 7.97 to 55.9%; walnut vinegar had the highest DPPH radical scavenging activity, followed by pomegranate, hawthorn, blackberry, and guelder-rose vinegars. Similarly to DPPH radical scavenging activity, the highest CUPRAC was determined in walnut vinegar, and it was significantly higher than in the other vinegars ($p<0.05$) that had high CUPRAC values, namely blackberry, rosehip, guelder-rose, and pomegranate vinegars. In terms of FRAP, the highest value was determined in blackberry vinegar, followed by hawthorn, walnut, and guelder-rose vinegars, though the difference between blackberry and hawthorn vinegars was not significant.

The bioactive properties of vinegars can vary depending on the type of raw material used. In our study, lemon, artichoke, jujube fruit, and fig vinegars showed the lowest values regarding all antioxidant activity tests. Different than our results, it was found that traditional home-made artichoke vinegar had higher DPPH radical scavenging activity than hawthorn and pomegranate vinegars [Ozturk *et al.*, 2015]. However, similar to our results, Bakir *et al.* [2017] deter-

TABLE 2. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities of vinegars.

Vinegar type	TPC (mg GAE/100 mL)	TFC (mg CE/100 mL)	DPPH$^{\bullet}$ scavenging activity (%)	CUPRAC (mg TE/100 mL)	FRAP (mg TE/100 mL)
Rosehip	81.4 ± 0.356^{b}	33.6 ± 0.315^{a}	47.4 ± 0.510^{b}	247 ± 4.64^{bc}	36.6 ± 0.404^{c}
Fig	46.9 ± 0.577^{i}	12.8 ± 0.010^{e}	19.2 ± 0.572^{d}	132 ± 0.814^{g}	10.7 ± 1.01^{f}
Lemon	26.8 ± 0.153^{j}	9.74 ± 0.185^{f}	9.09 ± 0.242^{f}	91.1 ± 6.75^{h}	15.5 ± 0.817^{f}
Jujube fruit	57.9 ± 0.456^{f}	9.84 ± 0.185^{f}	7.97 ± 0.716^{f}	135 ± 5.61^{fg}	16.1 ± 1.18^{ef}
Artichoke	25.0 ± 0.214^{k}	11.2 ± 0.010^{ef}	11.7 ± 0.557^{e}	62 ± 3.49^{i}	14.8 ± 1.26^{f}
Blackberry	88.1 ± 0.761^{a}	33.9 ± 0.543^{a}	54.4 ± 1.36^{a}	263 ± 0.814^{b}	58.1 ± 0.524^{a}
Guelder-rose	81.9 ± 0.384^{b}	34.9 ± 1.67^{a}	54.4 ± 1.04^{a}	233 ± 4.25^{c}	49.3 ± 2.96^{b}
Walnut	67.2 ± 0.410^{c}	16.2 ± 0.364^{d}	55.9 ± 0.840^{a}	315 ± 8.20^{a}	49.9 ± 2.14^{b}
Pomegranate	62.7 ± 0.064^{e}	21.4 ± 0.364^{c}	55.3 ± 0.916^{a}	214 ± 6.37^{d}	41.9 ± 1.95^{c}
Red grape	48.1 ± 0.100^{h}	20.2 ± 0.656^{c}	46.8 ± 0.159^{b}	163 ± 8.34^{e}	25.3 ± 1.32^{d}
Apple	50.7 ± 0.213^{g}	15.3 ± 0.010^{d}	39.4 ± 0.485^{c}	147 ± 5.21^{efg}	21.5 ± 1.01^{de}
Hawthorn	64.7 ± 0.115^{d}	26.1 ± 1.45^{b}	54.9 ± 0.399^{a}	151 ± 11.4^{ef}	54.4 ± 3.96^{ab}

GAE, gallic acid equivalent; CE, catechin equivalent; TE, Trolox equivalent; CUPRAC, cupric- reducing antioxidant capacity; FRAP, ferric reducing antioxidant power. Data are means ± standard deviations of triplicate determinations (n=3). Comparison is between values in rows, means with the same letter are not significantly different ($p>0.05$).

mined that blackberry, rosehip, and guelder-rose vinegars had higher antioxidant potential determined as CUPRAC, FRAP and DPPH$^{\bullet}$ scavenging activity than artichoke vinegar. Some studies have demonstrated that antioxidant activity of vinegars is correlated with their phenolic content since the aromatic phenolic ring in their structure can stabilize unpaired electrons [Verzelloni *et al.*, 2007; Xie *et al.*, 2017]. In our study, the Pearson correlation coefficients (r) for correlations of the results of antioxidant activity tests and TPC were 0.752, 0.844, and 0.802 when antioxidant potential was analyzed as DPPH$^{\bullet}$ scavenging activity, CUPRAC, and FRAP, respectively. The r values for correlations between the antioxidant activity and TFC were 0.767 (DPPH$^{\bullet}$ scavenging activity), 0.652 (CUPRAC), and 0.780 (FRAP).

Phenolic profiles

Gallic acid, protocatechuic acid, and caffeic acid were the main phenolics identified in vinegars (Table 3). In the study of Yun *et al.* [2016], eleven vinegars made out of fruits, cereals, and nuts were screened for their phenolic content, and gallic acid, protocatechuic acid, and caffeic acid were most abundant phenolics detected in their samples. In our study, regarding these three phenolic acids, their total content was significantly higher in blackberry and guelder-rose vinegar, followed by pomegranate, red grape, and walnut vinegar. Gallic acid content ranged from 7.41 to 22.3 mg/L, and the highest concentration was determined in blackberry vinegar, followed by red grape, guelder-rose, and pomegranate vinegars. The level of caffeic acid ranged from 10.8 to 14.1 mg/L, and the difference among the samples was not significant. The protocatechuic acid content of the samples varied between 5.63 and 9.08 mg/L, although the guelder-rose had the highest levels, its difference from pomegranate, hawthorn, and rosehip vinegars was only significant (Table 3).

Considering the content and number of individual phenolics identified in our samples, the content was the highest in walnut, blackberry, and guelder-rose vinegars. Kaempferol was only identified in blackberry vinegar, and syringic acid was determined in hawthorn vinegar (Table 3). Rutin was detected in hawthorn at the highest concentration and followed by apple and walnut vinegars. The presence of rutin in apple vinegars was also reported by Kelebek *et al.* [2017]. The content of *p*-coumaric acid in guelder-rose vinegar was significantly ($p<0.05$) higher than those of blackberry, walnut, and apple vinegars. Bakir *et al.* [2017] determined significantly higher *p*-hydroxybenzoic acid content in guelder-rose, pomegranate, and artichoke vinegars among their vinegar samples. In our study, although the content of *p*-hydroxybenzoic acid was also the highest in guelder-rose, the difference among other samples was not found as significant ($p>0.05$). It has already been reported that the different production methods (conventional or submerged), biotechnological process (alcoholic fermentation or acetous fermentation), or the raw materials involved in vinegar processing may have substantial effects on the bioactive components of the final product [Ho *et al.*, 2017]. For example, Kelebek *et al.* [2017] screened eight apple and grape vinegars with different brands and produced in different geographical regions for their bioactive components and revealed that the content of individual compounds varied in different grape and apple vinegar samples. In another study, unpolished rice vinegars contained more phenolic compounds compared to rice vinegar, because rice bran in the unpolished rice provided a higher amount of phenolic acids such as dihydroferulic acid, dihydrosinapic acid, sinapic acid, vanillic acid, and *p*-hydroxycinnamic acid [Shimoji *et al.*, 2002].

The PCA was conducted to reduce the number of dimensions and to obtain a small number of factors that show the maximum variability between the samples. Three princi-

TABLE 3. Contents (mg/mL) of individual phenolics in vinegars.

Vinegar type	Gallic acid	Protocatechuic acid	Caffeic acid	*p*-Hydroxybenzoic acid	Syringic acid	*p*-Coumaric acid	Rutin	Kaempferol
Rosehip	8.48±2.15^{de}	5.63±0.858^{c}	12.46±3.19^{a}	–	–	–	–	–
Fig	9.99±1.94^{de}	8.11±1.61^{ab}	13.65±1.72^{a}	1.55±0.679^{a}	–	–	–	–
Lemon	7.41±0.344^{e}	6.90±0.728^{abc}	10.8±0.532^{a}	3.04±1.00^{a}	–	–	–	–
Jujube fruit	10.9±0.869^{cde}	6.83±0.878^{abc}	10.9±0.744^{a}	–	–	–	7.72±0.244^{c}	–
Artichoke	7.87±0.348^{e}	7.96±0.393^{abc}	10.8±0.258^{a}	2.12±1.09^{a}	–	–	8.14±0.219^{c}	–
Blackberry	22.3±4.98^{a}	7.46±1.36^{abc}	14.1±0.879^{a}	–	–	1.21±0.344^{b}	–	2.84±0.548
Guelder-rose	14.9±2.55^{bc}	9.08±0.714^{a}	14.1±0.900^{a}	3.09±0.758^{a}	–	2.77±0.754^{a}	–	–
Walnut	11.3±0.380^{cde}	8.35±0.487^{ab}	14.1±0.125^{a}	2.59±1.04^{a}	–	1.30±0.170^{b}	8.59±1.46^{bc}	–
Pomegranate	13.2±2.49^{bcd}	6.18±0.878^{bc}	12.7±1.55^{a}	1.59±0.481^{a}	–	–	–	–
Red grape	16.9±1.06^{b}	6.99±0.876^{abc}	12.0±0.620^{a}	–	–	–	–	–
Apple	8.54±0.441^{de}	7.44±0.797^{abc}	13.2±1.61^{a}	2.00±0.756^{a}	–	1.14±0.437^{b}	10.19±0.785^{b}	–
Hawthorn	8.55±0.561^{de}	6.25±0.694^{bc}	12.28±1.04^{a}	–	1.71±0.163	–	14.7±1.48^{a}	–

Data represent the mean ± standard deviations of three measurements. "-" not detected. The comparison is between values in rows, means with the same letter are not significantly different ($p>0.05$).

pal components (PCs) with eigenvalues >1 accounted for 80.1% of the total variance. PC1 and PC2 explained 54.2% and 17.5% of the total variance, respectively. According to biplot in Figure 1, artichoke, lemon, and jujube fruit vinegars were located on the left side of the plot, whereas blackberry, walnut, and guelder-rose vinegars were located on the right side, which showed that they have roughly opposite responses. PC1 revealed the highest variation, the differences among the samples along the PC1 axis explained more, compared to the similar distances along the PC2 axis. The variables affecting PC1 were related to blackberry, guelder-rose, walnut, rosehip, and pomegranate vinegars. The last two most likely differed from the others based on the effects of the variables on PC2. The variables, including TPC, TFC, and antioxidant activity values were correlated with each other and contributed similar information on PC1. The color parameters a* and b* were also in the same group and were negatively correlated with L* values. Comparing the angles between variables, it could be evaluated that CUPRAC, IP, and p1 (gallic acid content) were more correlated with each other, and that TPC, TFC, DPPH$^{\bullet}$ scavenging activity, FRAP, a* and b* values were closely correlated. The content of p2 (protocatechuic acid), p4 (p-hydroxybenzoic acid), and p6 (rutin) did not show any correlation with the antioxidant activity values.

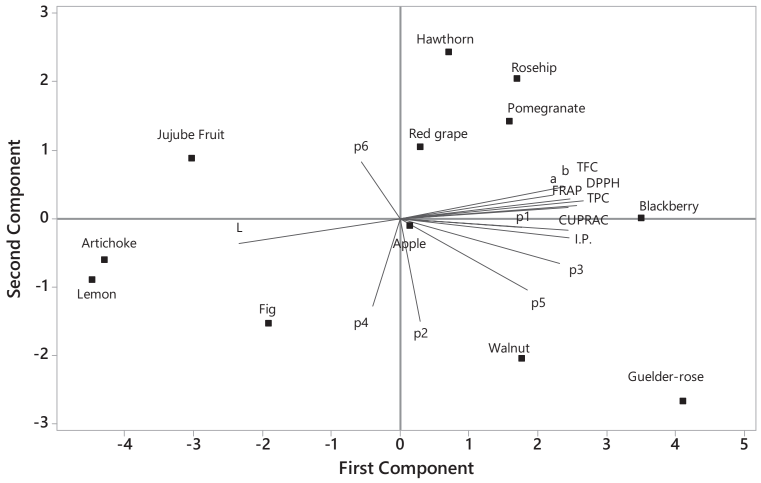


FIGURE 1. Principal component analysis (PCA) biplot for vinegars.

Variables are total phenolic content (TPC), total flavonoid content (TFC), DPPH$^{\bullet}$ scavenging activity (DPPH), cupric- reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP), induction period of mayonnaise oxidation by OXITEST (IP), color parameters: L* (lightness), a* (redness) and b* (yellowness), and content of gallic acid (p1), protocatechuic acid (p2), caffeic acid (p3), *p*-hydroxybenzoic acid (p4), *p*-coumaric acid (p5), and rutin (p6).

Volatile compounds

A total of 114 individual volatile compounds were identified in the vinegar samples and listed in Table 4. The volatile composition of vinegar is widely variable; it usually includes higher content of alcohols, esters, and some aldehydes and ketones. The compounds belonging to acetic acid esters and alcohol groups were the major volatiles. Among acetic acid esters, isoamyl acetate was determined in all vinegars at changing ratios. Except for blackberry, pomegranate, and lemon vinegar, acetic acid esters were more abundant, whereas the ratio of alcohols was more prominent in these samples. Isoamyl acetate and ethyl acetate are among the compounds with the highest odor activity value in vinegar [Baena-Ruano *et al.*, 2010]. It is related to the fruity aroma, and the production of isoamyl alcohol and acetic acid during fermentation. Similar to that, isoamyl alcohol was the most abundant volatile constituent belonging to the alcohol group, it was determined in all vinegars except for fig vinegar. It was also reported that isoamyl alcohol (3-methyl-1-butanol) was the most abundant volatile in vinegar samples [Callejón *et al.*, 2008]. α-Terpineol was only detected in lemon and jujube fruit vinegar. α-Terpineol has been proposed as an indicator for predicting the storage time of citrus fruits, and it was produced from its putative precursors in citrus juice (D-limonene and linalool). Its content was found higher in lemon juice than in orange or grapefruit juices stored for a month. Leonés *et al.* [2019] showed the decrease of α-terpineol content during the transformation of lemon juice to lemon vinegar. 3-Methyl-2-pentanone was only found in blackberry and pomegranate vinegars at high portions of total volatile compounds.

To study the possible similarities among the volatile compositions of the samples, the data (main peaks belonging to each group) was subjected to a hierarchical clustering analysis by taking the squared Euclidean as a distance measure and the Ward linkage method. The dendrogram showed two clusters (Figure 2). In one cluster, rosehip and artichoke vinegars showed the highest similarity, followed by blackberry and hawthorn vinegara. This similarity could be due to a higher isoamyl alcohol proportion in rosehip, artichoke, and blackberry vinegars compared to other samples. Additionally, rosehip and artichoke vinegars had a higher proportion of 1-methyl-propyl acetate, whereas blackberry had a lower one.

1-Methylpropyl acetate proportion of hawthorn vinegar was also approx. 75% of acetic acid esters and with this content hawthorn vinegar significantly differed from the other samples. The similarity of jujube fruit vinegar to other samples in their cluster was low, its differences are based on the proportion of 1-hexyl acetate (in acetic acid ester group), propyl propionate, and ethyl butyrate (ester group). In the study of Yang *et al*. [2019], it was determined that in the later storage period of fresh jujube fruits, hexyl acetate was one of the most important volatile components.

Oxidative stability of mayonnaise samples

The capacity of vinegars to delay the oxidation of mayonnaise was measured by the OXITEST method, and induction periods (IP) of mayonnaise samples prepared with different vinegars are given in Table 5. Compared to lemon vinegar, widely used in mayonnaise production, IP of the mayonnaise samples prepared with guelder-rose, pomegranate, fig, hawthorn, and a few other vinegars was significantly higher. IP of mayonnaise samples prepared with jujube fruit, artichoke, and red grape vinegar was not significantly different ($p>0.05$) compared to the sample prepared with lemon vinegar. The coefficients of Pearson correlations between IP and antioxidant activity of vinegars were found as 0.760 (DPPH$^{\bullet}$ scavenging activity), 0.627 (CUPRAC), and 0.598 (FRAP), whereas these determined between IP and TPC and TFC were at 0.694 and 0.623, respectively.

Oxidative stability of emulsions is one of the crucial factors determining the shelf life of products. Several factors such as types of oil, formulation, and pH, oxygen concentration,

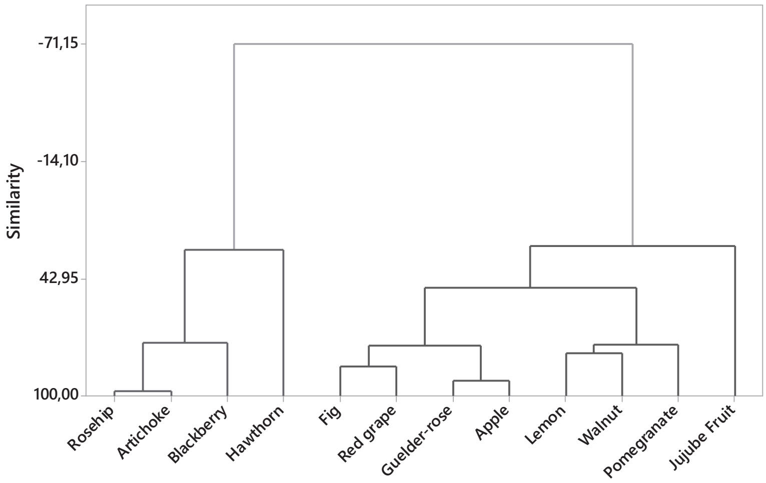


FIGURE 2. Hierarchical cluster analysis of volatiles of vinegars. The dendrogram was obtained using the squared Euclidean distance measure and Ward method as linkage.

The variables included are individual proportions of 1-methylpropyl acetate, isoamyl acetate, isobutyl acetate, 2-methylbutyl acetate, *n*-butyl acetate, 1-hexyl acetate, 3-methyl-2-pentanone, 2-methyl-1-butanol, isoamyl alcohol, α-terpineol, propyl propionate, ethyl isovalerate, ethyl butyrate, ethyl lactate, methyl propyl ether, and 1-chloromethane. The proportion of the variables was more than 90% of the total volatile area for each vinegar sample.

TABLE 4. Compositions and relative content (% area) of volatiles in vinegars.

Volatiles	Rosehip	Fig	Lemon	Jujube fruit	Artichoke	Blackberry	Guelder–rose	Walnut	Pomegranate	Red grape	Apple	Hawthorn
Acetic acid esters												
1-Methylpropyl acetate	30.6	–	–	–	22.2	5.44	27.9	20.3	4.65	–	14.7	54.9
Isoamyl acetate	20.9	25.6	28.3	18.2	23.4	19.7	16.9	38.9	19.5	23.4	17.1	6.77
2-Methylpropyl acetate	2.85	–	4.95	4.24	6.35	8.57	8.14	7.25	9.97	11.6	1.25	–
2-Methylbutyl acetate	–	25.7	–	–	–	–	18.3	–	–	23.7	22.8	8.64
n-Butyl acetate	–	10.6	7.03	–	–	–	–	0.74	–	–	7.49	2.57
2,3-Butanediol diacetate	–	2.21	–	0.27	–	–	–	–	–	–	–	–
Phenethyl acetate	–	0.16	–	–	–	0.18	–	–	–	–	–	–
1-Hexyl acetate	–	–	0.22	27.3	1.79	–	–	–	–	–	–	–
n-Propyl acetate	–	–	–	–	–	–	–	–	–	–	–	–
1,2-Dimethylpropyl acetate	–	–	–	–	–	–	–	–	–	–	0.69	–
Menthyl acetate	–	–	–	–	–	0.27	–	–	–	–	–	–
Acids												
2-Oxovaleric acid	0.13		–	–	–	–	–	–	–	–	–	–
2,3-Dibromobut-2-enedioic acid		0.41	–	–	–	–	–	–	–	–	–	–
Aldehydes												
3-Methylglucose	0.14	–	–	–	–	–	–	–	–	–	–	–
Tetradecanal	0.30	–	–	–	–	–	–	–	–	–	–	–
Octadecanal	0.29	–	–	–	–	–	–	–	–	–	–	–
n-Hexanal	0.14	–	–	–	–	–	–	–	–	–	–	–
1,6-Anhydro-β-D-glucopyranose	–	0.88	–	–	–	–	–	–	–	–	–	–
Benzeneacetaldehyde	–	0.15	–	–	–	–	–	–	–	–	–	–
Phenylacetaldehyde	–	–	–	1.08	–	–	–	–	–	–	–	–
Ketones												
2-Pentadecanone	0.13	–	–	–	–	–	–	–	–	–	–	–
Isomenthone	–	0.22	–	–	0.09	–	–	–	–	–	–	–
3,7,7-Trimethylbicyclo[4.1.0] heptan-4-one	–	0.21	–	–	–	–	–	–	–	–	–	–
Acetoin	–	–	–	–	–	–	0.72	0.76	1.07	0.20	–	–
3-Hydroxycyclohexanone	–	–	–	–	–	–	–	–	–	–	0.39	0.49

TABLE 4. Continued

Volatiles	Rosehip	Fig	Lemon	Jujube fruit	Artichoke	Blackberry	Guelder–rose	Walnut	Pomegranate	Red grape	Apple	Hawthorn
Ketones												
Tetrahydrothiopyran-4-one	–	0.24	–	–	–	–	–	–	–	–	–	–
p-Menthan-3-one	–	–	–	0.13	–	–	–	–	–	–	–	–
3-Methyl-2-pentanone	–	–	–	–	–	19.5	–	–	17.6	–	–	–
Alcohols												
2-Methyl-1-butanol	–	12.4	19.6	–	–	–	18.8	20.1	33.6	28.5	25.2	–
Isoamyl alcohol	33.1	–	12.4	6.44	34	39.9	6.62	7.33	10.4	9.08	8.22	13.3
trans-1,2-Cyclopentanediol	0.11	–	–	–	–	–	–	–	–	–	–	–
4-Ethyl-1-octyn-3-ol	0.14	–	–	–	–	–	–	–	–	–	–	–
Eucalyptol	–	–	1.44	–	–	0.24	–	–	–	–	–	–
Cyclopentanol	–	–	–	–	–	–	–	–	–	–	–	0.30
Phenethyl alcohol	0.15	0.17	–	–	0.19	0.32	–	0.13	–	–	–	–
l-Menthol	0.28	–	–	0.32	–	–	–	–	–	–	–	–
2-Ethyl-1-decanol	–	–	–	0.24	–	–	–	–	–	–	–	–
Fenchyl alcohol	–	–	0.78	0.28	–	–	–	–	–	–	–	–
6-Tridecanol	–	–	–	–	–	–	–	1.58	–	–	–	–
α-Terpineol	–	–	18.5	3.19	–	–	–	–	–	–	–	–
Terpinen-4-ol	–	–	1.23	0.26	–	–	–	–	–	–	–	–
2-Heptanol	–	–	–	–	–	–	–	–	0.33	–	–	–
2-Ethoxyethanol	0.21	–	–	–	–	–	–	–	0.17	–	–	–
Linalool	–	–	–	–	–	–	–	–	–	–	–	0.12
2-Propoxyethanol	–	–	–	–	–	–	0.42	–	–	–	–	–
3-Octanol	–	–	–	–	–	–	–	–	–	–	–	0.28
Neomenthoglycol	0.05	–	–	–	–	–	–	–	–	–	–	–
Carvomenthol	–	–	–	–	–	0.12	–	–	–	–	–	–
2-Methyl-2-methylamino-1-propanol	–	–	–	–	–	–	–	–	–	–	–	0.83
2,4-Dimethyl-3-hexanol	0.14	–	–	0.67	–	–	–	–	–	–	–	–
n-Hexanol	–	–	–	–	1.85	0.14	–	–	–	0.16	–	–
Borneol	–	–	0.29	–	–	–	–	–	–	–	–	–

TABLE 4. Continued

Volatiles	Rosehip	Fig	Lemon	Jujube fruit	Artichoke	Blackberry	Guelder–rose	Walnut	Pomegranate	Red grape	Apple	Hawthorn
Alcohols												
1-(4-Methoxyphenyl)ethanol	–	0.27	–	–	–	–	–	–	–	–	–	–
2-Isopropoxyethanol	–	0.17	–	–	–	–	–	–	–	–	–	–
2,3-Butanediol	–	–	–	1.83	–	–	–	–	–	–	–	–
Heptane-1,7-diol	–	0.18	–	–	–	–	–	–	–	–	–	–
1,3-Butylene glycol	–	0.49	–	–	–	–	–	–	–	–	–	–
2-Ethyl hexanol	–	0.22	–	–	–	–	–	–	–	–	–	0.18
Nonan-2-ol	–	0.33	–	–	–	–	–	–	–	–	–	–
Isomenthol	–	0.51	–	–	–	–	–	–	–	–	–	–
2-(4-Methylphenyl)-2-propanol	–	–	–	–	–	–	–	–	–	–	–	–
Esters												
Ethyl butanoate	0.20	–	–	–	–	0.31	–	–	0.37	0.10	–	0.73
Ethyl 2-methylbutyrate	–	–	–	0.79	0.49	–	–	–	–	–	–	–
Propyl butyrate	–	–	–	0.24	–	–	–	–	–	–	–	–
Methyl 5-hydroxypentanoate	–	0.22	–	–	–	–	–	–	–	–	–	–
Hexyl formate	–	–	–	0.50	–	–	–	–	–	–	–	–
2-Methylpropyl 2-hydroxypropanoate	0.40	–	–	–	–	–	–	–	–	–	–	–
Butyl propionate	0.23	–	–	–	–	–	–	0.16	–	–	–	–
Pentyl propionate	–	–	–	0.35	–	–	0.30	–	–	–	–	–
Ethyl octanoate	–	–	–	–	0.59	0.67	–	0.23	–	–	–	–
Ocimenyl acetate	–	–	–	0.22	–	–	–	–	–	–	–	–
Amylpropionate	–	–	–	–	–	–	–	–	–	–	0.18	0.19
Propyl propionate	–	–	–	8.06	0.59	–	–	–	–	–	0.13	2.40
sec-Butyl propionate	–	–	–	–	–	–	–	–	–	–	–	–
Diethyl carbonate	–	–	–	–	0.150	–	–	–	–	–	–	0.87
Ethyl isovalerate	0.29	0.71	0.31	2.86	0.67	0.85	0.25	0.16	0.58	0.66	–	0.51
sec-Butyl acetate	0.49	–	–	–	–	–	–	–	–	–	–	–
Methyl 3-hydroxyhexanoat	0.13	–	–	–	–	–	–	–	–	–	–	–
Ethyl hexanoate	0.22	–	0.91	1.31	1.36	0.78	–	0.41		0.19	0.39	0.73

TABLE 4. Continued

Volatiles	Rosehip	Fig	Lemon	Jujube fruit	Artichoke	Blackberry	Guelder–rose	Walnut	Pomegranate	Red grape	Apple	Hawthorn
Esters												
Ethyl butyrate	–	0.15		4.99	1.73	–	–	0.63		0.57	–	–
3-Hydroxypropyl prop-2-enoate	0.28	–	–	–	–	–	–	–	–	–	–	–
Isopropyl butanoate	–	–	–	–	–	–	–	–	–	–	–	0.17
Propyl valerate	–	–	–	0.20	–	–	–	–	–	–	–	–
Isobutyl lactate	–	–	–	0.46	–	–	–	–	–	–	–	–
Ethyl lactate	–	1.47	2.19		3.17	0.97	–	–	0.55	0.22	–	4.18
Ethyl heptanoate	–	0.27	–	0.11	0.15	–	–	–	–	–	–	–
Methyl 2-methoxy-3-methyl-2-butenoate	–	0.18	–	–	–	–	–	–	–	–	–	–
Bornylester	–	0.51	–	0.21	0.10	–	–	–	–	0.22	–	–
Isopropyl laurate	–	0.24	–	–	–	–	–	–	–	–	–	–
8-Bromo-3-oxo-octanoic acid methyl ester	–	0.18	–	–	–	–	–	–	–	–	–	–
Others												
2,4,5-Trimethyl-1,3-dioxolane	0.16	3.73	–	0.34	–	0.28	0.50	0.16	–	–	–	–
Cyclohexasiloxane	2.54	–	–	0.27	0.15	–	–	–	–	–	–	–
Vitispirane	0.77	–	0.16	–	0.25	0.33	0.33	–	–	0.50	–	0.88
Methyl propyl ether	–	–	–	14.6	–	–	–	–	–	–	–	–
1,1,2-Trimethylcyclohexane	0.30	–	–	–	–	–	–	–	–	–	–	–
1-Nonadecene	0.25	–	–	–	–	–	–	–	–	–	–	–
1,4-Cineole	–	–	0.77	–	–	–	–	–	–	–	–	–
2-Carene	–	–	0.24	–	–	–	–	–	–	–	–	–
2-Hydroxyheptane	–	–	–	–	–	–	–	–	0.33	–	–	–
5-Eicosene	0.23	–	–	–	–	–	–	–	–	–	–	–
6-(Methylthio)purine	–	0.27	–	–	–	–	–	–	–	–	–	–
Phenyl-2-nitropropene	0.31	–	–	–	–	–	–	–	–	–	–	–
Limonene	–	–	0.17	–	–	–	–	–	–	–	–	–
Linaloyl oxide	–	–	0.46	–	–	–	–	–	–	–	–	–
Nonylbenzene	0.35	–	–	–	–	–	–	–	–	–	–	–
2,2-Dimethyldecane	–	–	–	–	–	–	–	–	–	–	–	0.14

TABLE 4. Continued

Volatiles	Rosehip	Fig	Lemon	Jujube fruit	Artichoke	Blackberry	Guelder-rose	Walnut	Pomegranate	Red grape	Apple	Hawthorn
Others												
Methyl pentyl disulfide	–	0.21	–	–	–	–	–	–	–	–	–	–
1-Heptadecene	0.94	–	–	–	–	–	–	–	–	–	–	–
1-Chloropentane	–	6.98	–	–	–	–	–	–	–	–	–	–
[3-(2-Cyclohexylethyl)-6-cyclopentylhexyl]benzene	0.48	–	–	–	–	–	–	–	–	–	–	–
6-Phenylhexylamine	–	0.23	–	–	–	–	–	–	–	–	–	–
1-Nonadecene	0.27	–	–	–	–	–	–	–	–	–	–	–
2,2,4,6,6-Pentamethylheptane	–	0.27	–	–	–	–	–	–	–	–	–	–
2-Methyl-1,3-dioxolane	1.49	3.32	–	–	0.22	0.55	0.32	0.33	–	–	–	–

TABLE 5. Induction period (IP) and rate constant (*k*) of oxidation of the mayonnaises prepared with different kinds of vinegar.

	IP (min)	C_0 (bar)	*k*	R^2
Rosehip	356 ± 5.5^{abc}	6.40	1.89^{c}	>0.99
Fig	359 ± 2.0^{ab}	6.55	1.80^{d}	>0.99
Lemon	318 ± 2.5^{e}	6.81	2.11^{a}	>0.99
Jujube fruit	331 ± 4.0^{cde}	6.45	2.08^{a}	>0.99
Artichoke	325 ± 3.0^{de}	6.54	2.07^{a}	>0.99
Blackberry	355 ± 4.5^{bc}	6.34	1.90^{c}	>0.97
Guelder-rose	382 ± 7.0^{a}	6.69	1.91^{c}	>0.99
Walnut	358 ± 7.0^{abc}	6.68	1.80^{d}	>0.99
Pomegranate	363 ± 6.5^{ab}	6.70	1.76^{d}	>0.99
Red grape	340 ± 17.0^{bcde}	6.66	2.12^{a}	>0.99
Apple	351 ± 7.5^{bcd}	6.65	2.01^{b}	>0.98
Hawthorn	357 ± 21.5^{abc}	6.33	1.78^{d}	>0.99

Data are means ± standard deviations of triplicate determinations (n=3). R^2 is the coefficient of determination and C_0 is the inital presure in the sample vessel. The comparison is between values in rows, means with the same letter are not significantly different (p>0.05).

antioxidants presence, interfacial characteristics, and droplet characteristics affect the oxidative stability of the mayonnaise-type emulsions [Paraskevopoulou *et al.*, 2007]. In our study, the induction period (IP) and oxidation rate constant (*k*) were used in evaluating the oxidative stability of emulsions. IP value ranged from 318 to 382 min and differed significantly among vinegar types (p<0.05). The samples formulated with guelder-rose and lemon vinegars showed the highest and the lowest IP value, respectively. The high positive correlation (0.760) was observed between DPPH$^{\bullet}$ scavenging abilities of vinegar and IP value, meaning that the samples prepared with vinegar with high radical scavenging ability showed more resistance to the oxidation. Oxidation data, namely time *versus* pressure, were set to the first-order kinetic model to determine the oxidation rate of mayonnaise samples at 90°C and the effect of vinegar type on oxidation rate. The *k* values were used to compare the oxidation rate of the samples. They differed significantly among vinegar types and ranged from 1.78 to 2.12 (Table 5). The samples with high IP values showed lower *k* values. The higher *k* values were determined for the samples prepared with lemon, jujube fruit, red grape, and artichoke vinegars. Therefore, both oxidation rate and shelf life were closely related to vinegar types, and the oxidative stability of the mayonnaise type emulsions could be improved by the selection of vinegar showing high radical scavenging abilities.

CONCLUSION

In this study, different types of vinegars manufactured with the traditional method were characterized regarding their physicochemical properties, total phenolic and total flavonoid contents, antioxidant activity, individual phenolic content, and volatile composition. The capabilities of vinegars to delay lipid oxidation in mayonnaise samples, and its correlation with antioxidant activity values were also evaluated. In terms of the analyzed properties, blackberry, guelder-rose, and walnut vinegars can be recommended over the other vinegars. Due to their antioxidant properties, vinegar types should be accounted for an important factor in the production of mayonnaise to improve its oxidative stability. PCA and HCA presented similarities and differences among the vinegars based on the variables studied. The vinegars produced from different raw materials could be easily differentiated according to antioxidant activities, individual phenolics, and volatile compounds. This study suggests that fruit type should be considered as a crucial factor in the production of vinegars affecting not only sensory properties but also their physicochemical and bioactive properties.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID IDs

A. Karadag https://orcid.org/0000-0001-8615-7321
F. Bozkurt https://orcid.org/0000-0003-4905-095X
H. Bekiroglu https://orcid.org/0000-0003-3328-1550
O. Sagdic https://orcid.org/0000-0002-2063-1462

REFERENCES

1. Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC Method. *Journal of Agricultural and Food Chemistry*, *52*(26), 7970–7981. https://doi.org/10.1021/jf048741x
2. Baena-Ruano, S., Santos-Dueñas, I.M., Mauricio, J.C., García-García, I. (2010). Relationship between changes in the total concentration of acetic acid bacteria and major volatile compounds during the acetic acid fermentation of white wine. *Journal of the Science of Food and Agriculture*, *90*(15), 2675–2681. https://doi.org/10.1002/jsfa.4139
3. Bakir, S., Devecioglu, D., Kayacan, S., Toydemir, G., Karbancioglu-Guler, F., Capanoglu, E. (2017). Investigating the antioxidant and antimicrobial activities of different vinegars. *European Food Research and Technology*, *243*(12), 2083–2094. https://doi.org/10.1007/s00217-017-2908-0
4. Benzie, I.F.F., Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry*, *239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292
5. Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT – Food Science and Technology*, *28*(1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5
6. Budak, N.H., Aykin, E., Seydim, A.C., Greene, A.K., Guzel-Seydim, Z.B. (2014). Functional properties of vinegar. *Journal of Food Science*, *79*(5), R757–R764. https://doi.org/10.1111/1750-3841.12434
7. Callejón, R.M., González, A.G., Troncoso, A.M., Morales, M.L. (2008). Optimization and validation of headspace sorptive extraction for the analysis of volatile compounds in wine vinegars. *Journal of Chromatography A*, *1204*(1), 93–103. https://doi.org/10.1016/j.chroma.2008.07.064
8. Chen, H., Chen, T., Giudici, P., Chen, F. (2016). Vinegar functions on health: Constituents, sources, and formation mechanisms. *Comprehensive Reviews in Food Science and Food Safety*, *15*(6), 1124–1138. https://doi.org/10.1111/1541-4337.12228
9. Chou, C.-H., Liu, C.-W., Yang, D.-J., Wu, Y.-H. S., Chen, Y.-C. (2015). Amino acid, mineral, and polyphenolic profiles of black vinegar, and its lipid lowering and antioxidant effects *in vivo*. *Food Chemistry*, *168*, 63–69. https://doi.org/10.1016/j.foodchem.2014.07.035
10. De Leonardis, A., Macciola, V., Iorizzo, M., Lombardi, S.J., Lopez, F., Marconi, E. (2018). Effective assay for olive vinegar production from olive oil mill wastewaters. *Food Chemistry*, *240*, 437–440. https://doi.org/10.1016/j.foodchem.2017.07.159
11. Ho, C.W., Lazim, A.M., Fazry, S., Zaki, U.K.H.H., Lim, S.J. (2017). Varieties, production, composition and health benefits of vinegars: A review. *Food Chemistry*, *221*, 1621–1630. https://doi.org/10.1016/j.foodchem.2016.10.128
12. Horiuchi, J.-I., Kanno, T., Kobayashi, M. (1999). New vinegar production from onions. *Journal of Bioscience and Bioengineering*, *88*(1), 107–109. https://doi.org/10.1016/S1389-1723(99)80186-8
13. Kelebek, H., Kadiroğlu, P., Demircan, N.B., Selli, S. (2017). Screening of bioactive components in grape and apple vinegars: Antioxidant and antimicrobial potential. *Journal of the Institute of Brewing*, *123*(3), 407–416. https://doi.org/10.1002/jib.432
14. Lee, J.-H., Cho, H.-D., Jeong, J.-H., Lee, M.-K., Jeong, Y.-K., Shim, K.-H., Seo, K.-I. (2013). New vinegar produced by tomato suppresses adipocyte differentiation and fat accumulation in 3T3-L1 cells and obese rat model. *Food Chemistry*, *141*(3), 3241–3249. https://doi.org/10.1016/j.foodchem.2013.05.126
15. Leonés, A., Durán-Guerrero, E., Carbú, M., Cantoral, J. M., Barroso, C.G., Castro, R. (2019). Development of vinegar obtained from lemon juice: Optimization and chemical characterization of the process. *LWT – Food Science and Technology*, *100*(June 2018), 314–321. https://doi.org/10.1016/j.lwt.2018.10.096
16. Lopez-Huertas, E., Fonolla, J. (2017). Hydroxytyrosol supplementation increases vitamin C levels *in vivo*. A human volunteer trial. *Redox Biology*, *11*, 384–389. https://doi.org/10.1016/j.redox.2016.12.014
17. Mohamad, N.E., Keong Yeap, S., Beh, B.K., Romli, M.F., Yusof, H.M., Kristeen-Teo, Y. W., Sharifuddin, S.A., Long, K., Alitheen, N.B. (2018). Comparison of *in vivo* toxicity, antioxidant and immunomodulatory activities of coconut, nipah and pineapple juice vinegars. *Journal of the Science of Food and Agriculture*, *98*(2), 534–540. https://doi.org/10.1002/jsfa.8491
18. Nakamura, K., Ogasawara, Y., Endou, K., Fujimori, S., Koyama, M., Akano, H. (2010). Phenolic compounds responsible for the superoxide dismutase-like activity in high-brix apple vinegar. *Journal of Agricultural and Food Chemistry*, *58*(18), 10124–10132. https://doi.org/10.1021/jf100054n

19. Ozturk, I., Caliskan, O., Tornuk, F., Ozcan, N., Yalcin, H., Baslar, M., Sagdic, O. (2015). Antioxidant, antimicrobial, mineral, volatile, physicochemical and microbiological characteristics of traditional home-made Turkish vinegars. *LWT – Food Science and Technology*, *63*(1), 144–151.
https://doi.org/10.1016/j.lwt.2015.03.003
20. Paraskevopoulou, D., Boskou, D., Paraskevopoulou, A. (2007). Oxidative stability of olive oil–lemon juice salad dressings stabilized with polysaccharides. *Food Chemistry*, *101*(3), 1197–1204.
https://doi.org/10.1016/j.foodchem.2006.03.022
21. Samad, A., Azlan, A., Ismail, A. (2016). Therapeutic effects of vinegar: a review. *Current Opinion in Food Science*, *8*, 56–61.
https://doi.org/10.1016/j.cofs.2016.03.001
22. Shimoji, Y., Tamura, Y., Nakamura, Y., Nanda, K., Nishidai, S., Nishikawa, Y., Ishihara, N., Uenakai, K., Ohigashi, H. (2002). Isolation and identification of DPPH radical scavenging compounds in Kurosu (Japanese unpolished rice vinegar). *Journal of Agricultural and Food Chemistry*, *50*(22), 6501–6503.
https://doi.org/10.1021/jf020458f
23. Singleton, V.L., Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(3), 144 LP–158.
http://www.ajevonline.org/content/16/3/144.abstract
24. Ubeda, C., Callejón, R.M., Hidalgo, C., Torija, M.J., Troncoso, A.M., Morales, M.L. (2013). Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins. *LWT – Food Science and Technology*, *52*(2), 139–145.
https://doi.org/10.1016/j.lwt.2012.04.021
25. Verzelloni, E., Tagliazucchi, D., Conte, A. (2007). Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar. *Food Chemistry*, *105*, 564–571.
https://doi.org/10.1016/j.foodchem.2007.04.014
26. Xie, X., Zheng, Y., Liu, X., Cheng, C., Zhang, X., Xia, T., Yu, S., Wang, M. (2017). Antioxidant activity of chinese shanxi aged vinegar and its correlation with polyphenols and flavonoids during the brewing process. *Journal of Food Science*, *82*(10), 2479–2486.
https://doi.org/10.1111/1750-3841.13914
27. Yang, L., Liu, J., Wang, X., Wang, R., Ren, F., Zhang, Q., Shan, Y., Ding, S. (2019). Characterization of volatile component changes in jujube fruits during cold storage by using Headspace-Gas Chromatography-Ion Mobility spectrometry. *Molecules* (*Basel, Switzerland*), *24*(21), 3904.
https://doi.org/10.3390/molecules24213904
28. Yu, X., Yang, M., Dong, J., Shen, R. (2018). Comparative analysis of the antioxidant vapacities and phenolic compounds of oat and buckwheat vinegars during production processes. *Journal of Food Science*, *83*(3), 844–853.
https://doi.org/10.1111/1750-3841.14074
29. Yun, J.-H., Kim, Y.-J., Koh, K.-H. (2016). Investigation into factors influencing antioxidant capacity of vinegars. *Applied Biological Chemistry*, *59*(4), 495–509.
https://doi.org/10.1007/s13765-016-0185-4
30. Zhishen, J., Mengcheng, T., Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, *64*(4), 555–559.
https://doi.org/10.1016/S0308-8146(98)00102-2

Submitted: 26 May 2020. Revised: 16 August and 1 September 2020. Accepted: 9 September 2020. Published on-line: 15 October 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 361–375
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/128132
http://journal.pan.olsztyn.pl

Original research article
Nutritional Research Section

Effects of Four-Week Intake of Blackthorn Flower Extract on Mice Tissue Antioxidant Status and Phenolic Content

Vedran Balta[1], *Domagoj Đikić*[1]*, *Irena Crnić*[2], *Dyana Odeh*[1], *Nada Oršolić*[1], *Ivana Kmetič*[2], *Teuta Murati*[2], *Verica Dragović Uzelac*[2], *Irena Landeka Jurčević*[2]

[1]*Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia*
[2]*Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia*

Key words: pharmacokinetic, flavan-3-ol, flavonoids, bioavailability, phenolic compounds, *Prunus spinosa* L.

The study examined the antioxidative physiological effects of phenolics from an ethanol-water extract of blackthorn flowers orally administrated to C57/BL6 mice for 28 days in daily doses of 25 mg of total phenolics/kg body weight. Contents of phenolics in the intestine, liver, and kidneys collected after 1, 7, 14, 21, and 28 days of extract administration were analyzed by UPLC-MS/MS method. In the same tissues, the antioxidative properties were determined as ferric reducing antioxidant power (FRAP), $ABTS^{\bullet +}$ scavenging activity, content of reduced glutathione (GSH), and activity of superoxide dismutase (SOD) and catalase (CAT). The lipid peroxidation in tissues was also evaluated by thiobarbituric acid reactive substances (TBARS) assay. The exposed mice (compared to the control ones) had a lower content of TBARS in all tissues mostly on the third/fourth week of daily consumption. SOD activity and GSH content increased on the 28th day in tissues. CAT activity was higher only in the liver after one week of consumption but remained unchanged in other organs throughout the experiment. Phenolic profiles were different in individual tissues. The most prominent increases compared to the control were determined for contents of 3-*O*-feruloylquinic acid, 4-*O*-*p*-coumaroylqiunic acid, kaempferol pentoside, and quercetin rhamnoside in the intestine; for ferulic acid and quercetin 3-*O*-rutinoside in the liver; and for quercetin 3-*O*-rutinoside, ferulic acid, and 4-*O*-*p*-coumaroylquinic acid in the kidneys. The screened phenolics with different distribution in tissues could be responsible for slight differences in the recorded antioxidative effects.

INTRODUCTION

Blackthorn (*Prunus spinosa* L.) is a perennial shrub belonging to the rose family (Rosaceae), growing throughout Europe, western Asia, and northwest Africa [Elez-Garofulić *et al.*, 2018]. Recently, blackthorn has become interesting both industrially as a food plant and pharmacologically as a nutraceutical or a medicinal plant because it is a rich source of phenolic compounds [Meschini *et al.*, 2017; Mikulic-Petkovsek *et al.*, 2016; Pinacho *et al.*, 2015; Yuksel, 2015]. The fruits of blackthorn, known also as the "sloe", are small round with black skins covered in a blue waxy bloom and extremely acid-tasting, and must be thermally processed prior to consumption. They are used for jams, liqueurs, wines, juices, compote, and tea production. Blackthorn flowers, bark, and root have been traditionally used in folk medicine for diuretic and laxative properties, due to their abilities to remove excess sodium ions and harmful products of metabolism, to reduce blood vessels permeability, and against inflammation of the urinary tract [Elez-Garofulić *et al.*, 2018]. In western and northern Europe and Mediterranean countries, fruits were traditionally consumed but consumption declined in the 20th century [Alarcón *et al.*, 2015; Menendez-Baceta *et al.*, 2012]. There is archaeological evidence that fruits were consumed in distant past as well. In the book on palaeoethnobotany of the prehistoric food plants of the Near East and Europe, the blackthorn seeds are mentioned as leftover of fruits that have been found and recovered in a number of European prehistoric sites from the neolithic and iron age, sometimes in large quantities (in barrels) [Renfrew, 1973].

Mikulic-Petkovsek *et al.* [2016] compared the phenolic and other secondary metabolite contents of various *Prunus* spp. wild fruits and concluded that the blackthorn (*P. spinosa*) showed richness of various plant phenolics making it a good candidate species among the genus *Prunus* spp. for the studies of phenolic biological activity upon consumption. Although berries are traditionally consumed, other plant parts such as flowers were studied for the content of phenolics as well. For example, a study of various morphological parts of blueberries (*Vaccinium angustifolium* L.) and lingonberries (*Vaccinium vitis-idaea* L.) has found that the leaves have a much higher phenolic content and antioxidant capacity than fruits [Kelly *et al.*, 2017]. Olszewska and her co-authors were among the first who recorded the abundance of phenolics in *P. spinosa* leaf and also in the flower extract [Olszewska *et al.,* 2001; Olszewska & Wolbiś, 2001, 2002]. Marchelak *et al.* [2017] reported that blackthorn flower extract contained

---

* Corresponding Author: Tel.: 00 385 91 5898159;
E-mail: magistar_djikic1@yahoo.com (D. Đikić)

total phenolics up to 584.07 mg/g of dry weight (dw). Likewise, similar studies in *P. spinosa* flowers were done by Elez-Garofulić *et al.* [2018] and Lovrić *et al.* [2017], confirming the richness of phenolics and the antioxidative properties of the flower extract *in vitro*. However, although this plant phenolic extracts are recently scientifically examined, those experiments describing bioactivity (antioxidative properties and similar physiological modulations) were done mostly under *in vitro* conditions. *In vitro* studies showed that the phenolics from blackthorn extract were potent antioxidants that scavenged DPPH$^{\bullet}$, reduced ferric ions, and inhibited linoleic acid peroxidation and pro-inflammatory enzymes (lipoxygenase and hyaluronidase) activity, as well as effectively protected the isolated human plasma (outside the body, *in vitro*) against peroxynitrite-induced damage by reducing levels of oxidative stress biomarkers: 3-nitrotyrosine, lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) [Marchelak *et al.*, 2017]. Authors proposed the hypothesis concluded from *in vitro* results that the blackthorn extract might enhance the total antioxidant bioactivity if administrated within the organism. There are also *in vitro* antitumor assays as well as a study conducted by our group where the flower extract showed promising antitumor results [Murati *et al.,* 2019].

Based on the described *in vitro* evidence and lack of *in vivo* data, we have previously also conducted the preliminary *in vivo* study by applying only a single acute dose of the blackthorn flower extract in mice to establish whether the phenolics can be absorbed *via* the gastrointestinal system. A significant concentration of phenolics was found in plasma of animals after 24 h, indicating their good gut absorption [Đikić *et al.,* 2018]. Based on those results of blood pharmacokinetic study [Đikić *et al.,* 2018], indicating that the number of phenolics are being absorbed, we set foundations for this 28-day consumption study and evaluation of the antioxidative activity in the organs (intestine, liver, kidney). We wanted to establish, whether mice tissue antioxidative defense markers change due to the subchronic (28 day) intake of blackthorn flower extract.

MATERIALS AND METHODS

Chemicals and standards

HPLC grade formic acid and acetonitrile were purchased from BDH Prolabo, VWR (Lutterworth, England). The following commercial phenolic compound standards: quercetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, caffeic acid, gallic acid, ferulic acid, chlorogenic acid, and *p*-coumaric acid were purchased from Sigma–Aldrich (Steinheim, Germany). The (+)-catechin, (-)-epicatechin, (-)-epicatechin 3-gallate, (-)-epigallocatechin 3-gallate, apigenin, and luteolin were purchased from Extrasynthese (Genay, France) and quercetin 3-*O*-rutinoside from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Horse heart cytochrome C (type VI), human blood superoxide dismutase (SOD; type I, lyophilized powder, 2400 U/mg protein), xanthine and xanthine oxidase (200 U/mL), Ellman reagent (5,5'-dithiobis-(2-nitrobenzoic acid); DTNB), NADPH, glutathione reductase and hydrogen peroxide (30%) 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,4,6-tripyridil-s-triazine (TPTZ) were all purchased from Sigma-Aldrich. Deionized water of Milli-Q quality (Millipore Corp., Bedford, USA) was used throughout the experiment.

Preparation and analysis of blackthorn flower extract

The samples of dry blackthorn flowers were bought from Suban Ltd. company (Samobor, Croatia), a certified collector and producer of medicinal plants, and were part of the batch number 63451. The preparation of the blackthorn flower extract (ethanol/water extraction with microwave assistance) was described in detail by Elez-Garofulić *et al.* [2018] and Lovrić *et al.* [2017]. We have utilized the described methods because they yielded the best extraction of phenolics and proved 50% (*v*/*v*) ethanol/water used as a solvent to be safe for mammalian (mice) consumption in comparison to the other organic solvents which might remain as residues. For the *in vivo* experiment, the total phenolic (TP) content was analyzed in the original ethanol/water extract. The TP content of blackthorn flower extracts was determined using the method with the Folin-Ciocalteu reagent previously reported by Lovrić *et al.* [2017]. A volume of 100 μL of the extract (5-fold diluted) was mixed with 200 μL of the Folin–Ciocalteu reagent and 2 mL of distilled water, and after 3 min, 1 mL of 20% Na_2CO_3 was added. This mixture was incubated at 50°C for 25 min. The absorbance was measured at 765 nm using a spectrophotometer (model UV-1600PC; VWR International, Leuven, Belgium). The blank contained 100 μL of the solvent used for extraction instead of the extract. The TP content was calculated according to the gallic acid standard calibration curve, and expressed in mg of gallic acid equivalents (GAE) per mL of extract. The TP content of original extract was 0.5 mg GAE/mL. The extract was further evaporated under reduced pressure at 45°C to remove ethanol and concentrate the solution of polyphenolics. Before application to the mice, the concentrated solution was re-dissolved and further diluted with water to achieve the final applied solution with removed alcohol suitable for use in a dose of 25 mg TP GAE/kg body weight of mice (this water-based working solution for mouse doses, was applied in the volume of 0.2 mL per mouse weighing on average 30 grams). The phenolic composition of blackthorn flower extract and details of the UPLC-MS/MS method used for its determination were shown in our previous study [Đikić *et al.,* 2018; Elez-Garofulić *et al.,* 2018]. It was reported that the highest concentrations were detected for 3-*O*-caffeoylquinic, 3-*O*-*p*-coumaroylquinic, and 3-*O*-feruloylquinic acid among phenolic acids; then for (+)-catechin and (-)-epicatechin among flavan-3-ols; and finally for kaempferol glycosides (kaempferol pentoside and kaempferol rhamnoside) and quercetin glycosides (quercetin 3-*O*-rutinoside, quercetin pentoside, and quercetin rhamnoside) among flavonols.

Experimental animals and husbandry, study design *in vivo*

For this experiment, a total of 50 male inbred C57BL/6 mice, weighing 30±1.5 g were obtained from the Department of Animal Physiology, Faculty of Science University of Zagreb, Croatia. Animals were fed a standard laboratory diet, tap water *ad libitum*, and received 12 h of light per day. The standardized diet was 4 RF 21, Mucedola (Settimo Milanese, Italy). The composition of standardized pellet mouse feed included wheat, wheat straw, hazelnut skins, maize, soy bean hulled, corn gluten feed, fishmeal, dicalcium phosphate, sodium chloride, whey powder, soybean oil, yeast; and contained 12% moisture, 18.5% protein, 3% fats, 6% crude fibers, 7% crude ash, E672 (vitamin A), E671 (vitamin E), E1 (Fe), E2 (I), E3 (Co), E4 (Cu), E5 (Mn), and E6 (Zn). Phenolic content of Mucedola feed pellets was analyzed in our previous study and results are given in Đikić *et al.* [2018]. The analysis revealed that all phenolic compounds that were detected in Mucedola standard mouse feed were in substantially lower contents than in the blackthorn flower extract [Đikić *et al.,* 2018].

Bioethical standards in animal husbandry and experimental protocol

Maintenance and care of all experimental animals was performed according to the guidelines applicable in the Republic of Croatia [NN 55/2013]. The experimental procedures were approved by the Bioethics Committee of the Faculty of Science, University of Zagreb [Bioethic approval, 2014] and were conducted according to the Guidelines on *in vivo* experiments and accepted and international standards on the Guide for the care and use of laboratory animals [2011].

Treatment groups and doses

Animals were randomly divided according to treatment into two separate groups, namely the control group and the blackthorn flower extract group. Within those two groups, the subgroups were formed based on the time of sacrifice post treatment. Animals from both control or blackthorn flower extract groups were sacrificed on the day 1, 7, 14, 21, and 28 post treatment (the subgroups). Each subgroup contained 5 animals. Saline for the control and blackthorn flower extract for the exposed group were administered daily as a single oral dose for the period of 28 days by gavage in a volume of 0.2 mL per animal. Treatment of all animals took place between 8–10 a.m. in order to equalize circadian differences between treatments and avoid differences in metabolism. The blackthorn flower extract-treated groups were dosed 25 mg of TP of blackthorn extract per kg of body weight of mice (mg TP/kg bw). The dose was derived from pilot experiments.

Tissue preparation

At designated experimental days for organ collection, animals were anesthetized by halothane and perfused through with 10 mL of phosphor buffer saline (PBS) and sacrificed by cervical dislocation, 24 h after the last administered dose on the particular day of experiment. Intestine, liver, and kidneys were extracted. Such tissue samples were used for antioxidative activity assays and for the determination of individually bioaccumulated phenolics by the UPLC MS/MS method. Prior to the measurement of antioxidative parameters and UPLC-MS/MS analysis, the tissue samples were placed in 50 mM phosphate buffer (pH=7.4) and homogenized (10% of homogenate, by tissue mass per volume of PBS) with an ultrasonic homogenizer (SONOPLUS Bandelin HD2070, Bandelin Electronic GmbH & Co KG, Germany) using an MS73 probe (Bandelin, Electronic GmbH & Co KG Germany). Thereafter, homogenates were sonicated on ice for 30 s in three 10-s intervals, centrifuged at 20,000×g for 15 min at 4°C, and immediately frozen at -80°C until analysis. Further details of supernatant treatment for antioxidative activity determination or UPLC-MS/MS analysis are described in each section separately.

Antioxidant status of tissues

Tissue supernatant samples that were stored until analysis as described in the above section were slowly thawed at +6°C on cooling pads until liquid again. Afterwards, they were centrifuged at 20,000×g for 15 min at 4°C. The supernatants of the centrifuged tissue homogenates were further used for analysis of ferric reducing antioxidant power (FRAP), $ABTS^{\bullet+}$ scavenging activity, TBARS content, CAT and SOD activity, and GSH content following protocols described below.

Determination of the ferric reducing antioxidant power of tissues

The FRAP assay for tissue homogenates was conducted according to Katalinic *et al.* [2005] method adopted for animal organs and modified from the assay by Benzie & Strain [1996]. The FRAP reagent was prepared from 5 mL of a TPTZ solution (10 mM) in HCl (40 mM) and 5 mL of an $FeCl_3$ solution (20 mM) mixed with 50 mL of an acetate buffer (0.3 M, pH=3.6). Such freshly prepared FRAP reagent (1.5 mL) was mixed with 200 μL of water and 50 μL of the tissue sample or as a blank standard sample with 50 μL water, and incubated for 4 min at room temperature. After 4 min of incubation, the absorbance was measured at λ=595 nm with a Libro S22 spectrophotometer (Biochrom Ltd. Cambridge, UK). The results of the ferric reducing ability of the tissue homogenate were calculated according to the standard curve and expressed as nmol Fe^{2+} per mg of protein in a tissue homogenate.

Determination of the antioxidant capacity of tissues by ABTS assay

The ABTS assay for tissue homogenates was conducted according to Katalinic *et al.* [2005] method adopted for animal organs and modified from assay by Re *et al.* [1999]. The volume of 20 μL of the tissue supernatant was mixed with 2 mL of an $ABTS^{\bullet+}$ solution and after 6 min of incubation, the absorbance was measured at a wavelength of 734 nm with a Libro S22 spectrophotometer (Biochrom). The $ABTS^{\bullet+}$ solution was prepared by oxidizing the 7 mM $ABTS^{\bullet+}$ solution with a freshly prepared 140 mM potassium peroxydisulfate solution mixed in equal proportions. On the day of analysis, the solution was diluted with PBS (pH 7.4) and incubated at 30°C so that the absorbance of this solution was 0.700 (±0.020). As a blank sample-free mix without tissue sample, a 1 mL of $ABTS^{\bullet+}$ solution and 20 μL phosphate buffer was used and the mix of $ABTS^{\bullet+}$ solution with tissue sample was compared to it. The results are expressed as nmol Trolox equivalents per mg of protein in the tissue homogenate.

Markers of tissue oxidative stress defense systems

Protein concentration in tissues

Protein concentration in the samples was determined with the method of Lowry *et al.* [1951], with bovine serum albumin (BSA) used as the standard. Protein concentration in tissue samples was used to express the values of the measured oxidative stress parameters (TBARS, FRAP, $ABTS^{\bullet+}$, SOD, and CAT).

Lipid peroxidation in tissues

Lipid peroxidation was determined by measuring the content of TBARS using a modified method of Ohkawa *et al.* [1979] described in Landeka Jurčević *et al.* [2017]. A centrifuged sample of 200 μL of the homogenized tissue was mixed with 200 μL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid (pH=3.5), and 1.5 mL of 0.81% thiobarbituric acid, and incubated at 95°C for 60 min. After cooling on ice, the absorbance was measured at 532 and 600 nm with a Libro S22 spectrophotometer (Biochrom). The total absorbance was determined using the formula:

$$A_{\text{total}} = A_{532\text{ nm}} - A_{600\text{ nm}} \qquad (1)$$

TBARS levels were determined using the molar absorption coefficient for malondialdehyde-thiobarbiturate (MDA–TBA) complex of 1.56×10^5 $M^{-1}cm^{-1}$ and expressed as nmol/mg protein of tissues homogenate.

Superoxide dismutase activity in tissues

The SOD assay is a modification of the method by Flohé & Ötting [1984] described in Landeka Jurčević *et al.* [2017]. An undiluted sample of the tissue homogenate (25 μL) was mixed with 1.45 mL of a reaction solution (cytochrome C, 0.05 mM; xanthin, 1 mM mixed in a 10:1 (*v*/v) ratio with DTNB). A volume of 20 μL of xanthine oxidase (0.4 U/mL) was added to start the reaction. The absorbance of the reaction mixture was measured at 550 nm over 3 min with a Libro S22 spectrophotometer (Biochrom). One unit of SOD activity was defined as the amount of enzyme required to achieve 50% inhibition of superoxide anion formation within the sample which was started by the reaction of xanthine oxidase. The results were expressed as units per mg of protein in tissue homogenate (U/mg protein).

Catalase activity in tissues

The CAT activity was assayed by measuring the initial rate of H_2O_2 degradation according the method modified from Aebi [1984] and described in Landeka Jurčević *et al.* [2017]. The reaction mixture was prepared by mixing 33 mM H_2O_2 in 50 mM phosphate buffer, pH=7.0. This reaction mixture (900 μL) was mixed with the supernatant of the tissue homogenate (100 μL). The absorbance was measured at 240 nm for 3 min using the Libro S22 spectrophotometer (Biochrom). The CAT activity was calculated using the molar absorption coefficient of 43.6 $M^{-1}cm^{-1}$ for H_2O_2. The results were expressed as U/mg protein.

Reduced glutathione in tissues

The reduced glutathione (GSH) assay is a modification of the method first described by Tietze [1969] and then in Landeka Jurčević *et al.* [2017]. In a 96-well plate, 40 μL of 10 mM DTNB (Ellman's Reagent) was mixed with 20 μL of the tissue supernatant (obtained as described above) pretreated with 40 μL of 0.035 M HCl, incubated for 10 min. Then, 100 μL of the reaction solution prepared earlier by mixing 9980 μL of 0.8 mM NADPH and 20 μL of glutathione reductase, 0.2 U/mL, was added and the absorbance was read at 412 nm every minute for 5 min in an ELISA plate reader (Biorad Laboratories, Hercules CA, USA). The GSH levels were determined from the calibration curve of GSH standards. The results are expressed as μM/mg proteins.

Determination of the phenolic content in tissues

The UPLC-MS/MS analysis of phenolics in the tissues of mice post-sacrifice

The qualitative and quantitative analysis of individual phenolic compounds in mice tissues was performed with the Ultra High Performance Liquid-Chromatography Tandem Mass Spectrometry (UPLC-MS/MS). The methods are described in detail (with respective standard curves) by Elez-Garofulić *et al.* [2018]. Briefly, the Agilent 1290 UPLC system (Agilent Technologies, Santa Clara, CA, USA) with the Zorbax Eclipse Plus C18 column (100×2.1 mm, 1.8 μm; Agilent, Santa Clara, CA, USA) was used. Column oven temperature was set at 35°C and flow rate was 0.35 mL/min. The mobile phase consisted of 0.1% (*v/v*) formic acid (A) and acetonitrile with 0.1% (*v/v*) formic acid (B). The QQQ 6430 triple quadrupole mass spectrometer and the Agilent MassHunter Workstation Software (Agilent, Santa Clara, CA, USA) were used for mass spectrometry. The positive and negative electrospray ionization (ESI) mode and dynamic multiple reaction monitoring (dMRM) mode were applied. Capillary voltage was +4000/-3500 V, nitrogen drying temperature was 300°C, flow rate was 11 L/h, and the pressure of the nebulizer was 40 psi. The total analytical time was 12.5 min. The analytes were identified by comparing their retention times and mass spectra with the corresponding standards. For unavailable standards, the structural identification of phenolic compounds was done by comparing the mass fragment ions with the previously reported mass fragmentation patterns; and quantification was performed using the calibration curve of standards from the same phenolic group. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively.

For the UPLC-MS/MS analysis, the frozen samples that were stored until analysis as described in the above section were slowly thawed at +6°C on cooling pads until liquid again. Afterwards, they were centrifuged at 20,000×g for 15 min at 4°C. The supernatants (200 μL of the supernatant of the tissue homogenate) was then mixed with 10 μL of a mixture of β-glucuronidase (250 units) and sulfatase (20 units), and then incubated at 37°C for 45 min. The reaction mixture was extracted twice with ethyl acetate, to remove tissue debris of the homogenate. The combined ethyl acetate solutions were added to 10 μL of a 20% ascorbic acid solution and evaporated to dryness in a vacuum centrifuge concentrator. Prior to the chromatographic analysis, the samples were reconstituted in 300 μL of a 10% aqueous acetonitrile solution (*v/v*) and centrifuged at 6500×g for 5 min. The supernatant was transferred to an injection vial and then an aliquot of 2.5 μL of the supernatant was injected onto the UPLC column [Ganguly *et al*., 2016; Gonzales *et al*., 2015].

The results of the UPLC-MS/MS analysis were presented as AUClast, and C_{max} values. These were obtained from the curves of the phenolic content in the tissues *versus* the time (T, days) of blackthorn flower extract administration to mice as the area under the curve and the maximum concentration, respectively. To compute the mean AUClast, $C_{max,}$ and T_{max} values, a non-compartmental pharmacokinetic analysis was done using Phoenix WinNonlin 8.0. software (Certara, Princeton, NJ, USA).

Statistical analysis

Data of TBARS, FRAP, and ABTS assays; tissue content of antioxidative defense molecules; and the AUClast and C_{max} values were presented as means and standard deviation. All data were compared for statistically significant differences ($p \leq 0.05$) by the t-test between the control group and the blackthorn flower extract-treated group within each individual tissue. The Kruskal-Wallis ANOVA was used for testing the statistically significant differences ($p \leq 0.05$) between days of treatment for antioxidative parameters. All statistical analyses were conducted in the SPSS version 17.0 software (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Lipid peroxidation, antioxidative properties, and the content of antioxidative defense molecules in the mice tissues after blackthorn flower extract administration

Compared to the control animals, the administration of blackthorn flower extract to mice significantly ($p \leq 0.05$) lowered the lipid peroxidation (content of TBARS) in tissues (Figure 1 A-C). The inhibited lipid peroxidation was observed in all three assessed organs (intestine, liver, and kidneys) after three weeks of administration. Since no *in vivo* results can be found in literature for blackthorn, the closest study resembling ours was that evidencing the similar lipid peroxidation reduction recorded with *Aronia melanocarpa* extract [Broncel *et al.,* 2010].

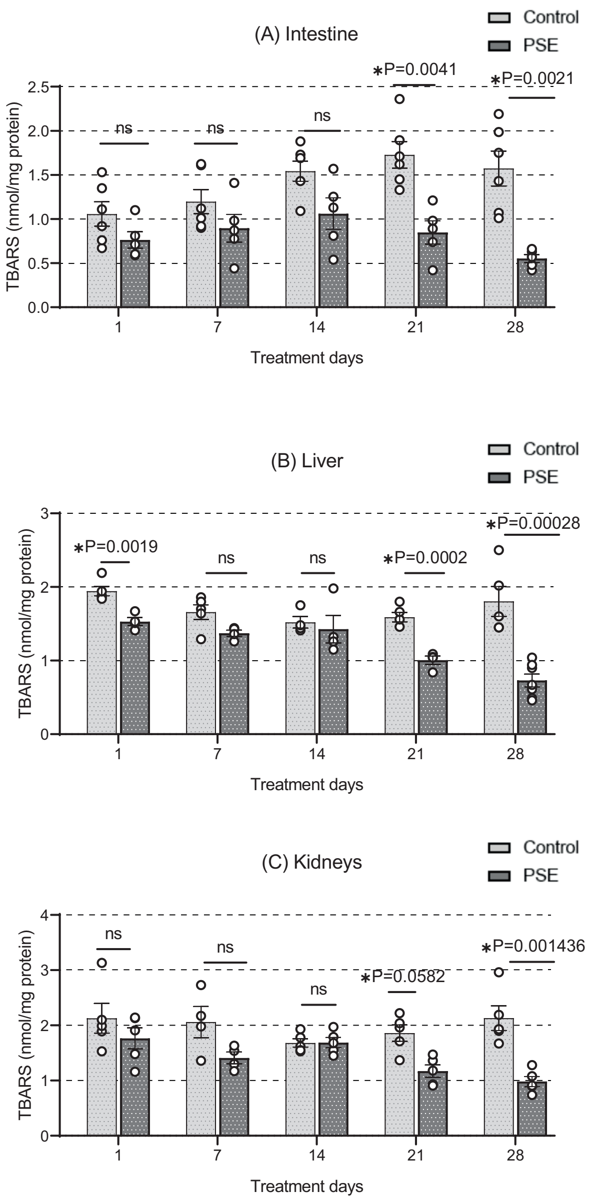


FIGURE 1. The lipid peroxidation expressed as the content of thiobarbituric acid reactive substances (TBARS) in the (A) intestine, (B) liver, and (C) kidneys; PSE – blackthorn flower extract-treated group. The values that are statistically different between control and PSE-treated group are marked with p value of t-test. ns – no statistically significant differences ($p > 0.05$).

The antioxidative capacity of the tissues (Figure 2 and 3) was measured as FRAP and $ABTS^{\bullet+}$ scavenging activity. Each method specifically showed slightly different mechanisms of tissue antioxidative capacity. In the intestine and liver, the FRAP value of blackthorn flower extract-treated group was slightly but significantly ($p \leq 0.05$) higher compared to the control group on the 28th day, while in the kidneys it did not differ statistically during the experiment. In turn, the antioxidant capacity determined as the $ABTS^{\bullet+}$ scavenging activity of the intestine was the highest ($p \leq 0.05$) already on the 7th and 28th day of the experiment. In the liver, FRAP was significantly higher in the exposed ($p \leq 0.05$) animals on the 28th day only. In the kidneys, the antioxidative capacity measured as both FRAP and $ABTS^{\bullet+}$ scavenging activity was not significantly different between the control and the blackthorn flower extract-treated animals. In general, it seems that at least four weeks of consumption is necessary to observe positive antioxidative effects in the intestine and liver using FRAP and/or $ABTS^{\bullet+}$ method. Similar trends were found for other phenolic- and polyphenol-rich plants. For example, Nakhaee *et al.* [2009] and Salahshoor *et al.* [2019] showed an increased antioxidative potential (determined with the FRAP method) of liver tissue of streptozotocin-induced diabetic rats after dosing *Eucalyptus globulus* and *Fucaria vulgaris*, respectively.

In concordance with the pattern of described TBARS, FRAP, and $ABTS^{\bullet+}$ scavenging activity changes, the SOD activity of intestine, liver, and kidneys (Table 1) showed somewhat different increase specific for each organ. The SOD activity of intestine and liver of the treated animals was significantly

TABLE 1. The activity of superoxide dismutase (SOD) and catalase (CAT), and content of reduced glutathione (GSH) in the mice tissues after 1–28 days of daily repeated oral administration of the blackthorn flower extract (PSE) compared to these of untreated control mice.

	Intestine		Liver		Kidneys	
	Control	PSE	Control	PSE	Control	PSE
SOD activity (U/mg proteins)						
1 day	6.03±0.54^{b}	8.17±1.63^{c}	14.26±1.87^{a}	10.43±3.08^{c}	10.83±3.53^{b}	12.38±2.04^{b}
7 day	9.06±1.23^{a}	6.68±0.46^{c}	11.42±4.97^{a}	12.4±0.84^{c}	10.41±1.53^{b}	12.51±1.93^{b}
14 day	7.85±1.33^{b}	7.32±1.76^{c}	10.86±2.11^{a}	13.31±2.04^{c}	16.97±1.54^{a}	21.38±1.17^{a}
21 day	5.55±2.42^{b}	10.39±1.00#^{b}	12.78±2.91^{a}	17.31±1.08#^{b}	11.18±1.94^{b}	14.04±1.92^{b}
28 day	10.17±2.31^{a}	14.53±2.17#^{a}	15.44±1.49^{a}	22.93±3.21#^{a}	12.78±1.81^{b}	21.42±3.93#^{a}
CAT activity (U/mg proteins)						
1 day	5.8±1.96^{b}	5.93±3.63^{b}	115.11±12.17^{a}	100.51±20.19^{a,b}	106.23±28.06^{a}	95.86±8.26^{a}
7 day	7.03±4.03^{a}	6.94±1.07^{d,a}	75.44±11.62^{b}	92.06±5.28#^{b}	117.16±30.49^{a}	91.26±9.46^{a}
14 day	6.12±3.64^{a}	11.1±1.07^{a}	66.95±19.21^{b}	80.61±13.76#^{b}	88.11±29.09^{a}	98.28±39.31^{a}
21 day	7.47±3.81	3.53±1.43^{b}	50.9±15.29^{a}	70.36±21.53#^{b}	78.37±44.21^{a}	86.48±25.37^{a}
28 day	9.49±4.94^{a}	9.78±3.89^{a}	93.91±25.84^{a}	151.89±27.96#^{a}	61.13±19.76^{b}	83.42±24.72^{a}
GSH content (μM/mg proteins)						
1 day	41.44±9.94^{b}	36.26±6.91^{c}	61.77±9.56^{b}	55.59±22.22^{b}	38.61±14.34^{b}	36.87±5.47^{b,c}
7 day	57.86±16.81^{a}	53.76±6.08^{b}	49.77±14.33^{b}	66.95±24.75^{b}	31.24±9.64^{b}	31.89±6.64^{c}
14 day	52.73±12.39^{a}	36.78±4.22^{c}	50.79±12.52^{b}	71.65±22.66^{b}	33.19±7.63^{b}	55.04±12.78# ^{b}
21 day	55.43±18.91^{a}	65.99±6.84^{b}	60.01±11.09^{b}	79.13±15.71#^{b}	38.61±16.47^{b}	56.94±14.76^{b}
28 day	76.79±12.69^{a}	114.12±9.94# ^{a}	109.81±25.45^{a}	138.15±26.51#^{a}	62.64±9.23^{a}	93.58±28.76#^{a}

The statistically significant differences (p≤0.05) between the control group and the PSE-treated group on the day of sampling (within rows). The different superscript letters (a-e) show statistically significant difference (p≤0.05) between the 1st, 7th, 14th, 21st, 28th day of sampling within the control or the PSE group (within columns). The values are expressed as means±standard deviation (SD).

higher compared to that of control groups (p≤0.05) after three weeks of consumption (from 21st until 28th day). In the kidneys, a significant increase (p≤0.05) in SOD activity occurred later and was recorded only after four weeks of treatment. Since there is no previous data on *in vivo* antioxidative effects of *P. spinosa* flower extract, the results could be only compared to other plant species rich in polyphenols. For example, Nardi *et al.* [2016] investigated and compared the anti-inflammatory and antioxidative effects of goji berry, blueberry, and cranberry extracts administrated to mice. Mice were treated for 10 days with 50 and 200 mg of extract per kg bw. The antioxidant status of liver was determined by testing GSH concentration and CAT activity. Both were the highest in the group that received goji berries extract and the lowest in the group that obtained the blueberries extract. Similarly, Jin & Yin [2012] investigated the antioxidant effect of polyphenols from leaves of bamboo plants on the aging process of mice. Three groups of mice were treated with the leaf extract at doses of 20, 40, and 80 mg/kg bw. Their antioxidant status was measured by determining SOD, glutathione peroxidase (GSH-Px), and CAT activity, and total antioxidant capacity with the ORAC method. Compared with the control group, the activity of all enzymes in the serum and liver were statistically significantly higher in the groups treated with the leaf extract. In turn, phenolics from grapes and wine modulated SOD, GSH, and CAT levels in mice liver and kidneys [Landeka Jurčević *et al.,* 2017]. Interestingly, the CAT activity (Table 1) increased (p≤0.05) only in the liver as a result of blackthorn flower extract treatment, compared to the control animals. The onset of activation in the liver was very early in the experiment, *i.e.* from the 7th day onwards. Intestine and kidneys did not show significantly different activity in the treated animals compared to their controls, respectively. The GSH contents (Table 1) in all tissues of blackthorn flower extract administered animals were higher compared to these of controls. They increased (p≤0.05) by the end of the four-week treatment (on the 28th day). However, only in the liver such a significant increase (p≤0.05) started earlier – on the 21st day of the experiment (Table 1). In general, liver was the most prominent organ where induced activity of antioxidative defense molecules was recorded, while kidneys were the least responsive organ for the induction of tissue antioxidative markers.

Does the blackthorn flower extract has antioxidant properties in mammalian organism?

This important fundamental question addressed in this work was based on the previous publications that demonstrated antioxidant and other bioactive properties of the blackthorn flower extract under *in vitro* conditions [Elez-Garofulić *et al.,* 2018; Lovrić *et al.,* 2017; Marchelak *et al*., 2017; Meschini *et al*., 2017; Mikulic-Petkovsek *et al*., 2016; Olszewska & Wolbis, 2001; Pinacho *et al*., 2015]. The results presented in Figure 1 A-C and Table 1 indirectly show the reduction of oxidative stress that occurred *in vivo* in all major entry and excretory organs after ingestion and application of the blackthorn flower extract in mice, but mainly after three to four weeks of daily extract consumption.

The results also prove that the blackthorn flower extract has a similarly significant oxidation-inhibiting potential (Figure 2 and 3) as other plants that are rich sources of phenolics and were previously verified as good antioxidants. Similar properties as in our work are shown for other numerous plants, for example *Aronia melanocarpa*, for whom it was demonstrated that the 8-week consumption of its extract by animals lowered the serum MDA concentration in the experimental model of the metabolic syndrome [Broncel *et al.,* 2010]. Those results obtained in mice were later confirmed in humans. For example, the consumption of *Aronia melanocarpa* juice prior to an ergonomic test in humans [Pilaczynska-Szczesniak *et al.,* 2005], caused a significant reduction in DNA damage and reactive oxidative species (ROS) concentration and significant improvement of oxidative stress markers, antioxidant enzyme activity, and mitochondrial performance. Therefore, we expect the *P. spinosa* flower extract to be equally efficient in humans as tested in this model. Similar effects, noticed first in animals and then in humans, were recorded for many nutritional products and fruits such as tea, black wine, apple juice and goji berries, *etc*., plants which are known to contain large amounts of phenolics [Ganguly *et al.,* 2016; Jin & Yin, 2012; Landeka Jurčević *et al.,* 2017; Nardi *et al.,* 2016; Olszewska & Wolbis, 2001; Teng & Chen *et al.,* 2019].

Definitely, based on the presented results, we can confirm that this major finding of antioxidative properties *in vivo*, supports the *in vitro* properties of antioxidant effects of blackthorn extract earlier reported by Marchelak *et al.* [2017] and other authors [Elez-Garofulić *et al.,* 2018; Lovrić *et al.,* 2017; Meschini *et al*., 2017; Mikulic-Petkovsek *et al*., 2016; Olszewska & Wolbis, 2001; Pinacho *et al*., 2015]. However, the prolonged intake of at least three weeks is necessary to achieve first observable antioxidative bioactivity in healthy (not pathologically challenged) mice at the dose of 25 mg of TP/kg bw. This result can serve as a guideline for future mechanistic studies in mice and based on estimations and literature it remains to be seen if a dose calculated to human equivalent dose (HED) would achieve a similar effect in humans.

What are the mechanistic and molecular explanations of the observed antioxidative properties of a blackthorn flower extract in mice tissues which might bring innovation to this and similar future studies?

We believe that the blackthorn flower extract in mice caused the boosting of beneficial physiological or antioxidant effects in the target tissues by molecular mechanism described previously [Bao *et al.,* 2018; Dominko & Đikić, 2018; Dominko *et al.,* 2020; Squillaro *et al.,* 2018]. Based on the literature, we provide two major mechanistic explanations and hypotheses that can explain the observed inhibition of lipid peroxidation and increased antioxidative activity in mice tissues after three to four weeks of consumption.

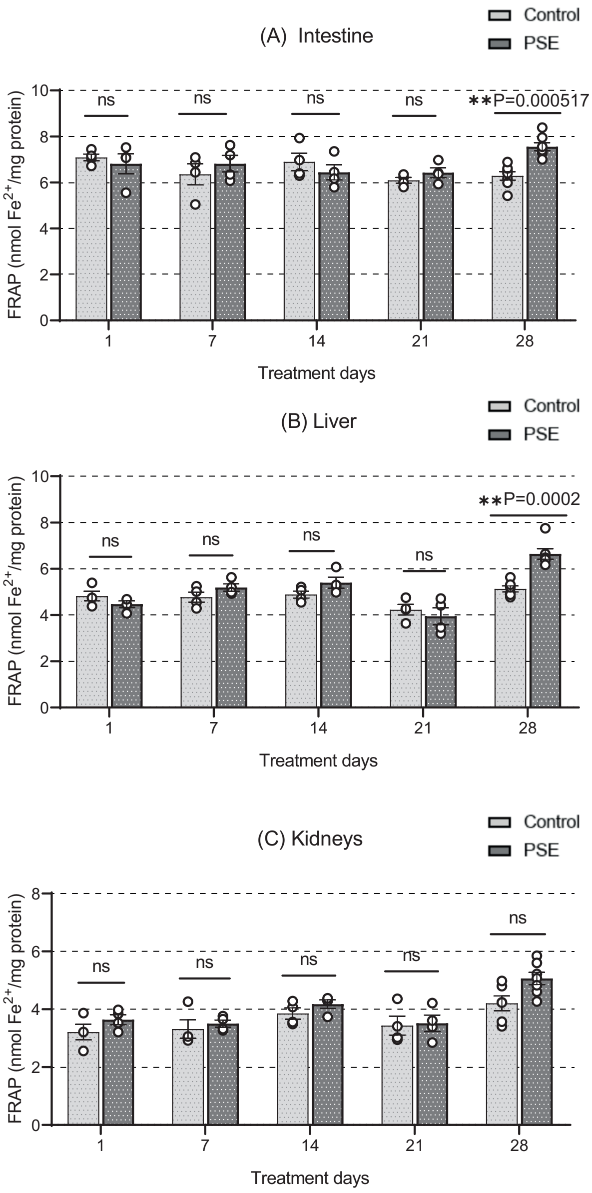


FIGURE 2. The antioxidant capacity of the tissue homogenates as ferric reducing antioxidant power (FRAP) of the (A) intestine, (B) liver, and (C) kidneys of mice treated with blackthorn flower extract and untreated control animals (Control). PSE – blackthorn flower extract-treated group. The values that are statistically different between control and PSE-treated group are marked with p value of t-test. ns – no statistically significant differences (p>0.05).

The first presumption and explanation is that the lowered lipid peroxidation (TBARS) is a consequence of the direct ROS scavenging properties of the bioabsorbed and bioavailable phenolics that were accumulated in the tissues (as listed for each tissue in Tables 2–4 and discussed in the next section). The second mechanistic explanation of the antioxidative effects could be that those phenolics all together synergistically indirectly induced the transcriptional mechanism (and other cellular mechanisms) for the production of in-

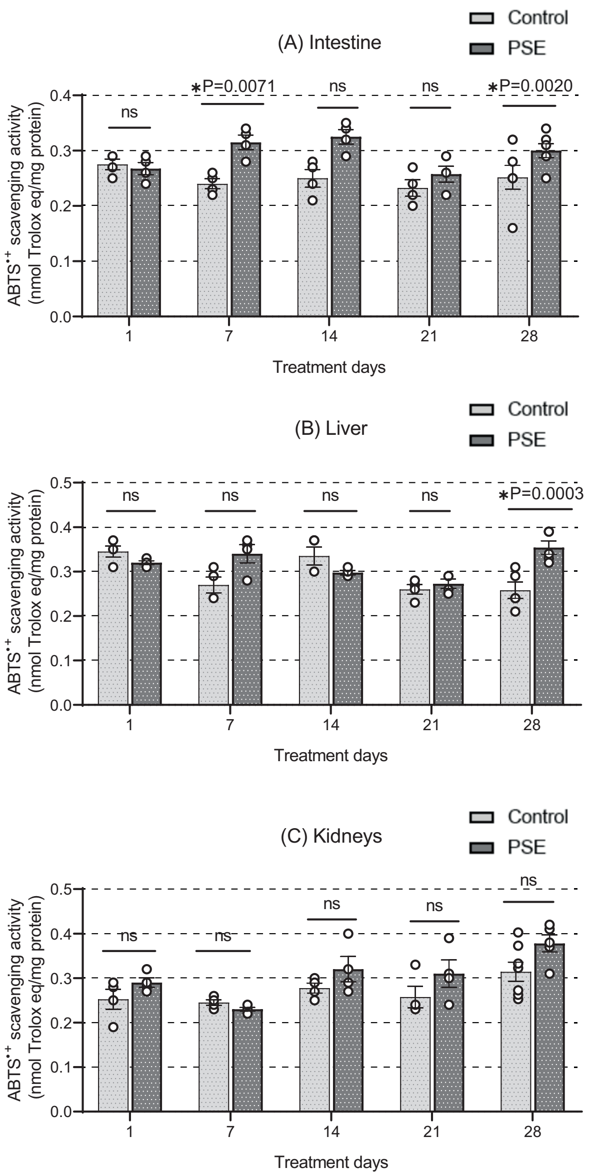


FIGURE 3. The ABTS•+ scavenging activity of the tissue homogenates (A) intestine, (B) liver and, (C) kidneys of mice treated with blackthorn flower extract and untreated control animals (Control). PSE – blackthorn flower extract-treated group. The values that are statistically different between control and PSE-treated group are marked with p value of t-test. ns – no statistically significant differences (p>0.05).

ternal cellular antioxidative defense enzymes and molecules (CAT, GSH, SOD, and others). There is also a possibility that both proposed mechanisms took place in parallel, probably by the early onset (third week) of scavenging properties and later (fourth week) jointly supported by induction. In support of the first mechanical explanation of the direct scavenging activities by organ-bioavailable phenolics, we hypothesized that the phenolic compounds bioabsorbed in tissues would increase the overall antioxidant capacity of tissue homogenates. However, both FRAP and ABTS•+ scavenging activity method did not yield similar positive results for all tissues – an increase in the antioxidative capacity was recorded only in liver and/or intestine after the fourth week of extract consumption. These results could support the proposed hypothesis only to the limited extent. Perhaps, the use of other antioxidative tests would give positive results in the kidneys, as in the case of Jin & Yin [2012] who investigated the antioxidant effect of phenolics from leaves of bamboo plants in mice tissues based on the oxygen radical absorbance capacity (ORAC). Thus, one important guideline for future studies on the similar model is to incorporate diverse antioxidative measurements in tissues.

The second explanation of the changes taking place in the tissues of mice after blackthorn flower extract administration may be the indirect effect of phenolics on cellular and molecular mechanisms and activation of SOD, GSH, or CAT cellular pathways of the antioxidative defense. Those antioxidative effects achieved by molecular mechanisms were described in the literature [Dominko & Đikić, 2018; Dominko *et al.,* 2020; Squillaro *et al.,* 2018]. Phenolics boost the activation of transcription factors Erk-Nrf2-HO1, GCLM, and TrxR1 signal pathway and enhance the antioxidant enzymes such as heme oxygenase-1, phase II detoxification enzymes, and enzymes involved in GSH metabolism [Bao *et al.,* 2018; Dominko & Đikić, 2018; Dominko *et al.,* 2020; Nardi *et al.,* 2016; Squillaro *et al.,* 2018]. Expressions of phase II detoxification and antioxidant enzyme genes are controlled by the antioxidant response element (ARE), which contains genes that are regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2). Thus, after activation, Nrf-2 dissociates from Keap1 and is transferred into the nucleus to activate the translation of antioxidant genes and phase II detoxification genes, such as HO-1, NAD(P)H quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase modifier (GCLM) [Dominko & Đikić, 2018; Dominko *et al.,* 2020]. Reduced glutathione (GSH) can efficiently eliminate electrophiles and ROS that are generated during the chemical metabolism within cells. It is known that the enhancement of endogenous antioxidant defense by flavonoids is associated with the direct elimination of reactive oxygen species, inhibition of lipid peroxidation, reduction of oxidized glutathione level, increase of reduced glutathione level, and restoration of activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione *S*-transferase, and glucose 6-phosphate dehydrogenase) [Dominko & Đikić, 2018; Dominko *et al.,* 2020]. Many studies have reported that herbal extracts modulate the expression of glutamate-cysteine ligase (GCL) which consists of catalytic (GCLC) and modifier (GCLM) subunits, which is a rate-limiting enzyme of GSH synthesis. The secondary mechanism could refer to the neutralization of oxidative species, and inhibition of the activation of the nuclear transcription factor-κB (NF-κB) signaling pathways [Bao *et al.,* 2018; Nardi *et al.,* 2016; Squillaro *et al.,* 2018].

In this study, the activation of SOD, CAT, and GSH in the group receiving the blackthorn flower extract was tissue-specific. In all organs, there was an increased SOD activity but it occurred on different days of the experiment. Such an increase in kidneys was observed on the 28th day but not earlier, whereas in the liver a slight (although still statistically not significant) increase was recorded already on the 14th day of the experiment that became significant between 21st-28th day. Therefore, the accumulation of superoxide anion was a consequence of mitochondrial activ-

TABLE 2. Phenolic compound profile in the mice intestine after 28 days of daily oral administration of the blackthorn flower extract compared to untreated control group expressed as area under the curve of phenolic content in the tissue *vs*. treatment period (AUClast), maximal concentration (C_{max}), and day of the experiment when it was achieved (T_{max}).

Phenolic compound		AUClast (h·µg/g)#	C_{max} (µg/g) #	T_{max} (day)
Phenolic acids				
Caffeic acid	Control	41.6±17.4	0.20±0.05	7
	PSE	111.8±13.9	0.37±0.02	7
p-Coumaric acid	Control	186.7±37.4	0.47±0.14	21
	PSE	444.9±6.3	1.66±0.94	1
3-*O*-*p*-Coumaroylquinic acid	Control	/	/	
	PSE	71.8±14.4	0.19±0.02	7
4-*O*-*p*-Coumaroylquinic acid	Control	627.7±4.2	1.22±0.05	1
	PSE	1095.0±27.8	2.16±1.27	21
Ferulic acid	Control	368.3±61.5	1.03±0.36	21
	PSE	740.9±9.7	1.68±0.95 ns	1
3-*O*-Feruloylquinic acid	Control	211.9±37.3	0.62±0.13	7
	PSE	768.4±6.3	4.60±0.42	7
Gallic acid	Control	215.1±96.7	1.27±0.67	1
	PSE	575.2±12.6	2.20±1.27 ns	7
Flavones				
Luteolin	Control	89.5±19.2	0.19±0.10	7
	PSE	171.8±30.6	0.64±0.28 ns	1
Apigenin	Control	188.3±17.8	0.88±0.14	1
	PSE	523.9±72.5	2.5±0.25	1
Flavan-3-ols				
(–)-Epigallocatechin 3-gallate	Control	6.3±0.8	0.02±0.01	21
	PSE	14.5±3.9	0.17±0.01	28
Flavonols				
Kaempferol 3-*O*-glucoside	Control	93.5±2.1	0.16±0.00	7
	PSE	146.1±3.8	0.29±0.17 ns	7
Kaempferol pentoside	Control	1344.3±6.1	2.74±0.01	7
	PSE	1559.4±74.7	5.29±2.90 ns	1
Kaempferol rhamnoside	Control	/	/	
	PSE	9.9±0.01	0.05±0.02	28
Kaempferol pentosyl-hexoside	Control	260.6±13.9	0.51±0.01	7
	PSE	329.4±10.2	0.85±0.45	1
Quercetin 3-*O*-glucoside	Control	63.9±10.2	0.16±0.01	7
	PSE	151.0±12.5	0.55±0.05	21
Quercetin 3-*O*-rutinoside	Control	270.3±4.29	0.77±0.01	7
	PSE	952.0±12.1	1.73±0.21	1
Quercetin acetyl-hexoside	Control	219.2±23.8	0.51±0.01	28
	PSE	429.9±0.8	0.67±39.00 ns	28
Quercetin rhamnoside	Control	387.7±11.8	0.95±0.10	14
	PSE	723.7±17.3	1.67±0.94 ns	1

#The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group for each individual phenolic compound were determined excluding values marked with ns – no statistically significant differences ($p > 0.05$). PSE – blackthorn flower extract-treated group; / – not detected. AUClast – area under the curve, C_{max} – maximal detected concentration, T_{max} – day of experiment when maximal concentration was detected.

TABLE 3. Phenolic compound profile in the mice liver after 28 days of daily oral administration of the blackthorn flower extract compared to untreated control group expressed as area under the curve of phenolic content in the tissue *vs*. treatment period (AUClast), maximal concentration (C_{max}), and day of the experiment when it was achieved (T_{max}).

Phenolic compound		AUClast (h·μg/g)#	C_{max} (μg/g) #	T_{max} (day)
Phenolic acids				
Caffeic acid	Control	54.5±0.2	0.23±0.01	21
	PSE	479.4±39.1	1.78±0.15	21
p-Coumaric acid	Control	/	/	
	PSE	38.4±0.1	0.16±0.03	7
3-*O*-*p*-Coumaroylquinic acid	Control	8.3±0.7	0.03±0.02	1
	PSE	68.5±0.1	0.25±0.01	7
4-*O*-*p*-Coumaroylquinic acid	Control	317.5±2.8	0.53±0.01	1
	PSE	798.1±0.1	1.42±0.00	21
Ferulic acid	Control	494.6±0.4	0.77±0.02	14
	PSE	835.8±3.6	1.64±0.02	28
Gallic acid	Control	76.9±88.1	0.58±0.04	28
	PSE	110.1±16.2	1.31±0.19	28
Flavones				
Luteolin	Control	51.3±3.3	0.18±0.01	7
	PSE	78.7±1.0	0.33±0.02	7
Flavonols				
(–)-Epicatechin 3-gallate	Control	169.2±2.4	0.70±0.05	14
	PSE	265.9±0.1	1.40±0.01	14
(–)-Epigallocatechin 3-gallate	Control	256.6±0.1	0.87±0.00	14
	PSE	400.0±61.5	1.06±0.25 ns	7
Flavonols				
Kaempferol 3-*O*-glucoside	Control	71.6±0.4	0.15±0.01	1
	PSE	108.3±0.3	0.25±0.01	7
Kaempferol rhamnoside	Control	8.0±1.1	0.02±0.04	7
	PSE	26.2±0.5	0.12±0.02	7
Kaempferol acetyl-hexoside	Control	4.1±0.8	0.02±0.05	7
	PSE	13.4±0.1	0.07±0.01 ns	7
Quercetin 3-*O*-glucoside	Control	70.6±0.8	0.14±0.09	7
	PSE	168.6±0.1	0.39±0.03	1
Quercetin 3-*O*-rutinoside	Control	715.1±78.9	1.55±0.03	14
	PSE	1113.1±0.6	2.57±0.03	14
Quercetin pentoside	Control	84.2±1.1	0.13±0.01	7
	PSE	670.0±1.6	1.44±0.02	7

The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group for each individual phenolic compound were determined excluding values marked with ns – no statistically significant differences ($p > 0.05$). PSE- blackthorn flower extract-treated group; / – not detected. AUClast – area under the curve, C_{max} – maximal detected concentration, T_{max} – day of experiment when maximal concentration was detected.

ity and boosted metabolism. For example, the liver was the only organ that from the earlier onset of experiment (7th day) had increased CAT activity and the only organ whose catalase antioxidative pathway was induced. Other organs did not show CAT activation due to the blackthorn flower extract treatment. The liver was the only organ with increases in the values of all three parameters and the only organ that had increased CAT activity within the time of extract administration (but only on the 28th day). Liver, opposite to other organs, has normally higher physiological expression of catalase, and is the organ with a high metabolic rate (biotransformation activity). Therefore, we are firmly convinced that,

TABLE 4. Phenolic compound profile in the mice kidneys after 28 days of daily oral administration of the blackthorn flower extract compared to untreated control group expressed as area under the curve of phenolic content in the tissue *vs.* treatment period (AUClast), maximal concentration (C_{max}), and day of the experiment when it was achieved (T_{max}).

Phenolic compound		AUClast (h·µg/g)#	C_{max} (µg/g) #	T_{max} (day)
		Phenolic acids		
4-*O*-*p*-Coumaroylquinic acid	Control	412.3±11.1	0.64±0.01	28
	PSE	1143.1±2.6	2.06±0.04	28
Ferulic acid	Control	261.6±2.8	0.84±0.56	21
	PSE	1354.5±0.3	5.45±0.00	21
		Flavones		
Luteolin	Control	11.3±3.5	0.13±0.15	28
	PSE	131.2±13.7	0.52±0.19	1
Apigenin	Control	128.2±1.0	0.45±0.01	1
	PSE	349.2±2.6	0.96±0.02	21
		Flavan-3-ols		
(+)-Catechin	Control	92.9±0.1	0.21±0.00	1
	PSE	776.2±1.4	1.41±0.01	7
(–)-Epicatechin	Control	0.3±0.0	0.02±0.03	1
	PSE	848.4±0.4	1.55±0.00	7
		Flavonols		
Quercetin 3-*O*-glucoside	Control	85.1±1.0	0.15±0.00	7
	PSE	121.0±0.6	0.19±0.00 ns	1
Quercetin 3-*O*-rutinoside	Control	240.1±1.0	0.64±0.35	7
	PSE	1764.0±6.7	2.96±0.02	14
Quercetin pentosyl-hexoside	Control	86.8±1.2	0.13±0.00	1
	PSE	193.2±1.10	0.40±0.00	1

The statistically significant differences ($p \leq 0.05$) between the control group and the PSE-treated group for each individual phenolic compound were determined excluding values marked with ns – no statistically significant differences ($p > 0.05$). PSE – blackthorn flower extract-treated group; / – not detected AUClast – area under the curve, C_{max} – maximal detected concentration, T_{max} – day of experiment when maximal concentration was detected.

physiologically, it is possible that after 28 days of continuous intake of xenobiotic molecules in a higher concentration, peroxides were accumulating in hepatocytes and the expression and activity of catalase was increased. The GSH content also increased in all organs predominantly on the 28th day. This significant change, although minor compared to the control animals, indicates that phenolics present in organs probably enhance the metabolic activity. Presumably, the metabolic processes of conjugation activated the elimination of xenobiotic molecules (phenolics) as normal physiological characteristics, since kidneys are the major organs where conjugated glutathione-xenobiotic complexes (in this case polyphenol residue conjugates) enter the excretory pathway, cleaved as mercapturic acids [Dominko & Đikić, 2018]. The present study results are consistent with literature data, for example with Nardi *et al*. [2016] who investigated and compared the anti-inflammatory and antioxidative effects of goji berry, blueberry, and cranberry extracts in mice. Mice were treated with the extract at 50 and 200 mg/kg bw for 10 days. The antioxidant activity was determined by testing the GSH content and CAT activity, which were the highest in the group that received goji berries extract and the lowest in the group that obtained the blueberries extract. Similarly, Jin & Yin [2012] investigated the antioxidant effect of phenolics from leaves of bamboo plants on the aging process of mice. Three groups of mice were treated with the leaf extract at doses of 20, 40, and 80 mg/kg bw. The authors reported that the CAT and SOD activity and the GSH content in the serum and liver decreased while the MDA levels increased significantly in the groups treated with the leaf extract, compared with the control group.

Content of phenolic compounds in individual tissue following four-week administration of blackthorn flower extract in mice

The selective and specific tissue content was observed for specific subgroups of phenolic compounds after the intake of the blackthorn flower extract (Tables 2–4). In the intestine

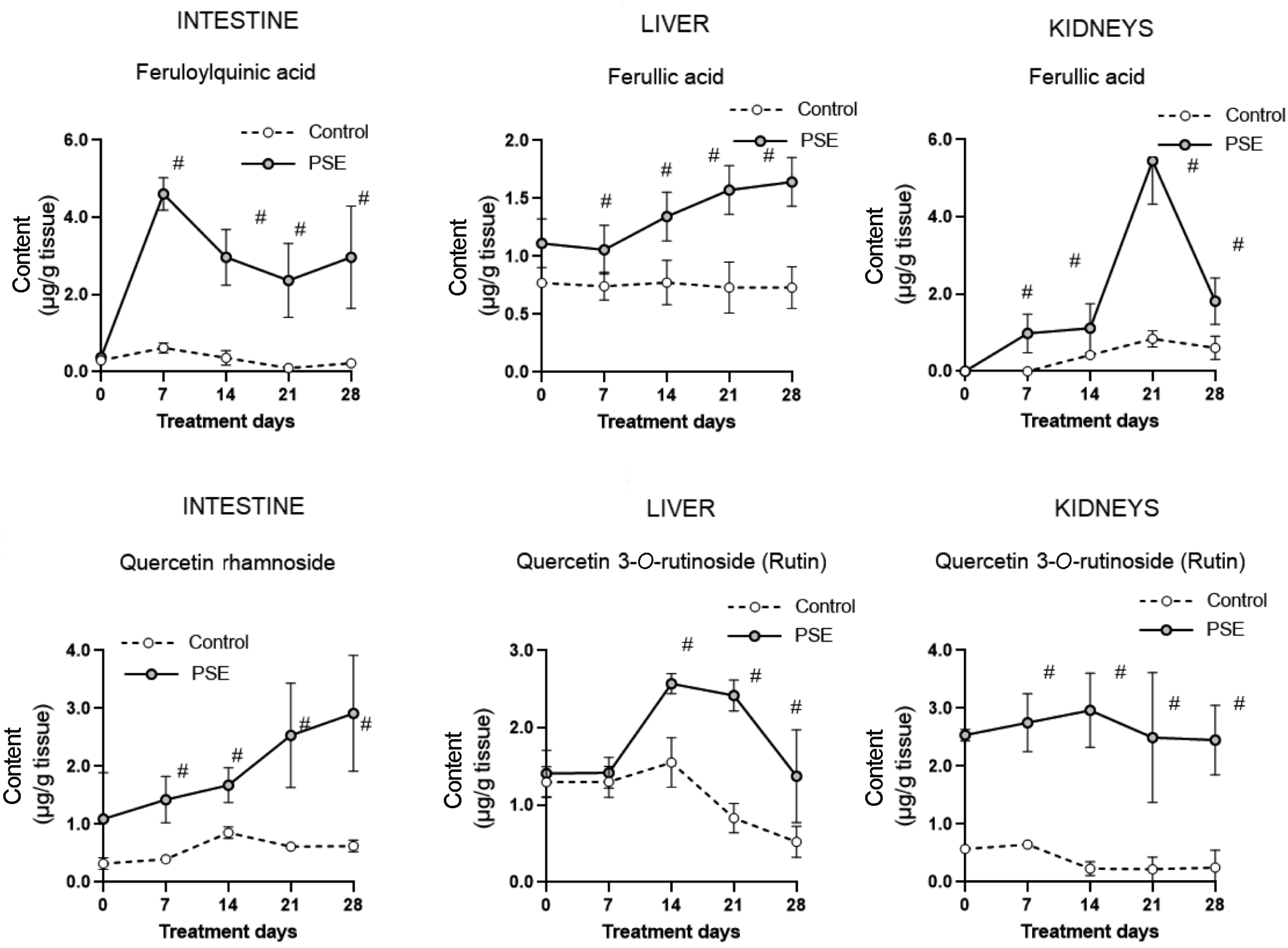


FIGURE 4. Individual phenolic content in the intestine, liver, and kidneys of mice treated with blackthorn flower extract and untreated control animals (control). PSE – blackthorn flower extract-treated group. #The values are statistically different ($p \leq 0.05$) between untreated control and PSE-treated groups.

and the liver, more phenolics were detected than in the kidneys and their composition was generally similar.

Among the seven phenolic acids detected in the intestine, 3-*O*-feruloylquinic acid was the only one absent in the liver. Opposite to those tissues, only two phenolic acids, namely 4-*O*-*p*-coumaroylquinic acid and ferulic acid, were determined in the kidneys. Ferulic acid was, therefore, the only one present in all three organs and its AUClast after 28 days was the highest in the kidneys of the treated animals.

Among the flavan-3-ols, (+)-catechin and (–)-epicatechin were found only in the kidneys. (–)-Epicatechin 3-gallate was present only in the liver and (–)-epigallocatechin 3-gallate was detected in the intestine and liver. Among the flavonols, the intestine contained four kaempferol glycosides, while three and four quercetin glycosides were present in the liver and intestine, respectively. The least varied phenolic composition was observed in the kidneys, with no detected kaempferol glycosides and only three quercetin glycosides. Interestingly, quercetin 3-*O*-glucoside and quercetin 3-*O*-rutinoside were present in all three organs.

The specific distribution of phenolic compounds in each tissue was observed (Tables 2–4) Namely, in the intestine, 3-*O*-feruloylquinic acid was present with most prominently different C_{max} that was approximately 4 times higher in the tissue of the treated animals than in the control ones. Intestine contained also other compounds in high quantities (either AUClast or C_{max}), such as 4-*O*-*p*-coumaroylqiunic acid, kaempferol pentoside, quercetin rhamnoside, and quercetin 3-*O*-rutinoside, but their difference from the control was less prominent than in 3-*O*-feruloylquinic acid (Table 2, Figure 4). In the liver, the highest AUClast values were determined for quercetin 3-*O*-rutinoside (1113.1 h·µg/g) followed by ferulic acid (835.8 h·µg/g) (Table 3, Figure 4). The content of phenolics in kidneys was generally lower. The highest C_{max} was determined for ferulic acid and quercetin 3-*O*-rutinoside followed by 4-*O*-*p*-coumaroylquinic acid (Table 4, Figure 4).

Does the distribution of phenolics occur in tissues after 28-day consumption of blackthorn flower extract?

We further hypothesized that the differences in the antioxidative defense activation and specific organ patterns were a consequence of differences in the bioaccumulation of various phenolic compounds from the extract. Therefore, we decided to screen major phenolics in the analyzed organs (Tables 2–4, Figure 4). Probably, they were jointly and synergistically responsible for the observed antioxidative effects and no individual compound among detected ones could be, with certainty, proclaimed to individually cause the bioactive effects measured in each analyzed organ.

The selective tissue content and distribution was observed for specific subgroups of phenolic compounds of the blackthorn flower extract. Since mouse has a faster metabolism, in humans the time at which similar concentrations can be reached could probably be longer. However, current scientific methodology does not have the means to estimate concentrations of absorbed phenolics in human tissues in a similar manner of time dynamics as shown here in the animal model.

The lowest number of phenolics with a low concentration was observed in the kidneys. It was probably not only a consequence of lesser vascularization (than in the intestine and liver) or low absorption, but also a consequence of the enhanced polyphenol excretion and their removal with urine. Thus, we believe that the measured antioxidative effects and activation of SOD, CAT, or GSH, probably followed the specific phenolic bioacummulation pattern in each organ. *In vivo* research has shown that phenolic compounds are absorbed in larger amounts than previously thought and especially after long-term consumption they are able to increase plasma antioxidant capacity [Teng & Chen, 2019]. Our study supports this observation. This effect was also noticed for many nutritional products such as tea, black wine and apple juice, which are known to contain large amounts of phenolics [Teng & Chen, 2019]. However, the bioabsorption and organ distribution of only a small number of phenolics is well known, as described in, *e.g*., historic works on quercetin [Olthof *et al.,* 2000; Wang *et al.,* 2016]. In our previous publication, Đikić *et al.* [2018], in the same model but with the single acute dose (analyzed in different times within 24 h), we have concluded that the serum does not give a true picture of phenolics content and distribution in body. Similar conclusions can be found in literature [Gonzales *et al.,* 2015]. The number of compounds and their bioavailable concentrations were very low in the acute (2 h) experiment compared to this experiment here with 28 days of exposure, and the recorded antioxidant bioactivity in tissue confirming that the molecular bioabsorption actually happened. The exact analysis of tissues showed that the concentrations up to approximately 5 μg/g in the intestine were approximately 2-fold higher *vs*. concentrations in the liver, but liver and intestine shared a similar pattern of compound types distribution. This balance is probably the reflection of normal physiological enterohepatic recirculation. The phenolic contents in the kidneys were detected by UPLC-MS/MS in the experiment of Ganguly *et al.* [2016] following oral administration of black tea as the aqueous infusion and alcoholic extract to guinea pigs for 14 days. The authors reported the contents of (–)-epigallocatechin (49.1 and 34.2 ng/g tissue, respectively), (–)-epicatechin (47.8 and 22.3 ng/g tissue), (–)-epigallocatechin 3-gallate (91.8 and 45.0 ng/g tissue), and (–)-epicatechin 3-gallate (22.5 and 12.8 ng/g tissue, respectively). If the content of flavan 3-ols in mice kidneys determined in our study was expressed in ng/g tissue, the range would be between 210–1553 ng/g tissue, depending on the compound. The higher values are probably due to the difference in experimental animals used (guinea pig *vs*. mice in this study) but also a longer (2 times) dosing regime in this study. The model used in this study shows that the consumption could be within line with safety standards for the concentration of bioabsobred phenolics from food supplements and medicinal plants [Harwood *et al*., 2007; Peng *et al*., 2016] and it the first published work on the antioxidative activity of blackthorn *in vivo*. Aside from fundamental knowledge of particular phenolics bioaccumulation and bioavailability and correlation to antioxidative effects, there is a practical value of the study in the way that the aqueous infusion in the form of an alcoholic extract can be used for production and consumption.

CONCLUSION

The contents of phenolics increased significantly in mice tissues after at least three weeks of blackthorn flower extract consumption. The extract had significant bioactive properties *in vivo* and induced antioxidative defense pathways in the organ-specific manner. Thus, the study demonstrates that the administration of *Prunus spinosa* flower extract to mice partially inhibited the oxidative stress in tissues. Among individual phenolic compounds of the extract, the study screened these bioavailable and absorbed. In the intestine, it was mainly 3-*O*-feruloylquinic acid, 4-*O*-*p*-coumaroylqiunic acid, kaempherol pentoside, and quercetin rhamnoside, in the liver – ferulic acid and quercetin 3-*O*-rutinoside, and in the kidneys – quercetin 3-*O*-rutinoside, ferulic acid, and 4-*O*-*p*-coumaroylquinic acid. Possibly, these phenolics in mixtures but together with other that were in lower contents, were responsible for the antioxidant effects of the blackthorn flower extract. It must be emphasized that, to the best of our knowledge, this is the first study on the antioxidative activity of blackthorn *in vivo* and there are no studies on human consumption conducted with the blackthorn flower extract.

ACKNOWLEDGEMENTS

We are grateful to the staff of the Animal Breeding Facility of the Department of Animal Physiology Faculty of Science, University of Zagreb, for their help during conduction of the *in vivo* experiment and to Dr. Zoran Zoric and Dr. Sandra Pedisic for their great effort during the preparation of UPLC-MS/MS data and results.

RESEARCH FUNDING

This work was supported by the project “Bioactive molecules of medical plants as natural antioxidants, microbicides, and preservatives” (KK.01.1.1.04.0093), co-financed by the Croatian Government and the European Union through the European Regional Development Fund-Operational Programme Competitiveness and Cohesion (KK.01.1.1.04.).

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

ORCID IDs

V. Balta https://orcid.org/0000-0002-0424-1173
D. Đikić https://orcid.org/0000-0003-0909-3488
I. Crnić https://orcid.org/0000-0002-7014-1103
D. Odeh https://orcid.org/0000-0002-6182-755X
N. Oršolić https://orcid.org/0000-0001-5102-3606
I. Kmetić https://orcid.org/0000-0001-5412-7128
T. Murati https://orcid.org/0000-0001-7157-400X
V. Dragović Uzelac https://orcid.org/0000-0002-4179-3727
I. Landeka Jurčević https://orcid.org/0000-0002-3785-1752

REFERENCES

1. Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology, 105*, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3
2. Alarcón, R., Pardo-de-Santayana, M., Priestley, C., Morales, R., Heinrich, M. (2015). Medicinal and local food plants in the south of Alava (Basque Country, Spain). *Journal of Ethnopharmacology, 176*, 207–224. https://doi.org/10.1016/j.jep.2015.10.022
3. Bao, Y., Qu, Y., Li, J., Li, Y., Ren, X., Maffucci, K.G., Li, R., Wang, Z., Zeng, R. (2018). *In vitro* and *in vivo* antioxidant activities of the flowers and leaves from *Paeonia rockii* and identification of their antioxidant constituents by UHPLC-ESI-HRMSn *via* pre-column DPPH$^\bullet$ reaction. *Molecules, 23*(2), art. no. 392. https://doi.org/10.3390/molecules23020392
4. Benzie, I.F., Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical Biochemistry*, *239*(1),70–76. https://doi.org/10.1006/abio.1996.0292
5. Bioethic approval of the experimental procedure within the project, (2014). Document No. 251–58–10617–14–37, Faculty of Science, University of Zagreb, Croatia (in Croatian).
6. Broncel, M., Kozirog, M., Duchnowicz, P., Koter-Michalak, M., Sikora, J., Chojnowska-Jezierska, J. (2010). *Aronia melanocarpa* extract reduces blood pressure, serum endothelin, lipid, and oxidative stress marker levels in patients with metabolic syndrome. *Medical Science Monitor, 16*(1), 28–34.
7. Đikić, D., Balta, V., Kmetić, I., Murati, T., Orsolić, N., Dragovic Uzelac, V., Landeka Jurčević, I. (2018). UPLC/MS analysis of plasma bioavailability of 32 polyphenols in C57BL/6 mice treated with single acute dose (24 h) of flower extract of the blackthorn *Prunus spinosa* L. *Molecular and Experimental Biology in Medicine, 2*, 23–31.
8. Dominko, K., Đikić, D. (2018). Glutathionylation: a regulatory role of glutathione in physiological processes. *Arhiv za Higijenu Rada i Toksikologiju, 69*(1), 1–24. https://doi.org/10.2478/aiht-2018-69-2966
9. Dominko, K., Đikić, D., Hecimovic, S. (2020). Enhanced activity of superoxide dismutase is a common response to dietary and genetically induced increased cholesterol levels. *Nutritional Neuroscience, 23*(5), 398–410. https://doi.org/10.1080/1028415X.2018.1511027
10. Elez-Garofulić, I., Zorić, Z., Pedisić, S., Brnčić, M., Dragović-Uzelac, V. (2018). UPLC-MS2 Profiling of blackthorn flower polyphenols isolated by ultrasound-assisted extraction. *Journal of Food Science, 83*(11), 2782–2789. https://doi.org/10.1111/1750-3841.14368
11. Flohé, L., Ötting, F. (1984). Superoxide dismutase assays. *Methods in Enzymology, 105*, 93–104. https://doi.org/10.1016/S0076-6879(84)05013-8
12. Ganguly, S.G., Mantha, S., Panda, K. (2016). Simultaneous determination of black tea-derived catechins and theaflavins in tissues of tea consuming animals using Ultra-Performance Liquid-Chromatography Tandem Mass Spectrometry. *PLoS One, 11*(10), art. no. e0163498. https://doi.org/10.1371/journal.pone.0163498
13. Gonzales, G.B., Smagghe, G., Grootaert, C., Zotti, M., Raes, K., Van Camp, J. (2015). Flavonoid interactions during digestion, absorption, distribution and metabolism: a sequential structure-activity/property relationship-based approach in the study of bioavailability and bioactivity. *Drug Metabolism Reviews, 47*(2), 175–90. https://doi.org/10.3109/03602532.2014.1003649
14. *Guide for the Care and Use of Laboratory Animals* (2011). Washington DC, USA, *National Academies Press.* 2011, 86–123.
15. Harwood, M., Danielewska-Nikiel, B., Borzelleca, J.F., Flamm, G.W., Williams, G.M., Lines, T.C.A. (2007). Critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food and Chemical Toxicology*, *45*(11), 2179–2205. https://doi.org.10.1016/j.fct.2007.05.015
16. Jin, S.L., Yin, Y.G. (2012). *In vivo* antioxidant activity of total flavonoids from indocalamus leaves in aging mice caused by D-galactose. *Food and Chemical Toxicology, 50*(10), 3814–3818. https://doi.org/10.1016/j.fct.2012.07.046
17. Katalinic, V., Modun, D., Music, I., Boban, M. (2005). Gender differences in antioxidant capacity of rat tissues determined by 2,2’-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. *Comparative Biochemistry and Physiology C. Toxicology & Pharmacology*, *140*(1), 47–52. https://doi.org/10.1016/j.cca.2005.01.005
18. Kelly, E., Vyas, P., Weber, J.T. (2017). Biochemical properties and neuroprotective effects of compounds in various species of berries. *Molecules, 23*(1), art. no. 26. https://doi.org/10.3390/molecules23010026
19. Landeka Jurčević, I., Dora, M., Guberović, I., Petras, M., Rimac Brnčić, S., Đikić, D. (2017). Polyphenols from wine lees as a novel functional bioactive compound in the protection against oxidative stress and hyperlipidaemia. *Food Technology and Biotechnology, 55*(1), 109–116. https://doi.org/10.17113/ftb.55.01.17.4894
20. Lovrić, V., Putnik, P., Kovačević, D.B., Jukić, M., Dragović-Uzelac, V. (2017). Effect of microwave-assisted extraction on the phenolic compounds and antioxidant capacity of blackthorn flowers. *Food Technology and Biotechnology, 55*(2), 243–250. https://doi.org/10.17113/ftb.55.02.17.4687
21. Lowry, D.H., Rosebrough, N.J., Farr, A.L. (1951). Protein measurement with the Folin-phenol reagent. *The Journal of Biological Chemistry, 193*(1), 265–275.
22. Marchelak, A., Owczarek, A., Matczak, M., Pawlak, A., Kolodziejczyk-Czepas, J., Nowak, P., Olszewska, M.A. (2017). Bioactivity potential of *Prunus spinosa* L. flower extracts: phytochemical profiling, cellular safety, pro-inflammatory enzymes inhibition and protective effects against oxidative stress *in vitro*. *Frontiers in Pharmacology, 8,* art. no. 680. https://doi.org/10.3389/fphar.2017.00680

23. Menendez-Baceta, G., Aceituno-Mata, L., Tardío, J., Reyes--García, V., Pardo de Santayana, M. (2012). Wild edible plants traditionally gathered in Gorbeialdea (Biscay, Basque Country). *Genetic Resources and Crop Evolution, 59*(7), 1329–1347. https://doi.org/10.1007/s10722-011-9760-z
24. Meschini, S., Pellegrini, E., Condello, M., Occhionero, G., Delfine, S., Condello, G., Mastrodonato, F. (2017). Cytotoxic and apoptotic activities of *Prunus spinosa* Trigno ecotype extract on human cancer cells. *Molecules, 22*(9), art. no. 1578. https://doi.org/10.3390/molecules22091578
25. Mikulic-Petkovsek, M., Stampar, F., Veberic, R., Sircelj, H. (2016). Wild *Prunus* fruit species as a rich source of bioactive compounds. *Journal of Food Science, 81*(8), C1928-C1937. https://doi.org/10.1111/1750-3841.13398
26. Murati, T., Miletić, M., Kolarić, J., Lovrić, V., Kovačević, D.B., Putnik, P., Jurčević, I.L., Đikić, D., Dragović-Uzelac, V., Kmetič, I. (2019). Toxic activity of *Prunus spinosa* L. flower extract in hepatocarcinoma cells. *Arhiv za higijenu rada i toksikologiju, 70*(4), 303–309. https://doi.org/10.2478/aiht-2019-70-3322
27. Nakhaee, A., Bokaeian, M., Saravani, M., Farhangi, A., Akbarzadeh, A. (2009). Attenuation of oxidative stress in streptozotocin-induced diabetic rats by *Eucalyptus globulus*. *Indian Journal of Clinical Biochemistry, 24*(4), 419–425. https://doi.org/10.1007/s12291-009-0075-1
28. Nardi, G.M., Farias Januario, A.G., Freire, C.G., Megiolaro, F., Schneider, K., Perazzoli, M.R., Do Nascimento, S.R., Gon, A.C., Mariano, L.N., Wagner, G., Niero, R., Locatelli, C. (2016). Anti-inflammatory activity of berry fruits in mice model of inflammation is based on oxidative stress modulation. *Pharmacognosy Research, 8*(1), 42–49. https://doi.org/10.4103/0974-8490.178642
29. NN 55/2013. Act on Animal Welfare, Croatia (2013). Official Gazette of the Republic of Croatia, *1129* (in Croatian).
30. Ohkawa, H., Ohishi, N., Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry, 95*(2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3
31. Olszewska, M., Głowacki, R., Wolbís, M., Bald, E. (2001). Quantitative determination of flavonoids in the flowers and leaves of *Prunus spinosa* L. *Acta Poloniae Pharmaceutical, 58*(3), 199–203. https://europepmc.org/article/med/11712737.
32. Olszewska, M., Wolbiś, M. (2001). Flavonoids from the flowers of *Prunus spinosa* L. *Acta Poloniae Pharmaceutical, 58*(5), 367–372.
33. Olszewska, M., Wolbiś, M. (2002). Further flavonoids from the flowers of *Prunus spinosa* L. *Acta Poloniae Pharmaceutical*, *59*(2), 133–137. https://europepmc.org/article/med/12365605
34. Olthof, M.R., Hollman, P.C., Vree, T.B., Katan, M.B. (2000). Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans. *The Journal of Nutrition, 130*(5), 1200–1203. https://doi.org/10.1093/jn/130.5.1200
35. Peng, K.Z., Zhang, S.Y., Zhou, H.L. (2016). Toxicological evaluation of the flavonoid-rich extract from Maydis stigma: Subchronic toxicity and genotoxicity studies in mice. *Journal of Ethnopharmacology*, *192*,161–169. https://doi.org/10.1016/j.jep.2016.07.012
36. Pilaczynska-Szczesniak, L., Skarpanska-Steinborn, A., Deskur, E., Basta, P., Horoszkiewicz-Hassan, M. (2005). The influence of chokeberry juice supplementation on the reduction of oxidative stress resulting from an incremental rowing ergometer exercise. *International Journal of Sport Nutrition and Exercise Metabolism, 15*(1), 48–58. https://doi.org/10.1123/ijsnem.15.1.48
37. Pinacho, R., Cavero, R., Astiasarán, I., Ansorena, D., Calvo, M. (2015). Phenolic compounds of blackthorn (*Prunus spinosa* L.) and influence of *in vitro* digestion on their antioxidant capacity. *Journal of Functional Foods, 19*, 49–62. https://doi.org/10.1016/j.jff.2015.09.015
38. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS$^{\bullet+}$ radical cation decolourization assay. *Free Radical Biology & Medicine, 26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3
39. Renfrew, J.M. (1973). Palaeoethnobotany: *The Prehistoric Food Plants of the Near East and Europe.* Methuen, London, UK.
40. Salahshoor, M.R., Mohammadi, M.M., Roshankhah, S., Najari, N., Jalili, C. (2019). Effect of *Falcaria vulgaris* on oxidative damage of liver in diabetic rats. *Journal of Diabetes and Metabolic Disorders, 18*(1), 15–23. https://doi.org/10.1007/s40200-019-00385-3
41. SPSS version 17.0 (SPSS Inc, Chicago, IL). Available at: https://www.hks.harvard.edu/.../A%20SPSS%20Manuals/SPSS%2.
42. Squillaro, T., Cimini, A., Peluso, G., Giordano, A., Melone, M.A.B. (2018). Nano-delivery systems for encapsulation of dietary polyphenols: An experimental approach for neurodegenerative diseases and brain tumors. *Biochemical Pharmacology, 154*, 303–317. https://doi.org/10.1016/j.bcp.2018.05.016
43. Teng, H., Chen, L. (2019). Polyphenols and bioavailability: an update. *Critical Reviews in Food Science and Nutrition, 59*(13), 2040–2051. https://doi.org/10.1080/10408398.2018.1437023
44. Tietze, F. (1969). Enzyme method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Analytical Biochemistry, 27*, 502–522. https://doi.org/10.1016/0003-2697(69)90064-5
45. Wang, W., Sun, C., Mao, L, Ma, P., Liu, F., Yang, J., Gao, Y. (2016). The biological activities, chemical stability, metabolism and delivery systems of quercetin: a review. *Trends in Food Science & Technology, 56*, 21–38. https://doi.org/10.1016/j.tifs.2016.07.004
46. Yuksel, A.K. (2015). The effects of blackthorn (*Prunus spinosa* L.) addition on certain quality characteristics of ice cream. *Journal of Food Quality, 38*(6), 413–421. https://doi.org/10.1111/jfq.12170

Submitted: 30 April 2020. Revised: 14 July, 18 September, and 29 September 2020. Accepted: 30 September 2020. Published on-line: 9 September 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 377–385
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/128613
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Effect of Boiling on Colour, Contents of Betalains and Total Phenolics and on Antioxidant Activity of Colourful Powder Derived from Six Different Beetroot (*Beta vulgaris* L. var. *conditiva*) Cultivars

Jan Bárta[1], *Veronika Bártová*[1]*, *Tereza Šindelková*[1], *Markéta Jarošová*[1], *Zuzana Linhartová*[3],
Jan Mráz[3], *Jan Bedrníček*[2], *Pavel Smetana*[2], *Eva Samková*[2], *Ivana Laknerová*[4]

[1]*Faculty of Agriculture, Department of Plant Production, University of South Bohemia,*
Na Sádkách 1780, 370 05, České Budějovice, Czech Republic
[2]*Faculty of Agriculture, Department of Food Biotechnology and Agricultural Products Quality, University of South Bohemia,*
Na Sádkách 1780, 370 05, České Budějovice, Czech Republic
[3]*Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity*
of Hydrocenoses, Institute of Aquaculture and Protection of Waters, University of South Bohemia,
Na Sádkách 1780, 370 05, České Budějovice, Czech Republic
[4]*Food Research Institute, Radiová 1285/7, 102 00, Prague, Czech Republic*

Key words: beetroot, *Beta vulgaris* L. *conditiva*, beetroot meal, cultivar, boiling, betalain pigments, antioxidant activity

Powders prepared from freeze-dried roots of six beetroot (*Beta vulgaris* L.) cultivars with red (cv. D'Egypte, Karkulka, Betina, Alexis), yellow (cv. Burpee's Golden) or red-white (cv. Chioggia) colour of root flesh were evaluated before and after boiling treatment (20 min, 100°C). Water and fat holding capacity (WHC and FHC), colour, contents of betalains and total phenolics (TPCs), and antioxidant activity were all significantly affected by cultivar as well as powder thermal treatment. WHC increased after boiling of the powder and varied between cultivars from 2.14 g/g (cv. D'Egypte) to 2.59 g/g (cv. Chioggia). The highest FHC of 1.22 g/g was observed in cv. Betina. Colour stability was optimal in the cultivars with red root flesh; with their lightness decrease between 6 and 10%. This corresponded with betalain content that was the highest in red cv. Betina; for which the values before and after boiling were 18.10 and 15.04 mg/g DM, respectively. Cultivar Betina significantly exceeded the other cultivars also in TPCs and antioxidant activity. The TPCs values of this cultivar were 3.73 and 3.32 mg gallic acid equivalents/g DM in the unboiled and boiled variant, respectively. Antioxidant activity of Betina powder before and after boiling was 11.13 and 11.69 mg ascorbic acid equivalents/g DM, respectively. The results indicated the significance of beetroot cultivar selection for thermal processing. Beetroots with red flesh significantly exceeded yellow and red-white cultivars in all the evaluated characteristics.

INTRODUCTION

Beetroot (*Beta vulgaris* L. *conditiva*) is a traditional and important vegetable all over the world that is usually consumed in traditional western cuisine [Nistor *et al*., 2017; Sawicki *et al*., 2016; Vasconcellos *et al*., 2016]. It contains over 80% of water, while such components as saccharides, dietary fiber, nitrogenous substances, and ash prevail in its dry matter (DM) [Vasconcellos *et al*., 2016; Wruss *et al*., 2015]. However, consumer interest in beetroot has arisen due to the content of minor compounds implicated in numerous health benefits. These compounds include particularly betalains, phenolic acids, flavonoids, and ascorbic acid, which may increase the total antioxidant capacity of beetroot [Chhikara *et al*., 2019; Ravichandran *et al*., 2012; Vasconcellos *et al*., 2016; Wruss *et al*., 2015].

Attractiveness of beetroot for consumers is mainly due to the colour of root flesh, which as a cultivar-specific trait, offers different shades of red and red-violet. Beetroot cultivars with yellow and red-white (alternate red and white rings in cross-section) root flesh colour are well-known (Plant Variety Database – European Commission). Root flesh colour is derived from betalains, being water-soluble nitrogenous pigments having a core structure known as betalamic acid [Ravichandran *et al*., 2013]. Average content of betalains in beetroot is 0.1 g/100 g of fresh root matter but it can be up to 2 g/kg of fresh matter in some cultivars [Velíšek, 2014]. Betalains consist of two main groups, the red-violet betacyanins (major pigment is betanin) and the yellow betaxanthins (major pigment is vulgaxanthin) [Celli & Brooks, 2017; Sawicki *et al*., 2016]. They are free radical scavengers and prevent oxygen-induced and free radical-mediated oxidation of biological molecules [Sawicki & Wiczkowski, 2018]. However, betalains exhibit also anticarcinogenic [Chhikara *et al*., 2019; Gengatharan *et al*., 2015] and antimicrobial activities [Celli & Brooks, 2017]. Stability of betalain pigments is influenced by betalain structure and composition, pH value, water activity, oxygen, light, metal and antioxidant

---

* Corresponding Author: vbartova@zf.jcu.cz (V. Bártová)

presence, and temperature that represents the most important factor [Güneşer 2016].

Beetroot can be consumed as fresh vegetable, beetroot juice, after heat cooking or as a component of, *e.g.*, food products [Ranawana *et al.*, 2016]. Powder derived from red beetroot or beetroot pomace [Porto Dalla Costa *et al.*, 2017] can be applied in bakery [Kohajdová *et al.*, 2018], or meat products [Sucu *et al.*, 2018]. Functional properties, including water and fat holding (absorption) capacity, gelling ability, emulsifying activity and others are important for these types of beetroot application. Changes in these functional characteristics occurring during heat treatment are crucial for the use of beetroot in food applications. The attractiveness of beetroot powder is due to its red colour, pigment content, and total antioxidant activity, all of which can be influenced by heat treatment [Nistor *et al.*, 2017; Ravichandran *et al.*, 2013; Sawicki & Wiczkowski, 2018].

The aim of this study was to evaluate the impact of boiling on colour, antioxidant activity, and betalain pigment and phenolic contents in different colourful powders prepared from several beetroot cultivars.

MATERIAL AND METHODS

Plant material and beetroot powder processing

Plants of six beetroot (*Beta vulgaris* var. *conditiva*) varieties (Table 1) were cultivated in south-bohemian region (GPS N 49° 39' 56'', E 14° 88' 68'') under organic crop management in 2018. No mineral fertilizers, pesticides or other chemical preparations were applied during the growing season. Roots of ten average beet plants were used to prepare a freeze-dried powder. Roots were thoroughly washed, dried, and weighed. Afterwards, they were cut into 2-mm thin slices, frozen at -20°C, and subsequently freeze-dried using a freeze-drier (ALPHA 1–4 LSD, Martin Christ, Germany) under a temperature of -50°C, pressure of ≤0.420 mbar, and time period of 72 h. Freeze-dried samples were grinded using a planetary mono mill (Pulverisette 6, Fritsch, Germany) under 450 rpm for 2 min to obtain a beetroot powder.

Colour, functional properties, and antioxidant characteristics were analysed in the beetroot powders before and after powder heat treatment. Two g of the prepared beetroot powders were mixed with 20 mL of deionised water and carefully shaken. These powder mixtures were placed in a water bath with a temperature of 100°C for 20 min (time was measured after reaching the temperature of 100°C in sample interior). After centrifugation (15 min, 3,600×*g*, and 20°C) and supernatant removal, the beetroot powders were cooled, freeze-dried to constant weight, and pulverised by using a planetary mill (Pulverisette 6) under 450 rpm for 2 min to obtain a boiled variant of the beetroot powder.

Water solubility and water holding capacity

Water solubility (S) and water holding capacity (WHC) of unprocessed beetroot powders were analysed by weighing 300 mg of powder and mixing with 5 mL of deionised water. The tubes with the mixture were allowed to stand at room temperature for 30 min under continuous shaking. Samples were centrifuged (15 min, 3,600×*g*, and 20°C). The supernatant was discarded and tubes with pellet were weighed, freeze-dried (-50°C, <0.420 mbar, 48 h), and afterwards weighed again. Solubility and water holding capacity were computed from the obtained weight data according to the following formulas (Equations 1–3).

$$S\,(\%) = 100 * (m_s - m_{lp}) / m_s \qquad (1)$$

$$WHC_{powder}\,(g/g) = (m_{wp} - m_{lp}) / m_s \qquad (2)$$

$$WHC_{non\text{-}soluble\ part}\,(g/g) = (m_{wp} - m_{lp}) / m_{lp} \qquad (3)$$

where: S – a water solubility of beetroot powder DM (%), m_s – weight of the sample (g), m_{lp} – weight of the freeze-dried pellet (g), WHC_{powder} – water holding capacity of beetroot powder (g of water per g of powder DM), m_{wp} – weight of the wet pellet (g), and $WHC_{non\text{-}soluble\ part}$ – water holding capacity of DM of beetroot powder non-soluble part (g of water per g of non-soluble part DM).

Water solubility and water holding capacity of the heat-treated beetroot powder were determined by the same analytical procedure as described for the unprocessed powders and computed with Equation 1 and Equation 4, respectively.

$$WHC_{non\text{-}soluble\ part\ after\ boiling}\,(g/g) = (m_{wpab} - m_{lpab}) / m_{lpab} \qquad (4)$$

where: $WHC_{non\text{-}soluble\ part\ after\ boiling}$ – water holding capacity of non-soluble part of powder after boiling (g of water per g of non-soluble part DM), m_{wpab} – weight of the wet pellet after boiling (g), and m_{lpab} – weight of the freeze-dried pellet after boiling (g).

Fat holding capacity

Fat holding capacity (FHC) was determined analogously as WHC with the following differences: 5 mL of rapeseed oil were used instead of 5 mL of water. Supernatant (oil phase) was thoroughly discarded after centrifugation and tubes with pellet were weighed. The obtained weight of fatted pellet data allowed computing the FHC values (Eq. 5).

$$FHC\,(g/g) = (m_{fs} - m_s) / m_s \qquad (5)$$

where: FHC – fat holding capacity (g of oil per g of powder DM), m_{fs} – weight of the fatted pellet (g), and m_s – weight of the sample (g).

Colour analysis

Colour of beetroot powder was measured for all evaluated cultivars before and after boiling, using a colorimeter Color-Eye XTH (X-Rite, Grand Rapids, MI, USA) which is based on CIE (Commission Internationale de l'Eclairrage) system and provides L* a* b* parameters (L* lightness, 0% – black, 100 % – white; a* red – green; b* yellow – blue).

Betalain content

The betalain content was determined spectrophotometrically. Beetroot powder (100 mg) was extracted with 1 mL of 50% ethanol for 30 min. Supernatants were collected after centrifugation (20°C, 3,600×*g,* 15 min). The extraction procedure was repeated twice and the final total extract volume was 3 mL. The absorbance of the obtained and diluted (1:50) extracts was read (BioMate 5 spectrophotometer, Thermo-Fisher Scientific, Waltham, MA, USA) at λ=540 nm for betacyanins (BCs) and at λ=480 nm for betaxanthins (BXs). The absorbance was used to calculate the betalain, betacyanin, and betaxanthin contents for each sample (Eq. 6).

$$\text{BC or BX (mg/g)} = A_{540/480} \times DF \times MW \times V / \varepsilon \times L \times Wd \quad (6)$$

where: BC or BX – content of betacyanins (BCs) or betaxanthins (BXs) (mg/g), $A_{540/480}$ – absorbance at absorption maximum for BCs (λ=540 nm) or BXs (λ=480 nm), DF – dilution factor, MW – molecular weight: 550 g/mol for BCs and 308 g/mol for BXs, V – volume of sample solution (mL), ε – the molar extinction coefficient 60,000 1/(M×cm) for BCs and 48,000 1/(M×cm) for BXs, L – path length of the cuvette (1 cm), and Wd – weight of the dried sample (g).

Total phenolic content

The total phenolic content (TPC) was determined spectrophotometrically using the Folin-Ciocalteau's reagent after previous extraction of phenolics from freeze-dried matter using 80% ethanol with a solid-to-solvent ratio of 1:20 (*w/v*). The extraction was performed for 24 h under room temperature, and the mixtures were then centrifuged at 3,600×*g* for 10 min, filtered, and subsequently refrigerated at 4°C until analysed. The reaction mixture contained 20 μL of the beetroot extract, 1980 μL of distilled water, 100 μL of Folin-Ciocalteau's reagent, and 300 μL of 20% (*w/v*) sodium carbonate. Absorbance was read at λ=734 nm (BioMate 5 spectrophotometer) after 2 h. Gallic acid was used as a standard and the results obtained were expressed as mg of gallic acid equivalent (GAE) per g of sample DM [Hamouz *et al*., 2006].

Antioxidant activity

The beetroot powder extracts for determination of the antioxidant activity (AOA) were prepared as described for TPC determination. Antioxidant activity was determined using ABTS and DPPH methods according to Šulc *et al.* [2007] with modifications. Ascorbic acid was used as the standard for both of the methods and results were expressed as mg of ascorbic acid equivalent (AAE) per g of sample DM.

(2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was dissolved in the amount of 54.9 mg in 20 mL of a 5 mM phosphate buffer (pH 7.4), and BTS radical cations were activated by 1 g MnO_2. The filtered solution (PTFE 0.25 μm) was diluted in a 5 mM phosphate buffer to the absorbance of 0.800±0.01 at λ=734 nm. Absorbance of the reaction mixture (1 mL $ABTS^{\bullet+}$ solution and 100 μL of beetroot extract) was measured at λ=734 nm.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical in the amount of 0.025 g was dissolved in 100 mL of methanol to obtain a stock solution. The reaction mixture was prepared by mixing 975 μL of 10% (*v/v*) $DPPH^{\bullet}$ stock solution and 25 μL of the beetroot extract. Absorbance of the reaction mixture was measured at λ=515 nm after 30-min incubation under room temperature.

Statistical analysis

Data obtained were statistically examined by the one-way (water and fat holding capacity) or two-way (content of betalain pigments, TPCs, antioxidant activity) analysis of variance (ANOVA), and significant differences were determined using the Fisher LSD test at a significance level of $p<0.05$. Relations among selected parameters were tested using the correlation analysis. Statistical evaluation was performed with the STATISTICA software, version 12 (StatSoft Inc., USA). Three independent replicates were performed for each evaluated parameter.

RESULTS AND DISCUSSION

Functional properties of beetroot powder

Our study was carried out with the group of six beetroot cultivars with different colour of root flesh. The list of the analysed cultivars with their basic characteristics (root flesh colour, weight, and DM content) are shown in Table 1. Average weight of the roots ranged between 202 (cv. Alexis) and 404 g (Burpee's golden), while differences between cultivars in DM content were found to be in a narrow range from 13.3 (cv. Burpee's golden) to 15.0 g/100 g (cv. D'Egypte). Similar DM of beetroots were also found by Szopińska & Gaweda [2013] and Kazimierczak *et al.* [2014].

High WHC values were determined in powders from cultivars Chioggia and Betina (Table 2), *i.e.* 1.88 and 1.81 g/g powder, respectively. However, WHC of these cultivars did not differ significantly from WHC of cultivars Burpee's golden and Karkulka. In contrast, the lowest WHC was determined in the powder from beetroot of cv. D'Egypte. The content

TABLE 1. List of beetroot cultivars and description of root characteristics.

Beetroot cultivar	Country of origin	Root shape	Root flesh colour	Average root weight (g)	Dry matter content (g/100 g)
Alexis	CZ	cylindrical	deep red	202	13.40
Betina	CZ	spherical	deep red	269	13.52
Burpee's Golden	UK	spherical	yellow (golden)	404	13.29
Chioggia	IT	spherical	rings of red and white	379	14.91
D'Egypte	PL (CZ, FR, ES, IT)	flattened	deep red	323	15.03
Karkulka	CZ	cylindrical	deep red	390	14.69

TABLE 2. Functional properties of beetroot powders.

Beetroot cultivars	Water solubility (%)	Water holding capacity (g/g)		Water holding capacity after boiling (g/g)		Fat holding capacity (g/g)
		Powder	Non-soluble part	Powder	Non-soluble part	
Alexis	68.53 ± 2.65^{a}	1.65 ± 0.05^{bc}	4.71 ± 0.09^{c}	2.35 ± 0.10^{bc}	6.21 ± 0.07^{c}	1.09 ± 0.04^{b}
Betina	65.45 ± 0.95^{ab}	1.81 ± 0.01^{ab}	5.24 ± 0.12^{a}	2.59 ± 0.05^{a}	6.91 ± 0.12^{a}	1.22 ± 0.06^{a}
Burpee's Golden	66.75 ± 2.30^{ab}	1.69 ± 0.05^{ab}	5.07 ± 0.06^{ab}	2.23 ± 0.08^{c}	6.41 ± 0.06^{bc}	1.15 ± 0.03^{ab}
Chioggia	64.48 ± 2.36^{b}	1.88 ± 0.13^{a}	5.28 ± 0.28^{a}	2.56 ± 0.18^{ab}	6.60 ± 0.15^{b}	1.13 ± 0.05^{b}
D'Egypte	68.27 ± 1.54^{a}	1.53 ± 0.19^{c}	4.78 ± 0.32^{bc}	2.14 ± 0.07^{c}	6.22 ± 0.16^{c}	1.14 ± 0.05^{ab}
Karkulka	65.76 ± 1.90^{ab}	1.72 ± 0.06^{ab}	5.02 ± 0.10^{abc}	2.29 ± 0.18^{c}	6.24 ± 0.20^{c}	1.14 ± 0.07^{ab}

Means with different subscript letters for the water and fat holding capacity indicate significant differences according to the one-way ANOVA (factor cultivar), Fisher LSD test ($p<0.05$). Results are shown for the dry matter of powder/non-soluble part of powder.

of water-soluble components varied from 64.48 to 68.53%, depending on cultivar. Increasing the proportion of water non-soluble components resulted in WHC increase before and after powder boiling. The boiling process increased WHC of the powders made of all of cultivars tested. After boiling, the highest WHC values were determined for the powders made of cultivars Betina and and Chioggia and reached 2.59 and 2.56 g/g powder, respectively. Differences in WHC after boiling between these two cultivars were not statistically significant ($p>0.05$).

Similarly, high FHC value was determined in the powder of cv. Betina with a low water solubility. However, the determined FHC values did not differ significantly from these assayed in powders from beetroots of cultivars Burpee's golden, D'Egypte, and Karkulka. The FHC of Betina powder reached 1.22 g/g; the variability between cultivars was from 1.09 (Alexis) to 1.22 g/g (Betina). The WHC and FHC values of wheat powder have been usually determined at 1.1 and 2.5 g/g, respectively [Menon *et al*., 2015]. Data obtained in our study show beetroot powder to be a material with high values of WHC and FHC; thus suitable as a component of powder mixtures usable in products expected to absorb water and/or fat. Porto Dalla Costa *et al.* [2017] have presented high WHC (mean 10.14 g water/g powder) and FHC (mean 3.3 g/g powder) for beetroot powder prepared from waste pomace. Such a high WHC was probably due to the high contents of insoluble fiber and protein in this type of beetroot material. In agreement with this assumption, we found the highest content of total dietary fiber in powder from cv. Chioggia (11.6% of DM) (not published data of authors). This explains the highest value of WHC and the lowest value of water solubility found in the powder from this beetroot cultivar. The two components, fibers and proteins, are considered to be predominant in the powder ability to retain oil and water [Menon *et al*., 2015; Porto Dalla Costa *et al*., 2017], and responsible for increasing viscosity, preventing syneresis, and modifying product's texture [Porto Dalla Costa *et al*., 2017].

In this context, beetroot powder is a promising additive in food systems that can replace, at least partly, wheat and soybean powders as functional components in products expected to hold up water and/or oils [Kohajdová *et al*., 2018] or in reduced-energy products [Chau *et al*., 2004].

Colour of beetroot powder

The colour of beetroot powder differed significantly among the cultivars ($p<0.05$) and the colour parameters were significantly influenced by the boiling process (Figure 1). Four tested cultivars had different shades of deep red colour, roots of one cultivar had red-white strips and another one yellow (golden) colour (Table 1). The L* parameter values of beetroot powder of the non-boiled samples were (Figure 1) in the range from 46.86 (cv. Alexis) to 84.87 (cv. Burpee's Golden). The values of b* colour parameter ranged from 0.40 (cv. Karkulka) to 47.50 (cv. Burpee's golden). Deep red colour was observed in beetroot powder from cultivars D'Egypte, Karkulka, Betina, and Alexis. The beetroot powder made of all cultivars tested had a more intensive colour before than after boiling Colour parameters of beetroot samples changed after boiling and the L* parameter values were from 40.16 (cv. Betina) to 57.78 (cv. Burpee's Golden). The greatest decreases of lightness caused by boiling were found in the powders prepared from Burpee's Golden (decrease by 27%) and Chioggia (decrease by 18.6%) roots. The decrease of L* value noted for the root powder from the redo-coloured cultivar was only between 6% and 10%. Beetroot characteristic coloration and colour stability during thermal treatment depends on the content and stability of highly active pigments, especially betalains [Guldiken *et al*., 2016] and carotenoids [Ninfali & Donato, 2013]. Betalains (betacyanins and betaxanthins) may be degraded during heat processing mainly by isomerisation, hydrolysis, decarboxylation, and dehydrogenation to brown and yellow constituents [Bach *et al*., 2015; Herbach *et al*., 2004]. The main beetroot betaxanthin, *i.e.* vulgaxanthin I, is more degradable by heat than the main betacyaninsm such as betanin and isolbetanin [Herbach *et al*., 2004].

The effect of temperature on the visually-assessed colour of beetroot powder was reported by Bach *et al.* [2015] and Chandran *et al.* [2014]. Chandran *et al.* [2014] observed that temperature had no significant effect on L* value, but caused modification in a* and b* values. With an increase of temperature, a* value decreased with a corresponding increase in b* values. Similar trends were observed in our experiment with the exception of cv. Burpee's Golden with yellow colour of the original beetroot powder. However, data pre-

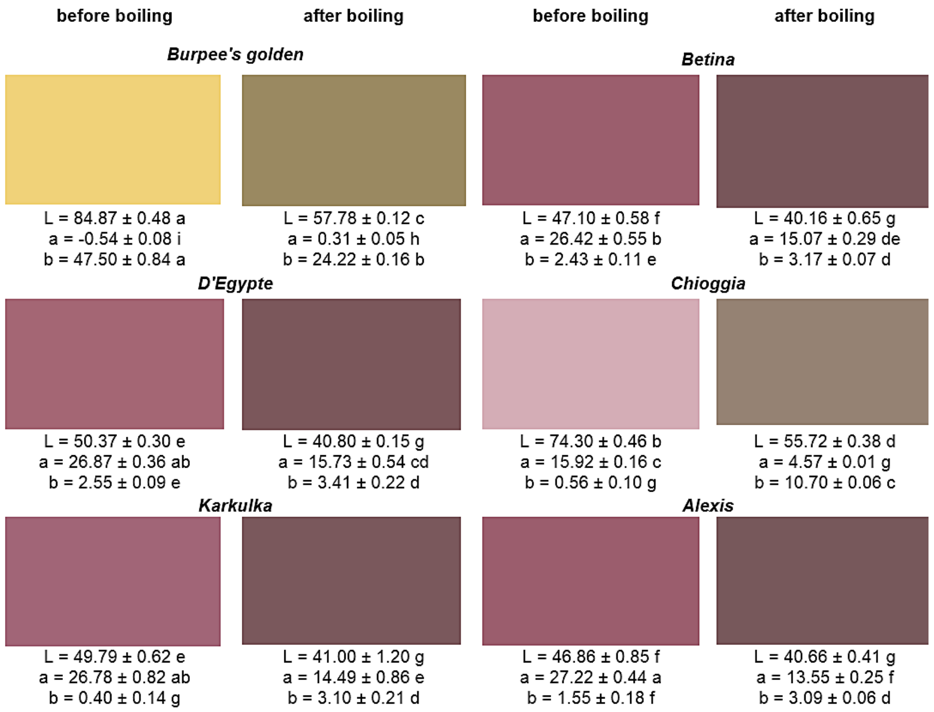


FIGURE 1. Colour of the powder from different beetroot cultivars before and after boiling treatment.

Means with different subscript letters for each colour parameter indicate significant differences according to the two-way ANOVA (evaluated factors: cultivar and sample treatment), Fisher LSD test (p<0.05).

sented by Chandran *et al.* [2014] were based on the analysis of one unknown deep purple cultivar. In turn, Bach *et al.* [2015] evaluated the effect of beetroot cultivar and culinary preparation on colour changes. The decrease of colour intensity was not so extensive in red beetroot cultivars (Taunus, Rosket a Pablo) as in the red-white beetroot cultivar Chioggia. In cv. Burpee's Golden, the authors did not observe such a great decrease of colour intensity as we found in our experiment.

Contents of betacyanins, betaxanthin, and betalains

As presented above, betalain content, composition, and thermal stability affect beetroot powder colour before and after boiling. Table 3 collates contents of betacyanins and betaxanthins, the two categories of betalains, in dependence on beetroot cultivar and thermal treatment of beetroot powder. Before boiling, the content of betalains (BLs) in powder of red beetroot cultivars ranged from 10.92 (cv. D'Egypte) to 18.10 (cv. Betina) mg/g DM. Powders of yellow beetroot cultivar Burpee's Golden and red-white cultivar Chioggia contained only 2.07 and 1.38 mg/g DM of BLs, respectively. A relative decrease in betalain content caused by boiling the beetroot powder ranged from 9% to 29%, depending on cultivar.

The content of red-violet pigments – betacyanins (BCs), in the tested powders followed the similar trend as it was found for BLs. The BCs content in beetroot powders of red root cultivars ranged from 6.63 (cv. D'Egypte) to 11.14 mg/g DM (cv. Betina). Red-white cultivar Chioggia and yellow root cultivar Burpee's Golden contained 0.90 and 0.54 mg/g DM of BCs, respectively. Boiling treatment led to a decrease in BCs content in the range from 3 to 28%.

The content of yellow-orange pigments – betaxanthins (BXs), was higher in the powder from the red root cultivars (interval within cultivars was found between 4.29 and 6.95 mg/g DM) than in the powder from the yellow cv. Burpee's Golden (1.53 mg/g DM) and red-white cv. Chioggia (0.48 mg/g DM). The lowest BCs/BXs ratio was demonstrated for the yellow cultivar in both types of powder before and after boiling. The highest ratio indicated the strong supremacy of betacyanins in roots of this cultivar. The BXs level was also reduced by the boiling treatment, the reduction varied between 8% and 36%. The content of BXs in the boiled powder samples varied between 0.44 (cv. Chioggia) and 5.31 mg/g DM (cv. Betina). Data of BCs/BXs indicate the lower thermal stability of BXs in comparison with BCs.

The BLs, BCs, and BXs contents found in our experiment were generally higher than literature data. The total content of BLs was mostly reported within the broad range of 4–17 mg/g DM, depending on the cultivar [Sawicki *et al.*, 2016; Slatnar *et al.*, 2015]. Sawicki *et al.* [2016] described, in a set of 13 red beetroot cultivars, the content of total BLs in the range from 10.26 to 17.15 mg/g DM, the contents of BCs and their derivatives from 7.18 to 13.50 mg/g DM, and BXs content within the range of 2.71–4.25 mg/g DM. The lowest content of betalains was found in red beetroot varieties from Finland (4.43–9.60 mg/g DM), as well as in those grown in the United States (0.65–0.80 mg/g FM) [Lee *et al.*, 2014]. Unfortunately, betalains are highly sensitive to light, heat, and oxygen [Kowalski *et al.*, 2014; Nistor *et al.*, 2017]. The colour and flavour of dried red beetroots are considered to be the most important quality attributes affecting their acceptability by consumers [Nistor *et al.*, 2017; Sorour

TABLE 3. Content of betalain pigments in beetroot powders before and after boiling treatment.

Beetroot cultivars	BC content (mg/g DM)		BX content (mg/g DM)		Betalains (mg/g DM)		BCs/BXs ratio	
	Before boiling	After boiling	Before boiling	After boiling	Before boiling	After boiling	Before boiling	After boiling
Alexis	10.63 ± 0.39^{a}	8.69 ± 0.07^{c}	6.15 ± 0.23^{b}	4.55 ± 0.03^{d}	16.78 ± 0.62^{b}	13.23 ± 0.10^{d}	1.73	1.91
Betina	11.14 ± 0.32^{a}	9.73 ± 0.51^{b}	6.95 ± 0.21^{a}	5.31 ± 0.28^{c}	18.10 ± 0.52^{a}	15.04 ± 0.79^{c}	1.60	1.83
Burpee's Golden	0.54 ± 0.01^{g}	0.50 ± 0.50^{g}	1.53 ± 0.05^{f}	0.98 ± 0.12^{g}	2.07 ± 0.05^{i}	1.48 ± 0.12^{i}	0.35	0.51
Chioggia	0.90 ± 0.02^{g}	0.72 ± 0.72^{g}	0.48 ± 0.01^{h}	0.44 ± 0.05^{h}	1.38 ± 0.02^{i}	1.16 ± 0.13^{i}	1.88	1.64
D'Egypte	6.63 ± 0.44^{e}	6.42 ± 0.62^{e}	4.29 ± 0.22^{d}	3.51 ± 0.34^{e}	10.92 ± 0.63^{f}	9.93 ± 0.96^{g}	1.55	1.83
Karkulka	7.72 ± 0.17^{d}	5.58 ± 0.66^{f}	4.45 ± 0.10^{d}	3.17 ± 0.39^{e}	12.17 ± 0.27^{e}	8.75 ± 1.05^{h}	1.73	1.76

Means with different subscript letters for contents of betacyanins (BC), betaxanthins (BX), and betalains indicate significant differences according to the two-way ANOVA (evaluated factors: cultivar and sample treatment), Fisher LSD test ($p<0.05$); DM – dry matter.

TABLE 4. Total phenolic content and antioxidant activity of beetroot powder before and after boiling treatment.

Beetroot cultivar	TPC (mg GAE/g DM)		Antioxidant activity (mg of AAE/g DM)			
			ABTS method		DPPH method	
	Before boiling	After boiling	Before boiling	After boiling	Before boiling	After boiling
Alexis	3.25 ± 0.21^{cb}	3.49 ± 0.13^{ab}	10.58 ± 2.64^{a}	10.7 ± 0.69^{a}	6.77 ± 1.30^{def}	16.90 ± 0.31^{ab}
Betina	3.73 ± 0.43^{a}	3.32 ± 0.21^{cb}	11.13 ± 1.19^{a}	11.69 ± 0.09^{a}	8.68 ± 0.90^{d}	17.59 ± 0.29^{a}
Burpee's Golden	1.47 ± 0.14^{f}	0.93 ± 0.05^{g}	5.65 ± 0.23^{c}	2.75 ± 0.07^{d}	4.90 ± 1.13^{fg}	13.45 ± 0.47^{c}
Chioggia	1.39 ± 0.14^{f}	0.64 ± 0.11^{g}	5.73 ± 0.23^{c}	2.03 ± 0.03^{d}	4.29 ± 0.85^{g}	8.36 ± 2.77^{d}
D'Egypte	2.58 ± 0.93^{d}	2.22 ± 0.10^{e}	7.67 ± 0.63^{b}	7.71 ± 0.24^{b}	6.11 ± 1.06^{efg}	16.44 ± 0.65^{ab}
Karkulka	3.09 ± 0.29^{c}	2.46 ± 0.18^{ed}	8.47 ± 0.66^{b}	7.88 ± 0.18^{b}	7.96 ± 0.52^{de}	15.22 ± 1.25^{bc}

Means with different subscript letters for total phenolic content (TPC) and antioxidant activity indicate significant differences according to the two-way ANOVA (evaluated factors: cultivar and sample treatment), Fisher LSD test ($p<0.05$); GAE – gallic acid equivalent; AAE – ascorbic acid equivalent.

et al., 2014]. However, the effect of cultivar on the betalain pigments and colour stability has not been described widely. Herbach *et al*. [2004] described novel yellow neobetanin structures and two orange-red betanin degradation products in thermally-treated (85°C) red beet juice explaining the yellow-orange shift of thermally-treated red beet powder. Nistor *et al.* [2017] similarly found a decreasing level of betacyanins and betaxanthins with increasing drying temperatures in beetroot material of cv. Cylindria and also confirmed betalains heat sensitivity.

Total phenolic content and antioxidant activity

Antioxidant activity was evaluated by the ability of beetroot powder extracts to scavenge $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ and was expressed as the equivalent of ascorbic acid. The extracts of all the cultivars demonstrated a strong potential to scavenge both radicals (Table 4). These results indicated that DPPH radicals could be strongly scavenged by degradation products released in beetroot powder during boiling (substances modification, fragmentation, *etc.*). This conclusion was confirmed by high correlation coefficients determined using DPPH and betalains characteristics in the boiled variant (Table 5). Both, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ are suitable for evaluating the antioxidant activity in individual items of fruit, vegetable and beverages; however, the DPPH method was presented as more suitable for the determination of the antioxidant activity of hydrophobic compounds [Kedare & Singh, 2011]. The results indicated that the shift of hydrophobicity of beetroot components after thermal degradation probably resulted in increased solubility of degradation products and their enhanced reactivity with DPPH radicals. Sawicki & Wiczkowski [2018] did not found a significant decrease of the antioxidant activity in red beet samples after boiling when the ABTS method was employed, however, if they used the DPPH method, the level of antioxidant activity was significantly lower. Nevertheless, the study of Sawicki & Wiczkowski [2018] was made with only one cultivar (Czerwona Kula), while our results indicate a significant effect of cultivar on betalain content, stability, and antioxidant activity. According to data presented by Sawicki & Wiczkowski [2018], boiling and fermentation of beetroot material increased the content of betanidin (aglycon of betanin), that showed activity in both aqueous and lipid bilayers [Slimen *et al*., 2017]. This fact could be responsible for the observed increase in the antioxidant activity of the boiled beetroot samples determined with the DPPH method.

TABLE 5. Correlation coefficients between betalain content or TPC and antioxidant activity of beetroot powders before and after boiling treatment.

	Before boiling	After boiling
BC content *vs*. AOA ABTS method	0.869***	0.986***
BC content *vs*. AOA DPPH method	0.794***	0.802***
BX content vs. AOA ABTS method	0.864***	0.987***
BX content *vs*. AOA DPPH method	0.806***	0.841***
BL content *vs*. AOA ABTS method	0.871***	0.988***
BL content *vs*. AOA DPPH method	0.801***	0.816***
TPC *vs*. AOA ABTS method	0.812***	0.982***
TPC *vs*. AOA DPPH method	0.831***	0.818***

*** correlation is significant at $p<0.001$; BC – betacyanins; BX – betaxanthins; BL – betalains; TPC – total phenolic content; AOA – antioxidant activity.

The antioxidant activity determined with $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ before boiling varied between cultivars from 5.65 (cv. Burpee's Golden) to 11.13 mg AAE/g DM (cv. Betina) and from 4.29 (cv. Chioggiea) to 8.68 mg AAE/g DM (cv. Betina), respectively. In turn, after boiling, the cultivar variability of antiradical activity against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ was between 2.03 (cv. Chioggia) and 11.69 mg AAE/g DM (cv. Betina) and between 8.36 (cv. Chioggia) and 17.59 mg AAE/g DM (Betina), respectively. The lowest values of the antioxidant activity were observed for the yellow and red-white cultivars Burpee's Golden and Chioggia in all evaluated variants. This indicates the significant effect of betalain content on the total antioxidant activity of both raw beetroot powders and beetroot products after thermal treatment. The highest content of betalains was determined in cv. Betina (Table 3). This cultivar had the highest antioxidant activity in both the treatment variants of the experiment. In general, the antioxidant activity of beetroot products is mostly defined as the same or the higher for thermally-processed variants in comparison with the original samples by most of the other authors [Gokhale & Lele, 2011; Ravichandran *et al*., 2012, 2013]. On the contrary, Vasconcellos *et al.* [2016] detected with the HPLC method about 10% reduction in the antioxidant activity of the samples of boiled red beetroot as compared with the freeze-dried ones. In the study of Gokhale & Lele [2011], the increased antioxidant activity found after beetroot thermal treatment was ascribed to a higher extractability of betaxanthin and total phenolic compounds. This conclusion was not confirmed in our study. The increase in the antioxidant activity found with the DPPH method is rather caused by degradation products because no increase was observed in BLs and TPCs after boiling. Our results concerning the significant effect of the cultivar on components exhibiting the antioxidant activity confirm data of Sawicki *et al.* [2016] who observed a similarly broad range of antioxidant activities with both ABTS and DPPH methods for the extracts of 13 red beetroot cultivars. These authors also observed a positive correlation between the antioxidant activity determined with ABTS and DPPH methods and total betalain and betacyanin content, which corresponds with our results presented in Table 5. The correlations between the antioxidant activity and contents of BCs, BXs, BLs, and TPCs were statistically significant and very similar in all of the comparisons. The highest correlation coefficients were found between antioxidant compound contents and results determined with the ABTS method for beetroot powders after boiling. Close correlations were also found between the content of total phenolics and antioxidant activity determined with ABTS and DPPH methods. Phenolic compounds belong to important beetroot antioxidant components [Chhikara *et al*., 2019; Guldiken *et al*., 2016], concentrated mainly in the peel and the crown [Kujala *et al*., 2002]. In our study, TPCs ranged from 2.58 to 3.73 mg GAE/g DM and below 1.5 mg GAE/g DM in the powder from red root cultivars and in the powder prepared from roots with yellow and red-white colour of root flesh, respectively. The highest content was found in the cultivar Betina, while the lowest in the red-white cultivars Chioggia and Burpee's Golden. This corresponded again with the content of betalains and total antioxidant activity of beetroot powder. The TPCs was significantly lower ($p<0.05$) after boiling in all the cultivars except for cultivar Alexis. The most intensive decrease of TPC was observed in cultivars with non-red colour of root flesh. Similarly, Guldiken *et al.* [2016] reported TPC values of 2.55 and 2.38 mg GAE/g FM for raw and boiled beetroot variants, respectively. However, the analyses were given for only one cultivar and on fresh weight basis. On the contrary, Vasconcellos *et al.* [2016] found TPC at 0.75 and 2.79 mg GAE/g DM for freeze-dried slices of red beetroot and for the boiled variant (100°C, 40 min), respectively. In generally, the content of total phenolics of the vegetables is significantly affected by cooking style. Results obtained by other authors [Naveena *et al*., 2016; Wen *et al*., 2010] indicated that phenolic compounds were sensitive to heat treatment, but Wen *et al.* [2010] and Raikos *et al.* [2018] reported that different types of the cooking process differently affected various vegetables and some combinations of vegetable species/cultivars and heat processing increased their phenolic contents while others decreased them. This phenomenon could be explained by direct interactions between phenolics and some food components or food matrix such as fibre, fat, proteins or carbohydrates, affecting their phenolic content and bioaccessibility [Şengül *et al*., 2013].

CONCLUSIONS

The obtained data indicated a significant effect of cultivar on betalain content of beetroot powders that varied for boiled and raw variant from 1.38 to 18.10 and from 1.16 to 15.04 mg/g DM, respectively. Interestingly, the content of betaxanthins was significantly lower in the powders from the red-white cultivar Chioggia and yellow cultivar Burpee's Golden compared to the powders from the red-coloured beetroot cultivars. The thermal treatment of beetroot powder resulted in a decrease of both betacyanin and betaxanthin contents, but also total phenolic content and antioxidant activity (except for the antioxidant activity of cv. Alexis) when assessed with the ABTS method. Interestingly, the use of the DPPH method resulted in a higher antioxidant activity determined in the boiled variant of beetroot powder in comparison with the non-boiled one. This indicates higher sensitivity of DPPH radical to degradation products exhibiting the antioxidant activity. Degradation of beetroot pigments during boiling treatment resulted in the shift of beetroot powder colour that was obvious especially for the yellow cultivar Burpee's Golden and red-white cultivar Chioggia. Beetroot powder before boiling was more intensive in all the evaluated cultivars. A decrease in L* parameter (lightness) was lower for cultivars Betina and Alexis. Especially the cultivar Betina with a high antioxidant activity, total phenolic and betalain contents, but also water and fat absorption and colour stability was found promising for thermal processing. The obtained data confirmed the importance of beetroot cultivar not only for their chemical composition but also for their suitability to thermal processing and subsequent use.

RESEARCH FUNDING

The present work was supported by the Grant Agency of the University of South Bohemia in České Budějovice, project GAJU 027/2019/Z and by the Ministry of Agriculture of the Czech Republic, project NAZV QJ1610324. Jan Mráz and Zuzana Linhartová were financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – project CENAKVA (LM2018099), CENAKVA Center Development (CZ.1.05/2.1.00/19.0380), and Biodiversity (CZ.02.1.01/0.0/0.0/16_025/0007370).

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ORCID IDs

J. Bárta http://orcid.org/0000-0002-6830-2369;
V. Bártová http://orcid.org/0000-0003-2865-6792;
M. Jarošová http://orcid.org/0000-0002-6846-3779;
Z. Linhartová http://orcid.org/0000-0001-8246-6020;
J. Mráz http://orcid.org/0000-0003-3545-1269;
J. Bedrníček http://orcid.org/0000-0003-0584-3923;
E. Samková https://orcid.org/0000-0002-9246-4456;
I. Laknerová http://orcid.org/0000-0001-7929-1127.

REFERENCES

1. Bach, V., Mikkelsen, L., Kidmose, U., Edelenbos, M. (2015). Culinary preparation of beetroot (*Beta vulgaris* L.): The impact on sensory quality and appropriateness. *Journal of the Science of Food and Agriculture, 95*, 1852–1859. https://doi.org/10.1002/jsfa.6886
2. Belhadj Slimen, I., Najar, T., Abderrabba, M. (2017). Chemical and antioxidant properties of betalains. *Journal of Agricultural and Food Chemistry, 65*(4), 675–689. https://doi.org/10.1021/acs.jafc.6b04208
3. Celli, G.B., Brooks, M.S.-L. (2017). Impact of extraction and processing conditions on betalains and comparison of properties with anthocyanins – A current review. *Food Research International, 100*, 501–509. https://doi.org/10.1016/j.foodres.2016.08.034
4. Chandran, J., Nisha, P., Singhal, R.S., Pandit, A.B. (2014). Degradation of colour in beetroot (*Beta vulgaris* L.): a kinetics study. *Journal of Food Science and Technology*, *51*, 2678–2684. https://doi.org/10.1007/s13197-012-0741-9
5. Chau, C., Chen, C., Lee, M. (2004). Comparison of the characteristics, functional properties, and *in vitro* hypoglycemic effects of various carrot insoluble fiber-rich fractions. *LWT – Food Science and Technology, 37*, 155–160. https://doi.org/10.1016/j.lwt.2003.08.001
6. Chhikara, N., Kushwaha, K., Sharma, P., Gat, Y., Panghal, A. (2019). Bioactive compounds of beetroot and utilization in food processing industry: A critical review. *Food Chemistry*, *272*, 192–200. https://doi.org/10.1016/j.foodchem.2018.08.022
7. Gengatharan, A., Dykes, G.A., Choo, W.S. (2015). Betalains: Natural plant pigment with potential application in functional foods. *LWT – Food Science and Technology, 64,* 645–649. https://doi.org/10.1016/j.lwt.2015.06.052
8. Gokhale, S.V., Lele, S.S. (2011). Dehydration of red beet root *(Beta vulgaris)* by hot air drying: process optimization and mathematical modelling. *Food Science and Biotechnology, 20*(4), 955–964. https://doi.org/10.1007/s10068-011-0132-4
9. Guldiken, B., Toydemir, G., Memis, K.N., Okur, S., Boyacioglu, D., Capanoglu, E. (2016). Home-processed red beetroot (*Beta vulgaris* L.) products: changes in antioxidant properties and bioaccessibility. *International Journal of Molecular Sciences*, *17*, art. no. 858. https://doi.org/10.3390/ijms17060858
10. Güneşer, O. (2016). Pigment and color stability of beetroot betalains in cow milk during thermal treatment. *Food Chemistry, 196*, 220–227. https://doi.org/10.1016/j.foodchem.2015.09.033
11. Hamouz, K., Lachman, J., Dvorak, P., Juzl, M., Pivec, V. (2006). The effect of site conditions, variety and fertilization on the content of polyphenols in potato tubers. *Plant Soil and Environment*, *52*, 407–412. https://doi.org/10.17221/3459-PSE
12. Herbach, K.M., Stintzing, F.C., Carle, R. (2004). Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparation. *Food and Chemical Toxicology, 69*, 491–498. https://doi.org/10.1111/j.1365-2621.2004.tb10994.x
13. Kazimierczak, R., Hallmann, E., Lipowski, J., Drela, N., Kowalik, A., Püssa, T., Matt, D., Luik, A., Gozdowski, D., Rembiałkowska, E. (2014). Beetroot (*Beta vulgaris* L.) and naturally fermented beetroot juices from organic and conventional production: Metabolomics, antioxidant levels and anticancer activity. *Journal of the Science of Food and Agriculture, 94*(13), 2618–2629. https://doi.org/10.1002/jsfa.6722
14. Kedare, S.B., Singh, R.P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science* and *Technology*, *48*, 412–422. https://doi.org/10.1007/s13197-011-0251-1
15. Kohajdová, Z., Karovičová, J., Kuchtová, V., Lauková, M. (2018). Utilisation of beetroot powder for bakery applications. *Chemical Papers*, *72*, 1507–1515.

https://doi.org/10.1007/s11696-018-0392-z

16. Kowalski, S.J., Szadzinska, J. (2014). Kinetic and quality aspect of beetroots dried in non-stationary conditions. *Drying Technology*, *32*, 1310–1318.
https://doi.org/10.1080/07373937.2014.915555
17. Kujala, T.S., Vienola, M.S., Klika, K.D., Lopocen, J.M., Pihlaja, K. (2002). Betalain and phenolic compositions of four beetroot (*Beta vulgaris*) cultivars. *European Food Research and Technology*, *214*(6), 505–510.
https://doi.org/10.1007/s00217-001-0478-6
18. Lee E.J., An, D., Nguyen, C.T., Patil, B.S., Kim, J., Yoo, K.S. (2014). Betalain and betaine coposition of greenhouse- or field--produced beetroot (*Beta vulgaris* L.) and inhibition of HepG2 cell proliferation. *Journal of Agricultural and Food Chemistry, 62*(6), 1324–1331.
https://doi.org/10.1021/jf404648u
19. Menon, L., Majundar, S.D., Ravi, U. (2015). Development and analysis of composite flour bread. *Journal of Food Science and Technology, 52*, 4156–4165.
https://doi.org/10.1007/s13197-014-1466-8
20. Naveena, N., Vishnuvardhana, R., Bhaskarachary, K. (2016). Effect of boiling and juicing on the content of polyphenols, *in vitro* bioaccessibility and antioxidant activity of commonly consumed vegetables and fruits. *The Indian Journal of Nutrition and Dietetics, 53*(4), art. no. 365.
https://doi.org/10.21048/ijnd.2016.53.4.8396
21. Ninfali, P., Donato, A. (2013). Nutritional and functional potential of *Beta vulgaris* cicla and rubra. *Fitoterapia*, *89*, 188–199.
https://doi.org/10.1016/j.fitote.2013.06.004
22. Nistor, O-V., Seremet Ceclu, L., Andronoiu, D.G. Rudi, L., Botez, E. (2017). Influence of different drying methods on the physicochemical properties of red beetroot (*Beta vulgaris* L. var. Cylindra). *Food Chemistry, 236*, 59–67.
https://doi.org/10.1016/j.foodchem.2017.04.129
23. Porto Dalla Costa, A., Hermes, V.S., de Oliveira Rios, Flôres, S.H. (2017). Minimally processed beetroot waste as an alternative source to obtain functional ingredients. *Journal of Food Science and Technology, 54*(7), 2050–2058.
https://doi.org/10.1007/s13197-017-2642-4
24. Raikos, V., McDonagh, A., Ranawana, V., Duthie, G. (2016). Processed beetroot (*Beta vulgaris* L.) as a natural antioxidant in mayonnaise: Effects on physical stability, texture and sensory attributes. *Food Science and Human Wellness, 5*(4), 191–198.
https://doi.org/10.1016/j.fshw.2016.10.002
25. Ranawana, V., Campbell, F., Bestwick, C., Nicol, P., Milne, L., Duthie, G., Raikos, V. (2016). Breads fortified with freeze-dried vegetables: quality and nutritional attributes: Part II: Breads not containing oil as an ingredient. *Foods*, *5*(4), art. no. 62.
https://doi.org/10.3390/foods5030062
26. Ravichandran, K., Ahmed, A.R., Knorr, D., Smetanka, I. (2012). The effect of different processing methods on phenolic acid content and antioxidant activity of red beet. *Food Research International, 48*(1), 16–20.
https://doi.org/10.1016/j.foodres.2012.01.011
27. Ravichandran, K., Saw, N.M.M.T., Mohdaly, A.A.A., Gabr, A.M.M., Kastell, A., Riedel, H., Cai, Z., Knorr, D., Smetanka, I. (2013). Impact of processing of red beet on betalain content and antioxidant activity. *Food Research International, 50*(2), 670–675.
https://doi.org/10.1016/j.foodres.2011.07.002
28. Sawicki, T., Bączek, N., Wiczkowski, W. (2016). Betalain profile, content and antioxidant capacity of red beetroot dependent on the genotype and root part. *Journal of Functional Foods, 27*, 249–261.
https://doi.org/10.1016/j.jff.2016.09.004
29. Sawicki, T., Wiczkowski, W. (2018). The effects of boiling and fermentation on betalain profiles and antioxidant capacities of red beetroot products. *Food Chemistry, 259*, 292–303.
https://doi.org/10.1016/j.foodchem.2018.03.143
30. Şengül, M., Yildiz, H., Kavaz, A. (2014). The effect of cooking on total polyphenolic content and antioxidant activity of selected vegetables. *International Journal of Food Properties, 17*(3), 481–490.
https://doi.org/10.1080/10942912.2011.619292
31. Slatnar, A., Stampar, F., Vebaric, R., Jakopic, J. (2015). HPLC-MS^n identification of betalains profile of different beetroot (*Beta vulgaris* L. ssp. *vulgaris*) parts and cultivars. *Journal of Food Science, 80*(9), C1952-C1958.
https://doi.org/10.1111/1750-3841.12977
32. Sorour, H., El-Mesery, H. (2014). Effect of microwave and infrared radiation on drying of onion slices. *International Journal of Applied Engineering Research, 2*, 119–130.
33. Sucu, C., Turp, G.Y., (2018). The investigation of the use of beetroot powder in Turkish fermented beef sausage (sucuk) as nitrite alternative. *Meat Science, 140*, 158–166.
https://doi.org/10.1016/j.meatsci.2018.03.012
34. Šulc, M., Lachman, J., Hamouz, K., Orsák, M., Dvořák, P., Horáčková, V. (2007). Selection and evaluation of methods for determination of antioxidant activity of purple- and red-fleshed potato varieties. *Chemické Listy*, *101*, 584–591.
35. Szopińska, A.A., Gaweda, M. (2013). Comparison of yield and quality of red beet roots cultivated using conventional, integrated and organic method. *Journal of Horticultural Research, 21*(1), 107–114.
https://doi.org/10.2478/johr-2013-0015
36. Vasconcellos, J., Conte-Junior, C., Silva, D., Pierucci, A.P., Paschoalin, V., Alvares, T.S. (2016). Comparison of total antioxidant potential, and total phenolic, nitrate, sugar, and organic acid contents in beetroot juice, chips, powder and cooked beetroot. *Food Science and Biotechnology, 25*(1), 79–84.
https://doi.org/10.1007/s10068-016-0011-0
37. Velíšek, J. (2014). *The Chemistry of Food*, 1st Edition, John Wiley & Sons, Chichester, West Soussex, United Kingdom, p. 1124.
38. Wen, T.N., Prasad, K.N., Yang, B., Ismail, A. (2010). Bioactive substance contents and antioxidant capacity of raw and blanched vegetables. *Innovative Food Science & Emerging Technologies*, *11*(3), 464–469.
https://doi.org/10.1016/j.ifset.2010.02.001
39. Wruss, J., Waldenberger, G., Huemer, S., Uygun, P., Lanzerstorfer, P., Müller, U., Höglinger, O., Weghuber, J. (2015). Compositional characteristics of commercial beetroot products and beetroot juice prepared from seven beetroot varieties grown in Upper Austria. *Journal of Food Composition and Analysis, 42*, 46–55.
https://doi.org/10.1016/j.jfca.2015.03.005

Submitted: 1 July 2020. Revised: 10 September and 15 October 2020. Accepted: 19 October 2020. Published on-line: 9 December 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 387–397
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/129015
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Sensory Assessment and Physicochemical Properties of Wheat Bread Supplemented with Chia Seeds

Stanisław Kowalski[1]*, *Anna Mikulec*[2], *Henryk Pustkowiak*[3]

[1]*Department of Carbohydrate Technology, Faculty of Food Technology, University of Agriculture in Krakow,*
ul. Balicka 122, 30–149 Krakow, Poland
[2]*State University of Applied Sciences in Nowy Sacz, ul. Staszica 1, 33–300 Nowy Sacz, Poland*
[3]*Department of Cattle Breeding, Faculty of Animal Science, University of Agriculture in Krakow,*
Al. Mickiewicza 24/28, 31–120 Krakow, Poland

Key words: wheat-chia bread, sensory profile, fatty acid profile, consumer acceptability test

Quantitative descriptive analysis, consumer acceptability as well as physicochemical characteristics were employed to analyse the effect of ground chia seeds on the texture and quality of wheat bread. Chia seed oil changes and its impact on selected characteristics were determined. The share of chia seeds did not contribute to bread quality deterioration. The addition of 7.5% chia seeds significantly reduced consumer acceptance as well as contributed to the development of undesirable features in the sensory profile, including fatty, rancid, and off-flavour. The share of chia seeds in an amount not exceeding 5% did not deteriorate the overall quality of bread. The composition of fatty acids of bread with chia seeds, especially with their 5 and 7.5% addition, could be valuable from the nutritional point of view. The fatty acid profile of bread with 5 and 7.5% chia seeds was characterised by over 50% higher polyunsaturated fatty acids to saturated fatty acids ratio compared to wheat bread. The *n*-6/*n*-3 ratio in the fatty acid profile of wheat bread was 18.77, while in the fatty acid profile of chia seed bread it ranged from 1.42 to 0.67. Oil extracted from bread exhibited better quality features as compared to oil extracted from chia seeds and subjected to the oil oxidative stability test.

INTRODUCTION

Chia (*Salvia hispanica* L.), a plant that belongs to the *Lamiaceae* family, has been known for many centuries. Due to nutritional and health effects, chia seeds become more and more popular in many countries, especially across the Europe. Nowadays, they have found various applications, such as functional food, animal feed, and cosmetics [Dinçoğlu & Yeşildemir, 2019]. Some studies have shown their consumption to yield beneficial effects on such health problems as insulin resistance, dyslipidemia, cardiovascular diseases, or inflammation [Vuksan *et al*., 2017]. In recent years, consumers expect food with additional health benefits resulting from its consumption. They seek for a healthy lifestyle, which is also associated with the growing food awareness.

In 1997, the European Parliament approved chia seeds as a novel food, which resulted in its increased use in food production. Consumption of chia seeds is not typical in the European culture, but bread is still a staple food product in the diet of many people. The addition of chia seeds to popular food products, including bread, can be a perfect dietary supplement and bring a number of health benefits for consumers. Additives used for food supplementation can, on the one hand, improve the nutritional value of bread, but on the other hand, contribute to its diminished sensory acceptance.

Chia seeds, due to their chemical composition, have a high nutritional potential. They contain protein of a high biological value with such essential amino acids as isoleucine, leucine, lysine, and valine. For this reason, chia seeds can be a supplement of cereal proteins that are deficient in essential amino acids, especially lysine. In addition, the seeds contain fatty acids, and in particular are a valuable source of *n*-3 and *n*-6 fatty acids [Sandoval-Oliveros & Paredes-Lopez, 2013; Vázquez-Ovando *et al*., 2010]. Due to the high content of fatty acids, including unsaturated ones, product with seeds of chia may be susceptible to rancid processes, which may lead to lowered consumer acceptance. Chia seeds are characterised by a high dietary fibre content, mainly the water soluble fraction [Vázquez-Ovando *et al*., 2010], and are an important source of natural antioxidants [Reyes-Caudillo *et al*., 2008]. They can be a valuable supplement in conventional therapy for overweight and obesity in diabetics because of the glycaemic control and a high dietary fibre content [Vuksan *et al*., 2017]. Mohd Ali *et al*. [2012] concluded that chia oil can maintain a balanced serum lipid profile, while Citelli *et al*. [2016] observed changes in the lipid profile in the liver caused by chia oil supplementation. Chia *inter alia* has been used for the production of bread [Coelho & Salas-Mellado, 2015; Hrušková & Švec, 2015; Miranda-Ramos *et al*., 2020; Romankiewicz *et al*., 2017], pasta [Oliveira *et al*., 2015], and even ice cream

---

* Corresponding Author: Tel.: +48 126624781;
E-mail: rrkowals@cyf-kr.edu.pl (S. Kowalski)

[Campos *et al*., 2016]. Several studies of bread with chia seeds showed that they contributed to the technological quality and texture parameters, depending on additive type – flour or seeds, and the method of their preparation. For example, Coelho & Salas-Mellado [2015] observed a decrease in the specific volume and the total score values, and an increase in the firmness of bread crumbs with chia seeds. In turn, Romankiewicz *et al*. [2017] noticed that the addition of chia seeds decreased baking losses, but also bread volume and crumb hardness, whereas Hrušková & Švec [2015] determined higher elasticity of dough containing hydrated chia resulting in firmer crumb compared to the bread with dry chia flour. All listed authors concluded that the addition of chia seeds increased the nutritional value of the bread, for example bread with chia contained more dietary fibre and mineral components. Products with chia seeds were characterised by a rich fatty acid composition and a higher content of phenolic compounds. Miranda-Ramos *et al*. [2020] pointed out that phytates of chia seeds may affect the bioavailability of such microelements like Zn and Fe. They also emphasised that the glycaemic index was lower in bread with chia ingredients compared to wheat bread, which in combination with nutritional benefits could be clinically important for the prevention of metabolic diseases. In sensory evaluation, the breads with chia seeds met with consumer acceptance. Romankiewicz *et al*. [2017] concluded that the substitution of wheat flour with chia seeds up to 6% did not negatively affect the final product acceptance.

Due to the composition of chia seeds, it was hypothesised that although their addition can improve the nutritional value of the bread, it may, on the other hand, contribute to a decreased consumer acceptance resulting from oxidative changes of fats. However, there is a lack of data regarding the influence of chia seeds on the oxidative stability of fats contained in the products with their addition. In this study, ground chia seeds were added to the dough, which can contribute to the better utilization of nutrients but also could result in faster oxidation changes of fats during bread baking. It should be emphasised that most of the studies on the nutritional, technological, and sensory value of bakery and pastry products are carried out with seeds or grains, defatted or refined flour. Considering that the intestinal transit time in humans is too short to digest whole seeds and grains, it seems important to supplement bread with crushed seeds or grains. In the present study, wheat flour was partially replaced with ground chia seeds, thanks to which the bread was enriched with all the valuable nutrients contained in the whole grain, which offers both technological, nutritional, and sensorial novelty. For this reason, the aim of this study was to evaluate the effect of substituting some part of wheat flour with ground chia seeds on selected physicochemical and textural properties, on the technological and sensory quality, as well as on the acceptance of bread with 2.5, 5, or 7.5% of ground chia seeds.

MATERIALS AND METHODS

Materials

Formulation and bread making method by Mikulec *et al*. [2019] was applied. Some part of wheat flour (WF) type 650 (PZZ Stoislaw, Poland) was replaced with ground chia seeds (Natura Food, Intenson Europe sp. z o. o., Poland) in the following wheat/chia ratios (*w/w*): 97.5/2.5 (WCh2.5); 95/5 (WCh5) and 92.5/7.5 (WCh7.5). The chia seeds were ground using a laboratory mill (KM13, Bosch, Gerlingen, Germany). Standard wheat bread (WS) was also prepared. Forty loaves were prepared for each sample from two batches.

Chemicals used in the experiment

The following chemicals were used for analyses: petroleum ether, chloroform, acetic acid glacial, potassium iodide, sodium thiosulfate, starch soluble phenolphthalein, sodium hydroxide, potassium hydroxide, sodium chloride, *n*-hexane, methyl orange, ethanol, sodium methylate (Avantor Performance Materials S.A., Gliwice, Poland), 12% boron trifluoride, Supelco 37 component FAME Mix and CLA isomers, MES hydrate, TRIZMA® base (Sigma-Aldrich Co., St. Louis, MO, USA), sulphuric acid, acetone (PPH Stanlab, Sp. z o.o., Lublin, Poland), Kjeltabs (Foss, Hillerød, Denmark), hydrochloric acid (PPUH Tarchem Sp. z o.o., Tarnowskie Góry, Poland), Tashiro indicator, boric acid, (Chempur, Piekary Śląskie, Poland), buffer solutions pH 4.0, 7.0, and 9.0 (Eurochem BGD. Sp. z o.o., Tarnów, Poland), and total dietary fibre assay kit (Megazyme, Bray, Ireland).

Analysis of the proximate composition of wheat flour and chia seeds

The contents of water (AOAC 925.10), protein (AOAC 950.36), crude fat (AOAC 935.38), starch (AOAC 996.11), ash (AOAC 923.03), total, and soluble and insoluble dietary fibre (AOAC 991.43) were determined following the Association of Official Analytical Chemists (AOAC) methods [AOAC, 2006]. Analyses was performed in duplicate. Results were expressed as g/100 g dry matter (d.m.).

Analysis of the basic quality features of bread

Two hours after baking, some instrumental quality parameters of bread, such as bread volume [AACC, 2000], total baking loss [Majzoobi *et al*., 2011], and crumb moisture (AOAC 925.10) [AOAC, 2006] were determined.

Texture analysis

Selected texture parameters of the bread crumb, such as hardness, cohesiveness, chewiness, gumminess, springiness, and resilience, were determined acording to the procedure described by Szcześniak [2002]. Cylindrical samples of crumb ($r = 40$ mm, $h = 30$ mm) were analysed with a TA.XT2.Plus texture analyzer (Stable Micro System, Surrey, UK) equipped with 5 kg load cell, using an aluminium, cylinder probe ($\varphi = 36$ mm), at the test speed of 2 mm/s, deformation of the entire sample height reaching 75%, and pause of 10 s. Measurements were done in ten replications.

Consumer acceptability

Consumer acceptability tests using a 9-point hedonic scale [Stone & Sidel, 2004] were performed by a panel consisting of 75 untrained potential consumers (20–60 years old, 49 females and 26 males) who were habitual consumers of bread. Consumers were recruited from different institutes of the State Higher Vocational School in Nowy Sacz, Poland (staff and students).

Sensory profile

The assessment was carried out in a sensory laboratory at the State University of Applied Sciences in Nowy Sącz, Poland, which meets the requirements of the Internatioanl Organization for Standarization (ISO) 8589 standard [ISO, 2010]. Descriptive sensory profiling of breads using a quantitative descriptive analysis (QDA), according to ISO 13299 standard [ISO, 2016] was carried out 4 h after baking by a panel of 8 assessors (in two independent repetitions) (n = 8, 7 women and 1 man, aged from 21 to 49), qualified as experts – according to ISO 8586 standard [ISO, 2014] and having appropriate methodological (theoretical and practical) preparation in the field of sensory methods and extensive experience in conducting assessments using quantitative descriptive analysis. Bread samples (35 g, a thickness of 12±2 mm) were placed on previously prepared and coded disposable paper trays. Pure water having a temperature of 20°C was used as a taste neutralizer between samples. Samples were presented to the assessors simultaneously.

The attributes of the analysed products were selected in accordance with the ISO 13299 procedure [ISO, 2016] and their definitions were established. Twenty qualitative attributes were selected and defined (Table 1). The 100-mm unstructured linear scale with marked boundary values from 0 to 10 conventional units (c.u.), where 0 meant no intensity of a given attribute, and 10 meant high intensity of a given attribute, was used. Results were calculated using Microsoft Excel 10.0 software (Microsoft, Redmond, WA, USA).

Analysis of oil extracted from bread and chia seeds

Chia seeds as well as bread supplemented with 7.5% chia seeds (WCh7.5) were analysed for oil fraction stability. The WCh7.5 sample was selected from among breads, due to the highest content of fat and possibly the greatest effect on the oxidative changes in fat. For this purpose, chia seeds/bread samples were milled using a laboratory mill (KM13, Bosch Germany), and the oil was extracted with petroleum ether (1:3, *w/v*) for 3 h with continuous stirring. After decanting, fresh petroleum ether was added to the solid residue, and the extraction procedure was repeated twice. After collection, all liquid phases were centrifuged and the supernatant was removed from the oil fraction by evaporation using a vacuum evaporator (RV 10 C S99, IKA, Königswinter, Germany).

The oils obtained were subjected to the following analyses:

Oil oxidative stability test

Chia oil extracted from chia seeds/bread was subjected to the oxidation stability test according to Pike [2001]. The analysis kit consisted of a purified compressed air cylinder with a flow regulator, and a thermostat. The air used for the measurement was drawn through the filter and the water vapour was removed by means of a molecular sieve. Oil sample was thermostated at 60°C under a constant air flow (20 L/h) and continuously aerated for 5 days. The samples were then analysed for peroxide and iodine values as well as free fatty acid content.

TABLE 1. Sensory attributes of breads used in the quantitative descriptive analysis (QDA).

Attribute	Definition	Word anchors (0–10 c.u.)
Appearance		
Beige colour of the crust	Visual perception of the bread crust colour	light → dark
Porosity	Visual perception of the bread crumb porosity/porous	poreless → porous
Odour		
Flour	Odour typical of flour	none → very intensive
Grain-seed	Odour typical of cereal grains, dried seeds, bran	none → very intensive
Alcoholic	Odour typical of the ethyl alcohol (ethanol)	none → very intensive
Buttery	Odour typical of butter	none → very intensive
Rancid	Odour typical of rancid oil	none → very intensive
Fatty	Odour typical of fat	none → very intensive
Off-odour	Smell of something other than "regular" smell of bread	none → very intensive
Flavour		
Sweet	Taste characteristic to sugar or substance containing or resembling sugar, as honey or saccharin (basic taste illustrated by sucrose diluted in water 1.5%)	none → very intensive
Bitter	The taste experience when quinine or coffee is taken into the mouth (basic taste illustrated by caffeine diluted in water, 0.5%)	none → very intensive
Grain-seed	Taste characteristic to cereal grains, dried seeds, bran	none → very intensive
Acidulous	The intensity of the acidulous taste	none → very intensive
Rancid	Taste typical of rancid oil	none → very intensive
Fatty	Taste typical of fat	none → very intensive
Off-flavour	Taste of something other than "regular" flavour of the bread	none → very intensive
Texture (mouth feel)		
Hardness	The force required to bite bread samples	not hard → hard
Chewiness	The energy required to chew a product until it is ready to be swallowed (the number of chews used to prepare the product to be swallowed)	not chewy → chewy
Adhesiveness	The force required to remove bread crumbs	low → high
Moistness	Slightly or moderately wet	dry → moist

Peroxide value determination

The analysis was done according to the 3960 ISO standard [ISO, 2017]. The peroxide value was expressed in mmol of active oxygen per kilogram of the sample. As a result, the mean of two parallel determinations was assumed.

Free fatty acid determination

Free fatty acid (FFA) content was determined according to Del Flores-Álvarez *et al*. [2012] procedure. The results were expressed as lauric acid equivalents. The assay was carried out in duplicate for each sample. FFA content was calculated as follows (Eq. 1):

$$\%\text{FFA as lauric acid} = \\ = \frac{\text{mL NaOH} \times \text{NaOH molarity} \times 20\ (\text{equivalent weights})}{\text{weight (g)}} \quad (1)$$

Iodine value determination

The test was made according to the European Committee for Standardization (CEN) DIN EN 14111 standard [CEN, 2003]. The iodine value was expressed as grams of I_2 absorbed by 100 g of the sample. As a result, the mean of two parallel determinations was assumed.

Determination of fatty acid profile

The extraction of total lipids was performed in duplicate according to the Folch *et al*. [1957] method, while the derivatisation and determination of total fatty acid composition were performed according to the AOAC – approved method 991.39 [AOAC, 2006]. Briefly, to the fat extracted (10 mg), 0.5 mL of 0.5 N KOH (methanol solution) was added and heated to 85°C, then 1 mL of 12% BF_3 (methanol solution) was added, and the mixture was reheated at 85°C. After cooling to room temperature, 1 mL of hexane and 5 mL of saturated NaCl solution were added. About 1 μL of the resulting sample was injected onto the column connected to the TRACE GC ULTRA chromatograph (Thermo Electron Corporation, Milano, Italy) with a flame ionization detector (FID). Operating parameters were as follows: FID temperature 250°C; dispenser temperature 220°C; oven temperature 160°C (3 min) to 210°C (3°C/min) (210°C, 35 min). SUPELCOWAX10 column (30 m, 0.25 mm, 0.25 μm) was used and helium was applied as a carrier gas at a flow rate of 1 mL/min. Split flow was 10 mL/min. Individual fatty acid methyl esters were identified by comparison to the standards of a mixture of Supelco 37 component FAME Mix, and of CLA isomers.

Nutritional properties

Nutritional properties of lipids were evaluated using three indexes: the atherogenicity index (AI), the ratio of hypocholesterolemic to hypercholesterolemic fatty acids (HH), and the thrombogenicity index (TI) (Eq. 2–4) [Ulbricht & Southgate, 1991]:

$$\text{AI} = \frac{\text{C12:0} + [4 \times \text{C14:0}] + \text{C16:0}}{\Sigma\text{MUFA} + \Sigma(\text{n}-6) + \Sigma(\text{n}-3)} \quad (2)$$

$$\text{HH} = \frac{\text{C18:1}n9 + \text{C18:2} + \text{C20:4} + \text{C18:3} + \text{C20:5} + \text{C22:5} + \text{C22:6}}{\text{C14:0} + \text{C16:0}} \quad (3)$$

$$\text{TI} = \frac{\text{C14:0} + \text{C16:0} + \text{C18:0}}{0.5 \cdot \text{C18:1} + 0.5(\text{MUFA-C18:1}) + 0.5 \times \text{PUFA}(n-6) + 3 \times \text{PUFA}(n-3) + \frac{\text{PUFA}(n-3)}{\text{PUFA}(n-6)}} \quad (4)$$

where: MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids.

Statistical analysis

The analysis of variance (ANOVA) and Duncan post-hoc test ($p < 0.05$) as well as principal component analysis (PCA) were performed using Statistica 13.3 (Stat Soft, Kraków, Poland).

RESULTS AND DISCUSSION

Analysis of the basic quality features of wheat flour and chia seeds

The wheat flour and chia seeds used for bread making differed significantly ($p<0.05$) in the content of proximate chemical components. In comparison with the wheat flour, chia seeds had approx. 1.5 times higher protein content, approx. 22 times higher crude fat content, approx. 10 times higher total dietary fibre content, and approx. 8 times higher mineral components content, which confirms their high nutritional value (Table 2). The chemical composition of chia seeds used in the present study is similar to that reported by other authors [Coelho & Salas-Mellado, 2015; Hrušková *et al*., 2015]. Therefore, substituting a part of wheat flour with ground chia seeds had to contribute to the increase in the nutritional value of the bread.

Analysis of the basic quality features of bread

The bread with chia seeds did not differ significantly in the total baking loss, which ranged from 14.25% for wheat bread to 14.75% for bread with 2.5% chia seeds (Table 3). No influence of chia seeds on bread volume was observed, which ranged from 883.25 mL for bread with 7.5% chia seeds to 897.67 mL for wheat bread. A decrease in crumb moisture was observed in the bread with chia seeds addition, on the day of baking (Table 3). Opposite results were obtained by Romankiewicz *et al*. [2017], who observed an increase in the baking loss after chia seeds addition and a reduction in bread volume, compared to wheat bread. However, Hrušková *et al*. [2015] obtained a larger volume of bread with chia flour, which was caused by the initial hydration of chia flour due to water absorption by chia polysaccharides. It is well known

TABLE 2. Chemical composition of chia seeds and wheat flour.

		Chia seeds	Wheat flour
Moisture (g/100 g)		6.87 ± 0.06^b	13.73 ± 0.06^a
Protein (g/100 g d.m.)		20.58 ± 0.01^a	14.12 ± 0.03^b
Crude fat (g/100 g d.m.)		33.99 ± 0.01^a	1.56 ± 0.01^b
Dietary fibre (g/100 g d.m.)	Soluble	3.45 ± 0.04^a	1.61 ± 0.01^b
	Insoluble	25.81 ± 0.05^a	1.36 ± 0.04^b
	Total	29.25 ± 0.01^a	2.97 ± 0.04^b
Ash (g/100 g d.m.)		4.96 ± 0.01^a	0.65 ± 0.00^b
Starch (g/100 g d.m.)		0.00 ± 0.00^a	73.13 ± 0.01^b

Values (mean ± standard deviation) in the same row marked with different letters are significantly different at $p<0.05$; d.m. – dry matter.

that free water evaporates quicker during baking and storage, compared to the bound one. Despite the reduction of the gluten protein in bread, a larger loaf volume was obtained as a result of the addition of a gluten-free raw material, such as chia flour. Bread volume depends on the type and amount of protein used in dough formulation, as well as on its interaction with starch. Although chia seeds have a high protein content, it cannot form a continuous gluten-like network, thus impairing water entrapment. Additionally, proteins compete with starch for water, delaying starch swelling and gelatinization [Gnanavinthan *et al*., 2010]. Therefore, in reference to our research and that of other authors, it can be concluded that the method of bread dough preparation, especially involving raw gluten-free and high-fibre content, can be very important for loaf volume, baking loss, or bread crumb moisture content.

Texture analysis

The hardness of the bread crumb is one of the most important and often determined parameters in bread texture studies. It is the main determinant of bread quality and is closely related to the perception of bread as fresh to consumers. The highest hardness on the day of baking was determined in the breads with 2.5 and 5% share of chia seeds, reaching 3.10 and 3.25 N, respectively (Table 3). There was no significant difference between the hardness of the wheat bread and bread with the 7.5% chia seed content. An increase in cohesiveness, gumminess, and chewiness of bread crumbs with chia seeds was observed, compared to wheat bread. A positive effect of 5 and 7.5% of chia seeds on the reduction of gumminess and chewiness of crumb was observed in comparison to other breads (Table 3). The changes in the texture of the bread crumb were mainly due to the decreased gluten content caused by the addition of chia seeds, an ingredient with a higher dietary fibre and fat content [Collar *et al*., 2007]. Bread dough is a complex system in which the interaction between the different ingredients may cause various effects. Depending on their amount, the additives used in bread making can change textural properties, for example fat restricts starch swelling during baking. During baking, anti-firming components interact with wheat flour starch molecules and decrease starch swelling. As a result, less surface area is exposed to gluten and weaker cross-linking with protein occurs; therefore, the firming rate is reduced [Scheuer *et al*., 2016]. For resilience, a significant increase ($p<0.05$) was observed in the bread with 7.5% share of chia seeds (Table 3). Coelho & Salas-Mellado [2015] obtained similar results; they observed an increase in the firmness of bread crumbs with chia on the baking day. Meanwhile, Romankiewicz *et al*. [2017] reported a 4 to 8% decrease in the crumb hardness, gumminess, and chewiness of bread with chia addition on the baking day, compared to the standard one. Comparing the texture profile assessed by the sensory panel to the results obtained from

TABLE 3. Characteristics of wheat bread and breads with addition of chia seeds.

Bread type	WS	WCh2.5	WCh5	WCh7.5
Physicochemical parameters				
Total baking loss (%)	14.25±0.55^{a}	14.75±0.60^{a}	14.28±0.60^{a}	14.60±0.46^{a}
Volume (mL)	898±13^{a}	892±12^{a}	885±10^{a}	883±10^{a}
Moisture of bread crumb (g/100 g)	45.70±0.01^{a}	45.28±0.09^{b}	45.17±0.13^{b}	45.30±0.08^{b}
Texture parameters				
Hardness (N)	2.74±0.12^{b}	3.10±0.13^{a}	3.25±0.12^{a}	2.79±0.12^{ab}
Springiness (-)	0.94±0.03^{ab}	0.89±0.01^{b}	0.89±0.04^{b}	0.96±0.02^{a}
Cohesiveness (-)	0.66±0.03^{d}	0.81±0.01^{b}	0.78±0.02^{c}	0.85±0.02^{a}
Gumminess (N)	2.12±0.09^{c}	3.54±0.07^{a}	2.49±0.16^{b}	2.52±0.09^{b}
Chewiness (N·mm)	1.96±0.09^{c}	3.34±0.13^{a}	2.69±0.14^{b}	2.84±0.17^{b}
Resilience (-)	0.40±0.01^{b}	0.40±0.02^{b}	0.41±0.01^{b}	0.51±0.01^{a}
Consumer acceptability				
Taste	8.9±0.30^{a}	8.8±0.47^{a}	8.3±0.48^{b}	7.5±0.45^{c}
Texture	8.9±0.30^{a}	8.8±0.40^{a}	8.2±0.31^{b}	7.6±0.32^{c}
Aroma	8.7±0.47^{a}	8.6±0.50^{a}	8.4±0.68^{b}	7.6±0.65^{c}
Crumb colour	8.8±0.40^{a}	8.7±0.67^{a}	8.1±0.53^{b}	7.1±0.48^{c}
Crust colour	8.8±0.42^{a}	8.7±0.51^{a}	8.0±0.42^{b}	7.2±0.67^{c}
Overall acceptability	8.9±0.30^{a}	8.8±0.52^{a}	8.2±0.45^{b}	7.3±0.42^{c}

WS – wheat bread, WCh2.5 – bread with 2.5% addition of chia seeds, WCh5 – bread with 5% addition of chia seeds, WCh7.5 – bread with 7.5% addition of chia seeds. Values (mean ± standard deviation) in the same row marked with different letters are significantly different at $p<0.05$.

instrumental tests, the evaluators did not evaluate the hardness of WCh7.5 at a similar level as for wheat bread (Table 4). It may be due to the fact that only crumb hardness was analysed using a texturometer, in comparison to whole bread slices in the QDA.

Consumer acceptability

Consumer preference assessments provide very important and valuable information for food and drink producers, however they are not sufficient. Food producers should meet the expectations and needs of consumers, because without consumer acceptance they will not achieve the intended success. A new product should meet the certain level of quality required by the consumers because only a few percent of new products are fitted on the market.

In the consumer acceptance test, wheat bread and bread with 2.5% of chia seeds obtained similar scores (Table 3) and did not differ significantly ($p \geq 0.05$). Other breads differed ($p<0.05$) by means of the organoleptic evaluation compared to WS and WCh2.5, as well as differed among each other. The lowest scores were given to the bread with a 7.5% share of chia seeds. This bread obtained much lower scores, especially for the colour of crumb and crust and overall acceptability, which ranged from 7.1 to 7.3 (Table 3).

Other authors observed deterioration in the organoleptic characteristics, lower rating and consumer acceptance of bread with chia seeds [Coelho & Salas-Mellado, 2015; Hrušková *et al*., 2015]. Hrušková *et al*. [2015] observed lower scores given for the appearance, texture, and overall acceptability to the bread with 8% addition of chia seeds. Deterioration of the organoleptic properties of bread enriched with such non-cereal additives like hemp [Mikulec *et al*., 2019], cistus extract [Mikulec *et al*., 2020], herbs [Adams *et al*., 2011], and others can be explained by the content of essential oils that cause its specific taste and smell. The same explanation can be applied to the changes observed in the present study.

Descriptive sensory profiling

The manufacturer must know not only how product is rated by consumers, but also why it has received a specific rating. Sensory profile analysis provides valuable answers to the "why" question. The method of sensory profiling (QDA) assumes that sensory characteristics are not homogeneous, but consist of a number of individual distinguishing attributes, a significant part of which can be identified and separately analysed.

In the sensory profile of the obtained breads, the flour and alcoholic odours were significantly less perceptible in the breads with 5% share of chia seeds, but significantly more grain-seed odour and flavour were detected in all breads with chia seeds (Table 4), compared to wheat bread. The share of chia seed contributed to the appearance of fatty flavour as well as rancid and off-flavour, mainly in WCh7.5 (Table 4). Regarding the textural properties, the assessors did not observe the difference between chia seed breads and wheat bread.

TABLE 4. Intensity of the sensory attributes of breads.

Attribute	WS	WCh2.5	WCh5	WCh7.5
Crust colour	3.0 ± 0.5^{c}	3.2 ± 0.5^{c}	4.5 ± 0.7^{b}	7.5 ± 0.2^{a}
Porosity	9.5 ± 1.6^{a}	9.2 ± 0.9^{a}	8.6 ± 1.9^{a}	8.4 ± 2.2^{a}
Flour (odour)	3.1 ± 0.4^{a}	2.8 ± 0.6^{a}	1.1 ± 0.3^{b}	0.9 ± 0.3^{b}
Grain-seed (odour)	0.6 ± 0.5^{d}	2.1 ± 0.5^{c}	3.9 ± 0.5^{b}	5.5 ± 0.9^{a}
Alcoholic (odour)	3.6 ± 1.0^{a}	2.3 ± 0.8^{a}	1.4 ± 0.5^{b}	1.3 ± 0.3^{b}
Buttery (odour)	0.5 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Rancid (odour)	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.5 ± 0.5^{a}
Fatty (odour)	0.0 ± 0.0^{d}	0.9 ± 0.2^{c}	1.5 ± 0.5^{b}	2.5 ± 0.5^{a}
Off-odour	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.5 ± 0.5^{a}
Sweet	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Bitter	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Grain-seed (flavour)	0.6 ± 0.2^{d}	3.5 ± 0.5^{c}	5.3 ± 1.1^{b}	6.6 ± 1.9^{a}
Acidulous	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Rancid (flavour)	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.6 ± 0.5^{a}	2.5 ± 1.5^{a}
Fatty (flavour)	0.0 ± 0.0^{d}	1.5 ± 0.6^{c}	$2.50+0.9^{b}$	4.5 ± 1.5^{a}
Off-flavour	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	2.1 ± 0.9^{a}
Hardness	3.6 ± 1.5^{a}	4.1 ± 2.2^{a}	4.3 ± 1.9^{a}	5.4 ± 2.3^{a}
Chewiness	4.5 ± 1.8^{a}	5.1 ± 2.5^{a}	4.8 ± 1.0^{a}	5.2 ± 0.9^{a}
Adhesiveness	8.6 ± 1.2^{a}	8.2 ± 0.9^{a}	7.8 ± 0.5^{a}	7.4 ± 0.8^{a}
Moistness	5.6 ± 0.5^{a}	6.1 ± 0.7^{a}	5.8 ± 0.9^{a}	4.9 ± 1.1^{a}

WS – wheat bread, WCh2.5 – bread with 2.5% addition of chia seeds, WCh5 – bread with 5% addition of chia seeds, WCh7.5 – bread with 7.5% addition of chia seeds. Values (mean ± standard deviation) in the same row marked with different letters are significantly different at $p<0.05$.

The PCA analysis allows finding dependencies in a data set, better data representation, classification, pattern searching, or dimension reduction. Values are not evenly distributed along all directions of the multidimensional coordinate system, but show concentrations in certain subspaces. Finding these subspaces allows seeing patterns that are invisible or unnoticeable in the original data layout. The PCA transformation determines the correlation that occurs between multiple variables in a data set. If the data are correlated, then knowing only some of them is enough to determine the characteristics of the tested product [Jolliffe & Cadima, 2016].

The PCA of textural and sensory data of the analysed breads shows that principal component 1 and principal component 2 described 91.42% of the total variation. The PCA plots (Figure 1a and 1b) indicate that standard wheat bread (WS) was closely related to porosity, alcoholic odour, and adhesiveness. WCh2.5 bread was well correlated with moisture content, hardness, and gumminess (both parameters from instrumental texture analysis). WCh7.5 bread was related to such sensory attributes like crust colour, grain-seed, rancid and off-odour, rancid and off-flavour, and with such instrumental texture parameters like springiness and resilience. In turn, WCh5 bread showed a poor correlation with the attributes correlated with the first principal component but correlated with grain-seed, and off-odour, grain-seed, and fatty taste as well as with hardness from sensory assessment.

TABLE 5. Quality characteristics of chia oil during oxidative stability test.

Sample	Time (day)	Iodine value (g I_2/100 g)	Peroxide value (mmol O_2/kg)	Free fatty acid content (mg NaOH/g)
Oil extracted from bread WCh7.5 (OFB)	0	86.12 ± 0.39^{a}	22.51 ± 0.49^{b}	3.41 ± 0.15^{c}
	5	86.66 ± 0.59^{a}	37.93 ± 0.84^{a}	6.24 ± 0.04^{b}
Oil extracted from chia seed (OCS)	0	85.70 ± 0.79^{a}	5.04 ± 0.17^{c}	0.85 ± 0.1^{d}
	5	88.73 ± 2.99^{a}	37.74 ± 0.22^{a}	8.79 ± 0.03^{a}

WCh7.5 – bread with 7.5 % addition of chia seeds. Values (mean ± standard deviation) in the same columns marked with different letters are significantly different at $p<0.05$.

Analysis of bread and chia oil oxidative stability

During storage, fat may undergo some changes as a result of hydrolysis, oxidation, and polymerization processes, which lead to undesirable organoleptic and chemical changes, lowering the nutritional value as well as consumer acceptance. Such changes can also result in the formation of compounds harmful to health. Especially changes among the fat fraction result in undesired sensory deterioration. There are many factors influencing the oil stability, like fatty acid composition, presence of thermally-oxidised compounds, oxidative enzymes, and antioxidants [Manzocco *et al*., 2016].

According to the Codex Alimentarius [Joint WHO/FAO Codex Alimentarius Commission, 2017] requirements, in cold-pressed oils, the content of free fatty acids resulting from fat hydrolysis should be ≤4.0 mg KOH/g and the peroxide value should be at <15 mmol O_2/kg. Oil samples from chia seed (OCS) and from bread with 7.5% of chia seeds (OFB), extracted on the baking day, met the requirements specified in the Codex Alimentarius [Joint WHO/FAO Codex Alimentarius Commission, 2017] for the free fatty acid content (Table 5). After five days of storage, the FFA content of OFB increased to 6.24 mg KOH/g (almost double) and in OCS to 8.79 mg KOH/g (more than 10-fold). The peroxide value of OCS did not exceed the allowable level on the first day of the oxidation stability test. The peroxide value of OFB was higher compared to OCS, which may be due to the thermal processes that take place in bread during baking. Qualitative changes in OCS after five days of aerating at 60°C corresponded to the changes that took place in OFB. The iodine value, which is a measure of the degree of unsaturation of fats used to identify them in products, did not change during bread storage, compared to the value obtained on the bread baking day. For OCS, it changed slightly from 85.70 to 88.73 g I_2/100 g (Table 5) and the increase observed was statistically insignificant ($p \geq 0.05$). The obtained results indicate that in breads after 5 day of storage, the process of chia seeds fat oxidation was less advanced as compared to pure chia seeds oil extracted from raw material and subjected to the oil oxidative stability test. The high value of the iodine value indicates a high content of unsaturated fatty acids in the tested samples, which was confirmed by the fatty acid profile. Similar observations were made by Maire *et al*. [2013] who investigated baked products enriched with raw materials, being the source of unsaturated

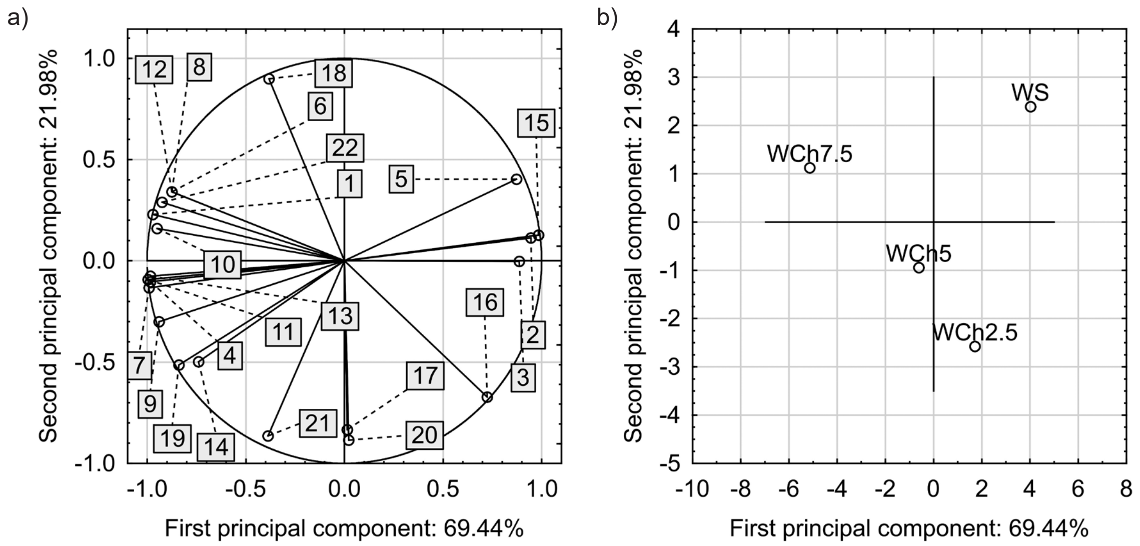


FIGURE 1. a) Projection of variables on the factor plane x: Crust colour (1), Porosity (2), Flour (3), Grain-seed (odour) (4), Alcoholic (odour) (5), Rancid (odour) (6), Fatty (odour) (7), Off-odour (8), Grain-seed (taste) (9), Rancid (taste) (10), Fatty (taste) (11), Off-flavour (taste) (12), Hardness (13), Chewiness (14), Adhesiveness (15), Moistness (16), Hardness txt (17), Springiness txt (18), Cohesiveness txt (19), Gumminess txt (20), Chewiness txt (21), Resilience txt (22); b) Projection of cases on the factor plane x: WS – wheat bread, WCh2.5 – bread with 2.5% of chia seeds, WCh5 – bread with 5% of chia seeds, WCh7.5 – bread with 7.5% of chia seeds.

TABLE 6. Fatty acid profile and fat nutritional quality of chia seeds and supplemented breads.

Fatty acids	Chia seeds	WS	WCh2.5	WCh5	WCh7.5
Content (% of total fatty acids)					
12:0	0.004±0.001^{d}	0.028±0.003^{a}	0.011±0.000^{b}	0.007±0.000^{c}	0.007±0.000^{c}
14:0	0.027±0.004^{d}	0.233±0.009^{a}	0.101±0.002^{b}	0.076±0.000^{c}	0.071±0.005^{c}
15:0	0.013±0.001^{e}	0.212±0.004^{a}	0.089±0.000^{b}	0.078±0.001^{c}	0.052±0.000^{d}
16:0	6.178±0.092^{e}	20.636±0.201^{a}	16.308±0.219^{b}	14.625±0.541^{c}	13.341±0.476^{d}
16:1*n*-9	0.046±0.007^{e}	0.560±0.0040^{a}	0.199±0.019^{b}	0.141±0.011^{c}	0.098±0.003^{d}
16:1*n*-7	0.062±0.004^{e}	0.298±0.004^{a}	0.221±0.002^{b}	0.149±0.009^{c}	0.119±0.006^{d}
17:0	0.028±0.000^{e}	0.091±0.004^{b}	0.072±0.001^{c}	0.103±0.000^{a}	0.063±0.000^{d}
17:1	0.011±0.001^{d}	0.042±0.006^{a}	0.034±0.000^{b}	0.027±0.003^{c}	0.022±0.009^{c}
18:0	2.178±0.095^{c}	0.801±0.021^{d}	2.161±0.012^{c}	3.166±0.065^{b}	3.376±0.035^{a}
18:1*n*-9	4.699±0.057^{e}	9.380±0.047^{a}	7.405±0.057^{b}	6.998±0.052^{c}	6.513±0.133^{d}
18:1*n*-7	0.623±0.033^{d}	0.817±0.044^{a}	0.724±0.006^{c}	0.619±0.012^{d}	0.741±0.005^{b}
18:2*n*-6	18.658±0.062^{e}	63.155±0.181^{a}	42.421±0.139^{b}	30.724±0.401^{c}	30.072±0.028^{d}
18:3*n*-3	67.216±0.085^{a}	3.365±0.042^{e}	29.876±0.025^{d}	42.813±0.136^{c}	45.097±0.263^{b}
20:0	0.172±0.004^{d}	0.078±0.013^{e}	0.180±0.001^{c}	0.318±0.009^{a}	0.277±0.003^{b}
20:1	0.088±0.000^{d}	0.308±0.008^{a}	0.200±0.008^{b}	0.160±0.009^{c}	0.155±0.005^{c}
Nutritional indexes of fat					
Σ SFA	8.600	22.079	18.922	18.373	17.187
Σ MUFA	5.529	11.405	8.783	8.094	7.648
Σ PUFA	85.874	66.520	72.297	73.537	75.169
AI	0.06	0.28	0.21	0.18	0.17
TI	0.04	0.46	0.16	0.12	0.11
HH	14.6	3.64	4.86	5.48	6.09
PUFA/SFA	9.98	3.01	3.82	4.00	4.37
n-6/*n*-3	0.28	18.77	1.42	0.72	0.67

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; AI – atherogenicity index; HH – ratio of hypocholesterolemic to hypercholesterolemic fatty acids, TI – thrombogenicity index; WS – wheat bread, WCh2.5 – bread with 2.5% addition of chia seeds, WCh5 – bread with 5% addition of chia seeds, WCh7.5 – bread with 7.5% addition of chia seeds. Values (mean ± standard deviation) in the same row marked with different letters are significantly different at $p<0.05$.

fats, and observed that lipid oxidation occurred at a very early stage (during dough preparation) and to a minor extent during the baking process.

Determination of fatty acid profile

Chia seed oil can be an alternative for vegetarians and people who are allergic to fish products [Zettel & Hitzmann, 2018]. PUFA *n*-6 and *n*-3 are essential fatty acids that must be derived from the diet. The human body does not have a suitable enzyme for the endogenic synthesis of α-linolenic and linoleic acid, due to the lack of desaturases introducing double bonds at the 3rd and 6th carbon atoms in the carbon chain. These acids are precursors for the synthesis of long-chain polyene fatty acids such as eicosapentaenoic acid and docosahexaenoic acid [Martynowicz *et al*., 2019].

In the fatty acid profile of chia seeds as well as bread supplemented with chia seeds, predominant were the 18-carbon chain fatty acids, with their contents reaching up to 82.42% in WCh7.5 and 93.37 in chia seeds (Table 6).

α-Linolenic acid (18:3n-3) had the greatest share in the fatty acid profile of chia seeds and the lowest one in the fatty acid profile of wheat bread (67.216 and 3.365%, respectively). Analyses showed a significant increase in the share of 18:3*n*-3 in the fatty acid profile of breads along with chia seed content increase from 29.876 in WCh2.5 to 45.097% in WCh7.5, and a significant decrease in the share of linoleic acid (18:2*n*-6) in the fatty acid profile of breads with increasing chia seed content. In the fatty acid profile of wheat bread, a much greater share of 18:2*n*-6 was observed compared to the profiles of other breads (Table 6). Osuna *et al*. [2016] and Melilli *et al*.

[2020] observed similar proportions in the fatty acid profile of wheat bread.

Chia oil is a valuable source of α-linolenic acid and phytosterols. The nutritional value of bread with chia seeds was evaluated by such nutritional indexes as AI, HH, TI, PUFA/SFA, and the *n*-6/*n*-3 ratio. These indexes evaluate the nutritional quality of foods based on their fatty acid compositions. AI and TI can be used as predictors or risk factors for cardiovascular diseases and should be kept at low levels in a healthy everyday diet [Ulbricht & Southgate, 1991]. The AI ranged from 0.17 for WCh7.5 to 0.28 for WS, meanwhile for chia seeds it reached 0.06. A decrease in the value of this index was observed along with an increase in chia seed content in bread. The TI ranged from 0.11 to 0.46 for WCh7.5 and wheat bread, respectively, and was 0.04 for chia seeds. The HH index indicates the effect of fatty acids on cholesterol metabolism and its high value is important from a nutritional point of view. The HH values ranged from 3.64 for WS to 6.09 for WCh7.5 (14.6 for chia seeds). An increase in the value of this index was observed with the increase in chia seed content in bread. Another increase was observed in the breads with chia seeds for the PUFA/SFA ratio. Its values ranged from 3.01 (WS) to 4.37 (WCh7.5), while for chia seeds it was 9.98 (Table 6). A low PUFA/SFA ratio in diets is considered a risk factor for increased blood cholesterol levels. In the present study, the PUFA/SFA ratio determined for the breads with chia seeds was by 0.8 to 1.4 times higher than that determined for wheat bread. Due to a high content of linolenic acid in chia seeds, their addition to bread formulations caused a significant decrease in the *n*-6/*n*-3 ratio which ranged from 1.42 (WCh2.5) to 0.67 (WCh7.5). While, for wheat bread, its value was as high as 18.77. Modern western diets provide excessive levels of *n*-6 but very low levels of *n*-3 fatty acids, leading to an undesired (from a nutritional point of view) *n*-6/*n*-3 ratio, characterised by 10- to 20-fold predominance of *n*-6 acids [Simopoulos, 2008]. Therefore, the ratio of 18:3*n*-3 to 18:2*n*-6 in chia seeds and hence bread supplemented with this seeds may have beneficial effects on the human health [Smith *et al*., 2011]. Other authors observed similar relationships regarding the improvement of the nutritional value of bread upon the addition of functional ingredients, like purslane flour [Melilli *et al*., 2020] or oilseeds, such as flax or soybeans [Osuna *et al*., 2016]. Therefore, consuming the bread with chia seeds would help increase the *n*-3 intake in the dietary habits of the population. Overall, the nutritional indexes used for quality evaluation indicated that breads with chia seeds, especially with their 5% and 7.5% content, could have beneficial health effects for consumers.

CONCLUSION

The wheat grain and chia seeds used for bread making differed significantly in the content of proximate chemical components. The addition of chia seeds to dough formulations at the three levels tested did not deteriorate bread quality. The total baking loss and loaf volume of the breads with chia seed addition did not differ significantly from these of the wheat bread. The sensory and organoleptic quality of food products is considered to be the key factor driving consumers' purchase decisions. The chia seed content not exceeding 5% did not contribute to reducing the overall sensory quality of bread. The addition of 7.5% chia seeds significantly reduced consumer acceptance due to the appearance of fatty, rancid, and off-flavour. The chemical composition of chia seeds, especially the composition of fatty acids, is very valuable from the nutritional point of view. Enrichment and fortification of bakery products with chia seeds could be justified for nutritional reasons. The results do not confirm the negative impact of chia seeds on shortening the bread shelf life resulting from oxidative changes of fats.

RESEARCH FUNDING

This research was financed by the Ministry of Science and Higher Education of the Republic of Poland from founds for year 2020.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ORCID IDs

S. Kowalski https://orcid.org/0000-0001-5269-0291
A. Mikulec https://orcid.org/0000-0002-2737-5967

REFERENCES

1. AACC. (2000). *Approved Methods of the AACC (10th ed.)*. St Paul, MN: USA: American Association of Cereal Chemists.
2. Adams, A., Kruma, Z., Verhé, R., De Kimpe, N., Kreicbergs, V. (2011). Volatile profiles of rapeseed oil flavored with basil, oregano, and thyme as a function of flavoring conditions. *Journal of the American Oil Chemists' Society, 88*(2), 201–212. https://doi.org/10.1007/s11746-010-1661-3
3. AOAC. (2006). *Official Methods of Analysis (18th ed.)*. Gaithersburg, MD: AOAC – Association of Analytical Chemists International.
4. Campos, B., Ruivo, T., Scapim, M., Madrona, G., Bergamasco, R. (2016). Optimization of the mucilage extraction process from chia seeds and application in ice cream as a stabilizer and emulsifier. *LWT – Food Science and Technology, 65*, 874–883. https://doi.org/10.1016/j.lwt.2015.09.021
5. Citelli, M., Fonte-Faria, T., Vargas-Silva, S., Barja-Fidalgo, C. (2016). Dietary supplementation with chia (*Salvia hispanica* L.) oil reduces the complications caused by high fat diet-induced obesity. *The FASEB Journal, 30*(1 Supplement), 907–920.
6. Coelho, M.S., Salas-Mellado, M. (2015). Effects of substituting chia (*Salvia hispanica* L.) flour or seeds for wheat flour on the quality of the bread. *LWT – Food Science and Technology, 60*(2), 729–736. https://doi.org/10.1016/j.lwt.2014.10.033
7. Collar, C., Santos, E., Rosell, C.M. (2007). Assessment of the rheological profile of fibre-enriched bread doughs by response surface methodology. *Journal of Food Engineering*, *78*(3), 820–826. https://doi.org/10.1016/j.jfoodeng.2005.11.026

8. Del Flores-Álvarez, C., Molina-Hernández, E.F., Hernández-Raya, J.C., Sosa-Morales, M.E. (2012). The effect of food type (fish nuggets or french fries) on oil blend degradation during repeated frying. *Journal of Food Science, 77*(11), C1136-C1143. https://doi.org/10.1111/j.1750-3841.2012.02930.x
9. Dinçoğlu, A.H., Yeşildemir, Ö. (2019). A renewable source as a functional food: chia seed. *Current Nutrition & Food Science, 15*(4), 327–337. https://doi.org/10.2174/1573401314666180410142609
10. European Committee for Standardization. (2003). *Fat and oil derivatives – Fatty acid methylesters (FAME) – Determination of iodine value* (DIN EN standard No.14111).
11. Folch, J., Lees, M., Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry, 226,* 497–509.
12. Gnanavinthan, A., Sun-Waterhouse, D., Quek, S., Perera, C. (2010). Properties of bread dough with added fiber polysaccharides and phenolic antioxidants: a review. *Journal of Food Science, 75*(8), R163-R174. https://doi.org/10.1111/j.1750-3841.2010.01815.x
13. Hrušková , M., Švec, I. (2015). Chemical, rheological and bread characteristics of wheat flour influenced by different forms of chia (*Salvia hispanica* L.). *Emirates Journal of Food and Agriculture, 27*(12), 872–877. https://doi.org/10.9755/ejfa.2015-04-073
14. Internatioanl Organization for Standarization. (2017). *Animal and vegetable fats and oils – Determination of peroxide value – Iodometric (visual) endpoint determination* (ISO standard No.3960).
15. Internatioanl Organization for Standarization. (2010). *Sensory analysis – General guidance for the design of test rooms* (ISO standard No.8589).
16. Internatioanl Organization for Standarization. (2016). *Sensory analysis. Methodology – General guidance for establishing a sensory profile* (ISO standard No.13299).
17. Internatioanl Organization for Standarization. (2014). *Sensory analysis – General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors* (ISO standard No.8586).
18. Joint FAO/WHO Codex Alimentarius Commission. (2017). *Codex Alimentarius*. *Standard for named vegetable oils*. Codex stan 210–1999, revision 2001, 2003, 2009, 2017. Amendment 2005, 2011, 2013 and 2015.
19. Jolliffe, I.T., Cadima, J. (2016). Principal component analysis: a review and recent developments. *Philosophical Transactions of The Royal Society A: Mathematical Physical and Engineering Sciences, 374*(2065), art. no. 20150202. https://doi.org/10.1098/rsta.2015.0202
20. Maire, M., Rega, B., Cuvelier, M.E., Soto, P., Giampaoli, P. (2013). Lipid oxidation in baked products: impact of formula and process on the generation of volatile compounds. *Food Chemistry*, *141*(4), 3510–3518. https://doi.org/10.1016/j.foodchem.2013.06.039
21. Majzoobi, M., Farahnaky, A., Agah, S. (2011). Properties and shelf-life of part and full-baked flat bread (Barbari) at ambient and frozen storage. *Journal of Agricultural Science and Technology, 13,* 1077–1090.
22. Manzocco, L., Calligaris, S., Anese, M., Nicoli, M. (2016). Determination and prediction of shelf life of oils/fats and oil/fat–based foods. In M. Hu, & C. Jacobsen (eds.), *Oxidative Stability and Shelf Life of Foods Containing Oils and Fats*. AOCS American Oil Chemists' Society, pp.133–156. https://doi.org/10.1016/B978-1-63067-056-6.00002-1
23. Martynowicz, H., Jodkowska, A., Nowacki, D., Mazur, G. (2019). A closer look at polyunsaturated fatty acids and hypertension. *Advances in Hygiene and Experimental Medicine, 73,* 102–108. https://doi.org/10.5604/01.3001.0013.0645
24. Melilli, M.G., Di Stefano, V., Sciacca, F., Pagliaro, A., Bognanni, R., Scandurra, S., Virzì, V., Gentile, C., Palumbo, M. (2020). Improvement of fatty acid profile in durum wheat breads supplemented with *Portulaca oleracea* L. Quality traits of purslane--fortified bread. *Foods, 9*(6), art. no. 764. https://doi.org/10.3390/foods9060764
25. Mikulec, A., Kowlaski, S., Makarewicz, M., Skoczylas, Ł., Tabaszewska, M. (2020). Cistus extract as a valuable component for enriching wheat bread. *LWT – Food Science and Technology, 118,* art. no. 108713. https://doi.org/10.1016/j.lwt.2019.108713
26. Mikulec, A., Kowlaski, S., Sabat, R., Skoczylas, Ł., Tabaszewska, M., Wywrocka-Gurgul, A. (2019). Hemp flour as a valuable component for enriching physicochemical and antioxidant properties of wheat bread. *LWT – Food Science and Technology, 102,* 164–172. https://doi.org/10.1016/j.lwt.2018.12.028
27. Miranda-Ramos, K., Millán-Linares, M.C., Haros, C.M. (2020). Effect of chia as breadmaking ingredient on nutritional quality, mineral availability, and glycemic index of bread. *Foods, 9(5),* art. no. 663. https://doi.org/10.3390/foods9050663
28. Mohd Ali, N., Yeap, S.K., Ho, W.Y., Beh, B.K., Tan, S.W., Tan, S.G. (2012). The promising future of chia *Salvia hispanica* L. *Journal of Biomedicine and Biotechnology, 2012*, art. no. 171956. https://doi.org/10.1155/2012/171956
29. Oliveira, M.R., Novack, M.E., Santos, C.P., Kubota, E., Severo da Rosa, C. (2015). Evaluation of replacing wheat flour with chia flour (*Salvia hispanica* L.) in pasta. *Semina: Ciencias Agrarias, 36*(4), 2545–2553. https://doi:10.5433/1679-0359.2015v36n4p2545
30. Osuna, M.B., Romero, A., Judis, M.A., Bértola, N.C. (2016). Enrichment of the fatty acids profile of breads replaced with functional flours blends. International *Journal of Research in Engineering and Technology*, *05*(09), 224–232. https://doi.org/10.15623/ijret.2016.0509036
31. Pike, O. (2001). Assessment of oxidative stability for lipids. In *Current Protocols in Food Analytical Chemistry.* John Wiley & Sons, Inc., pp. D2.3.1-D2.3.5. https://doi.org/10.1002/0471142913.fad0203s00
32. Reyes-Caudillo, E., Tecante, A., Valdivia-López, M.A. (2008). Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican chia (*Salvia hispanica* L.) seeds. *Food Chemistry, 107*(2), 656–663. https://doi.org/10.1016/j.foodchem.2007.08.062
33. Romankiewicz, D., Hassoon, W., Cacak-Pietrzak, G., Sobczyk, M., Wirkowska-Wojdyła, M., Ceglińska, A., Dziki, D. (2017). The effect of chia seeds (*Salvia hispanica* L.) addition on quality and nutritional value of wheat bread. *Journal of Food Quality, 2017,* 1–7. https://doi.org/10.1155/2017/7352631

34. Sandoval-Oliveros, M., Paredes-Lopez, O. (2013). Isolation and characterization of proteins from chia seeds (*Salvia hispanica* L.). *Journal of Agricultural and Food Chemistry, 61*(1), 193–201. https://doi.org/10.1021/jf3034978
35. Scheuer, P.M., Luccio, M., Zibetti, A.W., Miranda, M.Z., de Francisco, A. (2016). Relationship between instrumental and sensory texture profile of bread loaves made with whole-wheat flour and fat replacer. *Journal of Texture Studies, 47*(1), 14–23. https://doi.org/10.1111/jtxs.12155
36. Simopoulos, A. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine, 233*(6), 674–688. https://doi.org/10.3181/0711-MR-311
37. Smith, G., Atherton, P., Reeds, D., Mohammed, B., Rankin, D., Rennie, M., Mittendorfer, B. (2011). Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older adults: a randomized controlled trial. *American Journal of Clinical Nutrition, 93*(2), 402–412. https://doi.org/10.3945/ajcn.110.005611
38. Stone, H., Sidel. J.L. (2004). *Sensory Evaluation Practices* (3rd ed). Redwood City: Academic Press Inc., pp. 87–90. https://doi.org/10.1016/B978-012672690-9/50002-0
39. Szczesniak, A.S. (2002). Texture is a sensory property. *Food Quality and Preference*, *13*(4), 215–225. https://doi.org/10.1016/S0950-3293(01)00039-8
40. Ulbricht, T.L.V., Southgate, D.A.T. (1991). Coronary heart disease: seven dietary factors*. Lancet, 338*(8773), 985–992. https://doi.org/10.1016/0140-6736(91)91846-M
41. Vázquez-Ovando, A., Rosado-Rubio, J.G., Chel-Guerrero, L.A., Betancur-Ancona, A. (2010). Dry processing of chía (*Salvia hispanica* L.) flour: chemical characterization of fiber and protein. *CyTA – Journal of Food, 8*(2), 117–127. https://doi.org/10.1080/19476330903223580
42. Vuksan, V., Jenkins, A., Brissette, C., Choleva, L., Jovanovski, E., Gibbs, A., Bazinet, R.P., Au-Yeung, F., Zurbau, A., Ho, H.V., Duvnjak, L., Sievenpiper, J.L., Josse, R.G., Hanna, A. (2017). Salba-chia (*Salvia hispanica* L.) in the treatment of overweight and obese patients with type 2 diabetes: A double-blind randomized controlled trial. *Nutrition, Metabolism, and Cardiovascular Diseases, 27*(2), 138–146. https://doi.org/10.1016/j.numecd.2016.11.124
43. Zettel, V., Hitzmann, B. (2018). Applications of chia (*Salvia hispanica* L.) in food products. *Trends in Food Science & Technology, 80,* 43–50. https://doi.org/10.1016/j.tifs.2018.07.011

Submitted: 10 July 2020. Revised: 3 September and 12 October 2020. Accepted: 28 October 2020. Published on-line: 9 December 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 399–408
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/129719
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Effect of Lecithin and Mono- and Di-Glycerides on Quality and Shelf Life of Hazelnut Butter: Chemometric Approach

Amir Pourfarzad[1]*, *Rezvaneh Shokouhi Kisomi*[2]

[1]*Department of Food Science and Technology, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran*
[2]*Department of Food Science, Rasht Branch, Islamic Azad University, Rasht, Iran*

Key words: texture analysis, sensory evaluation, lecithin, hazelnut, mono- and di-glyceride

Nut butter can be recognized as a functional food substitute for the animal butter. This study aimed to investigate the effects of mono- and di-glycerides and lecithin on the physicochemical properties and sensory characteristics of hazelnut butter. For this purpose, mono- and di-glycerides, and lecithin were employed in the hazelnut butter formulation at 0, 1, and 2 g/100 g addition levels. The proximate composition, acidity, peroxide value, and texture parameters were evaluated. Although adding mono- and di-glycerides and lecithin to the hazelnut butter formulation did not significantly change the adhesiveness, it increased their hardness. The sensory analysis revealed that lecithin and mono- and di-glycerides did not significantly affect the color, taste, and flavor of the butters. The highest texture, spreadability, and overall acceptance scores were observed when lecithin was used at the level of 2 g/100 g. The lowest acidity had butter containing mono- and di-glycerides at the level of 1 g/100 g. The peroxide values showed no significant changes during the 90-day storage. The principal component analysis (PCA) allowed discriminating among the features. The partial least squares regression (PLSR) models were applied to find the relationship between sensory and instrumental data. Thus, chemometric approach appears to be a promising technique for the analysis of hazelnut butter.

INTRODUCTION

Hazelnut (*Corylus avellana* L.) is a shrub belonging to the Fagales order from the Betulaceae family and Coryluideae sub-family. By monitoring the regular development of nuts in the last five years, the global hazelnut-cultivated area greatly increased beyond 670,000 ha in 2017 [FAOSTAT, 2020]. Also, the hazelnut cultivation has recently extended beyond the native areas, namely Europe and West Asia [Ascari *et al*., 2020].

Hazelnuts can be a valuable component of a human diet due to their special nutritional value [Pourfarzad & Mehrpour, 2017]. Hazelnut kernels are important sources of tocopherols and water-soluble B-vitamins, as well as minerals [Capurso *et al*., 2018; Stuetz *et al*., 2017]. According to the daily microelement requirements, 100 g of hazelnut provide about 50% for Fe, 41% for Mo, 32% for Zn, 21% for Se, and 21% for Cr [Turan & Akccedil, 2011]. A hazelnut-rich diet can also easily improve a daily intake of Mn, Cu, and Co. The oil is a major component of hazelnut kernels (50–73 g/100 g) [Garcia *et al*., 1994]. It contains mainly unsaturated fatty acids, *i.e*., oleic and linoleic acids, while saturated fatty acids (palmitic and steric acids) constitute only ~9% of its total fatty acids [Taş & Gökmen, 2015]. Given the unique fatty acid profile, hazelnut can protect low-density lipoproteins against oxidation and thus prevent the development of the cardiovascular disease [Ros, 2010]. Hazelnut oil contains β-sitosterol, a natural plant sterol recognized to reduce cholesterol levels and defend against different types of cancer such as breast, colon, and prostate cancers [Akcicek *et al*., 2005]. The protein content of hazelnut kernel changes between 10 and 22 g/100 g [Taş & Gökmen, 2018]. Hazelnuts are a popular and important source of high-quality vegetable protein in vegetarian diets, where they rank high on the list of foods most frequently consumed, above meat substitutes. They have a high content of L-arginine. This amino acid is the precursor of the endogenous vasodilator (nitric oxide), and might help improve vascular reactivity [Huynh & Chin-Dusting, 2006]. Hazelnuts are also a good source of dietary fiber (10.4 g/100 g) [Ros, 2010].

Nut butter is one of the nut processing products that is obtained by milling the roasted kernels and mixing them with a small amount of sugar. It can be considered as a substitute for animal butter to be used as a breakfast food and also in different food products such as cookies, ice creams, and cakes [Dobhal *et al.*, 2018]. Nowadays, the hazelnut processing is of great importance due to the limited use of hazelnut in its raw form and the continuous increase in the number of hazelnut gardens. One of the possibilities for wider food use of hazelnuts is the production of hazelnut butter. In other words, butter can be a good new product for consumers to increase the consumption of highly

---

* Corresponding Author: Tel.: +98-13-33690282; Fax: +98-13-33690281; E-mail: amir.pourfarzad@gmail.com

nutritious hazelnuts. The formulation of hazelnut butter is the first step in commercializing this product. The intrinsic tendency of various phases to separate is an undesirable phenomenon in food products such as chocolates and nut butters and spreads. It needs to be eliminated using different processing operations and proper additives [Ardakani *et al*., 2009; Villarroel *et al*., 1993].

Emulsifiers are a wide class of additives that are employed in the food industry to improve the quality and prolong the shelf life of products [Pourfarzad *et al*., 2012, 2014, 2019; Pourfarzad & Habibi-Najafi, 2012]. They allow two different phases to combine and form a semi-homogenous and stable mixture that can last for a long time. Moreover, the addition of an emulsifier increases product tolerance to high temperature. Thus, it seems crucial to evaluate the effect of this type of additives on the hazelnut butter properties. The mono- and di-glycerides and lecithin are commonly used as emulsifiers. They were recently successfully applied in chocolate spread emulsion and cheese analogue obtained from raw peanut [Eudier *et al*., 2020; Said *et al*., 2019].

To the best of our knowledge, a limited number of studies have addressed the impact of emulsifiers on the quality and shelf life of hazelnut butter. Therefore, the present study aimed to investigate the effect of emulsifiers on the physicochemical and sensory properties of hazelnut butter. In the next step, the correlation between different features of hazelnut butter was evaluated using symmetric multivariate methods, such as correlation analysis and principal component analysis (PCA), and asymmetric method like partial least squares regression (PLSR).

MATERIALS AND METHODS

Materials

Raw hazelnuts (without outer shell) were provided from Eshkevarat (Rudsar, Guilan, Iran). Mono- and di-glycerides (CREMODAN® Super Mono-diglyceride) and lecithin (catalogue number 429415) were supplied from Danisco (Copenhagen, Denmark) and Merck (Darmstadt, Germany) Companies, respectively. All other chemicals, reagents, and solvents were of analytical grade.

Hazelnut butter production

The raw hazelnuts were roasted in a solar dome convection system (model 3855SCR, LG Company, Seul, South Korea) for 20 min at 150°C [Pourfarzad *et al*., 2016, 2018]. After cooling down, the brown skin was manually peeled off using a rough cloth, and the butter production was conducted by a nut butter mill made by Niri Organic Yazd (model N1, Yazd, Iran). Two batches were processed for each treatment and one batch contained 1 kg of hazelnuts. Then, 10 g/100 g of sugar was added to the prepared butter and kneaded to provide soft dough. In this step, the specified amounts of mono- and di-glyceride (0, 1, 2 g/100 g) and lecithin (0, 1, 2 g/100 g) were added to the hazelnut butter. The treatment levels were chosen by carrying out preliminary screening tests according to the literature reports and instrumental aspects.

Proximate composition analysis

The contents of moisture (926.12), ash (950.49), protein (955.04C), fat (922.06), total dietary fiber (978.10), and total sugars (984.22), as well as acidity (962.12) and peroxide value (965.33) were analyzed by the Association of Official Analytical Chemists (AOAC) methods [2005].

Texture analysis

The penetration test was conducted on 200-gram samples of butter to evaluate hardness and adhesiveness. For this purpose, a Brookfield texture analyzer (CT3, Middleboro, MA, United States) was employed, and the force required for the penetration of a thick elliptical bar probe with a flat cross-section and diameter of 0.48 cm (velocity of 200 mm/min and penetration depth of 1 cm) was determined [Ardakani *et al*., 2009].

Sensory evaluation

Ten trained panelists of both genders aged from 25 to 60 evaluated the hazelnut butter samples. All assessors of the internal sensory panel have undertaken the basic flavor test, odor test, and color vision test. They have been trained for sensory methods in several sessions and their evaluation aptitude has been routinely checked. The panel was particularly familiarized with the score of attribute intensities using the verbal meanings describing the ends of the intensity scales of the characteristics. Each sample contained 30 g of hazelnut butter, which were given to panelists in plastic containers at room temperature. The taste, flavor, texture, color, spreadability, and overall acceptance were assessed. Each parameter was scored in a 5-point scaling range from 1 (lowest) to 5 (highest) [Ardakani *et al*., 2009; Qaziyani *et al*., 2019].

Oil separation

The oil was collected using a Pasteur pipette, moved to a 50-mL graduated cylinder, and its volume was determined. The oil separation rate was calculated as follows [Gills & Resurreccion, 2000a]:

$$\text{Oil separation (\%)} = \text{(Volume of oil separated)}/\text{(Volume of hazelnut butter before oil separation)} \times 100 \quad (1)$$

Shelf life test

To determine the product shelf life, acidity, peroxide value, and oil separation of the samples were evaluated during 90-day storage at 25°C [AOAC, 2005; Ardakani *et al*., 2009].

Statistical analysis

The data on the physicochemical properties and sensory characteristics of hazelnut butter with and without emulsifiers were statistically analyzed. To investigate the significant difference between the means, the Duncan's multiple range test was carried out after the analysis of variance at the 95% confidence level. Each experiment was performed in six replications. The Pearson's correlation analysis, PCA, and PLSR modeling were performed on the data sets. The data analyses were taken using Minitab 15 (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Proximate composition of hazelnut butter

The contents of moisture, fat, protein, dietary fiber, total sugars, and ash of the control hazelnut butter (without emulsifiers) were as follows: 0.76±0.00, 59.09±0.21, 15.68±0.01, 5.51±0.12, 15.88±0.23, and 2.21±0.06 g/100 g, respectively. A daily intake of 25 g of hazelnuts is recommended in a healthy diet [Stuetz *et al*., 2017], which nearly corresponds to the standard portion size of 1 oz (28.35 g) displayed by the United States Department of Agriculture [USDA, 2015]. Based on this recommendation, it can be concluded that hazelnut butter could provide 3%, 17%, 53%, and 22% of the daily dietary requirement for carbohydrates, proteins, fat, and dietary fiber, respectively.

Texture analysis

The results of instrumental texture analysis of hazelnut butters are shown in Table 1. The addition of lecithin and mono- and di-glycerides increased the hardness of the butters, although no significant difference ($p>0.05$) was observed between hardness of the samples with different contents of emulsifiers (1 and 2 g/100 g). The higher hardness of butters with additives compared to the control one can be attributed to the hydrophilicity of lecithin and mono- and di-glycerides. They can strengthen the network structure of butter solids, which leads to the stabilization of oil in it [Totlani & Chinnan, 2007]. The development of this network resulted in the greater energy required for the probe penetration during the texture analysis. Also, the interactions between emulsifiers and hazelnut biopolymers produce large internal frictions and, as a result, higher hardness [Izidoro *et al*., 2009].

Adhesiveness was determined as the work required to pull the probe away from the sample in the penetration test. The values of this parameter for hazelnut butters ranged from 1.18 to 1.25 N·s (Table 1). However, no significant differences ($p>0.05$) were found between adhesiveness of the control samples and the butters containing emulsifiers. The level of both lecithin and mono- and di-glycerides added also did not significantly differentiate the adhesiveness of the butters.

Sensory evaluation

Table 2 shows the results of the sensory analysis of hazelnut butters. The taste, flavor, and color scores of the control sample were not significantly different from those containing various levels of lecithin and mono- and di-glycerides ($p>0.05$). As one of the dominant features in consumers' food preferences, the sensory texture plays a key role in product attractiveness, purchasing decisions, and ultimate consumption [Pellegrino *et al*., 2020]. Adding lecithin to the hazelnut butter formulation increased the texture score of the samples from 3.67 (control butter) to 4.36 (butter with 2 g/100 g addition level). In the case of adding the second emulsifier, although there was no significant difference between the texture scores of the control sample and butter with mono- and di-glycerides at level of 1 g/100 g ($p>0.05$), the sample with a higher content of mono- and di-glycerides (2 g/100 g) showed a significantly higher texture score ($p\leq0.05$). Spreadability is a particularly important characteristic of semi-solid food texture. It is a subjective term linked to the way a sample is uniformly distributed over a surface [Gills & Resurreccion, 2000b]. In our study, upon the addition of mono- and di-glyceride to butter, its spreadability was assessed by panelists as significantly better ($p\leq0.05$) than that of the control sample, although no significant difference ($p>0.05$) was observed between the spreadability of products with different contents of mono- and di-glycerides (1 and 2 g/100 g). The sample containing lecithin at 2 g/100 g showed higher spreadability than the control and butter with additive of 1 g/100 g. The trends

TABLE 1. Texture parameters of hazelnut butters with different contents of emulsifiers.

Emulsifier	Additive level (g/100 g)	Hardness (N)	Adhesiveness (N·s)
Lecithin	0	5.45±0.023^{b}	1.15±0.082^{a}
	1	5.72±0.041^{a}	1.21± 0.045^{a}
	2	5.81±0.078^{a}	1.18±0.324^{a}
Mono- and di-glycerides	0	5.48±0.043^{b}	1.21± 0.012^{a}
	1	5.72±0.067^{a}	1.08±0.213^{a}
	2	5.75±0.055^{a}	1.25±0.325^{a}

Each value is a mean ± standard deviation of three experimental replicates (n=6). Values in columns with different letters are significantly different (p≤0.05).

TABLE 2. Sensory characteristics of hazelnut butters with different contents of emulsifiers.

Emulsifier	Additive level (g/100 g)	Taste	Flavor	Texture	Color	Spreadability	Overall acceptance
Lecithin	0	3.23±0.232^{a}	3.67±0.322^{a}	3.31±0.032^{c}	4.03±0.434^{a}	3.43±0.032^{b}	3.21±0.031^{b}
	1	3.63±0.427^{a}	3.73±0.544^{a}	3.81±0.201^{b}	4.01±0.656^{a}	3.77±0.034^{ab}	3.63±0.033^{a}
	2	3.67±0.432^{a}	3.87±0.004^{a}	4.36±0.203^{a}	4.01±0.646^{a}	3.97±0.012^{a}	4.03±0.042^{a}
Mono- and di-glycerides	0	3.37±0.433^{a}	3.60±0.444^{a}	3.67±0.009^{b}	4.13±0.322^{a}	3.33±0.101^{b}	3.53±0.009^{b}
	1	3.37±0.302^{a}	3.80±0.323^{a}	3.57±0.101^{b}	3.91±0.111^{a}	3.81±0.323^{a}	3.33±0.004^{b}
	2	3.81±0.109^{a}	3.87±0.632^{a}	4.11±0.201^{a}	4.01±0.564^{a}	4.03±0.077^{a}	4.01±0.005^{a}

Each value is a mean ± standard deviation of three experimental replicates (n=6). Values in columns with different letters are significantly different (p≤0.05).

TABLE 3. Acidity (g oleic acid/100 g) of hazelnut butters with different contents of emulsifiers during storage.

Emulsifier	Additive level (g/100 g)	Storage time (day)			
		1	30	60	90
Lecithin	0	0.28±0.003^{a}	0.48±0.005^{a}	0.56±0.003^{a}	0.59±0.005^{a}
	1	0.28±0.003^{a}	0.42±0.006^{b}	0.53±0.005^{b}	0.55±0.003^{b}
	2	0.28±0.004^{a}	0.41±0.004^{c}	0.42±0.001^{c}	0.47±0.021^{c}
Mono- and di-glycerides	0	0.28±0.003^{a}	0.46±0.002^{b}	0.54±0.018^{b}	0.59±0.002^{b}
	1	0.28±0.002^{a}	0.38±0.003^{c}	0.37±0.022^{c}	0.45±0.001^{c}
	2	0.28±0.004^{a}	0.47±0.002^{a}	0.60±0.005^{a}	0.63±0.011^{a}

Each value is a mean ± standard deviation of three experimental replicates (n=6). Values in columns with different letters are significantly different ($p \leq 0.05$).

TABLE 4. Peroxide value (meq/kg) of hazelnut butters with different contents of emulsifiers during storage.

Emulsifier	Additive level (g/100 g)	Storage time (day)			
		1	30	60	90
Lecithin	0	0.08±0.003^{a}	0.09±0.004^{a}	0.16±0.002^{a}	0.19±0.014^{a}
	1	0.08±0.002^{a}	0.09±0.004^{a}	0.16±0.003^{a}	0.19±0.014^{a}
	2	0.08±0.001^{a}	0.09±0.006^{a}	0.16±0.004^{a}	0.19±0.024^{a}
Mono- and di-glycerides	0	0.08±0.002^{a}	0.09±0.006^{a}	0.16±0.015^{a}	0.19±0.016^{a}
	1	0.08±0.001^{a}	0.09±0.006^{a}	0.16±0.024^{a}	0.19±0.006^{a}
	2	0.08±0.001^{a}	0.09±0.006^{a}	0.16±0.017^{a}	0.19±0.004^{a}

Each value is a mean ± standard deviation of three experimental replicates (n=6). Values in columns with different letters are significantly different ($p \leq 0.05$).

of sensory texture and spreadability might be similar due to the effect of emulsifiers on the hardness [Ardakani *et al*., 2009; Saker Ardakani, 2007]. The addition of lecithin also improved the overall acceptance of the samples, although no significant difference ($p > 0.05$) was observed between butters containing emulsifier at the levels of 1 and 2 g/100 g. The overall acceptance of the butter containing mono- and di-glycerides at 2 g/100 g was significantly higher ($p \leq 0.05$) compared to the samples with lower additive contents (0 and 1 g/100 g). Also, no significant difference ($p > 0.05$) was observed between the scores given to the control sample and butter with mono- and di-glyceride addition at 1 g/100 g.

Shelf life test

Acidity is one of the major parameters in the quality control of oils and edible fats. Its value may increase as a result of hydrolysis and lipolysis. High acidity is an indicator of the low quality of oil. The oil storage can significantly change the acidity [San Martín *et al*., 2020]. In our study, the effect of adding emulsifiers to hazelnut butters on their acidity was monitored over the 90-day storage period (Table 3). There were no significant differences ($p > 0.05$) between the acidity of different butters on the first day. Adding lecithin to butter significantly reduced acidity increase during storage. The control butter had significantly higher acidity ($p \leq 0.05$) than the butters with lecithin at each monitored storage time (30–90 days). Also, the level of the lecithin additive significantly affected the acidity. Our notice is in line with previous studies, in which acidity of a similar product, *i.e*., pistachio butter, was determined during storage [Haghani Haghighi *et al*., 2008]. Authors recommend using lecithin as an emulsifier to improve the long-term storage stability of nut butter. The hazelnut batters containing mono- and di-glycerides at levels of 1 g/100 g and 2 g/100 g had the lowest and the highest acidity, respectively, at each monitored storage time. In other words, the mono- and di-glycerides added at the level of 1 g/100 g decreased the acidity compared to the control butter, but a higher addition level resulted in acidity increase. This can be attributed to the presence of fatty acids in the chemical structure of mono- and di-glycerides. With the higher level of mono and di-glyceride addition, more products of fatty acid hydrolysis were generated, which led to an increase in product acidity [Mirtajeddini *et al*., 2016].

The oxidation is the main process of fat spoilage, giving rise to undesirable odor and taste. One of the parameters commonly used to evaluate the state of oxidation of fats and oils is the peroxide value. The changes in the peroxide values during 90-day storage of hazelnut butter are shown in Table 4. As expected, the peroxide values increased during storage. However, at each storage time for which the measurement was taken, the peroxide value of the control butter

TABLE 5. Oil separation (mL) in hazelnut butters with different contents of emulsifiers during storage.

Emulsifier	Additive level (g/100 g)	Storage time (day)			
		1	30	60	90
Lecithin	0	0±0.001^{a}	5.79±0.211^{a}	6.74±0.054^{a}	8.42±0.021^{a}
	1	0±0.001^{a}	2.65±0.344^{b}	3.08±0.031^{b}	3.85±0.033^{b}
	2	0±0.001^{a}	2.11±0.223^{c}	2.46±0.022^{c}	3.07±0.054^{c}
Mono- and di-glycerides	0	0±0.001^{a}	5.82±0.213^{a}	6.77±0.043^{a}	8.46±0.021^{a}
	1	0±0.001^{a}	2.45±0.321^{b}	2.85±0.233^{b}	3.56±0.021^{b}
	2	0±0.001^{a}	1.62±0.175^{c}	1.88±0.322^{c}	2.35±0.087^{c}

Each value is a mean ± standard deviation of three experimental replicate experiments (n=6). Values in columns with different letters are significantly different ($p \leq 0.05$).

did not differ significantly ($p>0.05$) from the peroxide values of the butters containing lecithin and mono- and di-glycerides at both levels.

Oil separation can be used as an indicator of product structure preservation during storage [Deng *et al*., 2020]. The oil separation of hazelnut butters with and without emulsifiers during the storage is shown in Table 5. There was no oil separated in any of the samples on the first day of storage experiment. Oil separation increases with storage, but this increase was significantly lower in hazelnut butters with emulsifiers than in the control. The level of additive also affected the oil separation. Oil separation in butters with both emulsifiers added at the level of 1 g/100 g was significantly higher than that reported for the samples containing 2 g/100 g at each storage time (30, 60, or 90 days). This behavior was probably exhibited by the increased intermolecular interactions. The oil was detected as a non-emulsified (free) fluid in the voids between solid particles [Ereifej *et al*., 2005]. The oil separation is the result of the hazelnut matrix compression. Emulsifiers can immobilize the free oil and prevent its migration [Totlani & Chinnan, 2007]. The mechanism for avoiding the oil separation seems to be associated with the increased viscosity of the oil phase *via* hardness increase [Ereifej *et al*., 2005].

Relationship between physicochemical properties and sensory characteristics of hazelnut butter

Correlation analysis

The results of Person's correlations between texture parameters, quality properties at 30–90-day storage, and sensory scores of hazelnut butter are shown in Table 6. The correlation coefficients (r) of significant correlations ranged from -0.487 to 1. Within the texture parameters, the hardness highly correlated with sensory characteristic scores and oil separation at 30–90-day storage. The harder samples strongly corresponded to those with higher sensory scores of spreadability and texture. As indicated earlier, emulsifiers affect the sensory texture and spreadability due to a change in hardness. Also, the samples with high hardness showed lower oil separation during the storage. This may be due to the oil immobilization effect of emulsifiers [Totlani & Chinnan, 2007], leading to the increased hardness. Adhesiveness was highly positively correlated with acidity at 30–90-day storage. Within the sensory characteristics, the overall acceptance, spreadability, and flavor scores showed significant correlations with many other properties. The overall acceptance was highly positively correlated with taste and texture. Considering the significant relationships between sensory characteristics and shelf life properties, color and acidity showed positive correlations. Also, the higher the spreadability score, the lower the oil separation. Among the shelf life properties, acidity on the 30th day of storage was significantly correlated with oil separation during the storage. The results show that the sensory and texture analysis can potentially be used to estimate different properties of hazelnut butter. The findings deserve special attention and may have significant potential for optimizing and improving the product quality, shelf life properties, and sensory characteristics as well as for reducing the time and costs by minimizing the experiments.

Principal component analysis

Sometimes, too many variables are involved in calculations, and the principal component analysis (PCA) helps reducing them to a smaller group. PCA is a factor model in which the factors are based on the total variance, allowing for bargaining the fewest number of variables that clarify the variance [Tipping & Bishop, 1999]. The data of instrumental texture analysis, sensory analysis and quality of storage experiment of hazelnut butter were subjected to PCA (Figure 1). The multivariate treatment of the data obtained from the samples enabled reducing the variables to two principal components, which together explained 79.6% of the total variability. The first component (PC1) accounted for 53.7% and the second component (PC2) for 25.9%. According to the PCA biplot, hardness, adhesiveness, and sensory characteristics such as overall acceptance, spreadability, texture, flavor, and taste, were negatively correlated to PC1, whereas other hazelnut butter properties were positively correlated to PC1. Hardness, acidity of the fresh butter (1 day of storage experiment), and oil separation during the storage were negatively correlated to PC2, while other butter characteristics and properties were positively correlated to PC2. As clearly revealed in the PCA biplot (Figure 1), the butters with the best sensory scores of overall acceptance, taste, and texture were those

TABLE 6. Pearson's correlation coefficients of significant[a] correlations between features of hazelnut butters containing lecithin and mono- and di-glycerides.

	Hardness	Adhesiveness	Overall acceptance	Spreadability	Color	Texture	Flavor	Taste	Acidity 1	Acidity 30	Acidity 60	Acidity 90	O.S. 1	O.S. 30	O.S. 60	O.S. 90
Adhesiveness																
Overall acceptance	0.708***	0.655**														
Spreadability	0.953***		0.725***													
Color	-0.597**	0.626**		-0.619**												
Texture	0.786***	0.496*	0.974***	0.757***												
Flavor	0.899***		0.681**	0.974***	-0.646**	0.726***										
Taste	0.797***	0.648**	0.928***	0.828***		0.880***	0.729***									
Acidity 1													1.000***			
Acidity 30	-0.629**	0.592**			0.678**						0.910***	0.927***				
Acidity 60		0.772***			0.607**							0.985***				
Acidity 90	-0.487*	0.730***			0.624**											
O.S. 1									1.000***							
O.S. 30	-0.975***		-0.669**	-0.979***	0.654**	-0.711***	-0.920***	-0.811***		0.542**					1.000***	1.000***
O.S. 60	-0.975***		-0.668**	-0.979***	0.654**	-0.711***	-0.920***	-0.811***		0.541**						1.000***
O.S. 90	-0.975***		-0.668**	-0.979***	0.654**	-0.711***	-0.920***	-0.811***		0.542**						

[a] at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***);O.S. – Oil separation; O.S. 1, 30, 60 and 90 – oil separation on day 1, 30, 60, and 90 of storage; Acidity 1, 30, 60, 90 – acidity on day 1, 30, 60, and 90 of storage.

containing mono- and di-glyceride at the level of 2 g/100 g and lecithin at the level of 1 g/100 g. The results of PCA confirm our previous consideration that the addition of lecithin resulted in a decrease in oil separation during the storage, but increased spreadability, flavor, and hardness, and that the lowest acidity during storage was observed for the sample containing mono- and di-glycerides at the level of 1 g/100 g.

Partial least squares regression

PLSR is a technique that diminishes the predictors to a smaller series of uncorrelated components and makes least squares regression on the components rather than the original data. For instance, PLSR is often used to establish relationships between instrumental and sensory descriptive data. Based on this technique, the process-checking strategies primarily measure the unrealized variable models to explain the error recognition indices later. Today, such strategies have extraordinary potentials for industrial applications [Godoy *et al*., 2014; Martens & Martens, 2001]. Thus, the prediction of sensory characteristics by instrumental dimensions is of supreme importance in the food industry, as it provides much information about the appropriateness and quality of the final product. Several researchers have afforded to correlate various chemical and rheological properties describing the quality and shelf life of different food products with sensory characteristics [Hejri-Zarifi *et al*., 2013; Pourfarzad *et al*., 2012; Qaziyani *et al*., 2019]. Determining the quality profile by instrumental dimensions has the benefits of high convenience, reproducibility, and directness compared to the evaluation of sensory components [Sabanis *et al*., 2009]. Thus, the instrumental properties with the most significant interrelationships with other properties were used as the predictors. In our study, the instrumental texture parameters (hardness and adhesiveness), acidity and oil separation during storage were used to predict sensory scores of hazelnut butters. The output regression equations are given in Table 7.

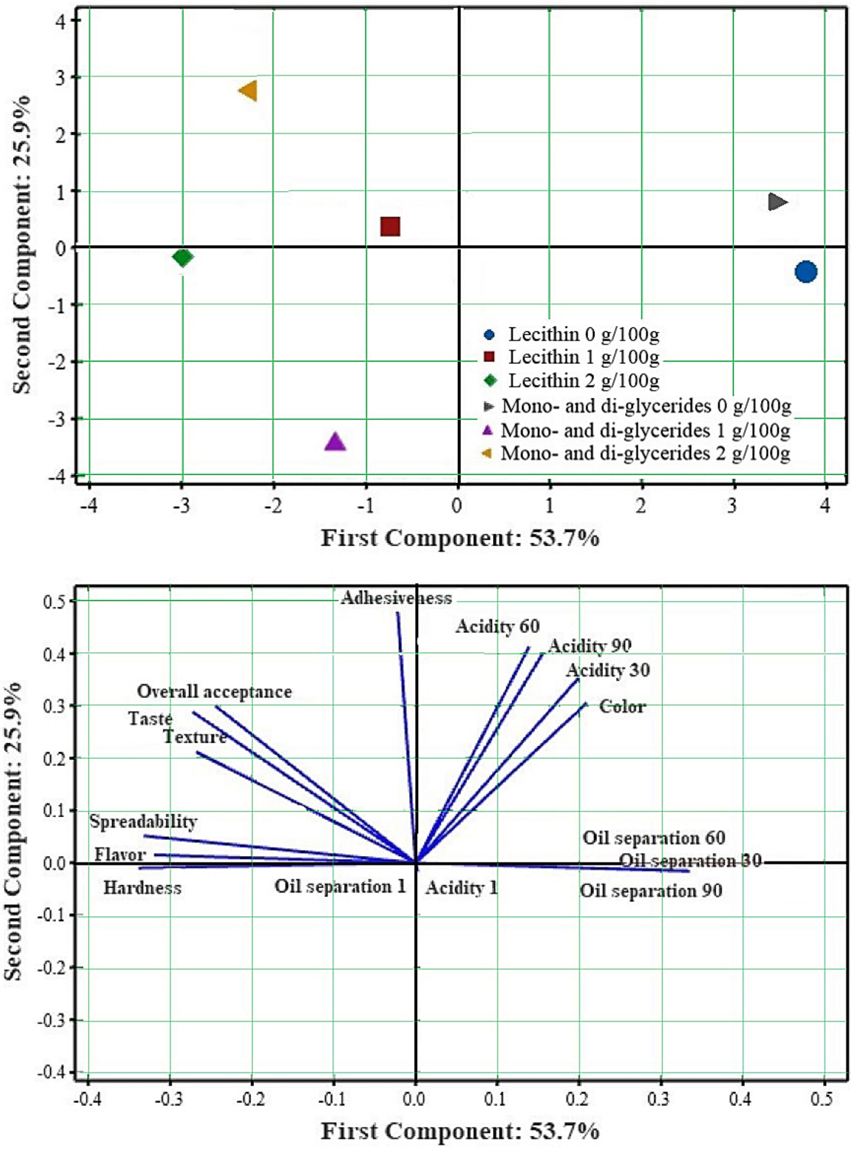


FIGURE 1. Biplot of principal component analysis of hazelnut butters containing lecithin and mono and di-glycerides and without emulsifiers. Variables are sensory characteristics (taste, flavor, texture, color, spreadability, and overall acceptance), texture parameters (hardness and adhesiveness), and quality properties (acidity and oil separation on day 1, 30, 60, and 90 of storage).

TABLE 7. Partial least squares regression (PLSR) models for sensory characteristics of hazelnut butters containing lecithin and mono- and di-glycerides.

Sensory attribute	Multiple regression equation	R^2
Taste	-3.84 + 0.86 Hardness + 1.94 Adhesiveness + 0.65 Acidity 30 – 0.029 O.S.30	99.92
Flavor	-3.49 + 1.32 Hardness – 1.25 Adhesiveness + 2.81 Acidity 30 + 0.014 O.S.30	98.96
Texture	-45.04 + 7.78 Hardness + 0.96 Adhesiveness + 5.28 Acidity 30 + 0.41 O.S.30	94.90
Color	0.39 + 0.43 Hardness + 0.95 Adhesiveness – 0.25 Acidity 30 + 0.063 O.S.30	96.92
Spreadability	-6.28 + 1.78 Hardness – 1.36 Adhesiveness + 3.89 Acidity 30 – 0.056 O.S.30	99.97
Overall acceptance	-32.92 + 5.58 Hardness + 1.61 Adhesiveness + 5.02 Acidity 30 + 0.26 O.S.30	94.73

O.S.30: Oil separation on day 30 of hazelnut butter storage; R^2: multiple determination coefficient.

The high coefficients of multiple determination (94.73%––99.97%) provide evidence for the applicability of PLSR model within the ranges of the variables involved.

CONCLUSIONS

Generally, the results of the present research showed that the hazelnut butter could be a good food product to increase the consumption of nutritious hazelnuts. The lecithin and mono- and di-glycerides as emulsifiers had no negative effect on the sensory characteristics and texture parameters of hazelnut butter. Both emulsifiers improved hazelnut butter stability. By optimizing the physicochemical properties and sensory characteristics of hazelnut butter, the best qualitative, sensory, and shelf life properties were observed in the samples containing lecithin at the level of 2 g/100 g and mono- and di-glycerides at the level of 1 g/100 g. Moreover, the sensory characteristics was significantly positively correlated with hardness and negatively with oil separation during storage. These findings were confirmed by PCA results. Combining the sensory scores and data of instrumental measurements in the PLSR analysis provided a valuable insight for predicting the effect of physicochemical properties on the sensory characteristics of hazelnut butters. In future study, the effect of other factors such as hazelnut roasting, content of antioxidants and sweeteners on the hazelnut butter quality, stability, and marketing can be considered.

ACKNOWLEDGEMENTS

The Department of Food Science and Technology at University of Guilan is warmly acknowledged for providing necessary research facilities.

RESEARCH FUNDING

Partial support for this research was provided by the Guilan Science and Technology Park under creativity project number 97/7145.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID IDS

A. Pourfarzad https://orcid.org/0000-0003-2913-9682

REFERENCES

1. Akcicek, E., Otles, S., Esiyok, D. (2005). Cancer and its prevention by some horticultural and field crops in Turkey. *Asian Pacific Journal of Cancer Prevention, 6*, 224–230.
2. AOAC. (2005). Official methods of analysis of AOAC International. In: Association of Official Analytical Chemists, Washington, DC.
3. Ardakani, A.S., Shahedi, M., Kabir, G. (2009). Optimizing formulation of pistachio butter production. *Journal of Science and Technology of Agriculture and Natural Resources, 13*(47 (A)), 49–60.
4. Ascari, L., Siniscalco, C., Palestini, G., Lisperguer, M.J., Huerta, E.S., De Gregorio, T., Bregaglio, S. (2020). Relationships between yield and pollen concentrations in Chilean hazelnut orchards. *European Journal of Agronomy, 115*, art. no. 126036. https://doi.org/10.1016/j.eja.2020.126036
5. Capurso, A., Crepaldi, G., Capurso, C. (2018). Nuts. In: Capurso, A., Crepaldi, G., Capurso, C. (Eds.) *Benefits of the Mediterranean Diet in the Elderly Patient,* Springer, pp. 263–284. https://doi.org/10.1007/978-3-319-78084-9_11
6. Deng, Y., Peng, C., Dai, M., Lin, D., Ali, I., Alhewairini, S.S., Zheng, X., Chen, G., Li, J., Naz, I. (2020). Recent development of super-wettable materials and their applications in oil-water separation. *Journal of Cleaner Production*, *266*, art. no. 121624. https://doi.org/10.1016/j.jclepro.2020.121624
7. Dobhal, K., Singh, N., Semwal, A., Negi, A. (2018). A brief review on hazelnuts. *Internationl Journal of Recent Scientific Research, 9*(1), 23680–23684. http://dx.doi.org/10.24327/ijrsr.2018.0901.1515
8. Ereifej, K., Rababah, T., Al-Rababah, M. (2005). Quality attributes of halva by utilization of proteins, non-hydrogenated palm oil, emulsifiers, gum arabic, sucrose, and calcium chloride. *International Journal of Food Properties, 8*(3), 415–422. https://doi.org/10.1080/10942910500267323
9. Eudier, H., Ben-Harb, S., Lorand, J.-P., Duthil, F., Negahban, M., Saiter, J.-M., Chan-Huot, M. (2020). Properties of an analogue cheese obtained from raw peanut. *Peanut Science, 47*(2), 81–88. https://doi.org/10.3146/PS20-1.1
10. FAOSTAT. (2020). FAO Statistics, Food and Agriculture Organization of the United Nations. In: Rome, Italy.
11. Garcia, J., Agar, I., Streif, J. (1994). Lipid characteristics of kernels from different hazelnut varieties. *Turkish Journal of Agricultural and Forestry, 18*, 199–202.
12. Gills, L.A., Resurreccion, A.V.A. (2000a). Overall acceptability and sensory profiles of unstabilized peanut butter and peanut butter stabilized with palm oil. *Journal of Food Processing and Preservation, 24*(6), 495–516. https://doi.org/10.1111/j.1745-4549.2000.tb00437.x

13. Gills, L.A., Resurreccion, A.V.A. (2000b). Sensory and physical properties of peanut butter treated with palm oil and hydrogenated vegetable oil to prevent oil separation. *Journal of Food Science, 65*(1), 173–180.
https://doi.org/10.1111/j.1365-2621.2000.tb15975.x
14. Godoy, J.L., Vega, J.R., Marchetti, J.L. (2014). Relationships between PCA and PLS-regression. *Chemometrics and Intelligent Laboratory Systems, 130*, 182–191.
https://doi.org/10.1016/j.chemolab.2013.11.008
15. Haghani Haghighi, H., Azar, M., Mazloumi, M.T., Komeyli Fonoud, R. (2008). Survey of formulation, production and sensory evaluation of pistachio butter. *Iranian Journal of Food Science and Technology, 5*(3), 19–26 (in Persian).
16. Hejri-Zarifi, S., Ahmadian-Kouchaksaraei, Z., Pourfarzad, A., Khodaparast, M.H.H. (2013). Dough performance, quality and shelf life of flat bread supplemented with fractions of germinated date seed. *Journal of Food Science and Technology*, *51*(12), 3776–3784.
https://doi.org/10.1007/s13197-013-0929-7
17. Huynh, N.N., Chin-Dusting, J. (2006). Amino acids, arginase and nitric oxide in vascular health. *Clinical and Experimental Pharmacology and Physiology, 33*(1–2), 1–8.
https://doi.org/10.1111/j.1440-1681.2006.04316.x
18. Izidoro, D.R., Scheer, A. de P., Sierakowsk, M.-R. (2009). Rheological properties of emulsions stabilized by green banana (*Musa cavendishii*) pulp fitted by power law model. *Brazilian Archives of Biology and Technology, 52*(6), 1541–1553.
https://doi.org/10.1590/S1516-89132009000600026
19. Martens, H., Martens, M. (2001). Analysis of two data tables X and Y: Partial Least Squares Regression (PLSR). In *Multivariate Analysis of Quality: An Introduction*. Wiley & Sons Ltd., London, UK, pp. 111–125.
20. Mirtajeddini, S.B., Azadmard-Damirchi, S., Peighambardoust, S.H., Rasouli-Pirouzian, H. (2016). Application of ester monodiglycerid citric acid (Citrem) in replacement of lecithin, on some qualitative characteristics of milk chocolate. *Journal of Food Research, 26*(2), 265–276 (in Persian).
21. Pellegrino, R., Cheon, B.K., Forde, C.G., Oleszkiewicz, A., Pieniak, M., Luckett, C.R. (2020). The contribution of texture contrasts and combinations to food acceptance across cultures. *Journal of Texture Studies, 51*(2), 225–231.
https://doi.org/10.1111/jtxs.12494
22. Pourfarzad, A., Ahmadian, Z., Tavassoli-Kafrani, M.H. (2019). The effect of sodium stearoyl lactylate on structural changes of wheat gluten in a model system fortified with inulin: Investigation with Fourier transform infrared spectroscopy. *Bioactive Carbohydrates and Dietary Fibre, 17*, art. no. 100175.
https://doi.org/10.1016/j.bcdf.2018.12.001
23. Pourfarzad, A., Gheibi, S., Ahmadian, Z. (2016). Evaluation of sensory characteristics and modeling of the kinetics of hazelnut color indices during infrared roasting. *Innovative Food Technologies, 4*(2), 81–99.
24. Pourfarzad, A., Gheibi, S., Ahmadian, Z. (2018). Effect of different roasting methods, blanching and pasteurization conditions on physicochemical and sensory characteristics of hazelnut milk. *Innovative Food Technologies, 5*(2), 301–318.
25. Pourfarzad, A., Habibi-Najafi, M.B. (2012). Optimization of a liquid improver for barbari bread: staling kinetics and relationship of texture with dough rheology and image characteristics. *Journal of Texture Studies, 43*(6), 484–493.
https://doi.org/10.1111/j.1745-4603.2012.00362.x
26. Pourfarzad, A., Haddad Khodaparast, M., Karimi, M., Mortazavi, S. (2014). Optimization of a novel improver gel formulation for Barbari flat bread using response surface methodology. *Journal of Food Science and Technology, 51*(10), 2344–2356.
https://doi.org/10.1007/s13197-012-0778-9
27. Pourfarzad, A., Mehrpour, G.R. (2017). Health benefits of hazelnut. *EC Nutrition, 8*, 101–105.
28. Pourfarzad, A., Mohebbi, M., Mazaheri-Tehrani, M. (2012). Interrelationship between image, dough and Barbari bread characteristics; use of image analysis to predict rheology, quality and shelf life. *International Journal of Food Science & Technology, 47*(7), 1354–1360.
https://doi.org/10.1111/j.1365-2621.2012.2980.x
29. Qaziyani, S.D., Pourfarzad, A., Gheibi, S., Nasiraie, L.R. (2019). Effect of encapsulation and wall material on the probiotic survival and physicochemical properties of synbiotic chewing gum: study with univariate and multivariate analyses. *Heliyon, 5*(7), art. no. e02144.
https://doi.org/10.1016/j.heliyon.2019.e02144
30. Ros, E. (2010). Health benefits of nut consumption. *Nutrients, 2*(7), 652–682.
https://doi.org/10.3390/nu2070652
31. Sabanis, D., Soukoulis, C., Tzia, C. (2009). Effect of raisin juice addition on bread produced from different wheat cultivars. *Food Science and Technology International, 15*(4), 325–336.
https://doi.org/10.1177/1082013209346581
32. Said, A., Nasir, N.A. M., Bakar, C.A.A., Mohamad, W.A.F.W. (2019). Chocolate spread emulsion: Effects of varying oil types on physico-chemical properties, sensory qualities and storage stability. *Journal of Agrobiotechnology, 10*(2), 32–42.
33. Saker Ardakani, A. (2007). Study on the possibility of utilization sunflower and sesame pastes in the formulation of pistachio butter *Iranian Food Science and Technology Research Journal, 3*(1), 1–6.
34. San Martín, E., Avenoza, A., Peregrina, J.M., Busto, J.H. (2020). Solvent-based strategy improves the direct determination of key parameters in edible fats and oils by 1H NMR. *Journal of the Science of Food and Agriculture, 100*(4), 1726–1734.
https://doi.org/10.1002/jsfa.10193
35. Stuetz, W., Schlörmann, W., Glei, M. (2017). B-vitamins, carotenoids and α-/γ-tocopherol in raw and roasted nuts. *Food Chemistry, 221*, 222–227.
https://doi.org/10.1016/j.foodchem.2016.10.065
36. Taş, N.G., Gökmen, V. (2015). Profiling triacylglycerols, fatty acids and tocopherols in hazelnut varieties grown in Turkey. *Journal of Food Composition and Analysis, 44*, 115–121.
https://doi.org/10.1016/j.jfca.2015.08.010
37. Taş, N.G., Gökmen, V. (2018). Profiling of the contents of amino acids, water-soluble vitamins, minerals, sugars and organic acids in Turkish hazelnut varieties. *Polish Journal of Food and Nutrition Sciences, 68*(3), 223–234.
https://doi.org/10.1515/pjfns-2018-0002
38. Tipping, M.E., Bishop, C.M. (1999). Probabilistic principal component analysis. *Journal of the Royal Statistical Society: Series B (Statistical Methodology), 61*(3), 611–622.
https://doi.org/10.1111/1467-9868.00196

39. Totlani, V., Chinnan, M. (2007). Effect of stabilizer levels and storage conditions on texture and viscosity of peanut butter. *Peanut Science, 34*(1), 1–9.
https://doi.org/10.3146/0095-3679(2007)34[1:EOSLAS]2.0.CO;2
40. Turan, G.Y.M., Akccedil, F. (2011). The importance of Turkish hazelnut trace and heavy metal contents for human nutrition. *Journal of Soil Science and Environmental Management, 2*(1), 25–33.
41. USDA. (2015). National nutrient database for standard reference, release 28. United States Department of Agriculture Beltsville.
42. Villarroel, M., Biolly, E., San Martin, S., Estrada, G. (1993). Chilean hazelnut butter, a new alternative for consumers. *Plant Foods for Human Nutrition, 44*(2), 131–136.
https://doi.org/10.1007/BF01088377

Submitted: 19 June 2020. Revised: 16 September, 11 October, and 29 October 2020. Accepted: 2 November 2020. Published online: 9 December 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 409–418
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/130255
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Aronia melanocarpa Leaves as a Source of Chlorogenic Acids, Anthocyanins, and Sorbitol, and Their Anti-Inflammatory Activity

Agnieszka Zielińska[1], Dorota Bryk[2], Katarzyna Paradowska[1], Iwona Wawer[3]*

[1]*Department of Physical Chemistry, Chair of Physical Pharmacy and Bioanalysis, Faculty of Pharmacy, Medical University of Warsaw, Banacha Str. 1, 02–097 Warsaw, Poland*
[2]*Chair of Biochemistry and Clinical Chemistry, Faculty of Pharmacy, Medical University of Warsaw, Banacha Str. 1, 02–097 Warsaw, Poland*
[3]*Herbology Department, Carpathian State University, Rynek 1, 38–400 Krosno, Poland*

Key words: *Aronia melanocarpa* leaves, sorbitol, chlorogenic acids, anti-inflammatory activity, NMR, HPLC

Aronia melanocarpa E. berries are a valuable component of the healthy diet. They are extremely high in phenolics exhibiting strong antioxidant properties. However, not much information is available on the chemical composition and bioactive potential of chokeberry leaves. Therefore, the analyses of sugars and phenolics of extracts from chokeberry leaves collected from June to October were performed using spectroscopic (NMR) and chromatographic (HPLC-DAD/RI, GC-MS) methods. The leaf extracts contained a significant amount of sorbitol, especially those made of leaves collected since June to July (avg. 145.2 mg/g d.w.). The average contents of Cya-3-Gal and chlorogenic acids in the extracts were at 0.52 mg g d.w. and up to 13.1 mg/g d.w., respectively. Chokeberry leaf extracts from green and red leaves were subjected to the *in vitro* study on human umbilical vein endothelial cells (HUVECs). Both extracts suppressed TNF-α-induced surface expression of ICAM-1 and VCAM-1 molecules, and exhibited anti-adhesive and anti-inflammatory properties. Green and red leaves may foster a therapeutic potential in the prevention of atherosclerosis and other pathological events involving leucocyte adhesion. That is why chokeberry leaves can be considered as a promising component of functional foods owing to the high content of chlorogenic acids and sorbitol.

ABBREVIATIONS

CGA – chlorogenic acid, 3-*O*-caffeoylquinic acid (CAS 327–97–9); nCGA – neochlorogenic acid, 5-*O*-caffeoylquinic acid (CAS 906–33–2); iCGA – isochlorogenic acid A, 3,5-dicaffeoylquinic acid (CAS 2450–53–5); Cya-3-Gal – cyanidin 3-*O*-galactoside (CAS 27661–36–5); TNFα – tumor necrosis factor alpha; ICAM-1 – intercellular adhesion molecule 1; VCAM-1 – vascular cell adhesion molecule 1; CAMs – cell adhesion molecules; HUVECs – human umbilical vein endothelial cells.

INTRODUCTION

Aronia melanocarpa [Michx.] Elliot [Hardin, 1973; Strigl *et al.*, 1995] is a member of the *Rosaceae* family; commonly called the black chokeberry, chokeberry or simply aronia. It is cultivated in Poland and other European countries as well as in the USA and South Korea. Its fruits are most frequently used to produce juice, syrup, jam, jellies, wine, and herbal teas. Earlier studies were focused on the chemical composition and biological activity of the fruits. Recently, an increased interest in aronia has been observed because of the nutritional benefits of the berries and their role in the prevention of degenerative diseases. The berry extracts and juice showed antioxidant, antiatherosclerotic, antidiabetic, anti-inflammatory, antiviral, and antimutagenic properties [Chrubasik *et al.*, 2010; Kokotkiewicz *et al.*, 2010; Kulling & Rawel, 2008].

There are only a few studies available on the composition and potential application of chokeberry leaves. As by-products of aronia cultivation, they are plentiful and cheap raw material. The leaves are green in the spring and summer, and turn red and become decorative in the autumn. Chokeberry leaves may exhibit interesting biological effects due to the content of phenolic compounds and their high antioxidant activity [Thi & Hwang, 2014]. Among their phenolic components, flavonoids (glycosides of cyanidin, quercetin, isorhamnetin and apigenin, and kaempferol derivative), hydroxycimmanic acids (chlorogenic, neochlorogenic, and dicaffeoylquinic acids), and proanthocyanidins (oligomers and polymers) were detected using HPLC-MS/MS [Lee *et al.*, 2014; Teleszko & Wojdyło, 2015].

A. melanocarpa fruits have an interesting free sugar profile. They contain significantly more sorbitol than fructose, glucose, and sucrose [Denev *et al.*, 2018; Yang *et al.*, 2019]. Sugar alcohols (polyols): sorbitol, mannitol and xylitol, were

---

* Corresponding Author:
E-mail: agnieszka.zielinska@wum.edu.pl (A. Zielińska)

found in several wild berries [Mäkinen & Söderllng, 1980]. Sorbitol and mannitol occurred most abundantly in rowan berries (*Sorbus aucuparia*), with their contents reaching up to 5.3 and 0.38 mg/g of fresh weight, respectively. Polyols are used as sugar replacers [Livesey, 2003]. They are low glycemic, low insulinemic, low-energy, and osmotic (colon-hydrating, laxative). The glycemic index (GI) and insulinemic index (II) of sorbitol are 9 and 11, respectively (sucrose 65 and 43, respectively, and glucose 100 and 100, respectively). An average diet provides 2.6–5.8 g of polyols per day [Lee, 2015]. Dried prunes (9–18 g of sorbitol/100 g) are one of the highest sorbitol-containing *Rosaceae* fruit. However, to the best of our knowledge, sugar composition of chokeberry leaf has not yet been determined. The Food and Drug Administration (FDA) has asserted that sorbitol is generally recognized as safe (GRAS, 21CFR184.1835).

Chlorogenic acids (CGAs) are the main phenolic compounds of chokeberry [Thi & Hwang, 2014]. They also occur in remarkable quantities in coffee beans, cocoa, apples, and berry fruits (blackberry, mulberry) [Clifford, 2000]. Chlorogenic acid exhibits high antioxidant activity [Marinova *et al.*, 2009] due to the ability to scavenge free radicals and modulate antioxidant enzyme gene expression [Gugliucci & Bastos, 2009]. CGAs reduce glucose absorption, act as a protective agent to liver cells, and may offer clinical benefits in the treatment of neurodegenerative diseases [Mikami & Yamazawa, 2015].

Anthocyanins are plant pigments classified as natural non-nutritive substances, their presence being indicated by dark red color of fruits and leaves. Young leaves deficient in chlorophyll as well as old leaves are red in color. Discoloration of old leaves results from atmospheric factors, *e.g.*, cold weather or ultraviolet radiation [Lee, 2002; Szopa *et al.*, 2017; Thi & Hwang, 2014; Zou *et al.*, 2012]. The dominant pigments in aronia fruits are cyanidin 3-*O*-galactoside and cyanidin 3-*O*-arabinoside [Zielińska *et al.*, 2020]. Anthocyanins provide a range of health benefits, they can reduce oxidative stress, act as anti-inflammatory agents, or regulate glucose concentration in the blood. It has been demonstrated that the intake of aronia juice and the extracts lowers blood pressure and reduces serum levels of total cholesterol, LDL cholesterol, and triglycerides in patients with the metabolic syndrome [Skoczyńska *et al.*, 2007]. Moreover, berry extracts were found to reduce the levels of cardiovascular risk markers, such as oxidized low-density lipoprotein (oxy-LDL), C-reactive protein (CRP), interleukin 6 (IL-6), soluble intercellular adhesion molecule-1 (sICAM-1), serum-soluble vascular cell adhesion molecule-1 (sVCAM-1), or monocyte chemoattractant protein 1 (MCP-1) in patients after myocardial infarction [Naruszewicz *et al.*, 2003, 2007]. Based on *in vitro* studies and animal experiments, it is assumed that most of the beneficial activity of *A. melanocarpa* extracts is due to the high anti-oxidative activity of polyphenols, especially anthocyanins [Chrubasik *et al.*, 2010]. The studies on endothelial cells have provided new insights into understanding the molecular mechanisms underlying their beneficial effects, besides antioxidative and radical scavenging ones [Xia *et al.*, 2009]. The *in vitro* studies have demonstrated that the *A. melanocarpa* fruit extract inhibits 7b-hydroxycholesterol-induced apoptosis of endothelial cells [Zapolska-Downar *et al.*, 2012]. Human umbilical vein endothelial cells (HUVECs) were treated with various concentrations of the commercial chokeberry fruit extract prior to the treatment with a tumor necrosis factor alpha (TNFα). The surface protein and mRNA expression of cell adhesion molecules ICAM-1 and VCAM-1, as well as the adhesion of peripheral blood mononuclear leucocytes (PBMLs) to HUVECs were evaluated [Chang *et al.*, 2010]. The endothelial adhesiveness to PBMLs and the expression of adhesion molecules in endothelial cells can also be influenced by chlorogenic acid [Bagchi *et al.*, 2002] or proanthocyanidins.

The aim of this study was to perform a qualitative and quantitative analysis of the phenolic compounds and sugars of leaf water extracts of chokeberry var. 'Nero', as well as to track their changes in the growing season (from flowering to fruit ripening) by means of chromatographic methods (high-performance liquid chromatography with UV/VIS diode array and refractive index detection, HPLC-DAD/RI; gas chromatography – mass spectrometry, GC-MS) and spectroscopic methods (nuclear magnetic resonance, NMR). A further goal was to investigate the anti-inflammatory activity of the extracts in the *in vitro* study on human umbilical vein endothelial cells (HUVECs). Water was chosen as the solvent because of the potential use of the leaves in herbal teas.

MATERIALS AND METHODS

Plant material

Aronia melanocarpa (Michx.) Elliott leaves were collected at an ecological plantation (Organic Nursery Aronia Eggert, Grójec, Poland), from June until October 2013. Plant material was authenticated by Mr. Piotr Eggert (M.Sc.). A voucher specimen (no 10192) was deposited at the herbarium of the Department of Biology and Pharmaceutical Botany, Medical University of Gdansk, Poland. The leaves were collected from the moment they were young but shaped until turning red, for a period of five months. Harvesting was performed on the following days : June 4 and 26, July 18 and 26, August 16, September 2 and 21, October 18, collecting about 250 g of leaves from three selected, healthy, and well-defined shrubs on the plantations (from the same shrubs at all collection periods). All leaf samples were frozen immediately after collection and lyophilized. The lyophilized leaves were ground into powder and stored at -70°C until extraction and analyses.

Chemicals

All standards, reagents, and solvents were of analytical or gradient grade. HPLC standards: chlorogenic acid (CGA, CAS 327–97–9), neochlorogenic acid (nCGA, CAS 906–33–2), isochlorogenic acid A (iCGA, CAS 2450–53–5), and cyanidin 3-*O*-galactoside (Cya-3-Gal, CAS 27661–36–5) were purchased from PhytoLab GmbH & Co. KG, (Vestenbergsgreuth, German), sold as a primary reference standard with certified absolute purity. D-Sorbitol (99%, CAS 50–70–4), d-(+)-glucose (≥99.5%), D-(−)-fructose (≥99%), and sucrose (≥99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Sigma-Aldrich and Avantor Performance Materials S.A. (Gliwice, Poland). Ultra-pure water (Millipore Milli-Q) was used for sample preparation.

Human umbilical vein endothelial cells (HUVECs), EBM-2 (Endothelial basal medium), EGM-2 Bulletkit = EBM-2 + all growth supplements, fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetate), and trypsin neutralizing solution (TNS) were obtained from LONZA (Basel, Switzerland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and non-enzymatic cell dissociation solution were provided by Sigma (St. Louis, MO, USA). Human recombinant TNFα, phycoerythrin (PE) – conjugated mouse monoclonal antibody anti-human ICAM-1, fluorescein isothiocyanate (FITC) – conjugated mouse anti-human VCAM-1 monoclonal antibody, and suitable conjugated mouse IgG isotypes were all purchased from Becton Dickinson (San Diego, CA, USA). All other chemicals were purchased from Sigma-Aldrich.

Extraction

The leaf samples of 0.5 gram were weighed out. Then, 50 mL of boiling deionized water was added and the samples were kept at 90°C for 30 min. Next, they were passed through a sintered glass filter under reduced pressure and evaporated to dryness using a Heidolph Hei-VAP Core rotary evaporator (Heidolph Instruments, Schwabach, Germany). Three extracts were prepared for each batch. Next, the dry samples were dissolved in 10 mL of 80% (*v/v*) MeOH (to remove polymerized sugars), filtered through a sintered glass filter, and evaporated to dryness using a rotary evaporator. To obtain carbohydrate fractions, the extracts were dissolved in 80% (*v/v*) MeOH, loaded on an RP-18 column (10×100 mm), and eluted under increased pressure of 80 bar (Waters HPLC pump 515, Waters Corp., Milford, MA, USA), with 50 mL of 80% (*v/v*) MeOH (first fraction, SF), later with 100% methanol (second fraction), eventually with chloroform to clean the column. The SF contained water-soluble carbohydrates and chlorogenic acids, the second fraction contained other phenolics. The fractions were evaporated to dryness and stored in a freezer for further analysis.

Sugar and sorbitol content analysis by HPLC-RI and GC-MS

The HPLC-RI quantitative analysis was carried out using a Hitachi Chromaster HPLC system with an RI detector and a Purospher STAR NH_2 (5 μm, 4×250 mm) column, under isocratic conditions, mobile phase: acetonitrile/water 75:25 (*v/v*), a flow rate: 1 mL/min. The concentration of sorbitol was determined using a calibration curve generated for sorbitol, glucose, fructose, and sucrose standards. The linearity of the method was assessed based on the value of the coefficient of determination (R^2) of the calibration curve obtained for each standard (Table 1). The limit of detection (LOD) and limit of quantification (LOQ) concentrations were set using equations: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$, where σ is the standard deviation of the y-intercept and S is the slope of the calibration curve (Table 1). The results of method accuracy analysis showed low values of % RSD (< 2) for interday variation. The recovery results were within the acceptable limit (recovery ranged from 97 to 103%) of the interday variation, which indicated that the method is accurate. There was no significant change observed for the chromatograms of the standard solution and the experimental solution.

For the GC measurement, trimethylsilyl derivatives of the extract compounds and sugar standards were obtained with the BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) mixture, according to Bstfa+Tmcs Spec. [1997]. The composition of derivatized samples was determined using the GC system coupled to an Agilent 7890A & 5975C MS detector (Santa Clara, CA, USA). The column and analytical parameters were as follows: HP-5MS capillary column (30 m×0.25 mm×0.25 μm, Agilent), carrier gas – helium (1 mL/min), temperature program – 150°C for 3 min, 150–300°C, 5°C/min. The compounds were identified using reference mass spectra from the NIST Standard Reference Database. All the HPLC and GC retention times were confirmed using respective standards.

Sorbitol analysis by NMR spectroscopy

^{13}C NMR spectra were recorded at room temperature on a Varian VNMRS 300 MHz spectrometer (Palo Alto, CA, USA) operating at 75 MHz for ^{13}C. The dry extracts were dissolved in DMSO-d_6. Chemical shifts (δ) are given in ppm with TMS as an internal standard. NMR peak assignment for sorbitol is in agreement with the reported data [Ulrich *et al.*, 2008].

CGA and anthocyanin content analysis by HPLC-DAD

Qualitative and quantitative analyses of the leaf extracts were performed using HPLC-DAD. Profiles of chlorogenic acids and anthocyanins were characterized simultaneously using the Hitachi Chromaster system (Tokyo, Japan) with a Purospher STAR RP-18e column (5 μm, 4×250 mm),

TABLE 1. Calibration curves, concentration range, limit of detection (LOD), and limit of quantification (LOQ) determined for sugar and sugar alcohol using the HPLC-IR method.

Compound	Calibration curve[a]	R^2	Linear range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
Sorbitol	A=114479c–709	0.999	20.0–50.0	0.51	1.55
Fructose	A= 437966c–923	0.997	0.25–2.5	0.06	0.18
Glucose	A=404226c+711	0.998	0.25–2.5	0.05	0.15
Sucrose	A=448100c+962	0.998	0.25–2.5	0.05	0.15

[a] A – peak area, c – concentration (mg/mL) of the compound.

according to the methods validated and described in our publication on the chokeberry research [Zielińska *et al.*, 2020]. A mobile phase gradient system consisting of 4.5% (*v/v*) formic acid (A) and acetonitrile (B) was employed for the analysis. The gradient conditions were as follows: 0–5 min, 5% B; 5–15 min, 5–8% B; 15–50 min, 8–25% B; 50–55 min, 25–50% B; 55–65 min, 5% B, flow rate: 1–15 min, 1 mL/min; 15–50 min, 0.8 mL/min; 50–65 min 1 mL/min. The anthocyanin chromatograms were monitored at 520 nm, CGAs at 330 nm, retention times were: nCGA 10.9 min, CGA 20.5 min, iCGA 22.3 min, and Cya-Gal 26.8 min. The concentrations of the compounds were determined using an appropriate calibration curve. All measurements were performed in triplicate.

Anti-inflammatory activity of green and red chokeberry leaves in human endothelial cells

Materials

Two extracts were selected for detailed testing of the anti-inflammatory activity: from green leaves (collected on August 16) and red leaves (collected on October 18). The aim of the anti-inflammatory activity tests was to compare the properties of green and red leaves. Therefore, the samples with a similar CGA acid content were selected, one of which additionally contained anthocyanins. The August 16 leaves were healthy with no signs of reddening or wilting. In the case of a sample collected in the autumn, fully red leaves were selected (only sample form October 18 met this criterion).

The extracts were dissolved before use in the phosphate-buffered saline (PBS) solution containing 10% DMSO (10 mg/mL) and further diluted with culture medium. All reagents were maintained at -20°C and further dilutions were made with culture medium prior to use.

Cell culture and experimental conditions

Human umbilical vein endothelial cells were cultured in endothelial cell growth medium with 2% FBS. The cells were maintained at 37°C in 5% CO_2 in humidified atmosphere and used for experiments between passages 3 and 4 (doubling population ≤10). For experiments, human umbilical vein endothelial cells were cultured in 6- or 12-well plates. When the HUVECs reached confluence, the cells were pretreated with the *A. melanocarpa* extract (50 μg/mL) for 2 h. The cells were then treated with TNFα (10 ng/mL) for the indicated period of time. Cell viability remained constant in all experiments (above 90%).

Cell viability assessment by MTT assay

Cell viability was assessed by determining the MTT salt conversion using mitochondrial dehydrogenase. Briefly, the cells were incubated for 24 h in 24-well plates at a concentration of 50 μg/mL of the tested extracts, and then for another 4 h with 0.5 mg/mL of the MTT solution, which is converted in living cells to insoluble formazan under the influence of mitochondrial dehydrogenase. The converted pigments were then dissolved in 0.04 M HCl in absolute isopropanol. The absorbance of dissolved formazan was measured spectrophotometrically at 570 nm using an Epoch microplate reader (BioTek Instruments Inc., Winooski, VT, USA) equipped with Gen5 software. Cell viability remained constant in all experiments (above 90%).

Measurement of ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells using flow cytometry

Resting HUVECs in 12-well plates were pretreated with the indicated concentration (50 μg/mL) of *A. melanocarpa* extracts for 2 h and then treated with TNFα (10 ng/mL) for 16 h. After the experiments, the cells were washed with a phosphate-buffered saline solution (PBS). Next, they were harvested and washed in PBS containing 1% FBS and resuspended in 100 μL of the labeling buffer. Immediately after that, the cells were incubated with PE-conjugated mouse anti-human ICAM-1 and FITC-conjugated mouse anti-human VCAM-1 antibodies, in a dark place for 1 h. For the isotype control, the cells were treated with PE-conjugated mouse anti-IgG1 antibody. The samples were washed again with PBS and analyzed (10,000 cells per sample) by flow cytometry using the FACSCalibur system (BD, Biosciences, San Jose, CA, USA) with the CellQuest Software. After correcting for non-specific binding using the isotype control, the mean fluorescence intensity was measured as an indicator of ICAM-1 and VCAM-1 surface protein expression.

Statistical analysis

The results were expressed as mean ± standard deviation (n=3). Statistica 10 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis of experimental results. One-way analysis of variance (ANOVA) was applied with Tukey test at a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Contents of sorbitol and sugars

Simple carbohydrates can be distinguished by thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC). These methods have specific advantages and drawbacks. For GC separations, sugars have to be derivatized. In the case of the HPLC method, the detection of simple carbohydrates from highly colored small fruit samples may be disturbed by anthocyanins. Therefore, we used NMR spectroscopy which does not require chemical preparation of the sample and yields information on all sugars in one experiment (NMR spectrum).

The GC-MS analysis, performed prior to ^{13}C NMR, indicated that the major sugar of chokeberry leaves may be one of the sugar alcohols: sorbitol or mannitol (Table 2). Among other trimethylsilyl (TMS) derivatives, two peaks of glucose (α- and β-glucopyranoside) and one of sucrose were identified, also quinic and caffeic acids – constituents of chlorogenic acid. However, the fructose peaks were not detected, probably due to the decomposition of fructose silyl derivatives.

The NMR analysis was performed to identify sugars in the leaf extracts. The ^{13}C NMR spectra of the sugar fraction of water extracts from aronia leaves showed six high resonances revealing the presence of sorbitol as the main

TABLE 2. GC-MS data for trimethylsilyl (TMS) derivatives of the sugar fraction (SF) of chokeberry leaf extract (sample collected on October 18).

Peak No	t_R (min)	Masses	Relative content in SF (% of total area)	Compound
1	22.181	345, 255	21.2	Quinic acid (5 TMS)
2	22.490	204, 191	6.4	D-Glucose (5 TMS)
3	22.987	319, 205, 103	44.9	Sorbitol/mannitol (6 TMS)
4	23.356	204, 191	10.2	D-Glucose (5 TMS)
5	24.615	396, 219	2.5	Caffeic acid (3 TMS)
6	28.232	217, 204	5.5	Derivatization by-product
7	28.266	217, 204	3.7	Derivatization by-product
8	29.055	437, 361, 217	5.5	Sucrose (8 TMS)

Note: mass 73 and 147 for TMS groups are omitted in the table.

component. An exemplary spectrum of the sugar fraction (sample from July 24) is shown in Figure 1a. For comparison, ^{13}C NMR spectra (in DMSO-d6) of pure sorbitol and mannitol are illustrated in Figures 1b and 1c, respectively. Chemical shifts are collected in Table 3. The spectra and data collected show that the sugar fraction of leaf extracts did not contain mannitol.

The ^{13}C NMR spectrum of the sugar fraction of water extract reveals two areas typical of saccharides (Figure 1a, Table 4). One of them is situated between δ 60 and 80 ppm (CH and CH_2 carbons) and the other between δ 80 and 106 ppm where the signals of anomeric carbons of glucose (α- and β-glucopyranose) and fructose (α- and β-fructofuranoside) can be observed.

The HPLC analysis with a refractive index detector (HPLC-RI) and NH_2 column was performed to determine the content of carbohydrates. Its results indicated that the minor soluble carbohydrates present in the water extract of chokeberry leaves at the levels of 1–4% were fructose, glucose, and sucrose (3.3±0.1, 2.2±0.1, 6.1±0.3 mg/g of lyophilized leaves, respectively). The main sugar component, sorbitol, represented *ca.* 80% of total carbohydrates, with the average content at 145.2±1.8 mg/g of lyophilized leaves. Its high content has also been determined in the leaves of other shrubs and fruit trees, *e.g.* at 60 – 80 mg/g d.w. on average in apples (depending on irrigation) [Naschitz *et al.*, 2010], and at 30 – 40 mg/g d.w. in peaches [Liu *et al.*, 2013]. On the other hand, its content in blueberries is as low as 0.02–0.05 mg/g d.w. [Fotiric Aksic *et al.*, 2019]. Thus, the sorbitol content can vary widely in plants.

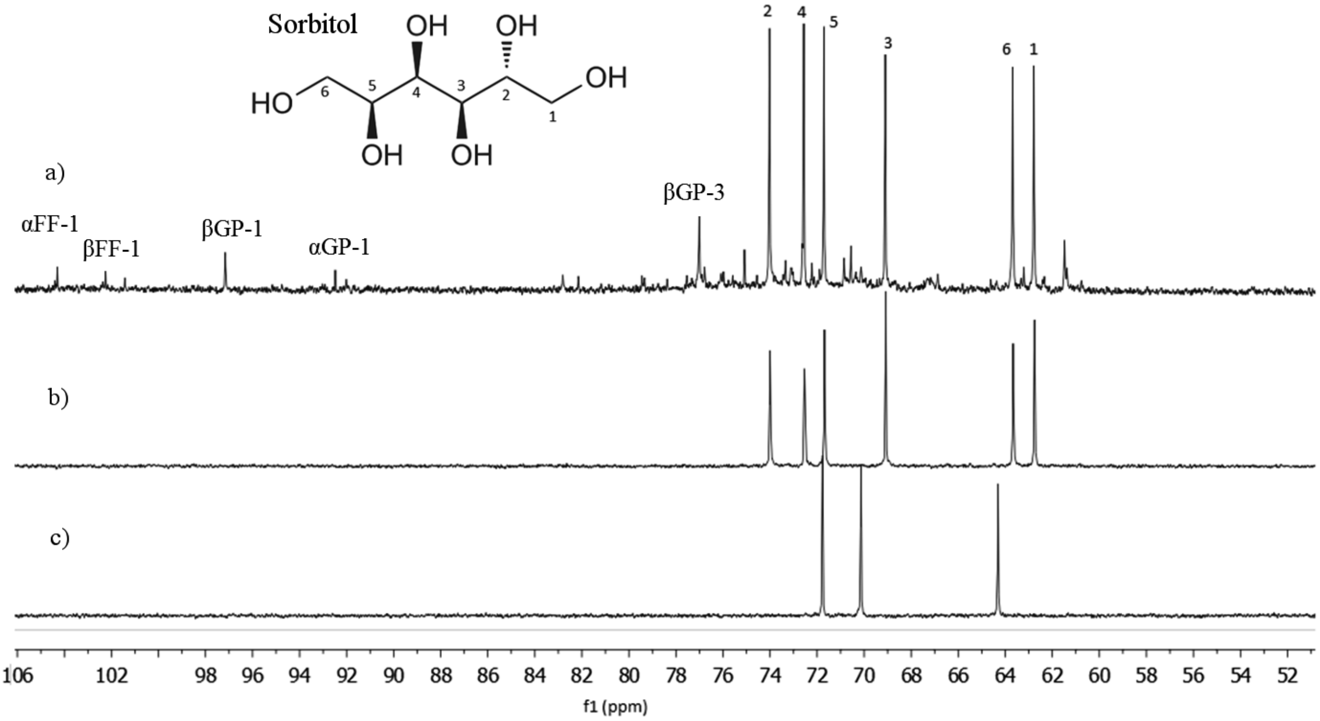


FIGURE 1. ^{13}C NMR spectra of a) the sugar fraction of the chokeberry leaf extract (sample from July 24) with the carbon numbering of sorbitol, b) sorbitol standard, and c) mannitol standard. GP – glucopyranose, FF – fructofuranoside.

TABLE 3. ^{13}C NMR chemical shifts (δ, ppm) of sorbitol of the sugar fraction (SF) of chokeberry leaf extract (sample collected on July 26), and standards: sorbitol and mannitol.

Carbon	δ Sorbitol (SF)	δ Sorbitol std	δ Mannitol std
1	62.98	62.52	64.31
2	74.22	73.77	71.77
3	69.30	68.85	70.14
4	72.92	72.30	70.14
5	71.91	71.45	71.77
6	63.88	63.43	64.31

TABLE 4. ^{13}C NMR chemical shifts (δ, ppm) of anomeric carbons C1 (α- and β-glucopyranose and fructofuranoside of the sugar fraction of chokeberry leaf extract; sample collected on July 26).

Glucose/fructose	δ C1
α-Glucopyranose (αGP-1)	92.68
β-Glucopyranose (βGP-1)	97.35
β-Glucopyranose (βGP-3)	77.20
α-Fructofuranoside (αFF-1)	104.49
β-Fructofuranoside (βFF-1)	102.44

The changes of sorbitol content in *A. melanocarpa* leaves were observed during seasonal growth (Figure 2). In the spring, the content of sorbitol was high, later it decreased and remained unchanged during the summer. An increase was observed after fruit collecting (September 20) and the content decreased slightly at the end of the season (October 18). The pattern of changes can be related to flowering, fruit ripening, and removal. In Poland, the harvest usually takes place between the last week of August and the first week of September. The highest content of anthocyanins, vitamin C, and reducing sugars in fruits was determined between August 29 and September 1, as reported by Andrzejewska *et al.* [2015] and Yang *et al.* [2019].

The fruit removal has a significant effect on the metabolism of the whole plant, including the leaves. The study on peach leaves [Nii, 1997] showed that the level of reducing and non-reducing sugars increased immediately after the harvest and then decreased successively, whereas the content of sorbitol also increased after fruit removal but remained high.

CGAs and anthocyanins in chokeberry leaves during seasonal growth

The HPLC studies have shown that Cya-3-Gal is responsible for the red color of the leaves. Other pigments present in chokeberry fruits, mainly cyanidin-3-*O*-arabinoside [Zielińska *et al.*, 2020], were below detection level in our study. The average content of anthocyanin Cya-3-Gal was found to be 0.52±0.01 mg/g dry weight (d.w.) of red leaves, which was relatively low compared to the fruit (2–3 g/100 g d.w.) [Gralec *et al.*, 2019]. This content is lower than in the study by Szopa *et al.* [2017], were it reached 1.9 mg/100 g d.w.. These differences may result from various weather conditions in a given year (temperature during development, amount of sunshine and rainfall, time of leaf coloring).

The content of chlorogenic acids (nCGA, CGA, and iCGA) was determined in chokeberry leaf extracts collected from June till October (Figure 3). The average total content of CGAs was 13.1 mg/g d.w. and 1.9 mg/100 mg of the extract (range 1.46–2.59 mg/100 mg extract). The iCGA was present in the smallest amount. The content of all CGAs varied with the growth stages of the leaves. The highest CGAs content was observed in the extract of leaves collected in the initial growth phase, for instance in June before fruit ripening. At a further stage of growth, a decrease in the content of chlorogenic acids could be observed, with a slight increase in September. The decrease in CGAs content can be correlated with the appearance of anthocyanins in fruit, which takes place in the middle of July. A similar tendency was observed dur-

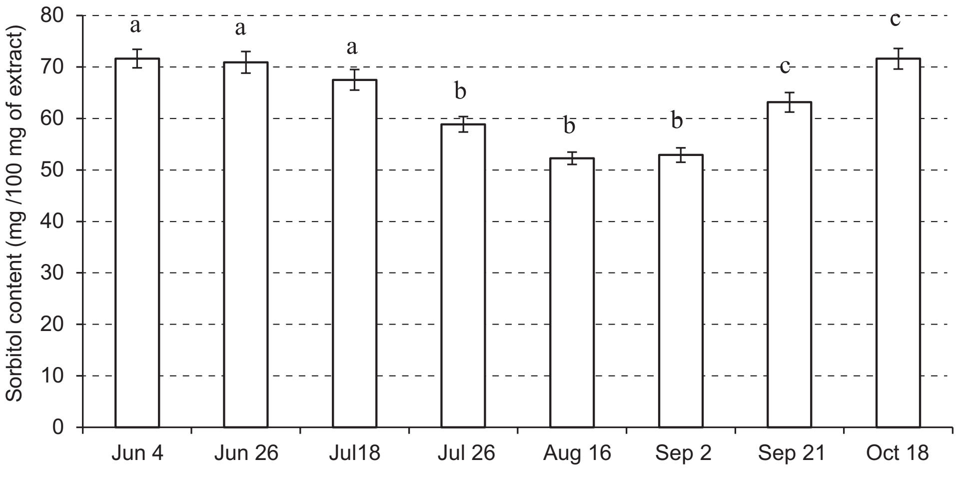


FIGURE 2. The sorbitol content in extracts from chokeberry leaves collected from June to October, Data are expressed as mean (n = 3). Error bars indicate standard deviation. Different letters above bars indicate significant differences (p≤0.05).

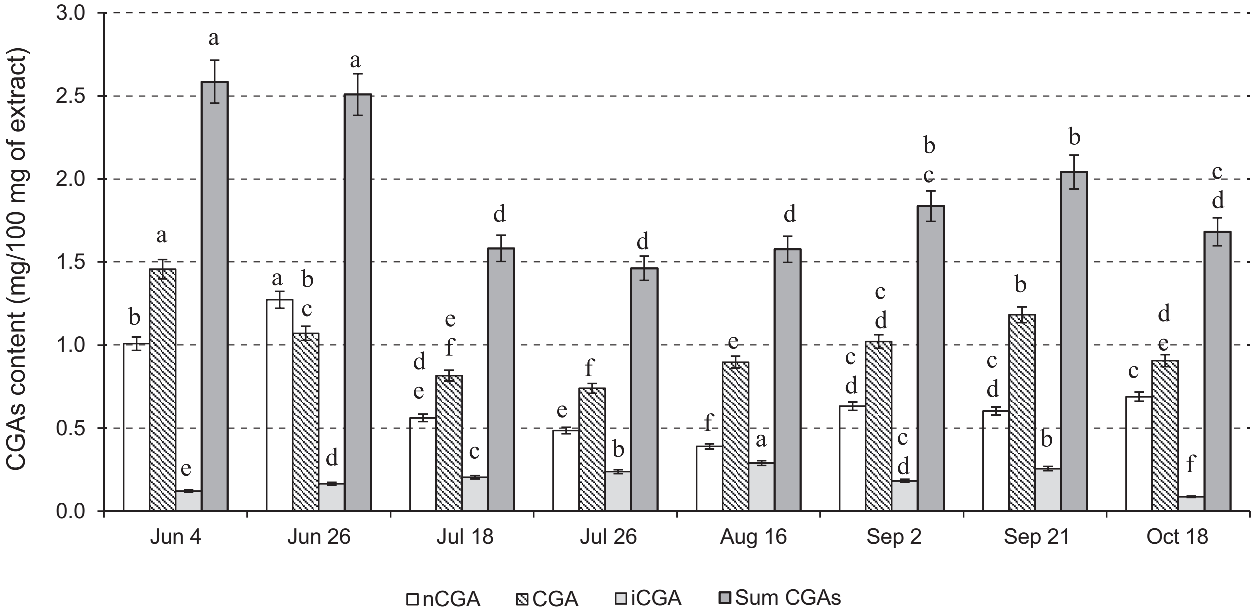


FIGURE 3. The chlorogenic acids (CGAs) content in water extracts of chokeberry leaves collected from June to October. Data are expressed as mean (n = 3). Error bars indicate standard deviation. Different letters above bars corresponding to the same compound indicate significant differences ($p \leq 0.05$). CGA – chlorogenic acid, nCGA – neochlorogenic acid, iCGA – isochlorogenic acid A.

ing the analysis of chokeberry fruit extracts during ripening [Zielińska *et al.*, 2020]. In green fruit, the content of CGAs was remarkably high, then it decreased and remained unchanged till the end of the season (October). However, the content of both isomers (CGA and nCGA) was fivefold higher (avg. 70 mg/g d.w.).

Our research confirms previous reports that the chlorogenic acids are the main phenolic compounds present in *A. melanocarpa* leaves. Korean researchers [Thi & Hwang, 2014] determined the content of CGAs in chokeberry leaves harvested at different stages of growth and extracted with two different solvents: distilled water and 80% ethanol, which turned out to have a significant impact on the CGAs content in the extract. The content of chlorogenic acid in distilled water and 80% ethanol extracts of chokeberry leaves was 17.2 mg/g and 22.8 mg/g, respectively. These findings correspond quite well with the results obtained in the present study. In turn, the research by Szopa *et al.* [2017] showed that the dominant compounds in all analyzed leaf extracts from Aronia species (*A. melanocarpa*, *A. arbutifolia*, and *A.* × *prunifolia*) were chlorogenic and neochlorogenic acids. Their contents ranged from 184.0 to 678.2 mg/100 g d.w. and from 143.5 to 482.7 mg/100 g d.w., respectively.

Anti-inflammatory activity of green and red chokeberry leaves in human endothelial cells

Adhesion molecules are strongly involved in the formation of atherosclerotic plaques by mediating leukocyte migration. Here, we observed that both chokeberry leaf extracts (green leaves collected on August 16 and red leaves collected on October 18) reduced TNF-α induced surface expression of ICAM-1 and VCAM-1 molecules. The extract from the green leaves from the August harvest caused 1.2-fold inhibition of ICAM-1, which is stimulated by TNFα. As for the surface expression of the TNFα-stimulated VCAM-1 molecule, it was 1.3-fold inhibited by the extracts from the green leaves (Figure 4). The red leaf extract inhibited ICAM-1, which is stimulated by TNFα (1.2-fold inhibition). As for the surface expression of the TNFα-stimulated VCAM-1 molecule, it was 1.3-fold inhibited by the extract from the red leaves The treatment of endothelial cells with leaf extracts alone had no effect on constitutively expressed levels of ICAM-1 or VCAM-1.

The red leaf extracts had a rather strong anti-inflammatory effect on endothelial cells. This is probably due to the presence of chlorogenic acids and anthocyanins. It should be noted that the content of Cya-3-Gal in the extracts was low (0.18 mg/100 g), whereas that of chlorogenic acids was ten times higher (1.9 mg/100 g). Surprisingly, the anti-inflammatory effect was similar to that of the green leaf extract which has no anthocyanins but contains chlorogenic acids as the main phenolic ingredient. Thus, the CGAs may be the key compounds determining these properties. These two extracts also contained sorbitol, but its role was not assessed. In conclusion, both leaf extracts are valuable and can be used in the future to prepare medicinal products against inflammation and degenerative diseases, such as cardiovascular diseases.

It seemed interesting to consider the results of the study on chokeberry leaves from South Korea [Lee *et al.*, 2014]. The antioxidant capacities of the leaf extracts were determined as the DPPH$^{\bullet}$ and ABTS$^{\bullet +}$ scavenging activities, which were the highest for young leaves, followed by the mature and aged ones. This antioxidant activity trend may be associated with the total phenolic content, particularly with that of the chlorogenic acid derivatives. Younger chokeberry leaves may be utile for processing into high-quality functional tea, as they had the highest phenolic content. Our results on the content of chlorogenic acids and anti-inflammatory activity support this assumption. However, there is no significant

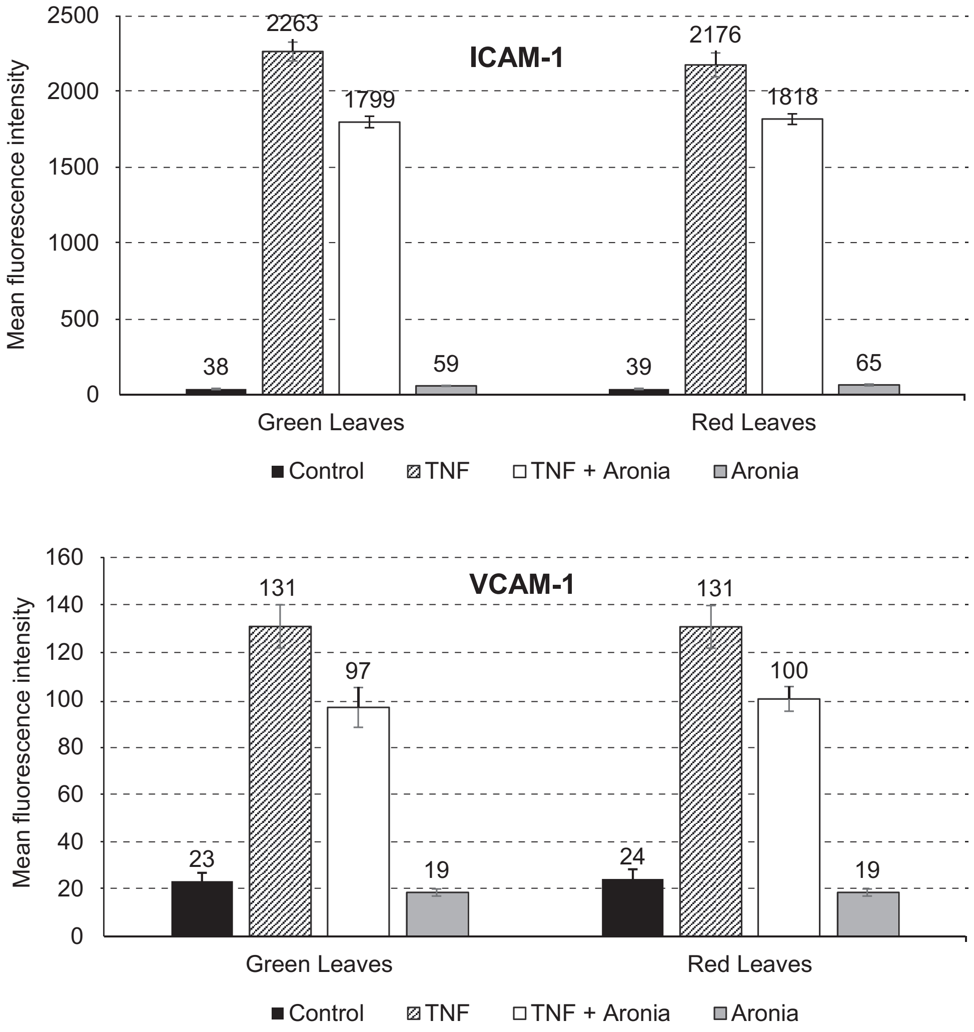


FIGURE 4. The effect of chokeberry leaf extract on ICAM-1 and VCA-1 expression after TNFα stimulation. Error bars indicate standard deviation (n = 3).

difference between the young and the aged leaves in the anti-inflammatory effect on endothelial cells. In this regard, red leaves can also be considered as a functional food ingredient.

CONCLUSION

Sorbitol is commonly used as a sweetening agent, an alternative to refined sugar. *A. melanocarpa* leaves appeared to be a rich source of sorbitol and therefore infusion (herbal tea) or the extract might be a valuable component of the diet, especially for diabetic patients. The content of sorbitol was higher at the beginning and the end of the season, but lower in the summer. The green and red leaf extracts have a rather strong anti-inflammatory effect on endothelial cells. This is probably due to the presence of chlorogenic acids and anthocyanins. Therefore, both the green leaf extract and the red leaf extract potentially can be used in functional teas, together with dried/lyophilized chokeberry fruits or fruit powders. The leaves are affordable raw material, however, it is worth remembering that collecting red leaves in the fall makes no harm to bushes, as opposed to removing them in the spring.

ACKNOWLEDGEMENTS

The authors thank Professor Włodzimierz Daniewski for his support in the experiments.

RESEARCH FUNDING

This work was supported by the Polish National Science Centre (grant number 2015/17/B/NZ7/03089).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID IDs

A. Zielińska https://orcid.org/0000-0002-2244-0627
D. Bryk https://orcid.org/0000-0002-2792-4153
K. Paradowska http://orcid.org/0000-0002-9613-856X
I. Wawer http://orcid.org/0000-0002-8852-4209

REFERENCES

1. Andrzejewska, J., Sadowska, K., Klóska, Ł., Rogowski, L. (2015). The effect of plant age and harvest time on the content of chosen components and antioxidative potential of black chokeberry fruit. *Acta Scientiarum Polonorum, Hortorum Cultus, 14*(4), 105–114.
2. Bagchi, D., Bagchi, M., Stohs, S., Ray, S.D., Sen, C.K., Preuss, H.G. (2002) Cellular protection with proanthocyanidins derived from grape seeds. *Annals of the New York Academy of Sciences, 957*(1), 260–270. https://doi.org/10.1111/j.1749-6632.2002.tb02922.x
3. Bstfa+Tmcs Spec. (1997). Product Specification T496021. Sigma-Aldrich Co.
4. Chang, W.C., Chen, C.H., Lee, M.F., Chang, T., Yu, Y.M. (2010). Chlorogenic acid attenuates adhesion molecules upregulation in IL-1beta-treated endothelial cells. *European Journal of Nutrition, 49*(5), 267–275. https://doi.org/10.1007/s00394-009-0083-1
5. Chrubasik, C., Li, G., Chrubasik, S. (2010). The clinical effectiveness of chokeberry: a systematic review. *Phytotherapy Research, 24*(8), 1107–1114. https://doi.org/10.1002/ptr.3226
6. Clifford, M.N. (2000). Chlorogenic acids and other cinnamates – nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science of Food and Agriculture, 80*(7), 1033–1043. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<1033::AID-JSFA595>3.0.CO;2-T
7. Denev, P., Kratchanova, M., Petrova, I., Klisurova, D., Georgiev, Y., Ognyanov, M., Yanakieva, I. (2018). Black chokeberry (*Aronia melanocarpa* (Michx.) Elliot) fruits and functional drinks differ significantly in their chemical composition and antioxidant activity. *Journal of Chemistry, 2018*, art.no. 9574587. https://doi.org/10.1155/2018/9574587
8. Fotiric Aksic, M., Tosti, T., Sredojević, M., Milivojević, J., Meland, M., Natić, M. (2019). Comparison of sugar profile between leaves and fruits of blueberry and strawberry cultivars grown in organic and integrated production system. *Plants, 8*(7), art. no. 205. https://doi.org/10.3390/plants8070205
9. Gralec, M., Wawer, I., Zawada, K. (2019). *Aronia melanocarpa* berries: Phenolics composition and antioxidant properties changes during fruit development and ripening. *Emirates Journal of Food and Agriculture, 31*, 214–221. https://doi.org/10.9755/ejfa.2019.v31.i3.1921
10. Gugliucci, A., Bastos, D.H. (2009). Chlorogenic acid protects paraoxonase 1 activity in high density lipoprotein from inactivation caused by physiological concentrations of hypochlorite. *Fitoterapia, 80*(2), 138–142. https://doi.org/10.1016/j.fitote.2009.01.001
11. Hardin, J.W. (1973). The enigmatic chokeberries (*Aronia, Rosaceae*). *Bulletin of the Torrey Botanical Club, 100*(3), 178–184. https://doi.org/10.2307/2484630
12. Kokotkiewicz, A., Jaremicz, Z., Luczkiewicz, M. (2010). Aronia plants: a review of traditional use, biological activities, and perspectives for modern medicine. *Journal of Medicinal Food, 13*(2), 255–269. https://doi.org/10.1089/jmf.2009.0062
13. Kulling, S.E., Rawel, H.M. (2008). Chokeberry (*Aronia melanocarpa*) – A review on the characteristic components and potential health effects. *Planta Medica, 74*(13), 1625–1634. https://doi.org/10.1055/s-0028-1088306
14. Lee, D. (2002). Anthocyanins in leaves: Distribution, phylogeny and development. *Advances in Botanical Research, 37*, 37–53. https://doi.org/10.1016/S0065-2296(02)37042-3
15. Lee, J. (2015). Sorbitol, Rubus fruit, and misconception. *Food Chemistry, 166*, 616–622. https://doi.org/10.1016/j.foodchem.2014.06.073
16. Lee, J.E., Kim, G.S., Park, S., Kim, Y.H., Kim, M.B., Lee, W.S., Jeong, S.W., Lee, S.J., Jin, J.S., Shin, S.Ch. (2014). Determination of chokeberry (*Aronia melanocarpa*) polyphenol components using liquid chromatography-tandem mass spectrometry: Overall contribution to antioxidant activity. *Food Chemistry, 146*, 1–5. https://doi.org/10.1016/j.foodchem.2013.09.029
17. Lee, S.Y., Ramirez, J., Franco, M., Lectez, B., Gonzalez, M., Barrio, R., Major, U. (2014). Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates protein homeostasis through the proteasomal shuttle Rpn10. *Cellular and Molecular Life Sciences, 71*(14), 2747–2758. https://doi.org/10.1007/s00018-013-1526-7
18. Liu, D., Ni, J., Wu, R., Teng, Y. (2013). High temperature alters sorbitol metabolism in *Pyrus pyrifolia* leaves and fruit flesh during late stages of fruit enlargement. *Journal of the American Society for Horticultural Science, 138*(6), 443–451. https://doi.org/10.21273/JASHS.138.6.443
19. Livesey, G. (2003). Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. *Nutrition Research Reviews, 16*(2), 163–191. https://doi.org/10.1079/NRR200371
20. Mäkinen, K.K., Söderllng, E. (1980). A quantitative study of mannitol, sorbitol, xylitol, and xylose in wild berries and commercial fruits. *Journal of Food Science, 45*(2), 367–371. https://doi.org/10.1111/j.1365-2621.1980.tb02616.x
21. Marinova, E., Toneva, A., Yanishlieva, N. (2009). Comparison of the antioxidative properties of caffeic and chlorogenic acids. *Food Chemistry, 114*(4), 1498–1502. https://doi.org/10.1016/j.foodchem.2008.11.045
22. Mikami, Y., Yamazawa, T. (2015). Chlorogenic acid, a polyphenol in coffee, protects neurons against glutamate neurotoxicity. *Life Sciences, 139*, 69–74. https://doi.org/10.1016/j.lfs.2015.08.005
23. Naruszewicz, M., Daniewski, M., Łaniewska, I., Pikto-Pietkiewicz, W., Millo, B., Zapolska-Downar, D. (2003). 2P-0473 Effect of anthocyanins from chokeberry (*Aronia melanocarpa*) on blood pressure, inflammatory mediators and cell adhesion molecules in patients with a history of myocardial infarction (MI). *Atherosclerosis Supplements, 4*(2), art. no. 143. https://doi.org/10.1016/S1567-5688(03)90615-5
24. Naruszewicz, M., Laniewska, I., Millo, B., Dłuzniewski, M. (2007). Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (MI). *Atherosclerosis, 194*(2), art. no. e179–184. https://doi.org/10.1016/j.atherosclerosis.2006.12.032
25. Naschitz, S., Naor, A., Genish, S., Wolf, S., Goldschmidt, E.E. (2010). Internal management of non-structural carbohydrate resources in apple leaves and branch wood under a broad range of sink and source manipulations. *Tree Physiology, 30*(6), 715–727. https://doi.org/10.1093/treephys/tpq028

26. Nii, N. (1997). Changes of starch and sorbitol in leaves before and after removal of fruits from peach trees. *Annals of Botany, 79*(2), 139–144.
https://doi.org/10.1006/anbo.1996.0324
27. Skoczyńska, A., Jedrychowska, I., Poręba, R., Affelska-Jercha, A., Turczyn, B., Wojakowska, A., Andrzejak, R. (2007). Influence of chokeberry juice on arterial blood pressure and lipid parameters in men with mild hypercholesterolemia. *Pharmacological Reports, 59*, Suppl. 1, 177–182.
28. Strigl, A.W., Leitner, E., Pfannhauser, W. (1995). Die schwarze Apfelbeere (*Aronia melanocarpa*) als natürliche Farbstoffquelle. *Dtsch Lebensmitt Rundsch, 91*, 177–180 (in German).
29. Szopa, A., Kokotkiewicz, A., Kubica, P., Banaszczak, P., Wojtanowska-Krośniak, A., Krośniak, M., Marzec-Wróblewska, U., Badura, A., Zagrodzki, P., Bucinski, A., Luczkiewicz, M., Ekiert, H. (2017). Comparative analysis of different groups of phenolic compounds in fruit and leaf extracts of Aronia sp.: *A. melanocarpa, A. arbutifolia*, and *A. ×prunifolia* and their antioxidant activities. *European Food Research and Technology, 243*(9), 1645–1657.
https://doi.org/10.1007/s00217-017-2872-8
30. Teleszko, M., Wojdyło, A. (2015). Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. *Journal of Functional Foods, 14*, 736–746.
https://doi.org/10.1016/j.jff.2015.02.041
31. Thi, N.D., Hwang, E.-S. (2014). Bioactive compound contents and antioxidant activity in aronia (*Aronia melanocarpa*) leaves collected at different growth stages. *Preventive Nutrition and Food Science, 19*(3), 204–212.
https://doi.org/10.3746/pnf.2014.19.3.204
32. Ulrich, E.L., Akutsu, H., Doreleijers, J.F., Harano, Y., Ioannidis, Y.E., Lin, J., Livny, M., Mading, S., Maziuk, D., Miller, Z., Nakatani, E., Schulte, C.F., Tolmie, D.E., Kent Wenger, R., Yao, H., Markley, J.L. (2008). BioMagResBank. *Nucleic Acids Research, 36*, D402–408.
https://doi.org/10.1093/nar/gkm957
33. Xia, M., Ling, W., Zhu, H., Ma, J., Wang, Q., Hou, M., Tang, Z., Guo, H., Li, Ch., Ye, Q. (2009). Anthocyanin attenuates CD40--mediated endothelial cell activation and apoptosis by inhibiting CD40-induced MAPK activation. *Atherosclerosis, 202*(1), 41–47.
https://doi.org/10.1016/j.atherosclerosis.2008.04.005
34. Yang, H., Kim, Y.J., Shin, Y. (2019). Influence of ripening stage and cultivar on physicochemical properties and antioxidant compositions of aronia grown in South Korea. *Foods, 8*(12), art. no. 598.
https://doi.org/10.3390/foods8120598
35. Zapolska-Downar, D., Bryk, D., Małecki, M., Hajdukiewicz, K., Sitkiewicz, D. (2012). *Aronia melanocarpa* fruit extract exhibits anti-inflammatory activity in human aortic endothelial cells. *European Journal of Nutrition, 51*(5), 563–572.
https://doi.org/10.1007/s00394-011-0240-1
36. Zielińska, A., Siudem, P., Paradowska, K., Gralec, M., Kaźmierski, S., Wawer, I. (2020). *Aronia melanocarpa* fruits as a rich dietary source of chlorogenic acids and anthocyanins: 1H-NMR, HPLC-DAD, and chemometric studies. *Molecules, 25*(14), art. no. 3234.
https://doi.org/10.3390/molecules25143234
37. Zou, Y., Liao, S., Shen, W., Liu, F., Tang, C., Chen, C.-Y.O., Sun, Y. (2012). Phenolics and antioxidant activity of mulberry leaves depend on cultivar and harvest month in Southern China. *International Journal of Molecular Sciences, 13*(12), 16544–16553.
https://doi.org/10.3390/ijms131216544

Submitted: 24 July 2020. Revised: 26 September, 8 October, and 3 November 2020. Accepted: 6 November 2020. Published on-line: 9 December 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 419–428
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/130256
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

Physicochemical Properties of Biscuits Enriched with Horseradish (*Armoracia rusticana* L.) Products and Bioaccessibility of Phenolics after Simulated Human Digestion

Lolita Tomsone, Ruta Galoburda, Zanda Kruma, Kristine Majore*

Department of Food Technology, Faculty of Food Technology,
Latvia University of Life Sciences and Technologies, Rigas street 22, Jelgava, Latvia

Key words: horseradish, pomace, microencapsulated juice, total phenolic content, radical scavenging activity, bioaccessibility

Health-conscious consumers are interested in products with an increased bioactive compound content. The nutritional quality is considered both as a stability and bioaccessibility concern. The aim of this study was to investigate the effect of horseradish products (horseradish root pomace, horseradish leaf pomace, microencapsulated horseradish root and leaf juice) on the physicochemical properties of biscuits, as well as to assess *in vitro* bioavailability and activity of phenolics. The bioactive compounds and antioxidant activity of horseradish products, which were used to replace the flour in biscuit dough, were analysed. Physicochemical parameters (pH, water activity, colour, phenolic compound contents, and antioxidant activities) were determined for the biscuits. Horseradish products compared to other cruciferous vegetables have a high content of phenolic compounds, as well as high antioxidant activity. Higher levels of bioactive compounds were found in microencapsulated horseradish leaf juice, compared to the other horseradish products used in the study. Enriched biscuits had a significantly higher total phenolic content (TPC) and antioxidant activity, compared to the control. After 180-day storage, TPC of biscuits with horseradish root products did not change significantly but in biscuits with horseradish leaf products it decreased by 27–29%. The bioaccessibility index of phenolic compounds after *in vitro* digestion of enriched biscuits ranged between 2.19 and 2.99. Microencapsulated horseradish leaf juice was more effective in enriching biscuits with bioactive compounds. The developed biscuits enriched with horseradish products could meet consumer expectations for healthy, functional food.

INTRODUCTION

No matter how healthy people want to eat, they also like to have some snacks, treats, and fast carbohydrates, such as biscuits. To make these snacks healthier, it is possible to use biologically active compounds from plants of natural origin in their preparation. Scientists around the world have already conducted studies on the addition of various plant products, by-products of food production, as well as plant extracts to foods, improving their nutritional value as well as biological value. For example, it has been possible to significantly increase the content of phenolic compounds and increase the antioxidant activity of bread with the addition of sweet-lupines [Villarino *et al.,* 2014], rice bran [Irakli *et al.*, 2015], cumin and caraway seeds and by-products [Sayed-Ahmad *et al.*, 2018b], chia seeds and cakes [Sayed-Ahmad *et al.*, 2018a], pasta with mango peel powder [Ajila *et al.*, 2010], *Hibiscus sabdariffa* L. calyxes' residue [Ahmed & Abozed, 2015], and biscuit with grape marc extract [Pasqualone *et al.*, 2014]. As each plant has a specific profile of individual phenolic compounds, their behaviour and efficiency in food matrices also differ; therefore in-depth research of new sources is advisable.

Plants are rich in many biologically active compounds, such as phenolics (phenolic acids and flavonoids), and essential oils, which possess various antioxidant properties [Naczk & Shahidi, 2004]. Therefore, local plants specific to each region, but not widely used, are also sought and identified. Horseradish (*Armoracia rusticana* L.) is a plant growing in temperate latitudes that does not require special growing conditions, and a previous study [Tomsone, 2015] has shown that these plants contain high amounts of biologically active compounds such as phenolic compounds with antioxidant properties. It was found that total phenolic content (TPC) of horseradish leaves was 711–5406 mg GAE/100 g dry matter (DM), but TPC of roots was on average 7–10 times lower. Other studies showed a smaller difference in TPC between leaves and roots, *i.e.*, 256–385 mg GAE/100 g DM and 174–289 mg GAE/100 g DM, respectively [Calabrone *et al.*, 2015]. These plants have long been used for medicinal purposes with a wide range of applications for the treatment of various diseases using both roots and leaves [Agneta *et al.*, 2013]. Although horseradish roots have a greater culinary value, horseradish leaves can also be used for food purposes. Like other cruciferous plants, besides phenolic compounds [Prieto *et al.*, 2019], horseradish is rich in volatile sulphur-containing compounds – glucosinolates [Kloucek *et al.*, 2012], and enzymes such as peroxidase and myrosinase [Belitz *et al.*, 2009; Mokdad *et al.*, 2009].

The high content of biologically active substances in certain food ingredients does not always ensure high bioacces-

---

* Corresponding Author: E-mail: lolita.tomsone@llu.lv (L. Tomsone)

sibility and bioavailability during digestion. Bioavailability, defined as the amount of compounds available for absorption, can depend on a number of factors. These factors are affected by the chemical structure of compounds caused by glycosylation, esterification and polymerization [Alminger *et al*., 2014; Gayoso *et al*., 2018]. The raw materials of the plant kingdom consist of many compounds, which could be unstable in the aggressive environment of the stomach. They can be stabilised using modern technologies. One of such solutions is microencapsulation. It is a physical solution to prevent premature degradation of these substances, where the microcapsule wall acts as a physical protective barrier to chemical compounds.

The aim of this study was to investigate the effect of horseradish root pomace, horseradish leaf pomace, microencapsulated horseradish root and leaf juice on the physicochemical properties of biscuits, as well as to assess *in vitro* bioavailability and activity of phenolics.

MATERIALS AND METHODS

Horseradish products

Horseradish (*A. rusticana* L.) was harvested in Latvia (latitude 56° 39' N, longitude 23° 44' E), washed, cut in 5 cm long pieces. Horseradish leaves and roots were packed in polyethylene bags (approximately 0.5 kg each, in total 50 kg roots and 25 kg leaves) and frozen at -18°C. Horseradish root and leaf juice was obtained by grinding the frozen sample, and then extracting in a basket press. Juice was microencapsulated in a Mini Spray Dryer B-290 (Büchi, Flawil, Switzerland) using maltodextrin as a wall material at the core-to-wall ratio of 50:50 [Tomsone, 2020b]. Horseradish root and leaf pomace was dried in a Musson-1 microwave-vacuum drier (Ingredient, St. Petersburg, Russia) as described by Tomsone *et al*. [2020a].

Biscuit preparation

Biscuits were prepared according to the formulation described in Table 1, and the calculation of the required amount of horseradish product to be added was based on the TPC in each product. The traditional recipe of butter biscuit dough was selected for a control. Horseradish products were used to enrich biscuits with phenolic compounds.

All ingredients were mixed using a Varimixer AR10 mixer (Wodschow & Co., Brondby, Denmark) for ±10 min. The dough was allowed to rest for ±10 min, and then rolled in a 0.7 cm thick sheet. Circles of 4.5 cm in diameter were then cut and were further divided into four equal segments. Biscuits were baked using the silicon baking sheet at 200±5°C for ±10 min in a rotary oven (Sveba Dahlen, Fristad, Sweden). Two batches (each 800 g) of biscuits were baked for each formulation and tempered to room temperature 22±2°C within 1 h. Biscuits were then stored in cardboard boxes at room temperature in the dark at 60±5% relative air humidity and analysed twice – on the next day after baking (further referred to as: before storage) and after 180 days storage (further referred to as: after storage). Ten biscuits were randomly selected from the batch, crushed and used as the average sample for further analysis.

Reagents

Gallic acid, Folin-Ciocalteu reagent, and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were purchased from Sigma-Aldrich (Buchs, Switzerland). All other chemicals were obtained from Acros Organics (Belgium, WI, USA).

Spectrophotometric assays of the total phenolic content and antioxidant activity

Extraction procedure

The extraction of phenolic compounds from horseradish root and leaf pomace was done according to Tomsone *et al.* [2020a]. Briefly, dried pomace was ground and twice extracted using acetone in an ultrasonic bath and then re-extracted with ethanol/water (80:20, *v/v*). Extraction was completed in triplicate for each type of pomace.

For the spectrophotometric analysis of microencapsulated horseradish root and horseradish leaf juice, 0.1 g of powder was dissolved in 100 mL of distilled water and stirred for 20 min on a magnetic stirrer (magnet size 4.0 × 0.5 cm) at 700 rpm. Three extracts were prepared for each type of microcapsules.

For biscuit extraction, 2.5 g of crushed biscuits were suspended in 10 mL of an ethanol/water mixture (80:20, *v/v*) in a glass flask. The extraction was completed at 20±1°C in an ultrasonic bath YJ5120–1 (Oubo Dental, St. Louis, MO, USA) for 30 min at 35 kHz. The clear top layer of the extract was decanted and centrifuged using a CM-6MT centrifuge (Elmi Ltd., Riga, Latvia) for 5 min at 3,500 rpm. The supernatant was collected in a 25-mL graduated flask. Biscuit sediment was re-extracted with 10 mL of a fresh solvent and treat-

TABLE 1. Formulation of biscuit doughs with different horseradish products.

Ingredients	Samples				
	C	B_HRP	B_HLP	B_HRM	B_HLM
Flour and horseradish products, incl.					
flour (g)	500	487.50	497.10	492.10	499.20
dried horseradish root pomace (g)	–	12.50	–	–	–
dried horseradish leaf pomace (g)	–	–	2.90	–	–
horseradish root microcapsules (g)	–	–	–	7.90	–
horseradish leaf microcapsules (g)	–	–	–	–	0.80
Butter (g)	270	270	270	270	270
Sugar (g)	125	125	125	125	125
Egg mass (g)	50	50	50	50	50
Salt (g)	5	5	5	5	5
Baking powder (g)	3	3	3	3	3

Abbreviations: C: control; B_HRP: biscuits with horseradish root pomace powder; B_HLP: biscuits with horseradish leaf pomace powder; B_HRM: biscuits with horseradish root juice microcapsules; B_HLM: biscuits with horseradish leaf juice microcapsules.

ed in an ultrasonic bath for an additional 10 min. The top layer was decanted and centrifuged for 5 min. Both supernatants were combined in a 25-mL graduated flask and filled with solvent till the mark. Extraction was completed in triplicate for each sample.

Spectrophotometric analysis

All spectrophotometric analyses were performed using a JENWAY 6300 spectrophotometer (Barloworld Scientific Ltd., Staffordshire, UK) and were used according to the procedure described by Tomsone *et al.* [2020a].

The total phenolic content (TPC) was determined according to the Folin–Ciocalteu spectrophotometric assay described by Singleton *et al.* [1999]. Calculations were done using a standard curve of a gallic acid solution in water, and results were expressed as mg gallic acid equivalents (GAE) per 100 g of DM. Each extract was analysed in triplicate.

$DPPH^{\bullet}$ scavenging activity was determined according to the method described by Yu *et al.* [2003]. The 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS) was used to determine $ABTS^{\bullet +}$ scavenging activity according to Re *et al.* [1999] procedure. Calculations were done using a standard curve, and results were expressed as mmol 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents per 100 g DM. Each extract was analysed in triplicate.

Determination of individual phenolic compounds and organic acids

Individual phenolics and organic acids were determined by HPLC as described by Tomsone *et al.* [2020a]. Briefly, 1 N HCl/ethanol/distilled water (1:80:19, *v/v/v*) was used for phenolics extraction in an ultrasonic bath YJ5120–1 (Oubo Dental) at 35 kHz and 20±1°C for 10 min. The supernatants obtained after centrifugation were then separated and quantified using a Prominence LC-20AD HPLC system (Shimadzu, Canby, OR, USA) with a photodiode array detector SPD M20A (Shimadzu, Kyoto, Japan). For determination of organic acids, samples were extracted with a solution of metaphosphoric acid in distilled water (pH 3.0), using a magnetic stirrer. The analytical C18 column (Perkin Elmer, 4.6 mm ×250 mm, 5 mm) and detection at a wavelength of 210 nm were used. The total organic acids were calculated as a sum of oxalic, tartaric, quinic, malic, malonic, ascorbic, citric, fumaric, and succinic acids. Each extract was analysed in triplicate.

Determination of moisture content, pH, and water activity

Moisture content in the horseradish products (dried horseradish root pomace, dried horseradish leaf pomace, microencapsulated horseradish juice, and microencapsulated horseradish leaf juice) and biscuits was determined according to the International Organization for Standardization (ISO) 6496 standard [ISO, 1999], which was used for the expression of results in dry matter. Each batch was analysed in triplicate.

The water activity (a_w) was measured directly at 25°C using the Novasina Thermoconstanter model Labswift-a_w (Novasina AG, Lachen, Switzerland). Each reading was completed according to the manufacturer's instructions and done in triplicate for each batch.

The pH value of the samples was measured according to Association of Official Analytical Chemists (AOAC) method No. 943.02 [AOAC, 2020] with some modifications. The biscuit sample (5 g) was weighed into an Erlenmeyer flask and 50 mL of distilled H_2O was added. The suspension was prepared by mixing for 10 min at 25°C. Then, the pH was measured using a Jenway-3505 pH-meter (Barloworld Scientific Ltd., Dunmow, Essex, UK) with a glass electrode standardized by buffer solutions of pH 4 and pH 7, both at 25°C. Each batch was analysed in triplicate.

Colour evaluation

Colour analyses were performed using a ColorTec-PCM instrument (Accuracy Microsensors, Inc., Pittsford, New York, USA), using the CIE L* a* b* colour space, where L* represents the sample's lightness in the range from 0 (black) to 100 (white), a* is the redness from green to red (-a* – +a*), and b* is the yellowness from blue to yellow (-b* – +b*). Each biscuit type was crushed and placed in a PE-LD ziplock bag (size 60 × 75 mm). The colour of each batch was measured on the bag surface in ten random places.

The total colour difference (ΔE) between the enriched biscuits and the butter biscuits without horseradish (control) on the day after baking and cooling was calculated using Equation (1):

$$\Delta E = \sqrt{(L_2^*-L_1^*)+(a_2^*-a_1^*)+(b_2^*-b_1^*)} \qquad (1)$$

where: L_2^*, a_2^*, and b_2^* – measured values of biscuit samples with horseradish products; and L_1^*, a_1^*, and b_1^* – the values of butter biscuit samples (control).

The total colour difference (ΔE_t) between the samples after 180-day storage and the respective biscuits before storage was calculated using Equation (2):

$$\Delta E_t = \sqrt{(L_{t2}^*-L_{t1}^*)+(a_{t2}^*-a_{t1}^*)+(b_{t2}^*-b_{t1}^*)^2} \qquad (2)$$

where: L_{t2}^*, a_{t2}^*, and b_{t2}^* – measured values of biscuit samples after 180-day storage; and L_{t1}^*, a_{t1}^*, and b_{t1}^* – measured values of the respective biscuit samples before storage.

Chroma (C*) was calculated according to Equation (3), and hue angle (h) according to Equation (4) [Dag *et al.*, 2017; Sant'Anna *et al.*, 2013].

$$C_{ab}^* = \sqrt{a^{*2}+b^{*2}} \qquad (3)$$

$$h_{ab} = \arctan\,(b^*/a^*) \qquad (4)$$

when + a* and +b* are in quadrant I.

In vitro digestion

The digestibility of the products was assessed in a gastrointestinal tract (GIT) model – bioreactor, in which nutrient transit and digestibility were simulated. The GIT model used in the study (Multifors 2, INFORS-HT, Bottmingen-Basel, Switzerland) consisted of a 500 mL bioreactor tank in which the processes were controlled with the Iris 6 Pallalel Bioprocess Control software (INFORS-HT, Bottmingen-Basel, Switzerland). The reactor was equipped with temperature and pH sensors, a magnetic stirrer, as well as pumps that controlled the rate of hydrochloric acid (HCl) and sodium bicarbonate ($NaHCO_3$) addition. The temperature in the bioreactor was maintained at 37±0.1°C to implement the simulation process. During the process, continuous mixing was ensured at 120–250 rpm.

The digestibility of the products was simulated based on Minekus *et al.* [2014] enzymatic *in vitro* digestibility protocol. The product (30 g) was placed in a bioreactor connected to a temperature and pH sensor, 30 mL of simulated saliva fluid (SSF) were added, and the sample was kept at 37°C for 2 min. The transition to the stomach was simulated by adding a simulated gastric fluid (SGF), pepsin, $CaCl_2$, and distilled water into the bioreactor. Gastric acid secretion was simulated by adding 1 M HCl and adjusting the pH to 3.0±0.2. Digestion in the stomach was simulated for 2 h.

The stomach content was then neutralised to pH 7.0±0.2 by 1 M $NaHCO_3$ and a simulated intestinal fluid (SIF) containing a concentrated electrolyte solution, enzymes (trypsin, chymotrypsin, α-amylase, lipase), bile salts, $CaCl_2$, and distilled water was added, simulating the transit of gastric contents into the duodenum. Digestion in duodenum was simulated for 2 h. The samples were frozen and stored at −18±1°C till further analysis. Each batch was analysed in duplicate.

Prior to the analysis, the samples were defrosted and centrifuged for 30 min. TPC and antioxidant activity (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity) were measured in the supernatant or the digested part. The bioaccessibility index (PAC) was calculated according to Equation (5) [Celep *et al.*, 2017; Świeca *et al.*, 2017]:

$$PAC = C_{GE}/ C_{BE} \qquad (5)$$

where: C_{GE} – the total phenolic content or antioxidant activity in the samples after gastrointestinal digestion; and C_{BE} – the total phenolic content or antioxidant activity in the non-digested samples.

Statistical analysis

All experiments were performed with at least three replicates and the results are shown as mean ± standard deviation. The analysis of variance (ANOVA) and Tukey's test or t-test were performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA) to determine parameter differences between samples. The differences were considered significant at $p \leq 0.05$. Linear correlation analysis was performed to analyse the relationship between the variables tested.

RESULTS AND DISCUSSION

Description of the horseradish products

Horseradish products can be used as a source of antioxidants. An important group of the natural antioxidants, due to their reduction-oxidation potential, is phenolic compounds – plant secondary metabolites. The horseradish products studied in the current research are also rich in phenolics.

The analysis of variance showed that the TPC, individual phenolic compounds, and antioxidant activity in horseradish products differed significantly (Table 2). TPC in the leaf pomace was four times higher, compared to the root pomace, but in the microencapsulated leaf juice it was 10 times higher, compared to the root juice. As previous studies reported, the horseradish leaves contained a higher amount of the phenolics, compared to roots [Tomsone, 2015]. In general, the microencapsulated horseradish leaf juice had a higher content of all the determined compounds, except for (+)-catechin (the highest content was detected in the horseradish root microcapsules). TPC ranged from 244.44 mg/100 g DM of root pomace to 3730.38 mg/100 g DM of encapsulated leaf juice, showing potential especially for horseradish leaf products to improve the health value of biscuits with small quantities added.

TABLE 2. Phenolic compound and organic acid contents, and antioxidant activity of horseradish products.

Parameters	Pomace		Microcapsules	
	Root HRP	Leaf HLP	Root juice HRM	Leaf juice HLM
TPC*	244.44±10.90^{d}	1028.57±27.64^{b}	376.64±8.05^{c}	3730.38±62.13^{a}
DPPH$^{\bullet}$ scavenging activity**	13.86±0.45^{d}	53.13±0.62^{c}	89.60±2.11^{b}	120.33±1.05^{a}
ABTS$^{\bullet+}$ scavenging activity**	23.64±0.79^{d}	298.84±7.96^{b}	120.47±3.10^{c}	631.32±13.56^{a}
(+)-Catechin***	6.96±0.32^{c}	2.39±0.10^{d}	42.04±1.15^{a}	9.90±0.43^{b}
Sinapic acid***	0.71±0.04^{d}	1.53±0.08^{c}	11.67±0.54^{b}	324.92±11.25^{a}
2-Hydroxycinnamic acid***	1.66±0.05^{d}	1.80±0.07^{c}	3.70±0.19^{b}	44.40±2.14^{a}
Rutin***	37.77±1.73^{d}	220.23±1.68^{b}	75.46±2.77^{c}	2033.52±101.68^{a}
Total organic acids***	760.04±28.00^{c}	772.66±28.63^{c}	2725.91±106.30^{b}	6879.92±244.01^{a}

All data are means ± standard deviation (n=9). ^{a,b,c}… – values with different superscripts in the same row are significantly different ($p \leq 0.05$). *mg GAE (gallic acid equivalent)/100 g DM, **mmol Trolox equivalent/100 g DM, ***mg/100 g DM. Abbreviations: HRP: horseradish root pomace powder; HLP: horseradish leaf pomace powder; HRM: horseradish root juice microcapsules; HLM: horseradish leaf juice microcapsules, TPC: total phenolic content, DM: dry matter.

Among the individual phenolic compounds quantified in horseradish product samples, the highest content of rutin reached 2033.52 mg/100 g DM of horseradish juice microcapsules. Rutin has a wide range of bioactivities including antioxidant and anti-inflammatory ones, and therefore scientists are searching for its potential sources [Camponogara *et al.*, 2020; Kherbache *et al.*, 2020]. A similar content of rutin was found in dried aerial parts of buckwheat (2.66%), but in extracts its content increased to 6.75% [Kim & Lim, 2019]. The banana leaf extract also reached 5% of rutin [Yingyuen *et al.*, 2020]. Results of the current study confirm that horseradish leaf juice can be a potential source of rutin.

Scientists have been testing the possibility of adding different phenolic-rich products to food, as each of the added products contains an individual and unique chemical profile, and compounds can react differently in each particular food matrix. Therefore, it is necessary to study the effect of phenolic compounds of horseradish on the food matrix. The obtained results allowed choosing the quantity of each horseradish product to be added, so that the same concentration of phenolic compounds would be added to each biscuit type based on the TPC.

Organic acids are important for plant metabolism and were found in all horseradish products analysed. Organic acids are closely related to the formation of phenolic compounds in plants, as they are part of the phenylpropanoid pathway biosynthesis. Horseradish leaf juice microcapsules (HLM) had the highest contents of organic acids among the studied products. There was a tendency that microencapsulated horseradish juice had 3–8 times higher content of organic acids, compared to the pomace. The main organic acids in all horseradish products were quinic, citric, fumaric, and succinic acids (data not shown).

Biscuit characteristics

Total phenolic content (TPC) and antioxidant activity in biscuits

The TPC values varied among the biscuit samples (Table 3). On the day after baking, the control biscuits (C) had a significantly ($p \leq 0.05$) lower TPC than the enriched biscuits. The addition of the horseradish products to biscuits increased their TPC by 21% (B_HRP) to 60% (B_HLM), compared to the control. Other researchers also succeeded in increasing the TPC of flour products by adding various plant materials rich in phenolics, such as rice bran by 200 to 400% [Irakli *et al.*, 2015], and cumin seeds and by-products by 50–116% [Sayed-Ahmad *et al.*, 2018b]. The same amount of phenolic compounds provided by different horseradish products was added to the biscuits in the current research. However, the individual phenolic compound profiles of additives differed significantly. The horseradish leaf juice microcapsules had higher contents of rutin, sinapic acid, and 2-hydroxycinnamic acid, compared to the other horseradish products (Table 2). Possibly, some of the individual compounds were more susceptible to the increased temperature and moisture vaporization during baking, resulting in the loss of TPC.

During baking, the antioxidant activity, like TPC, also increased in the biscuits containing horseradish products (Table 3). Compared to the control, the DPPH$^{\bullet}$ scavenging activity increased by 7% (B_HRP) to 26% (B_HRM), but the ABTS$^{\bullet+}$ scavenging activity by 18% (B_HRP) to 81% (B_HLM).

TABLE 3. Total phenolic content (TPC) and antioxidant activity of biscuits enriched with horseradish products one day after baking and after 180-day storage.

Samples	Before storage	After storage	Changes after storage
TPC (mg GAE/100 g DM)			
C	$14.82\pm0.71^{d,A}$	$15.60\pm0.61^{b,A}$	n.s.
B_HRP	$17.96\pm0.36^{c,A}$	$18.99\pm0.93^{a,A}$	n.s.
B_HLP	$23.15\pm0.93^{a,A}$	$16.94\pm0.45^{b,B}$	27% (↓)
B_HRM	$21.74\pm0.43^{b,A}$	$20.04\pm1.08^{a,B}$	n.s.
B_HLM	$23.75\pm0.43^{a,A}$	$16.98\pm0.28^{b,B}$	29% (↓)
DPPH$^{\bullet}$ scavenging activity (mmol TE/100 g DM)			
C	$1.58\pm0.08^{c,B}$	$2.11\pm0.03^{a,A}$	33% (↑)
B_HRP	$1.70\pm0.08^{b,c,B}$	$2.28\pm0.05^{a,A}$	34% (↑)
B_HLP	$1.86\pm0.06^{a,b,B}$	$2.18\pm0.01^{a,A}$	17% (↑)
B_HRM	$1.99\pm0.10^{a,A}$	$2.14\pm0.12^{a,A}$	n.s.
B_HLM	$1.93\pm0.03^{a,B}$	$2.23\pm0.08^{a,A}$	16% (↑)
ABTS$^{\bullet+}$ scavenging activity (mmol TE/100 g DM)			
C	$1.06\pm0.05^{d,A}$	$0.56\pm0.06^{e,B}$	47% (↓)
B_HRP	$1.25\pm0.06^{c,A}$	$0.68\pm0.01^{d,B}$	46% (↓)
B_HLP	$1.80\pm0.08^{a,b,A}$	$1.32\pm0.04^{a,B}$	27% (↓)
B_HRM	$1.71\pm0.09^{b,A}$	$1.15\pm0.05^{b,B}$	33% (↓)
B_HLM	$1.92\pm0.06^{a,A}$	$1.10\pm0.03^{b,c,B}$	43% (↓)

All data are means ± standard deviation ($n=18$). ^{a,b,c}… – values with different superscripts in the same column for the same parameter are significantly different ($p \leq 0.05$). ^{A,B}– values with different superscripts in the same row are significantly different ($p \leq 0.05$). Abbreviations: C: control; B_HRP: biscuits with horseradish root pomace powder; B_HLP: biscuits with horseradish leaf pomace powder; B_HRM: biscuits with horseradish root juice microcapsules; B_HLM: biscuits with horseradish leaf juice microcapsules; DM: dry matter; n.s.: change is statistically insignificant ($p > 0.05$). ↑: Percentage increase after 180-day storage. ↓: Percentage decrease after 180-day storage.

The increase in the antioxidant activity has been reported when food production by-products were added to flour products. Mango peel powder increased antioxidant activity 3 to 22 times [Ajila *et al.*, 2010], *Hibiscus sabdariffa* L. calyxes' residue by 18–140% [Ahmed & Abozed, 2015], as well as cumin and caraway seeds and by-products [Sayed-Ahmad *et al.*, 2018b], and chia seeds and cakes by 12–37% [Sayed-Ahmad *et al.*, 2018a]. In this study, higher DPPH$^{\bullet}$ scavenging activity was found for the biscuits with horseradish root microcapsules (B_HRM), horseradish leaf microcapsules (B_HLM), and horseradish leaf pomace (B_HLP), while higher ABTS$^{\bullet+}$ scavenging activity for the biscuits with horseradish leaf microcapsules (B_HLM) and horseradish leaf pomace (B_HLP). It may be due to the different individual phenolic compound profiles of the horseradish products and the differences in antioxidant activity determination methods. The DPPH assay is fast and easy to use in measuring the ability of compounds to act as radical scavengers or hydrogen donors, and is therefore often used to determine the antioxidant potential of food products [Szawara-Nowak *et al.*, 2016]. But still, the results could be interfered by different solubility of the compounds in alcohol and could depend on the chemical structure of the samples tested due to their different reaction kinetics with the radical. The ABTS assay, on the other hand, is another commonly used method for determining the antioxidant activity of foods containing a broader range of antioxidants, including hydrophilic and lipophilic compounds, but its results could also be affected by the chemical structure of compounds, pH, and oxygen concentration in media.

The correlation between TPC and DPPH scavenging activity in the analysed biscuits before storage was strong (r=0.869) and similar to these reported by Sayed-Ahmad *et al*. [2018b] and Moldovan *et al.* [2016]. The coefficient of correlation between TPC and ABTS$^{\bullet+}$ scavenging activity was r=0.818.

During storage, various biochemical reactions continue in the food. Antioxidants, including phenolic compounds, and their content change over time. It was observed that during storage, TPC did not change significantly ($p>0.05$) in the biscuits with added horseradish root products (B_HRP and B_HRM) (Table 3). However, TPC decreased significantly ($p\leq0.05$) in the biscuits with the added horseradish leaf products (B_HLP and B_HLM) after 180-day storage. A similar decrease in TPC during storage was observed in rye ginger cakes [Zieliński *et al.*, 2012], where TPC had decreased by 2–23% after storage. At the same time, TPC decreased significantly during storage of gluten-free rice-buckwheat biscuits [Sakač *et al.*, 2016], and after 6 months of storage the decrease reached 26%. The diversity of phenolic compounds in food matrixes and endogenous factors of food, such as pH and fat content, can affect the stability of phenolic compounds during storage [Ahmed & Abozed, 2015; Alminger *et al*., 2014].

All analysed biscuits, except for B_HRM, showed a significant ($p\leq0.05$) increase in DPPH$^{\bullet}$ scavenging activity during storage (Table 3). The largest changes were observed for the C and B_HRP samples. Changes in the antioxidant activity with DPPH$^{\bullet}$ could be explained by the development of Maillard reaction products, which have antioxidant properties. Zieliński *et al*. [2012] found that the content of Maillard reaction products increased by as much as 380% during storage of rye ginger cakes. The decrease in the TPC of the analysed biscuits during storage, and the increase in their DPPH$^{\bullet}$ scavenging activity can also be explained by the fact that the antiradical activity against DPPH$^{\bullet}$ may be provided by polar compounds other than phenolics.

The ABTS$^{\bullet+}$ scavenging activity of the experimental biscuits decreased until the end of the storage period (Table 3). The smallest changes were observed for B_HLP, but the largest ones for C and B_HRP samples. During storage, the ABTS$^{\bullet+}$ scavenging activity decreased similarly to TPC.

The pH and water activity (a_w) of biscuits

Horseradish products contained organic acids, therefore adding them to biscuits lowered their pH (Table 4). The pH of the biscuits without additives was 6.683 (sample C), whereas the pH of the biscuits containing horseradish products was significantly ($p\leq0.05$) lower compared to the control and ranged between 6.395 (B_HLP) and 6.476 (B_HRP). Since horseradish leaf products contained more organic acids (Table 2), their addition resulted in lowered pH of the biscuits (B_HLP and B_HLM). According to Ahmed & Abozed [2015], organic acids may act as preservatives, thus improving the microbiological and physicochemical stability of the baked products. However, the pH of biscuits in the current research was higher than the values reported for crackers produced with *Hibiscus sabdariffa* by-products [Ahmed & Abozed, 2015].

TABLE 4. pH and water activity of biscuits enriched with horseradish products one day after baking and after 180-day storage.

Samples	Before storage	After storage	Changes after storage
	pH		
C	$6.683\pm0.027^{a,A}$	$6.711\pm0.004^{a,A}$	n.s.
B_HRP	$6.476\pm0.009^{b,A}$	$6.485\pm0.005^{b,A}$	n.s.
B_HLP	$6.395\pm0.035^{c,d,A}$	$6.319\pm0.005^{d,B}$	n.s.
B_HRM	$6.434\pm0.008^{c,A}$	$6.380\pm0.005^{c,B}$	n.s.
B_HLM	$6.397\pm0.028^{d,A}$	$6.322\pm0.022^{d,B}$	n.s.
	a_w		
C	$0.394\pm0.001^{a,A}$	$0.385\pm0.001^{b,B}$	n.s.
B_HRP	$0.259\pm0.001^{b,B}$	$0.382\pm0.001^{d,A}$	47% (↑)
B_HLP	$0.188\pm0.001^{d,B}$	$0.387\pm0.001^{a,A}$	105% (↑)
B_HRM	$0.224\pm0.001^{c,B}$	$0.384\pm0.000^{c,A}$	71% (↑)
B_HLM	$0.177\pm0.001^{e,B}$	$0.386\pm0.001^{a,b,A}$	117% (↑)

All data are means ± standard deviation ($n=6$). $^{a,b,c\ldots}$ – values with different superscripts in the same column for the same parameter are significantly different ($p\leq 0.05$). ^{A,B} – values with different superscripts in the same row are significantly different ($p\leq 0.05$). Abbreviations: C: control; B_HRP: biscuits with horseradish root pomace powder; B_HLP: biscuits with horseradish leaf pomace powder; B_HRM: biscuits with horseradish root juice microcapsules; B_HLM: biscuits with horseradish leaf juice microcapsules. n.s.: change is statistically insignificant ($p>0.05$). ↑: Percentage increase after 180-day storage. ↓: Percentage decrease after 180-day storage.

After 180-day storage, no significant ($p>0.05$) pH changes were observed in the biscuits (Table 4).

Water activity (a_w) values of the control biscuits after baking were significantly higher ($p\leq0.05$) compared to the enriched biscuits (Table 4). The lowest water activity was measured in the biscuits with added horseradish leaves products (B_HLP and B_HLM) but these samples showed the highest increase in water activity after storage. Generally, there was an increase in water activity after 180-day storage, except for the control sample (sample C). The water activity of the biscuits during storage had the same characteristics as that of commercial biscuits [Romani *et al.*, 2016] and gluten-free biscuits with chestnut flour supplement [Paciulli *et al.*, 2018], when it increased slightly during storage.

TABLE 5. Colour parameters of biscuits enriched with horseradish products.

Parameter	C	B_HRP	B_HLP	B_HRM	B_HLM
L*	77.48 ± 0.98^{a}	69.54 ± 1.69^{b}	64.08 ± 0.96^{c}	$66.17\pm1.98^{b,c}$	64.04 ± 2.01^{c}
a*	1.53 ± 0.22^{c}	2.70 ± 0.14^{b}	$3.69\pm0.65^{a,b}$	4.15 ± 0.54^{a}	$3.91\pm0.65^{a,b}$
b*	22.16 ± 1.04^{a}	22.53 ± 1.94^{a}	22.33 ± 1.59^{a}	21.52 ± 1.29^{a}	23.81 ± 1.04^{a}
ΔE	–	8.24 ± 0.74^{c}	13.77 ± 1.45^{a}	$11.90\pm1.01^{a,b}$	13.96 ± 1.59^{a}
Chroma	$22.16\pm1.22^{a,b}$	22.69 ± 1.48^{a}	22.63 ± 1.70^{a}	$21.92\pm1.47^{a,b}$	24.12 ± 1.57^{a}
Hue angle (°)	1.55 ± 0.02^{a}	1.45 ± 0.02^{b}	$1.41\pm0.02^{b,c}$	1.38 ± 0.02^{c}	$1.41\pm0.03^{b,c}$
ΔE_t	2.06 ± 0.15^{c}	$2.69\pm0.24^{a,b}$	0.57 ± 0.06^{d}	2.12 ± 0.18^{c}	3.19 ± 0.36^{a}

Each measured value is the mean±standard deviation. ($n=20$). ^{a,b,c}… – values with different superscripts in the same row are significantly different ($p\leq0.05$). ΔE shows difference between respective sample and control, ΔE_t is totatl colour difference between samples after 180-day storage compared with respective sample before storage. Abbreviations: C: control; B_HRP: biscuits with horseradish root pomace powder; B_HLP: biscuits with horseradish leaf pomace powder; B_HRM: biscuits with horseradish root juice microcapsules; B_HLM: biscuits with horseradish leaf juice microcapsules.

Biscuit colour

The colour of food is an important visual aspect that can either attract or deter consumers from a product, regardless of its true value. Therefore, the average values of colour parameters of the experimental biscuits after baking are reported in Table 5. The results showed that the addition of horseradish products significantly ($p\leq0.05$) increased the darkness and redness of the biscuits, but did not affect the blue-yellow tones. It may be affected by the transformation of phenolic compounds to quinones during heat treatment, which are subsequently converted to dark coloured melanins [Taranto *et al.*, 2012]. Paciulli *et al.* [2018] also reported a similar trend in colour change upon baking when chestnut flour was added to gluten-free biscuits.

Pigments present in leaves increased the darkness of the experimental products. The darkest biscuits were obtained by adding horseradish leaf products (horseradish leaf pomace powder and microencapsulated horseradish leaf juice), which is indicated by the L* values of the biscuit colour. The heat treatment caused changes of the natural pigments colour, and as a result of various reactions, the tone of the enriched biscuits turned brownish red, which is also visible from the results of a* values.

Higher a* values were found for the biscuits with added horseradish root juice microcapsules (B_HRM), compared to the biscuits with horseradish root pomace. The differences between a* values are probably due to the use of maltodextrin as a wall material for the microcapsules. Similar observations have been made with the addition of cumin and caraway seeds and by-products [Sayed-Ahmad *et al.*, 2018b], and chia seeds and cakes [Sayed-Ahmad *et al.*, 2018a] to flour products.

The total colour difference (ΔE) indicates that the biggest differences compared to the control biscuits (sample C) were observed in the biscuit samples enriched with horseradish leaf products (B_HLM and B_HLP). The ΔE of B_HLM was by 9% higher than that of the control biscuits. The hue angle values indicate redness of the experimental biscuits, as the hue angle value is less than 10° (Table 5).

In general, the enrichment of the biscuits with various horseradish products resulted in darker biscuits, which could be associated with non-enzymatic chemical reactions such as caramelization and browning reactions. Similar to the addition of grape marc extract to biscuits [Pasqualone *et al.*, 2014], the colour of the experimental biscuits could be affected by the oxidation and degradation products of phenolics and chlorophyll compounds present in the horseradish products added.

Major colour changes after 180-day storage (ΔE_t, Table 5) were observed in B_HLM biscuits, whereas no significant changes were observed in B_HLP samples. Additionally, it was the only sample with smaller ΔE_t than control (C).

When assessing colour differences and changes in biscuit samples, it should be taken into account that the addition of natural plant products may cause changes in colour parameters due to the characteristic colour of these plant products, as well as mutual biochemical reactions.

Bioaccessibility of TPC and antioxidant activity after biscuit digestion *in vitro*

The high content and activity of phytochemicals in certain food ingredients does not always ensure high bioaccessibility and bioavailability during *in vitro* digestion. The results obtained in this study (Figure 1a) showed that TPC was significantly higher in all analysed biscuit samples after the digestion than before digestion, which is also consistent with other studies [Irakli *et al.*, 2019; Szawara-Nowak *et al.*, 2016; Zieliński *et al.,* 2020].

The *in vitro* digestive process in the gastrointestinal tract involves the addition of enzymes and the adjustment of the pH at each stage according to the physiology of the human gastrointestinal tract. The effect of this process depends on the nature of the original sample. The metabolism and absorption of phenolic compounds usually depend on their physicochemical properties – basic structure, molecular size, degree of polymerization or glycosylation, solubility, as well as conjugation with other phenolic compounds [Carbonell-Capella *et al.,* 2014].

Bioaccessibility indexes (PACs) after simulated gastric treatment were used in the study to evaluate the amounts of phenolics that are released from the relevant food matrix after digestion, making these compounds available for ab-

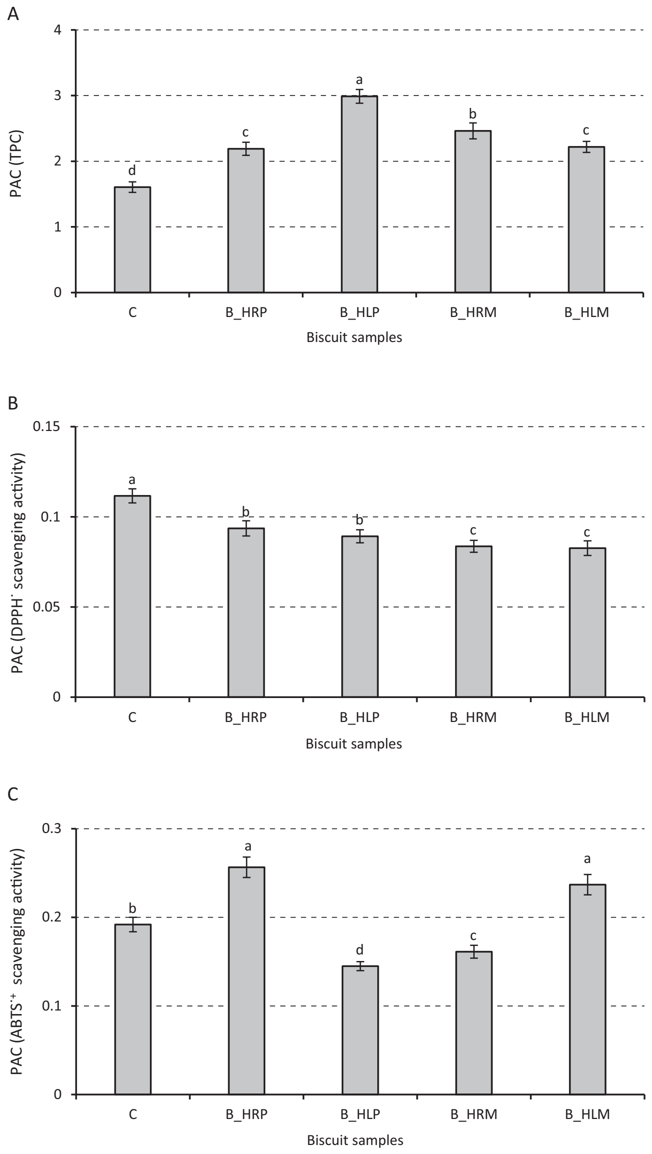


FIGURE 1. Bioaccessibility indexes (PACs) based on (A) TPC, (B) $DPPH^{\bullet}$ scavenging activity and (C) $ABTS^{\bullet +}$ scavenging activity after biscuit *in vitro* digestion. C – control; B_HRP – biscuits with horseradish root pomace powder; B_HLP – biscuits with horseradish leaf pomace powder; B_HRM – biscuits with horseradish root juice microcapsules; B_HLM – biscuits with horseradish leaf juice microcapsules; TPC – total phenolic content.

sorption through the gastrointestinal mucosa. Figure 1 shows the PACs based on the determination of TPC and antioxidant activity after digestion. In all biscuit samples, the PAC for TPC exceeded 1.6, showing a mean value of the phenolic bioavailability index of approximately 2.34. According to Zielinski *et al.* [2020], these values indicate high bioaccessibility. This can be explained by the fact that during digestion phenolics are released from cell wall matrices, as well as from their conjugated forms. There is also evidence in the literature that TPC of food matrices enriched with various natural substances increased after digestion [Irakli *et al.*, 2019; Szawara-Nowak *et al.*, 2016]. The highest PACs were observed for the biscuits with added horseradish leaf pomace powder (B_HLP), which were by 86% higher than these determined for the control biscuits.

Unfortunately, in all biscuit samples, the values of PAC based on the antioxidant activity were low (according to the criteria described by Zieliński *et al.* [2020]); *i.e.*, 0.09 in the case of $DPPH^{\bullet}$ scavenging activity and 0.20 in the case of $ABTS^{\bullet +}$ scavenging activity (Figure 1b,c). This could indicate that some phenolic compounds with antioxidant properties in the food matrix, which are exposed to the *in vitro* digestion, interact with other compounds to form indigestible complexes [Jakobek, 2015], while other compounds may be released from their bound state. The highest PAC for $DPPH^{\bullet}$ scavenging activity was observed in the control biscuits. However, a higher PAC for $ABTS^{\bullet +}$ scavenging activity was determined for the biscuits with added horseradish root pomace (B_HRP), which was by 33% higher than that of the control biscuits.

CONCLUSION

Pomace and microcapsules of horseradish root and leaves could potentially be used as the functional ingredients in foods due to their content of phenolic compounds with antioxidant activity. The addition of horseradish products to butter biscuits enriched the bakery product with phenolic compounds, as well as improved their antioxidant potential. Microencapsulated horseradish leaf juice was more effective in enriching the biscuits with bioactive compounds, thus opening up this raw material to new interesting applications in the food industry. The bioaccessibility indexes after biscuit digestion based on TPC indicated the high bioaccessibility of phenolic compounds from experimental products. Further research is required to optimise the functional recipe for horseradish-fortified flour confectionery, examining their potential health effects. It is also necessary to study the use of these raw materials in other products by assessing the properties and organoleptic attributes of these innovative functional raw materials that meet consumer expectations.

ACKNOWLEDGEMENTS

Authors acknowledge support of Dr. Ingmars Cinkmanis (Department of Chemistry, Faculty of Food Technology, Latvia University of Life Sciences and Technologies), as well as Engineer, Ph.D. Vanessa Durrieu (Laboratoire de Chimie Agro-industrielle, LCA, Université de Toulouse, INRAE, Toulouse, France). Authors also acknowledge a student of Jelgava secondary school No. 4 Anna Rusmane and her supervisor Aija Pridane for the co-operation within school research project, performing practical experiments.

RESEARCH FUNDING

This research was funded by the European Regional Development Fund Post-doctoral Research Support Program (project No.1.1.1.2/16/I/001), grant number 1.1.1.2./VIAA/1/16/187.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

ORCID IDs

L. Tomsone https://orcid.org/0000-0003-3879-3811
R. Galoburda https://orcid.org/0000-0002-5804-516X
Z. Kruma https://orcid.org/0000-0001-6123-1927
K. Majore https://orcid.org/0000-0001-8504-8473

REFERENCES

1. Agneta, R., Möllers, C., Rivelli, A.R. (2013). Horseradish (*Armoracia rusticana)*, a neglected medical and condiment species with a relevant glucosinolate profile: a review. *Genetic Resources and Crop Evolution*, *60*(7), 1923–1943. https://doi.org/10.1007/s10722-013-0010-4
2. Ahmed, Z.S., Abozed, S.S. (2015). Functional and antioxidant properties of novel snack crackers incorporated with *Hibiscus sabdariffa* by-product. *Journal of Advanced Research*, *6*(1), 79–87. https://doi.org/10.1016/j.jare.2014.07.002
3. Ajila, C.M., Aalami, M., Leelavathi, K., Rao, U.J.S.P. (2010). Mango peel powder: A potential source of antioxidant and dietary fiber in macaroni preparations. *Innovative Food Science and Emerging Technologies*, *11*(1), 219–224. https://doi.org/10.1016/j.ifset.2009.10.004
4. Alminger, M., Aura, A.-M., Bohn, T., Dufour, C., El, S.N., Gomes, A., Karakaya, S., Martínez-Cuesta, M.C., McDougall, G.J., Requena, T., Santos, C.N. (2014). *In vitro* models for studying secondary plant metabolite digestion and bioaccessibility. *Comprehensive Reviews in Food Science and Food Safety*, *13*, 413–436. https://doi.org/10.1111/1541-4337.12081
5. AOAC (2020) Association of Official Analytical Chemists International: Official Methods of Analysis. AOAC 943.02–1943, pH of flour, potentiometric method [http://www.aoacofficialmethod.org/index.php?main_page=product_info&products_id=937].
6. Belitz, H.D., Grosch, W., Schieberle, P. (2009). *Food Chemistry*. Springer Berlin Heidelberg. Retrieved from [https://books.google.co.in/books?id=xteiARU46SQC].
7. Calabrone, L., Larocca, M., Marzocco, S., Martelli, G. (2015). Total phenols and flavonoids content, antioxidant capacity and lipase inhibition of root and leaf horseradish (*Armoracia rusticana*) extracts. *Food and Nutrition Sciences*, *06*(01), 64–74. https://doi.org/10.4236/fns.2015.61008
8. Camponogara, C., Brum, E. da S., Belke, B.V., Brum, T.F., Jesus, R. da S., Piana, M., Bauermann, L. de F., Oliveira, S.M. (2020). *Casearia decandra* leaves present anti-inflammatory efficacy in a skin inflammation model in mice. *Journal of Ethnopharmacology*, *249*, art. no. 112436. https://doi.org/10.1016/j.jep.2019.112436
9. Carbonell-Capella, J.M., Buniowska, M., Barba, F.J., Esteve, M.J., Frígola, A. (2014). Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(2), 155–171. https://doi.org/10.1111/1541-4337.12049
10. Celep, E., İnan, Y., Akyüz, S., Yesilada, E. (2017). The bioaccessible phenolic profile and antioxidant potential of *Hypericum perfoliatum* L. after simulated human digestion. *Industrial Crops and Products*, *109*, 717–723. https://doi.org/10.1016/j.indcrop.2017.09.032
11. Dag, D., Kilercioglu, M., Oztop, M.H. (2017). Physical and chemical characteristics of encapsulated goldenberry (*Physalis peruviana* L.) juice powder. *LWT – Food Science and Technology*, *83*, 86–94. https://doi.org/10.1016/j.lwt.2017.05.007
12. Gayoso, L., Roxo, M., Cavero, Y.R., Calvo, I.M., Ansorena, D., Astiasarán, I., Wink, M. (2018). Bioaccessibility and biological activity of *Melissa officinalis*, *Lavandula latifolia* and *Origanum vulgare* extracts: Influence of an *in vitro* gastrointestinal digestion. *Journal of Functional Foods*, *44*, 146–154. https://doi.org/10.1016/j.jff.2018.03.003
13. Irakli, M., Katsantonis, D., Kleisiaris, F. (2015). Evaluation of quality attributes, nutraceutical components and antioxidant potential of wheat bread substituted with rice bran. *Journal of Cereal Science*, *65*, 74–80. https://doi.org/10.1016/j.jcs.2015.06.010
14. Irakli, M., Mygdalia, A., Chatzopoulou, P., Katsantonis, D. (2019). Impact of the combination of sourdough fermentation and hop extract addition on baking properties, antioxidant capacity and phenolics bioaccessibility of rice bran-enhanced bread. *Food Chemistry*, *285*, 231–239. https://doi.org/10.1016/j.foodchem.2019.01.145
15. ISO Standard 6496. (1999). Animal feeding stuffs — Determination of moisture and other volatile matter content.
16. Jakobek, L. (2015). Interactions of polyphenols with carbohydrates, lipids and proteins. *Food Chemistry*, *175*, 556–567. https://doi.org/10.1016/j.foodchem.2014.12.013
17. Kherbache, A., Senator, A., Laouicha, S., Al-Zoubi, R.M., Bouriche, H. (2020). Phytochemical analysis, antioxidant and anti-inflammatory activities of *Helichrysum stoechas* (L.) Moench extracts. *Biocatalysis and Agricultural Biotechnology*, *29*, art. no. 101826. https://doi.org/10.1016/j.bcab.2020.101826
18. Kim, D.S., Lim, S.B. (2019). Subcritical water extraction of rutin from the aerial parts of common buckwheat. *Journal of Supercritical Fluids*, *152*, art. no. 104561. https://doi.org/10.1016/j.supflu.2019.104561
19. Kloucek, P., Smid, J., Frankova, A., Kokoska, L., Valterova, I., Pavela, R. (2012). Fast screening method for assessment of antimicrobial activity of essential oils in vapor phase. *Food Research International*, *47*(2), 161–165. https://doi.org/10.1016/j.foodres.2011.04.044
20. Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food and Function*, *5*, 1113–1124. https://doi.org/10.1039/C3FO60702J
21. Mokdad, A., Mikšovská, J., Larsen, R.W. (2009). Photothermal studies of CO photodissociation from peroxidases from horseradish and soybean. *Biochimica et Biophysica Acta – Proteins and Proteomics*, *1794*(11), 1558–1565. https://doi.org/10.1016/j.bbapap.2009.06.021
22. Moldovan, B., Iasko, B., David, L. (2016). Antioxidant activity and total phenolic content of some commercial fruit-flavoured

yogurts. *Studia Universitatis Babes-Bolyai Chemia*, *LXI*(3, Tom 1), 101–108.

23. Naczk, M., Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, *1054*(1–2), 95–111. https://doi.org/10.1016/S0021-9673(04)01409-8
24. Paciulli, M., Rinaldi, M., Cavazza, A., Ganino, T., Rodolfi, M., Chiancone, B., Chiavaro, E. (2018). Effect of chestnut flour supplementation on physico-chemical properties and oxidative stability of gluten-free biscuits during storage. *LWT – Food Science and Technology*, *98*, 451–457. https://doi.org/10.1016/j.lwt.2018.09.002
25. Pasqualone, A., Bianco, A.M., Paradiso, V.M., Summo, C., Gambacorta, G., Caponio, F. (2014). Physico-chemical, sensory and volatile profiles of biscuits enriched with grape marc extract. *Food Research International*, *65*(PC), 385–393. https://doi.org/10.1016/j.foodres.2014.07.014
26. Prieto, M.A., López, C.J., Simal-Gandara, J. (2019). Chapter Six – Glucosinolates: Molecular structure, breakdown, genetic, bioavailability, properties and healthy and adverse effects. *Functional Food Ingredients from Plants, 90,* 305–350. https://doi.org/10.1016/bs.afnr.2019.02.008
27. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3
28. Romani, S., Rocculi, P., Tappi, S., Dalla Rosa, M. (2016). Moisture adsorption behaviour of biscuit during storage investigated by using a new Dynamic Dewpoint method. *Food Chemistry*, *195*, 97–103. https://doi.org/10.1016/j.foodchem.2015.06.114
29. Sakač, M., Pestorić, M., Mandić, A., Mišan, A., Nedeljković, N., Jambrec, D., Jovanov, P., Lazić, V., Pezo, L., Sedej, I. (2016). Shelf-life prediction of gluten-free rice-buckwheat cookies. *Journal of Cereal Science*, *69*, 336–343. https://doi.org/10.1016/j.jcs.2016.04.008
30. Sant'Anna, V., Gurak, P.D., Ferreira Marczak, L.D., Tessaro, I.C. (2013). Tracking bioactive compounds with colour changes in foods – A review. *Dyes and Pigments, 98*(3), 601–608. https://doi.org/10.1016/j.dyepig.2013.04.011
31. Sayed-Ahmad, B., Talou, T., Straumite, E., Sabovics, M., Kruma, Z., Saad, Z., Hijazi, A., Merah, O. (2018a). Evaluation of nutritional and technological attributes of whole wheat based bread fortified with Chia flour. *Foods*, *7*(9), art. no. 135. https://doi.org/10.3390/foods7090135
32. Sayed-Ahmad, B., Talou, T., Straumite, E., Sabovics, M., Kruma, Z., Saad, Z., Hijazi A., Merah, O. (2018b). Protein bread fortification with cumin and caraway seeds and by-product flour. *Foods*, *7*(3), art no. 28. https://doi.org/10.3390/foods7030028
33. Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M.B. (1999). [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1
34. Świeca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B. (2017). Wheat bread enriched with green coffee – *In vitro* bioaccessibility and bioavailability of phenolics and antioxidant activity. *Food Chemistry*, *221*, 1451–1457. https://doi.org/10.1016/j.foodchem.2016.11.006
35. Szawara-Nowak, D., Bączek, N., Zieliński, H. (2016). Antioxidant capacity and bioaccessibility of buckwheat-enhanced wheat bread phenolics. *Journal of Food Science and Technology*, *53*(1), 621–630. https://doi.org/10.1007/s13197-015-2074-y
36. Taranto, F., Delvecchio, L.N., Mangini, G., Del Faro, L., Blanco, A., Pasqualone, A. (2012). Molecular and physico-chemical evaluation of enzymatic browning of whole meal and dough in a collection of tetraploid wheats. *Journal of Cereal Science*, *55*(3), 405–414. https://doi.org/10.1016/j.jcs.2012.02.005
37. Tomsone, L. (2015). *Investigation of biologically active substances in horseradish and lovage*. Ph.D. Thesis, Latvia University of Life Sciences and Technologies, Jelgava, Latvia, p. 148 (in Latvian).
38. Tomsone, L., Galoburda, R., Kruma, Z., Cinkmanis, I. (2020a). Characterization of dried horseradish leaves pomace: phenolic compounds profile and antioxidant capacity, content of organic acids, pigments and volatile compounds. *European Food Research and Technology*, *246*(8), 1647–1660. https://doi.org/10.1007/s00217-020-03521-z
39. Tomsone L., Galoburda, R., Kruma, Z., Durrieu, V., Cinkmanis, I. (2020b) Microencapsulation of horseradish (*Armoracia rusticana* L.) juice using spray-drying. *Foods*, *9*(9), art. no. 1332. https://doi.org/10.3390/foods9091332
40. Villarino, C.B., Jayasena, V., Coorey, R., Chakrabarti-Bell, S., Johnson, S. (2014). The effects of bread-making process factors on Australian sweet lupin-wheat bread quality characteristics. *International Journal of Food Science and Technology*, *49*(11), 2373–2381. https://doi.org/10.1111/ijfs.12595
41. Yingyuen, P., Sukrong, S., Phisalaphong, M. (2020). Isolation, separation and purification of rutin from Banana leaves (*Musa balbisiana*). *Industrial Crops and Products*, *149*(March), art. no. 112307. https://doi.org/10.1016/j.indcrop.2020.112307
42. Yu, L., Perret, J., Harris, M., Wilson, J., Haley, S. (2003). Antioxidant properties of bran extracts from "Akron" wheat grown at different locations. *Journal of Agricultural and Food Chemistry*, *51*(6), 1566–1570. https://doi.org/10.1021/jf020950z
43. Zieliński, H., Del Castillo, M.D., Przygodzka, M., Ciesarova, Z., Kukurova, K., Zielińska, D. (2012). Changes in chemical composition and antioxidative properties of rye ginger cakes during their shelf-life. *Food Chemistry*, *135*(4), 2965–2973. https://doi.org/10.1016/j.foodchem.2012.07.009
44. Zieliński, H., Szawara-Nowak, D., Wronkowska, M. (2020). Bioaccessibility of anti-AGEs activity, antioxidant capacity and phenolics from water biscuits prepared from fermented buckwheat flours. *LWT – Food Science and Technology*, *123*, art. no. 109051. https://doi.org/10.1016/j.lwt.2020.109051

Submitted: 25 August 2020. Revised: 7 October and 4 November. Accepted: 6 November 2020. Published on-line: 9 December 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 429–443
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/130401
http://journal.pan.olsztyn.pl

Original research article
Food Quality and Functionality Section

In Vitro Characterization of Fluted Pumpkin Leaf Protein Hydrolysates and Ultrafiltration of Peptide Fractions: Antioxidant and Enzyme-Inhibitory Properties

Akinsola Albert Famuwagun[1,2], Adeola Monisola Alashi[1], Saka Olasunkanmi Gbadamosi[2], Kehinde Adekunbi Taiwo[2], Durodoluwa Joseph Oyedele[3], Odunayo Clement Adebooye[4], Rotimi Emmanuel Aluko[1]*

[1]Department of Food and Human Nutritional Sciences, University of Manitoba, R3T 2N2 Winnipeg, Canada
[2]Department of Food Science & Technology, Obafemi Awolowo University, 220002 Ile-Ife, Nigeria
[3]Department of Soil and Land Resources Management, Faculty of Agriculture,
Obafemi Awolowo University, 220002 Ile-Ife, Nigeria
[4]Department of Agronomy, Faculty of Agriculture, Obafemi Awolowo University, 220002 Ile-Ife, Nigeria

Key words: fluted pumpkin leaf, protein hydrolysates, membrane ultrafiltration, antioxidant activity, antidiabetic activity, antihypertensive activity

Hydrolysates were produced using Alcalase (AH), chymotrypsin (CH), pepsin (PH), and trypsin (TH), and also fluted pumpkin leaf protein isolate (FLI) as a substrate. AH had the lowest degree of hydrolysis (16.37%) but exhibited overall superior antioxidant and enzyme inhibitory properties. Therefore, it was fractionated by membrane ultrafiltration to give <1, 1–3, 3–5, 5–10, and >10 kDa peptide fractions. Gel permeation chromatography showed that the molecular weight of the FLI was 19.77 kDa and that of the hydrolysates was below 7.5 kDa. The hydrolysate peptides had a high content of hydrophobic amino acids but low levels of sulfur-containing amino acids, when compared to protein of FLI. Peptide sequence analysis showed that the hydrolysates consisted of dipeptides, tripeptides, and tetrapeptides with molecular weights below 500 Da. The hydrolysates were also stronger inhibitors of linoleic acid oxidation, α-amylase, α-glucosidase, and angiotensin converting enzyme (ACE) than FLI. Among the fractions, the <1 and 1–3 kDa were the most effective free radical scavengers and metal chelators in addition to their strong inhibitory activities against α-amylase, α-glucosidase, and ACE. We conclude that the AH and low molecular weight peptide fraction (<3 kDa) could find applications in formulating foods with various bioactive properties.

INTRODUCTION

Proteins are integral food compounds that provide nutritional, technological, and functional properties. In addition to these properties, proteins can give rise to bioactive peptides or hydrolysates, which show certain specific properties such as antioxidant, antihypertensive, and antidiabetic activities [Chalamaiah *et al*., 2017]. The various properties of these hydrolysates and peptides make them applicable as potential agents in the treatment, prevention, and amelioration of various diseases as well as in the shelf life extension of food products [Ortiz-Martinez *et al*., 2014]. Bioactive peptides from foods are obtained after enzymatic hydrolysis, because the active sequences lie within the primary structure of the proteins [Aluko, 2015; Ramada *et al*., 2017]. Based on this fact, the study of protein hydrolysates has become a new area in biochemical and nutritional research. Enzymatic hydrolysis has been an effective way of releasing these peptides from the precursor proteins because it preserves the functionalities of the peptides. The properties of enzymatic hydrolysates depend on the primary structure of parent protein(s), degree of hydrolysis (DH), nature of protease(s) used, and the type of peptides released during the hydrolysis such as their molecular weight and amino acid composition and sequence [Mirzaei *et al*., 2016]. Variations in the specificities of enzymes used during protein hydrolysis are important to the hydrolysates because the same protein chain can produce different peptides when cleaved by different proteases [Tavano, 2013]. After hydrolysis, peptides that were inactive in the intact proteins are released and can be further fractionated into peptides of varying molecular weights using an ultrafiltration membrane; the fractions may then be evaluated for bioactive functions.

The human body is constantly subjected to various activities, leading to oxidative stress, which sometimes leads to overpowering of the intrinsic antioxidant defense mechanism of the body. Overwhelming of the in-built defense system may occur as a result of many factors which may lead to reactive oxygen species accumulating as reactive radicals that cause severe damages to the body [Wei *et al*., 2001; Yoshikawa & Naito, 2002]. To expand the treatment options, antioxidant peptides that target the reduction of oxidative stress would be of interest [McCarthy *et al*., 2013]. Consumption of natural dietary supplements, such as peptides,

---

* Corresponding Author: Tel.: +2347038688258;
E mail: akinsolaalbert@gmail.com (Dr. A. Famuwagun)

with varieties of antioxidant compounds may help to augment the body defense mechanism against the free radicals. Antioxidant peptides exert their activity through multiple pathways such as scavenging free radicals, chelating transition metals, and reducing ferric irons [Ramada *et al*., 2017]. These peptides inhibit oxidative processes that lead to the formation of harmful radicals, which cause various diseases such as cardiovascular impairment (including hypertension and atherosclerosis), cancer, and diabetes [Yoshikawa & Naito, 2002]. Several peptides and hydrolysates with antioxidant properties have been produced from plant materials such as hemp seed [Girgih *et al*., 2011], canola seed [Alashi *et al*., 2014], *kariya* [Gbadamosi & Famuwagun, 2016], and alfalfa leaf [Xie *et al*., 2016].

Hypertension is a risk factor of cardiovascular disease, stroke, and end stage renal disease [Kim & Byun, 2012]. The angiotensin-I converting enzyme (ACE) in the renin-angiotensin system regulates blood pressure in the human body and catalyzes the cleavage of the C-terminal dipeptide from inactive angiotensin I to produce the active angiotensin II, in addition to inactivating bradykinin, a potent vasodilator [Cao *et al.*, 2010]. Therefore, ACE inhibition is important to controlling the blood pressure. ACE-inhibitory peptides have been produced from various plant proteins such as cotton seed [Filho *et al*., 2020], soy protein [Wang *et al*., 2019], canola [Alashi *et al*., 2014], oat [Bleakley *et al*., 2017], peanut [Gong *et al*., 2017], and rice [Pinciroli *et al*., 2019].

Diabetes mellitus is an endocrine system disease that causes disruption in the normal metabolic process arising from multiple organ damage syndromes [Arise *et al*., 2016]. Inhibition of enzymes, such as α-amylase and α-glucosidase, will slow down the digestion and absorption of glucose in the intestine and is therefore, one of the key approaches in controlling diabetes. Enzymatic hydrolysis of plant protein has been used to release many potential antidiabetic peptides such as pea seed [Awosika & Aluko, 2019], watermelon seed [Arise *et al*., 2016], and soy protein [Wang *et al*., 2019]. Therefore, peptides with multifunctional activities have been suggested to tackle the damaging effects of excessive levels of α-amylase, α-glucosidase, ACE, and toxic free radicals in the body [Gong *et al.,* 2017].

Proteins obtained from leaves represent a good source of nutrition for human [Aletor *et al*., 2002]. Fluted pumpkin leaf (*Telfairia occidentalis*) is one of the most widely consumed leafy vegetables in Nigeria [Odiaka, 2001]. The leaf is available all year round in Nigeria depending on water availability and is used to prepare various dishes. The dried leaf powder has about 30.05–32.82% protein content [Nnamezie *et al*., 2019; Udousoro & Ekanem, 2013], which is comparable with some seed proteins. This high level of protein in fluted pumpkin dried leaf could enhance its use in the production of high-quality protein products.

Protein has been isolated from fluted pumpkin dried leaf and characterized [Famuwagun *et al*., 2020]. However, there is no published work on fluted pumpkin leaf protein hydrolysates, especially on their antioxidant and enzyme inhibitory activities. This study determined the antioxidant and enzyme inhibitory activities of fluted pumpkin leaf protein hydrolysates produced using four different food grade proteases. The most active protein hydrolysate was then fractionated using the ultrafiltration membrane method. The fractions were tested for antioxidant, antidiabetic (α-amylase and α-glucosidase inhibition), and antihypertensive (angiotensin-converting enzyme inhibition) activities.

MATERIALS AND METHODS

Preparation of dried leaf powder

Fresh leaves of fluted pumpkin leafy vegetables were obtained from accredited Microveg farmer in Osun State, Nigeria. The leaves were sorted, destalked, rinsed, and subsequently oven-dried using a hot air oven (Uniscope SM9053 Laboratory Oven, Singerfried, England) at a drying temperature of 55±2°C for 8 h. The dried leaves were milled to powder with a laboratory blender (VLC sapphire grinder, IS-4930, Edinburgh, England). The milled powder was subjected to acetone extraction at a ratio of 1:10 (leaf powder:acetone, *w/v*). The purpose of this was to remove any form of interfering compound (such as polyphenol) during protein isolation. The leaf powder/acetone suspension was continuously stirred on a magnetic stirrer for 2 h and later filtered using muslin cloth. The residue obtained was again extracted with acetone and the resulting residue was air-dried in the fume hood at room temperature for 48 h to ensure the escape of residual acetone from the residue. This was then packaged as fluted pumpkin leaf powder and stored at -20°C.

Preparation of fluted pumpkin leaf isolate and enzymatic hydrolysates

The fluted pumpkin leaf isolate (FLI) was obtained by a combined method of solubilization, precipitation, and neutralization described by Malomo *et al*. [2014], which was slightly modified. A suspension of fluted pumpkin leaf powder in deionized water was prepared at a 5% (*w/v*) concentration. The suspension was adjusted to pH 10.0 with 2 M NaOH and then stirred continuously for 1 h at 37°C. The residue was separated from the solution by centrifuging at 3500×*g* for 30 min at 4°C. The supernatant was collected and adjusted to pH 4.5 using 2 M HCl and stirred for 20 min. The precipitate obtained after centrifugation was washed twice with distilled water to remove salt, re-dispersed in deionized water, and freeze dried to obtain the fluted pumpkin protein isolate. The FLI was hydrolyzed using four different proteases (purchased from Sigma-Aldrich, St. Louis, MO, USA), namely Alcalase (>2.4 U/g), chymotrypsin (>250 units/mg), pepsin (>250 units/mg), and pancreatin (>250 units/mg) at an enzyme:substrate ratio of 1:100. Different hydrolysis conditions were employed as follows: Alcalase (pH 8.0, 50°C); trypsin (pH 8, 37°C); pepsin (pH 2.0, 37°C), and chymotrypsin (pH 8.0, 37°C). The pH of the hydrolysis process was maintained using either 1 M NaOH or 1 M HCl as appropriate, while temperature was kept constant with a thermostat. After the 4-h hydrolysis, the enzymes were inactivated by heating and holding at 85°C for 15 min followed by centrifugation (9000×*g*, 4°C, 30 min), and the supernatant was freeze-dried to obtain the respective enzymatic protein hydrolysate and stored at -20°C.

Measurement of degree of hydrolysis (DH)

The method with *o*-phthalaldehyde (OPA) described by Charoenphun *et al*. [2013] was used to estimate DH with some modifications. The freshly prepared reagent consisted of 6 mM OPA (dissolved in methanol) and 0.2% (*v/v*) 2-mercaptoethanol in 50 mM sodium tetraborate containing 1% (*w/v*) sodium dodecyl sulphate (SDS). Acid hydrolysis was performed on the FLI using 6 M HCl (5 mg/mL concentration of the FLI in the acid) at 110°C for 24 h. A 200 μL aliquot of the OPA reagent was added to 5 μL of the standard (Gly-Gly-Gly), protein hydrolysates or acid-hydrolyzed isolate. The mixture was incubated for 100 s at room temperature and the absorbance was measured at 340 nm using a microplate reader (Multiskan Thermo Fischer Scientific, Waltham, MA, USA). The DH, defined as the percentage of cleaved peptide bonds, was calculated as follows:

$$DH\ (\%) = \frac{(NH_2)t_x - (NH_2)_{to}}{(NH_2)_{total} - (NH_2)_{to}} \times 100 \qquad (1)$$

where: $(NH_2)t_x$ is the number of free amino groups in the protein hydrolysate and $(NH_2)_{total}$ is the total number of amino groups in the protein (obtained from acid hydrolysate of FLI), and $(NH_2)_{to}$ is the amount of free amino groups of unhydrolyzed FLI.

Membrane ultrafiltration of fluted pumpkin leaf protein hydrolysate

Based on the bioactive properties, the Alcalase hydrolysate (AH) exhibited superior activities relative to the hydrolysates. As a result of this, AH was fractionated in an Amicon 8400 ultrafiltration stirred cell (Millipore Corp., Billerica, MA, USA) using 1, 3, 5, and 10 kDa molecular cut offs (MWCO) membranes. The Alcalase hydrolysate was passed through a 1 kDa membrane to collect the <1 kDa permeate while the retentate from the <1 kDa was passed through the 3 kDa membrane to obtain the 1–3 kDa. The retentate from the 3 kDa was passed through a 5 kDa to obtain 3–5 kDa permeates while the retentate from the 5 kDa was finally passed through a 10 kDa membrane to obtain the 5–10 kDa permeate. The final retentate (>10 kDa) and all the membrane permeates were freeze-dried and stored at –20°C.

Amino acid composition

The amino acid profile of each sample was determined according to the established methods described by Girgih *et al.* [2011] using a HPLC system after hydrolysis with 6 M HCl. The cysteine and methionine contents were determined after performic acid oxidation while the tryptophan content was determined after alkaline hydrolysis.

Determination of molecular weight distribution

The molecular weight (MW) distribution of fluted pumpkin leaf protein isolate and hydrolysates was evaluated using the method described by Alashi *et al*. [2014]. A 1000 μL sample aliquot, taken from the sample prepared at 5 mg/mL concentration in a phosphate buffer, pH 7.0, containing 0.15 M NaCl was loaded on the AKTA FPLC system (GE Healthcare, Montreal, PQ, Canada) equipped with a Superdex™ Peptide 10/300 GL column (10 × 300 mm) and the elution was carried out at room temperature using a phosphate buffer at 0.5 mL/min flow rate. Peptide molecular weight of each peak was estimated by extrapolating sample elution volume to a linear plot of log MW *versus* elution volume of standard proteins (cytochrome C, 12.38 kDa; aprotinin, 6.51 kDa; vitamin B12, 1.85 kDa; glycine, 0.075 kDa).

Determination of antioxidant properties

1,1-Diphenyl-2-picrylhydrazine (DPPH) radical scavenging activities

The method described by Girgih *et al.* [2011], with little modification, was used to determine the DPPH radical scavenging activities of the samples. The samples were made into solution using 0.1 M sodium phosphate buffer, pH 7.0, that contained 1% (*v/v*) Triton-X and prepared to a final concentration of 0.0156–1.0 mg/mL. A 100 μM DPPH radical concentration was prepared using 95% methanol. A 0.1 mL aliquot of each sample and 0.1 mL of the DPPH radical solution was pipetted into a 96-well plate. The mixture was incubated in the dark for 30 min at room temperature. The buffer and the reduced glutathione (GSH) served as the blank and positive control, respectively. After incubation, the absorbance was measured at 517 nm using a microplate reader. The percentage DPPH radical scavenging activity was calculated using Equation (2):

$$DPPH\ radical\ scavenging\ activity\ (\%) = \\ = \frac{(A1 - A2)}{A1} \times 100 \qquad (2)$$

where: A1 and A2, are absorbance of the blank and sample, respectively. The effective concentration that scavenged 50% of the free radicals (EC_{50}) was calculated for each sample by non-linear regression from a plot percent DPPH radical scavenging activity *versus* sample concentration (mg/mL).

Superoxide radical scavenging activity

The method described by Xie *et al*. [2008] was used to determine superoxide radical scavenging activity (SRSA) of the FLI, hydrolysates, AH fractions, and GSH as the standard. Each of the samples was dissolved in 50 mM Tris-HCl buffer, pH 8.3, containing 1 mM EDTA to concentrations between 0.25 and 1.5 mg/mL. The sample (80 μL) was transferred into a clear bottom microplate well while 80 μL of buffer was added to the blank well. This was followed by the addition of 40 μL of 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark, and the change in the rate of reaction was measured immediately at room temperature over a period of 4 min (1 min interval) using a microplate reader at a wavelength of 420 nm. The SRSA was calculated using the Equation 3:

$$SRSA = \\ = \frac{slope\ of\ blank\ for\ SRSA - slope\ of\ sample\ for\ SRSA}{slope\ of\ absorbance\ per\ minute\ of\ blank\ of\ SRSA} \times 100 \qquad (3)$$

The EC_{50} was calculated for each sample by non-linear regression from a plot of percent SRSA *versus* sample concentration (mg/mL).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity (HRSA) of the samples was determined by a modified method of Ajibola *et al*. [2011]. The samples (0.1–4 mg/mL) and 1,10 phenanthroline (3 Mm) were dissolved in 0.1 M phosphate buffer (pH 7.4) separately. Also, 3 mM $FeSO_4$ and 0.01% (*w/v*) hydrogen peroxide were each separately dissolved in distilled water. The mixture was incubated for 1 h at room temperature and centrifuged (3500×*g*, for 30 min). To a 96-well plate, 50 μL of the sample or GSH was first pipetted, followed by 50 μL of each of the 1,10-phenanthroline and $FeSO_4$ solutions. The reaction was initiated by adding 50 μL of hydrogen peroxide to the well that was then covered, incubated at 37°C for 1 h, and shaken constantly. The blank was prepared in a similar manner as the sample but was made up of 50 μL of the phosphate buffer in place of the sample. The absorbance of the colored reaction mixtures was measured at 10 min intervals for 1 h in a microplate reader at a wavelength of 536 nm. The reaction rate (ΔA/min) was then used to evaluate the HRSA value as follows:

$$HRSA = \frac{\Delta A/\min\ of\ blank - \Delta A/\min\ of\ sample}{\Delta A/\min\ of\ blank} \times 100 \quad (4)$$

The effective concentration that scavenged 50% of hydroxyl radicals was calculated for each sample using a non--linear regression from a plot of HRSA (%) activity *versus* sample concentration (mg/mL).

Ferric reducing antioxidant power

The modified method described by Benzie & Strain [1996] was used determine the ferric reducing antioxidant power (FRAP) of the samples. The FRAP working reagents were prepared as follows: acetate buffer (300 mM at pH 3.6); 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (10 mM); and $FeCl_3$ (20 mM) were mixed in the ratio of 5:1:1 (*v/v/v*) to obtain a straw-colored solution and the temperature was raised to 37°C. The samples were dissolved in water to the final concentration of 0.167 mg/mL. About 40 μL of the sample solution and 200 μL of FRAP reagent were pipetted into a clear 96-well microplate and the absorbance of the mixture was read at 593 nm. The ferric ion reducing ability of the samples were extrapolated from the calibration curve obtained from iron (II) sulfate heptahydrate ($FeSO_4 \times 7H_2O$) prepared at concentrations between 0.025 and 0.25 mM and presented as Fe^{2+} (mM).

Metal chelating activity

The metal chelating activity (MCA) of the samples was evaluated using the $FeCl_2$/ferrozine method described by Xie *et al*. [2008]. The samples and the standard (glutathione) were dissolved in distilled water to concentrations between 1 and 5 mg/mL and water was used as blank. One thousand microliters of the sample, standard or blank was transferred into different test-tubes and 50 μL of $FeCl_2$ (2 mM) was added, followed by 1.85 mL of distilled water, and then 100 μL of 5 mM ferrozine. The mixture was well vortexed and incubated for 10 min at room temperature. The reaction mixture (200 μL) was subsequently transferred from the reaction tube into a 96-well plate and absorbance values for the blank (Ab) and samples (As) were measured at 562 nm using a microplate reader. The MCA was determined using Equation (5):

$$Metal\ chelating\ activity\ (\%) = \frac{(Ab-As)}{Ab} \times 100 \quad (5)$$

The effective concentration that chelated 50% of the metals (EC_{50}) was calculated for each sample by non-linear regression from a plot of metal chelating activity (%) *versus* sample concentration (mg/mL).

Inhibition of linoleic acid oxidation

The inhibitory properties of the protein samples against linoleic acid oxidation were evaluated following the method described by He *et al.* [2013]. Sodium phosphate buffer (0.1 M, pH 7.0) was used to dissolve the samples and GSH (standard) to give sample concentration of 0.25 mg/mL, and then the sample was centrifuged. About 1 mL of the sample supernatant or blank (phosphate buffer) was mixed with 1 mL of linoleic acid (50 mM linoleic acid dissolved in 95% ethanol). The mixture was incubated at 60°C and the degree of color changes was measured after 24 h for seven days. Each day, the reaction tube contained 100 μL aliquot of the assay mixture, in addition to 4.7 mL of 75% (*v/v*) ethanol, 100 μL of 30% (*w/v*) ammonium thiocyanate, and 100 μL of 0.02 M ferric chloride dissolved in 1 M HCl. After the mixture was incubated for 3 min, 200 μL was transferred into a clear-bottom 96-well plate and the absorbance was measured at 500 nm using a microplate reader. An increase in the absorbance value suggested a high level of linoleic acid oxidation.

Inhibition of angiotensin-converting enzyme

The *in vitro* inhibitory properties of the FLI, hydrolysates, and AH fractions against the ACE (purchased from Sigma-Aldrich, St. Louis, MO, USA) were evaluated following the method described by Udenigwe *et al*. [2009], involving the use of *N*-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPPG) and Tris–HCl buffer. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5, and kept at 37°C) was mixed with 20 μL of ACE (final activity of 20 mU) and 200 μL of the samples (0.2 to 3 mg/mL assay concentration) dissolved in the Tris–HCl buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. Tris–HCl buffer served as the assay blank. ACE activity was expressed as the change in the rate of reaction (ΔA/min) and inhibitory activity was determined using Equation 6:

$$ACE\ ibhibition = \frac{\Delta A/\min(blank) - \Delta A/\min(sample)}{\Delta A/\min(blank)} \times 100 \quad (6)$$

where: (ΔA/min)(blank) and (ΔA/min)(sample) are ACE activities in the absence and presence of samples, respectively. The concentration that inhibited 50% of the ACE

(IC_{50}) was calculated for each sample by non-linear regression from a plot of ACE activity (%) *versus* sample concentration (mg/mL).

Inhibition of α-amylase

The porcine α-amylase inhibitory activity of the samples was evaluated using the 3,5-dinitrosalicylic acid (DNSA) method described by Kwon *et al*. [2008]. The samples and standard (acarbose) were dissolved using phosphate buffer (0.02 M, pH 6.9 containing 6 mM NaCl) to a final concentration of 200–500 μg/mL and 1–9 μg/mL for the samples and standard, respectively. α-Amylase (purchased from Sigma-Aldrich (St. Louis, MO, USA) was also dissolved in phosphate buffer (0.02 M, pH 6.9 containing 6 mM NaCl) to a final concentration of 1 mg/mL. One hundred microliters of the samples or the standard were mixed with 100 μL of α-amylase (200 units/mg) in a reaction tube and pre-incubated to 25°C. Two hundred microliters of a starch solution (1%, prepared in 0.02 M phosphate buffer, pH 6.9 containing 6 mM NaCl) were added to the assay mixture and incubated for 10 min at room temperature. The reactions were halted after the mixture was incubated in boiling water for 5 min. Then, 1000 μL DNSA was added and the mixture was cooled. Two hundred microliters of the mixture were pipetted into the 96-well microplate after diluting to the ratio of 1:5 with double distilled water. The absorbance was then measured with a microplate reader at 540 nm. The percentage inhibition was then determined using Equation (7):

$$\alpha\text{– }Amylase\ inhibition\ (\%) = \frac{(Absorbance\ of\ control - (Absorbance\ of\ sample - Absorbance\ of\ sample\ blank))}{Absorbance\ of\ control} \times 100 \tag{7}$$

Inhibition of α-glucosidase

The yeast α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (pNPG) were used to determine the inhibitory activity of the samples against α-glucosidase [Kim *et al*., 2004]. The samples, the standard (acarbose), and α-glucosidase enzyme (purchased from Sigma-Aldrich, St. Louis, MO, USA) were dissolved using a phosphate buffer (0.1 M, pH 6.9) to final concentrations of 1–6 mg/mL, 0.0312–0.25 mg/mL, and 1 mg/mL for the samples, acarbose and α-glucosidase respectively. Exactly 100 μL of the sample or the standard was added to 50 μL of the enzyme (200 units/mg). After pre-incubating for 20 min at 37°C, 100 μL of pNPG (5 mM, dissolved in 0.1 M phosphate buffer, pH 6.9) was added to a 96-well microplate. The mixture was incubated for 10 min at 37°C and the absorbance was read with a microplate reader at 405 nm for 30 min at the interval of 1 min. The percentage inhibitory activity was evaluated using Equation (8):

$$\alpha\text{– }Glucosidase\ inhibition\ (\%) = \ = \frac{(final\ absorbance\ of\ blank - (final\ absorbance\ of\ sample)}{final\ absorbance\ of\ blank} \times 100 \tag{8}$$

Mass spectrometry and identification of peptide sequences

Peptide sequences present in the protein hydrolysates were identified using the method of Malomo & Aluko [2019]. Sample solutions were diluted to 10 ng/μL using aqueous formic acid (0.1%, *v/v*) and passed through a 0.2 μm filter. Then, 10 μL of the filtrate was infused into an Absciex QTRAP® 6500 mass spectrometer (Absciex Ltd., Foster City, CA, USA) coupled with an electrospray ionization source. Operating conditions were as previously described: 3.5 kV ion spray voltage at 150°C, and 30 μL/min flow rate for 3 min in the positive ion mode with 2000 m/z scan maximum. The peptide sequences were obtained according to The Uniprot Consortium [2019] using the published primary sequences of *Telfairia occidentalis* Rubisco protein (Entry name = A5X4F4_9ROSI) at mass tolerance of +0.05 Da.

Statistical analysis

The experiments were carried out in triplicate determinations. Data were subjected to the analysis of variance using the statistical package for social sciences software, version 18. The statistical significance of difference between mean values of the data were chosen at $p<0.05$ level using the Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Degree of hydrolysis (DH)

After a 4-h enzymatic hydrolysis of FLI, the DH ranged between 16.37 and 20.66%. Chymotrypsin hydrolysate (CH) had the highest DH (20.66±0.37%). This value was not different significantly ($p \geq 0.05$) from the DH of 20.53±0.21% obtained for trypsin hydrolysate (TH) and 19.06±0.23% for pepsin hydrolysate (PH) but significantly ($p<0.05$) higher than the value (16.37±0.42%) obtained for Alcalase hydrolysate (AH). This may suggest that the peptide bonds of the protein (FLI) are more accessible to chymotrypsin, trypsin, and pepsin enzymes, since the DH depends on the number of broken bonds, which also reflects the enzyme specificity during digestion [Filho *et al*., 2020]. Muhamyankaka *et al*. [2013] reported 6–14% DH of pumpkin seeds protein hydrolysates, values that are lower than obtained in the current work. The differences in DH values may suggest variations in the amino acid sequence of the proteins (pumpkin seed *vs*. leaf), which will affect the number of peptide bonds broken by the proteases.

Amino acid composition

Table 1 shows that the protein hydrolysates had higher contents of aspartic, glutamic, proline, valine, and lysine amino acids, when compared to FLI. The essential amino acid contents were higher than the 32.14 to 34.81% earlier reported for pumpkin seed protein hydrolysates [Muyamyyankaka *et al*., 2013], which indicates that the samples are good sources of high-quality protein. All the samples had high (>35%) contents of hydrophobic amino acids, which were higher in the hydrolysates compared to FLI. The hydrophobic amino acid content is important due to the potential for increased peptide solubility in hydrophobic environments, which can lead to enhanced antioxidant activities [Rajapakse *et al*., 2005;

TABLE 1. Amino acid composition (%) of fluted pumpkin leaf protein and hydrolysates*.

Amino acid	FLI	AH	CH	PH	TH
Aspartic acid/asparagine	10.28	11.73	11.19	11.00	11.32
Threonine	4.22	2.21	2.90	4.05	2.71
Serine	5.22	2.91	3.74	5.24	3.45
Glutamic acid/glutamine	11.08	14.94	12.74	14.36	13.25
Proline	4.30	5.03	5.03	5.74	4.89
Glycine	5.22	4.72	4.79	5.12	4.81
Alanine	6.75	6.42	6.59	6.69	6.62
Cysteine	1.66	1.10	0.91	0.96	0.84
Valine	5.73	7.33	6.46	6.66	6.53
Methionine	2.22	1.79	1.97	1.21	1.73
Isoleucine	4.72	5.78	5.32	4.53	5.50
Leucine	9.04	9.30	9.81	8.08	9.14
Tyrosine	4.35	2.66	2.69	2.70	2.14
Phenylalanine	5.66	5.61	6.27	4.77	5.22
Histidine	3.53	3.07	3.16	3.44	3.04
Lysine	6.92	7.08	7.18	7.25	8.33
Arginine	6.88	6.43	7.24	6.94	9.04
Tryptophan	2.21	1.89	2.00	1.24	1.42
BCAA	19.49	22.41	21.59	19.27	21.17
HAA	37.98	40.37	39.97	38.03	39.22
AAA	12.22	10.16	10.96	8.71	8.78
PCAA	17.33	16.58	17.58	17.63	20.41
NCAA	30.80	31.79	30.57	34.65	30.73
SCAA	3.88	2.89	2.88	2.17	2.57
EAA	44.25	44.06	45.07	41.23	43.62

*FLI, protein isolate; AH, Alcalase hydrolysate; CH, chymotrypsin hydrolysate; PH, pepsin hydrolysate; TH, trypsin hydrolysate. BCAA: branched-chain amino acids (valine, leucine, isoleucine); HAA: hydrophobic amino acids (proline, glycine, alanine, leucine, isoleucine, valine, methionine); AAA: aromatic amino acids (tyrosine, phenylalanine, tryptophan); PCAA: positively-charged amino acids (arginine, lysine, histidine; NCAA: negatively-charged amino acids (aspartic acid/asparagine, glutamic acid/glutamine, serine, threonine); SCAA: sulfur-containing amino acids (cysteine and methionine); EAA: Essential amino acids (valine, leucine, isoleucine, phenylalanine, histidine, tryptophan, methionine, lysine, threonine).

Zhu *et al.*, 2006]. The content of branched-chain amino acids (BCAAs) was also higher in hydrolysates than in FLI with the exception of PH. The presence of BCAAs is reported to be important for enhanced ACE-inhibitory activity of peptides. Increased levels of negatively charged and positively charged amino acids were observed only for PH and TH, respectively. Amino acids with negative charges contain excess electrons, which may contribute to an increase in the free radical quenching ability of antioxidant peptides. However, there were decreases in aromatic and sulfur-containing amino acids, which have also been suggested to contribute to increased antioxidant activities [Udenigwe & Aluko, 2012].

Peptide sequencing

Amino acid sequences of identified peptides, the observed mass/charge and the calculated mases are shown in Table 2. Based on the protein database, six, seven, eleven, and four peptides were identified in Alcalase, chymotrypsin, pepsin, and trypsin hydrolysates, respectively. CH contained a higher number of dipeptides (5 out of 7 peptides identified) when compared with the other protein hydrolysates. This is consistent with the DH of the hydrolysates in this study, where CH had the highest value. The presence of shorter peptides such as the dipeptides may suggest greater advantages to interact with and neutralize free radicals more effectively to inhibit propagation. In addition, all the identified peptides have molecular weights <500 Da and this may enhance the ease of absorption of the peptides from the gastrointestinal tract and improve *in vivo* activities [Malomo & Aluko, 2019]. Alcalase and chymotrypsin hydrolysates shared three (PGV, GVP, and RP) common peptides (2 tripeptides and 1 dipeptide) while pepsin and trypsin hydrolysates showed two (STGT and FKA) similar peptides. The similarity in some of the peptides identified in the hydrolysates suggests that the catalytic activities of the enzymes can be well compared. With the exception of HAVI, all the peptides identified from AH and CH contained proline while BCAAs (L, I, V) were also present in most of the peptides. Most of the peptides were mainly composed of either hydrophobic or branched chain amino acids, which may have implications in their bioactivities. For instance, Chen *et al.* [2012] reported that the presence of glycine in egg-white peptide contributed to the antioxidant properties of the hydrolysate due proton donating ability of this amino acid. A previous report by Mirzaei *et al.* [2016] showed that the presence of hydrophobic amino acids such as proline in the peptide sequence obtained from *Saccharomyces cerevisiae* facilitated the scavenging of free radical species and the quenching of singlet oxygen.

A previous study has indicated that the dipeptide sequences (RP, VP, PV) have ACE-inhibitory activity [Sentandreu & Toldrá, 2007] while IP, PI, LP, VF, and FV peptides are dipeptidyl peptidase (DPP)-IV inhibitors in addition to their ACE inhibitory potentials [Lacroix & Li-Chan, 2012; Sentandreu & Toldrá, 2007]. Several other dipeptide subsequences that make up tripeptides and tetrapeptides identified in this study have been linked to various bioactivities. For instance, PGV (PG, GV subsequences), GVP (VP and GP subsequences), IEPV (IE, PV or EP subsequences), PLDL (PL, LD or DL subsequences), SSTG (ST and TG subsequences), YIA (YI and IA subsequences), and IAY (IA and AY subsequences) have been reported to function as ACE and DPP-IV inhibitors according to BIOPEP UWM database [http://www.uwm.edu.pl/biochemia/index.php/pl/biopep]. The peptide subsequences, HA and VI of the identified tetrapeptide HAVI have also been reported to possess renin inhibitory activity [Pihlanto & Mäkinen, 2017] while KA dipeptide, which is one of the subsequences of FKA, has been reported as DPP-III inhibitor [Dhanda *et al.*, 2008].

TABLE 2. Amino acid sequences of peptides identified in the enzymatic hydrolysates of fluted pumpkin leaf protein isolate.

Observed molecular weight (Da)	Calculated molecular weight (Da)	Peptide sequence	Location*
Alcalase hydrolysate (AH)			
272.200	272.160	PGV	f30–32, 356–358
272.200	272.160	GVP	f31–33, 245–247
272.200	272.172	RP	f151–152
439.300	439.266	HAVI	f282–285
457.300	457.266	IEPV	F71–74
457.300	457.266	PLDL	F883–91
Chymotrypsin hydrolysate (CH)			
229.200	229.155	PL	f88–89, 152–153
229.200	229.155	PI	f247–248
229.200	229.155	IP	f124–125
229.200	229.155	LP	f355–356, 359–360
272.200	272.160	PGV	f30–32, 356–358
272.200	272.160	GVP	f31–33, 245–247
272.200	272.172	RP	f151–152
Pepsin hydrolysate (PH)			
215.100	215.139	VP	f32–33, 246–247
215.100	215.139	PV	f73–74, 360–361
351.200	351.151	SSTG	f45–48
351.200	351.166	FQG	f132–134
351.200	351.166	QFG	f385–387
351.200	351.166	GFK	f110–112
365.200	365.167	STGT	f46–49
365.200	365.218	FKA	f111–113
365.200	365.202	YIA	f81–83
365.200	365.202	IAY	f82–84
365.200	365.202	AIY	f208–210
Trypsin hydrolysate (TH)			
265.200	265.155	VF	f108–109, 239–240
265.200	265.155	FV	f329–330, 337–338
365.200	365.167	STGT	f46–49
365.200	365.218	FKA	f111–113

*Within the primary structure of *Telfairia occidentalis* Rubisco; f = fragment.

Molecular weight distribution

Size exclusion chromatography was used to estimate the molecular weight distribution of fluted pumpkin leaf protein isolate and hydrolysates, as shown in Figure 1. The molecular weight of the hydrolysates was mainly between 2.1 and 7.2 kDa, which was lower than the molecular weight of FLI (19.11 kDa). The results show that the FLI was degraded to lower molecular weights by the hydrolyzing enzymes. A previous study on alfalfa leaf hydrolysate reported a peptide molecular weight range of 0.2 to 1.9 kDa [Xie *et al.*, 2008]. The estimated molecular weight of PH main peak that eluted early (~12 min) was 7.2 kDa, which suggests the presence of bigger peptides when compared to the other protein hydrolysates with longer elution times. The findings from these results showed that the hydrolysates have varying peptide molecular weights, which is consistent with the fact that the enzymes differ in protease specificity. This variation in molecular weights of the hydrolysates with respect to the hydrolyzing enzymes is expected to influence the functional and bio-active effects of the hydrolysates because these properties are dependent on the chain length of peptides closely correlated with their molecular weight [Wasswa *et al.*, 2007]. The smaller average peptide molecular weight of TH, AH, and CH is important because of the potential for escaping further enzyme degradation in the gastro-intestinal tract and improving absorption into blood circulation [Horton *et al.*, 2002].

Antioxidant properties

DPPH radical scavenging activity (DRSA)

The DRSA is based on a decolorization assay that measures the capacity of antioxidants to directly react with the DPPH radical by monitoring its absorbance at 517 nm. It is a stable organic nitrogen-centered free radical with a dark purple color but becomes colorless when reduced to its non-radical form by an electron-donating compound, which enables estimating antioxidant capacity [Saidi *et al.*, 2014]. The DRSA values of fluted pumpkin leaf isolate and hydrolysate are shown in Table 3. The EC_{50} values of the protein

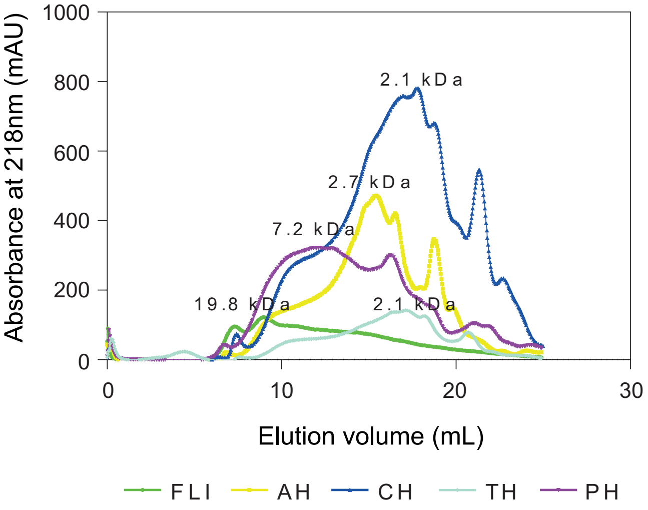


FIGURE 1. Comparative gel-permeation chromatograms of the fluted pumpkin leaf protein isolate (FLI) and Alcalase (AH), chymotrypsin (CH), pepsin (PH), and trypsin (TH) hydrolysates after passage through a Superdex Peptide12 10/300 GL column. Inserted values indicate estimated molecular weights.

TABLE 3. EC_{50} (mg/mL) determined for antioxidant activities and IC_{50} (mg/mL) determined for ACE-inhibitory property of fluted pumpkin leaf protein isolate, hydrolysates, and Alcalase hydrolysate ultrafiltration peptide fractions.

	DPPH radical scavenging	Superoxide radical scavenging	Hydroxyl radical scavenging	Metal chelation	ACE inhibition
Hydrolysates					
GSH	0.237 ± 0.019^{e}	0.785 ± 0.008^{c}	0.668 ± 0.023^{d}	4.961 ± 0.016^{a}	–
FLI	0.825 ± 0.029^{a}	0.899 ± 0.002^{b}	3.259 ± 0.087^{c}	4.655 ± 0.010^{b}	2.826 ± 0.099^{a}
AH	0.240 ± 0.001^{e}	0.914 ± 0.009^{b}	5.969 ± 0.041^{a}	4.903 ± 0.032^{a}	0.675 ± 0.035^{d}
CH	0.486 ± 0.014^{c}	0.939 ± 0.007^{a}	3.170 ± 0.035^{c}	4.894 ± 0.006^{a}	0.666 ± 0.042^{d}
PH	0.602 ± 0.153^{b}	0.965 ± 0.003^{a}	5.260 ± 0.062^{b}	4.756 ± 0.008^{b}	1.432 ± 0.014^{b}
TH	0.361 ± 0.009^{d}	0.873 ± 0.005^{b}	3.277 ± 0.025^{c}	4.839 ± 0.016^{ab}	0.889 ± 0.011^{c}
AH fractions					
<1 kDa	0.047 ± 0.005^{c}	0.676 ± 0.095^{g}	0.600 ± 0.023^{d}	3.283 ± 0.074^{b}	0.340 ± 0.036^{c}
1–3 kDa	0.049 ± 0.001^{c}	1.222 ± 0.012^{c}	1.259 ± 0.087^{c}	2.914 ± 0.165^{c}	0.217 ± 0.004^{d}
3–5 kDa	0.421 ± 0.014^{a}	1.112 ± 0.074^{d}	1.713 ± 0.029^{b}	3.342 ± 0.088^{b}	0.372 ± 0.039^{c}
5–10 kDa	0.053 ± 0.002^{c}	1.347 ± 0.013^{b}	1.711 ± 0.083^{b}	3.441 ± 0.078^{b}	0.356 ± 0.069^{c}
>10 kDa	0.054 ± 0.002^{c}	1.433 ± 0.037^{a}	1.456 ± 0.064^{c}	3.481 ± 0.015^{b}	0.446 ± 0.038^{b}
GSH	0.237 ± 0.019^{b}	0.785 ± 0.008^{f}	0.668 ± 0.023^{d}	4.961 ± 0.016^{a}	–

Note: Values are mean ± SD of triplicate determination. Values with different superscripts along the same column (separately for hydrolysates and AH fractions) are significantly ($p<0.05$) different from each other. Abbreviations: AH, Alcalase hydrolysate; FLI, fluted pumpkin leaf protein isolate; CH, chymotrypsin hydrolysate; PH, pepsin hydrolysate; TH, trypsin hydrolysate; GSH, reduced glutathione.

hydrolysates were between 0.240 and 0.602 mg/mL, which is significantly lower ($p<0.05$) compared to the 0.825 mg/mL determined for FLI. Therefore, the results suggest that the hydrolysates possess stronger electron donating ability when compared to the protein isolate. The results also reflect the beneficial effect of protein hydrolysis in enhancing the bioactive properties of proteins. These results also revealed that the hydrolysates exhibited different DRSA values, which is consistent with the varied proteolytic specificity of the enzymes used during protein hydrolysis. This is because the release of the peptides and the exposure of the amino acid residues depend on the specificity of the enzymes [Thamnarathip *et al.*, 2016]. A previous work also showed different DRSA values for different enzymatic cotton seed protein hydrolysates [Filho *et al.*, 2020]. Among the protein hydrolysates, Alcalase hydrolysate (AH) had stronger DRSA when compared to the other hydrolysates, which may be due to the higher contents of glutamine, valine, and isoleucine (Table 1) and this was the basis for separating Alcalase hydrolysate (AH) into lower molecular weight fractions using ultrafiltration membrane process.

The DRSA EC_{50} values for the AH ultrafiltration peptide fractions are shown in Table 3. With the exception of 3–5 kDa peptides, the peptide fractions had similar DRSA values. The DRSA EC_{50} values of <1 kDa (0.047 mg/mL), 1–3 kDa (0.049 kDa), and 5–10 kDa (0.053 mg/mL) were significantly ($p<0.05$) lower than those of the parent hydrolysate (0.240 mg/mL) and the GSH, the physiological peptide (0.261 mg/mL). The results showed <1 kDa peptide fraction exhibited stronger electron donating ability when compared to the other fractions, which may be related to the small peptide size, which enhanced interactions with the DPPH free radical. The results showing that the <1 kDa had stronger DRSA than the other peptide fractions are consistent with previous works on perilla seed hydrolysate [Park & Yoon, 2019] and pigeon pea hydrolysate [Olagunju *et al.*, 2018] peptide fractions.

Superoxide radical scavenging activity (SRSA)

The superoxide is usually unable to initiate lipid peroxidation without the presence of other free radicals. In addition to its ability to destroy cellular biopolymers, the superoxide radical may also engage in reactions that lead to the production of hydrogen peroxide (H_2O_2) and the highly toxic hydroxyl radical [Jamdar *et al.*, 2012]. As shown in Table 3, the SRSA EC_{50} values for the protein hydrolysates were between 0.873 and 0.965 mg/mL, which are significantly ($p<0.05$) higher than the 0.785 mg/mL determined for GSH. This suggests that the hydrolysates had weaker SRSA than GSH. The results suggest that enzymatic hydrolysis did not significantly enhance the SRSA of the hydrolysates, as evidenced in the data whereby the EC_{50} value obtained for FLI (0.899 mg/mL) was significantly ($p>0.05$) lower than the respective values determined for CH and PH. The SRSA of AH peptide fractions is shown in Table 3. The parent hydrolysate (AH) exhibited significantly ($p<0.05$) stronger SRSA than the >1 kDa peptide fractions. The results indicate that with exception of the <1 kDa peptides, strong peptide synergistic interactions within the protein hydrolysate were weakened after membrane separation. The strong SRSA of <1 kDa peptides may be due to the small size, which may have favored increased interactions with the free radical as previously reported for pigeon pea seed peptide fractions [Olagunju *et al*., 2018].

Hydroxyl radical scavenging activity (HRSA)

The hydroxyl radical is one of the most reactive species generated by biological systems, which is mainly generated *in vivo* by many reactions such as Fenton, superoxide-dependent, and the Haber-Weiss. The hydroxyl radical exerts cellular oxidative damage, which may cause several chronic diseases [Jia *et al*., 2013]. Therefore, scavenging this radical would reduce the risk for oxidative deterioration of tissues and associated negative effects on human health. The HRSA values for protein isolate and hydrolysates are shown in Table 3. The values showed that the protein hydrolysates had EC_{50} between 3.170 and 5.969 mg/mL while the protein isolate had EC_{50} at 3.259 mg/mL, suggesting that only the Alcalase and pepsin hydrolysates were less potent when compared with the protein isolate. There was no significant difference ($p>0.05$) in the HRSA of FLI, TH, and CH. Jia *et al*. [2013] reported that high contents of hydrophobic amino acids played an important role in the HRSA of mussel protein hydrolysates. The variations in HRSA of the protein hydrolysates may be related to differences in the proteolytic specificities of the enzymes used for digestion.

The HRSA EC_{50} values of AH peptide fractions were between 0.600 and 1.713 mg/mL (Table 3). The separated fractions showed higher radical quenching ability than the parent hydrolysate (AH), which suggests that ultrafiltration membrane separation enhanced the HRSA of the resulting peptides. This may be due to reduced antagonistic interactions between the peptides after membrane ultrafiltration separation. The results are consistent with an earlier report on rapeseed hydrolysates where the peptide fractions showed superior HRSA than the unfractionated hydrolysate [He *et al*., 2013]. The <1 kDa peptide fraction possessed significantly ($p<0.05$) higher HRSA than the >1 kDa peptide fractions. The stronger HRSA of the <1 kDa peptides may have been favored by its low molecular weight, which would have enhanced peptide interactions with the hydroxyl radical molecules [Koopman *et al*., 2009]. Similar findings showing that the <1 kDa peptides had better HRSA than the other peptide fractions were previously reported for chickpea peptides [Li *et al*., 2008].

Metal chelating activity (MCA)

The MCA principle involves the formation of colored complexes with ferrous iron molecules when a test compound competes with a synthetic chelator [Nam *et al*., 2008]. The extent of color change, which accompanies the complex compound formation, is used to estimate the chelating activity of the antioxidant test compound. Table 3 shows that FLI and PH were better metal chelators when compared to AH, CH, and TH, which suggests that enzymatic hydrolysis did not improve the MCA of the hydrolysates. The similar MCA values for FLI and PH may be attributed to the threonine and serine contents, which are higher than the levels present in AH, CH, and TH. This is because serine and threonine can ionize to produce negative charges that will then enable stronger interactions with the ferrous iron [Mirzaei *et al*., 2016]. The MCA values for AH peptide fractions (EC_{50}: 2.914 to 3.481 mg/mL) indicate stronger metal chelators when compared to the unfractionated hydrolysate (EC_{50}: 4.903 mg/mL) and GSH. The results suggest that membrane separation reduced the antagonistic peptide interactions present in AH. The 1–3 kDa peptides had the strongest MCA with a lower EC_{50} value while the chelating activities of <1 kDa, 3–5 kDa, 5–10 kDa peptide fractions were not significantly ($p>0.05$) different from each other. A previous report also reported that the 1–3 kDa peptide fraction of perilla seed protein hydrolysate exhibited greater metal chelating ability than the other fractions [Park & Yoon, 2019].

Ferric reducing antioxidant power (FRAP)

The FRAP assay estimates the potential of antioxidant compounds to donate electron/hydrogen ions and interrupt propagation of chain reactions involving free radicals [Dorman *et al*., 2003]. The FRAP values of the fluted pumpkin protein hydrolysates (0.166 to 0.287 mM) were significantly ($p<0.05$) higher compared to the protein isolate (Figure 2A), which suggests that enzymatic hydrolysis improved the reducing property of the protein hydrolysates. This may be due to protein fragmentation and the accompanying increased number of N- and C-terminals, which contain electron-donating groups (NH_2 and COOH, respectively) [Jia *et al*., 2013]. Previous works reported FRAP values of between 0.045 and 0.125 mM for alfalfa leaf protein hydrolysates [Xie *et al*., 2008] and between 0.01 and 0.35 mM for hawk tea hydrolysates [Jia *et al*., 2013]. Among the protein hydrolysates, chymotrypsin hydrolysate showed stronger reducing property when compared to the other protein hydrolysates. This is consistent with the smaller average molecular weight of CH as shown in Figure 1, which suggests that the reduced peptide chain length may have enhanced reduction of Fe^{3+} when compared to the longer peptides present in AH, TH, and PH.

The FRAP of the Alcalase peptide fractions ranged between 0.234 and 0.313 mM (Figure 2B). In general, FRAP values were higher in the fractions when compared with values of the unfractionated hydrolysate, which suggests that the ultrafiltration membrane process improved the capacity of the resulting peptides to donate electrons. The increased FRAP potency after membrane separation suggests a positive effect through the reduction of antagonistic peptide interactions within the unfractionated hydrolysate. The <1 kDa peptide fraction exhibited the highest ferric reducing antioxidant power among the membrane fractions, which is consistent with a previous report on fish hound peptide fractions [Bougatef *et al*., 2009]. This is an indication that low molecular weight peptides have stronger potentials than bigger peptides for effective reduction of Fe^{3+}.

Lipid peroxidation inhibition

Lipid oxidation is an important chain reaction process because of the damaging effects on food and biological systems. It is a major cause of quality deterioration, such as the rancidity development and loss in nutritional values of foods [Elias *et al*., 2008]. In human systems, lipid oxidation and its by-products have been linked to a number of chronic inflammatory and neurogenerative diseases,

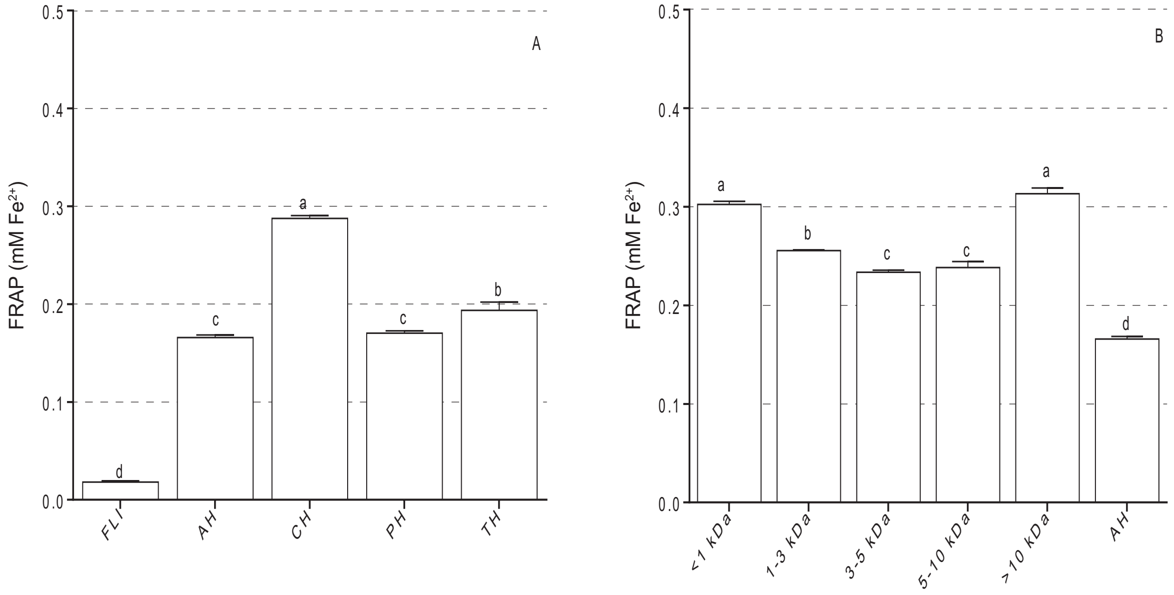


FIGURE 2. Ferric reducing antioxidant power (FRAP) of fluted pumpkin leaf protein isolate (FLI), Alcalase (AH), chymotrypsin (CH), pepsin (PH), trypsin (TH) hydrolysates (A), and AH peptide fractions (B). Different letters above bars indicate significant differences ($p<0.05$).

making it of great interest for human health. The inhibition of linoleic acid oxidation is a standardized method developed to evaluate the stability of fats and oils under elevated temperatures [Jia *et al.,* 2013]. Any antioxidant compound that delays or inhibits the oxidation of unsaturated fatty acids *in vitro* would probably show similar effects *in vivo*. The inhibition of lipid oxidation by fluted pumpkin leaf isolate and hydrolysates over a period of seven days of incubation is shown in Figure 3. Absorbance of the control (without antioxidant compound) increased on the 1st incubation day and attained its peak absorbance value on the 2nd day (0.881). The values, however, decreased progressively for the rest of the five days. The decrease in the absorbance of the control may be attributed to rapid depletion of linoleic acid coupled with decomposition of the hydroperoxides as the reaction progressed. This is consistent with data from a previous work on pumpkin seed protein hydrolysates [Venuste *et al*., 2013]. The peak absorbance values of oxidation of linoleic acid with glutathione (0.175) and fluted pumpkin leaf isolate (0.455) occurred on the first day of incubation and decreased for the rest of the incubation days. These values were higher than the peak values of oxidation of lipids with protein hydrolysates, which occurred on the 3rd day for CH (0.096), and 4th day for AH (0.082), PH (0.083), and TH (0.088). This pattern of results suggests that hydrolysis improved the inhibition of the linoleic acid oxidation. The stronger inhibition of linoleic acid oxidation exhibited by protein hydrolysates over the protein isolate may be related to protein fragmentation that would have increased charge density (for electron donation) and reduced peptide size (better interactions with free radicals). Moreover, the increased electron donating ability of the protein hydrolysates, as reflected in the stronger DRSA and FRAP, may have contributed to the better inhibition of lipid peroxidation when compared to the unhydrolyzed protein (FLI).

Enzyme inhibitory properties

α-Amylase inhibitory activities

α-Amylase is an enzyme responsible for hydrolyzing the linear chain of starch molecules, which results in the production of shorter chains (mainly di- and tri-saccharides) that are further broken down by other enzymes into absorbable glucose; therefore, inhibition of this enzyme activity would contribute to the management of diabetics and obesity in humans [Habtamu *et al*., 2019]. The α-amylase inhibitory activity of the hydrolysates ranged between 42.47 and 46.84%, and was significantly ($p<0.05$) higher

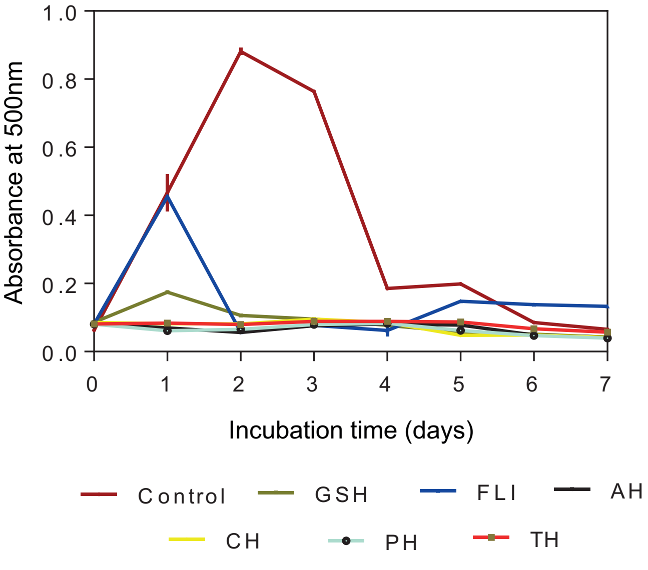


FIGURE 3. Inhibition of linoleic peroxidation by fluted pumpkin leaf protein isolate (FLI) and Alcalase (AH), chymotrypsin (CH), pepsin (PH), and trypsin (TH) hydrolysates.

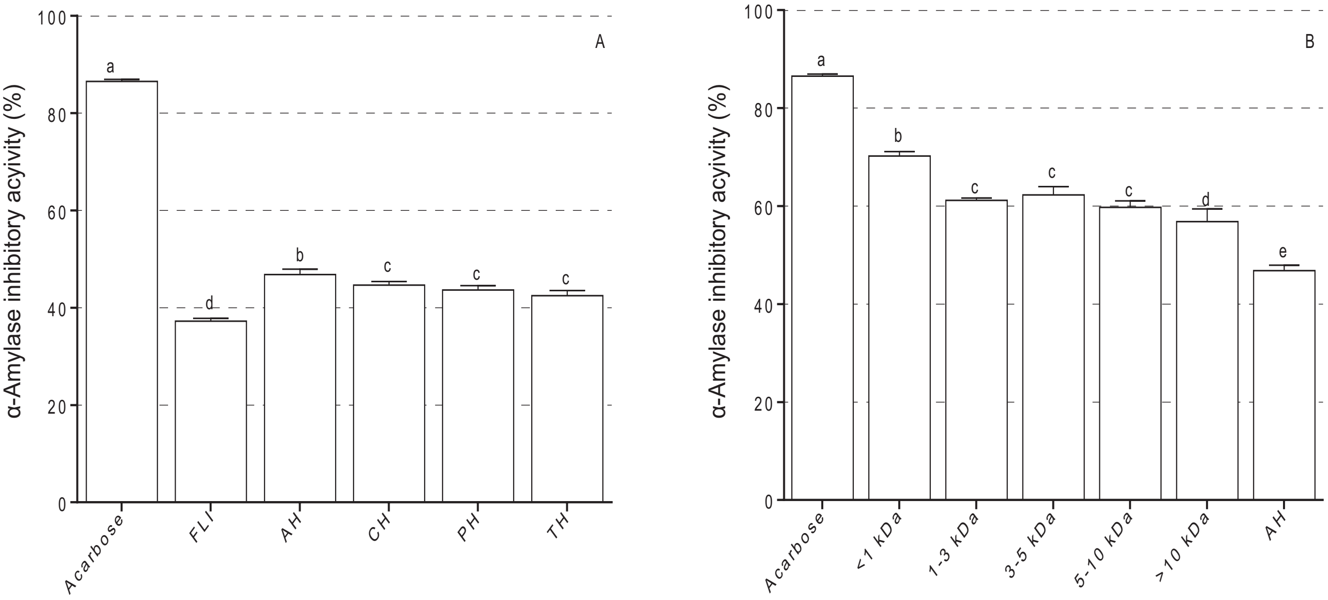


FIGURE 4. α-Amylase inhibitory activities of fluted pumpkin leaf protein isolate (FLI) and Alcalase (AH), chymotrypsin (CH), pepsin (PH), trypsin (TH) hydrolysates (A), and AH peptide fractions (B). Different letters above bars indicate significant differences ($p<0.05$).

than the 37.24% determined for FLI (Figure 4A). Therefore, proteolysis led to improved inhibitory properties of the hydrolysates against α-amylase. The positive effect of proteolysis on α-amylase inhibition may be due to the production of multiple peptide products that can interact with the enzyme protein when compared to the few polypeptide chains present in the FLI. The results show that AH was the most effective α-amylase inhibitor and that its inhibitory activity may be related to the higher content of BCAA when compared to CH, PH, and TH. However, the inhibitory values obtained in this work were lower than the 70–80% previously reported for cowpea protein hydrolysates [Olusola & Ekun, 2019] and 60 to 77% for watermelon seed protein hydrolysates [Arise *et al.,* 2016]. The α-amylase inhibitory properties of Alcalase hydrolysate peptide fractions are presented in Figure 4B. The 56.83 to 70.22% inhibitory levels are higher than the 46.84% obtained for the unfractionated protein hydrolysate. Improved α-amylase inhibition by the peptide fractions suggests reduced peptide antagonism, which enhanced interactions with the enzyme protein. However, the α-amylase inhibitory activity of the protein hydrolysates and peptide fractions is lower than the 86.54% noted for acarbose (a commercial drug). Among the fractions, the <1 kDa peptides showed greater inhibition, which is consistent with increased ability of small size peptide to interact with the α-amylase protein.

α-Glucosidase inhibitory activities

In the human system, α-glucosidase breaks down dietary short-chain carbohydrates to produce glucose for intestinal absorption, which leads to an increase in blood glucose levels. Therefore, the inhibition of α-glucosidase activity would slow down glucose absorption and attenuate blood glucose levels following meals, which is one of the effective ways to manage diabetes [Arise *et al.*, 2016]. The α-glucosidase inhibitory properties of the hydrolysates (13.87 to 23.76%) were higher than the 6.79% determined for fluted protein isolate (Figure 5A). The results also showed that the inhibition of α-glucosidase activity differed among the hydrolysates, which may be related to differences in the proteolytic specificity of the enzymes used during protein digestion. This pattern of results showing the enzyme-dependent activity of protein hydrolysates is consistent with previously published works on watermelon seed hydrolysates [Arise *et al.*, 2016] and soy protein hydrolysates [Wang *et al.*, 2019]. The <1 kDa, 1–3 kDa, and >10 kDa peptide fractions had stronger α-glucosidase inhibitory activities when compared with the unfractionated AH, while there was no significant difference ($p>0.05$) between the 3–5 kDa peptide fraction and the parent AH. This may suggest that some of the peptide fractions had reduced peptide-peptide interactions and better peptide-enzyme interactions when compared to the unfractionated hydrolysate (Figure 5B). Unlike the α-amylase inhibition, the <1 kDa (28.26%) and 1–3 kDa (27.38%) had similar α-glucosidase inhibitory activity but were weaker inhibitors than the >10 kDa peptide fraction. Therefore, peptide molecular weight does not seem to play an important role in the α-glucosidase inhibitory activity of the peptide fractions. The results suggest differences in the mode of action of α-amylase and α-glucosidase in addition to variations in how the enzymes interact with inhibitory peptides.

Angiotensin-converting enzyme (ACE)-inhibitory properties

ACE is a multi-functional enzyme that plays vital roles in the regulation of blood pressure, and inhibition of its activity can lead to a reduction in the angiotensin II concentration with concomitant attenuation of hypertension, which can cause many cardiovascular diseases [Zhang *et al.*, 2006]. The ACE-inhibitory IC_{50} values show significant ($p<0.05$) increase in potency after enzymatic hydrolysis of FLI

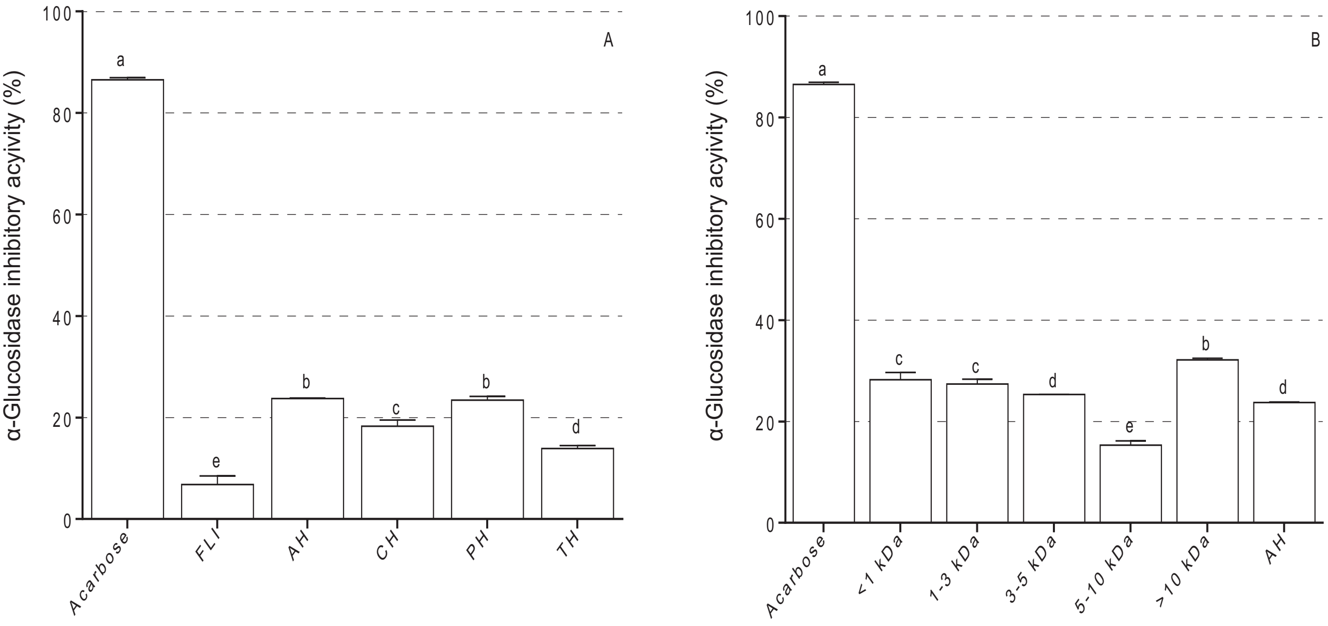


FIGURE 5. α-Glucosidase inhibitory activities of fluted pumpkin leaf protein isolate (FLI) and Alcalase (AH), chymotrypsin (CH), pepsin (PH), trypsin (TH) hydrolysates (A), and AH peptide fractions (B). Different letters above bars indicate significant differences ($p<0.05$).

(Table 3). This pattern may be attributed to an increased number of peptide molecules in the hydrolysates when compared to the few polypeptide molecules in FLI. Moreover, the smaller peptides may have better interactions with the enzyme active site than the big FLI protein molecules. There was no significant ($p<0.05$) difference in the ACE-inhibitory properties of CH and AH, but these two hydrolysates had lower IC_{50} values (0.666 and 0.675 mg/mL, respectively), which suggest their greater inhibitory potency when compared to PH (1.432 mg/mL) and TH (0.889 mg/mL). CH and AH also had higher contents of aromatic amino acids (AAA), BCAA, HAA, and sulfur-containing amino acids (SCAA) as shown in Table 1, which may have contributed to the superior ACE-inhibitory properties when compared to PH and TH. The results are consistent with previous works on chickpea [Medina-Godoy *et al.,* 2012] and lupin [Boschin *et al.*, 2014] protein hydrolysates with strong ACE-inhibitory activities attributed to the high level of hydrophobic amino acids.

The ACE-inhibitory IC_{50} values of Alcalase peptide fractions ranged from 0.217 to 0.446 mg/mL, and were significantly lower (more potency) when compared to the unfractionated hydrolysate (Table 3). The 1–3 kDa peptide fraction showed the strongest ($p<0.05$) ACE inhibition (IC_{50} = 0.217 mg/mL), whereas there were no significant differences between <1 kDa (0.340 mg/mL), 3–5 kDa (0.372 mg/mL), and 5–10 kDa fractions (0.356 mg/mL). In contrast, the >10 kDa peptide fraction was the weakest (0.446 mg/mL) ACE inhibitor. The ACE-inhibitory IC_{50} values obtained in this study for peptide fractions were lower when compared to the 3.00–3.64 mg/mL reported for *Leucopaxillus tricolor* [Geng *et al*., 2016], 0.50–2.00 mg/mL for *Fucus spirallis* [Paiva *et al*., 2017], and 0.615–1.975 mg/mL for sea cucumber *Acaudina molpadioidea* [Zhao *et al*., 2009] protein hydrolysates.

CONCLUSIONS

In this study, four proteases were used to hydrolyze fluted pumpkin leaf protein isolate into hydrolysates with a distinct degree of hydrolysis, peptide molecular weight distribution, and amino acid composition, which contributed to variations observed in antioxidant and enzyme (α-amylase, α-glucosidase, and ACE) inhibitory properties. The results of the peptide sequencing showed that the hydrolysate contained dipeptides, tripeptide, and tetrapeptides, and the presence of these short peptides may contribute to the bioactivities of the hydrolysates. The AH was fractionated into peptides of varying molecular weights with the <1 kDa showing better overall antioxidant and enzyme-inhibitory properties. The results support previous works in scientific literature that have shown stronger bioactive properties of small size peptides when compared to the bigger peptides. Membrane ultrafiltration separation of AH led to an increase in the *in vitro* bioactive properties, which supports the use of this method in enriching peptide fractions with active molecules. Therefore, the resulting peptides, especially the low molecular weight fractions, may find application as ingredients in the food and nutrition industries to formulate functional foods and nutraceuticals for the management of oxidative stress, hyperglycemia, and hypertension. However, *in vivo* studies that use animal models are required to validate bioactive properties of the peptides.

RESEARCH FUNDING

This research was supported by the International Development Research Centre and Global Affairs Canada through the Canadian International Food Security Research

Fund, Project 107983 on synergizing indigenous vegetables and fertilizer micro-dosing innovations among West African farmers.

CONFLICT OF INTERESTS

There is no conflict of interest in the publication of the article. All the authors agreed to the submission of this work.

ORCID IDs

A.A. Famuwagun https://orcid.org/0000-0003-1474-1785
K.A. Taiwo https://orcid.org/0000-0003-4353-8591
R.E. Aluko https://orcid.org/0000-0002-9025-2513

REFERENCES

1. Ajibola, C.F., Fashakin, J.B., Fagbemi, T.N., Aluko, R.E. (2011). Effect of peptide size on antioxidant properties of african yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions. *International Journal of Molecular Sciences*, *12*, 6685–6702. https://doi.org/10.3390/ijms12106685
2. Alashi, A.M., Blanchard, C.L., Mailer, R.J., Agboola, S.O., Mawson, A.J., Rong, H., Malomo, S.A., Girgih, A.T., Aluko, R.E. (2014). Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. *Food Research International*, *55*, 281–287. https://doi.org/10.1016/j.foodres.2013.11.015
3. Aletor, O., Oshodi, A.A., Ipinmoroti, K. (2002). Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. *Food Chemistry*, *78*(1), 63–68. https://doi.org/10.1016/S0308-8146(01)00376-4
4. Aluko, R.E. (2015). Antihypertensive peptides from food proteins. *Annual Review of Food Science and Technology*, *6*(1), 235–262. https://doi.org/10.1146/annurev-food-022814-015520
5. Arise, R.O., Yekeen, A., Ekun, O. (2016). *In-vitro* antioxidant and α-amylase inhibitory properties of watermelon seed protein hydrolysates. *Environmental and Experimental Biology*, *14*(4), 163–172. https://doi.org/10.22364/eeb.14.23
6. Awosika, T.O., Aluko, R.E. (2019). Inhibition of the *in vitro* activities of α-amylase, α-glucosidase and pancreatic lipase by yellow field pea (*Pisum sativum* L.) protein hydrolysates. *International Journal of Food Science and Technology*, *54*(6), 2021–2034. https://doi.org/10.1111/ijfs.14087
7. Benzie, I.F.F., Strain, J.J. (1996). Ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292
8. Bleakley, S., Hayes, M., O'Shea, N., Gallagher, E., Lafarga, T. (2017). Predicted release and analysis of novel ACE-I, renin, and DPP-IV inhibitory peptides from common oat (*Avena sativa*) protein hydrolysates using *in silico* analysis. *Foods*, *6*(12), art. no. 108. https://doi.org/10.3390/foods6120108
9. Boschin, G., Scigliuolo, G.M., Resta, D., Arnoldi, A. (2014). ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. *Food Chemistry*, *145,* 34–40. https://doi.org/10.1016/j.foodchem.2013.07.076
10. Bougatef, A., Hajji, M., Balti, R., Lassoued, I., Triki-Ellouz, Y., Nasri, M. (2009). Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. *Food Chemistry, 114*(4), 1198–1205. https://doi.org/10.1016/j.foodchem.2008.10.075
11. Cao, W., Zhang, W.C., Hong, P., Ji, H., Hao, J. (2010). Purification and identification of an ACE inhibitory peptide from the peptic hydrolysate of *Acetes chinensis* and its antihypertensive effects in spontaneously hypertensive rats. *International Journal of Food Science and Technology*, *45*(5), 959–965. http://dx.doi.org/10.1111/j.1365-2621.2010.02219.x
12. Chalamaiah, M., Yu, W., Wu, J. (2018). Immunomodulatory and anticancer protein hydrolysates(peptides) from food proteins: a review. *Food Chemistry*, *245*, 205–222. https://doi.org/10.1016/j.foodc hem.2017.10.087
13. Charoenphun, N., Cheirsilp, B., Sirinupong, N. (2013). Calcium binding peptides derived from tilapia (*Oreochromis niloticus*) protein hydrolysates. *European Food Research Technology*, *236*, 57–63. https://doi.org/10.1007/s00217-012-1860-2
14. Chen, C., Chi, Y.J., Zhao, M.Y., Lv, L. (2012). Purification and identification of antioxidant peptides from egg white protein hydrolysate. *Amino Acids*, *43*, 457–466. https://doi.org/10.1007/s00726-011-1102-0
15. Dhanda, S., Singh, H., Singh, J., Singh, T.P. (2008) Functional characterization and specific effects of various peptides on enzymatic activity of a DPP-III homologue from goat brain. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *23*(2), 174–181. https://doi.org/10.1080/14756360701450996
16. Dorman, H.J.D., Peltoketo, A., Hiltunen, R., Tikkanen, M.J. (2003). Characterization of the antioxidant properties of deodorised aqueous extracts from selected Lamiaceae herbs. *Food Chemistry*, *83*(2), 255–262. https://doi.org/10.1016/S0308-8146(03)00088-8
17. Elias, R.J., Kellerby, S.S., Decker, E.A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition, 48*(5), 430–441. https://doi.org/10.1080/10408390701425615
18. Famuwagun, A.A., Alashi, A.M., Gbadamosi, S.O., Taiwo, K.A., Oyedele, D.J., Adebooye, O.C., Aluko, R.E. (2020). Comparative study of the structural and functional properties of protein isolates prepared from edible vegetable leaves. *International Journal of Food Properties, 23*(1), 955–970. https://doi.org/10.1080/10942912.2020.1772285
19. Filho, J.G., Rodrigues, J.M., Valadares, A.C.F., de Almeida, A.B., Valencia-Mejia, E., Fernandes, K.F., Lemes, A.C., Alves, C.C.F., Sousa, H.A.F., da Silva, E.R., Egea, M.B., Dyszy, F.H. (2020). Bioactive properties of protein hydrolysate of cottonseed byproduct: Antioxidant, antimicrobial, and angiotensin-converting enzyme (ACE) inhibitory. *Waste and Biomass Valorization*, *2020.* https://doi.org/10.1007/s12649-020-01066-6
20. Gbadamosi, S.O., Famuwagun, A.A. (2016). Studies on the proximate, functional and antioxidant properties of fermented and unfermented *Kariya* (*Hildergardia barterii)* seed protein hydrolysates obtained by enzymatic hydrolysis. *British Journal of Biotechnology, 14,* 1–14. https://doi.org/10.9734/BBJ/2016/26685

21. Geng, X., Tian, G., Zhang, W., Zhao, Y., Zhao, L., Wang, H., Ng, T.B. (2016). A tricholoma matsutake peptide with angiotensin converting enzyme inhibitory and antioxidative activities and antihypertensive effects in spontaneously hypertensive rats. *Scientific Reports*, *6*, art. no. 24130.
https://doi.org/10.1038/srep24130
22. Girgih, A., Udenigwe, C., Li, H., Adebiyi, A., Aluko, R. (2011). Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa L.*) protein hydrolysates. *Journal of the American Oil Chemists' Society*, *88*(11), 1767–1774.
https://doi.org/10.1007/s11746-011-1841-9
23. Gong, K., Deng, L., Shi, A., Liu, H., Liu, L., Hu, H., Adhikari, B., Wang, Q. (2017). High-pressure microfluidisation pretreatment disaggregates peanut protein isolates to prepare antihypertensive peptide fractions. *International Journal of Food Science and Technology*, *52*(8), 1760–1769.
https://doi.org/10.1111/ijfs.13449
24. Habtamu, A.M., Abdalbasit, A., Gasmalla, R.Y., Wei, Z. (2019). Evaluation of the *in vitro* α-amylase enzyme inhibition potential of commercial dried Laver (*Porphyra* Species) Seaweed protein hydrolysate. *Turkish Journal of Fisheries and Aquatic Sciences, 18*(4), 547–556.
https://doi.org/10.4194/1303-2712-v18_4_06
25. He, R., Girgih, A.T., Malomo, S.A., Ju, X., Aluko, R.E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods*, *5*, 219–227.
https://doi.org/10.1016/j.jff.2012.10.008
26. Horton, H.R., Moran, L.A., Ochs, R.S., Rawn, J.D., Scrimgeour, K.G. (2002c). Properties of Enzymes. In *Principles of Biochemistry.* 3rd Edition. Prentice Education, NJ, pp. 242–244.
27. Jamdar, S.N., Rajalakshmi, V., Sharma, A. (2012). Antioxidant and ACE inhibitory properties of poultry viscera protein hydrolysate and its peptide fractions. *Journal of Food Biochemistry*, *36*(4), 494–501.
https://doi.org/10.1111/j.1745-4514.2011.00562.x
28. Jia, X., Ding, C., Dong, L., Yuan, S., Zhang, Z., Chen, Y., Yuan, M. (2013). Comparison of the chemical and functional properties of protein hydrolysates from different mature degree hawk teas. *Journal of Food and Nutrition Research, 1*(6), 138–144.
https://doi.org/10.12691/jfnr-1-6-5
29. Kim, S.R., Byun, H.G. (2012). The novel angiotensin I converting enzyme inhibitory peptides from rainbow trout muscle hydrolysate. *Fisheries and Aquatic Sciences*, *15*(3), 183–190.
https://doi.org/10.5657/FAS.2012.0183
30. Kim, Y.M., Wang, M.H., Rhee, H.I. (2004). A novel alpha glucosidase inhibitor from pine bark. *Carbohydrate Research*, *339*, 715–727.
https:/doi/org/10.1016/j.carres.2003.11.005
31. Koopman, R., Crombach, N., Gijsen, A.P., Walrand, S., Fauquant, J., Kies A.K., van Loon, L.J.C. (2009). Ingestion of a protein hydrolysate is accompanied by an accelerated *in vivo* digestion and absorption rate when compared with its intact protein. *American Journal of Clinical Nutrition*, *90*(1), 106–115.
https://doi.org/10.3945/ajcn.2009.27474
32. Kwon, Y., Apostolidis, E., Shetty, K. (2008). Inhibitory potential of wine and tea against alpha amylase and alpha glucosidase for management of hypertensive linked to type 2 diabetes. *Journal of Food Biochemistry*, *32(1)*, 15–31.
https://doi.org/10.1111/j.1745-4514.2007.00165.x
33. Lacroix, I.M.E., Li-Chan, E.C.Y. (2012). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in-silico* approach. *Journal of Functional Foods*, *4*(2), 403–422.
https://doi.org/10.1016/j.jff.2012.01.008
34. Li, Y., Jiang, B., Zhang, T., Mu, W., Liu, J. (2008). Antioxidant and free radical scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemistry, 106*(2), 444–450.
https://doi.org/10.1016/j.foodchem.2007.04.067
35. Malomo S.A., He, R., Aluko, R.E. (2014). Structural and functional properties of hemp seed protein products. *Journal of Food Science*, *79*, C1512–C1521.
https://doi.org/10.1111/1750-3841.12537
36. Malomo, S.A., Aluko, R.E. (2019). Kinetics of acetylcholinesterase inhibition by hemp seed protein-derived peptides. *Journal Food Biochemistry*, *43*(7), art. no. e12897.
https://doi.org/10.1111/jfbc.12897
37. McCarthy, A.L., O'Callaghan, Y.C., O'Brien, N.M. (2013). Protein hydrolysates from agricultural crops-bioactivity and potential for functional food development. *Agriculture, 3,* 112–130.
https://doi.org/10.3390/agriculture3010112
38. Medina-Godoy, S., Ambriz-Pérez, D.L., Fuentes-Gutiérrez, C., Germán-Báez, L.J., Gutiérrez-Dorado, R., Reyes-Moreno, C., Valdez-Ortiz, A. (2012). Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. *Journal of the Science of Food and Agriculture*, *92(9)*, 1974–1981.
https://doi.org/10.1002/jsfa.5570
39. Mirzaei, M., Aminlari, M., Hosseini, E. (2016). Antioxidant, ACE-Inhibitory and antibacterial activities of *Kluyveromyces marxianus* protein hydrolysates and their peptide fractions. *Functional Foods in Health and Disease*, *6*(7)*,* 425–439.
https://doi.org/10.31989/ffhd.v6i7.250
40. Mirzaei, M.M., Mirdamadi, S., Ehsani, M.R., Aminlari, M., Hosseini, E. (2015). Purification and identification of antioxidant and ACE-inhibitory peptide from *Saccharomyces cerevisiae* protein hydrolysate. *Journal of Functional Foods*, *19*, 259–268.
https://doi.org/10.1016/j.jff.2015.09.031
41. Muhamyankaka, V., Shoemaker, C.F., Nalwoga, M., Zhang, X.M. (2013). Physicochemical properties of hydrolysates from enzymatic hydrolysis of pumpkin (*Cucurbita moschata*) protein meal. *International Food Research Journal*, *20*(5), 2227–2240.
42. Nam, K.A., You, S.G., Kim, S.M. (2008). Molecular and physical characteristics of squid (*Todarodes pacificus*) skin collagens and biological properties of their enzymatic hydrolysates. *Journal of Food Science*, *73*(4), C243–C255.
https://doi.org/10.1111/j.1750-3841.2008.00722.x
43. Nnamezie, A.A., Famuwagun, A.A., Gbadamosi, S.O. (2019). Changes in some nutritional and sensory properties of treated and stored fluted pumpkin leaf. *Annals. Food Science and Technology, 20*(1), 65–81.
44. Odiaka, N.I. (2001). Survey on the production and supply of *Telfairia occidentalis* in Makurdi, Benue State Nigeria. *African Journal of Food Science*, *2*, 12–18.

45. Olagunju, A.I., Omoba, O.S., Enujiugha, V.N., Alashi. A.M., Aluko, R.E. (2018). Pigeon pea enzymatic protein hydrolysates and ultrafiltration peptide fractions as potential sources of antioxidant peptides: An *in vitro* study. *LWT – Food Science and Technology*, *97*, 269–278.
https://doi.org/10.1016/j.lwt.2018.07.003
46. Olusola, A.O., Ekun, O.E. (2018). Alpha-Amylase inhibitory properties and in vitro anti-oxidant potentials of cowpea seed protein hydrolysates. *AASCIT Communications, 6*(1), 1–12.
47. Ortiz-Martinez, M., Winkler, R., García-Lara, S. (2014). Preventive and therapeutic potential of peptides from cereals against cancer. *Journal of Proteomics*, *111*, 165–183.
https://doi.org/10.1016/j.jprot.2014.03.044
48. Paiva, L., Lima, E., Isabel, N., Baptista, J. (2017). Angiotensin I-Converting Enzyme (ACE) inhibitory activity, antioxidant properties, phenolic content and amino acid profiles of *Fucus spiralis* L. protein hydrolysate fractions. *Marine Drugs, 15*(10), 3–11.
https://doi.org/10.3390/md15100311
49. Park, B.Y., Yoon, K.Y. (2019). Biological activity of enzymatic hydrolysates and the membrane ultrafiltration fractions from perilla seed meal protein. *Czech Journal of Food Sciences*, *37*(3), 180–185.
https://doi.org/10.17221/145/2018-CJFS
50. Pihlanto, A., Mäkinen, S. (2017). The function of renin and the role of food-derived peptides as direct renin inhibitors. In A. N. Tolekova (Ed.), *Renin-Angiotensin System-Past, Present And Future*. InTech Open, London, UK, pp. 241–258.
https://doi.org/10.5772/intechopen.69513
51. Pinciroli, M., Aphalo, P., Nardo, A.E., Añón, M.C., Quiroga, A.V. (2019). Broken rice as a potential functional ingredient with inhibitory activity of renin and angiotensin-converting enzyme (ACE). *Plant Foods for Human Nutrition, 74*(3), 405–413.
https://doi.org/10.1007/s11130-019-00754-6
52. Rajapakse, N., Mendis, E., Byun, H.G., Kim, S.K. (2005). Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *Journal of Nutritional Biochemistry*, *16*(9), 562–569.
https://doi.org/10.1016/j.jnutbio.2005.02.005
53. Ramada, M.H.S., Brand, G.D., Abrão, F.Y., Oliveira, M., Filho, J.L.C., Galbieri, R., Gramacho, K.P., Prates, M.V., Bloch, C. (2017). Encrypted antimicrobial peptides from plant proteins. *Scientific Reports*, *7*, art. no. 13263.
https://doi.org/10.1038/s41598-017-13685-6
54. Saidi, S., Deratani, A., Belleville M.P., Amar, R.B. (2014). Antioxidant properties of peptide fractions from tuna dark muscle protein by-product hydrolysate produced by membrane fractionation process. *Food Research International*, *65*, 329–336.
55. Sentandreu, M.A., Toldrá, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chemistry, 97*(3), 546–554.
https://doi.org/10.1016/j.foodchem.2005.06.006
56. Tavano, O.L. (2013). Protein hydrolysis using proteases: an important tool for food biotechnology. *Journal of Molecular Catalysis and Biological Enzymology*, *90*, 1–11.
https://doi.org/10.1016/j.molcatb.2013.01.011
57. Thamnarathip, P., Jangchud, K., Nitisinprasert, S., Vardhanabhuti, B. (2016). Identification of peptide molecular weight from rice bran protein hydrolysate with high antioxidant activity. *Journal of Cereal Science*, *69*, 329–335.
https://doi.org/10.1016/j.jcs.2016.04.011
58. The UniProt Consortium (2019). UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Research, 47*(D1), D506-D515.
https://doi.org/10.1093/nar/gky1049
59. Udenigwe, C.C., Aluko, R.E. (2012). Food protein-derived bioactive peptides; Production, processing, and potential health benefits. *Journal of Food Science*, *77*(1), R11–R24.
https://doi.org/10.1111/j.1750-3841.2011.02455.x
60. Udenigwe, C.C., Lin, Y.S., Hou, W.C., Aluko, R.E. (2009). Kinetics of the inhibition of renin and angiotensin, I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods*, *1*, 199–207.
https://doi.org/10.1016/j.jff.2009.01.009
61. Udousoro, I., Ekanem, P. (2013). Assessment of proximate compositions of twelve edible vegetables in Nigeria. *International Journal of Modern Chemistry*, *4*(2), 79–89.
62. Venuste, M., Zhang, X., Shoemaker, C.F., Karangwa, E., Abbas, S., Kamdem, P.E. (2013). Influence of enzymatic hydrolysis and enzyme type on the nutritional and antioxidant properties of pumpkin meal hydrolysates. *Food and Function*, *4*, 811–820.
https://doi.org/10.1039/c3fo30347k
63. Wang, R., Zhao, H., Pan, X., Orfila, C., Lu, W., Ma, Y. (2019). Preparation of bioactive peptides with antidiabetic, antihypertensive, and antioxidant activities and identification of α-glucosidase inhibitory peptides from soy protein. *Food Science and Nutrition*, *7*(5), 1848–1856.
https://doi.org/10.1002/fsn3.1038
64. Wasswa, J., Tang, J., Gu, X.-H., Yuan, X.-Q. (2007). Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp (*Ctenopharyngodon idella*) skin. *Food Chemistry*, *104*, 1698–1704.
https://doi.org/10.1016/j.foodchem.2007.03.044
65. Wei, Y.-H., Lu, C.-Y., Wei, C.-Y., Ma, Y.-S., Lee, H.-C. (2001). Oxidative stress in human aging and mitochondrial disease-consequences of defective mitochondrial respiration and impaired antioxidant enzyme system. *Chinese Journal of Physiology*, *44*(1), 1–11.
66. Xie, Z., Huang, J., Xu, X., Jin, Z. (2008). Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. *Food Chemistry*, *111*, 370–376.
https://doi.org/10.1016/j.foodchem.2008.03.078
67. Yoshikawa, T., Naito, Y. (2002). What is oxidative stress? *Japanese Medical Association Journal*, *45*(7), 271–276.
68. Zhang, Y., Lee, E.T., Devereux, R.B., Yeh, J., Best, L.G., Fabsitz, R.R., Howard, B.V. (2006). Prehypertension, diabetes, and cardiovascular disease risk in a population-based sample: The strong heart study. *Hypertension, 47,* 410–414.
https://doi.org/10.1161/01.HYP.0000205119.19804.08
69. Zhao, Y., Li, B., Dong, S., Liu, Z., Zhao, X., Wang, J., Zeng, M.A. (2009). Novel ACE inhibitory peptide isolated from *Acaudina molpadioidea* hydrolysate. *Peptides, 30*, 1028–1033.
https://doi.org/10.1016/j.peptides.2009.03.002
70. Zhu, K.X., Zhou, H.M., Qian, H.F. (2006). Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with Alcalase. *Process Biochemistry, 41*, 1296–1302.
https://doi.org/10.1016/j.procbio.2005.12.029

Submitted: 25 August 2020. Revised: 2 October, 3 November, and 10 November 2020. Accepted: 12 November 2020. Published on-line: 9 December 2020.

http://journal.pan.olsztyn.pl
Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 445–446

VOLUME 70 AUTHOR'S INDEX

Author/No./Page

http://journal.pan.olsztyn.pl
Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 447–449

CUMULATIVE TABLE OF CONTENTS
PJFNS, 2020, VOL. 70

No. 1

No. 2

No. 3

No. 4

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 4, pp. 451–453
http://journal.pan.olsztyn.pl

Instruction for Authors
Section: Editorial News

INSTRUCTIONS FOR AUTHORS

SUBMISSION. Original contributions relevant to food and nutrition sciences are accepted on the understanding that the material has not been, nor is being, considered for publication elsewhere. **All papers should be submitted and will be processed electronically via Editorial Manager system (available from PJFNS web site: http://journal.pan.olsztyn.pl). On submission, a corresponding author will be asked to provide: Cover letter; Files with Manuscripts, Tables, Figures/Photos; and Names of two potential reviewers (one from the author's homeland – but outside author's Institution, and the other from abroad).** All papers which have been qualified as relevant with the scope of our Journal are reviewed. All contributions, except the invited reviews are charged. Proofs will be sent to the corresponding author or to the first author and should be returned within one week since receipt. No new material may be inserted in the text at proof stage. It is the author's duty to proofread proofs for errors.

Authors should very carefully consider the preparation of papers to ensure that they communicate efficiently, because it permits the reader to gain the greatest return for the time invested in reading. Thus, we are more likely to accept those that are carefully designed and conform the instruction. Otherwise, papers will be rejected and removed from the on-line submission system.

SCOPE. The Polish Journal of Food and Nutrition Sciences publishes original, basic and applied papers, and reviews on fundamental and applied food research, preferably these based on a research hypothesis, in the following Sections:

Food Technology:
- Innovative technology of food development including biotechnological and microbiological aspects
- Effects of processing on food composition and nutritional value

Food Chemistry:
- Bioactive constituents of foods
- Chemistry relating to major and minor components of food
- Analytical methods

Food Quality and Functionality:
- Sensory methodologies
- Functional properties of food
- Food physics
- Quality, storage and safety of food

Nutritional Research Section:
- Nutritional studies relating to major and minor components of food (excluding works related to questionnaire surveys)

"News" section:
- Announcements of congresses
- Miscellanea

OUT OF THE SCOPE OF THE JOURNAL ARE:
- Works which do not have a substantial impact on food and nutrition sciences
- Works which are of only local significance i.e. concern indigenous foods, without wider applicability or exceptional nutritional or health related properties
- Works which comprise merely data collections, based on the use of routine analytical or bacteriological methods (i.e. standard methods, determination of mineral content or proximate analysis)
- Works concerning biological activities of foods but do not provide the chemical characteristics of compounds responsible for these properties
- Nutritional questionnaire surveys
- Works related to the characteristics of foods purchased at local markets
- Works related to food law
- Works emphasizing effects of farming / agricultural conditions / weather conditions on the quality of food constituents
- Works which address plants for non-food uses (i.e. plants exhibiting therapeutic and/or medicinal effects)

TYPES OF CONTRIBUTIONS. *Reviews:* (at least: 30 pages and 70 references) these are critical and conclusive accounts on trends in food and nutrition sciences; *Original papers:* (maximally: 30 pages and 40 references) these are reports of substantial research; *Reports on post and forthcoming scientific events, and letters to the Editor* (all up to three pages) are also invited (free of charge).

REVIEW PROCESS. All scientific contributions will be peer-reviewed on the criteria of originality and quality. Submitted manuscripts will be pre-evaluated by Editor-in-Chief and Statistical Editor (except for review articles), and when meeting PJFNS' scope and formal requirements, they will be sent to a section Editor who upon positive pre-evaluation will assign at least two reviewers from Advisory Board Members, reviewers suggested by the author or other experts in the field. Based on the reviews achieved, Section Editor and Editor-in-Chief will make a decision on whether a manuscript will be accepted for publication, sent back to the corresponding author for revision, or rejected. Once a manuscript is sent back to the corresponding author for revision, all points of the reviews should be answered or rebuttal should be provided in the Explanation letter. The revised manuscripts will be checked by Section Editor and by the original reviewers (if necessary), and a final decision will be made on acceptance or rejection by both Section Editor and Editor-in-Chief.

Polish Journal of Food and Nutrition Sciences uses CrossCheck's iThenticate software to detect instances of similarity in submitted manuscripts. In publishing only original research, PJFNS is committed to deterring plagiarism, including self-plagiarism. Your manuscript may be screened for similarity to published materials.

COPYRIGHT LICENSE AGREEMENT referring to Authorship Responsibility and Acknowledgement, Conflict of Interest and Financial Disclosure, Copyright Transfer, are required for all authors, i.e. *Authorship Responsibility and Acknowledgement*: Everyone who has made substantial intellectual contributions to the study on which the article is based (for example, to the research question, design, analysis, interpretation, and written description) should be an author. It is dishonest to omit mention of someone who has participated in writing the manuscript ("ghost authorship") and unfair to omit investigator who have had important engagement with other aspects of the work. All contributors who do not meet the criteria for authorship should be listed in an Acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chairperson who provided only general support. Any financial and material support should also be acknowledged. *Conflict of Interest and Financial Disclosure*: Authors are responsible for disclosing financial support from the industry or other conflicts of interest that might bias the interpretation of results. *Copyright License Agreement*: Authors agree that papers accepted become the copyright of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland, and may not be published elsewhere without the Editor's permission in writing.

A manuscript will not be published once the signed form has not been submitted to the Editor with the manuscript revised after positive reviews.

ETHICAL APPROVAL OF STUDIES AND INFORMED CONSENT. For all manuscripts reporting data from studies involving human participants or animals, formal approval by an appropriate institutional review board or ethics committee is required and should be described in the Methods section. For those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be followed. For investigations of humans, state in the Methods section the manner in which informed consent was obtained from the study participants (i.e., oral or written). Editors may request that authors provide documentation of the formal review and recommendation from the institutional review board or ethics committee responsible for oversight of the study.

UNAUTHORIZED USE AND COPYRIGHT AGREEMENT. Published manuscripts become the property of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (IAR&FR PAS) and may not be published elsewhere without written permission. Unauthorized use of the PJFNS name, logo, or any content for commercial purposes or to promote commercial goods and services (in any format, including print, video, audio, and digital) is not permitted by IAR&FR PAS.

MANUSCRIPTS. A manuscript in English must be single-sided, preferably in Times New Roman (12) with 1.5-point spacing, without numbers of lines. The Editor reserves the right to make literary corrections and to make suggestions to improve brevity. English is the official language. The English version of the paper will be checked by Language Editor. Unclear and unintelligible version will be sent to the author(s) for correction.

Every paper should be divided under the following headings in this order: a **Title** (possibly below 150 spaces), **Running title** (up to 50 spaces, submitted under the Title); the Names(s) of the author(s) in full. In paper with more than one author, the asterisk indicates the name of the author to whom correspondence and inquiries should be addressed, otherwise the first author is considered for the correspondence. Current full postal address of the indicated corresponding author or the first author must be given in a footnote on the title page; the Place(s) where the work was done including the institution name, city, country if not Poland. In papers originated from several institutions the names of the authors should be marked with respective superscripts; the **Key words** (up to 6 words or phrases) for the main topics of the paper; an **Abstract** (up to 250 words for regular papers and reviews and 100 words for Short Reports) summarizing briefly main results of the paper, no literature references; an **Introduction** giving essential background by saying why the research is important, how it relates to previous works and stating clearly the objectives at the end; **Materials and Methods** with sufficient experimental details permitting to repeat or extend the experiments. Literature references to the methods, sources of material, company names and location (city, country) for specific instruments must be given. Describe how the data were evaluated, including selection criteria used; **Results and Discussion** presented together (in one chapter). Results should be presented concisely and organized to supplement, but not repeat, data in tables and figures. Do not display the data in both tabular and graphic form. Use narrative form to present the data for which tables or figures are unnecessary. Discussion should cover the implications and consequences, not merely recapitulating the results, and it must be accomplished with concise **Conclusions**; **Acknowledgements** should be made to persons who do not fill the authorship criteria (see: Authorship forms); **Research funding** should include financial and material support; and **References** as shown below.

REFERENCES each must be listed alphabetically at the end of the paper (each should have an Arabic number in the list) in the form as follows: **Periodicals** – names and initials of all the authors, year of publication, title of the paper, journal title as in Chemical Abstracts, year of publication, volume, issue, inclusive page numbers; **Books** – names and initials of all the authors, names of editors, chapter title, year of publication, publishing company, place of publication, inclusive page numbers; **Patents** – the name of the application, the title, the country, patent number or application number, the year of publication.

For papers published in language other than English, manuscript title should be provided in English, whereas a note on the original language and English abstract should be given in parentheses at the end.

The reference list should only include peer-reviewed full-text works that have been published or accepted for publication. Citations of MSc/PhD theses and works unavailable to international Editors, Reviewers, and Readers should be limited as much as possible.

References in the text must be cited by name and year in square parentheses (e.g.: one author – [Tokarz, 1994]; two authors – [Słonimski & Campbell, 1987]; more than two authors – [Amarowicz *et al*., 1994]). If more than one paper is published in the same year by the same author or group of authors use [Tokarz, 1994a, b]. Unpublished work must only be cited where necessary and only in the text by giving the person's name.

Examples:

Article in a journal:

Słonimski, B.A., Campbell, L.D., Batista, E., Howard B. (2008). Gas chromatographic determination of indole glucosinolates. *Journal of Science and Food Agriculture*, *40*(5), 131–143.

Book:

Weber, W., Ashton, L., Milton, C. (2012). *Antioxidants – Friends or Foes?* 2nd editition. PBD Publishing, Birmingham, UK. pp. 218–223.

Chapter in a book:

Uden, C., Gambino, A., Lamar, K. (2016). Gas chromatography. In M. Queresi, W. Bolton (Eds.), *CRC Handbook of Chromatography*, CRC Press Inc., Boca Raton, Florida, USA, pp. 44–46.

ABBREVIATIONS AND UNITS. Abbreviations should only be used when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. Metric SI units should be used. The capital letter L should be used for liters. Avoid the use of percentages (% g/g,% w/w; Mol-%; vol-%), ppm, ppb. Instead, the expression such as g/kg, g/L, mg/kg, mg/mL should be used. A space must be left between a number and a symbol (e.g. 50 mL not 50mL). A small x must be used as multiplication sign between numeric values (e.g. 5×10^2 g/mL). Statistics and measurements should be given in figures, except when the number begins a sentence. Chemical formulae and solutions must specify the form used. Chemical abbreviations, unless they are internationally known, Greek symbols and unusual symbols for the first time should be defined by name. Common species names should be followed by the Latin at the first mention, with contracting it to a single letter or word for subsequent use.

FIGURES should be submitted in separate files. Each must have an Arabic number and a caption. Captions of all Figures should be provided on a separate page "Figure Captions". Figures should be comprehensible without reference to the text. Self-explanatory legend to all figures should be provided under the heading "Legends to figures"; all abbreviations appearing on figures should be explained in figure footnotes. Three-dimensional graphs should only be used to illustrate real 3-D relationships. Start the scale of axes and bars or columns at zero, do not interrupt them or omit missing data on them. Figures must be cited in Arabic numbers in the text.

TABLES should be submitted in separate files. They should be as few in number and as simple as possible (like figures, they are expensive and space consuming), and include only essential data with appropriate statistical values. Each must have an Arabic number and a caption. Captions of all Tables should be provided on a separate page "Table Captions". Tables should be self-explanatory; all abbreviations appearing in tables should be explained in table footnotes. Tables must be cited in Arabic numbers in the text.

PAGE CHARGES. A standard publication fee has been established at the rate of 450 EUR + tax (if applicable) irrespective of the number of pages and tables/figures. For Polish Authors an equivalent fee was set at 1950 PLN + VAT. Payment instructions will be sent to Authors via e-mail with acceptance letter.

Information on publishing and subscription is available from:

Ms. Joanna Molga
Editorial Office of Pol. J. Food Nutr. Sci.
Institute of Animal Reproduction and Food Research
Tuwima 10 Str., 10–748 Olsztyn 5, Poland
phone (48 89) 523–46–70, fax (48 89) 523–46–70;
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

Nutrition