ISSN (1230-0322)

2021, Vol. 71, No. 2

Food

Polish Journal of Food and Nutrition Sciences

formerly Acta Alimentaria Polonica

Published

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

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

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

POLICY: Editors select submitted manuscripts in relation to their relevance to the scope. 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.

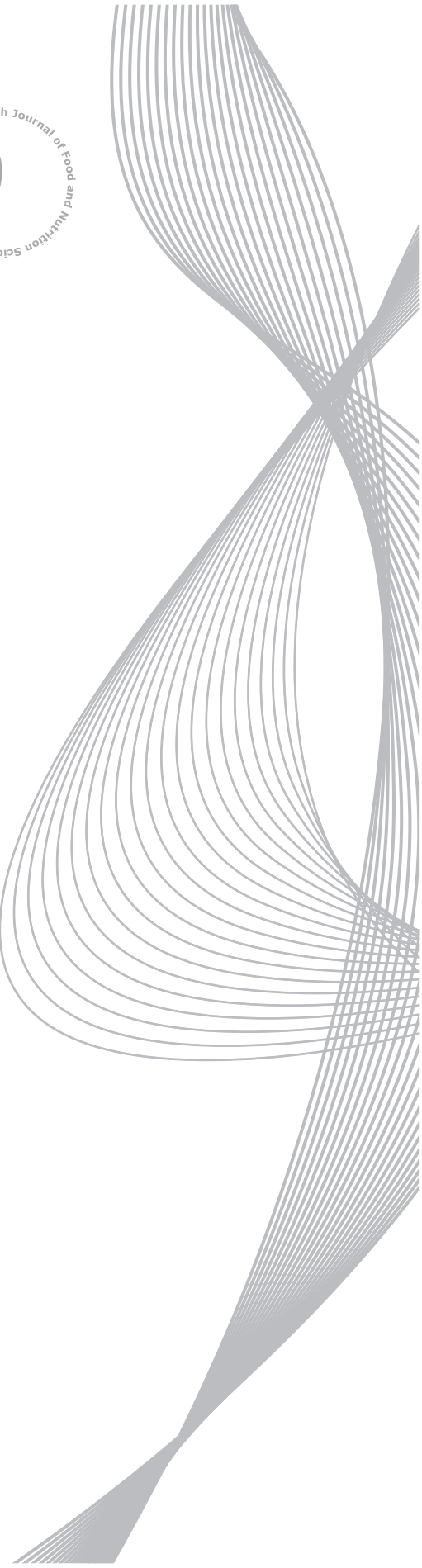
EDITORIAL AND BUSINESS CORRESPONDENCE: Submit contributions (see Instructions to Authors) and address all communications regarding subscriptions, changes of address, etc. to:

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)
2 0 2 1 , Vol. 71, No. 2

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. 2021, Vol. 71, No. 2

Advisory Board of PJFNS 2019–2022

Pol. J. Food Nutr. Sci. 2021, Vol. 71, No. 2

POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
covered by CURRENT CONTENTS/AGRICULTURE, BIOLOGY & ENVIRONMENTAL SCIENCES, JOURNAL CITATION REPORTS AND SCIENCE CITATION INDEX EXPANDED, SCOPUS, DOAJ, EBSCO, BIOSIS, IFIS Publishing and CAS abstracts, and AGRO-Librex, FROSTI, and AGRIS data bases
2021, VOL. 71, NO. 2

REVIEW

Crossroad of Tradition and Innovation – The Application of Lactic Acid Fermentation to Increase the Nutritional and Health-Promoting Potential of Plant-Based Food Products – a Review.
A.M. Ogrodowczyk, N. Drabińska 107

ORIGINAL PAPERS

Kinetic Modelling of Betalain Stability and Color Changes in Yogurt During Storage.
O. Guneser 135

Determination of Saponins in Leaves of Four Swiss Chard (*Beta vulgaris* L.) Cultivars by UHPLC-CAD/QTOF-MS/MS.
A. Mroczek, U. Klimczak, M. Kowalczyk 147

Development of New Gluten-Free Maize-Field Bean Bread Dough: Relationships Between Rheological Properties and Structure of Non-Gluten Proteins.
A. Fetouhi, A. Sujak, L. Bentallah, A. Nawrocka, M. Szymańska-Chargot, M. Tomczyńska-Mleko, A.Wójtowicz, M.N. Zidoune 161

Macro- and Micro-Nutrient Composition and Antioxidant Activity of Chickpea and Pea Accessions.
M. Costantini, C. Summo, M. Centrone, I. Rybicka, M. D'Agostino, P. Annicchiarico, F. Caponio, S. Pavan, G. Tamma, A. Pasqualone 177

Study of Interactions Between Individual Phenolics of Aronia with Barley β-Glucan.
L. Jakobek, P. Matić, J. Ištuk, A.R. Barron 187

Optimization of Distillation Conditions for Improved Recovery of Phthalides from Celery (*Apium graveolens* L.) Seeds.
A. Kokotkiewicz, A. Badura, Ż. Tabaczyńska, A. Lorenc, A. Buciński, M. Luczkiewicz 197

Effect of Composition and Processing Conditions on Selected Properties of Potato-Based Pellets and Microwave Expanded Snacks Supplemented with Fresh Beetroot Pulp.
K. Lisiecka, A. Wójtowicz, A. Sujak 211

Phenolic Composition, Antioxidant and Antineurodegenerative Potential of Methanolic Extracts of Fruit Peel and Flesh of Pear Varieties from Serbia.
A. Savić, A. Alimpić Aradski, J. Živković, K. Šavikin, S. Jarić, P.D. Marin, S. Duletić-Laušević 225

Instruction for Authors 237

Ministerstwo Nauki
i Szkolnictwa Wyższego

KOMUNIKAT

W latach 2019–2020 kwartalnik naukowy ***Polish Journal of Food and Nutrition Sciences*** realizował 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 były 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* accomplished 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 were 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

2021 – One volume, four issues per volume. Annual subscription rates are: Poland 150 PLN, all other countries 80 EUR. Prices are subject to exchange rate fluctuation. Subscription payments should be made by direct bank transfer to Bank Gospodarki Żywnościowej, Olsztyn, Poland, account No 17203000451110000000452110 SWIFT code: GOPZPLWOLA with corresponding banks preferably. Subscription and advertising offices at the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, ul. J. Tuwima 10, 10-747 Olsztyn, Poland, tel./fax (48 89) 5234670, fax (48 89) 5240124, e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

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

Wersja pierwotna (referencyjna) kwartalnika PJFNS: wersja papierowa (ISSN 1230–0322)
Nakład: 70 egz.; Ark. wyd. 17,7; Ark. druk. 17,7
Skład i druk: Mercurius, www.mercurius.com.pl

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 107–134
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/134282
http://journal.pan.olsztyn.pl

Review article
Section: Food Technology

Crossroad of Tradition and Innovation – The Application of Lactic Acid Fermentation to Increase the Nutritional and Health-Promoting Potential of Plant-Based Food Products – a Review

*Anna Maria Ogrodowczyk, Natalia Drabińska**

Division of Food Sciences, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10–747 Olsztyn, Poland

Key words: fermentation, lactic acid fermentation, lactic acid bacteria, plant food, fermented food

Lactic acid (LA) fermentation of plant-based products is a commonly used process all over the world. Recently, except for extending the shelf-life of food and improving its palatability, the popularity of fermented food increased because of their nutritional and health-promoting quality. In this review, the existing knowledge about the effects of LA fermentation in different types of plant-based food matrices on their nutritive and health-promoting potential is presented and discussed based on the most recent studies. Furthermore, the toxicological and unfavorable effects are addressed.

This review shows that although the biotransformation of several nutrients and bioactive compounds and the strain-dependent properties need more in-depth elucidation in several matrices, the recently published studies proved that LA-fermented cereals, fruits, vegetables, legumes, and seaweeds are good sources of health-promoting molecules. The obtained products offer a good alternative for a growing number of vegans, vegetarians, and flexitarians, who look for the plant-based, healthy alternatives. However, still more clinical trials evaluating the effect of their consumption on the human health are in demand.

LACTIC ACID FERMENTATION – OVERVIEW

Fermentation was a common method of food preservation since ancient times. The seasonal production of crops forced the processing of products to make food available throughout the year. Fermentation allows extending the shelf-life of the product, decreases its volume facilitating transportation, and destroys undesirable components. Now, except for extending the shelf-life of food and improving its palatability, the popularity of the fermented food increased because of their nutritional quality and health benefits. And although the role of microorganisms had not been discovered yet, the development of microbiological knowledge and the need for providing larger quantities of food led to understand the potential of fermentation. Recently, selected microbes have been isolated from fermented food matrices for the production of probiotics, considered beneficial for human health [Marco *et al*., 2017].

There is a wide range of materials, techniques and microorganisms used for fermentation. The fermentation can occur spontaneously or be triggered by the addition of specially selected starter cultures. During this metabolic process, carbohydrates are oxidized in the absence of an electron acceptor. There are only four main fermentation types: alcoholic (for wine and beer production, predominantly by yeast), acetic acid (for vinegar, ascorbic acid and cellulose production), alkaline (for the production of typical Asian and African products, such as dawadawa, ugba, bikalga, kinema, natto, and thuanao), and lactic acid (LA). In this review, the emphasis is put on the last-mentioned type. LA fermentation, as its name suggests, is carried out by lactic acid bacteria (LAB). LAB are Gram-positive, acid-tolerant, in general non-sporulating, catalase-negative bacteria which produce LA as the main product of their fermentation. For many years, LAB have been further divided into homofermentative and heterofermentative ones, which were used to impart the desired features of the fermented products. In the aforementioned classification, the homofermentative LAB include several genera, such as *Lactococcus, Pediococcus, Streptococcus*, and *Enterococcus*, which are able to convert glucose to LA with a more than 80% theoretical efficiency rate [Blajman *et al*., 2020]. In turn, the heterofermentative bacteria, including the following genera *Leuconostoc, Oenococcus*, and some of *Lactobacillus, e.g. Lactobacillus brevis* and *Lactobacillus fermentum*, except glucose produce significant quantities of ethanol, CO_2, and other acids [Moon *et al*., 2018]. Some LAB, like *Lactobacillus plantarum* and *Lactobacillus pentosus*, were classified as facultatively heterofermentative, depending on the environment [Zaunmüller *et al*., 2006].

* Corresponding Author:
E-mail: n.drabinska@pan.olsztyn.pl

Submitted: 11 February 2021
Accepted: 15 March 2021
Published on-line: 20 April 2021

Nevertheless, a new taxonomic cassification was introduced in 2020 under which 23 novel genera and a union of *Lactobacillaceae* and *Leuconostocaceae* were suggested [Zheng *et al*., 2020]. In this work, the new reclassification of the genus *Lactobacillus* was proposed based on the whole genome sequencing of *Lactobacillaceae* and *Leuconostocaceae.* The genus *Lactobacillus* was divided into 25 genera including the emended genus *Lactobacillus*, which includes host-adapted organisms that have been referred to as the *Lactobacillus delbrueckii* group, *Paralactobacillus*, and 23 novel genera named *Holzapfelia*, *Amylolactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, *Agrilactobacillus*, *Schleiferilactobacillus*, *Loigolactobacillus*, *Lacticaseibacillus*, *Latilactobacillus*, *Dellaglioa*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, *Secundilactobacillus*, and *Lentilactobacillus*. And despite the fact that in this review the taxonomic names of bacteria are as reffered by the original authors, we decided that the new classification is worth mentioning, because, for example, three groups: *Lactobacillus delbrueckii*, *Lactobacillus casei*, and *Lactobacillus salivarius*, now belong to the most diverse groups even though they had previously been included into the same homofermentative group of LAB.

Regardless of the used taxonomy, the ability of bacteria to produce lactic acid was superior from a technological point of view, and the diversity of the LAB during fermentation depends on the starter culture and can change during the fermentation process, affecting the properties of fermented foodstuff [Moon *et al*., 2018]. Due to a high enzymatic activity, LAB are able to decompose and detoxify many compounds present in various types of raw materials (*e.g.,* phenolic compounds, colourants, mycotoxins, acrylamide, pesticides) thanks to their high biogenic activity, they also produce many substances with various biological activities (*e.g.,* volatile fatty acids, exopolysaccharides, proteins). All these functions are briefly presented in Figure 1. Such a high biological activity of LAB and their adaptation abilities are, on the one hand, beneficial and, on the other hand, a risky phenomenon. LAB are able, *inter alia*, to receive plasmids carrying antibiotic resistance genes, they can also produce various types of proteins with activities that have not been fully described so far. In a hypersensitive organism, some of these proteins may be immunoreactive. Bacteria are also able to synthesize biogenic amines, the accumulation of which is a disadvantageous effect. The adverse effects of LAB activity are summarized in Figure 2. The current knowledge describing both types of phenomena will be described in detail in this paper.

Plant-based fermented food products are of special interest since they are proposed as a non-dairy alternative for a natural, probiotic administration [Martins *et al*., 2013]. The advantage of plant-based fermented foodstuff is that they are suitable for vegan and milk- and lactose-intolerant individuals as well as, beside the probiotic microorganisms, contain a wide variety of phytochemicals, minerals, and fibre [Gupta & Abu-Ghannam, 2012].

In this review, the existing knowledge about the effects of LA fermentation of different types of plant-based food matrices on their health-promoting potential and nutritional quality is presented and discussed based on the most recent studies. Moreover, the toxicological and unfavorable effects are addressed. Finally, the future studies needed to expand the potential of LA fermentation of plant-based food are underlined.

VEGETABLES AND FRUITS

Fruits and vegetables are sources of carbohydrates, dietary fibre, vitamins, minerals, and phytochemicals, including phenolics, carotenoids, betalains, glucosinolates and many

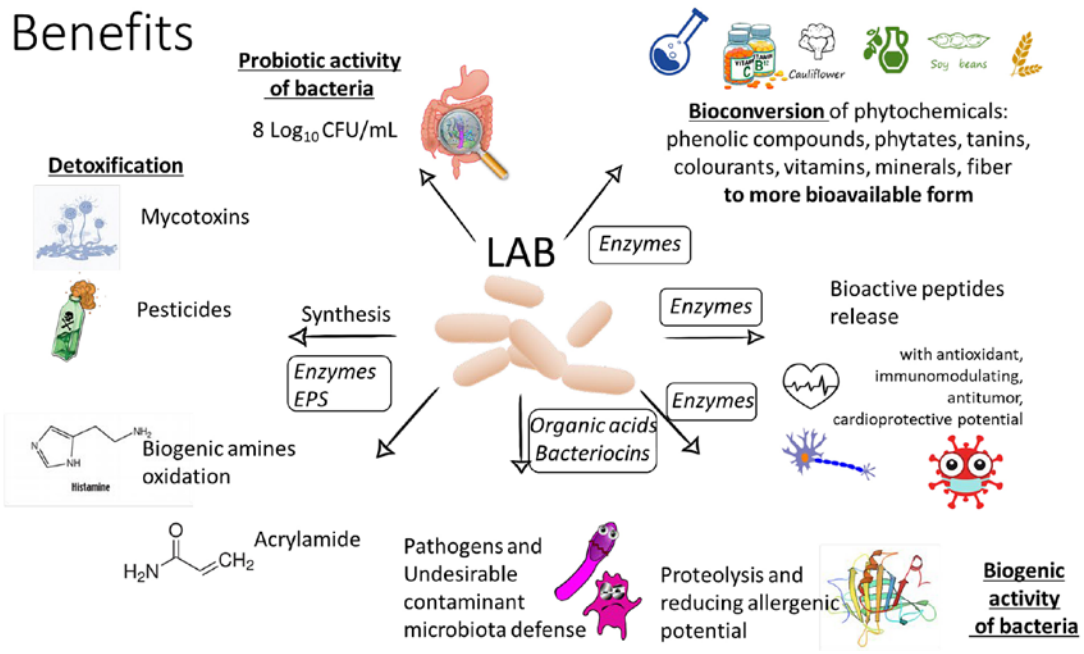


FIGURE 1. The summary of benefits caused by fermentation with lactic acid bacteria (LAB).

others; therefore, their consumption is highly recommended to maintain good health. To preserve the shelf-life of fruits and vegetables, fermentation was traditionally used worldwide, especially in Asia, Africa, and Eastern Europe. Examples of fermented vegetables include sauerkraut – fermented white cabbage in Eastern Europe, kimchi – Korean dish made of napa cabbage and Korean radish, gundruk – a dish made of fermented green leaves in Nepal, fermented cucumbers, beetroots, tomatoes, and many others including non-alcoholic beverages, summarized and technologically characterized in other reviews [Di Cagno *et al*., 2013; Garcia *et al*., 2020]. Fermented products can be manufactured using the LAB starter culture or *via* the spontaneous fermentation with natural microbiota, which is more frequent in traditional, homemade food products. The spontaneous LA fermentation of raw fruits and vegetables occurs under favourable conditions, such as anaerobiosis, and appropriate salt concentration, water activity, and temperature. LA fermentation results in the physicochemical changes of raw materials due to lower pH and microbial enzymatic activity. Therefore, fermented products have different nutritional characteristics and profile of bioactive compounds compared to fresh fruits and vegetables. Changes in the nutritional composition and the profile of phytochemicals are highly dependent on the matrix, the LAB strain, and the fermentation conditions.

Safety

Due to the contact with soil, and during the whole processing and transportation chain, fruits and vegetables can be contaminated with harmful viruses and faecal or coagulase-positive bacteria (Figure 2). Fortunately, these contaminations do not contribute to the foodborne outbreaks since the density of bacteria is too low and the microbial competition with positive microbiota occurs [Di Cagno *et al*., 2013]. However, in the highly contaminated areas and during inappropriate handling of fruit and vegetable outbreaks are likely. There have been many pathogens reported, however, the most frequent ones include *Salmonella* sp. in leafy vegetables and *Escherichia coli* in sprouted seeds and green vegetables [Da Silva Felício *et al*., 2015; Uyttendaele *et al*., 2015]. LA fermentation can be applied to reduce the risk of foodborne poisonings and health problems. Usually, the growth of Gram-negative bacteria is inhibited already at the beginning of fermentation [Di Cagno *et al*., 2013]. However, a recent study has shown that in the carrot juice spiked with foodborne pathogens, including *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* Typhimurium, and *Escherichia coli* O157:H7, the count of bacteria in the initial phase of fermentation increased [Van Beeck *et al*., 2020]. Fermentation for 8 days at 20°C resulted in the drop of the bacterial count below the detection limit, making the fermented juice safe to drink. Moreover, LAB-fermented products, like cider obtained from the fermentation of apple juice, have a high content of acids, resulting in pH decrease, which prevents the growth of patogenic bacteria [Guiné *et al*., 2021]. Contrary, the study with cauliflower subjected to the spontaneous fermentation and spiked with *Listeria monocytogenes* and *Salmonella typhimurium* showed that LAB were not able to compete with pathogenic bacteria present in the cauliflower until the end of fermentation [Paramithiotis *et al*., 2012]. This study underlines the need for safety reassessment of spontaneously fermented vegetables and suggests that the application of starter cultures can be a safer option. A recent study has demonstrated the potential of virulent phages infecting mesophilic aerobic bacteria producing nitrite to inhibit the growth of unwanted bacteria [Zheng *et al*., 2020]. The authors reported that the use of PhageMIX together with *Lactobacillus plantarum* M6 decreased the count of *Pseudomonas mendocina* and *Enterobacter cloacae* below the limit of detection after 48 h of fermentation in the cucumber juice

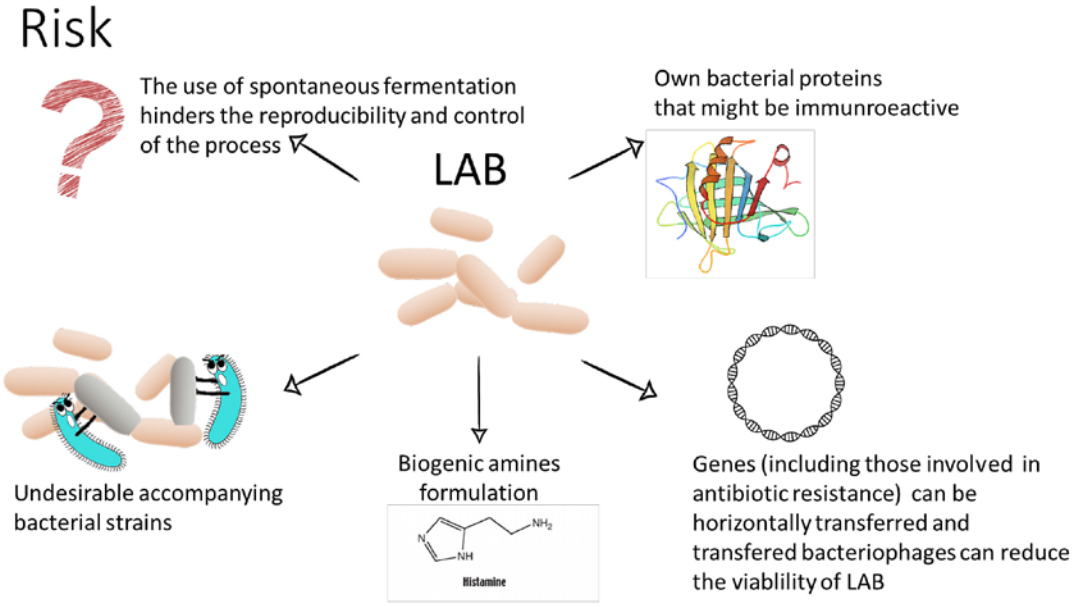


FIGURE 2. The summary of risks associated with fermentation by lactic acid bacteria (LAB).

artificially contaminated with mesophilic aerobic bacteria. This study suggests that the application of phages can not only improve safety but also reduce the spoilage and losses of fermented food products.

Another risk posed by fermented vegetable consumption is the formation of biogenic amines (BAs), which can be toxic to humans in high concentration. BAs are formed by the decarboxylation of free amino acids by LAB, with the quantities dependent on the bacteria strain [Garai *et al*., 2007]. Again, the most severe risk is in the homemade products fermented spontaneously, when the unpredictable concentration of biogenic amines can be produced. The study of Alan *et al.* [2018] showed that putrescine, cadaverine, and histamine were detected in almost all analysed samples of naturally fermented pickled cucumbers with a very wide range of concentrations varying between the samples. Histamine, tyramine, and putrescine are the major BAs in commercially available ciders; however, also high concentrations of cadaverine can be detected as well [Ladero *et al*., 2011]. The study using qPCR for the quantitative detection of BA producers showed that many bacteria belonging to LAB are responsible for the high BA content [Ladero *et al*., 2011].

Carbohydrates and organic acids

LAB fermentation and acidification cause changes in the profiles of carbohydrates and organic acids. In the apple, orange, and grape juices which were processed with freeze-dried *Lactobacillus plantarum* 49, *Lactobacillus brevis* 59, *Lactobacillus paracasei* 108, *Lactobacillus fermentum* 111, and *Lactobacillus pentosus* 129 strains, the contents of organic acids and sugars varied during storage, depending on the strain added and juice type [Garcia *et al*., 2018]. *Lactobacillus paracasei* and *Lactobacillus plantarum* were able to degrade glucose, malic, tartaric, and citric acids when these acids were available in the matrix, leading to an increase in the content of LA and succinic acid. A recent study has shown that fermentation of elderberry juice with twelve LAB strains resulted in the high LA production [Cirlini *et al*., 2020]. Glucose and fructose contents did not change during LA fermentation, while malic and citric acids were extensively metabolised, suggesting a switch in the microbial metabolism with the use of organic acids instead of sugars. The fermentation of chokeberry with LAB led to a significant reduction in the total sugar and fructose contents, while in sea buckthorn the content of sugars was not affected, which resulted in a change in the sugar/acid ratio between the fruits [Markkinen *et al*., 2019]. This suggested that the effect of the fermentation on sugar profile was dependent on the matrix used for fermentation.

Glucosinolates

Cruciferous vegetables are commonly fermented in many countries with popular products like sauerkraut, kimchi, fermented cauliflowers, *etc*. These vegetables, apart from the unique flavour and taste are a source of the special group of bioactive compounds – glucosinolates. Glucosinolates and their breakdown products are considered as chemopreventive, reducing the risk of cancer development [Quirante-Moya *et al*., 2020]. The enzymatic hydrolysis of glucosinolates occurs during the damage of plant tissues (cutting, shredding, chewing), but also through microbial activity. During cabbage fermentation, complete degradation of glucosinolates was observed in the majority of studies [Martinez-Villaluenga *et al*., 2009; Palani *et al*., 2016]. Surprisingly, a recent study has shown that the LAB-induced fermentation increased glucosinolate content in the autoclaved broccoli puree [Ye *et al*., 2019]. However, the authors explained it by improved extractability of glucosinolates from cell walls due to the enzymatic activity of LAB, especially that the content of glucosinolates in the raw material was very low. Glucosinolates are usually hydrolysed to isothiocyanates and nitriles, which are directly responsible for the bioactive potential. Fermentation leads to the formation of isothiocyanates and nitriles; however, the direction of hydrolysis is strain-dependent. Mullaney *et al.* [2013] showed that LAB hydrolysed glucosinolates mainly to nitriles, while *Enterobacteriaceae* hydrolysed them mostly to isothiocyanates. The opposite effect was reported by Cai *et al*. [2019], who observed an increase in sulforaphane, an isothiocyanate derived from glucoraphanine, in LAB-fermented broccoli puree. Sulforaphane, which is considered as one of the strongest anticancer isothiocyanates [Soundararajan & Kim, 2018], was found stable in fermented broccoli puree even up to four months of storage at 4°C [Cai *et al*., 2019]. The differences in the results obtained by various authors can be related to the stage of fermentation. The profile of glucosinolate breakdown products depends on fermentation conditions and the profile of glucosinolates in the raw material. In sauerkraut, the major breakdown products were ascrobigen and allyl isothiocyanate derived from glucobrassicin and sinigrin, respectively, *i.e.* two main glucosinolates in cabbage [Ciska *et al*., 2009; Palani *et al*., 2016], while in broccoli products, the major breakdown product was sulforaphane derived from the dominant glucoraphanine [Cai *et al*., 2019]. Nevertheless, it needs to be emphasised that glucosinolates and their breakdown products easily leach into water [Ciska *et al*., 2016]; therefore, the brine from fermentation can also be used as a source of bioactive compounds, with the confirmed health-promoting activity [Hallmann *et al*., 2017].

Carotenoids

Another important group of compounds are colourants, widely distributed in fruits and vegetables. Carotenoids are tetraterpenoids, imparting plants yellow, orange, and red colour. Besides being pigments, some of them are precursors of vitamins (β-carotene, the precursor of vitamin A) and they are strong antioxidants, thus confer several health benefits [Meléndez-Martínez, 2019]. LA fermentation can affect their content in plants. In a study of Oloo *et al.* [2014], different varieties of orange-fleshed sweet potatoes were fermented with *Lactobacillus plantarum* MTCC to measure the retention of β-carotene. Its initial content differed between the potato varieties from 0.031 to 17.02 mg/100 g. However, irrespectively of its initial content, the retention of β-carotene reached up to 93.97%, indicating LA fermentation to prove well in carotein preservation [Oloo *et al*., 2014]. In another study, LA fermentation was applied as a preservation method for a tomato puree, and the fate of lycopene and β-carotene was evaluated [Bartkiene *et al*., 2013]. Tomato pulp was fermented

with LAB, including *Lactobacillus sakei* KTU05–6, *Pediococcus acidilactici* KTU05–7, and *Pediococcus pentosaceus* KTU05–8, capable of producing bacteriocins. LA fermentation with *Pediococcus pentosaceus* and *Lactobacillus sakei* resulted in an apparent increase in lycopene content in some of the tomato varieties, causing a higher *cis/trans* lycopene ratio in fermented tomato, thereby increasing the bioavailability of these compounds in the human body [Unlu *et al*., 2007]. However, although the content of the carotenoids increased after fermentation, it does not mean that their degradation did not occur. The increase in carotenoid content was supposedly due to the enzymatic degradation of cell walls and better extractability, and not to *de novo* synthesis of carotenoids [Unlu *et al*., 2007]. From 15 to 45% degradation of α-carotene and β-carotene was noted in the carrot juice fermented for 24 h with *Bifidobacterium* strains (*Bifidobacterium lactis* Bb-12, *Bifidobacterium bifidum* B7.1 and B3.2) [Kun *et al*., 2008]. The degradation of carotenoids was also indirectly presented in the work of Lee *et al.* [2018], who analysed volatile organic compounds in red pepper fermented with *Lactobacillus parabuchneri*. These authors reported an increase in the contents of some degradation compounds derived from carotenoids, including β-ionone, β-cyclocitral, α-ionone, and β-damascenone after LA fermentation. β-Damascenone, a potential breakdown product of neoxanthin, was not detected in the raw red pepper and its content increased successively during fermentation, suggesting that the degradation of carotenoids did occur. While in a recent study, 120 h of acerola and guava fruit by-product fermentation by strains of *Lactobacillus* sp. had no effect on the total carotenoid content [de Oliveira *et al*., 2020]. In general, the impact of LA fermentation on the content of carotenoids seems to depend on the chemical structure, fermented matrix, and fermentation conditions.

Betalains

Another group of plant pigments is represented by betalains, water-soluble nitrogen-containing compounds, which can be further divided into red-violet betacyanins and yellow-orange betaxanthins. Betalains are not widely present in the plant world and are detected mainly in plants of the order *Caryophyllales*. Their main source among vegetables is a beetroot. Czyżowska *et al.* [2006] evaluated the effect of LA fermentation with six LAB strains on the stability of betalains in beetroot juice made of two beetroot varieties. They reported the presence of five betalains including four betacyanins: betanin, isobetanin, betanidin, isobetanidin, and neobetanin; and one betaxantin, *i.e.,* vulgaxanthin I. Interestingly, betanidin and isopetanidin were detected only in the fermented products. Two beetroot varieties and juices made of them differed in the betalain profiles. The Chrobry variety had a higher content of betanidin than betalain, while in Czerwona Kula variety, this ratio was reversed [Czyżowska *et al*., 2006]. The stability of the betalains in the fermented beetroot juice was evaluated in another study of these authors [Klewicka & Czyżowska, 2011]. Beetroot juice was fermented by *Lactobacillus brevis* 0944 and *Lactobacillus paracasei* 0920, stored for 180 days at 4°C, and determined for betalain content. Initially, betalains were relatively stable and after 7 days of LA fermentation, their content decreased by 12% and remained at the same level up to 30 days of fermentation. Drastic drops were observed after 90 and 180 days of fermentation, reaching only 32 and 25% of the initial betalain content. Similarly, the storage of LA-fermented, grated beetroot for seven months resulted in about three-fold decrease in betalain content [Czyżowska *et al*., 2020]. The content of betalains seems to depend on the form of product made from a vegetable. In a recent study, fresh, dried, and freeze-dried beetroot products sprayed with LAB were compared in terms of betalain content [Barbu *et al*., 2020]. Freeze-dried beetroot had seven-times more betalains than the control, unprocessed beetroot and other fermented beetroot products. Moreover, the ratio between betacyanin and betaxanthin differed between the food products, with betaxanthins prevailing in freeze-dried beetroot, betacyanins in fresh, control beetroot, and the equal ratio between both betalains in fresh LAB-processed beetroot and dried product [Barbu *et al*., 2020]. Importantly, fermented beetroot has a lower content of betanin compared to the fresh, boiled, jam-processed, and juice beetroot [Guldiken *et al*., 2016]. The level of isobetanin in the pickled beetroot was also lower than in the majority of the analysed beetroot products.

Next to beetroot, betalains can be found in other plants, which are however rarely subjected to LAB fermentation. The latest study has shown the effect of LA fermentation of *Opuntia ficus-indica* (prickly pear) juice by *Weissella cibaria* and *Pediococcus pentosaceus* on the content of bioactive compounds [Allendez *et al*., 2020]. The authors reported that the total phenolic and betalain contents were preserved after LA fermentation, thus exhibiting antioxidant potential. The study with fresh fruits of prickly pear fermented by *Leuconostoc mesenteroides* showed that the content of betalains was rather stable [Di Cagno *et al*., 2016]. After 21 days of fermentation, the content of betaxantins decreased slightly and the content of betacyanins was higher than in the control, unprocessed fruit. However, the differences in the betalain content were noticeable between the fruits fermented with different LAB strains. The highest content of both betaxantines and betacyanines was noted in prickly pear fermented with *Leuconostoc mesenteroides* OP23, while the lowest value was noted for that fermented with *Leuconostoc mesenteroides* OP4 [Di Cagno *et al*., 2016].

Phenolic compounds

Phenolic compounds are one of the most abundant classes of plant secondary metabolites, widely distributed in fruits and vegetables. They are associated with a high antioxidant activity, therefore are implicated in lowering the risk of diseases caused by oxidative stress, like cancer, cardiovascular and neurodegenerative disorders [Vauzour *et al*., 2010]. The effect of LA fermentation on the content of phenolic compounds and antioxidant capacity in fruits and vegetables has been extensively investigated. In general, with a very broad range of studies on the antioxidant activity and polyphenol content in LAB-fermented fruits and vegetables, no predictable trend of changes could be obtained. The discrepancies are associated with analysing different plants using different starter cultures *versus* spontaneous fermentation, different

conditions of the process, and also different assays used for the antioxidant capacity measurement. The only conclusion repeated in many works is that the controlled fermentation with fully-defined starter cultures is more beneficial than the spontaneous fermentation [Pistarino *et al*., 2013; Yang *et al*., 2014].

The content of phenolics and antioxidant capacity were determined in LA-fermented fruits including orange, pomegranate, cherry, mulberry, elderberry, cherimoya, kiwi, and many others, mainly in the form of juice [Chen *et al*., 2019; de la Fuente *et al*., 2021; Filannino *et al*., 2013, 2015; Isas *et al*., 2020; Kwaw *et al*., 2018; Ricci *et al*., 2019; Zhou *et al*., 2020]. The study of Filannino *et al.* [2013] evaluated the effect of LA fermentation on the organoleptic and nutraceutical potential of pomegranate juice. The total content of phenolics decreased during fermentation as compared to the baseline, however, remained stable and higher than in the pomegranate juice which was fermented without the starter culture. Differences were also observed between the effects of the individual LAB strains on the total phenolic content. The highest phenolic content was determined in juice fermented with *Lactobacillus plantarum* C2. A similar trend was observed for the antioxidant capacity determined by an assay using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$). The content of ellagic acid increased significantly in all LAB-fermented juices, however again, the highest content was determined in the juices fermented with *Lactobacillus plantarum* C2 [Filannino *et al*., 2013]. An attempt to understand the metabolism of protocatechuic, caffeic, and *p*-coumaric acids by phenolic acid decarboxylases and reductases of *Lactobacillus* strains in a strain-specific manner was undertaken in cherry juice and broccoli puree [Filannino *et al*., 2015]. The majority of *Lactobacillus* strains exhibited the ability to decarboxylate protocatechuic acid to catechol, while strains of *Lactobacillus plantarum* and *Lactobacillus spicheri* Lp38 decarboxylated caffeic acid to vinyl catechol. However, other strain of LAB metabolised the same acid in a different way. *Lactobacillus fermentum* FUA3165 reduced caffeic acid to dihydrocaffeic acid, while *p*-coumaric acid was metabolised to *p*-vinylphenol and phloretic acid by the majority of *Lactobacillus plantarum* and *Lactobacillus fermentum* strains [Filannino *et al*., 2015]. In another study, *Lactobacillus plantarum* was used to ferment kiwifruit pulp, and the changes in the antioxidant potential and the profile of phenolic compounds were detected [Zhou *et al*., 2020]. The fermentation led to an increase in the content of total phenolics, flavonoids as well as 6,7-dihydroxycoumarin, *p*-coumaric acid, and protocatechuic acid in kiwi product. The major phenolic acids were protocatechuic and chlorogenic acids, while gallic acid, chlorogenic acid, (–)-epicatechin, and (+)-catechin were degraded during LA fermentation. The increase in the polyphenolic compounds enhanced the antioxidant capacity measured using DPPH and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) assays [Zhou *et al*., 2020]. An innovative beverage based on orange juice and milk fermented with *Lactobacillus brevis* POM and *Lactobacillus plantarum* (TR-7, TR-71, TR-14) was proposed by de la Fuente *et al.* [2021]. All the strains used led to an increase in the antioxidant capacity; however, the effect on the content of phenolic compounds was more strain-dependent. The greatest amount of phenolic compounds was determined in the beverage fermented with *Lactobacillus plantarum* TR-71 and TR-14 for 72 h. Moreover, the production of compounds participating in food preservation, such as DL-3-phenyllactic acid and 3,4-dihydroxyhydrocinnamic acid, was detected in the LAB-fermented beverage [de la Fuente *et al*., 2021].

Moreover, a wide variety of vegetables has been fermented and the fate of phenolics and antioxidant capacity caused by these compounds in these products was evaluated. Spontaneously fermented asparagus was reported to have a ferric ion reducing antioxidant power (FRAP); however, LA fermentation did not affect DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities as well as total phenolic and flavonoid contents [Tabaszewska *et al*., 2018]. On the other hand, fermentation caused the reduction in contents of individual phenolic compounds, total sugars, and B vitamins. Broccoli purre fermented with seven strains of broccoli-derived LAB was used to track the biotransformation of polyphenolic compounds [Ye *et al*., 2019]. The authors reported the presence of ten phenolic compounds, which total content increased significantly after LA fermentation. In the broccoli purre fermented with *Lactobacillus plantarum*, the highest increase in phloretic acid was determined compared to that fermented with other LAB strains. Therefore, the authors suggested that LA fermentation could be a promising tool to increase the health-promoting potential of vegetables. Another factor that can affect bioactive compounds might be the conditions of storage. A study of Kapusta-Duch *et al.* [2017] compared the effect of four-month sauerkraut storage in low-density polyethylene bags (PE-LD) and metalised polyethylene terephthalate foil (PET met/PE) on the content of ascorbic acid, total phenolic content, and antioxidant activity. The ascorbic acid and total phenolic contents decreased during four-month storage; however, there was no difference between container types. Nevertheless, the type of container affected the antioxidant capacity of sauerkraut. The product kept in PET met/PE bag had a higher antioxidant capacity than the cabbage kept in PE-LD bag and the non-stored sauerkraut.

A special group of phenolics are anthocyanins, responsible, similarly to betalain, for the red-purple colour of fruits and vegetables. Red cabbage was found to be a source of twenty different non-acylated and acylated anthocyanins with the dominant cyanidin 3-diglucoside 5-glucoside [Wiczkowski *et al*., 2015]. The spontaneously fermented red cabbage was found to have a lower by 24% content of anthocyanins than the fresh cabbage; however, the loss of these compounds was lower than in stewed cabbage. Interestingly, fermentation reduced the bioavailability of anthocyanins from red cabbage, thus increasing the intestinal capacity measured in the plasma of subjects consuming a fermented product [Wiczkowski *et al*., 2016]. Shalgam, a traditional Turkish LA-fermented beverage made of black carrot, was found to be a source of bioactive compounds, including anthocyanins responsible for the colour of the drink [Toktaş *et al*., 2018]. During the initial stage of fermentation, the content of bioactive compounds was lower than in the raw red carrot and the final shalgam. Interestingly, even though sixteen polyphenolic compounds were detected in the beverage, only five phenolics were detected in bioavailability test [Toktaş *et al*., 2018].

Pro- and prebiotic activity

An important feature of fermented fruits and vegetables is their effect on the human gut. Fermented plant-based products can be a substitute to dairy probiotics, satisfying needs of vegans and individuals suffering from allergies. The effect of fermentation conditions on the survival of the probiotics in pomegranate juice has recently been reported [Mustafa *et al*., 2019]. The authors found that fermentation of *Lactobacillus casei* at 37°C without agitation resulted in the highest biomass density. Another alternative to dairy probiotics can be modified kombucha, which originally is a fermented tea beverage, prepared by fermenting sweetened black tea with tea fungus, yeasts, and acetic acid bacteria. A study of Cvetković *et al.* [2019] showed the potential of wild strains of LAB in kombucha preparation, without affecting the activity of tea fungus, thus forming grounds for the development of a new, probiotic beverage. Furthermore, fermented fruits can also be a source of prebiotics. The prebiotic activity of polysaccharides from LAB-fermented longan pulp was evaluated in a study of Huang *et al.* [2019]. These authors reported that logan pulp fermented for 12 h exhibited a stronger stimulatory effect on *Lactobacillus* strain proliferation, proving the prebiotic activity of the LAB-fermented product.

Fermented products have been reported to influence health parameters evaluated in animal and humans studies. Cactus pear juice fermented with autochthonous *Lactobacillus plantarum* S-811 was found to decrease body weight and to normalise insulin resistance, hyperglycemia, and hyperlipemia parameters in obese mice [Verón *et al*., 2019]. Moreover, the intake of asparagus polysaccharides fermented with *Lactobacillus plantarum* NCU116 was effective in alleviating cyclophosphamide-induced hepatotoxicity in mice [Zhang *et al*., 2020]. The fermented product reduced the level of hepatic biochemical markers (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactic dehydrogenase), and proinflammatory cytokines (tumour necrosis factor-α and interleukin-1β). Moreover, the fermented asparagus polysaccharides affected the metabolism of bile acids and short-chain fatty acids as well as glutathione homeostasis [Zhang *et al*., 2020]. A very interesting study was reported by Zhou *et al.* [2019]. They performed *in vitro* assays proving that persimmon juice fermented with co-culture of *Lactobacillus plantarum* C17 and *Lactobacillus pentosus* Lp-B exhibited antihangover and antihypertensive activity. The selected strains of bacteria were capable of synthesising γ-aminobutyric acid (GABA), which was suggested to be responsible for antihangover capacity evaluated by alcohol dehydrogenase activation rate, acetaldehyde dehydrogenase activity, and hydroxyl inhibition rate. All these studies showed that the consumption of fermented fruits, vegetables, and beverages can confer a very broad range of beneficial effects on human health.

CEREALS AND PSEUDOCEREALS

The staple human food all over the world consists of carbohydrate-rich products. It is estimated that approx. 60% of the calorie intake is derived from the three main kinds of cereals: wheat, maize, and rice [Hermann, 2009]. And despite the great importance of the cereal-based products, their nutritional quality is not always sufficient, since they have a lower protein content than other food matrices, like meat and dairy, are deficient in essential amino acids, like lysine, and can contain undesired compounds, such as amylase/trypsin inhibitors (ATIs), phytates, and others summarised in a recent, excellent review [Samtiya *et al*., 2020]. Moreover, the palatability of unprocessed cereals can be unsatisfactory. Fermentation of the cereals results in the reduction of the content of carbohydrates and non-digestible poly- and oligosaccharides [Albiac *et al*., 2020]. Moreover, the deficiency of amino acids can be alleviated through their synthesis by bacteria responsible for fermentation [Omoba & Isah, 2018]. The application of LA fermentation in the processing of starch-containing materials enables the formulation of several products, such as bread, noodles, pastries, confectionery, and beverages with typical sour-sweet taste and butterish aroma, thus reducing the need for flavourings and additives. Importantly, products obtained by fermentation with LAB confer additional health benefits related to the probiotic characteristics of many LAB alone [Bartkiene *et al*., 2019] and their ability to synthesise vitamins, amino acids, and short-chain fatty acids, considered as postbiotics.

LAB are commonly used as a starter culture for sourdough in breadmaking [Yagmur *et al*., 2016]. It is estimated that there are about 50 different species of LAB being a part of the sourdough microbiota. They differ around the world, but the most common ones include *Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Micrococcus*, and *Bacillus*, regardless of the flour type [Minervini *et al*., 2014]. LAB in the sourdough originate mainly from the flour used for breadmaking, but also from other ingredients and the production environment. The bread made with the sourdough features a better quality as compared to the bread processed only with Baker's yeast, which is more often used in the food industry for breadmaking. Sourdough-based bread has longer shelf-life and more pleasant sensory attributes. And, despite the large scale production of bread which should be quick and efficient, the consumer demand for more nutritious, natural, and healthy food forced the baking industry to go back to the traditions and to produce the sourdough-based products. Only in Italy, there are recognised more than 200 sourdough types, of which many are unique enough to be considered as protected geographical indication (PGI) or protected designation of origin (PDO) [Palla *et al*., 2017].

Safety

LAB not only modify the flavour and taste of the bread but also improve its safety. In the course of fermentation, the presence of undesired components, such as ATI, can be reduced, mitigating the immune response to the grain-based products [Huang *et al*., 2020]. The phytates can be significantly reduced after fermentation [Kheterpaul & Chauhan, 1991; Liang *et al*., 2008], improving the availability of minerals, including zinc, iron, and calcium. Furthermore, fermentation was found to be effective in detoxifying cereals, reducing the aflatoxin level in the maize-based product [Wacoo *et al*., 2019].

Bread may also contain acrylamide, a harmful and potential cancerogenic compound formed during the heat treatment *via* the Maillard reaction. Therefore, there have been attempts

to reduce its level in food. Nachi *et al.* [2018] showed that wheat bread obtained from sourdough inoculated with *Lactobacillus brevis, Lactobacillus plantarum, Pediococcus pentoseus*, and *Pediococcus acidilactici* had a lower acrylamide content than the bread prepared only with yeast. Similar findings were later confirmed in a study where the rye-wheat bread with LAB sourdough was compared to the bread acidified with LA, and the authors found that *Lactobacillus plantarum* sourdough decreased the acrylamide content in the bread, while the application of the acid itself did not show any effect on the bread quality [Bartkiene *et al*., 2017]. The acrylamide-reducing activity is suggested to be strain-dependent, and not every LAB can have a similar effect. In the study comparing the effectiveness of sourdough with four strains of *Lactobacillus, Lactobacillus rhamnosus* was found to be the most efficient in decreasing the acrylamide content in whole-wheat bread [Esfahani *et al*., 2017].

Another benefit of the LAB sourdough affecting the safety of cereal-based products is its ability to reduce toxins. Malting grains are often contaminated with *Fusarium* sp. and their mycotoxins [Habler *et al*., 2016]. The application of several LAB strains, such as *Lactobacillus sakei* KTU05–6, *Pediococcus acidilactici* KTU05–7, and *Pediococcus pentosaceus* KTU05–8, KTU05–09, and KTU05–10 decreased the content of deoxynivalenol, zearalenone, and toxins T-2 and HT-2 even by 70% in malting grains. The applied strains, not only reduced the content of the toxins in grains, but also inhibited *Fusarium* sp. growth [Juodeikiene *et al*., 2018]. The effectiveness of LA fermentation in reducing *Fusarium* mycotoxin content was also confirmed in a study with whole grain sorghum [Adebo *et al*., 2019]. These authors found that strains of *Lactobacillus*, especially *Lactobacillus fermentum* FUA 3321, were able to reduce the total content of mycotoxins even by 98%. The identification of strains effective in removing mycotoxins during sourdough fermentation is an important concern of the food industry. A recent study has shown that Lithuanian whole milled and fractionated wheat grains from 2017 and 2018 harvest were highly contaminated with mycotoxins [Zadeike *et al*., 2021]. The bran and coarse flour had a high content of several mycotoxins, which has to be considered while recommending the whole-grain products. The 48-h fermentation of wheat fractions with LAB led to a significant reduction in the content of mycotoxins; however, the rate of reduction was dependent on the fraction and mycotoxin type. Only enniatin B_1 was fully removed from the whole meal samples, while other mycotoxins were still detected [Zadeike *et al*., 2021].

Phenolic compounds

LA fermentation can also be beneficial to bioactive compounds. Starch-based crops are rich in different phytochemicals, including phenolic compounds. LA fermentation can affect their composition in the product; however, the extent and the direction of the changes depend on grain types, LAB species, and fermentation conditions (pH, temperature, and duration). Single-strain solid-state LA fermentation with *Lactobacillus rhamnosus* resulted in a lower bound phenolic content in fermented wheat bran compared to that in fermented autoclaved bran [Spaggiari *et al*., 2020]. In contrast, an increase in total free phenolic content was observed when the fermentation was conducted for 24 or 48 h, suggesting that *Lactobacillus rhamnosus* was able to metabolise the conjugated phenolic compounds. This phenomenon led to an increase in the antioxidant capacity in the wheat bran assessed using DPPH, FRAP, and ABTS assays. Similarly, an increase in the antioxidant capacity and total phenolic content was observed in maize mashes fermented with a Fresco DVS 1010 culture (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus*) [Matejčeková *et al*., 2019]. Interestingly, the effect of LAB on the profile of phenolic compounds is dependent on the plant matrix. A recent study of Garzón *et al.* [2020] evaluating the influence of LA fermentation on phenolics in red and white sorghum fermented products, has shown differences in phenolic acid profiles. In the fermented product made of red sorghum, an increase of *p*-coumaric acid content was observed, while the contents of caffeic, ferulic, and sinapic acid decreased. In turn, in the white sorghum-based product, the contents of free *p*-coumaric and bound sinapic acids increased [Garzón *et al*., 2020]. The authors explained changes in the phenolic acids by the activity of LAB enzymes to hydrolyse the complex forms of phenolics, increasing free forms of phenolic acids. The changes in the contents of phenolic acids are related to the cellular energy balance of LAB, which can use hydroxycinnamic acids as external acceptors of electrons [Filannino *et al*., 2014]. The metabolism of phenolic compounds by LAB is mediated by several enzymes, including decarboxylases, reductases, glycosidases, and esterases [Esteban-Torres *et al*., 2015; Santamaría *et al*., 2018]. The composition of low-molecular weight phenolic compounds in the fermented sorghum-based porridge, typical in Africa and called ting, was found to additionally depend on the tannin content [Adebo *et al*., 2018]. Fermented high tannin-sorghum had a higher content of (+)-catechin, gallic acid, and quercetin than the ting made of low tannin-sorghum. Interestingly, the single strain fermentation was more beneficial in terms of bioactive compounds than the co-culture of *Lactobacillus fermentum* strains, and the strain FUA 3321 yielded better properties of ting [Adebo *et al*., 2018].

LAB-fermented flours and their effect on bioactive compounds in baked products have recently gained much scientific attention. A study comparing spontaneous fermentation of quinoa flour and flour fermented with *Lactobacillus plantarum* ATCC 8014 showed that the controlled LA fermentation resulted in a higher content of flavonoids, total phenolics, organic acids, and folic acid in muffins [Chiş *et al*., 2020]. The higher content of phenolics resulted also in a higher antioxidant capacity of the finished product, which can be used to improve the quality of the gluten-free products, known for their poorer health-beneficial properties [Conte *et al*., 2019]. A recent study with biscuits made of buckwheat flour fermented individually with 14 LAB strains has shown that changes in the total phenolic content in flours were highly dependent on LAB strain [Zieliński *et al*., 2020]. The highest total phenolic content was determined in the flour fermented with *Lactobacillus plantarum* IB and in the water biscuits made of this flour. LAB fermentation contributed to the increased contents of *p*-coumaric, sinapic, protocatechuic, and caffeic acids,

and to decreased contents of vanillic acid in flours. However, the thermal processing during biscuit production decreased the content of the majority of phenolic acids, except the vanillic and protocatechuic ones. The changes in the contents of phenolic compounds, especially *p*-coumaric, sinapic, syringic, vanillic, and protocatechuic acids as well as kaempferol, quercetin, apigenin, and orientin contributed to a decrease in the angiotensin-converting enzyme (ACE) inhibitory activity of the flour, which was much higher in the digested biscuits [Zieliński *et al*., 2020]. Interestingly, the biscuits with the highest content of phenolics were characterised by the highest hardness [Wronkowska *et al*., 2018]. The same research group showed that water-biscuits made of buckwheat flour fermented with LAB inhibited formation of advanced glycation end-products (AGEs), which was highly strain-dependent [Zieliński *et al*., 2020]. Biscuits made of flours fermented with *Lactobacillus plantarum* W42, *Lactobacillus casei* 2K, and *Lactobacillus rhamnosus* GG had the highest anti-AGEs activity, significantly higher than the control, non-fermented biscuits. However, biscuits made of flours fermented with *Lactobacillus delbrucki* subsp. *bulgaricus* 151 and *Streptococcus thermophilus* MK-10 had a much lower anti-AGEs potential even compared to the control biscuits, confirming again that the effect of LA fermentation on the health properties is highly strain-dependent.

The processing of the cereals and pseudocereals results in the generation of the vast amount of by-products. The by-products of wheat, maize, and rice are rich sources of nutrients and bioactive compounds; therefore, attempts have been made to utilise them as innovative functional foods. One of the methods which can be used to process grain wastes can be LA fermentation. There is a recent comprehensive review summarising the latest attempts of exploiting the potential of the cereal industry by-products [Verni *et al*., 2019], therefore this aspect will not be described in this review article.

Probiotic activity

LA-fermented cereal-based products are of great importance from the gut health point of view. Many LAB are considered as probiotics, which are live organisms providing a health benefit to the host when ingested in adequate quantities [Hill *et al*., 2014]. An important part of this definition is "adequate quantities"; therefore, despite containing LAB classified as probiotics, many fermented products, cannot be considered as probiotic. A value of 6 $\log_{10}$ CFU/mL based on a daily dose of 100 mL, required for prebiotics, was obtained in LAB-fermented cereal-based beverages made of the mixture of oat, barley, and malt [Salmerón *et al*., 2015]. After 10 h of incubation, the count of individual LAB strains was around 8 $\log_{10}$ CFU/mL in all analysed beverages. However, the applied bacterial strains affected the sensory properties of the beverages, and the product fermented with *Lactobacillus plantarum* NCIMB 8826 was the most acceptable, which was related to the highest content of acetaldehyde [Salmerón *et al*., 2015]. The type of cereal significantly affects bacterial growth. Cell count of approx. 8 $\log_{10}$ CFU/mL could be reached within 6 h of fermentation of malt, which may be attributed to the presence of considerable amounts of monosaccharides (glucose and fructose) and disaccharides (maltose and sucrose) in the malt medium [Rathore *et al*., 2012]. The type of bacteria has to be considered while designing a probiotic product. Since there are LAB strains which are homofermentative, like *Lactobacillus acidophilus*, the main product of their metabolism will be LA formed during the glycolysis of carbohydrates in the Embden-Meyerhof pathway. On the other hand, facultatively heterofermentative bacteria, like *Lactobacillus plantarum*, produce other acids, like acetic acid, beside the LA in a pentose phosphate pathway. These differences in the LAB metabolism can affect consumer perception of LAB-fermented products, making some of them unacceptable to consumers. In a recent study, strains of *Pediococcus* spp. isolated from Iranian traditional fermented cereal-dairy product called Tarkhineh showed health beneficial properties [Vasiee *et al*., 2020]. Three isolated strains were successfully tested against several pathogenic bacteria, like *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PTCC 1707, *Salmonella typhimurium* PTCC 1609, and *Staphylococcus aureus* ATCC 25923. Moreover, one strain, *Pediococcus acidilactici* IAH-5, showed exceptional features of cholesterol removal rate, antioxidant capacity, and a high auto-aggregation potential, indicative of probiotic adhesion to the epithelial cells [Vasiee *et al*., 2020]. Some LAB can also feature amylase activity, increasing the availability of energy from starch-containing sources and improving the energy density of food products [Nguyen *et al*., 2007]. A recent study has evaluated the amylase activity of 132 LAB strains isolated from Chinese fermented food products [Xu *et al*., 2020]. Out of these 132 strains, only three strains of *Lactobacillus plantarum* were found to exhibit the amylase activity and were further characterised to confirm their potential as probiotics. More amylolytic LAB were isolated from the traditional Bulgarian sourdough [Petkova *et al*., 2020]. The authors reported the presence of 36 LAB with amylase activity confirmed with the presence of six amylolytic genes, and the expression of the α-*amy* gene.

Positive effects of LAB-fermented cereal products were confirmed in animal and human studies. *Lactobacillus plantarum* DSMZ16627 and *Pediococcus acidilactici* NCIMB3005-fermented animal feed consisting of a mixture of barley (45%), wheat (42%), and wheat feed (12%) was given to pigs for 55 days to evaluate its effects on pig growth, nutrient digestibility, and pig gut health [Torres-Pitarch *et al*., 2020]. LA-fermented feed increased total tract nutrient digestibility and average daily body weight gain, as well as reduced contents of caecal butyrate and propionate. Moreover, it caused changes in the intestinal microbiota. The pigs fed with LAB-fermented feed had a lower caecum abundance of taxa negatively associated with pig growth, including *Megasphaera, Bifidobacterium*, and *Streptococcus* [Torres-Pitarch *et al*., 2020]. A single-blinded and parallel clinical trial study where subjects were eating fermented barley-wheat flour noodle for ten weeks showed a lower level of fasting blood glucose, HbA1c, and triglycerides in subjects with the metabolic syndrome [Pan *et al*., 2020]. Moreover, improved satiety and the reduction of fat mass, without the loss of lean mass, was observed in a group consuming fermented noodles. The above-mentioned studies proved that the LA-fermented cereal-based products could be a good alternative to dairy probiotics.

Dietary fibre

Another important factor related to gut health is the intake of dietary fibre. LA fermentation of starch-based products was found to increase the ratio of soluble to insoluble fibre [Mihhalevski *et al*., 2013]. Interestingly, the measured content of dietary fibre was higher after fermentation than the one calculated from the content of fibre in individual raw materials used for rye bread production. However, in the same study, the LA fermentation resulted in the significant loss of B-complex vitamins, especially thiamine, riboflavin, and pyridoxine, which can be explained by microbial activity [Mihhalevski *et al*., 2013]. A similar finding regarding dietary fibre was found in a study characterising wheat-naked barley bread after sourdough fermentation with the commercial LV1 starter culture consisting of LAB and yeast [Pejcz *et al*., 2017]. The bread produced had not only improved technological properties but also higher contents of dietary fibre, arabinoxylans, and β-glucans. β-Glucans are β-D-glucose polysaccharides with mixed (1→3), (1→4) linkages consisting of a significant part of barley dietary fibre. They were repeatedly reported to lower serum cholesterol, glucose, and lipid profiles, regulate intestinal microbiota homeostasis, boost the immune system, support body weight maintenance, and even prevent cancer development [Chaichian *et al*., 2020; De Angelis *et al*., 2015; El Khoury *et al*., 2012; Tong *et al*., 2015]. The activity of β-glucan itself is also modified by LA fermentation. A recent study has shown that β-glucan fermented with *Lactobacillus plantarum* dy-1 had lower molecular weight as a result of degradation by LAB. The structural changes of β-glucan enhanced its *in vitro* physiological activities, like the α-amylase, α-glucosidase, and lipase-inhibitory activity as well as the cholesterol adsorption capacity [Xiao *et al*., 2020].

Development of free-from products

With the increasing number of food allergies, gluten-related disorders, and intolerances to individual food components, several attempts have been made to reduce the level of triggering components in cereal-based food. Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) are considered to be one of the factors inducing intestinal symptoms in the irritable bowel syndrome (IBS). The content of fructans in grain-based products is too high for the subjects sensitive to FODMAP, and LA fermentation was found to decrease it. Rye bread made with sourdough produced with *Lactobacillus plantarum* was found to have a much lower fructan content without compromising technological properties and consumer acceptance [Pejcz *et al*., 2020]. However, the optimisation of the fermentation conditions is crucial because, as the author stated, the prolonged fermentation failed to reduce fructan content. A separate group of LAB was distinguished, called fructophilic LAB, which prefers fructose over glucose as a growth substrate. These bacteria, especially *Apilactobacillus kunkeei* B23I and *Fructobacillus fructosus* MBIII5, used in sourdough were found to quickly metabolise fructose and efficiently degrade fructans in the wheat medium [Albiac *et al*., 2020]. A Finish research group evaluated the effect of LAB-sourdough bread *versus* traditional yeast sourdough bread on the severance of symptoms in IBS subjects [Laatikainen *et al*., 2016, 2017]. The consumption of low-FODMAP rye bread, made with LA-sourdough, resulted in a lower incidence of flatulence, abdominal pain, cramps, and stomach rumbling in IBS patients and a lower value of breath hydrogen [Laatikainen *et al*., 2016]. However, it did not affect the quality of life and symptom severity scoring system (IBS-SSS). Similarly, sourdough wheat bread had a lower content of ATIs and FODMAP as compared to yeast-based wheat bread; however, it did not affect IBS symptoms in a seven-day trial [Laatikainen *et al*., 2017]. Therefore the authors suggested that bread modification is not enough to reduce symptoms and that the holistic modification of dietary habits is required.

Cereals, including wheat, barley, and rye, are sources of substances triggering allergic and autoimmune disorders. Gliadins, glutenins, and albumin/globulin fraction are involved in IgE-dependent allergic reaction and development of autoimmune diseases, like celiac disease. Selected LAB strains were found to degrade wheat proteins belonging to albumin/globulin and gliadins as well as to hydrolyse IgE-binding epitopes of wheat allergens, potentially reducing the allergenicity of wheat [Stefańska *et al*., 2016]. Moreover, the 60-day challenge with sweet baked goods made of previously sourdough-fermented flour showed no changes in haematology, serology, and intestinal barrier markers in eight coeliac disease patients in remission [Di Cagno *et al*., 2010]. This study showed that complete degradation of gluten through fermentation of wheat flour can be a safe option for gluten-free diet followers. The screening of bacterial strains capable of reducing allergenicity has been performed in the last years. A recent study has shown that three strains of LAB, *i.e.*, *Pediococcus acidilactici* XZ31, *Pediococcus pentosaceus* GD4, and *Lactobacillus sakei* GS6, had the highest antiallergic potential [Fu *et al*., 2020], which can be used in the development of new, hypoallergic products.

LEGUMES

Legumes are rich sources of particularly desired ingredients like proteins, carbohydrates, dietary fibre, minerals, and phenolic compounds, including isoflavones (IFs). LA-fermentation of legumes brings several advantages since it decreases the content of non-nutritional factors, improves their digestibility, and reduces allergenicity [Çabuk *et al*., 2018; Frias *et al*., 2008]. Fermentation also improves the biological activity of legume components for example *via* protein proteolysis with the release of bioactive peptides [Gibbs *et al*., 2004], through bioconversion of IFs [de Camargo *et al*., 2019] or due to the potential of LAB for exopolysaccharide secretion in a matrix of legumes [Li *et al*., 2014]. Although the nutritional level of soybean is limited by the presence of several substances undesirable from a nutritional point of view, such as ATIs and phytates [Egounlety & Aworh, 2003], it is the most utilised legume for fermentation. Other legumes like black beans, green beans, peanuts, chickpea or lupine subjected to fermentation are also attractive materials for manufacturing health-beneficial food products.

Safety

Traditionally fermented soybean foods have a high content of BAs. Mah *et al.* [2019] reported that soybean-based products

may contain: tryptamine, β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine as an effect of staphylococci, *Bacillus* spp., and LAB strains fermentation. The effect of decarboxylase-positive microorganisms is enhanced by the LAB proteolytic capacity, resulting in a high content of amino acids and finally BAs. Especially putrescine, tyramine, and histamine were detected in almost all analysed samples [Mah *et al*., 2019]. However, the LAB strains capable of producing an opposite effect are also tested. One of the most promising LAB are starter cultures with amine oxidase activity. Three different classes of oxidases have been described: copper-containing, multi-copper containing, and flavin-containing monoamine oxidases. They catalyse the oxidative deamination of BAs, forming aldehydes, hydrogen peroxide, and ammonia. These oxidases were detected in *Bacillus* spp. but also in selected strains of *Lactobacillus plantarum* that are applied for miso production [Lee *et al*., 2016].

Another issue related to the safety of legume-based fermented products is the change in the immunoreactivity of proteins produced by LAB. Standard starter cultures applied for the fermentation of soy milk contain *Lactobacillus* and *Bifidobacterium* strains and for ease, commercial dairy starters are often used for fermentation. This is a risky protocol due to the different composition of raw material, *e.g*., different carbohydrate composition (legumes contain stachyose, raffinose, sucrose, glucose, and fructose, while dairy sugars include lactose, glucose, and galactose) [Champagne *et al*., 2009]. It has been already reported that LAB metabolic changes and decreased activity are minor problems compared to the changed expression of bacterial proteins and their further modifications that can cause different immunoreactive LAB features. It was reported that *Lactobacillus casei* GCRL163 cultured in the conditions of lactose starvation showed the expression of eleven glycolytic enzymes that were differentially regulated. This bacterial survival strategy was responsible for different, expected immune system response to this product [Hussain *et al*., 2009]. A recent study has confirmed that some shock factors, like the mentioned persistent carbohydrate starvation or extreme acidity in the final product, may up-regulate the expression of bacterial surface proteins (S-protein) in the tested *Lactobacillus paracasei* GCRL46. The S-protein is homologous to secreted glucan-binding (GpbB) and immunoglobulin-binding (SibA) proteins, which are expressed in pathogenic streptococci and may be immunoreactive with IgE, as are allergenic epitopes [Pepper & Britz, 2019]. This explains why well-adapted *Lactobacillus plantarum* strains are very often used for the fermentation of legumes. Therefore, the protective effect on the starter cultures used requires further studies, especially for a demanding matrix the legumes are.

LA fermentation in legume-based products can also reduce the content of mycotoxins (aflatoxin B_1, zearalenone, ochratoxin, fumonisins, patulin) and pesticides (glyphosate) but also inhibits the development of pathogens in fermented seeds and sprouts, which was summarised and explained in recent literature reviews [Anal *et al*., 2020; Licandro *et al*., 2020].

Probiotic activity

LAB are usually not considered as the most common bacteria in the traditional fermentation of legumes. In most products, fermentation of legumes is carried out with the use of alkaline fermentation microorganisms [Kwon *et al*., 2019; Seo *et al*., 2018]. Still, LA fermentation plays a pivotal role in the development of physicochemical properties and biological activity of legume products. LAB are successfully applied for fermenting soybeans, to obtain soy milk, soy sauce, the Vietnamese tuong, modified Japanese natto, Korean doenjang, and Cambodian sieng [Chen *et al*., 2021; Jung *et al*., 2016]. Many LAB strains used for the fermentation of legumes belong to the group of those with probiotic properties; therefore, the ways of maintaining their high count in products are widely explored. The technological process for legume-based, milk-replacing beverages preparation or soy sauce preparation includes boiling for 5 min or autoclaving at 121°C for 15 min [Li *et al*., 2014]. All these protocols result in the deactivation of natural microbiota. Thus, various starter cultures based on single strains or mixed co-cultures are used with different efficiencies in achieving adequate probiotic cell numbers.

It has been reported that the addition of *Streptococcus* and *Saccharomyces* to the soy milk can enhance the viability of *Lactobacillus helveticus* R0052 in a mixed culture [Champagne *et al*., 2009], but the mixed cultures need to be thoroughly tested in terms of their ability to compete in the raw material. Legumes have a moderate buffering capacity, which might affect the application of standard dairy starter cultures like *Lactobacillus delbrueckii* R0187 and *Lactobacillus rhamnosus* R0011, and probiotic strains like *Bifidobacterium longum* R0175 for fermentation. Contrary, studies based on other strains, *i.e.*, *Lactobacillus delbrueckii* subsp. *bulgaricus* IM025 and *Lactobacillus rhamnosus* GG, showed increased bacterial proliferation of those strains and acidic properties in soy milk co-cultured with *Streptococcus thermophilus* [Farnworth *et al*., 2007]. Therefore, the effectiveness of fermentation is largely determined by the precise selection of strains in starter cultures for the particular raw material.

The more combined the raw material is, the more complicated co-cultures are needed for fermentation to preserve its biological properties. This regularity was reported in the research on a mixed peanut-soy milk functional beverage fermented with six different LAB strains [Santos *et al*., 2014]. The peanut and soy milks were mixed in the 2:1 ratio. Desirable parameters for this matrix (appropriate count of bacteria and acidification level) were found in a binary culture of *Pediococcus acidilactici* UFLABFFCX 27.1 with *Lactobacillus acidophilus* LACA 4, and in the co-culture of those two with *Saccharomyces cerevisiae* UFLA YFFBM 18.03. In that raw material, probiotic *Lactobacillus* strains achieved higher quantity and viability when cultivated with *Saccharomyces cerevisiae*. LA production for those combinations of strains reached 9.03 and 8.51 g/L, respectively. Moreover, the reduction of fermentation time by half was observed due to the rapid metabolism of carbohydrates and the formation of free amino acids. The higher content of growth-stimulating metabolites for probiotic *Lactobacillus* strains enabled reaching 8 $\log_{10}$ CFU/mL in the product up to 8 h of fermentation [Santos *et al*., 2014]. The addition of yeast served as a source of protein and vitamin B, which was previously explained by Rekha & Vijayalakshmi [2010]. Similar quantity and kinetics of fermentation in mixed

co-cultures were also noted in products containing *Bifidobacterium* RBL 00079 that was able to metabolise stachyose in soy milk fermented with co-cultures with *Lactobacillus delbruecki* subsp. *bulgaricus* IM 025, in which stachyose content was by two logarithms higher than in the dairy material [Farnworth *et al*., 2007]. In other studies, LAB assigned to several *Lactobacillus* sp., including *Lactobacillus plantarum, Lactobacillus rhamnosus*, and *Lactobacillus casei,* and 50 other microorganisms grouped in 10 different microbial consortia were tested for the fermentation potential of both the pea protein and pea-milk protein emulsions. In the mixed emulsions, LAB significantly inhibited the growth of *Actinobacteria* and *Proteobacteria* but also inhibited *Bacillus* strains (*e.g*., *Bacillus subtilis* and *Bacillus licheniformis*) [Ben-Harb *et al*., 2019]. *Lactobacillus plantarum* strains were the most adapted for the pea protein emulsion fermentation and were able to reach 8.5 $\log_{10}$ CFU/g. The *Lactococcus lactis* and *Lactobacillus casei/rhamnosus* group were dominant in the tested consortia preferable for the fermented mixed pea-milk protein emulsion. Their quantity reached 9.1 $\log_{10}$ CFU/g. Moreover, this procedure enabled reducing the level of hexanal, which high content normally leads to an undesired green pea aroma, whereas LA fermentation promoted the release of volatile compounds responsible for a roasted/grilled aroma in the pea protein emulsion, and a fruity, lactic aroma for the pea-milk protein emulsion [Ben-Harb *et al*., 2019]. In another study, the application of mixed co-cultures for soy milk fermentation brought the antagonistic effect instead of the expected synergistic effect on the growth of bacteria [Champagne *et al*., 2010]. Nevertheless, the health-promoting effects, manifested by increased IF bioavailability and oligosaccharide digestibility, sustained stronger in mixed cultures. Champagne *et al.* [2010] reported that fermentation with *Lactobacillus helveticus* R0052 combined with *Streptococcus thermophilus* ST5 and *Bifidobacterium longum* R0175 resulted in the more than a tenfold reduction in R0175 strain viability in comparison to single strain cultures. The combination of those strains was used to achieve an increase in the content of folic acid, niacin, riboflavin, and vitamins B_{12} and B_6, previously suggested by other groups [Mo *et al*., 2013; Limón *et al*., 2015].

In the legume fermentation technology, the selection of solid *vs*. fluid state fermentation has a particularly significant effect on the count and viability, and thus on the probiotic activity of bacteria. In the production of legume-based foodstuff, a favourable solution seems to be co-fermentation using LAB and *Bacillus subtilis* from natto by solid-state fermentation [Chen *et al*., 2021]. Chen *et al.* [2021] reported that after fermentation of lentils, the best growth of LAB was observed in aerobic solid-state co-fermentation, whereby the viable counts of *Lactobacillus plantarum* TK9 and *Lactobacillus paracasei* TK1501 reached 8.41 and 8.77 $\log_{10}$ CFU/g, respectively. This study showed that both, the composition of applied strains but also the adjustment of the conditions (aerobic/anaerobic) and the solid/liquid state of the fermentation of legumes were important. The viability of those strains reached 5.90 and 5.23 $\log_{10}$ CFU/g in anaerobic conditions and 6.61 and 6.5 $\log_{10}$ CFU/g in the liquid state, respectively. The benefits of solid-state fermentation of soybean meal with *Lactobacillus strains* and *Clostridium butyricum* have also been reported [Su *et al*., 2018]. A successive increase in counts of both bacterial strains was noted during 48 h of fermentation, reaching 8 $\log_{10}$ CFU/g at spore production of 6 $\log_{10}$ CFU/g [Su *et al*., 2018]. The authors reported also that the pH value and sugar content (especially raffinose and stachyose) in fermented soybean meal were considerably reduced in the presence of *Lactobacillus* strains. Furthermore, this newly developed, fermented piglet feed showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* [Su *et al.* 2018]. These results suggest that LA fermentation improves the probiotic properties of legume matrix in many ways and that the fermented legumes are beneficial in both human nutrition and animal feeding.

Isoflavones

IFs belong to flavonoids and are structurally and functionally similar to the human estrogen, estradiol, therefore are called phytoestrogens. Due to this similarity, IFs were suggested to elicit preventive effects in many kinds of hormone-dependent diseases but also to exhibitit a beneficial effect in alleviating menopausal symptoms and minimising their undesirable consequences [Chiang & Pan, 2011].

Legumes, especially soybean, lentil, broad bean, and chickpea, are the richest sources of IFs [de Camargo *et al*., 2019]. Their native forms are conjugated with sugars. The β-glucoside forms are not absorbed and require hydrolysis, which increases their bioavailability and subsequent metabolism [Donkor & Shah, 2008]. LAB have β-glucosidase activity and can increase the aglycone IF content in fermented products, and thus they increase IF bioavailability in comparison to unfermented products. The potential of LAB to convert IFs was described by Donkor & Shah [2008], who reported it in soy milk fermented with *Lactobacillus acidophilus* LAFTI L10, *Bifidobacterium lactis* LAFTIB94, and *Lactobacillus casei* LAFTI L26. These authors observed significant differences between raw (0.2 μg/mL of aglycones) and fermented soy milk (0.6 μg/mL). However, the rate of conversion did not differ significantly between the tested strains, although the kinetics of conversion was strictly strain-dependent and correlated with the kinetics of bacterial growth. In another study, the size of soybean seeds was suggested to influence the efficacy of conversion and the composition of aglycones during the fermentation with *Lactobacillus plantarum* strain P1201 [Lee *et al*., 2018]. Consequently, the content of isoflavone aglycones in all five samples obtained from small-seeded cultivars (average 13.9 g/100 seeds), namely Pungsannamul, Pungwon, Janggi, Shinhwa, and Heapum, was significantly higher than those from the five medium-seeded cultivars (average 21.4 g/100 seeds), namely Saedanbaek, Daewon, Daepung, Neulchan, and Taekwang. The most abundant individual aglycone was daidzein (average 26.6→265.9 μg/g, ten times higher conversion), followed by genistein (average 19.9→169.5 μg/g, 8.5 times), and glycitein (average 12.3→18.2 μg/g, 1.5 times).

Another study reported that glucosides, especially in soybean, were derived from three main IFs (daidzein, geninstein, and glycitein) and each could have acetyl and malonyl glucoside conjugates [Champagne *et al*., 2010]. The deconjugation levels varied between the different glucosides. Moreover, each

strain had a different potential for deconjugation. Fermentation by *Lactobacillus helveticus* R0052 resulted in the 50% reduction in total glucosides of soybeen, with 6-*O*-malonyl glucosides being reduced by 64%. Fermentation by a single strain of *Streptococcus thermophilus* ST5 or *Bifidobacterium longum* R0175 had no significant effect on IF levels. Combining an ST5 strain with a R0052 in the culture reduced the effectiveness of the latter. In another study, five isolates of LAB, *Lactobacillus acidophilus* B4496, *Lactobacillus bulgaricus* CFR2028, *Lactobacillus casei* B1922, *Lactobacillus plantarum* B4495, and *Lactobacillus fermentum* B4655 in a combination with yeast *Saccharomyces boulardii* were used, and the ability to hydrolyse daidzein and genistein in approx. 98% during 24 h of fermentation was reported for all co-culture combinations [Rekha & Vijayalakshmi, 2010]. The authors concluded that the mixed cultures of LAB with non-lactic probiotic yeast *Saccharomyces boulardii* had a synergic effect on the IFs conversion. Similar results regarding the effects of different LAB co-cultures and the use of seeds from different cultivars have been demonstrated for lentil-based fermented products [Chen *et al*., 2021]. In this study, the profile of IFs in unfermented green lentil samples was different depending on their origin and was different from the profiles reported for soybean. In general, in the mixed co-cultures of LAB (*Lactobacillus plantarum* TK9 and *Lactobacillus paracasei* TK1501) with *Bacillus subtilis* from natto in the solid-type fermentation, anaerobic conditions were favourable for IFs conversion; however, some strains had a higher conversion activity in aerobic conditions. Alike findings were noted in an experiment where similar processes were monitored for fermented kidney bean extracts [Limón *et al*., 2015]. The authors reported synergistic conversion activity of *Lactobacillus plantarum* CECT 748T with *Bacillus subtilis* CECT 39T in solid-type fermentation. Again, the synergic activity of LAB with *Bacillus subtilis* to enhance the production of IF aglycones was observed, increasing four-fold the antioxidant activity of fermented legumes [Limón *et al*., 2015].

Fermentation is also an effective solution in improving the quality of legume seed sprouts that have become popular in recent years. LA fermentation of four types of legume sprouts with *Lactobacillus casei* 0979 was used to increase the content of IFs and to improve the microbiological safety of the final product [Budryn *et al*., 2019]. Fermentation of germinated seeds increased five times the content of IFs. The highest content was noted for clover and chickpea sprouts. Furthermore, during fermentation, the count of LAB increased by 2 $\log_{10}$ CFU/mL, that of mould decreased by 1 $\log_{10}$, whereas those of *E. coli* and *Klebsiella* sp. by 2 $\log_{10}$. In turn, *Salmonella* sp. and *Shigella* sp. did not occur after fermentation, similarly to *Staphylococcus epidermidis*, while *S. aureus* and *S. saprophyticus* counts decreased by 3 $\log_{10}$ or were not detected in some samples.

Another interesting issue is the effect of legume-derived IFs after LA fermentation on the gastrointestinal tract. The impact of the intestinal microbiota of host on a consumed product is not fully understood and thus intestinal fermentation can have an uncontrolled impact on IFs conversion and retention. The wide interindividual variability in the bioconversion of IFs is related to specific metabotypes, indicating how IFs are metabolised in humans. Latest data report that fermentation reduces the passive excretion of non-metabolised IFs and can reduce the impact of gut microbiota on further conversion [de Oliveira Silva *et al*., 2020]. In this study, the authors reported that women excreted higher amounts of colonic metabolites and lower amounts of aglycones than men. The prevalence of *O*-demethylangolensin-producer, equol-producer, and nonproducer metabotypes after consumption of unfermented products (soybean meal biscuits) accounted for 56, 11, and 36%, while after consumption of the fermented product reached 72, 11, and 17%, respectively. It was reported that these gender-microbiota-dependent parameters could influence the beneficial effect of IFs. One of the longitudinal epidemiologic studies indicated a beneficial effect of dietary soybean genistein and daidzein in the prevention of osteoporosis by stimulating bone formation and suppression of bone resorption in the menopausal period of life [Xiao *et al*., 2018]. Some randomised controlled clinical trials reported the beneficial effect of the daily consumption of fermented and unfermented soy milk products on serum lipid profiles in moderately hypercholesterolemic male participants. Reduced serum levels of total cholesterol, LDL-cholesterol, and non-HDL-cholesterol werereported in the groups receiving IF-supplemented and soy milk fermented with LAB compared to the participants consuming unfermented soy milk [Cavallini *et al*., 2016].

There are also reports referring to the beneficial effect of IFs from fermented soy milk on carcinogenesis. In a large trial with 306 women with breast cancer and 662 controls, the habitual consumption of soybean IFs fermented with *Lactobacillus casei* Shirota since adolescence was associated with a reduced risk of breast cancer [Toi *et al*., 2013]. Moreover, the immunoprotective effect of IFs of soy milk fermented with a mixed culture of 2 strains of *Lactobacillus plantarum* (DPPMA24W and DPPMASL33), *Lactobacillus fermentum* DPPMA114, and *Lactobacillus rhamnosus* DPPMAAZ1 was reported. This product was able to reduce NO release from human Caco-2/TC7 cell lines with induced inflammation with interferon-γ (INF-γ) and lipopolysaccharides [Toi *et al*., 2013]. It was also observed that the treatment with this product succeeded in the reduction of interleukin 8 (IL-8) expression, suggesting a direct beneficial effect of IF aglycones generated during fermentation and the formation of equol from the aglycone daidzein [Di Cagno *et al*., 2010]. All these health-promoting activities are promising; however, it has to be taken into consideration that many positive effects can be associated with LAB metabolites.

Other phenolic compounds

Legumes contain also other phenolic compounds different from IFs, such as phenolic acids, flavonols, flavanones, *etc*., which are responsible for a high antioxidant capacity. Their presence contributes to lowering the risk of diseases caused by oxidative stress, like cancer, cardiovascular and neurodegenerative disorders [Vauzour *et al*., 2010]. The effect of LA fermentation on the content of phenolics and antioxidant capacity of legumes has been extensively investigated and some mechanisms that were already referred to the section dedicated to fruits and vegetable fermentation are

actual here. Indeed, the controlled fermentation of legumes with fully-defined starter cultures is more beneficial and safer than the spontaneous fermentation [Licandro *et al*., 2020]. However, it is more complicated to distinguish the direct role of LAB in creating the phenolic profile of legumes. The vast majority of studies referred to the significant role of alkaline fermentation of legumes with *Rhizopus* sp. [Ali *et al*., 2016] and *Bacillus subtilis natto* [Duenas *et al*., 2012] in the production of phenolic compounds. The phenolic profile is also affected by other variables, like material type and process conditions, [Limón *et al*., 2015].

A study on the impact of LA fermentation with *Lactobacillus plantarum* CECT 748 on phenolic compounds in soybean and mung bean-based products reported major changes in IFs content, but only in the soybean products [Landete *et al*., 2015]. Surprisingly, IFs and kaempferol derivatives were not detected in the non-fermented green bean. The compounds detected in mung bean were mainly glycoside forms of flavanones (eriodictyol 7-*O*-galactoside, eriodictyol 7-*O*-glucoside) and flavones (apigenin hexose, apigenin 8-*C*-glucoside, and luteolin 7-*O*-glucoside) (total content – 486.64 μg/g), whereas the most abundant compounds extracted after fermentation were apigenin derivatives (apigenin 7-*O*-glucoside and apigenin 8-*C*-glucoside), whose content increased from 399.23 μg/g to 1519.98 μg/g. Moreover, a significant increase was determined in the contents of eriodictyol 7-*O*-galactoside from 5.49 to 95.74 μg/g and (+)-catechin derivative from non-detectable to 11. 47 μg/g. In the case of the soybean-based fermented products, a significant increase was detected in the contents of naringenin from the non-detectable to 7.72 μg/g and kaempferol diglucoside from 12.86 μg/g to 20.21 μg/g. Additionally, increased contents of hydroxybenzoic and hydroxycinnamic acids were found [Landete *et al.* 2015].

A study by Limón *et al.* [2015] also refers to the applicability of the above-mentioned *Lactobacillus plantarum* CECT 748 strain in single strain fermentation of kidney beans in comparison to naturally fermented and fermented with *Bacillus subtilis* products. It has been confirmed that CECT 748 strain hydrolyses β-glucosidic bonds of several phenolic compounds occurring in a conjugated form (phenolic glucosides) to the corresponding aglycones, increasing the content of free phenolics. A general increase in the content of hydroxycinnamic acid derivatives was observed in the product fermented with this strain. (+)-Catechin content decreased remarkably in all fermented products, reaching levels under the detection limit. The decrease of (+)-catechin (monomer) content could be attributed to the formation of procyanidins (flavan-3-ols oligomers) during fermentation. In this study, a significant decrease was reported in flavanone and flavonol contents, by 94 and 78%, respectively. Phenolic compounds in the samples fermented with *Bacillus subtilis* reached levels under the detection limit [Limón *et al*., 2015]. Noteworthy, most of the studies referring to the applicability of *Lactobacillus* strains for legume fermentation used strains of *Lactobacillus plantarum* which seems to be the most adapted to this matrix. In a study where the DSM 20174 strain was used to ferment grass pea, it was concluded that the observed increase in the level of phenolic compounds during fermentation might be due to the activity of bacterial laccase and peroxidase [Starzyńska–Janiszewska & Stodolak, 2011]. Moreover, the drop in pH upon fermentation could be the factor activating enzymes participating in the hydrolysis of phenolic glycosides and causing their liberation from the lignocellulosic matrix [Çabuk *et al*., 2018].

It was reported that the augmented amount of phenolic compounds, including phenolic acids, in fermented legumes, especially mung bean and soybean, enhanced their antioxidant, cytotoxic, and immunomodulatory effects evaluated in the *in vitro* studies on hormone-dependent breast adenocarcinoma MCF-7 and human non-tumorigenic breast MCF 10A cell lines [Ali *et al*., 2016]. Nevertheless, most of the available research can only partly support the hypothesis that the antioxidant potential is due to the content of phenolics and not to the other antioxidants, including tocopherols, carotenoids, and peptides, released from proteins in fermented products. Regardless of which compounds are responsible for the beneficial effects, the antioxidant potential of legume-based fermented products is significantly higher than that of the non-fermented ones [Limón *et al*., 2015].

Vitamins, γ-aminobutyric acid, and other bioactive compounds

There are many ambiguous results regarding the impact of LA fermentation on the content of vitamins in legume-based products. In general, it is considered that the LA fermentation results in their enrichment with valuable nutritional compounds, like vitamins and minerals. Legumes themselves are a rich source of vitamins, but the increase in their content is mainly through alkaline fermentation using *Bacillus subtilis* or *Rhisopus* sp. Mo *et al.* [2013] showed that the contents of vitamins in soy-based foods, including vitamin K_2 in natto and vitamin B_{12} in tempeh, were higher compared to the non-fermented material. Additionally, indigenous bacteria in soybeans, such as *Klebsiella pneumoniae* and *Citrobacter freundii*, which persist the warming up process, have been found to contribute to vitamin B_{12} increase compared to the non-fermented material. Fermentation with LAB can also improve the bioavailability of these vitamins from the product [Mo *et al*., 2013]. In several studies reported in this manuscript, the content of vitamins B_6 and B_{12} in fermented legume-based products did not increase significantly [Champagne *et al.*, 2009, 2010], except for the fermentation with *Saccharomyces boulardii* that was able to increase vitamin B_2 content in soy milk but failed to increase it in the co-cultures with LAB [Rekha & Vijayalakshmi, 2010]. One of the studies referring to LAB-fermented soy germ showed that despite the total contents of tocopherols and phytosterol were significantly reduced during fermentation, their antioxidant activity was high [Hubert *et al*., 2008]. Raw soy germs contained 0.36 mg/g of total tocopherols, with 47.5% of α-tocopherol, 0.3% of β-tocopherol, 2.2% of γ-tocopherol, and 50% of δ-tocopherol. At the end of the fermentation, the total content of tocopherols accounted only for less than 0.08 mg/g. A decreased total phytosterol content, from 4.17 mg/g to 1.09 mg/g, was also determined after 48 h of the process. Interestingly, the antioxidant properties of fermented soy germs were significantly improved due to higher contents of saponins, phytosterols, and tocopherols. It was found that δ-tocopherol was the most

effective in scavenging the DPPH radical with a 74% absorbance inhibition at a concentration of 0.5 mg/mL, followed by soy germ asapogenol and α-tocopherol which showed 44% and 41% DPPH$^{\cdot}$ scavenging activity, respectively, at the same concentration. In the tested products, the conjugation profile of soyasaponins B was modified during the incubation period but the total content of soyasaponins B remained unchanged. The content of 2,3-dihydro-2,5-dihydroxy-6-methyl-4H--pyran-4-one (DDMP)-conjugated soyasaponins decreased drastically from 14.8 to 2.1 mol/g when soy germs were fermented, indicating that all the tested LAB strains were able to cleave the DDMP-moieties, releasing maltol, which is often used as a flavour enhancer.

Another beneficial component that accumulates during LA fermentation is GABA. It is a non-protein amino acid produced from L-glutamic acid by glutamic acid decarboxylase (GAD). The potential to synthesise GABA is a strain--dependent feature, and it has been reported that LAB fermentation of legumes, including soybean and kidney bean, allows a significant increase in the content of this compound in fermented material [Limón *et al*., 2015]. GABA was found to regulate blood pressure, act as a strong secretagogue of insulin from the pancreas, and is considered as an antidepressant since it is a primary neurotransmitter [Del Toro-Barbosa *et al*., 2020].

Exopolysaccharides (EPS) are one of the bioactive components produced by LAB with a high efficacy in legume--based products. Because of the high content of carbohydrates in the raw material, many LAB strains are able to synthesise EPS loosely connected to the cell surface. The potential prebiotic activity of EPS may enhance the growth of certain microbial strains in the human gut. In addition, EPS have a plenty of other beneficial properties, including antimicrobial, anticancer, antioxidant, and immunomodulatory ones [Wang *et al.*, 2015]. The potential for the production of EPS in fermented legume-based products improves not only the health--promoting properties of the products, but also increases their technological usefulness. The consumption of legume-based beverages fermented with different *Lactobacillus* strains and fermented dough made of legume flour confers multiple health benefits. The fermentation with LAB strains producing EPS improves rheological properties, viscosity, and sensory values of final products [Li *et al*., 2014; Xu *et al*., 2017].

Protein bioavailability and other bioactive effects

Legumes represent a reach source of nutritious proteins and can restore soil nitrogen without fertilizers; hence, they are considered to be both environmentally and nutritionally friendly. Due to their protein content, they are often recommended in sustainable diets. Both unfermented and fermented legume-based products contain almost all the essential and non-essential amino acids.

Studies on the bioavailability and nutritional content of legume components have also emphasised the benefits of alkaline fermentation to proteins [Bautista-Expósito *et al*., 2018]. Alkaline pH is more suitable for the fermentation of various legumes, including soybeans, kidney beans or lentils, but LA fermentation is also widely used due to proteolytic enzymes and amine oxidase activity of LAB. The use of mixed cultures also allows for a shorter fermentation process. It has been reported that the fermentation with *Bacillus subtilis* or *Saccharomyces cerevisiae* is longer (over 48 h) due to their limited capability to cleave glycoproteins, phosphoproteins or domains containing a high number of disulphide bridges, like in lectins, and that the LAB can reduce the fermentation time to less than 24 h [Limón *et al*., 2015; Santos *et al*., 2014].

Currently, the FAO/WHO defines protein quality in terms of the amino acid profile and digestibility of a protein source based on an *in vivo* bioassay [FAO, 1991]. The high content of sulfur amino acids inhibits the digestibility of legume proteins. In fermented legume-based products, protein digestibility reaches a maximum even after 5 h of fermentation (87.4%), like in Çabuk *et al.* study [2018]; however, the score of most of the sulfur amino acids is not reduced. To ensure an optimal reduction in sulfur amino acid content, even 11-h fermentation is needed. The reduction in sulfur content was reported to alter the *in vitro* protein digestibility from 67.0% at 0 h to 54.6% at 11 h. These data suggest that while fermentation is a viable method for reducing certain non-nutritive compounds in a legume protein concentrate, an alternative bacterium should be used which metabolises sulfur amino acids to a lesser extent than *Lactobacillus plantarum* [Çabuk *et al*., 2018]. While soybean-based drinks are currently considered to be the best alternative to cow's milk because of their protein and amino acid content, still almond milk has very little proteins whereas rice and coconut drinks, besides lacking proteins, are also rich in sugar and fat hence, they were fermented with LAB in a limited scale. In terms of the usefulness of proteins, legumes are increasingly used in LA fermentation.

To increase the digestibility of legume proteins, presoaking and hydrolysis with various proteases (papain, bromelain, Veron HPP and Veron PS) followed by fermentation were used [Verni *et al*., 2020]. It was found that the content of peptides and amino acids of fermented lentil was significantly improved when fermentation was preceded by soaking seeds and hydrolysis, especially with bromelain at the enzyme to substrate ratio of 10%. Although the fermentation generally releases the peptides and amino acids to a small extent, the total free amino acid content (*e.g*., aspartic acid, glutamic acid, leucine, lysine, and arginine) in pre-treated lentils fermented with *Lactobacillus acidophilus* ATCC 4356 was shown to increase up to 18%. Verni *et al.* [2020] reported also a slight increase in the content of Cys and Trp after the fermentation with the mentioned ATCC 4356 and with *Lactobacillus ferment*um DSM 20052 strains. This finding was promising since all legume storage proteins are relatively poor in sulfur-containing amino acids (methionine, cysteine) and tryptophan. Unfortunately, a substantial arginine reduction was observed in the DSM 20052 fermented product, probably because the amino acids were used by the strain for growth. A significant increase in GABA (about 20%) in a *Lactobacillus acidophilus* ATCC 4356-fermented lentil-based product was noted which was consistent with the results previously reported for kidney bean [Limón *et al.* 2015]. It was also suggested that the much higher content of free amino acids and peptides had a positive effect on the viability of LAB in the final product and extended its shelf-life [Verni *et al*., 2020]. In the study of Hu [2020], the addition of legume protein hydrolysates to

fermented products increased the content of bacterial biogenic sulfur-containing amino acids and improved LAB viability in the final product. The addition of soybean peptides promoted better properties of dairy-based yoghurt fermented with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [Hu *et al*., 2020], which featured a higher content of volatile compounds and better bacterial viability.

Although fermentation with co-cultures has a positive effect on the profile and bioavailability of many biologically-active legume compounds, the use of co-cultures does not positively affect the release of amino acids and peptides from legume proteins. In fermentation of a peanut-soy milk mixture, pure cultures of *Saccharomyces cerevisiae* UFLA YFFBM18.03, *Lactobacillus rhamnosus* LR 32, *Lactobacillus acidophilus* LACA 4, and *Pediococcus acidilactici* UFLA BFF-CX 27.1 promoted the increase in total amino acids (48.02, 47.32, 46.21, and 44.07%, respectively). Among the tested microorganisms, not LAB strains but *Saccharomyces cerevisiae* was responsible for the greatest accumulation of histidine, lysine, and threonine but also methionine and tryptophan. However, a significant reduction in the content of free amino acids, including essential amino acids, was found in the co-culture. It was concluded that these amino acids promoted the growth of bacteria [Santos *et al*., 2014].

The proteolytic activity of LAB in a legume matrix is also used in the context of nisin production [Liu *et al.* 2017]. Nisin, a natural antimicrobial peptide, is produced by certain *Lactococcus lactis* strains, which generally require expensive high-quality nitrogen sources due to their limited ability to biosynthesise amino acids. In the reported study, defatted soybean meal was used as a sole nitrogen source to support nisin overproduction. Both medium optimization in the context of amino acid and peptide contents but also targeted enhancement of the proteolytic function in *Lactococcus lactis* F44 through rationally expressing the related enzymes were applied [Liu *et al.* 2017]. Heterologous protease (NprB), an oligopeptide transporter subunit (OppA), and two peptidases (PepF and PepM) were introduced. The constructed strain was capable of achieving efficient biomass accumulation and nisin yield with a 30% decreased amount of soybean meal hydrolysates [Liu *et al*., 2017].

A very widely analysed field of knowledge about the role of fermentation in products based on legumes is the production of novel bioactive peptides conferring specific health benefits. Their application in the development of functional foods and pharmaceuticals to replace synthetic drugs in the treatment of several diseases was previously well-reviewed [Sanjukta & Rai, 2016]. Due to the aforementioned low efficiency of LAB in the proteolysis of legume proteins, LA fermentation is rarely used separately but more often combined with alkaline or completely replaced by it [Nawaz *et al*., 2017; Wang *et al*., 2015]. It has been reported that peptides obtained from, *e.g*., fermented soybean, where the major sources of peptides are glycinin and β-conglycinin, may act like regulatory compounds and exhibit bioactive properties such as, *e.g*., antihypertensive, antimicrobial, antioxidant, antidiabetic, and anticancer. It is impossible to list precisely all possible functions, but among the sequences deposited in the databases of bioactive peptides with proven effect, short sequences of di- and tripeptides dominate. The sequences describing the mentioned proteins account for over 95% of the 303 already reported ones [FeptideDB: Web Application for New Bioactive Peptides from Food Protein].

Peptides obtained from soy milk fermented with *Lactobacillus casei* strains had strong ACE-inhibitory activity proven both in *in vitro* and *in vivo* studies. It was confirmed that the ability to generate health-promoting profiles of peptides is a strongly strain-dependent feature [Bao & Chi, 2016]. In the study by Bao & Chi [2016], soy milk fermented with ten different strains was tested in terms of its role in antihypertensive regulation in Wistar-Kyoto rats and spontaneously hypertensive rats. Results showed that soy milk fermented with *Lactobacillus casei* CICC 20280 and CICC 23184 had high ACE inhibitory activity in *in vitro* tests. Moreover, the administration of CICC 20280 and CICC 23184 fermented products by gavage could effectively lower the blood pressure of spontaneously hypertensive rats to a normal level, while there was no effect on blood pressure of Wistar-Kyoto rats. It was concluded that, based on multiple control groups with individual components, the bioactive substances responsible for this activity were peptides and GABA. Their contents in CICC 20280 and CICC 23184 fermented products were 3.97 mg/mL (peptide) and 1.71 mg/mL (GABA), as well as 5.17 mg/mL (peptide) and 1.57 mg/mL (GABA), respectively [Bao & Chi, 2016].

While searching for new sources of nutritious protein by, *e.g*., adapting plants, especially legumes, peptides obtained from bean fermentation carried out by *Lactobacillus plantarum* 299v also showed very favourable properties [Jakubczyk *et al*., 2017]. The authors were looking for potential inhibitory peptides of enzymes involved in the metabolic syndrome. They demonstrated that only the fraction with the molecular weight of 3.5–7 kDa obtained after fermentation at 22°C for 3 h had the α-amylase inhibitory activity. The optimal fermentation conditions to release peptides with molecular weights of 3.5–7.0 kDa and the highest lipase or ACE inhibitory activity were determined as 30°C and 3 days. The fractions with the highest inhibitory activity were identified by LC-MS/MS, and eight unique peptides were identified in that fraction. The results reported by these authors also suggest that optimal fermentation process conditions should be selected according to the expected specific activity of peptides [Jakubczyk *et al*., 2017].

Another study reported the anticarcinogenic effect of soybean powder fermented with *Lactobacillus plantarum* DGK-17, which was previously isolated from kimchi [Khan & Kang, 2017]. The treatment with a fermented soybean extract reduced cancer cell colony formation, morphological changes, and apoptotic cell death of HCT-116 colon cancer cells in a dose-dependent manner. It also resulted in a weakened mitochondrial membrane potential, which also caused the release of Cyto C, further activating caspase-mediated cancerous cell death. It was concluded that the mechanism of action of the peptide fraction was based on cytosolic and mitochondrial reactive oxygen species sensitization and activation of mitogen-activated protein kinase (JNK) and other apoptotic signaling molecules. Such solutions indicate a great potential that is still to be discovered, while an important issue

is the careful selection of LAB strains, sometimes the less obvious ones, that could be used for fermentation [Khan & Kang, 2017].

The role of bacterial selection is particularly important in terms of their ability to reduce allergenicity and immunoreactivity of proteins. As it was mentioned before, although the alkaline fermentation offered a significant potential to eliminate unavailable components of legumes, including reducing the immunoreactivity of major allergens especially in solid-state fermentation type, the LAB are still the most effective in conventional, liquid fermentation [Frias *et al*., 2008]. It has been a while since Frias *et al.* [2008] reported that *Lactobacillus plantarum*-fermented soybean flour showed the highest grade of reduction in IgE-immunoreactivity (96–99%) depending upon the sensitivity of the human allergic plasma. Among the solid-state fermentation products, those inoculated with *Bacillus subtilis* yielded an 81 and 86% reduction in immunoreactivity against both human plasma 97.5 IgE kUA/L and human pooled plasma samples, respectively. A current study conducted by Yang *et al.* [2018] demonstrated that the solid-state fermentation of a soybean meal by a starter culture containing *Lactobacillus casei* CGMCC1. 539, yeast, and *Bacillus subtilis* CICC 20641 affected the allergenicity in *in vitro* and *in vivo* studies. Soybean β-conglycinin and glycinin were degraded into low-molecular-weight polypeptides, in which allergenic epitopic sequences reduced IgE-binding capacity in the *in vitro* trials with human serum. It was confirmed in an animal model of sensitised BALB/c mice. The treatment with fermented soybean induced reduction of mast cell protease-1 (mMCP-1) level and specific to soybean IgE, and boosted the secretion of IFN-γ [Yang *et al*., 2018]. Analogous results were reported by Xia *et al.* [2019] in immunised BALB/c mice model get after the treatment with soy milk fermented only with LAB: *Lactobacillus brevis* CICC 23474 and *Lactobacillus* sp. CICC 23470 strains. IgE, mMCP-1, and Th2-related cytokines were decreased, while IFN-γ production increased in spleen cell cultures. However, the intestinal villus was slightly damaged after the challenge [Xia *et al*., 2019], like in the Yang *et al*. [2018] study.

The greatest discrepancies in the immunomodulatory and allergenic potential of soybean-based products are observed especially concerning soy sauce and natto. One of the production technologies considers two-step fermentation of mixed soybean and gluten proteins using *Aspergillus oryzae* and LAB strains. However, after fermentation, the product undergoes several steps of heating and filtration, which contribute to the reduction of its allergenic potential; however, tests with allergic sera have indicated that the soybean allergens were still present in this product after the fermentation. The presence of some residual soybean allergens in different commercial soy sauces, immunostained by patient sera, allowed identifying enhanced immunoreactivity of β-subunit of β-conglycinin and oleosin, which are not known as major soybean allergens [Magishi *et al*., 2017]. It might have been a side effect of fermentation, which caused that some hidden before epitopes became exposed after unfolding the conformational structures of proteins. However, in the context of the previously mentioned increase of IFN-γ expression and some abnormalities in epithelial tissue physiology reported by Yang *et al.* [2018] and Xia *et al.* [2019], the data about soy sauce properties to induce Th1/Th2 regulation are convergent. On the other hand, the protective role of soy sauce was demonstrated in a mouse model of dextran sulphate sodium (DSS)-induced colitis, where the expression of proinflammatory genes encoding enzymes, such as iNOS and cyclooxygenase-2 (COX-2), was decreased in the colonic mucosa, and the expression of genes encoding proinflammatory cytokines in serum, such as TNFα, IFN-γ, IL-6 and IL-17A, was reduced as well [Song *et al*., 2014].

In the context of undesirable responses to soybean components after fermentation, the cross-reactivity and role of those products in delayed response should be also mentioned. An IgE-independent response has been reported in the context of poly(γ-glutamic acid) (PGA), produced in natto fermented with *Bacillus subtilis* but also other strains in co-culture. It was reported that PGA caused late-onset anaphylaxis. The possible cross-reactivity might be significant to the sting of cnidarian jellyfish that also contains PGA [Inomata *et al*., 2018].

Another important issue is also the immunoreactivity of the bacterial proteins in the products. It was reported that in legume-based products, the bacterial proteins from bacterial biomass may gain even 16% rise during 11-h fermentation [Çabuk *et al*., 2018]. The last two referred studies, in particular, show how much unknown is still to be verified about the role of proteins in fermented legume-based products.

SEAWEED

Seaweeds are traditionally consumed in Asian countries as marine vegetables. Despite many changes in taxonomic affiliation, a very large number of seaweed species are still classified within the kingdom Plantae; that is why those species will be refered in this review. More than 140 species of macroalgae are used worldwide as foods and depending on their pigment composition, are classified into red algae (*Rhodophyta*), green algae (*Chlorophyta*), and brown algae (*Phaeophyta*). They contain various beneficial bioactive ingredients but owing to their low content of lipids, high content of polysaccharides, fibres, minerals, vitamins, and abundance of phenolic compounds, seaweeds are known for their antioxidant, anticoagulant, antiinflammatory, anticancer, antihypertensive, antiviral, and antimicrobial activities, which was well-reviewed by Chye *et al.* [2018]. The primary method to obtain biologically-active compounds from seaweed is chemical extraction but fermentation might be a promising alternative in this respect. LA fermentation improves the digestibility and bioavailability of the seaweed components, but also extends their stability and shelf-life. For some compounds, like fiber and vitamins, spontaneous and alkaline fermentation of seaweed with *Aspergillus niger* and *Mucor* sp. through solid-state fermentation were reported to be more efficient [Rodríguez-Jasso *et al*., 2013]. However, the fermentation of seaweed can be carried out by LAB or using co-cultures with LAB strains to improve the sensory attractiveness of new products [Bruhn *et al*., 2019]. It is also highly probable that studies on the potential role of fermented seaweed compounds might be soon reported due to the growing interest in possible antiviral

and antibacterial effects of seaweeds reported by traditional Eastern medicine. Due to COVID-19, an increasing number of studies are appearing in this context [Pereira & Critchley, 2020]. Some antiviral properties of unfermented algae have already been reported in the context of the action of polysaccharides and phlorotannin derivatives in inhibiting polymerase and ribonuclease activities of HIV-1 RT or HSV [Pereira *et al*., 2004] or in bacterial infections but still not much is known about those agents in fermented products.

Polysaccharides

Many beneficial properties of seaweed are related to the presence of polysaccharides but their bioavailability is limited due to their polymeric structure. The main constituents of these polysaccharides are fucose, mannose, galactose, and uronic acids depending on the seaweed types, which cross-linked with each other and formed complex structures with a high molecular weight that are resistant to degradation and utilisation by microorganisms. On the other hand, fermentation helps increase the solubility of polysaccharides through depolymerization. Bacterial extracellular enzymes modify or lead to degradation of, *e.g*., the sulfated polysaccharide of the seaweeds, such as sulfated galactan, or fucans which can then be extracted. The polysaccharides released during fermentation show exceptionally strong anticoagulant properties [Shobharani *et al*., 2013]. Shobharani *et al.* [2013] reported that LAB strains (*Lactobacillus plantarum* MTCC 1328, *Lactobacillus fermentum, Pediococcus pentosaceus* NCIM 5420, *Pediococcus acidilactici* NCIM 5424, *Enterococcus durans* NCIM 5427, and *Enterococcus faecium* NCIM 5363) of marine origin applied for the fermentation of *Sargassum* sp. caused an increase in the anticoagulating activity of preparations as compared with the control. The enhanced anticoagulating activity was found to be positively correlated with the total sugar content, indicating the role of polysaccharides in inhibiting coagulation cascade. Moreover, LAB cell quantity (8–9 $\log_{10}$ CFU/mL) and viability were reported to be surprisingly high and long, up to 12 days, during which the LA production was high. It was also reported that the increased production of LA in seaweed-based formulas could contribute to the reduction of the molecular mass of alginates without compromising their desired bioactivity [Ramnani *et al*., 2012]. The selection of LAB strains with limited acidifying properties in that context was a limiting agent in previous studies and resulted in the discrimination of LA fermentation as an effective method to isolate alginate. Ramnani *et al.* [2012] reported that some low molecular weight polysaccharides derived from agar and alginate-bearing seaweeds in acidified conditions exhibited great potential to be used as a novel source of prebiotics and caused a significant increase in bifidobacteria populations from 8.06 at 0 h to 8.55 $\log_{10}$ CFU/mL at 24 h ($p = 0.018$).

One of the reported fermented seaweed polysaccharide activities is their radio-protective effect. For this purpose, *Ecklonia cava* fermented with *Lactobacillus brevis*, *Saccharomyces cerevisiae*, and *Candida utilis* was used for the treatment of γ-ray-irradiated peripheral immune cells – splenocytes. It was proven that the application of polysaccharides isolated from the >30 kDa fraction, stimulated proliferation and reduced the number of apoptotic and necrotic cells, as well as markedly reduced the DNA damage and production of reactive oxygen species. *Ecklonia cava* fermented with LAB showed the strongest DNA protective activity [Lee *et al*., 2013]. Hence, it may be concluded that fermentation significantly improves the protective and probiotic properties of seaweed.

Phenolic compounds

Seaweeds are a rich source of phenolic compounds that are secondary defence metabolites synthesised by macroalgae in response to stress conditions. They have a whole spectrum of proven biological activities including antiinflammatory, antioxidant, and antidiabetic ones [Chye *et al*., 2018; Gupta & Abu-Ghannam, 2012]. LAB used for the fermentation of seaweeds are reported to be capable of metabolising phenolic compounds, which on the one hand reduces their content, and on the other hand, improves their bioavailability [Chye *et al*., 2018]. Several studies reported that in different seaweeds, like *Sargassum* sp., *Zostera marina, Himanthalia elongata, Laminaria digitata*, and *Laminaria saccharina*, the *Lactobacillus*-fermentable caffeic acid, *p*-coumaric acid, and ferulic acids are metabolised into their respective vinyl and ethyl derivatives [Gupta & Abu-Ghannam, 2011; Rianingsih & Sumardianto, 2020]. In the study of Rianingsih & Sumardianto [2020], fermented *Sargassum* sp. has been reported to be a promising material to produce a non-dairy probiotic beverage. The *Lactobacillus plantarum* 0027 and *Lactobacillus acidophilus* FNCC-0051 LAB strains used for fermentation were able to grow by 1 $\log_{10}$ during 24 h of fermentation of a seaweed:water homogenate (1:12 *v/v*). Moreover, LAB were capable of increasing the count of probiotic cells to >8 $\log_{10}$ CFU/mL but also to double the final total content of phenolic compounds compared to the non-fermented products. Importantly, fermentation with FNCC-0051 resulted in greater antioxidant activity, tested with the DPPH assay, than the fermentation with 0027 [Rianingsih & Sumardianto, 2020]. The metabolism of phenolic compounds in *Sargassum* sp. fermented with different strains of marine-isolated LAB differed significantly depending on the strain applied [Shobharani *et al.*, 2013]. The viability and activity of strains remained high up to 12 and even 18 days. Under the optimal fermentation period, the seaweed fermented with *Enterococcus faecium* P1–2CB-w1 exhibited the maximum antioxidant activity, tested with seven different assys, including ABTS and DPPH, which was positively correlated to the content of phenolics (r=0.82–0.92) [Shobharani *et al*., 2013].

Another beneficial activity of seaweed phenolic compounds regulated by LA fermentation is the colon-protective phlorotannin activity. It was tested both in the context of metabolism of these compounds in fermented products, but also in the context of their fermentation by intestinal microbiota [Charoensiddhi *et al*., 2016]. On the one hand, the LA fermentation seemed to result in a significant decrease in the content of phlorotannins in a fermented brown seaweed *Ascophyllum nodosum* and a reduced antioxidant capacity of the extracts noted in the ABTS assay but, on the other hand, the antigenotoxic activity and cell growth inhibitory effect in colon HT-29 cells was maintained and enhanced especially for

the high molecular weight fraction of phlorotannins extracted after fermentation [Corona *et al*., 2017].

Phenolics, such as phlorotannins, flavonoids, and phenolic acids, and their derivatives from seaweeds are already used in the treatment of diseases, especially those related to oxidative stress. Therefore, further studies on their fate after LA fermentation of seaweed are needed.

Protein and peptides

The quality of algae protein is relatively high compared to cereal and soybean proteins. More than 75% of seaweeds belonging to the Plantae kingdom have a higher relative content of protein essential amino acids than wheat flour and 50% higher than soybean, rice, and corn flours [Mæhre *et al*., 2014]. The relative content of methionine and lysine was comparable to their content in protein of traditional food-plants. The main limitation in using seaweed as a protein source is its lower content than in corn or soybean. Total protein content varies from about 10 to 40 g/100 g of seaweeds dry weight, and differs between seasons and species. The highest protein content in seaweed is found in the winter-early spring period, while not in the summer [Mæhre *et al*., 2014]. Moreover, the content of proteins in the fermented biomass of seaweed increases significantly due to bacterial protein production [Rianingsih & Sumardianto, 2020].

Bioactive peptides can be released from seaweed proteins by proteases. Seaweed-derived peptides are known for their antioxidant, anticarcinogenic, renin inhibitory, and antihypertensive activities [Cian *et al*., 2016; Fitzgerald *et al*., 2014], but not much is known about the direct role of LA fermentation in their release.

It is worth noting that among seaweed proteins, the presence of lectins might be important. Lectins are glycoproteins involved in many biological processes, like intercellular communication. They can agglutinate red blood cells but are also strong mitogens especially for B and T lymphocytes. Although algae seem to feature a low sensitising potential, a few case studies referred to hypersensitive reactions after seaweeds consumption [Thomas *et al*., 2019]. One of the indicated reasons may be the homology between seaweed lectins and those identified in some legumes, including peanuts, where they are reported to have the allergenic potential [Goodman, 2020]. LA fermentation may hypothetically reduce the allergenic potential. On the other hand, algal lectins exhibit plenty of bioactive properties, including antibiotic, cytotoxic, antinociceptive, antiinflammatory, antiadhesive, and antiviral ones [Singh *et al*., 2015].

SUMMARY AND FUTURE PERSPECTIVES

Fermented food products are gaining attention, not only due to their preservation feasibility and sensory attributes but also because of their nutritional quality and health-promoting potential. The probiotic properties of LA-fermented dairy products are well-established, while the parallel technology for plant-based products is much less developed. However, this review has shown that the area of LA fermentation of plants affords enormous opportunities for new, functional food developments. And although the biotransformation of several nutrients and bioactive compounds and the strain-dependent properties need more in-depth elucidation in several matrices, the recently published research proved that LAB-fermented cereals, fruits, vegetables, legumes, and seaweeds are a good source of health-promoting and nutritional molecules. The obtained products offer a fine alternative for a growing number of vegans, vegetarians, and flexitarians, who look for the plant-based, healthy alternatives. On the other hand, the process of fermentation should be properly designed and controlled to avoid diminishing product's quality and prevent risk to health.

Further studies are needed to evaluate the effect of the intake of LA-fermented foodstuff on the human health in clinical trials since the effects of fermented food are likely to be different than coupled activties of individual bioactive compounds, nutrients, and microbes. Moreover, the survival of probiotic strains in the human gut and the host-microbe interactions need comprehensive, fundamental research. And even though the human studies are limited, the emerging evidence shows that the health benefits of LA-fermented plant-based food products are well-beyond the native materials.

ORCID IDs

N. Drabińska https://orcid.org/0000-0001-5324-5982
A.M. Ogrodowczyk https://orcid.org/0000-0001-7350-4072

REFERENCES

1. Acín Albiac, M., Di Cagno, R., Filannino, P., Cantatore, V., Gobbetti, M. (2020). How fructophilic lactic acid bacteria may reduce the FODMAPs content in wheat-derived baked goods: a proof of concept. *Microbial Cell Factories*, *19*(1), art. no.182. https://doi.org/10.1186/s12934-020-01438-6
2. Adebo, O.A., Kayitesi, E., Njobeh, P.B. (2019). Reduction of mycotoxins during fermentation of whole grain sorghum to whole grain ting (a Southern African food). *Toxins*, *11*(3), art. no. 180. https://doi.org/10.3390/toxins11030180
3. Adebo, O.A., Njobeh, P.B., Kayitesi, E. (2018). Fermentation by *Lactobacillus fermentum* strains (singly and in combination) enhances the properties of ting from two whole grain sorghum types. *Journal of Cereal Science*, *82*, 49–56. https://doi.org/10.1016/j.jcs.2018.05.008
4. Alan, Y., Topalcengiz, Z., Dığrak, M. (2018). Biogenic amine and fermentation metabolite production assessments of *Lactobacillus plantarum* isolates for naturally fermented pickles. *LWT – Food Science and Technology*, *98*, 322–328. https://doi.org/10.1016/j.lwt.2018.08.067
5. Ali, N.M., Yeap, S.-K., Yusof, H.M., Beh, B.-K., Ho, W.-Y., Koh, S.-P., Abdullah, M.P., Alitheen, N.B., Long, K. (2016). Comparison of free amino acids, antioxidants, soluble phenolic acids, cytotoxicity and immunomodulation of fermented mung bean and soybean. *Journal of the Science of Food and Agriculture*, *96*(5), 1648–1658. https://doi.org/10.1002/jsfa.7267
6. Allendez, G.N., Alzogaray, M.S.L., Nazareno, M.A., Gupta, C. (2020). Lactic acid fermentation as a tool to enhance the antioxidant properties of *Opuntia ficus-indica* juice. *Indian Journal of Agricultural Biochemistry*, *33*(1), 20–24. https://doi.org/10.5958/0974-4479.2020.00003.9

7. Anal, A.K., Perpetuini, G., Petchkongkaew, A., Tan, R., Avallone, S., Tofalo, R., Nguyen, H. Van, Chu-Ky, S., Ho, P.H., Phan, T.T., Waché, Y. (2020). Food safety risks in traditional fermented food from South-East Asia. *Food Control*, *109*, art. no. 106922. https://doi.org/10.1016/j.foodcont.2019.106922
8. Bao, Z., Chi, Y. (2016). *In vitro* and *in vivo* assessment of angiotensin-converting enzyme (ACE) inhibitory activity of fermented soybean milk by *Lactobacillus casei* strains. *Current Microbiology*, *73*(2), 214–219. https://doi.org/10.1007/s00284-016-1051-7
9. Barbu, V., Cotârleț, M., Bolea, C.A., Cantaragiu, A., Andronoiu, D.G., Bahrim, G.E., Enachi, E. (2020). Three types of beetroot products enriched with lactic acid bacteria. *Foods (Basel, Switzerland)*, *9*(6), art. no. 786. https://doi.org/10.3390/foods9060786
10. Bartkiene, E, Vidmantiene, D., Juodeikiene, G., Viskelis, P., Urbonaviciene, D. (2013). Lactic acid fermentation of tomato: Effects on cis/trans lycopene isomer ratio, β-carotene mass fraction and formation of L(+)- and D(−)-lactic acid. *Food Technology and Biotechnology*, *51*(4), 471–478.
11. Bartkiene, E., Bartkevics, V., Pugajeva, I., Krungleviciute, V., Mayrhofer, S., Domig, K. (2017). The contribution of *P. acidilactici*, *L. plantarum*, and *L. curvatus* starters and L-(+)-lactic acid to the acrylamide content and quality parameters of mixed rye – wheat bread. *LWT – Food Science and Technology*, *80*, 43–50. https://doi.org/10.1016/j.lwt.2017.02.005
12. Bartkiene, E., Lele, V., Ruzauskas, M., Domig, K.J., Starkute, V., Zavistanaviciute, P., Bartkevics, V., Pugajeva, I., Klupsaite, D., Juodeikiene, G., Mickiene, R., Rocha, J.M. (2019). Lactic acid bacteria isolation from spontaneous sourdough and their characterization including antimicrobial and antifungal properties evaluation. *Microorganisms*, *8*(1), art. no. 64. https://doi.org/10.3390/microorganisms8010064
13. Bautista-Expósito, S., Peñas, E., Silván, J.M., Frias, J., Martínez-Villaluenga, C. (2018). pH-controlled fermentation in mild alkaline conditions enhances bioactive compounds and functional features of lentil to ameliorate metabolic disturbances. *Food Chemistry*, *248*, 262–271. https://doi.org/10.1016/j.foodchem.2017.12.059
14. Ben-Harb, S., Saint-Eve, A., Panouillé, M., Souchon, I., Bonnarme, P., Dugat-Bony, E., Irlinger, F. (2019). Design of microbial consortia for the fermentation of pea-protein-enriched emulsions. *International Journal of Food Microbiology*, *293*, 124–136. https://doi.org/10.1016/j.ijfoodmicro.2019.01.012
15. Blajman, J.E., Vinderola, G., Páez, R.B., Signorini, M.L. (2020). The role of homofermentative and heterofermentative lactic acid bacteria for alfalfa silage: a meta-analysis. *The Journal of Agricultural Science*, *158*(1–2), 107–118. https://doi.org/10.1017/S0021859620000386
16. Bruhn, A., Brynning, G., Johansen, A., Lindegaard, M.S., Sveigaard, H.H., Aarup, B., Fonager, L., Andersen, L.L., Rasmussen, M.B., Larsen, M.M., Elsser-Gravesen, D., Børsting, M.E. (2019). Fermentation of sugar kelp (*Saccharina latissima*)—effects on sensory properties, and content of minerals and metals. *Journal of Applied Phycology*, *31*(5), 3175–3187. https://doi.org/10.1007/s10811-019-01827-4
17. Budryn, G., Klewicka, E., Grzelczyk, J., Gałązka-Czarnecka, I., Mostowski, R. (2019). Lactic acid fermentation of legume seed sprouts as a method of increasing the content of isoflavones and reducing microbial contamination. *Food Chemistry*, *285*, 478–484. https://doi.org/10.1016/j.foodchem.2019.01.178
18. Çabuk, B., Nosworthy, M.G., Stone, A.K., Korber, D.R., Tanaka, T., House, J.D., Nickerson, M.T. (2018). Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technology and Biotechnology*, *56*(2), 257–264. https://doi.org/10.17113/ftb.56.02.18.5450
19. Cai, Y.X., Wang, J.H., McAuley, C., Augustin, M.A., Terefe, N.S. (2019). Fermentation for enhancing the bioconversion of glucoraphanin into sulforaphane and improve the functional attributes of broccoli puree. *Journal of Functional Foods*, *61*, art. no. 103461. https://doi.org/10.1016/j.jff.2019.103461
20. Cavallini, D.C.U., Manzoni, M.S.J., Bedani, R., Roselino, M.N., Celiberto, L.S., Vendramini, R.C., de Valdez, G.F., Abdalla, D.S.P., Pinto, R.A., Rosetto, D., Valentini, S.R., Rossi, E.A. (2016). Probiotic soy product supplemented with isoflavones improves the lipid profile of moderately hypercholesterolemic men: a randomized controlled trial. *Nutrients*, *8*(1), art. no. 52. https://doi.org/10.3390/nu8010052
21. Chaichian, S., Moazzami, B., Sadoughi, F., Haddad Kashani, H., Zaroudi, M., Asemi, Z. (2020). Functional activities of beta-glucans in the prevention or treatment of cervical cancer. *Journal of Ovarian Research*, *13*(1), art. no. 24. https://doi.org/10.1186/s13048-020-00626-7
22. Champagne, C.P., Green-Johnson, J., Raymond, Y., Barrette, J., Buckley, N. (2009). Selection of probiotic bacteria for the fermentation of a soy beverage in combination with *Streptococcus thermophilus*. *Food Research International*, *42*(5–6), 612–621. https://doi.org/10.1016/j.foodres.2008.12.018
23. Champagne, C.P., Tompkins, T.A., Buckley, N.D., Green-Johnson, J.M. (2010). Effect of fermentation by pure and mixed cultures of *Streptococcus thermophilus* and *Lactobacillus helveticus* on isoflavone and B-vitamin content of a fermented soy beverage. *Food Microbiology*, *27*(7), 968–972. https://doi.org/10.1016/j.fm.2010.06.003
24. Charoensiddhi, S., Conlon, M.A., Vuaran, M.S., Franco, C.M.M., Zhang, W. (2016). Impact of extraction processes on prebiotic potential of the brown seaweed *Ecklonia radiata* by *in vitro* human gut bacteria fermentation. *Journal of Functional Foods*, *24*, 221–230. https://doi.org/10.1016/j.jff.2016.04.016
25. Chen, K., Gao, C., Han, X., Li, D., Wang, H., Lu, F. (2021). Co-fermentation of lentils using lactic acid bacteria and *Bacillus subtilis natto* increases functional and antioxidant components. *Journal of Food Science*, *86*(2), 475–483. https://doi.org/10.1111/1750-3841.15349
26. Chen, Y., Ouyang, X., Laaksonen, O., Liu, X., Shao, Y., Zhao, H., Zhang, B., Zhu, B. (2019). Effect of *Lactobacillus acidophilus*, *Oenococcus oeni*, and *Lactobacillus brevis* on composition of bog bilberry juice. *Foods (Basel, Switzerland)*, *8*(10), art. no. 430. https://doi.org/10.3390/foods8100430
27. Chiang, S.-S., Pan, T.-M. (2011). Antiosteoporotic effects of *Lactobacillus*-fermented soy skim milk on bone mineral density and the microstructure of femoral bone in ovariectomized mice. *Journal of Agricultural and Food Chemistry*, *59*(14), 7734–7742. https://doi.org/10.1021/jf2013716

28. Chiş, M.S., Păucean, A., Man, S.M., Vodnar, D.C., Teleky, B.-E., Pop, C.R., Stan, L., Borsai, O., Kadar, C.B., Urcan, A.C., Muste, S. (2020). Quinoa sourdough fermented with *Lactobacillus plantarum* ATCC 8014 designed for gluten-free muffins—a powerful tool to enhance bioactive compounds. *Applied Sciences, 10* (20), art. no. 7140. https://doi.org/10.3390/app10207140
29. Chye, F.Y., Ooi, P.W., Ng, S.Y., Sulaiman, M.R. (2018). Fermentation-derived bioactive components from Seaweeds: Functional properties and potential applications. *Journal of Aquatic Food Product Technology*, *27*(2), 144–164. https://doi.org/10.1080/10498850.2017.1412375
30. Cian, R.E., Garzón, A.G., Ancona, D.B., Guerrero, L.C., Drago, S.R. (2016). Chelating properties of peptides from red seaweed *Pyropia columbina* and its effect on iron bio-accessibility. *Plant Foods for Human Nutrition*, *71*(1), 96–101. https://doi.org/10.1007/s11130-016-0533-x
31. Cirlini, M., Ricci, A., Galaverna, G., Lazzi, C. (2020). Application of lactic acid fermentation to elderberry juice: Changes in acidic and glucidic fractions. *LWT – Food Science and Technology*, *118*, art. no. 108779. https://doi.org/10.1016/j.lwt.2019.108779
32. Ciska, E., Drabińska, N., Narwojsz, A., Honke, J. (2016). Stability of glucosinolates and glucosinolate degradation products during storage of boiled white cabbage. *Food Chemistry*, *203*, 340–347. https://doi.org/10.1016/j.foodchem.2016.02.079
33. Ciska, E., Verkerk, R., Honke, J. (2009). Effect of boiling on the content of ascorbigen, indole-3-carbinol, indole-3-acetonitrile, and 3,3'-diindolylmethane in fermented cabbage. *Journal of Agricultural and Food Chemistry*, *57*(6), 2334–2338. https://doi.org/10.1021/jf803477w
34. Conte, P., Fadda, C., Drabińska, N., Krupa-Kozak, U. (2019). Technological and nutritional challenges, and novelty in gluten-free breadmaking: A review. *Polish Journal of Food and Nutrition Sciences*, *69*(1), 5–21. https://doi.org/10.31883/pjfns-2019-0005
35. Corona, G., Coman, M.M., Guo, Y., Hotchkiss, S., Gill, C., Yaqoob, P., Spencer, J.P.E., Rowland, I. (2017). Effect of simulated gastrointestinal digestion and fermentation on polyphenolic content and bioactivity of brown seaweed phlorotannin-rich extracts. *Molecular Nutrition and Food Research*, *61*(11), art. no. 1700223. https://doi.org/10.1002/mnfr.201700223
36. Cvetković, D., Ranitović, A., Savić, D., Joković, N., Vidaković, A., Pezo, L., Markov, S. (2019). Survival of wild strains of *Lactobacilli* during kombucha fermentation and their contribution to functional characteristics of beverage. *Polish Journal of Food and Nutrition Sciences*, *69*(4), 407–415. https://doi.org/10.31883/pjfns/112276
37. Czyżowska, A., Klewicka, E., Libudzisz, Z. (2006). The influence of lactic acid fermentation process of red beet juice on the stability of biologically active colorants. *European Food Research and Technology*, *223*(1), 110–116. https://doi.org/10.1007/s00217-005-0159-y
38. Czyżowska, A., Siemianowska, K., Śniadowska, M., Nowak, A. (2020). Bioactive compounds and microbial quality of stored fermented red beetroots and red beetroot juice. *Polish Journal of Food and Nutrition Sciences*, *70*(1), 35–44. https://doi.org/10.31883/pjfns/116611
39. Da Silva Felício, M.T., Hald, T., Liebana, E., Allende, A., Hugas, M., Nguyen-The, C., Johannessen, G.S., Niskanen, T., Uyttendaele, M., McLauchlin, J. (2015). Risk ranking of pathogens in ready-to-eat unprocessed foods of non-animal origin (FoNAO) in the EU: Initial evaluation using outbreak data (2007–2011). *International Journal of Food Microbiology*, *195*, 9–19. https://doi.org/10.1016/j.ijfoodmicro.2014.11.005
40. De Angelis, M., Montemurno, E., Vannini, L., Cosola, C., Cavallo, N., Gozzi, G., Maranzano, V., Di Cagno, R., Gobbetti, M., Gesualdo, L. (2015). Effect of whole-grain barley on the human fecal microbiota and metabolome. *Applied and Environmental Microbiology*, *81*(22), 7945–7956. https://doi.org/10.1128/AEM.02507-15
41. de Camargo, A.C., Favero, B.T., Morzelle, M.C., Franchin, M., Alvarez-Parrilla, E., de la Rosa, L.A., Geraldi, M.V., Maróstica Júnior, M.R., Shahidi, F., Schwember, A.R. (2019). Is chickpea a potential substitute for soybean? Phenolic bioactives and potential health benefits. *International Journal of Molecular Sciences*, *20*(11), art. no. 2644. https://doi.org/10.3390/ijms20112644
42. de la Fuente, B., Luz, C., Puchol, C., Meca, G., Barba, F.J. (2021). Evaluation of fermentation assisted by *Lactobacillus brevis* POM, and *Lactobacillus plantarum* (TR-7, TR-71, TR-14) on antioxidant compounds and organic acids of an orange juice--milk based beverage. *Food Chemistry*, *343*, art. no. 128414. https://doi.org/10.1016/j.foodchem.2020.128414
43. de Oliveira, S.D., Araújo, C.M., Borges, G. da S.C., Lima, M. dos S., Viera, V.B., Garcia, E.F., de Souza, E.L., de Oliveira, M.E.G. (2020). Improvement in physicochemical characteristics, bioactive compounds and antioxidant activity of acerola (*Malpighia emarginata* D.C.) and guava (*Psidium guajava* L.) fruit by-products fermented with potentially probiotic lactobacilli. *LWT – Food Science and Technology*, *134*, art. no. 110200. https://doi.org/10.1016/j.lwt.2020.110200
44. de Oliveira Silva, F., Lemos, T.C.C., Sandôra, D., Monteiro, M., Perrone, D. (2020). Fermentation of soybean meal improves isoflavone metabolism after soy biscuit consumption by adults. *Journal of the Science of Food and Agriculture*, *100*(7), 2991–2998. https://doi.org/10.1002/jsfa.10328
45. Del Toro-Barbosa, M., Hurtado-Romero, A., Garcia-Amezquita, L.E., García-Cayuela, T. (2020). Psychobiotics: mechanisms of action, evaluation methods and effectiveness in applications with food products. *Nutrients*, *12*(12), art. no. 3896. https://doi.org/10.3390/nu12123896
46. Di Cagno, R., Barbato, M., Di Camillo, C., Rizzello, C.G., De Angelis, M., Giuliani, G., De Vincenzi, M., Gobbetti, M., Cucchiara, S. (2010). Gluten-free sourdough wheat baked goods appear safe for young celiac patients: a pilot study. *Journal of Pediatric Gastroenterology and Nutrition*, *51*(6), 777–783. https://doi.org/10.1097/MPG.0b013e3181f22ba4
47. Di Cagno, R., Coda, R., De Angelis, M., Gobbetti, M. (2013). Exploitation of vegetables and fruits through lactic acid fermentation. *Food Microbiology*, *33*(1), 1–10. https://doi.org/10.1016/j.fm.2012.09.003
48. Di Cagno, R., Filannino, P., Vincentini, O., Lanera, A., Cavoski, I., Gobbetti, M. (2016). Exploitation of *Leuconostoc mesenteroides* strains to improve shelf life, rheological, sensory and functional features of prickly pear (*Opuntia ficus-indica* L.) fruit puree. *Food Microbiology*, *59*, 176–189. https://doi.org/10.1016/j.fm.2016.06.009

49. Di Cagno, R., Mazzacane, F., Rizzello, C.G., Vincentini, O., Silano, M., Giuliani, G., De Angelis, M., Gobbetti, M. (2010). Synthesis of isoflavone aglycones and equol in soy milks fermented by food-related lactic acid bacteria and their effect on human intestinal Caco-2 cells. *Journal of Agricultural and Food Chemistry*, *58*(19), 10338–10346. https://doi.org/10.1021/jf101513r
50. Donkor, O.N., Shah, N.P. (2008). Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. *Journal of Food Science*, *73*(1), M15–M20. https://doi.org/10.1111/j.1750-3841.2007.00547.x
51. Duenas, M., Hernandez, T., Robredo, S., Lamparski, G., Estrella, I., Munoz, R. (2012). Bioactive phenolic compounds of soybean (*Glycine max* cv. Merit): modifications by different microbiological fermentations. *Polish Journal of Food and Nutrition Sciences*, *62*(4), 241–250. https://doi.org/10.2478/v10222-012-0060-x
52. Egounlety, M., Aworh, O.C. (2003). Effect of soaking, dehulling, cooking and fermentation with *Rhizopus oligosporus* on the oligosaccharides, trypsin inhibitor, phytic acid and tannins of soybean (*Glycine max* Merr.), cowpea (*Vigna unguiculata* L. Walp) and groundbean (*Macrotyloma geocarpa* Harms). *Journal of Food Engineering*, *56*(2–3), 249–254. https://doi.org/10.1016/S0260-8774(02)00262-5
53. El Khoury, D., Cuda, C., Luhovyy, B.L., Anderson, G.H. (2012). Beta glucan: health benefits in obesity and metabolic syndrome. *Journal of Nutrition and Metabolism*, *2012*, art. no. 851362. https://doi.org/10.1155/2012/851362
54. Esteban-Torres, M., Landete, J.M., Reverón, I., Santamaría, L., de las Rivas, B., Muñoz, R. (2015). A *Lactobacillus plantarum* esterase active on a broad range of phenolic esters. *Applied and Environmental Microbiology*, *81*(9), 3235–3242. https://doi.org/10.1128/AEM.00323-15
55. FAO. (1991). Protein quality evaluation: report of the joint FAO/WHO expert consultation. *Food and Nutrition Paper, 92*.
56. Farnworth, E.R., Mainville, I., Desjardins, M.-P., Gardner, N., Fliss, I., Champagne, C. (2007). Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. *International Journal of Food Microbiology*, *116*(1), 174–181. https://doi.org/10.1016/j.ijfoodmicro.2006.12.015
57. *FeptideDB: Web application for new bioactive peptides from food protein*. http://www4g.biotec.or.th/FeptideDB/
58. Filannino, P., Azzi, L., Cavoski, I., Vincentini, O., Rizzello, C.G., Gobbetti, M., Di Cagno, R. (2013). Exploitation of the health-promoting and sensory properties of organic pomegranate (*Punica granatum* L.) juice through lactic acid fermentation. *International Journal of Food Microbiology*, *163*(2–3), 184–192. https://doi.org/10.1016/j.ijfoodmicro.2013.03.002
59. Filannino, P., Bai, Y., Di Cagno, R., Gobbetti, M., Gänzle, M.G. (2015). Metabolism of phenolic compounds by *Lactobacillus spp*. during fermentation of cherry juice and broccoli puree. *Food Microbiology*, *46*, 272–279. https://doi.org/10.1016/j.fm.2014.08.018
60. Filannino, P., Gobbetti, M., De Angelis, M., Di Cagno, R. (2014). Hydroxycinnamic acids used as external acceptors of electrons: an energetic advantage for strictly heterofermentative lactic acid bacteria. *Applied and Environmental Microbiology*, *80*(24), 7574–7582. https://doi.org/10.1128/AEM.02413-14
61. Fitzgerald, C., Aluko, R.E., Hossain, M., Rai, D.K., Hayes, M. (2014). Potential of a renin inhibitory peptide from the red seaweed *Palmaria palmata* as a functional food ingredient following confirmation and characterization of a hypotensive effect in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry*, *62*(33), 8352–8356. https://doi.org/10.1021/jf500983n
62. Frias, J., Song, Y.S., Martínez-Villaluenga, C., De Mejia, E.G., Vidal-Valverde, C. (2008). Immunoreactivity and amino acid content of fermented soybean products. *Journal of Agricultural and Food Chemistry*, *56*(1), 99–105. https://doi.org/10.1021/jf072177j
63. Fu, W., Rao, H., Tian, Y., Xue, W. (2020). Bacterial composition in sourdoughs from different regions in China and the microbial potential to reduce wheat allergens. *LWT – Food Science and Technology*, *117*, art. no. 108669. https://doi.org/10.1016/j.lwt.2019.108669
64. Garai, G., Dueñas, M.T., Irastorza, A., Moreno-Arribas, M.V. (2007). Biogenic amine production by lactic acid bacteria isolated from cider. *Letters in Applied Microbiology*, *45*(5), 473–478. https://doi.org/10.1111/j.1472-765X.2007.02207.x
65. Garcia, C., Guerin, M., Souidi, K., Remize, F. (2020). Lactic fermented fruit or vegetable juices: past, present and future. *Beverages, 6(1)*, art. no. 8. https://doi.org/10.3390/beverages6010008
66. Garcia, E.F., de Oliveira Araújo, A., Luciano, W.A., de Albuquerque, T.M.R., de Oliveira Arcanjo, N.M., Madruga, M.S., dos Santos Lima, M., Magnani, M., Saarela, M., de Souza, E.L. (2018). The performance of five fruit-derived and freeze-dried potentially probiotic *Lactobacillus* strains in apple, orange, and grape juices. *Journal of the Science of Food and Agriculture*, *98*(13), 5000–5010. https://doi.org/10.1002/jsfa.9034
67. Garzón, A.G., Van de Velde, F., Drago, S.R. (2020). Gastrointestinal and colonic *in vitro* bioaccessibility of γ-aminobutiric acid (GABA) and phenolic compounds from novel fermented sorghum food. *LWT – Food Science and Technology*, *130*, art. no. 109664. https://doi.org/10.1016/j.lwt.2020.109664
68. Gibbs, B.F., Zougman, A., Masse, R., Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. *Food Research International*, *37*(2), 123–131. https://doi.org/10.1016/j.foodres.2003.09.010
69. Goodman R.E. (2020). *AllergenOnline vesion 20*. http://www.allergenonline.org/
70. Guiné, R.P.F., Barroca, M.J., Coldea, T.E., Bartkiene, E., Anjos, O. (2021). Apple fermented products: an overview of technology, properties and health effects. *Processes, 9(2),* art. no. 223. https://doi.org/10.3390/pr9020223
71. Guldiken, B., Toydemir, G., Nur Memis, K., 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*(6), art. no. 858. https://doi.org/10.3390/ijms17060858
72. Gupta, S., Abu-Ghannam, N. (2011). Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science and Technology, 22(6),* 315–326. https://doi.org/10.1016/j.tifs.2011.03.011

73. Gupta, S., Abu-Ghannam, N. (2012). Probiotic fermentation of plant based products: possibilities and opportunities. *Critical Reviews in Food Science and Nutrition*, *52*(2), 183–199. https://doi.org/10.1080/10408398.2010.499779
74. Habler, K., Hofer, K., Geißinger, C., Schüler, J., Hückelhoven, R., Hess, M., Gastl, M., Rychlik, M. (2016). Fate of *Fusarium* toxins during the malting process. *Journal of Agricultural and Food Chemistry*, *64*(6), 1377–1384. https://doi.org/10.1021/acs.jafc.5b05998
75. Hallmann, E., Kazimierczak, R., Marszałek, K., Drela, N., Kiernozek, E., Toomik, P., Matt, D., Luik, A., Rembiałkowska, E. (2017). The nutritive value of organic and conventional white cabbage (*Brassica Oleracea* L. Var. Capitata) and anti-apoptotic activity in gastric adenocarcinoma cells of sauerkraut juice produced therof. *Journal of Agricultural and Food Chemistry*, *65*(37), 8171–8183. https://doi.org/10.1021/acs.jafc.7b01078
76. Hermann, M. (2009). The impact of the European Novel Food Regulation on trade and food innovation based on traditional plant foods from developing countries. *Food Policy*, *34*(6), 499–507. https://doi.org/10.1016/j.foodpol.2009.08.005
77. Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66
78. Hu, Y., Li, Y., Liu, X. (2020). Soybean peptides promote yoghurt fermentation and quality. *Biotechnology Letters*, *42*(10), 1927–1937. https://doi.org/10.1007/s10529-020-02912-2
79. Huang, F., Hong, R., Zhang, R., Dong, L., Bai, Y., Liu, L., Jia, X., Wang, G., Zhang, M. (2019). Dynamic variation in biochemical properties and prebiotic activities of polysaccharides from longan pulp during fermentation process. *International Journal of Biological Macromolecules*, *132*, 915–921. https://doi.org/10.1016/j.ijbiomac.2019.04.032
80. Huang, X., Schuppan, D., Rojas Tovar, L.E., Zevallos, V.F., Loponen, J., Gänzle, M. (2020). Sourdough fermentation degrades wheat alpha-amylase/trypsin inhibitor (ATI) and reduces pro-inflammatory activity. *Foods (Basel, Switzerland)*, *9*(7), art. no. 943. https://doi.org/10.3390/foods9070943
81. Hubert, J., Berger, M., Nepveu, F., Paul, F., Daydé, J. (2008). Effects of fermentation on the phytochemical composition and antioxidant properties of soy germ. *Food Chemistry*, *109*(4), 709–721. https://doi.org/10.1016/j.foodchem.2007.12.081
82. Hussain, M.A., Knight, M.I., Britz, M.L. (2009). Proteomic analysis of lactose-starved *Lactobacillus casei* during stationary growth phase. *Journal of Applied Microbiology*, *106*(3), 764–773. https://doi.org/10.1111/j.1365-2672.2008.03961.x
83. Inomata, N., Miyakawa, M., Aihara, M. (2018). Surfing as a risk factor for sensitization to poly(γ-glutamic acid) in fermented soybeans, natto, allergy. *Allergology International*, *67*(3), 341–346. https://doi.org/10.1016/j.alit.2017.11.001
84. Isas, A.S., Mariotti Celis, M.S., Pérez Correa, J.R., Fuentes, E., Rodríguez, L., Palomo, I., Mozzi, F., Van Nieuwenhove, C. (2020). Functional fermented cherimoya (*Annona cherimola* Mill.) juice using autochthonous lactic acid bacteria. *Food Research International*, *138*, art. no. 109729. https://doi.org/10.1016/j.foodres.2020.109729
85. Jakubczyk, A., Karaś, M., Złotek, U., Szymanowska, U. (2017). Identification of potential inhibitory peptides of enzymes involved in the metabolic syndrome obtained by simulated gastrointestinal digestion of fermented bean (*Phaseolus vulgaris* L.) seeds. *Food Research International*, *100*, 489–496. https://doi.org/10.1016/j.foodres.2017.07.046
86. Jung, W.Y., Jung, J.Y., Lee, H.J., Jeon, C.O. (2016). Functional characterization of bacterial communities responsible for fermentation of doenjang: a traditional korean fermented soybean paste. *Frontiers in Microbiology*, *7*, art. no. 827. https://doi.org/10.3389/fmicb.2016.00827
87. Juodeikiene, G., Bartkiene, E., Cernauskas, D., Cizeikiene, D., Zadeike, D., Lele, V., Bartkevics, V. (2018). Antifungal activity of lactic acid bacteria and their application for *Fusarium* mycotoxin reduction in malting wheat grains. *LWT – Food Science and Technology*, *89*, 307–314. https://doi.org/10.1016/j.lwt.2017.10.061
88. Kapusta-Duch, J., Kusznierewicz, B., Leszczyńska, T., Borczak, B. (2017). Effect of package type on selected parameters of nutritional quality of chill-stored white sauerkraut. *Polish Journal of Food and Nutrition Sciences*, *67*(2), 137–144. https://doi.org/10.1515/pjfns-2016-0014
89. Khan, I., Kang, S.C. (2017). Apoptotic activity of *Lactobacillus plantarum* DGK-17-fermented soybean seed extract in human colon cancer cells via ROS–JNK signaling pathway. *Journal of Food Science*, *82*(6), 1475–1483. https://doi.org/10.1111/1750-3841.13732
90. Kheterpaul, N., Chauhan, B.M. (1991). Effect of natural fermentation on phytate and polyphenolic content and *in-vitro* digestibility of starch and protein of pearl millet (*Pennisetum typhoideum*). *Journal of the Science of Food and Agriculture*, *55*(2), 189–195. https://doi.org/10.1002/jsfa.2740550204
91. Klewicka, E., Czyżowska, A. (2011). Biological stability of lactofermented beetroot juice during refrigerated storage. *Polish Journal of Food and Nutrition Sciences*, *61*(4), 251–256. https://doi.org/10.2478/v10222-011-0028-2
92. Kun, S., Rezessy-Szabó, J.M., Nguyen, Q.D., Hoschke, Á. (2008). Changes of microbial population and some components in carrot juice during fermentation with selected *Bifidobacterium* strains. *Process Biochemistry*, *43*(8), 816–821. https://doi.org/10.1016/j.procbio.2008.03.008
93. Kwaw, E., Ma, Y., Tchabo, W., Apaliya, M.T., Wu, M., Sackey, A.S., Xiao, L., Tahir, H.E. (2018). Effect of *Lactobacillus strains* on phenolic profile, color attributes and antioxidant activities of lactic-acid-fermented mulberry juice. *Food Chemistry*, *250*, 148–154. https://doi.org/10.1016/j.foodchem.2018.01.009
94. Kwon, Y.S., Lee, S., Lee, S.H., Kim, H.J., Lee, C.H. (2019). Comparative evaluation of six traditional fermented soybean products in east asia: a metabolomics approach. *Metabolites*, *9*(9), art. no. 183. https://doi.org/10.3390/metabo9090183

95. Laatikainen, R., Koskenpato, J., Hongisto, S.-M., Loponen, J., Poussa, T., Hillilä, M., Korpela, R. (2016). Randomised clinical trial: low-FODMAP rye bread vs. regular rye bread to relieve the symptoms of irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics*, *44*(5), 460–470. https://doi.org/10.1111/apt.13726
96. Laatikainen, R., Koskenpato, J., Hongisto, S.-M., Loponen, J., Poussa, T., Huang, X., Sontag-Strohm, T., Salmenkari, H., Korpela, R. (2017). Pilot study: comparison of sourdough wheat bread and yeast-fermented wheat bread in individuals with wheat sensitivity and irritable bowel syndrome. *Nutrients*, *9*(11), art. no. 1215. https://doi.org/10.3390/nu9111215
97. Ladero, V., Coton, M., Fernández, M., Buron, N., Martín, M.C., Guichard, H., Coton, E., Alvarez, M.A. (2011). Biogenic amines content in Spanish and French natural ciders: Application of qPCR for quantitative detection of biogenic amine-producers. *Food Microbiology*, *28*(3), 554–561. https://doi.org/10.1016/j.fm.2010.11.005
98. Lee, J.H., Kim, B., Hwang, C.E., Haque, M.A., Kim, S.C., Lee, C.S., Kang, S.S., Cho, K.M., Lee, D.H. (2018). Changes in conjugated linoleic acid and isoflavone contents from fermented soymilks using *Lactobacillus plantarum* P1201 and screening for their digestive enzyme inhibition and antioxidant properties. *Journal of Functional Foods*, *43*, 17–28. https://doi.org/10.1016/j.jff.2018.01.022
99. Lee, S.M., Lee, J.Y., Cho, Y.J., Kim, M.S., Kim, Y.-S. (2018). Determination of volatiles and carotenoid degradation compounds in red pepper fermented by *Lactobacillus parabuchneri*. *Journal of Food Science*, *83*(8), 2083–2091. https://doi.org/10.1111/1750-3841.14221
100. Lee, W., Ahn, G., Lee, B.-J., Wijesinghe, W.A.J.P., Kim, D., Yang, H., Kim, Y.M., Park, S.J., Jee, Y., Jeon, Y.-J. (2013). Radio-protective effect of polysaccharides isolated from *Lactobacillus brevis*-fermented *Ecklonia cava*. *International Journal of Biological Macromolecules*, *52*, 260–266. https://doi.org/10.1016/j.ijbiomac.2012.10.004
101. Lee, Y.-C., Kung, H.-F., Huang, Y.-L., Wu, C.-H., Huang, Y.-R., Tsai, Y.-H. (2016). Reduction of biogenic amines during miso fermentation by *Lactobacillus plantarum* as a starter culture. *Journal of Food Protection*, *79*(9), 1556–1561. https://doi.org/10.4315/0362-028X.JFP-16-060
102. Li, C., Li, W., Chen, X., Feng, M., Rui, X., Jiang, M., Dong, M. (2014). Microbiological, physicochemical and rheological properties of fermented soymilk produced with exopolysaccharide (EPS) producing lactic acid bacteria strains. *LWT – Food Science and Technology*, *57*(2), 477–485. https://doi.org/10.1016/j.lwt.2014.02.025
103. Liang, J., Han, B.-Z., Nout, M.J.R., Hamer, R.J. (2008). Effects of soaking, germination and fermentation on phytic acid, total and *in vitro* soluble zinc in brown rice. *Food Chemistry*, *110*(4), 821–828. https://doi.org/10.1016/j.foodchem.2008.02.064
104. Licandro, H., Ho, P.H., Nguyen, T.K.C., Petchkongkaew, A., Nguyen, H. Van, Chu-Ky, S., Nguyen, T.V.A., Lorn, D., Waché, Y. (2020). How fermentation by lactic acid bacteria can address safety issues in legumes food products? *Food Control*, *110*, art. no. 106957. https://doi.org/10.1016/j.foodcont.2019.106957
105. Limón, R.I., Peñas, E., Torino, M.I., Martínez-Villaluenga, C., Dueñas, M., Frias, J. (2015). Fermentation enhances the content of bioactive compounds in kidney bean extracts. *Food Chemistry*, *172*, 343–352. https://doi.org/10.1016/j.foodchem.2014.09.084
106. Liu, J., Zhou, J., Wang, L., Ma, Z., Zhao, G., Ge, Z., Zhu, H., Qiao, J. (2017). Improving nitrogen source utilization from defatted soybean meal for nisin production by enhancing proteolytic function of *Lactococcus lactis* F44. *Scientific Reports*, *7*(1), art. no. 6189. https://doi.org/10.1038/s41598-017-06537-w
107. Mæhre, H.K., Malde, M.K., Eilertsen, K.E., Elvevoll, E.O. (2014). Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. *Journal of the Science of Food and Agriculture*, *94*(15), 3281–3290. https://doi.org/10.1002/jsfa.6681
108. Magishi, N., Yuikawa, N., Kobayashi, M., Taniuchi, S. (2017). Degradation and removal of soybean allergen in Japanese soy sauce. *Molecular Medicine Reports*, *16*(2), 2264–2268. https://doi.org/10.3892/mmr.2017.6815
109. Mah, J.-H., Park, Y.K., Jin, Y.H., Lee, J.-H., Hwang, H.-J. (2019). Bacterial production and control of biogenic amines in Asian fermented soybean foods. *Foods (Basel, Switzerland)*, *8*(2), art. no. 85. https://doi.org/10.3390/foods8020085
110. Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligné, B., Gänzle, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E.J., Hutkins, R. (2017). Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology*, *44*, 94–102. https://doi.org/10.1016/j.copbio.2016.11.010
111. María Landete, J., Hernández, T., Robredo, S., Dueñas, M., de las Rivas, B., Estrella, I., Muñoz, R. (2015). Effect of soaking and fermentation on content of phenolic compounds of soybean (*Glycine max* cv. Merit) and mung beans (*Vigna radiata* [L] Wilczek). *International Journal of Food Sciences and Nutrition*, *66*(2), 203–209. https://doi.org/10.3109/09637486.2014.986068
112. Markkinen, N., Laaksonen, O., Nahku, R., Kuldjärv, R., Yang, B. (2019). Impact of lactic acid fermentation on acids, sugars, and phenolic compounds in black chokeberry and sea buckthorn juices. *Food Chemistry*, *286*, 204–215. https://doi.org/10.1016/j.foodchem.2019.01.189
113. Martinez-Villaluenga, C., Peñas, E., Frias, J., Ciska, E., Honke, J., Piskula, M.K., Kozlowska, H., Vidal-Valverde, C. (2009). Influence of fermentation conditions on glucosinolates, ascorbigen, and ascorbic acid content in white cabbage (*Brassica oleracea* var. capitata cv. Taler) cultivated in different seasons. *Journal of Food Science*, *74*(1), C62–C67. https://doi.org/10.1111/j.1750-3841.2008.01017.x
114. Martins, E.M.F., Ramos, A.M., Vanzela, E.S.L., Stringheta, P.C., de Oliveira Pinto, C.L., Martins, J.M. (2013). Products of vegetable origin: A new alternative for the consumption of probiotic bacteria. *Food Research International*, *51*(2), 764–770. https://doi.org/10.1016/j.foodres.2013.01.047
115. Matejčeková, Z., Mikulajová, A., Vlková, E., Liptáková, D., Mošovská, S., Hybenová, E., Valík, Ľ. (2019). Design of bacte-

rial cultures in fermented functional maize product formulation. *Polish Journal of Food and Nutrition Sciences*, *69*(4), 417–426. https://doi.org/10.31883/pjfns/112642

116. Meléndez-Martínez, A.J. (2019). An overview of carotenoids, apocarotenoids, and vitamin A in agro-food, nutrition, health, and disease. *Molecular Nutrition & Food Research*, *63*(15), art. no. 1801045. https://doi.org/10.1002/mnfr.201801045
117. Mihhalevski, A., Nisamedtinov, I., Hälvin, K., Ošeka, A., Paalme, T. (2013). Stability of B-complex vitamins and dietary fiber during rye sourdough bread production. *Journal of Cereal Science*, *57*(1), 30–38. https://doi.org/10.1016/j.jcs.2012.09.007
118. Minervini, F., De Angelis, M., Di Cagno, R., Gobbetti, M. (2014). Ecological parameters influencing microbial diversity and stability of traditional sourdough. *International Journal of Food Microbiology*, *171*, 136–146. https://doi.org/10.1016/j.ijfoodmicro.2013.11.021
119. Mo, H., Kariluoto, S., Piironen, V., Zhu, Y., Sanders, M.G., Vincken, J.-P., Wolkers-Rooijackers, J., Nout, M.J.R. (2013). Effect of soybean processing on content and bioaccessibility of folate, vitamin B12 and isoflavones in tofu and tempe. *Food Chemistry*, *141*(3), 2418–2425. https://doi.org/10.1016/j.foodchem.2013.05.017
120. Moon, S.H., Kim, C.R., Chang, H.C. (2018). Heterofermentative lactic acid bacteria as a starter culture to control kimchi fermentation. *LWT – Food Science and Technology*, *88*, 181–188. https://doi.org/10.1016/j.lwt.2017.10.009
121. Mullaney, J.A., Kelly, W.J., McGhie, T.K., Ansell, J., Heyes, J.A. (2013). Lactic acid bacteria convert glucosinolates to nitriles efficiently yet differently from *Enterobacteriaceae*. *Journal of Agricultural and Food Chemistry*, *61*(12), 3039–3046. https://doi.org/10.1021/jf305442j
122. Mustafa, S.M., Chua, L.S., El-Enshasy, H.A. (2019). Effects of agitation speed and kinetic studies on probiotication of pomegranate juice with *Lactobacillus casei*. *Molecules (Basel, Switzerland)*, *24*(13), art. no. 2357. https://doi.org/10.3390/molecules24132357
123. Nachi, I., Fhoula, I., Smida, I., Ben Taher, I., Chouaibi, M., Jaunbergs, J., Bartkevics, V., Hassouna, M. (2018). Assessment of lactic acid bacteria application for the reduction of acrylamide formation in bread. *LWT – Food Science and Technology*, *92*, 435–441. https://doi.org/10.1016/j.lwt.2018.02.061
124. Nasiri E.B., Kadivar, M., Shahedi, M., Soleimanian-Zad, S. (2017). Reduction of acrylamide in whole-wheat bread by combining lactobacilli and yeast fermentation. *Food Additives & Contaminants: Part A*, *34*(11), 1904–1914. https://doi.org/10.1080/19440049.2017.1378444
125. Nawaz, K.A.A., David, S.M., Murugesh, E., Thandeeswaran, M., Kiran, K.G., Mahendran, R., Palaniswamy, M., Angayarkanni, J. (2017). Identification and *in silico* characterization of a novel peptide inhibitor of angiotensin converting enzyme from pigeon pea (*Cajanus cajan*). *Phytomedicine*, *36*, 1–7. https://doi.org/10.1016/j.phymed.2017.09.013
126. Nguyen, T.T.T., Loiseau, G., Icard-Vernière, C., Rochette, I., Trèche, S., Guyot, J.-P. (2007). Effect of fermentation by amylolytic lactic acid bacteria, in process combinations, on characteristics of rice/soybean slurries: A new method for preparing high energy density complementary foods for young children. *Food Chemistry*, *100*(2), 623–631. https://doi.org/10.1016/j.foodchem.2005.09.080
127. Oloo, B.O., Shitandi, A., Mahungu, S., Malinga, J.B., Rose, O.B. (2014). Effects of lactic acid fermentation on the retention of beta-carotene content in orange fleshed sweet potatoes. *International Journal of Food Studies*, *3*(1), 13–33. https://doi.org/10.7455/ijfs/3.1.2014.a2
128. Omoba, O.S., Isah, L.R. (2018). Influence of sourdough fermentation on amino acids composition, phenolic profile, and antioxidant properties of sorghum biscuits. *Preventive Nutrition and Food Science*, *23*(3), 220–227. https://doi.org/10.3746/pnf.2018.23.3.220
129. Palani, K., Harbaum-Piayda, B., Meske, D., Keppler, J.K., Bockelmann, W., Heller, K.J., Schwarz, K. (2016). Influence of fermentation on glucosinolates and glucobrassicin degradation products in sauerkraut. *Food Chemistry*, *190*, 755–762. https://doi.org/10.1016/j.foodchem.2015.06.012
130. Palla, M., Cristani, C., Giovannetti, M., Agnolucci, M. (2017). Identification and characterization of lactic acid bacteria and yeasts of PDO Tuscan bread sourdough by culture dependent and independent methods. *International Journal of Food Microbiology*, *250*, 19–26. https://doi.org/10.1016/j.ijfoodmicro.2017.03.015
131. Pan, R., Xu, T., Bai, J., Xia, S., Liu, Q., Li, J., Xiao, X., Dong, Y. (2020). Effect of *Lactobacillus plantarum* fermented barley on plasma glycolipids and insulin sensitivity in subjects with metabolic syndrome. *Journal of Food Biochemistry*, *44*(11), art. no. e13471. https://doi.org/10.1111/jfbc.13471
132. Paramithiotis, S., Doulgeraki, A.I., Tsilikidis, I., Nychas, G.-J.E., Drosinos, E.H. (2012). Fate of *Listeria monocytogenes* and *Salmonella Typhimurium* during spontaneous cauliflower fermentation. *Food Control*, *27*(1), 178–183. https://doi.org/10.1016/j.foodcont.2012.03.022
133. Pejcz, E., Czaja, A., Wojciechowicz-Budzisz, A., Gil, Z., Spychaj, R. (2017). The potential of naked barley sourdough to improve the quality and dietary fibre content of barley enriched wheat bread. *Journal of Cereal Science*, *77*, 97–101. https://doi.org/10.1016/j.jcs.2017.08.007
134. Pejcz, E., Spychaj, R., Gil, Z. (2020). Technological methods for reducing the content of fructan in rye bread. *European Food Research and Technology*, *246*(9), 1839–1846. https://doi.org/10.1007/s00217-020-03537-5
135. Pepper, S.J., Britz, M.L. (2019). An acid up-regulated surface protein of *Lactobacillus paracasei* Strain GCRL 46 is phylogenetically related to the secreted glucan- (gpbb) and immunoglobulin-binding (siba) protein of pathogenic streptococci. *International Journal of Molecular Sciences*, *20*(7), art. no. 1610. https://doi.org/10.3390/ijms20071610
136. Pereira, H.S., Leão-Ferreira, L.R., Moussatché, N., Teixeira, V.L., Cavalcanti, D.N., Costa, L.J., Diaz, R., Frugulhetti, I.C.P.P. (2004). Antiviral activity of diterpenes isolated from the Brazilian marine alga *Dictyota menstrualis* against human immunodeficiency virus type 1 (HIV-1). *Antiviral Research*, *64*(1), 69–76. https://doi.org/10.1016/j.antiviral.2004.06.006
137. Pereira, L., Critchley, A.T. (2020). The COVID 19 novel coronavirus pandemic 2020: seaweeds to the rescue? Why does substantial, supporting research about the antiviral properties of seaweed polysaccharides seem to go unrecognized

by the pharmaceutical community in these desperate times? *Journal of Applied Phycology*, *32*(3), 1875–1877. https://doi.org/10.1007/s10811-020-02143-y

138. Petkova, M., Stefanova, P., Gotcheva, V., Kuzmanova, I., Angelov, A. (2020). Microbiological and physicochemical characterization of traditional Bulgarian sourdoughs and screening of lactic acid bacteria for amylolytic activity. *Journal of Chemical Technology and Metallurgy*, *55*(5), 921–934.
139. Pistarino, E., Aliakbarian, B., Casazza, A.A., Paini, M., Cosulich, M.E., Perego, P. (2013). Combined effect of starter culture and temperature on phenolic compounds during fermentation of Taggiasca black olives. *Food Chemistry*, *138*(2–3), 2043–2049. https://doi.org/10.1016/j.foodchem.2012.11.021
140. Quirante-Moya, S., García-Ibañez, P., Quirante-Moya, F., Villaño, D., Moreno, D.A. (2020). The role of brassica bioactives on human health: are we studying it the right way? *Molecules (Basel, Switzerland)*, *25*(7), art. no. 1591. https://doi.org/10.3390/molecules25071591
141. Ramnani, P., Chitarrari, R., Tuohy, K., Grant, J., Hotchkiss, S., Philp, K., Campbell, R., Gill, C., Rowland, I. (2012). *In vitro* fermentation and prebiotic potential of novel low molecular weight polysaccharides derived from agar and alginate seaweeds. *Anaerobe*, *18*(1), 1–6. https://doi.org/10.1016/j.anaerobe.2011.08.003
142. Rathore, S., Salmerón, I., Pandiella, S.S. (2012). Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. *Food Microbiology*, *30*(1), 239–244. https://doi.org/10.1016/j.fm.2011.09.001
143. Rekha, C.R., Vijayalakshmi, G. (2010). Bioconversion of isoflavone glycosides to aglycones, mineral bioavailability and vitamin B complex in fermented soymilk by probiotic bacteria and yeast. *Journal of Applied Microbiology*, *109*(4), 1198–1208. https://doi.org/10.1111/j.1365-2672.2010.04745.x
144. Rianingsih, L., Sumardianto (2020). Antioxidant activity in seaweed (*Sargassum sp.*) extract fermented with *Lactobacillus plantarum* and *Lactobacillus acidophilus*. *Earth and Environmental Science, 530,* art. no. 012011. https://doi.org/10.1088/1755-1315/530/1/012011
145. Ricci, A., Cirlini, M., Calani, L., Bernini, V., Neviani, E., Del Rio, D., Galaverna, G., Lazzi, C. (2019). *In vitro* metabolism of elderberry juice polyphenols by lactic acid bacteria. *Food Chemistry*, *276*, 692–699. https://doi.org/10.1016/j.foodchem.2018.10.046
146. Rodríguez-Jasso, R.M., Mussatto, S.I., Sepúlveda, L., Agrasar, A.T., Pastrana, L., Aguilar, C.N., Teixeira, J.A. (2013). Fungal fucoidanase production by solid-state fermentation in a rotating drum bioreactor using algal biomass as substrate. *Food and Bioproducts Processing*, *91*(4), 587–594. https://doi.org/10.1016/j.fbp.2013.02.004
147. Salmerón, I., Thomas, K., Pandiella, S.S. (2015). Effect of potentially probiotic lactic acid bacteria on the physicochemical composition and acceptance of fermented cereal beverages. *Journal of Functional Foods*, *15*, 106–115. https://doi.org/10.1016/j.jff.2015.03.012
148. Samtiya, M., Aluko, R.E., Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: an overview. *Food Production, Processing and Nutrition*, *2*(1), art. no. 6. https://doi.org/10.1186/s43014-020-0020-5
149. Sanjukta, S., Rai, A.K. (2016). Production of bioactive peptides during soybean fermentation and their potential health benefit. *Trends in Food Science & Technology*, *50*, 1–10. hhttps://doi.org/10.1016/j.tifs.2016.01.010
150. Santamaría, L., Reverón, I., de Felipe, F.L., de las Rivas, B., Muñoz, R. (2018). Ethylphenol formation by *Lactobacillus plantarum*: Identification of the enzyme involved in the reduction of vinylphenols. *Applied and Environmental Microbiology*, *84*(17), art. no. e01064–18. https://doi.org/10.1128/AEM.01064-18
151. Santamaría, L., Reverón, I., López de Felipe, F., de las Rivas, B., Muñoz, R. (2018). Unravelling the reduction pathway as an alternative metabolic route to hydroxycinnamate decarboxylation in *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, *84*(15), art. no. e01123–18. https://doi.org/10.1128/AEM.01123-18
152. Santos, C.C.A. do A., Libeck, B. da S., Schwan, R.F. (2014). Co-culture fermentation of peanut-soy milk for the development of a novel functional beverage. *International Journal of Food Microbiology*, *186*, 32–41. https://doi.org/10.1016/j.ijfoodmicro.2014.06.011
153. Seo, H.S., Lee, S., Singh, D., Shin, H.W., Cho, S.A., Lee, C.H. (2018). Untargeted metabolite profiling for koji-fermentative bioprocess unravels the effects of varying substrate types and microbial inocula. *Food Chemistry*, *266*, 161–169. https://doi.org/10.1016/j.foodchem.2018.05.048
154. Shobharani, P., Halami, P.M., Sachindra, N.M. (2013). Potential of marine lactic acid bacteria to ferment *Sargassum sp.* for enhanced anticoagulant and antioxidant properties. *Journal of Applied Microbiology*, *114*(1), 96–107. https://doi.org/10.1111/jam.12023
155. Singh, R.S., Thakur, S.R., Bansal, P. (2015). Algal lectins as promising biomolecules for biomedical research. *Critical Reviews in Microbiology*, *41*(1), 77–88. hhttps://doi.org/10.3109/1040841X.2013.798780
156. Song, J.L., Choi, J.H., Seo, J.H., Lim, Y.I., Park, K.Y. (2014). Anti-colitic effects of kanjangs (fermented soy sauce and sesame sauce) in dextran sulfate sodium-induced colitis in mice. *Journal of Medicinal Food*, *17*(9), 1027–1035. https://doi.org/10.1089/jmf.2013.3119
157. Soundararajan, P., Kim, J.S. (2018). Anti-carcinogenic glucosinolates in cruciferous vegetables and their antagonistic effects on prevention of cancers. *Molecules, 23*(11), art. no. 2983. https://doi.org/10.3390/molecules23112983
158. Spaggiari, M., Ricci, A., Calani, L., Bresciani, L., Neviani, E., Dall'Asta, C., Lazzi, C., Galaverna, G. (2020). Solid state lactic acid fermentation: A strategy to improve wheat bran functionality. *LWT – Food Science and Technology*, *118*, art. no. 108668. https://doi.org/10.1016/j.lwt.2019.108668
159. Starzyńska–Janiszewska, A., Stodolak, B. (2011). Effect of inoculated lactic acid fermentation on antinutritional and antiradical properties of grass pea (*Lathyrus sativus* 'Krab') flour. *Polish Journal of Food and Nutrition Sciences*, *61*(4), 245–249. https://doi.org/10.2478/v10222-011-0027-3
160. Stefańska, I., Piasecka-Jóźwiak, K., Kotyrba, D., Kolenda, M., Stecka, K.M. (2016). Selection of lactic acid bacteria strains for the hydrolysis of allergenic proteins of wheat flour. *Journal of the Science of Food and Agriculture*, *96*(11), 3897–3905. https://doi.org/10.1002/jsfa.7588

161. Su, L.-W., Cheng, Y.-H., Hsiao, F.S.-H., Han, J.-C., Yu, Y.-H. (2018). Optimization of mixed solid-state fermentation of soybean meal by *Lactobacillus* species and *Clostridium butyricum*. *Polish Journal of Microbiology*, *67*(3), 297–305. https://doi.org/10.21307/pjm-2018-035
162. Tabaszewska, M., Gabor, A., Jaworska, G., Drożdż, I. (2018). Effect of fermentation and storage on the nutritional value and contents of biologically-active compounds in lacto-fermented white asparagus (*Asparagus officinalis* L.). *LWT – Food Science and Technology*, *92*, 67–72. https://doi.org/10.1016/j.lwt.2018.02.003
163. Thomas, I., Siew, L.Q.C., Watts, T.J., Haque, R. (2019). Seaweed allergy. *Journal of Allergy and Clinical Immunology: In Practice*, *7*(2), 714–715. https://doi.org/10.1016/j.jaip.2018.11.009
164. Toi, M., Hirota, S., Tomotaki, A., Sato, N., Hozumi, Y., Anan, K., Nagashima, T., Tokuda, Y., Masuda, N., Ohsumi, S., Ohno, S., Takahashi, M., Hayashi, H., Yamamoto, S., Ohashi, Y. (2013). Probiotic beverage with soy isoflavone consumption for breast cancer prevention: a case-control study. *Current Nutrition and Food Science*, *9*(3), 194–200. https://doi.org/10.2174/15734013113099990001
165. Toktaş, B., Bildik, F., Özçelik, B. (2018). Effect of fermentation on anthocyanin stability and *in vitro* bioaccessibility during shalgam (şalgam) beverage production. *Journal of the Science of Food and Agriculture*, *98*(8), 3066–3075. https://doi.org/10.1002/jsfa.8806
166. Tong, L.-T., Zhong, K., Liu, L., Zhou, X., Qiu, J., Zhou, S. (2015). Effects of dietary hull-less barley β-glucan on the cholesterol metabolism of hypercholesterolemic hamsters. *Food Chemistry*, *169*, 344–349. https://doi.org/10.1016/j.foodchem.2014.07.157
167. Torres-Pitarch, A., Gardiner, G.E., Cormican, P., Rea, M., Crispie, F., O'Doherty, J.V., Cozannet, P., Ryan, T., Cullen, J., Lawlor, P.G. (2020). Effect of cereal fermentation and carbohydrase supplementation on growth, nutrient digestibility and intestinal microbiota in liquid-fed grow-finishing pigs. *Scientific Reports*, *10*(1), art. no. 13716. https://doi.org/10.1038/s41598-020-70443-x
168. Unlu, N.Z., Bohn, T., Francis, D.M., Nagaraja, H.N., Clinton, S.K., Schwartz, S.J. (2007). Lycopene from heat-induced cis-isomer-rich tomato sauce is more bioavailable than from all-trans-rich tomato sauce in human subjects. *British Journal of Nutrition*, *98*(1), 140–146. https://doi.org/10.1017/S0007114507685201
169. Uyttendaele, M., Jaykus, L.-A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Rao Jasti, P. (2015). Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. *Comprehensive Reviews in Food Science and Food Safety*, *14*(4), 336–356. https://doi.org/10.1111/1541-4337.12133
170. Van Beeck, W., Verschueren, C., Wuyts, S., van den Broek, M.F.L., Uyttendaele, M., Lebeer, S. (2020). Robustness of fermented carrot juice against *Listeria monocytogenes*, *Salmonella Typhimurium* and *Escherichia coli* O157:H7. *International Journal of Food Microbiology*, *335*, art. no. 108854. https://doi.org/10.1016/j.ijfoodmicro.2020.108854
171. Vasiee, A., Falah, F., Behbahani, B.A., Tabatabaee-Yazdi, F. (2020). Probiotic characterization of *Pediococcus strains* isolated from Iranian cereal-dairy fermented product: Interaction with pathogenic bacteria and the enteric cell line Caco-2. *Journal of Bioscience and Bioengineering*, *130*(5), 471–479. https://doi.org/10.1016/j.jbiosc.2020.07.002
172. Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M.J., Spencer, J.P.E. (2010). Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients*, *2*(11), 1106–1131. https://doi.org/10.3390/nu2111106
173. Verni, M., Demarinis, C., Rizzello, C.G., Baruzzi, F. (2020). Design and characterization of a novel fermented beverage from lentil grains. *Foods*, *9*(7), art. no. 893. https://doi.org/10.3390/foods9070893
174. Verni, M., Rizzello, C.G., Coda, R. (2019). Fermentation biotechnology applied to cereal industry by-products: nutritional and functional insights. *Frontiers in Nutrition, (6),* art. no. 42. https://doi.org/10.3389/fnut.2019.00042
175. Verón, H.E., Gauffin Cano, P., Fabersani, E., Sanz, Y., Isla, M.I., Fernández Espinar, M.T., Gil Ponce, J.V., Torres, S. (2019). Cactus pear (*Opuntia ficus-indica*) juice fermented with autochthonous *Lactobacillus plantarum* S-811. *Food & Function*, *10*(2), 1085–1097. https://doi.org/10.1039/C8FO01591K
176. Wacoo, A.P., Mukisa, I.M., Meeme, R., Byakika, S., Wendiro, D., Sybesma, W., Kort, R. (2019). Probiotic enrichment and reduction of aflatoxins in a traditional African maize-based fermented food. *Nutrients*, *11*(2), art. no. 265. https://doi.org/10.3390/nu11020265
177. Wang, J., Zhao, X., Tian, Z., He, C., Yang, Y., Yang, Z. (2015). Isolation and characterization of exopolysaccharide-producing *Lactobacillus plantarum* SKT109 from Tibet Kefir. *Polish Journal of Food and Nutrition Sciences*, *65*(4), 269–279. https://doi.org/10.1515/pjfns-2015-0023
178. Wiczkowski, W., Szawara-Nowak, D., Romaszko, J. (2016). The impact of red cabbage fermentation on bioavailability of anthocyanins and antioxidant capacity of human plasma. *Food Chemistry*, *190*, 730–740. https://doi.org/10.1016/j.foodchem.2015.06.021
179. Wiczkowski, W., Szawara-Nowak, D., Topolska, J. (2015). Changes in the content and composition of anthocyanins in red cabbage and its antioxidant capacity during fermentation, storage and stewing. *Food Chemistry*, *167*, 115–123. https://doi.org/10.1016/j.foodchem.2014.06.087
180. Wronkowska, M., Jeliński, T., Majkowska, A., Zieliński, H. (2018). Physical properties of buckwheat water biscuits formulated from fermented flours by selected lactic acid bacteria. *Polish Journal of Food and Nutrition Sciences*, *68*(1), 25–31. https://doi.org/10.1515/pjfns-2017-0027
181. Xia, J., Zu, Q., Yang, A., Wu, Z., Li, X., Tong, P., Yuan, J., Wu, Y., Fan, Q., Chen, H. (2019). Allergenicity reduction and rheology property of *Lactobacillus*-fermented soymilk. *Journal of the Science of Food and Agriculture*, *99*(15), 6841–6849. https://doi.org/10.1002/jsfa.9969
182. Xiao, X., Tan, C., Sun, X., Zhao, Y., Zhang, J., Zhu, Y., Bai, J., Dong, Y., Zhou, X. (2020). Effects of fermentation on structural characteristics and *in vitro* physiological activities of barley β-glucan. *Carbohydrate Polymers*, *231*, art. no. 115685. https://doi.org/10.1016/j.carbpol.2019.115685

183. Xiao, Y., Zhang, S., Tong, H., Shi, S. (2018). Comprehensive evaluation of the role of soy and isoflavone supplementation in humans and animals over the past two decades. *Phytotherapy Research*, *32*(3), 384–394. https://doi.org/10.1002/ptr.5966

184. Xu, Y., Coda, R., Shi, Q., Tuomainen, P., Katina, K., Tenkanen, M. (2017). Exopolysaccharides production during the fermentation of soybean and fava bean flours by *Leuconostoc mesenteroides* DSM 20343. *Journal of Agricultural and Food Chemistry*, *65*(13), 2805–2815. https://doi.org/10.1021/acs.jafc.6b05495

185. Xu, Y., Zhou, T., Tang, H., Li, X., Chen, Y., Zhang, L., Zhang, J. (2020). Probiotic potential and amylolytic properties of lactic acid bacteria isolated from Chinese fermented cereal foods. *Food Control*, *111*, art. no. 107057. https://doi.org/10.1016/j.foodcont.2019.107057

186. Yagmur, G., Tanguler, H., Leventdurur, S., Elmaci, S.B., Turhan, E.U., Francesca, N., Settanni, L., Moschetti, G., Erten, H. (2016). Identification of predominant lactic acid bacteria and yeasts of turkish sourdoughs and selection of starter cultures for liquid sourdough production using different flours and dough yields. *Polish Journal of Food and Nutrition Sciences*, *66*(2), 99–107. https://doi.org/10.1515/pjfns-2015-0041

187. Yang, A., Zuo, L., Cheng, Y., Wu, Z., Li, X., Tong, P., Chen, H. (2018). Degradation of major allergens and allergenicity reduction of soybean meal through solid-state fermentation with microorganisms. *Food and Function*, *9*(3), 1899–1909. https://doi.org/10.1039/C7FO01824J

188. Yang, J., Ji, Y., Park, H., Lee, J., Park, S., Yeo, S., Shin, H., Holzapfel, W.H. (2014). Selection of functional lactic acid bacteria as starter cultures for the fermentation of Korean leek (*Allium tuberosum* Rottler ex Sprengel.). *International Journal of Food Microbiology*, *191*, 164–171. https://doi.org/10.1016/j.ijfoodmicro.2014.09.016

189. Ye, J.-H., Huang, L.-Y., Terefe, N.S., Augustin, M.A. (2019). Fermentation-based biotransformation of glucosinolates, phenolics and sugars in retorted broccoli puree by lactic acid bacteria. *Food Chemistry*, *286*, 616–623. https://doi.org/10.1016/j.foodchem.2019.02.030

190. Zadeike, D., Vaitkeviciene, R., Bartkevics, V., Bogdanova, E., Bartkiene, E., Lele, V., Juodeikiene, G., Cernauskas, D., Valatkeviciene, Z. (2021). The expedient application of microbial fermentation after whole-wheat milling and fractionation to mitigate mycotoxins in wheat-based products. *LWT – Food Science and Technology*, *137*, art. no. 110440. hhttps://doi.org/10.1016/j.lwt.2020.110440

191. Zaunmüller, T., Eichert, M., Richter, H., Unden, G. (2006). Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. *Applied Microbiology and Biotechnology*, *72*(3), 421–429. https://doi.org/10.1007/s00253-006-0514-3

192. Zhang, Z., Fan, S., Huang, D., Xiong, T., Nie, S., Xie, M. (2020). Polysaccharides from fermented *Asparagus officinalis* with *Lactobacillus plantarum* NCU116 alleviated liver injury *via* modulation of glutathione homeostasis, bile acid metabolism, and SCFA production. *Food & Function*, *11*(9), 7681–7695. https://doi.org/10.1039/D0FO01435D

193. Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107

194. Zheng, X.-F., Yang, Z., Zhang, H., Jin, W.-X., Xu, C.-W., Gao, L., Rao, S.-Q., Jiao, X. (2020). Isolation of virulent phages infecting dominant mesophilic aerobic bacteria in cucumber pickle fermentation. *Food Microbiology*, *86*, art. no. 103330. https://doi.org/10.1016/j.fm.2019.103330

195. Zhou, C., Li, J., Mao, K., Gao, J., Li, X., Zhi, T., Sang, Y. (2019). Anti-hangover and anti-hypertensive effects *in vitro* of fermented persimmon juice. *CyTA – Journal of Food*, *17*(1), 960–966. https://doi.org/10.1080/19476337.2019.1680578

196. Zhou, Y., Wang, R., Zhang, Y., Yang, Y., Sun, X., Zhang, Q., Yang, N. (2020). Biotransformation of phenolics and metabolites and the change in antioxidant activity in kiwifruit induced by *Lactobacillus plantarum* fermentation. *Journal of the Science of Food and Agriculture*, *100*(8), 3283–3290. https://doi.org/10.1002/jsfa.10272

197. Zieliński, H., Honke, J., Topolska, J., Bączek, N., Piskuła, M.K., Wiczkowski, W., Wronkowska, M. (2020). ACE inhibitory properties and phenolics profile of fermented flours and of baked and digested biscuits from buckwheat. *Foods, 9(7),* art. no. 847. https://doi.org/10.3390/foods9070847

198. 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

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 135–145
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/134393
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Kinetic Modelling of Betalain Stability and Color Changes in Yogurt During Storage

Onur Guneser

Department of Food Engineering, Engineering Faculty, Uşak University, 1 Eylül Campus, 64000, Uşak-Turkey

Key words: natural pigments, betalains, yogurt, reaction kinetics, color

Assessment of the storage stability of betalains added to food during processing is crucial to estimate the shelf-life of colored food products and the potency of natural food colorants. The stability of beetroot betalains in yogurt during storage was evaluated in this study. Kinetic experiments were conducted at storage temperatures of 4°C, 10°C, and 20°C. The relationships were also determined between the betalain degradation and lightness (L^*), redness (a^*), and yellowness (b^*). First-order kinetics was observed in the betalain degradation, and the changes in color parameters of the yogurt samples fitted zero-order kinetics. The activation energy required for the degradation of betalains and changes in L^*, a^*, and b^* was found as 104.9, 67.6, 76.5, and 86.1 kJ/mol, respectively. The half-life period of the degradation of red beet betalains was found as 51.43, 30.91, and 4.54 days at 4°C, 10°C, and 20°C, respectively. Multiple linear regression models were also established for betalain content and color parameters. There was a decrease in betalain content and a^* color value in the yogurt colored with a beetroot extract during storage. A significant positive correlation was found between pH, a^* value, and betalain content in yogurt, while a significant negative correlation was found between betalain content and L^* and b^* values. Further studies need to be carried out to reveal the relationship between color parameters and natural pigments in food systems.

INTRODUCTION

Since the last decade, natural pigments have attracted attention of the manufacturers in the food industry. The trend has shifted from artificial colorants to natural pigments owing to scientific studies regarding the potential risks of synthetic colorants to consumer health. At present, many scientific studies are being performed on natural pigments, and food manufacturers are trying to use them in food systems [Amchova *et al.,* 2015; Galaffu *et al.,* 2015]. Anthocyanins are the most studied natural pigments originating from plants, while betalains, carotenoids, chlorophylls, and curcumin are some other natural pigments that also offer beneficial health effects, such as preventing obesity [Martins *et al.*, 2016].

Betalains are heterocyclic derivatives of betalamic acids. They are divided into two categories, namely: betacyanins and betaxanthins. Betacyanins exhibit red to purple hues, while betaxanthins exhibit yellow to orange hues. Beetroot (*Beta vulgaris* L. ssp. *vulgaris*), colored Swiss chard (*B. vulgaris* L. ssp. *cicla*), amaranth (*Amarathus* sp.), cactus fruit (*Opuntia* sp.), pitayas (*Stenocereu* ssp.), and pitahayas (*Hylocereus undatus*) are the main plant sources of betalains. *Amanita muscaria* (fly agaric, a higher fungus) is also their natural source [Azeredo, 2009; Bárta *et al.,* 2020; Delgado-Vargas *et al.,* 2000; Gengatharan *et al.,* 2015]. Betalains have also gained interest owing to their health-promoting properties such as anti-atherogenic, anti-carcinogenic, anti-inflammatory, and hypolipidemic activities, along with colorant properties in food applications [Bárta *et al.,* 2020; Delgado-Vargas *et al.,* 2000; Moreno *et al.,* 2008]. Red beetroot is the commercial source of red colored betalains including betanin and isobetanin. Therefore, a beetroot extract or/and juice concentrate is used as a food colorant in many food products, such as dairy-based snacks, with E number 162 (E-162) [Azeredo, 2009; Herbach *et al.,* 2006].

Although the potential use of natural pigments is high, their application in foods is limited due to their low stability, weak tinctorial strength, strong interactions with food ingredients, and inability to match desired hues [Sigurdson *et al.,* 2017]. In this context, although betalains have certain pharmacological activities and color properties, it can be said that their main drawback is their strong earth-like aroma. Several types of studies are ongoing for their applicability in food systems using various techniques, such as co-pigmentation. Betalains as natural colorants in real food systems are less explored. A recent study [Gengatharan *et al.,* 2017] has shown the effects of pH and refrigerated storage on the stability of a colorant extract obtained from red pitahaya in yogurt. The degradation rate of betacyanin in yogurts containing the colorant extract at 14 days of refrigerated storage was 1.0%, while a loss of 1.6% betacyanin was observed in yogurt colored with a commercial

* Corresponding Author: Tel: +90 276 221 21 21 Ext: 2754;
E-mail: onur.guneser@usak.edu.tr (Dr. O. Guneser)

Submitted: 16 December 2020
Accepted: 16 March 2021
Published on-line: 20 April 2021

colorant E-162. This study also showed that the extract from red pitahaya treated at pH 4 and 5 caused a lower reduction of betacyanins compared to E-162 in yogurt during ten weeks of refrigerated storage at 4°C.

Betalains are stable at pH ranging from 3 to 7 compared with anthocyanins, and are suitable coloring agents that can be stabilized by ascorbic acid [Sigurdson *et al.,* 2017]. Herbach *et al.* [2007] reported that the betacyanins present in purple pitaya (*Hylocereus polyrhizus*) could be easily stabilized by 1% ascorbic acid. In contrast, Karangutkar & Ananthanarayan [2021] found that 0.05% of ascorbic acid reduced betacyanin content in *Basella rubra* in a model beverage system during storage because of its pro-oxidant effect. Moreover, the addition of 5 mM (+)-catechin in a model beverage system was found to fulfill the maximum pigment retention at low temperature (4°C), and in the absence of light and oxygen.

Assessment of the thermal and storage stabilities of betalains added in real food systems during processing is crucial to estimate the shelf-life of colored food products and the potency of natural food colorants [Güneşer, 2016]. This study aimed to evaluate the stabilities of beetroot betalains in yogurt during storage at 4°C, 10°C, and 20°C using a chemical kinetics approach. The relationships between betalain content, color parameters, and pH during storage were determined using multiple linear regression and correlation analyses.

MATERIALS AND METHODS

Materials

Beetroot betalains used in the present study were acquired from a commercial natural liquid colorant from beetroot (*Wild Flavors,* ADM Wild GmbH, Eppelheim, Germany) that was obtained from NANTE Chemical Company (Istanbul, Turkey). Cow milk for yogurt production was obtained from a local producer (Usak, Turkey). The yogurt starter culture (*Büyüyo Yogurt Culture,* a mixed culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* + *Streptococcus thermophiles* + *Lactobacillus acidophilus*) was obtained from Danem Dairy Company (Isparta, Turkey). All chemicals were of analytical/chromatographic grade and were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Production of yogurt with beetroot betalains

Yogurt was prepared according to the procedure of Yiğit *et al.* [2011]. Cow milk (6 L) was blended using a hand blender to get a homogeneous matrix. Then, the milk was distributed in three glass jars. The glass jars were placed in a water bath and heated to 85±2°C for 30 min. Then, they were placed in an ice-water bath for cooling. When the temperature of the glass jars had reached at 43±2°C, the beetroot colorant (3 g/L) and the yogurt starter culture (0.25 g/L) were added into each glass jar aseptically and then mixed by a hand blender at a medium speed (2 g force). The inoculated milk was poured into 100-g plastic cups (polypropylene) with lids (polyethylene terephthalate) and incubated at 43±2°C in an incubator (Nüve-ES 120, Ankara, Turkey) until pH= 4.7. After incubation, the yogurt samples were immediately shifted to refrigerated incubators for further experimentation. The procedures for the preparation of the yogurt samples were established based on the standard yogurt production steps [Tamime & Robinson, 2007]. Yogurt production was performed in duplicate. The amount of commercial beetroot colorant added into yogurt was determined by pre-coloring experiments and the recommendations of the producer. Moreover, in the Turkish Food Codex [TFC, 2013], the limit of beetroot colorant use was determined as a *quantum satis*. The inoculation rate of the yogurt culture was based on the recommendations of the Danem Dairy Company.

Storage experiment

The storage stability and changes in the color parameters of beetroot betalains in yogurt were examined at 4°C, 10°C, and 20°C for 60, 40, and 20 days, respectively, in refrigerated incubators (Nüve-ES 120, Ankara, Turkey and Memmert IPP500, Schwabach, Germany). The temperatures for yogurt storage were chosen by considering possible facilities such as transport, storage, and retail temperature conditions that consumers and manufacturers generally use for yogurt (4°C as storage temperature, 10°C as refrigerator temperature, and 20°C as cool ambient temperature). During storage, the yogurt samples were taken at regular time intervals (8-day for 4°C; 4-day for 10°C; and 2-day for 20°C) for chemical and color analyses, which were performed in duplicate for each storage temperature.

Titratable acidity, pH, and proximate analysis

Physicochemical properties of the yogurt samples, including pH, titratable acidity (g lactic acid/100g), total solids (g/100g), contents of fat (g/100g), protein (g/100g), and ash (g/100g) were determined by the methods described by Bradley *et al.* [1992].

Determination of betalain content in yogurts during storage

The betalain fraction was separated from yogurt samples using the method proposed by Gandía-Herrero *et al.* [2012]. In brief, 10 g of yogurt was centrifuged at 3075×*g* in a falcon tube at 10°C, and next the upper part was filtrated using a 0.45 μ PTFE syringe filter. Using this separation method, the recovery rate was >95%. Quantification of betalains (betanin+iso-betanin) in the yogurt fraction was performed by the HPLC with the external standard method [Naderi *et al*., 2012]. The Agilent 1260 HPLC system with Agilent multiple wavelength UV detector (Agilent Technologies Inc., Folsom, CA, USA) was used. Separation of betalains was carried out using a Zorbax SB-C18 column (Agilent, 4.6×250 mm, particle size of 5 μm). The mobile phase consisted of 0.5 mL/L trifluoroacetic acid solution and acetonitrile (90:10). The flow rate was kept constant at 1.0 mL/min, and the column temperature was maintained at 20°C for a total run. The detector was set at 540 nm for monitoring betalains. Betanin (product no: 901266, Sigma–Aldrich, St. Louis, MO, USA) was used as an external standard. The limit of detection, the limit of quantification, and repeatability were determined to be 7.12 mg/kg, 23.75 mg/kg, and 1.05 %, respectively. The content of betalains in yogurts was expressed as mg/kg yogurt.

Measurement of color parameters

The color parameters (L^* – lightness, a^* – redness and b^* – yellowness) of the yogurts were measured by using Minolta Cr-400 colorimeter (Minolta Co. Ltd., Osaka, Japan). A standard white plate was used for colorimeter calibration. Standard illuminant C and a standard observer angle (2°) were used for color measurements [ISO-CIE, 2008; Wrolstad & Smith, 2017]. The measurement was taken twice per sample.

Calculation of kinetic parameters

The storage stability of betalains and changes in color parameters of the yogurt samples during storage were evaluated using kinetic parameters (reaction order, reaction rate constants (k), and half-life period ($t_{1/2}$)). The effect of temperature on betalain degradation was studied and expressed by both activation energy (E_a) and temperature quotient (Q_{10}) [van Boekel, 2008].

Statistical analysis

Pearson's correlation and multiple regression analysis were performed to evaluate the relationship between the color parameters and betalain content of the yogurts [Sheskin, 2004]. SPSS software version 15.0 for Windows (IBM, Armonk, NY, USA) was used for the statistical analysis.

RESULTS AND DISCUSSIONS

Proximate composition, pH, and titratable acidity of the fresh yogurt

The stability of several natural pigments is affected by the physicochemical characteristics of food containing them. Therefore, the proximate composition of the fresh yogurt was determined, and the results are given in Table 1. The yogurt contained 13.13 g/100 g of dry matter and 2.50 g/100 g of fat, while about 3.01 g/100 g of protein and 0.85 g/100 g of ash on average. Its pH was about 4.53 and its titratable acidity was 0.81 g lactic acid/100 g. The chemical composition of yogurt may be affected by many factors, such as milk composition, presence of additives, process types, *etc*. The chemical composition of the fresh yogurt in the present study was typical of this kind of product, as supported by the results of previous studies [Özoğlu *et al.,* 2020; Yiğit *et al.,* 2011]. Özoğlu *et al*. [2020] investigated proximate compositions of homemade probiotic and commercial non-probiotic yogurts. They found that the pH values and dry matter contents of the yogurts were at 4.45–4.48 and 11.25–11.45 g/100 g, respectively. Protein and fat contents of the yogurts were found to range between 2.95 and 4.0, and between 3.01 and 3.70 g/100 g, respectively. Similarly, Farinde *et al.* [2009] reported 15.9 g/100 g of dry matter, 2.4 g/100 g of protein, and 0.4 g/100 g of ash contents for the commercial cow's milk yogurts sold in Nigeria. Hence, an average pH and acidity values of the yogurts were determined as 4.1 and 0.1 g lactic acid /100 g, respectively. The moisture content of the yogurt samples ranged from 83.3% in cow's milk

TABLE 1. Proximate composition, pH, and titratable acidity of the fresh yogurt.

Parameter	Value
pH	4.53±0.12
Titratable acidity (g lactic acid/100 g)	0.81±0.05
Dry matter content (g/100 g)	13.13±0.02
Fat content (g/100 g)	3.01±0.01
Protein content (g/100 g)	2.50±0.01
Ash content (g/100 g)	0.85±0.01

The values are expressed as means±standard deviation (SD).

Changes of yogurt pH and titratable acidity during storage

In the present study, the pH and titratable acidity of yogurts during storage were also monitored. The pH of yogurt decreased significantly during storage at all storage temperatures (Figure 1). The pH decrease was higher at 20°C than at other storage temperatures probably due to the higher metabolic activity of lactic acid bacteria at this temperature, as indicated by titratable acidity (Table 2). Increasing acidity in yogurt during storage is known as a post-acidification effect caused by the metabolic activities of lactic acid bacteria that produce lactic acid, and decreasing the shelf-life of yogurt [Shah, 2000].

Storage stability of betalains and changes in color parameters of yogurts

The chromatographic separations of betalains in the yogurts are shown in Figure 2. Two betalains were identified in the yogurts colored with beetroot colorants. The peak 1 with retention time of 10.53 min corresponds to betanin, while the second peak with retention time of 14.04 min to iso-betanin (Figure 2). Moreover, betanin and iso-betanin are predominant betalains in beet root, as mentioned before. The findings of the present study are in good agreement with the literature data [Azeredo, 2009; Herbach *et al.,* 2006]. The content of betalains in the yogurts stored at 4–20°C is provided in Figure 3. It was 305.18 mg/kg of the fresh yogurt and decreased during storage by 49.36%, 58.55%, and 95.65% at 4°C, 10°C, and 20°C, respectively.

The degradation of betalains followed the first-order reaction kinetics with high determination coefficients (R^2 = 0.983, 0.971 and 0.969 at 4°C, 10°C, and 20°C, respectively). Kinetic parameters for the degradation of betalains are given in Table 3. The k values were found in the range from -13.4×10^{-3}/day to -152.3×10^{-3}/day. The degradation rate of betalains in the yogurt samples increased with the elevation of storage temperature. This finding was confirmed by the half-life ($t_{1/2}$) values. Values $t_{1/2}$ for betalains in the yogurt samples were found to be 51.43, 30.91, and 4.54 days at 4°C, 10°C, and 20°C, respectively. Similarly to present findings, several studies have described the first-order kinetics of betalain degradation in different types of food under various storage and process conditions [Caldas-Cueva *et al.,* 2016; Kayın *et al.,* 2019; Tobolková *et al.,* 2020]. Kayın *et al.* [2019] reported the first-order kinetics for betacyanin and betaxanthin degradations in red beet juice concentrates stored in glass jars with and without aluminum foil at 25–45°C.

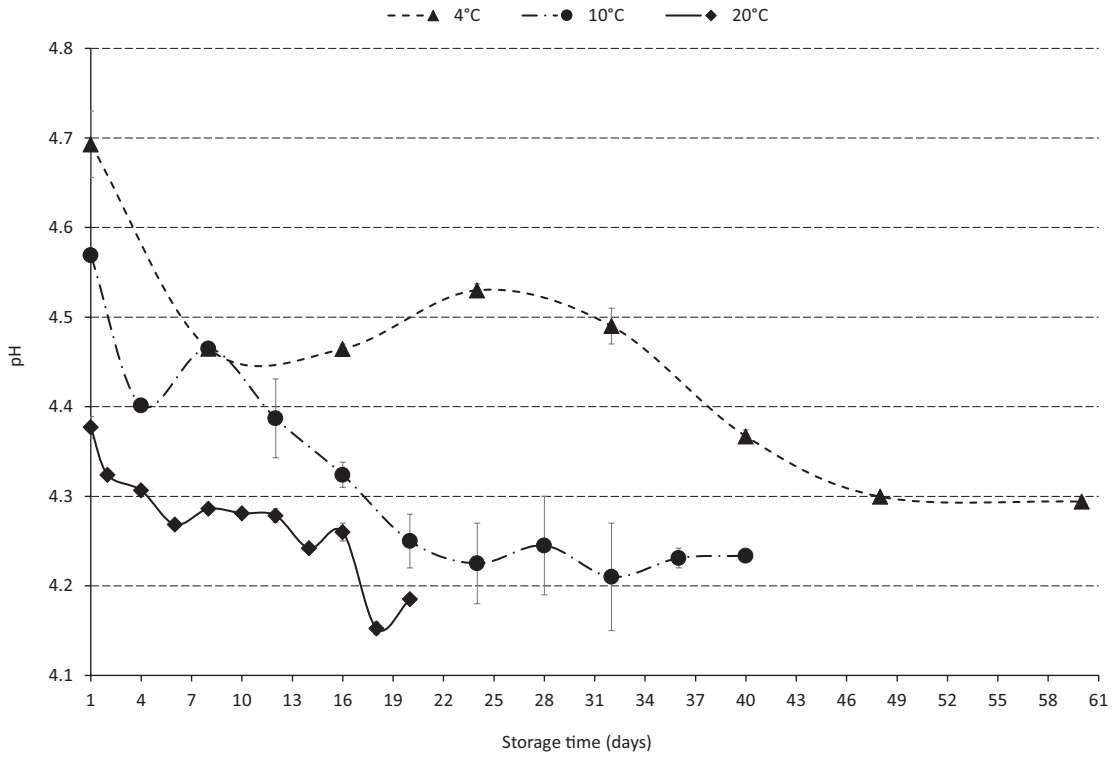


FIGURE 1. The pH value of the yogurts stored at 4–20°C.
The error bars represent the standard error.

TABLE 2. Titratable acidity (g lactic acid/100 g) of the stored yogurts.

Storage time (day)	Temperature (°C)		
	4	10	20
1	0.75±0.01	0.80±0.01	0.89±0.01
2	–*	–	0.98±0.05
4	–	0.84±0.03	0.97±0.01
6	–	–	0.96±0.01
8	0.82±0.02	0.92±0.01	1.01±0.01
10	–	–	1.03±0.01
12	–	0.99±0.01	0.99±0.01
14	–	–	1.02±0.01
16	0.92±0.02	0.91±0.02	0.96±0.04
18	–	–	1.03±0.02
20	–	0.92±0.01	1.01±0.01
24	0.91±0.13	0.92±0.01	–
28	–	0.99±0.01	–
32	0.90±0.01	0.95±0.01	–
36	–	1.01±0.01	–
40	0.86±0.01	0.90±0.04	–
48	0.90±0.01	–	–
60	0.91±0.01	–	–

*Analysis was not conducted on particular days. The values are expressed as means±standard error (SE).

They calculated k values reaching 14.8×10^{-3}/day, 29.4×10^{-3}/day, and 79.5×10^{-3}/day for betacyanin degradation at 25°C, 35°C, and 45°C, respectively, whereas low k values, such as 8.5×10^{-3}/day, 37.1×10^{-3}/day, and 90.2×10^{-3}/day, were calculated for betaxanthin degradation at the same storage temperatures.

In another study by Tobolková *et al.* [2020], the first-order reaction was observed for the degradation of both betacyanins and betaxanthins in apple-beetroot juice stored at 2°C, 7°C, and 20°C. The k values for betacyanin and betaxanthin degradations ranged from 7.3×10^{-3}/day to 47.1×10^{-3}/day and from 6.4×10^{-3}/day to 28.5×10^{-3}/day, respectively, which were lower than those observed in the present study. Similarly, Caldas-Cueva *et al.* [2016] reported that the degradation of betacyanins of an ayrampo (*Opuntia soehrensii*) seed extract and a red beet extract during the storage at 4°C and 25°C, at pH 4.5 followed the first-order reactions kinetics. The stability of betacyanins in the ayrampo seed extract was higher than that in the red beet extract at both temperatures tested. Color retention in yogurts containing the ayrampo seed extract and the red beet extract was not influenced by fat content, and the ayrampo seed extract showed better color retention over 4-week storage at 4°C compared with the red beet extract. Contrary to present findings, Moreno *et al.* [2007] reported the zero-order reaction for the degradation of betalains from tuna (*Opuntia elatior* Miller) and beetroot (*Beta vulgaris* L.) in four different citrus beverage formulations during storage at 7°C, with k values between 12.4 and 18.1 g/100 mL×day.

The differences in findings on betalain degradation could be attributed to the differences in the structure

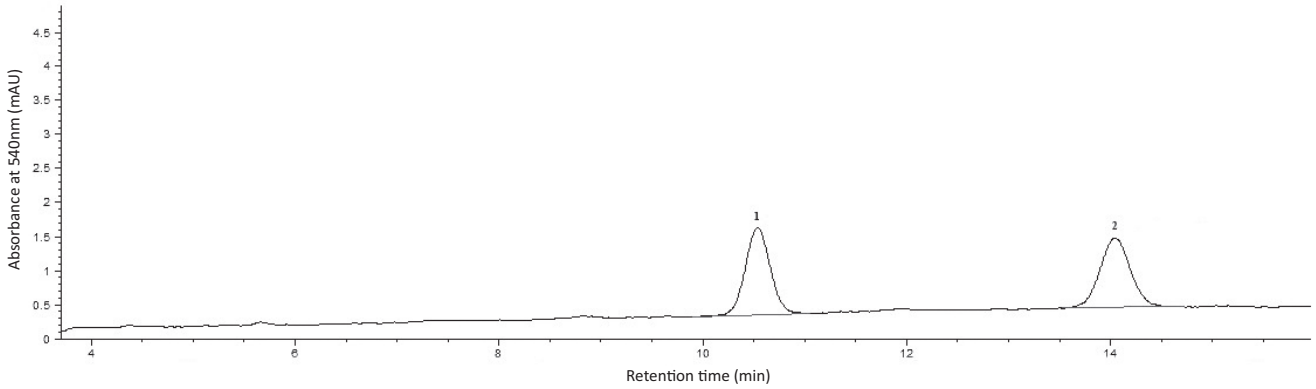


FIGURE 2. HPLC separation of the betalain fraction from yogurt with a red beet colorant. Peaks number 1 and 2 correspond to betanin and iso-betanin, respectively.

of individual betalains, food matrix and product formulation, process conditions, and storage temperatures [Azeredo *et al*., 2009; Khan, 2006]. Many types of reactions, such as hydrolysis, drive the degradation of betalains [Manchali *et al*., 2013]. Each reaction that has different responses regarding chemical kinetics occurs by various chemical mechanisms and depends on many factors. Betalains are considered as heat-labile pigments. Many researchers have reported [Herbach *et al*., 2004, 2007; Kayın *et al*., 2019] that their stability decreased by increasing temperature depending on pigment concentration, temperature level, exposure time of heating, or the presence of oxygen. It has been emphasized that betalains are considerably degraded between 50 and 75°C [Herbach *et al.*, 2006; Manchali *et al*., 2013]. They may be also degraded by isomerization at low temperatures, and their chemical structure and color change as a result of isomerization reactions [Herbach *et al.*, 2006]. Especially, betalains are transformed to the decarboxylated, dehydrogenated, or glycoside derivatives at high temperature. For instance, betanin (red) is conversed to neobetanin, which has yellow color, by dehydrogenation reaction, while 15-decarboxy-betanin (red), 17-decarboxy-betanin (orange-red), and cyclodopa-5-*O*-glycoside (colorless) are formed by the decarboxylation of betanin [Azeredo, 2009; Herbach *et al.*, 2006; Khan, 2016; Manchali *et al.*, 2013]. Fermentation was also determined to affect the stability of betalains. According to Czyżowska *et al.* [2006], betanidin and isobetanidin can be formed by izomerization in red beet juice during lactic acid fermentation. In addition, ratios of betanin+isobetanin/betanidin+isobetanidin, isobetanin/betanin, neobetanin/betanin, and vulgaxanthin I/betanin were found to change in the fermented red beet juice depending on the variety of beet processed (Czerwona Kula and Chrobry). The authors reported that these changes could be due to the activity of β-glucosidase sourced by lactic acid bacteria and the activities of certain

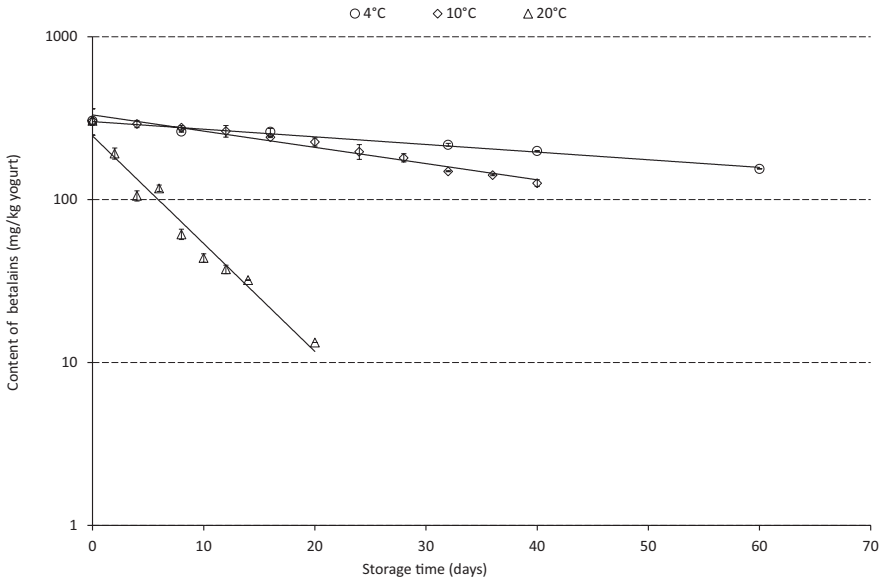


FIGURE 3. Betalain content in the yogurts stored at 4–20°C.
The error bars represent the standard error.

TABLE 3. The kinetic parameters determined for betalain degradation and for color parameter changes of the yogurts during storage.

Parameter	Temperature (°C)	Reaction order	Reaction rate constant (k)	$t_{1/2}$ (day)	Activation energy (E_a) (kJ/mol)	Q_{10}	
						4–10°C	10–20°C
			$\times 10^{-3}$ (1/day)				
Betalain content	4	First-order	-13.4±0.1 (0.983)[a]	51.43	104.9±0.1 (0.966)	2.43	6.61
	10		-23.0±0.9 (0.971)	30.91			
	20		-152.3±0.1 (0.969)	4.54			
			$\times 10^{-3}$ (L^*/day)				
L^*	4	Zero-order	28.8±1.6 (0.982)	-[b]	67.6±4.8 (0.988)	2.13	3.56
	10		45.5±1.7(0.952)	-			
	20		162.1±8.7 (0.852)	-			
			$\times 10^{-3}$ (a^*/day)				
a^*	4	Zero-order	-53.3±0.8 (0.958)	-	76.5±0.6 (0.994)	2.63	3.35
	10		-95.6±7.5 (0.982)	-			
	20		-321±1.4 (0.948)	-			
			$\times 10^{-3}$ (b^*/day)				
b^*	4	Zero-order	34.9±1.2 (0.906)	-	86.1±1 (0.990)	3.15	3.76
	10		69.7±6.5 (0.958)	-			
	20		262.6±1.9 (0.924)	-			

[a]Numbers in parentheses are determination coefficient (R^2). [b]The value was not calculated due to zero-order reaction. L^*: lightness, a^*: redness, b^*: yellowness, $t_{1/2}$: half-life, Q_{10}: temperature quotient. The values are expressed as means±standard deviation (SD).

enzymes including dehydrogenase, polyphenoloxidases, or peroxidase, which were found in the red beet juice medium [Czyżowska *et al*., 2006].

Unlike the changes in betalain content of the yogurt samples, changes in L^*, a^*, and b^* color parameters followed the zero-order kinetics during storage (Figure 4, Table 3). The L^* and b^* values increased, while the a^* values decreased during yogurt storage at all storage temperatures. Furthermore, k values determined for the changes in L^*, a^*, and b^* values were found in the range between 28.8×10^{-3} and 162.1×10^{-3} L^*/day; -53.3×10^{-3} and -321×10^{-3} a^*/day; as well as 34.9×10^{-3} and 262.6×10^{-3} b^*/day, respectively. According to this, the reaction rate determined for changes in the a^* values of the yogurts stored at all temperatures was higher than those determined for the L^* and b^* values (Table 3). This can be attributed to the changes in chemical structures and spectral properties of betalains as a result of several reactions. Indeed, degradation of betalains led to an increase in L^*, b^*, and hue angle values and to a decrease in a^* value, as commonly reported in previous research [Fernández-López *et al*., 2013; Gandia-Herrero *et al*., 2010; Herbach *et al*., 2004]. Different UV absorption and reflectance properties were reported for decarboxylated, dehydrogenated, or glycoside derivatives from betalains in different food matrices and solvents. According to this, betanin from red beet displays a maximum UV visible absorbance at 538 nm, while its dehydrogenated derivatives (neobetanins) have the maximum UV absorbance at 477 nm [Herbach *et al*., 2004].

Although several works are available on the kinetics of changes in the color parameters of anthocyanins in various foods [Reyes & Cisneros-Zevallos, 2007; Roidoung *et al.,* 2017], studies regarding the kinetics of color changes of betalains are sparse. Kayın *et al.* [2019] reported that L^*, a^*, and b^* value changes in a red beet juice concentrate stored at 25°C, 35°C, and 4°C followed the zero-order kinetics with k values lower than that found in the present study. Sonar *et al.* [2019] reported that L^*, a^*, and b^* values of beetroot puree packed in polymeric films with different oxygen transmission rates decreased during storage at 7°C, and the changes of overall color difference (ΔE) of beetroot puree were found to follow the zero-order reaction kinetics. In a study by Narkprasom *et al.* [2012], the first-order kinetics was reported for the degradation of Hunter a value of *Djulis* extract (*Chenopodium formosanum* Koidz.) in model systems containing various ethanol concentrations (0%–60%) during storage at 20–50°C. Similarly, Chandran *et al.* [2014] reported that the degradation of Hunter a/b values of beetroot at 50–120°C in an isothermal heating condition followed the first-order kinetics.

Activation energy (E_a) and temperature quotient (Q_{10}) values of betalain degradation and changes of the color parameters of the stored yogurts

The temperature dependency of the betalain degradation and color changes in the yogurt samples were expressed by E_a and Q_{10} values. The E_a value for the degradation of betalains in the yogurts during storage at 4–20°C was 104.9 kJ/mol,

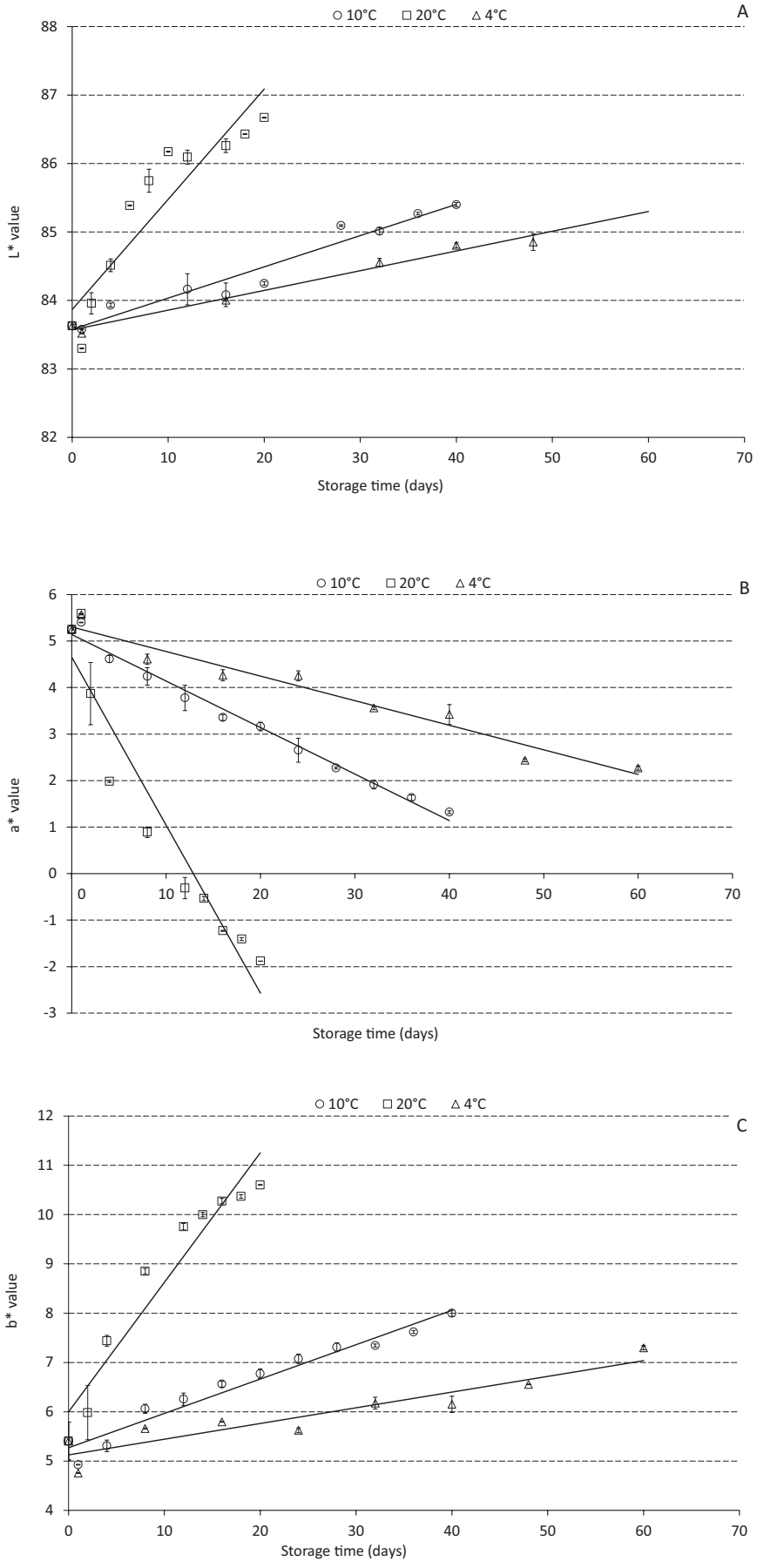


FIGURE 4. Color parameters – L^* – lightness (A), a^* – redness (B), and b^* – yellowness (C) – of the yogurts stored at 4–20°C. The error bars represent the standard error.

while calculated Q_{10} values were 2.43 and 6.61 at 4–10°C and 10–20°C, respectively (Table 3). Based on these results, it can be interpreted that the reaction rate for betalain degradation in the yogurts is more influenced by the temperature change from 10°C to 20°C compared with the temperature change from 4°C to 10°C. The E_a values denoting the color changes were 67.6, 76.5, and 86.1 kJ/mol for L^*, a^*, and b^* color parameters, respectively. Due to low E_a values, the changes in L^* and a^* values were more affected by the temperature elevation than b^* value. Indeed, Q_{10} values of the changes in b^* value were found to be higher than those of L^* and a^* values (Table 3).

Various E_a values for the betalain degradation and color changes in food systems have been reported [Das *et al.,* 2019; Kayın *et al.,* 2019; Siow & Wong, 2017]. In a study by Siow & Wong [2017], E_a values were calculated as 92.817 and 82.953 kJ/mol for the degradation of betacyanins in red-fleshed dragon fruit (*Hylocereus polyrhizus*) juice and its concentrate at 25°C, 37°C, and 45°C, respectively, and were lower than E_a value observed in the present study. Similarly, Kayın *et al.* [2019] reported lower E_a values, such as 66.07 and 93.27 kJ/mol, for betacyanin and betaxanthin degradations in a red beet juice concentrate stored in glass jars at 25–45°C. Those researchers have also reported E_a values at 23.00, 93.28, and 88.26 kJ/mol for L^*, a^*, and b^* color parameters, respectively [Kayın *et al.,* 2019]. Similarly to the present findings, Das *et al.* [2019] reported E_a values such as 119.75 and 125.34 kJ/mol for the degradation of betacyanins extracted from red amaranth using water and 50% ethanol at pH 1 and 3, respectively, at storage temperatures of 4°C and 30°C. In turn, the E_a value of 37.54 kJ/mol was reported by Chandran *et al.* [2014] for the changes of Hunter *a/b* value in beetroot heated at 50–120°C.

Relationships between the pH value, betalain content, and color parameter values in yogurts during storage

Unlike anthocyanins, betalains are color-stable in a food matrix in a wide range of pH values. The maximum UV absorption and color spectrum of betalains do not change significantly at pH from 3 to 7. According to Fu *et al*. [2020], the optimal stability of betalains was reached at pH 4–6. In the present study, the pHs of the yogurts ranged from 4.15 to 4.69 (Figure 1). Therefore, the color stability of betalains in the yogurts could be expected. However, changes in the color parameters were observed during storage (Figure 4). As explained earlier, these results could be related to the type of degradation, molecular interactions of betalains with the structural components of the yogurt matrix, and others factor, such as dissolved oxygen concentration, content of metal cations content, and enzymes, apart from the pH. Moreover, several types of acids and their amounts have different effects on the color of betalains [Khan, 2016]. In the present study, the titratable acidity (g lactic acid/100 g) of the yogurt samples increased gradually during the storage depending on the storage temperature (Table 2).

Synthetic or natural color pigments that are found in foods have different spectral characteristics. The ultraviolet/visible spectra of these compounds are of great importance because they provide valuable information about their structure and content in products [Sant'Anna *et al.*, 2013; Wrolstad & Smith, 2017]. Revealing the relationship between the content of color compounds and their spectral properties in foods by using low-cost and fast techniques is essential to make faster decisions in food processing and preservation [Pathare *et al*., 2013]. In this context, many studies have been conducted based on different foods [Alighourchi & Barzegar, 2009; Gonçalves *et al.,* 2007; Güneşer, 2016; Humphries *et al.,* 2004; Su *et al.,* 2016].

TABLE 4. Multiple linear regression models for pH, betalain content, and color parameters of the yogurts stored at different temperatures.

Temperature (°C)	Multiple regression equation	R^2	*P value*
4	Betalain (mg/kg) = 7.88 L^* + 88.23 a^* + 39.40 b^* + 72.50 pH – 1329	97.55	0.020
10	Betalain (mg/kg) = 3.5 L^* + 85.30 a^* + 41.50 b^* – 7.4 pH – 581	98.59	0.000
20	Betalain (mg/kg) = 6.10 L^* + 39 a^* – 12 b^* – 168 pH + 352	72.09	0.177

[a]Numbers in parentheses are *P* values for regression analysis. L^*: lightness, a^*: redness, b^*: yellowness.

The relationship between the content of betalains, color parameters, and pH of the stored yogurts was explored by multiple linear regression and correlation analyses in the present study. Significant fit of regression models was found for the storage temperatures of 4°C and 10°C with high determination coefficients (R^2 = 97.55 and 98.59) (Table 4). This indicates that the developed models have a reasonable predictive power to calculate the betalain content of the yogurts stored at 4–10°C. The relative weight of the redness (a^*) as a predictor variable had the highest value, followed by yellowness (b^*) in the models. Furthermore, a positive correlation was determined between the content of betalains, a^* value, and pH value, whereas L^* and b^* values showed a negative correlation with betalain content (Table 5).

These findings show that the effective assessment of betalain content in yogurt can be achieved by studying the color values of L^*, a^*, b^*, and pH instead of time-consuming chromatographic analysis. Similarly, in a previous study [Güneşer, 2016], a negative correlation was found for L^* values and betalain contents in milk with a beetroot colorant heated at 70–90°C. Moreover, a significant positive correlation was observed between chroma values and betalain contents. Liaotrakoon *et al.* [2013] showed that a^* and b^* values could be used as criteria to determine the quality of white and red-flesh dragon fruit purees and also that the betalain content of red-flesh dragon fruit purees could be estimated by using the total color change with a high prediction (R^2=0.94). In another study by Gonçalves *et al.* [2007], the total anthocyanin content of four different sweet cherry cultivars (Burlat, Saco, Summit, and Van) was reported to negatively correlate with L^*, a^*, b^*, chroma, and hue angle values during storage at 1.5°C and 15°C. In turn, Humphries *et al.* [2004] investigated the relationship between L^*, a^*, b^* values, and lutein and carotene contents in wheat and triticale samples. The positive correlation found between lutein contents and b^* values was

TABLE 5. Coefficients of correlations between pH, betalain content, and color parameters of the stored yogurts.

	Betalain content	L^*	a^*	b^*
L^*	-0.834 (0.001) [a]			
a^*	0.960 (0.001)	0.853 (0.001)		
b^*	-0.935 (0.001)	0.832 (0.001)	-0.990 (0.00)	
pH	0.720 (0.001)	-0.651 (0.001)	0.727 (0.001)	-0.692 (0.001)

[a]Numbers in parentheses are *P* values determined for correlation analysis. L^*: lightness, a^*: redness, b^*: yellowness.

stronger for durum wheat than that determined for other wheat varieties. In contrast, it was found that carotene and lutein contents were weakly correlated with L^* and a^* values in wheat and triticale samples. It can generally be concluded that such color parameters as L^*, a^*, and b^* are directly related to pigments present in food items. Thus, the color analysis is a useful tool to determine the content of natural pigments of various food items at various processing stages. Sensory color analyses should also be performed in this respect along with instrumental color and chromatographic analyses.

CONCLUSION

This study evaluated the stability of beetroot betalains in yogurts during storage. The pH values of yogurts containing the beetroot extract decreased during the storage, and the decrease was the highest at 20°C. First-order kinetics were reported for the degradation of betalains in yogurts stored at 4°C, 10°C, and 20°C. Moreover, changes in L^*, a^*, and b^* values followed the zero-order kinetics. Storage temperatures had the least effect on L^* value followed by a^* value of color of the yogurts. It was observed that the developed multiple linear regression models had a reasonable predictive power to calculate the betalain content of yogurt samples stored at 4–20°C. The highest value as a predictor variable was obtained for a^* in the models. Further studies should be performed by considering the matrix effect on natural pigments, color parameters, and sensory color properties. All obtained data can be analyzed further using advanced statistical methods to reveal the relationship between color value and natural pigments in food systems.

RESEARCH FUNDING

This study was supported by The Scientific Research Fund of Uşak University (2014/MF020).

ACKNOWLEDGEMENTS

The author would like to thank NANTE Chemical Com. (Istanbul, Turkey) for providing the beetroot extract. The author would also like to thank Danem Dairy Company (Isparta, Turkey) for providing the yogurt starter culture.

CONFLICT OF INTERESTS

The author declares that there is no conflict of interest.

ORCID ID

O. Guneser https://orcid.org/0000-0002-3927-4469

REFERENCES

1. Alighourchi, H., Barzegar, M. (2009). Some physicochemical characteristics and degradation kinetic of anthocyanin of reconstituted pomegranate juice during storage. *Journal of Food Engineering*, *90*, 179–185. https://doi.org/10.1016/j.jfoodeng.2008.06.019
2. Amchova, P., Kotolova, H., Ruda-Kucerova, J. (2015). Health safety issues of synthetic food colorants. *Regulatory Toxicology and Pharmacology*, *73*, 914–922. https://doi.org/110.1016/j.yrtph.2015.09.026
3. Azeredo, H.M.C. (2009). Betalains: properties, sources, applications, and stability – a review. *International Journal of Food Science & Technology*, *44*, 2365–2376. https://doi.org/10.1111/j.1365-2621.2007.01668.x
4. Bárta, J. Bártova, V., Šindelková, T., Jarošová, M., Linhartová, Z., Mráz, J., Bedrníček, J., Smertana, P., Samková, E., Laknerová, I. (2020). 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. *Polish Journal of Food and Nutrition Sciences, 70*(4), 377–385. https://doi.org/10.31883/pjfns/128613
5. Bradley, J.R.L., Arnold, J.E., Barbano, D.M., Semerad, R.G., Smith, D.E., Vines, B.K. (1992). Chemical and physical methods. In R. Marshal (Ed.), *Standard Methods for the Examination of Dairy Products.* American Public Health Association, Washington, DC, USA, pp. 433–531.
6. Caldas-Cueva, J.P., Morales, P., Ludena, F., Betalleluz-Pallardel, I., Chirinos, R., Noratto, G., Campos, D. (2016). Stability of betacyanin pigments and antioxidants in ayrampo (*Opuntia soehrensii* britton and rose) seed extracts and as a yogurt natural colorant. *Journal of Food Processing and Preservation*, *40*, 541–549. https://doi.org/10.1111/jfpp.12633
7. 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
8. Czyżowska, A., Klewicka, E., Libudzisz, Z. (2006). The influence of lactic acid fermentation process of red beet juice on the stability of biologically active colorants. *European Food Research and Technology*, *223*(1), 110–116. https://doi.org/10.1007/s00217-005-0159-y
9. Das, M., Saeid, A., Hossain, M.D.F., Jiang, G.H., Eun, J.B., Ahmed, M. (2019). Influence of extraction parameters and stability of betacyanins extracted from red amaranth during storage. *Journal of Food Science and Technology*, *56*, 643–653. https://doi.org/10.1007/s13197-018-3519-x
10. Delgado-Vargas, F., Jiménez, A.R., Paredes-López, O. (2000). Natural pigments: carotenoids, anthocyanins, and betalains –

characteristics, biosynthesis, processing, and stability. *Critical Reviews in Food Science and Nutrition*, *40*, 173–289. https://doi.org/10.1080/10408690091189257

11. Farinde, E.O., Adesetan, T.O., Obatolu, V.A., Oladapo, M.O. (2009). Chemical and microbial properties of yogurt processed from cow's milk and soymilk. *Journal of Food Processing and Preservation*, *33*(2), 245–254. https://doi.org/10.1111/j.1745-4549.2008.00336.x
12. Fernández-López, J.A., Angosto, J.M., Giménez, P.J., León, G. (2013). Thermal stability of selected natural red extracts used as food colorants. *Plant Foods for Human Nutrition*, *68*(1), 11–17. https://doi.org/10.1007/s11130-013-0337-1
13. Fu, Y., Shi, J., Xie, S.Y., Zhang, T.Y., Soladoye, O.P., Aluko, R.E. (2020). Red beetroot betalains: perspectives on extraction, processing, and potential health benefits. *Journal of Agricultural and Food Chemistry*, *68*(42), 11595–11611. https://doi.org/10.1021/acs.jafc.0c04241
14. Galaffu, N., Bortlik, K., Michel, M. (2015). An industry perspective on natural food colour stability. In: M.J. Scotter(Ed.), *Colour Additivies for Foods and Beverages.* Woodhead Publishing Series in Food Science, Technology and Nutrition, Oxford, UK, pp. 91–130. https://doi.org/10.1016/B978-1-78242-011-8.00005-2
15. Gandía-Herrero, F., Simon-Carillo, A., Escribano, J., Garcia--Carmona, F. (2012). Determination of beet root betanin in dairy products by high-performance liquid chromatography (HPLC). *Journal of Chemical Education, 89*, 660–664. https://doi.org/10.1021/ed200397q
16. Gandía-Herrero, F., Escribano, J., García-Carmona, F. (2010). Structural implications on color, fluorescence, and antiradical activity in betalains. *Planta*, *232*(2), 449–460. https://doi.org/10.1007/s00425-010-1191-0
17. Gengatharan, A., Dykes, G.A., Choo, W.S. (2017). The effect of pH treatment and refrigerated storage on natural colourant preparations (betacyanins) from red pitahaya and their potential application in yoghurt. *LWT – Food Science and Technology*, *80*, 437–445. https://doi.org/10.1016/j.lwt.2017.03.014
18. Gengatharan, A., Dykes, G.A., Choo, W.S. (2015). Betalains: Natural plant pigments with potential application in functional foods. *LWT – Food Science and Technology*, *64*, 645–649. https://doi.org/10.1016/j.lwt.2015.06.052
19. Gonçalves, B., Silva, A.P., Moutinho Pereir, J.M., Bacelar, E.A. (2007). Effect of ripeness and postharvest storage on the evolution of colour and anthocyanins in cherries (*Prunus avium* L.). *Food Chemistry*, *103*, 976–984. https://doi.org/10.1016/j.foodchem.2006.08.039
20. 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
21. Herbach, K.M., Maier, C., Stinzing, F.C., Carle, R. (2007). Effects of processing and storage on juice colour and betacyanin stability of purple pitaya (*Hylocereus polyrhizus*) juice. *European Food Research and Technology*, *224*, 649–658. https://doi.org/10.1007/s00217-006-0354-5
22. Herbach, K.M., Stintzing, F.C., Carle, R. (2006). Betalain stability and degradation – astructural and chromatic aspects. *Journal of Food Science*, *71*, R41–R50. https://doi.org/10.1111/j.1750-3841.2006.00022.x
23. Herbach, K.M., Stintzing, F.C., Carle, R. (2004). Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations. *Journal of Food Science*, *69*(6), C491–C498. https://doi.org/10.1111/j.1365-2621.2004.tb10994.x
24. Humphries, J.M., Graham, R.D., Mares, D.J. (2004). Application of reflectance colour measurement to the estimation of carotene and lutein content in wheat and triticale. *Journal of Cereal Science*, *40*, 151–159. https://doi.org/10.1016/j.jcs.2004.07.005
25. ISO-CIE (2008). *Colorimetry, part 4: CIE 1976 L* a* b* colour space,11664–4*.
26. Karangutkar, A.V., Ananthanarayan, L. (2021). Evaluating the effect of additives on stability of betacyanin pigments from *Basella rubra* in a model beverage system during storage. *Journal of Food Science and Technology, 58*, 1262–1273. https://doi.org/10.1007/s13197-020-04635-8
27. Kayın, N., Atalay, D., Akçay, T.T., Erge, H.S. (2019). Color stability and change in bioactive compounds of red beet juice concentrate stored at different temperatures. *Journal of Food Science and Technology*, *56*, 5097–5106. https://doi.org/10.1007/s13197-019-03982-5
28. Khan, M.I. (2016). Stabilization of betalains: A review. *Food Chemistry*, *197*, 1280–1285. https://doi.org/10.1016/j.foodchem.2015.11.043
29. Liaotrakoon, W., De Clercq, N., Hoed, V.V., de Walle, D.V., Lewille, B., Dewettinck, K. (2013). Impact of thermal treatment on physicochemical, antioxidative and rheological properties of white-flesh and red-flesh dragon fruit (*Hylocereus* spp.) purees. *Food and Bioprocess Technology*, *6*, 416–430. https://doi.org/10.1007/s11947-011-0722-4
30. Manchali, S., Murthy, K.N.C., Nagaraju, S., Neelwarne, B. (2013). Stability of betalain pigments of red beet. In B. Neelwarne (Ed.), *Red Beet Biotechnology*, Springer Science+Business Media, Springer, pp. 55–74. https://doi.org/10.1007/978-1-4614-3458-0_3
31. Martins, N., Roriz, C.L., Morales, P., Barros, L., Ferreire, C.F.R.I. (2016). Food colorants: challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices. *Trends in Food Science & Technology*, *52*, 1–15. https://doi.org/10.1016/j.tifs.2016.03.009
32. Moreno, D.A., Garcia-Viguera, C., Gil, J.I., Gil-Izquierdo, A. (2008). Betalains in the era of global agri-food science, technology and nutritional health. *Phytochemistry Reviews*, *7*, 261–280. https://doi.org/10.1007/s11101-007-9084-y
33. Moreno, M., Betancourt, M., Pitre, A., Garcia, D., Belen, D., Medina, C. (2007). Evaluación de la estabilidad de bebidas cítricas acondicionadas con dos fuentes naturales de betalaínas: tuna y remolacha. *Bioagro, 19*, 149–159 (in Spanish; English abstract).
34. Naderi, N., Ghazali, H.M., Hussin, S.A., Amid, M., Manap, M.Y.A. (2012).Characterization and quantification of dragon fruit (*Hylocereus polyrhizus*) betacyanin pigments extracted by two procedures. *Pertanika Journal of Tropical Agricultural Science*, *35*, 33–40.
35. Narkprasom, K., Wang, S.P., Hsiao, S.M., Tsai, P.J. (2012). Kinetics of color loss of Djulis (*Chenopodium formosanum* Koidz.) extracts during storage in different concentrations of alcohol and temperature. *APCBEE Procedia*, *2*, 32–36. https://doi.org/10.1016/j.apcbee.2012.06.007

36. Özoğlu, Ö., Şaşmazer, R.Ç., Erginkaya, Z., Korukoğlu, M. (2020). Surveying production and characteristics of homemade yogurt by using a commercial probiotic culture. *Gıda – The Journal of Food*, *45*, 814–824. https://doi.org/10.15237/gida.GD20028
37. Pathare, P.B., Opara, U.L., Al-Said, F.A.J. (2013). Colour measurement and analysis in fresh and processed Foods: a review. *Food and Bioprocess Technology*, *6*, 36–60. https://doi.org/10.1007/s11947-012-0867-9
38. Reyes, L.F., Cisneros-Zevallos, L. (2007). Degradation kinetics and colour of anthocyanins in aqueous extracts of purple- and red-flesh potatoes (*Solanum tuberosum* L.). *Food Chemistry*, *100*, 885–894. https://doi.org/10.1016/j.foodchem.2005.11.002
39. Roidoung, S., Dolan, K.D., Siddiq, M. (2017). Estimation of kinetic parameters of anthocyanins and color degradation in vitamin C fortified cranberry juice during storage. *Food Research International*, *94*, 29–35. https://doi.org/10.1016/j.foodres.2017.01.013
40. 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*, 601–608. https://doi.org/10.1016/j.dyepig.2013.04.011
41. Shah, N.P. (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science*, 83, 894–907. https://doi.org/10.3168/jds.S0022-0302(00)74953-8
42. Sheskin, D. (2004) *Handbook of Parametric and Nonparametric Statistical Procedures*. 3rd edition. Chapman and Hall/CRC Press, New York, USA, pp. 667–727. https://doi.org/10.1201/9781420036268
43. Sigurdson, G.T., Tang, P., Giusti, M.M. (2017). Natural colorants: food colorants from natural sources. *Annual Review of Food Science and Technology*, *8*, 261–280. https://doi.org/10.1146/annurev-food-030216-025923
44. Siow, L.F., Wong, Y.M. (2017). Effect of juice concentration on storage stability, betacyanin degradation kinetics, and sensory acceptance of red-fleshed dragon fruit (*Hylocereus polyrhizus*) juice. *International Journal of Food Properties*, *20*, 623–632. https://doi.org/10.1080/10942912.2016.1172086
45. Sonar, C.R., Rasco, B., Tang, J., Sablani, S.S. (2019). Natural color pigments: oxidative stability and degradation kinetics during storage in thermally pasteurized vegetable purees. *Journal of the Science of Food and Agriculture*, *99*, 5934–5945. https://doi.org/10.1002/jsfa.9868
46. Su, G., Songming, Z., Menglong, X., Hosahalli, S.R., Lin, Y., Yu, Y. (2016). Pressure degradation kinetics of anthocyanin pigment and visual color of chinese bayberry juice. *International Journal of Food Properties*, *19*, 443–453. https://doi.org/10.1080/10942912.2015.1038562
47. Tamime, R., Robinson, R.K. (Eds.) (2007). Yoghurt: Science and Technology. 3rd edition. Woodhead Publishing Limited, CRC Press, Cambridge, UK, pp. 13–161. https://doi.org/10.1533/9781845692612.13
48. TFC. (2013). *Turkish Food Codex Regulation on Food Additives.* Regulation No. 28693. Ankara: Republic of Turkey Ministry of Agriculture and Forestry.
49. Tobolková, B., Polovka, M., Dasko, L., Belajova, E., Durec, J. (2020). Evaluation of qualitative changes of apple-beetroot juice during long-term storage at different temperatures. *Journal of Food Measurement and Characterization*, *14*, 3381–3388. https://doi.org/10.1007/s11694-020-00592-0
50. van Boekel, M.A.J.S. (2008). Kinetic modeling of food quality: a critical review. *Comprehensive Reviews in Food Science and Food Safety*, *7*, 144–158. https://doi.org/10.1111/j.1541–4337.2007.00036.x
51. Wrolstad, R.E., Smith, D.E. (2017). Color analysis. In S.S Nielsen (Ed.), *Food Analysis,* Cham: Springer International Publishing, pp. 545–555. https://doi.org/10.1007/978-3-319-45776-5_31
52. Yiğit, K., Guneser, O., Yüceer, Y. (2011). Farklı ticari kültürler kullanılarak üretilen yağsız yoğurtların fiziksel kimyasal ve duyusal özellikleri. *Hasad Gıda*, 26, 30–35 (in Turkish).

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 147–159
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/134623
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Determination of Saponins in Leaves of Four Swiss Chard (*Beta vulgaris* L.) Cultivars by UHPLC-CAD/QTOF-MS/MS

Agnieszka Mroczek[1,*], *Urszula Klimczak*[1], *Mariusz Kowalczyk*[2]

[1]*Department of Plant Biochemistry, Institute of Biochemistry, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02–960 Warsaw, Poland*
[2]*Department of Biochemistry, Institute of Soil Science and Plant Cultivation, State Research Institute, Czartoryskich 8, 24–100 Puławy, Poland*

Key words: *Beta vulgaris*, Swiss chard, Amaranthaceae, triterpene saponins, oleanolic acid, hederagenin, UHPLC-MS

Swiss chard is a vegetable valued not only for the taste of its leaves but also because of its health-promoting properties. To date, nothing is known regarding the occurrence of saponins in the Swiss chard plant, even though they could be at least partially responsible for the nutraceutical activities of this plant. This research aimed to describe saponins from the leaves of four Swiss chard (*Beta vulgaris* L.) cultivars. Saponin structures were analyzed by UHPLC-CAD/QTOF-MS/MS. Based on the fragmentation patterns, we tentatively identified 16 triterpene saponins in *B. vulgaris*, including two that had not been detected previously. The observed compounds were glycosides of five different, tentatively identified aglycones, *i.e.*, oleanolic acid, hederagenin, gypsogenin, akebonoic acid, and serjanic acid. Moreover, the structure of four saponins detected in Swiss chard leaves included dioxolane-type and six acetal-type substituents. Eleven, eight, eleven, and eight saponins were observed in saponin fractions obtained from Rhubarb, Bulls Blood, Perpetual Spinach, and White Silver cultivars, respectively. Furthermore, the content of all identified triterpene derivatives in the investigated cultivars was estimated using a method based on the UHPLC coupled with QTOF-MS/MS and charged aerosol detector (CAD). The analyzed cultivars differed in the total and individual saponin content. The total saponin content ranged from 125.53 to 397.09 μg/g DW.

INTRODUCTION

Swiss chard (*Beta vulgaris* L.) is a plant grown strictly for its edible leaves. This vegetable is valued not only for the taste of its leaves but also because of its health-promoting properties [Ivanović *et al.*, 2019]. Swiss chard was used in folk medicine to treat diabetes, kidney diseases, and the immune system [Hashem *et al.*, 2016; Ninfali *et al.,* 2013]. In contemporary studies, extracts from its leaves have been demonstrated to reveal anticancer, anti-inflammatory, and antioxidant properties [Ninfali *et al.*, 2013; Pyo *et al.*, 2004]. Previous works have reported that Swiss chard contains flavonoids, such as vitexin, vitexin 2-*O*-rhamnoside, and vitexin 2-*O*-xyloside [Hashem *et al.*, 2016; Ninfali *et al.*, 2013], while its leaves to contain many phenolic acids, such as syringic acid, 4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, and vanillic acid, along with two aldehydes: 2,5-dihydroxybenzaldehyde and 2,4,5-trihydroxybenzaldehyde [Gennari *et al.*, 2011].

Swiss chard belongs to the Amaranthaceae family that includes many economically important plants utilized as herbal medicines or vegetables. The Amaranthaceae plants' phytochemical composition includes essential oils, betalains, phenolic compounds, and triterpene saponins [Mroczek, 2015]. From the various groups of secondary metabolites present in Amaranthaceae plants, triterpene saponins appear to be one of the most significant from the nutraceutical point of view, yet they are still undervalued.

Triterpene saponins are amphiphilic compounds characterized by their structure containing a triterpenoid aglycone, also called sapogenin, which consists of a pentacyclic C_{30} skeleton and one or more sugar chains [Sparg *et al.*, 2004]. The carbohydrate portion consists of one or more sugar moieties, and the most common monosaccharides include hexoses (D-glucose, D-galactose), 6-deoxyhexoses (L-furanose, L-quinovose, L-rhamnose), pentoses (L-arabinose, D-xylose), and uronic acids (mainly *d*-glucuronic acid). Saponin can also consist of non-carbohydrate acetyl, organic acid type, aminoacyl, or sulfuric substituents [Arslan & Cenzano, 2020; Mroczek, 2015]. Saponins are categorized according to the number of sugar chains in their structure as mono, di- or tridesmosidic. Monodesmosidic saponins have a single sugar chain, often attached through an ether linkage at C-3 of the aglycone, while bisdesmosidic saponins have an additional sugar chain attached through an ester linkage at

* Corresponding Author: Tel.: +48 22554 3318;
E-mail: mroczek@biol.uw.edu.pl (A. Mroczek)

Submitted: 13 January 2021
Accepted: 9 March 2021
Published on-line: 20 April 2021

C-28. Tridesmosidic saponins have three sugar chains and seldomly occur. The structural complexity of saponins, arising from the variable structure of the aglycone and the number and structure of the sugars or functional groups, results in many physical, chemical, and biological properties of this diverse group of compounds [Sparg *et al.*, 2004].

Saponins are associated with a wide range of other biological properties, *i.e.*, plant defence against pathogens [Sparg *et al.*, 2004], deterrence to insects [Tava & Odoardi, 1996], antifungal properties [Osbourn *et al.*, 1996], and low digestibility in ruminants [Troszyńska, 2004]. Widely distributed in medicinal herbs and edible plants, triterpene saponins are also interesting from a pharmaceutical point of view. Various studies have reported beneficial antimicrobial, anti-inflammatory, immunomodulatory, hepatoprotective, antidiabetic, hypolipidemic, anticancer, and adjuvant effects of saponins [Kirk *et al.*, 2004; Podolak *et al.*, 2010]. Due to their abilities as surfactants and antifungal agents, saponins or saponin-containing plant extracts can be used for industrial applications, such as in cosmetic or food industries.

Although these compounds have significant biological importance, little is known about their distribution in plants from the Amaranthaceae [Mroczek, 2015]. Triterpene saponins have recently been extensively studied in sugar beet roots and sugar pomace due to a floc problem during sugar production [Yoshikawa *et al.*, 1996]. They were also detected in red beet cultivars – Red Sphere [Mroczek *et al.*, 2012; Spórna-Kucab *et al.*, 2020], Rocket, Wodan [Mroczek *et al.*, 2012], Chrobry, and Nochowski [Mikołajczyk-Bator *et al.*, 2016a]. Recently, saponins have been qualitatively characterized in the leaves of the red beet cv. Wodan [Mroczek *et al.*, 2019]. What is worth noticing is that, in both *Beta vulgaris* groups, *i.e.*, sugar and red beet, glycosides of simple sugars moieties were reported along with saponins bearing dioxolane-type and acetal-type substituents [Mikołajczyk-Bator *et al.*, 2016a; Yoshikawa *et al.*, 1996, 1998].

To date, nothing is known regarding the occurrence of saponins in the Swiss chard plant, even though they could be at least partially responsible for its pharmacological and nutraceutical activities due to the broad spectrum of their biological activities. Thus, the aim of this study was a qualitative and quantitative analysis of saponins in the leaves of four cultivar of Swiss chard: Lukullus, Rhubarb, White Silver, and Perpetual Spinach.

Consequently, appropriate methods needed to be established for the qualitative and quantitative analysis of saponins in complex plant matrices. Mass spectrometry (MS), as an extremely sensitive and specific analytical technique, providing qualitative and quantitative data, is an essential tool for saponin characterization [Foubert *et al.*, 2010; Ge *et al.*, 2017; Gómez-Caravaca *et al.*, 2011; Kowalczyk *et al.*, 2011; Mikołajczyk-Bator *et al.*, 2016a, 2016b]. In the current study, we used ultra-performance liquid chromatography (UHPLC) coupled with quadrupole-time-of-flight mass spectrometry (QTOF-MS/MS) that has recently been widely applied to characterize natural compound structures, including saponins [Alara *et al.*, 2018; Mikołajczyk-Bator *et al.*, 2016a; Onlom *et al.*, 2017].

Although the mass spectrometry is considered a universal detection method, the mass detector's response, particularly with electrospray ionization, is highly dependent on the investigated compounds' chemical properties. Furthermore, significant matrix effects can affect the electrospray ionization of analytes, leading to inaccurate measurements. Thus, quantitative analyses employing mass spectrometry detection with electrospray ionization generally require an individual reference standard for each investigated analyte and careful investigation of the matrix effects, which are unavoidable if raw extracts are analyzed. The requirement for authentic standards is often difficult to fulfill in investigations of chemically diverse groups of compounds, especially in the phytochemical and environmental analyses. In the case of saponins, the inherent chemical diversity often practically precludes obtaining analytical standards for all possible congeners.

Therefore, various types of the so-called universal detectors are employed that generate responses independently of the investigated analytes' chemical properties and are less prone to matrix effects. Thus, the second detector utilized in this study was a charged aerosol detector (CAD), which is suitable for detecting semi-volatile and non-volatile analytes and has been used to analyze saponins for over a decade [Baker & Regg, 2018; Vehovec & Obreza, 2010; Wu *et al.*, 2019]. The use of a universal detector enabled the estimation of saponin contents without costly and time-consuming purification of individual saponins for reference standards.

Such a multi-detector approach allowed for the rapid, simultaneous characterization of saponins present in plant material based on their fragmentation pathways and subsequent estimation of detected compounds' content.

MATERIALS AND METHODS

Chemicals

Analytical-grade methanol, LC-MS-grade acetonitrile, diethyl ether, and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA); formic acid was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ultrapure water was obtained using a Merck Millipore (Burlington, MA, USA) system.

Sample preparation

Plants of Swiss chard cv. Rhubarb, Bulls Blood, Perpetual Spinach, and White Silver were cultivated in the field in the standard growing conditions in temperate climatic region with fertile, neutral soil in the central Mazovia region in Poland (coordinates 52°31'45.6"N 21°38'58.3"E) during the 2015 vegetational season. Ten plants were randomly collected per cultivar. Aerial parts were lyophilized, finely powdered, and used immediately for the successive extraction. The dried and finely powdered leaves (1 g) were defatted with diethyl ether *via* ultrasonic-assisted extraction for 1 h at room temperature. Defatted material was then extracted with 80% (*v/v*) aqueous methanol (3×50 mL) *via* ultrasonic-assisted extraction for 1 h at room temperature. This method was based on the method optimized in our previous studies [Mroczek *et al.*, 2012, 2019]. The extracts were collected and concentrated under a nitrogen atmosphere (extract mass: 32–49 mg) and subsequently redissolved in water.

The solution was applied to 1 g LiChroprep RP-18 SPE cartridges (40–63 μm, Merck, Germany), previously conditioned with water. The saponin fraction was monitored by TLC on silica gel 60 F-254 plates (Merck, Darmstadt, Germany) with an ethyl acetate/acetic acid/water (7:2:2, *v/v/v*) solvent system. The spots were visualized by spraying with the Liebermann-Burchard reagent and subsequent heating at 130°C. The column was first washed with water and then with 40% (*v/v*) methanol to remove sugars and phenolics. Saponins were eluted with 80% (*v/v*) methanol and dried under a vacuum.

UHPLC–CAD/QTOF-MS/MS analysis

Samples obtained after SPE fractionation were suspended in distilled water and diluted in the 1:1 (*v/v*) ratio with the internal standard solution (digoxin Sigma-Aldrich D6003, CAS 20830–75–5, final concentration 20 pmol/μL). Analyses were carried out using a UHPLC Thermo Ultimate 3000RS system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Thermo Corona Veo RS charged aerosol detector. Separations were performed using a Waters BEH C18 column (1.7 μm, 100×2.1 mm; Waters, Milford, MA, USA). A mobile phase consisting of 0.5% (*v/v*) formic acid in acetonitrile (B) and 0.5% (*v/v*) formic acid in water (A) was used for the separation. The elution profile was isocratic at 24% B, 0–1 min; linear from 25 to 55% B, 1–22 min; isocratic at 55% B, 22–24 min; and linear from 55 to 24% B, 24–30 min. The column was maintained at 30°C with a constant flow rate of 0.4 mL/min.

The eluate from the column was split in a ratio of 3 to 1 between the CAD and the ion source of the QTOF mass spectrometer (Bruker Impact II HD; Bruker, Billerica, MA, USA). The following instrumental parameters were used for QTOF-MS/MS analysis: capillary voltage, 2.8 kV; nebulizer pressure, 0.7 bar; drying gas flow, 6 L/min; drying gas temperature, 200°C; ion energy 4 eV; collision RF 700.0 Vpp; transfer time 100.0 μs; and pre-pulse storage 10.0 μs. Negative ions were acquired over the range of *m/z* 100–1500 with 5 Hz scanning frequency. MS/MS spectra were obtained using automated data-dependent acquisition, in which two of the most intense precursor ions were fragmented by collision-induced dissociation (CID, Ar collision gas). Collision energies were automatically selected from the pre-defined list based on the *m/z* of fragmented ions and ramped between 75 and 125% of the selected value. The QTOF analyzers' internal mass calibration was based on the sodium formate clusters, injected in the 10 mM solution in 50% (*v/v*) 2-propanol to the ion source through 20 μL directly before every analysis.

Tentative identifications of saponins were carried out using high-resolution measurements of *m/z* ratios with errors not exceeding 5 ppm, and chemical formulas were calculated on this basis. Due to the presence of isobaric compounds with identical formulas in the analyzed samples, additional identification data were obtained from the MS/MS fragmentation spectra.

For each tentatively identified saponin, extracted ion chromatograms corresponding to either the deprotonated molecule or the formic acid adduct ions were created with 0.01 Da width. Signals from QTOF-MS/MS and CAD detectors were aligned (-3.6 s delay of CAD vs. QTOF-MS/MS signal), and for each chromatographic peak detected on the extracted ion chromatograms, a corresponding peak (if present) on the signal from the CAD detector was manually integrated. The CAD response was calibrated in the concentration range from 5 to 35 pmol/μL using a series of dilutions from 1 mg/mL stock solutions of 3,28-diglucoside of medicagenic acid (medicoside G) and soyasaponin Bb. The calibrated ratio between peak area and internal standard peak area was linear in the utilized range of concentrations. All measurements were performed at least in triplicate. The results were expressed as μg of saponin per g of dry weight (DW) of leaves.

Statistical analysis

Microsoft Excel 2010 was used for statistical analysis. The resulting data were presented as means ± standard deviations. Means were compared by the analysis of variance (ANOVA) with Tukey's post-hoc test adopting the standard criterion of significance at $p \leq 0.05$.

RESULTS AND DISCUSSION

Characterization of saponin structures

After the leaves of four different Swiss chard cultivars were extracted under the optimized conditions, the SPE fractionation on RP-18 cartridges was carried out with the step gradient of methanol and water. SPE 80% (*v/v*) methanol fractions of saponins were analyzed by UHPLC-CAD/QTOF-MS/MS to identify their components. Figure 1 shows a chromatogram of leaves of Swiss chard (*Beta vulgaris* L.) cultivars with numerous peaks observed. Eleven, eight, eleven, and eight compounds, which could be identified as saponins due to their molecular mass, were observed in extracts from Rhubarb, Bulls Blood, Perpetual Spinach, and White Silver cultivars, respectively.

The compounds detected in this work were tentatively characterized using MS data, together with the interpretation of the observed MS/MS spectra. Table 1 shows the list of all compounds tentatively characterized through UHPLC-QTOF-MS/MS experiments along with their retention times (t_R), the accurately detected mass of each saponin and the MS/MS fragment ions, and the bibliographic references used in the identification process.

Based on the analysis of the MS^2 spectra, it could be concluded that Swiss chard saponins are derivatives of several different aglycones: oleanolic acid (*m/z* 455), hederagenin (*m/z* 471), gypsogenin (*m/z* 469), akebonoic acid (*m/z* 439), and serjanic acid (*m/z* 499) (Figure 2). Except for serjanic acid, the remaining sapogenins were previously identified in the red beet and sugar beet [Mikołajczyk-Bator *et al.*, 2016a,b; Mroczek *et al.*, 2012, 2019; Murakami *et al.*, 1999; Spórna-Kucab *et al.*, 2020; Yoshikawa *et al.*, 1995, 1996, 1998]. Nevertheless, all detected aglycones, including highly oxidized serjanic and akebonoic acids, were previously found in glycoside forms of other representatives of the Amaranthaceae family. Specifically, serjanic acid derivatives were detected in *Chenopodium quinoa* [Gómez-Caravaca *et al.*, 2011], while akebonoic acid was categorized as an aglycone of saponins in *Chenopodium quiona* [Gómez-Caravaca *et al.*, 2011],

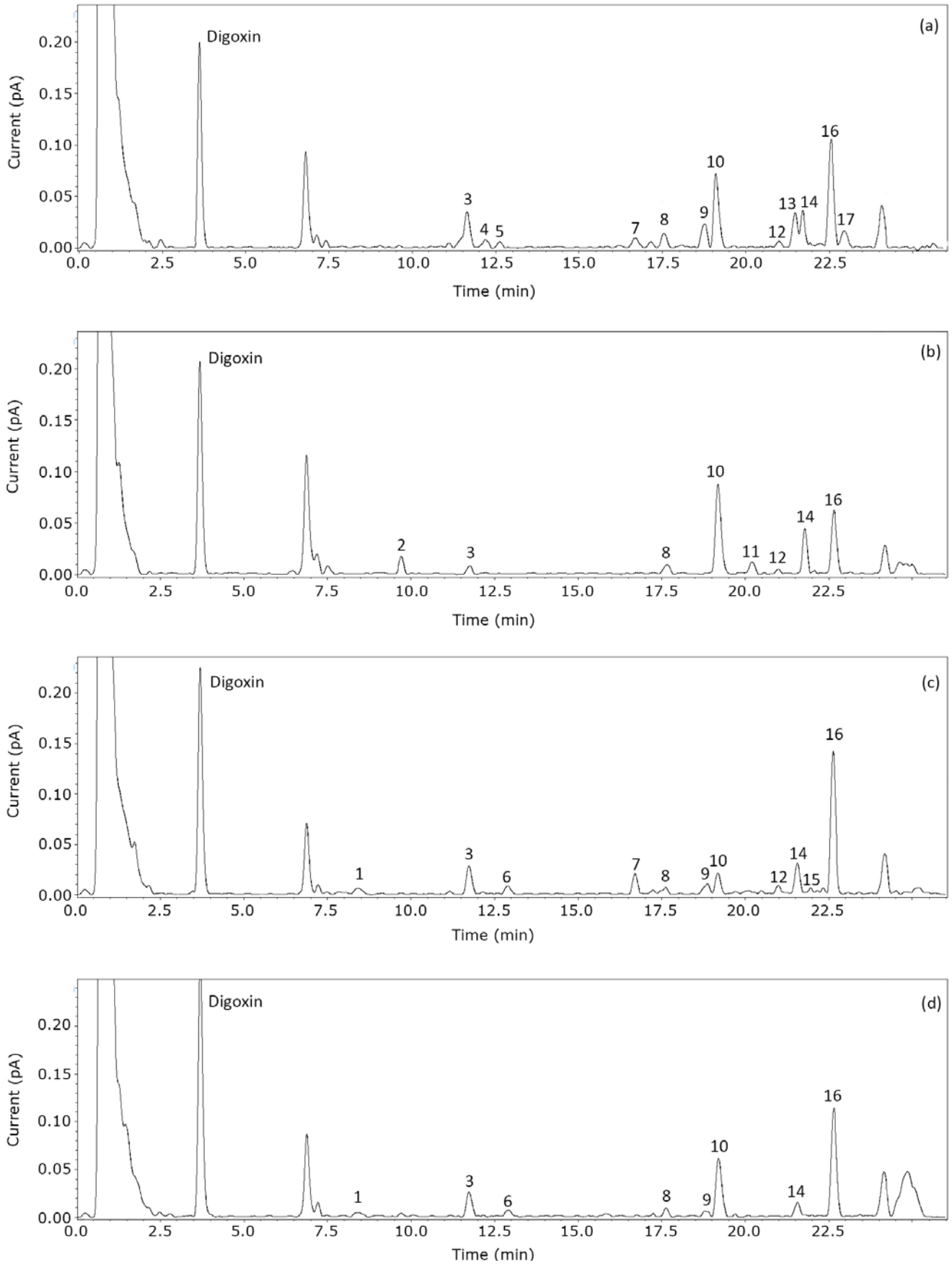


FIGURE 1. UHPLC-CAD chromatogram of saponin fractions from leaves of Swiss chard (*Beta vulgaris* L.) cultivars Rhubarb (a), Bulls Blood (b), Perpetual Spinach (c), and White Silver (d).

TABLE 1. Spectral characteristic of saponins detected by UHPLC-QTOF-MS/MS in Swiss chard leaves and their tentative identification.

Nr	t_R (min)	Precursor ion (*m/z*)[a]	Calculated formula	Error (ppm)	Isotopic fit (mSigma)	Primary product ions (*m/z*)	Tentative identification	Reference
1	8.4	1087.4914	$C_{53}H_{83}O_{23}$	4.8	21.8	925 [M-Hex-H]$^-$, 793 [M-Hex-Pen-H]$^-$, 731 [M-Hex-CO_2-hPen-H]$^-$, 455 [M-Hex-Hex-Pen-UrA-H]$^-$	Hex-Hex-Pen-UrA-oleanolic acid	Mroczek *et al.* [2012]; Mikołajczyk-Bator *et al.* [2016a]
2	9.1	1117.5019	$C_{53}H_{81}O_{25}$	4.8	72.8	835 [M-Act-$C_4H_8O_4$-H]$^-$, 793 [M-Act-Hex-H]$^-$, 731 [M-Act-hHex-CO_2-H]$^-$, 631 [M-Act-Hex-Hex-H]$^-$ 455 [M-Act-Hex-Hex-UrA-H]$^-$	Act-Hex-Hex-UrA-oleanolic acid	Yoshikawa *et al*. [1998]; Mikołajczyk-Bator *et al*. [2016b]
3	11.7	955.4489	$C_{47}H_{71}O_{20}$	5.7	16.3	835 [M-$C_3H_4O_5$-H]$^-$, 793 [M-Act-H]$^-$, 631 [M-Act-Hex-H]$^-$, 455 [M-Act-Hex-UrA-H]$^-$	Act-Hex-UrA-oleanolic acid	Yoshikawa *et al*. [1996]
4	12.5	837.3891	$C_{42}H_7O_{16}$	4.4	2.4	717 [M-$C_3H_4O_5$-H]$^-$, 675 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 499 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-serjanic acid	
5	12.9	925.4391	$C_{46}H_{69}O_{19}$	5.1	57.9	745 [M-Hex-H_2O-H]$^-$, 569 [M-Hex-CO_2-hPen-H]$^-$, 551 [M-Hex-CO_2-H_2O-hPen-H]$^-$	Pen-Hex-UrA-oleanolic acid	Yoshikawa *et al*. [1996]; Mikołajczyk-Bator *et al*. [2016a]
6	16.8	807.3795	$C_{41}H_{59}O_{16}$	3.8	17.5	647 [M-Diox-H]$^-$, 471 [M-Diox-UrA-H]$^-$	Diox-UrA-hederagenin	
7	17.6	809.3954	$C_{41}H_{61}O_{16}$	1.4	15.8	689 [M-$C_3H_4O_5$-H]$^-$, 647 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 471 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-hederagenin	Yoshikawa *et al*. [1998]; Mikołajczyk-Bator *et al*. [2016a]
8	18.7	779.3845	$C_{40}H_{59}O_{15}$	1.9	47.7	647 [M-Pen-H]$^-$, 471 [M-Pen-UrA-H]$^-$	Pen-UrA-hederagenin	Mikołajczyk-Bator *et al*. [2016a]
9	19.1	925.4779	$C_{46}H_{63}O_{19}$	2.5	18.2	763 [M-dAct-H]$^-$, 569 [M-dAct-HexA-H]$^-$, 631 [M-dAct-Pen-H]$^-$	Act-Pen-UrA-oleanolic acid	Mroczek *et al*. [2012]
10	20.1	925.4419	$C_{47}H_{73}O_{18}$	2.2	17.6	793 [M-Pen-H] $^-$, 745 [M-Hex-H_2O-H]$^-$, 731 [M-UrA-H_2O-H]$^-$, 455 [M-Pen-Hex-UrA-H]$^-$	Pen-Hex-UrA-oleanolic acid	Mroczek *et al*. [2012]
11	20.9	807.3815	$C_{41}H_{59}O_{16}$	-0.8	9.0	627 [M- $C_3H_4O_5$-C_2H_2O-H-H]$^-$, 469 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-gypsogenin	Mikołajczyk-Bator *et al*. [2016a]
12	21.5	809.3945	$C_{41}H_{61}O_{16}$	2.5	15.9	689 [M-$C_3H_4O_5$-H]$^-$, 647 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 471 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-hederagenin	Yoshikawa *et al*. [1998]; Mikołajczyk-Bator *et al*. [2016a]
13	21.7	763.4267	$C_{41}H_{63}O_{13}$	1.0	18.3	631 [M-Pen-H]$^-$, 455 [M-Pen-UrA-H]$^-$	Pen-UrA-oleanolic acid	Mroczek *et al*. [2012]; Mikołajczyk-Bator *et al*. [2016a]
14	19.6	777.3683	$C_{40}H_{57}O_{15}$	2.6	43.4	657 [M-$C_3H_4O_5$-H]$^-$, 615 [M-$C_3H_4O_5$-C_2H_2O-H-H]$^-$, 439 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-akebonoic acid	Mikołajczyk-Bator *et al*. [2016a,b]
15	22.5	793.4007	$C_{41}H_{61}O_{15}$	1.1	9.4	673 [M-$C_3H_4O_5$-H]$^-$, 631 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 455 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-oleanolic acid	Yoshikawa *et al*. [1998], Mikołajczyk-Bator *et al*. [2016b]
16	22.9	791.3838	$C_{41}H_{59}O_{15}$	2.7	5.7	631 [M-Diox-H]$^-$, 455 [M-Diox-UrA-H]$^-$	Diox-UrA-oleanolic acid	Yoshikawa *et al.* [1996], Mikołajczyk-Bator *et al.* [2016b]

Act – acetal substituent, aAct – deoxyactetal, Diox – dioxolane substituent Hex – hexose, hHex – hydrated hexose, Pen – pentose, UrA – uronic acid.

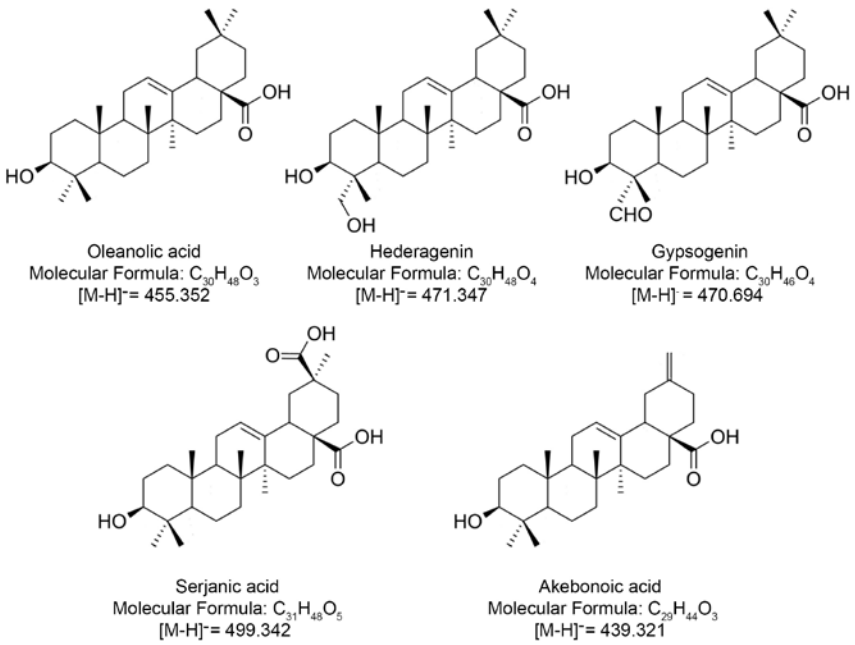


FIGURE 2. Chemical structures of saponin aglycones present in the leaves of Swiss chard *(B. vulgaris* L.).

Amaranthus hypochondriacus [Kohda *et al.*, 1991], and *Pfaffia glomerata* [Nakamura *et al.*, 2010].

Specifically, the majority of the saponins in the leaves contained oleanolic acid as an aglycone, including 5 (Rhubarb), 4 (Bulls Blood), 5 (Perpetual Spinach), and 6 (White Silver) in each cultivar. The number of hederagenin derivatives was 3 (Rhubarb), 1 (Bulls Blood), 3 (Perpetual Spinach), and 2 (White Silver). Only one gypsogenin derivative was detected in cv. Rhubarb, Bulls Blood, and Perpetual Spinach. Serjanic acid was present in the structure of one saponin of cv. Rhubarb and akebonoic acid in one compound of cv. Perpetual Spinach.

The differences in triterpene saponins' chemical structures are due to various aglycones, the saccharide units' composition, and the varied linkages of sugar moieties. The saccharide moieties detected in saponins from Swiss chard cultivars were composed of uronic acid (UrA), pentose (Pen), and hexose (Hex). However, different monosaccharide epimers cannot be differentiated using mass spectrometric data alone. Similarly, the determination of the number of sugar chains and their composition usually needs additional analytical steps.

Additionally, acetal (Act)- and dioxolane (Diox)-type dicarboxylic acids linked to the sugar moieties were detected in Swiss chard leaves. Acetal and dioxolane-type substituents were previously detected in sugar beet and red beet saponins and were linked by 1→2 (Glc→GlcA), 1→3 (Xyl→GlcA), and 3→3 (Act→GlcA) glycosidic bonds [Mikołajczyk-Bator *et al.*, 2016a; Murakami *et al.*, 1999; Yoshikawa *et al.*, 1995, 1996, 1998]. Moreover, an acetal type substituent was described in the saponin isolated from the *Chenopodium album* plant [Mroczek, 2015]. The presence of this type of residues in a carbohydrate chain, presumed to be derived by oxidative degradation of pentose and hexose units, seems to be unique for Amaranthaceae saponins.

The structures of 16 saponins of Swiss chard leaves tentatively proposed based on the detailed fragmentation patterns are shown in Table 1, whereas selected fragmentation spectra are presented in Figure 3.

The compound with the smallest molecular mass detected in all saponin fractions was **13** (t_R=21.7 min), which exhibited a deprotonated ion at *m/z* 763.4016. The fragmentation pattern of this compound indicated the presence of pentose (product ion at *m/z* 631 $[M–132–H]^-$) and uronic acid (product ion at *m/z* 455 $[M–132–176–H]^-$) in the structure. According to data found in the literature, in triterpene saponins, uronic acid is typically attached to the C-3 hydroxyl group of the aglycone [Mikołajczyk-Bator *et al.*, 2016b]. However, based on the MS/MS data alone, it is impossible to reliably detect whether hexuronic acid is attached to C-3 hydroxyl or C-28 carbonyl of the aglycone. Nevertheless, the C-3 linkage of hexuronic acid is also evident from the known structures of various *Beta vulgaris* saponins, such as betavulgarosides (Figure 4). Therefore, this compound and other saponins described in this study are presumably derivatives of C-3 glucuronides of the ascribed triterpene acids. Furthermore, an analogous sugar chain consisting of pentose and uronic acid also occurred in the glycoside of hederagenin **8** (t_R=18.7 min), which was present in all cultivars except Bulls Blood. Such a derivative of hederagenin was not described in *Beta vulgaris* plants before.

The MS^2 spectrum of saponin **15** (t_R=22.5 min), which was present in all cultivars and exhibited a deprotonated precursor ion at *m/z* 793.4016, yielded product ions at *m/z* 673 $[M–120–H]^-$ and *m/z* 631 $[M–120–42–H]^-$, which indicated the presence of an acetal moiety composed of tartaraldehyde and glycolic acid in the oleanolic glucuronide structure. Additionally, a substituent having such a structure was also present in the sugar chain of the derivatives of serjanic acid

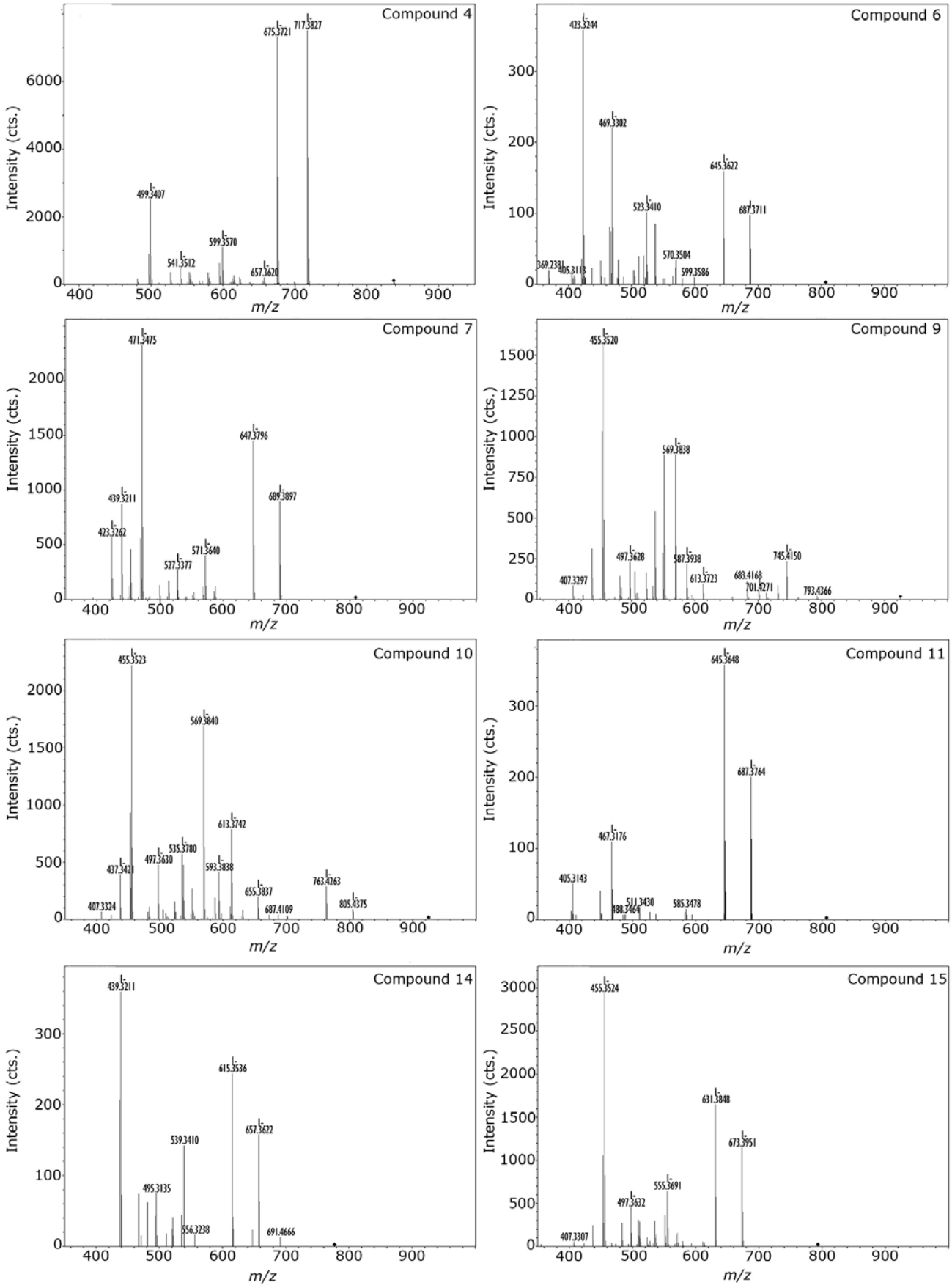


FIGURE 3. MS/MS spectra of compounds **4**, **6**, **7**, **9**, **10**, **11**, **14**, and **15** detected in leaves of Swiss chard (*B. vulgaris* L.) cultivars, tentatively identified as saponins.

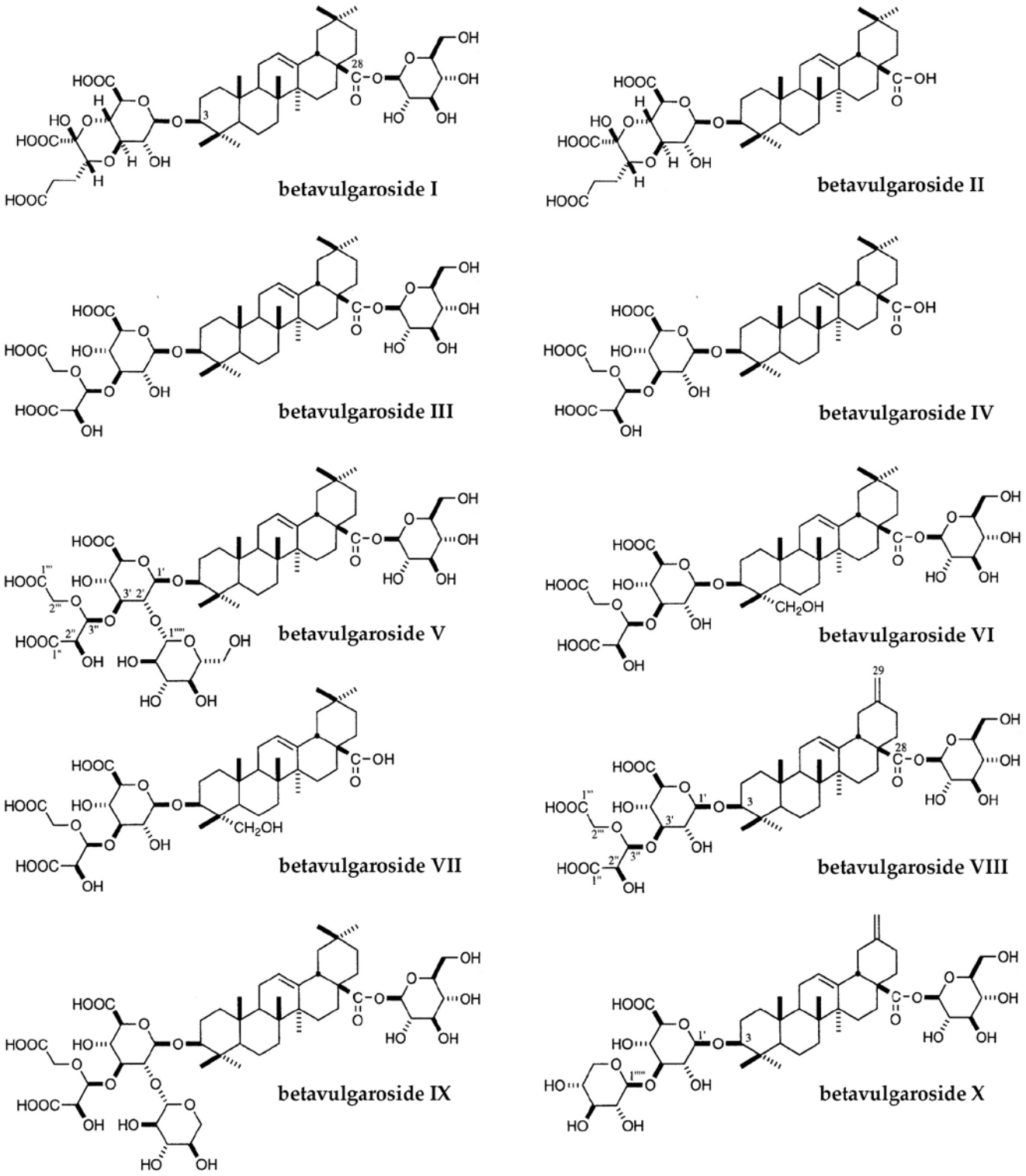


FIGURE 4. Chemical structures of betavulgarosides I–X [adopted from Murakami *et al.*, 1999; Yoshikawa *et al.,* 1995, 1996, 1998].

4 (t_R=12.5 min, detected only in the Rhubarb cultivar), gypsogenin derivative **11** (t_R=20.9 min, present in all cultivars except White Silver), akebonoic acid derivative **14** (t_R=19.6 min, present only in Perpetual Spinach), hederagenin derivative **7** (t_R=17.6 min, which was present in all cultivars except Rhubarb), and **12** (t_R=21.5 min, was present only in Rhubarb). It should be noted that the acetal group described in this study has also been identified in saponins of sugar beet and red beet roots [Mikołajczyk-Bator *et al.*, 2016a,b; Mroczek *et al.*, 2019; Yoshikawa *et al.*, 1995, 1996]. For saponin **3**, fragmentation of the precursor ion at *m/z* 955.4512 led to product ions at *m/z* 835, 793, and 631, resulting in an abundant aglycone ion at *m/z* 455 due to the loss of the acetal moiety, hexose, and uronic acid. According to the MS/MS spectra, saponin **3** is a triglycoside of oleanolic acid differing from compound **15** by the presence of additional hexose.

Another unusual substituent found in the *Beta vulgaris* species is dioxolane [Mikołajczyk-Bator *et al.*, 2016a; Murakami *et al.*, 1999; Yoshikawa *et al.*, 1995, 1996, 1998]. In Swiss chard leaves, this moiety was present in saponins **6** ($t_{R=}$16.8 min) and **16** (t_R= 22.9 min). The MS/MS fragmentation of both saponins showed some similarity. Concerning saponin **16** with the precursor $[M–H]^-$ ion at *m/z* 791.3859, MS/MS data showed the loss of 160 Da, forming a fragment at *m/z* 631, followed by the loss of an uronic acid residue. The formation of the 160 Da fragment was due to the cleavage of the dioxolane substituent. Similarly, the fragmentation of the deprotonated ion of saponin **6** at 807.3795 *m/z* clearly showed the loss of a dioxolane substituent (160 Da). As for saponin **16**, it showed a fragment at *m/z* 455, which is characteristic of oleanolic acid, and the fragmentation of **6** had shown a fragment ion at *m/z* 471, which corresponds to a hederagenin aglycone. Saponin **16** was previously detected in sugar beet [Yoshikawa *et al.*, 1996] and red beet [Mikołajczyk-Bator *et al.*, 2016a], while saponin **6** was detected in *Beta vulgaris* for the first time.

Saponins **9** (t_R=19.1 min) and **10** (t_R=20.1 min) were identified with similarity in the appearance of peaks exhibiting pseudomolecular ions $[M–H]^-$ at *m/z* 925.4439 and 925.4761. Saponin **10** showed fragments formed at *m/z* 745 $[M–Hex–H_2O–H]^-$, 569 $[M–Hex–CO_2–hPen–H]^-$, *m/z* 551 $[M–Hex–CO_2–H_2O–hPen–H]^-$ and *m/z* 455 $[M–Hex–CO_2–H_2O–hPen–H–UrA–H]^-$, which were due to the loss of hexose, pentose and, uronic acid from the oleanolic acid derivative. For saponin **9**, fragmentation of the precursor ion led to product ions at *m/z* 763, 569, and 631, resulting in an abundant aglycone ion at *m/z* 455 due to the loss of the deoxy-acetal moiety, pentose, and uronic acid. According to the MS/MS spectra, saponin **9** was a triglycoside of oleanolic acid differing from compound **11** by the presence of acetal instead of hexose.

The fragmentation of compound **1** (t_R=8.4 min), differing from compound **10** by 162 Da, resulted in the formation of product ions at *m/z* 925 $[M–Hex–H]^-$, *m/z* 793 $[M–Hex–Pen–H]^-$, and *m/z* 455 $[M–Hex–Hex–Pen–UrA–H]^-$ corresponding to the loss of two hexose, pentose, and uronic acid units. The sugar chain based on sugar moieties appears similar to that of the saponin with the same molecular mass described in the red beet roots [Mroczek *et al.*, 2012; Mikołajczyk-Bator *et al.*, 2016a].

The MS/MS spectrum of saponin **2** (t_R=9.1min) showed fragment ions at *m/z* 835, 925, 631, and 455 $[M–162–H]^-$, indicating the loss of an acetal moiety, two glucose units, and uronic acid, respectively. Therefore, based on this data, it can be speculated that saponin **2** has the same structure as betavulgaroside V present in the sugar beet [Yoshikawa *et al.*, 1998]. As described in the literature, betavulgaroside V is an oleanolic acid bidesmoside with a branched carbohydrate chain composed of glucose, an acetal substituent, and glucuronic acid attached to the C-3 hydroxyl group of the aglycone and another glucose attached in the C-28 position [Yoshikawa *et al.*, 1998].

Triterpenoid saponins were detected in *Beta vulgaris* for the first time by the Yoshikawa and Murakami teams and named betavulgarosides (Figure 4). The structures of these substances were determined by spectral methods and by chemical correlation [Yoshikawa *et al.*, 1995, 1996, 1998]. Among the substances tentatively identified in the present study, compounds **6**, **3**, **15**, **2**, **12**, and **1** have the same summary formulae and, based on MS/MS data, also similar structural features as betavulgarosides II, III, IV, V, VII, and IX, respectively. It cannot be excluded that these betavulgarosides are present in Swiss chard plants; however, this needs to be confirmed in additional analyses. Nevertheless, the structural similarity between the saponins from Swiss chard leaf and the sugar beet is in line with what could be expected considering both plants' close affinity – they are derived from a common ancestor – the wild beet [Biancardi *et al*., 2016].

Quantitative analysis

Because of structural features of dioxolane- and acetal-type substituents, *B. vulgaris* saponins are relatively difficult to separate using reversed-phase chromatography. The calculations of pKa for carboxylic groups of a dioxolane substituent carried out using Perkin-Elmer ChemDraw software (v. 19.1) indicate that the lowest acidic pKa is at 2.05. In the typical conditions used in LC-MS, the addition of 0.1% formic acid decreases the mobile phase's pH to approximately 2.7. This value is not low enough to suppress the dissociation of all carboxylic groups and results in very broad and often overlapping chromatographic peaks of dioxolane derivatives. Such difficulties were never reported in the literature addressing the *B. vulgaris* saponins, and they were, apparently, not perceived as a problem in the previously published qualitative and qualitative analyses of *B. vulgaris* saponins [Mikołajczyk-Bator *et al.*, 2016a; Mroczek *et al.*, 2012, 2019]. However, chromatograms presented in all these studies (for example, see Figure 1A, peaks 27–32 in Mikołajczyk-Bator *et al.* [2016a]) clearly demonstrate that resolving these issues is crucial for any reliable quantitative method (peak broadening results in increased detection limits) and that good separation of the analytes is often critical for many detection methods, including mass spectrometric detection. Various approaches can yield improved chromatographic resolution and peak shape for dioxolane- and acetal-containing saponins. A recent LC-MS quantitative study of saponins from roots of *Achyranthes* sp., including betavulgarosides, used an ion-pairing reagent, dihexylammonium acetate (DHAA), to increase chromatographic resolution [Kawahara *et al.*, 2016]. While this approach enabled significant improvement of the chromatographic resolution and excellent peak shapes, from our experience, DHAA is a relatively challenging to purge persistent contaminant of chromatographic systems that severely affects the performance and sensitivity during the positive-mode electrospray ionization analyses. For this reason, mobile phases containing DHAA may only be suitable for dedicated LC-MS systems.

Another possibility for improving the chromatography of betavulgarosides and related compounds is the acidification of the mobile phase. To this end, an acid with lower pKa (for example, trifluoroacetic acid – TFA) or a much higher formic acid concentration must be used. Our study used the latter approach with 0.5% (*v/v*) formic acid, even though

it required a decrease in capillary voltage to prevent excessive capillary current and arcing. The alternative application of 0.05% (*v/v*) TFA as a mobile phase additive was also tested (data not shown). However, it offered a better chromatographic resolution at the cost of significant signal suppression in the negative electrospray mode, to the point where obtaining high-quality MS/MS spectra, a crucial component of the proposed method, became impossible.

The method we propose uses two detectors to collect qualitative and quantitative data on Swiss chard saponins. This approach allows correlating the identification and relative quantification of constituents in complex mixtures of botanical origin [Baker *et al.*, 2018]. It can, by no means, replace classic LC-MS assays based on the individual reference standards. Instead, it can be considered as a viable alternative to the total saponin content (TSC) measurement carried out using other methodologies, such as the spectrophotometric assay [Le *et al.*, 2018], the macro lens-coupled smartphone assay [León-Roque *et al.*, 2019], or the UHPLC-UV assay [Wu *et al.*, 2019]. In turn, the approach we propose offers more reliable quantitation of individual saponin peaks (thus avoiding false-positive signals of spectrophotometric and foam-formation methods) as well as detection that is not dependent on the particular structural features of the investigated analytes. Structure-dependent detection is the weak point of the UHPLC-UV-QTOF method [Wu *et al.*, 2019], which is very similar to our methodology in general concept and application. However, it uses a calibration compound with a specific structure and a specific number of chromophores contributing to the absorbance at 210 nm used for quantitation. Any structural deviation resulting in a different number of chromophores (for example, an additional carboxyl group due to esterification) will change the molar absorbance coefficient and invalidate calibration for that specific compound. Similarly, saponins without chromophores, such as selected steroidal saponins or pseudoginsenoside F11, will produce no signal at all. Non-specific absorbance at 210 nm also restricts the choice of mobile phases for chromatography, practically excluding those with higher UV cut-off, such as methanol or acetone.

The CAD we applied in this study is considered a universal detector. However, like with all aerosol evaporative detectors, its response varies as a function of mobile phase composition [Hutchinson *et al.*, 2010]. Thus, in an ideal isocratic separation, all the analytes at the same concentration should produce identical responses. However, in gradient elution, the higher is the percentage of the organic solvent, the higher signal will be obtained for the analytes. Besides the isocratic elution, which is often not feasible for very complex samples, there are two ways of correcting this discrepancy. One possibility is to provide the detector with a constant mobile phase concentration throughout the analysis, utilizing the secondary pump running the reverse gradient through a separate, identical column. Both columns' outflows are mixed before the detector, providing the constant concentration of the mobile phase [Baker *et al.*, 2018; Górecki *et al.*, 2006]. While relatively simple in application and providing nearly perfect results [Baker *et al.*, 2018], this approach requires additional, careful equipment set up and uses significant volumes of solvents. As an alternative, the so-called 3-D calibration can compensate for signal changes during the elution [Hutchinson *et al.*, 2010].

TABLE 2. Details of the UHPLC-CAD/QTOF-MS/MS method validation.

Parameter	Medicoside G	Soyasaponin Bb
Range [μM]	5–35	5–35
Regression equation (*n=9*)	y = 0.065x+ 0.185	y = 0.076x+ 0.261
R^2	0.995	0.992
LOD [μM]	2.4 (1.8 μg/g DW)	3.6 (3.4 μg/g DW)
LOQ [μM]	7.3	10.69
Intra-day precision peak area ratio RSD% (*n=5*)		
7.5 μM	1.0	2.5
32.5 μM	0.8	2.4
Intra-day accuracy % (*n=5*)		
7.5 μM	91.9 (76.5)*	105.7 (92.8)*
32.5 μM	96.6 (80.3)*	102.0 (116.2)*
Inter-day precision peak area ratio RSD% (*n=10*)		
7.5 μM	5.1	6.7
32.5 μM	4.4	5.6
Inter-day accuracy % (*n=10*)		
7.5 μM	93.4	90.3
32.5 μM	91.6	99.4

* Cross-calibration result, LOD – Limit of Detection, LOQ – Limit of Quantitation, RSD – relative standard deviation, DW- dry weight.

We applied a slight modification of the latter approach. Using two calibration standards, medicoside G and soyasaponin Bb, eluting at two different t_R in the gradient (5.6 min and 21.8 min, respectively), we obtained a set of calibrations applicable to estimate several analytes eluting between these two points. Furthermore, elution during the separation was switched to isocratic with 55% acetonitrile at 22 min. Therefore the concentration of compounds eluting after that time could be estimated using soyasaponin Bb calibration with acceptable accuracy. Cross-calibration, estimation of medicoside G with soyasaponin Bb curve, and *vice versa* produced errors generally not exceeding 25% (Table 2), which in our view were acceptable. Our method's main disadvantage is the relatively narrow linear dynamic range, slightly under one order of magnitude. However, the obtained limits of detection were at acceptable levels of approx. 1–3 μg/g DW.

Nevertheless, the method's main limitation is that the detector's response is determined based on a single calibrant, which might lead to errors if the detector's response to the analyte is significantly different from the response toward the calibrant. Furthermore, because CAD is a 1D detector, it is difficult to assess the purity of integrated peaks in co-elution cases, although this can be aided by analyzing the corresponding MS signals. Needless to say, the method cannot be applied directly to raw extracts and requires some clean-up and fractionation before the UHPLC-CAD/QTOF-MS/MS analysis.

TABLE 3. The contents of individual and total saponins in Swiss chard (*B. vulgaris* L.) cultivars (μg/g DW).

No	Rhubarb	Bulls Blood	Perpetual Spinach	White Silver
1	nd	nd	6.02±0.85^{a}	2.95±0.39^{b}
2	nd	19.83±2.71	nd	nd
3	17.20±2.37^{a}	5.25±1.20^{c}	18.18±1.39^{a}	12.9±1.26^{b}
4	4.68±0.87	nd	nd	nd
5	nd	nd	18.88±3.88^{a}	5.47±2.92^{b}
6	11.01±1.90^{b}	nd	26.69±2.34^{a}	nd
7	5.90±0.97^{a}	4.99±0.26^{b}	3.10±0.39^{c}	3.46±1.10^{bc}
8	18.17±0.30^{a}	nd	19.59±2.99^{a}	3.24±0.27^{b}
9	30.57±4.48^{b}	63.11±3.97^{a}	29.61±2.52^{b}	34.58±2.20^{b}
10	nd	17.71±1.26	nd	nd
11	3.19±0.51^{b}	2.69±0.28^{b}	6.26±1.01^{a}	nd
12	10.57±1.46	nd	nd	nd
13	9.54±0.26^{a}	23.04±1.60^{b}	52.57±3.29^{c}	5.86±1.13^{a}
14	nd	nd	17.44±2.21	nd
15	77.76±6.22^{b}	42.39±4.21^{c}	178.92±24.36^{a}	57.07±2.96^{bc}
16	13.52±1.67	nd	nd	nd
TOTAL	202.11	159.18	397.09	125.53

Values are expressed as the means ± standard deviations of three independent samples. Results in rows not sharing a common letter are significantly different ($p \leq 0.05$); nd – not detected. The numbers (1–16) correspond to the numbers of compounds named in Table 1.

The developed method was then applied to quantify 16 individual saponins present in the leaves of Swiss chard cultivars. The quantitative data revealed differences in saponins' content thereof (Table 3). Specifically, relatively the high content of individual saponins was in the Perpetual Spinach cultivar's leaves, with a total content of 397.09 μg/mg DW. This content was approximately 2 and 2.5 times higher than that in cv. Rhubarb and White Silver leaves and approximately three times higher than in cv. Bulls Blood leaves.

All cultivars contained relatively large amounts of saponin **15**, which predominated in leaves of cultivars Rhubarb, Perpetual Spinach, and White Silver, accounting for 37, 45, and 46% of total saponin content, respectively. Moreover, saponin **15** was the second most abundant saponin in the Bulls Blood cultivar (27% of total saponin content). Saponin **9** was predominant in the Bulls Blood cultivar (40% of total saponin content) and was the second most abundant saponin in leaves of Rhubarb, Perpetual Spinach, and White Silver (15, 16, and 27% of total saponin contents, respectively). The significant accumulation of these two specific triterpene saponins suggests their biological function, but further research is necessary to resolve this issue.

The quantitative analysis of saponins in Swiss chard leaves showed significant differences between their contents in different cultivars. Our previous study demonstrated divergences in the saponin content in leaves of different red beet cultivars [Mroczek *et al.*, 2019]. In contrast to Swiss chard cultivars, showing strong divergences in saponin profiles, red beet leaves of all analyzed cultivars contained the same number of saponin, although in different proportions. The selection of new varieties of Swiss chard, aiming at improving such crop features like pest resistance, storage stability of the collected plant material, taste, or the content of betalains, influences possibly the content of saponins in different plant varieties indirectly or directly, *e.g.*, the antimicrobial activities of Swiss chard saponins can determine its resistance. The different saponin content can also impact the nutraceutical effects of these widely consumed plants. However, extensive studies are required to assess their biological bioactivity and beneficial health properties.

CONCLUSIONS

The UHPLC-CAD/QTOF-MS/MS method has been established for the simultaneous qualitative and quantitative analyses of saponins in Swiss chard leaves. For the qualitative analysis, a total of 16 compounds were tentatively characterized in different Swiss chard cultivars based on MS data and MS/MS fragmentation patterns. In turn, for the quantitative analysis, all saponins detected in plant material were separated and simultaneously determined. The results show that various cultivars differ in the total content of saponins, yet the major compounds are common for all tested cultivars. Specific composition patterns and content of individual saponins may also reflect their function, as saponins' biological activity is often associated with specific chemical constituents. However, further studies are required to provide the total structures of Swiss chard saponins and to elucidate their biological properties within the frame of structure-function relationship studies.

To the best of our knowledge, this work presents the first study of saponins in the Swiss chard plant. The data obtained indicate that Swiss chard is a vital source of these bioactive phytochemicals. As the relative distribution and composition of saponins vary between different cultivars, the developed method allows a complete analysis of Swiss chard saponins and can be of great significance for future investigations and applications. Furthermore, this analytical approach could be validated to determine and quantitate saponins in different Amaranthaceae family species.

RESEARCH FUNDING

This research was partly funded by the Ministry of Science and Higher Education through the Faculty of Biology, University of Warsaw intramural grant DSM nr. 501-D114-86-0117600.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ORCID IDs

M. Kowalczyk https://orcid.org/0000-0001-7454-4762
A. Mroczek https://orcid.org/0000-0002-1677-9413

REFERENCES

1. Alara, L.R., Abdurahman, N.H., Ukaegbu, C.I., Azhari, N.H., Kabbashi, N.A. (2018). Metabolic profiling of flavonoids, saponins, alkaloids, and terpenoids in the extract from *Vernonia cinerea* leaf using LC-Q-TOF-MS. *Journal of Liquid Chromatography & Related Technologies*, *41*, 722–731. https://doi.org/10.1080/10826076.2018.1511995
2. Arslan, I., Cenzano, A.M. (2020). Triterpene saponins in cancer therapy: a review of mode of action. *Revista Brasileira de Farmacognosia, 30*, 1–6. https://doi.org/10.1007/s43450-020-00033-5
3. Baker, T.R., Regg, B.T. (2018). A multi-detector chromatographic approach for characterization and quantitation of botanical constituents to enable *in silico* safety assessments. *Analytical and Bioanalytical Chemistry*, *410*, 5143–5154. https://doi.org/10.1007/s00216-018-1163-y
4. Biancardi, E., Panella, L.W., Lewellen, R.T. (2012). History and current importance. In Biancardi, E., Panella, L.W., Lewellen, R.T. (Eds.), *Beta maritima*. *The Origin of Beets*, 1st edition, Springer, New York, USA, pp. 1–41. https://doi.org/10.1007/978-1-4614-0842-0
5. Foubert, K., Cuyckens, F., Vleeschouwer, K., Theunis, M., Vlietinck, A., Pieters, L., Apers, S. (2010). Rapid quantification of 14 saponins of *Maesa lanceolata* by UPLC–MS/MS. *Talanta*, *81*, 1258–1263. https://doi.org/10.1016/j.talanta.2010.02.018
6. Ge, Y-W., Zhu, S., Yoshimatsu, K., Komatsu, K. (2017). MS/MS similarity networking accelerated target profiling of triterpene saponins in *Eleutherococcus senticosus* leaves. *Food Chemistry*, *227*, 444–452. https://doi.org/10.1016/j.foodchem.2017.01.119
7. Gennari, L., Felletti, M., Blasa, M., Angelino, D., Celeghini, C., Corallini, A., Ninfali, P. (2011). Total extract of *Beta vulgaris* var. cicla seeds *versus* its purified phenolic components: antioxidant activities and antiproliferative effects against colon cancer cells. *Phytochemical Analysis*, *22*, 272–279. https://doi.org/10.1002/pca.1276
8. Gómez-Caravaca, A.M., Segura-Carretero, A., Fernández-Gutiérrez, A., Caboni, M.F. (2011). Simultaneous determination of phenolic compounds and saponins in qinoa (*Chenopodium quinoa* Willd) by a liquid chromatography-diode array detection-electrospray ionization-time-of-flight mass spectrometry methodology. *Journal of Agricultural and Food Chemistry*, *59*, 10815–10825. https://doi.org/10.1021/jf202224j
9. Górecki, T., Lynen, F., Szucs, R., Sandra, P. (2006). Universal response in liquid chromatography using charged aerosol detection. *Analytical Chemistry*, *78*, 3186–3192. https://doi.org/10.1021/ac060078j
10. Hashem, A.N., Soliman, M.S., Hamed, M.A., Swilam, N.F., Lindequist, U., Nawwar, M.A. (2016). *Beta vulgaris* subspecies cicla var. flavescens (Swiss chard): flavonoids, hepatoprotective and hypolipidemic activities. *Pharmazie*, *71,* 227–232.
11. Hutchinson, J.P., Li, J., Farrell, W., Groeber, E., Szucs, R., Dicinoski, G., Haddad, P.R. (2010). Universal response model for a corona charged aerosol detector. *Journal of Chromatography A*, *1217*, 7418–7427. https://doi.org/10.1016/j.chroma.2010.09.056
12. Ivanović, L., Milašević, I., Topalović, A., Đurović, D., Mugoša, B., Knežević, M., Vrvić, M. (2019). Nutritional and phytochemical content of Swiss chard from Montenegro, under different fertilization and irrigation treatments. *British Food Journal*, *121*, 411–425. https://doi.org/10.1108/BFJ-03-2018-0142
13. Kawahara, Y., Hoshino, T., Morimoto, H., Shinizu, T., Narukawa, Y., Fuchino, H., Kawahara, N., Kiuchi, F. (2016). LC-MS-based quantification method for *Achyranthes* root saponins. *Journal of Natural Medicines*, *70*, 102–106. https://doi.org/10.1007/s11418-015-0942-4
14. Kirk, D.D., Rempel, R., Pinkhasov, J., Walmsley, A.M. (2004). Application of *Quillaja saponaria* extracts as oral adjuvants for plant-made vaccines. *Expert Opinion on Biological Therapy*, *4*, 947−958. https://doi.org/10.1517/14712598.4.6.947
15. Kohda, H., Tanaka, S., Yamaoka, Y., Ohhara, Y. (1991). Saponins from *Amaranthus hypochondriacus*. *Chemical and Pharmaceutical Bulletin*, *39*, 2609–2612. https://doi.org/10.1248/cpb.39.2609
16. Kowalczyk, M., Pecio, Ł., Stochmal, A., Oleszek, W. (2011). Qualitative and quantitative analysis of steroidal saponins in crude extract and bark powder of *Yucca schidigera* Roezl. *Journal of Agricultural and Food Chemistry*, *59*, 8058–8064. https://doi.org/10.1021/jf2022397
17. Le, A.V., Parks, S.E., Nguyen, M.H., Roach, P.D. (2018). Improving the vanillin-sulphuric acid method for quantifying total saponins. *Technologies*, *6*, art. no. 84. https://doi.org/10.3390/technologies6030084
18. León-Roque, N., Aguilar-Tuesta, S., Quispe-Neyra, J., Mamani-Navarro, W., Alfaro-Cruz, S., Condezo-Hoyos, L. (2019). A green analytical assay for the quantitation of the total saponins in quinoa (*Chenopodium quinoa* Willd.) based on macro lens-coupled smartphone. *Talanta*, *204*, 576–585. https://doi.org/10.1016/j.talanta.2019.06.014
19. Mikołajczyk-Bator, K., Błaszczyk, A., Czyżniejewski, M., Kachlicki, P. (2016a). Characterization and identification of triterpene saponins in the roots of red beet (*Beta vulgaris* L.) using two HPLC-MS systems. *Food Chemistry*, *192*, 979–990. https://doi.org/10.1016/j.foodchem.2015.07.111
20. Mikołajczyk-Bator, K., Błaszczyk, A., Czyżniejewski, M., Kachlicki, P. (2016b). Identification of saponins from sugar beet (*Beta vulgaris*) by low and high-resolution HPLC–MS/MS. *Journal of Chromatography B, 1029–1030*, 36–47. https://doi.org/10.1016/j.jchromb.2016.06.038
21. Mroczek, A. (2015). Phytochemistry and bioactivity of triterpene saponins from Amaranthaceae family. *Phytochemistry Reviews*, *14*, 577–605. https://doi.org/10.1007/s11101-015-9394-4
22. Mroczek, A., Kapusta, I., Janda, B., Janiszowska, W. (2012). Triterpene saponin content in the roots of red beet (*Beta vulgaris* L.) cultivars. *Journal of Agricultural and Food Chemistry*, *60*, 12397–12402. https://doi.org/10.1021/jf303952x
23. Mroczek, A., Kapusta, I., Janda, B., Stochmal, A., Janiszowska, W. (2019). MS/MS and UPLC-MS profiling of triterpenoid saponins from leaves and roots of four red beet (*Beta vulgaris* L.) cultivars. *Phytochemistry Letters*, *30*, 333–337. https://doi.org/10.1016/j.phytol.2019.02.015
24. Murakami, T., Matsuda, H., Inadzuki, M., Hirano, K., Yoshikawa, M. (1999). Medical foodstuffs. XVI. Sugar beet. (3): abso-

lute stereostructures of betavulgarosides II and IV hypoglycemic saponins having a unique substituent, from the root of *Beta vulgaris* L. *Chemical and Pharmaceutical Bulletin, 47*, 1717–1724. https://doi.org/10.1248/cpb.47.1717

25. Nakamura, S. Chen, G., Nakashima, S., Matsuda, H., Pei, Y., Yoshikawa, M. (2010). Brazilian natural medicines. IV. New noroleanane-type triterpene and ecdysterone-typesterol glycosides and melanogenesis inhibitors from the roots of Pfaffia-glomerata. *Chemical and Pharmaceutical Bulletin*, *58*, 690–695. https://doi.org/10.1248/cpb.58.690
26. Ninfali, P., Angelino, D. (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
27. Onlom, C., Nuengchamnong, N., Phrompittayarat, W., Putalun, W., Waranuch, N., Ingkaninan, K. (2017). Quantification of saponins in *Asparagus racemosus* by HPLC-Q-TOF-MS/MS. *Natural Product Communications, 12*(1), 7–10. https://doi.org/10.1177/1934578X1701200103
28. Osbourn, A.E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell*, 8, 1821–1831. https://doi.org/10.1105/tpc.8.10.1821
29. Podolak, J., Galanty, A., Sobolewska, D. (2010). Saponins as cytotoxic agents: a review. *Phytochememistry Reviews*, *9*, 425–474. https://doi.org/10.1007/s11101-010-9183-z
30. Pyo, Y.H., Lee, T.C., Logendra, L., Rosen, R.T. (2004). Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts. *Food Chemistry*, *85*, 19–26. https://doi.org/10.1016/S0308-8146(03)00294-2
31. Sparg, S.G., Light, M.E., Van Staden, J. (2004). Biological activities and distribution of plant saponins. *The Journal of Ethnopharmacology*, *94*, 219–243. https://doi.org/10.1016/j.jep.2004.05.016
32. Spórna-Kucab, A.E., Wybraniec, S. (2020). High-speed countercurrent chromatography in separation and identification of saponins from *Beta vulgaris* L. cultivar Red Sphere. *Polish Journal of Food and Nutrition Sciences*, *70*(1), 67–74. https://doi.org/10.31883/pjfns/116416
33. Tava, A., Odoardi, M. (1996). Saponins from *Medicago* spp.: Chemical characterization and biological activity against insects. In G. Waller, K. Yamasaki (Eds.), *Saponins Used in Food and Agriculture*, Plenum Press, New York, US, pp. 97–109. https://doi.org/10.1007/978-1-4613-0413-5_9
34. Troszyńska, A. (2004). Non-nutrient bioactive substances in food of plant origin causing bitterness and astirgency. *Polish Journal of Food and Nutrition Sciences, 54*, 65–73.
35. Vehovec, T., Obreza, A. (2010). Review of operating principle and applications of the charged aerosol detector. *Journal of Chromatography A, 1217*, 1549–1556. https://doi.org/10.1016/j.chroma.2010.01.007
36. Wu, X., Jia, L., Wu, J., Liu, Y., Kang, H., Liu, X., Li, P., He, P., Tu, Y., Li, B. (2019). Simultaneous determination and quantification of triterpene saponins from *Camellia sinensis* seeds using UPLC-PDA-QTOF-MS/MS. *Molecules*, *24*, art. no. 3794. https://doi.org/10.3390/molecules24203794
37. Yoshikawa, M., Murakami, T., Kadoya, M., Matsuda, H., Yamahara, J., Muraoka, O., Murakami, N. (1995). Betavulgarosides I, II, III, IV and V hypoglycemic glucuronide saponins from the roots and leaves of *Beta vulgaris* L. (sugar beet). *Chemical Pharmaceutical Bulletin*, *41*, 1621–1626. https://doi.org/10.3987/COM-95-7111
38. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1996). Medical foodstuffs. III. Sugar beet. (1): hypoglycemic oleanolic acid oligoglycosides, betavulgarosides I, II, III, and IV, from the root of *Beta vulgaris* L. (Chenopodiaceae). *Chemical and Pharmaceutical Bulletin*, *44*, 1212–1217. https://doi.org/10.1248/cpb.44.1212
39. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1998) Medical foodstuffs. XV. Sugar beet. (2): structures of betavulgarosides V, VI, VII, VIII, IX and X from the roots and leaves of sugar beet (*Beta vulgaris* L., Chenopodiaceae). *Chemical and Pharmaceutical Bulletin*, *46*, 1758–1763. https://doi.org/10.1248/cpb.46.1758

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 161–175
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/135800
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Development of New Gluten-Free Maize-Field Bean Bread Dough: Relationships Between Rheological Properties and Structure of Non-Gluten Proteins

Awatif Fetouhi[1], Agnieszka Sujak[2], Leila Bentallah[1], Agnieszka Nawrocka[3], Monika Szymańska-Chargot[4], Marta Tomczyńska-Mleko[5], Agnieszka Wójtowicz[6], Mohammed Nasreddine Zidoune[1]*

[1]*Institut de la Nutrition, de l'Alimentation et des Technologies Agro-Alimentaires, Université des Frères Mentouri, Constantine 1, Route de Ain El_Bey, Constantine, Algeria*
[2]*Department of Biosystems Engineering, Faculty of Environmental and Mechanical Engineering, Poznań University of Life Sciences, Wojska Polskiego 50, 60–637 Poznań, Poland*
[3]*Laboratory for Quality Assessment of Grain and Oilseed Raw Materials, Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4, 20–290 Lublin, Poland*
[4]*Department of Microstructure and Mechanics of Biomaterials, Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4, 20–290 Lublin, Poland*
[5]*Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin, Akademicka 15, 20–950 Lublin, Poland*
[6]*Department of Thermal Technology and Food Process Engineering, University of Life Sciences, Głęboka 31, 20–612 Lublin, Poland*

Key words: gluten-free dough, maize, field bean, non-gluten proteins, pasting properties, protein secondary structure, FT-Raman spectroscopy

This work aimed to examine the rheological properties and structural features of newly developed gluten-free doughs with maize (M), field bean (FB), maize-filed bean (MFB), and maize-field bean improved with hydrothermally-treated maize (IMFB), and compare them with soft wheat (SW) dough as a control. The relationships between viscoelastic characteristics, pasting properties of dough, and structure of non-gluten proteins analyzed using FT-Raman spectroscopy were investigated. All gluten-free doughs showed significantly higher values of the elastic modulus than SW dough. The low values of tan δ for doughs of M, MFB, and IMFB formulas indicated strong contribution of the solid character in their structural formation as compared to SW and FB doughs. Protein backbone of maize and maize-based doughs was characterized by the absence of pseudo-β-sheet structure and a high content of β-sheet accompanied with a low content of antiparallel-β-sheet. According to principal component analysis (PCA), a strong relationship was found between protein secondary structure, tan δ, gelatinization temperature, and between aromatic amino-acid chains, peak viscosity, and breakdown. The mechanism of non-gluten protein network establishment was based on the formation of β-sheet and α-helix structure. The study results indicate the significant involvement of trans-gauche-gauche (TGG) and trans-gauche-trans (TGT) disulfide bridges in the formation of the non-gluten protein matrix rather that gauche-gauche-gauche (GGG) conformation. PCA analysis showed that the water absorption of the starch granules increased with the greater exposition of the tyrosyl residues.

INTRODUCTION

Obtaining gluten-free bread (GFB) of superior quality is a technological challenge. Gluten is considered as a key component imparting wheat dough its unique properties in baked goods. It is composed of gliadins and glutenins – two proteins that contribute to the bread dough viscosity and elasticity [Barak *et al*., 2014; Belton, 1999]. According to Singh & MacRitchie [2001], gluten structure develops and becomes apparent when the wheat flour is hydrated and subjected to the energy of mixing. It is defined as a viscoelastic mass capable of forming structures that retain gases allowing dough to expand and become soft, light, and palatable after baking [Khatkar & Schofield, 2007].

The inability of gluten-free (GF) flour to form viscoelastic dough after kneading with water, makes production of GFB technologically difficult. The study of the mechanisms of development of the structure of gluten-free bread dough with or without improvement is thus the key to the understanding and controlling the functionality of components, and it brings up possible solutions to improve GF product quality.

* Corresponding Author:
agnieszka.sujak@up.poznan.pl (A. Sujak)

Submitted: 9 February 2021
Accepted: 14 April 2021
Published on-line: 5 May 2021

FT-IR and FT-Raman spectroscopy has been used in several studies to determine the dough structure development and to investigate the gluten-protein quality and structure relationships [Nawrocka *et al*., 2016b; Pourfarzad *et al*., 2015; Sivam *et al*., 2013; Wang *et al*., 2015]. Our recent study [Fetouhi *et al*., 2019] on the viscoelastic behavior of rice-field bean gluten-free dough using FT-IR concluded that the low viscoelastic quality of this type of GF dough is due to the different secondary structures developed by non-gluten as compared to gluten proteins. Accordingly, in non-gluten proteins, β-sheet content increased, albeit with the absence of β-turn structures. The reorganization of starch molecules also had an effect on the quality of GFB doughs. Moreover, a relationship between the structure of proteins and starch and the viscoelastic behavior of rice-field bean dough was clearly indicated. As rice and rice-field bean preparations showed low kneading quality, we started to search for other formulas.

In this context, our work aimed to study the structural features of newly developed types of GF dough based on maize-field bean formulas through examining correlations between rheological properties and structure of non-gluten proteins. Maize (M) and field bean (FB) flours were chosen in order to obtain a balanced composition of blended amino acid in GF bread. As the conformation of the S-S bridges plays a major role in the functional properties of gluten dough, the disulfide bridge regions were analyzed in GF doughs and compared with soft wheat dough. FT-Raman spectroscopy has not yet been adapted in GF dough investigation to identify the relationship between structural mechanisms and quality of GF bread dough development.

MATERIALS AND METHODS

Raw materials

Soft wheat (SW) flour (*Triticum aestivum*) (Młyn Piaski, Piaski, Poland) was purchased locally. Maize (*Zea mays* L.) (Alicampo Company, Del Viso, Buenos Aires, Argentina) and field bean (*Vicia faba* L.) (Al-behera, Tanta, Egypt) seeds were purchased from the Algerian market. The seeds were ground using an MG E3 grinder (UMA Rouiba, Ar_Ruwajba, Algeria) and sieved to obtain flours with a particle size less than 200 μm. Sodium chloride was purchased from Sigma Aldrich (Poznań, Poland). Distilled water was used for dough preparation (Hydrolab, Straszyn, Poland). The chemical composition of raw materials was evaluated according to American Association of Cereal Chemists (AACC) [1995] methods: AACC 46–10 for protein, AACC 30–10 for fat, and AACC 08–01 for ash content. Total dietary fiber was determined based on the Association of Official Analytical Chemists (AOAC) 993.21 procedure [AOAC, 2000]. Proximate composition of raw materials was: SW – protein 12.25 g/100 g, fat 0.98 g/100 g, ash 0.55 g/100 g, dietary fiber 1.30 g/100 g; M – protein 5.13 g/100 g, fat 1.41 g/100 g, ash 0.45 g/100 g, dietary fiber 2.00 g/100 g; and FB – protein 23.91 g/100 g, fat 3.91 g/100 g, ash 7.82 g/100 g, dietary fiber 9.33 g/100 g.

Dough preparation

All tested doughs were prepared using a Farinograph-E (model 81101142, Brabender, Germany) equipped with a 50 g mixer. Here, 50 g of SW or gluten-free flours (M, FB, and maize-field bean (MFB) formula) and aqueous solution of sodium chloride (2%, *w/w*) were kneaded for 20 min with an appropriate amount of distilled water determined according to the Farinograph optimal conditions (500 FU). MFB formula was obtained by mixing 33.34% of FB flour with 66.66% of M flour in order to obtain a balanced composition of blended amino acids [Benatallah *et al*., 2012]. An improved maize-field bean (IMFB) formula was prepared according to the procedure described by Bourekoua *et al*. [2016], where a portion of 6.9 g of maize flour was mixed with 34.5 mL of distilled water and heated to 65°C with continuous stirring. The obtained gel was stored at 4°C for 24 h and then added to a mixture of 16.67 g of field bean and 26.43 g of maize flours. Water amounts added to each sample, as well as dough water absorption (WA) are presented in Table 1. All doughs were allowed to rest for 20 min at room temperature before oscillatory tests. Samples for FT-Raman analysis and pasting proprieties determination were lyophilized for 24 h (0.04 mbar, -50°C). After freeze-drying, they were ground to powder in a laboratory grinder (MK100S, Katowice, Poland).

Rheological proprieties

Small strain oscillatory test

The oscillatory test was done to estimate the differences in the viscoelastic behavior of non-gluten doughs in comparison with control SW dough. The measurements were conducted using a Rheo-Stress 300 rheometer (Karlsruhe, Germany) equipped with parallel plates of 5 cm in diameter with the adjusted gap of 2 mm. After the resting time (20 min), dough was placed between the plates, the excess of dough was removed, and it was then subjected to scanning at 20°C with a frequency sweep ranging between 0.1–10 Hz and low strain value (0.1%) in order to keep the examined dough in a linear viscoelastic region determined *via* strain sweep tests performed at 1 Hz frequency according to Lazaridou *et al*. [2007]. The storage (G') modulus, loss (G") modulus, and the loss tangent (tan δ) were recorded. For each type of dough, the test was done in duplicate.

Pasting properties

Measurements were performed according to Dib *et al*. [2018], using a microvisco-amylograph (Brabender OHG, Duisburg, Germany) operated under constant conditions of speed (250 rpm) and sensitivity (235 cm×g). Freeze-dried dough powders (10 g) and an appropriate amount of distilled water (corrected to compensate of 14% moisture) were mixed and continuously stirred for 5 min to obtain a homogeneous dispersion. The sample was heated from 30°C up to 93°C, held at 93°C for 5 min, cooled from 93°C to 50°C, and held at 50°C for 1 min. The heating/cooling rate was 7.5°C/min. The estimated proprieties were: gelatinization temperature (GT, °C), initial viscosity (IV, mPa·s), peak viscosity (PV, mPa·s), final viscosity (FV, mPa·s), breakdown (BD, mPa·s), and setback (SB, mPa·s). For each sample, the test was repeated twice.

FT-Raman analysis and data manipulation

Raman spectra were acquired using the FT-Raman module (NXR FT Raman) of a Nicolet 6700 FT-IR bench with

TABLE 1. Water added, water absorption characteristics of doughs at 500 FU, and rheological parameters (G', G" and tan δ) of doughs in the linear viscoelastic domain (strain 0.1%) at 1 Hz frequency.

Dough	Water* (mL/50 g)	WA (%)	G' (Pa)	G" (Pa)	tan δ (-)
SW	25.9	52.5±0.2^{c}	32,490±689^{d}	14,780±309^{b}	0.449±0.009^{a}
M	40.5	79.6±0.2^{a}	71,720±6083^{a}	15,705±1562^{b}	0.218±0.003^{d}
FB	22.8	45.6±0.2^{d}	58,010±1213^{b}	26,350±551^{a}	0.454±0.009^{a}
MFB	29.5	58.0±0.2^{b}	50,320±3783^{c}	14,100±658^{b}	0.280±0.002^{b}
IMFB	19.5	38.2±0.2^{e}	96,420±20,195^{a}	24,802±5356^{a}	0.256±0.001^{c}

*±0.1 mL/50 g; SW – soft wheat, M – maize, FB – field bean, MFB – maize-field bean formula, IMFB – improved maize-field bean formula, WA – water absorption, G' – storage modulus; G" – loss modulus; tan δ – loss tangent. $^{a-e}$ – different letters in columns indicate significant differences at α=0.05.

an InGaAs detector and CaF_2 beam splitter (Thermo Scientific, Madison, WI, USA). Samples were placed in stainless cubes and illuminated by means of a Nd:YAG excitation laser operating at 1064 nm. The maximum laser power was 1 W. In a single measurement, the spectra were recorded over the range of 3500–150 cm^{-1} and each resulting spectrum was given an average of 200 scans at 8 cm^{-1} of resolution. The analyzed spectra were averaged over the five registered spectra. Spectral data from sample scans were baseline-corrected using OMNIC software (version 8.2, Thermo Fischer Scientific Inc., Madison, WI, USA). Amide I band (1590–1720 cm^{-1}), the S-S region (490–540 cm^{-1}), aromatic amino acids environment: ratio of tyrosine doublet (I(850 cm^{-1})/I(830 cm^{-1})) and tryptophan band (I(760 cm^{-1})) were analyzed. To eliminate the effect of starch absorbance from the S-S region, starch spectra corresponding to each type of dough were collected and subtracted from dough spectra in the region 450––550 cm^{-1} as follows: first starch band and that of the dough sample were peak normalized at maximum starch absorbance (479 cm^{-1}), then the normalized band of starch was subtracted from the dough sample. Here, the S-S region (490–540 cm^{-1}) band was baseline-corrected, surface normalized, and analyzed in order to estimate the structural conformation of disulfide bridges participating in the gluten-free protein network. The second derivatives of the amide I band and the S-S region were calculated using a five-point two-degree polynomial function in order to separate the overlapping bands and to identify the constituents of protein secondary structure and disulfide bridges conformation. In undertaking this, baseline-corrected and surface-normalized amide I bands and the S-S region were deconvoluted with Gaussian curves using ORIGIN software (version 8.0724 PRO, Origin Lab Corporation, Northampton, MA, USA). Differences in spectra were also calculated in order to confirm deconvolution results. The quality of the band deconvolution was indicated by $R^2>0.99$, solution convergence, and $\chi^2< 0.001$. The relative composition of amide I band secondary protein structures and types of structural conformation in the S-S region were expressed as percentage of the area of the fitted region manifested as a relative area of components centered at specific wavenumbers [Nawrocka *et al*., 2016b]. Amide I secondary structures: aggregates (AGR), pseudo β-sheet (Pβ-sh), β-sheet (β-sh), α-helix (α-hx), β-turn (β-trn), and antiparallel β-sheet (Aβ-sh), as well as disulfide conformations: gauche--gauche-gauche (GGG), trans-gauche-gauche (TGG), and trans-gauche-trans (TGT) were assigned according to Nawrocka *et al*. [2016b] and Gómez *et al*. [2013]. To study the behavior of hydrophobic interactions inside the aromatic amino acids environment, the ratio R of the intensities of I(850 cm^{-1})/I(830 cm^{-1}) characteristic of tyrosine doublet, and I – the intensity I(760 cm^{-1}) characteristic of tryptophan band, were calculated.

Statistical analysis

The analysis of variance ANOVA and significant differences test (Tukey HSD test) were done to find differences between the rheological behavior of tested doughs and to estimate the effect of maize-field bean dough improvement. Principal component analysis (PCA) was used to highlight the relationships between viscoelastic behavior, pasting proprieties, and protein structure to explain the rheological behavior of GF doughs *versus* SW dough. Both statistical tests were carried out using XLSTAT software (version 2009.1.01, Addinsoft, USA) at a confidence level of α=0.05. Results were expressed as means ± standard deviations.

Correlations between rheological and structural parameters were checked by calculation of Pearson's coefficients (r). The following interpretation was assumed: $|r|\geq0.8$ – very strong correlation, $|r|\geq0.7$ strong correlation.

RESULTS AND DISCUSSION

Rheological proprieties

The rheological properties were determined in order to explain the structural features of the non-gluten doughs in comparison with dough made of soft wheat (control).

Viscoelastic behavior

Dough viscoelastic behavior was tested by ascertaining storage modulus (G'), loss modulus (G"), and loss tangent (tan δ) as a function of frequency in the linear viscoelastic region (Figure 1). These three rheological properties show the nature of the food matrix. G' (elastic or storage modulus) relates to the material's ability to store energy elastically and G'' (viscous or loss modulus) is related to the material's ability to dissipate stress through heat. Loss tangent (tan δ)

is the ratio of G'' to G' which provides information on the relative contribution of viscous properties to elastic properties of the network [Tunick, 2011]. To compare the viscoelastic behavior of the different dough types, analysis of variance was conducted between G', G", and tan δ at 1 Hz of frequency. The results are summarized in Table 1.

All GF doughs had higher storage modulus than SW dough ($p<0.05$). These results were similar to those of Sivaramakrishnan *et al*. [2004] who reported higher storage modulus of GF rice-based doughs when compared with standard SW dough. FB supplementation of maize flour, however, significantly reduced the storage modulus from 71,720 Pa for M dough, to 50,320 Pa for the MFB formula. Treating maize hydrothermally as in the IMFB formula resulted in a significant increase in the solid character of GF dough compared to MFB dough. As stated by Lazaridou *et al*. [2007], the strengthening of GF dough by partly gelatinized ingredients improved elasticity and decreased extensibility as compared to SW dough. Here, the elasticity of GF doughs is directly related to the level of structuralization of the dough matrix and the value of G' increases with increasing level of structuralization. Both Lazaridou *et al*. [2007], and He & Hoseney [1992] showed that the presence of interactions between the proteins and other components of dough was manifested by low values of G'. This may indicate the low ability of non-gluten proteins to interact with other dough constituents, in comparison with gluten proteins, and might explain the low elasticity characterizing GF doughs.

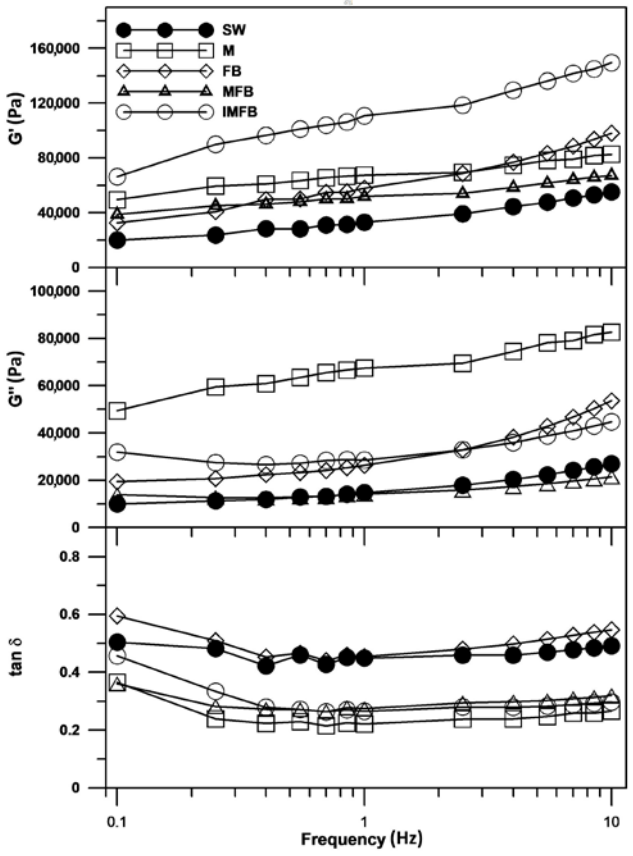


FIGURE 1. Rheological parameters (G' – storage modulus, G" – loss modulus, tan δ – loss tangent) of doughs of soft wheat (SW), maize (M), field bean (FB), maize-filed bean (MFB) formula and improved MFB formula (IMFB) as a function of frequency.

The results of variance analysis showed insignificant differences between the loss modulus G" of SW, M, and MFB doughs (Table 1). Field bean flour supplementation of maize did not affect the viscous behavior of maize dough. FB and IMFB formula doughs gave G" values significantly higher than that of control SW. Moreover, the hydrothermal treatment resulted in the significant increase of the viscous modulus of IMFB formula dough, and this dough behaved similarly to high-protein FB dough.

The FB dough alone had a similar value of tan δ to SW dough (Table 1) [Dus & Kokini, 1990]. The low value of tan δ registered for M dough indicates the strong contribution of the solid character in its structural formation. FB flour supplementation of M flour significantly increased the tan δ value, which indicates that the cereal–legume formulation is characterized by an improved GF dough viscous behavior induced by reducing the solid-state contribution during its formation. This could be explained by the decreased matrix structuralization and indicates that the FB proteins had developed weaker chemical bonds with other constituents than between themselves. The hydrothermal treatment of the part of maize flour resulted in a significant decrease of tan δ value of IMFB dough (1.09 times lower than that of MFB dough). Moreover, the addition of hydrothermally-treated maize flour to the GF bread recipe reduced the GF dough viscosity – as confirmed *via* the pasting characteristics of IMFB formula dough. The hydrothermally-treated maize flour may have acted as a binder of the dough matrix, improving its elastic component [Dib *et al.*, 2018].

Pasting proprieties

During dough formation, starch acts as an inert filler in the dough's continuous protein matrix [Hřivna, 2018]. A dough rheological behavior is significantly affected by the specific properties of starch granules present on its surface [Larsson & Eliasson, 1997]. Pasting properties of the studied doughs are presented in Table 2. As indicated, GT and IV values of all the tested doughs were similar. Here, SW and M doughs had the highest values of PV and BD, followed by the MFB formula and the improved IMFB doughs. FB dough showed the lowest PV and BD, indicating the highest stability of its paste due to a high protein content and the ability to absorb and hold water during heating. Ragaee & Abdel-Aal [2006] suggested that high PV and BD values were related to the degree of starch granule swelling during heating. Here, the greater swelling capacity, the higher the PV values, hence, SW and M dough starch granules had a higher swelling capacity than the other doughs. These results are similar to the finding reported by Ragaee & Abdel-Aal [2006] who found that SW flour gave high PV and BD values as compared to whole grain meals of durum wheat, barley, and sorghum.

During cooling, the viscosities of all doughs increased to an FV (Table 2), indicating the formation of a gel structure due to re-association between starch molecules. The high SB value is an indicator of the retrogradation and reordering

TABLE 2. Pasting characteristics of wheat and gluten-free doughs.

Dough	GT (°C)	IV (mPa·s)	PV (mPa·s)	FV (mPa·s)	BD (mPa·s)	SB (mPa·s)
SW	73.5±2.2^{a}	14.5±0.7^{a}	223.5±13.4^{a}	333.0±12.7^{b}	80.0±6.1^{a}	186.5±6.4^{a}
M	74.5±0.1^{a}	13.5±2.1^{a}	213.5±14.8^{a}	385.5±24.7^{a}	31.0±2.8^{b}	206.5±14.8^{a}
FB	72.5±0.3^{a}	13.0±1.4^{a}	117.5±4.9^{d}	185.0±8.5^{e}	2.5±0.7^{d}	78.0±4.2^{c}
MFB	74.8±0.1^{a}	14.0±0.0^{a}	166.0±5.6^{b}	276.0±7.1^{c}	26.0±2.8^{b}	141.5±4.9^{b}
IMFB	74.4±0.1^{a}	14.0±0.1^{a}	146.0±2.8^{c}	245.0±5.6^{d}	18.0±0.1^{c}	122.0±83.0^{b}

SW – soft wheat, M – maize, FB – field bean, MFB – maize-field bean formula, IMFB – improved maize-field bean formula, GT – gelatinization temperature, IV – initial viscosity, PV – peak viscosity, FV – final viscosity, BD – breakdown, SB – setback. $^{a\text{-}e}$– different letters in columns indicate significant differences at α=0.05.

of starch molecules attributable to syneresis [Ragaee & Abdel-Aal, 2006]. Accordingly, starch molecules of SW and M doughs with high SB values (186.5 and 206.5 mPa·s, respectively) induced high retrogradation rates. In contrast, FB dough with a low SB value (78.0 mPa·s) (Table 2) generated the lowest retrogradation rates due to having the lowest starch content.

Pasting properties of GF doughs were significantly affected by legume flour supplementation of maize. This brought about insignificant differences between MFB and IMFB doughs and significant differences to that of SW. Maize flour hydrothermal treatment in the IMFB dough resulted in a slight lowering of pasting parameter values compared to the MFB formula. This could be due to the alteration of the treated maize flour's pasting properties because of partial starch gelatinization resulting in bond formation between the chains of the amorphous region in starch molecules, as well as alteration of crystallinity induced by hydrothermal treatment, and thus by the starch retrogradation that occurred after cooling [Zavareze & Guerra Dias, 2011]. Similar results were found by Dib *et al*. [2018] who studied the effect of hydrothermally-treated corn flour addition on the quality of corn/field bean gluten-free pasta.

Structure of dough proteins

Secondary structure of proteins involved in dough matrix development

Raman spectroscopic protein structure analysis was based on the following vibrational modes: amide A (NH stretching ~3500 cm^{-1}), amide B (NH stretching ~3100 cm^{-1}), and amide I to VII (I: 1600–1700 cm^{-1}, II: 1480–1580 cm^{-1}, III: 1230–1300 cm^{-1}, IV: 625–770 cm^{-1}, V: 640–800 cm^{-1}, VI: 540–600 cm^{-1}, VII: ~200 cm^{-1}) [Rygula *et al*., 2013]. The amide I band is usually used to estimate the type and the percentage of protein secondary structure in gluten matrices [Sivam *et al*., 2013].

Figure 2 shows the curve-fitted amide I bands (1590––1720 cm^{-1}). Peak numbers and position used in the curve fitting of each type of dough were determined according to results indicated by the second derivative (Figure 3). Distribution of secondary structures of doughs protein, calculated by deconvolution of FT-Raman spectra of the examined doughs is summarized in Table 3. The content of secondary structures of proteins for all doughs was evaluated by analysis of amide I differential spectra between GF and SW doughs (Figure 4) and between various gluten-free doughs (Figure 5). Here, amide I bands of maize and maize-based doughs showed a different shape in comparison with that of SW and FB doughs. This could indicate differences in the type and distribution of protein secondary structures (Figure 2). All amide I bands demonstrated a high absorbance around ~1655 cm^{-1}. This revealed that the secondary structure involved in the formation of the tested doughs was dominated by α-helix conformation [Sivam *et al*., 2013].

Deconvolution of the amide I band of SW dough (Figure 2A) showed that the distribution of its secondary structures quantitative was predominated by α-helix (43%), followed by antiparallel β-sheet (18%), β-turn (15%), β-sheet (11%), pseudo β-sheet (6%), and finally the aggregates (7%) (Table 3). A previous study carried out by Nawrocka *et al.* [2015] uncovered differences in the secondary structure of native gluten proteins corresponding to α-helices (60%) and antiparallel β-sheet (8%) content, as compared to the results obtained in our study where a relatively small fraction of pseudo β-sheet structure was found. The absence of random coil structure was also noted in this study. In contrast, Gómez *et al*. [2013] showed the participation of the random coil structure in the structuring of the native gluten backbone. The α-helix structure was also predominant in this study.

The deconvolution of amide I band of maize dough (Figure 2B) revealed that its protein backbone was mainly formed by α-helix (46%) at levels close to that of SW dough [Pelton, 2000]. Aggregates (21% found at 1602 cm^{-1}) and β-sheets (19% at 1629 and 1639 cm^{-1}) followed α-helix in the maintaining of maize dough protein networks (Table 3). These two structures were, respectively, 3.0 and 1.7 times more abundant than in SW. Fractions of antiparallel β-sheet (8%), followed by β-turn structure (6%) were lower than in control SW dough, and pseudo β-sheet structures were not evident. The FT-IR spectroscopic work of Mejia *et al.* [2007] on the secondary structure of viscoelastic polymers of maize α-zein and wheat gluten proteins showed that the native structure of maize α-zein was mainly composed of α-helices (~68%). Similar results were reported by Matsushima *et al*. [1997].

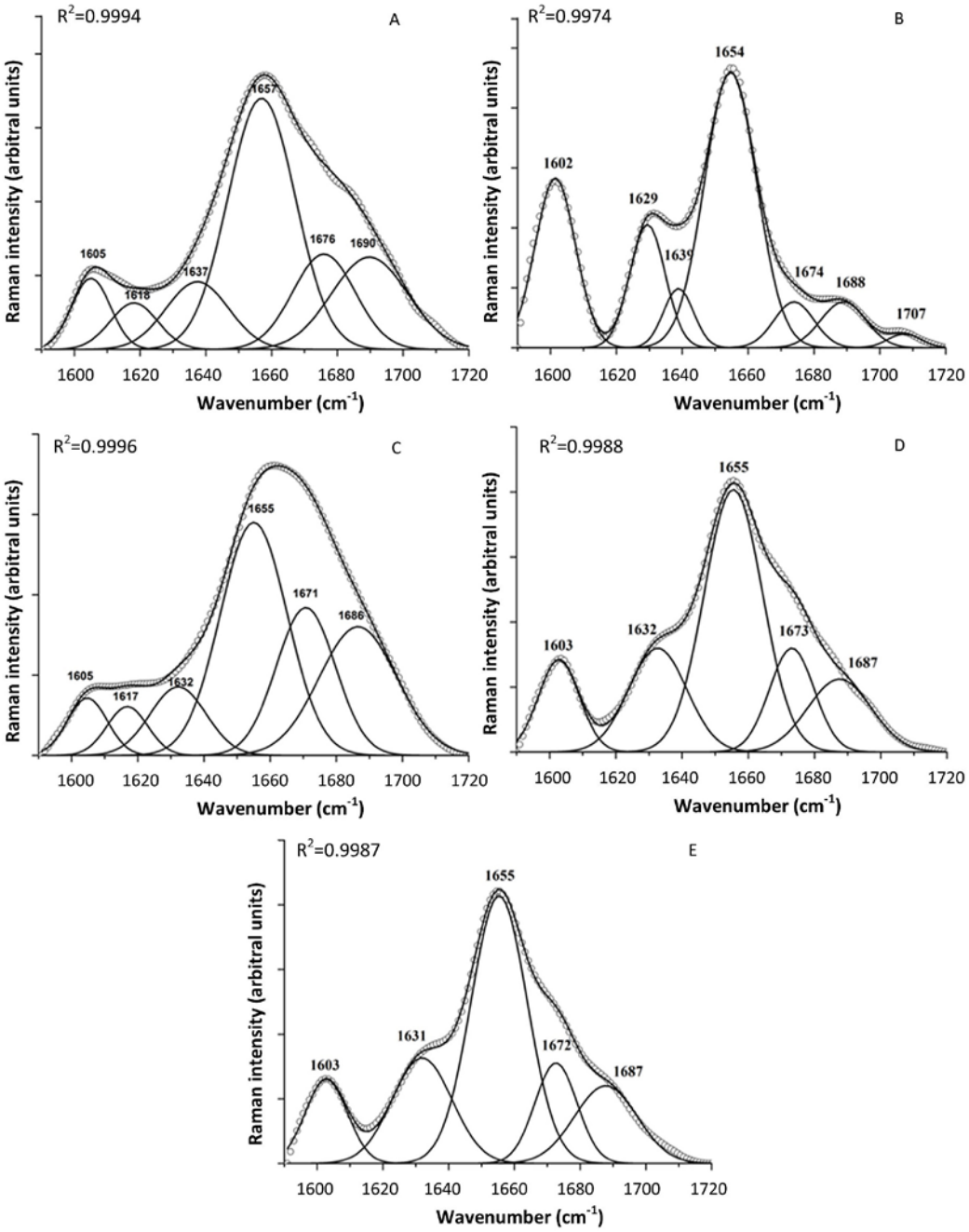


FIGURE 2. Deconvoluted Raman spectra in the area representing amide I band (1590- 1720 cm^{-1}) of doughs: (A) soft wheat, SW, (B) maize, M, (C) field bean, FB, (D) maize-filed bean, MFB, formula, (E) improved MFB formula, IMFB. Solid line – fitted curve, open circles – original data.

The secondary structure of FB dough (Figure 2C) consisted of the dominant α-helix structure at 37% (1.16 times lower than that in SW dough), 24% of antiparallel β-sheet (at 1685 cm^{-1}), and 21% of β-turn (1671 cm^{-1}) (1.33 and 1.4 times higher than in SW control dough). Although an increase was observed in the case of FB antiparallel β-sheet, no statistical differences were found between FB and SW. Amounts of aggregates (5%), pseudo β-sheet (4%), and β-sheet (9%) were close to that of the control dough (Table 3). FT-IR spectroscopy of field bean dough showed the dominance of the α-helix structure (45%) in the formation of its protein network [Fetouhi *et al.*, 2019].

The deconvolution of the amide I band of MFB dough (Figure 2D) indicated that the replacement of 1/3 of the M flour with FB led to a decrease in the aggregate fraction from 21 to 11%. Furthermore, antiparallel β-sheet (1687 cm^{-1}) and β-turn (1673 cm^{-1}) content increased, respectively, from 8 to 13% and from 6 to 14% as compared with M dough (Table 3). SW dough showed lower values of β-sheet than MBF and IMBF but comparable amounts of β-turn. Similar values of α-helix and β-sheet and pseudo β-sheet fractions were found in M, MBF, and IMBF doughs which differed significantly from FB and SW dough. Thus, the effect of maize structural composition seems to be important in this analysis.

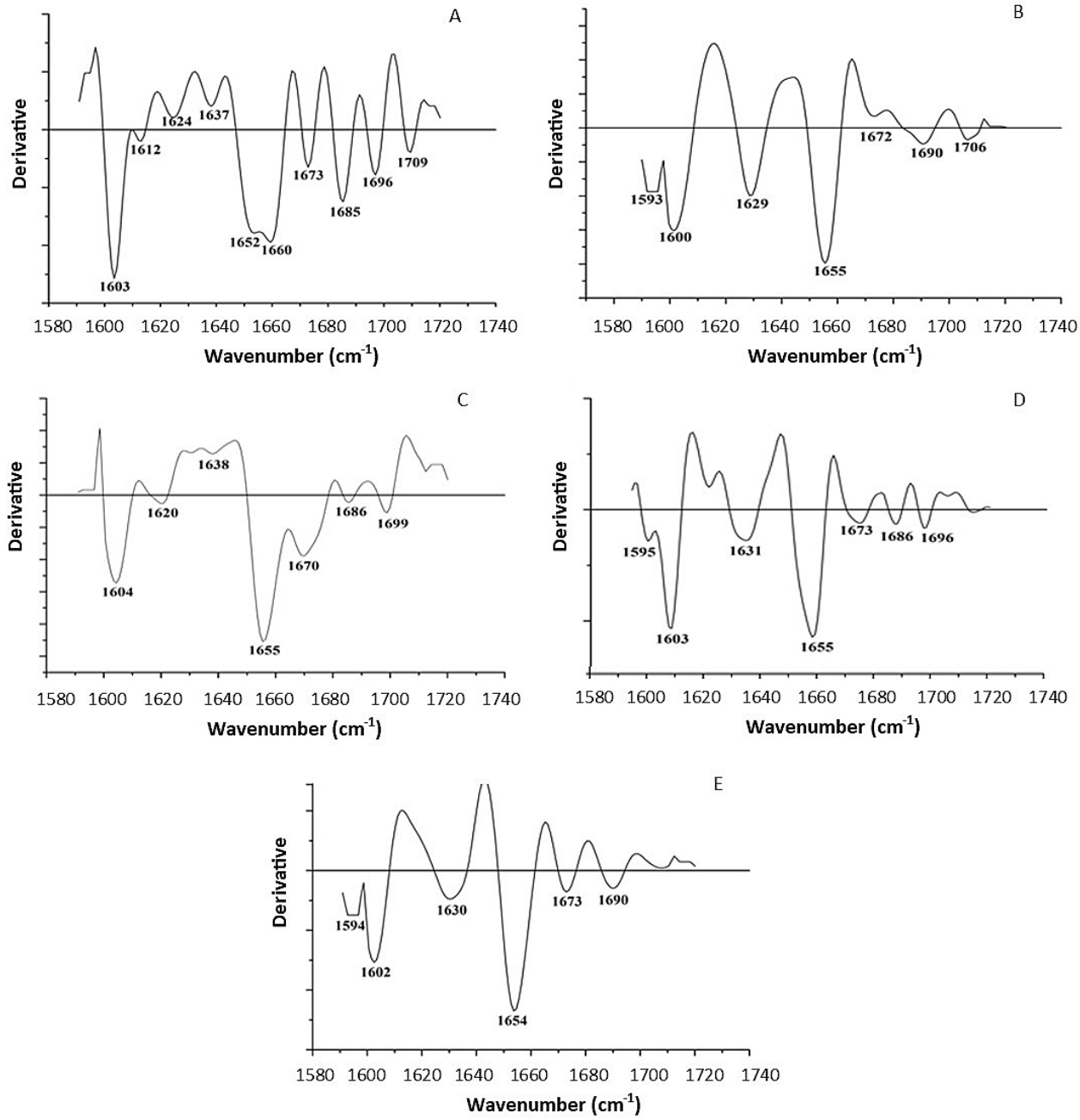


FIGURE 3. Second derivative of Raman spectra of doughs in amide I region: 1720–1590 cm^{-1}: (A) soft wheat, SW, (B) maize, M, (C) field bean, FB, (D) maize-filed bean, MFB, formula, and (E) improved MFB formula, IMFB, dough.

By comparing the curve fitting results of the IMFB dough amide I band (Figure 2E) with the formula without improver and with SW, it is noticeable that the incorporation of hydrothermally-treated maize flour in the GF bread formula caused a slight increase in the amount of antiparallel β-sheet structure (1687 cm^{-1}) from 13 to 15% (no statistical differences) and a slight decrease in the content of β-turn (12%–1672 cm^{-1}) approaching SW dough. No significant changes were noted for aggregates, β-sheet, and α-helix (Table 3). This effect was comparable to that of SW dough, but lower than that for M dough. The addition of hydrothermally-treated maize flour had no effect on the type of the secondary structure of dough.

Aromatic amino-acids chains

The behavior of the side groups is often used in conformational studies of proteins. In addition to cysteine, tyrosine and tryptophan residues were also involved in maintaining the dough protein backbone [Wieser, 2007].

The R ratio of the doublet intensity of tyrosine is a direct measure of the negative charge state of phenolic oxygen and that of the tyrosine environment. Overman *et al.* [1994] suggested that R was sensitive to the hydrogen bonding state of a phenoxyl tyrosine mixture. This can be interpreted as follows: if R=0.30, the OH proton functions as donor of a strong hydrogen bond; at R=2.5, the OH oxygen functions as an

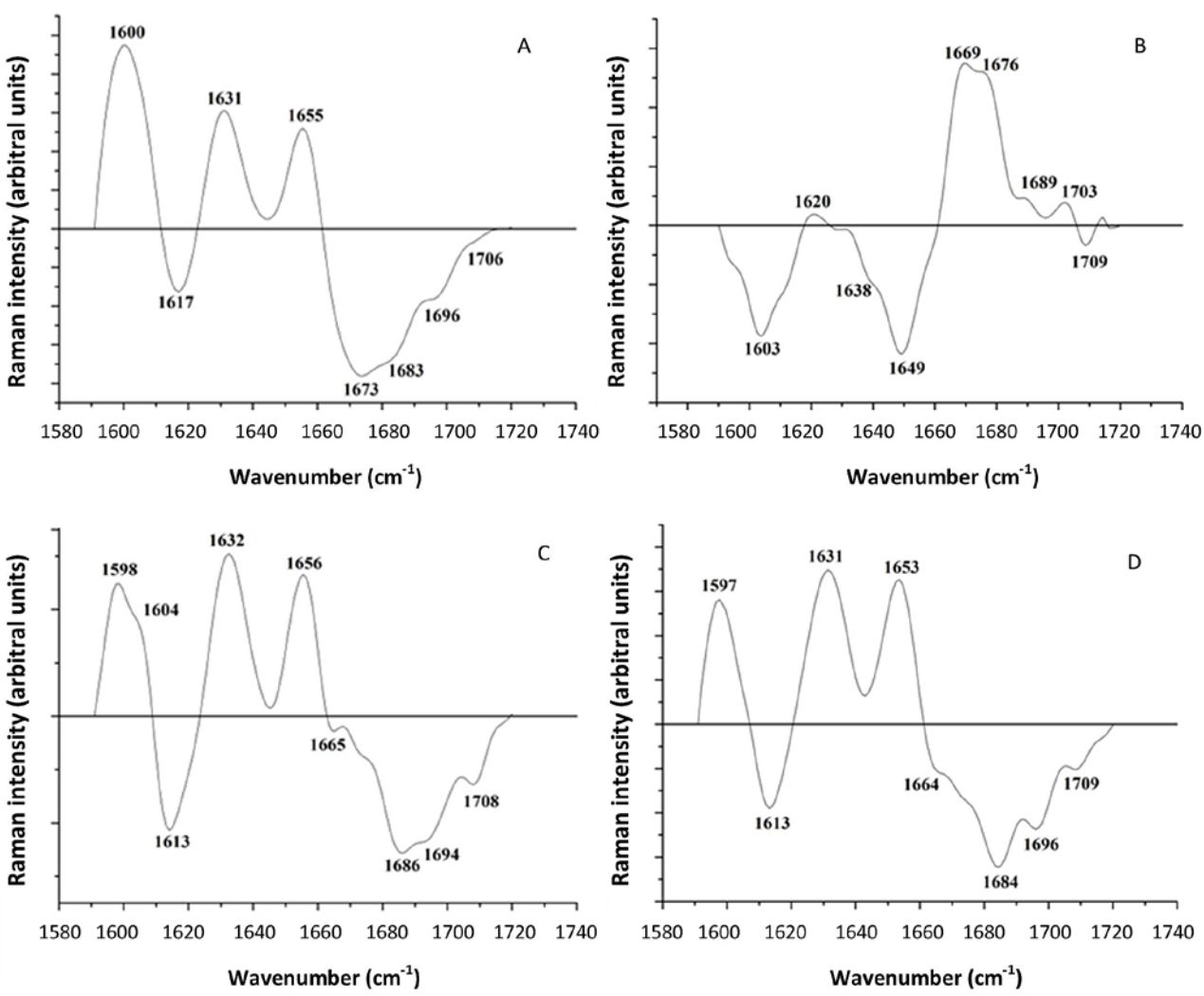


FIGURE 4. Raman differential spectra in amide I region between (A) maize, M; (B) field bean, FB; (C) maize-field bean, MFB, formula; and (D) improved MFB formula, IMFB, doughs and soft wheat, SW, dough.

acceptor of a strong hydrogen bond, while R=1.25 if the OH group functions as a donor and an acceptor, as for solvent--exposed tyrosine. Herrero [2008] demonstrated that the tyrosine residue OH group behaved as an acceptor in a strong H-bond when R was higher than 2.5 and as H-bond donor when R was lower than 0.3. The R value is also a good indicator of the location of the tyrosyl group exposed or buried within the protein structure [Ferrer *et al.,* 2011]. When the intensity I at 850 cm^{-1} is higher than that at 830 cm^{-1}, tyrosyl residues are exposed and act as a positive charge, facilitating local repulsion between protein molecules and inducing a change in the tertiary protein structure. If I at 850 cm^{-1} is smaller than that 830 cm^{-1}, this indicates that the tyrosyl groups are buried in the protein backbone and that these groups participate in protein folding by inter- and intra-molecular hydrogen bonds [Wang *et al*., 2017; Herrero, 2008].

The ratio of I(850 cm^{-1})/I(830 cm^{-1}) values (R) calculated for the tested doughs (Table 3) was ≥2.5 for SW (3.41), M (2.79), and IMFB formula (2.52) doughs. In the case of MFB dough, the ratio approximated 2.5. Only FB dough had an R ratio between 2.5 and 0.3 (2.37). These results indicate that for all dough types, the tyrosyl residues were exposed and acted as a positive charge facilitating local repulsion between the protein molecules responsible for tertiary protein structure changes. The SW sample showed the highest R value in this study (R≥2.5), indicating that tyrosyl residues in wheat dough behave as an exposed acceptor in a strong H-bond. This value was higher than those reported by Nawrocka *et al*. [2016b] and Ferrer *et al*. [2011] for gluten proteins (0.88 and 1.29, respectively). This difference could be due to the different character of molecular interactions triggered by the presence of various amounts of protein in raw materials applied in GF dough formulations that favor the exposition of tyrosine residues at protein structure surfaces.

All gluten-free doughs demonstrated lower R ratio values than the SW dough. The highest value was observed for maize dough, while the lowest one for field bean dough (Table 3). These results indicate that the tyrosine OH groups of gluten--free doughs, except for the protein-rich field bean doughs, behaved as acceptors in a strong H-bond. At the same time, the OH groups of tyrosine residues in field bean dough behaved as both proton donors and acceptors.

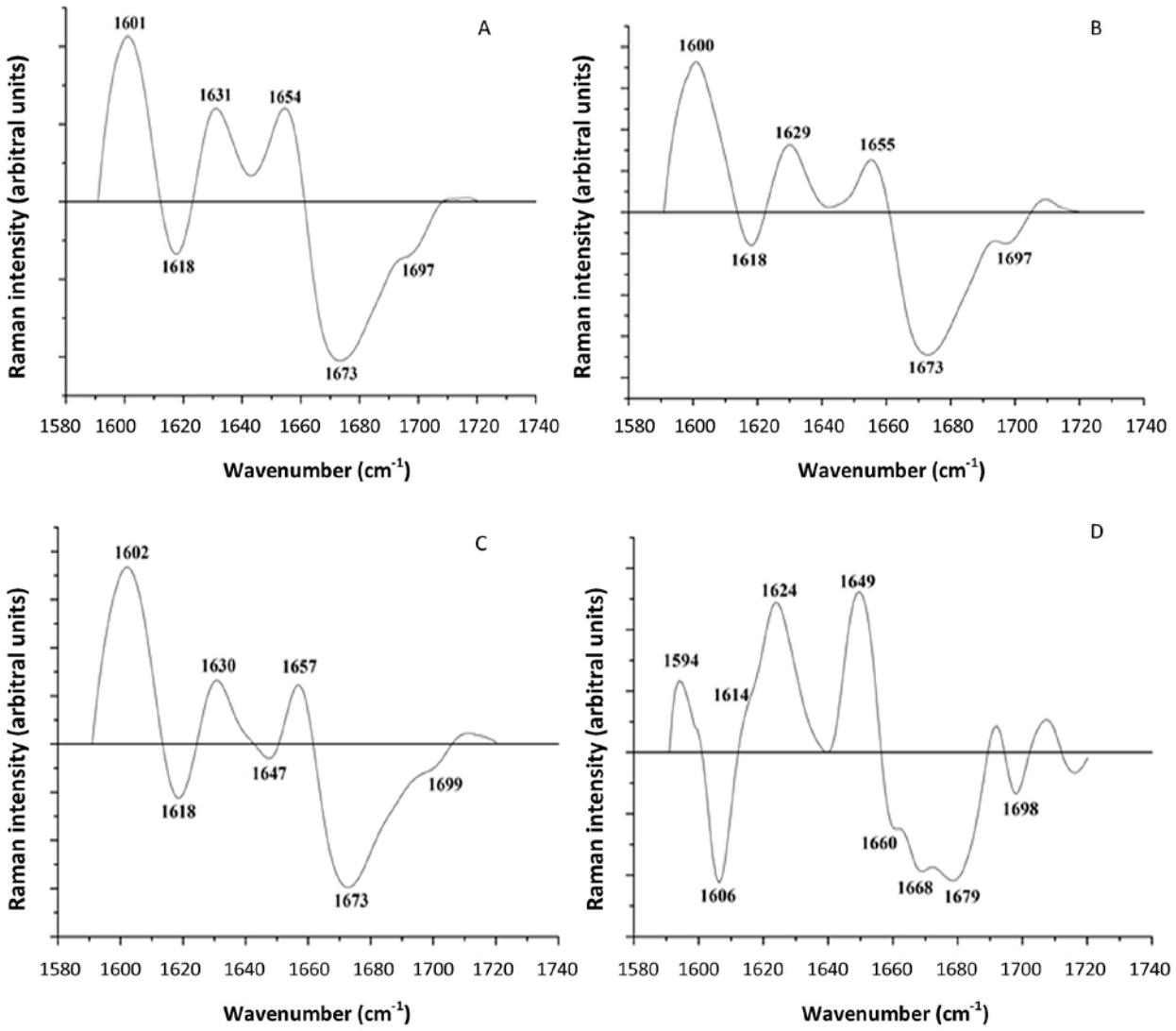


FIGURE 5. Raman differential spectra in amide I region between maize M and the following preparations: (A) field bean, FB; (B) maize-field bean, MFB; and (C) improved MFB formula, IMFB; (D) shows the differential spectrum between amide I region of IMFB and MFB doughs.

TABLE 3. Distribution of secondary structures of doughs protein calculated by deconvolution of FT-Raman spectra in the amide I region and aromatic amino acids (tyrosine and tryptophan) structure of doughs. Values calculated on the basis of 5 averaged spectra consisting of 200 scans. The quality of band deconvolution was indicated by $R^2>0.99$, solution convergence and $\chi 2$ were <0.001.

Sample	AGR (%)	Pβ-sh (%)	β-sh (%)	α-hx (%)	β-trn (%)	Aβ-sh (%)	R I(850)/I(830)	I (760)
SW	7^{b}	6^{a}	11^{b}	43^{b}	15^{b}	18^{ab}	3.41^{a}	0.37^{b}
M	21^{a}	0^{b}	19^{a}	46^{a}	6^{c}	8^{c}	2.79^{b}	0.44^{ab}
FB	5^{c}	4^{a}	9^{c}	37^{c}	21^{a}	24^{a}	2.37^{c}	0.60^{a}
MFB	11^{b}	0^{b}	17^{a}	45^{ab}	14^{b}	13^{bc}	2.46^{c}	0.60^{a}
IMFB	10^{bc}	0^{b}	19^{a}	44^{b}	12^{b}	15^{b}	2.52^{bc}	0.60^{a}

SW – soft wheat, M – maize, FB – field bean, MFB – maize-field bean formula, IMFB – improved maize-field bean formula, AGR – aggregates, Pβ-sh – pseudo β-sheet, β-sh – β-sheet, α-hx – α-helix, β-trn – β-turn, Aβ-sh – antiparallel β-sheet. Deconvolution granted accuracy higher than ± 1%, R –ratio of the doublet intensity of tyrosine, I – spectrum intensity characteristic of the tryptophan band. Ratios are taken from a spectrum resulting from an average of 5 spectra containing 200 scans at 8 cm^{-1} of resolution. $^{a-c}$ – different letters in column indicate significant differences at $\alpha=0.05$.

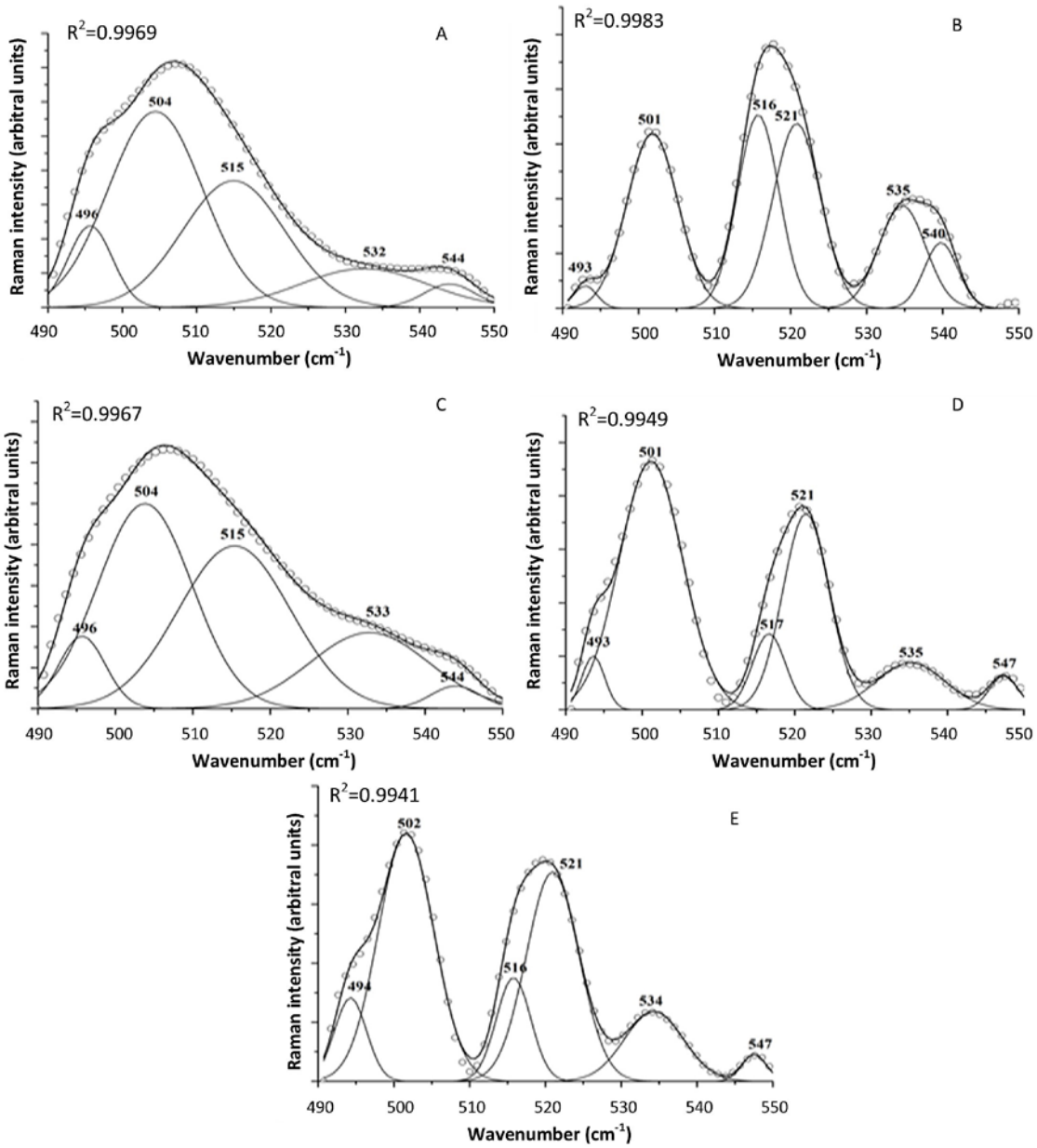


FIGURE 6. Deconvoluted Raman spectra in the area representing S-S region of doughs (490–550 cm^{-1}): (A) soft wheat, SW dough, (B) maize, M dough, (C) field bean, FB dough, (D) maize-filed bean formula, MFB dough, and (E) maize-filed bean improved formula, IMFB dough. Solid line – fitted curve, open circles – original data.

The tryptophan band with the maximum at 760 cm^{-1} is used to indicate the strength of H-bonding and the hydrophobicity of indole ring environments [Linlaud *et al*., 2011]. The increase in its intensity indicates the 'buriedness' of tryptophan residues inside protein molecules, whereas the decrease shows the release of these residues from the hydrophobic zone and their contribution in the formation of disordered protein structures [Nawrocka *et al*., 2015].

We found that the tryptophan band I(760) intensity shown by the SW dough (0.37) was higher compared to the gluten protein (0.087 or 0.167) presented by Nawrocka *et al*. [2015, 2016a]. FB, MFB, and IMFB formula doughs had similar I(760) intensities (0.60) (Table 3), which were significantly higher ($p<0.05$) than that of SW by 1.62 times and insignificantly higher ($p\geq0.05$) than that of M dough (0.44) by 1.36 times. This difference indicates that supplementing maize flour with field bean and the hydrothermal treatment of part of the maize flour in GF bread dough increased tryptophan residue buriedness inside the protein backbone.

Disulfide bridges conformation

In bread-dough-making, disulfide bridges participate in the formation and development of the protein network, especially the tertiary structure [Wieser, 2007]. Gómez *et al*. [2013] and Ferrer *et al*. [2011] stated that the S-S bridge conformation played a major role in the functional properties of gluten dough. Thus, we analyzed the disulfide bridge region in GF doughs and compared them with that of SW dough. Disulfide bridge conformation distribution is shown in Table 4, while Figure 6 reveals the deconvoluted S-S dough regions.

TABLE 4. Distribution of disulfide bridge conformations of the tested doughs with positions of band maxima (in cm^{-1}) in brackets. Values calculated on the basis of 5 averaged spectra consisting of 200 scans. The quality of band deconvolution was indicated by $R^2>0.99$, solution convergence and $\chi 2$ was <0.001.

Dough	GGG(%)	TGG(%)	TGT(%)
SW	51^{a} (496-504)	35^{b} (515)	14^{b}(532-544)
M	28^{c} (493-501)	51^{a} (516-521)	21^{a} (535-540)
FB	41^{b}(496-504)	36^{b} (515)	20^{a} (532-543)
MFB	51^{a} (493-501)	36^{b} (517-521)	13^{b} (535-547)
IMFB	45^{ab} (494-502)	41^{ab} (516-521)	14^{b} (534-547)

SW – soft wheat, M – maize, FB – field bean, MFB – maize-field bean formula, IMFB – improved maize-field bean formula, GGG – gauche--gauche-gauche, TGG – trans-gauche-gauche, TGT – trans-gauche-trans. $^{a-c}$ – different letters in column indicate significant differences at $\alpha=0.05$.

The analysis of the S-S bond conformation of the control SW dough showed the predominance of the GGG conformation (51%), followed by TGG (35%) (Table 4). Nawrocka *et al*. [2015] made the same observation and demonstrated the predominance of these two types of conformations in the formation of a gluten network. Moreover, Gómez *et al*. [2013] analyzed the effect of fiber addition on gluten quality and demonstrated that the S-S bridge structure of the gluten network was predominated by GGG and TGT structures and lacked TGG conformation. The predominance of the GGG conformation indicates that the SW dough protein network is more structurally stable [Nawrocka *et al*., 2016b].

When comparing the gluten-free doughs with the control, we noted that only the M dough differed significantly in the percentage distribution of the S-S bridge conformations and that it was characterized by a predominance of the TGG conformation (1.5 times higher than of SW). However, a small amount of GGG fraction (1.8 times lower than of SW) and TGT (1.5 times higher than of SW) conformations was observed as well. Nawrocka *et al*. [2016b] believed those results indicate that the maize dough protein network is characterized by less stable disulfide bridges. Moreover, the lower protein content in maize could explain the fragility of the M dough protein network when compared to SW dough.

The other types of doughs showed disulfide bridge conformation distribution close in type to SW dough, which was characterized by a predominance of the GGG conformation, followed by TGG, then TGT – but with different fraction ratios.

The quantitative distribution of the three types of conformations in FB dough was similar to SW control dough. When comparing distribution percentages of the three forms of S-S bridges, we observed that the fractions of TGG (36%) were similar while TGT (20%) conformations were higher than that of the control, but the GGG conformations (41%) were lower (Table 4). These results suggest that during FB dough protein network development, the formation of the GGT and TGT conformations was in detriment of GGG conformation, which, in turn, could explain the less stable structure of the field bean dough (beyond its greater protein content).

The conformation of MFB dough S-S bridges was closer to that obtained for the SW control: GGG (51 close to 53%), TGG (36 close to 35%), and TGT (13 close to 14%), respectively (Table 4). Therefore, the substitution of maize flour with field bean improved the S-S bridges distribution, and promoted the formation of more stable structures. The addition of hydrothermally-treated maize flour to the IMFB dough only slightly affected the distribution of disulfide bridge conformation.

Relationship between structural properties and rheological behavior of gluten-free bread doughs

The poor quality of the GF dough and its low rheological behavior could be due to the structural mechanisms of non--gluten protein network dough development. Thus, using PCA, we studied the relationships between the rheological parameters, the types of secondary structures, aromatic amino-acid environment properties, and disulfide bridge conformations.

PCA clearly resolved data into four principal components, which explained 49.21 (PC1), 30.31 (PC2), 15.75 (PC3), and 4.72% (PC4) of the variation. The results obtained for components PC1 and PC2 were retained because they accounted for 79.52% of the total variance. PC scores and loadings of PC1 against PC2 are shown in Figure 7. The correlation matrix between rheological and structural parameters of the tested doughs is presented in Table 5.

The predominance of the first principal component (PC1) was defined by the β-turn content because this parameter showed a high correlation with this factor (r=0.970) and divided the plane vertically (Figure 7). The second principal component (PC2) was defined by the BD parameter (r=0.923) and divided the plane horizontally. As seen in the PCA scores (Figure 7A), PC1 shows a great variability. Being on the positive side of PC1, FB and IMFB doughs were seen to have similar properties to those of SW. This indicates that they had similar properties dependent on β-turn content. Moreover, the application of hydrothermally-treated maize flour in the IMFB formula affected β-turn structure content. Regarding the PC2 axis as defined by BD, all gluten-free doughs were located in the negative side, hence demonstrating that the differences in the rheological behavior between SW and GF doughs were highly related to the pasting properties (BD) rather than to the viscoelastic properties as indicated by G' and G".

WA was considered as a mixing parameter and a factor that affected dough structure during its formation. It was strongly positively correlated with AGR (r=0.846) and strongly negatively correlated with Aβ-sheet content (r=-0.721) (Table 5). This indicates that the high water absorption of GF dough promotes the formation of aggregates to the detriment of Aβ-sheet fabrication. This suggestion is confirmed by the strong negative correlation between AGR and Aβ-sheet content. Here, a strong negative correlation was found between WA and AGR amount, indicating that differences do exist between mechanisms of gluten and non-gluten protein network development.

The storage modulus G' was influenced only by Pβ-sheet amount (r=-0.712). This indicates that the stiff and rigid skeleton of non-gluten doughs is due to the low capacity of GF doughs to form this kind of the secondary structure during matrix development.

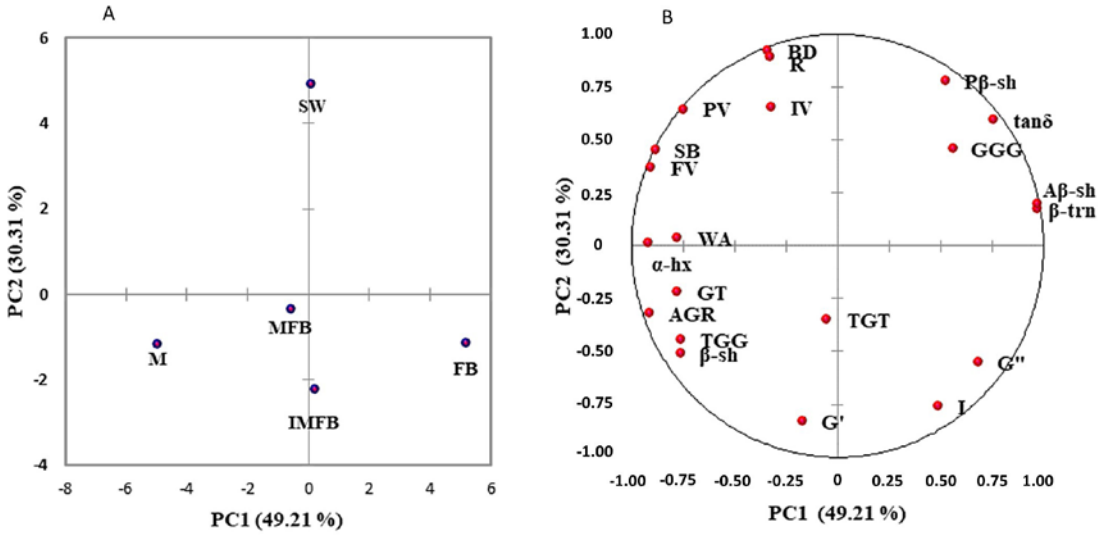


FIGURE 7. Principal component analysis (PCA) of rheological (A) and structural (B) characteristics of the tested doughs: soft wheat, SW; maize, M; field bean, FB; maize-filed bean formula, MFB; maize-filed bean improved formula, IMFB. WA – water absorption, G' – storage modulus; G" – loss modulus; tan δ – loss tangent, GT – gelatinization temperature, IV – initial viscosity, PV – peak viscosity, FV – final viscosity, BD – breakdown, SB – setback, AGR – aggregates, Pβ-sh – pseudo β-sheet, β-sh – β-sheet, α-hx –α-helix, β-trn – β-turn, Aβ-sh – antiparallel β-sheet, GGG – gauche--gauche-gauche, TGG – trans-gauche-gauche, TGT – trans-gauche-trans, R – ratio of the doublet intensity of tyrosine, and I – spectrum intensity characteristic of the tryptophan band.

Tan δ was strongly and positively correlated with the amounts of Pβ-sheet (r=0.947), Aβ-sheet (r=0.885), and β-turn (r=0.837), while a strong negative correlation was observed between AGR (r=-0.817), β-sheet (r=-0.983), and α-helix (r=-0.794) content (Table 5). Fetouhi *et al*. [2019] observed similar correlations between tan δ and the amounts of β-turn, β-sheet, and α-helix for GF doughs based on a rice--field bean formula. They explained that these results were due to the great tendency of gluten-free ingredients to form α-helix and β-sheet structures, hence strongly promoting protein structuralization. This is in direct relationship with low values of tan δ and the high inflexibility and rigidness displayed during gluten-free dough formation. In our study, low tan δ values of GF doughs could have occurred due to the low capacity of non-gluten proteins to form Pβ-sheet structures (positive correlation) where these types of structures may participate in the formation of β-sheet structuring (negative correlation). The positive correlation between tan δ and Aβ-sheet (r=0.885) and β-turn content (r=0.837) and the strong negative correlation between Aβ-sheet and α-helix (r=-0.940) content demonstrate that the mechanism of non-gluten protein network formation is based on the formation of β-sheet and α-helix structures, hence, not only to the detriment of β-turn, but also at the expense of the Aβ-sheet structure.

We noted the effect of the disulfide bridge conformational changes on the rheological behavior of gluten. According to the PCA score (Figure 7B) and correlation matrix (Table 5), the correlation between GGG and TGG (r=-0.946) and TGT (r=-0.818) content was strongly negative. This indicates the more intense participation of TGG and TGT disulfide bridges in the formation of the non-gluten protein matrix rather than the GGG conformation that was noted in the case of gluten proteins [Nawrocka *et al*., 2015].

No direct relationship between the S-S bridges conformation and rheological behavior of GF doughs was observed based on the PCA plot (Figure 7B) or correlation matrix. We presume that disulfide bridge conformation indirectly participated in the rheological behavior because this affected the type of secondary structure forming the non-gluten protein matrix. The strong positive correlation observed between TGG content and AGR (r=0.924) was accompanied by a negative correlation with Aβ-sheet (r=-0.751) and β-turn (r=-0.852). GGG conformation was also negatively correlated with AGR structure content (r=-0.813) (Table 5). All these results indicate the low tendency of non-gluten proteins to form β-turn and Aβ-sheet structures, and the high capacity of these types of proteins to form the AGR structures that are promoted by the high participation of the TGG disulfide bridges conformation that is characteristic for non-gluten dough matrices. This effect could explain the indirect participation of the S-S bridges conformation in the low viscoelastic behavior of gluten-free doughs. By analyzing analogous relationships, Nawrocka *et al.* [2015] formulated similar suggestions as obtained in our work, where the increase in the number of TGG and TGT conformations and a decrease in GGG conformation promoted the aggregation and folding of gluten proteins.

The effects of the structural aspect of non-gluten proteins on the pasting properties of GF doughs are presented in Figure 7B and Table 5. PCA analysis showed a high positive correlation between GT and β-sheet (r=0.913) and α-helix (r=0.940) values, and a negative relation with Aβ-sheet (r=-0.882) content. PV value was also negatively correlated with the tryptophan content as indicated by I(760 cm^{-1}) standards. This indicates that the PV value may be affected by the degree of buriedness of tryptophan residues. However, the BD parameter was positively correlated with

TABLE 5. Correlation matrix (Pearsons' coefficients r) between mixing parameter, pasting properties, viscoelastic characteristics and structure fractions of proteins of the tested doughs.

Variables	WA	G'	G"	tanδ	GT	IV	PV	FV	BD	SB	AGR	Pβ-sh	β-sh	α-hx	β-trn	Aβ-sh	GGG	TGG	TGT	R
G'	-0.173																			
G"	-0.676	0.559																		
tanδ	-0.444	-0.658	0.228																	
GT	0.375	0.333	-0.545	**-0.875**																
IV	-0.122	-0.288	-0.568	-0.030	0.458															
PV	0.646	-0.403	**-0.828**	-0.151	0.367	0.594														
FV	**0.806**	-0.178	*-0.795*	-0.417	0.518	0.416	**0.947**													
BD	0.190	-0.618	-0.679	0.269	0.096	**0.804**	**0.848**	0.637												
SB	*0.749*	-0.227	**-0.816**	-0.364	0.510	0.504	**0.973**	**0.994**	0.711											
AGR	**0.846**	0.346	-0.451	**-0.817**	0.651	-0.085	0.508	*0.757*	-0.016	0.691										
Pβ-sh	-0.283	*-0.712*	0.037	**0.947**	*-0.780*	0.155	0.154	-0.126	0.530	-0.066	-0.658									
β-sh	0.351	0.662	-0.242	**-0.983**	**0.913**	0.187	0.203	0.434	-0.159	0.398	0.759	**-0.905**								
α-hx	0.536	0.194	-0.692	*-0.794*	**0.940**	0.558	0.652	*0.769*	0.359	*0.767*	*0.744*	-0.600	**0.844**							
β-trn	-0.681	-0.394	0.487	**0.837**	-0.772	-0.235	-0.625	**-0.819**	-0.195	*-0.783*	**-0.930**	0.621	**-0.847**	**-0.888**						
Aβ-sh	*-0.721*	-0.300	0.611	**0.885**	**-0.882**	-0.251	-0.580	*-0.782*	-0.146	*-0.745*	**-0.922**	*0.714*	**-0.879**	**-0.940**	**0.958**					
GGG	-0.687	-0.449	-0.021	0.516	-0.133	0.526	-0.219	-0.470	0.269	-0.383	**-0.813**	0.413	-0.419	-0.248	0.650	0.534				
TGG	0.693	0.545	-0.122	*-0.728*	0.423	-0.275	0.347	0.608	-0.158	0.532	**0.924**	-0.582	0.670	0.529	**-0.852**	*-0.751*	**-0.946**			
TGT	0.485	0.154	0.268	0.002	-0.416	**-0.823**	-0.068	0.097	-0.390	0.012	0.389	0.000	-0.141	-0.318	-0.112	-0.002	**-0.818**	0.587		
R	0.221	-0.554	-0.563	0.314	-0.039	0.676	**0.851**	0.653	**0.970**	*0.717*	0.022	0.596	-0.215	0.267	-0.213	-0.102	0.106	-0.040	-0.193	
I	-0.499	0.497	0.602	-0.186	0.028	-0.452	**-0.914**	*-0.798*	**-0.865**	**-0.829**	-0.280	-0.484	0.136	-0.310	0.377	0.255	0.211	-0.235	-0.111	**-0.940**

WA – water absorption, G' – storage modulus; G" – loss modulus; tan δ – loss tangent, GT – gelatinization temperature, IV – initial viscosity, PV – peak viscosity, FV – final viscosity, BD – breakdown, SB – setback, AGR – aggregates, Pβ-sh – pseudo β-sheet, β-sh – β-sheet, α-hx – α-helix, β-trn – β-turn, Aβ-sh – antiparallel β-sheet, GGG – gauche-gauche-gauche, TGG – trans-gauche-gauche, TGT – trans-gauche-trans, R – ratio of the doublet intensity of tyrosine, I – spectrum intensity characteristic of the tryptophan band. In bold: very strong correlations r≥0.8; in Italics: strong correlations r≥0.7.

the R ratio (r=0.970), indicating the effect of tyrosyl residues on the swelling capacity of starch granules during heating and the distribution of starch granules in the protein matrix. This relationship suggests that if more tyrosyl residues are exposed, the swelling capacity of starch granules increases.

CONCLUSIONS

This study contributed to understanding the development mechanism of gluten-free GF doughs by examining protein structural features. The solid behavior that characterized GF dough was due to the absence of Pβ-sheet secondary structure. We demonstrated that the protein network of gluten-free MFB dough matrix was principally developed by the production of β-sheet and α-helix structures. At the same time, a decrease of β-turn and Aβ-sheet secondary structures was observed, greatly affecting the viscoelastic behaviour of the examined doughs. PCA also showed that the water absorption of the starch granules increased when more tyrosyl residues were exposed. Moreover, we noted that the TGG disulfide bridge conformation strongly participated in GF protein network development and promoted the high structuralization that contributed to the low bread-making quality of GF components. In addition, strong correlations were observed between pasting properties and structural composition of the tested GF doughs as compared to gluten SW dough. Finally, an improved IMFB formula with the addition of a fraction of hydrothermally-treated maize flour showed relatively good structural properties as compared to other doughs tested.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ORCID IDs

A. Nawrocka https://orcid.org/0000-0001-8618-2092
A. Sujak https://orcid.org/0000-0001-5616-3827
M. Szymańska-Chargot https://orcid.org/0000-0002-2251-4307
M. Tomczyńska-Mleko https://orcid.org/0000-0002-6870-1060
A. Wójtowicz https://orcid.org/0000-0001-5981-6109

REFERENCES

1. AACC. (1995). Approved Method of the AACC. 9th Edition, American Association of Cereal Chemists, St. Paul, USA.
2. AOAC. (2000). Official Methods of Analysis. 17th Edition, The Association of Official Analytical Chemists, Gaithersburg, USA.
3. Barak, S., Deepak, M., Khatkar, B.S. (2014). Influence of gliadin and glutenin fractions on rheological, pasting, and textural properties of dough. *International Journal of Food Properties,17*(7), 1428–1438. https://doi.org/10.1080/10942912.2012.717154
4. Belton, P.S. (1999). Mini review: On the elasticity of wheat gluten. *Journal of Cereal Science*, *29*(2), 103–107. https://doi.org/10.1006/jcrs.1998.0227
5. Benatallah, L., Zidoune, M.N.,Michon, C. (2012). Optimization of HPMC and water addition for a gluten-free formula with rice and field bean based on rheological properties of doughs. *International Review of Chemical Engineering, 4*(5), 1755–2035. https://doi.org/10.3390/foods8050156
6. Bourekoua, H., Benatallah, L., Zidoune, M.N., Rosell, C.M. (2016). Developing gluten free bakery improvers by hydrothermal treatment of rice and corn flours. *LWT – Food Science and Technology, 73,* 342–350. https://doi.org/10.1016/j.lwt.2016.06.032
7. Dib, A., Wójtowicz, A., Benatallah, L., Bouasla, A., Zidoune, M.N. (2018). Effect of hydrothermal treated corn flour addition on the quality of corn-field bean gluten-free pasta. *Contemporary Research Trends in Agricultural Engineering 10*, 1–9. https://doi.org/10.1051/bioconf/20181002003
8. Dus, S.J., Kokini, J.L. (1990). Prediction of the nonlinear viscoelastic properties of hard wheat flour dough using the Bird–Carreau constitutive model. *Journal of Rheology, 34*(7), 1069–1084. http://dx.doi.org/10.1122/1.550110
9. Ferrer, E.G., Gómez, E.G., Anon, M.C., Puppo, M.C. (2011). Structural changes in gluten protein structure after addition of emulsifier. A Raman spectroscopy study. *Spectrochimica Acta, A79*(1), 278–281. https://doi.org/10.1016/j.saa.2011.02.022
10. Fetouhi, A., Benatallah, L., Nawrocka, A., Szymańska-Chargot, M., Bouasla, A., Tomczyńska-Mleko, M., Zidoune, M.N., Sujak, A. (2019). Investigation of viscoelastic behaviour of rice-field bean gluten free dough using the biophysical characterization of proteins and starch: a FT-IR study. *Journal of Food Science and Technology*, *56*(3), 1316–1327. https://doi.org/10.1007/s13197-019-03602-2
11. Gómez, A.V., Ferrer, E.G., Añón, M.C., Puppo, M.C. (2013). Changes in secondary structure of gluten proteins due to emulsifiers. *Journal of Molecular Structure, 1033*, 51–58. https://doi.org/10.1016/j.molstruc.2012.08.031
12. He, H., Hoseney, R.C. (1992). Factor controlling gas retention in non-heated doughs. *Cereal Chemistry, 69(1)*, 1–6.
13. Herrero, A.M. (2008). Raman spectroscopy for monitoring protein structure in muscle food systems. *Critical Reviews in Food Science and Nutrition, 48*(6), 512–523. https://doi.org/10.1080/10408390701537385
14. Hřivna, L., Zigmundová, V., Burešowá, I., Maco, R., Vyhnánek, T., Trojan, V. (2018). Rheological properties of dough and baking quality of products using coloured wheat. *Plant, Soil and Environment, 64*, 203–208. https://doi.org/10.17221/62/2018-PSE
15. Khatkar, B.S., Schofield, J.D. (2007). Dynamic rheology of wheat flour dough I. Non-linear viscoelastic behavior. *Journal of the Science of Food and Agriculture, 82*(8), 827–829. https://doi.org/10.1002/jsfa.1109
16. Larsson, H., Eliasson, A.C. (1997). Influence of the starch granule surface on the rheological behaviour of wheat flour dough. *Journal of Texture Studies, 28*(5), 487–501. https://doi.org/10.1111/j.1745-4603.1997.tb00132.x
17. Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N., Biliaderis, C.G. (2007). Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of Food Engineering, 79*(3), 1033–1047. https://doi.org/10.1016/j.jfoodeng.2006.03.032
18. Linlaud, N., Ferrer, E., Puppo, M.C., Ferrero, C. (2011). Hydrocolloid interaction with water, protein, and starch in wheat dough. *Journal of Agricultural and Food Chemistry, 59(2)*, 713–719. https://doi.org/10.1021/jf1026197
19. Matsushima, N., Danno, G-I., Takazewa, I., Izumi, Y. (1997). Three-dimensional structure of maize α-zein proteins studied by small-angle X-ray scattering. *Biochimica et Biophysica Acta (BBA) – Protein Structure and Molecular Enzymology, 1339*(1), 14–22. https://doi.org/10.1016/S0167-4838(96)00212-9
20. Mejia, C.D., Mauer, L.J., Hamaker, B.R. (2007). Similarities and differences in secondary structure of viscoelastic polymers of maize a-zein and wheat gluten proteins. *Journal of Cereal Science, 45*(3), 353–359. https://doi.org/10.1021/jf203073a
21. Nawrocka, A., Szymańska-Chargot, M., Miś, A., Ptaszyńska, A.A., Kowalski, R., Waśko, P., Gruszecki, W.I. (2015). Influence of dietary fibre on gluten proteins structure – a study on model flour with application of FT-Raman spectroscopy. *Journal of Raman Spectroscopy, 46(3)*, 309–316. https://doi.org/10.1002/jrs.4648
22. Nawrocka, A., Szymańska-Chargot, M., Miś, A., Wilczewska, A.Z., Markiewicz, K.H. (2016a). Dietary fiber-induced changes in the structure and thermal properties of gluten proteins studied by Fourier Transform-Raman spectroscopy and thermogravimetry. *Journal of Agriculture and Food Chemistry, 64(10)*, 2094–2104. https://doi.org/10.1021/acs.jafc.5b05712
23. Nawrocka, A., Miś, A., Szymańska-Chargot, M. (2016b). Characteristics of relationships between structure of gluten proteins and dough rheology: influence of dietary fibres studied by FT Raman spectroscopy. *Food Biophysics, 11*(1), 81–90. https://doi.org/10.1007/s11483-015-9419-y
24. Overman, S.A., Aubrey, K., Vispo, N.S., Cesareni, G., Thomas Jr, G. (1994). Novel tyrosine markers in Raman spectra of wild-type and mutant (Y21M and Y24M) Ff virions indicate unusual environments for coat protein phenoxyls. *Biochemistry, 33,* 1037–1042. https://doi.org/10.1021/bi00171a001

25. Pelton, J.T., McLean, L.R. (2000). Spectroscopic methods for analysis of protein secondary structure. *Analytical Biochemistry, 277(2)*, 167–176. https://doi.org/10.1006/abio.1999.4320
26. Pourfarzad, A., Habibi Najafi, M.B., Haddad Khodaparast, M.H., Hassanzadeh Khayyat, M. (2015). Serish inulin and wheat biopolymers interactions in model systems as a basis for understanding the impact of inulin on bread properties: a FTIR investigation. *Journal of Food Science and Technology, 52*(12), 7964–7973. https://doi.org/10.1007/s13197-015-1939-4
27. Ragaee, S., Abdel-Aal, E.M. (2006). Pasting properties of starch and protein in selected cereals and quality of their food products. *Food Chemistry, 95(1)*, 9–18. https://doi.org/10.1016/j.foodchem.2004.12.012
28. Rygula, A., Majzner, K., Marzec, K.M., Kaczor, A., Pilarczyk, M., Baranska, M. (2013). Raman spectroscopy of proteins: a review. *Journal of Raman Spectroscopy, 44*(8), 1061–1076. https://doi.org/10.1002/jrs.4335
29. Singh, H., MacRitchie, F. (2001). Application of polymer science to properties of gluten. *Journal of Cereal Science, 33*(3), 231–243. https://doi.org/10.1006/jcrs.2000.0360
30. Sivam, A.S., Sun-Waterhouse, D., Perera, C.O., Waterhouse, G.I.N. (2013). Application of FT-IR and Raman spectroscopy for the study of biopolymers in breads fortified with fiber and polyphenols. *Food Research International, 50*(2), 574–585. https://doi.org/10.1016/j.foodres.2011.03.039
31. Sivaramakrishnan, H.P., Senge, B., Chattopadhyay, P.K. (2004). Rheological properties of rice dough for making rice bread. *Journal of Food Engineering, 62*(1), 37–45. https://doi.org/10.1016/S0260-8774(03)00169-9
32. Tunick, M.H. (2011). Small-strain dynamic rheology of food protein networks. *Journal of Agricultural and Food Chemistry, 59*(5), 1481–1486. https://doi.org/10.1021/jf1016237
33. Wang, K., Sun, D-W., Pu, H., Wei, Q. (2017). Principles and applications of spectroscopic techniques for evaluating food protein conformational changes: A review. *Trends in Food Science & Technology, 67*, 207–219. https://doi.org/10.1016/j.tifs.2017.06.015
34. Wang, Q., Li, Y., Sun, F., Li, X., Wang, P., Sun, J., Zeng, J., Wang, C., Hu, W., Chang, J., Chen, M., Wang, Y., Li, K., Yang, G., He, G. (2015). Tannins improve dough mixing properties through affecting physicochemical and structural properties of wheat gluten proteins. *Food Research International, 69*, 64–71. https://doi.org/10.3390/ijms161226132
35. Wieser, H. (2007). Chemistry of gluten proteins. *Food Microbiology*, *24*(2), 115–119. https://doi.org/10.1016/j.fm.2006.07.004
36. Zavareze, E.M., Guerra Dias, A.R. (2011). Impact of heat-moisture treatment and annealing in starches: A review. *Carbohydrate Polymers, 83*(2), 317–328. https://doi.org/10.1016/j.carbpol.2010.08.064

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 177–185
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/135813
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Macro- and Micro-Nutrient Composition and Antioxidant Activity of Chickpea and Pea Accessions

Michela Costantini[1], Carmine Summo[1], Mariangela Centrone[2], Iga Rybicka[3], Mariagrazia D'Agostino[2], Paolo Annicchiarico[4], Francesco Caponio[1], Stefano Pavan[1], Grazia Tamma[2], Antonella Pasqualone[1]*

[1]*Department of Soil, Plant and Food Science (DISSPA), University of Bari Aldo Moro, via Amendola, 165/A, I-70126 Bari, Italy*
[2]*Department of Bioscience, Biotechnologies and Biopharmaceutics (DBBB) University of Bari Aldo Moro, via Amendola, 165/A, I-70126 Bari, Italy*
[3]*Faculty of Commodity Science, Poznań University of Economics and Business, al. Niepodległości 10, 61–875 Poznań, Poland*
[4]*Research Centre for Animal Production and Aquaculture, Council for Agricultural Research and Economics (CREA), viale Piacenza, 29, I-26900 Lodi, Italy*

Key words: antioxidant activity, chickpeas, peas, phenolic extract, mineral composition, bioactive compounds

Epidemiological studies reported an inverse association between the consumption of legumes and the incidence of age-related diseases. This trend could be attributed to the presence of antioxidant compounds, especially phenolic and flavonoid compounds. In this paper, five pea (*Pisum sativum* L.) and twelve chickpea (*Cicer arietinum* L.) accessions, having different characteristics and geographical origin, were characterised in terms of antioxidant activity, as well as macro- and micro-nutrient composition. The antioxidant activity has been evaluated using both DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging capacity assays. Chickpea and pea accessions showed a different behaviour in the presence of these different radicals. Chickpea accessions were characterised by significantly higher DPPH$^{\bullet}$ scavenging activity, while peas showed a significantly higher value of antioxidant activity evaluated using the ABTS assay. Pea accessions had the highest content of total phenolic compounds, Zn, and Cu. A positive correlation was found between some minerals, such as Zn, Cu and P, and the ABTS$^{\bullet+}$ scavenging activity. Black and brown chickpea accessions showed significantly higher contents of anthocyanins, Mn, Mg, and Ca, which were positively correlated with the antioxidant activity assessed with the DPPH assay. Despite the dataset investigated in our study included a limited number of accessions, it was possible to highlight the influence of the chemical composition on the antioxidant activity due to the high phenotypic diversity found between the accessions, emphasising the importance of selecting the antioxidant activity assay according to the matrix to be evaluated.

INTRODUCTION

The demand for grain legume-based food is expected to increase in developing countries, as a consequence of the demographic growth, but also in the developed countries, given their contribution to a healthy diet and food safety. The increased awareness of risks associated with excessive consumption of animal proteins [Daryanto *et al.*, 2015] and with fat accumulation due to the high intake of energy-dense foods poor in micronutrients and bioactive compounds also plays a determinant role in prompting legume consumption.

In addition, greater cultivation and consumption of food legumes has high priority to increase the sustainability of agriculture in terms of soil fertility, greenhouse gas emissions, energy efficiency, pollution, and crop diversity [Annicchiarico, 2017].

Chickpea (*Cicer arietinum* L.) is the third grain legume species cultivated worldwide [FAOSTAT data, 2018]. It has been shown that the two commercial types of chickpea, *i.e. kabuli*, with large seeds and beige coat, and *desi*, with small seeds and dark-coloured, fall in different genetic clusters [De Giovanni *et al.*, 2017]. Furthermore, a black-pigmented chickpea type (*Apulian black*) traditionally cultivated in Apulia (Southern Italy), displayed peculiar phenotypic and genetic features [Pavan *et al.*, 2017]. From a nutritional point of view, chickpeas are characterised by high dietary fibre and lipid content [Jukanti *et al*., 2012]. The lipid fraction, in particular for the coloured types such as *desi* and *Apulian black* type, has a high content of essential unsaturated fatty acids [Summo *et al.*, 2019a,b] which elicit beneficial effects on human health [Jukanti *et al.*, 2012].

Pea (*Pisum sativum* L.) is the fourth grain legume cultivated worldwide [FAOSTAT data, 2018], grown for both

* Corresponding Author: Fax: +39 080 5443467;
e-mail: carmine.summo@uniba.it (Prof. C. Summo)

Submitted: 7 December 2020
Accepted: 15 April 2021
Published on-line: 19 May 2021

human nutrition and livestock feeding. Studies on pea genetic diversity highlighted clear differentiation between the pea wild progenitor, *P. sativum* subsp. *elatius*, and the main pea cultivated subspecies (*P. sativum* subsp. *sativum*). Within *P. sativum* subsp. *sativum*, geographical patterns of variation were identified, as landraces from the Mediterranean area, the Caucasus, Ethiopia, and Central Asia exhibited peculiar genetic features [Smýkal *et al.*, 2012].

As other legumes, pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) are characterised by low levels of lipids and high contents of proteins, complex carbohydrates, B group vitamins, and minerals. They represent a good source of minerals, such as iron, zinc, calcium, magnesium, potassium, sulphur, and selenium [Ashokkumar *et al.*, 2015] and carotenoids, such as β-carotene [Ashokkumar *et al.*, 2015].

Both pea and chickpea have been studied for agronomic [Fotiadis *et al.*, 2019], genetic [Pavan *et al.*, 2017], and nutritional features [Summo *et al.*, 2019a]. Furthermore, they have been proposed as functional ingredients of bakery products, such as bread and cakes [Millar *et al.*, 2019; Pasqualone *et al*., 2019a], as well as ready-to-eat foods, such as purée and burgers [Summo *et al.*, 2016, 2019c].

Moreover, epidemiological studies reported an inverse association between the consumption of legumes and the incidence of age-related diseases [Kris-Etherton *et al.*, 2002]. The beneficial effect of legumes on health could be attributed to their content of phenolics and flavonoids [Fidrianny *et al.,* 2016], which are the most active antioxidant compounds in foods [Dudonne *et al.*, 2009]. Furthermore, antioxidant defences rely heavily on minerals in the diet, such as Fe, Mn, Cu, Zn, and Mg [Evans & Halliwell, 2001]. Dietary antioxidant compounds can stimulate cellular defences and help prevent oxidative damage [Dudonne *et al.*, 2009]. There are numerous published methods measuring the *in vitro* total antioxidant capacity. They can be classified in hydrogen atom transfer (HAT) or electron transfer (ET) based assays. The ET-based assays include the total phenols assay by Folin-Ciocalteu reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzthiazoline--6-sulfonic acid) radical scavenging capacity assays [Huang *et al*., 2005]. Both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ can be used to predict the antioxidant activity of vegetables, fruits, pulses, and food products [Fidrianny *et al.,* 2016; Yao *et al*., 2013]. The principal aim of this study was to evaluate the antioxidant activity of chickpea and pea accessions by using two different radical scavenging capacity assays, such as DPPH and ABTS. Furthermore, proximate composition, contents of minerals, phenolic compounds, carotenoids, anthocyanins, and phytates of whole meal flour from the same accessions of chickpea and pea were determined. Finally, correlations between both antioxidant activities and legume flour composition were defined.

MATERIAL AND METHODS

Plant material and flour preparation

Twelve chickpea and five pea accessions were considered in this study, selected from the *ex situ* repositories of the United States Department of Agriculture (USDA), the Department of Plant, Soil and Food Science of the University of Bari, Italy (DiSSPA), and the Institute of Biosciences and Bioresources of the Italian National Research Council (CNR-IBBR) (Table 1). For each, type, country of origin, and phenotypic traits (seed size and colour) were indicated. Chickpea accessions encompassed the three genetic clusters previously identified, corresponding to the *desi*, *kabuli,* and *Apulian black* types [Pavan *et al.*, 2017]. Genetic diversity of pea germplasm was ensured by selecting one accession referable to the wild progenitor *P. sativum* subsp. *elatius* collected in Syria*,* and four *P. sativum* subsp. *sativum* accessions originating from the Mediterranean area (Italy and Turkey), the Caucasus (Georgia), and Ethiopia. Among them, the Italian landrace ROR12 was reportedly resistant to the parasitic weed *Orobanche crenata* [Pavan *et al.*, 2016]. All plants were grown in the experimental farm "P. Martucci" of the University of Bari "Aldo Moro", Italy (41°01'22.1'' N, 16°54'21.0'' E) during the growing season 2017–2018. They were harvested according to a randomised complete block design with two replicates, each replicate being formed by 30 individual plants. After harvesting at crop maturity, chickpea and pea seeds were milled (ETA mill, Vercella Giuseppe, Mercenasco, Italy) and sieved at 0.6 mm.

TABLE 1. Chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) accessions analysed.

Legume		Type	Seed size	Seed colour	Origin
Chickpea (*Cicer arietinum* L.)	PI292006	KC	Small	Beige	Jordan
	PI339154*	KC	Large	Beige	Turkey
	PI357648*	KC	Small	Beige	Serbia--Montenegro
	PI518255*	DC	Small	Brown	Afghanistan
	PI251514*	DC	Small	Black	Iran
	PI140293*	DC	Small	Brown	Iran
	PI358934*	DC	Small	Black	Iran
	PI533683*	DC	Large	Black	Spain
	W610046*	DC	Large	Black	Bulgaria
	110694*	AB	Large	Black	Italy
	MG_13*	AB	Large	Black	Italy
	MG_17*	AB	Large	Black	Italy
Pea (*Pisum sativum* L.)	IG116297		Medium	Green	Turkey
	ROR12		Large	Green	Italy
	IG52442		Medium	Green--pigmented	Syria
	IG134828		Medium	Green--pigmented	Georgia
	IG51520		Medium	Green--pigmented	Ethiopia

KC – *kabuli* chickpea, DC – *desi* chickpea, and AB – *Apulian black* chickpea.

*The chickpea accessions are part of a wide collection already characterised, whose data repository is in Summo *et al.* [2019a].

Preparation of extracts and determination of the antioxidant activity

Antioxidant activity was determined using both DPPH and ABTS radical (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) scavenging capacity assays. The determinations were performed for an aqueous-methanol extract (20/80, *v/v*) prepared as reported by Summo *et al*. [2019b]. The DPPH radical scavenging capacity assay was carried out following the procedure described in Pasqualone *et al.* [2015]. The ABTS assay was performed according to Difonzo *et al.* [2017]. The antioxidant activity values were expressed as μmol of Trolox equivalent per g of dry matter (d.m.) of seeds. Each analysis was done in triplicate.

Determination of nutritional composition and bioactive compounds in flours

Proteins (total nitrogen × 5.7), lipids, ashes, total dietary fibre, and moisture of the flours were determined according to the Association of Official Analytical Chemists (AOAC) methods 979.09, 945.38 F, 923.03, 991.43 and 925.10, respectively [AOAC, 2006]. Lipid content was determined. Carbohydrate content was calculated by difference.

Total carotenoid content was assessed using the method reported by Pasqualone *et al.* [2013] and was expressed as mg of β-carotene equivalent per kg of seed d.m.

Total anthocyanin content was determined as described by Pasqualone *et al.* [2015] and was expressed as mg of cyanidin 3-*O*-glucoside equivalent per kg of seed d.m.

Total phenolic compound (TPC) content was assessed as described by Summo *et al.* [2019b] using the extracts prepared as previously reported in section *Preparation of extracts and determination of the antioxidant activity*. The content of total phenolic compounds was expressed as mg of ferulic acid equivalent per g of seed d.m., considering a calibration curve prepared with ferulic acid at different concentrations.

Total phytate content was measured according to the method reported in Summo *et al.* [2019b].

Determination of minerals in flours

The determination of minerals in flours was performed by digesting using a microwave oven (CEM 6, Mars, CEM Corporation, Matthews, United States). Briefly, 0.5 g of each sample was weighed into a Teflon vessel, and 7 mL of HNO_3 (65%) and 1 mL of H_2O_2 (30%) were added [Rybicka & Gliszczyńska-Świgło, 2017]. After cooling, digests were diluted to 50 mL with demineralised water (Hydrolab System, Wiślina, Poland) and kept at 4°C until spectroscopic determinations. Three digests were prepared for each sample. Spectroscopic determinations of minerals were performed using atomic emission spectroscopy and the method described in detail by Ozbek & Akman [2016]. Analytical wavelengths for minerals were: 213.9 nm for Zn, 324.8 nm for Cu, 372.0 nm for Fe, 403.1 nm for Mn, 616.2 nm for Ca, 404.4 nm for K, and 589.0 nm for Na. The spectroscopic analysis was performed using two independent standard curves with a range from 0.05 to 1 μg/mL for microelements, 0.05 to 5 μg/mL for Na, and from 10 to 100 μg/mL for other macroelements. Due to the high limit of quantification (LOQ) of phosphorus in atomic spectroscopy, its content was determined using the spectrophotometric molybdenum blue method adopted for multiple analysis using 48-microwell plates and microplate spectrophotometer (BioTek PowerWave XS2, Biokom, Warsaw, Poland) [Murphy & Riley, 1962]. Briefly, 0.16 mL of the sample, then 0.08 μL of 5% ammonium molybdate, 0.08 μL of 0.5% hydroquinone, and 0.08 μL of 20% sodium sulphite were added to the well. The plate was shaken and left for 30 min in the dark; the absorbance was measured at 823 nm.

Statistical analysis

Data were subjected to one-way ANOVA followed by Tukey's HSD test, considering both the differences between the species (chickpea *vs.* pea) and those among the accessions. Significant differences among the values of all recorded variables were determined at $p<0.05$ by the XLStat software (Addinsoft SARL, New York, NY, USA). Correlation analysis was performed by the same software.

RESULTS AND DISCUSSION

Antioxidant activity evaluation

The antioxidant activity has been evaluated using two different radical scavenging capacity assays, namely DPPH and ABTS, and expressed as μmol Trolox/g of dry matter (Figure 1). A different activity was observed between chickpea and pea accessions depending on the assay. Chickpea accessions were characterised by a significantly higher DPPH$^{\bullet}$ scavenging activity, while peas showed a significantly higher value of antioxidant activity when the ABTS assay was performed. The same trend has been reported by other researchers in green bean (*Phaseolus radiates* L.) and peanut (*Arachis hypogaea* L.) extracts [Fidrianny *et al.*, 2016]. This result could be linked to the different chemical composition that characterised the two different legume species analysed. In fact, it has been shown that different phenolic compounds are responsible for quenching different free radicals [Xu *et al*., 2016]. In order to explain the different antioxidant activities between chickpea and pea species, correlations between *in vitro* radical scavenging capacity and compositional features of legume accessions were investigated.

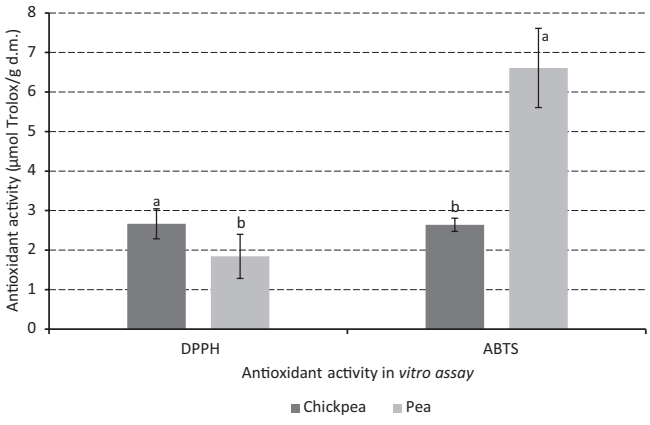


FIGURE 1. Mean values, standard deviation and results of the statistical analysis of antioxidant activity of chickpea and pea accessions assessed using both DPPH and ABTS radical scavenging assays. Different small letters indicate significant differences between the species ($p<0.05$) for the type of antioxidant activity method used (n=12 for chickpeas and n=5 for peas).

TABLE 2. Proximate composition and content of bioactive compounds in the chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) accessions analysed.

	Variety	Type	Proteins (g/100 g d.m.)	Lipids (g/100 g d.m.)	Ashes (g/100 g d.m.)	Carbohydrates (g/100 g d.m.)	Total dietary fiber (g/100 g d.m.)	Total phenolic compounds (mg ferulic acid/g d.m.)	Total carotenoids (mg β-carotene/kg d.m.)	Total anthocyanins (mg cyanidin 3-*O*-glucoside/kg d.m.)	Total phytates (mg phytic acid/g d.m.)
Chickpea*	MG_13	AB	20.29	3.36	3.37	54.32	18.67	1.08	34.21	121.99	14.97
	MG_17	AB	17.31	4.42	2.69	59.86	15.72	0.82	41.26	79.16	9.66
	110694	AB	22.71	3.35	3.90	52.84	17.20	0.96	42.76	119.00	11.36
	PI339154	KC	18.11	2.86	3.45	66.36	9.21	0.92	27.76	25.78	11.86
	PI292006	KC	20.34	4.16	3.64	60.60	11.26	0.69	29.46	32.45	14.72
	PI357648	KC	21.38	4.47	3.48	62.73	7.94	0.81	22.76	27.45	10.35
	PI518255	DC	17.80	2.83	3.75	45.81	29.82	0.71	46.85	44.37	15.29
	PI251514	DC	19.17	3.71	3.70	51.08	22.35	0.79	41.61	159.62	13.96
	PI140293	DC	19.32	2.80	3.47	49.39	25.03	1.08	26.01	46.52	11.79
	PI358934	DC	17.30	3.90	3.48	55.36	19.95	0.76	37.03	155.79	15.44
	PI533683	DC	20.19	3.35	3.70	52.70	20.06	1.04	42.27	115.40	11.02
	W610046	DC	25.92	3.41	3.65	48.87	18.15	0.83	48.92	103.23	11.95
Mean			*19.98*^{B}	*3.55*^{A}	*3.52*^{A}	*54.99*^{A}	*17.95*^{A}	*0,87*^{B}	*36.74*^{A}	*85.90*^{A}	*12.70*^{A}
DS			*2.50*	*0.59*	*0.30*	*6.20*	*6.37*	*0.14*	*8.59*	*49.63*	*2.06*
Pea	IG116297		26.82	1.68	2.89	50.95	17.66	1.12	25.03	33.36	13.23
	ROR12		24.42	1.52	3.32	58.07	12.67	1.22	16.72	19.26	10.71
	IG52442		27.76	1.63	3.68	47.84	19.09	1.10	59.39	78.30	14.78
	IG134828		26.75	2.06	3.45	53.69	14.04	1.05	33.59	72.63	13.99
	IG51520		26.02	1.30	3.49	55.56	13.63	1.03	23.56	35.92	16.09
Mean			*26.36*^{A}	*1.64*^{B}	*3.36*^{A}	*53.22*^{A}	*15.42*^{A}	*1.10*^{A}	*31.66*^{A}	*47.89*^{A}	*13.76*^{A}
DS			*1.25*	*0.28*	*0.30*	*3.98*	*2.79*	*0.07*	*16.62*	*26.03*	*2.01*

KC – *kabuli* chickpea, DC – *desi* chickpea, and AB – *Apulian black* chickpea.

Different letters indicate significant differences between the species at $p<0.05$. *The chickpea accessions are part of a wide collection already characterised, whose data repository is in Summo *et al.* [2019a].

Nutritional composition and bioactive compound content

Table 2 reports proximate composition and content of bioactive compounds of chickpea and pea accessions examined in this study. Significant differences ($p<0.05$) between the two species were found for protein, lipid, and carbohydrate contents. Chickpea showed a significantly higher lipid content and significantly lower protein and carbohydrate contents than pea. No significant differences ($p>0.05$) between species emerged for total dietary fibre content, possibly due to the high variability observed among the accessions within the species.

Data on the chemical composition and bioactive compound content of a collection of chickpea accessions, including the twelve ones tested in this study, were reported and discussed in our previous work [Summo *et al.*, 2019b]. Considering the data on the pea accessions (Table 2), a large variation was currently observed among the pea accessions, especially for bioactive compounds. In particular, the accession ROR12 showed the highest value of total phenolic compounds (1.22 mg ferulic acid/g d.m.). The observed mean value of total phenolic compounds was higher than the levels obtained by Zia-Ul-Haq *et al.* [2013], who reported 0.99 mg/g as a maximum value of total phenolic compounds detected in the cultivar Climax. Notably, at a high concentration, antioxidants can act as pro-oxidants by reacting with molecular oxygen [Sotler *et al.*, 2019]. Due to the high metabolic rate, reactive oxygen species (ROS) generation is incredibly high in transformed cells [Perillo *et al.*, 2020].

The total carotenoid content also varied considerably among pea accessions, ranging from 16.72 mg β-carotene/kg d.m. in the accession ROR12 to 59.39 mg β-carotene/kg d.m. in the IG52442. Ashokkumar *et al.* [2015], examining a collection of 94 pea genotypes, found a carotenoid content in pea ranging from 10 to 27 μg/g in accessions with green cotyledons, and from 5 to 17 μg/g in accessions with yellow cotyledons.

TABLE 3. Mineral composition of the chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.) accessions analysed.

	Variety	Type	Zn (mg/100 g d.m.)	Cu (mg/100 g d.m.)	Fe (mg/100 g d.m.)	Mn (mg/100 g d.m.)	Mg (mg/100 g d.m.)	Ca (mg/100 g d.m.)	K (mg/100 g d.m.)	Na (mg/100 g d.m.)	P (mg/100 g d.m.)
Chickpea	MG_13	AB	4.46	1.03	4.22	2.48	176.49	194.69	898.45	4.89	405.82
	MG_17	AB	2.81	0.29	3.78	4.78	153.95	290.81	756.18	2.89	297.10
	110694	AB	2.65	0.37	4.26	3.63	184.57	219.68	1055.07	7.00	425.91
	PI339154	KC	2.80	0.43	2.95	3.40	173.57	146.50	1159.11	7.53	456.10
	PI292006	KC	2.80	0.31	3.52	3.07	168.78	211.94	1009.69	9.09	406.16
	PI357648	KC	2.49	0.23	2.73	3.26	160.20	174.71	957.08	9.99	386.94
	PI518255	DC	3.05	0.36	4.22	4.32	179.79	431.83	1034.08	15.48	419.95
	PI251514	DC	2.15	0.25	4.11	3.66	183.79	334.91	1027.31	39.36	394.75
	PI140293	DC	2.38	0.20	3.20	3.84	188.73	279.21	984.70	19.91	386.19
	PI358934	DC	2.25	0.22	3.44	4.34	190.05	376.76	1005.02	16.69	364.63
	PI533683	DC	2.35	0.23	3.68	4.03	179.16	305.03	980.09	1.83	395.08
	W610046	DC	1.99	0.21	3.25	3.32	175.69	210.17	1007.91	2.11	395.15
Mean			*2.68*^{B}	*0.34*^{B}	*3.61*^{B}	*3.68*^{A}	*176.22*^{A}	*264.69*^{A}	*989.56*^{A}	*11.40*^{A}	*394.48*^{A}
DS			*0.64*	*0.23*	*0.52*	*0.63*	*10.92*	*86.70*	*95.99*	*10.59*	*38.37*
Pea	IG116297		3.74	0.87	4.00	0.96	157.63	117.07	884.55	3.14	474.17
	ROR12		3.16	0.56	3.69	1.12	153.90	90.96	937.62	5.02	412.93
	IG52442		4.39	0.84	5.23	1.07	177.15	119.61	996.19	2.97	543.83
	IG134828		4.08	0.72	4.66	0.99	163.56	97.07	979.05	3.15	503.97
	IG51520		2.56	0.70	3.87	1.02	166.99	129.17	971.88	1.64	421.20
Mean			*3.58*^{A}	*0.74*^{A}	*4.29*^{A}	*1.03*^{B}	*163.85*^{B}	*110.78*^{B}	*953.86*^{A}	*3.18*^{A}	*471.22*^{A}
DS			*0.73*	*0.12*	*0.64*	*0.06*	*9.01*	*16.10*	*44.21*	*1.21*	*55.35*

KC – *kabuli* chickpea, DC – *desi* chickpea, and AB – *Apulian black* chickpea. Different letters indicate significant differences between the species at $p<0.05$.

The total anthocyanin content varied from 19.26 mg cyanidin 3-*O*-glucoside/kg d.m. in the non-pigmented pea accession ROR12 to 78.30 mg cyanidin 3-*O*-glucoside/kg d.m in the pigmented accession pea IG52442. Notably, anthocyanin content was highly variable even within pigmented accessions with the minimum value (23.56 mg cyanidin 3-*O*-glucoside/kg d.m) displayed by the accession IG 51520.

Legumes contain non-nutritional factors, such as phytates, that can reduce the bioavailability of some compounds or inhibit the enzymes necessary for their digestion [Shi *et al.*, 2018]. As reported in Table 2, no significant differences were observed between the two species, although considerable variation was found among the individual accessions. Pea accessions under the study showed a higher content of phytic acid than green and yellow peas studied by other researchers. Millar *et al.* [2019] reported that phytic acid content was 543.41 mg/100 g in the green pea and 574.14 mg/100 g in the yellow one was 574.14 mg/100g [Millar *et al.*, 2019]. Phytic acid is the principal storage form of phosphorus in seeds; this compound and its salts are capable of forming complexes with minerals, such as Ca, Cu, Mg, Fe, and Zn, thereby having a negative effect on their gastrointestinal absorption [Shi *et al.*, 2018].

Mineral composition

Potassium (K) was the most abundant mineral found in both chickpeas and peas (Table 3), without significant differences between them. Instead, significant differences were observed for other minerals such as zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), calcium (Ca), and phosphorus (P) ($p<0.05$). Chickpeas had the highest contents of Mn, Mg, and Ca. Magnesium and calcium contents in chickpeas were higher than those (mean values of 1402 and 1040 μg/g, respectively) reported in Vandemark *et al.* [2018], but similar to those reported by Kaya *et al.* [2018]. Peas had significantly higher Zn, Cu, Fe, and P values

TABLE 4. Correlation table (correlation coefficient – *r* values) between the *in vitro* antioxidant activity (DPPH and ABTS assays) and chemical compound contents determined in the pulse accessions.

	Pr	L	A	C	DF	TPC	TC	TA	PH	Zn	Cu	Fe	Mn	Mg	Ca	K	Na	P
DPPH	-0.53	**0.58**	-0.04	-0.14	0.34	-0.31	**0.66**	**0.64**	0.13	-0.11	-0.13	0.14	**0.64**	0.45	0.53	-0.20	0.04	-0.48
ABTS	**0.73**	**-0.79**	-0.46	-0.02	-0.26	**0.59**	-0.50	-0.43	0.01	**0.59**	**0.61**	0.39	**-0.86**	**-0.59**	**-0.68**	-0.26	-0.31	**0.70**

Pr – Proteins; L – Lipids; A – Ashes; C – Carbohydrates; DF – Dietary fibers; TPC – Total phenolic compounds; TC – Total carotenoids; TA – Total anthocyanins; PH – Phytates; *r* values in bold indicate a significant correlation ($p<0.05$).

than chickpeas. The values found in our study agree with Wang & Daun [2004], who reported a range of 2.50–5.20, 0.40–0.90, 4.30–7.90, and 270.30–950.50 mg/100 g for Zn, Cu, Fe, and P, respectively.

At the intraspecific level, *Apulian black* and some *desi* chickpea accessions showed a high Fe content, with the highest value observed in the accession 110694 (4.26 mg/100 g d.m.). In contrast, *kabuli* chickpeas were characterised by a low Fe content, with the lowest value found in the accession PI357648 (2.73 mg/100 g d.m.). In accordance with the previous study of Jukanti *et al.* [2012], DC accessions showed the highest Ca content (mean value 322.99 mg/100 g d.m.), followed by AC (mean value 235.36 mg/100 g d.m.) and *kabuli (*mean value 177.72 mg/100 g d.m.). The DC accession PI518255 displayed the highest Ca content (431.83 mg/100 g d.m.), which was found to be far from the mean value shown for chickpeas (264.69 mg/100 g d.m.). The AB variety MG_13 had the highest Zn and Cu levels (4.26 and 1.03 mg/100 g d.m., respectively).

Peas were characterised by a low variability among different accessions. However, the pigmented variety IG51520 had the highest content of Ca (129.17 mg/100 g d.m.), which was higher than the maximum value (106.90 mg/100 g) reported by Wang & Daun [2004].

Correlations between antioxidant activity and flour composition

Correlations between *in vitro* radical scavenging capacity and compositional features of legume accessions are reported in the Table 4.

A positive correlation was observed between the total phenolic content and $ABTS^{\bullet+}$ scavenging activity ($r=0.59$; $p<0.05$). On the contrary, a negative, but not significant ($p>0.05$) correlation was observed between ABTS assay results and contents of both carotenoids and anthocyanins. Considering the same compounds, an inverse trend was found for $DPPH^{\bullet}$ scavenging activity. Bioactive compounds, such as phenolics, carotenoids, and anthocyanins, are recognised as antioxidants. Thus, they can prevent or reduce lipid peroxidation and scavenge free oxygen radicals through their high antioxidant activities [Ashokkumar *et al.*, 2015]. Moreover, phenolic compounds exhibit anti-tumoral, anti-inflammatory, and anti-allergic properties while anthocyanins are important due to their anti-carcinogenic properties and the ability to limit the incidence of hepatic steatosis and cardiovascular diseases, to control obesity, and to mitigate diabetes [Hernandez-Velazquez *et al.*, 2020]. Interestingly a significant association has been found between the total flavonoid intake and a high level of magnesium, paralleled by a reduction of the metabolic syndrome [Jin *et al.*, 2020].

By contrast, several flavonoids, known for their antioxidant features, were proved, instead, to act as prooxidants and mutagenic factors in the *in vitro* studies [Rahal *et al.*, 2014]. A study conducted to determine the potential of grape pomace extracts as a source of natural antioxidants reported a positive correlation between $ABTS^{\bullet+}$ scavenging capacity and total phenolic contents, as well as with the total flavonoid contents [Xu *et al.*, 2016]. Yao *et al.* [2013] confirmed these results in black mung beans, where a significant positive correlation was found between bound phenolic acids and $ABTS^{\bullet+}$ scavenging activity ($r=0.941$; $p<0.01$). Flavonoids and tannins have a relevant influence on the ABTS antioxidant activity, while anthocyanin compounds give a greater contribution to the antioxidant capacity measured by DPPH test, as shown by Xu *et al*. [2016] in pomace extracts. Furthermore, a negative correlation between $ABTS^{\bullet+}$ scavenging activity and β-carotene was reported by Thaipong *et al.* [2006] for methanol extracts from guava fruit. Considering the nutritional composition, the ABTS data showed a positive correlation with protein content ($r=0.73$; $p<0.01$), whereas the same data were negatively correlated with lipid content ($r=-0.79$; $p<0.01$) (Table 4). As previously reported by other researchers in leguminous seeds [Grela *et al.*, 2017], a positive correlation was found between $DPPH^{\bullet}$ scavenging activity and lipid contents ($r=0.58$; $p<0.05$). Grela *et al*. [2017] have reported a high correlation between $DPPH^{\bullet}$ scavenging activity and unsaturated fatty acid contents, especially polyunsaturated ones, in several legumes, namely lupines, peas, chickpeas, lentils, grass peas, and common beans. In contrast, no correlation between fatty acids and $DPPH^{\bullet}$ scavenging activity in 20 Canadian lentils cultivars was reported by Zhang *et al.* [2014]. Usually, the number of unsaturated bonds in the fatty acids induces an exponential increase in the susceptibility to oxidation. Therefore, the content of the individual double bonds of fatty acids may not be directly related in a linear way to the antioxidant activity. The positive correlation between the antioxidant activity (measured by DPPH test) and lipid content found in our study may suggest that other compounds have a significant influence on the resistance to oxidation of fatty acids [Grela *et al*., 2017]. For instance, as mentioned above, carotenoids, anthocyanins, and phenolic compounds can contribute to the increase in the antioxidant potential.

A significant negative correlation was found between $ABTS^{\bullet+}$ scavenging activity and Mn ($r=-0.86$; $p<0.01$), Mg ($r=-0.59$; $p<0.05$) and Ca ($r=-0.68$; $p<0.01$), whereas

the correlation was positive for Zn (r=0.59; p<0.05), Cu (r=0.61; p<0.05) and P (r=0.70; p<0.01). No significant correlation between DPPH$^{\bullet}$ scavenging activity and mineral compounds was found, except for Mn (r=0.64; p<0.05). Despite the large amount of information available in scientific literature on mineral content of legumes, to the best of our knowledge, there are no reports on the direct correlation between mineral content and antioxidant activity. However, several studies suggested that an imbalance of minerals would change the content of polyphenols and flavonoids [Grela *et al*., 2017; Sulaiman *et al.*, 2011]. This behaviour may explain the positive correlation found for the content of Mn and DPPH$^{\bullet}$ scavenging activity. In fact, Mn is involved in activating enzymes that enhance the biosynthesis of flavonoids [Gordon, 2007]. A significant correlation between Mn content and DPPH$^{\bullet}$ scavenging activity was reported by Sulaiman *et al.* [2011] in banana (*Musa* sp.) fresh pulps and peels. Furthermore, Zn-deficient or Zn-excess conditions cause changes in the antioxidant enzyme activities, as shown in bean plants by Prabhu Inbaraj & Muthuchelian [2011]. Tewari *et al.* [2006] reported, instead, an increase in the activity of the antioxidative enzyme superoxide dismutase (SOD) in mulberry (*Morus rubra* L.) Mg-deficient plants, suggesting an inverse relationship between Mg and antioxidant activity. Other researchers reported a significant correlation between the total flavonoid content and minerals due to the chelating role of polyphenols, especially condensed tannins [Rehecho *et al.,* 2011]. Therefore, these compounds may prevent or delay metal-catalised initiation and decomposition of lipid hydroperoxides. Rehecho *et al.* [2011] reported significant correlations between the total flavonoid content and minerals, such as K, Zn, Cu, Ca, and Mg in verbena extracts.

CONCLUSION

Chickpea and pea accessions showed a different antiradical activity against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$. In particular, chickpea accessions were characterised by significantly higher DPPH$^{\bullet}$ scavenging activity, while pea showed a significantly a higher value of antioxidant activity evaluated using the ABTS assay.

Pea accessions had the highest content of total phenolic compounds, Zn and Cu. A positive correlation was found between some minerals, such as Zn, Cu and P, and the ABTS$^{\bullet+}$ scavenging activity found. Black and brown chickpea accessions showed a significantly higher content of anthocyanins, Mn, Mg and Ca, which were positively correlated with the antioxidant activity assessed by the DPPH assay. Therefore, the high phenolic content found in pea accessions was linked to the higher ABTS$^{\bullet+}$ scavenging capacity, while chickpeas, especially *Apulian black* and *desi* types, having high carotenoid and anthocyanin contents, were able to quench the DPPH radical.

Furthermore, the content of minerals and their composition may influence the antioxidant activity, especially ABTS$^{\bullet+}$ scavenging. Indeed, a significant negative correlation was found between ABTS$^{\bullet+}$ scavenging activity and Mn, Mg, and Ca, whereas the correlation was positive for Zn, Cu, and P.

Despite the dataset investigated in our study included a limited number of accessions, it was possible to highlight the influence of the chemical composition on the antioxidant activity due to the high phenotypic diversity found between the accessions, emphasising the importance of selecting the antioxidant activity assay according to the matrix to be evaluated.

RESEARCH FUNDING

This research has been performed within the project "LEgume GEnetic REsources as a tool for the development of innovative and sustainable food TEchnological system" supported under the "Thought for Food" Initiative by Agropolis Fondation (through the "Investissements d'avenir" programme with reference number ANR-10-LABX-0001–01), Fondazione Cariplo, and Daniel & Nina Carasso Foundation.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ORCID IDs

P. Annicchiarico https://orcid.org/0000-0001-8027-1035
F. Caponio https://orcid.org/0000-0002-0740-7677
M. Centrone https://orcid.org/0000-0001-5469-3365
M. Costantini https://orcid.org/0000-0002-8273-1039
M. D'Agostino https://orcid.org/0000-0001-6127-9656
A. Pasqualone https://orcid.org/0000-0001-6675-2203
S. Pavan https://orcid.org/0000-0002-3666-7291
I. Rybicka https://orcid.org/0000-0003-1679-328X
C. Summo https://orcid.org/0000-0002-2736-7705
G. Tamma https://orcid.org/0000-0002-8890-0278

REFERENCES

1. Annicchiarico, P. (2017). Feed legumes for truly sustainable crop-animal systems. *Italian Journal of Agronomy, 12*, 151–160. https://doi.org/10.4081/ija.2017.880
2. AOAC International (2006). Official Methods of Analysis (17th ed.). Association of Official Analytical Chemists, Gaithersburg, MD.
3. Ashokkumar, K., Diapari, M., Jha, A.B., Tar'an, B., Arganosa, G., Warkentin, T.D. (2015). Genetic diversity of nutritionally important carotenoids in 94 pea and 121 chickpea accessions. *Journal of Food Composition and Analysis*, *43*, 49–60. https://doi.org/10.1016/j.jfca.2015.04.014
4. Daryanto, S., Wang, L., Jacinthe, P.A., (2015). Global synthesis of drought effects on food legume production. *Plos One, 10*(6), art. no. e0127401. https://doi.org/10.1371/journal.pone.0127401
5. De Giovanni, C., Pavan, S., Taranto, F., Di Rienzo, V., Miazzi, M.M., Marcotrigiano, R., Mangini, G., Montemurro, C., Ricciardi, L., Lotti, C. (2017). Genetic variation of a global germplasm collection of chickpea (*Cicer arietinum* L.) including Italian accessions at risk of genetic erosion. *Physiology and Molecular Biology of Plants*, *23*, 197–205. https://doi.org/10.1007/s12298-016-0397-4

6. Difonzo, G., Russo, A., Trani, A., Paradiso, V.M., Ranieri, M., Pasqualone, A., Summo, C., Tamma, G., Silletti, R., Caponio, F. (2017). Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity. *Journal of Functional Foods*, *31*, 63–70. https://doi.org/10.1016/j.jff.2017.01.039
7. Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., Mérillon, J.M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry*, *57*(5), 1768–1774. https://doi.org/10.1021/jf803011r
8. Evans, P., Halliwell, B. (2001). Micronutrients: oxidant/antioxidant status. *British Journal of Nutrition*, *85*(S2), S67-S74. https://doi.org/10.1079/BJN2000296
9. Food and Agriculture Organization of the United Nations (2018). Data of Crops Production. (Accessed 02 November 2020).
10. Fotiadis, S., Koutroubas, S.D., Damalas, C.A. (2019). Nitrogen utilization and yield determination of spring mediterranean chickpea as influenced by planting date and environmental conditions. *International Journal of Plant Production*, *13*(1), 59–72. https://doi.org/10.1007/s42106-018-0034-y
11. Fidrianny, I., Elviana, D., Ruslan, K. (2016). *In vitro* antioxidant activities in various beans extracts of five legumes from West of Java-Indonesia using DPPH and ABTS methods. *International Journal of Pharmacognosy and Phytochemical Research*, *8*(3), 470–476.
12. Gordon, B. (2007). Manganese nutrition of glyphosate-resistant and conventional soybeans. *Better Crops*, *91*(4), 12–13.
13. Grela, E.R., Samolińska, W., Kiczorowska, B., Klebaniuk, R., Kiczorowski, P. (2017). Content of minerals and fatty acids and their correlation with phytochemical compounds and antioxidant activity of leguminous seeds. *Biological Trace Element Research*, *180*(2), 338–348. https://doi.org/10.1007/s12011-017-1005-3
14. Hernandez-Velazquez, I., Sanchez-Tapia, M., Ordaz-Nava, G., Torres, N., Tovar, A.R., Galvez, A. (2020). Black bean protein concentrate ameliorates hepatic steatosis by decreasing lipogenesis and increasing fatty acid oxidation in rats fed a high fat-sucrose diet. *Food and Function*, *11*(12), 10341–10350. https://doi.org/10.1039/D0FO02258F
15. Huang, D., Ou, B., Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*(6), 1841–1856. https://doi.org/10.1021/jf030723c
16. Jin, S., Liu, J., Jia, Y., Han, T., Zhao, X., Sun, C., Na, L. (2020). The association of dietary flavonoids, magnesium and their interactions with the metabolic syndrome in Chinese adults: a prospective cohort study. *British Journal of Nutrition*, 1–11. https://doi.org/10.1017/S0007114520004754
17. Jukanti, A.K., Gaur, P.M., Gowda, C., Chibbar, R.N. (2012). Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition*, *108*(S1), S11-S26. https://doi.org/10.1017/S0007114512000797
18. Kaya, M., Kan, A., Yilmaz, A., Karaman, R., Sener, A. (2018). The fatty acid and mineral compositions of different chickpea cultivars cultivated. *Fresenius Environmental Bulletin*, *27*(2), 1240–1247.
19. Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E., Etherton, T.D. (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *The American Journal of Medicine*, *113*(9), 71–88. https://doi.org/10.1016/S0002-9343(01)00995-0
20. Millar, K.A., Barry-Ryan, C., Burke, R., McCarthy, S., Gallagher, E. (2019). Dough properties and baking characteristics of white bread, as affected by addition of raw, germinated and toasted pea flour. *Innovative Food Science and Emerging Technologies*, *56*, art. no. 102189. https://doi.org/10.1016/j.ifset.2019.102189
21. Murphy, J., Riley, J.P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5
22. Ozbek, N., Akman, S. (2016). Method development for the determination of calcium, copper, magnesium, manganese, iron, potassium, phosphorus and zinc in different types of breads by microwave induced plasma-atomic emission spectrometry. *Food Chemistry*, *200*, 245–248. https://doi.org/10.1016/j.foodchem.2016.01.043
23. Pasqualone, A., Bianco, A.M., Paradiso, V.M. (2013). Production trials to improve the nutritional quality of biscuits and to enrich them with natural anthocyanins. *CyTA – Journal of Food*, *11*, 301–308. https://doi.org/10.1080/19476337.2012.753113
24. Pasqualone, A., Bianco, A.M., Paradiso, V.M., Summo, C., Gambacorta, G., Caponio, F., Blanco, A. (2015). Production and characterization of functional biscuits obtained from purple wheat. *Food Chemistry*, *180*, 64–70. https://doi.org/10.1016/j.foodchem.2015.02.025
25. Pasqualone, A., De Angelis, D., Squeo, G., Difonzo, G., Caponio, F., Summo, C., (2019a). The effect of the addition of Apulian black chickpea flour on the nutritional and qualitative properties of durum wheat-based bakery products. *Foods*, *8*(10), art. no. 504. https://doi.org/10.3390/foods8100504
26. Pavan, S., Lotti, C., Marcotrigiano, A.R., Mazzeo, R., Bardaro, N., Bracuto, V., De Giovanni, C. (2017). A distinct genetic cluster in cultivated chickpea as revealed by genome-wide marker discovery and genotyping. *The Plant Genome*, *10*(2), 1–9. https://doi.org/10.3835/plantgenome2016.11.0115
27. Pavan, S., Schiavulli, A., Marcotrigiano, A.R., Bardaro, N., Bracuto, V., Ricciardi, F., Charnikhova, T., Lotti, C., Bouwmeester, H., Ricciardi, L. (2016). Characterization of low-strigolactone germplasm in pea (*Pisum sativum* L.) resistant to crenate broomrape (*Orobanche crenata* Forsk.). *Molecular Plant-Microbe Interactions*, *29*(10), 743–749. https://doi.org/10.1094/MPMI-07-16-0134-R
28. Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., Migliaccio, A. (2020). ROS in cancer therapy: the bright side of the moon. *Experimental and Molecular Medicine*, *52*(2), 192–203. https://doi.org/10.1038/s12276-020-0384-2
29. Prabhu Inbaraj, M., Muthuchelian, K. (2011). The effect of zinc stress combined with high irradiance stress on membrane damage and antioxidative response in bean seedlings. *Environmental and Experimental Botany*, *74*, 171–177. https://doi.org/10.1016/j.envexpbot.2011.05.016
30. Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., Dhama, K. (2014). Oxidative stress, prooxidants, and antioxi-

dants: the interplay. *BioMed Research International*, *2014*, art. no. 761264.
https://doi.org/10.1155/2014/761264

31. Rehecho, S., Hidalgo, O., De Cirano, M.G.I., Navarro, I., Astiasarán, I., Ansorena, D., Cavero, R.Y., Calvo, M.I. (2011). Chemical composition, mineral content and antioxidant activity of *Verbena officinalis* L. *LWT – Food Science and Technology*, *44*(4), 875–882.
https://doi.org/10.1016/j.lwt.2010.11.035
32. Rybicka, I., Gliszczyńska-Świgło, A. (2017). Minerals in grain gluten-free products. The content of calcium, potassium, magnesium, sodium, copper, iron, manganese, and zinc. *Journal of Food Composition and Analysis*, *59*, 61–67.
https://doi.org/10.1016/j.jfca.2017.02.006
33. Shi, L., Arntfield, S.D., Nickerson, M. (2018). Changes in levels of phytic acid, lectins and oxalates during soaking and cooking of Canadian pulses. *Food Research International*, *107*, 660–668.
https://doi.org/10.1016/j.foodres.2018.02.056
34. Smýkal, P., Aubert, G., Burstin, J., Coyne, C.J., Ellis, N.T.H., Flavell, A.J., Ford, R., Hýbl, M., Macas, J., Neumann, P., McPhee, K.E., Redden, R.J., Rubiales, D., Weller, J.L., Warkentin, T.D. (2012). Pea (*Pisum sativum* L.) in the genomic era. *Agronomy*, *2*, 74–115.
https://doi.org/10.3390/agronomy2020074
35. Sotler, R., Poljšak, B., Dahmane, R., Jukić, T., Jukić, D.P., Rotim, C., Trebše, P., Starc, A. (2019). Prooxidant activities of antioxidants and their impact on health. *Acta Clinica Croatica*, *58*(4), art. no. 726.
https://doi.org/10.20471/acc.2019.58.04.20
36. Sulaiman, S.F., Yusoff, N.A.M., Eldeen, I.M., Seow, E.M., Sajak, A.A.B., Ooi, K.L. (2011). Correlation between total phenolic and mineral contents with antioxidant activity of eight Malaysian bananas (*Musa* sp.). *Journal of Food Composition and Analysis*, *24*(1), 1–10.
https://doi.org/10.1016/j.jfca.2010.04.005
37. Summo, C., Centomani, I., Paradiso, V.M., Caponio, F., Pasqualone, A. (2016). The effects of the type of cereal on the chemical and textural properties and on the consumer acceptance of precooked, legume-based burgers. *LWT – Food Science and Technology*, *65*, 290–296.
https://doi.org/10.1016/j.lwt.2015.08.009
38. Summo, C., De Angelis, D., Ricciardi, L., Caponio, F., Lotti, C., Pavan, S., Pasqualone, A. (2019a). Data on the chemical composition, bioactive compounds, fatty acid composition, physico-chemical and functional properties of a global chickpea collection. *Data in Brief*, *27*, art. no. 104612.
https://doi.org/10.1016/j.dib.2019.104612
39. Summo, C., De Angelis, D., Ricciardi, L., Caponio, F., Lotti, C., Pavan, S., Pasqualone, A. (2019b). Nutritional, physico-chemical and functional characterization of a global chickpea collection. *Journal of Food Composition and Analysis*, *84*, art. no. 103306.
https://doi.org/10.1016/j.jfca.2019.103306
40. Summo, C., De Angelis, D., Rochette, I., Mouquet-Rivier, C., Pasqualone, A. (2019c). Influence of the preparation process on the chemical composition and nutritional value of canned purée of kabuli and Apulian black chickpeas. *Heliyon*, *5*(3), art. no. e01361.
https://doi.org/10.1016/j.heliyon.2019.e01361
41. Tewari, R.K., Kumar, P., Sharma, N. (2006). Magnesium deficiency induced oxidative stress and antioxidant responses in mulberry plants. *Scientia Horticulturae, 108*, 7–14.
https://doi.org/10.1016/j.scienta.2005.12.006
42. Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*, *19*(6–7), 669–675.
https://doi.org/10.1016/j.jfca.2006.01.003
43. Vandemark, G.J., Grusak, M.A., McGee, R.J. (2018). Mineral concentrations of chickpea and lentil cultivars and breeding lines grown in the US Pacific Northwest. *The Crop Journal*, *6*(3), 253–262.
https://doi.org/10.1016/j.cj.2017.12.003
44. Wang, N., Daun, J.K. (2004). Effect of variety and crude protein content on nutrients and certain antinutrients in field peas (*Pisum sativum*). *Journal of the Science of Food and Agriculture*, *84*(9), 1021–1029.
https://doi.org/10.1002/jsfa.1742
45. Xu, Y., Burton, S., Kim, C., Sismour, E. (2016). Phenolic compounds, antioxidant, and antibacterial properties of pomace extracts from four Virginia grown grape varieties. *Food Science and Nutrition*, *4*(1), 125–133.
https://doi.org/10.1002/fsn3.264
46. Yao, Y., Yang, X., Tian, J., Liu, C., Cheng, X., Ren, G. (2013). Antioxidant and antidiabetic activities of black mung bean (*Vigna radiata* L.). *Journal of Agricultural and Food Chemistry*, *61*(34), 8104–8109.
https://doi.org/10.1021/jf401812z
47. Zhang, B., Deng, Z., Tang, Y., Chen, P., Liu, R., Ramdath, D.D., Liu, Q., Hernandez, M., Tsao, R. (2014). Fatty acid, carotenoid and tocopherol compositions of 20 Canadian lentil cultivars and synergistic contribution to antioxidant activities. *Food Chemistry*, *161*, 296–304.
https://doi.org/10.1016/j.foodchem.2014.04.014
48. Zia-Ul-Haq, M., Amarowicz, R., Ahmad, S., Riaz, M. (2013). Antioxidant potential of some pea (*Pisum sativum* L.) cultivars commonly consumed in Pakistan. *OXID Community Edition*, *36*(4), 1046–1057.

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 187–196
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/136051
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Study of Interactions Between Individual Phenolics of Aronia with Barley β-Glucan

Lidija Jakobek[1]*, *Petra Matić*[1], *Jozo Ištuk*[1], *Andrew R. Barron*[2]

[1]*Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, Franje Kuhača 18, Osijek, Croatia*
[2]*Yale University, Department of Statistics and Data Science, 24 Hillhouse Avenue, New Haven, CT 06511, USA*

Key words: adsorption isotherms, adsorption capacity, non-linear models, chokeberry, interactions

Beneficial effects of aronia phenolics are determined by their interactions with dietary fibers, such as β-glucan. The aim of this research was to study interactions between aronia phenolics and β-glucan by investigating the adsorption process. Phenolic compounds were extracted from aronia, analyzed using high-performance liquid chromatography, and adsorbed onto β-glucan at pH 1.5. The adsorption data were modeled by using Langmuir, Dubinin-Radushkevich, and Hill isotherms with a novel non-linear regression developed especially for adsorption isotherms. Aronia phenolics adsorbed onto β-glucan in amounts 31–250 mg/g (individual anthocyanins), 44–123 mg/g (individual flavonols), and 51 mg/g (neochlorogenic acid). The correlation between adsorption capacities and phenolic content was high (r^2=0.94), which suggested that the adsorption might be concentration dependent. Modeling with a novel non-linear regression allowed more precise determination of adsorption isotherm parameters. Furthermore, there was a correlation between maximum adsorption capacities predicted by models and measured adsorption capacities (r^2=0.76, r^2=0.81 and r^2=0.34 for Langmuir, Dubinin-Radushevich, Hill isotherms, respectively). The suggested bonds involved in interactions are non-covalent bonds (H bonds, Van der Waals forces). Principal component analysis showed that anthocyanins, flavonols, and phenolic acids could differently behave in the adsorption process, which could be due to differences in the chemical structures (ionic nature of anthocyanins, nonionic nature of flavonols and phenolic acids at low pH). In conclusion, aronia phenolics interacted with β-glucan by adsorbing onto its surface, and the novel modeling developed by our team was helpful in the interpretation of this process. Interactions should be further studied due to their importance for the beneficial effects of aronia.

INTRODUCTION

Aronia (*Aronia melanocarpa*) (also called chokeberry) is a fruit with a high content of phenolics belonging to different phenolic subgroups: anthocyanins, flavonols, phenolic acids, and flavan-3-ols [Denev *et al.,* 2019; Sidor *et al.,* 2019]. Due to these large contents, potential positive bioactivities of aronia phenolics have been investigated, including their beneficial effects on hypertension, diabetes, and hypercholesterolemia [Sidor *et al.,* 2019]. Aronia phenolics might reduce the risk of a metabolic syndrome development [Sidor *et al.,* 2019] and inhibit the activity of pancreatic lipase, which can be helpful in obesity prevention [Sosnowska *et al.,* 2018]. Due to these beneficial effects, aronia phenolics are still being investigated.

Phenolic compounds can interact with carbohydrates, proteins or lipids from food in the digestive tract [Jakobek, 2015], and this interaction can lead to many effects, one of which is the influence on the amount of phenolics accessible for absorption in the digestive tract (bioaccessibility) [Palafox-Carlos *et al.,* 2011; Renard *et al.,* 2017; Saura-Calixto, 2011]. Moreover, certain food components, like dietary fibers, which are indigestible in the small intestine and ferment in the large intestine, can interact with phenolics, which might result in the "carrying" of these compounds to the lower parts of the digestive tract. Herein, phenolics might be released and elicit their beneficial activities [Palafox-Carlos *et al.,* 2011]. Since dietary fibers have the potential to "carry" bioactive components through the digestive tract, they have already been investigated as delivery systems for various phenolics [Tang *et al.,* 2020]. Due to the importance of phenolic-dietary fiber interactions, they are still being investigated.

Interactions between phenolic compounds and dietary fibers can be investigated *in vitro* through the study of the adsorption process, during which a substance from a gas or liquid is being adsorbed onto an adsorbent. The amount of the substance adsorbed can be compared to the amount of the substance non-adsorbed, and their relationship is called an adsorption isotherm [Foo & Hameed, 2010; Limousin *et al.,* 2007]. The data in adsorption isotherm graphs can be modeled with various equations, some of which are Langmuir, Freundlich, Dubinin-Radushkevich, or Hill adsorption isotherms [Al-Ghouti & Da'ana 2020; Foo & Hameed 2010; Limousin *et al.,* 2007]. The approach to study interactions between phenolic

* Corresponding Author:
e-mail: lidija.jakobek@ptfos.hr (L. Jakobek)

Submitted: 19 December 2020
Accepted: 23 April 2021
Published on-line: 11 May 2021

compounds and dietary fibers through the adsorption process has been applied previously for phenolics and cellulose [Liu *et al.,* 2017], phenolics and starch nanoparticles [Liu *et al.,* 2016], or phenolics and cell wall constituents [Le Bourvellec *et al.,* 2004; Le Bourvellec & Renard, 2005].

β-Glucans are soluble dietary fibers found in cereals like oats or barley with many potentially positive effects on human health [Mäkelä *et al.,* 2020]. Since they show beneficial effects and they are dietary fibers, β-glucans are good candidates for interacting with phenolics and carrying them through the digestive tract, as proved successful with various bioactive compounds [De Smet *et al.,* 2013; Hwang *et al.,* 2018; Lee *et al.,* 2019; Li & Cheung, 2019]. Hence, the interactions of β-glucans with phenolic compounds have attracted interest to know the fate of these compounds in the digestive tract after their interactions, and, in particular, whether the phenolics are carried to the lower parts of the digestive tract by β-glucans where they might show beneficial effects. Interactions between β-glucan and tea phenolics [Gao *et al.,* 2012; Wu *et al.,* 2011a,b] or apple phenolics [Jakobek *et al.,* 2020a] have been studied *via* the adsorption process. But, to the best of our knowledge, there have been no studies of the interactions between aronia phenolics and β-glucan through adsorption. Many studies have shown that aronia has a very high content of phenolics, especially anthocyanins [Denev *et al.,* 2019; Sidor *et al.,* 2019], which differentiates it from other fruit and raises the possibility of their different behavior in the adsorption/interaction with β-glucan. Furthermore, since aronia shows the already-mentioned potential beneficial effects [Sidor *et al.,* 2019; Sosnowska *et al.,* 2018], as well as lowers blood pressure [Hellström *et al.,* 2010] and reduces plasma cholesterol [Kim *et al.,* 2013], this fruit offers a high potential for being used as a dietary supplement. This makes it important to know the effects of aronia on the human health. But much remains unknown about the fate of aronia phenolics in the digestive tract. The investigation of interactions between aronia phenolics and dietary fibers (β-glucan) can contribute to the knowledge in one part of this landscape of unknowns – the possible bonding of aronia phenolics to fibers, and the potential of β-glucan for carrying the aronia phenolics through the digestive tract.

The aim of this study was to investigate interactions between aronia phenolics and β-glucan from barley through the adsorption process. Aronia phenolics were extracted and analyzed with reversed-phase high-performance liquid chromatography (RP-HPLC). After conducting the adsorption process between aronia phenolics and β-glucan, the adsorption data were modelled with adsorption isotherm equations (Langmuir, Dubinin-Radushkevich, and Hill) using a novel, improved non-linear regression, a process developed by our team to fit and to interpret adsorption isotherms with more precision.

MATERIALS AND METHODS

Chemicals

Potassium chloride was purchased from Gram mol (Zagreb, Croatia), hydrochloric acid from Carlo Erba (Val-de-Reuil, France), β-D-glucan from barley, chlorogenic acid, quercetin 3-glucoside, quercetin 3-rutinoside from Sigma-Aldrich (St. Louis, MO, USA), and cyanidin 3-galactoside chloride, cyanidin 3-glucoside chloride, quercetin 3-*O*-galactoside from Extrasynthese (Genay, France). Orto-phosphoric acid (85% HPLC-grade), methanol, and acetonitrile (HPLC grade) were from Fluka (Buchs, Switzerland), J.T. Baker (Gliwice, Poland), and Fisher Scientific (Loughborough, UK), respectively.

Samples

Aronia berries (around 1 kg) were harvested from a local producer in the town of Orahovica, Croatia, and frozen at -18°C. Around 300 g of aronia were grinded and homogenized with a stick blender. Then, 0.2 g of homogenized aronia were weighed into a plastic tube, 1.5 mL of 80% (*v/v*) methanol in water was added, the sample was vortexed, extracted for 15 min (ultrasonic water bath) (RK 100, Berlin, Germany), and then centrifuged for 10 min at 6739×*g* (Eppendorf, Hamburg, Germany). The extract was pipetted into a separate plastic tube. The residue was extracted one more time with the same procedure using 0.5 mL of 80% (*v/v*) methanol. The two extracts were then combined to get a measured final volume of the extract of about 2 mL. The same procedure was repeated to get a second parallel extract of aronia. Extracts were filtered through a PTFE syringe filter with 0.45 μm pores and then analyzed on the HPLC system two times to determine the content of phenolics before adsorption (n=4).

Adsorption

A β-glucan solution (190 mg/L) was prepared by dissolving β-glucan in distilled water heated at 80°C. Since anthocyanins make a high portion of total aronia phenolics and they are stable at low pH, a pH 1.5 was chosen for the adsorption process. This enabled us to correctly determine anthocyanins in the adsorption process. Also, a low pH corresponds to the part of the digestive process. In accordance to this fact, a solution of pH 1.5 was prepared from HCl and KCl solutions (0.1 M) to be used in the adsorption process. For the adsorption experiment, the reaction solution contained β-glucan (15 mg/L), an aliquot of aronia extract (50 mL), and the rest of the volume to the total volume of 1 mL was a buffer solution at pH 1.5. The mass ratio of total phenolics of the aronia extract to β-glucan was 4 mg/1 mg. The vessel with the reaction solution was then placed onto a shaker for 3 h, at room temperature. After adsorption, the reaction solution was centrifuged for 10 min at 6739×*g* (Eppendorf, Hamburg, Germany). An aliquot of 500 mL was taken, filtered through a PTFE syringe filter 0.45 μm, and injected into the HPLC system for the determination of un-adsorbed phenolics (c_e in mg/L). The c_e value was also recalculated in mg. Adsorption capacity (q_e) (mg of phenolics adsorbed onto g of β-glucan) was calculated, by the following equation which is a rearrangement of the expression of the conservation of mass (the amount adsorbed plus the amount not adsorbed equals the initial amount of phenolics):

$$q_e = \frac{(c_0 - c_e)V_m}{\gamma_a V_a} \quad (1)$$

where: c_0 is the initial phenolic concentration in the reaction solution (mg/L), c_e is the phenolic concentration in the reaction solution after adsorption or un-adsorbed phenolics (mg/L), V_m is the total volume of a reaction solution (L), γ_a is the β-glucan concentration (g/L), and V_a is the volume of added β-glucan in a reaction solution (L). The modeling of the adsorption process with adsorption isotherm equations requires the data from adsorption at varying mass ratios of total phenolics from aronia to β-glucan. That is why the adsorption was performed using five various volumes of aronia extract aliquots in the range of 50 to 150 mL (mass ratio of total phenolics from aronia/β-glucan ranged from 4/1 to 9/1 mg/mg). Adsorption experiment was performed once with each of the two prepared aronia extracts, and un-adsorbed phenolics were determined two times (n=4).

RP-HPLC analysis

Aronia phenolics before and after adsorption were analyzed by using an 1260 Infinity II HPLC system (Agilent Technology, Santa Clara, CA, USA) with a quaternary pump, a photodiode array (PDA) detector, a vialsampler, a Poroshell 120 EC C-18 column (4.6×100 mm, 2.7 mm) with a Poroshell 120 EC-C18 4.6 mm guard-column. Phenolic compounds were separated by using 0.5% (*v/v*) H_3PO_4 (mobile phase A), and 100% acetonitrile (mobile phase B) with the following gradient: 5% B 0 min, 11% B 5 min, 15% B 7.5 min, 17.5% B 17.5 min, 20% B 20 min, 30% B 30 min, 70% B 32 min, 70% B 34 min, 5% B 36 min, 5% B 38 min. The flow rate was set to 0.8 mL/min. Stock solutions of anthocyanin standards were prepared in 0.1% HCl in methanol. Flavonol and phenolic acid stock solutions were prepared in 100% methanol. Five dilutions of standards (10 to 100 mg/L) were analyzed to plot calibration curves (r^2 0.9939 to 0.9995), which were used to quantify phenolics. Limits of detection (LOD) and limits of quantification (LOQ) were as follows: cyanidin 3-glucoside (0.24 and 0.74 mg/L), cyanidin 3-galactoside (0.22 and 0.68 mg/L), chlorogenic acid (0.95 and 2.88 mg/L), quercetin 3-glucoside (0.25 and 0.74 mg/L), quercetin 3-rutinoside (0.32 and 0.97 mg/L), and quercetin 3-galactoside (0.10 and 0.32 mg/L). Precision expressed as the coefficient of variation was between 3.3 and 18.8%. Identification was done by comparing UV-Vis spectrum and retention times of phenolic standards with those of peaks on the chromatogram of the aronia extract. Additionally, extracts were spiked with phenolic standards to confirm the identification. Neochlorogenic acid, cyandin 3-arabinoside, and cyanidin 3-xyloside were tentatively identified with the help of literature data [Sosnowska *et al.,* 2018] and quantified by using the calibration curve of chlorogenic acid and cyanidin 3-glucoside. The content of individual phenolics was expressed in mg per kg of aronia fresh weight (FW).

Adsorption isotherms

Experimental data q_e and c_e were modeled with Langmuir, Dubinin-Radushkevich, and Hill isotherm equations explained further in the text:

$$q_e = \frac{q_m K_L c_e}{1+K_L c_e} \text{ (Langmuir isotherm)} \quad (2)$$

$$q_e = q_s exp(-\beta\varepsilon^2) \text{ (Dubinin-Radushkevich isotherm)} \quad (3)$$

$$q_e = \frac{q_m c_e^{n_H}}{K_D + c_e^{n_H}} \text{ (Hill isotherm)} \quad (4)$$

The improved non-linear regression was used for modeling. The code for improved non-linear regression was written in the R programming language by our group and used for data modeling in our recent papers [Jakobek *et al.,* 2020a,b].

Statistical analysis

The contents of phenolics in aronia were based on two parallel extracts, each measured twice (n=4). Adsorption experiment was done once with each of the two aronia extracts. Un-adsorbed phenolics after the adsorption were measured twice (n=4), and two mean values of un-adsorbed phenolics were created. Modeling of the adsorption data mean values (q_e and c_e) was conducted by using the R program. The standard error (*SE*) was calculated:

$$SE = \sqrt{\frac{\left(\sum_{i=1}^{n}\left(q_{e,measured} - q_{e,model}\right)^2\right)}{(n-a)}} \quad (5)$$

where: $q_{e,measured}$ and $q_{e,model}$ are the measured q_e and the q_e calculated by the model, respectively, n is the total number of data points, and a is the number of parameters of the model. Principal component analysis, analysis of variance, and post-hoc Tukey test were used to find differences between the results using Minitab (Minitab LLC., State College, PA, USA).

RESULTS AND DISCUSSION

Phenolic profile of aronia

Eight phenolic compounds were separated and tentatively identified in the aronia extracts (Table 1, Figure 1). Four of them were anthocyanins (cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, and cyanidin 3-xyloside), three were flavonols (quercetin 3-rutinoside, quercetin 3-galactoside, and quercetin 3-glucoside), and one was phenolic acid (neochlorogenic acid). Anthocyanins showed their maxima in the UV-Vis spectrum at 280 and 516 to 518 nm, which is typical of anthocyanins. Three flavonols showed their maxima in the UV-Vis spectrum at 254–266 nm and 354–356 nm, a typical spectrum of flavonols. Neochlorogenic acid had an UV-Vis maximum at 320 nm. The UV-Vis spectra agree with those of authentic standards and with those from literature [Sosnowska *et al.,* 2018]. Anthocyanins, flavonols, and phenolic acids were present in the total contents of 5905, 1707, and 550 mg/kg FW (Table 1), which are similar to literature data [Denev *et al.,* 2019].

Adsorption

The adsorption was conducted first with 50 mL of aronia extract, and the adsorption capacities of individual phenolics ($q_{e,measured}$) are shown in Figure 2. Cyanidin 3-galactoside and cyanidin 3-arabinoside adsorbed in significantly higher amounts than cyanidin 3-xyloside and cyanidin 3-glucoside

TABLE 1. Contents of individual phenolics in aronia and maxima of UV-Vis spectra (λ_{max}) of identified compounds.

Phenolics	Content (mg/kg FW)	λ_{max} (nm)
Anthocyanins		
Cyanidin 3-galactoside	3525±470[a]	280, 516
Cyanidin 3-glucoside	210±0[c]	280, 516
Cyanidin 3-arabinoside*	1838±261[b]	280, 516
Cyanidin 3-xyloside*	332±19[c]	280, 518
Total	5905±750	
Flavonols		
Quercetin 3-rutinoside	878±148[c]	266, 356
Quercetin 3-galactoside	473±84[c]	254, 354
Quercetin 3-glucoside	356±60[c]	254, 356
Total	1707±292	
Phenolic acids		
Neochlorogenic acid*	550±75[c]	320
TOTAL	8162±1117	

Analysis of variance showed that not all means (n=4) were equal (p=0.000). Tukey pairwise comparisons were done at a significance level of 0.05. Means that do not share a letter are significantly different. * tentatively identified. FW-fresh weight.

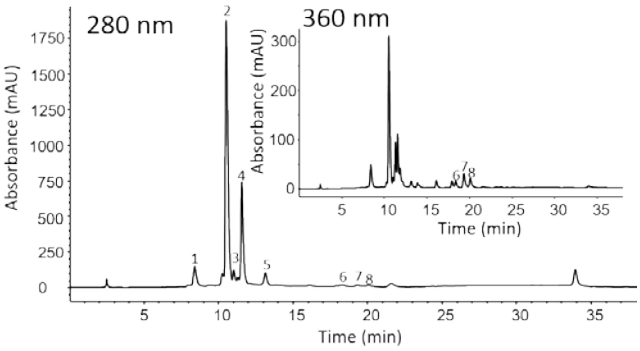


FIGURE 1. The chromatogram of aronia extract scaned at 280 and 360 nm with identified phenolics. Peak assignment 1* – neochlorogenic acid, 2 – cyanidin 3-galactoside, 3 – cyanidin 3-glucoside, 4* – cyanidin 3-arabinoside, 5* – cyanidin 3-xyloside, 6 – quercetin 3-rutinoside, 7 – quercetin 3-galactoside, 8 – quercetin 3-glucoside (* – tentatively identified).

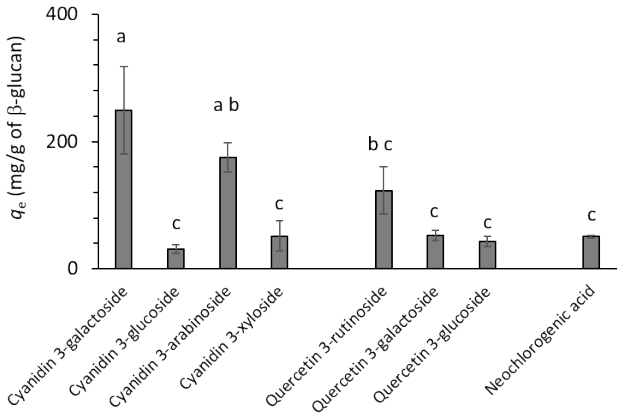


FIGURE 2. The adsorption capacity of individual aronia phenolics onto β-glucan, q_e (mass ratio of total aronia phenolics to β-glucan – 4/1) at pH 1.5. Analysis of variance showed that not all means were equal (p = 0.001). Tukey pairwise comparisons were done at a significance level of 0.05. Means that do not share a letter are significantly different.

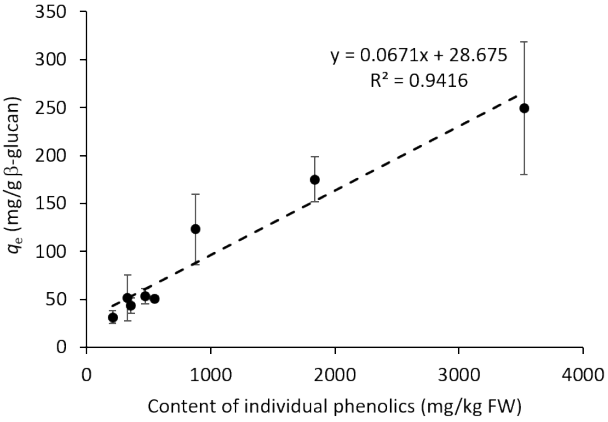


FIGURE 3. The correlation between the adsorption capacities of individual phenolics, q_e (mg/g β-glucan), and their content in the aronia extract (mg/kg of fresh weight (FW)). The data for q_e were obtained in the adsorption experiment with mass ratio of total aronia phenolics to β-glucan – 4/1, at pH 1.5.

(250, 175, 51, and 31 mg/g, respectively). In the flavonol subgroup, quercetin 3-rutinoside showed a higher adsorption capacity in comparison to galactoside and glucoside of quercetin (123, 53 and 44 mg/g, respectively) but those differences were not statistically significant. Neochlorogenic acid adsorbed in the amount 51 mg/g. The adsorption capacities of phenolics agree with literature data. Namely, phenolics extracted from tea adsorbed onto β-glucan from 0.7 to 40 mg/g, and a mixture of pure standards of phenolics from tea from 156 to 405 mg/g [Gao *et al.,* 2012]. At different pH values, tea phenolic compounds were adsorbed by β-glucan up to 116 mg/g [Wu *et al.,* 2011a]. Phenolic compounds adsorb onto cellulose too. Cyanidin 3-glucoside, ferulic acid, and catechin adsorbed onto cellulose up to 215, 56, and 102 mg/g, respectively [Phan *et al.,* 2016]. Various phenolics (cyanidin 3-glucoside, catechin, ferulic acid, chlorogenic acid, and gallic acid) adsorbed onto cellulose up to 600 mg/g [Phan *et al.,* 2015]. Similar amounts of phenolics were adsorbed by cell wall material (cyanidin 3-glucoside, ferulic acid, and catechin around 600, 150, and 400 mg/g, respectively [Phan *et al.,* 2017]). Furthermore, individual phenolics adsorbed onto β-glucan in higher amounts if they were present in the extract in a higher content, which is visible in the diagram $q_{e,measured}$ *vs* individual phenolics content (mg/kg FW) (Figure 3). The correlation was high (r^2=0.94). Accordingly, it can be suggested that the adsorption was concentration dependent. Phenolic compounds present in higher concentrations in the environment around β-glucan (cyanidin 3-galactoside and cyanidin 3-arabonoside) were

adsorbed by β-glucan in higher amounts, and those present in lower concentrations (cyanidin 3-xyloside, cyanidin 3-glucoside, flavonols, and neochlorogenic acid) were adsorbed in lower amounts. This agrees with the study of Phan *et al.* [2015] who investigated the adsorption of various phenolics onto cellulose and suggested that the adsorption capacity depended on the available phenolic molecules in the aqueous solution.

Adsorption isotherms

The adsorption experiment was conducted with several different initial concentrations of the aronia extract, which enabled determining more $q_{e,measured}$ for each phenolic compound. Those values were modelled in the q_e *vs* c_e diagrams with equations of Langmuir, Dubinin-Radushkevich, and Hill isotherms. The novel, improved non-linear regression was used for modeling. The improved modelling can be explained by first understanding how $q_{e,measured}$ values behave in q_e *vs* c_e diagram. If there are multiple values of $q_{e,measured}$ for one initial phenolic concentration, those multiple $q_{e,measured}$ values will lie on a diagonal line in the q_e *vs* c_e diagram. This is due to the mass balance equation (Eq. 1) as explained in our previous paper [Jakobek *et al.,* 2020b]. With a naive use of traditional non-linear regression, the $q_{e,model}$ is fitted to $q_{e,measured}$ to minimize the squares of vertical error, associated with a presumption of a model value of c_e matching its measured value. In contrast, for the improved non-linear modelling, the novelty is that the $q_{e,model}$ is fitted together with its corresponding c_e on the diagonal line, where the $q_{e,measured}$ values are positioned with their corresponding $c_{e,measured}$. This is the proper statistical modeling that recognizes that both q_e and c_e are measured values in response to the initial amounts with exact negative correlation in their measurements due to the mass balance. We used this improved way of non-linear regression in our recent paper [Jakobek *et al.,* 2020a]. An example of data modelled with the improved regression is shown in Figure 4. In the modelling of $q_{e,measured}$ data for cyanidin 3-arabinsoide, an improved non-linear regression gave a predicted $q_{e,model}$ (special curve point) on a diagonal line where $q_{e,measured}$ data are positioned (Figure 4). It can be seen that the improved modeling differs from that of customary modeling that fits the data in a standard way on the vertical line. Namely, the improved modeling gave lower standard errors (SE) in comparison to standard modelling. Indeed, the improved non-linear regression modeled the data in a better way, with lower SE. Lower SE allowed us to suggest that the improved non-linear regression ensures better prediction of adsorption isotherm parameters and, therefore, a more precise description of the adsorption process.

Langmuir isotherm

The Langmuir isotherm describes a monolayer adsorption onto the surface of an adsorbent. The sites on the adsorbent where the adsorption takes place are limited, identical and energetically independent [Limousin *et al.,* 2007]. The Langmuir equation is described by already mentioned equation (Eq. 2).

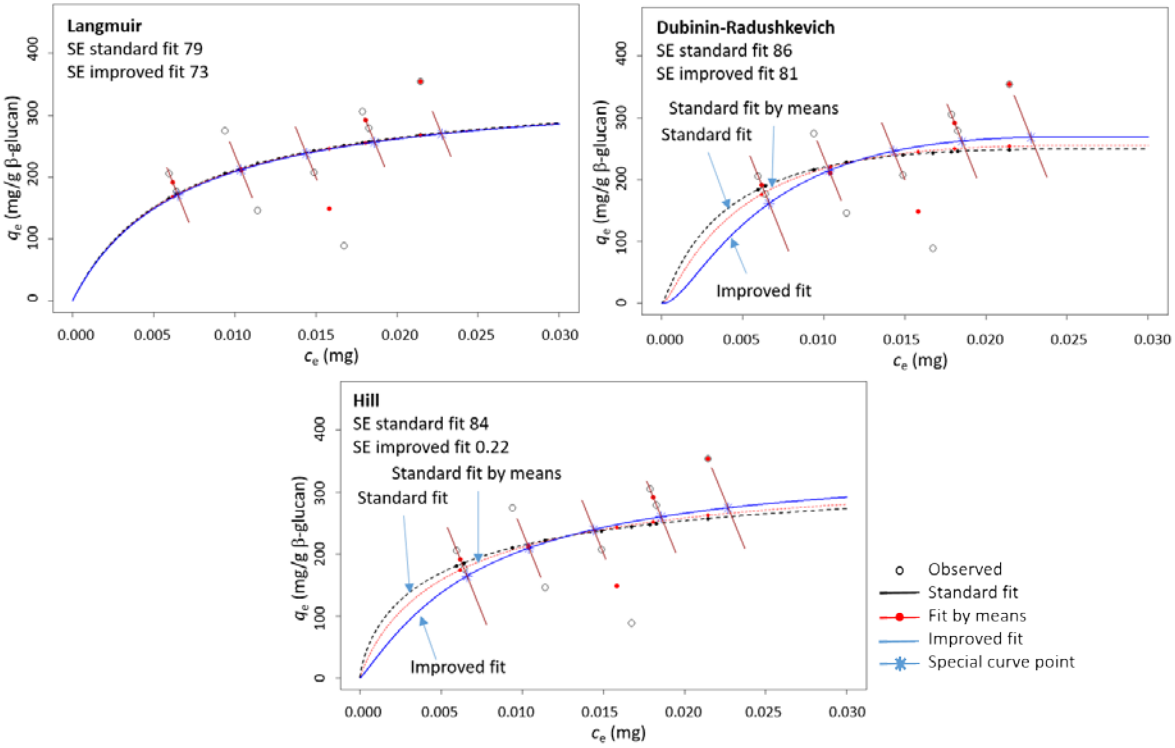


FIGURE 4. Modeling of adsorption capacity, q_e (mg/g of β-glucan), *vs* un-adsorbed amount of cyanidin 3-arabinoside, c_e (mg), by using the novel non-linear regression in R programing language. Curves were obtained for standard fit of measured values (black curve), standard fit of measured mean values (red curve), and a novel improved fit (blue curve). SE; standard error.

TABLE 2. Parameters of Langmuir, Dubinin-Radushkevich, and Hill adsorption isotherms obtained by the improved non-linear modeling of adsorbed phenolics from aronia onto β-glucan.

Phenolics	Langmuir			Dubinin-Radushkevich				Hill			
	q_m (mg/g)	K_L (1/mg)	SE	q_s (mg/g)	E (J/mol)	c_s (mg)	SE	q_m (mg/g)	n_H	K (mg)	SE
Anthocyanins											
Cyanidin 3-galactoside	356	193	105.0	309	8760	0.0600	104.0	554	0.10	0.0052	5.2
Cyanidin 3-glucoside	100	979	13.7	79	1816	0.0025	14.9	100	1.89	0.0012	0.1
Cyanidin 3-arabinoside	350	147	73.1	269	3248	0.0250	81.2	350	1.14	0.0073	0.2
Cyanidin 3-xyloside	238	353	33.4	190	4889	0.0190	2.7	614	0.66	0.0252	2.6
Flavonols											
Quercetin 3-rutinoside	200	181	31.8	232	2313	0.0250	2.3	200	2.73	0.0073	0.2
Quercetin 3-galactoside	80	263	12.9	99	3049	0.0260	2.4	80	2.02	0.0047	0.2
Quercetin 3-glucoside	50	509	10.3	40	2086	0.0070	11.3	50	1.78	0.0029	0.2
Phenolic acids											
Neochlorogenic acid	98	157	15.6	98	1107	0.0165	1.9	80	6.68	0.0088	0.1

SE – standard error; q_m is the Langmuir apparent maximum adsorption capacity of β-glucan (mg/g of β-glucan); K_L is the Langmuir equilibration constant of adsorption (1/mg); q_s is the theoretical saturation capacity or the maximum adsorption capacity of β-glucan (mg/g of β-glucan); E is the adsorption mean free energy (J/mol); c_s is theoretical saturation concentration or solubility (mg) for the given volume of solution; q_m is the Hill apparent maximum adsorption capacity (mg/g of β-glucan), n_H is the Hill cooperativity coefficient; and K is the Hill constant (mg).

$$q_e = \frac{q_m K_L c_e}{1+K_L c_e} \quad (2)$$

where: c_e is the phenolic amount in the reaction solution at equilibrium (un-adsorbed phenolics) (mg); q_e is the amount of phenolic adsorbed per g of β-glucan at equilibrium (mg/g); K_L is the Langmuir equilibration constant of adsorption (1/mg); and q_m is the apparent maximum adsorption capacity of β-glucan (mg/g) [Soto *et al.,* 2011]. All q_e and c_e for all phenolics were modeled with the Langmuir equation using the improved non-linear modelling. This enabled determining the parameters q_m and K_L (Table 2). The q_m represents the theoretical maximum adsorption capacity of a compound predicted with the model. The predicted q_m of two anthocyanins, cyanidin 3-galactoside, and cyanidin 3-arabinoside were higher than that of cyanidin 3-xyloside and cyandin 3-glucoside (356, 350, 238, and 100 mg/g, respectively). In the flavonol subgroup, quercetin 3-rutinoside showed a higher predicted q_m than galactoside and glucoside of quercetin (200, 80, and 50 mg/g, respectively). Neochlorogenic acid had a predicted q_m 98 mg/g. The predicted q_m values are in accordance with $q_{e,measured}$. To visualize this, Figure 5 shows the correlation between the predicted, theoretical q_m, and $q_{e,measured}$. A correlation was found between the measured values and those predicted by a model with the coefficient of determination (r^2) of 0.76.

Dubinin-Radushkevich isotherm

This isotherm describes an adsorption that takes place on a heterogeneous surface of an adsorbent, and involves a pore filling mechanism [Al-Ghouti & Da'ana, 2020]. It allows distinguishing between bonds involved in the adsorption (chemical or physical adsorption). The equation has already been mentioned as Eq. 3 [Hu & Zhang, 2019]:

$$q_e = q_s exp(-\beta\varepsilon^2) \quad (3)$$

The equation for the Polany potential (ε) is:

$$\varepsilon = RTln\left(\frac{c_S}{c_e}\right) \quad (6)$$

By inclusion of this ε in Equation 3, the Dubinin-Radushkevich equation becomes:

$$q_e = q_s exp\left(-\beta R^2 T^2 (\ln\left(\frac{c_S}{c_e}\right))^2\right) \quad (7)$$

where: q_s is the theoretical saturation capacity or the maximum adsorption capacity of β-glucan (mg/g); β is a constant related to the adsorption energy (mol^2/J^2); ε is the Polany potential (J/mol); R is the gas constant (8.314 J/mol K); T is the temperature (K); and c_s is theoretical saturation concentration or solubility (mg) for the given volume of solution [Foo & Hameed, 2010; Hu & Zhang, 2019]. The experimental data (q_e and c_e) were modeled using Equation 7 with the improved non-linear regression, and q_s, β, and c_s were determined. The parameter β was used to calculate the adsorption mean free energy E (J/mol) using Equation 8 [Foo & Hameed, 2010]:

$$E = \frac{1}{\sqrt{2\beta}} \quad (8)$$

Finally, q_s, E and c_s predicted with the model were reported (Table 2). The q_s that represents the theoretical saturation capacity of β-glucan showed the highest value for cyanidin 3-galactoside and cyanidin 3-arabionside, followed by cyanidin 3-xyloside and cyanidin 3-glucoside (309, 269, 190, and 79 mg/g, respectively). In the flavonol subgroup, the highest q_s was determined for quercetin 3-rutinoside, followed by quercetin 3-galactoside and quercetin 3-glucoside (232, 99, and 40 mg/g, respectively). Neochlorogenic acid had q_s of 98 mg/g. The q_s values predicted by the model followed the $q_{e,measured}$ (Figure 2). Namely, the compound with a higher $q_{e,measured}$ also had a higher fitted q_s and *vice versa*. This can be better seen in a diagram that shows q_s *vs* $q_{e,measured}$ (Figure 5). A high correlation was found between q_s and $q_{e,measured}$ (r^2=0.81), which indicates an agreement between the measured values and the predicted ones. Furthermore, the parameter E predicted by the model, which represents an apparent mean free energy of adsorption, was lower than 8,000 J/mol for most of the phenolics. This indicates a physical adsorption (physisorption) with the creation of non-covalent bonds like H bonds and Van der Waals forces, and agrees with earlier studies that suggested the physical adsorption between β-glucan and tea phenolics [Wu *et al.,* 2011a,b], tannic acid and β-glucan [Li *et al.,* 2019], phenolics and cellulose [Phan *et al.,* 2016] and procyanidins and cell wall materials [Le Bourvellec *et al.,* 2004]. In our study, only cyanidin 3-galactoside had an E value higher than 8,000 J/mol.

Hill isotherm

The Hill isotherm describes an adsorption of different species onto a homogenous adsorbent [Al-Ghouti & Da'ana, 2020], and allows suggesting whether the adsorption is cooperative or non-cooperative [Al-Ghouti & Da'ana, 2020]. The already mentioned Hill isotherm equation (Eq. 4) is:

$$q_e = \frac{q_m c_e^{n_H}}{K_D + c_e^{n_H}} \quad (4)$$

where: q_m is the apparent maximum adsorption capacity (mg/g), n_H is the Hill cooperativity coefficient; and K_D is the Hill constant (mg^{nH})) [Al-Ghouti & Da'ana, 2020]. In fitting a Hill model, we find it to be numerically more stable to represent the Hill constant in the form $K_D = K^{nH}$. Expressed in this form K is the half-max point (mg), corresponding to the value of c_e at which the Hill isotherm equation reaches half of its maximum value. From a stable fit to K we can then find K_D from K^{nH} using the fitted cooperativity coefficient n_H. All $q_{e,measured}$ values were modeled with Hill equation, and parameters of the model were determined (q_m, n_H and K) (Table 2). The parameter q_m which represents the predicted, theoretical maximum adsorption capacity was highest for cyanidin 3-xyloside, cyanidin 3-galactoside, and cyanidin 3-arabinoside (614, 554, and 350 mg/g respectively), followed by cyanidin 3-glucoside (100 mg/g), in the anthocyanin subgroup. In the flavonol subgroup, it was the highest for quercetin 3-rutinoside, followed by quercetin 3-galactoside and quercetin 3-glucoside (200, 80, and 50 mg/g, respectively). Neochlorogenic acid had a q_m of 80 mg/g. The correlation between predicted q_m and $q_{e,measured}$ was not as high (r^2=0.34) as for Langmuir and Dubinin-Radushkevich (Figure 5). Hill's parameter n_H describes a cooperative or non-cooperative bonding. When $n_H > 1$, the bonding could be a positively cooperative bonding. In this case, when a molecule bonds to an adsorbent, other molecules can bond more easily. If $n_H < 1$, the bonding is a negatively cooperative bonding (already bonded molecule makes the bonding of other molecules more difficult). If $n_H = 1$, the bonding is non-cooperative, where bonding is independent of the molecules already bound to the adsorbent [Al-Ghouti & Da'ana, 2020]. The bonding could be positively cooperated ($n_H > 1$ for cyanidin 3-glucoside, cyanidin 3-arabinoside; rutinoside, galactoside and glucoside of quercetin; neochlorogenic acid) but also negatively cooperated ($n_H < 1$ for cyanidin 3-galactoside, cyanidin 3-xyloside).

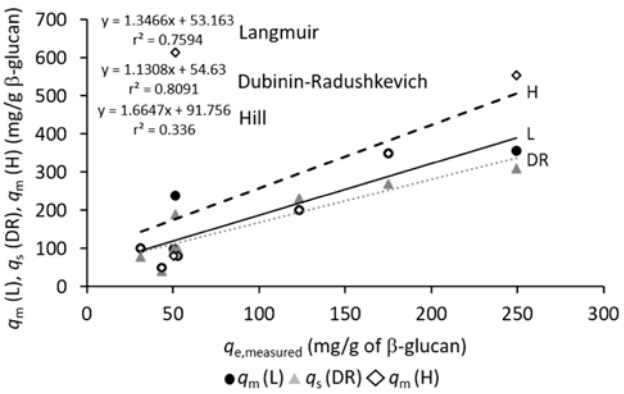


FIGURE 5. The correlation between predicted q_m from Langmuir (L), q_m from Hill (H), q_s from Dubinin-Radushkevich (DR), and $q_{e,measured}$ (mg/g of β-glucan). q_m is the Langmuir and Hill apparent maximum adsorption capacity of β-glucan (mg/g of β-glucan), q_s represents the Dubinin-Radushkevich theoretical saturation capacity or the maximum adsorption capacity of β-glucan (mg/g of β-glucan), $q_{e,measured}$ represents measured value of adsorption capacity (mg/g of β-glucan).

Principal component analysis (PCA)

The results obtained were subjected to PCA. The adsorption capacity ($q_{e,measured}$), the amount of phenolics in aronia, n_H and E, were chosen for PCA since they are important for the description of the adsorption process (Figure 6a). The following clustering of phenolic compounds according to their affiliation to a phenolic group can be suggested: anthocyanins, flavonols, and phenolic acids. Additionally, while performing PCA analysis, it became visible that n_H and E could be parameters that might have contributed to that difference and affect the clustering. Indeed, the scatterplot of n_H *vs* E (Figure 6b) shows that phenolic compounds are different according to n_H and E, and could be clustered according to their affiliation into a phenolic subgroup: flavonols, anthocyanins, and phenolic acids. Anthocyanins showed lower n_H and higher E than flavonols and phenolic acids. In the anthocyanin subgroup, as n_H goes to lower values, from positive toward negative cooperation in bonding, E becomes higher. Flavonols and phenolic acids have higher n_H for positive cooperation and lower E.

This behavior can be attributed to the chemical structure of phenolics. The aglycon in anthocyanin molecules is cyanidin, to which different glycosides are attached at

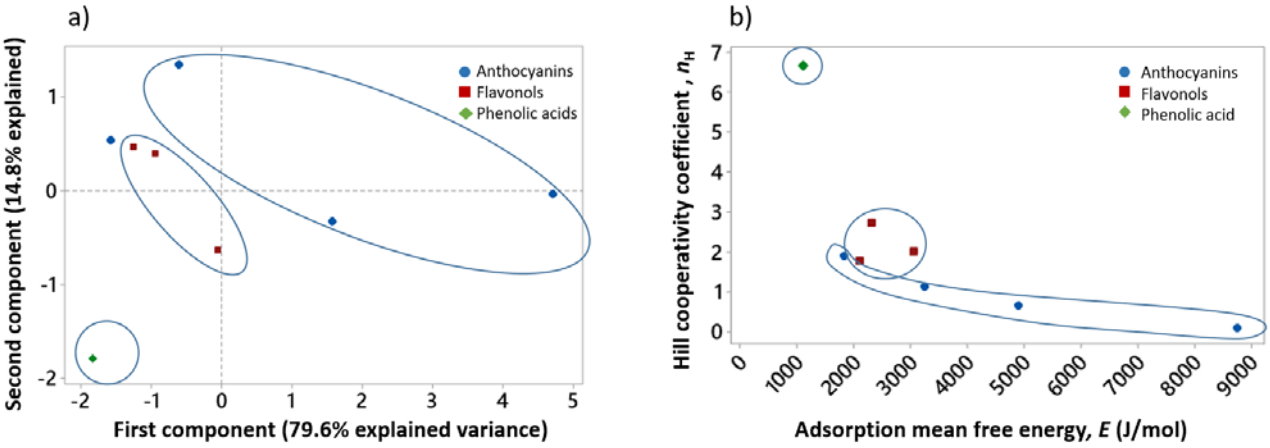


FIGURE 6. a) Principal component analysis of the measured value of adsorption capacity, $q_{e,measured}$ (mg/g β-glucan), content of individual phenolics in the extract (mg/kg fresh weight), Hill cooperativity coefficient, n_H and the Dubinin-Radushkevich adsorption mean energy, E (J/mol). b) Scaterplott of n_H and E (J/mol).

the C3 position of the C ring. At lower pH, as in our study (pH 1.5), cyanidin aglycon is present in the form of a flavylium cation which has an ionic nature. It has a positive charge at the oxygen atom of the C ring. The aglycon in quercetin derivatives is quercetin to which different glycosides are attached at the C3 position of the C ring. Quercetin does not have a charged atom. Furthermore, phenolic acid (neochlorogenic acid) is an acid and it changes the structure according to the pH value of the environment. At low pH (pH 1.5), the neochlorogenic acid is in a protonated, nonionic form [Uranga *et al.,* 2016], similar to flavonols. The bonding of anthocyanins onto β-glucan might be positively and then negatively cooperative, possibly due to the charged molecules. Namely, anthocyanin molecules can bond onto β-glucan, and once bonded (positive cooperation) they can repulse other molecules, which leads to more difficult bonding (a negative cooperation). And the adsorption mean free energy becomes higher. Flavonols and neochlorogenic acid are not charged at low pH, and thus a cooperative bonding with higher n_H values could be suggested.

CONCLUSION

The adsorption data can be modeled with a novel non-linear regression and this new approach gave lower SE than the standard non-linear modeling, with more precisely determined isotherm parameters. The experiments and modeling allowed us to suggest a description of the adsorption process. The bonds created between aronia phenolics and β-glucan could involve both H bonds and Van der Waals forces. H bonds could be created between OH groups of phenolics and β-glucan. Once H bonds are created, phenolics and β-glucan are closer, and Van der Waals attractive forces might additionally connect those molecules, as it was reported for the adsorption of tea phenolics onto β-glucan [Wu *et al.,* 2011a,b]. Furthermore, at pH 1.5 as in our study, the OH groups of all phenolics and β-glucan are protonated (nonionic), which is a prerequisite for the formation of H bonds [Li *et al.,* 2019]. This fact also supports the formation of H bonds.

Phenolics from aronia have different chemical structures at pH 1.5. Those structural differences might have affected the adsorption. Namely, flavonols and neochlorogenic acid are in nonionic forms in comparison to ionic anthocyanins. After the adsorption of all these phenolics, ionic anthocyanins might have caused some repulsions, which hindered further bonding.

Since the adsorption was studied at low pH (1.5), it might be suggested that aronia phenolics can interact with β-glucan at the low pH of the digestive tract. That behavior might be important for carrying phenolics through the digestive tract by β-glucan. These findings need to be supported by additional studies of adsorption at different pH values and using *in vitro* simulated digestion processes. Furthermore, β-glucan has already been studied as a delivery system for single strain DNA [Hwang *et al.,* 2018] or for doxorubicin molecules [Lee *et al.,* 2019]. That is why, it might be suggested that it has the potential to serve as a natural delivery system for aronia phenolics.

RESEARCH FUNDING

This work has been fully supported by Croatian Science Foundation under the project HRZZ-IP-2016-06-6777 and project HRZZ-IP-2016-06-6545. The work of J.I. was financed by European social fund, Operational Program 2014–2020, aim 10.II.3.

CONFLICT OF INTERESTS

The authors declare no potential conflicts of interests.

ORCID IDs

A.R. Barron https://orcid.org/0000-0002-8072-7351
J. Ištuk https://orcid.org/0000-0002-0675-5784
L. Jakobek https://orcid.org/0000-0002-4846-327X
P. Matić https://orcid.org/0000-0003-0804-1280

REFERENCES

1. Al-Ghouti, M.A., Da'ana, D.A. (2020). Guidelines for the use and interpretation of adsorption isotherm models: A review. *Journal of Hazardous Materials*, *393*, art. no. 122383. https://doi.org/10.1016/j.jhazmat.2020.122383
2. De Smet, R., Demoor, T., Verschuere, S., Dullaers, M., Ostroff, G.R., Leclerq, G., Allais, L., Pilette, C., Dierendonck, M., De Geest, B.G., Cuvelier, C.A. (2013). β-glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. *Journal of Controlled Release*, *172(3),* 671–678. https://doi.org/10.1016/j.jconrel.2013.09.007
3. Denev, P., Čiž, M., Kratchanova, M., Blazheva, D. (2019). Black chokeberry (*Aronia melanocarpa*) polyphenols reveal different antioxidant, antimicrobial and neutrophil-modulating activities. *Food Chemistry*, *284*, 108–117. https://doi.org/10.1016/j.foodchem.2019.01.108
4. Foo, K.Y., Hameed, B.H. (2010). Insight into the modeling of adsorption isotherm systems. *Chemical Engineering Journal*, *156(1),* 2–10. https://doi.org/10.1016/j.cej.2009.09.013
5. Gao, R., Liu, H., Peng, Z., Wu, Z., Wang, Y., Zhao, G. (2012). Adsorption of (-)-epigallocatechin-3-gallate (EGCG) onto oat β-glucan. *Food Chemistry, 132*(4), 1936–1943. https://doi.org/10.1016/j.foodchem.2011.12.029
6. Hellström, J.K., Shikov, A.N., Makarova, M.N., Pihlanto, A.M., Pozharitskaya, O.N., Ryhänen, EL., Kivijärvi, P., Makarov, V.G., Mattila, P.H. (2010). Blood pressure-lowering properties of chokeberry (*Aronia mitchurinii*, var. Viking). *Journal of Functional Foods*, *2*(2), 163–169. https://doi.org/10.1016/j.jff.2010.04.004
7. Hu, Q., Zhang, Z. (2019). Application of Dubinin-Radushkevich isotherm model at the solid/solution interface: A theoretical analysis. *Journal of Molecular Liquids,* 277, 646–648. https://doi.org/10.1016/j.molliq.2019.01.005
8. Hwang, J., Lee, K., Gilad, A.A., Choi, J. (2018). Synthesis of beta-glucan nanoparticles for the delivery of single strand DNA. *Biotechnology and Bioprocess Engineering, 23,* 144–149. https://doi.org/10.1007/s12257-018-0003-4
9. 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
10. Jakobek, L., Buljeta, I., Ištuk, J., Barron, A.R. (2020a). Polyphenols of traditional apple varieties in interaction with barley β-glucan: a study of the adsorption process. *Foods, 9(9),* art. no. 1278. https://doi.org/10.3390/foods9091278
11. Jakobek, L., Matić, P, Kraljević, Š., Ukić, Š., Benšić, M., Barron, A.R. (2020b). Adsorption between quercetin derivatives and β-glucan studied with a novel approach to modeling adsorption isotherms. *Applied Science*, *10*(5), art. no. 1637. https://doi.org/10.3390/app10051637
12. Kim, B., Ku, C.S., Pham, T.X., Park, Y., Martin, D.A., Xie, L., Taheri, R., Lee, J., Bolling, B.W. (2013). *Aronia melanocarpa* (chokeberry) polyphenol–rich extract improves antioxidant function and reduces total plasma cholesterol in apolipoprotein E knockout mice. *Nutrition Research*, *33*(5), 406–413. https://doi.org/10.1016/j.nutres.2013.03.001
13. Le Bourvellec, C., Guyot, S., Renard, C.M.G.C. (2004). Non-covalent interaction between procyanidins and apple cell wall material Part I. Effect of some environmental parameters. *Biochimica et Biophysica Acta*, *1672*(3), 192–202. https://doi.org/10.1016/j.bbagen.2004.04.001
14. Le Bourvellec, C., Renard, C.M.G.C. (2005). Non-covalent interaction between procyanidins and apple cell wall material. Part II: Quantification and impact of cell wall drying. *Biochimica et Biophysica Acta*, *1725*(1), 1–9. https://doi.org/10.1016/j.bbagen.2005.06.003
15. Lee, K., Kwong, Y., Hwang, J., Choi, Y., Kim, K., Koo, HJ., Seo, Y., Jeon, H., Choi, J. (2019). Synthesis and functionalization of β-glucan particles for the effective delivery of doxorubicin molecules. *ACS Omega, 4*(1), 668–674. https://doi.org/10.1021/acsomega.8b02712
16. Li, R., Zeng, Z., Fu, G., Wan, Y., Liu, X., McClemens, D.J. (2019). Formation and characterization of tannic acid/beta-glucan complexes: influence of pH, ionic strength, and temperature. *Food Research International, 120*, 748–755. https://doi.org/10.1016/j.foodres.2018.11.034
17. Li, X., Cheung, P.C.K. (2019). Application of natural β-glucans as biocompatible functional nanomaterials. *Food Science and Human Wellness, 8*(4), 315–319. https://doi.org/10.1016/j.fshw.2019.11.005
18. Limousin, G., Gaudet, J.P., Charlet, L., Szenknect, S., Barthès, V., Krimissa, M. (2007). Sorption isotherms: A review on physical bases, modeling and measurement. *Applied Geochemistry, 22*(2), 249–275. https://doi.org/10.1016/j.apgeochem.2006.09.010
19. Liu, C., Ge, S., Yang, J., Xu, Y., Zhao, M., Xiong, L., Sun, Q. (2016). Adsorption mechanism of polyphenols onto starch nanoparticles and enhanced antioxidant activity under adverse conditions. *Journal of Functional Foods, 26,* 632–644. https://doi.org/10.1016/j.jff.2016.08.036
20. Liu, D., Martinez-Sanz, M., Lopez-Sanchez, P., Gilbert, E.P., Gidley, M. J. (2017). Adsorption behavior of polyphenols on cellulose is affected by processing history. *Food Hydrocolloids*, *63*, 496–507. https://doi.org/10.1016/j.foodhyd.2016.09.012
21. Mäkelä, N., Brinck, O., Sontag-Strohm, T. (2020). Viscosity of β-glucan from oat products at the intestinal phase of the gastrointestinal model. *Food Hydrocolloids, 100,* art. no. 105422. https://doi.org/10.1016/j.foodhyd.2019.105422
22. Palafox-Carlos, H., Ayala-Zavala, J.F., González-Aguilar, G.A. (2011). The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *Journal of Food Science, 76*(1), R6–R15. https://doi.org/10.1111/j.1750-3841.2010.01957.x
23. Phan, A.D.T., D'Arcy, B.R., Gidley, M.J. (2016). Polyphenol-cellulose interactions: Effects of pH, temperature and salt. *International Journal of Food Science and Technology, 51(1)*, 203–211. https://doi.org/10.1111/ijfs.13009
24. Phan, A.D.T., Flanagan, B.M., D'Arcy, B.R., Gidley, M.J. (2017). Binding selectivity of dietary polyphenols to different plant cell wall components: Quantification and mechanism. *Food Chemistry, 233*, 216–227. https://doi.org/10.1016/j.foodchem.2017.04.115
25. Phan, A.D.T., Netzel, G., Wang, D., Flanagan, B.M., D'Arcy, B.R., Gidley, M.J. (2015). Binding of dietary polyphenols to cellulose: Structural and nutritional aspects. *Food Chemistry 171*, 388–396. https://doi.org/10.1016/j.foodchem.2014.08.118

26. Renard, C.M.G.C., Watrelot, A.A., Le Bourvellec, C. (2017). Interactions between polyphenols and polysaccharides: mechanism and consequences in food processing and digestion. *Trends in Food Science and Technology*, *60*, 43–51. https://doi.org/10.1016/j.tifs.2016.10.022
27. Saura-Calixto., F. (2011). Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. *Journal of Agricultural and Food Chemistry*, *59*(1), 43–49. https://doi.org/10.1021/jf1036596
28. Sidor, A., Drożdżyńska, A., Gramza-Michałowska, A. (2019). Black chokeberry (*Aronia melanocarpa*) and its products as potential health-promoting factors – An overview. *Trends in Food Science and Technology, 89*, 45–60. https://doi.org/10.1016/j.tifs.2019.05.006
29. Sosnowska, D., Podsędek, A., Redzynia, M., Kucharska, A.Z. (2018). Inhibitory effect of black chokeberry fruit polyphenols on pancreatic lipase –Searching for most active inhibitors. *Journal of Functional Foods, 49*, 196–204. https://doi.org/10.1016/j.jff.2018.08.029
30. Soto, M.L., Moure, A., Domíniguez, H., Parajó, J.C. (2011). Recovery, concentration and purification of phenolic compounds by adsorption. A review. *Journal of Food Engineering 105*(1), 1–27. https://doi.org/10.1016/j.jfoodeng.2011.02.010
31. Tang, HY., Fang, Z., Ng, K. (2020). Dietary fiber-based colon-targeted delivery systems for polyphenols. *Trends in Food Science and Technology, 100*, 333–348. https://doi.org/10.1016/j.tifs.2020.04.028
32. Uranga, J.G., Podio, N.S., Wunderlin, D.A., Santiago A.N. (2016). Theoretical and experimental study of the antioxidant behaviors of 5-*O*-caffeoylquinic, quinic and caffeic acids based on electronic and structural properties. *Chemistry Select, 1*, 4113–4120. https://doi.org/10.1002/slct.201600582
33. Wu, Z., Li, H., Ming, J., Zhao, G. (2011a). Optimization of adsorption of tea polyphenols into oat β-glucan using response surface methodology. *Journal of Agricultural and Food Chemistry*, *59*(1), 378–385. https://doi.org/10.1021/jf103003q
34. Wu, Z., Ming, J., Gao, R., Wang, Y., Liang, Q., Yu, H., Zhao, G. (2011b). Characterization and antioxidant activity of the complex of tea polyphenols and oat β-glucan. *Journal of Agricultural and Food Chemistry, 59*(19), 10737–10746. https://doi.org/10.1021/jf202722w

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 197–210
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/137612
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Optimization of Distillation Conditions for Improved Recovery of Phthalides from Celery (*Apium graveolens* L.) Seeds

Adam Kokotkiewicz[1,*], *Anna Badura*[2], *Żaneta Tabaczyńska*[2], *Andżelika Lorenc*[2], *Adam Buciński*[2], *Maria Luczkiewicz*[1]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Gdańsk, Hallera 107, 80–416 Gdańsk, Poland*
[2]*Department of Biopharmacy, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Jurasza 2, 85–089 Bydgoszcz, Poland*

Key words: alkylphthalides, enzyme pretreatment, hydrodistillation, salting-out effect, simultaneous distillation-extraction, size reduction

The essential oil of celery (*Apium graveolens*) is characterized by exceptionally high content of alkylphthalides. The mentioned compounds exhibit a number of biological effects (including hypotensive, lipid-lowering, neuroprotective, and cytotoxic) and are also responsible for distinctive aroma of the plant. In the current work, parameters of conventional hydrodistillation (HD) and simultaneous distillation-extraction (SDE) were optimized to obtain phthalide-enriched fractions of celery seeds. A positive correlation was shown between hydrodistillation time and improved essential oil and phthalide yields. The 6-h HD of comminuted seeds yielded essential oil (2.9%) with a higher total phthalide content (51%), as compared to the samples collected after 1.5–3.0 h, which gave 2.4–2.7% of oil containing 24.6–39.2% of total phthalides. The oil contained sedanenolide (36.7%), 3-*n*-butylphthalide (13.1%), and sedanolide (1.1%). A further increase in the total phthalide content was achieved by omitting the size reduction step prior to hydrodistillation (68.8%) and utilization of the salting-out effect (84.3%). Enzyme pretreatment had a negligible effect on essential oil and phthalide yields. The change of distillation mode from HD to SDE significantly increased the oil yield of whole seeds (from 2.8 to 5.8% for 6 h processing) while maintaining its high phthalide content (62.5%), which translated to an increase in the total phthalide yield from 19.4 to 36.0 g/kg.

INTRODUCTION

Celery (*Apium graveolens* L., Apiaceae) is a popular vegetable and aromatic medicinal plant. Its different varieties provide petioles, roots, leaves, and fruits (which are utilized as foods, spices, and medicinal raw materials [Salehi *et al.*, 2019; Turner *et al.*, 2021]. *A. graveolens* owes its characteristic flavor to the presence of an essential oil, composed of terpenes, sesquiterpenes and phthalides [Kokotkiewicz & Luczkiewicz, 2016]. Celery fruits (commonly referred to as 'seeds') are the major source of volatile oil (2–3% on average), which finds use in food flavoring, perfumery, and pharmaceutical industries [Kokotkiewicz & Luczkiewicz, 2016; Malhotra, 2006; Sowbhagya, 2014]. The size of celery oil market is substantial (50 tons/year), and large amounts are produced in India, China, Europe, and United States. India is the major producer of both celery seeds and celery oil, with annual production of 4000 and 25 tons, respectively [Sowbhagya, 2014]. Alkylphthalides, represented by 3-*n*-butylphthalide, sedanenolide, and sedanolide, are functionally among the most important constituents of *A. graveolens*, determining its specific spicy aroma [Kurobayashi *et al.*, 2006; Turner *et al.*, 2021] and thus being crucial for flavoring properties of celery. *A. graveolens* oils with high phthalide and low terpene content are preferred in the food industry because of their improved organoleptic properties. A high content of terpenes (up to 80% of limonene) renders celery oil unsuitable for culinary use, and thus high-terpene oils are usually deterpenated [Kokotkiewicz & Luczkiewicz, 2016]. Besides being flavoring agents, phthalides were shown to exhibit a plethora of biological activities [Sowbhagya & Srinivas, 2013]. For instance, 3-*n*-butylphthalide is well-known for its neuroprotective properties; it is currently used for the treatment of ischemic stroke and is also a promising therapeutic agent in Alzheimer's disease, vascular dementia, Parkinson's disease, and amyotrophic lateral sclerosis [Huang *et al.*, 2018; Salehi *et al.*, 2019]. Sedanenolide (aka senkyunolide A) was demonstrated to possess antiatherogenic [Lei *et al.*, 2019], neuroprotective [Gong *et al.*, 2018], and cancer-preventive activities [Liu *et al.*, 2018]. Another phthalide constituent of celery, sedanolide, showed a chemopreventive potential in mice [Zheng *et al.*, 1993] and human liver cancer cells [Hsieh *et al.*, 2015]. Apart from that, phthalide constituents of celery were shown to exhibit

* Corresponding Author:
E-mail: adamkokot@gumed.edu.pl (A. Kokotkiewicz)

Submitted: 4 February 2021
Accepted: 18 May 2021
Published on-line: 7 June 2021

antioxidant, larvicidal, nematicidal, and fungicidal activities [Rożek *et al.*, 2016].

Taking into account the flavoring and therapeutic properties of the above compounds, there is a good rationale to develop phthalide-enriched celery products for the use in nutraceutical and functional food industries [Sowbhagya, 2014]. Studies have shown that factors such as seed source, hydrodistillation time, and sample pretreatment affect essential oil yield and composition; however, these experiments were either preliminary [Jain *et al.*, 2003] or did not focus specifically on improving the phthalide content of the product [Dąbrowska *et al.*, 2020; El-Beltagi *et al.*, 2020; Sowbhagya *et al.*, 2007, 2010; Zorga *et al.*, 2020]. Sowbhagya & Srinivas [2013] demonstrated that both fractional distillation and liquid-liquid partitioning of celery essential oil can be used to obtain fractions enriched with phthalides but this approach requires an additional step following volatile oil hydrodistillation. Other approaches, based on salting-out effects and simultaneous distillation-extraction, have so far not been exploited, though data indicate that they could be useful for the isolation of phthalides from plant materials [Bartsch & Hammerschmidt, 1993; Chaintreau, 2001].

Given the available data, we assumed that distillation conditions can be optimized to maximize the recovery of phthalides from celery seeds. The aim of the present study was to develop a simple, efficient method for the isolation of a phthalide-enriched fraction from celery seeds. With this in mind, a series of experiments was designed to evaluate the influence of such factors as seed comminution, extraction mode (standard hydrodistillation *vs*. simultaneous hydrodistillation-extraction), process time, salting-out, and enzyme pretreatment, on the essential oil yield and phthalide content. The presented work is the first comprehensive report concerning the isolation of celery oil with an improved phthalide content.

MATERIALS AND METHODS

Plant material

The fruits of celery (*Apium graveolens* L.) were obtained in 2017 from Just Ingredients, Riverside Court, Beaufort Park, Chepstow, UK (country of origin: India; harvest date: 2017; batch number: LE12530/17; expiry date: 06.2019). The identity of the material was confirmed by the quality certificate provided, as well as by morpho-anatomical and phytochemical analysis conducted at the Department of Pharmacognosy, Medical University of Gdańsk, Poland. The plant material was stored in the dark in a hermetically-sealed container. The experiments described in the study have been conducted within one year since the material was acquired. The distillation products obtained in the course of the study were referred to as 'volatile oil', 'essential oil' or simply 'oil'.

Both whole (WS) and ground seeds (GS) were used in the study. The following sample preparation protocol was applied for seed grinding: 15 g of fruits were weighed, frozen using liquid nitrogen, and immediately ground to 0.2––0.63 mm size (15,000 rpm, 10 s; SM-450 grinder, Envisense, Lublin, Poland). The comminuted material was immediately transferred to a distillation flask and subjected to essential oil isolation as described in 'Essential oil isolation' section.

The moisture content of seeds was determined by distillation: The 7.5-g sample of seeds was ground as described above, transferred to 1000-mL round flask, to which 200 mL of chloroform were added. The material was then subjected to 3-h distillation using a Clevenger apparatus (see 'Hydrodistillation (HD) using Clevenger apparatus' section for details). The heating (EMEA3 stirred electromantle, Cole-Parmer, Stone, UK; 750 rpm) was adjusted to provide a condensate rate of 2 mL/min. The moisture content of celery seeds was 8.44±0.2% (*v/w*).

Reagents

Type 1 water was obtained using the Elix/Synergy system (Millipore, Billerica, US-MA). The reagents and solvents of analytical grade were from POCH, Gliwice, Poland (chloroform, disodium diphosphate dihydrate); Sigma-Aldrich, St. Louis, US-MO (acetic acid, anhydrous sodium sulfate, almond β-glucosidase, citric acid, LC-MS grade methanol, sodium chloride); Merck, Darmstadt, Germany (dichloromethane); and Fluka, Buchs, Switzerland (fungal pectinase). The reference compounds were obtained from Santa Cruz Biotechnology, Dallas, US-TX (3-*n*-butylphthalide, sedanolide, both ≥98%) and Chengdu Biopurify Phytochemicals, Chengdu, China (sedanenolide, 98%).

Essential oil isolation

Hydrodistillation (HD) using Clevenger apparatus

For essential oil isolation, a 15-g sample of celery seeds (whole or ground, as described in the '*Plant material*' section) was placed into a 1000-mL round flask; then 400 mL of water were added, and the flask was connected to the Clevenger apparatus (Carl-Roth, Karlsruhe, Germany; apparatus specification according to European Pharmacopoeia as presented by Bicchi [2000]). The mixture was brought to boiling (approx. 100°C), and heating was adjusted to provide a condensate rate of 2 mL/min (EMEA3 stirred electromantle, 750 rpm). The hydrodistillation was run for 1.5, 3.0, 4.5, and 6.0 h, and each experiment was repeated at least in triplicate. When the process had been completed, the apparatus was left to cool down for 30 min. The volatile oil content was determined volumetrically and expressed as % (*v/w*). The essential oil was collected, mixed with 100 mg of anhydrous sodium sulfate, and stored in a sealed vial at 8°C for 24 h. The dehydrated sample was moved to a clean vial and stored at 8°C. Prior to the HPLC analysis, the essential oil samples were diluted (1:1000, *v/v*) using LC-MS grade methanol.

Simultaneous distillation-extraction (SDE) using Likens--Nickerson apparatus

A 15-g sample of celery seeds (whole or ground, as described in the '*Plant material*' section) was placed in a 1000-mL round flask, then 400 mL of water were added, and the flask was connected to the Likens-Nickerson apparatus (Labit, Stare Babice, Poland; apparatus specification according to original design by Likens and Nickerson, as presented by Chaintreau [2001]). Dichloromethane (50 mL) was used as an extraction solvent, and the extract was collected into a 100-mL heart-shaped flask. The heating (water: EMEA3

stirred electromantle, 750 rpm; dichloromethane: non-stirred electric mantle) was adjusted to provide a condensate rate of 2 mL/min for both H_2O and CH_2Cl_2. The process was run for 1.5, 3.0, 4.5, and 6.0 h, and each experiment was repeated at least in triplicate. After the process had been over, the apparatus was left to cool down for 30 min. Dichloromethane was removed under reduced pressure (R-300 Rotavapor, Büchi, Flawil, Switzerland) according to the distillation program: 0–5 min, 600 mbar; 5–8 min, 400 mbar; 8–9 min, 300 mbar; and 9–10 min, 200 mbar. The volatile oil content was determined gravimetrically. In order to compare SDE results with conventional HD, the obtained values were converted to % (*v/w*) assuming the mean specific gravity of essential oil from celery fruits as 0.89 (according to EOA specification [Sowbhagya *et al.*, 2007]). After the solvent had been removed, 100 mg of anhydrous sodium sulfate was added to the remaining essential oil, and the sample was stored in a sealed flask at 8°C for 24 h. The dehydrated sample was transferred to a clean vial and stored at 8°C. Prior to the HPLC analysis, the essential oil samples were diluted (1:1000, *v/v*) using LC-MS grade methanol.

Evaluation of salting-out effect

In order to evaluate the effects of salting-out on the essential oil yield and phthalide content, a series of experiments analogous to those described in sections '*Hydrodistillation (HD) using Clevenger apparatus*' and '*Simultaneous distillation-extraction (SDE) using Likens-Nickerson apparatus*' were conducted. The experiments were run in the same manner except that sodium chloride (20 g) was added to a distillation flask along with the plant material. The HD and SDE processes employing the salting-out effect were run for 1.5, 3.0, 4.5, and 6.0 h, and each experiment was repeated at least in triplicate.

Enzyme pretreatment

The experiments were conducted with ground seeds only. To this end, 15 g of seeds were placed in a 1000-mL distillation flask, to which 400 mL of 0.15 M phosphate-citrate buffer (7.32 g disodium phosphate dihydrate + 3.73 g citric acid + 400 mL water, pH=5.0) and 2.0 g of pectinase (0.5%, *w/v*) or 0.1 g of β-glucosidase (0.025%, *w/v*) were added. The flask with the mixture was placed on a magnetic stirrer (750 rpm), incubated in the dark at 37°C for 24 h, and subsequently the mixture was subjected to hydrodistillation using the Clevenger apparatus (as described in the '*Hydrodistillation (HD) using Clevenger apparatus*' section). The processes were run for 3.0 and 6.0 h, and each experiment was repeated in triplicate.

LC-DAD-MS (ESI$^+$) analyses

The HPLC system used consisted of SIL-20AC autosampler (15°C), CTO-20AC column oven (30°C), two LC-20AD solvent pumps, DGU-20A3 degasser, SPD-M20A diode array detector (DAD), and 2010EV mass spectrometric (MS) detector (all components from Shimadzu, Kyoto, Japan). Separations were performed on Supelcosil LC-18 column (150× 4.6 mm, 3 μm particle size, Sigma–Aldrich) at a flow rate of 0.3 mL/min. The injection volume was 10 μL. The mobile phase consisted of: A, 0.25% (*v/v*) acetic acid; and B, methanol. The gradient program was as follows: 0–6 min, 32% B; 6–72 min, 32–85% B; 72–86 min, 85–100% B; 86–104 min, 100% B; 104–110 min, 100–32% B; 110–114 min, 32% B; 114 min, stop. LC–DAD data were recorded over the range of 190–800 nm. Mass spectrometric detection was performed in a positive ion mode over the range of *m/z* 100–800 using the scan acquisition mode. The following parameters of electrospray ionisation were applied: desolvation temperature, 250°C; heating block temperature, 270°C; capillary voltage, 2000 V; nebulizing gas flow rate, 1.5 L/min; drying gas flow rate, 15.0 L/min. The data were processed with the use of LC–MS solution 3.40 software (Shimadzu).

Phthalide constituents of essential oil samples were identified based on co-chromatography with reference substances and analysis of the recorded LC-DAD and LC-MS spectra. The DAD was employed for quantitative analyses: sedanenolide and 3-*n*-butylphthalide were quantified using the calibration curve of 3-*n*-butylphthalide based on the peak area at 280 nm, and sedanolide was quantified using the sedanolide calibration curve based on the peak area at 210 nm.

Statistical analysis

Statistical analysis was performed with PQStat 1.8.0 software (PQStat Software, Poznań, Poland). The data were presented as means ± standard deviations. In the case of normal data distribution and homogeneity of variance, the one-way ANOVA followed by Fisher LSD post-hoc test, was applied. Otherwise, the Kruskal-Wallis test followed by Dunn post-hoc test, was used.

RESULTS AND DISCUSSION

The effect of distillation mode, process time, and seed comminution on the essential oil yield and phthalide content

In the first part of the study, essential oil samples of ground celery seeds were obtained by conventional hydrodistillation using a standardized Clevenger-type apparatus recommended by European Pharmacopoeia [Bicchi, 2000]. Previous studies have indicated that both the degree of seed comminution and distillation time affect the celery oil yield and composition [Jain *et al.*, 2003; Sowbhagya *et al.*, 2007]; however, the so far conducted experiments did not focus specifically on improving the extraction efficiency of *A. graveolens* volatiles. The work by Jain *et al.* [2003] can be considered as preliminary since it did not include process optimization and detailed analysis of phthalide fraction. In turn, Sowbhagya *et al.* [2007] demonstrated that both the amount of volatile oil and its phthalide content increased in time during hydrodistillation. Nevertheless, the applied distillation times (0.5–2.0 h) were relatively short considering low volatility of phthalides in steam [Ludwiczuk *et al.*, 2001; Mukhopadhyay, 2000]. Given the above, it was decided to extend the time of hydrodistillation to 6 h, which was substantially longer than process times reported by Sowbhagya *et al.* [2007]. The samples were collected after 1.5, 3.0, 4.5, and 6.0 h, and results of essential oil yield determination were presented in Figure 1A.

The experiment proved that majority of the oil was isolated during the first 1.5 h of the process, which is in agreement with previous findings [Sowbhagya *et al.*, 2007]. Nevertheless, continued distillation resulted in a *ca*. 20% statistically significant increase in the essential oil yield. The amounts obtained after 1.5 h and 6.0 h were 2.44 and 2.94% (*v/w*), respectively. More importantly, the extended hydrodistillation time resulted in the substantial enrichment of a phthalide fraction, which confirmed our assumption that effective isolation of compounds of this group requires longer processing of the plant material. As shown in Figure 2A, C, E, G, there was a significant ($p<0.05$) increase in contents of individual compounds and total phthalides between 1.5 and 6.0 h of the process. During that time, sedanenolide content increased 2.3-fold (from 15.7 to 36.7%), 3-*n*-butylphthalide content increased 1.6-fold (from 8.3 to 13.1%), and the percentage share of sedanolide increased 1.8-fold (from 0.6 to 1.1%). Overall, there was a *ca*. 2-fold increase in the total phthalide content (from 24.6 to 51.0%, Figure 2G), which translated to a 2.5-fold increase in the total phthalide yield (from 6.0 to 15.0 g/kg, Figure 2H). Thus, it was demonstrated that phthalide-enriched oil of celery seeds can be obtained merely by extending the time of hydrodistillation beyond the 2.0–3.0 h employed in previous studies [Sowbhagya *et al.*, 2007, 2010]. Nevertheless, it has to be noted that the current study did not examine the effects of distillation time on the contents of other essential oil constituents present in celery (*i.e.*, terpenes and sesquiterpenes). Prolonged heating has been shown to affect the composition of volatile fractions of several aromatic plants [Venditti, 2020]. In the case of celery, thermal processing was shown to affect its flavor; however, the reported changes were attributed to the formation of thermally-generated compounds, but not to changes in the phthalide fraction [Kokotkiewicz & Luczkiewicz, 2016; Kurobayashi *et al.,* 2006]. Thus, further studies involving GC analysis and sensory evaluation are necessary to examine the effects of prolonged distillation on the properties of celery oil.

Further steps in the study involved determination of the effects of seed comminution on the essential oil yield and phthalide content. The research by Sowbhagya *et al.* [2007] demonstrated that employing seed flaking instead of full grinding resulted in an improved essential oil yield and faster release of phthalide constituents from the plant matrix. The milling of celery seeds also had drawbacks, such as material overheating and clogging. In the current work, we strived to examine whether essential oil can be efficiently isolated from whole celery seeds. From the industrial perspective, omitting the size reduction step would offer substantial benefits, saving both

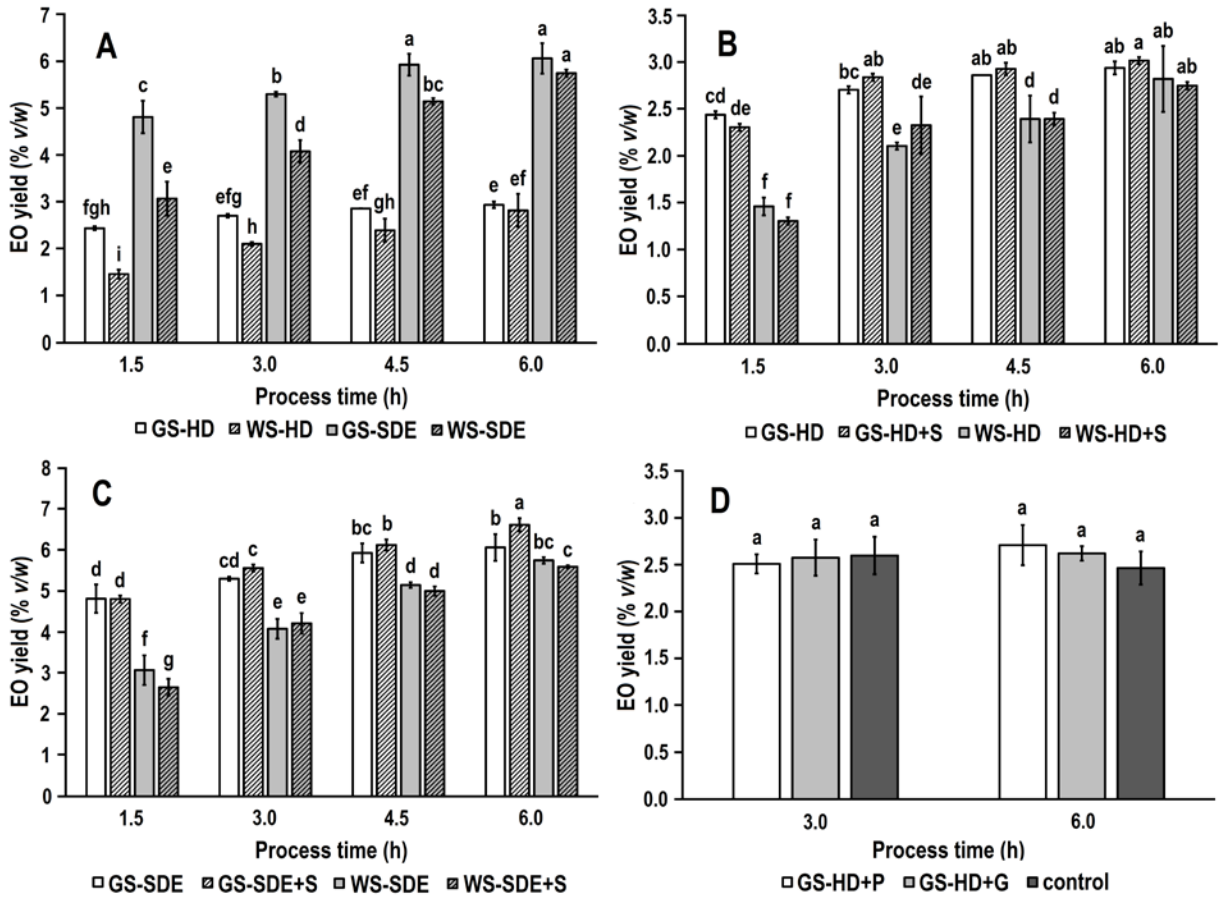


FIGURE 1. The yield of celery seed essential oil (EO), obtained under different distillation conditions: A – comparison of different distillation modes; B – salting-out effects in HD; C – salting-out effects in SDE; D – effect of enzyme pretreatment. Abbreviations: HD, hydrodistillation; HD+S, salt-assisted hydrodistillation; SDE, simultaneous distillation-extraction; SDE+S, salt-assisted distillation-extraction; HD+P, hydrodistillation preceded by pectinase treatment; HD+G, hydrodistillation preceded by β-glucosidase treatment; GS, ground seeds; WS, whole seeds. Values represent means ± standard deviations. Different letters indicate significant difference at $p<0.05$.

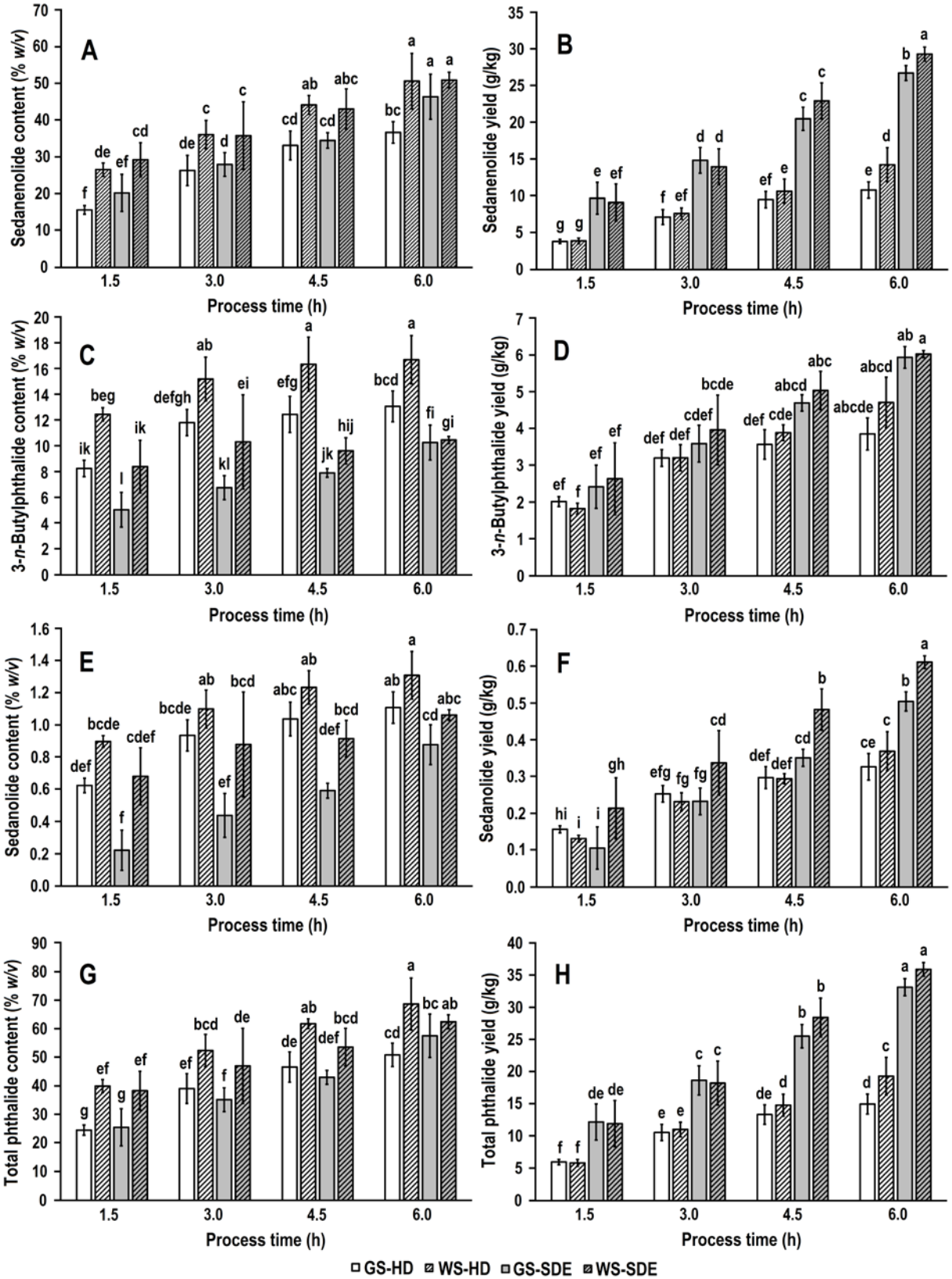


FIGURE 2. The contents and yields of phthalides in essential oil, obtained from whole and ground celery seeds using different modes and times of distillation: A – sedanenolide content; B – sedanenolide yield, C – 3-*n*-butylphthalide content; D – 3-*n*-butylphthalide yield; E – sedanolide content; F – sedanolide yield; G – total phthalide content; H – total phthalide yield. Abbreviations: HD, hydrodistillation; SDE, simultaneous distillation-extraction; GS, ground seeds; WS, whole seeds. Values represent means ± standard deviations. Different letters indicate significant difference at $p<0.05$.

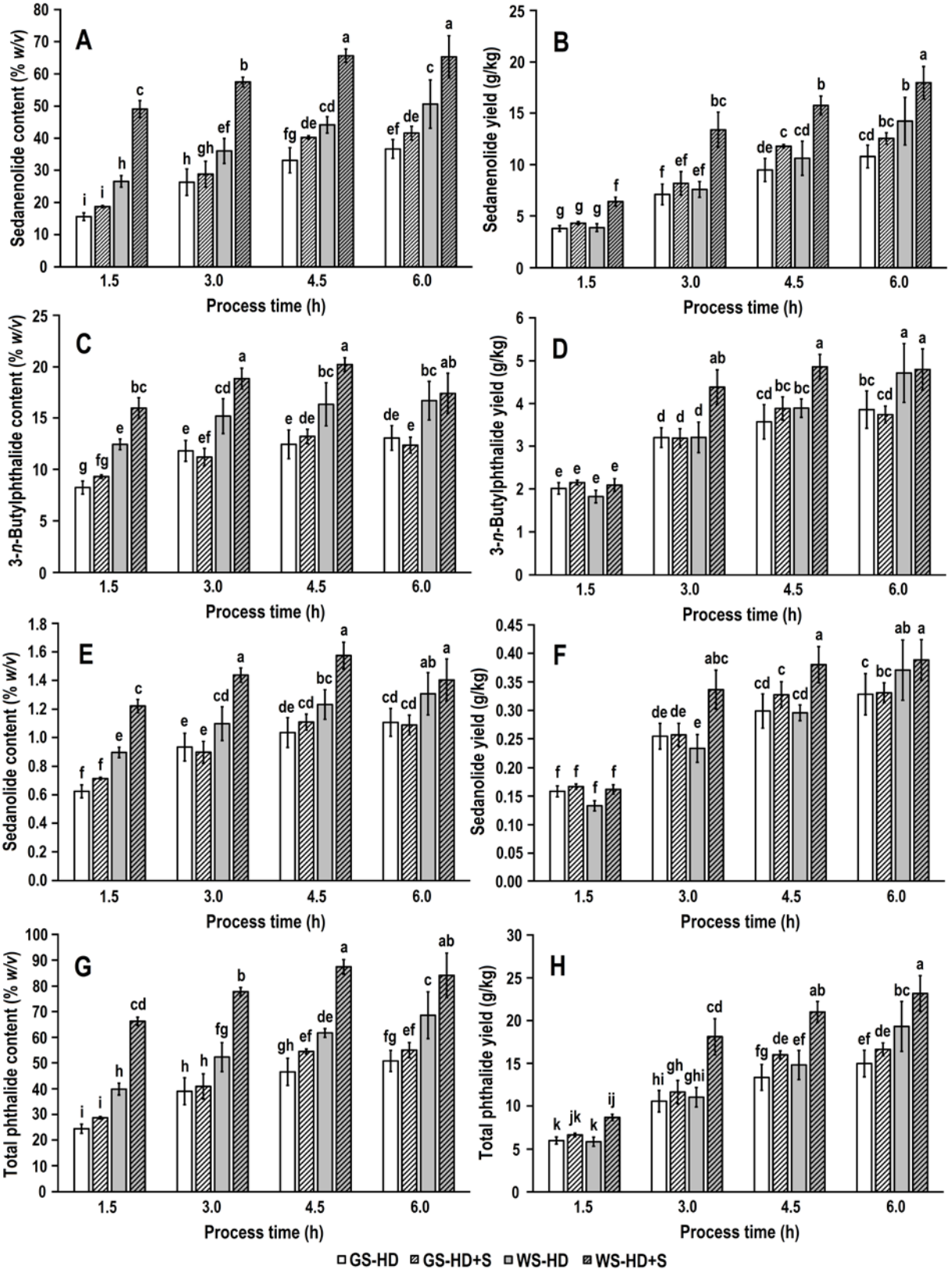


FIGURE 3. The contents and yields of phthalides in essential oil, obtained by salt-assisted hydrodistillation from whole and ground celery seeds: A – sedanenolide content; B – sedanenolide yield, C – 3-*n*-butylphthalide content; D – 3-*n*-butylphthalide yield; E – sedanolide content; F – sedanolide yield; G – total phthalide content; H – total phthalide yield. Abbreviations: HD, hydrodistillation; HD+S, salt-assisted hydrodistillation; GS, ground seeds; WS, whole seeds. Values represent means ± standard deviations. Different letters indicate significant difference at $p<0.05$.

time and energy required to isolate the oil. Celery seeds are small in size (1–2 mm) and thus it was assumed that good penetration of steam vapors through the plant matrix material could be achieved without prior comminution of the material. As presented in Figure 1A, the essential oil was released substantially slower from whole seeds, and the amount obtained after 1.5 h hydrodistillation was roughly 60% of the yield of comminuted seeds. However, the volatile oil yield recorded after 6.0 h (2.83%) was comparable to that from the ground plant material (2.94%). The differences in phthalide content of volatile oil samples obtained from both types of plant material were prominent. Regardless of process time, the amounts of major phthalides (sedanenolide and 3-*n*-butylphthalide), as well as the total phthalide content in essential oil from whole seeds were significantly ($p<0.05$) higher (1.3–1.6-fold increase) as compared to the ground seeds (Figure 2). Sedanolide was a minor phthalide constituent of the oil, and its content was not significantly ($p\geq0.05$) affected by the grinding process. The volatile oil obtained from whole seeds during 6-h hydrodistillation contained *ca*. 50% sedanenolide, 16.5% 3-*n*-butylphthalide, and 1.3% sedanolide (Figure 2A, C, E, G). Also, whole celery seeds provided significantly ($p<0.05$) higher total phthalide yields than the comminuted ones (19 *vs*. 15 g/kg, Figure 2H). In the case of 1.5-h hydrodistillation, both types of plant material provided essentially the same phthalide yields, which was because the lower essential oil yield of whole seeds (Figure 1A) was compensated by a higher content of these compounds in the volatile fraction (Figure 2A, C, E, G). Overall, the experiment proved the feasibility of omitting the size reduction step prior to hydrodistillation of celery seeds without compromising phthalide yield. Also, longer hydrodistillation times result in higher yields of essential oil with a higher phthalide content: during the 6-h HD process, whole celery seeds provided the same amount of oil as the comminuted ones (Figure 1A), but with a higher phthalide content (Figure 2G).

Besides examining the effects of hydrodistillation time and seed comminution on the celery oil yield and phthalide content, the study employed simultaneous distillation-extraction as an alternative mode of volatile oil isolation. In the SDE method, the distillate (consisting of water and essential oil constituents) is continuously flushed with an organic solvent, which extracts essential oil constituents. As compared to conventional hydrodistillation, SDE offers some advantages, including more efficient recovery of relatively polar and high-boiling constituents of essential oils, such as coumarin and β-phenylethyl alcohol [Bartsch & Hammerschmidt, 1993; Chaintreau, 2001]. Similarly to coumarin, alkylphthalides are lactones, which makes them relatively polar components of celery oil. Given the above, it was assumed that SDE could prove effective in obtaining a phthalide-enriched fraction from *A. graveolens* seeds. So far, SDE has only been employed to isolate volatiles from roots and petioles of celery [MacLeod & Ames, 1989; Van Wassenhove *et al.*, 1990], but the kinetics of the process has not been studied. As compared to celery seeds, the above materials contain only small amounts of volatiles and thus are rarely exploited as a source of essential oil.

As presented in Figure 1A, regardless of process time and the type of plant material used, the SDE extraction mode provided roughly a 2-fold increase is the essential oil yield as compared to HD. The release of volatiles from whole seeds was slower, and 6 h distillation was required to obtain the essential oil yield comparable ($p\geq0.05$) to the ground material (6.1 and 5.8% for ground and whole seeds, respectively). As shown in Figure 2A, C, E, G, the method of oil isolation affected phthalide contents of the samples. The contents of 3-*n*-butylphthalide and sedanolide were lower in the SDE samples obtained from both whole and ground seeds. On the contrary, the contents of sedanenolide and total phthalides were mostly unaffected by distillation mode. The exception were 6-h SDE samples obtained from ground seeds, which contained significantly ($p<0.05$) more sedanenolide (46.5%) than hydrodistilled oil (36.8%). The above differences may be due to different polarities of the examined phthalides, as well as the presence of non-phthalide constituents of the oil, which were not quantified in the current work. Similarly to HD experiments, whole celery seeds provided higher phthalide contents of the SDE samples, but the differences between the two types of material were less pronounced. After 6 h, the SDE samples obtained from whole and ground seeds did not differ significantly ($p\geq0.05$) with respect to individual and total phthalide contents (Figure 2A, C, E, G). The productivity of the SDE process, expressed as individual and total phthalide yields, was significantly ($p<0.05$) higher as compared to the conventional hydrodistillation. The highest values were obtained during 6-h processing of non-comminuted seeds (Figure 2B, D, F, H): the SDE procedure yielded 29.3 g/kg of sedanenolide (2-fold increase), 6.0 g/kg of 3-*n*-butylphthalide (1.3-fold increase), 0.61 g/kg of sedanolide (1.65-fold increase), and 36.0 g/kg of total phthalides (1.85-fold increase). The experiment has proved that SDE offers superior performance in terms of essential oil isolation from celery seeds. However, long (6 h) processing times should be applied to maximize volatile oil and phthalide yields. As in the case of conventional hydrodistillation, the size reduction step can be omitted without sacrificing productivity. In fact, seed comminution was shown to negatively affect the phthalide content of celery oil, regardless of the isolation method (HD or SDE). This is probably due to matrix effects, which are clearly different for the processes conducted using ground or non-damaged seeds. As described by Chaintreau [2001], lipid-containing matrices were shown to slow down the release of less volatile constituents during hydrodistillation of the essential oil. Our suggestion is that in the case of using whole celery seeds, the lipids stored inside them were released to a lesser extent than from the damaged matrix (*i.e.*, ground seeds). Consequently, the fats present in the seeds did not interfere with essential oil constituents, thus facilitating recovery of phthalides. The exact location of the volatiles might also play a role: essential oils in Apiaceae fruits can be found in the mesocarp whereas fats are stored deeper in the endosperm, and separated by the layers of testa. Given this, it seems that volatiles can be extracted from cremocarps more easily than fixed oils.

The effect of salting-out on the essential oil yield and phthalide content

Making use of the salting-out effect is simple, yet often effective means of improving productivity of the hydrodistillation process. The addition of salt to the distillation mixture

can increase the concentration of certain volatiles in the vapor phase, resulting in the improved essential oil yield [Naqvi *et al.*, 2002]. Salting-out can be employed in conventional hydrodistillation [Filly *et al.*, 2016; Naqvi *et al.*, 2002; Shamspur *et al.*, 2012] and SDE processes alike [Bartsch & Hammerschmidt, 1993; Chaintreau, 2001]. In particular, the procedure was shown to increase the recoveries of relatively polar constituents of essential oils, such as coumarin and 2-phenyl-ethyl alcohol [Bartsch & Hammerschmidt, 1993; Chaintreau, 2001]. Given the similarities in the structures and physico-chemical properties of coumarin and phthalides, it was decided to examine the effects of sodium chloride on the yield and composition of *A. graveolens* essential oil obtained by hydrodistillation or simultaneous distillation-extraction. So far, the effect of salting-out on the isolation of celery oil has not been extensively studied, and only preliminary experiments have been conducted in this respect [Jain *et al.*, 2003]. According to the cited work, the addition of salt during hydrodistillation of celery seeds failed to increase the essential oil yield and its phthalide content. However, the study can be considered as preliminary because neither standard deviations not statistical analysis of the data was provided [Jain *et al.*, 2003]. Given this, it was decided to investigate the effects of salting-out on the yield and composition of volatile oil, isolated by HD or SDE from whole and ground celery seeds. Sodium chloride was selected for the experiments as the most commonly used salting-out agent, and its amount was based on previous experiments [Filly *et al.*, 2016; Jain *et al.*, 2003; Naqvi *et al.*, 2002; Shamspur *et al.*, 2012].

Figure 1B presents the yield of salt-assisted hydrodistillation of celery oil. Regardless of the type of material used and process time, the addition of salt exerted no significant ($p \geq 0.05$) effect on the essential oil yield. As far as phthalide contents are concerned, salting-out did not significantly ($p \geq 0.05$) affect the composition of volatile fractions isolated from comminuted seeds (Figure 3A, C, E, G). These observations are in agreement with the results of Jain *et al.* [2003] who reported no substantial effect of salt on the yield and phthalide content of essential oil, obtained from ground seeds during 3-h hydrodistillation. However, whole celery seeds subjected to salt-assisted hydrodistillation yielded volatile oil samples with significantly ($p < 0.05$) elevated phthalide contents (Figure 3A, C, E, G). The addition of salt clearly facilitated phthalide release from the non-damaged seeds. The observed effect was the most prominent in the 1.5-h process and decreased with time. For 1.5-h hydrodistillation using whole seeds, the salting-out resulted in a 1.85-fold increase in sedanolide content (from 26.6 to 49.3%) and a 1.7-fold increase in the total phthalide content (from 40.0 to 66.4%), as compared to the process conducted without the use of salt. After 6-h HD, the recorded increase in sedanenolide content was 1.3-fold (from 50.7 to 65.4%), whereas the total phthalide content increased 1.2-fold (from 68.8 to 84.3%, the highest content reported in the study). These values translated to significantly ($p < 0.05$) higher yields of sedanenolide and total phthalides (Figure 3B, H). The contents and yields of 3-*n*-butylphthalide and sedanolide, on the other hand, were affected to a lesser extent (Figure 3C, D, E, F). The different effects of salting-out on the phthalide yield, observed for comminuted and whole seeds, indicate that the matrix effects (previously mentioned in the section '*The effect of distillation mode, process time, and seed comminution on the essential oil yield and phthalide content*') modulate the influence of salt on the release of these constituents from the plant material. It seems that in the case of comminuted seeds, the positive effect of salting-out is diminished by the negative effect of the fixed oils released from the plant matrix.

Similarly to conventional hydrodistillation, the yields of celery oil obtained by SDE were largely unaffected by salting-out (Figure 1C). After 6 h, there was only a small (1.1-fold), yet significant ($p < 0.05$) increase in the yield of essential oil isolated from ground seeds. In terms of individual and total phthalide contents, there were no significant ($p \geq 0.05$) differences between salt-assisted SDE and the reference group (Figure 4A, C, E, G). The yields of individual and total phthalides were also largely unaffected by salting-out. However, for the salt-assisted, 6-h SDE conducted with whole seeds, there was a small, but significant ($p < 0.05$) decrease in individual and total phthalide yields (Figure 4B, D, F, H). Thus, the use of salt offers no benefits during SDE of celery seeds. However, it has to be noted that even without the positive effect of salting-out, SDE of whole seeds still provides substantially higher phthalide yields (36.0 g/kg, Figure 4H) than the salt-assisted HD of the same type of material (23.2 g/kg, Figure 3H).

The effect of enzyme pretreatment on the essential oil yield and phthalide content

Enzyme pretreatment prior to hydrodistillation can increase essential oil yields either by chemically damaging plant tissues and thus facilitating the release of volatiles, or by releasing volatile oil constituents form their glycoside forms. Sowbhagya *et al.* [2010] demonstrated that preincubation with enzymes (including pectinase, cellulase, viscozyme, and protease) prior to steam distillation improved the essential oil yield of celery seeds; however, they did not state whether the reported values differed significantly ($p < 0.05$) from the control group. In the current study, celery seeds were pretreated with pectinase and evaluated for the essential oil yield and phthalide content. Another enzyme employed in the study was β-glucosidase which was used to check the presence of glucosidically-bound phthalides in celery seeds. Phytochemical studies on fresh stalk celery have shown that a small fraction of phthalides is bound as glycosides and thus cannot be isolated by direct hydrodistillation [Tang *et al.*, 1990]. Due to their lactone nature, phthalides can exist in the form of hydroxy acids, which enables them to form chemical bounds with sugars. After the glycoside bond is hydrolyzed, the aglycone undergoes cyclization and forms the respective phthalide [Tang *et al.*, 1990]. Since celery seeds have so far not been investigated for the presence of phthalide glucosides, it was decided to conduct hydrodistillation experiments using the material pretreated with β-glucosidase. The enzyme concentrations and experimental protocols employed in the current work were based on previous reports [Sgorbini *et al.*, 2015; Sowbhagya *et al.*, 2009; Tang *et al.*, 1990].

Figure 1D presents the yield of celery seed oil, obtained from the enzyme-pretreated material during 3 or 6 h

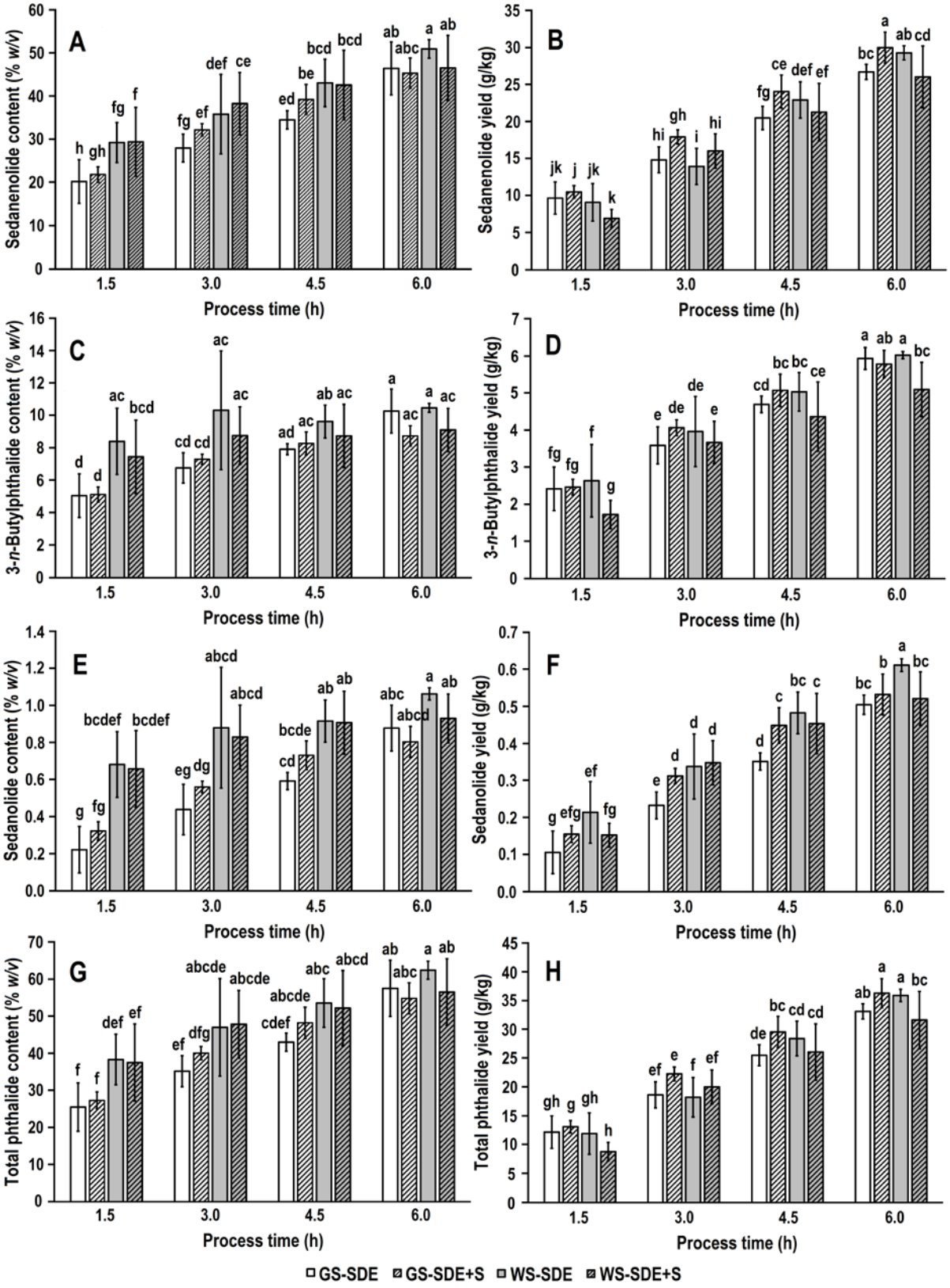


FIGURE 4. The contents and yields of phthalides in essential oil, obtained by salt-assisted distillation-extraction from whole and ground celery seeds: A – sedanenolide content; B – sedanenolide yield; C – 3-*n*-butylphthalide content; D – 3-*n*-butylphthalide yield; E – sedanolide content; F – sedanolide yield; G – total phthalide content; H – total phthalide yield. Abbreviations: SDE, simultaneous distillation-extraction; SDE+S, salt-assisted distillation-extraction; GS, ground seeds; WS, whole seeds. Values represent means ± standard deviations. Different letters indicate significant difference at $p<0.05$.

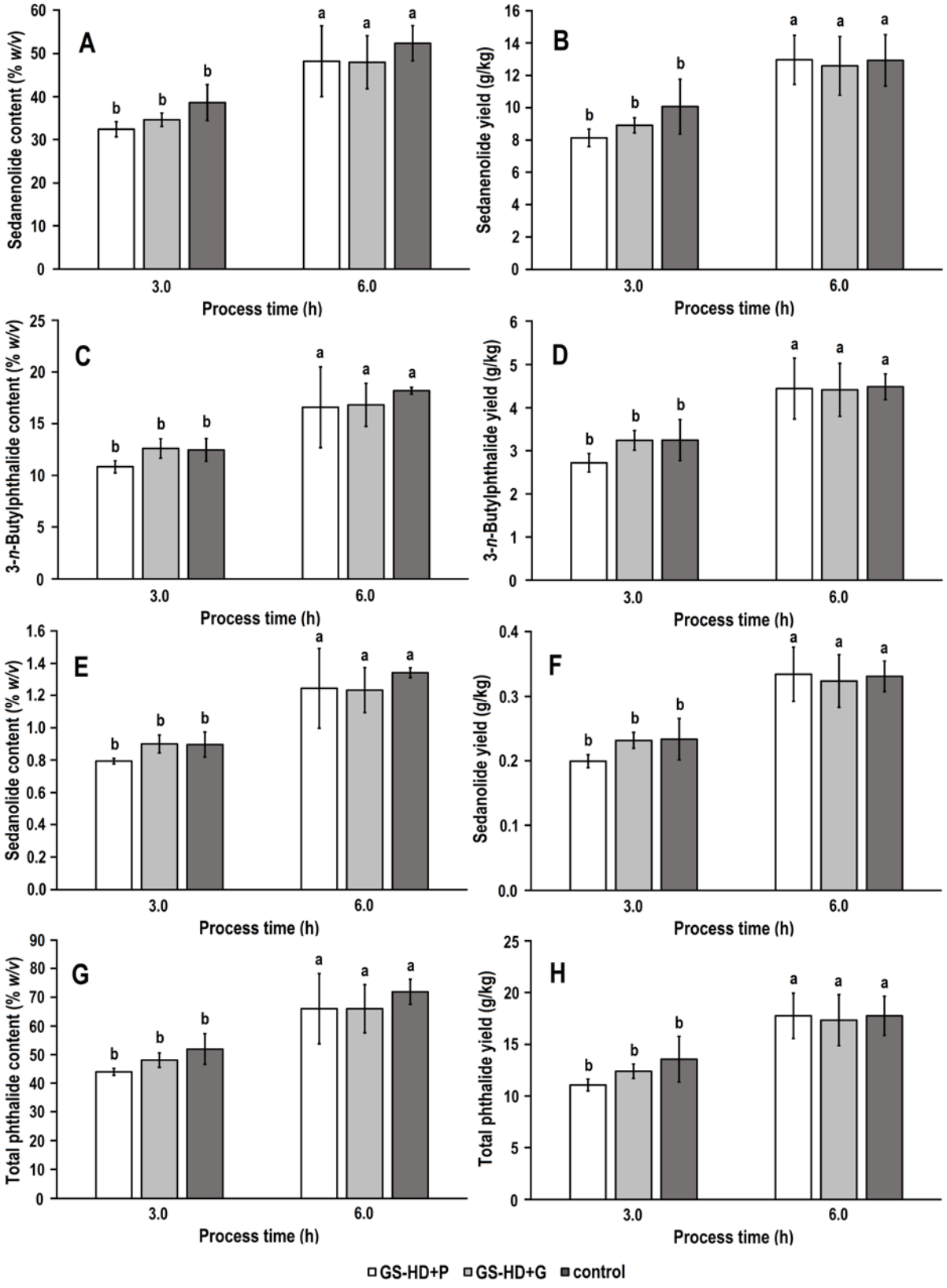


FIGURE 5. The contents and yields of phthalides in essential oil, obtained by hydrodistillation from enzyme-pretreated celery seeds: A – sedanenolide content; B – sedanenolide yield, C – 3-*n*-butylphthalide content; D – 3-*n*-butylphthalide yield; E – sedanolide content; F – sedanolide yield; G – total phthalide content; H – total phthalide yield. Abbreviations: HD, hydrodistillation; HD+P, hydrodistillation preceded by pectinase treatment; HD+G, hydrodistillation preceded by β-glucosidase treatment; GS, ground seeds; WS, whole seeds. Values represent means ± standard deviations. Different letters indicate significant difference at $p<0.05$.

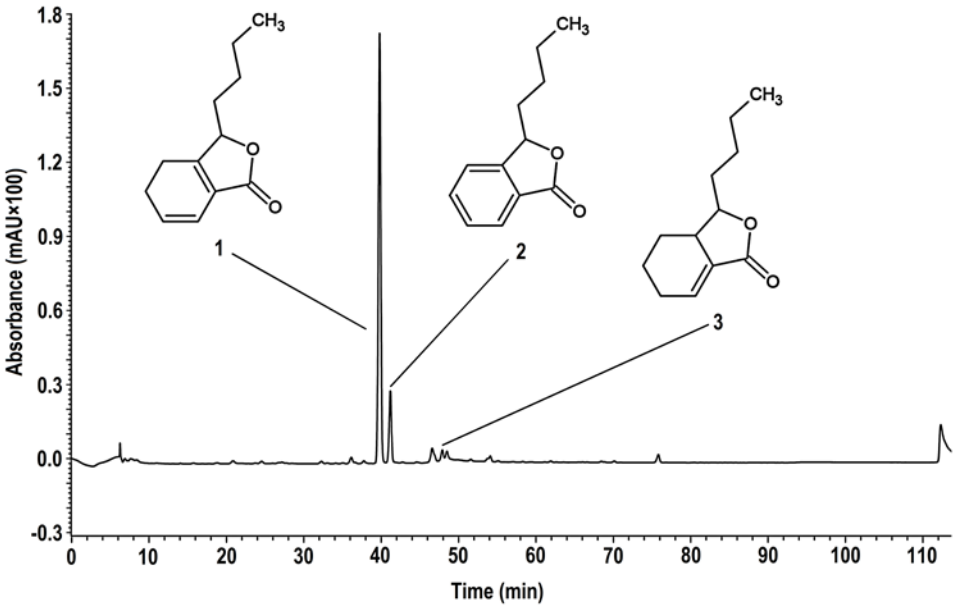


FIGURE 6. Exemplary chromatogram (λ=254 nm) of the phthalide fraction of *Apium graveolens* essential oil. Compounds: 1, sedanenolide; 2, 3-*n*-butylphthalide; and 3, sedanolide.

of hydrodistillation. Neither pectinase nor β-glucosidase significantly ($p \geq 0.05$) affected the essential oil yield. Individual and total phthalide contents of volatile oil samples obtained from enzyme-incubated seeds, and the corresponding phthalide yields, were not affected either (Figure 5A–H).

The results indicate that enzyme pretreatment is not a viable means of increasing the phthalide yield of celery seeds. As compared to conventional hydrodistillation, the procedure is more costly and time-consuming, which does not translate to improved productivity. Preincubation with β-glucosidase had no effect on essential oil and phthalide yields, thus indicating that similarly to aerial parts [Tang *et al.*, 1990], celery seeds either do not contain or have only negligible amounts of phthalide glycosides. However, since we did not directly analyze glucosidated forms of phthalides, the results have to be confirmed by other methods. It is also possible that the technique employed was not sensitive enough to detect a minor increase in phthalide content after pretreatment with β-glucosidase.

LC-DAD-MS (ESI$^+$) analyses of essential oil samples

In the current work, the modified method by Li *et al.* [2003] was adopted for qualitative and quantitative analyses of phthalide constituents of *A. graveolens* essential oil. The method was originally developed for the analysis of active components of *Ligusticum chuanxiong* [Li *et al.*, 2003] enabling complete separation of several compounds of the phthalide group. The analyses of essential oil samples conducted in the present work revealed the presence of three major alkylphthalide derivatives: sedanenolide (1, retention time, t_R = 39.66 min), 3-*n*-butylphthalide (2, t_R = 41.11 min), and sedanolide (3, t_R = 47.74 min). An exemplary chromatogram and structures of the analyzed compounds are presented in Figure 6. The qualitative composition of the phthalide fraction was the same in all samples, regardless of the distillation method applied.

The LC-DAD-ESI-MS data of compounds 1, 2, and 3 is presented in Table 1. The UV spectra of phthalides recorded during LC-DAD analyses were consistent with previous reports [Li *et al.*, 2003; Zhang X. *et al.*, 2003; Zhang L. *et al.*, 2012; Zuo *et al.*, 2011]. The UV spectrum of 1 showed a distinctive maximum at 281 nm, whereas 2 had maxima at 226 and 275 nm. Compound 3, bearing a single double bond, was characterized by a single maximum at 209 nm. The identity of phthalide constituents was further confirmed by LC-MS experiments. The ESI-MS spectra of compounds 1, 2, and 3 showed the presence of pseudomolecular ions at *m/z* 193, 191, and 195, respectively. A number of fragment ions was also observed, which reflected the fragmentation

TABLE 1. Retention times (t_R), absorption maxima of UV spectra (λ_{max}), and LC-MS (ESI$^+$) data of phthalide constituents of essential oil from *A. graveolens* seeds.

No	t_R (min)	λ_{max} (nm)	Observed ions (*m/z*)[a]	Compound
1	39.66	281	137 $[M+H-C_4H_8]^+$, 175 $[M+H-H_2O]^+$, **193** $[M+H]^+$, 215 $[M+Na]^+$, 407 $[2M+Na]^+$	sedanenolide
2	41.11	226, 275	135 $[M+H-C_4H_8]^+$, 145 $[M+H-H_2O-CO]^+$, 173 $[M+H-H_2O]^+$, **191** $[M+H]^+$, 213 $[M+Na]^+$, 403 $[2M+Na]^+$,	3-*n*-butylphthalide
3	47.74	209	149 $[M+H-H_2O-CO]^+$, 177 $[M+H-H_2O]^+$, **195** $[M+H]^+$, 217 $[M+Na]^+$, 411 $[2M+Na]^+$	sedanolide

[a] major ions are presented in bold.

TABLE 2. Validation parameters for quantitative HPLC determination of phthalides in essential oil samples from *A. graveolens* fruits.

Compound	λ (nm)	Regression equation[a]	Correlation coefficient[a]	Linearity (mg/mL)[a]	Intra-day precision (% RSD)[b]	Inter-day precision (% RSD)[c]	LOQ (mg/mL)[d]	LOD (mg/mL)[e]	Accuracy (% recovery)[f]
3-*n*-Butylphthalide	280	1.82×10^{6} + 2.30×10^{3}	0.9999	9.8×10^{-5} – 0.1	1.20	1.05	9.8×10^{-5}	3.2×10^{-5}	104.36 ± 0.67
Sedanolide	210	7.99×10^{6} + 3.48×10^{5}	0.9999	4.2×10^{-4} – 0.1	1.38	1.34	4.2×10^{-4}	1.4×10^{-4}	105.99 ± 0.97

[a] calculated using a series of standard dilutions for 10-point calibration curves – concentration (mg/mL) *vs.* peak area (mAu); [b] repeated analysis of the same sample on the same day (n=6); [c] repeated analysis of the same sample on different days (n=6); [d] levels corresponding to signal-to-noise (S/N) ratio of 3 (n=3); [e] levels corresponding to signal-to-noise (S/N) ratio of 10 (n=3); [f] estimated using the standard addition method at 3 different enrichment levels (+60%, +75%, +120%, n=3+3+3); expressed as means ± standard deviations. Abbreviations: RSD, relative standard deviation; LOQ, limit of quantification; LOD, limit of detection.

pattern characteristic for benzofuranone compounds [Niu *et al.*, 2008]. This included the loss of butylene, as well as dehydration and subsequent loss of carbonyl group. The formation of dimers and sodium adducts was also observed. The recorded spectra are in agreement with previous reports [Li *et al.*, 2003; Niu *et al.*, 2008; Wang *et al.*, 2018; Zhang X. *et al.*, 2003; Zhang L. *et al.*, 2012; Zuo *et al.*, 2011].

The identified phthalide derivatives have previously been demonstrated to be major non-terpenoid constituents of celery volatile oil [Kokotkiewicz & Luczkiewicz, 2016; Sowbhagya, 2014; Turner *et al.*, 2021]. The compounds were quantified using LC-UV. Given the similarities in UV characteristics of 1 and 2, quantitation of both these compounds was possible based on a series of dilutions of 3-*n*-butylphthalide. Sedanolide (3) exhibited different UV-absorption characteristics than 1 and 2, and as such was quantified using a separate calibration curve. The validation parameters of compounds used for quantitative studies are presented in Table 2.

CONCLUSIONS

The study demonstrated that conventional hydrodistillation and simultaneous distillation-extraction can be employed to obtain phthalide-enriched fractions of celery seeds. Process optimization enabled maximizing essential oil and phthalide yields. The experiments showed a clear positive correlation between hydrodistillation time and improved essential oil and phthalide yields. The 6-h hydrodistillation of comminuted seeds yielded essential oil with a significantly ($p<0.05$) higher phthalide content, as compared to the samples collected after 1.5–3.0 h. The obtained samples contained mainly sedanenolide and 3-*n*-butylphthalide, and a substantially lower amount of sedanolide. A further increase in phthalide contents was achieved by omitting the size reduction step prior to hydrodistillation and utilizing the salting-out effect. Enzyme pretreatment, on the other hand, had a negligible effect on essential oil and phthalide yields. The change of distillation mode from conventional hydrodistillation to simultaneous distillation-extraction significantly increased the essential oil yield while maintaining its high phthalide content. The results of the study are of practical importance and may be employed to produce celery oil with an exceptionally high phthalide content, which can be used as a flavoring agent in the food industry, or as a bioactive ingredient in nutraceutical products. An important finding is that the isolation process of celery oil can be simplified by omitting the size reduction step, thus saving time and energy without compromising product yield. However, it has to be noted that the current study focused solely on the phthalide content of celery oil. A more comprehensive analysis of the obtained samples, including GC and sensory evaluation, is needed in order to fully assess the benefits of the developed distillation method.

The positive effects of salting-out encourage further experiments in that field, aimed at the successive improvement of the productivity of the distillation process. Such a study would involve other types of salts, added to the distillation mixture at different concentrations. Moreover, it appears that the developed methods could be applied to other species. Apart from celery, no comprehensive SDE studies have so far been performed on other phthalide-bearing plants. However, alkylphthalides are present in several members of Apiaceae, including medicinal and/or flavoring plants such as *Angelica archangelica* L., *Angelica sinensis* (Oliv.), *Levisticum officinale* Koch., as well as representatives of the species *Ligusticum* and *Cnidium* [León *et al.*, 2017]. It would be particularly interesting to examine how SDE affects the isolation of phthalides of different polarities (including hydroxy-derivatives), which can be found in the above species. The phthalide-enriched fractions obtained from those plants could also gain interest as potential flavoring and bioactive agents.

RESEARCH FUNDING

This research received no external funding.

CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ORCID IDS

A. Badura https://orcid.org/0000-0002-6560-8231
A. Buciński https://orcid.org/0000-0002-0558-9139
A. Kokotkiewicz https://orcid.org/0000-0001-5025-7101
A. Lorenc https://orcid.org/0000-0002-1474-7864
M. Luczkiewicz https://orcid.org/0000-0001-5490-3588
Ż. Tabaczyńska https://orcid.org/0000-0003-1269-8330

REFERENCES

1. Bartsch, A., Hammerschmidt, F.-J. (1993). Separation of fragrance materials from perfumed consumer products. *Perfumer & Flavorist, 18*, 41–48.
2. Bicchi, C. (2000). Essential oils/Gas chromatography. In I.D. Wilson, C. Poole, M. Cooke (Eds.), *Encyclopedia of Separation Science*, Academic Press, Cambridge, UK, pp. 2744–2755. https://doi.org/10.1016/B0-12-226770-2/02471-6
3. Chaintreau, A. (2001). Simultaneous distillation–extraction: from birth to maturity—review. *Flavour and Fragrance Journal, 16*(2), 136–148. https://doi.org/10.1002/ffj.967
4. Dąbrowska, J.A., Kunicka-Styczyńska, A., Śmigielski, K.B. (2020). Biological, chemical, and aroma profiles of essential oil from waste celery seeds (*Apium graveolens* L.). *Journal of Essential Oil Research, 32*(4), 308–315. https://doi.org/10.1080/10412905.2020.1754937
5. El-Beltagi, H.S., Dhawi, F., Aly, A.A., El-Ansary, A.E. (2020). Chemical compositions and biological activities of the essential oils from gamma irradiated celery (*Apium graveolens* L.) seeds. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 48*(4), 2114––2133. https://doi.org/10.15835/nbha48412115
6. Filly, A., Fabiano-Tixier, A.S., Louis, C., Fernandez, X., Chemat, F. (2016). Water as a green solvent combined with different techniques for extraction of essential oil from lavender flowers. *Comptes Rendus Chimie, 19*(6), 707–717. https://doi.org/10.1016/j.crci.2016.01.018
7. Gong, S., Zhang, J., Guo, Z., Fu, W. (2018). Senkyunolide A protects neural cells against corticosterone-induced apoptosis by modulating protein phosphatase 2A and α-synuclein signaling. *Drug Design, Development and Therapy, 12*, 1865–1879. https://doi.org/10.2147/DDDT.S161748
8. Hsieh, S.L., Chen, C.T., Wang, J.J., Kuo, Y.H., Li, C.C., Hsieh, L.C., Wu, C.C. (2015). Sedanolide induces autophagy through the PI3K, p53 and NF-κB signaling pathways in human liver cancer cells. *International Journal of Oncology, 47*(6), 2240–2246. https://doi.org/10.3892/ijo.2015.3206
9. Huang, L., Wang, S., Ma, F., Zhang, Y., Peng, Y., Xing, C., Feng, Y., Wang, X., Peng, Y. (2018). From stroke to neurodegenerative diseases: The multi-target neuroprotective effects of 3-*n*-butylphthalide and its derivatives. *Pharmacological Research, 135*, 201–211. https://doi.org/10.1016/j.phrs.2018.08.007
10. Jain, M.P., Sama, J.K., Jain, S.M., Sharma, V.K., Singh, B. (2003). An improved method for the recovery of essential oil from celery seeds. *Indian Journal of Chemical Technology, 10*(4), 370–372.
11. Kokotkiewicz, A., Luczkiewicz, M. (2016). Chapter 37 – Celery (*Apium graveolens* var. *dulce* (Mill.) Pers.) oils. In V.R. Preedy (Ed.), *Essential Oils in Food Preservation, Flavor and Safety*, Academic Press, pp. 325–338. https://doi.org/10.1016/B978-0-12-416641-7.00037-7
12. Kurobayashi, Y., Kouno, E., Fujita, A., Morimitsu, Y., Kubota, K. (2006). Potent odorants characterize the aroma quality of leaves and stalks in raw and boiled celery. *Bioscience, Biotechnology, and Biochemistry*, 70(4), 958–965. https://doi.org/10.1271/bbb.70.958
13. Lei, W., Deng, Y.F., Hu, X.Y., Ni, J.N., Jiang, M., Bai, G. (2019). Phthalides, senkyunolide A and ligustilide, show immunomodulatory effect in improving atherosclerosis, through inhibiting AP-1 and NF-κB expression. *Biomedicine & Pharmacotherapy, 117*, 109074. https://doi.org/10.1016/j.biopha.2019.109074
14. León, A., Del-Ángel, M., Ávila, J.L., Delgado, G. (2017). Phthalides: Distribution in nature, chemical reactivity, synthesis, and biological activity. In A.D. Kinghorn, H. Falk, S. Gibbons, J. Kobayashi (Eds), *Progress in the Chemistry of Organic Natural Products,* Volume 104, Springer, pp. 127–246. https://doi.org/10.1007/978-3-319-45618-8_2
15. Li, S.-L., Chan, S.S.-K., Lin, G., Ling, L., Yan, R., Chung, H.-S., Tam, Y.-K. (2003). Simultaneous analysis of seventeen chemical ingredients of *Ligusticum chuanxiong* by on-line high performance liquid chromatography-diode array detector-mass spectrometry. *Planta Medica, 69*(5), 445–451. https://doi.org/10.1055/s-2003-39709.
16. Liu, M.M., Huang, K.M., Qian, L., Chatterjee, P., Zhang, S., Li, R., Zhou, S., Wang, Z., Luo, Y., Huang, Y. (2018). Effects of bioactive constituents in the Traditional Chinese Medicinal formula Si–Wu–Tang on Nrf2 signaling and neoplastic cellular transformation. *Phytomedicine, 40*, 1–9. https://doi.org/10.1016/j.phymed.2017.12.031
17. Ludwiczuk, A., Najda, A. B., Wolski, T., Baj, T. (2001). Chromatographic determination of the content and the composition of extracts and essential oils from the fruits of three varieties of stalk celery (*Apium graveolens* L. var. dulce Mill. Pers.). *Journal of Planar Chromatography—Modern TLC, 14*(6), 400–404.
18. MacLeod, G., Ames, J.M. (1989). Volatile components of celery and celeriac. *Phytochemistry, 28*(7), 1817–1824. https://doi.org/10.1016/S0031-9422(00)97866-X
19. Malhotra, S.K. (2006). 18 – Celery. In K.V. Peter (Ed.), *Handbook of Herbs and Spices,* Volume 3, Academic Press, pp. 317–336. https://doi.org/10.1533/9781845691717.3.317
20. Mukhopadhyay, M. (2000). Spice extracts. In M. Mukhopadhyay (Ed.), *Natural Extracts Using Supercritical Carbon Dioxide* (1st ed.), CRC Press, Boca Raton, pp. 177–200. https://doi.org/10.1201/9781420041699
21. Naqvi, A.A., Mandal, S., Chattopadhyay, A., Prasad, A. (2002). Salt effect on the quality and recovery of essential oil of citronella (*Cymbopogon winterianus* Jowitt). *Flavour and Fragrance Journal, 17*(2), 109–110. https://doi.org/10.1002/ffj.1059
22. Niu, Z.Q., Sun, Y.M., Niu, F., Han, J., Chen, D.W. (2008). Study of electrospray ionization tandem mass spectrometry of the benzofuranone compounds. *Chinese Chemical Letters, 19*(3), 324–328. https://doi.org/10.1016/j.cclet.2007.12.008
23. Rożek, E., Nurzyńska-Wierdak, R., Sałata, A., Gumiela, P. (2016). The chemical composition of the essential oil of leaf celery (*Apium graveolens* L. var. *secalinum* alef.) under the plants' irrigation and harvesting method. *Acta Scientiarum Polonorum. Hortorum Cultus, 15*(1), 149–159.
24. Salehi, B., Venditti, A., Frezza, C., Yücetepe, A., Altunta, Ü., Uluata, S., Butnariu, M., Sarac, I., Shaheen, S., Petropoulos, S.A., Matthews, K.R., Kılıç, C.S., Atanassova, M., Adetunji, C.O., Ademiluyi, A.O., Özçelik, B., Fokou, P.V.T., Martins, N., Cho, W.C., Sharifi-Rad, J. (2019). *Apium* plants: Beyond simple food and phytopharmacological applications. *Applied Sciences, 9*(17), art. no. 3547. https://doi.org/10.3390/app9173547.

25. Sgorbini, B., Cagliero, C., Pagani, A., Sganzerla, M., Boggia, L., Bicchi, C., Rubiolo, P. (2015). Determination of free and glucosidically-bound volatiles in plants. Two case studies: L-menthol in peppermint (*Mentha* x *piperita* L.) and eugenol in clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry). *Phytochemistry, 117*, 296–305. https://doi.org/10.1016/j.phytochem.2015.06.017
26. Shamspur, T., Mohamadi, M., Mostafavi, A. (2012). The effects of onion and salt treatments on essential oil content and composition of *Rosa damascena* Mill. *Industrial Crops and Products, 37*(1), 451–456. https://doi.org/10.1016/j.indcrop.2011.07.019
27. Sowbhagya, H.B. (2014). Chemistry, technology, and nutraceutical functions of celery (*Apium graveolens* L.): an overview. *Critical Reviews in Food Science and Nutrition, 54*(3), 389–398. https://doi.org/10.1080/10408398.2011.586740
28. Sowbhagya, H.B., Purnima, K.T., Florence, S.P., Appu Rao, A.G., Srinivas, P. (2009). Evaluation of enzyme-assisted extraction on quality of garlic volatile oil. *Food Chemistry, 113*(4), 1234–1238. https://doi.org/10.1016/j.foodchem.2008.08.011
29. Sowbhagya, H.B., Sampathu, S.R., Krishnamurthy, N. (2007). Evaluation of size reduction on the yield and quality of celery seed oil. *Journal of Food Engineering, 80*(4), 1255–1260. https://doi.org/10.1016/j.jfoodeng.2006.09.019
30. Sowbhagya, H.B., Srinivas, P. (2013). Enrichment of bio-active phthalides in celery seed oil. *Journal of Pharmacy and Nutrition Sciences, 3*(4), 250–257. http://dx.doi.org/10.6000/1927-5951.2013.03.04.6
31. Sowbhagya, H.B., Srinivas, P., Krishnamurthy, N. (2010). Effect of enzymes on extraction of volatiles from celery seeds. *Food Chemistry 120*(1), 230–234. https://doi.org/10.1016/j.foodchem.2009.10.013
32. Tang, J., Zhang, Y., Hartman, T.G., Rosen, R.T., Ho, C.-T. (1990). Free and glycosidically bound volatile compounds in fresh celery (*Apium graveolens* L.). *Journal of Agricultural and Food Chemistry, 38*(10), 1937–1940. https://doi.org/10.1021/jf00100a013
33. Turner, L., Lignou, S., Gawthrop, F., Wagstaff, C. (2021). Investigating the factors that influence the aroma profile of *Apium graveolens*: A review. *Food Chemistry, 345*, art. no. 128673. https://doi.org/10.1016/j.foodchem.2020.128673
34. Van Wassenhove, F., Dirinck, P., Vulsteke, G., Schamp, N. (1990). Aromatic volatile composition of celery and celeriac cultivars. *HortScience*, *25*(5), 556–559. https://doi.org/10.21273/HORTSCI.25.5.556
35. Venditti, A. (2020). What is and what should never be: artifacts, improbable phytochemicals, contaminants and natural products. *Natural Product Research, 34*(7), 1014–1031. https://doi.org/10.1080/14786419.2018.1543674
36. Wang, N., Zhao, X., Li, Y., Cheng, C., Huai, J., Bi, K., Dai, R. (2018). Identification of the absorbed components and metabolites of modified Huo Luo Xiao Ling Dan in Rat Plasma by UHPLC-Q-TOF/MS/MS. *Biomedical Chromatography, 32*(6), art. no. e4195. https://doi.org/10.1002/bmc.4195
37. Zhang, L., Zhu, L., Wang, Y., Jiang, Z., Chai, X., Zhu, Y., Gao, X., Qi, A. (2012). Characterization and quantification of major constituents of Xue Fu Zhu Yu by UPLC-DAD–MS/MS. *Journal of Pharmaceutical and Biomedical Analysis, 62*, 203–209. https://doi.org/10.1016/j.jpba.2011.12.026
38. Zhang, X., Xiao, H., Xu, Q., Li, X., Wang, J., Liang, X. (2003). Characterization of phthalides in *Ligusticum chuanxiong* by liquid chromatographic-atmospheric pressure chemical ionization--mass spectrometry. *Journal of Chromatographic Science, 41*(8), 428–433. https://doi.org/10.1093/chromsci/41.8.428
39. Zheng, G.Q., Kenney, P.M., Zhang, J., Lam, L.K. (1993). Chemoprevention of benzo[a]pyrene-induced forestomach cancer in mice by natural phthalides from celery seed oil. *Nutrition and Cancer, 19*, 77–86. https://doi.org/10.1080/01635589309514238
40. Zorga, J., Kunicka-Styczyńska, A., Gruska, R., Śmigielski, K. (2020). Ultrasound-assisted hydrodistillation of essential oil from celery seeds (*Apium graveolens* L.) and its biological and aroma profiles. *Molecules, 25*(22), art. no. 5322. https://doi.org/10.3390/molecules25225322
41. Zuo, A., Wang, L., Xiao, H., Li, L., Liu, Y., Yi, J. (2011). Identification of the absorbed components and metabolites in rat plasma after oral administration of *Rhizoma chuanxiong* decoction by HPLC–ESI-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis, 56*(5), 1046–1056. https://doi.org/10.1016/j.jpba.2011.08.010

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 211–224
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/138321
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Effect of Composition and Processing Conditions on Selected Properties of Potato-Based Pellets and Microwave-Expanded Snacks Supplemented with Fresh Beetroot Pulp

Katarzyna Lisiecka[1], *Agnieszka Wójtowicz*[1]*, *Agnieszka Sujak*[2]

[1]*Department of Thermal Technology and Food Process Engineering, University of Life Sciences in Lublin, Głęboka 31, 20–612 Lublin, Poland*
[2]*Department of Biosystems Engineering, University of Life Sciences in Poznań, Wojska Polskiego 50, 60–637 Poznań, Poland*

Key words: extrusion-cooking, snack pellets, beetroot, microwave expansion, chemical composition, molecular structure

The aim of this study was to evaluate the influence of the addition of fresh beetroot pulp in amounts from 2.5 to 30.0 g/100 g, as well as variable screw speeds (60, 80, 100 rpm) on the chemical composition, water absorption, water solubility index, density, expansion rate, and texture of potato-based pellets and expanded snacks. Their protein and starch structure was analyzed using the Fourier-transformed infrared (FTIR) spectroscopy. The snack pellets were made at a moisture content of 33 g/100 g of blends using a single-screw extruder equipped with a plasticizing unit having a length-to-diameter ratio (L/D) of 18. Processing temperature ranged from 65 to 105°C. The ready-to-eat snacks were expanded using microwave treatment (750 W, 40 s). Fresh vegetable pulp addition raised the protein content. The extrusion-cooking caused an increase in the content of insoluble dietary fiber and water absorption index of the pellets. In contrast, the microwave treatment reduced values of these parameters in expanded products and decreased the insoluble dietary fiber content of the expanded snacks. The addition of fresh beetroot pulp diminished the expansion ability and increased the content of β-sheet proteins. Additionally, an increase in the content of β-turn and β-sheet protein fractions was observed at the expense of random coil structure. This indicates that the protein structure underwent some form of ordering as the molecular interactions of the proteins intensified. The highest fraction of β-structures and the smallest content of random coil were observed when 80 rpm was applied during processing. It seems to be the most efficient screw speed in processing fresh beetroot pulp-supplemented snack pellets. Application of a fresh beetroot pulp as an additive in microwave expanded snacks made it possible to obtain products with an increased amount of fiber and a reduced fat content as an attractive and healthy alternative to deep-fried snacks.

INTRODUCTION

One of the most popular natural polysaccharides used in snack manufacture is starch, being a carbohydrate made of amylose and amylopectin fractions. Its botanical origin determines the main changes in starch processing and quality of final products [Sakač *et al*., 2020]. Starches of various biological origin differ in the degree of polymerization, the amylose to amylopectin ratio, and molecular organization of the crystalline regions of granules [Błaszczak *et al.,* 2005]. The knowledge of physicochemical, functional, and structural properties of starch, such as gelatinization, retrogradation, solubility, swelling power, water retention capacity, rheological behavior, and pasting properties are very useful in snack production [Sanchez-González *et al*., 2019].

The Fourier-transformed infrared (FTIR) spectroscopy is a universal analytical technique that can be applied to analyze structural changes in processed materials [Kowalczuk & Pitucha, 2019]. However, it has not yet been employed in the study of the properties of potato-based pellets or snack foods.

Products processed by means of extrusion-cooking are a significant element of the diet of people all over the world. Generally, extruded snacks are the most attractive products for children and young consumers. Therefore, very often, the snacks are enriched with extra ingredients, such as fruits, vegetables, herbs or other plant additives [Bhat *et al*., 2019; Lisiecka & Wójtowicz, 2019]. Potato-based snack pellets are the most popular snack items on the market, appearing in different shapes and flavors.

Extruded snack pellets need to be expanded in order to obtained ready-to-eat (RTE) snacks. Among the typical expansion methods applied are deep oil frying, hot air puffing, or microwave heating. Microwave treatment, as an alternative method of pellet expansion, reduces fat content in RTE products as compared to conventional expansion by frying in deep

* Corresponding Author:
Tel.: +(48) 81 4456127
E-mail: agnieszka.wojtowicz@up.lublin.pl (Prof. A. Wójtowicz)

Submitted: 25 March 2021
Accepted: 28 May 2021
Published on-line: 11 June 2021

oil [Lisiecka & Wójtowicz, 2021]. It is also an easy and inexpensive process to execute either on an industrial scale or at household conditions [Ruiz-Armenta *et al*., 2019].

Beetroot is a very popular vegetable in Poland, mostly consumed as cooked or baked or used as a soup ingredient. Dry beetroot and beetroot extracts are added to many food products as natural colorants, while other beneficial effects of beetroots are attributable to their antiviral, antidiabetic, cytotoxic, and allelopathic properties [Mikołajczyk-Bator *et al.*, 2016]. Fresh beetroot pulp is valued for its natural purple color and the high content of valuable nutritional components, not degraded by the drying process. Fresh beetroots are available on the market all year round due to the use of controlled atmosphere storage, which is an effective way to ensure an appropriate quality and microbiological safety. When used at home, beetroots are cleaned and cooked or baked without any microbiological risk. Moreover, because the extrusion-cooking is a high-temperature process, the processing by its means can be used as a method of thermal sterilization. As previously presented [Lisiecka & Wójtowicz, 2020], the use of fresh vegetables can significantly reduce the water volume needed in the technological process and the energy needed for the drying process. Thus, the use of fresh vegetables as additives to this type of indirectly expanded snacks has many advantages. After drying, the pellets have a moisture content below 11 g/100 g, which makes them stable and safe for storage.

This study aimed to examine the changes that take place in RTE snacks after extrusion of pellets at various screw speeds and following microwave expansion, with regard to the effect of replacing potato starch with increasing amounts of vegetable pulp. The novelty of this study lies in the use of fresh beetroot pulp rather than dry powdered vegetables to produce pellets, as in the case of most research performed [Bhat *et al*., 2019; Ruiz-Armenta *et al*., 2019].

MATERIALS AND METHODS

Raw materials

Potato flakes, grits, and starch were donated by Pol-Foods Sp. z o. o. (Prostki, Poland). Fresh beetroot was bought at a local market. The vegetables were washed with tap water, drained with paper towels, and ground with a laboratory knife mill LMN-100 TestChem (Radlin, Poland) with a sieve with opens below 400 μm. Next, after mixing with potato components, the blends were sifted again before moistening and processing.

Extrusion-cooking of snack pellets

The control blend composition was as follows: 25 g/100 g of potato flakes, 25 g/100 g of potato grits, and 50 g/100 g of potato starch. Other samples were fortified with the fresh beetroot pulp at a range from 2.5 to 30.0 g/100 g by replacing the respective amount of potato starch. The moisture content was assessed through employing a standard reference American Association of Cereal Chemists 44–15.02 air-oven method (130°C, 1 h) [AACC, 1995] both for dry components and for beetroot pulp after grinding. Calculation of water amount added was done in accordance to the previous paper [Lisiecka & Wójtowicz, 2020] to reach the moisture content of blends at 33 g/100 g (sum of water content in all components by weight). Firstly, dry components were mixed, then fresh pulp was added, mixed together, and the proper amount of water was added to each blend. During hydration, the components were mixed continuously for about 15 min.

A single screw extruder type TS-45 (ZMCh Metalchem, Gliwice, Poland) with a length-to-diameter ratio at L/D=18 of the plasticizing unit, with a screw of 3:1 compression ratio, and with an intensive glycol cooling installation of the last barrel section was employed to produce snack pellets. During processing, variable screw speeds were applied: 60, 80, and 100 rpm. The temperature profile during processing was set at 80°C in the dozing section, 105°C in the cooking section, and 65°C in the cooling section.

Snack pellets were shaped as dough strips using a 30×0.4 mm flat forming die and cut to approx. 30×30 mm pieces. The pellets were subsequently dried in a laboratory shelf dryer at 40°C for 10 h to their final moisture content of 11 g/100 g. The extrudates produced were kept in tightly closed foil packages before further testing.

Microwave expansion

Snack pellets were expanded by a treatment in a microwave oven (AVM-914/WH Philips/Whirlpool, Sweden). The optimum expansion conditions, *i.e*., microwave power of 750 W and exposure time of 40 s, were selected experimentally as treatment parameters. After expansion, the snacks were cooled to ambient temperature and stored in plastic bags before subsequent testing. Samples of both pellets and snacks were ground (if needed) before tests in a laboratory grinder (LMN-100, TestChem, Poland) to a particle size below 300 μm.

Water absorption index (WAI) and water solubility index (WSI) of pellets and RTE snacks

Water absorption index (WAI) and water solubility index (WSI) were determined according to the methods described by Lisiecka *et al*. [2021] in triplicate. The ratio of the gel weight to the dry sample weight was defined as WAI. The ratio of the weight of dry solids in the supernatant to the weight of the dry sample was derived as WSI.

Bulk density (BD) and volumetric expansion index (VEI) of snacks

The bulk density (BD) was specified as the weight of the sample divided by the occupied volume of the cylinder [Han *et al*., 2018]. Volumetric expansion index (VEI) was calculated as the ratio of the density of pellets and snacks after expansion. The measurements were performed in 5 replications.

Texture analysis of microwave-expanded snacks

The texture analysis was employed to determine selected properties of snacks, including hardness (H), crispness (CR), and fracturability (FR). It was conducted using a Kramer shear cell by a compression test on single extrudates. During the measurement, the head test speed was 100 mm/min. Texture measurements were performed in 5 replications [Alvarez *et al*., 2020; Shah *et al*., 2017].

FTIR analysis and data manipulation

FTIR spectroscopy was applied to check the protein and starch structures in the examined pellets and in the RTE microwave-expanded snacks. A Nicolet iS50 FTIR spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a diamond attenuated total reflectance attachment (GladiATR attachment, PIKE Technologies, Madison, WI, USA) was employed to collect spectra between 4000 and 400 cm^{-1} at a 4 cm^{-1} resolution. Before the FTIR measurements had been taken, ground samples were dried under a vacuum for 3 h. To obtain an optimal signal-to-noise ratio, 128 scans were collected. Each spectra was baseline-corrected using OMNIC software (version 8.2, Thermo Fischer Scientific, Waltham, MA, USA). The analyzed spectra were averaged over three registered spectra series. Spectral analyses were carried out by employing GRAMS AI Spectral Notebase (Thermo Fisher Scientific) [Pytlak *et al*., 2020].

In order to separate the bands representing certain protein structures in the amide I region and to allow identification of the protein secondary structural constitutes, the second derivative of the amide I band was calculated by applying 5-point, second-degree polynomial function. Following this, the derivative spectra were smoothed *via* 11-point second-degree polynomial Savitsky–Golay function according to Seabourn *et al*. [2008] and Susi & Byler [1983]. All amide I bands were baseline corrected and area normalized.

To estimate the fraction of different types of secondary structure, the amide I band (1590–1720 cm^{-1}) was deconvoluted with Gaussian curves using Grams 32 AI (version 9) software (Galactic, Salem, NH, USA) as indicated by the second derivative peak position identification. The quality of the band deconvolution was indicated by the following parameters: $R^2 > 0.99$, solution converged, and $\chi^2 < 0.001$. The composition of secondary protein structure participating in the amide I band was expressed as a percentage of the area of the fitted region denoted as a relative area of components centered at specific wavenumbers [Bock & Damodaran, 2013]. The secondary structure assignment was based on absorption wavenumbers as described previously [Fetouhi *et al*., 2019].

Amide I secondary structures: α-helix (1649–1659 cm^{-1}), β-sheet (1620–1644 cm^{-1}), β-turn (1660–1688 cm^{-1}), and random coils (1510–1619 cm^{-1}) were assigned. Crystalline and amorphous fractions of starch were studied by estimating ratio R equal to the proportion of the intensities I (1047 cm^{-1})/ I (1022 cm^{-1}) [Smits *et al*., 1998].

Chemical composition of pellets and RTE snacks

Based on the results of physical, textural, and structural characteristics, the optimum products were selected to perform chemical components analysis. Protein (AACC 46–10), fat (AACC 30–10), and ash (AACC 08–01) contents were determined in triplicate [AACC, 1995]. Contents of soluble and insoluble dietary fiber and its total content were duplicate-tested by applying the Association of Official Agricultural Chemists International 993.21 method [AOAC, 2000]. The final results were expressed on a wet basis.

Statistical analysis

The obtained results were subjected to statistical analyses using Statistica 13.3 software (StatSoft, Tulsa, OK, USA). Homogeneous groups were determined by ANOVA by using Tukey post-hoc test at $\alpha=0.05$. Moreover, principal component analysis (PCA) was performed for the tested characteristics of pellets and microwave-expanded snacks processed at 80 rpm as the optimum screw speed, by Statistica software. Additionally, during the analysis of the main PCA components, a correlation matrix was created between the examined features and expressed as Persons' coefficients.

RESULTS AND DISCUSSION

Physical properties of pellets and snacks

To prepare the expanded samples, the optimum microwave conditions were checked experimentally by several trials to avoid burning the pellets if the frequency or time were too intensive or too low, and if not the whole pellet surface was expanded uniformly. In our study, the microwave power of 750 W and the treatment time of 40 s were selected based on preliminary trials as the best conditions to avoid both burning the samples or uncomplete expansion. Chen *et al.* [2014] found that the drying time was significantly reduced with the microwave power increase when vacuum microwave drying was used to prepare fish crisps. But they also pointed out that the higher power density would easily lead to the burning of fish samples and to the deterioration of their quality. The microwave power over 4 W/g led to the cooking of samples, resulting in a significant deterioration of their quality, therefore they selected this microwave power for further study. In turn, Ruiz-Armenta *et al.* [2019] manufactured expanded snack products using a conventional microwave oven with a power of 1,450 W and a frequency of 2,450 MHz by selecting microwave heating for 20 s after testing different heating times (16, 18, 20, 22, 24, 26, and 28 s). Given the above findings, it can be concluded that the application of a higher microwave power allows reducing heating time to obtain properly expanded products. However, since the availability of microwave ovens with a power over 1000 W is limited, the conditions selected in our study make it possible to obtain expanded fat-free snacks at household conditions.

Several physical properties were evaluated for the extruded pellets and microwave-expanded snacks. The water absorption index indicates the ability of the product components to absorb and hold water [Lourenço *et al*., 2016] and measures the amount of gel which is formed by starch, protein, and fiber after swelling in the presence of extra water [Kaur *et al*., 2015]. During the extrusion-cooking, gelatinization of starch and protein denaturation occur under appropriate conditions. Gelatinized starch absorbs more water than it does in its native state. However, the presence of proteins or fibers in the extrudate composition may increase or decrease the WAI due to their conformational and structural changes affecting the hydrophilic/hydrophobic balance [Lourenço *et al*., 2016].

According to our research, the WAI of the control potato blend before extrusion was 3.71±0.04 g/g, whereas its WSI was 1.28±0.47%. The WAI and WSI values of fresh beetroot pulp-supplemented pellets and microwave-expanded snacks are presented in Table 1. The WAI and the WSI of all extruded samples were higher in comparison to the control potato

TABLE 1. Water absorption index (WAI) and water solubility index (WSI) of extruded pellets and microwave-expanded snacks supplemented with fresh beetroot pulp.

Content of beetroot pulp (g/100 g)	Screw speed (rpm)	WAI (g/g)		WSI (%)	
		P	S	P	S
0	60	4.62±0.14^{c}	5.28±0.03^{a}	5.12±0.78^{b}	7.05±1.57^{a}
	80	6.83±0.69^{ab}	5.35±0.20^{a}	1.42±0.53^{c}	5.75±1.23^{ab}
	100	7.26±0.20^{a}	5.19±0.01^{a}	3.12±0.53^{bc}	2.57±0.12^{de}
2.5	60	4.59±0.06^{c}	4.58±0.17^{cd}	3.70±0.65^{b}	4.24±0.58^{bc}
	80	6.20±0.09^{b}	5.00±0.08^{bc}	2.57±0.94^{bc}	3.91±0.19^{cd}
	100	6.00±0.11^{bc}	5.20±0.03^{a}	2.75±0.18^{bc}	2.27±0.30^{de}
5.0	60	6.21±0.02^{b}	4.73±0.03^{cd}	2.46±0.07^{bc}	3.96±0.58^{cd}
	80	6.77±0.04^{ab}	4.95±0.15^{bc}	4.48±1.47^{b}	4.48±0.65^{bc}
	100	7.32±0.08^{a}	5.02±0.05^{ab}	6.49±2.93^{ab}	2.85±0.81^{de}
7.5	60	7.10±0.11^{ab}	4.77±0.26^{cd}	7.08±2.85^{ab}	4.20±0.19^{cd}
	80	7.53±0.14^{a}	4.57±0.18^{cd}	2.04±1.55^{c}	3.19±0.48^{cd}
	100	5.96±0.10^{bc}	5.38±0.03^{a}	5.02±1.29^{b}	3.34±0.51^{cd}
10.0	60	6.70±0.08^{ab}	4.38±0.07^{cd}	6.98±3.37^{ab}	2.85±0.58^{de}
	80	6.83±0.07^{ab}	4.67±0.08^{cd}	8.63±2.93^{a}	4.18±0.06^{cd}
	100	6.94±0.03^{ab}	4.48±0.08^{cd}	4.74±0.85^{b}	3.55±1.61^{cd}
20.0	60	5.69±0.05^{bc}	3.73±0.04^{e}	6.21±0.31^{ab}	2.84±0.36^{de}
	80	6.01±0.14^{bc}	4.75±0.13^{cd}	5.79±0.62^{ab}	4.35±1.46^{bc}
	100	6.11±0.12^{bc}	4.98±0.01^{bc}	3.03±0.51^{b}	2.14±0.12^{e}
30.0	60	5.92±0.07^{bc}	4.60±0.07^{cd}	3.88±1.81^{b}	5.92±0.37^{ab}
	80	7.16±0.60^{a}	4.51±0.14^{cd}	6.27±1.10^{ab}	4.77±0.05^{bc}
	100	7.03±0.23^{ab}	4.67±0.13^{cd}	4.03±0.27^{b}	5.04±0.50^{bc}

P – extruded pellet, S – microwave-expanded snack. Values are presented as mean ± standard deviation (n=3); $^{a-e}$ means indicated with the same letters in columns do not differ significantly at α=0.05.

blend before extrusion. In contrast, microwave expansion decreased the WAI of all samples compared to the corresponding half-products (pellets), except for the control sample produced at 60 rpm. This could be due to the reactions between proteins, amylose, and amylopectin, leading to WAI reduction [Sharma *et al*., 2015].

The WSI is a very important parameter of extrusion-cooking intensity, and makes it possible to predict the material behavior during further processing [Rashid *et al*., 2015]. In our research, the maximum WSI was determined at 8.63% for the pellet with beetroot pulp at 10.0 g/100 g processed at 80 rpm and at 7.05% for the control potato snacks produced at the lowest screw speed. The minimum WSI was obtained for the control pellets extruded at 80 rpm (1.42%) and for the microwave-expanded snacks containing 20.0 g/100 g of fresh beetroot pulp processed at 100 rpm (2.14%).

Researchers who experimented with expanding corn starch-based pellets supplemented with naranjita and milk powder observed an increase in WSI after extrusion and after microwave treatment [Ruiz-Armenta *et al.*, 2019]. They concluded that this was linked to the loss of the semi-crystalline form of native starch granules after processing.

The bulk density (BD), volumetric expansion index (VEI), and texture parameters of the microwave-expanded snacks are presented in Table 2. The highest values of BD were recorded at each additive level when the highest screw speed was applied during pellet processing, except for the 20 g/100 g additive level; however, the results obtained for this level did not differ significantly from others. The highest BD values were determined at 76.03 kg/m^3, 76.80 kg/m^3 and 76.36 kg/m^3 for the snacks without additive and for the snack with 5.0 and 7.5 g/100 g additive content extruded at 100 rpm, respectively. Nevertheless, the differences between the tested samples were in most cases statistically insignificant ($p \geq 0.05$). The lowest bulk density was determined at 57.90 kg/m^3 for the snacks with 2.5 g/100 g of beetroot pulp extruded at 60 rpm. These snacks also featured a high value of the volumetric expansion index, but their hardness was surprisingly high. Moreover, the highest VEI value was recorded for the samples without the vegetable additive extruded at 100 rpm (6.17). The increasing

TABLE 2. Selected physical and textural features of microwave-expanded snacks supplemented with fresh beetroot pulp.

Content of beetroot pulp (g/100 g)	Screw speed (rpm)	BD (kg/m^3)	VEI (-)	H (N)	CR (N)	FR (N)
0	60	71.45±5.15^{abc}	4.77±0.45^{bcde}	170.40±15.96^{abc}	10.47±1.50^{c}	26.07±4.96^{c}
	80	70.52±7.76^{abc}	5.10±0.61^{abc}	158.00±14.06^{cbd}	34.97±5.62^{a}	37.58±6.76^{a}
	100	76.03±3.73^{b}	6.17±0.66^{a}	195.40±19.17^{a}	29.52±5.45^{ab}	36.30±6.12^{ab}
2.5	60	57.90±2.66^{e}	5.34±0.14^{ab}	183.00±4.97^{ab}	23.57±1.91^{abc}	27.63±3.95^{abc}
	80	67.84±1.81^{abcd}	5.04±0.54^{abcd}	145.33±7.41^{cbde}	16.73±3.83^{bc}	27.63±7.11^{abc}
	100	74.53±2.76^{abc}	4.87±0.37^{bcde}	160.00±6.98^{abcd}	14.58±3.27^{c}	26.00±5.00^{abc}
5.0	60	63.40±1.67^{cd}	4.11±0.19^{bcdef}	156.00±10.10^{cbd}	19.54±5.01^{bc}	26.40±5.37^{abc}
	80	71.31±0.93^{abc}	4.16±0.22^{bcdef}	139.63±9.61^{cdef}	14.48±3.21^{c}	19.56±2.11^{c}
	100	76.80±3.99^{ab}	4.74±0.36^{bcdef}	108.33±2.49^{ef}	19.40±4.43^{bc}	26.03±4.51^{abc}
7.5	60	64.01±3.66^{acd}	4.34±0.60^{bcdef}	111.07±11.48^{ef}	17.09±2.22^{bc}	19.17±2.90^{c}
	80	72.36±0.84^{abc}	4.63±0.11^{bcdef}	102.57±5.12^{f}	18.73±1.50^{bc}	24.83±2.90^{abc}
	100	76.36±3.15^{ab}	4.92±0.24^{abcde}	130.87±3.78^{def}	13.10±2.61^{c}	13.82±3.26^{c}
10.0	60	64.28±3.86^{acd}	3.85±0.21^{cdef}	174.33±9.10^{abc}	16.85±5.05^{bc}	21.98±2.16^{abc}
	80	63.09±2.04^{cd}	4.52±0.18^{bcdef}	159.00±11.58^{cbd}	15.62±2.85^{bc}	23.11±5.67^{abc}
	100	72.20±0. 87^{abc}	4.54±0.21^{bcdef}	161.33±14.52^{abcd}	21.22±5.82^{abc}	24.77±2.31^{abc}
20.0	60	64.39±0.79^{acd}	3.64±0.22^{ef}	174.67±9.84^{abc}	24.00±4.46^{abc}	27.43±4.77^{abc}
	80	64.68±0.69^{abcd}	4.29±0.25^{bcdef}	169.33±9.88^{abcd}	19.40±2.76^{bc}	19.50±2.76^{c}
	100	61.89±0.59^{cd}	5.35±0.21^{ab}	173.33±5.31^{abc}	15.75±2.67^{bc}	21.74±3.75^{bc}
30.0	60	64.60±1.65^{abcd}	3.79±0.22^{def}	107.93±10.82^{ef}	15.23±5.92^{c}	15.77±5.60^{c}
	80	70.99±3.25^{abc}	3.47±0.10^{f}	169.67±4.99^{abcd}	18.70±5.02^{bc}	19.37±4.60^{c}
	100	72.52±4.18^{abc}	4.54±0.43^{bcdef}	135.67±8.22^{cdef}	18.80±6.79^{bc}	24.90±4.80^{abc}

BD – bulk density; VEI – volumetric expansion index; H – hardness; CR – crispness; FR – fracturability. Values are presented as mean ± standard deviation (n=5); $^{a-f}$ means indicated with the same letters in columns do not differ significantly at α=0.05.

amount of fresh vegetable pulp lowered the VEI values, but at the same time lowered the hardness of the expanded snacks with up to 10 g/100 g of the additive; however, the differences were in most cases insignificant (p≥0.05). Further increasing the content of fresh beetroot pulp resulted in still low BD but lowered VEI, and after the microwave expansion the snacks supplemented with 10.0 and 20.0 g/100 g became harder than the samples with 5.0 or 7.5 g/100 g of beetroot pulp, probably due to the increased fiber content in RTE products. Expanded potato-based snacks should be characterized by low BD, suggesting good expansion after the final treatment, but the ultimate effect is directly connected with the expansion method used. Lisiecka & Wójtowicz [2021] demonstrated low BD of oil-fried snacks that ranged from 38.3 kg/m^3 for the control snacks produced at 60 rpm to 64.0 kg/m^3 for the fresh beetroot pulp-supplemented snacks. In most cases, BD of fried snacks was lower than of the microwave-expanded ones (Table 2) due to formation of an expanded internal structure with multiple empty pores inside snacks as influenced by steam evaporation during frying. Ruiz-Armenta *et al.* [2019] tested indirectly expanded corn starch-based snacks prepared by microwaves (1450 and 2450 W for 20 s) and found that the expansion rate of RTE products ranged from 8.61 to 9.83, and their density from 95.41 to 106.55 kg/m^3, which is higher than in our research. As reported by Lisiecka *et al.* [2021], after hot oil frying of extruded pellets supplemented with *Allium* genus vegetables, the bulk density of fried snacks varied from 38.3–56.2 kg/m^3 for the control potato snacks, while the highest BD reached 87.2 kg/m^3 if 20.0 g/100 g of leek was used and 70.7 kg/m^3 if 7.5 g/100 g of onion was added. When analyzing fried snacks made of composite flour prepared from barnyard millet, green gram, fried gram, and ajwain seeds, Krishnaraj *et al.* [2019] found that the water molecules were converted into steam during the quick heating and that oil was filling empty spaces, which could lead to much higher bulk density of the fried snacks compared to these treated with microwaves as low-fat products. Thus, several benefits can be found when expanding pellets with microwave heating, including both nutritional benefits because of the low energy value of the product as well as economic benefits for producers due its the low density.

The hardness of microwave-expanded snacks supplemented with fresh beetroot pulp ranged from 102.57 N to 195.40 N (for the snack with 7.5 g/100 g vegetable pulp in the recipe processed at 80 rpm and for the control sample extruded

at the maximum rotational speed of the extruder screw, respectively). The snacks from the control sample extruded at 80 rpm had the highest values of crispness and fracturability (34.97 N and 37.58 N), while the control snacks extruded at 60 rpm featured the lowest values of crispness (10.47 N). Moreover, the lowest fracturability was determined at 13.82 N for the snacks with 7.5 g/100 g fresh beetroot pulp when extruded at 100 rpm, but in most cases the results did not differ significantly ($p \geq 0.05$). The increasing level of beetroot pulp in snacks, as well as the variable screw speed, ambiguously affected their textural properties after microwaving, probably due to inhomogeneity in the pores inside the expanded snacks structure that resulted the inconclusive texture profile of RTE products. Nevertheless, the crispness and fracturability of the supplemented snacks expanded by microwaves were more similar than those of the control snacks.

FTIR spectra of pellets and snacks

Figures 1 and 2 show exemplary baseline-corrected and surface-normalized FTIR spectra of pellets (Figure 1a–c) and microwaved snacks (Figure 2a–c) registered over the wavelengths between 3900–450 cm^{-1}. There are six clear groups of bands registered in the wavelength ranges between 3700–3000, 2950––2800, 1750–1500, 1500–1250, and 1200–900 cm^{-1} and several peaks below 900 cm^{-1}, respectively. The first group of bands contains the NH stretching vibration of amide A (3600––3450 cm^{-1}) overlapped with OH stretching vibration (bounded water; 3550–3200 cm^{-1}). Another group represents the amide B (3000–2600 cm^{-1}) bands overlapped with peaks representing stretching vibrations of CH_2 and CH_3 groups (2950–2800 cm^{-1}).

The band between 1750 and 1500 cm^{-1} represents amide I and amide II bands centered at 1640 cm^{-1} (amide I) and 1540 cm^{-1} (amide II – conformationally sensitive) due to CO carbonyl stretch with a minor contribution from out-of-plane CN stretching vibration, as well as NH bonding and CH stretching of proteins, respectively. The band between 1350 cm^{-1} and 1200 cm^{-1}, characteristic of amide III region, reveals NH in-plane bending coupled with C-N-stretching and includes CH and NH deformation modes.

Furthermore, the spectral region of polysaccharides (1200–900 cm^{-1}) is characteristic for backbone vibrations of CO, CN, and CC groups (stretching modes), while the peak at 994 cm^{-1} is sensitive to water content and starch conformation [van Soest *et al*., 1995]. Finally, the low intensity peaks in the spectral region below 900 cm^{-1} belong to CH out-of-plane bending (900–675 cm^{-1}), OH bending (770–650 cm^{-1}), and to NH_2 and H_2 wagging vibrations (900–660 cm^{-1}). The collective analysis showed an increasing proportion of water with the increasing fresh beetroot pulp content in relation to polysaccharides and proteins in the case of pellets and the opposite in the case of snacks. The analysis of the region between 2950–2800 cm^{-1} indicated a lower lipid content in snacks compared to pellets. No major differences were found related to different screw speeds.

Analysis of the amide I region

Processing variables dependence of the relative composition of protein secondary structures calculated on the basis of deconvolution of FTIR spectra in the amide I region are presented in Figure 3. Figure 3a and 3b show the β-sheet

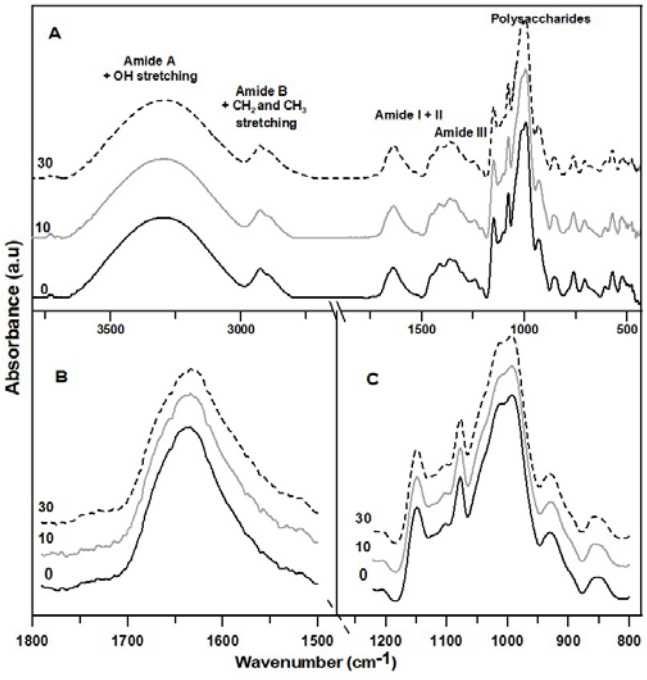


FIGURE 1. Baseline-corrected and surface-normalized FTIR spectra of pellets over the wavelengths 3900–450 cm^{-1} (a), 1750–1500 cm^{-1} (b), 1200––900 cm^{-1} (c). Black line: control potato pellets; Grey line: pellets with the addition of 10.0 g/100 g fresh beetroot pulp; Dotted line: pellets with the addition of 30.0 g/100 g fresh beetroot pulp.

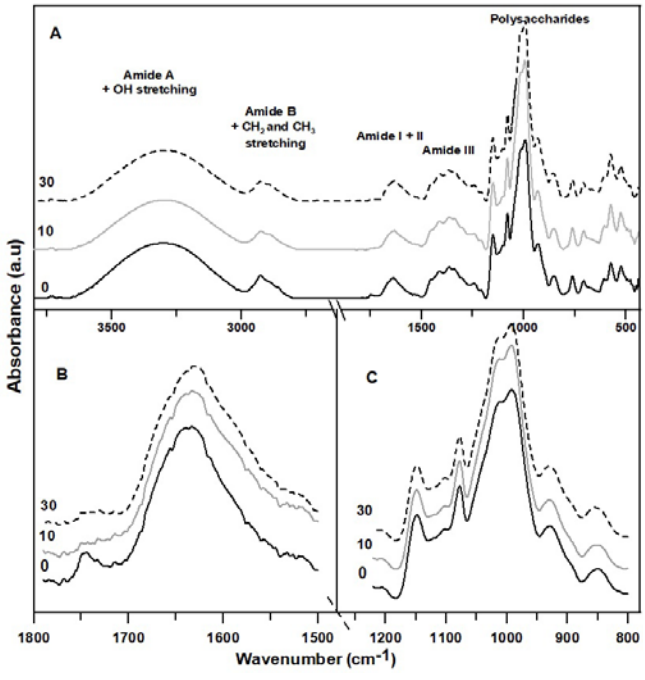


FIGURE 2. Baseline-corrected and surface-normalized FTIR spectra of microwave-expanded snacks over the wavelengths 3900–450 cm^{-1} (a), 1750––1500 cm^{-1} (b), 1200–900 cm^{-1} (c). Black line: control potato pellets; Grey line: pellets with the addition of 10.0 g/100 g fresh beetroot pulp; Dotted line: pellets with the addition of 30.0 g/100 g fresh beetroot pulp.

content of pellets and RTE snacks, respectively, while the β-turn content of the pellets and expanded snacks is presented in Figure 3c and 3d, respectively. In addition, the random coil content of the pellets and snacks evaluated by FTIR analysis is shown in Figure 3e and 3f, respectively.

As shown by the analysis of protein structure by deconvolution of amide I band, β-sheet was the main protein structure in the control sample, in the dried beetroot pulp, as well as in the examined pellets and snacks. Protein fractions in the control sample (not subjected to the extrusion process) containing potato flakes, potato grits, and starch had 8±0.07% of β-turn, 20±0.18% of α-helix, 55±0.50% of β-sheet, and 17±0.16% of random coil structures. In contrast, dried beetroot protein contained 8±0.06% of β-turn, no α-helix, 90±0.63% of β-sheet, and 1.7±0.01% of random coil structures. No α-helix structure was found in the samples regardless of screw rotation speed and additives – apart from the pellets containing 30.0 g/100 g of fresh beetroot pulp processed at 60 rpm where its content reached 13.6±0.01%. This phenomenon can be attributable to the extrusion-cooking

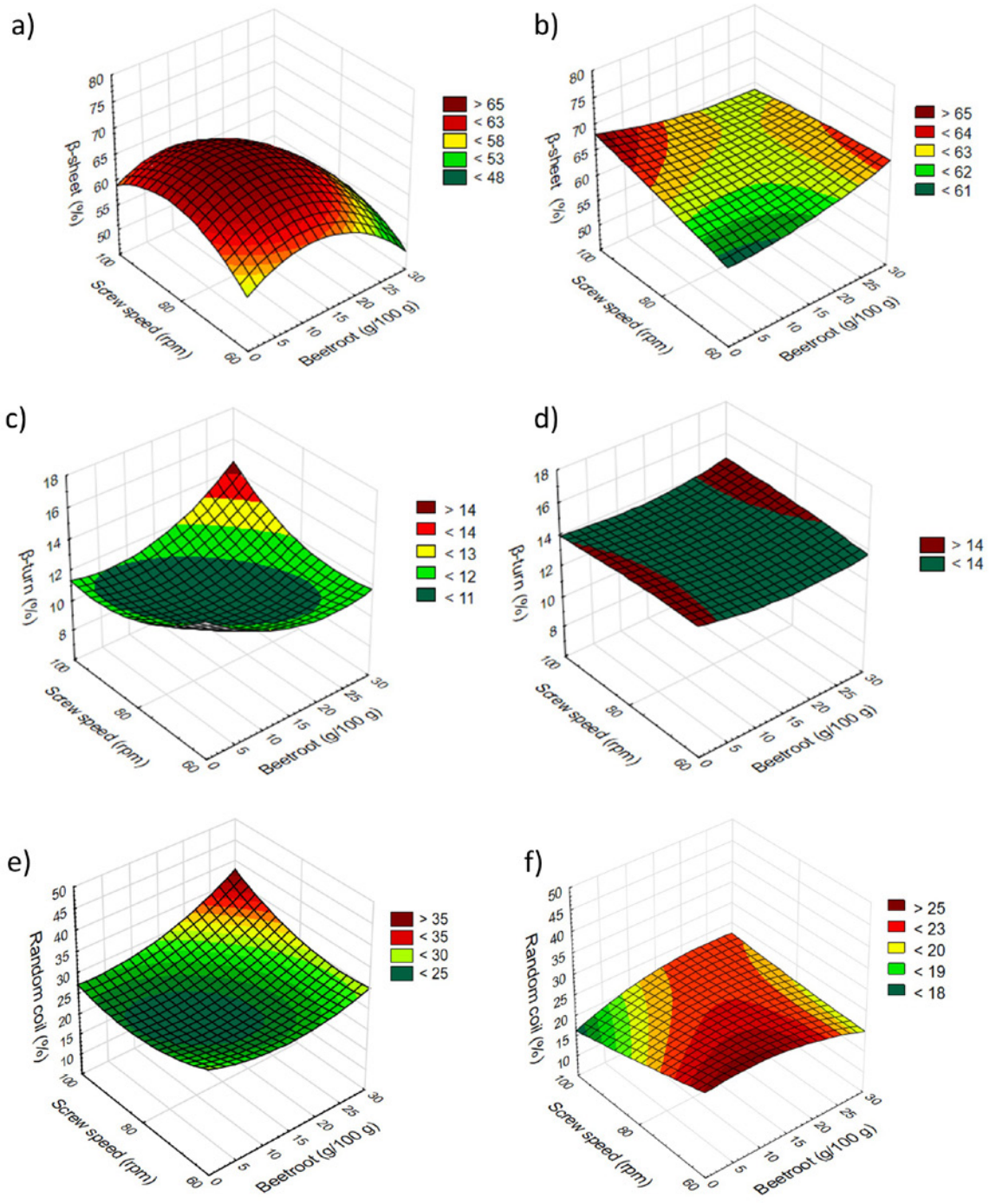


FIGURE 3. Processing variables dependence of relative composition of protein secondary structures calculated on the basis of deconvolution of FTIR spectra in the amide I region: a) β-sheet content of pellets, (b) β-sheet content of snacks, (c) β-turn content of pellets, (d) β-turn content of snacks, (e) random coil content of pellets, (f) random coil content of snacks.

process, which caused the disappearance of the α-helix structure. At this point, the extrusion process induced an ordering of the protein molecular structure. It should, however, be noted that strong interactions between neighboring α-helices can also produce an increase in the absorption region characteristic of the β-sheet structure.

The formed pellets contained between 8.2 and 16.4% of β-turn, and no α-helix (apart from above-mentioned sample), between 46.7 and 75.1% of β-sheet, as well as between 16.8 and 37.7% of random coil protein structures. Interestingly, the highest content of β-sheet was determined for the samples characterized by a relatively high beetroot pulp content (7.5 and 10.0 g/100 g) processed at all the applied screw speeds. This was accompanied by the highest β-sheet content (between 27.4% (60 rpm), 37.7% (80 rpm) and 35.9% (100 rpm)). Generally, an increase in the content of β-turn and β-sheet protein fractions was observed at the expense of random coil structure, indicating that the protein structure underwent some form of ordering as the molecular interactions in the proteins intensified. Therefore, the addition of fresh beetroot pulp resulted in an increase in the β-sheet structure content (the most stable protein structure). The pellets produced at the screw speed of 80 rpm had the highest content of β-sheet structure (from 46.7% to 75.5% in the control, and 61.5% on average in the pellets containing beetroot pulp) as compared to 60 rpm (from 47.5% in the control to 68.4% in the sample containing 7.5 g/100 g of fresh beetroot pulp; 58.2% on average in the pellets with beetroot pulp), and 100 rpm (from 50.5% in the control to 73.3% in the sample containing 10.0 g/100 g of fresh beetroot pulp; 58.5% on average in the pellets with beetroot pulp). Interestingly, the pellets containing between 5.0 and 10.0 g/100 g of fresh beetroot pulp had the highest fraction of this structure.

Our research indicated that the snacks contained less random coil protein structure and a little bit more of β-turn and β-sheet structure proteins, as compared to the not expanded pellets. This outcome points to the additional restructurization of the protein backbone. The microwave-expanded snacks contained between 12.6% and 14.4% of β-turn proteins, no α-helix structures, as well as between 49.8% and 67.7% of β-sheet and between 18.7 and 36.7% of random coil structure proteins. The ranges of the fractions representing protein structures of the snacks were also narrower. This result additionally indicates that microwave radiation resulted in greater protein stabilization. Although from the statistical point of view it was not relevant, the highest fraction of β-structures was found for 80 rpm of all the tested samples. The highest fraction of β-structures reached 66.8% for the snack containing 7.5 g/100 g of beetroot pulp. Simultaneously, the smallest fraction of random coil was observed for the above-mentioned screw speed. Such a result may be due to a relatively long time of production/extrusion in the case of 60 rpm and an increased temperature of the extrusion process in the case of 100 rpm screw speed.

Finally, the highest fraction of β-structures and the smallest content of random coil structure were determined when 80 rpm was applied during processing, which proves it to be the most efficient screw speed in the processing of fresh beetroot supplemented-snack pellets. Hence, these samples were subjected to the chemical composition analysis to evaluate the nutritional properties of snacks supplemented with fresh beetroot pulp.

Analysis of starch conformation

FTIR spectra in the region between 945 and 1195 cm^{-1} can be used to obtain information about starch structure [Meziani *et al*., 2011]. This part of the spectra represents COH bending and CH_2 related modes (1077, 1047, 1022, and 994 cm^{-1}) as well as CO and C-C stretching modes (1150 cm^{-1}). In addition, a peak at 994 cm^{-1} is sensitive to water content and starch conformation [van Soest *et al.,* 1995].

The absorbance intensity ratio (R) equal to I (1047 cm^{-1})/ I (1022 cm^{-1}) is frequently used as an indicator of the organization of different types of starch components [Hernández-Uribe *et al*., 2010; Meziani *et al*., 2011; Smits *et al*., 1998]. A high value of this ratio shows preponderance of the crystalline conformation over the amorphous conformation. This in turn indicates that starch has a strong tendency for retrogradation [Smits *et al*., 1998]. Additionally, in the region of 950–1200 cm^{-1} of FTIR spectra, starch shows sensitivity to the polymer conformation and hydration [Błaszczak *et al*., 2005]. In addition, the crystalline and amorphous zones of the starch are characterized by bands at 1047 cm^{-1} and 1022 cm^{-1}, respectively [Hernández-Uribe *et al*., 2010].

The absorbance intensity ratio (R) of the pellets and expanded snacks supplemented with fresh beetroot pulp is presented in Figure 4a and 4b, respectively. As indicated, the R value for the control blend before the extrusion-cooking was 0.68. In the case of pellets, the minimum value of R (0.64) was noted for the samples with 30.0 g/100 g of fresh beetroot pulp in the recipe when extruded at 100 rpm. In contrast, the maximum value (0.71) was determined for half-products with 10.0 g/100 g of vegetable additive processed at the screw speed of 80 rpm. After microwave treatment, the R value of the snacks decreased when compared to the results before expansion. The range of its values for the expanded snacks was from 0.60 (the samples with 7.5 g/100 g of beetroot processed at 60 rpm) to 0.65 (the control expanded snack extruded at the screw speed of 80 rpm).

In analyzing gluten-free cake, Fetouhi *et al*. [2019] obtained the R-ratio between 0.6 and 0.8 and concluded that its high values indicated a high tendency of starch for retrogradation [Fetouhi *et al*., 2019].

Analysis of the absorbance intensity ratio (R) shows a strong starch retrogradation ability for pellets as compared to snacks, with the most pronounced effect demonstrated at the screw speed of 80 rpm. This might have been due to a relatively longer time of production/extrusion when a low screw speed was applied and to an increased temperature of the extrusion process if the 100 rpm was used due to high friction and shear forces. On the other hand, starch of snacks showed much lesser tendency for retrogradation, which was the most visible at the screw speed of 60 rpm.

Chemical composition

Based on the results of physical, textural, and structural characteristics, the extruded pellets and microwave-expanded snacks processed at 80 rpm – chosen as the optimum

screw speed – were tested for their chemical composition. The control blend of potato components before extrusion had a protein content of 4.72±0.22 g/100 g, a fat content of 0.17±0.02 g/100 g, an ash content of 1.87±0.02 g/100 g, an insoluble dietary fiber content of 2.19±0.14 g/100 g, a soluble dietary fiber content of 3.75±0.15 g/100 g, and the total dietary fiber content at 5.94±0.16 g/100 g. The chemical composition of the pellets and expanded snacks supplemented with fresh beetroot pulp processed at 80 rpm is presented in Table 3.

In the extruded pellets, supplementation with the fresh beetroot pulp up to 10.0 g/100 g resulted in a lower protein content as compared to the control blend before extrusion. However, no significant ($p \geq 0.05$) differences were observed in the protein content of the extruded pellets supplemented with 0–10.0 g/100 g of the pulp. When the fresh beetroot pulp was used at 20.0 and 30.0 g/100 g, the protein content was significantly ($p < 0.05$) higher (Table 3). Based on the PCA of the samples extruded at 80 rpm screw speed (Figure 5a), a negative correlation was found between the protein content and the fat content (vectors' position on opposite sides of the layout and α-angle <90°) in the pellets with Pearsons' coefficient at r=-0.57, while a positive correlation was noticed between the protein content and contents of the remaining components of the chemical composition (vectors' position on the same side of the layout), with the correlation coefficient indicating a strong relation between protein content and ash contents (r=0.98).

Among the snacks expanded by the microwave treatment, there was an increase in protein content with the increasing addition of fresh beetroot pulp. However, a significant ($p < 0.05$) difference as compared to the control sample was determined only at 20.0 and 30.0 g/100 g of fortification level. The PCA (Figure 5c) results showed a negative correlation between the protein content in the expanded snacks and the fat content and insoluble dietary fiber content due to the positions of vectors for these features, as confirmed also by Persons' coefficients (r=-0.53 and r=-0.83, respectively). Similarly to pellets, a positive correlation was shown between the protein content and ash (r=0.97) and soluble dietary fiber (r=0.83) contents of the microwaved snacks. No significant effects of the extrusion-cooking processing variables or the addition of beetroot pulp on the fat content of the pellets were noticed. However, a negative correlation was found between the fat content and the soluble dietary fiber (r=-0.80) and the total dietary fiber (r=-0.60) contents in the pellets. In starch-rich extrudates in general, proteins act as diluents and reduce expansion due to their ability to affect water distribution in the matrix, and also because their macromolecular structure and conformation affect the rheological properties of extruded blends. Proteins also contribute to the formation of extensive networking through covalent links and electrostatic interactions that take place during the extrusion-cooking [Aguilar-Palazuelos *et al.,* 2012]. This explains the negative correlation demonstrated between VEI and protein content in our study.

The microwave-expanded snacks with the addition of a minimum of 5.0% fresh beetroot pulp contained significantly ($p < 0.05$) less fat than the control snacks. Additionally, there was a significant correlation between contents of both fiber fractions and the fat content in RTE snacks. The character of correlation was positive for the insoluble dietary fiber (r=0.59) and negative for the soluble dietary fiber (r=-0.57). The lower fat content in the expanded snacks could be due to the formation of protein-starch-fat complexes during thermal treatment both under extrusion-cooking and microwave conditions. Therefore, it may be concluded that every thermal treatment can successfully reduce fat content to produce low-fat snacks. Ruiz-Armenta *et al.* [2019] found a similar tendency for lowering fat content in both pellets and microwave-expanded snacks, and the differences they observed were significant (*e.g.*, 1.50–2.47% for unprocessed material, 0.28–1.10% for pellets, and 0.26–0.46% for RTE product). In turn, Lisiecka & Wójtowicz [2021] found a much higher fat content in fried snacks supplemented with beetroot pulp, ranging from 22.27 to 30.76 g/100 g, and concluded that fresh beetroot inclusion up to 30.0 g/100 g only slightly affected the fat content in the final puffs. Using the microwave treatment to expand snack pellets allowed achieving low-fat snacks, which is a nutritionally beneficial way to limit fat intake in the human diet.

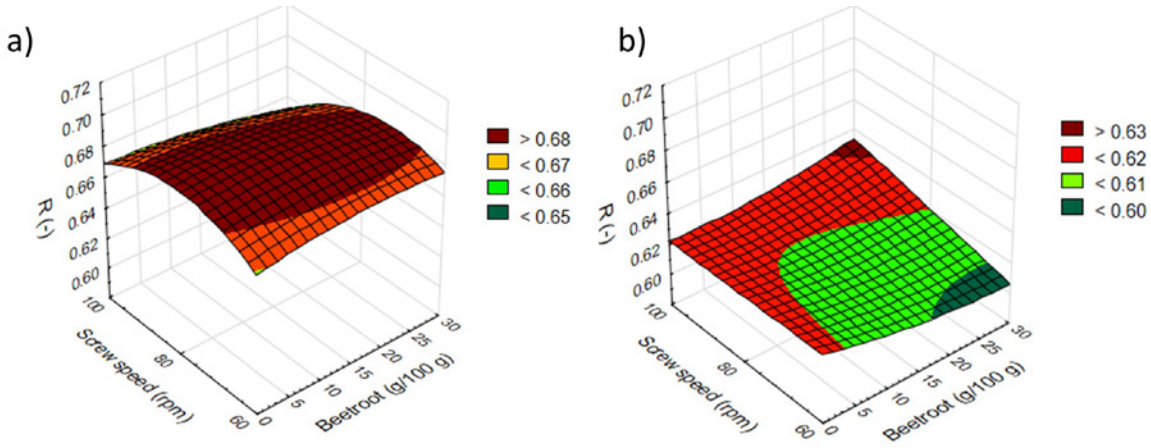


FIGURE 4. Absorbance intensity ratio R (-) of extruded pellets (a) and microwave-expanded snacks (b) supplemented with fresh beetroot pulp and processed at various screw speeds.

TABLE 3. Chemical composition of extruded pellets and microwave-expanded snacks supplemented with fresh beetroot pulp processed at the screw speed of 80 rpm.

Content of beetroot pulp (g/100 g)	Protein[1] (g/100 g)		Fat[1] (g/100 g)		Ash[1] (g/100 g)		Insoluble dietary fiber[2] (g/100 g)		Soluble dietary fiber[2] (g/100 g)		Total dietary fiber[2] (g/100 g)	
	P	S	P	S	P	S	P	S	P	S	P	S
0	4.07±0.19^{e}	4.22±0.29^{e}	0.34±0.18^{bc}	1.73±0.10^{b}	1.72±0.14^{ab}	1.83±0.02^{e}	4.77±0.09^{d}	3.21±0.10^{a}	0.66±0.10^{f}	5.63±0.11^{cd}	5.44±0.19^{d}	8.84±0.12^{c}
2.5	4.34±0.23^{e}	4.38±0.20^{e}	0.46±0.24^{a}	1.95±0.06^{a}	1.75±0.09^{ab}	2.00±0.01^{d}	5.12±0.11^{c}	3.13±0.12^{a}	0.77±0.04^{f}	5.35±0.12^{d}	5.49±0.15^{d}	8.88±0.14^{c}
5.0	4.57±0.20^{e}	4.49±0.20^{e}	0.15±0.08^{c}	0.18±0.03^{c}	1.82±0.11^{ab}	1.82±0.02^{e}	5.13±0.05^{c}	3.16±0.08^{a}	3.81±0.07^{b}	5.84±0.14^{c}	8.95±0.12^{b}	9.31±0.10^{b}
7.5	4.10±0.21^{e}	4.24±0.24^{e}	0.13±0.02^{c}	0.13±0.03^{cd}	1.69±0.07^{b}	1.80±0.04^{e}	5.08±0.08^{c}	2.43±0.10^{c}	1.55±0.05^{e}	5.26±0.11^{a}	6.63±0.13^{d}	7.69±0.14^{e}
10.0	4.62±0.21^{c}	4.71±0.21^{bc}	0.08±0.04^{c}	0.10±0.04^{cd}	2.00±0.20^{ab}	2.15±0.01^{c}	5.05±0.06^{c}	2.82±0.06^{b}	3.06±0.05^{c}	7.23±0.12^{a}	8.11±0.11^{c}	10.06±0.15^{a}
20.0	5.39±0.15^{b}	5.16±0.27^{b}	0.11±0.07^{c}	0.04±0.01^{d}	2.26±0.17^{ab}	2.51±0.02^{b}	9.74±0.06^{a}	1.14±0.08^{d}	4.02±0.07^{a}	6.53±0.13^{b}	13.75±0.13^{a}	7.67±0.16^{e}
30.0	5.76±0.12^{a}	5.90±0.22^{a}	0.06±0.01^{c}	0.02±0.01^{d}	2.51±0.24^{a}	3.57±0.05^{a}	5.75±0.08^{b}	1.24±0.06^{d}	2.83±0.05^{d}	7.31±0.11^{a}	8.58±0.13^{bc}	8.56±0.14^{d}

P – extruded pellet, S – microwave-expanded snack; Values are presented as mean ± standard deviation; [1] means of 3 replications, [2] means of 2 replications;
[a–e] means indicated with the same letters in columns do not differ significantly at α=0.05.

Pellets and snacks were, in general, characterized by a low ash content, while the maximum content of beetroot pulp in the recipe caused the highest ash content for both pellets and snacks (Table 3). The ash content of the pellets was positively correlated with the content of insoluble, soluble, and total dietary fiber contents, as shown by positions of vectors in Figure 5a, and as confirmed by Pearsons' coefficients (r=0.54, 0.58, and 0.63, respectively). In the case of microwave-expanded snacks, the ash content negatively correlated with the insoluble dietary fiber content (r=-0.80), and positively with the soluble dietary fiber content (r=0.75). The extrudates had significantly more insoluble dietary fiber compared to the control blend before extrusion (Table 3). However, the microwave treatment brought about a decrease in the insoluble dietary fiber and an increase in the soluble dietary fiber contents in the expanded snacks as compared to the pellets.

A positive correlation was found in the pellets between the insoluble dietary fiber content and the soluble (r=0.56) and total dietary fiber fractions (r=0.90). A strong correlation between soluble and total dietary fiber contents in the pellets (r=0.86) was noticed as well. In the case of the expanded snacks (Figure 5c), a negative correlation was noted between the insoluble and the soluble dietary fiber fractions (r=-0.57), whereas a positive correlation was found between the insoluble and the total dietary fiber fractions (r=0.53). The total fiber content in the pellets ranged from 5.44 g/100 g (control sample), to 13.75 g/100 g (the pellets with 20.0 g/100 g addition of fresh beetroot pulp) (Table 3), while in the blend before extrusion, it was at 5.94 g/100 g. In the microwave-expanded snacks, the total fiber content ranged from 7.67 g/100 g (the expanded snacks with 20.0 g/100 g addition of fresh beetroot pulp) to 10.06 g/100 g (the expanded snacks with 10.0 g/100 g addition of fresh beetroot pulp). Probably, due to the high amount of fiber-rich beetroot pulp, the samples with its 30 g/100 g content underwent lesser transformation during extrusion. After microwave expansion, the structure of snacks with a higher pulp content was different than that of the snacks with 20 g/100 g of the pulp content, which was also confirmed by the FTIR analysis, especially when looking at starch transformation after microwaving. During the extrusion-cooking, fibrous fractions of beetroot may disrupt the formation of starch and protein complexes, which could also affect contents of soluble and insoluble dietary fiber. Generally, the content of soluble dietary fiber increases after extrusion. It may be the effect of microwave treatment that is changing material structure, as confirmed by differences in FTIR spectra of pellets and expanded snacks. Moreover, it was found that VEI decreased with an increasing content of soluble dietary fiber in the extrudates. Delgado-Nieblas *et al.* [2012] concluded that a decrease in VEI could result from the high content of dietary fiber in winter squash, because fiber can make the cell walls collapse during the formation of steam bubbles, reducing the ability to retain air inside the formed cells and therefore reducing expansion. Ruiz-Armenta *et al.* [2019] reported lower crude fiber contents in pellets and microwave-expanded snacks, ranging from 0.94 to 1.94% for unprocessed material based on blends of corn starch/whole yellow corn flour with naranjita bagasse

and/or skimmed milk powder, through 0.83 to 1.88% for pellets, and to 0.78 to 1.75% for expanded products depending on the recipe variant. The high dietary fiber content of the snacks supplemented with fresh beetroot pulp means that they could be labelled as "high fiber products" according to the European Regulation (more than 6 g/100 g of fiber) [Regulation (EC) No 1924/2006, 2006].

PCA results

Figures 5a and 5c present variables for pellets and snacks, respectively (position of the load vectors in relation to the first two principal components). The distance and location of the studied properties between each other indicates the type of correlation between features (positive or negative – depending on the position of vector in an adequate square on graph and α-angle value). The position of vectors at the same part of the layout and an α-angle below 90° indicate a positive correlation, whereas opposite location of vectors indicate a negative correlation. In addition, together with the PCA, a correlation matrix was generated between the tested features showing the exact Pearsons' correlation coefficients cited in the manuscript. Approximately 74% of all the variance in the pellets data was explained by the first two principal components, PC1 (54.58%) and PC2 (19.14%). PC1 was strongly positively correlated with β-sheet protein and fat contents and strongly negatively correlated with random coil structure content, as well as with other components of chemical composition. In addition, PC2 was strongly positively correlated with R (Figure 5a). After placing the samples

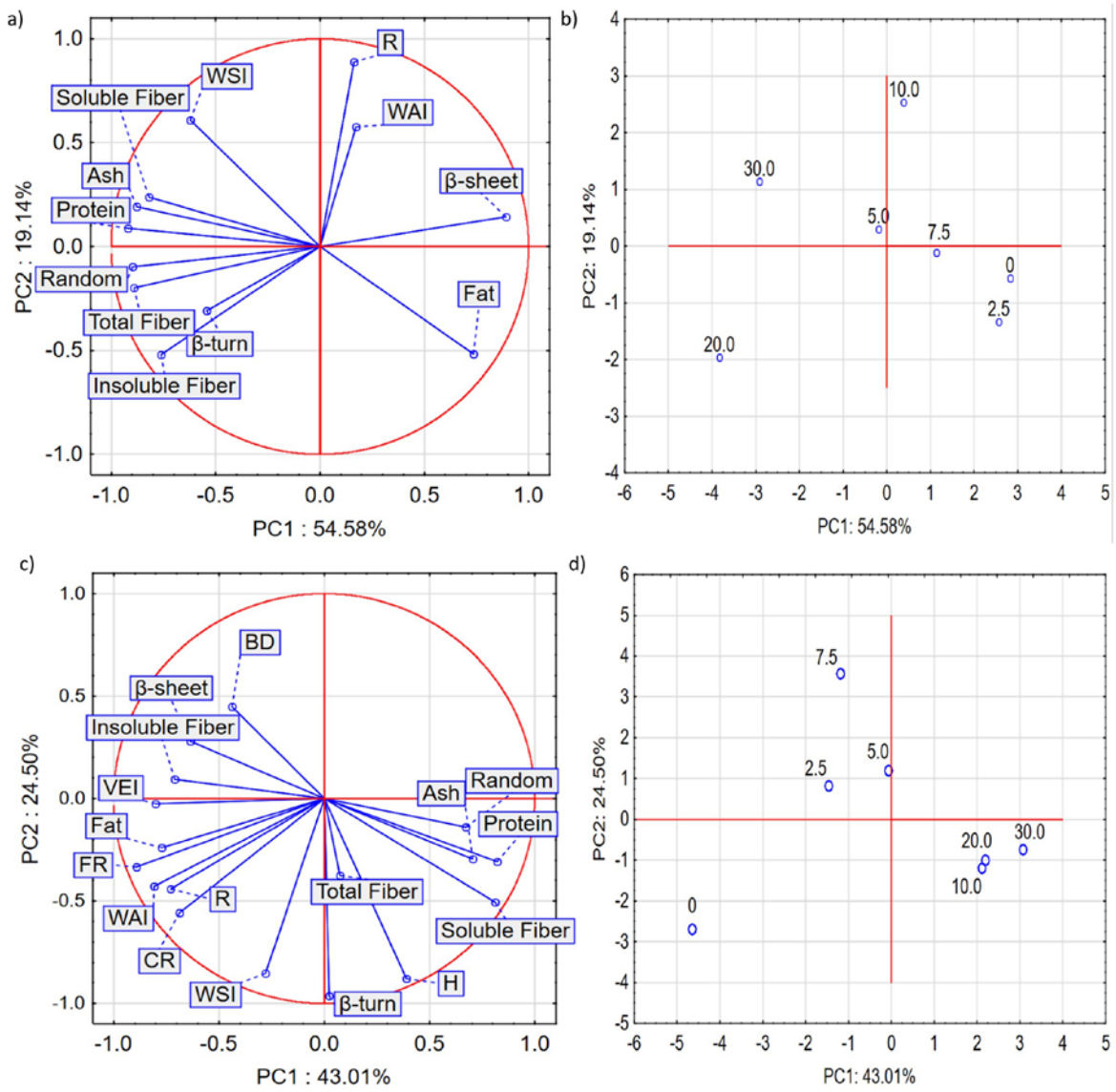


FIGURE 5. Principal component analysis (PCA) of extruded pellets (a) and expanded snacks (b) processed at 80 rpm, as well as objects characteristics of pellets (c) and snacks (d) in the space of the first two principal components (PC1 and PC2); 0–30.0 – content of fresh beetroot pulp (g/100 g). WAI – water absorption index; WSI – water solubility index; BD – bulk density; VEI – volumetric expansion index; H – hardness; CR – crispness; FR – fracturability; β-sheet – β-sheet content; β-turn – β-turn content; Random – random coil structure; R – absorbance intensity ratio; Protein – protein content; Fat – fat content; Ash – ash content; Insoluble fiber – insoluble dietary fiber content; Soluble fiber – soluble dietary fiber content; Total fiber – total dietary fiber content.

in the space of the first two components (Figure 5b), they differed from each other depending on beetroot pulp content. Significant differences can be observed between the pellets with 20.0 g/100 g and higher fresh beetroot pulp contents and those with lower pulp contents. From this plot, it can be concluded that the first principal component describes the system variability and determines the level of the additive in the pellets. In turn, in RTE snacks analysis, the first principal component (PC1) explained 43.01% of the total variance in the results and was strongly positively correlated with protein, ash, and soluble fiber contents as well as with random coil values, and negatively with WAI, fat content, insoluble fiber content, R ratio values, expansion index, and fracturability. What is more, the second principal component (PC2) explained 24.50% of the total variance of the results and was strongly negatively correlated with β-turn protein content, WSI, and hardness (Figure 5c). Placing the supplemented expanded snacks in the space of the examined principal component (Figure 5d) showed, in general, increasing differences between the samples as the fresh beetroot pulp amount increased, while some similarity was observed for the samples with the beetroot pulp content ranging from 10.0 to 30.0 g/100 g. From this graph, it can be concluded that the first principal component describes the system variability and determines the parameter of the fresh beetroot pulp content in the microwave-expanded snacks.

When assessing correlations of chemical composition and physical properties of the pellets and snacks by the PCA, it was shown that WAI of the pellets was negatively correlated (Figure 5a) with the content of soluble fiber, with a correlation coefficient at r=-0.60. In our research, a negative correlation was noticed between the WAI value and protein content (r=-0.60), soluble dietary fiber content (r=-0.52), and ash content (r=-0.55) of the snacks. In turn, a positive correlation (Figure 5c) was noticed between the WAI and fat content (r=0.79), insoluble dietary fiber content (r=0.66), and WSI (r=0.62) of the snacks. Additionally, PCA and correlation coefficients calculated showed the WSI of pellets was positively correlated (Figure 5a) with their contents of protein (r=0.65), ash (r=0.67), soluble dietary fiber (r=0.73), and the total dietary fiber (r=0.56), and negatively correlated with their fat content (r=-0.67). Our work confirmed the change in the crystallization of starch after processing of both extruded and microwaved samples as compared to native starch granules (Figure 5a and 5c, respectively).

PCA and correlation coefficients calculated for the snacks showed that their bulk density was negatively correlated (Figure 5c) with their content of random coil protein fraction (r=-0.87), and positively correlated with β-sheet protein fraction (r=0.93). While VEI was positively correlated with WAI (r=0.68), fat content (r=0.76), insoluble dietary fiber content (r=0.70), and fracturability (r=0.80). Additionally, VEI was negatively correlated with contents of protein (r=-0.86), ash (r=-0.80) and soluble dietary fiber (r=-0.69). Hardness was only positively correlated with β-turn protein fraction (r=0.80), WSI (r=0.67), protein content (r=0.64), ash content (r=0.60), and soluble dietary fiber content (r=0.70). Crispness was positively correlated with R-ratio of starch (r=0.96), WAI (r=0.66), WSI (r=0.72), and fracturability (r=0.84), and negatively correlated with random coil structure content (r=-0.63). Fracturability was negatively correlated with protein content (r=-0.62) and positively correlated with R-ratio of starch (r=0.76), WAI (0.77), fat content (r=0.82), and insoluble dietary fiber content (r=0.59).

The structure analysis of pellets and expanded snacks made by FTIR was also analyzed by PCA as the function of two main variables and correlation coefficients calculated, and was connected with the chemical composition as well as physical properties of the tested samples. We noted that the β-sheet content of pellet proteins was negatively correlated with β-turn (r=-0.71), random coil (r=-0.98) protein structure, the total protein content (r=-0.80), ash (r=-0.76), insoluble, soluble and total dietary fiber (r=-0.62, r=-0.60, and r=-0.69, respectively), while positively correlated with fat content (with r=0.64) in semi-products (Figure 5a). The β-sheet of microwave-expanded snack proteins was only negatively correlated (Figure 5c) with the random coil structure, with Pearsons' correlation coefficients expressed as r=-0.98 and with soluble dietary fiber content (r=-0.60), and positively correlated with bulk density (r=0.92) and crispness (r=0.52). Moreover, the pellet protein β-turn fraction was positively correlated with random coil structure (r=0.57), protein content (r=0.72), and content of insoluble dietary fiber (r=0.69), while the β-turn protein fraction of the microwaved snacks was positively correlated with WSI (r=0.72) and soluble dietary fiber content (r=0.52), hardness (r=0.80) and crispness (r=0.54) of snacks, and negatively correlated with BD (r=-0.59). The pellet protein random coil structure was correlated with all chemical components, but the strongest correlation was found between random coil structure and protein (r=0.75) and total dietary fiber (r=0.75) contents. In the case of expanded snacks supplemented with fresh beetroot pulp, random coil structure was positively correlated only with soluble dietary fiber content (r=0.52) and negatively with BD (r=-0.87), crispness (r=-0.63) and fracturability (r=-0.58). In turn, the R ratio of pellets was positively correlated with WSI (r=0.54) and negatively correlated with insoluble dietary fiber content (r=-0.58). Moreover, the R of the microwave-expanded snacks was positively correlated with WAI (r=0.70), WSI (r=0.68), β-sheet protein fraction (r=0.55), crispness (r=0.96), and fracturability (r=0.76), but negatively with random coil structure content (r=-0.64).

Hence, the application of PCA provided an overall view of the correlations between all the tested features of newly developed pellets and snacks supplemented with fresh beetroot pulp and identified the main components important in quality analysis of extruded pallets and microwave-expanded low-fat snacks.

CONCLUSIONS

Selected physical properties as well as texture and structure parameters were analyzed for both extruded snack pellets and microwave-expanded snacks. The use of fresh beetroot pulp in RTE snacks caused a reduction in their volumetric expansion due to increased contents of dietary fiber and protein instead of starch. The addition of vegetable pulp also resulted in the stabilization of the protein backbone structure, which was expressed by a decrease in random coil structure and an increase in β-turn protein fraction. Interestingly, the ranges

of the fractions representing protein structures in the snacks were narrower than those observed for the pellets by FTIR analysis. This indicates that microwave treatment also resulted in greater protein stabilization. The highest content of β-structure protein fraction and the smallest content of random coil was observed if 80 rpm screw speed was applied during pellet processing. In the microwave-expanded snacks supplemented with beetroot, an increase in the fresh vegetable pulp addition raised the protein content. Moreover, the addition of 5.0 g/100 g and more of fresh beetroot pulp as a starch substitute significantly reduced the fat content in the snacks compared to the potato control sample. We found that the extrusion-cooking caused an increase in the insoluble dietary fiber content and WAI in the obtained pellets, but the microwave treatment reduced the value of this parameter and increased insoluble dietary fiber content in the final RTE snacks compared to the semi-products. The highest fraction of β-structures and the smallest content of random coil were observed when 80 rpm was applied during processing, suggesting this screw speed to be the most efficient in processing fresh beetroot pulp-supplemented snack pellets. The use of a fresh beetroot pulp as an additive in microwave-expanded snacks made it possible to obtain products with an increased amount of fiber and a reduced fat content as an attractive and healthy alternative to deep-fried snacks. Moreover, application of fresh beetroot in extruded products allows minimizing the technological water and energy consumption due to the omission of beetroot drying step.

RESEARCH FUNDING

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

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

ORCID IDs

K. Lisiecka https://orcid.org/0000–0003–0659–8704
A. Sujak https://orcid.org/0000–0001–5616–3827
A. Wójtowicz https://orcid.org/0000–0001–5981–6109

REFERENCES

1. AACC. (1995). Approved Methods of the American Association of Cereal Chemists (9th ed.). St. Paul, USA.
2. Aguilar-Palazuelos, E., Zazueta-Morales, J., Harumi, E.N., Martínez-Bustos, F. (2012). Optimization of extrusion process for production of nutritious pellets. *Food Science and Technology, 32*(1), 34–42. https://doi.org/10.1590/S0101-20612012005000005
3. Alvarez, M.D., Paniagua, J., Herranz, B. (2020). Assessment of the miniature Kramer Shear Cell to measure both solid food and bolus mechanical properties and their interplay with oral processing behavior. *Foods, 9*, art. no. 613. https://doi.org/10.3390/ foods9050613
4. AOAC. (2000). Official Methods of AOAC International (17th ed.). Gaithersburg, USA.
5. Bhat, N.S., Wani, I.A., Hamdani, A.M., Gani, A. (2019). Effect of extrusion on the physicochemical and antioxidant properties of value added snacks from whole wheat (*Triticum aestivum* L.) flour. *Food Chemistry*, *276*, 22–32. https://doi.org/10.1016/j.foodchem.2018.09.170
6. Błaszczak, W., Valverde, S., Fornal, J. (2005). Effect of high pressure on the structure of potato starch. *Carbohydrate Polymers*, *59*, 377–383. https://doi.org/10.1016/j.carbpol.2004.10.008
7. Bock, J.E., Damodaran, S. (2013). Bran-induced changes in water structure and gluten conformation in model gluten dough studied by Fourier transform infrared spectroscopy. *Food Hydrocolloids*, *31*, 146–155. https://doi.org/10.1016/j.foodhyd.2012.10.014
8. Chen, F., Zhang, M., Mujumdar, A.S., Jiang, H., Wang, L. (2014). Production of crispy granules of fish: a comparative study of alternate drying techniques. *Drying Technology, 32*(12), 1512–1521. https://doi.org/10.1080/07373937.2014.903410
9. Delgado-Nieblas, C., Aguilar-Palazuelos, E., Gallegos-Infante, A., Rocha-Guzmán, N., Zazueta-Morales, J., Caro-Corrales, J. (2012). Characterization and optimization of extrusion cooking for the manufacture of third-generation snacks with winter squash (*Cucurbita moschata* D.) flour. *Cereal Chemistry, 89*, 65–72. https://doi.org/10.1094/CCHEM-02-11-0016
10. Fetouhi, A., Benatallah, L., Nawrocka, A., Szymańska-Chargot, M., Bouasla, A., Tomczyńska-Mleko, M., Zidoune M.N., Sujak, A. (2019). Investigation of viscoelastic behaviour of rice-field bean gluten free dough using the biophysical characterization of proteins and starch: a FT-IR study. *Journal of Food Science and Technology*, *56*(3), 1316–1327. https://doi.org/10.1007/s13197-019-03602-2
11. Han, Y., Tran, T., Le, V. (2018). Corn snack with high fiber content: Effects of different fiber types on the product quality. *LWT – Food Science and Technology, 96*, 1–6. https://doi.org/10.1016/j.lwt.2018.05.014
12. Hernández-Uribe, J.P., Ramos-López, G., Yee-Madeira, H., Bello-Pérez, L.A. (2010). Physicochemical, rheological and structural characteristics of starch in maize tortillas. *Plant Foods for Human Nutrition*, *65*, 152–157. https://doi.org/10.1007/s11130-010-0165-5
13. Kaur, G.J., Rehal, J., Singh, B., Singh, A.K., Kaur, A. (2015). Development of multigrain breakfast cereal using extrusion technology. *Asian Journal of Dairy & Food Research*, *34*(3), 219–224. https://doi.org/10.5958/0976-0563.2015.00043.3
14. Kowalczuk, D., Pitucha, M. (2019). Application of FTIR method for the assessment of immobilization of active substances in the matrix of biomedical materials. *Materials (Basel)*, *12*(18), art. no. 2972. https://doi.org/10.3390/ma12182972
15. Krishnaraj, P., Anukiruthika, T., Choudhary, P., Moses, J.A., Anandharamakrishnan, C. (2019). 3D extrusion printing and post-processing of fibre-rich snack from indigenous composite flour. *Food and Bioprocess Technology*, *12*, 1776–1786. https://doi.org/10.1007/s11947-019-02336-5
16. Lisiecka, K., Wójtowicz, A. (2021). Effect of fresh beetroot application and processing conditions on some quality features of new type of potato-based snacks. *LWT – Food Science and Technology, 141,* art. no. 110919. https://doi.org/10.1016/j.lwt.2021.110919

17. Lisiecka, K., Wójtowicz A. (2020). Possibility to save water and energy by application of fresh vegetables to produce supplemented potato-based snack pellets. *Processes*, *8*(2), art. no. 153. https://doi.org/10.3390/pr8020153
18. Lisiecka, K., Wójtowicz, A. (2019). The influence of fresh kale addition on selected properties of corn snacks. *International Journal of Food Engineering*, *15*(11–12), 1–11. https://doi.org/10.1515/ijfe-2018-0319
19. Lisiecka, K., Wójtowicz, A., Mitrus, M., Oniszczuk, T., Combrzyński, M. (2021). New type of potato-based snack-pellets supplemented with fresh vegetables from the *Allium* genus and its selected properties. *LWT – Food Science and Technology, 145*, art. no. 111233. https://doi.org/10.1016/j.lwt.2021.111233
20. Lourenço, L.F.H., Tavares, T.S., Araujo, E.A.F., Eder, A.F., Pena, R.S., Rosinelson, S., Peixoto Joele, M.R.G., Carvalho, A.V. (2016). Optimization of extrusion process to obtain shrimp snacks with rice grits and polished rice grains. *CyTA: Journal of Food*, *14*(2), 340–348. https://doi.org/10.1080/19476337.2015.1114025
21. Meziani, S., Jasniewski, J., Gaiani, C., Ioannou, I., Muller, J.M., Ghoul, M., Desobry, S. (2011). Effects of freezing treatments on viscoelastic and structural behavior of frozen sweet dough. *Journal of Food Engineering*, *107*, 358–365. https://doi.org/10.1016/j.jfoodeng.2011.07.003
22. Mikołajczyk-Bator, K., Błaszczyk, A., Czyżniejewski, M., Kachlicki, P. (2016). Characterisation and identification of triterpene saponins in the roots of red beets (*Beta vulgaris* L.) using two HPLC-MS systems. *Food Chemistry*, *1*(192), 979–90. https://doi.org/10.1016/j.foodchem.2015.07.111
23. Pytlak, A., Szafranek-Nakonieczna A., Sujak, A., Grządziel, J., Polakowski, C., Kuźniar, A., Proc, A., Kubaczyński, A., Goraj, W., Gałązka, A., Gruszecki, W.I., Bieganowski, A., Stępniewska, Z. (2020). Stimulation of methanogenesis in bituminous coal from the upper Silesian coal basin. *International Journal of Coal Geology*, *231*(1), art. no. 103609. https://doi.org/10.1016/j.coal.2020.103609
24. Rashid, S., Rakha, A., Anjum, F., M., Ahmed, W., Sohail, M. (2015). Effects of extrusion cooking on the dietary fibre content and Water Solubility Index of wheat bran extrudates. *Journal of Food Science and Technology, 50*(7), 1533–1537. https://doi.org/10.1111/ijfs.12798
25. Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. *Official Journal of the European Union*, OJ L 404, 30.12.2006. https://eur-lex.europa.eu/eli/reg/2006/1924/2014-12-13
26. Ruiz-Armenta, X.A., Zazueta-Morales, J.D.J., Delgado-Nieblas, C.I., Carrillo-López, A., Aguilar-Palazuelos, E., Camacho-Hernández, I.L. (2019). Effect of the extrusion process and expansion by microwave heating on physicochemical, phytochemical, and antioxidant properties during the production of indirectly expanded snack foods. *Journal of Food Processing and Preservation*, *43*(12), art. no. e14261. https://doi.org/10.1111/jfpp.14261
27. Sakač, N., Karnaš, M., Dobša, J., Jozanović, M., Gvozdić V., Kovač-Andrić, E., KraševacSakač, M., Šarkanj, B. (2020). Application of spectrophotometric fingerprint in cluster analysis for starch origin determination. *Food Technology & Biotechnology*, *58*(1), 5–12. https://doi.org/10.17113/ftb.58.01.20.6239
28. Sanchez-González, J.A., Echeverria, C., Lescano, L., Linares, G., Arteaga-Miñano H.L., Soriano-Colchado, J., Barraza-Jáuregui, G. (2019). Physico-chemical, thermal and rheological characteristics of starch isolated from four commercial potatoes cultivars. *Scientia Agropecuaria*, *10*(1), 63–71. https://doi.org/10.17268/sci.agropecu.2019.01.07
29. Seabourn, B.W., Chung, O.K., Seib, P.A., Mathewson, P.R. (2008). Determination of secondary structural changes in gluten proteins during mixing using Fourier transform horizontal attenuated total reflectance spectroscopy. *Journal of Agricultural and Food Chemistry*, *56*, 4236–4243. https://doi.org/10.1021/jf703569b
30. Shah, F.U.H., Sharif, M.K., Butt, M.S., Shahid, M. (2017). Development of protein, dietary fiber, and micronutrient enriched extruded corn snacks. *Journal of Texture Studies, 48*, 221–230. https://doi.org/10.1111/jtxs.1223
31. Sharma, S., Singh, N., Singh, B. (2015). Effect of extrusion on morphology, structural, functional properties and *in vitro* digestibility of corn, field pea and kidney bean starches. *Starch/Stärke*, *67*, 721–728. https://doi.org/10.1002/star.201500021
32. Smits, A.L.M., Ruhnau, F.C., Vliegenthart J.F.G., van Soest, U.F.G., van Soest, J.J.G. (1998). Ageing of starch based systems as observed with FT-IR and solid state NMR spectroscopy. *Starch/Stärke*, *50*, 478–483. https://doi.org/10.1002/(SICI)1521-379X(199812)50:11/12<478::AID-STAR478>3.0.CO;2-P
33. Susi, H., Byler, D.M. (1983). Protein structure by Fourier transform infrared spectroscopy: second derivative spectra. *Biochemical and Biophysical Research Communications*, *115*, 391–397. https://doi.org/10.1016/0006-291X(83)91016-1
34. van Soest, J.J.G., Tourois, H., de Wit, D., Veliegenthart, J.F.G. (1995). Short-ranges structure in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-transform IR spectroscopy. *Carbohydrate Research*, *279*, 201–214. https://doi.org/10.1016/0008-6215(95)00270-7

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 225–236
On-line ISSN: 2083-6007
Print ISSN: 1230-0322
DOI: 10.31883/pjfns/138320
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Phenolic Composition, and Antioxidant and Antineurodegenerative Potential of Methanolic Extracts of Fruit Peel and Flesh of Pear Varieties from Serbia

Aleksandra Savić[1,], Ana Alimpić Aradski[2], Jelena Živković[3], Katarina Šavikin[3], Snežana Jarić[4], Petar D. Marin[2], Sonja Duletić-Laušević[2]*

[1]*Natural History Museum in Belgrade, Njegoševa 51, 11000 Belgrade, Serbia*
[2]*Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia*
[3]*Institute for Medicinal Plant Research 'Dr Josif Pančić', Tadeuša Košćuška 1, 11000 Belgrade, Serbia*
[4]*Department of Ecology, Institute for Biological Research 'Siniša Stanković' – National Institute of the Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia*

Key words: pear fruit, traditional varieties, methanolic extracts, phenolic composition, antioxidant activity, antineurodegenerative activity

Pear (*Pyrus communis* L.) is consumed as fresh fruit, in numerous food products, and also used as a traditional remedy in various countries, including Serbia. In search of bioactive compounds, six traditional pear varieties ('Vidovača', 'Lubeničarka', 'Karamanka', 'Jeribasma', 'Lončara', 'Takiša') and wild pear from Serbia were investigated and compared with a commercial variety ('Williams Bartlett'). The aim of this study was to determine the total phenolic and flavonoid contents, phenolic composition, antioxidant capacity, and antineurodegenerative activities of methanolic extracts of peel, flesh, and mixed peel and flesh of pear fruits. Phenolic composition of extracts was determined with HPLC-DAD, while the antioxidant activity of extracts was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and ferric-reducing antioxidant power (FRAP) assays. Bearing in mind that oxidative stress is closely linked to neurodegeneration, the antineurodegenerative potential of the extracts was assessed by the inhibition of acetycholineserase (AChE) and tyrosinase (TYR) activities. The extracts of traditional varieties, particularly peel extracts, had a high content of phenolics, as well as significant antioxidant and moderate antineurodegenerative potential, compared to the commercial variety. The highest contents of total flavonoids and individual compounds, such as arbutin and chlorogenic acid, as well as the strongest antioxidant and TYR inhibitory activities were reported for the 'Takiša' peel extract. The performed analyses have revealed that fruits of traditional Serbian pear varieties are rich in bioactive components and could be used as functional food and for possible nutraceutical applications, to prevent diseases induced by oxidative stress.

ABBREVIATIONS

TPC, total phenolic content; TFC, total flavonoid content; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP, ferric-reducing antioxidant power; BHA, 2(3)-tert-butyl-4-hydroxyanisole; BHT, 3,5-di-tert-butyl-4-hydroxytoluene; TPTZ, 2,4,6-tripyridyl-s-triazine; L-DOPA, 3,4-dihydroxy-L-phenylalanine; AAE, ascorbic acid equivalents; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); AChE, acetylcholinesterase; TYR, tyrosinase; HPLC-DAD, high-performance liquid chromatography with diode array detection.

INTRODUCTION

Pear (*Pyrus communis* L.), belonging to the Rosaceae family, is one of the most widespread fruits in the world. It is the fifth most produced fruit worldwide, but, despite over 3000 pear cultivars, there are only a few dozen globally produced pear varieties [Brahem *et al.,* 2017]. Because of their desirable taste and good digestibility, pears are widely consumed as fresh or dry fruits, juices, and numerous products.

The pear has been used as a traditional remedy in China for more than 2000 years [Li *et al.,* 2012, 2014; Reiland & Slavin, 2015], and also in Ancient Greece [Parle & Arzoo, 2016]. Ethnobotanical surveys of the traditional use of *P. communis* L. fruit have shown similar usability of pear for consumption and medicinal purposes (against hypertension, diabetes, high cholesterol, constipation, as uroseptic,

* Corresponding Author:
E-mail aleksandra.savic@nhmbeo.rs (A. Savić)

Submitted: 22 January 2021
Accepted: 30 May 2021
Published on-line: 10 June 2021

antirheumatic, for body mass reduction) also in the Balkan region [Dajić-Stevanović *et al.,* 2014; Savić *et al.,* 2019]. Several previous studies investigated fruits, their nutritional components, and bioactivities of flesh and peel of different pear varieties grown worldwide [Kolniak-Ostek, 2016; Kolniak-Ostek *et al*., 2020; Li *at al.,* 2014, 2016b; Liaudanskas *et al*., 2017; Öztürk *et al.,* 2015; Reiland & Slavin 2015; Salta *et al.,* 2010; Sharma *et al.,* 2015]. Additionally, several investigations proved that, besides fruit, each part of the pear tree (leaves, bark, root) possesses phenolics with notable antioxidant activities [Carbonaro *et al.,* 2002; Imeh & Khokhar, 2002], providing numerous health benefits [Kolniak-Ostek, 2016; Öztürk *et al.,* 2015; Parle & Arzoo 2016].

The health-promoting, nutritional, and sensorial quality of the pear fruit is influenced by the pear species and variety from which it originates. Chemical composition analyses of pear fruits have demonstrated that they represent essential sources of sugars, vitamins, amino acids, minerals, phenolics, organic acids, and other chemical constituents important for human nutrition and health boosting [Li *et al.,* 2012, 2014; Salta *et al.,* 2010]. Phenolics in the pear fruit contribute to its sensory quality and are strongly linked to many health benefits and antioxidant activity [Brahem *et al.,* 2017]. Phenolic compounds are more concentrated in the peel of the fruit, compared to flesh [Brahem *et al.,* 2017; Li *et al.,* 2014; Kolniak-Ostek, 2016].

The studies of chemical composition of pear fruit revealed the presence of phenolics, such as arbutin, (–)-epicatechin, quercitrin, isoquercitrin, kaempferol, astragalin, tannins, as well as triterpenoids including friedelin, squalene, sterols, and saponins [Kaur & Arya, 2012; Kolniak-Ostek, 2016; Liaudanskas *et al*., 2017; Sharma *et al.,* 2015]. Phenolic acids (chlorogenic, syringic, ferulic, and coumaric acids) [Lin & Harnly, 2008; Salta *et al.,* 2010; Sharma *et al.,* 2015], organic acids (malic, citric, and shikimic acids), as well as vitamins C and E were also detected in pear fruits [Kolniak-Ostek, 2016; Öztürk *et al.,* 2015].

Methanol is commonly used to extract phenolics from fruit parts [Chel-Guerrero *et al.,* 2018]. The chemical analyses performed by Li *et al.* [2012] and Öztürk *et al.* [2015] revealed that methanolic extracts of pear fruit contained phenolic compounds, such as chlorogenic acid, arbutin, (+)-catechin, and (–)-epicatechin, followed by quercetin and rutin, predominantly in the peel. In addition to a high content of the phenolics, the peel is an excellent source of triterpenoids [Kolniak-Ostek, 2016; Li *et al.,* 2014].

The phenolic compounds are well known for their antioxidant activity. The antiviral activity of specific flavonoid groups, such as flavanones, flavonols, and catechins, has also been reported against various viral strains, as well as SARS-CoV and MERS-CoV, which suggests that functional food has a huge potential to strengthen the immune system and provide treatment for the ongoing epidemic of COVID-19 [Adem *et al.,* 2020]. Besides, phenolic compounds of various fruits can play a beneficial therapeutic role in the treatment of oxidative stress-induced neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease [Wilson *et al.,* 2017].

The aim of the present study was to evaluate and compare the total phenolic and flavonoid contents, the phenolic composition, as well as the antioxidant and antineurodegenerative activities of methanolic extracts of peel, flesh, and mixed peel and flesh of six traditional and rare pear varieties from Serbia ('Vidovača', 'Lubeničarka', 'Karamanka', 'Jeribasma', 'Lončara', and 'Takiša'), and wild pear, and comparing them to one commercial variety ('Williams Bartlett'). Additionally, the correlations were established between the content of phenolics and flavonoids and bioactivities tested. The mentioned pear varieties have never been studied before for chemical composition and bioactivities, hence they were subjected to detailed analyses.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, gallic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6--sulfonic acid) diammonium salt (ABTS), 2(3)-tert-butyl--4-hydroxyanisole (BHA), 3,5-di-tert-butyl-4-hydroxytoluene (BHT), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium acetate ($C_2H_3KO_2$), potassium-persulfate ($K_2S_2O_8$), dimethyl sulfoxide, aluminum nitrate nonahydrate ($Al(NO_3)_3 \times 9H_2O$), Folin--Ciocalteu reagent, sodium acetate (CH_3COONa), sodium carbonate anhydrous (Na_2CO_3), iron(III) chloride ($FeCl_3$), iron(II) sulfate heptahydrate ($FeSO_4 \times 7\ H_2O$), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetylcholine iodide, acetylcholinesterase from *Electrophorus electricus* (AChE), galanthamine hydrobromide from *Lycoris* sp., tyrosinase from mushroom, kojic acid, 3,4-dihydroxy--L-phenylalanine (L-DOPA), rutin hydrate (94%, HPLC), chlorogenic acid (95%, titration), hyperoside (97%, HPLC), arbutin (96%, HPLC), isoquercitrin (90%, HPLC), procyanidin B_1 (≥90%, HPLC) and procyanidin B_2 (>90%, HPLC), formic acid (98–100%), and acetonitrile (99.8%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Other used chemicals, such as ethanol, methanol, glacial acetic acid, and hydrochloric acid, were purchased from Zorka Pharma, Šabac (Serbia). Quercitrin (98.5%, HPLC) was purchased from Extrasynthese (Genay, France). Water was deionized using a MilliQ system (Millipore, Bedford, MA, USA).

Plant material

Fruits of eight different pear varieties were analyzed in this study (Figure 1). Among them, six are traditional varieties from organic production: 'Vidovača', 'Lubeničarka', 'Karamanka', 'Jeribasma', 'Takiša', 'Lončara' (from the hoods in central Serbia – Šumadija region), wild pear was collected from southwest Serbia, while fruits of commercial variety 'Williams Bartlett' were purchased at a local market. Fruits were sampled in 2016, at their optimal ripening stage recommended for consumption (from July till October). The collected material was stored at -20°C until the extracts preparation.

The fruit sizes varied between varieties: from approximately 20–25 g (wild pear and 'Takiša'); 30–50 g ('Vidovača' and Lubeničarka); 120–220 g ('Williams Bartlett', 'Karamanka', 'Jeribasma') to 220–280 g ('Lončara'). In the case of smaller fruits (wild pear, 'Takiša', 'Vidovača' and 'Lubeničarka'), 4–10 fruits were used for the extraction; and in the case of the larger ones ('Williams Bartlett', 'Karamanka', 'Jeribasma', 'Lončara'), 1–2 fruits were used.

Preparation of extracts

Before extracts preparation, the frozen fruits were peeled (skin thickness approx. 0.5 mm) for the peel sample, and mashed in the jar. The mixed peel and flesh, and pure flesh samples were cut into cubes (1 cm^3) and separately homogenized using a laboratory blender (No. 8010ES, Waring® Laboratory Science, Torrington, CT, USA). The pear extracts were prepared using methanol as followed: 10 g of peel were extracted with 10 mL of methanol; 30 g of flesh were extracted with 30 mL of methanol, and 30 g of peel and flesh were extracted with 30 mL of methanol. The extractions of the material were performed three times, every 24 h for 72 h at room temperature. The obtained liquid extracts were mixed and filtered (Whatman No.1) and subsequently concentrated under reduced pressure (rotavapor R-114, BÜCHI Labortechnik AG, Flawil, Switzerland). Yields of crude extracts were calculated and dry extracts were subsequently stored at +4°C. Stock solutions were prepared in methanol in a concentration of 1 mg/mL freshly before experiments.

Determination of total phenolic content

The total phenolic content of pear extracts was measured spectrophotometrically [Singleton & Rossi, 1965] with slight modification. The reaction mixture containing 0.2 mL of the extract in concentration of 1 mg/mL, 1.0 mL of 10% Folin-Ciocalteu reagent, and 0.8 mL of 7.5% Na_2CO_3 were incubated for 2 h, at room temperature in the dark. Distilled water was used as a blank, while control was prepared to contain the distilled water instead of the sample. Absorbances were recorded using a JENWAY 6305UV/Vis spectrophotometer (Jenway® Equipment for Analysis, Staffordshire, UK) at 740 nm. The phenolic content in extracts was determined using gallic acid as a standard and presented as gallic acid equivalents per gram of dry extract (mg GAE/g dry extract).

Determination of total flavonoid content

Flavonoid contents of the samples were measured spectrophotometrically [Park *et al*., 1997] with slight modification. The reaction mixture contained 1.0 mL of the pear extract (in the concentration of 1 mg/mL), 0.1 mL of 10% $Al(NO_3)_3 \times 9\ H_2O$, 0.1 mL of 1 M CH_3COOK, and 4.1 mL of 80% ethanol. Control was prepared using 96% ethanol instead of the extract, while 96% ethanol solution was used as blank. Absorbances were measured at 415 nm (JENWAY 6305UV/Vis spectrophotometer) after incubation for 40 min at room temperature. Sample flavonoid concentrations (mg/mL) were calculated using the standard curve equation, and expressed as quercetin equivalents per gram of dry extract (mg QE/g dry extract) from three measurements.

HPLC analysis

Phenolic compounds in the extracts were determined by comparing the retention times and absorption spectra (200–400 nm) of unknown peaks with those of pure standards (arbutin, rutin, chlorogenic acid, isoquercitrin, quercitrin, hyperosid, procyanidin B_1, procyanidin B_2) injected under the same conditions. The HPLC-DAD analysis was performed on an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Lichrospher® 100 RP 18e column (5 μm, 250×4 mm, Agilent Technologies, Palo Alto, CA, USA). Mobile phase A was formic acid in water (1%, *v/v*) and mobile phase B was acetonitrile. The concentration of injected extracts varied from 1.5 to 55 mg/mL. The injection volume was 30 μL, and flow rate was 1 mL/min with the gradient program as follows: 5–15% B 0–5 min, 15–20% B 5–8 min, 20% B 8–12 min, 20–30% B 12–15 min, 30% B 15–17 min, 30–35% B 17–20 min, 35% B 20–22 min, 35–100% B 22–25 min. Stop time of the analysis was 25 min. The column temperature was kept at 25°C during

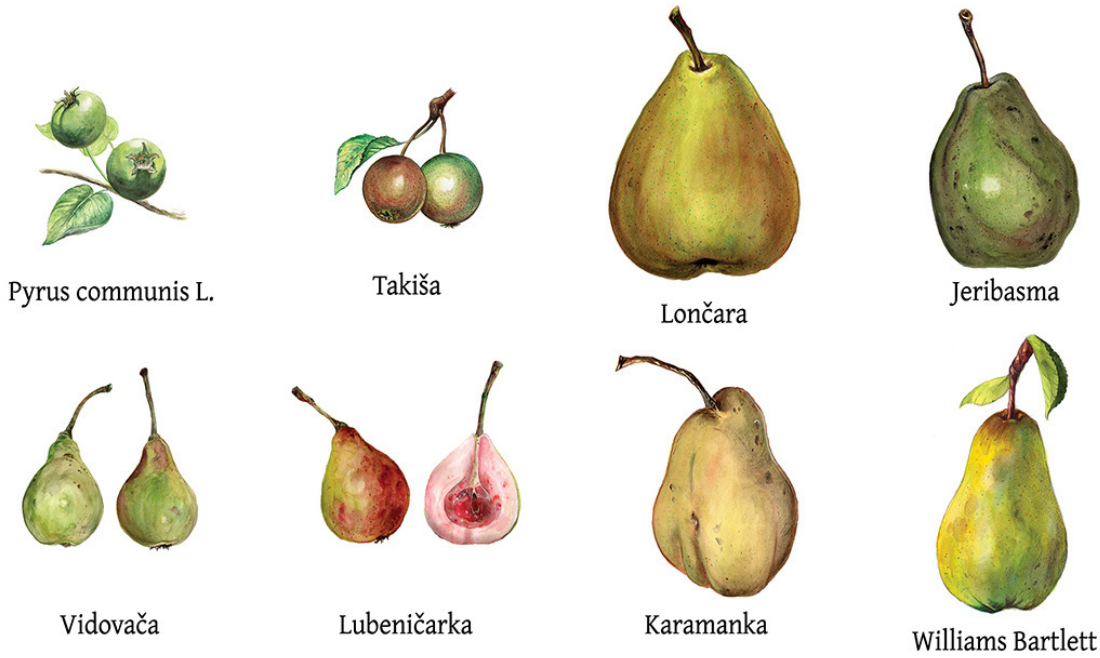


FIGURE 1. Traditional pear varieties ('Vidovača', 'Lubeničarka', 'Karamanka', 'Jeribasma', 'Takiša', 'Lončara'), commercial variety ('Williams Bartlett') and wild pear.

separation. The contents of individual phenolic compounds were expressed as μg/g of dry extract. The samples were analyzed in triplicate [Šavikin *et al.,* 2014].

Evaluation of antioxidant activity

DPPH assay

A slightly modified DPPH assay was employed [Blois, 1958] in order to evaluate free radical scavenging activity of the pear extracts. The reaction mixture (2000 μL) containing the pear extract and methanolic solution of DPPH• (40 μg/mL) was incubated in the dark at room temperature for 30 min. Ascorbic acid, BHA, and BHT were used as positive controls (tested at the concentrations from 1–20 μg/mL). The absorbances were measured using JENWAY 6305UV/Vis spectrophotometer at 517 nm. Methanol was used as a blank, while control was prepared to contain methanol instead of the pear extract/standard. DPPH• scavenging activity was determined using the following formula:

$$\text{DPPH}^{\bullet}\text{ scavenging activity (\%)} = [(A_C\text{-}A_S)/A_C] \times 100\% \quad (1)$$

where: A_C is absorbance of control and A_S is absorbance of the reaction mixture. The results were presented as extract concentration providing 50% inhibition of DPPH• (IC_{50} value, mg/mL).

ABTS assay

ABTS assay [Miller *et al.,* 1993] with slight modifications was also employed to evaluate radical scavenging activity of the pear extracts. $ABTS^{\bullet+}$ stock solution (7 mM) in 2.46 mM potassium-persulfate was prepared 12 h before experiment and stored at room temperature in the dark, and then subsequently diluted by distilled water to obtain a working solution which absorbance at 734 nm was 0.700±0.020. The reaction mixture consisted of 50 μL of extract (1 mg/mL) or methanolic solutions of BHA and BHT standards (0.1 mg/mL) and 2000 μL of a working $ABTS^{\bullet+}$ solution. Distilled water was used as a blank, while control contained distilled water instead of the sample. After incubation (30 min at 30°C), absorbances were measured using the JENWAY 6305UV/Vis spectrophotometer at 734 nm. $ABTS^{\bullet+}$ scavenging activity of the extracts was determined using ascorbic acid and presented as ascorbic acid equivalents (AAE) per gram of dry extract (mg AAE/g dry extract).

Ferric-reducing antioxidant power (FRAP) assay

In order to prepare FRAP reagent, sodium acetate buffer (300 mM, pH 3.6), TPTZ (10 mM) in HCl (40 mM) and $FeCl_3 \times 6\ H_2O$ (20 mM) were mixed in the proportion of 10:1:1 (*v/v/v*) and subsequently heated to 37°C before usage as previously described [Benzie & Strain, 1996]. Pear extracts (100 μL in concentration of 1 mg/mL) or positive controls (ascorbic acid, BHA, and BHT) in the concentration of 0.1 mg/mL, were added to 3000 μL of the FRAP reagent. After 4-min incubation at room temperature, absorbance of the reaction mixture was measured at 593 nm using the JENWAY 6305UV/Vis spectrophotometer. Distilled water was used as a blank, while control was prepared to contain distilled water instead of the sample. FRAP values were calculated using $FeSO_4 \times 7H_2O$ standard curve and expressed as μmol Fe (II)/g dry extract.

Antineurodegenerative activity

In the present study, AChE- and TYR-inhibitory effects of methanolic extracts of eight pear varieties were tested at concentrations of 25 μg/mL, 50 μg/mL, and 100 μg/mL.

Inhibition of acethylcholinesterase activity

Acethylcholinesterase (AChE) inhibition by the tested pear extracts was determined specrophotometrically using 96-well plates [Ellman *et al.,* 1961]. The reaction mixture (S) was prepared to contain 140 μL of a sodium phosphate buffer (0.1 M, pH 7.0), 20 μL of DTNB, 20 μL of a sample--buffer solution, and 20 μL of an AChE solution (5 units/mL). The commercial AChE inhibitor, galanthamine, was used as a positive control. Control (C) contained sodium buffer instead of the test sample, while blank (B) did not contain AChE. The acetylthiocholine iodide solution (10 μL) was added after incubation (25°C, 15 min) to initiate the reaction. Absorbances were measured at 412 nm using a Tecan Sunrise SN microplate reader equipped with XFluor4 software (Tecan Group Ltd., Männedorf, Switzerland). The recorded absorbances were used to calculate the percentage of inhibition of AChE according to the formula:

$$\text{AChE inhibition (\%)} = [\text{C-(S-B)/C}] \times 100 \quad (2)$$

Inhibition of tyrosinase activity

Inhibition of tyrosinase activity by the pear extracts was assessed spectrophotometrically using 96-well microtiter plates [Masuda *et al.,* 2005]. The wells were designed as: A (containing 120 μL of 0.1 M sodium phosphate buffer, (pH 7) and 40 μL of tyrosinase (46 U/L)), B (containing only phosphate buffer), C (containing 80 μL of sodium phosphate buffer, 40 μL of tyrosinase, and 40 μL of the pear extract), and D (containing 120 μL of phosphate buffer and 40 μL of the pear extract). Subsequently, 40 μL of L-DOPA was added and plates were incubated (30 min, 25°C). Absorbances were measured at 475 nm using the Tecan Sunrise SN microplate reader equipped with XFluor4 software. Kojic acid was used as a positive control. The recorded absorbances were used to calculate the percentage of inhibition of TYR according to the formula:

$$\text{TYR inhibition (\%)} = [\text{(A-B)-(C-D)/(A-B)}] \times 100\% \quad (3)$$

Statistical analysis

All measurements were performed in triplicate and presented as the mean±standard deviation. Analysis of variance (one-way ANOVA) and Tukey's post-hoc test were carried out to test the significance of differences among mean values using PAST (PAleontological STatistics) 3.21. [Hammer *et al.,* 2001]. Pearson's correlation coefficients were calculated between the content of phenolic components and values obtained from different bioactivity assays [Taylor, 1990].

RESULTS AND DISCUSSION

Extraction yield, and total phenolic and flavonoid contents of the pear extracts

Extraction yield of mixed flesh and peel extracts varied from 7.63 to 13.5%, that of flesh extracts from 7.40 to 15.7%, while that of peel extracts between 8.60 and 13.0% (Table 1).

Total phenolic content (TPC) and total flavonoid content (TFC) of the methanolic extracts of peel, flesh, and mixed flesh and peel samples were evaluated applying frequently used colorimetric methods (Folin-Ciocalteu and $AlCl_3$, respectively), which are described in literature in detail. The HPLC--DAD technique was subsequently employed for more precise quantitative-qualitative analysis of phenolic compounds.

The TPC and TFC of fruit extracts of eight pear varieties are presented in Table 1. The methanolic extracts obtained from peel, flesh, and mixed flesh and peel showed significantly ($p<0.05$) different TPC and TFC for all tested pear varieties. Generally, the results showed that the traditional varieties 'Lončara' and 'Takiša' had the highest total phenolic contents, while 'Takiša' had the largest amount of total flavonoids.

TPC for the peel samples ranged from 10.7 to 272 mg GAE/g dry extract for 'Lubeničarka' and 'Lončara' varieties, respectively. The TPC of flesh varied from 3.10 mg GAE/g dry extract ('Lubeničarka') to 198 mg GAE/g dry extract ('Lončara'). TPC of mixed flesh and peel extracts varied from 6.21 to 250 mg GAE/g dry extract with extreme values for the same varieties as for both peel and flesh. The highest values of TPC in flesh and peel separately, and mixed flesh and peel were determined in the samples of traditional 'Lončara' and 'Takiša' varieties, while the lowest values – in 'Lubeničarka' and 'Vidovača'. Comparing to the traditional varieties, TPC of wild pear and 'Williams Bartlett' displayed the intermediate values. A wide range of variations of TPC values was observed for the varieties analyzed in this study, as it was reported for other pear varieties of different origin [Kolniak-Ostek, 2016; Li *et al.*, 2014].

Lower TPC of European pear fruit extract of 14.5 μg GAE/g was achieved in the study of Sharma *et al.* [2015]. Abaci *et al.* [2016] investigated fruit of ten Turkish pear varieties and concluded that peel (300.1 to 687.2 mg GAE/100 g) had 2–3 times higher content of phenolic compounds than flesh (112.6 to 230.5 mg GAE/100 g). Kolniak-Ostek [2016] found that European pear peel (917.6 mg GAE/100 g dry matter) had 4 times higher amount of phenolics than flesh (234.2 mg GAE/100 g dry matter). Li *et al.* [2014] presented results of TPC determination in ten Chinese pear varieties and showed it was higher in the peel (263.6 to 1121.5 mg GAE/100 g dry weight) than in the flesh. In our study, peel was 2–3 times, or even 5 times richer in TPC than flesh, ranging overall from 3.10 mg GAE/g dry extract (flesh of 'Lubeničarka') to 272 mg GAE/g dry extract (peel of 'Lončara'). Imeh & Khohar [2002] reported the results of TPC determination in fruits of four commercial pear varieties (1795 to 2566 mg GAE/100 g dry weight), which displayed similar amounts as apple fruit of commercial varieties.

The TFC of eight pear peel extracts ranged from 2.52 to 9.94 mg QE/g dry extract with the lowest and the highest values determined for 'Vidovača' and 'Takiša', respectively.

TABLE 1. Extraction yield, total phenolic content (TPC), and total flavonoid content (TFC) of methanolic extracts of pear varieties.

Variety	Fruit part	Yield (%)	TPC (mg GAE/g dry extract)	TFC (mg QE/g dry extract)
Wild pear	flesh+peel	12.3	$16.4\pm0.5^{y,c}$	$2.54\pm0.10^{y,b}$
	flesh	12.0	$13.3\pm0.2^{z,c}$	$1.93\pm0.19^{z,b}$
	peel	9.00	$27.3\pm0.4^{x,c}$	$4.82\pm0.30^{x,b}$
Takiša	flesh+peel	7.63	$94.4\pm4.0^{y,b}$	$6.03\pm0.18^{y,a}$
	flesh	7.40	$79.5\pm2.1^{z,b}$	$3.21\pm0.20^{z,a}$
	peel	9.20	$242\pm6^{x,b}$	$9.94\pm0.19^{x,a}$
Lončara	flesh+peel	13.0	$250\pm7^{y,a}$	$1.83\pm0.11^{y,c}$
	flesh	8.40	$198\pm7^{z,a}$	$0.69\pm0.05^{z,d}$
	peel	10.9	$272\pm4^{x,a}$	$3.16\pm0.15^{x,d}$
Jeribasma	flesh+peel	9.10	$10.1\pm0.1^{y,d}$	$1.36\pm0.07^{y,d}$
	flesh	12.1	$3.78\pm0.05^{z,d}$	$1.22\pm0.09^{y,c}$
	peel	8.60	$23.2\pm0.7^{x,c}$	$2.73\pm0.13^{x,de}$
Vidovača	flesh+peel	11.1	$6.56\pm0.33^{y,d}$	$1.87\pm0.11^{y,c}$
	flesh	15.7	$5.39\pm0.06^{z,d}$	$1.32\pm0.08^{z,c}$
	peel	12.7	$14.4\pm0.1^{x,d}$	$2.52\pm0.07^{x,e}$
Lubeničarka	flesh+peel	13.5	$6.21\pm0.09^{y,d}$	$2.69\pm0.16^{y,b}$
	flesh	9.52	$3.10\pm0.02^{z,d}$	$1.72\pm0.01^{z,b}$
	peel	11.3	$10.7\pm0.1^{x,d}$	$4.30\pm0.09^{x,c}$
Karamanka	flesh+peel	11.4	$8.45\pm0.49^{y,cd}$	$0.98\pm0.03^{y,de}$
	flesh	11.3	$4.51\pm0.24^{z,d}$	$0.46\pm0.06^{z,d}$
	peel	13.0	$21.1\pm0.9^{x,cd}$	$2.98\pm0.19^{x,de}$
Williams Bartlett	flesh+peel	9.40	$10.1\pm0.1^{y,cd}$	$1.07\pm0.83^{y,d}$
	flesh	10.7	$3.78\pm0.05^{z,d}$	$0.70\pm0.00^{z,d}$
	peel	10.9	$23.2\pm0.7^{x,c}$	$2.57\pm0.10^{x,e}$

Within each pear variety, mean values with different superscript letters $^{(x\text{-}z)}$ differ significantly; for each fruit part separately, mean values with different superscript letters $^{(a\text{-}e)}$ differ significantly between varieties (one-way ANOVA, Tukey's post hoc; $p<0.05$). The superscript letters are assigned to show values in the descending order, where $^{(x)}$ and $^{(a)}$ present the highest values. GAE – gallic acid equivalents; QE – quercetin equivalents.

TFC of the flesh extracts ranged from 0.46 to 3.21 mg QE/g dry extract for 'Karamanka' to 'Takiša', and that of the mixed flesh and peel extracts ranged from 0.98 to 6.03 mg QE/g dry extract with the extreme values noted for the same varieties. Li *et al.* [2014] analyzed TFC of ten pear fruit extracts of Chinese varieties and results showed that TFC was 6–20 times higher in the peel (281.2 to 1682.7 mg rutin/100 g) than in the flesh. In turn, Sharma *et al.* [2015] recorded 10.30 μg catechin equivalents/mg in the sample of European pear fruit extract. As many researchers reported, pears contain high amounts of phenolics, including flavonoids, in the whole fruit, flesh, and especially in the peel. Brahem *et al.* [2017] presented the phenolic profile of peel and flesh crude extracts, and noted

TABLE 2. Content of individual phenolics in methanolic extracts of pear varieties (μg/g dry extract).

Variety	Fruit part	Arbutin	Chlorogenic acid	Rutin	Hyperoside	Isoquercitrin	Quercitrin	Procyanidin B_1	Procyanidin B_2
Wild pear	flesh+peel	$485\pm20^{z,b}$	$221\pm10^{x,d}$	tr	tr	$20.7\pm5.05^{y,c}$	$100\pm6^{y,d}$	$950\pm40^{x,a}$	$213\pm10^{z,b}$
	flesh	$695\pm31^{x,b}$	$61.2\pm4.8^{y,c}$	tr	tr	$8.01\pm1.03^{z,b}$	$92.8\pm4.6^{y,c}$	$972\pm58^{x,a}$	$275\pm14^{x,b}$
	peel	$555\pm29^{y,c}$	$25.8\pm4.0^{z,e}$	tr	38.4 ± 2.1^{c}	$125\pm7^{x,d}$	$264\pm13^{x,e}$	$258\pm10^{y,a}$	$245\pm11^{y,b}$
Takiša	flesh+peel	$1708\pm62^{y,a}$	$11763\pm63^{y,a}$	tr	5820 ± 76	$12216\pm46^{x,a}$	tr	$671\pm30^{x,b}$	$2065\pm62^{x,a}$
	flesh	$1157\pm44^{z,a}$	$10795\pm49^{z,a}$	tr	tr	$72.8\pm4.4^{z,a}$	nd	$476\pm29^{y,b}$	$393\pm17^{y,a}$
	peel	$3749\pm72^{x,a}$	$16147\pm60^{x,a}$	104 ± 6^{c}	215 ± 10^{a}	$657\pm21^{y,a}$	tr	$161\pm4^{z,b}$	$276\pm10^{z,b}$
Lončara	flesh+peel	$114\pm5^{y,d}$	$957\pm39^{y,b}$	tr	tr	$10.1\pm1.0^{y,c}$	77.6 ± 3.0^{e}	$63.3\pm2.4^{y,cd}$	$85.4\pm4.5^{z,c}$
	flesh	$100\pm5^{y,cde}$	$890\pm40^{y,b}$	tr	tr	$1.70\pm0.30^{y,d}$	tr	$50.1\pm2.1^{y,c}$	$134\pm6^{y,c}$
	peel	$767\pm21^{x,b}$	$1108\pm44^{x,b}$	84.9 ± 4.0^{cd}	118 ± 6^{b}	$216\pm9^{x,c}$	275 ± 11^{e}	$296\pm13^{x,a}$	$576\pm25^{x,a}$
Jeribasma	flesh+peel	$17.6\pm1^{z,e}$	$25.3\pm2.1^{z,f}$	24.2 ± 1.1	tr	3.55 ± 0.21^{c}	$264\pm12^{y,a}$	$40.9\pm3.0^{z,d}$	$86.1\pm3.5^{y,c}$
	flesh	$122\pm7^{x,cd}$	$36.3\pm2.9^{y,c}$	tr	tr	tr	$70.7\pm3.7^{z,d}$	$55.5\pm2.4^{y,c}$	$93.3\pm4.0^{y,d}$
	peel	$60.9\pm2.9^{y,f}$	$80.1\pm3.7^{x,e}$	571 ± 25^{a}	tr	93.8 ± 3.4^{f}	$1778\pm48^{x,a}$	$76.1\pm3.4^{x,c}$	$175\pm6^{x,c}$
Vidovača	flesh+peel	$175\pm6^{y,cd}$	$27.1\pm1.7^{y,f}$	tr	tr	$36.3\pm1.0^{y,c}$	$145\pm3^{z,c}$	37.0 ± 15.2^{d}	$25.1\pm1.1^{z,c}$
	flesh	$104\pm5^{z,cde}$	$37.5\pm2.0^{x,c}$	tr	tr	$2.15\pm0.21^{z,d}$	$192\pm7^{y,a}$	nd	$110\pm5^{y,cd}$
	peel	$374\pm14^{x,d}$	$30.2\pm1.1^{y,e}$	43.6 ± 1.6^{c}	tr	$134\pm4^{x,d}$	$376\pm12^{x,d}$	40.7 ± 2.2^{d}	$169\pm5^{x,c}$
Lubeničarka	flesh+peel	$86.9\pm3.8^{z,de}$	62.6 ± 3.1^{y}	tr	0.10 ± 0.03	$191\pm8^{x,c}$	$108\pm4^{y,d}$	$101\pm3^{y,c}$	$52.0\pm1.3^{z,c}$
	flesh	$141\pm5^{y,c}$	$64.4\pm2.9^{y,c}$	tr	tr	$14.4\pm0.7^{z,c}$	$95.1\pm3.0^{z,c}$	$105\pm4^{y,c}$	$76.0\pm2.8^{y,d}$
	peel	$308\pm13^{x,de}$	$88.4\pm4.0^{x,e}$	tr	38.3 ± 1.9^{c}	$80.7\pm3.2^{y,f}$	$131\pm5^{x,f}$	$150\pm6^{x,b}$	$99.2\pm3.3^{x,c}$
Karamanka	flesh+peel	$84.3\pm3.3^{y,de}$	$116\pm6^{y,e}$	tr	tr	20.0 ± 1.1^{c}	$210\pm8^{y,b}$	tr	tr
	flesh	$71.9\pm3.0^{y,de}$	$92.9\pm3.7^{z,c}$	tr	nd	tr	$134\pm6^{z,b}$	tr	tr
	peel	$247\pm8^{x,e}$	$395\pm15^{x,d}$	367 ± 13^{b}	tr	276 ± 10^{b}	$829\pm36^{x,b}$	tr	tr
Williams Bartlett	flesh+peel	$226\pm10^{y,c}$	$356\pm12^{y,c}$	tr	tr	36.9 ± 2.7^{c}	266 ± 10^{a}	tr	tr
	flesh	$64.4\pm2.5^{z,e}$	$38.6\pm2.8^{z,c}$	tr	tr	tr	tr	tr	tr
	peel	$710\pm21^{x,b}$	$883\pm22^{x,c}$	49.4 ± 2.7^{c}	tr	81.0 ± 3.2^{f}	463 ± 20^{c}	tr	tr

Within each pear variety, mean values with different superscript letters $^{(x\text{-}z)}$ differ significantly; for each fruit part separately, mean values with different superscript letters $^{(a\text{-}f)}$ differ significantly between varieties (one-way ANOVA, Tukey's post hoc; $p<0.05$). The superscript letters are assigned to show values in the descending order, where $^{(x)}$ and $^{(a)}$ present the highest values. tr – traces, nd – not detected.

a higher phenolic content in the peel than in the flesh of pear fruit. In the mentioned studies, the TPC and TFC were measured predominantly in methanolic extracts, and varied depending on species or variety and employed methods of extraction.

The TPC and TFC values determined in the peel of all pear varieties were higher than those found in the flesh and mixed peel and flesh, which could be important data for the consumption of the fruits, because the peel is often discarded, resulting in the loss of valuable compounds [Abaci *et al.,* 2016; Kolniak-Ostek, 2016; Lin & Harnly, 2008; Öztürk *et al*., 2015].

Phenolic composition of pear extracts

Determination of the qualitative and quantitative composition of phenolics of the extracts of eight pear varieties was performed using the HPLC technique and the results are presented in Table 2. Among the eight pear varieties, chlorogenic acid in the peel (25.8 to 16147 μg/g dry extract) and arbutin in the peel (122 to 3748 μg/g dry extract) were the major compounds, followed by quercitrin in the peel (131 to 1778 μg/g dry extract), and isoquercitrin (3.55 to 12216 μg/g dry extract) in the mixed flesh and peel extracts.

The peel of the most pear varieties had the higher content of chlorogenic acid compared to flesh, such as those obtained from 'Takiša', 'Lončara' and 'Williams Bartlett'. In the previous study of eight Chinese pear varieties, chlorogenic acid content in the whole fruits varied between 10.3 and 263.8 μg/g [Li *et al.*, 2012], while in our study the range was notably wider. Other varieties showed a high chlorogenic acid content in the peel; however, with the content differing among the analyzed varieties [Li *et al.,* 2014; Salta *et al.,* 2010]. As a potential chemoprotective agent, chlorogenic acid has been reported to elicit antioxidant, antitumor, and immune system-enhancing effects [Li *et al.,* 2014].

The content of arbutin in the pear methanol extracts varied, being predominantly higher in the peel compared to the flesh (except for wild pear), ranging from 60.9 to 3749 μg/g dry extract of peel; from 64.4 to 1157 μg/g dry extract of flesh; and from 17.6 to 1708 μg/g dry extract of the mixed flesh and peel. The highest content of arbutin was detected in 'Takiša' variety, while the lowest one in 'Jeribasma'. In the previous studies, some pear varieties showed arbutin contents 2 to 3 times higher in the peel than in the flesh [Kolniak-Ostek, 2016; Li *et al.,* 2014; Salta *et al.,* 2010]. As the major phenolic compound found in the different parts of plant and fruit, arbutin exhibits strong free radical scavenging properties. Additionally, it has been proved to elicit antibacterial, anti-inflammatory, antitussive, and skin-whitening effects and to be effective against urinary infections [Li *et al.,* 2014]. Dadgar *et al*. [2018] also demonstrated its ability to reduce oxidative stress in Parkinson's disease animal model.

In the present study, the contents of isoquercitrin, quercitrin, procyanidin B_1, and procyanidin B_2 differed depending on the variety and a fruit part used, with higher values determined in the peel. Quercitrin and isoquercitrin have been also identified in pears in some previous studies, which showed that their content varied greatly among cultivars and within different tissues [Liaudanskas *et al*., 2017]. Kaur & Arya [2012] identified quercitrin and isoquercitrin in *Pyrus communis,* while in this study, quercitrin was found in the highest amount in 'Jeribasma' peel extract and isoquercitrin in mixed flesh and peel extract of 'Takiša'. Li *et al*. [2016a] compared the antioxidant effects of quercitrin and isoquercitrin and concluded that isoquercitrin exhibited higher reactive oxygen species (ROS) scavenging activity than quercitrin, protecting mesenchymal stem cells from ROS-induced oxidative damage. Jeong *et al.* [2017] isolated five proanthocyanidins from *Pyrus pyrifolia* peel, while in the current study procyanidin B_2 was detected in the noticeably high amount in mixed flesh and peel extract of 'Takiša'. Fruits of wild pear were the richest in procyanidin B_1.

Rutin and hyperoside were detected in traces in all pear varieties. Some natural polyphenolics, such as rutin, have been shown as potent inhibitors of COVID-19 main protease (Mpro), which is considered a potential therapeutic drug target [Adem *et al.,* 2020].

Variations in the content of phenolics among different pear varieties were reported in the previous studies [Kolniak-Ostek *et al*., 2020; Li *et al*., 2014; Öztürk *et al.,* 2015]. The content of individual phenolic compounds in the extracts varied widely among the tested varieties, which could be attributed to the sampling locality, different pre- or post-harvest conditions, or, as it was demonstrated for apple cultivars, genetics can be supposed to play a major role causing very high phenolic content variability [McClure *et al*., 2019]. Additionally, the phenolics are not equally distributed in the fruit [Li *et al*., 2014].

Recent research indicates that long-term polyphenol consumption may play a vital role in promoting health through the regulation of metabolism and protection against various types of cancer, cardiovascular diseases, type 2 diabetes, gastrointestinal disorders, lung impairment, neurodegenerative diseases, *etc*. These benefits could be explained by the "biochemical scavenger theory", which suggests that phenolic compounds inactivate free radicals by forming stabilized chemical complexes, thus preventing further adverse reactions in the body [Cory *et al*., 2018].

The next step of the study was to investigate the antioxidant activity of the extracts and their potential to inhibit the enzymes involved in oxidative stress-induced neurodegenerative disorders.

Antioxidant activity of pear extracts

Three different assays: DPPH, ABTS and FRAP, were used to achieve more complete evaluation of the antioxidant activity of peel, flesh, and mixed peel and flesh fruit extracts. The results are presented in Table 3. In terms of the scavenging activity against DPPH$^{\bullet}$, all pear fruit extracts showed strong to low activity, depending on the variety. The DPPH$^{\bullet}$ scavenging activity was as follows: 'Takiša' > wild pear > 'Lončara' > 'Vidovača' > 'Jeribasma' > 'Lubeničarka' >'Karamanka' > 'Williams Bartlett'. The peel showed the highest DPPH$^{\bullet}$ scavenging activity (IC_{50} values ranging from 0.37 to 5.50 mg/mL). With some exceptions, methanolic extracts obtained from peel, flesh, and mixed flesh and peel showed significantly different IC_{50} values for all tested pear varieties (Table 3). The highest antioxidant potential was determined in the peel of 'Takiša' (IC_{50}=0.37 mg/mL), followed by wild pear peel (IC_{50} = 0.80 mg/mL), and 'Lončara' peel (IC_{50}=1.56 mg/mL). In all samples, the mixture of flesh and peel showed intermediate activity (0.79 to 6.91 mg/mL), while flesh extracts showed the lowest activity (3.71 to 22.39 mg/mL). Compared to BHA and BHT standards and particularly ascorbic acid, the tested pear extracts showed notably lower DPPH$^{\bullet}$ scavenging activity.

Similar DPPH$^{\bullet}$ scavenging activity to certain varieties in this study, was reported for the pear fruit extracts of some Chinese varieties [Li *et al*., 2012], *i.e.* Xuehua pear fruit (IC_{50}=0.59 mg/mL) and Nanguo pear fruit (IC_{50}=0.70 mg/mL). The extract of apple shaped pear (*P. pyrifolia* var. *pinggouli*) showed DPPH$^{\bullet}$ scavenging activity expressed as IC_{50}=38.30 mg/mL [Ma *et al.,* 2012], which is lower than the activity presented in this paper. Salta *et al.* [2010] investigated the scavenging activity of 'Rocha' pear fruit extract (IC_{50}=0.11 mg/mL) and also of a few commercial pear fruits, and obtained significantly higher antiradical activity against DPPH$^{\bullet}$ compared to results found in this research. Results of previous studies have indicated that pear fruit possesses a strong DPPH$^{\bullet}$ antiradical potential, and that peel has a stronger antioxidant activity than flesh [Kolniak-Ostek, 2016; Li *et al.,* 2012, 2014; Salta *et al.,* 2010].

In the ABTS assay, the highest values were achieved for the peel extracts (1.17 to 2.91 mg AAE/g dry extract); followed by mixed flesh and peel extracts (0.44 to 2.09 mg AAE/g dry extract) and flesh (0.26 to 1.18 mg AAE/g dry extract). For most of the tested pear varieties (Table 3), peel, flesh, and mixed flesh and peel methanolic extracts showed significantly ($p<0.05$) different antioxidant activities in the ABTS assay. The results obtained using this assay showed that 'Takiša' and 'Lončara' extracts exhibited the highest antioxidant activity. Compared to BHA and BHT standards, the tested extracts showed lower activity (Table 3). High ABTS$^{\bullet+}$ scavenging activity of the European pear fruit methanolic extract (IC_{50}=15.90 mg) was presented by Sharma *et al.* [2015].

TABLE 3. Antioxidant activity of methanolic extracts of pear varieties.

Variety/ Standard	Fruit part	DPPH• scavenging activity (IC_{50}, mg/mL)	$ABTS^{•+}$ scavenging activity (mg AAE/g)	Ferric-reducing antioxidant power (μmol Fe(II)/g)
Wild pear	flesh+peel	$2.85\pm0.19^{y,d}$	$0.77\pm0.05^{y,e}$	$743\pm19^{y,a}$
	flesh	$4.29\pm0.33^{x,d}$	$0.76\pm0.07^{y,c}$	$618\pm19^{z,a}$
	peel	$0.80\pm0.05^{z,d}$	$1.10\pm0.03^{x,d}$	$880\pm21^{x,b}$
Takiša	flesh+peel	$0.79\pm0.05^{y,e}$	$1.54\pm0.09^{y,b}$	$711\pm12^{y,a}$
	flesh	$3.85\pm0.23^{x,e}$	$1.18\pm0.01^{z,b}$	$606\pm8^{z,a}$
	peel	$0.37\pm0.03^{z,d}$	$2.91\pm0.10^{x,a}$	$933\pm22^{x,a}$
Lončara	flesh+peel	$2.90\pm0.16^{y,d}$	$2.09\pm0.10^{x,a}$	$739\pm16^{y,a}$
	flesh	$3.71\pm0.14^{x,e}$	$1.65\pm0.32^{xy,a}$	$631\pm7^{z,a}$
	peel	$1.56\pm0.12^{z,cd}$	$1.22\pm0.06^{y,d}$	$882\pm23^{x,b}$
Jeribasma	flesh+peel	$3.28\pm0.03^{x,d}$	$0.44\pm0.03^{y,f}$	$206\pm10^{y,d}$
	flesh	$3.71\pm0.07^{x,e}$	$0.41\pm0.03^{y,d}$	$190\pm9^{y,d}$
	peel	$2.72\pm0.09^{y,c}$	$2.67\pm0.04^{x,b}$	$737\pm14^{x,c}$
Vidovača	flesh+peel	$6.91\pm0.25^{x,a}$	$0.96\pm0.02^{y,d}$	$409\pm13^{y,c}$
	flesh	$5.97\pm0.52^{x,c}$	$0.59\pm0.03^{z,cd}$	$330\pm8^{z,b}$
	peel	$2.28\pm0.68^{y,c}$	$1.69\pm0.06^{x,c}$	$647\pm2^{x,d}$
Lubeničarka	flesh+peel	$4.99\pm0.07^{x,c}$	$0.95\pm0.04^{y,d}$	$393\pm18^{y,c}$
	flesh	$5.00\pm0.02^{x,cd}$	$1.00\pm0.06^{y,bc}$	$248\pm10^{z,c}$
	peel	$3.55\pm0.07^{y,b}$	$1.17\pm0.03^{x,d}$	$486\pm9^{x,e}$
Karamanka	flesh+peel	$5.06\pm0.26^{y,c}$	$1.18\pm0.06^{y,c}$	$391\pm8^{y,c}$
	flesh	$11.4\pm0.7^{x,b}$	$0.54\pm0.04^{z,cd}$	$178\pm2^{z,d}$
	peel	$5.50\pm0.38^{y,a}$	$2.52\pm0.03^{x,ab}$	$638\pm10^{x,d}$
Williams Bartlett	flesh+peel	$5.96\pm0.40^{y,b}$	$1.21\pm0.03^{y,c}$	$456\pm4^{y,b}$
	flesh	$22.4\pm1.1^{x,a}$	$0.26\pm0.04^{z,d}$	$221\pm14^{z,c}$
	peel	$4.57\pm1.09^{y,ab}$	$2.78\pm0.04^{x,bc}$	$645\pm10^{x,d}$
		(IC_{50}, μg/mL)		
BHT	–	17.9±0.2	27.5±0.2	4450±78
BHA	–	13.8±0.4	28.2±0.1	5840±53
Ascorbic acid	–	5.1±0.1	nt	1810±86

Within each pear variety, mean values with different superscript letters $^{(x\text{-}z)}$ differ significantly; for each fruit part separately, mean values with different superscript letters $^{(a\text{-}e)}$ differ significantly between varieties (one-way ANOVA, Tukey's post hoc; $p<0.05$). The superscript letters are assigned to show values in the descending order, where $^{(x)}$ and $^{(a)}$ present the highest values. AAE – ascorbic acid equivalents, BHT – 3,5-di-tert-butyl-4-hydroxytoluene; BHA – 2(3)-tert-butyl-4-hydroxyanisole. nt – not tested.

Considering the FRAP assay results, the peel extracts showed significantly higher values than flesh, and flesh and peel ones. The FRAP values of the tested extracts were lower than those of the tested standards (Table 3). The highest FRAP value was determined for the 'Takiša' peel extract (933 μmol Fe(II)/g dry extract) followed by 'Lončara' peel extract (882 μmol Fe(II)/g dry extract), and wild pear peel extract (880 μmol Fe(II)/g dry extract). With exception of 'Jeribasma' extracts, all methanolic extracts obtained from peel, flesh, and mixed flesh and peel of all other varieites tested showed significantly different FRAP values (Table 3). The FRAP of four commercial pears showed similar values to commercial apple fruit varieties, and slightly higher ones compared to those reported for peach and kiwi fruits [Imeh & Khokhar, 2012]. Kolniak-Ostek [2016] compared leaves, seeds, peel, and pulp of the 'Radana' pear and obtained the highest FRAP for leaves, probably because of the highest content of phenolics in this plant part.

To the best of our knowledge, there are a few studies on the ABTS and FRAP assays used to determine the antioxidant activity of pear fruits. However, due to the differences in the applied methods and presentation of results, it is difficult to directly compare our results with those obtained in other studies [Kolniak-Ostek, 2016; Kolniak-Ostek *et al*., 2020; Liaudanskas *et al*., 2017; Sharma *et al.*, 2015].

The highest antioxidant activity of 'Takiša', especially of the outer part of the fruit, could be attributed to the highest content of detected phenolics, particularly chlorogenic acid which is known for its antioxidant potential [Li *et al*., 2014].

Antineurodegenerative activity of pear extracts

The results of the evaluation of the antineurodegenerative activities of pear extracts are presented in Table 4. The extracts did not show enzyme inhibitory activity in the concentration-dependent manner. Methanolic extracts obtained from peel, flesh, and mixed flesh and peel of the pear varieties tested, showed significantly different AChE- and TYR-inhibiting activities (Table 4). No correlation was established between inhibiting effects against both enzymes and fruit parts of all varieties.

The AChE inhibition by the tested extracts, compared to galanthamine (42.38 to 57.11%), was low to moderate. The highest AChE inhibitory activity displayed mixed flesh and peel extracts of 'Lončara' (40.4%) at 50 μg/mL and 'Takiša' (39.5%) at 100 μg/mL. In some cases, the mixed flesh and peel extracts ('Takiša', 'Lončara', 'Vidovača') showed higher AChE inhibitory activity than flesh or peel, but in some cases the peel extract showed the highest activity (wild pear).

The TYR inhibition by the studied extracts was lower than the inhibition displayed by kojic acid (33.93 to 51.81%). The highest inhibition of TYR activity exhibited 'Takiša' peel extract (32.8%) at the concentration of 50 μg/mL, which is close to kojic acid at the same concentration.

Previous studies have shown the significant inhibition of AChE and TYR by various fruit extracts [Szwajgier & Borowiec, 2012; Šavikin *et al.*, 2018]. Antineurodegenerative activity of 70% ethanolic extract of peel of *Punica granatum* L. was studied by Šavikin *et al.* [2018], who obtained the highest AChE inhibition of 33.46% at extract concentration of 100 μg/mL, which is moderate compared to galanthamine (57.11%), while TYR inhibitory activity of 78.46% was higher than the inhibition ensured by kojic acid (51.81%).

TABLE 4. Antineurodegenerative activities of methanolic extracts with different concentrations obtained from pear varieties.

Variety/ Standard	Fruit part	AChE inhibition (%)			TYR inhibition (%)		
		25 μg/mL	50 μg/mL	100 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL
Wild pear	flesh+peel	21.8±0.7^{y,a}	34.5±3.0^{x,b}	31.6±3.1^{x,b}	17.7±1.4^{y,c}	21.2±3.2^{y,b}	13.4±0.8^{z,c}
	flesh	9.53±3.37^{z,d}	36.9±0.4^{x,a}	12.5±0.8^{y,c}	17.8±2.1^{y,b}	20.7±1.6^{y,b}	23.7±2.2^{x,a}
	peel	34.7±2.43^{x,a}	38.9±1.4^{x,a}	32.4±2.6^{x,ab}	24.6±1.8^{x,a}	27.4±2.1^{x,b}	19.4±1.8^{y,ab}
Takiša	flesh+peel	30.1±2.7^{x,a}	33.5±3.2^{x,b}	39.5±2.4^{x,a}	27.8±1.0^{xy,a}	28.2±2.5^{xy,a}	22.9±1.8^{x,ab}
	flesh	26.3±2.7^{x,c}	34.4±1.5^{x,a}	28.4±1.5^{y,b}	29.2±2.7^{x,a}	23.7±2.3^{y,ab}	21.1±2.1^{x,ab}
	peel	7.10±0.91^{y,e}	32.4±3.4^{x,b}	9.19±0.94^{z,d}	22.3±2.9^{y,ab}	32.8±0.7^{x,a}	24.6±2.8^{x,a}
Lončara	flesh+peel	31.5±3.1^{x,a}	40.4±0.9^{x,a}	30.9±2.2^{x,b}	24.5±2.1^{x,ab}	21.1±1.8^{y,b}	26.5±1.0^{x,a}
	flesh	31.3±1.0^{x,b}	36.1±4.6^{x,a}	35.7±4.5^{x,a}	26.6±1.7^{x,a}	27.0±1.9^{x,a}	17.8±1.7^{y,bc}
	peel	29.4±0.4^{x,b}	36.4±3.2^{x,ab}	30.2±3.3^{x,ab}	17.4±1.5^{y,b}	21.8±0.8^{y,c}	20.1±2.1^{y,ab}
Jeribasma	flesh+peel	7.66 ±0.95^{y,b}	36.3±0.6^{x,a}	10.3±1.7^{z,d}	22.8±1.6^{x,b}	25.0±2.1^{x,ab}	17.4±2.7^{x,bc}
	flesh	7.34±0.69^{y,d}	7.92±1.0^{y,b}	29.9±3.6^{x,ab}	21.1±1.8^{x,b}	11.8±0.5^{z,d}	16.8±1.1^{xy,bc}
	peel	37.8±1.0^{x,a}	35.6±2.6^{x,ab}	24.0±0.1^{y,bc}	18.5±2.2^{x,b}	16.6±2.1^{y,de}	12.7±1.1^{y,c}
Vidovača	flesh+peel	27.7±1.8^{x,a}	36.6±1.0^{x,a}	24.9±0.3^{x,c}	14.9±1.9^{y,cd}	20.7±3.0^{xy,b}	20.2±0.6^{x,b}
	flesh	8.47±0.44^{z,d}	10.3±2.0^{y,b}	27.6±1.7^{x,b}	27.4±2.1^{x,a}	27.1±2.4^{x,a}	21.7±2.2^{x,ab}
	peel	12.6±0.3^{y,d}	7.99±0.84^{y,d}	20.5±0.8^{y,c}	11.9±1.7^{y,c}	15.0±2.3^{y,de}	23.9±2.0^{x,a}
Lubeničarka	flesh+peel	35.0±0.8^{x,a}	37.5±1.4^{x,a}	24.2±0.8^{y,c}	25.3±0.9^{x,ab}	24.4±0.7^{x,ab}	25.0±2.7^{x,a}
	flesh	37.4±1.8^{x,a}	36.6±2.0^{x,a}	35.2±3.3^{x,a}	10.4±0.6^{z,c}	13.2±0.6^{y,cd}	16.5±0.7^{y,bc}
	peel	34.0±2.4^{x,ab}	28.8±2.5^{y,b}	28.7±1.8^{y,bc}	20.4±2.1^{y,ab}	13.7±1.0^{y,e}	8.56±1.00^{z,d}
Karamanka	flesh+peel	8.99±0.85^{y,b}	9.83±0.67^{y,c}	12.3±1.5^{y,d}	17.4±2.1^{x,c}	13.5±0.6^{z,c}	9.80±1.12^{y,d}
	flesh	9.57±0.31^{y,d}	10.7±1.1^{y,b}	9.60±0.12^{y,c}	18.7±1.9^{x,b}	17.2±1.2^{y,c}	16.5±1.3^{x,bc}
	peel	30.6±0.6^{x,b}	34.5±1.2^{x,ab}	36.7±2.1^{x,a}	19.4±0.7^{x,ab}	25.6±1.0^{x,bc}	14.8±0.8^{x,bc}
Williams Bartlett	flesh+peel	9.56±0.82^{y,b}	8.86±0.82^{y,c}	20.1±2.5^{z,c}	10.6±0.96^{y,d}	13.0±1.9^{y,c}	9.81±1.00^{y,d}
	flesh	23.8±2.9^{x,c}	34.3±1.1^{x,a}	39.4±1.1^{x,a}	15.1±1.66^{x,bc}	14.5±1.2^{xy,cd}	14.8±1.9^{x,c}
	peel	23.4±0.9^{x,c}	34.5±1.6^{x,ab}	28.4±1.7^{y,bc}	8.83±1.29^{y,c}	18.5±1.9^{x,d}	16.1±1.6^{x,b}
Galanthamine	–	42.4±0.7	50.6±0.5	57.1±1.7	–	–	–
Kojic acid	–	–	–	–	35.7±5.5	33.9±3.8	51.8±2.6

Within each pear variety, mean values with different superscript letters $^{(x\text{-}z)}$ differ significantly; for each fruit part separately and each tested concentration, mean values with different superscript letters $^{(a\text{-}e)}$ differ significantly between varieties (one-way ANOVA, Tukey's post hoc; $p<0.05$). The superscript letters are assigned to show values in the descending order, where $^{(x)}$ and $^{(a)}$ present the highest values. GAE – gallic acid equivalents; AChE – acetylcholinesterase; TYR – tyrosinase.

Szwajgier & Borowiec [2012] investigated anti-AChE activities of numerous fruits, and found significant activity of extracts of whole fruits, or juices. Peach juice (*Prunus persica* L.) and wild strawberry fruit extract (*Fragaria vesca* L.) showed the highest AChE inhibitory activity (6.10 eserine μM), followed by apple juice varieties (*Mallus domestica* Borkh.) (2.91 to 6.10 eserine μM), and plum extract (*Prunus domestica* L.) (3.09 eserine μM). Their results proved that different fruits can be promising sources of inhibitors of enzymes implicated in neurodegeneration, potentially preventing Alzheimer's and Parkinson's diseases [Szwajgier & Borowiec, 2012; Šavikin *et al.*, 2018].

Correlation among TPC, TFC, and individual phenolic compounds and bioactivity assays

As presented in Table 5, results of ABTS and FRAP assays were moderately correlated with TPC and TFC. Results of those assays showed weak correlation to arbutin and chlorogenic acid, while rutin and quercitrin and $ABTS^{\bullet+}$ scavenging activity were moderately correlated. A negative correlation was determined between $DPPH^{\bullet}$ scavenging activity and TPC, TFC and phenolic constituents because of presenting results of DPPH assay using IC_{50} values. The results obtained by DPPH assay were moderately correlated to TPC, TFC, and arbutin content. AChE inhibitory activity showed

TABLE 5. Correlation coefficients (*r*) among total phenolic content (TPC), total flavonoid content (TFC), and individual phenolic compound contents and results of bioactivity assays.

	DPPH• scavenging activity	$ABTS^{•+}$ scavenging activity	Ferric-reducing antioxidant power	AChE inhibition	TYR inhibition
TPC	-0.36	0.39	0.52	0.34	0.46
TFC	-0.50	0.44	0.43	0.30	0.54
Arbutin	-0.36	0.36	0.29	0.17	0.53
Chlorogenic acid	-0.30	0.30	0.06	0.16	0.53
Rutin	-0.13	0.61	0.35	0.18	0.04
Hyperoside	-0.20	0.08	-0.07	0.09	0.28
Isoquercitrin	-0.21	0.10	-0.06	0.09	0.30
Quercitrin	-0.13	0.55	0.33	0.06	-0.13
Procyanidin B_1	-0.29	-0.16	0.26	0.33	0.23
Procyanidin B_2	-0.34	0.08	0.08	0.18	0.36

According to Taylor (1990): $r \leq 0.35$ weak correlation; $0.36 < r < 0.67$ moderate correlation; $0.68 < r < 1$ strong correlation; AChE – acetylcholinesterase; TYR – tyrosinase.

weak correlation to TPC, TFC, and individual components. TYR inhibitory activity displayed moderate correlation to TPC, TFC, arbutin and chlorogenic acid contents. In some other studies of pear fruit extracts, peel-flesh phenolic content, peel phenolic content, and flesh phenolic content were significantly correlated [Abaci *et al.*, 2016]. Many researchers have found that antioxidant capacity of pear fruit appears to be strongly influenced by TPC and TFC, and highly significant linear correlations were shown between phenolic contents and antioxidant capacity [Abaci *et al.,* 2016; Imeh & Khohar, 2002; Kolniak-Ostek, 2016; Kolniak-Ostek *et al.*, 2020; Li *et al.,* 2012, 2014; Öztürk *et al.*, 2015; Salta *et al.,* 2010; Sharma *et al.,* 2016].

CONCLUSION

This study evaluated phenolic composition, total phenolic and flavonoid contents, and biological activities (antioxidant and antineurodegenerative) of peel, flesh, and mixed flesh and peel methanolic extracts of six traditional pear varieties ('Vidovača', 'Lubeničarka', 'Karamanka', 'Jeribasma', 'Takiša', 'Lončara'), one commercial variety ('Williams Bartlett'), and wild pear from Serbia. All varieties, as well as wild pear, were analyzed for the first time from this perspective. Methanolic extracts of traditional pear fruit varieties showed a high content of total phenolics and flavonoids, significant antioxidant potential, and moderate antineurodegenerative activity. The significant level of phenolics and flavonoids, and the highest antioxidant activity were shown by 'Takiša', 'Lončara', and 'Jeribasma'. Wild pear had average or high antioxidant activity, while commercial 'Williams Bartlett' displayed average or lower activities compared to traditional pear varieties from Serbia.

This study revealed that the fruits of the investigated traditional pears are rich in bioactive components and can be used in nutrition, for possible medical applications, and to prevent diseases induced by oxidative stress. The results are important to enhance their production as a significant source of functional food and potential medicinal remedy. Because of vulnerability of the gene pool, it is necessary to preserve the traditional pear varieties, as a favorable way to increase health benefits and agriculture sustainability. In the food processing industry, it is essential to develop technologies that will enable to process food, in order to keep high nutritional quality, but, also to retain its functional properties and biological activity. The COVID-19 pandemic affects the whole food sector, including food safety, bioactive food ingredients, food security, and sustainability. One of the promising alternatives in post-pandemic world includes the development of nutritional and immune-boosting plant-based products which could support the human health [Galanakis *et al*., 2021].

ACKNOWLEDGEMENT

The authors are grateful to Bora Milićević for drawings of *Pyrus* varieties.

RESEARCH FUNDING

This manuscript was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grants No 451–03–9/2021–14/200178, 451–03–9/2021–14/200003 and 451–03–9/2021–14/200007.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID IDs

A. Alimpić Aradski https://orcid.org/0000-0002-0879-6467
S. Duletić-Laušević https://orcid.org/0000-0002-4777-3989
S. Jarić https://orcid.org/0000-0002-9356-7334
P.D. Marin https://orcid.org/0000-0002-9460-1012
A. Savić https://orcid.org/0000-0002-9106-9980
K. Šavikin https://orcid.org/0000-0002-2086-9593
J. Živković https://orcid.org/0000-0002-9684-2637

REFERENCES

1. Abaci, Z.T., Sevindik, E., Ayvaz, M. (2016). Comparative study of bioactive components in pear genotypes from Ardahan/Turkey. *Biotechnology & Biotechnological Equipment*, *30*, 36–43. https://doi.org/10.1080/13102818.2015.1095654
2. Adem, S., Eyupoglu, V., Sarfraz, I., Rasul, A., Ali, M. (2020). Identification of potent COVID-19 main protease (Mpro) inhibitors from natural polyphenols: An *in silico* strategy unveils a hope against CORONA. Preprints, art. no. 2020030333. https://doi.org/10.20944/preprints202003.0333.v1

3. 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*, 70–76. https://doi.org/10.1006/abio.1996.0292
4. Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*, 1199–1200. https://doi.org/10.1038/1811199a0
5. Brahem, M., Renard, C.M.G.C., Eder, S., Loonis, M., Ouni, R., Messaoud, M., Le Bourvellec, C. (2017). Characterization and quantification of fruit phenolic compounds of European and Tunisian pear cultivars. *Food Research International*, *95*, 125–133. https://doi.org/10.1016/j.foodres.2017.03.002
6. Carbonaro, M., Mattera, M., Nicoli, S., Bergamo, P., Cappelloni, M. (2002). Modulation of antioxidant compounds in organic vs conventional fruit (Peach, *Prunus persica* L., and Pear, *Pyrus communis* L.). *Journal of Agricultural and Food Chemistry*, *50*, 5458–5462. https://doi.org/10.1021/jf0202584
7. Chel-Guerrero L.D., Sauri-Duch E., Fragoso-Serrano M.C., Pérez-Flores L.J., Gómez-Olivares J.L., Salinas-Arreortu, N., Sierra-Palacios, E.C., Mendoza-Espinoza, J.A. (2018). Phytochemical profile, toxicity, and pharmacological potential of peels from four species of tropical fruits. *Journal of Medicinal Food*, *21*(7), 734–743. https://doi.org/10.1089/jmf.2017.0124
8. Cory, H., Passarelli, S., Szeto, J., Tamez, M., Mattei, J. (2018). The role of polyphenols in human health and food systems: A mini-review. *Frontiers in Nutrition*, *5*, 87–96. https://doi.org/10.3389/fnut.2018.00087
9. Dadgar M., Pouramir, M., Dastan, Z., Ghasemi-Kasman, M., Ashrafpour, M., Moghadamnia, A.A., Khafri, S., Pourghasem, M. (2018). Arbutin attenuates behavioral impairment and oxidative stress in an animal model of Parkinson's disease. *Avicenna Journal of Phytomedicine*, *8*, 533–542.
10. Dajić-Stevanović, Z., Petrović, M., Aćić, S. (2014). Ethnobotanical knowledge and traditional use of plants in Serbia in relation to sustainable rural development. In: Pieroni, A., Quave, C.L. (Eds.), *Ethnobotany and Biocultural Diversities in the Balkans*, Springer, New York, Heidelberg, Dordrecht, London, pp. 229–252. https://doi.org/10.1007/978-1-4939-1492-0_12
11. Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, *7*, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9
12. Galanakis, C.M., Rizou, M., Aldawoud, T.M., Ucak, I., Rowan, N.J. (2021). Innovations and technology disruptions in the food sector within the COVID-19 pandemic and post-lockdown era. *Trends in Food Science & Technology*, *110*, 193-200. https://doi.org/10.1016/j.tifs.2021.02.002
13. Hammer, O., Harper, D.T., Ryan, P.D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*, 1–9.
14. Imeh, U., Khokhar, S. (2002). Distribution of conjugated and free phenols in fruits. *Journal of Agricultural and Food Chemistry*, *50*, 6301–6306. https://doi.org/10.1021/jf020342j
15. Jeong, D.E., Cho, J.Y., Lee, Y.G., Jeong, H.Y., Lee, H.J., Moon, J.H. (2017). Isolation of five proanthocyanidins from pear (*Pyrus pyrifolia* Nakai) fruit peels. *Food Science and Biotechnology, 26*(5), 1209–1215. https://doi.org/10.1007/s10068-017-0157-4
16. Kaur, R., Arya, V. (2012). Ethnomedicinal and phytochemical perspectives of *Pyrus communis* L. *Journal of Pharmacognosy and Phytochemistry*, *1*, 14–19.
17. Kolniak-Ostek, J. (2016). Chemical composition and antioxidant capacity of different anatomical parts of pear (*Pyrus communis* L.). *Food Chemistry*, *203*, 491–497. https://doi.org/10.1016/j.foodchem.2016.02.103
18. Kolniak-Ostek, J., Kłopotowska, D., Rutkowski, K.P., Skorupińska, A., Kruczyńska, D.E. (2020). Bioactive compounds and health-promoting properties of pear (*Pyrus communis* L.) fruits. *Molecules*, *25*(19), art. no. 4444. https://doi.org/10.3390/molecules25194444
19. Li, X., Jiang, Q., Wang, T., Liu, J., Chen, D. (2016a). Comparison of the antioxidant effects of quercitrin and isoquercitrin: Understanding the role of the 6 "-OH group. *Molecules, 21*(9), 1246–1257. https://doi.org/10.3390/molecules21091246
20. Li, X., Wang, T., Gao, W. (2016b). Nutritional composition of pear cultivars (*Pyrus* spp.). In V.R. Preedy, M.S.J. Simmonds (Eds.) *Nutritional Composition of Fruit Cultivars*, Academic Press, San Diego, CA, USA, pp. 573–608. https://doi.org/10.1016/B978-0-12-408117-8.00024-6
21. Li, X., Wang, T., Zhou, B., Gao, W., Cao, J., Huang, L. (2014). Chemical composition and antioxidant and anti-inflamatory potential of peels and flesh from 10 different pear varieties (*Pyrus* sp.). *Food Chemistry*, *152*, 531–538. https://doi.org/10.1016/j.foodchem.2013.12.010
22. Li, X., Zhang, J.Y., Gao, V.V.Y., Wang, H.Y., Cao, J.G., Huang, L.Q. (2012). Chemical composition and anti-inflammatory and antioxidant activities of either pear cultivars. *Journal of Agricultural and Food Chem*istry, *60*, 8738–8744. https://doi.org/10.1021/jf303235h
23. Liaudanskas, M., Zymonė, K., Viškelis, J., Klevinskas, A., Janulis, V. (2017). Determination of the phenolic composition and antioxidant activity of pear extracts. *Journal of Chemistry*, *2017*(1), 1–9. https://doi.org/10.1155/2017/7856521
24. Lin, L.Z., Harnly, J.M. (2008). Phenolic compounds and chromatographic profiles of pear skins (*Pyrus* spp.). *Journal of Agricultural and Food Chem*istry, *56*, 9094–9101. https://doi.org/10.1021/jf8013487
25. Ma, J.-N.., Wang, S.-L., Zhang, K., Wu, Z.-G., Hattori, M., Chen, G.-L., Ma, C.-M. (2012). Chemical components and antioxidant activity of the peels of commercial apple shaped pear (fruit of *Pyrus pyrifolia* cv. *pingguoli*). *Journal of Food Science*, *77*, C1097-C1102. https://doi.org/10.1111/j.1750-3841.2012.02899.x
26. Masuda, T., Yamashita, D., Takeda, Y., Yonemori, S. (2005). Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. *Bioscience, Biotechnology and Biochemistry*, *69*, 197–201. https://doi.org/10.1271/bbb.69.197
27. McClure, K.A., Gong, Y., Song, J., Vinqvist-Tymchuk, M., Palmer, L.C., Fan, L., Burgher-MacLellan, K., Zhang, Z.Q., Celton, J.M., Forney, C.F., Migicovsky, Z., Myles, S. (2019). Genome-wide association studies in apple reveal loci of large

effect controlling apple polyphenols. *Horticulture Research*, *6*(1), art. no. 107. https://doi.org/10.1038/s41438-019-0190-y

28. Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, *84*, 407–412. https://doi.org/10.1042/cs0840407
29. Öztürk, A., Demirsoy, L., Demirsoy, H., Asan, A., Gül, O. (2015). Phenolic compounds and chemical characteristics of pears *(Pyrus communis* L.). *International Journal of Food Properties*, *18*, 536–546. https://doi.org/10.1080/10942912.2013.835821
30. Park, Y.K., Koo, M.H., Ikegaki, M., Contado, J.L. (1997). Comparison of the flavonoid aglycone contents of *Apis melifera* propolis from various regions of Brazil. *Arquivos de Biologia e Tecnologia, 40*, 97–106.
31. Parle, M., Arzoo, (2016). Why is pear so dear. *International Journal of Research in Ayurveda and Pharmacy*, *7*, 108–113. https://doi.org/10.7897/2277-4343.07139
32. Reiland, H., Slavin, J. (2015). Systematic review of pears and health. *Food and Nutrition Today*, *50*(6), 301–305. https://doi.org/10.1097/NT.0000000000000112
33. Salta, J, Martins, A., Santos, R.G., Neng, N.R., Nogneira, J.M.F., Justino, J., Ruter, A.P. (2010). Phenolic composition and antioxidant activity of Rocha pear and other pear cultivars – a comparative study. *Journal of Functional Foods, 2*, 153–157. https://doi.org/10.1016/j.jff.2010.02.002
34. Savić, A., Jarić, S., Dajić-Stevanović, Z., Duletić-Laušević, S. (2019). Ethnobotanical study and traditional use of autochthonous pear varieties (*Pyrus communis* L.) in southwest Serbia (Polimlje). *Genetic Resources and Crop Evolution*, *66*, 589–609. https://doi.org/10.1007/s10722-018-00734-w
35. Sharma, K., Pasricha, V., Satpathy, G., Gupta, R.K. (2015). Evaluation of phytochemical activity of raw *Pyrus communis* L, an underexploited fruit. *Journal of Pharmacognosy and Phytochemistry*, *3*, 46–50.
36. Singleton, V.L., Rossi, J.A. (1965). Colometric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*, 144–158.
37. Szwajgier, D., Borowiec, K. (2012). Screening for cholinesterase inhibitors in selected fruits and vegetables. *Electronic Journal of Polish Agricultural Universities*, *15*, art. no. 6.
38. Šavikin, K., Živković, J., Zdunjić, G., Dojčinović, B., Menković, N. (2014). Phenolic and mineral profiles of four Balkan indigenous apple cultivars monitored at two different maturity stages. *Journal of Food Composition and Analysis*, *35*, 101–111. https://doi.org/10.1016/j.jfca.2014.05.004
39. Šavikin, K., Živković J., Alimpić, A., Zdunjić, G., Janković T., Duletić-Laušević, S., Menković, N. (2018). Activity guided fractionation of pomegranate extract and its antioxidant, antidiabetic and antineurodegenerative properties. *Industrial Crops & Products*, *113*, 142–149. https://doi.org/10.1016/j.indcrop.2018.01.031
40. Taylor, R. (1990). Interpretation of the correlation coefficient: a basic review. *Journal of Diagnostic Medical Sonography*, 6(1), 35–39. https://doi.org/10.1177/875647939000600106
41. Wilson, D.W., Nash, P., Buttar, H.S., Griffiths, K., Singh, R., De Meester, F., Takahashi, T. (2017). The role of food antioxidants, benefits of functional foods, and influence of feeding habits on the health of the older person: an overview. *Antioxidants*, *6*(4), art. no. 81. https://doi.org/10.3390/antiox6040081

Pol. J. Food Nutr. Sci., 2021, Vol. 71, No. 2, pp. 237–239
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 edition. 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