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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
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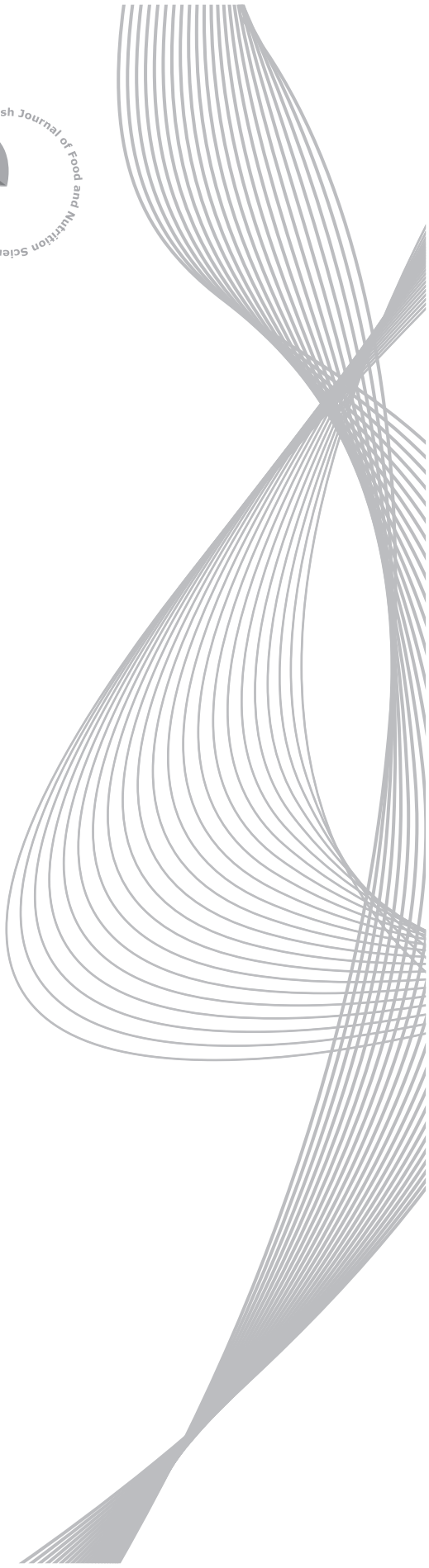

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REVIEW

ORIGINAL PAPERS

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.

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Review article
Section: Food Quality and Functionality

Recent Advances in Biotechnological Methods for Wheat Gluten Immunotoxicity Abolishment – a Review

Vijole Bradauskiene[1,2], Lina Vaiciulyte-Funk[1], Bakht Ramin Shah[3], Darius Cernauskas[1], Mihaela Adriana Tita[4]*

[1]Food Institute, Kaunas University of Technology, Radvilenu road 19C-413, Kaunas, Lithuania
[2]Food Technology Department, Klaipeda State University of Applied Sciences, Bijunu street 10–223, Klaipeda, Lithuania
[3]Institute of Aquaculture and Protection of Waters, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in Ceske Budejovice, Na Sádkách 1780, 370 05, České Budějovice, Czech Republic
[4]Faculty of Agricultural Sciences, Food Industry and Environmental Protection, Lucian Blaga University of Sibiu, Ion Ratiu street 5–7, Sibiu, România

Key words: celiac disease, wheat immunogenicity, detoxified wheat, gluten hydrolysis

Due to the increasing incidence of gluten intolerance, researchers are focusing on finding ways to eliminate immunotoxicity of wheat, this would allow the use of wheat products for gluten-intolerant consumers. The article reviews recent studies on biotechnological methods to eliminate and reduce the immunogenicity of wheat products. So far, many gluten removal methods have been proposed, but their efficacy levels were quite different. Enzymatic treatment of gluten fragments can be considered the simplest and non-invasive tool to eliminate the toxicity of gliadins and glutenins. For this purpose, various endogenous enzymes derived from cereals, and also those of bacterial, fungal, plant, and animal origin can be used in food processing. Some of the enzymes hydrolyze gluten, others block the action of toxic protein fragments. The majority of studies were carried out using lactic acid bacteria cultures, as single strains or in consortia. Satisfactory results have been achieved using bacterial and plant enzymes, but the complete elimination of gluten immunogenicity is still possible by using fungal proteases, engineered enzymes or combining several treatments, for example, by using lactic acid fermentation or germination with fungal proteases. However, the question of how degradation of gluten affects the quality of flour (dough) in practice remains unanswered. It is not clear whether the products of such wheat flour are better and safer than those made from starches and whether their price and quality are acceptable to consumers. The insights presented in this review will be helpful to other researchers and cereal-based food producers in choosing ways to reduce gluten immunogenicity.

ABBREVIATIONS

CD: coeliac disease; GF: gluten-free; GFD: gluten-free diet; HLA: human leukocyte antigen; LAB: lactic acid bacteria; NCGS: non-coeliac gluten sensitivity; PEP: prolyl endopeptidase; WA: wheat allergies.

INTRODUCTION

Wheat and other grains are one of the most important components of human nutrition on a global scale, and total wheat consumption has increased in recent years [FAOSTAT: Production/Yield quantities of Wheat in World, 2020]. However, for a large number of consumers, wheat gluten proteins cause severe intolerance, manifested by allergic reactions. Gliadins and glutenins are fractions of the immunogenic gluten protein [Giménez *et al*., 2019]. These toxic proteins can cause wheat allergies (WA) and development of coeliac disease (CD) in some consumers [Navarro *et al*., 2017]. CD is one of the most common diseases associated with food intolerance and its prevalence in the world is increasing [King *et al*., 2020]. CD is more pronounced for genetically predisposed people as an inflammatory disease of the upper small intestine. The manifestation of the disease is determined by glutamine- and proline-containing cereal gluten peptides, most of them with a minimum length of nine amino acids [Bromilow *et al*., 2017]. During digestion, these peptides are not sufficiently digested by digestive enzymes (pepsin, chymotrypsin, trypsin); they reach lymphatic tissue, meet antigenic cells HLA-DQ2 and HLA-DQ8, specific for coeliac disease, and stimulate intestinal T-cells. Typical clinical signs for CD are flat intestinal mucosa and malabsorption [McAllister *et al*., 2019]. In addition, an increasing number of cases of non-coeliac gluten sensitivity (NCGS) have been reported in consumers without CD or WA (gliadin does not cause mucosal inflammation of mucous membrane) [Catassi *et al*., 2015; Lionetti *et al*., 2017]. These cases are manifested by irritation

* Corresponding author: Tel.: +37067107872;
E-mail: vijole.bradauskiene@ktu.edu (V. Bradauskiene)

of the intestines and other symptoms associated with the use of gluten-containing foods [Tanveer & Ahmed, 2019].

According to the latest scientific data, the life-long (permanent) gluten-free diet (GFD) is an effective treatment for CD. This diet is popular among consumers with NCGS, and also among people who do not experience CD, but are seeking favorable health effect from consumption of a GFD [Krigel & Lebwohl, 2016]. Consumers seeking GFD face many challenges associated with cross-contamination, lack of clarity in food labeling policies, poor quality of gluten-free (GF) products, and higher prices compared to gluten-containing foods [Do Nascimento *et al*., 2017; Estévez *et al*., 2016]. Nutrition of GFD-compliant consumers is unbalanced, leading to a higher percentage of calories from fat and less from carbohydrates. In addition, there was found a deficiency of non-starch polysaccharides [Hopkins & Soon, 2019], which are very important for reducing the risk factors for developing chronic diseases and certain types of oncological diseases [Lovegrove *et al*., 2017]. GF products introduced in the market have a poorer taste than regular products, and may cause a nutritional deficiency among consumers due to the unbalanced composition of nutrients [Stantiall & Serventi, 2018]. Examination of patients with CD showed that those with the same energy intake as the control group had a lower intake of fiber, vitamin A, B-group vitamins: B_6, B_{12}, folic acid, thiamine and minerals: calcium, phosphorus, magnesium, and iron [Pellegrini & Agostoni, 2015; Vici *et al*., 2016]. Zinc and selenium deficiency may be also associated with the elimination of cereals from the diet [Stazi & Trinti, 2008; Tran *et al.*, 2011]. Bread and other products made from naturally gluten-free raw materials such as: buckwheat, rice, corn, quinoa, sorghum, or teff flours, often have lower textural and sensory properties compared to the corresponding gluten-containing bread products [Naqash *et al*., 2017]. The GF products usually have a high glycemic index [Vici *et al*., 2016], which is associated with an increased risk of obesity among consumers with CD. The increasing demand for high-quality GF bakery products leads to the search for new approaches in GF food producing. The production of nutritionally-balanced GF products is an important social and economic issue. To solve it, new strategies are being searched for to remove immunogenicity from wheat and other cereals and produce balanced, sensorially-acceptable to consumer products.

The diet should not only be gluten-free but also health-friendly to avoid nutritional imbalances [Chishty & Singh, 2017]. The setup of application of biotechnological tools based on enzyme treatments is an active field of research that may provide new possibilities to GF wheat product development. For products used in GFD, it is necessary to remove or degrade wheat prolamins that are harmful to gluten-non-tolerant users. Studies related to the elimination of gluten from wheat processing products by using biological methods have been performed [Scherf *et al*., 2018]. Unfortunately, there is a lack of scientific information about the efficiency of biological measures on different conditions, as well as on the possibilities of implementing these methods in the production of wheat products in order to modify their chemical composition and effectively eliminate gluten residues to improve product absorption.

The aim of this review article is to analyze the biotechnological methods for the elimination / reduction of immunogenicity of wheat products and evaluate the possibilities of their implementation. The insights of this review will be helpful to other researchers and wheat producers to choose ways for gluten immunotoxicity abolishment. The threshold set by the Codex Alimentarius [Standard 118–1979] for a gluten-free food claims at 20 mg gluten/kg product. However, despite the fact that manufacturers are subject to strict regulations, even consumers, who adher to GFD, were reported to consume more than the tolerable amount of gluten because of the contamination of products and inaccurate labeling [Bruins Slot *et al*., 2015]. Therefore, it is desirable to provide safe GF foods with a gluten level as low as possible. A reduction of the immunoreactivity of food proteins can be achieved by proteolysis occurring in food and degrading the immunoreactive protein fragments [El-Ghaish *et al*., 2011].

CLASSIFICATION OF BIOTECHNOLOGICAL MEASURES FOR GLUTEN HYDROLYSIS

Over the last decade, the use of biological measures in wheat products to eliminate or reduce the immunotoxicity of gluten proteins is being actively studied. Various enzymes such as those of bacterial, fungal, plant, and animal origin as well as recombinant enzymes expressed in microbial systems were investigated. The scheme of the sources of enzymes used for gluten hydrolysis, thus eliminating or reducing the gluten immunotoxicity, is shown in Figure 1.

Gluten can be hydrolyzed either by individual enzymes or by combining different biological measures [Scherf *et al*., 2018]. Enzymatic cleavage of gluten fragments is the easiest and non-invasive way to eliminate the toxicity of gliadins and glutenins. It can be applied in two ways. Firstly, in order to reduce the negative effects of gluten on patients with CD, enzymes should be taken with food [Janssen *et al*., 2015]. Secondly, the toxic effects of gluten can be eliminated before consumption during food processing [Jouanin *et al*., 2018].

POTENTIAL OF DIFFERENT ENZYMES TO ELIMINATE GLUTEN IMMUNOGENICITY

Different peptidases can be used to degrade gliadins and glutenins in food products [Wieser & Koehler, 2012]. Endoproteases attack internal peptide bonds, while exoproteases attack only the N-terminal (aminopeptidases) or C-terminal (carboxypeptidases) forms.

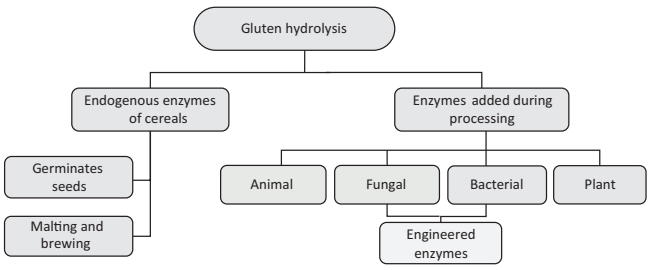

FIGURE 1. Sources of enzymes used for gluten hydrolysis.

TABLE 1. Cereal endogenous enzymes used for gluten hydrolysis.

Source of enzymes	Results	References
Germinating grains	Hydrolysis of prolamines.	Hartmann *et al.* [2006]
Proteases from germinating cereals	Hydrolysis of prolamines.	Stenman *et al.* [2009]
Wheat grains germinated for 8 days	Reduction of peptides eliciting immune response.	Boukid *et al.* [2017b]
Wheat kernels after 7-day germination	Degradation of gluten.	Michalcová *et al.* [2019]
Germination of wheat kernels	Gluten content in wheat germ (8–10 days) was lower than the limit of detection.	Adrianos *et al.* [2017]
Recombinant wheat cysteine protease Triticain-α	Triticain-α activated proteolytic enzymes *in vitro*.	Savvateeva *et al*. [2015]
Cysteine protease EP-B2 from germinated barley	EP-B2 has good specificity for the immunotoxic wheat gluten amino acid sequences.	Diaz-Mendoza *et al*. [2019]

Endogenous enzymes from cereals

Proteases are often synthesized as inactive specific proteases which are activated under the appropriate conditions of temperature, humidity, *etc*. One way to activate endoprotease is grain germination. Although this measure is mainly applied to barley – in the malt preparation and beer production [Guerdrum & Bamforth, 2012; Kerpes *et al*., 2016; Knorr *et al*., 2016], the immunogenicity of wheat can also be reduced in a similar way. Studies in which gluten is hydrolyzed by endopeptidases during grain germination are systematized in Table 1.

Hartmann *et al*. [2006] proved that after 2-h incubation (37°C, pH 6.5), the toxic wheat peptides were intensively degraded – peptides with more than eight amino acid residues were not detected. As Stenman *et al*. [2009] proved, the gluten content in specially prepared germinated wheat can be minimal. Michalcová *et al*. [2019] analyzed the conditions for digesting wheat gluten proteins by endoproteases during germination. In their experiment, the wheat kernels were germinated for up to 7 days at temperatures of 15–30°C (pH 3.0–8.0). The authors observed gluten degradation that started after 3 days and lasted for up to 7 days. The lowest content of gluten proteins was measured on the 7th day at a temperature of 20°C (pH 5.5). In turn, Adrianos *et al*. [2017] showed that the gluten content of wheat sprouts (8–10 days) in all preparations was lower than the limit of detection for both ELISA (4 mg/kg) and the thresholds established by Codex Alimentarius [Standard 118 – 1979]; therefore, the sprouts could be classified as GF. Wheat sprouts are rich in nutrients that might be beneficial for consumers with malabsorption disorders. They can be used for juicing, added to cocktails, or consumed as wheat grain dietary supplements in tablets, capsules, and in the liquid form. An isolate from germinated cereals or purified enzymes can also be used for gluten hydrolysis. Experiments showed that germinated cereal proteases were capable of cleaving intact gluten proteins [Geßendorfer *et al*., 2011]. Schwalb *et al*. [2012] conducted studies on different germinated grain crops and identified their specific peptidase activity. The studies were performed with peptidase-enriched solutions. They were obtained by extraction from germinated, lyophilized, and milled grains. A synthetic α-gliadin peptide was used as a peptide substrate. It was degraded under different conditions: 60–90 min at 40–50°C (pH 4.0; 6.5). Peptide degradation was found to be more intense at pH 4.0 than at pH 6.5. Peptide was degraded slowly and only about 30% was left after 90 min. Germinating barley (*Hordeum vulgare*) seeds secrete an enzyme, cysteine protease, which hydrolyzes hordein, *i.e.* the barley analog of wheat gluten. This glutenase, named EP-B2, has good specificity for the immunotoxic wheat gluten amino acid sequences. Besides, it was found to be most active at low pH, resistant to pepsin but digested at physiological concentrations of trypsin; therefore, it was proved be suitable for gluten hydrolysis [Diaz-Mendoza *et al.,* 2019]. Kiyosaki *et al*. [2009] and Savvateeva *et al*. [2015] studied abilities of recombinant wheat cysteine protease – Triticain-α to activate proteolytic enzymes (glutenase and collagenase) *in vitro*, which are optimally active at 37°C. Mass-spectrometry analysis showed that Triticain-α degraded immunotoxic peptides. Studies have shown that Triticain-α has a high glutenase activity under normal human physiological conditions (37°C) and can, therefore, be used in CD treatment.

Generally, proteases from germinated cereals can significantly reduce the amounts of toxic gluten proteins or peptides. Therefore, they may be used in a variety of areas: food supplements that help the body digest gluten without allergic reactions, as well as in the production of special foods for CD patients [Adrianos *et al*., 2017]. The quality and nutritional value of wheat products can be improved by choosing the optimal duration and conditions of grain germination [Ding *et al*., 2018], because by germinating wheat grains for up to 48 h, it is possible to bake improved-quality bread that will not be GF even though the rheological properties of the dough are poorer [Baranzelli, 2018; Cardone *et al*., 2020]. Long-term germination increases the activity of α-amylase (breaks down starch), can significantly reduce the quality of flour and the baking process: lower falling numbers can affect mixability, crumb strength, and loaf specific volume and sliceability [Thomason *et al*., 2019]. Therefore, germination of wheat is a complex way to eliminate gluten from baked goods. Such detoxified wheat can only be used to supplement GF products, while in the production process it is easier to use the isolate from sprouted cereal grains or purified enzymes.

Bacterial enzymes

Fermentation with probiotic strains

The most common way of applying bacterial enzymes is fermentation with *Lactobacillus* strains. Lactic acid bacteria (LAB) fermentation can improve the texture and palatability of various types of foods (whole grains, fiber-rich, gluten-free), stabilize or increase the amount of various biologically-active compounds, retard starch bioavailability, and improve mineral bioavailability [Katina *et al*., 2006]. LAB produce a variety of enzymes that degrade anti-nutritional compounds, thereby improving the texture of baked goods, ensuring the development of palatability and the formation of aromatic compounds, and prolonging the shelf life [Luz *et al*., 2019; Gobbetti *et al*., 2019; Sun *et al*., 2020]. Many studies have focused on the possibilities of probiotic strains to decrease the immunotoxicity of wheat products (Table 2). The main idea of those studies was to use bacterial enzymes during food processing to eliminate immunotoxic gliadins. Fermentation with LAB decreases the number of disulfide bonds in the gluten network, which causes an immune response in people with sensitivity to gluten [Gänzle *et al*., 2008]. Individual LAB species produce specific peptidases that are capable of hydrolyzing hardly degradable, immunoreactive, and coeliac disease-causing peptides [Vukotić *et al*., 2016]. It is important to select LAB strains with specific proteolytic effects for the successful breakdown of the gliadin complex structure [Stefańska *et al*., 2016].

The use of lactic acid cultures for the first time in the 21st century aimed to remove traces of gluten fragments from processed foods. Di Cagno *et al*. [2002] showed that selected LAB with proteolytic activity can effectively hydrolyze the toxic gliadin peptides in wheat sourdough. As Di Cagno *et al*. [2004] proved, *L. alimentarius 15M*, L. *brevis 14G*, *L. sanfranciscensis 7A*, and *L. hilgardii 51B* strains have peptidases capable of hydrolyzing all the different peptide bonds present in prolamins. The next study of Di Cagno *et al*. [2008] proved the effectiveness of selected LAB cultures to remove gluten residues and enhance the nutritional value of GF bread. Forty-six strains of LAB were tested for evaluating the proteolytic activity and medium acidification rate. Cultures of *L. sanfranciscensis LS40* and *LS41*, and *L. plantarum CF1* were selected as the most suitable for the production of GF bread from pseudocereals with gluten addition. During fermenting the bread, the initial gluten content of 400 mg/kg was degraded to below 20 mg/kg, and the content of free amino acids increased.

Several studies have also been carried out to assess the effectiveness of individual lactic acid cultures. Fermentation using *L. sanfranciscensis* [Thiele *et al*., 2004; Vermeulen *et al*., 2006] or *L. plantarum* [Gerez *et al.*, 2008; Rollan *et al*., 2005; Yin *et al*., 2015] promoted hydrolysis and increased solubility of wheat proteins. It has been shown that protein hydrolysis in sourdough is predominantly associated with pH-dependent activity of cereal enzymes and corresponding changes in proteolytic activity.

Several studies have been carried out to assess the effectiveness of the pool of selected probiotic lactobacilli. De Angelis *et al.* [2006] found the capacity of probiotic VSL#3 preparation to intensively hydrolyze wheat gliadins. Probiotic product VSL#3 consisting of strains of *Streptococcus thermophilus*, *L. plantarum*, *L. acidophilus*, *L. casei*, *L. delbrueckii spp*. *Bulgaricus*, *Bifidobacterium breve*, *B. longum*, and *B. infantis* was used in the fermentation of wheat flour dough to reduce the content of immunotoxic fragments, to hydrolyze gliadin peptides, and to achieve almost complete hydrolysis of gliadin. Patent application WO2006 / 097415 [2006] describes a method for gluten degradation using a complex mixture of at least six lactic acid bacterial cultures and / or bifidobacteria and long fermentation time (24–31 h). After hydrolysis, non-degraded gliadins remained, some gliadins were partially hydrolyzed, and others were insensitive hydrolyzed; therefore, this method is not the most suitable for complete gluten degradation. *L. plantarum CRL 775* and *Pediococcus pentosaceus CRL 792* also hydrolyzed gliadins during the fermentation of wheat dough. The cleavage of gliadins obtained using cell extracts was higher than using cell suspensions [Gerez *et al*., 2012]. Romanová & Urminská [2017] investigated growth characteristics of *L. plantarum CCM 3627* and *L. brevis CCM 1815* and the activity of aminopeptidases. In turn, Stefańska *et al.* [2016] investigated 11 LAB cultures that can hydrolyze gluten in baked goods. All sourdoughs have been found to contain some polypeptides with reactive epitopes. Two strains: *Enterococcus mundtii* and *Wickerhamomyces anomalus,* can be used as probiotics for leavening. *E. mundtii QAUSD01* and *W. anomalus QAUWA03* demonstrated the ability to tolerate low pH, resistance to bile salts, and hydrophobicity compared to other gluten-degrading yeast and bacterial strains. It is suitable to use them in cereal fermentation and, therefore, they can be used to produce bakery products for consumers with NCGS [Sakandar *et al*., 2018].

Sourdough-based biotechnology could contribute to the quality of life improvement in consumers suffering from CD [Nionelli & Rizzello, 2016]. However, this method is not suitable for complete gluten degradation. It is very important to select the optimally parameters of the fermentation process while preparing GF products. The results achieved in various studies showed that the proteolytic activity of the selected LAB strains was not high enough [Stefańska *et al*., 2016], long fermentation time worsened the technological properties of wheat bread [Katina *et al*., 2006], and that baked wheat products fermented with LAB were not safe for consumers with CD [Laatikainen *et al*., 2017]. Therefore, it is advisable to use these LAB to break down allergenic proteins in bakery products for consumers with CD in combination with other measures, and also to produce bakery products for consumers with NCGS [Sakandar *et al*., 2018].

Prolyl endopeptidases from microorganisms

A relatively new trend is gluten detoxification by breaking peptide bonds with prolyl endopeptidases (PEPs). PEPs are endoproteolytic enzymes secreted by microorganisms and plants. They hydrolyze gluten into smaller peptides that can be digested by intestinal enzymes or to amino acids [Heredia-Sandoval *et al*., 2016]. PEPs can be used as a dietary therapeutic tool for CD patients. The mechanism of their action is as follows: gluten is hydrolyzed by co-ingested peptidases in the consumer's stomach and stops (prevents) CD specific

TABLE 2. Enzymes of bacterial origin for reducing gluten immunogenicity.

Enzyme source	Enzyme/bacterial strain	Results	References
Fermentation with LAB cultures	*L. alimentarius, L. brevis, L. sanfranciscensis, L. hilgardii*	Gluten degradation.	Di Cagno *et al.* [2002]; Di Cagno *et al.* [2004]
	Probiotic product VSL # 3	Intense gliadin degradation.	De Angelis *et al*. [2006]; WO2006/097415
	L. sanfranciscensis	Gluten degradation.	Thiele *et al.* [2004]; Vermeulen *et al.* [2006]
	4 strains of *L. plantarum*	Gluten degradation – hydrolysis of wheat proteins.	Gerez *et al.* [2008] Rollan *et al.* [2005]
	L. plantarum	Hydrolysis and solubilization of wheat proteins.	Yin *et al*. [2015]
	47 strains of LAB were tested for evaluating proteolytic activity	Removed gluten residues in GF bread.	Giuliani *et al.* [2016]
	L. plantarum and *L. brevis*	Active proline aminopeptidase was produced.	Romanová *et al*. [2017]
	13 strains of probiotic bacteria	Partial gluten degradation.	Stefanska *et al.* [2016]
	18 commercial strains of LAB	The pool of LAB strongly hydrolyzed gluten in bread.	Francavilla *et al.* [2017]
	Enterococcus Enterococcus and *Wickerhanomyces* strains	LAB tolerated low pH and bile salts.	Sakandar *et al.* [2018]
	12 strains of LAB and yeasts isolated from Chinese traditional sourdough	Its ability to degrade wheat protein was limited.	Fu *et al.* [2020]
	Bifidobacterium species: *B. bifidum, B. longum, B. breve* and *B. animalis*	Proteolysis of intact gluten proteins, gliadins, and glutenins.	de Almeida *et al*. [2020]
Enzymes of bacterial origin	Prolylendopeptidase (PEP) from *Myxococcus xanthus*	PEP reduces the amount of immunoreactive gliadin peptides.	Alvarez-Sieiro *et al.* [2014]
	Proteases: *Bacillus stearothermophilus, B. thermoproteolyticus, Streptomyces griseus, B. licheniformis*	*B. licheniformis* and *B. thermoproteolyticus* work very effectively.	Socha *et al*. [2015]
	PEP from *F. meningosepticum, S. capsulata, M. xanthus* and *E. coli*	PEP partially degraded gliadin peptides.	Moreno Amador *et al.* [2019]
	Alcalase 2.4 L	Effectively removes gluten from unpurified starch.	Bassi [2016]
	Alcalase 2.4 L	Reduced the antigenicity of wheat gluten hydrolysates.	Cruz-Chamorro *et al.* [2020]
	New E40 glutenase from *Actinomycete* strain *Actinoallomurus A8*	Efficiently degrades the most immune-toxic gluten.	Cavaletti *et al.* [2019]
	Bacillus spp. isolated from sourdough	Reduced gluten content in wheat sourdough.	Rashmi *et al.* [2020]
Engineered enzymes	PEP enzyme of *Sphingomonas capsulate*	Effectively degraded CD-active peptides.	Ehren *et al.* [2008]
	Peptidase Kuma030	High activity of 99.97% gluten degradation.	Wolf *et al.* [2015]
	Peptidase KumaMax or Kuma010	116 times higher proteolytic activity compared to native.	Yoosuf & Makharia [2019]
Transglutaminase (mTG) of microbial origin	Transamidation with mTG in the presence of methyl ester of lysine	Reduced the ability to induce an immune response *in vitro.*	Luongo *et al.* [2020]
	MTG and chymotrypsin used to bind lysine or valine to gluten proteins	Reduced the specific immune response of gliadin.	Zhou *et al.* [2017]

immune responses in the small intestine. Likewise, PEPs may be used to produce GF products from gluten-containing raw materials. Enzymatic therapy for coeliac disease is promising; however, it is important to select the most active enzymes [Boukid *et al*., 2017a]. Matysiak-Budnik *et al*. [2005] found that PEPs partially degraded gliadin peptides at low concentrations (20 mU/mL) both *in vitro* and *ex vivo*, but could not protect the intestines from immunotoxic metabolites. High levels of PEPs and a longer time (at least 500 mU/mL for 3 h) were required for the complete detoxification of peptides. As Socha *et al*. [2015] proved, all the bacterial proteases of various origin were slightly different in the degree of proteolysis. *B. licheniformis* and *B. thermoproteolyticus* acted very effectively: molecular weight of fermentation products was low. The use of PEPs from *L. casei* with conventional food was proposed as a treatment method for CD patients [Alvarez-Sieiro *et al*., 2014]. Bassi [2016] described a method to produce GF starch, where the mass of unpurified starch is treated with an agent to degrade gluten. It should be noted that the effective removal of gluten (even up to 470 mg/kg) from starch can be achieved using Alcalase. Prolonged exposure to high concentrations of PEPs was shown to reduce the amount of immunoreactive gliadin peptides in wheat flours [Rashmi *et al*., 2020].

Engineered enzymes

An ideal peptidase for use in the oral enzymatic therapy should possess many qualities to meet high requirements of its application; therefore, it is unlikely that a single naturally occurring enzyme can afford this to the full extent. In a way to solve the problem, the computational protein design allows introducing new traits or significantly improve functional properties of native enzymes [Gordon *et al*., 2012]. Newly constructed enzymes, gluten hydrolases among them, exhibit higher specificity, much higher activity, solubility, and better performance in the required medium (for example, highly acidic environment of the stomach after meal) [Yoosuf & Makharia, 2019].

First works were carried out in this respect by Ehren *et al*. [2009]. They aimed to develop PEPs with higher activity and stability under gastric conditions, taking a PEP of *Sphingomonas capsulate* as a native template enzyme. The enzyme with as much as 20% enhanced specific activity at pH 4.5 and 200-fold greater resistance to pepsin was created. However, this peptidase was reported to have low to negligible levels of catalytic activity in a lower pH (in the actual pH range of the stomach) and was, thus, expected to be effective only in the small intestine region.

Best described engineered enzymes for gluten hydrolysis are peptidases KumaMax and Kuma030. KumaMax was designed as the improvement of kumamolisin-As (KumaWT, EC 3.4.21), an acidic serine endopeptidase of an acidophilic bacterium *Alicyclobacillus sendaiensis* [Gordon *et al*, 2012]. The cleavage specificity of the catalytically-active site of KumaWT was shifted to CD-active peptides. The KumaMax has more than 100-fold increased activity on the gluten tetrapeptide substrate. The next step in kumamolisin-As improvement was carried out using the same Rosetta Molecular Modeling Suite, which allowed redesign the active site of KumaMax [Wolf *et al*., 2015] to reach >99% activity of the enzyme. The new enzyme was referred to as Kuma030. It is 44-fold more active against peptides containing PQQ, and 11-fold more active against peptides containing PQL, than KumaMax. Kuma030 effectively (99.97%) degraded CD-active peptides in 30 min. This enzyme could also be applicable for gluten removal during food processing. Though the newly designed enzymes are still waiting for the clinical studies, they look as promising measures for preventing CD effects.

Transamidation with transglutaminase

Enzymatic hydrolysis (during the production of wheat flour dough) involves the degradation of the wheat proteins, including highly immunotoxic ones, to those with CD. It completely destroys the gluten structure, and reduces the technological properties (elasticity) of the dough and baked goods. In manufacturing practice, these problems are dealt with by using flour structure enhancers (gelatinized starch), emulsifiers, and hydrocolloids. Therefore, the scientific community has a strong interest in finding alternative methods for gluten degradation: strategies are being developed to eliminate harmful gluten peptides from gluten-free products to counteract the immunogenic effects of gluten fragments, as well as strategies to block gluten-induced inflammatory response [Heredia-Sandoval, 2016]. The essence of this gluten-blocking method is the specific blocking of toxic gluten fragments by microbial tissue-transglutaminase (*Streptomyces mobaraensis*) using lysine methyl ester. It has a great advantage because blocking the immunogenicity of T-cell epitopes does not damage the gluten network and preserves the technological properties of flour. Marino *et al*. [2017] assessed the safety of mTG-modified wheat flour for patients with CD and found that transamidated gluten reduced the number of clinical relapses to a limited extent. There would be a need for more extensive clinical trials to confirm the safety of enzymatic treatment of wheat flour for individuals with CD, including further investigation involving a large number of volunteers who consumed products from detoxified wheat flour.

Wheat flour transamidation can be a good alternative to a GFD, but there is not enough scientific evidence that this method is 100% safe. Scientists are worried and warned against the transglutaminase added to the industrially-processed food, doubting its safety and bringing up its putative role in CD pathogenesis [Aaron & Torsten, 2019]. There are ample studies that have shown that transglutaminase causes some neurodegenerative diseases, such as Alzheimer disease and Huntington disease [Amirdivani *et al*., 2018]. Furthermore, it is supposed that industrial processing of food using microbial transglutaminase to improve its properties has a negative effect on the gluten-sensitive population: it creates new immunogenic gluten peptides [Lerner & Matthias, 2015; Matthias *et al*., 2016] and may affect autoantigen occurrence in patients with CD.

Enzymes of fungal origin

Fungal proteases, commonly used as quality enhancers for baked goods, can cause primary proteolysis of gluten. Results of studies have shown that endoproteases from *Aspergillus niger* (AnP2), (AN-PEP) effectively degrade gluten proteins. Stepniak *et al*. [2006] noticed that *Aspergillus niger* (AnP2) prolyl endoprotease had optimal activity at pH 4–5 and remained stable at pH 2. Furthermore, the *A. niger*-derived enzyme effectively degraded all the T-cell stimulants and non-degraded gluten molecules. On average, the AnP2 degraded gluten 60 times faster than prolyl oligopeptidases. Low doses of pure AN-PEP can effectively reduce the amount of gluten fragments [Janssen *et al.*, 2015; Kumar Mohan *et al.,* 2019]. Toft-Hansen *et al.* [2014] tested 25 proteases and investigated 10 of them for their potential to degrade gluten *in vitro.* They proved that the protease from *Aspergillus niger* (AnP2) completely degraded gluten. In turn, Socha *et al*. [2015] tested the proteolytic activity of fungal proteases isolated from *Aspergillus sp.*, *Aspergillus oryzae*, and *Aspergillus niger*. The most effective proteolytic activity was also observed with the use of acid proteinase (AN-PEP) from *A. niger*, since wheat gliadins and peptides of low molecular weight were completely hydrolyzed. This prolyl endoprotease can also be used as an oral supplement to reduce the effect of gluten on consumers. AN-PEP can accelerate the digestion of gluten in the gastrointestinal tract [Salden *et al*., 2015]. This means that using AN-PEP in combination with gluten-containing food can eliminate the toxic effects of gluten, thus offering the possibility to refuse (occasionally) a strict GFD [Mitea *et al*., 2008; Montserrat *et al*., 2015]. Fungal proteases have the highest activity in

degrading gluten immunotoxic compounds compared to other proteases [Socha *et al*., 2015]. The composition of food affects the amount of AN-PEP required to eliminate gluten [Montserrat *et al*., 2015]. Studies have shown that single fungi-derived proteases can be effective for gluten degradation and that the combination of several fungal proteases allows a faster reduction in the content of toxic gluten fragments using only one enzyme [Ehren *et al*., 2009]. The ability of Flavourzyme (an enzyme complex from *Aspergillus oryzae*) to hydrolyze the prolamins of wheat was investigated. The results showed that Flavourzyme effectively degraded gliadins and could significantly reduce their immunotoxicity [Mickowska *et al*., 2018]. Schultz *et al.* [2018] investigated the ability of prolyl endopeptidases extracted from *Flammulina velutipes* (FvpP) to hydrolyze gluten. The FvpP hydrolyzed α-gliadin into small, less hydrophobic peptides after 20-h incubation. It was active at different pH values and higher salt concentrations, *i.e*., under similar conditions as in grain products, showed a moderate temperature stability, and slight thermal inactivation after use.

Studies have shown that fungi-derived proteases could be considered the most effective for gluten degradation. Further research should focus on using AN-PEP-treated starch in GF bakery products, and on degrading gluten in wheat bran or fermented food products to maintain a high nutritional value, and good technological and organoleptic properties, for example, in leavened products. However, doubts remain as to whether the use of these proteases is completely safe in the production of wheat products and acceptable to consumers.

Enzymes of plant origin

The use of plant enzymes is not a new concept in the baking industry. These enzymes play an important role in the production of some foods: syrups, alcoholic beverages, dairy products, bakery products, *etc*. [Meshram *et al*., 2019]. Plant proteases are enzymes that are commonly found in fruits, such as papaya, pineapples, figs, and kiwifruit. Sun *et al*. [2016] evaluated protease activity in 90 species of plants, including fruit and vegetables. Ten types of fruit and thirteen vegetables possessed high protease activity. Pineapples, figs, and papaya used to produce commercial proteases showed a high level of protease activity. In addition, high protease activity was detected in kiwifruit, broccoli, ginger, leek, and red pepper. Based on data above, it can be concluded that plants have high untapped potential as candidates for plant protease production.

Papain, bromelain, and actinidin belong to the cysteine protease family and exhibit a high hydrolytic potential. Papain was used to produce wheat gluten hydrolysates, a by-product of wheat starch production. During treatment with papain, the low molecular weight peptides were released from proteins [Wang *et al*., 2007]. Papain destroyed allergenic epitopes by hydrolyzing gliadins into small peptides [Buddrick *et al.*, 2015; Xue *et al.*, 2019] and exhibited great effects on gliadin hydrolysis even at its very low concentration [Li *et al.*, 2016]. Bromelain was used to produce hypoallergenic flour suitable for patients with wheat allergies. It can hydrolyze peptide bonds in proline residues and thereby alter the structure of gluten fragments [Watanabe *et al*., 2000]. Kiwifruit proteases are enzymes belonging to the cysteine protease family of papain. An *in vitro* study [Kaur *et al.,* 2010] showed that actinidine from a green kiwifruit affected protein digestion in the small intestine. Various food proteins, including cereal gluten, have been incubated with or without green kiwifruit extract using a two-stage *in vitro* digestive system consisting of an incubation with pepsin at stomach pH (mimicking gastric digestion) and then with pancreatin at low intestinal pH, imitating the human digestive tract. The kiwifruit extract affected gluten absorption. Actinidin has been shown to improve gluten digestion in experimental rats [Rutherfurd *et al*., 2011]. Jayawardana *et al.* [2019] analyzed the possibility of minimizing gluten intolerance by co-consumption of some fruits: papaya, pineapple and green kiwifruit, and highlighted the potential of green kiwifruit for consumption as a means of minimizing adverse effects of dietary gluten.

As Taga *et al.* [2017] proved, ginger protease can also hydrolyze gluten to peptides with an average molecular weight of <600 Da under weak acidic conditions. Data obtained from the studies performed by Bellir *et al.* [2014] and Gabr [2018] have confirmed that *Nigella sativa* (also known as blackseed or black caraway) had proteases that could be used in the food industry. The protease from the seeds of *N. sativa* can hydrolyze the gluten protein; therefore, it can be used to treat coeliac disease. *N. sativa* seed proteases, due to their ability to detoxify gluten, may offer an alternative treatment for CD in the future. Less common plants also have active proteolytic enzymes. Considerable attention was drawn to the study of digestive enzymes of an exotic fleshy plant *Nepenthes pitcher*, including protease nepenthesin [Ravee *et al*., 2018]. The *Nepenthes pitcher* fluid has a particularly strong ability to detoxify gluten, which can be associated with the formation of a new generation of prolyl endoproteases [Rey *et al*., 2016; Schräder *et al*., 2017]. Cumin (*Cuminum cyminum L.*) water extracts have high protease activity too. Cumin seed peptides significantly increased the proteolytic activity of pepsin (up to 400%) [Siow *et al*., 2016]; however experiments with gluten have not been performed.

Enzymes of animal origin and human digestive enzymes

Wheat gluten can be hydrolyzed using various enzymes of animal origin. A number of studies have been published in which wheat gluten has been degraded by commercial proteases of animal origin (pepsin, pancreatin, trypsin and chymotrypsin). All enzymes cleaved gliadin to peptides with a lower molecular weight (10–15 kDa), and chymotrypsin was the most effective; however, their effects have been found to be limited [Cao *et al*., 2020; Giorgi *et al*., 2020].

Insects and larvae have many strong proteolytic enzymes in the digestive tract [Grover *et al*., 2018; Pilon *et al*., 2017]. Insect enzymes have been shown to be specially adapted for the efficient hydrolysis of wheat proteins. A proline specific serine peptidase from the midgut of the yellow mealworm (*Tenebrio molitor*) can actively hydrolyze wheat gluten to polypeptides [Tereshchenkova *et al*., 2016]. Proteolytic bacteria from the gut of the velvetbean caterpillar (*Anticarsia gemmatalis*) showed increased activity at 40°C, and were active at pH 7.5–10 [Pilon *et al.*, 2017]. From among all the tested insects, *Rhizopertha dominica* showed the highest activity of

prolyl peptidase [Mika *et al*., 2015]. The proteolytic activity in *R. dominica* is owed to the trypsin-type enzymatic activity. This enzyme has been cloned and characterized but has not been used in the gluten-free food industry. Gutierrez *et al*. [2017] showed that enzymes of the gastrointestinal elastase 3B, elastase 2A, and carboxypeptidase A1 from human digestive tract could also degrade gluten. The investigation of the ability to degrade typical gluten peptides showed that, although they all were cleaved by proteases to a certain extent, the proteolysis products remained immunoreactive to coeliac T-cells and were likely to induce signs of CD [Tavano *et al*., 2018].

COMBINED APPROACH FOR WHEAT GLUTEN ALLERGENICITY ABOLISHMENT

Even if the enzyme treatment alone is unable to fully eliminate the immunogenicity of wheat products, the combined approach would allow applying these products in GF diet. There are various options for combining different methods. The results of studies on the combination of several biological treatments to eliminate the immunogenicity of wheat are presented in Table 3.

Combination of cereal germination with LAB fermentation

As reported by Loponen *et al*. [2007] and Montemurro *et al*. [2019], almost all wheat prolamins (gliadins and glutenins) were degraded during sourdough fermentation of sprouted wheat flour. Prolamin hydrolysis in sourdough of germinated wheat was more intense, possibly due to the high activity of cysteine proteinase in germinated wheat. Both of these methods are natural, and products made in this way are likely to be attractive to consumers, but they are not 100% effective.

Combination of cereal germination with fungal proteases treatment

Fungal proteases are often used to produce GF beer [Guerdrum & Bamforth, 2012]. Malt hydrolysis with AN-PEP resulted in a significant reduction in the residual prolamin content. There are previous studies [Walter *et al*., 2014] that fungal proteases successfully hydrolyzed gluten residues in sprouted wheat bran, but there have been no recent studies in this area.

Combination of selected *Lactobacillus* cultures with different proteases treatment

It has been shown that using selected LAB together with different proteases over a long time can reduce the residual content of gluten immunogenic sequences. The required fermentation time for gluten degradation was significantly reduced (up to 12–20 h at 30–37°C) using fungal proteases [Giuliani *et al*., 2016]. This effect was obtained using only two selected lactic acid bacteria (*L. sanfranciscensis DPPMA12* and *L. plantarum DPPMA125*) in combination with fungal proteases (isolated from *Aspergillus oryzae*, *A. niger*, or mixtures thereof). It has been found that LAB and fungal proteases can degrade the gluten of different wheat varieties, and the good tolerance to such treated wheat in coeliac patients has been demonstrated.

Curiel *et al.* [2014] have developed a technology for producing gluten-free pasta using entirely hydrolyzed wheat flour with good organoleptic characteristics and nutritional value. Wheat flour fermentation with LAB and fungal proteases reduces the content of gluten. The study by Arte *et al.* [2015] revealed the effects of various biological treatment methods, such as activation of endogenous bran enzymes, addition of an enzyme mixture, and microbial fermentation on wheat bran protein modification. The biological treatment in acidic media significantly increased the solubility of wheat bran protein. The study by Di Cagno *et al.* [2010] has shown that young coeliac patients are safe to eat sweet pastries made from wheat flours that have become GF during the fermentation. After fermentation, the wheat flour was dried in a spray dryer and used to produce sweet pastries. Selected LAB cultures and fungal proteases, commonly used for bakery products, degraded gluten to <10 mg/kg. Greco *et al.* [2011] have also proved that there is no immune response in CD patients who daily consume baked goods produced from hydrolyzed wheat flour. The tolerance to such treated wheat in coeliac patients has been proven on short-term consumption [Mandile *et al*., 2017]. This patented method encourages the already applied practice in Italy to produce GF bakery products with sourdough and pasta. However, more detailed clinical surveys are needed to demonstrate its long-term safety as well the technological properties of wheat flours with hydrolyzed gluten, and to elucidate its impact on the baking process. Whether the broken gluten network is replaced by hydrocolloids, the question arises – whether it is expedient to use such flour after gluten hydrolysis for baking, or might it be better to use wheat starch for bread making.

Combination of enzymes of different origins

Studies have shown that concomitant use of several enzymes leads to more efficient degradation of gluten than the use of individual enzymes. The use of several different enzymes together (of plant, bacterial, animal, or fungal origin), that cleave different peptide bonds has improved the efficiency of gluten hydrolysis [Brzozowski *et al*., 2020; Janssen *et al*., 2015]. Li *et al*. [2016] found that the sequential hydrolysis of wheat flour using several different enzymes was more effective in reducing the amount of gliadin than the hydrolysis by every individual enzyme. The sequential fermentation of wheat flour with Alcalase and papain almost completely eliminated the gluten under optimal conditions. It is obvious that, under suitable conditions, such hydrolysis is a promising way of producing low-allergenic wheat products. However, the organoleptic and functional characteristics of the Alcalase-papain-treated product have to be examined to determine the feasibility of this method for production. Although wheat gluten can be hydrolyzed using enzymes of different origins, it is necessary to carefully select them and manage the hydrolysis process to achieve the desired effect, since improper organization of the process can have the opposite effect [Tavano *et al*., 2018]. Therefore, some GF food additives can have a negative effect on consumers associated with wheat intolerance and even on patients with coeliac disease. The manufacturing process of GF products should be carefully designed and managed to avoid complications. It remains the challenge to

TABLE 3. Combined approach of several biological treatments to eliminate the immunogenicity of wheat.

Combined treatment	Enzyme source	Results	References
Malting or germinating + sourdough fermentation	*Lactobacillus rossiae, L.plantarum* and *L. sanfranciscensis* were used for fermentation of flours from germinated grains	Fermentation enhanced the nutritional and functional features of sprouted flours.	Montemurro *et al*. [2019]
Malting or germinating + fungal proteases	Bran extracts from germinated cereals + *Aspergillus niger* prolyl endopeptidase	AN-PEP exceeded the activities of germinated cereal in gluten degradation.	Walter *et al*. [2014]
Sourdough fermentation+ fungal proteases	*L. sanfranciscensis* and *L. plantarum* in combination with fungal proteases from *A. oryzae* and *A. niger*	The required fermentation time for gluten degradation significantly reduced.	Giuliani *et al*. [2016]
	White bread was prepared from wheat flour pre-treated with selected lactobacilli and fungal proteases	Such bread did not affect an immune response in patients with CD on short-term consumption.	Mandile *et al*. [2017]
Sourdough fermentation+ proteases of animal origin	Mixture of 5 different bacterial strains by a combination of pepsin, trypsin or chymotrypsin	Probiotic strains reduced the toxicity of gliadin that remains after peptic-tryptic digestion.	Giorgi *et al*. [2020]
Enzymes of different origin	Activation of endogenous wheat bran enzymes in addition of microbial fermentation	Biological treatment significantly increased solubility of wheat bran proteins.	Arte *et al*. [2015]
	Pepsin and other digestive supplements+ AN-PEP enzyme	AN-PEP enzyme enhanced the effects of digestive supplements.	Janssen *et al*. [2015]
	Chymotrypsin, Flavourzyme, Trypsin, Pepsin or Alcalase + Papain	Alcalase-papain significantly reduced allergenicity of wheat flour.	Li *et al*. [2016]
	The mixture of peptidases from *L. acidophilus* + *A. niger*	Coeliac-toxic peptides were hydrolyzed effectively after 3 h.	Brzozowski *et al.* [2020]

manage process factors such as enzyme type and its activity, content of proteins, pH, action mechanism, reaction time, and others. These conditions do not always correspond to the real conditions of wheat products manufacture in practice. Besides, additional measures are necessary to compensate for the reduction or elimination of gluten in order to ensure high product quality.

Combined approach of biotechnological and non-biotechnological measures

A number of studies have been performed to degrade gluten-immunotoxic compounds by physical treatments. Lamacchia *et al*. [2016] studied the effects of microwaves on soaked wheat kernels and reported that this modification could reduce the immunotoxic effects of wheat proteins by up to 99%, which would allow them to produce low-gluten bread. However, subsequent studies [Gianfrani *et al*., 2017] had used more accurate methods for the determination of gluten residues and observed that the microwave treatment did not reduce the celiac immunogenicity of gliadins. As Mahroug *et al*. [2019] confirmed, despite the significant changes observed in the gluten secondary structure, the microwave treatment was ineffective in decreasing the amount of potential celiac-toxic epitopes in wheat flour but even increased it when the flour was exposed to the low doses of energy [Leszczynska *et al*., 2003]. The reduction of the residual antigenicity of wheat proteins can be achieved during thermal treatment, by using high pressure, extrusion, and spray drying [Stănciuc *et al.*, 2018]; however, the effect was not sufficient to make wheat products suitable for gluten-sensitive people.

Another physical method – ultrasound treatment, can significantly improve emulsifying, foaming, and rheological properties of wheat gluten proteins, but slightly decreases their molecular weight [Zhang *et al*., 2011]. The ultrasound effects on the immunogenicity of wheat gluten are insignificant, but it can be used to activate enzymatic hydrolysis. Controlled ultrasound pretreatment can alter the microstructure, nano-mechanical properties, and secondary structures of wheat gluten, to increase the content of free amino acids and to improve the effects of enzymolysis [Zhang *et al*., 2015; Yang *et al.*, 2017]. Combined enzyme/ultrasound bioprocessing produces cavitation effects that enhance the transport of enzyme macromolecules to the surface of the substrate and thus activates the action of enzymes [Delgado-Povedano & De Castrož, 2015; Kwiatkowska *et al.,* 2011]. However, time and power control is very important in this process, because choosing the wrong cavitation parameters can reduce the degree of enzymatic hydrolysis [Islam *et al*., 2014; Yu *et al*., 2014].

Thus, although physical methods would be more accessible and do not require much energy and time, the enzymatic hydrolysis appears to be a more effective approach in minimizing allergenicity of wheat proteins [Rahaman *et al*., 2016]. However, the possibility of combining physical and biotechnological measures should be further explored.

CONCLUSIONS

The current gluten-free products available on the market have technological and sensory drawbacks. Due to the growing trend of gluten-free market in the last years, technologies for the production of gluten-free or reduced-gluten wheat products are being developed. Research on the use of the biological approach in wheat products in order to eliminate or reduce the immune toxicity of gluten proteins is being actively undertaken. Various enzymes, such as those of bacte-

rial, fungal, plant and animal origin, can be used to this end. Most of the studies were carried out using cultures of lactic acid, inlcuding their individual strains or various combinations. Selected strains that exhibit proteolytic activity, which reduces the allergenicity of wheat sourdough, can be used as specific starter LAB cultures to prepare foods for special purposes. Satisfactory results are achieved by using bacterial and plant enzymes, but the complete elimination of gluten immunogenicity in wheat products is still possible only by using fungal proteases, engineered enzymes or combining several treatments, for example, by using LAB fermentation or germination with fungal proteases. Despite numerous research in scientific laboratories, it is still impossible to offer patients with CD an alternative diet based on highly nutritious and tasty cereal GF products in practice.

The applicability of the used techniques in bread and bakery production is uncertain. Complete degradation of gluten requires long fermentation times, often in combination of several strains of different lactic acid bacteria. Moreover, with the addition of peptidases, gluten degradation must be controlled. This long and complicated process also leads to higher production costs of the final product. Furthermore, when wheat gluten is completely degraded, the viscoelastic properties are lost, which reduces the benefits of the process [Engström *et al*., 2015]. Without the use of additives, the optimal dough and cereal product cannot be prepared from such altered and processed wheat grains or flours. The safety of such products has not been fully proven. It has not been established whether the products of such wheat flour are better than those made from starches and whether their price and quality are acceptable to consumers. Furthermore, the question remains unanswered: Is it beneficial to degrade gluten in wheat flours?

As the majority of the studied biotechnological tools readily remove small amounts of gluten, it would be appropriate to use wheat by-products after physical removal of gluten by wet fractionation. However, further research should focus on using enzymatically-treated wheat starch and bran in gluten-free bakery products or fermented food products with a high nutritional value, and good technological and organoleptic properties, that can be consumed not only by people suffering from gluten intolerance, but also by other personalized groups of consumers.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

ORCID IDs

V. Bradauskiene https://orcid.org/0000-0003-1869-0807
D. Cernauskas https://orcid.org/0000-0002-7094-0183
L. Vaiciulyte-Funk https://orcid.org/0000-0002-2865-9523

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Elicitation with Sodium Silicate and Iron Chelate Affects the Contents of Phenolic Compounds and Minerals in Buckwheat Sprouts

Henryk Dębski[1], *Wiesław Wiczkowski*[2], *Dorota Szawara-Nowak*[2], *Marcin Horbowicz*[1]*

[1]*Institute of Biological Sciences, Siedlce University of Natural Sciences and Humanities, Prusa 14, 08–110 Siedlce, Poland*
[2]*Department of Chemistry and Biodynamics of Food, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10–748 Olsztyn, Poland*

Key words: *Fagopyrum esculentum*, common buckwheat, flavonoids, phenolic acids, iron, silicon

The study concerned the effect of elicitors on the contents of free flavonoids and phenolic acids, as well as their esters and glycosides, and contents of minerals in 7-day sprouts of common buckwheat. An aqueous solution containing a mixture of sodium silicate and Fe-EDTA (SIL-Fe) was compared with the same concentration of sodium silicate alone (SIL) and water (control). Phenolic compounds were analysed using an HPLC–MS/MS apparatus equipped with an ion-trap mass spectrometer, and analyses were conducted by multiple reaction monitoring of selected negative ions. The contents of macro- and microelements in sprouts were determined by the ICP-AES method, after sample mineralization in a mixture of HNO_3 and H_2O_2. The study showed that SIL-Fe influenced the accumulation of individual phenolics in buckwheat sprouts in different ways. Among the major flavonoids in the buckwheat sprouts treated with SIL-Fe, the content of free forms and esters of (-)-epicatechin and glycosides of quercetin as well as the total content of flavonoids decreased. Elicitation of buckwheat sprouts with SIL and SIL-Fe reduced the contents of calcium, potassium, copper, and zinc; however, the SIL-Fe treatment caused a 5-fold increase in iron content and a 2-fold increase in silicon content. The ratio of total flavonoids to the iron content of SIL-Fe-treated sprouts was 11.5, which was substantially lower than in the control, where it reached 64. The results of this study indicate that it is possible to produce buckwheat sprouts with a high content of flavonoids and fortified with iron, which may increase the availability of this element.

INTRODUCTION

Sprouts of various plant species are recognised as an important source of protein, mineral, dietary fiber, and vitamins in the human diet [Benincasa *et al*., 2019]. Germination has been reported to increase the macro- and microelements availability and also to significantly reduce the phytic acid content [Mbithi-Mwikya *et al.,* 2000; Sokrab *et al.,* 2012]. Compared with the seeds of common buckwheat (*Fagopyrum esculentum* Moench), the sprouts have a much higher content of phenolics [Kim S.-L. *et al.,* 2004; Kim S.-J. *et al.,* 2008; Kreft, 2016; Wijngaard & Arendt, 2006]. They are also rich in flavonoids representing several classes, *i.e.* flavonols, flavones, flavan-3-ols, and anthocyanins [Wiczkowski *et al.*, 2014], and in phenolic acids [Horbowicz *et al.,* 2015]. Buckwheat sprouts also contain derivatives of *trans*-cinnamic acid and benzoic acid [Wiczkowski *et al.,* 2016], while rapid accumulation of phenolic compounds is observed during their growth [Kim *et al.,* 2008; Koyama *et al.,* 2013; Terpinc *et al.*, 2016].

Elicitation is a method that effectively increases the accumulation of secondary metabolites in plants [Baenas *et al.,* 2014; Horbowicz *et al.,* 2015; Park *et al.,* 2017, 2019; Ruiz-Garcia & Gómez-Plaza, 2013]. Biotic elicitors have been shown to enhance the accumulation of flavonoids and other phenolic compounds in buckwheat sprouts [Park *et al.,* 2017, 2019]. Also sodium silicate affected the antioxidant system of ryegrass by enhancing phenolics production and antioxidant enzyme activation [Ribera-Fonseca *et al.,* 2018]. Silicon application increased phenolic compound contents in cucumber [Fawe *et al.,* 1998] and maize [Kidd *et al.,* 2001]. However, Chérif *et al.* [1994] reported that silicon had no effect on phenolic contents of plants in the absence of pathogen infection. On the other hand, Rogalla & Römeheld [2002] reported a decrease in the activity of phenylalanine ammonia-lyase (PAL) in Si-supplemented plants, and a decrease in phenolics content. They suggested that the decrease in phenolics content was caused by the mechanism of stress reduction through the formation of Si complexes with phenol moiety.

Mineral nutrient availability, uptake, and transport in plants are affected by a number of factors resulting in complex interactions between the elements [Marschner, 1980]. For instance, calcium is highly competitive with magnesium and potassium, while an excess Ca content in soil causes a deficiency of Fe, B, Mn, Mg, and K in plants. Silicon influences the accumulation of mineral nutrients in various plant species, and this influence depends on species examined as well as conditions of its growth [Greger *et al.,* 2018]. Si was added to the nutrient solution to diminish the uptake of Ca by rice plants [Ma & Takahashi, 1993], and Zn content in leaves of maize [Kaya *et al.,* 2009].

* Corresponding Author e-mail: mhorbowicz@uph.edu.pl (Prof. M. Horbowicz).

Silicon in soil was reported to have a major impact on the contents of calcium and microelements in grasses. A pot experiment with increased levels of silicon demonstrated a significant decline of calcium, manganese, iron, cobalt, copper, and zinc contents in leaf blades of common reed [Brackhage *et al*., 2013].

The germination of crucifer and legume seeds in the presence of Fe generated a considerable increase in the content of this element in sprouts, but their biomass accumulation slightly decreased [Przybysz *et al.,* 2016]. The cited authors have also shown that a higher Fe content increased the activity of antioxidative enzymes and contents of phenolic compounds.

Phenolics, as bioactive compounds occurring in plants and plant products, can play a beneficial role in human health, including the prevention of oxidative stress-related diseases and inflammatory conditions [Cory *et al.,* 2018; Cvejic *et al.,* 2018]. In turn, their cardioprotective properties are related to their antioxidant effect. In recent years, polyphenols have also been tested for their antiatherosclerotic properties [Santhakumar *et al.,* 2018].

Although flavonoids have many important properties for human health, they cannot be supplied in a diet in excessive quantities. This is because they can inhibit intestinal absorption of various elements, including the nonheme type of iron [Thankachan *et al.,* 2008]. Iron deficiency is one of the most extensive health disorders in the world, which may contribute to anemia development in a significant part of the global population [Stoltzfus *et al.,* 2004]. This deficiency still affects about a quarter of women and children worldwide [Cappellini *et al.*, 2020]. A high level of polyphenols is one of the causes of low absorption of iron by the human organism [Zijp *et al.,* 2000]. Nonheme iron is the major form of this element in plant foods and supplements. According to Hurrell *et al.* [1999], the excess consumption of tea or cocoa can significantly decrease the absorption of nonheme iron. Flavonoids can also inhibit intestinal heme iron absorption [Ma *et al.,* 2011]. Therefore, flavonoid-rich food and beverages should not be consumed together with high-Fe meals. This was an important reason for undertaking this study, the results of which may show the way to counteract the phenomenon of low iron bioavailability.

Therefore, the main research hypothesis advanced in this study was whether the presence of easily assimilable iron affects the content of phenolic compounds. Since iron was used in a mixture with sodium silicate, a separate set of sprouts was treated only with silicate for comparison. The detailed objectives of this study were to quantify free forms, esters, and glycosides of flavonoids and phenolic acids as a response to silicon and iron applied during germination and 7-day growth of common buckwheat sprouts. Another aim of the investigation was to determine the contents of iron and silicon and other macro- and microelements in these sprouts.

MATERIAL AND METHODS

Plant material

Seeds of common buckwheat (cv. Hruszowska) were soaked at 24°C in distilled water for 4 h. Initially, seeds without a seed coat were disinfected with 70% (*v/v*) ethanol for 1 min, and then with 2% sodium hypochlorite for 2 min, rinsed once in 0.01 N HCl and 3 times with distilled water. The disinfected seeds were placed on a layer of sterilized and moist cotton gauze stretched over an open 330-mL jar. In the next six days, the seeds and sprouts were soaked in distilled water (control) or elicitor solutions (SIL, SIL-Fe). The soaking lasting 15 min was carried out twice each day, at 9 am and 5 pm. After each treatment, the seeds were placed back on the gauze layer. The sprouts were grown under exposure to 100–120 μmol/(m^2·s) photosynthetically active radiation produced by high-pressure sodium lamps at 20±1°C (day, 16 h) and 16±2°C (night, 8 h). On the seventh day, the obtained sprouts were collected, cut into 2–3 mm pieces, freeze-dried in a laboratory freeze dryer (Alpha 1–2 LDplus, Martin Christ, Osterode am Harz, Germany) for 48 h, and used for analyses of flavonoids and phenolic acids, as well as micro- and macroelements.

Elicitor composition

The elicitors used in the study were solutions containing sodium metasilicate (Na_2SiO_3; SIL), and a mixture of sodium metasilicate and Fe-EDTA chelate (SIL-Fe, trade mark Optysil, Intermag, Olkusz, Poland). Buckwheat seeds or sprouts were immersed in 50 mL of distilled water (control), or in 50 mL of the solutions containing sodium metasilicate with a concentration of 4 mM (SIL), or with the Optysil, *i.e.* a mixture of Fe-EDTA and sodium metasilicate, 0.5 mM Fe and 4 mM of Na_2SiO_3 (SIL-Fe), respectively.

Analyses of free, esters and glycosides of flavonoids and phenolic acids

Sprout samples were analyzed with HPLC–MS/MS to determine various forms of phenolic acids and flavonoids. The profile and content of phenolic acids and flavonoids were determined according to the method of Płatosz *et al.* [2020]. Briefly, a crude extract was obtained from freeze-dried sprout samples with a mixture of methanol, water, and formic acid. The extraction was repeated five times, and from the obtained crude extracts free phenolic acids and flavonoids were isolated with diethyl ether after adjusting extract to pH 2 with 6 M HCl. After the isolation of free forms, esters were hydrolyzed at room temperature with 4 M NaOH, and glycosides in the residues were hydrolyzed with 6 M HCl. The released by hydrolysis free forms of phenolic compounds were extracted with diethyl ether. The obtained ether extracts were evaporated to dryness under stream of nitrogen and the residue was dissolved in 80% (*v/v*) methanol. The HPLC system used was equipped with a HALO C18 column (2.7 μm particles, 0.5 × 50 mm, Eksigent, Vaughan, Canada) which was kept at 45°C, and the eluent flow was 15 μL/min. The eluent was a mixture of A (water/formic acid; 99.05/0.95; *v/v*) and B (acetonitrile/formic acid, 99.05/0.95, *v/v*). The gradient was used as follows: 5% B for 0.1 min, 5–90% B in 1.9 min, 90% B for 0.5 min, 90–5% B in 0.2 min, and 5% B for 0.3 min. For the HPLC–MS/MS analysis, a QTRAP 5500 ion trap mass spectrometer (AB SCIEX, Vaughan, Canada) was applied. Qualitative and quantitative analyses were conducted in the negative mode by multiple reaction monitoring of selected ions. The following flavonoids (free, esters and glycosides) were analyzed: (-)-epicatechin, luteolin, orientin, vitexin, api-

genin, naringenin, kaempferol, *iso*-rhamnetin, and quercetin. Derivatives (free, esters, glycosides) of the following phenolic acids were analyzed as well: 4-hydroxybenzoic, caffeic, sinapic, *p*-coumaric, ferulic, syringic, and chlorogenic acids.

Determination of macro- and microelements

The contents of macro- and microelements were determined by the ICP-AES method using an Optima 8300 ICP--AES/OES spectrometer (Perkin Elmer, Waltham, MA, USA), after sample mineralization in a mixture of concentrated HNO_3 and 20% H_2O_2 (3:1) in a microwave system for 2 h [Barnes & Debrah, 1997].

Statistical analysis

Analyses of sprout tissue were performed in three replicates. The analysis of variance (one-way ANOVA) and Tukey's post hoc test were used to check the significance of differences. Calculations were performed using Statistica 12PL software (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

The use of iron chelate (SIL-Fe) and sodium silicate (SIL) during the germination and growth of buckwheat sprouts caused various changes in the content of individual phenolic compounds (Tables 1–5). Moreover, the use of iron chelate (SIL-Fe) resulted in about 20% lower fresh and dry matter contents of the buckwheat sprouts compared to those grown under control conditions (data not shown). Inhibition of growth under the influence of iron was previously demonstrated in the sprouts of crucifers [Przybysz *et al.,* 2016].

The quantitatively major flavonoid in 7-day buckwheat sprouts was (-)-epicatechin, which occurred mainly in the free form, representing more than 90% of its total content (Table 1). Regarding the content, the second group of flavonoids were their glycosides. Among them, quercetin glycosides appeared in the highest contents. However, (-)-epicatechin, apigenin, luteolin, and *iso*-rhamnetin did not appear as glycosides. The (-)-epicatechin content was many times higher compared to other flavonoids (Tables 1, 2, and 3). This confirms our earlier report in which 7-day sprouts of four buckwheat cultivars contained 3900–5400 μg/g dry weight (DW) (-)-epicatechin in cotyledons and 1000–1300 μg/g DW in hypocotyl [Wiczkowski *et al.,* 2014]. However, in other studies, in 10-day-old sprouts of Korean buckwheat, the (-)-epicatechin content was much lower, and reached only 455 μg/g DW [Park *et al.,* 2017]. Lower than presented here contents of (-)-epicatechin in buckwheat sprouts were also noted by Park *et al.* [2019]. On the other hand, similar epicatechin contents were found in 3-month-old leaves, stems, and roots of buckwheat [Uddin *et al.,* 2013].

A high (-)-epicatechin content is beneficial because the antioxidant activity of this flavonoid is higher than that of rutin, which is the major phenolic compound of buckwheat seeds [Watanabe, 1998]. Results from human trials indicate that (-)-epicatechin acts as an antioxidant both directly as a scavenger of free radicals and indirectly as a modulator of superoxide dismutase and glutathione peroxidase [Simos *et al.,* 2012]. It can also modulate macronutrient metabolism in normal and overweight subjects [Gutiérrez-Salmeán *et al.,* 2014].

TABLE 1. The contents of major flavonoids (free and released from ester and *O*-glycoside forms) in buckwheat sprouts (μg/g DW) treated with elicitors during growth.

Treatment	Free forms	Esters	*O*-glycosides	Total
(-)-Epicatechin				
Control	3529±78^a	77.05±3.25^b	nd	3606±81^a
SIL-Fe	2674±59^b	33.19±2.18^c	nd	2707±61^b
SIL	2652±45^b	132.5±2.99^a	nd	2784±48^b
Quercetin				
Control	81.23±4.11^a	96.33±5.23^b	749.3±11.5^a	926.9±20.6^a
SIL-Fe	93.45±3.89^a	80.33±6.45^b	511.2±16.2^c	685.0±26.5^c
SIL	59.65±2.56^b	143.0±4.89^a	590.4±8.9^b	793.1±16.4^b
Orientin				
Control	132.2±4.1^b	106.6±4.9^c	nd	238.8±9.0^b
SIL-Fe	133.1±3.2^b	141.3±4.8^b	nd	274.4±7.0^b
SIL	171.2±5.4^a	189.6±6.6^a	nd	360.8±7.8^a
Luteolin				
Control	6.62±0.22^a	9.70±0.33^a	99.17±2.99^b	115.5±3.5^b
SIL-Fe	6.17±0.17^a	5.28±0.25^b	93.71±3.11^b	105.2±3.5^b
SIL	6.47±0.11^a	6.35±0.33^b	151.6±4.4^a	164.4±4.8^a
Vitexin				
Control	47.37±2.01^b	52.02±2.09^b	nd	99.40±5.10^a
SIL-Fe	72.25±2.89^a	42.19±1.99^b	nd	114.4±4.9^a
SIL	62.35±3.11^a	62.92±1.33^a	nd	125.3±3.1^a

Results are shown as mean ± standard deviation (n=3). Means marked with the same letter are statistically insignificant at P>0.05 (post hoc Tukey's test). Comparisons were made within each column, for each flavonoid separately. nd – not detected; SIL-Fe – sodium metasilicate and Fe-EDTA chelate; SIL – sodium metalsilicate; DW – dry weight.

Besides (-)-epicatechin, derivatives of quercetin, orientin, vitexin, luteolin, apigenin, kaempferol, naringenin, and *iso*-rhamnetin were found in seven-day buckwheat sprouts (Tables 2 and 3). Similar composition of these flavonoids was found in 4-day sprouts of buckwheat by Terpinc *et al.* [2016]. Their results showed that the total content of C-glycosides of luteolin and apigenin exceeded the content of flavonols: quercetin-3-*O*-rutinoside and quercetin-3-*O*-sophoroside. In general, the contents of individual phenolics significantly increased during sprout growth from 6 to 10 days after germination [Kim *et al.,* 2008]. In turn, Koyama *et al.* [2013] have shown that the highest content of flavonoids in buckwheat sprouts was reached on day 6 of their cultivation. The results of the analysis of flavonoids in buckwheat sprouts are influenced by the fact that their content in cotyledons is many times higher than in hypocotyl and roots [Horbowicz *et al.,* 2015]. The mass proportions of these morphological parts change rapidly during sprout growth. Therefore, it is likely that the changing proportion of individual morphological parts of the sprout may be a major factor affecting its composition.

TABLE 2. The contents of minor flavonoids (free and released from ester and *O*-glycoside forms) in buckwheat sprouts (μg/g DW) treated with elicitors during growth.

Treatment	Free forms	Esters	*O*-glycosides	Total
		iso-Rhamnetin		
Control	47.56±2.10^{b}	nd	nd	47.56±2.10^{b}
SIL-Fe	109.1±4.4^{a}	nd	nd	109.1±4.4^{a}
SIL	24.36±1.25^{c}	nd	nd	24.36±1.25^{c}
		Apigenin		
Control	4.31±0.20^{a}	10.32±0.22^{a}	34.46±1.22^{a}	49.09±1.64^{a}
SIL-Fe	3.37±0.17^{a}	7.70±0.16^{b}	23.91±0.99^{b}	34.97±1.32^{b}
SIL	1.70±0.08^{b}	7.44±0.13^{b}	39.07±1.11^{a}	48.21±1.32^{a}
		Naringenin		
Control	2.92±0.10^{b}	1.25±0.06^{a}	1.58±0.11^{b}	5.75±0.27^{b}
SIL-Fe	3.34±0.09^{b}	1.39±0.08^{a}	4.79±0.25^{a}	9.53±0.42^{a}
SIL	4.84±0.09^{a}	1.17±0.05^{a}	4.59±0.18^{a}	10.60±0.32^{a}
		Kaempferol		
Control	nd	1.30±0.09^{a}	4.19±0.11^{a}	5.49±0.20^{a}
SIL-Fe	nd	1.41±0.05^{a}	3.50±0.08^{a}	4.91±0.13^{a}
SIL	nd	0.25±0.03^{b}	3.31±0.07^{a}	3.55±0.11^{b}

Results are shown as mean ± standard deviation (n=3). Means marked with the same letter are statistically insignificant at P>0.05 (post hoc Tukey's test). Comparisons were made within each column, for each flavonoid separately. nd – not detected; SIL-Fe – sodium metasilicate and Fe-EDTA chelate; SIL – sodium metalsilicate; DW – dry weight.

TABLE 3. The contents of total flavonols, flavones, and all flavonoids (free and released from ester and *O*-glycoside forms) in buckwheat sprouts (μg/g DW) treated with elicitors during growth.

Treatment	Free form	Esters	*O*-glycosides	Total
		Total flavonols		
Control	128.8±4.1^{b}	97.63±3.10^{b}	753.5±20.1^{a}	978.0±27.3^{a}
SIL-Fe	202.6±6.4^{a}	81.74±2.89^{c}	514.7±15.3^{b}	799.0±24.6^{b}
SIL	84.01±3.12^{c}	143.3±4.3^{a}	593.7±15.9^{b}	821.0±23.3^{b}
		Total flavones		
Control	190.5±4.6^{b}	178.7±3.9^{b}	133.6±3.8^{b}	502.8±12.3^{b}
SIL-Fe	214.9±5.1^{ab}	196.5±4.5^{b}	117.6±2.9^{b}	529.0±12.5^{b}
SIL	241.7±6.0^{a}	266.4±5.8^{a}	190.6±4.4^{a}	698.6±16.2^{a}
		Total flavonoids		
Control	3851±66^{a}	354.6±8.8^{b}	888.7±10.6^{a}	5094±85^{a}
SIL-Fe	3095±25^{b}	312.8±8.6^{b}	637.1±9.1^{c}	4044±43^{c}
SIL	2982±30^{b}	543.4±9.7^{a}	788.9±8.6^{b}	4314±48^{b}

Results are shown as mean ± standard deviation (n=3). Means marked with the same letter are statistically insignificant at P>0.05 (post hoc Tukey's test). Comparisons were made within each column, for each flavonoid group separately. SIL-Fe – sodium metasilicate and Fe-EDTA chelate; SIL – sodium metalsilicate; DW – dry weight.

Elicitation with a solution containing sodium silicate and Fe-EDTA (SIL-Fe) lowered the content of free (-)-epicatechin in buckwheat sprouts (Table 1). No effect of SIL-Fe was observed in other free flavonoids, except for *iso*-rhamnetin and vitexin, whose contents increased (Table 2). SIL-Fe had various effects on flavonoid esters. In some of them ((-)-epicatechin, luteolin, apigenin), it inhibited their accumulation, while in others it had no significant effect (Tables 1 and 2). SIL-Fe only favored the accumulation of orientin esters. However, SIL-Fe caused a significant reduction in quercetin and apigenin glycosides, but to a small extent in the glycosides of other flavonoids. In general, SIL-Fe decreased the total content of free flavonoids, as well as their esters and glycosides (Table 3).

Sodium silicate (SIL) caused a decrease in the contents of free (-)-epicatechin, quercetin, *iso*-rhamnetin, and apigenin, and an increase the contents of orientin, vitexin, and naringenin (Tables 1 and 2). The use of SIL increased also the content of esters of flavonoids, except for luteolin, apigenin, and kaempferol. Moreover, SIL decreased the contents of quercetin glycosides, but increased the contents of luteolin, apigenin, and naringenin glycosides. For total flavonoids, the use of SIL led to a reduction in their free forms and glycosides, but increased their ester content (Table 3).

In contrast to our results, in most studies conducted to date, the use of iron or silicon compounds has increased the content of phenolic compounds [Fawe *et al.,* 1998; Kidd *et al.,* 2001; Przybysz *et al.*, 2016; Ribera-Fonseca *et al.,* 2018]. However, Chérif *et al.* [1994] reported that silicon had no effect on phenolic contents of plants. In contrast, Rogalla & Römeheld [2002] reported a decrease in phenolic content and the activity of phenylalanine ammonia-lyase (PAL) in Si-supplemented plants. These authors suggest that it was due to the formation of silicon-phenol complexes in response to stress. Published data on the effects of Si and Fe concern sprouts and plants of species other than buckwheat, and at different stages of development. Moreover, the methods of Si and Fe application differ from the method used in our study. This makes it difficult to explain the effects of SIL-Fe and SIL in buckwheat sprouts.

Apart from a rich set of flavonoids, the described study of 7-day buckwheat sprouts confirmed the presence of seven phenolic acids (4-hydroxybenzoic, caffeic, sinapic, *p*-coumaric, ferulic, syringic, and chlorogenic) occurring as free forms, esters, and glycosides (Tables 4 and 5). The quantitatively major phenolic acid in 7-day buckwheat sprouts was 4-hydroxybenzoic acid (Table 4). It occurred in the free form and as esters and glycosides. The bound forms accounted for almost 97% of its total content. In the case of other acids, they occurred mainly as esters. In our previous study, *o*-, *m*-, and *p*-coumaric acids, sinapic acid, caffeic acid, and two isomers of ferulic acid were found in 11-day buckwheat sprouts [Wiczkowski *et al.,* 2016]. On the other hand, Zhang *et al.* [2015] detected there gallic, 3,4-dihydroxybenzoic, 2,3,4-trihydroxybenzoic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, and *trans*-3-hydroxycinnamic acids. However, Park *et al.* [2017] have

TABLE 4. The contents of major phenolic acids (free and released from ester and *O*-glycoside forms) in buckwheat sprouts (μg/g DW) treated with elicitors during growth.

Treatment	Free form	Esters	*O*-glycosides	Total
4-Hydroxybenzoic acid				
Control	155.3±2.89^b	628.5±8.8^a	3635±28^a	4419±40^a
SIL-Fe	167.1±1.99^b	325.5±4.6^c	3183±18^b	3675±25^c
SIL	211.6±2.15^a	441.8±4.4^b	3532±26^a	4185±33^b
Caffeic acid				
Control	61.30±2.66^b	914.8±13.3^c	85.60±3.20^c	1062±19^c
SIL-Fe	37.61±1.89^c	1458±23^b	186.5±2.77^a	1682±28^b
SIL	88.72±3.21^a	1654±26^a	155.2±2.44^b	1898±32^a
p-Coumaric acid				
Control	31.34±1.56^a	354.7±4.2^c	7.63±0.66^c	393.6±6.5^c
SIL-Fe	26.74±1.54^a	487.1±5.1^a	20.79±1.25^a	534.6±7.9^a
SIL	33.62±2.10^a	433.4±3.9^b	14.10±1.09^b	481.2±7.1^b
Sinapic acid				
Control	0.63±0.11^a	97.47±3.20^a	20.71±1.45^b	118.8±4.8^a
SIL-Fe	1.28±0.22^a	73.75±2.76^b	22.46±1.56^b	97.50±4.6^a
SIL	1.26±0.24^a	74.33±2.68^b	31.90±2.09^a	107.5±5.0^a

Results are shown as mean ± standard deviation (n=3). Means marked with the same letter are statistically insignificant at P>0.05 (post hoc Tukey's test). Comparisons were made within each column, for each phenolic acid separately. SIL-Fe – sodium metasilicate and Fe-EDTA chelate; SIL – sodium metasilicate; DW – dry weight.

TABLE 5. The contents of minor and total phenolic acids (free and released from ester and *O*-glycoside forms) in buckwheat sprouts (μg/g DW) treated with elicitors during growth.

Treatment	Free form	Esters	*O*-glycosides	Total
Ferulic acid				
Control	2.80±0.09^a	49.74±1.03^a	1.86±0.08^b	54.40±1.20^a
SIL-Fe	3.84±0.07^a	52.13±1.25^a	4.76±0.10^a	60.72±1.42^a
SIL	3.17±0.06^a	51.69±1.22^a	4.10±0.11^a	58.96±1.39^a
Syringic acid				
Control	nd	32.73±1.04^b	nd	32.73±1.04^b
SIL-Fe	nd	43.49±1.22^a	nd	43.49±1.22^a
SIL	nd	47.03±1.21^a	nd	47.03±1.21^a
Chlorogenic acid				
Control	33.86±1.15^b	nd	nd	33.86±1.15^b
SIL-Fe	53.48±2.09^a	nd	nd	53.48±2.09^a
SIL	20.94±0.99^c	nd	nd	20.94±0.99^c
Total phenolic acids				
Control	285.2±17.7^b	2078±24^c	3751±44^a	6114±86^b
SIL-Fe	290.1±12.3^b	2440±27^b	3417±35^b	6147±74^b
SIL	359.3±10.1^a	2702±31^a	3735±29^a	6799±70^a

Results are shown as mean ± standard deviation (n=3). Means marked with the same letter are statistically insignificant at P>0.05 (post hoc Tukey's test). Comparisons were made within each column, for each phenolic acid and total phenolic acids separately. nd – not detected; SIL-Fe – sodium metasilicate and Fe-EDTA chelate; SIL – sodium metalsilicate; DW – dry weight.

found only gallic, chlorogenic, caffeic, and benzoic acids in these sprouts. In the summary of so different results, it can be seen that many factors used in the production of buckwheat sprouts affect the composition and content of their phenolic compounds. The main factor seems to be the light conditions during the growth of buckwheat sprouts, but also an elicitor and the cultivar used [Horbowicz *et al.,* 2015; Kim *et al.,* 2008; Koyama *et al.,* 2013; Park *et al.,* 2017, 2019; Uddin *et al.,* 2013; Wiczkowski *et al.,* 2014; Zhang *et al.,* 2015].

The application of the SIL-Fe mixture decreased the contents of free *p*-coumaric and caffeic acids, and increased the contents of ferulic, chlorogenic, and sinapic acids (Tables 4 and 5). At the same time, the content of the esters of caffeic, *p*-coumaric, and syringic acids increased, while that of the esters of 4-hydroxybenzoic and sinapic acids decreased tangibly. Both elicitors have led to a considerable increase in the caffeic acid ester content. SIL-Fe also increased the content of glycosides of caffeic, *p*-coumaric and ferulic acids, but decreased the level of 4-hydroxybenzoic acid. In general, SIL-Fe did not affect the total content of free phenolic acids, but increased the level of their esters, and decreased the content of glycosides (Table 5). Elicitation with SIL increased the accumulation of free forms of phenolic acids and their total content (Table 5) as well as the contents of esters and glycosides of most phenolic acids and their total content.

Based on the weight of freeze-dried sprouts, it was calculated that 48% of the Fe was absorbed during the soaking of buckwheat sprouts in SIL-Fe, while only 8.1 and 8.6% of the Si during the soaking in SIL-Fe and SIL, respectively (data not shown). Elicitation with the mixture of SIL-Fe decreased the contents of calcium, potassium, sodium, copper, and zinc in buckwheat sprouts (Table 6). Similarly, in a pot experiment with increased levels of silicon in soil, a significant decline was found for calcium, copper, and zinc contents in leaf blades of common reed [Brackhage *et al.,* 2013]. Si present in the nutrient solution diminished the uptake of Ca by rice, and Zn content in maize [Kaya *et al.,* 2009; Ma & Takahashi, 1993]. However, a complex study by Greger *et al.* [2018] showed that Si increased the uptake of Mg, Ca, Fe, and Mn; and decreased the uptake of Cu, Zn, and K from the solution. Furthermore, the transport of Mg, Ca, Mn, and Mo to shoot increased, but that of Fe, Cu, and Zn decreased, while that of K, P, and B was not affected. These authors pointed out that Si influence depended on species examined, as well as conditions of its growth [Greger *et al.,* 2018]. The specificity of the experiment described here differs from other such studies on the influence of Si and Fe on plants. The seeds and then buckwheat sprouts were soaked in SIL-Fe and SIL solutions for 15 min in the morning and in the evening. This treatment might have caused some losses of minerals and phytochemicals from

TABLE 6. The contents of chosen macro- and microelements in buckwheat sprouts (μg/g DW) treated with elicitors during growth.

		Control	SIL-Fe	SIL
Macroelement	Calcium	1338±28[a]	920.6±12.5[b]	942.0±12.1[b]
	Magnesium	5068±54[b]	4919±45[b]	5506±29[a]
	Potassium	10304±123[a]	9407±98[b]	9135±66[b]
	Phosphorus	14522±125[a]	13883±134[a]	13575±129[a]
	Sodium	1427±27[b]	760.5±18.9[c]	2865±39[a]
Microelement	Copper	41.55±1.26[a]	21.72±0.99[b]	22.85±1.05[b]
	Silicon	152.6±8.8[b]	464.2±13.3[a]	481.2±12.9[a]
	Iron	81.12±1.89[b]	353.0±8.8[a]	76.08±1.77[b]
	Zinc	120.5±4.1[a]	98.39±1.66[b]	95.55±1.25[b]
	Manganese	53.74±1.51[b]	60.87±1.24[ab]	66.40±1.44[a]

Results are shown as mean ± standard deviation (n=3). Means marked with the same letter are statistically insignificant at P>0.05 (post hoc Tukey's test). Comparisons were made within each row, for each macro- and microelement separately. SIL-Fe – sodium metasilicate and Fe-EDTA chelate; SIL – sodium metasilicate; DW – dry weight.

soaked tissues. Therefore, discussion with data presented by other researchers is difficult.

The use of SIL-Fe has doubled the silicon content and more than five times the iron content of buckwheat sprouts. As a result of this procedure, the ratio of total flavonoids to iron content decreased from 64 in control sprouts to 11 in the SIL-Fe treated ones. In comparison, the use of silicate alone (SIL) also reduced the calcium, potassium, copper, and zinc contents, but increased magnesium and sodium contents, and almost doubled the silicon content. According to previous studies, a high level of flavonoids can inhibit intestinal absorption of the nonheme type of iron [Kim *et al.,* 2008; Thankachan *et al.,* 2008; Zijp *et al.,* 2000]. Therefore, it seems that a significant change in the flavonoid-iron ratio is beneficial for the bioavailability of plant-derived Fe.

CONCLUSION

The results of the study indicate that it is possible to produce buckwheat sprouts with a high content of flavonoids and fortified with iron. The research carried out showed also that the mixture of sodium silicate (SIL) and iron chelate (SIL-Fe) influenced the accumulation of individual phenolics in buckwheat sprouts in different ways. Among the major flavonoids of the SIL-Fe-treated buckwheat sprouts, the contents of free forms and esters of (-)-epicatechin and glycosides of quercetin significantly decreased. The reduction of the major flavonoids caused the total content of flavonoids to decrease as well. As a result, the ratio of total flavonoids to iron content decreased from 64 in control sprouts to 11 in the SIL-Fe treated ones. To the best of our knowledge, this is the first work which describes the influence of iron chelate and sodium metasilicate on the content of phenolic compounds and minerals in sprouts of common buckwheat.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID IDs

H. Dębski https://orcid.org/0000-0002-4145-1102
M. Horbowicz https://orcid.org/0000-0002-1789-4034
D. Szawara-Nowak https://orcid.org/0000-0003-2326-065X
W. Wiczkowski https://orcid.org/0000-0001-6021-5589

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Original article
Section: Food Quality and Functionality

Goji Berry and Whey Protein Concentrate Enriched Rice Extrudates – Physical Properties and Accessibility of Bioactives

Thomas Ménabréaz, Mathias Dorsaz, Dimitri Bocquel, Isabelle Udrisard, Agnieszka Kosińska-Cagnazzo, Wilfried Andlauer*

Institute of Life Technologies, University of Applied Sciences and Arts Western Switzerland Valais, Route du Rawyl 47, CH-1950 Sion, Switzerland

Key words: extrudates, goji berries, whey protein concentrate, HPLC, rutin, 2-*O*-β-D-glucopyranosyl-L-ascorbic acid

Extrudates are gaining popularity as convenient ready-to-eat products such as snacks or breakfast cereals. The nutritional limitation of extruded products is their low content of proteins, fibres, and phytochemicals. The challenge lies in increasing the nutritional value of extruded products while maintaining the quality of expansion. Goji berries are rich in bioactive compounds, such as polysaccharides, phenolic compounds, carotenoids, and an analogue of vitamin C. In the present study, rice flour-based extruded products were enriched with goji berries and whey protein concentrate. The varying addition of goji berries and whey protein concentrate affected expansion ratio, colour, and texture parameters of extrudates. The content and bioaccessibility of goji bioactives, *i.e.* 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (2-β-gAA) and the dominant phenolic compound – rutin, were evaluated for two extrudates with the highest addition of goji and whey protein concentrate. The extrusion process significantly reduced the content of 2-β-gAA both in formulations with and without whey protein concentrate by approximately 15%. The bioaccessibility of 2-β-gAA was negatively affected by the extrusion process, but not that of rutin. The addition of whey protein concentrate at a level of 7% had no significant effect on the bioaccessibility of neither 2-β-gAA nor rutin.

INTRODUCTION

Ready-to-eat products obtained by the extrusion process are gaining popularity as snacks or alternatives to breakfast cereals. They are eagerly consumed due to convenience and appealing texture characterised by crispiness and crunchiness [Brennan *et al*., 2013]. Extruded products might also respond to the increasing demand for gluten-free products [Alonso dos Santos *et al.*, 2019]. Efforts have been made to enhance their nutritional value by enriching with fibres and phytochemicals from fruits and vegetables [Brennan *et al.,* 2011; Obradović *et al.,* 2015; Wójtowicz *et al.*, 2018]. Extruded products enable also a decrease in sugar content, which is a pertinent requirement for a healthy nutrition [Faruque *et al.,* 2019]. It should be taken into account that the addition of fruits and vegetables decreases the amount of starch in the blend and might significantly influence the physical characteristics of extrudates, such as expansion and colour [Masatcioglu *et al.,* 2013; Obradović *et al.*, 2015; Yu *et al.*, 2017].

Extrusion is a high-temperature-short-time process which has an impact on the content and bioaccessibility of polyphenols [Zeng *et al.,* 2016], proteins, starch, carotenoids, and other compounds [Singh *et al.,* 2007]. The effect of extrusion on the content of phenolic compounds depends on two opposite outcomes: decomposition of heat-labile compounds and disruption of the cell wall followed by a release of covalently bound phenolic compounds [Wang *et al.,* 2014]. Stating about a potential health effect of food components should be preceded by bioaccessibility assessments confirming that after the digestion process the compound of interest is still present and available for absorption. Bioaccessibility is defined as the amount of a compound that is released from the food matrix and is considered to be available for absorption through the gut wall [Fernández-García *et al.,* 2009]. Bioaccessibility depends on processing and interactions with components of the food matrix, such as proteins for example. It may be determined by analysing the *digesta* after an *in vitro* gastro-intestinal digestion using enzymes under controlled conditions such as pH, temperature, ionic strength, and digestion time. Conducting human trials being costly and ethically disputable, simulated *in vitro* digestions have the advantage to be more rapid and less expensive [Minekus *et al.,* 2014].

Gou Qi Zi, *Lycii Fructus*, wolfberry or goji berry are the names given to fruit of *Lycium barbarum*, a plant from the *Solanaceae* family, growing in the temperate and subtropical zones of the world [Levin *et al.,* 2011]. In the traditional Chinese medicine, goji berries are recommended for their capacity to strengthen muscles, protect liver functions, regenerate the vital essence, and improve visual acuity [Huang, 1998]. Due to their potential benefits for human health

* Corresponding Author e-mail: kosinska.ag@gmail.com (A. Kosińska-Cagnazzo).

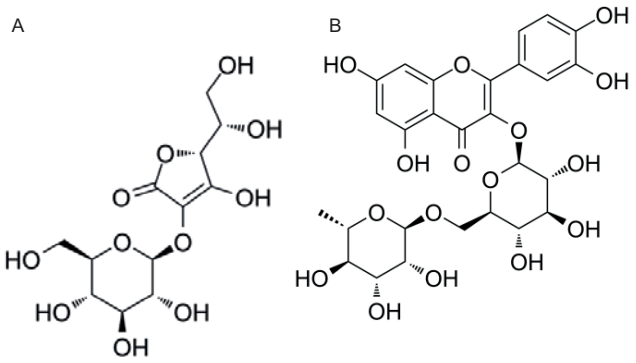

FIGURE 1. Chemical structures of goji bioactives (A) 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (2-β-gAA) and (B) rutin.

and thanks to efficient marketing strategies, goji berries are nowadays gaining popularity in western countries [Potterat, 2010]. It has been reported that goji berries contain 2-*O*-β--D-glucopyranosyl-L-ascorbic acid (2-β-gAA), called also an analogue of ascorbic acid [Toyoda-Ono *et al.,* 2004]. The 2-β-gAA molecule consists of an l-ascorbic acid bound to a d-glucose moiety by a β-glycosidic linkage (Figure 1A). The 2-*O*-α-D-glucopyranosyl-L-ascorbic acid, another derivative of ascorbic acid possessing a provitamin C activity and having a similar structure to 2-β-gAA, is widely used in Japan, particularly as a food and cosmetics additive [Yamamoto *et al.,* 1992]. Little is known about the potential provitamin C activity of 2-β-gAA in humans, as no clinical trial has been launched hitherto. Another bioactive compound of goji berry is rutin (Figure 1B), a predominant flavonoid [Potterat, 2010]. Rutin possesses antioxidative, antimicrobial, antifungal, and anti-allergic properties. It can also provide benefits for the treatment of cancer, diabetes, hypertension and hypercholesterolemia [Sharma *et al.,* 2013]. Clinical studies have shown that rutin, in combination with forskolin, a labdane diterpene produced by the Indian coleus plant, could reduce by 15% the intraocular pressure in patients with primary open-angle glaucoma [Vetrugno *et al.,* 2012].

The nutritional limitations of extruded products lie in their low protein contents. Thus, whey protein concentrate (WPC), as a valuable source of proteins and minerals, might be added in order to increase nutritional value of extrudates [Yu *et al.,* 2017]. WPC has a high nutritional quality resulting from its essential amino acid content, especially leucine and lysine, but also valine, threonine, methionine, and phenylalanine [Silva Teba *et al.,* 2017]. Supplementation with whey protein contributes to increased muscle protein synthesis and results in weight loss, satiety, and improved body composition [Fassina *et al.,* 2019]. Increasing the protein content of extruded products while maintaining the quality of expansion is challenging. It was reported that the addition of WPC (up to 7.36 g/100 g) positively influenced the quality of the final extruded product regarding physical properties [Silva Teba *et al.,* 2017].

The aim of this study was to evaluate the effect of goji and WPC addition on the expansion, colour, and texture parameters of extrudates and to determine the effect of extrusion on the content and the bioaccessibility of 2-β-gAA and rutin in goji and WPC enriched extrudates.

MATERIALS AND METHODS

Materials and reagents

Hydrochloric acid, formic acid, and sulfuric acid were obtained from Merck (Zug, Switzerland). Pepsin from porcine stomach mucosa, pancreatin from porcine pancreas, bile salts, sodium chloride, 2,6-di-*tert*-butyl-4-methylphenol (BHT), ascorbic acid, rutin, and acetone were acquired from Sigma-Aldrich (Buchs, Switzerland). Acetonitrile was purchased from Macron Fine Chemicals (Center Valley, PA, USA). Ethanol absolute and sodium hydroxide pellets were obtained from Cochimy (Martigny, Switzerland). All reagents used were of analytical grade or higher. Dry goji berries were purchased from Optymis (Morges, Switzerland). Rice flour was acquired from La Riseria Taverne SA (Taverne, Switzerland), salt from Saline de Bex (Bex, Switzerland), and LEDOR MO 80T WPC from Hochdorf (Hochdorf, Switzerland). Deionised water was obtained using a Milli-Q purification system (Millipore AG, Zug, Switzerland).

Extrusion process

The general flowchart of extrudate production process was depicted in Figure 2. Goji berries were ground in a Cut-o--mat H10 (Kneubühler Gastro Ltd, Luzern, Switzerland) for 10 s and passed through a sieve with a mesh aperture diameter of 2 mm. The powder with a particle diameter less than or equal to 2.0 mm (fine fraction) was stored in a metal bucket. The coarse particles were recovered, and the grinding and the sieving were repeated until the amount of mix was too low to be ground. The coarse particles were thrown away and the fine fractions were combined and stored in the same metal bucket at room temperature without humidity. Batches of 5 kg of raw mixture were prepared. The mixture for the production of rice extrudates (RE) were composed of 99.5% of rice flour and 0.5% of salt. In the mixture for the production of goji enriched extrudates, 3, 7, 10, 13, 17, and 20% of rice flour was replaced by goji powder, whereas for the production of goji and WPC enriched extrudates 2, 4 and 7% of WPC was added. All preparations were mixed in a powder mixer (Prodima, Saint-Sulpice, Switzerland) for 30 s before extrusion.

The extrusion conditions were based on the work of Kosińska-Cagnazzo *et al*. [2017]. Briefly, a K-Tron powder feeder (Coperion K-Tron, Niederlenz, Switzerland) was coupled with an Evolum 25 twin screw extruder (Clextral, Firminy, France). The screw configuration allowed applying a high shear to the material. The temperatures in the five first barrels from the feeder to the die were as follows: 20, 40, 60, 80, and 100°C. The last five barrels as well as the die, which was round and had a 2 mm diameter, were heated at 140°C. The dry mixture was fed at 13 kg/h and water was pumped at 1.4 L/h (DKM piston metering pump, Firminy, France). The speed of the extruder screws was set at 400 rpm and allowed to have a constant feed. A pelletizer EX21 (Clextral, Firminy, France) set at 2,000 rpm was placed in the front of the die and cut the extrudates at the exit. Die pressure and specific mechanical energy (SME) was constantly measured during the extrusion process. To prevent agglutination of the hot, humid and therefore sticky extrudates, a ventilation of 2000 L of air/min was applied at ambient temperature.

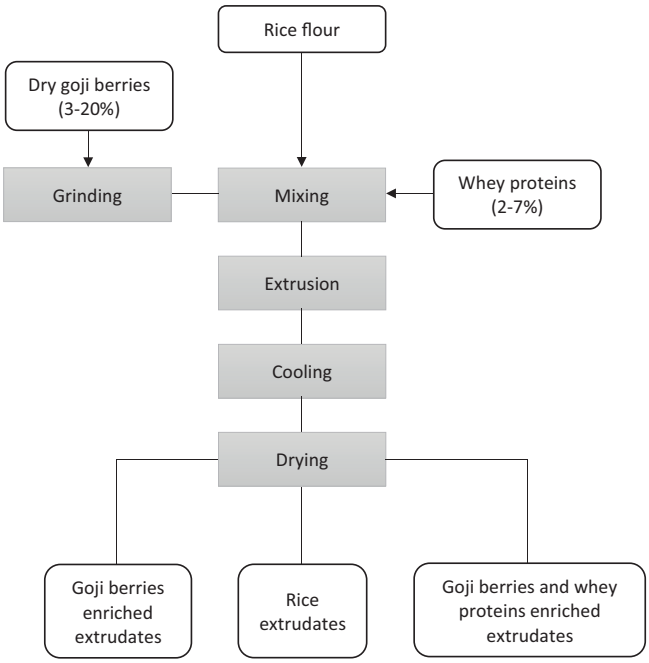

FIGURE 2. Flowchart of the extrusion process.

The cut extrudates were collected, cooled down, and dried in an Euromat B4 IS600 oven (Wiesheu, Grossbottwar, Germany) at 120°C for 10 min to obtain a final moisture level lower than 5%. Once the extrudates reached the room temperature, they were placed in plastic bags, sealed, and stored at 4°C until analysis. The water content of goji berries, pre-extrusion mixtures, and extrudates was evaluated using an HG53 Halogen moisture analyser (Mettler Toledo, Greifensee, Switzerland).

Physical characterization of extrudates

Expansion ratio

The diameters of 15 pieces of each extruded product were measured with a calliper (Mitutoyo, Urdorf, Switzerland). Diametric expansion ratio was calculated as a cross-sectional diameter of an extrudate divided by the diameter of the die opening and multiplied by 100.

Colour parameters CIELAB

Finely ground extrudates (5 g) were analysed in triplicate using a Konica Minolta CM-5 (Chiyoda, Tokio, Japan) colorimeter with standard illuminant D65 and 10° observer. The values of lightness (L*), greenness/redness (a*), and blueness/yellowness (b*) were recorded. Analysis was performed in 5 replicates.

Hardness analysis

Texture analyser TA-XT (Stable Micro Systems, Godalming, United Kingdom) was employed. An Ottawa cell of 9×7.1×7.1 cm was filled with extrudates and hardness analysis was performed by compression by 25% at a speed of 5 m/s. Analysis was performed in five replicates and the results were expressed in N.

Bulk density

An Ottawa cell was filled up with extrudates and the weight was evaluated in ten replicates. The results were expressed as kg/m^3.

In vitro digestion

A two-stage *in vitro* digestion model based on the procedure described by Xie *et al*. [2013] was used with some modifications. The gastric phase was initiated by weighing 4 g of ground samples (dry goji berries, pre-extrusion mixtures or extrudates) into a 50 mL tube. Then, 15 mL of 0.05 mol/L HCl solution (pH 1.3) was added to the samples (20 mL for the extrudates). A pepsin solution (8 mL; 3.5 g of pepsin from porcine stomach mucosa in 500 mL of 0.1 mol/L HCl) was added, the mixture was purged with nitrogen and placed in a Multitron PRO incubator (Blanc Labo, Lonay, Switzerland) at 37°C for 1 h with continuous shaking. After the gastric digestion, the pH was adjusted to 6.5 using an NaOH solution (1 mol/L). After addition of 8 mL of a pancreatin and bile salts solution (3.5 g and 3.7 g respectively in 500 mL of 0.1 mol/L $NaHCO_3$), the mixture was purged with nitrogen and placed at 37°C for 2 h with continuous shaking. The solution was centrifuged at 4,000 × *g* for 15 min using an Eppendorf Centrifuge 5810 (Hamburg, Germany) and the supernatant was recovered in a volumetric flask. The solution was adjusted to the volume of 50 mL with 0.9% NaCl solution and stored at 4°C until analysis. During the whole digestion process, the solutions were protected from light.

Chemical analyses

Extraction and analysis of 2-O-β-D-glucopyranosyl-L-ascorbic acid

The procedure of extraction was based on a previously published method [Kosińska-Cagnazzo *et al.,* 2017]. The samples of 1 g of dry goji berries, pre-extrusion mixture, and extrudates, respectively, were extracted with 10 mL of water in an ultrasonic bath for 10 min. Following centrifugation at 4,000 × *g* for 5 min, the supernatant was recovered in a 25 mL volumetric flask. The extractions from the residue were performed twice more with 5 mL of water and the supernatants were combined in the 25 mL graduated flask. Oxalic acid solution (1 mL, 4% *w/v* in water) was added to stabilize the solution and the volume was adjusted to 25 mL with water. The extractions were performed in quadruplicates for each sample. The extracts were filtered through a 0.20 μm PTFE syringe filter (Chromafil, Macheret-Nagel, Düren, Germany) in standard vials and stored at 4°C until analysis.

For digested samples, 2 mL of digesta was sampled and mixed with 2 mL of ethanol absolute in a 15 mL tube. The solution was then centrifuged at 4,000 × *g* for 3 min and the supernatant was collected and filtered through a 0.20 μm PTFE syringe filter (Chromafil, Macheret-Nagel) into a standard vial and stored at 4°C until analysis. The extractions were carried out in triplicates.

A volume of 5 μL of extracted and digested samples was injected onto an amino column (Aminex HPX-87H Ion exclusion, 300 mm × 7.8 mm i.d., particle size 5 μm, Bio-Rad, Hercules, CA, USA) coupled to a precolumn. The diode array detector (DAD) was set at 210 and 254 nm for the

detection of the analogue. The mobile phase was a 5 mmol/L sulfuric acid solution delivered in isocratic mode. The flow rate of 0.5 mL/min was applied and separation was carried out at 35°C. The quantification of 2-β-gAA was made using a calibration curve for ascorbic acid and a conversion factor reported by Tai & Gohda [2007]. The results were expressed as μg/g of goji berries dry matter (DM).

Extraction and analysis of rutin

The procedure was based on the method of Kosińska-Cagnazzo *et al.* [2017] with some modifications. Briefly, 600 mg of dry goji berries or 1,200 mg of pre-extrusion mixtures or extrudates was extracted with 5 mL of 70% (*v/v*) ethanol in an ultrasonic bath (VWR, Dietikon, Switzerland) at a 45 kHz working frequency for 10 min. Following centrifugation at 4,000 × *g* for 5 min, the supernatant was recovered in a 20 mL graduated flask. The extractions from the residue were performed twice more with 5 mL of 70% (*v/v*) ethanol and the supernatants were combined in the 20 mL graduated flask. The volume was adjusted to 20 mL using 70% ethanol. The extractions were performed in quadruplicates for each sample. The extracts were then filtered through an Exapure 0.45 μm nylon syringe filter (Alys Technologies, Bussigny, Switzerland) into vials and stored at 4°C until analysis. Digesta were extracted as described in the "*In vitro* digestion" section.

An Agilent 1220 infinity series liquid chromatograph (Agilent Technologies, CA, USA) coupled to an autosampler, a binary pump, and a G4294B UV-DAD detector (Agilent Technologies 110 Series) was used to carry out the HPLC analyses. A volume of 1 μL of extracts and 2 μL of digesta was injected onto a Kinetex C18 column (2.6 μm, 50 mm × 2.1 mm; Phenomenex, Torrance, CA, USA) heated at 40°C. The DAD was set at 320 and 340 nm for the detection of rutin. The mobile phase A was made of 1% aqueous formic acid and the mobile phase B of 1% (*w/v*) formic acid in acetonitrile. The elution gradient was as follows: 0 min: 100% A, 2 min: 100% A, 25 min: 90% A, 26 min: 90% A, 30 min: 40% A, 35 min: 40% A, 35.1 min: 100% A. The flow rate of 0.3 mL/min was applied. The identification was performed by comparison of UV-Vis spectra and retention time with those of standard compound of rutin. Quantification was done by external calibration. The results were expressed as μg/g DM of goji berries.

Statistical analysis

The extrusion process was performed in duplicate on two different days. The results are expressed as mean ± standard deviation. One-way ANOVA followed by a post hoc Tukey test was performed to determine if the differences between the results were statistically significant. The differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Extrusion process characteristics and physical properties of extrudates

The extrusion process was controlled by permanent measurement of die pressure and specific mechanical energy (SME). The collected dataset was compiled in Table 1. The maintaining of the process parameters at the desired values allows obtaining the final product with high overall quality [Moscicki, 2011]. The increasing addition of goji berries led to diminished values of die pressure and SME compared to plain rice extrudates (RE). It was reported that the addition of fruits can act as a lubricant and reduce friction in the extruder and shear forces, and thereby diminish the extrusion pressure [Moscicki, 2011]. WPC addition lowered die pressure even further, with low impact on SME values compared to goji extrudates (Table 1). A recent study evaluated the impact of WPC addition (0–40%) on the extrusion process of corn starch and reported that SME diminished significantly up to a WPC level of 20% [Yu *et al.,* 2017]. The authors attributed this observation to the lower starch content and the lower degree of gelatinization due to water competition between the WPC and corn starch in the blend.

TABLE 1. Extrusion process parameters.

	Goji berries (%)	WPC (%)	Pressure (bar)	SME (Wh/kg)
Rice extrudates	0	0	293.2±5.8^{a}	85.2±3.5^{a}
Rice extrudates with goji	3	0	280.5±4.4^{b}	85.2±3.5^{a}
	7	0	271.7±6.6^{b}	86.4±3.2^{a}
	10	0	243.1±4.3^{c}	78.2±1.7^{c}
	13	0	248.9±3.8^{c}	82.6±1.0^{b}
	17	0	233.6±4.0^{d}	70.2±1.7^{d}
	20	0	212.6±2.4^{e}	64.8±1.4^{e}
Rice extrudates with goji and WPC	20	2	200.2±3.2^{f}	67.6±1.2^{d}
	20	4	195.9±4.6^{fg}	67.7±4.0^{de}
	20	7	192.8±3.4^{g}	68.6±0.9^{d}

SME – specific mechanical energy, WPC – whey protein concentrate. The results with different letters within column are significantly different at $p \leq 0.05$.

The pelletizer employed in the presented study allowed obtaining ready-to-eat extruded products of round shape. A good expansion ratio of about 450% was noted for RE (Figure 3). The addition of 3 and 7% of goji berries to the extrudate blend caused an increase in the expansion ratio, whereas higher addition levels caused the expansion ratio to decrease significantly. Similarly, an initial increase in the expansion ratio with the addition of mango peel powder was observed by Mohamad Mazlan *et al.* [2019]. The linear expansion started to decrease when the addition of mango peel powder exceeded 8.34%. In the present study, 4 and 7% addition of WPC to the blends containing 20% of goji fruits increased the expansion ratio of the extrudates (Figure 3). Some literature data shows an increase in the expansion ratio with the addition of whey proteins up to 10% [Yu *et al.,* 2017].

The colour parameters of extruded products were significantly affected by goji and WPC addition (Table 2). RE showed a high L* value (85.0), which corresponds to lightness, whereas the intensiveness of red colour indicated by a* value was very low (0.96). The addition of goji berries decreased significantly the lightness of the extruded products and increased their

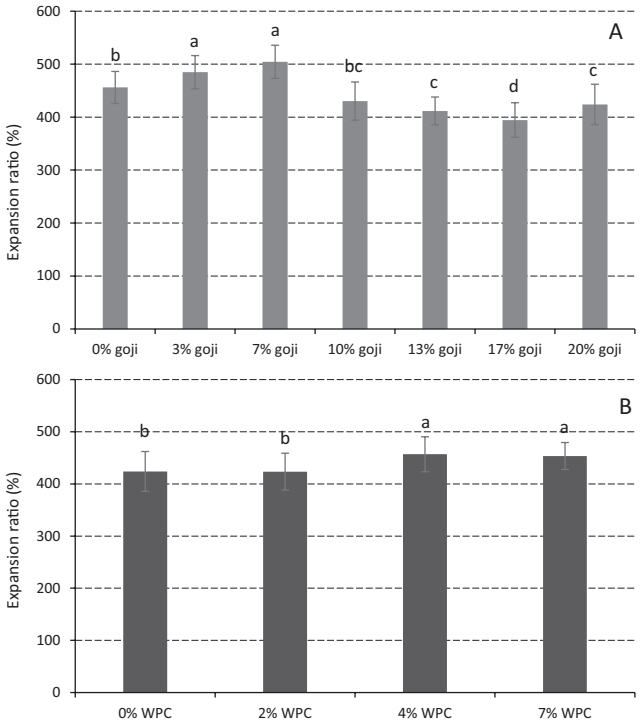

FIGURE 3. Expansion of extrudates with different percentage of goji berries and no whey protein concentrate (WPC) added (A) and with different percentage of WPC and 20% of goji berries (B) The results with different letters are significantly different at p≤0.05.

yellowness (b*) and redness (a*). This result is not surprising regarding the high content of orange-red carotenoids like zeaxanthin dipalmitate in goji berries [Potterat, 2010]. The addition of WPC led to a further decrease in lightness as well as a decrease in values of b* and a* parameters. Darker colour of the extrudates with WPC added might result from the reactions between reducing sugars and amino acids, including Maillard reaction and caramelization [Yu *et al.,* 2017].

Bulk density and hardness were determined for the rice extrudates (RE) and for the extrudates with the maximal addition of goji berries and WPC, *i.e.* rice extrudates with 20% of goji berries added (RGE) and rice extrudates with 20% of goji berries and 7% of WPC added (RGWE). The results were compiled in Table 3. The addition of goji berries as well as the addition of WPC increased significantly bulk density of rice extrudates. Masatcioglu *et al*. [2013] reported no change in bulk density of corn extrudates after adding 8% of ginseng powder and green tea, whereas Wójtowicz *et al*. [2018] found doubled values of bulk density after 10 to 20% addition of tomato powder to corn extrudates. The addition of 20% of goji berries increased the hardness of rice extrudates in comparison to RE. However, when goji berries were added together with whey proteins (RGWE) the increase in hardness was not significant. The addition of mango peel powder at 25% was observed to increase the hardness of extrudates up to 380 N [Mohamad Mazlan *et al*., 2019]. In the present study, a decrease in the expansion ratio was observed together with an increase in hardness in RGE in comparison to RE. A similar effect was observed by Wójtowicz *et al*. [2018] when corn extrudates were enriched with powdered tomato.

TABLE 2. Colour parameters of extrudates.

	Goji berries (%)	WPC (%)	*L**	*a**	*b**
Rice extrudates	0	0	85.0 ± 1.0^{a}	0.96 ± 0.1^{g}	16.7 ± 0.8^{d}
Rice extrudates with goji	3	0	68.1 ± 1.1^{b}	12.5 ± 0.4^{f}	44.7 ± 1.0^{b}
	7	0	61.6 ± 1.6^{c}	14.7 ± 1.1^{e}	44.6 ± 1.0^{b}
	10	0	59.4 ± 0.6^{d}	17.2 ± 0.5^{d}	48.2 ± 1.5^{a}
	13	0	58.2 ± 0.8^{d}	17.6 ± 0.3^{d}	47.2 ± 0.6^{a}
	17	0	56.8 ± 1.4^{e}	19.1 ± 0.8^{bc}	47.3 ± 1.0^{a}
	20	0	54.9 ± 0.5^{f}	21.6 ± 0.3^{a}	47.9 ± 1.0^{a}
Rice extrudates with goji and WPC	20	2	52.2 ± 1.0^{g}	19.4 ± 0.2^{b}	45.2 ± 1.1^{b}
	20	4	52.4 ± 1.0^{g}	19.6 ± 0.8^{b}	45.5 ± 1.7^{b}
	20	7	49.8 ± 1.0^{h}	18.8 ± 0.2^{c}	41.8 ± 1.4^{c}

WPC – whey protein concentrate. The results with different letters within column are significantly different at $p\leq0.05$.

Content of 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (2-β-gAA)

Goji berries, pre-extrusion mixture, RGE, and RGWE were analysed for the content and bioaccessibility of bioactives.

The content of 2-β-gAA in goji berries, pre-extrusion mixture, and extruded products was evaluated after aqueous extraction. Dry goji berries contained about 2,920 μg/g of 2-β-gAA. As expected, the pre-extrusion mixture of RE and RE itself contained no 2-β-gAA. The content of 2-β-gAA in the pre-extrusion mixture powder, amounted to 594 and 549 μg/g DM for RGE and RGWE, respectively (Figure 4A). The addition of 7% WPC, replacing rice flour, did not affect the content of 2-β-gAA in the pre-extrusion mixtures ($p>0.05$). On the other hand, the extrusion process significantly reduced the content of 2-β-gAA both in RGE and RGWE by approximately 15%. Similarly, as in the case of pre-extrusion mixtures, the content of 2-β-gAA did not differ significantly ($p>0.05$) between RGE and RGWE.

Compared to ascorbic acid, which is sensitive to heat and light, 2-β-gAA showed quite good stability during extrusion, as revealed previously [Kosińska-Cagnazzo *et al.,* 2017]. The die temperature, moisture content, speed and geometry of extruder screws influence significantly the content of ascorbic acid in extruded products with losses up to 76% [Obradović *et al.,* 2015]. The instability of ascorbic acid comes from the hydroxyl groups of the 2,3-enediol, which are easily oxidized, leading to dehydroascorbic acid. Numerous ascorbic acid derivatives possessing substituents at these positions, such as phosphate, sulphate, galactose or glucose [Yamamoto *et al.,* 1990], turn out to be more stable than ascorbic acid, which can explain why 2-β-gAA was more stable during extrusion.

Bioaccessibility of 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (2-β-gAA)

After *in vitro* simulated gastrointestinal digestion, 2,460 μg/g of 2-β-gAA was detected in dry goji berries digesta, which was not significantly different from the content of

TABLE 3. Bulk density and hardness of extrudates.

Extrudates	Bulk density (g/cm^3)	Hardness (N)
RE	35.5±3.24^c	167±24^b
RGE	50.3±4.35^a	215±28^a
RGWE	40.3±5.44^b	181±40^{ab}

RE – rice extrudates, RGE – rice extrudates enriched with goji berries (20%), RGWE – rice extrudates enriched with goji berries (20%) and whey protein concentrate (7%). The results with different letters within column are significantly different at $p \leq 0.05$.

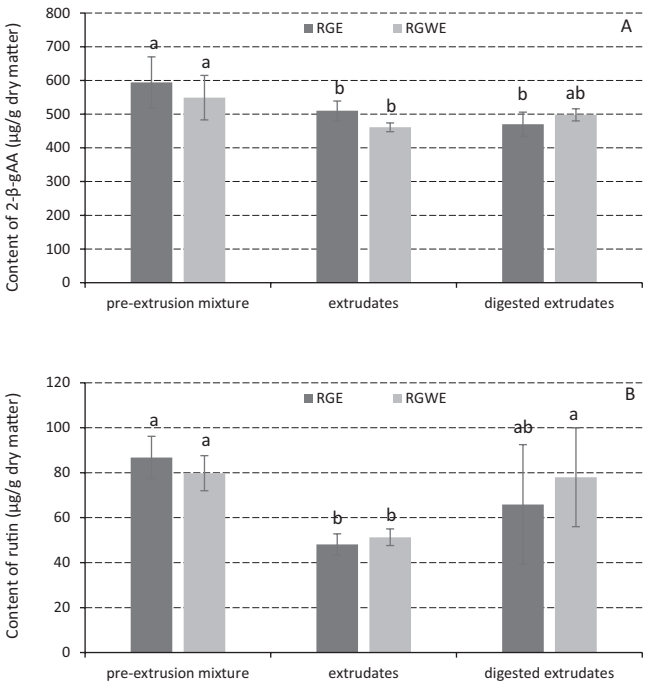

FIGURE 4. Content of 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (2-β-gAA) (A) and rutin (B) in extrudates as influenced by extrusion and digestion process RGE rice extrudates enriched with goji berries (20%) RGWE rice extrudates enriched with goji berries (20%) and whey protein concentrate (7%) The results with different letters are significantly different at p≤0.05.

2-β-gAA in goji berries before digestion ($p>0.05$). The addition of WPC at the level of 7% did not significantly affect the bioaccessibility of 2-β-gAA from extrudates (Figure 4A), which amounted to 470 and 498 μg/g DM for RGE and RGWE, respectively. There was no statistically significant difference in the content of 2-β-gAA before and after *in vitro* digestion for both RGE and RGWE. It might be due to the subcellular distribution of 2-β-gAA in fruits. It is assumed that ascorbic acid is synthesised by the plants in the cytosol through three different pathways and transported into chloroplasts by facilitated diffusion [Rautenkranz *et al.,* 1994]. If the synthesis of 2-β-gAA in goji berries occurs also in the cytosol, this means that the substance can easily diffuse from the cytosol to the extraction solvent. Thus, in general, 2-β-gAA is not bound to other compounds. Therefore, enzymes present during the *in vitro* digestion do not liberate more 2-β-gAA from the food matrix and, at the same time, 2-β-gAA is stable under digestion conditions and does not undergo degradation.

Concerning the nutritional values, ascorbic acid derivatives might be transformed to active vitamin C in the human body, due to the activity of such enzymes as phosphatase, sulfatase, α-glucosidase, and β-galactosidase [Nakamura *et al.,* 2009]. Three native β-glucosidases are present in the human body. Glucocerebrosidase and lactase phlorizin hydrolase are a part of the brush border enzymes, whereas the last β-glucosidase is a broad-specificity cytosolic enzyme present in the liver, kidney, and small intestine. The cytosolic β-glucosidase has been shown to cleave β-linkages in some isoflavonoids [Day *et al.,* 1998]. Nonetheless no studies have been performed on the ability of those enzymes to hydrolyse 2-β-gAA into ascorbic acid and glucose in human. Toyoda-Ono *et al.* [2005] reported an increased level of ascorbic acid and of intact 2-β-gAA in the portal vein blood of rats, after oral administration of the analogue. According to their findings, 2-β-gAA to some extent maintains the level of ascorbic acid in the rat tissues and hence acts as a provitamin C. They even proposed that the 2-β-gAA might serve as a stable ascorbic acid substitute for clinical applications. It remains to be evaluated whether 2-β-gAA might have provitamin C activity and, in consequence, whether goji berries and their extrudates are a reliable source of ascorbic acid in humans.

Content of rutin

The content of rutin in dry goji berries amounted to 408 μg/g DM. The addition of WPC at a level of 7% did not influence significantly ($p>0.05$) the extractability of rutin from pre-extrusion mixtures or extrudates (Figure 4B). The extrusion process decreased significantly the content of rutin in both types of extrudates, from 86.8 to 48.1 μg/g DM and from 79.8 to 51.3 μg/g DM for RGE and RGWE, respectively. The retention of around 60% of rutin from goji berries during the extrusion process corresponds with the existing literature [Kosińska-Cagnazzo *et al.,* 2017; Leyva-Corral *et al.,* 2016]. It is known that the extrusion cooking process might cause a decrease in the content of some compounds, such as polyphenols, which is generally attributed to the high temperature process [Ti *et al.,* 2015]. However, other parameters may influence the level of bioactive compounds in extruded products, such as the shear due to the screw configuration, the screws speed, and the moisture content [Brennan *et al.,* 2011]. For example, the stability of anthocyanins depends on die temperature and moisture content, *i.e.* the higher the die temperature, the lower the content of anthocyanins. The increase of moisture content has the opposite effect; the higher moisture content allows retaining more compounds after the extrusion cooking process [Durge *et al.,* 2013]. Oxidation of flavonoids in foods can occur through exposure to high pH, heat, and oxygen reactive species or when flavonoids come into contact with degradative enzymes, such as polyphenol oxidase, after cell wall degradation. Consequently, quinones are formed and may covalently bind to protein amine and amide groups, which may reduce the content of accessible flavonoids [Bordenave *et al.,* 2014]. Furthermore, during roasting, rutin may be converted into its aglycone, quercetin, which might also occur during the drying at 120°C for 10 min. The effect of roasting of flavonoid glycoside of noni leaves was investigated, and ru-

tin content was observed to decrease after a 20-min thermal treatment in an oven [Deng *et al.,* 2011]. A similar effect was observed for quercetin glycosides of onions during the roasting process, which suggests that flavonoid glycosides present in goji berries might follow the same path [Rohn *et al.,* 2007].

Bioaccessibility of rutin

The amount of rutin released from dry goji berries during *in vitro* simulated gastrointestinal digestion amounted to 227 μg/g and was significantly lower than its content in goji berries. The bioaccessibilty of rutin from RGE and RGWE amounted to 65.9 and 78.0 μg/g DM, respectively (Figure 4B). Interestingly, the amount of rutin released from RGWE during digestion was higher than that extracted from the same sample with a solvent. The addition of WPC at a level of 7% did not influence the bioaccessibility of extrudates.

Thermal processes have an impact on flavonoids structure and may also influence their bioaccessibility and bioavailability [Rohn *et al.,* 2007]. Only 17% of accessible rutin is absorbed by the small intestine, whereas the rest is hydrolysed into quercetin and metabolised by faecal microflora in phenylacetic acids such as 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid [Olthof *et al.,* 2003]. The content of rutin in the digesta of boiled potato accounted for 169% of the initial amount of rutin present. Polyphenol concentration in boiled potatoes issued from the chemical extraction may therefore underestimate the actual amount that can be released during digestion and that are bioaccessible [Miranda *et al.,* 2013]. Also in blended fruit juices the content of chlorogenic and *p*-coumaric acids, as well as naringenin and rutin increased after simulated gastrointestinal digestion [Rodríguez-Roque *et al.,* 2013]. On the other hand, in the same study, the content of ferulic and sinapic acids, hesperidin, quercetin, and (+)-catechin was lower in the digesta than in the juices before digestion.

The subcellular distribution of rutin on the goji shrub may play a role as well. The molecule is synthetised in the endoplasmic reticulum, possibly in a multi-enzyme complex [Falcone Ferreyra *et al.,* 2012]. Contrarily to ascorbic acid, which is synthetised in the cytosol and transported through facilitated diffusion, rutin and other polyphenols would be translocated from endoplasmic reticulum to vacuole or cell wall by an active process [Mintz-Oron *et al.,* 2008], possibly by the action of a multidrug resistance-associated protein transporter [Petrussa *et al.,* 2013], which could make it harder to extract. In food, non-covalent binding may occur between flavonoids and other macronutrients, such as proteins for example, involving Van der Waals forces such as hydrogen bonding as well as ionic and London interactions [Bordenave *et al.,* 2014]. This might reduce the amount of accessible rutin. Despite the above-mentioned potential interactions between proteins and flavonoids, a content of 7% WPC seems to have no significant influence on the extractable amount of rutin from extruded materials. However, according to Bordenave *et al.,* [2014], interactions between milk proteins and flavonoids might allow to have a better stability over storage time, by decreasing the availability of flavonoids for oxidative reactions.

CONCLUSION

The addition of goji berries at up to 7% improved the expansion ratio of the rice extrudates. Colour parameters were highly affected by the addition of goji berries and WPC. Extrusion is a high-temperature-short-time process that decreases significantly the content of 2-β-gAA and rutin coming from goji berry addition. 2-β-gAA was retained at 85% after the extrusion process, which suggests that the compound is more stable to heat, light, and oxygen than ascorbic acid. Bioaccessibility of 2-β-gAA from goji berries, but not that of rutin, was affected by the extrusion process. The addition of WPC at a level of 7% showed no significant effect on the bioaccessibility of neither 2-β-gAA nor rutin. The results suggest that the interactions between WPC and 2-β-gAA or rutin did not impact their bioaccessibility. The release of 2-β-gAA and rutin during *in vitro* simulated digestion was comparable to that of a chemical extraction carried out.

In view of the potential health benefits of the goji compounds, additional studies should be conducted, using the Caco-2 cell culture model, in order to predict their *in vivo* intestinal absorption. Provided that 2-β-gAA and rutin are well absorbed, the extrudates could be a reliable source of these beneficial compounds.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

ORCID IDs

W. Andlauer https://orcid.org/0000-0002-0507-1911
A. Kosińska-Cagnazzo https://orcid.org/0000-0003-2928-2403

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Original article
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Enhancement of the Stabilities and Intracellular Antioxidant Activities of Lavender Essential Oil by Metal-Organic Frameworks Based on β-Cyclodextrin and Potassium Cation

Ying Wang, Liang Wang, Jin Tan, Rong Li, Zi-Tao Jiang, Shu-Hua Tang*

Tianjin Key Laboratory of Food Biotechnology, College of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, China

Key words: microcapsulation, thermal stability, pH stability, cell viability, cellular antioxidant model, application extension

Lavender (*Lavandula angustifolia*) is an important medicinal and aromatic plant. However, the application of lavender essential oil (LEO) is limited by its instability, low solubility and high volatility. Therefore, to improve the stabilities and antioxidant activities of LEO and thereby expand the applications, LEO was microencapsulated by metal-organic frameworks based on β-cyclodextrin and potassium cation (K-βCD-MOFs) with different mass core/wall ratios. The results showed that the best inclusion rate was 96.67% with the ratio of 1:10. Then, the optimum inclusion product was characterized by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM). The thermal and pH stabilities and the intracellular antioxidant activities were also studied. The results showed that the stabilities of the oil with K-βCD-MOFs inclusions and their abilities to resist acid and alkali were significantly stronger than those of LEO itself. In addition, the intracellular antioxidant activities of LEO were also enhanced by the K-βCD-MOFs inclusion. These results suggested the potential of K-βCD-MOFs as carriers for essential oils in food industry applications.

INTRODUCTION

Lavender (*Lavandula angustifolia*), an aromatic plant belonging to the family Lamiaceae, is widely grown for essential oil production due to its aromatic property [Shafaei *et al.*, 2017]. This herbal plant is commonly utilized in many industrial fields including cosmetics, perfumes, pharmaceuticals, and foods [Yuan *et al*., 2019]. Taking foods as an example, it is widely used as flavoring in beverages, herbal teas, ice creams, sweets, cakes, and other aromatic plant products [Dong *et al*., 2020]. Essential oil, the most important and popular product of this aromatic plant, mainly exists in the glands of the aerial parts including flowers and leaves [Lis-Balchin, 2002]. The lavender essential oil (LEO) is believed to have anti-depressive, anti-inflammatory, antimicrobial (antibacterial/ antifungal) and antioxidant characteristics [Cavanagh & Wilkinson, 2005; Insawang *et al*., 2019; Kwiatkowski *et al*., 2020]. LEO is also prevalent in the pharmaceutical, cosmetic and food industries. However, the applications of LEO are limited by its highly unstable and volatile properties. As the essential oils are composed of various volatile and lipophilic components, they are susceptible to light, heat and air, and thus to conversion and degradation [Yuan *et al*., 2019]. Beyond that, their poor stabilities also lead to difficult storage and transportation.

Consequently, stability enhancement methods of LEO are essential to expand the applications. Microencapsulation technologies for essential oil have raised concern in biology, medicine and food researches in the last few years. As common wall materials for microencapsulation, cyclodextrins (CDs) are a class of cyclic oligosaccharides normally produced from starch under the effect of the enzyme catalytic reaction [Thombre *et al*., 2013]. CDs are characterized by their amphiphilic structure with hydrophobic inner cavity and hydrophilic outer wall, which could embed guest molecules to form relatively stable complexes [Tian *et al*., 2020]. They are mainly composed of 6, 7 or 8 glucose units, known as α-, β- and γ-CD, respectively [Thombre *et al*., 2013]. Among them, β-CD is one of the most important members and is derived from 7 D(+)-glucose units linked by α-1,4-glycosidic bonds [Thombre *et al*., 2013]. Comparing with other CDs, β-CD is the most frequently used CD due to the lowest price and relatively appropriate size cavity. The interest in β-CD regarding its food-related applications has been increasing since it was approved for food uses by FDA [Partanen *et al*., 2002]. With cavity, β-CD could act as a host and include some molecules as guests to form inclusion complexes by van der Waals and hydrophobic interaction forces, or hydrogen bonds [Deng *et al*., 2018]. However, the applications of β-CD are limited by its relatively low binding capacity and solubility. Therefore, the metal-organic frameworks (MOFs) with the utilization of the special structure of CDs were developed to solve the above problems. CD-MOFs are network-structure and crystalline porous materials derived from CDs and metal ions or clusters for biomedical applications [Li *et al*., 2017; Smaldone *et al*., 2010]. They have high porosity, large surface areas, and ver-

* Corresponding Author e-mail: tanjin@tjcu.edu.cn (J. Tan).

satility in terms of composition and functionalities [Li & Huo, 2015]. Particularly, K-βCD-MOFs could be synthesized from β-CD and K ions employing a vapor diffusion method [Smaldone *et al*., 2010]. The K-βCD-MOFs have good cutting structure, high drug loading capacity and strong inclusion ability, in addition, they are environment-friendly, biocompatible and without side-effects on the human body [Abuçafy *et al*., 2018; Liu *et al*., 2017]. Kumar *et al*. [2020] reviewed that MOFs could be widely applied to various areas including catalysis, energy storage, solar cells, air and water purification, gas storage, bioimaging, drug delivery, waste remediation and sensors. So far, MOFs are rarely applied in food, however, K-βCD-MOFs are recognized due to their non-toxicity and unique "food-grade synthesis" based on natural components [Kumar *et al*., 2020]. Therefore, the potential of K-βCD-MOFs as microcapsule wall materials is huge and the application prospects are also broad, especially possible in food.

To our best knowledge, limited researches were done on the essential oil stability improvement using CDs or MOFs related materials. For example, Yuan *et al*. [2019] reported LEO encapsulated in hydroxypropyl-β-cyclodextrin (HPCD) and Wu *et al*. [2019] investigated zinc metal-organic framework (Zn@MOF) as a carrier for thymol. There is no research hitherto on the stability improvement of LEO by microencapsulation by K-βCD-MOFs. Furthermore, whether the microencapsulation by K-βCD-MOFs affects the antioxidant activities of LEO is also important for further applications of the oil. Therefore, LEO was microencapsulated by K-βCD-MOFs to improve the stability and thereby expand the applications. The thermal/pH stabilities and intracellular antioxidant activities were also determined and compared, and the inclusion compound was further identified by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Apparatus

A blade homogenizer A10 (IKA, Staufen, Germany) was used to ground the plants. An ultrasonic-microwave assisted extraction (UMAE) apparatus CW-2000 (Xintuo, Shanghai, China) was used for the extraction of lavender essential oil. The absorbances for the inclusion rate and stability measurement were determined by an ultraviolet and visible spectrophotometer Lambda25 (PerkinElmer, Waltham, MA, USA). Cell proliferation was measured using a SpectraMax M5 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA). A Fourier transform infrared spectroscope IRAffinity-1 (Shimadzu, Tokyo, Japan) and a scanning electron microscope JSM-IT300 (JEOL, Tokyo, Japan) were used to characterize the essential oil included in the molecular microcapsules. Morphological characteristics of β-CD and K-βCD-MOFs were observed and photographed by an optical microscope (Smart, Chongqing, China).

Chemicals

β-CD and dimethylsulfoxide (DMSO) were purchased from Tianjin Chemical Factory (Tianjin, China). HeLa cells were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). The fetal bovine serum (FBS) and Dulbecco's modified eagle's medium (DMEM) were obtained from Gibco Co. (Carlsbad, CA, USA). Double-antibody (penicillin-streptomycin) was purchased from HyClone Co. (South Logan, UT, USA). Methyl thiazolyl tetrazolium (MTT) was purchased from Solarbio Biotechnology Co. (Beijing, China). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Distilled water was used throughout the experiment. KBr was of spectral purity and was purchased from Tianjin Chemical Factory (Tianjin, China). Other chemicals or reagents were all of analytical grade.

Plant materials and essential oil extraction

The lavender (*Lavandula angustifolia)* aerial parts were purchased from Xinjiang province, China. The plants were ground, sieved to 40 mesh, and stored in cool and dry place. LEO was then extracted by ultrasonic-microwave assisted extraction (UMAE) following our previous work [Wang *et al*., 2018] with modifications. The extraction temperature, microwave power, ultrasonic power, extraction time and the ratio of plant material to liquid were 100°C, 600 W, 50 W, 7 min and 1:10 (g/mL), respectively. The mean values of the LEO yields were 3.27% based on dry weights.

Synthesis of K-βCD-MOFs

The high purity β-CD crystals were obtained by the following steps. First, β-CD was dissolved in distilled water heated to 85°C to form a saturated solution, cooled down, and then the crystals were collected by filtration. The above operation was repeated three times and the crystals were combined and oven-dried at 100°C.

K-βCD-MOFs were synthesized based on the above purified β-CD crystals according to the methanol vapor diffusion method with some modifications [Smaldone *et al*., 2010]. Briefly, 1.1349 g of purified β-CD were dissolved with 0.4488 g of potassium hydroxide (KOH) in distilled water. This solution was transferred into a beaker after filtration and then sealed in a tank with 100 mL of methanol for one week. This operation aimed to allow the vapor of methanol diffuse into the solution and form K-βCD-MOFs. The formed K-βCD-MOFs are relatively rectangular, colorless and transparent crystals. The K-βCD-MOFs crystals were then washed twice with 30 mL of methanol. K-βCD-MOFs and β-CD were observed under an optical microscope for their basic morphological characteristics.

Formation of LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes

The lavender essential oil (LEO) as a core was microencapsulated by K-βCD-MOFs as wall materials to form the inclusion complexes by different mass core/wall ratios (1:3, 1:5, 1:8, 1:10 and 1:12) following Michida *et al*. [2015] with modification. Briefly, 1 mg/mL of the LEO solution was prepared by dissolving the oil in 45% ethanol, while K-βCD--MOFs were dissolved in isovolumetric distilled water. The oil solution was then added and dispersed into the β-CD-MOFs solution dropwise with stirring at 48°C to ensure thorough mixing and optimum inclusion. The mixture was cooled down

and freeze-dried to obtain the white powdered inclusion complex product. The inclusion rate was calculated as follows:

$$\text{Inclusion rate (\%)} = (1 - As_{LEO}/At_{LEO}) \times 100\% \quad (1)$$

where: As_{LEO} and At_{LEO} were the amount of LEO on the surface of inclusion complex and the total amount of LEO in the inclusion complex, respectively. The former was calculated by the following steps: 0.05 g of LEO/K-βCD-MOFs powder with each mass core/wall ratio was transferred into a 100 mL volumetric flask, and anhydrous ether was added, then the sample was shaken and made up to the volume. After sufficient mixing, the surface of the complex powder was thoroughly washed. After filtration, the absorbance of the supernatant was measured at 233 nm, which is the maximum absorption wavelength of LEO. At_{LEO} was determined as follows: 0.05 g of LEO/K-βCD-MOFs powder with each mass core/wall ratio was ultrasonically (60 W) dissolved by 100 mL of 45% ethanol for 30 min. After thorough dissolution and filtration, the absorbance of the supernatant was also measured at 233 nm. Both the amounts of the surface and total LEO were calculated using a standard curve of linalool, which is the main component of LEO [Dong *et al*., 2020]. The inclusion rate was also optimized by comparison among different mass core/wall ratios. The LEO/β-CD inclusion complex was also produced according to the previous steps with the core/wall ratio of 1:10, which was the optimum ratio for the LEO/K-βCD-MOFs production.

Properties of LEO, LEO/β-CD and LEO/K-βCD-MOFs

Thermal stability and pH stability measurements

The LEO, LEO/β-CD and LEO/K-βCD-MOFs solutions were prepared by dissolving them in 45% ethanol. Their concentration was 0.1 mg/mL (LEO equivalence). The actual concentrations of the latter two complexes were ten-fold of LEO, as the mass optimum core/wall ratio was 1:10. The three solutions were stored in an oven at 90°C in dark for 10 days. The absorbances of the above solutions were measured at 233 nm every 24 h to study the change of LEO amounts. The preservation rates of LEO, LEO/β-CD and LEO/K-βCD-MOFs to the original LEO amounts were calculated using the following equation:

$$\text{Preservation rate (\%)} = A_{thermal}/A_c \times 100\% \quad (2)$$

where: $A_{thermal}$ was the absorbance of LEO after heating, while A_c was the absorbance of LEO control sample without temperature treatment.

The pH values of LEO, LEO/β-CD and LEO/K-βCD-MOFs solutions were adjusted by phosphate buffer to 3, 7 and 10, respectively. The three solutions were prepared as mentioned above. The adjusted solutions were stored in the dark at 25°C for 10 days. The absorbances of the solutions were measured at 233 nm every 24 h to study the change of LEO amounts. The preservation rates of LEO, LEO/β-CD and LEO/K-βCD-MOFs to the original LEO amounts were calculated similarly to the above equation mentioned in the thermal stability measurement.

Intracellular antioxidant activity

No mycoplasma contaminations of HeLa cells were guaranteed by using Young *et al*.'s [2010] mycoplasma testing method. The cell viability and intracellular antioxidant activity were measured according to the previous method described in our earlier works [Wang *et al*., 2019; Yang *et al*., 2013] with some modifications. HeLa cells were transferred into a 96-well plate at a density of 1×10^5 per well in 100 μL of growth medium. The growth medium was pre-prepared by mixing 45 mL of DMEM, 5 mL of FBS and 500 μL of double-antibody. Cells were incubated with 5% CO_2 for 24 h at 37°C. After removal of the medium, the cells were washed by a phosphate buffer solution (PBS) (pH=7.4), and then treated with 100 μL of different concentrations (0.001–2 μL/mL, LEO equivalence) of LEO or included LEO (LEO/β-CD and LEO/K-βCD-MOFs) for another 24 h. For operation convenience in the cell experiments, the essential oil samples were measured in volume concentration *i.e*., "μL/mL". The mass of LEO can be calculated by its volume multiplied by its density (0.85 g/mL). The actual concentrations of LEO/β-CD and LEO/K-βCD-MOFs in all experiments were ten-fold of LEO, as the mass optimum core/wall ratio was 1:10. However, to enable further comparisons, the concentrations of these three were all expressed in "μL/mL, LEO equivalence". Different concentrations of the LEO were dissolved in different concentrations of DMSO, *i.e*., 0.001, 0.01 and 0.1 μL/mL in 0% DMSO, 0.2 μL/mL in 2% DMSO, 0.4 μL/mL in 4% DMSO, 0.5 μL/mL in 5% DMSO, 1 μL/mL in 10% DMSO, 2 μL/mL in 20% DMSO, respectively. For LEO/β-CD, 0.001–1 μL/mL were dissolved in 0% DMSO, and 2 μL/mL was dissolved in 2% DMSO. LEO/K-βCD-MOFs were directly dissolved in the medium. Therefore, the corresponding concentrations of DMSO were used as solvent controls for the cell viability and intracellular antioxidant activity experiments. The control was added with the growth medium instead of the samples. After PBS washing again, 5 mg/mL of MTT was added for another 4-h incubation. Subsequently, 150 μL of DMSO was added to dissolve the crystals, and the absorbance was then measured at 570 nm.

In the intracellular antioxidant activity experiment, HeLa cells were transferred and incubated according to the same procedures in cell viability assay. An aliquot of 100 μL of 20 μM DCFH-DA was mixed with 100 μL of 0.1 μL/mL of the LEO or included LEO (LEO/β-CD and LEO/K-βCD-MOFs) solution for 1 h. After the wells were washed by PBS (pH=7.4), 100 μL of 200 μM H_2O_2 was added into each well. The fluorescence intensities were measured every 5 min during the 1-h incubation time, and the integral area of the relative fluorescence intensity was calculated and marked as ∫SA. The excitation and emission wavelengths were 525 and 488 nm, respectively. When the growth medium was added instead of the samples, the integral area of the relative fluorescence intensity was ∫CA. The blank was prepared without both the samples and H_2O_2 solution. The intracellular antioxidant activity (CAA) was calculated according to the following equation:

$$\text{CAA (\%)} = 100 - (\int SA/\int CA) \times 100$$

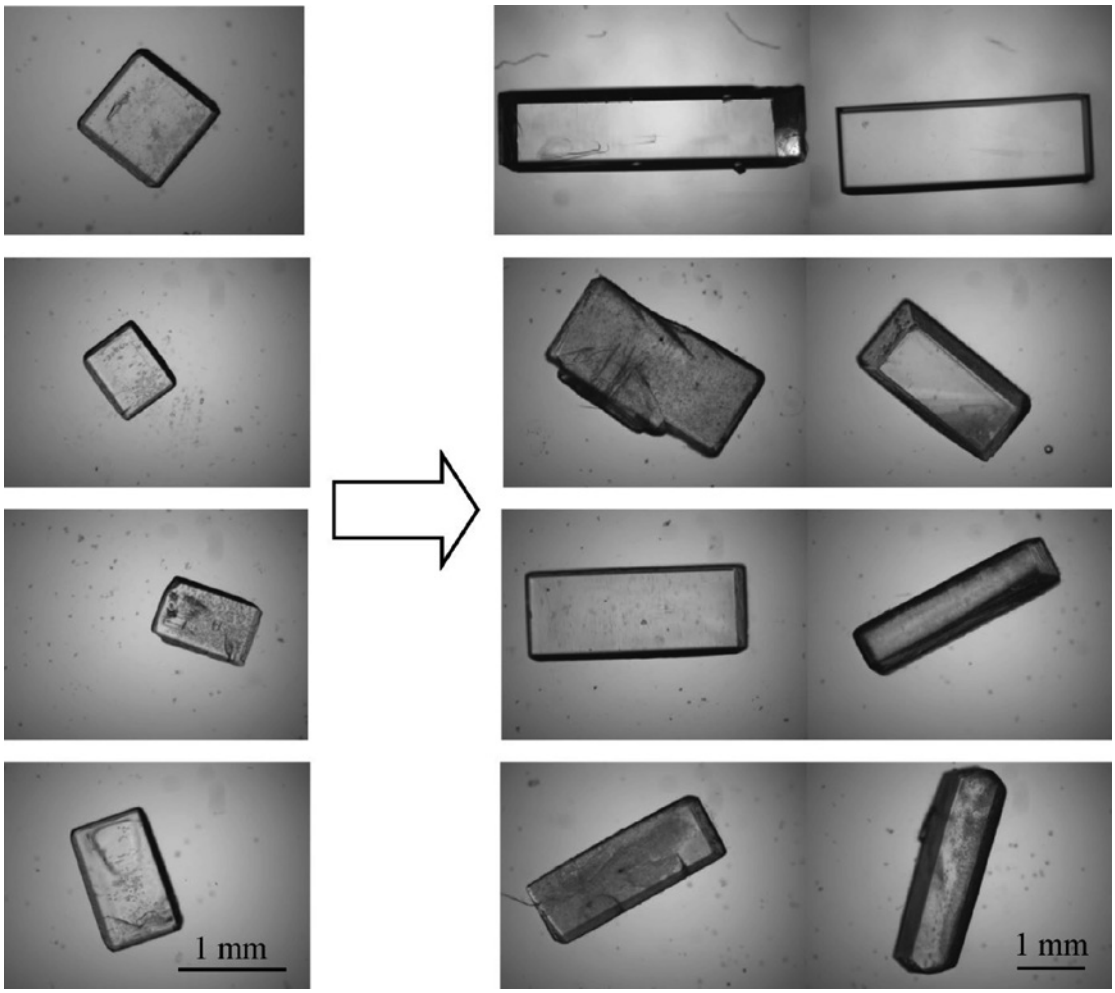

FIGURE 1. Optical microscope pictures of purified β-CD and metal-organic frameworks based on β-cyclodextrin and potassium cation (K-βCD-MOFs, magnification is 40 times).

Fourier transform infrared spectroscopy (FTIR)

The LEO, LEO/β-CD and LEO/K-βCD-MOFs inclusion, as well as the physical mixture of LEO and K-βCD--MOFs were analyzed by FTIR, which was conducted by KBr direct compression method, *i.e.*, 1 mg of the sample was ground together with KBr (about 100 mg) into a fine powder, and compressed into the holder using a compression gauge. The liquid LEO was given a drop on the surface of KBr. The inclusion complexes were cleaned with petroleum ether 3 times to ensure no presence of LEO on the surface. The spectra were scanned within the wavelength 4,000 to 450 cm^{-1}. The number of scans and spectral resolution were 5 scans and 4 cm^{-1}, respectively.

Scanning electron microscope (SEM)

Besides the microscope observations, the morphological characterization of K-βCD-MOFs and LEO/K-βCD-MOFs was further carried out by SEM analysis. Material particles were fixed on the silicon wafer and sprayed with a 100 nm-thick gold. The microstructures of the samples, such as the shapes and surface characteristics, were then observed and photographed using the SEM.

Statistical analyses

The inclusion rates of LEO to β-CD or K-βCD-MOFs in the inclusion complexes were compared among the different mass core/wall ratios by one-way ANOVA, followed by a post--hoc multiple comparison (Tukey's Test). One-way ANOVA was also performed to compare the differences among LEO, LEO/β-CD and LEO/K-βCD-MOFs at the same concentration (concentrations of the two including LEO were expressed as LEO equivalence) in thermal and pH stabilities, cell viabilities, as well as CAA. All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 16.0. Three replicates with mean values and standard deviations were carried out for all statistical analyses.

RESULTS AND DISCUSSION

Formation of K-βCD-MOFs

The K-βCD-MOFs were produced from the purified β-CD by the methanol diffusion method. The morphology features of their crystal structures were compared using an optical microscope (×40) and shown in Figure 1. They were both relatively regular and transparent with a neat appear-

ance. The β-CD showed shorter rectangular shape, while the K-βCD-MOFs were larger and longer.

Formation of LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes

Based on the formation of the wall material K-βCD--MOFs, LEO was microencapsulated as the core to produce inclusion complexes in different mass core/wall ratios. The inclusion rates of LEO/K-βCD-MOFs were shown in Figure 2. The inclusion rate increased significantly with the increase of the ratio, *i.e.*, with the increasing mass of wall material (K-βCD-MOFs), the inclusion rate of LEO to the wall significantly increased. The inclusion rate reached up to 93.45% with the core/wall ratio of 1:10, while no significant changes were observed for the rate of 95.67% with ratio of 1:12. Therefore, to save the resource and energy, the mass core/wall ratio of 1:10 was used for the future formation of LEO/K-βCD-MOFs complexes. In addition, the inclusion rates of LEO to K-βCD--MOFs and β-CD were also compared with the ratio of 1:10. The latter was only 78.42%, which was significantly lower than the rate of LEO/K-βCD-MOFs with the same ratio.

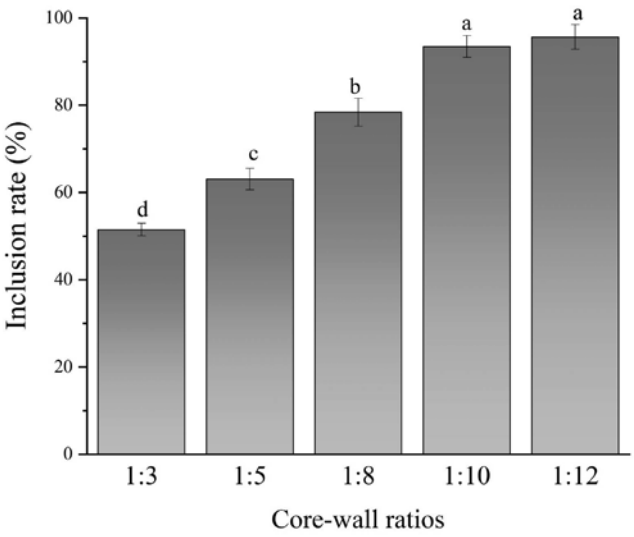

FIGURE 2. The inclusion rate of lavender essential oil (LEO) to metal-organic frameworks based on β-cyclodextrin and potassium cation (K-βCD-MOFs) under different core/wall ratios.

Bars with different lower-case letters show significant differences of inclusion rate among different core/wall ratios at p≤0.05 according to one-way ANOVA test. Data are reported as the mean ± SD of three replicates.

Properties of LEO, LEO/β-CD and LEO/K-βCD-MOFs

Thermal stability

The changes of LEO, LEO/β-CD and LEO/K-βCD--MOFs inclusion complexes under heat treatment at 90°C were studied during 10-day period with 1-day interval. After 24-h heat treatment, the preservation rate of LEO decreased to 53.27%, while the rate of LEO in the LEO/K-βCD-MOFs complex still remained at 90.13% (Figure 3A). As the heating time went on, the preservation rate of LEO and its inclusion complexes kept a gradually decreasing trend, with LEO's rate decreasing more rapidly and significantly than those of the complexes (Figure 3A). After 10 days, the preservation rate of LEO/K-βCD-MOFs and LEO/β-CD remained at 66.88 and 31.50%, respectively, while the rate of LEO was only 18.00% in the same environment (Figure 3A). The thermal stability of LEO could be significantly improved by embedding with β-CD and K-βCD-MOFs, especially the latter. These phenomena suggested that K-βCD-MOFs could provide better pro-

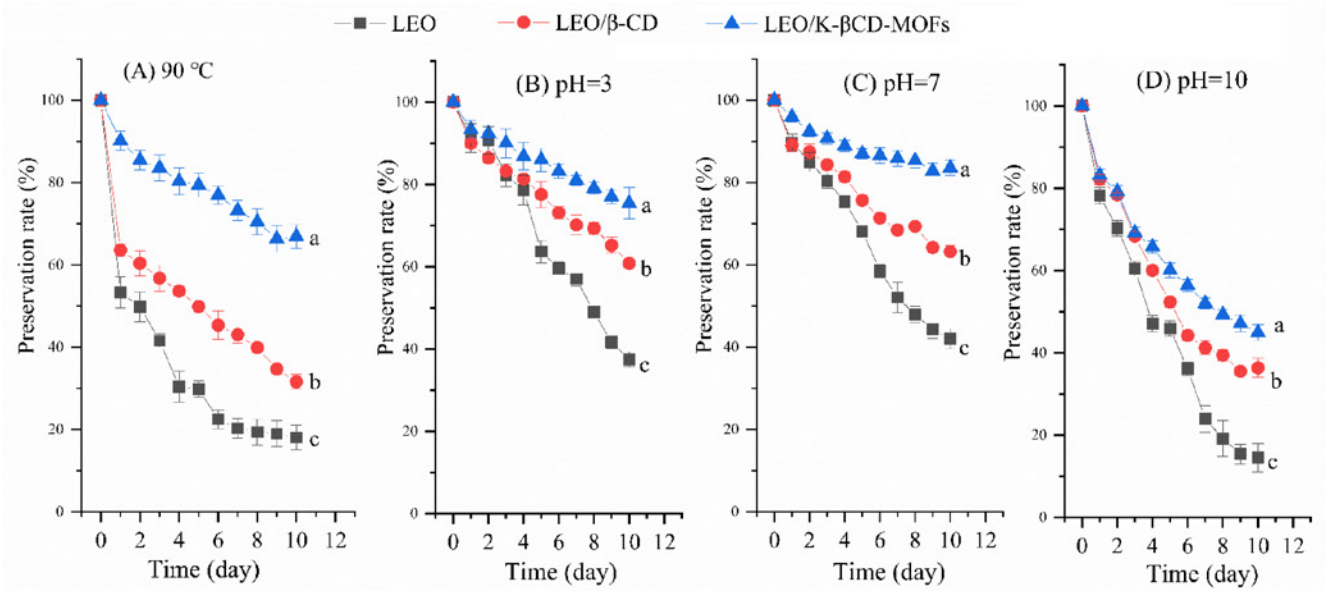

FIGURE 3. Thermal- (A) and pH-stabilities (B, C and D) of lavender essential oil (LEO), LEO/β-cyclodextrins (LEO/β-CD) and LEO/ metal-organic frameworks based on β-cyclodextrin and potassium cation (LEO/K-βCD-MOFs) inclusion complexes.

The LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes with core/wall ratio of 1:10 were used. Lines with different lower-case letters show significant differences of preservation rate at day 10 among LEO, LEO/β-CD and LEO/K-βCD-MOFs under the same conditions at p≤0.05 according to one-way ANOVA test. Data are reported as the mean ± SD of three replicates.

tection for LEO than β-CD against evaporation/degradation after LEOs were embedded. This could be explained by the improved thermal stability of both LEO and β-CD rendered by K-βCD-MOFs. Han *et al*. [2018] reported that the structures of CDs were modified, and the CD-MOFs were more thermally stable in an aqueous medium or in the physiological environment, as well as showed preferable solubility in water. Lv *et al*. [2017] reported that sucralose degraded very fast at 90°C for only one hour with about 14% left, while the stability of sucralose was dramatically enhanced by CD-MOFs, with only 13.7% decomposition under the same heat environment within 24 h. Furthermore, for future and better applications of LEO, the thermal stabilities of the inclusion complexes under many other thermal conditions and different working temperature (*e.g*., 50, 75, 100 and 121°C) should be studied.

pH stability

The results of the influence of different pH values (3, 7 and 10) on the stabilities of LEO, LEO/β-CD and LEO/β-CD-MOFs were obtained during 10-day period, and the variation trends of the preservation rate were shown in Figure 3B, C, D. Under acidic and neutral conditions, the stabilities of LEO and its inclusion complexes were better than under alkali condition, and the acid resistance of LEO/β-CD and LEO/β-CD-MOFs was much stronger than that of LEO itself (Figure. 3B, C). In strong alkali environment, the stabilities of LEO and LEO inclusion complexes decreased sharply. However, the stabilities of these complexes were still stronger than that of non-included LEO (Figure 3D). Therefore, in general, LEO/β-CD and LEO/β-CD-MOFs exhibited better acid-alkali resistance stabilities than the original oil, with better applicability under neutral and acidic conditions. This result can be attributed to the fact that the essential oils are always stable in weak acidic to neutral environment and are often applied in acidic conditions [Bensouda *et al*., 2019; Wang *et al*., 2009].

Intracellular antioxidant activities

Reduction reaction of MTT occurs with the presence of succinodehydrogenase in living cells, and MTT changes from yellow tetrazolium salt to purple formazan. Therefore, the absorbance at the maximum absorption wavelength of 570 nm of the purple product could indirectly reflect the amount of the living cells [Adach *et al*., 2016]. The effects of the different concentrations of LEO, LEO/β-CD and LEO/K-βCD-MOFs on the cell viabilities of HeLa cells were analyzed (Figure 4). Within the concentration range from 0.001 to 0.2 μL/mL, no significant difference was found among LEO, LEO/β-CD and LEO/K-βCD-MOFs. Both LEO and its inclusion complexes did not pose negative effects on the HeLa cell viabilities with concentrations lower than 0.2 μL/mL. However, the HeLa cell viabilities decreased with the concentration higher than 0.4 μL/mL. The inhibitory effects on cell viabilities of LEO/K-βCD-MOFs were significantly higher than those of LEO at concentration 0.4, 0.5 and 1 μL/mL, and also significantly higher than LEO/β-CD at 0.5 μL/mL. All the samples showed similar inhibitions on HeLa cells with the highest concentration at 2 μL/mL. The growth and viabilities of HeLa cells were inhibited, indicating the lavender essential oil could play an anticancer role with the concentrations reaching up to 0.4 μL/mL. Previous studies showed potential anticancer and antiproliferative activities of LEO by the induction of apoptosis and necrosis of cancer cells [Gezici, 2018]. LEO also exhibited on antitumor effects on the human prostate cancer, and its antitumor effect was associated with cell proliferation inhibition and apoptosis induction in xenograft tumors [Zhao *et al*., 2017]. Besides, the components of LEO also showed anticancer and antitumor effects. For example, one constituent of lavender oil – perillyl alcohol – has recently been identified as a potential anticancer agent, which may be useful in both treatment and prevention [Liston *et al*., 2003; Samaila *et al*., 2004]. Linalool and linalyl acetate also showed strong inhibitory effects on human prostate cancer PC-3 and DU145 cells [Zhao *et al*., 2017]. Usta *et al*. [2009] reported that linalool could decrease the viability of HepG20 to 50% and 0% by concentrations of 4 μM and 2 μM, respectively. Differences in the inhibitory activity of LEO and its microcapsules were in the order LEO/β-CD-MOFs > LEO/β-CD > LEO, and could be easily ascribed to the higher solubility of β-CD-MOFs than β-CD [Han *et al*., 2018], as well as the higher solubility of the complexes than LEO itself. All these related to the potential applications of LEO as potential anticancer/antitumor agents deserve further researches. Besides the anticancer role of LEO in killing HeLa cells, in the present study we focused on whether the intracellular antioxidant activities of LEO could be improved by microencapsulation by K-βCD-MOFs.

Although the chemical methods used to evaluate antioxidant activities *in vitro* are easy, convenient and inexpensive, they could reflect neither the antioxidant activities of the anti-

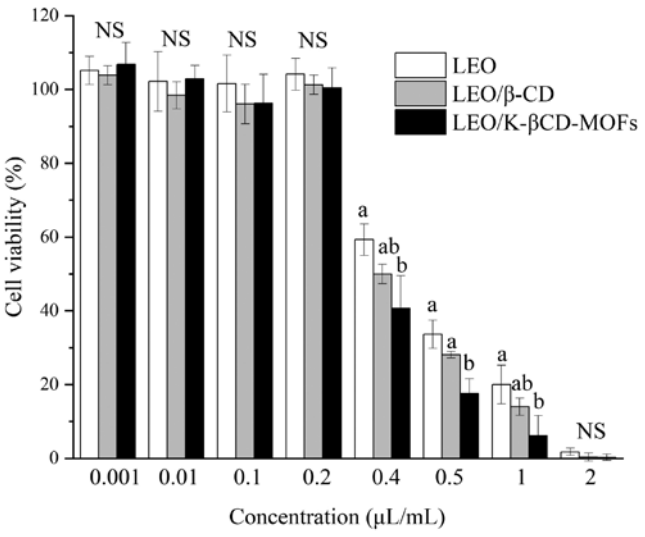

FIGURE 4. Effects of lavender essential oil (LEO), LEO/β-cyclodextrins (LEO/β-CD) and LEO/ metal-organic frameworks based on β-cyclodextrin and potassium cation (LEO/K-βCD-MOFs) inclusion complexes on cell viability in HeLa cells.

The LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes with core/wall ratio of 1:10 were used. Bars with different lower-case letters show significant differences among the effects of LEO, LEO/β-CD and LEO/K-βCD-MOFs on cell viability at $p \leq 0.05$ according to one-way ANOVA test. NS: Not significant difference. Data are reported as the mean ± SD of three replicates.

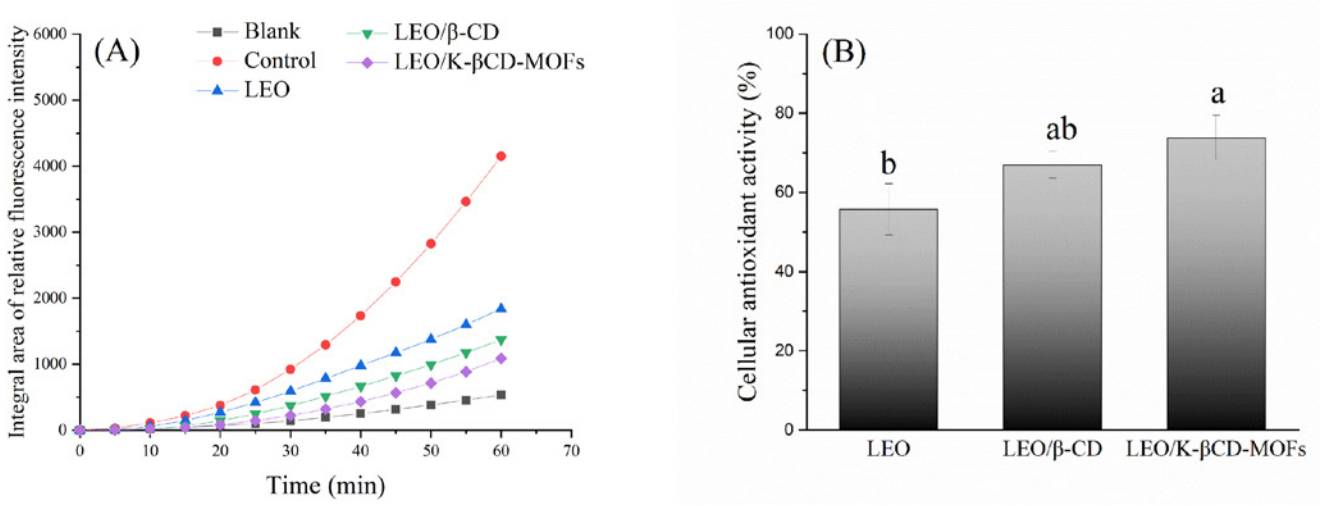

FIGURE 5. The integral areas of relative fluorescence intensity (A) and the intracellular antioxidant activity (CAA) of lavender essential oil (LEO), LEO/β-cyclodextrins (LEO/β-CD) and LEO/ metal-organic frameworks based on β-cyclodextrin and potassium cation (LEO/K-βCD-MOFs) (B) in HeLa cells.

The LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes with core/wall ratio of 1:10 were used. Bars with lower-case letters show significant differences among LEO, LEO/β-CD and LEO/K-βCD-MOFs at $p \leq 0.05$ according to one-way ANOVA. Data are reported as the mean ± SD of three replicates.

oxidants *in vivo* nor the real physiological environment of the human body. On the other hand, animal models or even human experiments are relatively expensive and time-consuming. Cell model could predict the antioxidant capacities of the antioxidants in the human body more accurately than chemical methods. Therefore, the cell model method is a good choice to simulate the internal physiological environment of the human body, study the intracellular antioxidant activities, and provide an economical and rapid method for better antioxidant research. Consequently, HeLa cells were chosen to simulate an *in vivo* environment to investigate the intracellular antioxidant activities of the LEO and its inclusion complexes with the concentration of 0.1 μL/mL, which was within the non-cytotoxic range (<0.2 μL/mL). In the living cells, non-fluorescent DCFH-DA could be hydrolyzed by esterases with the production of 2',7'-dichlorodihydrofluorescein (DCFH) [Labieniec & Gabryelak, 2007]. The non-fluorescent DCFH are then oxidized to fluorescent product 2',7'-dichlorofluorescein (DCF) by reactive oxygen species (ROS), which could be induced by H_2O_2. The fluorescent intensity of DCF correlates with the cellular ROS content, and this intensity decreases with the presence of antioxidants due to their ability to scavenge free radicals [Qian *et al*., 2012; Xu & Chang, 2012].

Figure 5A shows the integral areas of relative fluorescence intensity of HeLa cells (control) and HeLa cells with LEO and microcapsules of LEO/β-CD and LEO/ K-βCD-MOFs. After 1-h H_2O_2 stimulation, the intensity in the control was about 8 times as that of the blank without H_2O_2 stimulation. The relative fluorescence intensities of cells with LEO, LEO/β-CD and LEO/K-βCD-MOFs were all significantly lower than the control, indicating that both LEO and its inclusion complexes showed significant intracellular ROS scavenging activity in HeLa cells. The intracellular antioxidant activity (CAA) of LEO and its inclusion complexes were also shown in Figure 5B. LEO/K-βCD-MOFs showed significantly higher CAA than LEO. The lavender essential oil has been reported with high contents of oxygenated monoterpenes (31.53%) and monoterpene hydrocarbons (8.03%) [Dong *et al.*, 2020]. For example, linalool – which is the major oxygenated monoterpene of lavender – accounts for 19.71% of its essential oil [Dong *et al*., 2020]. The essential oils rich in monoterpenes and oxygenated monoterpenes generally show a high antioxidative potential [Deba *et al*., 2008; Tepe *et al*., 2004]. Monoterpenes are very active due to the presence of a double bond between two carbon atoms (C=C), and thus they could act as free radical scavengers [Mercier *et al*., 2009]. Therefore, lavender essential oil played a role in scavenging intracellular ROS, leading to the high CAA of LEO and its microencapsulated products.

The higher scavenging ability of LEO/K-βCD-MOFs is due to the higher solubility and availability in the cells compared to LEO and LEO/β-CD, which is in agreement with the reported higher solubility of K-βCD-MOFs than β-CD [Han *et al*., 2018]. Another possible reason might be the different microencapsulation capabilities of K-βCD-MOFs for different guest molecules. Yuan *et al*. [2019] reported that the microencapsulation by HPCD could change the relative contents of the major components of LEO dramatically. For example, contents of linalyl anthranilate and linalool changed from 42.18% to 24.05% and 34.94% to 60.87%, respectively, *i.e.*, they found that hydrocarbons and esters decreased, while conversely, carbonyl compounds and alcohols increased. Alcohols and carbonyl compounds were easier encapsulated by HPCD than other compounds of LEO. This phenomenon might be attributed to the properties of the hydrophobic cavity of HPCD [Yuan *et al*., 2019]. The similar explanation might apply to the present study. Therefore, future study should be carried on to analyze the LEO composition before and after microencapsulation by K-βCD-MOFs, and also to compare antioxidant activities among different compounds in LEO.

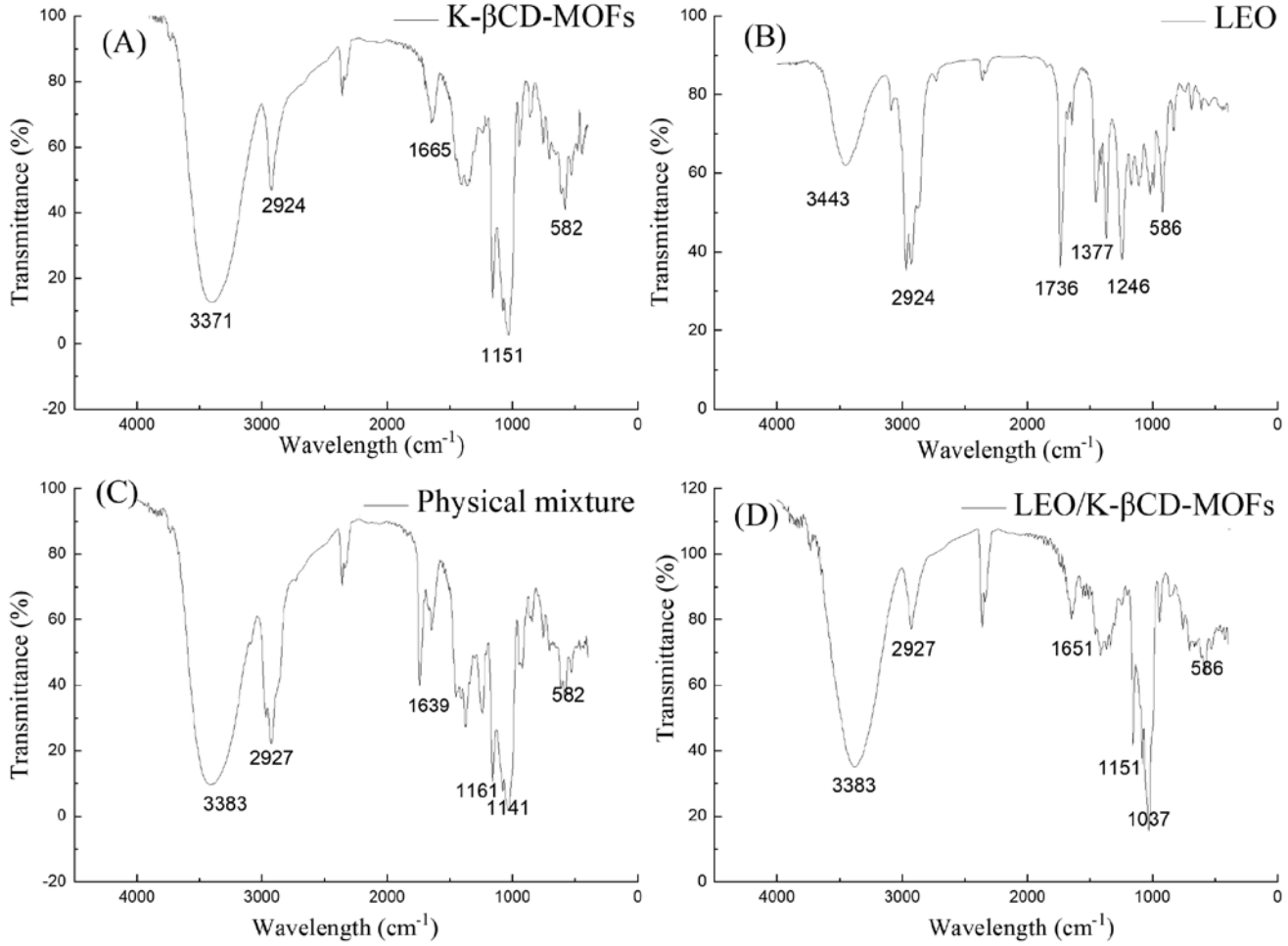

FIGURE 6. Infrared spectra of metal-organic frameworks based on β-cyclodextrin and potassium cation (K-βCD-MOFs) (A), lavender essential oil (LEO) (B), physical mixture of LEO and K-βCD-MOFs(C), and LEO/K-βCD-MOFs inclusion complex (D).

The LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes with core/wall ratio of 1:10 were used.

Wu *et al*. [2019] synthesized porous Zn@MOF and used it for microencapsulation of thymol for pathogen inhibition, and the T-Zn@MOF was proved to effectively inhibit *E. coli*. The present study was successful in synthesizing edible MOFs using β-CD and potassium ion. It is hopeful that K-βCD-MOFs could be applied to the applications of LEO with improved antioxidant activities in the food industries in the near future.

Characterization of LEO, LEO/β-CD and LEO/K-βCD--MOFs

Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared spectroscopy technology is usually helpful in detecting the interaction between cyclodextrin or MOFs as host and the guest molecules [Liu *et al*., 2019; Tu *et al*., 2020; Yuan *et al*., 2019]. The inclusion complexes could be confirmed by FTIR with the variation of position, intensity and even the shape of the peaks [Yuan *et al*., 2019]. Yuan *et al*. [2019] employed FTIR to provide the solid evidence for the formation of inclusion compounds of LEO by HPCD. Tu *et al*. [2020] also applied FTIR to support the formation of an inclusion complex of Mxene/carbon nanohorn with K-βCD-MOFs. FTIR was also applied to demonstrate the formation of inclusion complex in the present study. The infrared spectra of the K-βCD-MOFs, LEO, the physical mixture of LEO and K-βCD-MOFs, as well as LEO/K-βCD--MOFs inclusion complex were shown in Figure 6. Because the inclusion complexes were cleaned with petroleum ether 3 times, it could be considered that no LEO existed on the surface of the inclusion complex. If the absorption peak of the functional group of LEO could be found in the infrared absorption spectrogram of the inclusion complexes, we can deduce that LEO has been encapsulated by K-βCD-MOFs.

The spectrum of K-βCD-MOFs (Figure 6A) showed the characteristic absorption peaks of β-CD, suggesting the synthetic K-βCD-MOFs based on β-CD still kept the cavity structure of β-CD. The absorption peak between 3300–3400 cm^{-1} belongs to –OH, and the characteristic peak of $–CH_2$ is at 2924 cm^{-1}. The peaks around 1151 cm^{-1} represent the C–O stretching vibration absorption peak of K-βCD-MOFs and the inner cavity of the inclusion complexes.

Figure 6B showed the LEO infrared spectrum with the characteristic absorption peak of C=O at 1651 cm^{-1}. The spec-

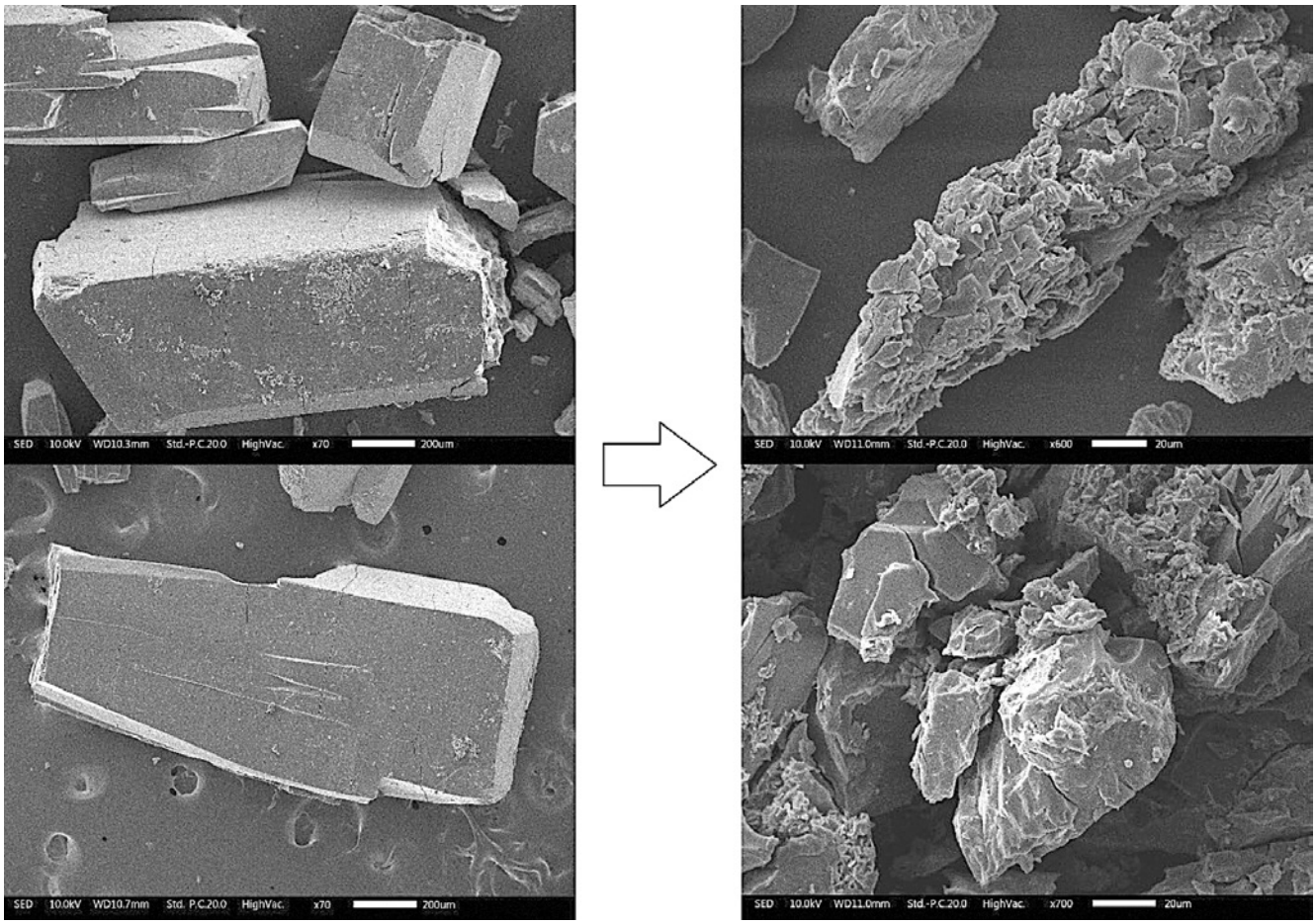

FIGURE 7. Scanning electron microscope pictures of metal-organic frameworks based on β-cyclodextrin and potassium cation (K-βCD-MOFs) and lavender essential oil (LEO)/K-βCD-MOFs inclusion complex.

The LEO/K-βCD-MOFs inclusion complexes with core/wall ratio of 1:10 were used. The magnifications are 70 and 700 times, respectively.

tra of the physical mixture and the inclusion complex were presented in Figure 6C, D, respectively. The main absorption peaks (–OH, C=O, C–O, $–CH_3$ and $–CH_2$) of the two substances were generally similar to those of K-βCD-MOFs. As the mass core/wall ratio was 1:10, the amounts of K-βCD-MOFs in both substances were much larger than that of LEO, resulting in the embedment of the LEO peaks in the bulk K-βCD-MOFs matrix. As the microencapsulation of LEO by K-βCD-MOFs was probably due to van der Waals force [Li *et al*., 2017], the absorption frequency and peak shape in the infrared spectra of the functional groups did not change after the microencapsulation. However, the peak intensities of the inclusion complexes were different from those of K-βCD-MOFs and LEO. This could be explained by the fact that LEO was bound and affected by the van der Waals force, which might cause the hinderance of its vibration and finally lead to the changes of intensity or even the disappearance of the peaks.

Finally, the spectrum of the inclusion compounds was obviously different from that of the physical mixture, especially regarding the C=O stretching vibration peak (around 1651 cm^{-1}) of LEO. This indicated that the LEO's environment in the inclusion compounds was different from that in the physical mixture. The weakening and the disappearance of some absorption peaks suggested that LEO has been successfully loaded as some groups have entered the cavity of CD. All the above infrared results proved the formation of LEO/K-βCD-MOFs.

Scanning electron microscope (SEM)

SEM has been commonly used to characterize the morphology and crystal structures of different MOFs [Wu *et al*., 2019]. To further characterize the inclusion complexes, the microstructures of K-βCD-MOFs and LEO/K-βCD-MOFs were observed by SEM. First, the representative SEM images of K-βCD-MOFs were shown in Figure 7. The crystalline K-βCD-MOFs has a long cuboid morphology, which is consistent with other researches [Liu *et al*., 2019; Tu *et al*., 2010]. The present study and the previous researches indicated K-βCD-MOFs were a rectangular solid with various sizes from millimeter to micron. For instance, Liu *et al*. [2019] showed the size of K-βCD-MOFs varied from 500 nm to 2 or several millimeters once examined by transmission electron microscope (TEM) and SEM. Figure 1 shows the size of K-βCD-MOFs was around several millimeters. Besides, by comparison with Figure 7, K-βCD-MOFs could be deemed smooth and long cubic crystals, while LEO/K-βCD-MOFs exhibited a relatively rough surface, which is a new image.

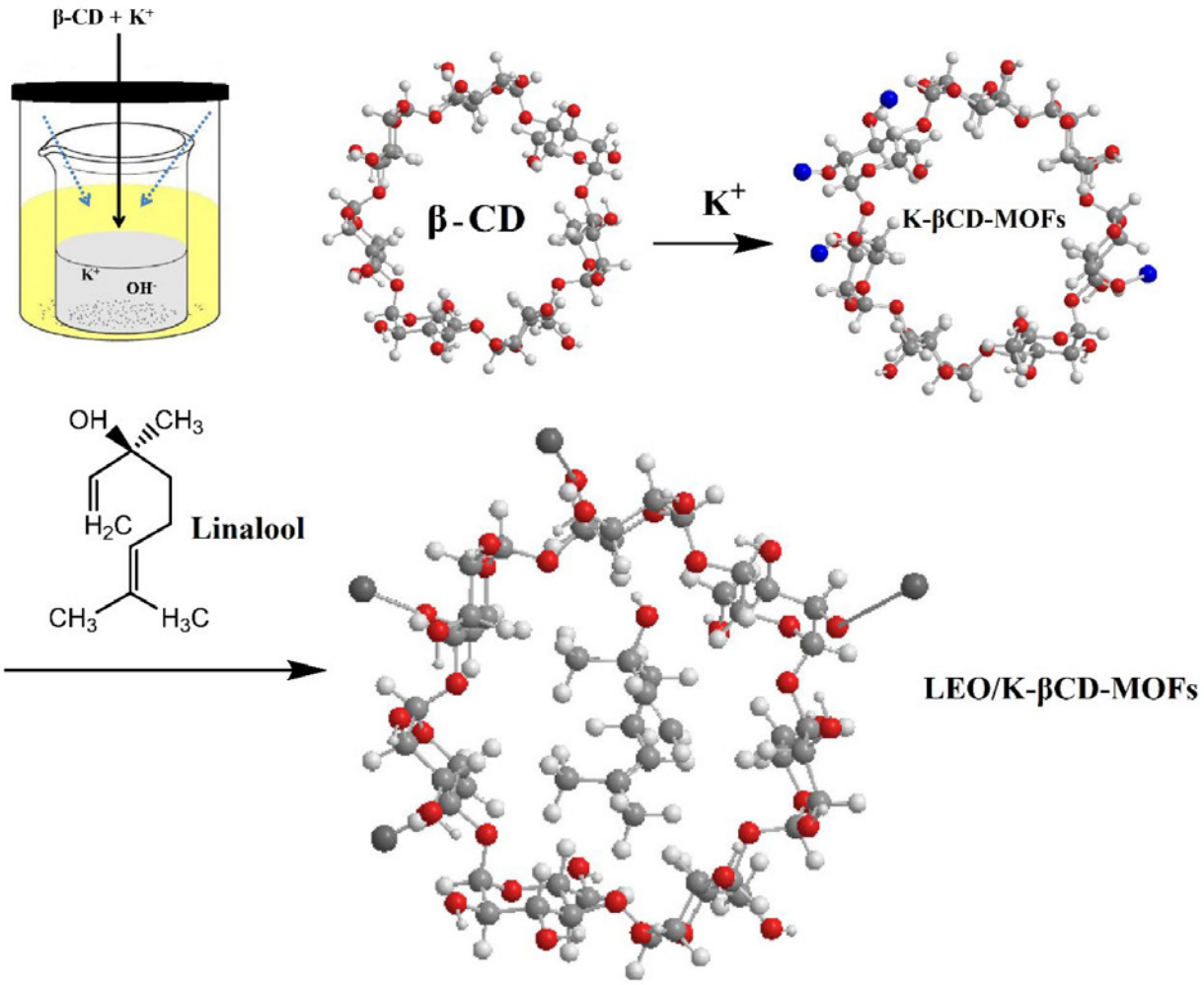

FIGURE 8. The scheme for the possible microencapsulation mechanism of lavender essential oil (LEO) by metal-organic frameworks based on β-cyclodextrin and potassium cation (K-βCD-MOFs) as the inclusion materials.

Linalool as the main component of LEO was taken as an example.

Possible mechanism of the production of LEO/K-βCD--MOFs microcapsule

The formation of K-βCD-MOFs was plotted in Figure 8. The potassium ion (K^+) is paired with seven oxygen atoms (O) of the four surrounding β-CD [Lu *et al*., 2015]. On the other hand, to further deduce the microencapsulation mechanism of the production of LEO/K-βCD-MOFs microcapsule, linalool is taken as an example as it is the main component of LEO [Dong *et al*., 2020]. The optimum mass core/wall ratio of linalool to K-βCD-MOFs at 1:10 is equaled to the molar ratio of about 1:1. The scheme for the possible microcapsule formation is shown in Figure 8. In detail, when linalool was encapsulated by K-βCD-MOFs, one K-βCD-MOFs molecules could encapsulate one linalool molecule (Figure 8). The specific reaction mechanism of the microencapsulation between LEO and K-βCD-MOFs deserves further research by single crystal X-ray diffraction, elemental analysis, and so forth.

CONCLUSIONS

Studies on the microencapsulation of lavender essential oil by β-CD and K-βCD-MOFs as well as analyses of the stability and antioxidant activity in HeLa cells of the LEO/β-CD and LEO/K-βCD-MOFs inclusion complexes were carried out. Our results showed the formation of a complex between LEO and CD-MOFs, as evidenced by FTIR and SEM. Furthermore, the microcapsules of LEO/K-βCD-MOFs were proved to be more thermally and acid-base stable than LEO, and its intracellular antioxidant effect was also significantly improved by encapsulation. The microencapsulation of LEO by K-βCD-MOFs not only did not inhibit the intracellular antioxidant activities of LEO, but also significantly improved their ROS scavenging abilities. All these indicated the application of lavender essential oil to food and medicine would be expanded, as the new functional K-βCD-MOFs materials prepared based on β-CD have great benefits to future. This information provides solid evidence and foundation for the future applications of LEO, and thus improves the production of tailor-made commodities by addressing different requirements of manufacturers of this herbal plant as medicines, herbal teas, food seasoning, cosmetics, and *etc*. The advantages of K-βCD-MOFs make them not only limited to chemical industry, but also applicable to new fields including foods, pharmaceuticals or health care products, *etc*.

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CONFLICT OF INTEREST

Authors declare no conflict of interest

ORCID IDs

J. Tan http://orcid.org/0000-0002-3515-7804
Y. Wang https://orcid.org/0000-0002-4933-9158

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Original article
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Synergistic Antimicrobial Effect of Raspberry (*Rubus idaeus* L., Rosaceae) Preparations and Probiotic Bacteria on Enteric Pathogens

Justyna Bauza-Kaszewska[1], Ewa Żary-Sikorska[1], Andrzej Gugolek[2], Anna Ligocka[1], Monika Kosmala[3], Elżbieta Karlińska[3], Bartosz Fotschki[4], Jerzy Juśkiewicz[4]*

[1]*Department of Microbiology and Food Technology, Faculty of Agriculture and Biotechnology, University of Science and Technology, Kaliskiego 7, 85–796 Bydgoszcz, Poland*
[2]*Department of Fur-bearing Animal Breeding and Game Management, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, Oczapowskiego 5, 10–718 Olsztyn, Poland*
[3]*Institute of Food Technology and Analysis, Łódź University of Technology, Stefanowskiego 4/10, 90–924 Łódź, Poland*
[4]*Department of Biological Functions of Food, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10–748 Olsztyn, Poland*

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Due to the increasing microbial tolerance to commonly used food preservatives, as well as growing consumer awareness of their adverse impact on human health, alternative methods of pathogens reduction in food are widely investigated. The aim of this research was to examine the antimicrobial activity of red raspberry pomace and seed preparations against enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Listeria monocytogenes*, and probiotic *Lactobacillus rhamnosus* strain. The combined action of LAB (lactic acid bacteria) and raspberry preparations on the pathogenic species was also evaluated. The results of our study showed no or weak antibacterial effect of raspberry preparations on the pathogenic bacteria tested. Regardless of preparation concentration (1.0 or 2.0 mg/mL), the bacteria number after 48-h incubation was usually higher than in the culture at the initial stage and varied from 10^5 to 10^7 cfu/mL. On the other hand, probiotic *Lactobacillus rhamnosus* strain caused a significant reduction in the enteric pathogen count after 24-h co-culture with LAB. The concentrations of both *Salmonella* serotypes were below the detection limit of the analytical methods applied. Moreover, the combined use of LAB and raspberry preparations resulted in the total elimination of *Salmonella* strains and the reduction in *L. monocytogenes* number from 10^5 to 10^2–10^4 cfu/mL after 24-h co-culture. EHEC revealed the highest resistance to the mixed culture effect. The synergic antimicrobial effect suggests the possibility of applying probiotic bacteria and berry preparations as natural antimicrobial agents in the food industry.

INTRODUCTION

Microbiological contamination of food is of great concern for the food industry. A considerable number of foodborne pathogens linked to serious illness and foodborne outbreaks (*e.g. Salmonella* spp., EHEC, *Listeria monocytogenes*, *Campylobacter jejuni*) have recently gained an intense attention of epidemiologists. There are various types of preservatives used to minimize the risk related to foodborne infections. However, due to the high prevalence of antibiotic-resistant microorganisms in food, increasing microbial tolerance to conventional food preservation methods is observed. Moreover, common consumer awareness of the adverse impact of chemicals on human health prompts a growing interest in the potential use of natural preservatives.

Natural preservatives of different origins; *e.g.* animal, plant, and microbiological; have been widely studied and their antimicrobial effectiveness remains undoubtful [Baptista *et al.*, 2020; Harich *et al.,* 2018; Salaheen *et al.,* 2016]. Their plant-derived representatives show direct antibacterial effects on the growth and metabolism of microorganisms or may indirectly modify their antibiotic resistance [Stefanovic *et al.,* 2012]. The application of essential oils and plant extracts as preservatives usually provides additional health benefits associated with the presence of specific active substances in their composition. The group of particular importance are phenolics, revealing high antibacterial and antifungal properties [Negi, 2012; Pandey *et al.,* 2017].

Fruits of red raspberry (*Rubus ideaus* L.), one of the most popular berry crops in the world, are commonly consumed both as fresh and as ingredients in processed food products. They are a rich source of various bioactive phenolic compounds, among which anthocyanins, ellagitannins, and conjugates of ellagic acid and quercetin are of great significance [Burton-Freeman *et al.,* 2016; Klewicka *et al.,* 2016; Stój *et al.,* 2006]. The total phenolic content of red raspberries varies from 200 to 500 mg/100 g of fresh matter; however,

* Corresponding Author: Tel.: +48 52 374 95 35; Fax: +48 52 374 95 05; E-mail: bauza@utp.edu.pl (J. Bauza-Kaszewska)

the content of anthocyanins varies between cultivars [Grumezescu, 2016; Simmonds & Preedy, 2015]. Among the anthocyanins found in red raspberries, cyanidin 3-*O*-sophoroside, cyanidin 3-*O*-glucoside, and pelargonidin 3-*O*-sophoroside are the most common and account for 79.8%, 14.2%, and 6.0% of the total anthocyanin content, respectively [Coultate, 2016; Shahidi & Alasalvar, 2016]. Sanguiin H-6 and lambertianin C are the major raspberry ellagitannins; however, their content and profile are genetically-determined and vary among different raspberry cultivars [Klewicka *et al.,* 2016; Vrhovsek *et al.,* 2009].

Fresh raspberries are the best source of bioactive compounds. Due to the short harvesting period, fresh raspberry fruits are mostly frozen or processed into concentrate, preserves, canned products, aseptic packs, and juice. During industrial juice production, a significant amount of the biologically-active ingredients remains in the fruit pomace and seeds [Schieber *et al.,* 2001]. These processed products still contain valuable substances and, therefore, increase the bio-potential of the by-products [Balasundram *et al.,* 2006]. The profiles of their phenolic compounds are similar to fresh fruits but the phenolic content of seeds and pomace is lower than that of fruit. Although anthocyanins in raspberries are quantitatively the most important polyphenols, they are accumulated mostly in the flesh and transferred to juice in the process of its production, which is why they are found in little quantities in the seeds and pomace. Contrary to the anthocyanins, ellagitannins accumulate mainly in the pomace. It should be emphasized that 80% of the dried raspberry pomace consists of the seeds. The fine grinding of the native pomace results in the damage to the seed coat, increasing the availability of the accumulated valuable seed compounds [Fotschki *et al.,* 2017].

Besides their beneficial effect on human health [Jimenez--Garcia *et al.,* 2013; Paredes-López *et al.,* 2010], phytochemicals in red raspberries show antimicrobial activity against many human pathogens [Nile &Park, 2014]. The complex phenolic polymers, such as ellagitannins, are particularly active in this respect [Heinonen, 2007; Małecka *et al.,* 2003]. The results of many research suggest various mechanisms related to the pathogen growth inhibition by berry phenolics, *e.g.* disruption of cytoplasmic membrane structure, permeabilization of the cell membrane, inhibition of extracellular enzyme secretion, or direct effect on microbial metabolism processes [Puupponen-Pimiä *et al.,* 2005a]. Since the colonization of intestinal epithelium is crucial for the food-borne bacterial infections, the anti-adherence activity of active substances in berries is also linked to their antimicrobial effect [Das *et al.,* 2017; Puupponen-Pimiä *et al.,* 2005b].

Among the natural substances used as natural food preservatives, lactic acid bacteria (LAB) metabolites demonstrate great antimicrobial potential [Adetoye *et al.,* 2018]. Organic acids, hydrogen peroxide, and bacteriocins inhibit the growth of intestinal and food-borne pathogens [Chen *et al.,* 2019].

Due to the antibacterial features of both, LAB and raspberry active substances (phenolic compounds), the putative synergic action of their combination, increasing the beneficial effect on human health, can be presumed. In this context, the co-application of these biocomponents in functional food (fermented milk products, juices) is an issue of a special interest.

The aim of this study was to investigate the antibacterial effects of dried preparations obtained from raspberry pomace, seed, and seedless pomace after industrial juice production against enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Listeria monocytogenes*, and probiotic *Lactobacillus rhamnosus* strain. The synergistic effect of LAB and raspberry preparations on the pathogenic species was also examined.

MATERIALS AND METHODS

Raspberry preparations

Native raspberry (*R. ideaus*) pomace (preparation P), seed preparation (preparation S; > 95% seed content), and raspberry seedless pomace (preparation W; <5% seed content) were supplied by Agro-Bio-Produkt Sp. z o.o. (Grodkowice, Poland). The native P pomace was a biomass residue after industrial juice production and drying in the SB-1.5 rotary drum dryer (AGROMECH Co., Rogozno Wlkp., Poland). The seed preparation was a commercial product intended for high-quality oil production. It was obtained by the producer with the aid of two layers of a mesh screen taking into account the average dimension (length, width, and thickness) of *R. ideaus* seeds. The W preparation was a residue obtained after the S preparation production. In order to standardize all three preparations, their samples were ground in a cryogenic environment (Freezer Mill 6870 SPEX, SamplePrep. Inc., Stanmore, UK) to obtain particles smaller than 0.65 mm. That process made it possible to get through the seed coat and to preserve the bioactive components on the preparations.

Proximate composition of the preparations

The official methods of the Association of Official Analytical Chemists [AOAC, 2007] method were used to determine the proximate analysis of the raspberry preparations: protein content, 920.152; dry m atter and ash content, 940.26; ether extract, 930.09; total dietary fiber (TDF) content, 985.29, and insoluble dietary fiber (IDF) content, 991.42. The soluble dietary fiber (SDF) content was estimated as the difference between TDF and IDF content. All analyses were performed in triplicate.

Phenolics content of the preparations

A three-step extraction procedure with acetone-water--formic acid (70:29.9:0.1, *v/v/v*) was applied to extract phenolics from preparations tested. First, 500 mg of ground material was vortexed with 4 mL of the solvent. After sonication for 15 min and centrifugation at 4,800 × g, the extract obtained was transferred into a flask. The vortexing was repeated twice with 3 mL of the solvent.

The content of ellagitannins was determined using a Smartline chromatograph (Knauer, Berlin, Germany) with a degasser (Manager 5000), two pumps (P1000), autosampler (3950), thermostat, and photodiode array detector (2800). Ellagitannins were separated on a Gemini C18 column: 250 × 4.6 mm, 5 μm, 110 Å (Phenomenex, Torrance, CA) by gradient elution with solvent A (0.05% [*v/v*] phosphoric acid--water) and solvent B (63:20:17 [*v/v/v*] acetonitrile-methanol-

-water with 0.05% phosphoric acid). The column temperature was set at 35°C, the flow rate was 1.25 mL/min, and the gradient program was as follows: 0–5 min, 5% (*v/v*) B; 5–30 min, 5–28% (*v/v*) B; 30–40 min, 28–73% (*v/v*) B; 40–45 min, 73% (*v/v*) B; 45–47 min, 73–5% (*v/v*) B; and 47–56 min, 5% (*v/v*) B. The injection volume was 20 μL.

ClarityChrom v. 3.0.5.505 software (Knauer, Berlin, Germany) was applied for data collection. The standards applied were ellagic acid, sanguiin H-6, lambertianin C, and bis-HHDP-glucose (Extrasynthese, Genay, France).

The excess phloroglucinol degradation method was applied to determine the content of procyanidins. A methanol solution (0.8 mL) with phloroglucinol (75 g/L) and ascorbic acid (15 g/L) were added to a 20 mg sample. To start the reaction, 0.4 mL of 0.2 M hydrochloric acid in methanol were added. The reaction proceeded at 50°C for 30 min and was stopped by adding 0.6 mL of a 40 mM sodium acetate solution in an ice bath. The samples were centrifuged at 3,600 × g for 5 min, diluted with a 40 mM sodium acetate solution, and analyzed using a Smartline chromatograph with a P2800 UV-Vis detector (both from Knauer, Berlin, Germany), an RF-10AXL fluorescence detector (FD) (Shimadzu, Tokyo, Japan), and a Gemini C18 column (110 Å, 5 μm, 250 × 4.60 mm) (Phenomenex, Torrance, USA). Phase A consisted of acetic acid and water (2.5:97.5, *v/v*), while phase B of acetonitrile and water (80:20, *v/v*). The applied gradient, with the flow rate of 1 mL/min at 25°C, was as follows: 0–10 min, 4–7% (*v/v*) B; 10–27 min, 7–30% (*v/v*) B; 27–29 min, 30–70% (*v/v*) B; 29–34 min, 70% (*v/v*) B; 34–35 min, 7–40% (*v/v*) B; and 35−40 min, 4% (*v/v*) B. Comparison of the retention times and UV-Vis spectra with those of standards: (−)-epicatechin, (+)-catechin, (−)-epicatechin−phloroglucinol adduct, and (+)-catechin−phloroglucinol adduct, was used for identification. The excitation and the emission wavelengths were 278 nm and 360 nm, respectively.

Microorganisms tested

The bacterial strains: *Salmonella* Typhimurium*, Salmonella* Enteritidis, EHEC, and *Listeria monocytogenes* from the collection of the Department of Microbiology and Food Technology, UTP University of Science and Technology in Bydgoszcz, Poland, were used in the study. *Lactobacillus rhamnosus* strain was obtained from a commercial probiotic preparation.

Antimicrobial activity of raspberry preparations

The liquid culture method was used to study the effect of P, W, and S raspberry preparations on the bacteria tested. The liquid growth medium LAPTg (10 mL) was inoculated with 100 μL of a bacterial overnight culture. The initial bacterial culture titer was 10^8 cfu/mL. Raspberry preparations were suspended in 10 mL of inoculated media to a final concentration of 1.0 or 2.0 mg/mL. Preparations doses were chosen according to Nohynek *et al.* [2006] and Puupponen-Pimiä *et al.* [2001] methodologies. The cultures were incubated at 37°C for 48 h. Bacterial culture samples were taken four times during an incubation time – immediately after inoculation (0) and after 6, 24, and 48 h. The samples were diluted by introducing 1 mL of the culture into test tubes containing sterile 0.9% NaCl solution (10^1–10^9). Next, 0.1 mL of each dilution was transferred and distributed on the medium surface, using the spread plate technique. The following selective growth media were used for bacteria isolation: EHEC – Endo Agar (Merck, 1.04044), *Salmonella* strains – BPL Agar (Merck, 1.10747), *L. monocytogenes* – ALOA (Merck, 1.00427), and *L. rhamnosus* – Rogosa Agar (Merck, 1.05413). The incubation of the tested microorganisms was conducted at 37°C for 24–48 h. Bacterial cultures with no raspberry preparations were used as controls. Each experiment was performed in triplicate.

Antimicrobial activity of probiotic *Lactobacillus* strain

The co-culture (mixed cultures, associated cultures) method was applied to estimate the interaction between *L. rhamnosus* and pathogenic strains. Co-cultures were obtained by adding 100 μL of a 24-h *L. rhamnosus* pure culture and 100 μL of an individual pathogen pure culture to 10 mL of the liquid growth medium LAPTg. The initial titer of both bacterial cultures was 10^8 cfu/mL. To examine the effect of LAB on the pathogens tested, the co-cultures were incubated at 37°C for 24 h. Culture samples were taken five times during the incubation time – immediately after inoculation (0) and after 15, 18, 21, and 24 h. The samples were diluted by introducing 1 mL of the culture into test tubes containing sterile 0.9% NaCl solution (10^1–10^9). Then, 0.1 mL of each dilution was plated out by spread plating on different selective solid media, allowing the growth of only one of the two microorganisms composing the mix. The cultures were incubated at 37°C for 24–48 h. Pure cultures of each bacteria were used as controls. The selective media and incubation conditions were similar to those described above. Each experiment was performed in triplicate.

Evaluation of the synergistic effect of raspberry preparations and *Lactobacillus* on the pathogens tested

Co-cultures of individual pathogenic bacterial strains and *L. rhamnosus*, enriched with raspberry preparations, were used to assess the combined action of LAB and raspberry compounds. The liquid growth medium LAPTg (10 mL) was inoculated with 100 μL of the *L. rhamnosus* overnight culture and 100 μL of the pathogen overnight culture. The initial titer of both bacterial cultures was 10^8 cfu/mL. Then, raspberry preparations were added to the inoculated media to a final concentration of 2.0 mg/mL. The co-cultures were incubated at 37°C for 24 h. Culture samples were taken five times during the incubation time – immediately after inoculation (0) and after 15, 18, 21, and 24 h. The samples were diluted by introducing 1 mL of the culture into test tubes containing sterile 0.9% NaCl solution (10^1–10^9). Then, 0.1 mL of each dilution was plated out by spread plating on the growth media as described above. The tested microorganisms were incubated at 37°C for 24–48 h. Pure cultures of each bacteria were used as controls. Each experiment was performed in triplicate.

Statistical analysis

Statistical analysis was performed using Statistica software (*TIBCO Software* Inc., Palo Alto, CA, USA). The results are presented as mean ± standard deviation (SD). Differences between control and experimental variants were analyzed by one-way ANOVA followed by the Dunnett's test ($p<0.05$).

RESULTS AND DISCUSSION

The chemical composition of raspberry preparations is summarized in Table 1. The content of total phenolics in raspberry preparations varied from 1665 mg/100 g in seed preparation (S) to 4253 mg/100 g in seedless pomace (W). The highest content of ellagitannins (4020 mg/100 g) was also noted in the seedless pomace preparation (W) (Table 1). Klimczak *et al*. [2011] demonstrated that most of the phenolics accumulate in the seedless fraction of the pomace; consequently, an increased concentration of these bioactive compounds was observed after the removal of seeds from the raspberry pulp.

All of the three preparations tested (W, S, P) showed no or low antimicrobial effect on the microorganisms tested, and bacterial species did not affect the raspberry inhibitory efficiency (Table 2). According to Nohynek *et al.* [2006], different bacterial species and strains demonstrate variable sensitivity to phenolic berry extracts. The results of their research showed a high efficiency of raspberry against *Helicobacter pylori* and *Bacillus cereus*. Growth inhibition of *Campylobacter jejuni* and *Candida albicans* was linked to the high content of ellagitannins in the composition of the tested extracts [Nohynek *et al.,* 2006]. In turn, Puupponen-Pimiä *et al.* [2001, 2005a] reported a low antimicrobial activity of raspberry against Gram-positive bacteria, including *L. monocytogenes.* On the other hand, raspberry extracts were strong inhibitors of Gram-negative intestinal bacteria, which might be the effect of different cell membrane structure. These findings were not confirmed by Velićanski *et al.* [2012], who reported a higher, compared to G-positive, resistance of G-negative bacteria, especially with *Escherichia coli* and *Salmonella* Typhimurium to raspberry pomace extracts in agar diffusion tests. Among all G-positive bacteria tested, *L. monocytogenes* showed the lowest sensitivity. Similarly, the study of Krstic *et al.* [2014] demonstrated the antibacterial activity of the raspberry ethanol extract and juice on Gram-positive bacteria. Rauha *et al.* [2000] observed that the inhibitory action of raspberry phenolics on bacteria tested was not limited to G-positive and G-negative species only. The raspberry active compounds inhibited the growth of *Bacillus subtilis* and *Micrococcus luteus* but had only limited effect on *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis.* The results of a study by Četojević-Simin *et al.* [2015] showed that Gram-positive and Gram-negative bacteria were similarly susceptible to raspberry pomace extracts, and that the growth inhibition of various bacterial species was neither strain- nor species-dependent.

Various internal and external factors might have caused the low antibacterial activity of the tested raspberry products in our study. The berry cultivar, as well as the procedure applied to obtain the plant preparations, influence the amount and composition of phenolic compounds and the antimicrobial action of a final product [Krisch *et al.,* 2008; Krstic *et al.,* 2014; Lima *et al.,* 2019]. The antibacterial efficiency of berry preparations is also due to their concentration applied in the experimental procedure. The doses used in our study were 1.0 or 2.0 mg/mL. Nohynek *et al.* [2006] observed 0.5 to 1.5 log reduction of *C. jejuni* number after 5-h incubation with lyophilized raspberry extracts in a 1.0 mg/mL concentration. A similar dose of raspberry extracts totally inhibited the growth of *S.* Typhimurium and *E. coli* CM 871 and decreased the number of *E. coli* strain 50 by 2 logs, compared to the control culture [Puupponen-Pimiä *et al.,* 2001]. Puupponen-Pimiä *et al.* [2005a] noted a strong inhibitory effect of raspberry extracts at a low concentration (1.0 mg/mL) at the initial phase of *Salmonella* strains cultivation, followed, however, by the increase in bacteria number after 12 and 24 h of incubation. Application of the 5.0 mg/mL dose resulted in the complete elimination of *S.* Typhimurium after 2-h incubation. In our study, the effect of raspberry preparations on pathogenic bacteria was dose-independent. The number of the microorganisms tested in the initial culture varied from 10^4 to 10^7 cfu/mL and, in the majority of experimental variants, increased after 6 and 24 h of incubation. During the next 24 h, a slight reduction was observed in bacteria culture density; however, the cell count was usually higher than in the culture at the initial stage (Table 2).

TABLE 1. Chemical composition of raspberry pomace and seed preparations.

Content	Native pomace (P)	Seedless pomace (W)	Seeds (S)
Dry matter (g/100 g)	95.4±0.0	95.3±0.0	96.5±0.2
Ash (g/100 g)	2.68±0.25	5.12±0.02	1.71±0.06
Protein (g/100 g)	11.4±0.0	18.9±0.1	10.5±0. 5
Ether extract (g/100 g)	12.2±0.1	6.00±0.01	14.1±0.2
TDF (g/100 g), including:	61.3±0.7	54.3±0.5	63.9±0.4
IDF (g/100 g)	59.2±0.7	51.4±0.5	62.7±0.4
SDF (g/100 g)	2.12±0.06	2.90±0.07	1.23±0.02
Total phenolics (mg/100 g), including:	2359±30	4253±23	1665±44
Ellagitannins	1949±18	4020±12	1211±34
bis-HHDP-glucoseisomer 1	36.5±4.0	32.7±2.2	34.6±3.2
bis-HHDP-glucoseisomer 2	41.8±2.1	3.40±0.30	38.9±2.4
Sanguiin H10 isomer 1	34.0±0.1	43.8±0.6	31.8±0.5
Lambertianin C without ellagic acid	44.9±0.9	84.8±0.5	29.1±1.4
SanguiinH10 isomer 2	0.0±0.0	65.0±1.0	31.1±1.0
Lambertianin C isomer 1	17.0±0.8	25.9±1.4	21.5±0.7
Lambertianin C isomer 2	56.4±0.0	75.7±1.0	41.9±0.0
Lambertianin C isomer 3	53.5±4.0	142±6	14.4±0.8
Lambertianin D	92.3±1.5	226±3	113±3
Lambertianin C	820±8	1998±0	375±7
Sanguiin-H6	752±5	1322±1	478±15
Ellagic acid	73.4±1.0	139±1	106±5
Procyanidins	397±12	229±11	439±9
Free catechins	13.1±0.1	3.40±0.04	14.9±0.5

TDF – total dietary fiber; IDF – insoluble dietary fiber; SDF – soluble dietary fiber.

TABLE 2. Effect of raspberry preparations on the bacteria count (cfu/mL).

Microorganism	Preparation dose and type		Incubation time (h)			
			0	6	24	48
EHEC	1 mg/mL	W	$3.20\times10^{6}\pm1.44\times10^{6}$	$4.00\times10^{8}\pm2.65\times10^{7}$	$2.80\times10^{8}\pm7.55\times10^{7}$	$7.67\times10^{7}\pm1.53\times10^{7*}$
		S	$3.00\times10^{6}\pm7.00\times10^{5}$	$3.47\times10^{8}\pm1.26\times10^{8}$	$4.43\times10^{8}\pm9.81\times10^{7}$	$5.53\times10^{7}\pm3.00\times10^{7}$
		P	$3.73\times10^{6}\pm9.29\times10^{5}$	$3.43\times10^{8}\pm5.77\times10^{7}$	$3.30\times10^{8}\pm1.15\times10^{8}$	$4.13\times10^{7}\pm1.69\times10^{7}$
	2 mg/mL	W	$4.00\times10^{6}\pm7.00\times10^{5*}$	$3.37\times10^{8}\pm2.08\times10^{7}$	$1.80\times10^{8}\pm7.21\times10^{7}$	$1.18\times10^{8}\pm3.05\times10^{6*}$
		S	$4.83\times10^{6}\pm1.25\times10^{6*}$	$5.13\times10^{8}\pm2.27\times10^{8}$	$2.63\times10^{8}\pm2.89\times10^{7}$	$9.03\times10^{7}\pm2.87\times10^{7*}$
		P	$6.40\times10^{6}\pm4.58\times10^{5*}$	$2.63\times10^{8}\pm5.77\times10^{6}$	$2.13\times10^{8}\pm7.64\times10^{7}$	$7.50\times10^{7}\pm1.51\times10^{7*}$
	Control		$1.90\times10^{6}\pm1.00\times10^{5}$	nd	nd	$1.50\times10^{7}\pm1.30\times10^{7}$
Listeria monocytogenes	1 mg/mL	W	$7.53\times10^{5}\pm1.74\times10^{5*}$	$6.83\times10^{6}\pm1.07\times10^{6}$	$7.93\times10^{5}\pm2.00\times10^{5}$	$4.43\times10^{7}\pm6.03\times10^{6*}$
		S	$7.27\times10^{5}\pm3.05\times10^{4*}$	$2.60\times10^{5}\pm3.46\times10^{4}$	$8.83\times10^{5}\pm2.75\times10^{5}$	$5.43\times10^{7}\pm2.08\times10^{6*}$
		P	$6.67\times10^{5}\pm5.77\times10^{4}$	$4.10\times10^{5}\pm1.73\times10^{4}$	$3.10\times10^{6}\pm1.23\times10^{6}$	$1.00\times10^{6}\pm3.61\times10^{5*}$
	2 mg/mL	W	$5.03\times10^{5}\pm9.50\times10^{4}$	$8.60\times10^{6}\pm3.49\times10^{6}$	$1.12\times10^{8}\pm1.50\times10^{7}$	$1.10\times10^{7}\pm2.65\times10^{6}$
		S	$5.20\times10^{5}\pm3.47\times10^{4}$	$7.53\times10^{6}\pm7.23\times10^{5}$	$1.68\times10^{8}\pm6.43\times10^{6}$	$2.57\times10^{5}\pm9.29\times10^{4*}$
		P	$5.53\times10^{5}\pm4.51\times10^{4}$	$6.40\times10^{6}\pm9.64\times10^{5}$	$1.36\times10^{8}\pm4.04\times10^{6}$	$3.17\times10^{7}\pm1.22\times10^{7*}$
	Control		$4.36\times10^{5}\pm1.21\times10^{5}$	nd	nd	$7.83\times10^{6}\pm3.01\times10^{6}$
Salmonella Enteritidis	1 mg/mL	W	$5.95\times10^{6}\pm1.40\times10^{6}$	$9.30\times10^{8}\pm2.33\times10^{8}$	$9.80\times10^{8}\pm2.44\times10^{8}$	$1.10\times10^{7}\pm7.60\times10^{6}$
		S	$5.90\times10^{6}\pm6.00\times10^{5}$	$8.37\times10^{8}\pm1.10\times10^{8}$	$6.83\times10^{8}\pm5.23\times10^{8}$	$2.17\times10^{6}\pm5.69\times10^{5*}$
		P	$6.02\times10^{6}\pm2.40\times10^{6}$	$7.17\times10^{8}\pm1.95\times10^{8}$	$1.33\times10^{9}\pm5.20\times10^{7}$	$9.83\times10^{6}\pm8.54\times10^{6}$
	2 mg/mL	W	$4.30\times10^{6}\pm1.04\times10^{6}$	$5.10\times10^{8}\pm7.94\times10^{7}$	$7.87\times10^{8}\pm6.35\times10^{7}$	$3.56\times10^{7}\pm2.70\times10^{7}$
		S	$4.00\times10^{6}\pm6.08\times10^{5}$	$4.47\times10^{8}\pm4.04\times10^{7}$	$8.43\times10^{8}\pm1.11\times10^{8}$	$1.61\times10^{7}\pm9.00\times10^{6}$
		P	$4.87\times10^{6}\pm1.08\times10^{6}$	$8.90\times10^{8}\pm2.01\times10^{8}$	$7.23\times10^{8}\pm5.51\times10^{7}$	$2.57\times10^{6}\pm2.62\times10^{6*}$
	Control		$7.53\times10^{6}\pm3.02\times10^{6}$	nd	nd	$4.00\times10^{7}\pm2.40\times10^{7}$
Salmonella Typhimurium	1 mg/mL	W	$4.70\times10^{6}\pm7.94\times10^{5}$	$4.50\times10^{8}\pm2.21\times10^{8}$	$9.90\times10^{8}\pm2.65\times10^{7}$	$3.80\times10^{8}\pm2.65\times10^{7}$
		S	$3.90\times10^{6}\pm1.31\times10^{6}$	$6.60\times10^{8}\pm2.13\times10^{8}$	$1.35\times10^{9}\pm3.23\times10^{8}$	$3.20\times10^{8}\pm2.00\times10^{7}$
		P	$4.57\times10^{6}\pm1.82\times10^{6}$	$5.27\times10^{8}\pm1.03\times10^{8}$	$7.57\times10^{8}\pm1.60\times10^{8}$	$2.27\times10^{8}\pm6.43\times10^{7*}$
	2 mg/mL	W	$6.30\times10^{6}\pm6.08\times10^{5}$	$3.00\times10^{8}\pm5.03\times10^{7}$	$8.00\times10^{8}\pm1.30\times10^{8}$	$1.01\times10^{8}\pm9.54\times10^{6*}$
		S	$5.50\times10^{6}\pm6.08\times10^{5}$	$3.23\times10^{8}\pm4.04\times10^{7}$	$8.03\times10^{8}\pm8.74\times10^{7}$	$3.63\times10^{8}\pm1.14\times10^{8}$
		P	$6.50\times10^{6}\pm1.80\times10^{6}$	$3.40\times10^{8}\pm5.20\times10^{7}$	$6.33\times10^{8}\pm3.51\times10^{7}$	$2.93\times10^{8}\pm2.09\times10^{7}$
	Control		$6.90\times10^{6}\pm1.10\times10^{6}$	nd	nd	$3.80\times10^{8}\pm7.21\times10^{7}$
Lactobacillus rhamnosus	1 mg/mL	W	$1.10\times10^{7}\pm2.00\times10^{6}$	$1.27\times10^{8}\pm6.93\times10^{7}$	$6.85\times10^{8}\pm5.07\times10^{7}$	$7.33\times10^{7}\pm2.52\times10^{7}$
		S	$9.00\times10^{6}\pm2.65\times10^{6}$	$9.20\times10^{7}\pm3.70\times10^{7}$	$6.99\times10^{8}\pm5.33\times10^{7}$	$8.33\times10^{7}\pm1.53\times10^{7}$
		P	$1.13\times10^{7}\pm3.21\times10^{6}$	$6.37\times10^{7}\pm3.51\times10^{7}$	$6.60\times10^{8}\pm4.00\times10^{7}$	$1.96\times10^{8}\pm4.51\times10^{7}$
	2 mg/mL	W	$1.27\times10^{7}\pm8.50\times10^{5*}$	$1.20\times10^{8}\pm9.50\times10^{6}$	$7.23\times10^{8}\pm1.63\times10^{8}$	$4.00\times10^{7}\pm2.00\times10^{6*}$
		S	$1.42\times10^{7}\pm2.33\times10^{6*}$	$1.50\times10^{8}\pm3.55\times10^{7}$	$8.83\times10^{8}\pm5.51\times10^{7}$	$3.87\times10^{7}\pm6.03\times10^{6*}$
		P	$1.57\times10^{7}\pm2.16\times10^{6}$	$1.23\times10^{8}\pm1.57\times10^{7}$	$1.01\times10^{9}\pm7.00\times10^{7}$	$3.97\times10^{7}\pm1.16\times10^{7*}$
	Control		$1.73\times10^{7}\pm1.53\times10^{7}$	nd	nd	$2.17\times10^{8}\pm7.64\times10^{7}$

EHEC – Enterohemorrhagic *E. coli*; W – raspberry seedless pomace preparation; S – seed preparation; P – native raspberry pomace preparation; nd – not determined; *significant difference ($p<0.05$) compared to control.

TABLE 3. Count of the pathogenic bacteria in the co-culture with *L. rhamnosus* (cfu/mL).

Microorganism	Culture conditions	Incubation time (h)				
		0	15	18	21	24
EHEC	Co-culture	$4.25\times10^{6}\pm3.51\times10^{5}$	$2.40\times10^{8}\pm4.65\times10^{7}$	$2.18\times10^{8}\pm7.21\times10^{6}$	$2.60\times10^{8}\pm3.46\times10^{7}$	$1.73\times10^{8}\pm1.89\times10^{7*}$
	Control	$4.00\times10^{6}\pm2.58\times10^{6}$	nd	nd	nd	$3.00\times10^{8}\pm4.00\times10^{7}$
Listeria monocytogenes	Co-culture	$7.00\times10^{5}\pm2.00\times10^{5}$	$1.27\times10^{7}\pm3.05\times10^{6}$	$7.27\times10^{6}\pm1.11\times10^{6}$	$6.20\times10^{5}\pm1.01\times10^{5}$	$1.33\times10^{3}\pm2.89\times10^{2*}$
	Control	$5.67\times10^{5}\pm1.15\times10^{5}$	nd	nd	nd	$4.03\times10^{8}\pm4.17\times10^{7}$
Salmonella Enteritidis	Co-culture	$4.00\times10^{6}\pm5.29\times10^{5}$	$2.33\times10^{7}\pm1.53\times10^{6}$	$1.53\times10^{5}\pm5.77\times10^{3}$	$9.77\times10^{3}\pm3.21\times10^{2}$	nf*
	Control	$3{,}63\times10^{6}\pm3.51\times10^{5}$	nd	nd	nd	$3.77\times10^{8}\pm3.79\times10^{7}$
Salmonella Typhimurium	Co-culture	$7.47\times10^{6}\pm1.07\times10^{6}$	$3.10\times10^{8}\pm4.00\times10^{7}$	$4.53\times10^{7}\pm1.53\times10^{6}$	$3.70\times10^{4}\pm8.88\times10^{3}$	nf*
	Control	$6.80\times10^{6}\pm1.06\times10^{6}$	nd	nd	nd	$6.37\times10^{8}\pm6.03\times10^{7}$

EHEC – Enterohemorrhagic *E. coli*; nd – not determined; nf – not found; *significant difference ($p<0.05$) compared to control.

Even though growth inhibition of pathogenic bacteria is a desirable result of the applied raspberry preparations, an opposite effect is expected towards probiotic LAB strains. Nohynek *et al.* [2006] observed no antagonistic action of raspberry extracts on probiotic *L. rhamnosus* strains. In the study by Puupponen-Pimiä *et al.* [2001], *Lactobacillus* strains in the liquid culture were not affected by raspberry extracts at low concentrations (1±1 mg/mL). However, the growth of these bacteria was inhibited, when a five times higher concentration of raspberry extracts was used. The present research showed no antimicrobial effect of the 1 mg/mL raspberry preparation dose on *L. rhamnosus* growth in the liquid culture. On the other hand, bacteria counts were significantly lower for all combinations of raspberry preparations (concentration 2 mg/mL) with *L. rhamnosus* compared to the control (Table 2).

The co-culture of probiotic lactobacilli and enteric pathogens is a laboratory method widely used to assess relationships between microorganisms tested. Chen *et al.* [2019] reported a significant growth inhibition of *E. coli* after 48-h co-culture with lactobacilli. A probiotic combination of *L. acidophilus* and *L. rhamnosus* caused a complete elimination of *E. coli* after 12 h [Bertuccini *et al.,* 2017]. The reduction in *Salmonella* Enterica number (from 8 $\log_{10}$ to no viable) occurred between 8 and 16 h of co-culture with the two LAB strains [Adetoye *et al.,* 2018]. Our research showed a high inhibitory effect of the probiotic *Lactobacillus* against *Salmonella* strains, resulting in the reduction of pathogen count below the inoculum level within 24 h (Table 3). After 24-h co-incubation, *L. monocytogenes* number decreased from the initial 7.00×10^{5} cfu/mL to 1.33×10^{3} cfu/mL, while in the control pure culture it exceeded 10^{8} cfu/mL (Table 3). Although the concentration of enterohemorrhagic *E. coli* cells in the co-culture and control culture remained at the same level of 10^{8} cfu/mL during the final analysis, a significant inhibitory activity of *L. rhamnosus* against EHEC was reported (Table 3).

Although various mechanisms, including the production of bacteriocins and H_2O_2, are linked to pathogen growth inhibition by LAB probiotic strains, the acidification of the growth medium is suggested to be the crucial factor responsible for the co-culture reduction efficiency [Chen *et al.,* 2019; Delley *et al.,* 2015]. On the other hand, Shen *et al.* [2014] reported a weak effect of the medium pH on the antibacterial activity of blueberry ethanol extract. A weak correlation between berry juices acidity and their antimicrobial effect was also noted by Krisch *et al.* [2008].

TABLE 4. Count of *Lactobacillus rhamnosus* (cuf/mL) in the co-culture with the pathogenic bacteria and raspberry preparations in the concentration of 2.0 mg/mL.

Co-culture conditions		Incubation time (h)	
		0	24
EHEC		$1.93\times10^{7}\pm3.51\times10^{6*}$	$6.27\times10^{8}\pm1.10\times10^{7}$
EHEC + raspberry preparations	W	$4.90\times10^{6}\pm1.25\times10^{6*}$	$1.37\times10^{9}\pm1.81\times10^{8*}$
	S	$6.63\times10^{6}\pm2.03\times10^{6}$	$1.45\times10^{9}\pm1.12\times10^{8*}$
	P	$5.83\times10^{6}\pm2.06\times10^{6*}$	$1.14\times10^{9}\pm1.11\times10^{8*}$
Listeria monocytogenes		$1.00\times10^{7}\pm1.70\times10^{6}$	$6.23\times10^{8}\pm4.15\times10^{7}$
Listeria monocytogenes + raspberry preparations	W	$4.53\times10^{6}\pm2.54\times10^{6*}$	$8.77\times10^{8}\pm1.00\times10^{8*}$
	S	$9.07\times10^{6}\pm2.57\times10^{6}$	$8.33\times10^{8}\pm1.27\times10^{8*}$
	P	$3.40\times10^{6}\pm5.29\times10^{5*}$	$1.01\times10^{9}\pm8.14\times10^{7*}$
Salmonella Enteritidis		$2.20\times10^{7}\pm7.00\times10^{6*}$	$7.33\times10^{8}\pm7.51\times10^{6}$
Salmonella Enteritidis + raspberry preparations	W	$1.10\times10^{7}\pm9.50\times10^{5}$	$1.30\times10^{9}\pm1.27\times10^{8*}$
	S	$8.10\times10^{6}\pm1.71\times10^{6}$	$1.02\times10^{9}\pm6.43\times10^{7*}$
	P	$3.63\times10^{6}\pm1.19\times10^{6*}$	$1.08\times10^{9}\pm1.77\times10^{7*}$
Salmonella Typhimurium		$1.23\times10^{7}\pm6.03\times10^{6}$	$6.03\times10^{8}\pm1.20\times10^{7}$
Salmonella Typhimurium + raspberry preparations	W	$5.77\times10^{6}\pm1.08\times10^{6}$	$9.60\times10^{8}\pm1.64\times10^{8*}$
	S	$4.93\times10^{6}\pm2.10\times10^{6}$	$9.77\times10^{8}\pm6.11\times10^{7*}$
	P	$5.23\times10^{6}\pm2.44\times10^{6}$	$1.29\times10^{9}\pm6.51\times10^{7*}$
Control		$1.04\times10^{7}\pm8.14\times10^{5}$	$5.63\times10^{8}\pm1.05\times10^{8}$

EHEC – Enterohemorrhagic *E. coli*; W – raspberry seedless pomace preparation; S – seed preparation; P – native raspberry pomace preparation; *significant difference ($p<0.05$) compared to control.

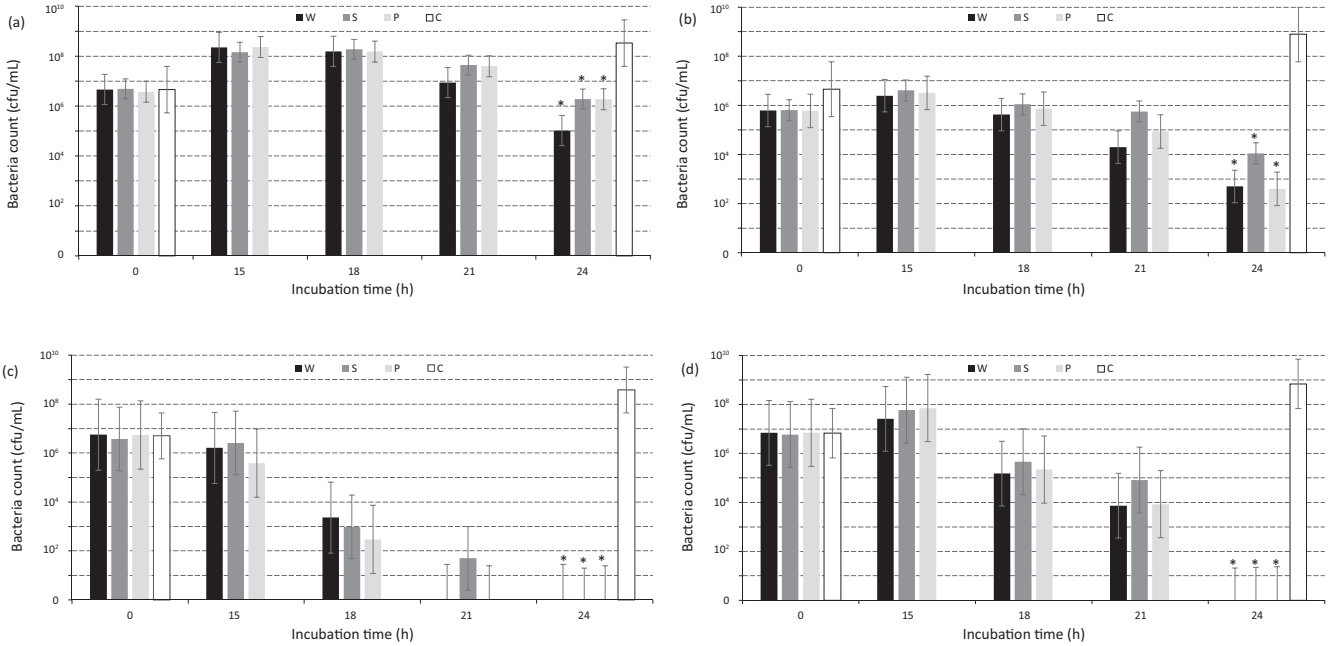

FIGURE 1. Count of the pathogenic bacteria (cuf/mL) in the co-culture with *L. rhamnosus* and raspberry seedless pomace, seeds and native pomace (W, S and P preparations, respectively) in the concertation of 2.0 mg/mL; (a) *Escherichia coli*, (b) *Listeria monocytogenes*, (c) *Salmonella* Enteritidis, and (d) *Salmonella* Typhimurium.

*Significant difference ($p<0.05$) compared to control.

The co-culture with enteric pathogens did not influence the growth of lactobacilli during the 24-h incubation. Probiotic strain cell count was comparable to that observed in the control culture and approximated 10^8 cfu/mL. A significant increase in *L. rhamnosus* number was observed in all of the mixed cultures enriched with raspberry preparations. Their number after 24-h incubation ranged from 10^8 cfu/mL to 10^9 cfu/mL and exceeded the values obtained in the co-culture including bacterial strains only, regardless of preparation type (Table 4). A lack of the inhibitory effect of pathogenic bacteria on lactobacilli was also confirmed in the above-mentioned studies [Chen *et al.,* 2019; Adetoye *et al.,* 2018].

The enrichment of the pathogen-probiotic mixed culture with raspberry preparations resulted in the reduction of pathogen cell count (Figure 1). In most cases, the addition of raspberry preparations to the co-culture caused a greater reduction in pathogen populations, compared to cultures with *Lactobacillus* only. Similarly to the results obtained in the experiment with bacterial co-cultures not mixed with berry preparations, complete inactivation of the tested *Salmonella* strains was observed within in 24 h (Figure 1). Moreover, the complete elimination of *Salmonella* Enteritidis was reported after 21 h of incubation with *L. rhamnosus* mixed with W and P preparations. The concentration of *S.* Typhimurium cells in the mixed cultures decreased after 21 h from 10^6 cfu/mL to 10^3–10^4 cfu/mL. *L. monocytogenes* proved less sensitive to *L. rhamnosus* and raspberry preparations; however, the reduction in cell number from 10^5 to 10^2 cfu/mL was found after 24 h of the co-culture with the addition of pomace (P) and seedless pomace preparations (W). On the other hand, EHEC revealed the highest resistance to the mixed culture effect.

The results of the study showed no influence of the raspberry preparations type and chemical composition on their antimicrobial efficiency. The higher concentration of phenolics and ellagitannins in the seedless pomace preparation (W) did not result in its stronger inhibitory effect on enteric pathogens and *Lactobacillus,* compared to other raspberry preparations tested (S, P) (Table 2, 4 and Figure 1).

The results from the present study demonstrate that the combination of *Lactobacilli* and raspberry preparations was synergic against the pathogens tested. The idea of the coupled application of berry products and LAB or their metabolites was also examined in other studies. Terpou *et al.* [2019] used berries as an immobilization carrier of the probiotic lactobacilli in the functional frozen yogurt production, resulting in the improved product flavor and microbiological stability. In the study on the malolactic fermentation effect on the berry juice composition, Markkinen *et al.* [2019] found that fermentation by *Lactobacillus plantarum* decreased the content of hydroxycinnamic acids but not of anthocyanins in chokeberry. Finally, the high antimicrobial potential of natural formulations based on plant oils or extracts, and lactic acid against *L. monocytogenes*, *E. coli*, and *S*. Typhimurium was reported by Harich *et al.* [2018].

CONCLUSIONS

Our study showed no or low antibacterial effect of raspberry preparations against the bacteria tested. Although other researchers generally confirmed the inhibitory action of raspberry-based products on microorganisms, heterogeneous and contrary results were obtained. The differences in the antimicrobial potential of the tested raspberry products are probably

caused by the composition and amount of active compounds in the tested materials, or susceptibility of the bacterial strains tested. The coupled use of the probiotic *Lactobacillus* strain and raspberry preparations resulted in the synergic inhibitory effect against enteric pathogens. The obtained results suggest the possibility of the combined use of probiotics and active substances found in berries as natural antimicrobial agents for the food industry in functional food production. However, due to the high unpredictability of the final effect of these factors on bacteria growth, further studies in this research area need to be continued and extended.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

ORCID IDs

J. Bauza-Kaszewska https://orcid.org/0000-0003-2972-6131
B. Fotschki https://orcid.org/0000-0002-9727-7481
A. Gugolek https://orcid.org/0000-0002-5360-9755
J. Juśkiewicz https://orcid.org/0000-0003-0068-5970
E. Karlińska https://orcid.org/0000-0002-7091-1278
M. Kosmala https://orcid.org/0000-0002-9018-3028
A. Ligocka https://orcid.org/0000-0002-7452-3213
E. Żary-Sikorska https://orcid.org/0000-0001-8140-3861

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Interplay Role of Heat-Moisture Treatment and Lipid from Egg yolk and Margarine on Functional and Pasting Properties of Banana Flour

Yana Cahyana[1], Tomi Nugraha[1], Nabila Aprilira[1], Karina Ayuningtias[1], Giffary Pramafisi Soeherman[1], Herlina Marta[2], Tensiska Tensiska[1]*

[1]Laboratory of Food Chemistry, Department of Food Technology, Universitas Padjadjaran, Indonesia
[2]Laboratory of Food Processing Technology, Department of Food Technology, Universitas Padjadjaran, Indonesia

Key words: heat-moisture treatment, lipid, egg yolk, margarine, complex

Egg yolk and margarine are commonly applied in starchy food processing as a lipid source. Both may affect the starch properties to different extent. Therefore, the effects of adding egg yolk (0.3, 1.0, and 1.7 g/100 g of flour) and margarine (1, 3, and 5 g/100 g of flour) to banana flour on the complex formation of gelatinized starch–lipid and on paste functional properties were investigated in this study. Native banana flour was used as a control. The complexing index (CI) value increased with the increasing egg yolk or margarine content in the paste. Egg yolk lipids formed complexes with flour starch more favorably than margarine lipids. The heat-moisture treatment (HMT) of banana flour increased its ability to form a complex. Complex formation may partly affect the swelling volume (SV) and freeze-thaw stability (FTS) of pastes. The decrease in SV was smaller in the paste with egg yolk than with margarine. Egg yolk also led to a lesser syneresis and solubility than margarine. For freeze-thaw stability, the interplay roles between the reducing effect of lipid addition and the increasing effect of HMT led to an increased syneresis suggesting that HMT factor prevailed. The reducing effect of lipid addition on egg yolk complex solubility predominated the increasing impact of HMT. Lipid source did not affect pasting properties except set back viscosity. HMT-pretreatment played a major role in the alteration of pasting properties compared to the lipid content.

ABBREVIATIONS

HMT – heat-moisture treatment, SV – swelling volume, FTS – freeze-thaw stability, CI – complexing index.

INTRODUCTION

Banana starch application in food has been explored in several studies [Cahyana *et al*., 2020; Fida *et al*., 2020]. In order to improve its properties in food application, native starch is commonly modified by various methods including physical [Adebowale *et al*., 2005; Bian & Chung, 2016], chemical [Aparicio-Saguilán *et al*., 2014; Cahyana *et al.*, 2018; Handarini *et al.*, 2020b; Wattanachant *et al*., 2003], and enzymatic ones [do Prado Cordoba *et al*., 2016], of which the physical treatment is promising due to the absence of chemical residues following the treatment. Amongst physical treatments, heat-moisture treatment (HMT) is of particular interest. HMT is carried out by heating starch above its gelatinisation temperature (normally ≥100°C) in a limited moisture content (normally ≤30%) [Marta *et al*., 2020]. The HMT treatment to banana flour results in remarkable changes of its pasting (an increase in pasting temperature but a decrease in peak, breakdown, and setback viscosity) and functional properties (a decrease in swelling volume and water absorption capacity) compared to the native starch [Cahyana *et al*., 2019; Cordeiro *et al*., 2018]. Therefore, the application of HMT-starch or flour in food application would be very advantageous.

When either native or HMT-starch is applied in food, other major components like lipid are often added as a product ingredient. A number of studies have reported the formation of complexes between lipid and starch. Fatty acids, such as lauric, myristic, palmitic, and stearic acids, form complexes with starch [Qin *et al*., 2019]. The lipid-starch complex was reported to affect starch properties, including a reduction in the final viscosity and *in vitro* starch digestibility [Mapengo & Emmambux, 2020].

Margarine and egg yolk are those which are often mixed with starch as a dough mixture prior to baking. The presence of both lipids and starch in a food matrix is expected to affect certain properties of starchy food due to the lipid-starch interaction. Given that both margarine and egg yolk are often present altogether in starchy food as the ingredients, it is interesting to study starch interaction with their lipids and its effect on complex formation as well as on starch properties. Considering that modified starch is favourably used in food application compared to the native starch, it is important to examine the effect of starch modification particularly that modified with HMT on its interaction with lipid using native starch as a control. Different content of margarine and egg

* Corresponding Author:
E-mail: y.cahyana@unpad.ac.id (Y. Cahyana)

yolk were applied to comprehend the effect of the content on studied parameters.

This study aimed to reveal the effect of lipid addition from egg yolk and margarine on functional properties, such as swelling volume, solubility and freeze-thaw stability, as well as pasting properties of HMT banana flour. Complexing index was analysed to measure the extent of complex formation between banana flour and lipids.

MATERIALS AND METHODS

Material

Banana cultivar Kapas harvested at 8–10 weeks after flowering was used in the present study (ripening stage 1, entirely green). The bananas were obtained from a local farmer in Jati Gede, Indonesia. Fruit fingers were selected for uniformity of colour, size, and shape. Margarine Palmia and egg of laying hens were purchased at a local market (Bandung, Indonesia). All chemical reagents used in this work were of analytical grade (SIGMA, Singapore) and used directly without further purification.

Lipid content and fatty acid profile determination

Lipid content and fatty acid profile of margarine and egg yolk were analysed at a commercial laboratory service of PT Saraswanti Indo Genetech (Jakarta, Indonesia). The laboratory has been certified by the National Accreditation Committee (KAN). Lipid content in margarine was determined using the Soxhlet method while lipids in egg yolk before extraction were hydrolysed (Weibull-Stoldt method) [Kolar *et al*., 1993]. Fatty acids were analysed using gas chromatography with flame ionization detection (GC FID Clarus 680 Perkin Elmer, USA). The analysis was carried out by injecting 1 μL of methylated (using BF_3-methanol reagent) samples at injection temperature of 240°C into the DB Fast-FAME (30 m × 0.25 mm, film thickness 0.25 μm) capillary column (Agilent, Santa Clara, CA, USA). Helium (H_2) was used as a carrier gas. The H_2 and air flow rates were 30 and 300 mL/min. An oven was set at temperature gradient of 50––230°C with running time being 24.67 min.

Banana flour preparation

Banana flour was prepared according to the previous method [Marta *et al*., 2019b]. The pulp from unripe banana fruit was sliced into pieces approx. 2 mm thick, dipped in water for 15 min, and then drained. The banana slices were then dried in a drying oven at 50°C for 24 h followed by milling to produce flour using a miller machine FCT Z-300 (Fomac, Jakarta, Indonesia). The flour was then passed through 100 mesh screens. Banana flour was stored in a polypropylene plastic bag as a primary packaging using aluminum foil along with silica gel as the secondary packaging, and stored at room temperature (26±2°C) for later analysis.

Heat-moisture treatment of banana flour

Heat-moisture-treated banana flour was prepared according to the previous method [Marta *et al*., 2020]. The flour was placed in a pan and its moisture content was adjusted to 30% by spraying distilled water homogenously. The pan was then covered and allowed to equilibrate at 4°C for 24 h in a refrigerator followed by heat treatment at 100°C for 8 h. The preparation was then dried in an oven at 50°C for 24 h, and ground prior to sieving through 100 mesh screen.

Formulating egg yolk or margarine mixtures with banana flour and measuring pasting properties

Rapid visco analyzer (RVA-SM2; Perten, Warriewood, Australia) was used to measure pasting properties of the mixture of egg yolk or margarine with banana flour. Margarine or egg yolk was weighed accurately on flour weight basis (1, 3, and 5 g/100 g of flour for margarine and 0.3, 1.0, and 1.7 g/100 g of flour for egg yolk) and placed in a viscometer followed by the addition of distilled water (25 mL) and 3.5 g of banana flour (native or HMT-flour). The mixture was then agitated by raising and lowering the plastic paddle through the canister 10 times before the canister was inserted into the instrument. Viscosity was recorded with the following profile: holding at 50°C for 1 min, heating from 50°C to 95°C for 3.7 min, holding at 95°C for 2.5 min, and cooling down to 50°C for 3.8 min. The gel was maintained for 2 min at 50°C with rapid stirring at 960 rpm for the first 10 s to disperse the starch sample, under a constant paddle rotating speed of 160 rpm throughout the entire analysis. The total run time for each sample was 13 min. Parameters of pasting properties were measured. The final resulting paste was used for other analyses.

Complexing index evaluation

The complexing index (CI) of gelatinised starch with lipids was determined using the method of Handarini *et al*. [2020a] with slight modifications. Immediately after the completion of the parameters of pasting properties, 5 g of starch paste was removed from the RVA canister and placed in a 50-mL capped tube. Distilled water (25 mL) was added into the tube and mixed with the paste at 50°C. The tube was the homogenised using vortex for 2 min, and 100 μL of the resulting dispersion was mixed with 15 mL of distilled water, followed by the addition of 2 mL of an iodine solution (2.0% KI and 1.3% of I_2 in distilled water). The absorbance was measured at 690 nm with a spectrophotometer (Rayleigh UV-9200, Beijing, China). Pastes without lipid addition were used as a reference. The experiment was carried out within 60 min to avoid starch retrogradation, and CI was calculated using the following equation:

$$CI\ (\%) = \frac{(A_{ref} - A_{sample})}{A_{ref}}\ x\ 100\% \quad (1)$$

where: CI is complexing index, A_{ref} and A_{sample} are respectively absorbance of reference and sample.

Swelling volume and solubility determination

The paste was cooled in iced water for 1 min and centrifuged at 3500 rpm for 30 min. The volume of the supernatant was measured, and solubility was determined from the supernatant previously dried in a hot air oven. The total volume was determined separately by measuring the mixture of the same quantity of flour, lipid, and distilled water as carried out in

RVA. The swelling volume and solubility were calculated according to Equations (2) and (3):

$$Swelling\ volume\ (^{mL}/_{g}) = \frac{(\text{Total volume} - \text{Supernatant volume})}{\text{Sample weight (dry basis)}} \quad (2)$$

$$Solubility\ (\%) = \frac{\text{Dried supernatant weight}}{\text{Sample weight}} x\ 100\% \quad (3)$$

Freeze-thaw stability determination

A 10 g of paste from RVA was taken, placed in a centrifuge tube, and cooled to room temperature in an iced shaking water bath. The tube was then subjected to a freeze–thaw cycle by storing at 4°C for 24 h, freezing at -15°C for 48 h, thawing at 25°C for 3 h, and centrifuging at 3500 rpm for 15 min. The supernatant removed from the gel was weighed. The extent of syneresis was expressed as the percentage of separated liquid per total weight of the sample in the centrifuge tube.

Statistical analysis

The experiments were conducted in triplicate. Statistical analysis was performed using Statistical Package for the Social Sciences (IBM, New York, USA). Data were expressed as mean ± standard deviation of triplicate determinations and were compared through one-way ANOVA using Duncan's Multiple Range test at $p<0.05$ significance level.

RESULTS AND DISCUSSION

Total lipid content and fatty acid profile

The egg yolk and margarine used in experiments were determined for their total lipid content and fatty acid profile. The total lipid content was 33.3 g/100 g and 81.0 g/100 g, respectively. Fatty acid profile of egg yolk and margarine is tabulated in Table 1. Approximately 25 different fatty acids were found in either egg yolk or margarine. Palmitic, stearic, oleic, and linoleic acids were the major fatty acids of egg yolk and margarine. However, unsaturated fatty acids were predominant in egg yolk and accounted for approximately 62.2% of total fatty acids. Meanwhile, 55.4% of total fatty acids of margarine were saturated fatty acids. Fatty acids of ω-9 and ω-6 were the unsaturated fatty acids predominantly present in both egg yolk and margarine.

Complexing indexes

Previous study has demonstrated that the maximum complex formation between starch and lipids takes place with the optimal ratio of both components [Tang & Copeland, 2007]. Lipids tend to self-associate when their addition to starch is above the optimal ratio. Therefore, it is important to choose an appropriate amount of lipid added to starch in experimental system used. Our preliminary study on the addition of egg yolk and margarine to banana flour at 1, 2, and 3 g/100 g showed that egg yolk at 2 g/100 g was above the optimal value while margarine at 3 g/100 g was below the optimal value. Further experimental work found that the addition of egg yolk and margarine less than 1.7 and 5 g/100 g, respectively, was within the acceptable range. To compare the effectiveness of margarine with egg yolk, their addition of 1 g/100 g of flour was selected.

TABLE 1. Fatty acid profile (% of total fatty acids) of egg yolk and margarine.

Fatty acid	Egg yolk	Margarine
α-Linolenic acid (18:3ω-3)	0.238	0.105
γ-Linolenic acid (18:3ω-6)	0.133	–
Linoleic acid (18:2ω-6)	16.3	8.32
Oleic acid (18:1ω-9)	41.1	35.9
Heptadecenoic acid (17:1)	0.159	0.017
Palmitoleic acid (16:1)	0.423	0.116
Arachidonic acid (20:4ω-6)	2.18	–
Caprylic acid (8:0)	–	0.675
Pentadecenoic acid (15:1)	0.090	–
Eicosatrienoic acid (20:3ω-6)	0.246	–
Myristoleic acid (14:1)	0.126	–
Eicosadienoic acid (20:2)	0.288	–
Docosahexaenoic acid (22:6ω-3)	0.469	–
Lignoceric acid (24:0)	–	0.077
Caproic acid (6:0)	–	0.049
Stearic acid (18:0)	9.34	5.57
Heptadecanoic acid (17:0)	0.213	0.099
Palmitic acid (16:0)	27.6	41.0
Pentadecanoic acid (15:0)	0.096	0.046
Behenic acid (22:0)	–	0.063
Myristic acid (14:0)	0.568	2.61
Heneicosanoic acid (21:0)	0.063	–
Lauric acid (12:0)	0.033	4.36
Eicosenoic acid (20:1)	0.303	0.120
Capric acid (10:0)	–	0.537
Arachidic acid (20:0)	0.027	0.386
Sum of ω-3 fatty acids	0.709	0.105
Sum of ω-6 fatty acids	18.8	8.32
Sum of ω-9 fatty acids	41.1	35.9
Sum of monounsaturated fatty acids	42.3	36.2
Sum of polyunsaturated fatty acids	19.8	8.42
Sum of saturated fatty acids	37.8	55.4
Sum of unsaturated fatty acids	62.2	44.6

The values of complexing indexes (CI) of starch of banana flour and lipids of egg yolk and margarine are presented in Table 2. The CI value for complexes of lipid-HMT flour starch increased with the increase in lipid content. The formation of lipid-starch complexes has been reported in previous studies using another lipid source such as palm oil [Farooq *et al*., 2018;

TABLE 2. Complexing indexes of gelatinised native and HMT-banana flours with various contents of egg yolk or margarine.

Lipid source	Content (g/100 g of flour)	Complexing index (%)	
		Native flour	HMT–flour
Egg yolk	0.3	–	2.03 ± 0.32^{c}
	1.0	2.56 ± 0.18^{B}	$8.23\pm0.19^{b,A,\alpha}$
	1.7	–	9.07 ± 0.32^{a}
Margarine	1	–	$2.80\pm0.26^{c,\beta}$
	3	9.38 ± 0.30^{B}	$11.2\pm0.65^{b,A}$
	5	–	15.9 ± 0.72^{a}

Uppercase letters compare values in the same row; lowercase letters compare values in the same column for egg yolk and margarine separately; Greek letters compare values for HMT-flours with egg yolk or margarine at the same addition level (1 g/100 g). Different letters denote significant differences at $p<0.05$.

Handarini *et al*., 2020a]. Our finding on the increase of the complexing index value with the increase in lipid content is in line with a previous study [Handarini *et al*., 2020a]. As mentioned above, when the ratio of lipids mixed with starch is above optimal value, CI may decrease due to the preference of lipids to self-associate [Tang & Copeland, 2007]. In the present study, the CI increased as the lipid content increased, confirming that the lipid contents in pastes did not exceed the optimal value.

In an inclusion model, the aliphatic part of lipids forming complexes with starch is located inside the helical cavity of amylose, whilst the polar group remains outside due to the steric and electrostatic repulsions [Godet *et al*., 1993]. Amylose can interact with two fatty acids in which the polar part of fatty acids is located at the end of each helix. Long amylose chains can even accommodate more than two fatty acids. The chains, however, are distorted to accommodate polar groups of fatty acids. The increase in CI in the present study with the increase of contents of egg yolk or margarine in pastes may indicate that the amylose chains might not be in a saturated state with the lipid so that they could accommodate more lipid to form a complex. At the content of 1 g/100 g, CI in egg yolk-HMT flour mixture was 8.23% which was much higher value than that of margarine-HMT flour (2.80%) (Table 2), suggesting that egg yolk containing more unsaturated fatty acids formed complexes with starch of banana flour more favourably than margarine. The CI value may also be affected by the presence of protein. Study on the effect of protein on starch-lipid complexes showed that the addition of protein promoted the formation of starch-lipid complexes [Cai *et al*., 2021]. Unfortunately, the content of protein in both egg yolk and margarine was not analysed in the present study. It is, therefore, difficult to assess the role of protein in the present study. The role of protein present in egg yolk on starch-lipid complex formation is worth further investigation.

The CI values of the complexes formed in pastes of native banana flour and egg yolk or margarine were 2.56 and 9.38%, respectively (Table 2). The CI increased to 8.23 and 11.2%, respectively, when HMT-flour was used at a corresponding lipid content. It suggests that more starch polymers are available to form complexes with lipids. HMT alters crystallinity and starch structure from B to A crystalline type [Cahyana *et al*., 2019; Marta *et al*., 2020]. These changes, however, may not be the driving force of the complex formation as the starch was mixed with lipid at high temperature (95°C) where the crystallites were melted and in amorphous phase when starch formed a complex with lipid. HMT also changes the content and chain lengths of the amylose fraction [Silva *et al*., 2017; Singh *et al*., 2011]. These changes may facilitate complex formation with lipid. Another study found that the other components present in the flour underwent an alteration following HMT treatment [Puncha-arnon & Uttapap, 2013], suggesting that apart from starch *per se*, the other components might affect the CI value of lipid-HMT banana flour compared to that of lipid-native flour.

Functional properties

The functional properties of pastes of native and HMT banana flour with different contents of lipids from two sources are presented in Table 3.

Swelling volume (SV)

The SV of pastes of HMT-flour and egg yolk or margarine decreased with the increase of their lipid contents (Table 3). The SV of HMT-flour with egg yolk ranged from 10.1 to 11.6 mL/g while that of HMT-flour with margarine from 8.27 to 9.25 mL/g. It suggests that the presence of both egg yolk and margarine lipids diminishes starch capacity to swell. The decrease in SV was concomitant with the increase in CI, therefore SV might be partly related to CI. A similar observation was also made for arrowroot starch-palm oil [Handarini *et al*., 2020a] and maize starch-stearic acid [Raphaelides & Georgiadis, 2006] mixtures.

The SV of paste of HMT-banana flour with egg yolk (1 g/100 g) was only slightly higher than that of HMT-flour mixed with margarine in the same proportion, even though the CI for the egg yolk complexes was significantly higher than that with margarine. This finding suggests that SV may be driven not only by complex formation but also by the nature of the lipid *per se*. Lipids in egg yolk are more hydrophilic due to the content of phospholipids. The presence of phospholipids may lead to higher water absorption and swelling volume.

Compared to the paste of native banana flour, the paste made of HMT- flour had lower SV values, *i.e.* 12.1 *vs.* 10.2 mL/g, respectively, when egg yolk was added and 11.6 *vs.* 8.92 mL/g, respectively, for pastes with margarine. This finding suggests that HMT plays a role in decreasing the swelling volume regardless of lipid type. Another study revealed that HMT of banana flour decreased its ability to swell compared to its native form along with a decrease in water absorption capacity [Cahyana *et al*., 2019], suggesting a link between the water absorption capacity and SV. In the present study, the CI values increased when the flour was pre-treated (HMT). This finding confirms that starch-lipid complex formation may play a role in decreasing SV of paste of banana flour with egg yolk or margarine.

Solubility

Trends in solubility of pastes of HMT-flour with egg yolk or margarine varied (Table 3). In the egg yolk-flour pastes,

TABLE 3. Functional properties of native and HMT-banana flour pastes with various contents of egg yolk or margarine.

Paste properties	Lipid source	Content (g/100 g of flour)	Banana flour	
			Native	HMT
Swelling volume (mL/g)	Egg yolk	0.3	–	11.6 ± 0.96^{a}
		1.0	12.1 ± 0.91^{A}	$10.2\pm0.19^{b,B,\alpha}$
		1.7	–	10.1 ± 0.73^{b}
	Margarine	1	–	$9.25\pm0.10^{a,\beta}$
		3	11.6 ± 0.32^{A}	$8.92\pm0.17^{b,B}$
		5	–	8.27 ± 0.10^{c}
Solubility (%)	Egg yolk	0.3	–	4.44 ± 0.28^{a}
		1.0	5.16 ± 0.95^{A}	$1.50\pm0.58^{b,B,\beta}$
		1.7	–	0.65 ± 0.48^{b}
	Margarine	1	–	$10.3\pm0.29^{b,\alpha}$
		3	11.5 ± 0.44^{A}	$10.7\pm0.49^{b,A}$
		5	–	12.4 ± 0.98^{a}
Freeze-thaw stability (% syneresis)	Egg yolk	0.3	–	32.4 ± 0.86^{a}
		1.0	8.34 ± 0.24^{B}	$20.9\pm0.45^{b,A,\beta}$
		1.7	–	21.0 ± 0.62^{b}
	Margarine	1	–	$42.8\pm1.37^{a,\alpha}$
		3	39.7 ± 0.53^{B}	$42.5\pm1.74^{a,A}$
		5	–	41.9 ± 1.45^{a}

Uppercase letters compare values in the same row; lowercase letters compare values in the same column for egg yolk and margarine separately; Greek letters compare values for HMT-flours with egg yolk or margarine at the same addition level (1 g/100 g). Different letters denote significant differences at $p<0.05$.

the solubility decreased from 4.44 to 0.65% with the increase in egg yolk content. Meanwhile, an opposite trend in solubility was observed with the increase in margarine content. Considering that CI of the mixture increased with the increase of egg yolk or margarine content, this opposite trend in solubility of egg yolk-banana flour pastes compared to margarine-banana flour pastes indicates that the solubility may not be linked to CI.

The solubility of gelatinised banana flour with a margarine content of 1 g/100 g was significantly higher than that with egg yolk added in the same amount. When measuring the solubility, supernatant was separated from the solid fraction following centrifugation, and then dried. Therefore, the remaining dry solid content following oven drying represents solids from margarine and amylose leaching into supernatant. The higher solubility of margarine-banana flour paste may be due to the formation of a lipid-starch complexes, which seems to be readily separated when solubility test was applied.

The solubility of pastes of native banana flour with lipids was higher compared to the solubility of pastes of HMT-flour either with egg yolk or margarine. In the absence of lipid, the solubility of breadfruit starch and banana flour was reported to increase following HMT [Cahyana *et al*., 2019; Marta *et al*., 2019a]. The solubility of HMT-banana flour in the presence of lipid may be affected by interplay factors of lipid and HMT effect. The interplay factors between HMT and lipid presence resulted in the decrease in solubility of egg yolk-HMT flour pastes suggesting that the lipid effect counteracted significantly the HMT effect. Meanwhile, in margarine-HMT flour pastes, the lipid effect on solubility was not statistically significant at margarine content below 3 g/100 g.

Freeze-thaw stability (FTS)

The increase of egg yolk content in the pastes of banana flour reduced significantly mixture syneresis from 32.4 to 21.0% (Table 3). However, the decrease of syneresis was not significant in the pastes of banana flour and margarine. The decrease of syneresis indicates an increase of FTS. Therefore the finding of syneresis reduction with the increase of lipid content, particularly egg yolk lipids, suggests that complex formation between lipids of egg yolk and starch of banana flour led to an increase of FTS.

The addition of egg yolk at 1 g/100 g to the banana flour caused 20.9% syneresis while the addition of margarine at the same content caused 42.8% syneresis, suggesting that egg yolk was more effective in increasing FTS than margarine. The higher FTS of pastes with egg yolk compared to these with margarine might be attributed to the extent of complex formation between banana flour and egg yolk or margarine. The CI of egg yolk-HMT flour was much higher than that of margarine. This higher CI means there were more egg yolk lipids to form a complex with starch polymers (amylose or amylopectin). During storage of the pastes at low temperature (as indicated in the FTS experiment), amylose and/or amylopectin move closer expelling water from the gel system (paste), which is quantified as a syneresis. The extent of complex formation may partly play a role in hampering the starch polymer from moving closer, hence in lower syneresis. In this context, egg yolk, which form complexes with banana flour starch with the higher CI than that of margarine, is effective in lowering syneresis. The effectiveness of egg yolk in reducing syneresis may also be linked to the different lipid composition in egg yolk compared to margarine.

The syneresis of paste of egg yolk-banana flour at the content of 1 g/100 g increased from 8.34% in the native flour to 21.0% in HMT-flour. A similar finding was also reported for margarine, suggesting that HMT of flour decreased FTS. Our finding is in line with previous studies on banana flour [Cahyana *et al*., 2019] and breadfruit starch in the absence of lipid [Marta *et al*., 2019a].

Pasting properties

The pasting properties of the mixtures of banana flour with egg yolk or margarine are presented in Figure 1 and tabulated in Table 4. The result shows that the increase in neither egg yolk nor margarine content affected pasting point. Similarly, the pasting points of mixtures with either egg yolk or margarine at 1 g/100 g did not differ significantly. This finding suggests that the addition of lipid did not play any role in determining the pasting point, and was in line with another study on arrowroot starch [Handarini *et al*., 2020a].

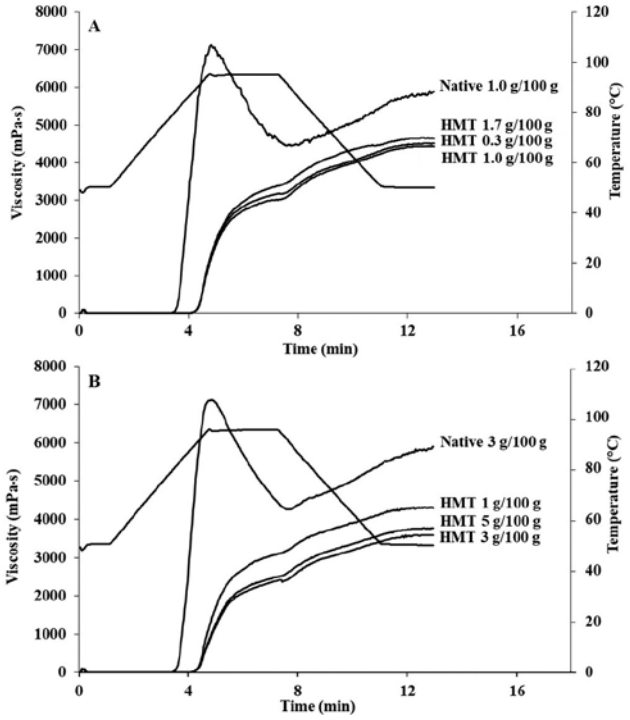

FIGURE 1. Pasting properties of native and heat-moisture-treated (HMT-flour) banana flour with various contents of (A) egg yolk (0.3––1.7 g/100 g) and (B) margarine (1–5 g/100 g).

Compared to the native flour-egg yolk mixture, the pasting point increased from 78.5°C to 87.3°C in HMT-flour. A similar finding was also made when margarine was used. It suggests that, contrarily to the lipid effect, HMT can change the pasting point. This change may relate to the modification of starch structure, which leads to the formation of more interactions and cross-links present within the granules following HMT. The more complex bonds may require higher temperature and energy to disrupt the starch structure [Huang *et al*., 2016; Zavareze & Dias, 2011]. Other studies reported that the pasting point of HMT-banana flour and breadfruit starch in the absence of lipid was higher than that of the native forms [Cahyana *et al*., 2019; Marta *et al*., 2019a].

Further examination on the other pasting properties showed that lipid addition either from egg yolk or margarine did not change the final and set back viscosity of their mixtures with banana flour. Hold viscosity was not affected by the presence of margarine while breakdown remained unchanged regardless of the egg yolk content. The addition of margarine at 5 g/100 g to HMT-flour increased the flour stability against heat and shearing during pasting, which can be attributed to the decrease in peak viscosity. The type of lipid was noted to affect the setback viscosity. Comparing the egg yolk to margarine addition at the same content (1 g/100 g), lipid from egg yolk resulted in higher set back viscosity than lipid from margarine. The other pasting properties were not affected by the type of lipid source.

In general, although a significant change was observed for several pasting properties at a certain level of lipid content, it is however considered to be small, particularly compared to the effect of HMT pretreatment. Compared to the mixture

TABLE 4. Pasting properties of native and HMT-banana flours with various contents of egg yolk or margarine.

Flour properties	Lipid source	Content (g/100 g of flour)	Banana flour	
			Native	HMT
Pasting point (°C)	Egg yolk	0.3	–	87.4 ± 0.32^{a}
		1.0	78.6 ± 0.09^{B}	$87.3\pm0.23^{a,B,\alpha}$
		1.7	–	87.2 ± 1.08^{a}
	Margarine	1	–	$87.5\pm0.47^{a,\alpha}$
		3	78.5 ± 0.29^{B}	$87.5\pm0.56^{a,A}$
		5	–	87.4 ± 0.23^{a}
Peak viscosity (mPa·s)	Egg yolk	0.3	–	$3139\pm140^{a,b}$
		1.0	7288 ± 230^{B}	$3030\pm155^{b,A,\alpha}$
		1.7	–	3375 ± 113^{a}
	Margarine	1	–	$3085\pm78.5^{a,\alpha}$
		3	6207 ± 30.1^{A}	$2724\pm291^{a,b,B}$
		5	–	$2730\pm21b^{b}$
Hold viscosity (mPa·s)	Egg yolk	0.3	–	$3098\pm147^{a,b}$
		1.0	4344 ± 210^{A}	$2979\pm162^{b,B,\alpha}$
		1.7	–	3328 ± 108^{a}
	Margarine	1	–	$3047\pm86.3^{a,\alpha}$
		3	4078 ± 307^{A}	$2688\pm293^{a,B}$
		5	–	2720 ± 238^{a}
Final viscosity (mPa·s)	Egg yolk	0.3	–	4477 ± 114^{a}
		1.0	5890 ± 172^{A}	$4436\pm101^{a,B,\alpha}$
		1.7	–	4647 ± 112^{a}
	Margarine	1	–	$4303\pm158^{a,\alpha}$
		3	5619 ± 14.2^{A}	$3973\pm334^{a,B}$
		5	–	4010 ± 215^{a}
Break down viscosity (mPa·s)	Egg yolk	0.3	–	40.8 ± 7.78^{a}
		1.0	2943 ± 192^{A}	$50.5\pm6.95^{a,B,\alpha}$
		1.7	–	47.3 ± 5.48^{a}
	Margarine	1	–	$37.5\pm7.78^{a,\alpha}$
		3	2129 ± 277^{A}	$36.0\pm3.22^{a,B}$
		5	–	10.3 ± 2.80^{b}
Set back viscosity (mPa·s)	Egg yolk	0.3	–	1379 ± 33.2^{a}
		1.0	1546 ± 127^{A}	$1457\pm81.1^{a,A,\alpha}$
		1.7	–	1319 ± 57.2^{a}
	Margarine	1	–	$1256\pm71.4^{a,\beta}$
		3	1541 ± 293^{A}	$1285\pm46.0^{a,A}$
		5	–	1288 ± 26.0^{a}

Uppercase letters compare values in the same row; lowercase letters compare values in the same column for egg yolk and margarine separately; Greek letters compare values for HMT-flours with egg yolk or margarine at the same addition level (1 g/100 g). Different letters denote significant differences at $p<0.05$.

in native flour, a remarkable decrease in peak viscosity was observed in HMT-flour either with egg yolk or margarine, suggesting the preponderant contribution of HMT pretreatment on the decrease of peak viscosity. Huge decreases were also noticed for other pasting properties, such as hold and final viscosity, and particularly breakdown viscosity. Breakdown decreased incredibly from 2943 mPa·s in egg yolk or 2129 mPa·s in margarine to 50.5 mPa·s or 36.0 mPa·s when the flour was subjected to HMT pretreatment. It suggests that HMT improves heat stability of the flour. The degree of break down changes due to the difference in lipid content was much smaller than due to HMT pretreatment, suggesting the main contribution of the change in starch structure following HMT. The role of HMT on the pronounced decrease of pasting properties was demonstrated in other studies on banana flour and breadfruit starch in the absence of lipid [Cahyana *et al*., 2019; Marta *et al*., 2019a].

CONCLUSIONS

Both egg yolk and margarine are capable of forming complexes with either native or HMT-banana starch. The CI value of lipid-starch increased with the increase of margarine or egg yolk content in pastes. Lipids from egg yolk were more favorable to form complexes compared to margarine. HMT increased flour ability to form complexes with lipids, which might be attributed to reduction in amylose chain lengths and the increase in amylose content following HMT.

The increase in the content of both egg yolk and margarine in HMT-banana flour pastes decreased SV and syneresis, while the solubility decrease was only affected by the increase in the egg yolk content. Comparing the native banana flour paste and HMT banana flour paste with the same lipid content, a decrease of SV, solubility, and FTS was found. Lipid source affected the extent of the change in the functional properties of banana flour pastes in which egg yolk resulted in higher SV but lower solubility and syneresis compared to margarine pastes. SV and FTS may be partly linked to CI.

The interplay role of HMT of banana flour and lipid content in pastes was clearly observed in the HMT-flour mixed with egg yolk in which the lipid effect thwarted the opposite HMT effect significantly, hence a decreased solubility. In respect of FTS, the reducing effect of lipid on syneresis was seemingly smaller than the increasing effect of HMT leading to an increased syneresis when lipid was mixed with HMT flour.

Although a statistically significant change was observed for certain pasting properties with the increase in lipid content, it was much smaller than that due to the HMT pre-treatment. HMT plays a major role in the alteration of pasting properties. Lipid source at 1 g/100 g of neither egg yolk nor margarine affected pasting properties except set back viscosity.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ORCID IDs

Y. Cahyana https://orcid.org/0000-0002-8615-9498
H. Marta https://orcid.org/0000-0003-4369-0016

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Exploring the Interactions Between Caffeic Acid and Human Serum Albumin Using Spectroscopic and Molecular Docking Techniques

*Ali Jahanban-Esfahlan[1,2], Leila Roufegarinejad[3], Mahnaz Tabibiazar[4,5], José M. Lorenzo[6,7], Ryszard Amarowicz[8]**

[1]Kidney Research Center, Tabriz University of Medical Sciences, Tabriz 5166–15731, Iran
[2]Department of Biology, Faculty of Fundamental Sciences, University College of Nabi Akram (UCNA), Tabriz, Iran
[3]Department of Food Sciences, Tabriz Branch, Islamic Azad University, Tabriz, Iran
[4]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[5]Department of Food Science and Technology, Faculty of Nutrition and Food Science, Tabriz University of Medical Sciences, Tabriz, Iran
[6]Centro Tecnológico de la Carne de Galicia, Parque Tecnológico de Galicia, 32900 San Cibrao das Viñas, Spain
[7]Área de Tecnología de los Alimentos, Facultad de Ciencias de Ourense, Universidad de Vigo, 32004 Ourense, Spain
[8]Department of Chemical and Physical Properties of Food, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10–486 Olsztyn, Poland

Key words: human serum albumin (HSA), fluorescence, caffeic acid, interaction, molecular docking

Ultraviolet-visible (UV–Vis) and fluorescence spectroscopy along with molecular docking were used to explore the interaction between human serum albumin (HSA) and caffeic acid (CA). CA is one of the major representatives of hydroxycinnamic acids in plants and is commonly present in plant-based foods. The mechanism by which CA quenched HSA fluorescence was determined to be static, and the values obtained for thermodynamic parameters indicated that the CA and HSA interaction was spontaneous. Hydrogen bonds and van der Waals forces were the main driving forces stabilizing the complex. The binding constant was in the order of 10^4/M and the number of binding sites for CA on HSA was calculated to be close to one. The results of fluorescence and UV–Vis spectroscopy showed that CA induced conformational changes in HSA structure. The distance of CA and the tryptophan residue of HSA, was determined to be ~2 nm by using Forster resonance energy transfer theory. The mode of binding and the binding site of CA on albumin were examined by performing molecular docking calculations. CA interacted with albumin in subdomain IA, and non–covalent interactions stabilized the complex. CA showed a high affinity for albumin, and thus this phenolic compound would be distributed in the body upon interacting with HSA.

ABBREVIATIONS

CA – caffeic acid; FRET – Forster resonance energy transfer; H–bonds – hydrogen bonds; HCAs – hydroxycinnamic acids; HAS – human serum albumin; IFE – internal filter effect; SV – Stern–Volmer; UV–Vis – ultraviolet-visible; and vdW – van der Waals.

INTRODUCTION

Caffeic acid (CA) with the chemical name of 3,4–dihydroxycinnamic acid is a natural, plant–derived phenolic compound that belongs to the class of hydroxycinnamic acids (HCAs). CA is commonly present in foods of plant origin, with fruits and products obtained from them being a particularly abundant source of this compound [El-Seedi *et al.*, 2012; Pirjo *et al*., 2006; Tomašević *et al*., 2019]. In fruits, it may constitute up to 70% of the total HCAs content [Sova & Saso, 2020]. The relatively good bioavailability of CA makes it possible to interact with human serum albumin (HSA) in the body [Rashmi & Negi, 2020]. In a cross-over study with 4 female and 3 male healthy ileostomy subjects, 95% of the ingested caffeic acid was absorbed from the small intestine in humans [Olthof *et al.,* 2001]. CA possesses different biological activities, such as antioxidant properties due to its high radical scavenging activity, and antimutagenic, anti-inflammatory, antidepressant, antimetastatic, anticarcinogenic, HIV replication-inhibitory, and antianxiety activities [Chen & Ho, 1997; El-Seedi *et al.*, 2012; Sova & Saso, 2020]. Additionally, CA has been reported to induce apoptosis in cancerous cells and inhibit tumor proliferation in animal models CA [Bhat *et al*., 2007; Chung *et al*., 2006].

Among the various serum proteins in the bloodstream of the human body, HSA is the abundant biomacro-

* Corresponding Author:
E-mail: r.amarowicz@pan.olsztyn.pl (R. Amarowicz)

molecule (~60%). Its role in the circulatory system is crucial because it functions as a transporter for various chemicals and pharmaceuticals [Jahanban-Esfahlan *et al*., 2016, 2019]. Immediately after transport into the blood, various bioactive substances interact with albumin and are efficiently distributed throughout the body [Roufegarinejad *et al*., 2019]. Thus, HSA has a high affinity for a broad spectrum of endogenous– and exogenous molecules, including food-derived bioactive compounds or other various chemicals. Furthermore, the solubility of poorly-soluble compounds increased upon their binding to HSA and, thus, the weak or strong interactions between chemicals and albumin will affect their fate in the blood [Roufegarinejad *et al.,* 2019]. HSA is categorized as a globular protein, and its molecular weight is 66.4 kDa. It has a heart–shaped structure, and includes 586 amino acids. Structurally, HSA includes three main homologous domains called domains I, II and III, and two A and B subdomains constructing each domain. Albumin contains two major binding sites named Sudlow's sites I and II. The first site is located in subdomain IIA, while the another is located in subdomain IIIA. Most of the molecules and chemicals bind to these regions of albumin [Jahanban-Esfahlan *et al*., 2015, 2019, 2020].

Although some data are available in the scientific literature for the interaction of CA and serum albumins [Adzet *et al*., 1988; Li *et al*., 2010; Min *et al*., 2004; Precupas *et al*., 2017; Sinisi *et al*., 2015; Skrt *et al*., 2012; Suryaprakash *et al*., 2000; Zhang *et al*., 2008], detailed information about the interaction of CA and HSA is scarce. Additionally, most of these investigations did not report full details regarding the binding of CA to albumin using fluorescence spectroscopy. Thus, the current study may be the first comprehensive and detailed report of the interaction of HSA and CA utilizing fluorescence, and UV–Vis spectroscopy approaches. Furthermore, molecular docking was performed using the ArgusLab software. The obtained results have been discussed to clarify the nature of the interaction between CA and HSA. The results of the present study are expected to fill the current gap in the protein binding properties of CA, which is an important plant–based phenolic compound in human health and nutrition.

MATERIALS AND METHODS

Materials

CA and fatty acid-free HSA were obtained from Sigma–Aldrich (Saint Louis, MO, USA) and used as received. The analytical-grade solvents and reagents were used without additional purification in the present study. Double distilled water was used in all the experiments.

Preparation of stock solutions

The phosphate buffer considered in this study was prepared at a concentration of 10 mM using potassium salts including KH_2PO_4 and K_2HPO_4 and then, NaOH was used for its pH adjustment. In the next step, phosphate buffer with physiological pH adjusted at 7.4 was used for the preparation of HSA stock and working solutions. Thus, the HSA stock solution was prepared by directly dissolving the protein powder in the prepared phosphate buffer. An ethanolic stock solution of CA with a concentration of 10 mM was prepared by dissolving specific amounts of CA powder in ethanol. The solutions used for fluorescence and UV–Vis spectroscopy were diluted appropriately from the prepared stock solutions.

Fluorescence spectroscopy

A Jasco FP-750 fluorescence spectrophotometer (Kyoto, Japan) was used to record fluorescence spectra. The light source of the apparatus was a xenon lamp, and the width of the quartz cell was 1 cm. An instrument composed of a stirrer and a cell holder with a water jacket was used as a temperature controller. The fluorescence spectroscopy experiments were carried out at temperatures of 290, 300 and 310 K. The corresponding fluorescence intensities for the emission wavelength (349 nm) were obtained using an excitation wavelength of 290 nm. A fixed slit width of 5 nm was applied for both excitation and emission wavelengths. The scan speed was 1200 nm/min. Using 2.5 μM HSA (a constant concentration) and increasing concentrations of CA (0, 2.5, 10, 30, 50, 70, and 90 μM), all fluorescence spectra were recorded at pH 7.4.

An experimental internal filter effect (IFE) may have reduced the emission intensity to some extent when spectra were recorded in the presence of increasing CA concentrations. This effect is an obvious issue affecting many fluorimetric methods, leading to a deviation of the results from the initial linearity, and therefore this effect must be considered. The fluorescence intensities were subsequently corrected for the absorption of the light at the excitation wavelength and reabsorption of the emitted light to reduce the IFE using the following equation:

$$F_{cor} = F_{obs}\, 10^{(A_{ex}+A_{em})/2} \quad (1)$$

where: F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively, and A_{ex} and A_{em} are the absorption of the CA at the excitation and the emission wavelengths [Roufegarinejad *et al*., 2019], respectively. The fluorescence intensity reported in this study is the fluorescence intensity that has been corrected.

The data obtained from the HSA fluorescence quenching experiment were evaluated using the Stern–Volmer (SV) equation to clarify the mechanism by which CA quenched HSA. The SV equation is presented as follows [Lakowicz, 2006]:

$$\frac{F_0}{F} = 1 + K_{SV}\,[Q] = 1 + K_q \tau_0\,[Q] \quad (2)$$

$$K_q = K_{SV}/\tau_0 \quad (3)$$

where: F and F_0 are the fluorescence emission intensities of HSA in the presence and absence of the quencher, respectively; K_q is the constant of the quenching rate for the biomolecule; τ_0 (equals 10^{-8} s) is the average lifetime of the biomolecule in the absence of quencher [Chen *et al*., 1990; Eftink, 1991]; [Q] and K_{SV} are the concentration of quencher and the con-

stant of SV quenching, respectively. Linear plots of F_0/F *vs*. [Q] are only expected for the static quenching mechanism.

Another SV equation was also used in this study for a further analysis of the fluorescence quenching process [Lehrer, 1971]:

$$\frac{F_0}{(F_0-F)} = \frac{1}{f_a} + \frac{1}{f_a K_a [Q]} \quad (4)$$

where: K_a and f_a are defined as effective quenching constant for the available fluorophores and the fraction of the nearby fluorophores, respectively.

The modified logarithmic SV equation was used to calculate the binding constant (K_b) and the number of binding sites (n) when ligands show an affinity to bind individually to similar binding sites on a biomolecule. The equation is presented as follows [Belatik *et al*., 2012; Ulrich, 1990]:

$$log \frac{(F_0-F)}{F} = log\ K_b + n log\ [Q] \quad (5)$$

For the determination of n and K_b values, the regression curves of $\log[(F_0–F)/F]$ *vs*. log[Q] were plotted. The y-coordinate and the slope of the obtained plots are n and K_b, respectively.

All the fluorescence experiments were performed in triplicate, and results were presented as the means (n=3).

UV–Vis spectroscopy

The UV–Vis spectra of HSA were obtained using T70 UV/Vis spectrophotometer (PG Instrument Ltd, Lutterworth, UK) in the presence and absence of CA at room temperature. The concentration of the protein was 20 μM, while four different concentrations of CA (0, 20, 50, and 100 μM) were analyzed. The range of recorded UV–Vis spectra was 200–430 nm.

Thermodynamic parameters

Changes in both entropy (ΔS) and enthalpy (ΔH) were obtained from the van't Hoff equation (Eq. 6) by assuming the critical point that the change in enthalpy is negligible at the studied temperature range.

$$ln\ K = -\left(\frac{\Delta H}{RT}\right) + \left(\frac{\Delta S}{R}\right) \quad (6)$$

$$\Delta G = \Delta H - T\Delta S = -RT\ lnK \quad (7)$$

In Eq. 6, K is the constant for the effective quenching and it corresponds to the K_a values obtained at the considered temperatures and R is the gas constant. The slope and the intercept of the plotted curves of lnK *vs.* 1/T were used to determine the values of ΔS and ΔH. The values of Gibb's free energy (ΔG) were obtained from Eq. 7.

Energy transfer studies

The overlapping region of the UV–Vis spectrum for the CA molecule and the HSA fluorescence spectrum was considered to determine the energy transfer and the distance (r) between CA as the acceptor and the tryptophan (Trp) residue of HSA as the donor. An equal concentration of 30 μM was used to obtain the UV–Vis spectrum of CA and HSA fluorescence spectrum, at wavelengths ranging from 200 to 500 nm.

According to the theory of energy transfer presented by Forster, the value of E (the efficiency of energy transfer) was obtained from the following equation [Lakowicz, 2006]:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (8)$$

where: R_0 is the critical distance with 50% energy transfer efficiency; r is the distance between the donor and acceptor molecules; and F and F_0 are the fluorescence intensities of the HSA in the absence and presence of CA , respectively. R_0 was obtained from Eq. 9:

$$R_0^6 = 8.8 \times 10^{-25}\ k^2 N^{-4} \Phi J \quad (9)$$

where: J is the overlap between the integral of acceptor absorption and the emission spectrum of donor fluorescence, Φ is the quantum yield of the donor fluorescence, N is the medium refractive index, and k_2 is the dipole spatial orientation factor [Lakowicz, 2006]. Accordingly, J was calculated by Eq. 10:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (10)$$

The molar absorption coefficient of the acceptor is ε (λ) at wavelength λ, and the fluorescence intensity of the fluorescent donor at wavelength λ is F (λ). Notably, 2/3, 0.118, and 1.336 are the values proposed for K_2, Φ, and N, respectively [Samari *et al.,* 2012].

Molecular docking studies

The binding sites for CA molecules and the binding energy of the formed CA–HSA complex were investigated using molecular docking. The crystal structure of HSA (PDB ID: 1AO6) was obtained from the Worldwide Protein Data Bank [wwPDB, https://www.rcsb.org/structure/1ao6]. Upon the removal of water and ligand molecules and the addition of hydrogen atoms, the docking calculations were performed using the ArgusLab 4.0.1 docking software [Jahanban-Esfahlan *et al*., 2017]. Notably, 0.4 and 80×80×80 angstroms were selected as the grid resolution and the size of the binding site bounding box, respectively. For all docking runs, a maximum of 200 candidate poses were used, and the docking engine was Argus Dock. A flexible form of the ligand, was selected, and the conformations were ranked to estimate the value of the binding energy using the Ascore scoring function. Ligand–receptor complexes resulting from molecular docking were comprehensively analyzed using PyMOL [Wang *et al*., 2008].

RESULTS AND DISCUSSION

Quenching of HSA fluorescence in the presence of CA

The fluorescence properties of aromatic amino acids, such as Trp, phenylalanine (Phe) and tyrosine (Tyr), in the structure of serum albumins enable researches to study the inter-

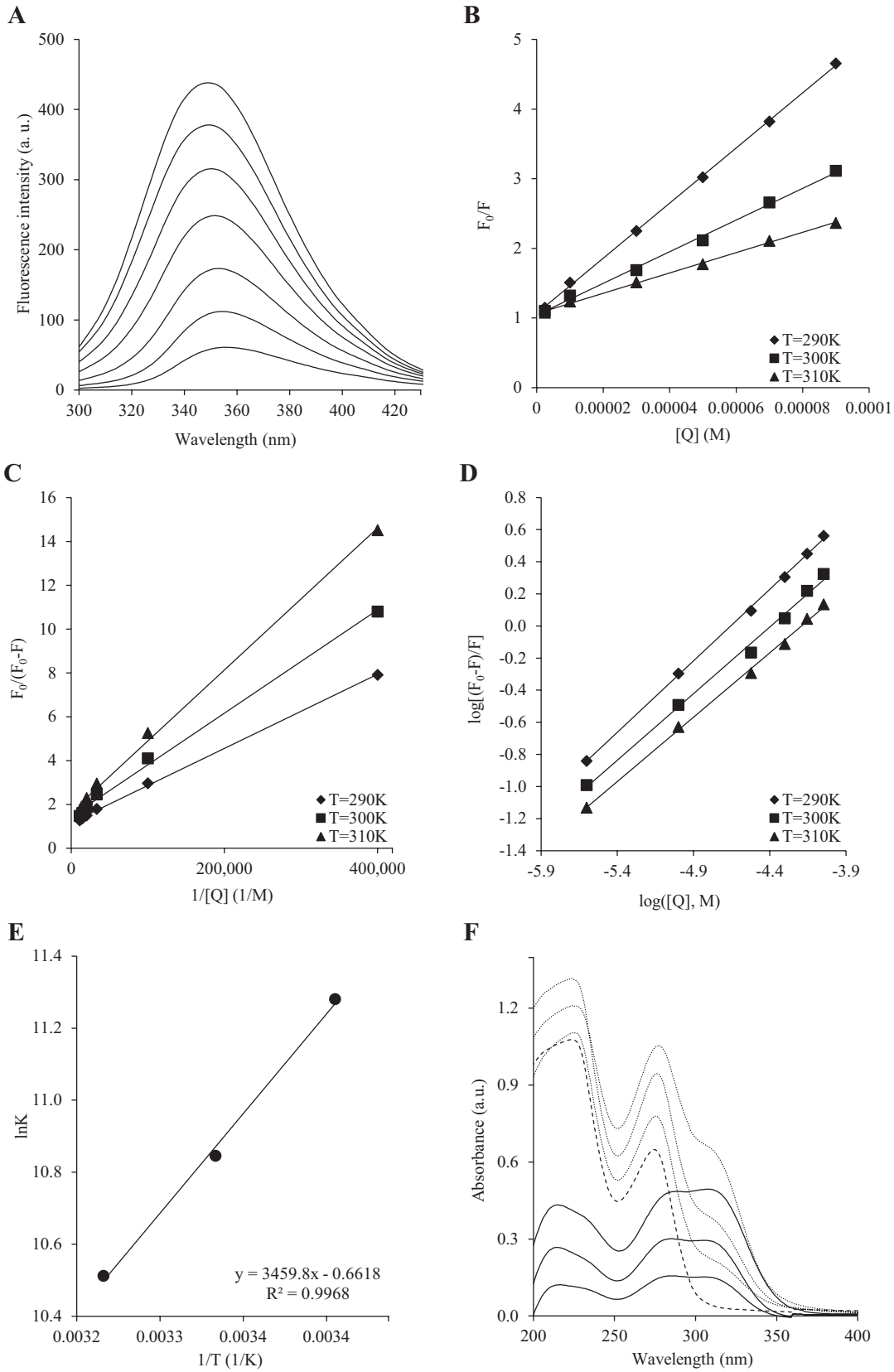

FIGURE 1. Fluorescence emission spectra of 2.5 μM human serum albumin (HSA) mixed with different concentrations of caffeic acid (CA) (λ_{ex}=290 nm) at 310±1 K. From top to bottom, CA concentrations were 0, 2.5, 10, 30, 50, 70, and 90 μM (A). The Stern–Volmer (SV) plots obtained at different temperatures for the fluorescence quenching of HSA by CA (B). Modified SV plots obtained at different temperatures for the CA–HSA complex (C). Logarithmic SV plots used to determine the number of biding sites for CA molecules per HSA molecule (D). The Van't Hoff plot obtained for HSA–CA complex in a phosphate buffer and pH 7.4 used to calculate the thermodynamic parameters (E). UV absorption spectra of HSA in the absence and presence of CA. The solid lines are the spectra of CA in the absence of HSA. The dashed line is the UV absorption spectrum of HSA alone. The dotted lines are HSA spectra in the presence of CA (F).UV-Vis absorption spectra of HSA in the absence spectrum of HSA alone, and the dotted lines represent the spectrum of HSA in the presence of CA (F).

actions between different ligands and HSA using a fluorescence quenching method. For the HSA molecule, its intrinsic fluorescence is related to the Trp residue because of the low quantum yield of Phe or the ionization of Tyr [Mrkalić *et al*., 2021; Sudhamalla *et al*., 2010]. Additionally, the fluorescence of Tyr is generally quenched when it is located in the vicinity of a carboxyl or amino groups, as well as other Trp residue [Jahanban-Esfahlan *et al*., 2017]. Figure 1A shows the fluorescence emission spectra recorded for HSA before and after the addition of different concentrations of CA. The sharp peak near 350 nm was related to the fluorescence emission of HSA. A regular reduction in the fluorescence intensity from the top to the bottom of the recorded peaks was observed in the presence of increasing CA concentrations, conforming the binding of CA to HSA and the quenching of the intrinsic fluorescence intensity of albumin.

Furthermore, upon CA addition, the maximum peak in the obtained HSA emission spectra exhibited a remarkable shift from 348 nm to 357 nm (9 nm), indicating a change in the polarity of the microenvironment surrounding the chromophore of HSA [Nair, 2018]. Similar results have been previously reported [Belatik *et al*., 2012; Cui *at al*., 2004; Mrkalić *et al.,* 2021; Razzak *et al.,* 2019]. This alteration is attributed to the loss of the compact albumin structure in the hydrophobic binding site pocket of subdomain IIA, and the location of Trp residue in this region of the protein is the reason of that alteration [Sułkowska 2002]. In the next steps, the obtained data for fluorescence quenching at a maximum peak of 349 nm and a temperature of 310±1 K were used for the quantitative analysis of CA and HSA interaction.

Fluorescence quenching mechanism analysis

Fluorescence quenching is caused by a reduction in the fluorescence quantum yield resulting from the interactions between the fluorophore and quencher molecules. Commonly, static and dynamic quenching are the two main mechanisms investigated when analyzing the type of fluorescence quenching. Usually, the formation of a ground–state complex without any fluorescence is recognized as static quenching, but a collisional encounter of the fluorophore and quencher is acknowledged as dynamic quenching [Lakowicz, 2006].

As illustrated in Figure 1B, the presented plots derived from the SV equation (2) for HSA in the presence of increasing concentrations of CA at three considered temperatures were linear. Thus, a static quenching mechanism underlies the HSA–CA interaction. Using the slope regression curve of F_0/F *vs*. [Q], K_q and K_{SV} values were obtained as tabulated in Table 1. In dynamic quenching, 10^{10}/M·s is the maximum value for the quenching constant in the scatter collision of various quenchers and biopolymers [Lakowicz & Weber, 1973]. Principally, fluorescence quenching depends on temperature changes, and thus, the main type of quenching is easily distinguished [Nair, 2018]. For the static quenching mechanism, K_{SV} values decreased with increasing temperature, but the opposite results are expected for dynamic quenching. As shown in Table 1, K_{SV} and K_q values clearly decreased as the temperature increased. Additionally, the calculated values for K_q are much larger than 10^{10}/M·s, indicating that the HSA quenching mechanism induced by CA is static quenching and not dynamic quenching. Also other phenolic acids (cinnamic acid), flavonoids (glabridin, diosmetin), and stilbenes (resveratrol) quenched the fluorescence of HSA through the static mode [Nair, 2018; Razzak *et al*., 2019; Sun *et al.,* 2018].

TABLE 1. Stern–Volmer quenching constant (K_{SV}) and a bimolecular quenching rate constant (K_q) for the binding of caffeic acid to human serum albumin at different temperatures.

T (K)	K_{SV} ($\times 10^4$/M)	K_q ($\times 10^{12}$/M·s)	r[a]
290	4.25	4.25	0.9997
300	2.43	2.43	0.9907
310	1.53	1.53	0.9949

[a]r is the linear correlation coefficient.

TABLE 2. Modified Stern–Volmer association constant (K_a) for the caffeic acid–human serum albumin (CA-HSA) interaction at different temperatures (T) and the values for enthalpy (ΔH), entropy (ΔS), and Gibb's free energy (ΔG) which were calculated as thermodynamic parameters for the binding of CA to HSA.

T (K)	K_a ($\times 10^4$/M)	r[a]	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔG (kJ/mol)
290	5.85	0.9992			-26.43
300	3.52	0.9992	-32.75	-21.78	-26.22
310	2.44	0.9996			-26.00

[a]r is the linear correlation coefficient.

TABLE 3. Binding constants (K_b) and the number of binding sites (n) for the interaction of caffeic acid with human serum albumin at different temperatures (T).

T (K)	K_b ($\times 10^4$/M)	n	r[a]
290	2.18	0.93	0.9981
300	1.14	0.92	0.9940
310	0.64	0.91	0.9962

[a]r is the linear correlation coefficient.

As observed in Figure 1C, plotting $F_0/(F_0–F)$ *vs*. 1/[Q] yields $1/f_a$ and $1/(f_aK_a)$ as the y-coordinate and the slope, respectively. Table 2 lists the calculated K_a values for the CA––HSA complex. Similarly, as the temperature increased, the obtained K_a values showed a decreasing trend verifying the decreasing trend observed for the K_{SV} (Table 1).

Binding constant and the number of binding sites

Figure 1D displays the $\log[(F_0–F)/F]$ *vs.* log[Q] plots of the CA–HSA complex investigated at the three temperatures, and Table 3 summarizes the corresponding values calculated for n and K_b. An increase in temperature led to a decrease in the values of K_b. The CA–HSA complex was not stable as the temperature increased, which might explain the decreasing values of K_b. According to the obtained values for the number of binding sites, n values were near unity, indicating that CA had one independent binding site on the HSA

molecule. The results obtained here were in good agreement with the values reported before. For example, comparative studies on the interaction of chlorogenic acid, caffeic acid, and ferulic acid with bovine serum albumin (BSA) using UV absorption spectroscopy, fluorescence spectroscopy, and synchronous fluorescence spectroscopy showed that the binding constant and n values were in the order of 10^4/M and ~1, respectively [Li *et al.,* 2010]. Similar results were also reported for CA when the interactions between different phenolics and BSA were investigated using fluorescence quenching and molecular docking [Skrt *et al.,* 2012].

UV–Vis spectroscopy

The structural changes and an understanding of the formation of a complex between different molecules and proteins are usually investigated with a suitable and effortless spectroscopic technique, such as UV–Vis spectroscopy. Therefore, UV–Vis spectra were recorded, and the results are shown in Figure 1F. At the wavelength of 280 nm, the intensity of the recorded UV–Vis spectra of HSA increased when increasing concentrations of CA were applied. The position of the maxima was also slightly shifted to higher wavelengths. These observations might be related to the change in the polarity and the hydrophobicity around the Trp residue [Ulrich, 1981, 1990; Tao *et al*., 1981], as observed in the fluorescence experiments. Hence, CA binds HSA and induces conformational changes in the albumin structure [Cui *et al*., 2004]. The transfer of energy during the collision between albumin molecules and the interacting substances is mainly mediated by dynamic quenching, with no alterations in the UV–Vis spectrum of albumin. However, for static quenching, the formation of complexes between various chemicals and proteins causes a decrease or an increase in the intensity of the UV–Vis spectrum for albumin [Jahanban-Esfahlan *et al*., 2017; Roufegarinejad *et al*., 2019]. In the present study, HSA fluorescence emission quenching was principally related to the formation of the CA-HSA complex. Thus, the results of fluorescence quenching obtained for CA–HSA were sufficiently supported by the changes in the UV–Vis spectra.

Thermodynamic parameters

Typically, vdW forces, electrostatic forces, H–bonding, and hydrophobic interactions are the key forces driving the interaction of various chemicals and biomacromolecules [Bourassa *et al*., 2011; Dan *et al*., 2019, Nair, 2018; Zou *et al*., 2019]. The changes in Gibb's free energy (ΔG), enthalpy (ΔH), and entropy (ΔS), the main thermodynamic parameters, should be considered to obtain a comprehensive understanding of the complexation mode between different ligands and proteins.

The process underlying the CA and HSA interaction was spontaneous because the value obtained for ΔG was negative, from –26.43 to –26.00 kJ/mol (Table 2). In protein–ligand interactions and according to the studies by Ross & Subramanian [1981], the amounts and the sign of thermodynamic parameters (ΔS and ΔH) are useful for studying the contribution of the main forces to the stability of the ligand–protein complexes. From the thermodynamic perspective, $\Delta H<0$ and $\Delta S>0$ represent support electrostatic forces, ΔH and $\Delta S>0$ suggest hydrophobic interactions, and ΔS and $\Delta H<0$ represent the H–bonding and vdW forces. As presented in Table 2, the calculated values for ΔS and ΔH in this study were –21.78 kJ/mol and –32.75 J/mol·K, respectively. The values calculated for ΔS and ΔH were negative indicating the enthalpy-driven interaction of CA and HSA; however, the entropy was unfavourable. Accordingly, the main driving forces stabilizing the complex were H–bonding and vdW forces.

Energy transfer

Forster resonance energy transfer (FRET) is known as the interaction between molecules with electronically excited states without the emission of a photon. The phenomenon is distance-dependent, and the energy of excitation is transferred from one donor molecule to another acceptor molecule [Lakowicz, 2006]. Several factors affect the FRET efficiency, such as: (1) the overlap region of the acceptor UV–Vis spectra and the donor emission, (2) the transition dipole orientation of the donor and acceptor, and (3) the distance between the donor and acceptor. The overlap in the spectroscopic region between the UV–Vis absorption spectrum of CA and the emission spectrum of HSA fluorescence is shown in Figure 1A.

Using equations (7), (8), and (9), $J=1.6637\times10^{-19}$ cm^3/M, $E=0.075$, $R_0=3.6616$ nm, and $r=1.92$ nm were calculated for the CA–HSA complex. Here, the calculated value of r for the interaction of CA with HSA was consistent with the value reported in a previous study [Li *et al*., 2010]. The probability of energy transfer is high when the average distance of the acceptor and donor molecules is less than 8 nm [Samari *et al*., 2012]. The calculated value for the distance between the donor molecule (Trp residue) in HSA and the acceptor molecules (the interacting CA) was approximately 2 nm. Additionally, the calculated values for r and R_0 followed the rule $0.5R_0<r<1.5R_0$ [Jahanban-Esfahlan *et al*., 2017; Roufegarinejad *et al.,* 2019.] Thus, energy was transferred during the CA and HSA interaction.

Molecular docking

CA was docked to HSA using ArgusLab software to determine the ideal binding site and the binding mode. ArgusLab is a useful docking program that performs computational molecular docking to provide researches an understanding of the interaction of different molecules with albumin. The best conformational binding mode for the interaction of CA and HSA is displayed in Figure 2B. CA binds to another site of the HSA molecule that differs from the known binding sites I and II. CA interacts with HSA within subdomain IA in domain I. Figure 2C shows the H–bonds that formed between the CA molecule and the amino acids of HSA. The amino acid residues Asn–9, Gly–248, Asp–249, Leu–251, and Glu–252 formed H–bonds with the CA molecule. Moreover, six other amino acid residues of HSA, including Tyr–30, Phe–102, Gly–71, Leu–250, His–67 and Leu–74, surrounded the CA molecule in its binding site (these amino acids are not shown in the figure). The binding energy for CA-HSA complex was determined to be –9.75 kcal/mol from the docking calculations. Both hydrophobic and hydrophilic

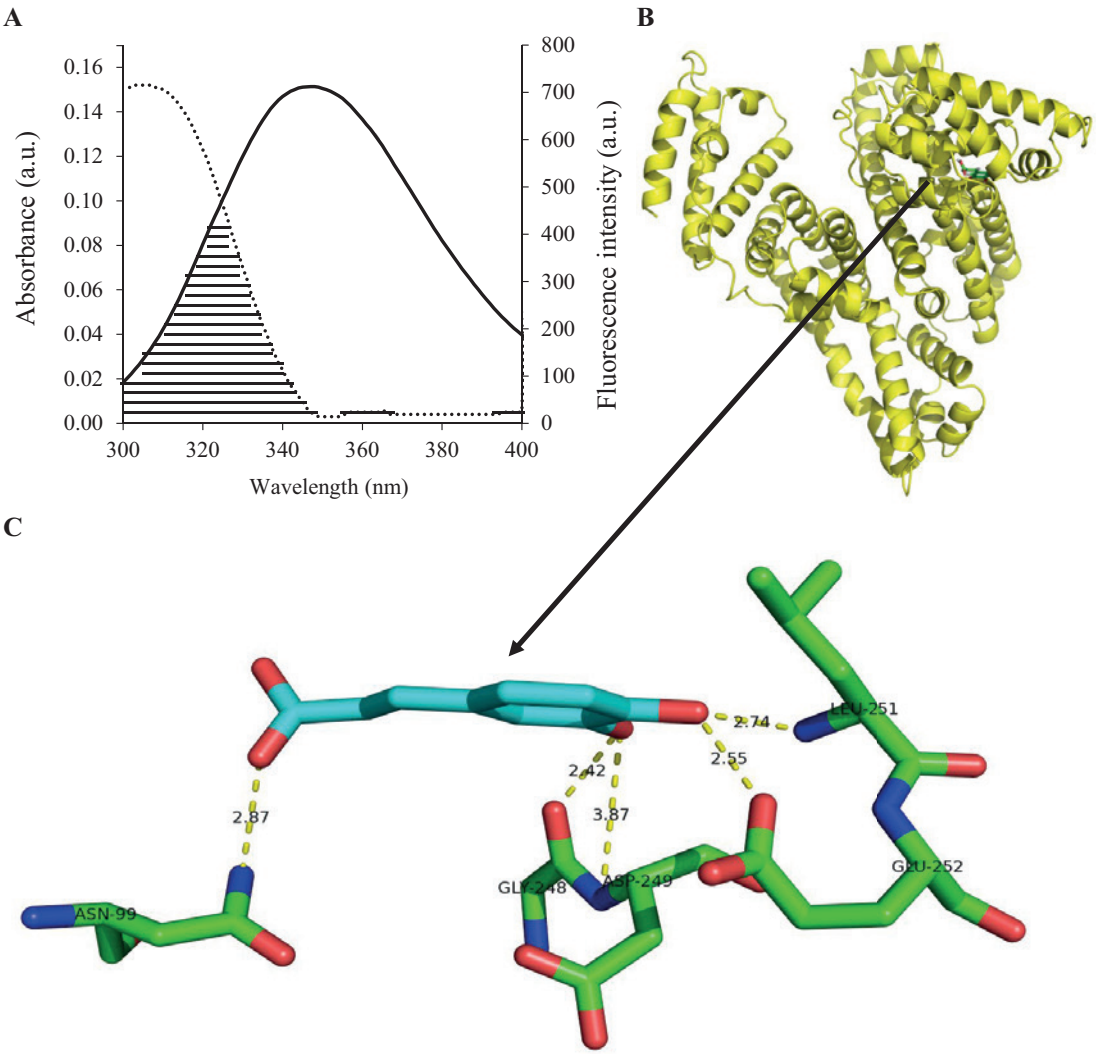

FIGURE 2. The overlap between the UV–Vis absorption spectrum of caffeic acid (CA) with human serum albumin (HSA) fluorescence emission spectrum at 25°C. The concentrations of CA and HSA were 30 μM. The spectrum of CA is shown as a solid dark line, and the spectrum of HSA is depicted as the dotted line. The overlap between the CA and HSA spectra is presented as the shaded part (A). CA docked with HSA, as illustrated in a cartoon image. CA and HSA molecules are represented in stick mode and yellow cartoon models, respectively (B). H–bonds between HSA amino acids and the docked CA molecule. Yellow dashed lines show H–bonds and the corresponding distances. The CA structure and amino acids are displayed in stick mode. Hydrogen atoms are not shown (C).

amino acids were located in the binding site of CA. Finally, molecular docking studies revealed the formation of numerous H–bonds in the CA-HSA confirming the results of fluorescence experiments.

CONCLUSIONS

In the present work, the interactions between CA and HSA have been analyzed using fluorescence spectroscopy, UV–Vis spectroscopy, and molecular docking methods, and numerous binding parameters have been obtained. A static quenching mechanism was identified for the intrinsic quenching of the HSA fluorescence by CA. The obtained values for the number of binding sites showed the presence of a single class of binding sites for the CA molecule on HSA. The interaction of CA and HSA was determined to be enthalpy-driven and spontaneous. The main driving forces stabilizing the complex were H–bonds and vdW forces. The molecular docking calculations indicated that CA binds to HSA in subdomain IA of domain I of HAS, and the detected H–bonds confirmed the results of fluorescence spectroscopy. According to the results of fluorescence and UV spectroscopy, CA induced conformational changes in the albumin structure.

Caffeic acid shows a high affinity for albumin, and thus this phenolic compound would be distributed in the body upon interacting with HSA.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

ORCID IDs

R. Amarowicz https://orcid.org/0000-0001-9731-0045
A. Jahanban-Esfahlan https://orcid.org/0000-0001-8693-3837
J.M. Lorenzo https://orcid.org/0000-0002-7725-9294

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Original article
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Starches Modified by Combination of Phosphorylation and High-Voltage Electrical Discharge (HVED) Treatment

Ivanka Grgić[1], Marijana Grec[2], Artur Gryszkin[3], Tomasz Zięba[3], Mirela Kopjar[2], Đurđica Ačkar[2], Antun Jozinović[2], Borislav Miličević[2,4], Sandra Zavadlav[5], Jurislav Babić[2]*

[1]*Institute of Public Health Brod-Posavina County, V. Nazora 2A, 35000 Slavonski Brod, Croatia*
[2]*Department of Food Technologies, Faculty of Food Technology Osijek, Josip Juraj Strossmayer University of Osijek, F. Kuhača 18, 31000 Osijek, Croatia*
[3]*Department of Food Storage and Technology, Wrocław University of Environmental and Life Sciences, ul. Chełmońskiego 37/41, 51–630 Wrocław, Poland*
[4]*Polytechnic in Požega, Vukovarska ulica 17, 34000 Požega, Croatia*
[5]*Department of Food Technology, Karlovac University of Applied Sciences, Trg J. J. Strossmayera 9, 47000 Karlovac, Croatia*

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Starch is extensively used in the food industry as a texture modifier, a fat substitute, and in other applications. To optimise starch functional properties for specific use, it is subjected to various modifications. High-voltage electrical discharge (HVED) treatment, as a non-thermal and rapid process, was applied in this research as a single method and in combination with phosphorylation in order to explore its potential for improving starch physicochemical properties. Maize, wheat, potato, and tapioca starches were modified, and $Na_5P_3O_{10}$ and Na_2HPO_4 were used for phosphorylation. Starch gelatinisation parameters (by DSC); paste clarity; and contents of amylose, damaged starch, and resistant starch were determined; and FTIR-ATR spectra were recorded. All modifications reduced the enthalpy of gelatinisation and decreased contents of amylose, resistant starch, and damaged starch. The effect of the HVED treatment on starch properties depended on starch type and combinations with chemicals. HVED could act as an aid in the starch phosphorylation process since the properties analysed were more effectively improved when HVED was combined with phosphorylation than by phosphorylation alone.

ABBREVIATIONS

HVED – High-voltage electrical treatment, DSC – Differential scanning calorimetry, FTIR-ATR – Fourier-transform infrared spectroscopy-attenuated total reflectance, and % T – % transmittance.

INTRODUCTION

Starch has an already well established role in food, pharmaceutical, paper, and packaging industries; however, in its native form it often does not come up to desired functional properties. Therefore, different modifications have been applied to improve its properties, including mainly chemical processes. Increasing demands for reducing chemical usage, waste generation, and energy exploitation have led to novel technique applications in all aspects of food processing and production. High-voltage electrical discharge (HVED) treatment has emerged as one of the non-thermal and rapid techniques applicable in decontamination of food, waste treatment, and extraction of bioactive compounds [Barišić *et al.*, 2020]. It is a process of releasing energy between two electrodes submerged in a liquid medium [Rosello-Soto *et al.*, 2015], which leads to the disruption of organic cells and enhanced extraction of different compounds. In addition, electrical discharge causes the formation of electrically-charged compounds (ions, free radicals), which induce chemical changes of the treated material. Most often, a pin-to-plate configuration is used, generating direct current glow or pulsed corona [Vanraes *et al.*, 2016]. If gas is available (air, N_2, CO_2, Ar, *etc.*) during the HVED treatment, plasma will be generated. There is a significant number of articles dealing with the influence of cold plasma on starch properties, with reported changes in starch structure, accompanied with cross-linking and/or formation of new functional groups [Bie *et al.*, 2016a,b; Khorram *et al.*, 2015; Thirumdas *et al.*, 2017]. However, to the best of authors' knowledge, there is no relevant data on the influence of other types of HVED or combination of HVED with chemical

* Corresponding Author:
E-mail: dackar@ptfos.hr (Đ. Ačkar)

modification of starch. The aim of this research was, therefore, to explore if HVED treatment in a water suspension induces changes in the physicochemical properties of starch, how it reflects on starch properties, and if it may be used as an aid in chemical modification of starch to increase process efficiency, by activating starch molecules. Phosphorylation of starch with $Na_5P_3O_{10}$ or Na_2HPO_4 was chosen for the research because it has been reported that these modifications also result in a combined effect of cross-linking and substitution [Sechi & Marques, 2017]. The additional treatment of HVED could favour one of the reactions, enabling easier control of the process and obtaining a desired compound (cross-linked or substituted).

MATERIAL AND METHODS

Materials and chemicals

Tapioca, potato, and maize starches were kindly provided by Cargill (Minneapolis, MN, USA). Wheat starch was isolated at the Faculty of Food Technology Osijek, Josip Juraj Strossmayer University of Osijek (Croatia) as described in our previous paper [Ačkar *et al*., 2010]. $Na_5P_3O_{10}$ or Na_2HPO_4 used for phosphorylation were obtained from Acros Organics (Geel, Belgium). Resistant starch assay kit, amylose/amylopectin assay kit, and starch damage assay kit were purchased in Megazyme (Bray, Ireland).

High-voltage electrical discharge (HVED) treatment of starch

Starch was suspended in demineralised water (1 g/mL) and treated with HVED (30 kV, 70 Hz, 30 min) with stirring at a magnetic stirrer. The HVED device was custom-made for the Faculty of Food Technology in Osijek by Inganiare CPTS1. It consists of a chamber, containing a stainless steel needle (diameter 2.5 mm) and a plate electrode (diameter 45 mm), connected to a high-voltage pulse generator. The distance between the electrodes during all treatments was 2 cm. After treatment, the starch suspension was centrifuged at 3000 rpm/5 min (Centra MP4R, IEC, Needham Heights, MA, USA) to remove excess water, air-dried overnight, and then dried in the oven at 130°C until the moisture content of <85 g/100 g was achieved.

Phosphorylation of starch with $Na_5P_3O_{10}$

Starch was phosphorylated with $Na_5P_3O_{10}$ according to the method described by Lim & Seib [1993]. Briefly, 2.5 g of $Na_5P_3O_{10}$ and 2.5 g of Na_2SO_4 was dissolved in 200 mL of demineralised water. Next, 100 g of starch was suspended in the solution by stirring at a magnetic stirrer for 20 min. The pH value of the suspension was set to 10.0 by drop-wise adding of NaOH (0.45 M), and the suspension was stirred for another hour. Then, it was centrifuged at 3000 rpm for 5 min (IEC Centra MP4R), water was discarded, and starch was air-dried overnight and thermally-treated at 130°C/2 h. Starch was then suspended in 250 mL of water, centrifuged, and washed three times with water. Thus obtained starch was dried at ambient temperature until the moisture content of <85 g/100 g was achieved.

Phosphorylation of starch with Na_2HPO_4

Phosphorylation with Na_2HPO_4 was conducted according to the method described by Sung *et al.* [2005] and Prasanthi & Rama Rao [2010] with slight modifications. Briefly, starch (100 g) and Na_2HPO_4 were suspended in 200 mL of demineralised water and stirred at a magnetic stirrer for 30 min. The suspension was centrifuged, starch was thermally treated and washed as described above.

Combinations of HVED treatment and phosphorylations

When starch was first treated with HVED, a suspension was prepared as described in the "High-voltage electrical discharge (HVED) treatment of starch" section and then phosphorylated with $Na_5P_3O_{10}$ or Na_2HPO_4. When starch was first phosphorylated, the chemical modification with $Na_5P_3O_{10}$ or Na_2HPO_4 was done prior to the HVED treatment.

Differential scanning calorimetry (DSC) analysis

A DSC 822E differential scanning calorimeter (Mettler Toledo, Giessen, Germany) was used to determine gelatinisation parameters of starches. Samples were analysed without pretreatment, as dry powders. Starch (on dry matter – d.m. – basis) and water were mixed in a ratio of 1:3 (*w/w*) in standard Al-crucibles (40 μL), hermetically sealed, and equilibrated at ambient conditions for 20 min. An empty aluminium pan was used as a reference. Starch samples were heated at the rate of 4°C/min from 25°C to 100°C and changes in enthalpy (ΔH in J/g d.m. of starch), onset temperature (t_o), peak temperature (t_p), and endset temperature (t_e) were obtained from the exotherm DSC curves. Analyses were done in triplicates.

Paste clarity determination

Paste clarity was determined (in triplicates) according to the method described by Raina *et al.* [2006]. To this end, 1% starch suspensions (on starch d.m. basis) were heated for 30 min in boiling shaking water bath (200 rpm). After 1-h holding at room temperature, % transmittance (%T) was measured at 650 nm against distilled water as a blank (Camspec M501 spectrophotometer, Leeds, UK).

Determination of contents of amylose, damaged starch, and resistant starch

Megazyme kits were used for analyses. Amylose content was determined according to the method described by Gibson *et al.* [1997]. Amylopectin was precipitated with concanavalin A and separated from amylose by centrifugation. Both amylose and amylopectin were enzymatically hydrolysed to glucose, which was measured spectrophotometrically (Camspec M501 spectrophotometer) after reaction with glucose oxidase with peroxidase and 4-aminoantipyrine reagent. Damaged starch content was determined according to the American Association of Cereal Chemists Method 76–31.01 [AACC, 2010] and resistant starch content according to the Association of Official Analytical Chemists Method 2002.02 [AOAC, 2005]. The contents of amylose (%), damaged starch (%), and resistant starch (g/100 g d.m.) were determined in triplicates.

Fourier-transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) analysis

FTIR-ATR spectra were recorded using a Cary 630 spectrometer (Agilent, Santa Clara, CA, USA) in the range of 4000–650 cm^{-1}. In total, 32 spectra of each sample were recorded and averaged with a spectral resolution of 16 cm^{-1}. Samples were analysed without pretreatment, as dry powders.

Statistical analysis

All modifications were repeated three times, and for each modification one composite sample was made and taken for further analysis. All analyses were done in triplicates, and the results obtained were statistically analysed by the analysis of variance and Fischer LSD test ($p<0.05$) in Statistica® 13 software (TIBCO Software Inc., Palo Alto, CA, USA). Results are expressed as mean value ± standard deviation.

RESULTS AND DISCUSSION

In order to explore HVED influence on starch properties and the potential of combining HVED with chemical modifications, four starch types (maize, wheat, potato, and tapioca) were treated with HVED alone, phosphorylated ($Na_5P_3O_{10}$ or Na_2HPO_4) alone, and phosphorylated in combination with HVED prior or after the chemical modification. Gelatinisation parameters (t_0, t_p, t_e, and ΔH), paste clarity, FTIR-ATR spectra, as well as contents of amylose, damaged starch, and resistant starch were determined to evaluate the effect of the treatments.

The gelatinisation parameters of native and modified starches are shown in Table 1. Generally, the HVED treatment resulted in a decrease in gelatinisation temperatures (with the exception of endset temperature of maize starch), but the change was not always statistically significant. Similarly, although not always statistically significant, the enthalpy of gelatinisation of the HVED-treated starches was lower compared to that of native ones (except for tapioca starch, where it was higher). This implies that the HVED treatment could cause changes in the morphology of starch granules. Other researchers reported the formation of fissures and cavities [Thirumdas *et al.*, 2017], enlargement of channels, and partial fractioning of starch granules [Bie *et al.*, 2016a]. All these changes facilitate water penetration into the granules and gelatinisation, reflected in decreased gelatinisation temperature and enthalpy. The difference observed for tapioca starch after the HVED treatment regarding gelatinisation enthalpy (Table 1) may be explained by low contents of fat, protein, and ash; a lower content of amylose; and higher molecular weights of amylose and amylopectin compared to all other starches [BeMiller & Whistler, 2009], which probably makes it less sensitive to granule damage.

Changes in the chemical structure have been reported as a result of plasma treatment as well. Depending on plasma type, treatment conditions, and starch type, depolymerisation, cross-linking, and formation of new functional groups have been reported [Bie *et al.*, 2016b; Khorram *et al.*, 2015]. The HVED treatment applied in this research is not as severe as the plasma treatment in the manner of energy input over time. However, the time was prolonged compared to the plasma treatment (which typically lasts from few seconds to several minutes), and this may have induced a low degree of depolymerisation, as indicated by the reduction in amylose content (Table 2), which, in turn may have contributed to the observed changes in gelatinisation parameters.

Both chemical modifications applied in this research (with $Na_5P_3O_{10}$ and Na_2HPO_4) resulted in decreased enthalpy of gelatinisation of most starch types (Table 1). Phosphorylation with Na_2HPO_4 had a more pronounced effect, probably because the smaller molecule of this reagent than $Na_5P_3O_{10}$ penetrated more easily through the active sites of starch. The decrease in gelatinisation enthalpy is typical of substitution reactions, along with a reduced content of resistant starch observed for all analysed samples, and increased paste clarity observed for maize and wheat starch paste (Table 2). Xie & Shao [2009] also found that mainly starch monoesters were formed over diesters during corn starch phosphorylation.

For most samples, the enthalpy of gelatinisation of the HVED-treated starches before chemical modifications was lower than that of only phosphorylated starches (Table 1). This indicates that starch phosphorylation may have been enhanced by the prior activation of starch molecules through the reaction with free radicals generated in water by HVED [Thirumdas *et al.*, 2018]. However, temperatures of gelatinisation did not follow this trend (Table 1), *i.e.* the HVED-treated maize starch phosphorylated with Na_2HPO_4 had significantly higher gelatinisation temperatures than the one that was only phosphorylated with Na_2HPO_4. Since reactions of phosphorylation involve electrically-charged compounds [Sechi & Marques, 2017], the formation of compounds by starch reaction with free radicals formed by HVED in water does not necessarily imply the activation of a starch granule towards the phosphorylation reaction (the phosphorylation reaction would be enhanced only if the cationic character of starch increases).

When phosphorylated starches were treated with HVED, the enthalpy of gelatinisation was lower compared to both HVED-treated and chemically-modified counterparts with a more pronounced decrease when Na_2HPO_4 was used (Table 1). It is apparent that the increase of the anionic character of starch makes it more prone to the reaction with active compounds formed in water by HVED.

Paste clarity is a physical property of starch that is also indicative of its water binding affinity. Substituted starches generally form more clear pastes than native counterparts due to more extensive hydration of granules, while cross-linked starches form more opaque pastes compared to native ones [Bhandari & Singhal, 2002]. Paste clarity of the investigated starches is shown in Table 2. It is evident that the HVED-treatment resulted in the increase of paste clarity of maize and potato starches, and its decrease for tapioca and wheat starches, although the statistical analysis does not show significance in all cases. The most significant change (decrease) was observed for tapioca starch, and this is consistent with the increase of gelatinisation enthalpy observed for native and HVED-treated tapioca starch (Table 1). Tapioca starch tends to form clearer pastes than cereal starches (which is observed in this research as well) and, unlike potato starch, it has a large number of thin layers of polymers

TABLE 1. Parameters of gelatinisation of starches modified by high-voltage electrical discharge (HVED), phosphorylation with $Na_5P_3O_{10}$, and Na_2HPO_4, and combination of the processes obtained using differential scanning calorimetry (DSC).

	Modification	t_o (°C)	t_p (°C)	t_e (°C)	ΔH (J/g)
Maize	Native	64.90±0.15^{c}	70.24±0.18^{c}	75.06±0.22^{c,d}	12.10±0.40^{a}
	HVED	64.71±0.03^{c}	70.13±0.19^{c}	75.42±0.18^{c}	11.74±0.07^{a,b}
	$Na_5P_3O_{10}$	63.32±0.05^{d}	69.40±0.11^{c,d}	74.87±0.18^{d,e}	11.19±0.20^{b,c}
	HVED+$Na_5P_3O_{10}$	63.53±0.28^{d}	69.19±0.01^{d}	74.44±0.06^{e}	12.31±0.03^{a}
	$Na_5P_3O_{10}$+HVED	63.63±0.16^{d}	69.16±0.12^{d}	74.47±0.19^{e}	10.96±0.43^{c,d}
	Na_2HPO_4	67.72±0.13^{a}	72.52±0.24^{b}	77.65±0.02^{b}	10.92±0.15^{c,d}
	HVED+Na_2HPO_4	69.12±0.11^{b}	74.39±0.02^{a}	79.19±0.04^{a}	10.24±0.08^{d}
	Na_2HPO_4+HVED	69.12±0.21^{b}	74.38±0.29^{a}	79.09±0.49^{a}	10.25±0.01^{d}
Wheat	Native	55.91±0.13^{C}	60.33±0.10^{C}	64.92±0.27^{C}	11.09±0.17^{A}
	HVED	55.60±0.01^{C}	60.03±0.01^{C,D}	64.56±0.12^{C}	10.25±0.18^{B}
	$Na_5P_3O_{10}$	55.00±0.22^{D}	59.38±0.14^{D,E}	63.84±0.03^{D}	9.66±0.27^{B,C}
	HVED+$Na_5P_3O_{10}$	54.42±0.14^{E}	59.04±0.33^{E}	63.20±0.03^{E}	9.05±0.51^{C,D}
	$Na_5P_3O_{10}$+HVED	53.97±0.07^{F}	58.74±0.17^{E}	63.40±0.07^{D,E}	9.46±0.18^{C}
	Na_2HPO_4	58.74±0.04^{B}	63.33±0.01^{B}	67.82±0.23^{B}	9.14±0.66^{C,D}
	HVED+Na_2HPO_4	60.14±0.07^{A}	65.16±0.70^{A}	69.08±0.09^{A}	7.90±0.16^{E}
	Na_2HPO_4+HVED	59.85±0.10^{A}	64.39±0.04^{A}	68.87±0.09^{A}	8.60±0.16^{D,E}
Potato	Native	57.23±0.21^{i}	63.12±2.07^{i}	66.33±0.08^{i}	18.68±0.15^{i}
	HVED	56.96±0.14^{i}	61.35±0.31^{ii}	66.36±0.48^{i}	17.88±0.36^{ii}
	$Na_5P_3O_{10}$	52.71±0.05^{iii}	57.17±0.09^{v}	62.15±0.06^{iv}	15.53±0.62^{iii}
	HVED+$Na_5P_3O_{10}$	52.66±0.23^{iii}	57.09±0.01^{v}	62.15±0.37^{iv}	15.15±0.19^{iii}
	$Na_5P_3O_{10}$+HVED	51.96±0.08^{iv}	56.62±0.07^{v}	61.75±0.08^{iv}	15.51±0.67^{iii}
	Na_2HPO_4	52.77±0.14^{iii}	58.10±0.21^{iv}	63.51±0.14^{iii}	14.04±0.51^{iv}
	HVED+Na_2HPO_4	54.10±0.01^{ii}	59.42±0.01^{iii}	64.57±0.04^{ii}	12.95±0.02^{v}
	Na_2HPO_4+HVED	54.10±0.22^{ii}	59.43±0.05^{iii}	64.79±0.24^{ii}	13.36±0.36^{iv,v}
Tapioca	Native	63.48±0.30^{III}	69.74±0.12^{II}	77.82±0.04^{II}	10.55±0.64^{V}
	HVED	61.56±0.18^{IV}	67.25±0.40^{III}	75.17±0.14^{IV}	15.19±0.29^{I}
	$Na_5P_3O_{10}$	59.85±0.06^{V}	66.04±0.05^{IV}	73.19±0.12^{V}	14.72±0.30$^{I, II}$
	HVED+$Na_5P_3O_{10}$	59.96±0.02^{V}	65.88±0.04^{IV}	73.03±0.20^{V}	14.15±0.47^{II}
	$Na_5P_3O_{10}$+HVED	59.73±0.01^{V}	65.83±0.11^{IV}	73.24±0.23^{V}	14.47±0.19^{IV}
	Na_2HPO_4	64.29±0.02^{II}	70.20±0.02^{II}	77.16±0.01^{III}	13.29±0.17^{III}
	HVED+Na_2HPO_4	66.01±0.29^{I}	71.83±0.62^{I}	78.70±0.64^{I}	12.13±0.41^{IV}
	Na_2HPO_4+HVED	65.77±0.21^{I}	71.76±0.07^{I}	78.77±0.14^{I}	12.03±0.47^{IV}

t_0, onset temperature; t_p, peak temperature; t_e, endset temperature; ΔH, enthalpy of gelatinisation. The results are expressed as mean±standard deviation (n=3). Values with different superscripts in the same column are different for the same starch type ($p<0.05$).

[Banura *et al.*, 2018]. This may be the reason for the unique effect of HVED on its clarity. However, Banura *et al.* [2018] reported that plasma treatment increased the swelling capacity of both maize and tapioca starches, which would imply that clarity should also increase. In turn, Bie *et al.* [2016a] reported that glow plasmas broke the hydrogen bonding network at the molecular level of tapioca starch and change starch crystallites. Unlike these researches, results obtained in the present study (increased enthalpy of gelatinisation, reduced paste clarity) imply that HVED, unlike plasma treatment, causes better alignment of starch chains and more ordered structural organisation of tapioca starch polymers.

TABLE 2. Paste clarity, contents of amylose, damaged starch, and resistant starch of starches modified by high-voltage electrical discharge (HVED) treatment, phosphorylation with $Na_5P_3O_{10}$, and Na_2HPO_4, and combination of the processes.

	Modification	Paste clarity (%T)	Amylose (%)	Damaged starch (%)	Resistant starch (g/100 g d.m.)
Maize	Native	8.31 ± 0.28^{d}	15.80 ± 0.05^{a}	2.41 ± 0.08^{a}	2.40 ± 0.54^{a}
	HVED	8.97 ± 0.16^{d}	15.20 ± 0.06^{a}	0.98 ± 0.04^{b}	$2.06\pm0.34^{a,b}$
	$Na_5P_3O_{10}$	18.90 ± 0.42^{a}	14.19 ± 0.11^{b}	0.82 ± 0.01^{d}	$1.04\pm0.89^{a,b}$
	HVED+$Na_5P_3O_{10}$	13.15 ± 0.21^{c}	$13.88\pm0.14^{b,c}$	1.02 ± 0.04^{b}	$0.80\pm0.19^{a,b}$
	$Na_5P_3O_{10}$+HVED	16.15 ± 0.07^{b}	12.54 ± 0.18^{d}	0.89 ± 0.00^{c}	$1.48\pm0.53^{a,b}$
	Na_2HPO_4	9.94 ± 0.03^{d}	13.15 ± 0.62^{d}	$0.86\pm0.03^{c,d}$	0.59 ± 0.06^{b}
	HVED+Na_2HPO_4	8.73 ± 0.06^{d}	$13.50\pm0.57^{b,c}$	0.68 ± 0.02^{e}	0.52 ± 0.16^{b}
	Na_2HPO_4+HVED	9.43 ± 0.01^{d}	15.75 ± 0.33^{a}	0.54 ± 0.00^{f}	0.50 ± 0.01^{b}
Wheat	Native	8.60 ± 0.03^{C}	20.27 ± 0.32^{A}	0.64 ± 0.01^{C}	0.66 ± 0.01^{A}
	HVED	7.66 ± 0.19^{C}	17.03 ± 0.93^{D}	0.67 ± 0.06^{C}	0.22 ± 0.01^{A}
	$Na_5P_3O_{10}$	17.50 ± 0.42^{A}	$18.12\pm0.02^{B,C}$	0.83 ± 0.05^{A}	0.29 ± 0.02^{A}
	HVED+$Na_5P_3O_{10}$	11.45 ± 0.64^{B}	$17.96\pm0.15^{C,D}$	0.75 ± 0.01^{B}	0.29 ± 0.17^{A}
	$Na_5P_3O_{10}$+HVED	11.90 ± 0.28^{B}	20.73 ± 0.91^{A}	0.75 ± 0.04^{B}	0.18 ± 0.01^{A}
	Na_2HPO_4	12.75 ± 0.07^{B}	19.04 ± 0.01^{B}	0.45 ± 0.00^{D}	0.15 ± 0.03^{A}
	HVED+Na_2HPO_4	11.70 ± 0.00^{B}	16.03 ± 0.21^{E}	0.35 ± 0.02^{E}	0.17 ± 0.00^{A}
	Na_2HPO_4+HVED	12.35 ± 0.35^{B}	$17.52\pm0.52^{C,D}$	0.37 ± 0.04^{E}	0.16 ± 0.01^{A}
Potato	Native	87.10 ± 0.57^{ii}	14.14 ± 1.54^{i}	1.89 ± 0.02^{i}	69.50 ± 3.09^{i}
	HVED	91.15 ± 0.35^{i}	11.87 ± 0.16^{ii}	0.35 ± 0.02^{iii}	69.39 ± 0.00^{i}
	$Na_5P_3O_{10}$	63.15 ± 0.92^{ii}	11.77 ± 0.10^{ii}	0.42 ± 0.03^{ii}	34.32 ± 0.99^{v}
	HVED+$Na_5P_3O_{10}$	82.00 ± 3.25^{iii}	9.73 ± 0.25^{iii}	$0.25\pm0.02^{iv,v}$	46.52 ± 0.39^{ii}
	$Na_5P_3O_{10}$+HVED	77.90 ± 0.99^{iv}	9.45 ± 0.18^{iii}	0.42 ± 0.03^{ii}	38.24 ± 1.29^{iv}
	Na_2HPO_4	28.20 ± 0.28^{iv}	11.63 ± 0.71^{ii}	0.41 ± 0.01^{ii}	47.44 ± 1.77^{ii}
	HVED+Na_2HPO_4	23.90 ± 0.14^{vii}	11.66 ± 0.02^{ii}	0.22 ± 0.01^{v}	41.57 ± 0.04^{iii}
	Na_2HPO_4+HVED	24.05 ± 0.35^{vii}	12.05 ± 0.20^{ii}	0.29 ± 0.02^{iv}	4.48 ± 0.08^{vi}
Tapioca	Native	48.85 ± 0.35^{I}	12.85 ± 0.36^{I}	0.02 ± 0.00^{V}	15.15 ± 0.10^{I}
	HVED	40.40 ± 1.70^{III}	$10.73\pm0.13^{II,III}$	0.01 ± 0.00^{V}	5.42 ± 0.57^{IV}
	$Na_5P_3O_{10}$	38.20 ± 0.57^{IV}	7.29 ± 0.10^{IV}	0.18 ± 0.00^{II}	3.16 ± 2.26^{V}
	HVED+$Na_5P_3O_{10}$	40.15 ± 0.21^{III}	10.06 ± 0.33^{III}	0.34 ± 0.03^{I}	11.30 ± 0.12^{II}
	$Na_5P_3O_{10}$+HVED	44.00 ± 0.99^{II}	10.99 ± 0.00^{III}	0.29 ± 0.01^{I}	2.81 ± 0.59^{V}
	Na_2HPO_4	26.95 ± 0.49^{V}	$10.17\pm0.04^{II,III}$	$0.13\pm0.01^{II,III}$	3.32 ± 0.05^{V}
	HVED+Na_2HPO_4	27.65 ± 0.35^{V}	$10.19\pm0.11^{II,III}$	$0.09\pm0.01^{II,III}$	$9.97\pm0.06^{II,\ III}$
	Na_2HPO_4+HVED	28.80 ± 0.85^{V}	11.52 ± 0.54^{II}	$0.04\pm0.01^{IV,V}$	9.54 ± 0.00^{III}

%T, transmittance of 1% starch suspension measured at 650 nm against distilled water. The results are expressed as mean±standard deviation (n=3). Values with different superscripts in the same column are different for the same starch type ($p<0.05$).

Generally, phosphorylation induced an increase of paste clarity of the investigated cereal starches, and a decrease of this property for tuber starches, as shown in Table 2. Here, a more pronounced effect on the increase of paste clarity in tuber starches was observed for starches phosphorylated with $Na_5P_3O_{10}$, than with Na_2HPO_4, probably due to larger molecules which keep starch chains more spaced and make light passing easier. Tuber starches, like potato and tapioca ones, are characterised by larger granules and a higher degree of polymerisation of amylose than maize and wheat starches [Banura *et al.*, 2018], which may be the reason for the observed opposite trend in paste clarity.

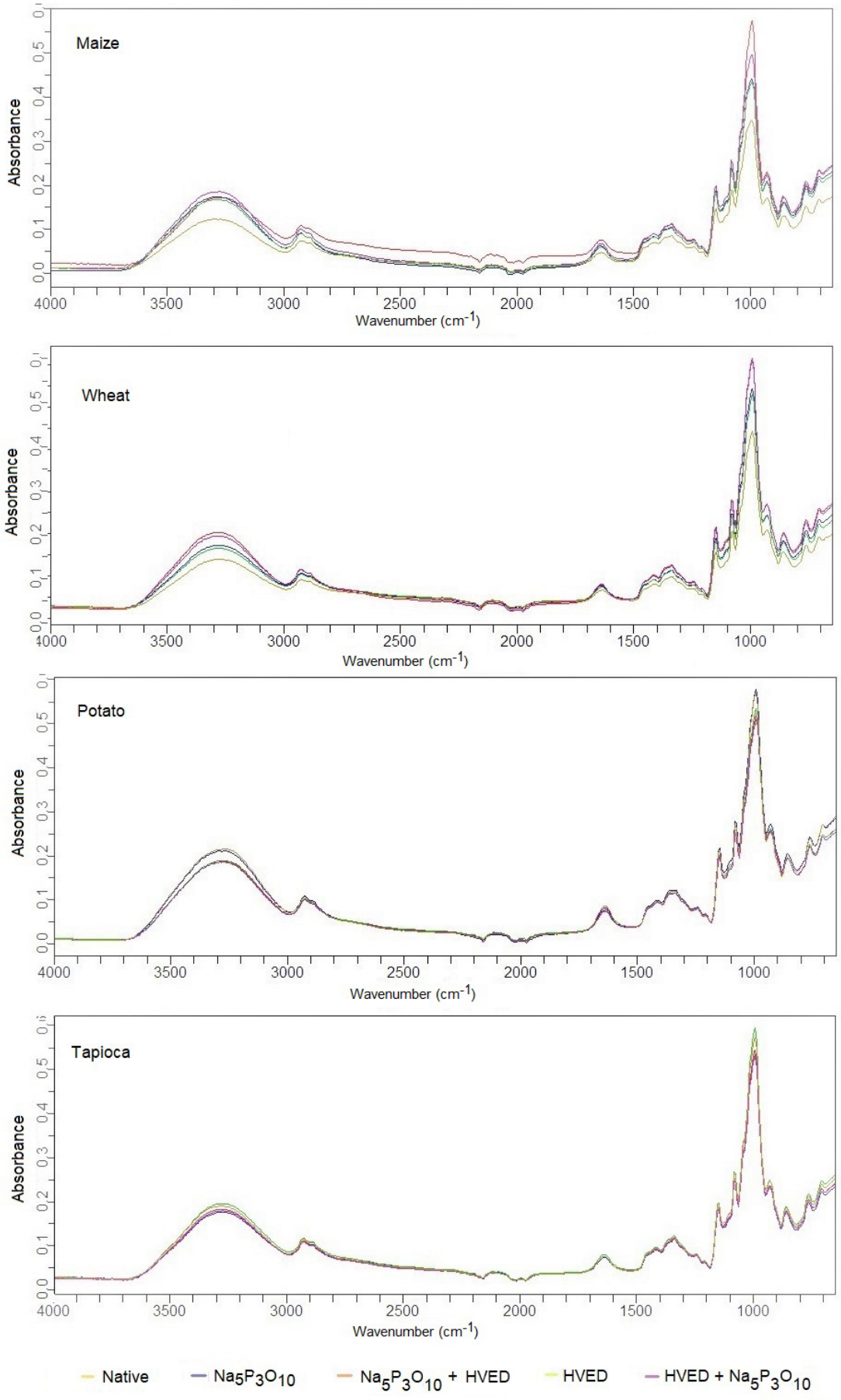

FIGURE 1. Fourier-transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) spectra of starches modified with $Na_5P_3O_{10}$, with and without high-voltage electrical discharge (HVED) treatment.

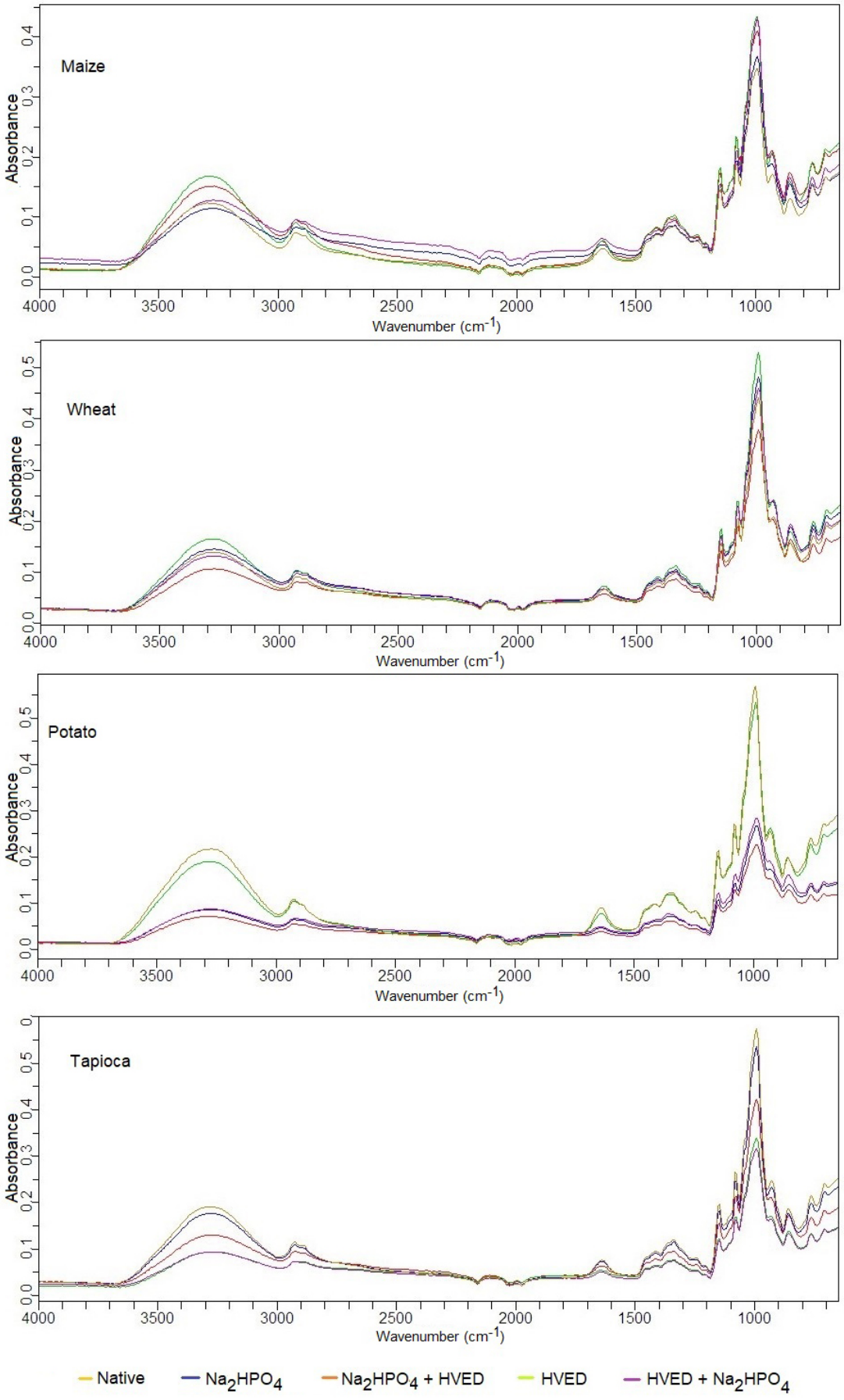

FIGURE 2. Fourier-transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) spectra of starches modified with Na_2HPO_4, with and without high-voltage electrical discharge (HVED) treatment.

The HVED treatment before phosphorylation resulted in a reduced paste clarity compared to the phosphorylated counterpart of cereal starches. Although statistical difference was not observed for wheat starch, the values are indeed lower (11.45 %T compared to 12.75 %T) for this sample as well. Again, for tuber starches the trend was opposite, *i.e.*, only the HVED-treated potato starch modified with Na_2HPO_4 revealed lower pasted clarity than the phosphorylated counterpart (Table 2). Generally, for cereal starches, paste clarity followed the order: HVED-treated starch < HVED-treated + phosphorylated < phosphorylated starch; and for tuber starches it followed the order: phosphorylated < HVED-treated + phosphorylated < HVED-treated starch. When HVED was applied after the phosphorylation, the clarity of pastes made of cereal starches decreased in the following order: phosphorylated > phosphorylated + HVED treated > HVED treated starch, while the clarity of pastes made of tuber starches did not show any trend.

Chaiwat *et al.* [2016] stated that variations in paste clarity of starch after HVED treatment depended on the final outcome of two competing reactions: cross-linking and depolymerisation. Cross-linking produces a more ordered structure and prevents the dissociation of starch chains during pasting, while depolymerisation increases paste clarity.

Amylose content in all analysed starches decreased after HVED treatment (Table 2) (although, the differences were not always statistically significant), which is consistent with results reported for plasma-treated starch [Bie *et al*., 2016b; Thirumdas *et al.*, 2018]. Thirumdas *et al.* [2018] ascribed this phenomenon to the depolymerisation of amylose, since physical modification predominantly affects amorphous regions of starch granules [Ascheri *et al.*, 2014]. In this research, a decrease in amylose content was observed after phosphorylation as well (Table 2). Ascheri *et al.* [2014] also reported decreased amylose content after phosphorylation of *S. lycocarpum* starch. In most samples treated by a combination of HVED and phosphorylation, the amylose contents were also lower compared to native starches (Table 2). However, it is difficult to reveal any trend regarding the coupled use of these methods. Starch damage was smaller after all modifications, except for tapioca starch, where virtually no damaged starch was present in native starch, and for wheat starch modified with $Na_5P_3O_{10}$ alone and in combination with HVED. Since damaged starch is a portion of starch that is physically broken or fragmented, and therefore has more affinity to water [Tian & Sun, 2020], it is possible that damaged starch was simply washed out during modification reactions.

Changes in the chemical nature of starches due to the HVED treatment and phosphorylation, both alone and in combination, are further supported by a reduction in resistant starch content determined in most samples (Table 2). Although the resistant starch content is typically linked to a higher amylose content (commonly high-amylose starches are used to produce resistant starch) [Liu *et al.*, 2020] and resistance to gelatinisation (temperatures and enthalpy of gelatinisation) [Apostolidis & Mandala, 2020], this research showed no obvious correlation between these parameters (results not shown). Furthermore, FTIR-ATR spectra (Figures 1 and 2) revealed that the number of –OH groups increased, which is visible from the increase in the heights of peaks at 993 cm^{-1} (C-O-H bending vibrations) and 3440 cm^{-1} (O-H stretching vibrations) [Deeyai *et al.*, 2013; Guntzler & Gremlich, 2006]. This implies that starch depolymerisation was the dominant reaction during the HVED treatment. However, a band at around 1000 cm^{-1} is also used as an indicator of crystallinity [Deeyai *et al.*, 2013]. The higher peak at 1000 cm^{-1} for modified starches would show that the crystalline order of starch granules was increased after all modifications, especially in the case of maize and wheat starches (Figures 1 and 2). Capron *et al.* [2007] reported that most authors use bands at 1047 and 1022 cm^{-1} to evaluate crystalline order of starch and link the band at 1000 cm^{-1} to intramolecular hydrogen bonds of hydroxyl groups, which "could allow inter-double helices associations", and concluded that the band at 1000 cm^{-1} was the result of hydrated crystalline regions. Since bands corresponding to phosphorus-containing groups of starches overlap with bands associated with C-O stretching vibrations of the glycosidic bond (1085 cm^{-1}), pyranose ring vibrations (929 cm^{-1}) [Deeyai *et al.*, 2013; Delval *et al.*, 2004], and the band showing the crystalline order of starch (around 1000 cm^{-1}) [Ispas-Szabo *et al.*, 1999], it is hard to confirm by FTIR-ATR that –OH groups were indeed substituted.

CONCLUSIONS

The HVED treatment of starch in an aqueous suspension may be used as an effective tool to modify starch properties both as a single method and in combination with its phosphorylation. As a result of the HVED treatment, generally, a reduction was observed in gelatinisation enthalpy, amylose content, and resistant starch content. The magnitude of these changes significantly depended on starch type. The HVED treatment prior to phosphorylation resulted in more pronounced changes of the investigated properties compared to phosphorylation alone, indicating that electrical discharges in water activate starch molecules, facilitate penetration of water to starch, and make them more prone to the reaction with phosphorylation agents. Additional examination of starch properties, such as swelling power, solubility, pasting properties, colour, morphology of starch granules, texture properties. *etc.* is, however, needed to get a better insight of the applicability of such modified starch in the food industry.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

ORCID IDs

Đ. Ačkar https://orcid.org/0000-0003-4257-2907
J. Babić https://orcid.org/0000-0002-6453-1850
M. Grec https://orcid.org/0000-0003-0915-2767
I. Grgić https://orcid.org/0000-0001-5166-1579
A. Gryszkin https://orcid.org/0000-0001-6139-0098
A. Jozinović https://orcid.org/0000-0001-9627-1013
M. Kopjar https://orcid.org/0000-0001-6864-4652
B. Miličević https://orcid.org/0000-0002-9680-6353
S. Zavadlav https://orcid.org/0000-0002-1163-1452
T. Zięba https://orcid.org/0000-0002-2791-342X

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Original article
Section: Food Technology

Molecular and Biochemical Characterization of the Greek Pepper (*Capsicum annuum*) Cultivars 'Florinis' and 'Karatzova'

Niki Mougiou[1], *Fotini Trikka*[1], *Sofia Michailidou*[1], *Marianna Pantoura*[1], *Anagnostis Argiriou*[1,2,*]

[1]*Institute of Applied Biosciences, Center for Research and Technology Hellas, 6th Km Charilaou Thermi Road, 57001 Thermi, Greece*
[2]*Department of Food Science and Nutrition, University of the Aegean, Myrina, 81400 Lemnos, Greece*

Key words: peppers, ISSR analysis, genetic diversity, nutrition, biochemical analysis

Peppers are the fourth most important vegetable in the global food economy. Greek pepper cultivars 'Florinis' and 'Karatzova' are especially popular because of the signature red color and sweet taste of the fruits. The economic interest in 'Florinis' peppers has led to many adulteration events. In that aspect, genetic profiles of 'Florinis', a 'Florinis'-type and 'Karatzova' peppers, were studied using Inter Simple Sequence Repeats (ISSR) molecular markers and an automated fragment detection system. Biochemical parameters, such as total dietary fiber, total phenolic and lycopene contents, and sugar profile that affect the fruit organoleptic and nutritional properties were evaluated. The molecular protocol established during this study may successfully discriminate the original 'Florinis' cultivar from the 'Florinis'-type peppers. 'Karatzova' cultivar, which fruits are similar to 'Florinis', presented also a unique profile. The biochemical evaluation revealed that 'Florinis' peppers had the highest sweetness index and total phenolic content. Such an analysis could be used for the discrimination of pepper cultivars sharing common morphological traits ensuring the unique identity of each cultivar and protecting farmers and consumers from fraud.

ABBREVIATIONS

PDO – protected designation of origin; ISSR – inter simple sequence repeats; RFU – relative fluorescence units; PCA – principal component analysis; PIC – polymorphic information content; MI – marker index; RP – resolving power; d.w. – dry weight.

INTRODUCTION

Pepper, *Capsicum sp.*, belongs to the *Solanaceae* family together with potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), and tobacco (*Nicotiniana tabacum*). The *Capsicum* genus includes about 30 species, 5 of which are domesticated; *Capsicum annuum*, *Capsicum pubescens, Capsicum baccatum, Capsicum frutescens*, and *Capsicum chinense*. *Capsicum annuum* is the widest cultivated species of pepper and includes hot and sweet varieties. Peppers are popular, not only for their distinct taste and aroma but also for the health benefits they offer upon consumption [Bagetta *et al.*, 2020; Sinisgalli *et al.*, 2020; Thuphairo *et al.,* 2019; Yokoyama *et al.*, 2020].

Studies have shown that peppers are an excellent source of ascorbic acid and phenolic compounds such as phenolic acids and flavonoids [Marín *et al.*, 2004]. Phenolics have antioxidant, anti-inflammatory, and antimicrobial properties [Shotorbani *et al.*, 2013]. In addition to phenolics, red peppers are rich in carotenoids, which are also considered to have antioxidant, cancer risk-reducing, and immune response-enhancing properties [Hornero-Méndez *et al.*, 2002]. One of the red pepper's carotenoids, lycopene, serves as the precursor of β-carotene and as a substrate for the biosynthesis of other carotenoids [Gómez-García & Ochoa-Alejo, 2013]. Furthermore, capsaicin, responsible for the pungent, spicy taste of hot peppers, has been tested for the treatment of migraine, chronic cough, diabetes, and as potent analgesic, anti-inflammatory, and anti-carcinogenic agent [Malagarie-Cazenave *et al.*, 2009]. Sweet peppers contain the non-pungent ester isostere of capsaicin, capsiate, that maintains the anti-inflammatory properties [Macho *et al*., 2003] and the same bio-potency as capsaicin without the sensory irritation [Sasahara *et al.*, 2010].

In Greece, 10–15 pepper varieties are cultivated [Thanopoulos, 2008]; in Northern Greece cultivar 'Florinis' is grown in the area of Florina, Region of Western Macedonia, greatly contributing to the economic activity of local farmers. The cultivar is very popular all over Greece and is in the process of getting a protected designation of origin (PDO) certificate. In another area of the Region of Central Macedonia, Aridea, local farmers grow the pepper cultivar 'Karatzova' which shares many common fruit traits with 'Florinis' cultivar. Fruits of both cultivars have a bright red color upon matura-

* Corresponding Author:
E-mail: argiriou@certh.gr (Prof. A. Argiriou)

tion, shiny and smooth surface, and sweet taste, while their size varies at around 20 cm for 'Karatzova' and 12–14 cm for 'Florinis' [Thanopoulos, 2008]. In the area of Drama, a 'Florinis'-type pepper variety is cultivated, used mainly for canned products. In many cases, it is falsely branded under the name 'Florinis' as it shares many common fruit traits with these peppers, like the bright red color, the shape, and sweet taste. The 'Florinis'-type peppers have thicker skin, slightly bigger fruit, and offer greater yield, thus farmers choose to use them over original 'Florinis' peppers to gain extra profit.

The aim of this study was to establish a protocol for the genetic characterization of the cultivars 'Florinis' and 'Karatzova', as well as for the discrimination between original 'Florinis' and 'Florinis'-type peppers, using inter simple sequence repeats (ISSR) molecular markers. The identification of the fragments was performed using an automated capillary electrophoresis system, which is a novelty of this study. In order to obtain a unique identity of each cultivar, the results of the genetic analysis were correlated with quantitative biochemical parameters.

MATERIALS AND METHODS

Plant material

The pepper (*Capsicum annuum*) samples analyzed in this study included 60 samples of 'Karatzova' cultivar (KAR1 – KAR60), collected from 6 different fields from the area of Aridea, Central Macedonia, Greece; 40 samples of 'Florinis' (FL1-FL40) cultivar collected from 5 different fields from the area Florina, Western Macedonia, Greece and 27 samples of 'Florinis'-type (DR1-DR27) collected from 7 different fields from Drama, Eastern Macedonia, Greece. All pepper plants were planted in greenhouses in February, 2020; plantlets where moved in open fields in April, 2020; and samples for analysis were collected in October, 2020, when fruits had reached full maturation.

For the biochemical analysis, three replicates of a pool of mature fruits from five plants per field was used. For total phenolic and lycopene contents determination, the fruits were freeze-dried (LyoQuest, Telstar, Terrassa, Spain), and milled to a fine powder. For sugar and total dietary fiber analysis, a pool of peppers was milled to a fine pulp.

Preparation of genomic DNA and PCR amplification

DNA was extracted from young leaves and/or mature dried fruits of 'Karatzova', 'Florinis', and 'Florinis'-type peppers using the NucleoSpin Plant II DNA extraction kit (Macherey- Nagel, Duren, Germany). Prior to extraction, the tissue was mechanically lysed in TissueLyser (Qiagen, Hilden, Germany) using zirconia beads (Biospec, Bartlesville, OK, USA). Five primers were used for ISSR amplification: UBC807 [Sequence (5'-3'): $(AG)_8T$, Tm: 47°C], UBC825 [Sequence (5'-3'): $(AC)_8T$, Tm: 50°C] [Lijun & Xuexiao, 2012], UBC811 [Sequence (5'-3'): $(GA)_8C$, Tm: 47°C], UBC823 [Sequence (5'-3'): $(TC)_8C$, Tm: 47°C], and UBC810 [Sequence (5'-3'): $(GA)_8T$, Tm: 47°C]. ISSR amplification was performed in PCR reactions of a total volume of 20 μL using 1 unit of MyTaq DNA polymerase (Bioline, London, UK), 15 ng of DNA template, 0.6 μM primer, and the following cycling program: 95°C for 2 min, 40 cycles of 95°C for 15 s, 20 s at annealing temperature (Tm), 72°C for 1 min, and one final extension step at 72°C for 7 min on a Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). ISSR fragments were separated in the Fragment Analyzer 5200 (Agilent Technologies, Santa Clara, CA, USA) genetic analyzer using the DNF-915 dsDNA reagent kit (Agilent Technologies). The results were acquired using the software ProSize 3.0 (Agilent Technologies).

ISSR data analysis

ISSR sharp and discrete fragments with a minimum peak height of 500 relative fluorescent units (RFU) and peak width above 5 s, were scored as present (1) or absent (0), creating a binary matrix for downstream analysis. The matrix was then analyzed using the GeneAlex 6.5 software [Peakall & Smouse, 2012]. Principal component analysis (PCA) was performed using the covariance-standardized method, and Neighbor-Joining phylogenetic trees [Saitou & Nei, 1987] were constructed using MEGAX software [Tamura *et al.*, 2007]. The information content of each primer was calculated based on the formula $PIC_i = 2f_i (1 - f_i)$ [Roldán-Ruiz *et al.*, 2000], where PIC_i is the polymorphic information content of marker 'i', f_i is the frequency of the amplified allele (band present), and $1 - f_i$ is the frequency of the null allele (band absent). Marker index (MI), also used as a measure of the utility of the markers, was calculated as the product of PIC and EMR for the specific marker. EMR was defined as the product of the fraction of polymorphic loci (n_p) and the number of polymorphic loci for an individual assay, meaning $EMR = n_p(n_p/n)$ [Milbourne *et al.*, 1997]. The resolving power (RP) of each primer was calculated as $RP = \Sigma Ib$, where Ib represents the informative fragments as $Ib = 1 - (2 \times |0.5 - p_i|)$, where p_i is the proportion of accessions containing the *i*th band [Prevost & Wilkinson, 1999]. Nei's gene diversity (H) and Shannon information index (I) were calculated using the software PopGene32 [Yeh & Boylet, 1997]. The average number of different alleles (Na), effective number of alleles (Ne), Shannon information index (I), expected heterozygosity (He), and unbiased expected heterozygosity (uHe) per locus were calculated using the GeneAlex 6.5 software [Peakall & Smouse, 2012].

Biochemical analysis

Total dietary fiber content of pepper fruits were measured with the Association of Official Analytical Chemists (AOAC) method 2009.01 [McCleary, 2007; McCleary *et al.*, 2010]. The results were expressed as g of dietary fiber per 100 g of fresh fruit.

Total phenolic content of the peppers was determined with a Folin-Denis reagent (Sigma–Aldrich, St. Louis, MO, USA) according to the method described by Lanza *et al.* [2010] with minor modifications. Briefly, 0.05 g of a dried fruit powder was extracted with 2 mL of methanol. The extraction was repeated five times and the extracts were combined. A small amount (50 μL) of the extract was mixed with an equal volume of the Folin-Denis reagent. In the mixture, 300 μL of a Na_2CO_3 saturated solution was added and brought to 1 mL volume with d.H_2O. The reaction was preceded for 60 min at room temperature. The solution was centrifuged

at 3000×*g* for 10 min and the absorbance of the supernatant was measured at 725 nm (UV-2600 spectrophotometer, Shimadzu, Kyoto, Japan). The quantification of total phenolics was determined by a calibration curve of caffeic acid (Fluorochem Ltd, Hadfield, U.K.) ranging from 20 to 100 μg/mL with a regression coefficient value (R) of 0.9982. Total phenolic contents were expressed as mg of caffeic acid equivalents (CAE) per g of dry weight (d.w.) of pepper fruits.

Lycopene content was measured with a modified method of Barrett *et al.* [2007]. Briefly, 0.05 g of a dried pepper powder was incubated in the dark for 1 h with occasional vortexing with 7.0 mL of a 4:3 (*v/v*) ethanol:hexane mixture. After 60 min, 1.0 mL of H_2O was added to each sample and shaken briefly. The samples were centrifuged at 3000×*g* for 2 min to allow a phase separation and dissipation of air bubbles. The organic layer was collected and the extraction was repeated with the addition of 3 mL of hexane. The extraction was repeated for 4 times. All hexane layers were combined, and the absorbance at 503 nm (A_{503}) was recorded. Lycopene content of pepper fruits d.w. was then calculated according to the following equation:

$$\text{Lycopene content (mg/g d.w.)} = \\ = (A_{503} \times 537 \times V_{extract})/(W_{sample} \times 172)$$

where: 537 M is the molecular weight of lycopene, $V_{extract}$ in mL is the volume of the hexane layer, W_{sample} in g is the weight of the extracted sample, and 172 1/(M·cm) is the extinction coefficient for lycopene in hexane.

Sugar analysis (glucose, fructose, saccharose) in pepper fruits was performed with a Dionex HPLC Ultimate 3000 equipped with a refractive index detector (Thermo Fisher Scientific), using a LiChrospher 100 NH_2 5 μm column (Sigma-Aldrich). The analysis was carried out according to Navarro *et al.* [2006] protocol. Briefly, a pool of peppers was milled to a fine pulp, centrifuged at 5000×*g* for 5 min, and in the supernatant the chromatographic determination of sugar profile was carried out with HPLC. Samples pretreatment was carried out according to Navarro *et al.* [2000]. The mobile phase was 85% (*v/v*) acetonitrile, with a flow rate of 0.9 mL/min. Solvent of HPLC grade was purchased from PanReac Applichem (Barcelona, Spain). Sweetness index was expressed as a sum of fructose and glucose.

Statistical analysis

For each chemical analysis triplicate measurements were conducted and data are expressed as mean value ± standard error (n=3). Statistical analysis was performed using paired t-test (GraphPad, San Diego, CA, USA).

RESULTS AND DISCUSSION

ISSR molecular markers have been widely used for the differentiation of *Capsicum* species and the genetic characterization of *Capsicum annuum* cultivars [Ibarra-Torres *et al.*, 2015; Lijun & Xuexiao, 2012; Tsaballa *et al.*, 2015]. The genetic analysis presented here resulted in a total of 53 amplicons in 127 pepper plants using 5 ISSR primers. To capture all information derived from ISSR fragments, the PCR products were analyzed in an automated capillary electrophoresis system. Studies have shown that the amount of data obtained using automated detection systems exceeds that obtained using the more conventional methods of agarose electrophoresis, eliminating also factors affecting the results originating from gel preparation, imaging and analysis as well as the subjectivity of each user at scoring [Bentley *et al.*, 2015].

To the best of our knowledge, this is the first time that ISSR bands were evaluated in a Fragment Analyzer system in plant studies, revealing an average of 10.6 bands amplified per primer. Primer UBC811 generated the maximum number of fragments (N=13) and primer UBC823 the lowest (N=8). The number of polymorphic loci where 31 out of 53, resulting in 58.5% mean percentage of polymorphic bands (Table 1). In fact, more alleles were scored in our research compared with the use of the same markers in other studies. For instance the use of UBC811 in other studies has resulted in 10 bands [Tsaballa *et al.*, 2015] or 6 bands [Hatami Maleki *et al.*, 2019], in contrast to 13 scored in our study. Moreover, Hatami Maleki *et al.* [2019] referred to 7 bands using the marker UBC823 and 6 using the UBC825, while in the present study these markers resulted

TABLE 1. Polymorphic information content and genetic diversity indices calculated for each primer used in the analysis of 'Florinis', 'Florinis'-type, and 'Karatzova' pepper cultivars.

Primer	No. of bands			%P[a]	Range (bp)	PIC[b]	MI[c]	RP[d]	H[e]	I[f]
	Total	Monomorphic	Polymorphic							
UBC807	12	4	8	66.7	202–833	0.13	0.71	21.1	0.15	0.24
UBC810	11	3	8	72.7	327–1159	0.18	1.05	18.66	0.20	0.30
UBC811	13	4	9	69.2	328–1615	0.12	0.73	22.48	0.15	0.25
UBC823	8	6	2	25.0	390–980	0.09	0.04	14.68	0.09	0.23
UBC825	9	5	4	44.4	369–1433	0.09	0.16	16.97	0.12	0.19
Total	53	22	31							
Average	10.6	4.4	6.2	58.5		0.12	0.54	18.75	0.14	0.22

[a]percentage of polymorphism; [b]polymorphic information content; [c]marker index; [d]resolving power; [e]Nei's gene diversity; [f]Shannon information index.

TABLE 2. Genetic diversity indices of pepper cultivars.

Cultivar	%P[a]	N[b]	Na[c]	Ne[d]	I[e]	He[f]	uHe[g]
'Karatzova'	18.87	58.64	6.75	6.81	0.66	0.45	0.48
'Florinis'	15.47	38.25	5.64	5.56	0.45	0.31	0.33
'Florinis'-type	4.31	23.70	6.83	7.20	0.17	0.12	0.14

[a]percentage of polymorphic loci; [b]sample size; [c]number of different alleles; [d]number of effective alleles; [e]Shannon information index; [f]expected heterozygosity; [g]unbiased expected heterozygosity.

in 8 and 9 bands, respectively. The percentage of polymorphic loci identified by the primers used in this study (58.5%) was lower than other relative studies in Greek *Capsicum annuum* landraces (83.6%) [Tsaballa *et al.*, 2015] or for the differentiation of pepper species (75%) [Lijun & Xuexiao, 2012]; however was sufficient for the discrimination of the cultivars of interest. The relatively small percentage of detected polymorphism indicates the narrow genetic pool of the tested samples.

Marker parameters regarding the information content and resolution power of each primer were calculated (Table 1). The mean PIC, MI, and RP values observed for all 5 primers were 0.12, 0.54, and 18.75, respectively. The primers that showed higher polymorphism had higher MI values, as expected [Najaphy *et al.*, 2011]. PIC reflects the discriminating ability of the marker and depends on the number of known alleles and their frequency distribution, thus representing genetic diversity. The average PIC value of the primers used in our study (0.12) was slightly lower than the PIC value of other studies, for instance regarding Bulgarian pepper cultivars (0.177) [Tsonev *et al.*, 2017] or Greek pepper cultivars (0.242) [Tsaballa *et al.*, 2015]. This suggests that there are few unique alleles detected by each primer, highlighting once again the genetic proximity of the cultivars under investigation.

Resolving power (RP) index indicates the number of genotypes identified by a primer; thus the highest value shows the most informative marker [Najaphy *et al.*, 2011]. In our study, the most informative markers for distinguishing the genotypes were UBC807, UBC811, and UBC810, exhibiting values of 21.1, 22.48, and 18.66, respectively. The average Nei's gene diversity index was 0.14 while the average Shannon index was 0.22. The highest Nei's gene diversity value was observed for the primer UBC810 (0.20), suggesting that this primer detected the most polymorphic loci, which is in agreement with the highest percent of polymorphism (%P), while the lowest was observed for primer UBC823 (0.09). The average number of alleles per locus (Na) was 1.585 and the effective number of alleles per locus (Ne) was 1.245.

Evaluation of genetic diversity indices of each cultivar were calculated for each field separately and then averaged per cultivar (Table 2). The highest percentage of polymorphic loci was observed for 'Karatzova' cultivar, followed by 'Florinis', while 'Florinis'-type samples had a very low percentage of this index. The same trend was observed for all the indices calculated. The higher the percentage of polymorphic loci, the higher the genetic diversity observed within a cultivar [Jiang & Liu, 2011]. Thus, 'Karatzova' cultivar appeared more genetically diverse while the samples from 'Florinis'-type cultivar started from a very narrow genetic base. This is also verified by the higher I and He value of 'Karatzova' samples.

Principal component analysis of the binary matrix for the 53 loci of *Capsicum annuum* showed that the two primary components accounted for 29.95% of total genetic variation (Figure 1). The analysis differentiated mostly the 'Katatzova' peppers which formed a tight cluster enclosing all samples of this cultivar analyzed. Although 'Florinis' peppers also formed a tight cluster enclosing the majority of 'Florinis' samples, a small nucleus, consisting of four samples, generated a different genetic profile, clustering away from the other

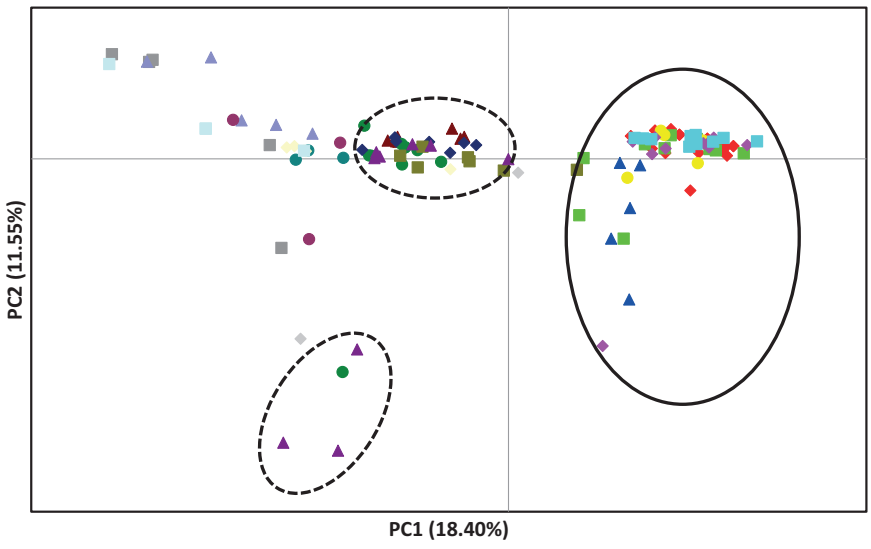

FIGURE 1. Principal component analysis based on the two first principal components derived from 53 bands amplified by five primers ISSR among 127 pepper samples. The samples in the black circle are 'Karatzova' peppers, those in the dashed circles are 'Florinis' peppers, and all the others are 'Florinis'-type samples.

TABLE 3. Sugar profile and contents of total dietary fiber, total phenolics, and lycopene of pepper cultivars.

Cultivar	Fructose (g/100 g fresh fruit)	Glucose (g/100 g fresh fruit)	Saccharose (g/100 g fresh fruit)	Total dietary fiber (g/100 g fresh fruit)	Total phenolic content (mg CAE/g d. w.)	Lycopene (mg/g d. w.)
'Karatzova'	1.78±0.16^{b}	1.36±0.20^{ab}	<0.5	2.20±0.13	1.11±0.18^{b}	1.14±0.27^{a}
'Florinis'	2.36±0.16^{a}	1.82±0.16^{a}	<0.5	2.93±0.53	7.26±0.39^{a}	0.33±0.06^{b}
'Florinis'-type	2.45±0.15^{a}	0.88±0.18^{b}	<0.5	2.04±0.16	7.48±0.25^{a}	0.60±0.08^{a}

Results are presented as means with their respective standard error (±SE). Different letters (a, b) in column indicate statistically significant differences ($p \leq 0.05$). CAE – caffeic acid equivalents; d.w. – dry weight.

samples. This could be an indicative that these are admixed samples and should not be included in 'Florinis' pepper breeding schemes. The PCA also highlights the genetic proximity of 'Florinis'-type peppers to 'Florinis' rather than 'Karatzova' cultivar. This confirms what was empirically known by some farmers that use them under the brand name 'Florinis'.

The phylogenetic tree constructed using the Neighbor-Joining method showed that the three cultivars formed distinct branches with the presence of some admixed samples (Figure 2). Three 'Florinis'-type samples, collected from two different populations, appeared genetically closer to 'Florinis' peppers than other plants of the same variety (DR4, DR5, DR13). *Vice versa*, the same was observed for one 'Florinis' sample (FL4). All the other samples clustered according to their cultivar. No particular grouping among populations (fields) of the same cultivar was observed.

Principal component analysis and phylogenetic tree highlighted the discriminatory power of the established protocol for the classification of 'Karatzova', original 'Florinis', and 'Florinis'-type samples. This is especially important for distinguishing between 'Florinis' and 'Florinis'-type as the latter is often branded under the PDO name 'Florinis' in canned products where it is difficult to identify it by the fruit morphology.

Since, the compositional quality and organoleptic properties of peppers have been shown to affect consumer preference [Bozokalfa & Eşiyok, 2011; Parisi *et al.*, 2017], the genetic characterization of the cultivars of interest was followed by the analysis of biochemical factors. Table 3 presents the results from the biochemical analysis including contents of total dietary fiber, total phenolics and lycopene, as well as sugar profile. The saccharose levels of all the samples were below 0.5 g/100 g of fresh fruit. Regarding the fructose values, 'Florinis'-type cultivar exhibited the highest value at 2.45 g/100 g of fresh fruit, slightly higher than 'Florinis' (2.36 g/100 g of fresh fruit), while 'Florinis' samples had higher glucose levels. As a result, 'Florinis' cultivar had the highest sweetness index, calculated as a sum of fructose and glucose, about 1.2-fold higher than the other two cultivars that were at the same level. 'Florinis' cultivar was also found rich in dietary fiber and phenolics, as along with 'Florinis'-type samples, exhibited 7-fold higher total phenolic content than 'Karatzova' samples. 'Karatzova' cultivar on the other hand, had the highest content of lycopene, 3.4 times higher than 'Florinis' that had the lowest.

The fact that 'Florinis' cultivar was characterized by high values in more than one biochemical trait was not a surprise as a similar phenomenon has been observed to Balkan cultivars [Nankar *et al.*, 2020]. Although there are several records on the biochemical traits or properties of various pepper cultivars of the Balkan region [Denev *et al.*, 2019; Nankar *et al.*, 2020], there is a limited number of studies about Greek peppers with emphasis on 'Florinis' cultivar [Niklis *et al.*, 2002; Papathanasiou *et al.*, 2020]. The high total phenolic content of 'Florinis' and 'Florinis'-type peppers was expected, as in full maturity the sweet peppers contain high amount of phenolic compounds [Papathanasiou *et al.*, 2020]. However, a direct comparison with previous studies might be controversial as either different measuring units were employed [Denev *et al.*, 2019; Papathanasiou *et al.*, 2020] or different cultivars were chosen [Denev *et al.*, 2019]. Nevertheless, considering that peppers contain 90–93% water, the values obtained in the present study are alike to previous measurements [Papathanasiou *et al.*, 2020]. Compared to other Balkan cultivars, 'Florinis' and 'Florinis'-type peppers had similar glucose contents to 'Pungent' and 'Sweet green' peppers [Denev *et al.*, 2019]. On the other hand, the contents of fructose were significantly higher to other Balkan varieties, while the high levels

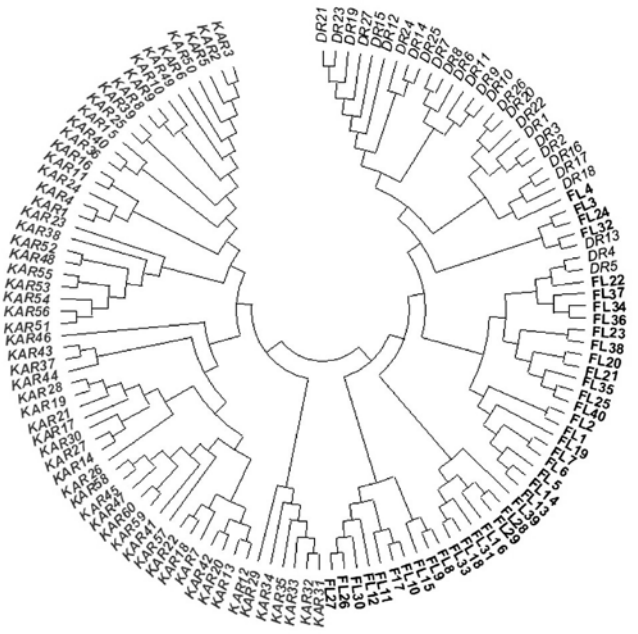

FIGURE 2. Neighbor-Joining phylogenetic tree representing the evolutionary relationships between the 127 pepper samples analyzed ('Karatzova' samples (KAR) in grey bold italic letters, 'Florinis' (FL) in black bold letters, 'Florinis'-type (DR) in black italic letters).

of total sugars resembled the values of 'Pumpkin' and 'Kapia' cultivars [Denev *et al.*, 2019]. Due to a lack of information regarding Greek cultivars, the biochemical parameters of total dietary fiber and lycopene content will be compared to other sweet peppers [Chávez-Mendoza *et al.*, 2015; Hernández--Carrión *et al.*, 2015]. Regarding total dietary fiber, the values of all three cultivars were significantly lower compared to red sweet Californian peppers [Hernández-Carrión *et al.*, 2015]. Furthermore, the lycopene contents of the three cultivars were lower than 'Sweet/Robusto' genotype that were harvested on September, still the fact that the sampling in the present study was carried out in October should be considered as it has been shown to influence the pigment content [Chávez-Mendoza *et al.*, 2015].

The biochemical profile of each cultivar underlined their differences in the sweetness value, the total dietary fiber, lycopene and total phenolic contents. 'Karatzova' and 'Florinis'--type cultivars are hard to distinguish based only on biochemical analysis, as their sweetness value and total dietary fiber content are similar and about 1–1.3-fold lower than 'Florinis' peppers. The quantification of both the total phenolics and lycopene level is important as studies have shown that the antioxidant properties of a plant are defined by multiple compounds [Chávez-Mendoza *et al.*, 2015]. It is well known that the biochemical properties of peppers are affected by various parameters, such as local environmental factors [Montalvo *et al.*, 2021], growth conditions [Ayodele *et al.*, 2015], and maturity stage [Bae *et al.*, 2014]. Therefore, in order to differentiate cultivars based solely on biochemical properties, it is important to cultivate them under identical conditions [Denev *et al.*, 2019; Nankar *et al.*, 2020]. However, the advantage of ISSR molecular markers is that they are not affected by such parameters, making the differentiation of phenotypically similar cultivars feasible.

CONCLUSIONS

Despite their broad presence in a human diet and their global economic importance, the taxonomic classification of *Capsicum* within and between species is still confusing. Until recently, their classification was based only on morphological traits of the fruit which requires effort and time for the full growth of the plant and is subject on the expertise of the agronomist. It is evident from this study that the cultivars 'Florinis' and 'Karatzova' can be characterized based on their DNA profile, following the established protocol, while the biochemical evaluation may further reinforce their identity. Moreover, it was shown that 'Florinis'-type peppers may be discriminated from the original cultivar, preventing consumers' fraud. The findings of this research project, the first one studying 'Florinis' and 'Karatzova' peppers combining genetic and biochemical methodologies, contribute to the effort made to monitor Greek pepper cultivars, not only as raw material but as processed food, throughout the food production chain. The developed protocols could be used in the protection of producers and consumers through the application of traceability systems that will protect PDO products and gain consumers' trust.

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CONFLICT OF INTERESTS

There is no conflict of interest to declare.

ORCID IDs

A. Argiriou https://orcid.org/0000-0002-9164-632X
S. Michailidou https://orcid.org/0000-0002-8250-0513
N. Mougiou https://orcid.org/0000-0003-3316-0361
M. Pantoura https://orcid.org/0000-0003-4979-8036
F. Trikka https://orcid.org/0000-0003-2445-2039

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70 VOLUME'S REVIEWERS

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Khwaldia K., Institut National de Recherche et d'Analyse Physico-Chimique (INRAP), Pôle Technologique de Sidi Thabet, Sidi Thabet, France
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Sandoval Torres S., Instituto Politécnico Nacional (IPN), Ciudad de Mexico, Mexico
Sawicki T., University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
Staroszczyk H., Gdańsk University of Technology, Gdańsk, Poland
Starowicz M., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

Tao Y., Nanjing Agricultural University, Nanjing, China
Terekhova I., G.A. Krestov Institute of Solution Chemistry of the Russian Academy of Sciences, Ivanovo, Russia
Totosaus A., Tecnologico de Estudios Superiores de Ecatepec (TecNM), Ecatepec de Morelos, Mexico
Tu M., Dalian Polytechnic University, Dalian, Liaoning, China
Turabi Yolaçaner E., Hacettepe University, Ankara, Turkey

Vulić J., University of Novi Sad, Novi Sad, Serbia

Wanasundara J., Agriculture & Agri-Food Canada, Saskatoon, Canada, Canada
Wiczkowski W., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland
Wierzejska R., National Institute of Public Health - National Institute of Hygiene, Warsaw, Poland
Wybraniec S., Cracow University of Technology, Cracow, Poland

You J., Huazhong Agricultural University, Wuhan, China
Yu Z., Bohai University, Jinzhou, China

Ziarno M., Warsaw University of Life Sciences, Warsaw, Poland

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Instruction for Authors
Section: Editorial News

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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.

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Ms. Joanna Molga
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