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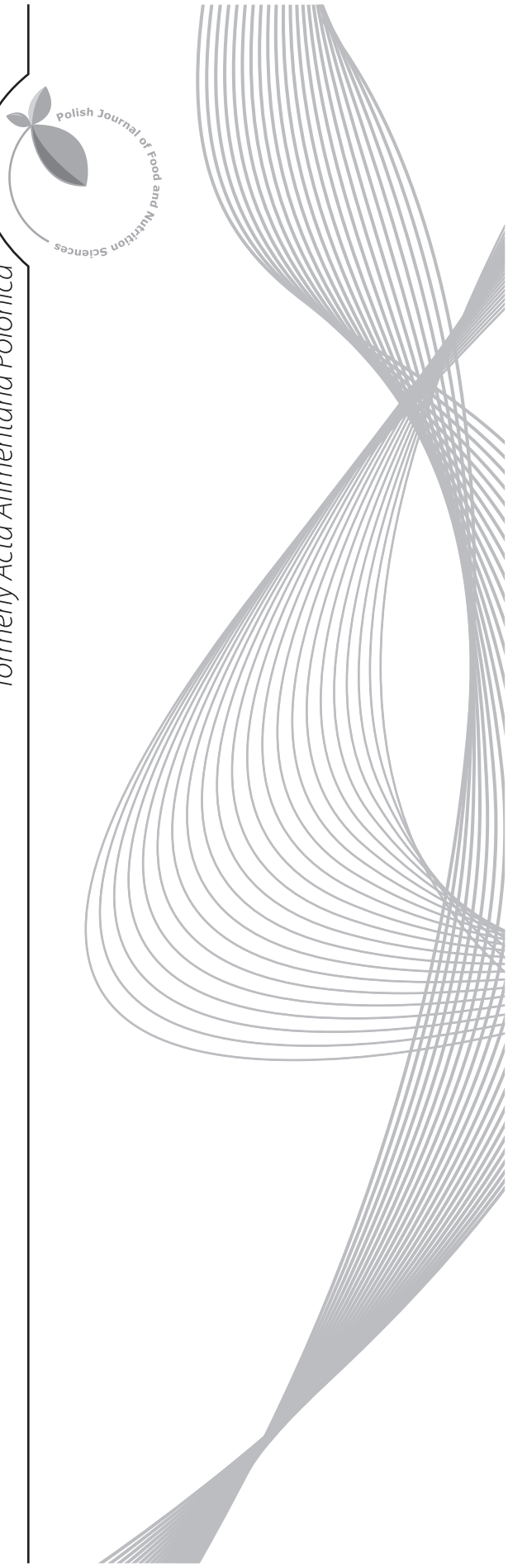

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REVIEW PAPER

ORIGINAL PAPERS

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Review article
Section: Nutritional Research

Health Benefits of Vegetarian and Mediterranean Diets: Narrative Review

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Diet is an important lifestyle factor influencing disease risk. Vegetarian and Mediterranean diets have their proponents and are promoted for various potential health benefits. Over the years, numerous cross-sectional and cohort studies and randomized clinical trials have been conducted to elucidate the relationship between the Mediterranean and vegetarian diet and cardiovascular, cancer, diabetes, and other disease risks. More recently, research has been conducted to compare both diets directly. In this narrative review, we discuss the effects of vegetarian and Mediterranean diets on lipid profile, blood pressure, inflammation markers, body weight, risk of cardiovascular disease, cancer, diabetes mellitus, metabolic syndrome, and chronic kidney disease, as well as their associations with gut microbiota and mental health. The paper also discusses the studies comparing vegetarian and Mediterranean diets and their health effects. It provides further evidence that both diets can be beneficial and advocates their promotion, especially in Westernized populations plagued by various chronic lifestyle-associated diseases. At the same time, the Mediterranean dietary model may appear to be a superior public health strategy, less prone to the risk of nutritional deficiencies and less challenging in implantation on a broader scale. However, further studies based on cross-over design and long-term observations are recommended to thoroughly compare vegetarian and Mediterranean diets and draw more firm conclusions on their effects on health.

INTRODUCTION

Diet, among other lifestyle factors, is known to significantly influence disease risk, including cancer, metabolic disorders, and cardiovascular diseases. The awareness of this association leads to a growing interest in diets that offer specific health benefits. However, these benefits require scientific evaluation not only in observational research but also through clinical trials. The latter study design is considered the gold standard for evaluating particular treatments, including dietary interventions, and can provide a causal relationship between diet, health benefits, and disease risk in humans [Lucey *et al.*, 2016; Mirmiran *et al.*, 2021; Staudacher *et al.*, 2022; Yao *et al.*, 2013]. The number of such studies in recent years is growing, enabling meta-analyses and comparing different diet interventions [Ge *et al.*, 2020]. However, similarly to the comparisons of the efficacy of various vaccines or other medical treatments, a direct comparison of health benefits related to particular diets should be made upon analyzing randomized controlled clinical trials involving the same group of volunteers. The number of such studies is still limited, although systematically increasing in recent years [Burrows *et al.*, 2022; Mellor *et al.*, 2022; Watson *et al.*, 2015, 2018].

Particular attention is given to the health benefits of Mediterranean and vegetarian diets, which show some similarities but also distinctive differences (Table 1). The former dietary pattern is predominantly characterized by the consumption of plant-derived foods such as fruits, vegetables, beans, cereals, nuts, and seeds, with olive oil used as a primary source of fat, moderate amounts of dairy foodstuffs, principally cheese and yogurt, moderate consumption of fish and seafood, and low consumption of meat, with avoidance or notably low intake of red meat [Davis *et al.*, 2015].

Traditionally embraced by the populations of Greece, Italy and Spain, the Mediterranean diet pattern has also been promoted outside the Mediterranean region in the hope that it may benefit Westernized populations plagued by various chronic diseases, many of which are associated with lifestyle factors, including diets [Sotos-Prieto *et al.*, 2022]. Despite these efforts, adherence to the Mediterranean diet in non-Mediterranean countries remains low [da Silva *et al.*, 2009]. Moreover, recent studies show that adherence to this diet in the Mediterranean region is only moderate and has decreased

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TABLE 1. Comparison of main characteristics of the vegetarian and Mediterranean diets.

	Vegetarian diet	Mediterranean diet
Main protein sources	Legumes, nuts; in some variations: fish, seafood, eggs, dairy	Legumes, nuts, fish, seafood, poultry, dairy
Typical protein intake	70 g/day (lacto-ovo-vegetarians) 64 g/day (vegans) [Mariotti & Gardner, 2019]	70 g/day [Tosti *et al.*, 2018]
Main carbohydrates sources	Cereals, fruits, vegetables, sweets	Cereals, fruits, vegetables, sweets
Main fat sources	Vegetable oils, nuts	Olive oil, nuts
Excluded products	Meat (and all animal-derived products in case of a vegan diet)	–
Methods of thermal processing	All; preferably raw fruits and vegetables	Avoidance of frying; preferably raw fruits and vegetables
Health risks	Calcium, iron, vitamin D_3, and B_{12} deficiencies	–
Distinguishing features	No meat consumption	Focusing on local products; olive oil as the main source of fat

over the decades [Obeid *et al.*, 2022; Veronese *et al.*, 2020]. Contrary to this, the interest in vegetarian diets appears to increase in high-income countries, as reflected by increasing interest in sales of plant-based alternatives to animal-derived products [Choudhury *et al.*, 2020; Paslakis *et al.*, 2020]. It is estimated that approximately 5% of Europeans may adopt vegetarian diets, compared to 8% of South Americans, over 15% of African and Middle East populations, and nearly 20% of Asians [Hargreaves *et al.*, 2021].

On the other hand, India, often perceived as a household with the largest population of vegetarians (estimated at 30–40% of the population), is currently experiencing the highest growth rates for meat consumption in the world [Arora *et al.*, 2020; Shridhar *et al.*, 2014]. Vegetarianism is characterized by total abstention from consuming meat. There are different variations of this diet that include eggs (ovo-vegetarianism), dairy (lacto--vegetarianism), or both (lacto-ovo-vegetarianism), or exclude them and any other animal-derived products, *e.g.*, honey (veganism) [Fontes *et al.*, 2022]. A diet such as flexitarianism, which limits but does not entirely exclude meat, is regarded as semi-vegetarianism. In addition, some individuals adhere to pesco-vegetarianism, which excludes all meats except fish [Wozniak *et al.*, 2020]. Although the motivations for transitioning to vegetarianism can be ethically- and environmentally driven, some individuals choose this dietary pattern for health-associated reasons [Dinu *et al.*, 2017; Rosenfeld, 2019; Rosenfeld & Burrow, 2017]. However, ceasing meat consumption or switching to a diet low in meat appears to be a challenge for many people [Macdiarmid *et al.*, 2016; O'Keefe *et al.*, 2016; Tucker, 2014].

Considering that vegetarian and Mediterranean diets both focus on plant-based foods but take a different approach regarding meat products, it is pivotal to understand their effects, separately and jointly, on health outcomes. It is particularly important given that both diets are gaining attention in some regions and populations, leading to public discussion on the superiority of one diet over another. Considering the discussion on dietary interventions as a non-pharmaceutical approach to decrease the risk of disease, summarizing the health benefits of both diets and comparing them may be beneficial for dieticians who work in the promotion of diets in various risk groups.

The objective of this narrative review was to present existing evidence on the potential associations between the Mediterranean and vegetarian diets and human health and to preliminary compare these diets. To this end, we have searched PubMed and Google Scholar databases using the following keywords: "Mediterranean diet", "vegetarian diet", "vegan diet", "plant-based diet", and "health". Peer-reviewed papers related to the search terms were assessed by titles, abstracts, and article content. We have preferentially selected meta-analyses and systematic reviews, if available. If not, other types of papers (observational and interventional studies) were included. Only English-language articles published between 2012–2022 were chosen to elicit the latest trends. The selected papers were categorized to construct subsequent review sections discussing the established effects of each diet on the lipid profile, blood pressure, inflammation markers, overall cardiovascular risk, as well as the risk of cancer, diabetes mellitus, metabolic syndrome, and chronic kidney disease, and associations with gut microbiome and mental health. We have included these effects and health conditions as they are the most studied in relation to diet. At the same time, they pose significant challenges to public health, with a need to seek non-pharmaceutical interventions that could decrease the associated morbidity and mortality. Separately, we have also selected the available studies designed to compare both diets regarding cardiovascular parameters, kidney function, metabolic profile, gut microbiome, and pain in rheumatoid arthritis. The exact metabolic and molecular mechanisms of how these two diets can affect different aspects of human health are not fully elucidated and remain subject to study (*e.g.*, see [Allen & Locasale, 2021; Tosti *et al.*, 2018] for reviews on this matter). Based on the conducted review, the present paper provides a base for promoting Mediterranean and vegetarian diets in different populations and groups of patients to benefit their health.

VEGETARIAN DIET AND HUMAN HEALTH

Vegetarian diet and lipid profile

The relationship between a vegetarian diet and lipid management has not been fully elucidated. Two meta-analyses

[Wang *et al.*, 2015; Yokoyama *et al.*, 2017] demonstrated the overall effect of a vegetarian diet on lowering the level of total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol and revealed no association with triglyceride levels. One of the meta-analyses involved 11 trials and a total of 832 participants from 28 to 56 years of age [Wang *et al.*, 2015]. The duration of the vegan, lacto-ovo-vegetarian, or lacto-vegetarian diet intervention ranged from 3 weeks to 18 months. Five studies involved participants with higher cardiovascular risk factors, such as obesity or type 2 diabetes. In three studies, participants were also in lipid-lowering therapy, which may have interfered with the results. Using omnivorous diets as comparators, vegetarian diets resulted in significant reductions in total cholesterol (–0.36 mmol/L), LDL cholesterol (–0.34 mmol/L), and HDL cholesterol (–0.1 mmol/L). However, there was no meaningful change in triglyceride levels (–0.04 mmol/L) [Wang *et al.*, 2015].

The second meta-analysis involved 49 studies (lasting more than 4 weeks) conducted on a total of 11,627 participants from 21 to 72 years of age [Yokoyama *et al.*, 2017]. Compared to omnivorous diet, the 30 observational studies and 19 clinical trials of vegetarians revealed reductions in total cholesterol concentration by 29.2 mg/dL and 12.5 mg/dL respectively, LDL cholesterol by 22.9 mg/dL and 12.2 mg/dL, and HDL cholesterol by 3.6 mg/dL and 3.4 mg/dL. In contrast, no significant effect of vegetarianism on triglyceride levels was observed [Yokoyama *et al.*, 2017]. The significant reduction in total serum concentrations of cholesterol and LDL cholesterol (–28.2 and –21.3 mg/dL, respectively, in vegetarians and vegans compared to omnivores was also demonstrated by the meta-analysis of 86 cross-sectional trials. A vegan diet was also associated with lowered levels of total cholesterol and LDL cholesterol, *i.e.*, –31.0 mg/dL and –22.9 mg/dL, respectively [Dinu *et al.*, 2017]. In contrast to Wang *et al.* [2015], the lowered concentrations of triglycerides in individuals adhering to a vegetarian but not vegan diet amounted to –11.4 mg/dL as compared to omnivores.

Another meta-analysis of 12 cross-sectional and cohort studies (1,300 participants) found that vegetarian diets followed for at least six months in four developing countries effectively reduced plasma triglyceride concentrations (standardized mean difference, SMD=–4.06 mmol/L). In contrast, vegetarians in eight developed countries showed a slight decrease in triglyceride levels (–0.31 mmol/L) compared to omnivores [Zhang *et al.*, 2013]. Contrary to the 2015 and 2017 publications, the 2014 meta-analysis, including 12 observational studies, demonstrated no difference in HDL cholesterol levels between vegetarians and omnivores (SMD=0.02 mmol/L) [Zhang *et al.*, 2014]. Lacto-ovo-vegetarianism, lacto-vegetarianism, and ovo-vegetarianism were the dietary models examined. The participants (n=4177, age group 30–52 years; 2,191 vegetarians, 1,986 omnivores) had no family history of vascular diseases (such as myocardial and cerebrovascular infarction, angina pectoris, and others), hyperlipoproteinemia, dyslipidemia, and other related diseases. There appeared to be no change in HDL cholesterol levels among vegetarians and omnivores even after the cultural conditions were considered (Asia, Latin and North America, and Europe). The authors of the meta-analysis indicated that information on blood pressure, weight, insulin, or diabetes could not be obtained for all studies, which limited any further analysis [Zhang *et al.*, 2014].

In general, the studies show the beneficial effect of vegetarian diets, including vegan, on lipid profiles encompassing mostly total cholesterol and LDL cholesterol levels. To some extent, the reduction in triglyceride concentrations was also found, but the findings in this regard are inconclusive and require further studies. Nevertheless, lipid profile is helpful in the determination of approximate cardiovascular risk, and its impairment is often seen in obesity. Interventional studies are required to understand the extent to which a vegetarian diet can lower the lipid profile in already diseased patients.

Vegetarian diet and blood pressure

Two meta-analyses have confirmed the beneficial hypotensive properties of vegetarian diets. The first, published in 2014, included 39 publications [Yokoyama *et al.*, 2014]. In six studies, a proportion of the participants were using antihypertensive medication; in twenty-two, the vegetarian dietary model was followed for more than one year. Seven of the works examined the impact of veganism, three focused on lacto-vegetarianism, fourteen on lacto-ovo-vegetarianism, and fifteen examined the diverse types (veganism, lacto-vegetarianism, lacto-ovo-vegetarianism, pesco-vegetarianism, and/or semi vegetarianism). The analysis of 7 controlled clinical trials (311 subjects aged 38–54), with a duration of six weeks or more, revealed that in comparison with a traditional diet, vegetarian diet consumption was associated with a decrease in mean systolic blood pressure by 4.8 mmHg (–6.6 to –3.1 mmHg) and diastolic blood pressure by 2.2 mmHg (–3.5 to –1.0 mmHg). The analysis of 32 observational studies (21604 participants aged 28 to 68 years) indicated that compared to an omnivorous diet, mean systolic blood pressure in individuals on a vegetarian diet decreased by 6.9 mmHg (–9.1 to –4.7 mmHg) and diastolic blood pressure decreased by 4.7 mmHg (–6.3 to –3.1 mmHg) [Yokoyama *et al.*, 2014].

The second meta-analysis, published six years later, including 15 randomized controlled trials with intervention periods ranging from 3 to 74 weeks, examined veganism (10 publications) and lacto-ovo vegetarianism (5 papers) [Lee *et al.*, 2020]. Adults were the participants in fourteen papers, and one paper involved children. Eight studies focused on diabetics, and seven on non-diabetics. Compared with an omnivorous diet, vegetarian diet consumption lowered systolic blood pressure by an average of 2.66 mmHg (–3.76 to –1.55) and diastolic blood pressure by 1.69 mmHg (–2.97 to –0.41). Subgroup analysis indicated that veganism reduced systolic blood pressure (–3.12 mmHg) more than lacto-ovo vegetarianism (–1.75 mmHg). A decrease in diastolic blood pressure was observed with the former diet (–1.92 mmHg), while no change was demonstrated for the latter. The authors reported the substantial heterogeneity of the studies used and noted that most were conducted in the USA. Despite the limitations, both meta-analyses suggest that vegetarian diets (mainly vegan diets) [Lee *et al.*, 2020] may be an effective non-pharmacological strategy to lower blood pressure [Lee *et al.*, 2020; Yokoyama *et al.*, 2014].

Vegetarian diet and body weight

The meta-analysis on the impact of a vegetarian diet on weight loss confirms its effectiveness in this regard [Huang *et al.*, 2016]. It included 12 randomized controlled trials involving 1,151 subjects from 18 to 82 years and examined the impact of a vegan diet (in 8 studies) and a lacto-ovo-vegetarian diet (in 4 studies). Obese or overweight participants were monitored for 8 weeks to 2 years. Subjects on vegetarian diets had significantly greater weight loss (2.02 kg on average) than those from the non-vegetarian groups. Compliance with the vegan diet resulted in more significant weight loss (–2.52 kg) than with the lacto-ovo vegetarian diet (–1.47 kg). Furthermore, weight loss was greater in subjects with <1 year of intervention (–2.05 kg) than in those with ≥1 year of intervention (–1.13 kg) [Huang *et al.*, 2016]. This indicates the beneficial effect of a vegetarian diet on weight reduction compared to non-vegetarian dietary practices. However, the attenuation observed after one year of adherence to a vegetarian diet requires further elucidation in future research and postulates a need to pursue more long-term studies.

Vegetarian diet and inflammation biomarkers

Three meta-analyses examining the effect of a vegetarian diet on inflammatory biomarkers confirm its key role in improving C-reactive protein (CRP) levels, whereas one of the analyses indicated a 2-year minimum intervention time [Craddock *et al.*, 2019; Haghighatdoost *et al.*, 2017; Menzel *et al.*, 2020]. The meta-analysis of 30 (cross-sectional or cohort) observational studies aimed to determine the association between vegetarianism and inflammatory and immunological markers (CRP, tumor necrosis factor α, fibrinogen, natural killer (NK) cells, leukocytes, lymphocytes, thrombocytes, interleukins, and immunoglobulins) [Craddock *et al.*, 2019]. The dietary models evaluated with an intervention period of 4 to 54 weeks involved lacto-ovo-vegetarianism (8 publications), lacto-vegetarianism (2), veganism (5), and a combination thereof. Participants included in two studies had chronic diseases: in one, patients were on dialysis; in the other, patients had cardiovascular disease and/or diabetes. Research on adult volunteers, with the exception of 1 study involving 2- to 18-year-old individuals, was conducted in Asia, Africa, North and South America, and Europe. As indicated, in comparison with the participants adhering to non-vegetarian dietary patterns, individuals on a vegetarian diet had lower levels of CRP (–0.61 mg/L), fibrinogen (–0.22 g/L), and a lower total leukocyte count ($-0.62\times10^3/\mu$L), thus suggesting a beneficial impact of vegetarianism on inflammation biomarkers [Craddock *et al.*, 2019].

These conclusions contradict the results of a meta-analysis published two years earlier, which reported no differences in high-sensitivity CRP levels while maintaining a vegetarian and non-vegetarian diet for less than two years. In addition, when compared to an omnivorous diet, vegetarianism was associated with increased levels of interleukin-6 (0.21 pg/mL), which has a dual role: proinflammatory as cytokine and anti-inflammatory as myokine [Haghighatdoost *et al.*, 2017]. As explained by Craddock *et al.* [2019], this is due to the inclusion of research on individuals treated with statins (reducing inflammation), which significantly altered the results. Moreover, the papers where participants consumed lesser amounts of meat in vegetarian groups, or the diet was not sufficiently described were also included in the meta-analysis conducted by Haghighatdoost *et al.* [2017]. Another meta-analysis (21 cross-sectional studies) evaluating the association of veganism and vegetarianism with biomarkers of inflammation was published in 2020 [Menzel *et al.*, 2020]. The vegan diet adoption ranged from 1 year to 20 years and the vegetarian diet from 1 year to 25 years. Most studies (12) were conducted in Asia, followed by Europe (6) and South America (3). The research focused on the following biomarkers: CRP, tumor necrosis factor α, interleukin-6, interleukin-18, interleukin-1 receptor antagonist, selectin E, intercellular adhesion molecule, monocyte chemoattractant protein-1, adiponectin, omentin-1, and resistin. The meta-analysis indicated that veganism, compared to an omnivorous diet, reduced CRP levels (mean difference, MD=–0.54 mg/dL) more than vegetarianism (–0.25 mg/dL). It was observed that the association between the vegetarian pattern and CRP was significantly stronger in patients with renal impairment (–3.91 mg/L). However, no significant changes were observed for the other inflammatory biomarkers [Menzel *et al.*, 2020].

Considering that inflammatory biomarkers, such as CRP, are associated with the pathogenicity of chronic diseases, such as type 2 diabetes and cardiovascular diseases, further investigations, including dietary interventional studies, are required to understand the effect of vegetarianism on reducing inflammation in specific groups of patients.

Vegetarian diet and cardiovascular disease

The impact of a vegetarian diet on cardiovascular disease (CVD) risk and mortality has been repeatedly examined [Matsumoto *et al.*, 2019; Pawlak, 2015; Petermann-Rocha *et al.*, 2021; Vahid *et al.*, 2022]. According to the analysis of 10 prospective cohort studies, the risk of ischemic heart disease morbidity and/or mortality in participants on a plant-based diet was reduced by 25%, although not for all cerebrovascular and cardiovascular diseases [Dinu *et al.*, 2017].

In 2021, two meta-analyses examining the impact of vegetarianism on CVD prevalence proved the key role of consumed plant-based food quality [Gan *et al.*, 2021; Quek *et al.*, 2021]. One analysis included 10 studies (9 cohorts) and a total of 698,707 participants, including 137,968 individuals with CVD, 41,162 with coronary heart disease (CHD), and 13,370 with stroke [Gan *et al.*, 2021]. As revealed, the strictest adherence to vegetarian diets (as measured by the plant-based diet index, PDI) remained associated with a lower risk of CVD (relative risk, RR=0.84) and CHD (RR=0.88), although not with stroke (RR=0.87). Similarily, the meta-analysis of seven prospective cohort studies, encompassing 29,705 individuals adhering to a vegetarian diet, did not find an association with the risk of stroke compared to non-vegetarians [Lu *et al.*, 2021].

It has also been reported that eating an unhealthy plant-based diet (rich in refined grains, sweets, and sweetened beverages) may increase CVD risk (RR=1.13) [Gan *et al.*, 2021]. The second meta-analysis included thirteen prospective cohort studies (410,085 participants; 78,671 vegans and vegetarians) and demonstrated that greater compliance with

a vegetarian diet appeared to be significantly associated with a lower risk of CVD morbidity (RR=0.90) and cardiovascular mortality (RR=0.92) [Quek *et al.*, 2021]. Unhealthy plant-based diets (by PDI) increased the risk of cardiovascular mortality (RR=1.05) but did not affect the volume of CVD morbidity. A healthy vegetarian diet was associated with reduced CVD incidence (RR=0.87), although no association with mortality was demonstrated. Overall, vegetarians, compared with individuals following an omnivorous dietary pattern, had a significantly lower prevalence of CVD (RR=0.81) but with similar mortality rates from CVD [Quek *et al.*, 2021].

The above-reviewed data confirm that a vegetarian diet may decrease cardiovascular risk; however, the quality of the plant-based products consumed is essential. It is also crucial for individuals adhering to vegetarian diets to improve their intake of B_{12} vitamin since its deficiency has been associated with elevated plasma levels of homocysteine, an independent risk factor for CVD [Feng *et al.*, 2020; Meleady & Graham, 1999; Obersby *et al.*, 2013]. Such deficiency can be prevented by supplementation of B_{12} vitamin or increased consumption of B_{12}-fortified foods [Damayanti *et al.*, 2018].

Vegetarian diet and cancer risk

The results of three meta-analyses focused on assessing the impact of a vegetarian diet on cancer risk are inconclusive. The first analysis included seven papers in which lacto-ovo-vegetarianism or veganism was the considered dietary model [Huang *et al.*, 2012]. A total of more than 124,000 participants from 10 to 90 years of age were under observation for 10 to 23 years. It has been reported that vegetarians had an 18% lower cancer morbidity than non-vegetarians [Huang *et al.*, 2012]. The subsequent meta-analysis also demonstrated a positive impact of the discussed diet on cancer risk. It included 86 cross-sectional surveys and 10 prospective cohort trials [Dinu *et al.*, 2017]. The analysis revealed a significant decrease in cancer risk compared to an omnivorous diet. Compliance with a vegetarian diet resulted in an 8% reduced risk and a 15% reduced risk with a vegan diet. However, no significant association was demonstrated for specific cancer types [Dinu *et al.*, 2017]. Likewise, in a study focusing on breast, colorectal, and prostate cancers, vegetarian diets had no significant impact on reducing the risk of the variations mentioned above of oncological diseases compared to a non-vegetarian diet [Godos *et al.*, 2017]. This meta-analysis included nine studies conducted on six cohorts. Among approximately 687,000 participants, there were 3,441 cases of breast cancer, 4,062 cases of colorectal cancer, and 1,935 cases of prostate cancer. However, during the analysis, a lower risk of colorectal cancer was noted with semi-vegetarian (–14%) and pesco-vegetarian (–33%) diets compared to a non-vegetarian pattern [Godos *et al.*, 2017].

Although there is evidence that vegetarian diets may beneficially impact cancer risk, further studies, including long-term observations, are required to understand their association with specific cancer types.

Vegetarian diet and type 2 diabetes mellitus risk

A meta-analysis of two cohort studies and twelve cross-sectional studies on the impact of a vegetarian diet on type 2 diabetes risk was published in 2017 [Lee & Park, 2017]. The combined odds ratio for diabetes in vegetarians *vs*. nonvegetarians was 0.726. Subgroup analyses suggested vegetarians were less likely to suffer from diabetes than omnivores. It has been estimated that individuals on a vegetarian diet were 27% less likely to develop diabetes than those on a traditional diet. Compared to four studies conducted in Southeast Asia, lower risks were observed in three studies conducted in the Western Pacific region and seven in Europe and North America. The meta-analysis suggests that a vegetarian diet may protect against diabetes by, among other things, increasing insulin sensitivity (especially in vegans) and lowering intramuscular lipid levels, which affect insulin resistance. The authors confirm the need for further research on the type of vegetarianism and diet duration in relation to diabetes risk [Lee & Park, 2017].

Moreover, numerous research papers have confirmed the beneficial influence of vegetarianism on individuals with diabetes. In the meta-analysis involving nine clinical trials (a total of 664 subjects), an association has been observed between vegetarian diet consumption (for at least three weeks) and glycemic control, along with other cardiometabolic risk factors [Viguiliouk *et al.*, 2019]. As demonstrated, this diet significantly reduced HbA1c (MD=–0.29%), fasting blood glucose (MD=–0.56 mmol/L), LDL cholesterol (MD=–0.12 mmol/L), non-HDL cholesterol (MD=–0.13 mmol/L), body weight (MD=–2.15 kg), body mass index (BMI) (MD=–0.74 kg/m^2), and waist circumference (MD=–2.86 cm) [Viguiliouk *et al.*, 2019]. The last three parameters were also analyzed in the 2021 meta-analysis of seven studies that examined the impact of plant-based diets on body weight in adults with type 2 diabetes and compared it to a regular meat diet [Austin *et al.*, 2021]. Studies included a vegan diet and one considered a lacto-vegetarian diet. Body weight, BMI, and waist circumference were measured in a total of 353 participants. Significant reductions in average differences in body weight, BMI, and waist circumference were found for plant-based diets *vs*. regular meat diet. Plant-based diet adoption by individuals with type 2 diabetes resulted in a 5.1% reduction in average body weight, a 5.4% reduction in BMI, and a 4.3% reduction in waist circumference. Dietary interventions without restricting energy intake also resulted in a significant reduction in body weight. Regardless of caloric content, plant-based diets have proved to be effective in reducing central adiposity in individuals with type 2 diabetes mellitus [Austin *et al.*, 2021]. Furthermore, adherence to a vegetarian diet was beneficial for diabetic patients to lower LDL cholesterol and non-HDL cholesterol, and also to ensure glycemic control [Viguiliouk *et al.*, 2019]. The existing evidence indicates that implementation of vegetarian diet can potentially represent cost-effective and low-risk interventions in type 2 diabetes mellitus.

Vegetarian diet and chronic kidney disease

To the best of our knowledge, there is no meta-analysis testing the effect of a plant-based diet on chronic kidney disease (CKD). Only one meta-analysis focused on selected biomarkers associated with kidney impairment. It was demonstrated, considering four available studies, that compared to

an omnivorous diet, adherence to vegetarianism was associated with lower CRP levels (–3.9 mg/L) [Menzel *et al.*, 2020].

In addition to this meta-analysis, there is one randomized controlled trial, two cross-over studies, and one cross-sectional study that tested vegetarianism's effect in the groups of non-dialyzed patients with CKD [Valim *et al.*, 2022]. A prospective, randomized, controlled trial of 207 participants showed that a very-low-protein vegetarian diet (VLPD; 0.4 g/kg/day) combined with amino acid ketoanalogues supplementation was more beneficial than a traditional low-protein diet (LPD; 0.6 g protein/kg/day) [Garneata *et al.*, 2016]. After 15 months of complying with dietary recommendations, 42% of patients in the LPD group reached the endpoint (initiation of renal replacement therapy or a >50% reduction in initial estimated glomerular filtration rate, eGFR), while in the vegetarian group, 13% patients did. Initiation of renal replacement therapy was more required in the first group (30%) than in the second group (11%). After adjusting for relevant variables (eGFR, body mass index, CPR, and angiotensin-converting enzyme inhibitor), a VLPD combined with amino acid ketoanalogues supplementation appeared to be associated with a lower probability of reaching the endpoint [Garneata *et al.*, 2016].

The other three studies (one cross-sectional study and two cross-over trials) did not show differences in renal function (measured parameters: eGFR or creatinine clearance) with vegan and meat-based diets [Chang *et al.*, 2018; Moe *et al.*, 2011; Soroka *et al.*, 1998]. However, two of these studies (both cross-over design studies) included a very small sample size of only 8–9 participants; therefore, the meaning results may be limited [Moe *et al.*, 2011; Soroka *et al.*, 1998; Valim *et al.*, 2022].

In summary, more studies on larger sample sizes and prolonged observation periods are required to draw final conclusions on vegetarian diets' role in CKD.

Vegetarian diet and gut microbiome

Research shows that the composition of the gut microbiota is different in individuals on a plant-based and omnivorous (meat-containing) diet. In one study, 268 non-diabetic volunteers (41 to 58 years old) were assessed for the effect of practicing vegetarianism for at least one year (66 people in the vegetarian group, 102 in the lacto-ovo vegetarian group and 100 in the omnivorous group) on gut bacteria counts [Franco-de-Moraes *et al.*, 2017]. Most of the studied population were women (54.2%) and 41.4% of the participants were overweight. The taxonomic composition and phylogenetic structure of the microbiota were obtained through the analysis of the 16S rRNA gene. Clinical, biochemical, and circulating inflammatory markers were compared. *Firmicutes* and *Bacteroidetes* were the most abundant bacterial types, with no differences in abundance between normal-weight and overweight subjects. Strict vegetarians had lower percentages of *Firmicutes* and *Bacteroidetes* than lacto-ovo vegetarians and omnivores.

Furthermore, a higher *Prevotella* abundance and *Prevotella*/*Bacteroides* ratio were noted in subjects on vegetarian diets than in the other groups. Both strict vegetarians and lacto-ovo vegetarians had a higher percentage of *Faecalibacterium* compared to omnivores, where excessive amounts of *Succinivibrio* and *Halomonas* from the Proteobacteria cluster were noted. Compared to the vegetarian groups, the latter group showed higher values of anthropometric data, insulin, insulin resistance index, and a worse lipid profile. Inflammatory markers exhibited a successive increase. Such findings suggest that the consumption of animal foods may trigger systemic inflammation and insulin resistance-dependent metabolic disorders [Franco-de-Moraes *et al.*, 2017].

A randomized cross-over study examining the effects of a vegetarian diet on, among other things, microbiota composition in individuals with ischemic heart disease was published in 2020 [Djekic *et al.*, 2020]. Participants (31 subjects; 29 men 63 to 70 years old), divided into two groups (vegetarian diet and omnivore diet), underwent a 4-week intervention. After a washout period (4 weeks), the groups swapped the isocaloric diets. Analysis showed that none of the dietary models affected the abundance or composition of the gut microbiota at the cluster level, while changes occurred in the abundance of several types of bacteria from the *Ruminococcaceae*, *Lachnospiraceae*, and *Eggerthellaceae* families. In vegetarian diet participants, the predominant *Ruminococcaceae* bacterial genera were associated with reduced oxidized LDL cholesterol levels and, subsequently, lower cardiometabolic risk. In addition, compared to the meat-eaters, the vegetarian group exhibited reduced fecal microbial taxa and plasma metabolites, associated with a lower risk of metabolic diseases, including cardiovascular diseases [Djekic *et al.*, 2020].

Differences in gut microbiota composition between vegetarians and omnivores were also demonstrated in a 4-week randomized controlled trial including healthy individuals (53 subjects; 33 women, 20 men), 18 to 60 years of age. The participants were divided into two groups: vegan and omnivore. As in other publications, stool samples were analyzed using 16S rRNA gene amplicon sequencing. It was shown that, in contrast to the omnivore group, the vegetarian group had a decrease in *Roseburia* and *Faecalibacterium* abundance and an increase in *Coprococcus*. These three types of bacteria may be associated with mental and physical health; hence, further research is recommended [Kohnert *et al.*, 2021]. The available data clearly show that different subtypes of vegetarian diets affect the abundance of other bacteria in the gut microbiota, resulting in improved physical and mental health.

Vegetarian diet and mental health

The effects of a vegetarian diet on mental health and the risk of depression have been analyzed numerous times in various populations, but the results of existing studies are inconsistent [Bègue & Shankland, 2022; Hopwood, 2022; Jin *et al.*, 2021; Lee *et al.*, 2021; Shen *et al.*, 2021]. The meta-analysis of thirteen observational or interventional studies (17,809 individuals) evaluated the association of vegetarianism and veganism with mental health and cognitive functions [Iguacel *et al.*, 2021]. It was demonstrated that vegans/vegetarians were more likely to suffer from depression (odds ratio, OR=2.1) but had lower anxiety levels (MD=–0.85) than those on a traditional diet. Subgroup analyses of anxiety showed higher risk in participants younger than 26 years and studies of higher quality. However, the heterogeneity of

the studies was considerable, although subgroup analyses showed numerous differences. The meta-analysis found no significant association between diet and depression, stress, mood, or cognitive impairment [Iguacel *et al.*, 2021].

Another meta-analysis of 13 studies (43,728 participants; 5,436 vegetarians and 38,292 non-vegetarians) found that vegetarians had higher depression scores (according to the PHQ-9 patient health questionnaire) than non-vegetarians [Ocklenburg & Borawski, 2021]. However, the authors stated that due to the considerable heterogeneity of publications, further empirical studies are needed before any definitive conclusions can be formed. Another meta-analysis (thirteen papers, including four cohort studies and nine cross-sectional studies) published in 2022 examined the association of vegetarianism with depression, anxiety, and stress symptoms in adults [Askari *et al.*, 2022]. Ten studies indicated no association of the dietary model in question with the incidence of depression. Four publications has suggested that adherence to a vegetarian diet is not associated with anxiety. Due to insufficient data, the authors could not collect stress scores; thus, the main conclusion of the meta-analysis was an indifferent effect of a vegetarian diet on depression and anxiety [Askari *et al.*, 2022].

Given the conclusions of the above-discussed link between a vegetarian diet and mental health, there is a need to pursue further studies. Importantly, there is a need to conduct studies on different populations before a generalization of the effect can be outlined. Moreover, some authors suggest that future research should also consider the effect of experimental diet manipulation on mental health outcomes [Lavallee *et al.*, 2019]. Last but not least, there are numerous confounding factors when testing the effect of any diet on mental health that need to be taken into account, *e.g.*, age, gender, socioeconomic status, education, physical activity, genetic predisposition, smoking, chronic health issues, environmental background (*e.g.*, trauma levels) [Alzahrani *et al.*, 2022; Sheldon *et al.*, 2021]. Controlling them all may be highly challenging when testing the effect of a particular diet.

MEDITERRANEAN DIET AND HUMAN HEALTH

Mediterranean diet and lipid profile

A cohort study of 4,740 participants 35–70 years of age examining the association between the Mediterranean diet and blood lipid levels in Iranian adults was published in 2021 [Panbehkar-Jouybari *et al.*, 2021]. After a four-year observation, the HDL cholesterol level in these subjects significantly increased (52.8±12.3 for the third *vs.* 51.6±11.6, first tercile). Furthermore, strict compliance with Mediterranean diet was associated with decreased LDL to HDL cholesterol ratio (OR=0.85), which promoted proper lipid metabolism. However, no alterations were observed in total cholesterol, LDL fraction, or triglyceride levels [Panbehkar-Jouybari *et al.*, 2021]. In the same year, a randomized cross-over study was published. It aimed to evaluate the effects of short-term (4-week) use of a caloric-restricted Mediterranean diet *vs*. a traditional caloric-restricted diet on lipid profile and other metabolic parameters in South Koreans with hypercholesterolemia [Son *et al.*, 2021]. After a two-week washout period, participants, randomly assigned to the caloric-restricted Mediterranean diet group or the control diet group (92 subjects), exchanged diets. It was shown that even after accounting for age, gender, changes in total energy intake, alcohol intake, smoking, and changes in physical activity; the caloric-restricted Mediterranean diet group significantly decreased total cholesterol levels and LDL and HDL cholesterol levels, thereby supporting the treatment of dyslipidemia. In addition, it has also been shown that the diet used had a beneficial effect on reducing the risk of cardiovascular diseases by decreasing anthropometric parameters, decreasing levels of white blood cells, fasting glucose, fasting insulin, homeostatic model assessment for insulin resistance (HOMA-IR) index, and hepatic steatosis index (FLI), regardless of energy intake, physical activity, and changes in body weight reduction [Son *et al.*, 2021].

Another cross-sectional study on the effect of the Mediterranean diet on lipid metabolism in subjects with familial hypercholesterolemia was conducted on Brazilian residents (n=92) and Spanish residents (n=98) [Antoniazzi *et al.*, 2021]. As shown, the majority of Brazilian residents (83.7%) had low adherence to the Mediterranean diet, which was associated with their higher LDL cholesterol levels than in the Spanish group: 179 (135–250) and 161 (133–193) mg/dL. After adjusting for socioeconomic parameters, caloric and fatty acid intake, and lipid-lowering pharmacological therapies, a significant association was reported between high adherence to the Mediterranean diet and lowering of LDL cholesterol, thus a beneficial effect on familial hypercholesterolemia [Antoniazzi *et al.*, 2021].

One multi-ethnic cohort study also examined the effects of the Mediterranean diet on the lipid profile of individuals from less-developed ethnic minorities [Zhang *et al.*, 2022]. As demonstrated, adherence to the Mediterranean diet (as measured by the alternative mediterranean diet scale – AMED) was negatively associated with total cholesterol, LDL, and HDL fraction levels. Comparing the highest quintiles with the lowest AMED scores, total cholesterol levels decreased by 0.082 (–0.092 to –0.049) mmol/L, LDL cholesterol by 0.030 (–0.048 to -0.012) mmol/L, and HDL cholesterol by 0.0275 (–0.036 to –0.019) mmol/L. Despite the absence of changes in triglyceride levels, the Mediterranean diet was found to effectively improve lipid metabolism in underdeveloped ethnic minorities [Zhang *et al.*, 2022].

Mediterranean diet and blood pressure

Many studies have demonstrated that the Mediterranean diet lowers blood pressure [Ahmed *et al.*, 2020; Dai *et al.*, 2022; Jennings *et al.*, 2019; Magriplis *et al.*, 2020; Septiadi *et al.*, 2021]. The meta-analysis encompassing six randomized controlled trials compared the Mediterranean diet with low-fat diets [Nissensohn *et al.*, 2016]. As shown, adherence to the Mediterranean diet for at least one year resulted in reduced systolic (–1.44 mm Hg) and diastolic (–0.70 mm Hg) blood pressure. However, due to the small number of studies included in the meta-analysis and their large heterogeneity, the authors did not have sufficient evidence to support the blood-pressure-lowering effects of the Mediterranean diet [Nissensohn *et al.*, 2016]. As many as three meta-analyses

(evaluating the association between the Mediterranean diet and blood pressure) were published in 2021 [Bakaloudi *et al.*, 2021; Cowell *et al.*, 2021; Filippou *et al.*, 2021]. One of these analyses, involving 19 randomized controlled trials (4,137 participants), showed that Mediterranean diet interventions reduced systolic and diastolic blood pressure by an average of 1.4 mmHg and 1.5 mmHg, respectively, compared with the control group [Cowell *et al.*, 2021]. As shown in the meta-regression, longer study duration and higher baseline systolic blood pressure were associated with more significant decreases in response to the Mediterranean diet. In the same publication, the meta-analysis of 16 observational studies (59,001 participants) showed that with higher adherence to the Mediterranean diet, the probability of developing hypertension was 13% less than at a lower [Cowell *et al.*, 2021].

Another meta-analysis of 54 observational studies noted that compared with the low adherence to the Mediterranean diet, greater adherence lowered systolic blood pressure (SMD=–0.08; –0.15 to –0.02) but did not significantly affect diastolic blood pressure (SMD=–0.07; –0.13 to 0.00) [Bakaloudi *et al.*, 2021]. These findings may be related to normal levels (<90 mmHg) of mean diastolic pressure in all study participants.

The other meta-analysis encompassed 35 randomized controlled trials (13,943 participants). It demonstrated that, compared with the usual diet and other intervention diets, the Mediterranean diet reduced systolic blood pressure by 1.5 mmHg and diastolic blood pressure by 0.9 mmHg [Filippou *et al.*, 2021]. Compared with the usual diet alone, the Mediterranean diet still caused both blood pressures to fall. However, the association disappeared when comparing the Mediterranean diet to all other intervention diets or a low-fat diet alone. Additionally, more significant reductions in diastolic blood pressure were observed in subjects with mean baseline systolic blood pressure ≥130 mmHg. It was noted that both systolic and diastolic blood pressure were lowered more in interventions with a mean follow-up period of ≥16 weeks [Filippou *et al.*, 2021].

Mediterranean diet and body weight

Two meta-analyses have shown that following a Mediterranean diet may support weight loss [Esposito *et al.*, 2011; Lotfi *et al.*, 2022]. The first analysis included 16 randomized controlled trials and 3,436 participants (1,848 with a Mediterranean diet and 1,588 with a control diet – in most studies, represented by a low-fat diet, but also high-carbohydrate, prudent or high-saturated fat diets) [Esposito *et al.*, 2011]. As shown, Mediterranean diet subjects had greater weight loss (–1.75 kg; –2.86 to –0.64 kg) and BMI (–0.57 kg/m^2; –0.93 to –0.21 kg/m^2), compared with the control group. Moreover, the weight loss was greater when following the Mediterranean diet with energy restriction (–3.88 kg; –6.54 to –1.21 kg), increased physical activity (–4.01 kg; –5.79 to –2.23 kg), and intervention longer than 6 months (–2.69 kg; –3.99 to –1.38 kg). What is important, none of the studies showed significant weight gain when following the Mediterranean diet, thus confirming the lack of negative effects of the substantial amounts of olive oil used in the Mediterranean diet [Esposito *et al.*, 2011].

The second meta-analysis included seven prospective cohort studies with a total of several hundred thousand participants [Lotfi *et al.*, 2022]. As shown, adherence to the Mediterranean diet was strongly associated with a 9% reduced risk of overweight and/or obesity (RR=0.91) only for studies on overweight and obesity (RR=0.92) and not for articles focusing only on obesity (RR=0.68). In addition, the analysis of 6 publications proved a risk reduction (RR=0.98) of overweight and/or obesity of 2% per 1 Mediterranean Diet Score (MDS). Each unit increase in the MDS was associated with a reduction in weight gain of 0.04 kg over five years [Lotfi *et al.*, 2022]. In summary, available meta-analyses suggest that the Mediterranean diet may support weight loss in adults.

Mediterranean diet and inflammation biomarkers

A meta-analysis of seventeen (lasting at least 12 weeks) randomized controlled trials on a total of 2,300 subjects evaluated the association of the Mediterranean diet with inflammation in adults [Schwingshackl & Hoffmann, 2014a]. Adherence to Mediterranean diet was shown to significantly increase adiponectin levels (weighted mean difference =1.69 μg/mL) and decrease high-sensitivity C-reactive protein (hs-CRP; –0.98 mg/L), interleukin-6 (–0.42 pg/mL), and intercellular adhesion molecule-1 (–23.73 ng/mL). The analysis results prove that adherence to the Mediterranean diet decreases inflammation and improves endothelial function [Schwingshackl & Hoffmann, 2014a].

The more recent meta-analysis, encompassing 13 publications (eight cross-sectional studies, three randomized clinical trials, 1 quasi-experiment, and 1 cohort study), examined the effect of the Mediterranean diet on inflammation in older people (≥65 years) [Wu *et al.*, 2021]. Two randomized controlled trials demonstrated that greater adherence to the Mediterranean diet among the elderly was associated with a decrease in CRP (–0.54 and –0.34 mg/dL) and interleukin 6 (–1.6 and –0.2 pg/mL). In contrast, in a 3-year cohort study, only a decrease in CRP values was noted (–0.10 pg/mL).

The meta-analysis of 5 cross-sectional studies also demonstrated that the Mediterranean diet significantly decreased CRP levels (SMD=–0.26). Data on other inflammatory markers varied in the included publications; thus, no significant correlations were found [Wu *et al.*, 2021].

Mediterranean diet and cardiovascular disease

The impact of the Mediterranean diet on cardiovascular risk and mortality associated with lipid and blood pressure was examined in five major meta-analyses. One of them included six studies and a total of 10,950 participants (477 major cardiovascular events, 693 deaths, 315 cardiovascular deaths) [Bloomfield *et al.*, 2016; Chen *et al.*, 2019; Liyanage *et al.*, 2016; Mayr *et al.*, 2018; Tang *et al.*, 2021]. As shown, adherence to the Mediterranean diet decreased the risk of major cardiovascular events (RR=0.63), coronary events (RR=0.65), stroke (RR=0.65), and cardiac failure (RR=0.30) but was not associated with mortality from cardiovascular causes (RR=0.90) or any causes (RR=1.00). After excluding a large (1,000 participants) study with non-integral data, there was still evidence of a significant association of the Mediterranean diet with cardiovascular events

(RR=0.69) and stroke (RR=0.66), although positive effects for coronary events (RR=0.73) and heart failure (RR=0.25) disappeared [Liyanage *et al.*, 2016].

In the second meta-analysis, two publications on primary prevention demonstrated no difference in mortality from any cause between subjects using the Mediterranean diet and other groups. At the same time, one study showed that the Mediterranean diet decreased the incidence of major cardiovascular events (hazard ratio=0.71) [Bloomfield *et al.*, 2016]. In one of the three publications on secondary prevention, adherence to the Mediterranean diet without fat intake restrictions appeared to decrease the risk of recurrent myocardial infarction and cardiovascular death [Bloomfield *et al.*, 2016].

The third meta-analysis, focusing on stroke-related data, included twenty prospective cohort studies encompassing 682,149 participants (16,739 stroke cases) [Chen *et al.*, 2019]. The RR for each 4-point increase in the MDS was 0.84 for all studies, 0.76 for studies on Mediterranean populations, and 0.86 for those on other populations. Adherence to the Mediterranean diet was associated with a lower risk of ischemic stroke (RR=0.86) and hemorrhagic stroke (RR=0.83). The data suggest that regardless of residence, the Mediterranean diet reduced the risk of both strokes [Chen *et al.*, 2019]. The most recent meta-analysis, including 7 cohort studies (37,879 participants with a history of CVD), found that the pooled RR for each 2-unit increment of the MDS was 0.85 for all-cause mortality and 0.91 for cardiovascular mortality [Tang *et al.*, 2021]. Moreover, according to the subgroup analysis for all-cause mortality, the association was stronger in trials of shorter duration (RR=0.75) and Mediterranean regions (RR=0.76) than in non-Mediterranean areas (RR=0.95). Thus, the meta-analysis proved that adherence to a Mediterranean diet improved survival in people with a history of CVD [Tang *et al.*, 2021].

The 2018 meta-analysis, including 11 studies, examined the effects of an olive oil-rich Mediterranean diet on inflammation in patients with coronary heart disease (CHD) [Mayr *et al.*, 2018]. Five clinical trials showed a slight reduction in CRP levels following adherence to the Mediterranean diet, and two noted a significant decrease. The random-effects model meta-analysis of four controlled trials did not prove any significant difference between the Mediterranean diet and low-fat diets in the final mean CRP levels.

Conversely, four observational studies showed a significant association between the Mediterranean diet and reductions in proinflammatory cytokines. Nevertheless, in most studies, the effect of the Mediterranean diet on inflammation in individuals with CHD was insignificant; thus, the effect of this diet cannot be conclusively determined [Mayr *et al.*, 2018]. In addition, a recent randomized controlled trial examined the association between long-term Mediterranean diet use by individuals with CHD and kidney function [Podadera-Herreros *et al.*, 2022]. Participants from the CORDIOPREV trial (n=1,002), were randomly assigned to the Mediterranean diet group or low-fat diet consumption group and underwent a 5-year dietary intervention. Kidney function was assessed at the beginning and at the end of the study by the determination of serum creatinine-based estimated glomerular filtration rate (eGFR). By distinguishing between participants with type 2 diabetes mellitus and healthy participants, multiple linear regression analysis showed that Mediterranean diet use was associated with a smaller decrease in eGFR than a low-fat diet in patients with type 2 diabetes mellitus and in the general population. However, no significant difference in eGFR was observed between the two dietary models in subjects without type 2 diabetes mellitus. Moreover, such an effect of the Mediterranean diet was observed in participants with mild eGFR impairment. Data suggest that long-term use of the Mediterranean diet may preserve kidney function in individuals with CHD and type 2 diabetes mellitus, especially in patients with mild eGFR impairment [Podadera-Herreros *et al.*, 2022]. It may therefore be concluded that the Mediterranean diet provides clinical benefits in preventing secondary cardiovascular diseases.

Mediterranean diet and cancer risk

According to research, the Mediterranean diet may reduce the risk of selected cancers [Gioxari *et al.*, 2021; Männistö *et al.*, 2021; Montano *et al.*, 2022; Schulpen & van den Brandt, 2021; Tayyem *et al.*, 2022]. In a pooled analysis of 3 Italian case-control studies, including 5,079 women (1,411 diagnosed with endometrial cancer and 3,668 in the control group) the 9-item MDS used showed that high adherence to this diet *vs*. low adherence was associated with a 57% reduction in endometrial cancer risk (OR=0.43) [Filomeno *et al.*, 2015]. The OR for an increment of one MDS unit, or one Mediterranean diet component, was 0.84. The findings suggest that a Mediterranean diet has a beneficial role in preventing endometrial cancer [Filomeno *et al.*, 2015]. The meta-analysis encompassing six studies (3,986 women; 2,321 with diagnosed breast cancer and 1,665 in the control group) evaluated the association between the Mediterranean diet and breast cancer (estrogen/progesterone receptor subtypes: ER/PR) in postmenopausal women [van den Brandt & Schulpen, 2017]. As shown, high adherence to the Mediterranean diet was associated with a 23% reduction in ER–PR– and ER– breast cancer risk by 27%, whereas for ER+, this relationship was not statistically significant, indicating a positive effect of the Mediterranean diet on specific breast cancer subtypes only [van den Brandt & Schulpen, 2017].

Another meta-analysis of 10 observational studies (33,451 prostate cancer cases) examined the effect of the Mediterranean diet on prostate cancer risk [Cheng *et al.*, 2019]. The RR was 0.95 in general for prostate cancer, 0.93 for advanced stage, and 0.92 for its lethal form. The results suggest that the Mediterranean diet is not associated with prostate cancer risk [Cheng *et al.*, 2019].

The meta-analysis of 21 cohort studies and 12 case-control studies (just under 1.5 million participants) evaluating the impact of the Mediterranean diet on various cancers was published in 2014 [Schwingshackl & Hoffmann, 2014b]. As shown, the highest adherence to the Mediterranean diet resulted in a 4% reduction in prostate cancer risk, a 14% reduction in colorectal cancer risk, a 56% reduction in laryngeal cancer risk, and a 10% reduction in cancer mortality risk. An association of the Mediterranean diet with breast, stomach, and pancreatic cancer was not observed [Schwingshackl & Hoffmann, 2014 b].

Conversely, a recent meta-analysis of 117 studies (encompassing a total of over 3 million participants) showed that the highest adherence to the Mediterranean diet reduced cancer mortality by 13% (based on eighteen cohort studies) and mortality from any cause among cancer survivors by 25% (based on eight cohort studies) [Morze *et al.*, 2021]. Furthermore, higher adherence to the Mediterranean diet resulted in a lower risk of breast cancer (by 6%; twenty-three observational studies), bladder cancer (by 13%; four observational studies), respiratory cancer (by 16%; five cohort studies), colorectal cancer (by 17%; seventeen observational studies), stomach cancer (by 30%; 7 observational studies), liver cancer (by 36%; four observational studies), and head and neck cancer (by 54%; nine observational studies). The association between the Mediterranean diet and the incidence of blood, esophageal, pancreatic, and prostate cancers was not observed, which is a finding consistent with the results of previous publications [Morze *et al.*, 2021].

Mediterranean diet and type 2 diabetes mellitus

In 2015, a large meta-analysis was published, including five randomized controlled clinical trials (lasting at least six months) and eight meta-analyses [Esposito *et al.*, 2015]. Meta-analyses showed that higher adherence to the Mediterranean diet was associated with a 19% [Schwingshackl *et al.*, 2015] and 23% reduction in type 2 diabetes risk, thus recommending the Mediterranean diet for the prevention of type 2 diabetes [Esposito *et al.*, 2015; Koloverou *et al.*, 2014]. The meta-analysis of 3 long-term clinical trials showed that the overall effect for HbA1c was –0.47% (–0.56 to –0.38), confirming the beneficial effect of the Mediterranean diet on glycemic control compared with traditional or low-fat diets. In addition, four other meta-analyses of randomized clinical trials also demonstrated lower HbA1c levels (–0.3% to –0.47%) in type 2 diabetes patients using the Mediterranean diet [Esposito *et al.*, 2015].

The meta-analysis published five years later included as many as 41 articles (3 randomized clinical trials and 38 prospective cohort studies) [Becerra-Tomás *et al.*, 2020]. The analysis of randomized controlled trials showed a beneficial effect of the Mediterranean diet on the prevalence of cardiovascular disease (RR=0.62) and myocardial infarction (MI; RR=0.65), whereas prospective cohort studies comparing the highest and lowest adherence to the Mediterranean diet proved an inverse association with CVD mortality (RR=0.79), coronary heart disease (RR=0.73), CHD mortality (RR=0.83) stroke incidence (RR=0.80), stroke mortality (RR=0.87), and MI incidence (RR=0.73). The above data suggest that the Mediterranean diet is highly beneficial in preventing CVD in individuals with type 2 diabetes [Becerra-Tomás *et al.*, 2020].

Mediterranean diet and chronic kidney disease

A meta-analysis of four studies (8,467 participants) which was published in 2020, assessed the association between adherence to the Mediterranean diet (assessed by standardized food frequency questionnaires) and prevention of chronic kidney disease [Hansrivijit *et al.*, 2020]. In all included articles, an index of the adopted diet's similarity to the Mediterranean diet was used (mean MDS=3.8±0.3). With the mean follow-up duration of 20.6±7.0 years, the pooled OR for CKD was 0.901 for each 1-point increment of MDS. The incidence of CKD equaled 0.026 events per person-year. Furthermore, the male sex was associated with the incidence of chronic kidney disease in an adjusted meta-regression analysis. However, there was no significant association between chronic kidney disease incidence and age, ethnicity, smoking, comorbidities, kidney function, and total daily energy intake. Adherence to the Mediterranean diet with a 1-point increase in MDS was associated with a 10% lower risk of chronic kidney disease. However, there were insufficient data for patients on dialysis or with preexisting chronic kidney disease [Hansrivijit *et al.*, 2020].

Mediterranean diet and gut microbiome

Mediterranean diet is characterized by high fiber intake, which is known to shift *Bacteroidetes* populations and maintain a reduced *Firmicutes* population, overall resulting in higher levels of short-chain fatty acids (SCFAs) in the gut [Haro *et al.*, 2017; Nagpal *et al.*, 2019]. A meta-analysis of seventeen studies was published in 2021 to determine whether the Mediterranean diet can prevent cancer and inflammatory bowel disease by modulating gut microflora [Illescas *et al.*, 2021]. The Mediterranean diet was compared with different dietary models, including the Paleolithic diet and the Western diet. Analysis of 1,563 stool samples showed that the microbiota of patients following the Mediterranean diet was enriched with beneficial bacteria promoting an anti-inflammatory environment (*Verrucomicrobia*, *Bacteroidetes*, *Actinobacteria*), whereas the bacterial community was reduced in those with inflammatory bowel disease, colonic adenocarcinoma, and colorectal cancer. An inverse relationship was also observed, with a decrease in the abundance of bacteria with pro-inflammatory properties (*Proteobacteria*, *Euryarchaeota*, *Fusobacteria*) in the Mediterranean diet and an increase in inflammatory bowel disease, colonic adenocarcinoma, and colorectal cancer. Moreover, subjects on the Mediterranean diet increased the abundance of *Akkermansia*, regarded as a marker of a healthy gut, and decreased that of *Fusobacterium*, a pathogenic bacterium associated with colorectal cancer and inflammatory bowel disease (IBD) [Illescas *et al.*, 2021]. Thus, the findings suggest that incorporating Mediterranean diet principles into lifestyle may prevent intestinal cancer.

Mediterranean diet and mental health

Of the twenty-two studies included in the 2013 meta-analysis, nine examined the effect of the Mediterranean diet on the risk of depression [Psaltopoulou *et al.*, 2013]. As shown, high and moderate adherence to the Mediterranean diet was significantly associated with a reduced risk (RR=0.68; 0.54–0.86). The protective effects of high adherence seemed independent of age, whereas the beneficial effects of moderate adherence to the Mediterranean diet reducing the risk of depression seemed to disappear with age [Psaltopoulou *et al.*, 2013]. A meta-analysis, published six years later, included a total of fourteen observational studies (four cohort and nine clinical-control studies) on a total of 56,043 participants [Shafiei *et al.*, 2019]. Analysis of the cohort studies, despite

the lack of heterogeneity, showed no significant association between adherence to a Mediterranean diet and risk of depression (RR=0.95; 0.79–1.16), whereas the cross-sectional studies found a 28% reduction in the risk (RR=0.72; 0.60–0.87). Given the use of food frequency questionnaires, the above differences in findings may be due to the misclassification of participants in some studies [Shafiei *et al.*, 2019]. Similarly as in the "Vegetarian diet and mental health" subsection, discussing the potential association between a vegetarian diet with mental health, one should note that there are numerous confounding factors and controlling them all, when testing the effect of diet [Alzahrani *et al.*, 2022; Sheldon *et al.*, 2021].

COMPARISON OF HEALTH BENEFITS OF VEGETARIAN AND MEDITERRANEAN DIETS

This section discusses the research allowing for direct comparison of the vegetarian and Mediterranean diets. It should be highlighted that the number of such investigations is limited. Therefore, more research, preferentially based on randomized cross-over studies in various populations, is encouraged in the future. The comparison of both diets discussed in subsequent subsections is summarized in Table 2.

TABLE 2. Comparison of health effects of vegetarian and Mediterranean diets emerging from comparative studies.

	Vegetarian diet	Mediterranean diet
Body weight control	↓	↓
Total cholesterol	↓↓	↓
Low-density lipoprotein cholesterol	↓↓	↓
Triglycerides	↓	↓↓
Blood pressure	↓	↓↓↓
Glycemic control	↓	↓↓
Total cancer risk	?	↓

Cardiovascular disease prevention

A randomized cross-over study performed as a part of the CARDIVEG Study examined the effect of the lacto-ovo vegetarian and Mediterranean diet on cardiovascular disease prevention [Sofi *et al.*, 2018]. Participants included overweight omnivores, at low or intermediate cardiovascular risk, with at least one additional risk factor (*i.e.*, abdominal obesity, high total cholesterol, high LDL cholesterol, high triglycerides, fasting glucose), and not on medications [Sofi *et al.*, 2016]. Subjects (118 individuals) were randomly divided into two groups (vegetarian diet $n=60$; Mediterranean diet $n=58$). Each dietary intervention phase lasted three months. After phase 1, the groups exchanged diets. Both low-calorie diets resulted in significant reductions in cardiovascular risk. A total of 81 subjects achieved the target values recommended by the European Society of Cardiology. Among them, 16 subjects in the lacto-ovo vegetarian group achieved target values for total cholesterol, 17 for LDL cholesterol, 6 for triglycerides, and 14 for BMI (body mass index). Meanwhile, from the Mediterranean diet group, target values for total cholesterol were achieved only by 7 participants, 6 for LDL cholesterol, 8 for triglycerides, and 10 for BMI. A low-calorie vegetarian diet was more effective in lowering total cholesterol and low-density lipoprotein levels, while a low-calorie Mediterranean diet led to greater reductions in triglyceride levels in overweight individuals [Sofi *et al.*, 2018].

Blood pressure in hypertension

The meta-analysis, involving 67 randomized trials and over 17,000 participants, estimated the effects of thirteen different (lasting at least 12 weeks) dietary interventions (including the Mediterranean and vegetarian diets) on blood pressure in individuals with hypertension and prehypertension [Schwingshackl *et al.,* 2019]. The Mediterranean diet ranked third as one of the most effective diets in reducing systolic and diastolic blood pressure. Although the vegetarian diet has not been distinguished in any way, the authors have found that an effective way to control blood pressure in populations with hypertension and prehypertension is to consume plenty of vegetables, fruits, grains, legumes, nuts, seeds, dairy products, and to follow a low intake of red meat, sugar-sweetened beverages, and sodium [Schwingshackl *et al.*, 2019]. This dietary pattern, with the exception of meat consumption, is consistent with both types of the discussed diets.

Cancer mortality

A meta-analysis examining the effect of vegetarian and Mediterranean diets on cancer mortality was published in 2020 [Molina-Montes *et al.*, 2020]. All papers included in the meta-analysis studied populations ranging from a few to several hundred thousand. Of the 13 articles, 5 focused on vegetarian/vegan diets and 8 on Mediterranean diets. The results indicated that the vegetarian/vegan diet did not exhibit significant preventive potential for overall cancer mortality compared with a non-vegetarian diet. However, the association between adherence to the Mediterranean dietary model and cancer mortality reached statistical significance. Nevertheless, none of the studies accounted for the influence of prognostic factors. Therefore, further analysis is needed to determine dietary guidelines for cancer survivors [Molina-Montes *et al.*, 2020].

Body weight control

One study simultaneously evaluated the effect of low-calorie lacto-ovo vegetarian and Mediterranean diets on weight loss [Sofi *et al.*, 2018]. A significant reduction occurred in both groups. The average weight reduction was –1.88 kg in the vegetarian group and –1.77 kg in the Mediterranean diet group. The decrease in BMI equaled –0.64 kg/m^2 in the vegetarian group and –0.67 kg/m^2 in the Mediterranean diet. In contrast, the reduction in fat mass reached –1.23 kg in the first group and –1.46 kg in the second group. The study confirms the beneficial effects of both vegetarian and Mediterranean diets in reducing body weight, BMI, and fat mass in overweight individuals. Given the comparable results, none of the diets could be determined as more effective [Sofi *et al.*, 2018].

Type 2 diabetes mellitus

Type 2 diabetes mellitus is the most common type of diabetes worldwide and appears to increasingly affect also younger

populations [Bellary *et al.*, 2021; Lawrence *et al.*, 2021]. Network meta-analysis was published in 2019, examining the effects of, among others, vegetarian and Mediterranean diets on blood lipid control in patients with type 2 diabetes [Neuenschwander *et al.*, 2019]. Fifty-two randomized controlled trials on adults with type 2 diabetes, with an intervention period ≥12 weeks and comparing dietary approaches for LDL cholesterol, HDL cholesterol, or triglycerides were included. It was shown that of the nine dietary approaches, the vegetarian diet was most effective in lowering LDL cholesterol levels compared to the control diet. Conversely, the Mediterranean diet favorably raised HDL cholesterol and lowered triglyceride levels compared to the control diet [Neuenschwander *et al.*, 2019]. Researchers assessing the effects of both diets on CVD prevention in overweight individuals reached similar conclusions [Sofi *et al.*, 2018]. Therefore, this implies that the diets similarly affect the lipid profile in healthy individuals and those with type 2 diabetes. However, a 2019 meta-analysis recognized the Mediterranean dietary model as the most effective dietary approach for the full control of diabetic dyslipidemia (79% according to the surface under the cumulative ranking curve (SUCRA) score) [Neuenschwander *et al.*, 2019].

Several studies have also reported the positive effects of Mediterranean and vegetarian diets on glycemic control in people with type 2 diabetes. A network meta-analysis of 56 studies comparing nine dietary approaches (including the two mentioned above) evaluated their effects on blood glucose levels in 500 people with type 2 diabetes [Schwingshackl *et al.*, 2018]. Considering HbA1c lowering, the surface under the cumulative ranking (SUCRA) score ranked Mediterranean diet (80%) as the second-best diet compared to the control group, and vegetarianism (60%) ranked fourth. The network analysis also indicated that the Mediterranean diet was the best dietary approach to reduce fasting glucose levels compared to a control diet (88%), followed by a vegetarian diet in the third place (63%). The authors' main conclusion is that the Mediterranean diet is the most effective dietary approach for improving glycemic control in patients with type 2 diabetes [Schwingshackl *et al.*, 2018]. However, both dietary approaches are considered beneficial for diabetics since they feature an abundance of vegetables, fruits, grains, and cereals – and therefore fiber – which positively affects blood glucose levels [Benson & Hayes, 2020].

A randomized controlled cross-over study in overweight/obese individuals with type 2 diabetes examined the effects of the diets in question in terms of hunger and satiety perception [Di Mauro *et al.*, 2021]. The Mediterranean and high-fiber vegetarian diets were compared. Participants (12 subjects; 5 women, 7 men), aged 54–72 years, consumed two types of isocaloric meals during two visits with trial investigators. Appetite, glucose, insulin, and gastrointestinal hormone levels were assessed during the appointments. Measurements were taken at fasting and every 30 min for 3.5 h after meal consumption. Glucagon-like peptide 1 and oxyntomodulin concentrations were significantly higher after the Mediterranean diet compared to the high-fiber vegetarian diet.

In addition, consumption of the Mediterranean meal was associated with a lower glycemic profile compared to a high-fiber vegetarian meal. However, there were no significant changes in self-reported scores on the visual analogue scale or insulin trend. In conclusion, in overweight/obese and type 2 diabetes participants, the Mediterranean diet was more effective than the high-fiber vegetarian diet in postprandial plasma glucose homeostasis and glucagon-like peptide 1 and oxyntomodulin release. However, the small number of participants must be considered [Di Mauro *et al.*, 2021].

Gut microbiome

The cardiovascular prevention with vegetarian diet (CARDIVEG) study examined the effects of low-calorie Mediterranean and vegetarian diets on gut microbiome composition and SCFA production [Pagliai *et al.*, 2020]. Omnivorous subjects (16 women and 7 men) with low to moderate cardiovascular risk were randomly assigned to the Mediterranean and vegetarian groups. After three months of dietary intervention, the groups exchanged. Next-generation 16S rRNA sequencing and SCFA analysis were performed on participants' stool samples. As the analysis showed, adherence to the Mediterranean diet affected the abundance of *Enterorhabdus*, *Lachnoclostridium*, and *Parabacteroides* strains, while a vegetarian diet significantly affected the abundance of *Anaerostipes*, *Streptococcus*, *Clostridium sensu stricto*, and *Odoribacter*. However, microbiome composition did not change significantly in either of the groups. A comparison of the mean variation of each SCFA between Mediterranean and vegetarian diets showed an opposite and statistically significant trend for propionic acid (+10% vs –28%, respectively, $p=0.034$).

In addition, changes in SCFAs were negatively correlated with changes in some inflammatory cytokines (vascular endothelial growth factor, monocyte chemoattractant protein-1, interleukin-17, interferon gamma-induced protein-10 and interleukin-12) [Pagliai *et al.*, 2020]. Correlation analyses showed a potential relationship between changes in strain types and changes in clinical and biological parameters. It was noted that with a Mediterranean diet, there were changes in the production of short-chain fatty acids, supporting their role in modulating the inflammatory response. However, short-term use of a Mediterranean or vegetarian diet did not result in major changes in the gut microbiota composition. It is suggested that such dietary interventions should last for more than three months, and it would be worthwhile to conduct the study on a greater sample size.

Metabolic profile

The recent study published in 2021 aimed to evaluate the effects of long-term vegetarian and Mediterranean dietary patterns on metabolic profile and salivary microbiota composition [Daniele *et al.*, 2021]. Participants (42 subjects; 20 men, 22 women) approximately 38 years of age completed a questionnaire assessing dietary habits for at least two years. Information from medical history, saliva sample analysis, and basal metabolic rate and respiratory rate values were used to assess the metabolic profile. It was shown that individuals on the Mediterranean diet had a higher species diversity of oral bacteria and a better metabolic profile compared to the vegan diet. Participants in the Mediterranean dietary patterns group had higher percentages of *Subflava* and *Prevotella* species, lower carbohydrate consumption and higher lipid intake than

individuals on the vegetarian diet. Followers of the Mediterranean diet achieved higher basal metabolic and lower respiratory rates. It was observed that *Prevotella* abundance was inversely related to the respiration rate and carbohydrate consumption, whereas *Subflava* abundance was positively correlated with basal metabolic rate.

Furthermore, *Lactobacillus* abundance, inversely related to the presence of *Subflava* in the Mediterranean diet group, was associated with decreased basal metabolic rate. The study proved the association of macronutrient consumption with metabolic profile and oral microbiota. It also confirmed the positive effect of the Mediterranean diet on basal metabolic rate and the abundance of microbial species associated with better protein, carbohydrate, and lipid metabolism. The analysis suggests that long-term adherence to the Mediterranean diet, with high contents of protein and lipids, is associated with higher oral microbial diversity and, therefore, a better metabolic profile compared to veganism [Daniele *et al.*, 2021].

Kidney function

In 2020–2-21, a randomized cross-over study was conducted as a part of the CARDIVEG Study to evaluate the effects of a lacto-ovo vegetarian diet versus the Mediterranean diet on kidney function in healthy individuals at medium to low cardiovascular risk [Dinu *et al.*, 2021]. Participants (107 subjects; 82 women, 25 men) aged 21–75 years were assigned to a lacto-ovo vegetarian or Mediterranean diet group for three months, and then switched the diets. The analysis included confounding variables such as age, sex, weight, physical activity, alcohol consumption, smoking, hypertension, LDL cholesterol, and glucose levels. As shown, adherence to the lacto-ovo vegetarian diet decreased creatinine levels by 5.3%, blood urea nitrogen by 8.7%, and urea by 5.8%. In contrast, the eGFR increased by 3.5%. Clinically significant improvement in the above parameters may positively affect the protection of renal function, at least with a short-term vegetarian diet. In the Mediterranean diet group, no significant changes were observed in the parameters considered. However, the study covered a period of three months; thus, the effect of dietary intervention with this diet for a longer time was not evaluated [Dinu *et al.*, 2021].

Pain in rheumatoid arthritis

A recent meta-analysis of seven randomized controlled trials (326 participants in total) was published to examine the effect of potentially anti-inflammatory diets (including, among others, vegetarian and Mediterranean) on pain in rheumatoid arthritis [Schönenberger *et al.*, 2021]. Visual analogue scale (VAS), CRP level, erythrocyte sedimentation rate, health assessment questionnaire, disease activity score-28 for rheumatoid arthritis (DAS28), number of tender/swollen joints, body weight, and BMI were used for the assessment. It was noted that better outcomes were achieved in dietary interventions lasting more than three months. Subgroup analysis showed that the Mediterranean diet tended to have a greater effect on pain reduction than the vegetarian diet. However, the former dietary interventions were only evaluated in two papers. Nevertheless, the authors of the meta-analysis conclude that anti-inflammatory diets, including vegetarian and Mediterranean diets, decrease pain in rheumatoid arthritis compared to traditional diets [Schönenberger *et al.*, 2021]. However, as indicated the risk of bias was high, while the evidence was very low, advocating further studies to understand the role of vegetarian and Mediterranean diets in rheumatoid arthritis and comparison of outcomes of their implementation.

FUTURE RESEARCH PROSPECTS AND CHALLENGES

Several gaps and challenges must be addressed in future assessments of the health benefits of vegetarian and Mediterranean diets, as well as their implementation as non-pharmaceutical methods of disease prevention.

1. As stated by the Academy of Nutrition and Dietetics, the vegetarian diet, including vegan, can provide health benefits if appropriately planned [Melina *et al.*, 2016]. However, unbalanced plant-based diets can be harmful and lead to nutritional deficiencies, weight gain, increase in triglyceride and glucose levels [Sabaté, 2003]. Therefore, epidemiological studies should attempt to distinguish individuals adhering to a vegetarian diet based on how well it is planned. This is particularly important given the fact that some plant-based products fall into the category of ultra-processed food [Gehring *et al.*, 2021; Ohlau *et al.*, 2022].
2. There is a need to distinguish and define different types of vegetarianism when testing its health effect and comparing it to other diets, *e.g.*, the Mediterranean diet. A vegetarian diet includes individuals restricting meat consumption but eating eggs (ovo-vegetarianism), dairy (lacto-vegetarianism), both (lacto-ovo-vegetarianism), and avoiding consumption of any animal-derived products (veganism). However, there appears to be an increased interest in flexitarianism, which assumes a primary focus on plant foods with the occasional inclusion of meat products (and can be regarded as semi-vegetarianism). The effects of this practice require further assessment [Derbyshire, 2017].
3. There are numerous confounding variables when addressing the health benefit of diet, encompassing age, BMI, physical activity, smoking, alcohol consumption, biochemical markers, clinical background, and genetic predispositions, while adjustment for all of them may often be a challenging or impossible task.
4. Some health effects of each diet are evidenced in a small sample size, advocating further research to provide additional data and improve the strength of evidence. Numerous studies include healthy subjects, and there remains a need to test whether specific diets can exert similar beneficial effects in individuals with active disease.
5. Pursuing cross-over interventional trials comparing the effects of vegetarian and Mediterranean diets in different groups is encouraged. This particularly concerns the studies designed to directly compare the effects of these diets on cardiovascular markers, gut microbiome, metabolic profile, kidney function, and course of autoimmune diseases.
6. Considering that a shift in diet may not produce immediate effects, there is a need to pursue long-term interventional trials for vegetarian and Mediterranean diets. This

is important to understand whether the effects evidenced so far are persistent or can attenuate over time (*e.g.*, years of adherence to a specific diet).

7. Implementation of diets, such as vegetarian or Mediterranean, in selected populations may be faced with several obstacles that include economic factors, difficulties in the availability of certain food products, additional time and effort to prepare meals adhering to a particular diet [Bonaccio *et al.*, 2016; Middleton *et al.*, 2015]. The broader implementation of vegetarian or Mediterranean diets in some populations, *e.g.*, the Western world, may also be challenging since both require limitation or complete abstinence from meat [Fehér *et al.*, 2020].

CONCLUSIONS

Both vegetarian and Mediterranean diets exhibit various health-beneficial effects. Both diets are beneficial for lipid management, reduce cardiovascular risk, promote weight loss, and improve glycemic control in patients with type 2 diabetes. However, in most studies, the Mediterranean diet shows a higher health-promoting potential than the vegetarian diet, particularly regarding triglyceride levels, control of glycemia and blood pressure, and total cancer risk. Additionally, adherence to the Mediterranean diet is related to a lower risk of nutritional deficiencies than vegetarianism. At the same time, adherence to the Mediterranean diet does not require complete exclusion of meat products which can be less challenging, contrary to the vegetarian diet. Hence, as long as vegetarian diets can provide numerous health benefits, the Mediterranean dietary model may preliminarily appear superior in public health strategies aiming to decrease the burden of various chronic diseases in which lifestyle factors play a significant role. More studies, primarily based on cross-over design and conducted on different populations and risk groups, are required to fully understand the difference in health outcomes between those two diets and draw definitive conclusions.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Original article
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Functional Properties and Bioactivities of Protein Powder Prepared from Skipjack Tuna (*Katsuwonus pelamis)* Liver Using the pH Shift Process

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Key words: tuna liver proteins, protein isolate, amino acid profile, functional properties, antioxidant capacity, ACE inhibitory activity

Skipjack tuna (*Katsuwonus pelamis*) liver (TL) contains high-quality proteins which can potentially serve as an excellent source of functional protein ingredients. Thus, this study was conceptualized to evaluate the physicochemical, functional, and biological properties of proteins from TL using the pH shift process. The pH shift process was conducted through solubilization of TL at pH from 1.5 to 12.5, and the solubilized proteins at pH 2.5, 3.5, 10.5 and 11.5 were precipitated at pH 5.5. Finally, the tuna liver protein powders after the processes at pH 2.5 and 11.5 (TLP 2.5 and TLP 11.5, respectively) were obtained by freeze-drying, *i.e.* those with the highest extraction and protein recovery yields under acidic and alkaline conditions. Protein and lipid contents of TLPs were higher and lower, respectively, compared to the TL powder (control). Glutamic acid, aspartic acid, and alanine were prominent amino acids found in both TLPs. Foaming properties and water/oil holding capacity were higher in TLP 11.5, while protein solubility and emulsion properties were greater in TLP 2.5 compared between groups. Additionally, the DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ scavenging activities, as well as the angiotensin I-converting enzyme inhibitory activity, were remarkably higher in TLP 11.5 than in TLP 2.5. On the other hand, significant ferrous-ion chelating activity was observed in TLP 2.5. In conclusion, TLP 11.5 could serve as an alternative functional protein ingredient that provides essential amino acids, functional properties, and bioactivities.

ABBREVIATIONS

ABTS – 2,2'-Azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid; ABTS$^{\cdot+}$ – 2,2'-Azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid radical cation; ACE – Angiotensin I-converting enzyme; DDW – Deionized distilled water; DPPH – 2,2-Diphenyl-1-picrylhydrazyl; EAA – Essential amino acid; EAI – Emulsifying activity index; ESI – Emulsion stability index; EW – Egg white powder; FC – Foaming capacity; FS – Foaming stability; HAA – Hydrophobic amino acid; HPAA – Hydrophilic amino acid; OHC – Oil holding capacity; PUFAs – Polyunsaturated fatty acids; SP – Soy protein concentrate; TL – Tuna liver; TLP – Tuna liver protein powder; TLP 2.5 – Tuna liver protein powder from solubilization at pH 2.5; TLP 11.5 – Tuna liver protein powder from solubilization at pH 11.5; WHC – Water holding capacity.

INTRODUCTION

Skipjack tuna (*Katsuwonus pelamis*) is a major fish species used by the canned tuna industry and other processed tuna products. In Thailand, the annual production of canned tuna has remarkably increased and reached approximately 445,000 tons, and the value exceeded 1,677 million US dollars in 2021 [Department of Fisheries, 2022]. Following the annual growth of the canned tuna industry, the discarding rate of fish processing by-products has also remarkably increased.

Tuna liver (TL) is an abundant solid by-product which has long been underutilized. Commonly, most TL is used in the production of low-market value products, such as fish feed meal, or left as waste, which creates huge economic and environmental concerns [Shen *et al*., 2022]. However, TL is nutritionally rich in protein, the content of which reaches 18 g/100 g [Kang *et al.*, 2007]. The most abundant essential amino acids of proteins include leucine, lysine, and valine and the prominent non-essential amino acids are aspartic acid, glutamic acid, and alanine [Kang *et al.*, 2007; Shen *et al.*, 2022]. Recently, there has been a great interest in increasing the utilization of TL as a potential source of protein-based food for functional ingredients [Shen *et al.*, 2022].

The pH-shift process, mainly acidic or alkaline solubilization followed by the isoelectric precipitation of proteins, is a potentially applicable technique that efficiently recovers functional and nutritional proteins from fish processing

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by-products that otherwise would be discarded from direct human consumption [Chomnawang & Yongsawatdigul, 2013; Kang *et al*., 2018; Kristinsson *et al*., 2013]. The recovered protein isolate can be directly dried into a shelf-stable fish protein powder for further utilization. To date, the pH shift process has been remarkably applied to recover protein from different varieties of fish processing by-products, such as salmon, cod, and herring, for subsequent development of alternative and functional food ingredients [Abdollahi & Undeland, 2019]. To the best of our knowledge, limited reports are available on the functional properties and bioactivities of protein powder produced from skipjack TL using the pH shift process.

From the above mentioned, by-products pose a significant problem for the canned tuna industry because it leads to environmental impact. However, tuna liver is characterized by a high protein content and can be used as a protein source in foods. The utilization of TL for human consumption could be an innovative strategy for eco-friendliness between industry and food sustainability for consumers. Therefore, this study aimed to evaluate the nutritional and functional properties as well as antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of protein powder prepared from skipjack TL using the pH shift process to enable future development of the use of TL protein as a valuable food.

MATERIALS AND METHODS

Raw materials and sample preparation

Frozen livers of skipjack tuna (*Katsuwonus pelamis*; body length of 43–53 cm and body weight of 4.5–5.5 kg) were kindly donated by a canned tuna processing plant (Samut Sakhon Province, Thailand), and transported in an icebox at approximately 4°C to the laboratory of the Department of Fishery Products, Kasetsart University, Bangkok within 2 h. After arrival, the tuna livers (TL) were immediately stored at –20°C until use and they were thawed for 24 h at 4°C prior to experiments. The liver was then cut into small pieces and ground using a laboratory blender (Waring Commercial, Torrington, CT, USA). A portion of ground liver was directly used as a raw material for protein isolation. The remaining portions of the ground liver were freeze-dried using freeze dryer (Coolsafe 95–15, Labogene, Lillerød, Denmark), ground, and kept as lyophilized powder in a sealed polyethylene bag at –20°C until further use in the analysis. The ground TL powder was marked as the control.

Determination of tuna liver protein solubility

To determine the protein solubility of TL at different pHs, the method described by Li *et al*. [2017] was used with slight modifications of the sample-to-solvent ratio. A flowchart of TL protein solubilization is shown in Figure 1A. Initially, the ground TL (20 g) was homogenized with six volumes of cold deionized distilled water (DDW) using a homogenizer (Ultra-Turrax T25; IKA®, Staufen, Germany) at 10,000 rpm for 1 min. The homogenate was adjusted to an acidic or alkaline pH from 1.5 to 12.5 (1.0 pH intervals) by adding 1 M HCl or 1 M NaOH during constant automatic stirring. After incubation for 10 min, the sample was centrifuged at 10,000×g for 10 min at 4°C using a refrigerated centrifuge (Tomy Seiko Co. LTD., Tokyo, Japan). The middle layer of the supernatant was collected, and the protein concentration was quantified by the Lowry method using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard protein [Lowry *et al*., 1951]. The protein solubility was calculated and expressed as mg/mL, and a solubility curve (solubility *vs*. pH) was generated.

Preparation of the tuna liver protein powder using the pH shift processes

The protein was recovered from TL using the pH shift process according to the method described by Kristinsson *et al*. [2013] with some modifications in the pH of protein solubilization step. A flowchart of TLP preparation is shown in Figure 1. Briefly, the ground liver was suspended in DDW at a ratio of 1:6 (*w/v*) and then homogenized in an ice bath at 10,000 rpm for 1 min. The homogenate was adjusted to the acidic pH (2.5 and 3.5) or alkaline pH (10.5 and 11.5) by the drop-wise addition of 1 M HCl or 1 M NaOH with continuous stirring at 4°C until reaching the maximum

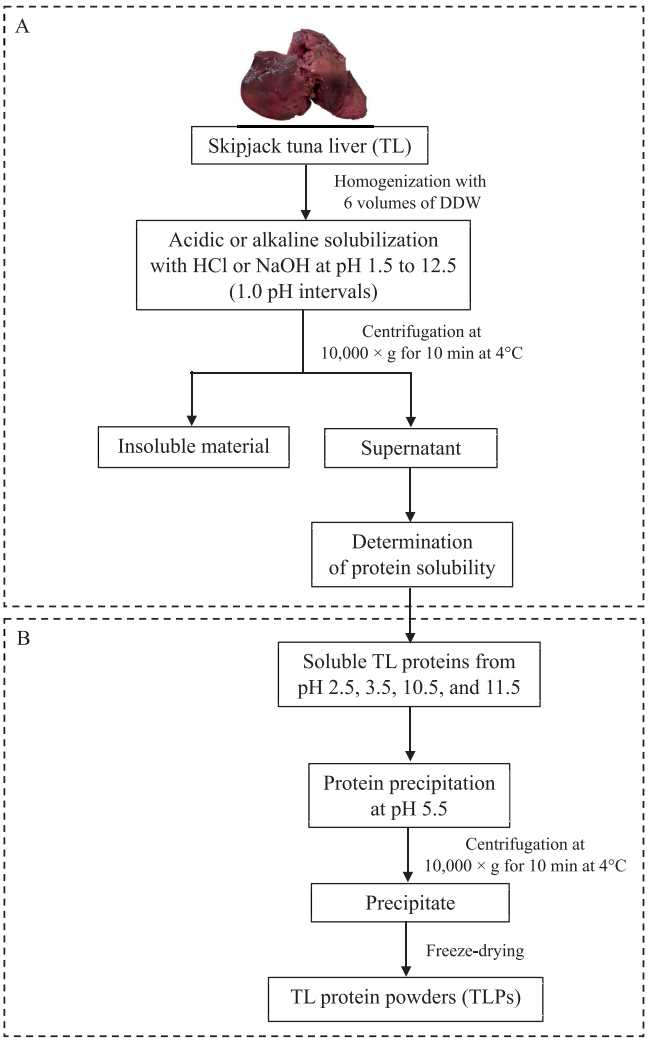


FIGURE 1. A flowchart of skipjack tuna liver protein powder (TLP) preparation. Acidic or alkaline solubilization (A) and soluble tuna liver (TL) protein precipitation prior to freeze-drying (B).

solubility points as determined by the solubility profile. The mixtures were left for 10 min at 4°C and then centrifuged at 10,000×g for 10 min at 4°C to separate the lipids (upper layer) and insoluble residues (sediment). The middle layer with soluble proteins was collected and pH was adjusted to 5.5 using 1 M HCl or 1 M NaOH to accomplish the precipitation of the solubilized proteins. Thereafter, the precipitated protein was recovered by centrifugation at 10,000×g for 10 min at 4°C and additionally suspended in DDW through homogenization for 1 min. The pH of the homogenate was neutralized (pH 7.0) and the protein was finally collected *via* centrifugation at 10,000×g for 10 min at 4°C. The TL proteins prepared by the pH shift process (pH 2.5, 3.5, 10.5, and 11.5) were freeze-dried, ground, and marked as TLP 2.5, TLP 3.5, TLP 10.5, and TLP 11.5, respectively. The TLPs were stored in a sealed polyethylene bag at –20 °C until further use in the analysis.

Determination of extraction yield and protein recovery yield

The extraction yield of the TLP samples (TLP 2.5, TLP 3.5, TLP 10.5, and TLP 11.5) was calculated as a percentage according to the following Equation (1):

$$Extraction\ yield = \frac{Weight\ of\ TLP}{Weight\ of\ TL} \times 100 \quad (1)$$

where, dry weight (g) of TLP and TL was used.

The protein recovery yield of the TLP samples was calculated as a percentage according to the equation (2) after determination of TLP and TL protein contents using the Kjeldahl method, specifically the conversion factor of 6.25 [AOAC, 2000].

$$Protein\ recovery\ yield = \frac{Weight\ of\ TLP \times Protein\ content\ of\ TLP}{Weight\ of\ TL \times Protein\ content\ of\ TL} \times 100 \quad (2)$$

Proximate composition analysis

The proximate composition (content of moisture, protein, lipid, and ash) of the TL powder and TLPs was analyzed according to the AOAC International [2000] following the analytical methods no. 950.46, 920.153, 960.39, and 928.08, respectively.

Color parameters analysis

The color attributes of the samples were determined using an UltraScan XE Hunter Lab tristimulus colorimeter (Hunter Assoc. Laboratory, Reston, VA, USA). Initially, the TL powder and TLPs were put in a transparent glass sample cup. The colorimeter was calibrated using a white standard plate and the lightness (L^*), redness (a^*), and yellowness (b^*) were measured. Whiteness was calculated using the following Equation (3):

$$Whiteness = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (3)$$

Visualization of appearance

The appearance of TL powder and TLPs was recorded using a digital camera (iPhone 11 Pro max; Apple, Cupertino, CA, USA).

Amino acid analysis

The amino acid profile analysis was conducted according to the method described by Jajic *et al*. [2013] with some modifications in the reagent for sample hydrolysis by excluding phenol. The TL powder and TLPs (0.5 g) were hydrolyzed with 5 mL of 6 M HCl using an oil bath (B-300, Buchi, Flawil, Switzerland) at 110°C for 24 h. The reaction mixture was finally diluted with water to 50 mL (HPLC grade) and then filtered through a 0.22-μm nylon membrane filter (Pall, Ann Arbor, MI, USA) prior to the amino acid analysis using high-performance liquid chromatography system (HPLC; Agilent 1200 infinity series LC system; Agilent Technologies, Palo Alto, CA, USA) equipped with a Poroshell-120 HPH--C18 column (4.6×100 mm, 2.7 μm particle size; Agilent Technologies). A 10-μL portion of the sample was injected at a flow rate of 1.0 mL/min. The column oven temperature was set at 40°C. Mobile phase A (pH 8.2) consisted of 10 mM disodium hydrogen phosphate (Na_2HPO_4), 10 mM sodium tetraborate ($Na_2B_4O_7 \times 10H_2O$), and 0.5 mM sodium azide (NaN_3). Mobile phase B consisted of acetonitrile, methanol, and water (45:45:10, *v/v/v*). All the reagents used for mobile phase preparation were of HPLC grade. The gradient conditions were set as follows: 2% B at 0–0.35 min, 2–57% B at 0.35–13.4 min, 57–100% B at 13.4–13.5 min, 100% B at 13.5–15.7 min, 100–2% B at 15.7–15.8 min, and 2% B at 15.8–18 min. The eluted amino acids were detected by monitoring at 230 nm for an excitation wavelength and at 450 nm for an emission wavelength using a fluorescence detector (FLD). Amino acids were identified and quantified based on the peak area integration using the peak area determined from a known amount of a mixed amino acid standard (0.2 mM solution; Agilent Technologies) for comparison. The data were expressed as g/100 g of protein.

Functional properties determination

Two commercial protein powders (soy protein concentrate, SP; and egg white, EW) were used as positive controls to estimate the solubility and functional properties of the TLPs from skipjack TL.

Protein solubility

The protein solubility of the TLPs was determined using the method described by Cha *et al*. [2020] with slight modifications in sample concentration. About 10 mg of freeze--dried sample was dispersed in 10 mL of distilled water, and the suspension was adjusted to pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 using 0.1 M HCl or 1 M NaOH, under continuous stirring at room temperature. The mixture was stirred magnetically for 30 min and centrifuged at 10,000×g for 15 min at 4°C. The protein content in the supernatant and total protein content in the sample after solubilizing with 0.1 M NaOH were determined according to the method described by Lowry *et al.* [1951] using bovine serum albumin (BSA) as a protein standard. The relative protein solubility (%) of the TLPs was calculated using the following Equation (4):

$$Relative\ protein\ solubility = \frac{Protein\ content\ in\ the\ supernatant}{Total\ protein\ content\ in\ the\ sample} \times 100 \quad (4)$$

Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) of TLPs were evaluated following the method explained by Han *et al.* [2019] with some modifications of oil type and amount of emulsion sample. Initially, an emulsion was prepared by homogenizing the mixture of palm oil and protein solution (10 mg/mL) at a 1:3 (*v/v*) ratio at a speed of 10,000 rpm for 1 min. Then, a 50 μL aliquot of the emulsion at the bottom layer of the container at 0 and 10 min after homogenization was collected and transferred to another tube containing 0.1% sodium dodecyl sulfate solution (5 mL). Thereafter, the mixture was mixed thoroughly for 30 s using a vortex mixer. The absorbance of the resulting emulsion was measured at 500 nm using a UV-Vis spectrophotometer (Evolution 300, Thermo Scientific, Waltham, MA, USA). The EAI (m^2/g) and ESI (min) values of the TLPs were calculated using the Equations (5) and (6), respectively:

$$EAI = \frac{2\times 2.303\times dil\times A}{C\times 1\ cm\times \Phi\times 10{,}000} \quad (5)$$

where: dil is the dilution factor (100), A is the absorbance measured immediately after emulsion formation, C is the protein concentration (g/mL) before emulsion formation in the aqueous phase, and Φ is the oil volume fraction of the emulsion (0.25).

$$ESI = \frac{A_0}{A_0 - A_{10}} \times t \quad (6)$$

where: A_0 is the absorbance at 0 min, A_{10} is the absorbance at 10 min after homogenization, and t is the time between measurements (10 min).

Foaming properties

The foaming capacity (FC) and foaming stability (FS) of the TLPs were analyzed following the method described by Cha *et al*. [2020]. To this end, 10 mL of sample solution (10 mg/mL) was homogenized at a speed of 10,000 rpm for 3 min and then the sample solution was immediately moved to a 50-mL cylinder. The sample was left undisturbed for 0 and 60 min. The FC (%) and FS (%) of each sample were calculated using the Equations (7) and (8), respectively:

$$FC = \frac{V_T}{V_0} \times 100 \quad (7)$$

$$FS = \frac{F_t/V_t}{F_T/V_T} \times 100 \quad (8)$$

where: V_0 is the initial volume before whipping, V_T is the total volume after whipping at 0 min, V_t is the total volume after whipping at 60 min, F_T is the foam volume after whipping at 0 min F_t is the foam volume after whipping at 60 min.

Water holding capacity (WHC)

The WHC of the TLPs was measured according to the method previously used by Han *et al*. [2019] with some modifications in centrifugation conditions. A 0.5-g portion of each sample was dispersed in 10 mL of distilled water and subsequently mixed for 60 s using a vortex mixer. After incubation at room temperature for 30 min, the solution was centrifuged at 6,000×g for 30 min and the volume of the resulting supernatant was measured. The difference between the initial volume of distilled water and the supernatant volume was calculated. The WHC was expressed as mL of absorbed water per g sample.

Oil holding capacity (OHC)

The OHC of the TLPs was determined following the method described by Han *et al.* [2019] with some modifications of oil type and the sample-to-oil ratio. Briefly, a 0.25 g portion of each sample was thoroughly mixed with 10 mL of palm oil for 60 s using a vortex mixer. After incubation at room temperature for 30 min, the sample was centrifuged at 6,000×g for 30 min and the difference between the supernatant and original volume of oil added to the sample was measured. The OHC was expressed as mL of oil per g sample.

Determination of antioxidant and antihypertensive activity

DPPH radical scavenging activity

The DPPH radical scavenging activity of TLPs was measured followed the method previously used by Yen & Hsieh [1995] with slight modifications in sample concentration. In brief, a reaction mixture of 1 mL of each sample solution (20 mg/mL) and 1 mL of 200 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich) in ethanolic solution was mixed thoroughly using a vortex mixer. The mixture was left at room temperature for 30 min in the dark. The absorbance of the resulting mixtures was measured at 517 nm against a blank without DPPH radical using a UV-Vis spectrophotometer. Trolox (0–1.8 μM) was used for the standard curve preparation. The DPPH radical scavenging activity was expressed as μmol Trolox equivalent/mg protein.

ABTS radical cation scavenging activity

The 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) radical cation scavenging activity was determined as described by Cha *et al*. [2020] with slight modifications in sample concentration. Briefly, the ABTS radical cation ($ABTS^{\bullet+}$) was generated by the reaction of ABTS stock solution (14.8 mM) and 5.2 mM potassium persulfate at room temperature for 12–16 h in the dark before use. The $ABTS^{\bullet+}$ solution was diluted with 200 mM phosphate buffer, pH 8.2 (1:1, *v/v*) to obtain an absorbance of 0.70±0.02 units using a UV-Vis spectrophotometer. About 0.5 mL of the protein sample solution (10 mg/mL) was mixed with 0.5 mL of DDW and 1 mL of $ABTS^{\bullet+}$ solution and the mixture was incubated at room temperature for 10 min in the dark. The absorbance was then measured at 734 nm. A standard curve of Trolox (0–200 μM) was plotted. The $ABTS^{\bullet+}$ scavenging activity was expressed as μmol Trolox equivalent/mg protein.

Ferrous-ion chelating activity

The ferrous-ion chelating activity was assessed using the method described by Torres-Fuentes *et al.* [2012] with slight modifications in sample concentration. To this end, 1 mL of the sample solution (0.1 g/mL) was mixed with

50 μL of 2 mM $FeCl_2$ solution and 3.7 mL of distilled water. Then, 0.1 mL aliquot of 5 mM ferrozine solution was added and vortexed vigorously. After incubation for 20 min at room temperature, the absorbance of the reaction mixture was measured at 562 nm using a UV-Vis spectrophotometer. A control was evaluated by replacing the sample solution with 1 mL of distilled water. EDTA (0–100 μM) was used as a reference standard. The ferrous-ion chelating activity was expressed as μmol EDTA equivalent/mg protein.

Angiotensin-converting enzyme (ACE) inhibitory activity

The ACE-inhibitory activity was elucidated using the method previously used by Kasiwut *et al.* [2019] with modifications in sample concentration. A mixture of 100 μL of the protein sample solution (50 mg/mL) and 150 μL of 8.3 mM hippuryl-histidyl-leucine (HHL) solution was pre-incubated at 37°C for 10 min. The reaction was initiated by adding 50 μL of 25 mU/mL ACE solution (prepared in 50 mM sodium borate buffer containing 300 mM sodium chloride and adjusted to pH 8.3) and the sample was then incubated at 37°C for 30 min. The reaction was stopped by adding 250 μL of 1 M HCl and mixing well. The resulting hippuric acid was extracted with 1 mL of ethyl acetate. Thereafter, 800 μL of the upper layer was transferred into a test tube and vacuum dried at 105°C for 2 h. The hippuric acid was dissolved in 5 mL of distilled water, and the absorbance was measured immediately at 228 nm using a UV-Vis spectrophotometer. The percentage of ACE inhibitory activity of TLP was calculated as a percentage using Equation (9):

$$\text{ACE inhibitory activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control} - A_{blank}} \times 100 \quad (9)$$

where: $A_{control}$ is the absorbance readings of the buffer, A_{sample} is the absorbance readings of the TLP solutions, and A_{blank} is the absorbance when the stop solution was added before the reaction occurred.

Statistical analysis

All analyses were performed in triplicate. The results were expressed as mean ± standard deviations (n=3). The statistical analyses were conducted using SPSS version 26 (SPSS Inc., Chicago, IL, USA). The data sets were subjected to one-way analysis of variance (ANOVA), followed by Duncan's multiple range post hoc test, and a significance level of p<0.05 was employed.

RESULTS AND DISCUSSION

Effects of pH on the protein solubility of tuna liver

To obtain a higher recovery of skipjack TLPs using the pH shift process, the solubility of proteins at various pH conditions (pH 1.5–12.5) was evaluated as shown in Figure 2. The protein solubility profile of the TL exhibited a rough V-shaped pattern. Protein solubility drastically increased when the pH was shifted to either side, ranging from pH 4.5 to 3.5 and pH 7.5 to 11.5. The maximum protein solubility was observed at pH 2.5 to 3.5 on the acidic side (0.46–0.49 mg/mL) and at pH 10.5 to 11.5 on the alkaline side (0.68–0.70 mg/mL), respectively. However, the minimum protein solubility was found at pH 5.5 (0.24 mg/mL) which may correspond to the isoelectric point of TLP due to the diminishment of electrostatic repulsions and the formation of large particles [Shen *et al*., 2022]. Similar findings were also reported in the protein solubility of fish muscle proteins such as tilapia [Chomnawang & Yongsawatdigul, 2013], rainbow trout [Lone *et al*., 2015], and yellowfin tuna liver [Shen *et al*., 2022]. In addition, it has been reported that the maximum solubility of chicken liver and goose liver proteins was observed at both extremely acidic and alkaline pH, ranging from pH 2.0 to 3.5 and 10.5 to 11.5, respectively, and the minimum solubility was observed at pH 5.0–5.5 [Li *et al*., 2017; Xiong *et al*., 2016]. The solubility of proteins has been increased in highly acidic or alkaline pH conditions due to an increase in the positive or negative charges of muscle proteins when the pH moved away from the isoelectric point which results in electrostatic repulsions of the protein molecules and the hydration of charged proteins [Xiong *et al*., 2016]. Based on the above results, the maximum solubility at pH 2.5 to 3.5 from the acidic side and pH 10.5 to 11.5 from the alkaline side were selected for the TL protein solubilizations, and pH 5.5 was chosen for the TL protein precipitations.

Extraction yield and protein recovery yield

The extraction yield of the TLPs under both acidic (pH 2.5 and 3.5) and alkaline (pH 10.5 and 11.5) pH conditions were determined as shown in Figure 3A. The yields of the TLP samples at pHs 2.5, 3.5, 10.5, and 11.5 were 13.85, 5.41, 39.61, and 48.82% (dry weight basis), respectively. This could be explained by the conformational changes of the proteins at extreme alkaline pHs causing the exposure of the buried groups in the protein structure which led to a higher yield [Abdollahi & Undeland, 2019]. Of note, the protein recovery yields of the TLPs were also evaluated. As shown in Figure 3B, the protein recovery yields in alkaline conditions were significantly (p<0.05) higher than those in acidic conditions. The maximum protein recovery yield was obtained from the alkaline pH shift at pH 11.5 (62.84%), whereas the recovery yield at the acidic pH shift was significantly (p<0.05) lower. This might be related to the increase in the electrostatic

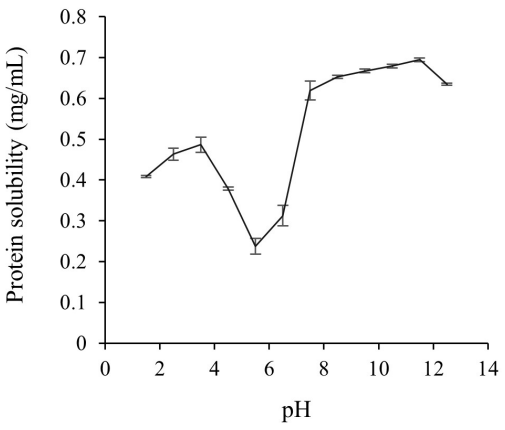


FIGURE 2. The solubility of tuna liver protein at different pHs. Values are reported as mean ± standard deviation (n=3).

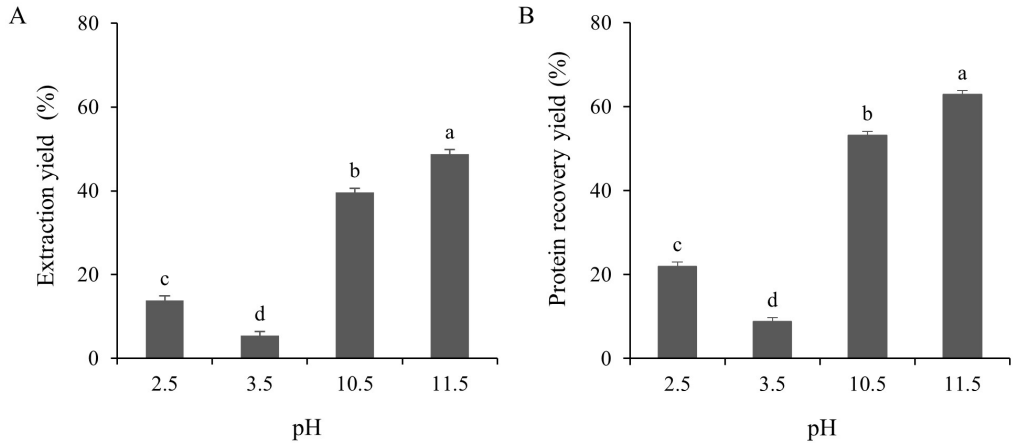


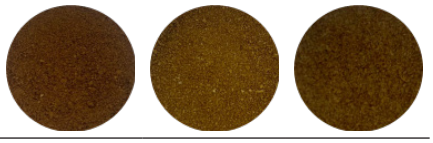
FIGURE 3. Extraction yield (A) and protein recovery yield (B) of tuna liver protein at different pHs. Values are reported as mean ± standard deviation (n=3). Different letters above bars indicate significant differences among treatments (p<0.05).

charge of the protein that consequently increased the protein solubility and yield. On the other hand, the lower protein recovery yield under acidic conditions might be caused by the trapping of solubilized protein in the sediment during the first centrifugation [Abdollahi & Undeland, 2019]. In addition, the higher protein recovery yields, as observed in the present study, were closely correlated to the solubility differences (p<0.05) of the TLPs under acidic or alkaline conditions (Figure 2). Even though the protein solubility at pH 3.5 was slightly higher than that at pH 2.5 (Figure 2), the protein recovery yield at pH 3.5 was the lowest among all treatments (8.74% dry weight basis; Figure 3B). This might be explained by the increase in the polarity of the protein at pHs below or above the isoelectric point which, in turn, raises the solubility of the proteins [Xiong *et al.*, 2016]. A similar finding has been reported in yellowfin tuna liver where alkaline conditions resulted in a higher protein recovery yield than acidic conditions [Shen *et al.*, 2022]. Different fish species and by-products, such as trout, silver carp, rohu, croaker, and anchovy, have shown a wide range of protein recovery yields ranging from 32–90% according to the pH shift process [Nolsøe & Undeland, 2009]. In addition, the yield of protein recovery was affected by many factors, such as the origin of raw material, centrifugal force, trapping of the solubilized proteins, protein amino acid composition, and the ratio between the raw materials and water [Marmon & Undeland, 2010]. Considering the above results, the optimal pHs to produce recovered proteins from TL using the pH shift process were pH 2.5 and pH 11.5.

Proximate composition

The proximate compositions of the TL powder (control) and TLPs (TLP 2.5 and TLP 11.5) are presented in Table 1. The moisture content of the TL powder, TLP 2.5, and TLP 11.5 was 9.74, 8.19, and 8.86 g/100 g, respectively. These results are important to consider as they strongly affect the protein powder properties [Pires *et al.*, 2012]. Both TLP 2.5 and TLP 11.5 had a protein content of 68.09 and 55.25 g/100 g, which were greater (p<0.05) than the control (42.93 g/100 g). This result could be explained by the loss of connective tissue during centrifugation under the alkaline pH shift process [Kristinsson *et al.*, 2006]. Therefore, the protein content was lower in TLP 11.5 than TLP 2.5. This result agreed well with the previous findings wherein acidic solubilization increased the protein content of pelagic fish and green crab compared to alkaline solubilization [Kang *et al.*, 2018; Kristinsson *et al.*, 2005]. The total lipid content changed from 14.98 g/100 g in the TL powder to 0.58 g/100 g in TLP 2.5 and 0.28 g/100 g in TLP 11.5 after the pH shift process due to the separation of lipids from proteins at very low or high pHs and a subsequent lipid migration into the processed water during centrifugation by the different solubility and density [Kristinsson *et al.*, 2005; 2006]. The ash content of TLP 11.5 was significantly (p<0.05) higher than that of the control and TLP 2.5

TABLE 1. Proximate composition, color parameters, and visual appearance of tuna liver powder (control) and tuna liver protein powders (TLPs) obtained following the pH shift process.

	Control	TLP 2.5	TLP 11.5
Proximate composition (g/100 g sample)			
Protein	42.93±1.49^{c}	68.09±1.74^{a}	55.25±0.81^{b}
Lipid	14.98±0.27^{a}	0.58±0.18^{b}	0.28±0.07^{b}
Moisture	9.74±0.29^{a}	8.19±0.86^{b}	8.86±0.23^{b}
Ash	4.68±0.15^{b}	2.30±0.18^{c}	6.63±0.14^{a}
Color parameter			
L^*	40.05±0.02^{c}	56.07±0.01^{a}	45.39±0.00^{b}
a^*	14.40±0.03^{a}	12.08±0.01^{b}	11.00±0.03^{c}
b^*	19.95±0.08^{b}	30.98±0.01^{a}	19.70±0.03^{b}
Whiteness	39.48±0.02^{c}	55.09±0.01^{a}	44.83±0.00^{b}
Visual appearance			

TLP 2.5, TLP from solubilization at pH 2.5; TLP 11.5, TLP from solubilization at pH 11.5. Values are reported as mean ± standard deviation (n=3). Different letters within the same row indicate significant differences among treatments (p<0.05).

(6.63, 4.68, and 2.30 g/100 g, respectively). This was probably due to the higher amount of NaOH and HCl used to adjust the pH at the alkaline conditions and during protein precipitation, resulting in a higher NaCl formation [Chomnawang & Yongsawatdigul, 2013]. Overall, the TLPs obtained following the pH shift process may have potential as an alternative source of protein with a low lipid content.

Color parameters and visual appearance

The color parameter differences among the TL powder and TLPs are shown in Table 1. The L^* of TLP 2.5 (56.07) was significantly ($p<0.05$) higher than that of the TL powder and TLP 11.5 (40.05 and 45.39, respectively). In addition, the whiteness of TLP 2.5 was significantly ($p<0.05$) higher than TLP 11.5, with values of 55.09 and 44.83, respectively. These results showed that TLP 2.5 obtained following the acidic solubilization had a much lighter coloration than TLP 11.5 obtained following the alkaline solubilization. Similar results were also reported by Marmon & Undeland [2010] who found that the recovered proteins from gutted herring using the acidic pH shift process were lighter and whiter than those using the alkaline pH shift process which was probably due to the better removal of pigments, melanin, hemoglobin, and myoglobin. Additionally, Kristinsson & Rasco [2000] reported that the connective tissue present in the fish protein isolates may cause an increase in brightness.

The a^* values of both TLP 2.5 and TLP 11.5 were decreased after the pH shift process, which contained 12.08 and 11.00, respectively (Table 1). However, TLP 2.5 had remarkedly ($p<0.05$) higher b^* values than TLP 11.5 and the control (30.98, 19.70, and 19.95, respectively). Of note, TLP 2.5 showed significantly ($p<0.05$) higher a^* and b^* values than TLP 11.5. Marmon & Undeland [2010] and Kang *et al*. [2018] reported that the a^* and b^* values of the recovered proteins from gutted herring and green crab obtained following the acidic pH shift process were higher than those of the alkaline pH shift process, which was probably due to the lipid retention in the recovered proteins. In addition, the presence of high levels of heme proteins, or the denaturation and oxidation of hemoglobin, might affect the a^* and b^* values of the recovered protein [Kristinsson & Rasco, 2000]. Overall, these results were consistent with the appearance of TLP 2.5 which showed as being lighter, whiter, redder, and yellower than TLP 11.5 and the TL powder (Table 1).

Amino acid profile

The amino acid profile of the TL powder and TLPs (TLP 2.5 and TLP 11.5) has been summarized in Table 2. The major amino acids found in the TL powder were cysteine (13.24 g/100 g protein), glutamic acid (12.56 g/100 g protein), aspartic acid (8.53 g/100 g protein), leucine (7.52 g/100 g protein), alanine (6.27 g/100 g protein), and lysine (6.22 g/100 g protein), whereas the dominant amino acids found in both TLP 2.5 and TLP 11.5 were glutamic acid (11.99–12.45 g/100 g protein), cysteine (11.99–12.39 g/100 g protein), aspartic acid (9.39–9.50 g/100 g protein), leucine (7.89–8.14 g/100 g protein), lysine (5.49–6.36 g/100 g protein), and alanine (5.59––5.71 g/100 g protein). Among the non-essential amino acids, the results revealed that TLP 2.5 and TLP 11.5 were rich in glutamic acid and aspartic acid, and alanine which provided umami and sweet flavor, respectively [Han *et al*., 2019]. Thus, both TLP 2.5 and TLP 11.5 could likely be considered as enhancers of flavor in food.

TABLE 2. Amino acid profile (g/100 g protein) of tuna liver powder (control), tuna liver protein powders (TLPs) obtained following the pH shift process and FAO reference pattern for adults.

Amino acid	Control	TLP 2.5	TLP 11.5	Reference pattern [FAO, 2013]
Aspartic acid[2]	8.53±0.13[b]	9.50±0.06[a]	9.39±0.02[a]	
Glutamic acid[2]	12.56±0.14[a]	11.99±0.11[c]	12.45±0.00[b]	
Serine[2]	4.00±0.03[c]	4.15±0.04[b]	4.35±0.00[a]	
Histidine*[2]	2.34±0.04[b]	2.56±0.03[a]	2.14±0.02[c]	1.5
Glycine[1]	4.54±0.09[a]	4.16±0.04[c]	4.29±0.01[b]	
Threonine*[2]	4.35±0.04[b]	4.43±0.03[b]	4.62±0.00[a]	2.3
Arginine[2]	4.73±0.09[c]	5.56±0.07[b]	5.64±0.01[a]	
Alanine[1]	6.27±0.06[a]	5.59±0.05[c]	5.71±0.01[b]	
Tyrosine	3.12±0.02[b]	3.40±0.03[a]	3.35±0.01[a]	
Cysteine	13.24±0.61[a]	11.99±0.17[b]	12.39±0.07[b]	
Valine*[1]	5.14±0.04[a]	5.01±0.04[b]	5.02±0.00[b]	3.9
Methionine*[1]	2.53±0.02[b]	2.68±0.03[a]	2.69±0.01[a]	2.2
Phenylalanine*[1]	4.32±0.02[b]	5.00±0.04[a]	4.94±0.01[a]	3.8
Isoleucine*[1]	4.30±0.02[b]	4.43±0.04[a]	4.43±0.01[a]	3.0
Leucine*[1]	7.52±0.03[c]	7.89±0.07[b]	8.14±0.01[a]	5.9
Lysine*[2]	6.22±0.04[a]	6.36±0.06[a]	5.49±0.01[b]	4.5
Proline[1]	4.85±0.07[a]	4.76±0.03[a]	4.32±0.03[b]	
Tryptophan*[1]	1.43±0.01[a]	0.53±0.01[c]	0.65±0.01[b]	0.6
EAAs	38.26±0.25[b]	38.89±0.05[a]	38.11±0.03[b]	
HAAs	40.90±0.15[a]	40.05±0.09[b]	40.18±0.04[b]	
HPAAs	42.73±0.27[c]	44.56±0.05[a]	44.08±0.08[b]	

*Essential amino acid. [1]Hydrophobic amino acid. [2]Hydrophilic amino acid. TLP 2.5, TLP from solubilization at pH 2.5; TLP 11.5, TLP from solubilization at pH 11.5; EAAs, total essential amino acids; HAAs, total hydrophobic amino acids; HPAAs, total hydrophilic amino acids. Values are reported as mean ± standard deviation ($n=3$). Different letters within the same row indicate significant differences among treatments ($p<0.05$).

Of note, the essential amino acid (EAA) content in both TLP 2.5 and TLP 11.5 was higher and similar to the control (38.89, 38.11, and 38.26 g/100 g protein, respectively) (Table 2). The results agreed well with those of the previous study by Marmon & Undeland [2010] who reported that the content of EAA in the recovered proteins from gutted herring increased significantly during the pH shift processing. Comparing between the TLP groups, the EAA content of lysine and histidine in TLP 2.5 were markedly ($p<0.05$) higher, whereas the content of leucine, threonine, and tryptophan were significantly ($p<0.05$) lower than in TLP 11.5. The prominent EAAs of the TLP groups were leucine, lysine, and valine. Similar findings were also reported by Shen *et al*.

[2022] who found that the most abundant EAAs in the recovered proteins from the liver of yellowfin tuna obtained following the acidic and alkaline pH shift processes were leucine, lysine, and valine. These EAAs are important as they promote brain functions and are associated with muscle metabolism and boost energy [Sarojnalini & Hei, 2019]. Overall, the content of all EAAs in both TLP 2.5 and TLP 11.5 meet the amino acid requirements for adults according to the suggestions of the Food and Agriculture Organization [FAO, 2013].

It is well-known that the nutritional and biological activities of protein are enhanced by the type, position in protein structure, and content of hydrophobic amino acid (HAA) and hydrophilic amino acid (HPAA) [Cha *et al*., 2020]. In the present study, according to the HAA content of all treatments ranging from 40.05 to 40.90 g/100 g protein, the predominant HAAs were leucine, alanine, and valine. On the other hand, the HPAA content obtained following all treatments ranged from 42.73 to 44.56 g/100 g protein where the major HPAAs were glutamic acid, aspartic acid, and lysine. A similar result was also observed by Cha *et al*. [2020] who found that the most remarkable HAAs in the recovered proteins from the roe of skipjack tuna obtained following the pH shift process were leucine, alanine, and valine, while the major HPAAs were glutamic acid and aspartic acid. Our results point out that the recovered proteins from skipjack TL obtained following the acid and alkaline pH shift processes could be considered a promising source of nutrients, particularly regarding amino acids.

Functional properties

Solubility of protein isolates

Solubility is an important functional property of proteins and protein-based formulations as it influences the usefulness of an ingredient in food and governs its physical characteristics [Freitas *et al.*, 2011]. The solubility of TLP 2.5 and TLP 11.5 in the pH range of 2 to 12 is depicted in Figure 4. There were significant ($p<0.05$) differences in the protein solubility between TLP 2.5 and TLP 11.5 at pHs 3–4 and 6–8 by which TLP 2.5 had a higher protein solubility (5.58–13.36%) than TLP 11.5 (0.13–5.65%). This could be explained by the prominent amount of polar and hydrophilic amino acid residues, such as lysine and histidine, in TLP 2.5 compared to TLP 11.5 ($p<0.05$) (Table 2) which could have hydrophilic interaction with water and consequently promote protein solubility [Cha *et al.*, 2020]. The highest solubility of TLPs was observed at pH 11 to 12 (38.41–42.45%). These results indicated that the extremely high alkaline conditions affected an increase in protein solubility which can be explained by the increasing net negative charges and hydrophilic amino acids leading to more binding sites for water [Kristinsson & Rasco, 2000]. However, both TLP 2.5 and TLP 11.5 showed minimum solubilities at pH 3–8 (0.13–13.36%) which could be explained by the proximity between the pH of the solution and the isoelectric point of the protein that could enhance protein precipitation. This result was consistent with the findings of Lone *et al.* [2015] and Cha *et al.* [2020] for rainbow trout and skipjack tuna roe protein which showed the lowest solubilities at pH 4–8 due to the proximity between the pH of the solution and the isoelectric point of the proteins. Generally, when the pH nearly reaches the isoelectric point, the protein net charge is minimized, resulting in protein aggregation [Lone *et al.*, 2015].

In this study, soy protein concentrate (SP) and egg white (EW) were used as positive controls, representing protein from plant and animal origins, respectively. Surprisingly, both TLPs showed a more similar pattern of protein solubility to SP which was totally different from that of EW (Figure 3). Compared to EW, TLP 2.5 and TLP 11.5 showed considerably ($p<0.05$) lower solubility at all pH values. The relatively low solubility of the TLPs might be due to the low solubility of the myofibrillar proteins of fish at pHs between 4.0 and 9.0 [Pires *et al.*, 2012]. Overall, both TLP 2.5 and TLP 11.5 exhibited a similar solubility to SP, suggesting the probability of TLPs application in food products.

Emulsifying properties

The emulsifying activity index (EAI) and the emulsifying stability index (ESI) of TLP 2.5 and TLP 11.5 compared to SP and EW are presented in Table 3. The results showed that TLP 2.5 had a significantly ($p<0.05$) higher EAI and ESI than TLP 11.5 (16.00 and 13.79 m^2/g, respectively; and 38.98, and 34.76 min, respectively). These results agreed well with the report by Panpipat & Chaijan [2017] who found that the recovered proteins of bigeye snapper head by-products obtained following the acidic pH shift process were higher in EAI than those of obtained following the alkaline pH shift process. This was probably due to the partial unfolding of muscle protein induced by the acidic condition which was responsible for the incorporation of protein into the oil droplet membrane and the positive effect on emulsification [Panpipat & Chaijan, 2017]. However, the EAI of SP and EW was higher than that of TLP 2.5 and TLP 11.5. This might be due to the higher hydrophobicity of SP and EW which promotes the interaction of protein with the oil surface, resulting in excellent emulsifying capacity [Pires *et al.*, 2012].

The higher ($p<0.05$) ESI of TLP 2.5 than TLP 11.5 may be attributed to the capability of the emulsion droplets to

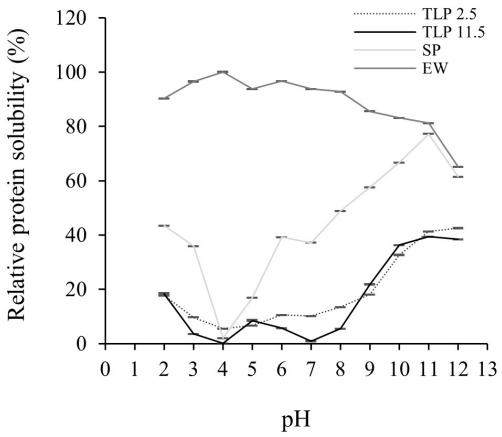


FIGURE 4. Relative protein solubility (%) at pH 2.0 to 12.0 of tuna liver protein powders (TLP) obtained following the pH shift process. TLP 2.5, TLP from solubilization at pH 2.5; TLP 11.5, TLP from solubilization at pH 11.5; SP, soy protein concentrate; EW, egg white powder. SP and EW were used as positive controls. Values are reported as mean ± standard deviation ($n=3$).

TABLE 3. Functional properties of tuna liver protein powders (TLPs) obtained following the pH shift process.

Functional properties	Protein powders			
	TLP 2.5	TLP 11.5	SP	EW
EAI (m^2/g)	16.00±0.03^c	13.79±0.43^d	23.46±0.03^a	17.36±0.15^b
ESI (min)	38.98±1.60^b	34.76±1.06^c	79.70±0.66^a	25.01±0.81^d
FC (%)	113.33±0.84^d	126.67±1.81^c	146.67±1.88^b	178.33±0.98^a
FS (%)	53.12±0.64^d	84.44±1.92^c	92.41±0.60^b	96.31±0.20^a
WHC (mL/g)	8.06±1.73^c	18.61±1.27^b	27.22±0.96^a	2.50±0.83^d
OHC (mL/g)	5.73±0.21^c	8.67±0.21^a	7.07±0.61^b	5.06±0.46^d

TLP 2.5, TLP from solubilization at pH 2.5; TLP 11.5, TLP from solubilization at pH 11.5; SP, soy protein concentrate; EW, egg white powder; EAI, emulsifying activity index; ESI, emulsion stability index; FC, foaming capacity; FS, foaming stability; WHC, water holding capacity; OHC, oil holding capacity. SP and EW were used as positive controls. Values are reported as mean ± standard deviation (n=3). Different letters within the same row indicate significant differences among treatments (p<0.05).

be dispersed without coalescence, flocculation, and cream. This result agreed well with the finding of Shen *et al.* [2022] for yellowfin tuna liver protein which showed a higher ESI obtained following the acidic rather than the alkaline pH shift process. The ESI of both TLP 2.5 and TLP 11.5 was considerably (p<0.05) higher than EW. This could be explained by the revelation of hydrophobic groups under the acidic and alkaline pH shift processes which stabilized the protein network formed and consequently led to the stability of the emulsion [Panpipat & Chaijan, 2017; Cha *et al.*, 2020]. However, the ESI of the TLPs was significantly (p<0.05) lower than that of SP. This might be correlated with the lower EAI of the TLPs.

Foaming properties

The foaming capacity (FC) and the foaming stability (FS) of the TLPs are shown in Table 3. The FC of TLP 11.5 was remarkably (p<0.05) greater than those of TLP 2.5 with values of 126.67% and 113.33%, respectively. In addition, the FS of TLP 11.5 was significantly (p<0.05) higher than that of TLP 2.5, which were 84.44% and 53.12%, respectively. The results for the FC from TLP 11.5 were similar to those reported by Shen *et al.* [2022] for the recovered proteins from yellowfin tuna liver through which the alkaline pH shift process showed a higher FC than the acidic pH shift process. This might be due to the exposure to a larger number of hydrophobic groups by the alkaline pH shift process which led to the better FC of the protein [Cha *et al.*, 2020; Shen *et al.*, 2022]. Additionally, the FS of TLP 11.5 was similar to that reported by Chanted *et al.* [2022] for the pig brain proteins obtained following the alkaline pH shift process which showed an excellent FS compared to the acidic pH shift process.

Both TLP 2.5 and TLP 11.5 had a significantly (p<0.05) lower FC than SP and EW (Table 3). This might be related to the protein solubility of the TLPs showing as being lower than SP and EW (Figure 4), which affected its ability to unfold at the air-water interface [Cha *et al.*, 2020]. This result was in accordance with the finding of Pires *et al.* [2012] who reported that the hake protein powder showed a lower FC than EW could be related to the lower protein solubility of the fish proteins. Interestingly, the FS of TLP 11.5 was greater than 80% which was comparable to that of SP and EW, indicating its potential as an excellent foam stabilizer. According to these results, the TLP prepared by the alkaline pH shift process possessed a high potency for foam stability which may be applicable in foam-based foods.

Water holding capacity

The water holding capacity (WHC) of TLPs is shown in Table 3. The WHC of TLP 11.5 was significantly (p<0.05) higher than that of TLP 2.5, accounting for 18.61 and 8.06 mL/g, respectively. These results agree well with Freitas *et al.* [2011] who found that the recovered protein of Argentine anchovy (*Engraulis anchoita*) obtained by the alkaline pH shift process had a higher WHC than the recovered proteins obtained following the acidic pH shift process. The lower WHC of TLP 2.5 might be related to the higher protein solubility (Figure 4) caused by the higher content of the polar group in the proteins which decreases the amount of adsorbed water [Han *et al.*, 2019]. The WHC of both TLPs was significantly (p<0.05) lower than that of SP but significantly (p<0.05) higher than that of EW. This might be due to the conformational changes of the TLPs caused by the acidic and alkaline pH shift processes which allowed accessibility between the hydrophilic amino acids and water, leading to the increasing WHC [Cha *et al.*, 2020].

Oil holding capacity

The oil holding capacity (OHC) of the TLPs is shown in Table 3. The OHC is an important characteristic required in the meat and emulsions industry as it affects the taste and functional properties of food [Freitas *et al.*, 2011]. The results showed that TLP 11.5 had a higher (p<0.05) OHC than TLP 2.5 with values of 8.67 and 5.73 mL/g, respectively. The high OHC of TLP 11.5 may be due to the larger amount of hydrophobic amino acids (*i.e.*, cysteine, alanine, glycine, and tryptophan) existing on the protein surface [Han *et al.*, 2019]. Additionally, both TLPs exhibited a higher (p<0.05) oil holding capacity than EW which may be related to the differences in protein content and hydrophobic amino acids such as valine, tryptophan, and phenylalanine, which can easily bind to oil [Han *et al.*, 2019].

Overall, the functional property analyses of the TLPs obtained through the acidic and alkaline pH shift process pointed out that these proteins are equivalent in many aspects to commercial proteins such as SP and EW, particularly in terms of solubility, emulsion properties, and water/oil holding capacity. Thus, TLPs could potentially be applied in various food systems and could potentially be used as alternative emulsifiers and in water/oil adsorption as part of emulsion-based food products.

Antioxidant properties

DPPH radical scavenging activity

DPPH$^{\bullet}$ is a stable free radical which can accept an electron or hydrogen radical and form a stable molecule [Yen & Hsieh, 1995]. It is widely used for the evaluation of the radical scavenging activity of primary antioxidants. In this study, the DPPH radical scavenging activity of both TLP 2.5 and TLP 11.5 are shown in Figure 5A. TLP 11.5 showed a significantly ($p<0.05$) higher activity than TLP 2.5, accounting for 0.73 and 0.55 μmol Trolox equivalent/mg sample, respectively. According to Zhang *et al.* [2018], the higher DPPH radical scavenging activity could be explained by the presence of hydrophobic amino acids or aromatic amino acids (alanine, leucine, valine, and isoleucine) in the recovered proteins obtained following the alkaline pH shift process. The pH shift process is based on acidic and alkaline solubilization and isoelectric precipitation of proteins [Kristinsson *et al*., 2005]. Since alanine, leucine, and isoleucine are more soluble in alkaline pH [Tseng *et al*., 2009], the higher scavenging activity of TLP 11.5 coincides with the significantly ($p<0.05$) higher leucine and alanine contents (Table 2) which were responsible for the DPPH radical scavenging activity [Zhang *et al.*, 2018]. Our TLP 11.5 showed higher DPPH radical scavenging activity under the same sample concentration compared to the cuttlefish (*Sepia pharaonis*) protein isolates obtained from alkaline pH shift process [Hamzeh *et al*., 2018].

ABTS radical cation scavenging activity

Value of ABTS radical cation scavenging assay is well accepted as an antioxidant index due to the applicability of both lipophilic and hydrophilic compounds [Cha *et al.*, 2020]. The ABTS$^{\bullet+}$ scavenging activity of the TLPs are shown in Figure 5B. The ABTS$^{\bullet+}$ scavenging activity of TLP 11.5 was 17.56 μmol Trolox equivalent/mg protein, which was greater ($p<0.05$) than that of TLP 2.5 (11.32 μmol Trolox equivalent/mg protein). This could be attributed to the higher amounts of some hydrophilic amino

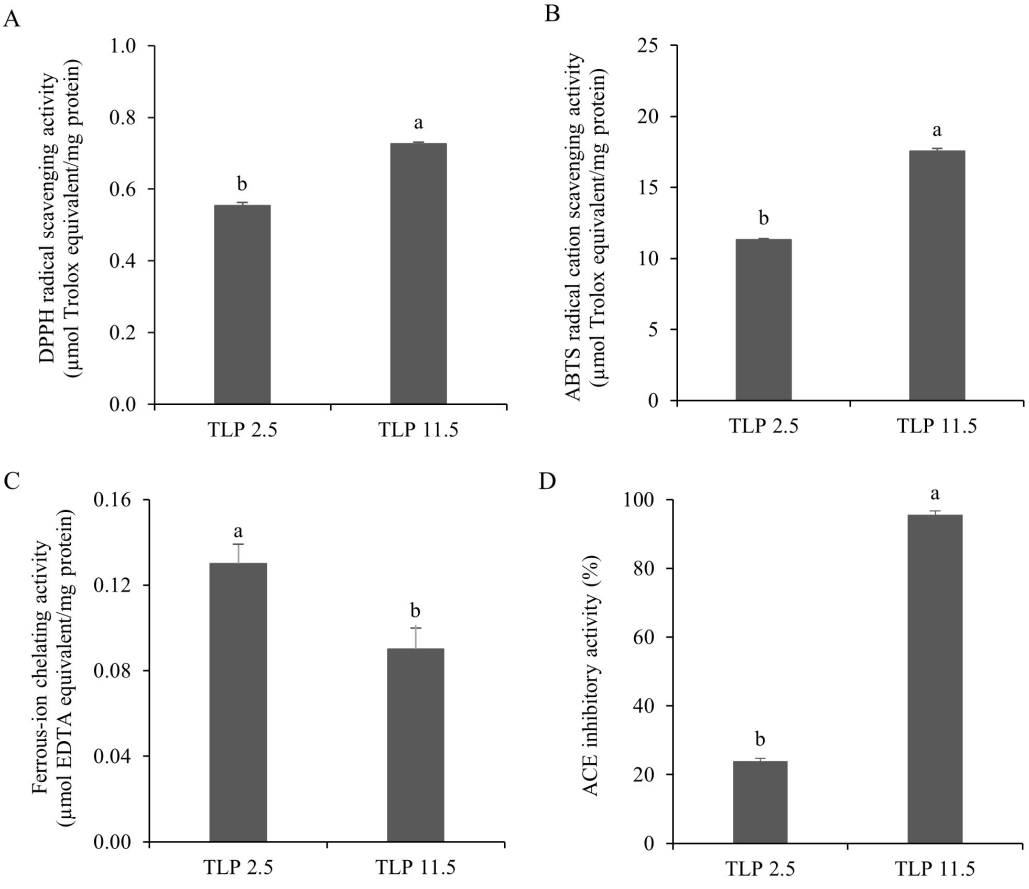


FIGURE 5. Antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of tuna liver protein powders (TLP) obtained following the pH shift process. DPPH radical scavenging activity (A), ABTS radical cation scavenging activity (B), ferrous-ion chelating activity (C), and ACE inhibitory activity (D). Values are reported as mean ± standard deviation ($n=3$). Different letters indicate significant difference among treatments ($p<0.05$).

acids, specifically glutamic acid, arginine, and threonine in TLP 11.5 compared to those in TLP 2.5 (Table 2). These amino acids typically exhibited high $ABTS^{\bullet+}$ scavenging activity [Cha *et al.*, 2020]. Additionally, the alkaline condition can induce the generation of negatively charged peptides [Binsan *et al*., 2008] which could in turn promote the $ABTS^{\bullet+}$ scavenging activity of TLP 11.5. A similar phenomenon was observed by Cha *et al.* [2020] who reported that the roe tuna recovered protein from the alkaline pH shift process exhibited high $ABTS^{\bullet+}$ scavenging activity. Compared to the rainbow trout by-product protein isolates obtained following alkaline pH shift process by Nikoo *et al*. [2019], TLP 11.5 showed greater $ABTS^{\bullet+}$ radical scavenging activity under the same sample concentration.

Ferrous-ion chelating activity

Ferrous-ion chelating activity is one of the most important indicators for determining the antioxidant properties of compounds. This activity is based on the bonding between the ferrous iron and functional carboxyl, amino, and hydroxy groups in antioxidants [Torres-Fuentes *et al.*, 2012]. The ferrous-ion chelating activity of the TLPs is shown in Figure 5C. Unlike the other antioxidant properties, namely the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities, TLP 2.5 exhibited a greater ferrous-ion chelating activity than TLP 11.5 (0.13 and 0.08 μmol EDTA equivalent/mg protein, respectively). This could be explained by the higher ($p<0.05$) content of polar amino acids (lysine and histidine) and other amino acids (proline) in TLP 2.5 than in TLP 11.5 (Table 2) which was implicated in its high iron-binding capacity [Torres-Fuentes *et al.*, 2012]. Sun *et al.* [2020] also reported a positive correlation between ferrous-ion chelating activity and histidine content which was higher in TLP 2.5 in this study. In addition, the NaCl content could affect the ferrous-ion chelating activity of protein by disrupting the chelating activity of specific peptides and amino acid side chain groups in protein [Zhu *et al*., 2014]. In the present study, the content of NaCl in TLP 11.5 was higher than in TLP 2.5. These resulted in the lower ferrous-ion chelating activity in TLP 11.5 than in TLP 2.5. However, TLP 11.5 still showed greater ferrous-ion chelating activity under the same sample concentration compared to the shrimp waste protein isolates obtained following pH shift process [Khumallambam *et al*., 2011].

ACE inhibitory activity

The inhibition of ACE, a key enzyme involved in the regulation of blood pressure, is an important pharmacological target to treat hypertension. Currently, ACE inhibitory peptides derived from food proteins have been well accepted as safe and excellent ACE inhibitors [Cha *et al.*, 2020]. The ACE inhibitory activity of both TLP 2.5 and TLP 11.5 is depicted in Figure 5D. The results showed a significantly ($p<0.05$) higher ACE inhibitory activity in TLP 11.5 (95.66%) than TLP 2.5 (24.03%) (0.13 and 2.28 mmol Captopril/mg sample, respectively). This is probably due to a higher content of some hydrophobic amino acids, such as leucine, and alanine, and some hydrophilic amino acids, such as arginine in TLP 11.5 than TLP 2.5 (Table 2). Similar results have been reported by Nakajima *et al.* [2009] according to whom the higher percentages of alanine and leucine, and to some extent methionine, led to the strong ACE inhibitory activity of fish muscle hydrolysate. The result obtained in the present study was in accordance with the findings of Cha *et al*. [2020] who reported that the ACE inhibitory activity of the recovered proteins from roe yellowfin tuna prepared by the alkaline pH shift process exhibited high ACE inhibitory activity which was probably due to the present of alanine and arginine. The alkaline pH shift process also affected the cleavage of the inter- and intra-molecular hydrogen bonds within and between the molecules which led to the faster release of ACE inhibitory peptides [Zhang *et al.*, 2017]. Furthermore, Kim *et al.* [2016] reported that the existence of NaCl could enhance the ACE inhibitory activity of peptides leading to the higher ACE inhibitory activity of TLP 11.5 than that of TLP 2.5. In comparison to the roe tuna protein isolates obtained following alkaline pH shift process by Cha *et al*. [2020], TLP 11.5 showed greater ACE inhibitory activity under the same sample concentration.

The results obtained in the present study indicate that TLP from the alkaline pH shift process (TLP 11.5) possessed better antioxidant activities, especially $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities, and ACE inhibitory activity than those obtained following the acidic pH shift process (TLP 2.5). Hence, the alkaline pH shift process is an excellent method for the development of TLP as a natural antioxidant and ACE inhibitor.

CONCLUSIONS

This study demonstrated the feasibility of producing recovered protein powder derived from valueless skipjack tuna liver through an acidic or alkaline pH shift process (TLP 2.5 or TLP 11.5). The protein recovery yield was higher in alkaline conditions (pH 11.5) than in acidic conditions (pH 2.5). Lipids were efficiently removed by the acidic and alkaline pH shift process. Both TLP 2.5 and TLP 11.5 were rich in essential amino acids, especially leucine, lysine, and valine. Compared with TLP 2.5, the TLP 11.5 from the alkaline pH shift process possessed better foaming properties and water/oil holding capacity. In addition, the TLP 11.5 had stronger bioactive properties, in terms of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities, and ACE inhibitory activity. Therefore, the TLP obtained by the alkaline pH shift process could potentially be applicable as an alternative functional protein ingredient. Further studies on the characterization of bioactive components responsible for antioxidant and ACE inhibitory activities could be valuable for TLP as nutraceutical and functional food ingredients for protein food and pharmaceutical applications.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Hydration Kinetics of Nixtamalized White Bitter Lupin (*Lupinus albus* L.) Seeds

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Key words: nixtamalization, bitter white lupin, hydration kinetics

Nixtamalization is usually performed on grains by cooking in an alkaline solution to improve the final product characteristics. White bitter lupin (*Lupinus albus*) seeds were nixtamalized at various concentrations of calcium hydroxide in the range of 0.16–3.33% at 50, 70, and 90°C for 35 min and steeped for 0, 8, 16, and 24 h, and the moisture uptake was determined to model seed hydration kinetics. Moisture uptake increased with increasing nixtamalization temperature regardless of calcium hydroxide concentration. The Page and Weibull models adequately described white bitter lupin hydration kinetics during nixtamalization. Model parameters K_p (Page model) and α (Weibull model) ranged from 80.2 to 410.03 and from 88.21 and 93.96, respectively, for nixtamalization at different calcium hydroxide concentrations, and from 58.55 to 662.88 and from 68.74 and 132.99, respectively, for nixtamalization at different temperatures. The alkaloid content of raw lupin flour was 1.08 g/100 g and it gradually decreased as a result of nixtamalization in increasingly concentrated calcium hydroxide solutions and higher temperatures. The cracks were visible in the microstructure of nixtamalized seed coats. Their number and size increased with the increase of processing temperature, calcium hydroxide concentration, and steeping duration. Overall, the presented results may be useful in optimizing the industrial nixtamalization of lupin seeds and increasing the possibility of their use as a valuable food ingredient.

INTRODUCTION

Lupin (*Lupinus* spp.) belongs to the Fabaceae (Leguminosae) family [Pastor-Cavada *et al*., 2009]. There are more than 400 species from the genus *Lupinus*; from which only four (*i.e.*, *L. albus* L.: white lupin, *L. angustifolius* L.: blue or narrow-leafed lupin, *L. luteus* L.: yellow lupin, and *L. mutabilis* L.: pearl or Tarrwi lupin) are of agronomic interest [Annicchiarico *et al*., 2010; Chiofalo *et al*., 2012; Gulisano *et al*., 2022]. The first three species originate from the Mediterranean area, while the native environment for *L. mutabilis* is South America. Lupin seeds are employed as a protein source for animal and human nutrition in various parts of the world [Chiofalo *et al*., 2012; Prusinski, 2017]. Lupin is cultivated not only due to the nutritional value of its seeds, but also to its adaptability to marginal soils and climates. Human consumption of lupins has increased in recent years [Lucas *et al*., 2015].

Lupins are high protein crops with an average protein content in the white lupin seeds ranging from 30.6 to 37.6 g/100 g [Martínez-Villaluenga *et al*., 2006; Sujak *et al*., 2006]. Furthermore, lupin has a minimum content of proteins with anti-nutritive properties (allergenic proteins) compared to other legumes (*i.e.*, peas, soybean, and bean) [Kurlovich *et al*., 2002]. The mean value of crude fat in *L. albus* of different varieties is 13 g/100 g [Martínez-Villaluenga *et al*., 2006]. As well as lupin seeds and seed coats contain various types of carbohydrates, mainly non-starch ones [Malekipoor *et al*., 2022]; which are the most abundant in seeds. Most carbohydrates are represented by soluble or insoluble fiber accounting for about 39.42 g/100 g of dry matter [Keller *et al*., 2022; Martínez-Villaluenga *et al*., 2006]. Starch content of lupin is very low and according to Borek *et al*. [2011] mature air-dried white lupin seeds are starch free.

The presence of non-nutritional compounds is considered a limiting factor for lupin consumption. The main non-nutritional substances found in lupin seeds are various alkaloids of the quinolizidine group [De Cortes Sánchez *et al*., 2005; Estivi *et al*., 2022; Sujak *et al*., 2006]. Such alkaloids are usually removed by either selecting genotypes with a low content of these components or through post-harvest treatments including germinating, cooking, soaking, fermentation and extraction [Estivi *et al*., 2022; Prusinski, 2017]. Therefore, alkaloid removal would enhance lupin consumption.

Nixtamalization is a pre-Columbian era process in which corn kernels are cooked in a calcium hydroxide solution [Ramírez-Araujo *et al*., 2019]. Nixtamalization is also applied to other grains and seeds including amaranth [Valdez-Niebla *et al*., 1993], sorghums [Díaz González *et al*., 2019; Gaytán-Martínez *et al*., 2017] and beans [Santiago-Ramos *et al*., 2018a]. This process improves the rheological properties

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of final products, which include viscoelasticity [Santiago-Ramos *et al*., 2018b], significantly increases the content of calcium and other minerals [Santiago-Ramos *et al*., 2018a; Vega Rojas *et al*., 2017] as well as affects the protein quality [Rincón-Aguirre *et al*., 2021]. Additionally, it was found that nixtamalization resulted in a discount of mycotoxin content and a reduction in the potential toxicity of maize contaminated with fumonisin [Voss *et al*., 2017]

Hydration kinetics of grain is a complex phenomenon and its exact mechanisms are largely not understood. The analysis of kinetics hydration is useful for process design and optimization [Augusto & Miano, 2017]. The hydration kinetics of lupin seeds was studied by Solomon [2007]. The author soaked the seeds in water at 20, 30, 40, and 50°C for up to 12 h and further fitted the Peleg model equation adequately to describe the hydration characteristics. In other study, Solomon [2009] determined the hydration kinetics of roasted lupin seeds soaked in water at 25, 35, 45, or 55°C for up to 8 h. The author also used Peleg model in addition to the first-order kinetic model, and hydration kinetics was described adequately. On the other hand, Miano *et al*. [2015] fitted a sigmoidal model when they studied the hydration kinetics and mathematical modeling of Andean lupin (*L. mutabilis* Sweet) seeds. There are limited studies on the hydration kinetics of bitter lupin seeds and – to the best of our knowledge – there are no studies on the effects of nixtamalization of lupin seeds on their hydration kinetics. Therefore, this work was initiated to evaluate the hydration kinetics of white bitter lupin seeds under various nixtamalization conditions (*i.e.*, nixtamalization temperature and calcium hydroxide concentration) and to determine changes in the microstructure and content of seed alkaloids as a result of the process conditions.

MATERIALS AND METHODS

Materials

Bitter lupin seeds (*i.e.*, Egyptian origin of the crop year 2020) were procured from a local market in Amman, Jordan. Before conducting the tests, seeds with defects, including breakage and color damage, were discarded and not included in testing to ensure seeds' uniformity in terms of maturity. The initial moisture content of lupin seeds was determined by grinding the seeds into flour before drying using a convection oven at 105°C (±2°C) until constant weight. The initial moisture content of lupin seeds was 6.47 g/100 g.

Nixtamalization of lupin seeds

The nixtamalization of lupin seeds was carried out using solutions of $Ca(OH)_2$ in different concentrations, *i.e.*, 0.16, 0.33, 0.50, 0.66, 1.33, 2.00, 2.66, and 3.33%. Parallel, seeds were treated in water (0% $Ca(OH)_2$). Seeds were cooked at 50°C, 70°C, and 90°C before being steeped in the same calcium hydroxide solutions or water for 0, 8, 16, and 24 h. A total of 216 samples ((9×3×4)×2) was achieved. For nixtamalization, 100 g of bitter lupin seeds were placed in a previously heated calcium hydroxide solution (or water for control) at the adequate temperature (*i.e.*, 50, 70 or 90°C) and cooked for 35 min. Beakers were covered during the process to prevent water evaporation and to maintain the exact calcium hydroxide concentration.

After steeping for the required duration, nixtamalized seeds were washed with distilled water to remove the excess calcium hydroxide. The water remaining on the surface of the seeds was removed using a paper towel. Moisture uptake by lupin seeds (M) was measured as the weight difference after and before the nixtamalization process using an analytical balance (BTD-323, Phoenix Instrument, Blomberg, Germany).

Mathematical modeling of hydration kinetics

Non-linear regression models (the Peleg, Lewis, Page, Weibull, Henderson, Kaptso-Njintang-Komnek-Hounhouigan-Scher-Mbolung, and Ibarz-Augusto models) were used to define the hydration kinetics of bitter lupin seeds [Augusto & Miano, 2017]. A list of models and their respective equations used in this study is presented in Table 1. Root mean square error (RMSE) and coefficient of determination (R^2) were determined to fit each model to the experimental data. The average percentage difference between the experimental and predicted values, also known as the mean relative deviation modulus (PMRD), was calculated according to Equation (1) and used as a measure of model adequacy. The model with the highest R^2 (that must be as close as possible to the one) and the least RMSE and PMRD (that must be as close as possible to zero) was chosen as the best [Saleh & Meullenet, 2013].

$$PMRD = \frac{100}{n}\sum_{i=1}^{n}\left(\frac{MR\ actual - MR\ predicted}{MR\ actual}\right) \quad (1)$$

where: n represents observations, *i* represents n=1, and MR is the rate of moisture uptake, given by Equation (2).

$$MR = \frac{M_t - M_z}{M_0 - M_z} \quad (2)$$

where: M_0 is the initial moisture content of the bitter lupin seeds, M_t is the moisture content of nixtamalized bitter lupin seeds at time t, and M_z is the final moisture content of nixtamalized bitter lupin seeds.

Microscopic analysis

The lupin seeds nixtamalized using the extreme and middle concentrations of calcium hydroxide solutions (3.33% and 0.66%, respectively), and water only (0% $Ca(OH)_2$) of the three temperatures and all steeping durations were photographed. The pictures were taken using an EZ4HD Leica microscope (Leica Microsystems, Singapore) and LAS EZ-V2-1 program.

Alkaloid content determination

The alkaloid content of selected samples was determined according to the method described by Shamsa *et al*. [2008] with some modifications. Alkaloids were extracted from dried (moisture content around 6 g/100 g) and ground into flour nixtamalized lupin seeds (10 g) with methanol (100 mL) in a water bath for 2 h, then sonicated for 30 min and left overnight at room temperature. The mixtures were filtered and methanol was evaporated under vacuum at 45°C. The dried extracts were dissolved in 2 M HCl and then filtered. One mL of the filtrate was washed twice with chloroform using a separatory funnel

TABLE 1. Equations of models used to fit bitter lupin seed hydration kinetics.

Model	Equation
Peleg	$M(t) = M_0 + \frac{t}{k_1 + k_2 \times t}$
Lewis	$\frac{M(t) - M_0}{M_\infty - M_0} = \exp(-k_l \times t)$
Page	$\frac{M(t) - M_0}{M_\infty - M_0} = \exp(-K_p \times t^n)$
Henderson	$\frac{M(t) - M_0}{M_\infty - M_0} = P_1 \times exp(-K_{H1} \times t) + P_2 \times exp(-K_{H2} \times t)$
Weibull	$\frac{M(t)}{M_\infty} = 1 - exp\left[-(\frac{t}{\alpha})^\beta\right]$
Kaptso-Njintang-Komnek-Hounhouigan-Scher-Mbolung	$M(t) = \frac{M_\infty}{1 + exp[-k_k \times (t - \tau)]}$
Ibarz-Augusto	$M(t) = \frac{M_\infty}{1 + \frac{M_\infty - M_0}{M_0} exp(-k_{IA} \times M_\infty \times t)}$

k_1, k_2, k_l, K_p, P_1, P_2, K_{H1}, K_{H2}, α, β, k_k, and k_{IA}: kinetic parameters of the mathematical models; M: product moisture content on dry basis, d.b. (g water/100 g solids); $M_{0:}$ initial moisture content (g water/100 g d.b.); $M_{\infty:}$ equilibrium moisture content (g water/100 g d.b.); t: time (min); (τ): parameter that describes the time (s) of the inflexion point, related to the lag phase.

and neutralized with 0.1 M NaOH. Then, 5 mL of 0.1 mM bromocresol green solution and 5 mL of phosphate buffer (pH 4.7) were added. The complex formed was extracted with 10 mL of chloroform by vigorous shaking. After separation of the chloroform phase, its volume was adjusted to 10 mL in a volumetric flask with chloroform. The absorbance of the mixture was measured at 470 nm. Alkaloid content was quantified on the basis of atropine standard curve and results were expressed as g of standard equivalent per 100 g of seeds.

Statistical analysis

To determine the effect of temperature, calcium hydroxide concentration, and steeping duration, one-way analysis of variance (ANOVA) with least significant differences (LSD) post-hoc test at a 5% level of probability was carried out using JMP statistical software release 10.0 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Lupin seed hydration

The hydration characteristics of nixtamalized lupin seeds in terms of moisture uptake at different calcium hydroxide concentrations, cooking temperatures, and steeping durations are presented in Table 2.

Discussing the effect of the temperatures used during the nixtamalization of bitter lupin seeds on moisture uptake, it can be mentioned that at the beginning of steeping (0 h), the water absorption of seeds treated at 50°C was lower ($p<0.05$) compared to that for seeds cooked at 90°C regardless of the calcium hydroxide concentration. Significant ($p<0.05$) differences were also noted between the nixtamalization at 50 and 70°C, but with the exception of processes carried out in 0.66%, 2.00%, and 2.66% $Ca(OH)_2$ solutions. No significant ($p\geq0.05$) differences were shown between treatments at 70°C and 90°C for low calcium hydroxide concentrations (0.16–0.66%). At a steeping duration of 8 h, nixtamalization in 0.16%, 0.33%, and 1.33% $Ca(OH)_2$ solutions showed no temperature effect on moisture uptake. In turn, water absorption of the samples treated without $Ca(OH)_2$ and at its concentrations of 0.50%, 0.66%, 2.66% and 3.33% were significantly ($p<0.05$) lower at 50°C than at 70°C and 90°C. In general, at the early stage of hydration, there was a difference in the moisture uptake by lupin seeds treated at different temperatures. Trends of increasing moisture uptake at higher temperatures could be due to the increase in seed coat pore size, which improved the water flow [Oliveira *et al*., 2013].

The results showing the effect of steeping duration on the hydration process are presented in Table 2. They indicated that the longer the steeping duration, the higher the moisture uptake. After 16 h of steeping, most of the samples reached their maximum moisture uptake (up to 130.9 g/100 g). Whereby, the highest rate of the water absorption occurred at the early stage of the process followed by a decrease in the rate. Similar trends have been reported for peas and beans [Ueno *et al*., 2015] and cereal grains [Thakur & Gupta, 2006]. In turn, the equilibrium moisture uptake of all the samples in our study was lower compared to the results reported by Solomon [2007] for hydrated lupin seeds (~140 to 170 g/100 g).

The decrease in moisture uptake of lupin seeds in some treatments after 24 h of steeping (Table 2) was attributed to the effect of calcium hydroxide, which softened lupin seed coat and increased the solid mass loss with the prolonged steeping. Similar results were reported by Castro-Muñoz & Yáñez-Fernández [2015] who found that the alkaline solution remaining after steeping of corn (that was not absorbed by grain) had a high content of soluble solids (polysaccharides, protein, lipids, as well as some bioactive compounds as polyphenols and carotenoids) and insoluble solids (including fiber fractions such as cellulose and hemicellulose).

TABLE 2. Moisture uptake of bitter lupin seeds (g/100 g, dry base) nixtamalized at different temperatures and calcium hydroxide concentrations, and steeped for different durations.

Temperature (°C)	Calcium hydroxide concentration (%)	Steeping duration (h)			
		0	8	16	24
50	0.00	43.5 ± 1.9^{cBb}	117.5 ± 0.1^{bAb}	130.3 ± 2.1^{aAa}	126.5 ± 0.3^{aAb}
	0.16	31.5 ± 1.8^{cDc}	118.0 ± 2.8^{bAa}	125.7 ± 0.6^{aBCb}	125.5 ± 1.6^{aABa}
	0.33	31.8 ± 0.3^{cDb}	114.2 ± 2.4^{bBa}	126.7 ± 3.2^{aABa}	127.7 ± 0.7^{aAa}
	0.50	36.4 ± 1.6^{cCDb}	114.9 ± 0.3^{bABb}	124.0 ± 2.8^{aBCDa}	126.7 ± 0.8^{aAa}
	0.66	42.0 ± 7.2^{cBCb}	113.2 ± 1.0^{bBb}	121.4 ± 1.3^{abDb}	126.5 ± 1.5^{aAa}
	1.33	49.9 ± 0.7^{cAc}	113.5 ± 0.7^{bBa}	122.9 ± 1.3^{aBCDa}	121.1 ± 1.4^{aCa}
	2.00	47.4 ± 2.1^{dABb}	111.9 ± 0.7^{cBb}	122.7 ± 0.7^{bBCDb}	128.0 ± 1.3^{aAa}
	2.66	42.4 ± 1.3^{cBCb}	113.9 ± 1.2^{bBb}	122.0 ± 1.1^{aCDb}	123.3 ± 1.2^{aBCb}
	3.33	41.5 ± 1.1^{cBCc}	112.4 ± 1.1^{bBc}	124.1 ± 1.8^{aBCDb}	125.8 ± 1.6^{aABa}
70	0.00	50.8 ± 1.7^{cBCa}	123.1 ± 1.7^{bAa}	130.0 ± 0.1^{aAa}	127.3 ± 0.2^{aAa}
	0.16	57.7 ± 3.3^{bAa}	123.5 ± 3.2^{aAa}	128.0 ± 0.1^{aABa}	122.4 ± 0.2^{aCDa}
	0.33	55.2 ± 3.9^{bABa}	121.2 ± 1.0^{aABa}	124.4 ± 1.6^{aCDa}	122.4 ± 1.2^{aCDb}
	0.50	49.6 ± 1.4^{cBCa}	121.5 ± 0.1^{bABa}	125.3 ± 0.5^{bBCa}	120.4 ± 0.4^{aDc}
	0.66	51.5 ± 2.0^{cBCab}	119.3 ± 1.3^{bABa}	125.0 ± 0.6^{aBCDa}	122.3 ± 2.1^{abCDa}
	1.33	52.0 ± 0.4^{cABb}	116.1 ± 4.7^{bBa}	122.1 ± 1.2^{abDa}	126.3 ± 2.3^{aABa}
	2.00	49.4 ± 3.6^{cBCb}	118.9 ± 3.5^{bABab}	127.3 ± 1.2^{aABCa}	124.3 ± 0.7^{abBCb}
	2.66	45.7 ± 4.4^{bCb}	122.9 ± 3.6^{aAa}	127.4 ± 1.9^{aABCab}	128.9 ± 0.5^{aAb}
	3.33	50.9 ± 0.7^{cBCb}	118.5 ± 2.9^{bABb}	127.9 ± 2.4^{aABab}	123.3 ± 1.8^{abCDa}
90	0.00	51.5 ± 2.3^{cCDa}	126.1 ± 0.2^{bAa}	129.6 ± 0.6^{aABa}	126.2 ± 0.2^{bAb}
	0.16	46.1 ± 0.7^{cDb}	121.8 ± 1.9^{bABa}	128.8 ± 0.2^{aABa}	125.2 ± 3.0^{abAa}
	0.33	52.5 ± 0.3^{cCa}	120.9 ± 3.5^{bBa}	126.6 ± 1.7^{aABa}	123.1 ± 0.3^{abAb}
	0.50	53.6 ± 0.8^{cCa}	121.8 ± 1.3^{bABa}	126.8 ± 1.4^{aABa}	123.6 ± 0.2^{bAb}
	0.66	56.2 ± 0.6^{cABCa}	121.8 ± 0.0^{bABa}	125.6 ± 0.7^{aABa}	123.7 ± 1.4^{abAa}
	1.33	54.8 ± 0.4^{bBCa}	122.9 ± 4.2^{aABa}	124.0 ± 8.7^{aBa}	123.6 ± 2.4^{aAb}
	2.00	61.2 ± 3.4^{bAa}	123.8 ± 1.3^{aABa}	129.0 ± 0.8^{aABa}	124.6 ± 1.2^{aAab}
	2.66	59.9 ± 6.0^{bABa}	123.3 ± 1.2^{aABa}	132.3 ± 4.4^{aAa}	125.4 ± 0.5^{aAa}
	3.33	62.2 ± 3.5^{cAa}	125.1 ± 0.8^{bABa}	130.9 ± 0.6^{aABa}	125.6 ± 0.2^{bAa}

Results are expressed as mean ± standard deviation. Different lowercase letters in the same row indicate significant ($p<0.05$) differences between the values determined for the samples treated at different steeping duration. Different capital letters in the same column (separately for each temperature) indicate significant ($p<0.05$) differences between the values determined for the samples treated with different concentrations of $Ca(OH)_2$. Different lowercase italic letters in the same column (separately for each $Ca(OH)_2$ concentration) indicate significant ($p<0.05$) differences between the values determined for the samples treated at different temperatures.

The effect of calcium hydroxide concentration on the hydration of lupin seeds did not follow a specific trend (Table 2). At the beginning of steeping (0 h), the lupin seeds treated with calcium hydroxide at concentrations of 1.33% and 2.00% had the highest moisture uptake at 50°C, while the calcium hydroxide concentration of 0.16%, 0.33% and 1.33% allowed to obtain the highest moisture uptake at 70°C. At 90°C, the highest values were determined at $Ca(OH)_2$ concentrations of 0.66% and 2.00–3.33%. In turn, at the end of steeping (24 h), the highest moisture uptake was found in the seeds treated with a wide range of calcium hydroxide concentrations (0–0.66%, 2.00% and 3.33%) at 50°Cas well as at concentrations of 0%, 1.33%, and 2.66% at 70°C; whereas $Ca(OH)_2$ concentration had no effect on water absorption at 90°C. The calcium hydroxide solubility in water is limited to about 1.2 g/L at 25°C [Farhad & Mohammadi, 2005]. The non-soluble part of calcium hydroxide could influence the hydration kinetics indirectly by disposing on the seed surface, which supposedly

TABLE 3. Root mean square error (RMSE) and coefficient of determination (R^2) of Peleg, Lewis, Henderson, Kaptso-Njintang-Komnek-Hounhouigan-Scher-Mbolung (KNKHSM), and Ibarz-Augusto models used to fit bitter lupin seed hydration kinetics.

Calcium hydroxide concentration (%)	Peleg		Lewis		Henderson		KNKHSM		Ibarz-Augusto	
	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2
0.00	4.36	0.98	0.35	0.62	0.39	0.45	9.14	0.98	8.94	0.98
0.16	7.48	0.96	0.36	0.59	0.45	0.35	10.36	0.96	10.24	0.96
0.33	104.84	0.96	0.35	0.61	0.39	0.40	11.66	0.95	20.96	0.96
0.50	104.52	0.97	0.35	0.61	0.05	0.97	10.27	0.97	10.19	0.97
0.66	4.86	0.98	0.34	0.63	0.04	0.97	11.18	0.97	11.05	0.96
1.33	4.02	0.98	0.33	0.64	0.03	0.98	10.72	0.98	10.51	0.97
2.00	5.12	0.98	0.33	0.64	0.05	0.97	11.74	0.96	11.56	0.96
2.66	6.38	0.97	0.34	0.62	0.92	0.41	10.87	0.97	10.67	0.97
3.33	6.35	0.96	0.33	0.62	0.51	0.35	10.99	0.96	10.77	0.96
Temperature (°C)										
50	2.44	0.98	0.38	0.68	0.20	0.97	12.74	0.97	17.79	0.98
70	3.01	0.98	0.32	0.61	0.34	0.98	8.88	0.98	8.93	0.98
90	1.58	0.98	0.31	0.59	0.19	0.98	7.87	0.98	7.83	0.98

contributed to the softness of the seed coat. The formation of gummy and sticky pericarp due to the calcium hydroxide treatment was reported by Martínez-Bustos *et al*. [2001] who indicated a significant role of maize pericarp modifications on the moisture kinetics.

Modeling the hydration kinetics of bitter lupin seeds

Water absorption data of the lupin treatments in terms of moisture uptake under the experimental conditions were fitted into several models by either concentration and/or by temperature (Table 1). The sigmoidal models (Kaptso-Njintang-Komnek-Hounhouigan-Scher-Mbolung and Ibarz--Augusto) that are used in the modeling of the hydration kinetics were not appropriate in describing the moisture uptake of bitter lupin seeds (Table 3). This inappropriate description by the sigmoidal models can be attributed to the effects of calcium hydroxide concentration that resulted in greater hydration. In a previous study on the hydration of Andean lupin seeds, it was found that the curve of hydration kinetics showed a sigmoidal shape [Miano *et al*., 2015]. The authors reported that the hydration lag phase was shortened as the temperature of the process increased, because at higher temperatures the minimum moisture content required for the seeds to change from glass to rubbery was reduced, resulting in increased water permeability.

In the exponential category, Page and Weibull models had the highest R^2 and the lowest values of RMSE and PMRD compared with other models evaluated (Table 3 and Table 4). The models adequately characterized the hydration behavior of bitter lupin seeds upon nixtamalization. The estimated parameters of Page and Weibull models for calcium hydroxide concentrations and temperatures, RMSE, R^2 and PRMD, are presented in Table 4. In the Page model, the value of the parameter related to diffusion coefficient and the sample geometry (K_p) ranged from 62.29 to 410.03 for different $Ca(OH)_2$ concentrations with the lowest value noted for the concentration of 2.00% and the highest one for the concentration of 0.16%. The n parameter of the Page model is related to the diffusion type and food microstructure [Miano & Augusto, 2017]. Our results showed a negative value of n for modeling the hydration kinetics of all samples regardless of the $Ca(OH)_2$ concentration. Since this model was originally developed for the drying process, this possibly explains the negative values of the n parameter in our study (*i.e.*, the water absorption being in the inverse direction to the drying process). The trend of the parameters of the Page model in function of temperature was very clear with K_p increasing with the increase in temperature while n had the invers function that reflects the diffusion phenomenon that increases with temperature elevation.

Weibull model is described by two parameters, *i.e.,* α – which is related to the reciprocal of the process rate constant, and the shape parameter β [Miano & Augusto, 2017]. The value of the α parameter determined for different calcium hydroxide concentrations ranged from 87.70 to 97.75 and for temperatures of 50, 70, and 90°C was 132.99, 81.73, and 68.74, respectively (Table 4); hence, its values increased along with decreasing temperature. In turn, the values of β determined for different $Ca(OH)_2$ concentrations and temperatures were below one and ranged from 0.65 to 0.81 for calcium hydroxide concentrations and from 0.70 to 0.79 depending on temperature. The correlations between the two parameters of each model determined to fit the hydration of lupin seeds at different calcium hydroxide concentrations and temperatures were analyzed. There was a high correlation between Page model parameters with R^2 of 0.93 (for $Ca(OH)_2$ concentrations)

TABLE 4. Equation parameters, root mean square error (RMSE), coefficient of determination (R^2), and mean relative deviation modulus (PMRD) of Page and Weibull models used to fit bitter lupin seed hydration kinetics.

Calcium hydroxide concentration (%)	Equation parameters*				RMSE		R^2		PMRD	
	Page		Weibull		Page	Weibull	Page	Weibull	Page	Weibull
	K_p	n	α	β						
0.00	213.74	-1.50	91.41	0.76	0.03	0.03	0.99	0.94	0.38	0.01
0.16	410.03	-1.66	93.05	0.81	0.06	0.05	0.97	0.91	2.57	2.42
0.33	126.28	-1.34	97.75	0.73	0.06	0.06	0.96	0.92	2.56	2.17
0.50	198.93	-1.47	92.82	0.77	0.05	0.04	0.97	0.93	1.37	1.02
0.66	80.02	-1.24	92.40	0.68	0.04	0.04	0.98	0.94	0.34	0.25
1.33	64.29	-1.19	87.70	0.65	0.03	0.03	0.98	0.95	0.11	0.65
2.00	62.29	-1.18	89.86	0.65	0.05	0.04	0.97	0.94	0.84	0.01
2.66	103.89	-1.30	93.96	0.71	0.05	0.04	0.97	0.94	0.59	0.10
3.33	98.70	-1.30	88.21	0.68	0.05	0.05	0.97	0.93	1.28	0.44
Temperature (°C)										
50	58.55	-1.08	132.99	0.70	0.04	0.03	0.85	0.99	0.14	0.00
70	208.65	-1.52	81.73	0.74	0.03	0.03	0.99	0.99	0.10	0.21
90	662.88	-1.86	68.74	0.79	0.03	0.03	0.98	0.99	0.08	0.30

*Equations of Page and Weibull models are shown in Table 1.

and 0.88 (for temperatures). In the Weibull model, the correlation between α and β was found only for modeling hydration at different temperatures with R^2 of 0.81.

The root mean square error (RMSE) of the two models describing the hydration of lupin seeds at different temperatures and $Ca(OH)_2$ concentrations was from 0.03 to 0.06 (Table 4). In turn, R^2 of the actual and estimated values ranged from 0.97 to 0.99 for calcium hydroxide concentrations following the Page model as well as from 0.91 to 0.95 when obtained using the Weibull model. Concerning temperatures, R^2 values were 0.85, 0.99, and 0.98 at 50°C, 70°C, and 90°C, respectively, following the Page model and 0.99 obtained by using the Weibull model at the three temperatures used in the present study. Thus, the Weibull model was more appropriate for describing the hydration kinetics in the function of temperatures for the nixtamalized bitter lupin seeds whereas the Page model proved more efficient in describing it in the function of $Ca(OH)_2$ concentrations.

PMRD which is the average percentage difference between the experimental and predicted values that is used as a measure of model adequacy was lower for most calcium hydroxide concentrations when analyzed using the Weibull model compared to the Page model (Table 4).

Microscopic pictures

Microscopic pictures of seeds nixtamalized at selected calcium hydroxide concentrations at 50°C, 70°C, and 90°C and steeped for 0–24 h are shown in Figure 1. From the pictures of nixtamalized seeds, it could clearly be noticed that with the increase in nixtamalization temperature, calcium hydroxide concentration and steeping duration, an increased number and size of cracks in seed coat were visible. The samples treated at 50°C without calcium hydroxide showed very small and countable cracks that were distributed on the edges of the seeds with a smooth surface. With the increase in calcium hydroxide concentration to 0.66%, the $Ca(OH)_2$ caused a small number of fissures with larger size on the surface of lupin seeds, which increased in size with the augmentation of steeping duration. For higher $Ca(OH)_2$ concentrations, the number of fissures increased and a great number of cleavages was noticed on the seeds treated in the 3.33% $Ca(OH)_2$ solution. Lupin seeds nixtamalized at 70°C debuted from very small cracks on the surface. The number of cracks and fissures increased with the increase of calcium hydroxide concentration from 0.66% to 3.33%. At 3.33% $Ca(OH)_2$ concentration, large fissures with a great number of lost parts were reported when the samples were steeped after nixtamalization for more than 16 h. Nixtamalization at greater temperature (*i.e.*, 90°C) resulted in similar trends of the seeds treated without $Ca(OH)_2$ to those nixtamalized at 70°C. However, with the increase in calcium hydroxide concentration from 0.66% to 3.33%, the number and size of cracks and fissures increased significantly. Our results are in accordance with the hydration of cowpeas that was reported by Sefa-Dedeh & Stanley [1979].

Alkaloid contents

The non-treated lupin seed flour had a total alkaloid content of 1.08 g/100 g. This value was lower than that obtained for *L. albus* L. seeds (14.4 g/kg) by Erbas [2010]. The alkaloid contents of the treated lupin seeds are presented in Table 5. For all those samples, the determined values were significantly

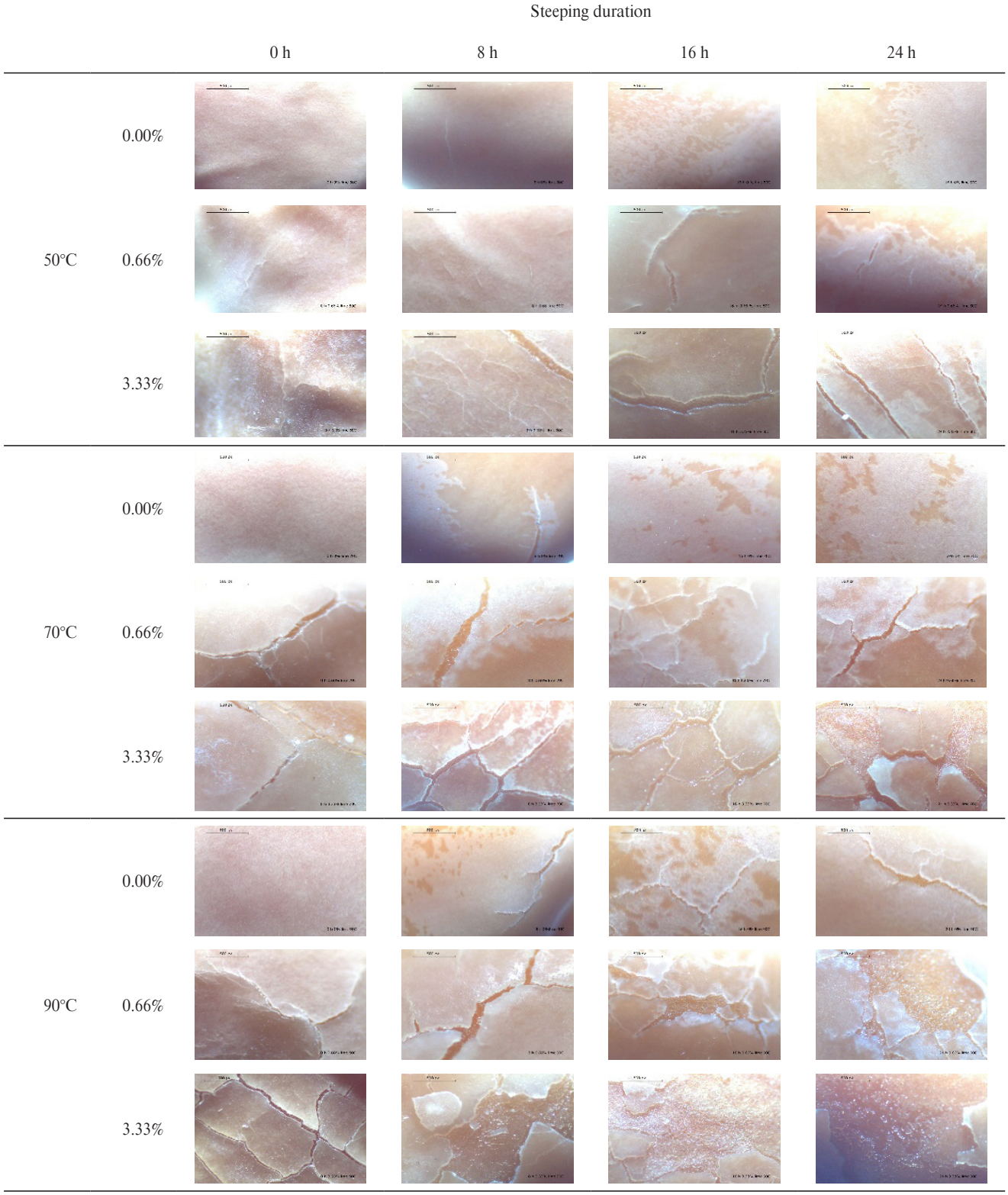

FIGURE 1. Microscopic pictures of bitter lupin seeds nixtamalized at different temperatures (50–90°C) and calcium hydroxide concentrations (0–3.33%) and steeped for durations of 0–24 h.

($p<0.05$) lower compared to the non-treated flour. The alkaloid contents of the nixtamalized seeds decreased significantly ($p<0.05$) with an increasing calcium hydroxide concentration and also with increasing cooking temperatures. Jiménez-Martínez *et al*. [2001] reported that the alkaline thermal treatment, in which the *L. campestris* seeds had a final alkaloid content of 0.002%, was more efficient than the aqueous thermal treatment. The steeping duration did not have a significant ($p<0.05$) effect on the elimination of alkaloids (Table 5). Their low content (0.39 g/100 g) was determined in the seeds treated at 90°C with 3.33% calcium hydroxide concentration and steeped for 16 h; however, it is still a high result within

TABLE 5. Alkaloid content of bitter lupin seeds (g/100 g) nixtamalized at different temperatures and calcium hydroxide concentrations, and steeped for different durations.

Steeping duration (h)	Calcium hydroxide concentration (%)	Temperature (°C)	
		50	90
0	0.00	0.99 ± 0.00^{aAa}	0.86 ± 0.01^{aAb}
	0.66	0.85 ± 0.00^{bAa}	0.52 ± 0.01^{bBb}
	3.33	0.70 ± 0.00^{cAa}	0.40 ± 0.01^{cAb}
16	0.00	0.99 ± 0.02^{aAa}	0.69 ± 0.02^{aBb}
	0.66	0.82 ± 0.01^{bAa}	0.61 ± 0.01^{bAb}
	3.33	0.68 ± 0.02^{cAa}	0.39 ± 0.01^{cAb}

Results are expressed as mean ± standard deviation. Different lowercase letters in the same column (separately for each stepping duration) indicate significant ($p<0.05$) differences between the values determined for the samples treated with different concentrations of $Ca(OH)_2$. Different capital letters in the same row indicate significant ($p<0.05$) differences between the values determined for the samples treated at different temperatures. Different lowercase italic letters in the same column (separately for each $Ca(OH)_2$ concentration) indicate significant ($p<0.05$) differences between the values determined for the samples treated with different steeping duration.

the range of 0.3% to 3% of bitter seeds that reported by Roberts & Wink [1998]. To eliminate more alkaloids from seeds, it is recommended to prolong the cooking duration with the addition of an excess amount of water.

CONCLUSIONS

The steeping duration of bitter white lupin seeds treated under the conditions of this study increases the hydration process until the equilibrium moisture content. The use of high nixtamalization temperatures accelerated the moisture uptake by seeds, and also, the high calcium hydroxide concentration increased the hydration, but the trend was not clear and needs more studies. A number of models were used to evaluate the shape of the hydration kinetics curve. Only two models, *i.e.*, Page and Weibull models, fitted to the experimental results of hydration of lupin seeds. Nixtamalization effectively reduced the content of alkaloids in the lupin seeds, which decreased with the increase of both the $Ca(OH)_2$ concentration and temperature applied during the process. On the other hand, steeping duration had no significant effect on the alkaloid content of the seeds. The microstructure of nixtamalized lupin seeds showed the increase in the number and size of cracks in the seed coat with the increase in the nixtamalization temperature, calcium hydroxide concentration, and steeping duration that accelerated the hydration process by the entrance of water through the hilum, cracks, and fissures.

This research may have practical application in the development of industrial production of lupin masa (*i.e.*, flour) and nixtamalized lupin flour with low alkaloid content for further use in bakery products or as a substitution for other flours. Furthermore, results may help optimize the industrial nixtamalization of lupin seeds and thus contribute to the reduction in energy costs of the process.

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The study received no external funding.

CONFLICT OF INTERESTS

The authors declare that they do not have any conflict of interests.

ETHICAL REVIEW

The study was approved by the Ethical Committee, the University of Jordan. Project approval number 9180366/2020/2022. No human or animal trails were used in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Original article
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β-Glucan and Aronia (*Aronia melanocarpa*) Phenolics: Interactions During *In Vitro* Simulated Gastrointestinal Digestion and Adsorption

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Key words: anthocyanin, bioaccessibility, chokeberry, flavonol, phenolic acid

Interactions between phenolics and dietary fibers, such as β-glucan, are important for the bioactivities of phenolics. However, interactions between aronia phenolics and β-glucan in the digestion process, both promoted for their health benefits, have not been studied. The aim was to study the interactions between aronia phenolics and β-glucan in an *in vitro* simulated digestion and in the adsorption process. After extracting aronia phenolics with chemically- and enzymatically-assisted extraction, and characterizing the phenolics, the aronia extract was subjected to simulated oral, gastric, and intestinal digestion without or with β-glucan. Flavonol release increased throughout oral, gastric, and intestinal digestion, while the recovery of phenolic acids and anthocyanins after an increase in the gastric phase, decreased in the intestinal phase. β-Glucan entrapped phenolics, lowering the quantities of recovered phenolics. It also adsorbed aronia phenolics at pH 1.5, 3.0, and 7.0. In comparison to 15 and 30 mg/L concentrations of β-glucan, a solution with the lower concentration (15 mg/L) allowed for the entrapment of the higher quantity of phenolics and had high adsorption capacity. Entrapment of aronia phenolics by β-glucan is important for the bioaccessibility and concentration of phenolics that reach the lower parts of the digestive tract which depends on β-glucan concentration.

INTRODUCTION

Phenolics have been shown to potentially elicit beneficial effects on human health [Hung *et al*., 2015; Khan *et al*., 2019], but they need to be released from the food matrix in the digestive tract and then absorbed to show those effects. The quantity of phenolics released in the digestive tract which become available for absorption is called bioaccessibility [Palafox-Carlos *et al.*, 2011]. Since bioaccessibility is important in the explanation of the beneficial effects of phenolics, it has been studied in recent years [Lingua *et al.*, 2019; Quatrin *et al.*, 2020; Solari-Godiño *et al.*, 2017; Stübler *et al.*, 2020]. The conditions in the digestive tract, such as pH, and the presence of various food components can affect the phenolics bioaccessibility [Palafox-Carlos *et al*., 2011]. Studies have shown that proteins, lipids or carbohydrates affect phenolics release in the digestive tract [Jakobek, 2015] due to their interactions with phenolics.

Dietary fiber intake is commonly part of a human diet. Plant fibers are resistant to digestion in the small intestine but can be digested in the lower parts of the digestive tract by the activity of microflora [Saura-Calixto, 2011] and can interact with various food components. Fibers can interact with phenolics, which was shown in the case of polyphenols from blackberry purée [Tomas *et al.*, 2020] or anthocyanins [Larsen *et al*., 2019]. By interacting with phenolics, fibers can "carry" them to the lower parts of the digestive tract [Saura-Calixto, 2011].

β-Glucans are soluble dietary fibers which are present in certain cereals [Mäkelä *et al.*, 2020] which also elicit health-beneficial effects. The presence of β-glucans can cause the reduction in the rate of intestinal transit of nutrients, which can be helpful in the attenuation of blood glucose levels [Mäkelä *et al.*, 2020]. Studies have also found that β-glucans can interact with phenolics [Gao *et al.*, 2012; Jakobek *et al.*, 2021]. During such interactions, phenolics might adsorb onto β-glucans [Gao *et al.*, 2012; Jakobek *et al.*, 2021], while β-glucans potentially provide a system that can "carry" phenolics through the digestive tract and "deposit" potentially high concentrations of phenolics to the lower parts of the digestive tract. There has been a study of the behavior of β-glucans and phenolics during *in vitro* digestion and colonic fermentation of barley and barley products containing both components [Mosele *et al*., 2018]. Knowledge of the interaction of phenolics from other sources with β-glucan in the digestion process is, however, limited.

Aronia or black chokeberry (*Aronia melanocarpa*) is rich in phenolics [King & Bolling, 2020; Sidor *et al.*, 2019], which

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makes this fruit popular to produce various food products and dietary supplements. Since aronia is used increasingly in the human diet due to its potential beneficial effects [King & Bolling, 2020; Sidor *et al.*, 2019], knowledge of the fate of aronia phenolics in the digestive tract and their bioaccessibility is needed. It is known that aronia phenolics can adsorb onto β-glucans [Jakobek *et al.*, 2021]. However, there are no previous studies of the interactions of β-glucans and aronia phenolics in the digestion process.

The aim of this study was, therefore, to investigate the interactions between phenolics from aronia and β-glucans by studying the *in vitro* simulated gastrointestinal digestion of aronia phenolics, with and without added β-glucans, and by studying the adsorption process between aronia phenolics and β-glucans.

MATERIALS AND METHODS

Study design

The *in vitro* simulated digestion of aronia was conducted without β-glucan and with 15 and 30 mg/L of β-glucan added in the digestion process. In addition, the adsorption of aronia phenolics onto β-glucan was conducted with β-glucan concentrations of 15 and 30 mg/L.

Chemicals

Reagents were purchased from Gram mol, Zagreb, Croatia (KCl, KH_2PO_4, $NaHCO_3$, $CaCl_2$, $MgCl_2 \times 6H_2O$); from Kemika, Zagreb, Croatia ($(NH_4)_2CO_3$, $Na_2HPO_4 \times 12H_2O$, $NaH_2PO_4 \times 2H_2O$, $CH_3COONa \times 3H_2O$); from Carlo Erba Reagents, Val de Reuil, France (NaCl); from Fluka, Buchs, Switzerland (*o*-phosphoric acid 85% HPLC-grade); from J.T. Baker, Gliwice, Poland (methanol, CH_3COOH); from Fisher Scientific, Loughborough, UK (acetonitrile); and from Avantor, Arnhem, Netherlands (HCl). α-Amylase (13 U/mg), pepsin (632 U /mg), pancreatin (8 USP), quercetin 3-rutinoside hydrate, quercetin 3-glucoside, chlorogenic acid, bile salt, and barley β-D-glucan were purchased from Sigma-Aldrich, St. Louis, MO, USA. Cyanidin 3-galactoside chloride, cyanidin 3-glucoside chloride, and quercetin 3-galactoside, were obtained from Extrasynthese, Genay, France.

Preparation of reagents

Stock solutions of electrolytes were dissolved in distilled water [Minekus *et al.*, 2014]: KCl (0.5 M), KH_2PO_4 (0.5 M), $NaHCO_3$ (1 M), $MgCl_2$ (0.15 M), $(NH_4)_2CO_3$ (0.5 M), NaCl (2 M), and $CaCl_2$ (0.3 M). Simulated salivary fluid, simulated gastric fluid, and simulated intestinal fluid were prepared in volumetric flasks by pipetting appropriate volumes of stock solutions of electrolytes and adding water to make up the final volume of flasks. The final concentrations of electrolytes in the simulated salivary fluid were: 18.87 mM KCl, 4.62 mM KH_2PO_4, 17 mM $NaHCO_3$, 0.056 mM $MgCl_2$, and 0.06 mM $(NH_4)_2CO_3$ [Minekus *et al.*, 2014]. In the simulated gastric fluids, the concentrations were: 8.62 mM KCl, 1.12 mM KH_2PO_4, 31.25 mM $NaHCO_3$, 0.15 mM $MgCl_2$, 0.62 mM $(NH_4)_2CO_3$, and 59.00 mM NaCl [Minekus *et al.*, 2014]. The simulated gastric fluid was adjusted to pH 3 with 1 M HCl. In the simulated intestinal fluid, the final electrolyte concentrations were: 8.5 mM KCl, 1 mM KH_2PO_4, 106.25 mM $NaHCO_3$, 0.41 mM $MgCl_2$, and 48.00 mM NaCl [Minekus *et al.*, 2014]. The simulated intestinal fluid was adjusted to pH 7 with 1 M HCl. Enzyme solutions, α-amylase (1.00 g/L), pepsin (31.66 g/L), and pancreatin (8.00 g/L), were prepared in simulated salivary, gastric and intestinal fluids, respectively. β-Glucan was prepared in distilled water (0.55 g/L and 1.10 g/L) and bile salt in the simulated intestinal fluid (25.00 g/L). All solutions were prepared daily and pre-incubated at 37°C before use. Solutions of pH 1.5 (HCl/KCl), pH 3.0 (citrate buffer, CH_3COONa/CH_3COOH), and pH 7.0 (phosphate buffer, Na_2HPO_4/NaH_2PO_4) were prepared as 0.1 M.

Phenolic extraction

One kg of aronia (*A. melanocarpa*) was harvested from orchards in Croatia (Orahovica), immediately frozen and stored at −18°C. Aronia was homogenized with a stick blender (FA 5295, First, Austria).

For chemical extraction [Jakobek *et al.*, 2021], aronia (3 g) and 22.5 mL of 80% (*v/v*) methanol were placed in a plastic tube, vortexed and extracted in an ultrasonic bath (Bandelin Sonorex RK 100, Berlin, Germany) for 15 min. The mixture was centrifuged (SL 8R, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 12,108×*g*. The liquid part was separated from the fruit residue, and the residue was extracted again using 10 mL of 80% (*v/v*) methanol, following the same procedure as above (ultrasonic bath 15 min, centrifuge 10 min at 12,108×*g*, supernatant separation from the residue). The two supernatants were combined. An aliquot was filtered (0.22 μm polytetrafluoroethylene (PTFE) filter) and analyzed using the high-performance liquid chromatography (HPLC) method as described below.

The fruit residue that remained after the chemical extraction was extracted with an enzyme-assisted extraction to extract the remained phenolics bound to dietary fiber [Bergantin *et al.*, 2017] as follows: 21 mL of distilled water, 1.2 mL of bile salt, 0.6 mL of pancreatin, and 0.3 mL of pepsin were added into the fruit residue. The solution was vortexed, incubated for 2 h at 37°C in a water bath while shaking (SW 22, Julabo, Seelbach, Germany), and centrifuged at 5°C at 12,108×*g* for 5 min. The supernatant was separated from the residue. The residue was extracted again with the same procedure. An aliquot (1 mL) of both supernatants obtained by enzymatic extractions was filtered through a 0.22 μm PTFE filter, put on ice, and analyzed separately using the HPLC method. The final total content of phenolics in aronia was calculated as a sum of contents determined after the chemically-assisted and the two enzyme-assisted extractions, and represented the content of phenolics before digestion.

Simulated digestion

For the oral phase digestion, a homogenized aronia was weighed (3 g) in a plastic tube where after 3.5 mL of simulated salivary fluid, 975 μL of H_2O, 25 μL of $CaCl_2$ (0.3 M), and 500 μL of α-amylase was pipetted. The solution was vortexed for 30 s and then put on ice. An aliquot of 500 μL was taken, filtered through a 0.22 μm PTFE filter, and analyzed using the HPLC method. To the solution remaining after the oral phase, 7.5 mL of simulated gastric fluid,

295 μL of H_2O, 5 μL of $CaCl_2$ (0.3 M), 200 μL of HCl (1 M), and 2 mL of pepsin were added to simulate the digestion. The solution was vortexed, incubated in a water bath with shaking for 2 h at 37°C, centrifuged at 5°C at 12,108×*g* for 5 min, and put on ice. An aliquot of 500 μL was taken, filtered through a 0.22 μm PTFE filter, and analyzed using the HPLC method. Then, the intestinal phase digestion was carried out. The solution remaining after the gastric phase digestion was treated by adding 11 mL of simulated intestinal fluid, 3.61 mL of H_2O, 40 μL of $CaCl_2$ (0.3 M), 150 μL of NaOH (1 M), 5 mL of pancreatin, and 0.2 mL of bile salt. The solution was vortexed, incubated for 2 h at 37°C in a water bath with shaking, centrifuged at 5°C at 12,108×*g* for 5 min, and put on ice. One mL was filtered through a 0.22 μm PTFE filter, and analyzed using the HPLC method. The simulated digestion of aronia with β-glucan was conducted with the same described procedure. The only difference was the addition β-glucan during all three steps of digestion so that its final concentration was 15 mg/L. A stock solution of β-glucan (0.55 g/L) was used. The digestion process was repeated without and with 30 mg/L of β-glucan with the same described procedure. The stock solution of β-glucan was 1.10 g/L.

The percentage of recovered phenolics was calculated using Equation 1:

$$Recovery\ (\%) = \frac{\gamma_{digestion\ phase}\ (mg/kg)}{\gamma_{before\ digestion}(mg/kg)}\ 100 \qquad (1)$$

where: $\gamma_{digestion\ phase}$ is the content of recovered phenolics after a particular digestion phase (mg/kg fresh weight (FW)) and $\gamma_{before\ digestion}$ is the phenolics content in fruit before digestion determined after chemically- and enzyme-assisted extraction (mg/kg FW).

Reversed-phase high performance liquid chromatography (RP-HPLC) analysis

Samples were analyzed on the HPLC system (1260 Infinity II, with a quaternary pump, a PDA detector and a vial sampler) (Agilent Technology, Santa Clara, CA, USA). Phenolics were separated using a Poroshell 120 EC C-18 column (4.6×100 mm, 2.7 μm) protected by a Poroshell 120 EC--C18 guard column (4.6 mm). The mobile phase consisted of 0.5% (*w/v*) H_3PO_4 (A) and 100% acetonitrile (B). Phenolics were separated with a gradient of 5–11% B 0–5 min, 11–15% B 5–7.5 min, 15–17.5% B 7.5–17.5 min, 17.5–20% B 17.5–20 min, 20–30% B 20–30 min, 30–70% B 30–32 min, 70% B 32–34 min, 70–5% B 34–36 min, and 5% B 36–38 min. The flow rate was set to 0.8 mL/min, and the volume of injection was 10 μL. The identification of phenolics was done by comparing the UV/Vis spectra and retention times of peaks with those of authentic standards. Samples were spiked with authentic standards to confirm the identification. Quantification was done by using calibration curves of authentic standards. Calibration curves were linear (r^2 0.9942 to 0.9991). Some phenolics (neochlorogenic acid, cyanidin 3-arabinoside and cyanidin 3-xyloside) were tentatively identified by using literature data [Sosnowska *et al.*, 2018], and quantified by using chlorogenic acid and cyanidin 3-glucoside calibration curves. Precision was determined by calculating the coefficient of variation of several measurements of phenolic standards (0.3 to 13.1%).

Adsorption capacity determination

The adsorption was studied at pH 1.5, 3.0, and 7.0, for 4 h (chosen due to the total duration of gastric and intestinal phase), at 37°C (the temperature of the human body and simulated digestion). The pH 3.0 and 7.0 were chosen for the adsorption study to match the pH values of simulated gastric and intestinal fluids, respectively. In addition, aronia contains high concentrations of anthocyanins whose chemical structures change depending on the pH. To cover wider pH range and different chemical structures of anthocyanins, the adsorption was also studied at pH 1.5. At such low pH, anthocyanins are considered stable and they take the form of a flavylium cation, while their structure changes at higher pH.

The reaction solution of 1.5 mL consisted of an aronia extract obtained with chemical extraction (50 μL), an aliquot of β-glucan to give a final concentration of 15 mg/L and an appropriate volume of buffer (pH 1.5, 3.0 and 7.0). Solutions were put in an incubator for 4 h at 37°C (IN 30, Memmert, Schwabach, Germany), and centrifuged at 6,739×*g* for 10 min (Eppendorf Minispin, Eppendorf, Hamburg, Germany). An aliquot of 1 mL was taken from the reaction solution, filtered through a 0.22 μm PTFE syringe filter, and analyzed *via* HPLC to determine un-adsorbed phenolics (c_e, mg/L). The adsorption was repeated for β-glucan at a concentration of 30 mg/L.

The adsorption capacity (q_e) (mg of phenolics adsorbed onto g of β-glucan) was calculated using Equation 2:

$$q_e = \frac{(c_0 - c_e)\cdot V_m}{\gamma_a \cdot V_a} \qquad (2)$$

where: c_0 is the initial phenolic concentration in the reaction solution (mg/L), c_e is the concentration of un-adsorbed phenolics (mg/L), V_m is the total volume of the reaction solution (L), γ_a is the β-glucan concentration (g/L), and V_a is the volume of added β-glucan in the reaction solution (L).

Zeta potential measurement

Aronia extract obtained after chemical extraction was diluted 1:30 (*v/v*) in buffers of pH 1.5, 3.0 and 7.0. β-Glucan was prepared in the same buffers with a final concentration of 15 and 30 mg/L. A mixture of aronia – β-glucan was prepared by mixing the aronia extract and β-glucan in the same buffers (aronia extract diluted 1:30 (*v/v*), β-glucan at 15 and 30 mg/L). All solutions were put in an incubator at 37°C for 4 h. Zeta potential was measured on a Zetasizer 2000 analyzer (Malvern, Malvern, UK).

Statistical analysis

All experiments were performed in triplicate each measured once (n=3). Analysis of variance (ANOVA) with post-hoc Tukey test was used to analyze differences between the results (Minitab LLC., State College, PA, USA). Principal component analysis was used for possible clustering of the data (Minitab LLC).

RESULTS AND DISCUSSION

Simulated digestion

Flavonols (quercetin 3-rutinoside, quercetin 3-galactoside, and quercetin 3-glucoside), anthocyanins (galactoside, glucoside, arabinoside, and xyloside of cyanidin) and phenolic acids (neochlorogenic acid and chlorogenic acid) were quantified in aronia extract (Table 1). This profile of phenolics was similar to that determined in previous studies [Denev *et al.*, 2019; Sosnowska *et al.*, 2018].

The aronia extract was subjected to the *in vitro* simulated digestion process without or with added 15 mg/L and 30 mg/L of β-glucan (Table 1). Individual phenolics were recovered in all phases of digestion significantly ($p<0.05$) below 100% (aronia before digestion) with expiation of chlorogenic acid in the oral phase with 15 mg/L β-glucan (94.1%) and the gastric phase without β-glucan (94.7%). This is similar to the simulated digestion of apples [Bouayed *et al.*, 2011; Fernández-Jalao *et al.*, 2020] or grapes [Lingua *et al.*, 2019] where the quantities of recovered phenolics were lower than the original quantities before the digestion.

Figure 1 shows the percentage of recovery of total phenolics in each step of digestion. Total phenolics were recovered in the oral digestion step in 22 and 26% without or with added 15 mg/L of β-glucan, respectively, and in 27 and 26% without or with added 30 mg/L of β-glucan, respectively. In comparison

TABLE 1. The percentage of recovered phenolics from aronia (before digestion) after the oral, gastric and intestinal phase of digestion without or with added β-glucan (15 and 30 mg/L).

Phenolic compounds	Before Digestion (%)	Oral phase (%)	Oral phase with β-glucan (%)	Gastric phase (%)	Gastric phase with β-glucan (%)	Intestinal phase (%)	Intestinal phase with β-glucan (%)
Concentration of β-glucan 15 mg/L							
Flavonols							
Quercetin 3-rutinoside	100^{a}	15.2±1.7^{e}	19.1±1.2^{e}	40.7±2.7^{b,c}	29.0±7.0^{d}	48.5±3.2^{b}	34.5±2.0^{c,d}
Quercetin 3-galactoside	100^{a}	12.5±0.5^{d}	14.0±1.1^{d}	38.0±1.8^{b,c}	31.2±3.6^{c}	45.6±5.8^{b}	38.6±2.0^{b,c}
Quercetin 3-glucoside	100^{a}	12.1±0.1^{f}	13.2±1.2^{f}	34.3±0.8^{d}	29.4±1.2^{e}	46.1±0.8^{b}	37.1±1.1^{c}
Anthocyanins							
Cyanidin 3-galactoside	100^{a}	21.2±0.7^{b}	17.1±1.2^{c}	7.6±0.4^{d}	2.7±1.1^{e}	1.0±0.0^{e}	1.0±0.0^{e}
Cyanidin 3-glucoside	100^{a}	11.2±0.4^{b}	2.1±0.1^{d}	5.3±0.1^{c,d}	6.1±3.1^{c}	11.7±0.4^{b}	11.2±0.9^{b}
Cyanidin 3-arabinoside	100^{a}	16.3±0.3^{d}	17.6±1.9^{d}	61.5±1.4^{b}	45.8±3.1^{c}	15.9±1.0^{d}	5.8±2.4^{e}
Cyanidin 3-xyloside	100^{a}	8.5±0.1^{c,d}	7.3±1.3^{d}	28.3±3.3^{b}	23.5±1.9^{b}	13.4±2.3^{c}	8.7±1.4^{c,d}
Phenolic acids							
Neochlorogenic acid	100^{a}	35.3±1.0^{e}	42.1±2.7^{d}	63.8±2.9^{b}	55.8±4.2^{c}	49.8±1.8^{c}	35.1±1.9^{e}
Chlorogenic acid	100^{a}	41.2±1.4^{d}	94.1±4.5^{a,b}	94.7±4.8^{a,b}	68.1±21.0^{c}	75.5±2.1^{b,c}	65.8±4.2^{c}
Concentration of β-glucan 30 mg/L							
Flavonols							
Quercetin 3-rutinoside	100^{a}	22.6±2.7^{d}	22.0±2.1^{d}	38.3±2.2^{c}	39.7±2.1^{c}	45.6±0.8^{b}	45.5±2.3^{b}
Quercetin 3-galactoside	100^{a}	16.0±1.4^{d}	15.0±1.6^{d}	37.5±1.1^{c}	37.7±1.2^{c}	46.7±2.3^{b}	46.0±1.4^{b}
Quercetin 3-glucoside	100^{a}	13.9±1.2^{d}	13.0±1.4^{d}	32.9±0.7^{c}	33.0±0.8^{c}	44.3±1.6^{b}	43.9±1.0^{b}
Anthocyanins							
Cyanidin 3-galactoside	100^{a}	22.0±0.7^{b}	21.9±0.4^{b}	3.8±0.8^{c}	2.9±0.1^{c}	1.5±0.0^{d}	1.5±0.0^{d}
Cyanidin 3-glucoside	100^{a}	21.0±4.6^{c}	19.8±2.9^{c}	51.0±8.9^{b}	50.3±10.4^{b}	16.4±2.0^{c}	14.4±2.6^{c}
Cyanidin 3-arabinoside	100^{a}	20.2±1.4^{c}	18.9±2.1^{c}	53.2±1.8^{b}	52.8±2.0^{b}	6.9±1.5^{d}	5.2±0.2^{d}
Cyanidin 3-xyloside	100^{a}	17.3±1.7^{d}	15.0±1.6^{d,e}	46.1±0.8^{b}	40.3±2.6^{c}	12.5±1.3^{e}	11.0±0.2^{e}
Phenolic acids							
Neochlorogenic acid	100^{a}	48.0±1.4^{c}	46.7±1.5^{c}	65.2±1.8^{b}	64.9±1.9^{b}	49.0±2.8^{c}	46.1±1.4^{c}
Chlorogenic acid	100^{a}	41.9±1.3^{c,d}	41.4±1.2^{d,c}	60.3±1.0^{b}	60.0±0.8^{b}	46.1±2.1^{c}	42.2±2.9^{c,d}

The values in rows with different letters are significantly different according to the post-hoc Tukey test ($p<0.05$). The data (mean ± standard deviation) was obtained in three independent experiments, each measured once ($n=3$).

to the oral digestion, the recovery in the gastric digestion phase increased significantly ($p<0.05$) to 44 and 33% without or with added 15 mg/L of β-glucan, respectively, and to 42 and 41% without or with added 30 mg/L of β-glucan, respectively. After the gastric phase, the recovery decreased significantly ($p<0.05$) in the intestinal phase to 24 and 17% without or with added 15 mg/L of β-glucan, respectively, and to 21 and 19 % without or with added 30 mg/L of β-glucan, respectively. In comparison to the total phenolic recovery in the oral phase, the total phenolic recovery in the gastric phase increased (Figure 1). This increase might be caused by the longer gastric phase, which lasted 2 h, in comparison to the oral phase, which lasted only 30 s. However, phenolics might also biodegrade/hydrolyze as they are sensitive to the alkaline environment of the small intestine, which can cause their recovery in the small intestine to decrease. The degradation of phenolics into products with different chemical structures can be suggested, as was mentioned in earlier studies [Bermúdez-Soto *et al.*, 2007; Bouayed *et al.*, 2011, 2012; Fernández-Jalao *et al.*, 2020]. In addition, the percentage recoveries are similar to other studies [Bouayed *et al.*, 2011; Lingua *et al.*, 2019; Solari-Godiño *et al.*, 2017]. The recoveries of total phenolics from grapes (34, 37, 13% after oral, gastric and intestinal phases, respectively) [Lingua *et al.*, 2019] or anchovy mince with added phenolics (30 to 54% after intestinal digestion) [Solari-Godiño *et al.*, 2017] are comparable to our study results. High percentages of phenolics have been also recovered from apples, *i.e.*, approx. 65 and 75% after gastric and intestinal phases, respectively [Bouayed *et al.*, 2011].

Flavonols

The recovery of total flavonols in the oral phase of digestion was 14 and 16% in the case of the digestion conducted without or with added 15 mg/L of β-glucan, respectively and 19 and 18 % without or with added 30 mg/L of β-glucan, respectively (Figure 1). In comparison to the oral phase, the recovery increased significantly ($p<0.05$) after the gastric phase to 38 and 29% without or with added 15 mg/L of β-glucan, respectively, and to 36 and 37% without or with added 30 mg/L of β-glucan, respectively. Further increase was observed after the intestinal phase to 47 and 36% without or with added 15 mg/L of β-glucan, respectively, and to 45 and 45% without or with added 30 mg/L of β-glucan, respectively. The percentage recovery of individual flavonols also increased through oral, gastric, and intestinal digestion, which was significant in most cases (Table 1). A lower pH and a longer period of digestion over the gastric phase in comparison to the oral phase, might have affected the increased quantity of flavonols. The increase in the intestinal phase might be the result of further release of flavonols entrapped in the matrix, as suggested for flavonols from jaboticaba fruit peel [Quatrin *et al.*, 2020]. The continuous increase of flavonols through three phases of digestion also suggests their stability in the conditions of the digestive tract, which agrees with the study of Bouayed *et al.* [2012]. However, some studies reported the decrease of flavonols after the digestion of chokeberry juice [Bermúdez-Soto *et al.*, 2007] or apples [Fernández-Jalao *et al.*, 2020]. Furthermore, a de-glycosylation of quercetin glycosides was suggested [Fernández

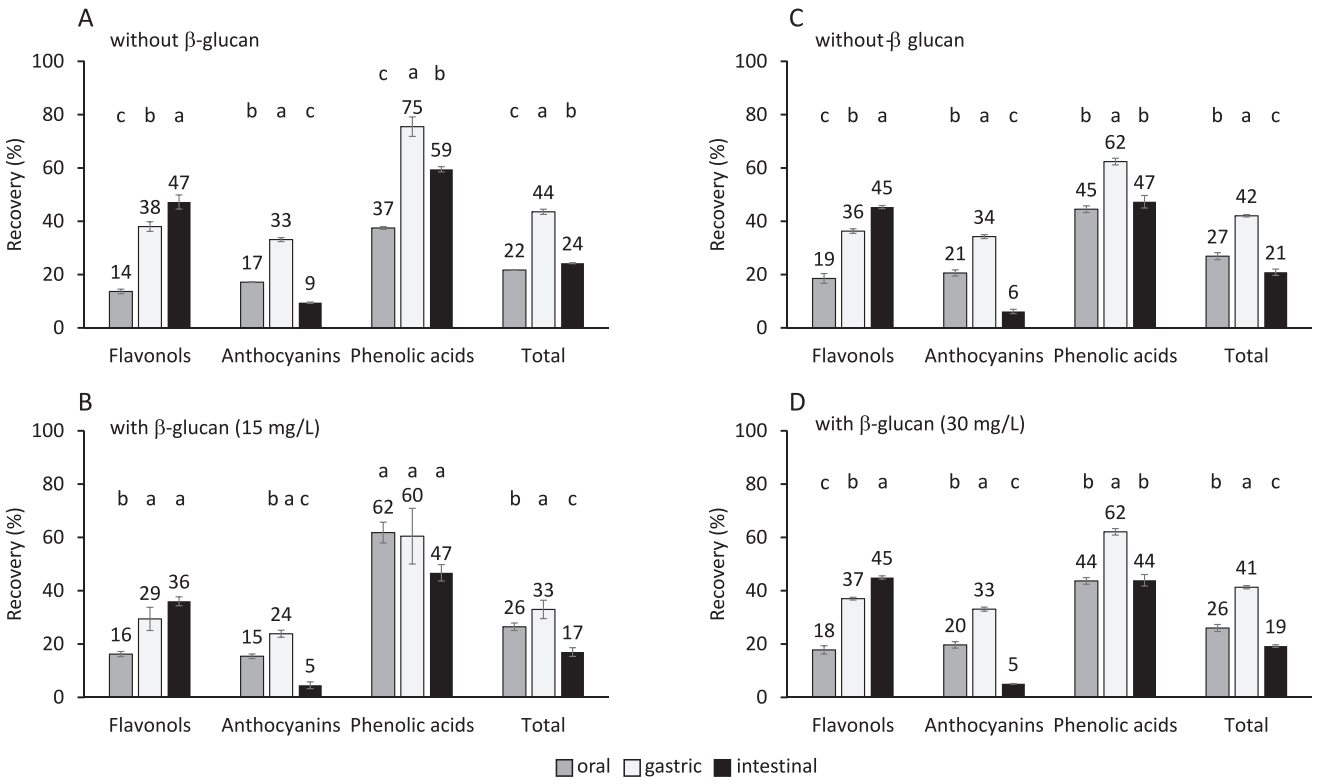

FIGURE 1. The percentage of recovered phenolics of individual subclasses and total phenolics from aronia after the oral, gastric, and intestinal phase of digestion A) without added β-glucan (part of the experiment with data on graph B), B) with added β-glucan at concentration of 15 mg/L, C) without added β-glucan (part of the experiment with data on graph D), and D) with added β-glucan at concentration of 30 mg/L. The significant differences between recovery in the oral, gastric, and intestinal phase according to post-hoc Tukey test ($p<0.05$) are marked by different letters above bars. The data (mean and standard deviation) was obtained in three independent experiments, each measured once ($n=3$).

Jalao *et al.*, 2020] since quercetin was found after digestion. Our study supports the resistance of flavonols to hydrolysis/degradation in the digestive tract. Recoveries of total flavonols in this study were lower than the recoveries after the digestion of apples (84, 84 and 62% after oral, gastric and intestinal digestion, respectively) [Fernández-Jalao *et al.*, 2020].

Anthocyanins

Anthocyanins were released in the oral phase of digestion conducted without or with added 15 or 30 mg/L of β-glucan and they were recovered in 17 and 15% without or with added 15 mg/L of β-glucan, respectively, and 21 and 20% without or with added 30 mg/L of β-glucan, respectively (Figure 1). The recovery increased significantly ($p<0.05$) in the gastric phase to 33 and 24% without or with added 15 mg/L of β-glucan, respectively, and to 34 and 33% without or with added 30 mg/L of β-glucan, respectively. Finally, anthocyanin release decreased significantly ($p<0.05$) after the intestinal phase (recovery decreased to 9 and 5% without or with added 15 mg/L of β-glucan, respectively, and to 6 and 5%, without or with added 30 mg/L of β-glucan, respectively). Most of the individual anthocyanins showed the same trend as total anthocyanins, the release increased from the oral phase to the end of the gastric phase, and decreased at the end of the intestinal phase (Table 1). The increase of recovery in the gastric phase can be explained by a longer digestion time. However, anthocyanins are also sensitive to pH change. At lower pH, such as the pH in the human stomach, anthocyanins can be released in the form of a flavylium cation, which explains the high quantities after the gastric phase. In the intestine, at higher pH, the anthocyanin chemical structure changes to quinonoidal anions, which will be explained further in the text. This might be the reason for the low quantities at the end of the intestinal phase. A similar decrease of anthocyanins in the intestinal phase has been shown after the digestion of apples [Bouayed *et al.*, 2011], chokeberry juice [Bermúdez-Soto *et al.*, 2007; Yu *et al.*, 2021], polyphenol-rich juice [Stübler *et al.*, 2020] or jaboticaba fruit peel [Quatrin *et al.*, 2020].

Phenolic acids

Phenolic acids showed similar behavior as anthocyanins. After the oral phase conducted without or with added β-glucan where phenolic acids were recovered in 37 and 62% without or with added 15 mg/L of β-glucan, respectively, and 45 and 44% without or with added 30 mg/L of β-glucan, respectively, the recovery significantly increased in the gastric phase in most cases to 75 and 60% without or with added 15 mg/L of β-glucan, respectively, and to 62 and 62% without or with added 30 mg/L of β-glucan, respectively, and then significantly decreased in the intestinal phase in most cases to 59 and 47% without or with added 15 mg/L of β-glucan, respectively, and to 47 and 44% without or with added 30 mg/L of β-glucan, respectively (Figure 1). At low pH, phenolic acids exist mostly in protonated, non-ionic forms. The low pH of the gastric phase favors non-ionic forms of phenolic acids and could have affected the quantities of the recovered total phenolic acids to increase in the gastric phase. At high pH, such as the pH of the intestine, phenolic acids dissociate, and the ratio of dissociated to non-dissociated phenolic acids becomes high. Phenolic acids in the non-ionic form at the end of the intestinal phase could be lower than in the oral or gastric phase, which can explain their decrease in the intestinal phase. Previous studies showed various trends [Bermúdez-Soto *et al.*, 2007; Fernández-Jalao *et al.*, 2020]. Total phenolic acids were stable in the gastric and intestinal digestion of chokeberry [Bermúdez-Soto *et al.*, 2007]; however, one study reported their decrease after the digestion of apples [Fernández-Jalao *et al.*, 2020]. Total hydroxycinnamic acids from apples recovered 63, 53 and 40% after oral, gastric and intestinal digestion, respectively [Fernández-Jalao *et al.*, 2020] or 84–109% and 32–57% after gastric and intestinal digestion [Bouayed *et al.*, 2012]. This is comparable to the results of this study.

Zeta potential

The zeta potential was determined to show the surface charge density of the aronia extract, β-glucan, and a mixture of aronia extract – β-glucan, which helps understand the behavior of phenolics and β-glucan during simulated digestion, and the bonding between them at different pH values. For the aronia extract, it changed from the net positive charge at pH 1.5 (30.1 mV) to the net negative charge at pH 3.0 (–14.5 mV) and pH 7.0 (–15.9 mV) (Table 2). Phenolics present in aronia can contribute to the charge of the aronia extract. At pH 1.5, anthocyanins exist predominantly in the positively-charged flavylium cation form, their structure transforms and at pH 3.0 hemiacetal and chalcone forms exist together with flavylium cations. At pH 7.0, anthocyanins are mostly transformed to negatively-charged quinonoidal anions. Furthermore, phenolic acids exist as non-dissociated forms at pH 1.5. At higher pH, dissociation takes place and the dissociated forms are negatively charged. It is suggested that the aronia extract had a net positive charge at pH 1.5 due to the positively-charged flavylium cations of anthocyanins. At pH 3.0, dissociated forms of phenolic acids might contribute to the net negative charge, and at pH 7.0, dissociated forms of phenolic acids and quinonoidal anions of anthocyanins might contribute to the negative charge of the aronia extract. The net charge of aronia extract confirmed the changes in the structures of anthocyanins and phenolic acids suggested for the intestinal digestion at pH 7.0, which caused the decreased recoveries. But those suggestions need to be confirmed in additional studies. The charge of β-glucan also changed

TABLE 2. Zeta potential (mV) of the aronia extract, β-glucan, and a mixture of aronia extract and β-glucan at different pH values.

Sample	pH 1.5	pH 3.0	pH 7.0
Aronia extract	30.1±25.6	–14.5±0.1	–15.9±1.3
β-Glucan 15 mg/L	8.5±12.9	1.6±0.2	–5.3±1.6
β-Glucan 30 mg/L	3.2±1.5	1.3±0.6	–2.1±0.2
Aronia – β-glucan mixture 15 mg/L	9.6±2.5	–1.5±1.0	–2.0±1.2
Aronia – β-glucan mixture 30 mg/L	6.5±4.3	–2.2±2.6	–1.1±0.7

The results are shown as mean ± standard deviation of three measurements.

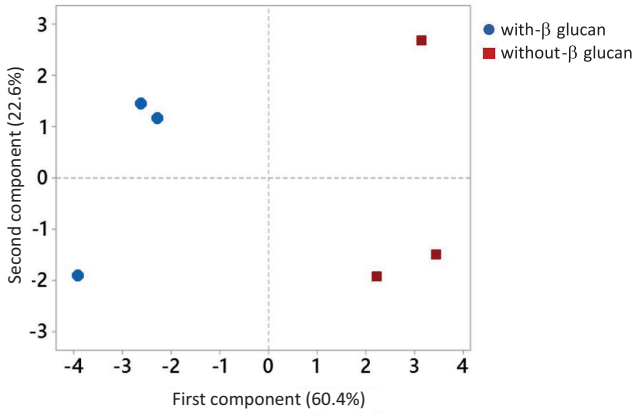

FIGURE 2. The principal component analysis of phenolics of individual subclasses (flavonols, anthocyanins, and phenolic acids, mg/kg) recovered in the oral, gastric, and intestinal phase of digestion conducted without or with β-glucan (15 and 30 mg/L).

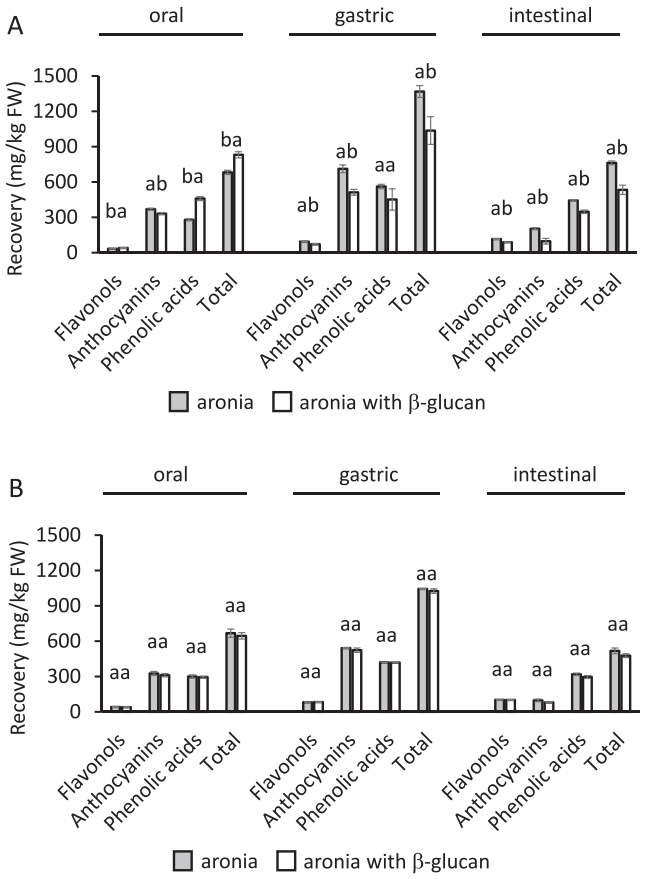

FIGURE 3. The quantity of recovered phenolics of individual subclasses from aronia (mg/kg fresh weight, FW) in each digestion step A) without and with 15 mg/L of β-glucan, B) without and with 30 mg/L of β-glucan. The significant differences between quantities with or without β-glucan according to post-hoc Tukey test ($p<0.05$) are marked by different letters above bars. The data (mean and standard deviation) was obtained in three independent experiments, each measured once ($n=3$).

from the net positive charge at pH 1.5 (8.5 and 3.2 mV for 15 and 30 mg/L, respectively) and pH 3.0 (1.6 and 1.3 mV for 15 and 30 mg/L, respectively) to the net negative charge at pH 7.0 (–5.3 and –2.1 mV for 15 and 30 mg/L, respectively) (Table 2). These values are still showing a system that was unstable with not enough charge to prevent repulsion between molecules, which allowed the aggregation of β-glucan particles. Considering the surface charge density of aronia phenolics and β-glucan, reaction between them could take place. At pH 3.0, which is the pH of the gastric simulated digestion, reaction could take place due to hydrogen bonds between OH groups of anthocyanins, phenolic acids, flavonols, and β-glucan, as suggested by Gao *et al.* [2012], but a positive charge of β-glucan and a negative charge of aronia phenolics could contribute to the bonding due to electrostatic forces. At pH 7.0, which is the pH of the simulated intestinal digestion, β-glucan and aronia phenolics both showed a negative charge, which could cause the repulsion forces and lower the possibility of further reactions. The mixture of the aronia extract and β-glucan showed a net positive charge at pH 1.5 (9.6 and 6.5 mV for 15 and 30 mg/L, respectively), and net negative charges at pH 3.0 (–1.5 and –2.2 mV for 15 and 30 mg/L) and pH 7.0 (–2.0 and –1.1 mV for 15 and 30 mg/L, respectively) (Table 2). The negative charge at pH 3.0 and 7.0 could be a result of negatively-charged phenolics and β-glucan.

Digestion with or without β-glucan

Principal component analysis (PCA) on the recovery of phenolics of individual subclasses in each digestion step was conducted to establish the difference between the release of phenolics in the digestion without or with β-glucan (Figure 2). The PCA showed the separation based on β-glucan present or absent in the digestion, which suggests that β-glucan affects the release of phenolics. The first principal component separated phenolics according to the β-glucan presence in the digestion and represented 60.4% of the data variability.

Furthermore, the quantities of recovered phenolics of individual subclasses and total phenolics were compared according to the presence or absence of β-glucan during digestion (Figure 3). Significantly ($p<0.05$) lower recoveries of the phenolics of individual subclasses and total phenolics were determined in the gastric and intestinal phases of digestion conducted with the lower concentration of β-glucan, *i.e.*, 15 mg/L, compared to process without β-glucan (Figure 3A). On the other hand, there were no significant differences ($p\geq0.05$) in the recovery of phenolics in the digestion of aronia without β-glucan and with β-glucan at a concentration of 30 mg/L (Figure 3B). This suggests that β-glucan in the low concentration had a stronger effect in entrapping phenolics. Greater contact area between β-glucan and phenolics when β-glucan was present in the lower concentration (15 mg/L) could be the reason for greater entrapment of phenolics.

Lower concentrations of phenolics released in the digestion conducted with β-glucan, even though not always statistically significant, suggest there were interactions between phenolics and β-glucan. Interactions resulted in the entrapment of phenolics in the structure of β-glucan, and in the lower concentrations of free phenolics. Studies have shown that phenolics could bond to β-glucans [Gao *et al.*, 2012], which can result in their low concentrations [Larsen *et al.*, 2019; Tomas *et al.*, 2020]. After the simulated digestion of blackberry phenolics, inulin, and pectin acted as an entrapping matrix

TABLE 3. Adsorption capacity of β-glucan for aronia phenolics (q_e, mg/g of β-glucan), at different pH values and different β-glucan concentrations.

Phenolic compounds	pH 1.5	pH 3.0	pH 7.0
Concentration of β-glucan 15 mg/L			
Flavonols			
Quercetin 3-rutinoside	7.5±1.1[a]	7.5±1.1[a]	7.4±1.1[a]
Quercetin 3-galactoside	2.3±0.2[a]	2.3±0.2[a]	2.3±0.2[a]
Quercetin 3-glucoside	4.0±0.3[a]	4.0±0.3[a]	4.0±0.3[a]
Anthocyanins			
Cyanidin 3-galactoside	95.6±2.8[a]	94.8±2.5[a]	98.2±2.7[a]
Cyanidin 3-glucoside	14.6±3.3[a]	14.5±3.6[a]	15.0±3.3[a]
Cyanidin 3-arabinoside	122.0±3.3[a]	121.4±3.0[a]	124.7±4.2[a]
Cyanidin 3-xyloside	20.3±0.2[a]	20.2±0.2[a]	20.7±0.2[a]
Phenolic acids			
Neochlorogenic acid	30.9±1.3[a]	30.9±1.3[a]	31.2±1.4[a]
Chlorogenic acid	47.6±0.8[a]	47.5±0.8[a]	47.9±0.8[a]
Concentration of β-glucan 30 mg/L			
Flavonols			
Quercetin 3-rutinoside	3.7±0.5[a]	3.7±0.6[a]	3.7±0.6[a]
Quercetin 3-galactoside	1.2±0.1[a]	1.2±0.1[a]	1.2±0.1[a]
Quercetin 3-glucoside	2.0±0.2[a]	2.0±0.1[a]	2.0±0.2[a]
Anthocyanins			
Cyanidin 3-galactoside	47.8±1.4[a]	47.7±1.4[a]	49.2±1.3[a]
Cyanidin 3-glucoside	7.3±1.7[a]	7.3±1.7[a]	7.5±1.7[a]
Cyanidin 3-arabinoside	60.9±2.2[a]	60.9±2.1[a]	62.4±2.1[a]
Cyanidin 3-xyloside	10.1±0.1[a]	10.1±0.1[a]	10.4±0.1[a]
Phenolic acids			
Neochlorogenic acid	15.4±0.7[a]	15.5±0.7[a]	16.6±0.7[a]
Chlorogenic acid	23.8±0.4[a]	23.8±0.4[a]	24.0±0.4[a]

Different letters in a row correspond to significant differences at different pH values according to post-hoc Tukey test ($p<0.05$). The data (mean ± standard deviation) was obtained in three independent experiments, each measured once ($n=3$).

and lowered the concentration of flavonoids at the end of intestinal digestion [Tomas *et al.*, 2020]. Larsen *et al.* [2019] also suggested that anthocyanins can form complexes with original and modified pectins, which lowers the concentration of free anthocyanins after short- and long-term incubation.

Enhanced entrapment of phenolics by dietary fibers in the small intestine could result in higher quantities of phenolics carried by fibers to the lower parts of the digestive tract where dietary fibers ferment and phenolics can be released [Solari-Godiño *et al.*, 2017]. In the large intestine, phenolics reveal potentially positive activities, like activities against colon cancer [Wu *et al*., 2021]. But according to the results of this investigation, the quantity of dietary fibers could be an important factor in the beneficial effects since only β-glucan in the lower concentration showed statistically significant interactions with phenolics.

Adsorption

Comparing the adsorption with low (15 mg/L) and high (30 mg/L) β-glucan concentration, β-glucan adsorbed more phenolics per gram when its concentration was low (15 mg/L) (Table 3). This agrees with the simulated digestion process in which lower quantities of β-glucan resulted in its interaction with phenolics, which lead to statistically significant lower quantities of the recovered phenolics at the end of the digestion process (Figure 3). At a low β-glucan concentration, there might be more chances of interactions between β-glucan and phenolics and larger surface area to which phenolics can adsorb, which leads to more phenolics being adsorbed per gram of β-glucan. At high β-glucan concentrations, there might be more interactions between β-glucan molecules, which might prevent the adsorption of phenolics. Furthermore, the viscosity of β-glucan solutions in high concentrations might prevent phenolic molecules to adsorb.

CONCLUSIONS

This study reports for the first-time the results on the simulated digestion of aronia phenolics with β-glucan. Aronia phenolics were recovered in the digestion process in a lower concentration than in the fruit before the digestion, indicating that some of the phenolics degrade during digestion. Different phases of digestion affected the release of total phenolics. In the oral and gastric phases, the recovery of total phenolics was gradual, and increased from oral to the end of the gastric phase. However, in the intestinal phase, the recovery of total phenolics decreased. Furthermore, the release of phenolics of individual subclasses was different in the three studied digestion phases. Flavonol recoveries increased from oral to gastric and intestinal phases, which might mean a high stability in the digestive tract. After the increase of total anthocyanins and phenolic acids in the gastric phase, the recoveries decreased after the intestinal phase. It can be suggested that the changes of chemical structures of phenolic acids and anthocyanins in the stomach and small intestine, could have affected their recovery, but this is not verified and still needs to be addressed in additional studies. β-Glucan did affect the release of phenolics. The presence of β-glucan (15 mg/L) significantly decreased the quantities of released phenolics at the end of the gastric and intestinal phases. However, with 30 mg/L of β-glucan, a similar but non-significant decrease was determined after oral, gastric, and intestinal digestion. The decrease could be explained by the interactions between β-glucan and phenolics, their entrapment by β-glucan, which finally caused their decreased release in the intestine. The concentration of β-glucan might be a significant factor in the entrapment of phenolics as its lower amounts seem to entrap significant amounts of phenolics.

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CONFLICT OF INTEREST

Authors declare no potential conflicts of interests

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Original article
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Antioxidant Capacity of Lentil Flour Hydrolysates Obtained with Pancreatin

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Key words: lentil proteins, degree of hydrolysis, radical scavenging activity, reducing power, ferrous ion chelating activity

Lentil is a valuable protein-rich crop, the health-promoting value of which can be further enhanced by its protein hydrolysis. In the present study, lentil flour was treated with pancreatin and the antioxidant capacity of the obtained hydrolysates with different degrees of hydrolysis (DH, 4–20%) was determined and compared with that of flour. The molecular weight (MW) distribution of the lentil proteins and their products of hydrolysis was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Size exclusion-high performance liquid chromatography (SE-HPLC) was deployed to show the profile of low MW compounds of the hydrolysates. Additionally, total phenolic contents were determined with the Folin-Ciocalteu's phenol reagent. The hydrolysates had higher antiradical activity against DPPH$^{\bullet}$, Trolox equivalent antioxidant capacity (TEAC) measured by the assay with ABTS$^{\bullet+}$, ferric reducing antioxidant power (FRAP), and ability to bind Fe^{2+} compared to lentil flour. Between the hydrolysates, the highest DPPH$^{\bullet}$ scavenging activity (EC_{50} of 0.298 mg/mL), TEAC (98.6 μmol Trolox/g), FRAP (109.2 μmol Fe^{2+}/g), antioxidant capacity of lipid-soluble compounds (ACL) determined by photoluminescence method (4.32 μmol Trolox/g), and Fe^{2+} chelating activity (80% at hydrolysate concentration of 0.3 mg/mL) were found for those with low DH (4–8%), which contained some subunits of proteins, polypeptides, and peptides with a wide MW range (0.556–66.0 kDa). The total phenolic content increased with increasing DH. In conclusion, the antioxidant capacity of lentil flour can be significantly improved by the limited hydrolysis of its proteins with pancreatin, as a result of the release of polypeptides and peptides with a wide range of MW. Thus modified lentil flour may be addressed and explored in future research as a functional food ingredient.

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is a valuable legume crop known worldwide and mainly used as a protein-rich component of the human diet. Crude protein content of lentil seed flour falls within the range of 25–30% [Barbana & Boye, 2013; Liu *et al*., 2020]. The two predominant fractions of lentil storage proteins, albumin and globulin, account for approximately 11% and 40% of the total protein content, respectively [Neves & Lourenço, 1995]. The share of glutelins in the protein pool is also significant [Osemwota *et al*., 2022]. All the essential amino acids were determined in the amino acid profile of the lentil proteins [Liu *et al*., 2022; Osemwota *et al*., 2022]. Their content was balanced well and only the contents of tryptophan and sulfur-containing amino acids (methionine and cysteine) were slightly lower than Food and Agriculture Organization/World Health Organization (FAO/WHO) requirement patterns.

In addition to a high nutritional quality, the enhancement of the health-promoting potential of lentil proteins by enzymatic hydrolysis thereof may be considered. The proteases used for hydrolysis can release peptides with different biological activities. In the case of lentil proteins treated with enzymes, several studies have shown the angiotensin I converting enzyme (ACE) inhibitory activity of hydrolysates [Barbana & Boye, 2011; Boye *et al*., 2010; Garcia-Mora *et al*., 2014; Rezvankhah *et al*., 2021a, b]. Moreover, this type of treatment allowed producing lentil protein hydrolysates or their fractions with anti-adipogenic potential, antiproliferative activity, and protective ability against protein glycation [Kuerban *et al*., 2020; Moreno *et al*., 2020]. Additionally, enzymatic hydrolysis by the sequential treatment with Alcalase and Flavourzyme decreased the immunoreactivity of lentil proteins [Cabanillas *et al*., 2010]. The radical scavenging activity of hydrolysates obtained with individual enzymes of microbial (Alcalase, Flavourzyme, Savinase, Protamex, and Corolase 7089) and animal (trypsin) origin or a combination of enzymes (Alcalase/Flavourzyme, pepsin/pancreatin) has also been reported [Garcia-Mora *et al*., 2014; Kuerban *et al*., 2020; Moreno *et al*., 2020; Rezvankhah

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et al., 2021a, b]. Although amino acid sequences of few multifunctional peptides with ACE inhibitory and radical scavenging activities released from lentil proteins had already been identified [García-Mora *et al*., 2017], to the best of our knowledge, hydrolysis of lentil proteins with pancreatin alone to release antioxidant peptides has not been studied so far. In turn, previous studies have shown that this enzyme was equally effective as Alcalase in hydrolyzing and enhancing antioxidant activity of plant seed proteins, including pea, flaxseed, fenugreek seeds, and more effective than many other proteases, such as Flavourzyme, pepsin, trypsin, chymotrypsin, and papain [Asen & Aluko, 2022; Karamać *et al*., 2016; Kaveh *et al*., 2022].

Most of the studies mentioned above have involved the hydrolysis of lentil protein isolates or concentrates, while protease treatment of lentil flour to release bioactive peptides has been less frequently explored. Lentil flour contains phenolic compounds, including phenolic acids, flavonoids and condensed tannins [Amarowicz *et al*., 2010; Paranavitana *et al*., 2021; Zhang *et al*., 2015]. Due to interactions with proteins, these compounds, especially tannins, may disturb hydrolysis. Recently, Boachie *et al*. [2022] have reported that the interaction of lentil proteins with tannic acid diminished the susceptibility of proteins to pepsin hydrolysis, as the enzyme had a limited access to the protein structure, which resulted in a modification of the profile of released peptides. On the other hand, phenolic compounds – as powerful antioxidants – contributed to antioxidant capacity of lentil flour subjected to simulated *in vitro* gastrointestinal proteolysis [Zhang & Chang, 2019]. Until now, lentil flour has been directly treated by Savinase under mild acidic and alkaline conditions, and the hydrolysates produced in both cases had higher oxygen radical absorbance capacity (ORAC) and better inhibited generation of reactive oxygen species in the murine macrophage cell line RAW 264.7 compared to lentil flour [Bautista-Expósito *et al*., 2018a, b]. Nevertheless, knowledge of the susceptibility of lentil flour as a whole to treatment with individual proteases and the changes in its antioxidant capacity resulting from such treatment is limited.

Therefore, the aim of our research was to treat lentil flour with pancreatin to obtain hydrolysates with different degrees of protein hydrolysis and to determine the antioxidant capacity of these hydrolysates compared to flour. Since the antioxidant activity of lentil protein hydrolysates has been studied so far mainly as radical scavenging activity, in addition to antiradical activity, also the reducing power and Fe^{2+} chelating activity were determined in our research.

MATERIALS AND METHODS

Plant material

Lentil seeds (*Lens culinaris*) of Tina cultivar were purchased at the Plant Breeding Station "Spójnia" (Śrem, Poland). Seeds were ground using a laboratory mill and sieved (mesh size 0.4 mm). The AOAC International approved methods were applied to evaluate the proximate composition of lentil flour: crude protein ($N\times6.25$), starch, lipids, ash, and moisture [AOAC, 1990], which amounted to 27.5±0.16, 48.3±0.71, 0.91±0.01, 3.68±0.12, and 5.91±0.14 g/100 g ($n=3$), respectively.

Reagents

Pancreatin from porcine pancreas (8×USP specifications), L-leucine, acrylamide, *N*,*N*'-methylene-bis-acrylamide, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), glycine, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) marker (SigmaMarker low range), size exclusion-high performance liquid chromatography (SE-HPLC) solvents and standards (bovine serum albumin (cat.-no. A8531), cytochrome C (cat.-no. C7150), bovine lung aprotinin (cat.-no. A3886), bovine insulin chain B oxidized (cat.-no. I6383), human angiotensin II (cat.-no. A9525), leucine encephalin (cat.-no. L9133), and Thr-Tyr-Ser (cat.-no. T0148)), Folin-Ciocalteu's phenol reagent (FCR), gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ferrozine, and 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). 2,4,6-Trinitrobenzene sulphonic acid and Coomassie brilliant blue R-250 were purchased from Serva (Heidelberg, Germany). The antioxidant capacity of lipid-soluble substances (ACL) and antioxidant capacity of water-soluble compounds (ACW) kits for the photochemiluminescence method were obtained from Analytik Jena (Jena, Germany). Ferrous chloride tetrahydrate ($FeCl_2\times4H_2O$), ferric chloride hexahydrate ($FeCl_3\times6H_2O$) and other reagents, all of analytical grade, were acquired from Avantor (Gliwice, Poland).

Enzymatic hydrolysis conditions and degree of hydrolysis determination

Lentil protein hydrolysis was carried out in a thermostatic vessel placed in an ETS 822 end-point titration system working in the pH-stat mode (Radiometer Analytical A/S, Copenhagen, Denmark). Lentil flour (3 g) was suspended in 30 mL of distilled water. The temperature was set at 50°C and the pH was adjusted to 8.0 using 0.2 M NaOH. Pancreatin was added at a ratio of 50 mAU/g of protein; its activity evaluated by Anson method was 0.74 AU/g. A stable pH 8.0 was maintained during hydrolysis by titration with 0.2 M NaOH. Subsequently, the curve of hydrolysis was plotted as a degree of hydrolysis (DH) *vs*. time.

The DH (%) was calculated according to Equation (1) [Adler-Nissen, 1986].

$$DH = \frac{B\times M_B}{\alpha\times P\times h_{tot}}\times 100 \quad (1)$$

where: B – volume of base used for titration (mL), M_B – molarity of the base (M), α – average degree of the α–NH_2 groups dissociation, P – weight of protein (g), and h_{tot} – total number of peptide bonds in the lentil flour proteins (meqv Leu-NH_2/g protein).

The h_{tot} was determined after acidic hydrolysis of lentil flour proteins in 6 M HCl at 105°C using the spectrophotometric

method with 2,4,6-trinitrobenzene sulphonic acid (TNBS) [Panasiuk *et al*., 1998].

Subsequently, the hydrolysates with designed value of DH: 4%, 8%, 12%, 16%, and 20%, were produced. The reaction was stopped by heating at 90°C for 5 min to inactivate the enzyme. The preparations were cooled down, neutralized with 0.2 M HCl, and lyophilized for ~48 h at –70°C and 0.013 mbar (Lyph Lock 6 freeze dry system, Labconco, Kansas City, MO, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of lentil flour proteins and their hydrolysates obtained with pancreatin was performed in 15% gel in the Laemmli's buffer system [Hames, 1990]. Proteins were denaturized by heating at 100°C for 5 min. The separation was carried out under reducing conditions (samples were dissolved in Tris-HCl buffer, pH 6.8, with 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) using a mini-PROTEAN tetra cell system (BioRad, Hercules, CA, USA) at amperage of 25 A. Sigma-Aldrich marker with molecular weights (MW) in the range of 6.5–66.0 kDa was used as a standard. Resolved proteins and polypeptides in gel were stained with Coomassie brilliant blue R-250. Their molecular weights were estimated by the Quantity One software (BioRad).

Size exclusion-high performance liquid chromatography (SE-HPLC)

The MW distribution of lentil flour proteins and products of their enzymatic hydrolysis was analyzed by SE-HPLC using a Shimadzu HPLC system (Kyoto, Japan) consisting of an LC-10AD$_{Vp}$ pump, an SPD-M10A$_{Vp}$ photo-diode array detector, and an SCL-10A$_{Vp}$ system controller. The samples (100 μL) were injected into a TSKgel G2000SW$_{XL}$ column (7.86×300 mm, 5 μm, Tosoh Bioscience, Tokyo, Japan). The mobile phase of 45% (*v/v*) acetonitrile with 0.1% (*v/v*) trifluoracetic acid was delivered at a flow rate of 0.2 mL/min. The detector was set at 220 nm. Bovine serum albumin (66.0 kDa), cytochrome C (12.4 kDa), bovine lung aprotinin (6.5 kDa), bovine insulin chain B oxidized (3.496 kDa), human angiotensin II (1.046 kDa), leucine enkephalin (0.556 kDa), and Thr-Tyr-Ser (0.369 kDa) were used as MW standards.

Total phenolic content determination

The total phenolic content (TPC) of lentil flour and hydrolysates was determined after their (50 mg) extraction with two portions of 80% (*v/v*) methanol (5 mL) at 65°C for 30 min [Karamać *et al*., 2020]. Filtrates from both extraction steps were pooled together and their volume was adjusted to 10 mL with extractant. An aliquot of 0.25 mL of the extract was vortexed with 0.25 mL of FCR, 0.5 mL of sodium carbonate saturated solution, and 4 mL of water. The reaction was carried out for 30 min at ambient temperature. The absorbance was measured at λ=725 nm using a Beckman DU-7500 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) after centrifugation of the reaction mixture. Results were expressed as mg gallic acid equivalents (GAE) per g of flour or hydrolysate.

Determination of DPPH radical scavenging activity

The DPPH$^{\bullet}$ scavenging activity of lentil flour and lentil flour protein hydrolysates was determined according to the procedure described by Karamać *et al*. [2014] with slight modifications. Samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, at a concentration ranging from 0.16 to 0.80 mg/mL. An aliquot of 1 mL of the methanolic solution of DPPH$^{\bullet}$ (0.15 mM) was added to 1 mL of the solution of hydrolysates and vortexed vigorously. Control samples were prepared in parallel with methanol. The mixture was left to stand at room temperature for 20 min before absorbance at λ=517 nm was measured using a DU-7500 spectrophotometer (Beckman Instruments). The percentage of DPPH$^{\bullet}$ scavenged by hydrolysate compounds was calculated using the Equation (2):

$$DPPH^{\bullet}\ scavenging\ activity = \frac{A_0-(A_S-A_C)}{A_0} \times 100\% \quad (2)$$

where: A_0 is the absorbance of the DPPH solution, A_S is the absorbance in the presence of the sample, and A_C is the absorbance of the control. The curves of DPPH$^{\bullet}$ scavenging activity *vs.* concentration of hydrolysate (mg/mL of reaction mixture) were plotted. The EC_{50} values, defined as the hydrolysate concentration effective to scavenge 50% of DPPH$^{\bullet}$, were read from the plot.

Determination of Trolox equivalent antioxidant capacity

Trolox equivalent antioxidant capacity (TEAC) of lentil flour and its hydrolysates was evaluated using the ABTS radical cation decolorization assay. ABTS$^{\bullet+}$ stock solution prepared according to Re *et al*. [1999] procedure was diluted with methanol up to a final absorbance of 0.70±0.02 at λ=734 nm. Hydrolysates were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, at a concentration of 10 mg/mL. Solutions of hydrolysates (40 μL) were added to 2 mL of ABTS$^{\bullet+}$ and vortexed. Mixtures were incubated for 6 min at 30°C using a block heater (TH-24, Meditherm, Warsaw, Poland) and the absorbance was measured at λ=734 nm. The results were calculated using the standard curve for Trolox (0.2–2.0 μmol/mL, r=0.999) and expressed in μmol of Trolox equivalents per g of hydrolysate or flour.

Photochemiluminescence assay

The photoluminescence (PCL) method [Popov & Lewin, 1999] was employed to evaluate the radical scavenging capacity of lentil flour and hydrolysates for superoxide anion radicals ($O_2^{\bullet-}$). The scavenging capacity of the hydrolysates was determined using the Photochem system and ACL and ACW kits (Analytik Jena), for integral measurement of lipid/methanol-soluble and water-soluble compounds, respectively. Briefly, for ACL determination, 2.3 mL of methanol (reagent 1), 200 μL of buffer solution (reagent 2), and 25 μL of luminol (reagent 3) were mixed. Then, 10 μL of the methanolic solution of hydrolysate (10 mg/mL) or standard (Trolox, 50–300 μM) were added. For ACW measurement, 1.5 mL of buffer solution, pH 10.5 (reagent 1), 1 mL of water (reagent 2), 25 μL of luminol (reagent 3), and 20 μL of the water solution of hydrolysate (0.5 mg/mL) or standard (L-ascorbic

acid, 10–150 μM) were mixed. The results were expressed as μmol of Trolox (ACL) or L-ascorbic acid (ACW) equivalents per g of hydrolysate or flour.

Determination of ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) was determined according to Benzie & Strain [1996] method. Lentil flour and lentil flour hydrolysate solutions (5 mg/mL) and FRAP reagent (10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \times 6H_2O$, and 0.3 M acetate buffer, pH 3.6; 10:1:1 *v/v/v*) were prepared. Sample solution (150 μL), FRAP reagent (2.25 mL), and distilled water (150 μL) were mixed and incubated for 30 min at 37°C using a TH-24 block heater (Meditherm). Absorbance readings were taken at λ=593 nm using a Beckman DU-7500 spectrophotometer. The results were calculated using the standard curve for $FeSO_4 \times 7H_2O$ (0.18–1.80 mM, r=0.996) and expressed as μmol of Fe^{2+} per g of hydrolysate or flour.

Determination of Fe^{2+} chelating activity

The Fe^{2+} chelating activity of lentil flour and lentil flour hydrolysates was evaluated using the assay with ferrozine [Karamać & Pegg, 2009]. Samples were suspended in distilled water at a concentration ranging from 0.1 to 0.8 mg/mL. Afterwards, 2.5 mL portions of sample solutions were vortexed with 0.25 mL of 0.4 mM solution of $FeCl_2 \times 4H_2O$. Then, 0.5 mL of 5 M ferrozine was added and after 10 min quiescent period at ambient temperature the mixtures were centrifuged for 5 min at 5,000×*g* using an MPW-210 centrifuge (MPW Med. Instruments, Warsaw, Poland). The absorbance of the supernatant was measured at λ=562 nm (Beckman DU-7500 spectrophotometer). A control sample was prepared in parallel by replacing the hydrolysate solution with distilled water. The percentage of Fe^{2+} bound was calculated.

Statistical analysis

The hydrolysis and antioxidant assays were performed in triplicate. The mean value and standard deviation were calculated using Microsoft Excel 2000 software (Microsoft Office Excel for Windows, Microsoft, Redmond, USA). The results of TPC and antioxidant assays were analyzed by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, USA). The differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Kinetics of hydrolysis of lentil flour proteins with pancreatin

The kinetic curve of hydrolysis of lentil flour proteins with pancreatin was depicted in Figure 1. After 120 min of the process, the DH reached 22%. The initial phase of the process was the most rapid, then the reaction rate slowed down gradually and stabilized after 100 min of hydrolysis. The maximal DH was in the range of 20–24%, which was previously achieved for lentil protein extract or concentrate hydrolyzed by Alcalase [Cabanillas *et al*., 2010; Garcia-Mora *et al*., 2014; Rezvankhah *et al*., 2021a]. Lower values were obtained using other proteases of microbial origin, like Flavourzyme, Savinase, Protamex, and Corolase 7089 (DH 8.5–15%) [Garcia-Mora *et al*., 2014; Rezvankhah *et al*., 2021a]. The digestive tract enzymes, *i.e.* chymotrypsin and trypsin, hydrolyzed lentil flour proteins up to DH of 13% and 4–57%, respectively [Avramenko *et al*., 2013; Karamać & Rybarczyk, 2008]. In the latter case, different enzyme to substrate ratios (1/100–1/1000, respectively) were used. Higher DH values were reported for lentil proteins treated sequentially with Alcalase and Flavourzyme (47.05–64.31%) [Barbana & Boye, 2011; Cabanillas *et al*., 2010; Rezvankhah *et al*., 2021a] and digested under simulated conditions of the gastrointestinal tract with pepsin-pancreatin or pepsin-trypsin-chymotrypsin (65.5–82.4%) [Aryee & Boye, 2016]. The DH determined in our research fell within the wide range of 12–55% reported for hydrolysates of other leguminous seeds and oilseeds obtained with pancreatin [Asen & Aluko, 2022; Betancur-Ancona *et al*., 2014; Karamać *et al*., 2014; Valdez-Ortiz *et al*., 2012]. This difference may be related to the protein profile of the seeds, but also to the hydrolysis carried out under different conditions (temperature, enzyme-substrate ratio, initial substrate concentration) and the use of different methods for DH determination.

Molecular weight distribution of proteins, polypeptides, and low MW compounds of lentil flour and hydrolysates

SDS-PAGE of lentil flour (DH 0%) under reducing conditions revealed more than 20 bands corresponding to proteins and protein subunits with MW in the range of 14.5–92.0 kDa (Figure 2). Bands with the highest intensity (48.8 and 47.4 kDa) were in the MW range previously distinguished as the subunit of vicilin [Boye *et al*., 2010; Garcia-Mora *et al*., 2014; Moreno *et al*., 2020]. Convicilin subunit might be recognized in a clear band corresponding to MW of 58.9 kDa [Boye *et al*., 2010]. Bands in the MW ranges of 34.0–39.0 kDa and 20.0–23.0 kDa originated probably from acidic and basic subunits of lentil 11S globulin, respectively. Few bands in the low MW range of 14.5–18.0 kDa might originate from γ-vicilin or some albumins. Bautista-Expósito *et al*. [2018c] showed the electrophoretic pattern of a lentil protein concentrate with two bands corresponding to MW of around 90 kDa and suggested that they distinguished lipoxygenase isoforms. Probably the analogous bands

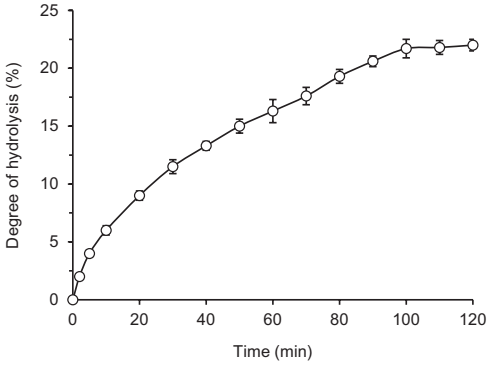

FIGURE 1. Kinetic curve of hydrolysis of lentil flour proteins with pancreatin. Error bars represent the standard deviations of the means (n=3).

in the electrophoretic separation of lentil flour were those of MW of 82.8 and 91.9 kDa (Figure 2). The SDS-PAGE pattern of lentil flour in our study corresponded well with those reported by Barbana & Boye [2011, 2013] and Aryee & Boye [2016], where bands ranging from 15 to 97 kDa were detected under reducing conditions for raw red and green lentil flours and protein concentrates.

As a result of pancreatin treatment, proteins with the highest molecular weights were degraded firstly (Figure 2). The electrophoretic pattern of the hydrolysate with DH 4% showed a lack of bands corresponding to MW higher than 65 kDa, and much weaker intensity of the bands in the range of 47.4–65.0 kDa compared to the SDS-PAGE pattern of lentil flour. An increase in DH of the hydrolysates resulted in a further decrease in the intensity or disappearance of bands corresponding to convicilin, vicilin, and acidic subunits of legumin. At the same time, bands originating from basic subunits of lentil 11S globulin did not lose their intensity completely and were apparent even in the electrophoretic pattern of the hydrolysate

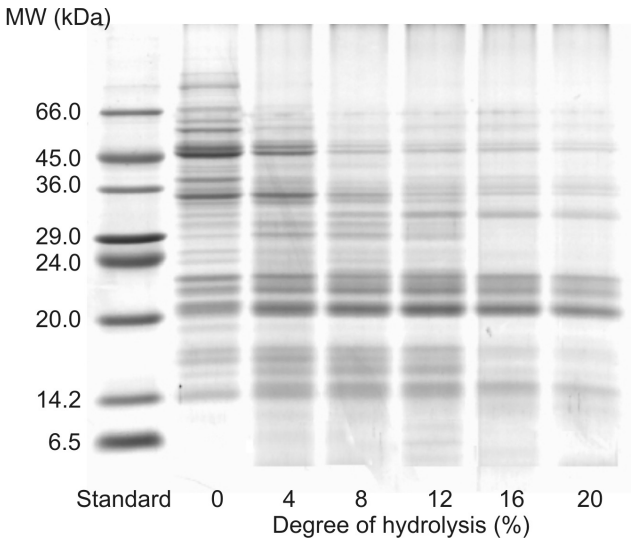

FIGURE 2. SDS-PAGE patterns of lentil flour and its hydrolysates obtained with pancreatin. MW – molecular weight.

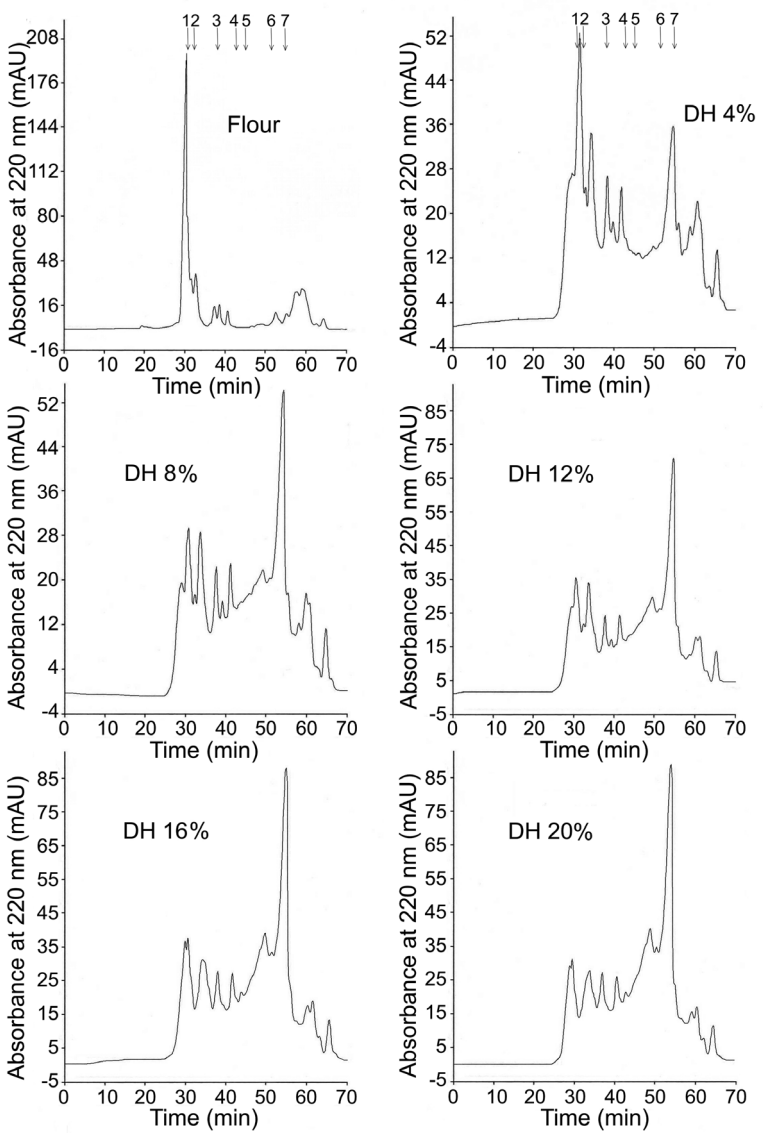

FIGURE 3. SE-HPLC chromatograms of lentil flour and its hydrolysates obtained with pancreatin. The arrows indicate the standards: 1 – bovine serum albumin (66.0 kDa) 2 – cytochrome C (12.4 kDa); 3 – bovine lung aprotinin (6.5 kDa); 4 – bovine insulin chain B oxidized (3.496 kDa); 5 – human angiotensin II (1.046 kDa); 6 – leucine enkephalin (0.556 kDa); 7 – Thr-Tyr-Ser (0.369 kDa). DH – degree of hydrolysis.

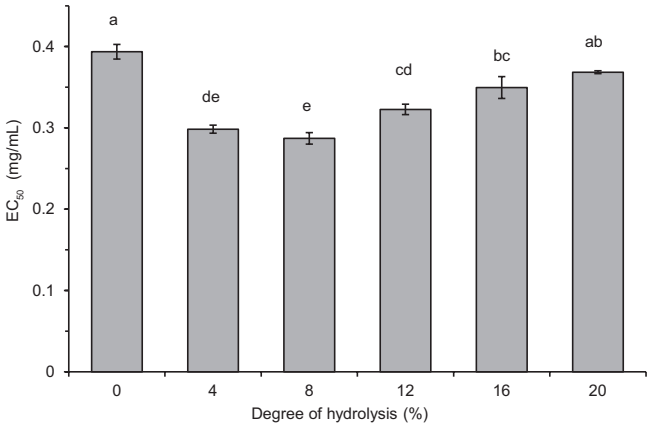

FIGURE 4. DPPH• scavenging activity of lentil flour and its hydrolysates obtained with pancreatin. Error bars represent the standard deviations of the means (n=3). Different letters above bars indicate significant differences among samples (p<0.05).

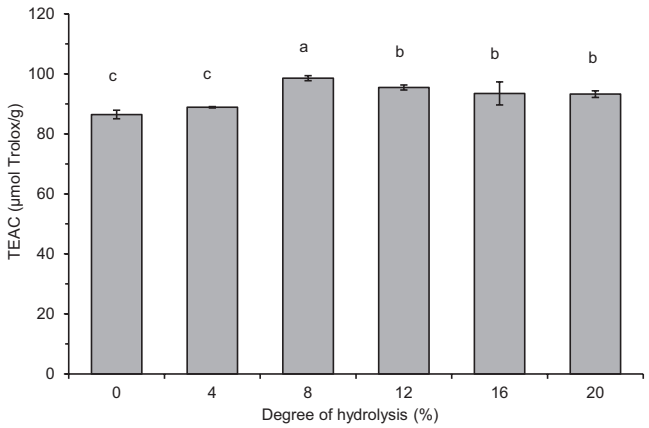

FIGURE 5. Trolox equivalent antioxidant capacity (TEAC) of lentil flour and its hydrolysates obtained with pancreatin measured by ABTS assay. Error bars represent the standard deviations of the means (n=3). Different letters above bars indicate significant differences among samples (p<0.05).

with DH 20%. Previous studies have reported the resistance of certain lentil protein subunits to hydrolysis with trypsin and chymotrypsin [Avramenko *et al*., 2013; Karamać & Rybarczyk, 2008; Neves & Lourenço, 1995]. SDS-PAGE separation of lentil globulin hydrolysates revealed that subunits with an MW below 21 kDa were not susceptible to trypsinolysis and those with the MW of 32–34 kDa and below 21 kDa were resistant to chymotrypsinolysis [Neves & Lourenço, 1995]. Similarly, our previous study [Karamać & Rybarczyk, 2008] showed that some lentil protein subunit bands were present in the electrophoretic pattern of a lentil flour hydrolysate obtained with chymotrypsin (DH 13%). In turn, Avramenko *et al*. [2013] determined the patterns of SDS-PAGE under non-reducing conditions of lentil protein isolate and its hydrolysates with trypsin (DH 4–20%). A band corresponding to an MW of around 30 kDa was found for the isolate and hydrolysates. These findings may explain the presence of bands from undigested protein in the hydrolysates obtained with pancreatin, which has trypsin and chymotrypsin activity.

The MW distribution of low MW compounds of lentil flour and hydrolysates was analyzed by SE-HPLC (Figure 3). A large peak eluting at short retention time was dominant in the chromatogram of lentil flour. It suggested that the molecules with the MW higher than 66.0 kDa were predominant in the sample. However, small peaks corresponding to lower MW were also present in the chromatogram. This was in line with the previous study, in which SE-HPLC fractions of green and red lentil flours with MW of 0.24–44 kDa were detected [Barbana & Boye, 2013]. The authors suggested that they could be derived from some subunits of 11S and 7S globulins, 2S albumin, polypeptides, γ-vicilin, and small peptides from the cleavage of the larger proteins. The peaks corresponding to the lowest MW (<0.556 kDa) could also be ascribed to the phenolic compounds, as provided in our earlier publication [Karamać & Rybarczyk, 2008]. The presence of phenolic acids (mainly hydroxycinnamates), flavonoids (including kaempferol and quercetin glycosides, (+)-catechin/(–)-epicatechin and their glycosides), and proanthocyanidins was demonstrated in lentil flour [Amarowicz *et al*., 2010; Bautista-Expósito *et al*., 2018b; Paranavitana *et al*., 2021; Zhang *et al*., 2015].

Protein hydrolysis up to DH 4% decreased the height of the SE-HPLC peak originating from the largest lentil proteins (Figure 3). At the same time, the presence of hydrolysis products with MW of 12.4–66.0 kDa, 3.5–6.5 kDa, and 0.369––0.556 kDa was apparent. Chromatograms of the hydrolysates with higher DH revealed successively smaller peaks corresponding to polypeptides with MW of 12.4–66.0 kDa and increasing peaks of compounds with MW of 0.369––1.046 kDa. The comparison of the MW profiles of the hydrolysates of lentil flour treated with pancreatin (Figure 3) to those previously published for hydrolysates obtained with chymotrypsin [Karamać & Rybarczyk, 2008] showed that pancreatin released considerably more products with low MW, especially peptides with MW of 0.369–0.556 kDa.

Radical scavenging activity of lentil flour and hydrolysates

Radical scavenging activity of lentil flour and its hydrolysates was investigated against two synthetic radicals (DPPH• and $ABTS^{\bullet+}$) and against superoxide ($O_2^{\bullet-}$) generated from luminol in the PCL assay, and results are shown in Figure 4, Figure 5 and Table 1, respectively. The ability of hydrolysates with DH 4–16% to scavenge DPPH• was significantly (p<0.05) higher than of the non-hydrolyzed sample (Figure 4). EC_{50} did not differ significantly (p≥0.05) only between the hydrolysate with DH 20% and lentil flour. Among the hydrolysates, the lowest EC_{50} (0.287–0.298 mg/mL) was determined for those with DH 4 and 8%. The $ABTS^{\bullet+}$ scavenging activity, expressed as TEAC, was also the lowest for lentil flour (86.5 μmol Trolox/g), although the value determined for the hydrolysate with DH 4% was similar (p≥0.05) (Figure 5). The strongest antiradical activity against $ABTS^{\bullet+}$ was noted for the hydrolysate with DH 8% (98.6 μmol Trolox/g). The TEAC of hydrolysates with DH 12–16% was significantly lower (p<0.05), *i.e.*, in the range of 93.2–95.5 μmol Trolox/g. The antioxidant capacity of lipid (ACL) and water (ACW) soluble compounds of lentil flour and hydrolysates was determined in the PLC assay. The ACL of the hydrolysate with DH 8% was significantly (p<0.05) higher than that of lentil flour (Table 1). High values were also noted for hydrolysates with DH 4 and 12%. In turn, ACW was in the range of 19.8–20.2 μmol L-ascorbic acid/g

TABLE 1. Antioxidant capacity of lipid (ACL) and water (ACW) soluble compounds determined by the photochemiluminescence assay as well as total phenolic content (TPC) of lentil flour and its hydrolysates obtained with pancreatin.

DH (%)	ACL (μmol Trolox/g)	ACW (μmol L-ascorbic acid/g)	TPC (mg GAE/g)
0	3.93±0.10^{bc}	19.9±0.58^{a}	1.129±0.013^{e}
4	4.14±0.15^{ab}	19.8±0.79^{a}	1.501±0.004^{d}
8	4.32±0.09^{a}	19.9±0.36^{a}	1.816±0.010^{c}
12	4.21±0.12^{ab}	20.2±0.49^{a}	1.879±0.024^{b}
16	3.78±0.13^{c}	19.9±0.32^{a}	1.884±0.030^{b}
20	3.53±0.08^{c}	20.1±0.64^{a}	2.168±0.015^{a}

Results are expressed as mean ± standard deviation (n=3). Values with different letters in the column differ significantly (p<0.05). DH – degree of hydrolysis; GAE – gallic acid equivalents.

and the difference between lentil flour and each hydrolysate was statistically insignificant (p≥0.05).

Generally, the hydrolysate with DH 8%, which contained polypeptides and peptides with a wide range of MWs, was a stronger free radical scavenger than the hydrolysates with a higher DH (12–16%) and containing low MW peptides (<0.556 kDa) as dominant. This finding does not coincide with previous reports indicating an increase in the radical scavenging activity of hydrolysates with an increase in DH [Karamać *et al*., 2014; Trigui *et al*., 2021] and with a decrease in MW of released polypeptides and peptides [Malomo *et al*., 2020]. On the other hand, some other reports have shown that hydrolysate fractions with peptides having intermediate MW (3–5 kDa, >0.5 kDa) or, comparable as in our study, with a wide range of MWs exhibited the highest antiradical properties [Hwang *et al*., 2010; Samaei *et al*., 2021; Tang *et al*., 2010]. These differences are due to the fact that the antioxidant activity of the released peptides is determined not only by their size, but also by their hydrophobicity as well as the composition and sequence of amino acids [Noman *et al*., 2022; Udenigwe & Aluko, 2011], which in turn depend on the structure of the parent proteins and the specificity of the proteases used. In the case of lentil protein hydrolysates, the structure of seven peptides (released by Savinase) with antiradical activity measured by the ORAC assay has been identified [García-Mora *et al*., 2017]. They consisted of 11–17 amino acids with MW in the range of 1.30–2.11 kDa and most of them contained C-terminal residues of Phe or Leu and hydrophobic amino acids (Trp, Tyr, Ile, Leu, Met, Phe) in the sequence. The release of antiradical hydrophobic polypeptides from lentil flour could also explain the PCL assay results in our research. Such peptides were involved in ACL but not in ACW, hence the increase in ACL due to limited hydrolysis and no significant differences in ACW between lentil flour and hydrolysates (Table 1). In turn, the highest radical scavenging activity of lentil flour hydrolysates with low DH (Figure 4, Figure 5 and Table 1) is consistent with the study of Garcia-Mora *et al*. [2014] who found out, while treating a lentil protein concentrate by Protamex and Savinase, that the highest ORAC was determined for hydrolysates with DH <5% (max. 11%) and ~9% (max. 15%), respectively. However, the hydrolysates obtained with Corolase 7089 and Alcalase exhibited the highest antiradical activity when DH was within a wide range of ~5–10% and ~17–23%, respectively.

In addition to the peptides released during hydrolysis, also phenolic compounds could affect the antiradical activity of lentil flour and hydrolysates. Both low MW phenolics and condensed tannins of lentil seeds are known for their ability to scavenge free radicals [Amarowicz *et al*., 2010]. In our research, the total content of lentil flour phenolics determined using the method with FCR was 1.129 mg GAE/g (Table 1). The TPC of hydrolysates was significantly higher (1.501–2.168 mg GAE/g) and dependent on DH. The increase in TPC of hydrolysates with their DH was in line with the report showing that treatments of a pinto bean concentrate with Alcalase and Savinase increased the TPC in a time-dependent manner [Garcia-Mora *et al*., 2015]. The enhanced TPC was also noted as a result of hydrolysis of a lentil protein concentrate with Alcalase and Flavourzyme [Rezvankhah *et al*., 2021a, b] and simulated *in vitro* gastrointestinal proteolysis of cooked lentil powder [Zhang & Chang, 2019]. However, when discussing the results of TPC obtained by the method with FCR, the limitations of this method should be taken into consideration. Some polypeptides and peptides released during hydrolysis could further reduce FCR and overestimate the real phenolic content. Indeed, Bautista-Expósito *et al*. [2018b] determined the phenolic profile of lentil flour and its hydrolysates by HPLC and found that the hydrolysates had a lower phenolic content calculated as a sum of contents of individual compounds than the flour. Nevertheless, the lack of correlation between TPC and the radical scavenging activity of flour and hydrolysates allowed us to speculate that rather the antioxidant polypeptides and peptides released by pancreatin affected the high TEAC, EC_{50}, and ACL of the hydrolysates with low DH.

Ferric reducing antioxidant power of lentil flour and hydrolysates

The FRAP of lentil flour and its hydrolysates with pancreatin are shown in Figure 6. The ability to reduce Fe^{3+} of all hydrolysates was significantly (p<0.05) higher than that of flour (68.5 μmol Fe^{2+}/g). The highest value was noted for the hydrolysate with DH 4% (109.2 μmol Fe^{2+}/g). For hydrolysates with increasingly higher DH, a successively lower FRAP was determined with the lowest value of 82.3 μmol Fe^{2+}/g (DH 20%). To the best of our knowledge, the reduction of $Fe^{3+}(TPTZ)_2$ by products of lentil protein hydrolysis obtained by any enzyme has not been determined so far. Whereas, an increase in FRAP as a result of protein hydrolysis has been widely reported for proteins of other sources hydrolyzed by various enzymes, *e.g.*, the FRAP values of hydrolysates obtained with papain, trypsin, pancreatin, Alcalase, and Flavourzyme were higher than that of flaxseed protein isolate [Karamać *et al*., 2016]. Hydrolysis of flour proteins of raw and roasted butterfly pea seeds with bromelain and trypsin also enhanced FRAP values [Ee *et al*., 2019]. Another example is fluted pumpkin leaf isolate treated with Alcalase, pepsin, trypsin and chymotrypsin, which had higher FRAP than protein substrate [Famuwagun *et al*., 2020]. The high FRAP of the hydrolysates with low DH was consistent with

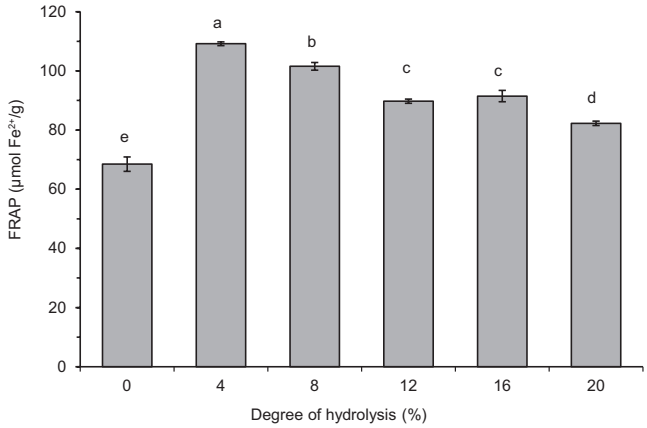

FIGURE 6. Ferric reducing antioxidant power (FRAP) of lentil flour and its hydrolysates obtained with pancreatin. Error bars represent the standard deviations of the means (n=3). Different letters above bars indicate significant differences among samples (p<0.05).

the results of the radical scavenging activity and, it could be speculated that peptides with a wide range of MWs contributed to the reducing power of lentil flour hydrolysates.

The FRAP values determined in our study were about two-three times lower than those reported for hydrolysates of flaxseed protein isolate produced with pancreatin (0.21––0.25 mmol Fe^{2+}/g) [Karamać *et al*., 2014]. Pancreatin treatment of the pigeon pea protein isolate also allowed obtaining a hydrolysate with high ability to reduce $Fe^{3+}(TPTZ)_2$ (0.05 mmol Fe^{2+}/mg) [Olagunju *et al*., 2018]. The lower reducing power of lentil flour hydrolysates compared to the aforementioned hydrolysates of protein isolates may be due to the different structure and amino acid composition of the proteins treated with pancreatin, but also to the high protein content of the isolates when compared with flour.

Fe^{2+} chelating activity of lentil flour and hydrolysates

The results of determinations of the ability of lentil flour and its hydrolysates obtained with pancreatin to bind ferrous ions are shown in Figure 7.

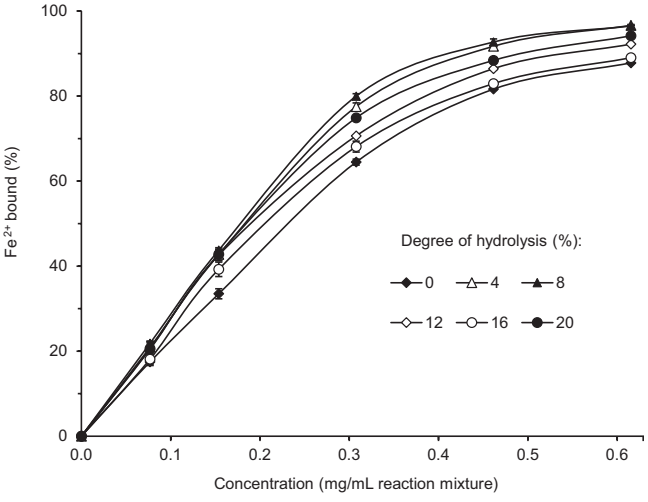

FIGURE 7. Fe^{2+} chelating activity of lentil flour and its hydrolysates obtained with pancreatin. Error bars represent the standard deviations of the means (n=3).

The Fe^{2+} chelating activity of both the flour and the hydrolysates increased with the increase of their concentration in the reaction mixture. This relationship was linear for up to approximately 0.3 mg/mL. At that concentration, the compounds of the hydrolysate with DH 8% bound 80% of ferrous ions, and the Fe^{2+} chelating activity decreased for hydrolysates with the following DH: 8% > 4% > 20% > 12% = 16%. The Fe^{2+} chelating activity of lentil flour was the lowest (64.5%). Although data on chelation of ferrous ions by lentil protein hydrolysis products are lacking in the literature, the improvement in Fe^{2+} binding capacity as a result of enzymatic hydrolysis is not surprising and was reported for various plant proteins treated with different enzymes [Famuwagun *et al*., 2020; Karamać *et al*., 2016; Zhang *et al*., 2014]. Also, the high Fe^{2+} chelating activity of hydrolysates with a relatively low DH was reflected in a previous study [Zhang *et al*., 2014]. The authors showed that the hydrolysate of soybean protein isolate with Flavourzyme with DH 6.79% had a significantly higher iron-binding capacity than hydrolysates with higher DH (up to 11.17%).

The percentage of ferrous ions bound (77.5–80.0%) by the most active hydrolysates (DH 4–8%) was relatively high considering their concentration in the reaction mixture (0.3 mg/mL) (Figure 7). Malomo *et al*. [2020] reported that the Fe^{2+} chelating activity of the hydrolysates of cashew nut and fluted-pumpkin proteins was approximately 20% and 95%, respectively, but at their higher concentration in the reaction mixture (1 mg/mL). Also, flaxseed protein hydrolysate obtained with pancreatin was used at a much higher concentration (1.54 mg/mL) to achieve the ferrous ion binding capacity of 71.5% [Karamać *et al*., 2016]. The high Fe^{2+} chelating activity in our study could be due to the presence of phenolic compounds in the samples. It is known that phenolic compounds, especially tannins, form chelates with ferrous ions [Karamać & Pegg, 2009].

CONCLUSIONS

Treatment of lentil flour with pancreatin allowed the hydrolysis of its proteins to DH 22%. Hydrolysates with increasingly higher DH contained polypeptides and peptides with increasingly lower molecular weights, but some basic subunits of lentil 11S globulin seemed to be quite resistant to digestion with pancreatin. The antiradical activity, reducing power, and Fe^{2+} chelating activity of lentil flour have been improved upon hydrolysis of its protein with pancreatin. Regardless of the mechanism of antioxidant action taken into account, the highest antioxidant capacity had hydrolysates with low DH (4–8%). The polypeptides and peptides with a wide range of molecular weights released from lentil proteins were responsible for enhanced antioxidant capacity of these hydrolysates. In turn, the total phenolic content of the hydrolysates determined with FCR increased with increasing DH. In conclusion, a limited hydrolysis with pancreatin could be recommended to improve the antioxidant capacity of lentil flour. Thus obtained hydrolysates with health benefits can be considered as an ingredient of functional foods. However, their use for this purpose requires future research.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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Original article
Section: Food Quality and Functionality

Effect of the Addition of Edible Insect Flour from Yellow Mealworm (*Tenebrio molitor*) on the Sensory Acceptance, and the Physicochemical and Textural Properties of Sponge Cake

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Key words: edible insects, sensory assessment, microbiological properties, amino acid profile, fatty acid profile

The aim of this study was to evaluate the possibility of using insect flour for sponge cake supplementation. Consumer acceptance, chemical composition, textural properties, and microbiological characteristics were determined. The addition of mealworm flour significantly increased the content of nutrients, especially protein, ash, lipid, and dietary fiber. Mealworm flour influenced the color parameters as well as textural properties. The fatty acid profile was dominated by monounsaturated fatty acids, ranging from 9.72 g/100 g for wheat flour sponge cake to 41.82 g/100 g for sponge cake with 20% addition of mealworm flour. The amino acid profile of mealworm flour was characterized by a significantly higher content of essential amino acids compared to wheat flour and sponge cakes. Sponge cake supplementation resulted in a good nutritional value of protein except for lysine which was the limiting amino acid in all samples. However, the limiting amino acid index was 63.04-63.10% compared to 30.38% for the mealworm and wheat sponge cake, respectively. The presence of insect flour reduced the organoleptic properties of the obtained sponge cakes, regardless of its quantity. The addition of mealworm flour contributed to a significant reduction in the hardness and fracturability of the sponge cakes on the baking day and during the 30-day storage. Insect flour addition did not reduce the microbiological safety of the final product. The study results indicate the possibility of using mealworm flour in the production of confectionery products.

INTRODUCTION

It is estimated that the world population will reach almost 10 billion by 2050, which causes a sharp increase in the demand for food, especially for proteins of animal origin, accounting for about 40% of the global demand. Production of animal protein is incredibly expensive and energy consuming. The Food and Agriculture Organization of the United Nations (FAO) estimates that it will only be possible to feed more than 9 billion people, the projected world population in 2050, if food production is increased by 70%. Almost 80% of the world's arable land is already used for livestock farming, and global meat consumption is projected to increase by 26% by the year 2050. World fish production would need to increase by 50% from 2006 levels to meet the demand identified for 2050 [FAO, 2009; Gladek *et al.*, 2017; van Huis *et al*., 2013]. On the other hand, climate change and food waste are other factors contributing to food decline worldwide [Tomaszewska *et al*., 2022]. In this context, insects can be seen as an alternative to existing food products [Skotnicka *et al.,* 2021]. They have a high feed conversion ratio: an average of 2 kg of feed per 1 kg of insect weight gain, while cattle production requires 8 kg of feed per 1 kg of weight gain [FAO, 2021; Oonincx & de Boer, 2012]. Their 1,900 species are now used as food [van Huis *et al*., 2013] in many parts of the world, mainly in Asia, Africa, and Latin America, by an estimated population of at least 2 billion people [FAO, 2021].

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Entomophagy may be the answer to one of the greatest challenges posed today, which is ensuring food security. The FAO calls for increased efforts to improve the acceptance and use of insects in food production, which is an element of sustainable development [van Huis *et al*., 2013]. It should be underlined that consumption of 100 g of caterpillars covers about 70% of the daily protein demand and 100% of the vitamin demand [Rumpold & Schlüter, 2013]. One of the many insects considered to be edible is the mealworm (*Tenebrio molitor*), which is used in the larval stage for food or feed purposes. These larvae are also used to obtain protein preparations, the fat fraction as well as the so-called insect meal or insect flour which is obtained by grinding freeze-dried or conventionally dried larvae [Bußler *et al*., 2016]. The mealworm larvae are rich in such nutrients as proteins (about 65 g/100 g), lipids (about 28 g/100 g), phosphorus and potassium (about 697 and 761 mg/100 g, respectively), and vitamin C (about 36 mg/100 g) [Jantzen da Silva Lucas *et al.*, 2020]. Considering the amino acid profile of the mealworm protein, it is worth noting that it contains more isoleucine, leucine, valine as well as phenylalanine and tyrosine than beef protein [Rumpold & Schlüter, 2013].

Therefore, edible insects may not only diversify the daily diet, contribute to food and nutritional security, but also have an impact on the reduction of the carbon footprint compared to the production of protein from farm animals [Vauterin *et al*., 2021]. Edible insects are known to be of use in variety of food products, including cricket powder for the production of muffin [Pauter *et al*., 2018]; *Hermetia illucens, Acheta domesticus, Alphitobius diaperinus* and *Tenebrio molitor* flours for bakery products [González *et al*., 2019; Kowalski *at al*., 2022; Wu *et al.*, 2020], *A. diaperinus* and *A. domesticus* for pancakes [Skotnicka *et al*., 2022] as well as mealworm larvae as a meat substitute in the production of hamburgers [Megido *et al*., 2016].

One of the key parameters of a food is its texture, which, in addition to technology, is influenced by the chemical composition of raw materials used in the production process. Kowalski *et al*. [2022] and Roncolini *et al.* [2019] reported a decrease in the hardness of bread baked with mealworm flour addition in comparison to bread with wheat flour. Whereas Pauter *et al*. [2018] observed an influence of cricket powder on a decrease in the hardness, springiness, chewiness, and resilience of muffins. Xie *et al*. [2022] also determined the influence of mealworm flour on a decrease in the hardness of sponge cakes and an increase in cohesiveness, chewiness, and resilience with an increase in the share of this flour from 5 to 15% in sponge cake formula. Reducing the hardness of products with insect flours may result from the weakening of the gluten structure as a result of the addition of a gluten-free ingredient. On the other hand, the increase in the value of other texture parameters may be due to the higher protein content, which may contribute to the strong binding of protein and starch by hydrogen bonds, which were formed during dough development and baking [Sriprablom *et al*., 2022; Xie *et al*., 2022].

The aim of our research was to evaluate impact of ground mealworm larvae on the sensory acceptance, and the physicochemical and textural properties of confectionary products, using sponge cake as a model. To eliminate the influence of the dough matrix, chicken eggs were replaced in formulations with a low-protein egg replacer, which made the analysis of amino acids and fatty acids more reliable.

MATERIAL AND METHODS

Materials used to sample preparation

The study samples were wheat sponge cake (WSC) and sponge cake in which part of the wheat flour type 450 (GoodMills Polska Sp. z o.o., Stradunia, Poland) was replaced by flour from ground mealworm (ZIRP Insects, Wien, Austria) in quantities of 10% (TMC10) and 20% (TMC20), on the initial wheat flour weight basis. The sponge cakes were prepared from a low-protein egg replacer (ER) (Natura-Werk Gebr. Hiller GmbH & Co.KG, Hannover, Germany) and sugar powder (Pfeifer & Langen Marketing Sp. z o.o., Poznan, Poland).

Sponge cake preparation

The sponge cakes were made using the cold method with the eggs:sugar:flour ratio of 2:1:1 (*w/w/w*) (Table 1). The dough was prepared using an Aristan 7 mixer (KitchenAid, Benton Harbor, Berrien, MI, USA). The low-protein egg replacer and water were mixed in the mixing bowl for 1 min at speed 2, then beaten for 5 min at speed 10. Sugar powder was added and the dough was again beaten for 3 min at speed 8, then sieved flour was added and the dough was mixed for 1 min at speed 2. The dough was allowed to stand for 10 min and then poured into silicone moulds (dimensions 30 mm) using a confectionery sleeve. The cakes were baked in a Miwe roll-in oven (Arnstein, Germany), at 200°C, for 12 min. When cooked, they were cooled down for half an hour at 19°C and used for further analyses.

Consumer acceptance analysis

Fifty participants aged 19 to 51 took part in the study. They were qualified from a randomly selected sample. Each participant assessed all cake variants proposed in the study. The study participants were healthy and did not take any food or medications that might affect their sensory evaluation. They took part in the research on a voluntary basis and the formal side of the research was approved by the Independent Bioethical Research Committee of the Medical University of Gdańsk (NKBBN/346/2021). Before the study, all

TABLE 1. Ingredients (g) used to produce different types of sponge cake.

Ingredient	WSC	TMC10	TM20
Low-protein egg replacer	75	75	75
Water	300	300	300
Wheat flour	187.5	168.75	150
Mealworm flour	-	18.75	37.50
Sugar powder	187.5	187.5	187.5

WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour

of them completed the food neophobia test (FNS), which was a condition for proceeding to the next stage of the study. They evaluated the appearance, aroma, taste, structure, and overall acceptance of the sponge cakes in a double-blind test, using a 5-point Likert scale (with 5 meaning excellent and 1 meaning extremely unsatisfactory).

Analysis of nutrient composition

The following parameters of wheat and mealworm flour, low-protein egg replacer and sponge cake were analyzed using AOAC International methods: ash content (AOAC 923.03); protein content (AOAC 950.36); crude lipid content (AOAC 935.38); water content (AOAC 925.10); total, soluble, and insoluble dietary fiber content (AOAC 991.43) [AOAC, 2006]. Analyses was performed in duplicate. Results were expressed as g/100 g dry matter (d.m.) ± standard deviation (SD).

Color analysis

The color of sponge cakes was analyzed using a Konica Minolta CM-5 spectrophotometer (Konica Minolta Sensing, Osaka, Japan) in the CIElab (L^*, a^*, b^*) system with 8 mm shutter, D65 illuminant, and 10° angle of measurement. The test temperature was 21°C. Measurements were done at ten different places on the surface of each sponge cake. The average color parameters from the measurements (were L^* value indicates lightness; the a^* value indicates red-green component (redness), and the b^* value yellow and blue components (yellowness) of a color) were determined, which were used to calculate the total color difference (ΔE) using the formula (1) [Fernández-Artigas *et al*., 1999]:

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

where: ΔL^* – lightness difference, Δa^* – redness difference, Δb^* – yellowness difference.

Based on the values of L^*, a^* and b^* parameters, the browning index (BI) was estimated [Buera *et al*., 1985] as follows (2):

$$BI = \frac{[100(X-0.31)]}{0.17} \quad (2)$$

where:

$$X = \frac{(a^*+1.75L^*)}{(5.645L^*+a^*-3.012b^*)} \quad (3)$$

Texture analysis

For the examination of the textural changes during storage, the sponge cakes were packed in polyethylene film pouches (HDPE) and stored for 30 days at ambient temperature (20±1°C, 65% relative humidity (RH)). Measurements of the snapping force and deflection of sponge cakes, such as hardness (peak force) and fracturability (the first significant break), were performed using a single-arm TA.XT2.Plus texture analyzer (stable Micro System Ltd, Godalming, Surrey, United Kingdom). A three-point bend rig (HDP/3PB) with the base's adjustable gap set at 20 mm using 5-kg load was used, with the trigger force 0.196 N, distance 5 mm. The texture parameter examination of the sponge cake was done after 1, 7, 14, 21, and 30 days of storage. Measurements were done in ten repetitions.

Scanning Electron Microscopy (SEM)

The structure of sponge cakes was observed using the HITACHI S-3400 N scanning electron microscope (Santa Clara, California, USA) Working parameters were as follows: Accelerating voltage 7.00 kV, emission current 60.0 μA, working distance 30.7 mm.

Determination of fatty acid profile

The derivatization and determination of fatty acid composition were performed according to the AOAC International approved method 991.39 [AOAC, 2006]. The analysis was performed as described by Kowalski *et al*. [2020] using a Shimadzu GC2010Plus chromatograph (Shimadzu corp., Kyoto, Japan) with a flame ionization detector (FID). Analyses was performed in duplicate, and results were expressed as g/100 g total fatty acids.

Nutritional quality indexes of lipids

The nutritional quality of sponge cake lipids was evaluated using five indexes: the ratio of hypocholesterolemic to hypercholesterolemic fatty acids (HH) (4), the atherogenic index (AI) (5), and thrombogenicity index (TI) (6), the ratio of polyunsaturated fatty acids to saturated fatty acids (PUFA/SFA), and the ratio of *n*–6 fatty acids to *n*–3 fatty acids (*n*6/*n*3) [Ulbricht & Southgate, 1991].

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

$$AI = \frac{C12{:}0+[4\times C14{:}0]+C16{:}0}{\sum MUFA+\sum n6+\sum n3} \quad (5)$$

$$\mathrm{TI} = \frac{C14{:}0+C16{:}0+C18{:}0}{0.5\times C18{:}1+0.5\times(\sum MUFA-C18{:}1)+0.5\times\sum n6PUFA+3\times\sum n3PUFA+\frac{\sum n3PUFA}{\sum n6PUFA}} \quad (6)$$

where: MUFA – monounsaturated fatty acids,

Amino acid analysis

Amino acid analysis was performed according to the method of Moore & Stein [1951]. Lyophilized samples were hydrolyzed in liquid 6 M HCl with 0.5% (*w/v*) phenol at 110°C for 24 h under an argon atmosphere. The hydrolysates were lyophilized, dissolved in buffer (0.2 M sodium citrate buffer, pH 2.2), filtered through a 0.45 μm syringe filter, and analyzed using the chromatographic amino acid analyzer (AAA400, Ingos, Prague, Czech Republic). Amino acids were analyzed by ion-exchange chromatography, with a cation exchanger and a sodium-citrate elution buffer system followed by post-column derivatization with ninhydrin and spectrophotometric detection at 570 and 440 nm, according to the standard protocol of the manufacturer. Sulphur-containing amino acids were analyzed as oxidation products obtained by performic acid oxidation followed by the standard hydrolysis procedure with HCl. For calibration of the amino acid analyzer, the amino acid analytical standard (mixture

of amino acids, Sigma-Aldrich, Saint Louis, Missouri, USA) was used in the concentration of 2.5 mM in 0.1 N HCl. Evaluation of the acquired data was performed using the software of the chromatographic device (Chromulan, Pikron, Prague, Czech Republic). Tryptophan was not determined as it is destroyed during acid hydrolysis, and asparagine and glutamine turn to aspartic acid and glutamic acid and in these forms are determined. Results were expressed as mg/g protein.

Nutritional quality of protein

Based on the amount and type of amino acids, the nutritional value of protein, expressed as the amino acid score (AAS), was calculated according to FAO/WHO [1991] (7):

$$AAS = \frac{mg\ of\ amino\ acid\ in\ 1\ g\ of\ test\ protein}{mg\ of\ amino\ acid\ in\ reference\ patern^*} \times 100 \quad (7)$$

*recommended amino acids scoring patterns for adolescents and adults, according to FAO [2013].

Microbiological analysis

For the examination of the microbiological changes during storage, the sponge cakes were packed in polyethylene film pouches (HDPE) and stored at ambient temperature (20±1°C, 65% RH). Their microbiological analyses were performed at the 1st and the 30th day of storage [Mikulec *et al*., 2020]. Samples were prepared according to International Organization for Standardization (ISO) 6887-1:2017 method [ISO, 2017]. In brief, 10 g of chipped sponge cakes were weighed and 90 mL of sterile saline were placed in a homogenizer bag, and homogenized in a BagMixer 400 W lab blender (Interscience, Saint Nom la Bretêche, France) for 3 min, speed 8 beats/s. The total number of aerobic amylolytic bacteria was determined by pouring 1 mL of each decimal dilution with Waksman medium (Biocorp Polska Sp. z o.o., Warsaw, Poland). After the incubation period (48 h, 37°C), the ability of the bacteria to produce amylase was observed by the appearance of a clear yellow zone around the colony by flooding it with Lugol's iodine solution according to PN-A-74134-4:1998 method [Polish Committee for Standardization, 1998]. To determine the total number of yeast and mold, 1 mL of appropriate decimal dilution was transferred into Sabouraud agar with chloramphenicol (Biocorp). The plates were incubated at 25°C for 3 to 5 days in line with ISO 21527-2:2008 method [ISO, 2008]. The number of β-glucuronidase-positive *Escherichia coli* was determined according to ISO 16649-2:2001 method [ISO, 2001]. The number of coagulase-positive staphylococci (*Staphylococcus aureus* and other staphylococci) was determined ISO 6888-1:2021 method with Baird-Parker agar medium [ISO, 2021], and the number of *Bacillus cereus* bacteria was determined according to ISO 7932:2004 procedure [ISO, 2004]. The results were expressed as cfu/g sponge cake. Measurements were done in two replicates.

Statistical analysis

Statistical analysis was carried out using Statistica 13.0 (StatSoft, Krakow, Poland). A one-way ANOVA was used to test sponge cake features at $p<0.05$. When ANOVA indicated significant differences, a post-hoc least significant difference (LSD) Fisher's test was performed. The results were presented as means ± SD.

In the consumer assessment, assuming that the simultaneous use of many explanatory variables will serve to increase the accuracy of prediction, the parameters of the equation describing the influence of sensory factors in the tested samples on the level of perceived acceptance after their consumption were estimated. For this purpose, the parameters of multiple regression were estimated, taking into account the concept of shared variability in the Excel 2010 PL (Microsoft Sp. z o.o., Warsaw, Poland) spreadsheet. At the stage of planning the experiment, the selection of the sample size at a level that would produce statistical conclusions, the appropriate accuracy and certainty as well as the probability of the test to detect the effects of the given size was examined on the basis of the test power analysis and the interval estimation of the sample size. Quantitative variables were characterized using the arithmetic mean and SD.

RESULTS AND DISCUSSION

Consumer acceptance

Consumer acceptance is undoubtedly a key parameter that determines the possibility of introducing a new product to the market. A high nutritional value must be accompanied by sensory attractiveness [Sun-Waterhouse & Wadhwa, 2013]. Taste, aroma, and appearance are some of the most important factors that influence the consumer's purchasing decision. Consumption of edible insects or products with their participation may be associated with nutritional neophobia and cultural prejudices. As already mentioned above, the consumer acceptance may be influenced by the texture and its relation to the taste sensations. As part of the undertaken research, the organoleptic acceptance of sponge cakes with mealworm flour was assessed. All of the considered descriptors (appearance, aroma, taste, and texture) were related to the acceptance level of the analyzed samples. Therefore, a hypothesis was formulated assuming that the acceptance of sponge cakes with the addition of mealworm flour is the resultant of the appearance, taste, aroma, and texture. The analysis of the values of the general quality ratings of the tested samples showed that traditional sponge cakes without the addition of insect flour received the highest level of acceptance (Table 2). The control sample containing no insects turned out to be the highest rated in terms of all traits. The results show that the addition of insect flour significantly deteriorated the acceptance of sponge cakes, both in the case of 10% of the addition and its double. This indicates that the amount of the additive was not the main determinant but the additive itself, which affected the appearance, aroma, taste, and texture of the products. However, the overall acceptance evaluation of the samples with the addition of mealworm flour was assessed at 3.82 and 3.80 for TMC10 and TMC20, respectively, which indicates some acceptability and gives information that the use of insect flour in human nutrition could be accepted and implemented in industrial production (Table 2).

The results of the organoleptic analysis became the basis for estimating the relationship between the acceptance

TABLE 2. Acceptance values for the characteristics of the organoleptic quality of sponge cakes.

Sponge cake	Overall acceptance	Appearance	Aroma	Taste	Texture
WSC	4.44±0.76[a]	4.92±0.44[a]	4.56±0.81[a]	4.52±0.70[a]	4.48±0.79[a]
TMC10	3.82±1.08[b]	4.26±1.05[b]	4.22±0.89[ab]	3.88±1.12[b]	3.92±1.07[b]
TMC20	3.80±1.01[b]	4.00±1.26[b]	4.16±1.08[b]	3.82±1.24[b]	3.64±1.17[b]

A 5-point Likert scale was used for the evaluation of sponge cakes. The results are presented as mean and standard deviation ($n=50$). Values in the same column marked with different letters are statistically significantly different at $p<0.05$. WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour.

of selected characteristics of the organoleptic quality of sponge cakes with the addition of insect flour and the general acceptance of sponge cakes. For this purpose, multiple regression analysis was used, which allowed for the development of acceptance models for the tested samples. The dependent variable was the sponge cake acceptance level, while the input independent variables were the acceptance ratings of individual organoleptic quality characteristics, such as taste, aroma, appearance, and texture. Independent variables were reduced to critical parameters. The significance of the generated acceptance models was assessed at the significance level of $p<0.05$. The model used to estimate the influence of particular variables on the assessment of the acceptance of sponge cake with the addition of 10% of insect flour turned out to be statistically significant, and all predictors explained 97% of the variability of the dependent variable ($R^2=0.97$). All the included predictors had a significant impact on the assessment of the acceptance of insect flour sponge cakes: acceptance of appearance ($p=0.014$), taste ($p=0.000$), aroma ($p=0.002$), and texture ($p=0.004$).

Taking the above descriptors into account, the regression equation took the following form (8):

$$\mathrm{ATMC10} = 0.3x_{1(texture)} + 0.53x_{2(taste)} - \\ - 0.008x_{3(aroma)} + 0.13x_{4(appearance)} \text{ aroma} \quad (8)$$

In the case of the evaluation of the acceptance of sponge cakes with the addition of 20% insect flour, the coefficient of determination assumed the value of $R^2=0.97$, which indicates that the generated model explained 97% of the variability of the dependent variable, *i.e.*, the acceptance of TMC20 sponge cakes, and 3% of the variability remained in the residual variable. As in the case of the smaller amount of mealworm flour addition, all the included predictors also had a significant impact on the sponge cake acceptance assessment: acceptance of appearance ($p=0.008$), taste ($p=0.002$), aroma ($p=0.012$), and texture ($p=0.000$).

Based on the large-scale regression analysis, an acceptance model for sponge cakes with 20% insect flour was generated. Taking the above descriptors into account, the regression equation took the following form (9):

$$\mathrm{ATMC20} = 0.30x_{1(texture)} + 0.41x_{2(taste)} - \\ - 0.15x_{3(aroma)} + 0.13x_{4(appearance)} \quad (9)$$

The obtained results indicate that the level of acceptance was the result of the predictors included in the regression equation. The taste and texture of both the TMC10 and TMC20 variants were of greatest importance for the overall acceptance of the sponge cakes with the addition of insect flour.

The present study results corroborate literature data. For example, Roncolini *et al*. [2019] reported a decrease in consumer acceptability of bread enriched with *T. molitor* flour; however, they found no effect of the additive level (5 and 10%) on the overall product acceptability. García-Segovia *et al*. [2020] analyzed breads with *T. molitor* and *A. diaperinus* at the same percentage of enrichment as in the previous work. However, they concluded that the decrease in consumer acceptability was directly proportionally correlated with the amount of insect flour added. Furthermore, breads with mealworm were rated higher than those with *A. diaperinus* flour. On the other hand, in our previous research on breads with cricket, buffalo worm and mealworm powder (from 10 to 30%), we found that the 10% share of these flours in breads did not lower consumer acceptance [Kowalski *et al*., 2022]. In the study by Pauter *et al*. [2018], consumers perceived the appearance and color of a muffin with 2, 5, and 10% cricket powder as unattractive. On the other hand, an increase in acceptance in the assessment of taste and texture was observed, compared to the control, even though some consumers indicated a noticeable "unpleasant" taste. The authors suggest that a 2% share would be acceptable to consumers [Pauter *et al*., 2018].

Nutrient composition

Food is a combination of various components that provides the necessary nutrients for cell growth and proliferation. They also fuel cellular metabolism. However, in addition to these roles, it is clear that nutrients and their metabolites are also active in facilitating, regulating, and coordinating the vast number of cellular processes that aim to maintain cellular homeostasis [Chen *et al*., 2018]. Therefore, the chemical composition of food determines its nutritional value and suitability for human nutrition. Based on the LSD Fisher's test, all analyzed samples differed significantly ($p<0.05$) in terms of nutritional value (Table 3). Mealworm flour had a significantly ($p<0.05$) higher ash, protein, and lipid content compared to wheat flour, which translated into a higher content of these ingredients in sponge cakes compared to the control. It should be emphasized that sponge cake with mealworm flour was also characterized by a significantly higher content of insoluble dietary fiber

TABLE 3. Nutritional value of raw materials and sponge cakes (g/100 g).

Sponge cake	Moisture	Protein	Ash	Fat	Dietary fiber		
					Insoluble fraction	Soluble fraction	Total
WF	9.84±0.04^{b}	11.52±0.01^{b}	0.44±0.00^{b}	1.76±0.03^{d}	0.43±0.01^{e}	1.34±0.05^{a}	1.76±0.04^{e}
TM	1.56±0.01^{e}	47.64±0.28^{a}	3.99±0.05^{a}	31.38±0.25^{a}	6.55±0.05^{a}	0.00±0.00^{d}	6.55±0.05^{a}
ER	10.45±0.09^{a}	0.23±0.01^{f}	0.29±0.01^{d}	0.42±0.01^{f}	0.00±0.00^{f}	1.18±0.01^{b}	1.18±0.01^{f}
WSC	6.16±0.01^{d}	5.08±0.03^{e}	0.22±0.01^{e}	0.58±0.03^{e}	0.75±0.03^{d}	1.14±0.02^{c}	1.89±0.05^{d}
TMC10	7.85±0.10^{c}	6.32±0.03^{d}	0.36±0.01^{c}	1.93±0.01^{c}	1.34±0.01^{c}	1.13±0.03^{c}	2.47±0.05^{c}
TMC20	6.17±0.12^{d}	8.04±0.03^{c}	0.46±0.00^{b}	3.50±0.06^{b}	2.09±0.07^{b}	1.13±0.02^{c}	3.22±0.02^{b}

The results are presented as mean and standard deviation ($n=2$). Values in the same column marked with different letters are statistically significantly different at $p<0.05$. WF – wheat flour; TM – mealworm flour; ER – low-protein egg replacer; WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour.

TABLE 4. Color parameters of sponge cakes.

Sponge cake	L^*	a^*	b^*	ΔE	BI
WSC	78.29±0.17^{a}	5.05±0.15^{c}	23.22±0.59^{b}	–	38.81±1.28^{c}
TMC10	71.93±0.63^{b}	6.33±0.17^{b}	25.18±0.57^{a}	6.79±0.79^{b}	48.19±1.86^{b}
TMC20	67.11±0.13^{c}	7.56±0.03^{a}	25.17±0.25^{a}	11.63±0.14^{a}	53.78±0.61^{a}

The results are presented as mean and standard deviation ($n=12$). Values in the same column marked with different letters are statistically significantly different at $p<0.05$. WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour; L^* – lightness; a^* – redness; b^* – yellowness; ΔE – total color difference, BI – browning index.

fraction. The results of the chemical composition of mealworm flour (Table 3) are consistent with those obtained by other authors, who determined a similar content of nutrients and especially protein, lipid, and ash (41.22 to 52.23%; 29.42 to 32.76 % and 3.57 to 4.30% respectively) [González *et al*., 2019; Khuenpet *et al*., 2020; Wu *et al*., 2020]. Other authors also observed an increase in the content of nutrients as a result of supplementing the products, like bread or muffins with an edible insects flour richer in individual nutrients [González *et al*., 2019; Khuenpet *et al*., 2020; Kowalski *et al*., 2020; Pauter *et al*., 2018].

Color parameters

Color is one of the key features of bakery products and, in addition to textural features, can affect consumer acceptance. In the one-way ANOVA, the LSD Fisher's test showed a significant ($p<0.05$) effect of edible insects flour on the CIELab parameters as well as on both total color difference and browning index (Table 4). With mealworm flour addition to cake formulation, a decrease in lightness (L^*), an increase in the content of redness (a^*) and yellowness (b^*) color components, an increase in the total color difference, and an increase in the BI value were observed. A similar effect in changing the values of color parameters (Table 4) was observed by Pauter *et al*. [2018], who used cricket flour to make muffins. They found a significant decrease in L^* and ΔE with an increase in the proportion of insect flour, while observed a significant decrease in the values of a* and b* parameters, in contrast to our study.

Texture parameters

All the mealworm flour sponge cakes were characterized by significantly ($p<0.05$) lower hardness compared to the control cake (WSC) (Table 5). The hardness of the control sponge cakes was at a similar level over the entire storage period. The lowest hardness was determined for the sponge cakes with 20% mealworm flour addition on the baking day and after 7 days of storage, and in the sponge cakes with 10% mealworm flour addition after 7 days of storage. Wheat sponge cake, on the baking day, showed significantly greater fracturability (75.43 N), compared to the other cakes, both on the baking day and during storage, which ranged from 27.70 N (TMC10 baking day) to 68.09 N (WSC after 30 days). Both the hardness and fracturability of the sponge cakes with insect flour increased from the 14th day of storage. Mealworm flour contributed to a significant reduction in their fracturability compared to WSC. Our results are in line with the literature data. Pauter *et al*. [2018] found lower hardness of muffins with cricket flour compared to muffins without additives. A significant effect on the reduction of hardness of breads with mealworm, regardless of the amount of the additive, was also observed in our previous work [Kowalski *et al*., 2022]. In turn, Feili *et al*. [2013] claimed that the hardness of bakery products depended mainly on their amylopectin and amylose contents. The protein content increase and the starch content decrease were reported by produce a significant decrease in hardness [Gómez *et al*., 2003; Martínez-Cervera *et al*., 2011]. Rodríguez-García *et al*. [2014] also concluded that hardness was related to the total air cell volume and crumb

TABLE 5. Texture parameters of sponge cake stored for 30 days.

Sponge cake	Storage period (day)	Hardness (N)	Fracturability (N)
WSC	1	89.33±11.84^{a}	75.43±10.85^{a}
TMC10		61.51±3.73^{cd}	42.87±6.73^{de}
TMC20		45.41±3.68^{fg}	27.70±3.19^{f}
WSC	7	91.78±11.44^{a}	53.27±14.37^{cd}
TMC10		44.11±3.68^{g}	38.04±3.42^{e}
TMC20		47.11±6.79^{fg}	39.04±4.77^{e}
WSC	14	79.82±5.54^{b}	53.42±4.19^{cd}
TMC10		66.78±5.23^{cd}	45.53±3.53^{de}
TMC20		52.85±5.43^{ef}	42.54±3.94^{de}
WSC	21	89.26±3.98^{a}	65.08±3.73^{b}
TMC10		57.01±3.68^{def}	56.01±3.68^{c}
TMC20		52.95±2.40^{ef}	50.91±3.57^{cd}
WSC	30	90.61±4.50^{a}	68.09±5.71^{ab}
TMC10		57.29±2.51^{def}	48.07±0.99^{cd}
TMC20		51.64±3.78^{f}	37.75±4.21^{e}

The results are presented as mean and standard deviation (n=10). Values in the same column marked with different letters are statistically significantly different at p<0.05. WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour.

volume, and as the gas cell size increased, the crumb structure of the product became softer and more delicate. The observed changes may stem from gluten dilution due to the substitution of a part of wheat flour with a gluten-free additive, which is mealworm flour. In addition to the gluten dilution effect, Xie *et al.* [2022] explained the reduction in hardness of cookies with the addition of mealworm by competition for water particles. The above-mentioned effects affected the structure of the sponge cakes, which seems to be confirmed by the SEM images (Figure 1), which clearly show that the structure of WSC was more porous and its pore walls were thinner compared to the structure of TM10 and TM20.

Fatty acid profile

Significant differences were observed in the content and profile of fatty acids both in the raw materials used to produce sponge cakes and in the finished products (Table 6). The fatty acid profile of mealworm flour and sponge cakes with its addition was dominated by monounsaturated fatty acids with the highest content of oleic and linoleic fatty acids. The nutritional value of flours and sponge cakes was evaluated by nutritional quality indexes of lipids such as AI, HH, TI, PUFA/SFA, and the *n*6/*n*3 ratio. AI and TI ranged from 0.24 and 0.38 for WSC respectively, to 0.56 and 0.78 for TMC20, respectively. HH of sponges cake lipids ranged from 2.56 for TMC20 to 4.25 for WSC. The PUFA/SFA ratio of the sponge cake with mealworm flour was lower compared to that of WSC, *i.e.*, 0.95 (TMC20) *vs*. 3.57 (WSC). In turn, the *n*6/*n*3 ratio increased from 15.85 for WSC to 27.10 for TMC20. Similar results of the fatty acid profile of the mealworm oil fraction were obtained by Wu *et al*. [2020]. AI and TI can be used as predictors

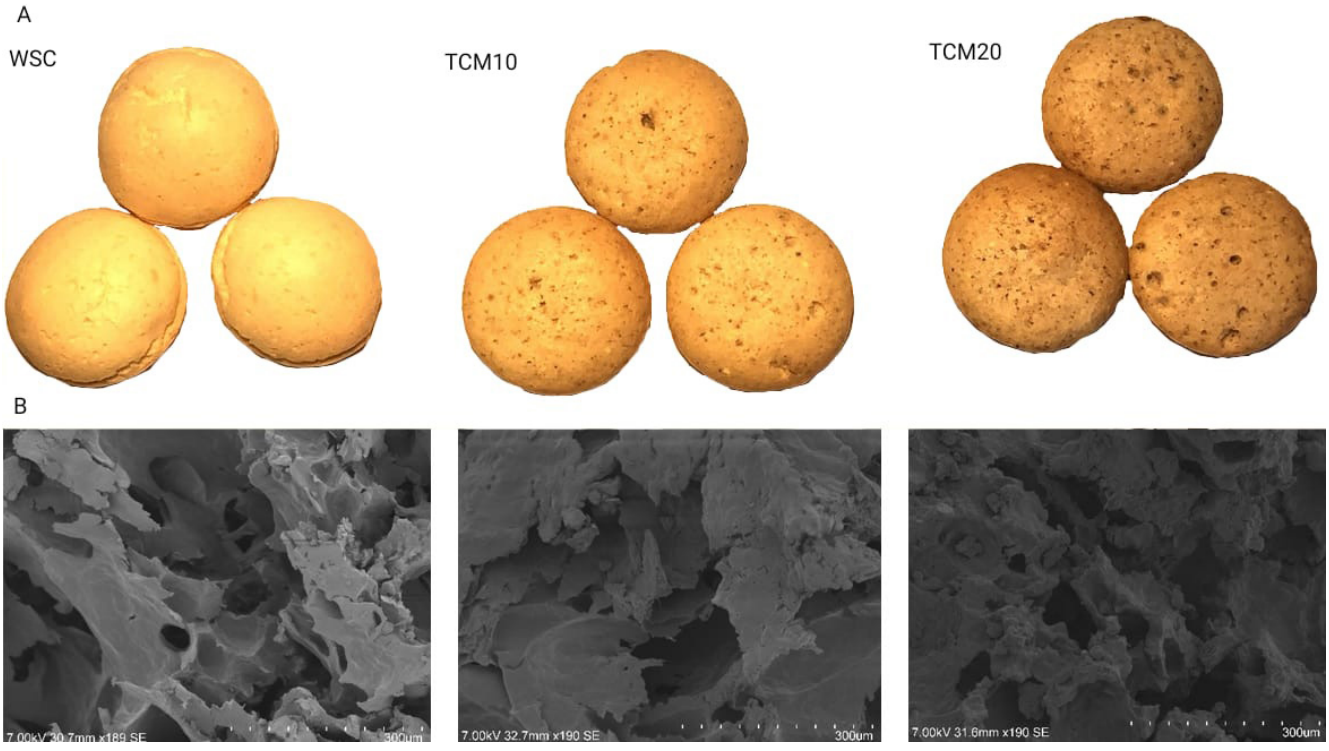

FIGURE 1. Images of wheat sponge cake (WSC) and sponge cakes with 10% addition of mealworm flour (TMC10) and with 20% addition of mealworm flour (TMC20) A) overall appearance B) scanning electron microscopy (SEM) images of the sponge cakes structure.

TABLE 6. Raw material and sponge cake fatty acid profiles (g/100 g total fatty acids) and nutritional quality indexes of lipids.

Fatty acid	WF	TM	ER	WSC	TMC10	TMC20
C12:0	0.02±0.00^{e}	0.38±0.00^{a}	0.31±0.00^{c}	0.06±0.00^{d}	0.36±0.00^{b}	0.36±0.00^{b}
C14:0	0.09±0.00^{e}	4.31±0.00^{a}	0.34±0.00^{c}	0.15±0.00^{d}	3.55±0.00^{c}	4.22±0.00^{b}
C14:1	0.00±0.00^{d}	0.16±0.00^{a}	0.00±0.00^{d}	0.00±0.00^{d}	0.14±0.00^{b}	0.15±0.00^{b}
C15:0	0.09±0.00^{e}	0.10±0.00^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.12±0.00^{a}
C16:0	17.62±0.00^{e}	18.98±0.04^{c}	2.40±0.00^{f}	18.52±0.00^{d}	19.01±0.00^{b}	21.96±0.00^{a}
C16:1*n*9	0.12±0.00^{f}	1.14±0.02^{a}	0.24±0.00^{d}	0.19±0.00^{e}	1.02±0.00^{c}	1.07±0.00^{b}
C16:1*n*7	0.13±0.00^{f}	1.88±0.02^{a}	0.33±0.00^{d}	0.16±0.00^{e}	1.69±0.00^{c}	1.77±0.00^{b}
C17:0	0.08±0.00^{e}	0.09±0.00^{b}	0.10±0.00^{a}	0.07±0.00^{d}	0.09±0.00^{b}	0.10^{a}±0.00^{a}
C17:1	0.04±0.00^{e}	0.11±0.00^{a}	0.03±0.00^{d}	0.04±0.00^{c}	0.10±0.00^{b}	0.10±0.00^{b}
C18:0	0.69±0.00^{f}	2.86±0.07^{b}	1.69±0.00^{d}	0.77±0.00^{e}	2.45±0.00^{c}	3.05±0.00^{a}
C18:1*n*9	9.18±0.00^{d}	44.68±0.05^{a}	7.28±0.00^{f}	8.94±0.00^{e}	37.72±0.00^{c}	38.67±0.00^{b}
C18:2*n*6	67.25±0.00^{a}	24.46±0.09^{f}	62.50±0.00^{c}	66.35±0.00^{b}	32.29±0.00^{d}	27.27±0.00^{e}
C18:3*n*3	4.16±0.00^{c}	0.74±0.00^{f}	4.48±0.00^{a}	4.19±0.00^{b}	1.31±0.00^{d}	1.01±0.00^{e}
C20:0	0.07±0.00^{b}	0.07±0.00^{b}	0.09±0.00^{a}	0.06±0.00^{c}	0.07±0.00^{b}	0.09±0.00^{a}
C20:1	0.45±0.00^{a}	0.05±0.00^{f}	0.11±0.00^{c}	0.38±0.00^{b}	0.10±0.00^{d}	0.07±0.00^{e}
Σ SFA	18.65±0.00^{f}	26.80±0.10^{b}	25.02±0.00^{d}	19.75±0.00^{e}	25.64±0.00^{c}	29.91±0.00^{a}
Σ MUFA	9.93±0.00^{d}	48.01±0.01^{a}	7.99±0.00^{f}	9.72±0.00^{e}	40.77±0.00^{c}	41.82±0.00^{b}
Σ PUFA	71.42±0.00^{a}	25.18±0.00^{f}	66.98±0.00^{c}	70.54±0.00^{b}	33.60±0.00^{d}	28.27±0.00^{e}
AI	0.22±0.00^{f}	0.50±0.00^{b}	0.32±0.00^{d}	0.24±0.00^{e}	0.45±0.00^{c}	0.56±0.00^{a}
TI	0.36±0.00^{f}	0.68±0.01^{b}	0.50±0.00^{d}	0.38±0.00^{e}	0.62±0.00^{c}	0.78±0.00^{a}
HH	4.55±0.09^{a}	3.00±0.03^{e}	3.27±0.03^{c}	4.25±0.01^{b}	3.16±0.02^{d}	2.56±0.02^{f}
PUFA/SFA	3.83±0.07^{a}	0.94±0.02^{c}	2.68±0.02^{b}	3.57±0.01^{a}	1.31±0.01^{b}	0.95±0.00^{c}
*n*6/*n*3	16.15±0.09^{d}	33.10±0.55^{a}	13.94±0.17^{e}	15.85±0.30^{d}	24.69±0.54^{c}	27.10±0.20^{b}

The results are presented as mean and standard deviation (n=2). Values in the same row marked with different letters are statistically significantly different at p<0.05. WF – wheat flour; TM – mealworm flour; ER – low-protein egg replacer; WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; AI – atherogenic index; HH – ratio of hypocholesterolemic to hypercholesterolemic fatty acids, TI – thrombogenicity index.

or risk factors for cardiovascular diseases and should be kept at low levels in a healthy everyday diet [Ulbricht & Southgate, 1991]. The HH index is an indicator of the effect of fatty acids on cholesterol metabolism and its high value is important from a nutritional point of view. According to nutritionists [Simopoulos, 2008], the *n*6/*n*3 ratio should range from 1:1 to 5:1, while the ratio of 10:1 has adverse health consequences. A low level of PUFA/SFA ratio in diets is deemed to be a risk factor for increased blood cholesterol levels [Pacetti *et al*., 2013]. Thus, the lipid fractions of raw materials and sponge cakes were characterized by too high values of the *n*6/*n*3 ratio (Table 6). Taking into account lipid indexes, the addition of mealworm flour to the formulations of studied sponge cakes diminished their nutritional quality.

However, it is difficult to unequivocally assess the nutritional value of insect lipids only on the basis of lipid indexes and the ratio of its individual fractions, because, for example, oleic acid, the content of which in the lipid profile of sponge cakes with mealworm was over 4 times higher than in the control cake, has a beneficial effect on insulin sensitivity [Palomer *et al*., 2018]. This acid not only prevents insulin resistance but also inhibits endoplasmic reticulum stress and anti-inflammatory effects, as well as prevents attenuation of the insulin signaling pathway, and improves β-cell survival [Palomer *et al*., 2018]. Research by Perona *et al*. [2004] demonstrated the effectiveness of oils rich in oleic acid in lowering blood pressure and low-density lipoprotein (LDL) cholesterol level [Perona *et al*., 2004]. Additionally, in the diet, a low ratio of palmitic to oleic acids also contributes to a reduced risk of diabetes development [Palomer *et al*., 2018]. The palmitic acid content of the sponge cakes, compared to oleic acid, was 0.50 (TMC10) and 0.57 (for TMC20), compared to 2.07 determined for WSC (Table 6), suggesting some potential for reducing the risk of diabetes development.

TABLE 7. Amino acid profiles and amino acid scores of raw materials and sponge cakes.

Amino acid	WH	TM	ER	WSC	TMC10	TMC20
Amino acid content (mg/g protein)						
Phe	45.87±0.47^{a}	36.64±1.08^{c}	28.5±0.28^{d}	42.94±2.23^{ba}	45.90±3.12^{ab}	42.14±0.55^{b}
Val	36.68±1.23^{d}	59.24±1.61^{a}	37.51±3.66^{d}	33.84±1.60^{e}	47.17±2.57^{cb}	49.37±1.51^{bc}
Thr	23.60±0.36^{c}	36.54±0.86^{a}	19.56±1.15^{e}	21.88±1.10^{d}	30.12±1.48^{b}	31.36±0.63^{b}
Ile	32.06±0.30^{c}	41.01±1.17^{a}	25.81±0.21^{e}	30.8±1.30^{d}	37.97±2.19^{b}	37.73±0.76^{b}
Met	17.79±0.14^{b}	16.22±3.92^{c}	18.66±0.63^{a}	16.53±0.20^{c}	16.32±0.26^{c}	15.60±0.14^{d}
His	19.50±0.24^{d}	33.47±0.81^{a}	16.42±0.61^{e}	19.68±0.81^{d}	28.35±1.81^{cb}	29.02±0.68^{bc}
Leu	63.01±0.60^{c}	69.09±1.98^{b}	58.36±0.46^{e}	60.66±2.91^{d}	70.44±4.64^{a}	68.63±1.30^{b}
Lys	19.29±0.20^{d}	53.21±1.59^{a}	32.13±0.45^{b}	14.58±0.66^{e}	30.26±0.97^{c}	30.29±0.72^{c}
Total EAA	257.78±2.46^{c}	345.43±12.70^{a}	236.98±4.97^{ed}	240.93±10.70^{de}	306.53±13.92^{b}	304.14±5.78^{b}
Asp	37.34±0.34^{e}	77.71±1.74^{a}	57.95±0.80^{c}	35.41±1.72^{f}	55.25±3.25^{dc}	60.73±1.27^{b}
Ser	44.88±0.40^{c}	43.65±0.85^{d}	25.45±0.36^{f}	42.71±2.36^{e}	48.54±2.70^{a}	45.79±0.75^{bc}
Glu	331.59±2.98^{a}	116.49±3.02^{e}	81.01±0.93^{f}	321.49±15.37^{b}	283.86±9.15^{c}	227.53±2.58^{d}
Pro	113.33±0.89^{a}	61.44±2.28^{d}	34.08±3.03^{e}	101.74±5.58^{b}	99.34±1.12^{b}	82.07±4.54^{c}
Gly	32.76±0.34^{c}	50.82±1.37^{a}	32.96±0.34^{c}	32.07±1.55^{c}	43.17±4.85^{b}	44.48±1.20^{b}
Ala	27.51±0.28^{e}	63.93±1.63^{a}	39.30±0.49^{d}	26.52±1.31^{e}	44.22±2.47^{c}	49.55±1.85^{b}
Tyr	27.94±1.27^{d}	81.72±2.09^{a}	25.07±0.58^{e}	23.70±0.72^{f}	44.22±2.48^{c}	51.09±2.67^{b}
Arg	34.88±0.71^{c}	50.88±1.65^{a}	33.76±0.20^{d}	31.51±1.45^{e}	42.22±2.66^{b}	41.49±0.89^{b}
Cys	21.36±0.25^{a}	7.62±1.84^{f}	9.96±1.26^{e}	19.17±0.31^{b}	16.22±0.27^{c}	13.86±0.09^{d}
Total non-EAA	671.58±3.19^{a}	554.26±6.12^{d}	340.17±3.51^{e}	634.33±9.84^{b}	677.05±9.89^{a}	616.59±7.59^{c}
Amino acid score (%)						
Val	91.70	148.10	93.78	84.60	117.93	123.43
Thr	94.40	146.16	78.24	87.52	120.48	125.44
Ile	106.87	136.70	86.03	102.67	126.57	125.77
His	121.88	209.19	102.63	123.00	177.19	181.38
Leu	103.30	113.26	95.67	99.44	115.48	112.51
Lys	40.19	110.85	66.94	30.38	63.04	63.10
AAA (Phe+Tyr)	180.02	288.68	130.73	162.56	219.80	227.39
SAA (Cys+Met)	170.22	103.65	124.43	155.26	141.48	128.13

The results are presented as mean and standard deviation (n=2). Values in the same row marked with different letters are statistically significantly different at p<0.05. WF – wheat flour; TM – mealworm flour; ER – low-protein egg replacer; WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour; EEA – essential amino acids; SAA – sulphur-containing amino acids; AAA – aromatic amino acids.

Amino acid profile

The quality of a protein depends on its amino acid profile and digestibility [van Huis *et al*., 2013]. Mealworm flour and sponge cakes with its addition were characterized by a significantly higher content of essential amino acids (Table 7) and the sum of aromatic amino acids (phenylalanine and tyrosine) which ranged from 345.43 to 304.14 and 118.36 to 93.23 mg/g protein, respectively (Table 7). Among the essential amino acids, the major ones were leucine, valine, lysine, and phenylalanine. The amino acid score was calculated by comparing the content of essential amino acids to the FAO standard [FAO, 2013]. The addition of mealworm flour did not change the limiting amino acid of the control sponge cakes, *i.e.* lysine, but the amino acid score was twice as high and amounted 63.04–63.10% for the mealworm sponge cakes compared to wheat sponge cake (30.38%) (Table 7). It should be emphasized that the addition of insect flour contributed to the improvement of the composition of most essential amino acids (except lysine) of sponge cake proteins

TABLE 8. Microbiological characteristic of raw materials and sponge cakes (cfu/g).

Sponge cake	TNB	TNF	NAB	Pathogens
Raw material				
WF	$1.2\times10^4\pm0.4\times10^{4b}$	$8.1\times10^4\pm0.70\times10^4$	15.0 ± 1.5^a	Nd
TM	$9.6\times10^4\pm1.0\times10^{4a}$	Nd	$1.9\times10^4\pm0.3\times10^{4b}$	Nd
Sponge cake in baking day				
WSC	Nd	Nd	Nd	Nd
TMC10	Nd	Nd	Nd	Nd
TMC20	Nd	Nd	Nd	Nd
Sponge cake stored for 30 days				
WSC	30.0 ± 4.5^a	50.0 ± 5.5^a	Nd	-
TMC10	10.0 ± 1.7^c	15.0 ± 3.5^b	Nd	-
TMC20	15.0 ± 2.0^b	5.0 ± 1.7^c	Nd	-

The results are presented as mean and standard deviation ($n=2$). Values in the same column (separately for raw material, sponge cake baking day and sponge cake 30 day storage) marked with different letters are statistically significantly different at $p<0.05$. WF – wheat flour; TM – mealworm flour; WSC – wheat sponge cake; TMC10 – sponge cake with 10% addition of mealworm flour; TMC20 – sponge cake with 20% addition of mealworm flour; TNB – total number of bacteria; TNF – total number of fungi; NAB – the number of amylolytic bacteria; Pathogens – *Escherichia coli, Bacillus cereus, Staphylococcus Aureus*; Nd – not detected.

to the values in line with the FAO recommendations [2013] (Table 7). The obtained results are consistent with those reported by other authors who also determined the amino acid profile in the mealworm flour [Jantzen da Silva Lucas *et al*., 2020; Wu *et al*., 2020].

Microbiological quality and stability

The microbiological assessment of food is essential to verify its suitability for safe human consumption. In 2015, the European Food Safety Authority (EFSA) issued a scientific opinion on the risk profile of the production and consumption of insects as food and feed [EFSA Scientific Committee, 2015]. Therefore, pursuant to the Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods and the commercialization of food containing insects and their parts, it must be authorized by the European Commission (EC) after EFSA has carried out a safety risk assessment. Edible insects and their products contain complex communities of microbes that originate from their digestive tract or from their breeding and processing environment. They are likely to contain saprophytic, spoilage or potentially pathogenic microorganisms [Osimani *et al*., 2018a, b, c]. In our study, microbiological analysis did not reveal the presence of pathogens in the raw materials and in the finished products. After baking, the sponge cakes were microbiologically clean, and after 30 days of storage, a slight increase was observed in the counts of fungi and bacteria (Table 8). The obtained results are similar to those reported by other authors who analyzed the microbiological quality of bread with mealworm [Roncolini *et al*., 2019] and cricket powder [Osmiani *et al*., 2018b]. Roncolini *et al*. [2019] found insignificant amounts of aerobic bacterial spores in bread crumb, which could be due to their temperature resistance, which when baked in the thermal center of the loaves was below 100°C. On the other hand, Osimani *et al*. [2018b] detected spore-forming bacteria in bread loaves with cricket powder, which may indicate a potential food safety problem. Small counts of bacteria and fungi were observed in the sponge cakes only after 30 days of storage. It is worth emphasizing, however, that these microorganisms were found in much higher numbers in the control sponge cakes. Thus, the low moisture content (from 6.16 for WSC to 7.85% for TMC10) (Table 3) and high temperature of baking probably protected the finished products from the development of pathogenic food microflora.

CONCLUSIONS

Replacing 10–20% of wheat flour with mealworm flour reduced the organoleptic properties of the sponge cakes. The level of acceptance was mainly affected by changes in the taste and texture of supplemented products. However, it should be emphasized that the acceptance of sponge cakes with mealworm flour was not much lower than that of the control wheat sponge cakes. The addition of mealworm flour contributed to the increase in the nutritional value of the final products. Sponge cakes prepared from flour with insect powder were characterized by lower hardness and fracturability both on the baking day and throughout the 30-day storage. Except for lysine, which was the limiting amino acid in all sponge cakes, the mealworm flour cakes were characterized by a higher content of all amino acids compared to the FAO recommended standard. The addition of mealworm flour did not diminish the microbiological quality of the sponge cakes, and after a 30-day storage period, TMC10 and TMC20 showed lower numbers of amylolytic bacteria and total bacteria compared to the control cakes.

ETHICAL STATEMENT

The study protocol was approved by the Independent Bioethical Research Committee of the Medical University of Gdańsk (NKBBN/346/2021).

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Original article
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Physicochemical and Sensory Properties with Special Emphasis on Thermal Characteristics of Whey Butter from Gouda Cheese Production Compared to Milk Butter

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Key words: whey butter, milk butter, differential scanning calorimetry, fatty acid profile, sensory attributes

The aim of this study was to characterise milk fat from whey butter and to identify potential differences between whey butter (WB) and sweet cream butter (milk butter – MB). The fatty acid (FA) profile, thermal properties, colour parameters, texture properties, and sensory attributes of MB and WB were compared. The values of texture properties (firmness, brittleness, and cohesiveness) and colour parameters (values of b^* and the yellowness index) of WB were lower than MB. The sensory analysis showed lower values of consistency descriptors (firmness, brittleness, cohesiveness), a less intense nutty and milky aroma, and a more intense cheesy aroma and taste in WB than in MB. WB was more abundant in monounsaturated, polyunsaturated, and long-chain FAs, including C18:0, C18:1 Σ*t*, C18:1 Σ*c*, C18:2, C18:3, and C18:2 *c*9, *t*11, and it was less abundant in saturated and medium-chain FAs, including C10:0, C12:0, C14:0, C14:1, C15:0, C16:0, and C16:1, relative to MB. Water content (MB *vs* WB and the corresponding fats) and thermal history (single *vs* repeated heating and cooling treatments) affected differential scanning calorimetry curves and phase transition peaks. The principal component analysis revealed that the FA profile influenced the crystallisation and melting peaks of MB fat (MBF) and WB fat (WBF). WBF crystallisation occurred at a lower temperature, was characterised by lower enthalpy, and proceeded more rapidly than MBF crystallisation. Various fat fractions had different melting characteristics, and most WBF fractions were characterised by lower melting enthalpy and a smaller maximum difference in heat flow than MBF. Whey butter and milk butter differed in physicochemical properties and sensory attributes, and their thermal profiles depended on the FA profile, water content, and thermal history.

INTRODUCTION

The coagulation of milk proteins in cheesemaking leads to the separation of milk components into two phases: curd and whey. Around 8 to 9 kg of whey is generated in the production of 1 kg of cheese. The global production of whey is estimated at 165 million tons, and it continues to increase by 1–2% every year [Panghal *et al.*, 2018]. Whey obtained during cheesemaking is utilised in 75% in Europe and in less than 50% on other continents [Macwan *et al.*, 2016]. Different types of whey are obtained in various cheese production technologies. Sweet whey is the by-product of rennet coagulation of milk, whereas acid whey is obtained during acid coagulation of milk. Sweet and acid whey differ in composition and parameters that determine their suitability for further processing [Nishanthi *et al.*, 2017]. Depending on the cheesemaking technology, 50–60% of milk solids are transferred to whey. Some of the separated solids have very high nutritional value. When whey is further processed (into whey protein concentrate, lactose, lactic acid), residual fat is separated from other components [Kasapcopur *et al.*, 2021; Macwan *et al.*, 2016]. Some fat is transferred to whey during cheesemaking. Concentrated whey fat in the form of whey cream can be processed into various products, such as whey butter [Jinjarak *et al.*, 2006; Panghal *et al.*, 2018].

The composition of milk fat is influenced by numerous factors, including seasonal variations, animal nutrition, lactation phase, and technology of dairy products. Differences in milk fat composition affect the physicochemical properties of butter [Amal, 2009; Anankanbil *et al.*, 2018; Couvreur *et al.*, 2006].

The phase transitions of milk fat, especially crystallisation and melting (which occur within a temperature range of –40°C to 40°C), are mainly affected by the physicochemical properties of fat, including the fatty acid (FA) profile, fat crystal structure, and the size of fat globules [Hokkanen *et al.*, 2021; Michalski *et al.*, 2004; Truong *et al.*, 2014]. The crystallisation of anhydrous milk fat was reported to differ between the hard (with a higher content of C14:0, C16:0, C18:0) and soft (with a higher content C18:1) milk fat fractions [Truong *et al.*, 2014]. Milk fat can be divided into three fractions based on melting temperature: the low-melting point

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fraction (LMPF) with a melting temperature below 10°C, the middle-melting point fraction (MMPF) with a melting temperature of 10–25°C, and the high-melting point fraction (HMPF) with a melting temperature higher than 25°C. The melting process of each fraction is represented by distinct endothermic peaks on differential scanning calorimetry (DSC) curves [Anankanbil *et al.*, 2018; Brożek *et al.*, 2022a, b; Hokkanen *et al.*, 2021]. The FA profile of milk fat, in particular the triacylglycerol (TAG) profile, determines the melting temperature of LMPF, MMPF, and HMPF, and influences the rheological properties of butter [Omar *et al.*, 2017]. According to Anankanbil *et al.* [2018], a high content of C14:0 and C16:0 increased the melting temperature of MMPF and HMPF, whereas a high content of C18:0, C18:1, and C18:2 decreased melting temperature. A higher C16:0/C18:1 ratio significantly increased the melting temperature of milk fat and butter firmness [Couvreur *et al.*, 2006]. Milk fat melting is a complex process due to the presence of three polymorphic crystal forms of fat with different properties and crystalline structure. The hexagonal α form (the least stable form that undergoes reversible crystallisation during rapid cooling) can be irreversibly transformed to a β' (more stable orthorhombic form) and β form (the most stable triclinic form) [Fredrick *et al.*, 2011; Hondoh & Ueno, 2016; Lopez & Ollivon, 2009; Staniewski *et al.*, 2021; Viriato *et al.*, 2019]. The thermal history of milk fat, which is determined during successive heating and cooling treatments, illustrates changes in the polymorphic structure of crystals and can be used to determine differences in melting behaviour relative to fat that was not subjected to thermal treatment. The thermal history of dairy products is recorded during thermal treatments, such as cream aging in butter production. Thermal history affects the ratio of solid to liquid fat phases, thus influencing the rheological properties of butter [Jukkola & Rojas, 2017; Staniewski *et al.*, 2021].

Milk and dairy products are complex matrices, where each component is characterised by unique phase transformations. In the results of DSC analysis, the phase transition peaks of different components can overlap if they occur within a similar temperature range. The above influences the identification of phase transition peaks in DSC curves and the corresponding peak parameters [Amara-Dali *et al.*, 2007; Lopez *et al.*, 2005; Tomaszewska-Gras, 2013]. Examples of overlapping phase transition peaks include fat crystallisation and melting peaks, and water freezing and ice melting peaks. Therefore, the presence of water in butter can significantly affect the observed phase transition peaks of butter fat in DSC curves. In DSC analyses, more complex thermal properties of milk fat can be observed in butter samples that do not contain water or have reduced water content than in butter samples with standard water content.

The aim of this study was to characterise milk fat from butter made from whey which was obtained from Gouda cheese production, and to identify potential differences between whey butter (WB) and sweet cream butter (milk butter – MB). The following research hypotheses were formulated: 1) MB and WB do not differ in selected physicochemical properties or sensory attributes; 2) the thermal profile of butter is not influenced by the type of butter, FA profile, presence of water or thermal history (DSC analysis of single *vs* multiple heating and cooling treatments). The research hypotheses were validated by measuring the FA profile, thermal properties, colour parameters, texture properties, and sensory attributes of MB and WB.

MATERIALS AND METHODS

Research materials

MB and WB were obtained from a dairy plant (Spółdzielnia Mleczarska Rospuda in Filipów, Warmia and Mazury voivodeship, Poland) as the final products of periodic churning performed under the same industrial conditions. WB was produced from whey obtained from Gouda cheese production. MB was produced from sweet milk cream. MB and WB were collected in triplicate during the week from different batches in the amount of 1 kg ($n=3$).

Water was removed from MB and WB to determine its influence on the thermal characteristics of fat. Butters were clarified at 60°C; the fat phase was separated from the aqueous phase, and the fat phase was filtered through filter paper containing anhydrous sodium bisulphate roasted at a temperature of 150°C. The above procedure was applied to obtain milk butter fat (MBF) and whey butter fat (WBF). The samples of MBF and WBF were obtained from one clarification process for each sample.

Samples of MB and WB were analysed for chemical composition, FA profile, colour parameters, texture properties, and sensory attributes. Samples of MB, WB, MBF, and WBF were used in the DSC analysis.

Chemical composition analysis

The content of total solids, solids-non-fat, and water of WB and MB was determined with the FoodScan Dairy Analyser (Foss, Hillerøed, Denmark). Fat content was determined with the gravimetric method by Rose-Gottlieb according to ISO 1211 [ISO, 2010].

Fatty acid profile analysis

Extraction of milk fat was conducted by the Rose-Gottlieb method [ISO, 2010]. Fatty acid esters were prepared according to ISO 15884 [ISO, 2002]. The 7890A gas chromatograph with a flame ionisation detection (Agilent Technologies, Santa Clara, CA, USA) was used to determine the FA profile on the CP-Sil 88 column (100 m × 0.25 mm, 0.20 μm; Agilent Technologies) with a thermal gradient from 60°C (1 min) to 180°C at 5°C/min; injector and detector temperature, 225°C and 250°C, respectively; flow rate of carrier gas (helium), 0.8 mL/min; split ratio, 1:50; injection volume, 1 μL; liner, 0.4 mm. The reference material was BCR-164 anhydrous milk fat (LGC Standards, Kiełpin, Poland). The proportions of fatty acids were calculated and divided into the following groups: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), short-chain fatty acids (SCFAs; C4–C8), medium-chain fatty acids (MCFAs; C10–C15), long-chain fatty acids (LCFAs; C16–C19), and branched-chain fatty acids (BCFAs; C13:0 *i*, C13:0 *ai*, C14:0 *i*, C15:0 *i*, C15:0 *ai*, C16:0 *i*). The following abbreviations were used in the description of fatty acid isomers: *ai – anteiso*, *i – iso*, *c – cis*, *t – trans*.

Differential scanning calorimetry analysis

The Q10 DSC analyser with a closed refrigerated cooling system (TA Instruments, New Castle, DE, USA) was calibrated with indium. The DSC analysis (sample weight: 10±1 mg; type of measuring crucibles: biconvex hermetically sealed aluminium crucible; gas: nitrogen; flow rate: 50 mL/min, reference sample: empty crucible) was divided into two consecutive and uninterrupted cycles to determine the influence of the thermal history of butter and butter fat on the melting behaviour of milk fat. In the first cycle, measurements were conducted in the following sequence: I – heating from –40°C to 95°C, II – cooling from 95°C to –40°C. The sequences from the first cycle were repeated three times in the second cycle. In each stage, temperature was changed at a rate of 10°C/min, and at the end of each stage, the samples were maintained at the final temperature for 1 min. The results of the DSC analysis were processed in the Universal Analysis 2000 program (TA Instruments). The following parameters of the identified phase transition peaks were determined according to Brożek *et al.* [2022b]: maximum peak temperature (T_{max}), phase transition onset temperature (T_{onset}), transition temperature range as peak width at half height ($\Delta T_{1/2}$), enthalpy (ΔH), peak height at maximum (P_{height}), starting (P_s) and ending (P_e) points of the peak.

Colour analysis

The colour analysis was performed in the CIELab space. Three colour parameters were measured: L^*, lightness from black (0) to white (100); a^*, greenness (–) to redness (+); b^*, blueness (–) to yellowness (+) with the CR-400 Chroma Meter (Konica Minolta Sensing Americas, Inc., Ramsey, NJ, USA) calibrated against a white plate (L^*=95.42; a^*=4.89 and b^*=–2.43). The results were used to calculate chromaticity (C^*), the whiteness index (WI), and the yellowness index (YI) according to equations (1), (2), and (3), respectively.

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

$$WI = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2} \quad (2)$$

$$YI = 142.86\, b^*/L^* \quad (3)$$

The total difference in colour chroma (ΔC^*) between MB and WB was calculated with the use of formula (4), and the total difference in colour (ΔE) between MB and WB was calculated with the use of formula (5).

$$\Delta C^* = {C_{MB}}^{*2} - {C_{WB}}^{*2} \quad (4)$$

$$\Delta E = [({L_{MB}}^* - {L_{WB}}^*)^2 + ({a_{MB}}^* - {a_{WB}}^*)^2 + ({b_{MB}}^* - {b_{WB}}^*)^2]^{1/2} \quad (5)$$

All parameters were calculated according to Pathare *et al.* [2013].

Texture analysis

Instrumental texture analyses were conducted with the use of the TA.XT.plus Texture Analyser (Stable Micro Systems, Godalming, United Kingdom) with Texture Exponent 32 software. Butter samples were stored in a refrigerator at 6±1°C, and texture analyses were conducted at the same temperature in the micro thermostatic chamber (Temperature Applied Sciences Ltd, West Sussex, United Kingdom) cooled with liquid nitrogen from the XL-120 cylinder (Taylor-Wharton Gas Equipment Sdn. Bhd., Shah Alam, Selangor, Malaysia). The sample was positioned centrally under a P/6 cylindrical flat probe. Butter firmness (N), consistency (N×s), cohesiveness (N), and the viscosity index (N×s) were determined in a penetration test (speed: 2 mm/s; distance: 20 mm). Firmness was measured as the maximum force needed to press the probe into the sample. Cohesiveness was described as the maximum force needed to overcome the resistance of the sample when the probe returns to its initial position. Consistency and the viscosity index were expressed by the area under the force *vs* time curve when the probe penetrated the sample and returned to its initial position, respectively. Butter cohesiveness and the viscosity index were described by negative values, and they were related to the direction of probe movement [Sánchez & Pérez, 2012; Tarapata *et al.*, 2021].

Sensory evaluation

The sensory profiles of MB and WB were determined on a descriptive scale according to ISO 13299 [ISO, 2016]. The evaluation was conducted by a panel of eight trained specialists with confirmed taste sensitivity according to ISO 8586 [ISO, 2014]. The descriptors were selected by the panel. Each panellist received a product sheet and a score sheet. The following quality descriptors were considered in the evaluation: appearance (1 descriptor – colour uniformity), aroma (4 descriptors – milky, pasteurisation, nutty and cheesy aroma), consistency (3 descriptors – firmness, cohesiveness, brittleness), and taste (4 descriptors – milky, pasteurisation, nutty and cheesy). Sensory attributes were evaluated on a five-point descriptive scale (1 to 5 points). The intensity of each sensory attribute was determined (1 – absence of descriptor, 5 – very intense).

Statistical analysis

The results of fatty acid profile, colour parameters, texture properties, and sensory attributes for MB and WB were analysed with the Student's *t*-test ($p \leq 0.05$). One-way ANOVA (normal distribution was verified using the Shapiro-Wilk test) was performed for the results of thermal properties, and the significance of differences between means was determined in Duncan's test at α=0.05. The relationship between the percentage content of individual FAs in total FAs in each sample and parameters of phase transition peaks was determined by principal component analysis (PCA). Data were processed statistically in StatSoft Inc. Statistica v. 13.1 software (Tulsa, OK, USA).

RESULTS AND DISCUSSION

In MB, the content of total solids was 85.20±0.03 g/100 g, solids-non-fat – 1.41±0.16 g/100 g, fat – 83.94±0.06 g/100 g, and water – 14.93±0.08 g/100 g. In WB, the content of total solids was 85.87±0.08 g/100 g,

solids-non-fat – 1.52±0.09 g/100 g, fat – 84.56±0.06 g/100 g, and water – 14.27±0.09 g/100 g. The obtained results were consistent with the values of Food and Agriculture Organization/World Health Organization standard [FAO/WHO, 2018] and the values given in the literature [Kasapcopur *et al.*, 2021; Nadeem *et al.*, 2014].

Fatty acid profile

An analysis of the FA profile revealed that C14:0, C16:0, C18:0, and C18:1 Σ*c* FAs dominated and accounted for 75.44 and 78.25 g/100 g of total FAs in WB and MB, respectively (Table 1). The remaining FAs were detected in smaller quantities. Samples of MB and WB differed significantly ($p \leq 0.05$) in the proportions of most FAs. Whey butter was significantly ($p \leq 0.05$) more abundant in C4:0, C15:0 *i*, C15:0 *ai*, C17:1, C18:0, C18:1 Σ*t*, C18:1 Σ*c*, C18:2, C18:3, C18:2 *c*9, *t*11, MUFAs, PUFAs, SCFAs, and LCFAs, but less abundant in C10:0, C10:1, C12:0, C13:0 *i*, C13:0 *ai*, C13:0, C14:0, C14:1, C15:0, C16:0 *i*, C16:0, C16:1, SFAs, and MC -FAs than MB. The FA profiles of both products were typical of MB and WB [Gómez-Mascaraque *et al.*, 2020; Nadeem *et al.*, 2014; Staniewski *et al.*, 2021].

The observed differences in the FA profiles of WB and MB probably resulted from the whey origin or whey cream production technology. The production of whey cream involves a larger number of processing operations than the production of milk cream, which promotes changes in milk fat globules (MFG) and milk fat globule membrane (MFGM) components in whey cream [Brighenti *et al.*, 2021; Jukkola & Rojas, 2017]. The FA profiles of whey depend on the cheese from which whey originates. WB was obtained from the whey of Gouda cheese, which is commonly produced in Europe. The available literature provides only reports from the studies of whey butter produced from the whey of regional cheeses or experimental products; therefore, some differences could be noticed between the FA profile of WB in the present study and the works of other authors. Çetinkaya [2021] found a higher percentage of unsaturated fatty acids (UFAs) in whey fat (from Kashar cheese) than in milk fat, but Kasapcopur *et al.* [2021] found no differences between whey fat (a mixture of whey from rennet-coagulated White-brined and Kashar and acid-coagulated Lor cheeses) and milk fat in the content of UFAs. Brighenti *et al.* [2021] reported greater differences in the size of MFG and a higher content of phospholipids in whey cream from soft cheese (Munster) than in milk cream. They observed that MFGM could be damaged during cheesemaking and whey processing operations, which can promote the formation of larger MFG aggregates. The FA profile of the MFG core differs from the FA profile of MFGM. The FA composition of MFGM phospholipids is characterised by a higher content of UFAs than the FA composition of core TAGs [Jhanwar & Ward, 2014]. According to Fauquant *et al.* [2005], MFGM are more abundant in UFAs, in particular PUFAs whose content is approximately six times higher than in the MFG core. Based on these studies, it can be assumed that the higher content of UFAs-rich phospholipids in WB compared to MB was responsible for the different FA profile in MB and WB, but this requires further studies.

TABLE 1. Fatty acid profile of milk butter and whey butter (g/100 g total fatty acids).

Fatty acid	Milk butter	Whey butter
C4:0	2.14±0.07^{b}	2.78±0.12^{a}
C6:0	1.72±0.08^{a}	1.81±0.02^{a}
C8:0	1.11±0.01^{a}	1.13±0.02^{a}
C10:0	2.65±0.04^{a}	2.56±0.03^{b}
C10:1	0.33±0.02^{a}	0.28±0.01^{b}
C11:0	0.02±0.01^{a}	0.02±0.01^{a}
C12:0	3.31±0.07^{a}	3.06±0.05^{b}
C12:1	0.05±0.01^{a}	0.02±0.01^{a}
C13:0 *i*	0.11±0.01^{a}	0.04±0.01^{b}
C13:0 *ai*	0.10±0.01^{a}	0.08±0.01^{b}
C13:0	0.11±0.01^{a}	0.09±0.01^{b}
C14:0 *i*	0.15±0.02^{a}	0.14±0.01^{a}
C14:0	12.03±0.17^{a}	10.79±0.14^{b}
C14:1	1.31±0.03^{a}	0.93±0.01^{b}
C15:0 *i*	0.28±0.01^{b}	0.33±0.01^{a}
C15:0 *ai*	0.46±0.01^{b}	0.57±0.02^{a}
C15:0	1.36±0.05^{a}	1.19±0.02^{b}
C16:0 *i*	0.29±0.01^{a}	0.28±0.01^{b}
C 16:0	36.20±0.53^{a}	30.61±0.92^{b}
C16:1	2.11±0.07^{a}	1.67±0.05^{b}
C17:0	0.61±0.06^{a}	0.64±0.02^{a}
C17:1	0.14±0.02^{b}	0.25±0.02^{a}
C18:0	9.71±0.32^{b}	11.33±0.44^{a}
C18:1 Σ*t*	1.13±0.16^{b}	3.31±0.14^{a}
C18:1 Σ*c*	20.31±0.34^{b}	22.71±0.69^{a}
C18:2 Σ*t*	0.11±0.03^{a}	0.07±0.01^{a}
C18:2	1.16±0.03^{b}	1.23±0.09^{a}
C18:2 *c*9, *t*11	0.38±0.03^{b}	1.08±0.04^{a}
C18:3	0.50±0.02^{b}	0.88±0.02^{a}
C19:0	0.12±0.02^{a}	0.11±0.04^{a}
SFA	72.48±0.45^{a}	67.57±0.78^{b}
MUFA	25.37±0.22^{b}	28.18±0.37^{a}
PUFA	2.27±0.07^{b}	4.38±0.12^{a}
SCFA	4.97±0.21^{b}	5.72±0.08^{a}
MCFA	22.26±0.23^{a}	20.10±0.11^{b}
LCFA	72.89±0.29^{b}	74.30±0.44^{a}
BSFA≤C16	1.39±0.02^{a}	1.44±0.03^{a}

Values are means ± standard deviations ($n=3$); values with different superscripts in row differ significantly at $p \leq 0.05$. *i*– *iso*; *ai* – *anteiso*; *c* – *cis*; *t* – *trans*; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; SCFA – short-chain fatty acids (C4–C8); MCFA – medium-chain fatty acids (C10–C15); LCFA – long-chain fatty acids, (C16–C19); BSFA – branched saturated fatty acids (C13:0 *i*, C13:0 *ai*, C14:0 *i*, C15:0 *i*, C15:0 *ai*, C16:0 *i*).

Differential scanning calorimetry (DSC)

The shape of DSC curves differed subject to butter type, water content, and the sample's thermal history (Figure 1, Figure 2, Figure 3). These differences affected the parameters of phase transition peaks describing both exothermic (Table 2) and endothermic (Table 3) transitions. Butter samples and the corresponding fat samples were compared to determine which phase transition peaks correspond to changes in milk fat and water. MBF and WBF were also used in the analysis to minimise differences in the fat content of samples which could have influenced the shape of DSC curves and the parameters of phase transition peaks.

The DSC curves plotted for MBF and WBF had a similar shape, but differed from the curves generated for MB and WB due to the presence of water in the analysed samples (Figure 1). The phase transitions of water in the butter samples (water freezing, ice melting, evaporation) induced much greater changes in heat flow than the phase transitions of fat. Phase transitions of water were visualised by large peaks in DSC curves (Figure 1, Figure 2, Figure 3). These peaks overlapped the peaks representing the phase transitions of milk fat, in particular endothermic peaks associated with fat melting (Figure 2). The parameters of crystallisation and ice melting peaks are consistent with those given in the literature [Tomaszewska-Gras, 2012].

Two exothermic peaks were observed in DSC curves in the range of –40°C to 16.5°C during cooling from 95°C to –40°C. A first peak (A) occurred in all samples between around –40°C to around 14°C, and a second peak (B) occurred between around 10°C to around 15°C (Figure 2).

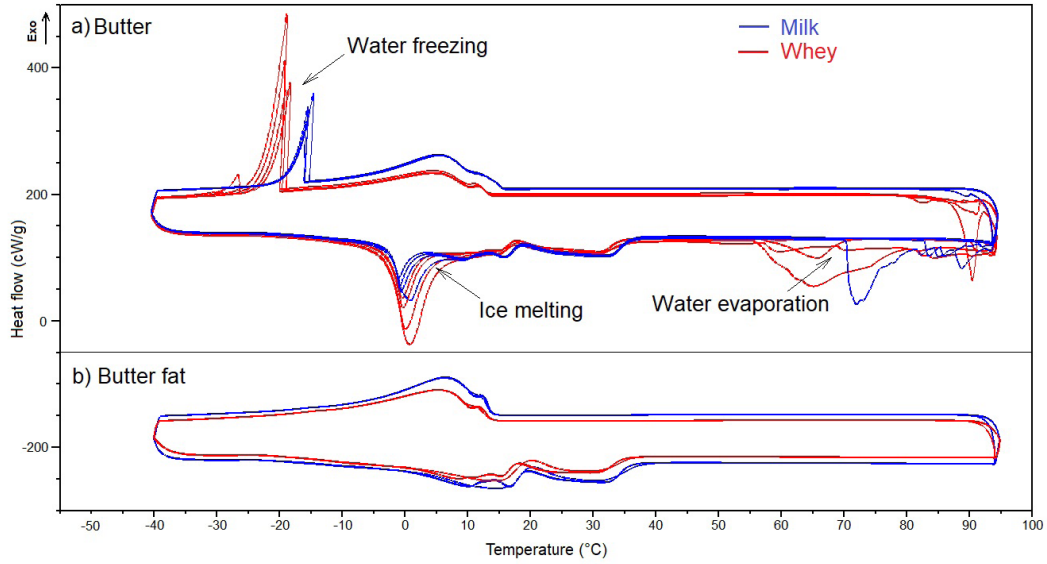

FIGURE 1. Differential scanning calorimetry (DSC) curves of milk butter and whey butter (a) and the corresponding fats (b) with an indication of the phase transitions of water, obtained in both cycles of the DSC analysis.

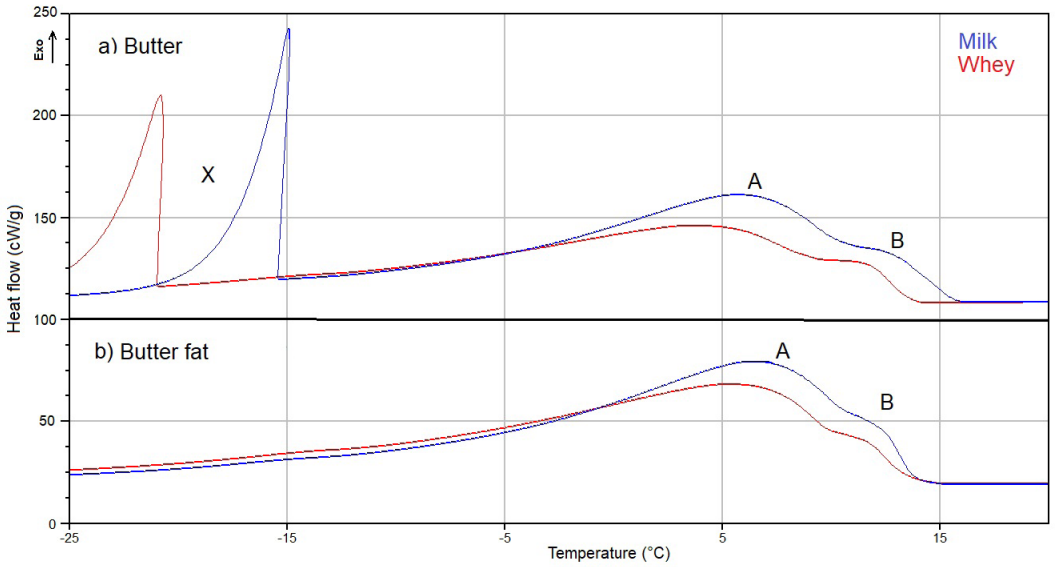

FIGURE 2. Exothermic peaks of fat crystallisation (A, B) and water freezing (X) in milk butter and whey butter (a) and the corresponding fats (b).

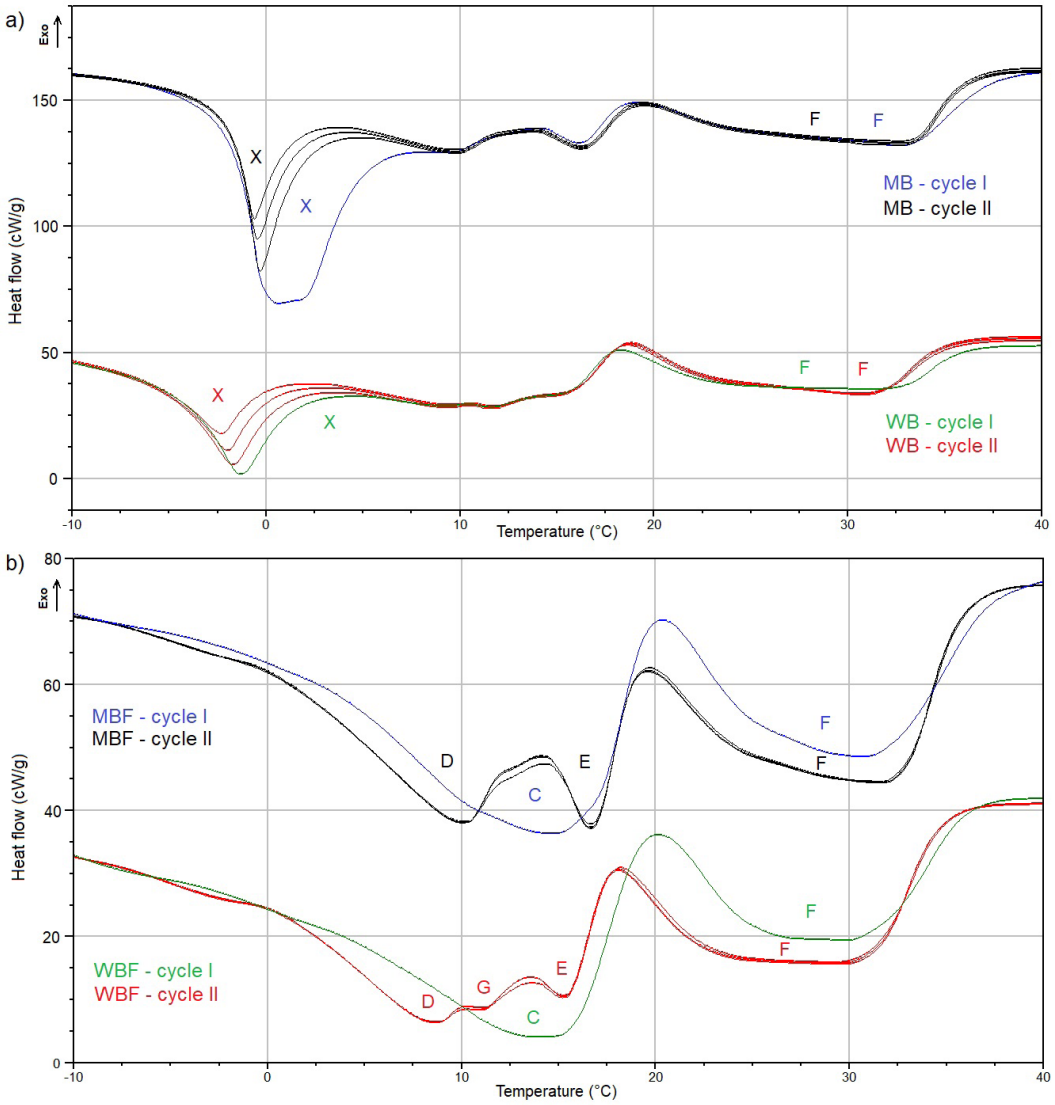

FIGURE 3. Melting curves of milk butter (MB), whey butter (WB) (a) and the corresponding fats (MBF and WBF, respectively) (b) in cycle I and cycle II of the differential scanning calorimetry (DSC) analysis. Endothermic peaks represent: C – low-melting point fraction (LMPF) and middle--melting point fraction (MMPF) melting; D and G – LMPF melting; E – MMPF melting; F – high-melting point fraction (HMPF) melting. Peaks X denote ice melting (a).

Peak B was not completely separated from peak A at a cooling rate of 10°C/min, therefore peaks A and B were determined jointly as a single exothermic peak (undifferentiated peaks A and B) (Table 2). Exothermic peaks (Figure 2) had similar characteristics to those reported by Smet *et al.* [2010]. In a mentioned study, onset temperature was determined at 15.35°C in the first crystallisation peak and at 12.07°C in the second crystallisation peak. Exothermic peaks (A–B) were associated with crystallisation that occurs within a temperature range of –40°C to 20°C [Brożek *et al.*, 2022a, b; Hokkanen *et al.*, 2021; Smet *et al.*, 2010]. Truong *et al.* [2014] noted two peaks between 0°C to 15°C in hard milk fat fraction. A first peak within the temperature range of 0°C to 10°C and a second additional smaller peak between 10°C to 15°C.

An analysis of the exothermic peaks of milk fat crystallisation demonstrated that water removal had no significant ($p>0.05$) effect on T_{max} values in MB samples or T_{max} and T_{onset} values in WB samples (Table 2). The remaining parameters of the exothermic peaks of fat crystallisation differed significantly ($p\leq0.05$) between MB and MBF as well as between WB and WBF. T_{onset} was the only parameter that did not differ significantly ($p>0.05$) between MBF and WBF. Values of parameters ΔH and P_{height} were higher in the crystallisation peaks of MBF and WBF than in the crystallisation peaks of MB and WB, respectively, due to a higher content of milk fat.

Endothermic peaks were observed in DSC curves in the range of –40°C to 40°C, especially in the range of –10°C to 40°C, during heating within a temperature range of –40°C to 95°C (Figure 3). The DSC curves presenting fat melting parameters differed across DSC cycles. Two endothermic peaks (C and F) were noted in cycle I, whereas four endothermic peaks (D–G) that did not change in successive heating and cooling treatments were observed in cycle II. The first peak (C) with an unclear onset temperature and its endset at 16–21°C was observed only in cycle I. Peak C was associated with unseparated LMPF and MMPF. In cycle II of the DSC

TABLE 2. Parameters of exothermic phase transition peaks – undifferentiated peaks A and B in cycle II of the differential scanning calorimetry (DSC) analysis of milk butter (MB), milk butter fat (MBF), whey butter (WB) and whey butter fat (WBF).

Sample	T_{max} (°C)	T_{onset} (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	P_{height} (cW/g)	P_s (°C)	P_e (°C)
MB	6.10±0.05[a]	15.75±0.12[a]	42.49±0.92[c]	13.42±0.08[c]	47.82±0.28[b]	16.24±0.19[a]	*
MBF	6.03±0.04[a]	14.02±0.14[b]	64.31±0.27[a]	15.33±0.18[b]	59.04±0.08[a]	15.68±0.05[b]	–37.55±0.49[a]
WB	4.93±0.04[b]	13.89±0.06[b]	36.11±0.17[d]	15.53±0.19[b]	36.87±0.07[c]	14.74±0.21[c]	*
WBF	5.05±0.03[b]	13.70±0.21[b]	59.17±0.29[b]	17.54±0.05[a]	48.38±0.30[b]	14.12±0.10[d]	–38.53±0.18[b]

The values are means ± standard deviations (n=3); values with different superscripts (a–d) in column differ significantly at $p \leq 0.05$. T_{max} – maximum peak temperature; T_{onset} – phase transition onset temperature; ΔH – enthalpy; $\Delta T_{1/2}$ – transition temperature range as peak width at half height; P_{height} – peak height at maximum; P_s – starting point of the peak; P_e – ending point of the peak.

*No clear endset temperature (the water crystallisation peak was not considered in the identification of the end of peak A).

analysis, peak C was transformed to peaks D and E. Peak D was identified as LMPF; it did not have a clear onset temperature, and its endset temperature was determined at 13–15°C. Peak G that was not clearly separated from peak D was observed only on WBF curves within a temperature range of 10–13°C. Peaks G and D represented LMPF melting; therefore, they were considered jointly as a single peak D during the determination of endothermic phase transition peaks (Table 3). Peak E (from 13–14°C to 18–19°C) was identified as MMPF and peak F (from 18–24°C to 34–42°C) was identified as HMPF. Endothermic peaks (C–F) were identified as overlapping LMPF and MMPF peaks (C), and as separate LMPF (D and G), MMPF (E), and HMPF peaks (F) based on published data [Brożek *et al.*, 2022a; Hokkanen *et al.*, 2021; Kasapcopur *et al.*, 2021; Smet *et al.*, 2010; Staniewski *et al.*, 2021]. Our previous study demonstrated that the characteristics of the crystallisation and melting peaks of fat from freeze-dried milk, cream and buttermilk were affected by the content of fat and FAs in samples [Brożek *et al*., 2022a]. Two fat crystallisation peaks in the DSC curves of buttermilk and milk, and three crystallisation peaks in the DSC curve of cream were reported due to a higher concentration of SFAs in cream than in buttermilk and milk. Małkowska *et al.* [2021] also observed three crystallisation peaks of the solid fraction of milk fat with a high content of SFAs.

The presence of water in MB and WB had a greater influence on the parameters describing the melting peaks of LMPF and MMPF than HMPF (Table 3) due to the presence of ice melting peaks overlapping LMPF and MMPF melting peaks in DSC curves (Figure 3). Ice melting peaks overlapped milk fat melting peaks C–E in the DSC curves of MB and WB; therefore, the corresponding parameters could not be determined. For this reason, only the parameters of endothermic peaks denoting HMPF melting (peak F) could be compared between cycle I and cycle II. The identification of peaks D and E in DSC curves depended on the size of the ice melting peak. Due to the higher water content in WB than in MB, ice melting peaks were larger on the DSC curve of WB, and they showed greater overlap with peaks C–E than on the DSC curve of MB (Figure 1). Tomaszewska-Gras [2012] found that ΔH of the ice melting peak and the water freezing peak were dependent on the water content of butter.

The parameters of endothermic peaks (C–F) (Figure 3) are presented in Table 3. A comparison of all products and products within each group (MB and MBF; WB and WBF) revealed that the parameters of the endothermic peaks (C–F) of butters and the corresponding fats differed significantly ($p \leq 0.05$) in most cases. In cycle I of the DSC analysis, the P_{height} of peak C differed between MBF and WBF ($p \leq 0.05$). In peak C, lower values of P_{height} indicate that the phase transition of MBF components was a less energy-intensive process than the phase transition of WBF components. In cycle I of the DSC analysis, the parameters describing peak F differed significantly ($p \leq 0.05$) across products (excluding P_e). In cycle I, differences ($p \leq 0.05$) in T_{onset} and P_s values were noted between MB and MBF and between WB and WBF, whereas no differences ($p > 0.05$) in the values of P_e were observed between MB and WB, or in the values of P_s and P_e between MBF and WBF. The above indicates that fat type did not determine the beginning and end of HMPF melting in cycle I, unlike water which influenced the beginning of HMPF melting in cycle I.

In cycle II, peak C was transformed to LMPF (D) and MMPF peaks (E) (Figure 3). A comparison of MBF and WBF did not reveal significant ($p > 0.05$) differences in the T_{onset} and P_s values of peak D (Table 3), which indicates that the type of butter fat had no effect on the onset of LMPF melting. In turn, MBF and WBF differed significantly ($p \leq 0.05$) in all parameters of peak E, which indicates that the type of butter fat determined the melting characteristics of MMPF. In cycle II of the DSC analysis, the parameters describing peak F differed ($p \leq 0.05$) between MBF and WBF (except for $\Delta T_{1/2}$ and P_e). The differences ($p > 0.05$) were found between MB and MBF (T_{max}, T_{onset} and P_s) and between WB and WBF (T_{max} and T_{onset}), which indicates that water content influenced the mentioned parameters of the HMPF peak in cycle II.

The thermal history of butters and the corresponding fats affected DSC curves showing their melting behaviour (Figure 3) and the parameters of endothermic peaks (Table 3). The greatest differences were noted between LMPF and MMPF. After the first stage of heating, cooling and reheating, the overlapping melting peaks of LMPF and MMPF (peak C) split into separate LMPF (peak D) and MMPF (peak E) peaks. Peak C was completely transformed to peak D (peaks D and G in WBF) and peak E; therefore, the corresponding parameters could not be accurately compared between cycle I and cycle II, and only a general change trend was confirmed. The applied thermal processing regime also influenced HMPF melting parameters (peak F). The endothermic peaks of MBF

TABLE 3. Parameters of endothermic phase transition peaks in cycle I and cycle II of the differential scanning calorimetry (DSC) analysis of milk butter (MB), milk butter fat (MBF), whey butter (WB) and whey butter fat (WBF).

Peak	Cycle	Sample	T_{max} (°C)	T_{onset} (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	P_{height} (cW/g)	P_s (°C)	P_e (°C)
C	I	MB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		MBF	15.25±0.58^{a}	2.88±1.16^{a}	19.66±1.63^{a}	10.97±0.57^{a}	–31.01±1.15^{b}	*	20.68±0.51^{a}
		WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		WBF	15.05±0.65^{a}	2.43±2.37^{a}	15.91±2.40^{a}	10.27±1.34^{a}	–27.48±1.52^{a}	*	20.14±0.14^{a}
D	II	MB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		MBF	9.41±0.08^{a}	1.48±0.33^{a}	5.51±0.49^{a}	6.33±0.21^{b}	–12.22±0.71^{b}	0.12±0.41^{a}	14.42±0.10^{a}
		WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		WBF	7.90±0.28^{b}	1.36±0.34^{a}	4.12±0.68^{b}	7.71±0.25^{a}	–9.93±1.58^{a}	0.02±0.32^{a}	13.82±0.36^{b}
E	II	MB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		MBF	17.17±0.03^{a}	14.98±0.08^{a}	2.22±0.14^{a}	2.43±0.13^{a}	–16.13±0.90^{b}	14.47±0.11^{a}	19.90±0.07^{a}
		WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		WBF	16.22±0.04^{b}	14.26±0.05^{b}	0.81±0.19^{b}	2.07±0.16^{b}	–7.02±0.85^{a}	13.91±0.06^{b}	18.46±0.04^{b}
F	I	MB	32.46±0.51^{aA}	19.71±0.47^{cA}	15.62±1.04^{aA}	11.32±0.45^{bA}	–25.26±1.61^{bA}	19.18±0.13^{bA}	41.84±2.32^{aA}
		MBF	31.64±0.61^{aA}	21.66±0.32^{aA}	15.76±0.82^{aA}	10.85±0.40^{cA}	–25.59±1.37^{bA}	21.11±0.81^{aA}	41.99±0.91^{aA}
		WB	30.21±0.80^{bA}	18.72±0.09^{dA}	12.52±0.91^{bA}	11.81±0.65^{aA}	–19.54±1.86^{aA}	18.25±0.08^{cA}	39.78±1.95^{aA}
		WBF	30.33±0.37^{bA}	20.90±0.32^{bA}	12.68±0.79^{bA}	11.76±0.42^{aA}	–19.73±1.59^{aA}	20.43±0.52^{aA}	39.97±1.07^{aA}
	II	MB	32.67±0.33^{aA}	19.96±0.26^{bA}	15.19±0.72^{aA}	11.52±0.21^{aA}	–26.48±0.69^{bA}	19.48±0.26^{bA}	41.25±0.91^{aA}
		MBF	32.04±0.21^{bA}	20.46±0.13^{aB}	15.56±0.37^{aA}	11.40±0.19^{aA}	–25.79±0.41^{bA}	19.95±0.17^{aB}	39.96±0.50^{abB}
		WB	31.05±0.35^{bA}	18.78±0.31^{cA}	13.36±0.62^{bA}	12.09±0.30^{aA}	–21.95±1.18^{aA}	18.35±0.24^{cA}	38.50±1.29^{bA}
		WBF	30.49±0.24^{cA}	19.30±0.20^{bB}	13.57±0.26^{bA}	11.63±0.15^{aA}	–21.35±0.47^{aA}	18.53±0.11^{cB}	40.11±0.17^{abA}

The values are means ± standard deviations (n=3); values with different lowercase letters (a–d) in superscripts in column separately for each peak and cycle differ significantly at $p \leq 0.05$; values with different uppercase letters (A–B) in superscripts for individual product in cycle I and cycle II of peak F differ significantly at $p \leq 0.05$. T_{max} – maximum peak temperature; T_{onset} – phase transition onset temperature; ΔH – enthalpy; $\Delta T_{1/2}$ – transition temperature range as peak width at half height; P_{height} – peak height at maximum; P_s – starting point of the peak; P_e – ending point of the peak; n.d. – not detected (the water freezing peak overlapped fat melting peaks).

and WBF differed in T_{onset} and P_s values, and the endothermic peak of MBF differed ($p \leq 0.05$) in the value of P_e between cycle I and cycle II (Table 3). In the DSC analysis, the peak parameters of all products were less scattered in cycle II than in cycle I, which indicates that the milk fat melting process was more stable in cycle II. The transition of peak C to peaks D and E (Figure 3) and the smaller scatter of results in cycle II than in cycle I (Table 3) could be explained by the transition of fat crystals from the polymorphic form α to forms β' and β. According to the literature, the hexagonal α form is irreversibly transformed to the more stable orthorhombic β' and triclinic β forms during thermal processing [Fredrick *et al.*, 2011; Hondoh & Ueno, 2016; Lopez & Ollivon, 2009; Staniewski *et al.*, 2021; Viriato *et al.*, 2019]. Our previous study demonstrated that thermal history influenced the parameters of phase transition peaks and fat melting peaks of cream, milk, and buttermilk [Brożek *et al.*, 2022b]. In turn, Staniewski *et al.* [2020] found that thermal history affected the melting process of anhydrous milk fat.

The following variables were subjected to PCA to identify variables that were most highly correlated with MBF and WBF, as well as variables that were bound by the strongest correlations: the FA profile of samples (g/100 g total FAs) (Table 1) and the peak parameters (Table 2 and Table 3). The first two principal components, PC1 and PC2, explained 80.50% of total variance in input data, but only PC1 explained 69.78% of the variance, whereas PC2 explained only 10.72% of the variance (Figure 4). Two clusters of variables (left and right) were formed at the ends of the PC1 axis. The variables from each cluster were strongly correlated with each other.

The loading vectors indicate that the variables were correlated. The greater the acute angle, the stronger the correlation between the variables in a given direction, whereas an obtuse angle points to an inverse correlation, and a straight angle indicates an absence of a correlation between variables. To facilitate the description of variables, peaks were marked with letters A and C–F, and the symbols I and II in the subscript denote cycles I and II, respectively. The cluster on the left side of the PC plot contains points representing variables with negative loadings on the PC1 axis in the range of –0.99 to –0.75 (Figure 4). These variables were higher in WBF than

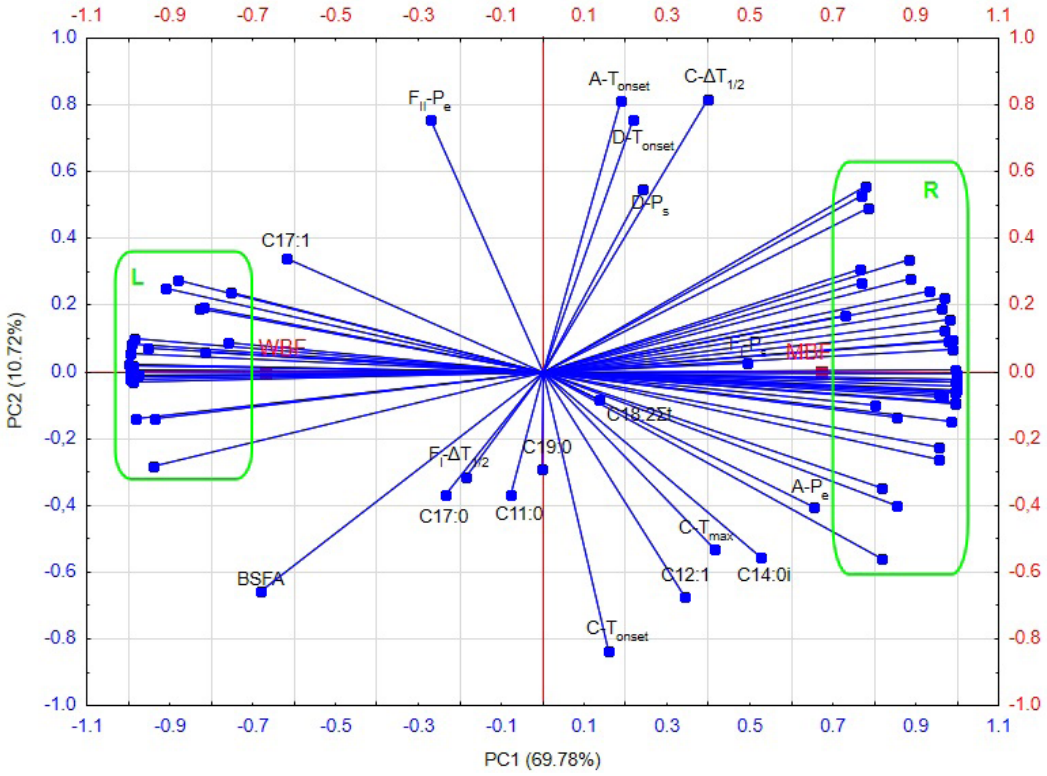

FIGURE 4. Principal component analysis biplot of milk butter fat (MBF) and whey butter fat (WBF), and the contribution of the parameters of peaks C and F in cycle I and peaks A, D, E and F in cycle II of the differential scanning calorimetry (DSC), and the content fatty acids (g/100 g total fatty acids) to principal component 1 (PC1) and principal component 2 (PC2). The variables were arranged in ascending (cluster left, L) and descending (cluster right, R) order based on loadings. The L cluster contains C15:0 *ai*, C18:1 Σ*t*, D-$\Delta T_{1/2}$, F_{II}-P_{height}, C18:3, C18:2 *c*9, *t*11, C15:0 *i*, MUFA, PUFA, C18:2, C18:1 Σ*c*, LCFA, E-P_{height}, A-$\Delta T_{1/2}$, C18:0, F_I-P_{height}, C-P_{height}, C4:0, SCFA, C8:0, D-P_{height}, C6:0, F_{II}-$\Delta T_{1/2}$. The R cluster contains C16:0, C14:1, C16:1, C14:0, F_{II}-T_{max}, C12:0, C13:0 *ai*, C13:0, SFA, MCFA, A-P_{height}, F_{II}-P_s, F_{II}-ΔH, C10:0, F_{II}-T_{onset}, C10:1, A-ΔH, D-T_{max}, C15:0, E-P_e, E-ΔH, F_I-ΔH, A-P_s, E-T_{max}, F_I-T_{max}, E-T_{onset}, A-T_{max}, C13:0 *i*, E-$\Delta T_{1/2}$, C16:0 *i*, D-ΔH, E-P_s, D-P_e, C-P_e, C-ΔH, F_I-P_e and F_I-T_{onset}. Symbols I, II in the subscript of peak F denote cycles I and II of the DSC analytical sequence, respectively.

in MBF. The left cluster includes the following variables: C15:0 *ai*, C18:1 Σ*t*, D-$\Delta T_{1/2}$, F_{II}-P_{height}, C18:3, C18:2 *c*9, *t*11, C15:0 *i*, MUFA, PUFA, C18:2, C18:1 Σ*c*, LCFA, E-P_{height}, A-$\Delta T_{1/2}$, C18:0, F_I-P_{height}, C-P_{height}, C4:0, SCFA, C8:0, D-P_{height}, C6:0, and F_{II}-$\Delta T_{1/2}$, which were arranged in an ascending order based on the loadings. The cluster on the right side of the PC1 axis contains points denoting variables that were bound by inverse correlations relative to the left cluster and were characterised by highly positive loadings in the range of 0.73 to 0.99. The values of these variables were higher in MBF than in WBF. The right cluster includes following variables: C16:0, C14:1, C16:1, C14:0, F_{II}-T_{max}, C12:0, C13:0 *ai*, C13:0, SFA, MCFA, A-P_{height}, F_{II}-P_s, F_{II}-ΔH, C10:0, F_{II}-T_{onset}, C10:1, A-ΔH, D-T_{max}, C15:0, E-P_e, E-ΔH, F_I-ΔH, A-P_s, E-T_{max}, F_I-T_{max}, E-T_{onset}, A-T_{max}, C13:0 *i*, E-$\Delta T_{1/2}$, C16:0 *i*, D-ΔH, E-P_s, D-P_e, C-P_e, C-ΔH, F_I-P_e, and F_I-T_{onset}, which were arranged in a descending order based on the loadings.

A higher content of SFAs and MCFAs, especially C12:0, C14:0 and C16:0, in MBF than in WBF increased the values of T_{max}, ΔH and P_s and decreased the value of $\Delta T_{1/2}$ in crystallisation peak A (which indicates that MBF crystallisation was characterised by higher enthalpy, occurred at a higher temperature, and proceeded less rapidly than WBF crystallisation), whereas an increase in the content of MUFAs, PUFAs, and LCFAs, including C18:1, and, to a much lesser degree, an increase in the content of SCFAs, including C4:0, C6:0 and C8:0, induced a reverse trend (which indicates that WBF crystallisation was characterised by lower enthalpy, occurred at a lower temperature, and proceeded more rapidly than MBF crystallisation). In our previous study, we found that T_{onset} increased, whereas $\Delta T_{1/2}$ decreased with a rise in the content of SFAs and MCFAs, in particular C14:0 and C16:0, in freeze-dried milk [Brożek *et al.*, 2022b]. In turn, Truong *et al.* [2014] reported that anhydrous milk fat had a crystallisation peak in the temperature range from –5°C to 10°C. The observed peak was smaller and characterised by higher values of T_{max} and T_{onset} in the hard fraction compared to soft fraction of milk fat.

The content of SFAs and MCFAs, especially C12:0, C14:0 and C16:0, influenced the value of P_e of the melting peak corresponding to the merged LMPF and MMPF peaks (peak C); the values of T_{max}, ΔH and P_e of the LMPF peak (peak D); the values of T_{max}, T_{onset}, ΔH, $\Delta T_{1/2}$, P_s and P_e of the MMPF peak (peak E); the values of T_{max}, T_{onset}, ΔH and P_e in cycle I; and the values of T_{max}, T_{onset}, ΔH and P_s in cycle II of the HMPF melting peak (peak F). The above implies that the maximum difference in heat flow in all endothermic peaks (based on the absolute values in cycles I and II), the enthalpy of LMPF, MMPF and HMPF (in cycles I and II), and the melting rate of MMPF were lower in WBF than MBF, whereas the beginning

TABLE 4. Colour parameters of milk butter (MB) and whey butter (WB).

Sample	L^*	a^*	b^*	C^*	WI	YI	ΔC^*	ΔE
MB	89.13±0.43[a]	–3.41±0.03[a]	32.08±0.11[a]	32.26±0.11[a]	65.96±0.15[b]	51.42±0.25[a]	2.25±0.11	2.33±0.27
WB	88.96±0.58[a]	–3.17±0.06[b]	29.84±0.23[b]	30.01±0.23[b]	68.02±0.02[a]	47.92±0.05[b]		

Values are means ± standard deviations (n=3); values with different superscripts in column differ significant at $p \leq 0.05$. L^* – lightness; a^* – position between redness and greenness; b* – position between blueness and yellowness; C^* – chromaticity; WI – whiteness index; YI – yellowness index; ΔE – total colour difference; ΔC^* – difference in saturation.

of MMPF (peak E) and HMPF (cycles I and II, peak F) melting and the end of melting in the overlapping peaks of LMPF and MMPF (peak C), LMPF (peak D) and MMPF (peak E) occurred at a lower temperature in WBF than MBF. The content of MUFAs, PUFAs, and LCFAs, including C18:0, and to a much smaller extent, the content of SCFAs, including C4:0, C6:0 and C8:0, induced a drop in the above parameters, but increased P_{height} values of all endothermic peaks, and $\Delta T_{1/2}$ values of LMPF and HMPF (cycle II) melting peaks. The above implies that LMPF and HMPF (cycle II) melted more rapidly in WBF than MBF. The presence of peak G can probably be attributed to a higher content of MUFAs, PUFAs, and LCFAs, including C18:0, in WBF than in MBF. According to Staniewski *et al.* [2021], the FA profile of TAGs can affect the melting behaviour of milk fat, in particular the value of P_s, and it can influence the textural properties of butter. Anankanblil *et al.* [2018] demonstrated that the P_s value of MMPF and HMPF crystallisation and melting peaks decreased with a rise in the content of C18:0, C18:1, C18:2, and C18:3, and increased with a rise in the content of C14:0 and C16:0. Larsen *et al*. [2014] and Smet *et al*. [2010] made similar observations also in the P_s value of the LMPF peak. The saturated FA C18:0 decreased the P_s values of milk fat crystallisation and melting peaks despite the fact that it has a higher melting temperature than C18:1, C18:2, and C18:3. The above could be attributed to mammary desaturase activity which transforms C18:0 into C18:1, thus lowering melting temperature [Anankanblil *et al.*, 2018; Larsen *et al.*, 2014]. Buldo *et al.* [2013] reported a correlation between the melting temperature (P_s) of MMPF and the content of C14:1, C16:0, C18:1 Σ*c*, C18:1 Σ*t*, and C18:2 *c*9, *t*11. In a PCA conducted in our previous study, the content of many FAs was correlated with the parameters of crystallisation and melting peaks of fat from freeze-dried milk, including the values of T_{onset} of the crystallisation peak, $\Delta T_{1/2}$ of the LMPF melting peak, and T_{max} and T_{onset} of the MMPF peak [Brożek *et al.,* 2022b]. These variables were inversely corelated with the $\Delta T_{1/2}$ of the crystallisation peak and the content of LCFAs, MUFAs, and PUFAs, including C18:0, C18:1, C18:2 and C18:3.

TABLE 5. Textural properties of milk butter (MB) and whey butter (WB).

Sample	Firmness (N)	Consistency (N×s)	Cohesiveness (N)	Viscosity (N×s)
MB	1.80±0.05[a]	30.90±0.74[a]	–0.86±0.07[b]	–1.54±0.19[a]
WB	1.68±0.06[b]	30.67±1.59[a]	–0.67±0.08[a]	–1.30±0.03[a]

Values are means ± standard deviation (n=3); values with different superscripts in column differ significantly at $p \leq 0.05$.

Colour

Samples of MB and WB differed significantly ($p \leq 0.05$) in the colour parameters a^*, b^*, C^*, WI and YI, but not in parameter L^* ($p > 0.05$) (Table 4). The values of b^* and YI were higher in MB, which indicates that it was more yellow in colour than WB. Colour chroma was also higher in MB, whereas the values of parameter a^* and the whiteness index were higher in WB.

The values of L^*, a^* and b^* in MB were similar to those given in the literature [Gómez-Mascaraque *et al.*, 2020]. In turn, WB was characterised by similar lightness, a higher contribution of greenness, and a significantly higher contribution of yellowness relative to other studies [Jinjarak *et al.*, 2006; Kasapcopur *et al.*, 2021]. The total colour difference between MB and WB reached 2.33 (Table 4). The detection of colour difference could be easily possible even by an inexperienced observer if the ΔE=2.0–3.5 [Dobrzańska & Cais-Sokolińska, 2014].

Texture

The textural properties of MB and WB are presented in Table 5. Milk butter and whey butter differed significantly ($p \leq 0.05$) in firmness. Internal stickiness (described by cohesiveness values) was significantly ($p \leq 0.05$) higher in MB than WB. The examined products did not differ significantly ($p > 0.05$) in consistency or viscosity. The obtained lower values of firmness and cohesiveness for WB compared to MB are in agreement with the fatty acid profiles of the samples. In turn WB was more abundant in MUFAs and PUFAs (Table 1) which contribute to butter softness. Amal [2009] and Jinjarak *et al.* [2006] reported lower hardness and cohesiveness in whey butter than in sweet cream butter. In the work of Staniewski *et al.* [2021], the rheological properties of butter were correlated with the composition of TAGs in the fat phase, and butter firmness decreased with a rise in the content of C18:1, C18:2, and C18:3 PUFAs in TAGs.

Sensory evaluation

The sensory evaluation revealed that MB and WB differed significantly ($p \leq 0.05$) in the values of consistency descriptors: firmness, brittleness, and cohesiveness (Figure 5) as well as in the intensity of milky and nutty aroma, and cheesy taste ($p \leq 0.05$). The remaining descriptors of taste, aroma, and colour uniformity did not differ significantly ($p > 0.05$) between the compared butters.

In a study by Mallia *et al.* [2008], butter enriched with UFAs was characterised by higher spreadability and a somewhat less intense milky taste and pasteurisation aroma than

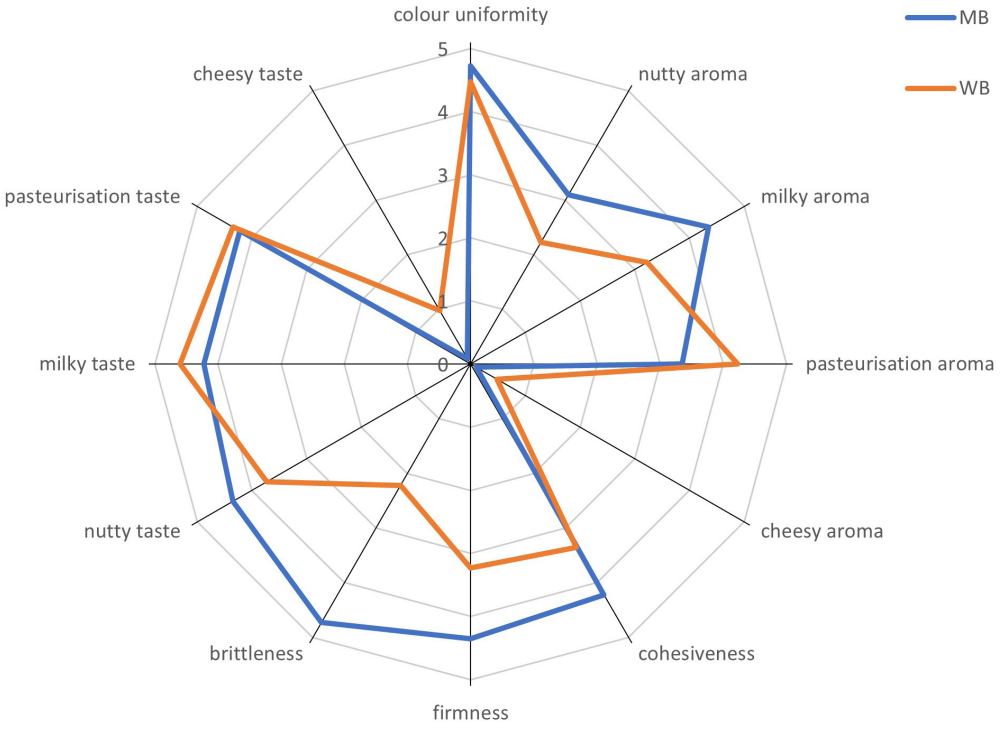

FIGURE 5. Sensory profile of milk butter (MB) and whey butter (WB).

conventional butter after 1 week of storage at 6°C. After 8 weeks of storage at 6°C, these differences, excluding spreadability, were levelled out between the samples. Jinjarak *et al.* [2006] found that sweet cream butter and whey butter differed significantly only in yellowness, shininess, porosity, melting rate, cardboard odour, and nutty flavour.

CONCLUSIONS

Whey butter had comparable sensory attributes to MB, but it was less yellow in colour. Whey butter was softer than MB due to a higher content of MUFAs, PUFAs, and LCFAs, leading to lower crystallisation onset temperature and higher melting onset temperature of fat. The thermal properties of butter were determined by butter type, FA profile, presence of water, and thermal history. Due to relatively high water content, the DSC curves of butter differed considerably from the DSC curves of butter fat. Water freezing and ice melting peaks overlapped fat crystallisation and melting peaks, which hindered their observations in DSC curves and affected their parameters. The phase transition peaks of milk fat and water, peak overlaps, and the extent to which water affected the parameters of milk fat phase transition peaks were determined based on an analysis of the DSC curves of butters and their fats. Thermal history influenced butter melting behaviour by inducing changes in DSC curves, including the number of peaks and peak parameters. The PCA revealed that a rise in the content of SFAs and MCFAs, especially C12:0, C14:0 and C16:0; increased the values of T_{max}, ΔH and P_s; decreased the value of $\Delta T_{1/2}$ of the crystallisation peak; increased the value of P_e of the merged melting peak of LMPF and MMPF; increased the values of T_{max}, ΔH and P_e of the LMPF peak; increased the values of T_{max}, T_{onset}, ΔH, $\Delta T_{1/2}$, P_s and P_e of the MMPF peak; increased the values of T_{max}, T_{onset}, ΔH and P_e in cycle I; and increased the values of T_{max}, T_{onset}, ΔH and P_s in cycle I of the HMPF peak. Reverse trends were observed with a rise in the content of MUFAs, PUFAs, and LCFAs, including C18:0, and to a smaller extent, with an increase in the content of SCFAs, in particular C4:0. These results were used to determine the influence of butter type on the crystallisation and melting characteristics of WBF and MBF. WBF crystallisation was characterised by lower enthalpy; it occurred at a lower temperature, and proceeded more rapidly than MBF crystallisation. In turn, LMPF and HMPF (cycle II) melted more rapidly in WBF than MBF, whereas the absolute maximum difference in heat flow in all fractions (cycles I and II), the enthalpy of LMPF, MMPF and HMPF (cycles I and II), the melting rate of MMPF, and the values at the beginning (MMPF and HMPF in cycles I and II) and the end of melting (overlapping LMPF and MMPF peaks in cycle I; separate LMPF and MMPF peaks in cycle II) were lower in WBF than MBF. Whey butter and milk butter differed in physicochemical properties and sensory attributes, and their thermal profiles depended on the FA profile, water content, and thermal history.

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CONFLICT OF INTERESTS

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

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Iron, Zinc, Copper, Manganese and Chromium in Green Teas, Their Transfer to Extracts and Correlations between Contents of Elements and Bioactive Compounds

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Green tea is used worldwide in the preparation of beverages, but also its extracts rich in bioactive compounds, especially flavan-3-ols, are of increasing interest. In addition to bioactive molecules, green tea represents a source of dietary elements. However, knowledge about their content in extracts is limited. The aim of our research was to determine the extent of transfer of selected elements, *i.e.*, iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), and chromium (Cr), from green teas to their extracts and to investigate whether the main bioactive compounds of the extracts affect this transfer. Twelve commercially available green teas were used in the study. The contents of elements in green teas and their extracts obtained with 80% acetone (*v/v*) were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES). High performance liquid chromatography in reverse phase (RP-HPLC) was used to determine contents of caffeine, (–)-epigallocatechin (EGC), (–)-epicatechin (EP), (–)-epigallocatechin gallate (EGCG), and (–)-epicatechin gallate (ECG). The element with the highest content in green teas was Mn (711–1402 μg/g), but its transfer to extracts was the lowest (0.269–0.646%). The mean Fe transfer, second abundant element in teas (115–725 μg/g), was 5.52%. The contents of Mn and Fe in extracts were 5.08–30.2 and 10.7–90.1 μg/g, respectively. Zn, Cu, and Cr were transferred with means of 10.4, 20.0, and 26.2%, respectively, which resulted in their contents in the extracts in the ranges of 5.03–12.6, 1.93–13.8, and 0.128–2.03 μg/g, respectively. The significant positive correlations of Zn content in extracts and/or transfer to extracts with EGCG, EGC and total flavan-3-ols as well as between the same Fe variables and EGC were determined, which suggested that these flavan-3-ols may positively affect the transfer of Fe and Zn from green tea to extracts. In turn, significant but negative correlations were found in the case of Mn and Cu. Future research is needed to identify the causes of the various transfer rate of elements from green teas to extracts.

INTRODUCTION

Tea plant (*Camellia sinensis* (L.) O. Kuntze) originates from China. Nowadays, the infusion of tea leaves is one of the most popular beverages in the world. It is made of leaves subjected to a different degree and nature of fermentation [Wong *et al*., 2022; Sun *et al*., 2022]. Nevertheless, green tea, *i.e.* fresh leaves treated only with steam, rolled and dried, is also a very popular commercial product. Production of green tea accounts for 20% of the total tea production [Sun *et al*., 2022]. During gentle leaf processing to obtain green tea, oxidative enzymes are inactivated and no fermentation occurs; therefore, the major compounds of the leaves remain in their native form [Wong *et al*., 2022]. The dominant compounds of leaves and green teas are flavan-3-ols, including (−)-epigallocatechin gallate, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epicatechin, (−)-gallocatechin gallate, and (+)-catechin [Jiang *et al*. 2019]; followed by (+)-gallocatechin and (−)-catechin gallate [Svoboda *et al*., 2015]. The flavan-3-ol content can be as high as 125.4 mg/g dry weight [Svoboda *et al*., 2015]. Phenolic acids, flavonols, flavones, flavanonols, proanthocyanidins and derivatives of compounds of each of these phenolic classes were also identified among green tea phenolics [Janiak & Amarowicz, 2018; Scoparo *et al*., 2012; Shi *et al*., 2022]. Moreover, green tea is a rich source of purine alkaloids with caffeine as a major compound [Jiang *et al*., 2019; Scoparo *et al*., 2012]. Green tea phenolics, especially flavan-3-ols are well known for their antioxidant activity [Carloni *et al*., 2013; Peluso & Serafini, 2017]. They are also responsible for a wide range of other bioactivities

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including anti-inflammatory, antidiabetic and anticancer activities, antiobesity and immunoregulatory effects, as well as cardiovascular- and hepato-protective properties [Tang *et al*., 2019]. Therefore, there is a growing interest in not only infusions but also green tea extracts as a health-promoting component of the diet [Bártíková *et al*., 2017; Tang *et al*., 2019]. Green tea extracts are also increasingly applied, as a natural alternative to chemical antioxidants, in food technology to extend the shelf life of food products [Senanayake, 2013]. Their effectiveness in lipid oxidation inhibition in meat products has been widely confirmed [Oswell *et al*., 2018; Shah *et al*., 2014]. To this end, antioxidants have been extracted from green tea using different methods [Vuong *et al*., 2011], including solvent extraction with water or organic solvents as the most popular one.

From a nutritional point of view, green tea can be considered a valuable source of elements. It is rich in macroelements including calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), and phosphorus (P) [Brzezicha-Cirocka *et al*., 2016; Koch *et al*., 2018]. Among essential trace elements, iron (Fe), copper (Cu), zinc (Zn), nickel (Ni), manganese (Mn), cobalt (Co), molybdenum (Mo), selenium (Se), and chromium (Cr) have been detected with the highest contents of Mn and Fe [Barone *et al*., 2016; Koch *et al*., 2018; Ma *et al*., 2019]. The transfer of individual elements from green tea to infusion has been shown to vary; however, Fe was less released from tea matrix to hot water than other trace elements [Koch *et al*., 2018; Wróbel *et al*., 2000]. Nevertheless, due to the high intake of tea beverages, it significantly contributes to meeting the daily dietary requirements of essential elements [Brzezicha-Cirocka *et al*., 2016; Chowaniak *et al*., 2021]. Although the trace element profile of green teas and their infusions has been studied depending on many variables, such as the geographical origin of the plants, their cultivar and growing conditions as well as leaf processing and infusion practices [Brzezicha-Cirocka *et al*., 2016; Deka *el al*., 2021; Na Nagara *et al*., 2022], the knowledge of the content of trace elements in the extracts and transfer of these elements to extracts is limited.

Elements, which are transition metals can bind to polyphenols forming stable complexes [Karamać & Pegg, 2009; Kejík *et al*., 2021; Samsonowicz & Regulska, 2017]. Considering essential trace elements of green tea, the complexation of iron, zinc, cooper, manganese, and chromium ions by flavan-3-ols has been demonstrated so far [Bronco *et al*., 2005; Cherrak *et al*., 2016; Lee & Heffern, 2022; Navarro *et al*., 2005]. Our previous study also showed the formation of complexes between ferrous ions and phenolic compounds of the high molecular weight fraction isolated from a green tea extract [Janiak & Amarowicz, 2018]. It has been suggested that interactions of polyphenols with metal ions may affect the transfer of these metals from green tea to infusions [Szymczycha-Madeja *et al*., 2012]. It seems that complexation may be even more important for the transfer and content of metals in green tea extracts, where phenolic compounds are concentrated. Therefore, the aim of our research was to determine the contents of selected essential trace elements, *i.e.*, Fe, Zn, Cu, Mn, and Cr, in commercially available green teas and their extracts to evaluate the transfer of these elements from teas to extracts. Moreover, our aim was to verify that the major bioactive compounds of the extracts affect this transfer.

MATERIALS AND METHODS

Materials and chemicals

Twelve green teas were purchased from local stores in Olsztyn, Poland. Tea No. 1 was bagged and the rest of the teas were bought in the form of tea leaves. Other characteristics of the green teas used are present in Table 1. The chemicals used for extraction and the standards: caffeine, (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (–)-epigallocatechin gallate (EGCG), and (–)-epicatechin gallate (ECG), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Nitric acid and ICP multielement standard solution IV CertiPUR (1000 mg/mL in nitric acid) were purchased from Merck (Darmstadt, Germany). The reference material – tobacco leaves ORIENTAL (CTA-OTL-1) – was supplied by the Institute of Nuclear Chemistry and Technology, Warszawa, Poland. Deionised water was obtained by the NANOpure Diamond water purification system (Barnstead, Dubuque, IA, USA).

Extraction

Green teas were powdered using a mortar and a pestle. Approximately 20 g of such prepared powders were extracted with 80% (*v/v*) acetone at a solid to solvent ratio of 1:10 (*w/v*) for 15 min at 50 C using the water bath JULABO SW22 (JULABO GmbH, Seelbach, Germany) [Slavova-Kazakova *et al*., 2021]. The liquids were filtered by Whatman No. 3 paper, and the residues were suspended in the next portions of solvent and re-extracted. The operation was done three times. Then, filtrates were combined. Organic solvent was removed using a rotary evaporator (R-210, Büchi Labortechnik AG, Flawil, Switzerland) and any remaining water residue was

TABLE 1. Characteristics of green teas used as research material and yield of their extraction with 80% (*v/v*) acetone.

No.	Form of tea	Country of origin	Type	Extraction yield (%)
1.	Bagged	More than one country	Not declared	32.43±2.70
2.	Leaves	Not declared	Mix	37.48±1.71
3.	Leaves	China	Chun Me	36.93±1.16
4.	Leaves	Sri Lanka	Gunpowder	27.92±2.14
5.	Leaves	China	”Long leaf”	37.65±1.22
6.	Leaves	China	”Classic”	30.72±1.28
7.	Leaves	China	Chun Me	42.34±1.54
8.	Leaves	China	Rolled	30.80±1.83
9.	Leaves	Japan	Sencha Makoto	28.05±1.72
10.	Leaves	China	Gunpowder	30.17±0.98
11.	Leaves	Japan	Sencha	25.64±1.37
12.	Leaves	China	”Long leaf”	34.01±1.69

Values are shown as mean ± standard deviation.

frozen and lyophilised (FreeZone 6 Liter Console System, Labconco, Kansas City, MO USA). The dried extracts were ground to a powder in a mortar and stored in tightly closed vials until analyses.

Mineralisation

Green teas and their extracts dried to a constant mass were placed in Teflon vessels. Portions of 0.16–0.29 g of green teas or extracts and 0.2841 g of tobacco leaves were weighed. Next, 6 mL of nitric acid were added and vials were sealed. The process of cold mineralisation spanned for 16 h. Afterwards, the material was degassed and transferred to a microwave-assisted mineraliser (MARS 5X, CEM Corporation, Matthews, NC, USA). Mineralisation (1200 W) was conducted for 57 min in constant pressure mode (max 55.19 bar). From 0–4 min, the temperature was raised to 160°C and maintained for 4 min, at 8–12 min it was raised to 180°C and maintained for 4 min, at 16–20 min it was raised to 200°C and kept for 7 min. Afterwards, the system was cooled for 30 min to restore initial conditions. The processed samples were degassed, diluted in deionised water, and transferred to 25-mL volumetric flasks. The same procedure was carried out for reference material and blank. Blank was prepared without sample addition. Thus obtained material was kept at 4°C until further use.

Element analysis

Mineralised green teas and extracts were analysed by inductively coupled plasma optical emission spectroscopy (ICP OES) using an iCAP 6000 series ICP-OES spectrometer coupled with a charge injection device (Thermo Electron, Waltham, MA, USA). Analysis was carried out in the plasma axial viewing mode with radio frequency generator set up to 1150 W. Samples were introduced to the system with a flow rate of nebulizer gas set to 0.5 L/min. Cu, Fe, Zn, Mn, and Cr were identified in the samples using spectral line wavelengths: 324.754 nm, 259.940 nm, 213.856 nm, 257.610 nm, and 284.325 nm, respectively. Samples were analysed in 3 repetitions. Calibration curves were prepared for each element from a stock standard solution (10 μg/mL) and appropriate dilutions (0.01, 0.2, 0.5, 1.0, and 2.0 μg/mL) to evaluate the content of metal ions in the samples, which was expressed in μg per g of green tea or extract. Additionally, the transfer (TR, %) of individual green tea elements to extract was calculated based on extraction yield.

Analysis of the contents of caffeine and flavan-3-ols using high performance liquid chromatography in reverse phase (RP-HPLC)

Green tea extracts were dissolved in the mixture of acetonitrile, water, and trifluoroacetic acid (5:95:0.1, *v/v/v*) (2 mg/mL) and filtered (0.22 μm, polyethersulfone membrane filter, TPP Techno Plastic Products AG, Trasadingen, Switzerland). Next, they were analysed after injection of 20 μL of each sample into the Luna C18 column (250×4.6 mm, 5 μm, Phenomenex, Torrance, St. Louis, MO, USA) attached to the chromatographic system consisting of two LC-10AD pumps, an SCL10A controller, a CTO-10AS column oven, and an SPD-M 10A detector (Shimadzu, Kyoto, Japan). Elution was conducted under binary gradient conditions [Janiak *et al*., 2017; Karamać *et al*., 2020]. The mobile phase consisted of two mixtures: A – acetonitrile:water:trifluoroacetic acid (5:95:0.1, *v/v/v*) and B – acetonitrile:trifluoroacetic acid (100:0.1, *v/v*). Gradient was set up as 0–40% B in 0–50 min. Flow rate was 1 mL/min. Peaks were recorded at 280 nm and their retention times were compared to those obtained for standards: EGC, caffeine, EC, EGCG and ECG. Calibration curves were prepared for each standard for quantification of compounds in extracts and green teas. Results were expressed as mg per g tea/extract.

Statistical analysis

All analyses were performed in triplicate. Data are presented as means ± standard deviations. Principal component analysis (PCA) allowed examining the relationships between element contents in green teas and in extracts, and their transfer to extracts. Content and transfer of each element were correlated with contents of the compounds detected by HPLC using Pearson's correlation. All those analysis were performed using STATISTICA 10 (StatSoft Inc., Tulsa, OK, UAS).

RESULTS AND DISCUSSION

The contents of Fe, Zn, Cu, Mn, and Cr in green teas and their extracts as well as percentage of these elements transferred from teas to extracts are shown in Table 2. Among the analysed elements, in both green teas and extracts, high contents of manganese (711–1402 and 5.08–30.2 μg/g, respectively) and iron (115–725 and 10.7–90.1 μg/g, respectively) and the low content of chromium (0.509–1.75 and 0.128––2.03 μg/g, respectively) were determined. The level of copper was estimated in the range of 11.7–18.6 μg/g green tea and 1.93–13.8 μg/g extract. Contents of zinc in teas and extracts were 19.3–31.2 and 5.03–12.6 μg/g, respectively. Our findings for green teas are in accordance with results obtained by other researchers [Brzezicha-Cirocka *et al*., 2016; Koch *et al*., 2018; Na Nagara *et al*., 2022]. For instance, Koch *et al*. [2018] analysed green teas originating from different countries (China, India, Japan, Kenia, and Sri Lanka) and reported contents of 15.4–33.6 mg/100 g for Fe, 3.11–4.07 mg/100 g for Zn, 1.34––2.03 mg/100 g for Cu, 39.0–126.0 mg/100 g for Mn, and 0.10–0.16 mg/100 g for Cr. However, other values were also noted in the literature, *e.g.*, a lower level of Fe (54.14––99.65 mg/kg) and a greater variation in the content of Cr (1.26–10.48 mg/kg) in green teas of different Indian cultivars [Deka *et al*., 2021]. Barone *et al*. [2016] determined a lower copper content (1.58–4.89 μg/g dry weight) and similar as in our study iron, chromium and zinc contents in green teas commercially available in Italy. In turn, Ma *et al*. [2019] reported much higher level of Zn (51.2–95.9 mg/kg) in Chine green teas, although the Mn, Cu, and Cr contents were within the ranges of those in our study. These differences may be due not only to the geographic origin and cultivar variation of the teas, but also to the plant growing conditions and method of post-harvest tea leaf processing [Brzezicha-Cirocka *et al*., 2016; Deka *el al*., 2021; Szymczycha-Madeja *et al*., 2012].

The transfer of green tea elements to extracts varied over a wide range of 6.23–70.8% for chromium (Table 2). The transfer ranges for iron and copper were also wide, 1.33–25.4%

TABLE 2. Content of Fe, Zn, Cu, Mn, and Cr in green teas (GT) and their extracts (EX), and transfer of green tea elements to the extracts (TR).

Tea No.	Fe			Zn			Cu			Mn			Cr		
	GT	EX	TR	GT	EX	TR	GT	EX	TR	GT	EX	TR	GT	EX	TR
	(μg/g tea)	(μg/g extract)	(%)	(μg/g tea)	(μg/g extract)	(%)	(μg/g tea)	(μg/g extract)	(%)	(μg/g tea)	(μg/g extract)	(%)	(μg/g tea)	(μg/g extract)	(%)
1	115±9	90.1±2.1	25.4	31.2±0.3	12.6±0.3	13.1	18.6±0.3	6.54±0.01	11.4	817±10	12.5±0.2	0.496	0.928±0.116	2.03±0.02	70.8
2	148±3	36.1±0.6	9.14	26.7±0.7	6.01±0.03	8.44	16.0±0.4	9.00±0.13	21.1	839±21	7.67±0.25	0.343	0.573±0.030	0.745±0.003	48.7
3	141±3	28.8±0.6	7.54	28.5±1.0	12.2±0.1	15.8	13.9±0.3	7.85±0.07	20.9	1053±27	10.8±0.3	0.379	0.554±0.127	0.587±0.093	39.1
4	725±1	41.2±0.3	1.59	27.6±0.1	7.94±0.02	8.03	15.1±0.2	11.0±0.2	20.3	1305±6	30.2±0.2	0.646	1.49±0.04	0.879±0.021	16.5
5	134±3	12.3±0.1	3.46	28.2±0.6	6.59±0.12	8.80	16.4±0.3	8.04±0.18	18.5	711±16	5.08±0.09	0.269	0.775±0.196	0.334±0.004	16.2
6	171±0	24.2±0.0	4.35	25.3±0.0	8.25±0.02	10.0	14.4±0.0	9.86±0.04	21.0	1402±6	22.7±0.1	0.497	0.648±0.023	1.08±0.04	51.2
7	141±0	11.2±0.1	3.36	24.0±0.1	7.04±0.01	12.4	14.6±0.0	6.32±0.01	18.3	717±1	6.60±0.02	0.390	0.509±0.004	0.272±0.015	22.6
8	426±1	23.3±0.3	1.68	25.1±0.0	6.80±0.03	8.34	17.0±0.0	13.8±0.9	25.0	1137±5	21.3±0.1	0.577	1.75±0.00	0.922±0.026	16.2
9	139±0	15.6±0.0	3.15	19.3±0.2	5.81±0.02	8.44	14.6±0.0	12.8±0.1	24.6	1391±6	23.3±0.1	0.470	0.544±0.014	0.128±0.022	6.60
10	243±1	10.7±0.0	1.33	20.9±0.0	5.03±0.01	7.26	14.0±0.0	12.5±0.1	26.9	880±16	15.0±0.1	0.514	0.877±0.067	0.181±0.045	6.23
11	196±0	21.7±0.0	2.84	22.1±0.1	10.1±0.0	11.7	14.3±0.0	1.93±0.06	3.46	983±5	21.6±0.0	0.563	1.08±0.057	0.308±0.063	7.31
12	213±0	14.7±0.0	2.35	21.2±0.0	7.65±0.01	12.3	11.7±0.0	9.55±0.03	27.8	1345±1	16.5±0.0	0.417	0.954±0.026	0.372±0.017	13.3

Values are shown as mean ± standard deviation (n=3).

and 3.46–27.8%, respectively, but in these cases the result for one sample significantly affected the scattering; excluding tea No. 1 for Fe and tea No. 11 for Cu, the transfer ranges were 1.33–9.14% and 11.4–27.8%, respectively. The values for zinc and manganese were the least differentiated, 7.26–15.8% and 0.269–0.646%, respectively. Thus, the lowest transfer of green tea manganese to extract was found with the mean value of 0.463%. The low value for iron (mean, 5.52%) can also be highlighted. Correlations between extraction yield and percentage of individual green tea elements transferred to the extract were assessed, and a significant negative correlation (correlation coefficient, r=–0.8018) was found only in the case of manganese. To the best of our knowledge, there is a lack of studies regarding the transfer of elements from green teas to their extracts. However, many authors have investigated the transfer of tea elements to infusions [Barman *et al*., 2020; Dambiec *et al*. 2013; Schulzki *et al*., 2017; Wróbel *et al*., 2000; Zhang *et al*., 2018]. Szymczycha-Madeja *et al*. [2012] grouped the elements taking into account the efficiency of their extraction from various types of teas to infusions, and classified iron as "poorly extractable" (<20%), while zinc, copper, manganese and chromium as "moderately extractable" (20–55%) group. In the case of green teas, the percentage of elements transferred to hot water during the preparation of beverages was 5.36–8.1% for Fe, 28.1–36.9% for Zn, 22.4–54.0% for Cu, 23.7–44.6% for Mn, and 25.0–45.4% for Cr [Koch *et al*., 2018; Schulzki *et al*., 2017; Wróbel *et al*., 2000; Yang *et al*., 2022]. Different values were noted by Chowaniak *et al*. [2021] who analysed the elements of green teas from Chinese cultivated and wild plants and their infusions: 11.5 and 11.9% for Cu, 11.1 and 11.0% for Fe, 9.9 and 9.8% for Mn, and 10.6 and 11.6% for Zn. Taking into account that the mean transfer of individual elements in our study accounted for 5.52% (Fe), 10.4% (Zn), 20.0% (Cu), 0.463% (Mn) and 26.2% (Cr), it can be stated that iron, copper, and chromium were extracted with 80% (*v/v*) acetone at levels similar to those obtained with hot water. The transfer of zinc and manganese to the extracts could be classified as poor according to the classification for infusions. A particularly large difference for manganese was evident in this comparison. The transfer of elements to infusions depends on the strength of their bonding to the tea matrix compounds and their hot water solubility [Szymczycha-Madeja *et al*., 2012]. It can be assumed that some manganese species were released from the tea matrix with 80% (*v/v*) acetone to the lesser extent than with water. Besides, due to a high Mn content in green tea (Table 2), its solubility in a solvent containing only 20% (*v/v*) of water could be hampered. Another reason can be found in the weak binding of Mn with the extracted compounds. Aqueous acetone was selected as an extractant in our study to maximise the extraction of green tea phenolic compounds. Perva-Uzunalić *et al*. [2006] reported that the use of hot aqueous acetone allowed obtaining a higher extraction efficiency of flavan-3-ols from green tea than aqueous ethanol, aqueous methanol or hot water.

The results of principal component analysis (PCA), that was conducted to highlight the differences in the content of elements in green teas and extracts as well as transfer of those elements to the extracts, are shown in Figure 1. The first two

principal components (PC1 and PC2) explained most of the total variance (65.35%). Whereas, four principal components (PC1–PC4) described 88.43% of the total variance. Figure 1A presents variables that differentiated distribution of the samples. Variables were positively or negatively correlated with PC1, which is visible in the two separate groups on the plot; positively correlated variables are shown on the right side and negatively correlated variables are grouped on the left side. In the case of Cu, Fe, and Cr contents in green teas, correlations were opposite to correlations of these element contents in the extracts and their transfer to extracts. Zn content and transfer to the extract correlated oppositely to the analogous variables for Mn. PC2 was correlated with variables mostly positively, which was indicated by their clustering in the upper part of the PCA plot. Cr and Fe contents in green teas, Mn content in green teas and extracts as well as transfer of Mn to the extract were strongly positively correlated with this component.

Figure 1B presents distribution of the samples between PC1 and PC2. Tea No. 1 was clearly separated from the rest due to its high Cu and Zn contents, Fe, Cr, Zn content in its extract, as well as high transfer of Cr and Fe. Tea No. 1 was in the form of bags; hence, its manufacturing technology was also an important factor in terms of the final quality of the product. All of the mentioned variables were negatively correlated with PC1 and inter-correlated with each other. The rest of the samples were distributed on the right from tea No. 1 on PCA plot. Position of teas No. 4 and 8 was influenced by their high content of Fe, Mn, and Cr and Mn transfer to the extract.

Figure 1C visualises distribution of variables correlated with PC3 and PC4 which explained 23.08% of the total variance (PC3 – 13.31% and PC4 – 9.77%). These values are not very high, nevertheless contribute to the overall description of the system. Distribution of samples based on PC3 and PC4 is shown in Figure 1D. Position of Tea No. 11, which is separated from other samples, can be justified by low transfer of Cu and high transfer of Zn and Mn to the extract of this tea.

Chromatographic separations of the extracts delivered information about the content of their main compounds, *i.e.*

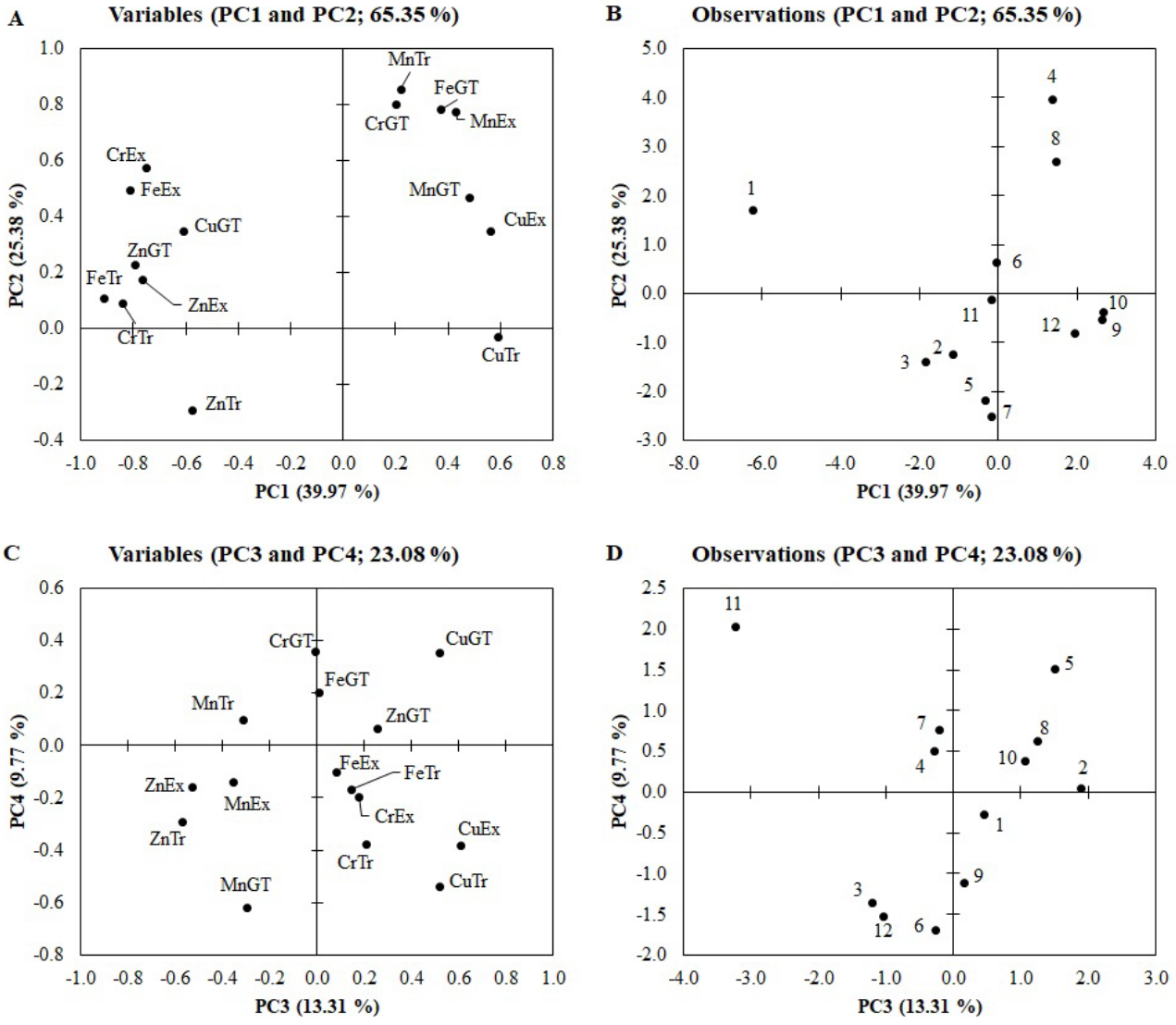

FIGURE 1. Principal component analysis (PCA) plots of distribution of variables (A for PC1 and PC2; C for PC3 and PC4) and distribution of observations (B for PC1 and PC2; D for PC3 and PC4). Numbers 1–12 correspond to the numbers of the tea samples. CuGT, FeGT, ZnGT, MnGT, and CrGT – contents of Cu, Fe, Zn, Mn, and Cr in green teas, respectively; CuEx, FeEx, ZnEx, MnEX, and CrEx – contents of Cu, Fe, Zn, Mn, and Cr in extracts, respectively; CuTr, FeTr, ZnTr, MnTr, and CrTr – transfers of Cu Fe, Zn, Mn, and Cr from green teas to extracts, respectively.

TABLE 3. Correlation of contents of Fe, Zn, Cu, Mn, and Cr in green teas (GT) and extracts (EX), and transfer of green tea elements to extracts (TR) with contents of individual flavan-3-ols and caffeine.

	Green teas						Green tea extracts					
	Caffeine	EGC	EC	EGCG	ECG	Total flavan-3-ols	Caffeine	EGC	EC	EGCG	ECG	Total flavan-3-ols
FeGT	-0.500	-0.453	**-0.586***	-0.565	-0.468	**-0.690***	-0.042	-0.221	-0.486	-0.219	-0.396	-0.566
FeEX	-0.359	0.460	-0.119	0.003	-0.084	0.063	**-0.576***	**0.587***	-0.110	0.122	-0.050	0.414
FeTR	-0.099	**0.592***	0.161	0.207	0.136	0.339	-0.500	**0.600***	0.125	0.137	0.130	0.567
ZnGT	0.048	0.199	0.327	-0.043	0.376	0.233	-0.351	0.119	0.278	-0.309	0.383	0.153
ZnEX	0.075	**0.764***	-0.021	0.550	-0.144	0.437	-0.248	**0.738***	-0.202	0.520	-0.224	**0.635***
ZnTR	0.536	**0.829***	0.237	**0.793***	-0.010	**0.703***	0.027	**0.598***	-0.106	**0.445***	-0.203	0.526
CuGT	-0.403	0.070	0.099	-0.199	0.273	-0.006	**-0.667***	0.187	0.219	-0.147	0.374	0.276
CuEX	-0.411	**-0.627***	-0.364	**-0.795***	-0.318	**-0.744***	0.314	-0.355	-0.020	-0.403	-0.164	-0.559
CuTR	0.046	-0.524	-0.084	**-0.596***	-0.155	-0.469	**0.614***	-0.449	0.121	-0.518	-0.097	**-0.629***
MnGT	-0.302	-0.113	**-0.598***	-0.210	**-0.579***	-0.444	0.240	0.127	-0.520	0.239	-0.506	-0.137
MnEX	**-0.696***	-0.209	**-0.864***	-0.303	**-0.747***	**-0.666***	-0.099	0.158	**-0.725***	0.370	**-0.633***	-0.144
MnTR	**-0.801***	-0.113	**-0.909***	-0.275	**-0.830***	**-0.672***	-0.242	0.275	**-0.752***	0.421	**-0.730***	-0.093
CrGT	-0.509	-0.172	-0.550	-0.435	-0.448	-0.531	-0.001	0.134	-0.370	0.005	-0.342	-0.154
CrEX	-0.366	0.322	-0.142	-0.129	-0.073	-0.037	-0.460	0.478	-0.075	0.012	0.002	0.312
CrTR	0.028	0.395	0.288	0.109	0.266	0.314	-0.358	0.335	0.261	-0.077	0.277	0.371

*Correlation is significant ($p<0.05$); EGC, (−)-epigallocatechin; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; ECG, (−)-epicatechin gallate.

caffeine, EGC, EC, EGCG, and ECG. The content of the caffeine was in the range of 53.2–70.3 mg/g extract. The flavan-3-ols were quantified in extracts at 56.4–126 mg/g (EGC), 26.1–44.4 mg/g (EC), 113–178 mg/g (EGCG), and 38.7––91.9 mg/g (ECG). Their sum was 259–365.4 mg/g extract. In the case of the contents in green teas, values for caffeine, EGC, EC, EGCG, ECG, and total flavan-3-ols ranged were at 16.1–25.0, 16.6–40.9, 7.28–16.7, 35.0–60.5, 10.8–34.6, and 88.3–148 mg/g, respectively. These results are in accordance with literature data concerning flavan-3-ols of commercially available green teas [Svoboda *et al*., 2015] and green teas obtained from fresh leaves by various processing methods (roasting, baking, steaming, and sun-drying) [Shi *et al*., 2022] as well as concerning caffeine of green teas from leaves of wild and cultivated plants [Chowaniak *et al*., 2021]. In turn, Jiang *et al.* [2019] reported slightly lower contents of both caffeine (15.6 mg/g) and flavan-3-ols (11.3, 4.5, 27.9, 10.3, and 52.24 mg/g for EGC, EC, EGCG, ECG, and total flavan--3-ols, respectively) in green tea prepared from fresh leaves compared to the values determined in our study.

Flavonoids with multiple hydroxyl groups and with the carbonyl group on ring C in their structures may form complexes with transition metal ions [Kejík *et al*., 2021; Samsonowicz & Regulska, 2017]. Green tea flavan-3-ols contain catechol and/or galloyl moieties that allow them to bind with iron, zinc, cooper, manganese, chromium, and other metal ions [Bronco *et al*., 2005; Cherrak *et al*., 2016; Lee & Heffern, 2022; Navarro *et al*., 2005]. We postulate that such complexes may affect the transfer of elements from green tea to extracts. To verify this hypothesis, the contents of individual compounds and total flavan-3-ols determined in green teas and extracts were correlated with contents of elements in green teas and extracts, and with their transfer from the teas to the extracts. The correlation coefficients are shown in Table 3. The most powerful positive correlations were found between Zn transfer to extracts and contents of EGC (r=0.829) and EGCG (r=0.793) in green teas. Consequently, a strong correlation occurred with total flavan-3-ols (r=0.703). High r value was also obtained for a correlation between the content of Zn in extracts and EGC in green teas. In the case of phenolic contents in extracts and Zn variables, there were again significant ($p<0.05$) positive correlations of EGC with Zn transfer, EGCG with Zn transfer, EGC with Zn content in extract, and additionally, total flavan-3-ols with Zn content in extract. Transfer of iron to extracts was positively ($p<0.05$) associated with contents of EGC in both extracts and green teas. The correlation between EGC (in both extracts and teas) and Fe content in the extract was also significant ($p<0.05$). Tolrà *et al*. [2020] reported that Fe content in tea leaves was positively related to galloylated flavan-3-ols (ECG and EGCG). Contrary to this finding, in our study, correlations between contents of Fe and mentioned phenolics in green teas were not significant ($p\geq0.05$), and moreover, significant ($p<0.05$) but negative correlations were found between Fe content and EC as well as between Fe content and total flavan-3-ols (Table 3). For Cu and Mn variables, generally, negative correlations were determined with caffeine and phenolic compound contents. Among them, the most powerful were relationships

between EC in teas and Mn content in extracts (r=–0.864) and Mn transfer to extracts (r=–0.909). Significant ($p<0.05$) correlations of the same Mn variables were found with ECG, total flavan 3-ols and caffeine in teas and EC and ECG in extracts. Moreover, Mn content in green teas significantly ($p<0.05$) negatively correlated with EC (r=–0.598) and EGC (r=–0.579) in teas. A previous study reported a negative (although statistically insignificant) correlation between total phenolic content (TPC) and Mn content in infusions of commercial available green, black, and red teas [Klepacka, 2022]. The author also noted a similar trend for the correlation between TPC and Cu content. In our research, Cu transfer to the extract significantly ($p<0.05$) negatively correlated with total flavan-3-ols in extracts and positively with caffeine in extracts (Table 3). Moreover, significant ($p<0.05$) correlations were determined between EGCG in teas and Cu content in the extract (r=–0.795) and its transfer to the extract (r=–0.596). In the case of Cr variables, all correlations were insignificant ($p\geq0.05$).

In general, positive significant correlations between the content of flavan-3-ols in the extracts and the content and transfer of Zn and Fe to extracts indicate that the complexes between these metal ions and flavan-3-ols could have been formed and have a significant effect on element transfer to extracts. Although flavan-3-ols are the predominant phenolic compounds [Wong *et al*., 2022], their polymeric forms (proanthocyanidins) have also been determined in small amounts in green tea [Janiak & Amarowicz, 2018; Shi *et al*., 2022]. These compounds, due to the multiple catechol and galloyl moieties in the structure, have been shown to exhibit stronger ability to bind metal ions than other classes of phenolics [Karamać & Pegg, 2009]. Moreover, these compounds are extracted with 80% acetone [Janiak & Amarowicz, 2018; Karamać & Pegg, 2009]. Proanthocyanidins could therefore contribute to the transfer of metal ions to the extracts and at the same time disrupt correlations of Fe and Zn contents with flavan-3-ols analysed in our study. This would explain that not all correlations were significant. In turn, positive correlations obtained for Zn and Fe variables and negative one for Cu and Mn variables could suggest their competitiveness to the hydroxyl groups of phenolic compounds. On the other hand, negative correlations shown for Cu and Mn may indicate the lack of interaction of these metal ions with flavan-3-ols and their presence in green teas in species that were not transferred to the extract. Indeed, Pongrac *et al*. [2020] found that part of manganese was allocated in tea leaf oxalate crystals, which are hardly soluble in water and in organic solvents. Nevertheless, these assumptions still need to be elucidated in future researches.

CONCLUSIONS

The results of this study demonstrate that among the analysed elements, manganese was the most abundant in commercial available green teas, followed by iron, zinc, copper, and chromium. The contents of these elements in green tea extracts were lower, especially for manganese; however, the extracts can still be considered as a significant source of iron, copper, chromium, and zinc. Iron turned out to be the predominant element in extracts. The transfer of elements to the extracts varied considerably. Manganese transfer was by far the lowest. Iron transfer may also be considered low. Comparable values were found for copper, zinc, and chromium. The use of 80% (*v/v*) acetone allowed obtaining an extract with a high content of flavan-3-ols (EGC, EC, EGCG and ECG) and caffeine. As contents of some of phenolics correlated positively with the Zn and Fe contents in extracts and the transfer of these elements to extracts, it was concluded that flavan-3-ols may positively affect the transfer of iron and zinc from green teas to extracts. Such relationships were not found for the remaining elements. More research is, however, needed to explain the reasons of differences in the transfer of elements from green teas to extracts and of low transfer of some of them. Nevertheless, the green tea extract was proved to represent a rich source of not only bioactive compounds but also trace elements.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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Original article
Section: Food Quality and Functionality

Effect of Cricket Powder Incorporation on the Profile of Volatile Organic Compounds, Free Amino Acids and Sensory Properties of Gluten-Free Bread

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Key words: sensory analysis, volatile compounds, edible insects, *Acheta domesticus*, house crickets, GC×GC-ToFMS

Scientists around the world are interested in edible insects as a source of valuable nutrients. Among the most often described are crickets, which represent a source of significant amounts of protein, lipids, vitamins, and minerals. This article reports results of a study into the effect of starch-to-cricket powder (CP) addition on the free amino acid profile as well as potential odorants in gluten-free (GF) bread. A significant 2.6-fold increase was observed in the content of essential amino acids in the CP-enriched GF bread. Moreover, the CP addition resulted in the formation of many volatile compounds, such as pyrazines, furans, and sulfur-containing compounds, which exhibit strong aroma-enhancing properties. The attractiveness of the CP-enriched bread was confirmed by the results of the sensory analysis, showing a significant improvement in its flavor when compared to the traditional GF bread without CP. GF bread enriched with CP was characterized by caramel, roasty, and cooked potatoes notes. On the basis of the obtained results, it was concluded that the use of CP not only improves the nutritional value but also aroma of GF bread.

INTRODUCTION

Bread is a staple source of carbohydrates commonly consumed worldwide, irrespectively of the country development [Conte *et al.*, 2016]. The basic bread recipe consists of wheat flour, water, salt, and a leavening agent. The presence of gluten proteins in wheat (gliadin), barley (hordeins), and rye (secalins) guarantees the typical viscoelastic properties of bread. However, the same proteins can be harmful to a growing number of consumers who suffer from gluten-related disorders [Scherf *et al.*, 2016]. In those individuals, the consumption of regular wheat bread can result in health complications and aggravation of the disease symptoms. Therefore, the demand for gluten-free (GF) alternatives has been increasing in the last few years. Gluten elimination from bread has a detrimental effect on its viscoelastic, technological, nutritional, and sensory properties [Conte *et al.*, 2019]. The available GF bread alternatives are mainly produced with corn, rice, and other GF cereals having a lower content of vitamins, minerals, and proteins than wheat flour [Aguiar *et al.*, 2021]. Importantly, these components also have a negative effect on the sensory properties of GF bread, including its aroma [Pico *et al.*, 2017a]. To date, many efforts have been undertaken to improve the nutritional value, structure, and aroma of GF bread. Among the most studied additives are hydrocolloids, proteins, lipids and emulsifiers, which were found to improve the technological and viscoelastic properties of dough [Conte *et al.*, 2020]. Moreover, the increasing popularity of fruit and vegetable by-products, additives rich in protein, fiber, and bioactive compounds, has been observed [Föste *et al.*, 2020]. However, additives can significantly influence the aroma of GF bread, which is of particular importance since flavor is the key to market success [Heenan *et al.*, 2008]. The pleasant aroma of traditional wheat bread is a result of fermentation, lipid oxidation, and Maillard reactions [Pico *et al.*, 2017a]. The Maillard reactions, which occur between amino acids and reducing sugars, are responsible for the browning of crust and the distinctive roasted and nutty aroma [Cerny, 2008]. The components of GF products, such as proteins, lipids as well as sugars, act as precursors and strongly affect the generation of volatile compounds [Pico *et al.*, 2017a]. However, because of the lower content of several amino acids, the aroma of GF bread is significantly different from that of wheat bread [Pico *et al.*, 2017a].

Edible insects (EIs) are considered a source of proteins, lipids, fiber, and minerals of a high nutritional value [Montowska *et al.*, 2019; Nissen *et al.*, 2020]. Previous reports have shown that the incorporation of EIs can significantly improve

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the nutritional value of GF food products [Kowalczewski *et al.*, 2021]. The Food and Agriculture Organization of the United Nations (FAO) emphasizes that EIs are highly nutritious and environmentally friendly due to their breeding conditions [FAO, 2013]. Regulation 2283/2015 on Novel Foods and its implementing Regulations 2468/2017 and 2469/2017 clarify the rules concerning edible insect's application in food in the European Union (EU) [UE, 2015]. According to the European Food Safety Authority, mealworm larvae can be used as a whole, dried as snacks, and powdered as an additive in other various food products [Turck *et al.*, 2021b]. Moreover, frozen and dried formulations from house crickets (*Acheta domesticus*) [Turck *et al.*, 2021a] and partially defatted house cricket powder are approved as a novel food [Turck *et al.*, 2022].

Edible insects likely to be accepted in Europe include mealworm and buffalo worm larvae as well as grasshoppers, crickets, and locusts [Skotnicka *et al.*, 2021]. However, the European population is reluctant to consume whole EIs, in both larval and adult forms [Mishyna *et al.*, 2020]. Recent studies have indicated that the growing consumer awareness of the environmental costs of meat production may improve the acceptance of alternative protein sources, including EIs [Ribeiro *et al.*, 2022]. Crickets are one of the most important among the many described EIs. The *A. domesticus* is one of the most produced species of crickets, owing to the ease of breeding and good flavor characteristics of the cricket powder (CP), known also as cricket flour, obtained from it. Published studies have demonstrated a high nutritional value of proteins and lipids obtained from crickets [Psarianos *et al.*, 2022]. Moreover, the biological activity of crickets has been widely described in the context of positive effects on human health. The most frequently reported are antioxidant and anti-inflammatory activities [Bernal, 2021], but the consumption of crickets has also been shown to improve the condition of intestinal microbiota [Stull *et al.*, 2018] or reduce insulin resistance [Escobar-Ortiz *et al.*, 2022].

CP-enriched GF bread is characterized by a unique bouquet of volatile organic compounds (VOCs) and its aroma resembles that of conventional bread [Nissen *et al.*, 2020]. Hence, it seems worth investigating how the addition of protein-rich insect flour affects flavor generation in GF bread. Therefore, the aim of this study was to analyze the changes of VOCs in the experimental GF bread fortified with different contents of CP halfway through the baking time (15 min) and after baking (30 min). The analyses were performed both for the crumb and the crust of the CP-enriched breads. The sensory properties and the content of precursors of several VOCs, namely free amino acids (FAA), were analyzed as well.

MATERIALS AND METHODS

Materials

Rice starch was purchased from BENEO GmbH (Mannheim, Germany), potato starch from PPZ Trzemeszno sp. z o.o. (Trzemeszno, Poland), guar gum and pectin from Agnex (Białystok, Poland), lyophilized yeast from Bakalland S.A. (Warsaw, Poland), sugar from Pfeifer & Langen Polska S.A. (Środa Wielkopolska, Poland), salt from Ciech Soda Polska S.A. (Janikowo, Poland), rapeseed oil from ZT 'Kruszwica' S.A. (Kruszwica, Poland), and cricket powder from Crunchy Critters (Derby, United Kingdom).

Preparation of gluten-free bread

The control GF bread, marked as CP0, was made of the following ingredients: 200 g rice starch, 50 g potato starch, 4.25 g guar gum, 4.25 g pectin, 15 g yeast, 5 g sugar, 4.25 g salt, 7.5 g rapeseed oil, and 275 g distilled water. Firstly, both types of starch were mixed together, then, in the test samples, starch mix was replaced with CP in three different quantities of 2, 6, and 10% (*w/w*), and breads containing cricket powder were denoted as CP2, CP6, and CP10, respectively. The amounts of other components were unchanged. All the ingredients, except rapeseed oil, were combined and mixed for 2 min at a speed of 70 rpm using the KitchenAid mixer (model 5KPM5EWH, KitchenAid, Benton Harbor, MI, USA), then the oil was added, and mixing was continued for the next 6 min. The dough was fermented in a fermentation chamber for 20 min (temperature 35°C, relative humidity 85%) and punched. Each sample of dough was divided into two parts (230 g each) and placed in baking forms. The final fermentation was carried out for 15 min at 35°C. The prepared dough was baked at 230°C for 30 min, then the bread was left at room temperature for 3 h to cool down and sliced (about 1.5 cm thick). The analyzed breads were baked twice, and three samples were taken from each baking. The samples were taken within 15 and 30 min of baking time. Afterwards, the crust and crumb were carefully separated and ground for later analysis.

Free amino acid analysis

The profile of individual FAAs was analyzed as described previously [Drabińska, 2022]. Briefly, 0.5 g of freeze-dried samples were extracted with 3.5 mL of 50% (*v/v*) methanol (ISO reagent purity, Supelco, Bellefonte, PA, USA) for 20 min at 50°C using a laboratory shaker S50 (CAT Germany GmbH, Lehrte, Germany) at a speed of 800 rpm/min. Extracts were centrifuged at 5,500×*g* for 15 min (Universal 320 R cooled centrifuge, Hettich Holding GmbH & Co. oHG, Kirchlengern, Germany), and supernatants were collected in 10 mL volumetric flasks. The extraction was performed three times.

The extracts were directly analyzed using the EZ: Faast™ kit for free (physiological) amino acids (Phenomenex, Aschaffenburg, Germany) according to the manufacturer's recommendations. The analytical procedure involves solid-phase extraction of 100 μL of the extract, followed by derivatization and liquid-liquid extraction. FAAs were separated using the ZB-AAA EZ Faast™ capillary column (10 m × 0.25 mm, Phenomenex) installed in an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler G45134 and an Agilent 5975C mass selective detector. Helium was used as a carrier gas with a flow rate of 1.5 mL/min. The samples (2 μL) were injected in the split mode at a 1:15 ratio. The initial oven temperature was 110°C, then it was increased to 320°C (30°C/min). The injector and ion source temperatures were 250 and 240°C, respectively. Amino acids were identified and quantified using standards for each amino acid, and normalized with reference to the internal standard (norvaline).

Analysis of volatile compounds in bread samples

To extract VOCs from the headspace (HS) of bread (crumb and crust), carboxen/divinylbenzene/polydimethylsiloxane (CAR/DVB/PDMS) fiber of a 2 cm length was used (Supelco, Bellefonte, PA, USA). A 1.5-g portion of each sample was placed in a 20-mL glass vial. The sample was preheated to 50°C for 5 min in a heating block. This was followed by a 30 min extraction at 50°C. The solid phase microextraction (SPME) fiber was desorbed in the gas chromatograph injection port at 250°C for 10 min.

Comprehensive two-dimensional gas chromatography coupled with time of flight mass spectrometry (GC×GC--ToFMS) analysis of the VOCs was performed using an Agilent Technologies 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a ZOEX cryogenic modulator (N_2) coupled to a PEGASUS 4 time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA). An Agilent Technologies GC sampler 80 was used to inject the samples. The compounds were separated on a nonpolar SLB-5 column (30 m × 250 μm × 0.250 μm; Supelco) as the first-dimension column, and a polar Supelcowax-10™ column (0.75 m × 200 μm × 0.1 μm; Supelco) as the second-dimension column. The oven program in the first dimension was as follows: initial temperature 40°C (1 min), 6°C/min to 200°C (0 min) and 25°C/min to 235°C/min (5 min). The oven program in the second dimension was: initial temperature 55°C (1 min), 6°C/min to 215°C (0 min) and 25°C/min to 250°C (5 min). Helium was used as the carrier gas at a flow rate of 0.8 mL/min. The modulation time was set to 4 s. The analyses were performed in the splitless mode. The temperature of the GC/MS transfer line was 260°C. Full spectral information (*m/z* range of 33–333) was acquired at a detector voltage of 1700 V at 150 spectra/s. The chromatograms were processed using LECO ChromaTOF® v.4.40 software, then the NIST 2.0 library was used for compound identification. The following parameters were used for the analyte match criteria: minimum similarity – 700, mass threshold – 10, and signal to noise ratio – 1000. Additionally, retention indexes were calculated and compared with literature data.

Sensory analysis

The aroma of the experimental GF products was evaluated by the quantitative descriptive analysis (QDA) method according to the International Organization for Standardization (ISO) 13299:2016 standard [ISO, 2016]. The evaluation was performed by 6 trained panelists. The samples were prepared by mixing crumbs with crusts in a 1:1 (*w/w*) ratio. Only the fully baked bread samples were analyzed. The odor attributes, determined in preliminary sessions, were: sour, roasty, cooked potato, caramel/chocolate, fatty, rice-like, floral, and earthy. The intensity of all attributes was ranked using a scale from 0 (not perceivable) to 3 (strongly perceivable).

Statistical analysis

For every test, three independent repeated measurements were made, unless stated otherwise. Results were analyzed using one-way analysis of variance independently for each dependent variable. The post-hoc Fisher's least significant difference (LSD) test was used to identify statistically homogeneous subsets at $\alpha=0.05$. Statistical analysis of the data was performed with the STATISTICA version 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). Moreover, to identify the differences in the VOCs distribution between the experimental GF breads, principal component analysis (PCA) was performed using SIMCA software package version 16 (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Free amino acids as precursors of volatiles

The contents of FAAs determined in the crumb and crust of the analyzed GF breads are presented in Table 1 and Table 2, respectively. Firstly, crust and crumb may be compared to determine any possible differences in the amino acids content between these parts. As shown in Table 1 and Table 2, nine essential amino acids (EAAs) were present in both the analyzed parts of the breads in a similar content range. Nevertheless, a minor lower content was determined in the crust part compared to the crumb for the majority of the EAAs. Tryptophan content was only notably lower in the crust than in the crumb. In terms of the composition of non-essential amino acids (NEAAs), no significant reduction was observed in the crust compared to the crumb. In fact, in some cases, the content of NEAAs was even larger in the crust of the bread.

The influence of baking time (15 *vs*. 30 min) on the amino acid composition of bread crumbs and crusts (Table 1 and Table 2) was also investigated. The prolonged baking time did not affect the total content of both EAAs and NEAAs of breads fortified with CP. The significant ($p<0.05$) differences in the total content of EAAs and NEAAs were noted for CP0 in crust as well as CP0 and CP2 in crumb. For individual FAAs, most of the changes in their content after 15 *vs*. 30 min of baking were minor and for the majority of them statistically not significant. Therefore, it was concluded that baking time did not affect the content of FAAs in crumb and crust of the analyzed breads.

The last studied factor was the amount of CP additive. Four different types of bread (without CP, with 2%, 6%, and 10% of CP), separately for crusts and crumbs, were compared (Table 1 and Table 2). Not all changes between CP0 and CP2 were statistically significant. In the crust of breads baked for 15 min, significant ($p<0.05$) changes were observed in the content of two EAAs: lysine and methionine; and three NEAAs: proline, asparagine, and aspartic acid, while in the crumb of breads baked for 15 min only the content of threonine, alanine, glycine, proline, and glutamic acid changed significantly. Insignificant differences ($p\geq0.05$) were noted for the total contents of both EAAs and NEAAs in crusts of CP0 and CP2 baked for 15 min, while in the crumb the statistically significant difference ($p<0.05$) was observed only for total NEAAs content. Significant differences were also noted between crusts of CP0 and CP2 baked for 30 min in respect of total content of EAAs and NEAAs . Interestingly, these differences were not always reflected for individual FAAs; a significant ($p<0.05$) difference was found for tryptophan (EAA), and five NEAAs: alanine, glycine, proline, aspartic acid, glutamic acid, and tyrosine in GF bread crust. A similar relationship was observed for isoleucine, lysine, alanine, glycine,

TABLE 1. Amino acid composition in crumbs of gluten-free bread without cricket powder (CP0) and with 2, 6, and 10% of cricket powder, presented as CP2, CP6, and CP10, respectively (μmol/g dry weight).

Amino acid	15 min of baking				30 min of baking			
	CP0	CP2	CP6	CP10	CP0	CP2	CP6	CP10
Essential amino acids (EAAs)								
Phenylalanine	11.9±0.3^{c}	11.5±2.8^{c}	15.9±1.3^{b}	20.7±1.3^{a}	11.0±0.4^{c}	11.7±0.7^{c}	13.6±3.5^{b,c}	21.5±1.9^{a}
Histidine	0.6±0.1^{c}	0.5±0.0^{c}	1.4±0.2^{b}	2.2±0.6^{a}	0.3±0.0^{e}	0.3±0.0^{e}	1.2±0.3^{b}	2.1±0.5^{a}
Isoleucine	1.0±0.1^{cd}	1.1±0.2^{c}	2.0±0.2^{b}	3.8±0.1^{a}	0.7±0.2^{d}	1.1±0.1^{c}	2.1±0.1^{b}	3.8±0.3^{a}
Lysine	1.5±0.2^{c}	1.6±0.1^{c}	1.9±0.6^{a}	3.5±0.2^{a}	1.2±0.1^{d}	1.4±0.1^{c}	2.0±0.2^{b}	3.7±0.5^{a}
Leucine	2.6±0.2^{d}	3.1±0.3^{cd}	4.1±0.7^{b}	6.2±0.3^{a}	2.8±0.5^{d}	2.7±0.4^{d}	3.6±0.3^{bc}	6.5±0.2^{a}
Methionine	0.8±0.2^{bc}	0.7±0.1^{c}	0.7±0.0^{bc}	1.1±0.1^{ab}	0.6±0.1^{c}	0.6±0.1^{c}	0.7±0.3^{bc}	1.5±0.6^{a}
Threonine	1.0±0.1^{c}	0.5±0.1^{d}	1.0±0.1^{c}	1.7±0.2^{b}	0.5±0.0^{d}	0.4±0.1^{d}	1.2±0.3^{c}	2.4±0.6^{a}
Tryptophan	12.6±3.8^{bc}	11.9±1.2^{cd}	17.0±5.9^{b}	22.7±5.3^{a}	6.4±0.8^{d}	6.0±0.4^{d}	16.2±2.1^{b}	15.6±1.3^{b}
Valine	6.7±0.7^{c}	5.9±0.6^{c}	10.4±1.3^{b}	14.6±0.3^{a}	4.9±0.9^{c}	4.9±0.8^{c}	9.8±0.7^{b}	16.4±4.1^{a}
Total EAA	**38.7±5.6^{c}**	**36.8±5.5^{c}**	**54.4±10.2^{b}**	**76.6±8.4^{a}**	**28.4±3.1^{d}**	**29.0±2.7^{d}**	**50.4±7.7^{b}**	**73.5±10.0^{a}**
Non-essential amino acids (NEAAs)								
Alanine	10.2±0.5^{d}	17.0±0.6^{c}	30.2±1.9^{b}	45.2±0.7^{a}	9.0±0.2^{d}	16.4±0.7^{c}	31.7±1.2^{b}	47.9±4.6^{a}
Glycine	3.9±0.4^{d}	8.2±0.6^{c}	17.0±2.3^{b}	25.0±0.5^{a}	4.3±0.4^{d}	6.6±1.2^{c}	16.1±1.0^{b}	26.5±1.6^{a}
Gamma aminobutyric acid	2.2±0.1^{bc}	2.6±0.2^{b}	2.7±0.4^{b}	4.0±0.4^{a}	2.0±0.3^{bc}	1.5±0.3^{c}	2.3±0.8^{bc}	4.2±0.7^{a}
Proline	7.4±0.3^{d}	9.9±0.3^{c}	14.2±1.5^{b}	19.4±0.2^{a}	7.0±0.1^{d}	9.9±0.4^{c}	14.1±0.6^{b}	19.9±0.9^{a}
Asparagine	23.7±1.8^{d}	21.3±2.9^{de}	29.0±2.0^{c}	47.1±1.7^{b}	16.9±0.1^{e}	21.7±1.8^{d}	29.0±3.4^{c}	53.2±4.9^{a}
Aspartic acid	1.9±0.2^{cd}	2.0±0.2^{c}	3.0±0.2^{b}	3.3±0.2^{b}	1.6±0.1^{d}	2.1±0.0^{c}	3.1±0.1^{b}	3.6±0.4^{a}
Glutamic acid	3.0±0.1^{b}	2.0±0.3^{cd}	2.3±0.5^{c}	4.0±0.2^{a}	1.4±0.1^{e}	1.7±0.2^{de}	2.9±0.3^{b}	4.3±0.6^{a}
Ornithine	0.7±0.1^{bc}	0.4±0.1^{cd}	0.9±0.2^{b}	1.3±0.2^{a}	0.6±0.0^{cd}	0.3±0.0^{d}	0.9±0.1^{b}	1.6±0.3^{a}
Tyrosine	0.4±0.1^{e}	0.6±0.1^{e}	1.7±0.2^{b}	2.6±0.1^{a}	1.0±0.3^{d}	0.5±0.0^{e}	1.4±0.1^{c}	2.7±0.2^{a}
Total NEAAs	**53.4±3.7^{d}**	**64.1±5.3^{c}**	**101.1±9.2^{b}**	**151.9±4.3^{a}**	**43.9±1.5^{e}**	**60.7±4.6^{c}**	**101.6±7.8^{b}**	**163.7±14.1^{a}**

Different letters in superscript in the same line indicate a significant difference ($p<0.05$).

proline, asparagine, aspartic acid, and tyrosine in GF bread crumb. In comparison to CP0, the addition of 6% of cricket powder resulted in significant differences in the content of the majority of FAAs, both in the crust and the crumb of model bread. In the CP10 bread, the content increased notably for all analytes. In the outer and the center of the bread baked for 15 min, the total content of EAAs doubled (CP0 *vs.* CP10), while the content of NEAAs increased 2.3 times. In the samples baked for 30 min, these changes were even larger; the total content of EAAs in CP10 crust and crumb was 2.6 times higher than in CP0, and the total NEAAs increased 4 times.

It is common knowledge that deficiencies of protein and EAAs, such as tryptophan, are common in individuals affected by gluten-related disorders. They may contribute to lower serotonin production and result in psychological symptoms observed in patients with celiac disease [Drabińska *et al.*, 2018]. These deficiencies are due to a coupled effect of disturbed intestinal absorption and lower protein quality of GF products. Therefore, the increment of both FAAs and protein content is preferable in the products dedicated to people with gluten-related disorders. It should be mentioned that CP was characterized by a high protein content of approx. 60%, which is significantly higher compared to different types of flour [Kowalski *et al.*, 2022] and its use in GF bread preparation significantly increased the total protein content of the final product [Kowalczewski *et al.*, 2021]. Moreover, FAAs are precursors of volatile compounds which might modify the aroma profile. As a consequence, their impact on the flavor is inevitable, which is discussed in the further parts of the manuscript.

Profile of VOCs in GF bread

The analysis of VOCs in the CP-enriched bread provided important information regarding its flavor quality. A wide range of VOCs can be formed depending on the available amino acids and the reaction conditions [Schwab *et al.*, 2008]. Among them are the products of Maillard reactions which occur at elevated temperatures and are especially important in breadmaking.

Comparing the composition of volatiles in the crust and crumb of analyzed breads multiple differences were observed

TABLE 2. Amino acid composition in crust of gluten-free bread without cricket powder (CP0) and with 2, 6, and 10% of cricket powder, presented as CP2, CP6, and CP10, respectively (μmol/g dry weight).

Amino acid	15 min of baking				30 min of baking			
	CP0	CP2	CP6	CP10	CP0	CP2	CP6	CP10
Essential amino acids (EAAs)								
Phenylalanine	11.9±1.2^{cd}	12.3±1.3^{cd}	18.5±1.0^{b}	24.0±2.1^{a}	10.6±1.8^{d}	11.9±0.1^{cd}	13.6±1.4^{c}	22.2±1.8^{a}
Histidine	0.5±0.1^{d}	0.6±0.2^{cd}	1.0±0.1^{b}	1.7±0.3^{a}	0.5±0.0^{d}	0.5±0.0^{d}	0.9±0.1^{bc}	1.9±0.3^{a}
Isoleucine	0.8±0.1^{c}	1.2±0.1^{c}	2.2±0.2^{b}	4.2±0.2^{a}	0.9±0.0^{c}	1.2±0.2^{c}	2.2±0.1^{b}	4.5±0.8^{a}
Lysine	2.4±0.4^{b}	1.9±0.2^{cd}	2.0±0.3^{bc}	3.3±0.2^{a}	1.1±0.1^{e}	1.4±0.3^{de}	1.7±0.4^{cd}	3.7±0.4^{a}
Leucine	2.7±0.2^{c}	2.6±0.4^{c}	3.8±0.6^{b}	6.9±0.2^{a}	2.7±0.5^{c}	3.0±0.8^{bc}	3.9±0.3^{b}	7.3±1.3^{a}
Methionine	1.2±0.2^{b}	0.7±0.1^{c}	1.0±0.1^{b}	1.4±0.1^{a}	0.7±0.1^{c}	0.5±0.1^{c}	0.7±0.1^{c}	1.5±0.2^{a}
Threonine	0.9±0.1^{cde}	1.1±0.2^{cd}	1.1±0.3^{cd}	1.9±0.2^{b}	0.5±0.1^{e}	0.8±0.2^{de}	1.3±0.3^{c}	2.6±0.6^{a}
Tryptophan	8.5±1.1^{cd}	8.0±1.3^{d}	9.2±0.6^{bc}	14.8±3.6^{a}	5.3±0.6^{e}	8.9±0.5^{c}	10.3±2.8^{b}	15.2±0.4^{a}
Valine	6.9±1.4^{c}	6.4±1.2^{c}	12.6±0.8^{b}	16.0±1.1^{a}	6.7±0.3^{c}	6.3±0.9^{c}	11.6±1.1^{b}	15.8±3.0^{a}
Total EAA	**35.8±4.8^{c}**	**34.7±5.0^{c}**	**51.4±4.1^{b}**	**74.1±8.0^{a}**	**28.9±3.7^{d}**	**34.5±3.1^{c}**	**46.1±6.7^{b}**	**74.7±8.7^{a}**
Non-essential amino acids (NEAAs)								
Alanine	11.9±1.7^{cd}	16.3±1.4^{c}	35.6±1.8^{b}	49.9±1.8^{a}	9.1±1.1^{d}	15.4±0.6^{c}	31.6±0.6^{b}	48.6±7.3^{a}
Glycine	4.4±0.5^{cd}	7.0±1.3^{cd}	17.6±0.7^{b}	26.4±0.8^{a}	3.6±0.8^{d}	8.1±2.2^{c}	15.7±1.0^{b}	28.4±5.2^{a}
Gamma aminobutyric acid	2.4±0.1^{cd}	3.0±0.6^{c}	2.8±0.7^{c}	4.1±0.5^{b}	1.8±0.1^{d}	2.4±0.4^{cd}	2.9±0.7^{c}	5.0±0.8^{a}
Proline	7.0±0.3^{de}	9.7±0.7^{c}	15.9±0.8^{b}	20.5±2.0^{a}	6.4±0.1^{e}	9.1±0.5^{cd}	14.6±0.0^{b}	21.0±2.9^{a}
Asparagine	36.9±0.6^{b}	23.6±5.4^{c}	37.8±7.0^{b}	47.6±9.6^{b}	19.2±1.1^{c}	22.1±5.8^{c}	40.2±4.3^{b}	64.8±14.3^{a}
Aspartic acid	1.6±0.2^{d}	2.8±0.2^{c}	3.3±0.4^{bc}	3.6±0.4^{ab}	1.8±0.1^{d}	2.9±0.1^{c}	2.8±0.4^{c}	4.2±0.7^{a}
Glutamic acid	2.9±0.2^{bc}	2.7±0.5^{c}	3.3±0.2^{b}	4.4±0.2^{a}	1.4±0.1^{d}	2.4±0.2^{c}	2.8±0.4^{c}	4.4±0.4^{a}
Ornithine	0.8±0.1^{bc}	0.9±0.1^{b}	0.8±0.2^{b}	1.2±0.1^{a}	0.6±0.1^{c}	0.6±0.1^{c}	0.6±0.1^{c}	1.4±0.1^{a}
Tyrosine	0.9±0.2^{c}	1.1±0.2^{bc}	1.4±0.1^{b}	2.3±0.1^{a}	0.5±0.1^{d}	1.3±0.0^{bc}	1.3±0.4^{b}	2.6±0.3^{a}
Total NEAAs	**68.7±4.1^{c}**	**67.1±10.4^{c}**	**118.4±11.9^{b}**	**160.0±15.3^{a}**	**44.5±3.6^{d}**	**64.1±9.8^{c}**	**112.4±7.9^{b}**	**180.3±32.0^{a}**

Different letters in superscript in the same line indicate a significant difference ($p<0.05$).

(Figure 1). Moreover, the time of baking influenced the profile of the analyzed volatile compounds. In the crumb of breads baked for 15 min, the dominant group of volatiles was alcohols. After a longer time of baking, the proportions changed in all the samples of crumbs. The abundance of alcohols was found to be lower in both crumbs and crusts of the samples treated with high temperature for a longer time. This was accompanied by an increase in the abundance of pyrazines and sulfur compounds. With prolonged baking, the content of ketones in the crumb increased, while it decreased in the crust. In both the crumb and crust, 30 min of baking resulted in a lower percentage of esters.

In the crust part of breads, the composition of volatiles after 15 min of baking was different than in crumbs. The abundance of alcohols was notably lower in crust than crumb, while aldehydes and esters were definitely more abundant in the crust. Major changes occurred in the crumbs of breads baked for 30 min, since longer baking time resulted in multiple Maillard reaction products. The most important observation, which differentiates the crust from crumb, is related to the content of pyrazines, the abundance of which was relatively low in the crumb.

Detailed results of the VOCs analysis are given in Table S1 (for crumbs) and Table S2 (for crusts). A total of 96 VOCs were identified in all the analyzed samples. These included: 3 acids, 16 alcohols, 13 aldehydes, 11 esters, 6 furans, 10 ketones, 18 pyrazines, 6 terpenes, 5 sulfur compounds, and 8 other components. Tables S1 and S2 also include the areas of peaks obtained in the GC×GC-ToF-MS analysis. A tentative identification was performed using GC×GC-ToF--MS data, and only the compounds with high scores of mass spectra (>90%) were presented. Additionally, confirmation of the identity was performed by comparing the obtained retention indexes with literature data. Acids were detected in the crumb of CP0, CP2, and CP6, while they were not present in the CP10. Acetic acid is most likely responsible for the sour aroma of rye bread since it was listed as one of its key odorants [Boeswetter *et al.*, 2019]. Because of the polar character of acids, they show poor yields when extracted by SPME from the HS. Acetic acid (1) was not visible in the chromatograms

of the CP10 samples. This might have been a result of its relatively low content since part of rice flour was replaced with CP. Another possible reason might have been competition phenomena [Wieczorek *et al.*, 2022], the result of increased content of non-polar VOCs present in the HS above the bread containing a larger amount of CP. Moreover, it was reported previously that acetic acid was one of the compounds whose content changed significantly depending on the yeast culture used in the process [Hansen & Hansen, 1996]. Although the yeast culture was not changed in this study, the modification of matrix composition caused by the partial replacement of starch by CP could have affected the metabolism of yeasts. It is yet another likely explanation for the lower acid content in the GF bread containing larger amounts of CP. The effect of CP on yeast fermentation has never been analyzed; therefore, it is only a speculation, which is, however, worth elucidation in the future. Additionally, the presence of acids was only detected in the crumbs of GF breads, while no peaks corresponding to acids were observed in their crust. This was probably the effect of their evaporation from the outer part of the bread.

Food fermentation by yeast is accompanied by the formation of aliphatic and aromatic alcohols, known as fusel alcohols [Hazelwood *et al.*, 2008]. When present in high contents, fusel alcohols cause the occurrence of off-flavor in food. However, low contents of these compounds in a product might result in a pleasant aroma. In the analyzed GF breads, the abundance of the majority of alcohols was found to decrease with a prolonged time of baking (Table S1 and S2). Moreover, larger peak areas for many of the individual alcohols were observed in the crumbs than in the crusts, especially for 1-hexanol (8). This can be explained by the evaporation of alcohols from the outer surface during heating. The addition of CP to the GF bread did not increase the content of alcohols. Moreover, their peak areas were lower in the breads with CP. A more detailed analysis reveals that 1-hexanol (8) was the predominant alcohol in the crumb of CP0 (15 min of baking) and CP10 (30 min of baking), while 1-pentanol (13) was the most abundant one in CP10 bread baked for 15 min. While in the crust part, the content of 1-hexanol (8) was lower than the contents of phenylethenyl alcohol (15), 1-propanol (16), and 2-methyl-1-propanol (17) in CP0. Some bias in the results was observed, probably because of the applied extraction technique. As presented in Table S1, the level of phenethyl alcohol (15) decreased with an increasing percentage of CP. Phenylethyl alcohol is a fusel alcohol that is produced from phenylalanine in the Ehrlich pathway during yeast fermentation [Pico *et al.*, 2017b]. The content of phenylalanine increased with greater CP addition (Table 1), therefore an increase of phenylethyl alcohol could be expected. Thus, there is no reasonable explanation for its lower abundance in the CP-enriched breads. The observed lower content of alcohols, including the latter, may be explained by the competitive

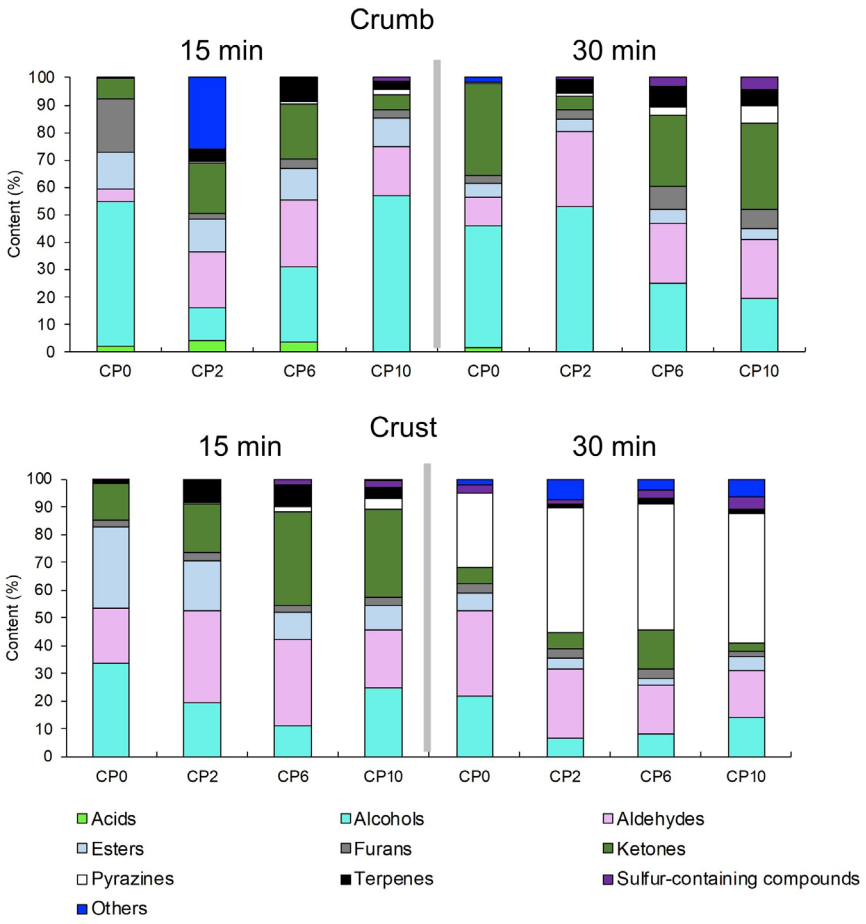

FIGURE 1. Percentage content of acids, alcohols, aldehydes, esters, furans, ketones, terpenes, pyrazines, and sulfur compounds in crumb and crust of breads supplemented with 2, 6, and 10% of cricket powder (CP) and baked for 15 and 30 min.

nature of SPME extraction. This point should be further investigated, since it is a common issue in food analysis [Spietelun *et al.*, 2013].

Next important group of compounds affected by the incorporation of CP are aldehydes (Figure 1). They are secondary breakdown products of unsaturated lipids [Shahidi, 2001] as well as precursors of alcohols in the Ehrlich pathway, formed from acids [Hazelwood *et al.*, 2008]. Their significance in the development of aroma of food products has been discussed recently [Dunkel *et al.*, 2014]. Some of them, like hexanal, 2-nonenal or 2,4-decadienal, were recognized as the main odorants in most of food products [Dunkel *et al.*, 2014]. Among the analyzed GF breads, the amount of hexanal (27) was found noticeably higher in the CP-enriched samples (Tables S1 and S2). Noteworthy, the content of this VOC was higher in the crust, compared to the crumb of the analyzed breads. The increment of its amount in the bread enriched with CP was probably a result of the high lipid content in CP. A previous study demonstrated lipid content of 29 g/100 g in CP [Montowska *et al.*, 2019]. Oxidation of fatty acids is one of the ways in which aldehydes are formed in food. This process is accelerated by heating, which is a likely reason behind the increased abundance of aldehydes in the crust compared to the crumb of the analyzed GF breads.

In terms of peak area comparison between breads with different CP contents, the major differences were observed in the level of pyrazines in the crust part (Table S1 and S2). These compounds are formed in bread from the reaction of amino acids with sugars *via* the Maillard reactions. This process is well documented [Yu *et al.*, 2021]. Pyrazines are known as important contributors to the pleasant aromas of bread; and many efforts have been made to promote their formation in GF bread [Pacyński *et al.*, 2015]. Therefore, they gained special attention due to their probable influence on the bread's flavor. Many pyrazines were formed in the crusts of breads with CP baking of 30 min, including: 2-ethylpyrazine (61), 3-ethyl-2-methylpyrazine (62), 2-ethyl-6-methylethylpyrazine (63), 2-isopropenylpyrazine (67), 5-ethyl-2,3-dimethylpyrazine (69), 2-methyl-3-(2-propenyl)pyrazine (70), 2-isobutyl--3-methylpyrazine (71), 3,5-diethyl-2-methylpyrazine (72), 2,3-diethylpyrazine (73), 2-ethyl-3,5-dimethylpyrazine (74), 2-acetyl-3-ethylpyrazine (75), 2,5-dimethyl-3-(2-methylpropyl)pyrazine (76), and 2-isoamyl-6-methylpyrazine (77) (Table S2). These compounds were not detected in the control bread – CP0. The increased content of pyrazines was determined in the breads with larger CP content, probably due to the higher content of FAAs. The abundance of pyrazines was higher in the crust than in the crumb, which can be explained by the greater exposure of the outer surface of bread to higher temperatures during baking. This intensified the Maillard reactions. However, some compounds, such as 2,3,5-trimethylpyrazine (68), were present in the crust part only after 15 min of baking; it was not detected after 30 min of baking. This suggests that this compound was further transformed or degraded at a quicker rate when exposure to heat was greater. Moreover, this compound was found in the crumb of the bread with its abundance increasing together with CP percentage (Table S1). A significant increase was also noted in the peak area of 2,5-dimethylpyrazine (65). Even though the affinity of this compound to the SPME fiber is low (its log P=0.6), it was present in high abundance, which indicates its high content. Therefore, despite its odor threshold being relatively high (800 ppb, according to Leffingwell & Associates [2018]) it might have been involved in the final aroma development. Its odor notes were described in the literature as chocolate, roasted, and earthy [Leffingwell & Associates, 2018]. Many of the pyrazines detected in this study (Tables S1 and S2) are characterized by low odor thresholds [Müller & Rappert, 2010]; therefore, their influence on the flavor seems inevitable.

The content of furans (described as furan and furan derivatives) was notably higher in bread without CP addition than in the ones containing CP (Figure 1). Furans are formed in the Maillard reactions [Srivastava *et al.*, 2018], but the mechanism of their formation remains unclear. It has been proposed that there are multiple precursors and alternative routes for the formation of furans in foods rather than a single mechanism. The major routes are the thermal degradation of carbohydrates and/or ascorbic acid and its derivatives, and the thermal oxidation of polyunsaturated fatty acids [Crews & Castle, 2007]. The abundance of furans increased with the increasing percentage of CP in GF formulations (Tables S1 and S2), which, similarly to the case of pyrazines, can be explained by the intensification of the Maillard reactions that resulted from to the greater availability of FAAs. Similarly, to pyrazines, furans were found more abundant in the crust, *i.e.*, the bread part that is more exposed to high temperatures. Some furan derivatives, such as 2-methylfuran (47), 2-ethylfuran (49), *etc.*, were present at different levels, without any unambiguous dependence on the CP content. Since there is a lack of literature data regarding the possibility of their mutual transposition, it is not possible to present a likely explanation of these results.

Many VOCs presented in Tables S1 and S2 show aroma activity, some of them, detected only in CP-fortified bread, were reported as key odorants in wheat bread [Rohleder *et al.*, 2019]. For instance, methional (88) was detected in the crumb of CP6and CP10, and in the crust part of all the breads with CP baked for 30 min. This is a further indication of the improvement of aroma quality of GF bread resulting from CP addition.

Due to the complexity of VOCs data, PCA was performed to reduce the dimensionality of the original data and the biplots are presented in Figure 2 separately for the crumb (A) and crust (B). For the crumb (Figure 2A), a good separation between the analyzed GF breads was observed. Breads CP0 and CP2 were positioned on the right side of the plot, while CP6 and CP10 – on the left side. CP0 was characterized by a higher abundance of the majority of alcohols and esters, while ketones, aldehydes, and sulfur compounds were associated with CP10. For crumbs, there was no differentiation between the baking times. For crusts (Figure 2B), there was no separation between the analyzed GF breads baked for 15 min, which all were placed in the bottom right quarter. In the case of the samples baked for 30 min, a clear separation of CP10 can be observed with its placement on the left side of the plot. Noteworthy, in accordance to the results described above, CP10 bread was characterized by the highest abundance of pyrazines and aldehydes.

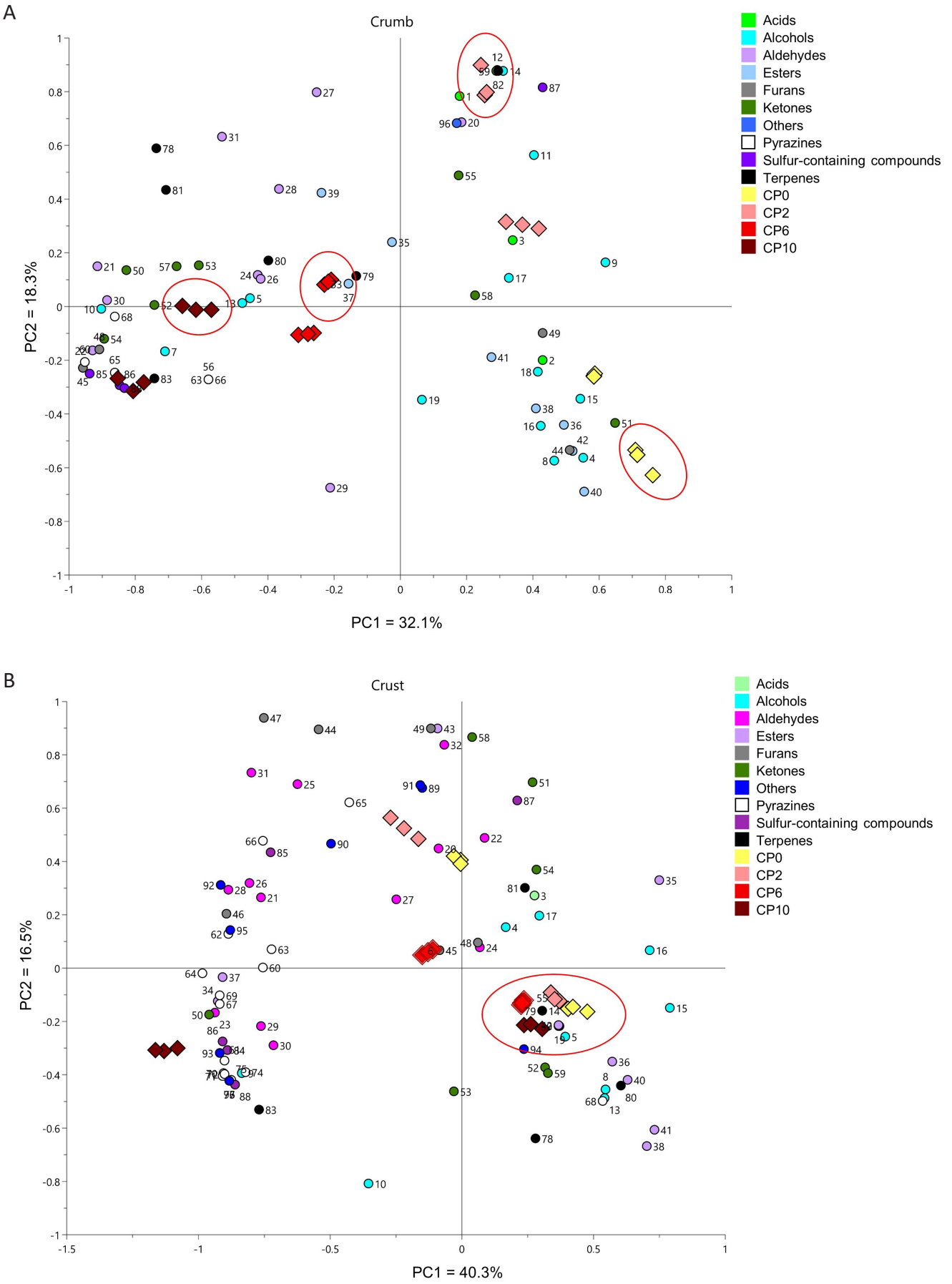

FIGURE 2. Principal component analysis biplots of volatile compounds in crumb (A) and crust (B) of control bread (CP0) and breads supplemented with 2, 6, and 10% of cricket powder, presented as CP2, CP6, and CP10, respectively. The results obtained after 15 min of baking are presented in red circles.

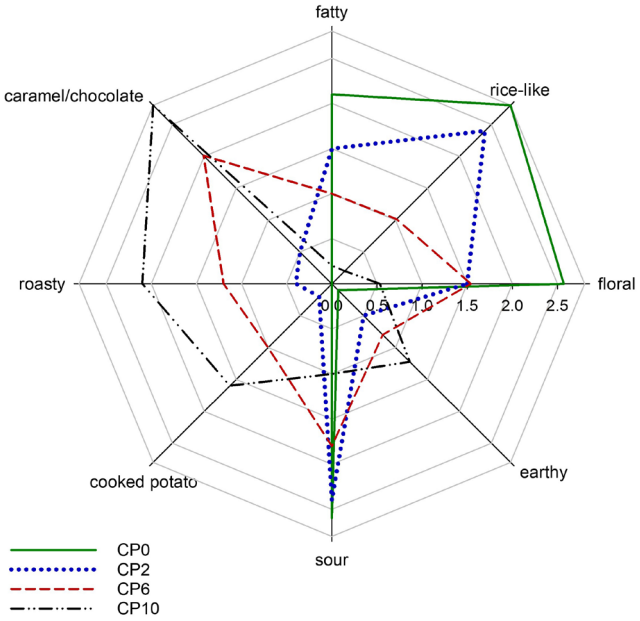

FIGURE 3. Radar chart showing the results of the sensory analysis of CP-enriched gluten-free (GF) breads. CP0, CP2, CP6, and CP10 – GF bread with 0, 2, 6, and 10% starch replacement with cricket powder, respectively.

Sensory analysis

The results of sensory analysis of the aroma of fortified GF breads are presented in Figure 3. The reference bread, with no CP addition, was characterized mainly by "rice-like", "sour", "fatty" and "floral" notes. These aroma notes were perceived with lower intensity as the CP percentage increased. The addition of CP resulted in increasing intensity of "caramel/chocolate", "roasty" and "cooked potato" notes. The GF breads enriched with CP were characterized by notes typical for wheat bread, such as nutty, caramel-like and roasted, that are associated with pyrazines. These results indicate that CP can be a valuable ingredient used for the manufacture of GF products with improved flavor characteristics, which currently are rather low [Alencar *et al.*, 2021]. Numerous literature data indicate that bakery products are often enriched with powdered forms of various EIs, *e.g.*, crickets, termites, grasshoppers, locusts, or mealworms [Yazici & Ozer, 2021]. The inclusion of edible insects into the recipe of these products affects not only the nutritional value but also the sensory properties of the end products. Zielińska *et al.* [2021] analyzed sensory acceptance of muffins enriched with different levels of cricket (*Gryllodes sigillatus*) and mealworm (*Tenebrio molitor*) powders. The results of their analysis showed that the EIs-enriched muffins were well-evaluated and fully accepted by consumers. Similar results were presented by Pauter *et al.* [2018], who also analyzed CP muffins. On the other hand, Smarzyński *et al.* [2021] showed that a small, 2% addition of CP to cookie formula improved the acceptance of final products.

CONCLUSIONS

The addition of CP influenced the sensory quality of GF breads. The nutritional quality in terms of the FAAs profile was also improved. This is of particular importance for the development of products dedicated to people on elimination diets. Content of both NEAAs and EAAs increased as a result of CP incorporation. Statistically significant changes were observed already at 4% CP addition. The higher content of FAAs contributed to the development of a richer aroma in the GF breads, which became similar to that of traditional wheat-based bread. This outcome is clearly related to the higher level of pyrazines in the CP- fortified GF breads, as the compounds of this group are responsible for the pleasant nutty and roasted notes. The analysis of VOCs in the experimental GF breads indicated that the addition of CP changed the composition of the volatile fraction in a significant way. Besides the case of pyrazines, major changes included increased aldehydes content, which was a likely result of the higher content of their precursors that were introduced with CP. The differences in the composition of volatiles affected the aroma of bread in a meaningful way which was confirmed by sensory assessment. Therefore, it can be concluded that CP is an attractive additive that shows potential for use in improving the sensory and nutritional properties of GF bread.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

The following are available online at http://journal.pan.olsztyn.pl/Effect-of-Cricket-Powder-Incorporation-on-the-Profile-of-Volatile-Organic-Compounds,156404,0,2.html; Table S1. Compounds tentatively identified based on results from GC×GC-ToF-MS analysis in the crumb part of breads subjected to the study (CP0, CP2, CP6, CP10), baked for 15 and 30 min. Table S2. Compounds tentatively identified based on results from GC×GC-ToF-MS analysis in the crust part of breads subjected to the study (CP0, CP2, CP6, CP10) baked for 15 and 30 min.

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Instruction for Authors
Section: Editorial News

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