DOCTORAL THESIS

Analytical Methods for Quantification of Bitter Compounds in Plant Protein Ingredients and Food Applications

Anastassia Zeinatulina

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Anastassia Zeinatulina

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Contents

List of publications

The list of author's publications, based on which the thesis has been prepared:

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II **Bljahhina, A.**, Pismennõi, D., Kriščiunaite, T., Kuhtinskaja, M., Kobrin, E.-G. (2023). Quantitative Analysis of Oat (*Avena sativa* L.) and Pea (*Pisum sativum* L.) Saponins in Plant-Based Food Products by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry. Foods, 12 (5), #991. https://doi.org/10.3390/foods12050991

III **Bljahhina, A.,** Kuhtinskaja, M., Kriščiunaite, T. (2023). Development of Extraction Method for Determination of Saponins in Soybean-Based Yoghurt Alternatives: Effect of Sample pH. Foods, 12 (11), #2164. https://doi.org/10.3390/foods12112164

IV **Zeinatulina[‡], A.**, Tanilas, K., Ehala-Aleksejev K., Viiard, E., Kriščiunaite, T. (2025). Digestibility of protein and estimated bioavailability of mineral compounds in plant-based yoghurt alternatives. Future Foods, 11, #100545. https://doi.org/10.1016/j.fufo.2025.100545

V **Zeinatulina[‡], A.**, Kaleda, A., Kuhtinskaja, M., Kriščiunaite, T. (2025). Optimisation of extraction method for quantification of free fatty acids in oat-, pea- and faba-bean-based protein sources by liquid chromatography coupled with mass spectrometry. NFS Journal, 39, #100228. https://doi.org/10.1016/j.nfs.2025.100228

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Author's contribution to the publications

Contribution to the papers in this thesis are:

I The author contributed to the idea formation and conceptualisation of the article, reviewed and edited the manuscript.

II The author developed an analytical method, performed all experiments, validated the method and analysed the data and wrote the manuscript.

III The author contributed to the idea formation, developed an analytical method, supervised validation experiments, analysed the data and wrote the manuscript.

IV The author supervised the saponin analysis, performed a quality check of all analytical data and wrote the manuscript.

V The author developed an analytical method, performed all experiments, analysed the data and wrote the manuscript.

Abbreviations

BLEND	Blend (52% pea protein isolate + 28% oat protein concentrate + 20% pea protein concentrate)
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C21:0	Heneicosanoic acid
C6:0	Hexanoic acid
DDMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one
DoE	Design of experiment
DoT	Dose-over-threshold
dwb	Dry weight basis
ESI	Electrospray ionisation
EtOH	Ethanol
EX–BLEND	Extruded blend (52% pea protein isolate + 28% oat protein concentrate + 20% pea protein concentrate)
FA	Formic acid
FB	Faba bean flour
F–F	Full factorial
FFAs	Free fatty acids
FPI	Faba bean protein isolate
GC–MS	Gas chromatography mass spectrometry
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IDL	Instrumental detection limit
IMS QTof	Ion Mobility Spectrometry Quadrupole Time-of-Flight
IPA	Isopropanol
IQL	Instrumental quantification limit
ISTD	Internal standard
LC–MS	Liquid chromatography coupled with mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge
MeCN	Acetonitrile
MeOH	Methanol
OD	Oat drink
OPC	Oat protein concentrate

P–B	Plackett-Burman
PBPAs	Plant-based protein applications
PD	Pea drink
PDA	Photo diode array
PPC	Pea protein concentrate
PPI	Pea protein isolate
SBD	Soybean-based drink
SIR	Selected ion recording
SPE	Solid phase extraction
SYA1; SYA2; VY4-S – VY12-S	Soybean-based yoghurt alternatives
RSD	Relative standard deviation
UV/Vis	Ultraviolet–Visible
v/v	Volume to volume
VY13-O – VY21-O	Oat-based yoghurt alternative
WGOF	Whole-grain oat flour
YP	Yellow pea flour
Z	Pooled sample composed of equal amounts of whole grain oat flour, yellow pea flour, faba bean isolate, oat protein concentrate, pea protein isolate and faba bean protein isolate

Introduction

Modern dietary patterns, increasing health awareness, ethical and environmental considerations have driven the food industry to intensify the development of plant-based dairy and meat alternatives alongside conventional animal-derived products. The global market for plant-based foods is projected to nearly triple within the next decade.[1] These alternatives are predominantly produced from protein-rich crops such as soybean, pea, faba bean, and oat, which undergo fractionation and purification to yield protein concentrates and isolates.[2–6] Compared with animal farming, plant-based protein production generates lower greenhouse gas emissions, requires fewer natural resources, and provides nutritional benefits such as reduced saturated fat and cholesterol and increased dietary fibre content. These products further address the needs of individuals with lactose intolerance or those following a vegan diet.[7–10] Despite these advantages, the development of high-quality plant-based alternatives remains challenging, primarily due to techno-functional limitations, reduced nutrient density and bioavailability, and the presence of undesirable sensory attributes, particularly beany and bitter flavours, that adversely affect consumer acceptance.[11,12]

Bitterness in plant-based dairy and meat alternatives is largely associated with crop-specific compounds inherent to the raw materials, most notably saponins and free fatty acids (FFAs). This is well documented in oats, peas, soybeans, and faba beans. Bitterness can be evaluated through descriptive sensory analysis[13] or quantified by specific bitter-tasting compounds such as saponins and FFAs instrumentally, using techniques such as liquid chromatography mass spectrometry.[14–28] Although various sample preparation protocols for saponins and FFAs have been proposed in recent decades, many are unsuitable for high-throughput workflows and routine laboratory implementation. Consequently, there is a clear need for rapid, simple, precise, and accurate analytical methods capable of quantifying bitterness-related compounds to support screening, monitoring, and optimisation during product development. Such methods would contribute to the production of more palatable and nutritionally adequate plant-based alternatives.

This thesis comprises five main sections. **Section 1** provides a comprehensive literature review summarising the nutritional, functional, sensory, and sustainability characteristics of selected plant protein crops used in the production of plant-based dairy and meat alternatives. It also outlines the structural features and sensory relevance of key bitter-tasting compounds, primarily saponins and FFAs, and critically evaluates existing extraction and detection methodologies. **Section 2** presents the research aims and underlying motivation, while **Section 3** details the materials, methods, instrumentation, and experimental conditions applied throughout the dissertation. **Section 4** synthesises the principal findings of publications. **Section 5** integrates the findings across all publications and outlines future research perspectives.

Overall, **Publication I** establishes the sensory importance and variability of bitterness perception in plant protein ingredients, justifying the need for employing analytical methods for quantification of the compounds responsible for that bitterness, while **Publications II, III and V** describe the development and application of liquid chromatography coupled with mass spectrometry (LC–MS) based methods including sample preparation procedure for quantification of bitter-tasting saponins and FFAs in various plant protein rich food ingredients and products. **Publication II** introduces a method enabling the simultaneous determination of oat and pea saponins in both

individual and mixed matrices, significantly simplifying previously established procedures, and **Publication III** further refines this method to ensure reliable recovery of soyasaponins from soybean-based yoghurt alternatives, where sample pH was shown to hinder extraction efficiency. **Publication IV** provides additional results on saponin quantification in plant-based yoghurt alternatives, which were achieved by applying methods developed in **Publications II and III**. **Publication V** employs a design of experiment approach to optimise extraction conditions for FFAs, particularly through fine-tuning solvent composition. Overall, the developed methods are characterised by their simplicity, robustness, suitability for high-throughput analysis, reduced environmental impact, and compatibility with routine laboratory applications due to the substantial shortening or elimination of extraction steps.

Taken together, the present dissertation offers a comprehensive quantitative assessment of saponin and FFA contents in various plant-based products, establishing clear links between chemical composition and sensory perception. These links support targeted strategies for managing bitterness in plant-based foods and inform future investigations into the sensory implications of these compounds.

Findings from this dissertation have been presented at international conferences, including the Biosystems Engineering conference 2023 (Tartu, Estonia), the 10th Recent Advances in Food Analysis in 2022 (Prague, Czech Republic), and the 27th NJF Congress 2022 organized by the Nordic Association of Agricultural Science (Selfoss, Iceland).

1 Literature overview

1.1 Plant-based protein applications (PBPAs)

The global demand for sustainable protein sources in food production has increased significantly in recent years. Modern diets are progressively shifting towards plant-based dairy and meat alternatives, driving innovation and diversification within the food industry. A wide range of crops are now utilized to produce these alternative protein foods. These crops can be broadly categorized into the following groups: cereals (wheat, corn, rice, oat, barley), legumes (peas, soy, beans, chickpeas, lentils, lupins, faba beans), oilseeds (sunflower, rapeseed, flaxseed, hemp seed, cotton seed, sesame seed, pumpkin seed), nuts (almond, pistachio, cashew, walnut, peanut), edible seeds (quinoa, buckwheat), pseudocereals (chia seed, amaranth), and tubers (potato).[29]

As of 2025, the global plant-based food market was valued at USD 14,225.3 million and is projected to nearly triple by 2035.[1] Among the leading plant-based protein sources, pea protein accounts for approximately 35.6% of the market share.[1] However, other reports indicate that soy remains the dominant protein source, representing 40.14% of the global plant-based food and beverage market in 2024.[30] Meanwhile, oat-based products have shown the most rapid growth, with a projected Compound Annual Growth Rate of 13.43% through 2030. These trends indicate that although soy continues to hold the largest market share, the demand for pea-based products is increasing rapidly, gaining competitive momentum within the plant-based protein sector.

To produce acceptable and palatable plant-based dairy and meat alternatives, various protein-rich ingredients derived from soybean, pea, faba bean and oat are used. The crops are processed through different protein purification techniques, such as air classification or wet fractionation under either alkaline or acidic conditions.[31] These methods yield protein-enriched ingredients including protein concentrates (protein content 50–75%), and protein isolates (protein content 80–90%).[2–6]

Compared to animal farming, the production of plant-based protein products generates significantly fewer greenhouse gas emissions and requires less land and water, addressing several key ethical and environmental concerns.[7] Furthermore, plant-based products generally contain lower levels of saturated fat and cholesterol while being richer in dietary fibre. They are also suitable for individuals who are lactose intolerant or who follow a vegan diet.[8–10]

Despite these benefits, food developers face several challenges when formulating and manufacturing plant-based alternatives to dairy and meat products. Product development can be complex, as the raw materials often present specific techno-functional limitations, lower nutrient density, and reduced nutrient bioavailability.[11,12] In addition, final products may inherit undesirable sensory characteristics from the raw materials, such as beany flavours or bitterness, which can negatively affect consumer acceptance.

1.2 Nutritional, functional, and sensory characteristics and sustainability aspects of selected plant protein crops

1.2.1 Soybeans (*Glycine max*)

Soybeans are the most commonly used crop in the production of plant-based alternatives, largely due to their nutritionally complete protein composition, which closely resembles that of animal protein.[32] In addition, soybeans are rich in isoflavones, low in saturated fat, and contain significant amounts of potassium, non-heme iron, and

B-group vitamins.[33] However, their use is limited for some consumers, as soybeans are among the top eight allergens worldwide.[34] Concerns have also been raised regarding phytoestrogens, such as isoflavones, which may mimic human oestrogens and potentially affect the health of children, individuals with hormonal conditions, or those with thyroid disorders.[35] Furthermore, raw soybeans contain antinutritional factors, including trypsin inhibitors, lectins, phytates, and saponins, which may impair protein and mineral absorption.[36] This contributes to the lower bioavailability of certain nutrients, such as calcium, vitamin B12, and vitamin D, in soybean-based dairy and meat alternatives compared to their animal-derived counterparts.[33]

From a functional perspective, soy protein concentrates and isolates exhibit excellent emulsifying properties and solubility, which contribute to a smooth mouthfeel, making them suitable for plant-based dairy alternatives.[13] Soy proteins also have high gelation and texturization properties, and extruded soybean protein fibres can closely mimic animal meat fibres.[37] Despite these advantages, sensory characteristics remain a limiting factor. Studies show that soy-based products are often associated with a bitter taste. Other sensory limitations include off-flavours caused by lipoxygenase activity and a greyish off-white colour, both of which may require flavour masking or additives to resemble dairy products.[38]

Soybeans are widely available and affordable, and their production generally results in a lower carbon footprint compared to dairy and meat.[39] However, large-scale soybean farming has been linked to deforestation and biodiversity loss, although the majority of global soybean production is used for animal feed rather than human consumption.[39] Additionally, a significant proportion of soybeans are genetically modified, which may be rejected by certain consumer groups.[40]

1.2.2 Peas (*Pisum sativum*)

The use of peas in plant-based meat production is increasing rapidly, driven by their favourable nutritional profile and consumer-friendly attributes. Pea protein concentrates and isolates exhibit a relatively balanced amino acid composition, making them an almost complete protein source.[41] They contain higher lysine content than many cereals, although they are low in sulphur-containing amino acids (methionine + cysteine), necessitating blending with cereals such as rice or oats to achieve a nutritionally balanced amino acid profile.[41,42] In terms of digestibility, pea protein has slightly lower protein digestibility compared to soy protein, with protein digestibility corrected amino acid score around 0.8–0.9 versus ~1.0 for soy.[41,43] Peas also contain antinutritional factors, such as phytates, tannins, and saponins, which can reduce mineral absorption.[20,44] Nevertheless, peas are rich in non-heme iron, as well as zinc, phosphorus, and calcium, providing nutritional benefits for plant-based consumers, although non-heme iron has lower bioavailability than heme iron.[42,45] Unlike soy, peas are not a major allergen, which enhances their acceptability among a broader consumer base.[46]

From a techno-functional perspective, pea proteins can form fibrous structures under extrusion, making them useful for plant-based meat analogues. They also exhibit good emulsifying and gelling properties, applicable in meat analogues, protein bars, and dairy alternatives. However, sensory challenges remain a key limitation, as pea proteins are often associated with “earthy,” “green,” or “bitter” off-flavours, which are difficult to mask in beverages and meat alternatives.[17,18,47–49] Additionally, pea proteins are less fibrous than soy, requiring blends or advanced extrusion technologies for meat analogues,

and may have lower solubility, which can cause separation in pea-based milk alternatives and necessitate the use of stabilizers.[13,50]

In terms of environmental sustainability, peas are nitrogen-fixing legumes that enrich soil fertility and reduce the need for synthetic fertilisers.[51] They also have a lower environmental footprint than soybeans and are generally non-GMO, which may be attractive to environmentally conscious consumers.[52] Historically, soybeans have primarily been cultivated for oil production, with the resulting protein-rich press cake treated as a by-product.[53] On the other hand, pea protein isolates are often more expensive than soybean proteins due to a less established processing infrastructure.[54]

One of the key reasons for their popularity is that pea protein is hypoallergenic, making it suitable for individuals with soy, dairy, or gluten intolerances. This broadens the consumer base and enables food manufacturers to label products as allergen-free, which is increasingly important in today's clean-label market.

1.2.3 Faba beans (*Vicia faba*)

Faba beans represent a novel source of plant protein for alternative foods. Faba bean protein isolates contain high protein levels, up to 85%, comparable to pea protein isolates, and possess a favourable amino acid profile.[42,55] Faba protein is particularly rich in essential amino acids, especially lysine, making it a strong complement to cereals such as oat or rice. However, like pea protein, faba protein is low in sulphur-containing amino acids (methionine + cysteine), and therefore blending with other protein sources is necessary for a complete amino acid profile.[42] Faba bean protein ingredients also contain antinutritional factors, including tannins, phytates, lectins, saponins, trypsin inhibitors, phenolics, oligosaccharides, and the pyrimidine glycosides vicine and convicine.[55] Vicine and convicine are responsible for favism in susceptible individuals, though the risk is much lower in protein isolates than in whole beans.[56] Oligosaccharides may cause bloating or flatulence, which is a minor digestive limitation.[55] Severe allergic reactions to the consumption of peas are rare, but possible, particularly in sensitive individuals.[57]

From a sensory and functional perspective, properly processed faba bean protein is blander than pea protein, with less "earthy" or "green" notes, making it more suitable for milk and meat alternatives.[58] Faba proteins also stabilize foams effectively and exhibit good emulsifying and gelling properties.[13] The light colour of faba protein isolates is advantageous for dairy alternatives.[13] However, some faba protein products can have noticeable bitterness and astringency, which remains a challenge for product development.[13] Commercial-scale faba bean protein isolates are still emerging, so their functional properties are less standardized compared to soybean proteins, which can complicate formulation and product consistency.[2,59,60]

In terms of sustainability, faba beans are nitrogen-fixing legumes, improving soil fertility and reducing fertilizer requirements.[61] They can also be cultivated in temperate climates, reducing dependence on tropical crops.[62] However, faba bean protein is currently less available and more expensive than soybean or pea protein, due to limited large-scale processing infrastructure.

1.2.4 Oats (*Avena sativa*)

Oats have a balanced macronutrient profile, providing carbohydrates, fibre, and a moderate amount of protein. However, typical oat-based milk production technologies do not concentrate protein, resulting in beverages with low protein content.[63] Although raw oats contain about 12–17% protein, much of it is lost or diluted during processing.[26]

Consequently, oats alone are less suitable as protein-rich dairy substitutes, unless the products are fortified with pea or soybean protein. Additionally, oat protein is incomplete, lacking essential amino acids such as lysine and methionine.[64] On the positive side, oats are rich in soluble fibres, particularly beta-glucans, which support heart health, lower cholesterol, and improve glycaemic response.[64–66] They also provide manganese, phosphorus, magnesium, and copper, although iron and zinc are present in smaller amounts. To match dairy nutritional profiles, oat-based products often require fortification with calcium, vitamin B12, and vitamin D.[64] Oats are generally hypoallergenic, though cross-contamination with gluten can occur during processing.[67]

From a sensory and functional standpoint, oats contribute a creamy mouthfeel and naturally sweet taste to milk alternatives without the need for extensive additives.[26] Their mild flavour profile and high foaming ability make oat-based milk alternatives more favourable than soybean-based counterparts.[68] Heat-processing of oats can cause starch gelatinization, which increases thickness and may be undesirable in some applications unless enzymes are applied to modify texture.[68,69] Oat protein exhibits weak gelation and limited fibrous texturization, making oats less suitable for meat analogues compared to soybean or pea proteins.[70–72] Furthermore, bitter compounds may co-extract with protein during the production of oat protein concentrates and isolates, potentially contributing to off-flavours in resulting products.[73,74]

In terms of sustainability, oats are resilient crops requiring less fertilizer and pesticides than many other grains.[75] Their cultivation generally has a lower carbon footprint and water use compared to other cereals and animal-based proteins.[76] Grown mainly in temperate regions, oats pose minimal deforestation risk and are widely accepted as non-GMO, natural products.[77,78]

1.3 Sensory perception and chemical basis of bitterness in plant protein ingredients and PBPAs

Flavour is a complex, multisensory perception shaped by the interplay of taste, aroma, and the food matrix.[79] In plant-based protein products, flavour perception plays a decisive role in consumer acceptance, as consumers often expect sensory profiles that resemble those of meat and dairy rather than legumes. Among the various flavour attributes, bitterness is one of the most prominent and challenging off notes, arising from intrinsic plant metabolites and their oxidation products and remains a key focus in efforts to improve the sensory quality of plant-based proteins.

1.3.1 Sensory analysis of bitterness

Sensory analysis is a key methodological tool for assessing bitterness. Quantitative descriptive analysis is one of the most frequently used sensory methods, in which trained panellists, working under strict and standardised conditions, identify and quantify the sensory properties of a product or ingredient.[80] The sensory data generated through quantitative descriptive analysis can be used to develop multidimensional quantitative models, which subsequently inform product marketing or product development activities.[80]

In recent years, quantitative descriptive analysis has been widely employed to evaluate bitter taste in plant-based foods and ingredients.[81–83] To further advance the understanding of sensory attributes in plant-based protein ingredients, Jakobson et al. (2023) conducted a comprehensive sensory characterisation of 24 protein concentrates

and isolates derived from soybean, pea, oat, faba bean, chickpea, mung bean, potato, wheat, and canola.[13] Sensory properties were evaluated using a 10-point scale (0–9). For instance, pea protein ingredients exhibited bitterness ratings ranging from 2 to 5, whereas faba bean protein powders ranged from 4 to 6, indicating notable variability both within and between crop sources.

Variability between products derived from the same crop may be attributed to differences in protein production techniques, which can influence the concentration of non-volatile bitter-tasting compounds present in protein-rich plant materials. Meanwhile, differences among products originating from different crops or different crop varieties are likely driven by the presence of crop-specific bitter compounds. These include saponins, phenolic compounds, tannins, bitter peptides, and lipid-derived molecules such as monoglycerides, FFAs, and oxidised fatty acids.[84,85] These compounds exhibit distinct bitter taste thresholds, meaning that perceived bitterness is influenced not only by their absolute concentration but also by their dose-over-threshold (DoT) value.[18,19,73,86] This highlights the importance of considering both chemical composition and sensory thresholds when evaluating bitterness in plant-based protein ingredients.

1.3.2 Bitter-tasting saponins in PBPAs

Soybeans, the legume studied in this thesis, are well-known for their characteristic beany and bitter off-taste, which has been primarily attributed to a large variety of soyasaponins naturally present in the beans.[14,87,88] Soyasaponins are classified into three groups, A, B, and E, based on their aglycone structures, with group B soyasaponins representing the predominant saponins in soybeans.[22,89–91] Previous research conducted in Japan analysed 39 samples of various soybean products, including seeds, soybean-based milk alternatives, tofu, yuba, natto, miso, douchi, and tofuyo, for their soyasaponin content.[15] That study monitored more than 100 soyasaponin compounds.[15] In contrast, the present dissertation focuses specifically on bitter-tasting **soyasaponin Bb (synonyms: soyasaponin I and saponin B) (1), soyasaponin Ba (3), soyasaponin Aa (4), and soyasaponin Ab (5)** (Figure 1), which together make up from 33 to 63% of all soyasaponins in soybean-based milk products.[15] Additionally, only compounds for which pure commercial standards were available were included in the present study.

Currently, the precise bitter taste threshold has been determined only for soyasaponin Bb. Previous studies reported that the bitter taste threshold of soyasaponin Bb is approximately 8.5 µmol/L.[20] Whereas a more recent investigation reported a higher threshold of 1620 µmol/L.[18]

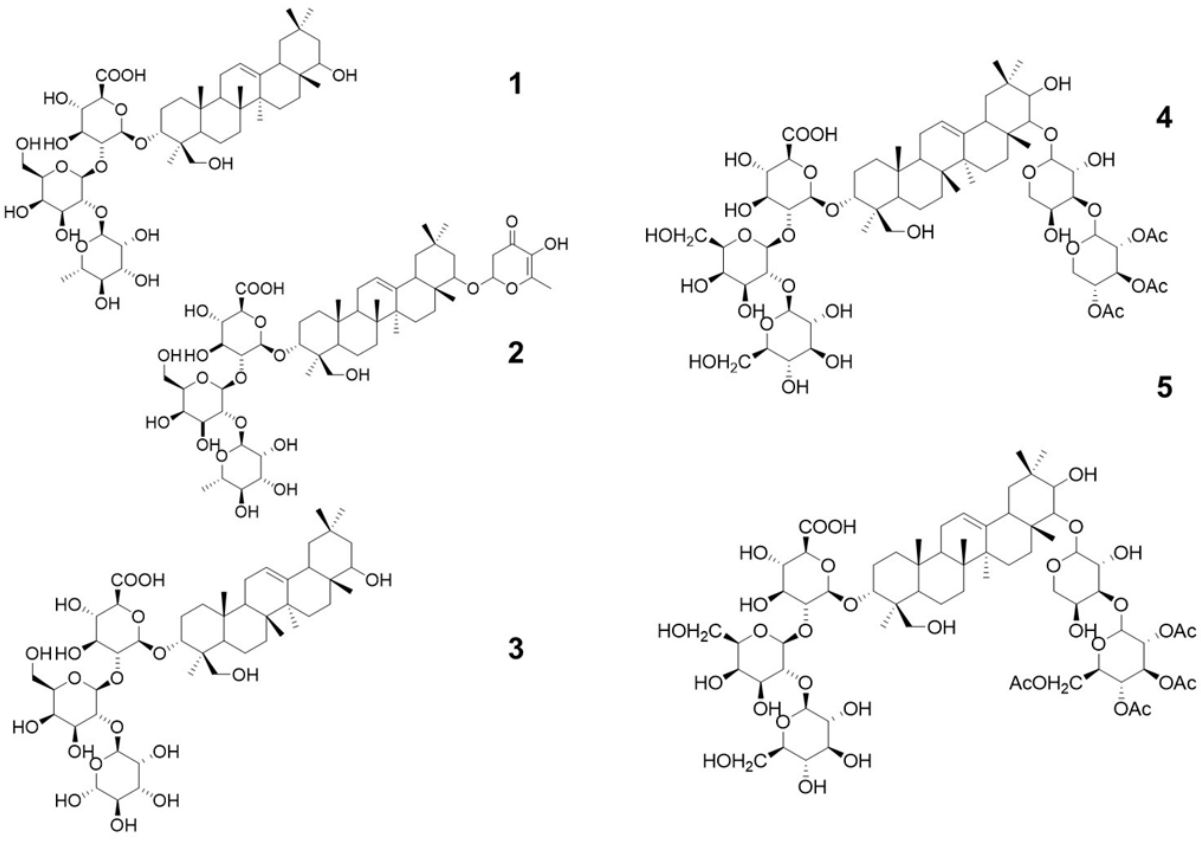

*Figure 1. Chemical structures of bitter-tasting soybean and pea saponins: soyasaponin Bb – $C_{48}H_{78}O_{18}$ (**1**), 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP saponin or soyasaponin βg) – $C_{54}H_{84}O_{21}$ (**2**), soyasaponin Ba – $C_{48}H_{78}O_{19}$ (**3**), soyasaponin Aa – $C_{64}H_{100}O_{31}$ (**4**), soyasaponin Ab – $C_{67}H_{104}O_{33}$ (**5**)*

Peas also contain triterpenoid saponins, including 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP saponin), which can degrade into soyasaponin Bb, which are the major contributors to bitter or astringent taste in peas.[20,86] While the bitter taste threshold of soyasaponin Bb has been reported to be approximately 8.5 µmol/L, the bitter taste threshold of DDMP saponin remains unknown because no food-grade reference standard is available, and the compound is highly unstable, making its extraction from food matrices challenging.[92] Consequently, the aforementioned study estimated the bitter taste threshold of DDMP saponin by evaluating a mixture of saponins (soyasaponin Bb + DDMP saponin in a ratio of approximately 1:4), which resulted in a threshold of approximately 2 µmol/L.[20] This suggests that DDMP saponin is roughly four times more bitter than soyasaponin Bb. Previous research has also demonstrated that during the production of pea protein concentrate and isolate, saponins become concentrated two- to threefold compared to the initial pea flour.[93,94] The present thesis focuses on monodesmosidic triterpenoid saponins (Figure 1), specifically **soyasaponin Bb (1)** and **DDMP saponin (2)** found in pea-containing matrices.[20,92]

Faba beans, another legume species examined in the present thesis, primarily contain soya-type triterpenoid saponins. These include both group B soyasaponins, which exist in several structural variants, and their DDMP-conjugated forms.[95] Both soyasaponin Bb and DDMP saponins have been identified in raw as well as soaked faba beans.[95] However, the analytical determination of saponins in faba bean ingredients were beyond the scope of the present dissertation.

Previous studies have identified avenacoside A and avenacoside B as key phytochemicals responsible for the characteristic astringent and bitter off-taste of **oats.**[19,73] Sensory experiments determining the oral threshold concentrations of oat saponins revealed a wide range of bitterness thresholds (avenacoside A: 6 ± 19 µmol/L; avenacoside B: 7 ± 20 µmol/L), indicating substantial inter-individual variability in sensory sensitivity among panellists.[73] Despite their relatively low concentrations in oat flour, avenacosides were shown to exert a pronounced sensory impact. This was further supported by the high DoT values (33.1 and 29.8 in oat flour), confirming that avenacosides are major contributors to oat bitterness due to their low sensory threshold levels within this matrix. The present thesis focuses on oat-specific steroidal saponins, namely the bisdesmosidic compounds **avenacoside A (6) and avenacoside B** (**7**), as well as the monodesmosidic steroidal saponin **26-desglucoavenacoside A (8)**, which are naturally present in oat grains and leaves (Figure 2).[27,28,73,96]

To address the need for quantitative data on saponins, **Publications II and III** describe the development and validation of saponin extraction methods. These methods were subsequently applied to both solid and liquid plant-based protein ingredients, as well as to intermediate and commercial products derived from oat-, pea-, and soybean (**Publications II**–**IV**).

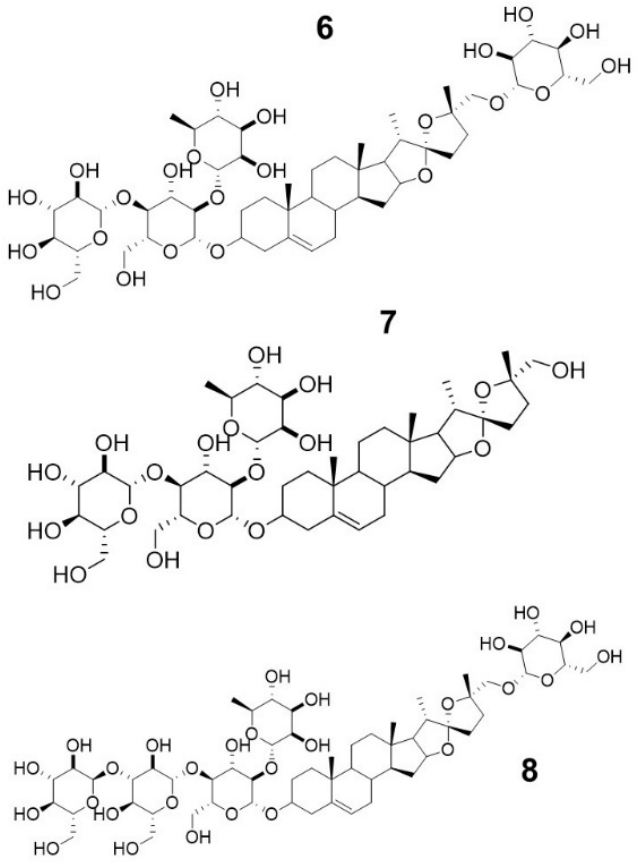

*Figure 2. Chemical structures of bitter-tasting oat saponins: avenacoside A – $C_{51}H_{82}O_{23}$ (**6**), 26-desglucoavenacoside A – $C_{45}H_{72}O_{18}$ (**7**), avenacoside B – $C_{57}H_{92}O_{28}$ (**8**).*

1.3.3 Bitter-tasting FFAs in PBPAs

A large number of plants contain a wide variety of triglycerides, which serve multiple functions, including energy storage, provision of a carbon source, reduction of water usage, and thermal insulation, among others.[97] These triglycerides are stored in different parts of plants, but the largest reserves are found in seeds, such as sunflower, soybean, canola, and coconut, and in fruits, including olives, avocados, and certain nuts.

During the production of protein ingredients from protein-rich crops, such as oat, pea, and soybean, the lipid-rich fraction can be removed through various extraction methods, including n-hexane soaking or supercritical CO_2 extraction, with or without the addition of ethanol (EtOH) or isopropanol (IPA).[53,98–102] However, the endogenous lipases and lipoxygenase might degrade remaining lipids into FFAs, which are considered bitter-tasting, non-volatile lipid compounds in oat, pea, and faba bean products.[86,103,104] The main FFAs of interest (Figure 3) in this dissertation are **myristic acid (C14:0; 9), palmitic acid (C16:0; 10), stearic acid (C18:0; 11), oleic acid (C18:1; 12), linoleic acid (C18:2; 13), and linolenic acid (C18:3; 14)**.[18,19,73,86,105,106] Among these compounds, C18:3 exhibits the most intense bitter taste, with the lowest bitter threshold of 277 µmol/L, whereas C18:1 is the least bitter, with a threshold of 2180 µmol/L.[86] Previous studies

have identified C18:2 and C18:3 as the dominant bitter-tasting FFAs in pea protein isolate and oat flour.[19,86]

In **Publication I**, we investigated the variability and correlation of bitterness sensitivity to C18:2, C18:3, oat flour, and pea flour among trained sensory panellists and consumers. Although the bitter taste thresholds of several compounds are known, assessing their actual impact on product bitterness requires accurate knowledge of their concentrations to calculate the DoT values. To address this, **Publication V** describes the development and validation of an extraction method for FFAs, which was subsequently applied to quantify these compounds in various plant-based protein ingredients.

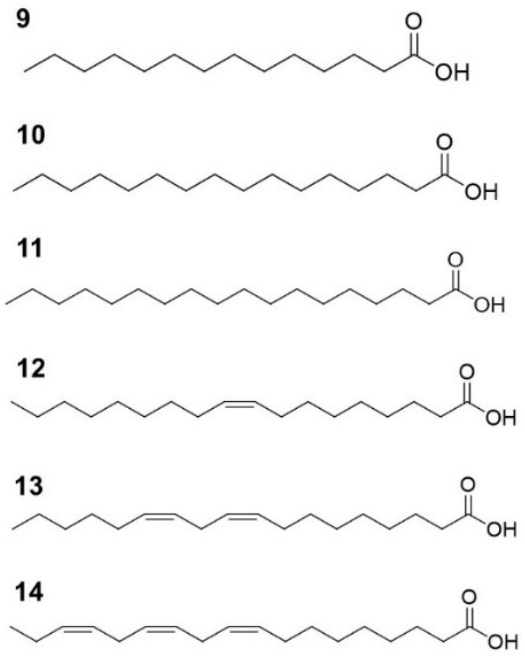

*Figure 3. Chemical structures of bitter-tasting FFAs: myristic acid [C14:0] – $C_{14}H_{28}O_2$ (**9**), palmitic acid [C16:0] – $C_{16}H_{32}O_2$ (**10**), stearic acid [C18:0] – $C_{18}H_{36}O_2$ (**11**), oleic acid [C18:1] – $C_{18}H_{34}O_2$ (**12**), linoleic acid [C18:2] – $C_{18}H_{32}O_2$ (**13**), and linolenic acid [C18:3] – $C_{18}H_{30}O_2$ (**14**).*

1.3.4 Other bitter molecules in plant protein crops

In addition to saponins, **legumes** (e.g., peas, soybeans, beans, chickpeas, lentils, lupins, and faba beans) contain other bitter-tasting compounds, such as phenolics, tannins, bitter peptides, and certain alkaloids.[106,107] Isoflavones (e.g., genistein, daidzein, and glycitein) subtly contribute to both the bitterness and astringency of soybean-based products. Small hydrophobic peptides, rich in leucine, valine, phenylalanine, and proline, generated during protein hydrolysis processes (e.g., fermentation or enzymatic treatments) can impart an intensely bitter taste.[108–110] Additionally, phenolic compounds such as chlorogenic acid derivatives may further enhance the bitterness or astringency of soybean-derived products.[111] Peas, another legume examined in this thesis, contain bitter-tasting phenolic acids and hydrophobic peptides that can form during enzymatic or natural proteolysis.[17,112] Similarly, faba beans products, also studied in the present

thesis, possess specific bitter peptides derived from vicilin- and legumin-type proteins during protein isolate production.[105] Although the antinutrients vicine and convicine found in faba beans are not directly associated with bitterness (being more relevant to favism), their related secondary metabolites can contribute undesirable off-notes.[84]

Cereal grains (e.g., wheat, corn, rice, oats, and barley) also contain phenolic compounds and tannins, including ferulic, caffeic, p-coumaric, and vanillic acids, and bitter peptides may form during protein hydrolysis.[19,73,113] Whole-grain flour fractions generally exhibit higher levels of phenolics, leading to a slightly more bitter and astringent sensory profile. In oats, besides the oat specific saponins called avenacosides, the unique oat phenolic compounds avenanthramides also contribute mild bitterness and astringency, particularly if they are not effectively removed during processing.[19,73,114,115] Moreover, lipid oxidation products such as aldehydes and ketones, generated through lipase or lipoxygenase activity, can impart bitter and oxidized notes in oats that have not undergone heat treatment.[116]

Protein can also be extracted from press cakes of **oilseed crops** (e.g., sunflower, rapeseed/canola, flaxseed, hemp, cottonseed, sesame, and pumpkin). These press cakes, as well as the protein concentrates and isolates produced from them, may contain characteristic bitter compounds, including phenolics and oxidized lipids.[117] A recent study identified a novel bitter compound, pinocarveol β-D-apiofuranosyl-(1→6)-β-D-(4-O-caffeoyl)glucopyranoside, in sunflower press cake.[117] Additionally, lipid oxidation products such as aldehydes and ketones contribute to harsh and bitter off-notes.[118] In rapeseeds, the key bitter compounds are glucosinolates and phenolic derivatives, including sinapine and kaempferol-sinapoyl glucosides, which are primarily responsible for the bitterness in rapeseed protein concentrates and isolates.[118,119]

Nuts (e.g., almonds, pistachios, cashews, walnuts, and peanuts) can exhibit bitterness and astringency due to the presence of oxidized lipids, tannins, and polyphenols.[120,121] Almonds, in particular, contain the bitter and toxic cyanogenic glycoside amygdalin.[122] Similarly, **edible seeds and pseudocereals** such as quinoa, buckwheat, chia, and amaranth contain species-specific triterpenoid saponins, phenolic compounds (e.g., rutin, catechins, and tannins), and flavonoids concentrated in the seed coat.[115,123,124] Moreover, proteins extracted from **tubers** such as potatoes can contain glycoalkaloids, primarily α-solanine and α-chaconine, which are known for their pronounced bitterness and toxicity.[125]

1.4 Analytical methods for determination of soy, pea and oat saponins in PBPAs

Commonly, the main methods used for determining total saponin content and for fingerprinting include spectrophotometric–colorimetric assays (e.g., vanillin–H_2SO_4 method), foam tests (afrosimetry, foam height measurement), hemolytic assays, and high-performance thin-layer chromatography.[126–128] These methods provide an overall estimation of total saponin content rather than quantifying individual compounds. In contrast, instrumental techniques such as high-performance liquid chromatography (HPLC),[19,20,27] gas chromatography (GC),[129–131] and quantitative nuclear magnetic resonance spectroscopy[132,133] enable the quantitative analysis and structural elucidation of individual saponins. The LC–MS instrumentation was applied In **Publications II–IV** for individual saponin compound quantification. Saponin extraction and purification methods are often time-consuming and depend on the complexity of the food matrix.[14,17,20–22,24,26,27]

A selection of extraction methodologies applicable with LC separation for soy, pea, and oat saponins is summarized in Table 1, Table 2, Table 3, respectively.

Typically, **the extraction of pea and soybean saponins** begins with sample defatting using hexane, followed by extraction with MeOH or EtOH aqueous solutions. The obtained extract is then evaporated to dryness, and solid-phase extraction (SPE) may be employed to further purify the sample (Table 1 and Table 2).[17,18,25,92] In most protocols (Table 1) for soybeans, dry samples are extracted with aqueous MeOH, concentrated under vacuum, and subsequently redissolved in an appropriate solvent for chromatographic analysis.[14,15,21,22]

The extraction of oat saponins (Table 3) is generally laborious and time-consuming.[24,26–28,134] Typically, sample preparation involves defatting with hexane for up to three days, while liquid samples are first freeze-dried before extraction. Extraction is usually performed using pure MeOH or aqueous MeOH under reflux or sonication conditions. The extracts are then concentrated to dryness under vacuum, and in some cases, additional SPE cleanup is applied before solvent evaporation. Quantification of oat saponins is further complicated by the limited availability of suitable external and internal standards (ISTDs).

Saponins from various plant origins have been analysed using liquid chromatography coupled with ultraviolet visible (UV/Vis), evaporative light scattering, refractive index, or mass spectrometry (MS) detectors.[135,136] However, quantification by UV/Vis detection is challenging because most saponins lack chromophoric groups, such as conjugated double bonds or aromatic rings, which are necessary for strong absorption in the UV/Vis region (typically 210–280 nm). Consequently, saponins exhibit very low molar absorptivity, resulting in weak and poorly defined signals, and therefore low quantitative accuracy with UV detection. To overcome this limitation, post-chromatographic derivatization with p-anisaldehyde–sulfuric acid can be employed to enable UV/Vis detection.[95] In contrast, liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI/MS) provides high sensitivity and specificity without the need for analyte derivatization, making it a preferred method for the quantitative and structural analysis of saponins.[19,20,27,87]

Despite the rapid advancement of analytical techniques, the simultaneous quantification of saponins originating from different plant species remains challenging. Current methodologies typically require labour-intensive sample preparation steps, which limit their suitability for routine laboratory analysis. Therefore, in **Publications II and III**, considerable effort was devoted to developing and optimising saponin extraction procedures aimed at enabling high-throughput analysis and ensuring their applicability in routine laboratory settings. The newly developed methods were designed to provide a simplified workflow in which samples do not require freeze-drying, and organic extraction solvents are not exchanged by rotary evaporation. Furthermore, the optimized extraction protocols were applied to both solid and liquid commercial products for saponin quantification, as described in **Publications II–IV**.

Table 1. A selection of extraction methods for soybean saponins.

Analytes	Matrix	Instrumentation	Brief description of extraction method	Reference
Various soyasaponins	Soybean flour, tofu, texturized soybean protein	HPLC–PDA/ESI/MS	• 4 g of finely ground dry samples (if soybean hypocotyls 0.2–1 g) + 100 mL of 70% EtOH (v/v) with stirring for 2.5 h at room temperature. • Filter extracts and evaporate to dryness at <30 °C • Redissolve residue in 10 mL of 80% MeOH (v/v). • Filter extract and analyse by HPLC–PDA/ESI/MS.	Hu et al., (2002) [21]
Soyasapogenol A and soyasapogenol B	Various soy-based foods	HPLC–MS/MS	• Perform 2-steps extraction with 3–5 g of soybean flour under sonication power with 70% EtOH (v/v) for 1h. • Centrifuge extract and combine. • Concentrate supernatant under vacuum to 50 mL under reduced pressure. • Filter prepared sample and analyse by HPLC–MS/MS	Kamo et al., (2014) [22]
106 soyasaponin compounds	39 products (including - soybean seeds, soy-based milk alternative, tofu	LC–PDA/MS/MS	• Freeze dry and mill liquid samples. Mill seeds. • Extract samples with 5-fold volume 80% MeOH (v/w) at RT for 1 h. • Extracts were centrifuged and applied to analysis by LC–PDA/MS/MS	Chitisankul et al., (2018) [14] and Chitisankul et al., (2021) [15]

Table 2. A selection of extraction methods for pea saponins.

Analytes	Matrix	Instrumentation	Brief description of extraction method	Reference
Soyasaponin Bb and DDMP[1] saponin	16 pea cultivars	HPLC–ELSD and HPLC–Q Ion-trap MS	• Defat pea flour by hexane refluxing for 6 h and air-dry overnight. • Extract 1 g of sample with 100 mL 70% EtOH (v/v) for 1 h at 25 °C at constant shaking at 200 rpm. ISTD equilenin was added before extraction. • Filter extract thought ashless paper. • Evaporate EtOH under vacuum. • Clean remaining water-sample solution using SPE cartridge. Elute saponins using MeOH. • Air-dry resulting MeOH-saponin extract and reconstitute in 1mL of 50% EtOH (v/v) before HPLC analysis.	Heng et al., (2006) [20]
Soyasaponin Bb	Pea protein isolate	UHPLC–MS/MS	• Extract 500 mg of sample using 5 mL aqueous MeOH solution (1:1, volume to volume, v/v), incubated on an orbital shaker for 1h • Membrane filter before LC–MS/MS analysis	Gläser et al., (2020) [18]
Soyasaponin Bb and DDMP[1] saponin	Pea flour, pe protein isolate and pea starch	HPLC–MS	• Extract 600 mg powder sample with 4 mL of 70% EtOH (v/v) containing 1 mg/L leucine-enkephalin (ISTD) • Stir at 350 rpm at room temperature for 1 h. • Centrifuge, dilute, filter before LC–MS analysis.	Noguera et al., (2022) [25]
Large group of analytes, including tentative identification of saponins using soyasapogenol B	Pea protein isolate and pea flour	UHPLC–DAD–MS	• Perform 3-step extraction with 6 g of sample and 30 mL MeOH-formic acid (FA) solution (99:1, v/v) for 2 h. Combine extracts (total 90 mL). • Keep supernatants at -20 °C for 10 min before centrifugation. • Evaporate supernatants at 40 °C under vacuum in darkness. Remove precipitates during concentration process using centrifuge and then evaporate supernatant completely. • Samples were reconstituted in 1 mL of 80% MeOH (v/v) and kept at -20 °C to promote precipitation. • Centrifuge, filter, and store at -80 °C in darkness until analysis.	Cosson et al., (2022) [17]

[1]2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one

Table 3. A selection of extraction methods for oat saponins.

Analytes	Matrix	Instrumentation	Brief description of extraction method	Reference
Avenacoside A, avenacoside B, 26-desglucoavenacoside A	16 oat cultivars (grains and husks)	UPLC–TQ–MS	• Grind sample and defat by Soxhlet (n-hexane, 72h) • A 2-step extraction of 1.0 g sample + 50 mL MeOH 1 h at boiling point under reflux, combine supernatants • Evaporate supernatants resuspend in 5% MeOH • SPE, exchange solvent, dilute before injection to LC–MS	Pecio et al., (2013) [27]
16 avenacosides (including isomers)	Oat bran (n = 3), oatmeal (n = 6), cold oat cereal (n = 6)	HPLC–MS	• A 3-step extraction of a one g of sample with 50 mL of MeOH under sonication for 30 min. • Combine supernatants, centrifuge. • Concentrate to dryness under *vacuo*. • Reconstitute in 2 mL 50% MeOH, centrifuge, dilute prior to LC–MS.	Yang et al., (2016) [28]
22 saponins including 7 new saponins identified	7 oat cultivars (oat grains)	UPLC–QTOF–MS, UPLC–MS/MS	• A 2-step extraction of 0.1 g of powdered samples. First with 1.5 mL and 1.0 mL of 70% MeOH (v/v) under sonication 30 min. • Combined supernatants, centrifuges, filter. • Concentrate by under N_2 gas, redissolved in 4 mL water. • ISTD was added (protodiscin). • SPE by applying C18 cartridge. Eluted saponins were concentrated by N_2 gas, redissolved in 70% MeOH prior to LC–MS analysis.	Kwon et al., (2024) [24]
Avenacoside A	Oat-based milk alternatives	LC–MS/MS	• Place 10 mL of liquid sample into a separating funnel, add 10 mL of hexane, shake and equilibrate for 15 min before removing fat. • Centrifuge, remove hexane + fat • Add 500 µL of the sample to 1.5 mL of MeCN containing 50 µL of FA. Shake for 1 h. • Centrifuged, filter. Analyse by LC–MS	McCarron et al., (2024) [26] and McCarron et al., (2025) [134]

1.5 Analytical methods for determination of bitter-tasting FFAs in PBPAs

Generally, **extraction methods for lipid compounds** in various food matrices can be divided into two main groups. The first group comprises classical liquid-liquid extraction (LLE) methods that employ immiscible solvents, such as the Bligh and Dyer, Folch, Soxhlet, and Matyash methods. These approaches typically use solvent systems that are incompatible with LC–MS instrumentation without an additional solvent exchange step.[137–140] The second group utilizes miscible solvents, such as MeOH and IPA, for lipid extraction. Extracts obtained using these solvents can be directly injected into LC–MS systems without further solvent modification.[18,19,73,86,104] In line with the growing emphasis on green chemistry principles, classical LLE solvents are increasingly being replaced with less hazardous alternatives, such as MeOH and IPA.[141] A summary of extraction methods for FFAs from different plant-based matrices is provided in Table 4. Previous studies have extracted bitter molecules from pea protein isolates,[18,86] whole grain oat flour,[19,73] and poppy seeds[104] using aqueous MeOH. Compared with traditional LLE procedures, these aqueous MeOH extraction methods provide milder extraction conditions that help preserve glycerides. Additionally, they are faster and more suitable for adaptation to high-throughput analytical workflows. In contrast, FFAs in plant oils have been extracted using a chloroform/IPA mixture, followed by sample cleanup via SPE cartridges.[23] However, this method involves several time-consuming solvent reconstitution steps, as chloroform-containing extracts cannot be directly introduced into LC–MS instrumentation.

Traditionally, **FFAs in food matrices** have been analysed using GC coupled with either a flame ionization detector or a MS detector.[142,143] However, these methods involve time-consuming steps, including hexane extraction, SPE, and chemical derivatization of the sample extracts prior to GC analysis.[144] Derivatization of FFAs into fatty acid methyl esters can generate undesirable by-products, such as decomposed glycerides, due to high temperatures and limited reaction specificity during lipid esterification.[145] Furthermore, the GC sample inlet and column are heated to high temperatures to facilitate sample evaporation, transfer, and separation, which can induce triglyceride breakdown and formation of additional FFAs, complicating selective quantification.[145] To summarize, GC analysis often requires laborious sample preparation and carries the risk of thermal degradation of analytes.

In contrast, HPLC coupled with MS offers a practical alternative, as it operates at ambient or moderately elevated temperatures, avoiding thermal degradation and derivatization steps.[19,23,73,86,104] The composition of FFAs in pea protein isolates and oat flour has been successfully analysed using LC–MS without derivatization or high temperatures.[19,86] Additionally, the ISTD can be added in minimal amounts during post-extraction steps, improving precision and cost-effectiveness while ensuring accurate quantification of analytes.[146,147] Although properly optimized derivatization procedures in GC–MS can provide higher sensitivity for volatile FFAs, HPLC–MS methods offer a more straightforward, reliable, and accurate approach for routine FFA analysis.

Today, mild extraction conditions are commonly employed for FFA isolation from various food matrices; however, these approaches often involve multiple steps and diverse conditions. In **Publication V**, we aimed to develop and validate a simple and more environmentally friendly extraction method that is directly compatible with LC–MS instrumentation. Such a method is essential for the routine screening, monitoring, and quality control of different plant-based protein production processes, intermediate and commercial products.

Table 4. A selection of extraction methods for free fatty acids in various foods.

Analytes	Matrix	Instrumentation	Brief description of extraction method	Reference
C14:0, C16:0, C18:0, C18:1, C18:2, C18:3	Oat flour	LC–MS/MS	• Weigh 12 g of sample, add ISTD (^{13}C–palmitic acid, $^{2}H_2$-9,10–oleic acid) • Perform 3-step extraction with 300 mL for 2 h under stirring • Combine extracts, concentrate to dryness under vacuum. • Redissolve residue in 5 mL in MeOH. Remove di- and triglycerides by means of SPE C18. • Elute and concentrate to dryness under vacuum, redissolve in starting conditions of the HPLC gradient. • Filter, dilute and perform HPLC–MS/MS analysis.	Günther-Jordanland et al., (2020) [19]
C18:2, C18:3 and other lipid derivate bitter compounds	Pea protein isolates	LC–MS/MS	• Weigh 500 mg of sample, add MeOH/H_2O (1:1, v/v, 5 mL), add ISTD ([$^{13}C_{18}$]-linoleic acid) to a cryogenic tube • Perform extraction using orbital shaker for 1 h at 300 U/min. • Membrane-filter extracts and inject to the UHPLC–DMS–MS/MS system.	Gläser et al., (2020) [18], and Gläser et al., (2021) [86]
Fatty acids from C6:0 to C21:0	Plant oils	LC–ESI($^-$)–MS	• Dilute 10 mg of oils in 1 mL chloroform/iso-propanol (2:1, v/v), add 10 µL of antioxidant (BHT 0.02 mg/mL in MeOH) and 10 µl of ISDT (ISTDs C8:0-d15, C12:0-d23, and C20:5 n3-d5). • Use SPE columns for FFAs quantification (aminopropyl columns) to remove triacylglycerols. Washing solvent - diethyl ether/acetic acid 98/2 (v/v) and chloroform/IPA 2/1 (v/v). • Load samples, wash with chloroform/ iso-propanol 2/1 (v/v). • Elute with diethyl ether/acetic acid 98/2 (v/v). Neutralized with 1 mL 1 M $NaHCO_3$, and collect upper layer. Evaporate to dryness under vacuum. • Reconstitute in EtOH, dilute and perform analysis on LC–MS.	Koch et al., (2021) [23]

C18:3—linolenic acid, C14:0—myristic acid, C16:0—palmitic acid, C18:2—linoleic acid, C18:1—oleic acid, C18:0—stearic acid, C6:0— hexanoic acid, C21:0—heneicosanoic acid

2 Motivation and aims for work

Protein fractions from oats, peas, faba beans, and soybeans are widely used in plant-based dairy and meat alternatives. However, raw-material-specific off-flavours and bitterness continue to hinder consumer acceptance. Consequently, there is growing interest in the reliable identification and quantification of bitter compounds in these matrices. This has driven the need for robust analytical methods applicable across diverse raw materials and commercial products. Such methods should be simple, sensitive, accurate, precise, high-throughput, time-efficient, environmentally sustainable, and suitable for routine laboratory analysis.

The main objective of this dissertation was to develop and apply analytical methods for quantification of bitter-tasing compounds in plant protein ingredients and food applications.

The specific aims of the current work were:

1. To characterize the bitterness perception variability of oat and pea protein ingredients using both quantitative descriptive analysis by trained sensory panellists and consumer tests
2. To develop quantification methods for bitter-tasting oat-, pea-, soyasaponins, and bitter-tasting FFAs which are suitable for screening, monitoring, and quality control applications in various plant-based matrices.
3. To apply the developed methods to screen commercially available PBPAs, including raw materials, protein concentrates, protein isolates, dairy alternatives, and extrusion-cooked protein materials. To estimate the bitterness potential of FFAs, soyasaponin Bb, and avenacosides in various matrices using DoT factor calculations.

3 Experimental

Below is a summary of the developed methodologies used in this work. All experimental procedures and results are detailed in the **Publications I–V**.

3.1 Food samples

Details of food samples and their relevant nutritional and compositional information are provided in the **Publications I–V**. Thereby, the list of food samples used in this thesis is provided below (Table 5).

Table 5. The list of analysed food ingredients, intermediate and commercial products.

Plant origin	Sample code	Sample name
Oat	OPC	oat protein concentrate
	WGOF	Whole-grain oat flour
	OD	oat drink
	VY13-O – VY21-O	oat-based yoghurt alternative
Pea	PPI	pea protein isolate
	PPC	pea protein concentrate
	YP	yellow pea flour
	PD	pea drink
Faba bean	FPI	faba bean protein isolate
	FB	faba bean flour
Soybean	SBD	soybean-based drink
	SYA1; SYA2; VY4-S – VY12-S	soybean-based yoghurt alternatives
Mixed	BLEND	blend (52% pea protein isolate + 28% oat protein concentrate + 20% pea protein concentrate)
	EX-BLEND	ex-blend (52% pea protein isolate + 28% oat protein concentrate + 20% pea protein concentrate)
	Z	pooled sample composed of equal amounts of whole grain oat flour, yellow pea flour, faba bean protein isolate, oat protein concentrate, pea protein isolate, and faba bean protein isolate

3.2 Sensory analysis of bitter stimuli (Publication I)

3.2.1 Participants

Descriptive sensory evaluations were performed with trained assessors (n = 12; mean age 31 ± 6 years) and consumer tests using a consumer-based descriptive approach (n = 100; mean age 32 ± 10 years; 20 males and 80 females). All participants gave written consent.

3.2.2 Samples

These tests aimed to assess sensory perception differences among WGOF, YP aqueous solutions, linoleic acid, linolenic acid, and established bitterness reference compounds (caffeine, L-tryptophan, and quinine).

A preliminary sensory test was carried out to confirm that the selected concentrations could distinguish bitterness sensitivity among assessors and samples. Concentrations were chosen based on previous studies and adjusted for oat and pea flours through preliminary testing.[19] As reported by Günther-Jordanland et al. (2020), linolenic and

linoleic acids differ in bitterness thresholds; therefore, the same DoT factor was applied for comparability.[19] The chosen levels were based on linolenic acid concentrations previously found in oat flour. Final concentrations were confirmed to be perceptible and suitable for the sensory scale. Solutions were freshly prepared on the test day using potable water (Saku Läte OÜ, Estonia); fatty acids were emulsified with 0.1% xanthan gum (Piprapood OÜ, Estonia). PROP test solutions were prepared a day earlier with distilled water and stored at +4 °C until evaluation.

3.2.3 Sensory analysis

A preliminary sensory test with nine trained panellists was conducted to assess the bitterness of all samples. Both the preliminary and main sensory tests were performed using nose clips, according to previous study,[16] to minimize retronasal interference, particularly from the strong odours of oat and pea samples.

All sensory analyses were conducted by 12 trained experts at the Centre for Food and Fermentation Technologies (Tallinn, Estonia) in a sensory room compliant with ISO 8589:2007, following the procedures of ISO 6658:2017. Panellists participated in two separate sensory procedures: a PROP test to characterize bitterness sensitivity, and a sensory evaluation of the samples

The PROP test, based on Tepper et al. (2001), assessed individual bitterness sensitivity using a 0.32 mmol/L PROP and 0.1 mol/L NaCl solution, rated on a General Labeled Magnitude Scale.[148] Panellists were categorized as supertasters (≥51), medium-tasters (15.5–51), or non-tasters (≤15.1).

The sensory analysis of the samples was performed in duplicate (two parallels) across four sessions over two days, with 45-minute breaks between sessions. Each session included five samples, evaluated for bitterness intensity on a 0–9 linear scale (0 = none, 5 = medium, 9 = very strong). Samples were served in 30 mL coded transparent cups at room temperature (21–22 °C) and presented in randomized order following a Williams Latin Square design to minimize carry-over effects. Quinine, consistently perceived as bitter, was included as the first sample in each session for calibration.

Bitterness intensity data were collected using RedJade software (RedJade Sensory Solutions LLC, USA). Since bitterness perception is time-dependent, each evaluation lasted 60s, allowing panellists to record peak intensity. To avoid cumulative effects, assessors were instructed to spit out all samples and take long breaks between tastings. Palate cleansing lasted 60s between samples, using spring water, water biscuits, and pears. Standardized intake procedures were followed, as swallowing can enhance perceived bitterness.[149]

3.2.4 Consumer test

The same oat and pea flours and concentrations used in the trained panel sensory analysis were applied in the consumer test. Sample preparation followed the same procedure described in Chapter 3.2.2. Consumer tests were conducted in the same ISO 8589:2007-compliant sensory room. Samples were served in lidded plastic cups, and participants were instructed to shake them before tasting to minimize precipitation. Nose clips were used during evaluation to reduce the influence of aroma. Bitterness intensity was rated on a 0–9 scale (“none” to “very strong”), identical to the sensory analysis. Each sample was evaluated for 60 seconds, after which participants recorded the highest perceived bitterness intensity. Further methodological details are provided in **Publication I**.

3.3 Measurement of saponins in oat, pea and soybean-based samples (Publications II–IV)

Novel methodologies were developed for the simultaneous quantification of avenacosides in oat products and pea saponins in pea products (**Publication II**), as well as for the quantification of soyasaponins in soybean-based yoghurt alternatives (**Publication III**). Developed methods were applied to analyse oat-based and soybean-based yoghurt alternatives in **Publication IV**.

3.3.1 Sample extraction procedures

Accurately weighed **oat- and pea-based powder samples** (100 mg, n = 3) were extracted in aqueous EtOH (70%, v/v) by applying ultrasonication treatment without additional heating during 30 min. The extracts were centrifuged, and supernatants were passed through PLD+ columns (Biotage Sweden AB, Uppsala, Sweden) to remove precipitated proteins and phospholipids. Obtained filtrates were diluted with acetonitrile (MeCN) and mixed with soyasaponin Ba and ^{13}C–oat extract working solutions before LC–MS analysis.

Liquid samples of **oat, pea drinks, and oat-based yoghurt alternatives** (0.25 g, n = 3) were weighed into a 5 mL volumetric flask and filled with ultrapure water. Thoroughly mixed samples were centrifuged. Sample supernatant was diluted with MeCN (sample:MeCN, 1:5) and centrifuged to remove precipitated proteins. The PLD+ columns were used to remove precipitated proteins and phospholipids. The filtrate was diluted with MeCN to achieve 50% MeCN solution of the sample. The achieved sample solution was combined with ISTD working solutions as described for solid samples and injected into the LC–MS.

In case of soyasaponin analysis in **soybean-based yoghurt alternatives** homogeneous liquid samples (0.35–0.40 g, n = 3) into a 5 mL volumetric flask. Ultrapure water was used to fill up the flask and mixed. The aqueous ammonia solution (5%, v/v) was used to alkalised (pH 8 ± 0.25) sample solution (native pH of yoghurt alternative was ~4.6). Samples were stabilised by incubation at room temperature for 30 minutes on a tube rotator Stuart SB3 (Bibby Scientific Ltd, Staffordshire, UK). After stabilisation, samples were centrifuged and diluted in 1:1 with MeCN in new tube. The mixture was centrifuged to remove precipitated proteins. The supernatant was passed through a PLD+ columns. The filtrate was combined with working solution of ISTD (molecular structure of asperosaponin VI is shown in Figure 4) and injected into the LC–MS.

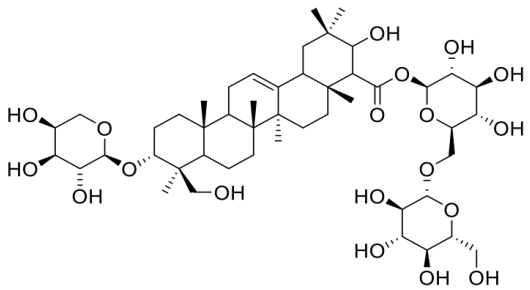

Figure 4. Chemical structure of asperosaponin VI–$C_{47}H_{76}O_{18}$.

3.3.2 Liquid chromatography and high-resolution mass spectrometry experiments

For the confirmation experiments of avenacosides and soyasaponins, an analytical UPLC® system (I-Class Plus, Waters Corporation, Milford, MA, USA) equipped with a BEH Amide column (1.0 × 150 mm, 1.7 µm, Waters Corporation) and a BEH Amide VanGuard pre-column (2.1 × 5 mm, Waters Corporation) was used. A 0.1% of FA (formic acid) in ultrapure water (A) and 0.1% FA in MeCN (B) were used as mobile phases at flow rate set at 200 µL/min during saponin separation. The gradient program was as follows: 0–0.5 min at 10% A; 0.5–10.5 min, linear gradient from 10% to 70% A; 10.5–12 min at 70% A; 12–12.5 min, linear gradient from 70% to 10% A; 12.5–15 min at 10% A. The flow rate was 0.2 mL/min.

For analytical detection, a Vion IMS QTof high-resolution mass spectrometer (HRMS) (Waters Corporation, Milford, MA, USA) was operated in HDMSe mode with negative ionization. The source parameters were as follows: capillary voltage −1.5 kV, cone voltage −80 V, source temperature 120 °C, cone gas flow 50 L/h, desolvation gas temperature 500 °C, and desolvation gas flow 800 L/h. Data were acquired in the HDMSe mode over an m/z range of 800–1300, using a scan time of 0.5 s, a custom quadrupole profile, low-energy collision energy of 6 V, and high-energy collision energy ramping from 30 to 80 eV. HRMS was routinely calibrated using leucine enkephalin as a lockmass, and mass accuracy across the m/z 50–2000 range was ensured with manufacturer-provided calibration solutions.

3.3.3 Liquid chromatography low-resolution mass spectrometry experiments

The samples were analysed using a Waters UPLC® system (Waters Corporation, Milford, MA, USA) coupled with a Waters Quattro Premier XE Mass Spectrometer (low resolution MS) equipped with ZSpray™ Source and controlled by Waters MassLynx™ 4.1 (V4.1 SCN805, Waters Corporation, Milford, MA, USA).

The mobile phase composition and flow rate was the same as described in Section 3.3.2. Saponin separation was performed by BEH Amide column (1.0 × 50 mm, 1.7 µm) coupled with BEH Amide VanGuard Pre-column (2.1 × 5 mm) from Waters Corporation (Milford, MA, USA). Two slightly different gradient programs were used to retain saponins. In case of oat and pea saponin method gradient was as follows: 0–0.17 min at 10% A, 0.17–3.5 min linear gradient 10–70% A, 3.5–4.0 min at 70% A, 4.0–4.5 min linear gradient 70–10% A, 4.5–6.0 min at 10% A. In case of soyasaponins gradient changed as follows: 0–0.17 min at 10% A; 0.17–1.5 min linear gradient 10–70% A; 1.5–4.17 min at 70% A; 4.18 min switch to 10% A; 4.18–6.0 min at 10% A. The chromatographic runs were 6 minutes long with 200 µL/min flow rate and column temperature was held at 50 °C during all experiments. Negative electrospray ionisation with optimised source conditions was applied to ionise all analytes. Mass spectrometer was used in single-ion-recording acquisition mode. Deprotonated molecules $[M–H]^-$ were chosen based on a scan-type experiments (Table 6).

Table 6. The mass-to-charge ratio (m/z), capillary and cone voltages of saponins and internal standard (ISTD) compounds.

Compound name	Matrix	m/z	Capillary voltage (kV)	Cone voltage (V)
avenacoside A	oat	1061.5	-1.5	80
$^{13}C_{51}$–avenacoside A	ISTD (oat matrix)	1112.5		
26-desglucoavenacoside A	oat	899.5		
avenacoside B	oat	1223		
DDMP[1] saponin	pea	1067		
soyasaponin Bb	pea	941.5		
	soybean		-2.5	100
soyasaponin Ba	ISTD (pea matrix)	957.3	-2.5	120
	soybean			
soyasaponin Aa	soybean	1364.3		
soyasaponin Ab	soybean	1435.6		
asperosaponin VI	ISTD (soybean matrix)	927.5		

[1]2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one

3.4 Measurement of FFAs in oat-, pea- and faba-bean-based protein sources (Publication V)

3.4.1 Sample extraction procedure

Powder sample (50 mg, n = 3) was weighed into 2 mL tubes and 1 mL of extraction solution was added (IPA:MeOH, 1:1 v/v) and incubated in an Eppendorf Thermomixer® C (Eppendorf AG, Hamburg, Germany) stirring motion set to 1400 rpm at 15 °C for 30 minutes. Samples were centrifuged and filtered using PTFE filter. The filtrates were diluted to fit into linear range of the calibration curve, receiving a solution containing H_2O:MeOH:IPA (1:1:1, v/v), which is the starting composition of the mobile phases in the chromatographic gradient. Diluted sample was combined with U–^{13}C–oat extract working solution of ISTD before injection into the LC–MS.

3.4.2 Liquid chromatography and mass spectrometry

FFAs were analysed using a Waters UPLC® system coupled with a Waters Quattro Premier XE Mass Spectrometer equipped with a ZSpray™ Source, controlled by Waters MassLynx™ 4.1 software.

The flow rate was set at 0.25 mL/min, the column heater was maintained at 55 °C, and mobile phases consisted of (A) 60% H_2O, 40% MeOH, and 10 mM ammonium acetate, and 0.005% acetic acid; and (B) 90% IPA, 10% MeOH, 10 mM ammonium acetate, and 0.005% acetic acid. Analytes were separated using an ACQUITY UPLC CSH C18 Premier column (2.1 × 100 mm, 1.7 µm) coupled with an ACQUITY UPLC CSH C18 VanGuard Pre-column (2.1 × 5 mm) from Waters Corporation. And the gradient was as follows: 0–0.5 min at 60% A, 0.5–8.0 min linear gradient from 60% to 20% A, 8.0–8.6 min at 2% A, 8.6–11.4 min linear gradient from 20% to 1% A, 11.4–12.0 min at 1% A, 12.0–12.1 min change to 60% A, and 12.1–17.00 min equilibrated at 60% A.

FFAs and their isotopically labelled counterparts were ionized using negative electrospray ionization mode under optimised source conditions. The deprotonated ^{12}C– and ^{13}C–FFAs $[M–H]^-$ were selected for the quantification of fatty acids, with mass-to-charge ratios (m/z ± 0.5 Da) for selected ion recording (SIR) channels provided in Table 7.

Table 7. The mass-to-charge ratio (m/z) of free fatty acids and internal standard compounds.

Analyte	Abbreviation	m/z
myristic acid	C14:0	227.2
palmitic acid	C16:0	255.2
stearic acid	C18:0	283.2
oleic acid	C18:1	281.2
linoleic acid	C18:2	279.2
linolenic acid	C18:3	277.2
^{13}C–myristic acid	^{13}C14:0	241.2
^{13}C–palmitic acid	^{13}C16:0	271.2
^{13}C–stearic acid	^{13}C18:0	301.2
^{13}C–oleic acid	^{13}C18:1	299.2
^{13}C–linoleic acid	^{13}C18:2	297.2
^{13}C–linolenic acid	^{13}C18:3	295.2

3.5 Quantitative analysis (Publications II–V)

The calibration curves of external standards were built by correlating the concentrations of external standards to the response factors (external standard peak area/internal standard peak area) by applying Equation 1.

$$response\ factor = \frac{external\ standard\ peak\ area}{internal\ standard\ peak\ area} \quad (Equation\ 1)$$

U–^{13}C–oat extract solution was prepared as described in relevant sample extraction procedure (section 3.4.1). In case of avenacosides the ISTD was fully isotopically labelled $^{13}C_{51}$-avenacoside A (details of ISTD solution preparation in **Publication II**). In addition, fully isotopically labelled FFAs, from ^{13}C–oat extract, were used as ISTD for FFAs quantification (details of ISTD solution preparation in **Publication V**). In case of quantification of soyasaponin Bb and DDMP saponin in pea samples, soyasaponin Ba was used as ISTD compound. Asperosaponin VI was used as ISTD in case of soyasaponin quantification in soybean-based yoghurt alternatives.

All compounds were quantified absolutely, except avenacoside B, 26-desglucoavenacoside A and DDMP saponin. Avenacoside B and 26-desglucoavenacoside A) were quantified relatively using the avenacoside A calibration curve. Avenacoside B and 26-desglucoavenacoside A results are presented in avenacoside A equivalents. The quantification of DDMP saponin was based on soyasaponin Bb curve, and the results are given in soyasaponin Bb equivalents.

3.6 Calculation of bitter-taste contribution

The impact of avenacosides A, avenacoside B, soyasaponin Bb and FFAs to the bitterness of the study samples was calculated using Equation 2.[19,150] The ratio of the concentration to the taste threshold (TC) determines the DoT. The TC values (avenacoside A—7 µmol/L, avenacoside B—6 µmol/L, soyasaponin Bb—1620 µmol/L, C14:0—1703 µmol/L, C16:0—1546 µmol/L, C18:0—726 µmol/L, C18:1—2180 µmol/L, C18:2—1810 µmol/L, C18:3—277 µmol/L) used as published in previous studies.[19,86]

$$dose-over-threshold\ (DoT) = \frac{concentration\ of\ FFA\ \left(\frac{\mu mol}{kg}\ "as\ is"\right)}{taste\ threshold\ \left(TC; \frac{\mu mol}{L}\right)} \quad (Equation\ 2)$$

3.7 Validation of the methods (Publications II–V)

The Eurochem and the European Medicine Agency validation guidelines were used to evaluate the following parameters during method validation: specificity, selectivity, the linear range, instrumental detection limit (IDL) , instrumental quantification limit (IQL), limit of detection (LOD), limit of quantification (LOQ), precision and recoveries of the whole quantification methods, and the matrix effects based on calibration standards and samples.[151,152]

Despite applying the developed methods to samples in various physical states and across a broad spectrum of analytes, the IDLs and IQLs were determined using standard solutions, thereby reflecting the inherent instrumental limitations. LODs and LOQs were calculated using Equation 3, where the minimum acceptable dilution factor was applied.

$$LOD\ or\ LOQ\ \left(\frac{mg}{100g}\ sample\right) = \left(\frac{IDL\ or\ IQL\ (\frac{mg}{L}) * dilution * volume\ (L)}{sample\ weight\ (g)}\right) * 100 * \left(\frac{mean\ Recovery\%}{100}\right) (Equation\ 3)$$

Total analyte recoveries (Equation 4) were evaluated by spiking with known amounts of analytes at four concentration levels (unspiked, lower IQL, middle IQL, and upper IQL) followed by extraction using the developed procedures.

$$total\ recovery\ (\%) = \left(\frac{C_{spiked}}{C_{unspiked} - C_{spike}}\right) \times 100 \qquad (Equation\ 4)$$

Matrix effects (Equation 5) were determined by post-extraction spiking of sample extracts with calibration standards and comparing the resulting matrix-matched calibration curve slopes to those obtained from solvent-based curves. The detailed information of exact validation procedures is provided in **Publications II, III and V.**

$$matrix\ effect\ (\%) = \left(\frac{slope_{matrix-matched}}{slope_{solvent-matched}}\right) \times 100\ (Equation\ 5)$$

3.8 Data acquisition, processing and analysis (Publications II–V)

Data acquisition was performed in Waters MassLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA). Data analysis was performed in Waters QuanLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA) and Microsoft Excel® 365 Apps for business (Microsoft Corporation, Redmond, WA, USA). HRMS data was processed, visualised and analysed using UNIFI (Waters Corporation, Milford, MA, USA).

3.9 Visualisation and statistics

For **Publication I**, sensory analysis, consumer test data processing, and visualization were conducted using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA).

For **Publications II–V**, statistical analyses of saponin and FFA data were performed in Microsoft Excel®. Data analysis related to the development of sample extraction methods (**Publications III and V**) was carried out using R software (version 4.2.2; The R Foundation for Statistical Computing, Vienna, Austria).

In **Publication IV**, data analysis and visualization were performed using R software (version 4.3.0; The R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was assessed using the Kruskal–Wallis test followed by the Conover–Iman multiple comparison test, implemented via the R package Conover.test (version 1.1.5). The significance threshold was set at $p < 0.05$.

For **Publication V**, the Chemometric Agile Tool (CAT, free software) was employed for experimental design, data analysis, and interpretation.[153] Linear models without interactions were fitted to the Plackett–Burman design, models with two-way interactions were applied to the full factorial design, and models including two- and three-way interactions were used for the mixture design. Data visualization was performed using R software (version 4.3.0) with the ggplot2 package (version 3.5.1). The statistical significance of coefficients was determined by t-tests, while ANOVA followed by the Tukey–Kramer post hoc test was conducted using the agricolae package (version 1.3–5). The level of significance was set at $p < 0.05$.

4 Results and discussion

The results presented in this dissertation are based on five peer-reviewed publications (**Publications I–V**). Overall, these studies describe the development and application of analytical methodologies for the quantification of bitter-tasting compounds in various plant-based protein ingredients, intermediate products, and final food applications. The first sub-chapter, corresponding to **Publication I**, focuses on the sensory evaluation of bitterness perception in plant-based products, providing the sensory context and rationale for the subsequent analytical investigations. The second sub-chapter, encompassing **Publications II, III, and V**, presents the development and optimization of sample preparation procedures and analytical methods for the quantification of bitter compounds, including saponins and FFAs, in different plant protein matrices. The final sub-chapter, based on **Publications II–V**, demonstrates the application of these methods to quantify bitter-tasting compounds in diverse plant-based ingredients and food products, and to assess the contribution of avenacosides, soyasaponin Bb, and FFAs to the overall bitterness of these products.

4.1 Perception of bitter molecules in plant-based products (Publication I)

The primary objective of **Publication I** was to investigate individual differences in the perception of bitterness in plant-based protein ingredients. A trained sensory panel evaluated the bitterness of selected reference compounds and water-based extracts of oat and pea flours. The results, presented in Figure 5, illustrate the variation in perceived bitterness among the tested samples. Among all stimuli, L-tryptophan elicited the most consistent bitterness ratings, indicating a uniform perception across panellists. In contrast, greater variability was observed in the bitterness scores for fatty acids and for the oat and pea flour extracts. This variation suggests that bitterness perception in oat and pea matrices is more strongly influenced by individual differences among assessors than by the characteristics of conventional bitter reference compounds.

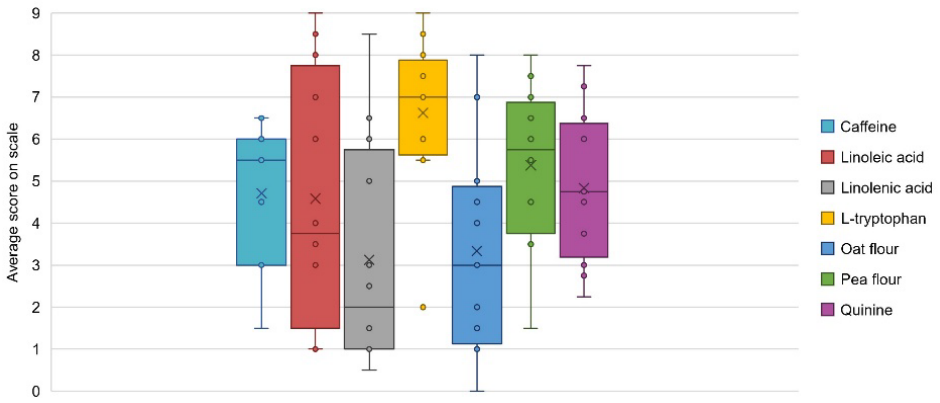

*Figure 5. Box & Whisker plot for bitterness by each stimulus in trained accessors sensory tests. Circles and whisker endpoints indicate individual scores of assessors. The average value is marked with "X", the median is marked with a horizontal line, and whiskers indicate lower and upper quartiles (**Publication I**).*

To complement the findings obtained from the trained assessor panel, consumer sensory tests were conducted. Figure 6 presents the distribution of bitterness intensity ratings for pea and oat flours based on these consumer evaluations, highlighting clear differences in perception between the two matrices. For pea flour, the bitterness ratings were evenly distributed across the scale, indicating a wide variation in perceived bitterness among consumers. In contrast, the oat flour ratings were concentrated toward the lower end, with most participants reporting little to no bitterness and only a few providing high-intensity scores. On average, oat flour was perceived as significantly less bitter (mean = 2.1 ± 2.0) than pea flour (mean = 4.4 ± 2.6). A comparable trend was observed among the trained assessors, who also rated oat flour as less bitter (mean = 3.3 ± 2.6) than pea flour (mean = 5.4 ± 2.0). These consistent findings indicate that oat flour generally elicits a milder and more variable bitterness perception compared to pea flour.

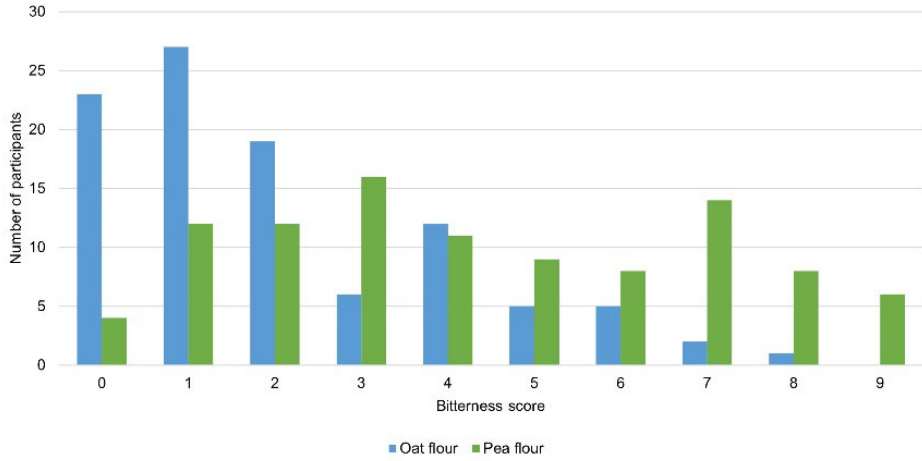

*Figure 6. Distribution of bitterness intensity ratings for pea and oat flour in consumer test (**Publication I**).*

The taste threshold values of fatty acids may be influenced by individual differences in the lipolytic activity of saliva.[154] A correlation between the bitterness of oat and pea flours was expected, as previous studies have identified linolenic acid and linoleic acid as key bitter compounds in both oat flour[19,73] and pea protein isolates.[18,86] In the present study, a correlation was observed between oat flour and linolenic acid, but not between oat flour and pea flour.

Although the fat content of oat flour was higher (average 6.5%) than that of pea flour (average 1.8%), the bitterness of pea flour was generally perceived as more intense. This suggests that compounds other than fatty acids, such as saponins or other secondary metabolites, may contribute more strongly to the bitterness of pea flour. However, this hypothesis could not be directly confirmed, as suitable food-grade reference compounds were not available for sensory testing.

While sensory analysis provided valuable insights into bitterness perception in oat and pea matrices, it also revealed clear limitations, as the sensory data alone could not explain the underlying chemical causes of bitterness or the substantial variability observed among individuals. To determine the concentrations of key bitter compounds

in plant-based products, reliable analytical quantification techniques are essential. The development of novel methods that complement sensory evaluation by providing quantitative data would enable more precise, standardised, and controlled sensory experiments. The absence of suitable food-grade reference compounds and the complex composition of plant-based matrices made it impossible to directly identify which molecules contributed most to the perceived bitterness, emphasizing the need for complementary analytical approaches capable of quantitatively determining key bitter compounds and linking them with sensory perception. In response to these challenges, the subsequent studies (**Publications II–V**) focused on the development and optimisation of chromatographic and mass spectrometric methods for the quantification of potential bitter compounds, such as saponins and FFAs, across various plant protein ingredients and food applications.

4.2 Analysis of bitter-tasting compounds in plant-based products

The sensory analysis conducted in **Publication I** highlighted the need to develop analytical methodologies for the quantification of bitter-related compounds. The following efforts involved the method development, optimisation and quantification of saponins in oat and pea matrices (**Publication II**) and in soybean-based yoghurt alternatives (**Publication III**), as well as the FFAs (C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3) in various plant-based protein matrices (**Publication V**).

4.2.1 Identification of bitter-tasting compounds

Avenacoside A, avenacoside B and 26-desglucoavenacoside A, soyasaponin Bb, DDMP saponin, soyasaponin Ba, soyasaponin Ab, soyasaponin Aa, and six fatty acids (C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3) were selected as primary bitter-tasting compounds in oat, pea, soya, and faba bean matrix based on previous studies.[14,19–21,27,86,87,104]

4.2.1.1 Identification of saponins in oat and pea matrices (Publication II)

Due to the limited availability of external standard compounds, the specific m/z values of avenacoside B, 26-desglucoavenacoside A, and DDMP saponin were determined by analysing OPC and PPI extracts together with the available standard compounds (avenacoside A and soyasaponin Bb) using HRMS. Figure 7 presents the HRMS chromatograms and spectra of the commercially available standard compounds. During ESI^- ionisation, soyasaponin Bb produced a deprotonated ion species $[M-H]^-$, whereas avenacoside A formed a formate adduct ion $[M+FA-H]^-$. Figure 8 shows the HRMS chromatograms and spectra of the OPC extract. The results confirmed that avenacoside A formed the same formate adducts during sample ionisation. In addition, the presence of avenacoside B and 26-desglucoavenacoside A was verified, both detected as deprotonated ion species $[M-H]^-$. Similarly, Figure 9 presents the HRMS chromatograms and spectra of the PPI extract, confirming the presence of soyasaponin Bb. However, as shown in Figure 9a,d, DDMP saponin was absent in the PPI extract. Instead, several unknown compounds with unsuitable m/z values were detected. To evaluate the precision of saponin identification for compounds without pure standards, the identification error was calculated based on the difference between the detected and theoretical m/z ratios. A summary of the HPLC–HRMS experiments is provided in Table 8, demonstrating high mass accuracy across all identified saponins.

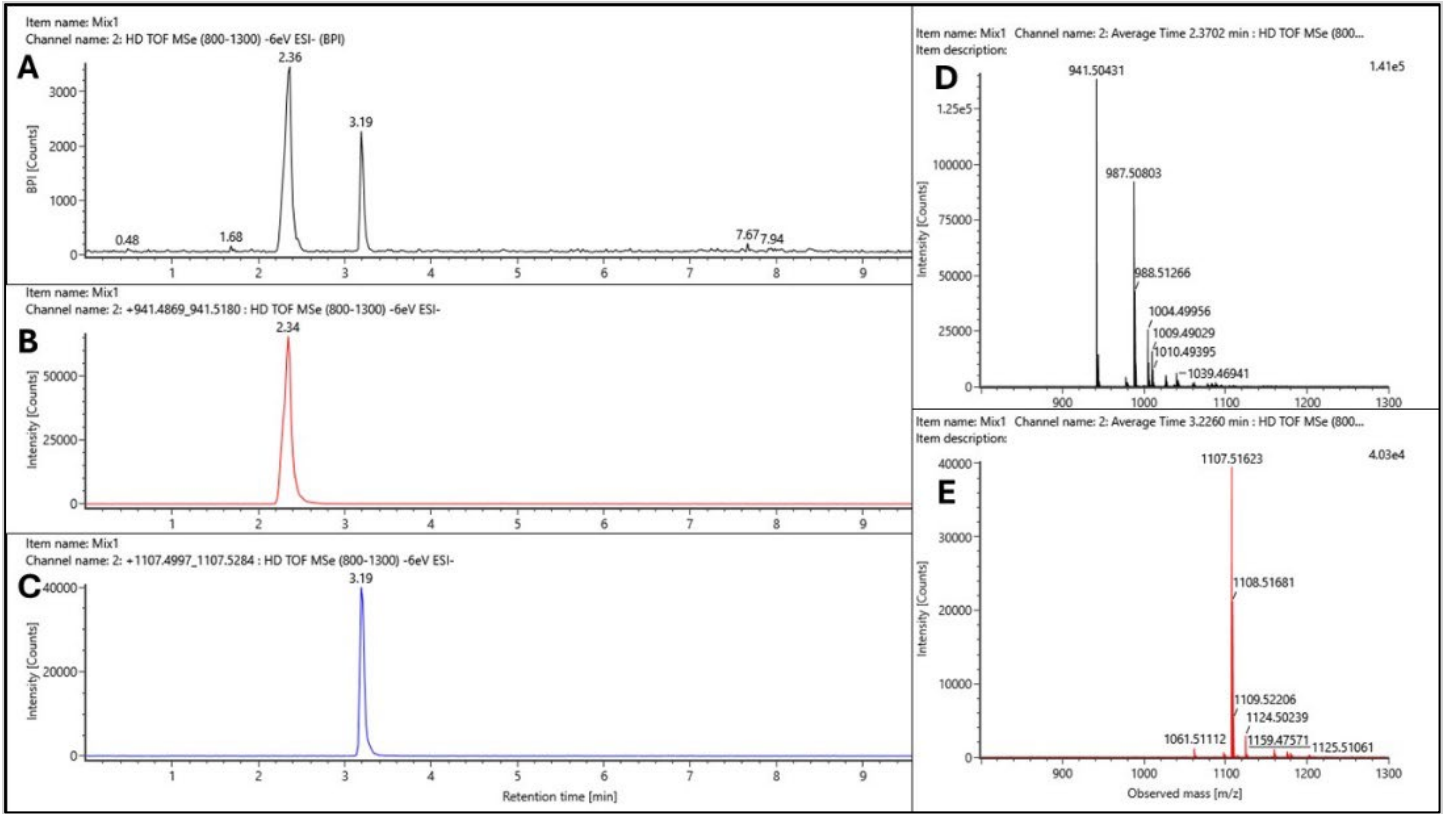

*Figure 7. The results of HPLC–HRMS experiments with commercially available standard compounds. **A**—avenacoside A and soyasaponin Bb standard solution BPI chromatogram (retention time vs intensity); **B**—extracted ion chromatogram (EIC) of soyasaponin Bb (retention time vs intensity); **C**—EIC of avenacoside A (retention time vs intensity); **D**—mass spectrum of soyasaponin Bb peak (RT 2.36, chart A) (mass-to-charge-ratio vs intensity); **E**—mass spectrum of avenacoside A peak (RT 3.19, chart A) (mass-to-charge-ratio vs intensity).*

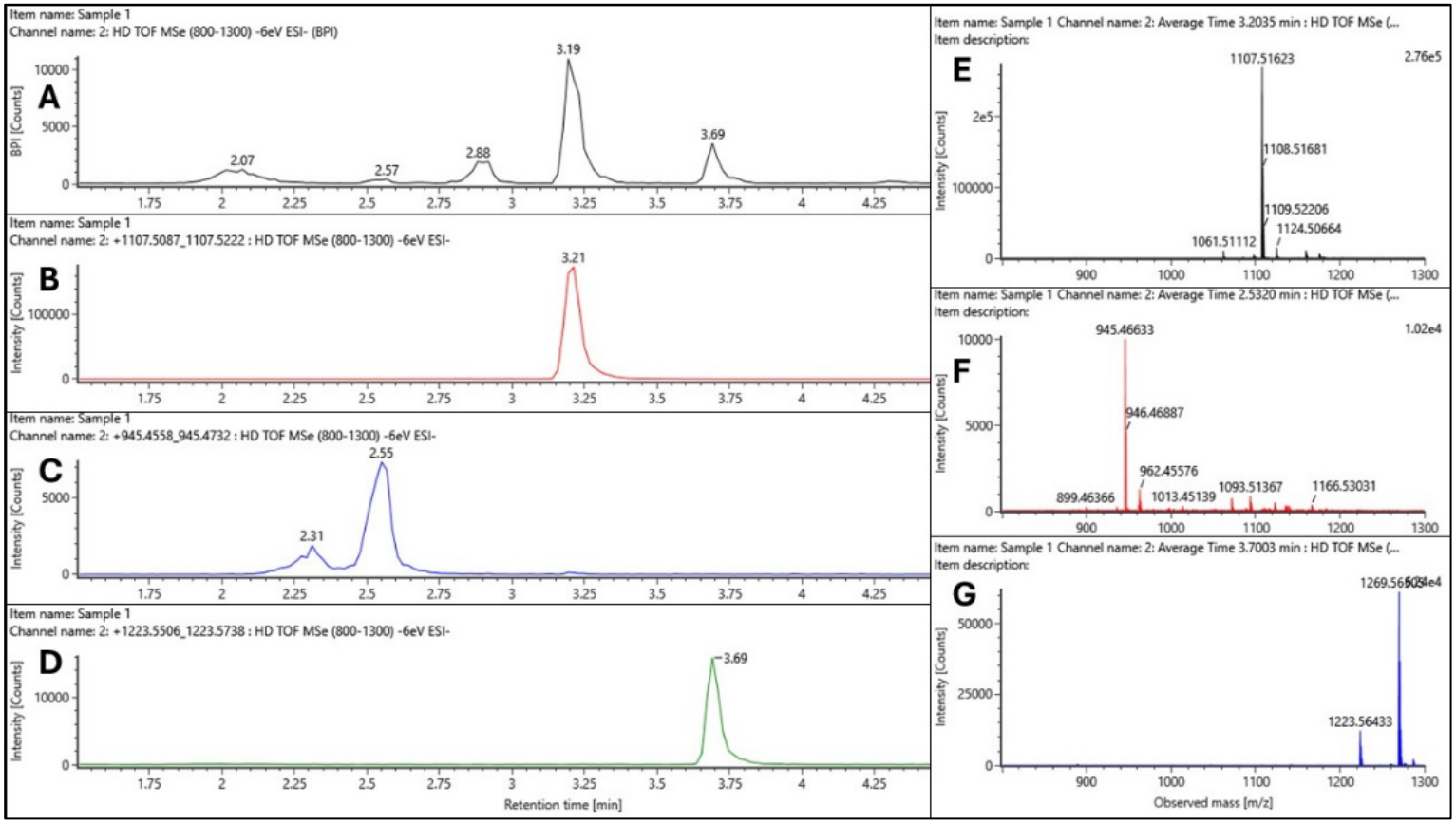

Figure 8. The results of HPLC–HRMS experiments with oat protein concentrate. ***A****—OPC BPI (base peak intensity) chromatogram (retention time vs intensity);* ***B****—extracted ion chromatogram (EIC) of avenacoside A (retention time vs intensity);* ***C****—EIC of 26-desglucoavenacoside A (retention time vs intensity);* ***D****—EIC of avenacoside B (retention time vs intensity);* ***E****—mass spectrum of avenacoside A peak (RT 3.19, chart A) (mass-to-charge ratio vs intensity);* ***F****—mass spectrum of 26-desglucoavenacoside A peak (RT 2.55, chart A) (mass-to-charge ratio vs intensity);* ***G****—mass spectrum of avenacoside B peak (RT 3.69, chart A) (mass-to-charge ratio vs intensity).*

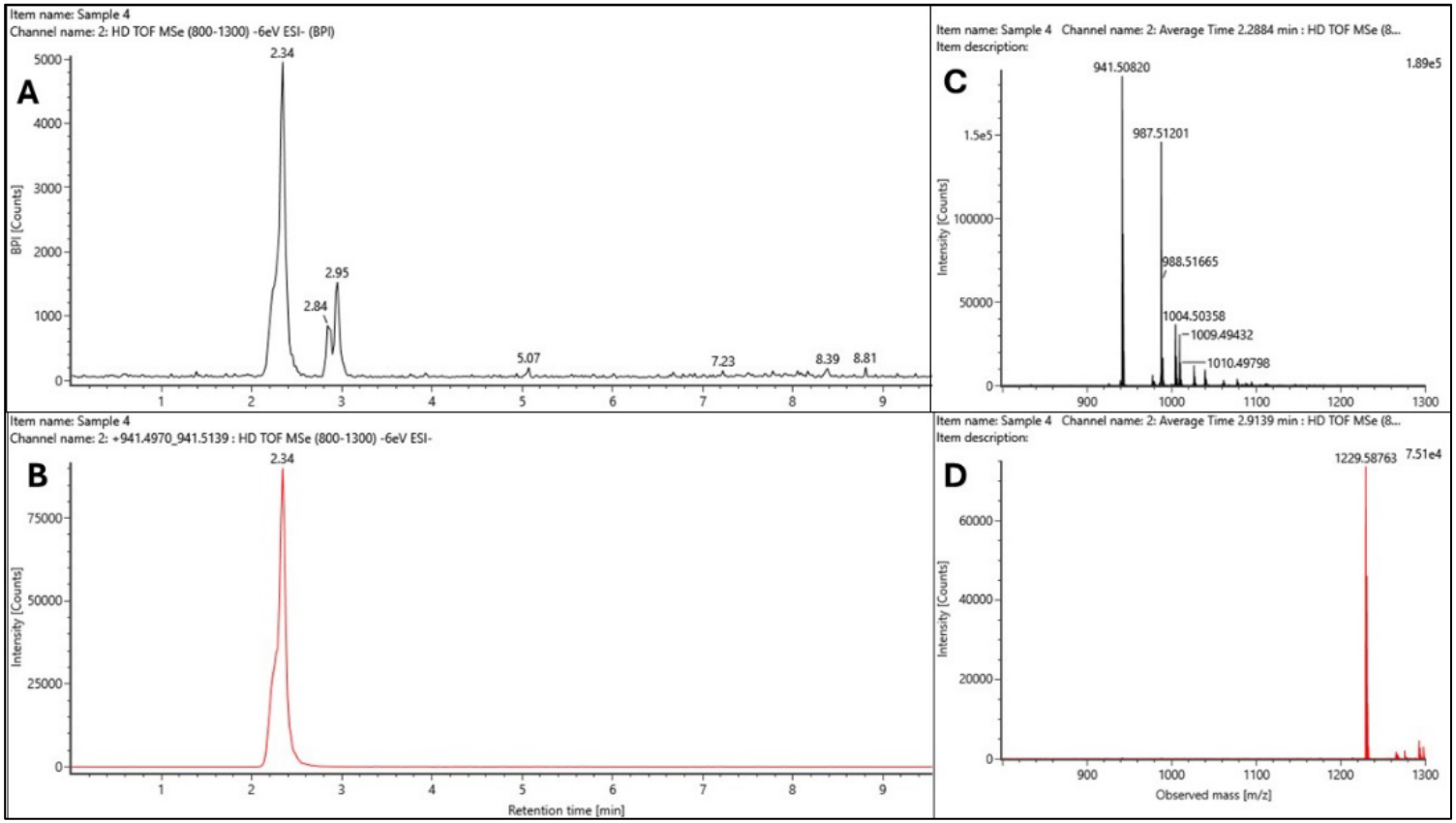

Figure 9. The results of HPLC–HRMS experiments with PPI (pea protein isolate). ***A****—PPI BPI (base peak intensity) chromatogram (retention time vs intensity);* ***B****—extracted ion chromatogram of soyasaponin Bb (retention time vs intensity);* ***C****—mass spectrum of soyasaponin Bb peak (RT 2.34, chart A) (mass-to-charge ratio vs intensity);* ***D****—mass spectrum of unknown peak (m/z 1229.58763, RT 2.84-2.95, chart A) (mass-to-charge ratio vs intensity).*

Table 8. The results of HPLC–HRMS experiments on saponins in oat and pea matrices.

Analyte	Molecular formula	Monoisotopic mass, Da	Calculated m/z	Observed m/z	Adduct	Error, ppm
avenacoside A	$C_{51}H_{82}O_{23}$	1062.5247	1107.5229	1107.51623	$[M+FA-H]^-$	-6.02
avenacoside B	$C_{57}H_{92}O_{28}$	1224.5775	1223.5697	1223.56433	$[M-H]^-$	-4.39
26-desgluco-avenacoside A	$C_{51}H_{82}O_{23}$	900.4719	945.4701	945.46633	$[M+FA-H]^-$	-3.99
soyasaponin Bb	$C_{48}H_{78}O_{18}$	942.5188	941.5110	941.50431	$[M-H]^-$	-7.11

Once the presence of avenacoside B and 26-desglucoavenacoside A was confirmed by HRMS, their specific m/z values were incorporated into the SIR acquisition program for LRMS. Figure 10 displays the SIR chromatograms obtained using LRMS for WGOF and YP extracts spiked with a working solution of the ISTD. The presence of DDMP saponin in the YP extract was confirmed by low-resolution MS, consistent with its high concentration in this sample.

Furthermore, $^{13}C_{51}$–avenacoside A was readily detected in the ^{13}C–oat flour extract using low-resolution MS, owing to its high concentration, complete isotopic labelling, and identical retention time to the external standard. In contrast, the concentration of $^{13}C_{57}$–avenacoside B in the ^{13}C–oat flour extract was too low, or the compound exhibited incomplete isotopic labelling, preventing its use as an ISTD for avenacoside B quantification. As the exact concentrations of the ISTD compounds in the ^{13}C–oat flour extract used as the ISTD working solution were unknown, a simplified calibration approach was employed (Equation 1) to quantify saponins.

Overall, the optimised LC–MS method enabled effective separation of oat and pea saponins, along with their respective ISTD, within six minutes, ensuring robust and reliable identification and quantification across all analysed samples.

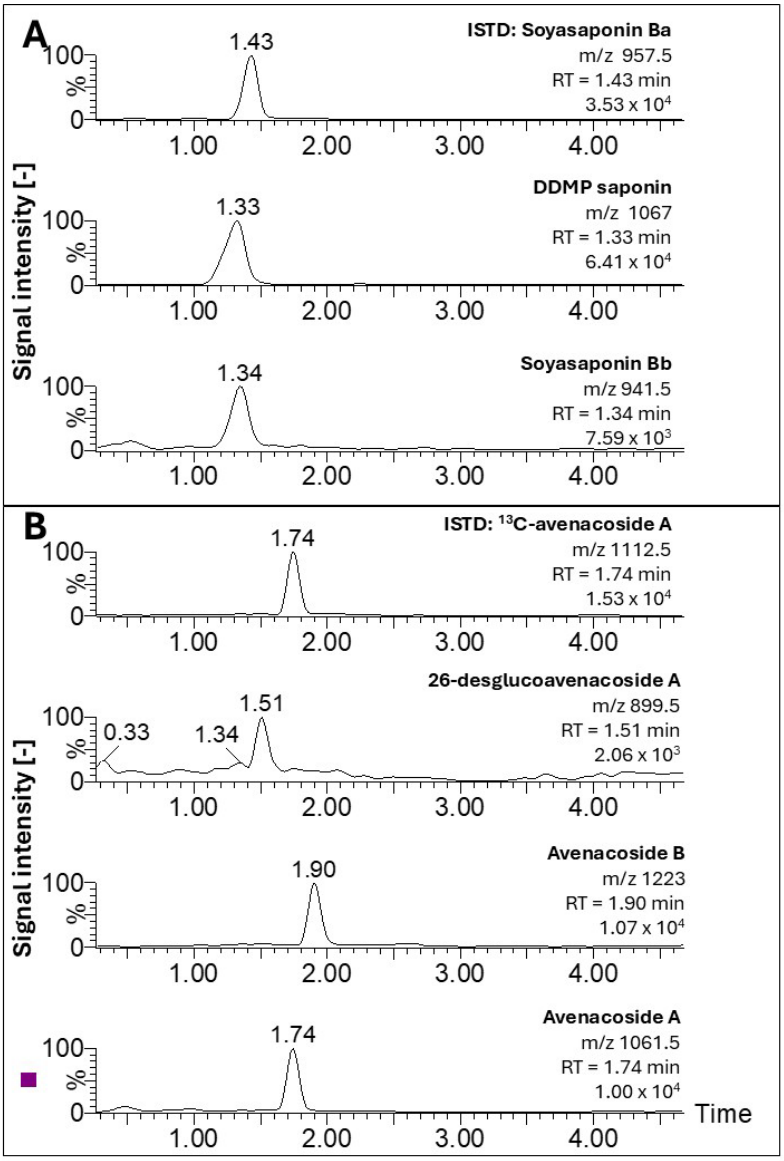

Figure 10. LC–MS chromatograms (SIR) of saponins in pea flour (A)—soyasaponin Bb; 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) saponin, and ISTD soyasaponin Ba; and whole grain oat flour (B)—avenacoside A, avenacoside B, 26-desglucoavenacoside A, and ISTD ^{13}C–avenacoside A. Specific details of chromatographic conditions are described in ***Publication II****.*

4.2.1.2 Identification of saponins in soybean matrix (Publication III)

The soyasaponins of interest were commercially available from Sigma-Aldrich; however, isotopically labelled soybeans, which could serve as a potential source of ISTD compounds, were not available. Asperosaponin VI was identified as being chemically similar to the target soyasaponins (Figure 4) and was therefore selected as the ISTD compound for their quantification. Figure 11 presents the chromatograms of the standard compounds in both the standard solution and the soybean-based yoghurt alternative sample extract. It can be observed that soyasaponin Aa was below the detection limit in

this sample. Asperosaponin VI and the soyasaponins exhibited identical retention times and therefore asperosaponin VI could be successfully employed as an ISTD, enabling a more precise quantification of soyasaponins (Equation 1).

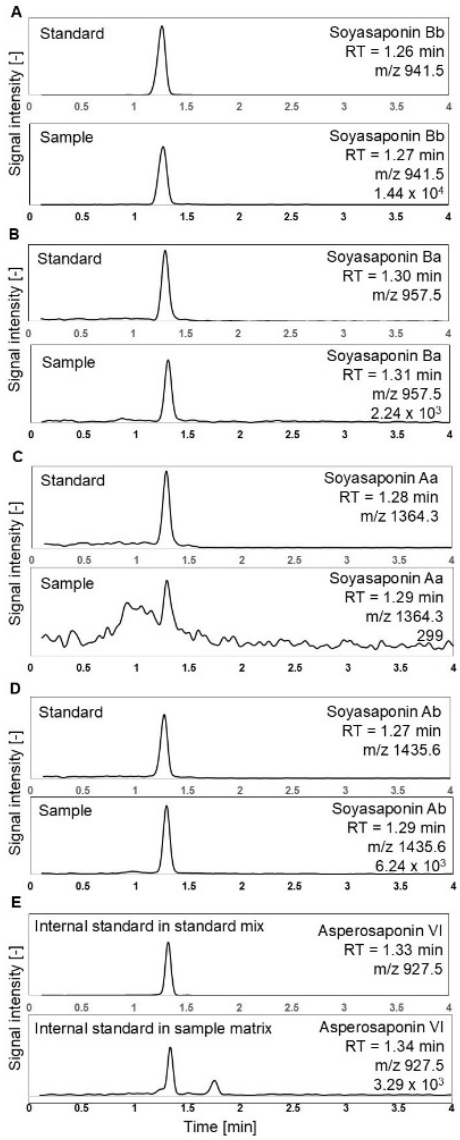

*Figure 11. LC–MS chromatograms (SIR) of soyasaponins in standard, soybean-based yoghurt alternative, and internal standard: (**A**) soyasaponin Bb, (**B**) soyasaponin Ba, (**C**) soyasaponin Aa, (**D**) soyasaponin Ab, and (**E**) asperosaponin VI—ISTD. Specific details of chromatographic conditions are described in **Publication III**.*

4.2.1.3 Identification of FFAs in various plant-based matrices (Publication V)

For the analysis of FFAs, all compounds were available as external standards. The ^{13}C–oat flour extract was used as the source of fully isotopically labelled FFAs. All labelled FFAs were identified based on their specific m/z values and verified by comparison with the

retention times of the corresponding external standard compounds (Figure 12). The extract contained fully labelled ^{13}C–fatty acids without cross-contamination from their naturally occurring, unlabelled counterparts. All ^{13}C–FFAs exhibited sufficient concentrations in the ^{13}C–oat flour extract, except for ^{13}C–myristic acid, which was present at too low a level to serve as an ISTD for myristic acid. To compensate, ^{13}C–linolenic acid was selected as an alternative ISTD due to its similar retention time. Since the exact concentrations of ISTD compounds in the ^{13}C–oat flour extract (used as the ISTD working solution) were unknown, a simplified calibration equation was applied (Equation 1). The suitability of using ^{13}C–linolenic acid as the ISTD for myristic acid was tested and validated. The results demonstrated satisfactory performance in terms of linearity, matrix effects (post-extraction spiking), and recovery (pre-extraction spiking) experiments (see Section 4.2.3.3, Table 13 for detailed results). Consequently, ^{13}C–linolenic acid was successfully employed for the calibration of myristic acid, alongside the external standard, to enable more accurate mass spectrometric quantification.

The optimised LC–MS method achieved effective separation of the fatty acids and their isotopically labelled counterparts within 15 minutes, ensuring robust and precise analytical performance.

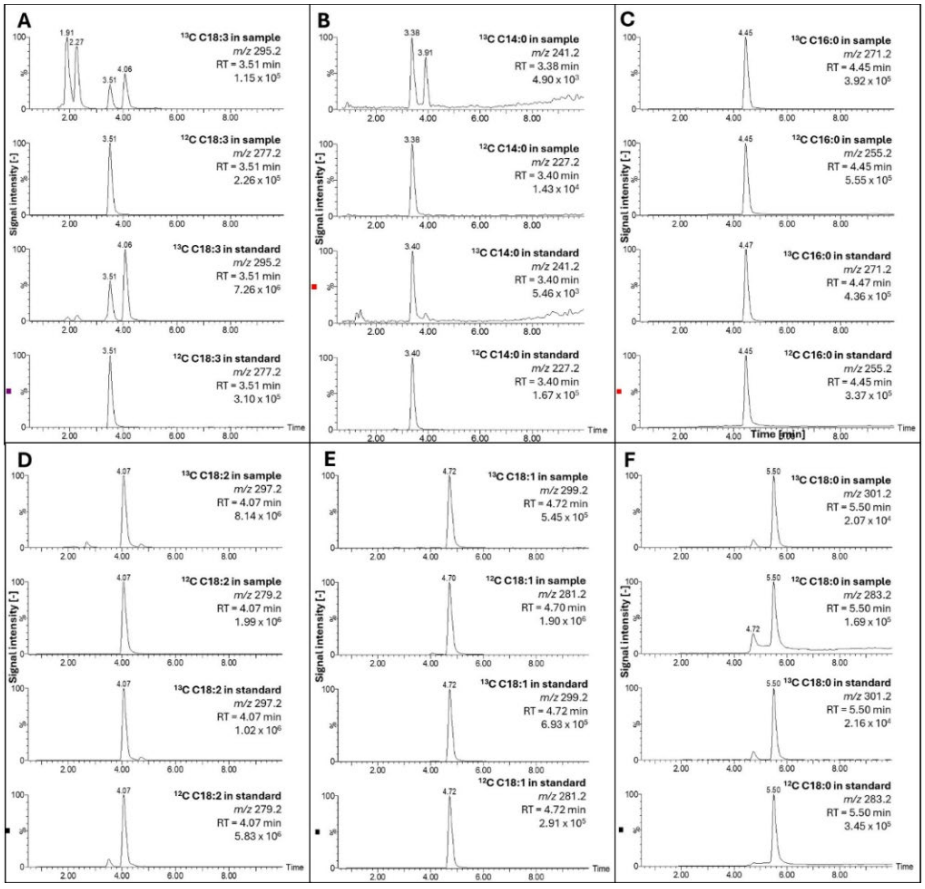

*Figure 12. LC–MS chromatograms of ^{13}C– and ^{12}C–fatty acids in pooled sample matrix and standard solutions (SIR): (**A**) linolenic acid—18:3; (**B**) myristic acid—C14:0; (**C**) palmitic acid—C16:0; (**D**) linoleic acid—C18:2; (**E**) oleic acid—C18:1; and (**F**) stearic acid—C18:0. Chromatographic conditions are described in **Publication V.***

4.2.2 Development of sample extraction procedures

Sample extraction procedures for oat, pea, and soybean saponins, as well as FFAs, were developed for both solid and liquid food products. During the development of these procedures, particular attention was given to simplifying the extraction process, improving environmental sustainability, ensuring compatibility with routine laboratory workflows, reducing cost, and minimizing processing time.

4.2.2.1 Simultaneous extraction of oat and pea saponins (Publication II)

The development of an extraction method for the analysis of oat and pea saponins from powder samples began with the application of two previously established approaches.[20,27] Table 9 presents the main steps of these extraction methods, along with the saponin extraction yields obtained using both the reference methods (1A and 2A) and the modified methods (1B, 2B, 2C, and 2D).

As both reference extraction procedures required defatted samples, defatted OPC and PPI were prepared using hexane Soxhlet extraction. Saponin extraction was then carried out on the defatted OPC (fat content 18.9%) and PPI (fat content 4.7%) using methods 1A, 1B, 2A, and 2B. For comparability, the results are expressed relative to the original fat-containing samples. In OPC, extraction using method 1B yielded 37% more avenacoside A compared to method 1A, while method 2B produced a 205% higher yield than method 2A. In the case of PPI, method 1B resulted in a twofold increase in soyasaponin Bb yield compared to method 1A, and method 2B achieved a 77% higher yield than method 2A. Therefore, the highest yields of avenacoside A and soyasaponin Bb were obtained using method 2B for OPC and PPI, respectively. Although methods 1B and 2B produced comparable saponin levels, method 2B was preferred due to its greater efficiency, as method 1B involved a time-consuming two-step MeOH reflux extraction. The second part of Table 9 outlines further sample extraction steps (methods 2B, 2C, and 2D) that were tested to optimise the saponin extraction procedure.

Table 9. The comparison of extraction steps of reference methods 1A[1] and 2A[2], and their modified versions (1B, 2B, 2C and 2D), and saponin yields. Detailed description of extraction methods 1A, 1B, 2A, 2B, 2C, and 2D are available in Supplementary Information in ***Publication II.*** *The quantitative data of methods 2B* and 2C, 2C** and 2D are presented in Figure 13 and Figure 14, respectively.*

Procedure steps	Sample extraction 1A	Modified sample extraction 1B	Sample extraction 2A	Modified sample extraction 2B	Modified sample extraction 2B*	Modified sample extraction 2C	Modified sample extraction 2C**	Modified sample extraction 2D
Hexane defatted sample (Soxhlet)	yes	yes	yes	yes	yes	no	no	no
Sample and solvent amount (g/mL)	0.5 g, 25 mL x 2 MeOH	0.5 g, 25 mL x 2 MeOH	0.5 g, 50 mL EtOH (70%, v/v)	0.1 g, 10 mL EtOH (70%, v/v)	0.1 g, 10 mL EtOH (70%, v/v)	0.1 g, 10 mL EtOH (70%, v/v)	0.1 g, 10 mL EtOH (70%, v/v)	0.1 g, 10 mL EtOH (70%, v/v)
Extraction	2-step reflux at boiling point	2-step reflux at boiling point	1 h at 25 °C in a shaking incubator	1 h at 25 °C in a shaking incubator	1 h at 25 °C in a shaking incubator	1 h at 25 °C in a shaking incubator	1 h at 25 °C in a shaking incubator	30 min ultrasonic bath
Clean-up	decant	centrifuge (17,000 *g* x 10 min at 10 °C)	ashless filter paper	centrifuge (17,000 *g* x 10 min at 10 °C)	centrifuge (17,000 *g* x 10 min at 10 °C)	centrifuge (14,000 *g* x 10 min at 10 °C)	centrifuge (14,000 *g* x 10 min at 10 °C)	centrifuge (14,000 *g* x 10 min at 10 °C)
Solvent evaporation	vacuum rotary evaporator	—	vacuum rotary evaporator	—	—	—	—	—
Resuspended	an aqueous MeOH (5%, v/v)	—	—	—	—	—	—	—
Centrifuge	17,000 *g* x 10 min at 10 °C	—	17,000 *g* x 10 min at 10 °C	—	—	—	—	—
Sample clean-up and concentration	SPE C18	—	SPE C18	—	—	—	—	—
Solvent evaporation	N_2 flow	—	N_2 flow	—	—	—	—	—
Post extraction clean-up	—	—	—	—	Tested: PLD+ column; 0.2 µm filter; 0.2 µm syringe filter; 3–, 10–, 30–, 50– kDa cut-off		PLD+ column	
Reconstitution/dilution in an aqueous MeCN (50%, v/v)	yes	yes	yes	yes	yes	yes	yes	yes
Obtained results (mg/100 g ± SD)[3]								
Avenacoside A[4]	19 ± 2[a]	26 ± 1[a]	18 ± 3[a]	37 ± 2[a]	Results in Figure 13		Results in Figure 14	
Soyasaponin Bb[5]	100 ± 3[a]	214 ± 5[a]	124 ± 10[a]	219 ± 8[a]				

[1] Reference: Pecio et al. (2013). [2] Reference: Heng, Vincken, Koningsveld, et al. (2006). [3] Each result represents mean ± SD (n = 2). [4]Measured in oat protein concentrate. [5]Measured in PPI. [a] Result is presented on fat-containing sample. *method 2B but testing filtering devices. **method 2C—shaking incubator extraction.*** method 2D—ultrasonication extraction.

The necessity of fat removal prior to saponin extraction was evaluated using four samples: WGOF, OPC, YP, and PPI. Saponins were extracted using methods 2B and 2C (as described in Table 9), and the results are shown in Figure 13. No notable differences were observed between the two methods regarding the impact of fat removal. However, the 3–kDa and 10–kDa cut-off centrifugal filters demonstrated poor performance across all sample matrices and saponin types. The highest analyte recoveries were achieved using 50-kDa, and in some cases 30–kDa, cut-off devices. The use of ISOLUTE® PLD+ columns and 0.2 µm syringe filters provided even better results than the 30–kDa or 50–kDa filters. While both options produced similar analyte recoveries, the PLD+ columns generated clearer MS chromatograms. Moreover, PLD+ columns can be easily integrated into high-throughput workflows via 96–well plate formats. These findings indicate that fat removal prior to saponin extraction is unnecessary and that PLD+ columns provide the most effective sample clean-up following extraction.

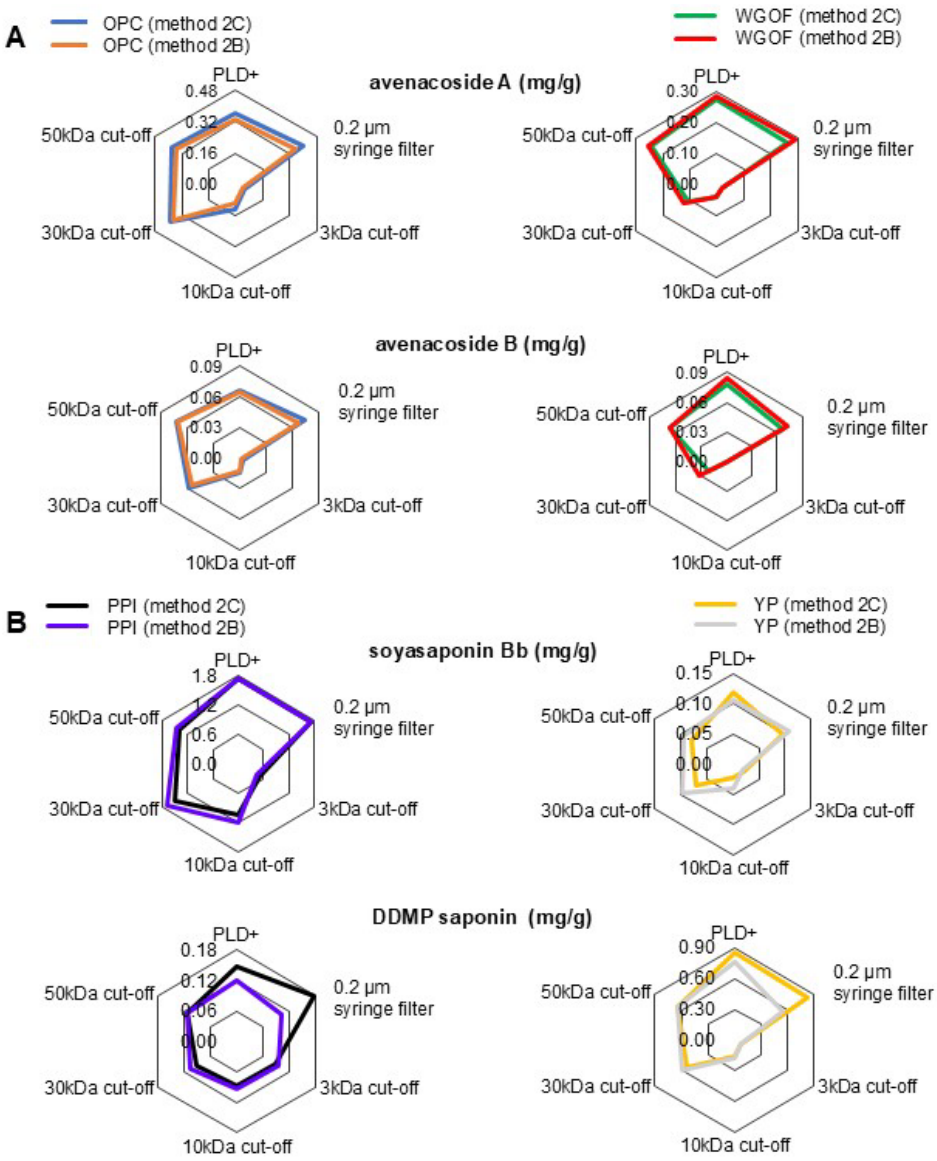

*Figure 13. The effect of different sample pre-extraction and post-extraction clean-up procedures (method 2B and 2C) on saponin yield. The samples: oat matrix (**A**)—avenacoside A and avenacoside B in oat protein concentrate (OPC) and whole grain oat flour (WGOF); pea matrix (**B**)—soyasaponin Bb and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) saponin in pea protein isolate (PPI) and yellow pea flour (YP). **Method 2B**—pre-extraction sample defatting by hexane. **Method 2C**—fat containing sample. The post-extraction sample clean-up devices: PLD+ columns for protein and phospholipid removal, 0.2 µm syringe filter, 3–50-kDa cut-off centrifugal filters. The results of avenacoside B are presented in equivalents of avenacoside A mg/g and DDMP saponin in equivalents of soyasaponin Bb mg/g. Each result represents mean of two replicates. (**Publication II**).*

The influence of ultrasonic power on saponin yield was evaluated using methods 2C and 2D on OPC, PPI, WGOF, and YP, as shown in Figure 14. Ultrasonication did not significantly affect saponin yield compared to the tube rotator method; however, its use remains advantageous due to the reduced extraction time.

Previous research has reported that DDMP saponins degrade rapidly at temperatures above 40 °C,[92] whereas another study found that pure DDMP saponin in MeOH solution only began to degrade at 65 °C.[21] In the present study, the heating observed during sonication (from 23 °C to 40 °C over 30 minutes) did not affect analyte stability. Considering both extraction time and yield, method 2D was therefore selected for the analysis and validation of all solid samples.

Liquid samples, such as OD and PD, were analysed without lyophilisation prior to extraction. Sample preparation involved only ISOLUTE® PLD+ cartridge purification before LC–MS analysis, as this approach had previously proven most effective for cleaning solid sample extracts.

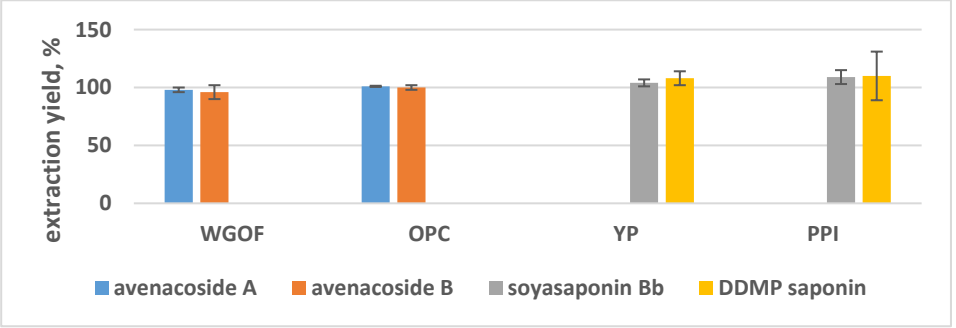

Figure 14. The effectiveness of ultrasonic bath extraction compared to reference extraction conditions using the tube rotator (extraction yield 100%). Each result represents mean ± standard deviation (n = 2). Samples: whole grain oat flour—WGOF, oat protein concentrate—OPC, yellow pea flour—YP, pea protein isolate—PPI. DDMP—2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one.

The sample extraction procedure developed and reported in **Publication II** enables the analysis of saponins from different plant species, specifically oat and pea, within the same matrix. The resulting methodologies for both powder and liquid samples are efficient and compatible with high-throughput analysis. Notably, saponin analysis from oat and pea drinks can be performed without time-consuming lyophilisation prior to analyte extraction. The procedure is further streamlined by eliminating the need for pre-extraction fat removal and by employing PLD+ columns for post-extraction clean-up. However, the methodology has certain limitations, particularly regarding absolute quantification. This limitation arises primarily from the lack of commercially available pure standards for all targeted saponins, which prevents precise determination of their absolute concentrations.

4.2.2.2 Extraction of soyasaponins from soybean-based yoghurt (Publication III)

As demonstrated in **Publication II**, liquid samples can be effectively used for saponin extraction without pretreatment steps such as lyophilisation. Building on these findings, the same approach was applied to SYA to analyse soyasaponins. However, it soon became evident that further optimisation of the extraction method was required.

The results revealed that soyasaponin recovery was adversely affected by acidic sample matrices. The native pH of the SBD was 8.8, whereas the pH of the yoghurt alternatives ranged between 4.6 and 4.7. The influence of both the native pH of the

products and the effect of pH adjustment prior to extraction on the final soyasaponin yield are summarised in Table 10. Samples were analysed both unspiked and spiked with the four target soyasaponins, with and without pH adjustment during the extraction procedure.

In samples analysed at their native pH, soyasaponin recoveries in the SBD ranged from 80% to 109%. In contrast, significantly lower concentrations and recoveries were obtained from both yoghurt alternatives, although their recoveries at native pH were comparable. These findings indicate that soyasaponin recovery efficiency is strongly influenced by the sample pH, demonstrating a clear pH dependency.

Since the SBD and SYA share a very similar composition and nutritional profile, it was hypothesised that the observed differences in soyasaponin recovery were primarily driven by pH. To test this hypothesis, experiments were conducted by simulating the pH environments of the respective products. Specifically, the pH of the SYA was adjusted to alkaline levels matching that of the SBD, while the pH of the SBD was reduced to resemble that of the SYA.

The results confirmed that samples at alkaline pH yielded satisfactory soyasaponin recoveries, whereas acidic samples resulted in notably lower recoveries. To determine the optimal extraction conditions, additional tests were performed by alkalising the yoghurt alternatives to three pH levels (7.5 ± 0.2, 8.0 ± 0.2, and 8.5 ± 0.2) and spiking them with all target soyasaponins. All alkaline conditions improved analyte recovery, with the most consistent and reproducible results observed at pH 7.5 ± 0.2 and pH 8.0 ± 0.2. Consequently, the optimal extraction pH was established at 8.0 ± 0.25 for subsequent analyses.

Although saponins are amphiphilic and generally more soluble in organic solvents, the aqueous solubility of soyasaponin Bb is highly pH-dependent—being minimal in acidic conditions and reaching its maximum around pH 7–8.[155] This behaviour explains the reduced recoveries observed in acidic matrices. By adjusting the pH of soybean-based yoghurt alternatives to mildly alkaline conditions, solubility limitations can be overcome, allowing direct analysis of liquid samples using a modified version of the saponin extraction method described in **Publication II**.

The method presented in **Publication III** provides a straightforward and high-throughput procedure for extracting soyasaponins directly from SYA. A key advantage of this approach is that it eliminates the need for time-consuming steps such as lyophilisation or rotary evaporation, which have been major limitations of previous methodologies. In addition, the method circumvents the use of SPE. This simplification reduces equipment requirements and enhances analytical throughput, making the procedure highly suitable for routine quality control applications.

The principal innovation of this method lies in recognising and implementing pH adjustment as a critical initial step in the extraction process. Specifically, the samples must be alkalised to substantially improve soyasaponin recovery, as their solubility is considerably reduced in acidic environments. This optimisation enables the direct analysis of liquid samples without additional preprocessing steps, thus streamlining the workflow while maintaining analytical accuracy.

*Table 10. The correlation between sample pH and recovery rates of soyasaponins across different extraction methods. The samples tested: soybean-based drink (SBD), soybean-based yoghurt alternatives (SYA1 and SYA2). Results are presented as mean ± standard deviation (n = 3). (**Publication III**).*

Sample	pH	Soyasaponin Bb		Soyasaponin Ba		Soyasaponin Aa		Soyasaponin Ab	
		mg/100 g	Recovery (%) spike 2.10 mg/L	mg/100 g	Recovery (%) spike 1.86 mg/L	mg/100 g	Recovery (%) spike 1.91 mg/L	mg/100 g	Recovery (%) spike 2.03 mg/L
SBD	8.8 (native)	12.60 ± 0.71^{a}	98 ± 4^{a}	2.43 ± 0.26^{a}	109 ± 8^{a}	<LOQ	85 ± 5^{a}	<LOQ	80 ± 5^{a}
	4.2 ± 0.2	1.20 ± 0.14^{b}	23 ± 5^{b}	0.82 ± 0.04^{b}	26 ± 5^{b}	<LOQ	54 ± 6^{b}	<LOQ	51 ± 5^{b}
SYA1	4.7 (native)	0.84 ± 0.03^{b}	20 ± 2^{b}	0.56 ± 0.02^{b}	25 ± 2^{b}	<LOQ	43 ± 3^{b}	0.58 ± 0.06^{b}	41 ± 3^{b}
	7.0 ± 0.2	5.39 ± 0.53^{a}	77 ± 2^{a}	1.03 ± 0.15^{a}	109 ± 3^{a}	<LOQ	104 ± 1^{a}	1.82 ± 0.1^{a}	115 ± 1^{a}
SYA2	4.6 (native)	1.30 ± 0.08^{b}	27 ± 2^{c}	0.54 ± 0.04^{b}	25 ± 1^{c}	<LOQ	48 ± 3^{c}	3.07 ± 0.07^{b}	63 ± 4^{b}
	7.0 ± 0.2	10.94 ± 0.63^{b}	85 ± 10^{b}	2.36 ± 0.03^{ab}	83 ± 8^{b}	<LOQ	91 ± 7^{ab}	8.09 ± 0.38b^{a}	98 ± 6^{a}
	7.5 ± 0.2	13.63 ± 1.63^{a}	89 ± 4^{ab}	2.68 ± 0.06^{a}	102 ± 5^{ab}	<LOQ	86 ± 4^{b}	9.93 ± 1.38^{a}	103 ± 1^{a}
	8.0 ± 0.2	14.43 ± 0.61^{a}	100 ± 14^{ab}	2.54 ± 0.34^{a}	114 ± 16^{a}	<LOQ	107 ± 15^{a}	9.18 ± 0.36^{a}	110 ± 15^{a}
	8.5 ± 0.2	13.51 ± 1.97^{a}	111 ± 1^{a}	2.79 ± 0.4^{a}	99 ± 8^{ab}	<LOQ	74 ± 7^{b}	9.01 ± 1.15^{a}	91 ± 8^{a}

<LOQ—below instrumental quantification limit

Statistical significance within each sample matrix was assessed using ANOVA; means marked with different letters indicate significant differences at P<0.05.

4.2.2.3 Extraction of FFAs from oat-, pea-, and faba bean protein sources (Publication V)

The extraction method for FFAs was adapted from previously published procedures for FFA isolation from plant-based matrices.[19,86,104] These methods were selected based on their applicability to similar matrices and their potential for substantial procedural simplification.

Initially, the key extraction steps were identified from the literature, and a Plackett–Burman (P–B) experimental design was constructed to evaluate seven extraction parameters: sample weight, extraction solvent, antioxidant use, extraction steps, solvent volume, extraction equipment, and extraction time (experimental matrix provided in **Publication V**). Preliminary screening identified antioxidant use, solvent type, and extraction time as significant factors, while the remaining parameters had minimal influence (Figure 15). Extraction with H_2O:MeOH (1:1, v/v) yielded C18:0 levels below the quantification limit, preventing coefficient calculation for this analyte.

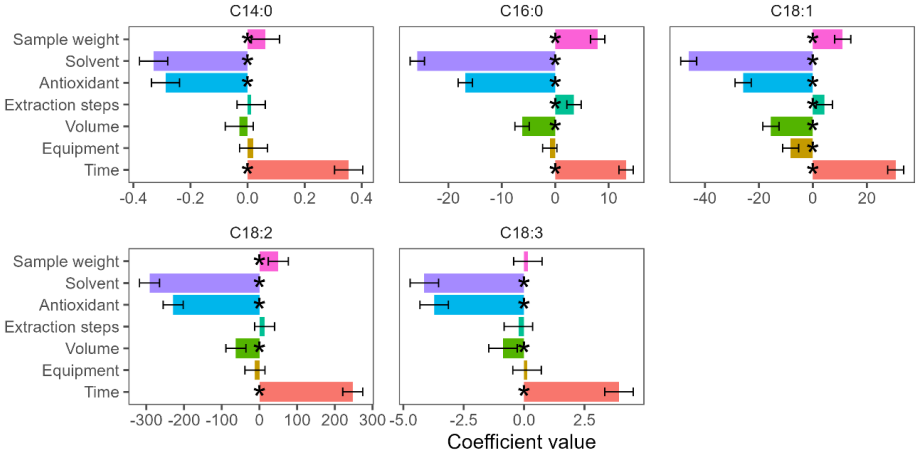

*Figure 15. Standardised effect plots from the Plackett–Burman experimental design, generated using Z sample to optimise the free fatty acid (FFAs) extraction. Statistically significant coefficients ($p<0.05$) are indicated by asterisks. R^2 0.96–0.99. C14:0—myristic acid, C16:0—palmitic acid, C18:1—oleic acid, C18:2—linoleic acid, C18:3—linolenic acid. (**Publication V**).*

To further optimise the extraction procedure, a full factorial (F–F) design was employed to refine the three key factors identified in the P–B design: solvent composition, antioxidant use, and extraction time (F–F experimental matrix presented in **Publication V**). The F–F experiment revealed solvent composition as the only statistically significant factor (Figure 16). Notably, a 50% aqueous IPA solution, not previously tested in the P–B design, provided the highest FFA extraction yields. A mixture of antioxidants (BHT, indomethacin, and t–AUCB), previously reported for FFA analysis,[23] was evaluated at different concentrations. Unlike in the P–B design, antioxidant presence had no measurable effect on fatty acid yield in the F–F experiment, likely due to shorter extraction times and differences in solvent composition. The apparent influence of antioxidants in the P–B design may therefore be attributed to confounding between main effects and two-way interactions, which that design cannot independently resolve.

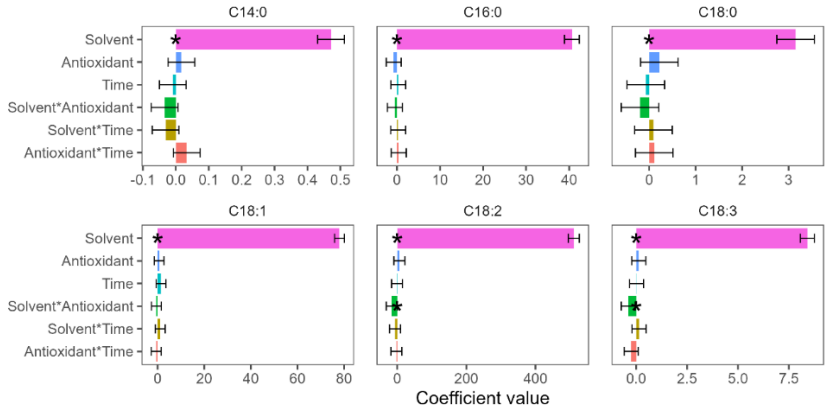

*Figure 16. Standardised effect plots from the full factorial experimental design, generated using Z sample to optimise the free fatty acid (FFAs) extraction. Asterisks indicate statistically significant coefficients ($p<0.05$). R^2 0.91–0.99. C14:0—myristic acid, C16:0—palmitic acid, C18:0—stearic acid, C18:1—oleic acid, C18:2—linoleic acid, C18:3—linolenic acid. (**Publication V**).*

Given the consistent identification of solvent composition as the critical factor in both the P–B and F–F experiments, a mixture design was subsequently conducted to further optimise the solvent system (design matrix available in **Publication V**). Water, MeOH, and IPA were chosen based on their common use in extraction protocols and compatibility with LC–MS mobile phases. The mixture design demonstrated that the highest FFA yields were obtained using a MeOH:IPA (1:1, v/v) solvent system (Figure 17), confirming the trends observed in the earlier experiments. Statistically, no significant differences in recovery were detected between the IPA:MeOH (1:1, v/v), H_2O:IPA:MeOH (1/6:4/6:1/6, v/v), and H_2O:IPA:MeOH (1/3:1/3:1/3, v/v) mixtures. Although aqueous IPA solutions have previously been reported for FFA extraction from oils,[156] the present findings demonstrate that solvent systems containing up to one-third water can still achieve efficient extraction while reducing solvent toxicity and overall solvent use.

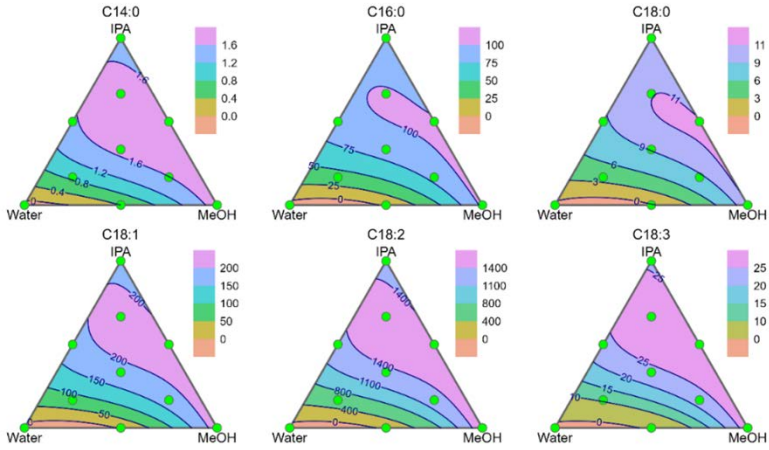

*Figure 17. Contour plots from the mixture design experiment using Z sample. Green points represent the experimental runs (n = 3). The values and colour gradient show free fatty acids yield (FFAs) ($p<0.05$). R^2 0.82–0.88. C14:0—myristic acid, C16:0—palmitic acid, C18:0—stearic acid, C18:1—oleic acid, C18:2—linoleic acid, C18:3—linolenic acid, IPA—isopropanol, MeOH—methanol. (**Publication V**).*

While the inclusion of water did not significantly affect total FFA yields, slightly higher recoveries of C16:0 and C18:0 were observed with the IPA:MeOH (1:1, v/v) mixture. Therefore, this binary solvent system was selected as the most practical and efficient option. Both IPA and MeOH effectively dissolve the target FFAs and are fully compatible with LC–MS analysis, eliminating the need for solvent exchange or evaporation steps often required when using immiscible solvents. During the P–B and F–F experiments, the aqueous MeOH solution (H_2O:MeOH, 1:1, v/v), recommended in earlier studies,[86,104] yielded significantly lower FFA amounts compared to other solvent systems. Conversely, the mixture design confirmed that IPA:MeOH (1:1, v/v) produced substantially higher yields.

The universal FFA extraction procedure developed and presented in **Publication V** was designed for application to oat, pea, and faba bean flours, as well as their corresponding protein concentrates and isolates. The matrix scope of this methodology could be extended to other plant species, although additional method control and validation would be required. High analytical accuracy and precision were achieved using isotopically labelled ISTD (^{13}C–oat flour extract), which significantly improve LC–MS quantification by compensating for MEs and enhancing data reliability.

The developed extraction procedure is streamlined, high-throughput, and time-efficient, requiring no derivatisation prior to detection, thus making it suitable for routine analysis. The optimised extraction solvent, consisting of a 1:1 (v/v) IPA and MeOH mixture, is fully miscible and directly compatible with LC–MS injection, eliminating the need for solvent evaporation or exchange steps. Through a systematic design of experiment approach, it was demonstrated that less hazardous solvents such as IPA and MeOH can effectively replace traditional LLE solvents like chloroform. Furthermore, the optimised solvent mixture can tolerate up to 33% water without compromising FFA recovery, thereby reducing solvent toxicity and overall solvent consumption. These improvements make the method more environmentally sustainable and fully aligned with the principles of green analytical chemistry.

4.2.3 Validation of developed methods

The methods developed for the simultaneous quantification of oat and pea saponins in both solid and liquid samples, the quantification of soyasaponins in SYAs, and the quantification of bitter-tasting FFAs were validated following the development and optimisation of their respective sample extraction procedures. The validation process involved the use of several sample matrices, including OPC, OD, PD, PPI, SYA, and Z samples. The linearity ranges, IQLs, and LOQs for avenacosides, soyasaponins, and FFAs showed as instrumental limits are summarised in Table 11 (more parameters available in Appendix 1). Method repeatability, encompassing retention time stability and peak area variability, is presented in (Table 12). Finally, Table 13 provides an overview of the overall method precision, recovery rates, and matrix effects.

*Table 11. The validation parameters of developed methods: linear range, calibration curve, instrumental quantification limits (IQLs), and limit of quantification (LOQs) of saponins and free fatty acids (FFAs). Data from **Publications II, III, and V**.*

Analyte	Plant origin	External standard	Internal Standard	Linear range (mg/L)	Calibration curve	R^2	IQL (µg/L)	LOQ (mg/100g)
avenacoside A	oat	avenacoside A	^{13}C–avena-coside A	0.01–2.44	Y = 0.2445x-0.0197	0.9998	15	0.62[a] 0.48[b]
26-desgluco-avenacoside A								n.a.
avenacoside B								
soyasaponin Bb	pea	soyasaponin Bb	soyasaponin Ba	0.01 –2.52	Y = 0.7699x + 0.0048	0.9930	12	0.44[c] 0.36[d]
	soybean		asperosaponin VI					0.18[e]
DDMP[1] saponin	pea		soyasaponin Ba					n.a.
soyasaponin Ba	soybean	soyasaponin Ba	asperosaponin VI	0.02–2.26	Y = 0.2949x + 0.0025	0.9975	33	0.52[e]
soyasaponin Aa		soyasaponin Aa	asperosaponin VI	0.02–2.33	Y = 0.3994x + 0.0021	0.9965	27	0.42[e]
soyasaponin Ab		soyasaponin Ab	asperosaponin VI	0.01–2.48	Y = 0.3259x + 0.0033	0.9943	25	0.39[e]
myristic acid	all	myristic acid	^{13}C18:3	0.03–3.66	Y = 5.0041x-0.0015	0.9975	24	0.19[f]
palmitic acid		palmitic acid	^{13}C16:0	0.05–6.51	Y = 0.3711x+0.0399	0.9880	121	0.95[f]
stearic acid		stearic acid	^{13}C18:0	0.08–5.18	Y = 9.5017x+1.0909	0.9967	83	0.59[f]
oleic acid		oleic acid	^{13}C18:1	0.04–5.12	Y = 0.3073x-0.0003	0.9932	21	0.17[f]
linoleic acid		linoleic acid	^{13}C18:2	0.05–6.25	Y = 0.0337x+0.0008	0.9955	50	0.39[f]
linolenic acid		linolenic acid	^{13}C18:3	0.05–6.53	Y = 4.2341x-0.0006	0.9943	39	0.30[f]

[1]2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one; [a]oat protein concentrate; [b]oat drink; [c]pea protein isolate; [d]pea drink; [e]soybean-based yoghurt alternative; [f]pooled sample Z; n.a. – not available

*Table 12. Repeatability as relative standard deviation (RSD) of retention times (RT) and peak areas of saponins, free fatty acids (FFAs) and internal standards (ISTD). Data from **Publications II, III, and V**.*

Analyte	Matrix	*m/z*	RT, min	RT RSD (%)		Peak area RSD (%)	
				Intra-day (n = 6)	Inter-day (n = 18)	Intra-day (n = 6)	Inter-day (n = 18)
Avenacoside A	Oat	1061.5	1.78	0.23	0.48	1.8	3.0
Avenacoside B	Oat	1223	1.93	0.20	0.27	4.1	4.5
26-desglucoavenacoside A	Oat	899.5	1.54	0.36	0.56	3.8	6.0
^{13}C–avenacoside A	ISTD (oat)	1112.5	1.78	0.28	0.54	2.9	4.2
Soyasaponin Bb	pea	941.5	1.42	0.29	0.98	2.6	3.1
	soybean		1.28	0.03	0.70	1.3	1.1
DDMP[1] saponin	pea	1067	1.4	0.23	0.86	3.9	6.3
Soyasaponin Ba	soybean ISTD (pea)	957.3	1.32	0.70	0.80	1.7	2.0
Soyasaponin Aa	soybean	1364.3	1.3	0.16	0.50	1.0	2.2
Soyasaponin Ab	soybean	1435.6	1.28	0.37	0.60	0.2	3.5
Asperosaponin VI	ISTD (soybean)	927.5	1.34	0.10	1.50	3.9	3.0
C18:3	Oat/pea/faba	277.2	3.37	2.50	3.90	1.4	6.1
C14:0	Oat/pea/faba	227.2	3.26	0.70	2.00	1.8	6.0
C16:0	Oat/pea/faba	255.2	4.31	0.40	1.00	2.7	8.0
C18:2	Oat/pea/faba	279.2	3.93	0.50	1.60	0.9	4.7
C18:1	Oat/pea/faba	281.2	4.56	1.00	2.00	0.3	4.5
C18:0	Oat/pea/faba	283.2	5.34	0.50	0.80	1.1	8.0
^{13}C18:3	ISTD Oat/pea/faba	295.2	3.38	1.50	2.00	0.9	7.1
^{13}C16:0	ISTD Oat/pea/faba	271.2	4.31	0.50	1.40	1.6	4.2
^{13}C18:2	ISTD Oat/pea/faba	297.2	3.93	0.50	1.10	0.4	4.3
^{13}C18:1	ISTD Oat/pea/faba	299.2	4.56	1.40	1.90	0.2	4.8
^{13}C18:0	ISTD Oat/pea/faba	301.2	5.36	0.60	1.00	2.8	6.4

[1]2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one

Table 13. The precision of the method, recoveries, and matrix effects of saponins and free fatty acids (FFAs) in different plant-based food ingredients and products. OPC—oat protein concentrate, OD—oat drink, PD—pea drink, PPI—pea protein isolate, SYA—soybean-based yoghurt alternative, Z—pooled sample composed of equal mix of WGOF (whole grain oat flour), YP (pea flour), FPI (faba bean protein isolate), OPC, PPI and FPI (faba bean protein isolate). Data from ***Publications II, III, and V****.*

Analyte	Sample	Precision RSD (%)		Recovery, %[2]	Matrix effect, %
		Intra-Day (n = 6)	Inter-Day (n = 9)		
Avenacoside A	OPC	11	11	90–115	100
	OD	12	12	96–104	107
	PD	n.a.	n.a.	94–106	105
Avenacoside B	OPC	13	9	n.s.	n.s.
	OD	3	8	n.s.	n.s.
26-desglucoavenacoside A	OPC	6	7	n.s.	n.s.
	OD	10	16	n.s.	n.s.
Soyasaponin Bb	PPI	6	7	82–100	110
	OD	n.a.	n.a.	98–113	105
	PD	6	7	89–98	102
	SYA	6	11	82–95	91
DDMP[1] saponin	PPI	8	11	n.s.	n.s.
Soyasaponin Ba	SYA	5	12	86–97	94
Soyasaponin Aa	SYA	<LOQ	<LOQ	81–101	99
soyasaponin Ab	SYA	4	12	88–96	94
Myristic acid	Z	5	8	93–103	102
Palmitic acid	Z	4	13	96–101	104
Stearic acid	Z	6	12	85–92	95
Oleic acid	Z	3	7	98–101	95
Linoleic acid	Z	4	8	97–99	93
Linolenic acid	Z	6	7	92–100	102

RSD—relative standard deviation
n.a.—not applicable for this sample matrix
<LOQ—below limit of quantification
n.s.—no standard for pre- and post-extraction spiking
[1]2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one
[2] range of mean recoveries, more detailed recovery values at three spiking levels could be found in respective publication.

4.2.3.1 Validation results of oat-containing matrices (Publication II)

In oat-containing matrices (OPC, WGOF, and OD), all three avenacosides were quantified using commercially available avenacoside A. This study successfully demonstrated the use of isotopically labelled avenacoside A, derived from a ^{13}C–oat flour extract, as the ISTD. In contrast, a recent study by Kwon et al. (2024) employed steroidal protodioscin as an alternative ISTD for avenacoside quantification by UPLC–MS/MS in MRM mode.[24] Due to its structural similarity to avenacosides, protodioscin represents a suitable substitute when isotopically labelled standards are unavailable.

The linearity of avenacoside A was established over the range of 0.01–2.44 mg/L (Table 11), with the IQL, based on the lowest calibration point, determined to be 15 µg/L. These IQL values are lower than those reported in previous studies.[19,24,27,28] Calculated avenacoside A LOQs in OPC and OD were 0.62 mg/100g and 0.48 mg/100g, respectively (Table 11). The repeatability of avenacoside retention times was ≤0.6%, while peak area repeatability showed intra-day variation ≤4.1% and inter-day variation ≤6.0% (Table 12). In OPC and OD matrices, intra-day precision ranged from 3% to 13%, and inter-day precision from 7% to 16%. Recovery and matrix effect experiments were conducted using

avenacoside A in OPC, OD, and PD matrices (Table 13). Recoveries ranged from 90% to 115%, and matrix effects from 100% to 107%. These results are consistent with previously published methods, which reported recoveries between 98% and 104%.[19,28]

4.2.3.2 Validation results of pea- and soybean-containing matrices (Publications II–III)

In pea-containing matrices (PPI, PD), soyasaponin Bb and DDMP saponin were quantified using commercially available soyasaponin Bb, with soyasaponin Ba employed as the ISTD. In contrast, in the SYA matrix, all available soyasaponins (soyasaponin Bb, soyasaponin Ba, soyasaponin Aa, and soyasaponin Ab) were quantified using external standards, with asperosaponin IV serving as the ISTD. Although these two quantification approaches differ, they could potentially be harmonised in future studies. Previous research has also successfully used the O-methylated isoflavone formononetin, naturally present in leguminous plants, as an ISTD for soyasaponin quantification in soybean products.[21]

In this study, the linear range for soyasaponins quantification was 0.01–2.5 mg/L, with IQLs from 13 to 33 µg/L (Table 11). LOQs of soyasaponin Bb in PPI, PD, and SYA were 0.44 mg/100g, 0.36 mg/100g, and 0.18 mg/100g, respectively (Table 11). LOQs of soyasaponin Ba, soyasaponin Aa, and soyasaponin Ab in SYA were 0.52 mg/100g, 0.42 mg/100g, and 0.39 mg/100g, respectively (Table 11). Retention time and peak area repeatability, expressed as relative standard deviation (RSD), were below 1.5% and 6%, respectively (Table 12). Intra- and inter-day precision were below 8% and 12%, respectively (Table 13). These results are consistent with previous studies, which reported intra-day and inter-day variation for soyasaponin Bb of 1.9–9.8%[21,87], 7.5–14.3%[21,87] in soybeans, respectively.

Recovery and matrix-effect experiments were conducted by pre- and post-extraction spiking of the PPI, OD, PD, and SYA matrices with pure standards. Recoveries ranged from 81% to 113%, and matrix effects from 91% to 110%, demonstrating excellent method performance (Table 13). These results are in line with previously reported recoveries for soyasaponin Bb in various soybean products, including soybean flour, textured vegetable protein, and tofu, which ranged from 94% to 121%.[21]

4.2.3.3 Validation results of FFAs-containing matrices (Publication V)

The quantification method for FFAs was validated using pure standards and a pooled sample composed of equal amounts of WGOF, YP, FPI, OPC, PPI, and FPI. All targeted fatty acids were quantified using pure standard compounds alongside isotopically labelled ^{13}C–FFAs derived from a ^{13}C–oat flour extract. Similarly, a recent study employed an isotopically labelled fatty acid (^{13}C18–linoleic acid) for the quantification of multiple fatty acids in commercial PPI products.[86] The linear range for FFA quantification was 0.03–6.50 mg/L, with IQLs from 21 to 121 µg/L and LOQs from 0.19 to 0.95 mg/100g in sample Z (Table 11). Repeatability, expressed as the RSD of retention times and peak areas, did not exceed 3.9% and 6.4%, respectively, for both FFAs and their isotopically labelled analogues. Intra- and inter-day RSDs ranged from 2.8% to 13.0% (Table 12). Analyte recoveries were between 93% and 103%, while matrix effects ranged from 93% to 104%, demonstrating high method accuracy and robustness (Table 13). These performance characteristics are consistent with those reported in previous studies.[19,86]

Overall, the methods developed and presented in **Publications II–III, and V** demonstrated high accuracy, precision, and sensitivity, owing to the use of isotopically labelled or otherwise appropriate ISTD for MS quantification. Moreover, the sample extraction procedures were designed to be simple and streamlined, enhancing overall method efficiency and making them suitable for routine analysis.

4.3 Quantification of bitter-tasting compounds in food ingredients, intermediate, and commercial products

LC–MS methods developed for the quantification of key bitter-tasting saponins and FFAs were applied to selected oat- (n = 3), pea- (n = 4), faba bean-based (n = 2), and mixed (n = 2) food ingredients, as well as intermediate products, and commercial products. The presented results are limited, as not all developed methods were applied across the entire range of samples. A summary of the quantitative data (mg/100 g) is provided in Table 14, which compiles results reported in **Publications II and V.**

Table 14. The content of saponins and free fatty acids (FFAs) (C14:0—myristic acid, C16:0—palmitic acid, C18:0—stearic acid, C18:1—oleic acid, C18:2—linoleic acid, C18:3—linolenic acid) in oat protein concentrate (OPC), whole grain oat flour (WGOF), oat drink (OD), pea protein isolate (PPI), pea protein concentrate (PPC), yellow pea flour (YP), pea drink (PD), pea/oat blend (BLEND), extruded pea/oat blend (EX-BLEND), faba bean protein isolate (FPI), faba bean flour (FB) (mean ± SD; n = 3). Data from ***Publications II and V****.*

Product	mg/100 g "as is"										
	Avenacoside A	Avenacoside B*	26-desgluco-avenacoside A*	Soyasaponin Bb	DDMP** saponin[1]	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
OPC	42.3 ± 3.0[a]	33.8 ± 0.7[a]	5.1 ± 0.2[a]	n.a.	n.a.	1.3 ± 0.1[b]	77.1 ± 4.4[b]	4.5 ± 0.2[b]	142.3 ± 5.4[b]	907.8 ± 48.7[b]	4.5 ± 0.3[b]
WGOF	23.4 ± 2.9[b]	14.0 ± 1.5[b]	≤LOQ	n.a.	n.a.	3.2 ± 0.2[a]	149.0 ± 20.7[a]	15.1 ± 2.0[a]	533.0 ± 47.2[a]	2748.4 ± 248.0[a]	17.1 ± 0.9[a]
OD	4.6 ± 0.1[d]	2.7 ± 0.2[d]	≤LOQ	n.a.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PPI	n.a.	n.a.	n.a.	243.8 ± 6.2[a]	10.8 ± 0.7[d]	≤LOQ	15.3 ± 1.8[c]	5.6 ± 1.3[b]	38.4 ± 0.3[c]	332.5 ± 4.9[c]	4.8 ± 0.1[b]
PPC	n.a.	n.a.	n.a.	20.3 ± 1.6[c]	107.6 ± 4.1[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YP	n.a.	n.a.	n.a.	6.2 ± 0.4[c]	61.1 ± 2.0[b]	≤LOQ	≤LOQ	≤LOQ	4.9 ± 0.2[c]	35.9 ± 0.4[d]	1.2 ± 0.1[c]
PD	n.a.	n.a.	n.a.	3.5 ± 0.2[c]	≤LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BLEND	13.5 ± 1.0[c]	10.9 ± 0.3[c]	1.3 ± 0.3[b]	123.9 ± 6.3[b]	27.1 ± 3.5[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EX-BLEND	10.6 ± 0.3[c]	9.6 ± 0.9[c]	1.1 ± 0.9[b]	132.9 ± 12.4[b]	11.4 ± 0.8[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
FPI	n.a.	n.a.	n.a.	n.d.	n.d.	≤LOQ	≤LOQ	≤LOQ	≤LOQ	4.2 ± 0.2[d]	≤LOQ
FB	n.a.	n.a.	n.a.	n.d.	n.d.	≤LOQ	8.0 ± 1.0[c]	≤LOQ	26.5 ± 0.7[c]	188.6 ± 1.1[cd]	2.1 ± 0.3[c]

n.a—not applicable for this plant origin.

n.d.—not determined.

≤LOQ—below limit of quantification

*avenacoside B and 26-desglucoavenacoside A are quantified in avenacoside A equivalents.

**2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one

[1]DDMP saponin is quantified in soyasaponin Bb equivalents

Statistical analysis was conducted within all product groups and means labelled with different letters were considered significantly different at $p<0.05$.

4.3.1 Quantification results of saponins in oat-based products (Publication II)

Avenacosides were analysed in oat-containing reference matrices (OPC, WGOF, OD, BLEND, and EX-BLEND). Avenacoside A was identified as the predominant compound in all tested oat products, with concentrations ranging from 4.6 to 42.3 mg/100 g. Avenacoside B was present at lower concentrations in oat products, ranging from 2.7 to 33.8 mg/100 g (Table 14). In contrast, 26-desglucoavenacoside A was detected at significantly lower levels in OPC, BLEND, and EX-BLEND, ranging from 1.1 to 5.1 mg/100 g. The highest total avenacoside content was observed in OPC.

The analysed WGOF contained 23.4 mg/100g of avenacoside A and 14.0 mg/100g of avenacoside B, representing 62% and 38% of the total avenacoside content, respectively. Comparably to the present study, previous research also identified avenacoside A as the dominant avenacoside in oat grains, accounting for 41.9–60.6% of the total avenacoside content, while avenacoside B contributed 35.8–55.2%.[27] The content of 26-desglucoavenacoside A was substantially lower, ranging from 2.4% to 6.4% (mean 3.5%) in oat grains.[27] Similarly, Günther-Jordanland et al. (2020) reported avenacoside A and avenacoside B as the most abundant saponins, each present at approximately 22 mg/100 g in oat flour.[19]

A recently published study demonstrated that during the production of oat-based milk alternatives from oat flour, under conditions simulating industrial processing, the concentration of avenacoside A increased by approximately 33%, reaching around 3.6 mg/100 g in oat-based milk alternatives.[134] This concentration is consistent with levels observed in OD analysed in the present study.

The BLEND sample, consisting of 52% PPI, 28% OPC, and 20% PPC, was analysed alongside its individual components. The recoveries of avenacoside A, avenacoside B, and 26-desglucoavenacoside A were 114%, 115%, and 90%, respectively. Following extrusion cooking, the content of avenacoside A in EX-BLEND decreased by 21%; however, this difference was not statistically significant compared with the uncooked product ($p < 0.05$). In contrast, the concentrations of avenacoside B and 26-desglucoavenacoside A remained largely unchanged.

The methods developed for the quantification of avenacosides in both dry and liquid matrices produced results consistent with previously published data, demonstrating their suitability for the analysis of real products containing avenacosides.

4.3.2 Quantification results of saponins in pea-based products (Publication II)

Matrices containing pea protein ingredients (PPI, PPC, YP, PD, BLEND, and EX-BLEND) were analysed for soyasaponin Bb and DDMP saponin (Table 14). The concentration of soyasaponin Bb varied widely, ranging from 3.5 to 243.8 mg/100 g. The content of DDMP saponin was significantly lower, ranging from 10.8 to 107.6 mg/100 g, while in the PD matrix it was below the LOQ.

Pea protein ingredients were also included in the BLEND sample (52% PPI+ 28% OPC + 20% PPC) in which the recoveries of soyasaponin Bb and DDMP saponin were 95% and 100%, respectively. During extrusion cooking, the soyasaponin Bb content increased from 123.9 to 132.9 mg/100 g, which may have resulted from the conversion of DDMP saponin into soyasaponin Bb under thermal processing conditions. The DDMP saponin is known to readily decompose into soyasaponin Bb during common food processing operations.[92]

Heng et al. (2006) reported that the total saponin content in dry peas ranges from 70 to 190 mg/100 g on a dry weight basis (dwb).[92] The present study revealed that saponins tend to concentrate during the pea protein purification process. An increase in total saponin content was associated with a higher protein content (YP < PPC < PPI). This trend is supported by a recent study by Tanambell et al. (2025), which applied a modified version of the saponin extraction methodology originally described in Publication II to investigate changes in saponin content during pea protein isolate production.[93] That study demonstrated that the combined content of soyasaponin Bb and DDMP saponins in pea flour (protein content 20%) was approximately 200 mg/100 g dwb. In contrast, pea protein isolates with protein contents ranging from 80% to 90% contained substantially higher total concentrations of soyasaponin Bb and DDMP saponins, ranging from 100 to 780 mg/100 g dwb, depending on the protein purification conditions. On the contrary, ultrafiltration was shown to remove a significant proportion of saponins from pea protein isolates.[93] Overall, the present study confirms that saponins generally concentrate during protein purification processes and may therefore significantly contribute to the perceived bitterness of novel pea-based protein products.

4.3.3 Quantification results of FFAs in oat-, pea- and faba bean-based protein sources (Publication V)

The developed LC-MS method was applied to analyse the content of FFAs in plant protein–based matrices (OPC, WGOF, PPI, YP, FPI, and FB). The dominant FFAs (C18:2 and C18:1) were detected across a broad concentration range in oat-, pea- and faba bean-based protein sources (values shown in Table 14). The predominance of C18:2 and C18:1 in oat- and pea-based products aligns with previous findings.[19,86] A study on naked oats identified C18:2, C18:1, and C16:0 as the major fatty acids.[157] The present study revealed a similar distribution pattern in the FFA profile of oat-based products. Faba bean-based products contained lower overall FFA levels, although the same dominant compounds (C18:2 and C18:1) were observed. Although protein purification techniques reduce FFA content, some FFAs remain and may contribute to bitter taste perception or to the formation of odour-active compounds in plant-based protein ingredients and end products.

4.3.4 Estimation of bitterness DoT in oat-, pea- and faba bean products

Sensory evaluation is commonly employed to assess the bitterness of food products. However, as demonstrated in **Publication I**, the assessors assigned highly variable bitterness rankings for WGOF and YP. It was also noted that the bitterness of oat flour may be associated with the presence of FFAs. Previous studies have reported DoT values for both saponins and FFAs to estimate their potential impact on the bitterness of pea protein isolates and oat flour.[19,86]

Given the availability of quantitative data of saponins and FFAs in the present study, bitter taste thresholds were used to calculate DoT values to link analytical results with potential sensory perception and to estimate the individual contributions of each bitter-tasting compound to the overall flavour profile. Bitterness perception was evaluated for selected powder- and liquid-based products using available taste threshold values for avenacoside A, avenacoside B, soyasaponin Bb, and FFAs (Table 15).

*Table 15. The results of calculated dose-over-threshold of avenacosides and free fatty acids (C14:0—myristic acid, C16:0—palmitic acid, C18:0—stearic acid, C18:1—oleic acid, C18:2—linoleic acid, C18:3—linolenic acid) bitterness in oat protein concentrate (OPC), whole grain oat flour (WGOF), oat drink (OD), pea protein isolate (PPI), pea protein concentrate (PPC), yellow pea flour (YP), pea drink (PD), pea/oat blend (BLEND), extruded pea/oat blend (EX-BLEND), faba bean protein isolate (FPI), faba bean flour (FB). Data from **Publications II, V** and unpublished data.*

Plant origin	Products	Avenacoside A	Avenacoside B	Soyasaponin Bb	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
O	OPC	56.84	45.98	—	0.03	1.95	0.22	2.31	17.89	0.58
O	WGOF	31.44	19.04	—	0.08	3.76	0.73	8.66	54.15	2.22
O	OD	6.18	3.67	—	—	—	—	—	—	—
P	PPI	—	—	1.60	—	0.39	0.18	0.62	6.55	0.63
P	PPC	—	—	0.13	—	—	—	—	—	—
P	YP	—	—	0.04	—	—	—	0.10	0.80	0.16
P	PD	—	—	0.02	—	—	—	—	—	—
P/O	BLEND	18.14	14.83	0.81	—	—	—	—	—	—
P/O	EX-BLEND	14.24	13.06	0.87	—	—	—	—	—	—
F	FPI	—	—	—	—	—	—	—	0.08	—
F	FB	—	—	—	—	0.13	—	0.43	3.72	0.27

DoT is ratio of the concentration of a compound to its taste threshold. The threshold values used were as follows: avenacoside A—7 µmol/L, avenacoside B—6 µmol/L, soyasaponin Bb—1620 µmol/L, C14:0—1703 µmol/L, C16:0—1546 µmol/L, C18:0—726 µmol/L, C18:1—2180 µmol/L, C18:2—1810 µmol/L, and C18:3—277 µmol/L, as reported in previous studies.[19,86]

"–" no data.

For avenacoside A and avenacoside B, the calculated DoT values varied widely in OPC, WGOF, OD, BLEND and EX-BLEND, ranging from 6 to 57 and 4 to 46, respectively. The highest DoT values were observed in OPC, while the lowest were found in OD. Both blends exhibited similar DoT values. Importantly, all DoT values for avenacosides in oat-based products were significantly greater than 1, indicating that these compounds likely contribute to the perceived bitterness of the products. The highest DoT values for C18:2 were observed in the oat-based products WGOF (54.15) and OPC (17.89). In contrast, the concentrations of C14:0 and C18:0 were too low (DoT<1) to significantly contribute to the bitterness of the oat-based products. These findings align with previous research demonstrating that FFA levels in non-heat-stabilised oats can increase up to tenfold during storage under high-moisture conditions due to lipolytic enzyme activity.[158,159] Overall, the highest DoT values for avenacoside A, avenacoside B, and C18:2 were observed in the oat-based products OPC and WGOF. These results are consistent with previous reports of bitter-tasting FFAs in whole-grain oat flour and pea protein isolates.[19,86] Günther-Jordanland et al. (2016, 2020) also reported that C14:0 and C18:0 were found at concentrations below their respective taste thresholds, indicating they do not contribute notably to bitterness in oat flour.[19,73]

For pea-containing products (PPI, PPC, YP, PD, BLEND, and EX-BLEND), a bitter taste threshold value is available only for soyasaponin Bb. Its taste threshold is approximately 230 times higher than that of avenacoside A, indicating that soyasaponin Bb is substantially less bitter. In the present study, the calculated DoT values for soyasaponin Bb in pea-based products ranged from 0 to 1.6, with the highest value observed in PPI. Previously, Gläser et al. (2020) reported that soyasaponin Bb had no major impact on the bitterness of pea protein isolates (DoT = 0.7).[18] The results of the present study indicate that the content of soyasaponin Bb may vary among pea-based products and should therefore be determined when estimating perceived bitterness.

In a subsequent study, Gläser et al. (2021) characterised 17 commercial pea protein isolates to evaluate their lipid composition and the individual contributions of different compounds to bitterness.[86] Their results showed that C18:3 and C18:2 were the predominant FFAs, exhibiting the highest DoT factors and thus representing the major contributors to bitterness in pea protein isolates. The present study confirms the contribution of C18:2 to bitterness; however, the content of C18:3 in the analysed PPI was too low to contribute to bitterness. In addition to their bitter taste, these FFAs are also responsible, for generating numerous odour-active compounds in peas, through their breakdown products.[48]

To summarize, avenacosides, soyasaponin Bb, and FFA were quantified in oat-, pea-, and faba bean-based ingredients and plant-based dairy alternatives. Avenacosides and C18:2 were the main contributors to bitterness in oat-based products, while soyasaponin Bb and other FFAs had minimal impact in legume-based products. DoT calculations confirmed that avenacosides and C18:2 exceeded taste thresholds in key oat products, particularly OPC and WGOF. These results provide new quantitative insights for processed products, linking chemical composition to sensory perception and offering a foundation for managing bitterness in plant-based foods.

4.3.5 Quantification of saponins in oat- and soybean-based yoghurt alternatives and estimation of bitterness DoT (Publication IV)

The developed LC–MS methods for the quantification of avenacosides and soyasaponins were applied in a study focusing on protein digestibility in plant-based yoghurt alternatives. Saponins were quantified in 19 commercially available oat- and soybean-based yoghurt alternatives, as they are known to contribute bitterness and act as antinutritional factors (**Publication IV**). The results of saponin quantification, together with DoT calculations, are presented in Table 16.

In soybean-based yoghurt alternatives, concentrations of soyasaponin Bb, soyasaponin Ba, and soyasaponin Ab ranged from 7.2 to 13.6 mg/100 g, 1.0 to 2.2 mg/100 g, and 1.4 to 7.1 mg/100 g, respectively. Soyasaponin Aa was below the LOQ in all analysed products. Until recently, limited information has been available on the soyasaponin content of soybean-based dairy alternatives, particularly yoghurt alternatives. A recent study by Chitisankul et al. (2021) expanded this knowledge by examining the soyasaponin composition in 39 food products, including 14 soybean-based milk alternatives.[15] That study reported soyasaponin Aa consistently below LOQ, while soyasaponin Bb, soyasaponin Ba and soyasaponin Ab ranged from 27 to 308 mg/100 g dwb, from 0 to 14 mg/100 g dwb, and from 1 to 44 mg/100 g dwb in soybean-based milk alternatives, respectively. Based on an estimated average dry matter content of 11% in the products analysed in the present study, the corresponding average concentrations of soyasaponin Bb, Ba, and Ab were calculated as 114, 29, and 54 mg/100 g dwb, respectively. These results are consistent with previously published data and confirm the presence of notable quantities of soyasaponins in commercial soybean-based yoghurt alternatives.

In oat-based yoghurt alternatives, avenacoside A, avenacoside B, and 26-desglucoavenacoside A were quantified at concentrations ranging from 0.8 to 5.5 mg/100 g, 0.2 to 2.4 mg/100 g, and 0.2 to 2.7 mg/100 g, respectively. The concentrations of avenacosides in oat-based products exhibited a 7- to 13-fold variation, which was substantially greater than the variability observed for soyasaponins in the soybean-based yoghurt alternatives. The production processes used for oat-based yoghurt alternative productions may be less standardised due to their relative novelty.

In addition, DoT values for bitterness were calculated for the plant-based yoghurt alternatives (Table 16). The DoT values for soyasaponins were consistently low (below 1), indicating that soyasaponin Bb is unlikely to contribute to the perceived bitterness of these products. In contrast, the DoT values for avenacoside A and avenacoside B exhibited greater variability, ranging from 1.07 to 7.39 and from 0.27 to 3.26, respectively. In eight out of the nine oat-based products analysed, the DoT values indicate that avenacosides may contribute significantly to the bitter taste. These results suggest that bitterness in oat-based products remains an unresolved issue and requires further efforts from food technology scientists.

*Table 16. The results of saponin analysis in the soybean- (VY3-S—VY12-S) and oat-based (VY13-O—VY21-O) yoghurt alternatives (mean ± SD; n = 3) and estimated dose-over-threshold (DoT) values of saponins in these products (**Publication IV,** and unpublished data).*

Soybean-based	mg/100 g Soya-saponin Bb	Soya-saponin Ba	Soya-saponin Ab	DoT Soya-saponin Bb	Oat-based	mg/100 g Avena-coside A	Avena-coside B*	26-desgluco-avenacoside A*	DoT Avena-coside A	Avena-coside B
VY3-S	13.1 ± 1.2 ^{ab}	2.2 ± 0.1 ^{a}	3.2 ± 0.0 ^{abc}	0.09	**VY13-O**	2.4 ± 0.1 ^{c}	1.3 ± 0.1 ^{de}	2.7 ± 0.5 ^{a}	3.22	1.77
VY4-S	13.6 ± 0.8 ^{a}	2.1 ± 0.1 ^{a}	2.4 ± 0.1 ^{bcd}	0.09	**VY14-O**	0.8 ± 0.1 ^{e}	0.2 ± 0.0 ^{g}	0.2 ± 0.0 ^{c}	1.07	0.27
VY5-S	9.8 ± 1.2 ^{cd}	1.0 ± 0.2 ^{c}	1.4 ± 0.1 ^{f}	0.06	**VY15-O**	4.0 ± 0.2 ^{a}	1.9 ± 0.2 ^{ab}	0.3 ± 0.1 ^{bc}	5.37	2.58
VY6-S	9.5 ± 1.1 ^{cd}	1.3 ± 0.2 ^{bc}	1.7 ± 0.1 ^{ef}	0.06	**VY16-O**	5.5 ± 0.3 ^{a}	2.4 ± 0.1 ^{a}	0.4 ± 0.1 ^{b}	7.39	3.26
VY7-S	12.1 ± 0.4 ^{ab}	1.6 ± 0.2 ^{ab}	2.2 ± 0.1 ^{cde}	0.08	**VY17-O**	1.5 ± 0.0 ^{de}	1.0 ± 0.0 ^{ef}	1.4 ± 0.1 ^{a}	2.02	1.36
VY8-S	10.9 ± 0.0 ^{bc}	1.6 ± 0.2 ^{ab}	2.3 ± 0.2 ^{cde}	0.07	**VY18-O**	2.2 ± 0.2 ^{cd}	0.6 ± 0.1 ^{fg}	0.4 ± 0.1 ^{b}	2.96	0.82
VY9-S	12.2 ± 0.1 ^{ab}	2.1 ± 0.0 ^{a}	3.5 ± 0.1 ^{ab}	0.08	**VY19-O**	2.8 ± 0.2 ^{b}	1.5 ± 0.1 ^{bc}	2.7 ± 0.4 ^{a}	3.76	2.04
VY10-S	9.1 ± 0.7 ^{cd}	1.0 ± 0.1 ^{c}	2.1 ± 0.1 ^{def}	0.06	**VY20-O**	2.3 ± 0.2 ^{c}	1.4 ± 0.1 ^{cd}	0.3 ± 0.0 ^{bc}	3.09	1.90
VY11-S	8.2 ± 0.9 ^{d}	1.0 ± 0.1 ^{c}	1.4 ± 0.0 ^{f}	0.05	**VY21-O**	2.8 ± 0.1 ^{b}	1.5 ± 0.1 ^{c}	1.3 ± 0.1 ^{a}	3.76	2.04
VY12-S	7.2 ± 0.7 ^{d}	1.5 ± 0.1 ^{abc}	7.1 ± 0.7 ^{a}	0.05						

*Avenacoside B and 26-desglucoavenacoside A are quantified in avenacoside A equivalent. DoT is ratio of the concentration of a compound to its taste threshold. The threshold values used were as follows: avenacoside A—7 µmol/L, avenacoside B—6 µmol/L, soyasaponin Bb—1620 µmol/L as reported in previous studies.[18,19] Statistical analysis was conducted within the product groups (S and O) and means labelled with different letters were considered significantly different at $p<0.05$.

5 Conclusions

The main objective of this dissertation was to develop and apply analytical methods to quantify bitter-tasting compounds in plant protein ingredients and food products. This involved exploring variability in individual bitterness perception of oat and pea flours, developing quantification methods for bitter-tasting saponins and FFAs, and applying these methods to screen commercial plant-based protein products. Additionally, the bitterness potential of key compounds was estimated using DoT factor calculations.
The main conclusions were as follows:

- Sensory tests with trained assessors and consumers showed high variability in bitterness perception of oat and pea flours.
    - Most respondents perceived bitterness in pea flour, whereas far fewer reported moderate or high bitterness in oat flour, indicating a smaller affected consumer segment. Overall, the results highlight the sensory relevance and variability of bitterness in plant protein ingredients, supporting the need for analytical methods to quantify the compounds responsible for it.
- Sample extraction procedures were comprehensively developed, and validated, and successfully combined with LC–MS methods for the analysis of avenacosides, soyasaponins and FFAs in powdered and liquid protein-based foods and ingredients derived from oats, peas, faba beans, and soybeans.
    - Simplified and high throughput saponin and FFAs extraction methods were developed for routine laboratory use, eliminating the need for freeze-drying and rotary evaporation. Shortened small-scale extraction procedures were shown to be effective for oat-, pea-, soyasaponins and FFAs in plant-based protein concentrates, isolates, and milk and yoghurt alternatives, enabling reliable saponin quantification in both solid and liquid commercial products.
    - A design-of-experiments approach was successfully applied to optimize the extraction procedure for FFAs, including the selection of the extraction solvent. The optimized sample preparation method for FFAs was faster and employed less hazardous solvents compared with traditional methods.
    - ^{13}C–oat flour proved to be an excellent source of ISTD for the quantification of avenacosides and FFAs.
- The concentrations of avenacosides, soyasaponin Bb, and DDMP saponin were higher in high-protein products, indicating that these compounds become concentrated during protein extraction processes. The developed method successfully quantified both saponin classes in mixed matrices. All studied soybean yoghurt alternatives contained similar amounts of soyasaponins, with soyasaponin Bb being the predominant form. The highest FFA content was observed in WGOF, whereas the lowest concentrations were found in pea and faba bean products. DoT calculations for quantified bitter-tasting compounds in various plant-based ingredients, intermediate, and commercial products suggested that oat products are generally more bitter. Avenacoside A and FFAs (C18:1 and C18:2) were identified as the key bitter compounds in the oat products studied, whereas soyasaponin Bb concentrations were too low to contribute significantly to bitterness.

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Мои самые искренние слова благодарности я хочу адресовать своей семье. Без моего мужа Георгия и сына Марка обучение в докторантуре не было бы таким радостным и вдохновляющим. Совмещать учёбу и семейную жизнь – задача не из лёгких. У этой диссертации есть один маленький, но очень важный соавтор – Марк.

Abstract

Analytical methods for quantification of bitter compounds in plant protein ingredients and food applications

The growing demand for plant-based foods, driven by health, ethical, and environmental considerations, has stimulated the development of alternatives from protein-rich crops such as soybeans, peas, faba beans, and oats. These ingredients offer nutritional and sustainability benefits, yet the sensory properties of plant-based products remain a major barrier to consumer acceptance. In contrast to consumers' expectations, many plant-based ingredients exhibit undesirable flavours, with bitterness being particularly problematic. Compounds such as saponins and free fatty acids (FFAs) are known contributors, but rapid and reliable analytical methods to quantify them in complex matrices are limited, constraining the optimisation of taste in product development.

This dissertation aims to develop and validate analytical methods for the quantification of bitter-tasting compounds in plant protein ingredients and food applications. It addresses three key questions: (1) How variable is individual perception of bitterness for oat and pea flours? (2) How can bitter-tasting oat, pea, and soy saponins, as well as FFAs, be quantified reliably in plant-based matrices? (3) What levels of FFAs, soyasaponin Bb, and avenacosides are present in food ingredients, intermediate, and commercial products, and what is their bitterness potential in these matrices?

The work is structured around five publications. Publication I uses quantitative descriptive sensory analysis with trained panellists and consumers to assess bitterness in oat and pea flours. Pea flour was generally more bitter than oat flour, with bitterness correlating strongly with fatty acid content. Considerable individual variability in perception was observed, highlighting the need for analytical methods to quantify the compounds underlying bitterness. Publications II–V focus on the development, optimisation, and application of extraction procedures for saponins and FFAs in oat, pea, soybean, and faba bean products, including powders and liquids. Extraction procedures were combined with liquid chromatography mass spectrometry to produce simple, high-throughput, eco-friendly methods suitable for liquid samples without lyophilisation. A design of experiments approach optimised free fatty acid extraction, particularly by varying solvent composition, and all methods were validated according to established guidelines.

The validated methods were applied to quantify bitter compounds in intermediate ingredients and commercial products, including plant-based drinks, yoghurt alternatives, flours, protein concentrates, and isolates. Saponin concentrations were highest in protein concentrates and isolates, with soyasaponin Bb predominant in soybean-based yoghurt alternatives. Whole-grain oat flour contained the highest levels of FFAs, whereas pea and faba bean products were lowest. Dose-over-threshold calculations indicated that oat-based products are generally more bitter, with avenacosides and the free fatty acids C18:2 and C18:3 identified as primary contributors. In contrast, soyasaponin Bb and other FFAs contributed minimally to the bitterness of soybean-based products.

These results provide new insights into the variability of bitter compound concentrations and their sensory relevance in processed plant-based foods. Avenacosides and C18:2 frequently exceed taste thresholds in oat-based matrices, particularly in oat protein concentrates and whole-grain oat flour, highlighting targets for taste optimisation. Mapping the distribution and sensory impact of saponins and FFAs

across ingredients and products informs strategies to reduce bitterness and improve palatability.

In summary, this dissertation advances both sensory and analytical understanding of bitterness in plant-based proteins. It establishes robust, validated methods for quantifying bitter-tasting saponins and FFAs across diverse matrices and links these compounds directly to sensory perception. The integration of sensory evaluation with chemical quantification provides a precise understanding of how specific compounds contribute to perceived bitterness. Collectively, the findings offer actionable insights for the food industry, supporting the development of more palatable plant-based foods and contributing to the growing market for sustainable, protein-rich alternatives.

Lühikokkuvõte

Analüütilised meetodid kibedate ühendite kvantitatiivseks määramiseks taimse valgu põhistes toidumaatriksites

Taimepõhiste toitude kasvav nõudlus, mida mõjutavad tervise-, eetilised ja keskkonnakaalutlused, on soodustanud taimsete liha- ja piimaalternatiivide väljatöötamist valgurikastest kultuuridest, nagu sojaoad, herned, põlduba ja kaer. Kuigi need toorained pakuvad nii toiteväärtuse kui ka jätkusuutlikkusega seotud eeliseid, kujutavad taimepõhiste toodete sensoorsed omadused endiselt olulist barjääri tarbijate vastuvõtlikkuse osas. Vastupidiselt tarbijate ootusele, ilmnevad paljudel taimepõhistel koostisosadel soovimatud maitseomadused, millest kibedus on eriti probleemne. Tuntud kibeduse põhjustajad on näiteks saponiinid ja vabad rasvhapped, kuid keerukates maatriksites nende kiire ja usaldusväärne analüüsimine on piiratud, mis omakorda piirab maitse optimeerimist tootearenduses.

Käesoleva doktoritöö eesmärk on välja töötada ja valideerida analüütilised meetodid kibedate ühendite kvantifitseerimiseks taimsetes valgu koostisosades ja nendest valmistatud toodetes. Töö käsitleb kolme peamist küsimust: (1) Kui suur on individuaalne varieeruvus kibeduse tajumisel kaera- ja hernejahude puhul? (2) Kuidas saab kibedaid kaera-, herne- ja sojasaponiine ning vabu rasvhappeid usaldusväärselt kvantifitseerida taimepõhistes maatriksites? (3) Millised on vabade rasvhapete, saponiinide ja avenakosiidide sisaldused toorainetes, pooltoodetes ja lõpptoodetes ning milline on nende kibeduse potentsiaal analüüsitud maatriksites?

Töö on struktureeritud viie publikatsiooni ümber. Publikatsioon I kasutab kvantitatiivset kirjeldavat sensoorset analüüsi, kasutades nii treenitud kui tarbijate paneeli, et hinnata kibedust kaera- ja hernejahudes. Üldiselt hinnati hernejahu kibedamaks kui kaerajahu, kus kibedus korreleerus tugevalt rasvhapete sisaldusega. Täheldati märkimisväärset individuaalset varieeruvust kibeduse tajumises, rõhutades vajadust analüütiliste meetodite järele kibedust põhjustavate ühendite kvantifitseerimiseks. Publikatsioonid II–V keskenduvad sojasaponiinide ja vabade rasvhapete ekstraheerimisprotseduuride väljatöötamisele, optimeerimisele ja rakendamisele kaera-, herne-, soja- ja põldoatoodetes, sealhulgas pulbrites ja vedelikes. Ekstraheerimisprotseduurid kombineeriti vedelikkromatograafia-massispektromeetriaga, et luua lihtsad, suure läbilaskevõimega ja keskkonnasõbralikumad meetodid, mis muuhulgas sobivad vedelate proovide analüüsiks ilma lüofiliseerimiseta. Vabade rasvhapete ekstraheerimist optimeeriti *design-of-experiment* abil, fokusseerides lahusti koostisele. Kõik meetodid valideeriti kehtestatud juhiste järgi.

Valideeritud meetodeid rakendati kibedate ühendite kvantifitseerimiseks pool- ja lõpptoodetes, sealhulgas taimepõhistes jookides, jogurti alternatiivides, jahudes, valgukontsentraatides ja -isolaatides. Saponiinide sisaldus oli kõrgeim valgukontsentraatides ja -isolaatides, kus sojaubadest valmistatud jogurtialternatiivides domineeris sojasaponiin Bb. Täisterakaerajahu sisaldas kõrgeimat vabade rasvhapete taset, samas kui herne- ja põldoatooted olid sisaldused madalaimad. *Dose-over-threshold* arvutused näitasid, et kaerapõhised tooted on üldiselt kibedamad, kus kibeduse peamisteks põhjustajateks olid avenakosiidid ja vabad rasvhapped C18:2 ja C18:3. Seevastu sojaubadest valmistatud toodete kibeduses mängisid sojasaponiin Bb ja vabad rasvhapped vaid vähest rolli.

Need tulemused annavad uusi teadmisi kibedate ühendite kontsentratsiooni varieeruvusest ja nende sensoorsest olulisusest töödeldud taimepõhistes toitudes. Avenakosiidide ja rasvhappe C18:2 kogused ületavad sageli maitseläveväärtusi kaerapõhistes maatriksites, eriti kaera valgu kontsentraatides ja täisterakaerajahus, mis kinnitavad antud ühendite olulisust maitse optimeerimisel. Saponiinide ja vabade rasvhapete jaotuse ning sensoorse mõju kaardistamine toorainetes ja toodetes loob aluse strateegiatele kibeduse vähendamiseks ja maitseomaduste parandamiseks.

Kokkuvõttes edendab käesolev doktoritöö nii sensoorset kui ka analüütilist arusaamist kibedusest taimsest valgust valmisatud toodetes. See kehtestab robustsed ja valideeritud meetodid kibedate saponiinide ja FFA-de kvantifitseerimiseks erinevates maatriksites ning seob need ühendid otseselt sensoorse tajuga. Sensoorse hindamise integreerimine keemilise kvantifitseerimisega võimaldab täpselt mõista, kuidas konkreetsed ühendid mõjutavad tajutavat kibedust. Tulemused pakuvad toiduainetööstusele praktilisi teadmisi, toetades maitsvamate taimepõhiste toitude väljatöötamist ja edendades jätkusuutlike valgurikaste alternatiivide turu arengut.

Table 11 (extended). *The validation parameters of developed methods: linear range, calibration curve, instrumental detection limits (IDLs), instrumental quantification limits (IQLs), as well as limit of detection (LODs), and limit of quantification (LOQs) of saponins and free fatty acids (FFAs). Data from* ***Publications II, III, and V***

Analyte	Plant origin	External standard	Internal Standard	Linear range (mg/L)	Calibration curve	R^2	IDL (µg/L)	IQL (µg/L)	LOD (mg/100g, sample code)	LOQ (mg/100g, sample code)
avenacoside A	oat	avenacoside A	^{13}C–avenacoside A	0.01–2.44	y = 0.2445x-0.0197	0.9998	4	15	0.164 (OPC) 0.128 (OD)	0.615 (OPC) 0.480 (OD)
26-desglucoavenacoside A	oat								—	—
avenacoside B	oat									
soyasaponin Bb	pea	soyasaponin Bb	soyasaponin Ba	0.01 –2.52	y = 0.7699x + 0.0048	0.9930	0.2	12	0.007 (PPI) 0.006 (PD)	0.437 (PPI) 0.359 (PD)
	soybean		asperosaponin VI						0.003 (SYA)	0.182 (SYA)
DDMP[1] saponin	pea		soyasaponin Ba						—	—
soyasaponin Ba	soybean	soyasaponin Ba	asperosaponin VI	0.02–2.26	y = 0.2949x + 0.0025	0.9975	8	33	0.125 (SYA)	0.518 (SYA)
soyasaponin Aa	soybean	soyasaponin Aa	asperosaponin VI	0.02–2.33	y = 0.3994x + 0.0021	0.9965	7	27	0.109 (SYA)	0.421 (SYA)
soyasaponin Ab	soybean	soyasaponin Ab	asperosaponin VI	0.01–2.48	y = 0.3259x + 0.0033	0.9943	1.4	25	0.022 (SYA)	0.394 (SYA)
myristic acid	all	myristic acid	^{13}C18:3	0.03–3.66	Y = 5.0041x-0.0015	0.9975	7	24	0.055 (Z)	0.188 (Z)
palmitic acid		palmitic acid	^{13}C16:0	0.05–6.51	Y = 0.3711x+0.0399	0.9880	36	121	0.284 (Z)	0.953 (Z)
stearic acid		stearic acid	^{13}C18:0	0.08–5.18	Y = 9.5017x+1.0909	0.9967	25	83	0.177 (Z)	0.588 (Z)
oleic acid		oleic acid	^{13}C18:1	0.04–5.12	Y = 0.3073x-0.0003	0.9932	6	21	0.048 (Z)	0.167 (Z)
linoleic acid		linoleic acid	^{13}C18:2	0.05–6.25	Y = 0.0337x+0.0008	0.9955	15	50	0.118 (Z)	0.392 (Z)
linolenic acid		linolenic acid	^{13}C18:3	0.05–6.53	Y = 4.2341x-0.0006	0.9943	12	39	0.092 (Z)	0.300 (Z)

[1]2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one; Samples: OPC—oat protein concentrate, OD—oat drink, PD—pea drink, PPI—pea protein isolate, SYA—soybean-based yoghurt alternative, Z—pooled sample composed of equal mix of whole grain oat flour, pea flour, faba bean protein isolate, oat protein concentrate, pea protein isolate, faba bean protein isolate; "–" not available.

Appendix 2

Publication I

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Individual differences in sensitivity to bitterness focusing on oat and pea preparations

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ABSTRACT

Growing interest in plant-based alternative products offers hope for a more sustainable future. Oat and pea are among the most potential raw materials, due to their nutritional value and their widespread growth. However, one of the key issues with the development of such products is their bitter off-taste due to ingredients like saponins, amino acids, and fatty acids. The perception of bitterness varies between compounds and depends on physiological differences among individuals, which also complicates the development of plant-based alternatives. This study aimed to investigate individual differences in bitterness sensitivity, with a particular focus on pea and oat products. Sensory evaluations (n = 12) and consumer tests (n = 100) showed that people were generally more sensitive to the bitterness of peas than oats. No statistically significant difference was observed in bitterness perception based on diet habits. Furthermore, findings demonstrated that no general standard for assessing a variety of product groups can be recommended for sensory analysis. Well-established bitter standards such as PROP, quinine, and caffeine, were not correlating with other bitter stimuli. Thus, the choice of the bitter standards to be used in sensory analysis depends on the purpose of the study and the samples included.

1. Introduction

Global issues related to environmental change have been a matter of concern for years in which the food industry holds an important role. According to a report by, "population growth of 2 billion people by 2050 will increase diet-related environmental pressures". One way to reduce the pressure is to decrease the share of crops grown for animal feed, which in turn could enhance agricultural efficiency and food availability for people (Foley et al., 2011). Since a sustainable diet involves increasing the consumption of plant-based foods (FAO and WHO, 2019), there would be greater demand for plant-based alternatives on the market. For example, the plant-based meat alternative market is estimated to increase at a compound annual growth rate (CAGR) of 7.91% from 2022 to 2027 (Mordor Intelligence, 2021b), whereas for the plant-based dairy alternative market CAGR forecast is 10.12% for the same period (Mordor Intelligence, 2021a).

Although there is a growing interest in plant-based alternatives on the market today, it is still challenging to meet consumer expectations. Consumption of alternative proteins is influenced by positive/negative emotions (Onwezen et al., 2022) or term associations (Michel et al., 2021), food neophobia (Allen, Goddard, Farmer, 2018), poor nutritional profile compared to conventional proteins (Silva et al., 2020), and accessibility in stores (Gravely and Fraser, 2018). Since taste is one of the most important drivers of food choice (Andersen et al., 2019; Boesveldt et al., 2018; De Pelsmaeker et al., 2017; Kourouniotis et al., 2016; Wedowati et al., 2020), the use of plant-based proteins can often be problematic due to their bitter aftertaste (Cosson et al., 2020; Gläser et al., 2020; Mittermeier-Kleßinger et al., 2021; Vaikma et al., 2021).

Bitterness has played an evolutionarily important role as a defense mechanism against toxin uptake (Shichida et al., 2013). Certainly, this has had a significant impact on human dietary patterns. However, bitterness does not always indicate harmfulness and may be related to other beneficial compounds as well (such as antioxidants, and phytonutrients) (Nissim et al., 2017; Shichida et al., 2013). It has been concluded that there is a variation in bitterness sensitivity between individuals and it can be related to generic genetic or specific genetic variation as well as environmental circumstances (such as diet) (Dsamou et al., 2012). The situation is further complicated by the fact that bitter compounds differ by raw material and their specific composition. Bitter off-taste in plant-based alternatives, however, can be related to various ingredients, such as glucosinolates, flavonoids, phenols, terpenoids, terpenes, mono-

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glycerides, fatty acids, peptides, and amino acids (Chigwedere et al., 2022; Gläser et al., 2020; Günther-Jordanland et al., 2016; Mittermeier-Kleßinger et al., 2021; Nissim et al., 2017; Tangyu et al., 2019).

One of the most promising raw materials for plant-based alternative producers are oats and peas. They are mainly grown in temperate regions, but can also be grown in other climates such as the Mediterranean (Canales et al., 2021; Djanaguiraman et al., 2020; Neugschwandtner et al., 2020). They also have good nutritional value, for instance, fiber and protein content (Dahl et al., 2012; Rasane et al., 2015; Sterna et al., 2016). Growing interest in pea and oat-based alternatives is also supported by the results of the Smart Protein (2021) consumer survey (n = 7578), according to which 32% of European consumers would like peas to be the main ingredient in a plant-based diet, whereas 29% would prefer oats. However, these raw plants often have issues with bitterness. Therefore, pea and oat proteins have been included in the current study together with some associated bitter compounds.

Saponins have been considered the key bitter compounds in some plant-based sources, although specific compounds may vary depending on the raw material. Pea bitterness is caused by soy saponin I and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) saponin (Heng et al., 2006) while oats have different steroidal and furostanol saponins (Günther-Jordanland et al., 2016, 2020). Avenanthramides have previously been considered as key bitter compounds in oats (Günther-Jordanland et al., 2016), although a more recent study indicates that their relation to oat bitterness is not significant (Günther-Jordanland et al., 2020). Research has shown that lipids are also contributing to the bitterness in plant-based products. A study by Gläser et al. (2021) investigated 17 pea-protein isolates and found that linoleic acid and α-linolenic acid had the highest impact on bitter taste among lipids. These fatty acids have great activeness for the bitter taste in oat flour (Günther-Jordanland et al., 2020). In addition to fatty acids, monoglycerides have a significant impact on oat bitterness (Günther-Jordanland et al., 2020), and trihydroxyoctadecenoic acids on pea bitterness (Gläser et al., 2020). Since plant proteins and their hydrolysates provide bitter peptides and amino acids (Mittermeier-Kleßinger et al., 2021), these compounds also hold an important role in the bitterness of plant-based products. Tryptophan has been found to be the most potent amino acid stimulating bitterness (Kohl et al., 2013) found widely in various foods such as soy, oat, wheat, egg, and cheese (FoodData Central, 2019; Laffitte et al., 2016).

The development of plant-based products that are pleasant for most consumers is often difficult. Not only because bitterness is perceived differently depending on the composition of the product, but also because of individual sensitivity to bitter compounds. There are approximately 25 different T2R receptors in the human gustatory system that can be activated by various bitter compounds in different concentration ranges (Roura et al., 2015; Shichida et al., 2013). As different receptors are involved in bitter stimuli, individual differences in taste thresholds of consumers affect each person's perception of bitterness. In the sensory field, reference compounds are often used to standardize the use of scales for assessing bitterness. One of the most frequently used compounds as bitter standard are caffeine (Chigwedere et al., 2022; Czepa and Hofmann, 2004; Kobue-Lekalake et al., 2012) and quinine (Caporale et al., 2004; Nath et al., 2022; Nissim et al., 2017). Among others propylthiouracil (PROP) is similarly often used to characterize individual differences in sensitivity to bitterness (Agovi et al., 2022; Cosson et al., 2020; Tepper et al., 2001). However, sensitivity to all these compounds (PROP, quinine, and caffeine) has been shown to depend on genetic variation to some extent (Hansen et al., 2006). There have also been studies showing bitterness linked to diet habits. For example, Cliceri et al. (2018) found that PROP responsiveness was lower in the vegetarian population (compared to flexitarians and omnivores) and Mozhdehi et al. (2021) demonstrated with a female sample group, that vegetarians had a lower detection threshold for caffeine (compared to omnivores and vegans).

Consumption of plant-based alternatives is often driven by sustainability motivators. Europe's Bioeconomy Strategy encourages innovation toward sustainable food production and consumption (European Commission, 2018). There are many different approaches to improving the flavor of plant protein products. Mittermeier-Kleßinger et al. (2021) provide an overview of strategies such as fermentation or hydrolysis methods, masking bitterness with other flavors or with bitter inhibitors, post-processing by marination or seasoning, choosing "pure" protein isolates or concentrates, changing the texture to induce the applicable interaction with flavor perception, etc. Before choosing a specific treatment technique, it is important to understand which compounds cause unwanted bitterness and how important this sensation is to individual sensitivity. For example, breaking down bitter molecules may lessen the health benefits of these specific compounds in some cases (Nissim et al., 2017).

The present work aimed to investigate individual differences in various bitter stimuli. Individual sensitivities were studied from the standpoint of trained assessors as well as consumers with different diet patterns. The focus was particularly on oat and pea preparations, which often have problems with bitterness despite the high market potential of these raw materials. It was hypothesized that oat and pea have greater variability in bitterness sensitivity than the well-established bitter standards (PROP, quinine, and caffeine), which underlines the importance of sensory panel selection and the use of proper bitter standards.

2. Materials and methods

2.1. Participants

Participants in the sensory evaluations were selected from a pool of highly trained evaluators. Training and monitoring of the assessors had previously been conducted according to ISO 8586:2012. Sensory tests were conducted with 12 trained panelists (average age 31 ± 6 years), who have had previous experience in evaluating plant proteins and plant-based products. Assessors were further familiarized with the samples and the methodology in preliminary tests described in chapter 2.3.

The consumer test was conducted with 100 untrained participants (average age 32 ± 10 years, 20 males and 80 females) of whom 43 were vegans/vegetarians and 57 were omnivores/flexitarians. The sample group was not balanced in terms of age or gender, as focus was only on dietary habits. Hayes et al. (2013) discuss that habits may override genetic differences in bitterness perception. Dietary groups were allocated on the assumption that omnivores and flexitarians consume conventional foods more than vegans/vegetarians who often include plant-based alternatives in their diets. Likewise, established in the previous literature (Cliceri et al., 2018; Mozhdehi et al., 2021), flexitarians and omnivores could be more responsive to bitterness compared to vegans and vegetarians.

All participants gave written consent to take part in the experiment. Participants were informed in advance of the purpose and the procedures of the study. Participants were assured of the confidentiality of their data. Taking part in the given study was voluntary and one could withdraw from the test at any time. Participants were in good health and had no known allergy to the components.

2.2. Samples

The final list of bitter stimuli (Table 1) was selected based on a focus on specific plant preparations as well as some of the well-established bitter taste standards with chemical diversity. Unrefined organic yellow pea and whole grain oat flour were used to understand the perception of bitterness in plant-based matrices. The nutritional content of pea and oat flour is given in Table A.1 in Supplementary Material. Based on the literature on the identification of bitter compounds, fatty acid standards for corresponding plants were included. Unfortunately, due to the

Table 1
Compounds evaluated in the sensory analysis.

Compound	Supplier	Concentration (g/l)	Reference	Purpose
Caffeine (99%)	Sigma-Aldrich, Burlington MA, United States	0.392	Roura et al. (2015)	Sensory analysis
Linoleic acid (95%)	Sigma-Aldrich, Burlington MA, United States	6.65	Günther-Jordanland et al. (2020)	Sensory analysis
Linolenic acid (99%)	Sigma-Aldrich, Burlington MA, United States	1.01	Günther-Jordanland et al. (2020)	Sensory analysis
L-tryptophan	Fitness Trading, Zambrów, Poland	3	Solms (1969)	Sensory analysis
Whole grain oat flour	Tammejuure Mahetalu OÜ, Tammejuure, Estonia	65	NA	Sensory analysis and consumer test
Yellow pea flour	Tammejuure Mahetalu OÜ, Tammejuure, Estonia	65	NA	Sensory analysis and consumer test
Quinine hydrochloride dihydrate (95%)	Sigma-Aldrich, Burlington MA, United States	0.0125	Roura et al. (2015)	Sensory analysis
Propylthiouracil (PROP)	Sigma-Aldrich, Burlington MA, United States	0.0544	Tepper et al. (2001)	PROP test
Sodium chloride (NaCl)	AAS Mozõrsol, Gomeli obl., Belarus	5.8	Tepper et al. (2001)	PROP test

limited availability of plant-derived organic chemicals, saponins, monoglycerides, and trihydroxy-octadecenoic acids, were not included in the study as pure standards.

A preliminary sensory test was conducted with all samples to make sure that the given concentrations can describe differences in bitterness sensitivity between assessors and samples (described in chapter 2.3). Concentrations for the compounds were selected based on previous literature (references in Table 1). For oat and pea flour, suitable concentrations were established with the preliminary tests. According to a paper by Günther-Jordanland et al. (2020), linolenic acid and linoleic acid have very different bitter threshold concentrations. To ensure comparability of the bitterness of fatty acids, the same dose-over-threshold (DoT) factor was used for both fatty acids. The chosen concentration was based on the concentration level of linolenic acid previously detected in oat flour (Günther-Jordanland et al., 2020). The final decision on the concentrations was made based on the preliminary tests when it was confirmed that all sample solutions were perceptible at the given concentrations and fully fit the scale.

The solutions for the sensory test were prepared on the same day of the experiments using potable water (Saku Läte OÜ, Estonia). Fatty acids were emulsified in the potable water using 0.1% xanthan gum (Piprapood OÜ, Estonia) solution for equal distribution. Solutions for the PROP test were prepared the day before evaluation using distilled water and were kept at +4 °C before the evaluation.

2.3. Sensory analysis

A preliminary sensory test was conducted with 9 trained panelists, in which participants evaluated the bitterness of all samples noted in chapter 2.2. Preliminary tests also included the evaluation of other basic tastes, as studies have shown that specific bitter stimuli often include astringency and/or acidity (Chalé-Rush et al., 2007; Gläser et al., 2021; Stephan and Steinhart, 2000). However, as these were not detected at all or with very low intensity, the risk of a dumping effect (Clark and Lawless, 1994) was minimal and these additional features were excluded. Both the preliminary test and the sensory analysis were conducted using nose clips as in other research (Cosson et al., 2020; Epke et al., 2009; Higgins et al., 2021; O'Mahony, 1991; Pirc et al., 2022) to minimize the effect of interfering retronasal sensations, especially the strong flavor of oat and pea samples. For example, it has been shown that bitterness in olive oil is in co-occurrence with cis-3-hexen-1-ol in the odor profile (Caporale et al., 2004) that as a compound may also be found in plant-based dairy alternatives (Vaikma et al., 2021).

All sensory analyses were carried out by 12 trained experts at the centre for Food and Fermentation Technologies (Tallinn, Estonia) in a dedicated sensory room in compliance with ISO 8589:2007. Sensory tests were conducted following ISO 6658:2017. Panelists participated in two separate sensory tests: the PROP test and the sensory analysis (see also Table 1). First, the PROP test was conducted in a session as a classical method to study bitterness status and to investigate whether it alone could explain bitterness sensitivity. One-solution PROP test was based on the study by Tepper et al. (2001) where the taste intensity of 0.32 mmol/l of PROP and 0.1 mol/l of NaCl was evaluated on a General Labeled Magnitude Scale (gLMS). Panelists were then grouped as supertasters (PROP value $\geq$ 51), medium-tasters (51 > PROP value > 15.5) and non-tasters (PROP value $\leq$ 15.1).

Secondly, samples for sensory analysis were evaluated in two parallels, in a total of four sessions. Sessions were conducted within two days. Two sessions were conducted on one day, with 45-minute breaks between. The assessments on each day were carried out at the same time of day to minimize variation in sensitivity. All sessions consisted of 5 samples and the assessment took about 10–15 min in total. The assessors were asked to evaluate the bitterness of each sample. The working linear scale was established at 0–9, where "0″ represents no stimulus, "1″ very low, "5″ medium, and "9″ very strong.

Samples were served in 30 mL transparent cups coded with three-digit random codes. Assessors were encouraged to stir the samples before testing to reduce possible precipitation in the samples (especially for sensory analysis that included flours). All the samples were at room temperature (21–22 °C) during the evaluation. Evaluations took place as a blind test. The samples for the sensory analysis were given to assessors in different order following a Williams' Latin Square design to reduce the carry-over effect (Macfie et al., 1989). Preliminary experiments showed that quinine was generally perceived as bitter by all the assessors and therefore it was used as the first sample in each sensory test to compare the different sessions with each other. Quinine is often used as a standard in other studies as well (Caporale et al., 2004; Nath et al., 2022; Nissim et al., 2017).

Data collection was done using RedJade sensory software (RedJade Sensory Solutions LLC, Martinez CA, USA). The intensity of bitterness is a temporal stimulus, the rate of which depends on the specific compound (Higgins et al., 2021; Leach and Noble, 1986). To reduce the effect of time intensity, the time for the assessment of each sample was set at 60 s after which it was possible to mark the highest perceived intensity on the evaluation sheet. Since the intensity of bitterness can also accumulate with increased exposure, such as the number of sips (Mura et al., 2018), long breaks between samples were also encouraged. Due to the ethical and safety aspects assessors were asked to spit out all the samples. It was also important to standardize the method of intake for all participants, as research shows that swallowing a sample can increase perceived bitterness (Running and Hayes, 2017). After each sample, palate-cleansing was set to 60 s until the next sample could be evaluated. Assessors were provided with spring water, water biscuits, and pears as palate cleansers.

2.4. Consumer test

The same oat and pea flours were used for the consumer test in the same concentrations as in the sensory analysis with trained panelists.

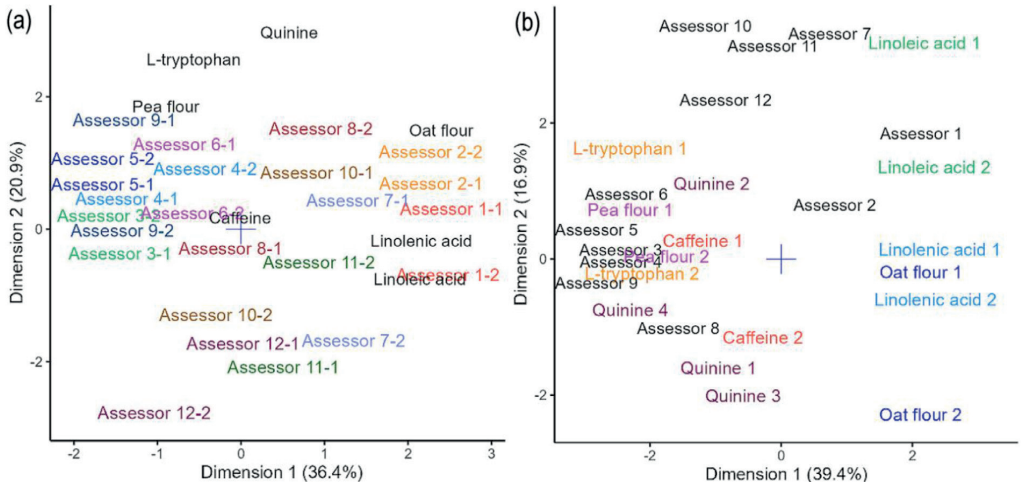

Fig. 1. PCA plot representing sensitivity to different bitter stimuli by assessors in replicate experiments (a) and by stimuli in replicate experiments (b). Numbers 1 and 2 indicate the replicate values. The numbers on the axis names indicate the variance explained by the component.

Sample preparation was done in the same way as for the sensory analysis described in chapter 2.2. The consumer tests were carried out in the same sensory room (in compliance with ISO 8589:2007) as the sensory analyses. The samples were served in plastic cups covered with a lid. Participants were instructed to shake the samples before consumption to reduce the possible precipitation. Nose clips were used during the evaluation of the samples. A scale from 0 to 9 was applied as in the sensory analysis, ranging from "none" to "very strong". The time for the assessment of each sample was set at 60 s and the participants were asked to mark the highest perceived intensity of bitterness on the scale.

2.5. Statistical analysis

Initial data analysis for sensory analysis and consumer test was conducted using MS Excel (Microsoft, Redmond WA, USA). For example, Relative standard deviation (RSD) was used to see whether the stability of different sessions for each assessor was improved by the standardization process. Similarly to other publications (Zhang et al., 2018; Zhi et al., 2016), RSD criteria for an acceptable variation was set as < 20%. PanelCheck (version 1.4.2, Nofima, Tromsø, Norway) software was used to check the performance of the panel and assessors building on the publication by Tomic et al. (2009). Panel stability and compound variation were further analyzed statistically using R version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria). Ordinations for Principal Component Analysis (PCA) were also calculated and visualized using the “prcomp” function".

Statistical analysis for the consumer test was done in R version 4.2.0 (The R Foundation for Statistical Computing, Vienna, Austria). The results were not normally distributed; thus, the Wilcoxon rank-sum test and Chi-square test of independence were used to check for statistically significant differences. Wilcoxon rank sum test was performed to see whether there would be a significant difference between the omnivore/flexitarian and vegan/vegetarian population’s sensitivity to pea and oat bitterness and whether there is a difference between the two flours. In addition, people were divided into groups based on given ratings to bitterness intensities (0–2 as low, 3–6 as medium, 7–9 as high), which was tested with Pearson’s Chi-squared. The purpose of the test was to see whether there would be statistically significant differences in the distribution of data in these bitterness groups between omnivores and vegans/vegetarians.

3. Results

3.1. Sensory analysis

3.1.1. Panel stability across sessions

It was important to minimize the possible differences coming from variations in sensitivity and scale usage across days/sessions. Since quinine was used as a reference, the results were initially standardized to the intensity of the compounds assessed in sessions against the quinine intensity for the same session. Comparing the results before and after the standardization showed that most RSD exceeded the criterion even after the standardization. Specifically, 45% of all RSD values exceeded the criterion before standardization, while this increased to 58% after standardization. It was revealed that the difference in evaluation scores across sessions was not in correlation with quinine scores, thus, the results were not standardized for further analysis. Meaning that the variations in scores were not caused by scale usage differences but instead could be related to the variations in sensitivity that might be different for each bitter stimulus.

PanelCheck was used to pre-check the performance of the panel and assessors. 3-way ANOVA confirmed that there were significant differences for bitter stimuli ($p < 0.001$), but no significant differences for the assessor effect nor replicate session effect. This was an acceptable result for this study, as the aim was not to achieve similar results, but rather to examine individual differences in the perception of bitterness. F values showed that most of the assessors were able to discriminate the samples on a significant level. Further, p*MSE (mean square error) showed that assessors were able to differentiate bitter stimuli with some variation in repeatability. The existence of large differences is supported by the explained variance shown in the PCA plots, where a total of PC1 and PC2 explained 57.3% and 56.3% of the variance (Fig. 1a and Fig. 1b accordingly). When comparing assessors (Fig. 1a), a wide variation in the sensitivity to different bitter stimuli was demonstrated. This seems to confirm that bitterness is a complex stimulus that can be perceived very differently depending on the person and the compound. Generally, the evaluation results from the parallel sessions were similar with some variation – assessors perceived bitterness largely in a similar way, regardless of the day. However, the variability of evaluation scores across replicate sessions was specific for different bitter stimuli. Pea flour had the most stable evaluation scores across sessions (Fig. 1b). The bitterness of pea flour was perceived generally with high intensity by most of

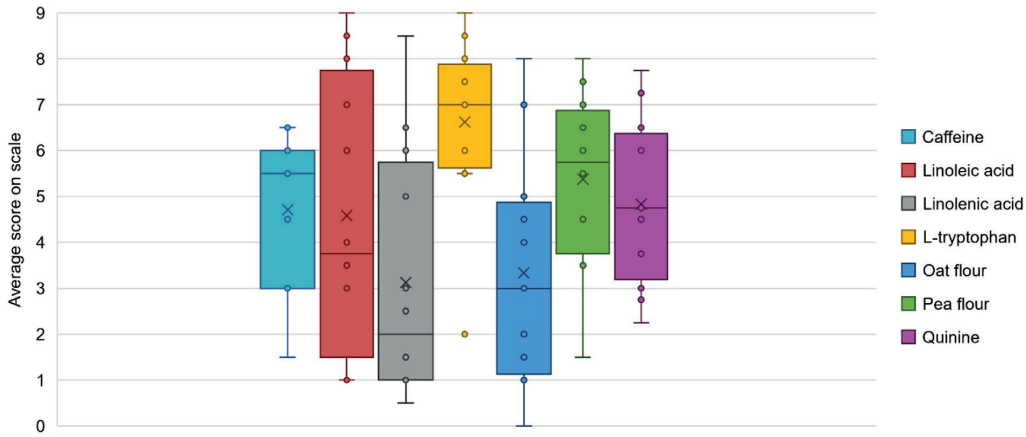

Fig. 2. Box & Whisker plot for bitterness by each stimulus. Circles and whisker endpoints indicate individual scores of assessors. The average value is marked with "X", the median is marked with a horizontal line, and whiskers indicate lower and upper quartiles.

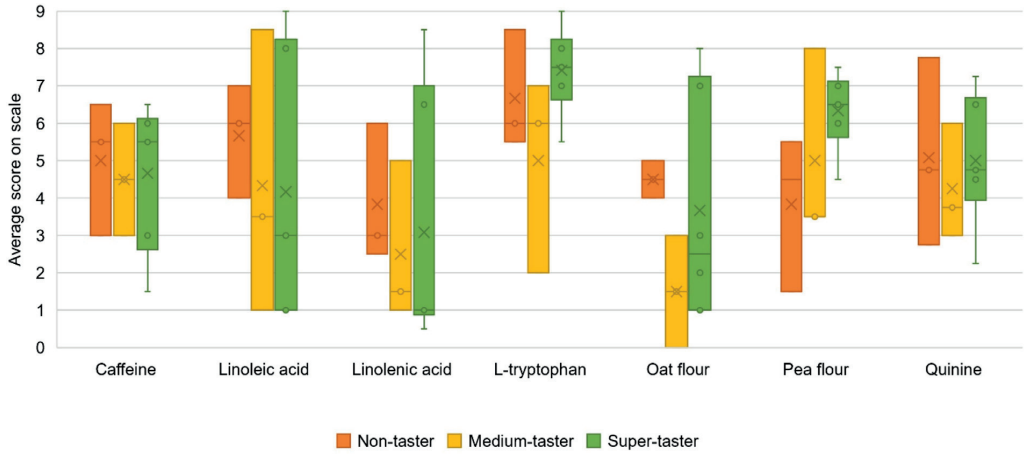

Fig. 3. Box & Whisker plot for the bitterness of compounds by PROP taster status. Circles and whisker endpoints indicate individual scores of assessors. The average value is marked with "X", the median is marked with a horizontal line, and whiskers indicate lower and upper quartiles.

the panelists. The highest variation between sessions was observed with fatty acids and oat flour.

3.2. Variation of bitterness perception for different stimuli and relationship to prop sensitivity

Fig. 2 demonstrates differences in bitterness perception by each stimulus (average scores and standard deviations for each sample and assessor can be found in Table A.2 in Supplementary Material). In general, L-tryptophan had the smallest variation of ratings among the panel, while fatty acids, oat, and pea flour had higher variations in bitterness evaluated by different assessors. This also seems to confirm the hypothesis that oat and pea bitterness has higher variability in the perception of bitterness than other well-established standards.

According to PROP test, panelists were divided into 3 groups: super-tasters (PROP value $\geq$ 51), medium-tasters (51 > PROP value > 15.5) and non-tasters (PROP value $\leq$ 15.1). The aim was to determine whether PROP taster status correlates with the sensitivity to different bitter compounds. Of the 12 panelists, 6 were categorized as super-tasters, 3 as medium tasters, and 3 as non-tasters. Fig. 3 demonstrates that there was no clear connection between compound sensitivity and PROP taster status. Only pea flour seemed to have some connection with taster status, where non-tasters evaluated bitterness as the lowest on average and super-tasters as the highest. With some compounds, the super-taster group had less variability, for instance, pea flour and L-tryptophan. Interestingly, the largest variations for super-tasters were in oat flour and fatty acid (linoleic and linolenic acid) bitterness, indicating that there may be a greater discrepancy in the bitter sensitivity of oats. Furthermore, the sensation of fatty acid bitterness was associated with an irritating mouthfeel, making it challenging to describe. Medium-tasters had the largest variations with linoleic acid, L-tryptophan, and pea flour bitterness. For non-tasters, however, quinine bitterness varied the most. Caffeine was the only bitter stimulus perceived similarly by all taster groups, as indicated by similar variability and average bitterness.

Correlations between different bitter stimuli are shown in Table A.3 in Supplementary Material. A strong association was seen between fatty acids. There was a very strong correlation (ρ = 0.95; p < 0.001) between linoleic acid and linolenic acid. Oat flour was also strongly correlated with linolenic acid (ρ = 0.61; p < 0.5) and moderately correlated (ρ = 0.48) with linoleic acid. This confirms that the fatty acids are

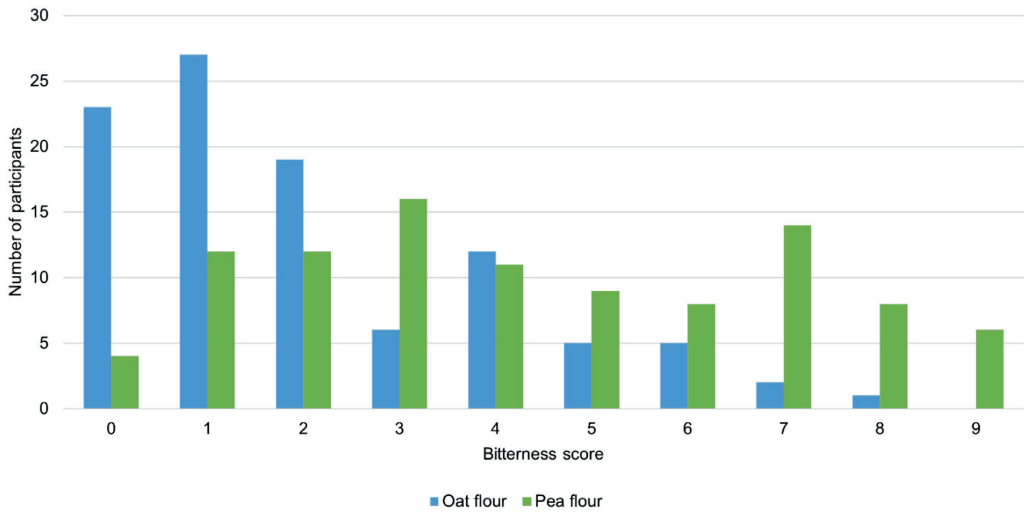

Fig. 4. Distribution of bitterness intensity ratings for pea and oat flour.

related to the bitter perception of oat. However, pea flour's bitterness showed a moderately negative correlation with the fatty acids' bitterness. This is also explained by the fact that the fat content in pea flour was considerably lower than in oat flour (Supplementary Table A.1).

3.3. Consumer test

Fig. 4 Distribution of bitterness intensity ratings for pea and oat flourshows the distribution of intensity ratings among pea and oat flour. The distribution of intensity scores for pea flour were rather uniform across the scale. For oat flour, however, a different distribution can be seen. Most participants perceived no or very low bitterness in oat flour, while only a few people rated the bitterness of oats as high intensity. Thus, oat flour was generally perceived as less bitter (average score 2.1 ± 2.0) than pea flour (average score 4.4 ± 2.6). A similar tendency was seen with trained assessors, who generally scored oat flour's bitterness (average score 3.3 ± 2.6) lower than for pea flour (average score 5.4 ± 2.0).

The distribution of oat and pea bitterness perceived by consumers is shown in Figure A.1 in Supplementary Material. Consumers were grouped into omnivores/flexitarians and vegans/vegetarians based on their dietary habits. Personal scores on the scale were categorized into three additional groups based on the level of bitterness: 0–2 as low, 3–6 as medium, and 7–9 as high. For oat samples, the distribution of the scores did not differ much based on dietary habits. There were only a few people in the omnivore/flexitarian group who were more sensitive to oat bitterness. For pea flour, however, omnivores/flexitarians had a higher proportion of participants highly sensitive to bitterness compared to vegans/vegetarians. Although more vegans/vegetarians were sensitive to the bitterness of pea flour compared to oats, most vegans/vegetarians had medium or low sensitivity to pea flour bitterness.

Wilcoxon rank sum test showed no significant difference in the average bitterness values for different diets. However, there was a significant difference between the bitterness score for pea and oat flours ($p < 0.001$). Further, people were divided into groups based on given ratings for bitterness intensities (0–2 as low, 3–6 as medium, 7–9 as high) as described in chapter 2.5. Pearson's Chi-squared test confirmed that there was no significant difference in bitterness groups for oat flour based on diet patterns. In the case of pea flour, there was a significant difference between bitterness groups ($p < 0.1$). Figure A.1 in Supplementary Material demonstrates that, overall, there were proportionally more omnivores/flexitarians who belonged to the "high" bitterness group compared to vegetarians/flexitarians. Thus, there is an indication of the effect of diet on the bitterness groups with pea products.

4. Discussion

Romano et al. (2008) discuss that there is always some scale variation, although trained panels generally have smaller variations. The present study shows that even when panelists are trained to respond similarly, there is always a possibility for differences due to specific stimulus. There may be bitter molecules that some assessors do not recognize at any level, thus they are not able to rate them on the given scale. It is important to understand whether the difference arose from inadequate training or physiological differences.

Differences in bitterness sensitivity are often investigated using PROP as a bitter standard. For example, Dinehart et al. (2006) showed in their study that the bitterness of vegetables is correlated to PROP sensitivity, whereas bitterness and sweetness of vegetables are associated together with quinine sensitivity. However, since the taste test was conducted with asparagus, brussels sprouts, and kale, these findings do not necessarily explain bitterness perception in other vegetables. Similarly, another study by Duffy et al. (2020) indicated that PROP status is associated with the bitterness of the same vegetables. As previously discussed, plant-based materials can contain various bitter compounds, as well as there are several bitter receptors for different compounds. Therefore, it is difficult to claim that one selected bitter compound can explain the bitterness of all other compounds. Since the TAS2R38 receptor is activated through PROP, research is often focused on this phenotypic variation (Nolden et al., 2020; Tepper, 2008). However, as Hayes & Keast (2011) discuss, the taster status and PROP perception are so intertwined in the scientific literature that it is difficult to change. Although the current study was also focused on plant-based products, no clear connection was shown between PROP taster status and compound sensitivities. Drewnowski and his colleagues (2007) investigated preference for 171 different food items and found that there was no significant relationship between food preference and PROP taster status. It has previously been suggested that the bitterness of different compounds can only be compared if they activate common bitter receptors (Roura et al., 2015). In our study, PROP sensitivity did not correlate with sensitivity to other stimuli either.

Different bitter stimuli varied in stability across sessions. The bitterness of fatty acids and oat flour was having the most variation across

assessors as well as sessions. Firstly, this may be because few assessors were sensitive to these stimuli and perceived them with high intensity. Secondly, the bitterness coming from fatty acids was also described as hot and irritating (Delompré et al., 2019), which may be confusing when interpreted on a bitterness scale. It could also mean that the assessors who evaluated bitterness high for these stimuli, were generally more sensitive to slightly different sensory properties of fatty acids. It must also be noted that the variation in taste threshold values of fatty acids is related to differences in the lipolytic activity of saliva (Delompré et al., 2019). A high salivary lipolytic activity generates high levels of free fatty acids in saliva, which can induce a high taste threshold for free fatty acids due to an adaptation of the taste receptors to basal salivary concentration in fatty acids (Neyraud et al., 2017). Thus, due to a correlation between fatty acid and oat bitterness, people with high sensitivity to oat flour bitterness may have been with high salivary lipolytic activity. However, the correlation between oat flour and fatty acids was expected, as earlier research has shown linolenic acid and linoleic acid to be key bitter compounds in oat (Günther-Jordanland et al., 2020). Pea flour bitterness was not correlated with fatty acids, even though α-linolenic acid and linoleic acid have been considered key bitter compounds among lipids in pea-protein isolates (Gläser et al. 2021). Although the fat content in pea flour was lower than in oat flour, the bitterness of pea flour was generally perceived as more intense. That could mean that saponins or other compounds are more influential on the bitterness of pea flour. Unfortunately, it was not possible to confirm it as the necessary standards were not available in this study.

There are some overlapping receptors (TAS2R7, TAS2R10, TAS2R14, TAS2R43, TAS2R46) for caffeine and quinine bitterness (Meyerhof et al., 2010), but the present study showed a weak negative correlation between sensitivity to these stimuli. Possibly this tendency can be related to the coffee consumption habits of the assessors. In their study, Lipchock et al. (2017) showed that the bitter taste of caffeine was influenced by coffee consumption habits, while quinine consumption is generally less frequent and played a smaller role in quinine sensitivity. It is also possible that all the mechanisms of the bitterness receptors are not yet known. For example, Meyerhof et al. (2010) identified 9 receptors for quinine bitterness, but later publications additionally indicated the existence of TAS2R31 and possibly TAS2R19 (Hayes et al., 2015). Reportedly, L-tryptophan can only bind to the TAS2R4 receptor, which can also be activated by quinine (Kohl et al., 2013). This supports the finding in the current study that L-tryptophan and quinine showed a weak positive correlation in bitterness intensity. There was also a strong positive correlation in the perception of bitterness for both fatty acids. HTC-8 cells have been shown to express both the bitter receptors TAS2R16 and TAS2R38 and the fat taste receptors CD36 and GPR120 (Brissard, 2018). Receptors CD36 and GPR120 are involved in fat taste perception (Ozdener et al., 2014), suggesting that fat and bitter taste share a common signaling mechanism. This implies that similar receptors may have been activated for both linoleic and linolenic acid, showing a strong positive correlation in the perception of bitterness.

Pea flour was generally perceived as significantly more bitter than oat flour. This trend was observed by both consumers and the trained sensory panel. There was no significant difference in the bitterness of oat flour based on dietary habits. However, the proportion of omnivorous/flexitarian consumers who perceived pea products as highly bitter (scores 7–9) was significantly higher than the proportion of vegetarian/flexitarian consumers. Furthermore, this indicates that the bitterness of pea protein products might be more of a limiting factor influencing the acceptance of plant-based products.

The findings reveal the importance of accounting for individual differences in bitter perception when selecting, training, and using sensory panels for different types of plant-based products. The same panel that is sensitive to bitterness in pea-based products may not always be a suitable panel for oat-based products. On one hand, the bitter compounds in the raw materials are generally different, but on the other hand, the concentration of the same bitter compounds is also different. As producers hope to bring well-received alternative products to the market, the use of accurate sensory methods is crucial to optimize production processes.

4.1. Limitations and future research

Due to the unavailability of food-grade saponin standards, major oat and pea saponins were not included in the study. The correlation between saponin bitterness compared to bitterness in oat and pea flour would provide information on the effect of saponins on overall bitterness. Especially, since fatty acids did not correlate with the bitterness of pea flour. As it is not known exactly how saponins affect the bitter taste at the receptor level (Shuntang, 2018), further studies should investigate this issue. The same suggestion applies to lipid compounds, only some of which were included in this study due to limited availability. In addition, previous research mentions other lipid compounds playing an important role in oat and pea bitterness. For example, monoglycerides in oat (Günther-Jordanland et al., 2020) and trihydroxyoctadecenoic acids in peas (Gläser et al. 2021).

There were some samples included in the study that were not fully soluble, specifically precipitating flours and hydrophobic fatty acids. Neyraud (2014) emphasizes that physical properties, such as solubility, can play a role in the perception of bitterness. The author explained that taste compounds must be dissolved in saliva to bind to the taste receptor. However, this does not seem to be an issue for fatty acids since Von Ebner gland protein (LCN1) found in salivary glands can transport hydrophobic fatty acids to taste receptors (Neyraud, 2014). Thus, only in the case of oat and pea flour, solubility could have influenced the perception of bitterness. However, this effect was minimized by selecting appropriate concentrations in preliminary tests and by encouraging stirring of the samples before tasting to ensure homogeneity of samples.

A sensory panel size could also be a limiting factor in this study. A sample size of 12 assessors may not be enough to draw strong conclusions regarding the correlations between PROP and other compounds. Thus, future research could use larger panels in similar studies. However, to address this shortcoming, a consumer test was carried out as a complementary experiment to test the findings of the sensory panel and to provide a larger scale.

This study involved a total of two flours, one from pea and one from oat. As there are many varieties of peas and oats, a more diverse sample could be explored to validate the results of this study. In addition to oat and pea, other raw materials could be investigated in the future. Oats and peas were included in the present study for environmental and nutritional considerations, as well as because the key bitter compounds have been studied previously. However, less is known about other potential raw materials, such as various legumes (e.g. fava beans, chickpeas, lupins), which are often used in plant-based alternatives, but their use is limited due to bitterness. Therefore, there is room for further research on bitterness in the context of plant-based alternatives.

Food liking and acceptability are not only related to bitterness but also other product characteristics. For example, odor profile has an important effect on food acceptability of plant-based alternatives. Duffy et al. (2020) demonstrated that a more intense flavor enhances the intensity of bitterness of the green vegetables tested. However, this was not the focus of this research as nose clips were used in this study. For example, cis-3-hexen-1-ol is found in various plant-based products (Caporale et al., 2004; Vaikma et al., 2021), but also can affect food preferences due to an individual's ability to detect this compound (Hayes et al., 2013). In the future, the interaction of bitterness with other product characteristics on the pleasantness of food (incl. plant-based alternatives) can be explored.

5. Conclusions

Bitterness is a complex mechanism, thus variation in bitterness perception should be considered when putting together a sensory panel and

conducting sensory tests for plant-based products. This is also demonstrated by the tendency that PROP sensitivity was not a general indicator of higher sensitivity to other bitter stimuli. Although the PROP test is often used to measure individual differences in bitterness perception, these results show that this is not always justified. Furthermore, it is debatable whether it is possible to use a general reference to bitterness, as is often done (e.g. quinine, caffeine). In the present study, it was not possible to standardize the sensory data using quinine because assessment scores for different stimuli were not in correlation with quinine scores. Variations in sensitivity varied depending on the bitter stimulus. Therefore, it seems that a more precise standard is needed for the assessment of the products made from a specific raw material. The need for different standards may already arise when comparing raw materials.

When comparing pea and oat samples, most people were sensitive to the bitterness of the pea samples. This trend was confirmed by both the sensory panel and the consumer test. There were also significantly more omnivores/flexitarians who perceived pea bitterness on a higher level than vegans/vegetarians. Fewer people perceived oat bitterness more intensely, but this was found to correlate with sensitivity to fatty acid bitterness. This implies that a high sensitivity to oat bitterness is related to individual sensitivity to fatty acids. In conclusion, developers of plant-based alternatives should take the individual differences of the general population into consideration.

Ethical statement

All participants from the sensory panel as well as the consumer panel gave written consent to take part in the experiment. Participants were informed in advance of the purpose and the procedures of the study. Participants were assured of the confidentiality of their data. Taking part in the given study was voluntary and one could withdraw from the test at any time. Participants were in good health and had no known allergy to the components. The ethical statement in the given manuscript is also included in chapter 2.1.

Declaration of Competing Interest

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.

CRediT authorship contribution statement

Helen Vaikma: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **Grete Metsoja:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Validation, Writing – original draft. **Anastassia Bljahhina:** Writing – review & editing. **Sirli Rosenvald:** Conceptualization, Supervision, Methodology, Writing – review & editing.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2022.100206.

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Appendix 3

Publication II

Bljahhina, A., Pismennõi, D., Kriščiunaite, T., Kuhtinskaja, M., Kobrin, E.-G. (2023). Quantitative Analysis of Oat (Avena sativa L.) and Pea (Pisum sativum L.) Saponins in Plant-Based Food Products by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry. Foods, 12 (5), #991.
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Article

Quantitative Analysis of Oat (*Avena sativa* L.) and Pea (*Pisum sativum* L.) Saponins in Plant-Based Food Products by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry

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Abstract: This work presents the sample extraction methods for solid and liquid sample matrices for simultaneous quantification of oat (*Avena sativa* L.) and pea (*Pisum sativum* L.) saponins: avenacoside A, avenacoside B, 26-desglucoavenacoside A, and saponin B and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) saponin, respectively. The targeted saponins were identified and quantified using a hydrophilic interaction liquid chromatography with mass spectrometric detection (HILIC-MS) method. The simple and high-throughput extraction procedure was developed for solid oat- and pea-based food samples. In addition, a very simple extraction procedure for liquid samples, without the need to use lyophilisation, was also implemented. Oat seed flour (U-^{13}C-labelled) and soyasaponin Ba were used as internal standards for avenacoside A and saponin B, respectively. Other saponins were relatively quantified based on avenacoside A and saponin B standard responses. The developed method was tested and successfully validated using oat and pea flours, protein concentrates and isolates, as well as their mixtures, and plant-based drinks. With this method, the saponins from oat- and pea-based products were separated and quantified simultaneously within 6 min. The use of respective internal standards derived from U-^{13}C-labelled oat and soyasaponin Ba ensured high accuracy and precision of the proposed method.

Keywords: oat; pea; avenacosides; saponin B; DDMP saponin; plant-based protein

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1. Introduction

The demand for sustainable protein sources in food production is continuously growing [1,2]. Oat (*Avena sativa* L.) and pea (*Pisum sativum* L.) proteins in the form of concentrates or isolates can act as an alternative to animal proteins due to their potential ability to provide desirable technological properties in plant-based meat and dairy substitutes [3,4]. Pea protein is an insufficient source of methionine but, on the other hand, has a high content of the essential amino acid lysine and branched-chain amino acids-leucine, isoleucine, and valine [4]. In contrast to pea, oat contains enough methionine but a scarce amount of lysine [3]. Blending oat and pea proteins in products is one way to achieve a complete essential amino acid profile [5], and such products are already available on the market. However, one of the main obstacles in the application of plant-based proteins in food production is their bitter and astringent off-taste [6–8]. It has been suggested that saponins might be the main cause of this sensation [9–14] influencing consumer acceptance.

Saponins are a diverse group of secondary defence metabolites widely spread in plant species [15]. Saponins investigated in this study are amphiphilic molecules, with polar water-soluble sugar moieties attached to a nonpolar, water-insoluble steroid or triterpene

core [16]. Oats, as the only cereals capable of accumulating saponins, contain bisdesmosidic steroidal saponins avenacoside A and B, and monodesmosidic 26-desglucoavenacoside A in their leaves and grains (Figure 1) [9,10,12,13,17]. Saponin B and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) saponin are monodesmosidic triterpenoid saponins found in peas [18,19]. Besides being taste-active bitter compounds, saponins have also been reported as antinutrients. As such, they may affect nutrient absorption by inhibiting metabolic and digestive enzymes [20] and by binding to minerals such as zinc and iron [21]. High concentrations of saponins in the diet may lead to hypocholesterolemic effect [22], hypoglycemia [23], inefficient protein digestion, vitamin and mineral uptake in the gut, and the development of a leaky gut [24]. Despite the reported negative nutritional impact, some studies have also shown positive cholesterol-lowering [25] and anticancerogenic [26] effects of saponins.

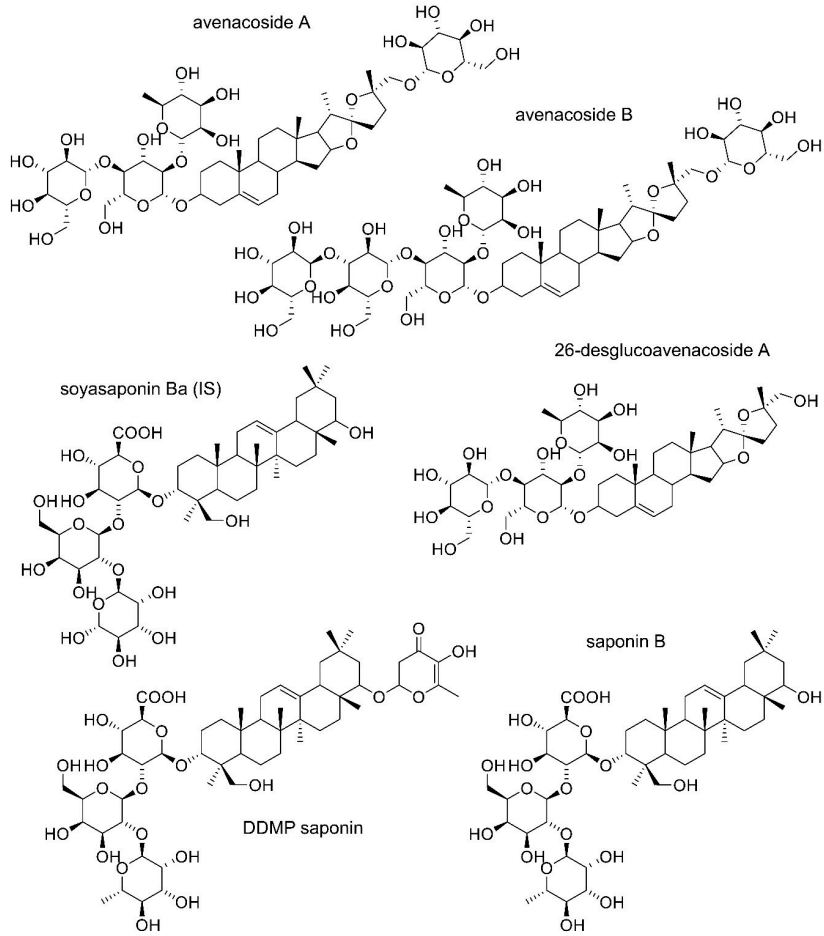

Figure 1. Chemical structures of saponins: avenacoside A, avenacoside B, and 26-desglucoavenacoside A from oat, saponin B and DDMP from pea, and soyasaponin Ba (used as internal standard [IS]).

The analysis of saponins could be performed using a wide range of classical methods such as gravimetry [15,27], hemolysis [28], bioassays [29], and spectrophotometry [30]. In addition, different saponins could be separated and analysed using chromatographic methods, e.g., thin-layer chromatography [31,32], gas chromatography [33], and high-

performance liquid chromatography (HPLC) [19]. The detection of the saponin class compounds could be carried out using the simplest optical detection methods [34,35], but these methods usually lack the selectivity and sensitivity of more advanced analytical techniques such as mass spectrometry [9,17,36–38]. The saponin extraction and the pre- and post-extraction sample clean-up before the LC analysis [9,17,19,39] are required to obtain a clean extract which would minimise matrix effect in mass spectrometric measurements. However, these sample preparation procedures are time-consuming and unsuitable for routine analysis of large amounts of samples. This creates the need for an improved, efficient, sensitive, more selective, and reproducible extraction method of saponins prior to the analysis. The use of liquid chromatography coupled with mass spectrometry (LC-MS) allows more precise and selective determination of the contents of different types of saponins in various plant species: oat [9,13,17], pea [19,32], and soya [39,40]. Although, the amounts of saponins have been quantified mainly from the seeds or husks of numerous oat and pea varieties [9,19,32], there is a lack of data concerning the concentrations of saponins in processed food ingredients, and the half- and end-products produced therefrom. To the best of our knowledge, there is no versatile method for the determination of saponins derived from different plant species in various food matrices.

The objective of this study was to develop simple sample extraction methods for solid and liquid plant-based food sample matrices for the selective and quantitative determination of five oat and pea saponins: avenacoside A, avenacoside B, 26-desglucoavenacoside A, saponin B, and DDMP saponin, using hydrophilic interaction liquid chromatography with mass spectrometric detection (HILIC-MS). To our knowledge, there are no reports on simultaneous HILIC analysis of the above-mentioned saponins in solid and liquid samples containing concurrent oat and pea ingredients.

2. Materials and Methods

2.1. Chemicals and Materials

HPLC-grade acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), hexane, propan-2-ol (IPA), and formic acid (FA) (for MS, 98%) were purchased from Honeywell (Charlotte, NC, USA). The standard compounds avenacoside A, saponin B (soyasaponin I), and soyasaponin Ba phyproof® were purchased from Sigma-Aldrich (Darmstadt, Germany). Uniformly isotopically labelled oat seed flour (U-^{13}C oat seeds, *Avena sativa* 97 atom%) was obtained from IsoLife BV (Wageningen, The Netherlands). Ultrapure water (18.2 MΩ·cm) was prepared with MilliQ® HX 7040SD equipped with MilliQ LC-Pak (Merck KGaA, Darmstadt, Germany). Biotage Isolute® PLD+ and C18 columns (100 mg/1 mL) were purchased from Biotage Sweden AB (Uppsala, Sweden). Amicon Ultra-0.5 centrifugal filter units (3, 10, 30, 50 kDa) and Millex-LCR filters (pore size 0.2 µm, filter dimension 13 mm) were obtained from Merck KgaA (Darmstadt, Germany).

2.2. Food Samples

Yellow pea flour, whole-grain oat flour, and oat and pea drinks were purchased from a local supermarket. Pea protein isolate (Bang & Bonsomer Estonia OÜ, Tallinn, Estonia), pea protein concentrate (Aloja-Starkelsen Ltd., Limbažu novads, Latvia), and oat protein concentrate (Lantmännen, Stockholm, Sweden) were obtained from producers. The composition and nutritional information available on the product label of these products is available in Supplementary Table S1. Untreated and extruded blends of pea protein isolate, oat protein concentrate, and pea protein concentrate (52:28:20, *w*/*w*) were produced in-house by following a previously published protocol [41].

2.3. Extraction Method for Solid Samples and for Liquid Samples

Sample extraction methods 1A, 1B, 2A, 2B, and 2C, which were tested during solid sample extraction method development, are described in the supplementary information.

Solid sample extraction (method 2D) was performed according to Heng et al. [19] with some modifications. Powdered non-defatted solid sample (100 mg) was weighed into a

10 mL volumetric flask (n = 3), filled with aqueous EtOH (70%, v/v), mixed thoroughly, and ultrasonicated for 30 min (without additional heating). After ultrasonication, samples were centrifuged (14,000× g for 10 min at 10 °C) to remove insoluble matter. The supernatant (500 µL) was passed through PLD+ columns by applying positive pressure to remove proteins and phospholipids. The obtained filtrate was diluted to receive an aqueous MeCN (50%, v/v) solution. The diluted filtrate (100 µL) was transferred to the LC-MS vials, mixed with 50 µL soyasaponin Ba working solution and 50 µL U-^{13}C-oat extract working solution, and injected into the LC-MS.

A homogeneous liquid sample was weighed (0.25 g) into a 5 mL volumetric flask (n = 3), filled with ultrapure water, and mixed thoroughly. Diluted sample solutions were centrifuged (14,000× g for 15 min at 10 °C) to remove insoluble matter. Sample supernatant (200 µL) and 800 µL MeCN were transferred into the next tube, mixed thoroughly, and centrifuged (14,000× g for 15 min at 10 °C) to remove precipitated proteins. The supernatant (500 µL) was passed through PLD+ columns. The obtained filtrate (300 µL) was transferred into a clear tube and diluted with 180 µL ultrapure water to obtain an aqueous MeCN solution (50%, v/v). The diluted sample filtrate was combined with internal standard solutions as described for solid samples and injected into the LC-MS.

2.4. Preparation of Standard Solutions

The stock solution of avenacoside A (500 mg/L) was prepared in ultrapure water and the aliquots were stored at −80 °C. The stock solution of saponin B (500 mg/L) was prepared in aqueous EtOH (60%, v/v) and aliquots were stored at −80 °C. The internal standard stock solution of soyasaponin Ba (100 mg/L) was prepared in MeOH.

The stock solution of U-^{13}C-oat seed flour extract containing $^{13}C_{51}$-avenacoside A was prepared using the previously described solid sample extraction method 2D with some modifications. U-^{13}C-oat seed flour (150 mg) was weighed into a 50 mL volumetric flask, filled with EtOH (70%, v/v), and mixed thoroughly. The flask was ultrasonicated for 30 min (without additional heating) and the obtained solution was centrifuged (17,000× g for 10 min at 10 °C) to remove insoluble matter. The supernatant was passed through PLD+ columns using a vacuum manifold. The cleaned extract was aliquoted and stored at −80 °C.

The internal standard working solutions were prepared freshly before the analysis. The working solution of internal standard soyasaponin Ba was prepared by diluting stock solution in the aqueous MeCN (50%, v/v). The U-^{13}C-oat extract working solution was prepared by diluting the stock solution two-fold with neat MeCN.

2.5. Liquid Chromatography Mass Spectrometry

Samples were analysed using a Waters UPLC® system (Waters Corporation, Milford, MA, USA) coupled with a Waters Quattro Premier XE Mass Spectrometer equipped with ZSpray™ Source and controlled by Waters MassLynx™ 4.1 (V4.1 SCN805, Waters Corporation, Milford, MA, USA). Mobile phases were as follows: (A) 0.1% FA in ultrapure water, (B) 0.1% FA in MeCN. Weak needle wash was composed of MeCN in ultrapure water (90%, v/v), and strong needle wash consisted of IPA in MeCN (50%, v/v). The seal wash solution was aqueous MeCN (50%, v/v). Samples were stored in an autosampler which was set at 8 °C. The injection volume was 2 µL. Saponins were separated using BEH Amide column (1.0 × 50 mm, 1.7 µm) coupled with BEH Amide VanGuard Pre-column (2.1 × 5 mm) from Waters Corporation (Milford, MA, USA). The final gradient was as follows: 0–0.17 min at 10% A, 0.17–3.5 min linear gradient 10–70% A, 3.5–4.0 min at 70% A, 4.0–4.5 min linear gradient 70–10% A, 4.5–6.0 min at 10% A. The column temperature was held at 50 °C during all experiments. The flow rate was set at 200 µL/min.

The analytes were ionised under negative electrospray ionisation (ESI-) and optimised source conditions. The source temperature was set to 120 °C, and high-purity nitrogen was fed into the source at 25 L/h (cone) and 600 L/h (desolvation) and desolvation gas was heated to 350 °C. The capillary voltage was set to −1.5 kV, cone voltage to 80 V, and extractor

voltage to 3 V. For measurement of analytes, a set of m/z values for single-ion-recording (SIR) experiments was recorded simultaneously during one chromatographic run. For saponin quantification, deprotonated molecules [M-H]- were chosen based on a scan-type experiment. Mass-to-charge ratios ($m/z \pm 0.5$ Da) for SIR channels were set as follows: avenacoside A—m/z 1061.5; avenacoside B—m/z 1223; 26-desglucoavenacoside A—m/z 899.5; $^{13}C_{51}$-avenacoside A—m/z 1112.5 (internal standard); saponin B—m/z 941.5; DDMP saponin—m/z 1067; soyasaponin Ba—m/z 957.5 (internal standard). Data acquisition was performed in Waters MassLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA). Data analysis was performed in Waters QuanLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA) and Microsoft Excel® (Microsoft 365 Apps for enterprise).

2.6. Calibration and Quantification

The working solution was prepared by diluting standard stock solutions 100 times with MeCN:H_2O:EtOH solution (50:36:14, v/v). Internal standards, soyasaponin Ba and U-^{13}C-oat extract, were added before injection to the autosampler vial, and their concentration in the vial was set at 0.75 mg/L and 0.3 mg/L, respectively. Calibration curve standard solutions (100 µL) were mixed with internal standards working solutions (50 µL U-^{13}C-oat extract working solution and 50 µL soyasaponin Ba working solution). Calibration curves were built for avenacoside A (0.01–2.44 mg/L) and saponin B (0.01–2.48 mg/L) using eight-point measurements of serially diluted standards, which were run in triplicate. The regression was found by fitting points to the linear equation. The external standard calibration curves were built by correlating the concentrations of external standards to the response factors, which were calculated according to Equation (1).

$$\text{response factor (RF)} = (\text{area of analyte})/(\text{area of internal standard}) \quad (1)$$

As only the avenacoside A standard was commercially available, other analytes of interest (avenacoside B and 26-desglucoavenacoside A) were quantified relatively using the avenacoside A calibration curve. Avenacoside B and 26-desglucoavenacoside A results are presented in avenacoside A equivalents. Avenacosides were quantified using isotopically labelled ^{13}C-avenacoside A as an internal standard. As DDMP saponin could not be sourced commercially, its quantification was based on the saponin B standard curve, and the results are given in saponin B equivalents. Both were quantified using soyasaponin Ba as an internal standard.

2.7. Validation of the Method

The following parameters were assessed during method validation: linearity, limit of detection (LOD), limit of quantification (LOQ), precision, specificity, sample extraction recoveries, and matrix effect (ME). Developed extraction methods for solid and liquid samples were validated separately. Oat protein concentrate and pea protein isolate were used to validate the solid sample extraction method. Saponin determination in liquid samples was validated using oat and pea drinks.

The linear range and linearity were evaluated via repeated measurements of standard solutions of avenacoside A and saponin B consisting of 8 individual points obtained from serial dilution of stock solutions. For the calculation of LOD and LOQ values for avenacoside A and saponin B compounds, the standard deviation (SD), obtained by analysing the peak areas of the lowest standard concentration point, was multiplied by three or ten, respectively [42].

To determine the intra-day precision of the instrumental method, oat protein concentrate and pea protein isolate extracts containing all analytes and internal standards were injected six times, and for inter-day precision, sample extracts were studied across three independent days to confirm the stability of the retention times and peak areas. The precision of the extraction methods was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was carried out by performing six repeated analyses of the samples on the same day, while the intermediate precision of the method was assessed

using samples that were analysed on three different days over two months under the same experimental conditions.

The total recoveries for avenacoside A and saponin B were evaluated by spiking the solid and liquid samples with a known amount of avenacoside A and saponin B at three different concentration levels. For estimation of solid sample extraction method recovery, oat protein concentrate and pea protein isolate (100 mg) were weighed into a 10 mL volumetric flask (n = 3). Aliquots of avenacoside A and saponin B standard solutions (10 mL) at three different concentrations were prepared in aqueous EtOH (70%, v/v) separately. These solutions were added to oat protein concentrate and pea protein isolate, mixed thoroughly and subjected to the solid sample extraction method as described above. The recoveries of avenacoside A and saponin B in oat and pea liquid samples were determined by cross-matrix spiking both sample matrices. For estimation of liquid sample extraction method recovery, separate standard stock solutions of avenacoside A and saponin B were prepared (200 mg/L). These solutions were added in different volumes to 0.25 g of liquid sample (oat and pea drink) (n = 3) weighed into a 5 mL volumetric flask, mixed thoroughly, and subjected to the liquid sample extraction method as described above. The total recovery was calculated using Equation (2) [43],

$$\text{total recovery (\%)} = (C_{\text{spiked}}/(C_{\text{unspiked}} + C_{\text{spike}})) \times 100\% \quad (2)$$

where C_{spiked} is the amount of saponin determined in the spiked sample, C_{unspiked} is the amount of saponin in the unspiked sample, and C_{spike} is the amount of saponins at three different concentration levels.

ME as one of the most problematic issues in LC-MS was evaluated for all four sample matrices (oat protein concentrate and pea protein isolate and plant-based drinks) by post-extraction sample spiking with calibration curve standard solutions, then constructing a calibration curve based on response factors and spiked standard concentrations, and comparing the matrix-matched calibration curve slope with the calibration curve slope in solvent (Equation (3)) [42]

$$\text{ME (\%)} = \text{slope}_{\text{matrix-matched}}/\text{slope}_{\text{solvent}} \times 100\%. \quad (3)$$

Statistical analysis was carried out using Excel® (Microsoft® 365 for enterprise). The results are presented as mean ± SD or relative standard deviation (RSD).

3. Results and Discussion

3.1. Development of Liquid Chromatography Method

The HPLC method was developed and assessed by analysing external standards and compounds available in oat and pea sample matrices. During development of the liquid chromatography method, two types of stationary phase chemistry were tested (C18 and HILIC) as well as different column dimensions. The best separation performance in terms of time of analysis, selectivity, and efficiency was achieved by the BEH Amide column (1.0 × 50 mm, 1.7 µm). Based on the literature [9,19] and scan-type experiments of oat flour and pea flour sample extracts, m/z values for SIR channels were chosen for the detection and relative quantification of targeted compounds without existing standard compounds in these sample matrices. Avenacoside B and 26-desglucoavenacoside A were found to be present in the oat sample matrix in addition to avenacoside A. DDMP saponin also occurred in the pea sample matrix besides saponin B. MRM experiments were conducted during development of a methodology but we have found that the MRM approach did not bring any more selectivity but significantly reduced sensitivity by not producing consistent fragments. The example of a chromatogram obtained by injecting the oat and pea flour extracts is shown in Supplementary Figure S1.

3.2. Development of Sample Extraction Methods

Two previously published extraction methods (avenacosides in grain and husks of oats [9] and saponins in peas [19]) were the starting points for the development of a method for simultaneous saponin extraction from oat and pea matrices. As both extraction methods were time-consuming, a more efficient sample preparation was proposed for saponin quantification. All samples were analysed using LC-MS method described in the Materials and Methods section.

Table 1 shows the main steps of extraction methods and saponin extraction yields obtained by reference methods (1A and 2A) and modified methods (1B, 2B, 2C, and 2D). To demonstrate the efficiency of the optimized methods, oat protein concentrate and pea protein isolate were analysed in duplicate.

Since both reference methods [9,19] started by fat elimination, defatted oat protein concentrate (fat 18.9%) and pea protein isolate (fat 4.7%) were extracted using methods 1A, 1B, 2A, and 2B. The oat protein concentrate extracted using method 1B gave 37% higher avenacoside A concentration compared to method 1A, and method 2B resulted in 50% higher yield than method 2A. Overall, the highest avenacoside A content in oat protein concentrate was achieved using extraction method 2B. Using method 1B, the pea protein isolate gave two times higher saponin B yield than using extraction method 1A, and method 2B gave a 76% higher yield than method 2A. Thus, the highest saponin B amount from pea protein isolate was extracted using method 2B. Although both improved methods 1B and 2B gave similar saponin yields in analysed matrices, it was decided to proceed with more process-efficient method 2B, as method 1B utilizing two-step methanol reflux extraction is very time-consuming.

The necessity for fat removal before saponin extraction from the matrix was determined. For this, saponins from four samples (oat flour and protein concentrate and pea flour and protein isolate) were extracted using extraction methods 2B and 2C, and lastly, the extracts were filtered through different filtering devices (the molecular weight cut-off filters with different membrane pore sizes (3, 10, 30, and 50 kDa), 0.2 µm syringe filter, and ISOLUTE® PLD+ Protein and Phospholipid Removal columns) before the LC-MS analysis. The results of this experiment are shown in Supplementary Figure S2.

No significant differences in avenacoside A, avenacoside B, saponin B, and DDMP saponin content were determined in Soxhlet-defatted and non-defatted oat and pea matrices. On the other hand, different molecular cut-off sizes had a significant impact on the recovery of saponins. The 3 kDa and 10 kDa cut-off filters showed inferior performance irrespective of the sample matrix and saponin type determined. The maximum recovery of analytes in the samples was achieved using 50 kDa and in some cases 30 kDa cut-off devices. In all sample matrices except oat protein concentrate, the application of PLD+ columns and syringe filters gave even better results than 30 kDa or 50 kDa cut-off filters. Although the PLD+ and 0.2 µm syringe filters gave quite similar analyte recovery, the application of PLD+ columns resulted in clearer MS chromatograms with a minimum number of interfering peaks in the chromatogram baseline. Moreover, filtering through the PLD+ column enables an easy transition of the procedure to a high-throughput workflow in the case of using 96-well PLD+ plates. The ISOLUTE® PLD+ proprietary multifunctional sorbent phase is optimised to selectively retain proteins and phospholipids [44]. The results indicated that pre-extraction fat removal is not necessary before saponin extraction and could be omitted and the application of PLD+ columns is the best solution for post-extraction clean-up of sample extracts. This resulted in a modified method 2C (described in Table 1).

Table 1. The extraction steps of reference methods 1A and 2A [9,19], their modified versions (1B, 2B, 2C, and 2D), and saponin yields obtained using these methods [a].

	Sample Extraction 1A	**Modified Sample Extraction 1B**	**Sample Extraction 2A**	**Modified Sample Extraction 2B**	**Modified Sample Extraction 2C**	**Modified Sample Extraction 2D**
Defatted sample	yes	yes	yes	yes	no	no
Sample and solvent amount (10 g/L)	0.5 g, 25 mL × 2 MeOH	0.5 g, 25 mL × 2 MeOH	0.5 g, 50 mL EtOH (70%, *v*/*v*)	0.1 g, 10 mL EtOH (70%, *v*/*v*)	0.1 g, 10 mL EtOH (70%, *v*/*v*)	0.1 g, 10 mL EtOH (70%, *v*/*v*)
Extraction	2-step reflux at boiling point	2-step reflux at boiling point	1 h at 25 °C in a shaking incubator	1 h at 25 °C in a shaking incubator	1 h at 25 °C in a shaking incubator	30 min ultrasonic bath
Clean-up	decant	centrifuge (17,000× *g* × 10 min at 10 °C)	ashless filter paper	centrifuge (17,000× *g* × 10 min at 10 °C)	centrifuge (14,000× *g* × 10 min at 10 °C)	centrifuge (14,000× *g* × 10 min at 10 °C)
Solvent evaporation	vacuum rotary evaporator	-	vacuum rotary evaporator	-	-	-
Resuspended	an aqueous MeOH (5%, *v*/*v*)	-	-	-	-	-
Centrifuge	17,000× *g* × 10 min at 10 °C	-	17,000× *g* × 10 min at 10 °C	-	-	-
Sample clean-up and concentration	SPE C18	-	SPE C18	-	-	-
Solvent evaporation	N_2 flow	-	N_2 flow	-	-	-
Filtering	-	-	-	-	PLD+ column	PLD+ column
Reconstitution/dilution in an aqueous MeCN (50%, *v*/*v*)	yes	yes	yes	yes	yes	yes
Obtained results (mg/100 g ± SD)						
Avenacoside A [b]	19 ± 2 [c]	26 ± 1 [c]	18 ± 3 [c]	37 ± 2 [c]	36 ± 3	37 ± 2
Saponin B [d]	100 ± 3 [c]	214 ± 5 [c]	124 ± 10 [c]	219 ± 8 [c]	229 ± 22	240 ± 19

[a] Detailed description of extraction methods 1A, 1B, 2A, 2B, and 2C is available in the supplementary information. Each result represents mean ± SD (n = 2). [b] Measured in oat protein concentrate. [c] Result is presented on fat-containing sample. [d] Measured in pea protein isolate.

The influence of ultrasonic power on the saponin extraction yields was also investigated. Saponins from oat protein concentrate, pea protein isolate, and oat and pea flours were extracted using methods 2C and 2D (results are shown in Supplementary Table S2). The results showed that ultrasonication did not have a statistically significant effect on saponin yield but considering the extraction time the application of ultrasonication is preferable. It should be noted that heating taking place during sonication had no effect on the analytes. During this experiment, the ultrasonic bath heated itself from ambient temperature (23 °C) to 40 °C in 30 min. Previous research has shown that the exposure of DDMP saponin to a temperature higher than 40 °C has a profound effect on its degradation into saponin B [18]. However, in another study, it was reported that the pure DDMP saponin in methanolic solution started to decrease in concentration when heated at 65 °C [45].

Based on the obtained results and considering the extraction time and yield, method 2D was utilized for analysis and validation of all solid samples.

Liquid food samples were analysed without the need to use lyophilisation before the sample extraction. The sample preparation method was based only on the application of ISOLUTE® PLD+ cartridges for sample extract purification before LC-MS analysis, previously chosen as the most efficient for cleaning the extracts of the solid samples.

3.3. Validation of the Method

When the chromatographic methods and sample extraction methods were developed, validation was performed to evaluate the linear range, LODs and LOQs, precision, recoveries, and matrix effect of the proposed method. The linearity of response and other calibration parameters for avenacoside A and saponin B are presented in Table 2. Linearity for these two saponin standards was obtained in the concentration range of 0.01–2.5 mg/L. The LOQs were estimated from the lowest point of the calibration curve ranging from 0.015 mg/L for avenacoside A and 0.014 mg/L for saponin B. The obtained LOQ results were lower than or in accordance with previous research [9,13,17,39].

Table 2. The linear range, calibration curve, limits of detection (LODs), and limits of quantification (LOQs) of avenacoside A and saponin B.

Analyte	Linear Range (mg/L)	Calibration Curve [1]	R^2	LOD (mg/L)	LOQ (mg/L)
avenacoside A	0.01–2.44	y = 0.2445x − 0.0197	0.9998	0.004	0.015
saponin B	0.01–2.48	y = 0.6350x − 0.0059	0.9999	0.004	0.014

[1] Calibration curve: y = ax + b, where x is the response factor and y is the concentration in mg/L.

After linearity was found to be acceptable for avenacoside A and saponin B, the repeatability of the method was appraised. Repeatability of retention times and peak areas were studied first with six replicate injections of oat protein concentrate and pea protein isolate extract. Table 3 shows the repeatability of retention times, peak areas, and the precision of solid and liquid sample extraction methods. RSDs of peak areas for all saponins did not exceed 6%. Intra- and inter-day RSDs were at a similar level, indicating that the methods are reproducible to an acceptable extent for the routine analysis of oat and pea products. Intra-day and inter-day RSDs were determined by extracting oat protein concentrate, pea protein isolate, and plant-based drinks on different days. The RSD of the intra-day precision ranged from 6 to 13% and inter-day precision from 7 to 11% in powdered oat and pea samples. For oat and pea plant-based drinks, the intra-day precision ranged from 3 to 12% and inter-day precision from 7 to 16%. The precisions for the DDMP saponin pea drink were not evaluable despite multiple measurements (DDMP saponin content in this sample was <LOQ).

Table 3. Repeatability of retention times (RT) and peak areas of saponins, and precision of the whole method.

Analyte	*m/z*	RT, min	RT RSD (%)		Peak Area RSD (%)		Precision RSD (%)			
							Powdered Samples		Plant-Based Drinks	
			Intra-Day (*n* = 6)	Inter-Day (*n* = 18)	Intra-day (*n* = 6)	Inter-Day (*n* = 18)	Intra-Day (*n* = 6)	Inter-Day (*n* = 9)	Intra-Day (*n* = 6)	Inter-Day (*n* = 9)
Avenacoside A	1061.5	1.78	0.23 [a]	0.48 [a]	1.8 [a]	3.0 [a]	11	11	12	12
Avenacoside B	1223	1.93	0.20 [a]	0.27 [a]	4.1 [a]	4.5 [a]	13	9	3	8
26-desglucoavenacoside A	899.5	1.54	0.36 [a]	0.56 [a]	3.8 [a]	6.0 [a]	6	7	10	16
^{13}C-avenacoside A [b]	1112.5	1.78	0.28	0.54	2.9	4.2	n.i.	n.i.	n.i.	n.i.
Saponin B	941.5	1.42	0.29 [c]	0.98 [c]	2.6 [c]	3.1 [c]	6	7	6	7
DDMP saponin	1067	1.40	0.23 [c]	0.86 [c]	3.9 [c]	6.3 [c]	8	11	n.a.	n.a.
Soyasaponin Ba [d]	957.5	1.48	0.30	0.68	2.5	3.0	n.i.	n.i.	n.i.	n.i.

[a] Measured in oat protein concentrate. [b] Internal standard for avenacoside A, avenacoside B, and 26-desglucoavenacoside A. n.i. Not implemented, no RSD calculable. [c] Measured in pea protein isolate. n.a. Not available in this matrix. [d] Internal standard for saponin B and DDMP saponin.

The recoveries were determined in oat protein concentrate and pea protein isolate powder by spiking the oat matrix with avenacoside A and the pea matrix with saponin B. The recovery of analytes in the case of the liquid sample extraction method was investigated separately. Table 4 shows the recovery results of powdered and liquid samples. The recoveries of avenacoside A and saponin B ranged from 90 to 115% and from 82 to 100% in oat protein concentrate and pea protein isolate, respectively. In the oat drink, the recovery of avenacoside A ranged from 96 to 113% and saponin B from 98 to 113%. In the pea drink matrix, the recoveries of avenacoside A and saponin B were from 94 to 106% and from 89 to 98%, respectively. According to validation guidelines, the acceptable recovery range for this method should be in the range of 80 to 110% [46]. Thus, the mean values of obtained recoveries were acceptable for both matrices. The recovery results obtained with the current procedure were similar to ones reported for previously proposed methods [9,13].

Table 4. Recoveries of saponins in solid (oat protein concentrate and pea protein isolate) and liquid samples (oat drink and pea drink) [1].

	Spiking Level	Spike (mg/L)	Recovery (%)	Spike (mg/L)	Recovery (%)	Recovery (%)
			Oat Protein Concentrate [2]		Oat Drink [3]	Pea Drink [4]
Avenacoside A	L1	1.2	115 ± 7	0.24	104 ± 6	106 ± 1
	L2	0.9	90 ± 7	0.12	113 ± 7	99 ± 3
	L3	0.4	107 ± 12	0.06	96 ± 13	94 ± 1
			Pea protein isolate [5]		Oat drink [4]	Pea drink [6]
Saponin B	L1	1.1	82 ± 2	0.23	105 ± 2	98 ± 5
	L2	0.5	100 ± 1	0.11	113 ± 8	89 ± 2
	L3	0.3	96 ± 10	0.06	98 ± 15	93 ± 6

[1] Each result represents mean ± SD (n = 3). [2] Unspiked matrix initial analyte concentration 0.70 mg/L. [3] Unspiked matrix initial analyte concentration 0.45 mg/L. [4] Analyte-free sample matrix. [5] Unspiked matrix initial analyte concentration 0.55 mg/L. [6] Unspiked matrix initial analyte concentration 0.15 mg/L.

Oat protein concentrate ME on avenacoside A was 100%, and pea protein isolate ME on saponin B was 110%. Avenacoside A and saponin B ME were 107% and 105% in the oat drink and 105% and 102% in the pea drink, respectively. All measured ME were in the optimal range between 90 and 110% [47].

The stock solution of U-^{13}C-oat seed flour extract was analysed for purity. The unlabelled avenacosides were not detected; thus, isotopically labelled avenacoside A was regarded as fully labelled. The working solution of ^{13}C-oat flour was added into the LC-MS vial before the analysis to assess the quantity of analytes and take into account ME. Moreover, recovery experiments confirmed that the method could be used even with internal standards added post-extraction.

Overall, the method has demonstrated acceptable validation performance in terms of recovery, sensitivity, specificity, and precision, and could be characterised as robust and effective and could potentially be applied in a high-throughput environment. Thus, the developed sample extraction method and the LC-MS method are suitable tools for the analysis of oat and pea saponins in different matrices, e.g., flours, protein concentrates and isolates, mixed matrices, and liquid plant-based drinks.

3.4. Determined Concentrations of Saponins in Food Ingredients, Half- and End-Products

High sensitivity and reproducibility as well as very short analysis time make the developed method suitable for routine quality analysis of oat- and pea-based food ingredients

and foods, as well as products containing oat and pea components. The results of saponin contents in various samples are shown in Table 5.

Table 5. Contents of saponins in different food samples [1].

	mg/100 g				
Sample	**Avenacoside A**	**Avenacoside B** [2]	**26-desglucoavenacoside A** [2]	**Saponin B**	**DDMP Saponin** [3]
Oat protein concentrate	42.3 ± 3.0	33.8 ± 0.7	5.1 ± 0.2	n.a.	n.a.
Whole-grain oat flour	23.4 ± 2.9	14.0 ± 1.5	<LOQ	n.a.	n.a.
Oat drink	4.6 ± 0.1	2.7 ± 0.2	<LOQ	n.a.	n.a.
Pea protein isolate	n.a.	n.a.	n.a.	243.8 ± 6.2	10.8 ± 0.7
Pea protein concentrate	n.a.	n.a.	n.a.	20.3 ± 1.6	107.6 ± 4.1
Pea flour	n.a.	n.a.	n.a.	6.2 ± 0.4	61.1 ± 2.0
Pea drink	n.a.	n.a.	n.a.	3.5 ± 0.2	<LOQ
Blend [4]	13.5 ± 1.0	10.9 ± 0.3	1.3 ± 0.3	123.9 ± 6.3	27.1 ± 3.5
Extruded blend [4]	10.6 ± 0.3	9.6 ± 0.9	1.1 ± 0.9	132.9 ± 12.4	11.4 ± 0.8

[1] Each result represents mean ± SD (n = 3). [2] Equivalent of avenacoside A. [3] Equivalent of saponin B. n.a. Not available in this sample matrix. [4] Blend: 52% pea protein isolate, 28% oat protein concentrate, and 20% pea protein concentrate.

In whole-grain oat flour, the contents of avenacoside A, avenacoside B, and 26-desglucoavenacoside A were 23.4 ± 2.9 mg/100 g, 14.0 ± 1.5 mg/100 g, and below LOQ, respectively. According to previous research, the concentrations of avenacosides and their ratios are different and depend largely on the variety of oats [9]. According to the latter study, the average avenacoside A content in oat grain in 16 analysed varieties was 36 ± 8 mg/100 g, avenacoside B content was in the range of 30 ± 4 mg/100 g, and 26-desglucoavenacoside A was 2.4 ± 0.8 mg/100 g [9]. Indeed, the contents of avenacoside A differed up to two-fold depending on the variety, and the ratios of avenacoside A to avenacoside B varied from 0.9 to 1.7 [9]. According to Günther-Jordanland et al. (2020), avenacoside A and avenacoside B content in oat flour has been reported to be 24.6 mg/100 g and 21.9 mg/100 g, respectively [13]. Thus, the concentration of avenacosides in the whole-grain oat flour determined in the present study is in a good correspondence with the results reported before [9,13]. In oat protein concentrate (53% protein; Table S1), avenacoside A content was 42.3 ± 3.0 mg/100 g, avenacoside B was 33.8 ± 0.7 mg/100 g, and 26-desglucoavenacoside A was 5.1 ± 0.2 mg/100 g. According to specification (Table S1), this product was manufactured from oat bran. Previous research has shown that the average content of avenacoside A and avenacoside B in three analysed oat bran products was 26 ± 7 mg/100 g and 8 ± 2 mg/100 g, respectively [17], which is similar to concentrations determined in the whole-grain flour in the current study. Thus, the increased content of avenacosides in oat protein concentrate should be ascribed to the partial concentration of the oat saponins together with the protein fraction during the production process of oat protein concentrate. In an oat drink, avenacoside A content was 4.6 ± 0.1 mg/100 g, avenacoside B was 2.7 ± 0.2 mg/100 g, and 26-desglucoavenacoside A was below LOQ. As it was a commercial liquid product with low dry matter content, it resulted in an apparently lower content of measured saponins. Nevertheless, according to specification (Table S1), the product contains only 1% of protein and the oat base is the only protein source in the oat drink. In this respect, considering the oat drink and, e.g., the whole-grain oat flour (12.5% of protein), the ratio of avenacosides to protein is much higher in the oat drink. One can suppose the considerable migration of saponins into the liquid phase when soaking the oats during the initial step of oat drink manufacture.

In pea flour (17.9% protein; Table S1), saponin B content was 6.2 ± 0.4 mg/100 g and relatively quantified DDMP saponin content was 61.1 ± 2.0 mg/100 g. In fact, our findings are inconsistent with the results of Reim and Rohn (2015), who analysed saponin B and DDMP saponin contents in hulls and peas in six different pea varieties using the HPTLC method [32]. They reported that saponin content in peeled peas was 10 to 40 mg/100 g of saponin B and 0 to 20 mg/100 g of DDMP saponin depending on pea variety [32]. Nonetheless, the present findings of high DDMP content in pea flour are comparable with

the results of Heng et al. (2006): the DDMP saponin content varied from 70 to 150 mg/100 g DM, whereas saponin B varied from 0 to 40 mg/100 g DM [19]. Our results confirm that the DDMP saponin is the predominant naturally occurring saponin present in pea. The high level of DDMP saponin in pea flour was observed in the current study most likely because it has not been thermally treated and DDMP saponin has not been converted into saponin B. In pea protein concentrate (46.9% protein; Table S1), the saponin B content was 80.3 ± 1.6 mg/100 g and DDMP content was 107.6 ± 4.1 mg/100 g. Saponins are found in the cotyledons and are often associated with the protein bodies of legumes [4]. Therefore, saponin accumulation in pea concentrate produced by dry milling and air classification is evident [4], which is in accordance with at least twice higher levels of saponins in pea protein concentrate compared to pea flour determined in our study. In pea protein isolate (75% protein; Table S1), saponin B content was 243.8 ± 6.2 mg/100 g and DDMP content was 10.8 ± 0.7 mg/100 g. These results show that protein wet extraction and isoelectric precipitation, likely performed to achieve protein isolate, degrade unstable DDMP saponin naturally occurring in peas into saponin B. In the pea drink, saponin B content was 3.5 ± 0.2 mg/100 g and DDMP saponin was below LOQ. According to the product specification (Table S1), it contains 2% of protein, and the only protein source is pea. Although the exact production process of the pea drink is unknown, taking into account the content of saponin B per 1 g of pea drink protein (1.75 mg), the probable pea protein source should contain at least 175 mg of saponins (sum of saponin B and DDMP saponin, as DDMP saponin is converted into saponin B during drink pasteurization) per 100 g of pure pea protein.

To test the applicability of the developed method for simultaneous determination of oat and pea saponins from one matrix, the blend of pea isolate, oat protein concentrate, and pea protein concentrate was used. In addition, the part of the mixture was extruded according to the previously published article [41]. Results show that avenacoside A, avenacoside B, 26-desglucoavenacoside A, saponin B, and DDMP saponin content in the blend were 13.5 ± 1.0 mg/100 g, 10.9 ± 0.3 mg/100 g, 1.3 ± 0.3 mg/100 g, 123.9 ± 6.2 mg/100 g, and 27.1 ± 3.5 mg/100 g, respectively. Considering that this blend was composed of 52% pea protein isolate, 28% oat protein concentrate, and 20% pea protein concentrate, which were also analysed separately, the recoveries of avenacoside A, avenacoside B, 26-desglucoavenacoside A, saponin B, and DDMP saponin were 114%, 115%, 90%, 95%, and 100%, respectively. In the extruded blend, avenacoside B and 26-desglucoavenacoside A content did not change significantly, avenacoside A content decreased by 21%, and saponin B content increased from 123.9 to 132.9 mg/100 g, which could potentially happen due to DDMP saponin conversion into saponin B during extrusion cooking.

4. Conclusions

In conclusion, the HILIC-MS-based method for oat and pea matrices, with a relatively simple extraction procedure for solid and liquid samples, allowing the simultaneous quantification of avenacoside A and saponin B, and the relative quantification of avenacoside B, 26-desglucoavenacoside A, and DDMP saponin, was employed for analysis of saponins in various food ingredients and products. Oat protein concentrate, pea protein isolate, and oat- and pea-based drinks were chosen for development and validation of the sample extraction methods. The optimised HILIC-MS method was able to absolutely quantify avenacoside A and saponin B in the matrices; other compounds were quantified based on existing standard compounds. The validation of the improved methods for both sample types (solid and liquid) showed the acceptable linear range, LODs and LOQs, precisions, recoveries, and MEs. Generally, an inter-day precision was below 20%. The accuracy and the precision of quantification were achieved by using the labelled internal standard (^{13}C-avenacoside A) obtained from U-^{13}C-labelled oat flour and with soyasaponin Ba as internal standards. The content of saponins was measured in different plant-based oat and pea products (ingredients, half- and end-products). This method could be potentially

extended for other plant-based sample matrices, and the absolute quantification of all analytes could be achieved if the missing saponin standards were to arrive on the market.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods12050991/s1. Description of solid sample extraction methods (1A, 1B, 2A, 2B, and 2C) used during the method development; Table S1: Nutritional information of analysed products; Figure S1: LC-MS chromatograms of oat and pea flours (SIR and ESI-). In oat flour: avenacoside A, avenacoside B, 26-desglucoavenacoside A, and internal standard ^{13}C-avenacoside A. In pea flour: saponin B, DDMP saponin, and internal standard soyasaponin Ba; Figure S2: Saponin yield in (a) oat and (b) pea matrices. The effect of sample clean-up: the pre-extraction of fat and six post-extraction filtration possibilities. The results of avenacoside B are presented in equivalents of avenacoside A mg/g and DDMP saponin in equivalents of saponin B mg/g; Table S2: The effectiveness of ultrasonic bath extraction compared to reference extraction conditions using the tube rotator (extraction yield 100%).

Author Contributions: A.B.: conceptualization, methodology, design of the experiments, carried out the experiments, investigation, validation, data curation, visualisation, writing—original draft, review and editing. D.P.: design of the experiments, methodology, writing—review and editing. T.K.: supervision, investigation, formal analysis, funding acquisition, project administration, resources, writing—review and editing. M.K.: writing—review and editing, supervision. E.-G.K.: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Appendix 4

Publication III

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Development of Extraction Method for Determination of Saponins in Soybean-Based Yoghurt Alternatives: Effect of Sample pH

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Abstract: The number of plant-based dairy alternative products on the market is growing rapidly. In the case of soybean-based yoghurt alternatives, it is important to trace the content of saponins, the phytomicronutrients with a disputable health effect, which are likely to be responsible for the bitter off-taste of the products. We present a new sample extraction method followed by hydrophilic interaction liquid chromatography with mass spectrometric detection (HILIC-MS) for identifying and quantifying soyasaponins in soybean-based yoghurt alternatives. Soyasaponin Bb, soyasaponin Ba, soyasaponin Aa, and soyasaponin Ab were quantified using commercially available standard compounds and with asperosaponin VI as the internal standard. As the recoveries of soyasaponins were unacceptable in yoghurt alternatives at their natural acidic pH, the adjustment of pH was performed as one of the first steps in the sample extraction procedure to achieve the optimum solubility of soyasaponins. The validation of the method included the assessment of linearity, precision, limit of detection and limit of quantification (LOQ), recovery, and matrix effect. The average concentrations of soyasaponin Bb, soyasaponin Ba, soyasaponin Ab, and soyasaponin Aa in several measured soybean-based yoghurt alternatives utilising the developed method were 12.6 ± 1.2, 3.2 ± 0.7, 6.0 ± 2.4 mg/100 g, and below the LOQ, respectively. This method provides an efficient and relatively simple procedure for extracting soyasaponins from yoghurt alternatives followed by rapid quantification using HILIC-MS and could find a rightful application in the development of healthier and better-tasting dairy alternatives.

Keywords: bitterness; *Glycine max*; plant-based foods; plant proteins; LC-MS

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1. Introduction

In recent decades, the market for plant-based dairy alternatives has vastly expanded. In addition to cereals, pseudocereals, and nuts, legumes are typically used to produce plant-based dairy alternatives. Due to their high protein content and quality, legumes such as soybeans (*Glycine max* L.) are widely used to manufacture dairy alternatives [1]. However, there is still a lack of quantitative data on the migration of phytonutrients during food processing from plant-based protein sources to the final consumable products. Along with macronutrients, soybeans contain several classes of biologically active compounds, including naturally occurring complex oleanane triterpenoid glycoside saponins [2]. Chitisankul et al. studied saponin content in nine soybean varieties and fourteen different soybean-based milk alternatives. The average total soyasaponin content reported was 246 ± 92 and 269 ± 140 µmol per 100 g dry weight basis (dwb), respectively [3,4], suggesting a transfer of saponins from the dry matter throughout the production chain of plant-based milk alternatives.

The dietary preferences of many consumers are shifting towards plant-based products due to environmental, health, and ethical reasons. Thus, from a nutritional point of view, it is important to quantify phytochemicals from emerging plant-based alternatives. Although human cells are not able to degrade saponins [5], some bacteria from gut microbiota convert saponins into sapogenols [6] and enter the bloodstream [7]. Until now, the data on the effects of saponins on human health are controversial. Negative consequences of high saponin consumption have been proven in livestock; e.g., health issues in the digestive tract of ruminants as well as decreases in wool, milk, and egg production were observed [8]. In addition, high concentrations of saponins may lead to the inefficient absorption of fat-soluble vitamins and damage the membrane of the intestinal inner epithelial wall [9]. On the contrary, several in vitro and in vivo studies have shown the positive immunological and antiviral effects of soyasaponins [10]. In addition, anti-cancerogenic [6], hepato-protective [11], anti-inflammatory [12], and anti-obesity effects [13,14] have been reported. The beneficial and deleterious nutritional properties of saponins are likely to be dose- and diet-dependent.

Soyasaponins are amphiphilic compounds composed of polar sugar moieties attached to a nonpolar pentacyclic ring [15,16]. Soyasaponins are generally distributed between group A and B depending on the glycosylation positions of soyasapogenol A and soyasapogenol B [17,18]. Soyasaponin Aa and soyasaponin Ab are glycosylated at the C-3 and C-22 position of soyasapogenol A (group A), while soyasaponin Ba and soyasaponin Bb are glycosylated at the C-3 position of soyasapogenol B (group B). The structures of the studied soyasaponins are shown in Figure 1.

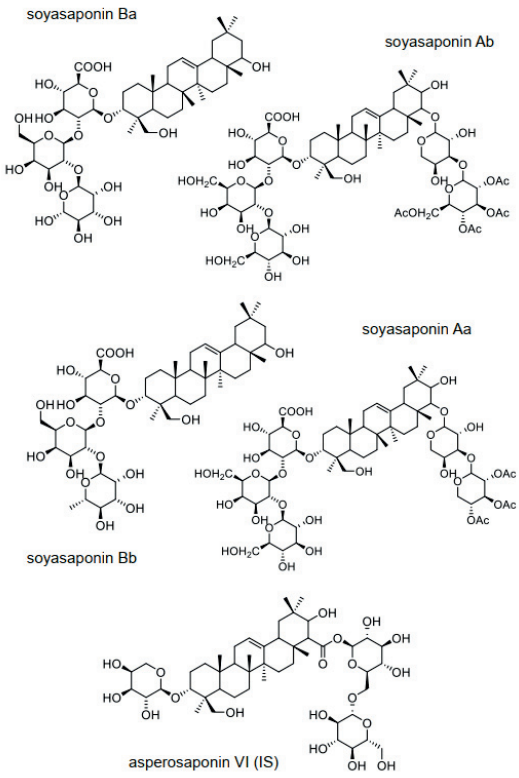

Figure 1. Chemical structures of soyasaponins (soyasaponin Aa, soyasaponin Ab, soyasaponin Ba, and soyasaponin Bb) quantified in this study and asperosaponin VI (used as internal standard (IS)).

In addition to structural differences, group A soyasaponins contribute more to a bitter sensation than group B saponins [19], causing a major unpleasant taste of soybean-based dairy alternatives [20,21]. The group A soyasaponins are located both in soybean seed hypocotyls and cotyledons [3]. The removal of hypocotyls is usually performed during the production of soybean-based milk alternatives but is not enough to fully discard group A soyasaponins from the end products [3,4]. Hence, the residual soyasaponin concentration might still influence the bitterness of a product and thus limit the consumer acceptance.

Researchers have widely characterised the molecular structures of several forms of soyasaponins and have reported different methods for their quantification [4,22–29]. Indeed, saponin quantification is considered challenging due to the lack of chromophores in their molecular structure, leaving out the possibility of using UV light at a specific wavelength for quantification. Liquid chromatography (LC) coupled to electrospray ionisation mass spectrometry (ESI/MS) is an alternative approach providing significant selectivity and specificity without a need for the derivatization of analytes [16,25]. Despite extensive research carried out on different soybean foods, only a few studies have identified or relatively quantified the levels of soyasaponins in soybean-based dairy alternatives [4,29]. In the case of previously published methods, the liquid samples were initially pre-processed before extraction by being either freeze-dried [4] or dried by rotary evaporation [29]. The time-consuming application of these techniques may be considered the major drawback of previously reported quantification methods impeding the direct analysis of liquid samples. New extraction procedures are required to mitigate the issues with traditional analysis methods, allowing to save on equipment resources, increase the analysis throughput, and overall, facilitate the implementation of quality control throughout the development of new soybean-based dairy alternative products.

This study aimed to develop a selective extraction and quantification method for the determination of soyasaponins (soyasaponin Aa, soyasaponin Ab, soyasaponin Ba, and soyasaponin Bb) from a soybean-based yoghurt alternative matrix using hydrophilic interaction liquid chromatography with mass spectrometric detection (HILIC-MS). To our knowledge, there are no studies presenting the soyasaponin quantification method in which sample extraction has been performed directly from liquid soybean-based dairy alternative samples.

2. Materials and Methods

2.1. Food Samples

Soybean-based drink (SBD) and five soybean-based yoghurt alternatives (YA1, YA2, YA3, YA4, and YA5) from different producers were purchased from the local supermarket. Supplementary Table S1 provides nutritional and compositional information available on the label of the products. Samples were aliquoted and stored at −20 °C.

2.2. Chemicals and Materials

All solvents were HPLC grade and were purchased from Honeywell (Charlotte, NC, USA). Formic acid (FA) (98% for MS) and the ammonia solution (25% for LC-MS) were from Honeywell (Charlotte, NC, USA) and Merck KGaA (Darmstadt, Germany), respectively. The standard compounds soyasaponin Aa, soyasaponin Bb, soyasaponin Ba, and asperosaponin VI Phyproof® Reference substances were from PhytoLab GmbH & Co. KG (Dutendorfer, Germany), and soyasaponin Ab was from MedChemExpress (Monmouth Junction, NJ, USA). Biotage Isolute® PLD+ (100 mg/mL) cartridges were obtained from Biotage Sweden AB (Uppsala, Sweden). Ultrapure water (18.2 mΩ·cm) was prepared using MilliQ® HX 7040SD equipped with MilliQ LC-Pak (Merck KGaA, Darmstadt, Germany).

2.3. Extraction Method for Samples

Soyasaponins were extracted according to the previously published method developed for pea and oat saponins [30] with some modifications. Briefly, the thawed homogeneous liquid sample was weighed (0.35–0.40 g) into a 5 mL volumetric flask (n = 3). Ultrapure

water was added to the line and mixed thoroughly. The sample solution (native pH of yoghurt alternative was ~4.6) was alkalised to reach the sample pH 8 ± 0.25 using aqueous ammonia solution (5%, *v/v*) or aqueous FA (25%, *v/v*). Samples were incubated on a tube rotator Stuart SB3 (Bibby Scientific Ltd, Staffordshire, UK) at room temperature for 30 min. After incubation, samples were centrifuged at 17,000 *g* at 10 °C for 10 min. After transferring the supernatant to a new Eppendorf tube, an equal volume of pure acetonitrile was added (MeCN, 1:1, *v:v*). The solution was mixed thoroughly and centrifuged at 14,800× *g* at 10 °C for 10 min to remove precipitated proteins. The supernatant (1000 µL) was passed through a PLD+ column using a vacuum manifold (VacMaster 10, Biotage Sweden AB, Uppsala, Sweden) at –0.5 bar. The filtrate (100 µL) was combined with an IS working solution (asperosaponin VI; 100 µL) and injected into the LC-MS.

2.4. Liquid Chromatography Mass Spectrometry

Analysis was performed as described previously [30] with adaptations to the analysis of soyasaponins. Briefly, a Waters UPLC® system (Waters Corporation, Milford, MA, USA) attached to a Waters Quattro Premier XE Mass Spectrometer equipped with ZSpray™ Source was used to analyse the samples. The equipment was controlled by Waters MassLynx™ 4.1 (V4.1 SCN805, Waters Corporation, Milford, MA, USA). Mobile phase A consisted of ultrapure water containing 0.1% FA, and mobile phase B consisted of MeCN containing 0.1% FA. The gradient was changed as follows: 0–0.17 min at 10% A; 0.17–1.5 min linear gradient 10–70% A; 1.5–4.17 min at 70% A; 4.18 min switch to 10% A; 4.18–6.0 min at 10% A. The mobile phases were pumped at 200 µL/min flow rate. A BEH Amide column (1.0 × 50 mm, 1.7 µm) coupled with BEH Amide VanGuard Pre-column (2.1 × 5 mm) from Waters Corporation (Milford, MA, USA) were used to retain saponins. The autosampler and column heater were set at 8 °C and 50 °C, respectively.

The MS part of the method proposed for determination of oat and pea saponins [30] was adapted to target the quantification of soyasaponins. Based on a scan-type experiment of external standards, the deprotonated molecules $[M\text{-}H]^-$ were chosen. The capillary voltage was set to −2.5 kV; cone voltages were optimised separately for every compound. The analysis was performed using negative electrospray ionisation (ESI^-) mode using single-ion-recording (SIR) mass-to-charge ratios shown in Table 1. High-purity nitrogen was set as a cone and as desolvation gas at a rate of 25 L/h and 600 L/h, respectively. The temperature of the desolvation gas was set to 350 °C. Data acquisition and target analyte quantification were performed in Waters MassLynx™ and QuanLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA) and Microsoft Excel® (Microsoft 365 Apps for enterprise, Microsoft Corporation, Richmond, WA, USA). Other parameters for MS were employed according to the description provided previously [30].

Table 1. The used m/z values and cone voltages for analytes.

Analyte	$[M-H]^-$ $m/z \pm 0.5$ Da	Cone Voltage (V)
Soyasaponin Bb	941.5	100
Soyasaponin Ba	957.3	120
Soyasaponin Aa	1364.3	120
Soyasaponin Ab	1435.6	120
Asperosaponin VI	927.5	120

2.5. Calibration and Quantification

The stock solutions of soyasaponin Aa, soyasaponin Ab, and soyasaponin Bb (1000 mg/L) were prepared in ethanol (EtOH; 99.9% purity). The stock solution of soyasaponin Ba (1000 mg/L) was dissolved in ethanol:methanol solution (EtOH:MeOH; 1:1, *v/v*). The stock solution of asperosaponin VI used as IS (1000 mg/L) was prepared in ultrapure water. All solutions were aliquoted and stored at −80 °C. The working solution of asperosaponin VI (30 mg/L) was made freshly before the analysis using the aqueous MeCN (50%,

v/v). The calibration curve standard solutions were diluted in MeCN:H_2O:EtOH solution (50:36:14, *v/v*).

The IS (asperosaponin VI) working solution was added to the calibration curve standard solutions and the sample solutions before the injection, keeping the concentration of the IS constant. Calibration curve solutions were built for all soyasaponins and were run in triplicate (0.01–2.5 mg/L). Seven-point calibration curves of soyasaponins were prepared by plotting peak area ratios of soyasaponins/IS against the concentration of the external standard compound. The linear regression approach led to linear responses showing correlation coefficients of >0.99 for all analytes.

2.6. Validation of the Method

The European Medicines Agency (EMA) validation guideline was used to evaluate the following parameters during method validation: selectivity, specificity, calibration curve and range, limit of detection (LOD), limit of quantification (LOQ), precision, sample extraction recoveries, and matrix effect (ME) [31].

The calibration curve and range were evaluated via repeated measurements of standard solutions of soyasaponins consisting of eight individual points obtained from serial dilution of stock solutions. The LODs and LOQs were calculated using a previously published tutorial [32].

To determine the intra- and interday precision of the instrumental method, the standard solution and the IS were both injected six times and across three independent days to affirm the stability of the retention times (RTs) and peak areas. In addition, the repeatability (intraday) and intermediate precision (interday) of the whole method was investigated using YA2. Repeatability analysis was performed by six replicate analyses of samples on the same day. The intermediate precision of the method was by analysis of six replicates on three different days over four weeks under the same experimental conditions.

The total recoveries of analytes were assessed by spiking YA2 with a known amount of soyasaponins at four different concentration levels (unspiked, lower LOQ, middle LOQ, and upper LOQ) and performing the extraction methods as described above [33].

ME was evaluated by post-extraction spiking of sample extracts with calibration curve standard solutions and comparing the solvent-matched calibration curve slopes with matrix-matched slopes [32].

2.7. Statistical Analysis

Data analysis for sample extraction method development was performed in R 4.2.2 (The R Foundation for Statistical Computing, Vienna, Austria). ANOVA followed by Tukey–Kramer post hoc test was performed with R package 'agricolae' 1.3–5. The significance level was set to 0.05. The results are presented as mean with standard deviation (SD) or relative standard deviation (RSD). All analyses were repeated in triplicate if not marked otherwise.

3. Results and Discussion

3.1. Development of Liquid Chromatography Mass Spectrometry Method

Previously reported LC-MS methods for the quantification of saponins varied from 6 to 80 min [2,30,34,35]. The shortest method with some modifications in the gradient and a total runtime of six minutes was used as a basis in our study. The SIR chromatograms shown in Figure 2 were obtained following an analysis of the soyasaponin standards, the sample of soybean-based yoghurt alternative, and the IS using the optimised analytical method described in Section 2.4. During the method development, we tested the multiple reaction monitoring (MRM) experimental conditions on our instrumentation. However, it did not enhance selectivity; instead, it notably decreased sensitivity by failing to generate consistent fragments. Nevertheless, the reasonably rapid retention of soyasaponins on the column and high-resolution peaks were achieved using the SIR mode. The proposed chromatographic method is more environmentally friendly and sustainable than previous approaches as i has a shorter duration, high-throughput nature, and reduced solvent usage.

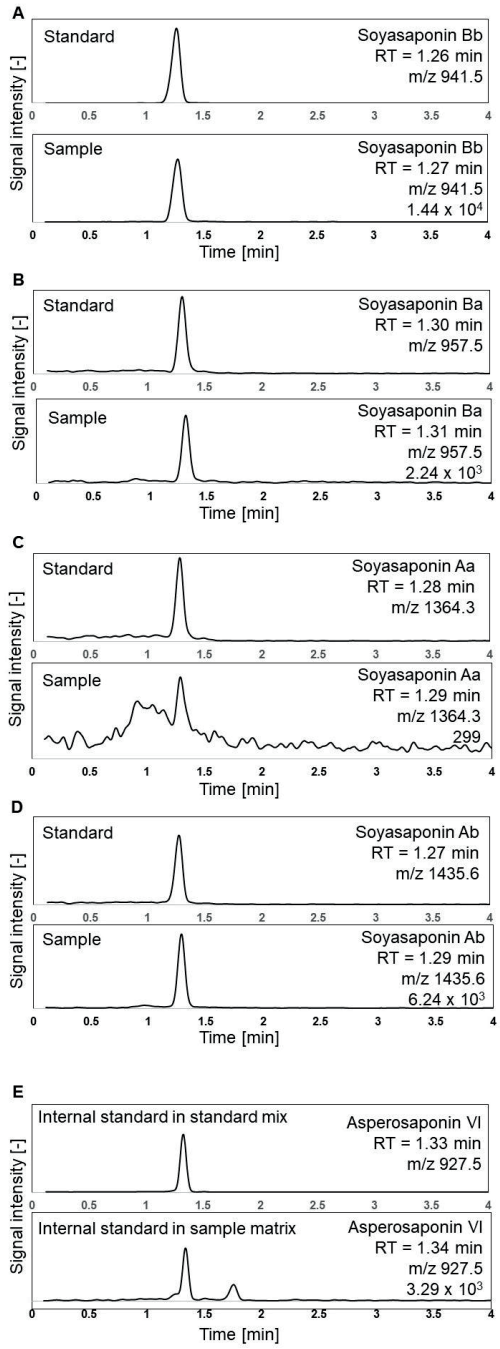

Figure 2. LC-MS chromatograms of external standard compounds and YA1 sample (SIR; ESI-): (**A**) soyasaponin Bb, (**B**) soyasaponin Ba, (**C**) soyasaponin Aa, (**D**) soyasaponin Ab, and (**E**) internal standard asperosaponin VI.

Although soyasaponins include over one hundred different compounds [4], only the forms relevant to soybean-based yoghurt alternatives, including soyasaponin Bb, soyas-

aponin Ba, soyasaponin Aa, and soyasaponin Ab, were selected for total quantification. During method development, we also screened soybean-based yoghurt alternatives for the possible semi-quantification of the DDMP-conjugated form, but these compounds were not identified in the matrix. Plant-based yoghurt alternatives are typically pasteurised at 95 °C or undergo an ultra-high-temperature treatment above 100 °C during the production process, which helps to manage microbiological concerns and prolong the shelf life [1]. Under heat treatment, the thermo-sensitive DDMP conjugates of group B soyasaponins may degrade into non-DDMP saponin species. Hu et al. showed that the DDMP-conjugated B group saponins started to decrease already when heated at 65 °C [2]. Indeed, the range of possible analytes to quantitate could be potentially expanded by total synthesis or fractioning other soyasaponin compounds from raw materials, but in both cases, it is time-consuming, not cost-effective, and impractical for routine analysis in laboratories.

Asperosaponin VI was chosen as the IS for soyasaponins in this method based on its structural similarity to the triterpenoid core [36] (Figure 1) and LC-MS retention similar to the targeted analytes. Ideally, each soyasaponin target compound should be quantified using its corresponding isotopically labelled internal standard when these become more readily available, without the need for the custom total synthesis of standards or the cultivation of isotopically labelled soybeans.

3.2. The Influence of Sample pH on Saponin Extraction

A previously published method for the measurement of saponins in oat- and pea-based drinks [30] was used as a starting point for the development of an extraction method for soyasaponins from soybean-based yoghurt alternatives. Traditionally, saponin extraction is performed using ethanol or methanol from a solid fat-free sample before subsequent LC-MS analysis, the whole procedure starting from a Soxhlet-assisted fat-removing step, followed by the solvent extraction. The simplified procedure in this recently proposed method allowed the extraction of saponins from liquid samples with a minimal number of extraction steps and a small volume of solvents. The comprehensive comparison of the performance of this extraction method with selected traditional ones has been provided elsewhere [30].

In the present study, we focused on the exploration of the effect of the pH of the soybean-based yoghurt alternatives on the quantification of soyasaponins as the pH of these products is considerably lower than that of the SBD. The native pH of the SBD and the soybean-based yoghurt alternatives (YA1 and YA2) were 8.8 and 4.6–4.7, respectively. The effect of the native pH of the products and the effect of the pH adjustment before extraction on the yield of the extracted saponins are reported in Table 2. The SBD and the soybean-based yoghurt alternatives were analysed as described in Section 2.3: unspiked and spiked with all four soyasaponins and with or without a pH adjustment included in the sample extraction protocol. Indeed, in the samples at their native pH, the recoveries of soyasaponins in the SBD ranged from 80 to 109%, while for both yoghurt alternatives, the concentrations and recoveries were significantly lower than those observed in the SBD. Moreover, the recoveries of soyasaponins at native pH were similar among yoghurt alternatives. These results suggest that soyasaponin recoveries could be pH-dependent.

Table 2. The relationship between sample pH and recovery (R) rates of soyasaponins during different extraction procedures. Samples: soybean-based drink (SBD); soybean-based yoghurt alternatives (YA1 and YA2). Each result represents mean ± standard deviation (n = 3). ANOVA statistical significance test was performed within sample matrices; means with different letters are significantly different at $p < 0.05$.

Sample	pH	Soyasaponin Bb		Soyasaponin Ba		Soyasaponin Aa		Soyasaponin Ab	
		mg/100 g	R [1] (%)	mg/100 g	R [2] (%)	mg/100 g	R [3] (%)	mg/100 g	R [4] (%)
SBD	8.8 (native)	12.6 ± 0.71 [a]	98 ± 4 [a]	2.43 ± 0.26 [a]	109 ± 8 [a]	<LOQ	85 ± 5 [a]	<LOQ	80 ± 5 [a]
	4.2 ± 0.2	1.2 ± 0.14 [b]	23 ± 5 [b]	0.82 ± 0.04 [b]	26 ± 5 [b]	<LOQ	54 ± 6 [b]	<LOQ	51 ± 5 [b]
YA1	4.7 (native)	0.84 ± 0.03 [b]	20 ± 2 [b]	0.56 ± 0.02 [b]	25 ± 2 [b]	<LOQ	43 ± 3 [b]	0.58 ± 0.06 [b]	41 ± 3 [b]
	7.0 ± 0.2	5.39 ± 0.53 [a]	77 ± 2 [a]	1.03 ± 0.15 [a]	109 ± 3 [a]	<LOQ	104 ± 1 [a]	1.82 ± 0.1 [a]	115 ± 1 [a]
YA2	4.6 (native)	1.3 ± 0.08 [b]	27 ± 2 [c]	0.54 ± 0.04 [b]	25 ± 1 [c]	<LOQ	48 ± 3 [c]	3.07 ± 0.07 [b]	63 ± 4 [b]
	7.0 ± 0.2	10.94 ± 0.63 [b]	85 ± 10 [b]	2.36 ± 0.03 [ab]	83 ± 8 [b]	<LOQ	91 ± 7 [ab]	8.09 ± 0.38b [a]	98 ± 6 [a]
	7.5 ± 0.2	13.63 ± 1.63 [a]	89 ± 4 [ab]	2.68 ± 0.06 [a]	102 ± 5 [ab]	<LOQ	86 ± 4 [b]	9.93 ± 1.38 [a]	103 ± 1 [a]
	8.0 ± 0.2	14.43 ± 0.61 [a]	100 ± 14 [ab]	2.54 ± 0.34 [a]	114 ± 16 [a]	<LOQ	107 ± 15 [a]	9.18 ± 0.36 [a]	110 ± 15 [a]
	8.5 ± 0.2	13.51 ± 1.97 [a]	111 ± 1 [a]	2.79 ± 0.4 [a]	99 ± 8 [ab]	<LOQ	74 ± 7 [b]	9.01 ± 1.15 [a]	91 ± 8 [a]

[1] soyasaponin Bb spike concentration: 2.10 mg/L. [2] soyasaponin Ba spike concentration: 1.86 mg/L. [3] soyasaponin Aa spike concentration: 1.91 mg/L. [4] soyasaponin Ab spike concentration: 2.03 mg/L.

As the composition and nutritional information of the SBD, YA1, and YA2 were very similar (see Table S1), the following experiments were conducted with adjusted pHs of the samples to test the hypotheses of pH effects on soyasaponin recoveries (the results are shown in Table 2). The pH of the SBD was acidified to mimic the pH of the yoghurt alternatives, while the yoghurt alternatives were alkalised to mimic the pH of SBD. The experiment indicated that the SBD acidified to pH 4.2 had unacceptable recoveries of soyasaponins, ranging from 23 to 54%. On the contrary, yoghurt alternatives that were alkalised (pH 7.0 ± 0.2) resulted in higher soyasaponin recoveries: from 77 to 115% and from 83 to 98% in YA1 and YA2, respectively.

Based on these findings, additional experiments were conducted to assess the pH value at which soyasaponins would result in the highest and most meaningful recovery. YA2 was analysed at three additional pH values: 7.5 ± 0.2, 8.0 ± 0.2, and 8.5 ± 0.2. The results indicated that pH values of 7–8.5 had a beneficial influence on the recovery of soyasaponins, but there was no strict pH optimum value. ANOVA showed a statistical difference in the recoveries of soyasaponins at analysed pH values in most cases. The most acceptable recoveries were achieved at pH 7.5 ± 0.2 and pH 8.0 ± 0.2. Therefore, for further analyses, the method's optimum pH value was chosen to be 8 ± 0.25.

Even though saponins are known as amphiphilic molecules, having a non-water soluble triterpene core and attached water-soluble sugar moieties, and are preferably soluble in organic solvents, soyasaponin Bb solubility is very low in the acidic region and increases drastically in the 6.5–7.3 pH region in aqueous buffers, having a solubility maximum in the range of 7 to 8 pH [37]. This fact elucidates the influence of different pH values of the samples on the soyasaponins recovery experiments. By adjusting the pH in soybean-based yogurt alternatives, the solubility issues of soyasaponins in acidic environments are overcome, enabling the direct analysis of liquid samples using a recently published method with modifications relevant to soyasaponins [30].

3.3. Validation of the Method

Validation was executed to assess the linear ranges, LODs and LOQs, precision, recoveries, and matrix effect of the proposed method for the determination of soyasaponins in yoghurt alternatives (Table 3). The calibration curves were constructed using a linear model with a weighing of 1/x. All four soyasaponins standards had high linearity ($R^2 > 0.99$) in the 0.01–2.52 mg/L concentration range. The estimated LOQs for soyasaponins were ≤33.4 µg/L. The results of the LOQs were either lower or in accordance with previous research [34,35].

Table 3. The linear range, calibration curve, limits of detection (LODs), and limits of quantification (LOQs) of soyasaponins.

Analyte	Linear Range (mg/L)	Calibration Curve	R^2	LOD (µg/L)	LOQ (µg/L)
Soyasaponin Bb	0.01–2.52	y = 0.7699x + 0.0048	0.9930	0.2	12.6
Soyasaponin Ba	0.02–2.26	y = 0.2949x + 0.0025	0.9975	8.0	33.4
Soyasaponin Aa	0.02–2.33	y = 0.3994x + 0.0021	0.9965	7.0	27.0
Soyasaponin Ab	0.01– 2.48	y = 0.3259x + 0.0033	0.9943	1.4	25.1

The repeatability of the method was investigated after the linearity of the soyasaponins was defined as acceptable. The results of the experiments are shown in the Supplementary Materials (Table S2). The RSDs of the peak RTs and the peak areas did not exceed 2% and 4%, respectively. It was observed that the intra- and interday RSDs for the whole method were lower than 12% and suitable for the routine analysis of soybean-based products. The precision observed using this method agreed with results reported by other LC-MS methods [34,35].

The recoveries of the soyasaponins were determined by spiking the YA2 with the analytes. Table 4 shows the results of the recovery of the YA2 at three spiking levels. The

recoveries ranged from 81 to 101%. The obtained recoveries were acceptable according to the guidelines [31] and comparable with the previously published methods [2,30,34,35].

Table 4. The recoveries of soyasaponins in soybean-based yoghurt alternative matrix (mean ± standard deviation (n = 3)).

Spiking Level	Soyasaponin Bb [1]		Soyasaponin Ba [2]		Soyasaponin Aa [3]		Soyasaponin Ab [4]	
	mg/L	R, %	mg/L	R, %	mg/L	R, %	mg/L	R, %
L1	0.03	95 ± 4	0.02	97 ± 5	0.02	101 ± 9	0.03	96 ± 3
L2	0.77	87 ± 4	0.68	87 ± 3	0.62	81 ± 1	0.8	90 ± 2
L3	1.54	82 ± 2	1.36	86 ± 2	1.23	81 ± 0	1.61	88 ± 0

[1] Unspiked matrix soyasaponin Bb concentration: 0.99 mg/L. [2] Unspiked matrix soyasaponin Ba concentration: 0.27 mg/L. [3] Unspiked matrix soyasaponin Aa concentration: 0.03 mg/L. [4] Unspiked matrix soyasaponin Ab concentration: 0.72 mg/L.

The experiment demonstrated that the soyasaponin Bb, soyasaponin Ba, soyasaponin Aa, and soyasaponin Ab MEs were 91%, 94%, 99%, and 94%, respectively. According to the guidelines, the achieved MEs were at an acceptable range [38], indicating sufficient sample clean-up.

According to validation guidelines, the method has confirmed sufficient validation performance regarding precision, recovery, sensitivity, and specificity. In addition, its efficiency and robustness for all the different yoghurt alternatives make the method valuable for screening and quality assurance.

3.4. Determined Concentrations of Soyasaponins in Soybean-Based Yoghurt Alternatives

We applied the developed and validated sample extraction method to quantify the soyasaponins in five soybean-based yoghurt alternatives (Table 5). All analysed samples had similar soyasaponin concentrations. The soyasaponin Bb, soyasaponin Ba, and soyasaponin Ab concentrations ranged from 11.7 to 14.5 mg/100 g, 2.6 to 4.2 mg/100 g, and 2.7 to 8.5 mg/100 g, respectively. The soyasaponin Aa concentration in all measured samples was below the LOQ.

Table 5. Soyasaponins content (mg/100 g) in soybean-based yoghurt alternatives (mean ± standard deviation (n = 3)). ANOVA statistical significance test was performed across all analysed samples; means with different letters are significantly different at $p < 0.05$.

Sample Code	mg/100 g			
	Soyasaponin Bb	Soyasaponin Ba	Soyasaponin Aa	Soyasaponin Ab
YA1	14.5 ± 0.4 ^{a}	2.8 ± 0.2 ^{b}	<LOQ	3.5 ± 0.4 ^{bc}
YA2	11.9 ± 0.8 ^{b}	2.9 ± 0.2 ^{b}	<LOQ	7.7 ± 0.3 ^{a}
YA3	12.6 ± 0.5 ^{b}	4.0 ± 0.4 ^{a}	<LOQ	8.5 ± 0.4 ^{a}
YA4	11.7 ± 0.4 ^{b}	2.6 ± 0.1 ^{b}	<LOQ	2.7 ± 0.3 ^{c}
YA5	13.3 ± 1.0 ^{ab}	4.2 ± 0.6 ^{a}	<LOQ	4.5 ± 0.5 ^{b}

Until now, limited data on the contents of soyasaponins in soybean-based dairy alternatives, including yoghurt alternatives, were available. A recently published study focused on characterizing the soyasaponin composition of 39 food products, including an analysis of 14 soybean-based milk alternative product [4]. The study showed that soyasaponin Aa was below the LOQ, soyasaponin Bb ranged from 27 to 308 mg/100 g dwb, soyasaponin Ba was quantified to be up to 14 mg/100 g dwb, and soyasaponin Ab ranged from 1 to 44 mg/100 g dwb in these products. Considering the average dry weight (~11%) of the samples in our study, our results showed that the average values of soyasaponin Bb, soyasaponin Ba, and soyasaponin Ab in the samples were 114 mg/100 g dwb, 29 mg/100 g dwb, and 54 mg/100 g dwb, which are in agreement with previously reported concentrations [4]. In another study, soyasaponin content was investigated [17], and the average sum of soyasaponin content in soybean-based milk alternatives was 39 µmol/100 g. The estimated average sum of quantified soyasaponins in the present study

was 21 µmol/100 g in soybean-based yoghurt alternatives. Considering different methods used for quantification and possible different soy varieties, our results supported both previously published soyasaponin studies [4,17].

Previously, nine varieties of soybean were studied [3]. They found that only four varieties contained soyasaponin Aa (from 22.3 to 97.5 mg/100 g dwb of seed), two varieties contained soyasaponin Ab (from 75.8 to 95.5 mg/100 g dwb of seed), seven varieties contained soyasaponin Ba (up to 6.4 mg/100 g dwb of seed), and all nine studied varieties contained soyasaponin Bb (from 8.7 to 21.3 mg/100 g dwb of seed) among other soyasaponins. Generally, hypocotyls contained larger quantities of soyasaponins than cotyledons. Since hypocotyls might be removed during the production of soybean-based dairy alternatives, there may be smaller amounts of soyasaponins than in the original soybean seed [3]. The seeds contained DDMP-conjugated soyasaponin Bb and soyasaponin Ba, which might degrade into respective non-conjugated forms during the production of soybean-based yoghurt alternatives [1,2], resulting in higher soyasaponin contents reported in our study.

In another study, the soyasaponin content was analysed in tofu, one of the popular consumed soybean-based foods [4]. The authors showed that tofu had a quite diverse soyasaponin composition. Among others, tofu contained soyasaponin Aa at 80 mg/100 g dwb, soyasaponin Ab from 23 to 136 mg/100 g dwb, soyasaponin Ba from 5 to 11 mg/100 g dwb, and soyasaponin Bb ranging from 112 to 312 mg/100 g dwb of the product. The quantities of soyasaponins in tofu were also similar to the results obtained in the current study in the soybean-based yoghurt alternatives.

Although the number of analysed samples in the present study was small, due to the limited number of soybean-based yoghurt alternatives available on the local market, it was possible to demonstrate the applicability of the developed method on real samples. In the case of commercial end products, it is not possible to make assumptions about the content of saponins in the soybean varieties used for the production or the effectiveness of starter culture bacteria involved in the technological process to degrade saponins. Analysing the entire production chain, from soybean seeds to the final dairy alternative products, would provide a more comprehensive understanding of the mitigation of phytonutrients and allow for a thorough investigation of the entire technological process.

4. Conclusions

In this study, a new sample extraction method for the direct analysis of liquid samples was developed for the determination of soyasaponins in soybean-based yoghurt alternatives using HILIC-MS. The rapid LC-MS method was able to quantify soyasaponin Bb, soyasaponin Ba, soyasaponin Aa, and soyasaponin Ab using asperosaponin VI as an internal standard. The results show that the acidic pH of the soybean-based yoghurt alternatives significantly affected the quantification of soyasaponins, leading to unsatisfactory soyasaponin recoveries. To address this issue, the effect of alkalisation on the extraction yield of saponins was evaluated, and the highest yield (from 100 to 114%) was achieved at pH 8.0 $\pm$ 0.25. By adjusting the pH at the beginning of the sample extraction process, it became possible to achieve satisfactory recoveries of soyasaponins in soybean-based yoghurt alternatives. The developed method was validated using a soybean-based yoghurt alternative as a test matrix. Overall, the inter-day precision of the method was below 12%. This validated method could be applied in the analysis of commercially available soybean-based yoghurt alternatives and used in technology and product development, e.g., for the high-throughput screening of fermentation processes to unveil the saponins-degrading ability of starter cultures. The application of the presented method has the potential to enhance the acceptance of emerging and developed plant-based dairy alternatives by consumers by improving the quality of the final product and the taste by controlling the taste-active compounds. This method could also be extended for analyses of soyasaponins in dairy alternative products produced from other legume species.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods12112164/s1. Table S1: Nutritional information of analysed products. Table S2: Repeatability of retention times (RTs), peak areas of soyasaponins, and precision of the whole method.

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Appendix 5

Publication IV

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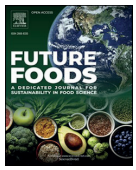

Digestibility of protein and estimated bioavailability of mineral compounds in plant-based yoghurt alternatives

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ABSTRACT

This study evaluated the nutritional quality of 25 commercial plant-based (PB) yoghurt alternatives (lupin-, soya-, oat-, coconut-based), focusing on protein digestibility and amino acid score and the content of the essential minerals calcium, iron, magnesium, and zinc. The phytates levels were also measured to estimate mineral bioavailability. The content of saponins was examined in soya and oat products. The findings of this study revealed a high variability of Protein Digestibility-Corrected Amino Acid Score (PDCAAS) within product groups, with values ranging from 0.21 to 0.82 for soya-based yoghurt alternatives and from 0.41 to 1.00 for oat-based yoghurt alternatives. Only one oat product exhibited high-quality protein (PDCAAS > 0.9). Most products had low mineral content, which makes them unsuitable as a mineral source. Phytates hindered the low mineral content, with levels ranging from 13 to 193 mg/100 g. However, products enriched with calcium showed satisfactory levels, and phytate content had minimal impact on the estimated calcium bioavailability. Oat- and soya-based products contained 1–8 mg/100 g of avenacosides and 11–18 mg/100 g soyasaponins, respectively. This research underscores the importance of paying more attention to the nutritional value during product development of PB yoghurt alternatives manufactured using various constituents and when incorporating these products into dairy-free diets.

1. Introduction

The fast-growing trend for plant-based (PB) dairy alternative products aims to meet the consumers' demand for delicious, healthy, nutrient-rich, and environmentally sustainable options. The number of consumers who prefer dairy alternatives has risen due to lactose intolerance, allergies, and health and environmental concerns (Silva et al., 2020). Additionally, some choose PB options as part of their lifestyle diets or due to ethical beliefs, like vegetarians and vegans. However, meeting nutritional needs with PB dairy alternatives can be challenging.

A variety of PB protein sources, including legumes [*e.g.* soya (*Glycine* max) and lupin (*Lupinus*)], cereals [*e.g.* oat (*Avena sativa*)], and other crops such as coconut (*Cocos nucifera*), have been employed to create a broad range of PB alternatives to traditional products (Montemurro et al., 2021). A recent study on cow's milk products and PB dairy alternatives on the New Zealand market revealed differences in their nutritional composition and found that PB dairy alternatives produced from different crops have varying amounts of macro- and micronutrients (Smith et al., 2022). This diversity in ingredient choices and utilized technologies has led to a wide variety of nutritional compositions within these products (Craig and Fresán, 2021; Fructuoso et al., 2021). Traditional dairy milk has been extensively studied and regarding its chemical composition is known to be rich in essential nutrients, still being low in iron, zinc, and magnesium (Antunes et al., 2023; Vanga and Raghavan, 2018; Walther et al., 2022). On the other hand, PB dairy alternatives are relatively new to the market and, as a very diverse group, have not yet received enough research attention regarding nutritional quality compared to traditional dairy milk.

Apart from variations in macro- and micronutrient composition, it is crucial to consider the bioaccessibility and bioavailability of nutrients in PB alternatives to ensure their effective absorption and utilization by the body. Bioaccessibility is a way to measure the part of consumed compound which is released from the food matrix and is available for absorption through the mucosa of the small intestine or transformed by the

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gut microbiota (Rodrigues et al., 2022). The term bioavailability refers to the portion of a nutrient which is absorbed and utilised through the digestive tract and reaches the target organ in the intact or metabolized form (Rodrigues et al., 2022; Wood, 2005). It is known that protein in PB dairy alternatives is significantly less bioavailable than in traditional dairy products (Mäkinen et al., 2016). For example, protein bioaccessibility and bioavailability can be described using Protein Digestibility-Corrected Amino Acid Score (PDCAAS), which includes the determination of protein quantity and digestibility, and amino acid score compared to reference protein amino acid contents (Schaafsma, 2000). Furthermore, PB protein sources include plant-specific compounds such as phytates, which are considered antinutrients (Montemurro et al., 2021). Phytates are known to hinder the absorption of minerals, as they can form poorly soluble complexes with multivalent cations such as calcium, iron, magnesium, zinc, and others (Foster and Samman, 2017; Raboy, 2001; Wood, 2005). In addition to phytates, PB protein sources also contain saponins, which can likewise impede the absorption of minerals and proteins (Francis et al., 2002; Potter et al., 1993).

Some recent studies have explored the nutrient content of PB dairy alternatives. According to statistical analysis of product labels and databases of nutrient content and product ingredients, it has generally been observed that PB dairy alternatives tend to be low in protein, and authors recommended performing more comprehensive chemical analyses of these substitutes (Craig and Fresán, 2021; Fructuoso et al., 2021; Moshtaghian et al., 2024). A considerable selection of PB milk alternatives was recently examined for macro- and micronutrients, including fat, protein, total carbohydrate, sugar profile, dietary fiber, mineral, and amino acid content, in comparison to animal-based milk types (Astolfi et al., 2020; Smith et al., 2022). The findings of the former study suggested that soya-based milk alternatives could be a suitable replacement for cow or goat milk in human diets (Astolfi et al., 2020). In another study, mineral composition and bioaccessibility were determined in PB yoghurt alternatives (pea-, soya-, and coconut-based) from the Brazilian market (Rebellato et al., 2023). Their results indicated that the bioaccessibility of essential minerals, measured according to INFOGEST 2.0 protocol, exhibited significant variation among the studied products (Rebellato et al., 2023). Despite the recent studies, a knowledge gap still exists concerning the current nutritional quality of PB yoghurt alternatives derived from various crops, particularly in terms of protein quality, bioavailability of mineral compounds, and the levels of antinutrients.

In this study, we aimed to characterize 25 commercially available PB yoghurt alternatives made from lupin, soya, oat, and coconut. We assessed protein quality through protein content, total amino acid profile, and PDCAAS analyses. Additionally, the levels of important mineral compounds such as calcium, iron, magnesium, and zinc were measured and their possible bioavailability with respect to the levels of phytates and saponins in the products was estimated.

2. Materials and methods

2.1. Collection and preparation of samples

PB yoghurt alternative products with a preference for samples without added flavouring (lupin, $n = 2$; soya, $n = 10$; oat, $n = 9$; coconut, $n = 4$; produced across Europe: Finland, United Kingdom, Estonia, Denmark, Sweden, France and Monaco, Belgium and Luxembourg) were commercially purchased (Table 1). The list of ingredients and nutritional information of studied products is provided in Supplementary Material Table S1. Each sample was aliquoted and stored at -20 °C. A portion of each sample was lyophilised using a Scanvac Coolsafe freeze dryer (LaboGene A/S, Denmark), ground with a mortar, and stored under vacuum at -20 °C. The dry weight of liquid samples was determined by incubation at 105 °C for 24 h using a Binder drying and heating oven (Binder drying and heating oven EDS-115, Tuttlingen Germany) while the dry weight of freeze-dried samples was determined using a halogen moisture analyser (HX 204 Mettler Toledo, USA) at 105 °C. The mineral, phytate, total amino acid, Kjeldahl, and PDCAAS analyses were performed on the freeze-dried samples, while saponin analysis was conducted from thawed liquid samples. All results are presented per ready-to-consume product.

Table 1
Analysed PB yoghurt alternatives.

Sample code	Product name	Main plant source
VY1-L	Lughurt Plain from Made with Luve	Lupin
VY2-L	Lughurt Plain from Made with Luve (blueberry)	Lupin
VY3-S	Sojade (So Soja, Natural)	Soya
VY4-S	Sojade (So Soja, alternative zu quark)	Soya
VY5-S	ProVamel. Soya. Coconut	Soya
VY6-S	ProVamel. Soya. Ohne Zucker	Soya
VY7-S	ProVamel. Soya. Skyr Style	Soya
VY8-S	ProVamel. Soya. Quarkalternative	Soya
VY9-S	Alpro High Protein	Soya
VY10-S	Alpro Natural	Soya
VY11-S	Alpro Natur ohne zucker	Soya
VY12-S	Rainbow Hapendatud mahesojatoode	Soya
VY13-O	BonSoya juuretisega kaerajook (õuna-kirsi)	Oat
VY14-O	Valio Oddlygood Mieto maustamaton	Oat[1]
VY15-O	Oatly Havregurt Turkisk	Oat[2]
VY16-O	Oatly Havregurt Natureli (Hapendatud maitsestamata kaeravahepala)	Oat[2]
VY17-O	Yosa Greek Style Natural	Oat[2]
VY18-O	Fazer Aito Kauravälipala Havrebaserad gurt	Oat
VY19-O	Planti Yog Oat Maiustamaton	Oat
VY20-O	Juustoportti Friendly Viking's O'gurt Natural	Oat[2]
VY21-O	Benecol Maustamaton kauragurtti	Oat[2]
VY22-C	The Coconut Collaborative Natural Coconut Yog	Coconut
VY23-C	Cocodeli vegan natural	Coconut
VY24-C	Koko Plain smooth&mellow	Coconut
VY25-C	Harvest Moon	Coconut

[1] minor protein ingredient – bean protein,.
[2] minor protein ingredient – potato protein.

2.2. Methods for protein characterization

2.2.1. Determination of protein content

The Kjeldahl method was employed to determine protein content according to previous publications (Mæhre et al., 2018). In summary, approximately 0.5 g of lyophilised sample ($n = 2$) was hydrolysed with 15 mL concentrated sulfuric acid (H_2SO_4) containing one catalyst tablet in a heat block at 420 °C for 1 h. After cooling, H_2O was added to the hydrolysates before neutralization and titration. The total nitrogen content in the lyophilised samples was then multiplied by the traditional conversion factor of 6.25 (for lupin- and coconut-based samples) or source-specific conversion factors of 5.71 and 5.83 for soya- and oat-based samples, respectively, to express the results as total protein (Mariotti et al., 2008). An in-house control sample was used to control the reproducibility of the analysis.

2.2.2. Determination of total amino acid profile

Total amino acid hydrolysis was performed from freeze-dried samples ($n = 3$) as described in the manufacturer's guide (Waters Corporation, 2023) and determined using a kit (AccQ•Tag™ Ultra Derivatization Kit; Waters, Milford, MA, USA) and the LC-UV method developed by Waters (Hewitson et al., 2007). Acid hydrolysis was conducted via vapor-phase acid hydrolysis under vacuum using 6 N HCl with 1% phenol for 20 h at 105 °C. Tryptophan was determined using alkaline hydrolysis in a nitrogen atmosphere with 4.2 N NaOH for 18 h at 105 °C. The hydrolysed samples were dissolved in a borate buffer, derivatized using a 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent (AQC reagent, SYNCHEM UG CO. KG, Felsberg, Germany) and then loaded onto an AccQ•Tag™ Ultra column. Amino acids were separated using a gradient of AccQ•Tag™ Ultra eluents A and B. Amino

acids were detected using a photodiode array detector, and data were processed using Empower® 3 SR4 FR5 Build 3471 software (Waters, Milford, MA, USA). The reference material (SRM 3234 soya flour, NIST, USA) was used to correct the amino acids that are susceptible to oxidative degradation (*e.g.*, cysteine, methionine, tryptophan, serine, tyrosine, arginine). The sum of all amino acids was used in PDCAAS calculation.

2.2.3. Determination of protein digestibility corrected amino acid score

The protein digestibility was assessed in freeze-dried samples ($n = 2$) using Megazyme kit K-PDCAAS (Megazyme Ltd., Wicklow, Ireland) according to manufacturer protocol (Megazyme, 2019). The protein contained in the products was enzymatically (pepsin, trypsin + chymotrypsin) broken down into α-amino acids and short peptides, and their reaction with ninhydrin was measured at 570 nm (Synergy H1 microplate reader, Agilent Technologies, Inc. USA). Primary amine concentration is determined using the l-glycine standard curve and corrected by dilution, sample weight and present amino acids. The result of the corrected primary amino acid concentration of standard samples provided by the kit manufacturer is fitted to the rat model and unknown samples are calculated based on achieved values. Results of total amino acid analysis and reference value of essential amino acids by the FAO in 2011 (histidine 20, isoleucine 32, leucine 66, lysine 57, methionine + cysteine 27, phenylalanine + tyrosine 52, threonine 31, tryptophan 8.5, valine 43 mg/g of protein) were used to calculate the amino acid score (Eq. (1)) (Megazyme, 2019). The PDCAAS value (Eq. (2)) for each sample was calculated by taking into account the results of *in vitro* digestibility and the amino acid score (Schaafsma, 2000). The limiting amino acid was the acid which was in highest deficiency (the smallest amino acid score value).

$$amino\ acid\ score = \frac{mg\ of\ limiting\ amino\ acid\ in\ 1g\ of\ test\ protein}{mg\ of\ the\ same\ amino\ acid\ in\ 1g\ of\ reference\ protein} \quad (1)$$

$$PDCAAS = amino\ acid\ score \times in\ vitro\ digestibility \quad (2)$$

2.3. Determination of mineral content

The analyses of calcium, iron, magnesium, and zinc content were conducted by the Estonian Veterinary and Food Laboratory (LABRIS). This analysis utilised inductively coupled plasma mass spectrometry (Agilent 7800 ICP-MS, Agilent Technologies, Inc. USA) using an in-house accredited method based on standard methods for this type of analysis (EVS, 2023a; 2023b). Samples were mineralised by wet ashing method (about 0.5 g sample + 4 mL conc. HNO_3, $n = 2$) using a microwave Anton Paar Multiwave PRO (Anton Paar GmbH, Graz, Austria). The standard methods were broadened to analyze calcium, iron, magnesium, and zinc. Depending on the concentration, some samples were further diluted in ultra-pure water. The quantification of the samples was accomplished by constructing a calibration curve by using a ratio (external to internal) and concentration of targeted mineral compound.

2.4. Determination of phytate and saponin content

Phytates (phytic acid [IP_6], inositol pentakisphosphate [IP_5], inositol tetraphosphate [IP_4], inositol trisphosphate [IP_3], inositol bisphosphate [IP_2] and inositol monophosphate [IP_1]) were quantified by LC-MS-ESI-TOF in freeze-dried samples ($n = 3$) following the methodology outlined in a previous publication (Tanilas and Krišč iunaite, 2022). The HILIC-MS methods were used to analyze saponins (soyasaponin Ab, soyasaponin Aa, soyasaponin Ba, and soyasaponin Bb in soya-based samples VY3-S to VY9-S; and avenacoside A, avenacoside B and 26-desglucoavenacoside A in oat-based samples VY13-O to VY21-O; $n = 3$) in the thawed, homogenous liquid samples, as described in recently published studies (Bljahhina et al., 2023a, 2023b). Avenacoside B and 26-desglucoavenacoside A were quantified in avenacoside A equivalents. The total contents of phytates and saponins were calculated by summing respective components.

2.5. Estimation of phytate inhibitory effect on bioavailability of mineral compounds

To estimate the inhibitory impact of phytates on the bioavailability of studied minerals, we calculated the molar ratios of these compounds (PHY:Fe, PHY:Zn, PHY:Ca, and PHY × Ca/Zn) using Eqs. (3)-6 (Dahdouh et al., 2019; Ma et al., 2007). These ratios give preliminary indication of mineral compound bioavailability in the presence of phytates. In cases where diets are high in both calcium and phytates, it is more informative to estimate zinc bioavailability by calculating PHY × Ca/Zn instead of PHY:Zn (Ma et al., 2007). Additionally, Eq. (7) was used to evaluate the molar ratio of PHY × Ca/Mg since phytates and calcium content can also influence magnesium absorption (Raboy, 2001). Finally, PHY × Ca/Fe was calculated to estimate the impact of phytates and calcium on iron bioavailability (Eq. (8)).

$$PHY:Fe = \frac{\frac{IP6(mg)}{660(MW)} + \frac{IP5(mg)}{580(MW)} + \frac{IP4(mg)}{500(MW)}}{\frac{Fe(mg)}{55.845(AtW)}} \quad (3)$$

$$PHY:Zn = \frac{\frac{IP6(mg)}{660(MW)} + \frac{IP5(mg)}{580(MW)} + \frac{IP4(mg)}{500(MW)}}{\frac{Zn(mg)}{65.38(AtW)}} \quad (4)$$

$$PHY:Ca = \frac{\frac{IP6(mg)}{660(MW)} + \frac{IP5(mg)}{580(MW)} + \frac{IP4(mg)}{500(MW)}}{\frac{Ca(mg)}{40.078(AtW)}} \quad (5)$$

$$PHY \times Ca \Big/ Zn = \frac{\left(\frac{IP6(mg)}{660(MW)} + \frac{IP5(mg)}{580(MW)} + \frac{IP4(mg)}{500(MW)}\right) \times \frac{Ca(mg)}{40.078(AtW)}}{\frac{Zn(mg)}{65.38(AtW)}} \quad (6)$$

$$PHY \times Ca \Big/ Mg = \frac{\left(\frac{IP6(mg)}{660(MW)} + \frac{IP5(mg)}{580(MW)} + \frac{IP4(mg)}{500(MW)}\right) \times \frac{Ca(mg)}{40.078(AtW)}}{\frac{Mg(mg)}{24.305(AtW)}} \quad (7)$$

$$PHY \times Ca \Big/ Fe = \frac{\left(\frac{IP6(mg)}{660(MW)} + \frac{IP5(mg)}{580(MW)} + \frac{IP4(mg)}{500(MW)}\right) \times \frac{Ca(mg)}{40.078(AtW)}}{\frac{Fe(mg)}{55.845(AtW)}} \quad (8)$$

2.6. Statistical analysis

Data analysis and visualization were performed in R 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was assessed using the Kruskal-Wallis test followed by the Conover-Iman test of multiple comparisons, performed with R package "Conover.test" 1.1.5. The significance level was set to 0.05.

3. Results and discussion

In this study, we conducted a nutritional characterization of 25 PB yoghurt alternatives. These products had previously undergone microbiological composition analysis, focusing on the determination of the relative abundance of live bacteria, along with the evaluation of their chemical composition and volatiles' profile, and sensory characteristics (Part et al., 2023). Significant differences were found in both chemical and microbiological composition, including the remarkable differences in the amount of live bacteria and sensorial attributes among products made from different protein sources as well as those produced based on the same crop (Part et al., 2023). In contrast, the main objectives of the present study were to investigate both the quantity and quality of

proteins, assess mineral, phytate and saponin content and estimate the inhibitory effect of phytates on mineral bioavailability in these products. The sample coding used in this study remains consistent with that of the previous research, with the addition of a plant origin marker.

3.1. Characterization of protein

PB dairy alternatives exhibit variability in protein content, as well as specific amino acid profiles based on their protein sources, resulting in varying amino acid scores and protein digestibility. To assess these parameters, we conducted analyses on the studied samples, including protein content, amino acid profile and *in vitro* digestibility. As a result of these evaluations, we calculated the amino acid score, the PDCAAS value, and the limiting amino acid for each product.

3.1.1. Protein content and sum of total amino acids

The studied PB yoghurt alternatives exhibited a substantial range in protein content, with a ten-fold difference observed, spanning from 0.4% to 5.4% (Fig. 1a, Supplementary Material Table S2). Specifically, soya-based products had the highest protein content, falling within the range of 3.2% to 5.4%. Meanwhile, oat- and coconut-based products displayed lower levels of protein, spanning from 0.5% to 2.6% and 0.4% to 1.2%, respectively. Lupin-based products showed protein content of 1.5%. These variations in protein content emphasize the variability in nutritional quality among different PB yoghurt alternatives. The sum of total amino acids (TAAs) (Fig. 1b, Supplementary Material Table S2) showed a similar trend to the protein content. A recent study of the Swedish market of PB yoghurt alternatives showed that protein content in these products ranged from 0.60 to 5.80 g/100 g (Moshtaghian et al., 2024). Additionally, a recent study on the products from the American market showed that the average plant-based soy, oat and coconut yoghurt protein content was 4, 3 and 0.2 g/100 g, respectively (Marlapati et al., 2024). The results of the present study are comparable with those previously published.

According to the regulation established by the European Parliament and the Council of the European Union products can only be labelled as

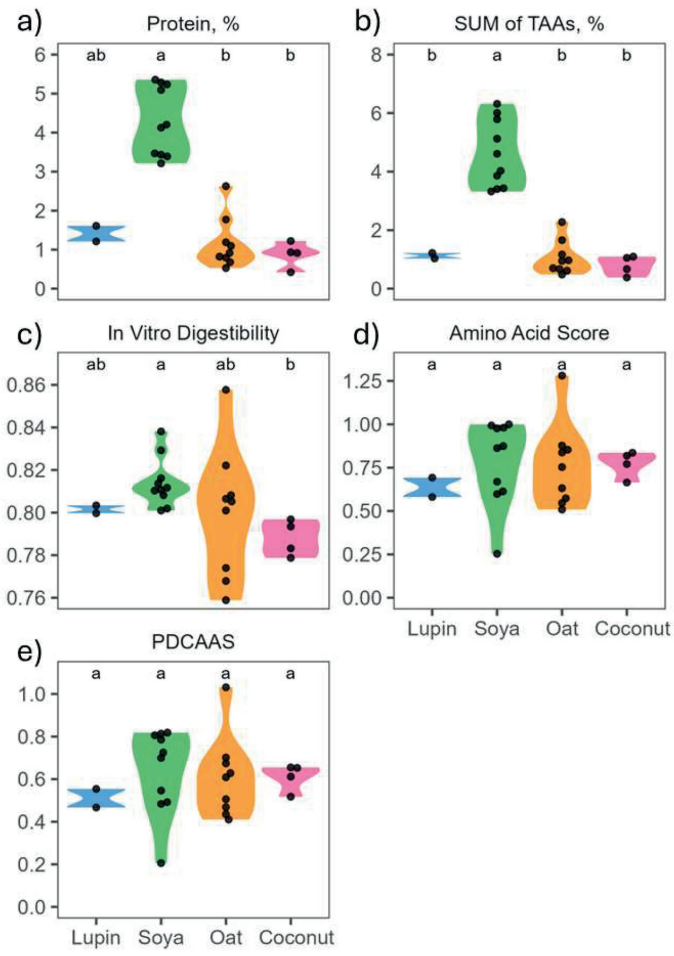

Fig. 1. The violin-plot distributions of studied PB yoghurt alternatives (lupin-, soya-, oat- and coconut-based) related to a) Protein (%); b) Sum of total amino acids (TAAs); c) In Vitro Digestibility of the protein in the studied products (with values ranging from 0 to 1.00); d) Amino Acid Score, a ratio calculated as the milligrams of the most limiting amino acid in 1 gram of test protein divided by the milligrams of amino acid in reference protein per 1 gram (Equation 1); and e) PDCAAS (Protein Digestibility-Corrected Amino Acid Score), calculated based on sum of TAAs, amino acid score, and *in vitro* digestibility (Eq. (2)), with values ranging from 0 to 1.0. The mean value of each product is denoted by a dot on the plot; groups with different letters are significantly different at $p < 0.05$.

"source of protein" or "high in protein" if protein accounts for at least 12% or 20% of the energy value, respectively (The European Parliament and the Council of the European Union. Regulation No 1924/2006, 2006). Our search in the Finnish Fineli Food Composition Database revealed a wide range of protein content in both plain and Greek-style dairy yoghurts spanning from 3 to 8.1 g/100 g (Fineli Food Database, 2024). These products could be classified both as "source of protein" and as "high in protein" because protein accounted for 11% to 37% of their energy value. In our study, only soya-based yoghurt alternatives met the criteria for being labelled as "high in protein products," with protein comprising 27–40% of their energy content (for total energy value see Supplementary Material Table S1). However, other PB yoghurt alternatives contained only 1 to 7% of their energy value from protein and could not be regarded as "source of protein". This comparison indicates that approximately half of the studied products did not match the protein content found in their dairy counterparts.

3.1.2. *In vitro digestibility of protein*

As PB dairy alternatives strive to establish themselves as viable substitutes for traditional dairy products, it becomes essential to consider not only the quantity of protein in these products but also the quality of that protein (Foster and Samman, 2017; Mäkinen et al., 2016; Rojas Conzuelo et al., 2022). In the present study, the analysis of the *in vitro* digestibility of PB yoghurt alternatives demonstrated that all the examined products exhibited a range between 0.77 and 0.86 (Fig. 1c). This indicates that approximately 80% of the protein in these products could be broken down into shorter peptides and amino acids during digestion, which could potentially be absorbed and utilised by the body. Comparatively, the true fecal digestibility of bovine milk protein has been previously determined to be 97% (Dupont and Tomé, 2020). This discrepancy highlights that the digestibility of protein in PB yoghurt alternatives studied is lower than that of bovine milk protein.

3.1.3. *Amino acid score*

Proteins must contain a sufficient amount of indispensable amino acids (His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val) to effectively support body development and growth through easy digestion and absorption (Chalupa-Krebzdak et al., 2018). The amino acid profile results can be found in Supplementary Material Figure S1 and Table S3. The amino acid score is calculated by comparing the amino acid content of the analysed protein (mg/g protein) with the requirements for indispensable amino acids (mg/g protein), and it is determined by the amino acid with the lowest ratio, which is known as the most limiting amino acid. Across a range of PB yoghurt alternatives, there was a significant variation in amino acid scores (Fig. 1d). In soya-based products, the scores ranged from 0.25 to 1.00. Similarly, in oat-based products, the scores spanned from 0.51 to 1.28, while in coconut-based products, they ranged from 0.66 to 0.82. Both lupin-based products amino acid scores range from 0.58 to 0.69. To provide a point of reference, the previous research has determined the amino acid score of traditional yoghurt to be 0.95 (Nadtochii et al., 2020).

3.1.4. *The PDCAAS values and first limiting amino acids*

The PDCAAS value serves as a quality indicator of a protein. It is derived from the calculation of the amino acid score, which is then adjusted based on the protein's true fecal digestibility (*in vitro* digestibility) (Schaafsma, 2000). The PDCAAS value reflects the degree to which the quantity of the most limiting amino acid is insufficient to reach the ideal level.

In our study, we observed a general fivefold variation in the PDCAAS values across the examined products (Fig. 1e). The results detailing the limiting amino acids for each product can be found in Supplementary Material Table S2. Among the studied oat-based products, the PDCAAS values had the highest variability from 0.41 to 1.00 and the most insufficient essential amino acids in these products were lysine, cysteine, methionine, tryptophan, threonine, and leucine. For soya-based products, the PDCAAS values ranged from 0.21 to 0.82, with valine, tryptophan, histidine, tyrosine, phenylalanine, cysteine, methionine, and lysine being the most deficient essential amino acids. In the case of lupin-based products, the PDCAAS was 0.47 to 0.55, and the first limiting amino acids were valine or lysine. Finally, in coconut-based products, PDCAAS values ranged from 0.52 to 0.65, with leucine and lysine identified as the first limiting amino acids. These results underscore the significant variability in the PDCAAS values of protein in soya- and oat-based yoghurt alternatives. Furthermore, they highlight the fact that the PB dairy alternatives produced using the same plant source may vary in the amino acid limiting their protein quality.

The high variability in PDCAAS and the identification of the first limiting amino acids among oat-based products may be tentatively attributed to the addition of other PB proteins, either bean or potato protein in the products VY14-O to VY17-O and VY20-O to VY21-O. In contrast, soya-based products have not been fortified with proteins from other sources. The differences in PDCAAS values among soya-based yoghurt alternatives can be attributed to various factors, including the specific choice and quality of protein source and processing methods used in the production of these alternatives (van den Berg et al., 2022). It's important to note that while soya is generally considered a good source of PB protein (Hughes et al., 2011; Rutherfurd et al., 2015), the present study showed that the PDCAAS value of a final product can vary widely.

It is indisputable that animal-based proteins possess high protein quality; for instance, the PDCAAS value for beef is 0.92, while in egg white and casein, it reaches the maximum score of 1.00 (Neira, 1991). In contrast, PB proteins are generally characterised by lower protein digestibility and definite plant-specific limiting amino acids, although there are exceptions. Low PDCAAS values have been assigned to lupin seeds (from 0.30 to 0.40) (Regina Pereira Monteiro et al., 2014), oat protein concentrate (from 0.58 to 0.69) (Abelilla et al., 2018; Nosworthy et al., 2023) and whole coconut meal (0.89) (Mepba and Achinewhu, 2003). In contrast, soya protein isolate has exhibited high PDCAAS values (1.00) (Hughes et al., 2011; Rutherfurd et al., 2015). Previous research lacks PDCAAS values for PB dairy alternatives. Therefore, the results obtained in this study can only be compared with that of respective protein concentrates or isolates. Our study showed that the PDCAAS value of protein fraction in soya- and oat-based yoghurt alternatives varied significantly in studied products, while only one oat-based product showed high protein quality with value over 0.90. Ten soya-based products exhibited lower PDCAAS values than stated in the literature for soya protein isolate (Rutherfurd et al., 2015). Half of the studied oat-based PDCAAS values were significantly lower than stated in the literature for oat protein concentrate (Nosworthy et al., 2023).

Generally, legume proteins (soya and lupin) are known to lack sulfur-containing amino acids methionine and cysteine and are high in leucine and lysine, while cereal proteins contain high levels of methionine and are low in lysine (Boye et al., 2012; Joint WHO/FAO/UNU Expert Consultation, 2007; Messina et al., 2022; Sobotka et al., 2016). Nevertheless, our findings revealed that methionine and cysteine were the limiting amino acids in only one of ten legume-based products, while lysine was the first limiting in four out of nine oat-based products. Previous reports on soya beans have shown that environmental factors can affect protein concentration as well as the amino acid proportions in the crop (Pfarr et al., 2018). Therefore, the limiting amino acids could potentially be different in studied products. In four other oat-based products, lysine was the second limiting amino acid. Some oat products contained other minor protein sources, which could have influenced the protein amino acid composition and the limiting amino acid results. On the other hand, legume products did not contain any obvious protein sources other than the main protein ingredient, which could have shifted the balance of amino acids. This emphasizes that using the information from existing literature for PDCAAS values and limiting amino acids of the main protein source during the technology and

product development of emerging PB dairy alternatives may not be a reliable approach.

3.2. Mineral content

The content of calcium, iron, magnesium, and zinc was evaluated in PB yoghurt alternatives due to the relatively low abundance of these minerals in vegan and vegetarian-based diets (Bickelmann et al., 2023; Neufingerl and Eilander, 2022). Additionally, it is important to consider that the bioavailability of these minerals in PB foods is decreased by the presence of antinutrients, such as phytates, oxalates, dietary fiber, polyphenols, etc. (Neufingerl and Eilander, 2022), which means that vegans and vegetarians may require higher dietary intake to meet their nutritional needs. Furthermore, according to product labels, samples VY5-VY25 contained added salt (marked on the product label as sea salt, salt, or iodised salt). It's important to acknowledge that the incorporation of different types of salts may have an impact on the mineral content of these products (Di Salvo et al., 2023).

According to the product labels, nine PB yoghurt alternatives (VY9-S to VY11-S; VY14-O to VY16-O; VY19-O; VY21-O; VY24-C) had been fortified with calcium to meet the recommended calcium nutritional requirements. Therefore, the studied yoghurt alternatives were divided into two groups: natural and enriched products. Our results showed that in natural products the calcium content ranged between 1.6 and 26.4 mg/100 g. While in the fortified PB yoghurt alternatives, the calcium content reached a target value of 120 mg/100 g, except for one product, VY10-S, which contained 108.5 mg per 100 g of calcium (Fig. 2 Supplementary Materials Table S4).

In general, the studied PB yoghurt alternatives exhibited variable levels of iron, magnesium, and zinc. The iron content showed a significant nine-fold variation among the evaluated products. Specifically, in lupin-, soya-, oat- and coconut-based products iron content ranged from 0.0 to 0.2 mg/100 g, 0.4 to 0.9 mg/100 g, 0.0 to 0.3 mg/100 g, and 0.2 to 0.5 mg/100 g, respectively. As for magnesium content, there was a remarkable 25-fold variation across the studied products. In lupin-, soya-, oat- and coconut-based yoghurt alternatives, magnesium content spanned from 1.2 to 1.7 mg/100 g, 16.6 to 29.8 mg/100 g, 1.2 to 9.9 mg/100 g, and 11.9 to 24.1 mg/100 g, respectively. The zinc content exhibited a six-fold variation across all products. In lupin-, soya-, oat- and coconut-based products zinc content ranged from 0.0 to 0.1 mg/100 g, 0.3 to 0.6 mg/100 g, 0.0 to 0.2 mg/100 g, and 0.1 to 0.3 mg/100 g, respectively. Statistical analysis revealed general differences in iron and zinc contents between soya-, oat-, and coconut-based products. Additionally, when considering magnesium, the studied samples could be categorized into two groups: lupin- and oat-based products with relatively higher and soya- and coconut-based products with lower magnesium content.

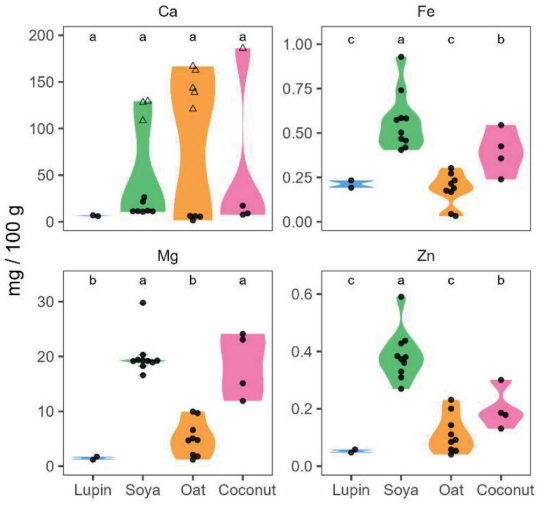

Fig. 2. The violin plot distributions of PB yoghurt alternatives (lupin-, soya-, oat- and coconut-based) related to calcium, iron, magnesium, and zinc content. Calcium-enriched products are marked with triangles on the Ca violin plot. The mean value of each product is denoted by a dot on the plot; groups with different letters are significantly different at $p < 0.05$.

The results of mineral content analysis in the current study align well with those from previous research on soya-, oat-, and coconut-based milk and yoghurt alternatives (Astolfi et al., 2020; Rebellato et al., 2023; Walther et al., 2022). The obtained results were compared with those of traditional dairy products, as PB yoghurt alternatives are often considered as substitutes. Buttriss and Rebelatto et al. have shown that cow's milk yoghurt contains 172 ± 26 mg/100 g of calcium, 0.1 ± 0.0 mg/100 g of iron, 16 ± 4 mg/100 g of magnesium, and 0.6 ± 0.1 mg/100 g of zinc (Buttriss, 2003; Rebellato et al., 2023). Our results indicated that the natural calcium content in the PB alternatives was approximately 18 times lower than that in cow's milk yoghurt. On the contrary, iron content was found to be 10 times higher in PB yoghurt alternatives compared to bovine milk yoghurt. The soya- and coconut-based yoghurt alternatives demonstrated magnesium content comparable to that of cow's milk yoghurt, with only a few soya-based products containing zinc amounts similar to those in traditional yoghurt.

The analysis of 25 PB yoghurt alternatives revealed that their levels of iron, magnesium, and zinc fell below the minimum requirements for a mineral nutrition claim (iron ≥ 2.1 mg/100 g, magnesium ≥ 56.3 mg/100 g, and zinc ≥ 1.5 mg/100 g), as specified in Regulation No 1924/2006 by (The European Parliament and the Council of the European Union. Regulation No 1924/2006, 2006) As a result, these products could not be marked as sources of iron, magnesium, or zinc. Nevertheless, the lower limit for calcium nutritional claim was achieved in almost all fortified products according to EU regulations (The European Parliament and the Council of the European Union. Regulation No 1924/2006, 2006).

3.3. Phytate content

Phytates (IP_6, IP_5, IP_4, IP_3, IP_2, IP_1) are widely distributed in the plant kingdom and are known to hinder the absorption of minerals (Schlemmer et al., 2009). In the present study, the IP_6 was the major phytate form ranging 20-fold across the studied products (Fig. 3a, Supplementary Materials Table S5). Phytates with a lower degree of phosphorylation, IP_5 and IP_4 were also found in minor amounts ranging 4-fold in the studied products (Fig. 3a). All other forms of IPs (IP_3, IP_2 and IP_1) were below the limit of quantification 3, 12, and 15 mg/100 g, respectively (Tanilas and Kriščiunaite, 2022). IP_6 concentration in lupin-, soya-, oat-, and coconut-based products was from 40.6 to 44.0 mg/100 g, 120.0 to 162.7 mg/100 g, 7.1 to 45.4 mg/100 g, and 20.0 to 53.5 mg/100 g, respectively. IP_5 concentration in lupin-, soya-, oat-, and coconut-based products was from 8.2 to 10.5 mg/100 g, 8.8 to 20.3 mg/100 g, 4.9 to 14.4 mg/100 g, and 4.6 to 9.3 mg/100 g, respectively. Each studied product contained up to 3 mg/100 g of IP_4.

As the mineral bioavailability is influenced by phytate content in consumed foods, the FAO/INFOODS/IZINCG Global Food Composition Database for Phytate has been compiled to address this issue (Dahdouh et al., 2019). It provides valuable nutritional information regarding phytate content, mineral composition, and calculated molar ratios of phytates to minerals for estimation of the effect on mineral bioavailability. This database contains multiple entries for soya-based milk alternatives ($n = 58$) with phytate content ranging from 91 to 173 mg/100 g (INFOODS, 2023). The results of our study on soya-based products align with these previously published findings (INFOODS, 2023). In contrast, previous study has shown that soy milk contained 935 mg/L of

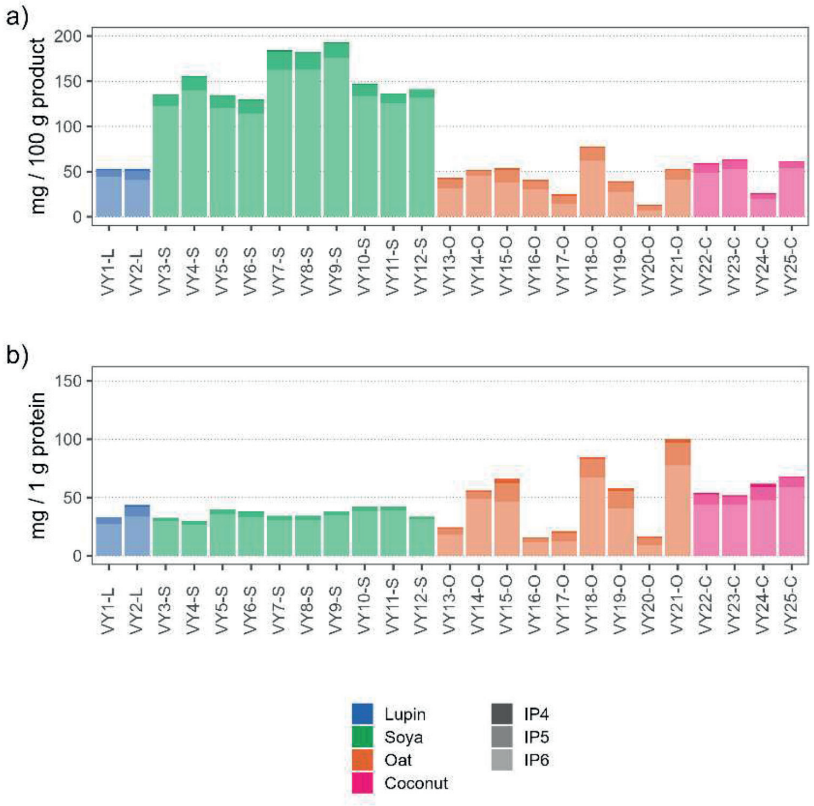

Fig. 3. The results of phytate analysis in the studied products: a) phytates per 100 g of product and b) phytates per 1 g of protein.

phytic acid (Burgos-Luján and Tong, 2015). However, limited data is available regarding phytate content in other plant sources used for yoghurt alternatives. Specifically, coconut kernel without any special production treatment were found to contain 136 mg/100 g (INFOODS, 2023). It has been reported that oat grains contained 279 ± 2 mg/100 g (Alemayehu et al., 2021), oat brans 2769 mg/100 g (INFOODS, 2023) and cereal cream 426 mg/L (Burgos-Luján and Tong, 2015) of phytates. Phytic acid content may vary in different lupin seed cultivars from 400 to 1200 mg/100 g (INFOODS, 2023; Trugo et al., 1993). Phytate levels in yoghurt alternatives are influenced not just by the source of plant ingredients but also by various factors, including the extraction methods, fermentation starter cultures and the technological processes used in final product manufacturing (Marklinder et al., 1995). Thus, a direct relationship between phytate content in yoghurt alternatives and their plant sources cannot be established.

Phytates, originating from the protein source, were also compared based on their density per gram of protein in the studied products, as illustrated in Fig. 3b. In the case of studied soya-based products, with soy being the only protein source, the phytate content is closely linked to the protein content, meaning that high protein brings about high phytates. On the contrary, the oat-based products exhibited the highest variability in phytates per gram of protein (6-fold difference), compared to lupin-, soya- and coconut-based products, which were fairly stable. This variability might suggest that the raw materials and production processes for lupin-, soya-, and coconut-based products are better standardized. In contrast, the utilization of oats as a novel raw material and its integration into new production techniques may still be evolving. Furthermore, the inclusion of minor protein components in the oat-based products may have contributed to further variability in the results.

3.4. Estimated phytate inhibitory effect on bioavailability of mineral compounds

3.4.1. Estimated bioavailability of iron and zinc

Phytates with higher phosphorylation state (IP_6, IP_5, P_4) are known to hinder the absorption of minerals (Schlemmer et al., 2009). Thus, we calculated the bioavailability of minerals in the studied products using Eqs. (2)-4. The PHY:Fe molar ratio in our study was found to range from 10 to 52 across all the products (Table 2). Specifically, for lupin-, soya-, oat-, and coconut-based products, the PHY:Fe molar ratio varied roughly by factors of 1, 3, 3, and 2, respectively. The recommended PHY:Fe value is <1, and preferably <0.4, to ensure that iron absorption remains unaffected by phytates (Hurrell and Egli, 2010). Unfortunately, none of the studied yoghurt alternatives met this recommended PHY:Fe value, as all of them exhibited high phytate and low iron content.

The PHY:Zn molar ratio varied significantly, ranging from 19 to 109 across all the products studied. In the case of lupin-, soya-, oat-, and coconut-based products the PHY:Zn molar ratio differed by factors of 1, 2, 3, and 2, respectively. According to the European Food Safety Authority (EFSA), PHY:Zn ratios should ideally be below five for high absorption efficiency or fall within the range of 5–15 for moderate absorption, and in case of exceeding 15 have low estimated bioavailability (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014). Nevertheless, all the yoghurt alternative products examined exhibited PHY:Zn values above the recommended range, indicating low estimated zinc bioavailability for all these products.

Table 2
The molar ratio of phytates to mineral compounds in studied PB yoghurt alternatives.

Ratio values	PHY:Fe < 0.4 preferred < 1 recommended	PHY:Zn < 5 very good 5 – 15 moderate > 15 low	PHY:Ca < 0.24 recommended	PHY × Ca/Zn < 200 recommended	PHY × Ca/Mg n.a.**	PHY × Ca/Fe n.a. **
code						
VY1-L	20	91	0.55	14	0.25	3
VY2-L	24	109	0.48	20	0.20	4
VY3-S	20	50	0.70	15	0.09	6
VY4-S	18	41	0.44	23	0.15	10
VY5-S	25	35	0.77	10	0.07	7
VY6-S	24	34	0.71	10	0.07	7
VY7-S	28	43	1.02	12	0.10	8
VY8-S	27	42	0.98	12	0.10	8
VY9-S*	33	54	0.09	172	1.20	105
VY10-S*	30	44	0.08	122	0.77	82
VY11-S*	29	44	0.06	143	0.90	93
VY12-S	13	24	0.33	16	0.12	9
VY13-O	16	73	0.46	12	0.21	2
VY14-O*	17	25	0.02	91	0.70	57
VY15-O*	29	62	0.02	239	1.57	103
VY16-O*	19	44	0.02	187	1.25	77
VY17-O	52	59	0.26	10	0.07	8
VY18-O	22	34	0.88	5	0.04	3
VY19-O*	20	35	0.02	154	1.35	83
VY20-O	37	24	0.55	1	0.01	1
VY21-O*	22	36	0.03	115	0.93	65
VY22-C	15	31	0.41	7	0.02	3
VY23-C	10	21	0.23	9	0.04	4
VY24-C*	10	19	0.01	95	0.39	44
VY25-C	13	34	0.50	7	0.03	2

* Product label statement - 120 mg of Ca contained in the 100 g of product, except VY24-C - 160 mg.
** n.a. – no available recommendations.

3.4.2. Estimated bioavailability of calcium

The PHY:Ca molar ratio exhibited a notable range in both natural and calcium-fortified PB yoghurt alternatives, spanning from 0.23 to 1.02 (a 5-fold difference) and 0.01 to 0.09 (a 10-fold difference), respectively. It's important to note that the recommended PHY:Ca value is <0.24 (Morris and Ellis, 1985). Accordingly, we observed that the molar ratios to phytate content were below 0.24 in ten samples, all of which were fortified with calcium. Among natural unfortified products, VY23-C stood out as an outlier with low phytates and calcium content. In contrast, all other products were low in calcium and high in phytate, which lowers the estimated bioavailability of a given mineral. To meet nutritional requirements, PB products must be fortified with calcium to provide an adequate amount of this mineral compound.

According to the FAO/INFOODS/IZINCG Global Food Composition Database for Phytate the average content of calcium and phytates in soya-based milk alternatives was 16 ± 4 mg/100 g and 132 ± 41 mg/100 g, respectively. As a result, the calculated PHY:Ca molar ratio for these products was 0.5, indicating low estimated calcium bioavailability. Thus, the findings of our study on soya-based yoghurt alternatives align well with previously reported data on milk alternatives (INFOODS, 2023).

The calculations of mineral bioavailability in our study show that, in general, PB yoghurt alternatives contain more minerals than cow's milk, but they are still low in available minerals. Furthermore, micronutrient bioavailability may be compromised by the presence of phytates in these products. Indeed, there were some exceptions in cases where the studied PB yoghurt alternatives had been enriched with calcium. The intentional enrichment with calcium and other minerals can be a valuable strategy to address the limitations associated with PB dairy alternatives, making such products a more favourable choice to maintain or improve mineral intake or degradation of higher phytates (IP_6, IP_5, and IP_4) into lower phytates (IP_3, IP_2 and IP_1) which do not exhibit an inhibitory effect on mineral bioavailability.

3.4.3. Estimated bioavailabilities of potentially competing mineral compounds

To assess the potential competition between calcium and other minerals like zinc, magnesium, and iron in terms of how well the body can absorb them, we calculated ratios (PHY × Ca/Zn, PHY × Ca/Mg, and PHY × Ca/Fe) for the studied products. For products without added calcium, these ratios ranged from 1 to 23, 0.01 to 0.25, and 1 to 10, respectively (Table 2). In contrast, products fortified with calcium had ratios ranging from 91 to 239, 0.39 to 1.57, and 44 to 105 respectively.

A recommended value for the PHY × Ca/Zn ratio is <200 (Ma et al., 2007). Except for one calcium-fortified product (VY15-O), all samples studied had ratios below 200, indicating that the presence of calcium, whether fortified or not, didn't significantly affect the estimated bioavailability of zinc. This trend was similarly observed for PHY × Ca/Mg and PHY × Ca/Fe ratios, suggesting that higher calcium content in fortified products might hinder the absorption of magnesium and iron. However, there are no established recommended ratio values for ensuring high bioavailability of magnesium and iron. Further research is needed to understand how the combined influence of calcium and phytates impacts the bioavailability of other mineral compounds in PB products and diets.

3.5. Saponin content

Saponins, a diverse class of chemicals, due to their amphiphilic molecular properties have different health properties, starting from health-promoting to antinutritional effects, and some of them are contemplated to be bitter-tasting (Li et al., 2022; Milgate and Roberts, 1995; Önning et al., 1996; Rao and Koratkar, 1997; Samtiya et al., 2020; Sang and Chu, 2017). Oat and soya crops as raw materials of food production have been studied for saponins distribution and content (Chitisankul et al., 2018; Günther-Jordanland et al., 2020; Pecio et al., 2013). In contrast, there is a lack of research on saponin levels in PB yoghurt alternatives.

In the current study, saponins were determined in soya- and oat-

based products by applying a recently published quantification method (Bljahhina et al., 2023b, 2023a). There was a 2- to 5-fold variability in the content of soyasaponins among soya-based products (Fig. 4a, Supplementary Materials Table S6). In the case of soya-based products soyasaponin Bb, soyasaponin Ba, and soyasaponin Ab were found to range from 7.2 to 13.6 mg/100 g, 1 to 2.2 mg/100 g, and 1.4 to 7.1 mg/100 g, respectively; soyasaponin Aa levels were below the limit of quantification. In oat-based products, the oat-specific saponins, namely avenacoside A, avenacoside B, and 26-desglucoavenacoside A were quantified. Avenacoside A content ranged from 0.8 to 5.5 mg/100 g, avenacoside B from 0.2 to 2.4 mg/100 g, and 26-desglucoavenacoside A from 0.2 to 2.7 mg/100 g. Hence, the concentration of individual avenacosides exhibited a more substantial 7- to 13-fold variability in oat-based products compared to the lower variability of soyasaponins observed in soya-based products.

Generally, all the studied soya-based products contained higher levels of protein along with elevated saponins concentrations. However, due to the absence of specific information regarding the crop varieties and production techniques employed in the creation of these products, it becomes challenging to precisely determine the extent of saponins migration into the final products. One potential approach is to standardize the protein content among the various products under investigation and then evaluate the potential saponins density within the protein of these products. Fig. 4b illustrates the saponin content per gram of protein in the products. Soya-based yoghurt alternative production technology processes are more standardised, therefore the variability of the soyasaponin content was 1.6-fold in 1 gram of protein. In contrast, the variability in avenacosides content within oat-based products per one gram of protein differed 8-fold within the product group. Possibly, the variability in saponin results (Fig. 4b) in oat-based products could be influenced by added minor protein sources (potato and bean protein, listed in Table 1; Supplementary Material Table S1). However, according to the ingredient list, these added protein components are likely to have a minimal impact on protein content.

Saponins are not digested by human digestive enzymes, only gut microbiota can degrade saponins (Amin et al., 2011; Hu et al., 2004). Therefore, saponins could potentially affect various aspects of human physiology, such as protein digestion, mineral and fat-soluble vitamin absorption, and intestinal mucosa permeability, and certain types of saponins and their secondary metabolites may have a positive preventive effect on Metabolic Syndrome (Luo et al., 2020). Soya and oat saponins have distinct chemical structures (triterpenoid and steroid, respectively), and the exact mechanism by which both saponins affect the bioavailability and digestibility of macro- and micronutrients remains unknown. Consequently, it is challenging to determine which saponins, whether from soya or oat, might have a more pronounced negative impact on protein digestibility and the bioavailability of mineral compounds. Health intervention studies are needed to elucidate the effects of saponins on humans under conditions relevant to food matrices.

The limited availability of standard compounds and quantification methods for saponins specific to plant protein sources has been a significant impediment to conducting comprehensive studies on saponin content in ready-to-eat products and throughout the production chain. Only limited data has been available on the contents of saponins in PB yoghurt alternatives until now. The saponins results obtained in the

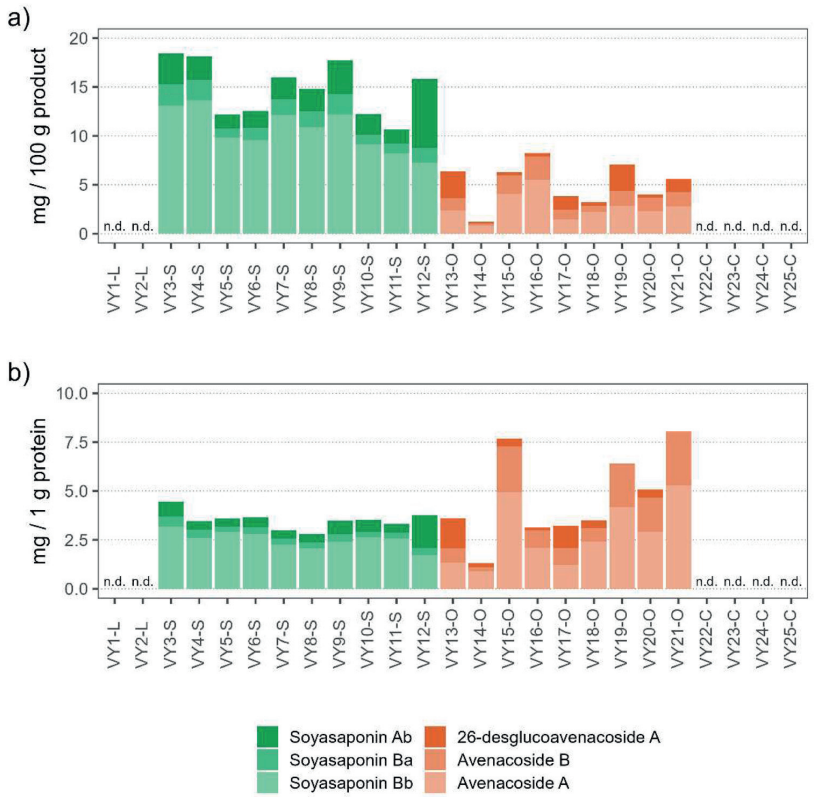

Fig. 4. The results of saponin analysis in the studied products: a) saponins per 100 g of product and b) saponins per 1 g of protein. n.d. not determined. *Avenacoside B and 26-desglucoavenacoside A are quantified in avenacoside A equivalents.

present study were consistent with recently published findings for oat drinks and soya-based yoghurt alternatives (Bljahhina et al., 2023b, 2023a). In the case of lupin- and coconut-based yoghurt alternatives, the quantification methods, or standard compounds for saponins determination were not available. Consequently, these matrices were excluded from the saponin analysis. In future studies, a more comprehensive assessment of saponin content, which includes the incorporation of various PB protein sources and specific analytes, could offer valuable insights into the presence and distribution of saponins in PB yoghurt alternatives.

4. Conclusion

Our study on a list of selected PB yoghurt alternatives currently available on the European market revealed that PB products do not meet the nutritional criteria to be considered full substitutes for traditional dairy-based yoghurt. This conclusion was drawn based on the analysis of protein quality, mineral content, estimated mineral bioavailability, and quantification of phytates and saponins. Even soya-based products with high protein levels failed to meet the required standards for protein quality. The PDCAAS values and the limiting amino acids varied significantly among PB yoghurt alternatives produced from different main protein sources. Generally, the content of essential minerals such as zinc, iron, magnesium, and calcium, was below requirements, except for those that had been fortified with calcium. Furthermore, the bioavailability of these minerals present at low concentrations would have been affected by the naturally occurring phytates in these products, as shown by the estimated mineral bioavailability results. Soya-based yoghurt alternatives contained the highest amounts of phytates and saponins among the studied products.

Studies like this offer crucial information for nutrition specialists on how to incorporate these products into dairy-free diets, taking into account their chemical and nutritive characteristics. The current study has certain limitations, primarily related to the present state of the PB market, including a limited range of protein sources and producers available. Globally, there is still a lack of knowledge regarding the interactions between some antinutrients and specific nutrients, and in many cases, the inhibitory concentrations have not been determined. For instance, more research effort is needed to better understand the interactions between saponins and proteins in food matrices. Nevertheless, the analysis of the essential nutritional profile of commercially available PB yoghurt alternatives provides an overview of the current situation on the PB market and highlights the aspects that require enhancement for optimizing the nutritional value of yoghurt alternatives.

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Ethical statement – studies in humans and animals

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Anastassia Zeinatulina: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kristel Tanilas:** Writing – review & editing. **Kristel Ehala-Aleksejev:** Writing – review & editing. **Ene Viiard:** Writing – review & editing. **Tiina Kriščiunaite:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100545.

Data availability

All data is provided in supplementary materials.

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Appendix 6

Publication V

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Original article

Optimisation of extraction method for quantification of free fatty acids in oat-, pea- and faba-bean-based protein sources by liquid chromatography coupled with mass spectrometry

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ABSTRACT

Plant-based protein sources, including oat, pea, and faba bean, are widely used in milk and meat alternatives. However, these crops can be inherently bitter, which limits their use in some food products. Processing into flours, protein concentrates, and isolates may cause changes in chemical composition and influence bitterness. We present an optimised and validated LC-MS method using isopropanol:methanol (1:1, *v/v*) solvent extraction to determine selected bitter-tasting free fatty acids (FFAs). Accurate quantification of FFAs (linolenic, myristic, palmitic, linoleic, oleic, and stearic acids) was achieved using isotopically labelled oat flour extract as an internal standard. The total FFA content in whole grain oat flour, oat protein concentrate, pea flour and protein isolate, and faba bean flour and protein isolate ranged from 4.4 to 3841 mg/100 g dry weight. FFA analysis aids in assessing bitterness and selecting processing techniques to reduce these compounds in plant-based foods.

1. Introduction

Consumers express significant interest in plant-based protein products, driven by health, environmental, and ethical concerns [1]. However, the broader acceptance of these products is impeded by multiple factors, including off-taste, over-processed flavour, texture, appearance, availability, and price [2]. Plant-based products made from oat (*Avena sativa* L.), pea (*Pisum sativum* L.), and faba bean (*Vicia faba* L.) are often described as having "green", "grassy", "beany", "fatty", and "bitter" taste [3]. Several molecule classes in these crops contribute to the bitter taste, including saponins, tannins, flavonoids, and free fatty acids (FFAs) [3–7]. Bitter-tasting FFAs, including myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), have been identified in oats [5,8], peas [4,9], and faba beans [7,10]. Previous sensory studies have shown that product developers cannot rely solely on sensory and consumer tests to assess bitterness in plant-based products made from oat and pea, as assessors often lack consensus [11]. Given the subjective nature of sensory tests for bitter taste, a more objective approach to estimate the perceived bitterness is to determine the content of FFAs and calculate their dose-over-threshold (DoT) values [8]. While eliminating bitterness is a key challenge in the production of plant-based dairy and meat alternatives [12,13], this study focuses on optimizing a method to quantify bitter-tasting FFAs. Accurate FFA quantification is crucial for assessing the effectiveness of processing techniques aimed at reducing these compounds.

FFAs have been extensively analysed in foodstuffs using gas chromatography (GC) coupled with a flame ionization detector or mass spectrometry [14,15]. This method typically requires hexane extraction, solid-phase extraction, and derivatisation of the sample matrix before analysis [16]. However, the labour-intensive chemical derivatisation of FFAs into fatty acid methyl esters can produce undesirable by-products from decomposed glycerides during lipid esterification [17]. Moreover, high column temperatures used during GC-MS analysis to volatilize sample components can lead to triglyceride breakdown, forming FFAs and complicating their selective determination [17]. GC analysis is complicated by extensive sample preparation, by-product formation, and thermal degradation, which can compromise the accuracy of FFA determination. To address these issues, high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) offers an alternative, operating at ambient or moderately elevated temperatures. This method enables more accurate analysis of bitter lipid-derived

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components without derivatization or excessive heat, as demonstrated in studies on pea protein isolate and oat flour [8,9]. Additionally, isotopically labelled internal standards can be added in minimal, cost-effective amounts to post-extraction samples, ensuring precise quantification of analytes using HPLC-MS [18–20]. While GC-MS often offers higher sensitivity for properly derivatized volatile FFAs, HPLC-MS provides a more straightforward and reliable method for accurate routine analysis of FFAs.

Previously published extraction methods for different lipid compounds in various matrices can be categorised in two groups. The first group includes classical liquid-liquid extraction (LLE) methods employing immiscible solvents, such as Bligh and Dyer, Folch, Soxhlet, and Matyash methods, which are not suitable for LC-MS analysis without solvent exchange [21–24]. The second group consists of methods using miscible solvents, like methanol (MeOH) and isopropanol (IPA), which are compatible with direct injection into LC-MS [4,5,8,9]. Additionally, as green chemistry principles are gaining popularity, traditional LLE solvents are increasingly being replaced with less hazardous alternatives like MeOH and IPA, where possible [25]. Aqueous MeOH solution has been used to extract and detect bitter molecules in pea protein isolate [4,9], whole grain oat flour [5,8], and poppy seeds [26]. The application of MeOH for extraction of FFAs provides milder extraction conditions than traditional LLE by keeping glycerides from decomposition and oxidation, it is less time-consuming and can increase the throughput of analysis [8]. Currently, mild extraction methods are used for FFA extraction from various food matrices, but they involve different conditions and extraction steps, posing challenges to result comparability. A simple and universally applicable extraction method that ensures reliable and reproducible results across different plant-based protein sources is still lacking.

Considering these aspects, this project aimed to develop a universal extraction procedure for the determination of FFAs from oat, pea, and faba bean flours, as well as their protein concentrates and isolates. These raw materials were targeted because they are frequently used in the production of dairy and meat alternatives, either alone or mixed together to achieve nutritionally balanced amino acid profile [20,27]. The criteria guiding the development of the FFA extraction method were that the final quantification method had to be simple, sensitive, precise, accurate, reliable for routine analysis, high throughput, time efficient, and with improved eco-friendliness. We hypothesised that using a mixture of solvents, specifically MeOH and IPA, would improve the efficiency of FFA extraction. A design of experiments (DoE) approach was selected to find the optimal sample extraction conditions and HPLC-MS analysis for the detection and quantification of selected bitter-tasting fatty acids (C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3).

2. Materials and methods

2.1. Materials

HPLC-grade purity solvents acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), and propan-2-ol (IPA) were purchased from Honeywell (Charlotte, NC, USA). Ultrapure water (18.2 MΩ·cm) was prepared with MilliQ® HX 7040SD equipped with MilliQ LC-Pak (Merck KGaA, Darmstadt, Germany). Mobile phase additives acetic acid (Ac) and ammonium acetate (AmAc) were purchased from Thermo Fisher Scientific (NH, US) and VWR (PA, US), respectively. Antioxidants butylated hydroxy toluene (BHT) and indomethacin were purchased from Sigma-Aldrich (Darmstadt, Germany), and trans-4-(-4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid (t-AUCB) from Cayman Chemical (Michigan, US). Fatty acid standards linoleic acid (C18:2) and stearic acid (C18:0) were purchased from Sigma-Aldrich (Darmstadt, Germany); linolenic acid (C18.3) from Acros Organics (Thermo Fisher Scientific Inc., MA, US); oleic acid (C18:1), myristic acid (C14:0), and palmitic acid (C16:0) from Dr. Ehrenstorfer™ (LCG Group, Middlesex, UK). Uniformly isotopically labelled oat seed flour (U-^{13}C oat seeds, *Avena sativa* 97 atom%) was obtained from IsoLife BV (Wageningen, The Netherlands). The PTFE filter (pore size 0.2 µm, filter dimension 13 mm) were obtained from Merck KgaA (Darmstadt, Germany).

2.2. Samples

Faba bean protein isolate (FPI; Tendra®, Cosun Protein, Dinteloord, The Netherlands), pea protein isolate (PPI; Careprotein pea 80, Caremoli SpA, Nova Milanese, Italy), and oat protein concentrate (OPC; PrOatein, Lantmännen, Stockholm, Sweden) were obtained as product examples from producers. Faba beans (FB; Kati, Estonia), yellow dehulled peas (YP; Veski Mati, Estonia) and whole grain oat flour (WGOF; Loona Talu Veski, Estonia) were commercially obtained from a local supermarket. FB, YP, and WGOF were finely milled using Thermomix® multicooker (Vorwerk & Co. KG, Wuppertal, Germany). A pooled sample (Z) was used for method optimisation and validation, and it was prepared by mixing equal weights of finely ground FPI, PPI, OPC, FB, YP, and WGOF.

2.3. Development of extraction method

Detailed experimental designs used for sample extraction optimisation (Plackett–Burman [P–B], full factorial [F-F], and mixture designs) are described in Supplementary Materials. The developed method of sample extraction was partially based on previously published methods [8,9].

2.4. The optimised extraction method

Finely milled samples (50 mg, $n = 3$) were weighed into 2 mL tubes in triplicate and 1 mL of extraction solvent was added (IPA:MeOH, 1:1 *v*/v), then mixed thoroughly and incubated for 30 min in an Eppendorf Thermomixer® C (Eppendorf AG, Hamburg, Germany) at 15 °C and stirring set to 1400 rpm. The samples were centrifuged at 14,000 x *g* at, 10 °C for 10 min and the supernatant was filtered using a 0.2 µm PTFE filter. The sample was diluted to fit into the linear range of the calibration curve, resulting in a solution containing H_2O:MeOH:IPA (1:1:1, *v*/v), which is the starting composition of the mobile phases in the chromatographic gradient. The diluted filtrate (100 µL) was pipetted into clear tubes containing 100 µL of U-^{13}C-oat extract working solution and then injected into the LC-MS on a 96-well plate (700 µL Round well, polypropylene, Waters Corporation, Milford, MA, USA).

2.5. Preparation of standard solutions

Fatty acid mixture stocks were prepared so that each compound was approximately 40 mg/L in IPA. Aliquots of mixture stocks were stored in glass vials with crimp cap with PTFE septa at −80 °C. All preparations were done under nitrogen atmosphere. Stock solution of internal standard (IS) for fatty acids quantification was prepared by weighing 50 mg of isotopically labelled oat flour into a 2 mL tube and processing in the same way as the samples (Section 2.4). The resulting filtrate was aliquoted and stored at −80 °C until the analysis. The working solution of the internal standard was prepared by diluting thawed stock 10-fold resulting in a solution with a composition of H_2O:MeOH:IPA (1:1:1, *v*/v).

2.6. Calibration and quantification

The stock of standards was diluted using equal amounts of stock, MeOH, and IPA, resulting in 3-fold dilutions in a solution consisting of H_2O:MeOH:IPA (1:1:1, *v*/v). Further dilutions were prepared in H_2O:MeOH:IPA (1:1:1, *v*/v). Solutions of fatty acids for calibration curves and sample solutions were additionally diluted with constant amount of working solution of internal standard before the injections. Eight-point calibration curves (measured in triplicate) were built for all fatty acids in the range of 0.02–6.5 mg/L by plotting peak area ratios of fatty acids/

internal standard against the concentration of the external standard compound. The linear regression approach led to linear responses showing correlation coefficients of $R^2 > 0.99$ for calibration curves of all analytes.

2.7. Liquid chromatography and mass spectrometry

Samples were analysed using a Waters UPLC® system (Waters Corporation, Milford, MA, USA) coupled with a Waters Quattro Premier XE Mass Spectrometer equipped with a ZSpray™ Source, controlled by Waters MassLynx™ 4.1 software (V4.1 SCN805, Waters Corporation, Milford, MA, USA). The mobile phases were: (A) 60 % H_2O, 40 % MeOH, 10 mM AmAc, and 0.005 % Ac; and (B) 90 % IPA, 10 % MeOH, 10 mM AmAc, and 0.005 % Ac (Monnin et al., 2018). The weak needle wash consisted of 60 % H_2O and 40 % MeOH, while the strong needle wash was composed of 80 % MeCN and 20 % H_2O. The seal wash solution was 50 % aqueous MeCN (*v*/v). Samples were stored in an autosampler set to 8 °C, with an injection volume of 5 µL. Fatty acids were separated using an ACQUITY UPLC CSH C18 Premier column (2.1 × 100 mm, 1.7 µm) coupled with an ACQUITY UPLC CSH C18 VanGuard Pre-column (2.1 × 5 mm) from Waters Corporation [29]. The final gradient was: 0–0.5 min at 60 % A, 0.5–8.0 min linear gradient from 60 % to 20 % A, 8.0–8.6 min at 20 % A, 8.6–11.4 min linear gradient from 20 % to 1 % A, 11.4–12.0 min at 1 % A, 12.0–12.1 min change to 60 % A, and 12.1–17.00 min equilibrated at 60 % A. The column temperature was maintained at 55 °C, and the flow rate was set at 0.25 mL/min.

The analytes were ionized using negative electrospray ionization mode (ESI^-) under optimised source conditions. The source temperature was set to 120 °C, with high-purity nitrogen supplied at 50 L/h for the cone and 700 L/h for desolvation, and the desolvation gas heated to 300 °C. The capillary voltage was set to −2.3 kV, cone voltage 30 V, and the extractor voltage to 3 V. During a single chromatographic run, a set of *m/z* values for single-ion recording (SIR) experiments was recorded simultaneously. For the quantification of fatty acids, the deprotonated analytes $[M\text{-}H]^-$ were selected based on a scan-type experiment, with mass-to-charge ratios (m/z ± 0.5 Da) for SIR channels provided in Table 1. Data acquisition was performed using Waters MassLynx™ V4.1 (SCN805, Waters Corporation).

2.8. Method validation

The Eurochem validation guideline was used to evaluate the following parameters during method validation: specificity, selectivity, the linear range, limit of detection (LOD), limit of quantification (LOQ), precision and recoveries of the whole quantification method, and the matrix effect (ME) based on calibration standards and sample Z [30].

The linear range and linearity were evaluated through repeated measurements of standard solutions of unlabelled fatty acids and isotopically labelled fatty acids at eight different concentrations obtained by serial dilution of stock solutions. Specificity and selectivity were determined by analysing blank solvents and sample matrix. The sensitivity of the method was evaluated by calculating the LOD and LOQ values for fatty acids, as well as the standard deviation (s) of the peak areas of the lowest standard concentration point ($n = 6$), and the calibration curve slope, as marked in Eq. 1 and Eq. 2 [30].

$$LOD = \frac{3s}{slope} \tag{1}$$

$$LOQ = \frac{10s}{slope} \tag{2}$$

To evaluate intra-day precision, a standard solution containing all analytes and internal standards was injected six times. For inter-day precision, the stability of retention times and peak areas was confirmed by analysing standard solutions over three separate days. The precision of the extraction method was determined using pooled sample Z by measuring repeatability (intra-day) and intermediate precision (inter-day). Repeatability involved conducting six repeated analyses of the samples on the same day. Intermediate precision was evaluated by analysing samples on three different days over a month under the same experimental conditions. To assess total recoveries of fatty acids, the Z sample was spiked with a known quantity of fatty acids at three different concentration levels ($n = 3$) before extraction. The initial concentration of FFAs was determined by analysing the unspiked sample Z matrix. The ME was evaluated by applying post-extraction spiking method to sample extracts with calibration standard solutions and comparing the slopes of solvent-matched calibration curves with those of matrix-matched curves [31].

Table 1
The m/z values of fatty acids and isotopically labelled internal standard compounds.

Analyte	Abbreviation	[M-H]
myristic acid	C14:0	227.2
palmitic acid	C16:0	255.2
stearic acid	C18:0	283.2
oleic acid	C18:1	281.2
linoleic acid	C18:2	279.2
linolenic acid	C18:3	277.2
^{13}C-palmitic acid	^{13}C16:0	271.2
^{13}C-stearic acid	^{13}C18:0	301.2
^{13}C-oleic acid	^{13}C18:1	299.2
^{13}C-linoleic acid	^{13}C18:2	297.2
^{13}C-linolenic acid	^{13}C18:3	295.2

2.9. Calculation of bitter-taste contribution of FFAs

The impact of FFAs on the perception of bitter taste in the study samples was calculated using Eq. 3 [8]. The DoT-factor was determined by calculating the ratio of the concentration to the taste threshold (TC). The TC values used were as follows: C14:0 – 1703 µmol/L, C16:0 – 1546 µmol/L, C18:0 – 726 µmol/L, C18:1 – 2180 µmol/L, C18:2 – 1810 µmol/L, and C18:3 – 277 µmol/L, as reported in previous studies [8,9].

$$Dose\text{-}over\text{-}threshold\ (DoT) = \frac{concentration\ of\ FFA\ \left(\frac{\mu mol}{kg}\ dwb\right)}{taste\ threshold\ \left(TC;\ \frac{\mu mol}{L}\right)} \tag{3}$$

2.10. Visualization and statistics

Waters QuanLynx™ V4.1 (SCN805, Waters Corporation, Milford, MA, USA) and Microsoft Excel® (Microsoft 365 Apps for enterprise) were used to performed data analysis. The free software CAT Chemometric Agile Tool was used for experimental design, data analysis, and interpretation [32]. Linear models without interactions were fit to the Placket-Burman design, linear models with two-way interactions were fit to the full factorial design, and linear models with two- and three-way interactions were used for the mixture design. Data visualization was performed in R 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria) using package "ggplot2" version 3.5.1. Statistical significance of coefficients was calculated using *t*-tests. ANOVA followed by Tukey–Kramer *post hoc* test was performed to the results of analysed samples with R package "agricolae" 1.3–5. The significance level was set to 0.05.

3. Results and discussion

3.1. Chromatographic and mass-spectrometric parameters

During method development CSH C18 column with acetic acid and

ammonium acetate was used as mobile phase additives [28]. Six fatty acids (C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3) were selected as target analytes based on previous studies [8,9,26]. Representative chromatograms of fatty acids from the standard solution and Z sample are shown in Supplementary Fig. S1. Isotopically labelled oat flour extract was used as a source of standard compounds, containing fully labelled ^{13}C-fatty acids without cross contamination from unlabelled fatty acids. The extract provided sufficient concentrations of fully labelled palmitic, oleic, linolenic, linoleic, and stearic acids. However, the concentration of ^{13}C-myristic acid was too low to serve as an IS for myristic acid (Supplementary Fig. S1B). Instead, ^{13}C-linolenic acid was tested as IS, as its retention time was similar to myristic acid. The usage of ^{13}C-linolenic acid as IS for myristic acid resulted in acceptable validation results of myristic acid (see Section 3.3). Therefore, the ^{13}C-linolenic acid was used for calibration in addition to the external standard to enable more precise mass spectrometric quantification of myristic acid. Similar approaches using isotopically labelled plants have been successfully used in quantitative analysis of avenacosides and phytates [18–20]. The optimised LC-MS method separated the fatty acids and their isotopically labelled counterparts within 15min.

3.2. Optimisation of sample preparation method

Three experimental series employing different design methodologies were conducted to optimise the sample extraction parameters.

The first experiment used the P–B experimental design to identify key factors in the sample extraction procedure for further optimisation. The main extraction approach was adapted from previously published methods for FFAs in plant-based matrices [8,9,26]. Seven extraction parameters were chosen to construct the experimental design matrix, namely sample weight, extraction solvent, antioxidant usage, number of extraction steps, extraction volume, extraction equipment, and extraction time, as shown in Supplementary Table S3. The results of this prescreening experiment showed that antioxidants, extraction solvent, and extraction time were the significant factors, whereas sample weight, extraction steps, volume, and equipment had minimal impact (Fig. 1). The data also revealed that using a H_2O:MeOH solution (1:1, *v*/v) resulted in C18:0 concentrations below the limit of quantification (Supplementary Table S3), making it impossible to calculate coefficients for this analyte in the experiment.

A F-F experimental design was conducted to verify and optimise the extraction solvent, antioxidant usage, and extraction time as they were identified as important parameters in the P–B design. The experimental design matrix for F-F is presented in Supplementary Table S4. This experiment revealed that the composition of the extraction solvent was the most critical factor, while the other parameters were not statistically significant (Fig. 2). Note that the extraction solvents used in the P–B and F-F experiments differed in composition. The highest fatty acid extraction efficiency was achieved with a 50 % aqueous IPA solution, which was not included in the P–B experiments. The mixture of antioxidants (BHT, indomethacin, and t-AUCB), previously used in FFA analysis of foodstuffs [33], was tested at varying concentrations in both P–B and F-F experiments. Unlike P–B, the results showed no difference in fatty acid yield regardless of the presence of antioxidants. This may be attributed to the shorter extraction time and different solvent composition used in the F-F design. Conversely, in the case of P–B design, this may be attributed to the aliasing of main effects with some two-way interactions that are not measured in this type of experimental design.

As both P–B and F-F experiments identified the extraction solvent as the most critical factor in the sample extraction procedure, a mixture design was employed to further optimise its composition. The corresponding experimental design matrix is shown in Supplementary Table S5. Three solvents were selected: water, MeOH, and IPA. Aqueous MeOH solution has been used in previously published studies [9,26], while IPA was used in the mobile phase and in the standard solution in this study. The results of the mixture design experiment showed that the highest FFAs yield was achieved with a MeOH:IPA (1:1, *v*/v) ratio (Fig. 3), aligning with the P–B and F-F experiment findings on solvent compositions. Statistically, there were no significant differences between extraction yield of FFAs in IPA:MeOH (1:1, *v*/v), H_2O:IPA:MeOH (1/6:4/6:1/6, *v*/v), and even in H_2O:IPA:MeOH (1/3: 1/3: 1/3, *v*/v). Previous studies have demonstrated the use of aqueous IPA solutions for extracting FFAs from oils [34]. However, our results show that a mixture containing up to 1/3 water can also be used, offering reduced toxicity and lower solvent consumption. Furthermore, it can be concluded that a small concentration of water in the sample does not compromise the yield of FFAs. However, taking into account that the yields of C16:0 and C18:0 in IPA:MeOH (1:1, *v*/v) were slightly higher (Fig. 3), the binary mixture was considered the more practical and manageable option for the extraction of FFAs. Both IPA and MeOH effectively dissolve the tested FFAs and are LC-MS compatible, eliminating the need for solvent evaporation and changes required when using immiscible solvents.

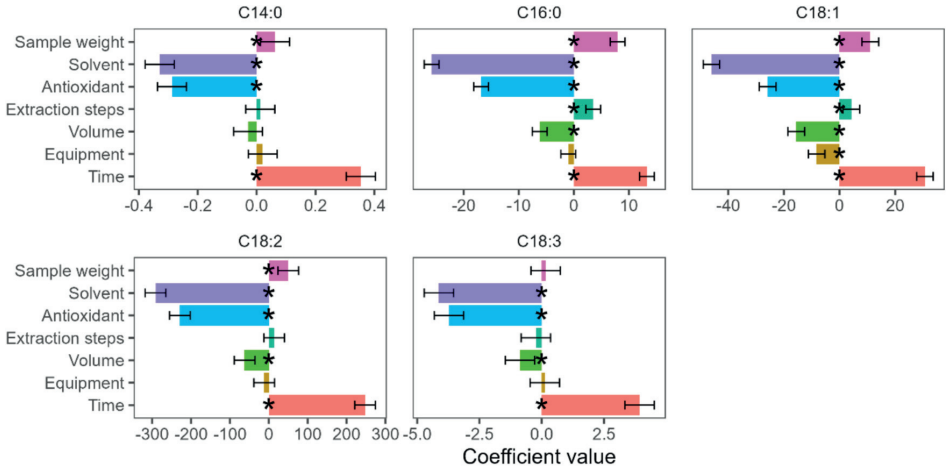

Fig. 1. Standardised effect plots from the Plackett–Burman experimental design, conducted using pooled sample Z, for the optimisation of the free fatty acid extraction step. Asterisks indicate statistically significant coefficients ($p < 0.05$). R^2 0.96–0.99. C14:0 – myristic acid, C16:0 – palmitic acid, C18:1 – oleic acid, C18:2 – linoleic acid, C18:3 – linolenic acid.

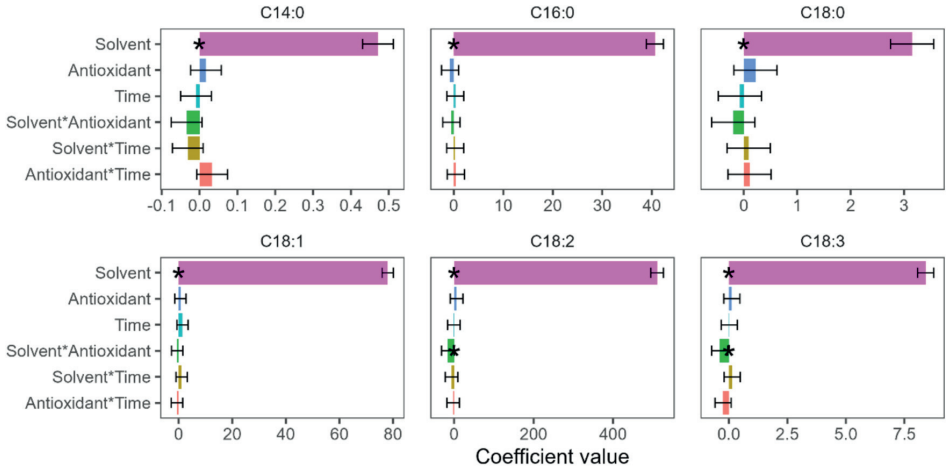

Fig. 2. Standardised effect plots from the full factorial experimental design, conducted using pooled sample Z, for the optimisation of the free fatty acid extraction step. Asterisks indicate statistically significant coefficients ($p < 0.05$). R^2 0.91–0.99. C14:0 – myristic acid, C16:0 – palmitic acid, C18:0 – stearic acid, C18:1 – oleic acid, C18:2 – linoleic acid, C18:3 – linolenic acid.

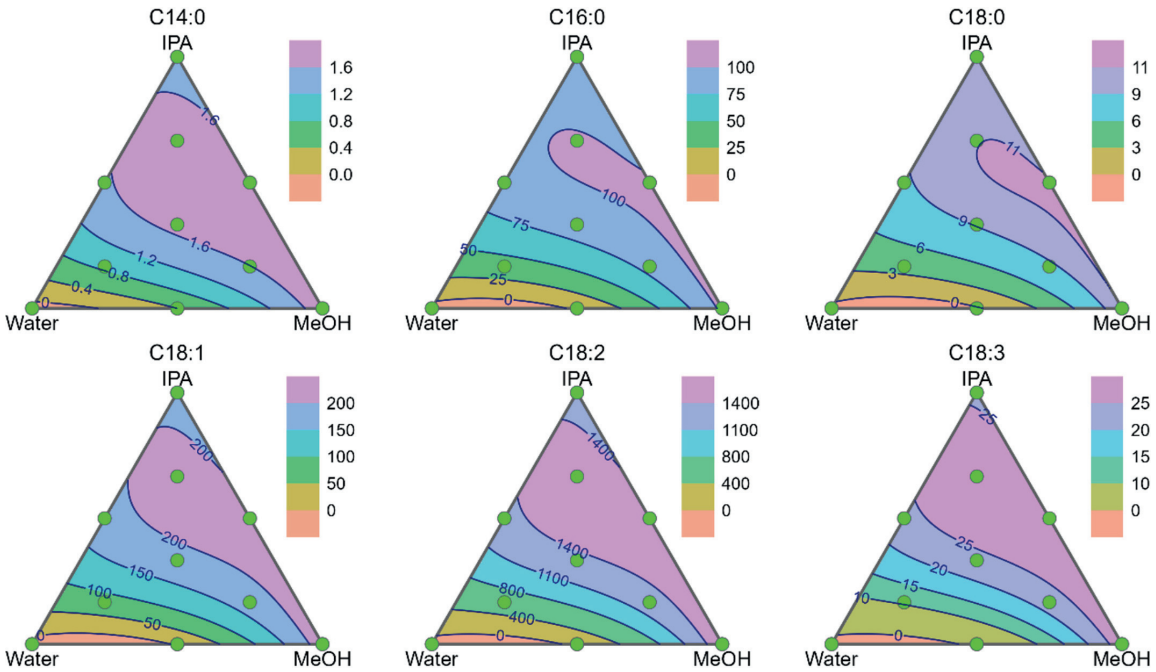

Fig. 3. The contour plots of mixture design experiment performed using pooled sample Z. Green points show the performed experiments ($n = 3$). The values and colours show free fatty acids yield ($p < 0.05$). R^2 0.82–0.88. C14:0 – myristic acid, C16:0 – palmitic acid, C18:0 – stearic acid, C18:1 – oleic acid, C18:2 – linoleic acid, C18:3 – linolenic acid, IPA – isopropanol, MeOH – methanol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2 provides a comparison of the previously published method for quantifying bitter-tasting lipid compounds in pea protein isolates [4,9] with the final optimised method. Testing the aqueous methanolic solution (H_2O:MeOH, 1:1, *v*/v), as recommended previously in the literature, during the P–B and F-F experiments revealed significantly lower FFA yields compared to other solvents. In contrast, the mixture design showed that an IPA:MeOH (1:1; *v*/v) extraction solution provided a higher FFA yield.

According to the Pfizer solvent selection guide, IPA and MeOH are classified as green solvents, offering a safer and less toxic alternative to chloroform and hexane [25]. The final method utilizes these less hazardous solvents instead of traditional LLE solvents (*e.g.,* chloroform). It is

Table 2
Comparison of sample extraction procedures from published methods by Gläser et al. (2020,2021) and the final optimised method.

	Gläser et al., 2020; Gläser et al., 2021 method	Optimised method
Sample weight (mg)	500	50
Extraction solvent composition	H_2O:MeOH (1:1, *v*/v)	IPA:MeOH (1:1, *v*/v)
Solvent volume (mL)	5	1
Pre-extraction internal standard	25 µL of [^{13}C18]-linoleic acid (IS1, 1.0 mM in MeOH), 25 µL of 18-hydroxyoleic acid (IS2, 1.0 mM in MeOH), and 25 µL of 1-myristoyl glycerol	–
Extraction conditions	Orbital shaker for 1 h at 300 U/min	Eppendorf Termomixer, 30 min, 15 °*C* at 1400 rpm
Centrifugation	–	14,000 × g at 10 °C for 10 min
Filtering	RC 15, 0.45 µm	PTFE 0.2 µm
Dilution	–	Dilute so that injectable solution contains H_2O: MeOH:IPA (1:1:1 *v*/v)
Internal standard usage (dilution 1:1)	–	^{13}C-oat flour extract (contains ^{13}C-fatty acids)

also less time-consuming and is easily adaptable to high-throughput applications. Handling immiscible solvents during LLE extraction is more complex than using LC-MS-compatible miscible solvents. Additionally, LLE extracts cannot be directly analysed with LC-MS equipment, requiring extra time for solvent exchange. Moreover, the final optimised method omits the need for solid-phase extraction, which is traditionally a part of the lipid analysis procedure. The use of isotopically labelled oat flour extract as a source of fully labelled IS compounds ensures reliable MS measurements and quantification of fatty acids with close *m/z* values. Isotopically labelled plants available on the market offer an economical alternative to synthesised isotopically labelled standard compounds. Using such extracts in calibration and sample solutions also allows to take matrix effects into account. Overall, the results demonstrate that the proposed method for the quantitative determination of FFAs by LC-MS in plant-based protein sources is fit for its intended purpose using a non-derivative and simple sample extraction procedure.

3.3. Validation of the method

The method was validated after development and optimisation, and the results are shown in Tables 3 and 4. The LOQs for the analytes were calculated from the lowest point of the calibration curve, ranging from 0.02 to 0.12 mg/L, and for LOQs, the corresponding values varied from 0.82 to 4.84 mg/100 g dry weight basis (dwb) (Table 3), consistent with values reported in previous research [35]. The repeatability of peak retention times (RTs), peak areas, and the precision of the entire method were investigated (Supplementary Table S6) following the verification of the acceptable linearity of the fatty acid responses (R^2 0.98–0.99). The RSDs of the peak RT and peak areas did not exceed 4 % and 8 %, respectively. The intra- and inter-day RSDs ranged from 2.8 % to 13.0 %, (Supplementary Table S6) which is in agreement with previous research [9,36]. The total recovery of fatty acids was determined by spiking the Z sample (Table 4). The recoveries ranged from 85 % to 103 % with %RSD less than or equal to 4 %, which is in accordance with [9]. The ME experiments showed that the MEs for the targeted fatty acids ranged from 93 % to 104 %. The results of the MEs were adequate, as specified in the guidelines [30]. The validation results indicated the method's suitability for routine analysis of FFAs in plant-based matrices.

3.4. Application of the method

The optimised extraction method was used to quantify FFAs in six plant-based matrices and their pooled sample Z (Table 5). The recoveries of C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3 in the composed pooled sample were 124 %, 121 %, 109 %, 113 %, 127 %, and 90 %, respectively. C18:2 and C18:1 were the predominant FFAs, with concentrations ranging from 4.5 mg/100 g to 3038 mg/100 g and from 5.4 mg/100 g to 589 mg/100 g, respectively.

The total sum of FFAs in analysed samples ranged from 4.4 to 3831

Table 3
The linear range, calibration curve, limits of detection (LODs), and limits of quantification (LOQs) of fatty acids in pooled sample Z.

Analyte	Linear range (mg/L)	Calibration curve	R^2	Internal Standard	LOD		LOQ	
					Instrumental (mg/L)	Method (mg/100 g dwb[g])	Instrumental (mg/L)	Method (mg/100 g dwb)
C14:0[a]	0.03–3.66	Y = 5.0041×-0.0015	0.9975	^{13}C18:3	0.01	0.29	0.02	0.96
C16:0[b]	0.05–6.51	Y = 0.3711× + 0.0399	0.9880	^{13}C16:0	0.04	1.45	0.12	4.84
C18:0[c]	0.08–5.18	Y = 9.5017× + 1.0909	0.9967	^{13}C18:0	0.02	0.99	0.08	3.31
C18:1[d]	0.04–5.12	Y = 0.3073×-0.0003	0.9932	^{13}C18:1	0.01	0.25	0.02	0.82
C18:2[e]	0.05–6.25	Y = 0.0337× + 0.0008	0.9955	^{13}C18:2	0.02	0.60	0.05	2.00
C18:3[f]	0.05–6.53	Y = 4.2341×-0.0006	0.9943	^{13}C18:3	0.01	0.46	0.04	1.54

[a] Myristic acid.
[b] Palmitic acid.
[c] Stearic acid.
[d] Oleic acid.
[e] Linoleic acid.
[f] Linolenic acid.
[g] Dry weight basis.

Table 4
Recoveries of fatty acids in the pooled sample Z (mean ± SD, n = 3).

	myristic acid		palmitic acid		stearic acid		oleic acid		linoleic acid		linolenic acid	
	C14:0		C16:0		C18:0		C18:1		C18:2		C18:3	
Content in unspiked matrix mg/L	0.20		1.30		1.11		2.70		4.21		2.80	
Spike level	spike mg/L	R*%	spike mg/L	R%	spike mg/L	R%	spike mg/L	R%	spike mg/L	R%	spike mg/L	R%
1	0.14	103 ± 2	0.10	96 ± 2	0.19	88 ± 5	0.07	98 ± 2	0.09	97 ± 0	0.27	100 ± 2
2	2.94	102 ± 2	1.00	97 ± 3	1.68	85 ± 0	0.95	100 ± 4	0.32	97 ± 1	2.90	95 ± 1
3	4.73	93 ± 1	2.33	101 ± 4	3.84	92 ± 4	1.92	101 ± 1	0.79	99 ± 4	4.41	92 ± 3

* Recovery.

Table 5
Concentration of free fatty acids (FFAs) in faba bean protein isolate (FPI), pea protein isolate (PPI), oat protein isolate (OPC), faba bean flour (FB), yellow pea flour (YP), whole grain oat flour (WGOF), and pooled sample Z (mean ± SD; *n* = 3), presented as mg/100 g on the dry weight basis (dwb).

Sample*	myristic acid	palmitic acid	stearic acid	oleic acid	linoleic acid	linolenic acid	SUM of FFAs
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	
FPI	<LOQ	<LOQ	<LOQ	<LOQ	4.5 ± 0.2d	<LOQ	4.4
PPI	<LOQ	16.3 ± 1.9 cd	5.9 ± 1.3b	41.0 ± 0.3c	354.5 ± 5.2c	4.8 ± 0.1b	423
OPC	1.5 ± 0.2b	19.2 ± 0.4b	5.5 ± 0.2b	76.2 ± 5.8b	546.5 ± 51.6b	5.1 ± 0.3b	1206
FB	<LOQ	9.0 ± 1.1d	<LOQ	30.0 ± 0.8c	213.1 ± 1.2cd	2.4 ± 0.4c	254
YP	<LOQ	<LOQ	<LOQ	5.4 ± 0.2c	40.1 ± 0.5d	1.4 ± 0.1c	47
WGOF	3.5 ± 0.2a	164.7 ± 22.9a	16.7 ± 2.2a	589.1 ± 52.2a	3038.0 ± 274.2a	18.9 ± 1.1a	3831
Z	1.0 ± 0.1b	42.3 ± 7.7c	5.1 ± 0.7b	139.3 ± 9.4b	886.4 ± 90.1b	4.9 ± 0.4b	1079
LOQ** mg/100 g dwb	1.0	4.8	3.3	0.8	2.0	1.5	–

ANOVA statistical significance test was applied across all samples; means with different letters are significantly different at $p < 0.05$.
* FPI, PPI, and OPC were not produced from the listed FB, YP, and WGOF.
** Limit of quantification.

Table 6
Results of calculated bitterness dose-over-threshold (DoT)* values in faba bean protein isolate (FPI), pea protein isolate (PPI), oat protein isolate (OPC), faba bean flour (FB), yellow pea flour (YP), and whole grain oat flour (WGOF).

Sample	myristic acid	palmitic acid	stearic acid	oleic acid	linoleic acid	linolenic acid
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
FPI	–	–	–	–	0.1	–
PPI	–	0.4	0.3	0.7	7.0	0.7
OPC	0.0	2.1	0.2	2.5	19.0	0.6
FB	–	0.2	–	0.5	4.2	0.3
YP	–	–	–	0.1	0.8	0.2
WGOF	0.1	4.2	0.8	9.6	59.9	2.5

* Dose-over-threshold (DoT) is ratio of the concentration of a free fatty acid to its taste threshold. The threshold values used were as follows: C14:0 – 1703 μmol/L, C16:0 – 1546 μmol/L, C18:0 – 726 μmol/L, C18:1 – 2180 μmol/L, C18:2 – 1810 μmol/L, and C18:3 – 277 μmol/L, as reported in previous studies [8,9].

mg/100 g dwb, with the highest content found in WGOF. Oats are high-fat cereals that contain a higher proportion of unsaturated fats and exhibit significant lipase activity, which makes their lipids more prone to oxidation and hydrolysis, leading to increased FFA levels [8,37,38]. The lipid content of 16 different oat varieties has been reported to range from 6.3 % to 16.0 % [39]. A study by Molteberg and Magnus reported an average total fatty acid content of 7.7 % in the dry matter of three Norwegian oat cultivars, with individual fatty acids C16:0 at 1.1 %, C18:0 at 0.1 %, C18:1 at 3.1 %, C18:2 at 3.3 %, and C18:3 at 0.1 % [37]. Lipase activity in raw oats and oat flour has been measured at 3.41 μmol min^{-1} g^{-1} and 0.41–0.77 μmol min^{-1} g^{-1}, respectively [38,40]. Storage studies have shown that the total FFA content in raw oat flour increased drastically from 0.45 to 6.41 g/100 g during 18 weeks, but the most significant release occurred within the first five weeks [37]. Similarly, our measured total FFA content in WGOF, approximately 3.8 g/100 g, falls within the same range. In contrast, the lipolytic activity of oat flour was shown to be significantly reduced after heat treatment [37,41], though total FFA levels still increased 2.5-fold over 42 weeks of storage [37]. The lower total FFA content in the OPC sample may be attributed to the various technological steps involved in its production, which could reduce lipase activity and limit FFA formation.

According to product specifications, OPC, PPI, and FPI contained 12 g/100 g, 10 g/100 g, and 0.3 g/100 g of total fat in the product, respectively, with estimated FFA content as a percentage of total fat was approximately 10 % in OPC, 4.2 % in PPI, and 1.4 % in FPI. Previously, faba bean flour has been shown to exhibit very high lipase activity (4.44–7.51 μmol min^{-1} g^{-1}) [40]. Nevertheless, the low absolute and relative FFA levels in the FPI sample underscore the role of processing in defining the potential bitterness of plant-protein ingredients.

Sensory assessments are used to evaluate bitterness. However, Vaikma et al. found that assessors disagreed on bitterness rankings for pea and oat flours, with highly varying scores [11]. That study highlighted that oat flour bitterness might be related to fatty acids [11]. Moreover, Gläser et al. and Günther-Jordanland et al. estimated the potential impact of FFAs on pea isolates and oat flour bitterness by calculating DoT factors [8,9], thereby disregarding the human factor of sensory tasting. The DoT results for the studied samples are presented in Table 6. Notably, the highest DoT values for C18:2 were found in both oat samples: WGOF (59.9) and OPC (19.0). Previous research has found that FFA content increased 10-fold during storage in high-moisture conditions, due to lipolytic enzyme activity in non-heat-stabilised oat [37,42]. However, the concentrations of C14:0 and C18:0 were insufficient ($DoT < 1$) to contribute to the bitter taste in the studied samples. Overall, these findings are consistent with previous studies on bitter-

tasting FFAs in whole grain oat flour and pea protein isolates [8,9].

4. Conclusion

In this study, an optimised sample extraction method for the analysis of bitter-tasting FFAs (C14:0, C16:0, C18:0, C18:1, C18:2, and C18:3) in oat-, pea-, and faba-based flours, protein concentrates, and isolates was developed and validated. Isotopically labelled oat flour extract served as a source of isotopically labelled standard compounds, enhancing the reliability and precision of LC-MS measurements and significantly reducing costs. The extraction method was optimised using a design of experiments approach, resulting in an improved composition of the extraction solvent. The mixture design indicated that a high yield of the FFAs was achieved with a IPA:MeOH (1:1, *v*/v) solution, with up to 33 % water tolerable without affecting the yield. The developed method is less toxic, less labour-intensive, and more environmentally friendly than previously published methods. Validation results demonstrated an overall precision RSD of less than 13 % and recoveries ranging from 85 % to 103 %. The method was successfully applied to selected oat, pea and faba-bean flours, protein concentrates and isolates. This approach could be extended to analyse other triglyceride-derived bitter compounds if these standard compounds are synthesised in-house or become commercially available.

Ethical statement - studies in humans and animals

Current study did not involve tests and experiments with and on humans and animals.

CRediT authorship contribution statement

Anastassia Zeinatulina: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Aleksei Kaleda:** Writing – review & editing, Visualization, Software, Data curation. **Maria Kuhtinskaja:** Writing – review & editing, Supervision, Methodology. **Tiina Krišciunaite:** Writing – review & editing, Resources, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Anastassia Zeinatulina reports financial support was provided by Estonian State Shared Service Centre. If there are other authors, they 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nfs.2025.100228.

Data availability

Data is contained within the article or supplementary material.

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Juhendatud väitekirjad

Grete Metsoja, MSc, 2023, Kibedust põhjustavate ühendite sensoorne ja instrumentaalne analüüs kaeras ja hernes, Tallinna Tehnikaülikool, Keemia ja biotehnoloogia instituut

Kristina Joarand, BSc, 2023, Ekstraktsiooni metoodika väljatöötamine saponiinide määramiseks sojabaasil jogurti alternatiivides. Tallinna Tehnikaülikool, Keemia ja biotehnoloogia instituut

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