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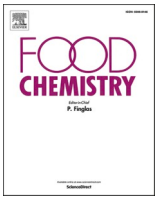

Minimizing anti-nutritional factors in wet protein extraction from Swedish faba beans through the application of response surface methodology

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ABSTRACT

Faba beans, rich in protein and ideal for Swedish cultivation, are limited in food industry use due to anti-nutritional factors (ANFs) that hinder nutrient absorption. An extraction method was developed in our study to mitigate ANFs in faba beans, using aqueous alkaline methods and isoelectric precipitation with differential salt concentration. This method yielded 15.8 g of protein per 100 g of flour, with a protein concentration exceeding 83% of the total extract. It reduced ANFs like phytic acid (28.0%), lectins (87.5%), vicine (98.5%), and convicine (99.7%). Extraction conditions were optimized using response surface methodology, identifying pH 6, 2 h, and 20 °C as the most effective parameters, achieving an 86% reduction in phytic acid, closely matched the model's predictions ($R^2 = 0.945$). This method effectively reduced ANFs, offering a sustainable approach for producing proteins suitable for diverse food products, including plant-based alternatives.

1. Introduction

Faba bean (*Vicia faba* L.), which is cultivated worldwide, is rich in protein, fiber, micronutrients (iron, zinc, folate), and bioactive compounds like flavonoids and phenolic acids (Bangar & Kajla, 2022; Lim, 2012). This bean has the potential to be a valuable ingredient in various food products such as baked goods, snacks, and pasta (Bouhadi, Belkhodja, & Benattouche, 2023). However, like most legumes, faba bean contains anti-nutritional factors (ANFs) such as phytic acid, lectins, vicine, and convicine, which can hinder nutrient absorption and pose health risks in humans and animals (Abbas & Ahmad, 2021; Dhull, Kidwai, Noor, Chawla, & Rose, 2022; Fekadu Gemede, 2014). Therefore, reducing these ANFs in faba flour is crucial to improve its nutritional value and safety.

Aqueous alkali protein extraction is a promising method for isolating protein from faba beans while reducing the levels of ANFs (Augustin & Cole, 2022). It involves soaking the beans in alkaline solution, usually NaOH or KOH, at room temperature for several hours (Karaca, Low, & Nickerson, 2011). The alkaline solution breaks down the cell walls of the beans, releasing proteins into solution. The solution is then neutralized and the proteins are precipitated out of solution by adjusting the pH. The resulting protein isolate is lower in ANFs and has improved nutritional value (Alireza Sadeghi, Appu Rao, & Bhagya, 2006; Deshpande & Cheryan, 1984).

For example, Amin, Petersen, Malmberg, and Orlien (2022) found that wet fractionation using aqueous alkali extraction significantly reduced the levels of ANFs in legumes, including phytic acid, tannins, and lectins. Similarly, Vioque, Alaiz, and Girón-Calle (2012) discovered that aqueous alkali extraction can practically eliminate vicine and convicine, two harmful substances in faba beans. However, process parameters in aqueous alkali extraction to lower anti-nutritional factors in faba bean products need to be optimized, since the efficiency of extraction and the quality of the resulting protein isolate are influenced by factors such as pH, soaking period, and temperature (Illingworth, Lee, & Siow, 2022; Jarpa-Parra et al., 2014). Therefore, additional research is required to determine the optimal conditions for aqueous alkali extraction to achieve maximum reductions in ANFs while maintaining the functional and nutritional characteristics of the protein isolate.

Response surface methodology (RSM) emerges as avital statistical tool in food science for optimizing food processing parameters (Ahmad et al., 2020; Tirado-Kulieva et al., 2021). In the case of aqueous alkali extraction of faba bean protein isolates, RSM can be used to optimize process parameters such as pH, soaking time, and temperature. This optimization aims to achieve the maximum reduction of ANFs while maintaining the functional and nutritional properties of the protein isolate. Use of RSM reduces the number of experiments required, saving time and resources while ensuring accuracy of results.

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The aim of the present study was therefore to optimize the process for aqueous alkali extraction of faba bean protein isolate using RSM. Specific objectives were to reduce the levels of main ANFs (phytic acid, lectins, vicine, and convicine) and to identify optimal conditions for maximizing ANF reduction while maintaining the functional and nutritional properties of the protein isolate. Such knowledge is needed to improve the quality of faba bean protein isolate and promote its broader use as a source of high-quality protein.

2. Materials and methods

2.1. Obtention of Faba bean

De-hulled and pre-milled faba bean flour (*Vicia faba* minor L., var. 'Tiffany') was obtained from Svensk Faba, Västra Götaland, Sweden. Tiffany is a summer faba bean variety low in anti-nutrients (such as phytic acid, vicine, and convicine), but rich in plant protein, nutrients, and dietary fiber (Mayer Labba, Frøkiær, & Sandberg, 2021). The provider supplied the crude chemical composition of the faba bean flour, indicating that it contains 45% carbohydrates, 29–32% protein, 2% fat, and 24% fiber.

2.2. Protein extraction and characterization

2.2.1. Protein extraction and content

Aqueous alkaline extraction followed by isoelectric precipitation with varying salt concentrations was used to extract total proteins from Swedish faba beans, as described by Langton et al. (2020) with modifications (Fig. 1). The flour was dispersed in distilled water at a ratio of 1:9 (*w/v*) and stirred overnight at room temperature (20 ± 2 °C) with a magnetic stirrer. The pH of the mixture was adjusted to 8 using a solution of 1 M NaOH (Sigma-Aldrich, Stockholm, Sweden) and it was then incubated with stirring at 40 ± 2 °C for 1 h, followed by centrifugation at 5000 ×*g* for 30 min. The supernatant was collected and pH was adjusted to 4.8 using 1 M HCl (VWR International, Stockholm, Sweden). The supernatant was then centrifuged at 5000 ×*g* for 20 min to precipitate the protein. The pellet obtained was collected and re-dissolved in 0.6 M NaCl (EMSURE, Darmstadt, Germany), and then dilutions with different concentrations of NaCl solution (0.1 M, 0.15 M, and 0.2 M) were investigated. Total protein concentration in the faba bean flour was determined via the Kjeldahl method. The nitrogen content in the extracted protein isolates was measured, and then converted to protein content using a conversion factor of 5.4 g nitrogen/g protein (Johansson et al., 2022).

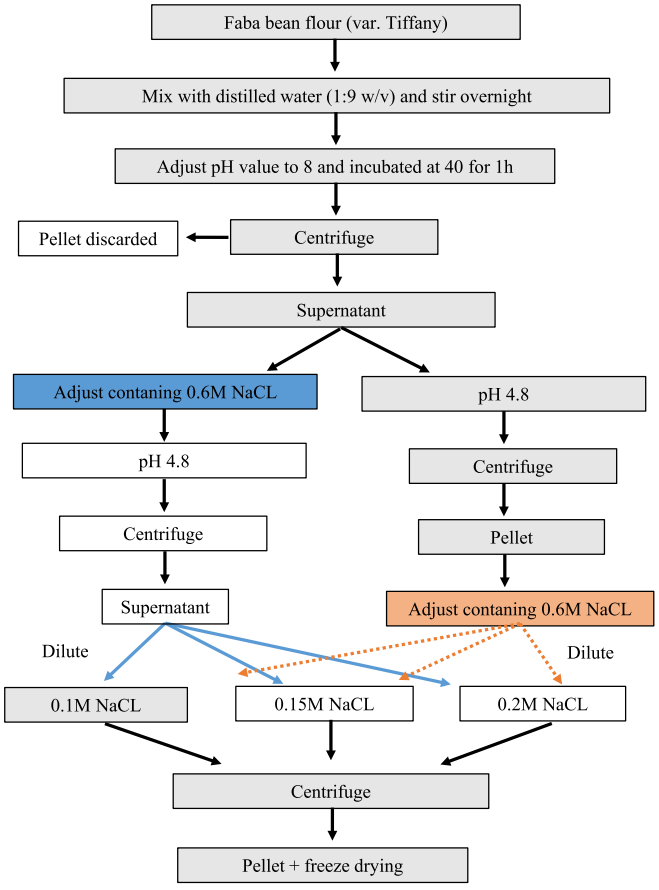

Fig. 1. Schematic outline of pre-optimized aqueous alkaline process combined with isoelectric point and salt precipitation for total protein extraction from Swedish fava beans.

2.2.2. Moisture content

The moisture content of the faba bean flour was determined using the moisture-air oven method (AACC 44 – 15 A) according to Akkad et al. (2021). Samples (1 g) were placed in an aluminum weighing dish, transferred to an oven (SHEL-LAB 1330F, Sheldon Manufacturing Inc., Cornelius, USA), and dried for 16 h at 105 °C. After drying, the dishes were cooled to 25 °C in a desiccator and weighed immediately. The moisture content was calculated as a percentage of the initial sample weight.

2.2.3. Protein solubility

Protein fractions (7S and 11S) were extracted according to the protocols outline by Suchkov, Popello, Grinberg, and Tolstogusov (1990) with some modifications. Detailed descriptions of these modified protocols have published by our group (Herneke, Lendel, Karkehabadi, Lu, & Langton, 2023; Johansson, Karkehabadi, Johansson, & Langton, 2023). Different protein solubility curves were generated by dissolving samples in deionized water, adjusting the pH to 10 with 0.1 M NaOH, and diluting to 10 mg/mL (Ralet & Guéguen, 2000). For a broad pH range, 100 μL of pH 10 solution were treated with 0.01 to 1 M HCl or 0.01 M NaOH and adjusted to 1 mL with deionized water. After vortexing and measuring the pH, samples were centrifuged at 13,500 ×*g* for 10 min and the absorbance of the supernatant was measured at 280 nm. Absorbance values of pH 10 samples were used to determine 100% solubility.

2.2.4. Size exclusion chromatography

A size exclusion chromatography (SEC) method described by Herneke et al. (2021) was used, with minor modifications, to assess the size and purity of faba bean protein. A 0.2 g portion of extracted faba bean protein isolate was dissolved in 20 mL deionized water. To ensure complete protein dissolution, the pH was adjusted to 8 and NaCl was added to a final concentration of 0.6 M. The protein solution was centrifuged at 3500 ×*g* for 5 min, and the resulting supernatant was filtered through a 45 μm sterile nylon syringe filter (Sigma-Aldrich, Stockholm, Sweden). A prepared protein solution (0.1 mL) was then loaded onto a Superdex/200 Hiload 16/600 size exclusion column using 50 mM Bicine (pH 8.7), 200 mM NaCl as running buffer.

2.3. Determination of ANFs during protein extraction

2.3.1. Phytic acid content

Phytic acid content in faba bean flour and extracted protein isolate was determined using a modified colorimetric method (Latta & Eskin, 1980). The method is based on the reaction between phytic acid, ferric ion (Fe^{3+}), and sulfosalicylic acid (Wade reagent) to form a complex, which can be measured at 500 nm. Samples were mixed with 3.5% HCl solution to achieve pH of 0.7–0.8 and then subjected to centrifugation after stirring with a magnetic stirrer for 1 h. The crude acid extract was purified by anion exchange chromatography using AG1-X8 resin (Bio-

Rad, Solna, Sweden), and phytate phosphorus ($P_{phy.}$) and inorganic phosphorus ($P_{inorg.}$) were separated. Eluate ($P_{phy.}$) was collected and adjusted to pH 3.0 with 0.1 M HCl. Sample (3 mL) mixed with 1 mL of Wade reagent (Sigma-Aldrich, Stockholm, Sweden) underwent vortexing and centrifugation at 3000 ×*g* for 10 min at room temperature. Absorbance at 500 nm was measured using a Shimadzu UV − 1800 spectrophotometer, calibrated with water. Background light interference was eliminated using a reagent blank of deionized water and Wade reagent. A standard curve was generated using standard solutions of commercial sodium phytate (Sigma-Aldrich, Stockholm, Sweden) mixed with Wade reagent to ensure the accuracy of the calibration for determining the phytic acid content in the samples.

2.3.2. Lectins

To estimate lectin (hemagglutinin) levels in faba bean flour and protein isolates, rabbit red blood cells (Merck KGaA, Darmstadt, Germany) were agglutinated by serially diluting the extract (Shi, Arntfield, & Nickerson, 2018). For this, 10 mL of saline were added to 1 *g* sample in a centrifuge tube. After vortexing and centrifugation, the clear supernatant was diluted in a 96-well plate, with a dilution range from 0 to 1:8192. Rabbit red blood cells were added to each well and left at room temperature for 2 h. Hemagglutinin activity was determined microscopically using saline and rabbit red blood cells, with negative agglutination served as the control. Positive agglutination indicated a higher concentration of lectins in the faba bean flour. The presence of at least five aggregated cells indicates positive evidence of agglutination. The lowest dilution containing one hemagglutinin unit (HU) was considered as specific hemagglutinin activity (HU/mg dry weight, DW) according to Liener and Hill (1953). The specific activity of hemagglutinin (HU/mg flour on a dry basis) was calculated as follows:

$$HU\Big/mg = \frac{Da{*}Db{*}S}{V}{*}\frac{100\%}{100\% - MC}$$

Where Da is the dilution factor of extract in well 1, Db is the dilution factor for the tube with 1 HU, S is the ratio of original extract volume (mL) to flour weight (mg), V is the extract volume in well 1, and MC indicates the moisture content of faba bean flour

2.3.3. Vicine and convicine

Vicine and convicine in faba bean flour and protein isolate were analysed with a method modified from Pulkkinen et al. (2019). To extract vicine and convicine, each sample (0.1 g) was mixed with MilliQ water (1.5 mL) followed by 10 min sonication (Branson Ultrasonics, Brookfield, Connecticut, US), and 10 min centrifugation at 12,535 ×*g* (Biofuge pico, Hereaus, Germany). The pellet was re-extracted and supernatants from both extraction cycles combined. The entire procedure was done in presence of uridine (1.0 mg, 99%, Sigma-Aldrich, Stockholm, Sweden) as an internal standard. To remove proteins, the combined supernatants were boiled (5 min), centrifuged (12,535 ×*g*, 10 min) and ultra-filtered (12,535 ×g, 10 min) though a 10 kDa Amicon® Ultra 0.5 mL centrifugal filter (Merck KGaA, Darmstadt, Germany). Each filtrate (400 µl) was diluted to 1.5 mL in MilliQ water, filtered through a 0.2 µm PTFE filter and stored at −20 °C until analysis on a Waters Acquity UHPLC system (Waters, Milford, USA). Vicine, convicine and uridine were separated on a Waters HSS T3 column (2.1 × 150 mm, 1.7 µm, C18, Milford, USA) with a mobile phase of 0.1% formic acid in MilliQ water (flow rate: 0.35 mL/min, column temperature: 25 °C, injection volume: 1 µL, detection wavelength: 273 nm). After 6.5 min, the column was washed with an acetonitrile gradient, the total run time being 11 min. Chromatographic data was recorded and processed in the Waters Empower 2 software. Vicine and convicine identification was done from retention times and UV absorbance maxima. A standard curve (0.001–0.056 µg vicine and 0.03 µg uridine per injection) was used for vicine quantification and convicine semi-quantification.

2.4. Enhanced phytic acid reduction using box-Behnken design (BBD)

Response surface methodology was used to assess the effect of three independent variables (pH (X_1), extraction temperature (X_2) and extraction time (X_3)) on the response function (Y), which represents the percentage reduction in phytic acid during protein extraction process. The workflow is depicted in Fig.2 and the independent variables are detailed in Table.A.1. A Box-Behnken design was selected for structuring the experimental data. The selected design variables and their actual and coded levels were paired with response variables. RSM was applied to the experimental data using a commercial statistical package, Design-Expert version 22.0.8 (Stat-Ease Inc., Minneapolis, MN, USA). Randomization of experiments was performed, to minimize the influence of unexplained variability in the observed responses caused by extraneous factors. The response variable was modeled by a second-order polynomial with the general form:

$$Y = \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{i=1}^{3} \beta iiX_i^2 + \sum_{i=0}^{2} \sum_{j=i+2}^{3} \beta ijXiXj$$

where Y is predicted response, β_0 is intercept coefficient, β_i is linear coefficient, β_{ii} is squared coefficient, β_{ij} is interaction coefficient, X_i, X_j are coded independent variables, X_iX_j are interaction terms, and X_i^2 are quadratic terms.

Statistical significance of the terms in the regression equations was assessed by analysis of variance (ANOVA) for each response. The adequacy of the models was determined based on model analysis, lack-of fit test, coefficient of determination (R^2), and adjusted-R^2 analysis. According to Joglekar and May (1987), R^2 should be at least 0.80 for good fit of a response model. Variables were considered more strongly significant if the absolute *t* value increased and the *p*-value decreased ($p < 0.05$). It should be noted that some non-significant variables ($p > 0.05$) were added to the model due to quadratic or interaction effects. The

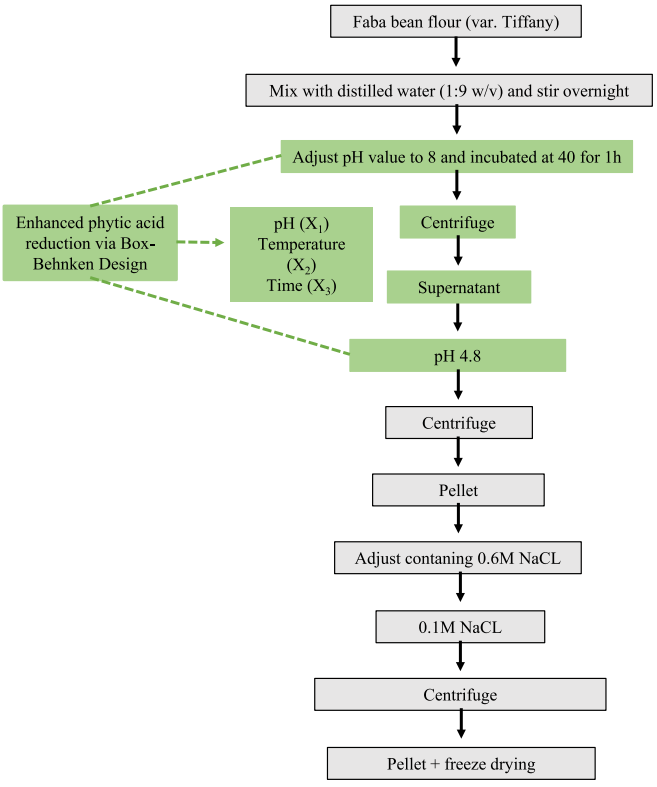

Fig. 2. Workflow of Box-Behnken Design (BBD) analyzing the impact of pH, extraction temperature and time on further phytic acid reduction in pre-optimized Swedish faba bean protein extraction.

correlation between the response and independent variables can be readily seen in contour and three-dimensional (3D) graphic surface plots, provided insights into the optimum levels and most influential variables for phytic acid reduction. The contour graphs illustrate whether the interactions between corresponding variables are significant, while response surface graphs (3-D) demonstrate how the response value sensitively changes with variable alterations (Li, Fang, & You, 2013). In these graphs, a single variable remains constant, enabling variation of the remaining two within experimental parameters. The contour plot morphologies reflect distinct variable interrelations. Optimal variable conditions correlate with maximal response values.

3. Results and discussion

3.1. Pre-optimized protein extraction and characterization

We pre-optimized the aqueous alkaline extraction process with varying salt concentrations, followed by isoelectric precipitation, to maximize the protein yield from Swedish faba beans. The results (Table 1) showed that protein yield increased when extracted using an alkaline solution at 40 °C, reaching isoelectric point at pH 4.8, and employing an optimal salt concentration of 0.1 M. Additionally, we found that the sequence of salt solution addition influenced the yield and concentration of the protein powder at both before and after pH adjustment to the isoelectric point of the protein. Adding salt prior to pH adjustment to 4.8 resulted in higher protein percentage (88.2%), but lower yield (9.28 g/100 g flour). In contrast, salt addition post-pH adjustment led to lower protein percentage (83.5%) but higher yield (15.8 g/100 g flour), as determinate by Kjeldahl methods (Table 1). As the objective of this project is to develop an end-product for food applications, we selected the method yielding the highest amount of extracted faba bean protein—utilizing post-pH adjustment salt addition—for further optimization of phytic acid reduction (Fig. 2).

The solubility profiles of protein in faba bean flour and protein isolate followed typical U-shaped curves (Fig. 3a), with the lowest solubility for all protein fractions occurring around pH 4–5, likely near their isoelectric point, and increasing solubility at pH outside this range. The faba bean flour displayed minimum solubility of 50% around the pH range 4–5. In contrast, the isolated protein from faba beans showed a markedly lower minimum solubility of 4.04% in a similar pH range. Among the globulins present in faba bean, legumin (11S) exhibited a more restricted pH range around its isoelectric point and a minimum solubility similar to that of the faba bean protein isolate. The minimum solubility of vicilin (7S) fell between that of the faba bean flour and the protein isolate. Size exclusion chromatography using a Superdex-200 column enabled identification of proteins by their size, with the chromatogram exhibiting distinct peaks at elution volumes of 58.05 mL and 67.27 mL (Fig. 3b). These peaks indicate the presence of 11S and 7S globulins, with estimated molecular weight close to 350 kDa and 150 kDa, respectively.

The results (Table 1) obtained in pre-optimization of protein extraction from Swedish faba beans offer insights into the effects of salt concentration and addition order. Alkaline pH and higher temperature (40 °C) can contribute to breakage of disulfide bonds in protein, facilitating the unfolding of protein structures and enhancing protein solubility and denaturation of proteins, which in turn improves protein recovery and yield (del Contreras et al., 2019; Du et al., 2018). Our results showed that adding salt prior to pH adjustment results in higher protein purity but lower yield, whereas adding salt post-pH adjustment has the opposite effect. Post-isoelectric point, addition of salt appears to facilitate more efficient precipitation, as indicated by the increased yield. This finding is particularly relevant given the growing interest in sustainable plant-based protein sources (Sussmann, Halter, Pickardt, Schweiggert-Weisz, & Eisner, 2013), and suggests that manipulating salt concentration may affect the yield and purity of extracted protein. This can be explained by the "salting-in" effect (Jiang et al., 2021), where salting-in-out extraction generates the highest protein yield due to increased overall protein solubility. The U-shaped solubility curves emphasize the impact of pH on protein solubility in faba beans. Isolated proteins showed increased sensitivity to pH, displaying different solubility levels across various pH ranges, with generally higher solubility in both acidic and alkaline conditions compared with faba bean flour, but notably lower solubility near their isoelectric point. At their isoelectric point, where they carry no net charge, these proteins are least soluble, resulting in heightened aggregation (Vogelsang-O'Dwyer et al., 2020). The reduction in solubility between faba bean flour (50%) and isolated protein (4.04%) may be attributable to removal of other constituents that assist in stabilizing protein structures during isolation. The reduced solubility of the isolated protein was primarily attributable to the presence of 11S globulins. In contrast, 7S globulins, characterized by their smaller size and globular structure, generally exhibited higher solubility (Johansson et al., 2023). This observation was further validated and supported by the SEC results, which aligned with molecular weight differences. Similar solubility trends have been reported previously (Ajibola & Aluko, 2022).

Table 1
Crude protein content analysed using Kjeldahl method.

Salt addition	post-pH adjustment			prior-pH adjustment		
Salt (Molar)	0.1	0.15	0.2	0.1	0.15	0.2
Protein yield (g/100 g flour)	15.8 ± 0.64	13.6 ± 0.35	13.2 ± 0.42	9.28 ± 0.52	9.31 ± 0.47	9.33 ± 0.67
Protein concentration %	83.5 ± 0.27	85.3 ± 0.00	85.8 ± 0.12	88.2 ± 0.69	91.0 ± 0.19	89.7 ± 0.58
Protein content (g)	13.2 ± 0.58	11.6 ± 0.30	11.3 ± 0.38	8.18 ± 0.41	8.47 ± 0.45	8.37 ± 0.66

The values obtained in triplicate for each of the two biological samples, representing the average of these measurements.

3.2. Reduction of ANFs in pre-optimized protein extraction

Concentration of four ANFs, phytic acid, lectins, vicine, and convicine, were determined during the pre-optimized protein extraction process (Table 2). The initial phytate concentration in faba bean flour was on average 1375.8 ± 28.00 mg/100 g DW. In contrast, the extracted protein had an average phytate concentration of 990.6 ± 63.68 mg/100 g DW, representing a 28.0% reduction in phytate concentration in the pre-optimized protein extraction process. The hemagglutinin activity of lectins in faba bean flour and the extracted protein isolate is shown in Fig. A.1. Compared with the negative and positive controls, hemagglutinin activity in faba bean flour (upper row in Fig. A.1) decreased with increasing dilution ratio, with one HU of the lowest dilution producing positive agglutination in wells 7–8 (W7–8). Similar positive agglutination patterns was found in wells 5–6 (W5–6) in extracted protein isolates (lower row in Fig. A.1). For greater clarity, the results for hemagglutinin activity are presented within the range of 7.1–28.4 HU for faba bean flour and 0.89–3.55 HU for the extracted protein isolates (Table 2). Based on these results, the pre-optimized protein extraction process significantly decreased the lectin level, by 87.5%. The vicine and convicine levels were reduced from an initial concentration of 1238.5 (vicine) and 37.9 (convicine) μg/g faba flour to 19.0 and < 1 μg/g extracted protein isolate, respectively (98.5% and 99.7% reduction, respectively) (Table 2).

These results demonstrate significant reductions in phytate, hemagglutinin activity, vicine, and convicine levels in extracted faba bean protein isolates following a pre-optimized wet protein extraction process. The concentration of phytic acid in faba beans typically ranges from 510 to 1770 mg/100 g DW in different varieties (Schlemmer, Frølich, Prieto, & Grases, 2009). This variation in phytic acid levels is believed to be influenced by several factors, such as the specific bean cultivar, soil characteristics, and the environmental conditions

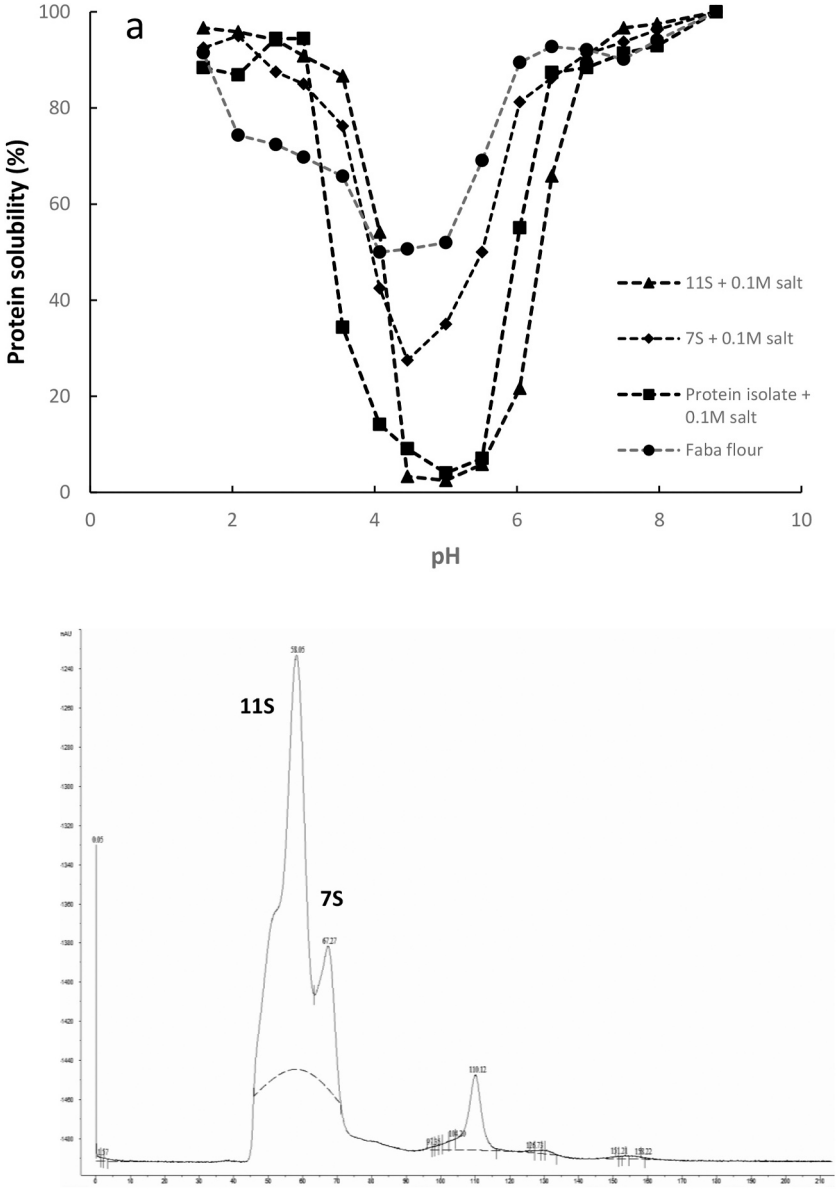

Fig. 3. Solubility of proteins in fava bean flour and protein isolates (a) and Size exclusion chromatography of extracted protein isolates (b).

Table 2
Reduction of anti-nutritional factors (ANFs) in pre-optimized wet protein extraction process.

ANFs	Phytic acid (mg/g)	Lectin HU/mg	Vicine and convicine (μg/g)	
Sample			vicine	convine
Faba bean flour	13.76 ± 0.28	7.1–28.4	1238 ± 53	37.9 ± 19
Faba protein isolates	9.91 ± 0.64	0.89–3.55	19 ± 4	<1
[a]**Reduction (%)**	28.0	87.5	98.5	99.7

* The faba flour from Svensk Faba, Västra Götaland, Sweden, is considered one biological sample. Two extractions were performed for faba bean protein, resulting in two biological samples. Anti-nutritional factors were analysed in triplicate for each sample.

[a] Reduction % = (ANF $_{\text{Faba flour}}$ − ANF $_{\text{Faba protein}}$) / ANF $_{\text{Faba flour}}$ *100%.

prevailing in the region where the beans are grown (Mayer Labba et al., 2021; Nicoletto, Zanin, Sambo, & Dalla Costa, 2019; Zehring, Walter, Quendt, Zocher, & Rohn, 2022). The initial concentration of phytate in the faba bean flour used in this study (var. 'Tiffany') was 1375.8 ± 28.00 mg/100 g DW. This falls within the previously reported range and aligns with existing research findings on phytate concentrations in faba beans (Sinkovič et al., 2023). After the pre-optimized protein extraction process, the phytate concentration in faba bean protein isolates reduced by 28.0%, which is consistent with findings in similar studies by e.g., Rahate, Madhumita, and Prabhakar (2021) and Samtiya, Aluko, and Dhewa (2020), who reported effectiveness of soaking (6-24 h) during protein extraction in reducing the phytic acid content in legumes by 27.9–36.0%.

Hemagglutinin activity, an indicator of lectins, showed a considerable decrease (87.5%) as observed from HU levels in dilution tests. Similar reductions have been observed previously by Ayyagari, Narasinga Rao, and Roy (1989) and Udeogu Ebere (2016). This emphasizes the effect of only using heat treatments to deactivate lectin activity in legumes, an important consideration due to the possible toxicity of lectins when ingested in large quantities. No previous study has focused exclusively on the impact of protein extraction methods in reducing lectin activity in legumes. This study is thus the first to demonstrate that ANFs, such as phytic acid and lectins, are reduced during extraction of plant-based proteins by various processes such as soaking and heating.

Table 3
Experimental runs, coded factors with the experimental and predicted response factor in the application of Response Surface Methodology (RSM).

	Coded variables			Actual values			Reduction of phytic acid (Y) %	
Run	x1	x2	x3	X_1	X_2	X_3	experimental	predicted
1	0	0	0	6	30	3.5	64	70.2
2	−1	1	0	5	40	3.5	67	65
3	0	−1	−1	6	20	2	86	83.5
4	−1	0	−1	5	30	2	53	55.5
5	1	1	0	7	40	3.5	36	36
6	0	0	0	6	30	3.5	66	70.2
7	0	1	−1	6	40	2	70	69.5
8	1	0	−1	7	30	2	55	55.5
9	−1	0	1	5	30	5	66	65.5
10	0	0	0	6	30	3.5	70	70.2
11	0	0	0	6	30	3.5	75	70.2
12	1	0	1	7	30	5	38	35.5
13	0	0	0	6	30	3.5	76	70.2
14	0	1	1	6	40	5	68	70.5
15	−1	−1	0	5	20	3.5	59	59
16	1	-1	0	7	20	3.5	56	58
17	0	-1	1	6	20	5	72	72.5

A significant decrease in vicine and convicine levels (by 98.5% and 99.7%, respectively) was achieved during protein extraction. This might be largely due to the solubility of these glycosides in aqueous environments and the methodologies used (Marquardt, Muduuli, & Frohlich, 1983). These results align with findings by Vioque et al. (2012) that vicine and convicine remain in the liquid phase during water-based protein extraction, while proteins are isolated. The present study confirmed the efficacy of wet protein extraction processes in diminishing specific ANFs in faba beans through techniques such as soaking and heating which break down the cellular structure of the beans, thereby facilitating separation. This has substantial implications for improving the safety and nutritional quality of faba bean-based foods, particularly for consumers prone to favism.

3.3. Enhanced reduction of phytic acid during protein extration

In the preliminary optimization phase of protein extraction, we achieved significant removal of lectins, vicine, and convicine, with the exception of phytic acid, which saw a reduction of 28%. To further optimize the reduction of phytic acid levels in extracted protein isolates, we employed response surface analysis utilizing the Box-Behnken Design (BBD) model. This approach optimized the activity of endogenous phytase, which breaks down phytic acid, across 17 variable combinations of optimal pH, temperature, and time. The resulted in a phytic acid reduction in extracted protein flour, compared to original faba bean flour, varying from 36% to 86% (Table 3).

3.3.1. Model fit

Applying RSM yielded a regression equation that empirically related the reduction in phytic acid to test variables in coded units:

$$Y = 70.2 - 7.50X1 - 4.0X2 - 2.5X3 - 7.0X1X2 - 7.5X1X3 + 3.0X2X3 - 18.35X1^2 + 2.65X2^2 + 1.15X3^2$$

where Y is predicted phytic acid reduction and Xi represents the independent variables: X1 (pH), X2 (temperature), and X3 (extraction time). The model suggests that an increase in pH diminishes the phytic acid reduction, with a negative quadratic term indicating a parabolic trend. Optimal reduction occurs at a higher pH, but reverses beyond pH 6. The predicted values of phytic acid reduction obtained using the regression model are compared with experimental values in Fig. A.2.

ANOVA was used to confirm the accuracy of the model, verify the significance of each coefficient, and evaluate the strength of interaction of each viable for the response factor during the extraction procedure (Table 4). The model exhibited a good fit, with determination coefficient (R^2) of 94.5% and a non-significant lack of fit, implying accuracy in the experimental domain (Basri et al., 2007; Lee, Yusof, Hamid, & Baharin, 2006). Model reproducibility was confirmed by low coefficient of variation (CV = 7.23%) (Mia, Khan, & Dhar, 2017; Rashid, Anwar, Ansari, Arif, & Ahmad, 2009). Statistical analysis indicated that linear, quadratic, and interaction terms were significant ($p < 0.05$), with pH being the most imfluential factor ($p < 0.01$), including its interactions with temperature and extraction time. Temperature also significantly influenced the model ($p < 0.05$). The peak activity of endogenous phytase was significantly affected by factors such as pH and temperature, which is consistent with previous findings (de Naves, Corrêa, Bertechini, Gomide, & dos Santos, 2012; Tijskens, Greiner, Biekman, & Konietzny, 2001).

3.3.2. Process optimization

The 3D response surface and 2D contour plots (Fig. 4a, b) clearly demonstrated the influence of extraction time and pH on the reduction in phytic acid concentration when the extraction temperature was maintained at a steady 30 °C. A marginal linear effect was observed with changes in extraction time. Notably, at pH levels below 6, extending the extraction time from 2 to 5 h did not significantly alter the phytic acid reduction. However, an important trend was observed when the pH rose above 6, where an increase in extraction time led to a decrease in phytic acid levels in the isolate. This effect was most pronounced at pH 7, where

Table 4
Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value	Significance
Model	2515.08	9	279.45	13.33	0.0013	significant
X_1	450.00	1	450.00	21.46	0.0024	**
X_2	128.00	1	128.00	6.10	0.0428	*
X_3	50.00	1	50.00	2.38	0.1665	NS
X_1 X_2	196.00	1	196.00	9.35	0.0184	*
X_1 X_3	225.00	1	225.00	10.73	0.0136	*
X_2 X_3	36.00	1	36.00	1.72	0.2315	NS
X_1^2	1417.78	1	1417.78	67.61	< 0.0001	**
X_2^2	29.57	1	29.57	1.41	0.2738	NS
X_3^2	5.57	1	5.57	0.2655	0.6222	NS
Residual	146.80	7	20.97			
Lack of Fit	34.00	3	11.33	0.4019	0.7601	NS
Pure Error	112.80	4	28.20			
Cor Total	2661.88	16				
R^2	0.9449					
R^2 (adjusted)	0.8739					
CV (%)	7.23					

Probability of F-test: ** P < 0.01, *P < 0.05, NS non-significant difference.

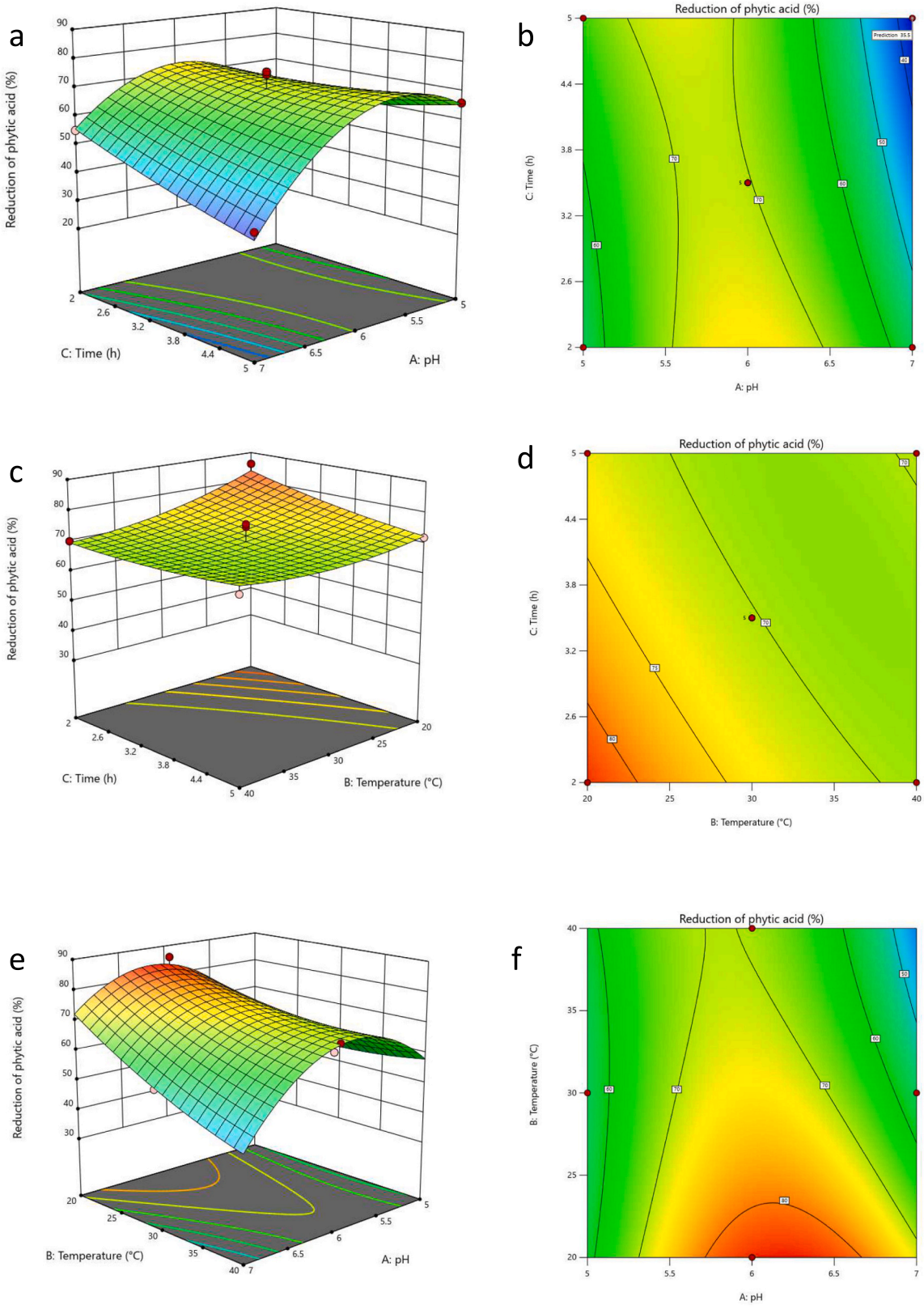

Fig. 4. Three-dimensional response surface and corresponding two-dimensional contour plots.

a 5-h extraction time yielded the lowest phytic acid reduction. The quadratic relationship of pH on extraction yield underscores the vital importance of carefully adjusting pH levels to optimize the extraction process for maximum reduction of phytic acid.

Fig. 4c and d illustrate the relationship between phytic acid reduction, extraction temperature, and extraction time at a constant pH of 6. The response surface in Fig. 4c showed a relatively flat slope, indicating a negligible interaction between extraction temperature and time. Analysis of Fig.4d reveals that both extraction temperature and time had linear impacts on phytic acid reduction. There was a marked decrease in phytic acid levels with an increase in both extraction time and temperature, leading to identification of the optimal conditions: extraction temperature 20 °C and extraction time 2 h, which corresponded to the greatest reduction in phytic acid.

Fig. 4e and f illustrate the effects of pH and extraction temperature on phytic acid reduction at a constant extraction time of 2 h. Similarly to earlier observations, the impact of extraction temperature on yield showed a linear trend. Notably, phytic acid reduction diminished as temperature increased, especially when pH values exceeded 5.5 and particularly at pH 7. The symmetrical configuration in Fig. 4e suggests that pH is a critical factor in the reduction in phytic acid. An initial increase in phytic acid reduction was observed as the pH rose to 6, especially at a lower temperature of 20 °C compared with 40 °C. However, when the pH exceeded 6, the phytic acid reduction decreased, regardless of the temperature.

Phytases, enzymes inherently present in faba beans, play a crucial role in degrading phytic acid, which is the primary phosphorus form in various plants, including legumes (Konietzny & Greiner, 2002). The activity of phytases can be influenced by several factors, including extraction time, ambient temperature, and pH level (Carlson & Poulsen, 2003; Esmaeilipour, Van Krimpen, Jongbloed, De Jonge, & Bikker, 2012; Greiner & Konietzny, 2006). Previous studies have shown that faba bean phytase demonstrates optimal activity under specific conditions: pH 5–6 and temperature of approximately 45–66 °C (Greiner et al., 2001; Habte-Tsion & Kumar, 2018; Luo, Xie, Min-Xu, & Luo, 2012). However, in this study we omitted this higher temperature range to avoid potential adverse effects on protein functionality. Our soaking treatments were thus conducted within a conservative range of pH 5 to 7, at temperatures between 20 °C and 40 °C, for a time of 2 to 5 h, integrated with the protein extraction process. We achieved a reduction in phytate concentration of 36–83%, which is consistent with findings by Greiner and Konietzny (2006) of a 26–100% reduction under similar process conditions. A key finding in our study was that the highest efficacy was achieved at pH 6 and 20 °C in extraction for 2 h, leading to an 86% reduction in phytate concentration.

Analysis of the combined influence of soaking time, temperature, and pH on phytate reduction during faba bean protein extraction revealed that extended extraction time had a minimal impact on phytic acid reduction at lower pH levels (<6). This suggests the existence of a pH threshold for the efficacy of phytic acid extraction. This is consistent with findings by Urbano et al. (2000) on the significant impact of optimal pH and extraction time on phytate hydrolysis during legume soaking. A linear trend was observed, where extended soaking period and higher temperature above a specific pH level (particularly beyond pH 6) decreased the effectiveness of phytate reduction. This finding partially contradicts findings by Shashego (2019) that prolonged soaking at higher temperatures significantly reduces phytic acid levels in soybean flour. This discrepancy may be due to e.g., differences in the bean variety and soaking environment, particularly the pH level. Additionally, prolonged exposure to heat may lead to effective thermal degradation of phytase activity, further influencing the reduction in phytic acid. A quadratic relationship between pH and phytic acid reduction was indicated to a certain extent, which emphasizes that pH plays a crucial role in the activity of endogenous phytases, with the optimal pH for these enzymes ranging from 4.6 to 6.0 (Frias, Doblado, Antezana, & Vidal-Valverde, 2003; Han & Gallagher, 1987). However, neutral or even slightly alkaline pH conditions can be optimal for phytases in legumes (Scott, 1991). In summary, our study highlights the importance of carefully balancing pH, extraction temperature, and extraction time to optimize the reduction in phytic acid in faba bean flour. Use of RSM provided valuable insights that can be used in improving the nutritional profile of plant-based proteins through optimized extraction processes.

3.3.3. Confirmative tests

The effectiveness of the model equation in predicting the optimal response was evaluated using the suggested conditions. When the optimal independent variable values (pH 6, extraction temperature 20 °C, and extraction time 2 h) were used in the regression equation, the predicted average reduction in phytic acid was 83.5%. Actual experiments under these optimal conditions resulted in a phytic acid reduction of 84.5%. This good correlation between predicted and observed values indicates good model precision and reliability. It could thus be a valuable tool for optimizing conditions in industrial processes, as discussed by Lee et al. (2019) in their review of process optimization in food technology. The good correlation between model predictions and experimental results also opens up avenues for further refining the model for even greater precision in future applications.

4. Conclusions

This study demonstrated that a combination of aqueous alkaline protein extraction and salt precipitation is an effective method for obtaining high yields of protein isolates with significantly reduced levels of ANFs (phytic acid, lectins, vicine, and convicine) from faba bean. Response surface methodology and a Box-Behnken design were used to optimize the extraction process for faba bean proteins, specifically targeting maximization of the phytic acid reduction in the resulting isolate. This optimization work, guided by a second-order model and 3D response surfaces, pinpointed pH as an important factor influencing phytic acid reduction, with notable effects in both linear and quadratic terms. Additionally, the interaction of pH with temperature and extraction time significantly influenced the reduction in phytic acid. However, under the conditions tested, extraction time did not significantly affect phytic acid level. The greatest reduction in phytic acid (~86%) was achieved at pH 6, combined with extraction temperature of 20 °C and extraction time of 2 h. There was a strong correlation between actual experimental values (84.5%) and values predicted (83.5%) using a model equation, indicating good accuracy of the model. The improved faba bean protein isolate obtained, with minimized levels of ANFs, offers potential for creating more nutritious plant-based foods to meet the growing preference for plant-based diets. Use of RSM in optimizing food processing could improve the area of application of food ingredients by enhancing their nutritional properties, leading to diverse food products that cater to specific dietary needs.

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CRediT authorship contribution statement

Jing Lu: Writing – review & editing, Writing – original draft, Investigation. **Galia Zamaratskaia:** Writing – review & editing, Supervision. **Maud Langton:** Writing – review & editing, Supervision. **Hanna Eriksson Röhnisch:** Writing – review & editing, Methodology. **Saeid Karkehabadi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Saeid Karkehabadi reports financial support was provided by Sveriges innovationsmyndighet. Saeid Karkehabadi reports a relationship with Sveriges innovationsmyndighet that includes: funding grants. 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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