Phytic acid in faba bean

– the effect of pH, soaking time and heat-treatment to reduce the phytic acid content of faba bean

Fytinsyra i åkerböna – effekten av pH, blötläggningstid och värmebehandling för att reducera halten av fytinsyra i åkerböna

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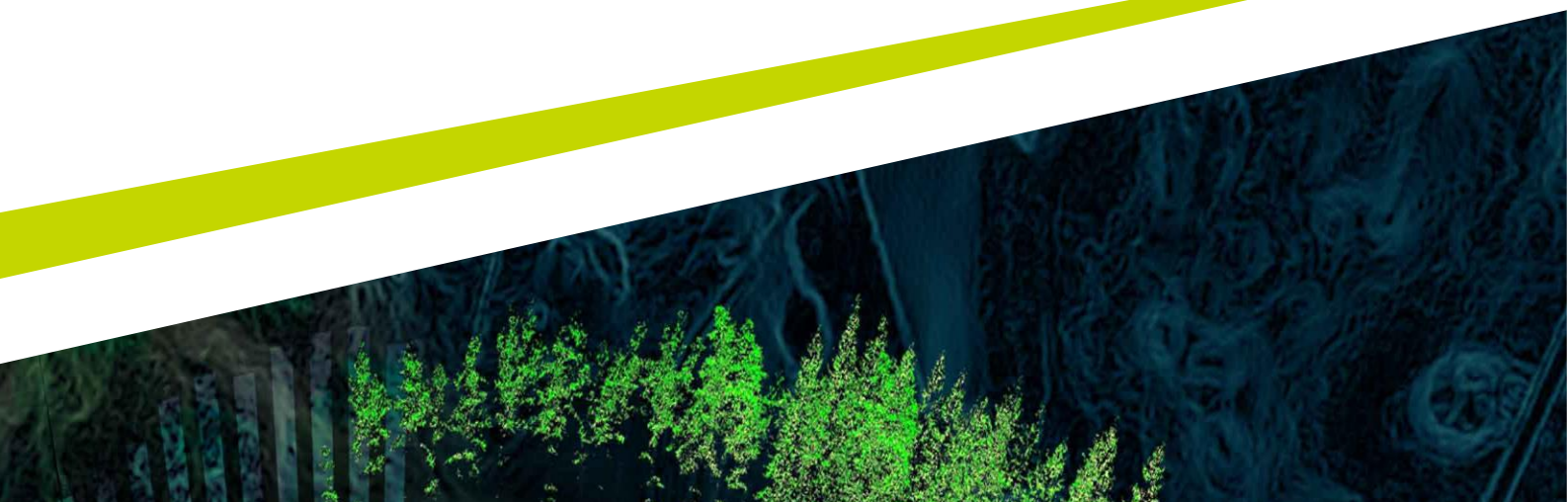

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Abstract

To feed a globally growing population, a challenging shift in the usage of today's food system is required. This includes a shift towards more sustainable human diets, with increased levels of protein from plant-based sources. Faba bean (*Vicia faba* L.) is a potentially attractive candidate to fulfil this purpose, not the least because of its qualitative nutrient content, but also for its cultivation potential in Sweden. However, faba beans have varying levels of antinutrients, including phytic acid. Phytic acid has metal chelating abilities with the potential to cause mineral deficiencies in humans. The compound can be reduced by different pre-treatments and processing techniques, such as soaking for various times in altered pH conditions and temperatures.

This master thesis had three aims. The first was to develop and optimise a method to evaluate the concentration of phytic acid in faba bean, variety Tiffany. The second was to identify and evaluate the effect of soaking treatment conditions, i.e., pH, soaking time and heat-treatment, with the purpose to minimize the concentration of phytic acid in the legume. Finally, the third aim was to establish the optimum treatment based on the conditions of the soaking treatments.

The faba bean proteins were extracted with alkaline extraction followed by isoelectric precipitation and differential NaCl solubility. The phytic acid was extracted and analysed using anion-exchange chromatography and a colorimetric method with Wade reagent.

The optimised method successfully evaluated the concentration of phytic acid throughout the protein extraction of faba bean, resulting in a 28 % reduction. All soaking treatments reduced the concentration of phytic acid. These results provide important indications of processing information on phytic acid in faba bean for the industry of plant-based foods. The optimum soaking treatment was treatment E, with a pH value of 6 at a temperature of 20 °C for 2 h. Treatment E reduced the concentration of phytic acid by 87 %.

Keywords: Plant-based food, *Vicia faba*, phytic acid, soaking treatment, anion-exchange chromatography, colorimetric method

Sammanfattning

För att mätta en globalt växande befolkning krävs ett skifte i hur man använder dagens livsmedelssystem. Skiftet skulle behöva innefatta en mer hållbar kosthållning, där en större andel av det protein som konsumeras kommer från växtbaserade alternativ. Åkerböna (*Vicia faba* L.) är ett potentiellt bra alternativ eftersom den har en god näringskvalitet och kan odlas i Sverige. Däremot innehåller åkerbönan olika nivåer av flertalet antinutrienter, varav en är fytinsyra. Fytinsyra har kelerande egenskaper som kan leda till mineralbrist hos människor. Mängden fytinsyra i åkerböna kan minskas genom olika förbehandlingsmetoder. Till exempel kan åkerbönan blötläggas i olika pH-värden, temperaturer och tidsperioder.

Det här projektet hade tre syften. Det första var att utveckla och optimera en metod för att undersöka fytinsyrainnehållet i åkerböna, av sorten Tiffany. Det andra var att identifiera och undersöka olika blötläggningsbehandlingar som skulle kunna minska koncentrationen av fytinsyra i åkerböna. Slutligen var det tredje syftet att fastslå vilken blötläggningsbehandling som var den optimala för att minska fytinsyrainnehållet i åkerböna.

Proteinerna från åkerbönan extraherades genom alkalisk extraktion, följt av isoelektrisk utfällning och tillsats av NaCl. Extraktionen och analysen av fytinsyra genomfördes med anjonbyteskromatografi och en kolorimetrisk metod som använde Wade reagens.

Den optimerade metoden minskade fytinsyrakoncentrationen i åkerböna med 28 % under proteinextraktionen. Samtliga blötläggningsbehandlingar hade en reducerande effekt på åkerbönans fytinsyrainnehåll. Resultaten kan anses ge viktiga indikationer till industrin av växtbaserade köttsubstitut. Den optimala blötläggningsbehandlingen var behandling E. Behandling E bestod av pH 6 i 20 °C under 2 h och minskade koncentrationen av fytinsyra med 87 %.

Nyckelord: Växtbaserade livsmedel, *Vicia faba*, fytinsyra, blötläggning, anjonbyteskromatografi, kolorimetrisk metod

Table of contents

List of tables **8**

List of figures **9**

Abbreviations **10**

1. Introduction **11**

1.1 Phytic acid and plants 12

1.1.1 Phytases and plants 13

1.2 Faba bean 14

1.2.1 Phytic acid in faba bean 15

1.3 Phytic acid and human nutrition 16

1.4 Soaking treatments 17

1.5 Analytical methods 18

1.5.1 Anion-exchange chromatography 18

1.5.2 Colorimetric method 18

1.6 Objective and aims 18

2. Materials and methods **20**

2.1 Material 20

2.2 Pre-trial and method optimisation 20

2.3 Protein extraction 22

2.4 Phytate extraction 24

2.5 Soaking treatment optimisation 24

2.6 Anion-exchange chromatography 25

2.7 Colorimetric method 26

2.8 Standard curve 26

2.9 Calculations 27

3. Results **28**

3.1 Phytic acid content in the protein extract 28

3.2 Phytic acid reduction and soaking treatments 29

3.2.1 Effect of pH 30

3.2.2 Effect of soaking time 30

3.2.3 Effect of heat-treatment 30

4. Discussion **30**

4.1 Phytic acid content in the protein extract 31
4.2 Phytic acid reduction and soaking treatments 32
4.3 Evaluation of analytical methods 33
4.4 Delimitations 34

5. Conclusion 36
5.1 Forward research question 36

References 37

Popular science summary 41

Acknowledgements 43

Appendix 1 – Raw Data 44

List of tables

Table 1. Phytic acid content (mg/100 g DW) in selected cereals and legumes* 13

Table 2. Optimum phytase conditions (pH and temperature) in different plant sources* . 14

Table 3. Phytate concentration (mg/100 g DW) in faba bean flour obtained with or without anion-exchange chromatography (AEC) and at different pH adjustments (0.75, 6 and 3) 22

Table 4. Soaking treatments applied to supernatant S3 (Figure 4) in the protein extraction 25

Table 5. Total phytate concentration (mg/100 g DW) obtained in pellet P3 treated with the different soaking conditions pH (5, 6 or 7), soaking time (2 h or 5 h) and temperature (20 °C or 40 °C). The samples were measured in duplicates or triplicates*. The results are presented as means ± standard deviations 29

List of figures

Figure 1. Chemical structure of phytic acid, IP_6. Adapted from PubChem (n.db)..... 12

Figure 2. Chemical structure of phytate, IP_6. Adapted from PubChem (n.da)..... 12

Figure 3. Flow scheme of the phytate extraction and analytical methods (anion-exchange chromatography and the colorimetric method)..... 21

Figure 4. Flow scheme of the protein extraction and the soaking treatment set up 23

Figure 5. Standard curve and the corresponding linear equation of sodium phytate concentration (0.1, 1, 2.5, 10, 20, 30, 45 µg/ml) mixed with Wade reagent measured spectrophotometrically at 500 nm 26

Figure 6. The total phytate concentration (mg/100 g DW) and percentual phytate reduction (%) throughout the protein extraction of faba bean. The results are presented in means ± standard deviations. The abbreviations S1, S2, S3, S5 and P3 represent different steps in the protein extraction where phytate was extracted and analysed (Figure 4) 28

Abbreviations

AEC	Anion-exchange chromatography
ANF	Antinutritional factor
DW	Dry weight
FAO	Food and Agriculture Organization of the United Nations
HPIC	High-performance ion chromatography
HPLC	High-performance liquid chromatography
IP_6	Inositol hexaphosphate
IP_5	Inositol pentakisphosphate
P_{inorg}	Inorganic phosphorus
P_{phy}	Phytate phosphorus
SDG	Sustainable development goal
UN	United Nations
WHO	World Health Organization

1. Introduction

In the year 2050, the world's population is estimated to reach almost ten billion people. This increase in population will have a considerable significance on the food supply (FAO 2018). Further, this demands a challenging shift in the usage of the current food system. The future food systems need to be more environmentally friendly and sustainable, and simultaneously provide populations with food that is palatably and nutritious. Fulfilling these criteria is essential to reach the 2030 Agenda, including many of the UN Sustainable Development Goals (SDG). The shift towards a more sustainable diet includes a reduction in the consumption of animal-based protein, i.e., red meat, and an increase in plant-based protein sources (Willett et al. 2019). In developing countries, plant-based protein sources, such as grain legumes, are a natural part of the everyday diet. However, the dominant protein source in developed countries is animal-based. In Sweden, the protein intake distribution of the population is estimated at 1, 21 and 28 % for grain legumes, cereals and meat respectively. The remaining 50 % mainly consists of dairy, egg and seafood (Röös et al. 2020).

In the search for new plant-based substitutes, and with specific consideration to the Swedish cultivation opportunities, the grain legume faba bean can potentially be considered an attractive ingredient. The crop has a favourable nutritional profile, partly because of its protein content, which benefits the potential of producing sustainable, novel plant-based meat substitutes. Though the faba bean is to this day under-utilised for human food purposes in Sweden and rather used for animal feed (Hushållningssällskapet 2018). Apart from the beneficial nutritive content of faba bean, the crop also consists of various levels of antinutritive compounds such as lectins, saponins and phytic acid (Mayer Labba et al. 2021).

The antinutrient phytic acid may contribute to nutritional implications in humans, of which one of the most prominently associated problem globally is deficiencies in micronutrients such as iron and zinc (FAO/WHO 2001; Samtiya et al. 2020). Knowledge about phytic acid in plant-based foods is therefore important in the light of the possible future protein shift and a greater demand for commercial meat substitutes. Further, it is necessary to monitor, and decrease, the concentration of phytic acid in faba beans to make the legume a suitable raw material to produce sustainable meat substitutes.

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1.1 Phytic acid and plants

Phytic acid (myo-inositol-1,2,3,4,5,6-hexa-dihydrogen phosphate; abbreviated IP6) is the main form of storage for phosphorus in plants, where it accounts for approximately 50-80 % of the total phosphorous. Phytic acid accumulates in protein storage vacuoles in the aleurone layer or the embryo of the plant seeds during seed development. Further, the compound is naturally found in metal complexes in plant foods including seeds, legumes, cereals, oilseeds and nuts (Sandberg & Scheers 2016; Damodaran et al. 2017; Kumar & Sinha 2018; Samtiya et al. 2020). The compound consists of an inositol ring with six esterified phosphate groups attached (Figure 1) (Damodaran et al. 2017). The acidic properties of phytic acid allow full protonation at pH >12, which makes the anionic form, phytate (Figure 2), and its associated salts the most prominent form of the compound found in plants (Sandberg & Scheers 2016).

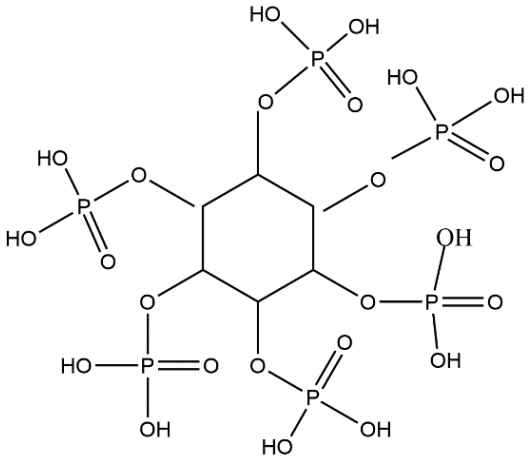

Figure 1. Chemical structure of phytic acid, IP_6. Adapted from PubChem (n.db)

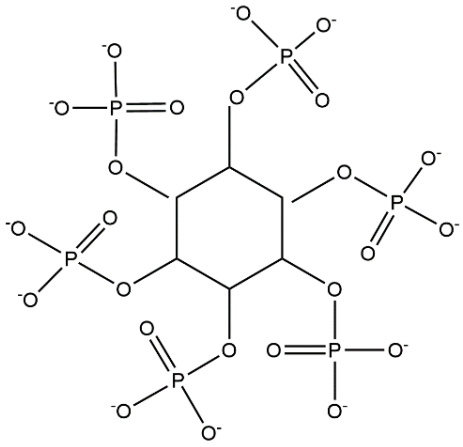

Figure 2. Chemical structure of phytate, IP_6. Adapted from PubChem (n.da)

The anionic form, phytate, has the property of chelating metal ions, which is beneficial for plant mineral storage. However, this has implications for human nutrition (Sandberg & Scheers 2016). These possible implications will be further described in section 1.3. The terms phytic acid and phytate are going to be used interchangeably throughout this thesis if nothing else is stated.

Inositol hexaphosphate, IP_6, is the predominant form of phytic acid found in plants. Although, lower inositol phosphates such as inositol pentakisphosphates,

IP_5, are also present because of IP_6 dephosphorylation caused by hydrolysis with phytases (Zhang et al. 2022). The quantity of phytate in legumes and cereals, as well as the occurrence of different phytate salts (IP_5-IP_1), varies between approximately 0.2-2 % of the dry weight (DW), depending on factors such as the plant source and the processing methods applied (Table 1) (Schlemmer et al. 2009). The levels can, however, be as high as 6 % in the germ and seed parts of the plant (Table 1).

Further, cereals, especially whole grain cereals, are relatively high in phytate because of their abundance in the bran and aleurone layers. To reduce the levels of the compound, milling can be used to separate the bran relatively easily from the rest of the cereal. However, milling is not an optimal option to reduce the concentration of phytate in legumes since it is more closely associated with the protein parts of the endosperm (Schlemmer et al. 2009; Samtiya et al. 2020). Instead, processing factors including soaking, heat-treatment, fermentation and germination are more associated with the reduction and elimination of phytic acid in legumes (Luo et al. 2009; Schlemmer et al. 2009; Samtiya et al. 2020; Ojo 2021). These processing and soaking techniques will be further in section 1.4.

*Table 1. Phytic acid content (mg/100 g DW) in selected cereals and legumes**

Cereals *Common names*	**Phytic acid** (mg/100 g DW)
Maize (germ)	720-2220 (6390)
Wheat (germ)	390-1350 (1140-3910)
Rice	60-1080
Barley	380-1160
Oat	420-1160
Rye	540-1460
Legumes *Common names*	**Phytic acid** (mg/100 g DW)
Kidney bean	610-2380
Faba bean	510-1770
Chickpeas	280-1600
Lentils	270-1510
Peas	220-1220

*Table adapted from Schlemmer et al. (2009)

1.1.1 Phytases and plants

Phytate containing plants have phytate degrading enzymes called phytases, myo-inositol (1, 2, 3, 4, 5, 6)- hexakisphosphate phosphohydrolases (Kumar & Sinha 2018). The major role of the endogenous enzyme is to catalyse the hydrolysis of phytate. The stepwise enzymatic degradation of phytate, IP_6, removes its phosphate groups, forming lower inositol phosphates, IP_5-IP_1, and further releases

phosphorous which can be used during germination and for the growth of the plants (Frias et al. 2003; Kumar & Sinha 2018).

There are four different groups of phytases. β-propeller phytases, cysteine phosphatases, purple acid phosphatases and histidine acid phosphatases. The last one is prominently used in animal feeds. The enzymes can further be classified according to their pH of activity. The classification includes acidic, neutral and alkaline phosphatase named depending on the pH activity optimum. Another way of classifying phytase is according to the position on the inositol ring where the phytate dephosphorylation is initiated (Kumar & Sinha 2018).

U is one unit used to express the activity of phytases. The definition of the unit is that one U of phytase is the quantity needed to free 1 µmol of inorganic phosphorus per minute from 0.0015 mol/L sodium phytate at pH 5.5 and 37 °C (Kumar & Sinha 2018).

*Table 2. Optimum phytase conditions (pH and temperature) in different plant sources**

Plant source	pH	Temperature (°C)	Specific activity at 37 °C (U/mg)
Faba bean	5	50	636
Mung bean	7.5	57	2.4
Soybean seeds	4.5-5	58	2.4
Barley	5	45	117
Oat	5	38	307
Rye	6	45	517
Wheat bran	6	45	127

*Table adapted from Kumar & Sinha (2018)

Further, phytases can be found in animals, e. g ruminants and vertebrates, as well as microorganisms. Microbial phytases, often extracted from moulds or engineered organisms, can be added to animal feed to decrease the concentration of phytate and increase the bioavailability of phosphorus (Gupta et al. 2015). However, these microbial phytases are not commercially used in human nutrition (Sandberg & Scheers 2016). Although, fungal phytases have successfully been evaluated and used as additives in the breadmaking process to decrease the phytate concentration of flours, doughs and bread (Jatuwong et al. 2020).

1.2 Faba bean

The grain legume faba bean (*Vicia faba* L.*)* is also known in the literature as field bean, broad bean or fava bean (Hebblethwaite 1983; Schlemmer et al. 2009; Mayer Labba et al. 2021). Further, it is divided into three groups depending on its seed size. The groups are var. *minor*, var. *equina* and var. *major* for the small-,

intermediate- and large-seeded variety respectively (Hebblethwaite 1983). The variety Tiffany (var. *minor)*, which was used within this project, has a considerably high protein content and harvest yield (Hushållningssällskapet 2018) making it suitable as a meat substitute or meat substitute ingredient.

Faba bean has a long tradition of human cultivation. The crop is grown worldwide today and can be found in more than 55 countries. The harvest area is estimated at 2.56 million acres which is giving rise to a harvest yield of 4.56 million tons (Crépon et al. 2010). Faba beans are not cultivated on large scale for human consumption today in Sweden (National Food Agency 2016) and the crop is more commonly used for animal feed purposes, rather than as a source of human food (Hushållningssällskapet 2018). However, in a global context, the crop is used for both feed and food purposes (Fouad et al. 2013).

Further, faba bean has several environmental benefits. Faba beans can be cultivated in many different climatic areas of Europe, which for instance the widely consumed soybean cannot (Crépon et al. 2010). This is one of the reasons why the faba bean could be particularly important in the Nordic regions, such as Sweden, in terms of the possibility of cultivating it and making it a part of the protein shift from animal-based protein to plant-based protein (Mayer Labba et al. 2021). Faba beans also have an important role in crop rotation practices with the potential ability to reassess nitrogen in the soil. This property is considered an attractive attribute, not the least in future sustainable food systems (Rubiales 2010).

Faba beans have a good nutritional content of carbohydrates, as well as fibres, vitamins, and minerals. The amino acid composition is similar to other grain legumes. When consumed together with cereals, which are commonly rich in tryptophan and sulphur amino acids, faba beans can be a good substitute for animal-based protein (Vioque et al. 2012). Further, faba beans are rich in minor components including bioactive compounds, with possible health-promoting properties such as reduced risk for cardiovascular disease and colorectal cancer, decreased blood cholesterol levels as well as improved gut health (Luo et al. 2009; Vioque et al. 2012; Mayer Labba et al. 2021). However, some bioactive compounds have antinutritive effects and can be considered antinutrient factors (ANF). These antinutrients can enable implications for human nutrition, which will be further addressed in section 1.3 (Mayer Labba et al. 2021).

1.2.1 Phytic acid in faba bean

The antinutritive bioactive compound phytic acid is found in faba beans (Mayer Labba et al. 2021). The function of phytic acid in faba bean is equal to the general function of phytic acid in all plants, i.e., as a phosphorus storage source (Sandberg & Scheers 2016). Examples of other antinutrients prevalent in various concentrations in faba beans are tannins, vicine and convicine (Crépon et al. 2010).

The approximate phytic acid concentration of faba bean ranges between 510-1770 mg/100 g DW (Table 1). Phytic acid can be present at various concentrations in the different varieties of the legume. The variation has been suggested to depend on the specific cultivar, soil factors and the growing conditions in the specific climate (Mayer Labba et al. 2021). According to the study by Zehring et al. (2022), conventional cultivation of faba bean is suggested to give rise to lower concentrations of phytic acid than organic cultivation of the legume.

1.3 Phytic acid and human nutrition

Naturally, the consumption of plant-based foods involves the consumption of phytate. In terms of human nutrition, phytate is considered a strong antinutritional factor (Damodaran et al. 2017). Antinutrients are compounds that affect, or impair, the absorption of other compounds or minerals (Cammack 2006). One antinutritional aspect of phytate is the compounds' ability to bind to, and further hinder the activity of, necessary food degrading enzymes, such as protease, amylase and trypsin, in the small intestine in humans. The possible consequence is restrained uptake of protein, fat and starch (Guo et al. 2015; Samtiya et al. 2020). Further, the compounds' ability to chelate, or bind, cations causing a prohibited absorption of the minerals, makes the compound least partly responsible for mineral ion deficiencies in humans (Sandberg & Scheers 2016; Samtiya et al. 2020).

Metals and mineral cations, such as Fe^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+}, are normally absorbed in the small intestine of humans at physiological pH (around pH 6-7). At the same pH, phytate carries eight out of twelve negative charges, which increases the phytate's affinity for cations. Since the formation of phytate chelates are pH-dependent, insoluble complexes with cations will be formed in the small intestine environment. Because monogastric animals and humans lack phytate degrading phytases, are these complexes not degraded and the bioavailability of the cations in the diet thus decreases (Sandberg & Scheers 2016; Samtiya et al. 2020). It is possible to estimate the relative bioavailability of cations in the presence of phytate. This is estimated by calculating the molar ratios of phytate to the specific mineral. These obtained molar ratios can be further used as indicators for the inhibitory effect of phytate on the mineral (Mayer Labba et al. 2021).

Additionally, chelating effects on minerals strongly depend on the phosphorylation level of the inositol phosphates. Highly phosphorylated IP_6 and IP_5 have a stronger effect on the mineral chelating properties than lower phosphorylated inositol phosphates (Haileslassie et al. 2016). The phosphorylation level of phytic acid depends on the hydrolysing capacity of the phytic acid degrading enzyme phytase. Further, lower inositol phosphates are more soluble than higher inositol phosphates and their negative effect on mineral absorption are less severe (Burbano et al. 1995).

It is important to highlight the possible beneficial biological role of phytic acid for humans and animal cells. The lower inositol phosphates, such as IP_4 and IP_3, have shown involvement in physiological functions such as the regulation of different intracellular processes (Frias et al. 2003; Marolt & Kolar 2020). Further, lower inositol phosphates may have intermediating functions in cell signalling, as well as beneficial involvement in preventing some forms of cancer such as colon cancer (Frias et al. 2003).

1.4 Soaking treatments

The nutritional value of a legume, including the bioavailability of minerals, is in general lower when the legume is consumed raw because of the prevalence of antinutrients. To reduce the presence of these antinutrients, including phytate, different food preparation techniques and processing methods can be used (Samtiya et al. 2020). Soaking is a simple preparative pre-treatment used to reduce the levels of ANFs in cereals and legumes before consumption. The magnitude of reduction depends on the soaking time, temperature and composition of the soaking solution, i.e., acidic, alkalic or water (Patterson et al. 2017). Not the least is this applicable to the grain legume faba bean. The effect of processing on faba bean to reduce phytate has been well-studied (Sharma & Sehgal 1992; Luo et al. 2009; Sharan et al. 2021). However, this master thesis had a special focus on evaluating the phytate concentration throughout the faba bean protein extraction, as well as evaluating the potential of reducing the phytate concentration in the final protein pellet P3, after applying different soaking treatments. The factors that made up the soaking treatments within this project were: pH, soaking time and modest heat-treatment (Table 4). The combinational usage of these parameters had the purpose of mimicking the environment where the phytate degrading enzyme phytase has its optimum activity. According to Luo et al. (2010), the endogenous faba bean phytates have a pH and temperature optima at approximately pH 6 and 50-55 °C, however, Greiner et al. (2001), as well as Kumar & Sinha (2018) establish an optimum for degradation of sodium phytate at pH 5 and 50 °C (Table 2).

Apart from the reduced phytate concentration caused by activating the endogenous phytase, the reducibility of phytate during soaking is also due to the water-soluble characteristics of the compound. Phytate diffuses from the legume, into the soaking water which will reduce the concentration of the compound in the legume (Patterson et al. 2017).

A combinational usage of soaking treatments has been established more effective to reduce the concentrations of ANFs compared to individual application of each factor (Hajos & Osagie 2004).

1.5 Analytical methods

1.5.1 Anion-exchange chromatography

Ionic chromatography is a method widely used to separate ions and polar analytes using its electrostatic interaction. The analyte ion has an opposite charge of the ionic functional groups on the chromatographical support. If the analyte ion, that is being separated, is an anion, the charge of the functional groups in the column support is positively charged. The mechanism of anion-exchange chromatography (AEC) starts with diffusion and binding of the analyte anions to the positively charged functional groups of the stationary phase. To unbind the analyte from the stationary phase, different amounts of energy are needed. Therefore, different concentrations of eluent ions are applied to the column, which will elute different column bounded ions. The ion separation occurs because less energy-requiring analyte ions are eluted earlier from the stationary phase than more energy-requiring analytes (Bhattacharyya & Rohrer 2012).

1.5.2 Colorimetric method

A colorimetric method with Wade reagent is used to analyse the amount of free, leaching phytate in a sample. The pink colour of the Wade reagent is due to the reaction between its contents of ferric ions and sulfosalicylic acid, which is spectrophotometrically monitored at an absorption maximum of 500 nm. When the reagent is applied to the phytate containing sample, a potential decrease in pink colour intensity indicates that the ferric ions have bound the free phytate, i.e., the phosphate esters of the phytate present in the sample, instead of reacting with the sulfosalicylic acid (Latta & Eskin 1980).

1.6 Objective and aims

Knowledge about phytic acid content in faba bean and methods to reduce the prevalence of the compound is important. Not least because of the globally prevalent iron deficiency, the compounds' ability to interfere with digestibility proteins which can lead to substantial health issues in humans, as well as to the rising demand for meat substitutes as a sustainable food choice. The objectives of this study were to evaluate the phytic acid concentration throughout the protein extraction of faba bean. Further, the optimal pre-treatment conditions for the reduction, or elimination, of phytic acid in the final protein fraction were evaluated since it has the potential to function as an ingredient for meat substitutes.

Consequently, three aims were formulated:

- The first aim of the study was to develop and optimise a method to evaluate the phytic acid concentration in faba bean flour (variety Tiffany).
- The second aim was to identify and evaluate the effect of soaking treatment conditions, i.e., pH, soaking time and heat-treatment, with the purpose to reduce the concentration of phytic acid in the legume.
- Finally, the third aim is to establish the optimum treatment for reducing the phytic acid concentration in faba bean, based on the condition of the soaking treatments.

2. Materials and methods

2.1 Material

The plant material used in this project was dehulled and pre-milled faba bean flour (*Vicia faba* L.*)*, of Tiffany variety, purchased from *svensk fava (*Marcus Nordgren). According to the manufacturer, the chemical composition of the flour was 1201 kJ (287 kcal), 45 % carbohydrates, 29-32 % protein, 2 % fat and 24 % fibre. The chemicals and reagents used were of analytical grade and the solutions were prepared with deionised water. HCl 37 % was obtained from VWR International AB (Sweden). NaCl and NaOH were from EMSURE. 5-Sulfosalicylic acid dihydrate, Iron (III) chloride hexahydrate and phytic acid sodium salt hydrate for the calibration curve were obtained from Sigma Aldrich.

2.2 Pre-trial and method optimisation

A combined pre-trial and method optimisation was implemented as a first step in the experimental outline of the project. The tests were performed on supernatant S1 in the protein extraction (Figure 4). The purpose was to get accustomed to the overall methodology and to evaluate the possible impact different pH adjustments throughout the phytate extraction and phytate analysis had on the final phytate concentration. The method optimisation was adapted from Fruhbeck et al. (1995), with modifications to suit this specific project.

In this project, the impact of using an AEC, as well as pH adjustment (pH 0.75, 6 and 3) throughout the phytate extraction and analysis procedure was evaluated. The procedures are described thoroughly in sections 2.4, 2.6 and 2.7, and an overview of the methods is seen in Figure 3.

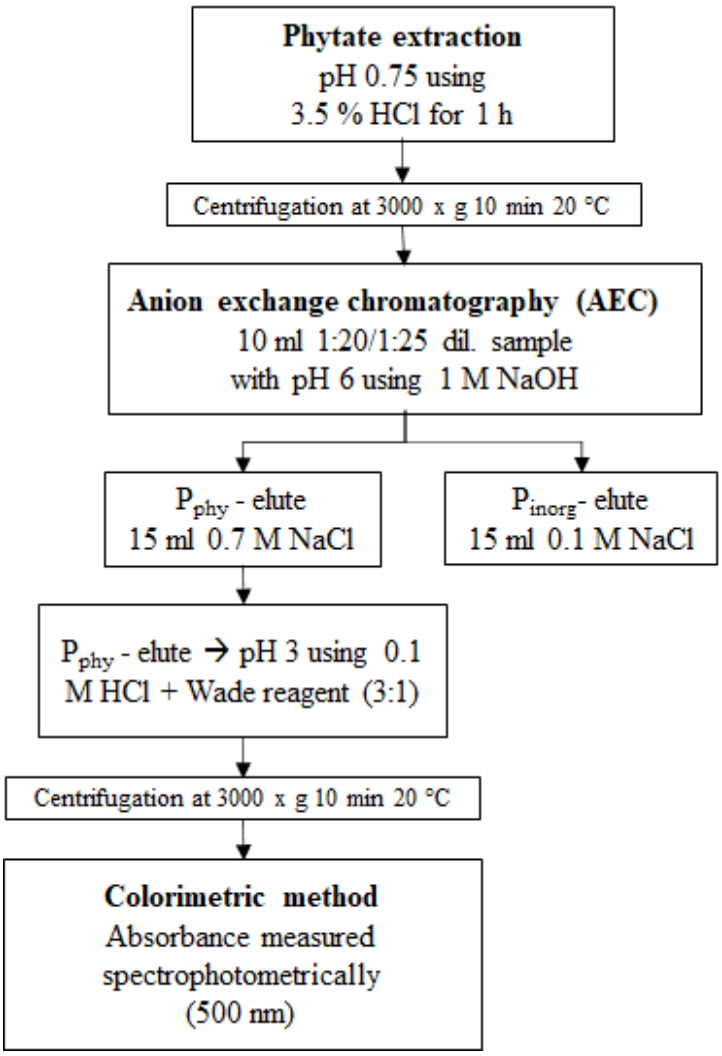

Figure 3. Flow scheme of the phytate extraction and analytical methods (anion-exchange chromatography and the colorimetric method)

Initially, the pH of the solution from the first step of the protein extraction, S1, was adjusted to 0.75 ± 0.03 using 3.5 % HCl, whereas the solution was kept for 1 h with constant stirring to allow the phytate to extract from the protein- and mineral complexes. Secondly, before adding the solution to the AEC column, the pH of the solution was changed to 6, above the protein's isoelectric point of 4.8. The purpose was to facilitate the binding to the column and thereby minimize the effect of metals and proteins. Finally, pH was changed to 3 before adding the Wade reagent, after elution with 15 ml 0.7 M NaCl. This step was performed to improve the binding capacity of phytate to the ferric ions in the Wade reagent and to decrease the variation among samples. pH 3 was used because the complex-forming ability of ferric ions is pH sensitive and has an optimum at pH 3 (Fruhbeck et al. 1995).

Table 3. Phytate concentration (mg/100 g DW) in faba bean flour obtained with or without anion-exchange chromatography (AEC) and at different pH adjustments (0.75, 6 and 3)

	pH adjustment and AEC					
	0.75		0.75 + 6		0.75 + 6 + 3	
	With	Without	With	Without	With	Without
Phytate concentration (mg/100 g DW)	794.5* ± 2.64	934.37**	1418.7* ± 75.88	1996.1**	1375.8* ± 19.80	2075.3* ± 26.39

*The impact of the pH adjustments and usage of AEC was evaluated in duplicates

** The impact of the pH adjustments and not using the AEC was evaluated in singles

The expected phytate concentration was believed to be between 510-1770 mg/100 g DW (Table 1). Values higher than 2000 mg/100 g DW were considered an overestimation of phytate, potentially caused by inorganic phosphorous or other interfering compounds being measured. The phytate concentration obtained at pH 0.75 + 6 and pH 0.75 + 6 + 3 without AEC was therefore considered an overestimation of phytate. pH 0.75 without AEC, measured a phytate concentration of 934.37 mg/100 g DW (Table 3) but was not further investigated because the values obtained with AEC at pH 0.75 + 6 and pH 0.75 + 6 + 3 were considered more reliable in the comparison to the literature (Latta & Eskin 1980).

In agreement with the results of Fruhbeck et al. (1995), pH adjustment to pH 6 made the concentration of assessed phytate increase compared to the concentration measured after only adjusting it to its phytate extracting pH of 0.75. The sample variation decreased when the pH was further adjusted to 3 compared to 0.75 + 6, which makes the results more accurate and precise. However, the influence of pH 3 adjustment was not greater at 0.75 + 6 + 3 with AEC compared to the phytate concentration at pH 0.75 with AEC. It was determined to continuously use the method where the pH was changed to 0.75 + 6 + 3 and with AEC because of the accurate phytate concentration obtained, together with a relatively low variation among the samples.

2.3 Protein extraction

The faba bean proteins were extracted with alkaline extraction, followed by isoelectric precipitation and differential NaCl solubility to increase the protein yield and functionality. The extraction method was adapted from Suchkov et al. (1990) with modifications to suit this specific project. Faba bean flour was mixed with distilled water (1:9 w/v) and it was stirred overnight using a magnetic stirrer. The pH of the solution was adjusted to pH 8 using 1 M NaOH and thereafter left for constant stirring on a magnetic stirrer with a heater at 40 °C for 1 h. The solution was centrifuged at 5000 x g for 30 min at 20 °C to remove insoluble materials. The

pellet was discarded and the pH of the supernatant was adjusted to the protein's isoelectric point, 4.8, with 1 M HCl. The supernatant was centrifuged at 5000 x g for 20 min at 20 °C. The obtained supernatant was discarded and the pellet was re-dispersed in 0.6 M NaCl which was further diluted to 0.1 M NaCl (dilution ratio 1:6), whereas it was centrifuged at 1500 x g for 15 min at 4 °C.

The supernatants of each protein extraction step were collected and further analysed for phytate content. The pellets throughout the protein extraction were collected, freeze-dried and further analysed for phytate content by redispersing the pellet in distilled water (1:100 w/v).

The overall protein extraction process, as well as when the soaking treatments were applied, is seen in Figure 4. In the figure, the solutions and supernatants are named S1-S6 and the pellets are named P1-P3. The phytate concentration was extracted and analysed in each step (except S4) and the complete results from the analyses are found in Appendix 1.

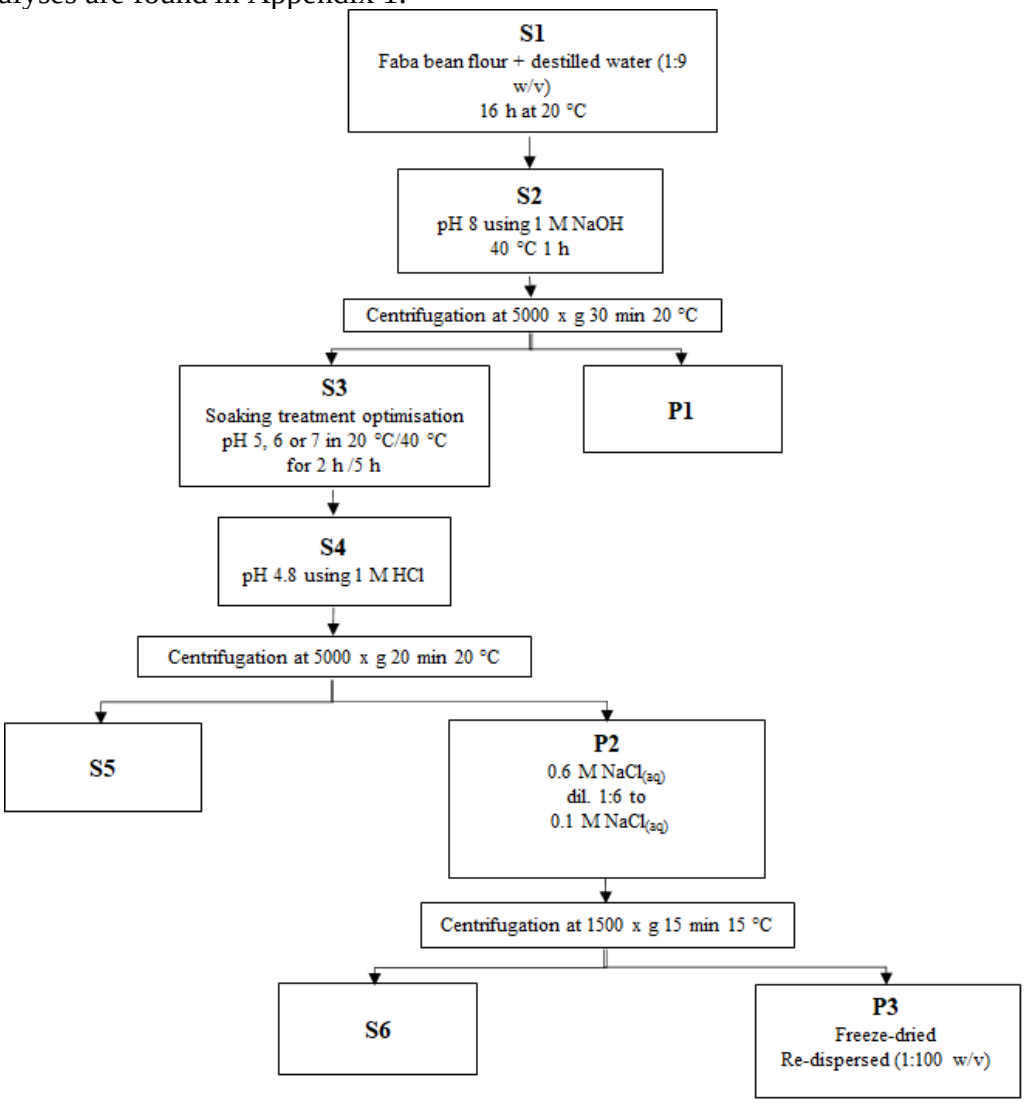

Figure 4. Flow scheme of the protein extraction and the soaking treatment set up

2.4 Phytate extraction

The phytate extraction procedure was adapted from Fruhbeck et al. (1995), with modifications to suit this specific project.

Phytate was extracted from the faba bean protein- and mineral complexes with the gradual addition of 3.5 % HCl until reaching the pH of 0.75 ± 0.03. The solution was continuously stirred for 1 h and it was centrifuged at 3000 x g for 10 min at 20 °C. The pellet was discarded, and the supernatant was collected for further phytate concentration analysis with anion-exchange chromatography and the colorimetric method using Wade reagent.

2.5 Soaking treatment optimisation

The soaking treatments evaluated to potentially benefit the activity of the endogenous phytase, and decrease the phytate concentration in faba bean, were pH 5, 6 or 7 at a temperature of 20 °C or 40 °C with a soaking time of 2 or 5 hours.

To obtain the desired pH values of the soaking treatments, the gradual addition of 1 M HCl or 1 M NaOH was used. The soaking treatments were either left at room temperature on a magnetic stirrer to maintain the desired temperature of approximately 20 °C, whereas a magnetic stirrer with heat was used to achieve a temperature of 40 °C.

The conditions for the different soaking treatments were applied to the supernatant S3 in the protein extraction (Figure 4) according to Table 4. Treatment A had a pH value of 5 at a temperature of 20 °C for 2 h, whereas treatment B had a pH of 5 at a temperature of 20 °C for 5 h etc (Table 4).

Table 4. Soaking treatments applied to supernatant S3 (Figure 4) in the protein extraction

Treatment name	pH	Temperature (°C)	Soaking time (h)
A	5	20	2
B	5	20	5
C	5	40	2
D	5	40	5
E	6	20	2
F	6	20	5
G	6	40	2
H	6	40	5
I	7	20	2
J	7	20	5
K	7	40	2
L	7	40	5

2.6 Anion-exchange chromatography

The separation between phytate phosphorus (P_{phy}) and inorganic phosphorus (P_{inorg}), together with other interfering compounds, was performed according to the anion-exchange chromatography methodology described in Fruhbeck et al. (1995) with modifications to suit this specific experiment. This separation is performed because the ferric ions of the Wade reagent are not able to discriminate between P_{phy} and P_{inorg} which could result in an overestimation of the phytate content (Naves et al. 2014). This was evaluated and further observed in the pre-trial of this project (Table 3). Separation was conducted in plastic columns (~1,3 x 6,5 cm; Avantor delivered by VWR International) packed with 1 g of anion-exchange resin (AG1-X8, 200-400 dry mesh, Sigma-Aldrich) and washed with 15 ml 0.7 M NaCl and 30 ml deionized water before use to ensure resin chloride saturation. 10 ml of 1:20 or 1:25 diluted sample with pH 6 adjusted with 1 M NaOH was added to the column, whereas the $P_{inorg.}$ was eluted with 15 ml 0.1 M NaCl. P_{phy} was eluted with 15 ml 0.7 M NaCl, collected, and saved for further analysis using the colorimetric method.

2.7 Colorimetric method

The colorimetric method was adapted from Latta & Eskin (1980) with modifications to suit this specific project.

Wade reagent was prepared by mixing 0.03 % $FeCl_3$ $6H_2O$ and 0.3 % sulfosalicylic acid (1:10) in a 100 ml volumetric flask with distilled water. The pH of the sample elute from the AEC method was adjusted to pH 3 with 0.1 M HCl. 3 ml sample and 1 ml Wade reagent were further added to a 15 ml falcon tube. After vigorous vortexing and centrifugation at 3000 x g for 10 min at 20 °C, the absorbance was read spectrophotometrically (Shimadzu UV-spectrophotometer UV-1800) at 500 nm using water to zero the machine. A reagent blank containing deionized water and Wade reagent (3:1) was used to subtract the background light from the samples.

2.8 Standard curve

Standard solutions (0.1, 1, 2.5, 10, 20, 30 and 45 µg/ml) with commercial sodium phytate and deionized water were mixed with the Wade reagent (3:1), vortexed and centrifuged at 3000 x g for 10 min at 20 °C. The absorbance was read spectrophotometrically (Shimadzu UV-spectrophotometer UV-1800) at 500 nm with water to zero the machine. A reagent blank containing deionized water and Wade reagent (3:1) was used to subtract the background light from the samples. The standard curve was plotted in Excel (Figure 5). To establish the accuracy of the standard curve, a 10 µg/ml sample was run each time a phytate analysis was performed. The coefficient of variation was between 5.5- 7.8 %.

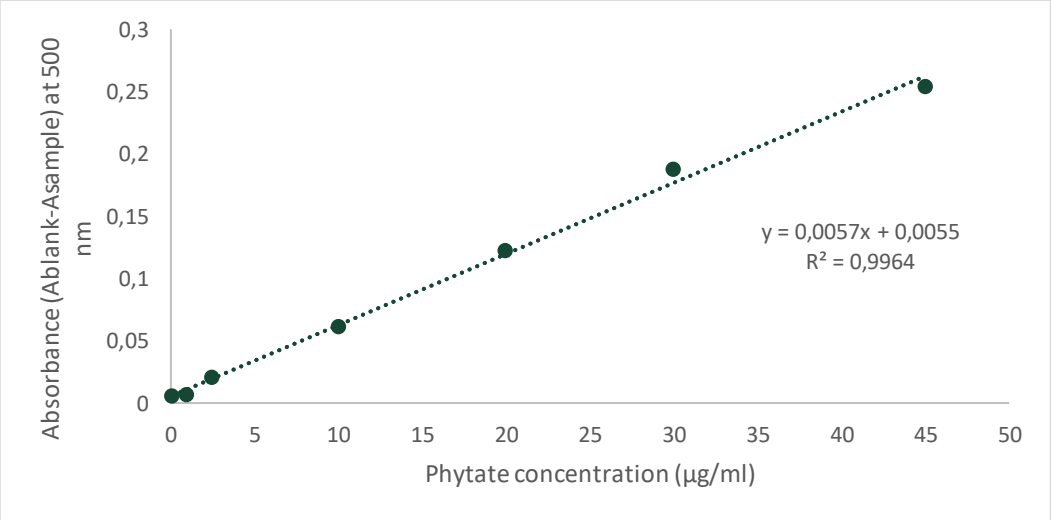

Figure 5. Standard curve and the corresponding linear equation of sodium phytate concentration (0.1, 1, 2.5, 10, 20, 30, 45 µg/ml) mixed with Wade reagent measured spectrophotometrically at 500 nm

2.9 Calculations

The phytate concentration in the faba bean flour was calculated using the standard curve equation (Figure 5), as well as equations 1, 2 and 3. Equation 1 was used to calculate the supernatants (S1-S6). Equations 2 and 3 were needed to calculate the phytate concentrations obtained in the protein pellets (P1-P3).

Equation 1. Phytate concentration (mg/100 g) in the faba bean flour

$$Phytate\ concentration1\ (mg/100\ g) = \left(\frac{C \times V}{v}\right) \times D \times d \times 0.001 \times \left(\frac{1}{W}\right) \times 100 \times 0.59$$

Equation 2. Phytate concentration (mg/X g) faba bean protein flour

$$Phytate\ concentration2\ (mg/X\ g) = \left(\frac{C \times V}{v}\right) \times D \times d \times 0.001 \times 0.59$$

Equation 3. Phytate concentration (mg/100 g) faba bean flour

$$Phytate\ concentration3\ (mg/100\ g) = \left(Phytate\ concentration2\ \times \frac{w}{0.2}\right) \times \left(\frac{100}{w}\right)$$

C= Phytate concentration (µg/ml) in eluent calculated from the equation obtained from the standard curve

V= Volume of eluent from AEC (ml)

v= Volume of sample added to AEC (ml)

D= Dilution factor

d= Volume of deionized water + volume of 3.5 % HCl added to the faba flour (ml)

W= Weight of faba flour (g)

w= Weight of faba protein flour (g

0.59= 59 % phytic acid in 100 g phytic acid sodium salt hydrate

3. Results

3.1 Phytic acid content in the protein extract

To get an overview of how the phytate concentration varied and decreased throughout the protein extraction of the faba bean flour, the phytate concentration was measured in each step (Appendix I). The initial mean phytate concentration from step S1 was established 1375.8 ± 28.00 mg/100 g faba bean flour. In comparison, the final protein pellet P3 had a mean phytate concentration of 990.6 ± 63.68 mg/100 g faba bean flour. This indicates a 28 % reduction in phytate concentration throughout the protein extraction, without applying any soaking treatments. The phytate concentration of the solutions and supernatants S1, S2, S3 and S5 decreased throughout the protein extraction process (Figure 6).

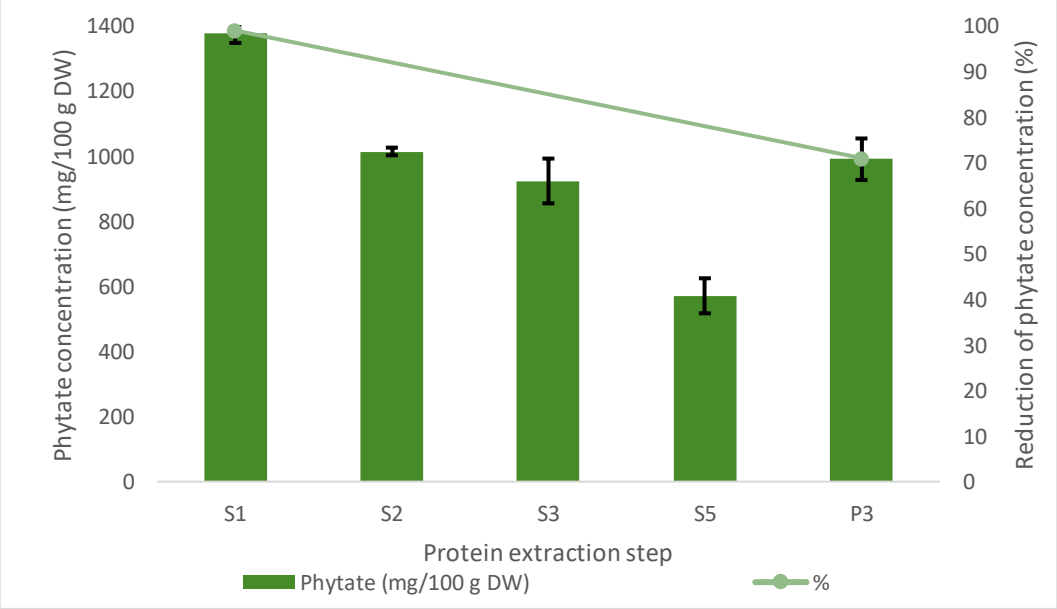

Figure 6. The total phytate concentration (mg/100 g DW) and percentual phytate reduction (%) throughout the protein extraction of faba bean. The results are presented in means ± standard deviations. The abbreviations S1, S2, S3, S5 and P3 represent different steps in the protein extraction where phytate was extracted and analysed (Figure 4)

3.2 Phytic acid reduction and soaking treatments

The purpose of applying the soaking treatments to the faba bean flour was to try to benefit the endogenous phytase of faba bean, as well as to influence the soluble phytate to leach out, which was believed to further decrease the phytate concentration. The soaking treatment conditions evaluated and applied in step S3 of the protein extraction were pH 5, 6 or 7 at a temperature of 20 °C or 40 °C with a soaking time of 2 or 5 hours (Table 4).

The soaking treatments (treatment A- L) influenced the phytate concentration to decrease in all observations (Table 5). The phytate extraction and analysis were performed in the final pellet, P3, of the protein extraction. The soaking treatment with the largest percentual phytate reduction was treatment E, indicating an 87 % reduction in phytate concentration compared to step S1 in the protein extraction. The soaking treatment with the smallest percentual phytate reduction was treatment K, indicating a 35 % reduction in phytate concentration.

Table 5. Total phytate concentration (mg/100 g DW) obtained in pellet P3 treated with the different soaking conditions pH (5, 6 or 7), soaking time (2 h or 5 h) and temperature (20 °C or 40 °C). The samples were measured in duplicates or triplicates. The results are presented as means ± standard deviations*

Treatment name	pH	Temperature (°C)	Soaking time (h)	Phytate concentration (mg/100 g DW)	Reduction (%) compared to S1 (1375.83 mg/100 g DW)
A	5	20	2	585.3 ± 142.72	↓ 57
B	5	20	5	435.7 ± 123.24	↓ 68
C*	5	40	2	734.9 ± 42.00	↓ 47
D	5	40	5	501.5 ± 223.97	↓ 64
E	6	20	2	180.1 ± 159.19	↓ 87
F	6	20	5	383.5 ± 0.00	↓ 72
G*	6	40	2	693.0 ± 84.00	↓ 50
H	6	40	5	445.3 ± 57.25	↓ 68
I*	7	20	2	678.9 ± 87.42	↓ 51
J*	7	20	5	371.0 ± 211.40	↓ 73
K	7	40	2	892.0 ± 92.22	↓ 35
L	7	40	5	293.9 ± 178.15	↓ 79

3.2.1 Effect of pH

To evaluate the effect of pH, the mean phytate concentration for pH 5, 6 and 7 was calculated from their four respective treatments in table 5. The mean phytate concentration for pH 5, 6 and 7 were 564, 425 and 559 mg/100 g respectively. This further indicates that pH 6 had the largest influence on decreasing the phytate concentration.

3.2.2 Effect of soaking time

To evaluate the effect of soaking time, the mean phytate concentration for the soaking times 2 and 5 h was calculated from their six respective treatments in table 5. The mean phytate concentration for the soaking times 2 and 5 h were 627 and 404 mg/100 g respectively. This further indicates that the 5 h soaking time had the largest influence on decreasing the phytate concentration.

3.2.3 Effect of heat-treatment

To evaluate the effect of heat-treatment, the mean phytate concentration for temperatures 20 and 40 °C was calculated from their six respective treatments in table 5. The mean phytate concentration for 20 and 40 °C were 439 and 593 mg/100 g respectively. This further indicates that the temperature of 20 °C had the largest influence on decreasing the phytate concentration.

4. Discussion

4.1 Phytic acid content in the protein extract

This master thesis aimed to develop and optimise a method to evaluate the phytate concentration in faba bean, variety Tiffany. The thesis had a special focus on the phytate concentration of faba bean in relation to the faba bean protein extraction, with the higher objective of producing ingredients for sustainable future meat substitutes. Pre-treatment conditions, i.e., soaking treatments, to potentially benefit the endogenous phytase and thereby reduce the phytate concentration further, were identified and evaluated.

The first phytate concentration (S1) was established after a soaking time of 16 h with the mean phytate concentration of 1375.83 mg/100 g DW. This was established to be the baseline for phytate concentration since the evidence for successfully reducing the phytate concentration of faba bean at soaking times >12 h in room temperatures, and slightly elevated temperatures, is contradictory to our knowledge. Patterson et al. (2017) soaked faba beans in double deionized water for 12 h at 30 °C, which decreased the phytate concentration by 26- 32 %. In comparison, Sharma & Sehgal (1992) dehulled and soaked two varieties of faba bean in 37 °C tap water for 12 h, which showed a 4 % decrease in phytate concentration. Also, Schlemmer et al. (2009) reported no reducing effect on the phytate concentrations in peas, lentils or beans after a 16 h soaking time at 22 °C.

Further, long soaking times may affect the leaching of other important water-soluble compounds, such as B-vitamins (Prodanov et al. 2004). Since the higher purpose of the study is to produce meat substitutes, this was considered an incitement to keep the soaking times shorter. The mean phytate concentration of 1375.83 mg/100 g DW further agrees with the range of phytate concentration likely to find in faba bean, 510-1700 mg/100 g DW (Table 1).

The mean phytate concentration obtained in the protein pellet (P3) from the protein extraction was 990.6 ± 63.68 mg/100g DW. This is a lower concentration compared to Latta & Eskin (1980), which obtained 1.4 % phytate in faba bean protein concentrate, using the same principle method. There are, however, some significant differences between the method of this master thesis and the study by Latta & Eskin (1980). One of the most important differences is that the study by

Latta & Eskin (1980) does not describe which faba bean variety they evaluate and how they obtained the protein concentrate. Knowledge about which variety has been used is important when one wants to compare specific phytate concentrations because the phytate content among the varieties has shown to vary a lot. In a study by Mayer Labba et al. (2021), eleven faba bean varieties were evaluated for phytate, IP_6, content using high-performance ion chromatography, HPIC. According to the study, the IP_6 concentration between the varieties ranged between 112-1281 mg/100 g, which shows a tenfold variation between the cultivars. Tiffany had an IP_6 concentration of 748 mg/100 g. Even though this master thesis, as well as Latta & Eskin (1980), did not use HPIC or evaluated the single IP_6 content, this proves the principle importance of comparing the same variety when comparing specific concentrations.

Another important difference between our study and the studies by Latta & Eskin (1980), as well as Fruhbeck et al. (1995), is that they use 2.4 % HCl compared to our 3.5 % HCl to extract the phytate. In the study by Latta & Eskin (1980), it was statistically established that there was no significant difference in phytate extraction using 2.4 % HCl compared to 4.8 % HCl. If this study was to be reperformed, the 2.4 % acid should be evaluated and further considered for improving the sustainability aspects of the methodology.

The supernatants of the protein extraction S1-S3 and S5 show a continuous decrease in phytate concentration (Figure 6). Analysis of supernatant S6 (Appendix 1) indicates results interpreted as there was no phytate left in that step. This indicates that the phytate is leaching out of the protein and mineral complexes to the supernatants throughout the protein extraction and the total phytate concentration ends up in the final protein concentration pellet P3. This agrees with the fact that phytate is water-soluble and has the potential to leach out of its protein- and mineral complexes (Schlemmer et al. 2009). This further enables that some of the phytates can be removed by removing the supernatants throughout the protein extraction. In this study, 28 % of the phytate was removed during the protein extraction without soaking.

4.2 Phytic acid reduction and soaking treatments

The purpose of applying the soaking treatments was to try to benefit the endogenous phytase of the faba bean and thereby decrease its phytate concentration. The faba phytase has been reported to have an optimum at pH 5 and 50 °C or pH 6 and 50-55 °C. Such high temperatures could not be evaluated in this study because they could interfere with the functional properties of the protein. This is further discussed in section 4.4.

Consequently, the soaking treatment conditions evaluated and applied in step S3 of the protein extraction were pH 5, 6 or 7 at a temperature of 20 °C or 40 °C with a soaking time of 2 or 5 hours.

The soaking treatments (treatment A- L) reduced the phytate concentration in all observations by 35- 87 %. The results from treatments C, D, G and H, are in agreement with the result range provided by Greiner & Konietzny (2005), which reported that 26-100 % of legume phytate can be hydrolysed at combinational soaking treatments with pH values of 5-6 in temperatures of approximately 45-65 °C.

The soaking treatment with the largest percentual phytate reduction was treatment E with pH 6 at 20 °C for 2 h, indicating an 87 % reduction in phytate concentration compared to step S1 in the protein extraction (Table 5). The pH condition of this treatment is in the range of pH values where the optimum activity for the endogenous phytase can be found (Greiner & Konietzny 2005; Luo et al. 2010).

Further, this study aimed to evaluate the effect of combinational soaking treatments on phytate concentration throughout faba bean protein extraction. Since the main focus of this study was to evaluate and optimise a method to fulfil that purpose, no statistical evaluation of the soaking treatments, i.e., pH, soaking time and heat-treatment, was performed. With that stated, it is difficult to make conclusions based on the percentual reduction of phytate from the single factors of pH, soaking time and heat-treatment. Though, one can interpret patterns in the results concerning for instance the soaking time. The result from this study suggests that a 5 h soaking time, at altered pH and temperature, was effectively reducing the phytate concentration. This is in agreement with Sattar et al. (1989) who stated that prolonging the soaking time in elevated temperatures was effective to reduce the concentration of phytic acid in legumes.

4.3 Evaluation of analytical methods

The results obtained from this master thesis indicate a decrease in phytate concentration from all soaking treatments. However, the treatments were measured in duplicates and the standard deviations from the samples are in general large, which indicates variation within the same soaking treatment sample. The cause of this variation could potentially be explained by poor adherence of the ferric ions in the Wade reagent to the phytate in the sample (Fruhbeck et al. 1995). If this would be the case, this might further indicate that the phytates in the treatments have been degraded to lower inositol phosphates, which are known to be less reactive with cations (Haileslassie et al. 2016). However, further studies on the composition of phytates in the treatment are needed to make any adequate conclusions on the matter.

Moreover, the treatments showed a large variation considering the reduction capacity of the phytate concentration (35- 87 %). This could be explained by the treatments themselves, which aimed to benefit the endogenous phytase. Though, it is not possible to establish whether it is the endogenous phytase that has been stimulated, activated and further reduced the concentration of phytate in the faba bean. Partly because no enzyme activity test was performed, but also because the reported temperatures on phytase activation could not be evaluated. Further, the decrease in phytate concentration throughout the protein extraction is not fully logical. P1 and S5 were discarded, corresponding to an approximate mean phytate concentration of 800 mg/100 g (Appendix 1). Simultaneously was the decrease in phytate concentration established 28 %, from S1 to P3, corresponding to a mean phytate concentration of approximately 400 mg/100 g faba bean flour.

Both considerations may have a potential methodological explanation which is connected to the usage of different columns, with varying flow rates, during the analysis with anion-exchange chromatography (AEC).

The columns, with samples applied, had different times of elution, or flow rates. The flow rate is an important attribute in AEC since the method depends on the binding and unbinding of the analyte ion, i.e., phytate. The ability of phytate to diffuse and bind to the resin in the column is permitted by the flow rate (Bhattacharyya & Rohrer 2012). Thus, the difference in flow rate among the treatments and throughout the protein extraction could have affected the adherence of the phytate to the resin, which further could be reflected in the resulting phytate concentrations.

Further, there was no consistency in flow rate observed among the samples that had been treated in different soaking conditions or throughout the steps of the protein extraction. I.e., samples with less phytate concentration did not consequently show faster or slower flow rates. This implies that the inconsistent flow rates of the columns might potentially have been caused by the resin packing practices, which were manually performed prior to each phytate analysis.

Further, the reproducibility of the project can be considered low because of the difficulties of monitoring a constant temperature during the soaking treatment optimisation. This could have affected the final phytate concentrations obtained from the protein and phytate extraction.

4.4 Delimitations

The benefit of the overall method used in this master thesis was that it is an easy-to-use method with relatively low expenses. Further, the materials and instruments are often available in many laboratories. However, the method had the delimitation of not being able to differentiate between the different inositol phosphates. This attribute has been established important since higher phosphorylation of phytate,

IP_6 and IP_5, allows for a stronger metal chelating capacity, potentially causing mineral deficiencies in humans. In practice, this means that this study cannot draw any conclusions on whether the resulting phytate concentration left in the faba bean protein after the soaking treatments are of a higher phosphorylation level. However, adding a SDS-PAGE step to the method would allow for the evaluation of the distribution and concentration of IP_6 in the treatments. Further, evaluation of the phosphorylation level of phytate could be done using, for instance, HPLC as well, however, this is a comparably expensive and time-consuming method (Raboy et al. 2020).

Another delimitation of the master thesis was that the effect of "high" heat-treatment temperatures on phytate concentration could not be studied. The reason was connected to the larger purpose of this study, to produce plant-based protein substitutes made of faba bean. Heating may initiate several physiochemical changes in the proteins (Preedy 2014). These changes could potentially affect the resulting functional properties of the protein and thereby impair faba beans' potential to function as an ingredient in sustainable meat substitutes.

5. Conclusion

To our knowledge, this is the first time soaking treatments with combinational usage of pH and temperature, have been evaluated and optimised during the protein extraction of faba bean flour, with the purpose to reduce the phytic acid concentration.

The optimised methodology successfully evaluated the concentration of phytic acid throughout the protein extraction, resulting in a 28 % reduction.

All soaking treatments reduced the concentration of phytic acid in the final extracted pellet, P3, which can further be considered a possible ingredient in plant-based meat substitutes. Consequently, important indications of processing information on phytic acid in faba bean can be provided to the industry of plant-based foods with the results of this research.

The optimum soaking treatment was treatment E, which had a pH value of 6 at a temperature of 20 °C for a soaking time of 2 h, resulting in an 87 % phytate reduction.

5.1 Forward research question

The majority of the soaking treatments had a larger percentual decrease in phytate concentration when the soaking time was 5 h compared to 2 h within the same temperature and pH. Evaluating the effect of prolonged soaking times in combination with altered pH and temperature conditions to reduce the phytate concentrations further might therefore be of interest in future research.

Further, since the higher purpose of this project was to use faba bean as a meat substitute or meat substitute ingredient, it is important to consider how the treatments influence the protein extraction process and thereby the amount of protein that can be obtained in the final protein pellet. A possible future research question would therefore be to investigate how the protein yield and the protein's functional abilities are affected by being exposed to the soaking treatments evaluated in this project.

Higher inositol phosphates, IP_6 and IP_5, are responsible for mineral chelation and deficiencies in humans. Further evaluation of how much of these are present in faba bean protein isolate after the different treatments are of interest. This could be evaluated using the analytical instruments HPLC or HPIC, as well as SDS-PAGE.

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Popular science summary

With a globally growing population, there is a need for a challenging shift from animal-based protein sources toward a diet with more sustainable plant-based protein sources. This commands for a wider range of commercially produced meat substitutes. Faba bean, with its high protein content, is a potentially suitable candidate, especially in a Swedish context. Though, faba beans also consist of various antinutrients, such as phytic acid, with properties that have the potential to impair the health of humans.

Faba beans have a long tradition of being cultivated by humans. The grain legume is used for both animal feed and human food purposes in a global context. The legume has several environmental and nutritional benefits, not the least since it can be cultivated in different climatic areas of Europe, including Sweden, and has a high protein content. Faba beans are also rich in several minor compounds, including phytic acid. The main function of phytic acid in plants is to function as phosphorous storage. The compound can be involved in health-promoting activities in humans, such as reducing the risk of some cancers. However, phytic acid is probably more associated with its antinutritive properties, which cause implications for human health. The implications include impaired absorption of necessary minerals, such as iron and zinc, as well as impaired uptake of carbohydrates and protein from the small intestine. The restricted mineral absorption can lead to mineral deficiencies in humans. The restricted absorption is connected to the fact that humans lack the phytic acid degrading enzyme phytase, which thereby prohibits humans from degrading the compound. Phytic acid's effect on humans depends on its chemical form, i.e, how degraded the compound is before consumption. A more degraded compound will not restrict the mineral absorption to an as high extent as a less degraded compound. Further, a more degraded compound has the potential to be involved in specific health-promoting activities in humans.

Knowledge about phytic acid in plant-based foods is therefore important in the light of the possible future protein shift and with the greater demand for commercial meat substitutes. The concentration of phytic acid in faba beans further needs to be monitored and decreased to influence the legume's potential to function as a sustainable raw material for the production of meat substitutes.

This master thesis was performed as a part of the Vinnova funded project "*Innovations to support for protein shift*". The overall aim of the thesis was to develop a method that would evaluate the phytic acid concentration in faba bean (variety Tiffany) throughout the extraction of its proteins. The project further aimed to investigate how different soaking treatments added to the protein extraction could influence a decrease in phytic acid concentration. Lastly, the project aimed to establish the optimum treatment for reducing the concentration of phytic acid based on the condition of the soaking treatments.

The result showed that the concentration of phytic acid in faba bean was reduced by 28 % throughout the protein extraction. All evaluated soaking treatments reduced the phytic acid concentration in the final protein fraction of the protein extraction, which could be considered a possible ingredient in plant-based meat substitutes. This research shows important indications of processing information on phytic acid for the industry of plant-based foods. Lastly, based on the conditions of the soaking treatments, the optimum soaking treatment was treatment E, which had a pH value of 6 at a temperature of 20 °C for a soaking time of 2 h, resulting in an 87 % reduction of phytic acid.

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Appendix 1 – Raw Data

Data obtained from the phytate extraction and analysis throughout each step (S1-S6 and P1-P3) of the protein extraction:

Sample	Mean diff. (abs_{blank}-abs_{mean})	Mean Abs_{blanc}	Abs_{mean}	Abs_1	Abs_2	Mean conc. (µg/ml)	Mean conc. (mg/0.2 g protein flour)	Mean conc. ± std (mg/100 g faba flour)
S1	0.214	0.538	0.324	0.321	0.327	36.6	-	1375.8 ± 28
S2	0.189	0.514	0.326	0.327	0.324	32.1	-	1014.4 ± 12
S3	0.167	0.496	0.329	0.337	0.320	28.3	-	923.7 ± 69
P1	0.010	0.504	0.495	0.494	0.495	0.70	9.29	209.6 ± 37
S4*	-	-	-	-	-	-	-	-
S5	0.103	0.4965	0.394	0.400	0.387	17.1	-	570.9 ± 54
P2	0.018	0.511	0.493	0.483	0.502	2.19	5.39	764.2 ± 821
S6	-0.002	0.4965	0.498	0.501	0.495	-1.23	-	-41.0 ± 25
P3	0.028	0.4985	0.471	0.472	0.470	3.86	178.6	990.6 ± 64

*Not measured

Data obtained from the phytate analysis and extraction from pellet P3 with soaking treatments applied:

Treatment name	pH	Temperature (°C)	Soaking time (h)	Weight (g) obtained from 5 g faba flour	Mean diff. (abs_{blank}-abs_{sample})	Mean Abs_{blank}	Mean Abs	Abs_1	Abs_2	Abs_3	Mean conc. (µg/ml)	Mean conc. (mg/0.2 g protein flour)	Mean conc. ± std (mg/100 g faba flour)
A	5	20	2	0.552	0.020	0.5005	0.481	0.478	0.483	-	2.54	1.17	585.3 ± 142.72
B	5	20	5	0.437	0.016	0.5005	0.485	0.487	0.483	-	1.75	0.87	435.7 ± 123.24
C*	5	40	2	0.510	0.010	0.508	0.499	0.502	0.495	0.481	3.07	1.47	734.9 ± 42.00
D	5	40	5	0.548	0.015	0.508	0.493	0.493	0.493	-	1.67	1.00	501.5 ± 233.97
E	6	20	2	0.567	0.022	0.503	0.481	0.479	0.483	-	0.70	0.36	180.1 ± 159.19
F	6	20	5	0.523	0.014	0.503	0.489	0.494	0.488	-	1.67	0.77	383.5 ± 0.00
G*	6	40	2	0.555	0.023	0.503	0.480	0.480	0.479	0.481	2.89	1.38	693.0 ± 84.00
H	6	40	5	0.524	0.015	0.504	0.489	0.492	0.486	-	1.93	0.89	445.3 ± 57.25
I*	7	20	2	0.545	0.022	0.503	0.481	0.481	0.479	0.483	2.84	1.36	678.9 ± 87.42
J*	7	20	5	0.347	0.017	0.5025	0.486	0.487	0.485	0.484	1.55	0.74	371.0 ± 211.40
K	7	40	2	0.602	0.026	0.4985	0.473	0.471	0.474	-	3.60	1.78	892.0 ± 92.22
L	7	40	5	0.603	0.0125	0.5045	0.492	0.495	0.489	-	1.23	0.59	293.9 ± 178.15

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