The impact of an ultrasound treatment on yellow peas on soaking time, phytic acid and protein digestibility

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Abstract

To increase the sustainability of the food sector and improve overall health, plant-based products have been advocated to be a solution. The plant-based products are often based on legumes. They contain a lot of nutrients and health benefits but also various antinutrients, including phytic acid which binds to several important minerals and proteins and reduces their bioavailability. Legumes are usually processed to reduce these antinutrients. However, these processes are often time consuming and reduce nutrients along the way. Therefore, it would be beneficial to find alternative methods to reduce these antinutrients and the processing time to make the plant-based alternatives more nutritious and cost effective.

This master thesis was made in collaboration with Axfoundation and Sevan. The aim of the thesis was to see if an ultrasound treatment of yellow peas at the beginning of the soaking had any impact on the soaking time, phytic acid content and protein digestibility. The soaking time was investigated by measuring the weight of the peas during soaking. The phytic acid were extracted and separated by an anion exchange followed by an UV spectrometry analyze. For the protein digestibility, an *in vitro* protein digestion was performed, and the protein content was measured by dynamic flash combustion.

The result showed that there was no significant difference in soaking time between the control and the ultrasound treated peas at 20 ºC, but at 55 ºC there was a significant difference after 20 hours of soaking. The average phytic acid content varied between 4.79-5.24 mg/g dry peas for the samples. However, there were no significant difference on phytic acid content between the controls and the ultrasound treated peas. Additionally, the average protein digestibility varied between 63.46-68.58% between the samples, but no significant difference between the samples was found.

Keywords: Ultrasound, Yellow peas, Soaking time, Phytic acid, Protein Digestibility

List of abbreviations

ANOVA	Analysis of Variance
DW	Dry weight
IP6	Myo-inositol hexaphospate
OD	Optical density
PA	Phytic acid
PD	Protein digestibility
STD	Standard deviation

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1. Introduction and problem formulation

1.1 Background

Over the past years, there has been a significant increase in the plant-based food market in Sweden. This increase has been driven by the consumers growing awareness of sustainability, health considerations, and ethical concerns for animal welfare. This aligns with the global goals to reduce climate change, where there is a need for a protein shift in the food sector to increase the sustainability. Plant-based diets have been suggested to be a part of the solution because of their lower environmental footprint due to the reduced need for land, water, and energy compared to animal-based diets.

However, despite the benefits offered by plant-based alternatives, there are challenges related to nutrient absorption due to the presence of antinutrients, particularly in legumes and grains. While traditional methods such as soaking and boiling can reduce the antinutrients, they are time consuming and can also lead to nutrient loss, impacting cost effectiveness and overall nutritional quality. This is why exploring alternative methods to effectively reduce antinutrient levels while preserving nutrients is of great interest both to enhance profitability and the nutritional values of plant-based foods.

1.2 Collaborations

This thesis was made in collaboration with both Axfoundation and Sevan.

1.2.1 Axfoundation

Axfoundation is an independent, non-profit business that is working for a more sustainable society. Together with over 300 partners and 70 scientists they are working to solve practical problems connected to the foods and resources used. To reach a more sustainable society, they are working with two different programs, Future Food and Future Material. In these programs experts investigate a problem and gives suggestion for solutions. If the solutions work in reality, the project is broadened, and relevant partners are invited to take a part of it. The goal to spread as much valuable knowledge as possible.

1.2.2 Sevan

Sevan is the market leading company in the category of hummus in the Nordic countries and started in 1998. As a company they value and work a lot with sustainability. Apart from hummus they are investing in more middle eastern food products, including falafel and sauces. They are currently working on a falafel made with at least 75% Swedish ingredients. In this falafel the traditional chickpeas are exchanged to yellow peas, as they are produced in Sweden.

1.3 Problem formulation and research questions

The currently under-progress product of falafel made from yellow peas is an interesting candidate when looking at plant-based alternatives. This is because the only treatment of the yellow peas in the production is a period of long soaking and a short frying. This product then potentially still contains some antinutrients which could disrupt the bioavailability of protein and minerals.

The following questions will be investigated in this thesis:

1. Can the soaking time be reduced with an additional ultrasound treatment in the beginning of the soaking?
2. Is there a difference in the content of phytic acid after the ultrasound treatment?
3. Does the bioavailability of protein change after the ultrasound treatment?

1.4 Limitations and focus area

This project will only look at the relationship between ultrasound and soaking of yellow peas, no other processes or pulses will be investigated. Regarding the antinutrients, only the phytate content will be measured, since this has the biggest prohibitive effect on the bioaccessibility of iron and zinc.

2. Aims

The aim of this thesis is to investigate the effects of an ultrasound treatment in the beginning of the soaking of yellow peas. However, the overall aim will be to test ultrasound as a technology to improve the processability during soaking and decrease antinutritional factors that may affect the nutritional values of the peas. This will be done by comparing the ultrasound treated peas with control treated peas.

There are three specific aims that is focused on:

- To evaluate if there is an effect on the soaking kinetics from temperature and ultrasound treatment.
- To assess if the concentration of phytic acid can be reduced by an ultrasound treatment.
- To assess whether an ultrasound treatment can improve protein digestibility.

3. Theoretical background

3.1 Meat substitutes

Meat substitutes, also known as plant-based products, offers a solution to environmental concerns, animal welfare, and health considerations compared to meat consumption. While meat substitutes offer a promising solution, there is also a lot of challenges. These challenges include, but are not limited to the bioavailability of nutrients, protein, and the knowledge about it.

Among others, iron and zinc are two of the minerals that risk to be deficient in a plant-based diet. One of the most known mineral deficiencies in Sweden is iron deficiency. Iron deficiency is common among fertile woman in Sweden who menstruate, especially in the teenage years (Hoppe et al. 2008, Hallberg et al. 1993). It can lead to a serious condition called anemia where there is not enough iron to produce hemoglobin (Turner et al. 2023). For someone eating a plant-based diet, it is therefore extra important to be aware of what you are consuming, since a lot of the plant-based options have less bioavailability of minerals because of phytic acid (PA). This also applies to zinc, which is an important mineral for the skin and immune system (Berger 2002). A study by Foster et al. (2013) found that vegetarians have a serum zinc level that is 1 µmol/L lower than nonvegetarian diets. This is not that much since a normal value is between 10-25 µmol/L, but it can have adverse effect for some.

When looking at the plant-based options available in stores in Sweden, a study by Mayer Labba et al. (2022) investigated 44 meat substitutes on the market and found that none of them had enough iron when looking at the phytate:iron molar ratio. They also found that only the products based on mycoprotein found in Quorn had a sufficient phytate:zinc ratio.

Another study made by Mariotti and Gardner (2019) found that the bioavailability of protein in animal-based foods is higher compared to plant-based foods. Even though it is rare to get a protein deficiency when consuming a plant-based diet, it is still important to be aware of, especially when you grow older since the protein absorption are worsening and the risk of less appetite increases (Donini et al. 2013).

3.2 Yellow peas

Yellow peas, also known as *Pisum sativum* L. belongs to the group garden peas. They are cultivated all over the world and is one of the most grown legumes for human consumption in Sweden (Tidåker et al. 2021, Gursak 2005). Yellow peas, just like other pulses, has been associated with many health benefits and is a great source of protein, fiber, nutrients, and bioactive compounds (Wang et al. 2008). However, the protein and minerals in raw pulses have low digestibility because of the presence of antinutrients like PA and raffinose. Yellow peas contain between 4.9-12.4 mg PA per dry weight (DW) gram of pea, which can be considered relatively high (Warkentin et al. 2020, Vojtíšková et al. 2010, Shi et al. 2018, Wang et al. 2008). Therefore, they are usually processed before consumption to make the nutrients more available.

Peas are usually stored and sold dry and contain in that state around 15% moisture (Livsmedelsverket 2023). Before consumption, they are usually hydrated by soaking. The hydration capacity on dry basis of yellow peas is around 100%. However, it can differ between different varieties (An et al. 2010).

The macro- and micronutrient content depend on various factors and moderately change between years, locations, and pea varieties (Santos et al. 2019). Therefore, the numbers mentioned in this section is not completely accurate and could slightly differ. From the macronutrient content shown in Table 1, it is shown that carbohydrates are the main component of yellow peas, followed by protein and a low amount of fat. The major fraction of carbohydrates is starch and it is also rich in dietary fiber (FAO/INFOODS 2017). The Protein Digestibility-Corrected Amino Acid Score (PDCAAS) is around 65-70% for cooked and baked peas (Nosworthy et al. 2017).

Table 1 – Macronutrient composition in yellow peas.

Macronutrient	**Protein**	**Carbohydrate**	**Fiber**	**Fat**
g/100 g DW	27.4	47.4	19.7	2.6

Yellow peas are also a potential source for several minerals, as can be seen in Table 2 (FAO/INFOODS 2017).

Table 2 – Mineral content in yellow peas.

Mineral	Ca	Cu	Fe	K	Mg	Mn	P	Zn
mg/100 g DW	47	0.21	5.6	982	116	1.2	325	3.7

3.3 Antinutrients

Antinutrients are naturally occurring compounds found in plants that is known to interfere with absorption or utilization of nutrients. There are two major groups of antinutrients in pulses, one group includes different proteins such as lectins, trypsin, and amylase inhibitors. The other group consists of non-proteins, including PA, phenolic compounds, saponins and several oligosaccharides, including raffinose and stachyose. Nevertheless, the so called antinutrients have shown to provide some health benefits. However, the name comes from their ability to reduce the bioavailability of nutrients by forming complexes with them or with enzymes that breaks them down. In most cases, traditional food processes reduce the levels of antinutrients, but it is dependent on the type of pulse and process used. In this thesis only PA will be investigated. (Han and Baik 2006)

3.3.1 Phytic acid

PA, also known as myo-inositol hexaphospate (IP6), and is as the name indicates, built up by six phosphate ester groups that acts as strong chelators and are attached to an inositol ring. It can bind up to 12 protons at once and chelates particularly to the cations Cu^{2+}, Ca^{2+}, Zn^{2+} and Fe^{3+}. When complexed with a cation, PA is referred to as phytate. (Andrews 2013) PA can be degraded to lower inositol phosphates (IP5-3), which has less binding capacity because of the loss of phosphate, which the cations primarily bind to (Sandberg and Scheers 2016). PA is attached to proteins of the kernel in pulses and consists of 60-90% of the total amount of phosphorus (Petroski and Minich 2020, Gibson et al. 2018).

The average consumption of PA for a western diet is 370 mg/day and 1 150 mg/day for vegetarians. However, a study recommended that the PA content should be less than 10 mg per meal to improve the absorption of iron. (Helstad et al. 2021) Another study by Hallberg et al. (1989) showed that the addition of 2 mg phytate to a meal inhibited the absorption by 18%, 25 mg by 64% and 250 mg by 82%. They claim that phytate content of 24 mg/100 g will provide an adequate absorption of non-heme iron from the meal while 220 mg/100 g will have negative effect. They also found that ascorbic acid counteracted the effect. Ascorbic acid, also known as

vitamin C, has shown to have an enhancing effect on the absorption of iron by reducing ferric to ferrous iron and its ability to chelate iron. This works for all inhibitors, and it is therefore recommended to consume vitamin C rich food together with plant-based sources of iron (Hurrell and Egli 2010).

By chelating cations and forming insoluble complexes with minerals, PA inhibits the absorption of them. Because of the lack of the enzyme phytase, humans cannot digest these complexes. The inhibition of iron and zinc is dose dependent. In theory, the phytate:mineral molar ratio should be low to correspond to a high bioavailability. Preferably, you want the phytate:zinc molar ratio to be below five and if the value is above 15, it corresponds to a low bioavailability (absorption is approximately 50% less). (Gibson et al. 2018, Fredlund et al 2006) The same principle works for iron, but there the phytate:iron should be below one, or ideally below 0.4 in a meal without iron absorption enhancers like ascorbic acid (Hurrell and Egli 2010).

The phytate complex stability depends on which cation is chelated, the pH of the solution, the phytate to cation molar ratio, and other compounds in the solution (Konietzny and Greiner 2003). A study found that the optimal pH to reduce phytate in faba beans was at pH 6 (Sterner 2021). IP5-3 has found to have a lower binding capacity to cations in the pH range 5-7. Which indicates that it is preferable to have a slightly lower pH to reduce the amount of phytate. Regarding interactions with proteins, PA forms insoluble complexes that strongly binds to the cationic group of the protein in pH below the isoelectric point of the protein. A pH below 3.5 is necessary to dissolve those complexes. (Konietzny and Greiner 2003) It can also bind to digestion enzymes that hinders the ingested protein to be absorbed (Mohan et al. 2016).

Some of the phytate usually degrades to lower inositol phosphates during processing, some of which no longer can form complexes with minerals (Sandberg and Scheers 2016). When the phosphate groups decrease, phytate seem to become more soluble (Konietzny and Greiner 2003). Furthermore, the released phosphorus group also becomes available for utilization by the human body (Konietzny and Greiner 2003).

Although, as mentioned previously, antinutrients also have some beneficial health effects. PA has shown to reduce glycemia, have antioxidant activity, and anti-cancer functions. (Gibson et al. 2018, López-Martínez et al 2017)

3.3.1.1 Phytase

Phytase is an enzyme that naturally occurs in tissues of animals, plants, and microorganisms (Ugwuanyi 2016). These enzymes catalyze the hydrolysis of the inositol ring in PA, leading to a release of the mineral or protein attached to it (Rizwanuddin et al. 2023). Phytase can therefore help to increase the absorption of these compounds in food containing PA. Phytase is commonly used in animal feed to increase the bioavailability of nutrients but are not allowed to be added to food (Zhu et al. 2011).

However, yellow peas contain its own phytase that can reduce the PA, during right conditions. Phytase is most effective in temperatures between 40-60 degrees and at pH 4-5.5 (Naves et el. 2012, Zhang et al. 2013, Rizwanuddin et al. 2023, Abdolshahi et al. 2021). The variety depends on the type of phytase and what source it comes from. Higher or lower temperatures will lead to inactivation and pH outside of the range will make the phytase unstable (Rizwanuddin et al. 2023). There is no information in the literature about which type of phytase there is in yellow peas.

3.4 Traditional processes for reduction of antinutrients

There are several processes that are commonly used today to both improve digestibility and to reduce antinutrients in pulses. However, these processes will also reduce some nutrients. Each process works a bit different depending on which pulse is used. Therefore, it is important to know which process or processes suits which pulse.

Among the traditional processes are dehulling, soaking, cooking, and fermentation but in this report only soaking will be further investigated.

3.4.1 Soaking

Soaking of pulses in water is a common process to soften the texture and reduce the boiling time. However, if the peas are stored in the wrong conditions it can cause textural defects that causes lack of hydration (Perera et al. 2023). It has been reported to be an effective method to reduce antinutrients and improve protein digestibility (PD). Although, it also causes minerals and water-soluble proteins to leak out in the soaking medium, which can make the phytate to mineral ratio unchanged (Petroski and Minich 2020). The beneficial or adverse effects depends

on the soaking time, temperature, and components in the soaking medium (El-Adawy et al. 2000, Huma et al. 2008).

The soaking time reduces when the temperature of the soaking medium is increased (Kon 1979). A study by Sattar et al. (1989) found that soaking at 55 °C decreased phytate more than soaking at 27 °C. It is common to soak the legumes in water together with salt or bicarbonate to reduce the cooking time and other properties (de León et al. 1992, Munthali et al. 2022). However, this will not be done in this project.

When soaking pulses, the reduction of PA takes place in several different ways including diffusion into the liquid media and activation of the enzyme phytase within the pulse who breaks it down. If the soaking takes place in an acidic environment, the phytase enzyme is triggered to reduce IP6 into lower forms with fewer phosphate molecules, which have a lower binding affinity to minerals. Additionally, the permeability of the cell wall also increases due to the acidic environment. (Sarkhel and Roy 2022) Nevertheless, pulses soaked in a low pH, makes them firmer and the cooking time increases (Kinyanjui et al. 2014).

There are two typical hydration behaviors, the first one initiates with a lag phase, followed by an exponential phase, a constant phase, and ending with a slow decrease in moisture uptake. This type is called a sigmoidal hydration behavior because of the curved s-shape. The second one initiates with a rapid moisture uptake, that gradually decreases because of the lower moisture gradient, it basically has the same pattern except the initial lag phase. This is called a downward concave shape pattern. The first one is more common for pulses due to restrictions of water-passing by seed coat, whereas the other one is more common for cereals. (Kumar et al. 2023)

Regarding soaking and reduction of PA, the studies made have found quite different results. Some studies showed that soaking had no effect on PA reduction in peas after being soaked for four and 24 hours respectively (Shi et al. 2018, Wang et al. 2008). However, one of the studies did find an increase of protein content by 2.6-5%, due to a loss of soluble solids. On the contrary, other studies made on other legumes have found reductions of PA after soaking for 12 hours (Mubarak 2005, Alonso et al. 2000).

3.5 Ultrasound

To create a more sustainable food system, thermal hydration of pulses needs to be reduced to lower the energy demand. Among the candidates to fulfill these conditions are high pressure processing, pulse electric field and ultrasound. Of these three candidates, ultrasound is the most promising option due to its cost effectiveness, lower energy need, and high upscaling potential (Kumar et al. 2023). In practice, there is both an ultrasonic bath and an ultrasound probe that can be used for this process (Singla and Sit 2021).

The use of ultrasound in processing of pulses is not a new thing, however, the lack of implementation of the ultrasound method is due to lack of research. Nevertheless, the research that has been conducted found that ultrasound treatment has minimal impact on bioactive components and accelerates the mass transfer rate during hydration, which can lower both the soaking and cooking time substantially. A study found that the time to reach sufficient moisture content reduced by up to 59% and that the cooking time reduced by up to 43%, depending on ultrasound exposure time and bean variety (Ulloa et al. 2015). Additionally, it can change the structure of different components within the pulses, leading to an increased bioavailability of macro and micronutrients (Kumar et al. 2023).

Ultrasonic waves have frequencies between 20 KHz to 100 MHz. In frequencies up to 100 kHz mostly physical effects are observed while chemical effects require frequencies between 200 to 500 kHz (Singla and Sit 2021). High intensity ultrasound (10-1000 W/cm^2) can affect the structures of biomolecules, both compositional and morphological, a higher intensity can be achieved with a higher amplitude (Kumar et al. 2023). When ultrasound is used in a liquid media, acoustic cavitation occurs in the media through a series of compressions and rarefactions. The cavitation produces and exothermically ruptures microbubbles and causes them to collapse and produce microturbulence, high pressure and shear conditions (Xu et al. 2016, Faizal et al. 2023). Following the cavitations are nuclear emissions, light emission, and hydrolytic reactions (Yasui 2017, Taleyarkhan 2002).

There are still limitations in the research about the benefits of sonication, but there are studies showing promising results. A study from Chiu (2021) have shown that ultrasound-assisted hydration retains 2-2.5 times the phenolic content compared to standard methods, and 1.5-2 times the flavonoid content. Miano et al. (2019) found that alkaloid content reduced by around 21% by ultrasound treatment compared to thermal hydration. Han and Baik (2006) found that soaking with ultrasound was more effective to reduce oligosaccharides in peas.

On the contrary, the cavitation also generates free radicals which may cause negative impact in the food. It could be both physical and chemical effects including lipid oxidation, protein denaturation and reduction of phenolic content (Faizal et al. 2023).

3.5.1 Ultrasound-mediated hydration

Hydration is primarily a mass transfer process. The hydration process in pulses is affected in two ways by ultrasound processing. Firstly, the direct effect where expansions and contractions are caused by the ultrasound waves causing a "sponge effect", where the pulses absorb water (Miano et al. 2016). Secondly, the indirect effect where acoustic cavitation creates micro-channels through the pulse coat and thereby accelerates the waters mass transfer rate (Kumar et al. 2023). The creation of these micro-channels is also known as sonoporation, which resembles the effects of pulse electric field treatments. The water activity of pulses dictates the rate and amount of channel formation. A higher water activity is more efficient since there is more water available to undergo cavitation. (Miano et al. 2016)

Even though ultrasound treatment is seen as a non-thermal process, Wong et al. (2019) found an increase in water temperature by 13%. This temperature increase can differentiate depending on the effect of the ultrasound. The research shows split opinions on the effect of sonication in water with higher temperature, where Patero and Augusto (2015) show that the effect vanishes while Yildirim (2021) found that it still shows an effect. Additionally, another study found that both a higher temperature and a higher power decreased the soaking time (Yildirim et al. 2011).

3.5.2 Ultrasound effect on proteins

In pulses, salt-soluble globulins, including legumin and vicilin, and water-soluble albumins are the main proteins (Derbyshire et al. 1976). In peas, 70-80% of the seed protein consists of legumins (Kumar et al. 2023). Proteins bioavailability is regulated by its solubility. Studies have found that ultrasound treatment increases the protein solubility by depolymerization and charge distribution of proteins, thereby also their bioavailability and emulsion stability (Sha and Xiong 2022). Ultrasound has also been shown to reduce the off flavors by changing the secondary and tertiary structures in the proteins (Zhang et al. 2021).

3.5.3 Ultrasound effect on antinutrients

As mentioned in section 2.3, there is a lot of different antinutrients available in pulses. Even though there has not been much research done, especially not on yellow peas, there are still some studies made on other crops that show the ultrasonic effect on some antinutrients. A study

found that ultrasound can reduce oxalate levels in elephant foot yam without phytochemical loss, which is a common phenomenon when using traditional methods (Srivastava et al. 2022). Another study showed that an ultrasound treatment significantly could reduce the amount of PA and tannins in finger millet (Yadav et al. 2021). Ultrasound also reduces the amount of trypsin inhibitors through conformation changes and reduction in disulfide bonds (Huang et al. 2008). Sharkhel and Roy (2022) found that ultrasound could reduce PA in pulses, while on the contrary, another study by Kaya et al. (2017) showed that the PA content in pulse hulls was not affected by ultrasound treatment.

Ultrasound could also have an indirect effect by activating the phytase enzyme. Several studies have shown that an ultrasound treatment increases the activity and immobilization efficiency of different enzymes (Dik et al. 2023). However, there is no study made on phytase.

3.5.4 Ultrasound parameters used in previous studies

The ultrasound parameters used in previous studies is very diverse, probably due to different accomplishment goals. In Table 3 some of the previous studies are documented with their used parameters.

Table 3 – Parameters used in previous studies.

Author	Frequency (kHz)	Power (W)	Time (min)	Pulse (s)	Temp (°C)	Crop	Effect
Polachini et al. (2021)	N/A	400	Up to 300	0.6 on 0.4 off	50	Cassava	Enhanced hydrolysis
Kaya et al. (2017)	22	100, 200, 400	90-210	5 on 25 off	45	Green lentil, red lentil, faba beans	Reduction of phenolic values
Lafarga et al. (2018)	40	250	30-60	N/A	4	Ganxet bean	Improved protein solubility, yield and emulsifying properties
Miano et al. (2016)	25	41 W/L	600	N/A	25	Mung bean	Reduced soaking time by 25%
Ye et al. (2016)	20	80	1-40	N/A	20	Pea protein isolate	Increased protein solubility
Rahman and Lamsal (2023)	20	2 200	2	30 on 3 off	50	Mung bean	Gelation temp reduced
Sha and Xiong (2022)	20	39	5	5 on 5 off	35	Pea	Increased solubility and emulsion capacity

4. Materials and methods

4.1 Materials

Dry yellow peas were provided by Sevan from the producer “Kalmar Öland”, (Färjestaden, Sweden) in a box of 10 kg and is the same that Sevan uses. The peas were stored in the box in a dry storage room at room temperature when they were not used.

4.2 Initial trials

To decide which settings to use on the ultrasound equipment for best effect, a few trials were made together with a control soaking curve at room temperature and a soaking curve at 55 ºC for comparison. The 55 ºC samples was chosen to try to activate the phytase.

4.2.1 Soaking curve

A soaking curve for non-treated peas were produced as a control by measuring the weight of 20 peas soaked in 200 ml tap water at room temperature each hour up to approximately 30 hours, this was performed in duplicates. However, the measurement was not performed 30 hours straight. Instead, some peas were ultrasound treated in the morning, followed by a few hours of measurements. Another batch of peas were treated and put in water in the evening and the measurements were continued the day after, to be able to get a record of all hours.

For the other control curve, almost the same procedure was performed again, but the yellow peas were soaked in 55 ºC water the first 30 minutes, then they were placed at room temperature for 30 hours. The temperature was kept at 55 ºC using a magnetic stirrer with heat (IKA RCT standard).

4.2.2 Ultrasound parameters

The ultrasound machine used was a Hielscher UP200St with a 14 mm sonotrode and a frequency around 26 kHz. The adjustable parameters were amplitude, power, and pulses. The pulses could be set to 10-100%, were 10% equals 0.1 second on and 0.9 second off, while 100% equals continuous operation. Both the amplitude and the power were chosen to always be at 100%, which corresponds to an amplitude of 90 µm and a power of 200 W.

For all trials, 20 peas were placed in a 250 ml beaker filled with 200 ml of tap water. The sonotrode was emerged into the water to a depth of 3.5 cm in the center of the beaker and the

temperature were measured continuously. The trials were designed to match the temperatures in the control curves. This was done by altering the pulse parameters and by adding ice or using a water bath.

4.3 Preparation of samples

The samples that seemed to soak faster was chosen to investigate further. The chosen samples can be seen in Table 4 with a description on how they were treated, all samples can be seen in Appendix 3. However, the temperature for the ultrasound treated samples is only valid after 10-15 minutes of treatment. All samples were made in duplicates except US& and R45 that only been made once. Additionally, samples US6 and R45 lack soaking curves but will nevertheless be used in further investigations. Those conditions were chosen in a later state of the project to try to optimize the activation of phytase.

Table 4 – Nomenclature of samples with treatment description.

Sample name	Treatment	Pulse (%)	Time (min)	Temperature during treatment (°C)	Additional
US2	Ultrasound	70	35	55	N/A
US5	Ultrasound	80	60	20	Ice bath
US6	Ultrasound	70	35	45	pH 5.2
R20	Control	N/A	N/A	20	N/A
R45	Control	N/A	30	45	pH 5.2
R55	Control	N/A	30	55	N/A
DR	Dry control	N/A	N/A	N/A	N/A

To prepare the samples for later analyzes, approximately 50 g of yellow peas were placed in a 400 ml beaker filled with 250 ml of cold water (to have the same height of the water in the beaker as the other samples). The water had a pH around 7 for all samples except US6 and R45 that had a pH of 5.2 after and addition of citric acid. Then each sample was treated as seen in Table 4 and placed to soak for a total of 20 hours in room temperature. After soaking, the peas for each sample were patted dry with a paper towel and placed in the freezer. The samples were later freeze dried, milled (Perten mill feeder 3170) into a powder with a mesh size of 0.8 mm and stored in airtight containers until used. Ten peas of each sample were placed in an oven for minimum 16 hours to determine dry matter.

4.4 Phytic acid content

The PA content for all samples was measured in at least duplicates according to the method described by Makkar et al. (2007).

4.4.1 Standard curve

A standard curve was produced by dissolving 10 mg PA sodium salt hydrate in 100 ml of MilliQ water. Then a dilution series was made with the dilutions seen in Appendix 2. Thereafter, 3 ml of each liquid was mixed with 1 ml of wade reagent. The wade reagent was made from 30 mg FeCl3 6H2O, 300 mg sulfosalicylic acid and was filled up to 100 ml by MilliQ water. The solution was vortexed and centrifuged at 3000 rpm for 10 minutes at 20 ºC (Beckman Coulter Allegra X-15R). The absorbance was measured with a UV-spectrophotometer (BMG Labtech SPECTROstar Nano) at 500 nm and was recalculated to concentration from the dilution series. The relationship that 100 g of sodium phytate equals 59.9 g of PA was incorporated. The equation for the standard curve was achieved from Excel and could be used later to determine the PA content in samples with unknown concentration.

4.4.2 Measurement of samples

To measure the PA content from the prepared pea samples, 2 g of each sample was continuously mixed with 50 ml 3.5% HCl for 1 hour at room temperature. Thereafter, the samples were centrifuged at 3000 rpm for 10 minutes at 20 ºC (Beckman Coulter Allegra X-15R). 2.5 ml of the mixture was diluted with 22.5 ml MilliQ water. Then 10 ml of that mixture was passed through a glass column containing approximately 1 cm glass wool and 0.5 g of AmberChrom 1X8 chloride anion exchange (200-400 mesh), followed by 15 ml 0.1 M NaCl and finally 15 ml of 0.7 M NaCl. Only the last solvent mentioned was collected, the others were discarded. Then, 3 ml of the collected liquid was taken to another test tube and 1 ml of the wade reagent was added. The sample was vortexed and centrifuged at 3000 rpm for 10 minutes at 20 ºC. The absorbance was measured with a UV-spectrophotometer (BMG Labtech SPECTROstar Nano) at 500 nm and the PA content was calculated from the standard curve and the moisture content was considered.

4.5 *In vitro* protein digestibility

The *in vitro* PD was measured in duplicates according to dumas method which is based on the INFOGEST protocol. A protein analyzer was used to measure the protein content.

4.5.1 Preparations

A stimulated salivary fluid (SSF), a stimulated gastric fluid (SGF) and a stimulated intestinal fluid (SIF) were prepared according to Table 5, filled up to 500 ml with MilliQ water and stored in the refrigerator until used. To be noted, $CaCl_2(H_2O)_2$ was not added until during the process.

Table 5 – Proportions of chemicals used in SSF, SGF and SIF.

Chemicals	Concentration (g/L)	Molarity (M)	SSF (ml)	SGF (ml)	SIF (ml)
KCl	37.3	0.5	18.9	8.6	8.5
KH_2PO_4	68	0.5	4.6	1.1	1
$NaHCO_3$	84	1	8.5	15.6	53.1
NaCl	117	2	0	14.8	12
$MgCl_2(H2O)_6$	30.5	0.15	0.6	0.5	1.4
$(NH_4)2CO_3$	48	0.5	0.1	0.6	0
HCl	36	5	0.12	1.92	1.08
$CaCl_2(H_2O)_2$	44.1	0.3	0.025	0.005	0.04

The enzyme solutions of pepsin, pancreatin and bile salt were prepared on the day of the experiment according to Table 6 and was stored in ice until used. The concentration was calculated with Equation 1.

$$Concentration\ \left(\frac{mg}{ml}\right) = \frac{Enzyme\ acticity\ \left(\frac{U}{ml}\right) \cdot Total\ V\ (ml)}{Specific\ activity\ \left(\frac{U}{mg}\right) \cdot V\ (ml)} \quad (1)$$

Table 6 – Properties of enzymes used.

Enzyme	Concentration (mg/ml)	Enzym activity (U/ml)	Specific activity (U/mg)	V of enzyme (ml) per sample
Pepsin	5	2000	5988	0.667
Trypsin	4	100	200	5
Bile salt	52	10 mM	2.55 mmol/g	3

4.5.2 Measurement

The experiment started with weighing 5 g of freeze-dried sample were put in a glass flask together with 4 ml SSF, 0.025 ml $CaCl_2(H_2O)_2$ and 0.975 ml MilliQ water for the salivary phase. The mixture was incubated at 37 ºC for 2 minutes. For the gastric phase, 8 ml SGF and 0.005 ml $CaCl_2(H_2O)_2$ was added to the flask and the pH was adjusted with 0.5 M HCl/1.0 M NaOH or 5 M HCl/5 M NaOH to get a pH of 2. Thereafter, 0.667 ml of the pepsin solution was added, and the flask was filled up to a total volume of 20 ml. This was incubated for 2 hours at the same temperature, the pH was checked and adjusted every 30 minutes to remain a constant pH. For the intestinal phase, 8 ml SGF and 0.04 ml $CaCl_2(H_2O)_2$ was added, and the pH was changed to 7 by using the same HCl and NaOH as before. Then 3 ml bile solution and 5 ml pancreatin was added and the total volume was filled to 40 ml by adding MilliQ water. This was incubated in a water bath for 2 hours at the same temperature and the pH was checked and adjusted every 30 minutes. Thereafter, the samples were put on ice for 10 minutes to stop the reaction and the pH was increased to above 9 to ensure completion of digestion. The samples were centrifuged at 4000 rpm for 20 minutes at 20 ºC (Beckman Coulter Allegra X-15R) and 20 ml of each sample together with 1 g of starch were freeze dried to measure the protein content.

4.5.3 Protein content

The freeze-dried samples from the *in vitro* PD and the freeze-dried samples used in the *in vitro* digestibility were analyzed as a control. The content of protein was determined by dynamic flash combustion. A protein analyzer (Thermo Scientific™ FLASH™, EA 1112 series, USA) equipment was used to analyze the samples, this was done in duplicates.

4.5.4 Calculations

To get the PD the protein content from the protein analyzer had to be recalculated. This was done by Equation 2 and 3, where *Protein fraction* equals the weight percent left after removal of starch in the samples from the *in vitro* PD, *Protein content* is the protein content of the *in vitro* PD samples with the blank subtracted, and *Protein content (ref)* is the protein content of the samples that has not been through the *in vitro* PD process.

$$Protein\ content\ (PD) = Protein\ fraction \cdot Protein\ content \quad (2)$$

$$Protein\ digestibility = \frac{Protein\ content\ (ref) - Protein\ content\ (PD)}{Protein\ content\ (ref)} \cdot 100 \quad (3)$$

4.6 Statistical analysis

Statistical analysis was performed for all different experiments. For the soaking curves, t-tests were performed in excel for each measured hour, to see if/when a significant difference would appear. The standard deviation (STD) was also calculated for each measured hour and from those, an average value could be calculated which will be referred to as a *pooled standard deviation*. For the PA and PD, a one-way Analysis of Variance (ANOVA) were performed in excel with a p-value of 0.05.

5. Results

5.1 Initial trials

During the initial trials, a lot of different trials were conducted, with different parameters. The ones that were not chosen to be investigated further can be seen in Appendix 3.

5.2 Soaking curves

The soaking of the peas was measured as percent of weight gain. They should approximately double their weight (gain 100%) to be considered properly soaked. The maximal soaking is around 130% and was achieved after 6 days. However, after 3 days they had started to sprout, at that time they had soaked around 120%.

In Figure 1, the soaking curves for each sample are shown. In each plot, there is one line representing the average and in Table 7, the pooled standard deviation for each curve is shown.

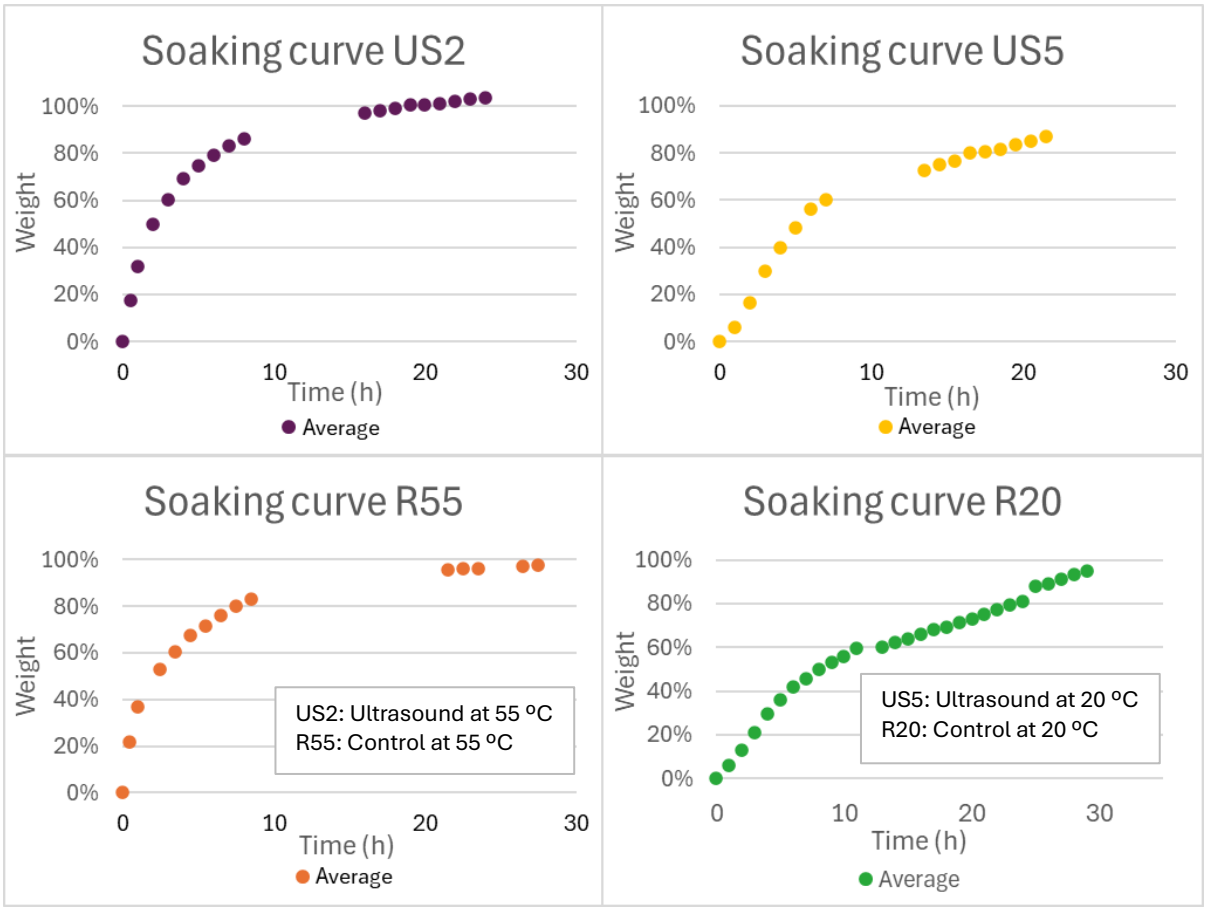

Figure 1 – Average soaking curves for each treatment. R55, R20, US2 and US5 correspond to the treatments described in Table 4, except for US6 and R45, since soaking curves were not made on those.

Table 7 – Pooled STD for each curve.

Sample	US2	R55	US5	R20
Pooled STD	3.12	2.33	5.58	2.95

In Figure 2 the average values from the control curves (R55 and R20) are plotted with the average value of its corresponding ultrasound curve (US2 and US5).

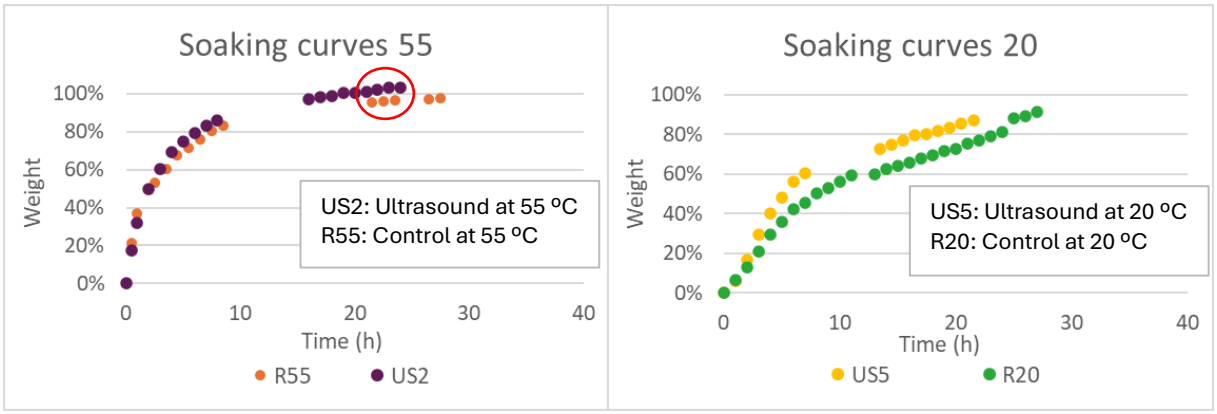

Figure 2 – Comparison of the control curve and its corresponding ultrasound treated curve, US2 with R55 and US5 with R20.

When performing statistical analyzes on the soaking curves, the t-test showed no significant difference between the control and the corresponding ultrasound treated soaking curve except after 21-23 hours in the 55 ºC plot, marked with a red circle.

During the ultrasound treatment, the temperature increased. This was documented and is shown in Table 8 for US2 and in Table 9 for US5.

Table 8 – Temperature measurements during ultrasound treatment for US2.

Time (min)	Temp 1 (ºC)	Temp 2 (ºC)	Temp 3 (ºC)	Temp 4 (ºC)	Average (ºC)
0	15	13	13	14	13.75
5	27	32	26	30	28.75
10	41	44	40	42	41.75
15	52	54	51	55	53
20	55	55	55	55	55
25	55	55	55	55	55
30	55	55	55	55	55
35	55	55	55	55	55

Table 9 – Temperature measurements during ultrasound treatment for US5.

Time (min)	Temp 1 (ºC)	Temp 2 (ºC)	Temp 3 (ºC)	Temp 4 (ºC)	Average (ºC)
0	12	13	12	13	12.5
10	20	21	20	19	20
20	19	20	20	20	19.75
30	20	19	20	19	19.5
40	20	19	21	19	19.75
50	20	20	20	20	20
60	20	20	20	20	20

5.2.1 Observations

During the soaking, it was observed that all peas did not behave equally. After both the ultrasound treatment and the control soakings, usually some peas looked fully hydrated, while most looked partly hydrated and some not at all. This can be visualized in Figure 3, there the fully hydrated peas are to the left, the partly hydrated peas are in the middle and the unsoaked peas are to the right. It was also observed that for R20, even after 10 hours there was still peas that looked like they have not started to get hydrated yet, but after 24 hours, all peas looked fully hydrated.

Figure 3 – Picture of peas after 30 minutes treatment in 55 ºC.

A secound observation was that the power output from the ultrasound machine was not 200 W, even though that was as a set parameter on the machine. The real values and their STD are shown in Table 10, where "1" indicates the first set of peas and "2" indicates the second set of peas.

Table 10 – Power output and STD from ultrasound machine, 1 indicates the first set of peas and 2 indicates the second set of peas.

Sample	**US2**	**US5**
1 (W)	78.78	81.96
1 (W)	75.26	85.93
2 (W)	77.24	92.94
2 (W)	76.77	91.73
Average 1 (W)	77.02 ± 1.76	83.95 ± 1.99
Average 2 (W)	77.01 ± 0.23	92.34 ± 0.60
Average total (W)	**77.01 ± 1.26**	**88.14 ± 4.44**

The power outputs for some other soaking trials that were not used for further analyzes can be seen in Appendix 4.

5.3 Phytic acid content

The standard curve together with its equation can be seen in Figure 4.

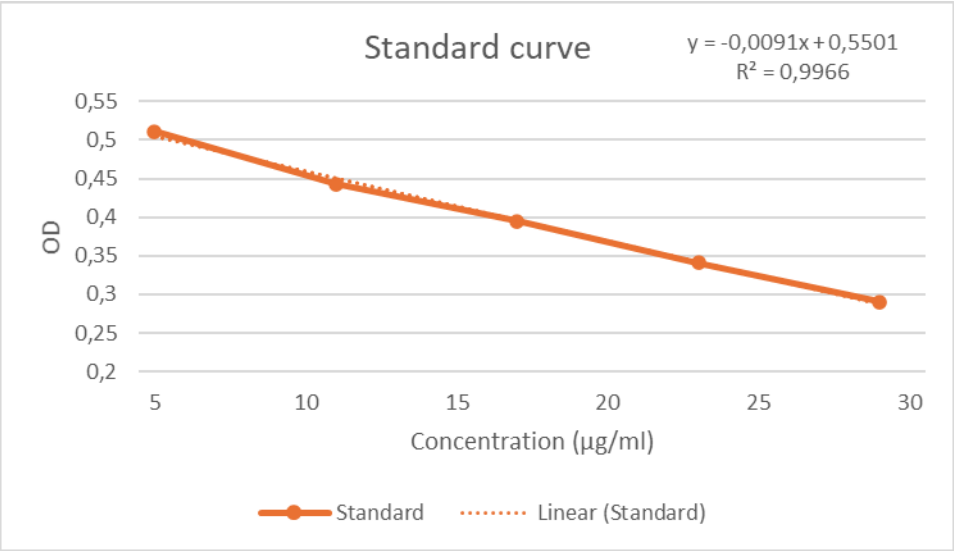

Figure 4 – Standard curve.

In order to get the amount of PA present in the peas, the dilution factors and moisture content (see Appendix 1) were taken into account. In Figure 5 the amount of PA per gram of dry pea is reported. The samples US6 and R45 have only been made in duplicates while the other samples have been made in sextuplicates. However, when performing statistical analyzes, the ANOVA showed no significant difference between the samples.

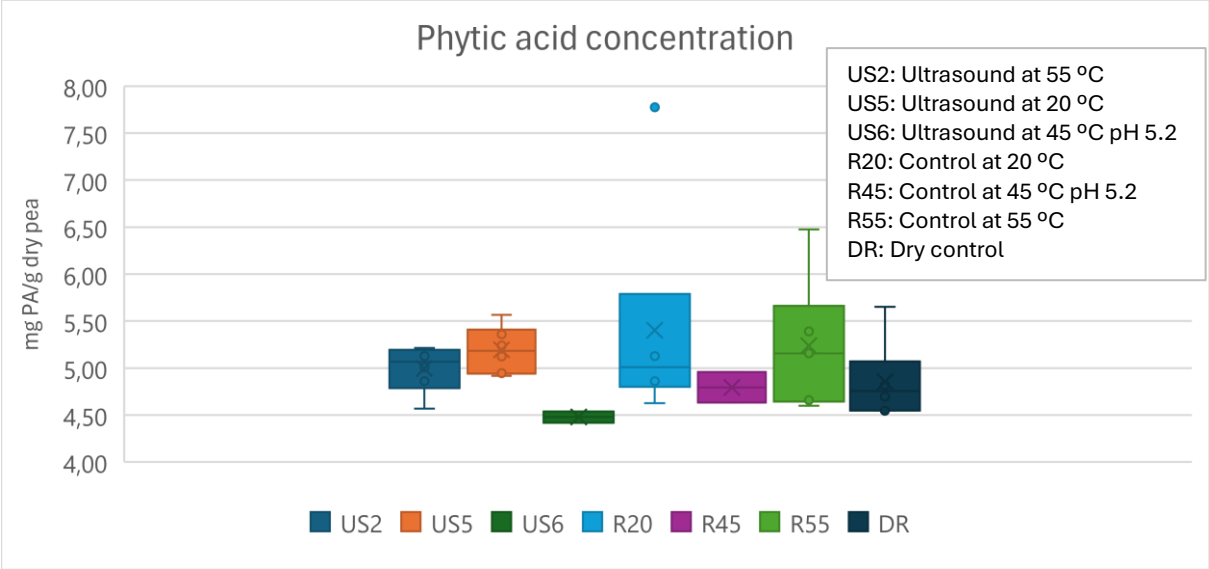

Figure 5 – Boxplot of PA concentration.

5.4 *In vitro* protein digestibility

The protein content from the control and ultrasound treated samples can be seen in Table 11.

Table 11 – Protein content of control and ultrasound treated samples.

Sample	US2	US5	US6	R20	R45	R55	DR
Trial 1 (% protein)	24.41	24.73	23.61	24.28	24.08	23.79	22.89
Trial 2 (% protein)	22.89	23.58	22.82	23.07	22.93	22.91	20.69
Average (% protein)	23.65 ± 0.76	24.15 ± 0.58	23.21 ± 0.39	23.67 ± 0.60	23.50 ± 0.57	23.35 ± 0.44	21.79 ± 1.10

The protein fraction from the *in vitr*o PD samples can be seen in Table 12. Since each sample from the *in vitro* digestibility and protein analysis were made in duplicates, there are four values for each treatment method. Sample 1 and 2 comes from one batch and sample 3 and 4 from another. The blank only have two samples since the other two got contaminated.

Table 12 – Protein content of the *in vitro* PD samples.

Sample	US2	US5	R20	R55	DR	Blank
1 (% protein)	23.18	18.66	18.73	19.57	19.80	3.39
2 (% protein)	23.46	18.94	18.92	19.96	18.72	3.38
3 (% protein)	19.09	21.06	20.37	19.36	18.69	
4 (% protein)	20.79	21.48	20.20	21.83	19.08	
Average (% protein)	21.63 ± 1.80	20.04 ± 1.25	19.56 ± 0.74	20.18 ± 0.98	19.07 ± 0.45	3.39 ± 0.01

The average PD for each sample can be seen in Table 13 and in Figure 6.

Table 13 – Average PD for each sample.

Sample	US2	US5	R20	R55	DR
PD (%)	63.46 ± 3.63	67.70 ± 2.61	68.58 ± 1.74	67.19 ± 1.72	65.71 ± 1.22

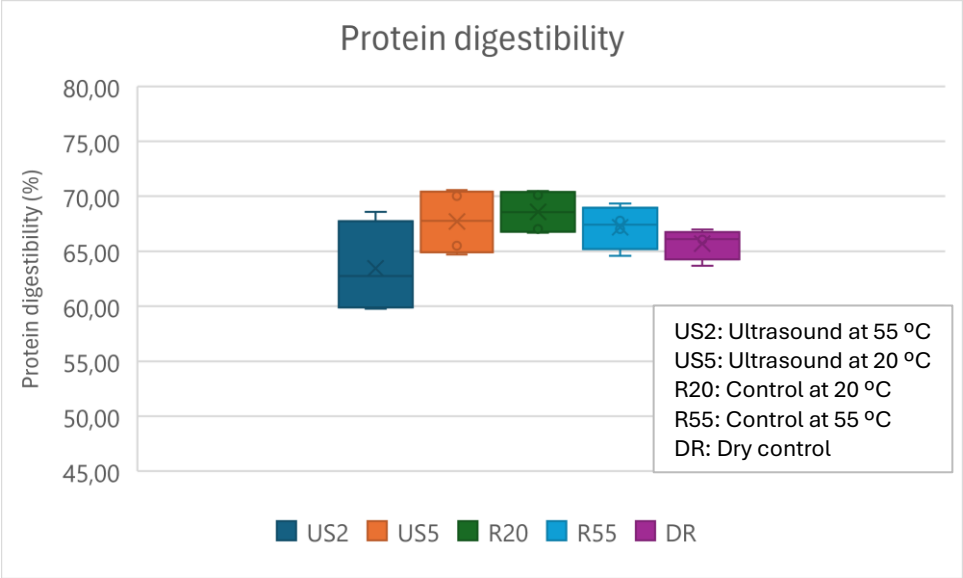

Figure 6 – Boxplot of PD.

Since there is quite a difference between the values from the two batches the STD gets relatively large. Therefore, the ANOVA showed no statistical difference between the samples.

6. Discussion

Overall, a major source of error for the entire project is the big variation between the peas. Some peas gain weight within 30 minutes when hydrated while others take over 10 hours. This can be partly visualized in the figures, where there is a visible gap between the different batches used. The reason for this is unknown but is probably due to permeability restrictions from the seed hulls that could have been an effect from wrong storage conditions.

It should also be noted that there are true replications for all samples except US6 and R45. Those samples are made from the same ultrasound treatment batch, while for the others, the samples are made from two ultrasound treatment batches. It could be a reason to why those samples have less standard deviation than the others except from the fact that there are less replications of them.

6.1 Soaking curves

Regarding the soaking curves, there are multiple sources of error. Firstly, the power output from the ultrasound machine is not consistent, which could affect the results. The reason to the big deviations is unknown but could be a limitation from the machine. However, the power output is larger when the machine is operating continuously without any pulses. To be noted, the continuous treatment did have a significant lower soaking capacity compared to both R55 and US2 which was treated with pulses, this can be seen in Appendix 3.

The study made by Miano et al. (2016) showed that an ultrasound treatment of mung beans could decrease the soaking time by 25%. Nevertheless, the treatment time in that study was six hours, so it could be that the treatment time has been too short in this study. Or it could just be that those legumes are more susceptible to the ultrasound treatment. However, when comparing the graphs in this study, the soaking time could potentially be reduced by 7 hours (35%) for the ultrasound treated samples. Indicating that a shorter treatment time might be enough. However, since there is no significant difference between the samples on the data available, this cannot be concluded.

The literature refers to ultrasound treatments as a non-thermal process. Even though there is no added temperature, the ultrasound still generates heat. It could therefore be argued that ultrasound treatments are in fact a thermal process. Depending on if this is wanted or not, it could be interesting to try longer pulse intervals, as used in other studies, to see if that can

control the increased temperatures. It is easy to control the temperature with ice or water bath in small scale, but if this was to be implemented in the industry it is not as easy and other solutions need to be found.

Another source of error is that the soaking curves was made with 20 peas (approximately 5 g) and 200 ml of water while the peas used for the experiments was made with 50 g of peas and 250 ml of water. The assumption was made that the peas would behave equally, but it is not certain. It would probably have been better to use bigger samples for the soaking curves since there is a big variation in the peas.

The control curves had in general a lower STD compared to the ultrasound treated peas. This could indicate that the ultrasound treatment does affect the peas somehow, but that it is not that consistent. It was also found that there was a lower STD for the samples treated in higher temperature. This could be because the effect of the heating has similar effects as the ultrasound treatment. Therefore, when both processes are used simultaneously, the effect of the ultrasound is reduced.

Another reason to why there is no significant difference found is because the temperatures used during the treatment is not consistent between the controls and the ultrasound treatments. In the ultrasound treatments, the water temperature is initially low, and then increases to the wanted temperature within 15 minutes. Therefore, the real treatment time at the set temperature is actually 10-15 minutes less. Nevertheless, for the controls, the wanted temperature is set from the beginning. This could lead to lower soaking capacities since the temperature has a big impact on the soaking capacity.

Finally, it would have been better to use MilliQ water instead of tap water during the soaking, this was not done since the experiments were to mimic the conditions at Sevan as much as possible. However, it would be preferable to have a better controlled environment for this project, since the water quality could have affected the results.

6.2 Phytic acid content

The method for PA content is probably not so trustworthy since you would expect a higher concentration of PA in DR than in the soaked samples. The PA content found was also in the lower spectrum compared to the literature and the big variation between the replications indicate that something could be wrong. It could be due to a problem with the extractions.

During the project, a few things were made to try to optimize the extraction, like finely milling the peas and vortex the samples for longer, but with no result.

A limitation with the method used by Makkar et al. (2007) is that it only measures the total phytic acid content. Therefore, it is possible that the IP6 present has been degraded to lower inositol phosphates (IP5-3) which has a lower inhibitory effect. Those results could have been achieved through a high-performance liquid chromatography, so it would be recommended to try that method instead.

Sample US6 were designed to reduce the PA content the most with its lower pH and moderate temperature to activate the phytase. Looking at the results, it seems like US6 could have been promising, since it has the lowest PA content. However, since the other samples has quite high STD and since not enough replicates were made, it could just have been a coincidence.

Regarding the literature, there were some mixed results regarding PA content and ultrasound where Sharkhel and Roy (2022) found that it could reduce PA in pulses, while on the contrary, Kaya et al. (2017) showed no effect in pulse hulls. Therefore, it is still unknown whether ultrasound can be used for this purpose or not.

The PA content was measured to be 479-524 mg/100 g pea in this project. Compared to the literature (Hallberg et al. 1989) where they advocated that 220 mg/100 g pea will have a negative impact. Therefore, it seems like it could be a problem with bioavailability of nutrients if these peas were to be consumed at this stage.

6.3 *In vitro* protein digestibility

The PD measured in this report matches the amounts of 65-70% in yellow peas that is mentioned in previous literature by Nosworthy et al. (2017). However, the peas used in that study had also been cooked which usually increases the PD. There is still no significant difference between the samples, and it is expected to at least be a 5-10% lower protein content for the DR. Additionally, the study from Ye et al. (2016) shown that ultrasound significantly improved the PD for pea protein in similar conditions as used in this study.

While analyzing the samples in the protein analyzer, a few standard samples were put in now and then to see if the calibrations still were ok. These standards indicated that there could be an error up to 3% for the samples, which could explain the deviations and why there were no significant difference. Additionally, after freeze drying the *in vitro* PD samples, some of the

samples bubbled up and got stuck to the aluminum foil surrounding the samples, which could have caused a lower protein content.

7. Conclusions and Future work

As a conclusion, none of the research questions investigated showed any statistical differences with the methods used in this project. More data is needed to be able to see if there is a significant difference between the treatments or if it is just the peas that differ. It could be of interest to investigate a longer or shorter treatment time to see if that has any impact. It would also be preferable to try a different method to measure PA content or optimize the method used in this project in some way.

The amount of PA measured in the peas indicates that it could negatively inhibit the absorption of both protein and minerals if they were to be consumed after the soaking process. It would therefore be recommended to add an additional processing step to reduce it. Another finding is that ultrasound treatment with pulses is much more effective for the soaking capacity than ultrasound treatment with continuous operation.

Since no significant difference was found for neither PA content nor PD, there was no point in making the falafel during this project. However, for future work if a significant difference is found, it would be intriguing to investigate whether this difference persists after the frying step, indicating that an ultrasound treatment could enhance the nutritional value of plant-based foods. Additionally, it would also be of interest to examine the effects of altering the parameters of the ultrasound machine or if the time of treatment have any impact. Furthermore, analyzing other antinutrients and the mineral content of the peas would also be worthwhile.

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Appendix

Appendix 1 - Moisture content

In Table 14, the weight lost during freeze drying and drying in oven is showed. The percentages are based on that the initial value is the weight of the untreated dry peas. The peas used in FD 1 and Oven 1 are the peas used for the first 4 PA trials, and FD 2 and Oven 2 are the peas used for trial 5 and 6.

Table 14 – Percentage loss of weight after freeze drying (FD)/drying in oven.

Sample	**US2**	**US5**	**US6**	**R20**	**R45**	**R55**	**DR**
FD 1 (%)	-12	-11	-	-10	-	-12	-4
Oven 1 (%)	-15	-13	-	-13	-	-15	-10
FD 2 (%)	-11	-12	-10	-10	-10	-11	-4
Oven 2 (%)	-14	-15	-13	-12	-12	-14	-8

Appendix 2 - Dilution series

The dilution series for standard curve is presented in Table 15. The stock solution named 100X contained 10 mg PA sodium salt hydrate and was filled up to 100 ml with MilliQ water. The correlation that 100 g PA sodium salt hydrate = 59.5 g PA was used to calculate the concentration of PA in the table.

Table 15 – Dilution series for the standard curve.

Dilution	100X (ml)	MilliQ (ml)	Final V (ml)	Conc P (µg/ml)
50X	10	10	20	29.95
40X	10	15	25	23.96
30X	15	35	50	17.97
20X	10	40	50	11.98
10X	5	45	50	5.99
5X	5	95	100	2.995
	50X (ml)			
2.5X	5	95	100	1.4975
	5X (ml)			
1X	10	40	50	0.599
0.5X	5	45	50	0.2995
	1X (ml)			
0.1X	5	45	50	0.0599

Appendix 3 - Soaking curves

In Table 16 the nomenclature for all trials is presented. If a temperature set and remain stable within 10-15 minutes, only one temperature is reported. Otherwise, the whole interval is reported.

Table 16 – Nomenclature for all samples.

Sample name	Treatment	Pulses (%)	Time (min)	Temperature during treatment (°C)	Additional
US1	Ultrasound	100	30	55	Water bath
US2	Ultrasound	70	35	55	N/A
US3	Ultrasound	10	30	11-21	N/A
US4	Ultrasound	80	30	20	Ice bath
US5	Ultrasound	80	60	20	Ice bath
US6	Ultrasound	70	35	45	pH 5.2
US7	Ultrasound	50	30	13-53	N/A
US8	Ultrasound	50	60	12-60	N/A
US9	Ultrasound	10	60	13-27	N/A
US10	Ultrasound	50	30	14	Ice bath
US11	Ultrasound	50	60	13	Ice bath
R	Control	N/A	N/A	10-15	N/A
R20	Control	N/A	N/A	20	N/A
R45	Control	N/A	30	45	pH 5.2
R55	Control	N/A	30	55	N/A
DR	Dry control	N/A	N/A	N/A	N/A

Sample US7-US11 were the initial trials. However, since the temperatures were not consistent with the controls, those trials were not investigated further. New trials with better adaptations (longer pulses to faster increase temperature and added water/ice bath) were applied for US1-US6. Initially, R was used as a control curve to mimic the conditions at Sevan where they apply cold water to their soaking. However, R20 was chosen to be the used control to better compliment the ultrasound treated samples.

In Figure 7 trial US1-US5 are shown together with the corresponding control curve. A t-test showed that there is a significant difference between sample R55 and US1 for the first set of hours marked with blue. There is also a significant difference between sample US2 and US1 in

hour 4-8 marked in red. However, there is no significant difference between any of the curves in “Soaking curve RT”.

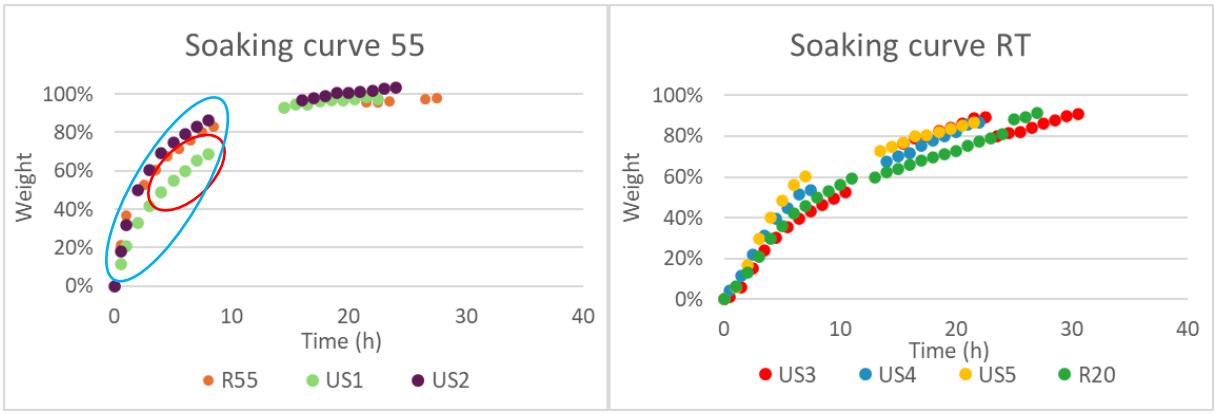

Figure 7 – Soaking curve for US1-US5 with corresponding control curve.

In Figure 8 trial US7-US11 is shown together with the R55 and the old control curve R.

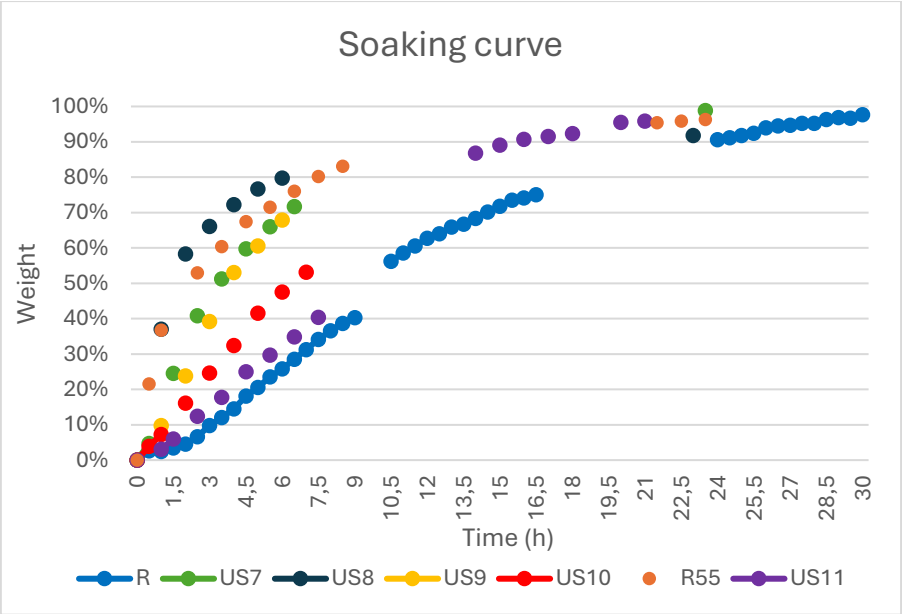

Figure 8 – Soaking curve for US7-US11 and control curve R and R55.

Appendix 4 - Average power

The average power output from the ultrasound machine for samples US1-US5 are shown in Table 17 and Figure 9.

Table 17 - Average power output for sample US1-US5.

Sample	US1	US2	US3	US4	US5
1 (W)	148.54	78.78	72.32	84.85	81.96
1 (W)	140.98	75.26	74.55	84.90	85.93
2 (W)	140.97	77.24	70.46	87.54	92.94
2 (W)	139.97	76.77	79.65	87.78	91.73
Average 1 (W)	144.76 ± 3.78	77.02 ± 1.76	73.44± 1.12	84.87 ± 0.03	83.95 ± 1.99
Average 2 (W)	140.47 ± 0.50	77.01 ± 0.23	75.06 ± 4.60	87.66 ± 0.12	92.34 ± 0.60
Average tot (W)	**142.62 ± 3.45**	**77.01 ± 1.26**	**74.25 ± 3.44**	**86.27 ± 1.40**	**88.14 ± 4.44**

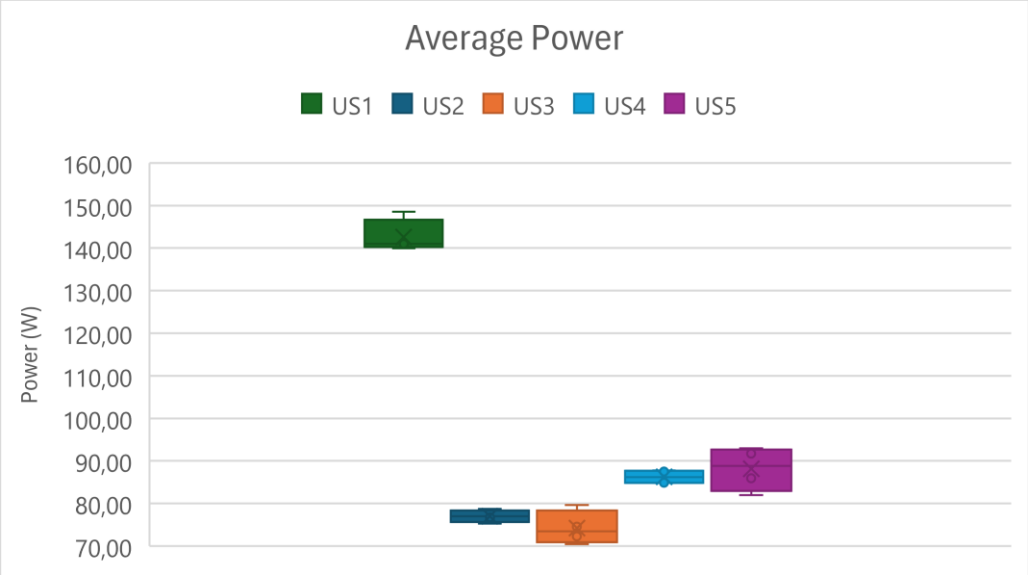

Figure 9 – Average power output for sample US1-US5.

The average power output from the ultrasound machine for samples US7-US11 are shown in Table 18. There is less data for these samples since these trials were only made ones.

Table 18 – Average power output for sample US7-US11.

Sample	US7	US8	US9	US10	US11
FM (W)	75.86	69.28	82.17	85.19	80.57
EM (W)	75.73	66.69	79.67	81.64	96.02
Average tot (W)	**75.80**	**67.98**	**80.92**	**83.42**	**88.29**
STDAV tot	**0.06**	**1.30**	**1.25**	**1.77**	**7.73**