

**Monitoring the Aroma Profile During the Manufacturing of Pea Protein  
Isolates**

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## **Dedication**

*I dedicate this research to my mother, my brother (in his memory), my husband and every teacher that helped me define and realize this dream.*

## Abstract

There is currently a growing interest for the use of plant proteins in food products. Among the plant proteins options, pea protein ingredients have rapidly gained popularity in the food industry. This increased interest for pea protein ingredients by the food industry is driven mainly by the consumer demands for healthier, non-genetically modified and more sustainable food products. However, challenges persist in producing a high-quality protein that possesses a bland flavor profile and that can be used broadly in new product development efforts. There has been little work published on the impact of the processing conditions used during the production of pea protein isolates on their aroma profile. This research project focuses on the measurement and characterization of the aroma compounds present in pea protein isolates and attempts to determine their sources, i.e. are they inherent to the plant, or formed during the production of pea protein isolates. The objectives of this study were: (1) develop a protocol for the extraction and identification of the volatile aroma compounds present in pea flour (PF); and (2) monitor the aroma profile during the manufacturing of pea protein isolates produced by (a) salt solubilization coupled with membrane filtration (salt-extraction) and (b) alkaline solubilization with isoelectric precipitation (pH-extraction).

Three methods for the isolation of volatile compounds were compared based on the ability to provide a complete aroma profile of pea flour: Stir Bar Sorptive Extraction (SBSE), Solvent Assisted Flavor Evaporation (SAFE) and Headspace Solid-Phase Microextraction (HS-SPME). Three and four aroma compounds – previously reported by

other authors- were identified in the SBSE and SPME isolates respectively, whereas 12 compounds were identified in the SAFE extract. SAFE showed to provide a much more complete aroma profile than either competing method tested in this study. Therefore, SAFE was selected for the evaluation of the aroma compounds present in the samples collected throughout the protein isolation processes.

Pea protein isolates were produced by salt-extraction and pH-extraction on a pilot plant scale. Samples were taken at several steps throughout the protein isolation processes. The aroma compounds were extracted from each sample using the SAFE method and were identified by Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O) and Gas Chromatography – Time-of-Flight Mass Spectrometry (GC-TOF-MS). In order to look for relationships between the GC-MS-O data and overall sensory character, a sensory evaluation was conducted on pea flour and the final pea protein isolate.

During the production of pea protein by salt-extraction, 60 different aroma compounds were isolated and identified. Out of the 60, 12 compounds were perceived with a “moderate” odor intensity by panelists through the sniffing port of the GC-MS-O in at least one of the processing steps. None of the major odorants were newly formed or completely lost during protein isolation suggesting that the processing steps do not completely remove existing or generate significant new aroma compounds. From the sensory evaluation, the aroma descriptors used to describe the pea flour and PPI testing solutions were also used to describe individual compounds eluting from the sniffing port.

This supports the hypothesis that the 12 compounds identified in this study are likely to be the significant contributors to the aroma profile of the samples analyzed.

Similar results were obtained during the production of pea protein by pH-extraction. Thirteen aroma compounds were found to be likely the most significant contributors to the aroma profile of the samples examined. This hypothesis was also supported by the sensory data which showed that the pea flour and pea protein isolate aqueous solutions were described with similar odor descriptors as those used during the instrumental analysis. Similar to the salt-extraction process, no new aroma compounds appear to be produced via the optimized pH-extraction; no existing compounds were completely removed from making a sensory contribution as determined by the olfactory analysis.

Most of the major aroma compounds were present from the very first step of the process (PF) which indicates that they are likely derived from the peas themselves as plant metabolites or are formed at some point before/during the production of PF. Additionally, the origin of these compounds was associated mainly with enzymatic degradation of unsaturated fatty acids and the Maillard reaction.

The current research will provide ingredient manufacturers with insights on the compounds that are likely to contribute to unpleasant aroma of pea protein isolates, their potential precursors and source and, approaches that can be explored in the future in order to reduce them and/or prevent their formation.

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# Chapter 1: Introduction

The emergence of “flexitarian”, vegetarian and vegan consumers as well as the increasing consumer demands for healthier and more sustainable food products has led to rapid growth in the plant protein market (Bloom, 2021) (Ahuja & Singh, 2019). Among different plant protein sources, peas have received the greatest interest by the food industry. Peas have a high protein content (20-30%), are not genetically modified, have low occurrence of allergenicity (USA Pulses, 2016), and are suited to grow in much of North America. However, peas are currently underutilized in food applications. Challenges persist in producing a high-quality protein that possesses a bland flavor profile that can be used broadly in new product development efforts. A recent survey by Mintel showed that flavor continues to be one of the barriers for plant-based meat companies seeking wider adoption of their products (Formanski, 2020).

The off notes in pea protein ingredients are commonly associated with enzymatic degradation of unsaturated fatty acids which originate mainly from the peas themselves as plant metabolites, as a result of processing and/or by deterioration during storage (Roland, Pouvreau, Curran, Van De Velde, & De Kok, 2017). Additionally, certain components such as carbohydrates, lipids and particularly proteins have the ability to bind flavor compounds (Paravisini & Guichard, 2016). These interactions have an impact on the inherent pea aroma compounds which are difficult to remove (Macleod, Ames, & Betz, 1988).

The aroma-protein interactions are influenced by intrinsic factors such as protein structure, amino acid profile and nature of the aroma (Wang & Arntfield, 2016). Additionally, extrinsic factors including temperature, pH and ionic strength lead to conformational changes of the proteins which can also impact the binding of aroma compounds and therefore, overall sensory perception (Wang & Arntfield, 2016). There is a need to understand if the processing conditions used during protein extraction favor the formation of aroma compounds and/or impact the binding of the existing aroma compounds to the proteins inhibiting their removal during processing.

Currently, the most commonly used method for protein extraction is alkaline solubilization with isoelectric precipitation which can damage the structural conformation of the protein and therefore, functional properties. There exists a need to optimize not only the conditions used for the extraction of pea protein but also the methods of extraction in order to produce pea protein isolates with high protein purity and yield.

In preliminary work within our research group, the extraction conditions to maximize protein purity and yield following a salt solubilization coupled with membrane filtration (salt-extraction) and alkaline solubilization with isoelectric precipitation (pH-extraction) were optimized (Hansen, 2020) . Therefore, this study aims to identify the aroma compounds present at different steps during the optimized salt- and pH- protein isolation process and attempts to determine their sources, i.e. are they inherent to the plant, or formed at various steps in the isolation of pea protein.

## 1.1. Hypotheses

1) Previous studies have shown that SAFE provides a more complete profile of the volatile aroma compounds present in a food matrix than any other method for the isolation of volatiles. *It is hypothesized that SAFE will show a more complete analytical aroma profile compared to SBSE and SPME techniques.*

2) A few researches have reported that objectionable aroma compounds might form during the protein isolation process. *It is hypothesized that objectionable flavor notes come from two sources: those inherent to peas and those that arise during the production of pea protein isolates.*

## 1.2. Objectives

The objectives of this research are to:

(1) Develop a protocol for the extraction and identification of the volatile aroma compounds present in pea flour

(2) Monitor the aroma profile during the manufacturing of pea protein isolates produced by (a) salt solubilization coupled with membrane filtration (salt-extraction) and (b) alkaline solubilization with isoelectric precipitation (pH-extraction).

## **Chapter 2: Literature Review**

### **2.1. Plant Protein Ingredients Market**

The demand for plant-based food products across the globe is on the rise. The plant protein ingredients market size for food application is expected to reach USD 8 billion by 2025 (Ahuja & Singh, 2019). This growth has been mainly driven by the emergence of consumers that avoid animal products (flexitarians, vegetarians and vegans) and an increased perception that plant-based products are healthier, more natural and environmentally friendly compared to animal products. In a 2020 study by Mintel (Formanski, 2020), 1684 participants were asked why they eat plant-based proteins. The results showed that 56% of this population eat plant-based proteins to be healthier, and 16% responded to lower their impact on the environment. Although sustainability and environment are not the main motivators to eat plant-based proteins, analysts predict that concerns over sustainability are likely to continue to increase as more people become aware of the impact of animal agriculture to the environment. One particular plant protein source that has shown an important increase in the market is peas. The global pea protein market size was valued at USD 130 million in 2019 and it is estimated to reach USD 235 million by 2026 (Ahuja & Mamtani, 2020). This increased interest is mainly due to different factors: peas do not require nitrogenous fertilizers as peas can draw nitrogen from the air, peas can be grown both as a cover crop (cultivated before another plant species) or as a rotational crop. Both types of crops reduce the amount of fertilizer needed for the next round of harvest (Trenton et al., 2018) and peas are suited to grow in much of

North America. Additionally, one of the major advantages of pea protein compared to one of the most widely used plant protein in meat alternatives, soy protein, is that pea protein is not genetically modified, and it has a low occurrence of allergenicity (USA Pulses, 2016). Unlike pea protein, soy protein is on the list of the “Big Eight” allergens in the United States (FDA, 2021). Although the pea protein market continues to expand, there are still a few challenges to overcome including off-flavor. A 2020 Mintel survey reported that 27% of the population interviewed do not consume plant-based meat substitutes because they do not like the taste (Formanski, 2020). More research needs to be conducted to improve the flavor of pea protein and make it more competitive to other plant proteins in the market.

## **2.2. Pea Protein Ingredients**

Currently, there are four pea protein ingredient forms available in the market: (1) pea flour (PF) which is produced by grinding roasted yellow field peas; (2) pea protein concentrate (PPC), which is produced by pin-milling and air classification of whole or dehulled peas that removes starch granules; (3) pea protein isolate (PPI), which is commonly obtained by milling of the peas followed by pH or salt precipitation; and (4) pea protein hydrolysate (PPH), which is produced by enzymatic or chemical hydrolysis of PPI (Sandberg, 2011).

### 2.2.1. Chemical Composition of Pea Ingredients

Table 1 shows that pea flour contains between 20-28% protein. The two major protein fractions of pea are globulins and albumins. Globulins constitute 60-75% of total protein and are separated into two major types: legumins and vicilins. Albumins are extracted by water whereas globulins are extracted using salt solutions at neutral pH (Lampart-Szczapa, 2001).

**Table 1.** Chemical Composition of pea ingredients (g/100g DM)\*

<b>Component</b>	<b>Pea flour</b>	<b>Protein Concentrate</b>	<b>Protein Isolate</b>	<b>Protein Hydrolysate</b>
<b>Crude protein (N x 6.25)</b>	20.0 – 28.0	47.2 - 59.2	80.3 - 90.8	84.2- 90
<b>Starch</b>	39.0- 55.7	7.0 - 7.9	1.2- 2.7	**
<b>Crude Fat</b>	1.1-1.3	2.6 - 3.8	1.7 - 5.1	**
<b>Crude Fiber</b>	1.7-1.9	2.5 - 2.8	0.1 - 1.3	**
<b>Total ash</b>	2.7-3.5	5.2- 5.5	4.4 – 5.2	1.9 -7.4

\*Data taken from (Sosulski & McCurdy, 1987), (Bhatty & Christison, 1984), (Øverland et al., 2009), (Urbano et al., 2003), (Hall, 2018)

\*\*Not found

Tömösközi et al. (2001) analyzed the amino acid composition of pea flour, PPC and PPI. They found that the amino acid profiles of these products were similar overall. Table 2 shows that glutamine is present in the greatest amount followed by aspartic acid, arginine and lysine, and lower quantities of tryptophan and sulfur-containing amino acids, methionine and cysteine.

**Table 2.** Amino acid composition (g AA/100g protein) of protein products\*

Amino Acid (In Percent)	Pea Flour	Pea protein Concentrate	Pea protein Isolate
Asp	10.46	11.58	11.52
Tre	3.66	3.12	3.69
Ser	4.37	4.96	6.09
Glu	16.60	16.39	17.03
Pro	5.56	4.30	5.01
Gly	4.43	4.50	4.68
Ala	4.53	4.13	4.41
½ Cys	0.34	0.35	0.73
Val	5.20	5.13	4.81
Met	0.86	0.85	0.78
Ile	3.80	3.48	3.68
Leu	6.36	6.94	8.16
Tyr	3.05	3.35	3.79
Phe	4.54	4.67	5.18
Lys	8.58	8.12	8.96
His	3.40	3.39	3.81
Trp	0.50	0.51	0.51
Arg	13.76	14.22	7.15

\*Data taken from (Tömösközi et al., 2001)

Pea flour (PF) also contains a significant amount of starch (39-56%). The amount of this component is significantly lower in PPC and PPI mainly because of its removal during the previously mentioned extraction processes. According to Dahl et al. (2012) uronic acids, cellulose and arabinose are the most abundant constituent carbohydrates found in pea seed meal followed by galactose, mannose, xylose and rhamnose. They also found traces of these sugars in the pea protein fractions (legumins, vicilins, and albumins).

Only one report was found on the fatty acid composition of peas. Villalobos Solis et al., (2013) identified the fatty acids present in pea oil samples. Linoleic acid (C18:2) was found to be the major component in pea oil (39.45%) followed by oleic (C18:1) and linolenic acid (C18:3) with 26.90% and 14.01% respectively.

### **2.2.2. Applications of Pea Protein Ingredients in Food**

Among different plant protein sources options, pea protein has rapidly gained interest among consumers and in the food industry mainly because it is non-GMO, and it is perceived as more environmentally friendly and healthier compared to animal proteins by consumers. Different forms of pea protein have been developed and used in a variety of food applications.

Pea protein ingredients have been used in the production of bakery products. Currently, many gluten-free products lack important nutrients and contain high levels of starch. Adding gluten-free proteins such as pea protein improves the nutritional quality of these products (Arntfield & Maskus, 2011). Additionally, pea flour can be used for baking. However, pea flour is often combined with other gluten-free flours such as rice for a complete amino acid profile. Pea protein has high concentration of lysine, but it is low in methionine, whereas rice protein has high amounts of methionine and low levels of lysine. When they are blended, they can make a complete protein and provide all the essential amino acids that are necessary in human diet (Lu, He, Zhang, & Bing, 2019). One major challenge that pea protein ingredients is facing today is their off-flavor and taste. When high concentrations of pea protein are required in order to increase the protein content of a bakery application, the use of precooked or deflavored pea flours is necessary (Tulbek, Lam, Wang, Asavajaru, & Lam, 2017). Comer, 1977, patented a method to remove bitter taste and pea flavor from pea flour for food application. This method consists of putting the flour in contact with moist steam for a time duration sufficient only to debitter the pea flour and remove the volatile compounds before a cooked pea flavor develops in the flour.

There has also been an increased interest in using pea protein ingredients in meat and meat analog applications. Pea protein ingredients are known to be effective binders and fillers in meat applications. This is mainly due to their emulsifying, gelling and water-holding properties (Tulbek et al., 2017). Many of the meat analogs present in the market today including chicken, beef and seafood (Formanski, 2020), have been created by processing pea protein through a cooking extruder or shear cell technology in order to create the fibrous strands of protein resembling to meat (Arntfield & Maskus, 2011).

Pea protein has also increasingly become popular in the beverage industry. In addition to sustainability, nutritional value is also emerging as an important consideration for consumers when making food purchases. Consumers are looking for food options that allow them to add protein into their diet (Cernivec, 2019). This trend has fueled beverage innovation with plant-proteins. Although pea protein ingredients are being used to satisfy the demand for plant-based beverages, these ingredients still face challenges as it relates to flavor and texture. Protein beverages usually require thermal treatments for safety and shelf life reasons. At high temperatures pea protein aggregates and precipitates creating a sedimentation in the finished product. Additionally, protein beverages are generally formulated around pH 4-6 in order to prevent undesirable astringency. At pH close to its isoelectric point, the protein has neutral charge leading to aggregation and precipitation (Lu et al., 2019). Arntfield & Maskus, 2011; Lu et al., 2019; and Tulbek et al., 2017 have provided a comprehensive review of these and other applications of pea protein ingredients.

The market of pea protein ingredients and their applications is expected to continue to gain popularity in the upcoming years (Ahuja & Mamtani, 2020). Although important

advancements on composition, functional properties, optimization of extraction methods and modification of pea protein have been attained, more research needs to be conducted in order to overcome current challenges.

## **2.3. Pea Protein Extraction**

Pea protein can be extracted by dry and wet fractionation processes. In dry fractionation, after the seeds are milled into flour, the protein and starch-rich fractions are separated by their different densities. During wet fractionation, the protein separation is based on solubilization of the protein (Yang, Zamani, Liang, & Chen, 2021). The most commonly used wet methods are alkaline solubilization with isoelectric precipitation and salt solubilization coupled with membrane filtration.

### **2.3.1. Alkaline Solubilization with Isoelectric Precipitation**

Protein isolation typically begins with pea flour. Pulse proteins are most soluble at alkaline pHs. Therefore, pea flour is first solubilized in an alkaline solution at a pH between 7 and 11 (Boye, Zare, & Pletch, 2010) (Arntfield & Maskus, 2011). The solution is then centrifuged in order to separate insoluble components including starch and insoluble fibers from the protein which remains in the supernatant. The pH of the supernatant is adjusted to its isoelectric point. The isoelectric point (pI) is the pH at which the net charge of a protein becomes zero. At a pH above the pI, the protein is predominantly negatively charged. These negative charges repel each other making the protein more soluble.

Similarly, at a pH below the pI, the protein is predominantly positively charged which create repulsion forces and also make the protein more soluble. At the pI the net charge of the protein is zero, which reduces the repulsive forces and induces the attraction forces causing the aggregation and precipitation of proteins (Novák & Havlí, 2016). The pI of pea protein has been found to be between 4-5 (Boye et al., 2010). Following protein precipitation, the protein solution is centrifuged, neutralized and dried (Boye et al., 2010).

### **2.3.2. Salt Solubilization Coupled with Membrane Filtration**

This process starts by solubilizing the proteins in a dilute salt solution. In this process, also known as “salting in”, small concentrations of salt increase the solubility of a protein in water. This occurs because charged groups on a protein bind the anions and cations of the salt solution more strongly than water. The ions, in turn, bind the water and therefore the protein is dispersed in water more easily (Vaclavik & Christian, 2007). Once the protein is solubilized in the aqueous phase, this mixture is centrifuged which causes the precipitation of starch and insoluble fibers while the protein and other small soluble components remain in the supernatant. The supernatant is then passed through a membrane in order to further concentrate the protein. The proteins having a molecular weight larger than the molecular weight cut-off of the membrane remain in the retentate whereas the small components (salts and sugars) pass through the membrane in the permeate and are consequently removed (Fredrikson, Biot, Larsson Alminger, Carlsson, & Sandberg, 2001). After membrane filtration, the retentate is dried to obtain pea protein isolate.

When comparing membrane filtration with other concentration methods (isoelectric precipitation), membrane filtration has shown to achieve higher protein yields and to preserve better the protein structure. Additionally, protein isolates obtained through membrane filtration have shown to have better solubility and functionality than isolates extracted through isoelectric precipitation (Lam, Karaca, Tyler, & Nickerson, 2018) (Gueguen, 1983).

## **2.4. Flavor Challenges of Pea Protein Ingredients**

Flavor, aroma, odor and taste are terms that are often used interchangeably. However, there is a clear difference between each of these terms. Taste is the sensory response to a substance by the receptors that are located in the oral cavity, mainly on the tongue. Taste compounds are generally non-volatile, water-soluble, low molecular weight (<2,000 amu) compounds. There are five basic tastes: sweet, bitter, sour, salty and umami (Guichard et al., 2016). Odor refers to volatile compounds that can be perceived by the sense of smell (ortho-nasal olfaction), whereas aroma is used to describe chemical compounds that are detected via the back of the throat when tasting a food (retro-nasal olfaction) (Peña y Lillo et al., 2005). The third sense that contributes to flavor perception is known as chemesthetic sensations. The most relevant are heat-related irritative sensations (from chili pepper and other spices), the non-heat related irritations (from mustard, horseradish and wasabi), the cooling sensations from menthol and other cooling agents, and astringency (Lawless & Heymann, 2010). Flavor is then a complex combination of the olfactory,

gustatory and chemesthetic sensations perceived during oral processing (ISO, 2008). The term off-flavor refers to the perception of an unpleasant taste, aroma, and other effects such as astringency (Roland et al., 2017).

Even though the popularity continues to grow, pea protein ingredients are facing major challenges related to off notes. These off-notes have been described as beany, earthy, and, grassy (Klein & Raidl, 1986). In some food applications, when the levels at which pea protein ingredients are incorporated are minimal, the flavor is not a concern. However, when higher amounts of this plant protein are to be added in order to reach the target protein claim, this often results in flavor issues. Flavor after all is an important driver for food and beverage purchases. If the product does not taste good the first time that is purchased, there is a low chance that the consumer will buy it again. Therefore, it is critical to use pea protein ingredients that have a clean flavor which would facilitate their incorporation in a wider range of products without impacting their overall flavor quality.

The off-notes associated with pea protein ingredients are due to the volatile aroma compounds which originate mainly from the peas themselves as plant metabolites, as a result of processing and/or by deterioration during storage (Roland et al., 2017). Additionally, certain components such as carbohydrates, lipids and particularly proteins have the ability to bind flavor compounds (Paravisini & Guichard, 2016). These interactions have an impact not only on the inherent pea aroma compounds which are difficult to remove but also, on the aroma profile of added flavor formulations (Macleod et al., 1988).

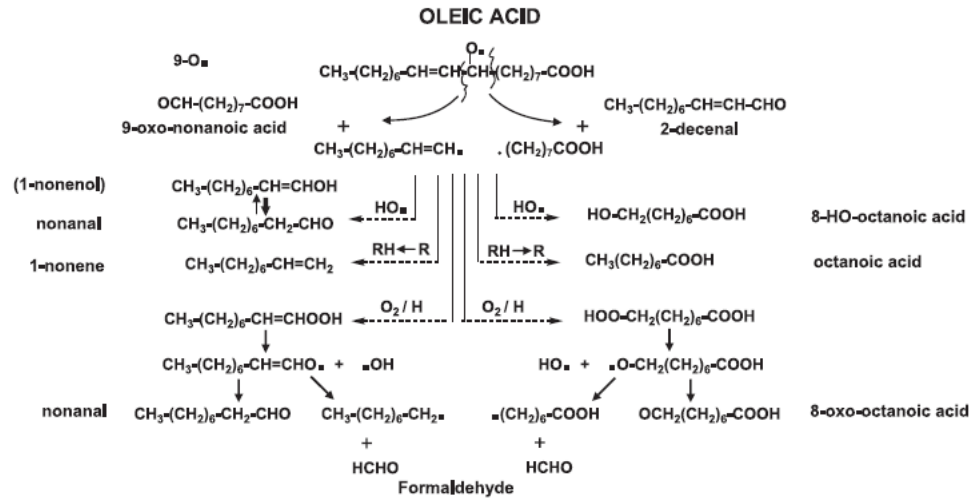
Today, the off-flavor of pea protein ingredients is one of the most important factors limiting the use of pea protein in a wider variety of food applications. While efforts have been made to mask and suppress off-notes in food formulation, this approach has had little success mainly because high amounts of these masking agents are often necessary which increases the cost of the product and also because it can create an imbalanced flavor profile. Therefore, there is a need to identify approaches that can eliminate or reduce the problematic off-flavors from the source or through processing methods instead of attempting to mask them (Ismail, Senaratne-Lenagala, Stube, & Brackenridge, 2020).

## **2.5. Formation of Volatile Compounds in Peas and Pea Ingredients**

### **2.5.1. Lipid Oxidation**

Oxidation of unsaturated fatty acids is a phenomenon that occurs in the presence of oxygen. Lipids can be oxidized by different mechanisms including, autoxidation and enzymatic oxidation. Autoxidation consists of the reaction of triplet oxygen ( $^3\text{O}_2$ ) with organic compounds whereas enzymatic oxidation occurs by the action of enzymes such as lipoxygenase, which is commonly found in plants. As shown in Figure 1 (Schaich, Shahidi, Zhong, & Eskin, 2013) in the initial stage of lipid oxidation, free radicals are formed after the lipid substrate is exposed to heat, light, or metal ions. These free radicals are unstable and therefore, react with oxygen leading to the formation of a peroxy radical. This peroxy radical subtracts a hydrogen atom from another unsaturated fatty acid generating primary oxidation products. These products are also unstable and further breakdown into more

stable low-molecular-weight volatile compounds including aldehydes, ketones and alcohols (Ahmed et al., 2016). These volatile compounds are known to adversely affect the flavor quality of food products (St. Angelo, Vercellotti, Jacks, & Legendre, 1996).



**Figure 1.** Scission pathways for oleic acid. (Source: Schaich, Shahidi, Zhong, & Eskin, 2013 with slight modifications)

The majority of the aroma compounds formed from lipids arises via lipoxygenase activity. Lipoxygenase attacks cis-cis double bonded fatty acids (most commonly linoleic and linolenic acid). This enzyme triggers lipid oxidation by abstracting a hydrogen atom from fatty acids. After lipid oxidation has been initiated by this enzyme the process follows a classic auto-oxidation process leading to off-flavors. Lipoxygenase is commonly found in plant tissues. When these plant tissues are damaged due to mechanical bruising during harvesting, this enzyme is released catalyzing the insertion of oxygen into polyunsaturated fatty acids (Hsieh, 1994). Murat et al. 2013 analyzed lipoxygenase activity as a basic chemical analysis at different steps along the pea protein extraction process and they found

that lipoxygenase activity was mainly detected in pea flour. Another enzyme involved in the decomposition of lipids in legumes is lipase. Lipase hydrolyses lipids into free fatty acids, which are then more prone to oxidation (Roland et al., 2017).

Low amounts of free fatty acids have been reported in fresh peas. However, these concentrations increase during storage or freezing due to enzymatic action (Azarnia, Boye, Warkentin, Malcolmson, et al., 2011). Villalobos Solis et al., 2013 studied the fatty acid profile from the oil of field pea and reported that the major unsaturated fatty acids found were oleic acid, linoleic and linolenic acids.

Hexanal has commonly been found in peas and its origin has been associated with the oxidation of linoleic acid catalyzed by lipoxygenase (Eriksson, 1975). Hexanal has been recognized as one of the main contributors to the off-aroma in peas and has been described as having green, grassy notes (Sessa & Rackis, 1977). Similarly, (E)-2-heptenal and (E)-2-octenal and (E)-2-nonenal have been found in peas. According to Azarnia, Boye, Warkentin, Malcolmson, et al., 2011, these compounds could originate from enzymatic oxidation of linoleic acid after tissue disruption or frost damage. (E)-2-heptenal has a pungent green fatty odor, (E)-2-octenal has been described as green-leafy whereas (E)-2-nonenal has a musty, oily, cucumber odor (Azarnia, Boye, Warkentin, Malcolmson, et al., 2011) (Sun, Cadwallader, & Kim, 2010).

Some authors (Azarnia, Boye, Warkentin, & Malcolmson, 2011) (Murray, Shipton, Whitfield, Kennett, & Stanley, 1968) have reported that alcohols including 1-octen-3-ol, 1-hexanol, 1-penten-3-ol have a distinct odor characteristic that likely contribute to the flavor of peas. 1-Octen-3-ol is a dominant alcohol in peas and has a mushroom, earthy,

green aroma (Murat et al., 2013). Likewise, the presence of 1-hexanol has been reported in peas and it has been characterized as having green, grassy and beany notes (Macleod et al., 1988). 1-Octen-3-ol and 1-hexanol are both products from oxidation of linoleic acid (Sun et al., 2010). 1-Penten-3-ol originating from oxidation of linoleic or linolenic acids has a penetrating green, grassy aroma (Murray, Shipton, Whitfield, & Last, 1976) (Oomah, Liang, & Balasubramanian, 2007).

Besides the normal metabolism of legumes, there are other factors that influence the formation of flavor. These factors include, plant genetics, soil nutrition, stage of maturity and conditions of storage from harvesting until the final destination. In a study by Jakobsen, Hansen, Christensen, Brockhoff, & Olsen, 1998, significant differences in the concentration levels of aroma compounds were detected among different genotypes and pea sizes. In their study, results showed that the hexanal content in two genetic selections was higher in small peas compared to larger sizes. They suggested that this difference could have been due to the fact that small size peas are more tender and therefore, could get easily damaged in the skin tissue by mechanical treatments. Similarly, Azarnia, Boye, Warkentin, Malcolmson, et al., 2011 compared the flavor profile of selected yellow and green pea cultivars. They concluded that the concentrations of the volatile compounds were affected by cultivar and crop year and that these concentration differences could impact the taste and flavor of peas. In another study by Azarnia, Boye, Warkentin, & Malcolmson, 2011, the impact of storage conditions (4°C, 22°C and 37°C for 12 months) on volatile aroma compounds of peas was studied. The authors found that the total area of aroma compounds

was lower in peas stored at 4°C compared to higher temperatures and therefore inferred that storage at 4°C could prevent oxidative degradation of lipids in peas.

Lipids may also undergo changes during processing conditions. Azarnia, Boye, Warkentin, Malcolmson, et al., 2011, evaluated the effect of processing (dry milling, cooking and dehulling) on volatile aroma compounds in peas. An increase in the concentration levels of aldehydes after milling and dehulling was observed whereas the aldehyde levels decrease after cooking the dehulled and whole seeds. On the other hand, an increase in the ketones levels was observed after cooking the whole and dehulled seeds. In another study by Trikusuma, Paravisini, & Peterson, 2020, the impact of UHT (Ultra High Temperature) processing on the aroma profile of a pea protein beverage was evaluated. The authors of this study found that UHT processing significantly impacted the concentration of 19 aroma compounds identified in the pea protein beverage sample. Out the 19 compounds, 15 were significantly increased and most of these compounds were associated with oxidative degradation.

### **2.5.2. Maillard Reaction**

Another mechanism leading to flavor formation is the Maillard reaction and associated Strecker degradation. The Maillard reaction is a chemical reaction between carbonyls and amines. The carbonyls in foods are typically reducing sugars, whereas the amines come from either free amino acids or proteins (Reineccius, 2005). These

mechanisms consist of complex reactions and decompositions resulting in the production of a large number of volatile compounds that contribute to the flavor of foods.

Three 3-alkyl-2-methoxypyrazines have been identified as naturally occurring in green peas: 3-isopropyl-2-methoxypyrazine, 3-sec-butyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine (Murray & Whitfield, 1975). Although these compounds occur at extremely low concentrations, they are significant contributors to the perceived green pea, bell-pepper aroma in peas due to their low sensory threshold values (Roland et al., 2017). Considering the low levels at which the 3-alkyl-2-methoxypyrazines are present in peas, their precursors (Table 3) could be at similar low levels and are not easily detectable (Murray & Whitfield, 1975).

**Table 3.** Possible precursors of naturally occurring methoxypyrazines

Compound	Possible precursors
3-Isopropyl-2-methoxypyrazine	Valine + glyoxal
3-Sec-butyl-2-methoxypyrazine	Isoleucine + glyoxal
3-Isobutyl-2-methoxypyrazine	Leucine + glyoxal

Source: Taken from (Murray & Whitfield, 1975) with slight modifications

Phenylacetaldehyde has been found in green pea and commercial pea protein extract and it contributes a floral and honey note (Murat et al., 2013) (Sheibani et al., 2016). This compound is formed by the Strecker degradation of aromatic amino acids, especially phenylalanine. Likewise, 3-methylthiopropional is the result the Strecker degradation of methionine and contributes to the fatty, potato flavor in pea protein extract (Murat et al., 2013).

Flavor formation due to the Maillard reaction is influenced by several different factors including: the reactants (type of sugar and amino acid), temperature, pH, and water activity. Pentoses (ribose, arabinose or xylose) which are generally not very abundant in foods, are very reactive in the Maillard reaction whereas hexoses (glucose, or fructose) are less reactive and reducing disaccharides (maltose or lactose) react rather slowly (Arnoldi, 2004). With regards to the reactivity of amino acids, lysine, glycine, tryptophan and tyrosine are the most reactive amino acids (Provost, 2019).

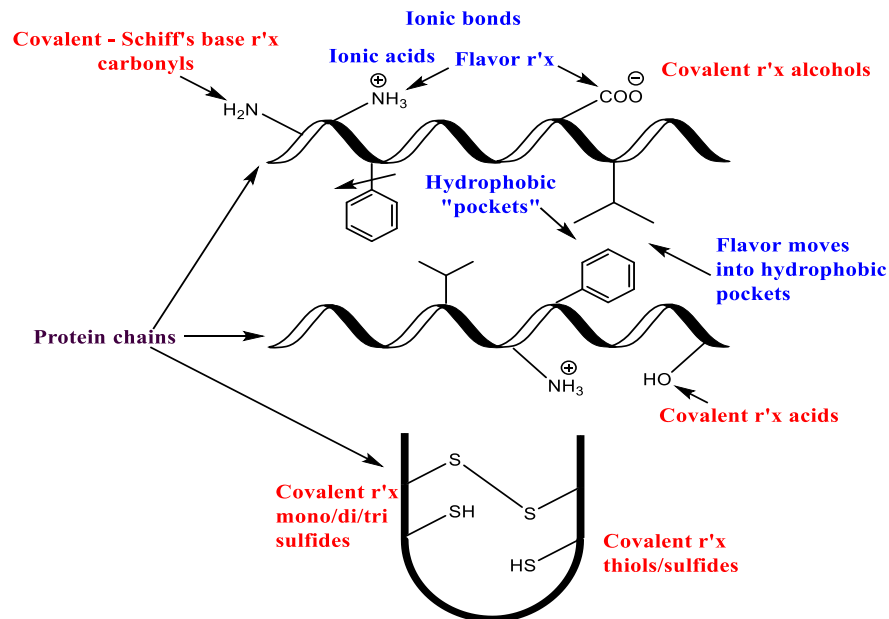
The rate of the Maillard reaction increases with temperature and as does off-flavor. However, the Maillard reaction occurs at room temperature, but at a much slower rate (Frank B Whitfield, 1992) (Nielsen, Ismail, & Sadler, 2010). In a study by Trikusuma et al., 2020, the impact of Ultra High Temperature (UHT) processing (140°C for 6s) and subsequent storage (7 weeks at 5°C) on the aroma profile of a pea protein beverage was studied. In this study, no significant changes in the concentration levels of 2-acetyl-1-pyrroline (2AP) were observed. However, these levels significantly increased during storage. The authors suggested that UHT processing could have initiated sugar degradation which resulted in accumulation of reactive carbonyl species leading to the formation of 2AP during storage. In the same study, methional, which is also a Maillard product, significantly increased after UHT processing. Methional is formed by the Strecker degradation of methionine.

pH also influences the rate of the Maillard reaction resulting in changes in the volatile compounds formed. Aaslyng et al., 1998 compared the volatile composition of acid and enzymatic hydrolysates from soy. They found that the low pH of the acid-hydrolyzed

vegetable protein did not favor pyrazine formation whereas the high pH of enzyme-hydrolyzed vegetable protein from soy lead to the formation of pyrazines. Additionally, the authors concluded that more Maillard reaction products - such as furfurals, furans and sulfur-containing volatiles – were produced in the acid-hydrolyzed vegetable protein than in enzyme-hydrolyzed vegetable protein due to the high processing temperatures during the acid hydrolysis.

## 2.6. Aroma-Protein Interactions

Food proteins have no odor on their own. However, they can bind and/or trap aroma compounds reducing their release and therefore, impacting their sensory perception (Paravisini & Guichard, 2016). Reversible weak hydrophobic, electrostatic, hydrophilic interactions as well as irreversible covalent bonds may be formed between aroma compounds and proteins (Figure 2).



**Figure 2.** Opportunities for aroma compounds to interact with proteins. Taken from (Reineccius & Anantharamkrishnan, in press)

Aroma-protein binding is influenced by several factors including amino acid composition of the protein, protein structure, types of aroma components, water activity, temperature, ionic strength, and pH.

According to Damodaran (2017) the mechanism of aroma-protein binding depends on the moisture content of the protein sample. In high-moisture foods, the volatile compounds are bound to proteins primarily through hydrophobic interactions. For instance, proteins such as soy and milk protein have been found to interact with volatile compounds primarily through hydrophobic interactions. On the other hand, in dry products, proteins are proposed to bind volatile compounds mainly via van der Waals hydrogen bonding, and electrostatic interactions.

The effect of the molecular weight of a volatile compound on protein-volatile compound interaction has also been previously studied. Long-chain volatile compounds seem to have a higher affinity for protein than short-chain aroma compounds. In a study by Heng et al., 2004, it was reported that ketones and aldehydes with a longer chain-length had higher affinities to vicilin than those compounds with shorter chain-length.

Several studies have been conducted to understand the effect of temperature on aroma-protein interactions. However, conflicting findings have been found in the literature. Processing conditions like heat treatment affect the conformational structure of proteins and therefore, the aroma - protein interactions, and the rates at which these reactions will occur. Wang & Arntfield, 2015a found that heat treatment (95°C for 30 min) enhanced the “binding” of all aldehydes studied (hexanal, heptanal, and octanal) to both salt-extracted canola protein isolate (CPI) and pea protein isolate (PPI). However, in the same study, they

reported that the retention of ketone flavors (2-hexanone, 2-heptanone and 2-octanone) by CPI and PPI was less affected compared to the aldehyde flavors under the same heat treatment conditions. While they suggested that the increased binding of aldehydes to the protein was due to the unfolding of the protein that occurs during heating leading to the exposure of previously buried hydrophobic residues that bind flavors, recent findings on covalent bonding of flavorings by Anantharamkrishnan, Hoye, & Reineccius, 2020 must also be considered. Anantharamkrishnan, Hoye, and Reineccius 2020 found that hexanal covalently reacts very rapidly with  $\beta$ -lactoglobulin even at ambient temperature whereas ketones were not reactive at ambient temperature. In contrast, Heng et al., 2004 found that heating (90°C for 30 min) decreased the binding of 2-octanone and octanal to vicilin. This decrease has been attributed to protein aggregation which promotes protein-protein interactions rather than protein-aroma interactions (Kühn, Considine, & Singh, 2008).

Likewise, studies have been carried out to evaluate the effect of pH on aroma-protein interactions. Dumont & Land, 1986 reported that decreasing pH leads to a decrease in the binding of diacetyl to pea protein. Wang & Arntfield, 2015b investigated the effect of pH on the binding properties of salt-extracted pea protein to selected mono-ketones and saturated aldehydes and they found that binding decreased in the order: pH 5 > pH 7 > pH 9 > pH 11 > pH 3. They suggested that the strong hydrophobic associations between proteins at pH 5 could have created additional flavor binding sites which increased flavor retention. Additionally, they explained that at extreme pH values (pH 3 and 11) the protein is heavily denatured or unfolded which could have caused loss of flavor binding sites and therefore, reduction in flavor retention.

Part of this apparent disagreement in results may now be explained by the work of Anantharamkrishnan and Reineccius 2020. Dumont and Land 1986 were studying the interaction of diacetyl with pea protein while Wang & Arntfield, 2015b, considered a ketone mixture (2-hexanone, 2-heptanone and 2-octanone). Diacetyl, as a diketone, reacts very rapidly with proteins via covalent bond formation whereas mono-ketones do not undergo covalent bond formation. Thus, studies considering only mono-ketones would measure hydrophobic interactions which would be strongly influenced by protein folding while studies with diacetyl (diketone) would not be influenced by protein denaturation (i.e. folding). Also, covalent bonds would not be formed at low pH explaining the low reactivity of the diacetyl at low pH as well. According to Anantharamkrishnan & Reineccius, 2020, at low pH, the intermediate step for forming covalent interactions (by the Schiff base mechanism) does not happen, as the amine groups would be protonated.

The type of salt and the amount of salt used for protein extraction can affect the conformational structure of the protein which could influence the aroma-protein interactions and therefore, sensory perception. Wang & Arntfield, 2015b studied the effects of salts on the binding of a selected ketone flavor mixture to salt-extracted pea proteins. They found that when comparing NaCl (Univalent) and CaCl<sub>2</sub> (Divalent) cations, higher concentrations of NaCl (0.25-1M) increased protein-flavor binding compared to CaCl<sub>2</sub>. However, lower concentrations (0.05-0.1M NaCl and 0.25M CaCl<sub>2</sub>) reduced flavor binding. Similar results were found by Andriot, Marin, Feron, Relkin, & Guichard, 1999. The authors reported that the binding of benzaldehyde to  $\beta$ -lactoglobulin decreased from 25 to 18% when 0.05M NaCl was added to the water. According to Wang & Arntfield,

2015b, high concentration of salt could cause exposure of nonpolar residues promoting intramolecular hydrophobic interactions. These hydrophobic interactions may enhance hydrophobic association of proteins but also hydrophobic association between protein and flavors leading to increased flavor binding. Wang & Arntfield, 2015b also reported that binding of flavors (at a concentration of 0.5M) was dependent upon the position of the anions in the lyotropic series and decreased in the order:  $\text{Na}_2\text{SO}_4 \gg \text{NaCl} > \text{NaCH}_3\text{COO} = \text{no salt} > \text{NaSCN}$ . As stated by Wang & Arntfield, 2015b, the increased flavor binding after adding high concentration of  $\text{Na}_2\text{SO}_4$  and  $\text{NaCl}$  was associated with a protein stabilizing/non-chaotropic effect which enhances hydrophobic interactions between protein and hydrophobic flavor compounds. On the other hand, protein de-structuring salts (e.g.,  $\text{NaSCN}$ ) promote protein denaturation which results in a loss of hydrophobic regions and therefore reduced flavor binding.

## **2.7. Current Approaches to Diminish Off-flavor Compounds in Pea Ingredients**

Due to the current sensory challenges of pea protein, much effort has been focused on finding strategies that reduce the off-flavors in peas and pea ingredients. While various methods have been investigated including fermentation, thermal treatment, solvent extraction, masking, etc., it is necessary to explore and develop other techniques that allow for flavor improvement but without compromising the structural conformation

and functionality of the protein. Some of the most recent methods investigated are reviewed as follows.

### **2.7.1. Fermentation**

Fermentation is one of the oldest methods used for preserving perishable foods and to improve their organoleptic properties. Fermentation is an anaerobic process where bacterial or yeast enzymes are used to break down compounds such as carbohydrates into alcohol or organic acids. Previous studies have reported the use of lactic fermentation as a potential tool for the improvement of the sensory perception of pea protein and pea ingredients. For instance, Schindler et al., 2012, evaluated the potential of lactic acid fermentation to reduce or mask-off flavors in spray-dried pea protein extracts. The authors found that fermentation changed the (analytical) aroma profile of pea protein by either decreasing the volatile content or by masking undesirable notes. It is possible that flavors generated by the fermentation resulted in masking the green/beany off-flavors. However, the impact of these changes on the protein quality and sensory perception were not considered in this study. More recently, Shi, 2020 conducted a similar research but unlike the previous study, they evaluated the impact of lactic acid fermentation on sensory perception and protein quality. The results were consistent with what it was reported by Schindler et al., 2012 and the descriptive analysis test performed supported the analytical data obtained. However, some changes in the functional properties of the protein were observed. In another study by Youssef et al., 2020, the impact of using co-cultures of lactic acid bacteria and yeast on the flavor profile of a pea protein-based product was studied. The authors concluded that while the fermentation with lactic acid

bacteria and yeast led to the degradation of some of the off-flavors, the presence of yeasts triggered the formation of esters which might have generated a masking effect on the sensory defects in peas.

### **2.7.2. Spray-Drying and Cyclodextrins**

In recent reports the potential of using solid dispersion-based spray drying as a technique to improve the off-flavor of pea protein isolate has been studied. Lan, Xu, Ohm, Chen, & Rao, 2019, investigated the influence of gum arabic and maltodextrin on the physical properties, solubility and flavor profile of pea protein isolate processed by spray-drying. The authors suggested that this technique enhanced solubility and led to a decrease in the content of two out of the three beany flavor markers (1-pentanol and 1-octen-3-ol). This decrease was attributed to: 1) The reduction of intermolecular hydrophobic interactions of peptide groups which could have resulted in the decrease of binding with hydrophobic volatile compounds and therefore their elimination during spray-drying and 2) the possible inactivation of lipoxygenase due to the high temperatures used during spray-drying. However, an increased in hexanal content was observed suggesting that lipid oxidation can occur during spray-drying.

Recently, the same research team published another work where they evaluated the impact of co-spray drying with cyclodextrin on the pea protein structures, aroma profile and functional attributes (Cui, Kimmel, Zhou, Rao, & Chen, 2020). This study reported no major changes on the protein structure and functional attributes and a

reduction in the content of off-flavors. This reduction was associated to the ability of cyclodextrin to entrap volatiles that are originally bonded to pea protein.

### **2.7.3. Solvent Extraction**

Another technique commonly used to eliminate or reduce the off-flavors in pea protein and pea ingredients is solvent extraction. Lipids are known to be involved in flavor formation via both its own degradation and/or interaction with aroma compounds. One approach that has been explored is to remove the lipids prior to protein extraction. In a study by Heng, 2005, hexane was used to remove lipids. However, they found that hexane extraction may not be sufficient to remove polar lipids and therefore chloroform/methanol was used instead. Although chloroform/methanol removed polar lipids, this solvent is not food grade and cannot be used in food ingredient production. More recently, Gohl, 2019, investigated high-pressure combined with ethanol solvent extraction and its impact on pea flour functionality and aroma profile. The authors concluded that high-pressure solvent extracted samples showed significantly lower pea flavor compared to untreated pea flour. However, the pea flavor was further decreased when no pressure was used. The use of this method was found to alter the conformational structure of the protein leading to a reduction of some of its functional properties, including foaming capacity and stability. These results are consistent with the results reported by Wang, Guldiken, Tulbek, House, & Nickerson, 2020. In this study, the efficiency of ethanol and isopropanol to remove off-flavors as well as their impact on the

functionality and quality of pea protein-enriched flour were evaluated. The authors concluded that 80% ethanol or isopropanol showed to be the most effective treatment to reduce volatile flavor compounds. However, some of the functional properties of the protein were negatively impacted with higher alcohol concentrations.

Ethanol has also been used as a co-solvent during supercritical carbon dioxide extraction (SC-CO<sub>2</sub>) mainly because of its high affinity with polar compounds allowing for extraction of both polar and non-polar aroma compounds (Vatansever & Hall, 2020) (Vatansever, Xu, Magallanes-López, Chen, & Hall, 2021). Vatansever & Hall, 2020, used a response surface methodology to find the optimal conditions for the SC-CO<sub>2</sub> + Ethanol extraction. The authors concluded that the data obtained in their study showed that this method can be used for the removal of off-flavors from pea flour. However, the impact of this technique on the structure and functional properties of the pea flour has not been investigated yet.

#### **2.7.4. Masking**

Masking agents are widely used today in different food products containing pea protein in order to suppress or cover up undesirable notes. One option is to use a congruent flavor, which means using a flavor system that complements that inherent off-note of a functional ingredient. Pea protein flavor has been described as earthy, nutty. An example of a congruent flavor would be using nut flavors such as peanut which can help complement the flavor of pea protein. Another commonly used method is flavor insertion. In this

method, instead of masking undesirable notes, these inherent notes are used as part of the flavor system. For example, pea protein has some green notes. If, for instance, a strawberry flavor is desired, instead of using a strawberry with green notes, a strawberry with cooked notes and a low level or no green notes should be used. In this way, the flavor attributes of the pea protein would be used to complement the green/fresh character needed (Mittelheuser, 2018).

As mentioned previously another undesirable flavor note present in pea protein is bitterness. The use of stronger aromas such as heavier peach and vanilla notes may help masking these bitter off-notes from pea protein (Eckert & Riker, 2007). Another common approach to reduce bitterness is to add sucrose or a sweetener. This can be explained by a phenomenon called mixture suppression, where tastes in a mixture are perceived as less intense than the individual components (Lawless & Johnson, 1987). Another method that has been effective to block bitter taste receptors is adding sodium chloride. Sodium can inhibit bitterness at the taste receptor site by interfering with the signal being transferred to the brain. Other salts commonly used for this purpose are monosodium glutamate and adenosine monophosphate (Hazen, 2003).

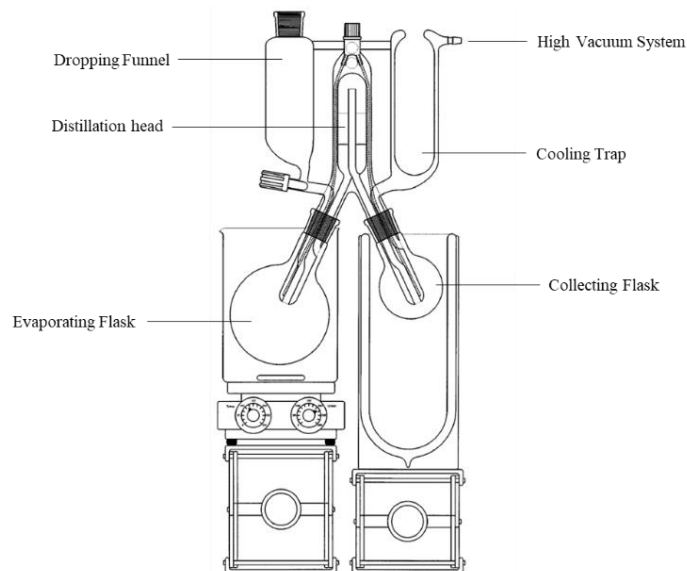
Although formulators can offer a wide variety of masking agents that help reduce, inhibit, or hide undesirable flavors in pea protein, significant challenges remain such as offering masking solutions that are natural or organic in origin which meet current consumer needs. Likewise, the addition of a large amount of flavor in order to suppress off-notes, not only is expensive but also, the flavor might reach a threshold where it becomes undesirable (Hazen, 2003).

## **2.8. Volatile Extraction Techniques for Analytical Purposes**

When developing a method to extract volatile compounds from a food product for study, it is extremely important to select, among other parameters, the appropriate extraction technique. No extraction method will accurately reflect the aromatic compounds and their proportions that are present in a food. Each method has its strengths/weaknesses. Several considerations must be taken into account when choosing a methodology, the most important is to have an analytical objective in mind (Reineccius, 2006). There are numerous methods available that can be used for volatile compound extraction, including static headspace, dynamic headspace (purge and trap), distillation (high vacuum distillation, steam distillation, thermal desorption), solvent extraction (Solvent Assisted Flavor Evaporation (SAFE)), sorptive extraction (Stir Bar Sorptive Extraction (SBSE) and Solid Phase Microextraction (SPME)). For this particular study, SAFE, SBSE and HS-SPME have been chosen.

### **2.8.1. Solvent Assisted Flavor Evaporation (SAFE)**

Of the aroma isolation methods developed to date, SAFE is considered to provide the most accurate aroma profile of a food product. SAFE employs low extraction temperatures which minimize the formation of thermally produced artifacts that are not characteristic of the sample (Majcher & Jeleń, 2009) (Elmore, 2014) (Roth et al., 2014)



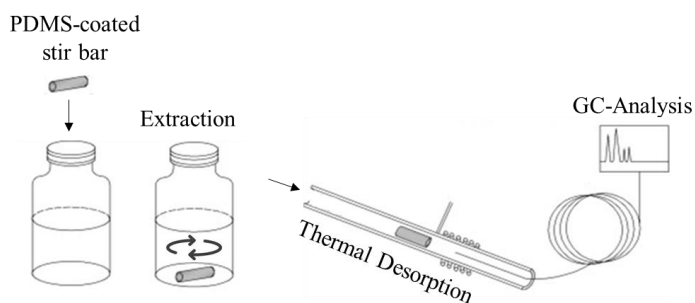
**Figure 3.** Solvent Assisted Flavor Evaporation (SAFE) apparatus (Source: Engel, Bahr, & Schieberle, 1999)

Prior to SAFE, a solvent extraction process is necessary. A food is combined with 2-3 volumes of a solvent, usually dichloromethane or diethyl ether, and that mixture is stirred for a specific time. The solvent extract is then filtered and run through the SAFE apparatus. The distillation starts by adding a small amount of sample from the dropping funnel into the evaporating flask which is under high vacuum ( $10^{-4}$  and  $10^{-5}$  mbar) and warmed to between 30-50°C. The sample evaporates in the distillation head which contains two propeller-shaped barriers in order to remove non-volatile material from the vapor. The extract condenses on the walls of the collecting flask, which is immersed in liquid nitrogen (Figure 3). The collected aroma distillate is then dried with anhydrous magnesium or sodium sulfate in order to remove the water. Finally, the aroma extract is concentrated by applying a stream of nitrogen until obtaining a final volume of around 0.1 -1 mL (Engel et al., 1999). The sample is stored in a freezer until further analysis.

Even though SAFE extraction is time-consuming, labor intensive, and requires a significant amount of starting material, it has good reproducibility (Elmore, 2014). In fact, Murat, Gourrat, Jerosch, & Cayot, 2012, analyzed the aroma profile of pea flour by using three different extraction methods: SAFE, SPME and Purge and Trap. They concluded that the SAFE extraction was the most suitable method as it has good extraction capacities and high sensory representativity of the global odor of pea flour.

### 2.8.2. Stir Bar Sorptive Extraction (SBSE)

Although SAFE provides the most accurate aroma profile among other methods, it is a time-consuming technique and it requires a large amount of sample. When a large number of samples need to be analyzed and there is limited time and amount of sample, other techniques are used such as SBSE. While not as rigorous as SAFE, it has been found that SBSE has good sensitivity and reproducibility (High et al., 2019) (Bicchi et al., 2009).



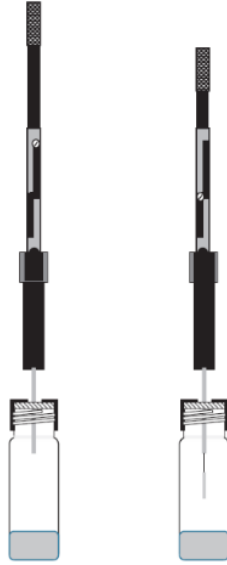
**Figure 4.** Stir Bar Sorptive Extraction (SBSE) (Source: Spietelun et al., 2013 with slight modifications )

In SBSE, the stir bar, commercially known as a Twister®, has a layer of PDMS (polydimethylsiloxane) sorbent. This layer acts as an extraction solvent rather than an

adsorbent. The stir bar is immersed in the product to be analyzed, allowed to equilibrate, then is rinsed carefully with water and finally dried with a Kimwipes®. The extracted volatile compounds are desorbed by placing the stir bar into a thermal desorption device (either a commercial piece of equipment or the GC injection port) where it is thermally desorbed into the injection port of a GC for analysis (Figure 4) (Sánchez-Rojas et al., 2009) (Bicchi et al., 2009). To the best of our knowledge this method has not been explored for the study of aroma compounds in peas and pea ingredients.

### **2.8.3. Headspace Solid -Phase Microextraction (HS-SPME)**

Similar to SBSE, this technique is based on the principle of adsorption. SPME uses an inert fiber which is coated with an adsorbent material. The fiber is initially placed in the headspace of a sample or in the sample itself if liquid and allowed to adsorb aroma compounds (Figure 5). The SPME fiber is then inserted directly into the GC injector where the compounds are thermally desorbed and analyzed. Although this technique is rapid, solvent-free and requires small amount of sample (Merkle, Kleeberg, & Fritsche, 2015), it has some disadvantages including the competition between volatiles for binding sites on the fiber which may introduce errors (Reineccius, 2005) and its low surface area which limits adsorption capacity.



**Figure 5.** Headspace Solid -Phase Microextraction (Source: Elmore, 2014)

SPME has been previously used to evaluate the impact of cultivar, crop year, processing and storage conditions on the volatile flavor compounds of field peas (Azarnia, Boye, Warkentin, & Malcolmson, 2011). Additionally, SPME was used to investigate the impact of co-spray drying pea protein solutions with cyclodextrins on their aroma profile (Cui et al., 2020) and to assess the applicability of supercritical CO<sub>2</sub> + Ethanol extraction as a method to improve the flavor of pea flour (Vatansever & Hall, 2020) (Vatansever et al., 2021).

## **Chapter 3: Development of a Protocol for the Extraction and Identification of Aroma Compounds Present in Pea Flour**

### **3.1. Overview**

The purpose of this study was to develop a protocol for the extraction and identification of the volatile aroma compounds present in pea flour. Three methods for the isolation of volatile compounds were selected: Stir Bar Sorptive Extraction (SBSE), Solvent Assisted Flavor Evaporation (SAFE) and Headspace Solid-Phase Microextraction (HS-SPME). Volatile isolates in pea flour were prepared using the noted methods and subsequently analyzed by Gas Chromatography (GC) and/or Gas Chromatography-Mass Spectrometry (GC-MS). Results showed that 3 and 4 aroma compounds with detectable odor previously reported by other authors were identified in the SBSE and SPME isolates, respectively, whereas 12 compounds were identified in the SAFE extract. SAFE provided a more complete aroma profile of pea flour than SBSE and SPME and thus SAFE was selected for the analysis of the aroma compounds present in pea flour.

## 3.2. Introduction

Peas have become a popular source of protein. Yellow field peas contain 20-30 % protein (Hall, 2018) (Wang, 2019), have a low occurrence of allergenicity and are viewed as a healthier and more environmentally friendly source of protein compared to meat and dairy products (USA Pulses, 2016). However, the inherent pea flavor that accompanies pea protein isolates remains a challenge since it hinders its application in food products. The aroma compounds present in peas have been extensively studied over the past 50 years. The major chemical families that have been identified in peas are alcohols, aldehydes, ketones and esters. Different extraction techniques and instrumental analysis have been used to identify the aroma compounds in peas. In early studies by (Ralls, McFadden, Seifert, Black, & Kilpatrick, 1965) (Whitfield & Shipton, 1966) (Murray et al., 1968) (Shipton, Whitfield, & Last, 1969), the isolation of volatile components in raw peas was done by methods such as adsorption on charcoal, solvent extraction, distillation and vacuum sublimation. Although these methods offer good recovery of volatiles with medium to high boiling points, the isolation of highly volatile aroma constituents is poor.

Methods have evolved over time and new extraction techniques have been introduced to evaluate the flavor of foods. A brief summary of the methods used to isolate volatiles from pea or pea products follows. **Static headspace** allows for a direct analysis of the equilibrium headspace above a food product. It is simple and easily automated. There are two issues with headspace methodologies: they lack sensitivity especially for compounds of low volatility and it is difficult to relate headspace

concentrations to actual concentrations in a food (Crocket et al., 2003) (Reineccius 2006). However, this technique has been previously used to study pea protein-flavor interactions and to understand the influence of saponins on these interactions (Heng et al. 2004). Likewise, static headspace has been utilized to study the interactions of carbonyl-containing flavorings with salt and alkaline-extracted pea protein isolates and to evaluate the effects of salts, pH, and heat treatment on these interactions (Wang and Arntfield 2014) (Wang & Arntfield, 2015a) (Wang & Arntfield, 2015b). **Dynamic headspace analysis** has also been commonly used in flavor research (aka Purge and Trap). In Purge and Trap (P&T), a Tenax trap (vinyl benzene-based polymer) is commonly used for capturing volatiles purged from a food sample. Although this method reduces matrix effects and increases sensitivity relative to the static headspace method (Crocket et al., 2003), it has a low surface area and therefore, a low adsorption capacity. Additionally, Tenax has low affinity for polar compounds (Reineccius, 2005). P&T has been used to evaluate the presence of 3-alkyl-2-methoxypyrazines in raw vegetables including peas (Murray & Whitfield, 1975). Similarly, in a study by Jakobsen et al. 1998, this method was used to identify the volatile compounds present in blanched green peas, to study the effect of pea size and genotype on aroma composition and, to determine important contributors to the aroma profile of peas by gas chromatography- olfactometry (GC-O). In a more recent study, P&T was used to identify the release of pea protein-bound volatile compounds from protein preparations and to study the effect of pH and degree of purification on the release of these volatile compounds (Heng, 2005). **Headspace Solid-Phase Microextraction** (HS-SPME) is a relatively new method for the extraction of volatile components. This method is simple, rapid and it does not require the use of

solvents. However, similar to other methods, SPME has some limitations, including competition of volatile components for the adsorption sites in the SPME fiber which can introduce errors (Reineccius, 2005) and low surface area which limits adsorption capacity. SPME has been used to evaluate the impact of cultivar, crop year, processing and storage conditions on the volatile flavor compounds of field peas (Azarnia, Boye, Warkentin, & Malcolmson, 2011) (Azarnia, Boye, Warkentin, Malcolmson, et al., 2011).

**Solvent Assisted Flavor Evaporation (SAFE)** has been used for flavor research of peas and pea ingredients. SAFE is a gentle isolation process as it uses low extraction temperatures, preserving the native aroma profile of the sample to be characterized (Pico, Oduber, Gómez, & Bernal, 2018). The drawbacks of SAFE are that it is a time-consuming method and it requires large amounts of sample. SAFE was compared to SPME and P&T for the evaluation of volatile compounds in pea flour (Murat et al., 2012), and it was also recently used to evaluate the impact of UHT (Ultra High Temperature) processing and subsequent storage on the aroma profile of a pea protein beverage (Trikusuma et al., 2020).

**Stir Bar Sorptive Extraction (SBSE)** is another technique for volatile isolation from food products. It is recognized for being simple, fast and requires small amount of sample amounts. To the best of our knowledge this method has not been explored for the study of aroma compounds in peas and pea ingredients. Therefore, the purpose of this study was to develop a protocol for the extraction and identification of volatile aroma compounds present in pea flour.

### **3.3. Materials and Methods**

#### **3.3.1. Samples and Chemicals**

Yellow field pea flour (PF) was supplied by AGT Foods (Regina, Canada). The flour was stored at room temperature in glass jars until analysis. Acetone (SupraSolv®) (99.8%) was obtained from Supelco (Darmstadt, Frankfurt, Germany). Anhydrous magnesium sulfate and chemical standard of methyl hexanoate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane (DCM) (GC Resolv™) (99.9%) was obtained from Fisher Scientific (Fair Lawn, NJ, USA).

#### **3.3.2. Extraction of Volatile Compounds by SBSE**

A SBSE method was performed by using a coated stir bar – commercially known as Twister® - with extraction time (30 min, 1 and 2 hrs) as a variable. SBSE was carried out in duplicate for each extraction time. A 20% PF solution in deionized (DI) water was initially prepared. 15 mL of the 20% PF solution was immediately transferred into a 20-mL glass vial. This solution was stirred with a polydimethylsiloxane (PDMS) coated stir bar (10mm length, 0.5 mm film thickness, Gerstel GmbH, Mülheim and der Ruhr, Germany) with an agitation rate of 900 rpm for 30 min, 1 and 2 hrs at room temperature. Thereafter, the Twister® was removed from the vial, quickly rinsed with DI water and dried with a clean tissue.

Two methods to desorb the aroma compounds from the PDMS coating were used: the injection port of a GC-O (Gas Chromatography-Olfactometry) and a Thermal

Desorption System (TDS) (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany) that was interfaced with a GC-MS (Gas Chromatography – Mass Spectrometry) system.

For the first desorption method, the aroma compounds were desorbed in the injection port of a GC-O at 220°C. For the second method, the temperature of the TDS was raised from 40°C to 240°C at a rate of 50°C/min, and then held for 4min. The desorbed analytes from the TDS system were cryofocused in a Cooled Injection System (CIS) (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany) by using liquid nitrogen (5°C cold trap). The trap temperature was then increased to 240°C at a rate of 10°C/sec and held for 1 min.

### **3.3.3. Extraction of Volatile Compounds by SPME**

Forty g of pea flour (PF) were combined with 200mL of DI water in a 300mL Erlenmeyer. This Erlenmeyer was closed with a cork and the mixture was stirred with an agitation rate of 350 rpm for 5min at room temperature. The Erlenmeyer was then placed in an ultrasonic water bath with a frequency of 40KHz at 45°C for 45 min. The SPME sampling was carried out by exposing the Divinylbenzene/Carboxen/Polydimethylsiloxane fiber (50/30µm film thickness and 2cm length; Supelco, Bellefonte, PA) in the headspace of the Erlenmeyer for 45 min under the same ultrasonic conditions mentioned before. The desorption of the aroma compounds was conducted by inserting the SPME fiber into the GC injection port at 220°C for 2 min.

### 3.3.4. Extraction of Volatile Compounds by SAFE

SAFE extraction was performed with different solvent systems (water, acetone-water (96:4 v/v), DCM, or DCM-Water (98:2 v/v)) and number of solvent extractions (one, two or three times) as variables. The effect of solvent type was first analyzed by keeping the number of solvent extractions constant at two as illustrated in Table 4.

One hundred g of PF and 250mL of solvent were placed in an Erlenmeyer flask. This mixture was stirred for one hr at room temperature. The solvent extracts (other than water) were filtered by using Whatman<sup>TM</sup> paper N°1. As the solids and liquid in the PF-water mixture could not be separated with Whatman<sup>TM</sup> paper, this extract was centrifuged at 1500rpm for 10 min at 20°C. For the second solvent extraction, the recovered solids from the first extraction were re-dispersed in 250mL of the corresponding solvent and then stirred for one hr at room temperature. The solids were again separated from the solvent following the procedure previously mentioned. The pooled solvent extract obtained was introduced into the SAFE apparatus. SAFE extraction was carried out at 45°C under vacuum ( $10^{-5}$  torr). Once the SAFE extracts were obtained, they were dried over anhydrous magnesium sulfate in order to remove water. Since the SAFE water extract could not be concentrated without loss of the volatiles (high boiling point), it was extracted with DCM prior to concentration. This was done in a separatory funnel (3 extractions). Finally, the ‘dried’ solvent extracts (without water residue) were concentrated to ca. 50µL by using a gentle stream of high purity nitrogen.

**Table 4.** Overview of the variables examined (type of solvents and number of solvent extractions)

Type of solvent	Number of solvent extractions
Water	2
Acetone-Water (96:4 v/v)	2
DCM	2
DCM-Water (98:2 v/v)	2
DCM	1
ibid	2
ibid	3

### 3.3.5. Analysis of Volatiles by Gas Chromatography (GC-FID)

A Hewlett-Packard 5890 Series II Gas chromatograph was used to perform GC analysis. It was equipped with a DB-WAX column (30m length x 0.25mm I.D. x 0.25um film thickness, serial #9930886, J&W Scientific). Hydrogen was used as carrier gas at a constant flow of 2.0 mL/min and a split ratio of 10:1. The oven temperature was programmed from 40°C to 85°C at a 3°C/min rate, from 85°C to 220°C at a 5°C/min rate and a final hold time of 3 min. A Flame Ionization detector was used.

### 3.3.6. Analysis of Volatiles by Gas Chromatography-Mass Spectrometry (GC-MS)

An Agilent 6890N gas chromatograph equipped with a 5973 MSD (mass selective detector) was used for GC-MS analysis. The separation of volatile compounds was performed using a fused silica capillary column DB-WAX (30m length x 0.25mm I.D x 0.25um film thickness, serial #UST510456H, Agilent Technologies, Inc). High-purity helium was used as carrier gas at a constant flow of 3.5 mL/min. Two µL of sample was injected in splitless mode. The oven temperature was programmed from 40°C to 85°C at a

rate of 3C°/min and from 85°C to 220°C at a rate of 5°C/min and a final hold time of 3 min. The injection port and transfer line temperatures were 220°C and 250°C respectively. The ionization energy was 70eV and the scan range was programmed to m/z 29 to 550.

The aroma compounds were tentatively identified using MS library matching. Relative quantification was carried out by integrating the area under the curve (AUC) for each identified aroma compound. The area of each aroma compound was then normalized using the average area of the internal standard (Methyl hexanoate).

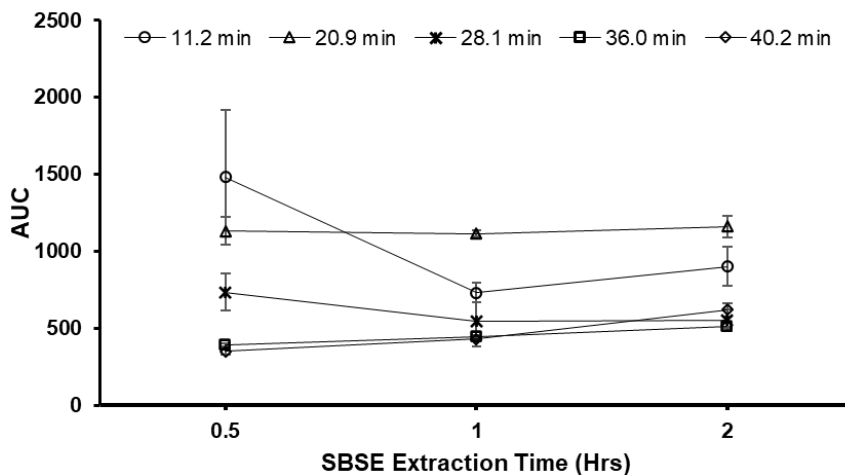
### **3.4. Results and Discussion**

#### **3.4.1. SBSE**

The impact of the extraction time (30min, 1 and 2 hrs) on the extraction efficiency of aroma compounds was studied. The extracted aroma compounds were initially analyzed by GC. Five aroma compounds present in PF eluting respectively, at 11.2, 20.9, 28.1, 36.0, 40.2 min were selected as indicators of extraction efficiency. The area under the curve (AUC) of each of these components was plotted against extraction time in Figure 6.

The results showed that for most of the compounds the extraction time did not have a significant effect on the relative amount extracted. The variation in results were generally acceptable (i.e. low) except for 0.5 hr for the compound eluting at 11.2 min. These results were obtained without using a specially designed cryo trap or thermal

desorber – this likely resulted in the variability found in the most volatile test compound (that at 11.2 retention time).



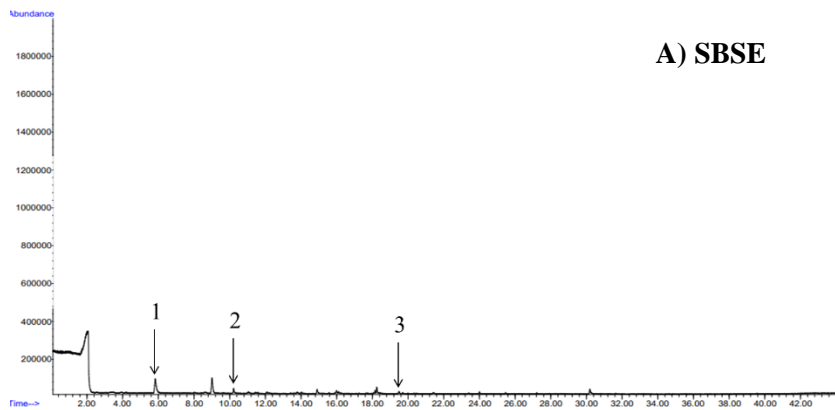
**Figure 6.** Impact of extraction time on relative amount of aroma compounds present in PF. Error bars represent the standard error of the mean (n=2)

The method was repeated using a TDS (Thermal Desorption System) equipped with a commercial CIS (Cooled Injection System) on our GC-MS instrument. The TDS-CIS system concentrates highly volatile compounds at the front of the column (by cryo focusing) which consequently reduces peak broadening, increases peak resolution and quantitative precision (Prieto et al., 2010).

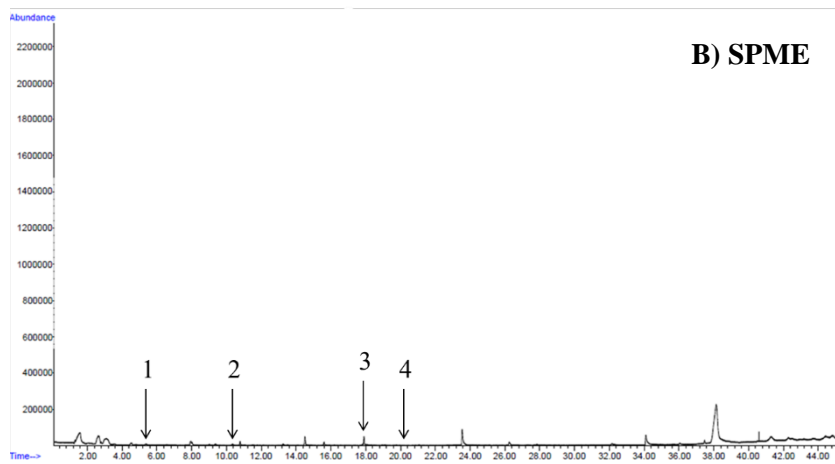
An effort was made to determine the best TDS-CIS conditions for volatile isolation. However, under the best conditions (SBSE extraction time: 2hrs, TDS-CIS method as discussed earlier in the Materials and Methods section), only 3 compounds - with detectable odor previously reported by other authors (Murat et al., 2013) were identified by GC-MS (Hexanal, 1-pentanol and 2-ethyl-1-hexanol) (Figure 7A) in the SBSE isolate. Hexanal has been described as having “green, grassy notes”, 1-pentanol

has been characterized with “grilled, dust” notes and 2-ethyl-1-hexanol with “floral” notes (Murat et al., 2013) (Trikusuma et al., 2020). A better sensitivity was expected with the TDS-CIS system. However, a direct comparison between using cry focusing and not using cry focusing cannot be made since the detectors in the GC and GC-MS are different. The flame ionization detector in the GC instrument responds to all organic compounds whereas MS measures the charged ions created as a result of fragmentation. If there is not a good fragmentation, no ions are detected. Also, the MS was operated in the full scan mode which reduces its sensitivity compare to selected ion monitoring. Therefore, this can explain the fact that the TDS-CIS coupled to our GC-MS instrument did not show a better sensitivity as one would have expected.

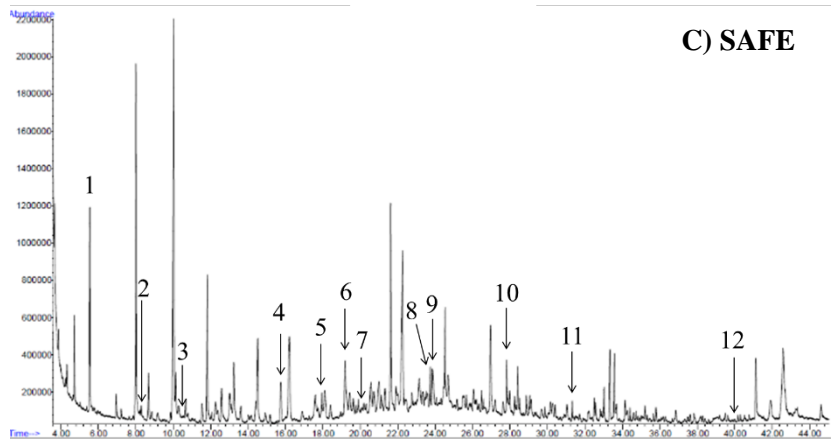
Additionally, the low SBSE extraction efficiency could be explained by two reasons: 1. SBSE can be affected by the complexity of the sample where the volatile compounds are extracted from. According to Prieto et al. 2010, the variability in ionic strength, pH, or sugar and fat contents in food may have a significant impact in the SBSE extraction efficiency (De Jager, Perfetti, & Diachenko, 2009). 2. The recovery of the polar compounds was poor. PDMS is known to be suitable for the extraction of low molecular weight volatiles and semi-volatile non-polar compounds (Li et al., 2020). Although, the majority of the aroma compounds are non-polar, a few of the compounds present in PF have high affinity with water and therefore, they might not be adsorbed by the PDMS coating (Prieto et al., 2010).



N°	Aroma compound
1	Hexanal
2	1-Pentanol
3	2-Ethyl-1-Hexanol



N°	Aroma Compound
1	Hexanal
2	1-Pentanol
3	1-Octen-3-ol
4	1-Octanol



N°	Aroma compound
1	Hexanal
2	Heptanal
3	1-Pentanol
4	Nonanal
5	1-Octen-3-ol
6	2-Ethyl-1-Hexanol
7	1-Octanol
8	1-Nonanol
9	Isovaleric acid
10	Hexanoic acid
11	2(3H)-Furanone, dihydro, 5-pentyl
12	Vanillin

**Figure 7.** Chromatograms obtained from GC-MS analysis of aroma compounds extracted by SBSE, SPME and SAFE

### **3.4.2. HS-SPME**

In total 4 aroma compounds previously reported by other authors (Murat et al. 2013) were identified in the HS-SPME extract: hexanal, 1-pentanol, 1-octen-3-ol, and 1-octanol (Figure 7B). 1-Octen-3-ol and 1-octanol have both been previously found in peas as well as in pea flour. 1-Octen-3-ol has been described as having “mushroom” notes where 1-octanol was described with “vegetable” notes (Murray et al., 1968) (Jakobsen et al., 1998) (Murat et al. 2013). In contrast to our findings, Murat et al. 2012, identified 26 compounds in a HS-SPME extract from PF. The authors found that 2-methyl-heptan-3-one and benzene derivatives were the most abundant in the extract. However, to the best of our knowledge, these compounds have not been reported in PF or pea ingredients before. Unlike our study, they used a non-polar column for the GC-MS analysis which could have allowed for the detection of some high boiling, non-polar compounds.

### **3.4.3. SAFE**

Under the best conditions, which will be explained in the following section, 12 aroma compounds - with detectable odor previously reported by other authors (Murat et al., 2013)- were identified in the SAFE extract (Figure 7C). Heptanal has been previously found in green peas and in pea protein extract and has been described as having “green, vegetable” notes (Murray et al., 1976) (Jakobsen et al., 1998) (Murat et al., 2013). The presence of nonanal has been reported in a pea protein beverage as well as in pea flour and its odor was described as “waxy, solvent and plastic”(Murat et al., 2013)(Trikusuma et al., 2020). 1-Nonanol has been previously detected in protein fractions (legumin and

vicilin) and in pea flour and has been characterized with a “pea, vegetable and silt” odor character (Murat et al., 2013) (Heng, 2005). In a previous study, isovaleric acid was found in pea flour and was described by panelists as “animal”. In the same study, hexanoic acid was also found but its odor was described “feces, meat broth and sewer notes” (Murat et al., 2013). 2(3H)-Furanone, dihydro-5-pentyl and vanillin were both found in pea flour in a study by Murat et al., 2013. 2(3H)-Furanone, dihydro-5-pentyl was described as having “sweet, coconut” notes whereas vanillin was described as “sweet, vanilla”.

#### **3.4.4. SAFE optimization**

Water, acetone-water (96:4 v/v), DCM-water (98:2 v/v) and DCM were used to extract the aroma compounds from PF for SAFE analysis. Water was chosen because it was assumed that it will make the PF more permeable facilitating volatile migration through the pea flour structure into an extracting solvent. Acetone and DCM were selected as these solvents have a high affinity for most aroma compounds.

The resulting SAFE extracts were then analyzed by GC-MS. Acetone was found not to be a suitable solvent mainly because it was not possible to remove the water for final concentration (water would be distilled over in the SAFE with the acetone) and acetone is not a “clean” solvent. It contains numerous impurities that would interfere with the GC analysis.

Table 5 shows the compounds found in the SAFE water (SAFE-E1), DCM – Water (SAFE-E2) and DCM (SAFE-E3) extracts that have been found to have a detectable aroma in previous literature (Murat et al., 2013). The results show that the same number of aroma compounds were identified in the DCM and DCM-Water extracts whereas a lower number of compounds were detected in the water extract. The hypothesis that the volatiles are more readily extracted from pea flour if there is some water present was not supported. The use of water alone was detrimental to volatile recovery - this can be explained since most of the volatile compounds are non-polar and therefore, they have a greater affinity for DCM than for water.

Even though water seems to extract some volatile compounds from PF, foaming of the water extracted sample in the SAFE system resulted in very long SAFE distillation times. Water would extract some of the proteins and saponins from PF which are both good surfactants (Chao & Aluko, 2018) (Kharkwal et al., 2012). Additionally, the presence of water in the system may increase enzymatic activity in the PF which may introduce artifacts and thereby alter the aroma profile of PF (Barbosa-Canovas et al., 2003). These preliminary observations demonstrated that water is not a suitable solvent to extract volatile compounds from PF because of excessive foam formation during SAFE distillation and, the active enzymes present in the PF may cause the development of artifacts along the process.

**Table 5.** Volatile compounds identified by GC-MS from different extraction solvents.

N°	SAFE -E1 (Water)		SAFE- E2 (DCM-Water)		SAFE- E3 (DCM)		Descriptor found in literature <sup>a</sup>
	Volatile Compound	AUC	Volatile Compound	AUC	Volatile Compound	AUC	
1	Hexanal	**	Hexanal	***	Hexanal	***	Green, grassy, fatty, leafy
2			Heptanal	*	Heptanal	*	Green, fatty, herbal
3	1-Pentanol	***	1-Pentanol	**	1-Pentanol	*	Bready, yeasty, balsamic, sweet
4			Nonanal	**	Nonanal	***	Waxy, aldehyde, citrus
5			1-Octen-3-ol	*	1-Octen-3-ol	**	Mushroom, fungal, musty
6	2-Ethyl-1-Hexanol	***	2-Ethyl -1-Hexanol	**	2-Ethyl-1-Hexanol	***	Citrus, fatty, floral, sweet
7			1-Octanol	*	1-Octanol	*	Green, waxy
8	1-Nonanol	***	1-Nonanol	***	1-Nonanol	**	Floral, waxy, fresh, citrus
9	Isovaleric acid	***	Isovaleric acid	*****	Isovaleric acid	***	Cheesy, sweaty, sour, pungent
10	Hexanoic acid	****	Hexanoic acid	***	Hexanoic acid	***	Cheesy, fatty, sweaty
11	2(3H)-Furanone, dihydro-5-pentyl	***	2(3H)-Furanone, dihydro-5-pentyl	**	2(3H)-Furanone, dihydro-5-pentyl	**	Sweet, coconut, lactic
12			Vanillin	**	Vanillin	*	Vanilla, caramel

<sup>a</sup> The Good Scents Company Information System (Luebke, 2021)

“\*” represents the area under the curve of volatile compounds < 499 999

“\*\*\*” represents the area under the curve of volatile compounds between 500 000 - 999 999

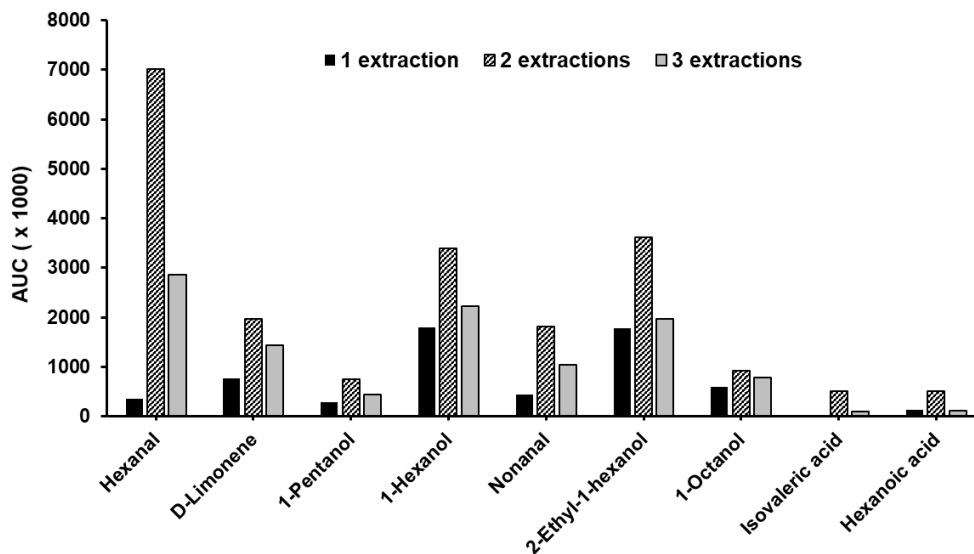
“\*\*\*\*” represents the area under the curve of volatile compounds between 1 000 000 – 4 999 999

“\*\*\*\*\*” represents the area under the curve of volatile compounds between 5 000 000- 9 999 999

“\*\*\*\*\*” represents the area under the curve of volatile compounds >10 000 000

On the other hand, 12 aroma compounds were identified in the DCM-water and DCM extracts compared to 7 compounds found in the water extract. In previous studies, DCM has shown to be effective at extracting a large number of volatile compounds representing a range of molecular classes (Prososki, Etzel, & Rankin, 2007). As DCM-water and DCM extracts did not show an improvement in terms of the number of volatile compounds extracted, DCM alone was chosen to extract aroma compounds from PF.

After optimizing the type of solvent used for the extraction of aroma compounds, the number of solvent extractions was optimized. One, two and three solvent extractions were tested. Nine aroma compounds eluting at different time points were selected as markers for obtaining quantitative data. The relative amount of these aroma compounds was calculated for each extraction protocol and plotted in Figure 8.

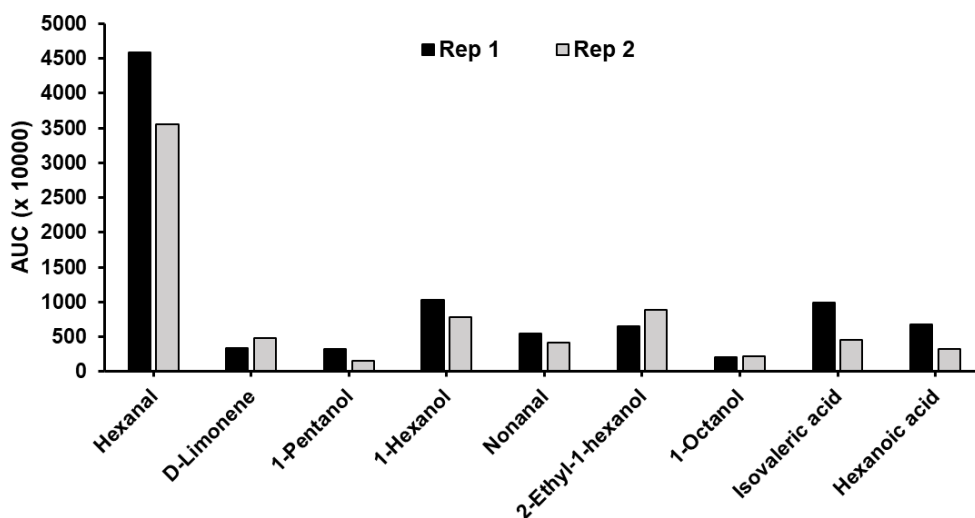


**Figure 8.** Relative amount of aroma compounds (peak areas) isolated from PF using 1, 2 and 3 solvent extractions

The relative amount of the aroma compounds extracted conducting two solvent extractions appeared to be higher than the relative amount of the compounds extracted

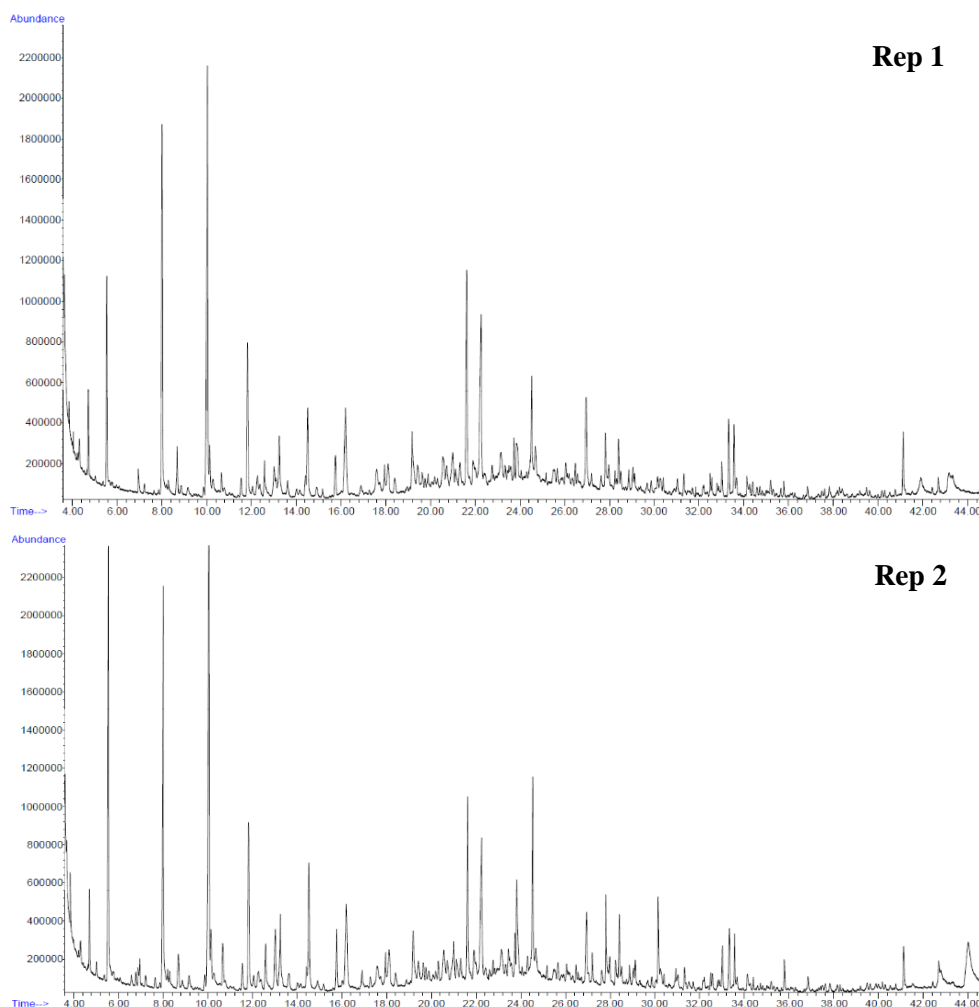
with one and three solvent extractions. This suggests that two solvent extractions may extract a higher amount of aroma compounds present in PF. It might seem surprising that the relative amount of the aroma compounds extracted using three solvent extractions was lower, but this can be explained by the fact that the amount of solvent that had to be evaporated to concentrate the sample was higher than in two and one solvent extractions. Extensive evaporation will result in greater losses of the aroma compounds. Using a more efficient method of concentration, e.g. Kuderna-Danish, might have resulted in a difference in the relative concentration of aroma compounds, but this difference would have likely been very small, especially since the sample was concentrated to a low volume (50uL).

The reproducibility of the SAFE method was evaluated by conducting two SAFE extractions following the same procedure and analyzing the samples by GC-MS. Nine aroma compounds that eluted at different retention times were selected and the area under the curve (AUC) of the peaks corresponding to each aroma compound was integrated (Figure 9).



**Figure 9.** Relative amount of aroma compounds (peak areas) isolated from PF using 2 solvent extractions. Rep 1 = Repetition 1 and Rep 2= Repetition 2.

The results indicated that although the AUC of hexanal, isovaleric acid and hexanoic acid was different between repetition 1 and 2, the AUC of the majority of the aroma compounds was very similar between repetition 1 and 2. This suggests that SAFE appears to have a good reproducibility at extracting volatile compounds from PF. Figure 10 shows the chromatograms of the SAFE extractions conducted in duplicate. Overall, it can be observed that although the areas under the peak of some of the compounds appear to be different, both chromatograms present a very similar aroma profile of PF.



**Figure 10.** Chromatograms obtained from analysis of aroma compounds isolated from PF by SAFE following the procedure in duplicate. Rep 1 = Repetition 1, Rep 2= Repetition 2

### 3.4.5. Comparison of SBSE, SPME and SAFE

When comparing the three methods, 3 compounds previously identified in PF by Murat et al. 2013, were detected using SBSE, 4 compounds using SPME and 12 compounds using SAFE. The identified compounds were classified into 4 chemical groups: alcohols, aldehydes, carboxylic acids and lactones. Alcohols and aldehydes are commonly found in raw peas and pea ingredients (Ralls et al., 1965) (Murray et al., 1976) (Heng, 2005) (Azarnia, Boye, Warkentin, Malcolmson, et al., 2011). Carboxylic acids and lactones have been reported in a few recent studies (Trikusuma, 2018) (Murat et al., 2013).

Based on the results obtained, SBSE, SPME and SAFE extracted some of the more volatile compounds. However, SAFE, unlike the other two methods, also extracted some of the larger molecular weight compounds (e.g. 2(3H)-Furanone, dihydro-5-pentyl, and vanillin). These findings are supported by Lau et al. 2018. In their study, HS-SPME and SAFE were compared for the evaluation of volatiles in tea. The authors suggested that SAFE facilitates high recoveries of semi-volatiles while HS-SPME is better at extracting highly volatile compounds. Similarly, High et al. 2019 found that when comparing SPME, SBSE and SAFE for the evaluation of volatile compounds in spray-dried sheep milk, SAFE extracted both small, polar compounds and large more non-polar compounds. One might think that large molecular weight compounds are less important for the aroma profile of PF due to their low volatility. However, in a previous work by Murat et al. 2013, several higher molecular weight compounds were detected by GC-O (Gas chromatography-Olfactometry) analysis, which suggests that these compounds can

play an important role in the perceived aroma of PF. In the present study, SAFE extracted a larger number of aroma compounds and a larger range of chemical groups compared to SBSE and SPME, and it also demonstrated good reproducibility.

Although SPME, and SBSE can be considered attractive extraction techniques because of their simplicity, and the small amount of sample that is required, these methods have some limitations. The PDMS coating of SBSE is known to extract mostly non-polar and weakly polar compounds, and therefore, this method might not be suitable to extract more polar compounds (Sánchez-Rojas et al., 2009).

In previous reports, SPME was shown to have a better extraction efficiency compared to SBSE which might be due to the Divinylbenzene/Carboxen/PDMS coating of the fibers used for SPME. This coating will adsorb more polar volatile compounds than the PDMS coating on the SBSE (Li et al., 2020). However, in some studies, SBSE has shown some advantages in comparison with SPME mainly because the larger phase volume (PDMS) on the stir bar compared to SPME fiber. The larger phase volume improves its capacity and recovery of the analytes (Castro & Ross, 2015) (Bader, 2018). On the other hand, when comparing SAFE with SBSE and SPME, the main drawback of SAFE is that it is time consuming, requires a large amount of sample and it uses substantial quantities of solvents.

SAFE has been shown to provide a more complete aroma profile of the sample than other methods of volatile isolation. Additionally, SAFE extracts can be stored frozen for a long period of time and can be used many times for GC-O analyses purposes, whereas SBSE and SPME extracts can be used only once (Murat et al., 2012).

There is a fair amount of literature dedicated to compare methods to extract aroma compounds from foods (Reineccius, 2005) (Elmore, 2015) (Marsili, 2012). The conclusions in terms of which method is the most effective extraction technique differs among studies. There is no method that will provide an accurate analytical aroma profile of a food. Each method has its strengths and weaknesses. Some authors have recommended that depending on the complexity of the food to be characterized, one should use more than one extraction method which can provide a more complete qualitative view of the aroma profile of a food (Xu, Fan, & Qian, 2007) (Sun, Zhang, & Song, 2021) (Wieczorek, Majcher, & Jelen, 2020). Nevertheless, the extra labor and input costs that this entails should also be recognized.

### **3.5. Conclusion**

In conclusion, three methods (SBSE, SPME and SAFE) were used to isolate the aroma compounds from PF. While only 3 and 4 aroma compounds – with detectable odor previously reported by other authors- were identified in the SBSE and SPME isolates respectively, 12 compounds were identified in the SAFE extract. SAFE appears to provide a much more complete aroma profile of PF than either competing method. Therefore, for the purpose of this study, SAFE was selected for the evaluation of the aroma compounds present in the samples to be analyzed in this research project.

# **Chapter 4: Monitoring Aroma Profile During the Production of a Pea Protein Isolate by Salt Solubilization Coupled with Membrane Filtration**

## **4.1. Overview**

The volatile profile was monitored during an optimized salt-extraction process (salt solubilization coupled with membrane filtration) to produce pea protein isolates (PPI). Aroma compounds from samples collected at different steps of the manufacturing process were isolated using Solvent Assisted Flavor Evaporation (SAFE) and analyzed by Gas chromatography-Mass Spectrometry-Olfactometry (GC-MS-O) and Gas Chromatography – Time-of-Flight Mass Spectrometry (GC-MS-TOF). A sensory evaluation of pea flour (PF) and PPI aqueous solutions was also conducted. Twelve aroma compounds were perceived with a “moderate” odor intensity by panelists from the sniffing port of the GC-MS-O. From the sensory evaluation, the majority of the aroma descriptors used to describe the PF and PPI testing solutions were similar as those used to describe individual compounds eluting from the sniffing port. This observation supports the hypothesis that the 12 compounds identified in this study are likely to be the main contributors to the aroma profile of the samples analyzed.

## 4.2. Introduction

In recent years, there has been an increase in demand for plant protein ingredients across the globe (Grand View Research, 2021). Among different plant protein sources, peas (*Pisum sativum L.*) have rapidly gained interest mainly because peas are not genetically modified, they fix nitrogen in the soil which reduces the need for fertilizers (Pulses, 2020), and they are suited to grow in much of North America. Additionally, peas have a low occurrence of allergenicity and are viewed as a healthier source of protein compared to meat and dairy products (USA Pulses, 2016) (FAO, 2016). Despite the perceived health and environmental benefits, pea protein ingredients possess characteristic beany, grassy and green notes, which have limited their utilization in food applications (Klein & Raidl, 1986). These off-notes are either generated by the plant itself (lipid, protein and carbohydrate metabolism), or result from harvesting, processing conditions, and/or storage (Sessa & Rackis, 1977).

Several reports on the intrinsic flavor of unblanched raw peas have been published as early as the late 1960s and 70s (Murray et al., 1968) (Shipton et al., 1969) (Murray & Whitfield, 1975) (Murray et al., 1976). More recently, due to an increased demand for plant proteins as an alternative to animal proteins, there has been further interest in studying the aroma profile of blanched peas, pea flour, pea protein isolate, legumin and vicilin preparations (major pea protein fractions), and in understanding plant protein-aroma interactions (Jakobsen et al., 1998) (Heng, 2005) (Wang and Arntfield 2015). Additional studies have focused on analyzing the impact of processing conditions on the aroma profile

of pea ingredients and pea protein based products. For instance, Trikusuma, Paravisini, and Peterson 2020, characterized the changes in the aroma profile of a pea protein beverage submitted to Ultra High Temperature (UHT) processing. They concluded that UHT processing significantly changed the volatile aroma composition and sensory profile of the pea protein beverage. They mainly attributed these changes to two reaction pathways: Lipid oxidation and the Maillard reaction. Murat et al. 2013, identified the aroma compounds present at different steps during a pH-based extraction of pea protein by Gas Chromatography-Mass Spectrometry (GC-MS) but only analyzed the first and last step (pea flour & pea protein isolate) by Gas Chromatography-Olfactometry (GC-O). Based on the GC-MS analysis and relative quantification, they indicated that the aroma profile evolved during the extraction process. As one would expect, some compounds appeared, and others disappeared at different steps of the process.

Processing conditions have an important impact not only on the flavor quality but also on protein structural and functional properties. In order to make pea protein competitive in the market with other plant protein sources, such as soy protein, it is necessary to optimize the processing conditions to obtain pea protein isolates with a clean flavor, high protein purity and good functionality. To the best of our knowledge, there are no reports focused on monitoring the aroma profile during an optimized manufacturing process to produce pea protein isolates. In preliminary work within our research group, the extraction conditions to maximize protein purity and yield following a salt extraction method were optimized (Hansen, 2020). Thus, the main objective of the present study was to monitor the aroma profile *during* this optimized salt-extraction method to produce pea protein isolates.

## **4.3. Materials and Methods**

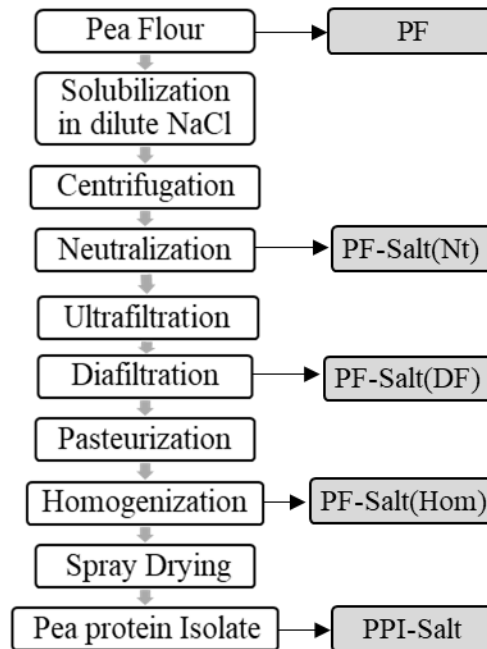
### **4.3.1. Samples and Chemicals**

Yellow field pea flour was kindly supplied by AGT Foods (Regina, Canada). The flour was stored at room temperature in closed glass jars until use. Chemical standards, of methyl hexanoate, hexanal, (Z)-4-heptenal, (Z)-6-nonenal, methional, 1-octen-3-ol, (E)-2-nonenal, 3-methylbutanoic acid (isovaleric acid), hexanoic acid, and maltol, were purchased from Sigma-Aldrich (St. Louis, MO). 2-Isobutyl-3-methoxypyrazine (IBMP) and (Z)-2-octanol were obtained from AstaTech (Bristol, PA). (E)-2-Octenoic acid was purchased from TCI America (Portland, OR). A homologous series of straight-chain alkanes (C<sub>5</sub>-C<sub>27</sub>) and anhydrous magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM) (GC Resolv<sup>TM</sup>) (99.9%) was obtained from Fisher Scientific (Fair Lawn, NJ).

### **4.3.2. Extraction of Pea Protein Isolate by Salt Extraction and Sample Collection**

Figure 11 outlines the optimized protein extraction method that was developed following a salt solubilization coupled with membrane filtration (Hansen, 2020). The process started by solubilizing the pea flour in dilute sodium chloride (0.5M NaCl). This solution was agitated in a jacketed tank equipped with a stirrer (Vektor Series, Lightnin®, Rochester, NY, USA) for one hour at room temperature (23°C). The solution was passed through a decanting centrifuge (Westfalia Separator AG, CA 220-01-30, Oelde, Germany)

and clarified with a desludging centrifuge (Westfalia Separator AG, SB 7-06-076, Oelde, Germany) in order to remove insoluble material, such as starch and fibers. The separated liquid containing protein was set aside. The insoluble material was extracted again by adding dilute NaCl and agitation in a jacketed tank with automated stirrer for 30 minutes. The solution was passed through the decanter centrifuge and desludging centrifuge. The combined liquid fractions were placed in a jacketed tank, the solution was neutralized with NaOH (6.25N) and agitated until the pH was stable at pH 7.



**Figure 11.** Samples collected along the extraction of pea protein following salt solubilization coupled with membrane filtration

The protein solution was then ultrafiltered (103- 138kPa inlet, 70-103 kPa outlet, PTI Advanced Filtration, PTI Technologies, St. Louis, MO, USA) with tangential (cross) flow and a spiral wound membrane (3 kDa MWCO) and diafiltered to further concentrate

the proteins by separating soluble fibers and small sugars. The retentate was pasteurized by passing the solution through a high temperature, short time (HTST; 73°C for 15 seconds) processing system (MicroThermics ® Electric Model 25HV Hybrid, 60-170 L/hr, MicroThermics ® Inc, Raleigh, NC, USA), followed by a two-stage homogenization (Gaulin 125 L; 17,200 kPa, 230 L/hr, Manton-Gaulin Mfg. Co. Inc., Everett, MA, USA). The homogenized retentate was then spray dried using a SPX Flow Anhydro Spray Dryer (9.5% TS, 180°C inlet, 90°C outlet, ca. 15kg water evaporation per hour) with a wheel type atomizer (24,500 rpm) (SPX Flow Inc., Charlotte, NC, USA).

Samples for flavor analysis were collected at different processing steps where we expected that the process may alter the aroma profile (Figure 11) (i.e. Pea flour (PF), after neutralization (PF-Salt(Nt)), after diafiltration (PF-Salt(DF)), after homogenization (PF-Salt(Hom)) and final product or pea protein isolate (PPI-Salt)). The samples were collected in glass jugs (3.8 L) and stored at -18°C until further analysis.

#### **4.3.3. Isolation of Volatile Aroma Compounds by Solvent-Assisted Flavor Evaporation (SAFE)**

The amount of sample used in flavor extraction at each processing step varied due to the dilution required by the process (some samples were very dilute while others were concentrated). Thus, the following sample amounts were used in extraction: 100.0g for PF, 252.7g for PF-Salt(Nt), 24.2g for PF-Salt(DF), 21.8g for PF-Salt(Hom) and 25.6g for PPI Salt (sample calculations can be found in Appendix A, Salt Extraction). For the extraction of dry samples (i.e. PF and PPI Salt), sample was weighed into a 500mL

Erlenmeyer flask containing 250mL of DCM and 100uL of methyl hexanoate (0.2mg/mL DCM) as internal standard (ISTD). The suspension was stirred with a magnetic stirrer for one hour at room temperature (23°C). The suspension was then filtered to recover the DCM fraction (with extracted volatiles).

The method was modified slightly to work with a liquid sample. For liquid samples (PF-Salt(Nt), PF-Salt(DF), PF-Salt(Hom)) DCM was added to the noted sample, the slurry was stirred for an hour, the DCM collected and the solvent collected and set aside. The extracted pea slurry was transferred back to the 500mL Erlenmeyer flask, 250mL of DCM were added again, and the solution was stirred for another hour at room temperature (23°C). The solution was filtered (or decanted if clear), and the two solvent fractions were pooled. The pooled solvent extract obtained was introduced into a SAFE apparatus. SAFE extraction was carried out at 45°C under vacuum ( $10^{-5}$  torr). Once the SAFE extract was obtained, it was dried with anhydrous magnesium sulfate in order to remove water residue. Finally, the 'dried' SAFE extract (without water residue) was concentrated to 50µL by using a gentle stream of high purity nitrogen.

#### **4.3.4. Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O)**

##### **Analysis**

An Agilent 6890N gas chromatograph-5973 MSD (mass selective detector) mass spectrometer equipped with a sniffing port was used for GC-MS-O analysis. The separation of volatile compounds was performed by using a fused silica capillary column DB-WAX (30m length x 0.25mm I.D x 0.25um film thickness, serial #UST510456H, Agilent

Technologies, Inc). High-purity helium was used as carrier gas at a constant flow of 3.5 mL/min. 2 $\mu$ L of sample was injected in splitless mode. The oven temperature was programmed from 40°C to 85°C at a rate of 3°C/min and from 85°C to 220°C at a rate of 5°C/min and a final hold time of 3 min. The eluent was divided at a 1:1 split ratio between the MS and the olfactometry port. The injection port and transfer line temperatures were 220°C and 250°C respectively. The ionization energy was 70eV and the scan range was programmed to m/z 29 to 550.

Three trained panelists were recruited and instructed to record the retention time, the sensory descriptors of the volatile aroma compounds detected through the olfactometry port, and to rate the odor intensity of each odorant using a general labeled magnitude scale (gLMS) (Appendix B) where “no sensation” is at the left end and “strongest imaginable sensation” at the right end (Green, Shaffer, & Gilmore, 1993).

The aroma compounds were tentatively identified using MS library matching and by comparison of the calculated retention index (RI) with published values. Absolute identification was performed only for the aroma compounds that were rated with an average odor intensity  $\geq 16.2$  (corresponding to the descriptor “moderate” on the gLMS) and detected by at least two panelists, as it was hypothesized that these compounds are likely to be the most significant contributors to the aroma profile of the samples. Absolute identification was conducted by comparing mass spectra, the RI of the compounds in the sample with those of the pure aroma standards, and odor descriptors with their corresponding standards and with literature.

Relative quantification was carried out by integrating the area under the curve (AUC) for each identified aroma compound. The area of each aroma compound was then normalized using the average area of the ISTD across all samples. Each aroma isolate was run in triplicate in the GC-MS-O.

#### **4.3.5. Gas Chromatography – Time-of-Flight Mass Spectrometry (GC-TOF-MS) Analysis**

In order to confirm the identity of the aroma compounds that had an average odor intensity  $\geq 16.2$  and that were found through GC-MS-O, a GC-TOF-MS analysis was carried out. The SAFE extracts obtained from each sample (PF, PF-Salt(Nt), PF-Salt(DF), PF-Salt(Hom) and PPI-Salt) were combined and concentrated to 50 $\mu$ L by using a gentle stream of high purity nitrogen. An Agilent 7890A Gas Chromatographic system (Agilent Technologies, Santa Clara, CA), coupled to Pegasus® 4D TOF-MS (LECO Corporation, St. Joseph, MI) was used. The separation of volatile compounds was performed using a fused silica capillary column DB-WAX (30m length x 0.25mm I.D x 0.25 $\mu$ m film thickness, serial #US0570343H, Agilent Technologies, Inc). High-purity hydrogen was used as carrier gas at a constant flow of 3 mL/min. 1 $\mu$ L of sample was injected in splitless mode. The oven temperature was programmed from 40°C to 85°C at a rate of 3°C/min and from 85°C to 220°C at a rate of 5°C/min and a final hold time of 3 min. The injection port and transfer line temperatures were 220°C and 250°C respectively. The ionization energy was 70eV and the scan range was programmed to m/z 29 to 400 at a scan rate of 20 scan/s. Data processing was carried out by ChromaTOF software (version 3.4).

The compounds were tentatively identified by comparison with mass spectrometric data from the NIST (National Institute of Standards and Technology) library version 2.2.

#### **4.3.6. Sensory Evaluation**

This sensory evaluation was conducted in compliance with the University of Minnesota Institutional Review Board (STUDY00011991). The samples used for the sensory evaluation were: 10% pea flour (PF) aqueous solution and 10% pea protein isolate (PPI-Salt) aqueous solution. Thirty mL of each aqueous solution were placed in a clear 120 mL sample cup with lid and were served at room temperature (28°C). The samples were assigned 3-digit codes. Eight panelists (37% men, 63% women) from the Department of Food Science and Nutrition at the University of Minnesota served as judges, all of which have experience in sensory analysis. Consent forms were provided to all the panelists prior to the sensory evaluation. The sensory evaluation of the samples occurred over one session for one hour. Participants were provided the two solutions and were instructed to smell the samples and record the odor descriptors as well as each odor descriptor's intensity for each sample. The intensity of each attribute was rated by using a general labeled magnitude scale (gLMS) (Appendix B) where 0 corresponds to "no sensation" (at the left end of the scale) and 100 corresponds to "strongest imaginable sensation" (at the right end of the scale) (Green et al., 1993).

#### **4.3.7. Statistical Analysis**

Analysis of variance (ANOVA) was performed using R Studio software version 1.4.1103 (RStudio, Inc., Boston, MA, USA). Significant differences ( $p \leq 0.05$ ) between the means ( $n=3$ ) of the samples were determined by using a Tukey-Kramer Honest Significant Difference (HSD) multiple means comparison test.

### **4.4. Results and Discussion**

#### **4.4.1. Volatile Aroma Compounds Identified by GC-MS-O During Pea Protein Extraction.**

The aroma compounds extracted from samples and identified by GC-MS-O analysis are shown in Table 6. In total, 60 volatile aroma compounds were identified in PF-Salt(Nt), 58 in PF-Salt(DF), PF-Salt(Hom) and PPI-Salt and 57 in PF. The aroma compounds extracted from the samples belonged to several different chemical classes with aldehydes being the most abundant followed by alcohols, carboxylic acids and ketones. These four chemical groups are often found in peas and have been previously reported in other studies (Murray et al., 1976) (Jakobsen et al., 1998) (Heng, 2005). Other chemical species including lactones, terpenes, furans, pyrazines, esters, sulfur compounds and pyrans were identified in the samples in smaller proportions.

**Table 6.** Volatile aroma compounds extracted by SAFE and tentatively identified by instrumental analysis collected at different steps of the pea protein extraction process (PF: Pea Flour, PF-pH(Sol): after double solubilization, PF-pH(Ovn): after pH adjusted to 3, PF-pH(Nt): after neutralization, PF-pH(DF): after diafiltration, PF-pH(Hom): after homogenization, PPI-pH: pea protein isolate)

Average RT (min)	Aroma compound	Calculated RI	Literature RI <sup>a</sup>	LogP <sub>ow</sub> <sup>b</sup>	Steps					
					PF	PF_Salt(Nt)	PF_Salt(DF)	PF_Salt(Hom)	PPI_Salt	
<i>Aldehydes</i>										
3.68	Pentanal	977	958-995	1.31	+	+	+	+	+	
5.55	Hexanal*	1077	1070-1099	1.78	+	+	+	+	+	
6.64	(E)-2-Pentenal	1119	1114-1144	1.28	+	+	+	+	+	
7.07	(Z)-3-Hexenal	1135	1127-1154	1.43	+	+	+	+	-	
8.31	Heptanal	1176	1176-1196	2.44	+	+	+	+	+	
9.29	(E)-2-Hexenal	1207	1197-1234	1.79	+	+	+	+	+	
10.13	(Z)-4-Heptenal*	1231	1227-1253	2.17	+	+	+	+	+	
11.84	Octanal	1277	1274-1301	2.95	+	+	+	+	+	
13.04	(E)-2-Heptenal	1310	1306-1339	2.30	+	+	+	+	+	
15.76	Nonanal	1384	1378-1403	3.46	+	+	+	+	+	
16.90	(E)-2-Octenal	1416	1398-1436	2.81	+	+	+	+	+	
17.27	(Z)-6-Nonenal*	1427	1425-1465	3.11	+	+	-	-	+	
17.58	Methional*	1440	1440-1471	0.44	+	+	+	+	+	
18.86	(E,E)-2,4-Heptadienal	1477	1474-1510	1.89	+	+	+	+	+	
19.65	Benzaldehyde	1502	1496-1535	1.48	+	+	+	+	+	
20.19	(E)-2-Nonenal*	1520	1518-1552	3.32	+	+	+	+	+	
23.08	(E)-2-Decenal	1631	1625-1659	3.83	-	+	+	+	-	
24.44	(E,E)-2,4-Nonadienal	1683	1670-1717	2.91	+	+	+	+	+	

25.94	(E,E)-2,4-Decadienal	1749	1706-1820	3.18	+	+	+	+	+
27.16	Tridecanal	1806	1801-1833	5.49	-	+	+	+	-
31.50	Pentadecanal	2030	2022-2050	6.51	-	-	-	+	-
33.47	Hexadecanal	2121	2111-2150	7.03	-	+	+	+	-
40.50	Vanillin	2538	2537-2581	1.58	+	+	+	+	+
<b>Alcohols</b>									
4.72	2-methyl-3-Buten-2-ol	1033	1024-1057	0.66	+	+	+	+	+
7.65	1-Penten-3-ol	1153	1142-1174	0.99	+	+	+	+	+
7.98	3-Penten-2-ol	1165	1164-1184	0.99	+	+	+	+	+
10.72	1-Pentanol	1247	1241-1263	1.51	+	+	+	+	+
13.22	(Z) 2-Penten-1-ol	1316	1301-1330	1.15	-	+	+	+	+
13.24	3-methyl-2-Buten-1-ol	1316	1299-1336	1.06	+	+	-	-	+
14.52	1-Hexanol	1350	1336-1367	2.03	+	+	+	+	+
15.60	(Z)-3-Hexen-1-ol	1381	1366-1396	1.69	-	+	-	-	-
17.05	2-Octanol*	1406	1392-1423	2.90	+	+	+	-	+
17.94	1-Octen-3-ol*	1448	1428-1458	2.52	+	+	+	+	+
18.12	1-Heptanol	1453	1439-1465	2.62	+	+	+	+	+
19.18	1-Hexanol, 2-ethyl	1486	1478-1498	2.82	+	+	+	+	+
21.11	1-Octanol	1554	1455-1591	3.00	+	+	+	+	+
23.73	1-Nonanol	1657	1641-1672	3.77	+	+	+	+	-
28.32	1-Undecanol	1856	1830-1871	4.40	+	-	-	-	-
28.42	Benzyl alcohol	1861	1837-1882	1.10	+	+	+	+	+
29.11	Phenethyl alcohol	1894	1886-1929	1.36	+	+	+	+	+
30.39	1-Dodecanol	1958	1944-1979	5.13	+	+	+	+	+

36.09	1-Pentadecanol	2268	2249-2285	6.44	-	+	+	+	+	
<b><i>Carboxylic Acids</i></b>										
22.83	Butanoic acid	1617	1605-1642	0.79	+	+	+	+	+	
23.82	Isovaleric acid*	1659	1659-1686	1.16	+	+	+	+	+	
26.05	Methyl Salicylate	1754	1743-1792	2.55	+	+	+	+	+	
25.44	Pentanoic acid	1726	1715-1752	1.39	+	+	+	+	+	
27.81	Hexanoic acid*	1834	1829-1847	1.92	+	+	+	+	+	
30.05	Heptanoic acid	1939	1926-1968	2.42	+	+	+	+	+	
32.12	Octanoic acid	2046	2042-2083	3.05	+	+	+	+	+	
34.10	Nonanoic acid	2153	2145-2190	3.42	+	+	+	+	+	
36.02	n-Decanoic acid	2263	2255-2296	4.10	+	+	-	-	+	
<b><i>Ketones</i></b>										
5.04	2,3-Pentanedione	1049	1047-1074	-0.85	+	+	+	+	+	
8.22	2-Heptanone	1173	1169-1195	1.98	+	+	+	+	+	
11.70	2-Octanone	1274	1274-1302	2.37	+	+	+	-	+	
15.60	2-Nonanone	1379	1375-1403	3.14	+	-	-	-	+	
16.17	3-Octen-2-one	1395	1394-1423	2.18	-	+	+	+	+	
27.02	2-Tridecanone	1799	1795-1822	5.05	-	-	+	+	-	
27.99	Geranyl acetone (E)	1843	1841-1872	4.13	+	-	-	+	+	
39.19	Benzophenone	2454	2423-2484	3.18	+	+	+	+	+	
<b><i>Lactones</i></b>										
24.26	Gamma-caprolactone	1678	1674-1717	0.41	+	-	-	+	+	
31.31	Gamma-nonolactone	2005	2005-2047	1.94	+	+	+	+	+	
<b><i>Terpenes</i></b>										

8.69	D-Limonene	1189	1183-1210	4.57	+	+	+	+	+
<b>Furans</b>									
9.87	2-Pentylfuran	1224	1219-1244	3.82	+	+	+	+	+
<b>Pyrazines</b>									
20.08	2-Isobutyl-3-methoxypyrazine or IBMP*	1518	1507-1534	2.55	+	+	+	+	+
<b>Esters</b>									
25.51	Undecanoic acid, ethyl ester	1731	1726-1759	5.37	+	-	+	+	+
<b>Sulfur compounds</b>									
29.85	Benzothiazole	1930	1589-1952	2.01	+	+	+	+	+
<b>Pyran</b>									
30.13	Maltol*	1949	1949-1995	0.09	+	+	+	+	+
<b>Others</b>									
34.40	(E)-2-Octenoic acid*	2176	2182	2.70	+	+	+	-	+

<sup>a</sup> Retention Index range from the National Institute of Standards and Technology Mass Spectrometry Data Center (NIST, 2018)

<sup>b</sup> LogP<sub>ow</sub> values from The Good Scents Company Information System (Luebke, 2021)

“+” Compounds detected by GC-MS in the sample

“-” Compounds not detected by GC-MS in the sample

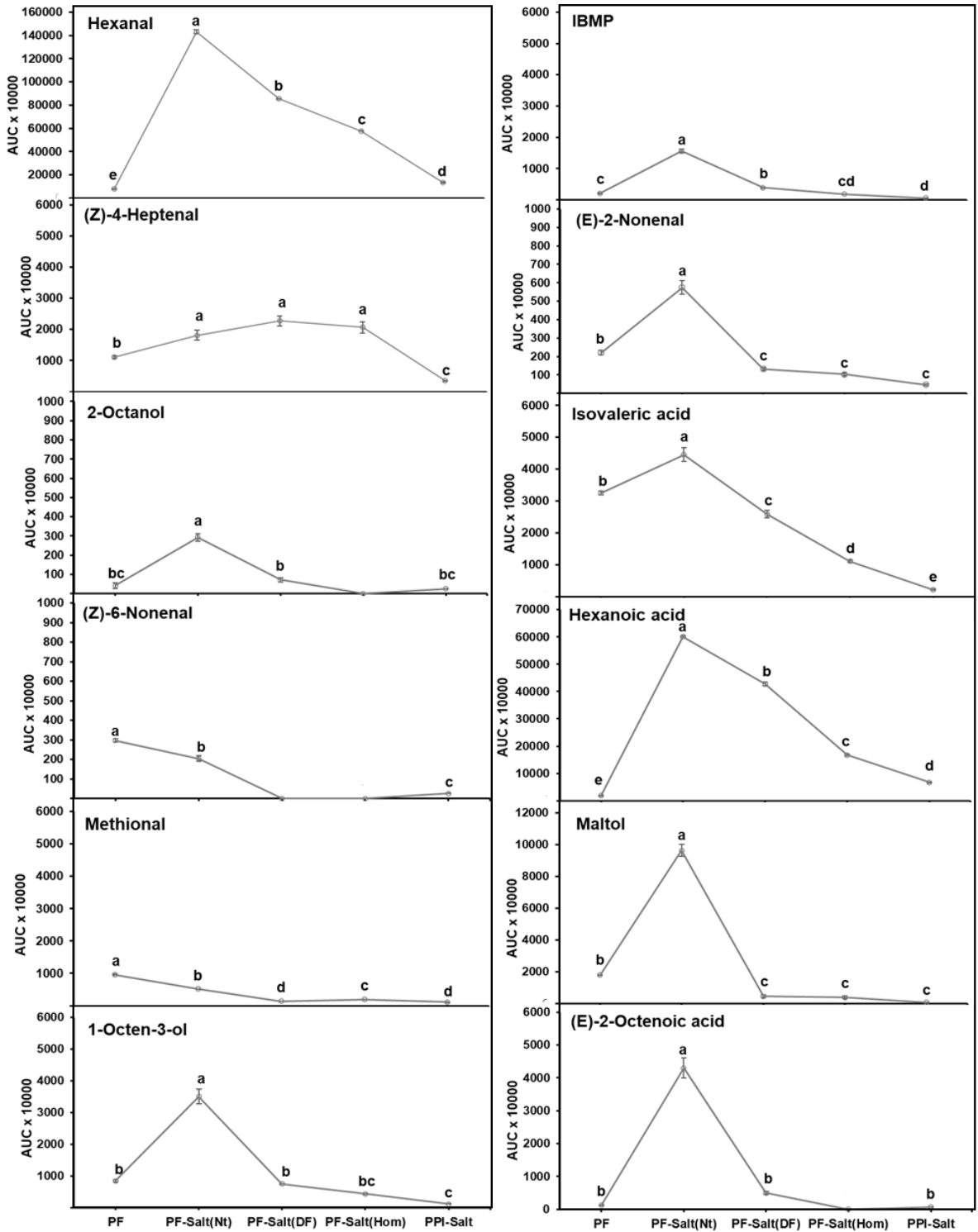
“\*” Compounds identified by: 1) Comparing mass spectra, 2) GC-TOF-MS, 3) Comparing retention indices of standards and 4) Comparing the odor descriptions with their corresponding standards and with literature

In this study, the focus was put on the aroma compounds that were rated with an average odor intensity  $\geq 16.2$  (moderate) and detected by at least two panelists in at least one of the steps of the process, as these compounds are likely to be the most significant contributors to the aroma profile of the samples. The twelve volatile compounds that met these requirements are presented in Table 7. The quantitative data on these compounds at various steps in processing are also shown in Figure 12.

**Table 7.** Aroma compounds rated with an average odor intensity  $\geq 16.2$  by at least 2 panelists in at least one of the steps of the pea protein extraction process

#	Aroma Compound	Description by panelists	Description found in literature
1	Hexanal	Green, grassy	Green, grassy, leafy
2	(Z)-4-Heptenal	Oily, fatty, fishy, oxidized oil	Oily, fatty, cream-like, fishy
3	2-Octanol	Grassy, musty, moldy, earthy	Green, woody, herbal, earthy
4	(Z)-6-Nonenal	Raw cucumber, celery, beany	Green, cucumber, vegetable
5	Methional	Raw potato, vegetable	Potato, vegetable, musty
6	1-Octen-3-ol	Mushroom, brothy	Mushroom, fungal, musty
7	2-Isobutyl-3-methoxypyrazine or IBMP	Bell pepper, earthy, soil	Green bell pepper, pea
8	(E)-2-Nonenal	Cucumber, nutty	Fatty, cucumber
9	Isovaleric acid	Cheesy, sour, pungent	Cheesy, sweaty
10	Hexanoic acid	Cheesy, pungent, rancid	Fatty, cheesy
11	Maltol	Sweet, caramel	Sweet, caramellic
12	(E)-2-octenoic acid	Musty, moldy, dirty	Musty, fatty, dirty, cheesy

From pea flour (PF) to the following step, neutralization (PF-Salt(Nt)), the concentrations of most of the aroma compounds increased (except for methional and (Z)-6-nonenal). These apparent increases in concentration are likely due to the pea flour being a solid material (structurally intact) which would limit the extraction efficiency of the volatile compounds as compared to later samples in which the pea plant structure was broken down by the extraction process.



**Figure 12.** Amount of aroma compounds present in samples (peak areas) collected at different steps of the pea protein extraction process. Error bars represent the standard error of the mean (n=3) values of three injections of the same aroma isolate in the GC-MS-O. Different lowercase letters above the bars indicate significant differences of each aroma compound across processing steps and according to the Tukey-Kramer multiple means comparison test ( $P < 0.05$ ).

Data interpretation on volatile concentrations at subsequent processing steps may be complicated by changes in sample pH, protein denaturation, and/or presence of salt in solution (Wang and Arntfield 2016). There is substantial information in the literature on how the binding of aroma compounds by plant proteins is influenced by the noted factors, thus, some of the variation in measured volatile concentration may reflect issues in volatile extraction from the protein (solution) rather than the absolute amount of aroma compounds in the sample.

We are not aware of any research which has investigated the ability of SAFE extraction to recover aroma compounds when bound to proteins. Since there is no way to correct for this potential analytical complication, we will continue the discussion of the data as obtained.

After neutralization, a two-step filtration (PF-Salt(DF) sample) was performed in order to concentrate the protein and remove low molecular weight components (salts and sugars). Along with these low molecular weight components, some of the aroma compounds appeared to also be lost except for (Z)-4-heptenal which remained constant. When sample dilution is done, compounds with significant water solubility would also be diminished.

Following the two-step filtration, the protein solution was subjected to pasteurization (for food safety reasons) and homogenization. These two processes were carried out in a closed system where one might think there is no loss of volatile compounds. However, the level of some of the aroma compounds including hexanal, 2-isobutyl-3-methoxypyrazine (IBMP), isovaleric acid and hexanoic acid, decreased as indicated in the PF-Salt(Hom) sample. The levels of methional increased, and the

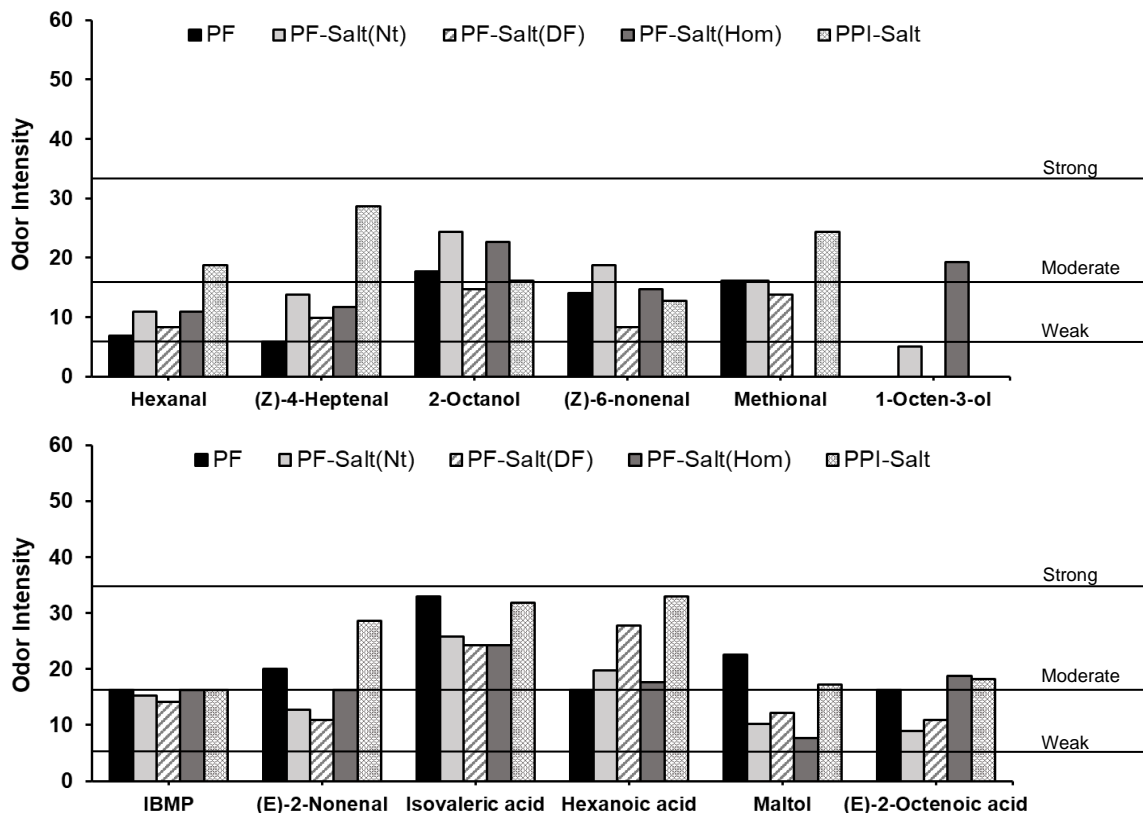
concentration of (Z)-4-heptenal, 1-octen-3-ol, (E)-2-nonenal, and maltol remained unchanged. At this stage of the process, protein is the main component that is left in the solution as most of the other components have been already removed. The last step of the process was spray drying. During this process, a decrease in the levels of some of the aroma compounds was observed. Losses may be attributed to evaporation during spray drying or enhanced binding with the proteins reducing volatile recovery.

Overall, when comparing PF (beginning material) with PPI-Salt (final PPI), it is observed that the levels of most of the aroma compounds significantly decreased. This observation is in agreement with previous reports on soy proteins (Wolf, 1970). The authors of this early study suggested that soy protein concentrates have a reduced flavor level compared to soy flour due to the removal of flavor compounds during the concentration processes.

#### **4.4.2. Odor Description and Intensity of Aroma Compounds Identified During Pea Protein Extraction Process**

Each of the samples collected at different steps along the manufacturing process of protein isolates were analyzed through the sniffing port coupled to the GC-MS system. As mentioned previously, the compounds shown in Table 7 were the main focus of this research due to their odor intensity of  $\geq 16.2$ .

Isovaleric acid, hexanoic acid and 2-octanol were detected with an average odor intensity ranging between 16.2 and 33.1 (which corresponds to “moderate” and “strong”, respectively on the gLMS) by the panelists as shown in Figure 13.



**Figure 13.** Mean of perceived odor intensity for the 12 most significant aroma contributors present in samples collected at different steps during the pea protein extraction process. Intensity ratings are from 100-point general labeled magnitude scale; a rating of 5.8 corresponded to the descriptor “weak”, a rating of 16.2 corresponded to the descriptor “moderate”, a rating of 33.1 corresponded to the descriptor “strong”.

The two carboxylic acids were detected by the panelists in all the samples and were described by the panelists as “cheesy, sour, pungent and rancid”. In a previous study, isovaleric acid was found in pea flour and was described by panelists as “animal”. In the same study, hexanoic acid was also found but its odor was described “feces, meat broth and sewer notes” (Murat et al., 2013). 2-Octanol was similarly detected by the panelists in all the samples and was described as having “grassy, musty, moldy and earthy” notes. This compound has been previously found by other researchers in frozen

green peas (Murray et al., 1976). Methional and IBMP were rated with an average odor intensity close to 16.2 (which corresponds to “moderate” on the gLMS). Methional was detected by the panelists in all the samples except in PF-Salt (Hom) and was characterized as “raw potato” notes. In previous studies, methional was detected by panelists in pea protein extract (Murat et al., 2013) and pea protein beverages (Trikusuma et al., 2020). In both studies this compound was described by the panelists as “potato” and “boiled potato”. IBMP was detected by panelists in all the samples and was described as “bell pepper, earthy and soil”. This compound has previously been found in frozen green peas, blanched green peas, pea flour and pea protein beverages (Murray et al., 1976) (Jakobsen et al., 1998) (Murat et al., 2012) (Trikusuma et al., 2020).

Aroma compounds including hexanal, 1-octen-3-ol, (E)- 2-nonenal and maltol were rated with an average odor intensity between 5.8 and 16.2 (which corresponds to “weak” and “moderate” on the gLMS scale). Hexanal was detected by the panelists in all the samples and was characterized with “green and grassy” notes. Hexanal has been the most common compound found in raw peas and pea ingredients by previous researchers. Hexanal is often described as having “fresh, grassy” notes (Ralls et al., 1965) (Murray et al., 1976) (Jakobsen et al., 1998) (Heng, 2005) (Azarnia, Boye, Warkentin, Malcolmson, et al., 2011) (Murat et al., 2013) (Trikusuma et al., 2020). 1-Octen-3-ol was detected only in two samples (PF-Salt(Nt) and PF-Salt(Hom)) and was characterized with “mushroom and brothy” notes. 1-Octen-3-ol was detected in pea flour and pea protein isolate by panelists in a previous report, and was characterized with “mushroom and vegetable notes” (Murat et al., 2013). (E)- 2-Nonenal was detected by panelists in all the samples.

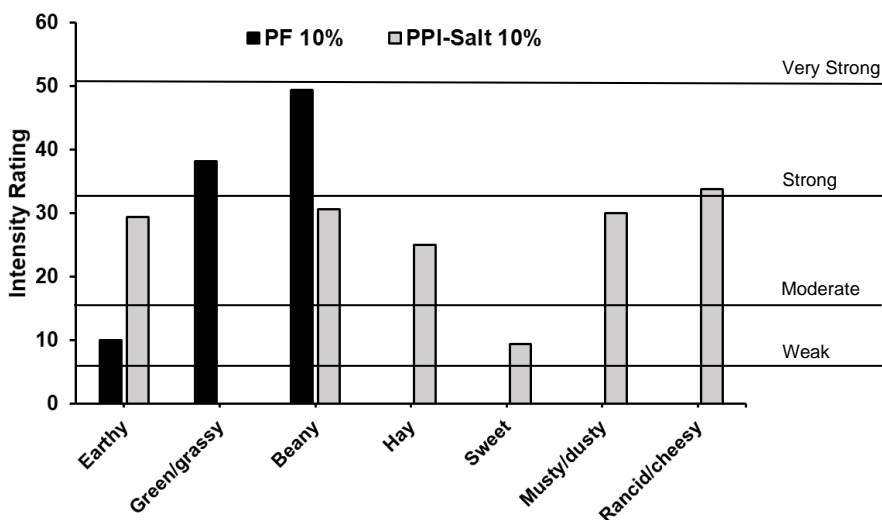
This compound was characterized as “cucumber and nutty” notes. The presence of (E)-2-nonenal in frozen green peas and pea flour has previously been reported (Murray et al., 1976) (Heng, 2005). Maltol was detected by panelists in all the samples. Panelists characterized this compound as having “sweet and caramel” notes. The presence of maltol in pea protein beverages has been previously reported (Trikusuma et al., 2020).

Other aroma compounds including (Z)-6-nonenal, (E)-2-octenoic acid, and (Z)-4-heptenal are reported for the first time in this study. These compounds were detected by all panelists in all of the samples. (Z)-6-Nonenal was described as “raw cucumber, celery and beany”, (E)-2-octenoic acid as “musty, moldy and dirty” and (Z)-4-Heptenal as “oily, fatty, fishy and oxidized oil”.

#### **4.4.3. Sensory Evaluation**

A sensory evaluation of aqueous solutions of the starting material (PF) and the final product (PPI-Salt) was conducted in order to look for relationships between the GC-MS-O data and overall perception. In the sensory evaluation, the aroma descriptors listed by at least two panelists are shown in the bar graph presented in Figure 14. Predicting a final overall sensory character of a food based on individual odors eluting on GC-MS-O analysis is highly unlikely. However, it is encouraging to see that the majority of aroma descriptors used in sensory analysis were also used to describe individual aroma compounds eluting from the sniffing port – some individual sensory notes could be linked to individual aroma compounds (Table 7). For example, the compounds responsible for the “earthy” notes in the tasting solutions were likely 2-octanol and

IBMP. The “green/grassy” aroma was likely from the presence of hexanal and 2-octanol. The “beany” odor character was due to (Z)-6-Nonenal and the “sweet” note was from maltol. The “musty/dusty” aroma could be attributed to 2-octanol and (E)-2-octenoic acid. The “rancid/cheesy” note was likely due to isovaleric and hexanoic acid.



**Figure 14.** Mean intensity ratings of PF and PPI-Salt aqueous solutions tested for aroma. Intensity ratings are from 100-point general labeled magnitude scale; a rating of 5.8 corresponded to the descriptor “weak”, a rating of 16.2 corresponded to the descriptor “moderate”, a rating of 33.1 corresponded to the descriptor “strong” and a rating of 50.1 corresponded to the descriptor “very strong”

Figure 14 shows that the hay, sweet, musty/dusty and rancid/cheesy were present in PPI-Salt but not in PF. Two possible explanations for these results are: 1) The *free* amounts of the compounds responsible for these aromas were present at low concentration in PF but increased during the salt-extraction of the protein to the point that panelists were able to detect it in PPI-salt. However, this cannot be correlated with the analytical data of this study because DCM was used for the extraction of the compounds rather than water. 2) Suppression effect: The relatively high intensity of green/grassy and

beany notes in PF could have caused a suppression effect on the other aromas (hay, sweet, musty/dusty and rancid/cheesy).

#### 4.4.4. Theoretical Pathways of Aroma Compound Formation

As shown in Table 8, most of the volatile compounds are known to originate from either enzymatic or autoxidative degradation of lipids.

**Table 8** Formation pathway of volatile aroma compounds detected during manufacturing of pea protein isolate

Aroma compound	Formation Pathway	Sources
Hexanal	Lipid oxidation	(Eriksson 1975)
(E)-2-Nonenal	Lipid oxidation	(Eriksson 1975)
1-Octen-3-ol	Lipid oxidation	(Forss, 1973) (Murray et al., 1976)
Hexanoic acid	Lipid oxidation	(Rowan, Allen, Fielder, & Hunt, 1999)
(Z)-6-Nonenal	Lipid oxidation	(Tressl, Bahri, & Engel, 1981)
2-Octanol	Lipid oxidation	(Wang, Xing, Chin, Ho, & Martin, 2001)
(Z)-4-Heptenal	Lipid oxidation	(Josephson, 1991) (Ong & Liu, 2018)
(E)-2-Octenoic acid	Lipid oxidation	(Matthews, Scanlan, & Libbey, 1971) (Kim Ha & Lindsay, 1991)
Methional	Strecker degradation	(Taylor, McDougall, & Steward, 2007)
2-Isobutyl-3-hydroxypyrazine (IBMP)	Maillard Reaction	(Murray & Whitfield, 1975) (Trikusuma et al., 2020)
Maltol	Maillard Reaction	(Pischetsrieder & Severin, 2005) (Yaylayan & Mandeville, 1994)
Isovaleric acid	Amino acid metabolism	(Bader, Czerny, Eisner, & Buettner, 2009) (Park, Choi, Kwon, & Kim, 2007)

Lipoxygenases are enzymes that occur naturally in peas (Murat et al., 2013). These enzymes catalyze the oxidation of fatty acids which, after undergoing a series of reactions, result in the formation of secondary products including aldehydes, ketones, furans, and

alcohols (Ho & Shahidi, 2020). The lipid content of field peas ranges between 1.2 and 6.3%. Linoleic acid (C18:2) the major fatty acid in pea oil (46%) followed by oleic (C18:1) and linolenic acid (C18:3) with 31% and 11%, respectively (Villalobos Solis et al., 2013). Despite the low lipid content in peas, degradation of these fatty acids during the manufacturing of pea protein isolates is likely responsible for the formation of most of the aroma compounds found in pea protein isolates.

A few other compounds including methional, IBMP and maltol have been reported to be products of the Maillard reaction and associated Strecker degradation. These compounds were detected in the samples before any thermal treatment was applied which would seem unusual. However, the Maillard reaction does take place at low temperatures, but at a much slower rate (Whitfield, 1992) (Nielsen et al., 2010).

#### **4.5. Conclusion**

In conclusion, processing treatments used during an optimized salt-extraction of pea protein led to variations in the levels of the most significant contributors to the aroma profile of the samples examined. The variations in the levels of some of the aroma compounds at various stages of the isolation process appeared to be noted by the panelists sniffing the GC-MS effluent. Additionally, the majority of the descriptors used in the sensory evaluation were similar as those used during the olfactory analysis. This finding supports our hypothesis that the 12 aroma compounds identified through instrumental analysis likely contribute to the aroma profile of the samples. None of the major odorants

were newly formed or completely lost during the protein isolation process suggesting that the processing steps do not completely remove existing or generate significant new aroma compounds. These observations suggest that the aroma compounds identified in the samples may come from the normal metabolism of the peas, are produced during the storage of the peas and/or produced during the process to obtain pea flour.

#### **4.6. Acknowledgement**

The authors would like to acknowledge Dr. Zata Vickers and the Sensory Center at the University of Minnesota for her assistance on the sensory evaluation methodology, Dr. Jean-Paul Schirlé-Keller and Dr. Vaidhyanathan Anantharamkrishnan for their technical support on the development of the SAFE methodology and GC-MS-O analysis and, Reynault Miller and Mitchell Maher, for their assistance conducting the protein extractions in the pilot plant at the Department of Food Science and Nutrition at the University of Minnesota.

## **Chapter 5: Monitoring Aroma Profile During the Production of a Pea Protein Isolate by Alkaline Solubilization coupled with Isoelectric Precipitation**

### **5.1. Overview**

The aroma profile was monitored during an optimized pH-extraction method (Alkaline solubilization coupled with isoelectric precipitation) to produce pea protein isolates (PPIs). Samples were taken at different steps throughout the protein extraction. The aroma compounds were isolated from these samples using Solvent Assisted Flavor Evaporation (SAFE) and were identified by GC-MS-O and GC-TOF-MS. A sensory evaluation of pea flour (PF) and PPI aqueous solutions was also conducted. From the instrumental analysis, 13 compounds were found to be likely the main contributors to the aroma profile of the samples examined. This hypothesis was also supported by the sensory data which showed that the PF and PPI aqueous solutions were described with some of the odor descriptors used during the instrumental analysis. No new aroma compounds appear to be produced via the optimized pH-extraction; no existing compounds were completely removed from making a sensory contribution as determined by the olfactory analysis.

## 5.2. Introduction

The interest in developing novel plant-based protein isolates has grown remarkably in recent years. This increased interest is mainly due to concerns over the welfare of animals and the environment as well as the perception among consumers that plant-based foods are a healthier source of protein when compared to animal products (Formanski, 2020). Although peas (*Pisum Sativum L.*) have been studied for years (Koyoro & Powers, 1987), (Johnson and Brekke 1983), it is not until recently that peas have been recognized as an important source of protein. Peas have a high protein content (20-30%), are not genetically modified and have low occurrence of allergenicity (USA Pulses, 2016). However, pea protein ingredients also possess strong beany, grassy and green notes, which have limited their utilization in food applications (Klein & Raidl, 1986). A few studies have reported the effect of specific processing treatments on the flavor profile of peas. Azarnia et al. 2011 analyzed the effect of dry milling, cooking and dehulling on volatile aroma compounds in peas. They found that the concentration levels of aldehydes increased after milling and dehulling and decreased after cooking the dehulled and whole seeds. Additionally, an increase in the ketones levels was observed after cooking the whole and dehulled seeds. Trikusuma, Paravisini, and Peterson 2020, characterized the changes in the aroma profile of a pea protein beverage submitted to an Ultra High Temperature (UHT) process. They concluded that UHT processing significantly changed the volatile aroma composition and sensory profile of the pea protein beverage. The flavor profile of a food may also be influenced by heat treatment, water activity, pH, salts and oxidation. These conditions often lead to the formation of

aroma compounds via the Maillard reaction, oxidation or fermentation (Reineccius, 2005).

Another factor that may be extremely important in determining aroma profile is food composition. Food components such as proteins, lipids and carbohydrates can modify aroma perception due to specific interactions with aroma compounds. Unlike lipids and carbohydrates, proteins may possess very complex structures (Paravisini & Guichard, 2016). Proteins are known to interact with aroma compounds and these interactions are influenced by intrinsic factors such as protein structure, amino acid profile and nature of the aroma. Numerous studies on the binding of aroma compounds to leguminous proteins have been reported in the literature (Macleod et al., 1988) (Wang & Arntfield, 2016). Additionally, extrinsic factors including temperature, pH and ionic strength lead to conformational changes of the proteins which can also impact the binding of aroma compounds. Wang and Arntfield 2015a, studied the effect of pH and salts on the binding properties of aroma compounds to pea protein. Similarly, Wang and Arntfield 2015b, investigated the impact of heat treatment on flavor binding of pea protein.

Soybeans have traditionally been the dominant source of protein in plant-based foods. However, soy protein has some limitations. Soy protein is one of the “Big Eight” allergens and it is sourced from GM (Genetically Modified) crops (Herman, 2003). In order to make pea protein more competitive with soy protein in the market, the functional properties of pea protein have to be improved. These properties as well as protein purity and yield may be improved by optimizing the method and conditions used during protein extraction. Maintaining the native protein structure during manufacturing may be

extremely important as this structure tends to have better functionality compared to a denatured protein. Alkaline solubilization coupled with isoelectric precipitation is the most common method for manufacturing pea protein isolates. In preliminary work within our research group, pea protein extraction conditions were optimized to maximize protein purity and yield following an alkaline solubilization coupled with isoelectric precipitation (pH-extraction) (Hansen, 2020). Currently, there is a lack of knowledge regarding the effect of the processing conditions used *during* an optimized pH-extraction on the aroma profile of pea protein isolates. Therefore, the main purpose of this study was to monitor the aroma profile during an optimized pH-extraction process to produce pea protein isolates.

## **5.3. Materials and Methods**

### **5.3.1. Samples and Chemicals**

Yellow field pea flour was kindly supplied by AGT Foods (Regina, Canada). The flour was stored at room temperature in closed glass jars until analysis. Chemical standards of methyl hexanoate, hexanal, (Z)-4-heptenal, (Z)-6-nonenal, methional, 1-octen-3-ol, (E)-2-nonenal, 3-methylbutanoic acid (isovaleric acid), hexanoic acid, and maltol were purchased from Sigma-Aldrich (St. Louis, MO). 2-Isobutyl-3-methoxypyrazine (IBMP) and (Z)-2-octanol were obtained from AstaTech (Bristol, PA). (E)-2-Octenoic acid was purchased from TCI America (Portland, OR). A homologous series of straight-chain alkanes (C<sub>5</sub>-C<sub>27</sub>) and anhydrous magnesium sulfate were purchased from Sigma-Aldrich

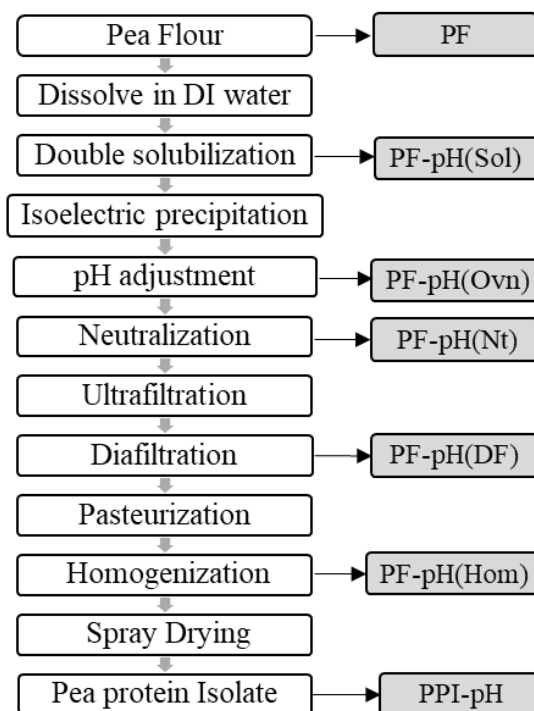
(St. Louis, MO, USA). Dichloromethane (DCM) (GC Resolv™) (99.9%) was obtained from Fisher Scientific (Fair Lawn, NJ).

### **5.3.2. Extraction of Pea Protein Isolate by pH Extraction and Sample Collection**

The optimized alkaline solubilization/isoelectric precipitation process developed for pea protein extraction (Hansen, 2020) is shown in Figure 15. The pea flour was first solubilized in DI water, adjusted to pH 7.5 with concentrated NaOH (6.25N) for one hour at room temperature (23°C), and then agitated in a jacketed bulk tank equipped with a stirrer (Vektor Series, Lightnin®, Rochester, NY, USA). The solution was passed through a decanting centrifuge (Westfalia Separator AG, CA 220-01-30, Oelde, Germany) and clarified with a desludging centrifuge (Westfalia Separator AG, SB 7-06-076, Oelde, Germany) to remove insoluble material, such as starch and fibers. The separated liquid containing protein was set aside. The insoluble material was re-suspended in DI water and the solution was adjusted to pH 7.5 with NaOH (6.25N). The solution was again agitated in a jacketed tank for one hour and passed through a decanting centrifuge and desludging centrifuge. The combined liquid fractions were placed in a jacketed tank, the pH was adjusted to pH 4.5 with HCl (4N) and agitated for 10 min. The solution was again passed through a decanting centrifuge and the precipitate (protein solution) was collected. The precipitate was then transferred to a jacketed tank with a mixer and re-solubilized in DI water. Due to the duration of the extraction process (two days), the sample had to be held overnight. The pH was adjusted to pH 3 with HCl (4N) and held in a cold room (6-8°C) to insure no microbial growth and prevent protein denaturation.

The next day, the protein solution was neutralized (pH 7) and agitated for one hour at room temperature (23°C). The protein solution was then ultrafiltered (103- 138kPa inlet, 70-103 kPa outlet, PTI Advanced Filtration, PTI Technologies, St. Louis, MO, USA) with tangential (cross) flow and a spiral wound membrane (3 kDa MWCO) and diafiltered in order to further concentrate the proteins. The high solids retentate was pasteurized by passing the solution through a high temperature, short time (HTST; 73°C for 15 seconds) processing system (MicroThermics ® Electric Model 25HV Hybrid, 60-170 L/hr, MicroThermics ® Inc, Raleigh, NC, USA), followed by a two-stage homogenization (Gaulin 125 L; 17,200 kPa, 230 L/hr, Manton-Gaulin Mfg. Co. Inc., Everett, MA, USA). The homogenized retentate was then spray dried using a SPX Flow Anhydro Spray Dryer (9.5% TS, 180°C inlet, 90°C outlet, ca. 15Kg water evaporation per hour) with a wheel type atomizer (24,500 rpm) (SPX Flow Inc., Charlotte, NC, USA).

Samples for flavor analysis were collected at different processing steps where we expected that the process may alter the aroma profile (Figure 15) (i.e. Pea flour (PF), after double solubilization (PF-pH(Sol)), after pH was adjusted to 3 and stored overnight (Ovn) (PF-pH(Ovn)), after neutralization (PF-pH(Nt)), after diafiltration (PF-pH(DF)), after homogenization (PF-pH(Hom)) and final product or pea protein isolate (PPI-pH)). The samples were collected in glass jugs (3.8L) and stored at -18°C until further analysis.



**Figure 15.** Optimized pea protein extraction by following alkaline solubilization with isoelectric precipitation. Sampling points are listed in the second column.

### 5.3.3. Isolation of Volatile Aroma Compounds by Solvent-Assisted Flavor Evaporation (SAFE)

Volatiles were extracted by SAFE following the protocol and parameters described earlier in Chapter 4 without modifications. Briefly, the amounts of sample used in flavor extraction were: 100.0g for PF, 59.8g for PF-pH(Sol), 281.3g for PF-pH(Ovn), 20.1g for PF-pH(Nt), 23.8g for PF-pH(DF), 22.0g for PF-pH(Hom) and 22.2g for PPI-pH (Sample calculations can be found in Appendix A, pH Extraction). For the extraction of dry samples (i.e. PF and PPI Salt), the sample and 250mL of DCM were transferred into an Erlenmeyer flask. 100uL of methyl hexanoate solution (0.2mg/mL DCM) was added as an internal standard (ISTD). The suspension was stirred for one hour at room temperature (23°C) and

was then filtered to recover the DCM fraction (with extracted volatiles). The method was modified slightly to work with a liquid sample. For liquid samples (PF-pH(sol), PF-pH(Ovn), PF-pH(Nt), PF-pH(DF), PF-pH(Hom)), DCM was added to the noted sample, the slurry was stirred for an hour, the DCM collected and the solvent collected and set aside. The extracted pea slurry underwent a second solvent extraction following the same process as in the first extraction. The pooled solvent fractions obtained were introduced into a SAFE apparatus. SAFE extraction was carried out at 45°C under vacuum ( $10^{-5}$  torr). The SAFE extract was then concentrated to 50 $\mu$ L by using a gentle stream of high purity nitrogen.

#### **5.3.4. Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O) Analysis**

The GC-MS-O analysis was conducted following the procedure and parameters previously described in Chapter 4. Briefly, an Agilent 6890N gas chromatograph-5973 MSD (mass selective detector) mass spectrometer equipped with a sniffing port was used for GC-MS-O analysis.

Three trained panelists were recruited and instructed to record the retention time, the sensory descriptors of the volatile aroma compounds detected through the olfactometry port, and to rate the odor intensity of each odorant using a general labeled magnitude scale (gLMS) (Appendix B) where “no sensation” is at the left end and “strongest imaginable sensation” at the right end (Green et al., 1993).

The aroma compounds were tentatively identified using MS library matching and by comparison of the calculated retention index (RI) with published values. Absolute identification was performed only for the aroma compounds that were rated with an average odor intensity  $\geq 16.2$  (corresponding to the descriptor “moderate” on the gLMS) and detected by at least two panelists, as it was hypothesized that these compounds are likely to be the most significant contributors to the aroma profile of the samples. Absolute identification was conducted by comparing mass spectra, the RI of the compounds in the sample with those of the pure aroma standards, and odor descriptors with their corresponding standards and with literature.

Relative quantification was carried out by integrating the area under the curve (AUC) for each identified aroma compound. The area of each aroma compound was then normalized using the average area of the ISTD across all samples. Each aroma isolate was run in triplicate in the GC-MS-O.

### **5.3.5. Gas Chromatography – Time-of-Flight Mass Spectrometry (GC-TOF-MS) Analysis**

In order to confirm the identity of the aroma compounds that had an average odor intensity  $\geq 16.2$  and detected by GC-MS-O, a GC-TOF-MS analysis was also carried out. The GC-TOF-MS analysis was conducted following the procedure and parameters previously described in Chapter 4. The SAFE extracts obtained from each sample PF, PF-pH(Sol), PF-pH(Ovn), PF-pH(Nt), PF-pH(DF), PF-pH(Hom), PPI-pH were combined and concentrated to 50 $\mu$ L by using a gentle stream of high purity nitrogen. An Agilent 7890A Gas Chromatography system (Agilent Technologies, Santa Clara, CA), coupled to

Pegasus® 4D TOF-MS (LECO Corporation, St. Joseph, MI) was used. The compounds were tentatively identified by comparison with mass spectrometric data from the NIST (National Institute of Standards and Technology) library version 2.2.

### **5.3.6. Sensory Evaluation**

This sensory evaluation was conducted in compliance with the University of Minnesota Institutional Review Board (STUDY00011991). The samples used for the sensory evaluation were: 10% Pea flour aqueous solution, and 10% pea protein isolate (PPI-pH) aqueous solution. Thirty mL of each aqueous solution were placed in a clear 120 mL sample cup with lid and were served at room temperature (28°C). The samples were assigned 3-digit codes. Eight panelists (37% men, 63% women) from the Department of Food Science and Nutrition at the University of Minnesota served as judges, all of which have experience in sensory analysis. Consent forms were provided to all the panelists prior to the sensory evaluation. The sensory evaluation of the samples occurred over one session for one hour. Participants were provided the two solutions and were instructed to smell the samples and record the odor descriptors as well as each odor descriptor's intensity for each sample. The intensity of each attribute was rated by using a general labeled magnitude scale (gLMS) (Appendix B) where 0 corresponds to "no sensation" (at the left end of the scale) and 100 corresponds to "strongest imaginable sensation" (at the right end of the scale) (Green et al., 1993).

### **5.3.7. Statistical Analysis**

Analysis of variance (ANOVA) was performed using RStudio software version 1.4.1103 (RStudio, Inc., Boston, MA, USA). Significant differences ( $p \leq 0.05$ ) between the means ( $n=3$ ) of the samples were determined by using a Tukey-Kramer Honest Significant Difference (HSD) multiple means comparison test.

## **5.4. Results and Discussion**

### **5.4.1. Volatile Aroma Compounds Identified by GC-MS-O During the Pea Protein Extraction**

The aroma compounds detected by GC-MS-O in the samples collected through the extraction process are shown in Table 9. The most represented chemical families in all the samples were aldehydes, alcohols, carboxylic acids, and ketones whereas only a few lactones, terpenes, furans, pyrazines, esters, sulfur compounds and pyrans were detected. Fifty nine compounds were detected in PF-pH(Ovn), 58 in PF and PPI-pH, 54 in PF-pH(DF), 52 in PF-pH(Nt) and PF-pH(Hom) and 50 in PF-pH(Sol).

**Table 9.** Volatile aroma compounds extracted by SAFE and tentatively identified by instrumental analysis in samples collected at different points during the production of pea protein. PF: Pea Flour, PF-pH(Sol): after double solubilization, PF-pH(Ovn): after pH adjusted to 3, PF-pH(Nt): after neutralization, PF-pH(DF): after diafiltration, PF-pH(Hom): after homogenization, PPI-pH: pea protein isolate.

Average RT (min)	Aroma compound	Calculated RI	Literature RI <sup>a</sup>	LogP <sub>ow</sub> <sup>b</sup>	Steps							
					PF	PF-pH (Sol)	PF-pH (Ovn)	PF-pH (Nt)	PF-pH (DF)	PF-pH (Hom)	PPI-pH	
<i>Aldehydes</i>												
3.70	Pentanal	978	958-995	1.31	+	+	+	+	+	+	+	
5.63	Hexanal	1079	1070-1099	1.78	+	+	+	+	+	+	+	
6.62	(E)-2-Pentenal	1119	1114-1144	1.28	+	-	+	+	+	+	-	
7.05	(Z)-3-Hexenal	1135	1127-1154	1.43	+	+	+	+	+	+	-	
8.29	Heptanal	1177	1176-1196	2.44	+	+	+	+	+	+	+	
9.27	(E)-2-Hexenal	1208	1197-1234	1.79	+	-	+	+	+	+	+	
10.13	(Z)-4-Heptenal*	1231	1227-1253	2.17	+	+	+	-	-	-	+	
11.83	Octanal	1279	1274-1301	2.95	+	+	+	+	+	+	+	
12.99	(E)-2-Heptenal	1310	1306-1339	2.30	+	+	+	+	+	+	+	
15.76	Nonanal	1384	1378-1403	3.46	+	+	+	+	+	+	+	
16.89	(E)-2-Octenal	1416	1398-1436	2.81	+	+	+	+	+	+	+	
17.27	(Z)-6-Nonenal*	1428	1425-1465	3.11	+	+	+	-	+	+	+	
17.70	Methional*	1441	1440-1471	0.44	+	+	+	+	+	+	+	
18.87	(E,E)-2,4-Heptadienal	1477	1474-1510	1.89	+	+	+	+	+	+	+	
19.65	Benzaldehyde	1502	1496-1535	1.48	+	+	+	+	+	+	+	
20.19	(E)-2-Nonenal*	1521	1518-1552	3.32	+	+	+	+	+	+	+	
24.42	(E, E)-2,4-Nonadienal	1683	1670-1717	2.91	+	+	+	+	+	+	+	

25.94	(E,E)-2,4-Decadienal	1749	1706-1820	3.18	+	-	+	-	-	-	+
27.12	Tridecanal	1805	1801-1833	5.49	-	-	-	+	+	+	-
33.46	Hexadecanal	2121	2111-2150	7.03	-	-	-	-	+	+	-
40.50	Vanillin	2539	2537-2581	1.58	+	+	+	+	+	+	+
<i>Alcohols</i>											
4.71	2 methyl, 3-Buten-2-ol	1034	1024-1057	0.66	+	-	+	+	-	+	+
7.65	1-Penten-3-ol	1154	1142-1174	0.99	+	+	+	+	+	+	+
8.01	3-Penten-2-ol	1166	1164-1184	0.99	+	+	+	+	+	+	+
10.70	1-Pentanol	1247	1241-1263	1.51	+	+	+	+	+	+	+
13.20	(Z)-2-Penten-1-ol	1316	1301-1330	1.15	-	+	+	+	+	+	+
13.22	3-Methyl-2-Buten-1-ol	1317	1299-1336	1.06	+	+	-	+	+	-	+
14.50	1-Hexanol	1350	1336-1367	2.03	+	+	+	+	+	+	+
15.57	(Z)-3-Hexen-1-ol	1381	1366-1396	1.69	-	+	+	+	-	-	-
17.13	2-Octanol*	1423	1392-1423	2.90	+	-	+	-	-	-	+
17.94	1-Octen-3-ol*	1449	1428-1458	2.52	+	+	+	+	+	+	+
18.11	1-Heptanol	1454	1439-1465	2.62	+	+	+	+	+	+	+
19.18	1-Hexanol, 2-ethyl	1487	1478-1498	2.82	+	+	+	+	+	+	+
21.11	1-Octanol	1554	1455-1591	3.00	+	+	+	+	+	+	+
23.70	1-Nonanol	1656	1641-1672	3.77	+	+	-	+	+	+	-
28.28	1-Undecanol	1857	1830-1871	4.40	+	+	-	+	+	+	+
28.40	Benzyl alcohol	1861	1837-1882	1.10	+	+	+	+	+	+	+
29.10	Phenethyl alcohol	1893	1886-1929	1.36	+	+	+	-	+	-	+
30.36	1-Dodecanol	1958	1944-1979	5.13	+	+	+	+	+	+	+

36.07	1-Pentadecanol	2265	2249-2285	6.44	-	-	-	-	-	-	+
<b><i>Carboxylic acids</i></b>											
22.81	Butanoic acid*	1618	1605-1642	0.79	+	-	+	+	+	+	+
23.80	Isovaleric acid*	1658	1659-1686	1.16	+	+	+	+	+	+	+
25.43	Pentanoic acid	1727	1715-1752	2.55	+	-	+	+	-	-	+
26.05	Methyl Salicylate	1754	1743-1792	1.39	+	+	+	+	+	+	+
27.82	Hexanoic acid*	1834	1829-1847	1.92	+	+	+	+	+	+	+
30.03	Heptanoic acid	1939	1926-1968	2.42	+	-	+	+	-	-	+
30.06	(E)-3-Hexenoic acid	1941	1918-1956	1.34	-	-	+	-	-	-	-
32.11	Octanoic acid	2050	2042-2083	3.05	+	+	+	+	+	+	+
34.10	Nonanoic acid	2161	2145-2190	3.42	+	+	+	+	+	+	+
35.99	n-Decanoic acid	2263	2255-2296	4.10	+	+	+	+	-	+	+
<b><i>Ketones</i></b>											
5.02	2,3 Pentanedione	1049	1047-1074	-0.85	+	+	+	+	+	+	+
8.22	2-Heptanone	1174	1169-1195	1.98	+	+	+	+	+	+	+
11.71	2-Octanone	1275	1274-1302	2.37	+	+	+	-	+	-	+
15.60	2-Nonanone	1380	1375-1403	3.14	+	-	-	-	-	-	+
16.19	3-Octen-2-one	1395	1394-1423	2.18	-	-	+	-	+	-	+
26.98	2-Tridecanone	1798	1795-1822	5.05	-	+	-	+	+	+	-
27.99	(E)-Geranyl acetone	1842	1841-1872	4.13	+	-	+	-	+	+	+
33.66	Hexahydrofarnesyl acetone	2128	2116-2137	7.13	-	-	+	-	-	-	-
39.19	Benzophenone	2451	2423-2484	3.18	+	+	+	+	+	+	+
<b><i>Lactones</i></b>											

24.30	Gamma-caprolactone	1678	1674-1717	0.41	+	+	+	+	+	+	+
31.33	Gamma-Nonalactone	2005	2005-2047	1.94	+	+	+	+	+	+	+
<b>Terpenes</b>											
8.68	D-Limonene	1189	1183-1210	4.57	+	+	+	+	+	+	+
<b>Furans</b>											
9.90	2-Pentylfuran	1225	1219-1244	3.82	+	+	+	+	+	+	+
<b>Pyrazines</b>											
20.07	2-Isobutyl-3-methoxypyrazine (IBMP)*	1517	1507-1534	2.55	+	+	+	+	+	+	+
<b>Esters</b>											
25.54	Undecanoic acid, ethyl ester	1732	1726-1759	5.37	+	+	-	+	+	+	+
<b>Sulfur compounds</b>											
29.85	Benzothiazole	1930	1589-1952	2.01	+	+	+	-	+	+	+
<b>Pyran</b>											
30.13	Maltol*	1944	1949-1995	0.09	+	+	+	+	+	+	+
<b>Others</b>											
34.50	(E)-2-Octenoic acid*	2175	2182	2.70	+	-	-	+	+	+	+
31.70	Unknown	2024			+	+	+	-	+	+	+

<sup>a</sup> Retention Index range from the National Institute of Standards and Technology Mass Spectrometry Data Center (NIST, 2018)

<sup>b</sup> LogP<sub>ow</sub> values from The Good Scents Company Information System (Luebke, 2021)

“+” Compounds detected by GC-MS in the sample

“-” Compounds not detected by GC-MS in the sample

“\*” Compounds identified by: 1) Comparing mass spectra, 2) GC-TOF-MS, 3) Comparing retention indices of standards and 4) Comparing the odor descriptions with their corresponding pure standards and with literature.

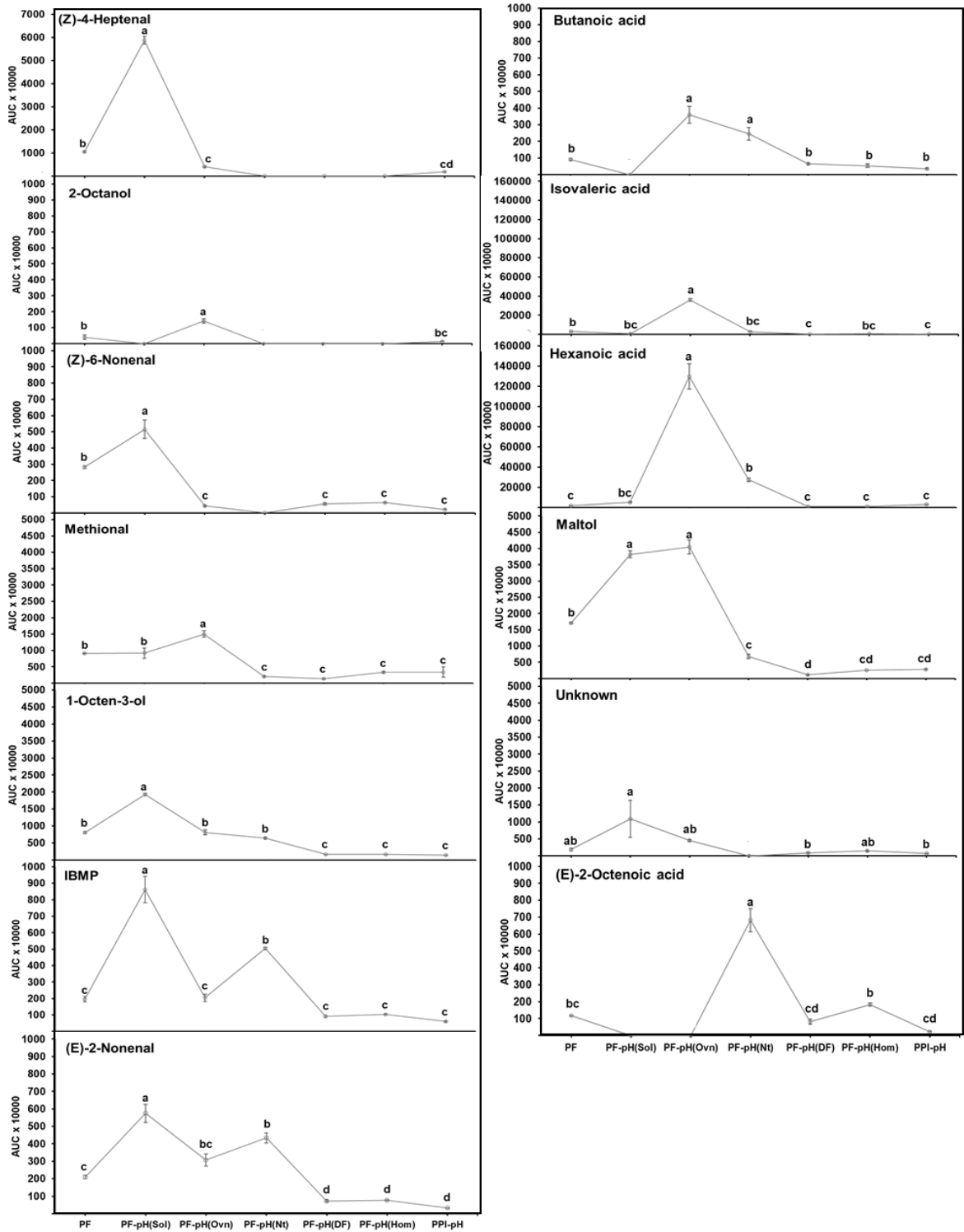
In this study it was hypothesized that the aroma compounds rated with a high odor intensity are likely to be the most significant contributors to the aroma profile of the samples. Therefore, efforts were focused on identifying the aroma compounds that were detected by at least two panelists, rated with an average odor intensity  $\geq 16.2$  (“moderate”) and detected in at least one of the samples. In total, 13 aroma compounds had these characteristics and are reported in Table 10. Out of the 13 compounds, the identity of one compound (labelled “Unknown”) could not be determined.

**Table 10.** Aroma compounds detected and rated with an average odor intensity  $\geq 16.2$  by at least 2 panelists in at least one step of the pH-extraction.

#	Compound	Description by panelists	Description found in literature
1	(Z) 4-Heptenal	Oily, fatty, fishy, oxidized oil	Oily, fatty, cream-like, fishy
2	2-Octanol	Grassy, musty, moldy, earthy	Green, woody, herbal, earthy
3	(Z)-6-Nonenal	Raw cucumber, celery, beany	Green, cucumber, vegetable
4	Methional	Raw potato	Potato, vegetable, musty
5	1-Octen-3-ol	Mushroom, Brothy	Mushroom, fungal, musty
6	2-Isobutyl-3-methoxypyrazine (IBMP)	Bell pepper, earthy, soil	Green bell pepper, pea
7	(E)-2-Nonenal	Cucumber, nutty	Fatty, cucumber
8	Butanoic acid	Cheesy, spoiled milk, saliva	Sharp, Acetic, Cheese
9	Isovaleric acid	Cheesy, sour, pungent	Cheesy, sweaty
10	Hexanoic acid	Cheesy, pungent, rancid	Fatty, cheesy
11	Maltol	Sweet, caramel	Sweet, caramellic
12	Unknown	Woody, floral, sweet, toasty	
13	(E)-2-octenoic acid	Musty, moldy, dirty	Musty, fatty, dirty, cheesy

#### 5.4.2. Relative Amounts of the Key Aroma Compounds at Various Processing Steps

Figure 16 shows how the key aroma contributors changed in recovered amounts during the protein isolation steps.



**Figure 16.** Peak areas of aroma compounds present in samples collected at different steps of the pea protein extraction process. Error bars represent the standard error of the mean ( $n=3$ ) values of three injections of the same aroma isolate in the GC-MS-O. Different lowercase letters above the bars indicate significant differences of each aroma compound across processing steps and according to the Tukey-Kramer multiple means comparison test ( $P < 0.05$ ).

Significant increases in the levels of some of the aroma compounds were observed after the PF was double solubilized (PF-pH(Sol)), however, the concentrations of methional, isovaleric and hexanoic acid and the “Unknown” remained constant. 2-Octanol, butanoic acid and (E)-2-octenoic acid dropped below the detection limit of the instrument. This apparent increase in some volatiles on solubilization of the pea flour is likely explained by the fact that some aroma compounds were not readily extracted from the solid cellular material structure of the PF.

There is a great deal of literature reporting on how aroma compound-protein binding is influenced by changes in temperature, sample pH and/or protein denaturation (Wang & Arntfield, 2016). Therefore, data interpretation at various processing steps is complicated as the variation in measured volatile concentration may reflect issues in volatile extraction from the protein (solution) rather than the absolute amount of aroma compounds in the sample. To the best of our knowledge there has been no research done on investigating the ability of SAFE extraction to recover aroma compounds when bound to proteins. Since there is no way to correct for this potential analytical complication, we will continue the discussion of the data as obtained.

In the two steps following the double solubilization, substantial changes in pH were made. After the double solubilization, the protein was precipitated (pH 4.5), collected and then the pH was adjusted to 3 which corresponds to the PF-pH(Ovn) sample. After leaving the solution at pH 3 overnight, the pH was brought to pH 7 which corresponds to the PF-pH(Nt) sample. Figure 16 shows that the pH adjustments caused a significant increase or decrease in the levels of the aroma compounds. These fluctuations

may be due to the changes in the net charge and in the structure of the protein which may have impacted the interactions with the aroma compounds. Dumont and Land 1986 reported that decreasing pH leads to a decrease in the binding of diacetyl to pea protein. Wang and Arntfield 2015a investigated the effect of pH on the binding properties of salt-extracted pea protein to selected mono-ketones and saturated aldehydes and they found that binding decreased in the order: pH 5 > pH 7 > pH 9 > pH 11 > pH 3. They suggested that the strong hydrophobic associations between proteins at pH 5 could have created additional flavor binding sites which increased flavor retention. Additionally, they explained that at extreme pH values (pH 3 and 11) the protein is heavily denatured or unfolded which could have caused loss of flavor binding sites and therefore reduction in flavor retention.

Part of this apparent disagreement in results may now be explained by the work of Anantharamkrishnan and Reineccius 2020. Dumont and Land 1986 were studying the interaction of diacetyl with pea protein while (Wang & Arntfield, 2015b) considered a ketone mixture (2-hexanone, 2-heptanone and 2-octanone). Diacetyl, as a diketone, reacts very rapidly with proteins via covalent bond formation whereas mono-ketones do not undergo covalent bond formation. Thus, studies considering only mono-ketones would measure hydrophobic interactions which would be strongly influenced by protein folding while studies with diacetyl (diketone) would not be influenced by protein denaturation (i.e. folding). Also, covalent bonds would not be formed at low pH explaining the low reactivity of the diacetyl at low pH as well. According to Anantharamkrishnan &

Reineccius, 2020, at low pH, the intermediate step for forming covalent interactions (by the Schiff base mechanism) does not happen, as the amine groups would be protonated.

The levels of isovaleric acid and hexanoic acid significantly increased when the pH was adjusted to pH 3 (PF-pH(Ovn)). This phenomenon may be an artifact of the volatile isolation process. At this pH, most of the acids are in their neutral form (not ionized) and most of the protein is positively charged. Therefore, one would expect that little to no interactions take place between the acids and the protein which makes the acids more accessible to be extracted by DCM. Additionally, in the neutral form, acids are more likely to be soluble in a non-polar solvent like DCM and consequently, they will be easily isolated from the sample. When the pH was increased from 3 to 7.5, a significant decrease in the levels of these acids (isovaleric and hexanoic acid) was observed. This again could be explained by the fact that at pH 7.5, most of the acids molecules are in the ionized form and therefore, they do not have a strong affinity with DCM hindering their extraction.

After neutralizing the pH, the solution was concentrated by following a two-step filtration: Ultrafiltration and diafiltration. This step corresponds to the PF-pH(DF) sample. During the two-step filtration small components like salts and sugars are removed. Along with these components, some of the aroma compounds may also be eliminated. This may explain the significant decrease in the levels of most of the aroma compounds: 1-octen-3-ol, IBMP, 2-nonenal, butanoic acid, hexanoic acid, maltol and (E)-2-octenoic acid. (Z)-4-Heptenal and 2-octanol were below detection levels while the other measurable volatile compounds remained constant. The volatiles removed from the

system in this manner would be dependent upon their competitive binding with the respective protein fractions.

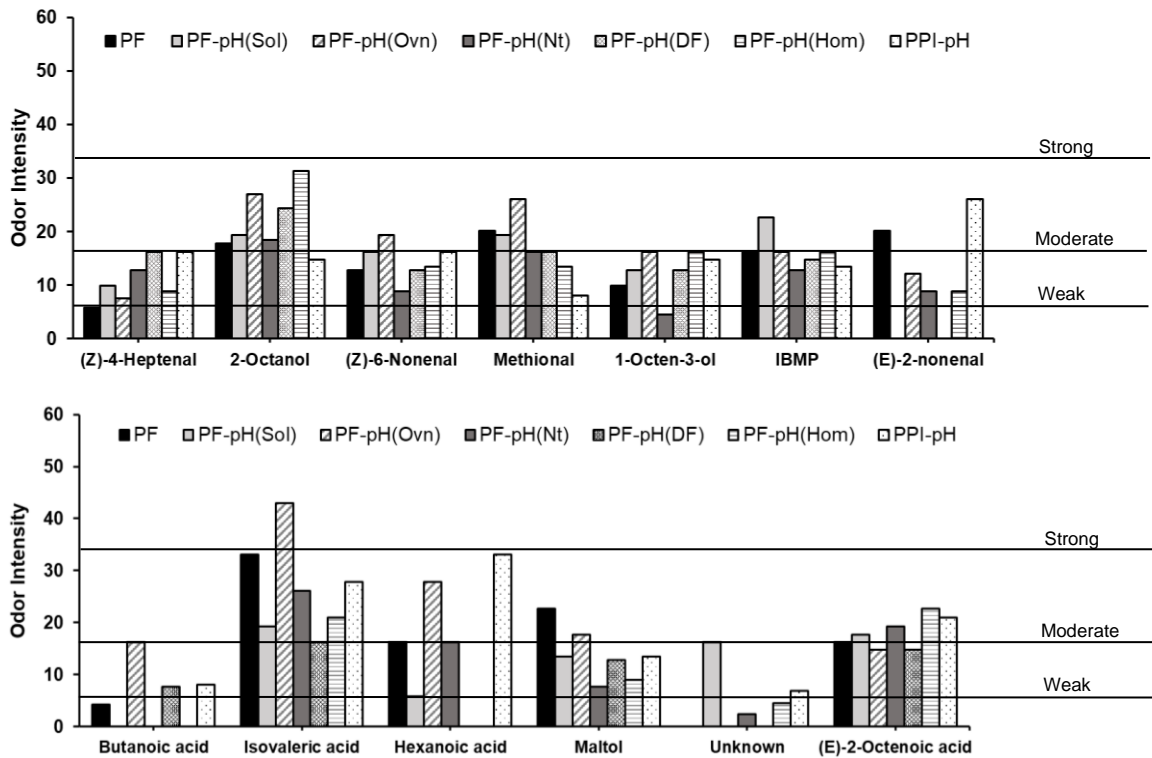
After the two-step filtration step, the protein solution was pasteurized (in order to kill pathogens) and homogenized. The pasteurization and homogenization steps were performed in a close system and therefore, one might expect no significant changes in the levels of the aroma compounds. While there were no significant changes in the levels of most of the aroma compounds from the filtration through the pasteurization/homogenization step (PF-pH(Hom) sample), the concentrations of (E)-2-octenoic acid decreased and (Z)-4-heptenal and 2-octanol dropped below instrument detectable levels.

After homogenization, the protein solution was spray dried. Figure 16 ((PPI-pH) sample) shows that the levels of most of the aroma compounds remained ca, constant, except for (E)-2-octenoic acid, which decreased. Spray drying involves both a heat treatment and a loss of water via evaporation. Due to the high boiling point of (E)-2-octenoic acid, one would not expect a significant loss of this compound during spray drying.

When comparing the starting material (PF) and the final product (PPI-pH), the concentrations of the aroma compounds either decreased or remained constant, which suggests that the pH-extraction method would remove some proportion of the aroma compounds originally present in the PF.

### 5.4.3. Odor Description and Intensity of Aroma Compounds Identified During the Production of Pea Protein by pH Extraction

The odor intensity of the aroma compounds detected by panelists sniffing at the GC-MS-O sniffing port in each sample are presented in Figure 17. 2-Octanol, (Z)-6-nonenal, methional, IBMP, (E)-2-nonenal, isovaleric acid, hexanoic acid, maltol and (E)-2-octenoic acid were detected and had an average odor intensity >16.2 (“moderate”) in at least one of the sampling points. 2-Octanol was detected at each step of the process and was described as having “grassy, musty, moldy and earthy” notes by the panelists. This compound was also found in unblanched green peas by Murray et al. 1976.



**Figure 17.** Mean intensity ratings of the 13 most potent aroma contributors extracted by SAFE and found during the isolation of pea protein. Intensity ratings are from 100-point general labeled magnitude scale (gLMS); a rating of 5.8 corresponded to the descriptor “weak”, a rating of 16.2 corresponded to the descriptor “moderate”, and a rating of 33.1 corresponded to the descriptor “strong”.

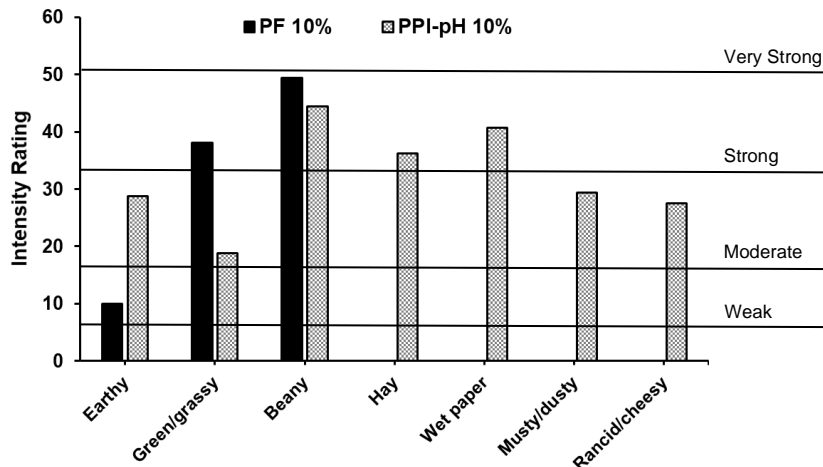
(Z)-6-nonenal was detected in all the samples and contributed to a “raw cucumber, celery and beany” aroma. This compound is reported for the first time. Methional was detected in all the samples and was described as having a “raw potato” aroma by the panelists. In a previous study by Murat et al. 2013, methional was found in pea protein extract. Similarly, Trikusuma, Paravisini, and Peterson 2020 found methional in a pea protein beverage. These two studies agreed that methional contributed to a “potato and boiled potato” aroma in the samples. IBMP was described by the panelists with “bell-pepper, earthy, soil” notes. Several reports have noted the presence of this methoxy-pyrazine in frozen green peas, blanched green peas, pea flour and in a pea protein beverage (Murray et al., 1976) (Murat et al., 2012) (Trikusuma et al., 2020). (E)-2-Nonenal was described with “cucumber and nutty” notes and was detected in all the samples by panelists, except in PF-pH(Sol), PF-pH(DF). This compound has been previously reported in frozen green peas and pea flour (Murray et al., 1976) (Heng, 2005). Isovaleric acid was perceived at each step of the process by panelists whereas hexanoic acid was detected in all the samples except in PF-pH(DF) and pF-pH(Hom). These compounds were both described as having a “cheesy, pungent, rancid” aroma. Murat et al. 2013, reported the presence of isovaleric acid in pea flour and hexanoic acid in pea protein extract. In their study, isovaleric acid was described with “animal” notes and hexanoic acid with “feces, meat broth and sewer” notes. Maltol was perceived at each step of the process and was described as having “sweet and caramel” notes similar to what was found by Trikusuma, Paravisini, and Peterson 2020 in a pea protein beverage. (E)-2-Octenoic was perceived in all samples and was described as “musty,

moldy and dirty”. To the best of our knowledge, the presence of this compound in peas or pea ingredients has not been reported in the literature.

The other compounds (Z)-4-heptenal, butanoic acid, 1-octen-3-ol and “Unknown” were perceived with an odor intensity of 16.2 (“moderate”) in at least one of the samples. (Z)-4-Heptenal was detected at each sampling point and was described as having an “oily, fatty, fishy” notes. (Z)-4-Heptenal is reported by the first time in this study. Butanoic acid was only detected in PF, PF-pH(Ovn), PF-pH(DF), PPI-pH and was described as “cheesy, spoiled milk and saliva”. The presence of butanoic acid was previously reported by Kryachko et al. 2020 in fermented pea protein enriched flour but no olfactory analysis was conducted in this study. 1-Octen-3-ol was described with “mushroom and brothy” notes and was perceived in all the samples by the panelists. Murat et al. 2013, reported the presence of 1-octen-3-ol in both pea flour and pea protein isolate. Panelists were able to detect the “unknown” compound in most of the samples except in PF, PF-pH(Ovn), PF-pH(DF) and was described as having a “woody, flora, sweet, and toasty” aroma.

#### **5.4.4. Sensory evaluation**

In order to look for relationships between the GC-MS-O data and overall perception, a sensory evaluation of aqueous solutions of PF (starting material) and PPI-pH (final product) was conducted. Seven odor descriptors were used by panelists to characterize the aroma of the PF and PPI-pH solutions samples (Figure 18).



**Figure 18.** Mean intensity ratings of PF and PPI-pH solutions tested for aroma. Intensity ratings are from 100-point general labeled magnitude scale (gLMS); a rating of 5.8 corresponded to the descriptor “weak”, a rating of 16.2 corresponded to the descriptor “moderate”, a rating of 33.1 corresponded to the descriptor “strong”, and a rating of 50.1 corresponded to the descriptor “very strong”.

The majority of these descriptors were also used to describe the aroma compounds eluting from the sniffing port coupled to the GC-MS system. Some of these odor descriptors could be linked to individual aroma compounds (Table 10). For instance, the aroma compounds primarily responsible for the “earthy” notes in the tasting solutions were likely 2-octanol and IBMP. The “green/grassy” aroma was likely due to 2-octanol. The “beany” odor character could be attributed to (Z)-6-nonenal. The “musty/dusty” aroma was likely from the presence of 2-octanol and (E)-2-octenoic acid, and the “rancid/cheesy” notes were likely due to butanoic acid, isovaleric acid and hexanoic acid. Figure 18 also illustrates that unlike PPI-pH, PF was characterized only with three out of the seven odor descriptors (earthy, green/grassy and beany). The other descriptors (hay, wet paper, musty/dusty and rancid/cheesy) were used to describe the aroma of PPI-pH. Based on the analytical data, no compounds having hay and wet paper notes were

identified in this study. It may be possible that the compound(s) responsible for these notes were not extracted in adequate quantities to be noted by panelists sniffing at the GC-MS-O sniffing port. Regarding the musty/dusty and rancid/cheesy descriptors, these aromas might have been formed during the pH-extraction of pea protein (pH extremes) or their concentrations might have been increased to the point that they were detectable by the panelists in PPI-pH.

#### **5.4.5. Theoretical Pathways of Aroma Compound Formation**

The theoretical pathway of formation of the aroma compounds found in this study were discussed earlier in Chapter 4, except for butanoic acid. The theoretical formation pathway of butanoic acid is discussed as follows.

The presence of butanoic acid in pea protein has been reported by (Kryachko et al., 2020). In this study, they evaluated the potential antimicrobial(s) produced by *Lactobacillus plantarum* during fermentation of pea protein enriched flour. The authors found that butanoic acid was one of the most predominant organic acids produced by *L. plantarum* and indicated that this compound might have been produced as a result of amino acid catabolism. Shukla et al. 2010, found butanoic acid in different samples of Doenjang, a traditional Korean fermented soybean paste. The authors suggested that butanoic acid might be synthesized by *Lactobacillus plantarum* which converts lipids into butanoic acid through intracellular enzymes activity.

## 5.5. Conclusion

In conclusion, the majority of the odor descriptors used during the sensory evaluation to describe the PF and PPI-pH aqueous solutions were also used during the GC-MS-O analysis. This supports our hypothesis that the 13 aroma compounds identified through instrumental analysis are likely to be significant contributors to the aroma profile of the samples examined. Additionally, this study found that the processing steps used during an optimized pH-extraction of pea protein tended to alter the concentration of the volatiles and some of these variations appeared to affect the odor intensity perceived by panelists through the sniffing port. While no new aroma compounds appear to be produced via the protein isolation process, no existing compounds were completely removed from making a sensory contribution as determined by the olfactory analysis. This is not a surprise in the sense that most of the aroma compounds tended to be hydrophobic and thus, they would stay with the protein through the extraction process rather than be lost to aqueous washes.

These findings suggest that most of the aroma compounds identified in the samples come from the normal metabolism or during storage of the peas. Therefore, approaches to reducing the inherent undesirable aroma compounds from the peas could involve plant breeding programs or processing approaches that include the extraction of the lipids to remove them as precursors and/or super critical extraction (Vatansever & Hall, 2020) (Vatansever et al., 2021) at the beginning or end of the protein isolation process.

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## Chapter 6: Overall Conclusions and Future Work

This research project was focused on the measurement and characterization of the volatile aroma compounds present in pea protein isolates and attempted to determine their sources, i.e. inherent to the plant, or formed during optimized salt- and pH- extractions.

In the first part of this study, three methods for the isolation of volatile compounds were compared based on the ability to provide a complete aroma profile of pea flour: SBSE, SAFE and HS-SPME. SAFE was found to provide a much more complete aroma profile of pea flour than either competing method and was therefore, selected to isolate the aroma compounds from the samples collected throughout the salt- and pH extractions.

Using the protocol developed for the isolation and analysis of aroma compounds in the first part of this study, the aroma profile was monitored following key processing steps during the optimized salt- and pH- methods to produce pea protein isolates. The analytical data showed that 12 and 13 aroma compounds were detected and rated with a “moderate” odor intensity by panelists through the sniffing port of the GC-MS-O in at least one of the processing steps during the salt- and pH- extractions, respectively. The majority of the most potent aroma compounds detected during the salt-extraction were also detected during the pH-extraction except for: 1) Hexanal which was detected with a high odor intensity during the salt-extraction process but not during the pH-extraction process and, 2) butanoic acid and the compound labelled as “unknown”: which were detected with a high odor intensity during the pH-extraction but not during the salt-

extraction. These results indicated that these compounds are likely to be the most significant contributors to the aroma profile of the samples examined. These initial findings were also supported by the sensory evaluation on the pea flour and pea protein (produced by salt- and pH-extractions) aqueous solutions.

Sensory results showed that the majority of the aroma descriptors used in sensory analysis of the starting flour and finished protein isolate were also used to describe individual odors eluting from the sniffing port of the GC-MS-O. In total, 3 odor descriptors were used to describe the odor of the PF testing solution, 6 to describe the PPI-Salt solution and 7 to describe the PPI-pH solution (Figure 14 & Figure 18). When comparing the odor descriptors used to describe the aqueous solutions of PPI-salt and PPI-pH, most of them were similar with the exception of: 1) “green/grassy” which was used to describe the odor of PPI-pH solution but not for the PPI-Salt solution, 2) “sweet” which was used to describe the PPI-Salt solution but not for the PPI-pH solution and 3) “wet paper” which was used to describe PPI-pH solution but not for PPI-Salt solution. Additionally, the odor intensity of the “beany” and “hay” notes appeared to be lower in the PPI-Salt solution compared to the PPI-pH solution, the intensity of “rancid/cheesy” was higher in the PPI-salt solution compared to the PPI-pH solution whereas the intensity of the “earthy” and “musty/dusty” odors was the same in both testing solutions. Neither protein extraction process appeared to yield a completely bland protein isolate. Some aroma compounds appeared to remain throughout the processing steps and could be detected by panelists in both finished protein isolates (PPI-salt and PPI-pH).

Contrarily to what it has been found by other researches, none of the major odorants were newly formed or completely lost during the protein isolation process suggesting that the processing steps do not completely remove existing or generate significant new aroma compounds. The origin of most of the major odorants was associated with lipid degradation and, the Maillard reaction and associated Strecker degradation. The later mechanisms are known to occur at high temperatures; however, they can also take place at low temperatures, but at a much slower rate. Most of the aroma compounds were found from the very first step of the process (PF) which suggests that the compounds are likely derived from the peas themselves as plant metabolites or are formed at some point before/during the production of PF.

Procedures such as plant breeding programs or processing methods that include the removal of lipids as precursors and/or super critical extraction at the beginning or end of the protein isolation process may seem attractive to reduce the inherent off-aroma in pea protein isolates. However, supercritical extraction may be cost prohibitive at a production scale and agronomy research is a long-term goal. Other approaches that have shown the potential to improve the aroma profile of pea protein isolates and that could be further investigated are 1) the use of cyclodextrin which has shown to have the ability to trap some of the off-aroma compounds present in pea protein and/or 2) fermentation which has demonstrated to change the aroma profile of pea protein by either decreasing volatile content or developing new pleasant aroma compounds that could mask the off-notes.

This research has demonstrated that proteins have the ability to bind aroma compounds which makes their extraction problematic. This aroma-protein binding is influenced by changes in temperature, sample pH and/or protein denaturation. Therefore, data interpretation at various processing steps was complicated as the variation in measured volatile concentration may reflect issues in volatile extraction from the protein (solution) rather than the absolute amount of aroma compounds in the sample. What was measured using our approach (SAFE extraction) is the amount of free or “extractable aroma compound”. To the best of our knowledge there has been no research done on investigating the ability of SAFE extraction to recover aroma compounds when bound to proteins. Volatile isolation may be improved by mild hydrolysis or pH adjustment. Hydrolysis or pH adjustment may open the protein structure providing fewer opportunities for flavor volatiles to bind to the protein. This would increase volatile recovery for analysis.

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## Appendices

### Appendix A: Sample Calculation for Determining the Amount of Sample Used for Flavor Analysis

#### Salt Extraction

**Equation:**

$$\begin{aligned} & \textit{Total Sample Amount – Lab Scale} \\ & = \frac{100g \times \textit{Total Sample Amount – Pilot Plant (kg)} \textit{ of specific processing step}}{\textit{Total Sample Amount – Pilot Plant (kg)} \textit{ of PF}} \end{aligned}$$

Example calculation for PF-Salt (Nt):

$$\textit{Total Sample Amount – Lab Scale} = \frac{100.0g \times 566.0 \textit{ kg}}{28.0 \textit{ kg}} = 2021.4g$$

Sample	Total Sample Amount – Pilot Plant (kg)	Total Sample Amount - Lab Scale (g)	Factor*	Amount of Sample Used for Flavor Analysis (g)
PF	28.0	100.0	-	100.0
PF-Salt(Nt)	566.0	2021.4	8	252.7
PF-Salt(DF)	20.3	72.5	3	24.2
PF-Salt(Hom)	18.3	65.4	3	21.8
PPI-Salt	1.8	6.4	4	25.6

\*Factor: Number of times the “Total Sample Amount – Lab Scale” value had to be multiplied or divided by in order to obtain the “Amount of Sample Used for Flavor Analysis” value.

## **pH Extraction**

<b>Sample</b>	<b>Total Sample Amount – Pilot Plant (kg)</b>	<b>Total Sample Amount - Lab Scale (g)</b>	<b>Factor*</b>	<b>Amount of Sample Used for Flavor Analysis (g)</b>
PF	37.9	100.0	-	100.0
PF-pH(Sol)	453.6	1196.8	20	59.8
PF-pH (Ovn)	106.6	281.3	-	281.3
PF-pH (Nt)	106.6	281.3	14	20.1
PF-pH (DF)	22.5	59.4	2.5	23.8
PF-pH (Hom)	20.9	55.1	2.5	22.0
PPI-pH	1.4	3.7	6	22.2

\*Factor: Number of times the “Total Sample Amount – Lab Scale” value had to be multiplied or divided by in order to obtain the “Amount of Sample Used for Flavor Analysis” value.

## Appendix B: General Labeled Magnitude Scale

General Labeled Magnitude Scale (gLMS)

