The Effect of Alcohol Washing on the Flavour Profiles, Functionality and Digestibility of Dry Processed Pea Protein Fractions

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ABSTRACT

In this research, the potential of aqueous-alcoholic solvent washing on removing off-flavours in air-classified pea protein enriched flour (PPEF) was investigated. PPEF was treated with aqueous ethanol or aqueous isopropanol at three different concentrations (20%, 50%, and 80%) to remove the unpleasant beany, earthy and astringent flavours. Headspace solid phase microextraction followed by GC-MS was used to identify the flavour compounds in untreated and treated PPEF. Besides the flavour profile, changes to their proximate composition, colour, functionality and protein quality were compared among untreated and treated samples.

There were 28 volatile compounds extracted from untreated PPEF. Total peak area of the compounds extracted from untreated PPEF was 261×10^6 with 64% contributed by aldehydes and 30% from alcohols. Most alcohol treatments reduced the total volatile compounds extracted of PPEF expect for the 20% isopropanol treatment. The level of flavour compound reduction rose with an increase in solvent polarity. Higher concentrations of ethanol and isopropanol (50% and 80%) showed greater effectiveness in removing flavour compounds by reducing the total peak area by 82%–94%, whereas solvents at the lowest concentration (20%) were less efficient. Treatment with 80% isopropanol resulted in the removal of 20 compounds from PPEF to below the detection level, followed by 50% isopropanol, 80% ethanol and 50% ethanol. However, there was no significant difference among the 50% and 80% treatments in the total peak area extracted.

The protein contents of all treated samples (58.2%–64.3% d.b.) increased compared to untreated PPEF (55.5% d.b.) due to the decrease in ash, lipid and carbohydrate content. All treated samples were significantly darker in colour compared to the untreated sample. The surface charge of the protein in the PPEF decreased after alcohol treatment. Also, the protein solubility was negatively affected by solvent treatment, *i.e.* reduced from 85% to 21%–52%, with isopropanol treatments and lower alcohol concentration treatments having greater negative impact on the solubility. Water hydration capacity was positively affected by alcohol washing, while oil holding capacity was negatively affected. Emulsion stability of PPEF was maintained in all isopropanol-treated samples but reduced in 50% and 80% aqueous-ethanol-treated samples.

Although *in vitro* protein digestibility was improved with the solvent treatments, from 79% to 82%–85%, the amino acid scores became lower with the treatments. Amino acid scored dropped from 0.9 in untreated PPEF to 0.66–0.84, due to the decrease in methionine and cystine in the

treated PPEF. The more significant drop in amino acid score compared to the rise in protein digestibility resulted in lower *in vitro* protein digestibility corrected amino acid scores (PDCAAS).

Both aqueous ethanol and aqueous isopropanol at 50% and 80% concentration were proved to be effective in removing flavour compounds with PPEF with remarkable modification of the chemical composition, protein functionality and protein quality in both positive and negative way. There is potential to use aqueous alcohol to treat pea protein enriched flour to obtain a functional high protein pulse ingredient with milder volatile flavour profile.

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LIST OF ABBREVIATIONS

PPEF	Pea protein enhanced flour
AC	Air classified
CHO	Carbohydrates
G6PD	Glucose-6-phosphate dehydrogenase
THR	Threonine
CYS	Cysteine
VAL	Valine
MET	Methionine
ILE	Isoleucine
LEU	Leucine
TYR	Tyrosine
PHE	Phenylalanine
HIS	Histidine
LYS	Lysine
TRP	Tryptophan
PER	Protein Efficiency Ratio
PDCAAS	Protein Digestibility Corrected Amino Acid Score
DIAAS	Digestible Indispensable Amino Acid Score
WHC	Water hydration capacity
OHC	Oil holding capacity
LOX	lipoxygenase
NSI	Nitrogen solubility index
GC	Gas-Chromatography
MS	Mass Spectrometry
HS-SPME	Headspace Solid-Phase Micro-Extraction
ES	Emulsion stability
ANOVA	Analysis of variance
PCA	Principal component analysis

d.b.	dry basis
CP	Crude protein
ppm	parts per million
ppb	parts per billion

1. INTRODUCTION

1.1 Overview

Pulses are the dry edible seeds of *Leguminosae* crops and include lentil, pea, faba bean, chickpea and edible bean (Hoover *et al.*, 2010). Pulses and pulse ingredients are considered highly nutritious as they are rich in protein, carbohydrates, fibre and micronutrients, and are low in fat (with the exception of chickpea, which contains 5%–7%) (Tiwari and Singh, 2012). However, their use in the food industry has been limited for the most part because of their beany or vegetable-like flavour (Ma *et al.*, 2011). Other reasons that limit their widespread adoption are their difficulty in cooking, often requiring a soaking stage and a multi-hour cook time, and the presence of bioactive compounds that may have negative health associations (Dahl *et al.*, 2012). There are different types of antinutritional components found in pulses an

d they include enzyme inhibitors (trypsin, chymotrypsin and amylase enzyme inhibitors) which inhibit the digestion of protein and starch, respectively. These enzyme inhibitors exist at low levels in most pulses and can be inactivited by various process treatments. Oxalates and phytates in pulses can interfere with mineral absorption, while phenolic compounds and condensed tannins interfere with protein digestion as they induce protein crosslinking. Lectins in raw pulses may induce hemolysis via red blood cell agglutination (Dahl *et al.*, 2012; Roy *et al.*, 2010). The pyrimidine glycosides, vicine and convicine, in the case of faba bean have been associated with favism, an acute haemolytic anaemia, in individuals with glucose-6-phosphate dehydrogenase deficiency (Khazaei *et al.*, 2019).

Pulses are typically sold as whole or split products. However, there has been growing interest in the food industry in value-added pulse fractions such as flours, air-classified protein and starch concentrates, isolates, peptides and fibre-rich fractions. Dry processing, such as by air classification, involves the physical separation of pulse flour into protein- and starch-rich fractions based on their differences in particle size and density (Schutyser *et al.*, 2015). Air-classified pulse protein fractions can range 30% to 65% protein content depends on the type of pulses (Schutyser *et al.*, 2015; Sosulski and Youngs, 1979; Tecklenburg *et al.*, 1984). Wet processing of pulse flours into a wet protein concentrate (65%–80% protein) or an isolate (>85%–90% protein) fraction is

also done commercially using techniques such as alkaline extraction (recovering protein by isoelectric precipitation and/or ultrafiltration), salt extraction, micelleular extraction and alcohol extraction. Compared to wet processing, dry processed pulse ingredients are cheaper but are inferior in functionality and flavour profile (Tiwari and Singh, 2012).

The beany, green, earthy flavour of pulse ingredients remains one of the biggest challenges during product development (Ma *et al.*, 2011). There are several different volatile chemical groups responsible for the beany and green taste of pulses such as alcohols, aldehydes, hydrocarbons, ketones, sulphur compounds, terpenes, esters and pyrazines (Azarnia *et al.*, 2011a). Among these volatile compounds, the majority are formed via lipid hydrolysis and fatty acid oxidation during processing and storage (Azarnia *et al.*, 2011a).

There are several strategies for addressing flavour issues in pulse ingredients including: a) cultivar selection, choosing cultivars with lower levels of lipoxygenase and flavour compounds; b) soaking followed by thermal treatments, where soaking allows for leaching of unwanted compounds from the seed, whereas cooking (*e.g.*, boiling, roasting, infrared heating and extrusion) helps to liberate the volatile compounds; c) germination, which reduces the levels of flavour compounds produced via lipid oxidation/lipoxygenase, but may enhance the off-flavour caused by non-volatile compounds such as saponin; d) fermentation, which may alter the subsitiuents attached to the saponin backbone, which makes saponin taste sweet or netural insead of bitter; and e) enzymatic treatments, which have been demostrated to reduce some off-flavours but to enhance other off-flavours at the same time (Roland *et al.*, 2017). In addition, alcohol-washing of pulse protein has shown some promise in improving the flavour profile (Tiwari and Singh, 2012), as many of the flavour volatiles are soluble in alcohol (Lee and Morris, 1963). Organic solvents have been proved successful in removing the beany, green flavour from soybeans, peanuts and groundnuts (Eldridge *et al.*, 1971; Johnson *et al.*, 1979; Madhavi *et al.*, 1989); however, this is typically at the expense of protein functionality (Roland *et al.*, 2017).

The overall goal of this research was to examine the impact of aqueous alcohol washing on the removal of volatile flavour compounds from air-classified pea protein enriched flour (PPEF), and the resulting impact that had on protein functionality and digestibility.

1.2 Objectives

1) To evaluate the impact of aqueous alcohol (ethanol and isopropanol) washing at different concentrations on the flavour profiles of pea protein enriched flours.
2) To evaluate the impact of aqueous alcohol (ethanol and isopropanol) washing at different concentrations on the functional properties of pea protein enriched flours.
3) To evaluate the impact of aqueous alcohol (ethanol and isopropanol) washing at different concentrations on the *in vitro* protein digestibility of pea protein enriched flours.

1.3 Hypotheses

The following hypotheses were tested in the study:

1) A higher concentration of aqueous alcohol will be more effective in removing the flavour compounds from pea protein enriched flours.
2) Isopropanol will be more effective at removing the flavour compounds since it's less polar than ethanol.
3) Functionalities of all pea protein enriched flours will be reduced with alcohol washing; to a greater extent with isopropanol and as the concentration of alcohol increases.
4) The digestibility of all pea protein enriched flours will be improved with alcohol washing; to a greater extent with isopropanol and as the concentration of alcohol increases.

2. LITERTURE REVIEW

2.1 Pulses

Pulses are *leguminous* crops (*e.g.*, chickpeas, peas, faba beans, lentils and edible beans) that are consumed globally for their nutritional benefits such as their ability to reduce blood insulin response, lower blood cholesterol, and reduce the risk of cardiovascular disease and hypertension (Asif *et al.*, 2013; Boye *et al.*, 2010a). They also help contribute to weight loss as consumption leads to satiety (Dahl *et al.*, 2012). Pulses can be described as the edible dry seeds with two cotyledons that have the ability to fix atmospheric nitrogen into the soil and increase the soil's fertility, making their use in crop rotations crucial for good agronomic practices (Hoover *et al.*, 2010; Schutyser *et al.*, 2015). Unlike other legumes (*e.g.*, peanuts and soybeans), pulses tend to be much lower in fat (<2%, with the exception of chickpea which is comprised of 5%–7% fat). However, they are rich in protein (20%–31%), carbohydrates (58%–70%) and total dietary fibre (10%–30 %), along with vitamins and minerals (Table 2.1). Regarding their protein, pulses are dominated by globulins (50%–60% of the total protein) (salt-soluble) and albumins (15%–25% of the total protein) (water-soluble) (Park *et al.*, 2010). The two main globulin-type proteins found in pulses are: a) legumin, which is a hexamer of a molecular mass of 350–400 kDa (11S protein, 'S' is a Svedberg Unit). Each of the six subunits is comprised of an α-chain (40 kDa) and β-chain (20 kDa) held together by a disulfide linkage, and then associated into its quaternary structure via hydrophobic interactions, hydrogen bonding and van Der waals attractive forces (Barac *et al.*, 2010; Gueguen *et al.*, 1988; Mosse and Pernollet, 1983); and b) vicilin, which is a trimer with a molecular mass of 150 kDa (7S protein), has no disulfide linkages and is held together via non-covalent forces (Stone *et al.*, 2015). A third, more minor globulin-type protein is convicilin, which has a molecular mass of 270 kDa (Boye *et al.*, 2010a). Albumin proteins range in molecular mass between 10–80 kDa, and include enzyme inhibitors, lectins and enzymes (Boye *et al.*, 2010a). Pulse proteins also tend to be rich in lysine, but tend to be deficient in the thiol-containing amino acids (*e.g.*, cysteine and methionine) (Asif *et al.*, 2013; Dahl *et al.*, 2012). In contrast to pulses, cereal proteins are rich in the prolamin-type proteins (alcohol soluble) which are rich in the thiol-containing amino acids but tend to be deficient in lysine (Asif *et al.*, 2013). As such, pulses and

cereals are often consumed together as part of a complementary diet around the world to ensure that one is consuming all the essential amino acids needed to support growth and development (Asif *et al.*, 2013).

Table 2.1. Macro- and micronutrient contents of pulses (per 100g).

Composition	Pea	Chickpea	Lentil	Faba bean	Navy bean
a) Macronutrients					
Protein (g)	25.7[c]	20.5[a]	24.6[a]	26.1[a]	22.3[a]
Carbohydrates (g)	68.6[c]	63.0[a]	63.3[a]	58.3[a]	60.8[a]
Total dietary fibre (g)	28.9[a]	12.2[a]	10.7[a]	25.0[a]	15.3[a]
Fat (g)	1.2[b]	6.0[a]	1.1[a]	1.5[a]	1.5[a]
b) Micronutrients					
Iron (mg)	6[a]	4[a]	6[a]	7[a]	5[a]
Calcium (mg)	89[a]	57[a]	35[a]	103[a]	147[a]
Potassium (mg)	460[b]	718[a]	677[a]	1062[a]	407[a]
Thiamine (µg)	726[b]	477[a]	873[a]	555[a]	775[a]
Riboflavin (µg)	215[b]	212[a]	211[a]	333[a]	164[a]
Niacin (µg)	2889[b]	1541[a]	2605[a]	2832[a]	2188[a]
Vitamin B_6 (µg)	174[b]	535[a]	540[a]	366[a]	428[a]
Folate (µg)	274[b]	557[a]	479[a]	423[a]	364[a]

Data obtained from USDA database[a]; Tiwari and Singh, 2012[b]; Asif *et al.*, 2013[c].

Canada is one of the major pulse producers in the world, along with India, China, Brazil, Myanmar and Australia (Tiwari and Singh, 2012). In 2016, Canada produced over 5 million tons of pulses and exported $4.1 billion worth of pulses, which made Canada the largest exporter of pulses to 124 countries (Agriculture and Agri-Food Canada, 2017). Pulses are gaining much interest in developing countries for food security purposes. However, in North America and Europe, much of the focus has been related to the innovation of protein ingredients and fractions for greater integration into the food industry. The food protein ingredient industry was worth

US$25.62 billion in 2016, with an expected annual growth of over 7% until at least 2025 (Grand View Research, 2018). Although the market is currently dominated by animal-derived proteins (*e.g.*, casein, whey, ovalbumin and gelatin) and soy, the industry is seeking healthy, lower cost alternatives that could meet industry demands into the future. Further to this, consumers are looking towards plant-based alternatives because of ethical, religious or moral reasons, and because of perceived safety concerns (Can Karaca *et al.*, 2011; Toews and Wang, 2013). Pulses also have the added market advantage of being non-genetically modified and low in allergens, with the exception of faba beans where vicine and convince are known to cause favism, a type hereditary disease, resulted in haemolysis and anemia in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency (McMillan *et al.*, 2001; Cappellini and Fiorelli, 2008).

2.2 Pulse processing

Pulses can be processed into flours (20%–30% protein) and protein enriched flours (30%–65% protein) by dry milling and fractionation. While in order to achieve ingredients with higher protein content, such as concentrates (65%–80% proteins) and/or isolates (>85% protein), wet extraction processes need to be employed. The demand for protein fractions, starch fractions, and fibre start to arise in the food industry due to increased interests from various applications.

2.2.1 Dry processing

Processing of pulses typically involves cleaning (*i.e.*, to remove any foreign materials including stones, insects, husk, straws, and other crops), dehulling (*i.e.*, to remove the hulls), sorted (*i.e.*, to remove the broken seed) and splitting (*i.e.*, splitting two cotyledons into individual pieces) followed by milling into a flour (Wood and Malcolmson, 2011). The hulls can also be milled to be used as a high fibre ingredient in the food industry. Its removal reduces the level of colour and flavour issues in further processed pulse fractions as the majority of the polyphenol compounds are located there. Whole or split pulses can be milled into grits, semolinas or flours by an impact mill such as a hammer mill. Sieves of different mesh size are used to separate the fine fragment from the coarse (Wood and Malcolmson, 2011). Milled pulse products with different particle sizes can be used for various purposes, as they display different functional attributes (*e.g.*, water or fat binding).

Pulse flour can be milled into ultrafine powder for starch granules and protein to be separated physically (Pelgrom *et al.*, 2013). Then, protein and starches can further be concentrated by air classification which is a dry separation technique that suspends finely milled flour in a flow of air allowing for the larger starch particles (known as the coarse fraction) and smaller protein particles (known as the fine fraction) to settle out based on size and density differences (Schutyser *et al.*, 2015). The lighter protein rich fragment leaves the classifier from the top, and the heavier starch-rich fragment is collected from the bottom (Schutyser *et al.*, 2015; Pelgrom *et al.*, 2013). Depending on the type of pulse being processed, flours may be transformed into starch-enriched or protein-enriched flours. Table 2.2 gives the relative amounts of protein within the flour prior to air classification, followed by both the coarse and fine fractions afterward. In both fractions, there are significant contaminants of the other. Depending on the levels of protein in the coarse fraction, the starch-rich ingredient may act as an enriched flour. Protein enriched fractions (fine fraction) typically range in protein between 28.9%–69.9% (Table 2.2). The higher amount in the faba beans reflects the greater amount of protein in the starting material. Meanwhile, due to the high-fat content, chickpeas tend not to air classify very well. Less energy and water are expected in the air classification process compared to those needed in wet extraction, such as isoelectric precipitation, which became the major reason of the rejuvenated interests in this technology (Schutyser *et al.*, 2015).

Table 2.2. The composition of pulse flours and air classified (AC) fractions (g /100g dry matter).

Pulse	**Flour**		**AC fine fraction**		**AC course fraction**	
	Protein, g	**CHO, g**	**Protein, g**	**CHO, g**	**Protein, g**	**CHO, g**
Pea	23.8[a]	70.9[b]	58.5[a]	30.7[b]	14.5[b]	82.7[b]
Lentil	23.7[a]	72.2[b]	57.6[a]	34.2[b]	12.2[b]	85.1[b]
Faba bean	31.0[a]	65.5[b]	69.9[a]	25.4[b]	14.4[b]	82.7[b]
Chickpea	19.5 [b]	70.1[b]	28.9[b]	56.4[b]	15.3[b]	76.3[b]
Navy bean	26.9[c]	65.7[c]	48.6[c]	41.4[c]	20.9[c]	72.3[c]

Data obtained from Schutyser *et al.*, 2015[a]; Sosulski and Youngs, 1979[b]; Tecklenburg *et al.*, 1984[c].

2.2.2 Wet processing

Protein concentrates (65%–80%) and/or isolates (>85% protein) can be prepared by a number of processes including: a) alkaline extraction followed by isoelectric precipitation, ultrafiltration or diafiltration; b) salt extraction; c) micellar extraction or d) alcohol precipitation. Depending on the final protein level within the process, ingredients are classified as a concentrate or an isolate. Concentrates produced from dry and wet processing tend to have different protein compositions and functionality.

(a) Alkaline extraction – isoelectric precipitation, ultrafiltration or diafiltration: Alkaline extraction followed by isoelectric precipitation is the most widely used industrial process for creating protein isolates. During this process, pulse flours/protein concentrates are dispersed in water and pH adjusted to pH 8.0–9.0 to solubilize the proteins. Proteins become more soluble in the solution with a pH away from the isoelectric point (Hall, 1996). In addition, the temperature of the solution can be elevated to around 50°C to improve the protein solubility (Boye *et al.*, 2010a). The insoluble materials (*e.g.*, insoluble fibre, carbohydrates and prolamin-type proteins) are removed via centrifugation, leaving a clarified supernatant. Then, pH is adjusted to pH 4.5 to precipitate the protein. At pH close to the protein's isoelectric point, the net charge of the protein is neutral and the repulsion between proteins is the lowest. Therefore, proteins aggregate via non-covalent bonding and hydrophobic interactions. The precipitated protein is centrifuged, washed and neutralized, then dried into a powder (Boye *et al.*, 2010a). Chakraborty *et al.* (1979) used this process to develop pea protein isolate (90.5% protein), lentil protein isolate (89.5% protein), Great Northern bean isolate (89.3% protein), chickpea protein isolate (90.5% protein) and mung bean (88.3% protein) protein isolate. While, Fernan dez-Quintela *et al.* (1997) extracted faba bean protein isolate (81.2% protein) and pea protein isolate (84.9% protein) with this method. The difference of protein content in the isolates is due to the variations in the process such as flour slurry concentration, pH, temperature and extracting time (Boye *et al.*, 2010a).

Alternatively to isoelectric precipitation, pulse proteins can also be recovered from the supernatant using membrane separation technology. Protein separated with this method shows better functionality (Fredrikson *et al.*, 2001). Microfiltration separates out proteins that are larger in size than 0.1 µm, whereas ultrafiltration separates out proteins in the size range of 0.001–0.02 µm (Koros *et al.*, 1996). The retentate from ultrafiltration is often combined with excess water and

then re-filtered in a process called diafiltration. Vose (1980) used membrane separation technologies to develop faba bean protein isolate (94.1% protein), and pea protein isolate (89.5% protein). Boye *et al.* (2010b) used a combination of ultrafiltration and diafiltration to recover pea protein (83.9% protein), lentil protein (82.7%–88.6% protein) and chickpea protein (68.5%–75.5%). In the same study, Boye *et al.* (2010b) found that protein isolates recovered by membrane separation technologies had 2.2% to 9.5% more protein compared to isolates produced by isoelectric precipitation.

(b) Salt extraction: Pulse proteins can also be separated by differences in salt solubility according to the Osborne classification scheme for characterizing protein types (Osborne, 1924). For instance, globulin- and albumin-type proteins are salt and water-soluble, respectively. Differences in solubility stem from their inherent surface characteristics and the relative proportion and spatial distribution of hydrophilic and hydrophobic amino acid moieties. 'Salting-in' of a protein occurs at relatively lower ionic strengths (*e.g.*, NaCl (0.1 to 1M)) in which ions act to help improve the structure of the hydration layers surrounding the proteins in solution (Boye *et al.*, 2010a; Hall, 1996). In this instance, the ionic conditions are such that protein-water interactions are favoured over protein-protein interactions. During extraction, pulse flour is firstly dispersed in a NaCl solution at neutral pH, and then clarified via centrifugation to remove insoluble material (Boye *et al.*, 2010a). Proteins remaining in the supernatant can then be recovered via membrane separation or precipitated via dialysis to remove the salt followed by centrifugation and drying (Boye *et al.*, 2010a; Martínez-Maqueda *et al.*, 2013; Can Karaca *et al.*, 2011). The high ionic strength promotes ion-water interactions and disrupts the hydration layers surrounding the proteins. As water molecules are pulled away from the surface of the proteins, hydrophobic amino acid moieties are exposed leading to protein-protein aggregation via hydrophobic interactions. Depending on the concentration of ammonium sulphate used, selected separation can be done based on the proteins hydrophobicity to induce precipitation of various protein fractions (Martínez-Maqueda *et al.*, 2013). Proteins that are more hydrophobic in-nature tend to require higher percentages of ammonium sulphate solutions to cause 'salting out' of a protein (Deak *et al.*, 2006). The salting in or out process depends on the type and concentration of salt present in solution, as described by the Hofmeister series (Hofmeister, 1888). Can Karaca *et al.* (2011) used salt extraction to concentrate pea protein, lentil protein, faba bean protein, and chickpea protein to 81.8%, 74.7%, 82.0% and 81.6%, respectively. Meanwhile, this group of researchers found that

protein isolate produced with salt extraction had 2.1% to 7.7% lower protein content when compared to isoelectric precipitated pulse protein isolates (Can Karaca *et al.*, 2011).

(c) Micellar extraction: During this process, 50 g flours are dispersed in 500 mL NaCl solution (1.0 N) (Stone *et al.*, 2015). This causes both globulin- and albumin-type proteins to become soluble. The solution is then clarified via centrifugation to remove insolubles. The supernatant is then diluted with 10 volumes of cold water and allowed to settle at refrigerated temperatures (4°C) under static conditions (*i.e.*, no stirring). In this process, the dilute NaCl becomes even diluter allowing for the globulin fraction to start coming out of solution and forming micelles via hydrophobic interactions. As the size of the micelles grows, proteins begin to settle (Murray *et al.*, 1978). The precipitate is then recovered via centrifugation and dried. Mwasaru *et al.* (1999) produced an enriched pigeon protein with 40.2% protein content and cowpea protein with 36.7% protein content; while Stone *et al.* (2015) used micelleular extraction to develop pea protein with (81.9–87.8% protein).

d) *Alcohol washing*: Alcohol extraction can be used in extracting prolamins from various cereals, such as extracting zein from distiller's grain (Natarajan *et al.*, 2009; Cookman and Glatz, 2009). During alcohol extraction, the flours are dispersed in various alcohol solutions at basic conditions to help solubilize the prolamin-type proteins (Cookman and Glatz, 2009). The insoluble fraction of the process, globulin and albumin proteins, are collected. Alcohol denatures the protein and washes away oligosaccharides, and some volatile compounds, which results in a protein with better taste profile but worse functionality and lower solubility than other extraction methods (Tiwari and Singh, 2012). Depending on the concentration of aqueous ethanol used, differences in protein solubility can occur within the soluble/insoluble fractions based on the protein's hydrophobicity.

Depending on the mode of extraction, different protein compositions are achieved leading to differing functionality in the final powdered ingredient. For instance, Papalamprou *et al.* (2009, 2010) and Kiosseoglou and Paraskevopoulou (2011) reported that isolates prepared using ultrafiltration contain a mixture of both globulin and albumin-type proteins, whereas those prepared by isoelectric precipitation are dominated by only the globulin-type proteins. Stone *et al.* (2015) stated that both globulin and albumin-type of proteins were present in their isolate prepared via salt extraction. Micellular precipitated protein undergoes less denaturation compared to protein precipitated in alkaline extraction with isoelectric precipitation (Stone *et al.*, 2015).

With alkaline extraction, the recovery methods may influence the protein properties and functionality. Ultrafiltrated pea protein and red lentil protein were more soluble at neutral pH and capable of holding more oil compared to isoelectric precipitated protein isolates (Boye *et al.*, 2010b). These different recovery methods did not significantly affect the proteins' water holding capacity, foaming properties and emulsifying properties (Boye *et al.*, 2010b). Among alkaline extraction, salt extraction and micellular precipitation, micellular precipitated pea protein had the lowest solubility (46%) while salt extraction had the highest (89%) (Stone *et al.*, 2015). Salt extracted pea protein isolate had the highest oil holding capacity, while micellular precipitated protein had the highest water holding capacity (Stone *et al.*, 2015). Pea protein extracted with salt extraction had the highest foaming capacity (163.3%–263.3%) compared to isoelectric precipitation (155.0%–183.3%) and micellular precipitation (133.3%–193.3%); while isoelectric precipitated pea protein isolate generated more stable foam (68.0%–69.2%) than the other two methods. Meanwhile, Can Karaca *et al.* (2011) produced several different pulse proteins with better solubility (61.42%–96.53%) via isoelectric precipitation than the proteins produced via salt extraction (solubility 30.16%–96.8%). In the aspect of emulsion properties, salt extracted proteins were not as efficient as isoelectric precipitated proteins as the emulsion prepared with salt extracted protein had lower emulsion activity index, shorter emulsion stability index, and bigger droplet size (Can Karaca *et al.*, 2011).

2.3 Protein quality

Protein quality refers to the amount of essential amino acids within the protein itself, as well as their bioavailability or ability for the protein to be uptaken into the metabolic processes (Nosworthy *et al.*, 2017). In contrast to animal-derived proteins, pulse proteins have a lower protein quality (Tiwari and Singh, 2012). Essential amino acids include histidine, isoleucine, leucine, methionine, lysine, phenylalanine, threonine, tryptophan and valine. They also include arginine, cysteine and tyrosine for immune compromised individuals and infants. Amino acid score is used to evaluate a protein's amino acid composition by comparing the mg of essential amino acids in 1 g of the protein of interest to that the amino acid reference pattern of 2-5-year-old children (FAO, 1991). The amino acid with the lowest score below 1.0 in the test protein is considered as the first limiting amino acid in this protein. In pulse proteins, the first limiting amino acids are thiol-containing amino acids (*e.g.*, cysteine and methionine) or tryptophan, while in

cereal, lysine is the first limiting amino acid (Asif *et al.*, 2013). Amino acid pattern and amino acid score of common pulses and cereals are listed in Table 2.3.

Besides the incomplete amino acid profile, pulses also contain bioactive compounds that can adversely affect protein and carbohydrate digestion, and mineral absorption. For instance, phenolic compounds act to cross-link proteins to reduce their digestibility, whereas the presence of enzyme inhibitors (trypsin, chymotrypsin and amylase inhibitors) can reduce the activity of digestive enzymes for proteins and carbohydrates (Dahl *et al.*, 2012; Nosworthy *et al.*, 2017). Phytates and oxalates can act as chelators to reduce mineral absorption (Dahl *et al.*, 2012), whereas lectins can cause diarrhea, vomiting, bloating and red blood cell agglutination (Roy *et al.*, 2010). The presence of oligosaccharides can be fermented by bacteria within the gastrointestinal tract leading to the production of gas, abdominal pain and flatulence (Granito *et al.*, 2005). And in the case of faba beans, the presence of vicine and convicine, which are glycosides, can be associated with favism in certain individuals (McMillan *et al.*, 2001). Processing, such as extrusion (Nosworthy *et al.*, 2017), roasting (Nosworthy *et al.*, 2017; Khattab *et al.*, 2009), boiling (Nosworthy *et al.*, 2017; Khattab *et al.*, 2009), fermentation (Granito *et al.*, 2005; Khattab *et al.*, 2009), autoclaving (Khattab *et al.*, 2009), microwave (Khattab *et al.*, 2009), infrared heating (Khattab *et al.*, 2009) and so on, can be used as a method to reduce or eliminate levels of these bioactive compounds. Processing also improves protein digestibility by causing changes to the protein's quaternary and tertiary structures during partial denaturation (Park *et al.*, 2010). For instance, Park *et al.* (2010) found that cooked peas had an average increase of *in vitro* protein digestibility of 4.1% as compared to raw peas. However, not all conditions lead to a positive impact on protein quality and accordingly are process and pulse dependent. Carbonaro *et al.* (1997) compared the *in vitro* protein digestibility between some raw and cooked pulses and observed an improvement in chickpea and dry bean, whereas digestibility declined after cooking for faba beans and lentils. The authors hypothesized that the addition of heat also resulted in a greater amount of protein-protein aggregation and disulfide bond formation which lead to reduced digestibility overall, despite the reduction in protease inhibitors (Carbonaro *et al.*, 1997).

Table 2.3. Amino acid pattern (mg amino acid per g of protein) (USDA database, 2015) and amino acid score (comparing against FAO 1991 reference pattern) of common pulses and cereals.

	Yellow Pea	Green Lentil	Faba Bean	Chickpea	Brown Rice	Wheat	Corn	Egg	FAO 1991 Reference Pattern
THR	35	36	36	37	37	28	38	44	34
VAL	45	50	44	42	59	44	51	68	35
MET+CYS	20	22	21	27	35	41	39	52	25
ILE	43	43	40	43	42	35	36	53	28
LEU	73	73	75	71	83	67	123	86	66
PHE+TYR	72	76	74	79	89	76	90	94	63
HIS	77	70	64	67	38	26	28	73	58
LYS	35	36	36	37	37	28	38	44	34
TRP	7	9	9	10	13	13	7	13	11
Amino acid score	0.73	0.72	0.84	0.89	0.66	0.45	0.49	1.00	N.A.
limiting amino acid	TRP	MET+CYS	MET+CYS	TRP	LYS	LYS	LYS	N.A.	N.A.

Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan).

Both amino acid profile and protein digestibility are directly or indirectly considered for protein quality assessment. In Canada, Protein Efficiency Ratio (PER) is used to evaluate protein quality by measuring the unit weight gain of a male rat after consuming the test protein (Health Canada, 1981). Both amino acid composition and protein digestibility can affect the result of this evaluation. Protein rating is calculated by multiplying PER with protein in a reasonable daily intake (Canadian Food Inspection Agency, 2018). In Canada, food products with a protein rating with 20 to 40 can be claimed as a 'source of protein', and product with a protein rating with 40 or above can be claimed as 'high in protein' (Canadian Food Inspection Agency, 2019). Meanwhile, Protein Digestibility Corrected Amino Acid Score (PDCAAS) is used in US to assess protein quality. PDCAAS is calculated by multiply the first limiting amino acid score by true fecal protein digestibility. True fecal protein digestibility as well as PER are both *in vivo* methods, which involve the use of animals (*e.g.*, rats or pigs) (Nosworthy *et al.*, 2017). Although more accurate than *in vitro* methods, *in vivo* methods are more expensive, labour-intensive, time-consuming and involving ethical issues regarding to animal testing. Hsu *et al.* (1977) found that there was a good correlation between the digestibility predicted with an *in vitro* multienzyme system and the true fecal digestibility (correlation coefficient = 0.9). This multienzyme system was sensitive enough to differentiate the influence of trypsin inhibitor, chlorogenic acid and thermal process (Hsu *et al.* 1977). This ecomonic, time-saving, and accurate *in vitro* method tend to be favoured. Currently, the PDCAAS method is approved for use by the FAO/WHO as the international standard, however, the DIAAS method is being considered for its replacement since it allows high-quality protein sources to be better differentiated (Nosworthy *et al.*, 2017).

2.4 Pulse protein functionality

*(a) Protein solubility***.** The solubility of a protein is related to both intrinsic and extrinsic factors. The former relates to the inherent characteristics of the protein itself, including amino acid composition and sequence, surface hydrophobicity and hydrophilicity, molecular mass, isoelectric point, protein conformation, protein profile, etc. (Zayas, 1997). In general, smaller proteins with higher surface charge tend to be the most soluble. However, this trend depends highly on the extrinsic environment the protein is in (Tiwari and Singh, 2012). Extrinsic factors include: solvent type, pH and ionic strength of the medium, along with other ingredient interactions such as with lipid or polysaccharides (Zayas, 1997). Protein solubility is correlated with protein surface charge;

therefore, solubility in pulse proteins tend to be greatest at pHs away from the isoelectric point where charge of protein and electrostatic repulsion is highest, and lowest at pHs close to the isoelectric point (close to ~4.5) were repulsion is minimal and aggregation is favoured because of non-covalent attractive forces and hydrophobic interactions (Hall, 1996; Can Karaca *et al.*, 2011). The presence of some salts can improve solubility through the 'salting in' effect or decrease solubility through the 'salting out' effect or by charge screening to promote protein aggregation (Hall, 1996; Martínez-Maqueda *et al.*, 2013). As a result, the same protein can behave differently in various applications. Protein solubility is considered as an index for determining protein functionality, as it can affect other functional properties including foaming and emulsifying (Toews and Wang, 2013). The high solubility of a protein ingredient widens the range of applications considerably. Boye *et al.* (2010b) concluded that greatest solubility of pulse protein was observed at pH away from the protein's isoelectric point at pH 1 to 3 and pH 7 to 10. Chickpea protein isolate showed greater solubility (60% and 65%) at pH 1 and 10, respectively, and minimal solubility at pH 4 to 6 (less than 10%). Similarly, the same authors found greater solubility (90% and 70%) of pea protein at pH 1 and 9, respectively, and nearly zero solubility at pH 4 to 6. Tiwari and Singh (2012) stated thermal treatment including moist and dry heat treatment, retort, extrusion and boiling could result in a decrease in protein solubility, whereas Beck *et al.* (2017) observed in their study that dry heat treatment at 130°C for 20 minutes decreased the protein solubility by 80%.

(b) Water and oil holding capacities. Water hydration (WHC) and oil holding (OHC) capacities of protein ingredients refers to the amount of water (or oil) that can be held by 1 g of protein (or protein ingredient) (Boye *et al.*, 2010a), and tends to be related to the amount of hydrophilic and hydrophobic amino acids on the surface, respectively and the capillaries formed within the protein aggregates (Tiwari and Singh, 2012; Toews and Wang, 2013). These properties are also related to particle size, where smaller particles tend to bind more water (or oil) due to their greater surface area for wetting. The ability of a protein to tie up water or oil helps to prevent cook loss from occurring within a product during processing or storage, and improves tenderness and moisture retention (Zayas, 1997; Boye *et al.*, 2010a). WHC has been reported for pea flour (0.76g/g) (Ma *et al.*, 2011), chickpea flour (0.8 g/g) (Ma *et al.*, 2011), lentil (0.68 g/g) (Ma *et al.*, 2011), faba bean (0.72 g/g) (Sosulski and McCurdy, 1987) and navy beans (1.39 g/g) (Du *et al.*, 2014). In contrast, OHC has been reported for pea (1.75/g) (Ma *et al.*, 2011), chickpea (1.78 g/g) (Ma *et al.*, 2011), lentil (1.7 g/g) (Ma *et al.*, 2011), faba bean (0.47 g/g) (Sosulski and McCurdy, 1987) and

navy beans (1.15 g/g) (Du *et al.*, 2014).

(d) Foaming properties. Foams are described as air-in-water emulsions, where air represents inclusions in the continuous water phase containing the protein. Foams are generated through the addition of mechanical energy in the form of high shear from sparging, homogenization or whipping (Zayas, 1997). During this process, proteins migrate from the water phase to the air-water interface where they then reorient to position the hydrophobic moieties towards the gas phase and hydrophilic moieties towards the water phase to lower the interfacial tension (Boye *et al.*, 2010a). Proteins at the interface (also known as the lamalle in foams) then act to form a viscoelastic film around the air bubbles to offer a physical barrier (Zayas, 1997). Foam stability is found to be the best near the isoelectric point of the protein, where the lack of electrostatic repulsion leads to a greater amount of protein-protein interactions and network formation in-between neighboring bubbles (Lam *et al.*, 2017). This network also helps inhibit draining of the continuous phase, which would ultimately lead to foam breakdown. The major driver leading to the destabilization of a protein-based foam is Oswald Ripening, a process whereby small air bubbles diffuse through the continuous phase and absorbs with a larger air bubble to make that one bigger (Damodaran, 2005). Foams are characterized by their foaming capacity, which refers to the volume of foam generated after mechanical shear for a given amount of protein, whereas foam stability refers to the ability of the foam to retain its structure and resist separation over time (Boye *et al.*, 2010a). Foaming capacity has been reported for pea protein (150%) (Toews and Wang, 2013), defatted chickpea protein (201%) (Toews and Wang, 2013), lentil protein (414 %) (Toews and Wang, 2013), faba bean (162 %) (Singhal *et al.*, 2016) and navy beans protein (622 %) (Toews and Wang, 2013). In contrast, foaming stability has been reported for pea protein (55%) (Toews and Wang, 2013), defatted chickpea protein (81 %) (Toews and Wang, 2013), lentil (80 %) (Toews and Wang, 2013), faba bean (65%) (Singhal *et al.*, 2016) and navy beans (63%) (Toews and Wang, 2013).

(e) Emulsifying properties. An emulsion refers to a thermal dynamically unstable mixture of an oil and water phase, in which one phase becomes dispersed within the continuous phase of the other in response to mechanical shear (*e.g.*, homogenization or ultrasonication) and in the presence of an emulsifier (*e.g.*, protein) (Damodaran, 2005). During emulsion formation, proteins act to migrate in the water phase to the oil-water interface, where like foams, proteins re-orient such that the hydrophobic moieties are positioned towards the oil phase and the hydrophilic

moieties are positioned towards the water phase. Protein-protein interactions then occur to form a viscoelastic film that protects against droplet coalescence (Kiosseoglou and Paraskevopoulou, 2011). Emulsions are most stable at pHs away from the isoelectric point where electrostatic repulsion is significant between droplets (McClements, 2004). The presence of salts increases ionic strength and acts to destabilize emulsions by screening charges on the protein's surface to allow for closer interactions and droplet flocculation and coalescence (McClements, 2004). Emulsion capacity measures the amount of oil that can be stabilized in an emulsion mixture per g of protein material, while emulsion activity index measures the total surface area of the oil droplets being stabilized in the emulsion (Nickerson, 2016; Tiwari and Singh, 2012). The stability of an emulsion over time is measured as emulsion stability index. (Tiwari and Singh, 2012). Emulsion capacity has been reported for isoelectric precipitated protein from pea (477.78 g/g), chickpea (504.43 g/g), lentil (484.44 g/g), and faba bean (513.33 g/g) (Can Karaca *et al.*, 2011). While, the emulsion activity index has been reported by Can Karaca *et al.* (2011) for pea (42.9 m^2/g), chickpea (47.9 m^2/g), lentil (44.51 m^2/g), and faba bean (44.3 m^2/g) proteins. And the emulsion stability index has been reported for pea protein (12.4 min), chickpea protein (82.9 min), lentil protein (86.8 min), and faba bean protein (69.4 min) (Can Karaca *et al.*, 2011).

2.5 Pulse flavours

Despite the nutritional and functional benefits of pulse ingredients, their widespread integration into the food industry has been hindered by their beany and/or grassy tastes, or aromas given off during cooking (Ma *et al.*, 2011). There are both volatile and non-volatile compounds contribute to the undesired flavour of pulses. Volatile compounds in pulses, such as aldehydes, ketones, alcohols and others, are often described as "green", "beany", "earthy", or "hay-like"; while the non-volatile compounds, including isoflavones, saponins, phenolic acids or peptides contribute to the bitterness and astringency in pulses (Roland *et al.*, 2017).

There are several classes of volatile compounds that were extracted from pulses in pervious literatures: alcohols, aldehydes, hydrocarbons, ketones, sulphur compounds, esters and pyrazines (Table 2.4.). For instance, Azarnia *et al.* (2011a) reported for pea, the composition of total volatile compounds extracted include: 19.9%–25.1% alcohols, 2.0%–2.5% aldehydes, 14.8%–20.2% hydrocarbons, 1.9%–2.1% ketones, 13.7%–16.6% sulphur compounds, 1.6%–2.9% esters and 1.0%–2.0% pyrazines.

Table 2.4. Flavour related studies involving pulses found in the literature.

Pulses	# of Compounds Identified	Compounds chemical Families	Reference
Frozen fresh green peas	22	Alcohols	Murray *et al.*, 1968
Uncooked dry beans	23	Alcohols and aldehydes	Lovegren *et al.*, 1979
Frozen fresh green peas	47	Aldehydes, ketones, alcohols, esters and pyrazines	Jakobsen *et al.*, 1998
Dry field peas	N/A	Alcohol, aldehydes, hydrocarbons, ketones, sulphur compounds, terpenes, esters and pyrazines	Azarnia *et al.*, 2011a
Dry field peas	32	Alcohol, aldehydes, hydrocarbons, ketones, sulphur compounds, alkanes, terpenes, esters and pyrazines	Azarnia *et al.*, 2011b
Dry pea flour	73	Alcohol, aldehyde, alkane, alkene, benzene derivative, ketone	Murat *et al.*, 2012
Low-tannin faba bean	45	Alcohols, hydrocarbons, aldehydes, alkanes and ketones	Oomah *et al.*, 2014
Navy bean, red kidney bean, green lentil, yellow pea with different heat treatment	79	Alcohol, aldehyde, alkane, aromatic compounds, terpenes, ester, sulphur compounds and nitrogen compounds	Ma *et al.*, 2016

Although the fat content in pulses is generally low, the decomposition of unsaturated fatty acids after harvesting, during process, and storage results in significant amounts of volatile flavour compounds being generated including alcohols, aldehydes, hydrocarbons, and ketones (Azarnia *et al.*, 2011a, Roland *et al.*, 2017). Lipid can be hydrolyzed by the lipolytic enzyme, such as lipase,

to free fatty acids, which will be more readily to be oxidized (Azarnia *et al.*, 2011a). The oxidation of unsaturated fatty acids can be both enzymatic and nonenzymatic, however the enzymatic degradation involves lipoxygenase (LOX) is believed to be the major mechanism generating undesired volatile compound in pulses (Roland *et al.*, 2017). Hydroperoxides, the initial products of LOX-catalyzed reaction, will be further degraded into various volatile compounds (Azarnia *et al.*, 2011a, Roland *et al.*, 2017).

According to Azarnia *et al.* (2012) and Malcolmson *et al.* (2014), the total volatile compounds in pea can be influenced by crop year, growing region, cultivar, and marketing class. A cultivar with lower volatile compounds can be a breeding target to produce raw material with a milder off-flavour. In additional, a cultivar with lower enzymatic activity can result in crop and ingredient with low off-flavour precursors, as lipid oxidization during harvest, process and storage would be limited. There were cultivars of soybeans with low-LOX or no LOX activity being recognized and selected for flavour improvement in applications (Roland *et al.*, 2017; Schindler *et al.*, 2012). Storage temperature also can have a significant effect on pulse flavour development post-harvest. At 4℃, there were minimum volatile flavour development due to low enzymatic activity (Azarnia *et al.*, 2011a). Meanwhile, LOX and lipase were more active at 22℃ and 37℃ which resulted in significant higher total volatile compounds. Among all the chemical families, aldehydes had the greatest increase in relative peak area at higher storage temperature (Azarnia *et al.*, 2011a).

However, breeding and storage control may not be sufficient to eliminate the off-flavour in pulses, there are several technologies can be used to treated whole pulse or pulse ingredients to reduce or mask the undesired pulse flavour.

2.6 De-flavouring strategies

2.6.1 Thermal treatments

Ma *et al.* (2016) evaluated the volatile compound modification in pulses including navy bean, red kidney bean, green lentil and yellow pea after cooking and drying. In this study, the pulses were boiled in water and dried with freeze drying or spray drying. The volatile compounds were greatly removed from green lentil with boiling and both drying methods. In navy bean and yellow pea, the total volatile compounds were reduced significantly after spray drying and slightly after freeze drying. After spray drying, the total volatile compounds remained the same in red

kidney bean. Boiling and freeze-drying removed a statistically significant amount of volatile compounds from red kidney beans.

The total amount of each chemical family either increases or decreases after the treatments, which depends on the cooking/drying methods and pulse species. Alcohols in all pulses are greatly decreased after spray drying but remained the same after freeze drying. Ma *et al.* (2016) suggests that the difference in formation and removal rates of alcohol during the cooking and drying treatments result in the various quantitative changes of total alcohol. The enzyme, alcohol oxidoreductase, involved in the alcohol formation in pulses is heat sensitive and becomes inactive after heat treatment. Meanwhile, lipoxygenase, an enzyme-aided in aldehyde and ketone formation, is heat stable and remained active after 40 minutes under 80°C (Ma *et al.*, 2016). The relative peak area of aldehydes increases remarkably in all pulses after spray drying, while relative peak area of aldehydes in pulses remains statistically the same after freeze drying. However, ketones are absent in navy bean, green lentil, and yellow pea after freeze-drying (Ma *et al.*, 2016). There is no pyrazine detected in raw pulses, while after roasting, there are several different pyrazines found in navy bean, green lentil and yellow pea due to Maillard reaction. Pyrazines provide a roasted nut and chocolate flavour, which is a pleasant and desired flavour profile (Ma *et al.*, 2016).

Although thermal treatments can alter flavour compound profile by removing undesired compounds and generated desired ones, the process can impact protein functionality in a positive, neutral or negative way, as proteins partially denature and unravel to expose buried reactive groups to give new surface properties (Pietrysiak *et al.*, 2018; Ma *et al.*, 2011). Pietrysiak *et al.* (2018) reported the functionality of a pea-rice protein isolate blend to improve after direct steam injection, where solubility increased from 3% to 41% at neutral pH, the emulsion activity index increased from 5.9 to 52.5 m^2/g, the foaming stability (after 30 min) increased from 68.2 to 82.8%, and the OHC increased from 1.8 to 4.9 g/g (Pietrysiak *et al.* 2018). However, Ma *et al.* (2011) reported after boiling and freeze-drying, protein solubility in lentils, chickpeas and pea decreased. Meanwhile, OHC and WHC were improved after cooking. OHC of lentil flour and yellow pea flour increased from 1.7 to 2.9 g/g and 1.7 to 2.3 g/g respectively. The increased porous structure of the flour and gelatinized starch granule in the flour was thought to contribute to the increased water holding capacity. WHC of lentil flour increased from 0.6 to 1.6 g/g, and that of pea flour increased from 0.8 to 1.8 g/g. Emulsion activity index increased in pea flour after cooking from 13.0 to 23.0 m^2/g, while emulsion stability was not affected. Foaming expansion was negatively

impacted by the cooking process. With boiled and dried chickpea and pea, there was no measurable foam formation (Ma *et al.*, 2011).

2.6.2 Germination

Germination has been used as a technology to reduce the antinutritional factors and improve nutritional quality of pulses (Roland *et al.*, 2017, Xu *et al.*, 2019). Germination has also shown improvement on the aroma and flavour profile in lupin and soybean flours as well as muffins enriched with these flours (Kaczmarska *et al.*, 2017, Kaczmarska *et al.*, 2018). However, the findings regarding modification on flavour profile is not consistent, which is largely depend on the type of pulses being germinated and the analytical method. Troszynska *et al.* (2011) found after a 7-day germination, the beany and green taste in green lentil were decreased, while the astringency and bitterness increased according to the sensory evaluation. Xu *et al.* (2019) employed headspace solid phase microextraction gas chromatography mass spectrometry/olfactory (HS-SPME-GC-MS/O) and found volatile compound responsible for beany flavour, such as hexanal and 1-hexanol, started to increase on the second day of the germination in green lentil and yellow pea. The rising level of lipids decomposed compounds found in germinated peas and lentils was a result of the increased activity of LOX. Based on the principal component analysis and hierarchical cluster analysis, Xu *et al.* (2019) selected hexanal (grassy flavour), 1-hexanol (green flavour), 2-pentylfuran (green bean flavour) and (E,E)-2,4-nonadienal (rancid flavour) as quantifiable markers can be used to represent beany flavour development in yellow pea and green lentil. During germination, the increase of LOX activity was responsible for the development of flavour precursors, but the alteration of macronutrient structure also made the flavour compounds more susceptible to be released (Xu *et al.*, 2019). Lipolysis, amino acid degradation and hydrolysis of starch and protein can be reason for increased beany flavour in germinated legumes (Xu *et al.*, 2019).

2.6.3 Fermentation

Schindler *et al.* (2012) investigated the possibility of using *L. plantarum* and *P. pentosaceus* to ferment pea protein isolate to improve the ingredient's flavour profile. Although the pasteurization before the fermentation generated a significant amount of hexanal, the hexanal content in the pea protein isolate after 48-hour fermentation was similar to that in the untreated

pea protein isolate. During fermentation, volatile compounds in pea protein isolate were not eliminated, but the volatile compound profile was altered. The more pleasant volatile compound(s) developed during fermentation facilitates the masking of the undesired pulse flavour and improves the overall ingredient flavour (Schindler *et al.* 2012, Roland *et al.*, 2017).

2.6.4 Solvent washing

Aqueous-ethanol washing is commonly applied in soy protein concentrate production to remove oligosaccharides, minerals, soluble nitrogenous components, and other constituents (Peter, 2019). As many flavour precursors are in the chemical families of alcohols, aldehydes, ketones, esters, and pyrazines, and are soluble in polar solvents, this protein concentrating process employing alcohol washing has showed effect in improving flavour as well. Several studies demonstrated that aqueous-alcohol solvents could improve the flavour profile by removing flavour compounds in lentil protein isolate, soybean, peanut and groundnut (Chang *et al.*, 2019, Eldridge *et al.*, 1971; Johnson *et al.*, 1979; Madhavi *et al.*, 1989). Eldridge *et al.* (1971) found that flavour of pentane: hexane-defatted soybean flakes was improved after 6-h of Soxhlet extraction. The intensity of the beany, bitter, astringent, cereal and cardboardy flavour was also reduced. Flavour scores of the defatted soybean flakes were increased from 4.2–4.3 to 7.0–7.2, 6.1–6.2 and 5.0–5.4 after washing with hexane: ethanol azeotrope, hexane: methanol azeotrope, and hexane: 2-propanol azeotrope, respectively. Solvents with higher polarity were used to wash the hexane-extracted peanut flour to remove residual lipid (Johnson *et al.*, 1979), reducing the total lipid levels from 0.75% to 0.18%–0.28% along with the green and beany flavours. According to the panelists, odour and flavour scores for the peanut flour significantly increased after 6-h extraction with absolute ethanol, hexane: ethanol azeotrope and hexane: methanol azeotrope; whereas the extraction with hexane: propanol azeotrope did not improve the flavour and odour of the peanut flour (Johnson *et al.*, 1979). Absolute ethanol, 95% isopropanol, hexane: ethanol azeotrope, and hexane: isopropanol azeotrope was used as secondary extraction solvents to remove the nutty flavour in groundnut seed. After 16-h of extraction, absolute ethanol removed 90.8% of the nutty note in groundnut seed followed by 95% isopropanol, hexane: ethanol and hexane: isopropanol removing, 86.6%, 76.9% and 70.8% of the nutty flavour respectively (Madhavi *et al.*, 1989). Chang *et al.* (2019) utilized acetone, ethanol and isopropanol to improve the flavour profile of lentil protein isolate (LPI). They found that there were significantly more volatile compounds

generated after the acetone treatment, especially ketones; while both ethanol and isopropanol at concentration 35% to 75% showed proficiency in removing off-flavour compounds from LPI.

Although solvent washing can remove these compounds, the process can adversely impact ingredient functionality. Both hexane: methanol azeotrope extracted peanut flour and soybean flakes showed the lowest nitrogen solubility index (NSI) among all extracted samples in the formerly mentioned studies. Meanwhile, the NSI of peanut flour and soybean flakes decreased much less after hexane: ethanol azeotrope and hexane: propanol azeotrope extraction (Johnson *et al.*, 1979; Eldridge *et al.*, 1971). Protein solubility of groundnut flour was not affected after the secondary extraction at pH 4, 7 or 9 (Madhavi *et al.*, 1989). The foaming capacity of peanut flour increased 200% after a secondary wash. However, Johnson *et al.* (1979) concluded this was because of the removal of lipid residue in the hexane-defatted peanut flour. Lipid in the flour limited the foam expansion; therefore the improvement of foaming capacity was not related to the interaction between the solvent and protein. Chang *et al.* (2019) observed decreased in LPI protein solubility after solvent treatments, while the LPI treated with 75% ethanol and isopropanol had the least decline in solubility and emulsion stability remained the same when compared to the untreated LPI.

2.7 Gas chromatography - mass spectrometry (GC-MS) with headspace solid phase micro-extraction (HS-SPME) for volatile compounds evaluation

Sensory evaluation conducted by selected panelists as well as analytical instruments, such as gas chromatography (GC), can be used to investigate the flavour profile and modification in products (Azarnia, *et al.*, 2012). A mixture of volatile compounds extracted from a food product can be separated in the GC column due to their various affinity towards the stationary phase inside of the column. The isolated compounds will exist the column with different retention time can be identified by a mass spectrometry (Azarnia *et al.*, 2012).

Quantity of volatile compounds in pulse protein concentrate is too little to be detected by GC directly, therefore an extraction and concentration of volatile compounds from pulse protein concentrate is necessary for accurate GC detection. Headspace solid phase micro-extraction (HS-SPME) appears to be a suitable technique for flavour compounds extraction, as it is reproducible, simple, fast, solvent-free and relatively cheap (Ducki, 2008, Azarnia 2012). HS-SPME has been widely used in analysis flavour compounds in various food products including wine,

cocoa/chocolate, legume, and dairy products (Ducki, 2008, Azzi-Achkouty, 2017, Azarnia 2012,). A coated silica fiber is exposed to the headspace of the sample for certain amount of time until the equilibrium between sample, headspace and fiber is reached.

Excessive time and temperature will not help increase the quantity of analytes absorbed into the fiber but may cause analyte desorption from the fiber and therefore reduce the analytes intensity (Azarnia, 2011b). Excessive heat may also induce enzymatic reaction and chemical changes which affects the results (Ducki, 2008). After extraction, the SPME fiber will be injected into a split injector connected to GC column. Analytes will be desorbed into GC column with high heat (Azarnia, 2011b).

Coating (stationary phase) on the SPME silica fiber affects the absorption of compounds. Several studies compared the sample extraction condition with PDMS (Polydimethylsiloxane), CAR-PDMS (Carboxen-polydimethylsiloxane), PDMS-DVB (Polydimethylsiloxane-divinylbenzene) and DVB/CAR-PDMS fibers (Divinylbenzene/Carboxen- Polydimethylsiloxane). PDMS alone favored non-polar compounds and resulted in very few flavour compounds with low intensity detected. CAR-PDMS was the most polar fiber and absorbed more volatile compounds but few semi-volatile compounds, while PDMS-DVB trapped more semi-volatile compounds and fewer volatile compounds (Ducki *et al.*, 2008; Xu *et al.*, 2019 Azarnia et al, 2011a). The DVB/CAR-PDMS fiber was demonstrated to extract both volatile and semi-volatile compounds with similar efficiency as this dual-layered fiber had characteristic of both CAR-PDMS and PDMS-DVB fiber. Flavour compounds include both volatile and semi-volatile compounds with molecule weight range from 40 to 275 (Ducki et al, 2008; Supelco, 2018). In addition, Xu *et al.* (2019) found that although CAR-PDMS gave larger peak area when compared to DVB/CAR-PDMS, however the latter one extract more identifiable compounds than the more polar fiber. As a result, DVB/CAR-PDMS was concluded to be the most suitable fiber for flavour compounds extraction for pulse ingredients (Xu *et al.*, 2019; Azarnia *et al.*, 2011a).

The condition of the sample can affect the release of volatile compounds from sample. Ducki *et al.* (2008) found significantly higher total peak area of alcohol and acid extracted from dry cocoa powder compared to those from wet cocoa slurry with salt, while peak area of aldehydes and ketones was found to be lower in dry condition than in brine condition. In addition, more volatile compounds from all groups were extracted from cocoa powder in salted water compared to distilled water.

3. MATERIALS AND METHODS

3.1 Materials

Two separate lots (lot 1 produced on April 20, 2017 and lot 2 produced on July 12, 2017) of commercial air-classified PPEF were obtained from AGT Food and Ingredients (Regina, SK, Canada). Ethanol and isopropanol (at 95% v/v) were purchased from Fisher Scientific (Ottawa, ON, Canada). Milli-Q water, generated via a Millipore Milli-Q™ water purification system (Millipore Corporation, Milford, MA, USA), was used in this study.

3.2 Aqueous alcohol washing

Aqueous alcohol washing of PPEF was performed using diluted alcohols (ethanol and isopropanol) at different concentrations (20, 50 and 80% v/v, with water) using the method mentioned by Chang *et al*. (2019) with minor modifications. Pea protein dispersions were prepared by mixing the PPEF with aqueous ethanol or aqueous isopropanol at a solid-to-liquid ratio of 1:10 (w/v) at 600 rpm for 5 min, followed by shaking on a shaking plate (at 500 rpm) for 1 h at room temperature (20°C). The alcohol-washed samples were obtained after centrifugation (Sorvall RC Plus Superspeed Centrifuge, Thermo Fisher Scientific, Asheville NC, USA) at 4000 × *g* for 20 min at room temperature (20°C), followed by vacuum drying of the precipitate (Model 5861 vacuum oven, NAPCO Scientific Co., USA) at 50°C overnight. The dried sample was milled into powder form with an analytical grinder (Model A11, IKA Works Inc., USA) and sifted through 50 mesh (Retsch GmbH, German).

3.3 Physicochemical properties

Composition

Proximate analysis of untreated and alcohol-washed PPEF samples was carried out according to the Association of Official Analytical Chemists (AOAC) official methods 925.10, 923.03, and 920.85, 920.87 (%N x 6.25), 984.13A for moisture, ash, protein and crude fat, respectively (AOAC, 2005). Ash, fat and protein values are reported on a % dry weight basis.

Colour

The colour of the untreated and alcohol-washed PPEF samples was measured using a Colorimeter (ColorFlex EZ 45/0, Hunter Associates Laboratory, Inc., Reston, VA, USA) as L* [lightness], a* [red (+)- green (-))] and b* [yellow (+)- blue(-)]. Colour change between untreated and treated PPEF was indicated by ΔE calculated according to the equation below.

$$\Delta E = \sqrt{(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2} \quad \text{(Eq. 1)}$$

Surface charge

The surface charge of all untreated and treated PPEF samples was determined by measuring the electrophoretic mobility (U_E) using a Zetasizer Nano (Malvern Instruments, Westborough, MA, USA) according to Stone *et al.* (2015) at a concentration of 0.05% (w/w) under pH 7.0. U_E is the velocity of a particle within an electric field and is related to the zeta potential (ζ), which can be determined using the Henry equation:

$$U_E = \frac{2\varepsilon \,.\, \zeta \,.\, f(\kappa\alpha)}{3\eta} \quad \text{(Eq. 2)}$$

where η is the dispersion viscosity, ε is the permitivity, and $f(\kappa\alpha)$ is a function related to the ratio of the particle radius (α) and the Debye length (κ). $f(\kappa\alpha)$ equaled 1.5 using the Smoluchowski approximation.

3.4 Determination of volatile compound profile

Headspace solid phase microextraction (HS-SPME) was applied to extract flavour compounds from the PPEF samples (untreated and alcohol-washed) using 2-cm-long, 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME StableFlex™ fibres (Supelco, Bellefonte, PA, USA). Briefly, the PPEF (1.5 g) was dispersed in saturated NaCl solution (6 g) for 1 h with stirring at room temperature. The protein solution (5 g) was then transferred into an amber bottle (10 mL) and incubated at 50°C in an ultrasonic water bath for 25 min to release the volatile compounds. After incubation, the SPME fibre was inserted into the headspace of the sample vial to absorb the volatile compounds in the bottle at 50°C for another 20 min. After extraction, the volatiles were thermally desorbed at 250°C for 3 min in the injection

port of a gas chromatography-mass spectrometer (GC-MS, GC: 7890A, Agilent Technologies, USA; MS: AccuTOF 4G GCv, Jeol, Japan). The volatile compounds were analyzed via an Agilent DB-5MS column (30 m × 0.25 mm, 0.25μm film thickness, Agilent Technologies, USA) via a slightly modified version of Oomah *et al.* (2014), described in detail by Chang *et al.* (2019). The identification of volatile compounds was performed using the National Institute of Standards and Technology NIST (v. 02) and Wiley (v. 138) libraries. The concentrations of volatile compounds in untreated and alcohol-washed PPEF samples were expressed as the total peak area. One measurement was made on each of duplicate washes from both lots (*i.e.*, four samples). Data was reported as the mean ± one standard deviation (n = 4).

3.5 Protein functionality

Solubility

Nitrogen solubility was tested according to the method of Can Karaca *et al.* (2011) on all samples (untreated, 20%, 50% and 80%, w/v, alcohol-washed). In brief, 0.2 g of sample was dispersed in 19.8 mL of 10 mM sodium phosphate buffer at pH 7.0. The 1.0% solution was stirred overnight at 4^{o}C to extract the soluble portion of the protein into the solution. On the second day, the solution was centrifuged to separate the insoluble components and the supernatant. Nitrogen solubility was calculated as the portion of nitrogen in the supernatant divided by the total nitrogen in the sample. The nitrogen content (which indicates the protein content) of the supernatant was measured using a micro-Kieldahl unit. The nitrogen solubility was calculated using Eq. 3.

$$\text{Nitrogen solubility} = \frac{nitrogen\ content\ of\ the\ supernatant}{nitrogen\ content\ of\ the\ sample} \times 100\% \qquad \text{(Eq. 3)}$$

Water hydration and oil holding capacities

Water hydration capacity (WHC) and oil holding capacity (OHC) were determined by mixing 1.0 g of sample in 10.0 g of water/oil in a weighed 15-mL, screw-cap centrifuge tube, according to Stone *et al.* (2015). Samples were vortexed for 10 s or until no dry powder was visible. Then samples were vortexed every 5 min for 30 min in total and lastly centrifuged (VWR clinical centrifuge 200, VWR International, Mississauga, ON, Canada) for 15 min at 2,000 × *g*. After removing the top liquid, the weights of the tube and the wet sample were recorded. The WHC and OHC were reported as the amount of water/oil absorbed per g of sample.

Emulsion stability by creaming

Emulsion stability (ES) was determined according to Stone *et al.* (2015) with modifications. Protein solutions were prepared by mixing 2% protein (w/w) in Milli-Q water. An 80:20 (8 mL protein solution: 2 mL canola oil) oil-in-water emulsion was made in a 50-mL, screw cap centrifuge tube by homogenizing with an Omni Macro homogenizer at 15,000 rpm for 5 min with a 20-mm saw-tooth generating probe positioned at the oil-water interface. Immediately after homogenization, the emulsion was transferred into a 10-mL graduate cylinder and allowed to separate for 24 h. ES was determined by Eq 4.

$$\%\mathrm{ES} = \frac{V_\mathrm{B} - V_\mathrm{A}}{V_\mathrm{B}} \times 100\% \qquad \text{(Eq. 4)}$$

where V_B is the volume of the original solution prior to emulsification (V_B = 8 mL) and V_A is the volume of the separated aqueous layer after 24 h.

3.6 Protein quality

(a) Amino acid composition

With the exception of the methionine, cysteine and tryptophan, samples were prepared as per AOAC Official Method 982.30, using 6 N hydrochloric acid hydrolysis over 24 h. Methionine and cysteine were analyzed according to AOAC Official Method 985.28, where prior to 6N HCl hydrolysis, samples were first oxidized with performic acid. The amino acids from both hydrolysis sets were derivatized and separated (AccQ-Tag Ultra C18, 1.7 µm column) using the AccQ-Tag Ultra system (Waters Ltd., Mississauga, ON) chemistry (Astephen, 2018) on a Shimadzu UPLC system, complete with an SIL-30AC autosampler. For tryptophan, samples were subjected to alkaline hydrolysis, and analyzed using ISO protocol 13904 (International Organization for Standardization, 2016). The NIST soy flour Standard Reference Material 3234 was used for all amino acid analyses as quality control. The hydrated molecular weights of the amino acids were used for quantitation.

(b) Determination of the amino acid score

Amino acid score was calculated by comparing the amino acid pattern of the sample (mg/g protein) against the FAO 1991 amino acid pattern reference for children from age 2 to age 5.

(Histidine, 19; Isoleucine, 28; Leucine, 66; Lysine, 58; Methionine + Cysteine, 25; Phenylalanine + Tyrosine, 63; Threonine, 34; Tryptophan, 11; Valine, 35) (FAO, 1991). The amino acid score was determined based on the first limiting amino acid in the sample which was the amino acid with the lowest score among these essential amino acids. .

(c) In vitro protein digestibility

In vitro protein digestibility was measured using the method described by Hsu *et al.* (1977) with slight modifications. A flour suspension was prepared to deliver 6.25 mg protein per mL; 50 mL of the suspension was adjusted to pH 8.0 with 0.1N NaOH or HCl and kept in a 37°C water bath. A multi-enzyme solution was prepared with 1.6 mg of trypsin (15 units/ mg), 3.1 mg chymotrypsin (60 units/mg) and 1.3 mg peptidase (40 units/mg) per mL. This enzyme solution was adjusted to pH 8.0 with 0.1N NaOH or HCl.and kept in an ice bath before use. One millilitre of the multi-enzyme solution was added to the 10mL of flour suspension with agitation in the 37°C water bath. The pH of the solution, which drops due to protein hydrolysis, was monitored over a 10-min period. The drop in pH reflected the degree of *in vitro* protein digestibility.

$$IVPD\ (\%) = 65.66 + 18.10 \times \Delta pH_{10min} \qquad \text{(Eq. 5)}$$

where, $\Delta pH_{10\ min}$ refers to the difference between pH 8.0 at time zero and the pH of the solution at the end of 10 minutes.

(d) In vitro Protein Digestibility Corrected Amino Acid Score (IV-PDCAAS)

The IV-PDCAAS was calculated as the product of IVPD and the amino acid score (Bai *et al.*, 2018).

3.7 Statistics

All aqueous alcohol washes were conducted four times (two washes of each lot of sample). All measurements were made once for each washed sample, with duplicates on each lot of the untreated sample. All results were reported as the mean ± standard deviation. The volatile flavour analysis was performed by GC-MS four times in total for each sample (two washes x two lots). One-way analysis of variance (ANOVA) followed by a Duncan *post hoc* test was conducted to

compare the statistical differences among untreated and treated samples using SPSS software (version 21.0, SPSS, Chicago, IL, USA). Cluster analysis was performed using the method described by Chang *et al.* (2019) to compare the similarity of the overall volatile compounds profile among untreated and treated samples using SPSS (version 25, IBM, Amonk, USA) and Gene Cluster 3.0/Java Treeview (Stanford University, CA, USA), respectively. Principal component analysis (PCA) and data processing (auto scaling) were performed using MetaboAnalyst, version 4.0 software (http://www.metaboanalyst.ca/) (Zhang *et al.*, 2016; Lim *et al.*, 2017; van den Berg *et al.*, 2006).

4. RESULTS AND DISCUSSION

4.1 Physicochemical properties

The physicochemical properties of untreated and alcohol washed PPEF are given in Table 4.1. In previous studies, it was reported that alcohol washing may be responsible for the elimination of simple sugars, oligosaccharides, fat and ash from the sample, raising its protein content in the process (Chang *et al.*, 2019; Gulewicz *et al.*, 2000; Hua *et al.*, 2005; Johnson & Lusas, 1983; Peter, 2018). Similarly, in the present study, protein levels were raised, and ash and lipid levels were reduced with the alcohol washing relative to the untreated PPEF ($p<0.05$), to a magnitude dependent on the type and concentration of alcohol used. In the case of ethanol, protein levels increased from ~55% to ~62% at the 20% and 50% concentration, then declined slightly to ~58% once the concentration increased to 80%. At this higher alcohol concentration, more crude fat was removed and less ash was in the final ingredient. Washing with isopropanol was much the same, except slightly higher protein levels were reached (~64% with 20/50% isopropanol, and ~59% with 80% isopropanol). Isopropanol was more effective at reducing crude fat than the ethanol was, which led to the higher protein levels. At the higher alcohol concentration, polarity is the least, leading to greater affinity to fat molecules and more efficient removal (Peter, 2018). Similar observations were made with alcohol washing by Chang *et al.* (2019) and Peter (2018) working with lentil protein isolate and PPEF, respectively. Peter (2018) reported that 50% ethanol or isopropanol was more effective in removing oligosaccharides than at 70%. In the former, 8.0 and 7.9% of the raffinose family oligosaccharides were removed upon washing, respectively, whereas at the higher level of alcohols, only 3.2% and 0.2%, respectively was removed. In the present study, protein contents were slightly reduced relative to lower alcohol concentrations hypothesized because of fewer amounts of oligosaccharides being removed in the washing step.

The effect of alcohol washing treatment on pigments in the PPEF were presented in Table 4.1. L* indicates the level of lightness (L*=0, black; L*=100, white), the positive a* and b* values indicates redness, yellowness, respectively. According to results, alcohol washing caused darkening of the PPEF samples especially with isopropyl alcohol. After the alcohol washing

Table 4.1. Physicochemical properties of alcohol washed air classified pea protein enriched flour.

PPEF sample	Protein (%, d.b.)	Ash (%, d.b.)	Lipid (% d.b.)	Colour				Surface charge (mV)
				L*	a*	b*	ΔE	
Untreated	55.5 ±1.1^{d}	5.6 ± 0.1^{a}	2.9 ± 0.1^{a}	89.9 ± 0.4^{a}	1.1 ± 0.0^{d}	14.7 ± 0.7^{d}	-	-29.4 ± 0.4^{a}
Ethanol washed								
20%	62.4 ± 1.7^{b}	4.0 ± 0.0^{d}	2.1 ± 0.2^{b}	80.9 ± 0.7^{c}	5.4 ± 0.7^{b}	22.0 ± 0.9^{b}	12.4±0.6^{bc}	-26.0 ± 1.0^{cd}
50%	61.4 ± 0.5^{b}	4.9 ± 0.1^{b}	2.3 ± 0.1^{b}	84.9 ± 0.4^{b}	2.4 ± 0.2^{c}	19.0 ± 1.4^{c}	6.8±1.3^{de}	-27.4 ± 1.4^{b}
80%	58.2 ± 1.5^{c}	5.7 ± 0.1^{a}	1.4 ± 0.2^{c}	86.1 ± 0.6^{b}	1.8 ± 0.6^{cd}	14.9 ± 2.5^{d}	4.1±0.6^{e}	-25.4 ± 0.8^{de}
Isopropanol washed								
20%	64.2 ± 1.3^{a}	4.1 ± 0.1^{d}	1.6 ± 0.1^{c}	74.3 ± 1.0^{e}	8.8 ± 0.3^{a}	24.5 ± 1.6^{a}	20.0± 0.6^{a}	-26.3 ± 1.1^{bcd}
50%	64.3 ± 0.9^{a}	4.8 ± 0.0^{c}	0.6 ± 0.1^{d}	78.2 ± 1.9^{d}	6.2 ± 1.5^{b}	24.5 ± 0.9^{a}	16.1±1.2^{ab}	-27.1 ± 1.0^{bc}
80%	59.3 ± 0.5^{c}	5.7 ± 0.1^{a}	0.9 ± 0.2^{d}	80.1 ± 1.4^{c}	5.1 ± 01.0^{b}	18.0 ± 1.1^{c}	10.4±2.0^{cd}	-24.4 ± 0.8^{e}

Data represent the mean of duplicates from two lots ± one standard deviation (n = 4).

L*= Lightness (0 =black/ 100 =white); a*=redness/greenness (“+” =red/ “–“=green); b*=yellowness/blueness (“+” =yellow/ “-“blue).

Data represent average values ± standard deviation of each treatment. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

treatment all samples showed lower L*, higher a* and b* values. However, it was reported that alcohol washing with 95% ethanol or 91% isopropyl alcohol led to light colored soybean flakes (Mustakas *et al.*, 1961). Furthermore, 65% ethanol washed soybean products had lighter color than the control samples (Wu *et al.*, 2011). In our study, a reason of the darker colors might be the drying process (vacuum drying at 50°C) after the alcohol treatment. Similarly, the darkening effect of alcohol washing treatment using acetone, ethanol, and isopropanol on color properties of lentil protein isolates were found (Chang *et al.*, 2019). In addition, the efficiency of alcohol washing on pigment removal can be seen in the samples with higher alcohol concentration. Higher alcohol concentrations of both ethanol and isopropyl alcohol limited darkening of samples. The lowest ΔE values obtained with 50% and 80% ethanol 6.8 and 4.1, respectively. Therefore, ethanol usage at higher concentrations ≥50% may restrict the generation of dark pigments during the alcohol washing treatment.

Both intrinsic and extrinsic factors can affect the protein surface charge including amino acid composition, protein conformation, pH and ion strength of the solution (Cheung, 2014). Alcohol washing also was shown to reduce overall surface charge on the protein (at pH 7) relative to the untreated PPEF (-29.4 mV), likely due to the removal of some alcohol soluble prolamin-type proteins which altered the protein profile (Table 4.1). The biggest reduction in charge occurred at the 80% alcohol level where zeta potentials were -25.4 mV and -24.4 mV for ethanol and isopropanol, respectively. Since the polarity is least at these levels, it is presumed more prolamins were solubilized and removed during the washing step. In addition to amino acid composition change due to the alcohol washing, the protein conformation was likely changed during the alcohol treatment. With more hydrophobic group move to the surface of protein in the less polar treatment with 80% alcohol concentration, this also resulted in the most significant reduction of surface charge.

4.2 Effect of alcohol washing on the volatile compound profiles

Based on structure of ethanol (CH_3CH_2OH) and isopropanol ($CH_3CH(CH_3)OH$), polarity of the solution relative to water in the present study is ranked as following:

Water > 20% ethanol > 50% ethanol/20% isopropanol > 80% ethanol/50% isopropanol > 80% isopropanol.

In another words, 20% ethanol had the highest polarity and 80% isopropanol had the least polarity. The volatile compounds of untreated and washed PPEF samples identified by GC-MS are presented in Table 4.2. An example of a GC result graph was presented as Figure 4.1. Volatile compound was semi-quantified by measuring the size of the compound peak in the graph. Total peak area reported for untreated air-classified PPEF was 261 $\times 10^6$ with 64% contributed by aldehydes and 30% from alcohols. Twenty-eight volatile compounds were extracted from untreated PPEF, which were composed of 11 alcohols, 8 aldehydes, 4 ketones, 2 acids, 1 ester, 1 furan, and 1 alkene. Flavour and aroma description of some of the identified compounds were listed in Table 4.3. According to the results of a one-way ANOVA test of total peak area, alcohol treatments at all concentrations had a statistically significant impact ($p<0.05$) on the volatile compounds of air-classified PPEF (Figure 4.2.a-A). All alcohol treatments resulted in a reduction in the total volatile compounds present, with the exception of 20% isopropanol which gave 29% higher levels than the untreated PPEF samples (Figure 4.2.a-A). Overall, as the concentration of alcohols increased, the washing treatments became more effective at removing compounds (Figure 4.2.a-A), hypothesized caused by the decrease in polarity of the solvent. For instance, the total peak area ($\times 10^6$) was reduced by 25%, 82% and 92% as the concentration of ethanol increased from 20, 50 and 80%, respectively; and increased by 29% or reduced by 93% and 94% as the concentration of isopropanol increased, respectively.

Within this, there were compounds extracted from all samples (untreated and treated), such as hexanal, octanal, nonanal, decanal, 1-hexanol, 1-octanol, furan, 2-pentyl-. In a similar manner, hexanal, 1-penten-3-ol, 2-hexenal, 2-pentylfuran, 1-pentanol, 2-heptanal, hexanol, nonanal, and 1-octen-3-ol, which are degradative oxidation products of polyunsaturated fatty acids, was reported as main volatiles in soy protein isolates (Samoto *et al.*, 1998). Some compounds were completely removed in certain treatments (*e.g.* heptanal, 1-penten-3-ol, 2-penten1-ol, butanone, etc.). On the other hand, the alcohol treatment and the drying process generated some compounds; such as, hexanoic acid, 2-nonanone, 1-(3-ethyloxiranyl)-ethenone, 1-nonen-4-ol in 20% alcohol treatments, and benzaldehyde in all alcohol treated samples. Among the volatile compounds extracted from PPC, aldehydes, alcohols and ketones are reported to be the main compounds that affect the flavour of pea (Roland *et al.*, 2007).

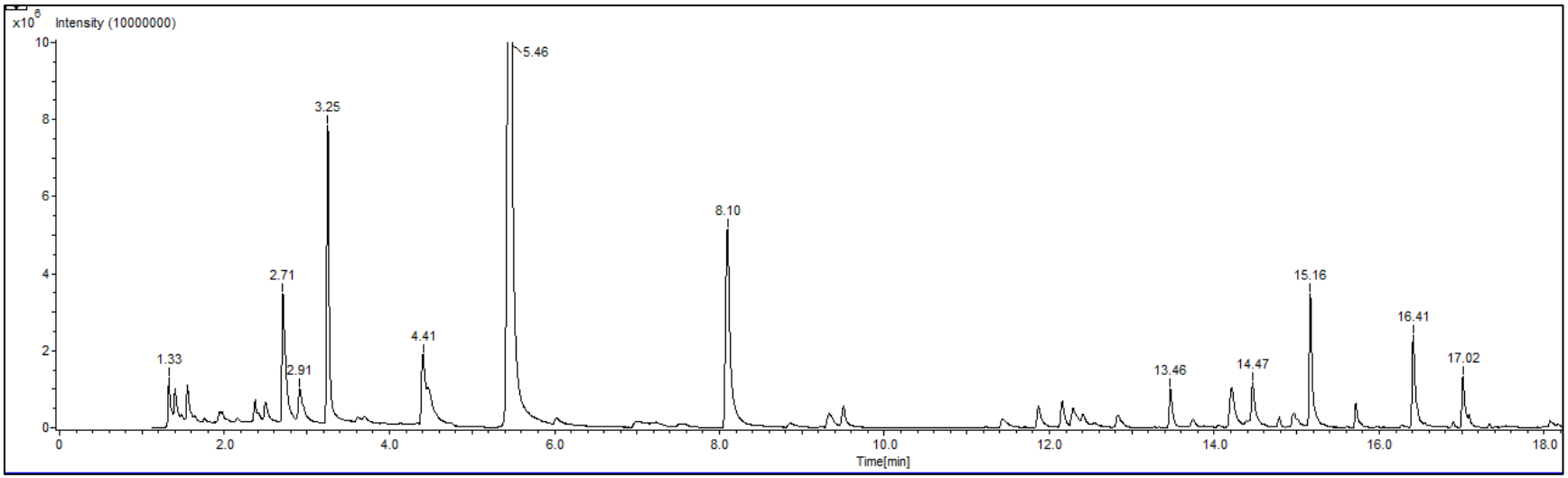

Figure 4.1 A gas chromatography result graph of untreated PPEF.

Each peak represented a volatile compound. For instance: 2.71: 1-Penten-3-ol, 4.41: 1-Pentanol, 5.46: Hexanal, 8.1: 1-Hexanol, 13.46: 1-Hexanol, 2-ethyl-, 14.47: 1-Octanol, 15.16: Nonanal, 16.42: 1-Nonanol, 17.02: Decanal.

Table 4.2. Volatile compounds of original pea protein concentrate (untreated) and pea protein concentrated treated by ethanol and isopropanol washing.

	Untreated	Ethanol			Isopropanol		
		20%	50%	80%	20%	50%	80%
Total Peak Area	261.02±11.31^{b}	195.67±13.76^{c}	47.70±11.64^{d}	21.98±7.10^{de}	335.96±44.23^{a}	19.08±4.23^{de}	16.07±2.10^{e}
Acid							
Butanoic acid, 3-methyl-	1.08±0.32^{ab}	1.73±0.93^{a}	0.94±0.42^{abc}	0.26±0.19^{bc}	1.49±0.29^{a}	1.11±1.34^{ab}	n.d.
Butanoic acid, 2-methyl-	0.58±0.23^{ab}	0.94±0.40^{a}	0.37±0.15^{bc}	0.37±0.42^{bc}	0.40±0.46^{bc}	0.21±0.14^{bc}	n.d.
Hexanoic acid	n.d.	1.95±1.15^{b}	n.d.	n.d.	34.64±12.83^{a}	n.d.	n.d.
Total Acid	1.65±0.55^{b}	4.61±1.69^{b}	1.30±0.54^{b}	0.63±0.26^{b}	36.53±13.33^{a}	1.32±1.39^{b}	n.d.
Aldehyde							
Butanal, 3-methyl-	0.27±0.24^{b}	9.78±3.17^{a}	1.36±0.58^{b}	0.58±0.12^{b}	n.d.	n.d.	n.d.
Butanal, 2-methyl-	1.30±0.51^{b}	13.00±4.11^{a}	0.60±0.66^{b}	0.32±0.07^{b}	3.31±3.82^{b}	n.d.	n.d.
Hexanal	141.29±10.57^{a}	117.45±11.89^{b}	29.29±10.19^{c}	9.94±2.76^{c}	108.58±34.35^{b}	7.06±3.43^{c}	2.01±1.88^{d}
Heptanal	2.96±0.85^{b}	0.85±0.09^{c}	0.20±0.15^{c}	n.d.	4.62±1.26^{a}	n.d.	n.d.
2-Heptenal	1.53±0.97^{a}	0.50±0.27^{cd}	0.23±0.47^{cd}	n.d.	0.93±0.73^{ab}	n.d.	n.d.
Octanal	1.54±0.10^{ab}	1.01±0.33^{b}	0.90±0.49^{b}	0.60±0.81^{b}	3.30±2.06^{a}	0.14±0.20^{b}	0.23±0.35^{b}
Nonanal	13.70±1.16^{a}	2.38±0.50^{d}	5.03±1.77^{c}	3.49±1.64^{cd}	8.86±1.67^{b}	4.92±1.22^{c}	3.89±1.94^{cd}
Decanal	3.48±1.81^{a}	0.28±0.16^{b}	2.15±1.55^{ab}	1.87±2.07^{ab}	1.85±1.33^{ab}	1.58±1.18^{ab}	1.25±0.43^{ab}
2-Octenal, 2-butyl-	n.d.	n.d.	n.d.	n.d.	2.19±0.61^{a}	n.d.	n.d.
Total Aldehyde	166.07±9.93^{a}	145.26±10.16^{ab}	39.77±10.40^{c}	16.80±5.01^{d}	133.63±33.43^{b}	13.71±4.23^{d}	7.37±3.12^{d}
Alcohol							
1-Penten-3-ol	14.59±1.77^{a}	1.04±2.08^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
1-Butanol, 3-methyl-	0.58±0.15^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

1-Butanol, 2-methyl-	0.89±0.30[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Hexanol, 3-methyl-	n.d.	n.d.	n.d.	n.d.	2.87±1.92[a]	n.d.	n.d.
1-Pentanol	10.16±2.21[a]	1.33±1.54[b]	0.49±0.35[b]	n.d.	n.d.	n.d.	n.d.
2-Penten-1-ol	10.03±0.63[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Hexanol	24.88±3.52[b]	10.92±0.45[c]	0.75±0.19[d]	0.60±0.13[d]	48.58±8.29[a]	2.03±2.72[d]	0.57±1.14[d]
1-Heptanol	1.73±0.42[a]	0.58±0.05b[c]	0.11±0.13[c]	0.17±0.34[c]	1.37±1.00[ab]	n.d.	n.d.
1-Octen-3-ol	3.23±0.89[b]	1.81±0.58[c]	0.77±0.28[cd]	0.29±0.08[d]	5.47±1.81[a]	n.d.	0.20±0.07[d]
1-Hexanol, 2-ethyl-	3.65±1.02[a]	1.47±0.35[b]	0.54±0.15[c]	0.36±0.11[c]	0.76±0.88[bc]	n.d.	n.d.
1-Octanol	3.37±0.27[a]	2.04±0.47[abc]	0.60±0.77[cd]	0.81±0.51[bcd]	2.36±1.99[ab]	0.87±1.21[bcd]	0.10±0.16[d]
1-Nonen-4-ol	n.d.	1.09±0.83[b]	n.d.	n.d.	2.99±2.43[a]	n.d.	n.d.
1-Nonanol	7.03±2.12[a]	2.74±1.51[b]	0.93±0.55[bc]	0.89±0.98[bc]	1.40±1.39[bc]	n.d.	n.d.
Total Alcohol	80.13±4.73[a]	23.08±2.37[c]	4.20±1.81[d]	3.21±2.00[d]	65.89±9.73[b]	2.94±3.87[d]	0.88±1.31[d]
Ketones							
Butanone	2.55±0.57[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Heptanone	0.67±0.22[b]	6.52±2.02[b]	0.48±0.18[b]	0.23±0.21[b]	46.33±19.87[a]	0.19±0.13[b]	n.d.
3-Octen-2-one	0.75±0.39[b]	1.24±0.60[b]	n.d.	n.d.	3.25±1.96[a]	n.d.	n.d.
4-Penten-2-one	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.57±0.30[a]
Isobutyl-2-heptenone	0.45±0.04[a]	0.18±0.12[ab]	n.d.	n.d.	0.22±0.44[ab]	n.d.	n.d.
2-Nonanone	n.d.	0.32±0.14[b]	n.d.	n.d.	4.20±3.23[a]	n.d.	n.d.
Ethanone, 1-(3-ethyloxiranyl)-	n.d.	1.36±0.32[b]	n.d.	n.d.	2.80±0.42[a]	n.d.	n.d.
2-Decanone	n.d.	n.d.	n.d.	n.d.	5.51±1.88[a]	n.d.	n.d.
Total Ketone	4.41±0.38[b]	9.62±1.36[b]	0.48±0.18[b]	0.26±0.25[b]	62.32±19.40[a]	0.19±0.13[b]	0.57±0.30[b]

Ester							
Hexanoic acid, 1-methylethyl ester	n.d.	n.d.	n.d.	n.d.	3.48±0.66[a]	n.d.	n.d.
Hexanoic acid, 4-octyl ester	4.59±0.49[a]	1.69±0.77[c]	n.d.	n.d.	3.27±1.72[b]	n.d.	n.d.
Total Ester	4.59±0.49[b]	1.69±0.77[c]	n.d.	n.d.	6.75±2.07[a]	n.d.	n.d.
Aromatic Hydrocarbons							
p-Xylene	n.d.	n.d.	n.d.	n.d.	2.07±1.27[a]	0.10±0.13[b]	0.75±0.77[b]
Benzaldehyde	n.d.	1.37±0.42[c]	1.20±0.35[c]	0.84±0.51[c]	6.01±0.99[a]	0.47±0.19[c]	2.83±2.18[b]
Furan, 2-pentyl-	2.38±0.69[c]	9.54±2.43[b]	0.50±0.38[c]	0.14±0.28[c]	22.60±7.30[a]	0.26±0.34[c]	0.57±0.36[c]
Benzeneacetaldehyde	n.d.	0.49±0.36[b]	0.18±0.12[c]	0.10±0.12[c]	1.19±0.21[a]	n.d.	n.d.
Total Aromatic Hydrocarbons	2.38±0.69[c]	11.40±2.29[b]	1.88±0.49[c]	1.08±0.62[c]	31.88±7.65[a]	0.83±0.59[c]	4.19±1.90[c]
Other							
(S)-Isopropyl lactate	n.d.	n.d.	n.d.	n.d.	1.13±2.27[b]	0.10±0.18[b]	3.04±2.39[a]
2-Pentene, 5-(pentyloxy)-, (E)-	1.80±0.31[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Other	1.80±0.31[ab]	n.d.	n.d.	n.d.	1.13±2.27[ab]	0.10±0.18[b]	3.04±2.39[a]

Data represent the mean of duplicates from two lots ± one standard deviation (n = 4) of each treatment. Different small letters in the rows represent statistically significant differences ($p < 0.05$).

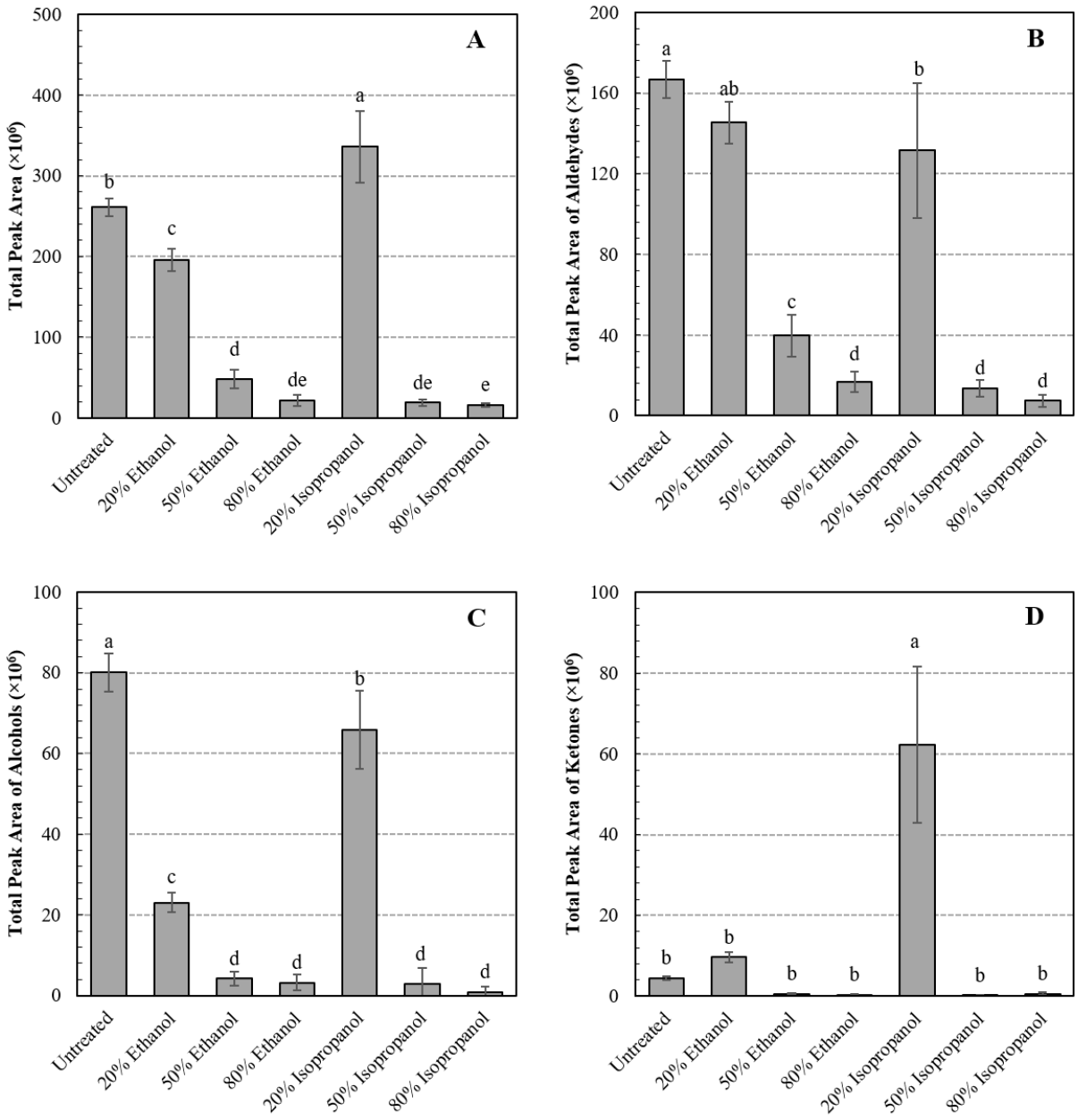

Figure 4.2.a. Peak area of total and classes of volatile compounds in untreated and treated air-classified pea protein concentrate samples. A: total peak area, B: peak area of aldehydes, C: peak area of alcohols, D: peak area of ketones.

Data represent the mean of duplicates from two lots ± one standard deviation (n = 4) of each treatment. Different small letters represent statistically significant differences ($p < 0.05$).

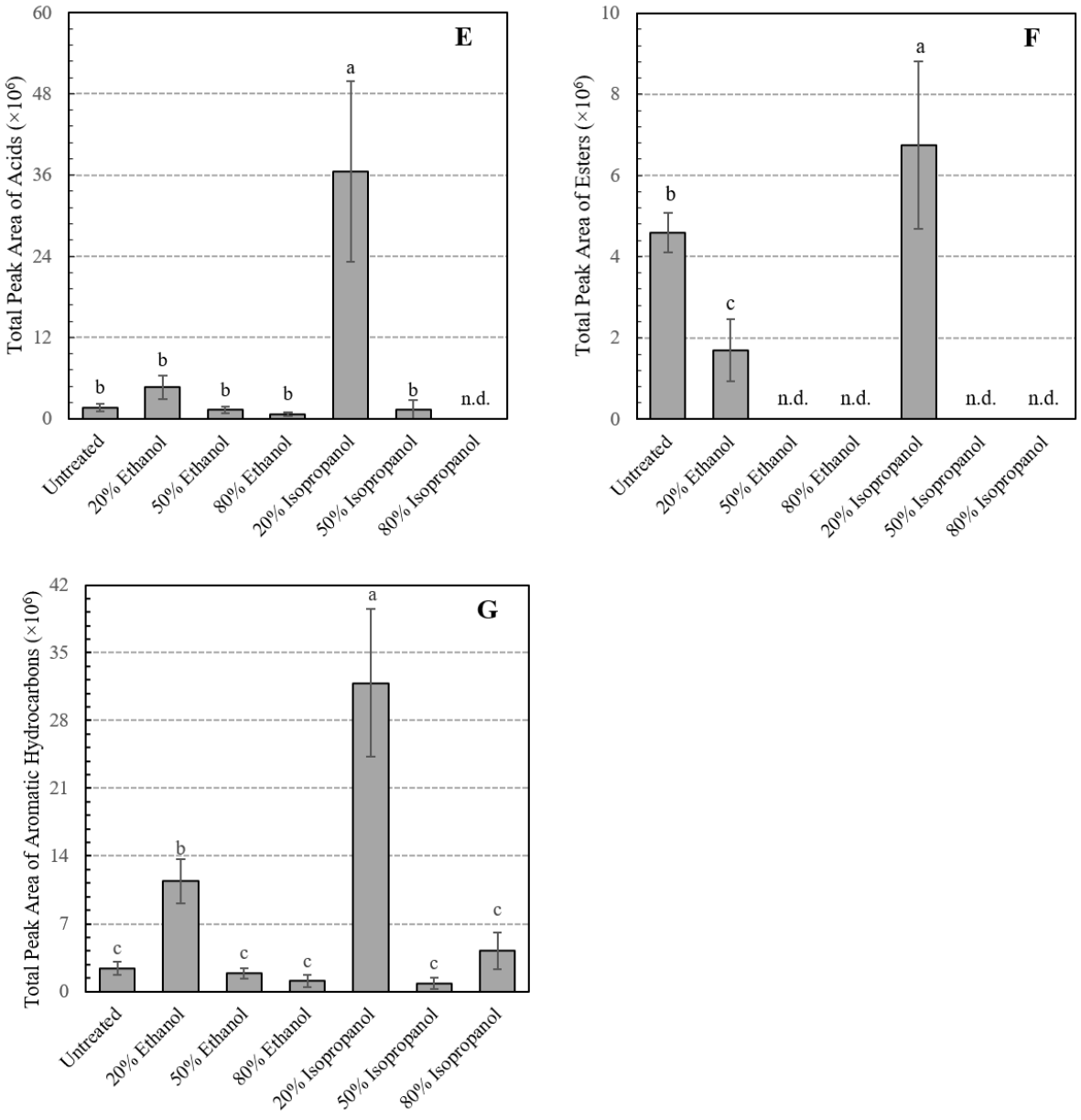

Figure 4.2.b. Peak area of total and classes of volatile compounds in untreated and treated air-classified pea protein concentrate samples. E: peak area of acids, C: peak area of esters, D: peak area of aromatic hydrocarbons.

Data represent the mean of duplicates from two lots ± one standard deviation (n = 4) of each treatment. Different small letters represent statistically significant differences ($p < 0.05$).

Aldehydes were the biggest class of volatile compounds extracted from untreated air-classified PPEF with a peak area ($\times 10^6$) of 167 in the untreated sample (Figure 4.2.a-B), and represents 63.7% of the total peak area. Removal of aldehydes was limited to 13%–21% with lower alcohol concentrations (20%), whereas at higher concentrations, aldehydes were reduced by 90-96% from what was in the untreated PPEF. Overall, isopropanol was more effective than ethanol at removing the aldehyde compounds at all corresponding concentrations. The most effective treatment in reducing aldehydes was 80% isopropanol. This treatment decreased the peak area ($\times 10^6$) of total aldehydes from 167 to 7, which is 96% reduction, in PPEF.

Hexanal is the most abundant aldehyde, accounting for 53.9% of the total peak area of the untreated sample (Table 4.2). Hexanal is a compound generated during the enzymatic oxidation of unsaturated fatty acid (linoleic acid) in legumes (Matoba *et al.*, 1989, Ma *et al.*, 2016). After harvesting, lipoxygenase reacts with linoleic acid in the presence of oxygen at neutral pH to form hydroperoxides. Then, the linoleic acid hydroperoxide (13-hydroperoxylinoleic acid) is cut by lyases into n-hexanal (Matoba *et al.*, 1989, Ma *et al.*, 2016). This compound was described to give green, beany, and hay-like off-flavour in peas (Roland *et al.*, 2017) and was reported to be the principle off-flavour compounds in several other legumes including soy, beans, lentils and faba beans (Matoba *et al.*, 1989, Ma *et al.*, 2016, Oomah *et al.*, 2014). All alcohol treatments reduced the peak area of hexanal significantly ($p<0.05$) (Table 4.2). Twenty percent ethanol and isopropanol removed 17% and 23% hexanal, respectively. The treatment with 80% isopropanol had the highest efficiency by reducing 99% of hexanal peak area, followed by 50% isopropanol, 80% and 50% ethanol treatments reducing 95%, 93% and 79% hexanal peak area, respectively (Table 4.2). Hua *et al.* (2005) found 88% reduction in hexanal in soy bean flake after 30 minutes 85% ethanol treatment at room temperature. Nonanal was determined to be the second largest aldehyde present total peak area ($\times 10^6$) of 14, and can be described as "floral, rose and soapy" (Table 4.2, Table 4.3) (Schindler et a, 2012, Burdock, 2002). All alcohol treatments, except for 20% isopropanol, reduced nonanal by 60%. There was no significant difference ($p>0.05$) among the higher alcohol concentrations (50%–80%) (Table 4.2). In general, at 50% and 80% concentration isopropanol was found more effective than ethanol in removal of all aldehyde compounds.

Alcohols was the second largest class of volatile compounds found within PPEF, having a peak area ($\times 10^6$) of 80, which is 31% of total peak area of untreated PPEF (Table 4.2, Figure 4.2.a-

C). The total peak area of alcohols was reduced by 90% with higher alcohol concentrations, but the lower concentrations was less effective (Figure 4.2.a-C). For instance, total peak area of alcohols was reduced by 71% and 17% with 20% ethanol and isopropanol. The latter was less effective because during the treatment and drying process, the peak area ($\times 10^6$) of 1-hexanol increased from 25 to 49 and 1-octen-3-ol increased from 3.2 to 5.5. The principal alcohol compound found in the untreated PPEF was 1-hexanol which is reduced from n-hexanal in the presence of alcohol oxidoreductase (Ma *et al.*, 2016). This compound is responsible for the green and herbaceous flavour in peas (Schindler *et al.*, 2012, Widjaja *et al.*, 1996). The peak area ($\times 10^6$) of 1-hexanol in 20% isopropanol treated sample (49) was twice as much as that in the untreated sample (25), which indicates that more 1-hexanol was generated than removed during the treatment (Table 4.2). It was hypothesized that the alcohol oxidoreductase in the 20% isopropanol treated sample was not inactivated and accelerated transforming hexanal to 1-hexanol during the drying process at 50°C. All 50% and 80% concentration treatments resulted in over 90% reduction in 1-hexanol peak area ($p > 0.05$). The alcohol oxidoreductase was expected to be inactivated in these treatments. The second largest alcohol found in the untreated PPEF was 1-Penten-3-ol was (peak area of 15×10^6), which gives bitter, mild green odour and taste (Table 4.2). All treatments were efficient in reducing or diminishing this compound, with 93% reduction after 20% ethanol treatment and no detectable 1-penten-3-ol found all the rest of treatments. Overall, alcohol compounds can be readily removed by alcohol treatments. All 50% and 80% treatment resulted in over 75% reduction of each alcohol compounds. No detectable 1-butanol, 3-methyl-, 1-butanol, 2-methyl-, and 2-penten-1-ol was found in any treated sample. Most alcohol compounds, namely 1-pentanol, 1-heptanol, 1-octen-3-ol, 1-hexanol, 2-ethyl-, and 1-nonanol, was more effectively removed with 50% and 80% isopropanol compared to the ethanal treatment at the corresponding concentration. Among these compounds, except for 1-octen-3-ol, the other ones were completely removed with 50% and 80% isopropanol treatments. Besides, commercial oil free soy products include 1-octen-3-ol as one of the main off flavour compounds (Samoto *et al.*, 1998).

Ketones only accounted for a small proportion (1.6%) of the total peak area (Figure 4.2.a-D). However, these compounds can have a large impact on the overall flavour in pea, because they had strong odour and flavour (Roland *et al.*, 2017). Total peak area was found to decrease significantly when washed at the 50% and 80% concentrations, whereas peak area increased for both alcohols at the 20% level (Figure 4.2.a-D) The largest peak in the ketone class was identified

as butanone, having a peak area of 3 $\times 10^6$, which had pungent, cheese-like and sweet apricot odor and taste (Schindler *et al.*, 2012, Burdock, 2002). Butanone was reduced to negligible levels with all treated samples. The presence of fat and active lipoxygenase with the 20% alcohol treated samples may be responsible for the increasing quantity of 2-heptanone after the treatment and drying process, as Ma *et al.* (2016) observed generation of 2-heptanone after heat treatment in peas, dry beans and lentils. Same theory may be used to explain the formation of several other ketones in the low concentration treated samples including 3-octen-2-one, 2-nonanone, ethanone, 1-(3-ethyloxiranyl)-, and 2-decanone. None of these compounds were found in 50% and 80% alcohol treatments (Table 4.2).

There were negligible hexanoic acid, 1-methylethyl ester found in the untreated PPEF, while the only sample carried detectable hexanoic acid, 1-methylethyl ester was 20% treated isopropanol with peak area of 3.5 $\times 10^6$ (Figure 4.2.b-E, F). Another hexanoic acid ester found in this study was hexanoic acid, 4-octyl ester. The peak area of ester was reduced from 4.6 to 1.7 in 20% ethanol treated sample, 3.3 in 20% isopropanol treated sample and undetectable in the rest of solvent treated PPEF. Furan, 2-pentyl-, reported as an autooxidation product of soybean and cottonseed oils, was the only aromatic hydrocarbons detected in the untreated PPEF (Krishnamurthy *et al.*, 1967) (Figure 4.2.b-G). Basically, alcohol washing of samples with high protein content removes lipid like materials that also eliminates unwanted flavour compounds (Eldridge *et al.*, 1963). However, higher amount of this compound was found both 20% solvent treated samples. This may be a result of a maillard reaction in samples with higher water content during vacuum drying or ultrasonic extraction of flavour compounds. Since, heterocyclic compounds like pyrazines and furans are the products of maillard reactions in sunflower seeds (Chen *et al.*, 2019). For instance, the peak area ($\times 10^6$) of furan, 2-pentyl was increased from 2.4 to 9.5 and 22.6 in 20% ethanol treated and 20% isopropanol treated PPEF, respectively. It is also known that ethanol-water or isopropyl alcohol-water mixtures form azeotropes which is dominated by hydrogen bonded water clusters in water rich mixtures (Wakisaka *et al.*, 2011). This may explain the loss of efficiency of elimination of flavour compounds with 20% alcohol including solvents. On the other hand, with higher concentration of solvents, the peak area of furan, 2-pentyl was reduced to <0.6. There were different levels of benzaldehyde and benzeneacetaldehyde generated during the solvent washing and drying process.

In summary, higher levels of alcohols (>50%) were more effective at reducing levels of volatiles in PPEF probably due to the inactivation of lipoxygenase and lower polarity which allowed for greater affinity to the flavour compounds. It is proposed at the 20% level, the inactivation of this enzyme is less effective, resulting in the generation of some volatile compounds during treatment and the drying process. There were 20 compounds reduced to below the level of detection in 80% isopropanol treatment, 17 and 6 compounds in 50% and 20% isopropanol treatments, while 11, 8, and 4 compounds were removed according to the GC result in 80%, 50% and 20% ethanol, respectively. Although isopropanol eliminated more volatile compound compared to ethanol at the corresponding concentration, there was no significant difference in total peak area extracted among the 50% and 80% treatments.

To better visualize the difference in volatile compound profile among untreated/treated samples, a cluster analysis (Figure 4.3) and principal component analysis (PCA) (Figure 4.4) were conducted. On the heatmap, black blocks indicated that no detectable compounds were found in the PPEF sample. With the red color become brighter, the peak area increases. Dark red blocks indicate peak area from 0 to 1 $\times 10^6$, maroon blocks indicate peak area from 1 to 2 $\times 10^6$, and bright red blocks indicate peak area from 2 to 3 $\times 10^6$ and above. The heat map shows that there were a large amount of volatile compounds generated in the 20% isopropanol treated sample. Some compounds were generated in the 20% ethanol treated sample and some were slightly decreased. The darker columns of 50% ethanol, 80% ethanol, 50% isopropanol, and 80% isopropanol presented the efficiency to remove the volatile compound in counts and amount. PCA analysis was performed on control and alcohol washed samples after the data processing, therefore, auto-scaling was applied to GC-MS data to normalize (van den Berg *et al.*, 2006). In PCA analysis, the first and second principal components accounted for 65.6% of the variance. Only the volatile flavour compounds of control sample positively correlated with second principal component. Except 20% ethanol washed, and 20% isopropanol washed samples, all alcohol treated samples overlapped and negatively correlated with both first and second principal components. In addition, all samples were separated into four groups (control, 20% isopropanol, 20% ethanol, and other alcohol treatments) regarding 95% confidence regions in PCA scores plot. This result summarizes the results presented in Figure 4.2. and Table 4.2.

Table 4.3. Common compounds found in PPEF with flavour/aroma description and detection threshold level.

Compound	Description	Detection Threshold Value	Reference
1-Butanol, 3-methyl-	Fruity, banana, sweet, bittersweet	250 ppb to 4.1 ppm	1
1-Heptanol	Woody, fatty	3 ppb	1
1-Hexanol	Herbaceous, woody, fragrant, mild, sweet, green, and fruity	200ppb to 2.5 ppm	1, 2, 3
1-Nonanol	Rose-orange,	50 to 90 ppb	1
1-Octanol	Fresh, orange-rose	42 to 480 ppb	1
1-Octen-3-ol	Mushroom-like, earthy	14 ppb	1, 4
1-Pentanol	Fusel-like, sweet	1.6 to 70 ppm	1
1-Penten-3-ol	Bitter, mild green	400 ppb	1, 4
2-Decanone	Orange like	n.a.	1, 3
2-Heptanone	Fruity, spicy, cinnamon, banana	1ppb to 1.33 ppm	1
2-Heptenal	Pungent, green, fatty	n.a.	1
2-Nonanone	Fruity, herbaceous	5 to 200 ppb	1, 3
3-Octen-2-one	Fruity, lemon	n.a.	1
Benzaldehyde	Bitter almond	100 ppb to 4.6 ppm	1
Benzeneacetaldehyde	Harsh green, fruity	4 ppb	1
Butanal, 2-methyl-	Pungent, fruity, chocolate, coffee-like	n.a.	1
Butanal, 3-methyl-	Acrid, apple like, fatty, almond,	n.a.	1
Butanone	Pungent, cheese, sweet apricot	n.a.	1, 2
Decanal	Waxy, floral, pronouced fatty, woody	0.1 to 6 ppb	4
Furan, 2-pentyl-	Green bean, metallic, vegetable	6 ppb	1
Heptanal	Strong, fatty, harsh, pungent	3 to 60 ppb	1
Hexanal	Green, Grassy, fatty, fruity	4.1 to 22.8 ppb	1, 2
Hexanoic acid	Sweaty, pungent, fatty, acrid, plastic, rancid	93 ppb to 10 ppm	1
Hexanoic acid, 1-methylethyl ester	Sweet, delicate, fruity	n.a.	1
Nonanal	Floral, rose soapy	1 to 8 ppb	1
Octanal	Fruity, citrus, honey	1.4 to 6.4 ppb	1

[1] Burdock, (2002); [2] Schindler, *et al.* (2012); [3] Widjaja *et al.* (1996); [4] Oomanh *et al.* (2007).

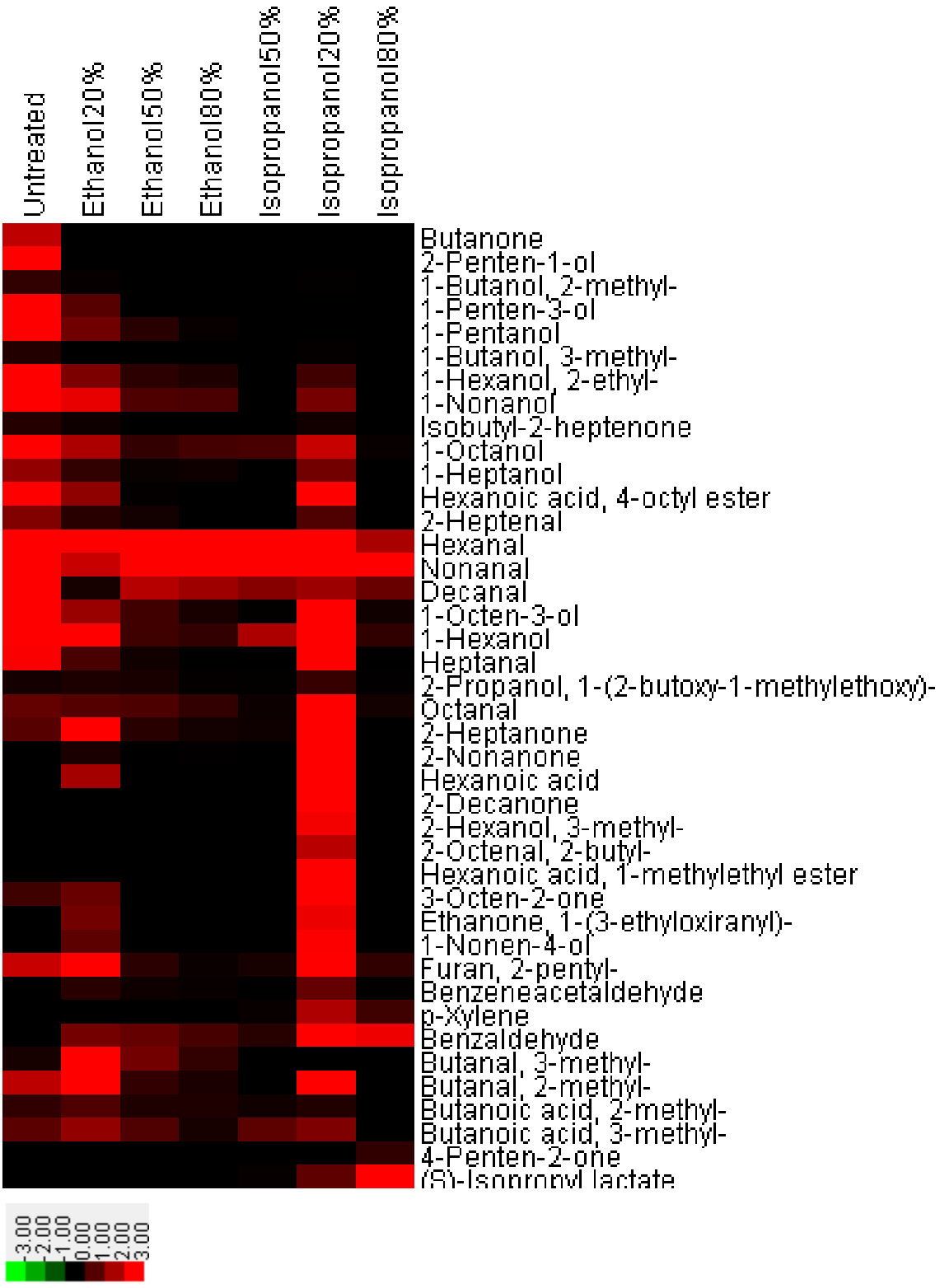

Figure 4.3. Heat map of volatile compound in untreated and treated air-classified pea protein concentrate samples.

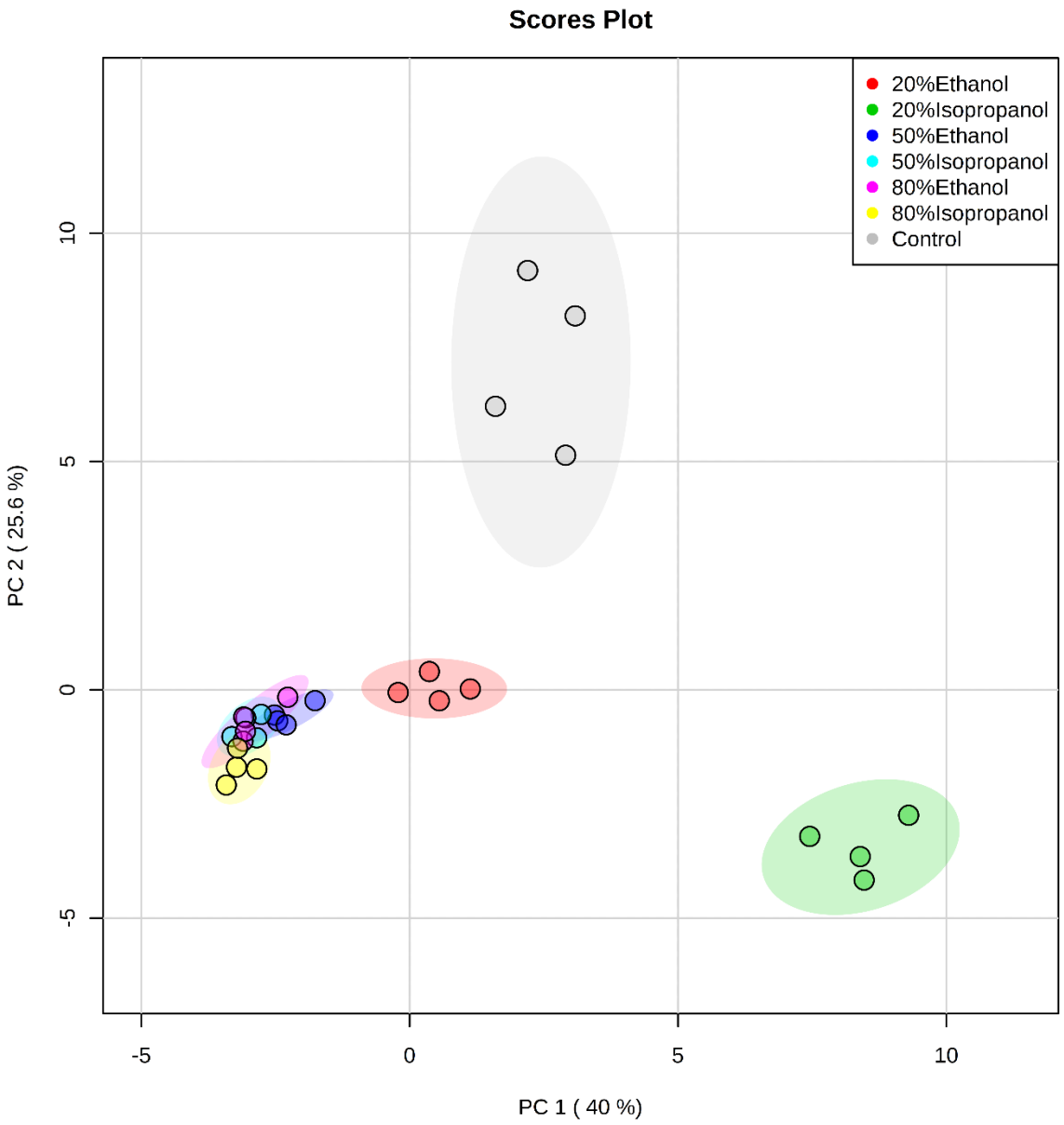

Figure 4.4. Principal component analysis (PCA) of volatile flavour compound profile for untreated (control) and alcohol washed samples.

4.3 Effect of alcohol washing on protein functionality

The functional properties of untreated and treated PPEF are given in Table 4.4. For solubility, all washes resulted in a significant reduction in protein solubility of PPEF. For instance, solubility was reduced from 85.4% (untreated) to 44.7%–52.7% and 20.6%–38.0% when washed with ethanol and isopropanol, respectively. The protein solubility increased with the addition of

alcohols in the solvent. Highest amount of soluble protein (52.7%) was observed among ethanol treated sample in 80% ethanol treated PPEF, similarly, 80% isopropanol treated PPEF had the highest solubility of 38% among the isopropanol treated sample. Bader *et al.* (2011) suggested a similar effect when studying ethanol and isopropanol-washed lupin seeds. In the case of both alcohols, a greater amount of proteins was soluble after the 80% alcohol wash than at the 20%, since more of the prolamin-type proteins were washed away. In addition to the removal of prolamin-type of proteins in the more concentrated solvent treatment, it was hypothesized there were more water-soluble albumin-type and salt-soluble globulin-type of protein washed away in 20% solvent treatments, which resulted in lower protein solubility. In general, PPEF washed with isopropanol had lower solubility compared to samples treated with ethanol at corresponding concentration level (Table 4.4). In the present study, it is assumed the lower solubility in PPEF when washed with isopropanol relative to ethanol is due to a greater amount of protein denaturation caused by the lower polarity of the solvent. Chang *et al.* (2019) observed the opposite results for alcohol washed lentil protein isolate, where ethanol washing led to slightly lower solubility than isopropanol. But like our study, they showed that solubility increased as the concentration of alcohol increased most likely due to the loss of prolamin proteins during the washing step.

The water hydration capacity (WHC) all treated PPEF was significantly increased relative to the untreated sample ($p < 0.05$). WHC of ethanol washed PPEF increased 1.18 g/g to 2.00–2.60 g/g, whereas isopropanol washing caused it to increase to 2.07–2.54 g/g (Table 4.4). Overall, WHC was similar when washed with 20% or 50% alcohols, then declined as the concentration increased to 80%, which might reflect the reduced charge on the protein's surface (Table 4.1). The oil holding capacity (OHC) of untreated PPEF was statistically ($p < 0.05$) higher than all treated sample. OHC reduced from 0.96 g/g with untreated PPEF to 0.67 to 0.80 g/g in solvent washed sample (Table 4.4). In general, the capacity of holding oil was similar among all treated sample.

In terms of emulsion stability (ES), there was no significant difference in ES between the untreated sample and the alcohol washed samples at the 20% level. ES the dropped to ~28%–29% with further addition of ethanol. In the case of the isopropanol wash, there was no statistical difference in ES relative to the untreated sample. The emulsion forming properties is closely related to the protein and fat contents, solubility, and viscosity of the protein solution (Peter, 2018; Toews & Wang, 2013; Chang *et al.*, 2019). In the present study, although the solubility of protein in treated PPEF samples was lower, the reduced fat content and increased viscosity of the protein

solution of treated samples, as suggested by Chang *et al.* (2019), may help maintain a certain level of ES.

Table 4.4. Functional properties of alcohol washed air classified pea protein enriched flour.

Sample	Solubility (%)	Water holding capacity (g/g)	Oil holding capacity (g/g)	Emulsion stability (%)
Untreated	85.4 ±2.5^{a}	1.18 ± 0.03^{d}	0.96 ± 0.06^{a}	40.00 ± 4.44^{ab}
Ethanol				
20%	44.7 ± 1.7^{c}	2.53 ± 0.05^{a}	0.80 ± 0.02^{b}	40.31 ± 3.87^{ab}
50%	42.6 ± 3.2^{cd}	2.60 ± 0.05^{a}	0.77 ± 0.02^{bc}	29.06 ± 3.13^{c}
80%	52.7 ± 2.9^{b}	2.00 ± 0.04^{c}	0.76 ± 0.03^{bc}	28.13 ± 2.98^{c}
Isopropanol				
20%	20.6 ± 3.4^{f}	2.39 ± 0.07^{b}	0.72 ± 0.03^{cd}	46.88 ± 6.33^{a}
50%	30.9 ± 2.8^{e}	2.54 ± 0.11^{a}	0.67 ± 0.02^{d}	45.31 ± 5.81^{a}
80%	38.0 ± 3.4^{d}	2.07 ± 0.03^{c}	0.73 ± 0.03^{c}	37.19 ± 4.49^{b}

Data represent the mean of duplicates from two lots ± one standard deviation (n = 4) of each treatment. Different small letters represent statistically significant differences ($p < 0.05$).

4.4 Effect of alcohol washing on protein quality

The amino acid profile (g per 100 g flour), essential amino acid content (mg per g protein) and the amino acid scores for untreated and treated PPEF are given in Tables 4.6 and 4.7. The untreated PPEF was found to be limiting in both methionine + cysteine and tryptophan, with the latter being the most limiting (amino acid score of 0.90) (Table 4.7). With alcohol washing, differences in polarity will impact the protein losses in solution. For instance, at the 20% level the solvent may have washed out some albumins and globulin proteins, along with prolamin-types. At this level, regardless of the alcohol type, threonine, methionine + cysteine, leucine, histidine, lysine and tryptophan were all limiting. The essential amino acid profile in pea albumins and globulins

Table 4.5. Protein quality summary for untreated and alcohol washed air classified pea protein enriched flour.

Sample	Limiting amino acid[1]	Limiting amino acid score[1]	IVPD[2] (%)	IV-PDCAAS [1] (%)
Untreated	Tryptophan	0.90	78.5 ± 0.3	70.6 ± 0.2
Ethanol				
20%	Methionine and cysteine	0.66	82.8 ± 1.5	54.8 ± 1.0
50%	Methionine and cysteine	0.77	84.6 ± 1.2	65.1 ± 0.9
80%	Tryptophan	0.84	81.5 ± 1.5	68.1 ± 1.2
Isopropanol				
20%	Methionine and cysteine	0.60	84.9 ± 0.2	51.0 ± 0.1
50%	Methionine and cysteine	0.72	84.8 ± 2.1	61.3 ± 1.5
80%	Methionine and cysteine	0.84	82.8 ± 1.8	69.4 ± 1.2

[1]Measurements were performed once on one sample from a composite sample of triplicate batches.
[2]Data represent the mean of duplicates from two lots ± one standard deviation (n = 4) of each treatment. Different small letters represent statistically significant differences ($p < 0.05$).
Abbreviations: IVPD (*In vitro* protein digestibility); IV-PDCAAS (*In vitro* protein digestibility corrected amino acid score)

Table 4.6. Amino acid profile (g per 100 g of flour, on an *as is* basis) for untreated and alcohol washed air classified pea protein enriched flour.

Amino Acid	*Untreated*	*Ethanol*			*Isopropanol*		
		20%	50%	80%	20%	50%	80%
CP (%)	52.69	59.70	58.83	55.77	61.78	61.98	57.10
Moisture (%)	6.04	4.34	4.24	4.17	3.72	3.61	3.64
Aspartic Acid	6.0	5.70	6.53	6.57	5.51	7.10	6.69
Glutamic Acid	8.89	8.66	9.93	9.61	8.28	10.38	9.54
Serine	2.64	2.44	3.10	2.87	2.41	3.04	2.89
Glycine	2.25	2.04	2.74	2.41	2.01	2.55	2.44
Histidine‡	1.13	0.99	1.31	1.26	0.95	1.27	1.17
Arginine	4.26	3.88	5.45	4.72	3.77	5.09	4.73
Threonine‡	2.02	1.79	2.43	2.20	1.79	2.38	2.17
Alanine	2.20	1.97	2.38	2.35	1.93	2.51	2.35
Proline	2.25	2.08	2.53	2.43	2.05	2.54	2.41
Tyrosine	1.83	1.69	2.30	2.12	1.70	2.34	2.22
Valine‡	2.46	2.33	2.86	2.66	2.31	2.88	2.64
Methionine*‡	0.56	0.48	0.57	0.57	0.47	0.58	0.55
Cysteine*	0.67	0.51	0.56	0.69	0.46	0.54	0.65
Isoleucine‡	2.30	2.18	2.64	2.48	2.14	2.72	2.48
Leucine‡	3.86	3.71	4.58	4.24	3.76	4.65	4.19
Phenylalanine‡	2.68	2.59	3.44	2.97	2.53	3.25	2.99
Lysine‡	3.93	3.15	3.79	4.02	2.91	4.05	3.66
Tryptophan‡	0.52	0.48	0.58	0.51	0.49	0.62	0.58

Measurements were performed once on each flour sample.
(*) Indicates sulfur amino acid. (‡) Indicates essential amino acids.
Abbreviations: CP (crude protein, wet weight basis)

Table. 4.7. Essential amino acid concentration (mg/g protein) and amino acid scores for untreated and alcohol washed air classified pea protein enriched flour.

	Amino acids								
	THR	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
***a)* Essential amino acid concentration (mg/g protein)**									
Untreated	38	47	23	44	72	87	21	75	10
Ethanol									
20%	30	39	17	37	62	72	17	53	8
50%	41	49	19	45	78	98	22	64	10
80%	39	48	23	45	76	91	23	72	9
Isopropanol									
20%	29	37	15	35	61	68	15	47	8
50%	38	46	18	44	75	90	20	65	10
80%	38	46	21	43	73	91	20	64	10
FAO Reference[1]	34	35	25	28	66	63	19	58	11
Pea Protein Albumin[2]	43	42	58	34	46	84	21	84	22
Pea Protein Globulin[2]	31	41	11	38	81	67	23	66	9
b) Amino acid score									
Untreated	1.13	1.33	0.94	1.58	1.10	1.38	1.12	1.29	*0.90
Ethanol									
20%	0.88	1.11	*0.66	1.31	0.94	1.14	0.87	0.91	0.73
50%	1.21	1.39	*0.77	1.60	1.18	1.55	1.17	1.11	0.90
80%	1.16	1.36	0.91	1.59	1.15	1.45	1.19	1.24	*0.84
Isopropanol									
20%	0.85	1.07	*0.60	1.24	0.92	1.09	0.81	0.81	0.72
50%	1.13	1.33	*0.72	1.57	1.14	1.43	1.08	1.13	0.90
80%	1.12	1.32	*0.84	1.55	1.11	1.45	1.08	1.10	0.92

Measurements were performed once on each flour sample.

(*) Indicates the first limiting amino acid.

Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); TRP (tryptophan); and PPEF (pea protein enhanced flour).

1. FAO, 1991.
2. Mariotti *et al.*, 2001.

are listed in Table 4.7. The methionine + cysteine content (mg/g of protein) of pea albumins was more than double that observed in pea protein. This may be related to the significantly lower sulfur-containing amino acid content in the in the 20% alcohol treated products compared to untreated and 80% alcohol washed PPEF, as more albumins may be washed out in these low alcohol treatments. All isopropanol treatments had lower methionine + cysteine content (mg/g of protein) compared to the corresponding ethanol treatments. This indicated that more albumins might be washed out in the isopropanol treatment then the ethanol treatment. This hypothesis was supported by the solubility results in Table 4.4. At 80%, higher amounts of prolamin-types were believed to be washed away rather than albumins and globulins. Except for 20%, regardless of the treatment, only methionine + cysteine and tryptophan were limiting. In all cases, the most limiting amino acid for treated PPEF was methionine + cysteine, with tryptophan always being the second most limiting. Untreated PPEF and 80% ethanol treatment were the two exceptions where the order was reversed.

However, the *in vitro* protein digestibility values were found to increase significantly from 78.5% (untreated) to 81.5%–84.6% and 82.8%–84.9% for ethanol and isopropanol, respectively. It was hypothesized this was due to the partial protein denaturation that occurred during alcohol washing. This would lead to an opening up of the protein structure to make them more susceptible to attack by digestive enzymes. Although, protease inhibitors are heat labile, however the mild heat application during drying (50°C) in this research was not sufficient to inactivate the inhibitors (Perez-Maldonado *et al.*, 2003). However, protease inhibitors include trypsin and chymotrypsin inhibitors are in the class of albumin protein with low molecular mass of 10–15 kDa. (Roy et al, 2010). During the alcohol treatment the protease inhibitors may be washed out or be denatured by alcohol. Reduced protease inhibitor activity would improve the protein digestibility.

Because the level of the most limiting amino acid decreased with alcohol washing at the 20% and 50% concentrations, the corresponding *in vitro* protein digestibility-corrected amino acid scores (IV-PDCAAS) were also reduced relative to the untreated PPEF (70.6%), despite their higher digestibility (Table 4.5). In contrast, when washed with 80% ethanol or isopropanol, similar IV-PDCAAS values to those observed in the untreated samples were obtained.

5. SUMMARY AND CONCLUSIONS

Impact of aqueous alcohol washing on physicochemical properties

All aqueous-alcohol-washed PPEF samples showed decreased crude fat and ash contents, which resulted in an increase in protein levels in the samples. However, the protein content decreased with increased alcohol concentration. As isopropanol was less polar than ethanol, it removed more fat compared to ethanol at the same concentration. In addition, with the lower solvent polarity at a higher alcohol concentration, the lowest fat content was observed in the sample treated with 80% isopropanol. The solvent treatments also resulted in darker-coloured samples (lower L* values) compared to untreated PPEF, which was in contrast to other solvent-washing studies. The darkening in samples in this study may be a result of the drying process. Among the treated samples, PPEF treated with higher alcohol concentrations had the highest lightness values (L*) and the lowest overall colour (ΔE) compared to untreated PPEF. Surface charge on the protein in PPEF at neutral pH was found to decrease after alcohol treatment, with the greatest decline found in the samples treated with 80% alcohol.

Impact of alcohol washing on flavour profiles

Overall, 28 volatile compounds were extracted from the untreated PPEF. The largest chemical group of the compounds extracted was aldehydes (64%), including the most abundant compound, hexanal and seven others. Hexanal accounted for 54% of the total peak area extracted. There were 11 different alcohols extracted, which accounted for 30% of the total peak area. All alcohol-washing treatments resulted reduction in the total peak area of volatile compounds extracted, excepted for 20% isopropanol treatment. The polarity of the solvent decreased with an increase in the concentration of alcohol, and the efficiency of flavour compound removal increased with the decrease in solvent polarity. Ethanol and isopropanol treatments at 80% concentration were the most proficient in reducing the total peak area of volatile flavour compounds in PPEF. Total peak area of aldehydes was reduced by 96% in PPEF extracted with 80% isopropanol. In addition, the most abundant compound, hexanal, was reduced to 1% with 80% aqueous

isopropanol treatment. Isopropanol was more efficient than ethanol in removing aldehydes at 50% and 80% concentration.

At the lowest concentration level (20%), ethanol removed more total alcohols than did isopropanol. In contrast, there were more 1-hexanol and 1-octen-3-ol generated than removed during treatment with 20% isopropanol treatment, which resulted in lower total alcohol peak area reduction. The enzyme, alcohol oxidoreductase, might not be inactivated with the 20% aqueous isopropanol treatment and would have assisted in accelerating the generation of these alcohol compounds during the washing and drying process. The compound found in the second highest amount, 1-hexanol, in PPEF was reduced by over 90% with 50% and 80% aqueous alcohol treatments. Although isopropanol washed out completely more types of alcohol compounds compared to ethanol at 50% and 80%, there was no significant difference in the total peak area of alcohols detected. Since a 20% alcohol concentration might not be sufficient to inactive the enzymes involved in the generation of volatile compounds, several ketones exhibited increased quantities after the 20% alcohol treatments. Both 20%-ethanol- and 20%-isopropanol-treated PPEF had significantly higher contents of 2-heptanone, a compound often found in heat-treated products. Meanwhile, solvents with higher alcohol concentrations were able to remove 90% or more of the ketones in the samples. There were several aromatic hydrocarbons generated during the alcohol washing and drying processes, including p-xylene, benzaldehyde, furan, 2-pentyl-, and benzeneacetaldehyde. Overall, eight compounds were reduced to below detectable levels with 50 ethanol treatment, 11 in 80% ethanol, 17 in 50% isopropanol, and 20 in 80% isopropanol. Although isopropanol was more efficient in eliminating volatile compounds at 50% and 80% concentration compared to ethanol, there was no significant difference in total peak area extracted among the four treatments.

Impact of alcohol washing on protein functionality

Protein solubility in PPEF was reduced with all alcohol-washing treatments. Overall, protein solubility also decreased with an increase in alcohol concentration, which may result from more prolamin and lesser amounts of albumin and globulin proteins being washed out. Isopropanol treatments had a more negative affect on protein solubility compared to ethanol treatments at the same concentration. In other words, among all treated samples, 80%-ethanol-treated PPEF had the highest protein solubility, while 20%-isopropanol-treated samples had the lowest solubility. Water

hydration capacity was increased after aqueous alcohol treatment, whereas oil holding capacity decreased. Among the treated PPEFs, the samples treated with 80% alcohol had the lowest water hydration capacity and oil holding capacity and there was no significant difference between ethanol and isopropanol treatments. There was no significant impact on emulsion stability with the 20% ethanol treatment or 20% and 50% isopropanol treatment, whereas samples washed with 50% or 80% ethanol or 80% isopropanol showed decreases in emulsion stability.

Impact of alcohol washing on protein quality

Untreated PPEF was limiting in sulfur-containing amino acids and tryptophan, with tryptophan being the first limiting amino acid. There are higher contents of methionine and cysteine per unit of albumin-type protein compared to those per unit of other types of protein in pea. As a result, the proposed greater loss of sulfur-amino-acid-rich albumin-type proteins with the 20% ethanol treatment caused the sulfur-containing amino acid score to drop significantly. PPEF treated with higher concentrations of aqueous alcohol had higher sulfur-containing amino acid scores and overall amino acid scores compared to 20%-alcohol-treated samples, although all treated samples had poorer amino acid profiles compared to the untreated sample. The alcohol treatments might have inactivated the protease inhibitor as well as opened up the protein structure. These modifications resulted in improved *in vitro* protein digestibility. With the counter-effect of amino acid score, the overall IV-PDCAAS was not improved. PPEF samples treated with 80% aqueous alcohol were the only ones remaining PDCAAS values similar to that of the untreated sample.

6. FUTURE STUDIES

This research investigated the effect of aqueous alcohol washing on the removal of volatile flavour compounds in pea protein enriched flour, as well as the functionality modifications associated with the alcohol treatments. Each flavour compound has a particular threshold value for detection. For example, decanal can be detected at a concentration as low as 0.1 ppb and will have a significant impact on flavour at low concentration, whereas 1-hexanol can be detected at a concentration over 200 ppb and may not affect the overall flavour profile at a much higher concentration compared to decanal. In addition, different combinations of compounds can affect sensory differently. Furthermore, in this study HS-SPME GC-MS was used to measure the volatile flavour compounds, hence the non-volatile compounds such as isoflavones, saponins, phenolic acids and peptides were not measured. Moving forward, it will be critical to perform sensory evaluation in order to correlate reductions in specific flavour compounds with sensory attributes.

The higher proportions of total volatile compounds extracted from the 20%-isopropanol-treated sample compared to the untreated sample indicated there might be more flavour compounds generated than removed by the 20% isopropanol treatment. It was hypothesized that the heat during drying induced enzymatic lipid degradation. To distinguish the effect of alcohol washing from that of the drying process on volatile compounds, the flavour profile of a negative blank sample, which would be treated with water, should be evaluated.

To better understand the changes in protein solubility and amino acid score, the protein content and fraction profile (*e.g.*, albumin, globulin, prolamin and gliadin) in the supernatant from the alcohol treatment should be analyzed. The finding could be used to verify the hypothesis of more albumin being washed out with the 20% alcohol treatment and the resultant lower solubility and amino acid score. An SDS-PAGE gel may be used in identify the protein fractions in the supernatant.

Lipoxygenase-catalyzed lipid degradation was the major cause of the generation of undesirable flavour compounds. Since the alcohol washing not only reduced the lipid concentration in pea protein enriched flour but also inactivated LOX (Roland *et al.*, 2017), the development of flavour compounds in treated PPEF would be minimized. Therefore, it would be

important to analyze the LOX activity and flavour compound modification over time by carrying out a storage stability evaluation of untreated and treated PPEF to study the effect of alcohol washing on shelf life.

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