Pea Protein – Volatile Compound Interactions:

Effects of Binding, Heat and Extraction on Protein Functionality

by

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Abstract

Binding of volatile flavour compounds to plant proteins is known to be an issue, particularly for developers of flavoured gluten-free snacks made with pea protein. This project used a model system to describe the effects of extraction and heat on the binding of hexanal (Hex), hexyl acetate (HxAc) and 2-octanone (2-Oct) to pea protein isolate and to evaluate any resulting change in protein functionality.

Yellow field peas were roasted, micronized or unheated. Proteins were isolated using either alkaline (PPIa) or salt (PPIs) extraction. Binding was evaluated using headspace-gas chromatography (HS-GC), where protein isolate (1%) was mixed with a volatile compound (100, 200 or 500 ppm). Thermal properties were examined with differential scanning calorimetry (DSC) and rheological properties with small-strain oscillatory deformation. For DSC, samples contained 10% PPI, for rheology, samples of 14.5% PPI were used; both with 500 ppm of volatile compounds. All experiments were at pH 7.6.

GC revealed binding difference with a four way interaction involving extraction, heating methods, volatile type and concentration. Of note was that roasted PPIa showed less binding than all other samples with HxAc at 200 ppm, while at 500 ppm HxAc showed trends toward more binding. Enthalpy of denaturation was reduced by heat and alkaline extraction while volatile compounds also appeared to influence thermal behaviour. Rheological behaviour of PPI was affected by extraction, heating and volatile compound. PPIs had gel points during cooling; the delay in gelation may be due to formation of a temporary primary structure. Unheated and micronized PPIs had later gel points than their PPIa counterparts. PPIa formed stronger gels as did the absence of volatile compound compared to Hex.

GC showed a decrease in volatile compounds in the presence of protein and an impact was seen both thermally and rheologically. Extraction method, heat method and volatile compounds all had measurable effects on the functionality of pea protein. Developers of novel foods using pea protein isolates should be aware of the binding of flavours by pea protein and effects on protein functionality.

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Melissa

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List of Abbreviations

A	Alkaline extraction
ANFs	Antinutritional factors
ANOVA	Analysis of variance
β-Lg	β-lactoglobulin
B	Boiled
CHO	Carbohydrate
DF	Degrees of freedom
DSC	Differential scanning calorimetry
d.w.b.	Dry weight basis
FS	Frequency sweep
G′	Storage modulus
G″	Loss modulus
GC/MS	Gas chromatography/Mass spectrophotometery
ΔH	Enthalpy
Hex	Hexanal
HOV	Homogeneity of variance
HPLC	High performance liquid chromatography
HS-GC	Headspace – gas chromatography
HxAc	Hexyl acetate
LSM	Least squared means
M	Micronized
2-Oct	Octanone
PPI	Pea protein isolate
PPIa	Pea protein isolate – alkaline extracted
PPIs	Pea protein isolate – salt extracted
R	Roasted
S	Salt extraction
SD	Standard deviation
SE	Standard error
Td	Temperature of denaturation
U	Unheated

1. Introduction

1.1. Pulses

Pulses are low-oil seeds from leguminous plants (compared with leguminous oil seeds); some examples are lentils, peas, beans, chickpeas, faba beans and cowpeas (World Health Organization, 2007). Because leguminous plants use nitrogen from the air (Latham, 1997), they are a lower-input agricultural crop that leaves the soil enriched rather than depleted. There are indigenous varieties throughout the world and many people depend on pulse proteins particularly in places where meat is inaccessible.

Plant proteins contain less methionine than meat and in order to maximize nutrition from them (i.e. increase protein digestibility and decrease anti-nutritional factors (ANFs)) processing treatments such as soaking, cooking and fermenting are necessary (Khattab, Arntfield & Nyachoti, 2009). In North America, pulses are promoted as a healthy food choice because of high fibre, lysine (an essential amino acid), vitamin and mineral content (iron, potassium, magnesium, zinc and B vitamins) as well as low fat content and glycemic index (Pulse Canada, 2012).

Protein is an essential macronutrient. It also provides functional properties to create texture, emulsions, foams and gels in food. These abilities depend on proteins' amino acid composition, structure, conformation and solubility, which can be altered by processing parameters such as temperature, pH and presence of solutes. Protein structure is formed and maintained by hydrophobic interactions, electrostatic interactions, hydrogen bonds and Van der Waals forces.

In 2012, Canada was the top producer and exporter of dried peas; accounting for 28% of the 9.9 million metric tonnes of dried peas produced worldwide and exporting over 80% of their peas produced, Canadians also consume peas. In 2009, we ate, on average, 1.3 kg/person compared with the worldwide average of 0.9 kg/person (Food and Agriculture Organization, 2014). As a consumer and primary

producer of dried peas, it is advantageous for Canada to invest in pea research to increase the value of peas produced both for export and domestic use.

1.2. Flavour, Volatiles and Legumes

Flavour is perceived by humans through three primary paths: taste, smell and nerve sensation. Compounds that activate receptors in the mouth correspond to salty, sweet, bitter, sour, umami and fat tastes. Aroma is sensed from volatile compounds that enter the nasal passage through the nose or the mouth (retronasally) to trigger the olfactory receptors and a response in the brain. Chemesthesis sensations are feelings of burning (spicy) or cooling (e.g. mint) from a stimulated trigeminal nerve. These three pathways intersect and overlap with each other and with other signals such as colour, sound, memory and texture to create, enhance or supress overall flavour. An adequate amount of flavour compounds from the food must be released and travel to the receptors to be perceived.

A word regarding terminology: 'flavour' encompasses taste, smell and sensation as perceived by humans. 'Aroma' is the perception of volatile compounds received by the olfactory receptors in humans. The term 'volatile compound' is most appropriate in this report as the compounds discussed have the ability to travel to human olfactory receptors but are detected using instruments.

Not all flavours are desirable. Legumes are often associated with flavour problems and off-flavours. Kinsella and Damodaran (1980) reported the compounds that contribute to beany, grassy and bitter flavour in soybeans included furans, aldehydes, alcohols, fatty acids and others. The formation of off-flavours can occur from enzymatic degradation of lipids by lipoxygenase or result during processing with high heat (Maillard reaction). In order to control off-flavour formation, researchers and processors have implemented specific parameters for milling, heating, water addition, soaking and roasting of soybeans (the most commonly used plant protein). Other methods considered to remove off-flavours include

steam or solvent extraction (Kinsella & Damodaran, 1980) and competitive binding with β-cyclodextrin (Arora & Damodaran, 2010).

1.3. Protein-Flavour Interactions

Although the main compounds (carbohydrate, fat, protein) in food do not contribute directly to food flavour, they can trap flavour-active compounds (Tromelin, Andriot & Guichard, 2006). With protein, binding is affected by type and conformation of the protein as well as the flavour compound length and chemical class (or functional group) (Li, Grün & Fernando, 2000; Tromelin et al., 2006). While interactions between protein and flavour can be reversible or irreversible, most are reversible involving hydrophobic interactions and hydrogen bonds (Tromelin et al., 2006). Reversible binding is important for protecting desirable flavour compounds during processing and releasing them during consumption (Zhou, Lee & Cadwallader, 2006). For undesirable flavours, little to no binding is preferred.

1.4. Objectives

This project sought to expand the knowledge of pea proteins regarding binding behaviour of volatile compounds. Specifically, the objectives were: to use a model system to describe the effect of heat treatments and protein extraction method on the binding of hexanal (Hex), hexyl acetate (HxAc) and octanone (2-Oct) to pea protein isolate using headspace-gas chromatography (HS-GC), and to evaluate any change in protein functionality due to the interaction between protein and the volatile compounds with thermal and rheological investigations.

2. Literature Review

2.1. Characteristics of Pea Protein

In pulses, there are two major protein fractions: globulins (salt soluble) and albumins (water soluble). The globulins, often called storage proteins, are legumin, vicilin and convicilin. Legumin (11S) has six subunits each composed of an acidic (40 KDa) and basic (20 KDa) polypeptide joined with a disulfide bridge to form a hexameric quaternary structure with a molecular weight of ~390 KDa (Croy, Derbyshire, Krishna & Boulter, 1979). Vicilin (7S) has a trimeric structure with a molecular weight of 175-180 KDa. The subunits are ~50 KDa with some splintered fragments. Vicilin contains no sulphur residues (Croy, Gatehouse, Tyler & Boulter, 1980; Gatehouse, Croy, Morton, Tyler & Boulter, 1981; Boye, Zare & Pletch, 2010). Convicilin is related to vicilin and often contaminates vicilin isolates. It is also trimeric with subunits ~71 KDa in molecular weight (Croy et al., 1980). Legumin and vicilin are common to all legumes although the quaternary structure may differ by plant (Tromelin et al., 2006).

Dried peas have 20-27% protein and the globulin fraction is 65-70% of the total protein (Sun & Arntfield, 2012). The isoelectic point of the pea globulin fraction is between pH 4 and 5 (Boye, Zare et al., 2010). Alongside the protein, dried peas also contain moisture (8%), crude fat (2%), ash (3%), fibre (5%), and carbohydrate (68%) (Khattab et al, 2009).

2.2. Protein Extraction Methods

In order to exploit the functionality of plant proteins they are often extracted from the biological material in which they are found. For pea proteins, there are two common ways to solubilize or extract the protein (salt and alkaline) and a number of ways to precipitate or concentrate the protein (using acid, isoelectrically, sudden dilution, dialysis, lyophilization).

Salt extraction followed by acid precipitation has been used by Bora, Brekke and Powers (1994) and Elmer, Karaca, Low and Nickerson (2011). A low concentration salt buffer solution provides counter ions to increase the electrostatic free energy which increases protein solubility. Isoelectric or acid precipitation is done by decreasing the pH to the isoelectric point where the repulsive forces are minimal causing the protein to aggregate. Protein, once precipitated, can be removed using centrifugation. Liu, Low and Nickerson (2009) also used a salt buffer (pH 8) to extract protein but then removed the salt with dialysis and recovered the protein via lyophilisation. Similarly, Sun and Arntfield (2010) developed a salt extraction procedure that did not require any pH alteration. Protein was extracted with a 0.3 M NaCl solution and then precipitated by a sudden dilution with cold water. The dilution encourages hydrophobic interactions within the protein causing aggregation and precipitation. The protein is recovered through centrifugation, de-salted via dialysis and concentrated with lyophilization.

Another extraction method uses an alkaline solution. As the pH is adjusted away from the isoelectric point, protein becomes highly charged increasing its interaction with water. Boye, Aksay et al. (2010) used this method to solubilize protein by adding NaOH to the protein solution to a pH of 9.5. Then they precipitated the protein isoelectrically by decreasing the pH to 4.5.

Because most methods used to solubilize or precipitate protein rely on a change in pH (and thus conformation), protein isolates are not often in their native form. Karaca, Low and Nickerson (2011) demonstrated this difference by comparing the emulsifying capabilities of protein isolates prepared by salt extraction with dialysis concentration (no pH change) to an alkaline extraction with acid precipitation (pH change). They found that the isolation method impacted the properties of the protein: namely surface hydrophobicity, surface charge and solubility.

2.3. Protein Functionality

Evaluating the physicochemical properties of proteins gives insight to how they may function in a food product. Thermal response and gelling ability are critical to texture and are influenced by changes in structure due to heat treatment, protein isolate preparation and perhaps binding of volatiles.

2.3.1. Thermal behaviour. While heat in general affects the conformation of proteins, there are differences between heat treatments, particularly dry and moist heat.

Micronization, an infrared heat process, has been shown to reduce the cooking time of legumes (Bellido, Arntfield, Cenkowski & Scanlon, 2006; Cenkowski & Sosulski, 1998). After tempering to a specific moisture content the legumes are exposed to the infrared heat for a short period of time (1-1.5 min) and can reach temperatures around 120°C. This was sufficient to denature the protein and pregelatinize the starch, reducing the cooking time by up to 1/3 (Cenkowski & Sosulski, 1998).

Heat treatments, including micronization, were compared as to the effects on protein quality and ANFs. Micronization was shown to increase the protein quality parameters of peas but neither micronization nor roasting (without added moisture) improved *in vitro* protein digestibility (Khattab et al., 2009). Dry heat, while it decreased ANFs, was not as effective as boiling, so ANFs still interfered with protein digestibility. Dry heat may also catalyze the Maillard reaction which involves protein and may contribute to a lower digestability (Khattab & Arntfield, 2009).

Functional and structural properties of heat treated flours were examined and compared by Ma et al. (2011). All heat treatments, including roasting and boiling, decreased trypsin inhibitor activity and increased emulsifying activity index. Boiling also caused a decrease in protein solubility and foaming while increasing fat absorption, water holding capacity and gelation.

Similarly, heat treating the protein isolates affected conformational and functional properties (Tang, Sun & Yin, 2009). Boiling vicilin-rich isolates from *Phaseolus* increased the emulsifying activity index, the protein solubility, hydrophobicity and fluorescence and decreased the free sulphydryl contents. In other words, boiling induced denaturation and disulfide bond formation.

Differential scanning calorimetry (DSC) is a calorimetric approach that monitors the energy required to maintain a temperature (Semenova, Antipova, Wasserman, Misharina, & Golovnya, 2002). Structural and conformational changes due to binding can be followed as such transitions require or release energy. Important parameters evaluated include the denaturation temperature (Td) and enthalpy (ΔH).

Thermal properties of pea protein have been studied by a few researchers. Pea protein thermograms are endothermic and show one peak as the 7S and 11S denaturation peaks overlap (Bora et al., 1994; Mession, Sok, Assifaoui & Saurel, 2013). Exothermic processes, like interruption of hydrophobic interactions and aggregation, occur alongside endothermic hydrogen bond breakage, but are not of the same magnitude. It is thought that Td is controlled by hydrophobic interactions (Arntfield & Murray, 1981) and that it is less sensitive to change than ΔH (Semenova, Antipova, Belyakova et al., 2002).

An early look at field pea micelle isolate (20%) by DSC indicated a Td of 86°C and ΔH of 3.72 cal/g (Arntfield & Murray, 1981). Similarly over the years, pea protein has been found to denature between 80-87°C in a low ionic strength aqueous solution near native or neutral pH (Sosulski, Hoover, Tyler, Murray & Arntfield, 1985; Bora et al., 1994; Semenova, Antipova, Wasserman et al., 2002; Shand, Ya, Pietrasik & Wanasundara, 2007; Sun & Arntfield, 2010; 2011; Mession et al., 2013).

Other important conclusions about pea protein thermal properties are that the addition of NaCl increases the Td and ΔH as it stabilizes the conformation of the protein by reducing repulsion within the protein and by ordering the water (Shand et al., 2007; Sun & Arntfield, 2010). Extraction method and the pH of the isolate are also important for thermal properties (Mession et al., 2013; Sun & Arntfield, 2011).

For example, Sun and Arntfield, 2010 found a commercial isolate to have both lower Td and ΔH due to the denaturing nature of the isolation process. Denaturation due to heat was also found to lower ΔH but increased Td as the heat 'knocked out' the lower Td proteins, leaving those more resistant to heat (Arntfield & Murray, 1981). In soy, a decrease in pH led to a decrease in Td. The gels formed at pH 3.8 were more granular and stiff than the gels at pH 7.6 indicating a coarse gel due to larger aggregates and more protein-protein interactions (Renkema, Lakemond, de Jongh, Gruppen & van Vliet, 2000). Tang et al. (2009) found that boiling the isolate prior to thermal analysis resulted in a 91% reduction in ΔH for some samples and in others there was no transition present.

Semenova and colleagues investigated the thermal effects of binding of aroma compounds, specifically HxAc, to legumin (Semenova, Antipova, Belyakova et al., 2002; Semenova, Antipova, Wasserman et al., 2002). First, there was an increase in ΔH as the concentration of HxAc increased to saturation, indicating improved conformational stability, attributed to extra bond formation inside the protein globule. Td was independent of HxAc concentration and, being less sensitive, indicated that the binding effects were small (Semenova, Antipova, Wasserman et al., 2002). In the third report, they found an increase in ΔH with an increase in the chain length of the volatiles as, perhaps, binding created more new bonds with longer compounds. This increase had a maximum and then declined as the protein may have compensated for the large compound by partially unfolding (Semenova, Antipova, Belyakova et al., 2002).

2.3.2. Gelation capacity. The ability to create gels and form networks is a key function of plant proteins. They can contribute to texture and flavour by creating structures that retain and release moisture and flavour compounds (Uruakpa, 2009). In order to create new cross-links, the protein must be partially denatured (often by heat) to expose reactive areas. The currently accepted theory of gelation is the corpuscular theory where the native protein unfolds into a strand of spherical structures

which then interact linearly and cross-link to form a network (Clark, Kavanagh & Ross-Murphy, 2001; Uruakpa, 2009). The three steps can also be described as denaturation, aggregation and agglomeration (Sun & Arntfield, 2010). The gel and mechanism of gelation is governed by a balance of attraction and repulsion, hydrophobic interactions, hydrogen bonds and electrostatic interactions. Gelation is dependent on the unfolding parameters (technique, temperature, rate, etc.), pH, presence of solutes (e.g. salt) and other macromolecules, etc.

Minimum gelling concentration is the least amount of protein required to create a gel. Values for pea protein have been reported between 3-20% depending on pH, salt concentration and method of extraction (Adebiyi & Aluko, 2011; Boye, Aksay et al., 2010; Sun & Arntfield, 2010; Wong, Vasanthan& Ozimek, 2013). Generally the more harsh (alkaline) the extraction method, the more protein required to create a gel.

A popular technique to investigate gelation is small strain oscillatory testing because it follows gel formation and characterizes the final gel. Storage (G') modulus refers to the elastic component, indicating the interactions that lead to the 3D network. The loss (G") modulus represents the viscous component showing the interactions that do not contribute to solidity. The ratio of G"/G' is known as tan δ and describes the nature of the gel. For example a strong gel will have a low tan δ as G'>G", i.e. the matrix is more elastic than viscous. The point of gel formation can be determined in two ways: First when G'>G", the cross-over point or when the elastic component dominates the matrix. Alternatively, the sudden increase in G' during heating can be extrapolated to the temperature axis.

Sun and Arntfield (2010, 2011, 2012) have thoroughly investigated the gelation properties of pea protein. They found a typical gel formation pattern during a heating profile of 25°C – 95°C – 25°C started with steady G'<G" indicating a liquid. At 80°C there was sharp rise in both G' and G" with G' increasing more and crossing-over G" (gel point, G'>G"). This was due to heat denaturation of the protein where

buried hydrophobic groups were exposed. For the rest of the test, G' remained greater than G" as crosslinks continued to be made and the network strengthened. During cooling, G' and G" both continued to rise slightly as the network stabilized. Tan δ decreased through the heating step with a sharp decrease to <1 at the gel point where it stabilized and stayed low throughout cooling.

By varying the rate of temperature change and concentration of protein, they concluded that: Gel point (84.5-85.7°C) was not affected by concentration (Sun & Arntfield, 2010). An increase in heating rate up to 2°C/min increased the gel point but there was no significant difference between 2°C/min and 4°C/min. Compared to commercial extracts, salt extracted protein gave stronger gels due to the lower degree of denaturation during extraction; however pea protein gels were still quite weak (Sun & Arntfield, 2010).

Subsequently, they explored the effects of pH and salt content. G' was found to be pH dependent and the further from the isoelectric point, the stronger the gel (Sun & Arntfield, 2011). Between pH 6 and pH 7, the G' was low as the temperature of denaturation (as determined by DSC) was greater than the maximum heat used (95°C) so the protein did not properly denature at these pH levels. The gels formed here were not very strong and the gel point was the highest (89.1 ± 0.1°C, pH 6). At the more extreme pHs, acid and alkali denaturation was thought to increase the gel strength. Salt was found to stabilize the quaternary structure leading to an increase in gel point. Without salt, the proteins were, perhaps, unstable and aggregated quickly upon heating, leading to a low G' and a poor gel. Alternately, without salt the electrostatic repulsion was too high to allow a gel to form. As such, pea protein required salt to gel between pH 6 and pH 8; in fact, at pH 7 the high standard deviation for tan δ led to issues in finding significant differences in the data (Sun & Arntfield, 2011).

Wong et al. (2013) blended pea and soy protein to optimize gel formation. They found the best synergy was at a pea:soy ratio of 2:8 at pH 6 with 10% protein concentration. O'Kane, Vereijken, Gruppen and

van Boekel (2005) also analyzed the gelation behaviour of pea protein isolates, looking particularly at differences in cultivar and legumin and vicilin content. These experiments are summarized in Table 1.

Table 1. Rheological explorations of pea protein

Protein (%)	Salt	pH	G′ (Pa)	Tan δ	Gel point (°C)	Reference
14.5	0.3M	5.65	3212.5 ± 0.71	0.17 ± 0	85.1 ± 0.56	Sun & Arntfield, 2010
14.5	0.0M	5.65	0.35 ± 0.17	1.36 ± 0.65	60.15 ± 1.91	Sun & Arntfield, 2011
10, 16, 22	2.5%	4	4881	nd[1]	nd	Wong et al., 2013
10, 16, 22	2.5%	6	2509	nd	nd	Wong et al., 2013
10, 16, 22	2.5%	8	3569	nd	nd	Wong et al., 2013
18	0	7.6	~1000	nd	nd	O'Kane et al., 2005

[1] No data reported

2.4. Flavour and Volatile Compounds

Flavour compounds come in all shapes and sizes with the only requirements that they are volatile and activate the olfactory system ('odour-active'). Typical compounds are small and can be aldehydes, ketones, esters, alcohols, compounds with aromatic rings or others. Many occur naturally in food and are added alongside synthetic flavouring agents to most processed foods. I have chosen to investigate the effects of three compounds: hexanal, hexyl acetate and 2-octanone. They represent different chemical classes of odourants and have 6-8 carbon molecules. They are described in further detail below.

2.4.1. Hexanal (Hex). A six-carbon aldehyde as shown in Figure 1, Hex (CAS 66-25-1) has a molecular weight of 100.1589 g/mol. It is slightly soluble in water. It has characteristic 'grassy' or 'unripe fruit' odour, often used in fruit flavour preparations and perfumes (Dictionary of Food Compounds, 2014). The odour threshold has been reported as 0.03 ppm (Schnabel, Belitz & von Ranson, 1988). It is generally regarded as safe.

Figure 1. Chemical structure of hexanal

2.4.2. Hexyl acetate (HxAc). Also known as hexyl ethanoate, HxAc (CAS 142-92-7) has a molecular weight of 144.2114 g/mol (Figure 2). It is a hexyl ester and is not very soluble in water. Generally regarded as safe, HxAc has a 'fruity' or 'herby' odour and is used in fruit aromas (Dictionary of Food Compounds, 2014). The odour threshold is 0.06 ppm according to Schnabel et al. (1988).

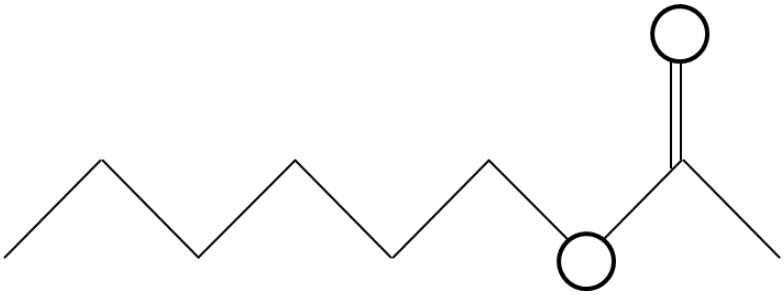

Figure 2. Chemical structure of hexyl acetate

2.4.3. 2-Octanone (2-Oct). An eight carbon ketone with a molecular weight of 128.2120 g/mol, 2-Oct (CAS 111-13-7) (Figure 3) is slightly soluble in water. Used as a flavouring agent, 2-Oct is said to have a 'green' or 'fruit' odour (Dictionary of Food Compounds, 2014). It is also generally regarded as safe with an odour threshold of less than 0.03 ppm (Schnabel et al., 1988).

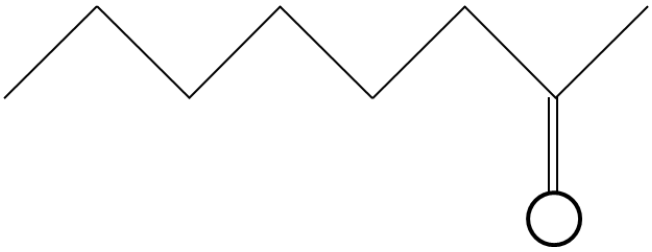

Figure 3. Chemical structure of 2-octanone

2.5. Protein-Volatile Compound Interactions

2.5.1. β-lactoglobulin (β-Lg). The primary whey protein, β-Lg, is widely used in food products as an emulsifier, foaming agent and fat replacer. It is soluble across a wide pH range and has a simple, well understood structure. For these reasons, β-Lg is one of the most studied proteins in volatile compound binding studies. From 1994-1999, a network of scientists (COST Action 96 and the French DGAL programme) collaborated to examine the interactions of food matrices with small ligands influencing flavour and texture in four areas: kinetics, modeling, sensory and instrumental analysis (Guichard, 2000; Guichard & Langourieux, 2000). They used β-Lg with a variety of volatile compounds in different model systems. Some of the investigations included: the effects of pH (Jouenne & Crouzet, 2000), the binding of benzaldhyde by static headspace and high performance liquid chromatography (HPLC) (Andriot, Marin, Feron, Relkin & Guichard, 1999), odour perception of vanillin and eugenol (Reiners, Nicklaus & Guichard, 2000), modeling of interaction between β-Lg and methyl ketones (Andriot, Harrison, Fournier & Guichard, 2000) and more. In addition to this particular project, other researchers have explored interactions of: whey protein with volatile compounds as influenced by fat and lactose (Mills & Solms, 1984); β-Lg with various aroma compounds of different lengths (van Ruth & Villeneuve, 2002); chemically modified β-Lg with β-ionone (Dufour & Haertlé, 1990); and native β-Lg compared to molten (via high pressure) β-Lg to aromatic and aliphatic ligands (Yang, Powers, Clark, Dunker & Swanson, 2003).

Some important conclusions were: Binding affinity was affected by conformational changes due to heat (Andriot et al., 1999), pressure (Yang et al., 2003), chemical modification (Dufour & Haertlé, 1990), pH (Jouenne & Crouzet, 2000; Mills & Solms, 1984; van Ruth & Villeneuve, 2002) and the presence of salt or other compounds (Andriot et al., 1999). Generally there was an increase in affinity with an increase in length and hydrophobicity of the volatile compound (Andriot et al., 2000; Guichard & Langourieux,

2000; Jouenne & Crouzet, 2000; Mills & Solms, 1984; van Ruth & Villeneuve, 2002) as well as with an increased concentration of protein (Andriot et al., 2000; van Ruth & Villeneuve, 2002). There was no binding with alcohols (van Ruth & Villeneuve, 2002).

2.5.2. Soy and pea. Gremli (1974) examined the interaction of soy protein with a series of aldehydes, ketones and alcohols using headspace analysis. They found significant interaction with aldehydes that increased as the molecular weight of the aldehyde increased. Similarly for ketones, retention increased with molecular weight up to 2-decanone and then decreased. There was no interaction observed with alcohols. Soy protein interaction with alcohols was further investigated (Chung & Villota, 1989; King & Solms, 1979) and soy protein was found to bind alcohols. It is possible the different results were due to the method of analysis: Chung and Villota (1989) used equilibrium dialysis while King and Solms (1979) used radioactive labeling.

Other papers in the area of soy protein-volatile compound interactions have studied carbonyl compounds (including 2-nonanone) with equilibrium dialysis method (Damodaran & Kinsella, 1981ab; O'Neill & Kinsella, 1987). They showed (in agreement with Gremli, 1974) that binding affinity increased with an increase in chain length and also observed that partial denaturation increased binding. Damodaran and Kinsella (1981ab) concluded the binding was hydrophobic in nature, thermodynamically favourable, and dependent on both the conformation of the protein and the position and type of the ligand's functional group. They also considered soy protein in fractions and found the carbonyl compounds bound more to the 7S fraction (vicilin). O'Neill and Kinsella (1987) corrected some assumptions and calculation errors in the earlier experiments by Damodaran and Kinsella (1981ab), but still used only a small section of the binding curve and sodium azide and mercaptoethanol in the buffer which raised concern for O'Keefe, Wilson, Resurreccion and Murphy (1991). O'Keefe et al. (1991) evaluated the binding of hexanal to soy fractions under a variety of conditions to account for the buffer

compounds. They concluded that there was increased binding when sodium azide was present and that 10 mM of 2-mercaptoethanol had no significant effect. Results reported in the literature for the number of binding sites vary widely due to the variety of sources of protein, method of analysis, breadth of analysis and presence of other compounds (O'Keefe et al., 1991).

Legumin extracted from broad beans was the source of protein for investigations on the binding of HxAc (Semenova, Antipova, Belyakova et al., 2002; Semenova, Antipova, Misharina & Golovnya, 2002; Semenova, Antipova, Wasserman et al., 2002). They compared the binding properties of native legumin to heat and acid denatured protein, reporting the highest binding for native protein and no binding with acid denatured legumin. Heat denatured legumin showed some binding but with a different mechanism than native legumin (Semenova, Antipova, Misharina et al., 2002). HxAc also affected the thermodynamic properties of native legumin; as the HxAc concentration increased the native protein increased in thermodynamic stability up to the saturation point where the protein aggregated (Semenova, Antipova, Wasserman et al., 2002). In the third paper (Semenova, Antipova, Belyakova et al., 2002), the authors concluded that, of the multiple binding sites on legumin, compounds had greater affinity for the sites that were more structurally suitable such that there was a higher binding affinity for methyl esters of carbonic acids than alkyl acetates.

With peas, the research is limited to the work of Dumont (1984; Dumont & Land, 1986) and Heng (2005; Heng et al., 2004). The former investigated retention of diacetyl in emulsions and concluded that lipid interferes with pea protein binding of diacetyl (Dumont, 1984). Dumont and Land (1986) calculated that there were 18 binding sites on pea protein and found that isoelectrically precipitated protein (compared to soluble protein) had less retention ability and released the bound compounds. Heng's work examined the importance of volatile compound binding in the development of novel protein foods, exploring the effect of heat as well as the presence of saponins (Heng, 2005; Heng et al., 2004). Legumin

bound aldehydes at pH 7.6 while vicilin bound both aldehydes and ketones at pH 7.6 and 3.8. Heating appeared to decrease the binding of the studied volatile compounds.

In protein-volatile compound binding studies, the protein concentration used ranged from 0.1-5% (Heng et al., 2004; Gremli, 1974 respectively). In the same way researchers chose different methods, they also chose different compounds and amounts to study. In general, the compounds were either an array from various chemical classes or of various lengths within a group (see Table 2 for a summary).

Table 2. Experimental materials and methods for evaluating protein-volatile compound interactions

Protein; Method	Volatile Compound				Reference
	Aldehyde	Ketone	Other	Amount	
Pea fractions (0.1-1%); HS-GC	Pentanal, Hexanal, Heptanal, Octanal	2-Pentanone, 2-Hexanone, 2-Heptanone, 2-Octanone		0.006-1.2 mM (0.8-103 ppm)	Heng et al., 2004
Pea protein, fractions (2500-15000 M); HS-GC		Diacetyl (butane-2,3-dione)		0.1-100 µM	Dumont & Land, 1986
Broadbean Legumin 11S (1%); Ultrafiltration/GC			Ester: HxAc		Semenova, Antipova, Misharina et al., 2002
Soy glycinin, β-conglycinin (20 mg/ml); HS-GC	Hexanal			1-1000 ppm	O'Keefe et al., 1991
Whole soy isolate, 7S, 11S fractions (1%); Equilibrium Dialysis		2-Nonanone			Damodaran & Kinsella, 1981a; O'Neill & Kinsella, 1987
Soy protein (1%); Equilibrium Dialysis	Nonanal	2-Heptanone, 2-Nonanone, 2-Octanone, 5-Nonanone			Damodaran & Kinsella, 1981b
Soy protein (5%); HS-GC	Hexanal, Heptanal, Octanal, Nonanal, Decanal, Undecanal, Dodecanal, 2-Hexenal, 2-Heptenal, 2,6-Nonadienal, 2,4-Nonadienal, 2-Decenal, 2-Dodecenal,	2-Hexanone, 2-Heptanone, 2-Octanone, 2-Nonanone, 2-Decanone, 2-Undacanone 2-Dodecanone, 2-Phenylmethylketone (4-Methyl-2-phenyl)-methylketone, (3,5-Dimethyl-2-phenyl)-methylketone, 4-Methyl-3-penten-2-one, 6-Methyl-5-hepten-2-one, 3-Octen-2-one 6-Nonen-2-one, 2-Furylmethylketone, (5-Methyl-2-furyl)-methylketone, (2,5-Dimehtyl-3-furyl)-propylketone, 2-Furylbutylketone	Alcohols: Isopentanol, Hexanol, 2-Octenol, 1-Octen-3-ol, 2-Phenylethanol, 1-Phenylethanol, Benzylalcohol,	10 mg/ 100 ml	Gremli, 1974

2.5.3. Methodology. The studies mentioned above aid in an overview of methods for use in protein-volatile compound binding experiments where researchers chose a variety of ways to monitor binding and structural changes. These methods include HPLC, affinity chromatography, dynamic equilibrium, HS-GC, DSC and fluorescence. While Tromelin et al. (2006) provided a comprehensive review of various techniques in their chapter; I will highlight the theory of HS-GC and DSC in brief. HS-GC (used by Andriot et al., 1999; 2000; Dumont, 1984; Gremli, 1974; Guichard & Langourieux, 2000; Heng, 2005; Heng et al., 2004; Jouenne & Crouzet, 2000; Mills & Solms, 1984; Semenova, Antipova, Belyakova et al., 2002; Semenova, Antipova, Misharina et al., 2002; Semenova, Antipova, Wasserman et al., 2002; van Ruth & Villeneuve, 2002) shows the partitioning of volatile compounds between the liquid and vapour phases thus accounting for any binding on the protein. From these measurements the binding constants and number of sites can be determined (Tromelin et al., 2006). DSC (Semenova, Antipova, Wasserman et al., 2002) uses a calorimetric approach that considers the energy required to maintain the sample at a particular temperature. Structural and conformational changes due to binding can be followed as transitions require or release energy.

2.5.4. Effects of heat on protein-volatile interactions. As the binding of volatiles is dependent on protein conformation, anything that alters conformation affects binding. Heating is common in food processing and is recommended to improve the nutrition and functional properties of legumes (Khattab et al., 2009). Heat is also known to alter the conformation of the protein and may change protein-volatile association. O'Neill and Kinsella (1988) found an increase in binding sites available in β-Lg with an increase in the time (1-30 min) the system was held at 75°C. For Andriot et al. (1999), heat treatment (76°C for 10 min) did not affect binding in water, but with heating and the addition of NaCl or ethanol, the amount of binding was halved because the salt and ethanol caused protein aggregation hiding potential binding sites.

For Heng et al. (2004) the amount of binding decreased with heat treatment (90°C for 30 min) from 17% to 5% for 2-octanone and from 45% to 10% for octanal. The heating caused aggregation (seen using gel filtration chromatography) and thus reduced the surface area. They also posited that because the reduction in binding was less than the reduction in surface area it was the change in conformation that altered either the place of binding sites and/or the affinity for the volatiles.

Alternately, Damodaran & Kinsella (1981b) found that at 5°C (compared to 25°C and 45°C) there was a reduction in the number of binding sites but higher binding affinity. Low temperatures decrease the strength of hydrophobic interactions and the authors suggested that this led to a change in conformation or structure, relocating the binding sites. They also examined partially denatured protein (90°C for 1 h) and found an increase in binding affinity.

3. Materials and Methods

3.1. Materials

Two sources of pea flour were used. The first was flour made from yellow whole peas, CDC Meadow, (locally sourced, Winnipeg, MB, Canada) that had been previously heat treated by roasting or micronizing. To compare with other heat treatments and non-heated flour, yellow pea flour (*Pisum sativum)* was obtained from Best Cooking Pulses, Portage La Prairie, MB, Canada The volatile compounds under investigation were hexanal (Hex), 2-octanone (2-Oct) and hexyl acetate (HxAc) (all Sigma-Aldrich).

All chemical reagents were of analytical grade.

3.2. Heat Treatment and Milling

Whole peas were used to create the roasted (R) and micronized (M) samples. Peas were tempered to 14% moisture overnight with distilled water in a Big Cat Mixer (model Red Lion, Canada). To ensure uniform water absorption the peas were tumbled 15 minutes twice in the first hour, 15 min/h for four hours and then held for an additional 16 hours at room temperature. The tempered peas were stored in sealed zippered bags until heat treatment the next day.

For roasting, the tempered peas were spread into a single layer on an aluminum dish and place in a 100°C convection oven for 15 min (Moffat Deltarex, Canada). After heating they were cooled to room temperature and stored in sealed zippered bags until grinding.

For micronization, a gas-fired, pilot-scale MR2 micronizer unit (Micronizer Ltd. Co., Suffolk, UK) was used. Peas were placed in the hopper and the feed gate gap (7 mm) adjusted so the peas were fed onto the conveyor in a single layer at vibrator speed 45. The slope of the conveyor was adjusted so the surface temperature of the peas exiting the micronizer was 100 ± 10°C. The temperature was tested

using an infrared thermometer 3 cm away from the peas. After heating, the peas were spread on a metal tray in a single layer to cool to room temperature and stored in sealed zippered bags until grinding. The Canadian International Grain Institute used a roller mill to create flour.

The pea flour was used for unheated (U) and boiled (B) samples. The boiled sample was only used in HS-GC analysis as boiling was shown to denature the protein 'erasing' the endotherm in DSC (Tang et al., 2009) and in rheological analysis, boiling would have created a gel prior to the gel preparation step. For HS-GC, the boiling treatment was done after the samples were prepared (volatile compounds added and capped). The vials were placed in a hot water bath (90°C) and held for 30 min (Heng et al., 2004).

3.3. Protein Extraction

For each roasted, micronized and unheated pea flour, two methods of protein extraction were used to elucidate any effects of extraction. After extraction the pea protein isolate (PPI) was lyophilized and stored at -18°C until analysis.

3.3.1. Salt extraction (S). As per Sun and Arntfield (2010), the pea flour (typically in 300g batches) was mixed with a 0.3 M NaCl (Fisher Scientific) solution in a 3:10 ratio (w/v). After stirring for 30 min at room temperature, the mixture was centrifuged at 4250 x g for 15 min at 4°C (Thermo Scientific Sorvall RC6+). The protein in the supernatant was concentrated by sudden dilution with cold (4°C) distilled water at a ratio of 1:2, allowed to rest at 4°C for 2 h and then centrifuged at 680 x g for 15 min at 4°C. The salt was removed from the protein pellet by dialysis at 4°C for 72 h against distilled water using a SpectraPor Membrane, molecular weight cut-off of 12000-14000 Da. This pea protein isolate – salt extracted will be referred to as PPIs.

3.3.2. Alkaline extraction (A). As per Boye, Aksay et al. (2010) with modifications by Karaca et al. (2011), pea flour (typically in 40g batches) was mixed with distilled water (1:15) and adjusted to pH

9.5 (Accumet AB15+ Basic pH Meter, Fisher Scientific) with 1.0 M NaOH (Fisher Scientific). The protein solubilized while stirring at room temperature for 1 h. The pH was maintained at 9.5 (adjusted at 20 and 40 min). Insoluble matter was removed with centrifugation (4500 x g, 20 min, 4°C). Protein was precipitated by decreasing the pH to 4.5 with 1.0 M HCl (Fisher Scientific) and recovered with centrifugation (4500 x g, 20 min, 4°C). The pellet was washed in acidified water (made with water and HCl, pH 4.5) and centrifuged (4500 x g, 20 min, 4°C). This pea protein isolate – alkaline extracted will be referred to as PPIa.

3.4. Solution Preparation

Potassium phosphate buffer (10 mM, pH 7.6) was prepared from monobasic and dibasic potassium phosphate (Fisher Scientific) and the pH was adjusted to 7.6. This buffer was used for all sample preparation and was stored at room temperature for no more than 3 months. Samples for analysis were made by mixing (vortex 1 min, VortexGenie 2, Fisher Scientific) protein stock, volatile stock and any additional buffer required to desired concentration. Table 3 contains the protein and volatile percentages in the samples for each analysis.

Table 3. Sample contents and process of analysis

Analysis	PPI (%)	Volatile (ppm)	Handling
DSC	10	500	Rested overnight at 4°C, analyzed at room temp
HS-GC calibration	10 0	100, 200, 500 50, 300, 600	Shaken at room temp for 2.5-17.5 hours
AR-2000	14.5	500	Rested overnight at 4°C, analyzed at room temp

3.5. Proximate Analysis

3.5.1. Protein. Nitrogen content of the PPI was determined by Crude Protein – Micro-Kjeldahl method (AACC Method 46-13.01) with some adjustments. Sample (100 mg) was digested at 350°C with catalyst and 3 mL of concentrated H_2SO_4 for 45 minutes. The solids were dissolved in 10 mL of distilled

water and distilled into 10 mL of H_3BO_3 until 50 mL of distillate was collected. The sample was titrated with a standardized HCl solution until pink. The total organic nitrogen was calculated and converted to protein using a conversion factor of 5.7 (Sun & Arntfield, 2012). The calculations are below:

$$\%\ Total\ organic\ Nitrogen = \frac{(S - B) \times N \times 14.007 \times 100}{sample\ weight\ (mg)}$$

$$S = mL\ of\ HCl\ for\ the\ sample$$
$$B = mL\ of\ HCl\ for\ the\ blank$$
$$N = Normality\ of\ HCl$$

$$\%\ Protein = \%\ Nitrogen\ \times 5.7$$

3.5.2. Moisture. The moisture content was determined by AACC Method 44-15.02 with the following changes. PPI samples, ~2 g portions, were dried in an air-oven at 100°C for 16 hours. Moisture loss was determined as weight loss and calculated as below:

$$\%\ Moisture = \frac{Initial\ Weight - Final\ Weight}{Initial\ Weight} \times 100$$

3.5.3. Ash. The ash content of the PPI samples was determined by AACC Method 08-01.01 after moisture analysis due to sample amount constraints. PPI samples, ~2 g portions, were pre-ashed over an open flame and then ashed in an ashing oven at 525°C for 16 hours. Total ash was calculated:

$$\%\ Ash = \frac{Final\ Sample\ Weight}{Initial\ Sample\ Weight} \times 100$$

3.5.4. Lipid. AACC Method 30-25.01 Crude Fat in wheat, Corn, and Soy Flour, Feeds, and Mixed Feeds was used to determine crude fat content in the PPI. The sample (3g) was previously freeze-dried and extracted for 16 hours with 160 mL of hexane using the Soxhlet apparatus. Calculation was:

$$\%\ Crude\ Fat = \frac{Final\ Weight}{Initial\ Sample\ Weight} \times 100$$

3.5.5. Carbohydrate. The amount of carbohydrate (CHO) in the PPI samples was calculated by difference using the following calculation:

$$\%\, CHO = 100 - \%\, Moisture - \%\, Protein - \%\, Ash - \%\, Lipid$$

In cases where lipid analysis was not done on the sample due to sample amount constraints, the lipid value used in calculating CHO was from the same pea source.

3.6. Headspace Gas Chromatography – Mass Spectrophotometer (GC/MS)

Samples (2 mL) for HS-GC/MS analysis contained 1% PPI and 100, 200, or 500 ppm volatile compound. This level of volatile compound appeared to be within, but at the lower range of detection for the method and equipment. The samples were prepared from 1.0 mL of 2% protein isolate stock solution in buffer which had been mixed by sonication (30 min) and the pH adjusted to 7.6; 0.2, 0.4 or 1.0 mL of 1000 ppm volatile compound stock solutions in buffer which had been sonicated (30 min); and 0.8, 0.6, or 0 mL additional buffer. Protein stock was made fresh each day while volatile solutions were refrigerated (4°C) and used within 24 h. The samples were mixed in 20 mL HS-GC vials (MicroLiter Analytical Supplies, 22 x 75 mm) and immediately capped (MicroLiter, Metal Seal 20 mm PFTE/Silicone septa). They were shaken at room temperature for 2.5 – 17.5 hours prior to analysis as preliminary experiments showed a minimum requirement of 1 hour for the volatile compound to equilibrate between the liquid and air phases (results not shown). Differences in time of equilibration are off-set by a random ordering of the samples each test run.

Analysis was as per Heng et al. (2004). Samples were analyzed using a Varian Gas Chromatograph (3800GC) and Mass Spectrophotometer (320MS, triple quad). After shaking for 14 min at 40°C, 1 mL of headspace was injected (splitless mode, injector temperature: 265°C) into the column with a CombiPal auto sampler after one absorption cycle on the Itex2 absorber attachment (CTC Analytics). The column

was a VF-5ms (length 30 m, diameter 0.25 mm, film thickness 20 μm) with helium as the carrier gas flowing at 4 mL/min. The oven heating program used was: 1 min hold at 40°C, ramped to 265°C at a rate of 25°C/min followed by a 3 min hold at 265°C. The identity of the peaks was confirmed with the MS in single quad mode using an electron ion source at 70 eV. The mass range was 25 Da to 250 Da.

Calibration curves for each volatile compound were prepared using 50, 300 and 600 ppm volatile compound in buffer analyzed under the same experimental conditions.

The peak area was integrated and the ratio of the treatment headspace response to the calibration was calculated. The control or no binding was represented as the value one. The ratio of response subtracted from one gave the percent of binding.

Each sample was analyzed at least in duplicate. Samples contained each volatile (Hex, 2-Oct, HxAc) at concentrations of 100, 200, 500 ppm, paired with each heat treatment (B, M, R, U) of the two pea protein extractions (PPIa, PPIs).

3.7. Differential Scanning Calorimetry

Samples (3 mL) for DSC analysis contained 10% PPI and 500 ppm volatile compound. The samples were prepared from 2.0 mL of 15% protein isolate stock solution in buffer which had been mixed by ultra-sonication for 30 min (Branson 3200) and the pH adjusted to 7.6; 0.75 mL of 2000 ppm volatile compound stock solutions, prepared in buffer and sonicated (30 min); and 0.25 mL additional buffer. PPI and volatile stock solutions were made fresh daily. The samples were vortexed (1 min) and rested overnight at 4°C. Prior to loading, the sample was tempered to room temperature (1 h) and vortexed for 1 min.

DSC analysis was according to Sun and Arntfield (2012) using a DSC Q200 (TA Instruments, New Castle, DE, USA). The instrument was calibrated with indium. Approximately 9-15 μg of the sample was weighed

into an aluminum TA Instrument TZero Pan and sealed with TZero Hermetic Lid. The heating rate was 10°C/min over the range of 40-120°C. An empty sealed pan was used as a reference. The resulting curves were integrated and the enthalpy (ΔH) and temperature of denaturation (Td) were calculated using Thermogram software.

Each sample was analyzed at least in duplicate. Samples contained each volatile (Hex, 2-Oct, HxAc, none) paired with each heat treatment (M, R, U) of the two extracted pea protein (PPIa, PPIs).

3.8. Rheology

Samples (3 mL) for rheological analysis contained 14.5% PPI and 500 ppm volatile compound. The protein concentration was selected to ensure sufficient protein to form a gel. The samples were prepared from 2.5 mL of 17.4% protein isolate stock solution in buffer which had been mixed by sonication (30 min) and the pH adjusted to 7.6 and 0.5 mL of 3000 ppm volatile compound stock solutions, prepared in buffer and sonicated (30 min). Protein stock solution was made ahead of time and frozen in 2.5 mL aliquots and volatile stock solutions were prepared and refrigerated for no more than 24 h. The samples were mixed using the vortex (1 min) and rested overnight at 4°C.

With few changes, rheological analysis was as per Sun and Arntfield (2010). Prior to loading, the sample was tempered to room temperature (1 h) and vortexed for 1 min. Approximately 1.3 mL of sample was transferred to the bottom plate of the parallel plate geometry of the AR 2000 Rheometer (TA Instruments, New Castle, DE, USA). The top plate was lowered to a 1.000 mm gap. Mineral oil was added to the tray on the top geometry and a solvent trap was placed to prevent the sample from drying out and create a water saturated environment. The heating profile was from 25°C up to 95°C and back to 25°C at a rate of 2°C/min with a 2 min hold at the beginning, middle (95°C) and end. After cooling, the gel was subjected to a frequency sweep (FS) from 0.01 – 10 Hz at 25°C. Data was collected

approximately every 10 s throughout the procedures. The input amplitude strain was set at 0.02 which was reported to be in the linear viscoelastic region (Sun & Arntfield, 2010).

Parameters of interest were the storage (G') and loss (G") moduli as well as their ratio, tan δ (G"/G'). Gel point was determined as the temperature at which G' became greater than G" after an increase in G' (cross-over point) or when tan δ equalled 1.

Each sample was analyzed at least in duplicate. Samples contained each volatile (Hex, 2-Oct, HxAc, none) paired with each heat treatment (M, R, U) of the two extracted pea protein (PPIa, PPIs).

3.9. Statistical Analysis

Data was analyzed using SAS 9.0 computer software. Homogeneity of variance (HOV) was tested with Bartlett's test which was chosen because it can accommodate data with less than three replicates unlike Levene's. If the data was determined to vary significantly ($p<0.05$), it was transformed using log base ten. A log transformation was chosen as the data requiring transformation was from the rheological experiment where values are reported on a log scale. In one case, homogenous variance could not be achieved with a transformation so after an evaluation of the objectives and experimental method, it was decided to not include some of the data in the statistical analysis. Once the assumption of HOV was met, data was subjected to analysis of variance (ANOVA) with a full factorial model. Main effects and interactions that showed potential significance ($p<0.10$) were re-entered into the model while clearly non-significant ($p>0.10$) interactions were not included. This 'cleaned-up' the ANOVA table and allowed significance previously attributed to non-significant interactions to be properly assigned to a significant effect. The data was re-analyzed with ANOVA using Tukey's adjustment. Means and standard deviations (SD) of all data are reported with least square means (LSM) and standard error (SE) reported for significant effects and interactions, $p<0.05$. HOV, full factorial ANOVA and significant ANOVA for all the data has been tabulated (refer to List of Tables p. vi).

4. Results and Discussion

4.1. Proximate Analysis

Table 4. Proximate analysis of heat treated and extracted PPI on an as is basis[1, 2]

Heat[3]	Extraction	Moisture (%)	Ash (%)	Protein[4] (%)	Lipid (%)	CHO[5] (%)
M	Alkaline	3.54 ± 0.01[bc]	1.79 ± 0.83	60.7 ± 0.8	-[6]	33.5*[7]
R	Alkaline	5.14[d]	1.09	61.0 ± 2.3	-	32.3*
U	Alkaline	4.10[cd]	2.00	60.8 ± 0.6	2.92 ± 0.22[b]	30.1
M	Salt	2.59 ± 0.04[b]	1.36 ± 0.03	67.3 ± 0.4	-	28.3*
R	Salt	1.39 ± 0.44[a]	0.975 ± 0.387	66.6 ± 4.1	0.46 ± 0.12[a]	30.6
U	Salt	2.62 ± 0.03[b]	1.91 ± 0.23	61.4 ± 1.6	5.14 ± 0.18[c]	28.9

[1] Analysis was done in duplicate where possible. Values reported are means ± standard deviations
[2] Values in a column are significantly different ($p<0.05$) where followed by different superscript letters
[3] For heat treatment peas were micronized (M), roasted (R), or unheated (U)
[4] Protein content was converted from total organic nitrogen using 5.7 as a conversion factor
[5] Carbohydrate by difference
[6] – Indicates no data due to insufficient sample
[7] *Indicates lipid value used in CHO calculation was from the same pea source (roasted sample)

Pea protein isolates were very similar regardless of the heat treatment or extraction method. The same samples were used for moisture and ash and duplicates were not possible for all. Moisture varied between 1.39 – 5.14% and ash from 0.975 – 2.00%. The difference in ash content was not significant. Protein content of the samples was from 60.7 – 66.6%; this is less than the 81.9% protein in the PPIs samples prepared by Sun and Arntfield (2012). Lipid content (0.46% - 5.14%) was expected to be low as peas do not contain a lot of lipid and it should not have passed through the extraction process to the isolate. However the unheated samples had significant lipid content. As only three samples were analyzed, it is unclear if the differences in lipid content in the isolates are due to the original pea lipid content, the extraction process or the heat treatment. Carbohydrate content was calculated by difference. It was higher than expected (28.3% - 33.5%) due to the lower protein content and varied where the lipid content used in calculations was not from the sample. The carbohydrate was most likely fibre as starch, another carbohydrate found in peas, should not be extracted in these processes.

4.2. GC/MS Results and Discussion

The reported values are ratios of headspace peak areas, calculated as shown in the equation below. Peak area without protein is based on calibration curves of each volatile compound at 50, 300 and 600 ppm in buffer (Appendix A).

$$Ratio = \frac{Peak\ Area\ _{with\ protein}}{Peak\ Area\ _{without\ protein}}$$

A ratio of one indicated no change in volatile concentration in the headspace. A ratio greater than one could be indicative of release of volatile. In other words, in the presence of protein there is more of the added volatile compound in the headspace than without the protein. Ratios less than one indicated a decrease of the added volatile compound in the headspace, perhaps due to binding of the volatile compound to the protein or increased solubility of the volatile compound. The volatile compound may have also degraded or changed in some way (e.g. became non-volatile) such that it was no longer identifiable with the method; although in no GC result were there any other visible peaks unlike Wang and Arntfield (2014).

It is known that protein can bind volatile compounds and if we assume that this is the primary reason for the decrease in the amount of volatile in the headspace, binding can be calculated as follows:

$$\%\ Binding = (1 - Headspace\ Ratio) * 100$$

4.2.1. Binding of volatile compounds by pea protein. Bartlett's test of HOV (not shown) of all the reported data (Table 5) showed that the assumption of homogenous variance was not met, even after a transformation. In investigating this heterogeneity, I plotted the residuals by each main effect (Appendix B). When the data was analyzed for volatile compound, HxAc showed greater variance than Hex and 2-Oct. When analyzing for each concentration 100 ppm was more variable than the others.

Changes due to heating and extraction showed comparable variability. As a primary goal of this investigation was to look at different volatile compounds and the effects of heating, variability due to HxAc was accepted. The variation seen due to the concentration of the volatile compound indicated that the method and instrument were not sensitive enough to reliably use at the lowest concentration for the chosen volatile compounds. I have chosen to not use the data at 100 ppm of the volatile compounds. The following results and statistics use only 200 and 500 ppm data.

Bartlett's test of HOV (Table 6) indicated that without the 100 ppm data, the assumption of homogenous variance was met ($p>0.05$), even with the variability of the HxAc samples, and ANOVA could be confidently used as a statistical analysis. The ANOVA (Table 7) showed significant interactions. As there is a significant interaction between all four factors, all data combinations with significant differences by Tukey's test can be seen in Figure 4.

Table 5. GC/MS ratio of headspace response of heated PPIa and PPIs with volatile compounds at various concentrations [1]

			Concentration (ppm)		
Heating[2]	Extraction[3]	Volatile[4]	100	200	500
B	A	Hex	0.6144 ± 0.4089	0.5260 ± 0.1638	0.7052 ± 0.0006
B	A	HxAc	0.8621 ± 0.3024	0.7288 ± 0.0711	0.7897 ± 0.0459
B	A	2-Oct	0.8341 ± 0.0328	0.8440 ± 0.0907	0.8812 ± 0.0127
B	S	Hex	0.5555 ± 0.0028	0.5885 ± 0.2111	0.6877 ± 0.0091
B	S	HxAc	0.7285 ± 0.3739	0.8040 ± 0.2575	0.4591 ± 0.1290
B	S	2-Oct	0.7689 ± 0.0201	0.7945 ± 0.0791	0.7708 ± 0.0938
M	A	Hex	0.6435 ± 0.1676	0.6742 ± 0.0627	0.9282 ± 0.1935
M	A	HxAc	1.146 ± 0.629	1.237 ± 0.258	0.5849 ± 0.2199
M	A	2-Oct	0.7940 ± 0.0751	0.9645 ± 0.1487	0.9386 ± 0.0317
M	S	Hex	0.7149 ± 0.1929	0.7577 ± 0.2604	0.9397 ± 0.0062
M	S	HxAc	0.4660 ± 0.0971	0.8150 ± 0.2312	0.5326 ± 0.1282
M	S	2-Oct	0.7438 ± 0.0434	0.8973 ± 0.0349	0.8747 ± 0.0104
R	A	Hex	0.6606 ± 0.0575	0.8590 ± 0.0579	0.8741 ± 0.0923
R	A	HxAc	2.199 ± 0.299	2.169 ± 0.261	0.6980 ± 0.0233
R	A	2-Oct	0.7528 ± 0.0904	0.8299 ± 0.0651	0.9303 ± 0.0414
R	S	Hex	0.6761 ± 0.1766	0.8626 ± 0.0979	0.8780 ± 0.1133
R	S	HxAc	0.9566 ± 0.3699	0.9095 ± 0.1846	0.7676 ± 0.0357
R	S	2-Oct	1.074 ± 0.097	1.217 ± 0.195	1.130 ± 0.064
U	A	Hex	0.4011 ± 0.0914	0.6169 ± 0.1112	0.7354 ± 0.0517
U	A	HxAc	0.5210 ± 0.1204	0.5906 ± 0.2488	0.3748 ± 0.1530
U	A	2-Oct	0.6860 ± 0.0930	0.7855 ± 0.1626	0.8056 ± 0.0431
U	S	Hex	0.4668 ± 0.0226	0.8133 ± 0.1767	0.7322 ± 0.0416
U	S	HxAc	0.9304 ± 0.9329	0.7460 ± 0.0106	0.3961 ± 0.0432
U	S	2-Oct	0.6450 ± 0.0215	0.7211 ± 0.0528	0.7942 ± 0.1111

[1] Values are means ± SD of two or more replications

[2] PPI were unheated (U) or treated with heat by boiling (B), micronizing (M) or roasting (R)

[3] Extractions were alkaline (A) or salt (S)

[4] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc) or 2-octanone (2-Oct)

Table 6. Bartlett's test of HOV in the GC/MS ratio of headspace response

Source	DF[1]	Chi-Square	P > ChiSq
Treatment	47	49.61	0.3695

[1] Degrees of freedom

Table 7. ANOVA for ratio of headspace response of volatile compounds with PPI in GC/MS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	0.0599	0.0599	3.13	0.0827
Volatile	2	0.2863	0.1431	7.49	0.0014
Heating	3	1.710	0.570	29.82	<.0001
Concentration	1	0.2772	0.2772	14.50	0.0004
Heating*extraction	3	0.0825	0.0275	1.44	0.2423
Extraction*volatile	2	0.3538	0.1769	9.25	0.0004
Heating*volatile	6	0.5213	0.0869	4.54	0.0009
Extraction*concentration	1	0.0162	0.0162	0.85	0.3616
Heating*concentration	3	0.2350	0.0783	4.10	0.0110
Volatile*concentration	2	1.290	0.645	33.73	<.0001
Heating*extraction*volatile	6	0.6480	0.1080	5.65	0.0001
Heating*extraction*concentration	3	0.2938	0.0979	5.12	0.0035
Extraction*volatile*concentration	2	0.1778	0.0889	4.65	0.0139
Heating*volatile*concentration	6	0.3564	0.0594	3.11	0.0112
Heating*extraction*volatile*conc	6	0.6620	0.1103	5.77	0.0001
Error	52	0.9943	0.0191		
Total	99	8.075			

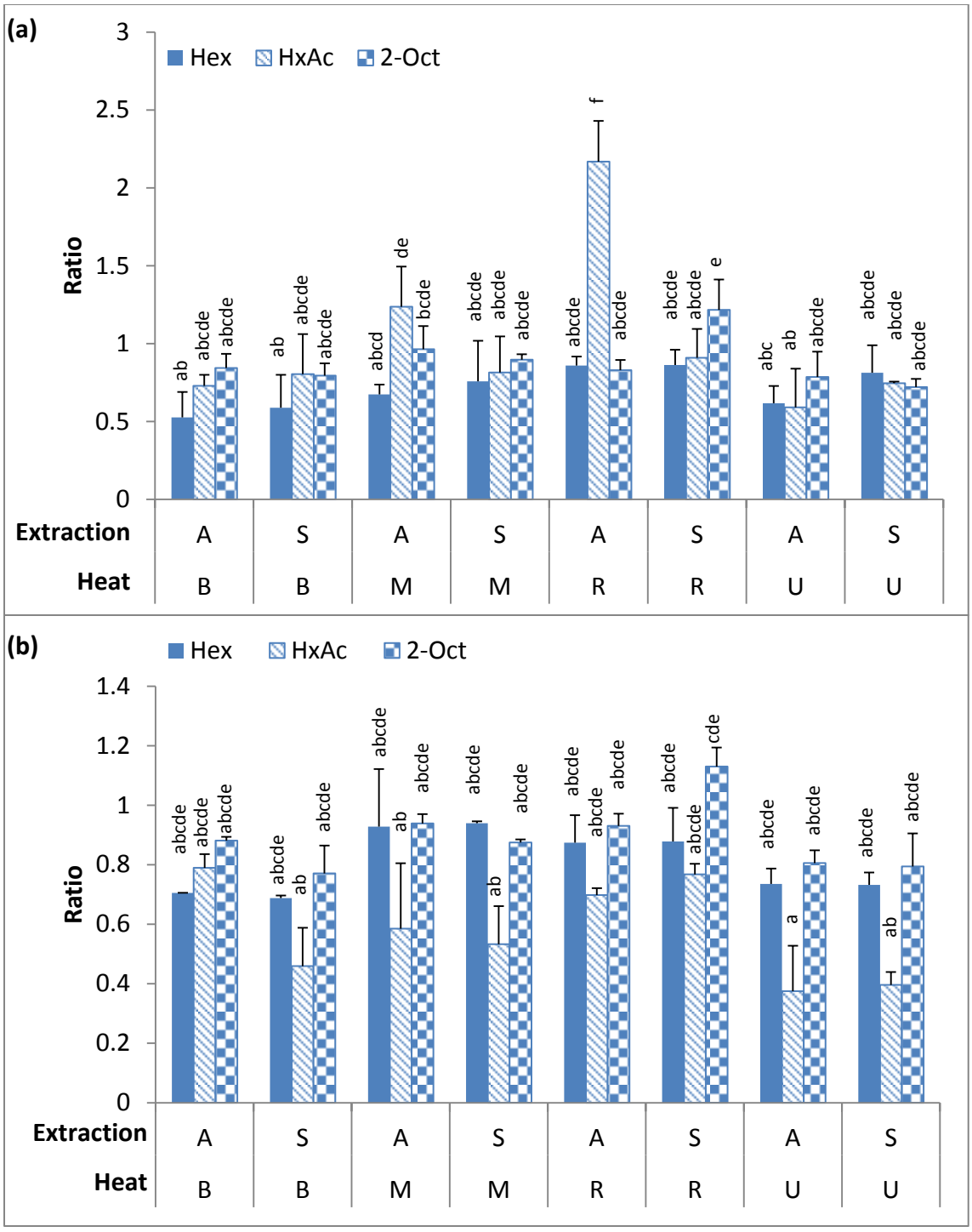

Figure 4. GC ratio of headspace response of heated PPIa and PPIs with volatile compounds at (a) 200 ppm and (b) 500 ppm. Bars in figure followed by different letters are significantly different by Tukey's test $p<0.05$, indicating a four way interaction. Volatile compounds used were hexanal (Hex), hexyl acetate (HxAc) or 2-octanone (2-Oct). PPI were extracted by alkaline (A) or salt (S) and unheated (U) or treated with heat by boiling (B), micronizing (M) or roasting (R).

Four samples had a headspace response ratio greater than one indicating release of a volatile, or no binding. These were roasted PPIa and micronized PPIa with HxAc at 200 ppm and roasted PPIs with 2-Oct at 200 and 500 ppm. These four samples had a significantly larger ratio than samples which had ratios less than 0.62 (~ >40% binding). These latter samples were boiled PPIs, micronized PPIs, unheated PPIs, micronized PPIa, and unheated PPIa with HxAc 500 ppm; unheated PPIa with HxAc 200 ppm; and boiled PPIs, boiled PPIa and unheated PPIa with Hex 200 ppm. While a four way interaction (heat x extraction x volatile x concentration) is interesting, it is difficult to ascribe meaning to such interactions. I will discuss the interactions by focusing on individual effects while keeping in mind that these effects are better demonstrated for some conditions than others.

4.2.1.1. Extraction effects. Extraction method was only significant for one sample: the PPIa (when roasted) with HxAc (at 200 ppm) gave a greater headspace ratio than with the same conditions for PPIs (Figure 4). It appeared for this volatile under these conditions, the alkaline extraction process changed the protein structure such that no binding was possible while the salt extraction had some binding. The alkaline extraction is thought to be harsher (greater pH alteration) than the salt extraction as seen in the lower enthalpy (Table 16 and 17) and would affect the protein structure more thereby altering the interactions between the protein and volatile compound to a greater extent. Some unfolding would lead to increased binding due to more and/or better access to binding sites. If the denaturation is to the extent that aggregation occurs, then there would be less binding as the sites would be physically masked. Wang and Arntfield (2014) reported differences in binding of volatile compounds by PPIa and PPIs by volatile chemical class. PPIa unexpectedly had a higher binding capacity for aldehydes than PPIs and the opposite for ketones. They postulated that the harsh nature of the alkaline extraction and the lower pH (unaltered after extraction) of the PPIa altered the conformation of the pea protein such that it had a higher affinity for aldehydes than ketones. The PPIs extraction process, being less harsh, thus represents a higher affinity for ketones by the native pea protein.

4.2.1.2. Volatile compound effects. It is known that both the functional group and carbon chain length of a volatile compound can impact binding affinity (Damodaran & Kinsella, 1981b; Jouenne & Crouzet, 2000; Gremli, 1974; Guichard & Langourieux, 2000; Mills & Solms, 1984; van Ruth & Villeneuve, 2002). This was shown again by Wang and Arntfield (2014) who found an increase in binding as the carbon number increased (about 2.3 fold per carbon) as well as a higher binding affinity for aldehydes than for ketones (2.5 fold) with PPI. Damodaran and Kinsella (1981b) showed a similar increase in binding constant per carbon (3 fold) as well as a 1.2 fold difference between aldehyde and ketone binding (9 carbon compounds) to soy protein. Gremli (1974), using soy protein found around a 1.6 fold increase by carbon number and a greater increase moving from ketone to aldehyde. For Heng et al. (2004) the chemical class also was a much more important factor as aldehydes bound about 9 fold more than the ketones while the increase in binding per carbon was only about 0.5 fold. Heng et al. (2004) used purified vicilin from pea protein at pH 7.6 which may account for the difference in binding compared with Wang and Arntfield (2014) who used a whole protein isolate and did not adjust the pH. Binding percentages for these discussed studies are summarized in Tables 8 and 9. It is also important to note that volatile compounds, themselves, are sensitive to pH.

Table 8. Binding percent of aldehydes (pentanal to nonanal) to proteins

Carbon no.	Soy protein 5%	Pea vicilin, pH 7.6	PPIa	PPIs
5	-[1]	75	-	-
6	37-44	-	14.4	6.4
7	62-70	-	27.2	24
8	83-85	88	56	48
9	90-93	-	-	-
Source	Gremli, 1974	Heng et al., 2004	Wang & Arntfield, 2014	

[1] – Indicates no data reported

Table 9. Binding percent of ketones (2-pentanone to 2- nonanone) to proteins

Carbon no.	Soy protein 5%	Pea vicilin, pH 7.6	PPIa	PPIs
5	-[1]	1	-	-
6	5-16	-	3.5	3.5
7	9-22	-	9.8	15
8	29-43	17	17.3	40
9	54-61	-	-	-
Source	Gremli, 1974	Heng et al., 2004	Wang & Arntfield, 2014	

[1] – Indicates no data reported

Damodaran and Kinsella (1981b) posit that the difference in binding affinity between aldehydes and ketones is due to steric hindrance and hydrophobicity. They found that as the keto group moved toward the middle of the chain there was increased steric hindrance affecting the access of hydrophobic binding sites. Hydrophobicity also increases as the number of carbons increase. In general, aldehydes are more hydrophobic and more reactive than ketones; this is thought to contribute to the increased binding affinity of aldehydes.

In the current study, only three volatile compounds were used, all from different chemical classes. In general, Hex and HxAc tended to have lower ratios (more binding) than 2-Oct. Of note, in Figure 4, is that at both 200 ppm and 500 ppm, 2-Oct, in combination with the roasted PPIs, led to significantly higher headspace ratios (less binding) than HxAc (500 ppm) with boiled PPIs, micronized PPIs, unheated PPIs, micronized PPIa and unheated PPIa (also at 200 ppm), as well as Hex (200 ppm) with boiled PPIs, boiled PPIa and unheated PPIa. Also, HxAc at 500 ppm showed a trend of lower ratios in each heated PPI than the other volatile compounds. There are two cases where these general conclusions do not hold, that is with HxAc (200 ppm) the micronized and roasted PPIa samples had large ratios (> 1) which were significantly larger than many of the other samples indicating, not only, a lack of binding, but also, release of volatile compound from the matrix.

The difference in binding between 2-Oct and Hex cannot be explained solely by the difference in volatile structure. Ketones, like 2-Oct, are less hydrophobic and have more steric hindrance compared with aldehydes like Hex (Damodaran & Kinsella, 1981b). The increased hydrophobicity due to the longer carbon chain of 2-Oct (compared to Hex), is not enough to offset the steric hindrance of the keto group at position 2 which may lead to less binding with 2-Oct. Although it has the same 6 carbon chain, HxAc is more hydrophobic than 2-Oct and Hex due to the ester group. This ester group would also contribute to steric hindrance decreasing the binding ability. While the characteristics of the volatile compound contribute to the ability to bind, the interaction with concentration of the volatile compound, extraction method and heat treatment is significant

4.2.1.3. Heat treatment effects. Interestingly, proteins that were boiled or not heated had a greater effect (more binding) on the headspace volatile content than the dry heat methods of roasting and micronizing for some samples (Figure 4). This can be seen as the samples with significantly larger headspace ratios (values that are not part of families 'ab' in Figure 4), were all roasted or micronized. Of the samples that have significantly smaller ratios (values not part of families 'def') most were either boiled or unheated. The exception is the micronized PPIa and PPIs, with HxAc (500 ppm) had over 40% binding (ratio < 0.60).

Because binding ability is dependent on the access to and 'fit' between the volatile compound and the binding site on the protein, heat treatments that alter the conformation of the protein will affect binding. These effects may either increase or decrease interactions depending on the protein conformation. Some heat would open up the protein so more sites were accessible, while harsh heat would unfold the protein such that the exposure of hydrophobic areas would lead to aggregation masking potential binding sites. Heng et al. (2004) found decreased binding with heat treated vicilin; boiling (90°C for 30 min) reduced binding at least 3.5 fold for both octanone and octanal. They found

that volatile compounds shorter than eight carbons had no binding to heat treated protein. The decrease in binding was thought to be due to aggregation of the protein and subsequent decrease in surface area. Andriot et al. (1999) also suspected that aggregation after heat denaturation limited access to the binding sites on β-LG for benzaldhyde as there was no change in binding due to heat unless salt or ethanol (known to encourage protein aggregation) were added. For Semenova, Antipova, Misharina et al. (2002), heating (90°C for 30 min) increased the binding of HxAc to legumin by opening up or increasing access to binding sites although the affinity for these sites was decreased.

In the current study, a whole protein isolate was used and heating tended to decrease the interaction between the protein and the volatile compound, except for the boiled sample. Micronizing has been shown to decrease the percentage of soluble proteins as a result of heat denaturation (and aggregation) (Bellido et al., 2006). It appeared that both roasting and micronizing led to protein structure changes that masked binding sites resulting in increased headspace ratio (decreased binding). Boiling also should have denatured the protein enough to cause aggregation and decreased binding; however it did not. Perhaps the boiling, which was done after the volatile addition, did not interrupt binding that had already occurred or the boiling may have led to a physical structure that trapped the volatile compounds. The heat treatment may have also destroyed or altered the volatile compound. Heng et al. (2004) did investigate the effect of boiling the protein before or after the volatile addition on binding and found that both ways decreased the binding. This may have been because their work was with purified vicilin which would denature and aggregate differently than the whole protein isolate used in the current study.

4.2.1.4. Concentration effects. Within the four way interaction, the effect of concentration of volatile compounds was only seen with HxAc and PPIa, where the samples had been either roasted or micronized (Figure 4). In these two cases, more volatile compound (500 ppm) had a greater effect on

headspace ratio; when the volatile concentration in the system was higher, a higher percentage of it bound to the protein. Other research on protein-volatile interactions has also shown increase in binding with increase in volatile concentration at concentrations less than 105 ppm (Heng et al., 2004), although without any explanation. Gremli (1974) found that the retention of the volatile compound was independent of the amount added up to 500 ppm. They speculated that an equilibrium occurred between the bound and free volatiles and 500 ppm was not enough to saturate the protein. Solms, Osman-Ismail and Beyeler (1973) indicated that if the binding of volatile compounds to food components altered the conformation then a simple equilibrium cannot be assumed. Perhaps, they suggested, the interaction at one binding site altered the conformation of the protein to allow an addition interaction that was previously inhibited. Nawar (1971) also suggested that while an equilibrium that is not concentration dependent may occur in pure water, it is not the case for more complex solutions. At the concentrations used it cannot be said if more sites were exposed at the higher concentration or if the sites were not saturated. Evaluation over a wider concentration range would be required to investigate this relationship.

4.3. DSC Thermal Properties of PPI: Effect of Volatile Compounds, Heat and Extraction

Differential scanning calorimetry was performed on the PPI samples mixed with 500 ppm of volatile compound. The resulting enthalpy curves were integrated and the areas are reported as ΔH while the peaks provide denaturation temperature (Td). The data (Table 10) were analyzed with Bartlett's test of HOV (Table 11) to ensure that ANOVA could be used. Full factorial ANOVA was done for each parameter (ΔH in Table 12; Td in Table 14). After removal of non-significant interactions, $p>0.10$, another ANOVA was performed with only main effects and potentially significant interactions. Both tables are shown and their difference is indicated in the titles. The second ANOVA showed significance in heating, extraction and their interaction for both enthalpy (Table 13) and Td (Table 15). Enthalpy also was affected by an

interaction between extraction and volatile (Table 13) and volatile compound effects had a p-value of 0.0536 for Td (Table 15). Significant ($p<0.05$) results by Tukey's test are given in Tables 16, 17 and 18.

Table 10. Enthalpy and Td as determined by DSC for heat treated PPIa and PPIs mixed with a volatile compound at 500 ppm[1]

Heating[2]	Extraction[3]	Volatile[4]	ΔH (J/g)	Td (°C)
M	A	Hex	2.547 ± 0.055	80.64 ± 0.62
M	A	HxAc	3.130 ± 0.624	80.86 ± 0.42
M	A	2-Oct	3.566 ± 1.010	80.49 ± 0.50
M	A	None	2.856 ± 0.062	80.06 ± 0.41
M	S	Hex	8.205 ± 0.427	80.45 ± 0.17
M	S	HxAc	6.700 ± 0.354	80.94 ± 0.50
M	S	2-Oct	8.405 ± 0.820	80.76 ± 0.29
M	S	None	8.015 ± 0.601	81.10 ± 0.48
R	A	Hex	3.122 ± 0.151	79.72 ± 0.60
R	A	HxAc	5.119 ± 1.918	80.69 ± 0.84
R	A	2-Oct	4.424 ± 0.796	79.76 ± 0.44
R	A	None	3.402 ± 0.634	80.40 ± 0.54
R	S	Hex	5.452 ± 0.226	79.55 ± 0.56
R	S	HxAc	4.847 ± 1.172	79.88 ± 0.90
R	S	2-Oct	4.811 ± 0.520	79.51 ± 0.55
R	S	None	6.660 ± 1.694	80.43 ± 0.70
U	A	Hex	4.678 ± 1.098	79.99 ± 0.03
U	A	HxAc	3.850 ± 0.012	80.35 ± 0.40
U	A	2-Oct	4.780 ± 0.120	80.53 ± 0.61
U	A	None	3.724 ± 0.449	79.95 ± 0.13
U	S	Hex	7.974 ± 0.428	81.56 ± 0.75
U	S	HxAc	8.615 ± 0.182	82.28 ± 0.70
U	S	2-Oct	8.356 ± 0.694	81.74 ± 1.05
U	S	None	8.128 ± 0.521	82.16 ± 0.21

[1] Values are means ± SD of two or more replications
[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)
[3] Extractions were alkaline (A) or salt (S)
[4] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

Table 11. Bartlett's test of HOV in ΔH and Td

		Enthalpy		Td	
Source	DF	Chi-Square	P > ChiSq	Chi-Square	P > ChiSq
Treatment	23	30.85	0.1265	12.62	0.9598

Table 12. Full factorial ANOVA for ΔH determined by DSC

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	159.8	159.8	208.0	<.0001
Volatile	3	1.259	0.420	0.55	0.6542
Heating	2	21.49	10.75	13.98	<.0001
Heating*extraction	2	30.22	15.11	19.66	<.0001
Extraction*volatile	3	5.786	1.929	2.51	0.0752
Heating*volatile	6	4.422	0.737	0.96	0.4672
Heating*extraction*volatile	6	8.152	1.359	1.77	0.1355
Error	34	26.13	0.769		
Total	57	257.2			

Table 13. ANOVA of main effects and significant interactions in ΔH

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	159.8	159.8	189.9	<.0001
Volatile	3	1.021	0.340	0.40	0.7503
Heating	2	19.95	9.98	11.86	<.0001
Heating*extraction	2	32.26	16.13	19.17	<.0001
Extraction*volatile	3	7.286	2.428	2.89	0.0456
Error	46	38.70	0.841		
Total	57	257.2			

Table 14. Full factorial ANOVA for Td determined by DSC

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	4.528	4.528	13.48	0.0008
Volatile	3	2.114	0.7045	2.10	0.1194
Heating	2	11.05	5.524	16.45	<.0001
Heating*extraction	2	9.723	4.861	14.48	<.0001
Extraction*volatile	3	1.259	0.4196	1.25	0.3076
Heating*volatile	6	1.348	0.2246	0.67	0.6753
Heating*extraction*volatile	6	0.7952	0.1325	0.39	0.8771
Error	33	11.08	0.3358		
Total	56	42.43			

Table 15. ANOVA of main effects and significant interactions in Td

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	4.344	4.344	14.48	0.0004
Volatile	3	2.465	0.8216	2.74	0.0536
Heating	2	10.99	5.493	18.31	<.0001
Heating*extraction	2	10.38	5.188	17.29	<.0001
Error	48	14.40	0.3001		
Total	56	42.43			

Table 16. Enthalpy (J/g) of heat treated PPI for two extraction methods[1]

	Extraction	
Heating[2]	Alkaline	Salt
M	2.996 ± 0.271[a]	7.831 ± 0.324[d]
R	4.083 ± 0.292[ab]	5.446 ± 0.278[c]
U	4.258 ± 0.324[bc]	8.200 ± 0.307[d]

[1] Values (LSM± SE) in table followed by different superscript letters are significantly different (p<0.05) by Tukey's test

[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)

Table 17. Enthalpy (J/g) of PPIa and PPIs for each volatile compound[1]

	Extraction	
Volatile[2]	Alkaline	Salt
Hex	3.449 ± 0.374[a]	7.210 ± 0.374[b]
HxAc	4.093 ± 0.309[a]	6.698 ± 0.348[b]
2-Oct	4.280 ± 0.329[a]	7.095 ± 0.348[b]
None	3.295 ± 0.348[a]	7.633 ± 0.326[b]

[1] Values (LSM± SE) in table followed by different superscript letters are significantly different (p<0.05) by Tukey's test

[2] Volatile compounds, added at 500 ppm, were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

Table 18. Denaturation temperature (°C) of heat treated PPI for two extraction methods[1]

	Extraction	
Heating[2]	Alkaline	Salt
M	80.54 ± 0.17[b]	80.81 ±0.19[b]
R	80.18 ±0.17[ab]	79.84 ±0.16[a]
U	80.20 ±0.19[ab]	81.94 ±0.18[c]

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly different (p<0.05) by Tukey's test

[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)

The enthalpy and temperature of denaturation determined using DSC are measures of the degree of structure in a protein. The greater the ΔH the more energy required to disrupt existing structure; similarly the temperature of denaturation is an indication of thermal stability, although enthalpy is thought to be the more sensitive measure (Semenova, Antipova, Belyakova et al., 2002).

Table 16 shows that PPIa had lower enthalpies for all heat treatments than PPIs. This is because the alkaline extraction process relies on pH changes from native to pH 9.5 for extraction and then to pH 4.5

for precipitation. This change alters the charge on the protein, interrupting the electrostatic interactions that contribute to the quaternary structure resulting in a partially denatured protein. Acid denaturation leaves the protein with less structure which requires less heat (i.e. enthalpy) to disrupt it. Arntfield and Murray (1981) investigated the effects of pH on fababean protein with DSC. They found that as pH approached extreme values ΔH decreased until no DSC curve was distinguishable.

Similar to acid denaturation, heating the PPI prior to analysis should lead to a decrease in ΔH due to heat denaturation. This was seen in the PPIs roasted sample, but the ΔH for the PPIs micronized sample did not decrease from the unheated sample. The roasted PPIs enthalpy decreased to levels seen in the PPIa unheated sample. Heat effects were also seen in the PPIa where, although the extraction was harsh, micronizing heat significantly lowered the enthalpy of the PPIa compared to the unheated PPIa. The two heat treatments had different effects depending on the extraction method. The roasted PPI was not further denatured with an alkaline extraction while the micronized PPI was. With salt extraction, a less harsh process, roasted PPI showed more denaturation with micronized PPI not being different from unheated. Arntfield and Murray (1981) demonstrated the effect of heat on the DSC thermogram of fababean protein. As the temperature was increased, ΔH decreased and there was a slight increase in Td.

Table 18 shows similar trends in Td where there was again an interaction between extraction and heat treatment. The unheated PPIs had a higher Td than PPIa. However, with micronizing and roasting, the Td's of the PPIs samples were not significantly different from the PPIa samples. Micronizing may have been a less severe heat treatment than roasting as seen in both the ΔH and Td of the PPIs samples. Pre-heating the protein for Arntfield and Murray (1981) resulted in an increased Td because the heat labile structures were destroyed leaving a more thermally stable protein. The Td results are within a 4°C range (Table 10) and although there are significant differences, they are not of practical value.

The effect of volatile compounds was not significant ($p<0.05$) in either ΔH or Td. However, enthalpy was affected by an interaction between extraction and volatile compound. In the full factorial ANOVA (Table 12) the interaction was potentially significant and did indeed show significance in the second ANOVA (Table 13). The F-value was low compared to the other significant factors and Tukey's test shows that within an extraction, there is no difference due to volatile compound (Table 17). However, considering the p-value of 0.0536 for the main effect of volatile compound on Td (Table 15), a discussion regarding the effect of volatile binding on protein thermal properties is required.

Wang and Arntfield (2014) recently reported on their investigations of the binding of volatile compounds to pea protein (among others) and the effects on thermal properties. They saw a decrease in ΔH with an increase in concentration (100, 250, 500 ppm) of the added volatile compounds in the PPIs samples. They posited that the volatile compounds had 'unfolding capacity' which led to increased binding. The protein unfolded as the volatile compound concentration increased, perhaps to accommodate the volatiles, decreasing the protein structure and enthalpy required to disrupt it, along with increasing the amount of volatile that bound to the protein. This change was not reflected in the temperature of denaturation. In the current study, the interaction between volatile compound and extraction in enthalpy values was significant. In the PPIs the absence of volatile compound had the highest enthalpy, in the PPIa samples, the control had the lowest enthalpy (Table 17). These differences were not significant because of the overwhelming effect of extraction; within an extraction, all values are part of the same families. The presence of volatile compounds may elicit some unfolding in the PPIs samples as speculated by Wang and Arntfield (2014) yielding a lower ΔH. It may also be that for the PPIa some of the structure lost in extraction is restored or replaced by the binding of volatile compounds to the protein.

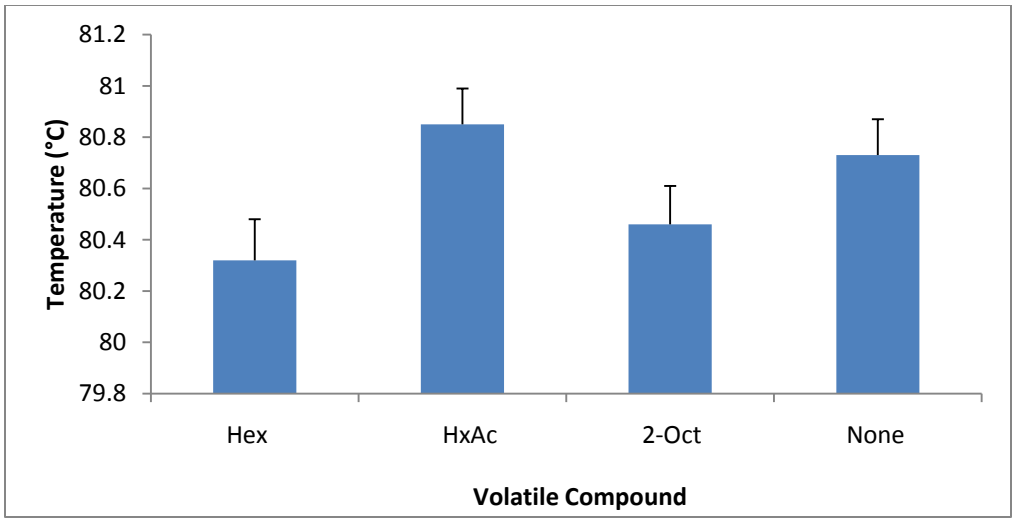

Figure 5. Temperature of denaturation of PPI by volatile compound (500 ppm). Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none. Values are LSM ± SE, p=0.0536 for volatile compound as main effect.

Temperature of denaturation also showed a trend regarding volatile compounds (p=0.0536), which are plotted in Figure 5. While the volatile compounds varied in their effect on Td; the presence of Hex led to a lower Td than that of HxAc. ΔH and Td are both measures of degree of structure, but they do not always correspond. Enthalpy changes reflect breaking of hydrogen bonds, disruption of hydrophobic interactions and protein aggregation; both endothermic and exothermic processes (Arntfield & Murray, 1981). Td is controlled more by the nonpolar hydrophobic interactions. It is difficult to ascribe meaning to a p value of 0.0536, especially with a low F-value, but perhaps the HxAc stabilizes the protein against thermal denaturation by adding more hydrophobic interactions while Hex destabilizes the protein by its tendency to unfold protein. It would be interesting to investigate these effects on thermal stability at higher concentrations of volatile compound.

Wang and Arntfield (2014) report ΔH values in the range of 9-15 J/g. This is similar to others including Mession et al. (2013); Murray and Arntfield (1981); Sun and Arntfield (2010, 2011, 2012). In the current study, enthalpies were found in the 3-9 J/g range: lower than others. This difference can be partially explained by the difference in pH. Arntfield and Murray (1981) showed that ΔH is pH dependent as the

protein structure is sensitive to pH changes. I adjusted the pH of the samples to pH 7.6 while others used a native pH (Wang & Arntfield, 2014; Sun & Arntfield, 2010; 2012). Mession et al. (2013) used pH 7.5 and the enthalpies are 9.2-9.5 J/g which is comparable to the current study. Other factors contributing to the difference would include the heat denaturation, differences in extraction procedures and inherent heterogeneity among proteins (Mession et al., 2013).

4.4. Small Strain Dynamic Rheology

Typical rheological curves obtained in this study are shown in Figure 6. The storage modulus (G') and loss modulus (G") were measured for a protein solution (14.5%) heated at a constant rate (2°C/min) under a 0.02 amplitude strain from 25°C to 95°C and then cooled back to 25°C at the same rate . G' represents the storage modulus or those interactions contributing to a 3D network. G" is the loss modulus or the viscous component where interactions do not advance solidity. A strong gel will have a high G'. As can be seen in Figure 6(a) the protein solution started out as a liquid where G' < G". As the solution was heated, G' and G" both generally increased with a sharp increase beginning around 70°C (in this example) where G' increased more rapidly than G". As G' became greater than G" it overtook the matrix so the solution became more solid than liquid, in other words, gelled. This could also be seen visually at the end of the experiment; the protein solution looked and felt like 'jelly'. Heat induced gelation was expected at the PPI concentration used (14.5%) as heat is known to disrupt the protein structure leading to aggregation and agglomeration as explained by Clark et al. (2001) and others (Sun & Arntfield, 2010; Uruakpa, 2009). During cooling, G' and G" both gently increased again, this time with G' > G", as the interactions and cross-links were strengthened. This is a similar curve shape to that reported by Sun and Arntfield (2010; 2011).

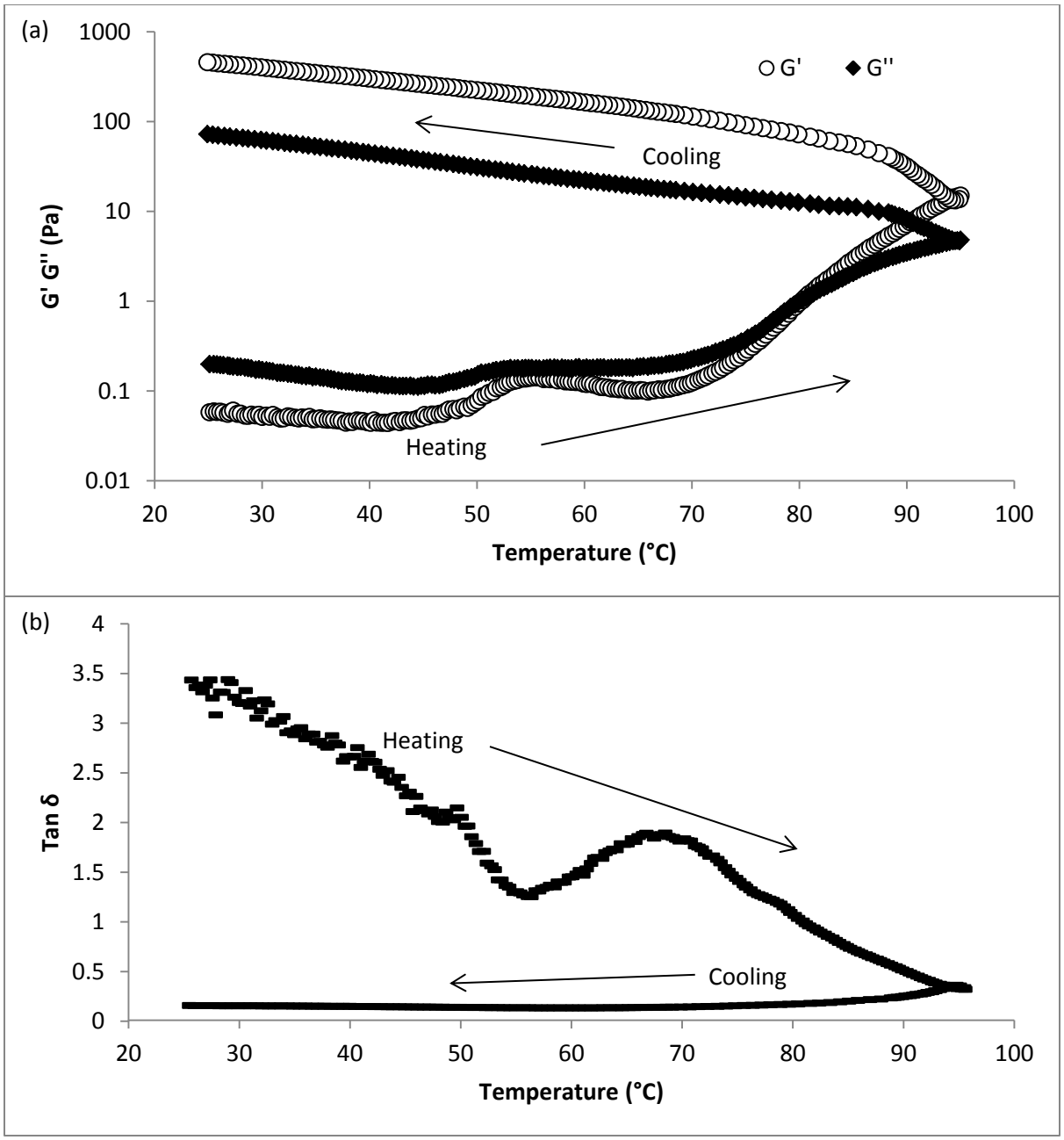

Figure 6. Typical curve obtained during rheological analysis with (a) storage (G') modulus, loss (G") modulus, and (b) their ratio tan δ. Sample shown is for unheated PPIa (14.5%) with 500 ppm 2-octanone.

Another descriptive parameter for the rheological examination is tan δ. Tan δ is the ratio of G' and G" (G"/ G') and helps characterize the gel. A well cross-linked gel will have a low tan δ. A typical tan δ result during heating and cooling is presented in Figure 6(b). Tan δ tended to decrease during heating. When

G”=G’, tan δ=1; this indicated the protein solution was dominated by the elastic component and formed a gel. Tan δ continued to decrease and stayed low during cooling.

In many of the samples, there was an unexpected dip and bump in tan δ during heating, peaking around 70°C for PPIa and 90°C for PPIs. This was interesting and corresponded to the unexpected elevated gel points seen in the PPIs samples (discussed further in section 4.3.1.). It appeared that for these samples some type of structure formed and then dissolved with a more stable gel starting to form at the end of the bump. This structure (at lowest dip point) still had a high tan δ (>1) and G’ < G” at this point, indicating that it was a weak, poorly linked matrix.

Rheological results are difficult to compare across studies where the equipment and experimental parameters vary widely and play a significant role in the results. However, the most similar experiments (Sun & Arntfield 2010; 2011; 2012) did not report any dip/bump in tan δ. It may be that due to the pH adjustment of the protein samples, the protein structure was altered in some way that upon heating a network formed briefly before dissolving and then gelling. Visually, the PPIs gels were ‘grainy’ and appeared to have particles of gel. This may have been due to solubility issues, although the PPIa were more difficult to solubilize. Another possibility is that the structure is from starch gelatinization. The proximate analysis results (Table 4) showed carbohydrate content to be between 28.3 – 33.5%. This is most likely fibre but some starch may have been extracted and gelatinized in the rheological experiment. There was no indication of the presence of starch during the thermal analysis, where, if starch was present, a curve would be expected around the same temperature as the dip in the tan δ curve.

More rheological research, perhaps at varying pH conditions, or structural analysis like microscopy or surface hydrophobicity may elicit some explanation. I also found in other rheological work that the results, particularly G’ and G”, varied widely within a sample. Similar to Wong et al (2013), some

standard deviations were greater than the mean itself and may point to a sensitivity of the experiment to sample preparation. Sun and Arntfield (2011) found that for gels with high tan δ values, the standard deviation was such that it limited finding significant difference.

Gel point and gel characteristics (from both the gel formation step and frequency sweep), with numerical results, from the rheological experiment are shown and discussed below. All data was subject to HOV testing and then transformed if needed. The first ANOVA table included all possible main effects and interactions. Interactions that were clearly not significant ($p>0.10$) were omitted and a second ANOVA was performed. Interactions, or main effects if there were no interactions, that were significant in the second ANOVA are tabulated and differences noted by Tukey's test, significance was defined as $p<0.05$.

4.4.1. Gel point of PPI during dynamic gel formation. As mentioned above, the gel point can be described as the point where G' surpasses G" (tan δ drops below 1) sometimes referred to as the cross-over point. As the elastic component becomes dominant, the matrix becomes a gel. This generally will occur after the onset of denaturation during heating, before the temperature of denaturation (peak in DSC) is reached (Uruakpa, 2009). In this experiment, the cross-over points occurred during heating and cooling. It was mostly the PPIs samples for which the average cross-over point occurred during the cooling. Because of this, gel time (instead of temperature) was used to accurately represent when the cross-over occurred (time of heating was 0-2100s; cooling 2100-4200s). Time was used for all statistical analysis and the results were converted back to temperature (as this is the parameter of interest) for graphical representation. Gel time and temperature data is reported for all samples in Table 19. The data conformed to a HOV (Table 20) and ANOVA (Tables 21 and 22) showed a significant interaction between heating and extraction, described in Table 23, as well as extraction by volatile (Table 24). The main effect of volatile had a p-value of 0.0616 (Table 22).

Table 19. Gel time and temperature of heat treated PPIa and PPIs in dynamic gel formation as indicated by G' > G" (cross-over point)[1]

				Temp (°C)[2]	
Heating[3]	Extraction[4]	Volatile[5]	Time (s)	Heating	Cooling
M	A	Hex	1830 ± 255	86.0	
M	A	HxAc	1806 ± 382	85.2	
M	A	2-Oct	1862 ± 337	87.1	
M	A	None	1645 ± 172	79.8	
M	S	Hex	2292 ± 110		88.6
M	S	HxAc	2241 ± 199		90.3
M	S	2-Oct	2164 ± 117		92.8
M	S	None	1938 ± 229	89.6	
R	A	Hex	2114 ± 19		94.6
R	A	HxAc	2112 ± 17		94.6
R	A	2-Oct	2100 ± 0	95.0	
R	A	None	1891 ± 214	88.0	
R	S	Hex	2362 ± 100		86.2
R	S	HxAc	2170 ± 66		92.6
R	S	2-Oct	1842 ± 119	86.4	
R	S	None	2277 ± 81		89.1
U	A	Hex	1538 ± 95	76.2	
U	A	HxAc	1448 ± 112	73.3	
U	A	2-Oct	1667 ± 6	80.6	
U	A	None	1450 ± 142	73.4	
U	S	Hex	2556 ± 369		79.8
U	S	HxAc	2067 ± 47	93.9	
U	S	2-Oct	2304 ± 4		88.2
U	S	None	2366 ± 112		86.2

[1] Data is mean ± SD of two or more replicates
[2] Corresponds to the gel time, calculated after averaging the time of gelation
[3] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)
[4] Extractions were alkaline (A) or salt (S)
[5] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

Table 20. Bartlett's test of HOV in gel time

Source	DF[1]	Chi-Square	P > ChiSq
Treatment	22	30.26	0.1123

[1] Test (and DF) does not include treatments with no or minimal variance

Table 21. Full factorial ANOVA for gel time

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	2382000	2382000	68.94	<.0001
Volatile	3	258800	86260	2.50	0.0787
Heating	2	318800	159400	4.61	0.0179
Heating*extraction	2	1053000	526600	15.24	<.0001
Extraction*volatile	3	256100	85370	2.47	0.0809
Heating*volatile	6	312600	52100	1.51	0.2093
Heating*extraction*volatile	6	167600	27940	0.81	0.5713
Error	30	1036000	34540		
Total	53	5978000			

Table 22. ANOVA of main effects and significant interactions in gel time

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	2357000	2357000	65.58	<.0001
Volatile	3	285000	94990	2.64	0.0616
Heating	2	268900	134400	3.74	0.0320
Heating*extraction	2	1113000	556600	15.49	<.0001
Extraction*volatile	3	320000	106700	2.97	0.0426
Error	42	1509000	35940		
Total	53	5978000			

Table 23. Gel time (s) of heat treated PPI extracted by alkaline or salt[1]

Heating[2]	Alkaline	Salt
M	1785 ± 60^{a}	2159 ± 67^{bc}
R	2051 ± 64^{b}	2142 ± 64^{bc}
U	1531 ± 64^{a}	2327 ± 64^{c}

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly different ($p<0.05$) by Tukey's test

[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)

Table 24. Gel time (s) of PPIa and PPIs with a volatile compound (500 ppm)[1]

Volatile[2]	Alkaline	Salt
Hex	1828 ± 72^{abc}	2408 ± 72^{e}
HxAc	1788 ± 77^{ab}	2160 ± 77^{cde}
2-Oct	1883 ± 72^{abcd}	2076 ± 72^{bcd}
None	1656 ± 67^{a}	2194 ± 77^{de}

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly different ($p<0.05$) by Tukey's test

[2] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

4.4.1.1. Importance of gel point. Gel point is an important parameter as it describes the point after which the protein is aggregated. This point also says something about the mechanism of gelation. If the protein begins to associate before the protein is unfolded, the gel will be more coagulum like and less structured (Sun & Arntfield, 2010). The gel point often follows the onset of heat denaturation, as the hydrophobic groups exposed begin to associate. If aggregation waits for the unfolding to occur, i.e. a higher (later) gel point, then the gel formed may be stronger (higher G'). One example of this is that with an increase in salt content (0-0.7 M), Sun and Arntfield (2011) found an increase in gel point and G' as the salt stabilized the structure against denaturation and then contributed to the network formation. However with 2.0M salt, PPI did not show a cross-over nor did it 'gel' (tan δ>2), as the gel point was elevated beyond the temperature of the experiment. So it does not follow that higher gel points always yield better gels. Gel point and gel strength are both dependent on many factors including salt content, pH, protein characteristics and structure and gelation mechanism.

With and without volatile compounds, PPIa samples (gel points between 80.2-87.8°C, Figure 9) were in range of the gel point of 85.1°C that Sun and Arntfield (2010) reported for their sample with salt. However without salt (Sun & Arntfield, 2011) the gel point was lower at 60.15°C. The presence of salt protects the structure leading to an increase in gel temperature. All of the current samples showed Td less than 83°C in the DSC analysis (Table 10), however, most did not gel (cross-over) before that temperature was reached in the rheology. All of the samples in this experiment 'gelled' with higher than expected gel times. Because my PPIa samples were in the range (and higher) than the samples with salt from Sun and Arntfield (2011), even though no salt was added, the extraction process may have created some salts or ionic compounds that protected the structure. However, this would not explain the gelling during cooling of the PPIs samples. The proximate analysis results showed that the PPI samples did not differ significantly in ash content (Table 4).

Samples that had gel points during the cooling phase, well beyond the Td, appeared to have some delay in gelation. By the maximum heat (95°C), the protein should have been entirely heat denatured with as many exposed groups as possible. The rheology curve for a sample that gels upon cooling still generally initiated gelling during heating. As it was the PPIs which had a structure formed that then dissolved late in heating (peaked at 90°C) as apparent in the tan δ curve (Figure 6b) as well as the gel point in cooling, it may have been that the formation of the first structure delayed the final gel formation. I did not come across this in any other reports. Perhaps after denaturation began the first structure that formed was not stable against higher temperatures and when it dissolved, the protein, being exposed again, aggregated and gelled. This final gel formed late but still saw the characteristic increase in G' and G" with a cross-over and then setting during the rest of the cooling period. This could also apply to the PPIa samples which also had a higher than expected gel point and a primary, temporary, structure that dissolved before the final gel was formed (although at a lower temperature than in the PPIs samples).

4.4.1.2. Interactions influencing gel point. Heating by extraction was a significant interaction for the cross-over point. Figure 7, with Tukey's significance noted ($p<0.05$), shows the mean PPIa gel temperatures (open symbols) were all during the heating period while PPIs (closed symbols) had gel temperatures in the cooling period. With the exception of roasted PPIa, all alkaline extracts had significantly lower gel times than PPIs. There was no difference between heating methods in the PPIs samples (Table 23) but with the alkaline extraction, roasted PPIa had a higher gel point than the micronized and unheated PPIa samples. In DSC, the interaction between heating and extraction was also significant for Td (Table 18) and relates to the gel point of the unheated PPI, where alkaline extraction had both a lower gel point and Td than unheated PPIs.

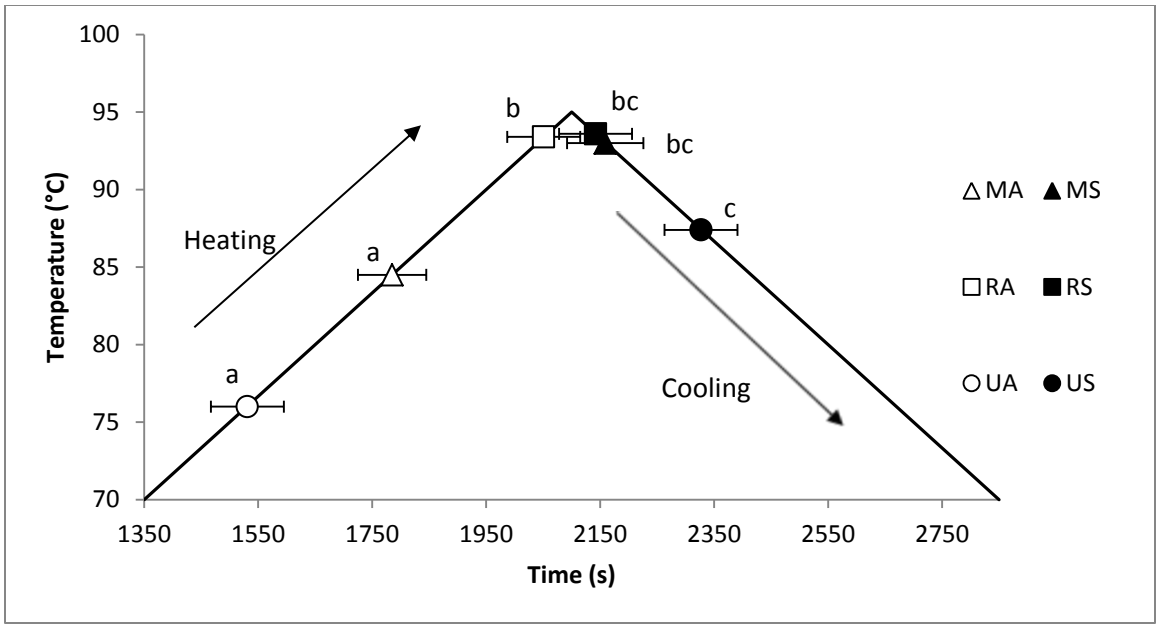

Figure 7**.** Gel time and temperature during small scale dynamic oscillatory rheological testing of PPI by heat treatment and extraction methods. Solid line represents the heating and cooling curve. Values (LSM ± SE) indicated by different letters are significantly different ($p<0.05$) by Tukey's test. PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R). The extraction method was either alkaline (A) or salt (S).

Extraction by volatile compound was also significant for the gel point. Figure 8 relates the gel times in Table 24 to temperature. Except for 2-Oct (diamonds), the control (no volatile) and other volatile compounds showed a difference between PPIa and PPIs (which had gel points in cooling). There was no difference within the PPIa samples. For PPIs, 2-Oct had an earlier gelation than Hex. Volatile compound was not a significant main effect although the p-value was 0.0616 (Table 22). Both volatile compound and the interaction between extraction and volatile had low F-values compared to extraction and heating by extraction (Table 22). It may be that while differences due to volatile compounds (and their interactions with other effects) are, if not significant, then important, they are being overshadowed by extraction. There is opportunity to learn more about the effects of volatile compounds on the gelation behaviour of PPI by using more compounds at varying concentrations.

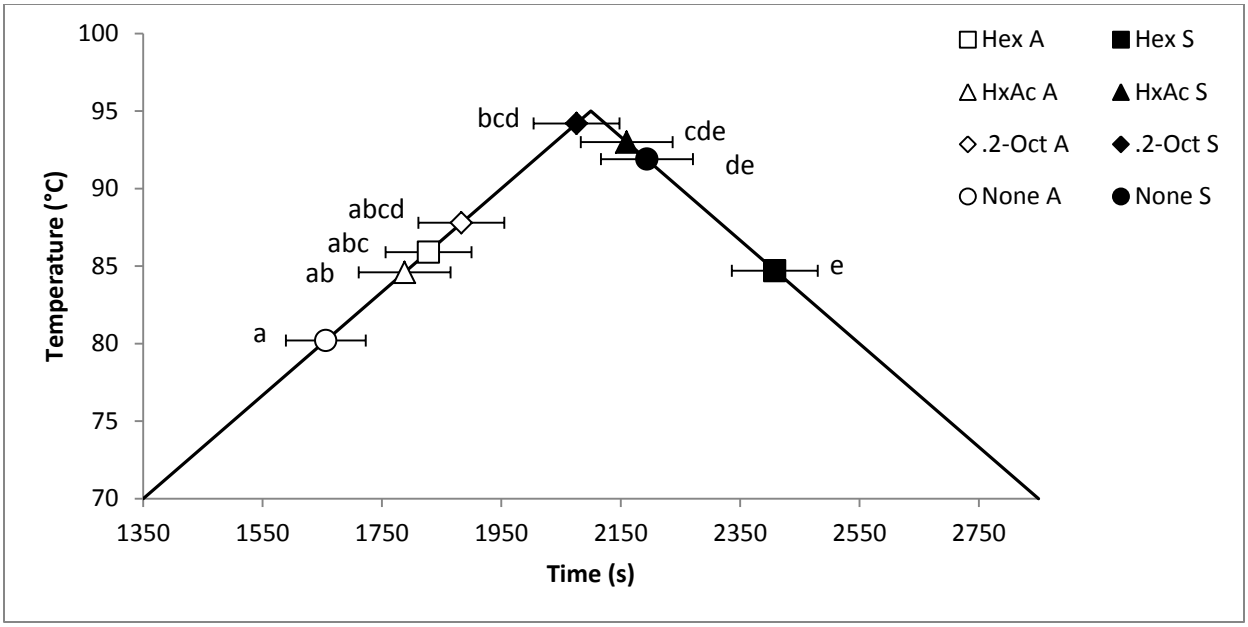

Figure 8. Rheological gel point (with temperature) of PPIa and PPIs with volatile compounds (500 ppm). Solid line represents the heating and cooling curve. Values (LSM ± SE) indicated by different letters are significantly different ($p<0.05$) by Tukey's test. The extraction method was either alkaline (A) or salt (S) and volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none.

4.4.2. Gel characteristics. The rheology experiment monitored the gel formation process and then looked at how the gel responded at various frequencies. The storage (G') and loss (G") moduli as well as their ratio are parameters that were recorded and tell us something of the gel that was formed. These characteristics help describe the gel. A strong gel will have a high G' values, G'>G", and be independent of frequency. Low tan δ values indicate good cross-linking.

First I will give the results from the gel formed during the dynamic gel formation step (final G', G", tan δ values). Second I will look at the characteristics of that gel at 1 Hz during the frequency sweep (FS). As it is the same gel, the characteristics should be essentially the same. The one difference is that the FS was performed after the dynamic step allowing the gel to age (~20 min). Third, the main effects, followed by interactions will be discussed. Again all data was subject to a HOV test and then transformed if needed to meet the assumptions of ANOVA. The first ANOVA table included all possible main effects and interactions. Interactions that were clearly not significant ($p>0.10$) were omitted and a second ANOVA

was performed. Interactions, or main effects if there were no interactions, that were significant in the second ANOVA were tabulated and differences noted by Tukey's test (significance was $p<0.05$).

4.4.2.1. Characteristics of the gel formed during the dynamic step. The final points (G' and G") on the rheology curve during the dynamic gel formation were used to characterize the gel. This data is reported in Table 25. For tan δ, Bartlett's test of HOV was met (Table 26) and the subsequent ANOVA analysis is reported in Tables 27 and 28. Heating by extraction was found to be significant and those means can be found in Table 29. Extraction by volatile was also significant (Table 30). The main effect of volatile had a p-value of 0.0584 (Table 28).

For G', the test for HOV was not met ($p<0.05$) so the data was analysed following a log transformation which yielded a data set for which HOV was met (Table 31). ANOVA results for G' are in Tables 32 and 33. Similarly, for G" the test for HOV (Table 35) was not met until a log transformation of the data. ANOVA results for G" are in Tables 36 and 37. The ANOVA on the transformed data revealed a significant main effect of extraction for G' (Table 34). In addition to extraction, G" showed volatile compounds and the interaction between extraction and volatile to be significant (Table 38). While for G' volatile compound was significant in the first ANOVA (Table 32), after the second ANOVA it was not ($p=0.060$, Table 33).

Table 25. Final gel characteristics of heat treated PPIa and PPIs with different volatile compounds (500ppm)[1]

Heating[2]	Extraction[3]	Volatile[4]	Tan δ	G′ (Pa)	G″ (Pa)
M	A	Hex	0.1595 ± 0.0001	2400 ± 2041	382.8 ± 325.8
M	A	HxAc	0.1708 ± 0.0122	852.4 ± 565.2	149.1 ± 107.0
M	A	2-Oct	0.1612 ± 0.0015	778.2 ± 255.0	125.3 ± 39.80
M	A	None	0.1985 ± 0.0347	6850 ± 6170	1363 ± 1140
M	S	Hex	0.1839 ± 0.0035	421.2 ± 34.44	77.42 ± 4.844
M	S	HxAc	0.1844 ± 0.0152	920.2 ± 931.6	162.7 ± 157.9
M	S	2-Oct	0.1915 ± 0.0112	2277 ± 2682	450.9 ± 538.8
M	S	None	0.1832 ± 0.0075	4606 ± 3059	855.4 ± 594.9
R	A	Hex	0.1512 ± 0.0028	1067 ± 241.8	161.6 ± 39.67
R	A	HxAc	0.1476 ± 0.0009	998.8 ± 63.92	147.4 ± 8.556
R	A	2-Oct	0.1519 ± 0.0031	1105 ± 550.9	167.0 ± 80.26
R	A	None	0.1718 ± 0.0211	7691 ± 7655	1412 ± 1444
R	S	Hex	0.2020 ± 0.0139	214.0 ± 91.22	42.58 ± 15.44
R	S	HxAc	0.1729 ± 0.0045	952.8 ± 329.9	165.4 ± 61.31
R	S	2-Oct	0.1861 ± 0.0167	4298 ± 4886	789.1 ± 926.1
R	S	None	0.1869 ± 0.0090	355.9 ± 180.0	65.70 ± 30.42
U	A	Hex	0.1996 ± 0.0052	585.9 ± 19.23	117.0 ± 6.930
U	A	HxAc	0.1798 ± 0.0001	2647 ± 2542	475.9 ± 456.9
U	A	2-Oct	0.1656 ± 0.0112	471.3 ± 16.83	78.12 ± 8.068
U	A	None	0.1819 ± 0.0006	3772 ± 4093	687.2 ± 746.5
U	S	Hex	0.2056 ± 0.0309	251.3 ± 245.2	46.63 ± 38.37
U	S	HxAc	0.1691 ± 0.0151	2528 ± 3149	451.3 ± 570.9
U	S	2-Oct	0.1806 ± 0.0033	308.6 ± 108.7	55.59 ± 18.61
U	S	None	0.1777 ± 0.0088	410.2 ± 296.5	70.98 ± 48.15

[1] Values are means ± SD of two or more replications

[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)

[3] Extractions were alkaline (A) or salt (S)

[4] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

Table 26. Bartlett's test of HOV in tan δ

Source	DF[1]	Chi-Square	P > ChiSq
Treatment	21	28.80	0.1190

[1] Test (and DF) does not include treatments with no or minimal variance

Table 27. Full factorial ANOVA for tan δ

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	0.003085	0.003085	12.63	0.0013
Volatile	3	0.001786	0.000595	2.44	0.0840
Heating	2	0.001175	0.000587	2.40	0.1075
Heating*extraction	2	0.001989	0.000995	4.07	0.0273
Extraction*volatile	3	0.001976	0.000658	2.70	0.0636
Heating*volatile	6	0.002574	0.000429	1.76	0.1423
Heating*extraction*volatile	6	0.000652	0.000109	0.44	0.8428
Error	30	0.007328	0.000244		
Total	53	0.02058			

Table 28. ANOVA of main effects and significant interactions in tan δ

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	0.003014	0.003014	12.16	0.0012
Volatile	3	0.002000	0.000667	2.69	0.0584
Heating	2	0.001279	0.000640	2.58	0.0877
Heating*extraction	2	0.001952	0.000976	3.94	0.0271
Extraction*volatile	3	0.002490	0.000830	3.35	0.0278
Error	42	0.01041	0.000248		
Total	53	0.02058			

Table 29. Tan δ of heat treated PPIa and PPIs[1]

	Extraction	
Heating[2]	Alkaline	Salt
M	0.1735 ± 0.0056^{ab}	0.1858 ± 0.0056^{b}
R	0.1556 ± 0.0056^{a}	0.1867 ± 0.0053^{b}
U	0.1818 ± 0.0056^{b}	0.1837 ± 0.0048^{b}

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly different (p<0.05) by Tukey's test

[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)

Table 30. Tan δ of PPIa and PPIs with volatile compounds (500 ppm)[1]

	Extraction	
Volatile[2]	Alkaline	Salt
Hex	0.1700 ± 0.0064^{a}	0.1985 ± 0.0060^{b}
HxAc	0.1660 ± 0.0064^{a}	0.1755 ± 0.0064^{ab}
2-Oct	0.1595 ± 0.0064^{a}	0.1858 ± 0.0060^{ab}
None	0.1857 ± 0.0056^{ab}	0.1818 ± 0.0057^{ab}

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly different (p<0.05) by Tukey's test

[2] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

Table 31. Bartlett's test of HOV in G' and transformed G'

		G'		Log G'	
Source	DF	Chi-Square	P > ChiSq	Chi-Square	P > ChiSq
Treatment	23	75.31	<.0001	27.24	0.2458

Table 32. Full factorial ANOVA for log G'

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.449	1.449	6.72	0.0146
Volatile	3	1.904	0.635	2.94	0.0490
Heating	2	0.784	0.392	1.82	0.1800
Heating*extraction	2	0.231	0.116	0.54	0.5907
Extraction*volatile	3	1.425	0.475	2.20	0.1085
Heating*volatile	6	1.770	0.295	1.37	0.2597
Heating*extraction*volatile	6	0.620	0.103	0.48	0.8189
Error	30	6.473	0.216		
Total	53	15.82			

Table 33. ANOVA of main effects in log G'

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.722	1.722	7.56	0.0084
Volatile	3	1.806	0.602	2.64	0.0600
Heating	2	1.167	0.583	2.56	0.0879
Error	47	10.70	0.228		
Total	53	15.82			

Table 34. Storage modulus (G') in gel formation of PPI by extraction[1]

Extraction	G' (Pa)
Alkaline	2624 ± 604[b]
Salt	1435 ± 584[a]

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly different (p<0.05) by Tukey's test

Table 35. Bartlett's test of HOV in G" and transformed G"

		G"		Log G"	
Source	DF	Chi-Square	P > ChiSq	Chi-Square	P > ChiSq
Treatment	23	76.63	<.0001	26.10	0.2960

Table 36. Full factorial ANOVA for log G"

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.130	1.130	5.19	0.0300
Volatile	3	1.948	0.649	2.98	0.0470
Heating	2	0.742	0.371	1.70	0.1991
Heating*extraction	2	0.245	0.122	0.56	0.5758
Extraction*volatile	3	1.544	0.514	2.36	0.0911
Heating*volatile	6	1.783	0.297	1.36	0.2609
Heating*extraction*volatile	6	0.564	0.094	0.43	0.8518
Error	30	6.536	0.218		
Total	53	15.61			

Table 37. ANOVA of main effects and significant interactions in log G"

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.173	1.173	5.70	0.0213
Volatile	3	1.773	0.591	2.87	0.0470
Heating	2	0.850	0.425	2.07	0.1388
Extraction*volatile	3	1.873	0.624	3.03	0.0391
Error	44	9.055	0.206		
Total	53	15.61			

Table 38. Loss modulus (G") of PPIa and PPIs with volatile compounds (500 ppm)[1]

	Extraction	
Volatile[2]	Alkaline	Salt
Hex	220.5 ± 218.6[a]	71.70 ± 203.0[a]
HxAc	257.4 ± 218.6[a]	259.8 ± 218.6[a]
2-Oct	123.5 ± 218.6[a]	478.0 ± 203.0[ab]
None	1197 ± 190[b]	296.3 ± 191.1[a]

[1] Values (LSM ± SE) in table followed by different superscript letters are Significantly different ($p<0.05$) by Tukey's test

[2] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

4.4.2.2. Gel characteristics at 1 Hz in the frequency sweep analysis. A frequency sweep looks at the dependence of G′ and G″ on frequency and provides another opportunity to characterize the gel. The gels were generally independent of frequency (Figure 9). Gel data (G′, G″, and their ratio tan δ) from 1 Hz in the frequency sweep is reported in Table 39.

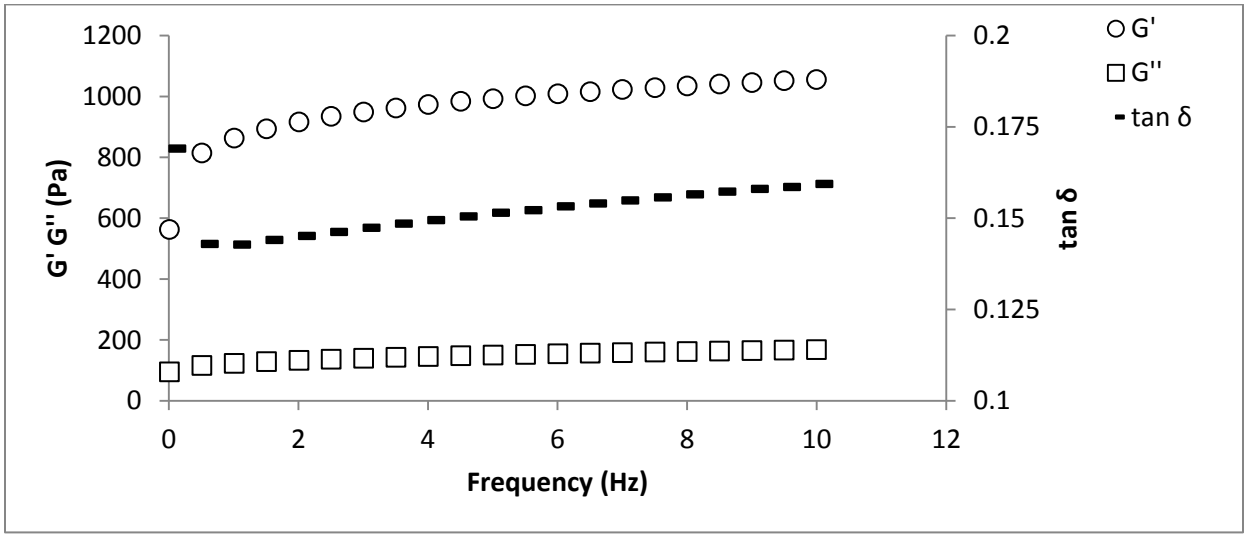

Figure 9. Typical rheological curve during the frequency sweep. Storage modulus (G′) and loss modulus (G″) are plotted along with their ratio (tan δ). The sample shown is roasted PPIa (14.5%) with no volatile compound.

Table 39. Frequency sweep (FS) gel characteristics at 1 Hz[1]

Heating[2]	Extraction[3]	Volatile[4]	Tan δ	G′ (Pa)	G″ (Pa)
M	A	Hex	0.1499 ± 0.0020	2398.0 ± 2018.2	357.5 ± 297.8
M	A	HxAc	0.1678 ± 0.0124	843.8 ± 558.9	145.0 ± 104.2
M	A	2-Oct	0.1572 ± 0.0015	778.1 ± 260.8	122.1 ± 39.7
M	A	None	0.1860 ± 0.0303	6585.0 ± 5779.2	1210.4 ± 970.5
M	S	Hex	0.1783 ± 0.0020	411.2 ± 45.8	73.3 ± 7.4
M	S	HxAc	0.1784 ± 0.0188	908.9 ± 919.4	153.5 ± 146.9
M	S	2-Oct	0.1802 ± 0.0022	2083.6 ± 2410.4	378.2 ± 439.0
M	S	None	0.1707 ± 0.0059	4463.0 ± 2918.9	770.2 ± 524.3
R	A	Hex	0.1468 ± 0.0010	1069.1 ± 238.9	157.0 ± 36.1
R	A	HxAc	0.1421 ± 0.0008	1020.4 ± 75.8	145.0 ± 9.9
R	A	2-Oct	0.1456 ± 0.0042	1117.8 ± 558.8	161.6 ± 76.7
R	A	None	0.1661 ± 0.0210	7525.9 ± 7399.6	1310.1 ± 1278.6
R	S	Hex	0.1967 ± 0.0146	210.8 ± 78.7	40.9 ± 12.4
R	S	HxAc	0.1638 ± 0.0036	944.9 ± 332.5	155.4 ± 57.9
R	S	2-Oct	0.1739 ± 0.0190	3408.2 ± 4063.2	577.9 ± 706.6
R	S	None	0.1808 ± 0.0139	345.0 ± 157.0	61.3 ± 23.6
U	A	Hex	0.1942 ± 0.0064	568.6 ± 22.1	110.5 ± 7.9
U	A	HxAc	0.1678 ± 0.0042	2502.8 ± 2386.1	415.2 ± 390.1
U	A	2-Oct	0.1609 ± 0.0088	462.3 ± 4.4	74.4 ± 4.8
U	A	None	0.1710 ± 0.0002	3672.8 ± 4008.2	627.8 ± 684.8
U	S	Hex	0.1996 ± 0.0308	234.4 ± 244.4	41.8 ± 37.3
U	S	HxAc	0.1616 ± 0.0087	2430.2 ± 3020.5	405.9 ± 509.4
U	S	2-Oct	0.1730 ± 0.0019	290.0 ± 113.8	50.1 ± 19.1
U	S	None	0.1668 ± 0.0070	482.2 ± 278.7	79.2 ± 42.9

[1] Data is mean ± SD of two or more replicates
[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)
[3] Extractions were alkaline (A) or salt (S)
[4] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

For tan δ, Bartlett's test of HOV was not met ($p<0.05$) so a log transformation was applied which yielded a data set for which HOV was met (Table 40). ANOVA analysis for tan δ is reported in Tables 41 and 42 while the significant interaction between heating and extraction can be found in Table 43. Effect of volatile compound ($p=0.0518$) was also found to interact with extraction at $p= 0.0565$ and heating $p=0.0576$ (Table 42).

Table 40. Bartlett's test of HOV in tan δ and transformed tan δ in the FS[1]

	Tan δ			Log tan δ		
Source	DF	Chi-Square	P > ChiSq	DF	Chi-Square	P > ChiSq
Treatment	22	34.34	0.0454	23	34.99	0.0521

[1] Test (and DF) does not include treatments with no or minimal variance

Table 41. Full factorial ANOVA for log tan δ in the FS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	0.01756	0.01756	13.18	0.0010
Volatile	3	0.00986	0.00329	2.47	0.0814
Heating	2	0.00665	0.00332	2.49	0.0995
Heating*extraction	2	0.01176	0.00588	4.42	0.0209
Extraction*volatile	3	0.01061	0.00354	2.65	0.0665
Heating*volatile	6	0.01759	0.00293	2.20	0.0706
Heating*extraction*volatile	6	0.00370	0.00062	0.46	0.8303
Error	30	0.03997	0.00133		
Total	53	0.1170			

Table 42. ANOVA of main effects and significant interactions in log tan δ in the FS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	0.01679	0.01679	13.84	0.0007
Volatile	3	0.01032	0.00344	2.83	0.0518
Heating	2	0.00720	0.00360	2.97	0.0641
Heating*extraction	2	0.01151	0.00576	4.75	0.0148
Extraction*volatile	3	0.01003	0.00334	2.76	0.0565
Heating*volatile	6	0.01658	0.00276	2.28	0.0576
Error	36	0.04367	0.00121		
Total	53	0.1170			

Table 43. Tan δ in the FS for heat treated PPI by alkaline or salt extraction[1]

	Extraction	
Heating[2]	Alkaline	Salt
M	0.1671 ± 0.0056^{ab}	0.1771 ± 0.0059^{b}
R	0.1516 ± 0.0056^{a}	0.1788 ± 0.0054^{b}
U	0.1736 ± 0.0059^{b}	0.1758 ± 0.0053^{b}

[1] Values (LSM ± SE) in table followed by different superscript letters are significantly Different ($p<0.05$) by Tukey's test

[2] PPI were unheated (U) or treated with heat by micronizing (M) or roasting (R)

For G', HOV is shown for raw and transformed data in Table 44, followed by ANOVA in Tables 45 and 46. For G", HOV is shown for raw and transformed data in Table 47, followed by ANOVA in Tables 48 and 49. The ANOVA on transformed G' and G" values revealed a significant main effect of extraction (Table 50) as well as volatile compound (Table 51) for both.

Table 44. Bartlett's test of HOV in G' and transformed G' in the FS

		FS – G'		FS – log G'	
Source	DF	Chi-Square	P > ChiSq	Chi-Square	P > ChiSq
Treatment	23	72.72	<.0001	26.64	0.2717

Table 45. Full factorial ANOVA for log G' in the FS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.469	1.469	7.12	0.0122
Volatile	3	2.064	0.688	3.33	0.0325
Heating	2	0.763	0.381	1.85	0.1750
Heating*extraction	2	0.214	0.107	0.52	0.6013
Extraction*volatile	3	1.280	0.427	2.07	0.1254
Heating*volatile	6	1.696	0.283	1.37	0.2588
Heating*extraction*volatile	6	0.574	0.096	0.46	0.8296
Error	30	6.191	0.206		
Total	53	15.25			

Table 46. ANOVA of main effects in log G' in the FS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.492	1.492	6.94	0.0114
Volatile	3	2.133	0.711	3.31	0.0280
Heating	2	1.016	0.508	2.36	0.1052
Error	47	10.10	0.215		
Total	53	15.25			

Table 47. Bartlett's test of HOV in G" and transformed G" in the FS

		FS – G"		FS – log G"	
Source	DF	Chi-Square	P > ChiSq	Chi-Square	P > ChiSq
Treatment	23	71.13	<.0001	25.14	0.3429

Table 48. Full factorial ANOVA for log G" in the FS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.176	1.176	5.78	0.0226
Volatile	3	2.072	0.691	3.40	0.0305
Heating	2	0.729	0.364	1.79	0.1841
Heating*extraction	2	0.212	0.106	0.52	0.5988
Extraction*volatile	3	1.349	0.450	2.21	0.1075
Heating*volatile	6	1.596	0.266	1.31	0.2840
Heating*extraction*volatile	6	0.528	0.088	0.43	0.8514
Error	30	6.104	0.203		
Total	53	14.72			

Table 49. ANOVA of main effects in log G" in the FS

Source	DF	Type III SS	Mean Square	F Value	P > F
Extraction	1	1.206	1.206	5.67	0.0213
Volatile	3	2.104	0.701	3.30	0.0283
Heating	2	0.964	0.482	2.27	0.1148
Error	47	9.995	0.213		
Total	53	14.72			

Table 50. Storage and loss moduli in the FS at 1 Hz for PPI by extraction[1]

Extraction	G' (Pa)	G" (Pa)
Alkaline	2554 ± 575[b]	436 ± 100[b]
Salt	1448 ± 554[a]	249 ± 96[a]

[1] Values (LSM ± SE) in each column followed by different superscript letters are significantly different (p<0.05) by Tukey's test

Table 51. Storage and loss moduli in the FS at 1 Hz for PPI with volatile compounds (500 ppm)[1]

Volatile[2]	G' (Pa)	G" (Pa)
Hex	872 ± 811[a]	141 ± 140[a]
HxAc	1442 ± 842[ab]	237 ± 146[ab]
2-Oct	1678 ± 786[ab]	283 ± 136[ab]
None	4012 ± 754[b]	708 ± 130[b]

[1] Values (LSM ± SE) in each column followed by different superscript letters are significantly different (p<0.05) by Tukey's test

[2] Volatile compounds were hexanal (Hex), hexyl acetate (HxAc), 2-octanone (2-Oct) or none

4.4.2.3. Main effects on the PPI gels. The gelation behaviour and characteristics were affected by extraction, volatile compound and heating. As heating was also seen to interact with extraction it will be discussed in this context in the next section.

Extraction method was significant for the storage and loss moduli in both the dynamic formation (Table 34) and FS (Table 50). Main effect of extraction is not shown for G" as there was also an interaction between extraction and volatile (Table 38). The mean G' value for PPIa was almost twice that for PPIs showing PPIa led to stronger gels (Table 34). The alkaline extraction process may have led to stronger gels because the denaturing power led to more reactive areas that were available prior to heat denaturation and gel formation. This is not what others have found. Sun and Arntfield (2010) compared commercial (generally alkaline extracted) PPI to PPIs and found PPIs to create stronger (lower tan δ) gels. Commercial PPI is thought to be more denatured than PPIs so that its ability to form gels in diminished. However, our alkaline extraction may have been less harsh (from pH 9.5 to 4.5) than a commercial extraction, aiding in the gel formation. I used PPI at 14.5% to ensure that there was enough protein to form a gel. Boye, Aksay et al. (2010) found that PPI precipitated isoelectrically required more protein to form a gel than ultra-filtrated PPI; 14% compared with 12%. This again would be because the degree of denaturation during isoelectric precipitation does not leave as much structure so more protein is required. They did not do rheological exploration, although least gelation concentration is, in some ways, a measure of gel strength.

Volatile compound was also shown to be a significant main effect for the moduli, particularly in the FS (Table 51). In the final gel, G" showed effects of an interaction between extraction and volatile compound (Table 38) while volatile compound alone had an effect on G' at significance level of p=0.06 (Table 33). Additionally, effects of volatile compound on tan δ were close to significant with p-values of 0.0584 (Table 28) and 0.0518 (Table 42) which warrants some discussion. In the frequency sweep, PPI

samples with no volatile compounds had higher G values than those samples with Hex (Table 51). There was also no difference between the volatile compounds or the other compounds compared to the sample with no volatile compound added (Table 51). Similar trends are in the final gel characteristics of the PPIa samples, where the control has a larger G" than any volatile compound (Table 38). Hex tended toward greater binding ability than 2-Oct (as discussed in section 4.2.1.2.) and it is possible that the presence, or binding of Hex, limited the gel strength by occupying sites that would have been used for protein-protein interactions. That this difference was seen primarily in the frequency sweep indicated that the gel was still 'curing' or changing throughout the hold and frequency sweep. With no volatile compound, the network continued to strengthen but with Hex present, the continued strengthening of the network as the gel 'aged' was limited. The order of tan δ values from smallest to largest by volatile compound was HxAc, 2-Oct, None, Hex. PPI, in the presence of Hex, was the weakest and least cross-linked gel. With no volatile compound, the G' values were highest indicating a strong gel; however the gel with the lowest tan δ, or best linked was HxAc. This indicated that the presence of volatile compounds altered both the strength and type of structure that was formed.

4.4.2.4. Interactions between main effects characterizing PPI gels. While there were no three way interactions, two-way interactions were determined to be significant for some parameters.

An interaction between heating and extraction was demonstrated in the final tan δ (Table 29) and FS tan δ (Table 43). The roasted PPIa had a significantly lower tan δ than roasted PPIs but no extraction effects were seen for the micronized or unheated samples. The roasted PPIa was also the only sample where heat created a more networked gel (lower tan δ) than the unheated sample. This may have been because roasting, a harsh heat treatment, disrupts the native structure of the protein, exposing more reactive groups, leading to a better cross-linked gel. When compared to the enthalpy results, a measure of structure, there was also an interaction between heating and extraction (Table 16). Roasting was seen

to decrease the enthalpy from unheated, but only with the PPIs. Micronizing was also seen to decrease the enthalpy, but only with PPIa. The heat treatments affect the level of structure as seen in enthalpy and presumedly in tan δ; however, in combination with extraction, the results are not consistent between enthalpy and tan δ. The level of structure or denaturation is not the only determinant of the ability of a protein to cross-link. The differences due to heat were not reflected in the G' and G" values.

Extraction by volatile was seen to be significant in tan δ (Table 30) and G" (Table 38), while for FS tan δ the interaction had a level of significance of p=0.0565 (Table 42). In the presence of Hex, PPIs created a less elastic (greater tan δ) gel than PPIa with Hex. With no volatile compound, PPIs were weaker (lower G") than PPIa. This may again point to the overwhelming effects of extraction.

Furthering the importance of volatile compounds, the interaction between heating and volatile compound had a p value equal to 0.0576 in the FS tan (Table 42). More volatile compounds and concentrations should be examined to better elucidate the effects of volatile compound on gelation behaviour.

4.4.2.5. Comparison of gel characteristics to other studies. In comparing the current results to other studies, the values obtained for G' (1400-2700 Pa) and tan δ (0.155-0.190) are in the range of samples with salt examined by Sun and Arntfield (2010) and Wong et al. (2013) (Table 1). Sun and Arntfield (2011), without salt, obtained a much lower G' (0.35 Pa) and higher tan δ (1.36); as did O'Kane et al. (2005). While it is difficult to compare across studies, it appears that both pH and salt content have an effect on the rheological properties. Without salt, protein is unstable and may aggregate before forming the cross-links that lead to a strong gel (Sun & Arntfield, 2011). However pH also has an effect on gel strength. Strongest gels are formed with some charge, i.e. away from the isoelectric point, but not at pH extremes (Sun & Arntfield, 2011). Sun and Arntfield (2011) found low G' values between pH 6-7 due to inadequate heating during the experiment. The current study was done at pH 7.6 where there

should be some charge which encourages interaction. While this explains the differences in part, other contributing factors may be the extraction methods and sample preparation.

5. Conclusions and Future Research Opportunities

The results of this project showed evidence of binding of volatile compounds to pea protein isolates. Using GC-MS there was a four way interaction between heating, extraction, volatile compound and concentration. In some cases, PPI tended to reduce the amount of Hex and HxAc in the headspace (bound) more than 2-Oct. Heat treatments of micronizing and roasting also showed trends of decreasing the binding of volatile compounds by the protein. This binding capacity of the protein, alongside extraction and heating effects, was accompanied by changes in the functional properties of the protein. Enthalpy, using DSC, was reduced for the PPIa samples (compared with PPIs) for all heat treatments. Roasting decreased the enthalpy in the PPIa sample while micronizing did the same in the PPIs sample. Temperature of denaturation was sensitive to heat (in PPIs) and extraction. The presence of Hex led to a lower Td than HxAc (volatile compound effects significant at $p=0.0536$). The effects of volatile compounds were seen keenly in the rheological portion of this study, where it appeared that the presence of volatile compounds weakened the gels. Gel point in the presence of 2-Oct was not sensitive to extraction method but with the PPIs, Hex had a later gel point than 2-Oct. Hex also tended toward weaker gels (lower G values) than gels with no volatile compound, particularly in the frequency sweep indicating the importance of time or curing. With no volatile compound, PPIa gels were stronger than with volatile compounds. Perhaps binding mechanism led to different effects of volatile compound on gelation behaviour (strength and cross-linking ability).

Also of interest in the rheology of the PPI was the temporary first structure formed that appeared to delay the final gel formation, most markedly in the PPIs samples which had gel points in the cooling period. This could be explored further.

I would also recommend investigations into the mechanisms of binding to learn more about the changes in structure that occur due to binding of volatile compounds. With GC/MS a wider concentration range of volatile compounds is need to elucidate the effects of concentration on binding. As well, the thermal effects of the volatile compounds may be clearer at higher concentrations of volatile compound, although one must keep in mind realistic concentrations of volatile compounds used in food products. There are other functional properties as well as volatile compounds that could be used to broaden the scope of a similar experiment.

Heating, extraction and volatile compounds all had measurable effects on the functionality of pea protein. This is important for developers of novel foods using pea protein isolates and they should carefully consider the effects of prior processing and flavour addition to optimize performance.

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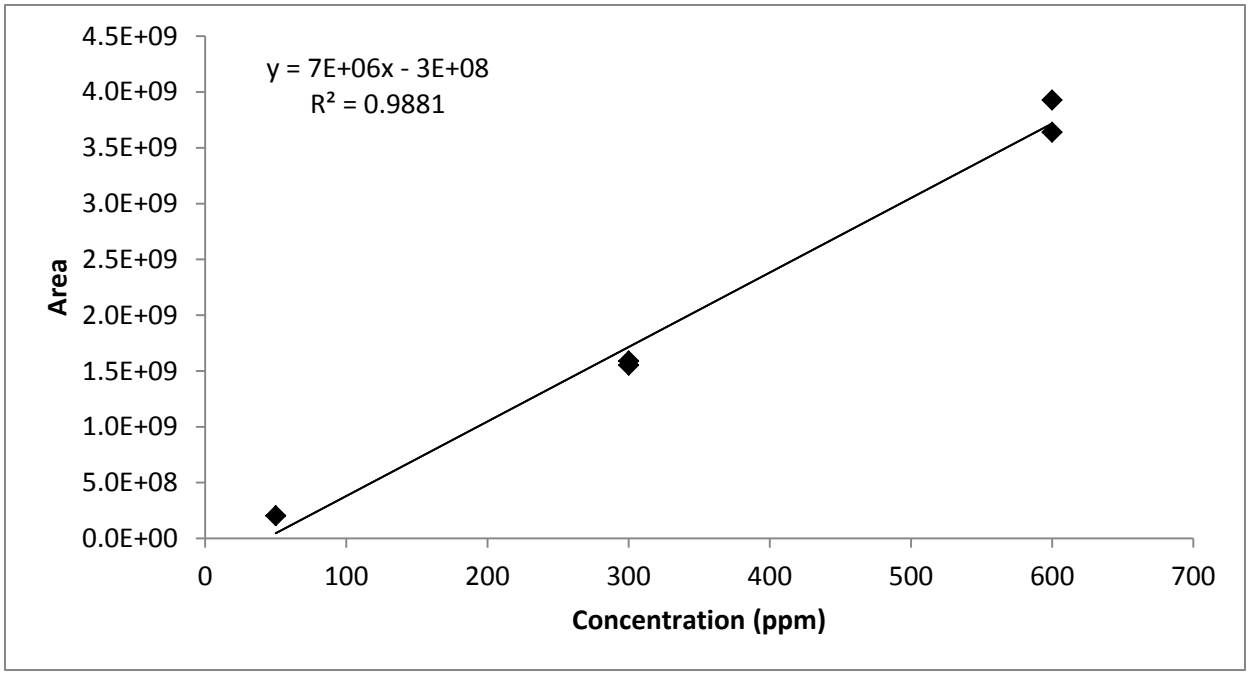
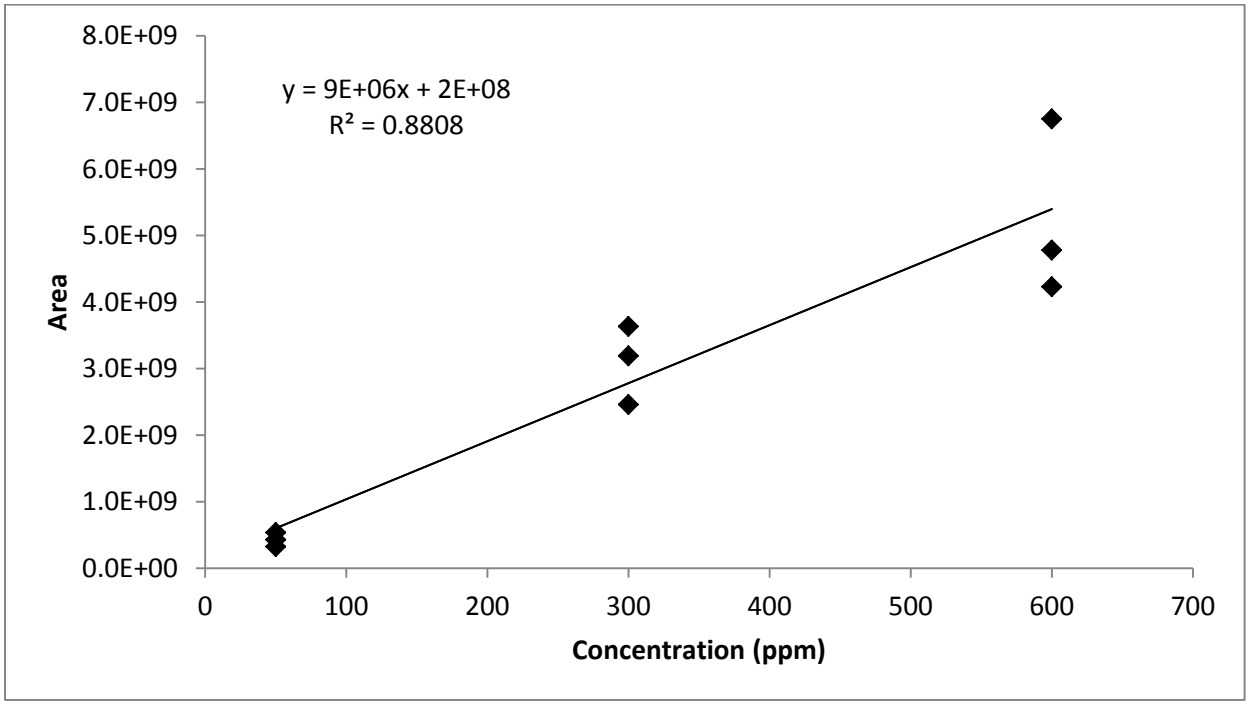
7. Appendices

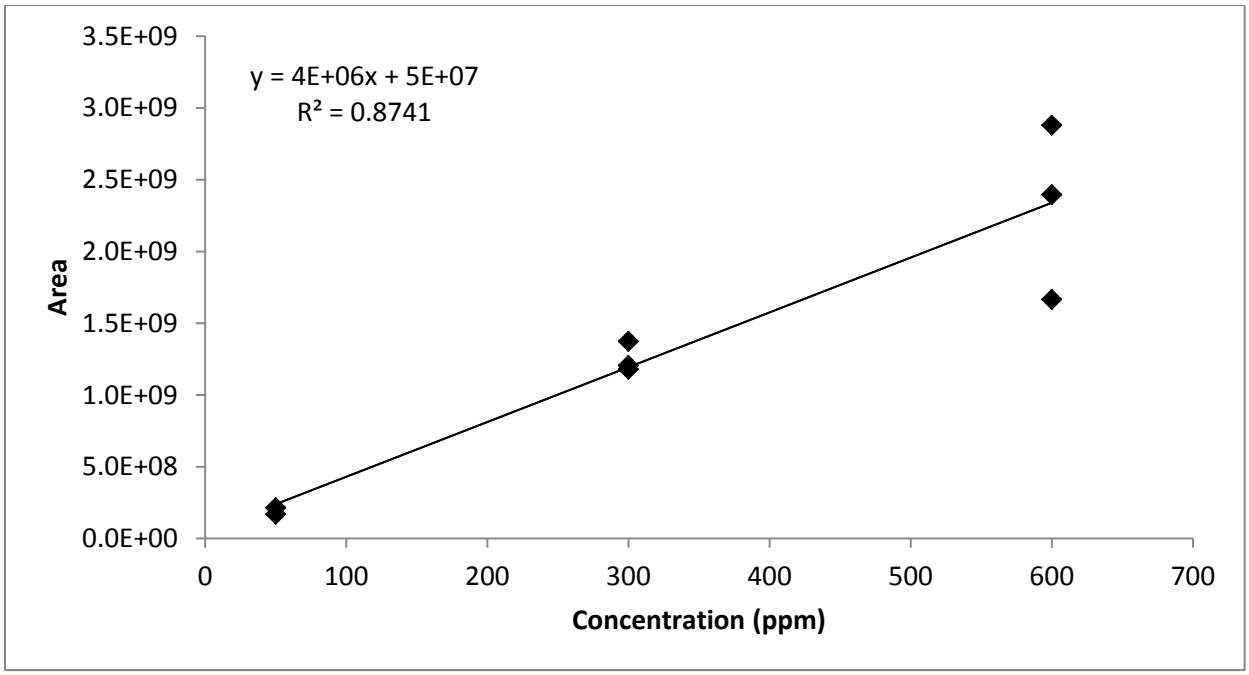
Appendix A. GC/MS Calibration Curves



Calibration curve for hexanal.

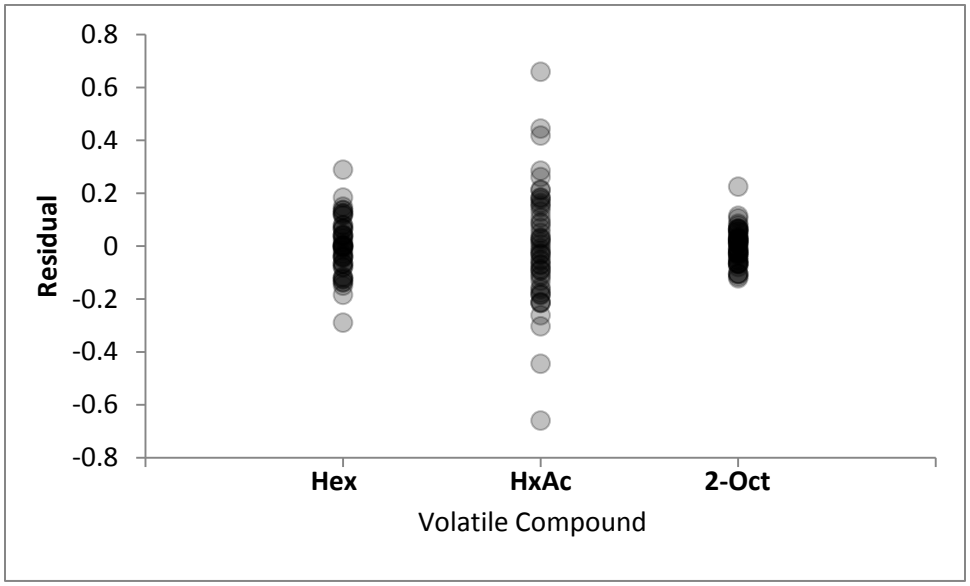


Calibration curve for hexyl acetate.

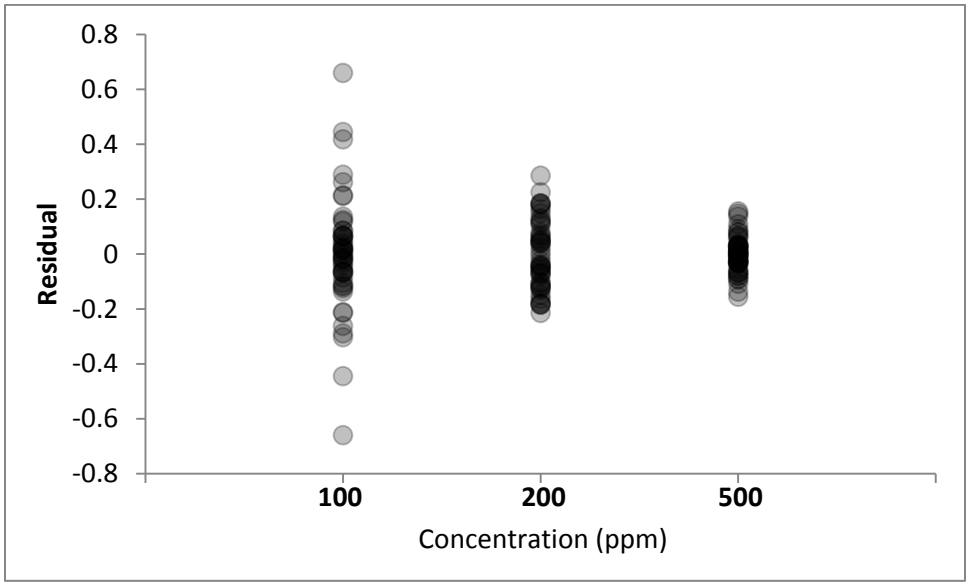


Calibration curve for 2-octanone.

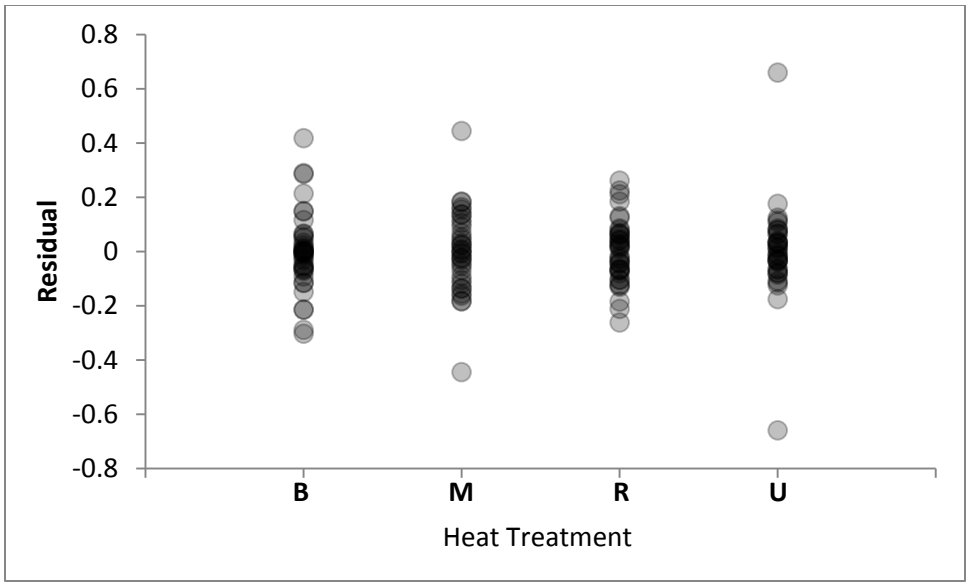
Appendix B. Residual plots of GC/MS data to confirm homogeneity of variance



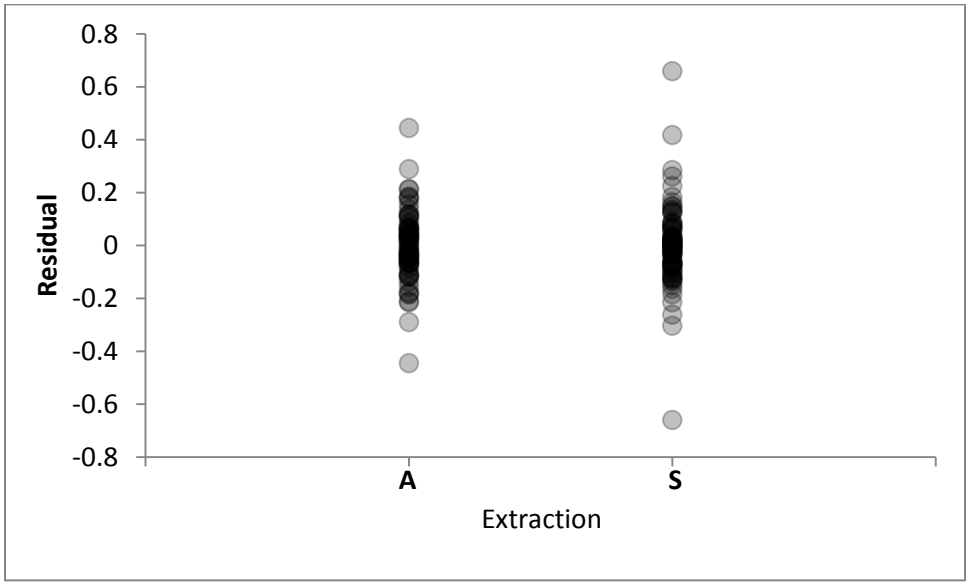
Residuals of all GC/MS data plotted by volatile compound, hexanal (Hex), hexyl acetate (HxAc), or 2-octanone (2-Oct).



Residuals of all GC/MS data plotted by concentration.



Residuals of all GC/MS data plotted by heat treatment, boiling (B), micronizing (M), roasting (R), or unheated (U).



Residuals of all GC/MS data plotted by extraction method, alkaline (A) or salt (S).