Evaluation of Protein-Flavour Binding on Flavour Delivery and Protein Thermal-Gelation Properties in regards to Selected Plant Proteins

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
of the Degree of

DOCTOR OF PHILOSOPHY

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ACKNOWLEGEMENTS

I would like to express my deepest gratitude to Dr. Susan D. Arntfield, my advisor, for her countless hours of reading, dedicated guidance, and thoughtful ideas throughout the entire process. This piece of work could not be accomplished without her support, patience and encouragement, whenever I came up with new ideas and discoveries. Thank you for allowing me being your student and inspiring me to continue my research as a PhD student. Sincere thanks are extended to Drs. Dave Hatcher and Michel Aliani for being members of my thesis committee and for their interest and support as well as encouragement for me to further pursue a PhD degree. It has been the most delightful thing after I made this choice. I would also like to express my gratitude to my external examer Dr. Gary Reineccius from University of Minnesota for coming to Winnipeg to join my defense and provide great recommendations for my thesis.

A very special thank you to Wayne D. Buchannon and Dr. Tom Ward in the Chemistry Department, without their guidance and persistent assistance, the GC/MS analysis could not have been accomplished. I am also thankful to Pat Heritage, Alison Ser, Yang Qiu and Prakash Sharma (Animal Science for amino acid analysis) whose technical support are most appreciated. I am also grateful to Dr. Rotimi Aluko (Human Nutritional Sciences) for his kindness to allow me to use his fluorimeter. In addition, I want to thank my office mates, Veji, Priya, Nancy and Buky and all the people in the lab and department who shared their thoughts and great moments with me during breaks and special lunch occasions.

This project was funded by the Natural Science and Engineering Research Council of Canada (NSERC). I also would like to thank University of Manitoba Faculty of Graduate Studies to provide several travel grants and scholarships as well as James W. Barlow Graduate Fellowship and Garson N. (Gerry) Vogel Memorial Award from Food Science Department for the financial support.

Finally, I want to thank my mom, dad and my girlfriend, Yujie, for their motivation and encouragement through my entire process to pursue this degree. Words cannot express how important you are for me to the completion of this degree.

I will never forget the days and nights I spend here.

PREFACE

This thesis has been written in a paper style format. It consists of ten chapters. Chapter 1 is an introduction, followed by Chapter 2, which presents a literature review that covers isolation and utilization of plant proteins, general knowledge of flavour compounds, flavour food matrix interactions as well as methodology, principles and factors affecting protein-flavour interactions. Research sections include Chapters 3, 4, 5, 6, 7 and 8 which were prepared as they were presented in paper style for submission except for formatting changes. Discussion, conclusion and some considerations about future research are presented in Chapter 9 and 10 respectively.

Chapter 3, "Binding of Carbonyl Flavours to Canola, Pea and Wheat Proteins using GC/MS Approach" was published in Food Chemistry, Issue 157, Pages 364-372, 2014 with authorship by Wang, K., and Arntfield, S. D.

Chapter 4, "Binding of Selected Volatile Flavour Mixture to Salt-Extracted Canola and Pea Proteins and Effect of Heat Treatment on Flavour Binding" was published in Food Hydrocolloids, Issue 43, Pages 410-417, 2015 with authorship by Wang, K., and Arntfield, S. D.

Chapter 5, "Effect of Salts and pH on Selected Ketone Flavours Binding to Salt-Extracted Pea Proteins: The Role of Non-Covalent Forces" was published in Food Research International (In Press), with authorship by Wang, K., and Arntfield, S. D.

Chapter 6, "Probing the Molecular Forces Involved in Binding of Selected Flavours to Salt-Extracted Pea Proteins" has been prepared to be submitted for

publication in Food Chemistry, with authorship by Wang, K., and Arntfield, S. D.

Chapter 7, “Modulation of interactions between selected volatile flavour compounds and salt-extracted pea protein isolates using chemical and enzymatic approaches” has been prepared to submit for publication in Journal of Agricultural and Food Chemistry, with authorship by Wang, K., and Arntfield, S. D.

Chapter 8, “Interaction of Selected Volatile Flavour Compounds and Salt-Extracted Pea Proteins: Effect on Protein Structure and Thermal-Induced Gelation Properties” was published in Food Hydrocolloids, Issue 51, Pages 383-394, 2015 with authorship by Wang, K., and Arntfield, S. D.

ABSTRACT

This work was undertaken to evaluate interactions between plant proteins and selected volatile flavour compounds on flavour delivery and heat-induced gelation properties for canola, pea and wheat proteins. An automated dynamic headspace GC/MS approach was adopted to monitor the change in flavour intensity in aqueous model systems. The extent of flavour binding was a function of protein source, protein isolation method and stereochemistry of the flavour compound. Using Differential Scanning Calorimetry and intrinsic fluorimetry, potential conformational changes due to partial denaturation of proteins were observed. Aldehyde flavours exhibited much higher "unfolding capacity" than ketones, which accounted for their remarkable binding affinities. Two volatile flavour by-products, 2-butyl-2-octenal and 2-pentyl-2-nonenal, were detected from the interactions between salt-extracted canola protein isolates (CPIs) with hexanal and heptanal, respectively, due to aldolisation reactions. Competitive bindings among homologous ketones and between heterologous aldehyde and ketone mixture were observed, while a synergistic effect was noted for aldehyde flavour mixtures. Environmental changes such as heating and addition of non-chaotropic salts increased binding for ketones; however, protein aggregation following continuous heating and denaturation of protein by chaotropic salt and at extreme pH values reduced ketone retention. Apart from molecular interactions, dramatic increases in flavour binding were monitored when physical adsorption of flavours on aggregated proteins was employed. By adding bonding disrupting agents, the molecular forces responsible for the interactions were probed

with hydrophobic interactions, hydrogen bond and ionic interactions being prominent for benzaldehyde, 2-octanone and hexyl acetate, whereas covalent interactions were implicated for octanal and dibutyl disulfide. Selectively modifying proteins via chemical (acetylation and succinylation) and enzymatic (Alcalase) approaches significantly altered protein-flavour binding affinities and this was influenced by the type of flavours selected and associated type of binding. In general, addition of flavour compounds diminished protein heat-induced gel forming properties by disrupting protein inter- and intra-molecular hydrophobic interactions. However, gel strength was regained with increasing concentration and chain length of aldehydes possibly due to the additional unfolding effect on proteins due to aldehyde binding. This facilitated the gel formation process, consequently resulting in formation of stronger gels.

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LIST OF ABBREVIATIONS

ANS……..8-anilino-1-naphthalene sulfonic acid

BSA……..Bovine serum albumin

CPI…….Canola protein isolate

CPIa…….……..Alkaline-extracted canola protein isolates

CPIs……..…….Salt-extracted canola protein isolates

C_g ……...….Proportion of flavour in the air

C_p ……..……..Proportion of flavour in the product

DH…….. Degree of hydrolysis

DSC…….……Differential scanning calorimetry

DTNB…….…….. 2-nitrobenzoic acid

DTT ……...Dithiothreitol

EDTA.……...…..…..Ethylenediaminetetraacetic acid

FI……..Fluorescence intensity

GC…….. Gas chromatography

GC-O ……..Gas chromatograph olfactometry

GuHCl……..……Guanidine hydrochloride

ΔG ……...…….....Free energy

ΔH ……....Enthalpy of denaturation

IEP …….....Isoelectric point

ITEX……..…..In tube extraction

K.……..Gas/product partition coefficient

K_b ……..……..Intrinsic binding constant

k……...…….....Mass transfer coefficient

log *P* ……..Hydrophobicity of flavour

λ_{max} ……...Wavelength of maximum fluorescence emission

2-mercaptoethanol ……..2-ME

MS ……...……..Mass spectrometer

MWCO ……..……..Molecular weight cut-off

nK ……...……..Overall binding constant

PES ……..……..Polyethersulfone

PG ……...……..Propylene glycol

PPIa ……...Salt-extracted pea protein isolates

PPIs ……..…..Salt-extracted pea protein isolates

PMM ……...……..Protein micellar mass

RFI ……...……..Relative fluorescence intensity

S_0 ……....Protein surface hydrophobicity

SDS-PAGE……....Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SPME ……..……..Solid phase micro-extraction

T_d ……..……..Denaturation temperature

TNBS ……..2,4,6-Trinitrobenzene sulfonic acid

WHO ……..……..World Health Organization

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Chapter 1 INTRODUCTION

Flavour is one of the most determinant factors for acceptance of foods by consumers. Aroma, taste, texture, and mouth feel all contribute to the perception of flavours (van Ruth and Poozen, 2002). Proper flavouring of foods to provide a substantial amount of free flavour molecules, plays an important role in headspace flavour concentration and resulting perception of aromas (olfaction). Unfortunately, the processes of food flavouring, flavour release and perception are not that simple. It is known that flavour release and delivery are affected by the intrinsic natures of flavour, flavour concentration in the food, and the availability of flavour to be perceived from the food matrices (Bakker et al., 1995). The latter closely relates to the interactions between flavour compounds and major food constituents such as proteins, lipids, and carbohydrates (Guichard, 2002; Reineccius, 2006a; Solms, Osman-Ismail, & Beyeler, 1973).

Plant proteins including sources from canola/rapeseed protein, pulse (dried peas, edible beans, lentils and chickpeas) proteins, cereal proteins (wheat), and soy protein have been widely recognized as potential ingredients in manufacturing novel foods and natural health products. With continuous improvement on their suitability to be incorporated into food systems, some functional properties of pulse and canola proteins have been found to be promising and comparable to those of animal and plant proteins such as casein, soybean or wheat proteins (Boye, Zare, & Pletch, 2010; Khattab & Arntfield, 2009; Pinterits & Arntfield, 2007).

Although various benefits of plant proteins have been raised, it is found that

when adding flavours to plant protein food matrices, flavour compounds are able to interact with protein, suppressing and unbalancing the flavour release thereby affecting their overall perception in foods (Gremli, 1974; Heng et al., 2004). This specific binding is considered to be undesirable and significantly impacts the performance of desirable flavours and aromas. From another perspective, when adding flavour to health-promoting low-fat foods, proteins and carbohydrates, which are the primary components of reduced-fat foods, interact differently with the flavour compounds than fat does (Kúhn, Considine, & Singh, 2006; Plug & Haring, 1993). Using flavour systems that are appropriate for full fat food may not be able to deliver a satisfactory sensory profile in reduced fat products (Reineccius, 2006a). Therefore, understanding of protein-flavour interactions is essential to formulate novel protein foods with desired flavour perception. More importantly, creation of new flavour formulations may be necessary to compensate for the impact of protein-flavour binding on flavour delivery.

In the scientific literature, interactions of volatile flavour compounds with animal and plant proteins in model food systems have been studied for over 40 years, although mechanisms responsible are still not explicit. Less emphasis has been put on plant proteins; especially deficient in the literature are canola and pulse proteins. To the author's knowledge, no data on binding of flavours with canola protein have been reported. With increased use of canola and pulse proteins in traditional and novel protein foods, a comprehensive understanding of their flavour binding properties and the nature of these interactions, are of great importance for improving their usability.

Therefore, this work was undertaken to provide a fundamental study of protein-flavour binding on flavour delivery with respect to some intrinsic features of different flavours (flavour chain length and functional group), protein sources (canola, pea and wheat proteins), protein isolation methods (salt *vs.* alkaline extraction) and extrinsic parameters including heating conditions, pH and the presence of salts. Competitive binding between homologous and heterological classes of flavours to these proteins was monitored. Protein-flavour binding mechanisms were examined by following the associated structural changes in proteins and probing the molecular forces using various bond disrupting agents. The effect of several non-enzymatic and enzymatic modifications of proteins on resulting flavour release was also investigated. The effect of protein-flavour binding on thermally-induced gelation properties was also determined to demonstrate potential effect of flavour-protein interaction on protein functionality.

It is expected that the level of protein-flavour binding is dependent upon the structural state of protein molecules and type of flavour selected, based on their functional groups and associated types of bonding. By monitoring the molecular forces and following the potential structural changes of proteins, future clues and prospective methods to control the release of bound volatile compounds can be provided.

Chapter 2 LITERATURE REVIEW

The aim of this review is to cover the fundamental information on proteins (plant sources) and flavours and the impact of their interactions on flavour retention and release. Flavour-food matrix interactions were overviewed with a special concern on their relation with flavour perception. Current knowledge on methodologies involved in protein-flavour binding studies, binding mechanisms and factors affecting the interaction were discussed. The implication of protein-flavour interaction on protein functionality especially protein thermal gelation properties was considered.

2.1 Utilization of plant proteins (canola and pulses) in food systems

Not just an essential source of nutrients, proteins from vegetable and plant sources have a long history of being incorporated into foods to impart a wide range of functionalities (Boye & Maltais, 2011; Foegeding & Davis, 2011; Tan et al., 2011). A great deal of research has been conducted on evaluating and optimizing their functions and unveiling the underlying mechanisms (Aider & Barbana, 2011; Boye et al., 2010; Boye, Zare, & Pletch, 2010; Moure et al., 2006).

Taking pulse protein as an example, in the last ten years, research conducted on incorporating pulse proteins in various food applications have included doughs (Mariotti et al., 2009), breads (Des Marchais et al., 2011; Villeneuve & Mondor, 2014), pastas (Mercier et al., 2011; Petitot et al., 2010), biscuits (Rababah, Ai-Mahasneh, & Ereifej, 2006), crackers (Han, Janz, & Gerlat, 2010), comminuted meat products (Drakos, Doxasakis, & Kiosseoglou, 2007), gels (Nunes, Raymundo, & Sousa, 2006) and extruded snack foods (Hood-Niefer & Tyler, 2010).

Canola (*Brassica napus, Brassica rapa or Brassica juncea*) is Canada's most valuable crop contributing $ 19.3 billion to the Canadian economy each year (Canola Council of Canada, 2015). From its roots in rapeseed, canola refers to those seeds comprising low levels of erucic acid (< 2 %) and glucosinolates (< 30 mol/g meal). According to Canola Council of Canada (2015), 18 million tonnes of canola were produced in Canada in 2013 with 7.1 million tonnes of seeds being crushed within Canada, producing about 2.37 million tonnes of canola oil (45 % of seed mass) and 3.16 million tonnes of canola meal (a by-product of canola oil extraction). About 40 % of canola seed and over 80 % of canola oil and meals are exported. Canola is mostly grown in southern Alberta, Saskatchewan and Manitoba.

Major uses of canola include oil for human consumption and meal for livestock feed. As 36-44 % of canola seed and more than 35 % of meal are high value protein, potential to isolate canola protein from seed and meal and then formulate it into foods as a value added ingredient is an area for emerging industrial use.

From an industrial perceptive, canola protein isolate (CPI) is obtained when the protein is dissolved while most of the other impurities are removed. In general, methods traditionally used to isolate canola protein involve aqueous extraction with alkaline or NaCl solutions and recovery of CPI by isoelectric precipitation (Blaicher et al., 1983; Nockrashy, Mukherjee, & Mangold, 1977). The efficacy of this method is greatly dependent upon the specific fraction of proteins that precipitates at the isoelectric points. Alternatively, as canola proteins are high molecular weight molecules (> 10,000 Da), a more recent methodology to obtain CPI is to use

ultrafiltration membranes to selectively remove small molecular compounds and retain the high-molecular-weight solutes (canola proteins) (Xu & Diosady, 2013). Developments in this process include combining ultra- and diafiltration with alkaline extraction and isoelectric precipitation (Tzeng, Diosady, & Rubin, 1990). An alternative approach is that patented by Burcon NutraScience Corporation who recovered CPI from a extraction that has been passed through an ultrafiltration membrane and diluted the concentrated retentate in chilled water to form a protein micellar mass (PMM) (Barker, Martens, & Murray, 2010).

Pulses and special crops may be Canada's most rapidly growing industry for which production increased from 1 million tonnes in 1990s to 5.7 million tonnes in 2010 (Pulse Canada, 2014a). Canada is the world's top pea and lentil producer accounting for 32 and 38.5 % of world production, respectively. Also as an active exporter, Canada exported a record 4.7 million tonnes of pulses worth nearly $ 2.7 billion in 2010 (Pulse Canada, 2014a). According to Codex Alimentarius (first edition) which is published by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) in 2007, pulses are defined as "dry seeds of leguminous plant which are distinguished from leguminous oil seeds by their low fat content". Major pulse crops include beans, lentils, peas, chickpeas, field beans and cow peas.

Pulses contain high amounts of protein ranging from 17-30% (~40g/100g dry matter) which is twice the amount of protein in whole grain cereals such as wheat, oats, barley and rice (Boye, Zare, and Pletch, 2010). Pulses contain a higher amount

of lysine (6-8 times of wheat) but are low in sulphur containing amino acids (methionine and cysteine) and tryptophan. This makes inclusion of pulses in traditional wheat products (which are high in sulphur amino acids and tryptophan) more reasonable and outstanding from a nutritional perspective.

Pulse protein can be separated or extracted using a wide range of methods based on density differences or solubility differences in water, salt, alkaline and acidic solutions (Tiwari & Singh, 2012). After being milled, finer flour particles, which are rich in proteins, can be separated from a heavier coarse fraction, which rich in starch, using air classification. Water is used to extract water-soluble proteins (albumin). When salt is used, protein 'salting in' occurs at low salt concentration due to formation of ionic layers on charged groups; at higher concentration of salt (e.g., ammonium sulfate), protein tends to "salting out' as a result of decreased availability of water to solubilize proteins. When milled flour is extracted at acidic or alkaline pH values, acidic precipitation or hydrocyclones can be applied to separate extracted proteins. Based on the density difference, hydrocyclones can be used to separate the protein-rich fractions (lighter overflow with protein content of 64.02 - 88.31 %) from the starch rich fractions (heavy underflow with starch content of 89.8 - 99.7 %) (Tiwari & Singh, 2012).

After plant proteins have been isolated, their utilization in food systems is not that simple. The major factors restricting the use of plant protein materials include presence of anti-nutritional and undesirable factors, limited ability to impart structure and desired functionality, high processing cost, unappealing color as well as

unpleasant taste and off-flavour issues, including saponin induced-bitterness and a beany flavour (Heng et al., 2006). Therefore, continuous research is still required to optimize the use of plant protein materials in various food systems.

2.2 Flavour compounds and their application

According to Leffingwell & Associates (2014), the worldwide flavour and fragrance market was worth $24 billion in 2013. This value was anticipated to exceed $30 billion in 2017 with an annual growth rate of 5.6 % (BCC Research, 2012). The major sales of flavours consist of flavour blends/compounds (41 %), fragrance blends/compounds (35 %), aroma chemicals (12 %) and essential oils (12 %) (Short, 2002). In 2013, beverages (34 %), dairy (13 %) and savory (10 %) products accounted for the greatest sales of flavours with the Asia-Pacific region appearing to be the biggest market (32 %), followed by North America (23 %) and Western Europe (20 %) (IAL Consultants, 2014). Some of the largest producers of flavours and fragrances include Givaudan (20.3 %), Firmenich (13.8 %), IFF (12.3 %), Symrise (10.0 %), Takasago (6.0 %) and Wide Flavors (4.8 %) which shared 67.2 % of the total sales in 2013 (Leffingwell & Associates, 2014).

Up to 10,000 different flavour molecules have been found in foods and beverages with around 2000-3000 flavour chemicals being used commercially (Cheetham, 2002). Usually, at least 20 to 50 (another view says 10 to 30) are routinely used to flavour food or beverage products (Cheetham, 2002; Reineccius, 2006b). Productions of these flavour chemicals can come from a wide variety of methods including Maillard reaction, roasting, enzyme catalyzed reaction, fermentation, pyrolysis and

biotechnology (Reineccius, 2006a).

Properties of flavour compounds and their behaviors in diverse food environments are dependent upon the chemical nature of the flavour molecules. Most flavours rely on one or more functional groups for them to be active (Cheetham, 2002). Fundamental classes of flavour compounds comprise hydrocarbons, carboxylic acids, acetals, alcohols, carbonyls (aldehydes and ketones), esters, ethers, heterocyclic compounds, lactones, phenols as well as nitrogen and sulfur-containing compounds (Reineccius, 2006a).

In practice, the concentration of a flavour molecule must be above its odor threshold to be perceived as an aromatic compound. As flavour thresholds can be very small, the final concentrations of a single flavour chemical in the end food or beverage product usually range between 0.01-0.05g/kg.

Either in liquid or powdered form, flavour compounds can be classified into a number of major types. Major flavour forms and their related application have been summarized in Table 2.1 based on Wright (2002).

When formulating and creating flavours, flavour compounds can be used alone or together with other flavour compounds to represent primary or secondary aroma characteristics (Wright, 2002). Some compounds contribute to the basic skeleton of the flavours, known as primary character of flavours, such as benzaldehyde for cherry flavours while others are beneficial for an optional secondary character such as ‘leaf green’ (*cis*-3-hexenol) in strawberries (Cheetham, 2002).

Table 2.1 Summary of major flavour forms and related application (Wright, 2002)

Major flavour forms	Applications
Water-soluble liquid flavours	Most common type of flavours used. Usually used in propylene glycol, ethanol or triacetin as simple solvent.
Oil-soluble liquid flavours	Suitable for end product which is fat or oil or which cannot tolerate water such as chocolate.
Dispersed flavours	Powered form flavours are dispersed or spread on a carrier before other ingredients are mixed in.
Spray-dried flavours	Flavours are emulsified in an aqueous gum solution followed by spray-drying. Powered flavours, prefer to be used in dry mixing and are unsusceptible to evaporation and oxidation.

All flavours used in end products require some consideration of what is needed to produce finished foods; these can be categorized into ingredients, processing, storage and consumption factors (Wright, 2002). Of the ingredients, fat content is considered to be the most crucial. Due to variations in flavour compounds with respect to fat solubility, retention or release of flavour comounds could be greatly dependent on solubility in fat (Wright, 2002). The most important processing factor by far is heat which usually leads to loss of volatiles or chemical changes in flavours. If the key components of flavours varied in boiling points, heat could cause the flavour to become unrecognizable (Wright, 2002). The storage factor is frequently related to the stability of flavours. Oxidation and migration of flavour through or into food packaging materials are two major factors responsible for changes in flavour during storage. Both factors could cause detectable loss of flavours and off-notes can be produced from flavour oxidation. For consumption factors, caution needs to be taken in terms of the temperature at which the food is consumed. The release of more volatile chemicals at higher temperature makes them harder to detect compared with the foods consumed cold.

Overall, understanding flavours and their application are very complex procedures that require knowledge, practice and expertise in both flavour and the food in which they are used.

2.3 Flavour-food matrix interaction and flavour perception

2.3.1 Release and perception of flavour compounds

Flavour perception is a multi-factor experience which involves olfactory (aroma), gustatory (taste), texture, mouthfeel and the integration of all of these senses (van Ruth & Roozen, 2002). It takes place when stimulants from foods contact the receptor cells which are located in the mucous membranes of the nose and mouth as well as breaking down the food structure during mastication (texture) or contacting with mouth lining to elicit heat or cooling sensations (mouth feel) (Taylor, 1996). Among the different sensory and flavour attributes, aroma or smell is the most important component of perceived flavour and has been considered to be closely related to consumers' purchase intent and acceptance of foods (O'Neill, 1996; Preininger, 2006).

Practically, aromatic compounds or odorants need to vaporize into the gaseous phase prior to entering the nasal cavity in order to be perceived (Preininger, 2006). In other words, the perceived aroma intensity is greatly dependent upon the amount of aroma compounds released from food matrices. Fig. 2.1 illustrates the potential factors that may affect the release and perception of aromas.

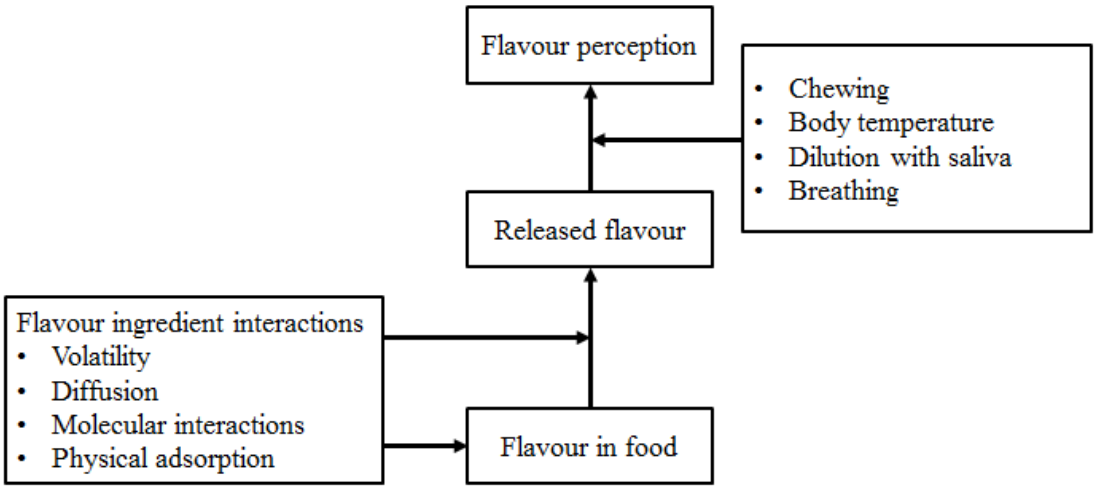


Figure 2.1 Factors impacting flavour release and perception
(Adapted from Preininger, M. (2006))

Thermodynamic and kinetic factors are two major aspects that have been noted to control the rate of flavour release from food matrices (Guichard, 2011; van Ruth & Roozen, 2002). At a static equilibrium state, flavour phase partitioning, volatility, and binding with ingredients as well as the interactions among these factors mainly contribute to the thermodynamic aspect of flavour delivery. Gas/product partition coefficient (K), defined as the proportion of flavor in the air (C_g) and product (C_p) phase at equilibrium, has been utilized to provide quantitative information about volatility and phase partitioning behaviours of flavours.

The kinetic aspect of flavour delivery focuses on determining the rate required for flavour to reach equilibrium under dynamic conditions (van Ruth & Roozen, 2002). As foods, especially solid food, are rapidly surrounded by a thin layer of saliva after being ingested, the interfacial mass transfer or diffusion of flavour compounds involves a three-phase partitioning between food, interface (i.e., saliva) and gas phase. Such a transfer is driven by the concentration difference of flavour compound

between the product and gas phases with the diffusion rate being controlled by the mass transfer coefficient (*k*) of flavour compound in each phase as well as concentration gradients (van Ruth & Roozen, 2002).

2.3.2 Types of binding between flavour and major food constituents

Foods are complex systems due to their diverse chemical composition and structure. The major food components, such as proteins, lipids, and carbohydrates, are known to interact with aromatic compounds, directly affecting their release, thus, the perception of flavours (Guichard, 2000, 2002, 2006, 2011; Preininger, 2006). Understanding the behaviour of aromatic compounds in food matrices and the strength and nature of the interactions between major food ingredients and aromatic compounds are therefore becoming particularly important for improving overall sensory profile (aroma) and quality of food products (Guichard, 2011).

In general, the interaction is directly influenced by the nature of the aromatic compounds and food matrices. van Ruth and Roozen (2002) categorized the types of binding between flavour substances and major food ingredients into physical trappings and molecular interactions. Physical trappings involve adsorption of flavour compounds onto inner or outer surface of dry food particles as well as absorption or "dissolution" of flavour compounds in food particles. Molecular interactions are composed of covalent chemical bindings as well as non-covalent physicochemical bindings including van der Waals forces, hydrogen bonds, hydrophobic interactions and ionic bonds (Kim and Min, 1989; Solms, Osman-Ismail, & Beyeler, 1973). It is important to address that when discriminating bound, dissolved and total flavour

concentrations, only the free dissolved unbound flavour molecules possess a vapor pressure that makes them of great interest in flavour research (de Roos, 2000).

It has been generally recognized that fat plays a more important role than proteins and carbohydrates in flavour partitioning as aromatic compounds differ in polarity, thus, in fat solubility (Guichard, 2011). This effect was more pronounced for hydrophobic aromatic compounds than the hydrophilic ones. For instance, non-polar hydrophobic compounds such as esters possessed significantly lower partition coefficients in oil and oil/water emulsions than the polar compound such as diacetyl (Guichard, 2002; van Ruth et al., 2002).

Carbohydrate matrices possess a diverse capacity to interact with volatile flavour compounds due to their different levels of hierarchy varying from simple sugars to very complex polysaccharides (Naknean & Meenune, 2010). Small sugar molecules or sweeteners affect flavour partitioning indirectly via binding with water molecules, leading flavours to be concentrated in the remaining available water which favours their release (Hansson, Andersson & Leufven, 2001; King et al., 2006; Lubbers & Guichard, 2003). Cyclic oligosaccharides especially cyclodextrin and polysaccharides such as starch, gum and pectin substances are famous for their ability to form inclusion complexes with aromatic compounds; therefore, they are recognized as good candidates for flavour carriers and materials for flavour encapsulation (Conde-Petit, Escher, & Nuessli, 2006; Ordonez & Herrera, 2014; Reineccius, Reineccius & Peppard, 2004).

Compared with flavours binding to lipids and carbohydrates, flavour-protein

interactions have been the most diverse due to the wide range of chemical structures the protein is able to provide, including varying amino acid side chains, terminal ends and hydrophobic pockets (Reineccius, 2006a). Reversible weak hydrophobic interactions, stronger ionic bonds, and irreversible covalent linkages have been reported between volatile flavour compounds and proteins (Zhou, Lee, & Cadwallader, 2006).

2.4 Protein binding with volatile flavour compounds

2.4.1 Source of proteins and flavours used in previous studies

For studies on the interactions of volatile flavour compounds with primarily animal and plant proteins for over 40 years (Damodaran & Kinsella, 1981a, 1981b; Gremli, 1974; Kúhn, Considine, & Singh, 2006), the major emphasis has been put on animal proteins especially milk proteins including β-Lactoglobulin (Guichard, 2000; O'Neill & Kinsella, 1987a), α-Lactalbumin (Kúhn et al., 2007), whey protein and caseins (Gkionakis et al., 2007; Kúhn et al., 2007; Kúhn, Considine, & Singh, 2008). Other animal proteins investigated include bovine serum albumin (Damodaran & Kinsella, 1980a, 1980b), fish actomyosin (Damodaran & Kinsella, 1983), ovalbumin (Ebeler, Pangborn, & Jennings, 1988), myoglobin (Gianelli, Flores, & Toldra, 2005), and pork muscles in dry-cured ham (Pérez-Juan, Flores, & Toldrá, 2006, 2007, 2008).

Compared with animal proteins, less emphasis has been put on plant or vegetable proteins including canola and pulse proteins. Soy proteins have been the most popular plant proteins used in flavour binding studies due to the presence of beany or grassy off-flavours from lipoxygenase-catalyzed oxidation of unsaturated fatty acids (Arora

and Damodaran, 2010; Damodaran & Kinsella, 1981a, 1981b; Gremli, 1974; O' Keefe et al., 1991a, 1991b). Pulse proteins are rarely studied; only pea protein (Dumont & land, 1986; Heng et al., 2004) and fababean protein (Ng, Hoehn, & Bushuk, 1989a, 1989b) have been evaluated. There have been no data reported for canola protein. With an increase of incorporation of plant protein materials in traditional and novel foods, more investigations are required to look at the way these proteins interact with volatile flavour compounds.

From a practical perspective, any volatile flavours that have been utilized in food applications should be evaluated. The principal volatile flavour compounds which have been studied are variable but mainly involve hydrocarbons (alkanes), carboxylic acid, unsaturated and saturated aldehydes and ketones, alcohols, esters, lactones, and phenols (Aspelund & Wilson, 1983; Li, Grun, & Fernando, 2000). A homologous series of flavours within the same class has been used to reveal the nature of binding (Guichard & Langourieux, 2000; Gkionakis et al., 2007). Typically, the particular flavour being selected should be a good representative in its chemical class and widely used in the flavour and food industry.

2.4.2 Methodology involved in analyzing flavour binding by proteins

Both instrumental and sensory analyses have been used to analyze flavour binding with the priority being given to the instrumental methods (Kúhn, Considine, & Singh, 2006; Ng, Hoehn, & Bushuk, 1989b). Among instrumental techniques, approaches being applied include static headspace-GC (Aspelund & Wilson, 1983; O'Keefe et al., 1991a, 1991b), equilibrium dialysis (Damodaran & Kinsella, 1981a,

1981b), solid-phase microextraction (Fabre, Aubry, & Guichard, 2002; Kúhn et al., 2007, 2008; Gkionakis et al., 2007), high performance liquid chromatography (Ng, Hoehn, & Bushuk, 1989a; Li, Grun, & Fernando, 2000), fluorimetry (O'Neill & Kinsella, 1987b) and NMR (Jung et al., 2002; Tavel, Guichard, & Moreau, 2008).

2.4.2.1 Flavour recovery and isolation

As a wide range of techniques for aroma isolation exist, proper selection of flavour recovery and isolation methods become critical for isolating and recovering volatiles that are of particular interest (Reineccius, 2002). Table 2.2 summarizes the potential flavour isolation techniques that have been used (Reineccius, 2002; 2006b). It must be kept in mind that there is no universal flavour isolation method that can yield an accurate analytical profile for all foods. Each technique will provide a unique view of the volatiles with some degree of bias (Leahy & Reineccius, 1984; Reineccius, 1993).

Considering the relationships between protein and flavours, static headspace has been the most adopted aroma recovering/isolation method for quantifying flavours; it is based on the direct analysis of the equilibrium headspace above a food product and the inherent volatility of an aroma compound (Reineccius, 2002). However, due to direct headspace injection volumes and detection limits of GC and mass spectrometry, flavour compounds with low volatility can be hard to partition into the headspace resulting in a poor sensitivity (Reineccius, 2006b).

Table 2.2 Strengths and weakness of the major flavour isolation techniques

Flavour isolation method	Strengths	Weaknesses
Static headspace analysis	Isolate most volatile and most abundant aroma compounds. Easily automated, precise, allows analysis of great number of samples.	Not useful if volatiles of interest are present in trace amounts or have very low vapor pressure/volatility. Headspace enrichment by distillation or addition of salts may be needed.
Purge and trap (dynamic headspace method)	Obtain less volatile and abundant constituents. Stable over time.	Weaknesses can result from the selection of trapping system. A *cryogenic trap* will trap water causing a primary problem. A *Tenax trap* has a low adsorption capacity but has a high affinity for nonpolar compounds and a low affinity for polar compounds. *Charcoal traps* make it hard to release aromatic components.
Solvent extraction	Most efficient and simplest method for aroma isolation. Obtain least volatile aroma compounds. Stable over time.	Food cannot contain lipids. Solvent must be of highest purity. Biases can be introduced due to the inherent solubility of various aroma compounds in different solvents (e.g., pentane, dichloromethane or ether).
High-vacuum (molecular) distillation	Can be applied to pure fats or oils, solvent extracts of fat-containing foods or aqueous-based foods. Use product moisture to co-distill volatiles.	As water is a major part of the distillate, a secondary extraction is required. The distortion of aroma profile can come from distillation and subsequent solvent extraction.
Simultaneous distillation/solvent extraction (Likens-Nickerson)	A liquid concentrated isolate is obtained which contains nearly all the volatiles in foods. Medium and high boilers are recovered well. The obtained liquid concentrate assists mass spectrometric work and further analysis. Stable over time.	Tedious, labor intensive, limited to 1-2 samples/day. Volatile profile obtained is dependent upon the volatility of aroma compounds during initial distillation, solubility during solvent extraction of distillate and volatility again when the solvent extract is concentrated. Flavour profile may not represent the true profile of food.
Solid phase microextraction	Simple, sensitive, has been automated and rapid, no solvent contamination.	Competition of flavour compounds could exist on the fibre coating creating biases in the quantitative analysis. Adsorbents may deteriorate over time and use, isolated volatile profile may change.

[1] Summarized based on Reineccius (2002, 2006b)

A purge and trap system or dynamic headspace method can be applied to deal with aroma compounds with low volatility (Reineccius, 2002). In dynamic headspace sampling, an inert gas such as nitrogen or helium is used to purge the sample. The striped aromas are subsequently captured in the trapping system which is cryogenic, or contains Tenax or charcoal (Reineccius, 2002).

More popular, solid phase micro-extraction (SPME) has been used a flavour recovery method which combines sampling, extraction, concentration, and sample introduction in a single step (Zhang & Pawliszyn, 1993). It was developed in 1990's by Pawliszyn and co-workers (Arthur & Pawliszyn, 1990) and has been extensively used as a fast and appropriate extraction method in quantifying flavour binding with proteins (Fabre, Aubry, & Guichard, 2002; Kúhn et al., 2007; Kuhn, Considine, and Singh, 2008; Gkionakis et al., 2007). Typically, a food sample is placed in a sealed vessel to equilibrate; whereafter an inert fused silica fiber coated with an adsorbent, usually carboxen-poly (dimethylsiloxane) (Adams et al., 2001), is then exposed to the headspace or immersed into the sample to adsorb volatiles (Yang & Peppard, 1994, Reineccius, 2006b). The 'loaded' fibre is then thermally desorbed in the GC injection port for further analysis. Figure 2.2 illustrates a schematic model of adsorption and desorption procedure involved in SPME process.

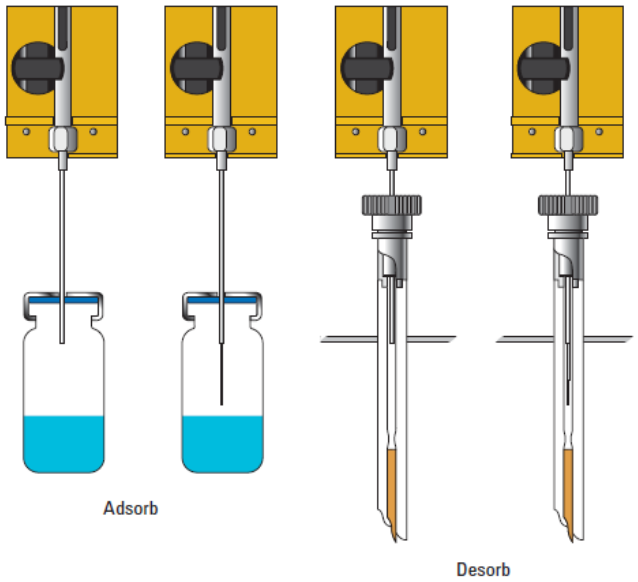


Figure 2.2 Schematic model of adsorption and desorption procedure involved in SPME process.
(adapted from Agilent Technologies, Inc., 2006, CTC automated sample injectors for gas chromatography, distributed by Agilent Technology, p.5)

Similar to SPME and other flavour enrichment methods, a relatively advanced flavour isolation technique adopted in the current analysis was the PAL COMBI-*xt* ITEX-2 Option (ITEX: In-Tube-Extraction) (CTC Analytics AG, Switzerland). This method was based on the idea from SPME using an equilibrium system but applying the flavour isolation procedure in a dynamic headspace manner with a *Tenax* trap, also called *In-Tube-Extraction*. In other words, it can be considered as an advanced automated dynamic headspace flavour isolation method. Figure 2.3 demonstrates the systemic flow of a PAL ITEX-2 cycle step by step. After the sample is shaken to equilibrium (Step 1), the analytes are adsorbed (loaded) onto the sorbent trap located within a side-hole needle attached to a gas-tight syringe (Step 2). By moving the syringe plunger up and down, the gas phase above the sample are pumped (‘purged’) through the sorbent trap inside the needle (dynamic headspace); consequently, loading

of the analyte takes place. Afterwards, analytes are thermally desorbed into the GC injection port (Step 3). The trap material is cleaned in a trap cleaning program between each sampling (Step 4).

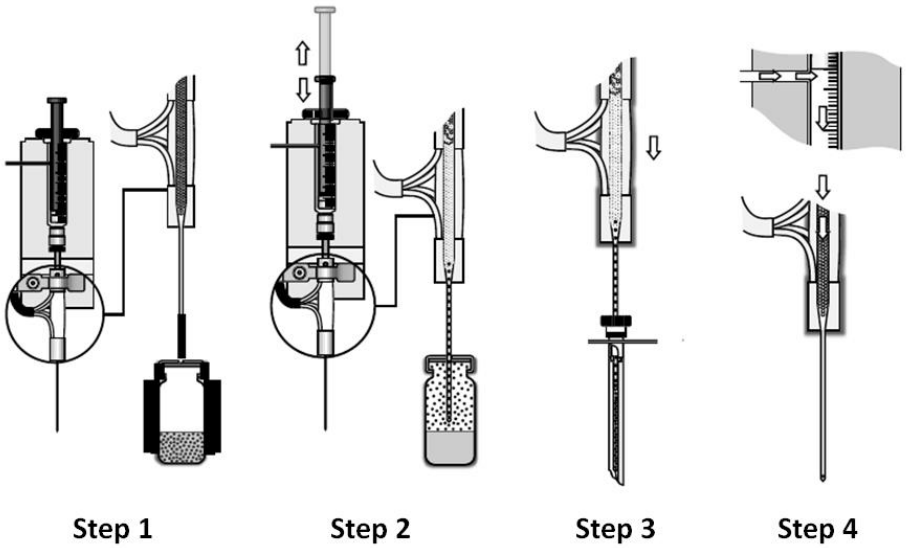


Figure 2.3 Step by step illustration of PAL ITEX-2 cycle during isolation and desorption of aroma compounds
(adapted from CTC Analysis AG (2013), 4th edition, Addendum to PAL User Manual PAL ITEX-2 Option Installation and Operation, p40-41)

By using this advanced 'dynamic headspace' extraction method, one is able to concentrate the analyte from the headspace in the *Tenax* trap, thereby increasing the GC detection limit as compared to a static headspace method. A representative profile of headspace above the sample can be obtained without competition of flavours on an adsorbent trap. It is suggested in the user manual that to reach the detection limit of a ppm to ppb concentration, three to twenty strokes are recommended, while 40 to 60 pumping strokes may be applied if the concentration is aimed at the ppt level. In a practical application, the number of strokes should be kept constant throughout the experiment.

2.4.2.2 Flavour quantification

Gas chromatography (GC) has been the standard and single most widely used detection method in flavour analysis (Reineccius, 2002). It is able to separate the volatile flavours captured in the headspace above the sample and create a chromatogram in which peak area has a linear relationship with the concentration of volatile in the headspace while retention time of each peak helps identify a single volatile or multiple volatiles with the same boiling point.

In flavour protein binding analyses, the concentration of free ligand in an aqueous solution is proportional to the concentration of volatiles in the headspace. When flavour binds to the protein in the aqueous solution, the change in concentration of volatiles in the headspace results; this can be monitored by a reduction of the peak area in the chromatogram (Gkionakis et al., 2007). Therefore, the two parameters of most interest are the percentage of flavour bound and moles of ligand bound per mole of protein.

When connected with a mass spectrometer (MS), the identity of separate flavours can be confirmed or further structural information of these volatiles can be provided (Reineccius, 2002). In addition, mass spectrometer can recognize the potential volatile byproducts generated (Kúhn, Considine, and Singh, 2008).

A unique and alternative method to characterize the isolated volatile compounds is to use a human nose; in this GC-Olfactometry or GC-O method, people are linked to instrument (Reineccius, 2006a, 2006b). This method has been widely used to identify the key aroma components that contribute to the sensory properties of foods

(Blank, 1997; Leland et al., 2001). After isolated volatiles are separated by GC, humans assess the potential importance of individual volatile compounds and their likelihood to contribute to sensory quality. The chromatogram derived from odour profiling is called an ‘aromagram’. Ideally, a well-trained personal is able to tell the potency (sensory intensity), character and even the chemical identity of a GC peak (Reineccius, 2002).

Recently, NMR Spectroscopy has been used to study flavour release and perception (Tavel, Guichard, & Moreau, 2008). It is known as an efficient tool to characterize the mechanisms of aroma-macromoleculr interactions at a molecular level. The most commonly used methods include monitoring the changes in NMR parameters between spectra for free macromolecules or flavour in flavour-macromolecule complexes; parameters measured include chemical shifts, relaxation rates and diffusion coefficients (Jung et al., 2002). Two types of diffusion-based NMR techniques were adapted by Dr. Ebeler’s group in UC-Davis to investigate the bindng of flavour comounds with bovine serum albumin (Jung et al., 2002). By monitoring changes in apparent diffusion coefficient as a function of BSA/flavour ratio, binding constant (affinity) and binding stoichiometry were obtained using a pulsed field gradient NMR mtheod. In addition, a diffusion-based nuclear Overhauser effect method was able to screen the flavour compounds and identify those which possessed binding affinity to proteins. Both of the above methods exhibit great potential for using NMR techniques to quantify flavour binding to proteins.

2.4.3 Methodology involved in monitoring structural change of proteins during flavour binding

Another methodology that can be used in monitoring protein-flavour binding is to follow the potential change in protein conformation as a result of interaction with flavour. Theoretically, any method that is suitable to monitor protein conformation can be utilized; these include differential scanning calorimetry and fluorimetry.

2.4.3.1 Differential Scanning Calorimetry (DSC)

DSC is one of the classical methods to evaluate denaturation of plant proteins (Arntfield & Murray, 1981; Léger & Arntfield, 1993). The measuring principle is to compare the rate of heat flow of a sample to an inert material which are heated and cooled at the same rate. In other words, it measures the energy required to sustain a constant temperature between an inert reference material and a studied substance (Tromelin, Andriot, & Guichard, 2006). As a result, changes in the samples that are associated with absorption, release or evolution or heat lead to a change in the differential heat flow which is then recorded as a peak (Biliaderis, 1983). It is known that the area under the peak indicates the enthalpic change in the heating or cooling phases and its direction indicates whether the thermal event is endothermic or exothermic.

When following protein thermal denaturation, ΔH represents the amount of energy required to completely denature a protein molecule; therefore, it provides crucial information about protein structural changes. On the other hand, denaturation temperature (T_d) indicates the temperature required to denature a protein molecule.

This method has been successfully used to evaluate some processing parameters such as pH, salts, chemical reagents to the structure and denaturation of proteins (Arntfield, Ismond, and Murray, 1990). In this way, the structural changes in plant proteins relating to binding with small molecular weight flavour compounds could be studied by differential scanning calorimetry which measures the thermal transitions of native and denatured proteins.

2.4.3.2 Fluorimetry

Spectrofluorometric methods have been extensively used in protein-ligand binding studies (Damodaran & Kinsella, 1980a; Dufour & Haertlé, 1990; Liu et al., 2005; Meynier et al., 2004; Muresan, van der Bent, & der Wolf, 2001). The wavelength shifts and changes in the intensity of the fluorescence emission peak of tryptophan residues have been used to monitor the environmental changes associated with these residues (aromatic amino acids: tryptophan, tyrosine and phenylalanine) in proteins, thereby providing information on protein structure (Cho, Batt, & Sawyer, 1994).

Damodaran and Kinsella (1980b) detected structural changes in BSA upon ligand binding based on both UV absorption and fluorescence emission behaviour. The peak of the BSA emission spectrum in fluorescence gradually shifted from 287 to 292 nm and was accompanied by a gradual decrease in maximum fluorescence intensity when molar ratios of binding increased from 1.1 to 7.0 for 2-nonanone. Liu et al. (2005) also observed decreased fluorescence intensity of whey protein concentrate with an increase in concentrations of benzaldehyde and methyl ketones (0 to 2.5 μM). The

authors explained that the exposure of previously buried tryptophan residues to the surrounding polar solvent (water) as a result of conformational change (unfolding) in protein due to flavour binding induced this quenching effect (Damodaran & Kinsella, 1980b).

2.4.4 Current understanding of protein-flavour binding mechanisms

The affinity of a flavour compound to protein can be considered as a multi-factor function related to the protein amino acid profile, protein overall conformation and stereochemistry of the flavour compound. Hence, no universal mechanisms to explain protein-flavour bindings have been attained. In other words, different aroma compounds, based on their chemical structure and functional group, could possess different binding affinities to proteins due to the nature and type of interactions involved. As a flavour compound could possess multiple functional groups which react differently with proteins (such as vanillin containing benzaldehyde, hydroxyl and ether groups), this could make the nature of interaction more complicated. As a result, when a complex flavour formulation is used in the presence of protein, the resulting flavour profile can be easily imbalanced and become very hard to predict and control. Figure 2.4 illustrates the potential opportunities for flavours to interact with protein molecules.

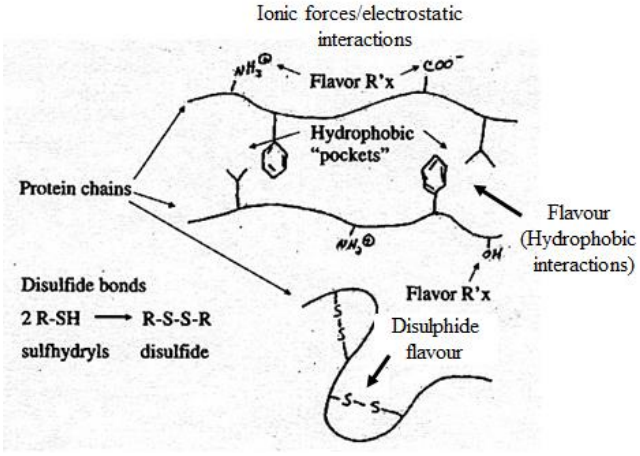


Figure 2.4 Opportunities for flavour to interact with protein molecules
(Schematic model adapted and modified from Reineccius (2006a))

In general, most flavour chemicals bind reversibly with protein via hydrophobic interactions with its non-polar interior region due to the presence of a non-polar (aliphatic) segment in the flavour compounds (Fig. 2.5).

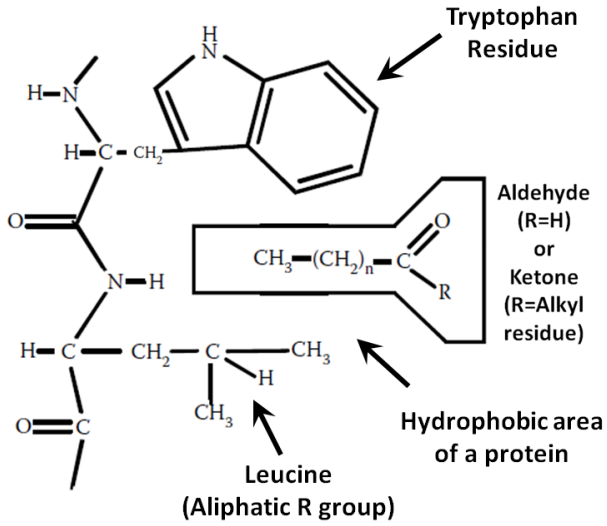


Figure 2.5 Illustration of protein hydrophobic interactions with aldehydes and ketones
(Schematic model adapted and modified from Matheis (1993))

In this situation, any factors that modify the conformation of proteins, thereby exposing or disrupting the interior hydrophobic area, would alter protein-flavour binding affinities. In addition to hydrophobic associations, Chung and Villota (1989) attributed retention of alcohols by soy proteins to hydrogen bonds as n-butanol

showed a higher binding constant than long chain n-hexanol indicating involvement of the secondary binding mechanism. Zhou, Lee and Cadwallader (2006) deduced that the greater binding of butyric acid with soy protein compared to the corresponding aldehyde and ketone, resulted from the ionic bonds between volatile acid and charged/polar groups on soy proteins.

Table 2.3 summarizes the current knowledge on binding mechanisms between protein and flavours. Compared with physicochemical interactions via non-covalent forces, chemical bonding via covalent linkages is more specific and associated with specific groups on proteins (e.g., $-NH_2$, -SS-, and -SH) (van Ruth and Rozzen, 2002; Reineccius, 2006a). These interactions could lead to the formation of salts, amides, esters, and aldols when reacting with proteins via amino ($-NH_2$) and sulfhydryl (-SH) groups (Mills & Solms, 1984; van Ruth and Roozen, 2002). One of the most famous examples is Schiff-base formation between aldehyde and ε-amino group of lysine residue (lys-NH_2 + R-CHO → lys-N=CH-R + H_2O) (Preininger, 2006).

Table 2.3 Current understanding of protein-flavour binding mechanisms

Type of interactions	Secondary molecular interactions	Regions or groups of proteins involved	Reversibility	Example of flavours
Physicochemical interactions	Hydrophobic interactions	Interior hydrophobic area of proteins	Reversible	Ketones[1, 2, 3], aldehydes[2], alcohols[4], ester[5, 6]
	Hydrogen bonds	-OH, -COOH, -SH	Reversible	Aliphatic alcohols[4, 7], lactone[8], volatile acids[8]
	Ionic bonds/ electrostatic linkages	$-NH_2$, -OH	Reversible	Volatile acid[8, 9]
	van der Waals forces		Reversible	Hydrocarbons[7, 10]
Chemical bondings	Covalent linkages	-S-S-, -SH,$-NH_2$	Irreversible	Aldehydes[11], vanillin[12], sulphur containing flavours[13]

[1] Damodaran & Kinsella (1981a);
[2] Gremli (1974);
[3] Andriot et al. (1999);
[4] Chung & Villota (1989);
[5] Landy, Druaux, & Voilley (1995);
[6] Pelletier, Sostmann, & Guichard (1998);
[7] Aspelund & Wilson (1983);
[8] Zhou, Lee, & Cadwallader (2006);
[9] Beyeler & Solms (1974);
[10] Plug & Haring (1994);
[11] Mills & Solms (1984);
[12] Hansen & Heinis (1991);
[13] Mottram & Nobrega (2000).

When sulfur-containing aroma compounds are involved, the potent disulfides contributing to meat flavour could interact with sulfhydryl and disulfide groups on proteins leading to a loss of disulfide aroma accompanied by the formation of the corresponding thiols (Mottram et al., 1996; Mottram & Nobrega, 2000). As a

consequence, intensity (potency) and character of the desired aroma profile can be altered. A higher number of sulfhydryl groups from cysteine and disulfide groups from cystine in proteins could also promote changes which result from sulfhydryl disulfide interchange reaction (R-S-S-R' + Protein-SH → R-SH + Protein-S-S-R').

Greater flavour binding has always been noted when covalent bonds were involved (Gremli, 1974). Aldehyde flavours which bind by partly reversible and partly irreversible mechanisms with proteins have been found to be retained by proteins at levels 2-5 times higher than ketones, whose binding only includes reversible non-covalent interactions (Heng et al., 2004).

From a thermodynamic aspect, the negative change in free energy ($\Delta G = -RT \ln K$) indicates that the binding is thermodynamically favorable and spontaneous (Damodaran and Kinsella, 1981a; Li, Grun, and Fernando, 2000). A higher (intrinsic) binding constant (K_b) and lower free energy (ΔG) result in higher binding affinity and increased flavour retention.

2.4.5 Factors influencing protein-flavour interactions

In addition to the mechanisms of binding, the interactions between protein and flavours are affected by the intrinsic properties of proteins and flavours and how these parameters react to environmental factors (extrinsic factors) such as temperature, pH, nature and concentration of salts as well as protein modification.

2.4.5.1 Protein source and concentration

Systematic comparison between proteins from different plant sources and their flavour binding capacities is limited. One report has shown that flavour binding

capacity decreased in the order: soy protein > gelatin > ovalbumin > casein > corn (Reineccius, 2006a). Based on the fact that covalent interactions were more effective than non-covalent associations, proteins containing a higher level of lysine, arginine and cysteine content could potentially exhibit higher flavour binding capacities if covalent bonds are involved.

Different protein fractions have been shown to have different binding affinities to different flavours. O'Keefe et al. (1991a, 1991b) studied the binding of soy glycinin and β-conglycinin to a series of aldehydes, ketones, alcohols, and hexane and found out that soy glycinin exhibited higher overall binding capacities to all flavours than soy β-conglycinin. The authors determined that different flavour binding patterns could be attributed to the different intrinsic molecular structure of the soy protein fractions.

Increasing protein concentrations increased the percentage of total flavour bound (Dumont & Land, 1986; Ng, Hoehn, and Bushuk, 1989a). Land (1994) found that percentage decrease in flavour headspace concentration was enhanced with an increase of the percentage added proteins, irrespective of the type of proteins and flavours involved. Similar greater bindings of methyl ketones and ester flavours were seen with increasing β-lactoglobulin and sodium caseinate concentrations, respectively (Andriot et al., 1999; Landy, Druaux, & Voillley, 1995).

2.4.5.2 Steochemistry of flavour compound and concentration

With respect to the structure of flavour molecules in different chemical classes, aldehydes usually possess a higher binding affinity to proteins than the corresponding

ketones (Heng et al., 2004). Conversely, Zhou, Lee and Cadwallader (2006) compared adsorption of diacetyl, hexanal, γ-butyrolactone and butyric acid to soy protein-based crackers and rated the relative binding strength as follows: acid > lactone > diketone > aldehyde. The potential involvement of ionic bonds between polar flavour compounds and soy proteins and the presence of two interaction centers (carbonyl oxygens) in diacetyl may account for greater adsorption when compared to hexanal.

Within the same chemical class of flavours, greater degree of flavour retention or higher value of binding constants usually resulted from increasing aliphatic chain length of the flavour molecules. Such flavours include aldehydes (Heng et al., 2004; O'Keefe et al., 1991a), ketones (Andriot et al., 1999; Damodaran and Kinsella, 1981a; O'Keefe et al., 1991a), alcohols (Chung & Villota, 1989) and esters (Landy, Druaux, & Voilley, 1995; Pelletier, Sostmann, & Guichard, 1998). It was found that a positive correlation existed between the hydrophobicity of ligands (within same class) and resulting flavour binding affinities further inferring the presence of hydrophobic interactions (Guichard, 2002).

Damodaran and Kinsella (1981a) found that moving the keto group to the middle of carbonyls decreased the binding constant. This was possibly due to the steric hindrance of the keto group limiting access to the binding sites necessary for hydrophobic interactions. Soy proteins provided a stronger binding capacity with unsaturated aldehydes than saturated ones (Gremli, 1974; Kühn, Considine, & Singh, 2008). A similar phenomenon was partially observed in the retention of unsaturated ketones.

In terms of the effect of flavour concentration on flavour binding, it was shown that with increase of flavour concentration, the total flavours bound increased but the percentage of flavours bound remained constant (Dumont & Land, 1986; Ng, Hoehn, & Bushuk, 1989a).

2.4.5.3 Heat treatment

Heat treatments usually result in denaturation of proteins when temperatures are high enough. Upon heating, unfolding of protein may increase the chance of flavour binding by exposing the interior binding sites or hydrophobic cores that were buried previously (Chung and Villota, 1989; Ng, Hoehn, & Bushuk, 1989a). In contrast, the aggregation of unfolded protein molecules upon heating could liberate bound flavours, thereby decreasing the protein flavour binding capacity (Kúhn, Considine, and Singh, 2006). However, the quaternary structure of proteins (e.g. presence of disulfide bonds) and their resistance to heat denaturation must not be overlooked (Damodaran and Kinsella, 1981a). These may explain the conflicting results in the literatures regarding the effect of heat on flavour-protein interactions (Gkionakis et al., 2007; Kühn, Considine, & Singh, 2008; Ng, Hoehn, & Bushuk, 1989b; O'Neill & Kinsella, 1987a).

Kühn, Considine and Singh (2008) treated whey protein isolate with heat and high pressure and measured subsequent binding of trans-2-nonenal, 1-nonanal, and 2-nonanone. They found that binding, involving covalent irreversible bonds, was greater than binding via hydrophobic interactions. The results indicated that covalent interactions were enhanced upon heat denaturation, while hydrophobic interactions

were weakened.

It has been noted that previous studies on temperature effects in relation to flavour-protein interactions did not consider protein denaturation temperatures (Kúhn, Considine, and Singh, 2006). Most studies were based on a single temperature and heating time. Systematic studies of the development of interactions with respect to increasing heating temperature and time are essential to provide comprehensive information about protein-flavour binding behaviour during heating.

2.4.5.4 pH

Jouenne and Crouzet (2000) investigated the binding of three methyl ketones, three ethyl esters and two terpenes to β-lactoglobulin at pH values of 3, 6, 9 and 11. All flavours exhibited increasing affinity to protein as pH increased from 3 to 9, whereas a decrease of flavour binding was noted when the pH increased from 9 to 11. The authors proposed that the increased flavour retention could be attributed to a change in the structural flexibility of the protein promoting better access to the hydrophobic binding sites, while the decreased flavour retention at pH 11 was due to alkaline denaturation of protein. Weel et al. (2003) also reported that by varying the pH (pH values of 3, 5, 6, 7 or 9) of a mixture of aldehydes in an *in vivo* release test, a dramatic drop in aldehyde binding was noted between pH 7 to 9 for whey protein. Conversely, van Ruth and Villeneuve (2002) only observed increased binding of 20 aroma compounds (expect α-pinene and alcohols) to β-lactoglobulin when increasing the pH from 3 to 9. All of the above results indicated a considerable effect of pH on protein-flavour binding and suggested structural changes to the proteins at different

pH values may account for the differences in flavour retention (Tromelin, Andriot and Guichard, 2006). However, the mechanisms responsible for different flavour binding patterns with proteins at different pH values were not explicit.

2.4.5.5 Ionic strength and species

Ionic strength and salt type can also affect protein conformation which could modify the interactions between protein and flavour. Damodaran and Kinsella (1981c) found that affinity of 2-nonanone to bovine serum albumin increased due to the protein stabilizing/structuring effect of certain sodium salts. Increasing concentrations of sodium salts (Na_2SO_4 and NaCl) from zero to 4 M promoted binding of 2-nonanone. The opposite result was seen for Cl_3COONa as it destabilizes proteins. Pérez-Juan, Flores and Toldrá (2007) evaluated the influence of different chloride salts (NaCl, KCl, $CaCl_2$ and $MgCl_2$) on the volatility of different flavour compounds and resulting binding affinity to porcine protein. They reported that KCl and NaCl increased the volatiles in the headspace, while the same effect was not observed for $CaCl_2$ and $MgCl_2$. It was shown that NaCl and KCl reduced binding affinity of porcine proteins to branched aldehydes, hexanal and methional. No effect was noted for octanal and 2-pentanone.

Using NaCl only, Jouenne and Crouzet (1997) found that the retention of 2-octanone to β-lactoglobulin increased between 0 and 1 M NaCl. However, when NaCl concentration was increased from 0 to 1 M during the binding with limonene at pH 3, binding decreased was seen between 0 and 0.25 M and then it increased from 0.25 M to 1 M (Jouenne and Crouzet, 1997). Andriot et al. (1999) reported binding of

benzaldehyde to β-lactoglobulin decreased from 25% to 18% by adding 0.05 M NaCl to the water. Overall, it seems that some conflicting results have been reported with respect to the effects of salts and further investigation is required.

2.4.5.6 Effect of protein modification

As protein flavour interactions are greatly influenced by the structure of protein molecules, altering protein structure could modify the protein flavour binding capacity (Damodaran & Kinsella, 1981b). In particular, the release of the bound volatile compounds as a result of protein modification has been of interest in the development of efficient flavour delivery systems (O'Neill, 1996).

Few modifications of proteins have been conducted where the resulting effect on protein-flavour binding efficacy has been evaluated. Damodaran and Kinsella (1981b) treated soy protein with urea. With increasing urea concentration from zero to 5 M, the overall 2-nonanone binding affinity (*nK*) significantly decreased, accompanied by gradual disassociation of soy proteins as manifested by an increase in the wavelength of maximum (λ_{max}) fluorescence emission. Additionally, Damodaran and Kinsella (1981b) studied effect of succinylation on soy protein binding with 2-nonanone and found that the treatment destroyed half of the binding sites (from 4 to 2) and reduced the binding constant (K_b) from 930 M^{-1} to 850 M^{-1}. As the fluorescence emission peak shifted from 337 to 353 nm, destabilization of the protein's hydrophobic region with exposure of tryptophan residues to a more polar environment resulted in the removal of bound volatile compounds.

O'Neill (1996) reported that chemical modification of β-lactoglobulin by ethyl

esterification of protein carboxyl side chains and reduction of disulfide bonds with sodium sulfite lowered the apparent binding constant to 2-nonanone while forming a large number of binding sites. Interestingly, the increased turbidity and optical density of ethyl-esterified proteins inferred potential protein aggregation; this introduced the possibility of multiple binding mechanisms including physical adsorption as well as hydrophobic interactions. Dufour and Haertle (1990) found that compared with methyl esterified β-lactoglobulin, ethyl esterified β-lactoglobulin had a much lower apparent binding constant.

More recently, Suppavorasatit and Cadwallader (2012a) successfully demonstrated that enzymatic deamidation by glutaminase decreased the overall binding affinity of vanillin and maltol with soy proteins. As the ultimate goal is to recover the bound volatile compounds to restore a complete flavour profile of aroma compounds during food consumption, methods that were able to destabilize protein hydrophobic interior regions by removing or substituting protein reactive functional groups (e.g., free $-NH_2$ or -SH groups) warrant attention. More importantly, the potential to release the reversibly bound flavours would be of great benefit. However, the effect of chemical and enzymatic modifications on protein nutritional quality should not be overlooked. Several of the most popular non-enzymatic and enzymatic approaches to modify proteins with an emphasis on their potential to improve protein-flavour binding behaviours are summarized in Table 2.4.

Table 2.4 Effect of some chemical modifications of proteins and underlying mechanisms of treatments

Treatments	Underlying mechanisms	References
Acylation	Introduction of an acetyl (CH_3-CO-) or succinyl group (-CO-CH_2-CH_2-CO-) into protein lysine residues	El-Adawy, 2000; Gruener & Isomond, 1996;
Esterification	Block protein negative charge thus increasing the net positive charge and isoionic points of proteins	Dufour & Haertlé, 1990; Sitohy, Chobert & Haertlé, 2000
Alkylation	Adding aldehyde to amino, hydroxyl, and thiol groups of proteins and increase negative charge on the protein	Chobert et al., 1990; Sikorski, 2001
Enzymatic hydrolysis/ proteolysis	Cleave peptide bonds, destabilize protein secondary and tertiary structure, increase free amino and carboxyl groups concentration, and reduce protein molecular masses	Claver and Zhou, 2005; Liu et al., 2010
Deamidation	Alter secondary and tertiary structure of protein by removing amide group from glutamine	Suppavorasatit, De Mejia & Cadwallader, 2011

As the impact of these non-enzymatic and enzymatic protein modifications on protein-flavour binding have been rarely studied and systematic comparisons of these treatments are limited, it is of great interest to investigate their effects and select the most appropriate method as a pretreatment to proteins prior to the addition of flavours.

2.5 Influence of protein-flavour binding on protein functionality

Plant proteins are used in foods to impart functionalities. Limited information has been found on the impact of binding of flavours by proteins on protein functional properties, including thermal gelation properties. To the author's knowledge, only the impact of protein-flavour binding on foaming properties of whey proteins has been studied (Relkin & Vermersh, 2001).

2.5.1 Functionality of proteins

When considering protein functionality, not only nutritional and biological properties are of importance, but the molecular properties as relate to protein structure and function within a food must be included (Moure et al., 2006). Moure et al. (2006) classified the functionality of vegetable proteins into three major groups based on potential mechanisms. Protein hydration is mainly responsible for protein-water interaction, water retention capacity and solubility. Viscosity, thickening and gelation properties are closely related to protein structure and rheology. Finally, foaming capacity, emulsifying activity, and stability are related to protein surface.

2.5.2 Protein thermal gelation and flavour binding

Protein gelation has been one of the most important functionalities of plant proteins with respect to the protein structure and rheological characteristics (Moure et al., 2006; Sun & Arntfield, 2010). By using a range of methods, including heat treatments, protein sols can form gels due to an increase of molecular interactions. It is known that hydrophobic interactions and hydrogen bonding are major forces responsible for gelation of canola and pea protein (Léger & Arntfield, 1993; O' Kane et al., 2004). After the intermolecular linking between protein molecules reaches a stage where a continuous network is established, a gel with a high degree of crosslinking and three dimensional structure can be formed (Foegeding & Davis, 2011). A wide range of factors including temperature, pH, ionic strength, reducing agents, urea, and the presence of non-protein substances can influence protein gel network formation (Damodaran, 1988).

The impact of flavour binding on protein thermal gelation is of interest. As flavour-protein interactions are known to involve a wide range of linkages (Reineccius, 2006a), in thermally-induced protein gel formation with flavour added, the increased temperature could thermally disrupt the reversibly bound flavours which are linked via hydrophobic interactions, whereas the irreversible bound flavours such as covalently linked aldehydes will not be disrupted. In both situations, there is great potential to impact protein gel formation. Therefore, a connection between flavour-protein interaction and protein gel formation (protein-protein interaction) could exist. It is expected that protein-flavour interactions could give some insight into its potential effects on the protein gel formation and gel properties.

Chapter 3 Binding of Carbonyl Flavours to Canola, Pea and Wheat Proteins using GC/MS Approach

3.1 Abstract

Interactions of homologous aldehydes (hexanal, heptanal, and octanal) and ketones (2-hexanone, 2-heptanone, and 2-octanone) to salt and alkaline-extracted canola and pea proteins and commercial wheat gluten were studied using GC/MS. Long chain aldehyde flavours exhibited higher binding affinity regardless of protein type and isolation method. Salt-extracted canola protein isolates (CPIs) revealed the highest binding capacity to all aldehydes followed by wheat gluten and salt-extracted pea protein isolates (PPIs), while binding of ketone flavours decreased in the order: PPIs > wheat gluten > CPIs. Two aldolization products, 2-butyl-2-octenal and 2-pentyl-2-nonenal, were detected from the interactions between CPIs with hexanal and heptanal, respectively. Protein thermal behaviour in the presence of these compounds was analyzed by differential scanning calorimeter, where decreased ΔH inferred potential conformational changes due to partial denaturation of PPIs. Compared to ketones, aldehyde flavours possessed much higher "unfolding capacity" (lower ΔH), which accounted for their higher binding affinities.

3.2 Introduction

Having little flavour of their own, proteins are known to bind flavour compounds, leading to a decrease in flavour intensity (Tromelin, Andriot, & Guichard, 2006). Major investigations over 40 years have focused on evaluating potential binding mechanisms and effect of flavour composition and processing parameters on protein-flavour binding performance (Gremli, 1974; Kühn, Considine, & Singh, 2006, 2008). It has been agreed that protein-flavour interactions are mainly based on reversible hydrophobic interactions (Damodaran & Kinsella, 1981a, b). Other interactions responsible for binding involve reversible hydrogen bonding, Van der Waal's forces, ionic bonds and irreversible chemical binding via covalent linkages (Reineccius, 2006c; Tromelin, Andriot, & Guichard, 2006; van Ruth & Poozen, 2002).

Although various approaches have been used to elucidate the nature of these interactions, the mechanisms underlying the phenomena are still not explicit. A classical thermodynamic approach with headspace analysis has been mostly applied to evaluate the extent of the interaction (Kühn, Considine, & Singh, 2006). However, this methodology does not provide information on proteins' behaviour in mixed flavour systems. It is known that binding depends not only on the intrinsic properties of proteins, but also the conformational state of proteins is critical (Damodaran & Kinsella, 1980b; Damodaran & Kinsella, 1981b; Kim & Min, 1989; O'Neill & Kinsella, 1987b). In addition, structural changes to the proteins as binding occurs are not well-known and information is currently limited. As differential scanning

calorimetry (DSC) has been utilised successfully to investigate transitions between native and denatured state of protein, it is a tool that can assess protein conformational changes in relation to protein-flavour interactions (Arntfield & Murray, 1981).

Milk and soy proteins have been most extensively evaluated in protein-flavour interaction studies, with less emphasis on canola and pulse proteins (Gremli, 1974; Stevenson, Chen, & Mills, 1996). Binding studies with pea (Dumont & Land, 1986; Heng et al., 2004) and fababean proteins (Ng, Hoehn, & Bushuk, 1989a, b; Semenova, Antipova, Misharina, & Golovnya, 2002) have been reported. No data has been presented on canola protein. Since isolation of plant proteins suitable for human consumption has increased (Arntfield, 2011; Sun, 2010), a better understanding of the behaviour of canola and pea proteins in the presence of flavours will provide essential information for formulating traditional or novel protein foods with desired flavour perception. In addition, as there is some potential to replace wheat proteins with pulse proteins in baked goods, wheat protein was also included for comparison.

Commercially, alkaline extraction followed by acid precipitation and spray drying have been utilised to prepare plant protein isolates (i.e., soy and pea proteins) (Sun, 2010). This method strongly impacts the protein's native structure and diminishes related protein functional properties (Sathe & Salunkhe, 1981; Sun & Arntfield, 2010). As an alternative to alkaline extraction, salt extraction combined with the subsequent formation of a micelle mass has become increasingly popular, since it retains protein functionality and induces little change of protein conformation (Burgess, 1991; Léger & Arntfield, 1993; Sun, 2010). Previously, researchers utilized

either isoelectric precipitation (Aspelund & Wilson, 1983; Damodaran & Kinsella, 1981a, 1981b) or salt-extraction (including PMM method) (Heng et al., 2004; Ng, Hoehn, & Bushuk, 1989a, 1989b) to prepare plant proteins for their studies. Therefore, of particular interest is to compare and evaluate the potential effects of these two protein isolation methods on protein-flavour binding properties.

The objectives of this study were, therefore, to investigate the effect of some intrinsic features of different flavours and proteins on protein-flavour binding with particular reference to canola, pea and wheat proteins. To investigate the impact of flavour structure on the interactions, six homologous series of aldehydes (C_6, C_7 and C_8) and ketones (C_6, C_7 and C_8) were chosen. The influence of both salt and alkaline protein extraction methods were examined for canola and pea proteins. Protein thermal behaviours with addition of these compounds were also determined, to evaluate changes in protein structure.

3.3 Materials and methods

3.3.1 Source of materials

Analytical grade flavours were purchased from Sigma-Aldrich Co. (St. Louis, MO). Commercial yellow pea flour (*Pisum sativum* L.) and canola meal were kindly supplied by Best Cooking Pulses Inc. (Portage la Praire, Canada) and BMW Canola (AL018, Winnipeg, Canada), respectively. Commercial vital wheat gluten was obtained from local market (Arrowhead Mills, Inc., Melville, NY). All other chemicals including NaCl, K_2HPO_4, KH_2PO_4, HCl and NaOH were analytical grade and purchased from Fisher Scientific (Ottawa, Canada).

3.3.2 Salt-extracted canola protein isolates (CPIs)

A protein micellar mass (PMM) method from Ser, Arntfield, Hydamaka, and Slominski (2008) was adapted with minor modifications. Firstly, 50 g of finely ground (Grind Central Coffee Grinder, Guisinart) and sieved (500 μm opening, USA Standard No. 35) canola meal were mixed with 500 mL of 0.5 M NaCl by constantly stirring at medium speed on a corning PC-353 stirrer (Scientific Support, Inc., Hayward, CA) for 1 h. The mixture was then centrifuged (3000*g*, 4℃, 15 min) and the supernatant (soluble protein solution) was successively filtered through four layers of Cheesecloth Wipes™ (Fisher Scientific) and two layers of each Whatman™ No. 4, 40 and 42 filter papers (90 mm ∅) under vacuum to remove any possible debris. Clarified canola protein solution was then concentrated to 3-5 times its original volume using a Vivaflow 200 ultrafiltration unit (Vivascience AG, Hannover, Germany) equipped with a 10,000 Da molecular weight cut-off (MWCO) polyethersulfone (PES) membrane at constant pressure of 250 kPa. The retentate (concentrated protein solution) was subsequently diluted 15 times using cold distilled water and let stand in a refrigerator at 3℃ for 16 h. Instantaneous protein precipitation upon dilution indicated formation of PMM which was recovered by a second centrifugation (6000*g*, 4℃, 15 min). The pellet was collected and kept frozen (-30℃) before freeze drying (Genesis SQ Freeze Dryer, Gardiner, NY).

3.3.3 Alkaline-extracted canola protein isolates (CPIa)

For the alkaline extraction, one part of meal was mixed with 10 parts of distilled H_2O with pH adjusted to 8 using 1.0 M NaOH and stirred for 1 h. pH was checked

and adjusted if necessary every 15 min. The slurry was centrifuged (4500*g*, 4℃, 20 min) and filtered through 4 layers of Cheesecloth followed by adjusting the pH of the supernatant to 4 using 0.1 M HCl. pH 4 was selected rather than 5 or 6 based on immediate protein precipitation and optimum yield of protein precipitate. After a second centrifugation (4500*g*, 4℃, 20 min), protein precipitate was collected and kept at -30℃ before being freeze dried.

3.3.4 PPIs extraction

Following the method previously described by Sun and Arntfield (2010, 2011), salt-extracted pea protein isolates (PPIs) was extracted from sieved (500 μm opening, USA Standard NO. 35) yellow pea flour using 0.3 M NaCl (pea flour: sodium chloride solution = 3:10, w/v) under constant stirring for ½ hour. After the first centrifugation (4260*g*, 4℃, 15 min), pea protein was recovered by diluting the supernatant in two times its volume of cold distilled H_2O and leaving in a refrigerator (3℃) for 2 h. The precipitated protein sediment was collected after a second centrifugation (680*g*, 4℃, 15 min) and re-suspended in small amount of distilled H_2O. The resulting protein suspension was then dialyzed using 12-14,000 Da MWCO dialysis tubing (Spectra/Por Dialysis Membrane, Rancho Dominguez, CA) against 20 times cold distilled H_2O for 72 h in a refrigerator. Distilled H_2O was changed every 24 h. The desalted protein isolates were stored at -30℃ until they were freeze dried.

3.3.5 PPIa isolation

Alkaline-extracted pea protein isolates (PPIa) were prepared according to Karaca, Low and Nickerson (2011). Essentially, 40 g of ground sieved yellow pea flour were finely dispersed into 600 mL of distilled H_2O adjusted to pH 9.5 with 1.0 M NaOH and constantly stirred for 1 h. The pH was checked every 15 min and adjusted of necessary. After centrifugation (4500*g*, 20 min, 4℃) to remove any insoluble materials, the pH of the supernatant (protein extract) was adjusted to 4.5 using 0.1 M HCl to initiate protein precipitation. Following a second centrifugation (4500*g*, 4℃, 20 min), the pellet was washed with acidified water (pH 4.5) followed by a third centrifugation at the same condition (Ghodsvali, Khodaparast, Vosoughi, & Diosady, 2005). The recovered protein isolates were stored at -30℃ prior to freeze drying.

Preparation of protein isolates occurred over a period of approximately 1 month; the protein isolate samples were then well mixed so that all further analyses were conducted on the bulk sample. The freeze dried protein samples including CPIs, CPIa, PPIs, PPIa and commercial wheat gluten contained 87.32%, 75.35%, 82.68%, 82.82% and 76.01% protein respectively using an N-to-protein conversion factor of 5.7 with a Dumas method and a FP-528 Nitrogen/Protein Determinator (LECO Corporation, St. Joseph, MI).

3.3.6 Flavour binding to plant proteins

To bind proteins and flavours, the method of Gkionakis, Taylor, Ahmad, and Heliopoulos (2007) was followed. Basically, each protein and flavour stock solution were firstly prepared and then mixed at specific ratio, to produce an aqueous sample

of protein isolate with desired concentration of flavour compounds. Samples were shaken gently to reach equilibrium of binding of flavours with proteins; headspace gas chromatography was then used for determining the binding of protein isolates with selected volatile flavours compounds.

3.3.6.1 Preparation of protein and flavour stock solutions

2% (w/v) CPIs and PPIs solutions were prepared in 0.01 M potassium phosphate buffer (pH 8) and subsequently placed into an ultrasonic water bath (Branson 3200, Bransonic Ultrasonic Cleaner, Shelton, CT) for 20 min to ensure a complete dispersion of the protein isolates. The ionic strength was kept as low as possible to minimize the effect of salt on protein conformation.

Stock solutions of each volatile flavour compound were prepared in phosphate buffer solution at 1000 ppm (0.1mL/100mL) and stored in amber glass bottles to prevent decomposition. Flavour stock solutions were put in an ultrasonic water bath for 1 h to ensure a thorough mixing before each use.

3.3.6.2 Preparation of GC/MS samples

In a typical experiment, to produce a 1% (w/v) final protein solution with 250 ppm flavour concentration, 1 mL of 2% (w/v) protein solution was carefully loaded into a 20-mL reaction vial (22×75 mm, Product No.: 20-2100, Microliter Analytical Supplies, Inc., Suwanee, GA) followed by addition of 0.5 mL of buffer and 0.5 mL of flavour stock solution to reach an aliquot volume of 2 mL. The flavour solution was added subsequently. As alkaline-extracted proteins (CPIa and PPIa) and wheat gluten

were not highly soluble in the desired buffer; these proteins (0.02g) were weighed directly into GC vials followed by addition of 1.5 mL of buffer and 0.5 mL of flavour stock solutions. The vial was then immediately sealed with Tan PTFE/silicone septa and magnetic metal crimp caps (Product No.: 20-0051M, Microliter Analytical Supplies, Inc., Suwanee, GA) and mixed by a Julabo SW22 shaking water bath (Julabo Labortechnik GMBH, Seelbach, Germany) at 30℃ and 125 rpm for 3 h prior to headspace sampling. Preliminary testing found that 3 h was adequate to reach equilibrium. Samples were prepared in duplicate and each sampled once.

3.3.6.3 GC/MS

Measurement of flavour binding to proteins was conducted using a Varian CP-3800 gas chromatography (Varian Chromatography Systems, Walnut Creek, CA) coupled with a Varian 320-MS triple quadrupole mass spectrometer (Varian, Inc., Walnut Creek, CA) operated in splitless and single quad mode. After mixing, samples were incubated and shaken for 14 min at 40℃ and 1 mL of sample headspace was aspirated into GC injector port by a CombiPal autosampler unit with PAL Itex-2 (In-Tube-Extraction) absorber attachment (CTC Analytics AG, Switzerland) after one absorption cycle. A VF-5ms column which was 30 m in length, had 0.2 mm inner diameter and 20 μm film thickness was run at 4 mL/min constant helium flow. The temperature was programed by heating the GC column at a rate of 25 ℃/min to 265℃ and then held for three minutes.

A mass spectrometer was used to confirm the identity of volatile flavour compounds and further determine the potential volatile flavour by-products generated.

Operating conditions for the mass spectrometer were 70 eV EI source with a mass range between 25 Da to 250 Da.

Binding percentage of flavours was determined from the difference between the peak areas of flavored samples in the absence and presence of proteins such that: Binding % = (1 - $\frac{Peak\ area_{\ with\ protein\ added}}{Pek\ area_{\ without\ protein\ added}}$) × 100%.

3.3.7 Differential scanning calorimetry (DSC)

To evaluate the effect of volatile flavour compounds on the thermal properties of PPIs suspensions, a thermal test was performed using a DSC Q200 (TA Instruments, New Castle, DE) following the method of Sun and Arntfield (2010). Flavour concentrations of 100, 250 and 500 ppm were selected to evaluate conformational changes. Enthalpy of denaturation (ΔH) and denaturation temperature (T_d) were obtained from the endothermic peaks in the thermograms using Universal Analysis 2000 software (Version 4.5A).

In a typical experiment, a 10% (w/v) PPIs suspension in 0.3 M NaCl with desirable flavour concentration was prepared by shaking the samples on a RKVSD rotary shaker (ATR, Laurel, MD) for 1 h at speed 40, followed by mixing for 1 min using a Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY) before being loaded into DSC pans. Pre-mixed protein suspension (10-15 μL) was accurately weighed in a Tzero pan (TA Instruments, New Castle, DE) and hermetically sealed. The thermal curve was obtained by heating the sample from 30 to 120℃ at 10℃/min in a standard DSC cell. The DSC had been calibrated against both sapphire and indium standards. An empty pan was used as reference. Each sample was tested in duplicate.

3.3.8 Experimental design

In the first study, a full $2 \times 3 \times 2 \times 2$ factorial experiment was employed to evaluate the influences of flavour class (aldehyde and ketone), flavour carbon number (C_6, C_7 and C_8), protein source (canola and pea) and protein isolation method (salt and alkaline extraction) on flavour retention by proteins. Commercial wheat gluten was also included for comparison. Each data point in the factorial design was duplicated.

In the second study, a full $2 \times 3 \times 3$ factorial experiment with flavour class (aldehyde and ketone), flavour carbon number (C_6, C_7 and C_8) and flavour concentration (250, 350 and 500 ppm) as the three factors was implemented. Each point in the factorial design was repeated at least twice. Protein thermal properties including both enthalpy of denaturation (ΔH) and denaturation temperature (T_d) were determined.

3.3.9 Statistical analysis

All data were analyzed using Microsoft Excel and SAS 9.3 program (SAS Institute Inc., Gary, NC). Tukey's test followed the analysis of variance indicated significant differences with a level of $p < 0.05$.

3.4 Results and discussion

3.4.1 Evaluation of intrinsic features of different flavours and proteins on protein-flavour binding performance

Different proteins possess different binding capacities to individual volatile flavour compound. In other words, one protein may possess relatively higher binding

capacity to one specific flavour or to all flavours compared with other proteins, and an individual protein does not bind all flavour compounds with equal affinity. All four factors tested including flavour class, flavour carbon number, protein source, and protein isolation method were found to significantly impact the level of flavour retention ($p < 0.05$). In addition to the independent effects, two way interactions were detected between the four factors expect flavour class and carbon number. More than that, three way interactions existed among four factors and a four way interaction was also observed. Data have been presented to show these interactions but the main factors will be discussed.

3.4.1.1 Effect of flavour chain length

The effect of all intrinsic features of different flavours and proteins on protein-flavour binding performance is summarized in Fig. 3.1. From Fig. 3.1, it is clear that all proteins were able to bind the volatile flavour compounds used in this study. Regardless of protein source or isolation method, retention of both aldehydes and ketones was significantly enhanced with an increase in flavour carbon number indicating the interaction was predominantly hydrophobic in nature, as has been generally recognized (Damodaran and Kinsella, 1981a). Gremli (1974) recorded a significant increase in the binding of aldehydes and ketones to 5% soy protein (w/v), when the carbon number increased from six to ten. Heng et al. (2004) also reported that with 0.1% pea vicilin (w/v), the retention of pentanal (C_5) and octanal (C_8) increased from 75% to 88%, meanwhile 2-octanone (C_8) was bound 16% more than 2-pentanone (C_5).

3.4.1.2 Effect of flavour functional group

In the current study, all proteins exerted higher binding capacities to aldehydes than ketones; binding capacities were 2-5 times higher (Fig. 3.1 a *vs.* b). Gremli (1974) found that 5% soy proteins (w/v) strongly bound aldehydes at a level 2-4 times higher than the ketones with the same carbon number. A similar trend was also reported by Heng et al. (2004) who investigated a series of aldehydes and ketones bound with pea vicilin.

Such observed differences may be due to the chemical nature of interactions where aldehydes form both reversible hydrophobic interactions and irreversible covalent bonds with amino (-NH_2) and sulfhydryl (-SH) groups of proteins, while only reversible weak hydrophobic interactions exist between ketones and proteins (Gremli, 1974; Tromelin, Andriot, & Guichard, 2006). Reineccius (2006c) concluded that loss of flavours can be dramatic when reaction with functional groups (e.g., -NH_2, -SH, -OH and -COOH) on proteins are involved.

Damodaran and Kinsella (1980b) noted that as the aldehyde groups are located at the terminal 1-position of aldehydes, there was steric hindrance to hydrophobic interactions; whereas the more steric hindrance resulting from the keto group at the 2-positon in ketones may not merely limit hydrophobic interactions but prevented these flavours from binding in general. More than that, the dramatic retention of aldehydes has also been attributed to the higher hydrophobicity (log *P*) of aldehydes as compared to ketones.

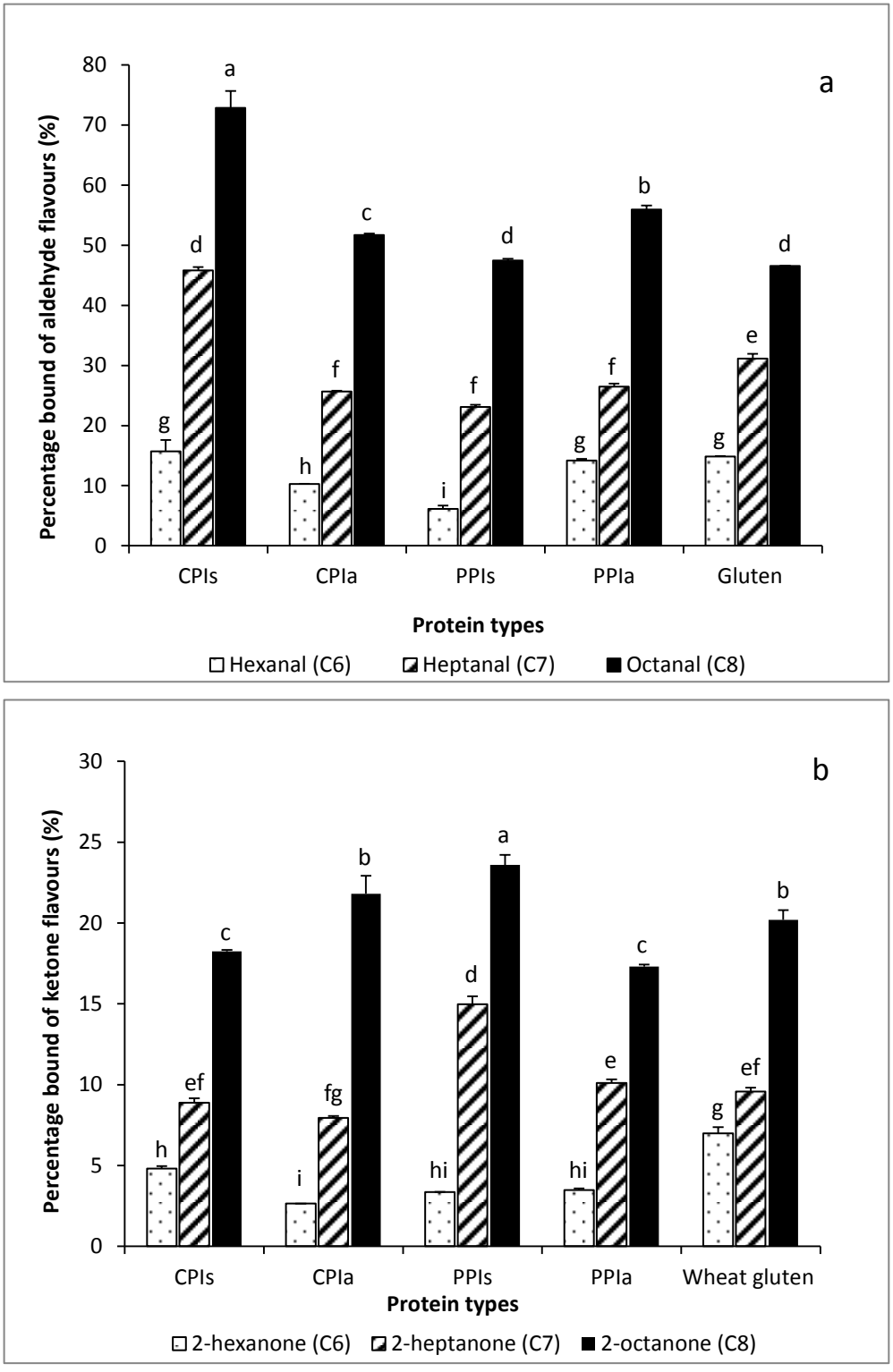


Figure 3.1 Effect of some intrinsic features of different proteins and flavours on protein-flavour binding performance at a protein concentration of 1% (w/v) and 250 ppm flavour level (a) percentage bound of aldehyde flavour; (b) percentage bound of ketone flavour

[a~i] In each figure, bar values followed by the same superscript are not significantly different ($p < 0.05$). To demonstrate the statical analysis, ANOVA table of Fig. 3.1 A was presented in Appendex A as an example.

3.4.1.3 Effect of protein sources

Due to the disruption of protein native structure by alkaline or acidic conditions, only salt-extracted proteins and wheat gluten will be considered when comparing protein source. CPIs revealed the highest binding capacity to aldehydes, followed by wheat gluten and PPIs (Fig. 3.1 a). In contrast, ketone flavours bound with proteins following the order: PPIs > wheat gluten > CPIs, although 2-hexanone was an exception and showed the lowest affinity to PPIs (Fig. 3.1 b). In a similar study investigating protein conformational effects by Damodaran and Kinsella (1981b), distinct binding of soy 7S and 11S protein fractions to 2-nonanone was noted and attributed to the different spatial arrangements of these two proteins. As the salt-soluble globular proteins are the major proteins extracted by salt-extraction methods (Shewry, 2004), it is speculated that the different binding patterns of proteins seen in the current study may directly result from the distinct quaternary structure of canola and pea globulins and wheat glutens.

Schwenke, Raab, and Damaschum (1983) reported the 12S globulin from canola is composed of six subunits; each subunit consists of two polypeptide chains linked by a single disulfide bond and a cavity buried inside, while the vicilin (7S) and legumin (11S) are the major globular proteins found in peas with vicilin being predominant (Sun, 2010). Conformationally, vicilin possesses a trimeric structure without disulfide bonds formation, whereas legumin contains six disulfide linked acidic and basic subunits. In contrast to canola and pea globulins, wheat glutens are composed of gliadins and glutenins which are characterized by inter- and

intra-molecular disulfide linkages.

Relating the potential of proteins to bind with aldehydes, functional groups of protein amino acids, especially -SH and -NH_2, play an important role in participating covalent interactions with flavours. The lower sulfur-containing amino acids of Cys and Met (0.35 and 1.60 g/100g protein) in PPIs and less disulfide linked structure may contribute to its low aldehyde binding affinity compared with 12S canola globulin (1.07 g Cys and 1.84g Met/100g globulin) (Khattab, Arntfield, & Nyachoti, 2009; Schwenke, et al., 1981). In contrast, the high level of disulfide bonds in wheat gluten is responsible for the stability of gluten structure and may create a steric hindrance to flavours by making the gluten subunits coming close together. Conversely, the native canola 12S globulin was stabilized by hydrophobic interactions rather than covalent disulfide bonds, which may in turn make these disulfide structures more favourably available for flavour binding (Wu & Muir, 2008).

Unexpectedly, the high Lys residue (contain free -NH_2 group) found in peas (6.25g/100g protein) did not increase PPIs aldehyde-binding capacity; more aldehyde binding was seen with CPIs, even though the Lys content of 12S canola globulin (3.45 g/100 g protein) was lower. This may infer that the affinity of aldehydes to Cys and Met residues was higher than Lys residues; therefore, the influence of Lys residues on aldehyde flavour retention was weakened. Kühn, Considine and Singh (2008) compared the amount of accessible amino acids in whey protein isolates with and without the addition of *trans*-2-nonenal. They found that amount of accessible Cys and Met was greatly reduced compared to Lys, suggesting sulfur-containing amino

acids may be more involved in protein-flavour interactions than Lys. This is in reasonable agreement with the findings in the current study.

As the binding of ketones with proteins is known to be hydrophobic in nature (Damodaran & Kinsella, 1981a), the hydrophobic regions in the cavity of canola 12S globulin may not be available for protein-flavour hydrophobic interactions, resulting in its lower binding capacity to ketones. Damodaran and Kinsella (1981b) explained the binding of 2-nonanone to soy 11S protein was much lower than soy 7S protein due to the inaccessibility of the buried hydrophobic regions of soy 11S proteins. In addition, the presence of intra-molecular disulfide bonds in 12S canola globulin may limit the accessibility of flavour to the hydrophobic core, while the lack of disulfide structure in PPIs may create less stearic hindrance for flavour to enter hydrophobic region, promoting protein-flavour hydrophobic interactions.

Moreover, van Ruth and Roozen (2002) stated that the amount of flavour bound increased with the hydrophobicity of proteins. As wheat protein is rich in hydrophobic amino acids (e.g., proline), the large repetitive hydrophobic regions in high molecular weight glutenin subunits could act as good canditates to retain hydrophobic flavours such as aldehydes and ketones (Shewry et al., 2009).

3.4.1.4 Effect of protein isolation methods (alkaline vs. salt extraction)

Alkaline-extracted canola proteins bound much less aldehyde flavours than salt-extracted ones (Fig. 3.1a); however, PPIa had a higher binding capacity to aldehydes compared with PPIs. With the exception of the 2-octanone, which had a higher binding affinity to CPIa than CPIs, all other ketone flavours showed higher

binding affinities to salt-extracted canola and pea proteins (Fig. 3.1b). Previously, it was noted that protein-flavour interactions were highly dependent on structural state of the protein (Kühn, Considine, & Singh, 2006). The harsh environment during alkaline extraction may have led to severe changes in protein conformation and solubility that are responsible for the differences in protein-flavour binding capacities.

A protein has zero net charge and least solubility at its isoelectric point (IP). A decreased electrostatic force between molecules results in an increase in protein-protein interactions which serve as favorable conditions for protein aggregation or precipitation (Vojdani, 1996). During the alkaline extraction of canola and pea proteins, the final product pH values were 4 and 4.5 during protein isoelectric precipitation. It is known that volatile compounds only can bind to protein when binding sites are available (Kim & Min, 1989). For these reasons, acid precipitated proteins may limit the accessibility of flavour compounds to the primary binding sites as the increased protein-protein interactions (aggregation) replace protein-flavour interactions. Dumont and Land (1986) found that a dramatic decrease of binding of diacetyl when the pH of pea protein solution was adjusted to the IP of pea proteins (~pH 4) which corresponds well with our observation.

For the unexpected increased binding of aldehydes to PPIa, it may be possible that modifications of proteins by alkaline extraction at pH 9.5 and acidic precipitation at pH 4.5 led to the exposure of new binding sites with the particularly higher binding affinity to aldehyde flavours.

3.4.1.5 Generation of new volatile flavour by-products

Interestingly, two volatile flavour by-products, 2-butyl-2-octenal ($C_{12}H_{22}O$) and 2-pentyl-2-nonenal ($C_{14}H_{26}O$), were formed when hexanal ($C_6H_{12}O$) and heptanal ($C_7H_{14}O$) were added to CPIs at room temperature. The additional peaks in CPIs were observed at elution times of 11.77 and 12.72 min, respectively. No volatile flavour by-products were detected in other protein-flavour mixtures. The chromatograms of hexanal and heptanal with and without CPIs addition and related mass spectral information are presented in Fig. 3.2 and Fig. 3.3. It clearly shows that 2-butyl-2-octenal and 2-pentyl-2-nonenal did not appear in the standards, which contained hexanal or heptanal in buffer (Fig. 3.2 a and Fig. 3.3 a) but were present in samples when CPIs were included (Fig. 3.2 b and Fig. 3.3 b), suggesting that the generation of new volatile flavour by-products can only be attributed to the protein-flavour interactions and was not the result of flavour degradation. The production of these peaks was confirmed in subsequent work on mixed systems.

Kim and Min (1989) systematically summarized the potential chemical interactions between protein and volatile aldehydes and stated that volatile aldehydes can react with either a free amino group of proteins or free amino acids to form Schiff bases by a condensation reaction. Nonetheless, the formed Schiff bases can undergo several condensation and polymerization processes, subsequently generating aldolization products under the condition of hydrolytic cleavage.

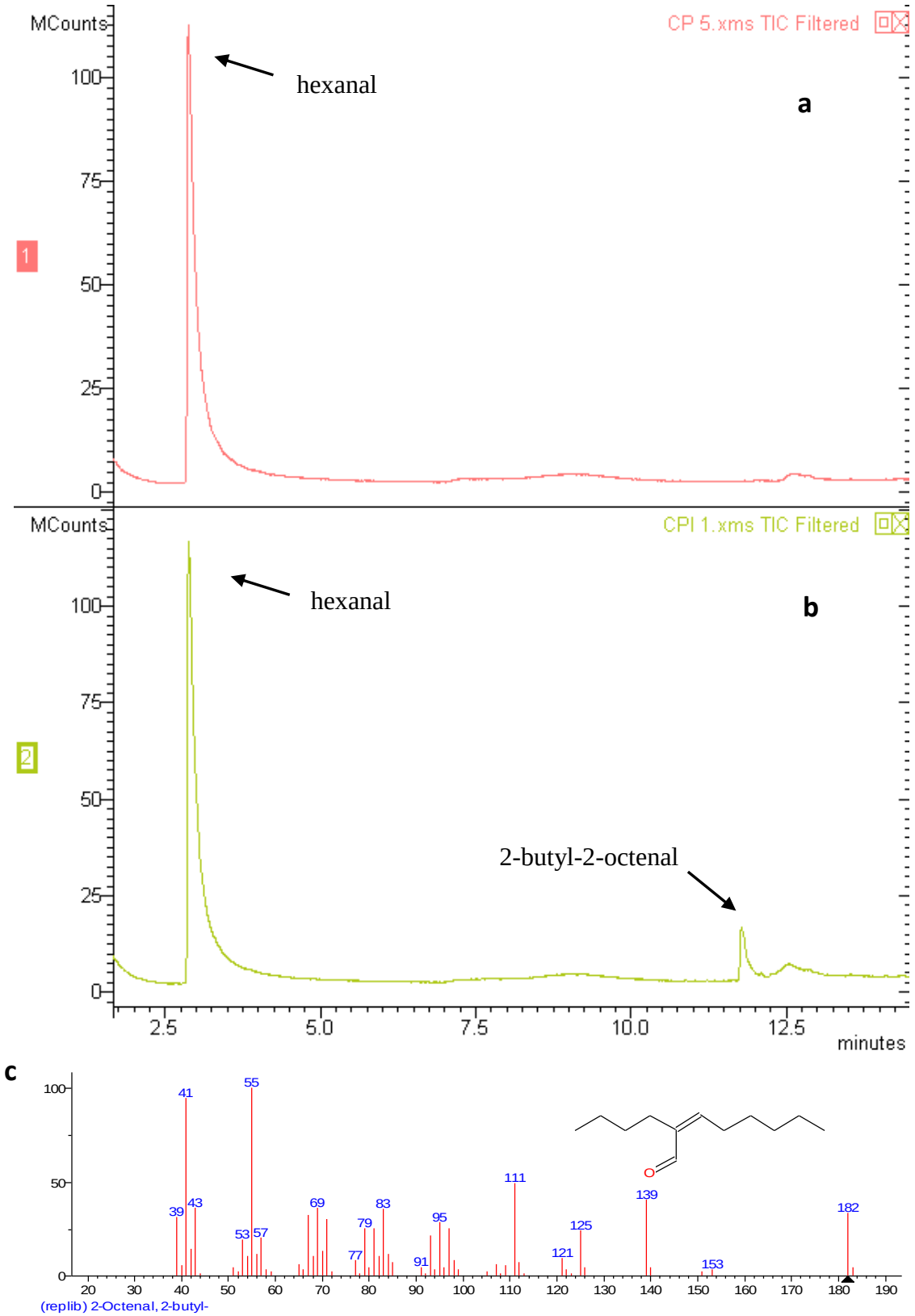


Figure 3.2 Illustration of the chromatograms of hexanal flavour without (a) and with (b) presence of CPIs and (c) structure and mass spectrometer information about 2-butyl-2-octenal.

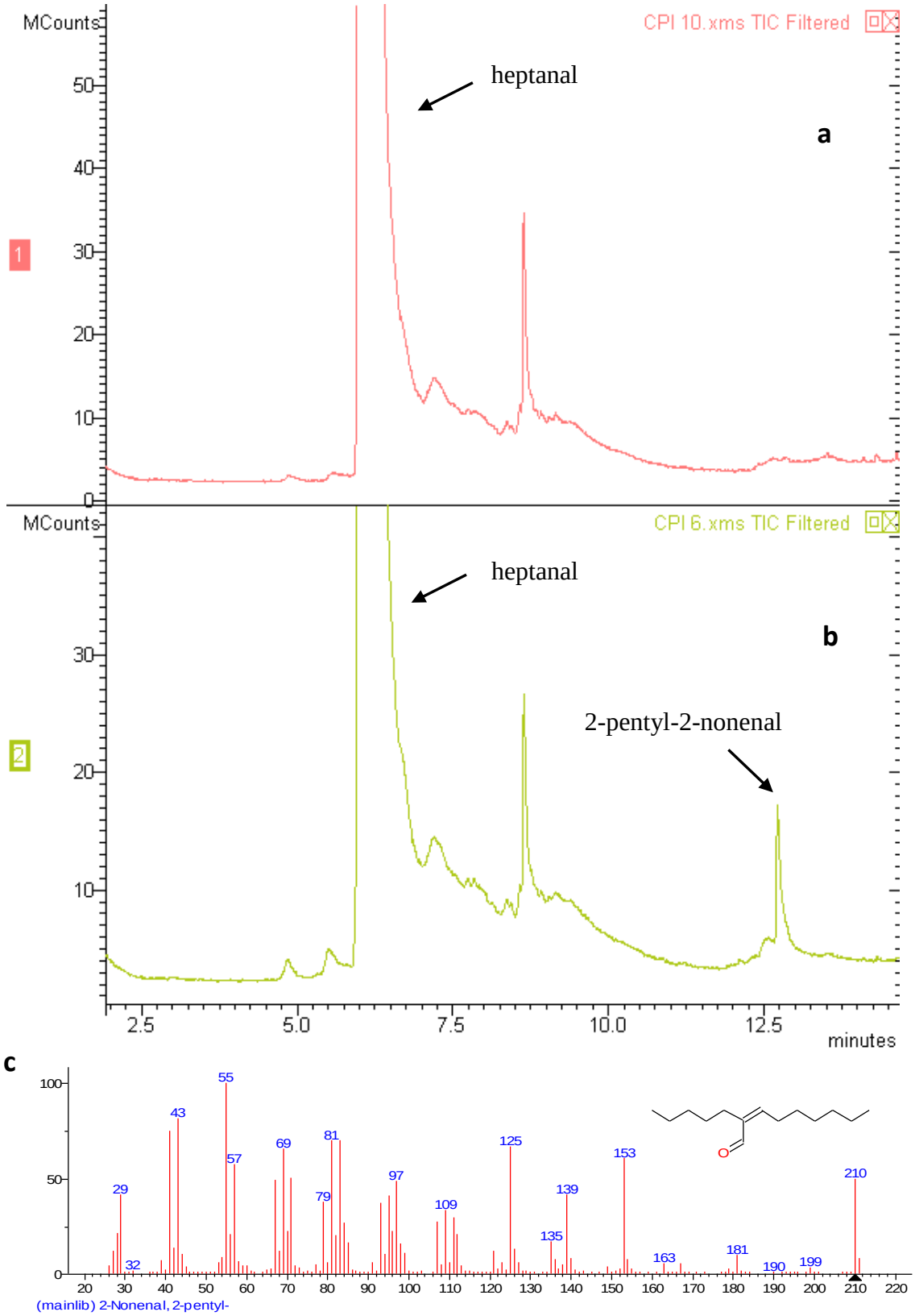


Figure 3.3 Illustration of the chromatograms of 1-heptanal flavour without (a) and with (b) presence of CPIs and (c) structure and mass spectrometer information about 2-pentyl-2-nonenal.

Following the pathway of interaction between propanal and a free amino group presented by Kim and Min (1989), the reaction sequence of hexanal interacting with a free amino group is shown in Fig. 3.4.

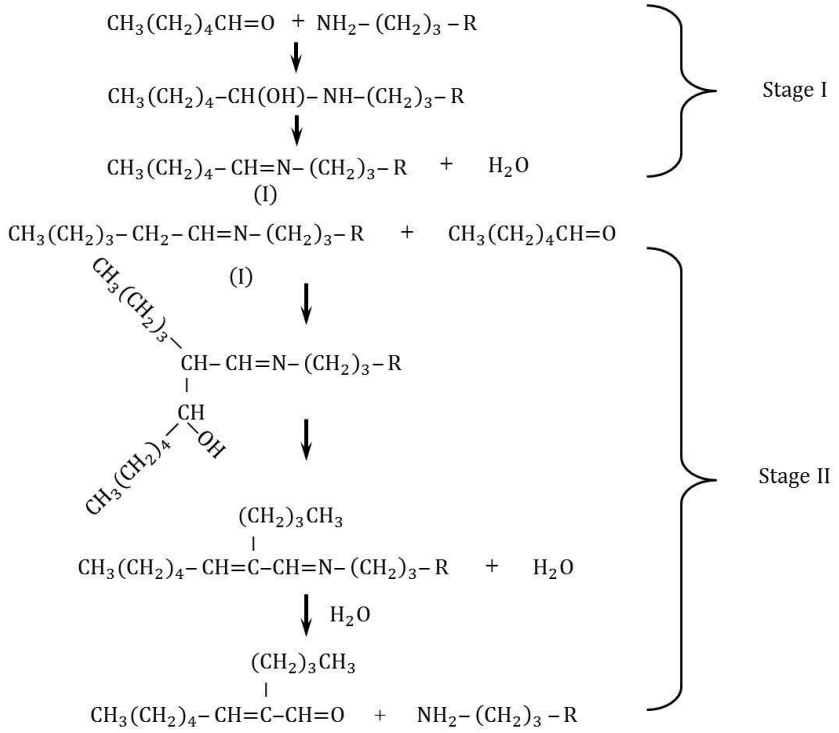


Figure 3.4 Pathway of the formation of volatile flavour by-product 2-butyl-2-octenal from hexanal with CPIs.

As illustrated, after forming a Schiff base (I) by condensation reaction between the aldehyde group of hexanal and the primary amino group of the protein (stage I), the formed Schiff base (I) was subsequently involved in an aldol reaction by interacting with another hexanal molecule (stage II). This was followed by hydrolytic cleavage, producing the oligomeric compound 2-butyl-2-Octenal which was detected in GC/MS. In a similar way, the formation of 2-pentyl-2-Nonenal from heptanal with CPIs may be attributed to the same reaction sequence. It has been noted previously this interaction does not affect the composition of protein molecules and can be

repeated as long as there is a free amino group and alkanals present, leading to the formation of more complicated aldolization products (Kim & Min, 1989). No such products were observed when octanal was the added aldehyde.

Gremli (1974) mentioned that the addition of flavours to soy protein foods not only led to their loss but also caused a change in the flavour profile. A gluey odor in casein-based foods has been attributed to the aldolization products of aldehydes (Pokorny, Thienluan, Kondratenko, & Janicek, 1976). Kühn, Considine, and Singh (2008) noted the generation of heptanal when *trans*-2-nonenal was added to whey protein isolate after heat treatment. They explained that the double bonds in *trans*-2-nonenal may have been responsible for the formation of heptanal, as heptanal was not formed in the samples containing nonanal under the same condition. However, the underlying mechanism was not clearly elucidated by the authors. According to Kühn, Considine, and Singh (2008), future research focusing on comparing flavour binding with proteins or specific amino acid with blocked N-terminal may be able to further explain the potential mechanism behind the formation of new volatile flavour compounds.

3.4.2 Effect of flavour binding on protein thermal properties

Salt-extracted pea protein isolates (PPIs) were chosen as examples to illustrate the effect of flavour binding on protein thermal properties. From the current analysis, adding volatile flavours did not significantly change the denaturation temperature of PPIs (data not presented); however, it was noted that addition of flavours to the protein samples resulted in significantly lower enthalpies of denaturation (ΔH), compared with the control sample to which flavour was not added. The ΔH of PPIs samples obtained with the addition of a homologous series of aldehydes and ketones at 250 ppm is plotted in Fig. 3.5.

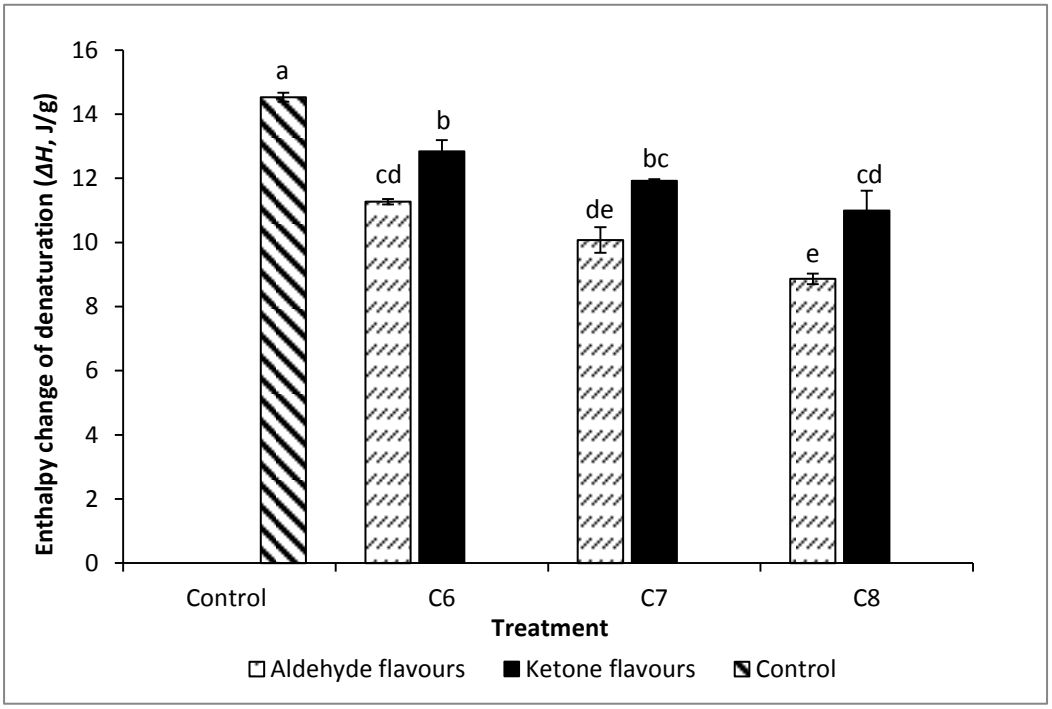


Figure 3.5 Effect of flavour binding on conformational change of PPIs (10%, w/v, 0.3M NaCl) at 250ppm flavour level.
[a~d] Bar values followed by the same superscript are not significantly different (P<0.05). Note: control was not added with flavour compounds.

It is clear that all ketone flavours exhibited a higher ΔH than the corresponding aldehydes, although both were lower than the control. Differential scanning

calorimetry (DSC) measures the energy required to sustain a constant temperature between an inert reference material and a studied substance (Tromelin, Andriot, & Guichard, 2006). When following protein thermal denaturation, ΔH represents the amount of energy required to completely denature a protein molecule; therefore, it provides crucial information about protein structural changes. A decreased ΔH infers that flavour molecule may be involved in unfolding the protein structure leading to protein partial denaturation, as evidenced by less energy is required to denature the protein molecule. Due to this partially denatured state, more binding sites may be revealed allowing for more severe binding of flavours. Compared with ketones, the greater decrease of ΔH for aldehydes indicates aldehyde flavours may possess much higher "unfolding capacity" than ketones, which may account for their higher binding affinities observed in Fig. 3.1 (a *vs* b).

This observation is similar to that seen by Kim and Min (1989) and Solms, Osman-Ismail, and Beyeler (1973) who noted as binding proceeds, more binding sites become available, due to changes in protein conformation. It can be speculated that, due to less steric hindrance resulted from the terminal carbonyl group for aldehyde as compared to ketone (two hydrocarbon groups link to carbonyl group), aldehydes could be more effective than ketones to enter hydrophobic core of proteins by competing intra- or intermoleucar hydrophobic interactions and hydrogen bonds, such that promoting a higher degree of protein unfolding.

Fig. 3.6 shows the relationship between the concentrations of added flavour and the resulting enthalpy of denaturation of PPIs. With an increase in the flavour

concentration, there was a linear decrease in the enthalpy of denaturation. This indicates that higher levels of volatile flavours leads to increased protein unfolding, with both flavour concentration and carbon number of flavour in a same class. Similar results were reported by Grinberg, Grinberg, Mashkevich, Burova, and Tolstoguzov (2002) who investigated interaction of ovalbumin with vanillin in a calorimetric study. At pH values of 6.7 and 3.0, both the enthalpy and temperature of ovalbumin denaturation were dramatically decreased by increasing vanillin concentration. This corresponds well with the finding in the current analysis.

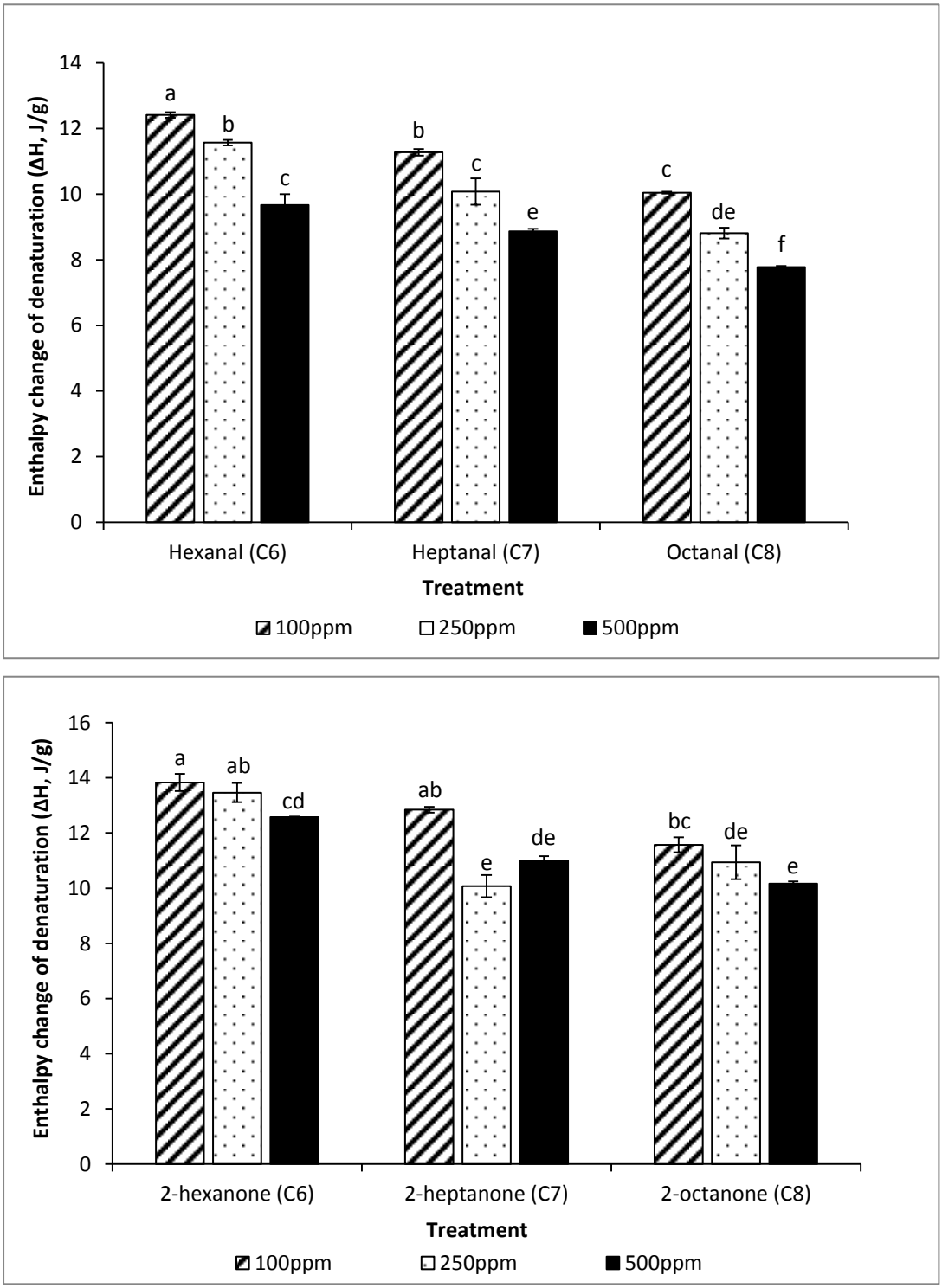


Fig. 3.6 Effect of concentration of homologous series of aldehydes and ketones on Δ*H* of PPIs (10%, w/v, 0.3M NaCl). [a~e] In each figure, bar values followed by the same superscript are not significantly different ($P<0.05$).

3.5 Conclusion

From the current investigation, flavour class, flavour carbon number, protein source, and protein isolation method were all found to have a significant impact on protein-flavour binding performance. Ketone flavours with lower carbon number were retained least by proteins, leaving a high volatile flavour concentration both at sample headspace and within food matrix. Different sources of proteins showed different flavour binding behaviours as a result of their inherent protein structure and amino acid composition. As different protein isolation methods were used, salt-extracted proteins exhibited higher flavour binding abilities than alkaline extracted ones, except for PPIa retained more aldehydes than PPIs. For wheat gluten, the capacity to bind with aldehydes or ketones fell between CPIs and PPIs. Formation of new volatile flavour by-products showed the potential to distort the original food flavour profile due to the formation of new volatile compounds. The changes observed in protein thermal properties indicated that protein conformational changes due to protein-flavour interactions increase protein flavour binding. Careful selection and use of flavour compounds and protein sources are necessary when manufacturing plant protein-based food products.

Connections between Chapter 3 and Chapter 4

Not only is it necessary to understand protein-flavour interaction in a single flavour-protein system, evaluating how a mixture of flavour compounds behaves in the presence of protein could provide more insight into this interaction at a condition more close to a commercialized flavour formulation where a number of flavour compounds are usually used together. In Chapter 3, the different proteins were compared in relation to flavour and clearly the source of the proteins was an important factor. Working with wheat protein, however, presented a challenge due to it poor solubility and as Chapter 4 was designed to systematically evaluate the binding behaviours in aqueous model systems, only CPIs and PPIs were used in combination with mixutres of homologous and heterologous classes of volatile flavour compounds. In addition, the effect of heat on protein-flavour binding was evaluated as a function of heating time under conditions in which a number of flavour compounds were present. The potential structural modification of proteins in the presence of these flavours were followed using differential scanning calormetry.

Chapter 4 Binding of Selected Volatile Flavour Mixture to Salt-Extracted Canola and Pea Proteins and Effect of Heat Treatment on Flavour Binding

4.1 Abstract

Binding of homologous and heterological classes of flavours with salt-extracted canola protein isolates (CPIs) and pea protein isolates (PPIs) and the effect of heat treatment on their binding were investigated using GC/MS. Competitive binding was observed when homologous ketones were added to CPIs and PPIs and when homologous aldehydes were mixed with CPIs. Ketone mixtures performed differently than aldehydes in that 2-octanone retained more effectively than 2-heptanone and 2-hexanone by CPIs and PPIs, whereas CPIs exhibited incremental affinity to hexanal rather than heptanal and octanal. For PPIs, the presence of aldehydes increased the proteins' overall flavour-binding capacities probably due to partial unfolding of proteins revealing more binding sites as manifested by the decreased ΔH from the DSC studies. Binding of hexanal to CPIs was significantly increased with increased heating time at 95 ℃, while a transition of 2-octanone retention from increasing to decreasing inferred heat-induced protein association released previously bound 2-octanone. Heat treatment at 95 ℃ for 30 min promoted greater competitive binding when mixed ketones and hexanal and 2-hexanone mixtures interacted with CPIs and PPIs, respectively, while dramatic increases of binding of aldehyde mixtures was observed thought out the heating processes.

4.2 Introduction

Among many factors influencing the mobility and release of flavour compounds within and from a food matrix, a major consideration is the chemical interaction between the food and the flavouring. A successful flavour formulation must be designed to survive a range of interactions with the food and eventually afford a sensory profile that is acceptable by consumers (Reineccius, 2006c). Unfortunately, such a flavour releasing profile is not easy to be achieved especially in health-oriented low-fat foods where proteins or carbohydrates perform differently when interacting with volatile flavours compared with fat (Guichard, 2011).

Unlike carbohydrate or lipids, proteins provide complex chemical structures for interacting with flavour compounds; these include hydrophobic pockets, amino acid side chains and terminal ends (Arora & Damodaran, 2010; Reineccius, 2006c; Wang & Arntfield, 2014). Both reversible weak hydrophobic interactions and irreversible strong covalent bonds may be formed between proteins and flavour compounds (Suppavorasatit & Cadwallader, 2012; Tromelin, Andriot, & Guichard, 2006). These interactions between flavour and proteins have led to dramatic reductions in desirable flavour intensity, thereby affecting the perception of flavour in various protein-based food products including soymilk (Suppavorasatit, Lee, & Cadwallader, 2012), vanillin-fababean protein slurries (Ng, Hoehn, & Bushuk, 1989a, 1989b), soy-containing crackers (Zhou, Lee, & Cadwallader, 2006), skim milk (Meynier et al., 2003; Meynier et al., 2004), milk protein sweetened drinks (McNeill & Schmidt, 1993) and dry-cured hams (Pérez-Juan, Flores, & Toldrá, 2006).

In previous studies relating flavour binding by proteins, normally only one flavour compound was studied in an aqueous model system (Kühn, Considine, & Singh, 2006). However, when two or more flavours are mixed, compatible, cooperative or competitive binding patterns can be observed (Guichard & Langourieux, 2000). Compared with simple protein-flavour system, less emphasis has been put on competitive binding studies (Sostmann & Guichard, 1998). Only studies on β-lactoglobulin (Jouenne, Chalier & Crouzet, 2000; Muresan & Leguijt, 1998) and 11S globulin of broad beans have been reported (Semenova et al., 2002). It can be hypothesized that when different volatile flavours are mixed, flavours with higher protein binding affinities should be retained more extensively than the flavours possessing lower binding affinities. Of particular interest in this work is to systematically evaluate how homologous and heterologous classes of aldehyde and ketone flavours behave when combined in a single system.

Protein-flavour interactions are also highly dependent on the protein's structural state (Li, Grün, & Fernando, 2000). Any changes in protein conformation may influence flavour binding characteristics. Heat treatments are most widely used to prepare food products. The effect of heat, however, has led to conflicting results with respect to flavour binding as increases (Gkionakis, Taylor, Ahmad, & Helipoulos, 2007; Ng, Hoehn, & Bushuk, 1989b) and decreases (Kühn, Considine, & Singh, 2008; O'Neill & Kinsella, 1987a) having been reported. Kühn, Considine, and Singh (2006, 2008) explained that increased binding during protein unfolding and decreased binding due to protein aggregation may account for the differences. In most studies, a

single heating time or temperature was used; systematically studying the development of protein flavour interactions with respect to heating time or temperature should provide additional insight into these different behaviours.

Therefore, one of the objectives of this study was to systematically evaluate the potential competitive binding phenomenon between selected volatile flavour compounds to both salt-extracted canola and pea protein isolates. In addition, clarifying the effect of heat treatment on flavour binding using the typical aldehyde and ketone flavours was another purpose of this investigation.

4.3 Materials and methods

4.3.1 Source of materials

Analytical grade flavours were purchased from Sigma-Aldrich Co. (St. Louis, MO). Homologous series of aldehydes (hexanal, heptanal, and octanal) and ketones (2-hexanone, 2-heptanone, and 2-octanone) were selected. Commercial yellow pea (*Pisum sativum* L.) flour was kindly supplied by Best Cooking Pulses Inc. (Portage la Praire, MB). Canola meal was obtained from Burcon NutraScience Co. (AL018, Winnipeg, Canada). All other chemicals including NaCl, K_2HPO_4, KH_2PO_4, HCl and NaOH were analytical grade and purchased from Fisher Scientific (Ottawa, Canada).

4.3.2 Salt-extracted canola protein isolates (CPIs)

A protein micellar mass (PMM) method from Ser, Arntfield, Hydamaka, and Slominski (2008) was adapted with minor modifications. Firstly, 50 g of finely ground (Grind Central Coffee Grinder, Guisinart) and sieved (500 μm opening, USA Standard

No. 35) canola meal was mixed with 500 mL of 0.5 M NaCl by constantly stirring at medium speed on a corning PC-353 stirrer (Scientific Support, Inc., Hayward, CA) for 1 h. The mixture was then centrifuged (3000*g*, 4℃, 15 min) and the supernatant (soluble protein solution) was successively filtered through four layers of Cheesecloth Wipes™ (Fisher Scientific) and two layers of each Whatman™ No. 4, 40 and 42 filter papers (90 mmØ) under vacuum to remove any possible debris. Clarified canola protein solution was then concentrated to 3-5 times its original volume using a Vivaflow 200 ultrafiltration unit (Vivascience AG, Hannover, Germany) equipped with a 10,000 Da molecular weight cut-off (MWCO) polyethersulfone (PES) membrane at constant pressure of 250 kPa. The retentate (concentrated protein solution) was subsequently diluted 15 times using cold distilled water and left in the refrigerator for 16 h. Instantaneous protein precipitation upon dilution indicated formation of PMM which was recovered by a second centrifugation (6000*g*, 4℃, 15 min). The pellet was collected and kept frozen (-30℃) before freeze drying (Genesis SQ Freeze Dryer, Gardiner, NY).

4.3.3 Salt-extracted pea protein isolates (PPIs)

A method previously described by Sun and Arntfield (2010, 2011) was followed to prepare salt-extracted pea protein isolates (PPIs). Pea protein was extracted from sieved (500 μm opening, USA Standard NO. 35) yellow pea flour using 0.3 M NaCl (pea flour: sodium chloride solution = 3:10, w/v) under constant stirring for ½ hour. After the first centrifugation (4260*g*, 4℃, 15 min), pea protein was recovered by diluting the supernatant in two times volume of cold distilled H_2O and refrigerating

(3℃) for 2 h. The precipitated protein sediment was collected after a second centrifugation (680*g*, 4℃, 15 min) and re-suspended in small amount of distilled H_2O. The resulting protein suspension was then dialyzed in 12-14,000 Da MWCO dialysis tubing (Spectra/Por Dialysis Membrane, Rancho Dominguez, CA) against 20 times cold distilled H_20 for 72 h in a refrigerator. Distilled H_2O was changed every 24 h. The desalted protein isolate was stored at -30℃ until freeze dried.

The freeze dried protein samples of salt-extracted canola and pea proteins contained 87.32 and 82.68 % protein respectively using a N-to-protein conversion factor of 5.7 according to Uruakpa and Arntfield (2006a) and Sun and Arntfield (2010) with a Dumas method and a FP-528 Nitrogen/Protein Determinator (LECO Corporation, St. Joseph, MI).

4.3.4 Flavour binding to plant proteins

To bind proteins and flavours, the method of Gkionakis, Taylor, Ahmad, and Heliopoulos (2007) was followed. Basically, protein and flavour stock solutions were first prepared and then mixed at specific ratio to produce an aqueous sample containing the desired concentrations of protein isolate and flavour compounds. Samples were shaken gently to reach equilibrium for binding of flavours with proteins. This was followed by the headspace gas chromatography technique for determining the binding of protein isolates with selected volatile flavour compounds.

4.3.4.1 Preparation of 2% protein and flavour stock solutions

2% (w/v) solutions of CPIs and PPIs were prepared in 0.01 M potassium phosphate buffer (pH 8) and subsequently placed into an ultrasonic water bath (Branson 3200, Bransonic Ultrasonic Cleaner, Shelton, CT) for 20 min to ensure a complete dispersion of the protein isolates (Gkionakis et al., 2007). The ionic strength was kept as low as possible to minimize the effect of salt on protein conformation.

Stock solutions of each volatile flavour compound were prepared in phosphate buffer as mentioned above at both 1000 (0.1mL/100mL) and 1500 ppm (0.15mL/100mL) and stored in amber glass bottles to prevent decomposition. These flavour stock solutions were put in an ultrasonic water bath for 1 hour to ensure a thorough mixing before each use.

4.3.4.2 Preparation of GC/MS samples

In a typical experiment, to produce 1% (w/v) final protein solution with a flavour concentration of 250 ppm, 1 mL of 2% (w/v) protein solution was carefully loaded into a 20 mL reaction vial (22×75 mm, Product No.: 20-2100, Microliter Analytical Supplies, Inc., Suwanee, GA) followed by addition of 0.5 mL of buffer and 0.5 mL of flavour stock solution (1000 ppm) to reach an aliquot volume of 2 mL. The flavour solution was added last. For the competitive binding study using three different flavours, the 1500 ppm flavour stock solutions were employed and 1/3 mL of each was mixed with the 1 mL of 2% protein to obtain a final concentration of 250 ppm for each flavour. The vial was then immediately sealed with Tan PTFE/silicone septa and magnetic metal crimp caps (Product No.: 20-0051M, Microliter Analytical Supplies,

Inc., Suwanee, GA). Samples were mixed by a Julabo SW22 shaking water bath (Julabo Labortechnik GMBH, Seelbach, Germany) at 30℃ and 125 rpm for 3 h before headspace sampling. Preliminary testing found that 3 h was adequate to reach equilibrium. Samples were prepared in duplicate and each sampled once.

For the heat treatment, reaction vials were placed into an Isotemp Water Bath 2320 (Fisher Scientific, Marietta, OH) at 95℃ and heated for the specified heating time. The control was not heated.

4.3.4.3 GC/MS

Measurement of flavour binding to proteins was conducted using a Varian CP-3800 Gas Chromatography (Varian Chromatography Systems, Walnut Creek, CA) coupled with a Varian 320-MS Triple Quadrupole Mass Spectrometer (Varian, Inc., Walnut Creek, CA) operated in splitless and single quad mode. After mixing, samples were incubated and shaken for 14 min at 40℃ and 1 mL of sample headspace was aspirated into the GC injector port by a CombiPal autosampler unit with PAL Itex-2 (In-Tube-Extraction) absorber attachment (CTC Analytics AG, Switzerland) after one absorption cycle. A VF-5ms column, with 30 m in length, had 0.2 mm inner diameter and 20 μm film thickness, was run at 4 mL/min constant helium flow. The temperature was programmed by heating the sample at a rate of 25℃/min to 265℃ and then held at this temperature for three minutes.

A mass spectrometer was used to confirm the identity of volatile flavour compounds and further determine the potential volatile flavour byproducts that have been generated (Kühn, Considine, & Singh, 2008). The EI source for the mass

spectrometer described above was operated at 70 eV with a mass range between 25 Da to 250 Da.

Binding percentage of flavours was determined from the difference between the peak areas of flavoured samples in the absent and presence of proteins such that: Binding % = (1 - $\frac{Peak\ area_{\ with\ protein\ added}}{Peak\ area_{\ without\ protein\ added}}$) × 100%.

4.3.5 Differential scanning calorimetry (DSC)

Protein conformational changes in the competitive flavour binding system were evaluated using a DSC Q200 (TA Instruments, New Castle, DE) following the method of Sun and Arntfield (2010). Thermal properties were determined for PPIs only. A flavour concentration of 250 ppm was selected for DSC analyses. Enthalpy of denaturation (ΔH) and denaturation temperature (T_d) were obtained from the endothermic peaks in the thermograms using Universal Analysis 2000 software (Version 4.5A).

In a typical experiment, 10% (w/v) PPIs suspension with the desirable flavour concentration was produced in 0.3 M NaCl. Samples were then shaken on a RKVSD rotary shaker (ATR, Laurel, MD) for 1 h at speed 40 and completely mixed for 1 min using Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY) before being loaded into DSC pans. 10-15μL of pre-mixed protein suspension was accurately weighed in a Tzero pan (TA, Netherland) and hermetically sealed. The thermal curve was obtained by heating the sample from 30-120℃ at a heating rate of 10℃/min in a standard DSC cell which had been calibrated against both sapphire and indium standards. An empty pan was used as reference. Each sample was tested in duplicate.

4.3.6 Experimental design

To investigate the effect of competition of flavours when bound with proteins, three competitive binding studies were implemented using both headspace gas chromatography and differential scanning calorimetry techniques. Homologous series of each of aldehydes (C_6, C_7, C_8), ketones (C_6, C_7, C_8) as well as a heterological system containing aldehyde (hexanal) and ketone (2-hexanone) were evaluated and compared to a protein-flavour system containing a single flavour. A fixed concentration of 250 ppm of each flavour was used to produce a competitive binding environment.

To investigate the effect of heat treatment on flavour binding, two experiments were conducted. Firstly, a systematic study involving heating CPIs-flavour mixtures at 95℃ and following binding as a function of heating time at 0, 0.5, 1, 2, 5, 10, 20, 40, to 60 min was carried out (Kühn, Considine, & Singh, 2008). 1-hexanal and 2-octanone were selected as the representatives for aldehydes and ketones, respectively. Secondly, a single heat treatment (95℃ for 30 min) was applied where homologous aldehydes, homologous ketones as well as heterological hexanal and 2-hexanone flavour mixtures were added to the protein samples to evaluate how bindings of flavours were affected by heat under the stress of competitive binding. Based on previously reported, denaturation temperature of CPIs and PPIs were ~ 89 ℃ (Uruakpa and Arntfield, 2005) and ~ 86 ℃ (Sun and Arntfield, 2012a), respectively, lower than the 95 ℃ that was used in the heating regime.

4.3.7 Statistical analysis

All data were analyzed using Microsoft Excel and SAS 9.0 program (SAS Institute Inc., Gary, NC). Tukey's test following the analysis of variance indicated significant different with a level of $p < 0.05$.

4.4 Results and discussion

4.4.1 Competitive binding between selected volatile flavours to CPIs and PPIs

4.4.1.1 Competitive binding between homologous ketones

Fig. 4.1 illustrates the binding pattern of a homologous series of ketones and aldehydes to CPIs (A) and PPIs (B) with and without heat treatment. In the ternary binding system which is composed of three ketones (Fig. 4.1 a & c), 2-octanone exhibited the highest binding affinity to proteins followed by 2-heptanone and 2-hexanone. This observation correlated well with order of ketone flavour retention in the simple protein-flavour system (control). It appears that the individual protein-ketone binding affinities are beneficial to predict the behaviour of retention of ketone flavours in a ternary competitive binding environment.

A trend towards competitive binding was observed for the mixtures of ketones (Figs. 4.1 a & c). While the binding of 2-hexanone and 2-heptanone to CPIs and PPIs were not significantly different and comparable to the control, there was a significant increase in the binding of 2-octanone by PPIs. This would suggest that 2-octanone can compete more effectively for available binding sites on proteins than 2-hexanone and 2-heptanone. Preferential binding of 2-octanone was previously reported in a protein-flavour system when only one flavour was included (Heng et al., 2004).

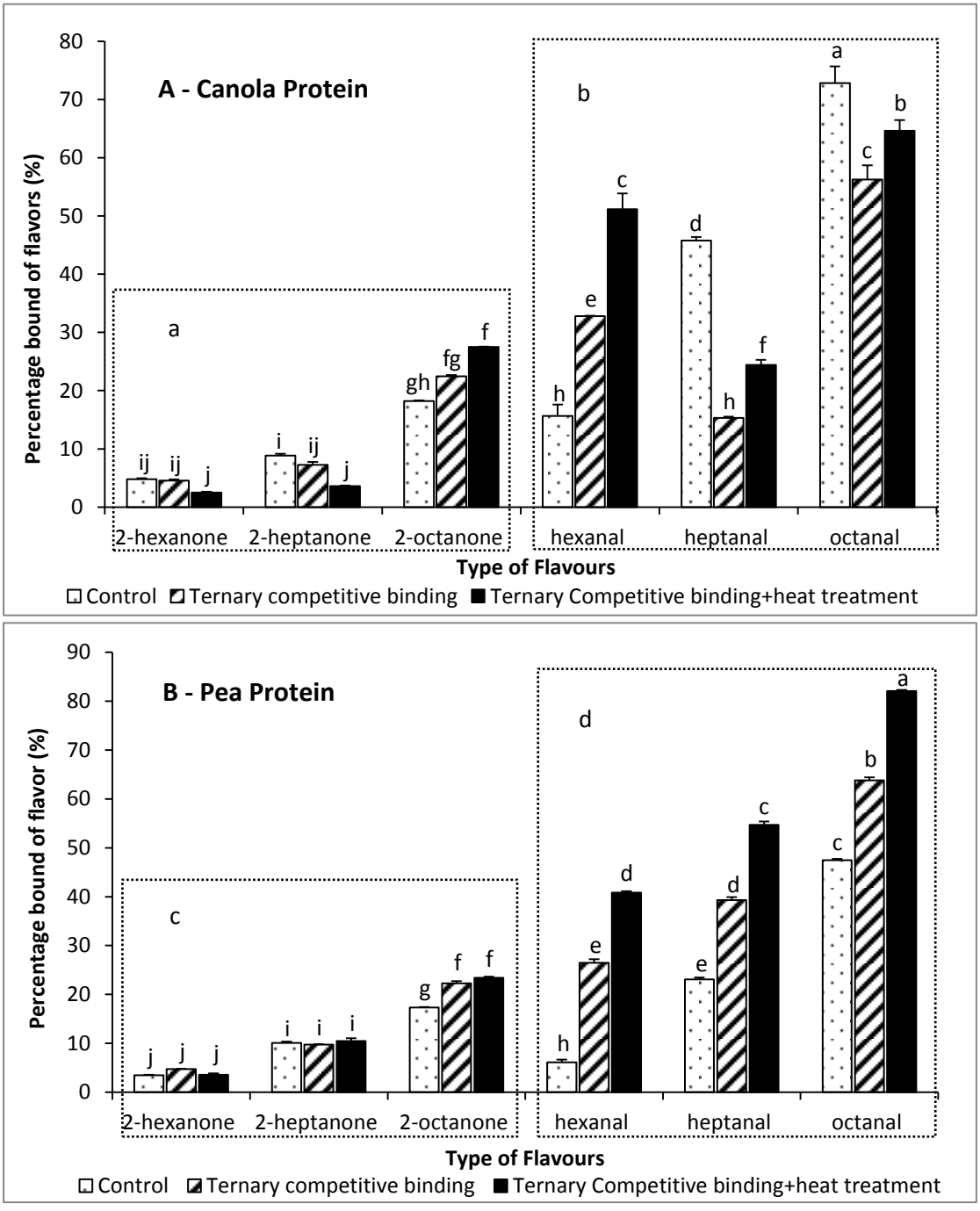


Figure 4.1 Percentage retention of homological series of ketone and aldehyde flavours at 250 ppm in a competitive binding environment with and without heat treatment (95℃, 30 min) (A) flavour binding to CPIs; (B) flavour binding to PPIs.

[a~j] In each figure, bar values followed by the same superscript are not significantly different (P<0.05) To demonstrate the statical analysis, ANOVA table of Fig. 4.1 A was presented in Appendex B as an example.

Note: control represents the percentage of bound flavour when the protein was mixed with a single flavour. The dash lines have been included to clearly identify the two types of flavours such that the ketones are shown in Fig. 4.1a and c and the aldehydes in 1b and 1d.

In the ternary system, despite the fact that the three ketones were mixed to give a total concentration of 750 ppm, the binding of all ketones was relatively stable (Fig. 4.1 a & c). It may be speculated that different ketone flavours bound to different sites on CPIs or PPIs (e.g. central cavity vs. hydrophobic surface pocket) such that increasing overall flavour concentration did not affect individual flavour binding affinities (Tromelin & Guichard, 2006). Tromelin and Guichard (2006) found that β-ionone and γ-decalactone preferentially bind to different sites on β-lactoglobulin uing 2D-NMR techniques. In contrast, binding could take place on same sites on proteins (e.g. central cavity or hydrophobic surface pocket). In this case, a flavour concentration of 750 ppm may not have been sufficient to fully saturate existing binding sites on proteins and only a limited competitive binding resulted. Kinsella (1990) proposed that increasing initial flavour concentration leads to an increase of the amount of binding as long as there are free binding sites available. This is speculative as the actual saturation point for these proteins is unknown at this time.

To better probe the binding sites and unveil the relations between type of flavours and binding sites involved, it would appear that more comprehensive studies involving methods which were traditionally used to study β-lactoglobulin-ligand interactions such as NMR (Tromelin & Guichard, 2006; Tavel et al., 2008), Four transform infrared spectroscopy (Kanakis et al., 2013; Li, Ma, & Ngadi, 2013), fluorescence techniques (Gholami & Bordbar, 2014; Kanakis et al., 2013), molecular docking (Gholami & Bordbar, 2014; Sahihi, Ghayeb, & Bordbar, 2013; Kanakis et al., 2013) and molecular dynamics methods (Gholami & Bordbar, 2014; Sahihi, Ghayeb,

& Bordbar, 2013) may be necessary.

4.4.1.2 Competitive binding between homologous aldehydes

Fig. 4.1 b shows that there is a significant increase in binding of hexanal to CPIs when included as part of a mixture rather than as a single compound; however, the binding of heptanal and octanal were dramatically decreased inferring a competitive situation was created by the mixture. Compared to the homologous series of ketone flavours binding with CPIs (Fig. 4.1 a), larger decreases or increases in flavour retention were observed in Fig. 4.1 b. It would appear that there was more competition between aldehyde flavours for binding sites on CPIs.

The increased binding for hexanal may be attributed to the high affinity of CPIs to a smaller molecular weight volatile. Gremli (1974) showed that the retention of ketones to 5% (w/v) soy protein increased from 2-hexanone up to 2-decanone but decreased when flavours larger than 2-decanone. Damodaran and Kinsella (1981a) also found the position of the keto group affected flavour binding affinities to soy proteins decreasing in the order: nonanal (1094 M^{-1}) > 2-nonanone (930 M^{-1}) > 5-nonanone (541 M^{-1}). Less binding was obtained when a keto group was in the centre of the molecule. The authors attributed this to the steric hindrance of keto group limiting access of flavours to the binding sites necessary for hydrophobic interactions. In a similar way, the conformational hindrance effect resulting from the structure of the larger flavour molecule could prevent access of heptanal and octanal to the binding sites further reducing their binding. In addition, the higher binding affinity of octanal control compared to the heptanal control led to a much lower

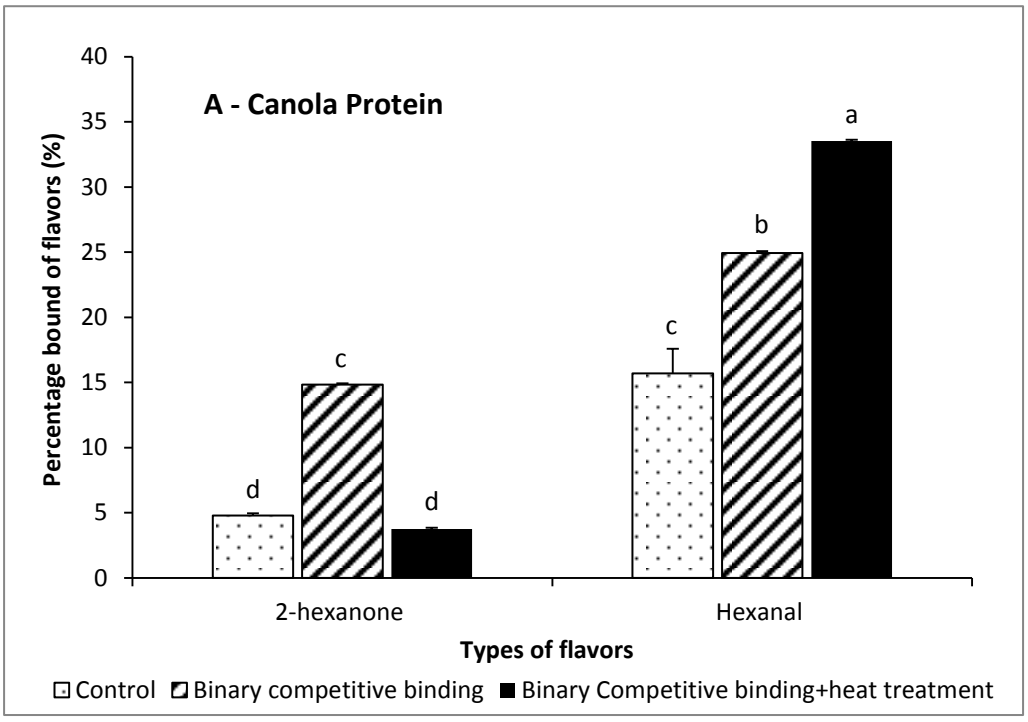
decrease in the flavour retention for octanal (16.57%) compared to heptanal (30.45%) in the ternary system indicating the importance of flavour intrinsic affinity to proteins even in the competitive binding environment.

In contrast, the binding of all aldehydes was significantly increased by 16.36 to 20.40 % for PPIs compared with the control (Fig. 4.1 d). It appears that cooperative effects rather than competition exist between aldehydes with this protein. Solms, Osman-Ismail, and Beyeler (1973) proposed a model and mechanism of flavour binding to proteins in which strong hydrophobic interactions along with protein conformational change are the major reasons accounting for the binding reactions. This was further demonstrated by Wang and Arntfield (2014) and emphasized that upon reaction of aldehyde flavours with pea proteins, the protein itself tended to lose its structure (partially unfolding) and exposed previously buried nonpolar residues. These revealed nonpolar residues were stabilized by further binding with hydrophobic flavours, which in turn, initiate more binding (Tanford, 1964). Based on this theory, protein conformational changes (partial denaturation) could account for the dramatic increase in the binding of aldehyde mixtures to PPIs (Fig. 4.1 d).

Even so, a thorough understanding of the different binding behaviours of mixed aldehydes between CPIs and PPIs would require more comprehensive approaches as indicated above.

4.4.1.3 Competitive binding between heterological 2-hexanone and hexanal

When 2-hexanone and hexanal were added together, increased binding for both types of flavours resulted, indicating possible cooperative effects (Fig. 4.2 A & B). It was noted that these cooperative effects were not found when ketones alone bound with proteins, but were observed when homologous aldehydes were mixed and heterological hexanal and 2-hexanone were added together. This implies that the presence of aldehyde may shift the protein-flavour binding characteristics from a competitive binding pattern to a situation where flavour retention is enhanced for all flavours. All these observations could be attributed to the potential unfolding of the protein induced by aldehyde flavours (Wang & Arntfield, 2014), creating more binding sites which are not only available for aldehyde flavour itself but also be available to other flavours such as ketones.



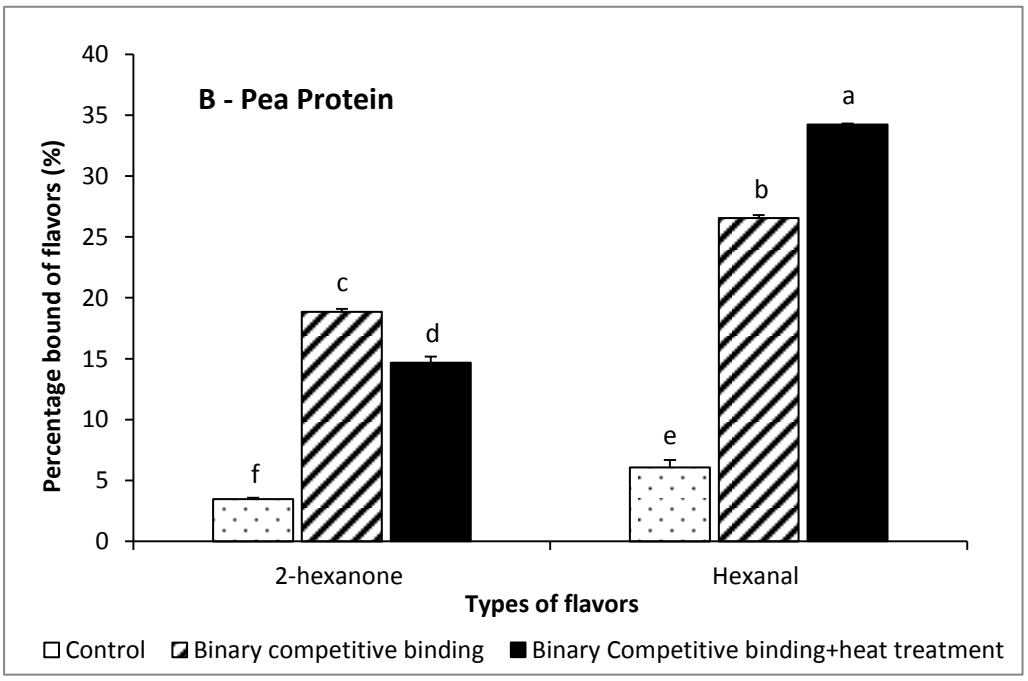


Figure 4.2 Percentage retention of heterological 2-hexanone and hexanal at 250 ppm in a competitive binding environment with and without heat treatment (95℃, 30min) (A) flavour binding to CPIs; (B) flavour binding to PPIs

[a~f] In each figure, bar values followed by the same superscript are not significantly different (P<0.05) To demonstrate the statical analysis, ANOVA table of Fig. 4.2 A was presented in Appendex A as an example.

Note: control means percentage of bound flavours in the simple protein-flavour system (only one flavour exists)

4.4.1.4 Protein thermal behaviour in competitive binding systems

Salt-extracted pea protein isolates (PPIs) were chosen as examples to illustrate the effect of flavour binding on protein thermal behaviours in competitive binding systems. Table 4.1 showed that addition of carbonyl flavour compounds did not significantly alter the pea protein denaturation temperature (T_d); however, compared with control, significant decreases of enthalpies of denaturation (ΔH) were observed inferring potential conformational changes in PPIs. In DSC analysis, as enthalpy of denaturation (ΔH) indicates amount of energy required to completely denature a protein molecule, it appears that partial denaturation (unfolding) of PPIs has resulted from the presence of these flavour compounds; hence, less energy was necessary to denature the protein molecule during DSC analysis. This effect has been previously reported to be more evident for aldehyde flavour with a longer chain length (Wang & Arntfield, 2014).

When the three aldehydes (at 250 ppm each) were mixed with pea proteins, the PPIs exhibited a significantly lower ΔH (7.64 J/K) compared with when the respective singe aldehyde was added at 250 ppm (C_6: 11.27 J/K, C_7: 10.28 J/K, C_8: 8.87 J/K). This inferred that the aldehyde mixture resulted in greater protein denaturation compared to the individual aldehyde flavours. This increased protein denaturation could result in more binding sites being revealed and available for flavour binding. This supports the results seen in Figure 4.1. d where the addition of the three aldehydes at the same time resulted in greater flavour retention, possibly due to greater unfolding of the pea proteins.

Table 4.1 Effect of flavour binding on the thermal properties of salt-extracted pea proteins

Flavour types	Carbon number	Denaturation temperature (T_d, ℃)	Enthalpy of denaturation (ΔH, J/K)
Control[a]		89.98 ± 2.18a	14.53 ± 0.14a
Aldehyde flavours[b]	C_6	88.56 ± 0.10a	11.27 ± 0.08cd
	C_7	91.12 ± 4.23a	10.28 ± 0.40de
	C_8	86.59 ± 0.02a	8.87 ± 0.16e
Ketone flavours[b]	C_6	92.91 ± 0.99a	12.85 ± 0.35b
	C_7	91.61 ± 2.84a	11.93 ± 0.05bc
	C_8	89.76 ± 0.19a	11.00 ± 0.62cd
Mixture of three aldehydes[c]		86.22 ± 0.09a	7.64 ± 0.47f
Mixture of three ketones		88.18 ± 0.47a	11.35 ± 0.04c
Mixture of hexanal and 2-hexanone		88.38 ± 0.70a	10.76 ± 0.08cd

a~f Column values followed by the same superscript are not significantly different (P<0.05)

[a] Control was not added with flavour compounds

[b] In the simple protein-flavour system, only one flavour was added at 250 ppm. C_6 indicates the respective aldehyde or ketone flavour with six carbon number.

[c] In the binary or ternary protein-flavour systems, each flavour was added at 250 ppm.

In contrast, this cumulative effect of decreasing in ΔH was not found for the mixture of ketone flavours (Table 4.1), where the ΔH (11.35 J/K) was noted to be in the middle of the enthalpy of 2-hexanone (12.85 J/K) and 2-octanone (11.00 J/K). This result supports the binding patterns for mixed ketones observed in Fig. 4.1 c where the level of ketone binding was similar to that obtained when they were present individually.

The ΔH of the mixture of hexanal and 2-hexanone (10.76 J/K) was significantly lower than 2-hexanone (12.85 J/K) but was not significantly different from hexanal (11.27 J/K) possibly due to the lower "unfolding capacity" of 2-hexanone with respect to protein conformational change (Wang & Arntfield, 2014). The decrease in ΔH of this flavour mixture compared with the individual flavour compounds may have

accounted for the dramatic increases in the binding of both flavours seen in Fig 4.2.

4.4.2 Effect of heat treatment on flavour binding

4.4.2.1 Effect of heating time on binding of hexanal to CPIs

Salt-extracted canola protein isolates (CPIs) was selected to demonstrate the effect of heat on flavour binding as a function of heating time. In Fig. 4.3, headspace signal of hexanal gradually decreased with time suggesting increasing binding of hexanal by CPIs upon heating. In the first 30 seconds, the percentage of hexanal bound to CPIs significantly increased by about 32% from 14.34 to 44.25% showing an extremely high affinity to the revealed binding sites exposed with protein unfolding during heat treatment. With further heating, the retention of hexanal continuously increased up to 66.85% over the first 10 min and then remained constant.

Overall, the result was in agreement with Damodaran and Kinsella (1981a) and Li, Grün, and Fernando (2000) who investigated the thermodynamics of flavour binding to soy and pea proteins and found that the interaction was thermodynamically favourable and spontaneous. This was supported by the continuous increase of binding of hexanal under heating seen in Fig 4.3.

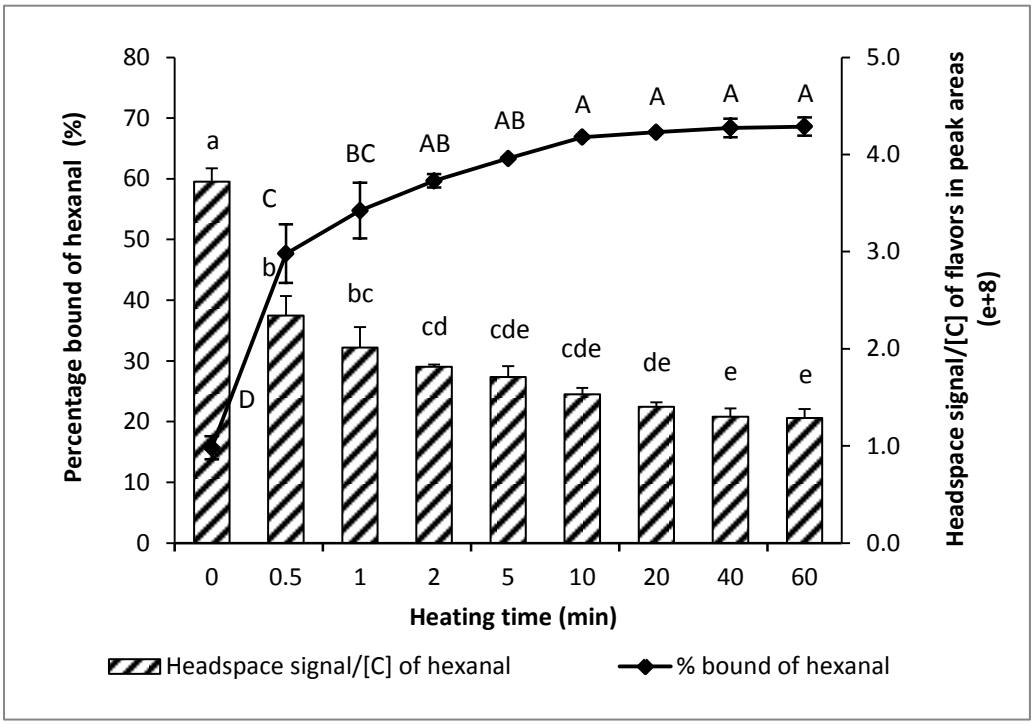


Figure 4.3 Percentage bound of hexanal to CPIs at 95℃ with increasing heating time and headspace signals of hexanal.

[a~e, A~D] In each legend, bar values followed by the same superscript are not significantly different (P<0.05)

In a similar study investigating the effect of heat on 1-octanal bound to whey protein isolate with increasing heating time at 80℃ (Kühn, Considine, and Singh, 2008), flavour retention was constant for the first 10 min followed by a slight increase after 20 minutes of heating. As aldehyde flavours bind to proteins via both reversible hydrophobic and irreversible covalent interactions (Tromelin, Andriot, & Guichard, 2006), the authors attributed this to the equilibrium between the release of reversibly bound 1-nonanal and retention of irreversibly bound 1-nonanal. If their hypothesis was to be applied to the results in the current study, the amount of irreversibly covalently bound hexanal upon heating should be much more than the amount of reversibly hydrophobically bound hexanal released, thereby accounting for the overall increase in flavour retention. Furthermore, Mills and Solms (1984) proposed the idea

that increasing temperature tended to reinforce the binding of "reactive" flavours such as aliphatic aldehydes.

4.4.2.2 Effect of heating time on binding of 2-octanone to CPIs

Contradictory results have been reported in terms of the effect of heat on protein binding with ketone flavours. Increased binding during early stages of protein unfolding and decreased binding at later stages when protein aggregation occurs have been suggested by Kühn, Considine, and Singh (2006, 2008) to explain these differences. In this current study, this initial increase in flavour binding followed by a decrease was clearly observed when 2-octanone was added to CPIs while heating at 95℃ with increasing heating time (Fig. 4.4).

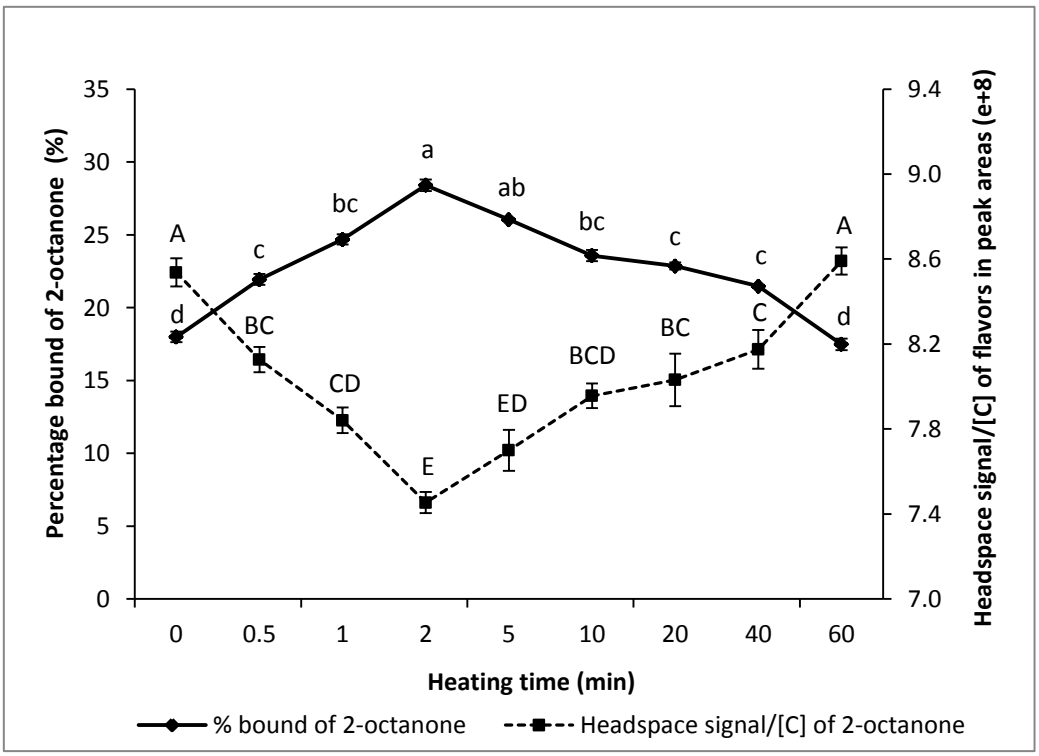


Figure 4.4 Percentage bound of 2-octanone to CPIs at 95℃ with increasing heating time and headspace signals of 2-octanone.

[a~d, A~D] For each parameter, points followed by the same superscript are not significantly different (P<0.05)

In the first 2 min of heating, the retention of 2-octanone increased from 17.99 to 28.4% possible due to exposure of previously buried hydrophobic regions that allowed more binding. As heating proceeded, binding dropped from 28.40 to 17.48% in the following 58 min. This transition of flavour binding from increasing to decreasing indicated that increased protein-protein interactions resulting from heat-induced aggregation of proteins replaced protein-flavour interactions which consequently led to release of previously bound volatile flavour compounds. Yüksel and Erdem (2005) demonstrated that, using a constant heating time of 5 min, the affinity of ANS towards milk proteins and number of protein surface hydrophobic sites decreased when temperature exceeded 90 ℃ compared with 70 and 80 ℃ indicating possible heat-induced protein aggregation. A similar pattern was observed for salt-extracted beef protein where protein surface hydrophobicity gradually increased with temperature from 20℃ to 70℃ but abruptly dropped when temperature reached 70℃ (Li-Chan, Nakai, & Wood, 1984). In addition, it was noted that this typical flavour releasing pattern was not observed when heating CPIs-hexanal flavour mixtures under the same condition, demonstrating that the major interactions between protein and aldehydes, predominantly covalent bindings, are irreversible while the hydrophobic interactions between protein and ketones are reversible.

As noted, the heating times presented in previous research were generally longer than two minutes (Ng, Hoehn, & Bushuk, 1989b; Gkionakis, Tayor, Ahmad, & Helipoulos, 2007; O'Neill & Kinsella, 1987a; Kühn, Considine, & Singh, 2008). According to the time dependent changes in the current study, the large difference in

the previous binding studies may simply be a function of heating time and the time when protein-protein interactions start to replace protein-flavour interactions.

Kühn, Considine, and Singh (2008) evaluated 2-octanone bound to a whey protein isolate with increasing heating time at 80℃. A continuous decrease of binding was observed from 1 to 80 min. The authors attributed this to the aggregation of protein molecules, resulting in exclusion or release of the flavour compounds previously bound to the proteins. They also proposed that the retained flavour binding ability of large aggregated whey proteins possibly on the protein surface may account for the bound 2-octanone (26%) that remained after the heat treatment. Based on the continuing increase of the headspace signal of 2-octanone up to 60 min (Fig. 4.4), it seemed that the heat treatment (95℃, 60 min) did not completely denature CPIs; further heating may cause higher extend of protein aggregation and greater release of 2-octanone until the protein is completely denatured. However, a certain level of flavour retention should be maintained due to presence of binding sites on the protein surface.

4.4.2.3 Effect of heat treatment on binding of composite aldehydes

Regardless of the competitive binding behaviour, the retention of all aldehydes by CPIs and PPIs were dramatically increased as a result of the heat treatment (Fig. 4.1b, d and Fig. 4.3). For CPIs, heating enhanced binding of hexanal by 18.35% and heptanal and octanal by about 11.06 and 8.33%, respectively. CPIs had a higher affinity to the small molecular weight volatile as noted previously without heating. Under heat treatment, aldehyde flavour retention was also higher for PPIs, with

increases between 14.35 to 18.25%. These observations can be explained based on the heat unfolding the protein structure revealing previously buried hydrophobic residues that were able to bind flavours (Kühn, Considine, & Singh, 2006). Our results related well with those of Ng, Hoehn and Bushuk (1989a) and Gkionakis, Heliopoulos, Taylor, and Ahmad (2006) who found heat-denatured fababean protein micelliar mass (95℃, 15 min) and soy protein (60℃) possessed 35.6% and 20% higher binding capacity to vanillin and lactones than the unheated form of these proteins.

4.4.2.4 Effect of heat treatment on binding of composite ketones

Heat treatment further promoted competitive binding between ketone flavours to CPIs (Fig. 4.1 a), while the binding of ketones to PPIs was nearly unaffected (Fig. 4.1 c). The competitive binding seen for ketones with CPIs can be attributed to heat-exposed binding sites first selectively binding with stronger 2-octanone rather than other ketones. As noted above (Section 4.4.2.2), ketones reversibly bind with proteins and previously bound ketones can be released upon heating. The higher individual affinity of 2-octanone than 2-heptanone and 2-hexanone to proteins may cause newly bound 2-octanone to be more resistant to be released when the protein aggregated, resulting in its higher retention after heat treatment.

In general, overall binding of ketone flavours to protein (Fig. 4.1 a & c) was less affected compared to aldehyde flavours in the ternary system (Fig. 4.1 b & d) after being heated at 95℃ for 30 min. This may be explained by the fact that the protein binding sites revealed upon heating showed relatively lower binding affinity to ketones than to aldehydes as well as the tendency of bound ketones to be released

upon heating. The relatively steady binding, especially for PPIs, suggested that ketone flavours may exhibit similar binding affinities to the previously existing hydrophobic pockets and the rearranged binding sites after heat treatment either on protein surface or in buried hydrophobic regions. In addition, the amount of ketones bound during heat-induced protein unfolding and the amount of ketones released due to thermal-induced protein aggregation might appear to be similar. It can be speculated that further heating of mixed ketones may result in greater releases of the low affinity 2-hexanone and 2-heptanone. Other researchers have observed a decrease binding of ketones when subjected to heat treatments (O'Neill & Kinsella, 1987a; Heng et al., 2004; Kühn, Considine, & Singh, 2008).

4.4.2.5 Effect of heat treatment on binding of heterological 2-hexanone and hexanal

As shown in Fig. 4.2, even though the presence of aldehyde flavour enhanced overall binding of 2-hexanone and hexanal, the heat treatment dramatically altered the overall flavour binding behaviours. It seemed that competitive binding between these two flavours was initiated. Upon heating, binding of hexanal to CPIs increased from 25% to 33% but this was accompanied by a dramatic decrease in the binding of 2-hexanaone from 15% to 4% (Fig. 4.2 A). A similar pattern was followed by PPIs (Fig. 4.2 B). This dramatic transformation of flavour retention can be attributed to the higher protein binding affinity of hexanal compared to 2-hexanone and the nature of the interactions where irreversible covalent linkages associated with hexanal flavour binding appear to make it much more competitive than 2-hexanone which associate

through reversible non-covalent hydrophobic interactions. Additionally, the potential release of 2-hexanone as protein aggregation occurs under heating may also contribute to the decrease of binding of 2-hexanone when mixed with hexanal.

4.5 Conclusion

In a competitive binding situation, ketone flavours generally followed the trends that they exhibited in simple protein-flavour systems. More complex binding behaviour was observed when a mixture of aldehyde flavours was analyzed. The cumulative effects for homologous series of aldehydes of decreased ΔH values indicated their possible cooperative effect on changes in protein conformation. This trend was not seen for composite ketones. More importantly, the ΔH values for mixed aldehydes and mixed ketones were both correlated well with the level of flavour binding. A continuous increase in binding of hexanal when heating CPIs with time indicated binding of aldehyde was irreversible, while the initial increase in binding with 2-octanone followed by a decrease with longer heating times demonstrated ketone was reversibly bound to CPIs. In general, heat treatment at 95℃ for 30 min significantly promoted binding of aldehydes with proteins regardless of the binding behaviour; however, retention of ketones was not largely affected indicating heat exposed binding sites did not favour ketone binding and binding of ketones during heating process was most likely reversible. When creating flavours in the food industry, the sensory profile cannot be simply considered as a combination of each flavour compound. In addition, heat treatment heavily modified the original flavour profile. In a competitive binding system, the individual affinities of flavours to

proteins (e.g. hexanal *vs.* 2-hexanone) as well as the reversibility of flavours need to be considered when determining the effect of heat on protein-flavour interactions.

Connections between Chapter 4 and Chapter 5

Chapters 3 and 4 evaluted effects of some intrinsic factors including flavour class, flavour carbon numner, protein source and isolation method and extrinsic parameter such as heat treatment on protein-flavour binding performance. However, two more practical extrinsic parameters which are frequently involved in protein analysis are the addition of salts and varying of pH. These two parameters could alter protein structure and thus greatly impact protein-flavour binding affinities. As some conflicting results have been reported previosuly, the objective of Chapter 5 was to provide a fundenmental evaluation of the effect of salt and pH on protein-flavour interactions and relate how the changes in proteins connect to resulting flavour binding properties. In addition, by including a series of salts in the lyotropic series, the molecular forces involved in the protein-flavour interactions could also be analyzed. Because of the size of the experiment it was felt that working with only the pea protein would provide the basis for understanding the relationship between, salt, pH and protein flavour interactions. As a result, the canola protein isolate was not included in the next or subsequent studies

Chapter 5 Effect of Salts and pH on Selected Ketone Flavours Binding to Salt-Extracted Pea Proteins: The Role of Non-Covalent Forces

5.1 Abstract

The effects of salts and pH on a mixture of homologous ketone flavours (2-hexanone, 2-heptanone and 2-octanone) binding to salt-extracted pea protein isolates (PPIs) were studied using GC/MS in an aqueous model system. Ionic strength, the specific flavour compound and pH were found to significantly affect protein-flavour binding. Comparing the univalent (NaCl) and divalent ($CaCl_2$) cations, higher concentrations of NaCl (0.25-1 M) greatly promoted protein-flavour binding compared to $CaCl_2$, while lower concentrations (0.05-0.1 M NaCl and 0.25 M $CaCl_2$) decreased flavour retention. Considering the effect of anions (0.5 M), flavour binding by PPIs was dependent upon the position of the anions in the lyotropic series such that binding decreased in the order: Na_2SO_4 >> NaCl > $NaCH_3COO$ = no salt > NaSCN. In terms of the effect of pH, overall flavour binding followed the order: pH 5 > pH 7 > pH 9 > pH 11 > pH 3. Competitive binding between the selected flavour mixture and PPIs was also observed as evidenced by 2-octanone more effectively binding to PPIs than 2-heptanone and 2-hexanone. This research supported existing knowledge on the effects of salts and pH on flavour partitioning with and without protein present. This further promotes utilization of plant proteins in aqueous food systems to achieve desired flavour attributes.

5.2 Introduction

When creating and formulating flavours, product formulation warrants consideration because interactions with proteins can influence flavours in the finished foods (Wright, 2002). This specific interaction has been known to reduce desirable aroma intensity (Heng et al., 2004; Kühn, Considine, & Singh, 2006; Wang & Arntfield, 2014, 2015) and possesses the potential to produce volatile flavour by-products (Kühn, Considine, & Singh, 2008; Wang & Arntfield, 2014). It has been agreed that non-covalent interactions including hydrophobic and electrostatic interactions, hydrogen bonds and van der Waals forces as well as covalent chemical linkages are the major forces responsible for binding between protein and flavours (Kim and Min, 1989; Solms, Osman-Ismail, & Beyeler, 1973; Tromelin et al., 2006; van Ruth & Roozen, 2002). By investigating protein-flavour binding patterns in an aqueous model system, information will be obtained that could aid flavour chemists and food product developers to flavour protein-containing beverage products so as to provide desirable flavour attributes (Suppavorasatit & Cadwallader, 2012; Suppavorasatit, Lee, & Cadwallader, 2013).

When considering the influence of solvent on protein-flavour binding characters, the impact of salts and pH are of particular interest not only because of their practical application, but also because they can be used to conduct fundamental investigations on molecular forces between protein and flavours. Moreover, limited information exists on plant proteins in terms of the effect of salts and pH on protein-flavour interactions. Most of the literature has focused on milk (Jouenne & Crouzet, 2000;

Mills & Solms, 1984; van Ruth & Villeneuve, 2002) and porcine protein extracts (Pérez-Juan, Flores, & Toldrá, 2007, 2008).

Ionic strength and salt type in the medium can affect protein conformation such that the interactions between protein and flavours may be modified (Lubbers, Landy, & Voilley, 1998). Conflicting results with different protein-flavour binding behaviours have been reported at different NaCl levels (Andriot et al., 1999; Guichard & Langourieux, 2000). Limited research is available on how protein-flavour interactions are affected as a result of different type and concentration of salts for plant proteins, and specifically salt-extracted pea protein isolates.

Particularly, anions of sodium salts tend to stabilize or destabilize proteins following the lyotropic/Hofmeister series: (stabilizing) $F^- > SO_4^{2-} > Cl^- > Br^- > SCN^- > Cl_3COO^-$ (destabilizing) (Arntfield, Murray, & Ismond, 1990). As adding salt in lyotropic series directly relates to the charge profile (electrostatic interactions) and hydrophobic interactions of proteins (Damodaran & Kinsella, 1981c; Melander & Horváth, 1977; Sun & Arntfield, 2012; Zhang & Cremer, 2006), the influence of different salts on protein-flavour interactions could help explain the conflicting results and address the importance of both hydrophobic and electrostatic interactions in protein-flavour association.

A strong influence of pH on protein-flavour binding has been shown for β-lactoglobulin and whey proteins (Jouenne & Crouzet, 2000; van Ruth & Villeneuve, 2002; Weel et al., 2003). It has been suggested that structural modification of proteins may account for the different protein-flavour binding behaviours. However, the

mechanisms responsible for binding differences have not been explicit. As altering pH can lead to denaturation and a change in protein charge (Vojdani, 1996), these changes, which influence intermolecular forces, may be crucial in elucidating the underlying mechanism of the influence of pH on protein-flavour binding profiles.

The present paper investigated the effects of salts and pH on the flavour binding properties of salt-extracted pea proteins to a selected ketone flavour mixture. The role of non-covalent forces was inferred based on responses at different environmental conditions (salts and pHs). A homologous series of ketone flavours was selected as it was previously found that ketone flavours possessed minimum effects on steric unfolding of protein structure (Wang & Arntfield, 2014, 2015). In addition, potential competitive binding between three ketones to pea proteins was also evaluated (Wang & Arntfield, 2015).

5.3 Materials and methods

5.3.1 Source of materials

The homologous series of ketone flavours (2-hexanone, 2-heptanone and 2-octanone) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Commercial yellow pea flour (*Pisum sativum* L.) was kindly supplied by Best Cooking Pulses Inc. (Portage la Prairie, MB). Na_2SO_4, NaCl, NaSCN and $CaCl_2$ were obtained from Fisher Scientific (Fair Lawn, New Jersey). CH_3COONa was acquired from BDH Chemicals Ltd. (Toronto, ON, Canada). All other chemicals, including K_2HPO_4, KH_2PO_4, HCl and NaOH, were analytical grade and purchased from Fisher Scientific (Ottawa, Canada).

5.3.2 Isolation of salt-extracted pea proteins

Following the procedure previously described by Wang and Arntfield (2014, 2015), salt-extracted pea protein isolates (PPIs) was extracted from sieved (500 μm opening, USA Standard No. 35) yellow pea flour using 0.3 M NaCl. After the first centrifugation (4260*g*, 4℃, 15 min) using a Thermo Scientific Sorvall RC6 Plus Centrifuge (Langenselbold, Germany), pea protein was recovered by diluting the supernatant in two times its volume of cold distilled H_2O and leaving in a refrigerator (3℃) for 2 h. The precipitated protein was collected after a second centrifugation (680*g*, 4℃, 15 min) and then dialyzed against cold distilled H_2O. The desalted protein isolates were stored at -30℃ until freeze dried. The freeze dried protein sample contained 82.68 % protein using an N to protein conversion factor of 5.7 with a Dumas method and a FP-528 Nitrogen/Protein Analysis System (LECO Corporation, St. Joseph, MI).

5.3.3 Preparation of gas chromatographic samples

The method of Wang and Arntfield (2014, 2015) was followed with minor modifications to suit the purpose of the current research. In a typical experiment, to produce 1% (w/v) final protein solution with an individual flavour concentration of 250 ppm at desired salt concentration (e.g. 0.5 M NaCl), 0.5 mL of 4 % (w/v) protein solution (in 0.01 M potassium phosphate buffer at pH 8) was carefully loaded into a 20 mL reaction vial (22×75 mm, Product No.: 20-2100, Microliter Analytical Supplies, Inc., Suwanee, GA) followed by addition of 0.25 mL of phosphate buffer and 0.25 mL of 4 M NaCl stock solution (in buffer). To get different pH values (e.g.

pH 3), 25 mL of 4 % (w/v) PPIs were firstly prepared by mixing 1 g of PPIs with 10 mL of 0.05 M NaCl followed by adjusting the pH to 3 using 1 M HCl (in 0.05 M NaCl) and then filled to 25 mL with 0.05 M NaCl (pH 3). After that, 0.5 mL of resulting protein suspension (4%, w/v) was mixed with 18.75 μL of 4 M NaCl (pH 3) and 481.25 μL acidified water (pH 3) to an aliquot volume of 1 mL. The vials were sealed with Parafilm® and attached onto a Belly Dancer® Mixer (Stovall Life Science Inc., Greensboro, NC) with gentle shaking of the protein mixture at speed 6 for 1 h.

The flavour solutions were added last. As competitive bindings were evaluated, 1/3 mL of each 1500 ppm flavour stock solutions (0.15mL/100mL) in potassium phosphate buffer or pH adjusted water were added to the premixed 1 mL of 2% (w/v) protein dispersion to obtain a final concentration of 250 ppm for each flavour. The vial was immediately sealed. Samples were mixed using a Julabo SW22 shaking water bath (Julabo Labortechnik GMBH, Seelbach, Germany) at 30℃ and 125 rpm for 3 h before headspace sampling. Preliminary testing found that 3 h was adequate to reach equilibrium. Samples were prepared in duplicate and each sampled once.

5.3.4 Gas chromatographic and mass spectrometric analysis

Measurement of flavour binding to proteins was conducted using a Varian CP-3800 Gas Chromatography (Varian Chromatography Systems, Walnut Creek, CA) coupled with a Varian 320-MS Triple Quadrupole Mass Spectrometer (Varian, Inc., Walnut Creek, CA) operated in splitless and single quad mode. After mixing, samples were incubated and shaken for 14 min at 40 ℃ and 1 mL of sample headspace was aspirated into GC injector port by a CombiPal autosampler unit with PAL Itex-2

(In-Tube-Extraction) absorber attachment (CTC Analytics AG, Switzerland) after one absorption cycle. A VF-5ms column, which was 30 m in length, had 0.2 mm inner diameter and 20 μm film thickness, was run at 4 mL/min constant helium flow. The temperature was programed by heating the sample at a rate of 10℃/min to 265℃ and then held for three minutes.

A mass spectrometer was used to confirm the identity of volatile flavour compounds and further identify any volatile flavour by-products generated. Operating conditions for the mass spectrometer were 70 eV EI source with a mass range between 25 Da to 250 Da.

Percentage of flavours bound for each treatment was determined from the difference between the peak areas of flavoured samples in the absence and presence of proteins such that:

$$\text{Binding \%} = \left(1 - \frac{Peak\ area_{\ with\ protein\ added}}{Peak\ area_{\ without\ protein\ added}}\right) \times 100\%.$$

As all three flavours were mixed at the same time, the overall percentage of flavours bound was obtained from the difference between sum of peak areas of flavoured samples with and without presence of proteins. A separate control sample in which the proteins were not included but contained salt or varied in pH was included for each treatment to account for the potential effect of solvent on flavour headspace volatility.

5.3.5 Experimental design

To evaluate the effect of neutral salts and pH on flavour binding (after the addition of protein), three experiments were implemented. First, the impact of cations was studied by following the extent of flavour retention after adding NaCl and $CaCl_2$ at concentrations of 0, 0.05, 0.1, 0.25, 0.5 and 1 M. This was followed by examining the impact of anions where sodium salts in the lyotropic series (Na_2SO_4, $NaCH_3COO$, NaCl and NaSCN) were incorporated at concentrations of 0.05 and 0.5 M. Finally, the effect of different pHs on protein-flavour interactions was investigated by adjusting the pH to 3, 5, 7, 9 and 11. To accurately calculate the extent of flavour retention as a result of protein addition, it was necessary to determine the effect of solvent (salts and pHs) on flavour headspace concentration without protein. Once these values were obtained, the same conditions were evaluated in the presence of proteins and the results corrected for the volatility observed with protein as noted above.

5.3.6 Statistical analysis

All data were analyzed in duplicate using Microsoft Excel and SAS 9.0 program (SAS Institute Inc., Gary, NC, USA). Two way factorial experiments considering type of flavours (C6, C7 and C8) with concentration of salts (0, 0.05, 0.1, 0.25, 0.5 and 1 M), or type of salts (no salt, NaSCN, $NaCH_3COO$, NaCl and Na_2SO_4) or pH values (pH 3, 5, 7, 9 and 11) were conducted. Tukey's test following the analysis of variance indicated significant different with a level of $p < 0.05$.

5.4 Results and discussions

5.4.1 Effects of solvent (salts and pHs) on flavour headspace concentration

It was found that both type and concentrations of salt and flavour types in the absence of protein significantly affected flavour headspace volatility ($p < 0.05$) (Fig. 5.1 & Fig. 5.2). In addition, two-way interactions were noted between salt concentration and flavour types (Fig. 5.1) and between different types of salts and flavours (Fig. 5.2). As a result, it was necessary that each treatment of PPIs (protein + flavour + salt/pH) be coupled with a control sample (flavour + salt/pH) which contained no protein to account for this change in flavour headspace volatility simply due to the addition of salts and alteration of pH. The calculation used to determine the percentage of flavours bound, therefore, has accounted for this change in volatility due to the solvent alone.

When looking at the solvent effect alone, it is clear from Fig. 5.1 that overall headspace concentrations (signals in triangle) of added flavours were significantly increased when concentrations of NaCl and $CaCl_2$ increased above 0.25 and 0.5 M, respectively, inferring the addition of chloride salts facilitated partitioning of volatile flavours from the aqueous phase to the gaseous phase up to 1.5 times higher than the sample to which no salt was added. This effect became more evident when 0.5 M Na_2SO_4 was added (Fig. 5.2B) where a 2.5 times increase in overall volatile headspace signal was seen. The increases in volatiles in the headspace were also seen with 0.5 M $NaCH_3COO$ (1.4 times) and 0.5 M NaCl (1.3 times).

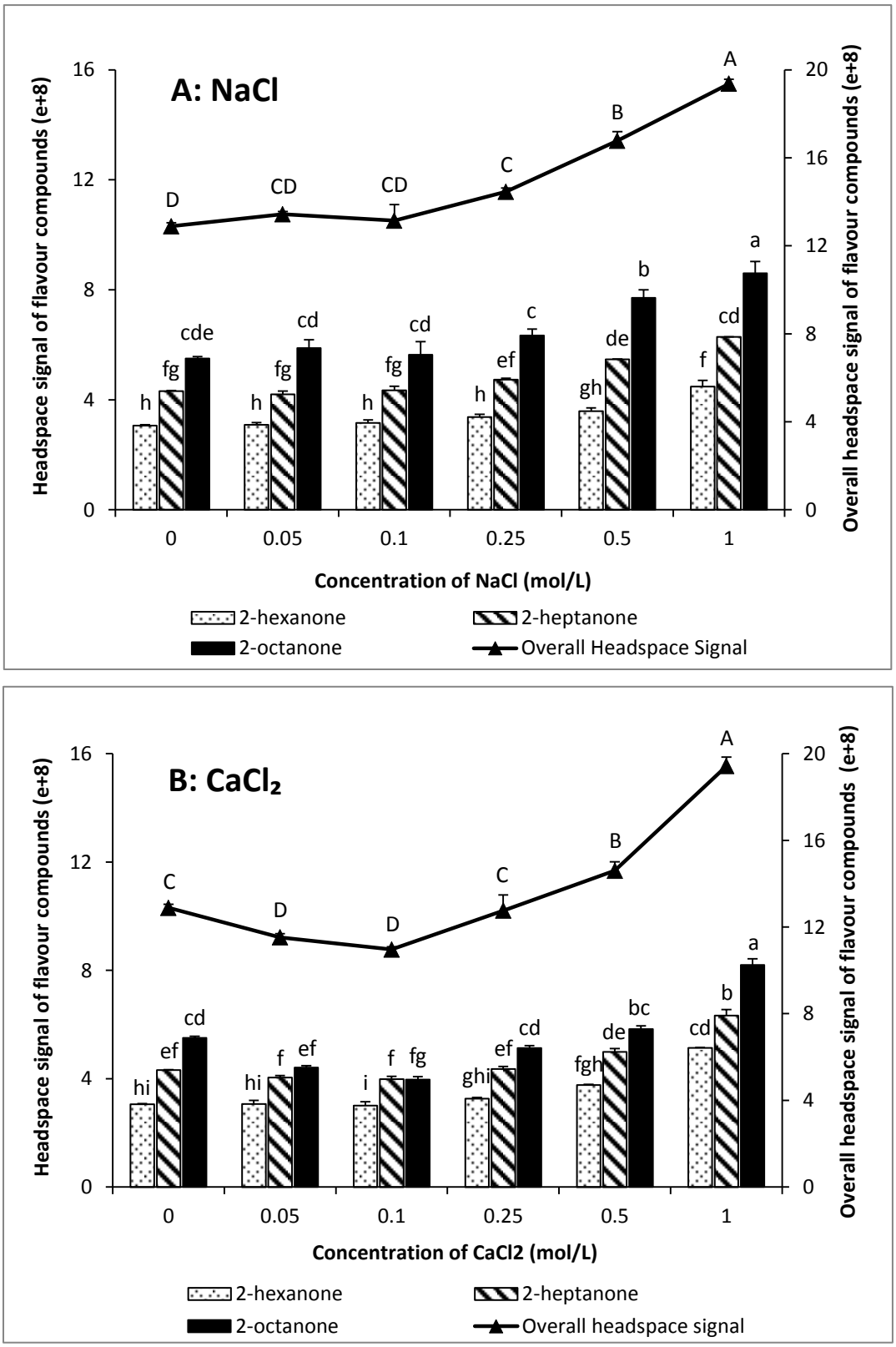


Figure 5.1 Effect of NaCl (A) and $CaCl_2$ (B) on headspace volatility of selected ketone flavour mixture at 250 ppm flavour concentration (without protein).

[A-D, h-i] In each figure, values followed by the same superscript are not significantly different ($p < 0.05$).

Pérez-Juan, Flores and Toldrá (2007) investigated different chloride salts on headspace concentration of aliphatic aldehydes and ketones in water. They found that presence of NaCl and KCl increased volatile headspace concentrations up to 5-10 times. $MgCl_2$ and $CaCl_2$ had a smaller effect and increased flavour volatility up to 2 times. Similar effects have also been reported by Poll and Flink (1984) who found, when NaCl was added to apple juice, volatile headspace concentrations of alcohols, aldehydes and esters increased more than 4 times, 1.75-3.5 times and less than 1.75 times, respectively. It has been concluded by Guichard (2002) and Reineccius (2006b) that addition of soluble salts to solution was able to drive organic volatiles from the aqueous phase into the gaseous phase such that the volatile concentration in the headspace was enriched. The different extents of enrichment may be attributed to the different types and concentrations of salts and flavours used. It is possible that the mixed flavour system in the current analysis minimized the effect of salt on individual headspace volatility.

In terms of salts in the lyotropic series, NaSCN at both 0.05 (Fig. 5.2A) and 0.5 M (Fig. 5.2B) were found to significantly decrease the overall flavour headspace concentration, although when looking at individual flavours only 2-octanone in 0.05 M NaSCN was significantly lower than the sample without salt. As a chaotropic salt, its influence on water and flavour compounds was not the same as that seen with the chloride anion and therefore did not have the same effect on flavour release.

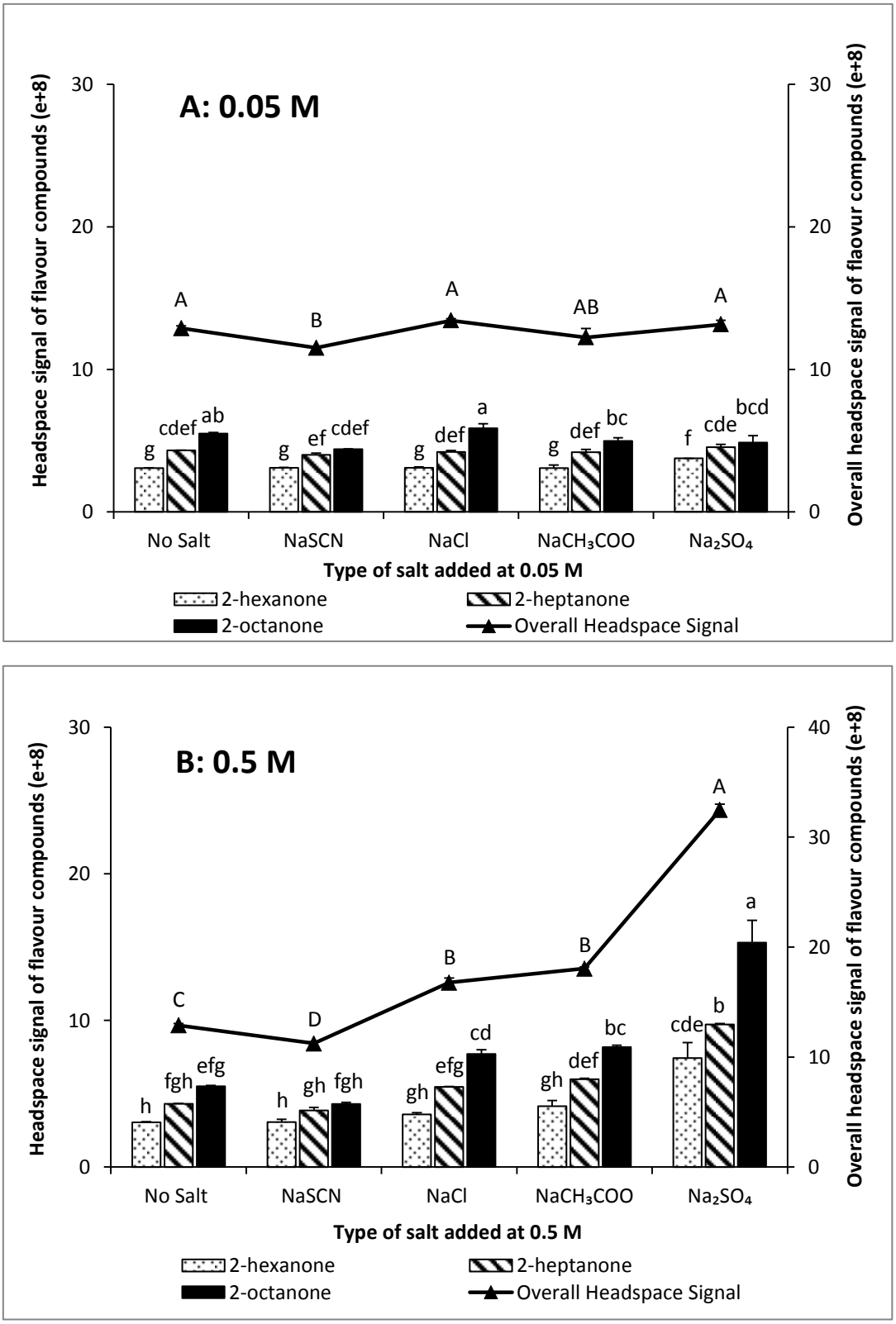


Figure 5.2 Effect of anions (NaSCN, NaCl, NaCH3COO and Na2SO4) at 0.05 (A) and 0.5 M (B) on headspace volatility of selected ketone flavour mixture at 250 ppm flavour concentration (without protein).

[A-D, a-h] In each figure, values followed by the same superscript are not significantly different ($p < 0.05$).

While increases in the total release of volatiles, similar to that noted for NaCl was seen for NaCl, $NaCH_3COO$ and Na_2SO_4 at a concentration of 0.5 M (Fig. 5.2B), at 0.05 M there was no significant change in the overall release of volatiles for these salts (Fig. 5.2A). The lack of effect with 0.05 M salts may simply indicate the concentration was too low to affect the partitioning of the ketones used in this study. These effects of NaSCN, $NaCH_3COO$ and Na_2SO_4 have not been reported in literature.

Additionally, pH was also noted to significantly alter the partitioning of volatiles from aqueous phase to the gaseous phase ($p < 0.05$) with pH 5 being shown to be the most effective at increasing flavour headspace concentration (Table 5.1). It is still not completely clear why no significant differences were found between pH 3, 7, 9 and 11 regarding to flavour headspace volatility.

5.4.2 Evaluation of effect of cations on protein-flavour binding performance

To investigate and compare the impact of cations on protein-flavour binding, NaCl (Fig. 5.3A) and $CaCl_2$ (Fig. 5.3B) at concentrations of 0, 0.05, 0.1, 0.25, 0.5 and 1 M were chosen. Results presented for volatiles in the presence of proteins have accounted for the effect of solvent on flavour headspace volatility by including a control sample (flavour + salt) for each treatment. For NaCl (Fig. 5.3A) and $CaCl_2$ (Fig. 5.3B), both salt concentration and flavour carbon number were found to significantly influence the binding between PPIs with the selected ketone flavours ($p < 0.05$).

5.4.2.1 Effect of NaCl

Fig. 5.3A shows that the retention of all three ketone flavours generally followed the same trend with a slight drop in degree of flavour retention being observed at 0.05 and 0.1 M NaCl, after which increasing NaCl concentration facilitated binding of all three ketones. At all conditions, 2-octanone bound PPIs more than 2-heptanone and 2-hexanone corresponding to the trend that was reported previously (Damodaran & Kinsella, 1981a, 1981b; Heng et al., 2004).

To understand the influence of NaCl on flavour binding, the impact of salt on protein stability and intermolecular forces must not be overlooked. At low NaCl concentrations, salts are believed to possess a non-specific ion effect involving electrostatic interactions between charged groups on the proteins (Damodaran & Kinsella, 1981c; Sun & Arntfield, 2010). When charged side chains on amino acid residues are neutralized by NaCl, proteins are known to be surrounded by a double layer of counter ions, which decreases inter-protein electrostatic interactions and promotes solubility of proteins due to charge repulsion (Damodaran, 1988; Ismond, Murray, & Arntfield, 1986; Vojdani, 1996). As Reineccius (2006c) stated that ionic interactions may be present between aroma compounds and proteins, the subsequently formed ionic layers could limit the interactions between proteins and flavours, causing the previously ionic-linked flavours to be released. This would account for the decreased retention of flavours at low salt concentrations and infer potential ionic interactions between PPIs and ketones. Andriot et al. (1999) reported that binding of benzaldehyde by β-lactoglobulin decreased from 25 to about 18 % when 0.05 M NaCl

was added.

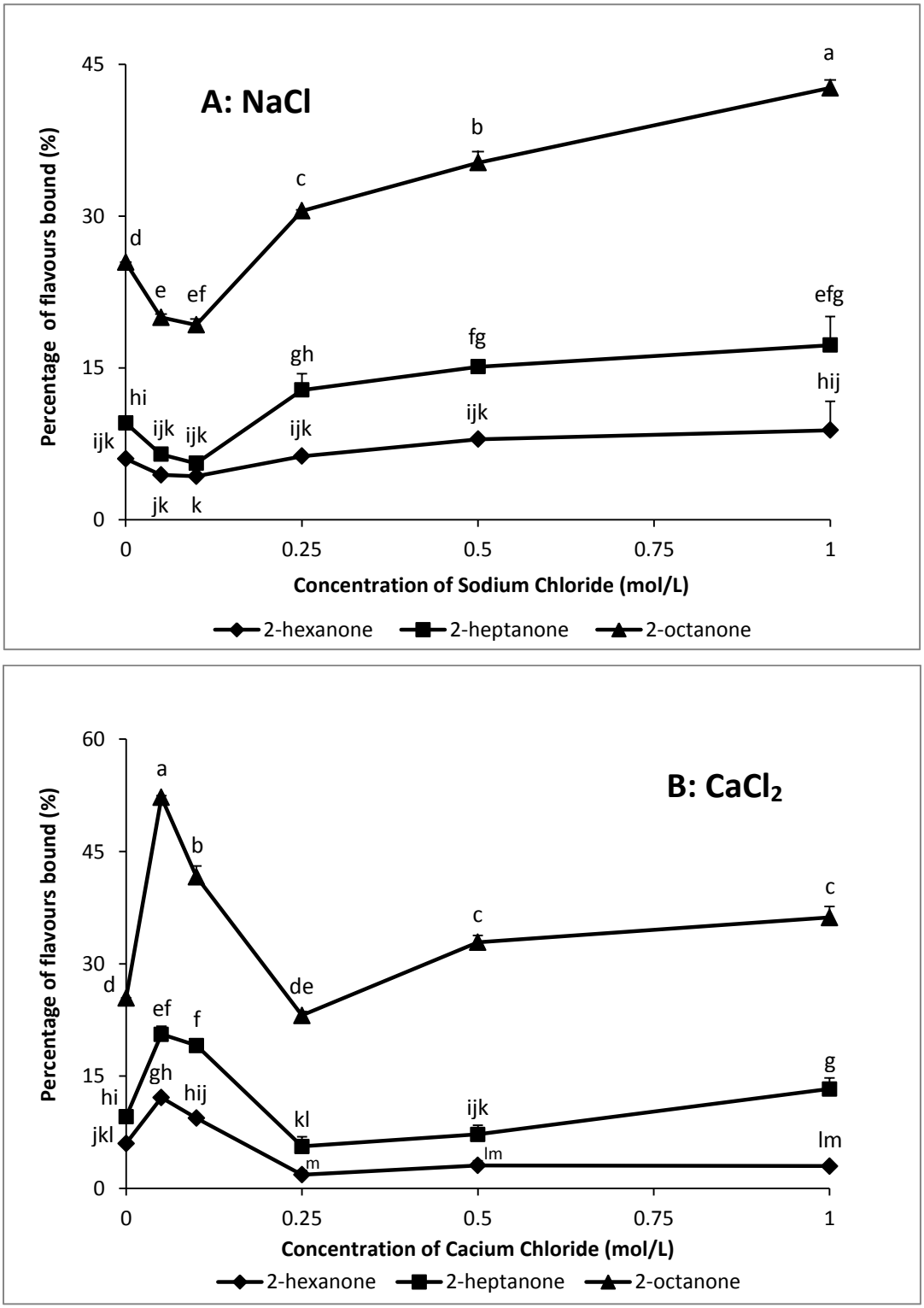


Figure 5.3 Effect of NaCl (A) and CaCl2 (B) on binding of selected ketones (at 250 ppm of individual flavour) to salt-extracted pea protein isolates (PPIs, 1%, w/v) as a function of added salt concentration.

[a-m] In each figure, points followed by the same superscript are not significantly different ($p < 0.05$).

More importantly, it was noted in Fig.5.3A that the decrease in binding at a NaCl concentration < 0.25 M was only statistically significant for 2-octanone. In spite of this, 19.25% of total added 2-octanone was retained by the protein at 0.1 M NaCl inferring that ionic interaction may only play a minor role in contributing to the binding of ketones to PPIs; other interactions such as hydrophobic interactions, hydrogen bonds and van der Waals forces may also be responsible for the retention of ketones by PPIs (van Ruth & Roozen, 2002).

At higher salt concentrations, it has been widely accepted that ion specific effects, also known as lyotropic effects, are predominant (Damodaran & Kinsella, 1981c). Increasing NaCl concentration promotes protein thermal stability which has been mainly attributed to the change in orientation of water molecules and subsequent ion-specific effect on protein hydrophobic association (Arntfield, Murray, & Ismond, 1990; Damodaran, 1988; Sun & Arntfield, 2012a). In other words, when high concentrations of salt were added, salt could perturb bulk water structure by causing preferential hydration on protein surface with exposure of thermodynamically unfavourable nonpolar residues consequently leading to protein nonpolar aggregation with increased hydrophobic interactions (Damodaran, 1988).

As protein-flavour interactions have been reported to be mainly hydrophobic in nature (Damodaran & Kinsella, 1981a; van Ruth and Roozen, 2002; Preininger, 2006), it is possible that this promotion of intramolecular hydrophobic interactions not only enhanced the hydrophobic association of proteins, but also promoted hydrophobic association between protein and flavours leading to increased flavour binding at high

salt concentrations. Kinsella (1990) observed a constant increase in binding of 2-octanone to bovine serum albumin when NaCl concentration increased from 0 to 4 M.

In addition, it can be seen from Fig. 5.3A that after the concentration of NaCl exceeded 0.1 M, the increase in binding of 23.43% for 2-octanone as the NaCl concentration went from 0.1 M (19.25%) to 1 M (42.68%) was much higher than similar increases for 2-heptanone (11.68%) and 2-hexanone (3.26%). This demonstrates the potential for competitive binding between three ketones to PPIs; long chain aliphatic ketone (2-octanone) competed more effectively than the other two compounds for binding sites on PPIs.

5.4.2.2 Effect of $CaCl_2$

During the analysis, it was seen that protein mixture became turbid when 1% (w/v) PPIs was prepared with 0.05 and 0.1 M $CaCl_2$. As shown in Fig. 5.3B, this phenomenon was accompanied by a sharp increase in binding of all three ketones. With a further increase in $CaCl_2$ concentration, the cloudiness decreased and gradually became clear as the $CaCl_2$ concentration reached 1 M.

As most plant proteins possess an isoelectric point between pH 4 and 6 (Boye, Zare, & Pletch, 2010; Vojdani, 1996), PPIs solution would expected to be negatively charged when prepared in phosphate buffer solution (pH 8) with a final pH value of 7.6 as recorded using a AB15 *Plus* pH Meter (Accumet® BASIC, Fisher Scientific). Based on the work of Arntfield, Murray and Ismond (1990), initial addition of $CaCl_2$ increased positive charge on proteins via calcium binding which counteracted the net

negative charge on proteins. As a consequence, this would prompt intermolecular electrostatic interactions between proteins causing proteins to aggregate or precipitate similar to what is seen at the isoelectric point, resulting in turbidity (Arntfield, Murray, & Ismond, 1990). As a result, it is possible that such strong hydrophobic association between proteins increased the magnitude of hydrophobic interaction with flavours.

On the other hand, Damodaran and Kinsella (1983) reported actomyosin, a fish protein, possessed higher affinity to 2-nonanone at 0.15 M NaCl than 0.6 M. They stated that at 0.15 M NaCl, actomyosin was in a form of insoluble protein fibres that promoted nonspecific surface adsorption (physical sorption) of 2-nonanone to the protein aggregates rather than a true binding at molecular level. Based on the turbidity that was seen at low $CaCl_2$ concentrations, the physical sorption of flavours onto the surface of aggregated PPIs may also contribute to the overall increase in the binding of selected ketones to PPIs (van Ruth & Roozen, 2009).

Despite the significant increase in flavour binding at 0.05 and 0.1 M $CaCl_2$, a release of bound flavours was noted at 0.25 M $CaCl_2$ and then the binding similar to that seen in buffer alone was restored when the $CaCl_2$ concentration reached 1 M. Similar to the impact of NaCl, the promotion of non-specific ionic effect due to calcium binding and increased hydrophobic association resulting from ionic specific effect may account for the changes in flavour binding as a function of added $CaCl_2$ concentration. In addition, further addition of $CaCl_2$ increased the net positive charge on the proteins causing protein molecules to repel which accounts for the increase in protein solubility.

Interestingly, at 0.25 M $CaCl_2$, the binding for 2-octanone was not significantly different from the control (no salt) while binding of 2-heptanone and 2-hexanone were significantly lower. The higher affinity of 2-octanone for PPIs could minimize its release at 0.25 M $CaCl_2$ inferring competitive binding between three flavours. In contrast, when the concentration of $CaCl_2$ increased from 0.25 to 1 M, PPIs exhibited a higher increase in binding for 2-octanone (13.07%) from 23.12 % (0.25 M) to 36.19% (1 M) than for 2-heptanone (6.06%) and 2-hexanone (1.16%) further demonstrating the preference for 2-octanone in the competition between selected flavours.

5.4.2.3 Comparison of effect of NaCl vs. $CaCl_2$

Comparing the effects of NaCl and $CaCl_2$ on protein-flavour binding efficacy (Fig. 5.4), it is clear that 0.05 to 0.1 M NaCl significantly decreased flavour binding; however, significant increases in flavour binding were observed above 0.25 M NaCl. For $CaCl_2$, the extent of flavour binding increased at 0.05 and 0.1 M $CaCl_2$, and then fell (0.25 M $CaCl_2$) and rose again (1 M $CaCl_2$). After the increases in flavour retention at lower concentration for $CaCl_2$, the effect of salts on protein flavour binding characters generally followed the same trend with that the degree of flavour binding by proteins decreased at low concentrations and then increased.

More importantly, it was noted that the overall percentage of bound flavours were significantly higher for NaCl than $CaCl_2$ when the concentration of cations varied from 0.25 to 1 M. Arntfield, Murray and Ismond (1986) systematically studied a variety of cationic species on protein thermal stability of fababean protein and ranked the relative influence of cations on protein stability as: K^+, $Na^+ > Li^+ > Ca^{2+}$. In a

similar fashion, the higher stabilizing effect of Na^+ compared to Ca^{2+} would result in the hydrophobic association between proteins and with flavours being more effectively promoted by NaCl than $CaCl_2$ which would account for the higher retention of flavours by NaCl at higher salt concentrations.

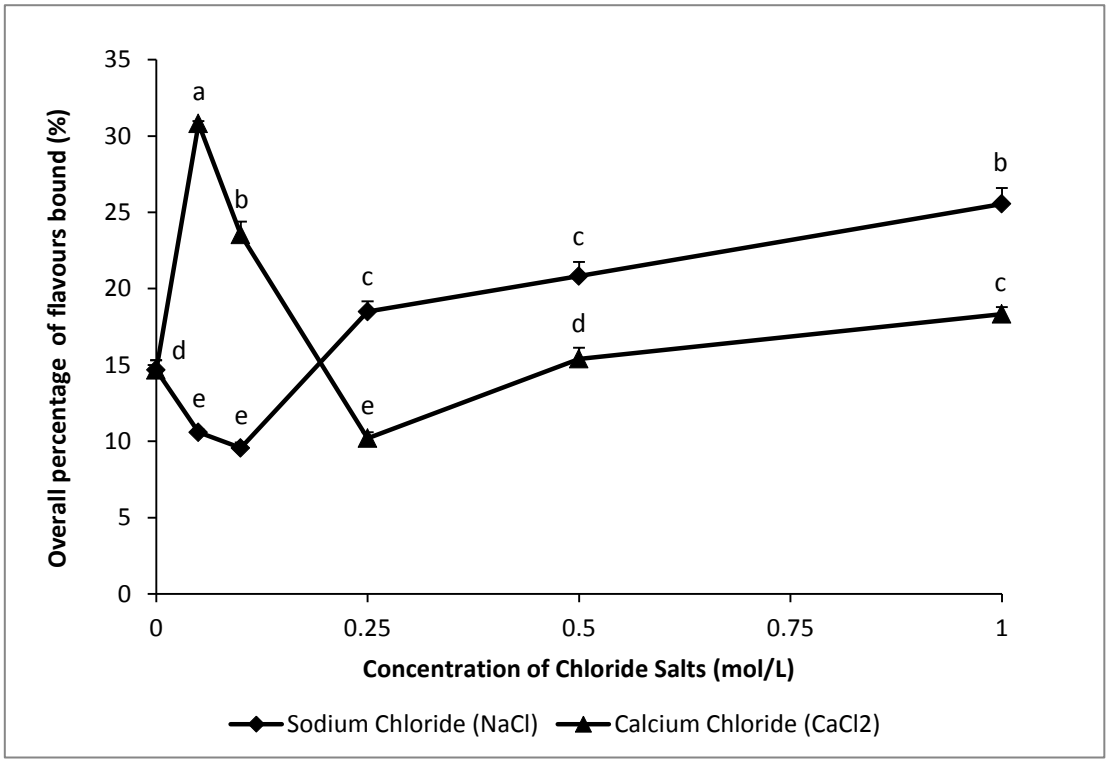


Figure 5.4 Comparison of effect of cations (Na^+ vs. Ca^{2+}) on overall binding of selected ketone flavour mixtures to PPIs (1%, w/v).
[a~e] Points followed by the same superscript are not significantly different ($p < 0.05$).

5.4.3 Evaluation of effect of anions on protein-flavour binding performance

To examine the effect of anions, sodium salts in lyotropic series including protein-structuring salts (Na_2SO_4 & NaCl) and protein-destructuring salts ($NaCH_3COO$ & NaSCN) were evaluated at both 0.05 and 0.5 M to account for the potential charge effect at low salt concentration and address the lyotropic effect at high salt concentration (Uruakpa & Arntfield, 2006b). It was found that salt type ($p < 0.05$) but not salt concentration ($p = 0.0526$) significantly affected protein-flavour

binding characteristics. A significant interaction between salt type and salt concentration was also noted.

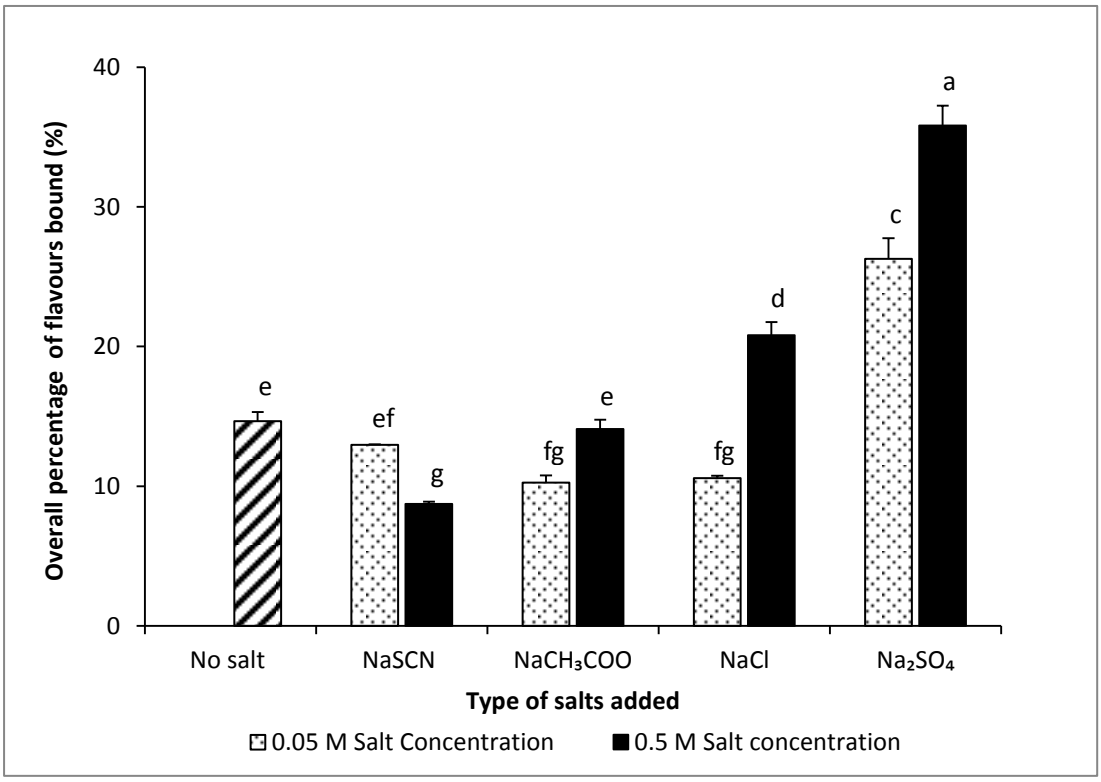


Figure 5.5 Comparison of effect of anions (SCN^-, $CHCOO^-$, Cl^- and SO_4^{2-}) at 0.05 and 0.5 M on overall binding of selected ketone flavour mixtures to PPIs (1%, w/v). $^{a\sim g}$ Bar values followed by the same superscript are not significantly different ($p < 0.05$).

When salts were present at 0.5 M (Fig. 5.5), protein-flavour binding affinity was dependent upon the position of the salt in lyotropic series. Specifically, the efficacy of anions to promote flavour retention followed the order: SO_4^{2-} >> Cl^- > CH_3COO^- = Control (no salt) > SCN^- which corresponds to the Hofmeister/lyotropic series reported by Damodaran (1996) and von Hippel and Schleich (1969). Based on the lyotropic effect, the addition of sodium salts at 0.5 M with greater protein stabilizing/non-chaotropic effect would enhance hydrophobic associations; these can

occur within PPIs and between PPIs and flavours. This strengthening of hydrophobic interactions and the presences of accessible sites for flavour compounds resulted in increased flavour binding. For example, the percentage overall binding of flavours significantly increased from 14.66 to 35.83 % in the presence of the non-chaotropic salt Na_2SO_4 in comparison to the control to which no salt was added.

In contrast, NaSCN at 0.5 M significantly decreased overall flavour retention by PPIs from 14.66 to 8.73 %. As a protein de-structuring or chaotropic salt, NaSCN is known to possess a direct ionic interaction with proteins, which promotes protein denaturation, decreases protein thermal stability and prevents protein aggregation (Zhang & Cremer, 2006). These adverse effects on protein structure could result in a loss of protein integrity in the protein's hydrophobic interior regions which were considered to be crucial for flavour binding (Damodaran & Kinsella, 1981a). It would appear that the retention of desirable or undesirable volatile flavour compounds to proteins can be prevented by disrupting the protein's native structure by using chaotropic salts. On the other hand, maintaining a certain level of protein structure is critical for flavour binding.

When $NaCH_3COO$ and NaCl were added at 0.05 M, binding between PPIs with flavours significantly decreased possibly due to electrostatic interactions between salts and proteins limiting sites for flavour binding. However, this effect was not seen for 0.05 M NaSCN, possibly because the changes in electrostatic interaction with the chaotropic salt also cause structural changes in the protein so that the overall percentage of flavours bound remained unchanged. On the other hand, 0.05 M sodium

sulfate remarkably increased flavour binding which may be due to its potential non-chaotropic effect even at the lower salt concentrations.

5.4.4 Evaluation of effect of pH on protein-flavour binding performance

To evaluate the impact of pH on protein-flavour binding, proteins were adjusted to the required pH values instead of using buffered systems (Heng et al., 2004; Gkionakis et al., 2006; Semenova et al., 2002;). In this manner, pH induced changes in proteins can be directly related to the resulting flavour binding without being affected by buffer ions (Jouenne & Crouzet, 2000).

Generally, the impacts of pH on protein-flavour binding support the hypothesis that hydrophobic interactions are the major forces responsible for the binding of ketone flavours to PPIs. As was shown in Table 5.1, increased retention of all three flavours was observed at pH 5, which was followed by pH 7, 9, 11 and 3. Proteins are known to have their lowest solubility and zero net charge at their isoelectric point (IEP). A decrease in repulsive electrostatic forces between protein molecules leads to an increase in protein-protein interactions which promotes aggregation and possibly precipitation (Vojdani, 1996; Wang & Arntfield, 2014). Under these conditions, strong hydrophobic associations between proteins could create additional binding sites which may account for the large increase in flavour retention at pH 5. Druaux et al. (1995) found that bovine serum albumin (BSA) which had an IEP of 4.9 bound 33.9% of γ-decalactone at pH of 5.3 higher than the 17.9% found at pH 3.5. In addition, the physical sorption of flavours onto the surface of acid precipitated protein may also be responsible for the increase in bound ketones (van Ruth & Roozen, 2002).

Table 5.1 Impact of pH on volatile headspace concentration and binding of selected ketones with PPIs (1%, w/v, 0.05 M NaCl) for individual and overall flavour retentions

pH values	Carbon number of ketone flavour	Volatile headspace signal	Overall headspace signal	Percentage of flavours bound (%)*	Overall percentage of flavours bound (%)**
3	C_6	4.98 ± 0.03^{f}	20.66 ± 0.92^{A}	0^{h}	7.71 ± 0.70^{D}
	C_7	7.29 ± 0.24^{cdef}		0^{h}	
	C_8	8.38 ± 0.72^{bc}		7.71 ± 0.70^{f}	
5	C_6	6.28 ± 0.12^{cdef}	31.44 ± 2.04^{B}	11.5 ± 1.61^{e}	32.62 ± 1.55^{A}
	C_7	10.7 ± 0.57^{b}		26.2 ± 0.46^{c}	
	C_8	14.4 ± 1.35^{a}		48.1 ± 1.00^{a}	
7	C_6	5.41 ± 0.26^{def}	21.97 ± 2.67^{A}	7.15 ± 0.68^{f}	19.32 ± 0.09^{B}
	C_7	7.94 ± 0.87^{cd}		13.9 ± 0.20^{e}	
	C_8	8.63 ± 1.54^{bc}		31.9 ± 1.27^{b}	
9	C_6	5.24 ± 0.05^{ef}	20.96 ± 0.13^{A}	3.64 ± 0.20^{g}	12.43 ± 0.09^{C}
	C_7	7.34 ± 0.16^{cdef}		6.79 ± 0.44^{f}	
	C_8	8.38 ± 0.16^{bc}		23.8 ± 0.17^{cd}	
11	C_6	5.07 ± 0.07^{f}	19.66 ± 0.62^{A}	3.45 ± 0.59^{g}	12.57 ± 0.25^{C}
	C_7	7.00 ± 0.16^{cdef}		7.06 ± 0.53^{f}	
	C_8	7.64 ± 0.39^{cde}		23.3 ± 0.81^{d}	

[a-h, A-D] Column values followed by the same superscript are not significantly different ($p < 0.05$).

* Percentage of flavours bound was based on the difference between the peak areas of flavoured samples in the absence and presence of protein

** Overall percentage of flavours bound was the difference between sum of peak areas of flavoured samples with and without proteins

When pH values are below and above the IEP (~pH 5), proteins exhibit a net positive or negative charge which promotes protein solubility (Vojdani, 1996). According to Table 5.1, lower flavour retention was noted at extreme pH values, such as pH 3 and pH 11. Sun and Arntfield (2011) measured the thermal properties of PPIs at different pH values and found that PPIs was more heavily denatured at lower pH value than higher pHs. In fact, PPIs (14.5 %, w/v) at pH 3 was completely denatured showing no thermal parameters, while PPIs at pH 11 had a denaturation temperature (T_d) of 94.2℃ and enthalpy of denaturation (ΔH) of 9.5 J/g compared to the T_d of 94.4℃ and ΔH of 13.6 J/g for PPIs at pH 5. As a consequence, the more severely denatured or unfolded state of PPIs at acidic environment may have caused greater loss of hydrophobic area and flavour binding sites, thereby accounting for the greater reduction in flavour retention for PPIs at pH 3 in comparison to the basic pH values.

Semenova et al. (2002) observed that native 11S globulin (legumin) isolated from broad bean exhibited the most binding affinity for hexyl acetate at pH 7.2 due to its unique quaternary structure; however, the acid denaturation of protein at pH 3.0 resulted in a complete loss in the ability to retain hexyl acetate. Similarly, Heng et al. (2004) evaluated interactions between homologous aldehydes and ketones with pea legumin. They noted that legumin only exhibited affinity to aldehydes at pH 7.6 and did not bind to aldehydes or ketones at pH 3.8.

5.5 Conclusion

Flavours headspace concentrations were significantly altered as a result of change in solvent environment (addition of salts and varying the pH of the systems). Protein-flavour binding was favoured under the condition that limited the charge on the protein and increased hydrophobic association (aggregation/precipitation). Promoting electrostatic interactions at extreme pH values and addition of neutral salts weakened protein flavour-binding capacity. The extent of flavour binding can be controlled by varying the type and concentration of salt added. Addition of higher concentrations of non-chaotropic salts stabilized the protein hydrophobic regions further enhanced protein-flavour hydrophobic association. In contrast, destabilization or denaturation of the protein's native structure (e.g., adding chaotropic salt or adjusting pH towards extreme values) decreased protein flavour-binding capacity. The lower flavour binding affinity of PPIs found at acidic environment can be beneficial to the flavouring of acidic protein-fortified beverage system. This improved understanding of the impact of salts and pHs on flavour retention could assist flavour chemists or product developers to select more appropratie environments for formulating protein-based food products.

Connections between Chapter 5 and Chapter 6

From previous literature, the majority of investigations have focused on evaluating the effect of different intrinsic and extrinsic factors on protein-flavour binding performance. Chapters 3, 4 and 5 have followed that trend using both individual flavour compounds and mixtures for flavours. Less emphasis has been put on determining the mechnisms underlying these interactions. By including various bond distrupting agents, Chapter 6 systematically evlauted the moleulcar forces between different classes of volatile flavour compounds and salt-extracted pea proteins. This evaluation provided critical information on the nature and type of molecular forces involved, which can be used to guide further modificaiotn of the interactions and thereby control the extent of protein-flavour binding.

Chapter 6 Probing the Molecular Forces Involved in Binding of Selected Volatile Flavours to Salt-Extracted Pea Proteins

6.1 Abstract

Molecular interactions between heterologous classes of flavour compounds with salt-extracted pea protein isolates (PPIs) were determined by involving various bond disrupting agents followed by GC/MS analysis. Flavour bound by proteins followed the order: dibutyl disulfide > octanal > hexyl acetate > 2-octanone = benzaldehyde. Benzaldehyde, 2-octanone and hexyl acetate bound non-covalently to PPIs, whereas octanal interacted with PPIs via covalent and non-covalent forces. Dibutyl disulfide reacted with PPIs only covalently as its retention was not diminished by urea and guanidine hydrochloride. By using propylene glycol, H-bonding and ionic interactions were implicated for hexyl acetate, benzaldehyde and 2-octanone. A protein-destabilizing salt ($Cl_3CCOONa$) reduced bindings for 2-octanone, hexyl acetate and benzaldehyde; however, retention for octanal and dibutyl disulfide increased. Conversely, a protein-stabilizing salt (Na_2SO_4) enhanced retention for benzaldehyde, 2-octanone, hexyl acetate and octanal. Formation of a volatile flavour by-product, 1-butanethiol, from dibutyl disulfide when PPIs was treated with dithiothreitol indicated the potential occurrence of sulfhydryl-disulfide interchange reactions.

6.2 Introduction

The affinity of a flavour compound to protein can be considered as a multi-factor function related to protein amino acid profile, protein overall conformation and stereochemistry of the flavour compound. From previous literature, irreversible chemical binding via covalent bonds and reversible physicochemical binding through van der Waals forces, hydrogen bonds, hydrophobic interactions and ionic bonds (electrostatic linkages) or a combination of the above have been generally accepted as the major forces contributing to the binding of aromatic compounds with proteins (Kim & Min, 1989; van Ruth & Rozzen, 2002; Kühn, Considine & Singh, 2006; Reineccius, 2006c). Although the mechanisms of interaction have been studied, understanding of the forces involved was mainly based on empirical or extrapolated data. Direct, qualitative studies focusing on the specific roles of these molecular forces between proteins and selected types of flavour compounds are limited.

One investigation was conducted by Chobpattana et al. (2002) who examined vanillin binding with milk proteins using various bond disrupting agents. They considered that urea was a hydrogen-bond disrupting agent, whereas SDS disrupted hydrophobic interactions. Addition of urea (0.8 M) significantly increased the free vanillin concentration for a vanillin/sodium caseinate mixture, while SDS (0.05%) did not. The effect of a combination of urea and SDS was the same as urea alone. Thus, the authors deduced hydrogen bonding was the major force leading to the binding of vanillin to sodium caseinate. For bovine serum albumin (BSA), both SDS and urea significantly enhanced the free vanillin concentration with their combination having

the greatest effect followed by SDS and urea. These observations indicated both hydrophobic interactions and hydrogen bonding were critical for the interaction between vanillin and BSA with hydrophobic interaction exhibiting a stronger effect.

By including bond disrupting agents, this methodology can be informative as it provides critical information about the bonds involved and is able to qualitatively predict the specific roles of different molecular forces between protein and flavour compounds. In addition, by using a specific reagent, the amount of flavour released from proteins could indicate the relative importance of that particular molecular force in protein-flavour binding reactions.

This methodology has been previously used to study of the interactions involved in heat-induced protein networks and protein-polysaccharide interactions (Arntfield, Murray & Ismond, 1991; Imeson, Ledward & Mitchell, 1977; Sun & Arntfield, 2012a; Uruakpa & Arntfield, 2006a, b; Ustunol et al., 1992; Utsumi & Kinsella, 1985). In a similar manner, if the retention of a flavour compound by proteins is not affected by the bond disrupting agent, the corresponding force or that specific protein structure is not required for the protein-flavour interaction. On the other hand, if the previously bound flavour compounds were released as the result of the addition of bond disrupting agent, this would indicate the retention of added flavour compounds was strongly dependent upon that particular interaction. Table 6.1 illustrates the potential methods and bond disrupting reagents that can be used to identify the specific forces and structure of proteins that responsible for the binding reactions.

Table 6.1 Effect of various reagents on molecular interactions/bonds between proteins and with flavours

Type of reagents	Non-covalent bonds			References
	Hydrophobic interactions	Hydrogen bonding	Ionic effects/ Electrostatic interactions	
Urea	Weaken	Diminish (mainly)	---	Uruakpa & Arntfield, 2006b; Ustunol et al., 1992
Propylene glycol (PG)	Disrupt	Enhance	Enhance	Ustunol et al., 1992; Utsumi & Kinsella, 1985;
Guanidine hydrochloride (GuHCl)	Weaken	Inhibit	Inhibit	Léger & Arntfield, 1993; Sun & Arntfield, 2012a; Tanford, 1968
Protein stabilizing salt (e.g., Na_2SO_4)	Promote	---	---	Damodaran & Kinsella, 1981b; Zhang & Cremer, 2006;
Protein destabilizing salt (e.g., $Cl_3CCOONa$)	Destabilize protein structure	---	Lower concentration relates to electrostatic interactions	Damodaran & Kinsella, 1981b; Kinsella, 1985; Ustunol et al., 1992; Utsumi & Melander & Horváth, 1977; Zhang & Cremer, 2006
Covalent bonds (-S-S-, -SH)				
Dithiothreitol (DTT)	Reducing agent cleave inter- and intramolecular S-S bonds			Léger &Arntfield, 1993; Sun & Arntfield, 2012a; Ustunol et al., 1992; Wolf, 1993
2-mercaptoethanol (2-ME)				

Therefore, the objective of this investigation was to systematically evaluate the molecular forces involved in binding between selected volatile flavour compounds and salt-extracted pea protein isolates (PPIs) using various bond disrupting agents including 5 M Urea, 1 M guanidine hydrochloride (GuHCl), 20% propylene glycol (PG), 0.5 M sodium sulfate (Na_2SO_4), 0.5 M sodium trichloroacetate ($Cl_3CCOONa$), 0.15 M dithiothreitol (DTT) and 0.3 M 2-mercaptoethanol (2-ME). Heterologous classes of flavour compounds including octanal, 2-octanone, hexyl acetate, benzaldehyde and dibutyl disulfide were selected. By identifying the molecular forces between protein and flavour compounds, this study contributes to qualitative understanding of protein-flavour binding mechanisms. This could aid flavour chemists and food product developers in optimizing desirable aroma and sensory attributes during the formulation of plant protein-based foods.

6.3 Materials and Methods

6.3.1 Source of materials

Analytical grade flavours, including 2-octanone, octanal, hexyl acetate, benzaldehyde and dibutyl disulfide, were purchased from Sigma-Aldrich Co. (St. Louis, MO). Commercial yellow pea flour (*Pisumsativum* L.) was supplied by Best Cooking Pulses Inc. (Portage la Praire, MB). Sodium sulfate, propylene glycol (PG), guanidine hydrochloride (GuHCl) and dithiothreitol (DTT) were received from Fisher Scientific (Fair Lawn, New Jersey). Sodium trichloroacetate ($Cl_3CCOONa$) and 2-mercaptoethanol (2-ME) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Urea was a product from Mallinckrodt Canada Inc. (Pointe-Claire, Quebec). All other

chemicals including K_2HPO_4, KH_2PO_4, HCl and NaOH were analytical grade and purchased from Fisher Scientific (Ottawa, Canada).

6.3.2 PPIs extraction

Following the method previously described by Sun and Arntfield (2010, 2011), salt-extracted pea protein isolates (PPIs) was extracted from sieved (500 μm opening, USA Standard NO. 35) yellow pea flour using 0.3 M NaCl (pea flour: sodium chloride solution = 3:10, w/v) under constant stirring for ½ hour. After the first centrifugation (4260*g*, 4℃, 15 min), pea protein was recovered by diluting the supernatant in two times its volume of cold distilled H_2O and leaving in a refrigerator (3℃)for 2 h. The precipitated protein sediment was collected after a second centrifugation (680*g*, 4℃, 15 min) and re-suspended in small amount of distilled H_2O. The resulting protein suspension was then dialyzed using 12-14,000 Da MWCO dialysis tubing (Spectra/Por Dialysis Membrane, Rancho Dominguez, CA) against 20 times cold distilled H_2O for 72 h in a refrigerator. Distilled H_2O was changed every 24 h. The desalted protein isolates were stored at -30℃ until they were freeze dried.

6.3.3 Flavour binding to plant proteins

To bind proteins and flavour compounds, the method of Gkionakis, Taylor, Ahmad, and Heliopoulos (2007) and Wang and Arntfield (2014, 2015a, 2015b) was followed with minor modification. Basically, each protein, bond disrupting agent and flavour stock solutions were firstly prepared separately and then mixed at specific ratio to produce an aqueous sample of protein isolate with desired concentration of

flavour compound and chemical reagent. Samples were shaken gently to reach equilibrium for binding of flavours with proteins, where after the headspace gas chromatography technique was used for determining the binding of protein isolates with selected volatile flavour compounds.

6.3.3.1 Preparation of protein, bond disrupting agent and flavour stock solutions

Four % (w/v) PPIs solutions were prepared in 0.01 M potassium phosphate buffer (pH 8) and subsequently placed into an ultrasonic water bath (Branson 3200, Bransonic Ultrasonic Cleaner, Shelton, CT, USA) for 20 min to ensure a complete dispersion of the protein isolates. The ionic strength was kept as low as possible to minimize the effect of salt on protein conformation.

All chemical stock solutions, including 10 M Urea, 4 M GuHCl, 80% PG, 2 M Na_2SO4, 2 M $Cl_3CCOONa$ and 1.2 M DTT, were prepared in the same potassium phosphate buffer as mentioned above.

Stock solutions of each volatile flavour compound were prepared at 500 ppm (0.05mL/100mL) in the same phosphate buffer and stored in amber glass bottles to prevent decomposition. Before combining with the proteins and bond-disrupting chemicals, flavour stock solutions were put in an ultrasonic water bath for 1 h to ensure a thorough dispersion of the flavour.

6.3.6.2 Preparation of GC/MS samples

In a typical experiment, protein, chemical and flavour were combined to produce the required concentrations. For example, to produce a 1% (w/v) final protein solution

with 250 ppm flavour in the present of desired concentration of chemical reagent (e.g., 1 M GuHCl) the experiment proceeded as follows: 0.6 mL of 4 M GuHCl was mixed with 0.6 mL of 4% (w/v) PPIs dispersion using a RKVSD rotary shaker (ATR, Laurel, MD) at speed 40 for 1 h to allow an interaction, after which 1 mL of the treated protein mixture was carefully loaded into a 20 mL reaction vial (22×75 mm, Product No.: 20-2100, Microliter Analytical Supplies, Inc., Suwanee, GA) and 1 mL of flavour stock solution was added to reach an aliquot volume of 2 mL. The flavour solution was added last. The vial was immediately sealed with Tan PTFE/silicone septa and magnetic metal crimp caps (Product No.: 20-0051M, Microliter Analytical Supplies, Inc., Suwanee, GA) and mixed by a Julabo SW22 shaking water bath (Julabo Labortechnik GMBH, Seelbach, Germany) at 30℃ and 125 rpm for 3 h prior to headspace sampling. Due to the limited solubility of urea, 2 % (w/v) pea protein dispersion was directly prepared in 10 M urea in which 1 mL of flavour stock solution was added. Preliminary testing found that 3 h was adequate to reach equilibrium. Samples were prepared in duplicate and each sampled once.

6.3.6.3 GC/MS

Measurement of flavour binding to proteins was conducted using a Varian CP-3800 Gas Chromatography (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled with a Varian 320-MS Triple Quadrupole Mass Spectrometer (Varian, Inc., Walnut Creek, CA, USA) operated in splitless and single quad mode. After mixing, samples were incubated and shaken for 14 minutes at 40℃ and 1 ml of sample headspace was aspirated into GC injector port by a CombiPal autosampler unit

with PAL Itex-2 (In-Tube-Extraction) absorber attachment (CTC Analytics AG, Switzerland) after one absorption cycle. A VF-5ms column which was 30 m in length, had 0.2 mm inner diameter and 20 μm film thickness was run at 4 mL/min constant helium flow. The temperature was programed by heating the sample at a rate of 10℃/min to 190℃ and then held for two minutes.

A mass spectrometer was used to confirm the identity of volatile flavour compounds and further determine the potential volatile flavour by-products generated (Wang & Arntfield, 2014). Operating conditions for the mass spectrometer were 70 eV EI source with a mass range between 25 Da to 250 Da.

Binding percentage of flavours was determined from the difference between the peak areas of flavoured samples in the absence and presence of proteins such that: Binding % = $(1 - \frac{Peak\ area_{\ with\ protein\ added}}{Peak\ area_{\ without\ protein\ added}}) \times 100\%$.

As all flavours were added at 250 ppm, the remaining free unbound flavour concentration can be expressed as: $250\ ppm \times \frac{Peak\ area_{\ with\ protein\ added}}{Peak\ area_{\ without\ protein\ added}}$.

Due to the fact that addition of salts or other chemical reagents may directly affect the headspace partitioning of volatile compounds (Wang & Arntfield, 2015b), each treatment of PPIs (protein + reagent+ flavour) was coupled with a control sample (reagent + flavour). By not including the protein, any change in flavour headspace concentration due to the addition of chemical reagents could be accounted for.

6.3.4 Statistical analysis

All data were analyzed using Microsoft Excel and SAS 9.3 program (SAS Institute Inc., Gary, NC). Tukey's test followed the analysis of variance indicated

significant differences with a level of $p < 0.05$.

6.4 Results and discussion

6.4.1 Effect of chemical classes of flavor compounds on protein-flavour binding performance

Limited information has been reported on flavour binding to pea proteins as a function of different classes of flavour compounds. As a result, the binding affinities of pea proteins with a wide range type of volatile flavour compounds were selected for investigation in the current research; these include aldehyde, ketone, ester, disulfide and aromatic flavours with a similar number of carbon atoms. The binding of these flavours in the absence of any additional chemicals is shown in Figure 6.1.

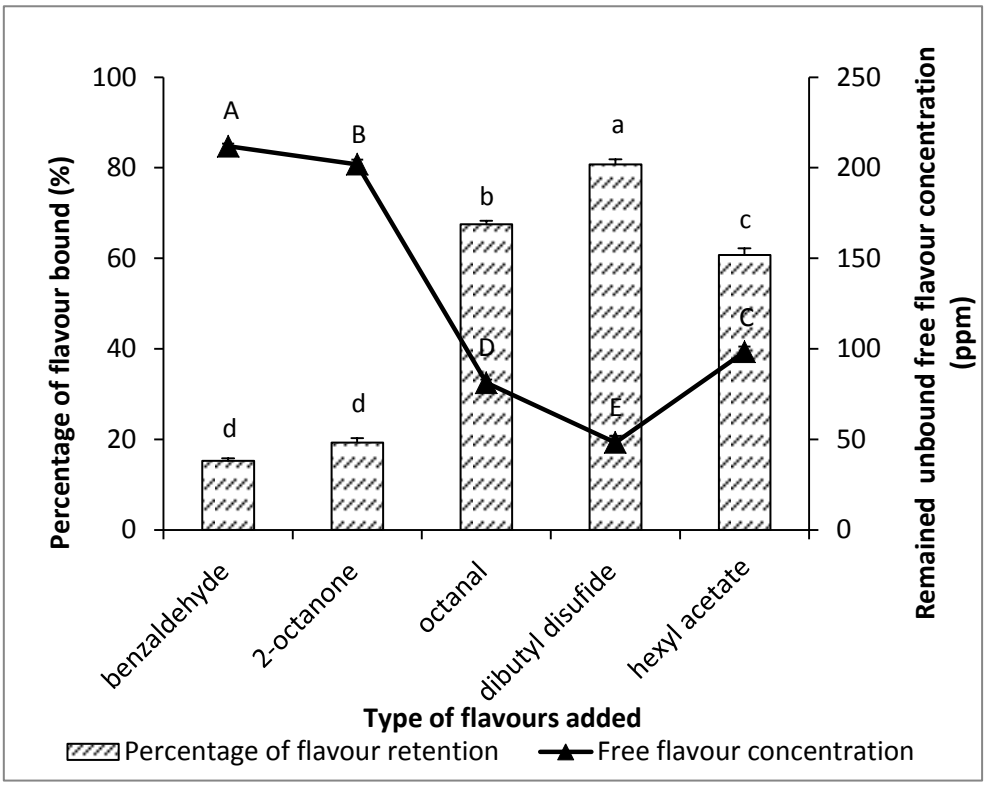


Figure 6.1 Effect of chemical classes of flavour compounds on protein-flavour binding performance and remained unbound free flavour concentrations in the aqueous mixtures containing 1% (w/v) salt-extracted pea protein isolates and 250 ppm flavour concentration.

$^{a\sim d, A\sim D}$ Values followed by the same subscript are not significantly different ($p < 0.05$).

It is clear from Figure 6.1 that all the selected flavours interacted with pea proteins with dibutyl disulfide (81%) being retained most extensively followed by octanal (68%), hexyl acetate (61%), 2-octanone (19%) and benzaldehyde (15%). A significant impact of flavour chemical class on protein-flavour binding performance was noted ($p < 0.05$). In this assay, dissolved unbound/free flavour compound which possesses a vapour pressure contributed to the headspace aroma concentration (de Roos, 2000). As all flavour compounds were added at 250 ppm, a headspace concentration of 212 ppm for benzaldehdye indicated that it was least affected by proteins. Lower values were obtained for 2-octanone (202 ppm), hexyl acetate (98 ppm), octanal (81 ppm) and dibutyl disulfide (50 ppm). The 80% retention of dibutyl disulfide in the presence of protein at the low end of the scale may severely influence the perception of this aroma.

The binding of octanal by PPIs was about 3.5 times higher than the corresponding ketone flavour (2-octanone). A similar trend was also noted by Heng et al. (2004) where pea vicilin strongly retained aldehydes at a level 2-5 times greater than the ketones with the same carbon number. This may be attributed to the different type of molecular interactions involved, where ketones form only reversible weak hydrophobic interactions with proteins, whereas both irreversible strong covalent bonds and reversible hydrophobic interactions have been reported between aldehydes and proteins (Mills & Solms, 1984; van Ruth & Roozen, 2002; Wang & Arntfield, 2014). About 60% of the total hexyl acetate was retained by pea proteins, indicating strong molecular forces between esters and pea proteins.

Surprisingly, the aromatic aldehyde flavour (benzaldehyde) was retained by PPIs at a level similar to the aliphatic ketone (2-octanone), which may suggest presence of weak bonding forces rather than strong covalent interactions. This agrees with the work of Relkin, Molle and Marin (2001) who, using spectrofluorimetry and electrospray ionization mass-spectrometry, observed benzaldehyde bound non-covalently with β-lactoglobulin. In addition, the steric hindrance resulting from the large molecular size of aromatic compounds may also contribute to its lower affinity to pea proteins (Gremli, 1974; Damodaran & Kinsella, 1980b, 1981a).

6.4.2 Effects of various chemical reagents on binding of selected volatile flavor compounds and potential involvement of molecular forces

6.4.2.1 Effect of urea

To determine the involvement of various non-covalent forces in pea protein binding with selected volatile flavour compounds, different chemical reagents including 5 M urea, 1 M guanidine hydrochloride (GuHCl) and 20 % propylene glycol (PG) were employed. The effects of chemical reagents (except 0.15 M DTT and 0.3 M 2-ME) on protein-flavour binding characteristics are presented in Fig. 6.2.

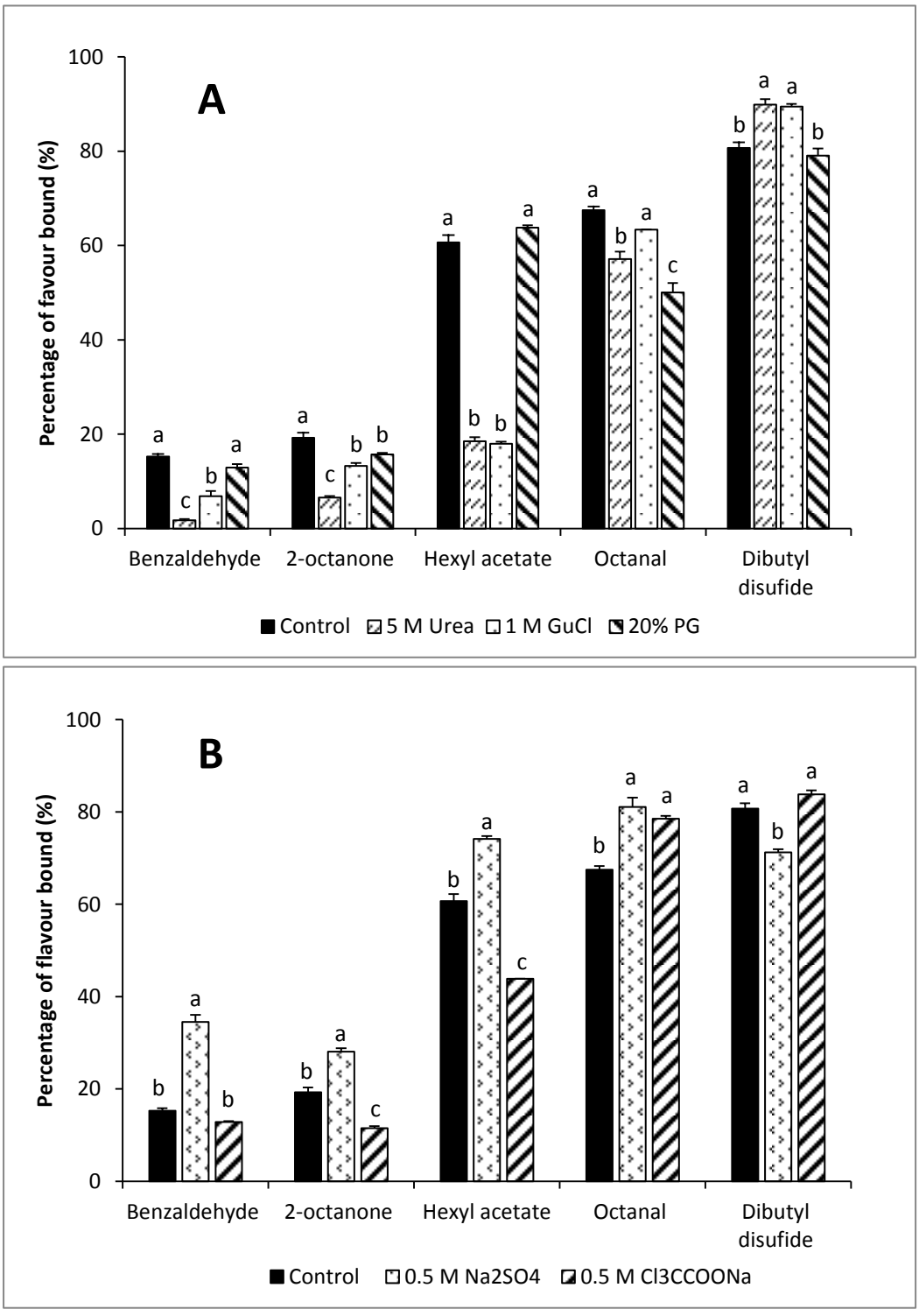


Figure 6.2 Effect of (A) urea, guanidine hydrochloride, propylene glycol, (B) 0.5 M Na_2SO_4 and $Cl_3CCOONa$ on binding of benzaldehyde, 2-octanone, hexyl acetate, octanal and dibutyl disulfide flavours to PPIs (1%, w/v) at 250 ppm flavour concentration.

Note: control represents the percentage of flavour bound by PPIs when no reagent was added. $^{a\sim e}$ For each flavour compound, bar values followed by the same subscript were not significantly different ($p < 0.05$).

As a protein denaturing agent, urea severely denatures proteins by disrupting hydrophobic interactions and hydrogen bonds (Uruakpa & Arntfield, 2006b; Ustunol et al., 1992). Therefore, by monitoring the changes in flavour retention in the presence of urea, the importance of hydrophobic interactions and hydrogen bonds in protein-flavour interaction can be elucidated. From Figure 6.2A, addition of 5 M urea significantly reduced the bindings for benzaldehyde, 2-octanone, hexyl acetate and octanal.

About 88% ((15% - 1.8%)/15% ×100%) of the benzaldehyde that bound to the protein when only the flavour and protein were included, was released in the presence of 5 M urea. This infers that benzaldehye retention relied greatly on the non-covalent forces, similar to what was observed by Relkin, Molle and Marin (2001). This value was followed by those for hexyl acetate (69%), 2-octanone (66%) and octanal (8%). Damodaran and Kinsella (1981a) stated that the interactions between ketone and soy proteins were hydrophobic in nature. The low level of flavour release for octanal upon addition of urea suggests its binding was mainly dependent on stronger bonding forces with only a small portion retained non-covalently. This observation agreed with what has been reported previously in that both irreversible covalent linkages and reversible physicochemical interactions are responsible for the binding between aldehyde and proteins with covalent interaction playing a more predominant role (Wang & Arntfield, 2015a). Binding of dibutyl disulfide by PPIs was not diminished by urea indicating its retention was independent of the protein retaining its intact hydrophobic structure. It is possible that the exposed interior regions of proteins

attributed to protein unfolding in the presence of urea provided additional binding sites that were favorable for volatile disulfide; this resulted in a higher level of disulfide retention.

6.4.2.2 Effect of guanidine hydrochloride

Guanidine hydrochloride (GuCl) is known to denature proteins by weakening hydrophobic interactions and inhibiting hydrogen and ionic bonds (Sun & Arntfield, 2012a). When GuHCl was added at a concentration of 1M, similar decreases in flavour binding were observed for all selected flavour compounds except octanal and dibutyl disulfide. Such a decrease in flavour retention further demonstrated the involvement of hydrophobic interactions and/or hydrogen and/or ionic bonds in the binding between hexyl acetate, benzaldehyde and 2-octanone with pea proteins.

About half of the previously bound benzaldehyde was released after GuHCl was added (Fig. 6.2A). As GuHCl inhibits hydrogen and ionic bonds, It can be deduced that the contribution of hydrogen bonds and/or electrostatic/ionic interactions may less or equivalent to that for hydrophobic interactions when evaluating the binding between benzaldehyde and PPIs. As the retention for octanal was not affected by GuHCl, it appears that hydrogen bonds and electrostatic interactions may only play a minor role in octanal retention. However, the drastic decrease in binding for hexyl acetate suggested there was potentially a large contribution by hydrogen bonds and electrostatic interactions to interactions between esters and pea proteins.

6.4.2.3 Effect of propylene glycol

Unlike urea and GuHCl, propylene glycol (PG) disrupts hydrophobic interactions but promotes hydrogen and ionic bonds (Ustunol et al., 1992; Utsumi & Kinsella, 1985). Therefore, it can be used as an effective reagent to probe the roles of hydrogen bonds, ionic forces and hydrophobic interactions between protein and flavour compounds (Sun & Arntfield, 2012).

Compared with the effects of urea and GuHCl, the overall flavour retention for hexyl acetate and benzaldehyde after addition of PG did not change in comparison to the control sample in which no reagent was added even though the hydrophobic interactions were anticipated to be weakened. This strongly suggests that hydrogen bonds and/or ionic forces between PPIs and hexyl acetate and benzaldehyde compensating the potential decrease of flavour binding resulting from the chaotropic effect of PG. An increase in flavour binding were seen for 2-octanone in the presence of PG when compared to the effect of urea and GuHCl on flavour binding. However the level remained lower than that of the control, suggesting hydrogen bonds and/or ionic forces were less critical for the binding of 2-octanone to PPIs than they were for hexyl acetate and benzaldehyde. These results are supported by previous literature. Reineccius (2006a) stated that ionic interaction may exist between flavour compounds and proteins. Wang and Arntfield (2015b) deduced the presence of ionic interactions between ketones and PPIs by increasing the ionic strength of the medium. They considered that the dramatic decrease of flavour retention at 0.05 M NaCl resulted from the formation of ionic layers between salt and proteins weakened proteins from

interacting with the previously ionic linked flavours, such that causing them to be released. In contrast, addition of PG did lead to a reduction in flavour binding for the aldehyde octanal and dibutyl disulfide, compared to the control and the urea and GuHCl treated samples. This indicated the absence of hydrogen and ionic forces for the interaction between proteins and these two flavours.

6.4.2.4 Effect of non-chaotropic (protein stabilizing) salt: Na_2SO_4

Three mechanisms have been used to account for the effect of salts on protein conformation; these are electrostatic shielding effects, non-specific charge neutralization and direct ion-macromolecule interactions (Zhang & Cremer, 2006). At low salt concentrations (< 0.5 M), salts exhibit non-specific ion effects by interacting with charged groups on the proteins, consequently influencing electrostatic interactions (Wang & Arntfield, 2015b). At high salt concentrations, (≥ 0.5 M), ion specific effects, also known as lyotropic effects, become predominant (Wang & Arntfield, 2015b). Following the lyotropic/Hofmeister series: (stabilizing) F^- > SO_4^{2-} > Cl^- > Br^- > SCN^- > Cl_3CCOO^- (destabilizing) anions of sodium salts tend to stabilize or destabilize proteins with Na_2SO_4 being well recognized as a protein stabilizing salt that can be used in foods (Arntfield, Murray & Ismond, 1990).

From Fig. 6.2B, 0.5 M Na_2SO_4 significantly enhanced the binding for benzaldehyde, 2-octanone, hexyl acetate and octanal to PPIs as compared to the control sample in which no salt was added. As a protein stabilizing salt, Na_2SO_4 is known to promote protein thermal stability by enhancing intra- and intermolecular hydrophobic associations (Sun & Arntfield, 2012a). By promoting hydrophobic

interactions between proteins, it could create additional areas that were more favorable for flavour retention. Increased flavour binding under these conditions supports the presence of protein-flavour hydrophobic interactions. In particular, the extent of binding for benzaldehyde by pea proteins increased about two fold from 15% to 34% suggesting hydrophobic interactions were instrumental to its retention. A great increase (~46%) in flavour binding was also noted for 2-octanone providing further evidence of the occurrence of non-covalent hydrophobic interactions between ketones and PPIs. Interestingly, addition of 0.5 M Na_2SO_4 led to a 20% increase in the binding of hexyl acetate and octanal compared to the respective control samples. This infers hydrophobic forces may be equally important for these two flavour compounds. On the other hand, the significant decrease in retention of dibutyl disulfide (from 80% for the control to 71%) can be attributed to the increased protein intermolecular interactions limiting accessibility of covalent binding sites on proteins.

6.4.2.5 Effect of chaotropic (protein destabilizing) salt: $Cl_3CCOONa$

As a chaotropic or protein destabilizing salt, a direct ionic interaction could be expected between $Cl_3CCOONa$ and PPIs (Damodaran & Kinsella, 1981b, Melander & Horváth, 1977; Ustunol et al., 1992; Utsumi & Kinsella, 1985; Zhang & Cremer, 2006). Zhang and Cremer (2006) specified that the specific ion-macromolecule interactions could denature proteins resulting in exposure of the reactive groups and a decrease in protein thermal stability. Sun and Arntfield (2012a) determined the thermal properties of PPIs in 0.3 M NaSCN, a protein destabilizing salt, and found that the enthalpy of denaturation (ΔH) significantly decreased from 15.8 to 10.7 J/g

indicating partial denaturation of PPIs. Similarly, when $Cl_3CCOONa$ was included, the partial unfolding of protein could lead to a loss of the interior hydrophobic binding area which may account for the diminished retention of flavours such as 2-octanone and hexyl acetate for which binding partially relies on hydrophobic interactions. This finding agrees with Wang and Arntfield (2015b) who observed the extent of binding for composite ketone flavours to PPIs was significantly reduced after adding 0.5 M NaSCN. It shows that denaturing or destabilizing a protein's native structure can partially release the non-covalently linked volatile flavour compounds. It was surprising, however, that binding of benzaldehyde was not reduced more; this may be attributed to the presence of ionic or hydrogen bonds as noted previously.

As octanal is known to bind PPIs via covalent and non-covalent forces, the increased flavour retention after the addition of the protein destabilizing salt $Cl_3CCOONa$ can be attributed to increased binding of this aldehyde with newly exposed covalent binding sites; this contribution was greater than that from non-covalently bound aldehyde released during partial protein denaturation. Changes in the structure of PPIs by $Cl_3CCOONa$ did not diminish the retention for dibutyl disulfide; this is likely because the binding was covalent in nature.

6.4.2.6 Effect of disulfide reducing agents

Sulfur-containing flavour compounds play an important role in contributing to meat aromas; therefore, it is necessary to understand their binding properties with pea proteins as there is a potential to use pea proteins as gelling agents in meat products (Sun, 2011). By including disulfide reducing agents, the role of disulfide bonds and

cysteine residues in binding with disulfide flavours can be examined. From Fig.6.2E, addition of 0.15 M dithiothretol (DTT) significantly enhanced retention for dibutyl disulfide from 80.07 ± 1.17% to 91.38 ± 1.08% ($p < 0.05$), whereas binding decreased to 62.44 ± 0.32% when 0.3 M 2-mercaptoethanol (2-ME) was added.

DTT reduces the existing disulfide bonds between cysteine residues and prevents inter- and intramolecular disulfide formation (Léger & Arntfield, 1993; Sun & Arntfield, 2012a). Legumin, one of the major components in salt-extracted pea protein, is known to contain 2-7 cysteine residues per 60 KDa subunit, which consists of a basic and an acidic subunit linked by one or more disulfide bonds (O' Kane et al., 2004). It is possible that the formation of cysteine residues from disulfide bond in pea legumin resulted from addition of DTT and further promoted sulphydryl disulfide interchange reactions, consequently resulting in greater flavour retention (Fig. 6.2E). On the other hand, 2-mercaptoethanol is known to prevent formation of disulfide bonds by competing or reacting with sulfhydryl groups on proteins, subsequently making -SH groups less accessible for the disulfide flavours, consequently reducing their retention.

Interestingly, a volatile flavour by-product, 1-butanethiol, was found when DTT was added to the mixture of PPIs and dibutyl disulfide. The chromatograms of dibutyl disulfide with and without addition of DTT and mixture of dibutyl disulfide, DTT and PPIs are presented in Fig. 6.3. The additional peak was observed at an elution time of approximately 2 min. It was noted that no formation of volatile flavour by-product was detected when dibutyl disulfide was present alone (Fig. 6.3A) or in the presence

of PPIs without any DTT (data not shown). This volatile by-product was observed when DTT was added to dibutyl disulfide (Fig. 3B) as well as in the presence of DTT and the protein (Fig. 3C). It is possible that the formation of this thiol compound from dibutyl disulfide was due to the reducing effect of DTT on disulfide flavour alone. However, addition of 1% (w/v) PPIs to the mixture of DTT and dibutyl disulfide led to a greater retention of dibutyl disulfide (93%) as well as increased formation of 1-butanethiol compared to DTT and disulfide flavour mixture (Fig. 6.3 B *vs.* C).

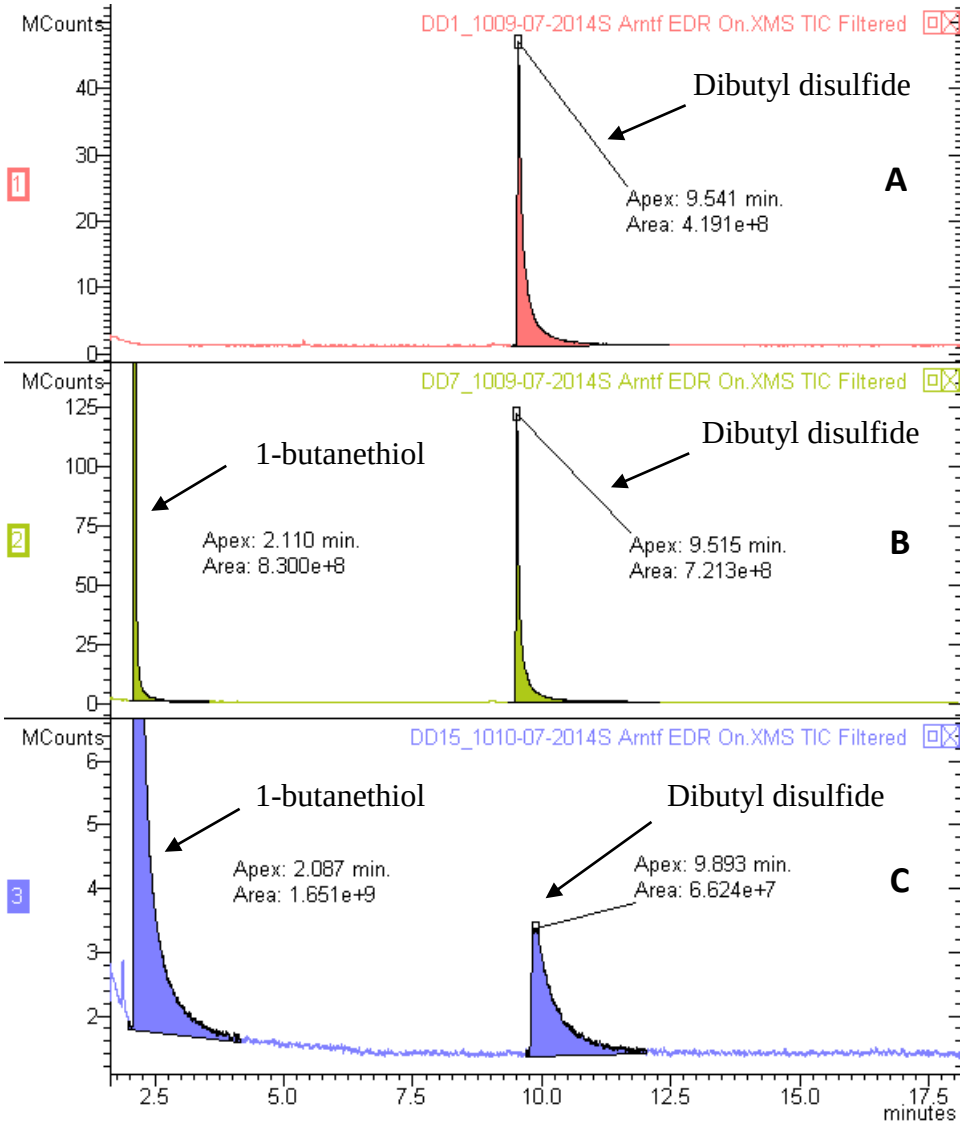


Figure 6.3 Illustration of the chromatograms of (A) dibutyl disulfide, (B) dibutyl disulfide and DTT and (C) dibutyl disulfide, DTT and PPIs.

This strongly suggests that presence of pea protein promoted sulfhydryl-disulfide interchange reactions and formation of the corresponding thiol compound as shown in the following reaction sequence (Fig. 6.4). It appears that the newly formed -SH groups resulting from the reducing effect of DTT on PPIs-S-S-PPIs were more available to disulfide flavour than the -SH groups on the native PPIs. This may explain why the corresponding thiol was only formed when PPIs was treated with DTT and not with PPIs alone. More importantly, the newly formed disulfide bonds could interact with free sulfhydryl groups further contributing to additional thiol formation. This process

seems to be repetitive as long as there is disulfide flavour available.

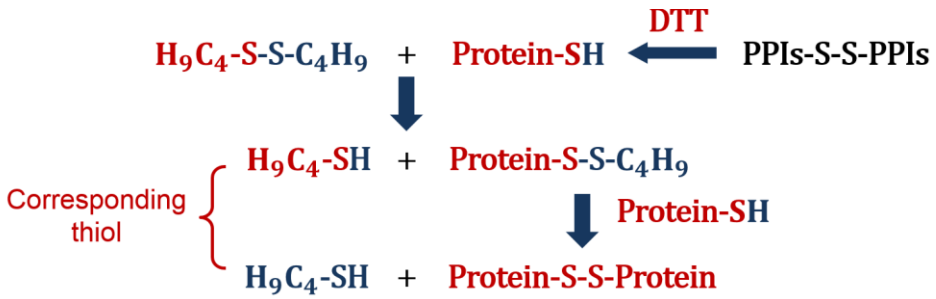


Figure 6.4 Pathway of formation of volatile flavour by-product (1-butanethiol) from dibutyl disulfide and salt-extracted pea proteins under DTT treatment.

It has been previously observed that sulfhydryl-disulfide interchange reactions occurred between ovalbumin and volatile disulfide flavours (Adams et al., 2001). Adams et al. (2001) found that addition of a series of sulfur-containing flavours including probyl, diethyl, diallyl, dipropyl, 2-furfuryl methyl and dibutyl disulfide to ovalbumin led to great reduction of headspace aroma concentration. Mottram et al. (1996) and Mottram and Nobrega (2000) found that the potent disulfides flavour

could interact with sulfhydryl and disulfide groups on proteins leading to a loss of disulfide aroma accompanied by the formation of the corresponding thiols. As a consequence, intensity (potency) and character of the desired aroma profile can be altered. The sulfhydryl disulfide interchange reaction is also promoted by a higher number of sulfhydryl groups from cysteine and disulfide groups form cysteine. While peas are known to be low in sulfur containing amino acids, there appear to be sufficient to participate in this interchange reaction.

6.4.3 Summary of molecular forces involved in binding of selected volatile compounds with PPIs

Table 6.2. Summary of molecular interactions/forces involved in binding between selected flavour compounds and PPIs

Type of flavours	Hydrophobic interactions	Hydrogen bonds	Ionic/electrostatic interactions	Covalent bonds
Benzaldehyde	Mostly	Lower involvement		No evidence
2-Octanone	> 70%	< 30%		No evidence
Hexyl acetate	Lower involvement	~ 70%		No evidence
Octanal	~15%	No evidence		~85%
Dibutyl disulfide	No evidence	No evidence		Strong evidence

Table 6.2 summarizes the potential molecular forces and interactions that were involved in the binding of selected volatile flavour compounds with salt-extracted pea proteins. As stated previously, when specific reagent was used, the amount of flavour released relative to the total amount of flavour retained by proteins was used to determine the relative importance of a particular molecular force in protein-flavour

binding reactions. It is clear from Table 6.2 that no single reagent was able to identify all molecular forces involved in protein-flavour interactions. A combination of different bond disrupting agents was necessary to address the various forces. In general, benzaldehyde, 2-octanone and hexyl acetate mainly bound non-covalently with pea proteins with benzaldehyde possessing the most extensive reliance on hydrophobic forces, followed by hydrogen bonds and ionic interactions. Similar to benzaldehyde, hydrophobic interactions appeared to be more important for binding between 2-octanone and PPIs than hydrogen bonds and ionic forces. The degree of retention for octanal (68%) was close to that of hexyl acetate (61%); however, the underlying forces and nature of bindings were distinctly different. Covalent interactions was more prominent for octanal (~85%), whereas hydrogen bonds and ionic forces were more critical for ester flavour (70%). Due to the similar increases (~20%) in flavour binding when Na_2SO_4 was employed, hydrophobic forces may play a similar role in binding of aldehyde (octanal) and ester (hexyl acetate) flavours with PPIs. The retention of dibutyl disulfide was not diminished by including protein destabilizing agents such as urea, GuHCl, PG and $Cl_3CCOONa$. Therefore, it can be concluded that covalent bonds are more likely to play a dominant role in its interaction with PPIs. In addition, the exposure of previous buried reactive binding sites on proteins can be critical for disulfide retention particularly if the protein is denatured or in an unfolded state.

6.5. Conclusion

By using a combination of chemical reagents, the molecular interactions/forces between salt-extracted pea proteins and various selected volatile flavour compounds were probed. However, it needs to be stated that no universal mechanism has been attained between protein and flavour compounds. A combination of different molecular forces/interactions as a function of the chemical composition of the flavour is necessary to explain binding mechanisms (Table 6.2). From a practical perspective, knowing the molecular forces between protein and flavour compounds provides information that will help predict the extent of flavour retention by proteins. More importantly, the systematic understanding of binding can provide insight into potential methods to selectively release the bound volatiles. Disrupting specific types of interactions or modifying a particular structure of proteins may help maintain a balanced desired flavour profile for protein-containing foods. In this way, the interactions between protein and flavour compounds may be controlled making it easier for product developers to create flavour formulations for protein-containing foods without saccrificing the sensory attributes of the product.

Connections between Chapter 6 and Chapter 7

In Chapter 6, the molecular forces involved in binding of a heterologous series of volatile flavour compounds and pea proteins have been elucidated. The next stage is to control the interaction by modifying proteins based on this information. Several structural modifications of pea protein were performed in Chapter 7. These included enzymatic hydrolysis using Alcalase and acylation. The effects of these modificaitons on protein-flavour binding performance were evaluated. It is clear from work in earlier chapters that protein structure plays an important role in maintaining flavour retention and controlling release of flavour compounds. Therefore, the structure of proteins at each stage of protein modification was monitored using different methods including DSC, SDS and non-reducing PAGE as well as measuring the surface hydrophobicity of the protein. As differnet classes of flavours bind proteins differently, the resulting flavour binding properties by modified proteins are discussed in relation to the potential binding mechanisms of each selected flavour compound as well as the changes in protein structure.

Chapter 7 Modification of Interactions between Selected Volatile Flavour Compounds and Salt-Extracted Pea Protein Isolates Using Chemical and Enzymatic Approaches

7.1 Abstract

Effects of chemical (acetylation and succinylation) and enzymatic (Alcalase) modifications on the binding properties of salt-extracted pea protein isolates (PPIs) to 2-octanone, octanal, hexyl acetate and dibutyl disulfide were monitored and related to changes in protein structure, based on differential scanning calorimetry and native-PAGE analyses. Addition of acetic and succinic anhydrides from zero to one gram per gram of protein gradually released the bound octanal and hexyl acetate mainly due to the loss of reactive -NH_2 and -OH groups and dissociation of protein inherent structure. Initial addition of these dicarboxylic acid anhydrides (< 0.1 g) resulted in partial denaturation of PPIs and increased binding for 2-octanone and dibutyl disulfide; however, further addition of anhydrides reduced retention of these flavour compounds probably due to extensive protein denaturation and masking of free -SH groups on the protein. The binding of dibutyl disulfide was found to be positively correlated with the number of free sulfhydryl groups on acylated PPIs inferring potential sulfhydryl-disulfide interchange reactions. Enzymatic hydrolysis of PPIs by Alcalase released bound ketone and ester flavours; however, the retention levels for aldehyde and disulfide flavours were enhanced. A strong relationship was found between protein-flavour binding affinities, the type of flavour and the structure of the protein as affected by different protein modification methods.

7.2 Introduction

To effectively utilize plant proteins in food systems, the loss of flavours due to interactions between protein and added flavours should be examined from all perspectives, including the influence of protein structure. Previously, chemical treatments including non-enzymatic and enzymatic methods have been used to improve the functionality of proteins (Shih, 1992). However, evaluating how different protein modification methods influence protein-flavour binding performance has received less attention (Damodaran & Kinsella, 1981b; Dufour & Haertle, 1990; O'Neill, 1996; Suppavorasatit & Cadwallader, 2012). As protein-flavour binding is greatly dependent upon the inherent structure of the protein, it can be hypothesized that any method that alters a protein's hydrophobic flavour binding region will reduce the number of accessible binding sites thereby reducing protein-flavour binding affinities.

As one of the most common chemical methods used for modifying food proteins, acylation has been shown to increase protein solubility (Paulson & Tung, 1987; Jamdar et al., 2010; El-Adawy, 2000; Yin et al., 2009a; Mirmoghtadaie, Kadivar, & Shahedi, 2009), improve emulsifying properties (Mirmoghtadaie, Kadivar, & Shahedi, 2009; Yin et al., 2009a; El-Adawy, 2000) and increase protein digestibility (El-Adawy, 2000; Yin et al., 2009b). It has been shown that high levels of acetylation effectively mask lysine residues, expose hydrophobic interiors and result in subunit dissociation (Franzen and Kinsella, 1976). As aldehyde flavours bind strongly to the ε-amino groups of lysine residues (Tromelin, Andriot, & Guichard, 2006), the acetylation

process could potentially reduce the protein's reactivity with these volatile aldehydes (Preininger, 2006). On the other hand, succinylation increases a protein's net charge by converting the cationic amino groups to anionic residues. This effect has been known to destabilize proteins and promote subunit dissociation (Shih, 1992; Damodaran & Kinsella, 1981b; El-Adawy, 2000). Therefore, it appears that acylation treatments accompanied by dissociation of a protein's native structure could become a method to reduce the binding capacity of proteins to volatile flavour compounds. Additionally, no direct comparison of acetylation and succinylation and the degree of acylation on protein-flavour binding performance has been evaluated.

Compared with chemical modification of proteins, enzymatic treatments are of great interest due to their mild processing condition, ease of control, reduced side reactions and by-products formation, and most importantly, high specificity (Liu et al., 2010; Claver and Zhou, 2005). Numerous studies have been conducted to evaluate the effect of enzymatic hydrolysis on protein functionalities (Liu et al., 2010). During enzymatic hydrolysis, peptide bonds are cleaved resulting in increased free amino and carboxyl groups leading to a reduction in the molecular weight and enhanced protein solubility. In addition, the tertiary structure of protein becomes disorganized thereby influencing the integrity of protein's hydrophobic region (Kristinsson and Rasco, 2000). It was suggested by Arail et al. (1970) that limited protein hydrolysis showed promise in loosening the interactions between proteins and flavour compounds. However, no systematic evaluation of enzymatic hydrolysis on protein-flavour binding has been found in the literature. With this purpose, Alcalase (subtilisin), a

widely used serine protease from Bacillus subtilis which is known for attacking the peptide bond through serine residues and having reative high efficiency than other enzymes was used in this study (Tang, Wang & Yang, 2009).

With an increasing interest in expanding the commercial use of plant proteins, including pea proteins, in foods (Boye, Zare, & Pletch, 2010), the objective of the present study was to evaluate the effect of several protein structural modifications on the resulting flavour-binding properties of salt-extracted pea protein isolates (PPIs). Both chemical (acetylation and succinylation) and enzymatic (Alcalase) modifications were performed. Heterologous classes of volatile flavour compounds were selected and include 2-octanone, octanal, hexyl acetate and dibutyl disulfide. The level of flavour binding was determined using GC/MS and conformational changes in the proteins were followed using differential scanning calorimetry, fluorescence spectroscopy, and native and reducing polyacrylamide gel electrophoresis. Resulting flavour binding properties of modified PPIs have been discussed in relation to the changes in the conformation of PPIs.

7.3 Materials and methods

7.3.1 Source of materials

Analytical grade flavours, including 2-octanone, octanal, hexyl acetate and dibutyl disulfide, were purchased from Sigma-Aldrich Co. (St. Louis, MO). Commercial yellow pea flour (*Pisum sativum* L.) was kindly supplied by Best Cooking Pulses Inc. (Portage la Praire, MB). Succinic anhydride was obtained from Anachemia Canada (Montreal, Canada). Acetic anhydride, 5,5’-Dithiobis

(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), Alcalase (protease from *Bacillus licheniformis*), D-lysine (monohydrate), 2-mercaptoethanol, Coomassie brilliant blue (R-250) and sodium phosphate dibasic dihydrate were purchased from Sigma-Aldrich Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS), hydroxylamine hydrochloride (NH_2-OH-HCl), ferric chloride, Tris(hydroxymethyl)aminomethane, glycine, glycerol, methanol, acetic acid, sodium phosphate monobasic monohydrate, 2,4,6-Trinitrobenzene sulfonic acid (TNBS) (in 5% methanol solution, w/v), NaCl, K_2HPO_4, KH_2PO_4, HCl and NaOH were supplied by Fisher Scientific (Fair Lawn, NJ).

Bromophenol blue (3',3",5',5"-Tetrabromophenolsulfonphthalein) and 8-anilino-1-naphthalene sulfonic acid (ANS) were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ) and Calbiochem-Behring (La Jolla, CA), respectively.

7.3.2 Preparation of salt-extracted pea protein isolates

Following the method previously described by Sun and Arntfield (2010, 2011), salt-extracted pea protein isolates (PPIs) was extracted from sieved (500 μm opening, USA Standard NO. 35) yellow pea flour using 0.3 M NaCl (pea flour: sodium chloride solution = 3:10, w/v) under constant stirring for ½ hour. After the first centrifugation (4260*g*, 4℃, 15 min), pea protein was recovered by diluting the supernatant in two times its volume of cold distilled H_2O and leaving in a refrigerator (3℃) for 2 h. The precipitated protein sediment was collected after a second centrifugation (680*g*, 4℃, 15 min) and re-suspended in small amount of distilled H_2O. The resulting protein suspension was then dialyzed using 12-14,000 Da molecular

weight cut-off (MWCO) dialysis tubing (Spectra/Por Dialysis Membrane, Rancho Dominguez, CA) against 20 times cold distilled H_2O for 72 h in a refrigerator. Distilled H_2O was changed every 24 h. The desalted protein isolates were stored at -30℃ until they were freeze dried (Genesis SQ Freeze Dryer, Gardiner, NY).

7.3.3 Acylation of pea proteins

Acetylated and succinylated pea proteins were obtained by covalently attaching the acetyl and succinyl groups to the amino ($-NH_2$) and sometimes hydroxyl (-OH) and thiol (-SH) groups on proteins with dicarboxylic acid anhydride.

Following the method of Yin et al. (2009a), pea protein dispersion (2.5 %, w/v) in 0.075 M potassium phosphate buffer (pH 8) was firstly prepared by constant stirring for 1 h on a Corning PC-353 Stirrer (Scientific Support, Inc., Hayward, CA). After adjusting the pH to 8, small increments of acetic or succinic anhydride were gradually added to the rapidly stirring pea protein dispersion at levels corresponding to 5, 10, 50 and 100% of the weight of PPIs. The pH was maintained between 7.5 and 8.5 using 2 M NaOH throughout the process. Completion of the reaction was indicated by no further change in pH. Protein was kept for an additional 1 h to ensure the reaction had gone to completion. To remove any undesirable salt, acylated proteins were dialyzed against 40 times distilled water for 72 hours at 4℃ using regenerated cellulose dialysis tubing with MWCO of 12,000 to 14,000 Da (Fisherbrand®, Pittsburgh, PA). Distilled H_2O was changed every 24 h. A control sample was prepared in the same manner without addition of anhydrides. Dialyzed proteins were kept frozen (-30℃) before being freeze dried.

7.3.3.1 Determination of degree of N-acylation

Measurement of degree of acylation of amino groups (N-acylation) on proteins followed the method of Yin et al. (2009a). A 0.1 % (w/v) PPIs dispersion was prepared in 0.05 M potassium phosphate buffer at pH 8 in the presence of 0.05 M NaCl. After adding 1 mL of 0.1 % (w/v) TNBS to 1 mL of protein dispersion, the resulting mixture was incubated at 60℃ for 1 h in the dark. Subsequently, 1 mL of 10% SDS and 0.5 mL of 1 M HCl were added. Absorbance of the mixtures was read at 335 nm using an Ultrospec 4300 pro UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, England) against a reagent blank which did not contain protein but had all other reagents. The degree of N-acylation was expressed as the relative decrease in the absorbance in comparison to the control sample to which no anhydride was added.

7.3.3.2 Determination of degree of O-acylation

According to Schwenke et al. (1998) and Yin et al. (2010), the degree of O-acylation (esterification of hydroxyl amino acids) can be measured based on the alkaline hydroxylamine method in which the formed ester bond is split by hydroxylamine with subsequent formation of a hydroxamic acid/Fe^{3+}complex. To use this method, 2 mL of alkaline hydroxylamine solution (2 M NH_2-OH-HCl : 3.5 M NaOH: deionized water in the volume fraction ratio of 2:1:1) were added to 1 mL of 0.05 % (w/v) protein solution prepared in 0.05 M potassium phosphate buffer (pH 8), followed by incubation of the mixture at 40℃ for 2 h. The reaction was then stopped by addition of 1 mL of concentrated HCl (diluted 3 times with distilled H_2O, v/v). One mL of 0.37 M $FeCl_3$ in 0.1 M HCl was then added and the mixture filtered

through Whatman™ No. 40 filter paper (90 mm ∅). The absorbance of the filtrate was measured at 540 nm against a reagent blank and considered as an index for the extent of O-acylation (Yin et al., 2010).

7.3.3.3 Determination of degree of S-acylation and free sulfhydryl concentration

The degree of S-acylation of PPIs was determined by measuring the decrease of total number of sulfhydryl groups on proteins using Ellman's reagent (Yin et al., 2010; Zhao et al., 2011). Ellman's reagent was prepared by dissolving 40 mg of DTNB in 10 mL of Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, pH 8.0). In a typical experiment, 50μL of Ellman's reagent was added to a protein mixture which consisted of 50 mg of protein sample and 5 mL of Tris-glycine buffer containing 8 M urea. The resulting mixture was mixed and incubated at room temperature for 1 h with occasional shaking. After centrifugation at 13,600*g* for 10 min at room temperature, the absorbance of the supernatant was measured at 412 nm against a reagent blank. The relative decrease in absorbance of the treated sample compared with the control sample without addition of anhydride was used to estimate the degree of S-acylation. To determine the concentration of free sulfhydryl groups, a similar process was followed using the untreated and treated PPIs in the abscence of 8 M urea. Without addition of urea, only the free sulfhydryl groups on the protein surface were measured. Based on Shimada and Cheftel (1988) and Zhao et al. (2011), a molar extinction coefficient of 1.36×10^4 $M^{-1}cm^{-1}$ was used to calculate the concentration of -SH groups which was expressed as μM/g of protein.

7.3.4 Enzymatic hydrolysis of PPIs

Enzymatic hydrolysis of pea proteins by Alcalase 2.4L (2.4 AU/g) was conducted at an enzyme to substrate (E/S) ratio of 1:8 (0.3 AU/g of protein) (Barbana & Boye, 2011). In a typical experiment, 107 µL of Alcalase was added to 40 mL of 2.5 % (w/v) PPIs dispersion at pH 8 and pre-warmed at 50℃ for 0.5 h. Proteolysis was carried out at 50℃ for various periods of time (0, 0.5, 1, 2.5 and 5 h) maintaining the pH at 8 using 1 M NaOH (Jamdar et al., 2010). At the end of the specified time, the enzyme was inactivated by heating the mixture in a boiling water bath for 10 min. A control sample was prepared following the same procedure without the addition of Alcalase but was heated at 50℃ for 5 h. The hydrolysates obtained were stored at -30℃ before freeze drying.

7.3.4.1 Determination of degree of hydrolysis

Quantification of the degree of protein hydrolysis followed the classical method of Adler-Nissen (1979) using the TNBS reagent. Typically, aliquots (0.25 mL) of sample and standard solutions were prepared in 1% SDS, followed by the addition of 2 mL of sodium phosphate buffer (0.215 M at pH 8). After mixing, 2 mL of TNBS reagent was added to the resulting mixture and incubated in a covered water bath (to exclude light) at 50℃ for 60 min (Spellman et al., 2003). The reaction was terminated by addition of 4 mL of 0.1 M HCl. After cooling at room temperature for 0.5 h, the absorbance of sample and standard solutions were recorded at 340 nm.

The degree of hydrolysis (DH) was calculated based on the number of peptide bonds broken within the total number of peptide bonds following the equation

(Spellman et al., 2003):

$$\text{DH \%} = 100 \times \left(\frac{AN_2 - AN_1}{Npb}\right),$$

where AN_1 represents the amino nitrogen content of protein substrate prior to hydrolysis (mg/g protein), AN_2 reflects the amino nitrogen content of protein after hydrolysis (mg/g protein) and Npb indicates the amino nitrogen content of the total number of peptide bonds in the protein substrate (mg/g protein). AN_1 and AN_2 were derived from the standard curve of absorbance plotted as a function of the milligrams of amino nitrogen content per litre using D-lysine as a standard (0-2.5mM). The total amino nitrogen content of peptide bonds per weight unit in pea proteins can be calculated from its protein content (82.68%), total nitrogen content (1/5.7) and amino nitrogen content of protein before hydrolysis (8.48 mg/g). An approximate value of 137 mg/g was used.

7.3.5 Measurement of Flavour binding to plant proteins

7.3.5.1 Preparation of protein and flavour stock solutions

Native or treated pea protein solutions (2 %, w/v) were prepared in 0.01 M potassium phosphate buffer (pH 8).The ionic strength was kept as low as possible to minimize the effect of salt on protein conformation.

Stock solutions of each volatile flavour compound were prepared at 500 ppm (0.05mL/100mL) in phosphate buffer solution and stored in amber glass bottles to prevent decomposition. Before use, flavour stock solutions were put in an ultrasonic water bath for 1 h to ensure a thorough distribution of flavour compounds (Gkionakis, Taylor, Ahmad, & Heliopoulos, 2007).

7.3.5.2 Preparation of GC/MS samples

Following the method previously described by Wang and Arntfield (2014, 2015a, 2015b), to produce a 1% (w/v) final protein solution with 250 ppm flavour concentration, 1 mL of 2% (w/v) protein solution was carefully loaded into a 20 mL reaction vial (22×75 mm, Product No.: 20-2100, Microliter Analytical Supplies, Inc., Suwanee, GA) followed by the addition of 1 mL of flavour stock solution to reach an aliquot volume of 2 mL. The flavour solution was added at last. The vial was then immediately sealed with Tan PTFE/silicone septa and magnetic metal crimp caps (Product No.: 20-0051M, Microliter Analytical Supplies, Inc., Suwanee, GA) and mixed by a Julabo SW22 shaking water bath (Julabo Labortechnik GMBH, Seelbach, Germany) at 30℃ and 125 rpm for 3 h prior to headspace sampling. Preliminary testing found that 3 h was adequate to reach equilibrium. Samples were prepared in duplicate and each sampled once.

7.3.5.3 GC/MS

Measurement of flavour binding to proteins was conducted using a Varian CP-3800 Gas Chromatography (Varian Chromatography Systems, Walnut Creek, CA) coupled with a Varian 320-MS Triple Quadrupole Mass Spectrometer (Varian, Inc., Walnut Creek, CA) operated in splitless and single quad mode. After mixing, samples were incubated and shaken for 14 minutes at 40℃ and 1 ml of sample headspace was aspirated into GC injector port by a CombiPal autosampler unit with PAL Itex-2 (In-Tube-Extraction) absorber attachment (CTC Analytics AG, Switzerland) after one absorption cycle. A VF-5ms column which was 30 m in length, had 0.2 mm inner

diameter and 20 μm film thickness was run at 4 mL/min constant helium flow. The temperature was programed by heating the sample at a rate of 10℃/min to 190℃ and then held for two minutes.

A mass spectrometer was used to confirm the identity of volatile flavour compounds and further determine the potential volatile flavour by-products generated (Wang & Arntfield, 2014). Operating conditions for the mass spectrometer were 70 eV EI source with a mass range between 25 Da to 250 Da.

Binding percentage of flavours was determined from the difference between the peak areas of flavored samples in the absence and presence of proteins such that: Binding % = $(1 - \frac{Peak\ area_{\ with\ protein\ added}}{Peak\ area_{\ without\ protein\ added}}) \times 100\%$.

7.3.6 Native and SDS-PAGE

Native and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed on a discontinues buffered system using a 7.5 % separating gel and a 4% stacking gel for untreated and acylated PPIs; only SDS-PAGE experiments were carried out for hydrolyzed PPIs based on the method of Laemmli (1970). Under reducing condition, proteins were solubilized in the sample buffer containing 0.0625 M Tris-HCl, 2 % SDS, 10% glycerol, 5% 2-mercaptoethanol (2-ME) and 0.02% bromophenol blue and heated at 95℃ for 5 min. After centrifugation at 2200*g* for 10 min at room temperature to remove any particulate matter (Sun & Arntfield, 2012b), 5 μL of the supernatant were loaded into each well of the gel (Mini PROTEAN® 3 System, Bio-Rad, Hercules, CA). Electrophoresis was conduct at a constant voltage of 200 V using a Bio-Rad Model 250/2.5 Power Supply

(Bio-Rad, Hercules, CA) with 0.025 M Tris, 0.192 M glycine and 0.1% SDS as the running buffer. Protein gels were stained in Coomassie brilliant blue (R-250), 40% methanol and 10% acetic acid and then destained in distilled H_2O. A range SDS-PAGE of low molecular weight standards from Bio-Rad (Hercules, CA) was used for both native and SDS-PAGE. Basically, 2 μL of standards was dissolved in 20 μL of sample buffer with 5 μL of the mixture being applied into one well. For native-PAGE, SDS, 2-ME in sample and running buffer as well as the initial heating of the sample were excluded.

7.3.7 Differential scanning calorimetry (DSC)

To evaluate the thermal properties of native and modified PPIs, a thermal test was performed using a DSC Q200 (TA Instruments, New Castle, DE) following the method of Sun and Arntfield (2010) and Wang and Arntfield (2014). Enthalpy of denaturation (ΔH) and denaturation temperature (T_d) were obtained from the endothermic peaks in the thermograms using Universal Analysis 2000 software (Version 4.5A).

In a typical experiment, a 15% (w/v) protein suspension in 0.3 M NaCl was prepared by shaking the samples on a RKVSD rotary shaker (ATR, Laurel, MD) for 1 h at speed 40 followed by mixing for 1 min using a Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY) before being loaded into DSC pans. 10-15μL of the pre-mixed protein suspension were accurately weighed in a Tzero pan (TA Instruments, New Castle, DE) and hermetically sealed. The thermal curve was obtained by heating the sample from 30 to 120℃ at 10℃/min in a standard DSC cell.

The DSC had been calibrated against both sapphire and indium standards. An empty pan was used as reference. Each sample was tested in duplicate.

7.3.8 Protein surface hydrophobicity measurement

Surface hydrophobicity (S_0) of protein samples was measured using a Synergy H4 Microplate reader (BioTek Instruments, Inc., Winooski, VT) following the procedure of Uruakpa and Arntfield (2006c) with minor modifications. ANS was used as the fluorescence probe.

Typically, pea protein solution was firstly prepared in potassium phosphate buffer solution (0.01M, pH 8) and diluted to series of protein concentrations: 0.001, 0.01, 0.02, 0.03, 0.04% (w/v). For the samples containing ANS, 10 μL of 8 mM ANS (prepared in the same phosphate buffer) was added to 2 mL of diluted protein dispersion and completely mixed. Samples were placed in the dark for 0.5 h to allow the binding between ANS and proteins to equilibrate and then mixed using a Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY) for 5 seconds prior to measurement of fluorescence intensity (FI). By measuring the FI at 470 nm after excitation at 390 nm, the relative FI was derived from subtracting the FI of samples without addition of ANS from the samples with ANS at each corresponding protein concentration. Relative FI was plotted against protein concentrations (w/v) and the resulting slope from the linear regression analysis was used as an index of the protein surface hydrophobicity. All measurements were conducted in duplicate.

7.3.9 Experiment design

A 4 × 3 × 5 full factorial experiment was adopted to evaluate the influences of chemical and enzymatic modifications of proteins on protein-flavour interactions using salt-extracted pea protein as an example. The main factors included four types of flavour compounds (2-octanone, octanal, hexyl acetate and dibutyl disulfide), three types of treatment (acetylation, succinylation and enzymatic hydrolysis using Alcalase) and five levels of modification for each acylation and hydrolysis treatment (anhydride to protein ratio at 0, 0.05, 0.1, 0.5 and 1 g/g for acetylated PPIs and hydrolysis times of 0, 0.5, 1, 2.5, and 5 h for hydrolyzed PPIs). Each protein modification was conducted in duplicate with the degree of modification for each treatment being tested in triplicate and the average value was recorded.

2-octanone, hexyl acetate, octanal and dibutyl disulfide were chosen as the heterologous series used in this studies as they were shown to exhibit different molecular interactions/forces when binding with pea proteins (Chapter 6).

7.3.10 Statistical analysis

All data were analyzed using Microsoft Excel and SAS 9.0 program (SAS Institute Inc., Gary, NC). Tukey's test following the analysis of variance indicated significant different with a level of $p < 0.05$.

7.4 Results and discussion

7.4.1 Degree of protein modification

7.4.1.1 Degree of acylation

Unlike many investigations that reported the degree of acylation based on the substitution of ε-amino groups of lysine residues only (Paulson & Tung, 1987; Gruener & Ismond, 1996; Lawal, 2005; El-Adawy, 2000; Yin et al., 2009a, 2009b), the degree of O-acylation for hydroxyl groups (serine and threonine) and degree of S-acylation for sulfhydryl groups (cysteine) were also determined in the current investigation to address the potential effect of these reactive functional groups on protein-flavour interactions (Fig. 7.1).

It is clear from Fig. 7.1A that gradual addition of acetic and succinic anhydride led to sharp increases in the degree of N-acylation. Acetic anhydride proved to be a more effective acylating agent than succinic anhydride at any given anhydride to protein ratio below 0.5 g/g. A plateau of 93 to 95% of N-acylation was then reached and further addition of anhydrides above 0.5 g did not lead to significant increases in the extent of N-acylation. It has been previously reported that acetic anhydride (liquid) was more effective in reacting with lysine residues on proteins than succinic anhydride (solid) due to its high solubility and low steric hindrance (Eisele & Brekke, 1981; El-Adawy, 2000; Ponnampalam et al., 1990). Similar results have been reported for acylated red kidney bean protein (Yin et al., 2009a; Yin et al., 2010), mung bean protein (El-Adawy, 2000), canola protein (Gruener & Ismond, 1996) and hemp protein isolates (Yin et al., 2009b).

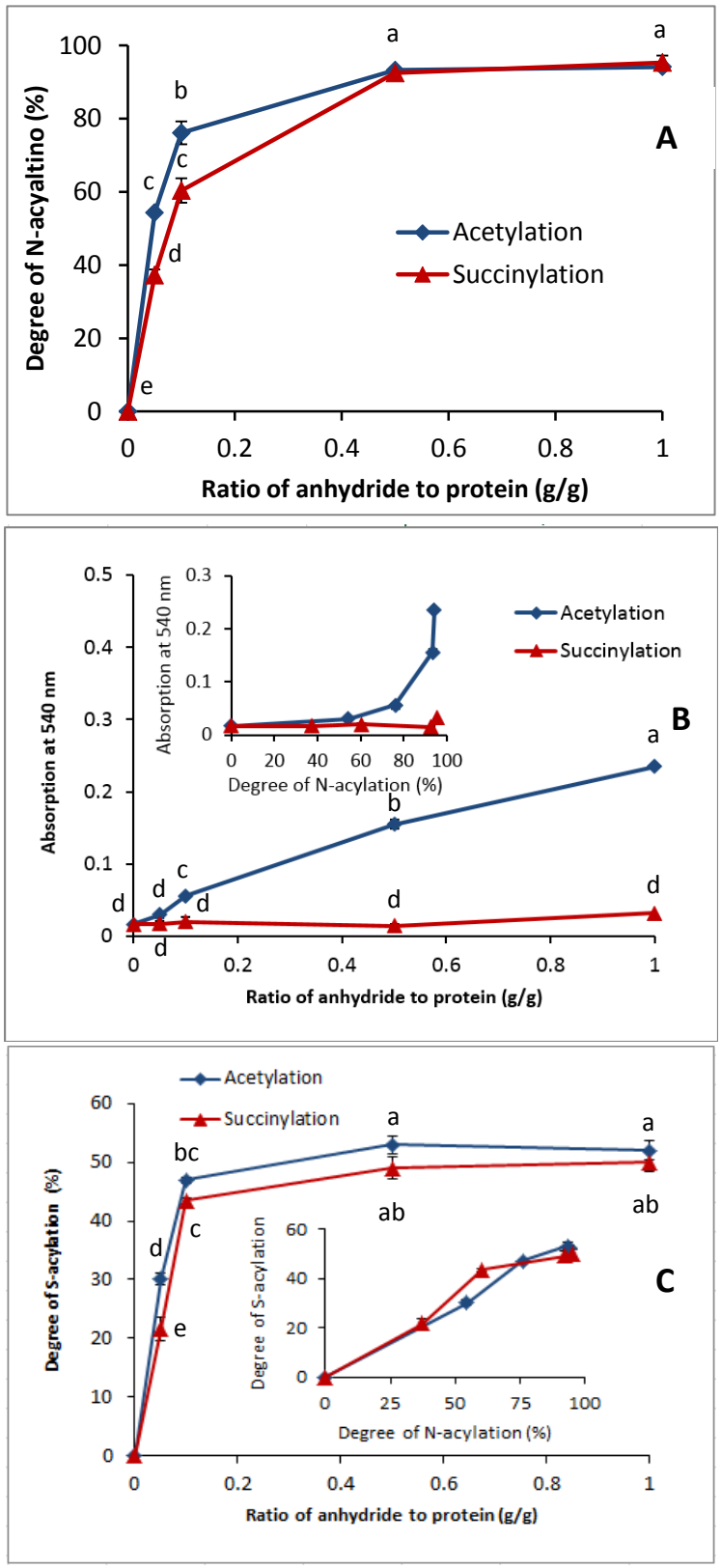


Figure 7.1 Degree of N-acylation (A), O-acylation (B) and S-acylation (C) of acetylated and succinylated pea protein isolates as a function of anhydride-to-protein ratio and degree of N-acylation.

[a~e] In each figure, points followed by the same superscript are not significantly different ($p < 0.05$).

From Fig. 7.1B, it can be seen that absorption at 540 nm that was used to monitor O-acylation significantly increased for acetylated PPIs as a function of anhydride to protein ratio; however, the degree of O-acylation for succinic anhydride remained unchanged. It was noted that the degree of O-acylation for acetylated PPIs was highly dependent upon the degree of N-acylation in that a sharp increase of absorbance was seen when the degree of N-acylation exceeded 93-95% (inset). This inferred that esterification of hydroxyl amino acids did not occur until after the hydroxyl groups on lysine. This is consistent with the findings of Yin et al. (2010) who observed that acylation of hydroxyl groups on red kidney bean proteins only occurred when the degree of N-acylation was greater than 93-94% with acetylated red kidney bean proteins possessed a distinctly higher level of O-acylation than the succinylated counterpart. It appeared that it was more difficult to acylate hydroxyl amino acids than lysine residues, which was attributed to the lower steric availability and higher p*Ka* value for hydroxyl groups compared with ε-amino groups on proteins (Franzen & Kinsella, 1976). Similar findings have been shown by Krause, Mothes and Schwenke (1996) and Schwenke and colleagues (1998; 2000) for acetylated legumin from faba bean protein.

From Fig. 7.1C, the degree of S-acylation increased greatly at low anhydryide to protein ratios, but once that ratio was above 0.2, values leveled off. S-acylation was affected equally by succinlyation and aceylation. Interestingly, there was a linear relationship between the degree of N-acylation with the level of S-acylation (inset).

Yin et al. (2010) reported that 40-60% S-acylation of red kidney bean protein was shown when N-acylation increased to 93-94%.

7.4.1.2 Degree of protein hydrolysis

Figure 7.2 shows a time dependent increase of degree of protein hydrolysis from zero to 16.5% as the hydrolysis time increased from 0 to 5 h. A sharp increase in the degree of protein hydrolysis was observed during the first hour, after which the rate of hydrolysis decreased. This pattern agrees with the typical protein hydrolysis curves that have been reported previously by Jamdar et al. (2010), Spellman et al. (2003) and Tang, Wang and Yang (2009).

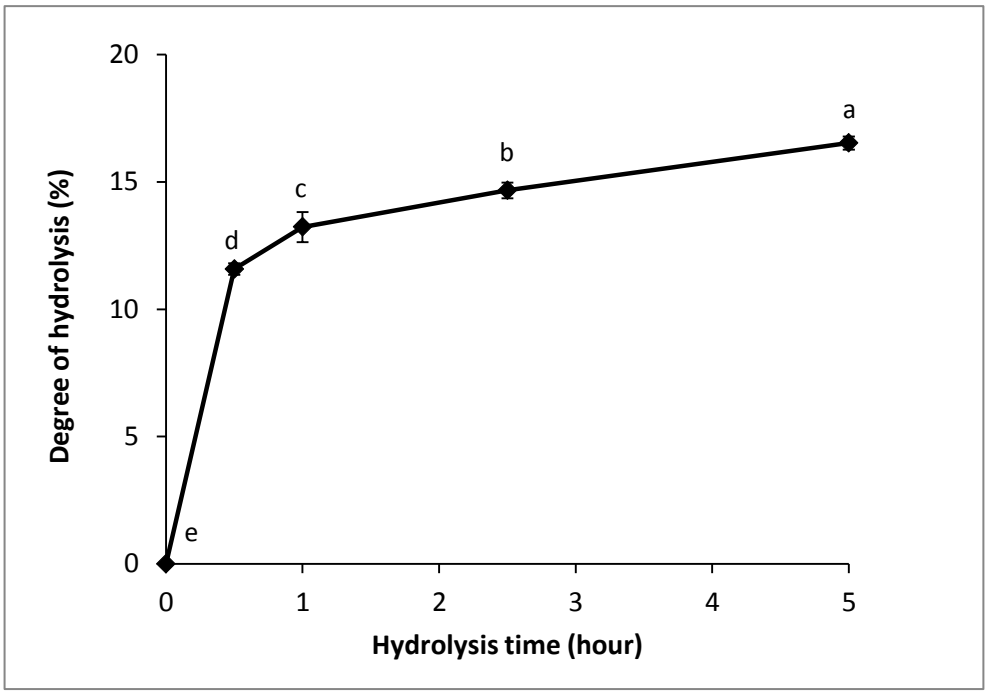


Figure 7.2 Degree of hydrolysis of pea protein isolates using Alcalase as a function of hydrolysis time from zero to five hour

[a~e] Points followed by the same superscript are not significantly different ($p < 0.05$).

7.4.2 Native and SDS-PAGE analyses

The effect of acylation on the molecular mass and mobility of PPIs were characterized by the native and dissociating PAGE displayed in Fig. 7.3A and Fig. 7.3B, respectively. It is clearly shown in Fig. 7.3A that acylation caused dissociation of native pea proteins by reducing the molecular weight of a major protein band (line 2) accompanied by formation of a number of lower molecular weight protein fractions (Gruener & Ismond, 1996). Based on the work of Schwenke et al. (1990), the major band that was observed in the native PAGE profile of untreated PPIs (line 2) may correspond to the high molecular weights of 7S pea vicillin and 11S legumin (300 to 400 kDa). With addition of acetic and succnic anhydrides at 0.05 g/g protein, an intense band was shown at molecular weight of ~50 kDa with several lighter bands were visualized at ~20, ~40, ~70 and ~97 KDa. The band at 50 KDa, was likely a pea vicilin subunits (Gatehouse et al., 1981), while other bands at 20, 40 and 70 kDa could represent the 11S basic and acidic subunits and convicilin, respectively (O'Kane et al., 2004; Sun, 2012b). It would appear that the addition of acetic and succinic anhydrides released the convicilin, 7S, 11S acidic and 11S basic subunits so that they migrated on the electrophoresis gel. The addition of 0.5 g and 1 g of succinic anhydrides led to the dissociation of all high molecular weight bands between 60 to 100 KDa seen at lower levels of succinylation (line 7 and 8) with formation of multiple lower molecular weight fractions around 50 to 66.2 KDa and around 25 kDa (line 9 and 10). Surprisingly, a gradual disappearance of acetylated proteins was observed after 0.5 g and 1 g of acetic anhydride were added, which may indicate

complete dissociation of pea protein structure. Another potential explanation is that neutralization of the positively charged amino groups due to extensive acetylation limited the charge on proteins, which subsequently inhibited the mobility of acetylated pea proteins in native gel electrophoresis and preventing them from entering the gel. Overall, these results are in line with those of Gruener and Ismond (1996) and Yin et al. (2009a) who observed that the major protein bands of canola and kidney bean proteins tended to dissociate and migrate farther into the gel with increasing the level of N-acylation when analyzed using native-PAGE analyses.

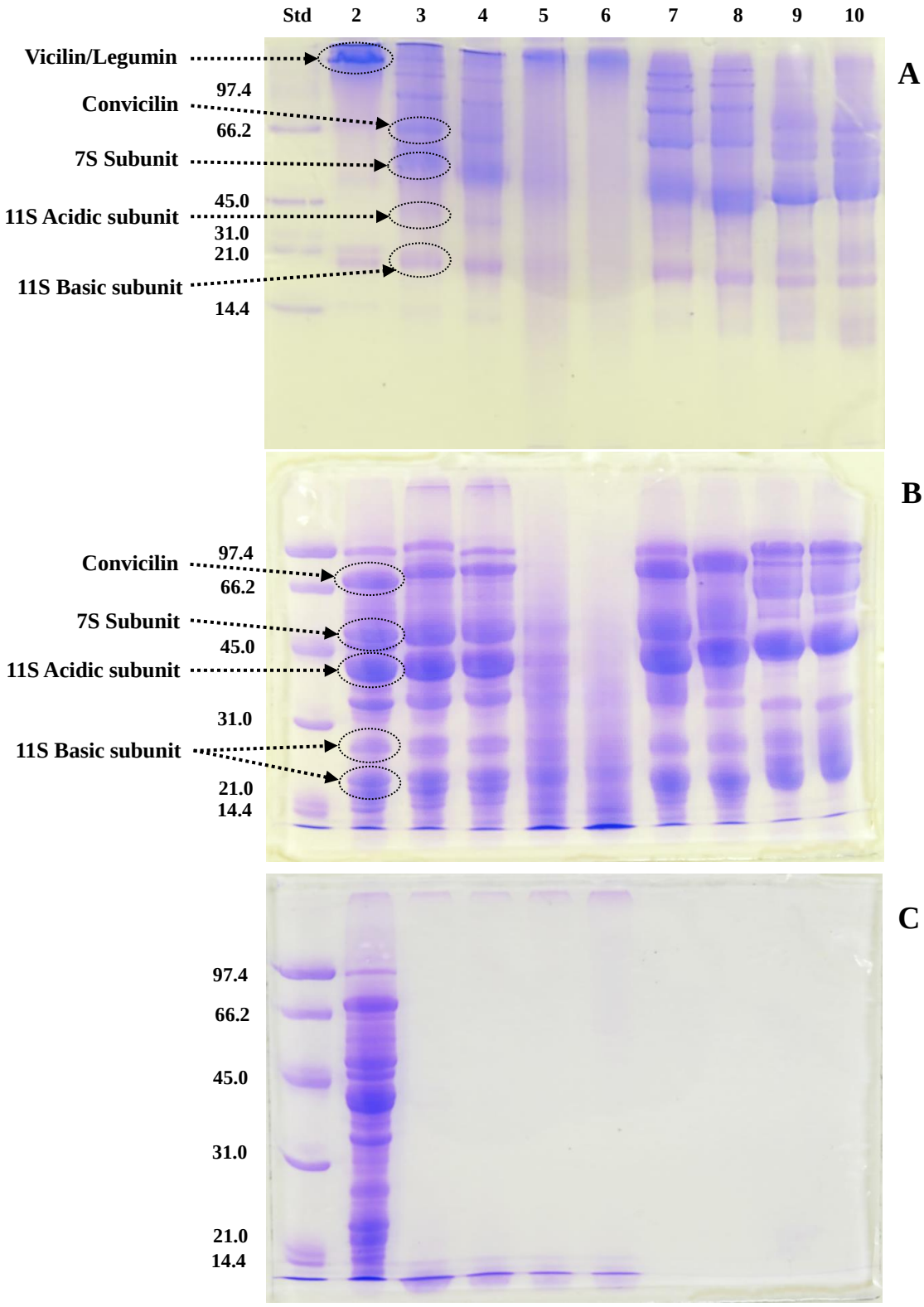


Figure 7.3 Electrophoretic patterns of acylated PPIs using native (A) and reducing PAGE (B) analysis and SDS-PAGE profile of hydrolyzed PPIs (C).
In Fig. 3A and 3B, Lane 1: MW standard marker, Lane 2: PPIs (original, without treatment), Lane 3-6, PPIs with addition of 0.05, 0.1, 0.5 and 1 g of acetic anhydride/ g protein, Lane 7-10, PPIs with addition of 0.05, 0.1, 0.5 and 1 g of succinic anhydride/ g protein. In Fig. 3C, Line 1: MW standard marker, Lane 2: PPIs (original, without treatment), Line 3-6: PPIs with hydrolysis time of 0.5, 1, 2.5 and 5 h.

The SDS-PAGE results for acylated PPIs samples shown in Fig. 7.3B are characterized by multiple major protein bands ranging in the size from approximately 10 to 97 kDa. With increasing ratios of succinic anhydride to protein, the SDS-PAGE profile (Fig. 7.3B) was characterized by a gradual increase in the molecular weight of the protein bands for PPIs (line 7 to 10). This increase in the molecular weights of protein subunits has been attributed to the binding of succinic anhydride increasing the molecular weight of proteins by 100 with each addition of succinic moiety (Yin et al., 2009). As a result, the major protein bands were hindered in their migration downward due to their larger size. Similar patterns have been noted by Yin et al. (2009a), Mirmoghtadaie et al. (2009) and Sheen (1991) for succinylated red kidney bean protein, oat protein and soluble tobacco leaf proteins, respectively. For acetylated PPIs, the molecular weight of PPIs subunits slightly increased at acetic anhydride to protein ratios of 0.05 g/g and 0.1g/g; however, a nearly complete reduction in the band intensity was observed after addition of acetic anhydate above 0.5 g/g (lines 5 and 6). It is possible that the high level of acetylation interferes with the binding of the Commassie brilliant blue and led to the protein almost not being stained as has been reported previously (Yin et al., 2009). Our observation was constant with Yin et al. (2009) and Sheen (1991) who found the similar weak staining of acetylated red kidney protein and acylated tobacco F-2-p protein using the same dye.

The molecular weights of the hydrolyzed PPIs using Alcalase are shown in Fig. 7.3C. Regardless of the time of hydrolysis, compared with the control sample which

contained no Alcalase (line 2), all hydrolyzed pea proteins were completely dissociated. Due to the impact of protein hydrolysis on the size and therefore the amino acid composition of peptides (Jamdar et al., 2010), it was shown that hydrolysis of pea protein using Alcalase for 0.5 h and higher (degree of hydrolysis higher than 12%) was effective in dissociating pea protein into peptides with molecular weights lower than 14.4 kDa. Similar complete disappearance of protein subunits was also observed when Ribotta et al. (2012) treated pea proteins using Alcalase at 0.0491 AU/g of protein.

7.4.3 Differential Scanning Calorimetry (DSC)

By measuring the thermal stability of proteins using differential scanning calorimeter, change in protein structure as a result of acylation and enzymatic hydrolysis could be progressively monitored based on changes in the enthalpy of denaturation (ΔH) and denaturation temperature (T_d). Table 7.1 illustrates the thermal properties of acylated PPIs as a function of anhydride to protein ratio

Table 7.1 Thermal properties of acylated salt-extracted pea protein isolates (15%, w/v, 0.3 M NaCl).

Type of treatment	Anhydride to protein ratio (g/g)	Enthalpy of denaturation (ΔH, J/g)	Denature temperature (T_d, ℃)
Control[a]	0	10.68 ± 0.09a	90.58 ± 0.40a
Acetylation	0.05	3.12 ± 0.06d	85.48 ± 0.37c
	0.1	1.34 ± 0.02e	81.28 ± 0.05d
	0.5	-	-
	1	-	-
Succinylation	0.05	8.70 ± 0.07b	88.38 ± 0.27b
	0.1	3.96 ± 0.04c	87.46 ± 0.13b
	0.5	-	-
	1	-	-

a~e Column values followed by the same online letters are not significantly different ($p < 0.05$).

[a] Control did not contain acetic or succinic anhydrides.

As seen in Table 7.1 the changes in the thermal properties of acylated PPIs reflected the structural changes for pea proteins using native electrophoresis (Fig. 7.3A). In general, with the addition of acetic and succinic anhydrides, enthalpy of denaturation and denaturation temperature continuously decreased, suggesting progressive denaturation of acylated pea proteins and after 0.05 and 0.1 g of anhydrides/g protein were added, no endothermic events occurred inferring complete unfolding or denaturation of the protein (Gruener & Ismond, 1996). As ΔH directly reflects the energy required to denature a protein molecule, the greater decrease in ΔH for acetylated PPIs indicates less structural integrity and more denatured than the succinylated PPIs.

The denaturation temperature indicates the temperature required to denature a protein molecule. With the addition of 0.1 g of acetic anhydride/g protein, T_d sharply reduced to 81.28℃, whereas a T_d value of 87.46℃ was recorded for succinyated PPIs

at the same anhydride to protein ratio. These corresponded well with the changes in enthalpy of denaturation, suggesting the structural loss noted for acetylated PPIs also produced a less thermally stable protein. A similar pattern was observed by Yin et al. (2010) in that the denaturation temperature of acetylated red kidney bean protein was lower than the succinylated one for a given level of treatment. Schwenke et al. (1998) found that the T_d values for faba bean legumin fractions (11S, 7S and 3S) continuously decreased as a liner function to the degree of N-succinylation.

As expected, no endothermic peaks were observed for any of the hydrolyzed pea proteins (data not shown), indicating complete protein denaturation and loss of the protein tertiary and quaternary structure due to formation of small peptides during protein hydrolysis by Alcalase (Fig. 7.3C).

7.4.4 Protein surface hydrophobicity (S_0)

Surface hydrophobicity (So) reflects the number of hydrophobic groups on the protein surface. The surface hydrophobicity of the control, as well as acylated and hydrolyzed PPIs were measured using ANS and are presented in Fig. 7.4. Both acetylation and succinylation resulted in significant changes in protein surface hydrophobicity and these changes depended on both the type and concentration of anhydrides involved ($p < 0.05$).

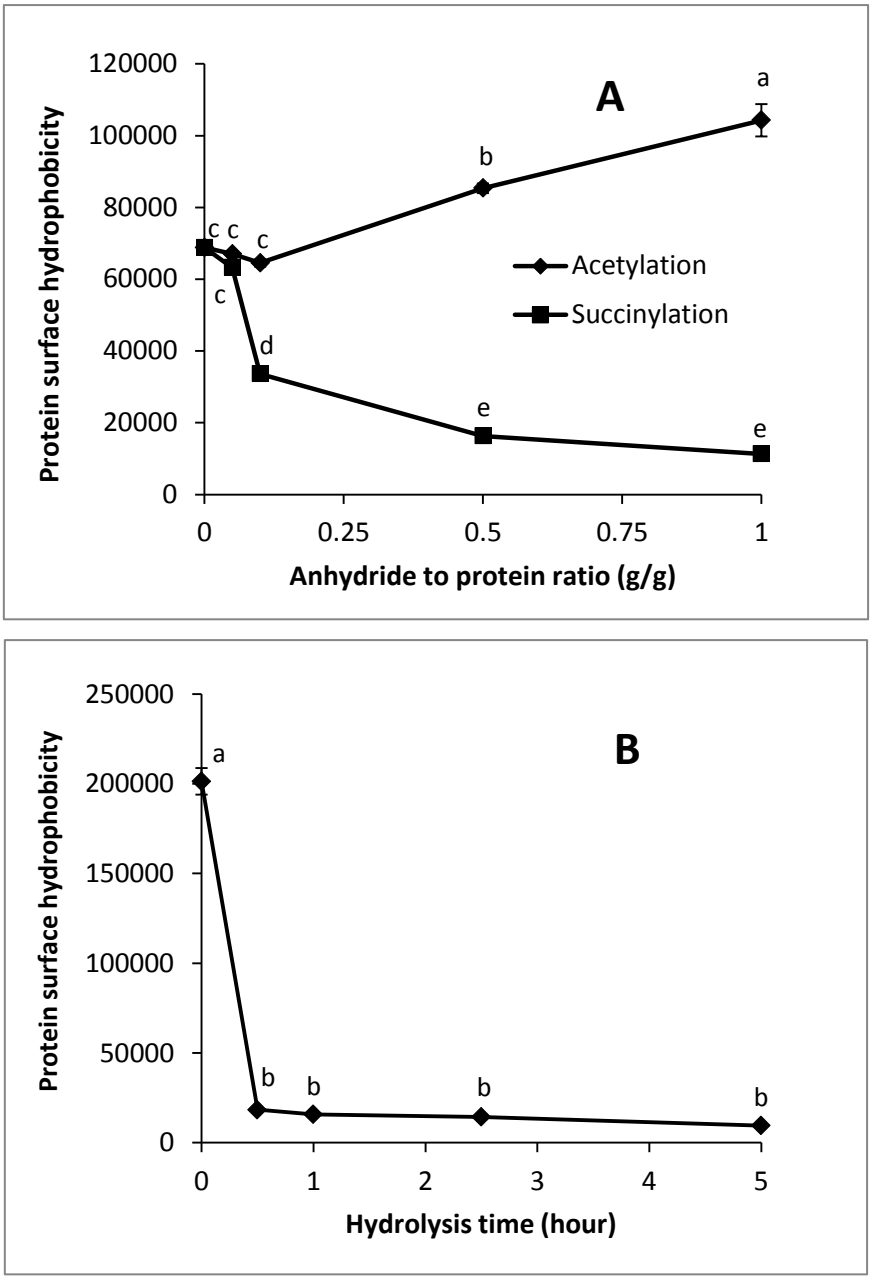


Figure 7.4 Effect of acylation (A) and protein hydrolysis (B) on the surface hydrophobicity of salt-extracted pea protein isolates

[a~e] In each figure, points followed by the same superscript are not significantly different ($p < 0.05$).

Similar to the influence of acetylation on the S_0 of red kidney protein (Yin et al., 2010), legumin from fababean (Krause, Mothes, & Schwenke, 1996; Schwenke et al., 2000) and canola protein (Gruener & Ismond, 1996), S_0 of acetylated PPIs initially decreased and then increased with the addition of increasing levels of acetic anhydride

(Fig.7.4A). The increase in the hydrophobic nature of acetylated PPIs can be attributed to the increased number of hydrophobic acetyl moiety as well as unfolding of the proteins upon acylation (Krause, Mothes, & Schwenke, 1996). As positive charges on proteins were replaced by the neutral charges with the addition of acetic anhydride, repulsion among protein subunits could reveal the previously buried hydrophobic patches, which became accessible for binding to aromatic fluorescence probes such as ANS (Gruener & Ismond, 1996).

In the case of succinylation, significant decreases of S_0 values were observed for the succinylated PPIs throughout the entire process. When succinic anhydrides were added to pea proteins, the increase in negative charges on proteins due to succinic moiety would lead to an overall increase of the negatively charged groups. In addition, this increase in electronegativity of proteins could inhibit the access of ANS^- to the unfolded protein subunits thereby lowering the protein S_0 (Yin et al., 2010). A similar effect of succinylation on the surface properties of red kidney bean protein (Yin et al., 2010) and canola proteins (Gruener & Ismond, 1996; Paulson & Tung, 1987) have been reported previously.

In terms of the effect protein hydrolysis on the S_0 of pea proteins, a sharp drop of the surface hydrophobicity of protein was observed after 0.5h of Alcalase hydrolysis and increasing hydrolysis time did not significantly decreased this value. Similar observations have been found by Avramenko, Low and Nickerson (2013), Jung, Murphy and Johnson (2005) and Celus, Brijs and Delcour (2007) for trypsin treated lentil protein isolate, soy flour and alcalase-treated brewers' spent grain protein

concentrate, respectively. It was proposed by Jung et al. (2005) that aggregation of proteins following exposure of the previously buried hydrophobic groups could contribute to the decrease in protein surface hydrophobicity.

7.4.5 Effect of acylation on protein-flavour binding performance

To demonstrate the potential effect of structural modification on protein-flavour binding properties, aldehyde, ketone, ester and disulfide flavours with potentially different protein-flavour binding mechanisms were selected for evaluation using similar flavours as in Chapter 6. Figure 7.5 illustrates the effect of acylation on pea protein binding with 2-octanone (Fig. 7.5A), octanal (Fig. 7.5B), hexyl acetate (Fig. 7.5C) and dibutyl disulfide (Fig. 7.5D).

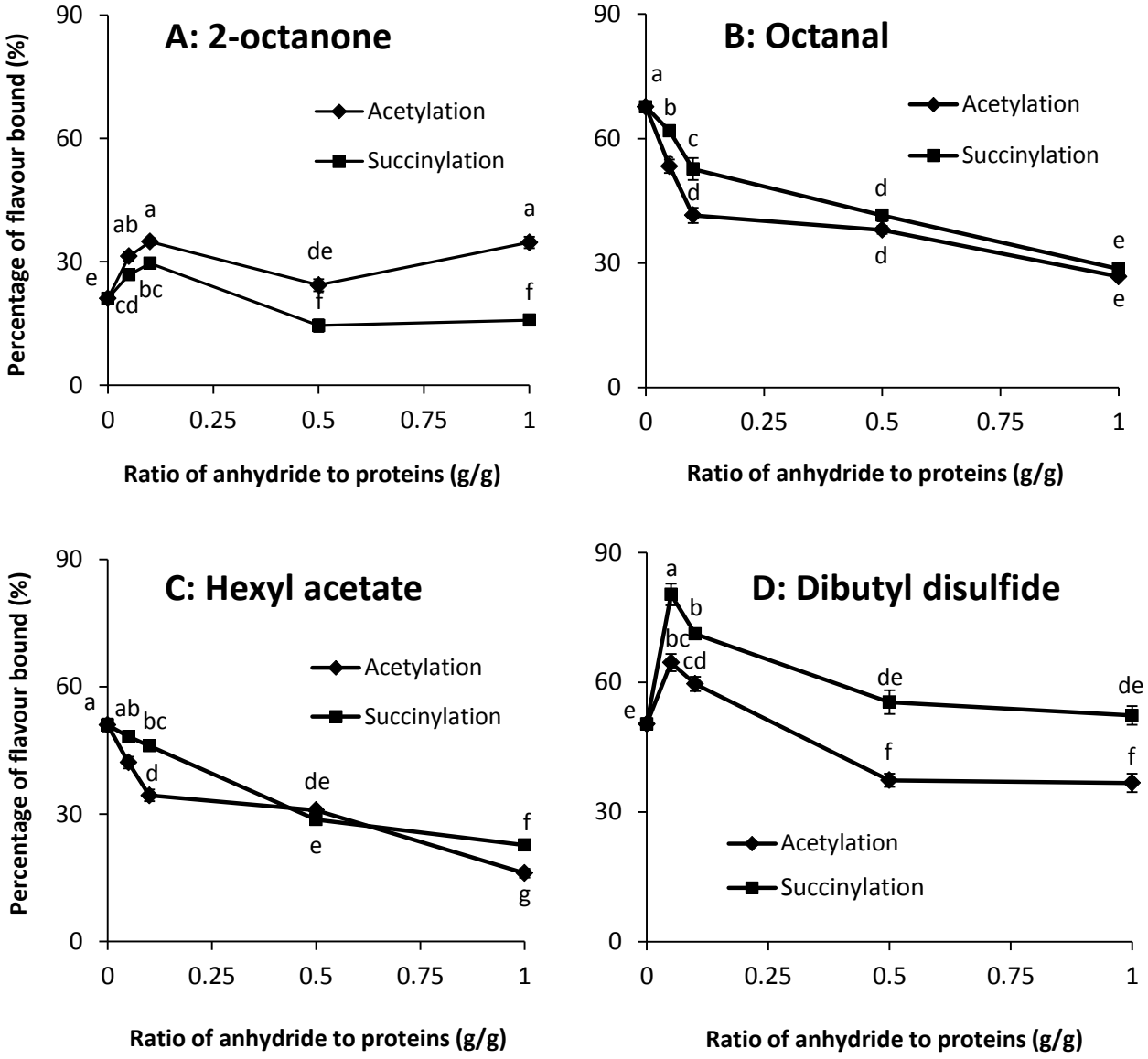


Figure 7.5 Effect of acylation on pea protein binding to 2-octanone (A), octanal (B), hexyl acetate (C) and dibutyl disulfide (D) flavours as a function of anhydride to protein ratio.

[a~g] In each figure, points followed by the same superscript are not significantly different ($p < 0.05$).

7.4.5.1 Effect of acylation on pea protein binding to 2-octanone

As one of the most commonly studied type flavour compounds in protein-flavour binding analyses, ketones have been known to interact with proteins mainly via reversible hydrophobic interactions (Damodaran & Kinsella, 1981a). Increasing the

anhydride to protein ratio up to 0.1g/g caused an initial increase in the binding of 2-octanone, which was followed by a reduction in the retention of this ketone (Fig. 7.5A). Due to partial denaturation of PPIs as seen in the DSC data (Table 7.1) and native PAGE analyses (Fig. 7.3A), the initial addition of anhydrides (0.05 and 0.1 g) could be the result of more flavours being bound to exposed hydrophobic areas. In addition, as acetylated PPIs was more severely denatured compared to the succinylated material, more hydrophobic binding sites could be revealed, thus contributing to its higher retention of 2-octanone. However, the extensive denaturation of protein resulting from further addition of acetic and succinic anhydrides at 0.5 g/g could lead to disruption of the original and exposed hydrophobic areas such that the affinity of acylated PPIs to ketone flavours was reduced. An unexpected increase in the binding of 2-octanonoe was observed at an acetic anhydride to protein ratio of 1g/g. Damodaran and Kinsella (1981b) evaluated the conformational effect of succinylation on binding of 2-nonanone to soy proteins. They found that only half of the binding sites were destroyed by succinylation. It was suggested by the authors that acylated proteins may only be partially destabilized such that still retain certain flavour-binding capacities. Another possibility is binding of flavour onto the protein surface. As an increase in protein surface hydrophobicity was observed when acetic anhydride increased from 0.5 to 1 g/g (Fig. 7.4A), these hydrophobic area may contribute to the increase of ketone retention noted in Fig. 7.5A. It appears that not only the interior hydrophobic region, but also the protein surface, play important roles in ketone retention.

7.4.5.2 Effect of acylation on pea protein binding to octanal

When considering the effect of acylation on PPIs binding with octanal, both acetylation and succinylation were effective in reducing the percentage of octanal bound from 67 to 27%. As previously reported, aldehyde is retained by proteins via both irreversible covalent bonds and reversible hydrophobic interaction (van Ruth and Roozen, 2002). Both the release of reversibly bound aldehyde due to protein dissociation as well as the direct inhibition of covalent linkages between aldehyde and ε-amino groups on lysine residues could contribute to the overall reduction in aldehyde retention.

The greater release of the bound aldehydes for acetylated PPIs at anhydride levels below 0.5 g/g could be attributed to the higher level of N-acetylation compared to its succinylated counterpart (Fig. 7.1A). No significant differences in octanal binding were noted after anhydrides were added above 0.5 g/g even though the acetylated PPIs exhibited much greater O-acylation (Fig. 7.1B). As the degree of N-acylation for acetylated and succinylated PPIs approached at a similar level after the addition 0.5 g/g of anhydrides (Fig. 7.1A), it is possible that the amino groups on the proteins played a more important role than hydroxyl groups when considering the protein-flavour binding efficacy to aldehyde flavours.

7.4.5.3 Effect of acylation on pea protein binding to hexyl acetate

A continuous decrease in the retention of hexyl acetate was observed with increasing anhydride to protein ratios (Fig. 7.5C), indicating a release of bound ester flavours as a result of acylation. For most treatment levels, acetylated PPIs exhibited

lower affinity to hexyl acetate than its succinylated counterpart; an exception was seen at an anhydride to protein ratio of 0.5 g/g, where the binding was not significantly different for the two anhydrides. At 1 g/g, the addition of acetic anhydride reduced the percentage of flavour bound from 51 to 16%. As shown in Chapter 6, hydrophobic interactions, hydrogen bonds and electrostatic interactions were mainly responsible for the retention of this ester by pea proteins (Chapter 6). At anhydride levels of < 0.5 g/g, the greater unfolding effect of acetic anhydride compared to succinic anhydride could be responsible for the greater flavour release. At anhydrides levels above 0.5 g/g, the higher degree of O-acylation (Fig. 7.1B) may inhibit the formation of hydrogen bonds and electrostatic interactions between esters and free charged/polar hydroxyl groups on proteins, further decreasing the affinity of proteins to ester flavours. This effect was greated for acetylated PPIs.

7.4.5.4 Effect of acylation on pea protein binding to dibutyl disulfide

From Fig. 7.5D, it is clear that acetylated PPIs exhibited lower binding affinities to dibutyl disulfide than succinylated PPIs throughout the entire acylation process. A sharp increase of disulfide retention was observed followed by a gradual reduction in disulfide binding when anhydrides were added up to a level of 0.05 g/g, after which no significant changes were observed. The initial increase in the binding for dibutyl disulfide to PPIs can be attributed to partial denaturation of proteins exposing previously buried free sulfhydryl groups that became available for covalent interactions. The subsequent decrease in the retention of dibutyl disulfide could be due to the increased level of S-acylation that reduced the number of accessible

sulfhydryl groups thereby limiting binding. The greater extent of S-acylation for acetic anhydride compared with succinylated PPIs could lead to fewer free sulfhydryl groups being available resulting in lower disulfide binding affinity for acetylated PPIs (Fig. 7.1C).

Interesting, it was found that the extent of flavour binding for dibutyl disulfide was positively correlated to the concentration of free/exposed sulfhydryl groups (Fig. 7.6). Based on these results, it is likely that sulfhydryl-disulfide interchange reactions between added disulfide flavour and available sulfhydryl groups took place. However, no corresponding thiol compounds were detected in the GC/MS analysis. This also supports the previous finding in Chapter 6 that disulfide flavours reacted with pea proteins via covalent interactions.

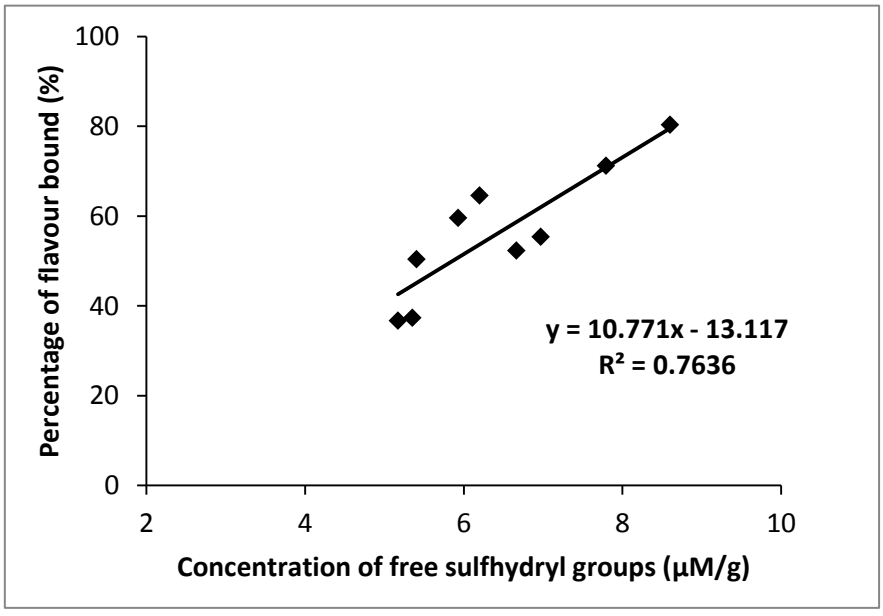


Figure 7.6 Correlation between percentage of dibutyl disulfide bound at 250 ppm to acetylated and succinylated PPIs (1%, w/v) and concentration of free sulfhydryl groups on pea proteins

7.4.6 Effect of enzymatic hydrolysis on protein-flavour binding performance

Using Alcalase, the impact of enzymatic hydrolysis on pea protein binding with aldehyde, ketone, ester and disulfide flavours was evaluated (Fig. 7.7). It was shown that both type of flavour and hydrolysis time significantly affected protein-flavour binding; an interaction was also noted between flavour type and hydrolysis time ($p < 0.05$). With increasing time and degree of hydrolysis, retention for aldehyde and disulfide increased during the first hour and then remained constant. In contrast, binding of the ketone and ester flavours gradually weakened with increased hydrolysis time. Based on Fig. 7.3C and DSC analysis, it was shown that complete dissociation of protein subunits was not able to fully release the selected volatile flavour compounds. As a result, some binding was expected. The increased binding for octanal and dibutyl disulfide may be a result of increased interaction with previously buried functional groups that were exposed during hydrolysis. However, the partial release of both 2-octanone and hexyl acetate flavours may reflect a decrease in hydrophobic regions during hydrolysis. The fact that 13 and 29% of added ketone and ester flavours, respectively, were still retained by PPIs, indicates there is some flavour binding to the protein surface.

Table 7.2 Effect of enzymatic hydrolysis on pea protein binding to 2-octanone, octanal, hexyl acetate and dibutyl disulfide flavours as a function of protein hydrolysis time

Type of flavours	Time of hydrolysis (h)				
	Control	0.5	1	2.5	5
2-octanone	23.76 ± 1.06a	19.66 ± 0.91b	16.98 ± 0.18bc	14.90 ± 0.47cd	13.31 ± 0.57d
Octanal	61.87 ± 0.46c	62.85 ± 0.57c	77.35 ± 0.35a	75.49 ± 0.29b	73.49 ± 0.44b
Hexyl acetate	46.95 ± 0.25ab	48.31 ± 0.08a	44.69 ± 0.25c	42.12 ± 0.22c	29.32 ± 1.74d
Dibutyl disulfide	81.19 ± 0.57c	84.10 ± 0.69b	91.22 ± 0.13a	92.79 ± 0.14a	92.90 ± 0.30a

For each flavor, values followed by the same superscript are not significantly different ($p < 0.05$). Control means PPIs has been treated at 50°C for 5 h without addition of Alcalase.

7.5 Conclusion

Compared with the enzymatic hydrolysis, chemical modification by acylation exhibited higher specificity and increased potential to reduce the affinity of proteins to volatile aldehydes and esters. This could be attributed to dissociation of protein structure and masking of free amino and hydroxyl groups. The retention for the ketone flavour was dependent on a balance between exposed hydrophobic areas and the integrity of protein structure as both the interior hydrophobic region and protein surface properties play important roles in ketone retention. Binding of dibutyl disulfide was positively correlated with the amount of free sulfhydryl groups existing on the protein surface. This provides evidence of potential sulfhydryl-disulfide interchange reactions. Protein hydrolysis during enzymatic modification increased the retention of octanal and dibutyl disulfide, while retention for 2-octanone and hexyl acetate decreased. The decreased binding for 2-octanone and hexyl acetate relied on protein inherent hydrophobic areas, some of which were lost during hydrolysis. As

there was no single mechanism that could account for the protein-flavour interactions, to control release the specific types of bound volatile flavour compounds, different strategies would be needed to modify the proteins. In this way, the negative impact of protein-flavour interaction on flavour quality may be minimized.

Connections between Chapter 7 and Chapter 8

In the preceeding chapters, the emphasis has been on the binding of flavours and how the properties of the flavours and protein structure influence the level of binding. However the effect of protein-flavour binding goes beyond flavour delivery and as a result the influence of these interactions on protein functionality cannot be overlooked. Therefore, the effect of protein-flavour binding on protein functionality, with a particular focus on thermal-indued gelation properties, was investigated in Chapter 8.

Chapter 8 Interaction of selected volatile flavour compounds and salt-extracted pea proteins: effect on protein structure and thermal-induced gelation properties

8.1 Abstract

Characterization of the impact of protein-flavour interactions on protein structure and thermally-induced salt-extracted pea protein gels with selected volatile flavours was studied using a fluorometric method (intrinsic) and small-strain dynamic oscillatory rheology. By monitoring intrinsic fluorescence of protein-flavour mixtures, conformational changes in proteins due to flavour binding were noted with long chain aldehydes resulting in higher degrees of protein unfolding in comparison with ketones. Protein gel forming properties were significantly altered as a function of flavour class (aldehyde and ketone), chain length (6－8 carbon number) and flavour concentrations (0, 250, 500, 1000 ppm). Addition of homologues aldehydes and ketones at 250 ppm decreased gel storage (G') and loss (G") moduli with long chain aldehydes possessing more prominent effects. Interestingly, protein gel strength was restored with increasing concentration and chain length of aldehydes accompanied by gradual decreases of gelling points, while elasticity and gelling points of protein-ketone mixtures remained constant. The additional protein denaturation observed in the fluorometric study could account for the formation of stronger gels during thermally-induced gel formation. A flavour-induced protein structure/function relationship was presented. In addition, flavour binding resulted in changes in G' during both the heating and cooling phases of gelation. For aldehydes, the change in G' during heating was more predominant, whereas the cooling phase was more

responsible for the decrease in G' for ketone flavours.

8.2 Introduction

Pulse proteins including those from dried peas, edible beans, lentils and chickpeas have been recognized as potential ingredients in manufacturing novel protein foods and natural health products (Boye and Maltais, 2011). In Canada, about 5.7 million tonnes of pulses were produced in 2010 and a record of 4.7 million tonnes of pulses was exported in 2011 (Pulse Canada, 2014a). From a nutritional perspective, pulses contain high amount of proteins ranging from 17-30% which is twice the amount of protein found in whole grain cereals (Boye, Zare, & Pletch, 2010; Pulse Canada, 2014b). More than that, some functional properties of pulse proteins have shown promise and were comparable to those of animal and plant proteins such as whey and soy proteins (Boye, Zare, & Pletch, 2010).

Although various benefits of pulse proteins have been recognized, it has been shown that when adding volatile flavouring compounds to plant protein-based food matrices, proteins could interact with flavouring components causing changes in desirable flavour profiles often leading to reductions in aroma intensity (Gremli, 1974; Heng et al., 2004; Zhou, Lee, & Cadwallader, 2006; Suppavorasatit & Cadwallader, 2012; Suppavorasatit, Lee, & Cadwallader, 2012; Wang & Arntfield, 2014, 2015a, 2015b) and generation of potential volatile flavour by-products (Kühn, Considine, & Singh, 2008; Wang & Arntfield, 2014).

When looking at the interactions between protein and flavours, most researchers focused on exploring retention and release of flavours as affected by a number of

intrinsic (e.g. structure and composition of proteins and flavours) and extrinsic (e.g. heat, pH, salt and high pressure treatment) factors (Tromelin, Andriot, & Guichard, 2006; Suppavorastit & Cadwallader, 2010). Little work has been conducted on how protein-flavour interactions affect protein structure and functionality. As protein functionality strongly impacts how proteins can be utilized in the food systems, the potential influence of flavouring components on protein behaviour and resulting functionality must not be overlooked.

Considering the nature of protein-flavour interactions, the mechanisms underlying the phenomena are still not explicit (Wang & Arntfield, 2014). One perspective to gain further insight into the nature of these interactions is to monitor protein-flavour binding using small hydrophobic ligands and spectroscopic techniques. As spectrofluorometric method has been extensively used in protein-ligand binding studies (Damodaran & Kinsella, 1980b; Dufour & Haertlé, 1990; Muresan, van der Bent, & der Wolf, 2001; Liu et al., 2005; Meynier et al., 2004), the wavelength shifts (λ_{max}) and changes in fluorescence intensity (FI) of emission peak of protein tryptophan residues can be used to monitor the environmental changes of these residues in proteins, consequently providing information on protein structure (Cho, Batt, & Sawyer, 1994). To date, no systematic comparison of the influence of aldehydes and ketones on protein structure has been made before using this spectrofluorometric technique.

Protein thermal gelation is an important functionality for plant proteins and is influenced by protein structure (Moure et al., 2006; Sun & Arntfield, 2010). Limited

information has been found on performance of proteins in gel formation in the presence of volatile flavour compounds. It was shown that protein-flavour interactions involve a wide range of linkages, including reversible interactions (hydrogen and ionic bonds, van der Waals forces and hydrophobic interactions) and irreversible covalent linkages (-NH_2 and -SH groups of proteins) (Kim & Min, 1989; Kühn, Considine, & Singh, 2006; Reineccius, 2006a). Hydrophobic interactions and hydrogen bonding are the major forces responsible for gelation of pea proteins (O' Kane et al., 2004; Sun & Arntfield, 2012a). In thermally induced protein gel formation with flavour added, it is possible that interactions between flavour molecules and proteins could disturb the intermolecular hydrophobic interactions between protein molecules that contribute to gel formation. As a result, a weaker gel could be formed. In addition, it can be further speculated that the irreversibly bound aldehyde flavours would be less likely to be affected by the heating process and would therefore have a greater chance of influencing protein gel formation than the reversibly bound ketone flavours.

The objective of this research was to evaluate the rheological characteristics of heat-induced PPIs gels in a mixed system with selected volatile flavour compounds. Both aldehydes and ketones were chosen to address the potential reversible and irreversible binding effect. In addition, protein conformational changes as a function of flavour added were monitored using a spectrofluorometric method. The potential correlation between the effect of flavour binding on protein structure and associated changes in protein thermal gelation properties were evaluated and discussed.

8.3 Materials and methods

8.3.1 Source of materials

Homologous series of aldehyde (hexanal, heptanal, and octanal) and ketone (2-hexanone, 2-heptanone, and 2-octanone) flavours were selected and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Commercial yellow pea (*Pisum sativum* L.) flour was kindly supplied by Best Cooking Pulses Inc. (Portage la Prairie, MB). All chemicals used were of reagent grade if not stated otherwise.

8.3.2. Preparation of salt-extracted pea protein isolates (PPIs)

Following the method previously described by Wang and Arntfield (2014, 2015a), salt-extracted pea protein isolates (PPIs) was extracted from sieved (500 μm opening, USA Standard NO. 35) yellow pea flour using 0.3 M NaCl (pea flour: sodium chloride solution = 3:10, w/v) under constant stirring for ½ hour. After the first centrifugation (4260*g*, 4℃, 15 min), pea protein was recovered by diluting the supernatant in two times its volume of cold distilled H_2O. After leaving in a refrigerator (3℃) for 2 h, the precipitated protein sediment was collected after a second centrifugation (680*g*, 4℃, 15 min) and re-suspended in a small amount of distilled H_2O. The resulting protein suspension was then dialyzed against 20 times cold distilled H_2O using 12-14,000 Da molecular weight cut-off dialysis tubing (Spectra/Por Dialysis Membrane, Rancho Dominguez, CA) for 72 h in a refrigerator. Distilled H_2O was changed every 24 h. The desalted protein isolates were stored at -30℃ until freeze dried (Genesis SQ Freeze Dryer, Gardiner, NY, USA).

By using a N to protein conversion factor of 5.7 (Sun & Arntfield, 2010), the

freeze dried PPIs contained 82.68 % protein using a Dumas method and a FP-528 Nitrogen/Protein Determinator (LECO Corporation, St. Joseph, MI, USA). The PPIs contained 3.53 % (w/w) of crude fat content determined using the AOAC Official Method 2003.06 (2003) using Soxhlet apparatus.

8.3.3 Amino acids composition of PPIs

Prior to spectrofluorometric analysis, the amino acid composition of PPIs (combined material that was used throughout the project) was determined to ensure the existence of hydrophobic amino acids such as tryptophan residues; this was done using an amino acid analyzer (Sykan Germany, Model S2100, S4300, Gewerbering, Eresing) in duplicate. After samples were hydrolyzed in 6 M HCl, the amino acid profiles of PPIs were determined following AOAC Official Method (Method 994.12; AOAC, 1995). Contents of cysteine and methionine were obtained by an oxidized hydrolysis procedure with performic acid (Andres & Bldar, 1985). Tryptophan content was measured after alkaline hydrolysis (Hugli & Moore, 1972).

8.3.4 Preparation of flavour stock solutions

Stock solutions of each volatile flavour compound (hexanal, heptanal, octanal, 2-hexanone, 2-heptanone and 2-octanone) were prepared in 0.3 M NaCl solution at 1500 ppm (0.15 mL/100mL) and sealed in amber gastight glass bottles to prevent decomposition. Following the method of Gkionakis, Taylor, Ahmad, and Heliopoulos (2007), flavour stock solutions were put in an ultrasonic water bath (Branson 3200, 50 Hz, Bransonic Ultrasonic Cleaner, Shelton, CT, USA) for 1 h to ensure a thorough

mixing before each use. Previous headspace GC/MS analysis (Wang & Arntfield, 2014, 2015a, 2015b) found that no change of flavour structure/composition was detected after this ultrasonication step while a significant and stable amount of flavour was retained in aqueous phase of flavour stock solutions.

8.3.5 Spectrofluorometric measurement

Potential conformational changes to PPIs upon flavour binding were followed by monitoring intrinsic fluorescence of tryptophan residues. Following the reaction conditions previously described by Wang and Arntfield (2014), 10 % (w/v) PPIs suspension was prepared in 0.3 M NaCl at the desired flavour concentration, followed by completely mixing the protein-flavour mixture on a RKVSD rotary shaker (Appropriate Technical Resources, Inc., Laurel, MD) for 1 h at speed 40 to allow an interaction between protein and flavours. As a complete clear solution was required to conduct spectroscopic measurement, the resulting protein-flavour suspension was well mixed and then centrifuged at 5000 *g* for 5 min at room temperature using an IEC Micromax centrifuge (IEC International Equipment Company, MA, USA) to create a clear supernatant which was subsequently diluted 100 times with 0.3 M NaCl prior to spectrofluorometric analysis (Mundi & Aluko, 2013; Malomo, He & Aluko, 2014). The spectroscopic characteristics of PPIs in the supernatant were measured to reflect potential changes of protein structure resulting from adding flavours.

Fluorometric measurements were conducted using a FP-6300 Spectrofluorometer (JASCO Corp., Tokyo, Japan) equipped with a FMM-100 fused quartz rectangular microcell (3×3 mm) at constant temperature of 25 ℃ regulated by a thermostatic cell

holder with circulating water (GHAAKE Water bath, Berlin, W. Germany). Based on the work of O'Neill and Kinsella (1987b), diluted protein samples were excited at 280 nm. The instrument was set at fast response and high sensitivity using a band width of 2.5 nm, 0.5 nm data pitch and 500 nm/min scanning speed. Emission spectra were obtained from 300 to 400 nm while recording emission peak wavelength (λ_{max}) and maximum fluorescence intensity (FI). Between samples, the microcell was washed twice with 0.3 M NaCl and then two times with subsequent samples before the next reading was recorded. Each sample was prepared in triplicate and each triplicate was measured twice with the average FI being used in subsequent calculations. It was noted that there was no difference between the FI of 0.3 M NaCl and 0.3 M NaCl with flavours added. Therefore, relative fluorescence intensity (RFI) was obtained by subtracting FI of 0.3 M NaCl from that of samples containing flavours and proteins.

8.3.6 Dynamic oscillatory rheology measurements

To look at the potential of volatile flavour compounds to impact the gel formation of salt-extracted pea protein isolates (PPIs), small amplitude oscillatory tests were conducted using an AR2000 Advanced Rheometer (TA Instruments, Inc., Newcastle, DE, USA).

Following the standard procedure described by Sun and Arntfield (2010, 2011), 14.5% (w/v) PPIs suspensions were produced in 0.3 M NaCl at the desired flavour concentration; the protein-flavour mixtures were shaken on a RKVSD rotary shaker (Appropriate Technical Resources, Inc., Laurel, MD) for 1 h at speed 40. After completely mixing the sample for 1 min using a Vortex-Genie 2 (Scientific Industries,

Inc., Bohemia, NY, USA), approximately 1.3 mL of protein suspension was transferred onto the lower plate of the parallel plate geometry. The upper plate (4 cm diameter flat plate, TA Instruments) was then lowered to a 1 mm gap width. To prevent solvent evaporation during heating, about 0.3 mL Mineral Oil (Light white oil, Sigma Chemical Co., St. Louis, MO) were added to the solvent well of upper plate and 4-6 cm solvent trap split covers were placed over the geometry to attain a saturated vapor.

Pea protein gels were formed by heating samples from 25℃ to 95℃ and then cooling from 95℃ to 25℃ at the ramp rate of 2 ℃/min and 1 Hz frequency. A 2 min equilibrium time was applied in between each oscillatory step. After cooling, a frequency sweep at 25℃ from 0.01 to 10 Hz was conducted to evaluate the stiffness of the final protein gel. A 2% strain was used for all the analyses. Samples were run at least in duplicate. Storage modulus (G'), loss modules (G"), and tan δ (G"/G') were monitored to reflect the elastic and viscous components of the gel as well as type of gel formed. Protein gelling temperature was determined based on the crossover point of storage and loss modulus during increasing of the temperature.

8.3.7 Experimental design

In the current study, 2×3×4 factorial experiments were implemented to evaluate the influences of flavour class (aldehyde and ketone), flavour carbon number (C_6, C_7 and C_8) and flavour concentration (0, 250, 500 and 1000 ppm) on overall structure and heat-induced gel formation properties of PPIs. Homologous series of aldehydes (hexanal, heptanal and octanal) and ketones (2-hexanone, 2-heptanone and 2-octanone)

were selected as the flavours.

In the first study, a spectrofluorometric method was used to monitor changes in protein overall conformation based on a change in the wavelength of the emission peak of tryptophan residues and maximum fluorescence intensity. All samples were prepared in triplicate.

In the second study, dynamic oscillatory testing was employed to monitor the development of gel structure and final gel strength in the presence of volatile flavour compounds. G', G'', tan δ and protein gelling temperature with and without presence of volatile compounds were reported. Each point in factorial design was repeated at least twice.

8.3.8 Statistical analysis

All data were analyzed using Microsoft Excel and SAS 9.0 program (SAS Institute Inc., Gary, NC, USA). Tukey's test following the analysis of variance indicated significant different with a level of $p < 0.05$.

8.4 Results and discussions

8.4.1 Amino acid composition of PPIs

Table 8.1 illustrates the amino acid composition of salt-extracted pea protein isolates. It can be seen that PPIs possessed great amounts of asparagine/aspartic acid (11.79%) and glutamine/glutamic acid (19.59%), was rich in arginine (10.619%) and lysine (7.09%), but had low levels of tryptophan (0.737%) and the sulphur containing amino acids cysteine (0.603%) and methionine (0.636%). These results agreed with

Pownall, Udenigwe and Aluko (2010) who found pea protein isolates contained 11.81% asparagine/aspartic acid, 16.54% glutamine/glutamic acid, 8.6% arginine, 7.35% lysine, 0.83% tryptophan, 0.87% cysteine and 1.12% methionine. Generally, hydrophobic amino acid such as tryptophan plays an important role to produce steric unfolding of protein structure. However, due to the small percentage (0.737%) of tryptophan residues as shown in Table 8.1, higher concentrations (250 to 1000 ppm) of flavours (aldehydes or ketones) is necessary in order to evaluate potential effect of carbonyl flavours on protein conformation by interacting with PPIs.

Table 8.1 Amino acid composition of salt-extracted pea protein isolates (PPIs)

Protein amino acid	Percentage of amino acid (%)
Asp [a]	11.79
Thr	3.128
Ser	5.254
Glu [b]	19.59
Pro	4.638
Gly	3.639
Ala	3.475
Cys	0.603
Val	4.281
Met	0.636
Ile	3.842
Leu	8.039
Tyr	3.318
Phe	4.883
His	2.716
Lys	7.09
NH_3	1.721
Arg	10.619
Trp	0.737
Total	100

[a] Asp include aspartic acid and asparagine

[b] Glu include glutamic acid and glutamine

8.4.2 Evaluation of flavour binding on conformational properties of PPIs

Following the changes in maximum fluorescence intensity for the protein emission spectra, Figure 8.1 illustrates the effect of flavour binding on PPIs overall conformation as manifested by changes in PPIs intrinsic fluorescence with increasing concentration of homologous aldehyde (Fig. 8.1a) and ketone (Fig. 8.1b) flavours.

From Fig. 8.1, it is clear that addition of volatile carbonyl compounds at all selected concentration led to significant reductions ($p < 0.05$) in the RFI, inferring binding of added flavour ligands resulted in quenching of PPIs fluorescence (Dufour & Haertlé, 1990; Muresan, van der Bent, & de Wolf, 2001; Liu et al., 2005). However, no change in the wavelength of PPIs emission peak (λ_{max}) was observed (data not shown). Interactions between PPIs (1%, w/w) and homologous aldehydes and ketones at 250 ppm were observed using a headspace GC/MS approach with long chain aldehyde possessing a higher ability to bound proteins (Heng et al., 2004; Wang & Arntfield, 2014, 2015a). Regardless of the type of flavour involved, increasing concentrations of volatile compounds resulted in gradual decreases of RFI with aldehydes exhibiting more prominent effects than ketones. Increasing aldehyde concentration to 250 ppm showed decreases of RFI for all aldehydes from 100 (arbitrary unit) to about 87.97; with long chain octanal obtaining a greater reduction (to 72.66) than heptanal (80.85) and hexanal (84.87) when flavour concentration reached 1000 ppm. However, decreases of RFI reached a plateau value of ~87 for ketones indicating limited conformational change in PPIs due to ketone binding in comparison with aldehydes.

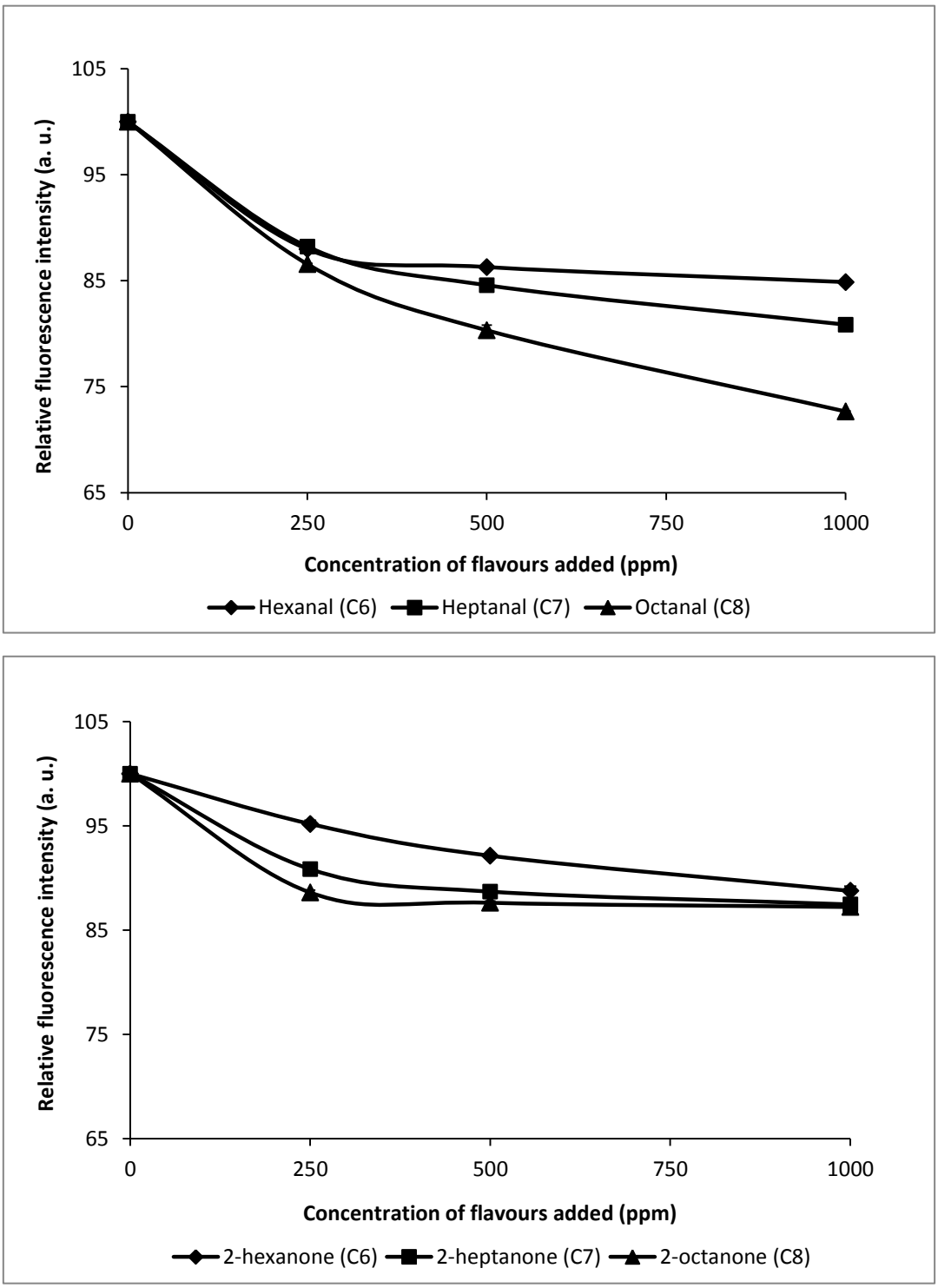


Figure 8.1Relative fluorescence emission intensity for 10% (w/v) PPIs at 312 nm with increasing concentration of homologous series of aldehyde (a) and ketone (b) flavours.

Note: All fluorescence intensity was normalized to 100 arbitrary units (a. u.) on the basis of the RFI of PPIs in the absence of flavour compounds.

It is known that fluorescence quenching is associated with polarity changes in the neighbourhood of tryptophan (Trp) residues (Lakowicz, 1999). The decrease of RFI (fluorescence quenching) inferred changes around the indoles (Trp) from a non-polar (hydrophobic) environment to a more polar environment indicating exposure of previously buried Trp residues resulted from conformational changes (unfolding) in proteins due to flavour binding (Damodaran and Kinsella, 1980b, 1981b). This finding correlated well with previous studies conducted by Wang and Arntfield (2014, 2015a) who demonstrated an unfolding effect of flavour on PPIs by measuring thermal properties of proteins in the presence of volatile carbonyls using differential scanning calorimetry. Wang and Arntfield (2014, 2015a) stated that, as enthalpy of denaturation (ΔH) of PPIs was significantly decreased when flavours were added, flavour molecules might be involved in unfolding of protein structure leading to protein partial denaturation, and as a result less energy was required to completely denature the protein molecule.

When comparing aldehydes and ketones, more fluorescence quenching was seen with aldehydes than corresponding ketones, particularly at higher flavour concentrations (Fig. 8.1a *vs*. 8.1b). It appears that aldehydes may possess a higher ability to denature protein than ketones as larger decreases of RFI suggested greater protein unfolding. It has been widely agreed that proteins preferentially bind aldehydes than ketones and the degree of binding increases with flavour chain length (Wang & Arntfield, 2014; 2015a). As a result, additional flavour binding sites can be created due to the unfolding effect of aldehydes which further promote binding of

flavours. In other words, a positive correlation could exist between the degree of interactions between proteins and flavours and the level of protein unfolding resulting from flavour binding.

Similar fluorescence quenching effects have also been reported for binding of several other hydrophobic molecules including β-ionone, retinol, curcumin, benzaldehyde, aliphatic aldehydes and methyl ketones to bovine serum albumin (Damodaran & Kinsella, 1980), whey protein concentrate (Liu et al., 2005) and β-lactoglobulin (Wang, Allen, & Swaisgood, 1997; Muresan, van der Bent, & de Wolf, 2001; Relkin, Molle, & Marin, 2001; Dufour & Haertlé, 1990; Stapelfeldt & Skibsted, 1994; Sneharani et al., 2010). It appears that the behaviour previously reported for milk proteins also results in unfolding of plant proteins such as PPIs and using spectrofluorometric method can follow changes in protein structure due to protein-flavour interaction and thus provide critical information on the nature of the binding.

8.4.3 Evaluation of flavour binding on thermal gelation properties of PPIs

The effect of flavour on PPIs thermal gelation properties was initially analyzed at a fixed flavour concentration of 250 ppm with a focus on added flavour structure. Subsequently, the impact of flavour binding on gel formation with gradual increases of flavour concentration was investigated for homologous aldehyde and ketone flavours.

8.4.3.1 Effect of flavour chain length and functional group (at flavour concentration of 250 ppm)

As shown in Figure 8.2, when flavours were mixed at 250 ppm, the resulting effect on protein thermal gelation properties generally coincided well with the previously stated hypothesis that inclusion of volatile flavour compounds in a mixed system with proteins produced lower G' and G" values suggesting decreased interaction between protein molecules and potential disruption of protein inter- and intra-molecular forces that contribute to network formation (Matsumura & Mori, 1996). However, no significant changes in tan δ (~0.15) were observed in the presence and absence of flavours indicating the type of gel formed remained constant.

For aldehydes, G' and G" gradually decreased with increasing flavour carbon number such that the long chain aldehyde exhibited a greater effect on protein gelation. A similar trend was seen for the C6 and C7 ketones, but values for the C8 ketone were not significantly different from those at C6 and C7. It has been previously noted that flavour binding generally increased upon heating (Ng et al., 1989a; Gkionakis, Taylor, Ahmad, & Helipoulos, 2007; Wang & Arntfield, 2015a). As flavour compounds bind with proteins via non-covalent or covalent linkages or a combination of the two (Tromelin, Andriot, & Guichard, 2006), the association of flavours with the reactive groups exposed during heating of proteins could interfere with the interactions between unfolded proteins, subsequently disrupting protein network formation and producing a weaker gel. It has also been reported that increasing carbon number of flavours led to a significant increase in the extent of

flavour binding (Gremli, 1974; Heng et al., 2004; Wang & Arntfield, 2014) and therefore would be expected to have a greater impact on gel structure.

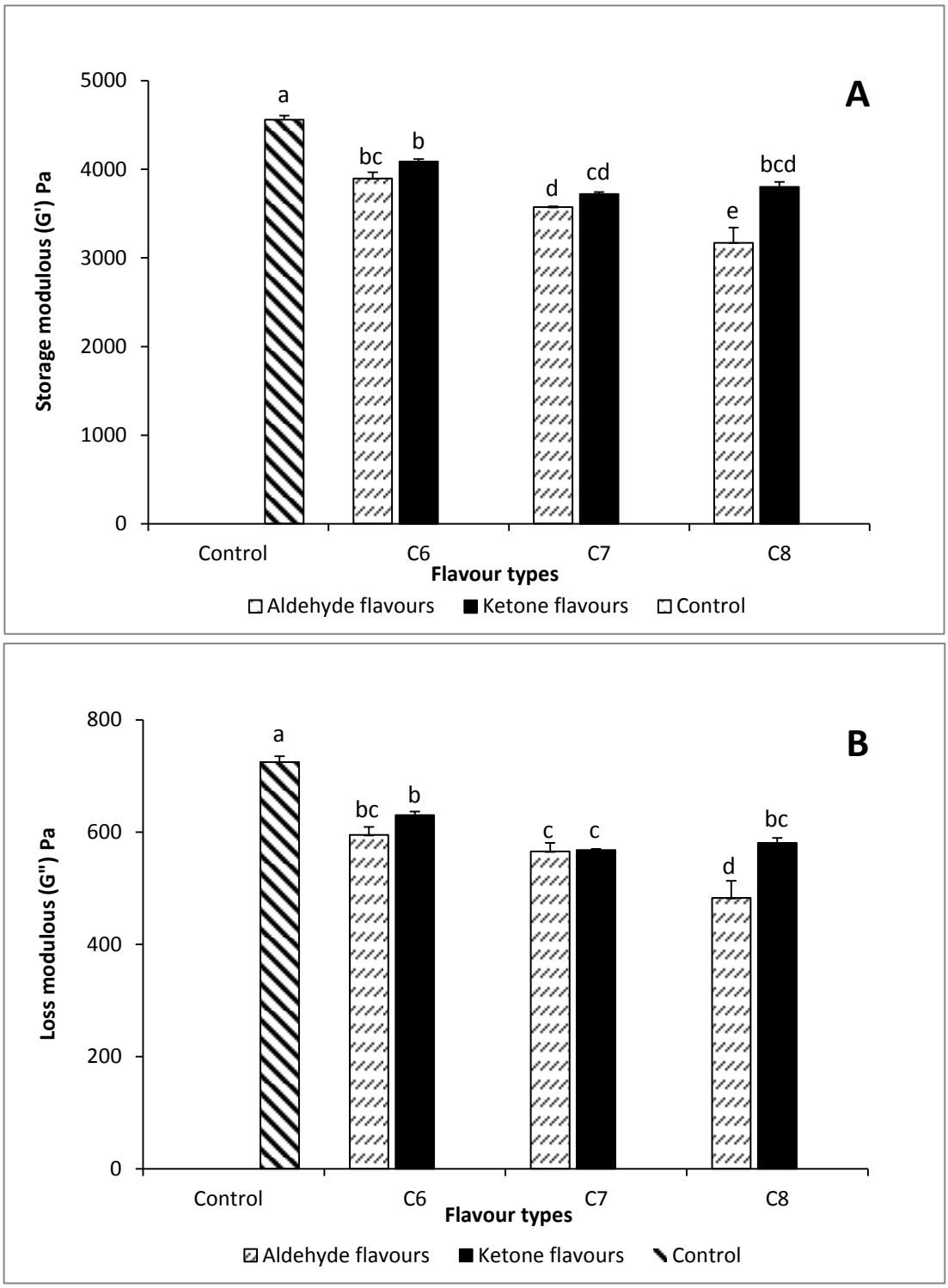


Figure 8.2 Effect of flavour binding on the storage (A) and loss (B) moduli of heat-induced pea protein gel (14.5%, w/v) at 250 ppm flavour concentration

[a-e] In each figure, bar values followed by the same superscript are not significantly different ($P < 0.05$).

As expected, this negative impact on protein gelation was more evidently shown for aldehydes than for ketones especially when flavour carbon number increased to eight. Wang and Arntfield (2015a) reported that percentage binding of hexanal dramatically increased with increasing heating time from 0 to 60 min at 95℃, while 2-octanone retention initially increased but then decreased after 2 min of heating. A release of previously bound 2-octanone as a result of replacement of protein-flavour interaction by heat-induced protein-protein associations was reported. Therefore, when considering the thermally-induced protein gel formation in the presence of flavours, the heating process could disrupt the reversibly bound flavours such as ketones; however, flavours such as aldehydes which were irreversibly bound through covalent linkages would be less likely to be disrupted by the thermal treatment during the gelling process. In addition, the ability of aldehydes to bind proteins at a level 2-5 times higher than ketones, which has been reported previously (Heng et al., 2004; Wang & Arntfield, 2014a), also helps explain why aldehyde flavours exhibited higher ability to impact protein gel strength in comparison with ketones at 250 ppm flavour concentration.

8.4.3.2 Effect of aldehydes as a function of added flavour concentration

As shown in Fig. 8.3, the decreases in PPIs gel elastic (G') and loss (G") moduli were reversed at concentration of aldehydes above 250 ppm. For octanal, the elastic modulus of PPIs was significantly improved from a low 3169.5 Pa to 5705 Pa when octanal concentration increased from 250 to 1000 ppm; the latter was higher than the 4559 Pa of the control sample to which no flavour was added. Similar trends were

seen for G' and G" of both heptanal and hexanal but differences were not as great as was seen with octanal.

For globular proteins, a prerequisite to protein gelation usually requires protein being denatured, often induced by heat (Sun & Arntfield, 2010). In other words, to form a gel, a protein is required to be denatured as a precondition for intermolecular crosslinking between heat-exposed residues of proteins. It has been noted that volatile carbonyl compounds possessed an unfolding effect on PPIs and could cause PPIs to be partially denatured (Wang & Arntfield, 2014; 2015a). This unfolding effect due to flavours was further confirmed by the fluorescence quenching of PPIs as illustrated by the decrease in intrinsic fluorescence intensity in the current study (Fig. 8.1). Therefore, in the presence of volatile carbonyl compounds, the resulting unfolding of PPIs due to flavour binding may reduce the heat required to denature PPIs to a point (protein gelling temperature) that is suitable for protein-protein crosslinking. As a result, network formation may begin sooner and greater gelling may be achieved (Fig. 8.5).

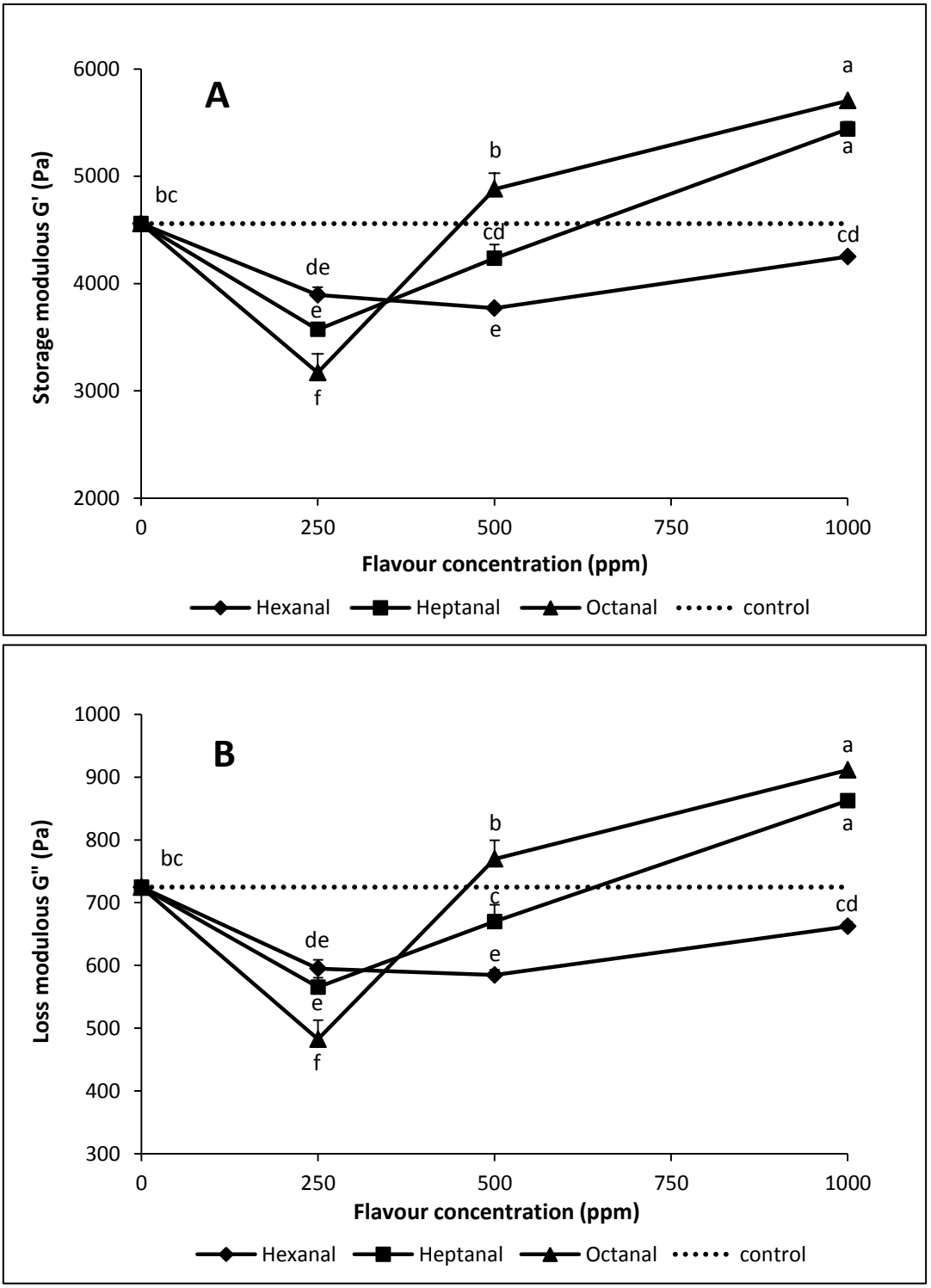


Figure 8.3 Effect of flavour binding on the storage (A) and loss (B) moduli of heat-induced pea protein gel (14.5%, w/v) with increased concentration of homologous aldehyde flavours.

[a-f]In each figure, points followed by the same superscript are not significantly different ($P < 0.05$). Note: Dash line which represents the G' and G" of the PPIs in the absence of flavours was added to facilitate comparison of impact of flavour binding on G' and G".

Results varied depending on the concentration of flavours employed. At 250 ppm, binding of aldehydes was able to weaken protein gel formation by inhibiting protein intermolecular hydrophobic forces (Fig 8.3); this factor appeared to have a greater influence than the previously identified unfolding effect of flavours as changes in protein structure were limited at this concentration (Fig. 8.1a). However, when concentration of aldehydes increased above 500 ppm, it is possible that the impact of flavour-induced "protein unfolding" become more predominant such that it exceeded the influence of flavour "binding effect" leading to an overall increase of the protein gel strength. As the effect of flavours on protein conformation was more evident for long chain aldehydes, particularly at higher flavour concentrations (Fig. 8.1a), the change in PPIs gel formation patterns seen in Fig. 8.3 can be attributed to the increased protein unfolding in the presence of flavours and is dependent on both flavour structure and concentration.

8.4.3.3 Effect of ketones as a function of added flavour concentration

Different from the gel formation patterns that was observed in Fig. 8.3, the G' and G" of the gel produced from protein-ketone mixtures decreased from 4559 and 724.70 Pa of control sample to a value that below 4089 (G') and 630.05 Pa (G") at ketone concentration of 250 ppm and remained stable even at elevated flavour concentrations (Fig. 8.4). This can be explained by the limited influence of ketones on protein structure as increasing concentration of ketones above 250 ppm did not lead to further decrease of PPIs RFI as shown in Fig. 8.1b. It appeared that, with increasing of flavour concentration, the potential unfolding of PPIs by ketones was overwhelmed

by the binding of flavours throughout the flavour concentration range used in the current study. As a consequence, a weaker gel was formed regardless of concentration and type of ketones involved.

Furthermore, as ketones have been shown to bind reversibly with proteins (Tromelin, Andriot, & Guichard, 2006), heating may disrupt the association between protein and ketones resulting in fewer ketones being bound and the influence on protein structure reduced. In addition, the release of previously or heat-induced bound ketones may not be complete. Wang and Arntfield (2015a) monitored the retention of 2-octanone flavour at 250 ppm to 1 % (w/v) salt-extracted canola protein isolate at 95℃ continuously for 1 h. The retention of 2-octanone increased from 17.99 to 28.4 % in the first two minutes of heating following by gradual decrease of flavour binding to 17.48 % in the following 58 min. About 17 % of the 2-octanone remained bound to canola proteins after 60 min of heating. In the current experiment, there may be some continuous release and binding of ketones during the heating process such that the ability of the protein to form a strong network remained low.

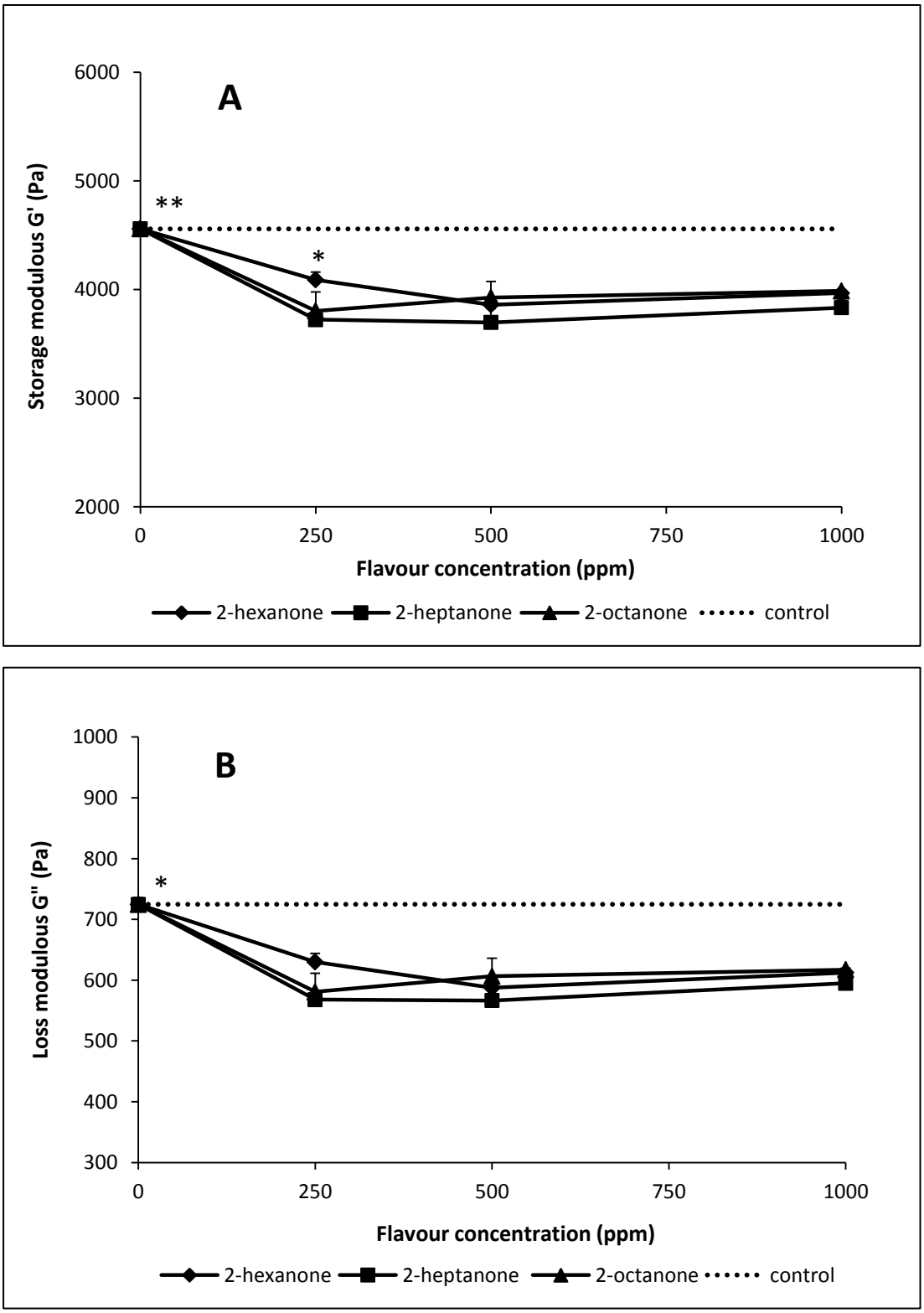


Figure 8.4 Effect of flavour binding on the storage (A) and loss (B) moduli of heat-induced pea protein gel (14.5%, w/v) with increased concentration of homologous ketone flavours.

* and ** indicate the number was significantly different from the others. Note: Dash line which represents the G' and G" of the PPIs in the absence of flavours was added to facilitate comparison of impact of flavour binding on G' and G".

8.4.3.4 Effect of flavour binding on gelling temperature

Traditionally, both the G'- G" crossover point and extrapolation of the rapidly rising phase of the storage modulus back to the temperature axis have been reported as being used to indicate the onset temperature of heat-induced protein gelation (Winter, 1987; Hsieh, Regenstein, & Rao, 1993; Sun & Arntfield, 2010). In the current research using dynamic oscillatory measurements to monitor the evolution of G' and G" as a function of cross-linking time, the G'- G" crossover point was selected to represent the onset gelling temperature (gelling point), as it was a well-defined number (Winter, 1987).

It can be seen from Table 8.2 that the gelling temperature of PPIs dispersions with presence of different concentrations of ketones fluctuated between 82 to 83℃ significantly lower than that of control (85.75 ℃), but were not significantly different from each other. However, significant decreases in gelling points for protein-aldehyde mixtures were noted with increasing aldehyde concentration with the greatest decreases occurring for octanal at 1000 ppm.

Table 8.2 Effect of flavour binding on gelling temperature (℃) of heat-induced PPIs gel (14.5%, w/v) as a function of added flavour structure and concentrations

Flavour types	Carbon number	Flavour concentration (ppm)			
		0	250	500	1000
Aldehyde flavours	C_6	85.75 ± 0.28[a]	84.25 ± 0.21[b]	81.10 ± 0.42[de]	81.20 ± 0.85[d]
	C_7		83.95 ± 0.21[bc]	80.25 ± 0.21[def]	79.50 ± 0.28[f]
	C_8		83.15 ± 0.49[bc]	79.75 ± 0.07[ef]	77.30 ± 0.28[g]
Ketone flavours	C_6		83.25 ± 0.21[bc]	82.65 ± 0.21[c]	82.60 ± 0.14[c]
	C_7		83.75 ± 0.49[bc]	83.80 ± 0.42[bc]	83.65 ± 0.21[bc]
	C_8		83.60 ± 0.14[bc]	83.85 ± 0.49[bc]	83.65 ± 0.21[bc]

[a~g] Column and row values followed by the same superscript are not significantly different ($P < 0.05$).

To demonstrate how the binding of aldehydes and ketones influence the development of the storage modulus (G'), Fig. 8.5 displays a portion (70 and 95℃) of the typical gel formation patterns for PPIs with and without the addition of octanal and 2-octanone at a concentration of 1000 ppm. The gel formation behaviour for PPIs in the presence of 1000 ppm octanal was distinctly different from that of control with a lower gelling point of 77.30 ℃ being observed. As increases in G' began at temperatures below 75 ℃, PPIs started to gel much earlier with a higher G' value was maintained throughout the heating phase in the presence of octanal than the control or the sample with 2-octanone. This observation supports our previous statement that partial unfolding of protein due to aldehyde binding reduced the energy barrier to dissociate proteins, resulting in a lower gelling point such that the time available for protein-protein crosslinking during the heating stage was increased.

As no decrease in gelling point was observed among ketone flavours (Table 8.2), it was clear that the gelling point of protein-ketone mixture was independent of type and concentration of ketones added. This observation can be attributed to the limited effect of ketones on protein structure (Fig. 8.1b).

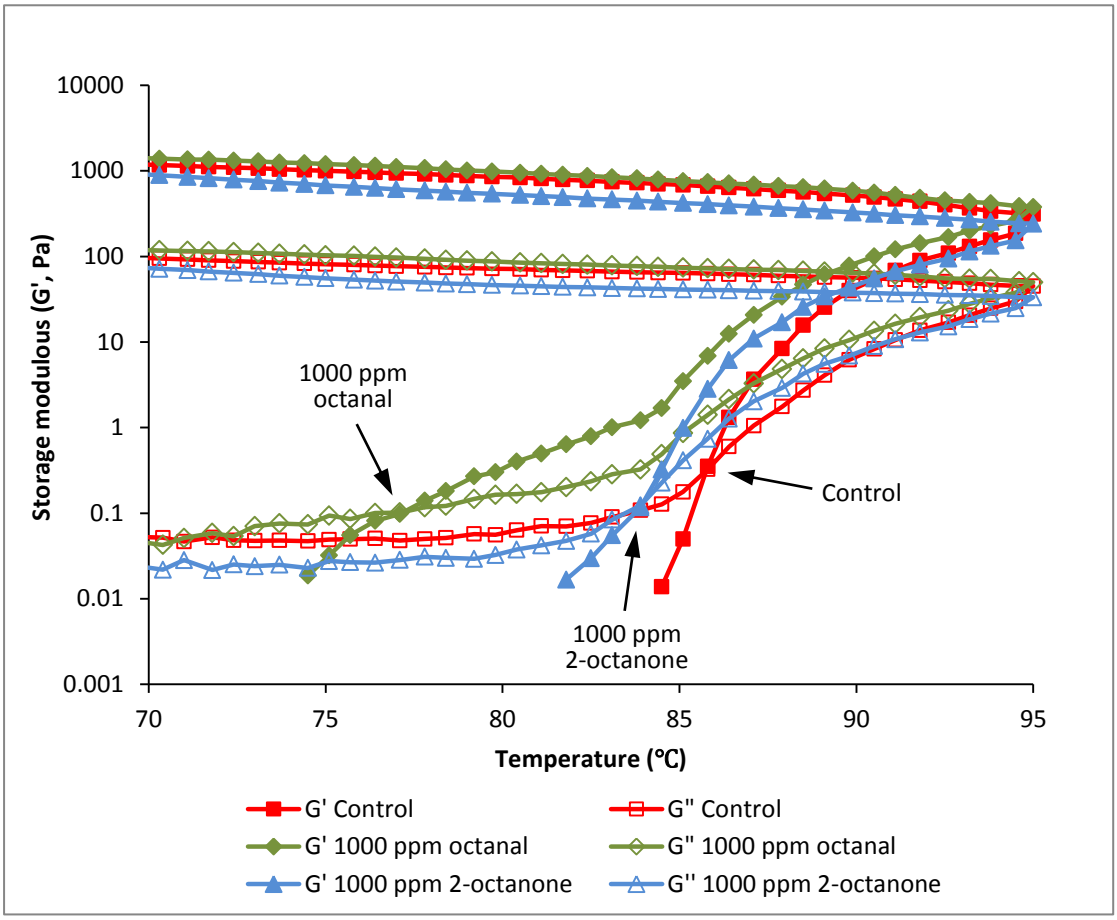


Figure 8.5 Typical gel formation patterns of pea proteins and pea proteins with the presence of 1000 ppm octanal and 2-octanone flavours.
Note: gelling points were shown as crossover points of G' and G" with increasing of heating temperature and the respective gelling point of the three illustrated samples was pointed out by arrows.

8.4.3.5 Determination of role of flavours during heating versus cooling

To elucidate the impact of protein-flavour association on protein gel formation, it is worthwhile to consider in which phase (heating phase, cooling phase or both phases) of gelling process that flavours were primarily interacting with proteins. By knowing at which stage proteins were most affected, it may be possible to manipulate the heating and cooling conditions to optimize the effect of flavour binding in terms of subsequent gel formation properties.

A method which calculates the percentage (%) relative change of G' during

heating and cooling phases of PPIs with and without addition of flavours was developed and presented as the following:

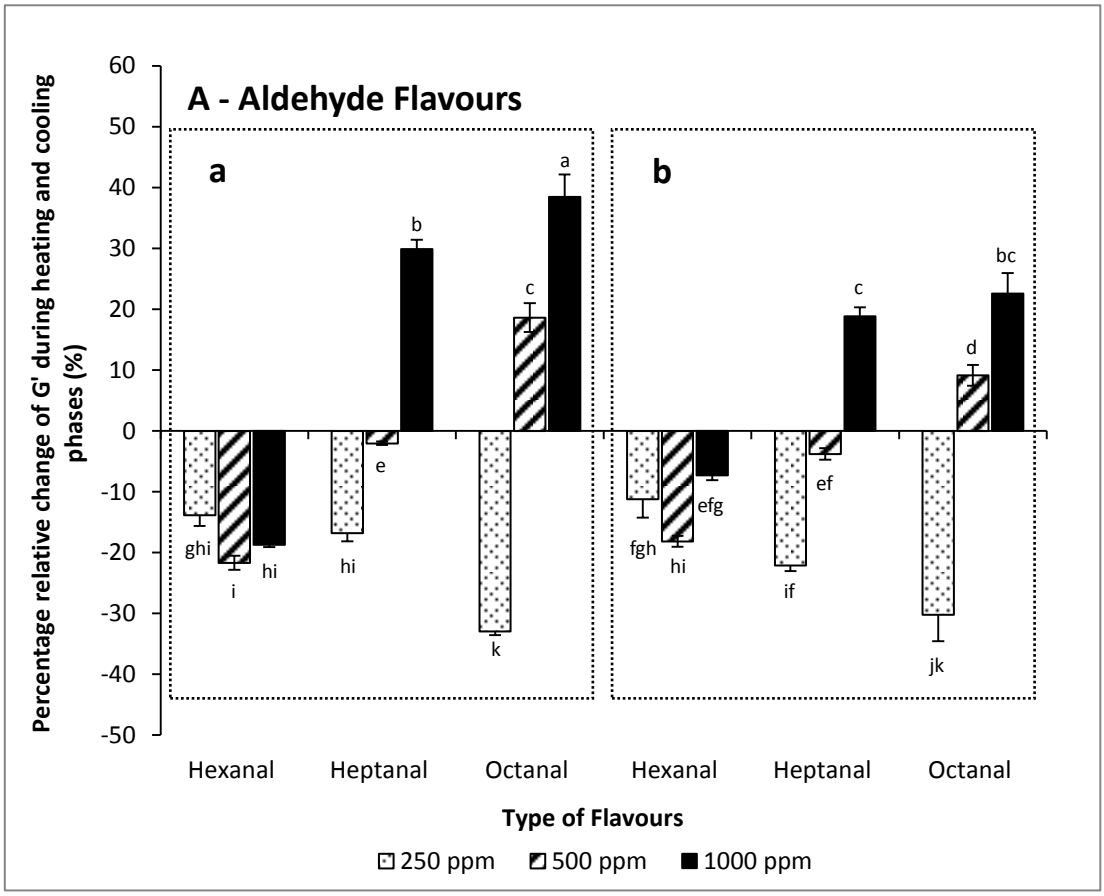
Percentage relative change of G' during heating phase =

$$\frac{(G'_{95℃} - G'_{25℃})\ of\ sample - (G'_{95℃} - G'_{25℃})\ of\ control}{(G'_{95℃} - G'_{25℃})\ of\ control} \times\ 100\ \%;$$

Percentage relative change of G' during cooling phase =

$$\frac{(G'_{25℃} - G'_{95℃})\ of\ sample - (G'_{25℃} - G'_{95℃})\ of\ control}{(G'_{25℃} - G'_{95℃})\ of\ control} \times\ 100\ \%.$$

By comparing the changes of G' during heating (from 25 to 95℃) and cooling (from 95 to 25℃) for PPIs in the presence of flavours to the corresponding change of G' for the control sample, the relative extent of increase or decrease of G' for PPIs as a result of binding with flavours can be determined. In other words, by comparing the relative change of elasticity in percentage, this method allows comparison of the effects of flavour binding during both the heating and cooling phases thus providing an indication as to which is more affected by the different flavours.


A - Aldehyde Flavours
a
b
Percentage relative change of G' during heating and cooling phases (%)
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
ghi
i
hi
hi
e
b
k
c
a
fgh
hi
efg
if
ef
c
jk
d
bc
Hexanal
Heptanal
Octanal
Hexanal
Heptanal
Octanal
Type of Flavours
250 ppm
500 ppm
1000 ppm

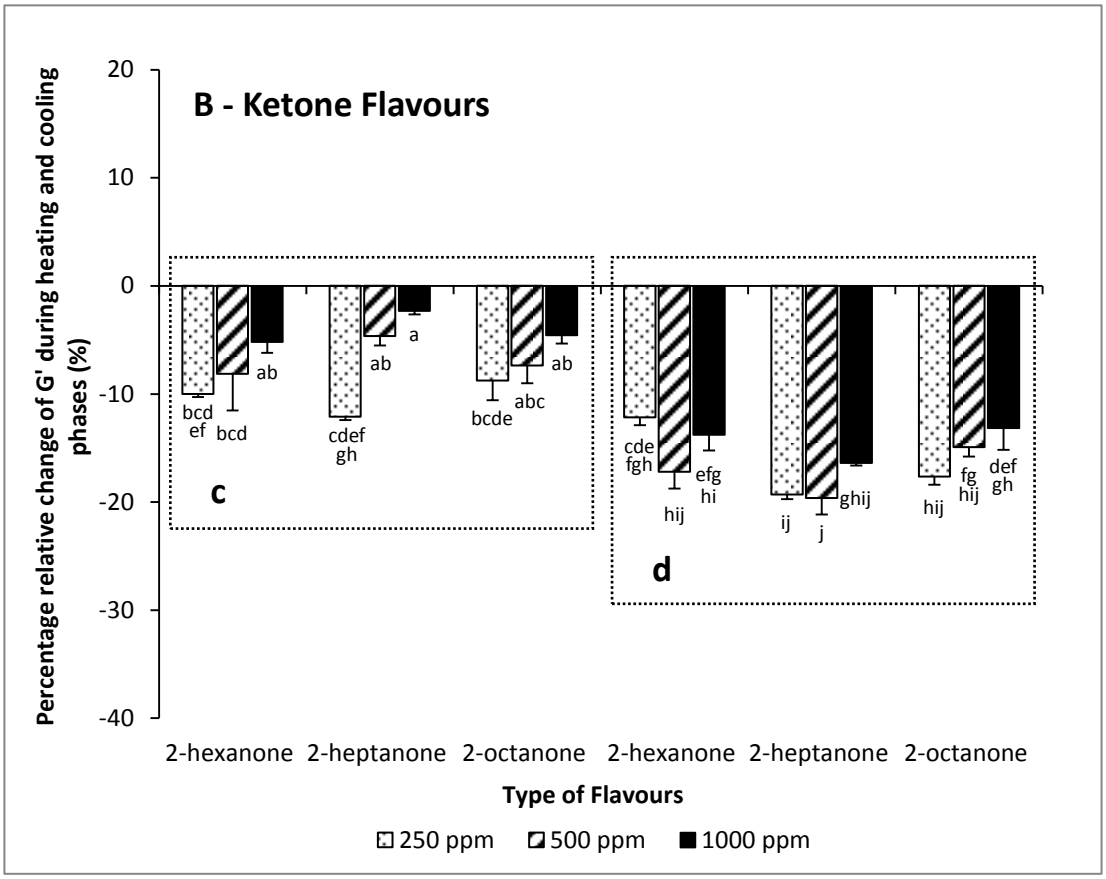

B - Ketone Flavours
c
d
Percentage relative change of G' during heating and cooling phases (%)
20
10
0
-10
-20
-30
-40
bcd
ef
bcd
ab
cdef
gh
ab
a
bcde
abc
ab
cde
fgh
hij
efg
hi
ij
j
ghij
hij
fg
hij
def
gh
2-hexanone
2-heptanone
2-octanone
2-hexanone
2-heptanone
2-octanone
Type of Flavours
250 ppm
500 ppm
1000 ppm

Figure 8.6 Percentage relative changes of G' during heating (Fig. 6Aa & 6Bc) and cooling (Fig. 6Ab & 6Bd) phases for homologous series of aldehyde (A) and ketone (B) flavours.
[a-j]In each figure, bar values followed by the same letters are not significantly different ($P < 0.05$). Note: the dashed lines have been included to clearly identify the heating and cooling stages of protein gelation such that the heating phases are shown in Fig. 6Aa and Bc and the aldehydes in 6Ab and 6Bd.

Fig. 8.6A illustrates the % relative changes of G' for homologous aldehydes during the heating (Fig. 8.6Aa) and cooling (Fig. 8.6Ab) phases. The positive value indicates the gel elasticity was increased, while negative values indicate gel strength was weakened by adding flavours. For aldehydes at 250 ppm, decreased gel elasticity during heating was comparable to the decrease of G' during cooling according to statistical analysis indicating both heating and cooling phases contributed to the overall decrease in G' when 250 ppm aldehydes were involved (Fig. 8.6A). It is possible that aldehydes continually interacted with PPIs throughout the entire gelling process.

With increasing aldehyde concentration, the impact of hexanal binding on G' was evident for both heating and cooling (Fig. 8.6A). However, when concentration of heptanal was increased to 1000 ppm and octanal concentration was above 500 ppm, the relative % increase in G' during the heating phase was significantly higher than the corresponding % increase of G' in cooling phase (Fig. 8.6Aa *vs.* 8.6Ab). This suggested that the improved G' values at higher concentrations, as shown in Fig. 8.

3, likely occurred during the heating phase rather than in cooling phase. It would appear that unfolding of the protein structure by aldehydes had a greater impact during the initial development of the gel network structure. This idea was further

supported by the lower protein gelling point (Table 8.2) and subsequent rapid increase of G' during the heating phase (Fig. 8.5). As protein-flavour associations have been reported to be thermodynamically favourable and spontaneous (Li, Grün, & Fernando, 2002), this also adds support to the idea that the impact of aldehydes on PPIs was more focused on the initial heating stage.

The % relative changes of G' for homologous ketones during the heating (Fig. 8.6 Bc) and cooling (Fig. 8.6 Bd) phases are all negative as expected since the addition of ketones resulted in decrease in gel elasticity as noted previously (Fig. 4). Except for % relative decrease of G' for 250 ppm 2-hexanone where values for the heating and cooling phases were close, the % relative decreases of G' for protein-ketone mixtures in heating phases were significantly lower than the corresponding changes in cooling phase; differences of 2 to 7 folds indicated the impact of ketone binding on gel strength was mainly occurred during the cooling rather than heating. During the cooling phase, it is believed that hydrogen bonding is important in gel stiffening and favored at low temperatures (Sun & Arntfield, 2012a). Chung and Villota (1989) and Chobpattana et al. (2002) found out that not only hydrophobic interactions, but also hydrogen bonding was responsible for the interactions of alcohols with soy protein and vanillin with sodium caseinate and bovine serum albumin. In consequence, it is possible that some degree of hydrogen bonding between selected ketones and PPIs could exist and interfere with the hydrogen bonds that contribute to the further establishment of gel network during the cooling stage. This could account for the diminished gel elasticity in the final material.

8.5 Conclusions

In summary, PPIs gel formation behaviours were significantly affected by addition of volatile flavouring compounds. All factors tested including flavour class, flavour carbon number and flavour concentration were found to significantly impact the protein gel formation characteristics ($p < 0.05$). Higher concentrations of aldehyde flavours with long chain lengths exhibited positive effect on protein gel structure development, while a general weaker gel was obtained when there was ketone binding. Binding of flavours altered protein native structure and environment of the tryptophan residues resulting in quenching of PPIs tryptophan fluorescence; this quenching was dependent on the type and concentration of flavours added. The transition of the influence of flavours from binding to unfolding of protein conformation could explain the change from a weaker gel to a stronger gel when aldehydes were involved; however, the limited unfolding ability of ketones may contribute to the constantly lower gel elasticity. More importantly, the impact of flavour binding on protein structure correlated well with the associated changes in protein thermal gelation properties. A method which successfully estimated the stage of protein gelation where flavours extended the greatest influence was established. When adding flavours to gel-based protein products, both the class and concentration of flavours need to be carefully selected and considered to produce a product that is acceptable for consumers.

Chapter 9 General discussions and conclusions

As the loss of free volatile compounds in aqueous protein systems has been known to greatly influence the quality, and therefore the consumer acceptability, of protein-containing foods (Guichard, 2002; Heng et al., 2004; Kúhn, Considine & Singh, 2006), examining the ability of different proteins to react with volatile flavour compounds as well as how these interactions respond to different intrinsic and extrinsic processing parameters become critically important to promoting the utilization of these proteins in food systems. In addition, unveiling the mechanisms of binding helps understand the interactions and provides perspective in ways to control the retention and release of these volatile flavour compounds. Therefore, this research was designed to systematically evaluate the interactions between selected volatile flavour compounds and extracted proteins from canola meal and pea flour in comparison to commercial vital wheat gluten. In addition to analyzing the qualitative change of flavour intensity as has been done previously (Gkionakis et al., 2006; Heng et al., 2004), structural change in proteins as a result of flavour binding as well as potential molecular forces involved were analyzed in the current investigations (Chatper 3, 4 and 8). Furthermore, selective modifications of protein structure, using chemical and enzymatic approaches, were also examined to potentially control the release of bound volatile compounds (Chapter 7).

An automated GC/MS approach for monitoring the retention of flavour compounds by measuring the change in flavour headspace concentration using an in-tube-extraction technique (ITEX) was developed (Chapter 3). By examining

ketones and aldehydes with different carbon numbers, it was found that flavour class, carbon chain length, protein source and protein isolation method significantly affected protein-flavour binding performance; long chain aldehyde flavours possessed a higher affinity to proteins compared to the corresponding ketone (Chapter 3). Considering the stereochemistry of flavour compounds, both hydrophobicity (within a similar class of flavour compounds) and functional groups of flavour compounds (between different flavour classes) contribute to protein-flavour binding affinities (Guichard, 2002). When more flavour classes were included, it was noted in that for flavours with a similar number of carbon atoms, the ability of proteins to bind flavours decreased in the order: disulfide > aldehyde > ester > ketone > aromatic flavour compound (Fig. 6.1). This order strongly reflected the type of molecular interactions involved for the different flavour functional groups (Chapter 6).

Comparing the affinity of flavour to different protein isolates, CPIs possessed higher affinity to aldehydes, while more ketone was retained by PPIs. This indicates that the higher level of sulfur-containing amino acids (Cys and Met) in CPIs may contribute the greater retention of aldehyde for CPIs, whereas the lack of intra-molecular disulfides bonds in PPIs may promote access of ketones to hydrophobic regions, thereby enhancing protein-flavour hydrophobic interactions (Khattab, Arntfield, & Nyachoti, 2009). In general, salt-extracted proteins bound more flavour compounds compared to the alkaline-extracted ones, with the exception of PPIa exhibiting a higher affinity to aldehydes than PPIs (Chapter 3). Although isoelectric precipitation of proteins may limit the accessibility of flavour compounds

to the primary binding sites due to increased protein-protein interactions (aggregation) (Chapter 3), the physical sorption of flavour compounds onto the surface of the aggregated proteins may contribute to the higher flavour retention when protein pH was reduced to its isoelectric point (Damodaran and Kinsella, 1983; van Ruth & Roozen, 2009). This increased binding of flavours was again observed when 0.05 or 0.1 M of $CaCl_2$ was added to PPIs, forming a precipitate due to a decrease in net electric charge on the proteins (Chapter 5; Fig. 5.3).

In addition to these intrinsic factors, extrinsic parameters such as heating conditions, addition of salts and changing of pH values greatly affected protein-flavour binding performance (Chapters 4 and 5). To elucidate the effect of heat on protein-flavour interactions, binding of aldehydes and ketones were followed as a function of heating time. A continuous increase in the binding of hexanal from 14% (no heating) to 67% was observed when the protein-flavour mixture was heated at 95ºC for 60 min (Fig. 4.3). According to Kühn, Considine and Singh (2008), the initial increase in binding of 2-octanone within the first 2 min of heating was due to protein unfolding while decreased binding after 2 min of heating could be attributed to heat-induced protein aggregation replacing protein-flavour interactions (Fig. 4.4). This may account for the conflicting results that were reported previously in terms of the effect of heat on protein binding with ketone flavours (Gkionakis, Taylor, Ahmad, & Helipoulos, 2007; Kühn, Considine, & Singh, 2008; Ng, Hoehn, & Bushuk, 1989b; O'Neill & Kinsella, 1987a).

While salt and pH can have direct practical applications in terms of using proteins

with flavours, they can also be used to fundamentally investigate molecular forces between protein and flavours (Arntfield, Murray, & Ismond, 1990). As observed in Chapter 5, at low salt concentrations (0.05 M), formation of ionic layers around proteins, due to the non-specific ion effect through electrostatic interactions, could limit the affinities of proteins to the previously ionic-linked flavour compounds consequently reducing their retention. Conversely, higher concentration of sodium salts (> 0.5 M) could lead to protein non-polar aggregation which not only enhanced protein intra-molecular hydrophobic interactions, but also promote hydrophobic associations between protein and flavours resulting in higher binding of flavours (Wang & Arntfield, 2015b). This increased flavour retention due to lyotropic effect of sodium salts was further demonstrated by evaluating the effect of anions of sodium salts in lyotropic series. It was found that flavour retention followed the position of the sodium salt in lyotropic series with protein stabilizing/non-chaotropic salt (e.g., Na_2SO_4) possessing the highest ability to promote flavour retention, possibly a result of the strengthening of hydrophobic interactions. In contrast, the loss of protein integrity due to the destabilizing effect of NaSCN contributed to the reduced retention of flavours. These results further emphasize the importance of protein structure in flavour binding. The influence of protein structure on flavour binding was also observed during protein acylation and hydrolysis processes (Chapter 7).

The greater reduction in flavour retention in an acetic environment could be attributed to more severe denaturation of proteins at lower pH values in comparison to the basic conditions (Sun & Arntfield, 2011). The increase in binding of flavours

when the pH was close to the IEP could result from both the enhanced hydrophobic association between protein and flavours as well as the surface sorption of flavour compounds on the aggregated proteins.

In previous investigations, most researchers did not consider flavour-protein interactions in which a composite of different types and concentrations of flavour compounds was used (Guichard & Langourieux, 2000; Kühn, Considine, & Singh, 2006). Due to the variability of flavour formulation applied in different food applications, more complex flavour profile could be created, making the binding system more difficult to predict and control. With this in mind, competitive binding between homologous and heterologous classes of volatile flavour compounds as well as the influence of heating, salts and pH values on these interactions were evaluated (Chapters 4 and 5). It is clear from that competitive binding between homologous ketones occurred with flavour compounds exhibiting higher affinity to proteins (e.g., 2-octanone) being retained more extensively than those with lower affinity (e.g., 2-hexanone) regardless of the presence of salts (Fig. 4.1 and Fig. 5.3). However, aldehydes behaved differently in that small molecular weight hexanal possessed higher affinity to CPIs than heptanal and octanal possibly due to steric hindrance of larger flavour molecules inhibiting their retention. In general, heating promoted competitive binding between flavours by increasing the retention of flavour that had a greater affinity to proteins. Surprisingly, a cooperative effect was observed when homologous aldehydes and a heterologous mixture and aldehyde and a ketone were mixed as ternary and binary systems, respectively (Fi.g 4.1 and 4.2). It was speculated

that the additional binding sites formed due to the “unfolding effect” of aldehyde on protein structure promoted the overall flavour retention. In addition, the thermal properties of PPIs in the presence of mixed flavours supported the change in flavour binding as shown in Table 4.1.

To investigate the nature of protein-flavour interactions, both the potential change in protein structure and molecular forces employed were investigated (Chapter 3, 4, 6 and 8). By monitoring the protein structure in the presence of different volatile flavour compounds using differential scanning calorimeter, the partial denaturation of proteins that was observed with aldehydes was much greater than that seen for the corresponding ketones (Fig. 3.5). This trend was further supported by the fluorometric study in which less quenching was produced by ketones than aldehydes at varying levels of flavour concentration (Fig. 8.1). It was proposed by Wang & Arntfield (2014) that upon reaction with flavour compounds, the protein tended to loss its original structure and expose previously buried non-polar residues. These residues were then stabilized by interacting with hydrophobic flavours, consequently resulted in increased flavour binding. As this ability to unfold the protein structure was more evident for aldehydes, this further accounted for their higher affinities to proteins in comparison with ketones. In addition, the effect of aldehydes on protein structure could account for the overall increase of flavour retention in the homologous ternary (hexanal, heptanal and octanal) and heterologous aldehyde and ketone (binary hexanal and 2-hexanone) systems (Table 4.1; Fig. 4.1d and Fig. 4.2).

Various bond disrupting agents were used to probe the molecular forces involved

in binding of selected volatile flavours to PPIs (Chapter 6). In terms of non-covalent forces, hydrophobic associations were more prominent for binding of benzaldehyde, as well as ketone and ester flavours, while retention of the ester hexyl acetate was relied mainly on hydrogen bonds and ionic interactions; the role of hydrogen and ionic interactions was lower for benzaldehyde and ketone. For covalent forces, aldehydes reacted with pea proteins via both reversible hydrophobic interactions and irreversible covalent bonds with covalent interaction exhibiting a more predominant effect. Binding for disulfide was not diminished by protein destabilizing agents suggesting its retention was covalent in nature and independent of a protein's inherent structure. The formation of thiol, 1-butanethiol, when dithiothreitol was added to PPIs strongly implicates the occurrence of sulfhydryl-disulfide interchange reactions (Fig. 6.3 and Fig. 6.4). No universal mechanism for protein flavour interactions can explain binding of different flavour compounds with pea proteins. Therefore, different bond disrupting agents were necessary to probe all forces that could be involved for individual classes of flavours.

Selective chemical and enzymatic modification of proteins were investigated with an idea of controlling the release of bound volatile flavour compounds (Chapter 7). It was shown that selective modification of free amino, hydroxyl and sulfhydryl groups and subsequent changes in protein quaternary structure were effective in reducing the affinity of aldehyde and ester to PPIs. However, binding of ketone flavours was dependent upon the integrity of protein structure and the accessibility of hydrophobic areas. A positive correlation was found between retention of disulfide flavour and free

sulfhydryl groups on the protein (Fig. 7.6), which further supported the existence of interchange reactions between free sulfhydryl groups and disulfide bonds as noted in Chapter 6.

While the effect of protein-flavour binding on flavour delivery has been the main focus of this work, the impact of the interactions between proteins and flavours on protein functionality was also investigated (Chapter 8). It was found that flavour class (aldehyde and ketone), number of carbon atoms (C6, C7 and C8) and flavour concentration (0, 250, 500 and 1000 ppm) significantly affected the gel forming properties of the pea protein isolate. Addition of low concentration of aldehydes and ketones resulted in formation of weaker gels mainly due to interference with protein inter- and intra-molecular forces that contribute to gel network formation. Protein gel strength was recovered with the addition of higher concentrations of long chain aldehyde flavours (Fig. 8.3). Due to the unfolding effect (partial denaturation) of aldehydes on protein structure, less heat may be required to denature a protein molecule to a point that is necessary for protein-protein crosslinking; this accounted for the stronger gel with a lower gelling point that was formed (Fig. 8.5). In general, it is clear that in addition to the impact on flavour release, interactions between proteins and flavours can result in changes in protein functionality.

In summary, this research provided fundenmental knowledge of the interactions between selected flavour compounds and salt- and alkaline-extracted canola and pea proteins and vital wheat gluten. The effects of heat treatment, differnet ionic conditions and the impact of pH values on protein-flavour binding were reported with

the underlying mechanisms being clearly presented. In terms of the nature of protein-flavour interactions, different affinities of flavours to protein could be attributed to different degree of protein partial unfolding by flavour compounds. Potential molecular forces between protein and selected flavours were successfully predicated using bonding disrupting agents. This method can be extremely useful to predict the nature of binding between proteins and flavour compounds containing multiple functional groups. Binding of flavours with proteins was selectively altered by choosing different chemical and enzymatic protein modification methods. With addition of flavour componds, gel strength of the system was modifed and the extent of modification was a function of the type and concentration of flavour compounds applied.

Chapter 10 Future research considerations

Based on previous literature, findings in the current research and observations during the experiment, some considerations and suggestions are proposed for future research projects that can be conducted in this field.

According to visual observations, it was noted in the DSC analysis that when adding flavour compounds (0~1000 ppm) to PPIs (10%, w/v, 0.3 NaCl), the protein tended to precipitate especially when high concentrations of long chain aldehyde were present. Moreover, the turbidity resulting from protein precipitation (in 0.01 M potassium phosphate buffer, pH 8) also made it impossible to examine the effect of protein-flavour binding on protein surface hydrophobicity as of a series concentration of protein solutions that would allow the passage of light were required. Therefore, a high precision density meter could be used to provide additional evidence of protein changes in the presence of flavour compounds. Meynier et al. (2004) investigated covalent bond formation between hexanal and t-2-hexenal with whey proteins and sodium caseinate. They found that a higher molecular weight band was revealed for whey proteins in the presence of aldehydes, which infers protein modification. Moreover, with addition of high concentration of t-2-hexenal, sodium caseinate could not enter the stacking gel of SDS-PAGE due to formation of protein aggregates. These electrophoretic patterns were shown again when 2-ME was added, indicating the formed aggregates were not disulfide bond linked (Meynier et al., 2004).

It was clearly demonstrated that aldehyde was able to partially unfold proteins with exposure of previously buried hydrophobic areas (Chapter 3 & 4). It is possible

that unfolded proteins were covalently linked by aldehydes and this led to formation of protein aggregates. This may also account for the formation of stronger gels with addition of high concentration of long chain aldehyde which was seen in Fig. 8.3. To verify the crosslinking between PPIs subunits, future research should be conducted on evaluating the molecular mass and mobility of PPIs with and without presence of aldehyde and ketone flavours using SDS-PAGE analysis.

From a practical aspect of flavour application, a wider range of flavour compounds should be investigated. Levels of flavour concentrations that range from 1-100 ppm, which are closer to the concentrations of flavours used in practical flavouring application, should be adopted. If working at lower levels of flavour concentration, multiple pumping stokes during flavour recovery step may be needed to optimize the peak in the GS/MS analysis. It was noted in the current investigation that the ITEX probe, which contains the *Tenax* trap, was not able to absorb alcohol, vanillin and matol flavours as they are highly water soluble. To obtain a complete flavour profile, different flavour isolation or recovery methods such as cryogenic or charcoal trap or solid-phase micro-extraction systems can be applied to evaluate a wider range of compounds (Reineccius, 2002).

It was noted that binding studies were conducted using simplied model systems. However, when considering the effect of protein-flavour binding on flavour retention and release, the presence of other major food components as well as the role of humans in flavour preception (e.g. oral manipulation) need to be considered.

Additionally, as a wide range type of flavour compounds are typically used in

flavour formulations, a greater emphasis should be put on evaluating competitive binding between flavour compounds and proteins instead of only looking at a single flavour system. It might be necessary to involve a formulated flavours which typically contains 20 to 50 different types of volatile flavour compounds in protein-flavour binding analysis. However, the resulting binding behaviours may be difficult to interpret.

From an instrumental perspective, a relatively innovative method to examine aroma profile of foods is GC-Olfactometry, from which the separated eluents from GC were evaluated by a trained personal and the potency or chemical identity of flavour compounds were reported. This methodology may be a good candidate to evaluate the flavour profile of protein containing foods, especially when multiple flavour compounds are present such as in competitive binding systems. In addition, it would provide the opportunity to verify the efficacy of different protein modifications to selectively release the bound volatiles from a sensory perspective.

Another important factor which should not be overlooked is the reversibility of protein-flavour interactions. As the release of the reversibly bound flavours directly contributes to the recovery of the original flavour profile, understanding the reversibility of bound volatiles could also provide critical information on the flavour release properties. However, in recent years, no systematic approach has been developed to quantify the reversibility/recoverability of the bound volatiles from protein systems.

Last but not least, it was found that a major limitation of this study was lack of

sensory evaluation data. To support the findings presented in this research and link the sensory and instrumental analyses together, sensory study using either a discriminative test or quantitive descriptive analysis should be considered in the future research.

References

Adams, R. L., Mottram, D. S., Parker, J. K., and Brown, H. M. (2001). Flavor-protein binding: disulfide interchange reactions between ovalbumin and volatile disulfides. *Journal of Agricultural and Food Chemistry*, 49, 4333-4336.

Adler-Nissen, J., and Olsen, H. S. (1979). The influence of peptide chain length on taste and functional properties of enzymatically modified soy protein. In Pour-El, A (Ed.) *Functional and Protein Structure* (pp. 638-641). Washington, DC: American Chemical Society.

Agilent Technologies, Inc. (2006). CTC automated sample injectors for gas chromatography, distributed by Agilent Technologies. Retrieved on Feb 2nd, 2015 from www.microprecision.com/repair_view/88544-.html.

Agilent Technologies, Inc. (2013). Addendum to PAL user manual PAL ITEX-2 Option (ITEX: In-Tube-Extraction) Installation and operation. Retrieved on Feb 2nd, 2015 from www.aquilantscientific.com/assets/.../ITEX2AddendumEdition3.pdf.

Aider, M., & Barbana, C. (2011). Canola proteins: composition, extraction, functional properties, bioactivity, applications as a food ingredient and allergenicity - A practical and critical review. *Trends in Food Science and Technology*, 22, 21-39.

Andriot, I., Marin, I., Feron, G., Relkin, P., and Guichard, E. (1999). Binding of benzaldehyde by β-lactoglobulin, by static headspace and high performance liquid chromatography in different physic-chemical conditions. *Lait*, 79 (6), 557-586.

Arai, S., Noguchi, M., Yamashita, M., Kato, H., & Fujimaki, M. (1970). Studies on flavor components in soybean. Part VI. Some evidence for occurrence of protein-flavour binding. *Agricultural and Biological Chemistry*, 34 (10), 1569-1573.

Arntfield, S. D. (2011). Canola and other oilseed proteins. In G. O. Phillips & Williams (Eds.), *Handbook of Food Proteins* (pp. 289-311). Cambridge: Woodhead Publishing Limited.

Arntfield, S. D., & Murray, E. D. (1981). The influence of processing parameters on food protein functionality I. Differential scanning calorimetry as an indicator of protein denaturation. *Canadian Institute of Food Science and Technology*, 14 (4), 289-294.

Arntfield, S. D., Murray, E. D., & Ismond, M. A. H. (1986). Effect of salt on the thermal stability of storage proteins from fababean (*Vicia faba*). *Journal of Food Science*, 51 (2), 371-377.

Arntfield, S. D., Murray, E. D., & Ismond, M. A. H. (1990). Influence of salts on the microstructural and rheological properties of heat-induced protein networks from ovalbumin and vicilin. *Journal of Agricultural and Food Chemistry*, 38, 1335-1343.

Arntfield, S. D., Murray, E. D., and Ismond, M. A. H. (1991). Role of disulfide bonds in determining the rheological and microstructural properties of heat-induced protein networks from ovalbumin and vicilin. *Journal of Agricultural and Food chemistry*, 39, 1378-1385.

Arora, A., & Damodaran, S. (2010). Competitive binding of off-flavour compounds with soy protein and β-cyclodextrin in a ternary system: a model study. *Journal of American Oil Chemists' Society,* 87, 673-679.

Arthur, C. L., and Pawliszyn, J. (1990). Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry*, 62, 2145-2148.

Aspelund, T. G. & Wilson, L. A. (1983). Adsorption of off-flavor compounds onto soy protein: a thermodynamic study. *Journal of Agricultural and Food Chemistry*, 31, 539-545

Avramenko, N., Low, N. H., & Nickerson, M. T. (2013). The effects of limited enzymatic hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate. *Food Research International*, 51, 162-169.

Bakker, J., Langley, K. R., Elmore, J. S., Brown, W., Martin, A., and Salvador. D. (1995). Release of flavour from food matrices and effect on flavour perception. In M. Rothe and H. P. Kruse (Eds) *Aroma: Perception, formation, evaluation* (pp. 191-207). Deutsche Institut fur Ernahrungsforschung, Potsdam-Rehbrucke.

Bakker, L. D., Martens, R. W., & Murray, E. D. (2010). Production of oil seed protein isolate. US Patent 7,687,087 B2.

Barbana, C., & Boye, J. I. (2011). Angiotensin I-converting enzyme inhibitory properties of lentil protein hydrolysates: determination of the kinetics of inhibition. *Food Chemistry*, 127, 94-101.

Beyeler, M., & Solms, J. (1974). Interaction of flavour model compounds with soy protein and bovine serum albumin. *Lebensmittel-Wissenschaft und-Technologie*, 7 (4), 217-219.

BCC Research. (2012). Global Markets for Flavors & Fragrances. Retrieved Jan 20th, 2015 from http://www.bccresearch.com/market-research/chemicals/flavors-fragrances-global-markets-chm034c.html.

Blaicher, F. M., Elstner, F., Stein, W., & Mukherjee, K. D. (1983). Rapeseed protein isolates: effect of processing on yield and composition of protein. *Journal of Agricultural and Food Chemistry*, 31, 358-362.

Blank, I. (1997). Gas-chromatography-olfactometry in food aroma analysis. In R. Marsili (Ed), *Techniques for Analyzing Food Aroma* (pp. 293-330). Marcel Dekker.

Boye, J. I., & Maltais, A. (2011). Pulses: a novel protein source. *AgroFood Industry hi-tech*, 22 (1), 24-26.

Boye, J. I., Aksay, S., Roufik, S., Ribereau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, 43, 537-546.

Boye, J., Zare, F., and Pletch, A. (2010). Review: Pulse proteins: processing, characterization, functional properties and applications in food and feed. *Food Research international*, 43, 414-431.

Burgess, D. A. (1991). *Purification, characterization and the micelle response of the 12S canola globulin.* M. Sc. Thesis. University of Manitoba, Winnipeg, Manitoba.

Canola Council of Canada (2015). Industry overview. Retrieved Feb 1st, 2015 from http://www.canolacouncil.org/markets-stats/industry-overview/.

Celus, I., Brijs, K., & Delcour, J. A. (2007). Enzymatic hydrolysis of brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates. *Journal of Agricultural and Food Chemistry*, 58, 8703-8710.

Cho, Y., Batt, C. A., & Sawyer, L. (1994). Probing the retinol-binding site of bovine β-lactoglobulin. *Journal of Biological Chemistry*, 269 (15), 11102-11107.

Chobert, J., Touati, A., Bertrand-Harb, C., Dalgalarrondo, M., Nicolas, M., and Haertle, T. (1990). Ethyl-substituted β-casein. Study of differences between esterified and reductively alkylated β-casein derivatives. *Journal of Agricultural and Food Chemistry*, 38, 1321-1326.

Chobpattana, W., Jeon, I. J., Smith, J. S., and Loughin, T. M. (2002). Mechanisms of interaction between vanillin and milk proteins in model systems. *Journal of Food Science*, 67 (3), 973-977.

Chung, S., and Villota, R. (1989). Binding of alcohols by soy protein in aqueous solutions. Journal of Food Science, 54 (6), 1604-1606.

Claver, I. P., and Zhou, H. (2005). Enzymatic hydrolysis of defatted wheat germ by proteases and the effect of the functional properties of resulting protein

hydrolysates. *Journal of Food Biochemistry*, 29, 13-26.

Conde-Petit, B., Escher, F., & Nuessli, J. (2006). Structural features of starch-flavour complexation in food model systems. *Trends in Food Science & Technology*, 17, 227-235.

Damodaran, S. (1988). Refolding of thermally unfolded soy proteins during the cooling regime of the gelation process: effect of gelation. *Journal of Agricultural and Food Chemistry,* 36 (2), 262-268.

Damodaran, S., and Kinsella, J. E. (1980a). Stabilization of proteins by solvents. Effect of pH and anions on the positive cooperativity of 2-nonanone binding to bovine serum albumin. *Journal of Biological Chemistry*, 255, 8503-8508.

Damodaran, S. & Kinsella. J. E. (1980b). Flavor protein interactions. Binding of carbonyls to bovine serum albumin: thermodynamic and conformational effects. *Journal of Agricultural and Food Chemistry*, 28, 567-571.

Damodaran, S. & Kinsella. J. E. (1981a). Interaction of carbonyls with soy protein: thermodynamic effects. *Journal of Agricultural and Food Chemistry*, 29, 1249-1253.

Damodaran, S., and Kinsella, J. E. (1981b). Interaction of carbonyls with soy protein: conformational effects. *Journal of Agricultural and Food Chemistry*, 29, 1253-1257.

Damodaran, S., & Kinsella, J. E. (1981c). The effects of neutral salts on the stability of macromolecules. *The Journal of Biological Chemistry*, 256 (7), 3394-3398.

Damodaran, S. & Kinsella, J. E. (1983). Binding of carbonyls to fish actomyosin. *Journal of Agricultural and Food Chemistry,* 31, 856-859.

Damodaran, S. (1988). Refolding of thermally unfolded soy proteins during the cooling regime of the gelation process: effect of gelation. *Journal of Agricultural and Food Chemistry,* 36 (2), 262-268.

Damodaran, S. (1996). Amino acids, peptides, and proteins (Chapter 6). In O. R. Fennema (Ed.), *Food Chemistry* (3rd ed.). (pp. 350) New York: Marcel Dekker, Inc.

de Roos, K. B. (2000). Physiochemical models of flavor release from foods. In D. D. Roberts and A. J. Taylor (Eds), *Flavor Release* (pp. 126-141). Washington, DC: American Chemical Society.

Des Marchais, L., Foisy, M., Mercier, S., Villeneuve, S., & Mondor, M. (2011). Bread-making potential of pea protein isolate produced by a novel ultrafiltration/dilfiltration process. *Procedia Food Science,* 1, 1425-1430.

Drakos, A., Doxastakis, G., & Kiosseoglou, V. (2007). Functional effects of lupin proteins in comminuted meat an emulsion gels. *Food Chemistry*, 100, 650-655.

Druaux, C., Lubbers, S., Charpentier, C., & Voilley, A. (1995). Effects of physic-chemical parameters of a model wine on the binding of γ-decalactone on bovine serum albumin. *Food Chemistry*, 53, 203-207.

Dufour, E., & Haertlé, T. (1990). Binding affinities of β-ionone and related flavor compounds to β-lactoglobulin: effects of chemical modifications. *Journal of Agricultural and Food Chemistry*, 38, 1691-1695.

Dumont, J. P. & Land, D. G. (1986). Binding of diacetyl by pea proteins. *Journal of Agricultural and Food Chemistry*, 34, 1041-1045.

Ebeler, S. E., Pangborn, R. M., & Jennings, W. G. (1988). Influence of dispersion medium on aroma intensity and headspace concentration of menthone and isoamy acetate. *Journal of Agricultural and Food Chemistry*, 36, 791-796.

Eisele, T. A., & Brekke, C. J. (1981). Chemical modification and functional properties of acetylated beef heart myofibrillar proteins. *Journal of Food Science*, 46, 1095-1102.

El-Adawy, T. A. (2000). Functional properties and nutritional quality of acetylated and succinylated mung bean protein isolate. *Food Chemistry*, 70, 83-91.

Fabre, M., Aubry, V., & Guichard, E. (2002). Comparison of different methods: static and dynamic headspace and solid-phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. *Journal of Agricultural and Food Chemistry*, 50, 1497-1501.

Foegeding, E. A., and Davis, J. P. (2011). Food protein functionality: a comprehensive approach. *Food Hydrocolloids*, 25, 1853-1864.

Franzen, K. L., Kinsella, J. E. (1976). Functional properties of succinylated and acetylated soy protein. *Journal of Agricultural and Food Chemistry*, 24 (4), 788-795.

Gatehouse, J. A., Croy, R. R. D., Morton, H., Tyler, M., & Boulter, D. (1981). Characterization and subunit structures of the vicilin storage protein of pea (*Pisum sativum L.*). *European Journal of Biochemistry*, 118, 627-633.

Gholami, S., & Bordbar, A. (2014). Exploring binding properties of naringenin with bovine β-lactoglobulin: A fluorescence, molecular docking and molecular dynamics simulation study. *Biophysical Chemistry*, 187-188, 33-42.

Ghodsvali, A., Khodaparast, M. H. H., Vosoughi, M., & Diosady, L. L. (2005). Preparation of canola protein materials using membrance technology and

evaluation of meals functional properties. *Food Research International*, 38, 223-231.

Gianelli, M., Flores, M., & Toldra, F. (2005). Interaction of soluble peptides and proteins from skeletal muscle with volatile compounds in model systems as affected by curing agents. *Journal of Agricultural and Food Chemistry*, 53, 1670-1677.

Gkionakis, G. A., Heliopoulos, G., Taylor, A. K. D., and Ahmad, J. (2006). Factors affecting the flavour binding properties of lactones with soya protein. British Food Journal, 108 (6), 456-474.

Gkionakis, G. A., Taylor, K. D. A., Ahmad, J., and Heliopoulos, G. (2007). The binding of the flavour of lactones by soya protein, amino acids and casein. *International Journal of Food Science and Technology*, 42, 165-174.

Gremli, H. A. (1974). Interaction of flavor compounds with soy protein. *Journal of American Oil Chemists' Society*, 51, 95A-97A.

Grinberg, V. Y., Grinberg, N. V., Mashkevich, A. Y., Burova, T. V., & Tolstoguzov, V. B. (2002). Calorimetric study of interaction of ovalbumin with vanillin. *Food Hydrocolloids*, 16, 333-343.

Gruener, L., and Ismond, M. A. H. (1996). Effects of acetylation and succinylation on the physicochemical properties of the canola 12S globulin. Part I. *Food Chemistry*, 60 (3), 357-363.

Guichard, E. (2000). COST action 96: interaction of food matrix with small ligands influencing flavour and texture. *Food Research International*, 33, 187-190.

Guichard, E. & Langourieux, S. (2000). Interactions between β-lactoglobulin and flavor compounds. *Food Chemistry*, 71, 301-308.

Guichard, E. (2002). Interactions between flavor compounds and food ingredients and their influence on flavor perception. *Food Review International*, 18, 49-70.

Guichard, E. (2006). Flavour retention and release from protein solutions. *Biotechnology Advances*, 24, 226-229.

Guichard, E. (2011). Binding and release of flavour coumpouds. In Protein-flavour interactions. In H. Jeleń. (Ed.), *Food flavours: chemical, sensory and technological properties* (pp. 137-154). Boca Raton: CRC Press.

Han, J., Janz, J. A. M., & Gerlat, M. (2010). Development of gluten-free cracker snacks using pulse flours and fractions. *Food Research International*, 43, 627-633.

Hansen, A. P., & Heinis, J. J. (1991). Decrease of vanillin flavour perception in the presence of casein and whey protein. *Journal of Dairy Science*, 74, 2936-2940.

Hansson, A., Andersson, J., & Leufvén, A. (2001). The effect of sugars and pectin on flavour release from a soft drink-related model system. *Food Chemistry*, 72, 363-368.

Heng, L., van Koningsveld, G. A., Gruppen, H., van Boekel, M. A. J. S., Vincken, J. P., Roozen, J. P., & Voragen, A. G. (2004). Protein-flavour interactions in relation to development of novel protein foods. *Trends in Food Science & Technology*, 15, 217-224.

Heng, L., Vincken, J., van Koningsveld, G., Legger, A., Gruppen, L., van Boekel, T., Roozen, J., & Voragen, F. (2006). Bitterness of saponins and their content in dry peas. *Journal of the Science of Food and Agriculture*, *86*, 1225-1231.

Hood-Niefer, S. D., & Tyler, R. T. (2010). Effect of protein, moisture content and barrel temperature on the physicochemical characteristics of pea flour extrudates. *Food Research International*, 43, 659-663.

Hsieh, Y. L., Regenstein, J. M., & Rao, M. A. (1993). Gel point of whey and egg proteins using dynamic rheological data. *Journal of Food Science*, 58 (1), 116-118.

IAL Consultants. (2014). An overview of the global flavours & Fragrances market, 9th edition. Retrieved Jan 20th, 2015 from http://www.ialconsultants.com/uploads/CUBE_press_release/2014-11-06/F_FPressRelease2014.pdf.

Imeson, A. P., Ledward, D. A., and Mitchell, J. R. (1977). On the nature of the interactions between some anionic polysaccharides and proteins. *Journal of the Science of Food and Agriculture*, 28 (8), 661-668.

Ismond, M. A. H., Murray, E. D., & Arntfield, S. D. (1986). The role of non-covalent forces in micelle formation by vicilin from *Vicia faba*. II. The effects of stabilizing and destabilizing anions on protein interactions. *Food Chemistry*, 21, 27-46.

Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., & Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chemistry*, 121, 178-184.

Jouenne, E., & Crouzet, J. (1997). Influence of sodium chloride and urea on volatile compounds β-lactoglobulin interactions. In A. O. Zacharoff (Ed) *Interaction of Food Matrix with small ligands influencing flavour and texture* (pp. 37-43), Office for Official Publications of the European Communities.

Jouenne, E., & Crouzet, J. (2000). Effect of pH on retention of aroma compound by β-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 48, 1273-1277.

Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science C: Food Chemistry and Toxicology*, 104, 180-187.

Jung, D., de Popp, J. S., & Ebeler, S. E. (2002). Application of pulsed field gradient NMR techniques for investigating binding of flavour compounds to macromolecules. *Journal of Agricultural and Food Chemistry*, 50, 4262-4269.

Kanakis, C. D., Tarantilis, P. A., Polissiou, M. G., & Tajmir-Riahi, H. A. (2013). Probing the binding sites of resveratrol, genistein, and curcumin with milk lactoglobulin. *Journal of Biomolecular Structure and Dynamics*, 31 (12), 1455-1466.

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean, lentil, and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research International*, 44, 2742-2750.

Khattab, R. Y. and Arntfield, S. D. (2009). Functional properties of raw and, processed canola meal. *LWT-Food Science and Technology*, 42, 1119-1124.

Khattab, R. Y., Arntfield, S. D., & Nyachoti, C. M. (2009). Nutritional quality of legume seeds as affected by some physical treatments, part 1: Protein quality evaluation. *LWT-Food Science and Technology*, 42, 1107-1112.

Kim, H., & Min, D. B. (1989). Interaction of flavour compounds with protein. In D. B. Min, & T. H. Smouse (Eds.), *Flavour Chemistry of Lipid Foods* (pp. 404-420). Champain: American Oil Chemists' Society.

King, B. M., Arents, P., Bouter, N., Duineveld, A. A., Meyners, M., Schroff, S. I., & Soekhai, S. T. (2006). Sweetener/sweetness-induced changes in flavour perception and flavour release of fruity and green character in beverages. *Journal of Agricultural and Food Chemistry*, 54, 2671-2677.

Kinsella, J. E. (1990). Flavour perception and binding. *International News on Fats, Oils and Related materials*, 1, 215-221.

Krause, J., Mothes, R., & Schwenke, K. D. (1996). Some physicochemical and interfacial properties of native and acetylated legumin from Faba Bean (Vicia faba L.). *Journal of Agricultural and Food Chemistry*, 44, 429-437.

Kristinsson, H. G., and Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40, 43-81.

Kúhn, J., Considine, T., and Singh, H. (2006). Interactions of milk proteins and volatile flavor compounds: implications in the development of protein foods. *Journal of Food Science*, 71, R72-R82.

Kúhn, J. Zhu, X., Considine, T., and Singh, H. (2007). Binding of 2-nonanone and milk proteins in aqueous model systems. *Journal of Agricultural and Food Chemistry*, 55, 3599-3604.

Kúhn, J. Considine, T., and Singh, H. (2008). Binding of flavor compounds and whey protein isolate as affected by heat and high pressure treatments. *Journal of Agricultural and Food Chemistry*, 56, 10218-10224.

Jouenne, E., Chalier, P., & Crouzet, J. (2000). Interactions between aroma compounds and β-lactoglobulin: competition phenomena. In R. Ofstad, *COST action 96. Proceedings of the meeting in Oslo* (Vol. 6, pp. 75-85). Luxembourg: European Commission.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Lakowicz, J. R. (1999). Protein fluorescence. In J. R. Lakowicz (Ed.), *Principles of fluorescence spectroscopy* (pp. 466-485). New York: Kluwer Academic/Plenum Publishers.

Land, D. G. (1996). In J. R. Piggott and A. Paterson (Eds) *Understanding Natural Flavours* (pp. 298-306). Chapman and Hall, London.

Landy, P., Druaux, & Voilley, A. (1995). Retention of aroma compounds by proteins in aqueous solution. *Food Chemistry*, 54 (4), 384-392.

Lawal, O. S. (2005). Functionality of native and succinyated Lablab bean (*Lablab purpureus*) protein concentrate. *Food Hydrocolloids*, 19, 63-72.

Leahy, M. M., & Reineccius, G. A. (1984). Comparison of methods for the analysis of volatile compounds from aqueous model systems. In (P. Schreier Ed) *Analysis of Volatiles: New Methods and Their Application* (pp. 19-47). DeGruyter, Berlin.

Leffingwell & Associates. (2014). 2009-2013 Flavor & Fragrance Industry Leaders. ` Retrieved Jan 20th, 2015 from http://www.leffingwell.com/top_10.htm.

Leland, J. J., Schieberle, P., Buettner, A., & Acree, T. E. (2001). *Gas chromatography-olfactometry: the state of the art*, ACS Symposium Series 782, Washington DC: American Chemical Society.

Léger, L. W., & Arntfield, S. D. (1993). Thermal gelation of the 12S canola globulin. *Journal of American Oil Chemists' Society*, 70 (9), 853-861.

Li, Z., Grun, I. U., and Fernando, L. N. (2000). Interaction of vanillin with soy and dairy proteins in aqueous model systems: a thermodynamic study. *Journal of Food Science*, 65(6), 997-1001.

Li-Chan, E., Nakai, S., & Wood, D. F. (1984). Hydrophobicity and solubility of meat proteins and their relationship to emulsifying properties. *Journal of Food Science*, 49, 345-350.

Liu, Q., Kong, B., Xiong, Y. L., and Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118, 403-410.

Liu, X., Powers, J. R., Swanson, B. G., Hill, H. H., and Clark, S. (2005). High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. *Journal of Food Science*, 70 (9), C581-C585.

Lubbers, S., & Guichard, E. (2003). The effects of sugars and pectin on flavour release from a fruit pastille model system. *Food Chemistry*, 81, 269-273.

Lubbers, S., Landy, P., & Voilley, A. (1998). Retention and release of aroma compounds in foods containing proteins. *Food Technology*, 52 (5), 68-74.

Mariotti, M., Lucisano, M., Pagani, M. A., & Ng, P. K. W. (2009). The role of corn starch, amaranth flour, pea isolate, and *Psyllium* flour on the rheological properties and the ultrastructure of gluten-free doughs. *Food Research International*, 42, 963-975.

Matheis, G. (1993). Interactions between volatile flavouring substances and food constituents. Part I: Carbohydrates, proteins and amino acids. *Dragoco Rep* (Flavouring Information Service), 3, 98-114.

Matsumura, Y., & Mori, T. (1996). Gelation. In G. M. Hall (Ed.), *Methods of testing protein functionality* (pp. 76-106). London: Blackie Academic & Professional.

McNeill, V. L., & Schmidt, K. A. (1993). Vanillin interaction with milk protein isolates in sweetened drinks. *Journal of Food Science*, 58 (5), 1142-1147.

Melander, W., & Horváth, C. (1977). Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: an interpretation of the lyotropic series. *Archives of Biochemistry and Biophysics*, 183, 200-215.

Mercier, S., Villeneuve, S., Mondor, M., & Des Marchais, L. (2011). Evolution of porosity, shrinkage and density of pasta fortified with pea protein concentrate during drying. *LWT-Food Science and Technology*, 44, 883-890.

Meynier, A., Garillon, A., Lethuaut, L., & Genot, C. (2003). Partition of five aroma compounds between air and skim milk, anhydrous milk fat or full fat cream. *Le*

Lait, 83 (3), 223-235.

Meynier, A., Rampon, V., Dalgalarrondo, M., and Genot, C. (2004). Hexanal and t-2-hexenal form covalent bonds with whey proteins and sodium caseinate in aqueous solution. *International Dairy Journal*, 14, 681-690.

Mills, O. E., & Solms, J. (1984). Interaction of selected flavour compounds with whey proteins. *Lebensmittel-Wissenschaft & Technologie*, 17, 331-335.

Mirmoghtadaie, L., Kadivar, M., & Shahedi, M. (2009). Effects of succinylation and deamidation on functional properties of oat protein isolate. *Food Chemistry*, 114, 127-131.

Mottram, D. S., & Nobrega, I. C. C. (2000). Interaction between sulfur-containing flavor compounds and proteins in foods. In D. D. Roberts and A. J. Taylor (Eds), *Flavor Release: Proceedings of a Symposium, New Orleans, August 1999* (pp. 274-281). Washington DC: American Chemical Society.

Mottram, D. S., Szauman-Szumski, C., & Dodson, A. (1996). Interaction of thiol and disulfide flavor compounds with food components. *Journal of Agricultural and Food Chemistry*, 44 (8), 2349-2351.

Moure, A., Sineiro, J., Domínguez, H., and Parajó, J. C. (2006). Functionality of oilseed protein products: A review. *Food Research International*, 39, 945-963.

Muresan, S. & Leguijt, T. (1998). Fluorimetric analysis of interactions between β-lactoglobulin and flavour compounds. In A. Zacharoff, *COST action 96. Proceedings of the meeting in Göteborg* (Vol. 2, pp. 45-52). Luxembourg: European Commission.

Muresan, S., van der Bent, A., and de Woft, F. A. (2001). Interaction of β-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: nonlinear quenching effects related to protein-protein association. *Journal of Agricultural and Food Chemistry*, 49, 2609-2618.

Naknean, P., & Meenune, M. (2010). Factors affecting retention and release of flavour compounds in food carbohydrates. *International Food Research Journal*, 17, 23-34.

Ng, P. K. W., Hoehn, E., & Bushuk, W. (1989a). Binding of vanillin by fababean proteins. *Journal of Food Science*, 54 (2), 105-107.

Ng, P. K. W., Hoehn, E., & Bushuk, W. (1989b). Binding of vanillin by fababean proteins. *Journal of Food Science*, 54 (1), 324-326.

Nockrashy, E. A. S, Mukherjee, K. D., & Mangold, H. K. (1977). Rapeseed protein isolates by conuntercurrent extraction and isoelectric precipitation. *Journal of*

Agricultural and Food Chemistry, 25, 193-197.

Nunes, M. C., Raymundo, A., & Sousa, I. (2006). Rheological behavior and microstructure of pea protein/κ-carrageenan/starch gels with different setting conditions. *Food Hydrocolloids*, 20, 106-113.

O'Neill, T. E. (1996). Flavor binding by food proteins: an overview. In R. J. McGorrin and J. V. Leland (Eds), *Flavor-Food Interactions* (pp. 59-74). Washington DC: American Chemical Society.

O'Neill, T., and Kinsella, J. E. (1987a). Effect of heat temperature and modification on conformation and flavor binding by β-Lactoglobulin. Journal of Food Science, 53 (3), 906-909.

O'Neill, T. E., & Kinsella, J. E. (1987b). Binding of alkanone flavours to β-Lactoglobulin: effects of conformational and chemical modification. *Journal of Agricultural and Food Chemistry,* 35, 770-774.

O'Kane, F. E., Vereijken, J. M., Gruppen, H., and van Boekel, M. A. J. S. (2004). Heat-induced gelation of pea legumin: comparison with soybean glycinin. *Journal of Agricultural and Food Chemistry*, 52, 5071-5087.

O'Keefe, S. F., Resurreccion, A. P., Wilson, L. A., & Murphy, P. A. (1991a). Temperature effect on binding of volatile flavor compounds to soy protein in aqueous model systems. *Journal of Food Science*, 56(3), 802-806.

O'Keefe, S. F., Wilson, L. A., Resurreccion, A. P., & Murphy, P. A. (1991b). Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique. *Journal of Agricultural and Food Science*, 39, 1022-1028.

Ordonez, M., & Herrera, A. (2014). Morphologic and stability cassava starch matrices for encapsulating limonene by spray drying. *Powder Technology*, 253, 89-97.

Paulson, A., & Tung, M. A. (1987). Solubility, hydrophobicity and net charge of succinylated canola protein isolate. *Journal of Food Science*, 52 (6), 1557-1561.

Pelletier, E., Sostmann, K., & Guichard, E. (1998). Measurement of interactions between –Lactoglobulin and flaour compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. *Journal of Agricultural and Food Chemistry*, 46, 1506-1509.

Pérez-Juan, M., Flores, M., and Toldrá, F. (2006). Model studies on the efficacy of protein homogenates from raw pork muscle and dry-cured ham in binding selected flavor compounds. *Journal of Agricultural and Food Chemistry*, 54, 4802-4808.

Pérez -Juan, M., Flores, M., and Toldrá F. (2007). Effect of ionic strength of different

salts on the binding of volatile compounds to porcine soluble protein extracts in model systems. *Food Research International*, 40, 687-693.

Pérez -Juan, M., Flores, M., and Toldrá, F. (2008). Effect of pork meat proteins on the binding of volatile compounds. *Food Chemistry*, 108, 12266-1233.

Petitot, M., Boyer, L., Minier, C., & Micard, V. (2010). Fortification of pasta with split pea and faba bean flours: pasta processing and quality evaluation. *Food Research International*, 43, 634-641.

Plug, H., and Haring, P. (1993). The role of ingredient-flavour interactions in the development of fat-free foods. *Trends in Food Science and Technology*, 4, 150-152.

Pinterits, A., and Arntfield, S. D. (2007). The effect of limited proteolysis on canola protein gelation. *Food Chemistry*, 102, 1337-1343.

Pokorny, J., Luan, N. T., Kondratenko, S. S., & Janicek, G. (1976). Changes in sensory value by interaction of alkanals with amino acids and proteins. *Nahrung*, 20, 267-272.

Poll, L., & Flink, J. M. (1984). Aroma analysis of apple juice-influence of salt addition on headspace volatile composition as measured by gas-chromatography and corresponding sensory evaluations. *Food Chemistry*, 13, 193-207.

Ponnampolam, R., Delisle, J., Gagne, Y., & Amiot, J. (1990). Functional and nutritional properties of acetylated rapeseed proteins. *Journal of American Oil Society*, 67, 531-536.

Preininger, M. (2006). Interactions of flavor components in foods. In A. G. Gaonkar and A. McPherson (Eds), *Ingredient Interactions: Effects on Food Quality* (pp. 478-528). Boca Raton: CRC Press.

Pulse Canada. (2014a). Pulse Industry. Retrieved July 13th, 2012 from http://www.pulsecanada.com/pulse-industry.

Pulse Canada. (2014b). Food & Health-Nutritional benefits. Retrieved July 13th, 2012 from http://www.pulsecanada.com/food-health/nutritional-benefits.

Rababah, T. H., Al-Mahasneh, M. A., &Ereifej, K. I. (2006). Effect of chickpea, broad bean, or isolated soy protein additions on physicochemical and sensory properties of biscuits. *Journal of Food Science*, 71, S438-442.

Reineccius, G. (1993). Biases in analytical profiles introduced by isolation method. In C. T. Ho and C. Manley (Eds), *Flavor Measurement* (pp. 61-76). New York: Marcel Dekker.

Reineccius, G. (2002). Instrumental methods of analysis. In A. J. Taylor (Ed), *Food Flavour Technology* (pp. 210-247). Sheffield: Sheffield Academic Press Ltd.

Reineccius, T. A., Reineccius, G. A., & Peppard, T. L. (2004). Utilization of β-cyclodextrin for improved flavour retention in thermally processed foods. *Journal of Food Science*, 69 (1), 58-62.

Reineccius, G. (2006a). *Flavour Chemistry and Technology*. Boca Raton: CRC Press.

Reineccius, G. (2006b). Choosing the correct analytical technique in aroma analysis. In A. Voilley and P. Etiévant (Eds), *Flavour in Food* (pp. 81-95). Cambridge: Woodhead Publishing Limited.

Reineccius, G. (2006c). Flavor release from foods. In *Flavour chemistry and technology* (pp. 139-159). Boca Raton: CRC Press.

Relkin, P., Molle, D., & Marin, I. (2001). Non-covalent binding of benzaldehyde to β-lactoglobulin: characterization by spectrofluorimetry and electrospray ionization mass-spectrometry. *Journal of Dairy Research*, 68, 151-155.

Relkin, P., & Vermersh, J. (2001). Binding properties of vanillin to whey proteins: effect on protein conformational stability and foaming properties. In E. Dickinson & R. Miller (Eds), *Food Colloids: fundamentals of formulation* (pp. 282-292). Cambridge: The Royal Society of Chemistry.

Ribotta, P. D., Colombo, A., & Rosell, C. M. (2012). Enzymatic modifications of pea protein and its application in protein-cassva and corn starch gels. *Food Hydrocolloids*, 27, 185-190.

Sahihi, M., Ghayeb, Y., & Bordbar, K. (2013). Interaction of β-lactoglobulin with resveratrol: molecular docking and molecular dynamics simulation studies. *Chemical and Biochemical Engineering Quarterly*, 27 (4), 417-422.

Sathe, S. K., & Salunkhe, D. K. (1981). Preparation and utilization of protein concentrates and isolates for nutritional and functional improvement of foods. *Journal of Food Quality*, 4, 233-245.

Schwenke, K. D., Knopfe, C., Mikheeva, L. M., & Gringerg, V. Y. (1998). Structural changes of legumin from Faba Beans (*Vicia faba* L.) by succinylation. *Journal of Agricultural and Food Chemistry*, 46, 2080-2086.

Schwenke, K. D., Knopfe, C., Seifert, A., Gornitz, E., & Zirwer, D. (2000). Acetylation of faba bean legumin: conformational changes and aggregation. *Journal of the Science of Food and Agriculture*, 81, 126-134.

Schwenke, K. D., Raab, B., & Damaschun, G. (1983). The structure of the 12S globulin from rapeseed (*Brassica napus L.*). *Die Nahrung*, 27 (2), 165-175.

Schwenke, K. D., Raab, B., Linow, K. J., Pahtz, W., and Uhlig, J. (1981). Isolation of the 12S globulin from rapeseed (*Brassica napus L.)* and characterization as a "neutral" protein. *Nahrung,* 25, 271-280.

Schwenke, K. D., Zirwer, D., Gast, K., Gornitz, E., Linow, K., & Gueguen, J. (1990). Changes of the oligometic structure of legumin from pea (Pisum sativum L.) after succinylation. *European Journal of Biochemistry,* 194, 621-627.

Semenova, M. G., Antipova, A. S., Misharina, T. A., and Golovnya, R. V. (2002). Binding of aroma compounds with legumin. I. Binding of hexyl acetate with 11S globulin depending on the protein molecular state in aqueous medium. *Food Hydrocolloids,* 16, 557-564.

Ser, W. Y., Arntfield, S. D., Hydamaka, A. H., and Slominski, B. A. (2008). Use of diabetic test kits to assess the recovery of glucosinolates during isolation of canola protein. *LWT,* 41, 934-941.

Sheen, S. J. (1991). Effect of succinylation on molecular and functional properties of soluble tobacco leaf proteins. *Journal of Agricultural and Food Chemistry,* 39, 1040-1074.

Shewry, P. R. (2004). Protein chemistry of dicotyledonous grains. In Cereals: Protein Chemistry. Pp. 466-472. UK: Elsevier Ltd.

Shewry, P. R., D'Ovidio, R., Lafiandra, F., Jenkins, J. A., Clare Mills, E. N., & Bekes, F. (2009). Wheat grain proteins. In K. Khan and P. R. Shewry (Eds), *Wheat chemistry and thechnology* (pp. 223-298). St. Paul, Minnesota: AACC International, Inc.

Shih, F. F. (1992). Modification of food proteins by non-enzymatic methods. In B. J. F. Hudson (ed.), *Biochemistry of Food Proteins* (pp.235-248). Verlag: Springer.

Shimada, K., & Cheftel, J. C. (1988). Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *Journal of Agricultural and Food Chemistry,* 36, 147-153.

Short, P. L. (2002). Reshaping flavors and fragrances. *Chemical & Engineering News Archive,* 80 (18), 32-37.

Sikorski, Z. E. (2001). Chemical reactions in proteins in food systems. In Z. E. Sikorski (ed.), *Chemical and functional properties of food proteins* (pp. 191-215), Lancaster, PA: Technomic Publ. Co. Inc.

Sitohy, M., Chobert, J. M., and Haertlé, T. (2000). Study of factors influencing protein esterification using β-lactoglobulin as a model. *Journal of Food Biochemistry,* 24, 381-398.

Sneharani, A. H., Karakkat, J. V., Singh, S. A., & Appu Pao, A. G. (2010). Interaction of crucumin with β-Lactoglobulin-stability, spectroscopic analysis, and molecular modeling of the complex. *Journal of Agricultural and Food Chemistry*, 58, 11130-11139.

Solms, J. Osman-Ismail, and Beyeler, M. (1973). The interactions of volatiles with food components. *Canadian Institute of Food Science and Technology*, 6 (1), A10-16.

Sostmann, K., & Guichard, E. (1998). Immobilized β-lactoglobulin on a HPLC-column: a rapid way to determine protein-flavor interactions. *Food Chemistry*, 62 (4), 509-513.

Spellman, D., McEvoy, E., O'Cuinn, G., FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, 13, 447-453.

Stapelfeldt, H., & Skibsted, L. H. (1994). Modification of β-lactoglobulin by aliphatic aldehydes in aqueous solution. *Journal of Dairy Research*, 61, 209-219.

Stevenson, R. J., Chen, X. D., Mills, O. E. (1996). Modern analyses and binding studies of flavour volatiles with particular reference to dairy protein products. *Food Research International*, 29 (3-4), 265-290.

Sun, X. D. (2010). *Gelation properties of protein mixtures catalyzed by transglutaminase crosslinking*. PhD. thesis. University of Manitoba, Winnipeg, Manitoba.

Sun, X., and Arntfield, S. D. (2010). Gelation properties of salt-extracted pea protein induced by heat treatment. *Food Research International*, 43, 509-515.

Sun, X., and Arntfield, S. D. (2011). Dynamic oscillatory rheological measurement and thermal properties of pea protein extracted by salt method: effect of pH and NaCl. *Journal of Food Engineering*, 105, 577-582.

Sun, X. D., & Arntfield, S. D. (2012a). Molecular forces involved in heat-induced pea protein gelation: effects of various reagents on the rheological properties of salt-extracted pea protein gels. *Food Hydrocolloids*, 28, 325-332.

Sun, X. D., & Arntfield, S. D. (2012b). Gelation properties of myofibrillar/pea protein mixtures induced by transglutaminase crosslinking. *Food Hydrocolloids*, 27, 394-400.

Suppavorasatit, I., and Cadwallader, K. R. (2011). Optimization of the enzymatic deamidation of soy protein by protein-glutaminase and its effects on the functional properties of the protein. Journal of Agricultural and Food Chemistry,

59, 11621-11628.

Suppavorasatit, I., & Cadwallader, K. R. (2012a). Effect of enzymatic deamidation of soy protein by protein-glutaminase on the flavour-binding properties of the protein under aqueous conditions. *Journal of Agricultural and Food Chemistry*, 60, 7817-7823.

Suppavorasatit, I., Lee, S., & Cadwallader, K. R. (2012b). Effect of enzymatic protein deamidation on protein solubility and flavour binding properties of soymilk. *Journal of Food Science*, 78 (1), C1-C7.

Tan, S. H., Mailer, R. J., Blanchard, C. L., & Agboola, S. O. (2011). Canola protein for human consumption: extraction, profile, and functional properties. *Journal of Food Science*, 76 (1), R16-28.

Tanford, C. (1964). Isothermal unfolding of globular proteins in aqueous solutions. *Journal of the American Chemical Society*, 86, 2050.

Tanford, C. (1968). Protein denaturation. *Advances in Protein Chemistry*, 23, 121-282.

Tang, C., Wang, X., & Yang, X. (2009). Enzymatic hydrolysis of hemp (*Cannabis sativa* L.) protein isolate by various protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry*, 114, 1484-1490.

Tavel, L., Andriot, I., Moreau, C., & Guichard, E. (2008). Interaction between β-lactoglobulin and aroma compounds: different binding behaviours as a function of ligand structure. *Journal of Agricultural and Food Chemistry*, 56, 10208-10217.

Tavel, L., Guichard, E., & Moreau, C. (2008). Contribution of NMR spectroscopy to flavour release and perception. *Annual Reports on NMR Spectroscopy*, 60, 173-188.

Taylor, A.J. (1996). Volatile flavor release from foods during eating. *Critical Reviews in Food Science and Nutrition*, 36, 765-784.

Tiwari, B., & Singh, N. (2012). Protein isolates and concentrates. In *Pulse Chemistry and Technology* (pp. 85-106). Cambridge: The Royal Society of Chemistry.

Tromelin, A., Andriot, I., & Guichard, E. (2006). Protein-flavour interactions. In A. Voilley and P. Etievant (Eds.), *Flavour in food* (pp. 172-207). Cambridge: Woodhead Publishing Limited.

Tromelin, A., & Guichard, E. (2006). Interaction between flavour compounds and β-lactoglobulin: approach by NMR and 2D/3D-QSAR studies of ligands. *Flavour*

and Fragrance Journal, 21, 13-24.

Tzeng, Y. M., Diosady, L. L., & Rubin, L. J. (1990). Production of canola protein materials by alkaline, extraction, precipitation, and membrance processing. *Journal of Food Science*, 55, 1147-1151.

Uruakpa, F., & Arntfield, S. D. (2005). The physic-chemical properties of commercial canola protein isolate-guar gum gels. *International Journal of Food Science and Technology*, 40, 643-653.

Uruakpa, F., & Arntfield, S. D. (2006a). Structural thermostability of commercial canola protein-hydrocolloid mixtures. *LWT*, 39, 124-134.

Uruakpa, F. O., and Arntfield, S. D. (2006b). Network formation of canola protein-κ – carrageenan mixtures as affected by salts, urea and dithiothreitol. *LWT*, 39, 939-946.

Uruakpa, F. O., and Arntfield, S. D. (2006c). Surface hydrophobicity of commercial canola proteins mixed with κ-carrageenan or guar gum. *Food Chemistry*, 95, 255-263.

Ustunol, Z., Xiong, Y. L., Means, W. J., and Decker, E. A. (1992). Forces involved in mixed pork myofibrillar protein and calcium alginate gels. *Journal of Agricultural and Food Chemistry*, 40, 577-580.

Utsumi, S., and Kinsella, J. E. (1985). Forces involved in soy protein gelation: effects of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S, and soy isolate. *Journal of Food Science*, 50, 1278-1282.

van Ruth, S. M., & Roozen, J. R. (2002). Delivery of flavours from food matrices. In A. J. Taylor (Ed), *Food Flavour Technology* (pp. 168-184). Sheffield: Sheffield Academic Press Ltd.

Villeneuve, S., & Mondor, M. (2014). Processing and bread-making potential of proteins isolated from malted and non-malted pea seeds by untrafiltration/diafiltration. *Food Bioscience*, 8, 33-36.

Vojdani, F. (1996). Solibulity. In G. M. Hall (Eds.), *Methods of testing protein functionality* (pp. 11-55). London: Blackie Academic & Professional.

von Hippel, P. H., & Schleich, T. (1969). The effects of neutral salts on the structure and conformational stability of macromolecules in solutions. In S. N. Timasheff and G. D. Fasman (Eds.), *Structure and conformational stability of macromolecules* (pp. 417). New York: Marcel Dekker Inc.

Wang, Q., Allen, J. C., & Swaisgood, H. E. (1997). Binding of retinoids to β-Lactoglobulin isolated by bioselective adsorption. *Journal of Dairy Science*, 80,

1047-1053.

Wang, K., & Arntfield, S. D. (2014). Binding of carbonyl flavour to canola, pea and wheat proteins using GC/MS approach. *Food Chemistry*, 157, 364-372.

Wang, K., & Arntfield, S. D. (2015a). Binding of selected volatile flavour mixture to salt-extracted canola and pea proteins and effect of heat treatment on flavour binding. *Food Hydrocolloids*, 43, 410-417.

Wang, K., & Arntfield, S. D (2015b). Effects of salts and pH on selected ketone flavours binding to salt-extracted pea proteins: the role of non-covalent forces. Food Research International. http://dx/doi/org/10.1016/j.foodres.2015.03.017. In Press.

Wang, K., & Arntfield, S. D. (2015c). Interaction of selected volatile flavour compounds and salt-extracted pea proteins: effect on protein structure and therml-induced gelation properties. *Food Hydrocolloids*, 51, 383-394.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Claassen, N. E., Gruppen, H., Voragen, A. G. J., and Smit, G. (2003). Effect of whey protein on the in vivo release of aldehydes. *Journal of Agricultural and Food Chemistry*, 51, 4746-4752.

WHO & FAO (2007). Codex Alimentarius: cereals, pulses, legumes, and vegetable proteins. Retrieved May 22, 2014 from http://www.fao.org/docrep/010/a1392e/a1392e00.htm.

Winter, H. H. (1987). Can the gel point of a cross-linking polymer be detected by the G'-G" crossover? *Polymer Engineering and Science*, 27 (22), 1689-1702.

Wolf, W. J. (1993). Studies on the cold-insoluble fraction of the water-extractable soybean proteins. II. Factors influencing conformation changes in the 11S components. *Archives of Biochemistry and Biochemistry and Biophysics*, 76, 377-393.

Wright, J. (2002). Creating and formulating flavours. In A. J. Taylor (Ed), *Food Flavour Technology* (pp. 1-26). Sheffield: Sheffield Academic Press Ltd.

Wu, J. & Muir, A. D. (2008). Comparative structural, emulsifying, and biological properties of 2 major canola proteins, cruciferin and napin. *Journal of Food Science*, 73 (3), C210-C216.

Xu, L., & Diosady, L. (2013). Processing of canola proteins: a review. In U. Thiyam-Hollander, N. A. M. Eskin, & B. Matthaus (Eds), *Canola and rapeseed: production, processing, food quality, and nutrition* (pp. 79-98). Boca Raton: CRC Press.

Yin, S., Tang, C., Wen, Q., & Yang, X. (2009a). Effects of acylation on the functional

properties and In vitro trypsin digestibility of red kidney bean (*Phaseolus vulgaris L.*) protein isolate. *Journal of Food Science,* 74 (9), E488-494.

Yin, S., Tang, C., Wen, Q., & Yang, X. (2009b). Functional and structural properties and in vitro digestibility of acylated hemp (Cannabis sativa L.) protein isolates. *International Journal of Food Science & Technology*, 44, 2653-2661.

Yin, S., Tang, C., Wen, Q., Yang, X., & Yuan, D. (2010). The relationships between physicochemical properties and conformational features of succnylated and acetylated kidney bean (*Phaseolus vulgaris L.)* protein isolates. *Food Research International*, 43, 730-738.

Yüksel, Z., & Erdem, Y. K. (2005). The influence of main milk components on the hydrophobic interactions of milk protein system in the course of heat treatment. *Journal of Food Engineering*, 67, 301-308.

Yang, X. G., and Peppard, T. (1994). Solid-phase microextraction for flavor analysis. *Journal of Agricultural and Food Chemistry*, 42, 1925-1930.

Zhang, Y., & Cremer, P. S. (2006). Interactions between macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology,* 10, 658-663.

Zhang, Z. Y., and Pawliszyn, J. (1993). Headspace solid-phase microextraction. *Analytical Chemistry*, 65, 1843-52.

Zhao, G., Liu, Y., Zhao, M., Ren, J., & Yang, B. (2011). Enzymatic hydrolysis and their effects on conformational and functional properties of peanut protein isolate. *Food Chemistry*, 127, 1438-1443.

Zhou, Q., Lee, S., & Cadwallader, K. R. (2006). Inverse gas chromatographic evaluation of the influence of soy protein on the binding of selected butter flavour compounds in a wheat soda cracker system. *Journal of Agricultural and Food Chemistry*, 54, 2216-5520.

Appendix A. Illustration of selected ANOVA table for Figure 3.1A

To illustrate the SAS analyses used in this research, several ANOVA tables were presented as examples as follows:

ANOVA of Figure 3.1 A Effect of some intrinsic features of different proteins and flavours on protein-flavour binding performance at a protein concentration of 1% (w/v) and 250 ppm flavour level (a) percentage bound of aldehyde flavour

The GLM Procedure

Dependent Variable: percent bound (percentbind)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	10895.57385	778.25527	831.48	<.0001
Error	15	14.03990	0.93599		
Corrected Total	29	10909.61375			

R-Square	Coeff Var	Root MSE	percentbind Mean
0.998713	2.976945	0.967467	32.49867

Source	DF	Type I SS	Mean Square	F Value	Pr > F
protein	2	420.594605	210.297303	224.68	<.0001
isolation	1	116.732704	116.732704	124.72	<.0001
flavorclass	0	0.000000	.	.	.
carbon	29	184.035707	4592.017853	4906.04	<.0001
prot*isol*flav*carbo	9	1174.210831	130.467870	139.39	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
protein	2	473.102029	236.551015	252.73	<.0001
isolation	1	116.732704	116.732704	124.72	<.0001
flavorclass	0	0.000000	.	.	.
carbon	29	184.035707	4592.017853	4906.04	<.0001
prot*isol*flav*carbo	9	1174.210831	130.467870	139.39	<.0001

Appendix B. Illustration of selected ANOVA table for Figure 4.1A

ANOVA of Figure 4.1 A Percentage retention of homological series of ketone and aldehyde flavours at 250 ppm in a competitive binding environment with and without heat treatment (95℃, 30 min) (A) flavour binding to CPIs; (B) flavour binding to PPIs.

The GLM Procedure

Dependent Variable: percent bound (percentbind)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	17144.90065	1008.52357	608.11	<.0001
Error	18	29.85225	1.65846		
Corrected Total	35	17174.75290			

R-Square	Coeff Var	Root MSE	percentbind Mean
0.998262	4.840436	1.287811	26.60528

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	226.68469	113.34234	68.34	<.0001
flavor	5	14510.08718	2902.01744	1749.83	<.0001
trt*flavor	10	2408.12878	240.81288	145.20	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	226.68469	113.34234	68.34	<.0001
flavor	5	14510.08718	2902.01744	1749.83	<.0001
trt*flavor	10	2408.12878	240.81288	145.20	<.0001

Appendix C. Illustration of selected ANOVA table for Figure 4.2A

ANOVA of Figure 4.2 A Percentage retention of heterological 2-hexanone and hexanal at 250 ppm in a competitive binding environment with and without heat treatment (95℃, 30min) (A) flavour binding to CPIs

The GLM Procedure

Dependent Variable: percent bound (percentbind)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1326.921667	265.384333	430.82	<.0001
Error	6	3.696000	0.616000		
Corrected Total	11	1330.617667			

R-Square	Coeff Var	Root MSE	percentbind Mean
0.997222	4.828897	0.784857	16.25333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	220.0611167	110.0305583	178.62	<.0001
flavor	1	859.1976333	859.1976333	1394.80	<.0001
trt*flavor	2	247.6629167	123.8314583	201.03	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	220.0611167	110.0305583	178.62	<.0001
flavor	1	859.1976333	859.1976333	1394.80	<.0001
trt*flavor	2	247.6629167	123.8314583	201.03	<.0001

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Intercept	33.52000000	B	0.55497748	60.40	<.0001
trt control	-17.83500000	B	0.78485667	-22.72	<.0001
trt nonheat	-8.58000000	B	0.78485667	-10.93	<.0001
trt heat	0.00000000	B	.	.	.
flavor 2-hexano	-29.76500000	B	0.78485667	-37.92	<.0001
flavor hexanal	0.00000000	B	.	.	.
trt*flavor control 2-hexano	18.87500000	B	1.10995495	17.01	<.0001
trt*flavor control hexanal	0.00000000	B	.	.	.
trt*flavor nonheat 2-hexano	19.65000000	B	1.10995495	17.70	<.0001
trt*flavor nonheat hexanal	0.00000000	B	.	.	.
trt*flavor heat 2-hexano	0.00000000	B	.	.	.
trt*flavor heat hexanal	0.00000000	B	.	.	.