ELUCIDATION OF THE INTERACTION BETWEEN SOY PROTEIN ISOLATE AND SIMULATED BEEF FLAVOUR

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA
April 2007

Abstract

The objective of this research was to explore interactions between aroma compounds in simulated beef flavour (SBF) and soy protein isolate (SPI) that may be involved in the suppression of beefy notes in SBF by SPI. A sensitive and reproducible headspace solid phase microextraction (HS-SPME) was established to isolate volatile compounds in SBF for analysis by gas chromatography (GC). Volatile and odour-active compounds in SBF were qualitatively evaluated by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC-O) using detection frequency method, respectively. A total of 70 compounds were tentatively identified including three furans, six S-heterocyclic compounds, ten N-heterocyclic compounds, six aldehydes, three alcohols, and two esters. Of 49 volatile compounds detected in the sniffing port of GC-O, the most odour-active included 2-methyl-3-furanthiol, delta-3-carene, alpha-terpinene, 2-ethyl-3,6-dimethylpyrazine, and several unidentified odourants. Descriptive analysis (DA) along with GC analysis was conducted to investigate changes in SBF aroma characteristics upon addition of SPI. Five attributes (beefy, roasted, yeasty, soymilk-like and cereal) were selected to assess various mixtures of SBF and SPI. The results from DA confirmed that "roasted", "beefy" and "yeasty" notes were highly positively correlated with SBF concentration, and the beefy related notes were substantially suppressed by increasing SPI content. Fifteen peaks from GC analysis were selected as indicator peaks to represent beef attribute in the mixtures of SPI and SBF. Changes in the release of beefy aroma components of SBF by addition of ingredients (glucosamine, sucrose, ascorbic acid, and/or polyethylene glycol) to SPI and the changes in SPI protein structure induced by the ingredients were investigated. The reduction of disulfide bonds, increased surface hydrophobicity and increased unordered structure in SPI containing ascorbic acid alone or with polyethylene glycol, along with increased GC peak areas of indicator peaks in those SPI-SBF mixtures, were found to be associated with an increase in the perceived beef characteristic attributes in descriptive analysis. These results provide the basis for further research to elucidate strategies maximizing perception of beefy aroma in soy based products.

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List of Abbreviations

A	SPI containing ascorbic acid
AEDA	Aroma Extract Dilution Analysis
AF	SBF with SPI containing ascorbic acid
ANOVA	Analysis of Variance
ANS	1-Anilinonaphthalene-8-sulfonic acid
AP	SPI containing ascorbic acid and polyethylene glycol
APF	SBF with SPI containing ascorbic acid and polyethylene glycol
CAR/PDMS	Carboxen/polydimethylsiloxane
CHARM	Combined Hedonic Aroma Response Measurements
CPA	cis-Parinaric acid
CW/DVB	Carbowax/divinylbenzene
DA	Descriptive Analysis
DF	Detection Frequency
df	Degree of Freedom
DH	Degree of Hydrolysis
DSC	Differential Scanning Calorimetry
DTNB	5,5′-Dithio-bis-2-nitrobenzoic acid
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
FD	Flavour Dilution
FID	Flame Ionization Detector
FT-Raman	Fourier-Transform Raman
G	SPI containing glucosamine
g	Grams
g	Gravitational Force
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GC-O	Gas Chromatography-Olfactometry
GF	SBF with SPI containing glucosamine

HS-SPME	Headspace-Solid Phase Microextraction
IP	Indicator Peak
kDa	Kilo Dalton
LSD	Least Significant Difference
MSD	Mass Selective Detector
MSE	Mean Squares of Error
NIR	Near-Infrared
P	SPI containing polyethylene glycol
PC	Principal Component
PCA	Principal Component Analysis
PF	SBF with SPI containing polyethylene glycol
PRODAN	6-Propionyl-2(N,N-dimethyl-amino)naphthalene
RFI	Relative Fluorescence Intensity
RSAP	Raman Spectral Analysis Package
S	SPI containing sucrose
SBF	Simulated Beef Flavour
SF	SBF with SPI containing sucrose
SPI	Soy Protein Isolate
SPIF	SBF with SPI
SPME	Solid Phase Microextraction
TIC	Total Ion Chromatogram

Acknowledgements

My deepest and most sincere gratitude goes to my supervisor, Dr. Eunice Li-Chan, for her outstanding guidance and thoughtful encouragement to complete this thesis. She is not only a knowledgeable academic supervisor but also a mentor in my mind who is devoted and caring with warm heart. It is a blessed pleasure to be her student.

I also sincerely appreciate my supervisory committee members, Dr. M. Cliff, Dr. C. Scaman and Ms. W. Willis, for their invaluable advice and suggestions to my studies. Their wisdom offered great help to proceed to each step. I appreciate their continuous support throughout the studies. I would also like to express my thanks to Dr. T. Durance, Dr. D. Kitts, Dr. S. Nakai and Dr. B. Skura for their academic advice and comments during classes and work.

My special thanks are extended to Ms. T. Nguyen, Dr. A. Pedro, Ms. V. Skura, Dr. P. Yaghmaee, and Mr. S. Yee in Food Science at UBC, Dr. T. Cottrell at the Pacific Agri-Food Research Station of Agriculture and Agri-Food Canada, Ms. L. Madilao in the Wine Research Centre at UBC as well as Mr. K. Cummings, Ms. A. McCannel, and Dr. G. Sandberg for their technical support and considerate help.

I would also like to thank all the graduate students and fellows in Food Science at UBC especially members in Dr. Li-Chan's group, Andrea, Anusha, Bo, Crystal, Imelda, Judy, Tam, Tom, and Wendy as well as Dr. Alizadeh-Pasdar, Dr. Kim and Dr. Meng for showing faithful friendship and sharing their experience with me. I will also remember friendship of Sung-Yon, Soon-Hye, Kyu-Won, Ji-Eun, Ro-Mee and other friends in Korea.

This research was supported by research funding and postgraduate scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC), Graduate Research Engineering and Technology (GREAT) scholarship granted by Science Council of British Columbia, and the University Graduate Fellowship provided by the University of the British Columbia.

Last but not least, my parents, parents-in-law, brother and sister in Korea, who have shown endless support, love, and prayers to me, receive my deepest gratitude. They always have been protective background surrounding me.

Dedication

This thesis is dedicated to:

My husband, Kwangsoo
Daughter, Jennifer (Yewon)
Son, Maric (Jongwon)

Your understanding and encouragement based on sacrifice
made this dissertation possible.

Co-Authorship Statements

The work presented in Chapter 2 of this thesis was published in "*Food Chemistry*" (2004), 88, 141-149, entitled "Development of solid-phase microextraction methodology for analysis of headspace volatile compounds in simulated beef flavour". Soo Yeun Moon, the thesis author, was the principal author and Eunice C. Y. Li-Chan, Soo Yeun Moon's supervisor, was the co-author.

The work presented in Chapter 3 of this thesis was published in "*Food Research International*" (2006), 39, 294-308, entitled "Odour-active components of simulated beef flavour analysed by solid phase microextraction and gas chromatography-mass spectrometry and –olfactometry". Soo Yeun Moon, the thesis author, was the principal author in the publication. Margaret A. Cliff, member of Soo Yeun Moon's supervisory committee, and Eunice C. Y. Li-Chan, Soo Yeun Moon's supervisor, were the co-authors.

The work presented in Chapter 4 of this thesis will be submitted for publication in the "*The Journal of the American Oil Chemists' Society*", entitled "Changes in aroma characteristics of simulated beef flavour by adding soy protein isolate as assessed by descriptive sensory analysis and gas chromatography-olfactometry". Soo Yeun Moon, the thesis author, was the principal author and Eunice C. Y. Li-Chan, Soo Yeun Moon's supervisor, was the co-author.

The work presented in Chapter 5 of this thesis will be submitted for publication in the "*The Journal of the American Oil Chemists' Society*", entitled "Assessment of added ingredient effect on the interaction of simulated beef flavour and soy protein isolate by gas chromatography and spectroscopic techniques". Soo Yeun Moon, the thesis author, was the principal author and Eunice C. Y. Li-Chan, Soo Yeun Moon's supervisor, was the co-author.

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CHAPTER 1. OVERVIEW AND LITERATURE REVIEW

1.1 General Introduction

For centuries, soybean has been one of the major sources of edible plant oil, while the remaining defatted soy flakes provide nutritional and economical advantages to food with its high protein content and relatively low cost. The little round bean has a long history with the first reference dating back to 2838 BC in a book "Pen-Ts'ae-Kung-Mu" written by Shen Nung (Hoogenkamp, 2006). It has been used as the starting material for soy flour, soymilk and tofu and various fermented foods such as soy sauce, miso, natto, and tempeh, which are part of the staple diet in oriental countries (Smith and Circle, 1978). Apart from being an important source of protein in food, soy proteins have the capacity to be functional ingredients in foods with the practical functionalities such as gelation, emulsification, stabilization, water adsorption, fibre formation, film formation, elasticity, and foaming properties (Ohren, 1981). Although soybeans have been part of the staple diet in many Asian countries for a long period, it has only been commercially grown in the North America since 1922 as an inexpensive source of edible oil. Significant production started since the 1960s but still a limited quantity of soybeans is consumed directly in North America and Europe (Hoogenkamp, 2006). In recent years, hypocholesterolemic effects of soy protein were reported and much evidence has been gathered demonstrating that consumption of soy protein helps to reduce risks of cardiovascular disease (Anderson et al., 1995). In October 1999, the Food and Drug Administration approved health claims for soy protein and coronary heart disease (USFDA, 1999), followed by a similar health claim by the Joint Health Claim Initiative in 2002 indicating "the inclusion of at least 20 grams of soy protein per day, as part of a diet low in saturated fat, can help reduce blood cholesterol levels" (Hoogenkamp, 2006). In addition, soy protein has become popular as meat substitutes with increasing number of vegetarians and health-conscious consumers wishing to reduce their intake of meat.

However, in spite of the high nutritional quality and great functionalities of soy proteins, flavour-associated problems have been a major practical hindrance to expand the usage of soy proteins in food products and consumer acceptance of soy products (MacLeod and Ames, 1988; Maheshwari et al., 1995; Rah et al., 2004; Schutte and Van den Ouweland, 1979). In addition to the

indigenous undesirable soy aroma components that are problematic to eradicate, interaction of flavour compounds with soy proteins has been reported (Damodaran and Kinsella, 1981a and 1981b; Gremli, 1974; Malcolmson and McDaniel, 1987). Considerable research has been performed to understand the flavour-binding nature of soy protein (Aspelund and Wilson, 1983; Beyeler and Solms, 1974; Damodaran and Kinsella, 1981a and 1981b; O'Keefe et al., 1991a and 1991b; Li et al., 2000; Zhou and Cadwallader, 2004). However, most of these studies used model systems of single ingredient or selective volatile model compounds associated with flavour or off-flavour, such as a series of aldehydes, ketones, alcohols, or alkanes. Even though valuable thermodynamic information was obtained from these studies, the knowledge may not be directly applied to the real food system, in which the flavour ingredients usually include combinations of a broad array of subclasses of compounds. Soy proteins bind with certain desirable flavour compounds, which could have an impact on flavour suppression or alteration of flavour profiles in the mixture or final food products. Therefore, it is imperative to elucidate the nature of the interactions of soy proteins with flavour compounds for the development of soy products with acceptable flavour quality.

In this thesis, interaction between soy protein isolate and simulated beef flavour used as commercially available ingredients in the food industry was investigated to elucidate integral flavour holding properties of the soy protein isolate and changes in sensory characteristics of the mixture.

1.2. Soybean Protein Isolate (SPI)

1.2.1. Specification and production

Soy protein isolate (SPI), also known as soybean protein isolate or isolated soybean protein, is the most concentrated form of commercially available soybean protein products. According to the specification defined by the Association of American Feed Control Officials, Inc. (AAFCO), "soy protein isolate is the major proteinaceous fraction of soybeans prepared from dehulled soybeans by removing the majority of non-protein components and must contain not less than 90 % protein on a moisture-free basis". Soy protein concentrate, another commercially available soy protein product, "is prepared from high quality sound, clean, dehulled soybean seeds by

removing most of the oil and water soluble non-protein constituents and must contain not less than 70 % protein on a moisture free basis" (Berk, 1992). The average chemical composition of SPI, compared to defatted soy flour and soy protein concentrate, is presented in Table 1.1.

To produce soy-related products, whole beans are soaked in water until they contain about 11 % moisture. After removal of the hulls, dehulled beans are milled into flakes or more fine particles to produce full-fat soy flour, which consists of 43 % protein, 23 % lipids, 29 % carbohydrate, and 5 % ash (MacLeod and Ames, 1988). To produce defatted soy flour, coarse flakes are extracted with a solvent such as hexane, and then deodourized by treatment at about 85 °C before milling to produce a fine powder. SPI is prepared by extracting defatted flakes or flours with dilute alkali solution (pH 7-10) at 50 - 55 °C, followed by centrifugation to eliminate the insoluble polysaccharide residue, as shown in Figure 1.1. After the alkali extract is clarified, it is acidified to pH 4.5, which is the isoelectric point of the major soy proteins, resulting in precipitation of the soy protein. The precipitate is neutralized to a pH about 6.8 and then spray-dried, leading to a highly soluble proteinate form of SPI. Alternatively, the precipitate may be centrifuged, washed, and made into a slurry with water and then spray-dried to produce the isoelectric form of SPI (Hettiarachchy and Kalapathy, 1997).

1.2.2. Major proteins in SPI

Most of the proteins in soybean are categorized as globulins, which are soluble in salt solution. According to their sedimentation properties, they can be classified into four fractions namely 2S, 7S, 11S, and 15S. The two major globulin components in SPI are glycinin (11S globulin) and β-conglycinin (7S globulin), which account for about 80 % of the total storage proteins in soybean (Moriyama et al., 2005). The average calculated molecular weight of SPI is 237 kDa based on 15 %, 34 %, 42 %, and 9 % of 2S (18-33 kDa), 7S (180-210 kDa), 11S (300-350 kDa), and 15S (600 kDa) in SPI, respectively (Li et al., 2000).

1.2.2.1. Glycinin

Glycinin is the purified form of the 11S fraction, which accounts for over 40 % of the total seed globulin in soybeans (Liu, 1997). At ambient temperature and pH 7.6, glycinin is believed to exist in the form of hexameric complexes (11S) with a MW of about 360 kDa; each monomeric

subunit is composed of an acidic polypeptide (ca. 34-44 kDa) and a basic polypeptide (ca. 20 kDa) with one single disulfide bond between the two polypeptides, which can be represented as A-S-S-B or A-B (Liu, 1997). Six monomeric subunits form a glycinin molecule $(A\text{-}S\text{-}S\text{-}B)_6$, with two hexagonal rings stacked one on top of the other (Yamauchi et al., 1991). However, a trimeric complex (7S) form was also found at pH 3.8 and a 15S fraction is thought to be a polymer form of the β-conglycinin (Renkema et al., 2002). The secondary structure of glycinin was reported by Abbott et al. (1996) to be 24 % α-helix, 30 % β-sheet, 42 % turns and 12 % unordered. Glycinin has a complex quaternary structure, which depends on pH and ionic strength (Renkema et al., 2002). The bonds responsible for the quaternary structure can be disrupted by urea, strong acid, strong base, heat, or sodium dodecylsulfate in combination with a reducing agent, leading to dissociation into the subunits and further into the acidic and basic polypeptides, and resulting in altered structure of glycinin as shown in Figure 1.2.

Five major subunits G1-G5 of glycinin that have been purified from the soybean cultivar CX635-1-1-1 are presented in Table 1.2. Those subunits can be divided into two groups (I and II) according to their physical and chemical properties. Compared to group II subunits, group I subunits have more uniform MW (58 kDa), contain more methionine, and exhibit about 90 % sequence homology among members, while the group II subunits show 60-70 % sequence homology between members. Among the five major subunits, the G5 subunit ($A_5A_4B_3$) is most unique in its general structure, consisting of acidic components composed of two different polypeptides (A_5A_4). Except for A_4 in G_5 subunit, all acidic and basic polypeptide are linked via disulfide bonds, while polypeptide A_4 is not covalently linked with a basic polypeptide. Interestingly, the G5 subunit was not found in Raiden cultivar; instead a subunit with a different acidic polypeptide A_6 was discovered (Liu, 1997).

1.2.2.2. β-Conglycinin

β-Conglycinin, the other major constituent in SPI, is a glycoprotein containing 4-5 % carbohydrate and is generally called 7S globulin. It is a trimer with a MW of about 180 kDa comprised of three subunits, α′, α, and β, with MW of 57-72 kDa, 57-68 kDa, and 42-52 kDa, respectively (Yamauchi et al., 1991). However, another subunit, called β′, was also reported in some soybean varieties (Morita et al., 1996). Unlike the polypeptides in glycinin, the subunits in

β-conglycinin are non-covalently associated through hydrophobic and hydrogen bonding without any disulfide bonds. As shown in Table 1.3, all three major subunits are abundant in aspartate, asparagine, glutamate, glutamine, leucine and arginine, and the amino acid composition is especially similar between the α and α′ subunits (Liu, 1997). Results from recent research showed important physiological functions of β-conglycinin such as hypocholesterolemic and hypotriglycemic activities in rats or suppression of serum triglyceride, insulin and glucose levels in normal and genetically obese mice (Moriyama et al. 2005).

1.2.3. Soy protein isolates used for functional ingredients

SPIs have been applied extensively in meat products as extenders and meat analogs, bakery products, and dairy products. The use of soy proteins as a food ingredient arises from their functional properties such as solubility, viscosity, gelation, emulsification, and foaming properties, as shown in Table 1.4, which are based on structural and conformational attributes of the proteins. Since non-covalent forces such as ionic or electrostatic interaction, hydrogen bonding and hydrophobic interaction of amino acid side chains as well as covalent disulfide bonds are responsible for formation and stability of the protein conformation, any factor that changes these interactions could affect the secondary and tertiary structure along with the exposure of amino acid side chains to the surface. In turn, these structural changes could lead to alteration in the functional properties of the protein.

Differences in amino acid composition between β-conglycinin and glycinin are shown in Table 1.3, including the higher contents of cysteine and methionine in glycinin than β-conglycinin. Aside from the compositional difference, the structural difference between the two proteins also results in considerable variation in functional properties of the proteins (Yamauchi et al., 1991). While glycinin was reported to have superior gel formation property, β-conglycinin had greater emulsifying power and emulsion stability. Both β-conglycinin and glycinin formed gels by heat and/or a coagulant during tofu manufacture but glycinin needed higher heating temperature to form a gel than β-conglycinin due to different heat denaturation characteristics (Hettiarachchy and Kalapathy, 1997). Utsumi and Kinsella (1985) stated that hydrogen bonding and disulfide bonds were important in maintaining gel network structures. It was also reported that disulfide cleavage weakened the gel strength at low protein concentration while it helped to reinforce

gelation at high protein concentration (Hettiarachchy and Kalapathy, 1997).

1.2.4. Structural changes in soy proteins

Protein denaturation is "any modification in conformation (secondary, tertiary, or quaternary structure) not accompanied by the rupture of peptide bonds involved in primary structure" (Cheftel et al., 1985). The conformation of a protein involving its secondary and tertiary structure is susceptible to be changed by physical causes such as heat, pressure, irradiation, or mechanical stress, and by chemical agents such as acids, alkalis, salts, organic solvents, surfactants, or reducing agents. Among those factors, heat-induced structural changes in soy proteins have been extensively researched as heating is one of the most frequently applied methods during processing.

Heating glycinin with β-conglycinin resulted in complex formation between dissociated β-conglycinin subunits and glycinin subunits (German et al., 1982). Thermal aggregation of glycinin in the presence of a reducing agent such as 10 mM 2-mercaptoethanol at 80 °C due to aggregation of the basic subunits was observed by Damodaran and Kinsella (1982). However, the thermal aggregation of glycinin was prevented by addition of isolated conglycinin, and formation of a soluble complex between the subunits of β-conglycinin and the basic subunits of glycinin was suggested (Damodaran and Kinsella, 1982). Involvement of breakage and formation of disulfide bonds between basic subunits of glycinin as a result of heating treatment was also reported by Utsumi et al. (1984). In addition, dissociation of both β-conglycinin and glycinin by heat treatment at 80 °C was detected, followed by subsequent interaction between the β-conglycinin and glycinin globulins; in particular, the basic subunits of glycinin and the β subunit of β-conglycinin were predominantly found in the aggregates.

The heat-induced subunit interaction between β-conglycinin and glycinin was schematically represented by Yamauchi and coworker (1991) as shown in Figure 1.3. Relatively mild heating triggered dissociation of both glycinin and β-conglycinin and further heating caused subsequent interaction with each other, resulting in the formation of soluble polymerized polypeptides from dissociated glycinin and β-conglycinin subunits. Although the occurrence of precipitation depends on the concentration of the proteins, the basic and β subunits tend to be located in the

precipitate while the acidic and α, α′ subunits are likely to be found in the supernatant through disulfide bonding among the acidic, α, and α′ subunits.

Plant proteins including soy proteins contain high contents of asparagine and glutamine residues, and the side chain amide groups are known to be important to stabilize the protein structure through hydrogen bonding, which are easily modified by relatively mild treatment (Matsudomi et al., 1985b). Changes in conformation and functional properties of soy protein by mild acid treatment (2 % soy protein solution in 0.05 N HCl) were reported (Matsudomi et al., 1985b). In the dilute acid condition, preferential deamidation without significant cleavage of the peptide bonds resulted, leading to an increase in functional properties such as solubility, emulsifying properties and foaming properties as well as increased surface hydrophobicity which was considered to be associated with conformational changes due to deamidation and acid –induced denaturation.

Although most soy protein studies have been conducted at neutral pH, it was reported that β-conglycinin exhibited association-dissociation behavior depending on the pH and ionic strength of the solution. β-conglycinin (7S) was found as a trimer structure at pH 7.6 at high ionic strength ($I > 0.5$) or acidic pH ($pH < 4.8$), while it existed as a hexamer (10S) at low ionic strength ($I < 0.2$) in the pH region 4.8-11.0 (Thanh and Shibasaki, 1979). Dissociation of glycinin hexamers to their constituent polypeptides has also been reported. Glycinin is mainly present in a hexameric form (11S) but with lowering of the ionic strength to 0.01 at pH 7.6, it was dissociated from the 11S form mainly into the 7S form, which was believed to be the trimeric form and less structured conformation (Utsumi et al., 1987). Lakemond et al. (2000) also confirmed that glycinin formed hexameric complexes (11S) at pH 7.6 and an ionic strength of 0.5 while it existed as trimers (7S) at pH 3.8 and at an ionic strength of 0.03. They also examined tryptophan fluorescence spectra using fluorescence spectroscopy to determine differences in the tertiary interactions within glycinin and reported that no significant structural changes were found among different ionic strengths in the range of 0.03-0.5 at pH 7.6, but a more tightly packed fluorophore environment was observed when the pH is lowered from 7.6 to 3.8

Simultaneous heat and reducing treatment may also affect structural characteristic of SPI.

Remondetto et al. (2002) reported that sulfite at low temperature promoted denaturation by reducing disulfide bonds. Cleavage of disulfide bonds by sulfite at 67-75 °C caused increase in molecular flexibility of SPI leading to formation of new disulfide bonds. Enhanced functional properties such as solubility, gelation, foaming and emulsification by increasing molecular flexibility due to partial reduction of disulfide bonds were also reported (Petruccelli and Añón, 1995).

1.2.5. Modification

Food proteins including SPI generally need some modification of their composition and structure to be used as food ingredients with suitable functional properties. Although various chemical modifications such as acylation, alkylation, esterification, phosphorylation, glycosylation, deamidation and reduction of disulfide bonds have been used to improve functionalities of food proteins, acylation seemed to be most widely researched. For instance, acylation increased the solubility of soy protein in acid solutions for use in coffee whiteners (Meyer and Williams, 1977). Acylation with either acetic anhydride or succinic anhydride significantly decreased the texture of SPI extrudate but increased its solubility (Simonsky and Stanley, 1982). SPI treated with increasing concentrations of Na_2SO_3, which could cause cleavage of disulfide bond, NaCl, or Na_2SO_4 showed decreased viscosity and adhesive strength (Kalapathy et al., 1996).

1.3. Flavour of soy products

1.3.1. Volatile compounds in soybean and soy products

Soybean foods such as soy flour, soymilk, and textured soy protein have been reported to have a typical odour, which is often described as green (raw/fresh), grassy, and beany (Cowan et al., 1973). In many cases, these beany odours detected in soy products are regarded as undesirable and even offensive, resulting in unpleasant soy products. The attributes could be detected by 80 % of the sensory panel even when soy flour was diluted with non-odorous wheat flour at 1:750 in the study of Moser et al. (1967). The off-flavour associated with soybean is a major barrier in the increased usage of soy proteins in human foods.

There have been several reports regarding the effect of heat treatment on the sensory properties of

soybean odour. The grassy/beany notes were decreased during the heating of soy flours such as texturization while cereal /grain-like and toasted attributes were retained or developed (Warner et al., 1983). Kato et al. (1981) also reported that the odour note of soybeans changed remarkably when dry heat was applied to soy flour at 200 °C with considerable weight loss due to decomposition of nonvolatile precursors and water evaporation. The subsequently roasted soy flour showed a pleasant odour with toasted note, which could mask other off-odours. However, when defatted soy proteins were retorted or sterilized, an unpleasant cooked odour was produced (Greuell, 1974).

The volatile compounds identified from soybeans, flours, soy protein concentrates, SPIs, or textured soy proteins were described in the comprehensive review by MacLeod et al. (1988) where a total number of 334 individual components were listed comprising 18 aliphatic hydrocarbons, 3 alicyclic hydrocarbons, 14 terpenoids, 31 aliphatic alcohols, 31 aliphatic aldehydes, 32 aliphatic ketones, 4 alicyclic ketones, 10 aliphatic carboxylic acids, 10 lactones, 13 aliphatic esters, 1 alicyclic ester, 6 aliphatic ethers, 7 aliphatic amines, 1 aliphatic nitrile, 5 chlorine-containing compounds, 67 benzenoids, 12 aliphatic sulfur compounds, 24 furanoids, 12 thiophenoids, 5 pyrroles, 1 pyridine, 19 pyrazines, 3 thiazoles, and 5 other sulfur heterocyclic compounds.

Recently, there have been several reports that were conducted using gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS) to determine the major volatile compounds that contribute to the beany odour. The most potent odourants in dry commercial SPIs determined by GC-O were butyric acid, 2-methyl butyric acid methyl ester, 2-pentyl pyridine and hexanal (Boatright et al., 1997), while dimethyl trisulfide, *trans, trans*-2,4-decadienal, 2-pentyl pyridine, *trans,trans*-2,4-nonadienal, hexanal, acetophenone and 1-octen-3-one were identified in aqueous solution of SPIs (Boatright et al., 1999). Lei et al. (2001) reported that acetaldehyde, methanethiol, hexanal, dimethyl trisulfide, and 2-pentyl furan were detected by GC-O and GC-MS in the headspace of the aqueous slurries of soy protein concentrate, and methionine was reported as the methyl group donor for formation of sulfite-associated methanethiol in aqueous slurries of SPI (Lei and Boatright, 2006).

The beany odour compounds in soy products have been reported to include aliphatic carbonyls, volatile fatty acids, alcohols, furans and amines. Hsieh et al. (1981) reported that the major volatile compounds of soy products were 1-hexanol, 1-pentanol, hexanal, 1-octen-3-ol, and 2-pentylfuran. Medium-chain aldehydes such as pentanal, hexanal, and heptanal were also reported to be the key class of compounds contributing to beany odours of soy proteins (Maheshwari et al., 1995). These volatile components could arise from the beans themselves by the action of soybean lipoxygenase and subsequent formation of lipid oxidation products, which are of critical importance in soybean off-flavour (Sessa et al., 1977). Oleic (18:1), linoleic (18:1), and linolenic (18:2) acids are the most important precursors since soy lipids are characterized by a relatively high content of unsaturated fatty acids, especially linoleic and oleic acids, which comprise 53.2 % and 23.4 %, respectively of the fatty acids in soybean oil. Other possible reactions include the result of heat on sugars and/or amino acids, thermal decomposition of phenolic acids and thiamine, and the oxidative and thermal degradation of carotenoids (MacLeod et al., 1988). Even though it is not easily recognized due to the complexity and variety of the reactions, secondary reactions can be induced from the products of primary reactions. Moreover, the grassy and objectionable odour could also be developed as a result of processing and during storage of soybeans and flour (Warner et al., 1983).

MacLeod et al. (1988) drew several conclusions in terms of the volatile compounds of commercially produced materials such as full-fat flakes/flours, defatted flakes/flours, toasted defatted flakes/flours, concentrates, isolates, and textured soy protein (TSP). Firstly, the commercially produced full-fat flakes/flours or soy concentrate did not show additional volatile components compared to those reported from ground beans and heated ground beans. Secondly, significantly fewer alcohols, aldehydes, and benzenoids were found in defatted flakes/flours. Thirdly, toasted defatted flakes/flours contained a great number of pyrazines. However, those had already been identified in either heated ground beans or defatted flours. Lastly, considerably fewer benzenoids and aliphatic alcohols were found in soy isolate, while a large number of aliphatic sulfur compounds, furans, and thiophens were recognized.

1.3.2. Interaction of soy protein with flavour compounds

1.3.2.1. Typical flavour binding model

The composition and concentration of volatile compounds in the headspace above food rather than in the food itself more directly affects the aroma profile of the food. Research to investigate binding behavior of proteins to flavour compounds has been characterized using gel filtration, equilibrium dialysis, and headspace method, and interpreted most widely by the Scatchard equation (Scatchard, 1949) and Klotz plot (Klotz, 1946) as described by O'Neill (1996).

Interaction of a protein (P) with a ligand (L; flavour molecule) may be represented as:

$$P + L = PL \qquad \text{[equation 1]}$$

As P(total) = PL + P, the equation 1 can be expressed as below with the association constant, K

$$(PL) = K(P)(L) \qquad \text{[equation 2]}$$

$$(PL) = K(L)[P(total)-(PL)] \qquad \text{[equation 3]}$$

The equation 3 can be converted using υ, the number of moles of ligand bound per mole of protein, as below.

$$\upsilon = (PL)/P(total) \qquad \text{[equation 4]}$$

$$\upsilon = K(L)/[1+K(L)] \qquad \text{[equation 5]}$$

The equation 5 can be extended with a protein having indistinguishable and independent binding sites (n)

$$\upsilon = nK(L)/[1+K(L)] \qquad \text{[equation 6]}$$

$$\upsilon/L = nK - K\upsilon \qquad \text{[equation 7]}$$

Therefore, plotting υ/L versus υ gives the Scatchard plot with the slope, -K, and y-intercept, nK. The binding equation also can be transformed into the double reciprocal equation (Klotz plot) to linearize the binding data:

$$\frac{1}{\upsilon} = \frac{1}{nK(L)} + \frac{1}{n} \qquad \text{[equation 8]}$$

Experimental determination of the value of the K as a function of temperature allows determination of thermodynamic parameters such as the Gibb's free energy of binding (ΔG°), the enthalpy of binding (ΔH°), and the entropy of binding (ΔS°).

$$\Delta G^\circ = -RT\ln K \qquad \text{[equation 9]}$$

$$\Delta H^\circ = -R d\ln K / d(1/T) \quad \text{[equation 10]}$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad \text{[equation 11]}$$

where T is the absolute temperature in °Kelvin (°K), and R is the gas constant.

1.3.2.2. Binding of flavour model compounds to soy protein

A number of studies have examined the interaction between proteins and flavour compounds in aqueous model systems. Most of the research was conducted using simple model compounds or a series of alcohols, aldehydes, ketones or carboxylic acid, which are recognized as off-flavour related volatile compounds in soybean.

Binding of n-hexanal and n-hexanol to native, partially denatured, denatured, and enzymatically hydrolyzed soy protein was compared using vacuum distillation and gel filtration techniques to investigate the effectiveness of enzymatic proteolysis in removing off-flavours from soy protein concentrate (Arai et al., 1970). In the study, binding constants of n-hexanal and n-hexanol for native soy protein were determined to be 173.4 and 80.3 M^{-1}, respectively (Table 1.5), and the interactions increased with the degree of denaturation and decreased with protein hydrolysis.

Gremli (1974) studied the effects of adding flavour compounds to SPI by headspace analysis and a high vacuum transfer system. In contrast to the results of Arai et al. (1970), aldehydes, especially unsaturated compounds, reacted more strongly with the proteins than ketones while alcohols did not interact with soy protein. The interactions showed both reversible and, of less importance, irreversible features and the retention of aldehydes by 5 % soy protein solution was positively correlated to the chain length of the aldehydes ranging from hexanal (C6) to dodecanal (C12) (Gremli, 1974).

The binding constant of SPI investigated with an equilibrium dialysis method was reported by Beyeler and Solms (1974) to decrease in the order of aldehydes, ketones, and alcohols (Table 1.5). No binding affinity was found with carboxylic acids, dimethylpyrazine, aniline or phenylalanine. The binding effects of SPI were found to be rather independent of pH and temperature, and characterized by weak and unspecific binding forces.

Damodaran and Kinsella (1981a) investigated the interaction of carbonyl compounds with soy protein using an equilibrium dialysis method. Binding affinities of ketones (C7 to C9) to soy protein increased with increasing chain length. The binding constant increased by nearly 3 times with the change of about –600 cal/CH_2 residue for each additional methylene group on the ligand (Table 1.5). The binding was suggested to be spontaneous and thermodynamically favorable due to the negative value of ΔG. In terms of the position of the keto group among nonanal, 2-nonanone, and 5-nonanone, shift of keto group toward the middle of the chain was observed to be associated with a decrease in binding affinity (Table 1.5). Steric hindrance of the relatively polar keto group to the hydrophobic interaction of ligand to the binding site of the protein was suggested to explain the result. Partial denaturation of soy protein induced by heat treatment increased the binding affinity for 2-nonanone compared to native soy protein (Table 1.5). The change in quaternary structure of soy protein was proposed to occur through certain reorganization of the subunits, which may affect the hydrophobicity of the sites resulting in increasing of binding affinity for the ligand (Damodaran and Kinsella, 1981a)

Different binding affinities were observed in glycinin and β-conglycinin in isolated systems. Damodaran and Kinsella (1981b) reported that the binding constant and the total number of binding sites of β-conglycinin for 2-nonanone were about the same as those of whole soy protein whereas almost no affinity was found in glycinin indicating β-conglycinin might be the responsible component for off-flavour binding in soy protein. The difference was assumed to be due to the different spatial arrangement of the subunits in the two proteins i.e. possible hydrophobic regions accessible for ligand binding in β-conglycinin versus not available or buried hydrophobic regions inside the glycinin. The authors also found an increase in the binding affinity of glycinin when ionic strength was increased from 0.03 M to 0.5 M, and suggested that the lack of affinity of glycinin for 2-nonanone was due to the dissociated form of glycinin isolated in low ionic strength. Both the binding affinity and the binding capacity of soy protein for 2-nonanone were considerably affected by urea or chemical modification with succinic anhydride (Damodaran and Kinsella, 1981b). Decreased fluorescence intensity of tryptophan residues with increasing urea concentration or succinylation treatment was explained by destabilization of the hydrophobic regions in soy protein. However, O'Neill and Kinsella (1987) reported binding constants (K) of whole soy protein, β-conglycinin, and glycinin for 2-nonanone

of 570, 3050, and 540 M^{-1}, respectively, and approximately 5, 2, and 3 primary binding sites per 100,000 daltons of whole soy protein, β-conglycinin, and glycinin, respectively, demonstrating that β-conglycinin had greater affinity for 2-nonanone than soy protein as well as glycinin. The discrepancy was partly explained by differences in the composition of the soy proteins used and different absorption coefficient applied in estimation of the β-conglycinin concentration.

The thermodynamics of binding for soybean glycinin and β-conglycinin with flavour ligands such as butanal, pentanal, hexanal, octanal, 2- and 3-hexanone, 2- and 5-nonanone, hexanol, and hexane was studied using a headspace technique by O'Keefe et al. (1991a). The number of binding sites and binding constants were greater for glycinin than β-conglycinin for all flavour ligands (including 2-nonanone) at all three temperatures, namely 5 °C, 20 °C, and 30 °C, in disagreement with the results from Damodaran and Kinsella (1981b) and O'Neill and Kinsella (1987). O'Keefe et al. (1991a) also reported that affinity for aldehydes increased with increasing chain length for glycinin, whereas it remained constant for β-conglycinin.

Binding affinities of hexanal to soy glycinin and β-conglycinin under different conditions were evaluated by O'Keefe et al. (1991b). In this study, in contrast to the results reported by O'Keefe et al. (1991a), similar binding constants of 270 and 303 M^{-1} were observed for glycinin and β-conglycinin, respectively, and the number of binding sites of hexanal to soy glycinin and β-conglycinin were 108 and 26, respectively, in 0.3 M Tris buffer (pH 8.0). The binding parameters changed by addition of 0.5 M NaCl, 0.02 % NaN_3 or 10 mM β-mercaptoethanol (Table 1.6). However, the reported numbers of binding sites for glycinin and β-conglycinin were much higher than those reported in previous studies, as shown in Table 1.6, and the researchers explained this gap by the differences in the model systems used and more careful approaches to saturation of the system with ligand used in the study (O'Keefe et al., 1991a).

Comparison of the reported literature on binding of flavour compounds to soy proteins reveals some apparently conflicting data, which may be attributed to the differences in methods used (gel filtration technique, equilibrium dialysis method, or headspace analysis), origin and degree of denaturation of soy protein material during preparation and the experimental conditions applied. Most of the research was performed in aqueous systems, where pH and other soluble component

may alter the binding affinity of flavour compounds to soy protein. For example, salts from different buffers and presence of sodium azide may possibly affect the equilibria in the sample evaluated. Inclusion of a reducing agent in some of the studies could significantly influence the conformational structure of soy protein resulting in possible changes in binding constant and the number of binding sites. The concentration of soy protein and the flavour compounds in each study were different, which may lead to different binding behavior of the soy protein. In addition, experimental errors should be considered as binding constants and the numbers of binding sites in some studies were acquired by extrapolation of very small portions of the binding curves, as indicated by O'Keefe (1991b).

In summary, even though the results from these previous studies have shown interaction of flavour compounds with soy protein data, the mode of the interactions has not been clearly elucidated as some conflicting results in terms of binding constant or the nature and magnitude of the protein flavour binding behavior were observed.

1.3.2.3. Soy protein isolate on binding of flavours

The binding affinity of SPI with vanillin in aqueous model system was compared with those of casein and whey protein isolates (Li et al., 2000). It was concluded that SPI demonstrated lower affinity to vanillin than whey protein isolates and casein; binding of vanillin to soy protein might be increased by conformational change, which could be induced by any process causing denaturation of soy protein.

Recently, Zhou et al. (2006) used inverse gas chromatography to examine the binding of selected butter flavour compounds such as diacetyl, hexanal, γ-butyrolactone, and butyric acid in wheat based soda crackers, compared to the binding in soda crackers where 25 % of the wheat was replaced with soy protein isolate. Binding of diacetyl and hexanal was not affected by soy protein isolate in the wheat cracker but increased binding was observed with γ-butyrolactone and butyric acid.

Comparison of the flavour binding properties of SPIs from three different commercial sources produced by different methods i.e., membrane processing versus traditional extraction techniques

was evaluated at different relative humidity levels with hexane, hexanal, and 1-hexanol (Zhou and Cadwallader, 2006). Although the SPIs showed similar flavour binding patterns, differences in absolute flavour binding potential of individual volatile compounds across the SPIs were observed probably due to slightly different lipid content and degree of denaturation resulting from variation in the extent of heat and chemical treatments during the processing.

1.4. Beef flavour

1.4.1. Addition of flavours to soy based products to simulate beef flavour

Beef flavourings have been increasingly required for use in meat analogues as well as for the more traditional use in convenience or processed beef foods. In recent years, soy protein has become popular as a material for meat substitutes with increasing number of vegetarians and the reported hypocholesterolemic effects of the protein (Anderson et al., 1995). Due to the difficulty of removing the indigenous undesirable soy aroma components, attention has turned to masking the residual off-flavour with simulated beef flavours in meat analogue products.

1.4.2. Natural beef flavours

Depending on the temperature and method of cooking, over 1000 volatile compounds in meat have been isolated and identified since the 1960s including various hydrocarbons, aldehydes, ketones, alcohols, carboxylic acids, esters, lactones, furans, pyridines, pyrazines, alkylphenols, thiols, thiophenols, thiazoles, other nitrogen compounds, halogenated compounds, and sulfur-containing compounds (Shahidi et al., 1986). However, the search for a specific character impact compound has not succeeded (Imafidon and Spanier, 1994). Chang and Peterson (1977) reported lactones, non-aromatic heterocyclic compounds, acyclic sulfur-containing compounds, and aromatic heterocyclic compounds as important contributors to beefy aroma notes. In their studies on the neutral fraction of roast beef, Min et al. (1977 and 1979) suggested that lactones, substituted aromatics, furans, and sulfur-containing compounds contribute to roast beef flavour. The review by MacLeod et al. (1981) described more than 450 compounds identified from cooked beef but no single character compound was reported to be uniquely responsible for cooked beef aroma. Even though some compounds have been thought to contribute more than others, it seems to be very difficult to reconstitute the beef flavour by combination of a few

compounds. Shahidi et al. (1986) concluded that, in contrast to fruits or chocolates, a particular class of compounds did not in itself result in the meat flavour and that a number of volatiles of different chemical classes existing in specific quantitative proportions were responsible for the meat flavours. The researchers proposed several reasons for the difficulty in obtaining a comprehensive understanding about the contribution of flavour compounds to cooked beef aroma such as the non-existence of a true character impact compound, either a single compound or a particular class of compounds, qualitative rather than quantitative nature of the reported studies, either too small or not easily assessable threshold values for some flavour compounds, and possible involvement of additive, antagonistic, and synergistic effects.

Generally, beef flavours are derived from the complex interactions of flavour precursors such as amino acids, peptides, sugars, thiamine, metabolites of nucleotides, lipids and products of lipid oxidation (Imafidon and Spanier, 1994). In particular, the Maillard reaction has been known to be important to cooked meat flavour and several research studies have been done to develop meat-like flavour by Maillard reaction with various amino acids and sugars (Ko et al., 1997a and 1997b).

1.4.3. Simulated meat flavours

Although there have been various types of simulated meat flavourings such as simply blended spices (Hill, 1973), meat extracts from a byproduct from the corned beef industry (Pyke, 1975), hydrolyzed vegetable protein (HVP) by enzymatic, alkaline or acid hydrolysis (Prendergast, 1974) and hydrolyzed yeast by autolysis with proteolytic enzymes naturally present in the yeast (Cogman and Sarant, 1977), the most common types are thermally produced simulated meat flavours, the so called "reaction product" meat flavours (Wilson, 1975). Generally, cooked beef flavours are obtained by heating several amino acids with a reducing sugar i.e. by the Maillard reaction. In particular, the reaction of cysteine as a "sulfur-donor" compound with a reducing sugar was reported to be important (May, 1974). As individual amino acids are relatively expensive, protein hydrolysates which contain free amino acids, peptides, nucleotides, reducing sugars, carbonyl compounds, and sulfur compounds, have been used to produce beef flavours. The time and temperature of the reaction are critical for producing different flavours and there have been a great number of "reaction product" patents (MacLeod et al., 1981).

1.5. Analysis of aroma compounds

1.5.1. Adsorption of aroma compounds - Headspace solid phase microextraction

Solid phase microextraction (SPME) is a sample preparation technique that has been gaining popularity in recent years. Traditionally, liquid-liquid extraction, solid phase extraction, supercritical fluid extraction, static headspace sampling or dynamic headspace (purge-and-trap) methods have been used to extract and concentrate volatile compounds for analysis by GC, but one or more drawbacks of each of the sample preparation method have been reported such as high cost, multi-step preparation, low sensitivity, prolonged extraction time, artifact formation or solvent contamination (Braggins et al., 1999). Compared to the previous methods, the SPME technique has been regarded as a simple, rapid, and economical method requiring no solvent (Yang and Peppard, 1994). It is a useful technique to isolate volatile components from a sample matrix, purify and concentrate the analytes, in which a fused silica fibre coated with a polymeric organic liquid is exposed into the sample headspace. The extracted volatiles in the coating are transferred to analytical equipment such as GC or HPLC for desorption and analysis.

Volatiles are captured by SPME based on the theory of equilibrium partitioning of the analytes between extraction medium i.e. the solid phase of SPME, and sample matrix (Zhang and Pawliszyn, 1993). At equilibrium, the amount of analyte absorbed by a liquid coating is directly related to its concentrations in the sample

$$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + V_s}$$

where n is the mass of an analyte absorbed by the coating; V_f and V_s are the volumes of the coating in the fibre and the sample, respectively; K_{fs} is the partition coefficient of the analyte between the coating in the fibre and the sample matrix; and C_0 is the initial concentration of the analyte in the sample (Zhang et al., 1994). This equation shows the linear relationship between the amount of analytes absorbed by the fibre coating and the initial concentration of the analytes. In headspace SPME, the driving force for analytes to transfer from sample matrix to fibre coating

involves three phases, which are matrix, headspace, and coating. In the case of aqueous samples, the headspace/water partition coefficients (K_{hs}) for most compounds are directly related to Henry's constants, determined by volatility and hydrophobicity of the compounds. The sensitivity of headspace SPME was reported to be almost the same as that of direct SPME (Zhang et al., 1994). When ion trap mass spectrometry was used, the detection limits of the headspace SPME and direct SPME techniques were reported to be at the 1 ppt level (Zhang and Pawliszyn, 1993). Agitation of the matrix, reduction of headspace volume, or temperature increase of samples can be employed to shorten the equilibration time for less volatile compounds.

Originally, the SPME technique was developed for analysis of pollutants in environmental water samples, by immersing the SPME fibre in an aqueous sample (Arthur and Pawliszyn, 1990; Arthur et al., 1992). It has also now been applied to flavour analysis, by employing headspace SPME sampling in foods such as cheese (Lecanu et al., 2002), edible oils (Steenson et al., 2002), coffee (Akiyama et al., 2003), ginger (Shao et al., 2003), kimchi (Lee et al., 2003), and sweet wines (Rodriguez-Bencomo et al., 2003).

1.5.2. Identification of aroma compounds

A wide array of techniques using a variety of equipment has been developed to isolate and identify aroma components in food commodities and the usage and applications are well established. A great deal of research on food flavour has been done with gas chromatography (GC) in conjunction with various detectors including flame ionization detector (FID) and mass selective detector (MSD) to identify volatile compounds in food matrix. With the increased sensitivity and precision of GC instruments along with development of techniques for adsorption of volatiles from food, a great number of volatile compounds have been listed as aroma components in foods and beverages (Mistry et al., 1997).

1.5.3. Detection of aroma compounds - gas chromatography-olfactometry (GC-O)

Even though the location of peaks in GC chromatograms gives us a clue to identify volatiles in the foods, the areas of the peaks do not necessarily reflect the aroma intensity of the foods. It is difficult to decide the relative importance of each aroma component in terms of relative contribution to the overall flavour of food as key aroma compound(s). It is also not a

straightforward process to select a few specific aroma chemicals responsible for the characteristic flavour notes including off-flavour, existing at very low concentration and/or with low odour thresholds in foods.

In that sense, GC analysis in conjunction with an olfactometric technique can be a useful tool to detect potent odour-active components from a complex mixture, which may contribute to the characteristic aroma of a given food. Several GC-olfactometry (GC-O) methods have been reported, which can be divided into three categories, namely, dilution methods, intensity methods, and detection frequency methods.

1.5.3.1. Dilution methods

The two most commonly used techniques of the dilution methods are aroma extract dilution analysis by Grosch (1993) and CHARM analysis by Acree et al. (1984).

1.5.3.1.1. Aroma extract dilution analysis (AEDA)

In AEDA, volatile compounds in the sample extract are isolated though an appropriate GC column and analyzed, more specifically, sniffed in the olfactometric detector (sniffing port of GC) generally in combination with a FID detector. The extract is subjected to stepwise serial dilution, typically in a 1:2 or 1:3 series, to be sniffed by panelists. Each panelist is asked to perceive odour components emerging in GC effluents and to provide a sensory descriptor for each perceived aroma. Subsequent analysis of each dilution in the series is performed until the odourants of interest cannot be perceived in the sniffing port. The results can be expressed as flavour dilution (FD) factor, which is the highest dilution value where the odourants can be still detected by GC-O. For instance, if a two times dilution series was conducted and an odourant was detected at the sixth dilution but not detected at the seventh dilution, the FD factor for the compound would be 2^6=64. The flavour profile of a sample in GC-O can be presented in a FD chromatogram consisting of the logarithm of the FD factor on the Y axis and the Kovats retention index (RI) on the X axis (Figure 1.4). The retention index of each odourant can be calculated through a subsequent experiment where a series of *n*-paraffins is analyzed under identical conditions as the odourant analysis, and retention times of paraffins are converted to retention indices according to their carbon number. For example, retention time of paraffin with 6 carbons

is converted to 600 as retention index.

AEDA has been reported as a useful method to screen potent odourants in sample. However, this technique is very time consuming (Mistry et al., 1997) as several dilutions have to be analyzed to obtain FD factors of odourants. Therefore, it can be a big obstacle to perform duplicate or triplicate analyses or to check reproducibility for a considerable number of panelists. In addition, Abbott et al. (1993) observed the "gaps" in the coincident responses i.e. inconsistent responses for a series of dilutions of the beer samples for 4 out of the 6 panelists in the study; a panelist did not detect an aroma compound at a particular dilution but detected it again at a higher dilution.

1.5.3.1.2. Combined Hedonic Aroma Response Measurements (CHARM) analysis

Like AEDA, the CHARM (Combined Hedonic Aroma Response Measurements) analysis is a dilution method based on odour-detection threshold, requiring several injections of stepwise dilutions of the original extract until odour-active compounds are no longer detected. In CHARM analysis, panelists are asked to press a button when an odour is perceived and to hold it down until the characteristic of the odour changes or disappears. The analysis can be conducted in conjunction with a commercially available system called CharmAnalysis™ along with Charmware™ software. The final results can be presented in a Charm chromatogram, which is created by accumulating the areas of square-shaped peaks generated from samples in all dilution series at a given retention index (Figure 1.5). Summed peak areas can be plotted with dilution value (Y axis) and retention index (X axis) and the areas under the curve are referred to as "charm values". Consequently, odour intensity is measured over the whole analysis time in CharmAnalysis and expressed as log "charm value", which refers to the area under the curve, vs. Kovats retention index while maximum odour intensity for each odour compound at a certain retention index is measured in AEDA and presented as log FD factors vs. Kovats retention index.

1.5.3.2. Intensity methods: Osme

While AEDA and CharmAalysis have been used as screening tools for ranking odour-active compounds in foods based on detection thresholds of odour components, the Osme method has been developed by McDaniel and co-workers (Miranda-Lopez et al., 1992) to directly measure odour intensity of a compound perceived in sniffing port. Therefore, theoretically, Osme needs

only one injection per sample if panels are well trained. In the Osme method, a group of panelists is used to rate aroma intensity of odour components in the undiluted extract eluting from GC-O by using a computerized time-intensity device with 16 point scale, which would provide an aromagram called an osmegram, in which Y axis and X axis represent average intensity by panels and Kovats retention index, respectively (Figure 1.6). The panelists are also asked to describe the sensory characteristic of each odour compound, which is recorded. However, very high variability of intensity evaluations within and between panelists (Guichard et al., 1995) as well as day-to-day variation in sensitivity (Pollien et al., 1997) were reported, indicating the importance of panel training in the Osme method.

1.5.3.3. Detection frequency method

Detection frequency methods were originally developed by Pollien et al. (1997), in which sniffing runs are replicated by a group of panelists (minimum 6-8, ideally 8-10 assessors). Panelists are asked to evaluate the effluents of the undiluted extract and record by a computerized device the beginning and the end of elution for each odour compound. In this method, the number of panelists detecting an odour compound at a certain retention index is employed to quantify the aroma intensity of the compound, rather than the dilution values measured in ADEA and CharmAnalysis or the perceived intensity measured in Osme. An aromagram of odour compounds is obtained by plotting the detection frequency (DF) or nasal impact frequency (NIF) on the Y axis and the Kovats retention index on the X axis (Figure 1.7). A value of 100 % DF or NIF means detection by all panelists. Satisfactory repeatability between independent panels was reported to be achieved without any panel training before analysis (Pollien et al., 1997).

Comparison of the three GC-O methods, which were AEDA (dilution method), Osme (intensity method), and olfactometry global analysis (detection frequency method), was performed by Le Guen and co-workers (Le Guen et al., 2000) to evaluate the main impact odourants of cooked mussels. The results from the three olfactometric methods were very similar and significantly positively correlated (p values ≈ 0.00001).

1.6. Analysis of structural properties of SPI

1.6.1. Sulfhydryl and disulfide groups

The most widely used method to determine the content of SH and SS groups in proteins is Ellman's method (Ellman, 1959), in which SH groups in proteins react with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) to produce 5-thiobis(2-nitro)benzoic acid (TNB), which has an extinction coefficient of 13,600 $M^{-1}cm^{-1}$ at 412 nm. To analyze the total SH groups in proteins, denaturants are used to expose or make the buried SH groups accessible (Beveridge et al., 1974). For total content of SS+SH groups in proteins, the SS bonds are first cleaved by the excess sodium sulfite in the presence of guanidine thiocyanate or sodium dodecyl sulphate (SDS) as a denaturant, and the liberated thiols can be measured by subsequent reaction with disodium-2-nitro-5-thiobenzoate (Thannhauser et al., 1984)

The sulfhydryl (SH) group has been regarded as the most reactive and important functional group in various proteins as it affects many characteristics of food proteins including emulsifying activity, foaming, whipping, gelation, solubility, swelling, water binding, antioxidant action, bread baking quality, and surface chemistry of food proteins (Owusu-Apenten, 2005). Cysteine residues with SH groups are found in almost all the storage and structural proteins while disulfide bond (SS) associated with cystine residues are also observed as essential structural units in most proteins (Friedman, 2001).

A broad range of SH+SS contents of soy proteins have been reported. Nakamura et al. (1984) reported the average values of surface, internal, and total SH contents of glycinin from five soybean cultivars as 0.6, 1.3, and 1.9 mole SH/mole glycinin, respectively. Total SH contents of SPI in buffer at pH 8 and pH 3 were reported to be 0.29 and 5.4 μmole/g protein, respectively, and these values were decreased with high pressure treatment between 400 and 600 MPa (Puppo et al., 2004). In addition, total SH content of SPI was reported as 8.3 μmole/g protein and it was increased to 10.1 μmole/g protein with addition of the antioxidant Tenox 22, while the total SH + SS content of 52.9 μmole/g protein was decreased to 43.9 μmole/g protein (Boatright and Hettiarachchy, 1995).

1.6.2. Surface hydrophobicity

Surface hydrophobicity (S_0), which can be measured using hydrophobic fluorescent probes as the initial slope of relative fluorescence intensity versus protein concentration, should be distinguished from total hydrophobicity, which is the calculated average hydrophobicity of a protein multiplied by the number of residues in the molecule. Several methods to assess hydrophobicities in proteins have been developed aside from using hydrophobicity scales of the constituent amino acids e.g. partition methods where partition coefficients of the proteins were measured from the solubility ratio between a polar and an immiscible nonpolar solvent, binding methods with hydrophobic ligand, and spectroscopic methods measuring either intrinsic fluorescence intensity of aromatic amino acids or fluorescent probes bound to proteins (Li-Chan, 1991). Many anionic fluorescent probes such as 1-anilinonaphthalene-8-sulfonate (ANS) and cis-parinaric acid (CPA) have been widely used, which have low quantum yield of fluorescence in aqueous solution but amplify upon binding to accessible hydrophobic regions of proteins. However, Alizadeh-Pasdar and Li-Chan (2000) reported that interpretation of measured hydrophobicity based on these anionic probes was not easy due to the influence of electrostatic interactions between anionic probe and protein under various pH conditions. They suggested the use of uncharged fluorescent probe, 6-propionyl-2(N,N-dimethyl-amino)naphthalene (PRODAN) based on the comparison of surface hydrophobicity of proteins at various pH using those different probes i.e. ANS, CPA, and PRODAN.

S_0 is regarded as one of the important parameters used to investigate the hydrophobic nature relating to functionality of proteins including soy protein since hydrophobic regions at the protein surface have been suggested as the main structural factor governing the functional properties of food proteins by many researchers (Hayakawa and Nakai, 1985; Molina et al., 2001; Molina Ortiz et al., 2004; Rickert et al., 2004). For instance, emulsion stability correlated linearly with the surface hydrophobicity of glycinin at each ionic strength (0.01, 0.1, and 0.5) while foaming power correlated curvilinearly with the surface hydrophobicity implying influences by other factors (Matsudomi et al., 1985a). The reported S_0 values for SPI and SPI treated by mild acid treatment with 0.05 N HCl, enzymatic deamidation with chymotrypsin, or heat treatment at 100 °C for 10 minutes measured using ANS were 37, 143, 250, and 1025, respectively (Boatright and

Hettiarachchy, 1995; Li-Chan, 1991; Molina Ortiz et al., 2004; Rao et al., 2002). The S_0 values of soy protein, β-conglycinin, and glycinin by CPA were measured to be 105, 99, and 44, respectively (Kato et al., 1987; Matsudomi et al., 1985b). Generally, the variability of the S_0 values previously reported in the literature is probably due to differences of SPI source, processing, purity or composition, and/or diverse analysis conditions in terms of pH, ionic strength, and temperature in addition to different fluorescent probes or methods for surface hydrophobicity.

1.6.3. Raman spectroscopy

1.6.3.1. Characterization and application of Raman spectroscopy

Raman spectroscopy has proved to be a practical tool to investigate the relationship between structure and function as the Raman effect depends on interactions between atomic positions, electron distribution and intermolecular forces (Carey, 1999). In Raman spectroscopy, molecules are irradiated by intense laser beams in visible, UV or near infrared (NIR) region (ν_0) and excited up to a "virtual state", which is between the ground state and the first excited electronic state (Figure 1.8). Although most of the excited molecules return to the original energy level, which is called Rayleigh scattering, a very small fraction of photons arrives at different vibration energy levels from the excitation laser, which is known as the Raman effect. Therefore, vibrational frequency (ν_m) is measured as a shift from the incident beam frequency in Raman spectroscopy, and depends on the chemical structure of the molecules responsible for the scattering (Ferrato and Nakamoto, 1994).

Although both Raman and IR spectroscopies provide information on vibrational bending and stretching modes of molecules, there are unique features to each spectroscopic technique. Some particular vibrations are intrinsically strong in Raman spectra while weak in IR. In general, strong vibrations are observed in Raman if the bond is non-polar (C≡C, C=C, C–C, and S–S) whereas strong vibrations are shown in IR if the bond is polar (O–H and N–H) and stretching vibrations are commonly stronger than bending vibrations in Raman spectra (Ferraro and Nakamoto, 1994). Due to a strong interfering band from water, generally dry or non-aqueous samples are applied in IR but Raman spectra of samples in aqueous solution can be analyzed without major interference as water is a weak contributor to Raman signal (Li-Chan, 1996). Another great advantage

compared to other spectroscopic methods is that Raman spectroscopy does not need optically clear samples, which can be aqueous or non-aqueous liquids, gels, films, powders, crystals or even gasous state, and also only a small sample area is required as the diameter of the laser beam is only 1-2 mm. In addition, Raman spectra can be obtained with samples such as hygroscopic or air-sensitive component, simply placed in sealed glass tubing, which is not possible in IR spectroscopy as IR radiation is absorbed by glass tubing (Ferraro and Nakamoto, 1994).

However, Raman spectroscopy has several disadvantages. Compared to strong Rayleigh scattering, Raman scattering is inherently weak as only one out of each million photons experiences such energy shift, requiring relatively high concentration of analyte or considerable excitation laser power. Recent improvement in detectors and optical filters has minimized the limitation (Reipa, 2005). Another frequent problem is the possible interference by fluorescence in some samples, which sometimes overlap with the Raman scattering signal. Fluorescence can sometimes be alleviated with cautious sample purification or photo bleaching before data acquisition (Reipa, 2005). The limitation due to the background fluorescence can be also resolved by using a newer technique known as Fourier-transform (FT) Raman spectroscopy that uses near-infrared (NIR) excitation with the Nd:YAG laser line at 1064 nm; this technique also offers improved resolution, shorter spectral acquisition time, and improved signal to noise ratios over conventional Raman spectroscopy (Li-Chan et al., 2002).

1.6.3.2. Protein structure analysis using Raman spectroscopy

Raman spectroscopy has been reported as a valuable tool to understand the secondary structure of proteins and reveal the information of protein side chains of amino acid residues such as tryptophan, tyrosine, phenylalanine, and sulfhydryl groups. Structural changes of various food proteins have been investigated using Raman technique such as SPI, spray-dried egg white and whey protein isolate (Zhao et al., 2004a and 2004b), whey protein (Nonaka et al., 1993), cod myosin (Careche and Li-Chan, 1997), oat globulin (Ma et al., 2000), cod collagen (Badii and Howell, 2003), red bean globulin (Meng et al., 2003), yam proteins (Liao et al., 2004), hake muscle protein (Herrero et al., 2004). In addition, interaction of trehalose with egg white lysozyme (Belton and Gil, 1994), interactions of lysozyme with whey proteins (Howell and Li-Chan, 1996), and interaction of lysozyme with corn oil (Howell et al., 2001) were also examined

using Raman spectroscopy.

The frequency and the relative intensity in Raman spectra provide information on vibrational motions of various amino acid side chains and –CO–NH– polypeptide back bone, which are sensitive to chemical changes and the surrounding microenvironments. The side chains of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine demonstrate characteristic Raman bands at 760 cm^{-1}, 850/830 cm^{-1}, and 1003 cm^{-1}, respectively. The changes in band intensity of tryptophan or the ratio from tyrosine doublet are useful to monitor the polarity of the microenvironment, or hydrogen bond involvement. The peak intensity of phenylalanine is not dependent on microenvironment and hence is often used as an internal standard. The C-H stretching and bending mode of aliphatic amino acid residues shown at 2800-3000 cm^{-1} and 1440-1465 cm^{-1}, respectively, can be applied to monitor hydrophobic interactions between aliphatic residues. In addition, disulfide and sulfhydryl groups may be detected in bands near 500-550 cm^{-1} and 2550-2580 cm^{-1}, respectively (Li-Chan, 1996). Among several distinct vibrational modes of –CO-NH- amide or peptide bond, amide I (near 1660 cm^{-1}) and amide III bands (near 1240 cm^{-1}) are the most powerful for the investigation of secondary structure and conformation of the protein molecule containing the propensity of α-helix, β- sheet, unordered and turn structures (Li-Chan et al., 2002). Williams and Dunker (1981) reported that Raman spectra in amide I region were useful to determine the detailed and reasonably accurate estimates of secondary structure of proteins by demonstrating less than 6 % absolute difference between X-ray and Raman estimates of the secondary structure of 17 protein samples.

Determination of the extent of acetylation in 3 protein samples (SPI, spray-dried egg white, and whey protein isolate) by Raman spectroscopy was investigated (Zhao et al., 2004a). A new C=O stretching vibration at 1737 cm^{-1} appeared during acetylation, which was used to quantify the extent of modification. Correlation coefficients obtained by linear regression analysis of the calibration curves for the Raman intensity ratio (1737 cm^{-1}/1003 cm^{-1}) to the extent of O-acetylation in SPI, egg white, and whey protein were 0.9984, 0.9979, and 0.9989, respectively. In addition, while random coils and β-sheet at 1246 cm^{-1} and 1667 cm^{-1} were reported to be major secondary structures in unmodified SPI, protein denaturation and dissociation at the initial stages of modification and transformation from random coil to β-sheet structure at the later stages of

acetylation were speculated according to the Raman spectral data. A similar approach was used to examine the effect of chemical modification of proteins with succinic anhydride (Zhao et al., 2004b). In the study, conformational changes such as transition of ordered into disordered structures, relocation of tryptophan residues from interior hydrophobic microenvironment to less hydrophobic exterior region, and conformational shift of cystine residue were reported.

1.7. Hypotheses

1. Changes in releasing of individual volatile compounds in simulated beef flavour (SBF) from soy protein isolate (SPI) can be monitored effectively by using gas chromatographic techniques coupled with headspace-solid phase microextraction (HS-SPME).

2. Reduction in the perceived aroma intensity of SBF when added to a soy-based matrix results from binding of aroma impact compounds to SPI.

3. Aroma intensities of volatile compounds in SBF, which are results of perception by the human nose, can be depicted using gas chromatography-olfactometry.

4. Aroma intensities of SBF-SPI mixtures, as evaluated by gas chromatography and descriptive analysis, are affected by the structural properties of the proteins, which can be altered by addition of ingredients through changes in disulfide bond, hydrogen bond, or hydrophobic interaction.

5. Structural changes in SPI induced by added ingredients can be detected using spectroscopic techniques such as fluorescence and FT-Raman spectroscopy or sulfhydryl group measurement.

1.8. Objectives

The overall objective of this thesis is to elucidate the interaction between SPI and aroma components of SBF. The specific objectives are to:

1. Develop a sensitive and reproducible analytical method for HS-SPME that is able to monitor changes in the headspace composition of samples containing SPI and SBF.

2. Identify the volatile compounds in the headspace of the SBF.

3. Determine aroma impact volatile compounds in the headspace of SBF.

4. Characterize the sensory perception of samples with SPI and SBF in terms of describing the attribute and intensity of the beefy note and explore the correlation between the instrumental chromatographic data and sensory evaluation information.

5. Investigate the changes in flavour binding behavior of the SPI in terms of beef attribute related aroma compounds as affected by added ingredients.

6. Monitor changes in the protein structure in SPI induced by the ingredients to understand the effect of each ingredient on the reduction of SBF binding to SPI.

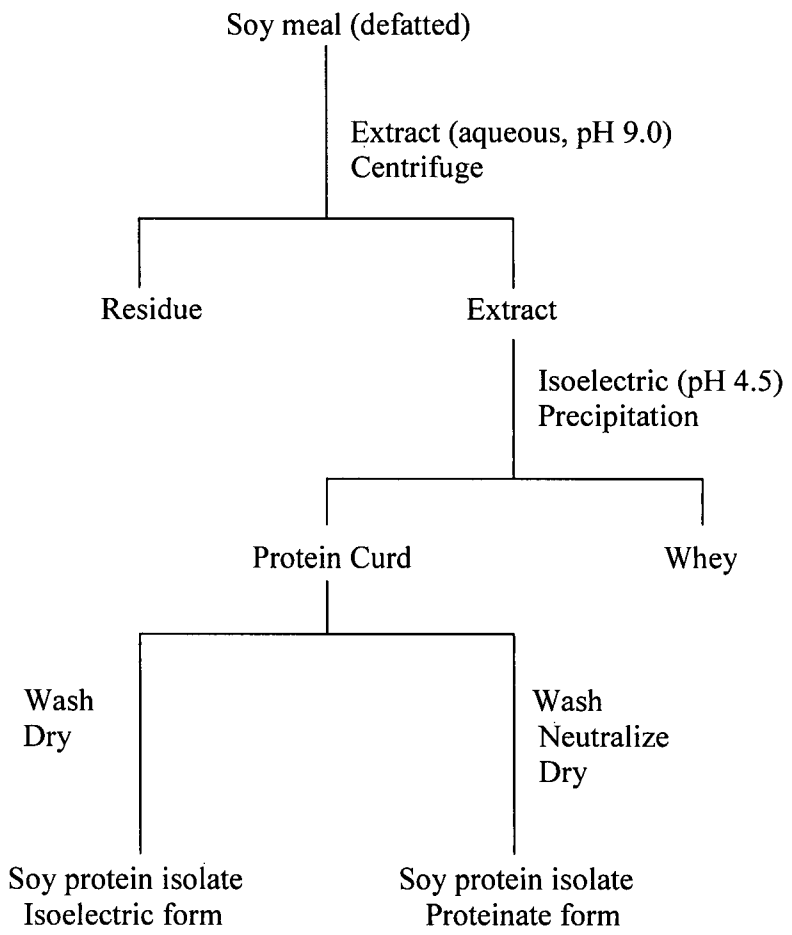

Figure 1.1. Preparation of soy protein isolates from defatted soy meal (Adapted from Hettiarachchy and Kalapathy, 1997).

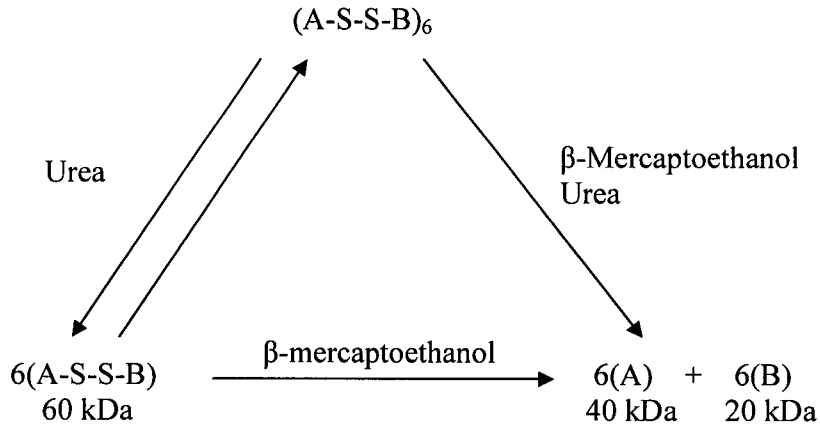

Figure 1.2. Mechanisms for dissociation of soybean glycinin from the hexameric form possessing one disulfide bond (SS) into its subunits and further into the acidic (A) and basic (B) polypeptides (Adapted from Nielson, 1985).

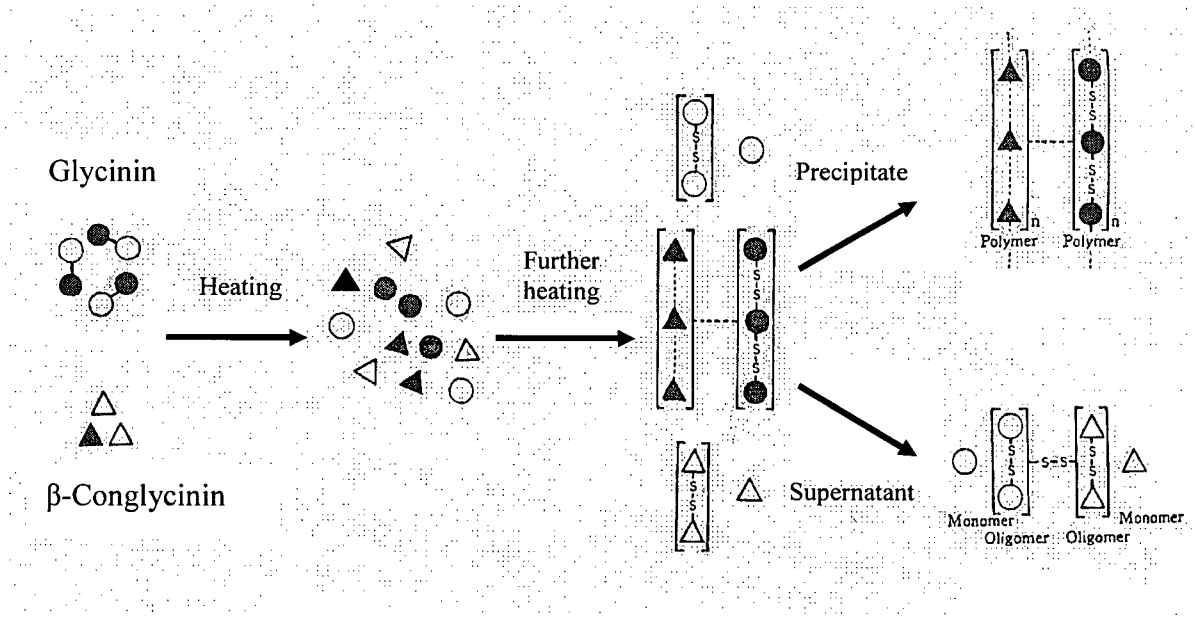

Figure 1.3. Schematic subunit interaction between β-conglycinin (7S globulin) and glycinin (11S globulin) on heating (Adapted from Yamauchi et al., 1991).

-------, interaction by secondary force; S-S, disulfide bond; ○, acidic polypeptide; ●, basic polypeptide; △, α, α′-polypeptide; ▲, β-polypeptide

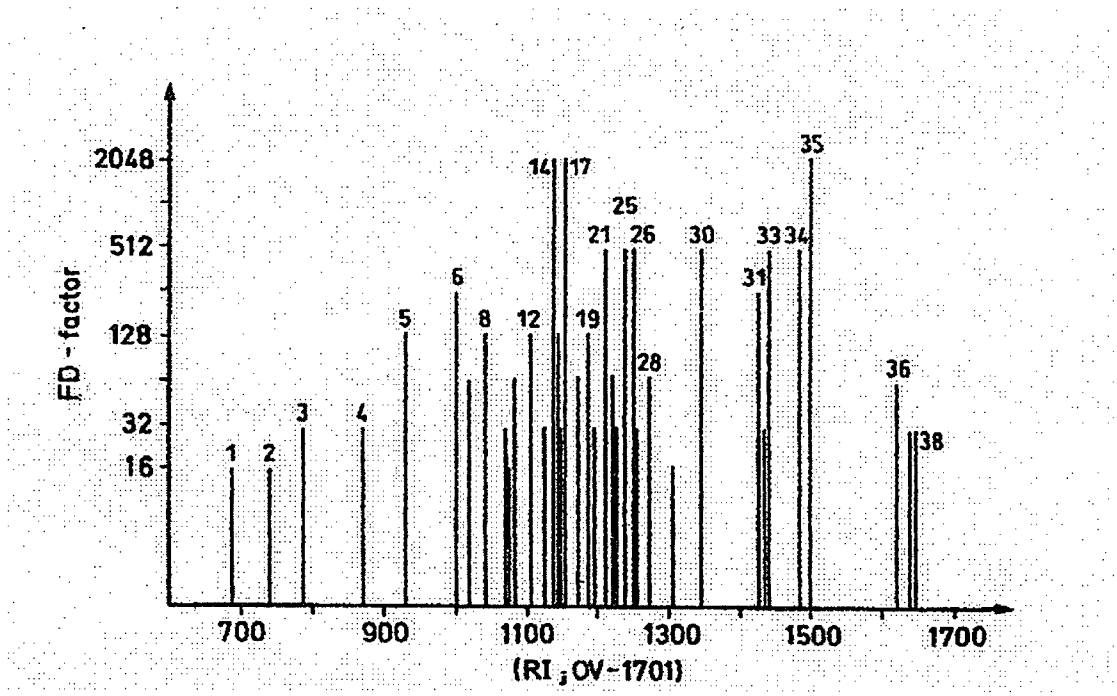

Figure 1.4. Example of flavour dilution chromatogram from aroma extract dilution analysis (Source: Blank, 1997).

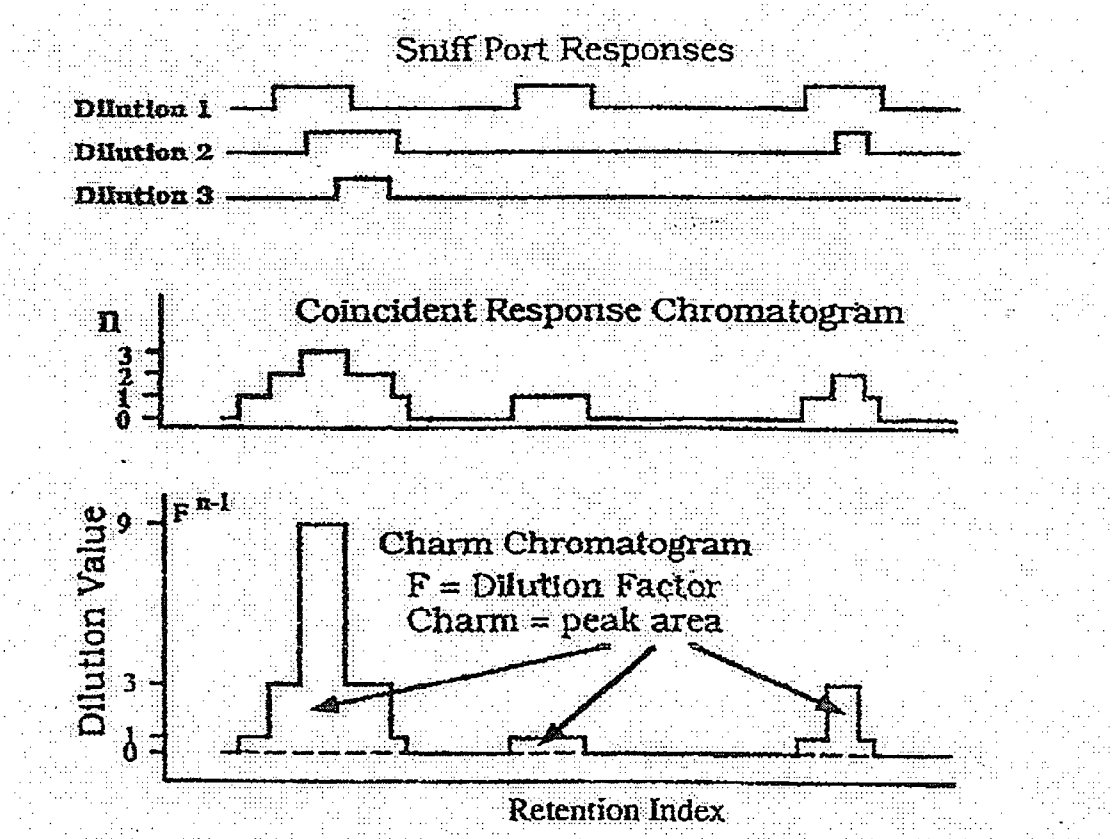

Figure 1.5. Example of Charm chromatogram from CharmAnalysis (Source: Mistry et al., 1997).

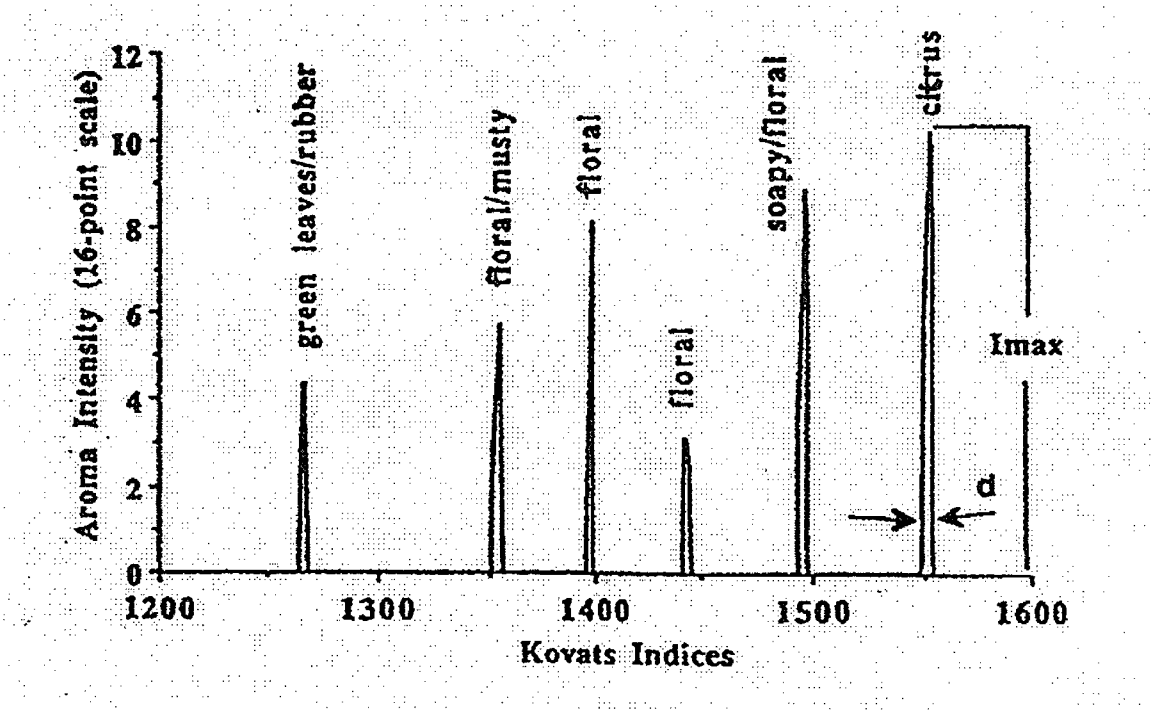

Figure 1.6. Example of osmegram from intensity methods (Source: Blank, 1997).

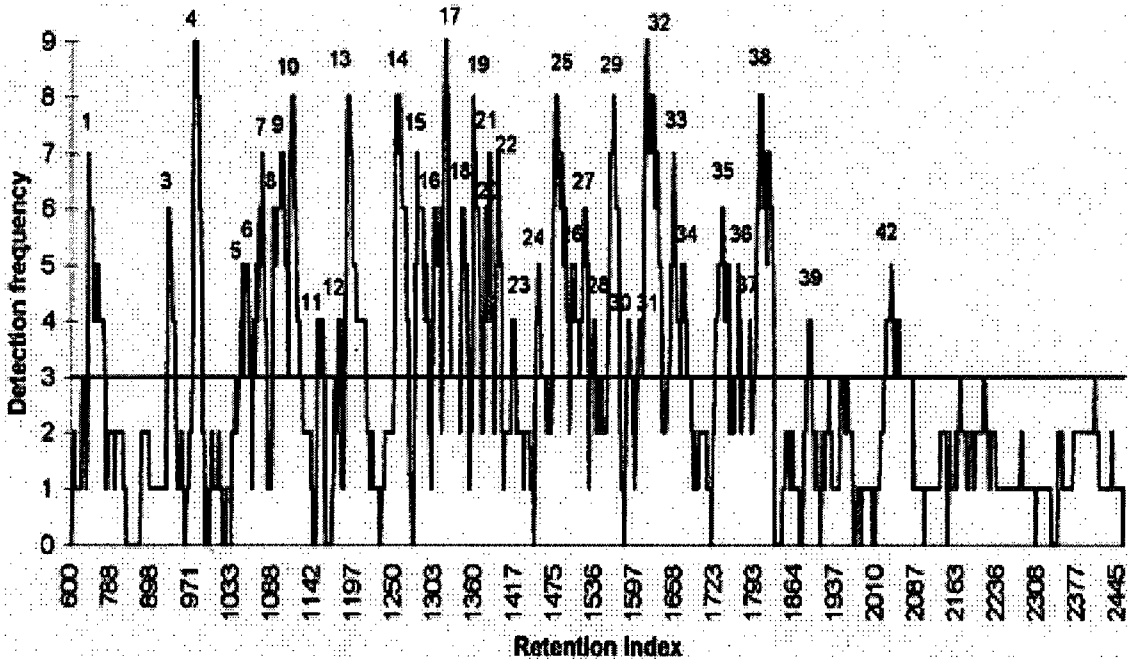

Figure 1.7. Example of aromagram from detection frequency method (Source: Le Guen et al., 2000).

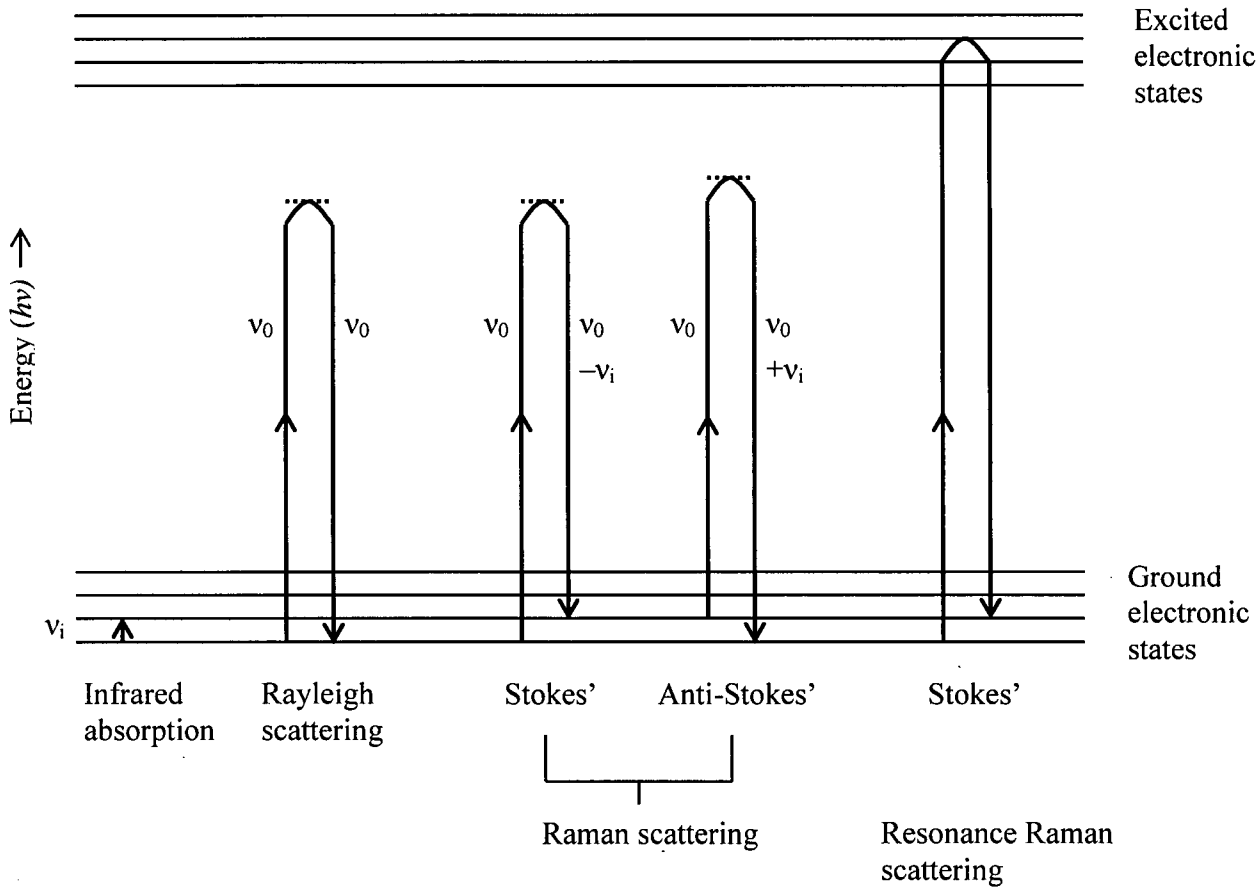

Figure 1.8. The relationships between infrared absorption, Rayleigh scattering and Raman scattering; Dotted line indicates a "virtual state" (Adapted from Li-Chan, 1996).

Table 1.1. Average chemical compositions (% by weight, dry basis) of defatted soy flour, soy protein concentrate, and soy protein isolate (Adapted from MacLeod and Ames, 1988).

Component	Defatted soy flour (%)	Soy concentrate (%)	Soy isolate (%)
Protein	56.0	72.0	96.0
Lipids	1.0	1.0	0.1
Carbohydrate			
Soluble	14.0	2.5	0
Insoluble	19.5	15.0	0.3
Fibre	3.5	4.5	0.1
Ash	6.0	5.0	3.5

Table 1.2. Five major soybean glycinin subunits (G1-G5) and their paired acidic (A) and basic (B) polypeptides (Adapted from Liu, 1997).

Group	Subunit	Structure of subunit	Mw (kDa)
I	G1	$A_{1a}B_2$	58
I	G2	$A_{1b}B1_b$	58
I	G3	A_2B_{1a}	58
II	G4	A_3B_4	62
II	G5	$A_5A_4B_3$	69

Table 1.3. Amino acid composition of β-conglycinin and glycinin of soybean[1] (Adapted from Krishnan, 2005).

Amino acid	β-Conglycinin (%)			Glycinin (%)				
	α′	α	β	*Gy1*[2]	*Gy2*	*Gy3*	*Gy4*	*Gy5*
Alanine	4.2	4.5	5.3	5.7	6.6	5.8	4.3	3.7
Arginine	6.7	7.9	7.0	5.7	6.2	5.6	7.3	6.3
Asparagine	6.5	7.1	8.0	7.8	8.6	7.5	6.1	6.7
Aspartic acid	4.5	4.8	5.1	3.6	3.9	3.4	6.0	4.7
Cysteine	0.8	0.9	0.0	1.7	1.7	1.7	1.1	1.2
Glutamine	8.9	8.3	8.0	10.1	10.9	10.5	9.5	9.5
Glutamic acid	13.6	13.6	8.9	8.6	7.9	8.6	9.9	8.7
Glycine	4.7	4.1	4.3	7.4	7.3	7.3	6.3	7.9
Histidine	3.5	1.4	1.9	1.7	0.9	1.3	2.8	3.2
Isoleucine	4.7	5.3	6.3	5.5	4.9	5.2	3.9	3.7
Leucine	7.2	8.3	9.9	6.9	7.1	6.9	6.9	6.9
Lysine	7.2	6.2	4.8	5.0	3.9	3.9	4.8	3.7
Methionine	0.3	0.2	0.0	1.3	1.5	1.1	0.4	0.6
Phenylalanine	4.5	4.6	6.8	4.2	4.1	5.2	3.0	3.2
Proline	5.7	6.7	5.1	6.1	5.6	6.2	6.9	7.3
Serine	7.4	7.2	7.5	6.7	6.4	7.3	7.3	7.7
Threonine	2.0	1.9	2.4	4.2	3.9	3.9	3.5	3.9
Tryptophan	0.5	0.3	0.0	0.8	0.9	0.9	0.9	0.8
Tyrosine	2.5	2.6	2.9	2.3	2.4	2.4	2.6	3.2
Valine	4.5	4.1	5.8	4.8	5.6	5.4	6.5	7.1

[1]Calculated amino acid composition of mature proteins from the sequences obtained from protein database.
[2]*Gy1, Gy2, Gy3, Gy4, and Gy5* are genes encoding glycinin G1, G2, G3, G4, and G5, respectively.

Table 1.4. Functional properties of soy protein isolates in food systems (Adapted from Hettiarachchy and Kalapathy, 1997).

Functional Properties	Mode of Action	Example of food applications
Solubility	Protein solvating	Beverages
Viscosity	Thickening, water binding	Soups, gravies
Gelation	Protein matrix forming and setting	Meats, curds, cheeses
Cohesion-adhesion	Protein acting as adhesive material	Meats, sausages, baked goods, pasta products
Elasticity	Disulfide linking in deformable gels	Meats, bakery items
Emulsification	Forming and stabilizing of fat emulsions	Sausages, bologna, soups, cakes
Fat absorption	Binding of free fat	Meats, sausages, doughnuts
Flavour-binding	Adsorbing, entrapping, releasing	Simulated meats, bakery items
Foaming	Forming film to entrap gas	Whipped toppings, chiffon desserts, angel cakes

Table 1.5. Thermodynamic constants for the binding of model flavour compounds to soy protein.

Ligand	Type of soy preparation	No. of binding sites (*n*)	Binding constant (K_{eq}, M^{-1})	ΔG, kcal/mol	Reference[1]
1-Hexanal	Native	-[2]	173.4	-3.007	1
1-Hexanol	Native	-	80.3	-2.558	1
Butanal	Native	-	10916	-	2
2-Butanone	Native	-	4975	-	2
1-Butanol	Native	-	2100	-	2
2-Heptanone	Native	4	110	-2.781	3
2-Octanone	Native	4	310	-3.395	3
2-Nonanone	Native	4	930	-4.045	3
2-Nonanone	Partly denatured	4	1240	-4.215	3
2-Nonanone	Succinylated	2	850	-3.992	3
5-Nonanone	Native	4	541	-3.725	3
Nonanal	Native	4	1094	-4.141	3
2-Nonanone	Native	5.5	570	-	4

n: the total number of binding sites per mole of protein

K: the intrinsic binding constant

[1]Reference from 1 - Arai et al. (1970); 2 – Beyeler and Solms (1974; 20 °C, pH 4.5); 3 – Damodaran and Kinsella (1981a; 25 °C); 4 - O'Neill and Kinsella (1987, 25 °C).

[2]- : not reported

Table 1.6. Number of binding sites on glycinin and β-conglycinin for various volatile flavour compounds at three different temperatures (Adapted from O'Keefe et al., 1991a and 1991b).

Compound	No. of binding sites[1]					
	at 5 °C		at 20 °C		at 30 °C	
	glycinin	β-conglycinin	glycinin	β-conglycinin	glycinin	β-conglycinin
Butanal[4]	163±32	30±13	205±73	22±8	171±46	21±46
Pentanal[4]	242±82	28±9	196±24	37±6	193±36	49±36
Hexanal[4]	149±16	23±4	96±6	32±7	108±10	26±10
Octanal[4]	101±5	18±3	76±6	38±5	66±3	59±3
2-Hexanone[4]	165±49	23±3	58±6	24±2	67±7	38±7
3-Hexanone[4]	165±14	35±18	54±5	36±3	60±6	22±5
2-Nonanone[4]	189±33	18±2	71±4	46±42	96±14	30±14
5-Nonanone[4]	245±48	19±2	67±8	44±14	65±31	20±31
Hexanol	79±13	12±3	42±12	8±5	42±10	10±10
Hexane	14±1	45±12	NA[2]	NA	NA	NA
Hexanal[4]	-[3]	-	108±10	26±10	-	-
Hexanal[5]	-	-	38±3	68±8	-	-
Hexanal[6]	-	-	76±3	32±2	-	-
Hexanal[7]	-	-	112±10	32±2	-	-

[1]Mean number of binding sites ± standard deviation per mole of protein from triplicate analysis

[2]No affinity

[3]- : not reported

[4]0.03 M Tris buffer (pH 8.0)

[5]0.03 M Tris buffer with 0.5 M NaCl

[6]0.03 M Tris buffer with 0.02 % NaN_3

[7]0.03 M Tris buffer with 10 mM β-mercaptoethanol

1.9. References

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CHAPTER 2. DEVELOPMENT OF SOLID-PHASE MICROEXTRACTION METHODOLOGY FOR ANALYSIS OF HEADSPACE VOLATILE COMPOUNDS IN SIMULATED BEEF FLAVOUR[1]

2.1. Introduction

A characteristic beef odour is of prime importance to the quality of processed beef products as well as beef analogue products such as vegetable protein based meat substitutes for vegetarian consumers. Among the vegetable protein materials, soy protein has become especially popular as meat substitutes over the past few years due to the reported hypocholesterolemic effects of soy protein resulting in reducing risks of cardiovascular disease (Anderson et al., 1995). However, flavour problems have been a major technical impediment to the increased usage of soy proteins in human foods (Maheshwari et al., 1995). Many studies have been reported on flavour of soy protein products with respect to the "beany" odour (Boatright and Lei, 1999; Lei and Boatright, 2001; Wolf 1975), and on reducing the off-flavour (Inouye et al., 2002; Maheshwari et al., 1995; McDaniel and Chan, 1988). In addition to indigenous undesirable soy aroma components that are difficult to remove, soy proteins may interact with desirable components of added flavour formulations such as simulated beef flavour. The presence of soy protein in aqueous systems has been reported to increase the retention of volatile components in samples (Gremli 1974), and suppression of chicken flavour in a formulated soup at high levels of soy protein has also been reported (Malcolmson and McDaniel, 1987). Moreover, a significant reduction in perceived flavour intensity was observed when simulated beef flavours were added at the supplier's recommended dosage to formulate soy protein based beef substitutes (Yves Veggie Cuisine, 2002, personal communication). However, there is little information on the specific underlying mechanism for these observations, which could provide potential solutions for the problem. To elucidate the interaction between soy protein and simulated beef flavours, it is crucial to use a sensitive and reproducible but simple method for the beef flavour analysis.

[1] A version of this chapter has been published. Moon, S. -Y., and Li-Chan, E. C. Y. (2004). Development of solid-phase microextraction methodology for analysis of headspace volatile compounds in simulated beef flavour. *Food Chemistry*, 88:141-149.

Headspace solid phase microextraction (HS-SPME) is a sample preparation technique that has been gaining popularity in recent years. Volatiles are captured by SPME based on the theory of equilibrium partitioning of the analytes between extraction medium i.e. the solid phase of SPME and liquid or solid sample matrix (Zhang and Pawliszyn, 1993), and can be analyzed by gas chromatography (GC). Traditional methods for the extraction and concentration of volatile compounds for analysis by GC such as liquid-liquid extraction, solid phase extraction, supercritical fluid extraction, static headspace sampling or dynamic headspace (purge-and-trap) methods have one or more drawbacks such as high cost, multi-step preparation, low sensitivity, prolonged extraction time, artifact formation or solvent contamination (Braggins et al., 1999). Compared to the previous methods, the SPME technique has been regarded as a simple, rapid, and economical method requiring no solvent (Yang and Peppard, 1994). Although the SPME technique was originally developed for analysis of pollutants in environmental water samples (Arthur et al., 1992), it has also now been applied to flavour analysis, by employing headspace SPME sampling in various food commodities such as cheese (Lecanuet al., 2002), edible oils (Steenson et al., 2002), coffee (Akiyama et al., 2003), ginger (Shao et al., 2003), kimchi (Lee et al., 2003b), and sweet wines (Rodriguez-Bencomo et al., 2003).

However, it has also been reported that the SPME analysis is drastically affected by several factors such as the nature of the SPME solid phase, adsorption time, adsorption temperature, salt addition, stirring condition, and sample size (Lee et al., 2003b; Rodriguez-Bencomo et al., 2002; Steenson et al., 2002). Generally 'one factor at a time' experiments have been conducted in most of the previous studies to determine the analysis conditions with SPME, but "one factor at a time' designs often overlook interactions among the factors. In contrast, Liu and Yang (2002) used response surface methodology (RSM) coupled with a two-factor central composite rotatable design to study the interaction between adsorption time and adsorption temperature. However, RSM is not the best choice when dealing with multiple factors due to the large number of experiments required. In order to investigate the effects of multiple factors as well as potential interactions between these factors in a time and cost effective manner, fractional factorial design based on Taguchi's orthogonal array can be considered (Arteaga et al., 1994). To date, there has been no report on important factors for optimum adsorption condition to analyze headspace volatile compounds in simulated beef flavour.

As a result, Taguchi's fractional factorial experimental design was proposed in this study to screen significant factors that would have a great impact on adsorption condition for headspace analysis of simulated beef flavour.

Therefore, the objectives of this study were to investigate the conditions that may influence HS-SPME of volatile compounds from simulated beef flavour by means of the Taguchi's method, and to investigate the effects of the factors along with potential interactions between these factors. The achievement of a sensitive and reproducible analytical method that is able to monitor changes in the headspace composition of the flavour sample would be the basis of future research to elucidate the mechanism of soy protein – flavour interactions that may be responsible for suppression of perceived intensity of beef flavour in soy protein products.

2.2. Experimental methods

2.2.1. Materials

The simulated beef flavour (SBF) used in this study was a commercially produced blended flavour ("vegetarian beef type flavour F96x49" from Mastertaste (Arlington Heights, IL), containing maltodextrin, autolyzed yeast extract, natural flavours, onion powder and silicon dioxide). Its beef character is primarily derived from Maillard reaction during roasting of the materials and it has been used in the industry to provide beefy flavour in vegetarian products. The solid phase assembly holder and four commercially available fibres, 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB), 65 μm carbowax/divinylbenzene (CW/DVB), 75 μm carboxen/polydimethylsiloxane (CAR/PDMS), and 50/30 μm stableflex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) were purchased from Supelco (Sigma-Aldrich Canada, Oakville, ON). Before use, each fibre was exposed to a splitless/split injection port under helium flow and conditioned for the recommended time at recommended temperatures according to the manufacturer's instruction (i.e. at 250 °C 30 minutes for PDMS/DVB, at 220 °C 30 minutes for CW/DVB at 300 °C 120 minutes for CAR/PDMS, and at 270 °C 180 minutes for DVB/CAR/PDMS). GC sample vials with 15 mL capacity and polypropylene hole cap with PTFE/silicone septa were purchased from Supelco.

2.2.2. Fractional factorial design

A Taguchi's $L_{27}(3^{13})$ orthogonal array was used to evaluate potentially significant factors affecting the adsorption of SBF compounds onto SPME fibres (Table 2.1). The main effects of 4 factors were investigated: adsorption temperature (A), adsorption time (B), salt concentration (C), and SPME phase (D). The 3 levels selected for each factor were 30 °C, 45 °C, and 60 °C for adsorption temperature, 20, 40, and 60 minutes for adsorption time, 0, 3, and 6 % of added sodium chloride concentration, and 65 μm PDMS/DVB, 65 μm CW/DVB, and 50/30 μm DVB/CAR/PDMS for SPME phase.

In addition to the 4 main factors, 3 interactions were also investigated: adsorption temperature × adsorption time (A×B), adsorption temperature × salt concentration (A×C), and adsorption temperature × SPME phase (A×D). The linear graph used for this experimental design is illustrated in Figure 2.1 and the column assignment and the levels of each factor are shown in Table 2.2. In this design, columns 1, 2, 5, and 8 were assigned to the main factors (A, B, C, and D). Columns 3+4, 6+7, and 9+10 were used to investigate three of the possible interactions between these factors, while columns 11, 12, and 13 were employed for the estimation of the error term. The levels of each factor were selected within the applicable range of the processing for commercial meat substitutes (Ross, 1996) based on the feedback by a development staff at a food company in Vancouver.

2.2.3. Solid-phase microextraction procedure

Flavour stock solution consisting of 10 g of SBF and 40 g of 30 mM Tris-HCl buffer (pH 6.0) was prepared fresh daily and 2.5 g of flavour stock solution and 3 g of buffer solution were mixed in a 15 mL capacity GC sampling vial with a magnetic stirring bar to provide a flavour concentration similar to that of commercial beef flavour products. To examine the salt effect, 0 g, 0.165 g, or 0.330 g NaCl was added to give 0 %, 3.0 %, and 6 % (w/w) added salt concentration, respectively. Since the SBF originally had 20 % salt, this resulted in 1.8 %, 4.8 % and 7.8 % final salt concentration in the samples. The range of salt levels of beef substitute products (500-800 mg/100 g of finished product) was considered to select these salt concentrations. The vial was tightly capped with a polypropylene hole cap with a PTFE/silicone septum. The SPME fibre (either 65 μm PDMS/DVB, 65 μm CW/DVB, or 50/30 μm

DVB/CAR/PDMS) was exposed to the headspace above the sample solution for 20, 40, or 60 minutes at 30 °C, 45 °C, or 60 °C according to the experimental design shown in Table 2.2. Stirring with a magnetic stirring bar was consistently applied for all samples.

2.2.4. Optimization of adsorption time

To study the effect of adsorption time at 60 °C on SPME by 50/30 μm DVB/CAR/PDMS fibre, samples, as prepared as in section 2.2.3, were maintained for 20, 40, 60, 80, 100 or 120 minutes with stirring. All treatments were made in triplicate, and results are expressed as the average and standard deviation values.

2.2.5. Gas chromatography

A Hewlett-Packard 5890 gas chromatograph with flame ionization detector (FID) and a DB-5 analytical fused silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness from J&W Scientific, Folsom, CA) were used for analysis of the volatile compounds. The injection was conducted in a splitless mode for 3 min at 250 °C. The oven temperature was held at 40 °C for 3 min, ramped to 180 °C at the rate of 3 °C/min and then to 260 °C at 10 °C/min, and maintained at 260 °C for 2 min. Helium was used as a carrier gas at a column-head pressure of 12 p.s.i. (1 p.s.i.=6894.76 Pa). The temperature of the FID detector was 280 °C, and 35 mL/min of hydrogen, 350 mL/min of air and 30 mL/min of helium as a make-up gas were used. To increase reliability in terms of number of peaks and peak area, several parameters in the chromatogram were set. Peak width was set to 0.04 and initial threshold was set to 1. The peaks with peak area under 10,000 were not regarded as reliable peaks.

A 0.75 mm I.D. inlet liner was employed to minimize broadening effect, and resulted in decreased peak width compared to a 2.0 mm injection glass liner. The type of injection mode (splitless/split), desorption time (0.5 - 3 min) and desorption temperature (220 °C - 260 °C) along with oven temperature programs were also tested to improve peak shape and sensitivity while reducing carry-over from the previous analysis. The detection of the analytes was improved using splitless mode, 3 minutes as desorption time, and 250 °C as desorption temperature.

2.2.6. Statistical analysis

General linear model of analysis of variance (ANOVA) was performed by using Minitab (version 13.30, Minitab inc. PA USA) to analyze the significance of the 4 main factors, namely, adsorption temperature (A), adsorption time (B), salt concentration (C), and SPME phase (D), and 3 interactions between the factors (A×B, A×C, and A×D). Tukey's multiple-range test was conducted for comparison of mean values of the data at the 95 % confidence level.

2.3. Results and Discussion

2.3.1. Screening of significant factors on the headspace analysis of the beef flavour

The FID responses in terms of total area and number of peaks for the chromatograms of each of the 27 experimental runs are shown in Table 2.2. Typical total ion chromatograms (TICs) are illustrated in Figure 2.2, which demonstrates the variation in both total peak area and number of peaks in the TICs of the headspace volatile compounds in SBF under three different adsorption conditions corresponding to experiment numbers 25, 26, and 27 (Table 2.2).

Table 2.3 shows the results of ANOVA for significance of the main factors and the selected interactions between the factors, on the total peak area and the number of peaks of headspace volatile compounds in SBF. For both total peak area and number of peaks, adsorption temperature and adsorption time were significant factors ($p \leq 0.05$) whereas SPME phase was highly significant ($p \leq 0.001$). Salt concentration did not have a significant effect ($p > 0.05$) within the studied range.

The number of interaction effects that can be examined is limited by the number of columns in a Taguchi's fractional factorial design, and interactions that are higher than second order are assumed not to be important. In this experiment, in addition to the 4 main factors, 3 selected interactions were investigated, which were adsorption temperature × adsorption time, adsorption temperature × salt concentration, and adsorption temperature × SPME phase. These interactions were selected based on previous research results demonstrating that adsorption temperature was one of the most important factors affecting adsorption efficiency by SPME (del Castillo and Dobson, 2002; Diaz et al., 2002; Liu et al., 2002; Steenson et al., 2002). None of the selected

interactions was statistically significant at the 5 % significance level.

2.3.2. Effects of SPME fibres

Based on the response of the total peak area obtained in preliminary experiments (data not shown), 3 types of SPME fibres (i.e. 65 μm PDMS/DVB, 65 μm CW/DVB, and 50/30 μm DVB/CAR/PDMS) were chosen from the 4 fibres mentioned above to conduct the fractional factorial experiment. Among the 3 SPME fibres tested, 50/30 μm DVB/CAR/PDMS fibre coating showed remarkably higher signal response ($p \leq 0.001$) than the other fibres for both total area and number of peaks (Figure 2.3). Figure 2.2 also illustrates the larger total peak area and number of peaks obtained using the 50/30 μm DVB/CAR/PDMS fibre. This fibre has improved stability of coating materials compared to other commercially available fibres (Supelco catalog 2003/2004). A coefficient of variation of 2.9 % was observed for five replicate analyses of the total area counts of the headspace volatiles in beef flavour, using the 50/30 μm DVB/CAR/PDMS fibre for 60 min at 60 °C. In addition, no considerable carry-over from the previous extraction was observed after 3 min desorption in the injector. Therefore, 50/30 μm DVB/CAR/PDMS fibre was selected as the most suitable fibre due to the high sensitivity with good reproducibility as well as durable property of the fibre.

2.3.3. Effects of salt concentration

The addition of salt at concentrations up to 6 % (which is equivalent to 7.8 % of total salt concentration considering the indigenous salt content of the flavour) did not significantly affect either total peak area or the number of peaks of the beef flavour samples as shown in Table 2.3. Generally, the presence of salt has been reported to stimulate adsorption of the volatile components from samples by changing the phase border properties and decreasing the solubility of hydrophobic components in the aqueous phase, the so called "salting out" effect (Yang et al., 1994). However, a salt concentration of 20-30 % (w/v) was suggested to be required to yield an adsorption effect for most flavour compounds (Lee et al., 2003a). Liu et al. (2002) reported that low concentrations of salt in the range of 0 % to 10 % did not affect adsorption efficiency of volatiles such as ethyl isovalerate and isoamyl acetate while adsorption of both compounds significantly increased at 20 % salt. In our study, an upper level of up to 6 % of sodium chloride was added since higher salt concentration would not be relevant in the context of the

type of food systems under consideration, i.e. beef flavoured vegetable products. High concentration of salt is also known to stimulate denaturation of proteins (Cheftel et al., 1985).

2.3.4. Effects of adsorption temperature

As adsorption temperature increased from 45 °C to 60 °C, both total peak area ($p < 0.1$) as well as the number of total peaks ($p \leq 0.05$) in the GC chromatograms of the samples increased while there was no significant difference between 30 °C to 45 °C (Figure 2.3). The increases of volatile compounds can be explained by the fact that higher temperature tends to drive the volatiles from the liquid phase to the gas phase. Diaz et al. (2002) also demonstrated that the adsorption efficiency of brominated analogs in water increased by increasing the temperature up to 50 °C and Steenson et al. (2002) found an increase of volatile compounds in vegetable oils with higher extraction temperature. In this study, 60 °C was proposed as the adsorption temperature for the beef flavour due to the high sensitivity of both total area counts and number of peaks. Furthermore, beef analogue products such as vegetarian hot dogs are frequently served at temperatures close to 60 °C (Yves Veggie Cuisine, 2002, personal communication).

2.3.5. Effects of adsorption time

Table 2.3 shows adsorption time was also one of the significant factors in the analysis of headspace volatile compounds of the beef flavour. The effect of adsorption time on the analysis of headspace volatile compounds is illustrated in Figure 2.3. Even though there were no statistically significant differences between the responses at 20 min and 40 min or at 40 min and 60 min, significant difference was found between 20 min and 60 min ($p \leq 0.05$) in total peak area in addition to the number of total peaks. The increase of volatile compounds with adsorption time was also reported by other research groups (Steenson et al., 2002; Tombesi and Freije, 2002). In contrast, Lee et al. (2003b) reported that the isolation of volatile compounds in kimchi adsorbed onto SPME increased with longer time up to 30 min, while it tended to decrease at times longer than 30 min depending on the adsorption temperature.

Since there was no interaction between factors, subsequent experiments were conducted using 50/30 μm DVB/CAR/PDMS fibre at 60 °C to investigate the adsorption time required to reach maximum total area responses of the samples. Figure 2.4 shows the effect of adsorption time at

60 °C by 50/30 μm DVB/CAR/PDMS fibre on the headspace volatile compounds of the beef flavour. The total area of volatile compounds was increased with longer adsorption times up to 80 min ($p \leq 0.05$) when equilibrium between the headspace of the beef flavour sample and the SPME fibre was reached. However, it was suggested that the SPME sampling time should be no longer than the total GC run time for maximum productivity, because good precision can be accomplished without achieving equilibrium as long as the adsorption time is controlled accurately (Penton, 1999). Therefore, the adsorption time of 60 min was selected, considering a good reflection of transferring of volatile compounds from sample solution to headspace, a suitable analysis time for the routine analysis, and a low coefficient of variation (2.9 %).

2.4. Conclusion

This study has demonstrated the application of a fractional factorial experimental design based on Taguchi's orthogonal array for screening the significant factors in SPME for analysis of the headspace volatile compounds in SBF. Out of 4 main factors and 3 potential interactions researched, adsorption temperature, adsorption time, and SPME phase were the factors that significantly affect the headspace analysis of the SBF in terms of the total peak area and the number of peaks. The selected adsorption conditions for SPME were adsorption by 50/30 μm DVB/CAR/PDMS fibre for 60 minutes at 60 °C. The establishment of this reproducible and representative analytical method by HS-SPME paves the way for ongoing research to monitor changes in the headspace composition of the SBF, to elucidate the basis for the interactions between flavour compounds and soy protein in vegetarian food products.

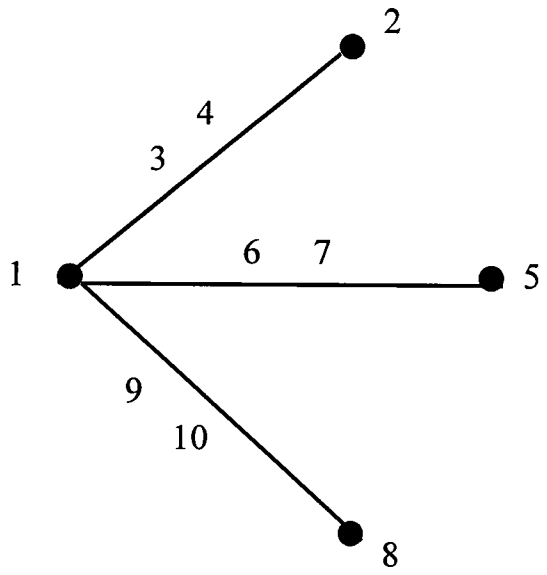


Figure 2.1. The standard linear graph of orthogonal array $L_{27}(3^{13})$ used in this study.

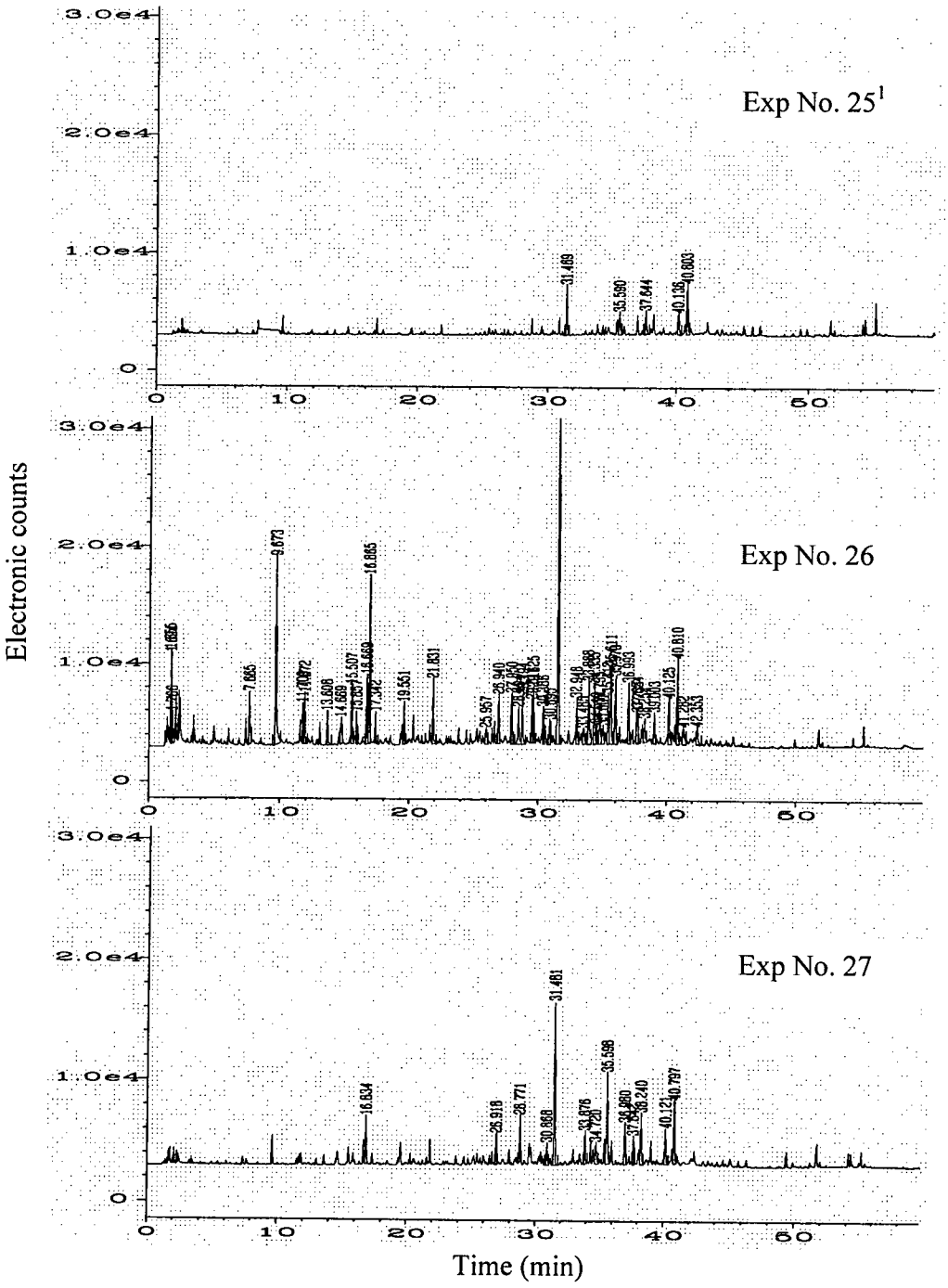
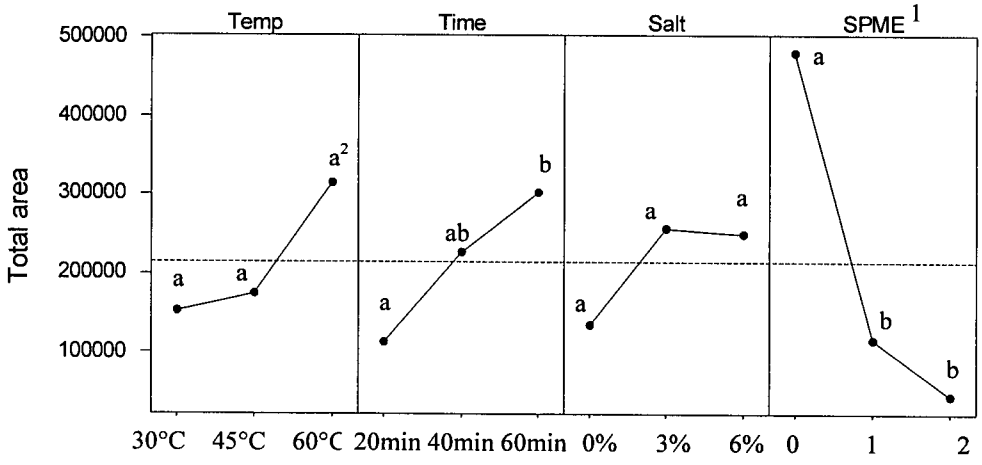
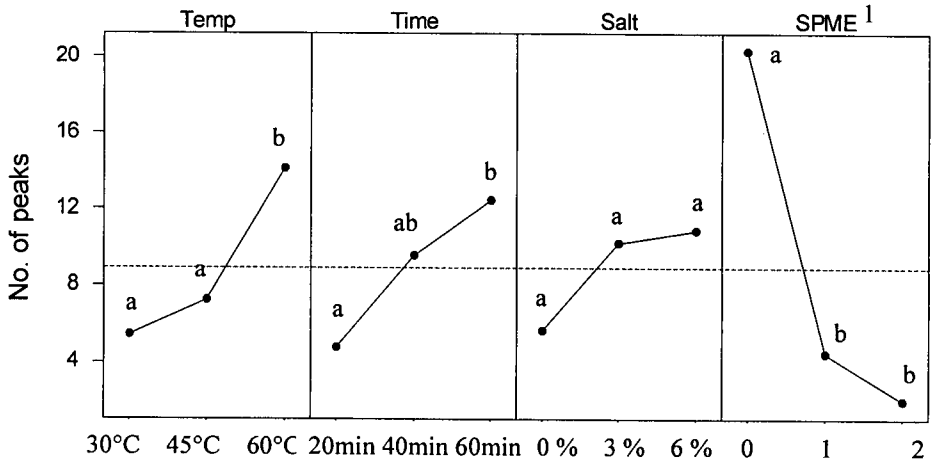


Figure 2.2. Total ion chromatograms (TICs) of headspace volatile compounds in simulated beef flavour using the SPME adsorption conditions described in Table 2.2 for experiment number 25, 26, and 27.

[1]Experiment numbers correspond to those of Table 2.2.



(a)



(b)

Figure 2.3. Effects of adsorption temperature, adsorption time, salt concentration, and SPME phase on (a) means of total area counts and (b) means of the number of peaks obtained in the analysis of headspace volatile compounds in simulated beef flavour.

Different letters (a and b) within each plot indicate significant difference ($p \leq 0.05$).

[1]Levels 0, 1, and 2 of the SPME phase refer to 50/30μm DVB/CAR/PDMS, 65μm PDMS/DVB, and 65μm CW/DVB, respectively.

[2]Different at 10 % significance level ($p = 0.0571$).

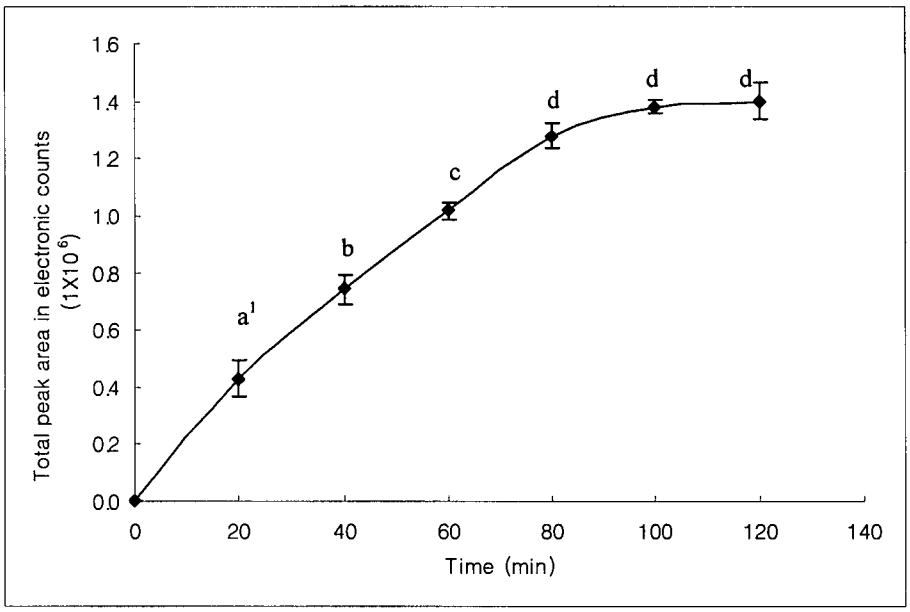


Figure 2.4. Effect of adsorption time on the headspace volatile analysis of simulated beef flavour by 50/30μm DVB/CAR/PDMS SPME at 60 °C. Points are averages from triplicate analyses and error bars are ± standard deviation.

[1]Different letters indicate significant difference ($p \leq 0.05$).

Table 2.1. Column assignment for the 4 factors and 3 interactions in this study based on Taguchi's $L_{27}(3^{13})$ orthogonal array.

Exp. No.	Exp. Order	Column number												
		1	2	3	4	5	6	7	8	9	10	11	12	13
		A	B	A × B	A × B	C	A × C	A × C	D	A × D	A × D	e	e	e
1	17	0	0	0	0	0	0	0	0	0	0	0	0	0
2	19	0	0	0	0	1	1	1	1	1	1	1	1	1
3	24	0	0	0	0	2	2	2	2	2	2	2	2	2
4	23	0	1	1	1	0	0	0	1	1	1	2	2	2
5	2	0	1	1	1	1	1	1	2	2	2	0	0	0
6	27	0	1	1	1	2	2	2	0	0	0	1	1	1
7	18	0	2	2	2	0	0	0	2	2	2	1	1	1
8	8	0	2	2	2	1	1	1	0	0	0	2	2	2
9	7	0	2	2	2	2	2	2	1	1	1	0	0	0
10	1	1	0	1	2	0	1	2	0	1	2	0	1	2
11	20	1	0	1	2	1	2	0	1	2	0	1	2	0
12	22	1	0	1	2	2	0	1	2	0	1	2	0	1
13	25	1	1	2	0	0	1	2	1	2	0	2	0	1
14	5	1	1	2	0	1	2	0	2	0	1	0	1	2
15	6	1	1	2	0	2	0	1	0	1	2	1	2	0
16	13	1	2	0	1	0	1	2	2	0	1	1	2	0
17	12	1	2	0	1	1	2	0	0	1	2	2	0	1
18	26	1	2	0	1	2	0	1	1	2	0	0	1	2
19	9	2	0	2	1	0	2	1	0	2	1	0	2	1
20	3	2	0	2	1	1	0	2	1	0	2	1	0	2
21	14	2	0	2	1	2	1	0	2	1	0	2	1	0
22	15	2	1	0	2	0	2	1	1	0	2	2	1	0
23	21	2	1	0	2	1	0	2	2	1	0	0	2	1
24	11	2	1	0	2	2	1	0	0	2	1	1	0	2
25	16	2	2	1	0	0	2	1	2	1	0	1	0	2
26	4	2	2	1	0	1	0	2	0	2	1	2	1	0
27	10	2	2	1	0	2	1	0	1	0	2	0	2	1

A: adsorption temperature (30 °C, 45 °C, and 60 °C as levels 0, 1, and 2)
B: adsorption time (20 min, 40 min, and 60 min as levels 0, 1, and 2)
C: salt concentration (0 %, 3 %, and 6 % as levels 0, 1, and 2)
D: SPME phase (50/30μm DVB/CAR/PDMS, 65μm PDMS/DVB, and 65μm CW/DVB as levels 0, 1, and 2)
e : error term

Table 2.2. Experimental design based on Taguchi's $L_{27}(3^{13})$ orthogonal array and the measured responses of total area count and the number of peaks in the gas chromatogram for each experimental run.

Exp. No.	Factors[1]				Responses[2]	
	A	B	C	D	Total Area	No. of Peaks
1	30	20	0	DVB/CAR/PDMS	228801	9
2	30	20	3	PDMS/DVB	36325	1
3	30	20	6	CW/DVB	30013	1
4	30	40	0	PDMS/DVB	68431	1
5	30	40	3	CW/DVB	63933	2
6	30	40	6	DVB/CAR/PDMS	369733	15
7	30	60	0	CW/DVB	43944	1
8	30	60	3	DVB/CAR/PDMS	383206	14
9	30	60	6	PDMS/DVB	143972	5
10	45	20	0	DVB/CAR/PDMS	236792	11
11	45	20	3	PDMS/DVB	47968	1
12	45	20	6	CW/DVB	37258	2
13	45	40	0	PDMS/DVB	87752	2
14	45	40	3	CW/DVB	27000	1
15	45	40	6	DVB/CAR/PDMS	432501	19
16	45	60	0	CW/DVB	24796	1
17	45	60	3	DVB/CAR/PDMS	524510	22
18	45	60	6	PDMS/DVB	154745	6
19	60	20	0	DVB/CAR/PDMS	287959	13
20	60	20	3	PDMS/DVB	78910	3
21	60	20	6	CW/DVB	33728	2
22	60	40	0	PDMS/DVB	156345	8
23	60	40	3	CW/DVB	60726	3
24	60	40	6	DVB/CAR/PDMS	777677	35
25	60	60	0	CW/DVB	79574	5
26	60	60	3	DVB/CAR/PDMS	1089718	45
27	60	60	6	PDMS/DVB	271640	13

[1]Factors were assigned to columns as shown in Table 2.1, where A: adsorption temperature (°C), B: adsorption time (min), C: salt concentration (%), and D: SPME phase

[2]Area reject:10,000, initial threshold: 1 and peak width: 0.04

Table 2.3. Analysis of variance of the main factors and the selected interactions between the factors on the total peak area and the number of peaks of headspace volatile compounds in simulated beef flavour.

Source	Degree of Freedom	Total peak area		No. of Peaks	
		F-value	Probability	F-value	Probability
A	2	5.14	0.050*	9.13	0.015*
B	2	5.95	0.038*	6.53	0.031*
C	2	3.10	0.119	3.52	0.098
D	2	36.13	0.000***	43.15	0.000***
A×B	4	1.05	0.455	1.53	0.304
A×C	4	0.56	0.702	0.43	0.780
A×D	4	2.46	0.156	2.82	0.124
error	6	-	-	-	-

*, **, and *** significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ respectively.

A: adsorption temperature, B: adsorption time, C: salt concentration, and D: SPME phase.

2.5. References

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CHAPTER 3. ODOUR-ACTIVE COMPONENTS OF SIMULATED BEEF FLAVOUR ANALYZED BY SOLID PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY AND -OLFACTOMETRY[2]

3.1. Introduction

Generally, fresh raw meat gives off very little odour. However, over a thousand flavour compounds have been reported to be generated during cooking (Warriss, 2000). Among these, about 880 different volatile compounds have been identified in cooked beef (Mottram, 1994), including hydrocarbons, alcohols, phenols, aldehydes, ketones, carboxylic acids, esters, lactones, furans, pyrans, pyrroles, pyridines, pyrazines, oxazoles, oxazolines, thiophenes, thiazoles, thiazolines and other nitrogen or sulfur containing compounds. So far, the postulated primary reactions for beef flavour development on heating include pyrolysis of amino acids and peptides, degradation of carbohydrates, ribonucleotides, thiamin and lipids, and interaction of sugars with amino acids or peptides, as extensively reviewed in the publications by Mottram (1991) and MacLeod (1994). It also has been reported that heating of the lean portions of beef, pork, chicken and lamb resulted in non-species-specific meaty flavour, while heating of the fat in meats, especially the phospholipids and of less importance the triglycerides, led to species-specific flavour of meat (Warriss, 2000).

Beef flavour seems to have been researched more widely than any other meat flavour (Mottram, 1994). Even though a great number of volatile compounds have been reported in beef, only some of them are important in terms of determining the beef flavour characteristics. Therefore, more and more, great effort has been made to find odour-active compounds and furthermore to identify key aroma compounds in beef. 2-Acetyl-2-thiazoline, furaneol, guaiacol, 2-ethyl-3,5-dimethyl pyrazine and 2,3-diethyl-5-methylpyrazine were identified as the character impact compounds for the roasty, caramel-like, burnt and earthy notes in roasted beef (Cerny and Grosch, 1992) and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, 12-methyltridecanal, methional, 3-hydroxy-4,5-dimethyl-

[2] A version of this chapter has been published. Moon, S. -Y., Cliff, M. A., and Li-Chan, E. C. Y. (2006). Odour-active components of simulated beef flavour analysed by solid phase microextraction and gas chromatography-mass spectrometry and -olfactometry. *Food Research International*, 39:294-308.

2(*5H*)-furanone, and 2-furfurylthiol were demonstrated as odour impact volatile compounds in stewed beef (Guth and Grosch, 1993). 2-Methyl-3-furanthiol, 2-acetyl-1-pyrroline, methional, 1-octen-3-one, phenylacetaldehyde, (*E*)-2-nonenal, (*E,E*)-2,4,decadienal, beta-ionone, and *bis*(2-methyl-3-furyl) disulfide were identified as potent odourants with high aroma values from boiled beef (Gasser and Grosch, 1988) and Kerscher and Grosch (1997) reported that 2-furfurylthiol, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, and 2-methyl-3-furanthiol were recognized to be the most potent odourants of boiled beef by aroma extract concentration analysis. In addition, 2-butanone, ethyl acetate, 2- and 3-methylbutanal, 2-octanone, and benzothiazole were identified with high detection frequency in cooked Irish beef (Machiels et al., 2003). Aside from the effects of cooking conditions, method of isolating volatile compounds and technique of analyzing gas chromatography olfactometric data, significant differences were also observed in the odour-active compounds of cooked beef due to breed and diet (Machiels et al., 2004).

In the meantime, there has been an ongoing interest to develop a simulated beef flavour to meet the consumer demand for non-meat based or vegetarian products. Great effort has been applied to imitate beef flavour using blended spices, by-products from the corned beef industry, flavour enhancers such as monosodium-L-glutamate (MSG), and protein hydrolysates (MacLeod and Seyyedain-Ardebili, 1981). In particular, there has been an increase in protein hydrolysates used as materials to produce meat-like savoury flavourings, including hydrolysed vegetable proteins produced from soy (Aaslyng et al., 1998), thermally treated yeast extracts (Münch and Schieberle, 1998), extruded enzyme-hydrolysed soybean protein (Baek et al., 2001) and soybean-based enzyme-hydrolysed vegetable protein (Wu and Cadwallader, 2002). In addition, processed meat flavour or so-called "reaction flavour" obtained by thermal treatment of a mixture of food components, was employed to more closely simulate beef-like flavour (MacLeod et al., 1981). However, the products of thermal processing have varying degrees of meaty aroma, depending not only on heating temperature but also reactant ratio and heating time (Wasserman, 1979).

May (1974) proposed the main reactions occurring in the various model systems to synthesize meat flavours were reactions of reducing sugars with amino compounds consisting of condensations, rearrangements, dehydrations and degradation, in addition to the reaction between reducing sugars and a sulfur donor such as cysteine. Due to the complexity of the reactions, much

work on reaction flavours has been done with various model systems (Mussinan and Katz, 1973; Güntert et al., 1990; Hofmann and Schieberle, 1995; Hofmann and Schieberle, 1997; Mottram and Nobrega, 2002). However, in comparison to flavour compounds produced in model systems consisting of reducing sugars and amino compounds, not enough information is available in terms of odour-active components in simulated meat flavourings. Specifically, there is a lack of published literature describing odour activities of the volatile compounds and character impact compounds in commercially-available simulated meat flavour, compared to the reported flavour components in actual meat systems.

A great number of research studies on food flavour have been conducted with gas chromatography (GC) in conjunction with flame ionization detector (FID) and mass selective detector (MSD) to identify volatile compounds in food commodities. However, the analysis of the chemical components is not sufficient to determine the aroma profile because not all of the compounds are odourants and their contributions to flavour are not usually directly related to their abundance. In other words, although the relative retentions of peaks in GC chromatograms give us clues to identify volatiles in the foods, the areas of those peaks do not necessarily reflect the aroma intensity of the foods. In that sense, GC analysis in combination with an olfactometric technique can be a useful tool to detect potent odour-active components from a complex mixture (Blank, 1997; Mistry et al., 1997), which may considerably contribute to the characteristic aroma of a given food. Dilution methods such as AEDA (aroma extract dilution analysis; Grosch, 1993) and CharmAnalysis™ (Acree et al., 1984), intensity method such as Osme (Miranda-Lopez et al., 1992), and the more recently developed detection frequency method (Linssen et al., 1993; Pollien et al., 1997), are the most commonly used GC-olfactometry (GC-O) techniques.

In the detection frequency method, the number of panelists detecting an odour at a certain retention index is utilized to quantify the aroma intensity of the effluent, rather than dilution values or perceived intensity as measured in the other methods. It was shown that detection frequency was directly related to the odour intensity (van Ruth and O'Connor, 2001). This method has been applied to determine odour-active compounds in foods such as bell peppers, dried leeks, dried French beans (van Ruth et al., 1995), cucumber (Marsili and Miller, 2000) and cooked beef (Machiels et al., 2003). Recently, Le Guen et al. (2000) compared three GC-O

methods, which were AEDA (dilution method), Osme (intensity method), and olfactometry global analysis (detection frequency method), to evaluate the main impact odourants of cooked mussels. The results from the three olfactometric methods were very comparable and well correlated positively (with p values of ~ 0.00001), indicating that all of the methods were significantly interrelated.

The objectives of this research were to identify headspace volatile compounds in simulated beef flavour by GC-MS analysis and to compare the profile of the simulated beef flavour to the flavour compounds found in cooked beef in this study and in previous reports. Flavour compounds were analyzed by GC-MS coupled with a headspace SPME method, which has been considered as a simple, rapid, solvent-free and economical method for extracting flavour components (Moon and Li-Chan, 2004). In addition, GC-O analysis based on detection frequency method was performed to evaluate the relative importance of the odour-active components and to determine the character impact compounds that contribute to the overall aroma of the simulated beef flavour.

3.2. Experimental methods

3.2.1. Materials

The simulated beef flavour (SBF; Mastertaste, Arlington Heights, IL) used in this study was a commercially-available blended flavour, as described in Section 2.2.1 of this thesis. The solid phase assembly holder, 50/30 μm stableflex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 15 mL capacity GC sample vials and polypropylene hole cap with PTFE/silicone septa were purchased from Supelco (Sigma-Aldrich Canada, Oakville, ON). All the fibres were conditioned by inserting them into a splitless/split injection port under helium flow for the recommended time at recommended temperatures according to the manufacturer's instruction before use and cleaned between analyses. Three *n*-paraffin mixes from Supelco (Cat. No. 47100, 47102 and 502243), specifically mixtures of C5 to C8 (C5, C6, C7 and C8), C10 to C16 (C10, C12, C14, and C16), and C12 to C60 (C12, C14, C16, C18, C20, C22, C24, C26, C28, C30, C32, C36, C40, C44, C50, and C60), were used to determine the Kovats indices of the volatiles for this study.

3.2.2. Headspace solid phase microextraction (HS-SPME)

The sensitive and reproducible HS-SPME method that was previously established for analyzing SBF, as described in Chapter 2 of this thesis (Moon and Li-Chan, 2004), was applied to investigate the volatile components of SBF, boiled beef and roasted beef samples. Two grams of SBF and 5 g of distilled water were mixed in a 15 mL capacity GC sampling vial with a magnetic stirring bar. The vial was tightly capped with a polypropylene hole cap with a PTFE/silicone septum. To prepare roasted beef, 100 g of raw beef (Canada AAA steak, well marbled) was chopped into 0.8 cm^3 and roasted in an oven at 190 °C for 15 minutes. Five grams of roasted beef was put in a 15 mL GC vial as the "roasted beef" sample. To prepare boiled beef, 300 g of raw beef from the same package as used for roasted beef was boiled with 600 g water for 15 minutes then chopped. Five grams of chopped beef was put in a 15 mL GC vial as the "boiled beef" sample. The 50/30 μm DVB/CAR/PDMS SPME fibre was exposed to the headspace above the sample for 60 minutes at 60 °C in a thermostat controlled water bath (± 2°C) to extract headspace volatile compounds. Stirring with a magnetic stirring bar was consistently applied. The selection of 60 °C as the adsorption temperature for HS-SPME was based on high sensitivity in terms of total area counts and number of peaks in the GC profile, as well as on the basis that beef analogues such as vegetarian hot dogs are usually served at temperatures close to 60 °C (Moon and Li-Chan, 2004).

3.2.3. GC-FID analysis

A Hewlett-Packard (HP) 5890 gas chromatograph with flame ionization detector (FID) and a DB-5 analytical fused silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness from J&W Scientific, Folsom, CA) were used for analysis of the volatile compounds. The injection was conducted in a splitless mode for 3 min at 250 °C. The oven temperature was held at 40 °C for 3 min, ramped to 180 °C at the rate of 3 °C/min and then to 260 °C at 10 °C/min, and maintained at 260 °C for 2 min. Helium was used as a carrier gas at a column-head pressure of 12 psi (1 psi=6894.76 Pa). The temperature of the FID detector was 280 °C, and 35 mL/min of hydrogen, 350 mL/min of air and 30 mL/min of helium as a make-up gas were used. Several parameters in the chromatogram were adjusted to increase reliability in terms of number of peaks and peak area.

Peak width was set to 0.04 and initial threshold was set to 1. The peaks with peak area under 10,000 were not regarded as reliable peaks.

3.2.4. GC-O analysis

The GC-O was comprised of an HP 5890 GC including a sniffing port (SGE, Texas, USA) with glass detection cone in addition to FID detector. At the end of the capillary column the effluent was split 1:3 for FID and sniffing port, respectively, using deactivated and uncoated fused silica capillaries as transfer lines, and the sniffing cone was purged with humidified air to help in maintaining olfactory sensitivity by reducing dehydration of mucous membranes in the nasal cavity. The ratio of 1:3 was used in order to allow enough flow to the FID detector while maximizing the effluent available to the sniffing port for detection by the panelist. The same column (DB-5) was used for GC-FID and GC-O analyses, and the equipment conditions such as injection port temperature, oven temperature programming, carrier gas (helium) flow, detection temperature and gases flow (hydrogen and air) for GC-O were identical with the GC-FID conditions as described above.

Detection frequency method using a panel of eight panelists was applied to obtain an odour profile for SBF. There was no specific training session for the SBF sample, but 5 of the 8 panelists had extensive experience with GC-O from other research. Each of the eight judges participated in perceiving the aroma compounds separated from SBF at the sniffing port, and the number of panelists detecting odour components during GC-O was summed to acquire an aromagram for SBF. The judges were asked to state odour characteristics, if possible, whenever they detected an odour. A separate GC run was conducted for each panelist and the odour perception was recorded during the first 45 minutes, and any odour recognition at the sniffing port that was reported by fewer than four of the eight judges was considered as noise.

3.2.5. GC-MS analysis

The GC-MS consisted of an Agilent 6890N GC, equipped with the DB-5 capillary column as above, in conjunction with Agilent HP 5973N mass selective detector (MSD) system. Separation was performed under the same conditions described above, using the same column as in the GC-FID and GC-O analyses. The MSD was operated in the electron impact ionization mode with 70

eV and mass range was from 35 to 600 amu. The temperature of capillary direct interface, source, and quadrupole were 280 °C, 230 °C, and 150 °C, respectively.

The aroma compounds were identified by matching mass spectra with spectra of reference compounds in Wiley 275 Mass Spectral libraries (version D.01.00, Wiley, New York, 2000; from Agilent Technologies) and verified on the basis of mass spectra obtained and Kovats retention indices on DB-5 stationary phase from analysis of the aroma standards and FlavorNet by Acree and Arn (2004).

3.3. Results and Discussion

3.3.1. Identification of volatile compounds

A typical GC-FID chromatogram obtained from the electrical responses of FID signals for the volatile compounds in the SBF is shown in Figure 3.1(a). The GC-FID chromatogram is significantly different from the aromagram obtained from the human response, as demonstrated in Figure 3.1(b). Among 88 peaks of volatile compounds in SBF detected by GC-MS, a total of 70 volatile compounds were either positively or tentatively identified by the corresponding mass spectra and Kovats indices, as shown in Table 3.1. A total of 40 peaks in roasted beef and 33 peaks in boiled beef were positively or tentatively identified and comparison with SBF in terms of the composition of the volatile compounds was demonstrated in Table 3.2. Overall, volatile compounds in SBF included more sulfur and nitrogen containing compounds and various alicyclic hydrocarbons while those in cooked beef in this study contained more aldehydes and ketones compared to SBF.

3.3.1.1. Furans and sulfur containing compounds

3-Methylfuran, 2-ethylfuran and 2-acetylfuran were identified in SBF (peaks 1, 2, and 7, respectively, in Table 3.1) and they were also reported to be found in cooked beef i.e. 3-methylfuran in canned beef (Persson and von Sydow, 1973), 2-ethylfuran in roast beef (Min et al., 1979), and 2-acetylfuran in roast beef and shallow fried beef (Min et al., 1979; Watanabe and Sato, 1972). 2-Pentylfuran, which seemed to be commonly found in beef under various conditions such as canned beef (Persson and von Sydow, 1973), boiled beef (Hirai et al., 1973),

roast beef (Min et al., 1979), canned and frozen stew (Peterson and Chang, 1982), roast beef, canned beef and canned beef stew (Shibamoto, 1980) and cooked ground beef (MacLeod and Ames, 1986) was also identified in roasted and boiled beef in this study but it was not found in SBF (Table 3.2). Furans can be produced by sugar caramelization and carbohydrate degradation (Shibamoto, 1980). Even though none of the various furan compounds has been attributed as being crucial to meaty flavour, they have been regarded to contribute to the overall odour of broiled or roasted meat (Shahidi et al., 1986).

In addition, sulfur containing compounds such as 2-methylthiophene, 2-methyl-3-furanthiol, 2,5-dimethylthiophene, 4,5-dimethylthiazole, 3-methyl-2-thiophenecarboxaldehyde, and dihydrothienothiophene were identified (peaks 4, 5, 6, 9, 28, and 41, respectively, Table 3.1) while methanethiol was found in boiled beef (Table 3.2). Methanethiol, which smells like cooked cabbage, was also reported to be found in cooked ground beef (MacLeod and Ames, 1986) and it can be perceived at a very low concentration due to its low threshold (0.2 μg/kg). Guth and Grosch (1994) concluded that methanethiol is one of the character impact odour compounds of stewed beef juice. Sulfur containing compounds have been reported to be important in cooked beef flavour since some heterocyclic sulfur compounds were described as possessing meat-like aromas (Mottram, 1994). It has been postulated that these sulfur containing heterocyclic compounds could be generated either from thermal degradation of cystine or cysteine (Shu et al., 1985), through the interaction between carbonyl compounds and sulfur containing amino acids (Zhang and Ho, 1989) or as a result of thermal decomposition of thiamin (Güntert et al., 1990). The former two pathways might be more important in flavour development in the SBF.

Most of the thiophenes found in meats were identified to be substituted at the 2-position (Werkhoff et al., 1993), similar to 2-methylthiophene and 2,5-dimethylthiophene found in this study. 2-Methylthiophene was isolated from pressure-cooked beef (Wilson et al., 1973; Madruga and Mottram, 1995) as well as roasted beef (Min et al., 1979), and reported to have sulfur (Acree et al., 2004) or green/sweet odour (Shahidi et al., 1986). 2,5-Dimethylthiophene was also identified in the neutral fraction of roasted beef (Min et al., 1979). However, it has been stated that 3-thiol-substituted thiophenes are the only thiophenes with meaty aroma (Werkhoff et al., 1993). Meanwhile, van den Ouweland and Peer (1975) reported that a complex mixture of

compounds including mercapto-substituted furan and thiophene derivatives possessing roasted meat odours were generated from the reaction of 4-hydroxy-5-methyl-3(*2H*)-furanone or its thio analog with hydrogen sulfide such as 4-hydroxy-5-methyl-3(*2H*) thiophenone. 4-Hydroxy-5-methyl-3(*2H*)-furanone could be a degradation product of ribonucleotides such as ribose-5-phosphate, indicating that beefy aromas could possibly be generated from sugar degradation in addition to Maillard reaction.

Thiol, sulfide or disulfide group substituted furans at the 3-position have been regarded to be associated with meat-like aroma even though not many of the compounds in fact have been detected in cooked or roasted meat (Werkhoff et al., 1993). 2-Methyl-3-furanthiol was identified in SBF and confirmed to possess meaty flavour in the sniffing port of GC-O in this study. MacLeod (1994) reported through a search of the literature that only 25 chemical compounds of the 880 cooked beef aroma components had been described as meaty. Most of the compounds (20 out of 25) in the list were sulfur-containing compounds including 2-methyl-3-furanthiol, which was present in the SBF in this study. Gasser and Grosch (1988) also demonstrated that 2-methyl-3-furanthiol, along with *bis*(2-methyl-3-furyl) disulfide, was one of the odour compounds with high aroma values in cooked lean beef and which possessed meat-like odour quality based on aroma extract dilution analysis (AEDA). The researchers compared odour thresholds between 2-methyl-3-furanthiol (0.005-0.01 μg/kg) and 2-methyl-3-(methylthio)furan (25-30 μg/kg), which has been suggested to be a character impact compound in cooked beef (MacLeod and Ames, 1986) and suggested that due to the 2500 times lower odour threshold than 2-methyl-3-(methylthio)furan, 2-methyl-3-furanthiol had far greater importance to the cooked beef flavour. Werkhoff et al. (1993) also reported that 2-methyl-3-furanthiol, *bis*(2-methyl-3-furyl)disulfide, and 2-methyl-3-(methylthio)furan were found in relatively large amounts in beef extracts while these compounds existed in pork and chicken in only trace amounts.

4,5-Dimethylthiazole, which was found in the SBF (peak 9), is known to possess roasted or grilled notes (Mottram, 1994) while 2,4-dimethylthiazole was reported to have meaty flavour (MacLeod, 1994). 4,5-Dimethylthiazole was reported in beef cooked at 140 °C (Madruga and Mottram, 1995), but 3-methyl-2-thiophenecarboxaldehyde and dihydrothienothiophene (peaks 28 and 41) have not been previously reported in beef. However, 3-methyl-2-

thiophenecarboxaldehyde was identified in chicken (Werkhoff et al., 1993) and 5-methyl-2-thiophenecarboxaldehyde was reported to be found in cooked beef (Wilson et al., 1973).

3.3.1.2. Nitrogen containing compounds

Among the 10 nitrogen containing compounds tentatively identified in the SBF, most components, except acetylpyrrole (peak 23), belonged to the pyrazine class of compounds. These include 2-ethyl-6-methylpyrazine, trimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, 2,5-diethyl-3,6-dimethylpyrazine, and 3-isopentyl-2,5-dimethylpyrazine (peaks 14, 15, 24, 30, 37, and 43, respectively, in Table 3.1), which were also reported to be found in cooked beef (MacLeod and Ames, 1986; Cerny and Grosch, 1994). In addition, the SBF contained 2,5-dimethyl-3-isobutylpyrazine, 2,6-diethyl-3,5-dimethylpyrazine, and 2-isoamyl-6-methylpyrazine (peaks 34, 36, and 38, respectively), which have not been reported as volatile components in cooked beef (Table 3.1). 2-Acetylpyrrole (peak 23) was reported to be found in fresh, frozen beef stew and canned beef stew (Peterson et al., 1982), cooked ground beef (MacLeod and Ames, 1986) and shallow fried beef (Watanabe and Sato, 1972). It was also found in both roasted and boiled beef in this study (Table 3.2).

Pyrazines have been reported to be one of the main components in the volatiles of cooked meats; almost 41 % of the volatile constituents of pressure-cooked pork liver were pyrazines (Mussinan and Walradt, 1974). A variety of pyrazines can be produced from the Strecker degradation, which involves interaction of nitrogen containing molecules (e.g. α-amino acids) with dicarbonyls resulting from carbohydrate decomposition in a classic Maillard reaction. MacLeod and Seyyedain-Ardebili (1981) listed 46 pyrazines identified as volatile components of natural beef aroma in their review article and Mussinan et al. (1973) isolated 33 pyrazines from pressure-cooked beef. Substantial similarities in aroma profile and roasted notes were found in the volatile compounds from beef heated with temperatures over 100 °C such as roasted beef, shallow fried beef, pressure-cooked beef and canned beef, and the roasted notes have been frequently derived from alkylpyrazines (MacLeod and Seyyedain-Ardebili, 1981). The odour of pyrazines has been traditionally regarded as nutty, roasted, or burnt; therefore Mussinan et al. (1973) proposed that the pyrazines contributed to characteristics of cooked foods due to the fundamental roasted aroma.

In this study, roasted beef included 2,6-dimethyl pyrazine and 2-ethyl-3,6-dimethylpyrazine whereas boiled beef did not have any pyrazine identified (Table 3.2).

3.3.1.3. Hydrocarbons and carbonyl compounds

Both saturated and unsaturated aliphatic hydrocarbons were identified in SBF, such as 7-methyl-3-methylene-1,6-octadiene, 1-tetradecene, tetradecane, and pentadecane (peaks 13, 51, 53, and 68, respectively, in Table 3.1), in addition to the aromatic hydrocarbons such as toluene and 4-isopropyltoluene (peaks 3 and 19). With the exception of 7-methyl-3-methylene-1,6-octadiene, all of these compounds have also been reported to be found in cooked beef (MacLeod and Seyyedain-Ardebili, 1981). Meanwhile, tridecane, tetradecane and toluene were identified in both roasted and boiled beef in this study while dodecane and 4-isopropyl toluene was found in roasted beef but not in boiled beef (Table 3.2).

Although one terpene, delta-3-carene, was found in roasted beef (Table 3.2), a great number of alicyclic hydrocarbon compounds were tentatively identified in SBF, as shown in Table 3.1. Some of them have been reported as volatile aroma components in cooked beef, such as alpha-phellandrene, gamma-terpinene, and beta-caryophyllene in canned beef stew (Chang and Peterson, 1977), limonene in roasted beef (Min et al., 1979), 2-pinene in heated ground beef (Ramarathnam et al., 1993). Peterson and Chang (1982) identified allo-ocimene, limonene, alpha-phellandrene, gamma-terpinene, and beta-caryophyllene in fresh, frozen beef stew, and delta-3-carene was found in roasted beef of this study (Table 3.2). Although a great number of saturated and unsaturated hydrocarbons in the range of C4 to C15 have been reported in beef, they are not believed to play an important role in roasted beef flavour (Min et al., 1979) due to their relatively weak, non beef-like odours. Compared to volatile compounds identified in cooked beef, the SBF contained a great number of alicyclic hydrocarbons including 25 terpenoids as shown in Table 3.1, which may contribute to differences in aroma characteristics between natural cooked beef and the SBF. Generally, terpenoids originate from herbs or plants. For example, the unique intricate terpenic odour of blackcurrant berries was given off from the terpene-containing glands in the plant (Ruiz del Castillo and Dobson, 2002). Mitiku et al. (2001) investigated the enantiomeric distribution in forty cold-pressed *Citrus* oil samples of alpha-pinene, beta-pinene, sabinene and limonene, which are some of the same terpenoids found in our study, and reported

that the species or varieties showed significant differences in the enantiomeric purity of the terpenoids. It can be postulated that the existence of various terpenoids in the SBF in this study are most likely derived from plant origin ingredients.

Formation of carbonyl compounds may result from oxidation of the unsaturated fatty acids and/or thermal oxidative decomposition of lipids in food material. In this study, benzaldehyde, octanal, phenylacetaldehyde, nonanal, decanal, and 5-methyl-2-phenyl-2-hexenal were identified in SBF (peaks 11, 16, 21, 27, 35, and 64 in Table 3.1). The first five of these 6 aldehydes found in SBF have been reported to be identified in cooked beef, while 5-methyl-2-phenyl 2-hexenal has been reported to be found in cooked pork by Mussinan and Walradt (1974).

Aldehydes are believed not only to contribute to the odour of foods including beef (Moody, 1983), but also to react with other compounds to produce flavour through amino-carbonyl reactions. Various research groups have reported a wide range of saturated and unsaturated aldehydes and ketones in cooked beef, consistent with the findings of this study in which 18 aldehydes and 5 ketones were identified in roasted beef and 17 aldehydes and 4 ketones were found in boiled beef (Table 3.2). Although most of the aldehydes and ketones found in roasted and boiled beef were common, acetaldehyde, butanal, 2- and 3-methyl butanal, and phenylacetaldehyde and 2- propanone appeared only in roasted beef while 2,4-nonadienal, (*E,E*)-2,4-decadienal, undecenal, and 4-dodecen-1-al were found only in boiled beef. The major aldehydes found in roasted beef were hexanal, heptanal, octanal, nonanal, and benzaldehyde, while pentanal, hexanal, heptanal, octanal, nonanal, nonenal, and benzaldehyde were the major aldehydes found in boiled beef. It is interesting to note that most of the aldehydes and ketones found in the cooked beef were not identified in SBF. Of particular interest was the absence in SBF of hexanal, which was the most and the second most dominant compound in boiled and roasted beef, respectively, based on the response by mass detector, as shown in Table 3.2.

Among 55 alcohol compounds listed as components of cooked beef volatiles in a review by Shahidi et al. (1986), relatively large amounts of unsaturated alcohols such as 1-penten-3-ol and 1-octen-3-ol were reported as components of boiled-beef flavour. In this study, 2-3,-butanediol, 1-heptanol, and 1-octen-3-ol were identified in roasted beef while 1-penten-3-ol, pentanol, 1-

hexanol, 1-heptanol, 1-octen-3-ol, and 1-octanol were found in boiled beef. In addition, linalool and nerolidol (peaks 26 and 76) were found as straight-chain primary alcohols and an aromatic alcohol, 4-methyl-2,6-di-*tert*-butylphenol (peak 70), which is also known as BHT, was identified in SBF. Generally esters have been associated with fruity flavours, but a considerable number of esters were found in beef stew (Peterson and Chang, 1982). Two esters, bornyl acetate and *cis*-11-hexadecen-1-yl acetate (peaks 40 and 79), were identified in the SBF.

From a quantitative point of view, based on the FID response in SBF, aldehydes (peaks 11 and 27 in Figure 3.1(a)) and terpenoids (peaks 44, 45, 48, 54, 58, 71 and 72 in Figure 3.1(a)) were abundant among a total of 70 volatile compounds tentatively identified in the SBF. However, this does not necessarily mean that they play an important role in the characteristic of the SBF, which is dependent on the odour characteristics and threshold of the volatile compounds.

3.3.2. Determination of odour-active compounds

The volatile compounds extracted by SPME from SBF were isolated and detected by panelists at the sniffing port of GC-O. The results, presented as retention time and detection frequency by the 8 panelists, are shown in Figure 3.1(b). Of a total of 49 odour-active compounds in SBF, 21 compounds were identified, mainly consisting of heterocyclic sulfur or nitrogen compounds, aldehydes and terpenoids: 3-methyl furan, 2-methyl-3-furanthiol, 2,5-dimethylthiophene, 2-acetylfuran, 4,5-dimethylthiazole, benzaldehyde, trimethylpyrazine, delta-3-carene, alpha-terpinene, 2-ethyl-3,6-dimethylpyrazine, nonanal, 2,3-diethyl-5-methyl pyrazine, beta-fenchyl alcohol, 2,5-dimethyl-3-isobutylpyrazine, decanal, 2-isoamyl-6-methylparazine, 3-isopentyl-2,5-dimethylparazine, delta-elemene, beta-cubebene, calamenene, and caryophyllene oxide (peaks 1, 5, 6, 7, 9, 11, 15, 17, 18, 24, 27, 30, 33, 34, 35, 38, 43, 44, 49, 71, and 78, respectively, in Figure 3.1(b)).

Among the 49 odour-active compounds, 17 components (10 identified and 7 unidentified) were revealed to have high aroma values (detected by 6 or more out of 8 panelists). The most powerful odour impact compounds identified in the SBF were 2-methyl-3-furanthiol, delta-3-carene, alpha-terpinene, and 2-ethyl-3,6-dimethylpyrazine (peaks 5, 17, 18, and 24, respectively). In addition, two unidentified peaks were noted with high detection frequency; one was detected before peak 1

by 7 panelists, and the other, between peaks 24 and 27, was detected by 8 panelists. It could be hypothesized that these two peaks are sulfur-containing aroma compounds which did not appear as peaks in GC-FID chromatogram due to their relatively low concentration in SBF along with low sulfur sensitivity of the FID detector, but could be detected by most of the panelists owing to the nature of low thresholds of sulfur-containing compounds.

2-Methyl-3-furanthiol is the compound that was found by Gasser and Grosch (1988) to have high aroma values in cooked lean beef, along with methional, 2(*E*)-nonenal, 2(*E*),4(*E*)-decadienal and *bis*(2-methyl-3-furyl) disulfide. Kerscher and Grosch (1997) also proposed 2-methyl-3-furanthiol as one of the most potent odourants in boiled beef. Among the pyrazines identified in this study (including trimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, 2,3-diethyl-5-methyl pyrazine, 2,5-dimethyl-3-isobutylpyrazine, 2-isoamyl-6-methylparazine, and 3-isopentyl-2,5-dimethylparazine), 2-ethyl-3,6-dimethylpyrazine was the most powerful odour-active compound to contribute to the aroma profile of the SBF. Cerny and Grosch (1992) stated that the character impact compounds with high aroma value from roasted beef included 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine, while Specht and Baltes (1994) suggested that the differences in pleasant flavour qualities in shallow-fried beef samples were mainly due to the combination of 2-ethyl-3,5-dimethylpyrazine and 2-propyl-3-methylpyrazine, and 2-ethyl-3,6-dimethylpyrazine with lesser importance.

It can be speculated that 2-methyl-3-furanthiol, which has been reported to be responsible for beef broth or roasted meat odour (Gasser and Grosch, 1988; Macleod, 1994; Kerscher and Rosch, 1997), plays a major role in conferring beef-like characteristics to the SBF, while 2-ethyl-3,6-dimethylpyrazine along with other pyrazines appeared to provide the roasted note in the SBF. The importance of 2-methyl-3-furanthiol to contribute to meaty note in meat-like process flavouring has also been demonstrated by several other research groups, which examined the odour-active compounds of the process flavouring. Baek et al. (2001) reported 2-methyl-3-furanthiol as the most intense compound with a cooked rice/vitamin-like/meaty aroma note in a beef-like process flavour, which was produced from hydrolysed vegetable proteins. Münch and Schieberle (1998) described that 2-methyl-3-furanthiol along with 2-furfurylthiol, 3-methylbutanal, and methional showed the highest odour activity values in a thermally treated

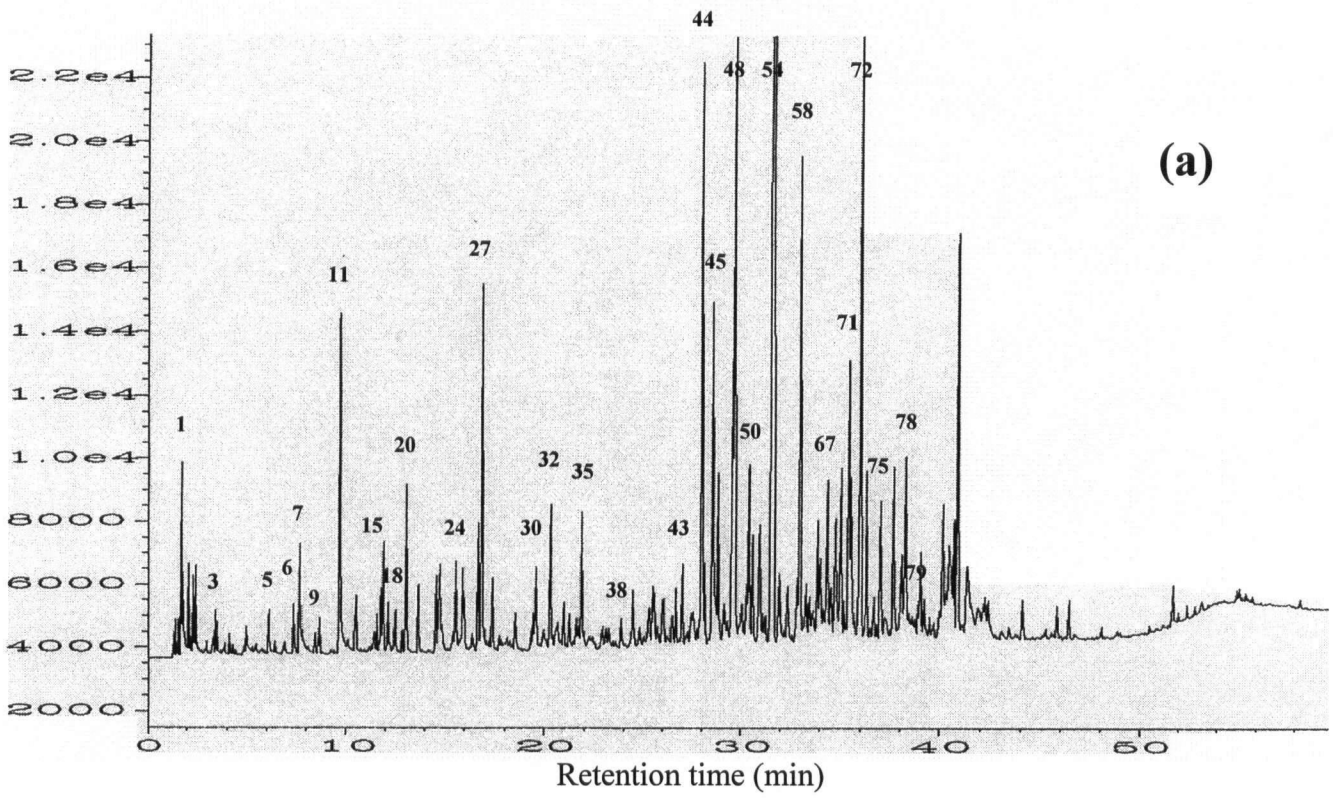
commercial yeast extract and a self-prepared bakers' yeast extract possessing roasty, meat-like odours. In addition, Wu and Cadwallader (2002) indicated that 2-furfurylthiol followed by 2-methyl-3-furanthiol, 3-mercapto-2-pentanone, and 3-(methylthiol)propanal were the most important odour-active compounds in the overall aroma of the meatlike process flavouring from hydrolysed vegetable protein.

On the other hand, along with many other alicyclic hydrocarbons found in SBF, delta-3-carene and alpha-terpinene, both of which are known to have a lemon odour (Acree et al., 2004), might contribute to some perceived difference between the SBF and cooked beef flavour. Moreover, the aldehydes and ketones that were found in the cooked beef but absent in SBF, may also be responsible for the different odour profile of SBF from that of cooked beef. According to Gasser and Grosch (1988), 2-octenal, 2-nonenal, 2,4-nonadienal, 2,4-decadienal (fatty, like fried potato), 1-octen-3-one (mushroom-like), 2-octanone, 2-decanone and 2-dodecanone (musty, fruity) and phenylacetaldehyde (honey-like) in addition to sulfur containing compounds were included in the 17 odour compounds having high aroma values found in cooked beef. 2-Nonenal and 2,4-decadienal were aldehydes with odour activity reported in roasted beef (Cerny and Grosch, 1992) and 3-mercapto-2-pentanone, 1-octen-3-one and 2-nonenal were indicated to be a part of the most potent odourants in boiled beef (Kerscher and Grosch., 1997). Although these aldehydes and ketones may not be directly responsible for beefy flavour in cooked beef, they might more than likely contribute to the fatty notes of the cooked beef, resulting in subtle differences between SBF and cooked beef.

3.4. Conclusion

Application of GC-FID, GC-MS and GC-O to the research of volatile compounds in SBF revealed an intricate combination of aroma compounds from several sub-classes. A total of 70 aroma compounds were identified in the SBF by combined use of SPME headspace analysis with GC-FID and GC-MS. Moreover, GC-O was useful to identify the main odour-active compounds which contribute to the aroma profile of SBF. Among these, several sulfur and nitrogen containing compounds as well as various terpenoids were proposed to be of essential importance for the flavour profile of the SBF. The most powerful odour-active compounds identified in SBF

were 2-methyl-3-furanthiol, delta-3-carene, alpha-terpinene, and 2-ethyl-3,6-dimethylpyrazine. It was postulated that the meaty characteristic of the SBF was provided by 2-methyl-3-furanthiol with its meat-like odour, along with various pyrazines contributing roasted notes, while other aroma compounds including terpenoids, along with the absence of various aldehydes and ketones, resulted in the subtle difference between the SBF and cooked beef. It is anticipated that the knowledge gained from this study on the potential impact odourants in SBF and comparison with odour-active compounds reported in cooked beef may provide some foundation for further research or applications by scientists and food or flavour companies that are interested in the chemistry and aroma characteristics of vegetarian simulated meat flavours.



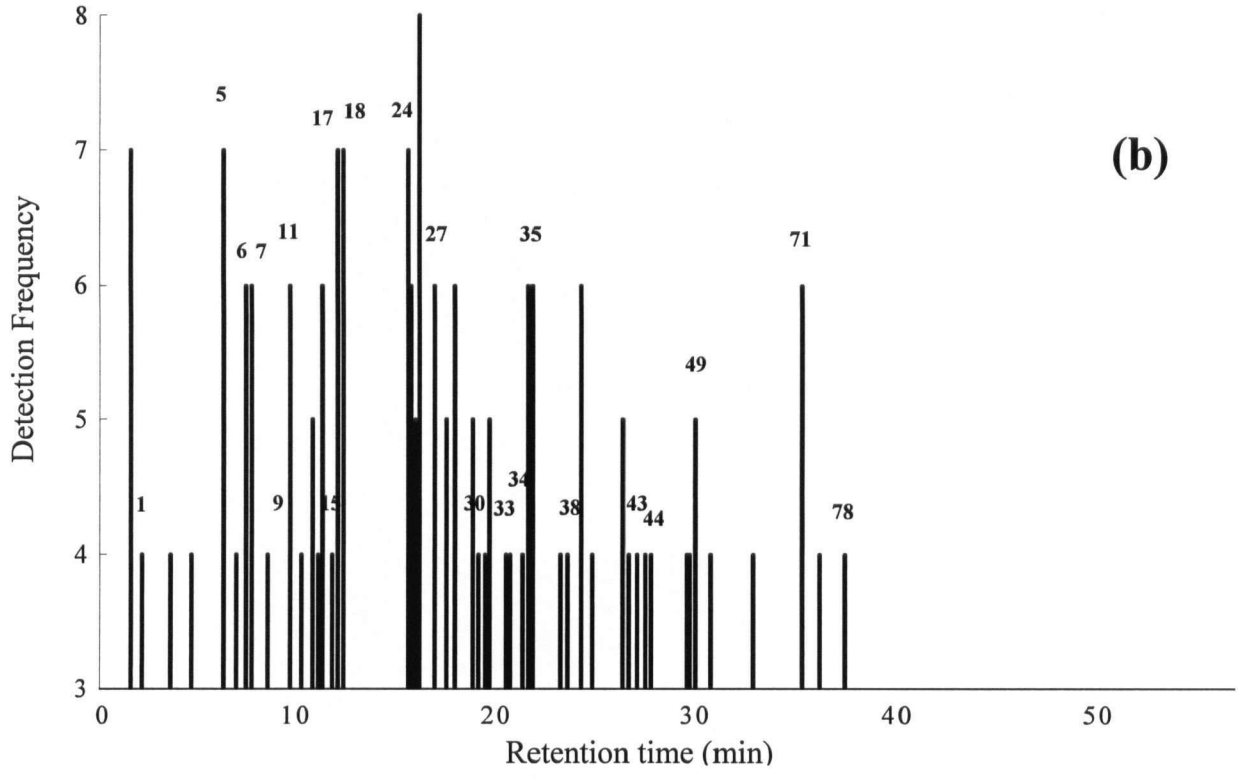


Figure 3.1. (a) GC-FID chromatogram of simulated beef flavour and (b) aromagram of volatile compounds of simulated beef flavour expressed as retention time and detection frequency by GC-O (number of panelists = 8). Peak numbers correspond to the peak numbers in Table 3.1.

Table 3.1. Volatile compounds in simulated beef flavour tentatively identified by mass spectral-search (MS) and linear retention index (LRI).

Peak No.[1]	Volatile compound	LRI[2]	Basis for identification	Previously reported in cooked beef[3]	Odour descriptor reported in the literature	Odour descriptor from panelists during GC-O[4]
1	3-Methylfuran	646	MS	*1*		Stinky, candle, beef
2	2-Ethylfuran	713	MS, LRI	*1,2,3*	Acid, sour, whey butter-like	Perfume, lemony, beef
3	Methylbenzene	762	MS, LRI	*1,3,4,5*	Paint	
4	2-Methylthiophene	819	MS	*1,3,6,7*	Sulfur, green, sweet	Floral, grass, burnt beef
5	2-Methyl-3-furanthiol	849	MS, LRI	*1,6,8,9*	Meaty	Meaty (2), oxo
6	2,5-Dimethylthiophene	881	MS, LRI	*3*		Beef, sweet, ham, rancid
7	2-Acetylfuran	888	MS, LRI	*1,3,10*	Balsamic	Sulfur, sweet, toffee, rancid
8	alpha-Phellandrene	907	MS	*1,11,12*	Fresh	
9	4,5-Dimethylthiazole	910	MS, LRI	*6*	Earthy, roasted, nutty, green	Meaty, sulfur, caramelized, floral
10	2-Pinene	912	MS, LRI	*5*	Pine	
11	Benzaldehyde	938	MS, LRI	*1,2,3,4,13*	Almond, musty, sweet, metallic	Pop corn, caramel, herby, sulfur, chemical, spicy
12	Sabinene	957	MS, LRI		Woody	
13	7-Methyl-3-methylene-1,6-octadiene	959	MS, LRI		Balsamic	
14	2-Ethyl-6-methylpyrazine	986	MS, LRI	*1,4,6,10,14*	Fruity	
15	Trimethylpyrazine	990	MS, LRI	*1,4,10,14,15*	Nutty, roasted	Burnt, smokey, painty, solvent
16	Octanal	995	MS, LRI	*1,2,4,6,13,16, 17*	Soapy	
17	delta-3-Carene	1000	MS, LRI		Lemon	Floral (3), sugar, honey, smokey, beef
18	alpha-Terpinene	1007	MS, LRI	*1*	Lemon	Grassy, plastic, smokey, cod liver oil. beef
19	4-Isopropyltoluene	1014	MS, LRI	*1*		
20	Limonene	1018	MS, LRI	*1,3,5,11*	Lemon	Grassy, rancid, rubbery

Table 3.1. continued

Peak No.[1]	Volatile compound	LRI[2]	Basis for identification	Previously reported in cooked beef[3]	Odour descriptor reported in the literature	Odour descriptor from panelists during GC-O[4]
21	Phenylacetaldehyde	1030	MS, LRI	*1,2,10,13,16*	Rosy, perfume	Herb, oil, burning
22	gamma-Terpinene	1049	MS, LRI	*1,11,12*	Gasoline	
23	Acetylpyrrole	1052	MS, LRI	*1,4,10,11,18*	Unpleasant, plastic, antiseptic	
24	2-Ethyl-3,6-dimethylpyrazine	1069	MS, LRI	*1,4,6,8,14*	Nutty, roasted, potato	Meat, smokey (2), sulfur, natural gas, soil
26	Linalool	1093	MS, LRI		Lemon, floral	
27	Nonanal	1097	MS, LRI	*1,2,4,6,13,16, 17*	Soapy, green, moldy	Grassy, tea, vegetable, lemony, sour, beefy
28	3-Methyl-2-thiophenecarboxaldehyde	1105	MS			Ham, sweet, beefy, savoury
29	2,3-Dihydro-3,5-dihydroxy-6-methyl-*4H*-pyran-4-one	1130	MS			
30	2,3-Diethyl-5-methylpyrazine	1148	MS, LRI	*1,4,6,14,15*	Nutty, roasted, potato	Burnt, cured meat, popcorn (2), buttery (2)
32	4-Terpeneol	1165	MS			Fried, barbecue, beefy
33	beta-Fenchyl alcohol	1179	MS, LRI		Camphor	Bleach, chlorine, wood, dandelion, meat
34	2,5-Dimethyl-3-isobutylpyrazine	1193	MS			Roasted meat, floral
35	Decanal	1198	MS, LRI	*1,2,4,6,10,16, 17*	Soapy	Rubber tubing, cooked veggie, sweet popcorn, smokey
36	2,6-Diethyl-3,5-dimethylpyrazine	1221	MS			
37	2,5-Diethyl-3,6-dimethylpyrazine	1225	MS	*1,14*		
38	2-Isoamyl-6-methylpyrazine	1242	MS			Rubbery (2), cabbage, sweet, mint
39	*trans*-Anethole	1276	MS			Sweet, sulfur, hot wing, beefy
40	Bornyl acetate	1278	MS			
41	Dihydrothienothiophene	1285	MS			
43	3-Isopentyl-2,5-dimethylpyrazine	1310	MS	*10*		Yeasty (2), fermented, floral

Table 3.1. continued

Peak No.[1]	Volatile compound	LRI[2]	Basis for identification	Previously reported in cooked beef[3]	Odour descriptor reported in the literature	Odour descriptor from panelists during GC-O[4]
						perfume
44	delta-Elemene	1331	MS			Barn yard, smelly socks, freshly cut wood, sulfur, burnt
45	alpha-Cubebene	1342	MS			
48	alpha-Copaene	1367	MS			
49	beta-Cubebene	1381	MS			Rotten, dusty, yeasty, rubbery
50	beta-Elemene	1384	MS			Sewage, green tea, meaty (2), MSG
51	1-Tetradecene	1390	MS	*1*		
52	*cis*-Caryophyllene	1395	MS	*1,11*		
53	Tetradecane	1398	MS, LRI	*1,3*	Alkane	
54	beta-Caryophyllene	1411	MS, LRI	*11,19*	Woody	Soil, meaty
55	epi-Bicyclosesquiphellandrene	1419	MS			Ashy, yeast, sulfur
56	allo-Aromadendrene	1427	MS			
57	alpha-Amorphene	1440	MS			
58	alpha-Humulene	1443	MS			
61	gamma-Cadinene	1469	MS		Woody	Herb, burnt, sulfur
62	beta-Selinene	1476	MS		Herbaceous	
64	5-Methyl-2-phenyl-2-hexenal	1482	MS			Wax, cured meat, roasted
65	alpha-Selinene	1485	MS			
66	gamma-Muurolene	1488	MS			
67	alpha-Muurolene	1494	MS			
68	Pentadecane	1500	MS, LRI	*1,3*	Alkane	
69	beta-Bisabolene	1505	MS			
70	4-Methyl-2,6-di-*tert*-butylphenol	1508	MS	*1,3,12,20*		
71	Calamenene	1515	MS			Herbal, savoury, spicy, yeasty, MSG, oxo, beef
72	delta-Cadinene	1518	MS		Woody	
73	1,2,3,4,4a,7-Hexahydro-1;6-dimethyl-	1524	MS			Medicine, caramelized,

Table 3.1. continued

Peak No.[1]	Volatile compound	LRI[2]	Basis for identification	Previously reported in cooked beef[3]	Odour descriptor reported in the literature	Odour descriptor from panelists during GC-O[4]
	4-(1-methylethyl)-naphthalene					roasted
75	Elemol	1542	MS			
76	Nerolidol	1559	MS			
77	Ionol	1562	MS			
78	Caryophyllene oxide	1571	MS			Matches, musty, earthy, butter, meaty
79	*cis*-11-Hexadecen-1-yl acetate	1576	MS			

[1]Peak numbers refer to all the peaks detected by GC-MS, as shown in Figures 3.1(a) and 3.1(b); only the 70 identified compounds are shown in this table.

[2]Linear retention index on DB-5 capillary column.

[3]References for volatile components previously reported in cooked beef: *1* MacLeod and Seyyedain-Ardebili (1981); *2* Drumm and Spanier (1992); *3* Min et al. (1979); *4* MacLeod and Ames (1986); *5* Ramarathnam et al. (1993); *6* Madruga and Mottram (1995); *7* Wilson et al. (1973); *8* Grosch (1993); *9* Werkhoff et al. (1993); *10* Cerny and Groschl. (1994); *11* Peterson and Chang. (1982); *12* Chang and Peterson (1977); *13* Moody (1983); *14* Mussinan and Katz. (1973); *15* Kerler and Grosch (1996); *16* Guth Grosch. (1993); *17* Ramarathnam et al. (1991); *18* Misharina et al. (1994); *19* Shahidi et al. (1986); *20* Hirai et al. (1973).

[4]Odour descriptor expressed by panelists at a given retention index in GC-O which was the same as that in GC-MS. Numbers in parentheses refer to the number of panelists using the same descriptor.

Table 3.2. Comparison of volatile compounds in simulated beef flavour, boiled beef and roasted beef tentatively identified by GC-MS coupled with HS-SPME.

Compound	Peak size[1]		
	Simulated beef flavour	Roasted beef	Boiled beef
1. Aliphatic and aromatic hydrocarbons			
7-Methyl-3-methylene-1,6-octadiene	M		
Dodecane		xS	
Tridecane		S	S
Tetradecane		S	S
Tetradecane	M		
1-Tetradecene	S		
Pentadecane	S		
2-Isopropyl-5-methyl-9-methylene-bicyclodec-1-ene	xS		
Toluene	S	S	xS
4-Isopropyl toluene	S	S	
Calamenene	xS		
trans-Anethole	xS		
alpha-Amorphene	xS		
2. Alicyclic hydrocarbons			
alpha-Phellandrene	S		
2-Pinene	S		
Sabinene	xS		
delta-3-Carene	S	S	
alpha-Terpinene	M		
Limonene	M		
gamma-Terpinene	S		
delta-Elemene	L		
alpha-Cubebene	M		
alpha-Copaene	L		
beta-Cubebene	S		
beta-Elemene	M		
cis-Caryophyllene	M		
beta-Caryophyllene	xL		
epi-Bicyclosesquiphellandrene	M		
allo-Aromadendrene	S		
alpha-Humulene	L		
gamma-Cadinene	M		
beta-Selinene	M		
alpha-Selinene	M		
gamma-Muurolene	xS		
alpha-Muurolene	M		
beta-Bisabolene	M		

Table 3.2. continued

Compound	Peak size[1]		
	Simulated beef flavour	Roasted beef	Boiled beef
delta-Cadinene	L		
1,2,3,4,4a,7-Hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	M		
3. Alcohols			
2,3-Butanediol		xS	
1-Penten-3-ol			S
Pentanol			S
1-Hexanol			S
1-Heptanol		xS	S
1-Octen-3-ol		S	M
1-Octanol			M
Linalool	M		
4-Terpeneol	M		
beta-Fenchyl alcohol	M		
4-Methyl-2,6-di-*tert*-butylphenol	M		
Elemol	M		
Nerolidol	xS		
Ionol	M		
4. Aldehydes			
Acetaldehyde		S	
Butanal		S	
3-Methyl butanal		S	
2-Methyl butanal		S	
Pentanal		S	M
Hexanal		M	L
Heptanal		M	M
Octanal	M	M	M
Octenal		xS	S
Nonanal	M	M	L
Nonenal		S	M
2,4-Nonadienal			S
Decanal	S	S	S
2-Decenal		xS	S
(*E,E*)-2,4-Decadienal			S
Undecanal		xS	S
Undecenal			S
Dodecanal		S	S
4-Dodecen-1-al			S
Hexadecanal		S	S
Benzaldehyde	M	M	M
Phenylacetaldehyde	S	S	

Table 3.2. continued

Compound	Peak size[1]		
	Simulated beef flavour	Roasted beef	Boiled beef
5-Methyl-2-phenyl-2-hexenal	M		
5. Ketones			
2-Propanone		S	
2-Butanone		M	M
3-Hydroxy-2-butanone		S	M
2-Heptanone		xS	S
2,3-Octanedione		S	M
2,3-Dihydroxy-6-methyl-*4H*-pyran-4-one	S		
6. Carboxylic acids and esters			
Acetic acid		S	
Hexanoic acid		xS	
Octanoic acid		xS	
Butyl butyrate			S
Benzyl acetate		xS	
Bornyl acetate	M		
cis-11-Hexadecen-1-yl acetate	S		
7. Furans and S-heterocyclic compounds			
3-Methylfuran	M		
2-Ethylfuran	S		
2-Acetylfuran	M		
2-Pentylfuran		S	M
Methanethiol			S
2-Methylthiophene	S		
2-Methyl-3-furanthiol	S		
2,5-Dimethylthiophene	S		
4,5-Dimethylthiazole	S		
3-Methyl-2-thiophenecarboxaldehyde	M		
Dihydrothienothiophene	S		
8. N-heterocyclic compounds			
2,6-Dimethyl pyrazine		xS	
Acetylpyrrole	S	S	
2-Ethyl-3,6-dimethyl pyrazine	M	S	
2-Ethyl-6-methylpyrazine	S		
Trimethylpyrazine	S		
2,3-Diethyl-5-methylpyrazine	S		
2,5-Dimethyl-3-isobutylpyrazine	S		
2,6-Diethyl-3,5-dimethylpyrazine	S		
2,5-Diethyl-3,6-dimethylpyrazine	S		
2-Isoamyl-6-methylpyrazine	S		

Table 3.2. continued

Compound	Peak size[1]		
	Simulated beef flavour	Roasted beef	Boiled beef
3-Isopentyl-2,5-dimethyl-pyrazine	M		
9. Miscellaneous			
Caryophyllene oxide	M		

[1]Peak size was considered to be "xS" (extra small) when the log value of the peak area was below 6, "S" (small) between 6 and 7, "M" (medium) between 7 and 8, "L" (large) between 8 and 9, "xL" (extra large) above 9.

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CHAPTER 4. CHANGES IN PERCEIVED AROMA CHARACTERISTICS OF SIMULATED BEEF FLAVOUR BY ADDING SOY PROTEIN ISOLATE AS ASSESSED BY DESCRIPTIVE SENSORY ANALYSIS AND GAS CHROMATOGRAPHY-OLFACTOMETRY[3]

4.1. Introduction

A characteristic beef odour is one of the most important parameters to determine the quality of beef analogue products such as vegetable protein based meat substitutes for vegetarian consumers. Among the vegetable protein materials used in meat substitutes, soy protein in particular has become more and more popular due to its reported health benefits such as hypocholesterolemic effects (Anderson et al., 1995) and cancer prevention (Fournier et al., 1998). More interest has been gained after the approval of a health claim by the U.S. Food and Drug Administration stating the cardiovascular advantages of soy consumption (USFDA, 1999).

Flavour related problems including "beany" odour (Boatright and Lei, 1999; Lei and Boatright, 2001; Wolf 1975) and off-flavour (Inouye et al., 2002; Maheshwari et al., 1995; McDaniel and Chan, 1988) have created technical obstacles to be overcome for the increased usage of soy proteins in human foods (Maheshwari et al., 1995). Aside from these undesirable yet hard-to-remove soy aromas, the interactions of soy proteins with desirable aroma components of added flavour formulations have presented a different challenge concerning soy based products. Gremli (1974) reported that the presence of soy protein in aqueous systems increased the retention of volatile components in samples, while Malcolmson and McDaniel (1987) observed the suppression of chicken flavour in a formulated soup at high levels of soy protein.

Thermally produced simulated meat flavours, so called "reaction flavours", are often employed in vegetarian products to more closely simulate meat-like flavour. Generally, this type of flavour ingredient can be generated by heating amino acids or protein hydrolysates with various sugars to construct varying degrees of meaty aroma according to the reactant ratio, heating temperature and

[3] A version of this chapter will be submitted for publication. Moon, S. -Y., and Li-Chan, E. C. Y. Changes in perceived aroma characteristics of simulated beef flavour by adding soy protein isolate as assessed by descriptive sensory analysis and gas chromatography-olfactometry. *The Journal of the American Oil Chemists' Society.*

heating time (Wasserman, 1979). May (1974) proposed the main reactions occurring in the various model systems to synthesize meat flavours, namely condensations, rearrangements, dehydrations and degradation between reducing sugars and amino acids including cysteine as a sulfur donor. Different types of protein hydrolysates as starting materials for meat flavour were reviewed by the same author (May, 1974).

Due to the complexity of the reactions involved in their creation, simulated meat flavours used in vegetarian products are likely to have a multifaceted aroma profile. Nevertheless, it is imperative to elucidate the influence of other ingredients such as soy protein on the sensory characteristics of the simulated meat flavours, in order to gain an understanding and provide potential strategies for overcoming the diminution in meaty aroma intensity observed in the presence of soy proteins.

In a previous study, development of a solid phase microextraction (SPME) technique for gas chromatographic (GC) analysis of simulated beef flavour was achieved (Moon and Li-Chan, 2004). Odour-active components in the simulated beef flavour were identified by the combination of GC-mass spectrometry (GC-MS) and GC-olfactometry (GC-O) (Moon et al., 2006). The present research was undertaken as an extension of these earlier studies, in order to identify "indicator peaks" in the GC profiles that could be used to monitor soy-protein induced changes in the aroma of simulated beef flavour, which would be correlated to the perceived aroma characteristics of soy protein-simulated beef flavour mixtures as assessed by panelists.

Therefore, the specific objectives of this study were (1) to apply descriptive analysis (DA) to express the aroma characteristics of the simulated beef flavour (SBF) and soy protein isolate (SPI), (2) to monitor changes in these sensory attributes as a function of different ratios of SBF and SPI, (3) to elucidate the relationship between the sensory response and GC data for the various mixtures of the SBF and SPI, and (4) to select GC indicator peaks that are correlated to beefy characteristics. Successful investigation of changes in aroma profile of SBF upon addition of SPI and selection of indicator peaks could lead to potential application to monitor the retention or release of beef flavour due to SPI-SBF interactions in samples containing SPI.

4.2. Experimental methods

4.2.1. Materials

The simulated beef flavour (SBF; Mastertaste, Arlington Heights, IL) was a commercially-available blended flavour, as described in Section 2.2.1 of this thesis. The soy protein isolate (SPI, lot # 02060631-532) used in this study was a commercially available product from Solae (St. Louis, MO). Protein content of the SPI analyzed by the nitrogen combustion method using a LECO FP-428 (LECO Corporation, Joseph, MI) was 89.3 ± 0.1 % using 6.25 as a conversion factor (Renkema et al., 2002; Puppo et al., 2004). The solid phase assembly holder, 50/30 μm stableflex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 15mL capacity GC sample vials and polypropylene hole cap with PTFE/silicone septa were purchased from Supelco (Sigma-Aldrich Canada, Oakville, ON).

4.2.2. Sensory analysis

Descriptive analysis was conducted by adapting the method of Zook and Pearce (1988) to obtain data describing the sensory attributes of SBF and SPI.

4.2.2.1. Panelist training

Ten subjects consisting of 8 women and 2 men with an interest in descriptive sensory evaluation were selected from students in the Food Science graduate program at UBC and from food development staff at a food company in Vancouver producing soy-based products for vegetarians and consumers preferring meatless products. The panelists received 8 hours of training, consisting of four 2-h sessions conducted over 2 weeks.

Training session I

The objectives of the first training session were to discuss the aroma characteristics of the SBF and SPI and to select potentially appropriate attributes describing them through open discussion. Two SBF samples, which were low SBF (150 mg in 5 g water) and high SBF (500 mg SBF in 5 g water), one SPI sample (500 mg SPI in 5 g water) and one mixture sample (500 mg SBF with 500 mg SPI in 5 g water) were prepared. Samples were provided in capped 15 mL vials covered with aluminum foil to prevent any potential bias from the appearance of the samples and held at 60 °C

for 20 minutes before serving. The aroma descriptors for the SBF suggested by the panel at the first training session were (1) brothy/oxo/miso-like, (2) roasted/dry/cooked/barbecue, (3) beefy (identity of beef), (4) rare/raw/bloody/uncooked, (5) browned caramel/sweet/candy-like and (6) cardboardy/rancid/off-flavour/yeasty. The aroma descriptors suggested for the SPI were (1) soymilk-like, (2) cooked cereal, and (3) straw/hay-like.

Training session II

The aim of the second training session was to reach consensus among panelists on the aroma descriptors. The explored aroma terms, along with their characteristics and reference materials prepared for the training session, are presented in Table 4.1. OxoBeef (Knorr OXO Beef Bouillon, Toronto, Ontario) solution was prepared as specified in Table 4.1 to explain the attribute of "brothy/oxo beef-like/miso-like". Fresh beef (1.25 kg of Canada AAA steak, 2.5 cm thick, purchased at Safeway) was cooked with 5 g of Club House seasoned salt (McCormick Canada Inc., London, Canada) in a conventional oven as indicated in Table 4.1. The surface crust (2 mm thickness) of the roasted beef was separated from the inner part of the beef; the surface crust was used for the attributes of "roasted/dry cooked beef/barbecue" while the interior of the cooked beef was employed for the "beefy" attribute. Fresh extra lean ground beef (Safeway) was employed for the attribute of "rare/raw/uncooked". As very little odour was perceived from fresh raw beef, 100 g of the ground beef was put in each 125 mL Erlenmeyer flask to amplify the odour intensity of raw beef; the flasks were covered with Parafilm and aluminum foil and kept at 4 °C before serving. Werther's Original Caramel (Storck, Mississauga, Ontario) was used for the attributes of "browned caramel/sweet/candy-like". Wet cardboard (15 cm × 30 cm) as well as yeast extract powders (supplied by the Hain Celestial Group, Delta, B.C., Canada) were used as references for the "cardboardy/rancid/off-flavour/yeasty" attribute.

Soy milk (Sunrise Soya Beverage, unsweetened) was used as a reference standard for "soymilk-like" attribute. Cooked oatmeal (Quaker Oatmeal Regular) prepared as shown in Table 4.1 was used for the attribute of "cooked cereal". Hay supplied from the Dairy Education and Research Center of UBC at Agassiz was put in a 200 mL beaker covered with Parafilm and aluminum foil and used for "straw/hay-like" attribute.

Except for the reference standards for "rare/raw/bloody/uncooked", "cardboardy/rancid/off-flavour/yeasty" and "straw/hay-like", which were provided at room temperature due to the sample characteristics, 20 g of each of the reference standards was placed in 15 mL vials, providing about half headspace volume, and held at 60 °C for 20 minutes. After discussing the odour characteristics of the references, the panelists were asked to perceive and differentiate those attributes in prepared samples consisting of 500 mg SBF in 5 g water and 500 mg SPI in 5 g water. By the end of the training session, the panelists agreed to select 3 attributes for SBF ("roasted", "beefy", and "yeasty") and 2 attributes for SPI ("soymilk-like" and "cereal") and recognized these attributes in SBF and SPI sample mixtures.

Training session III

The objectives of the third training session were to train the panelists to assess the 5 selected aroma attributes for samples containing various concentrations of SBF with or without SPI, and to discuss the results in order to reach a consensus on the intensity of the perceived attributes. The definitions which were agreed upon for the 5 selected descriptors, namely "roasted", "beefy", "yeasty", "soymilk-like", and "cereal", are listed in Table 4.2. Six samples containing 0, 250 or 500 mg of SBF with or without 250 mg of SPI were prepared as training samples and evaluation sheets with unstructured 15 cm line scales anchored at the ends by marks 1.3 cm from either end were used. Open discussion proceeded during evaluation of each sample, until consensus was reached among the panelists.

Training session IV

The objective of the fourth training session was to practice assessing various mixtures of SBF and SPI. A total of 12 samples (3 sets each with 4 samples) were assessed using the same sensory sheets to be used in the main sensory evaluation. A reference comprised of 150 mg of SBF and 250 mg of SPI was provided with each sample set. After the individual assessment for the samples, open discussion was held to assess the evaluation results and reach agreement for perceived aroma intensity of the provided samples.

4.2.2.2. Descriptive sensory analysis

Eight of the 10 trained panelists were able to participate in the main evaluation, which was carried out in individual sensory booths in a sensory room with air circulation. A total of 24 test samples were evaluated in two blocks of replications consisting of 12 samples, which were randomly ordered within the replication blocks. Four samples and a reference were provided for each set of the 6 evaluations. The 24 test samples (12 different samples in duplicate, coded with 3-digit random numbers) and the references (designated "R") were covered with aluminum foil and held at 60 °C for 20 minutes before serving. The 12 samples examined were 150 or 500 mg of SBF (i.e. "low" or "high" SBF) with 0, 250 or 500 mg of SPI (i.e. "no", "medium" or "high" SPI) in 5 g of distilled water, each with or without heat treatment (98 °C, 30 minutes), as shown in Table 4.3. The panelists were asked to evaluate the samples for the 5 aroma attributes that had been developed during the 4 training sessions. The intensity of each attribute in a sample was rated on an unstructured 15 cm line scale anchored at 1.3 cm from each end and labeled with "Slight" on the left, "Moderate" in the center, and "Intense" on the right. Panelists were requested to mark the intensities of the attributes in comparison with the reference, which were identified as "moderate" in the unstructured line scale. Data were quantified by measuring the distance of the panelists' mark from the origin in centimeters. In addition, a container including coffee beans was provided for each panelist, who was asked to sniff the coffee beans to minimize sensory adaptation.

4.2.3. Gas chromatography

In a 15 mL capacity GC sampling vial with a magnetic stirring bar, 150 or 500 mg of SBF and 0, 250 or 500 mg of SPI in 5 g of distilled water were mixed. The vial was tightly capped with a polypropylene hole cap with a PTFE/silicone septum. A 50/30 μm DVB/CAR/PDMS SPME fibre was then exposed to the headspace above the sample solution for 60 minutes at 60 °C in a thermostat controlled water bath (± 2 °C) to extract headspace volatile compounds. Stirring with a magnetic stirring bar was consistently applied. Detailed information on the headspace solid phase microextraction (HS-SPME) methodology and analysis conditions were described in earlier reports (Moon and Li-Chan, 2004; Moon et al., 2006). All treatments were prepared in triplicate and results were expressed as the average of the triplicate values.

4.2.4. Statistical analysis

Data from the descriptive analysis was evaluated by analysis of variance (ANOVA), Pearson correlation analysis, and principal component analysis (PCA) using Minitab software (version 13.30, Minitab inc. PA USA). ANOVA with Duncan's multiple comparison tests were performed to determine whether there were differences among individual samples and panelists for each sensory attribute. When the interaction between sample and replication was found to be significant, an adjusted F test was subsequently conducted based on using mean square of the interaction instead of mean square of error as the denominator (O'Mahony, 1986). Panel performance was also reviewed in terms of concordance among the panelists and repeatability of the individual panelists for each attribute using Pearson correlation and ANOVA analysis, respectively. In addition, Pearson correlation analysis on mean sensory scores for each attribute in the DA and mean values of peak area in GC chromatogram was conducted to determine correlation among panelists and between peak areas and sensory scores. PCA was performed on the mean sensory scores of the 12 samples for 5 attributes. The vector coefficients for each sensory attribute were rescaled by a factor of 5 to depict PCA bi-plots, and ellipses were employed to provide a visual aid for describing samples.

4.3. Results and Discussion

4.3.1. Sensory analysis

The results of the ANOVA of the attribute ratings across the 12 samples for 8 panelists are summarized in Table 4.4. Samples were highly significantly different at $p \leq 0.001$ for all attributes. Although "panelist" was a significant source of variation in all attributes except "beefy", this result is not unusual in descriptive analysis, and may mean that panelists were using different parts of the scale due to physiological differences in perceived intensity or differences in personal style of scoring, such as central or extreme raters. A significant replication effect was found in the "beefy" attribute, which might be partly influenced by the block effect of the replications in this study. There was no significant interaction between sample and panelist, indicating that the panelists were scoring consistently for each attribute. In addition, no significant interaction between panelist and replication was found, showing that the panelists on the whole were reproducible in the duplicate trials for each attribute. However, a significant

interaction between sample and replication was observed for the soymilk-like and cereal attributes. This implies that intensities of soymilk-like and cereal in the samples were not rated similarly when they were replicated. Because of the significant replication effect on beefy attribute and significant interaction effect between sample and replication in soymilk-like and cereal attributes, an adjusted F test was performed using the mean square of sample × replication interaction instead of mean square of error as a denominator, dealing with replications as a random effect (O'Mahony, 1986), as shown in Table 4.5. The results showed that samples were still significantly different in all attributes after taking the variation of replication into account, even though the level of significance of both soymilk-like and cereal attributes changed from $p \leq 0.001$ to $p \leq 0.01$.

4.3.1.1. Panel performance

The mean scores of each panelist for the 5 attributes and the grouping of the panelists by Duncan's multiple tests are shown in Table 4.6. The ranges between lowest and highest mean scores for soymilk-like (4.41), cereal (4.41), and yeasty (5.69) notes were relatively large compared to those of roasted (2.23) and beefy (1.97) notes. This result may be due to either different perception of panelists in each of the attributes or difficulty of consensus in characterization of soymilk-like, cereal, and yeasty notes compared to roasted and beefy notes. Obviously, panelist 4 evaluated the samples in a different way from the other panelists, giving low scores for all the samples especially in soymilk-like and yeasty notes. Panelist 5 may be a potential outlier in soymilk-like, cereal, and beefy notes.

In sensory analysis, panelists are considered as an instrument to measure sensory attributes of the samples. The degree of reliability of the panel immensely affects the results of the sensory evaluation and further data interpretation; therefore, panel performance was reviewed (Jeong et al., 2004; Labbe et al., 2004) in terms of concordance among the panelists and repeatability of the individual panelist. Pearson correlation coefficients and probabilities of sample mean for each sensory attribute between individual panelist and the other 7 panelists are presented in Table 4.7. The correlation coefficients between each panelist 1, 2, 3, 6, 7 or 8 with the rest of the panel were generally very high for each sensory attribute with the exception of the "yeasty" attribute. However, correlation coefficients corresponding to panelists 4 and 5 were relatively low ($p >$

0.05) for most of the attributes. In addition, the F ratio and probability for the replication effect for each of the panelists shown in Table 4.8 indicate that Panelists 1, 2, 3, 6, 7, and 8 were highly reliable in terms of repeatability, while the replicates were significantly different for panelist 4 in the cereal attribute ($p \leq 0.05$) and for panelist 5 in beefy and yeasty attributes ($p<0.1$). Therefore, further analysis was conducted without including the ratings from panelists 4 and 5.

4.3.1.2. Sensory characteristics of the samples

Correlation between the 5 sensory attributes and the concentration of SBF and SPI in the samples are shown in Table 4.9. Roasted, beefy, and yeasty attributes were positively correlated with SBF concentration ($p \leq 0.05$) and negatively correlated with SPI concentration ($p \leq 0.05$) while soymilk-like and cereal notes were highly positively correlated with SPI ($p \leq 0.01$) and negatively correlated with SBF concentration ($p \leq 0.05$).

ANOVA indicated significant differences ($p \leq 0.001$) among different samples in the intensity of all 5 attributes. The mean intensity values of the 5 attributes and the results of Duncan's multiple comparison tests are shown in Table 4.10. For more effective visual comparison, changes in the 5 sensory attributes in SBF by adding SPI are illustrated in Figure 4.1(a).

Samples S1, S2, and S4 were strong in beefy, roasted and to a lesser extent, yeasty notes, while they were weak in soymilk-like and cereal notes. Samples S3 and S6 were relatively even in all the 5 attributes. Sample S5 was very strong in soymilk-like and cereal notes but very weak in roasted, beefy, and yeasty notes. In particular, S2, S4, and S6, showed stronger roasted, beefy, and yeasty characteristics compared to S1, S3, and S5, respectively, due to increased SBF concentration at the same SPI content. Among high SBF samples the 3 beef flavour attributes (roasted, beefy, and yeasty) decreased while soymilk-like and cereal notes increased significantly, in the order of S2, S4, and S6, corresponding to increasing SPI content. A similar trend was observed as a function of increasing SPI content in the low SBF samples S1, S3, and S5, but with even more extensive changes (decreasing roasted, beefy and yeasty notes, and increasing soymilk-like and cereal notes). Although there were some minor differences, the general trends

for heat treated samples were very similar to those observed for the non-heat treated samples, as shown in Figure 4.1(b).

PCA was performed and the principal component (PC) loadings and scores of the sensory attributes and the samples are depicted in Figure 4.2(a) and Figure 4.2(b). PC1, PC2 and PC3 explained 97.8 %, 1.0 % and 0.7 % of the total variance, respectively. The eigenvalues of PC2 and PC3 were relatively small, 0.0478 and 0.0342, respectively, compared to PC1, which had an eigenvalue of 4.8892. All the attributes were highly loaded on PC1, which was characterized by positive loadings for cereal and soymilk-like attributes and negative loadings for roasted, yeasty, and beefy attributes. As a result, the 5 sensory attributes can be largely divided into 2 groups. One group includes roasted, yeasty, and beefy notes, which have negative values for PC1 and are attributed to SBF, while the other group contains cereal and soymilk-like notes, which come from SPI and have positive values for PC1. The 12 samples can be described by positive or negative PC1 values, as shown in Figure 4.2(a). Roasted, beefy and yeasty characteristics were stronger while cereal and soymilk-like notes were weaker in the order of S2, S4, S1, S6, S3, and S5. By adding SPI, the roasted, beef and yeasty notes decreased while cereal and soymilk-like notes increased in both low SBF and high SBF samples. In fact, PC 1 could be used as a tool to differentiate the aroma characteristics among samples. From the comparison between S1 (low SBF and no SPI) and S4 (high SBF with medium SPI), it was noted that although the amount of SBF in S4 was increased more than 3-fold compared to S1, the beefy characteristics of S4 were suppressed by the addition of SPI resulting in similar degree of beefy characteristics between the two samples. A similar although less marked trend was observed in the comparison between S3 (low SBF with medium SPI) and S6 (high SBF with high SPI). It can be postulated that interaction between SBF and SPI considerably hindered the beefy notes from releasing in the samples.

While all the attributes were negatively loaded in PC2, yeasty notes were positively loaded in PC3, as shown in Figure 4.2(b). Consequently, PC3 dimension was mainly defined by the yeasty note, which was described as being related to cardboardy/rancid/off-flavour of the samples during the sensory training session, in contrast to the other 4 attributes as demonstrated in Figure 4.2(b). It was interesting to see that all the heat treated samples except sample S2H tended to be located

on upper position of the PC3 in Figure 4.2(b), implying stronger yeasty note of heated samples compared to non heat treated samples.

4.3.2. Selection of indicator peaks (IPs) for the beef flavour

It would be useful to select indicator peaks to represent the beefy attributes in samples in order to evaluate changes in beefy characteristics under varying treatment conditions. Indicator peaks have been applied in various ways. For instance, propanal analyzed by GC-MS was used as an indicator peak to determine antioxidant effect of spices in sardines (Kasahara 2004). Galactose determined by GC-FID was used as an index of heat treatment in milk (Chiesa et al., 1999), and filbertone ((E)-5-methylhept-2-en-4-one) analyzed by GC-FID was used to verify adulteration of olive oils in hazelnut oils (Blanch et al., 2000). Indicator peaks could be used as a multiple-compound-quality index, such as the application of several selected bacterial metabolites identified by GC-MS and GC-O to predict spoilage off-flavours in packed or smoked salmon (Jørgensen et al., 2001). In the study from Narziβ et al. (1999), a group of volatile compounds was selected as a "forcing index" that appeared or increased in the early stage of beer ageing while several Strecker aldehydes and furfural were chosen as an "ageing index" that increased mainly late in ageing of beer. In this study, indicator peaks consisting of multiple aroma compounds to represent beefy attribute in sample were considered since beefy characteristics arise from not one or a few compounds, but a combination of various subclasses of components.

To qualify as an indicator peak, first of all, the peak appearing in the GC chromatogram should be positively correlated (at $p \leq 0.05$) with attributes related to the characteristic beefy flavour in descriptive sensory analysis. Correlation analysis was conducted between the DA scores of the 5 sensory attributes with the concentrations of SBF and SPI and the area of peaks in GC chromatogram. The results are attached in Appendix I. Among 112 GC peaks, the areas of 75 peaks were significantly correlated with the specific sensory attributes ($p \leq 0.05$). Of these 75 peaks, 64 peaks were positively correlated with roasted, beefy, and yeasty notes while negatively correlated with soymilk-like and cereal notes. Eleven peaks were positively correlated with soymilk-like and cereal notes and negatively correlated with roasted, beefy, and yeasty notes.

However, even though the areas of peaks give us information on the intensity of electrical response by flame ionization detector, the size of a peak according to the concentration of the compound does not necessarily reflect the aroma intensity of the peak in a sample due to the different odour threshold and/or differences in detector sensitivity for different compounds. Hence, another criterion was added to select indicator peaks; it must be odour-active with greater than 50 % detection frequency as assessed by GC-O. The present results were analyzed in the context of the odour-active components in SBF previously tentatively identified by GC in conjunction with olfactometry (Moon et al., 2006). The results of the analysis showed that among the 64 peaks that were positively correlated with roasted, beefy, and yeasty notes, only 15 peaks were identified as odour-active compounds in SBF, with greater than 50 % detection frequency (i.e. 4 or more detection frequency out of 8 panelists) by GC-O analysis. However, 5 out of the 15 peaks were not able to be identified by GC-MS due to either small peak area or low quality of identification.

Therefore, based on the two criteria, 15 peaks, namely peaks with retention times 1.717, 7.832, 12.046, 15.772, 16.133, 16.864, 18.199, 19.744, 22.062, 24.103, 25.374, 25.703, 28.204, 30.291, and 35.680 minutes, were selected from the 112 peaks detected in the sample including SBF (Table 4.11). Investigation of the selected indicator peaks under various environmental conditions may provide useful information to monitor changes in beef attribute related flavour binding or releasing in sample mixture of SPI and SBF.

4.4. Conclusion

DA along with GC analysis was applied to investigate changes in aroma characteristics of SBF upon addition of SPI. Three attributes (beefy, roasted, and yeasty) for SBF and 2 attributes (soymilk-like and cereal) for SPI were selected by DA and used to assess various mixtures of SBF and SPI. The results from the sensory analysis confirmed that roasted, beefy and yeasty notes were highly positively correlated with SBF concentration in the samples containing mixtures of SBF and SPI, and the beefy related notes were severely suppressed by increasing SPI content. Heat treated samples showed generally similar tendency to those observed for the non-heat treated samples. Principal component analysis of the data revealed that the aroma

characteristics among samples could be differentiated by PC1, which was characterized by positive loadings for cereal and soymilk-like attributes and negative loadings for roasted, yeasty, and beefy attributes. Moreover, PC 3 was also useful to detect increased yeasty notes in heated samples compared to non heat treated samples.

Given the observation that addition of SPI to SBF significantly hindered the release of the beefy related aroma in the samples, indicator peaks were selected, which would represent beefy characteristics in the sample. By considering information obtained by GC-O analysis on aroma-impact compounds in SBF, 15 peaks were categorized as indicator peaks, which were all recognized as odour-active compounds in GC-O analysis, and at the same time were significantly positively correlated with beefy characteristics analyzed by DA. The indicator peaks may form the basis of further research to elucidate the mechanism of SPI-SBF interactions to explain the suppression of beef volatile flavour components in samples containing SPI.

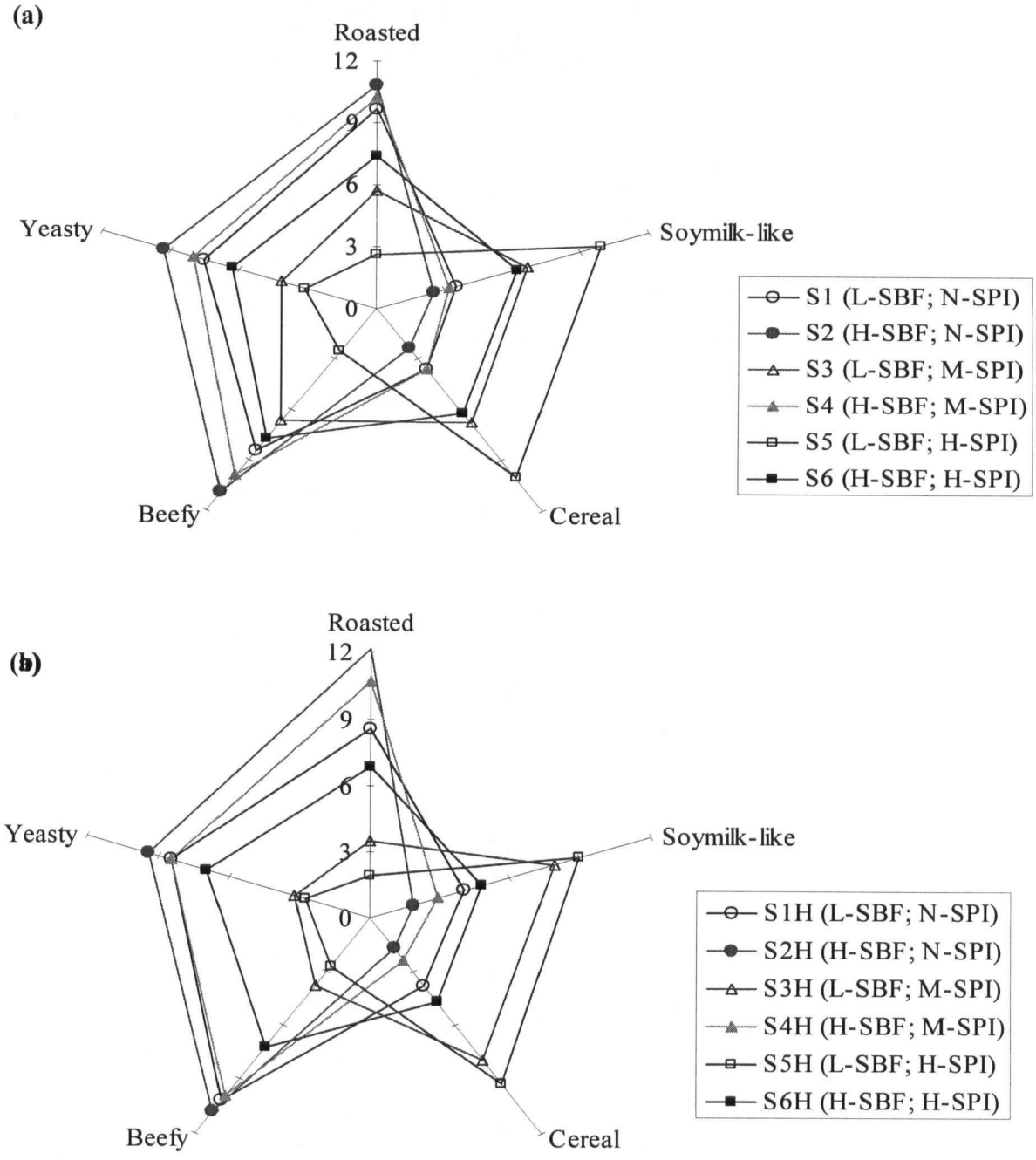

Figure 4.1. Cobweb diagram of the sensory scores from the descriptive analysis data for (a) 6 unheated mixtures of beef flavour and soy protein isolate (b) 6 heated (98 °C, 30 minutes) mixtures (n=12; 6 selected panelists with 2 replications).

L-SBF and H-SBF represent low (150 mg) and high (500 mg) dose of simulated beef flavour while N-SPI, M-SPI, and H-SPI symbolize no (0 mg), medium (250 mg), and high (500 mg) amount of soy protein isolate in the sample. Refer to Table 4.3 for the sample codes.

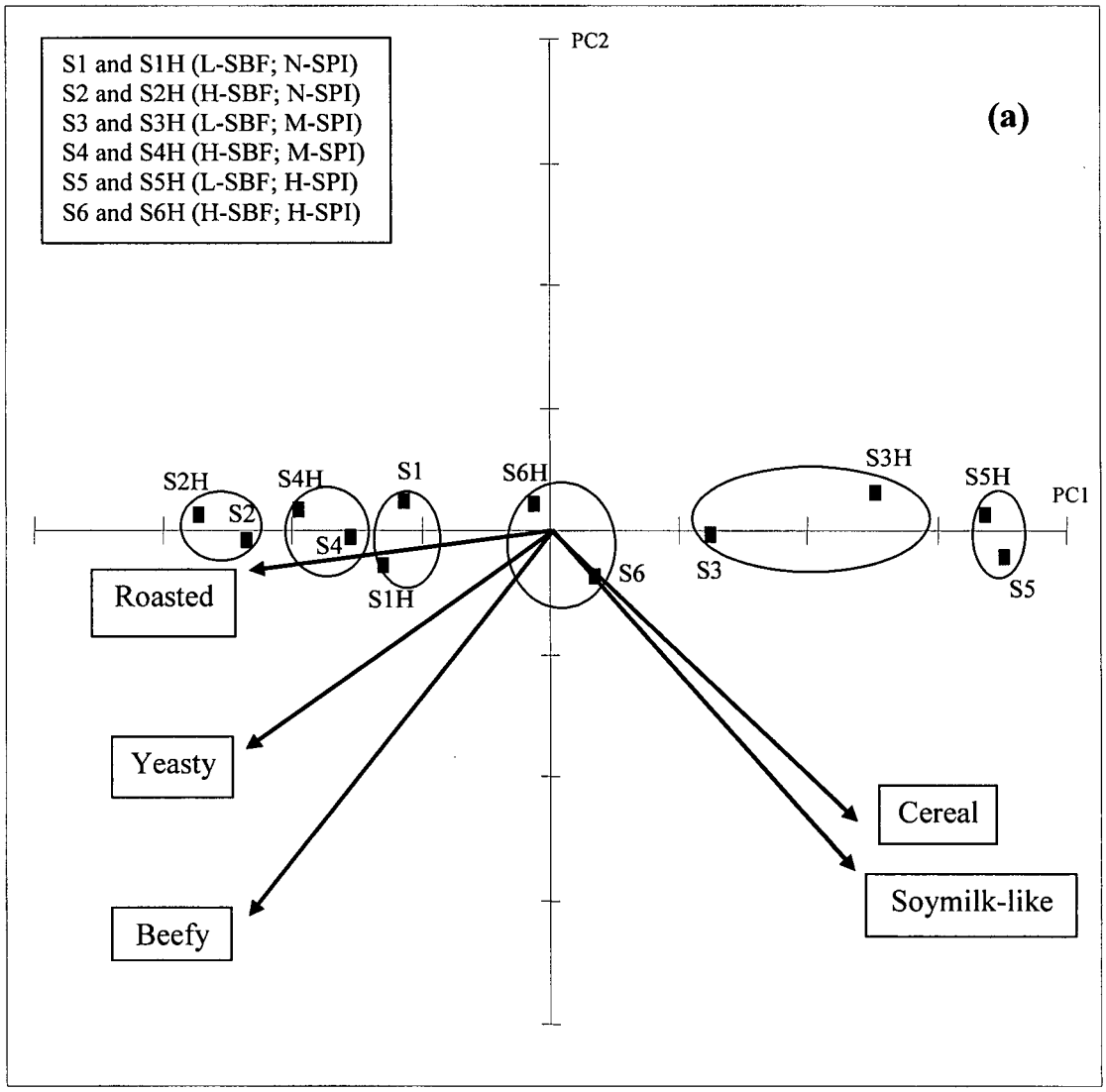

Figure 4.2(a). PC loadings and scores of the sensory attributes and the sample mixtures by principal component analysis; PC1 versus PC2.

L-SBF and H-SBF represent low (150 mg) and high (500 mg) dose of simulated beef flavour while N-SPI, M-SPI, and H-SPI symbolize no (0 mg), medium (250 mg), and high (500 mg) amount of soy protein isolate in the sample. Refer to Table 4.3 for the sample codes.

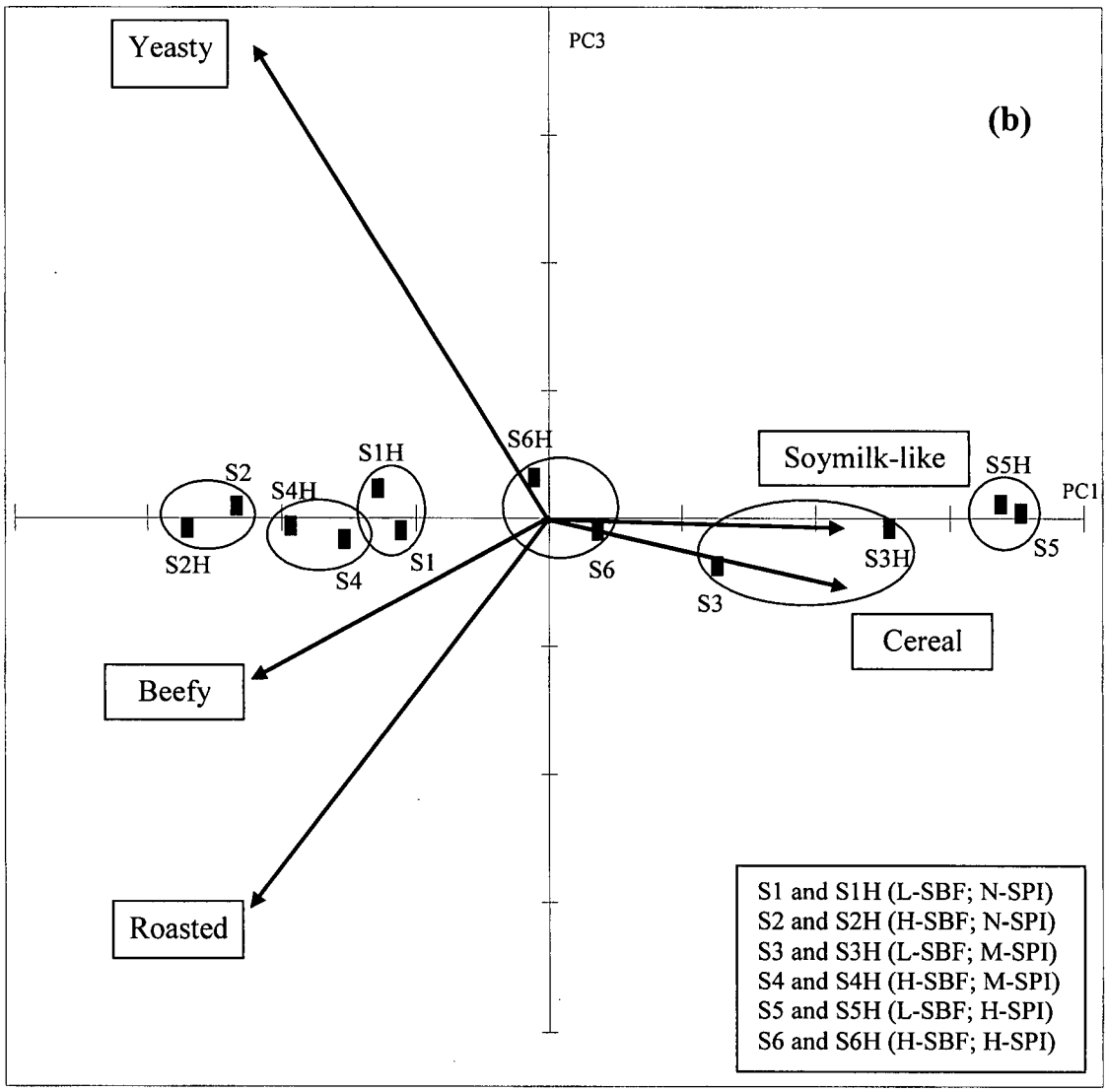

Figure 4.2(b). PC loadings and scores of the sensory attributes and the sample mixtures by principal component analysis; PC1 versus PC3.

L-SBF and H-SBF represent low (150 mg) and high (500 mg) dose of simulated beef flavour while N-SPI, M-SPI, and H-SPI symbolize no (0 mg), medium (250 mg), and high (500 mg) amount of soy protein isolate in the sample. Refer to Table 4.3 for the sample codes.

Table 4.1. Description and references for aroma terms used in the sensory training session for descriptive analysis of simulated beef flavour and soy protein isolate.

Terms	Characteristics	Reference
Brothy, oxobeef-like, miso-like	The smell associated with beef broth or oxo-beef solution.	4.5 g of OxoBeef powder (Knorr OXO Beef Bouillon) in 175 mL water
Roasted, dry cooked beef, barbecue	The smell associated with roasted or barbecued beef.	Surface crust of roasted beef cooked with Club House seasoned salt (McCormick) at 190 °C for 25 minutes and roasted 10 more minutes after turning over
Beefy	The smell associated with beef, as differentiated from pork or chicken meat.	Interior of roasted beef cooked at 190 °C for 25 minutes and cooked 10 more minutes after turning over
Rare, raw, uncooked	The smell associated with bloody raw beef.	Fresh extra lean ground beef
Cardboardy, rancid, off-flavour, yeasty	The smell associated with off flavour when beef has gone bad.	Wet cardboard (15 cm × 30 cm) and yeast extract powder
Browned caramel, sweet, candy-like	The smell associated with caramel.	Melted Werther's Original Caramel
Soymilk-like	The smell associated with volatile matter from soymilk.	Soymilk (Sunrise Soya Beverage, unsweetened)
Cooked cereal	The smell associated with hot cooked cereal.	Instant oatmeal (Quaker Oatmeal Regular; 1 packet in 150 mL boiling water)
Straw, hay-like	The smell associated with dry straw or hay.	Hay supplied from the Dairy Research Center at UBC

Table 4.2. Definition of the 5 selected sensory attributes agreed upon by panelists through the training sessions in the descriptive analysis for simulated beef flavour and soy protein isolate.

Descriptors	Definition
Roasted	A beef-related top note, associated with the degree of "roast beef crusty surface" that the sample represented.
Beefy	The lingering characteristic note, associated with as "beef" in contrast to pork or chicken meat.
Yeasty	The off-flavour agar-like note and as a roasted smell gone bad.
Soymilk-like	The green note associated with cooked soybeans.
Cereal	The note associated with cooked oatmeal or cooked pasta.

Table 4.3. Samples used in the sensory evaluation and GC analysis.

Code	SBF[2]	SPI[3]	Distilled de-ionized water	Heat Treatment[1]
S1	150 mg	0 mg	5 g	No
S1H	150 mg	0 mg	5 g	Yes
S2	500 mg	0 mg	5 g	No
S2H	500 mg	0 mg	5 g	Yes
S3	150 mg	250 mg	5 g	No
S3H	150 mg	250 mg	5 g	Yes
S4	500 mg	250 mg	5 g	No
S4H	500 mg	250 mg	5 g	Yes
S5	150 mg	500 mg	5 g	No
S5H	150 mg	500 mg	5 g	Yes
S6	500 mg	500 mg	5 g	No
S6H	500 mg	500 mg	5 g	Yes

[1]Sample vials were heated in water bath at 98 °C for 30 minutes.

[2]150 mg SBF and 500 mg SBF were also expressed as low SBF and high SBF as well as L-SBF and H-SBF throughout this thesis.

[3]0 mg of SPI, 250 mg SPI and 500 mg SPI were also expressed as no SPI, Medium SPI, and high SPI as well as N-SPI, M-SPI, and H-SPI throughout this thesis.

Table 4.4. Summary of analyses of variance with F values, mean squares of error (MSE), and degrees of freedom (df) for main effects and their interactions for each of the five attributes.

	F values						MSE
	Sample (S) (df = 11)	Panelist (P) (df = 7)	Replication (R) (df = 1)	S×P (df = 77)	P×R (df = 7)	S×R (df = 11)	
Roasted	21.06***	2.33*	3.35	1.19	0.70	0.86	6.42
Soymilk	17.31***	6.94***	0.09	1.18	0.67	3.16**	5.19
Cereal	14.18***	7.42***	2.49	0.96	0.86	2.32*	6.28
Beefy	15.74***	1.54	4.34*	1.24	1.05	1.26	6.66
Yeasty	8.53***	11.66***	2.02	1.44	0.89	0.90	7.27

*, **, and ***, significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Table 4.5. Adjusted F values of the sample effects using mean squares of sample by replication instead of mean squares of error for each of the five attributes.

Attributes	MS_{sample}	MS_{error}	$MS_{sample \times replication}$	Original F value	Adjusted F value
Roasted	135.24	6.42	5.52	21.06***	24.49***
Soymilk-like	89.94	5.19	16.41	17.31***	5.48**
Cereal	88.97	6.28	14.53	14.18***	6.12**
Beefy	104.77	6.66	8.39	15.74***	12.48***
Yeasty	61.98	7.27	6.57	8.53***	9.44***

** and ***, significant at $p \leq 0.01$, and $p \leq 0.001$, respectively.

Table 4.6. Results of Duncan's multiple comparison test on mean sensory scores of each panelist for 5 attributes[1].

Roasted		Soymilk-like		Cereal		Beefy		Yeasty	
Panelist	Mean score	Panelist	Mean score	Panelist	Mean score	Panelist	Mean score	Panelist	Mean score
8	6.36 a	4	2.55 a	4	3.17 a	5	5.98 a	4	2.63 a
6	6.41 ab	3	4.15 b	6	3.82 ab	6	6.79 ab	6	3.38 b
5	7.12 abc	7	4.90 bc	3	4.13 abc	3	7.41 ab	8	5.31 c
2	7.58 abc	6	5.13 bc	7	5.27 bcd	2	7.47 ab	5	5.95 cd
3	7.77 abc	8	5.30 bc	8	5.52 cd	4	7.49 ab	2	6.76 cde
4	8.09 bc	2	5.83 cd	1	5.96 de	7	7.81 b	1	7.22 de
1	8.26 c	1	5.98 cd	2	6.45 de	1	7.90 b	3	7.80 e
7	8.59 c	5	6.96 d	5	7.58 e	8	7.95 b	7	8.31 e

[1]Mean scores for each attribute within a column with different letters are significantly different ($p \leq 0.05$) using Duncan's multiple comparison test. Scores are listed in ascending order (n=24; 12 samples with 2 replications).

Table 4.7. Pearson correlation coefficients and probabilities of sample means for each sensory attribute between individual panelist and the other 7 panelists[1].

Panelist		Attributes				
		Roasted	Soymilk-like	Cereal	Beefy	Yeasty
Panelist 1	Coefficient	**0.914**	**0.918**	**0.904**	**0.735**	**0.835**
	(P)	(0.000)	(0.000)	(0.000)	(0.006)	(0.001)
Panelist 2	Coefficient	**0.873**	**0.867**	**0.954**	**0.878**	**0.850**
	(P)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
Panelist 3	Coefficient	**0.965**	**0.884**	**0.869**	**0.953**	**0.870**
	(P)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
Panelist 4	Coefficient	0.419	**0.680**	0.375	0.228	-0.098
	(P)	(0.175)	(0.015)	(0.230)	(0.476)	(0.774)
Panelist 5	Coefficient	**0.726**	**0.670**	0.517	0.493	0.478
	(P)	(0.008)	(0.017)	(0.085)	(0.103)	(0.116)
Panelist 6	Coefficient	**0.944**	**0.681**	**0.777**	**0.904**	0.137
	(P)	(0.000)	(0.015)	(0.003)	(0.000)	(0.671)
Panelist 7	Coefficient	**0.907**	**0.845**	**0.867**	**0.896**	**0.875**
	(P)	(0.000)	(0.001)	(0.000)	(0.000)	(0.000)
Panelist 8	Coefficient	**0.808**	**0.744**	**0.820**	**0.896**	0.470
	(P)	(0.001)	(0.006)	(0.000)	(0.000)	(0.123)

[1]Bold letters indicate Pearson correlation coefficients greater than 0.600, which are statistically significant at $p \leq 0.05$ (n=12; means of 12 samples).

Table 4.8. F ratio and probability of replication effect on the 5 attributes for individual panelist[1].

Panelist		Attributes				
		Roasted	Soymilk-like	Cereal	Beefy	Yeasty
Panelist 1	F ratio	0.13	0.34	0.00	0.16	0.28
	(P)	(0.717)	(0.567)	(0.963)	(0.693)	(0.599)
Panelist 2	F ratio	0.15	0.20	0.16	0.09	0.01
	(P)	(0.698)	(0.657)	(0.691)	(0.768)	(0.934)
Panelist 3	F ratio	0.73	0.07	0.06	1.17	0.69
	(P)	(0.403)	(0.795)	(0.812)	(0.292)	(0.414)
Panelist 4	F ratio	1.16	0.15	5.06	0.47	0.80
	(P)	(0.294)	(0.706)	**(0.036)**	(0.502)	(0.383)
Panelist 5	F ratio	0.01	2.23	0.75	2.99	3.04
	(P)	(0.928)	(0.150)	(0.395)	**(0.098)**	**(0.095)**
Panelist 6	F ratio	0.04	0.01	0.36	0.24	2.51
	(P)	(0.847)	(0.918)	(0.558)	(0.632)	(0.128)
Panelist 7	F ratio	0.58	0.14	0.26	1.34	0.40
	(P)	(0.454)	(0.713)	(0.612)	(0.259)	(0.533)
Panelist 8	F ratio	0.59	0.07	0.37	0.74	1.22
	(P)	(0.451)	(0.788)	(0.549)	(0.399)	(0.2810

[1]Bold letters indicate statistically significant replication effects at $p \leq 0.1$ (n=24; 12 samples with 2 replications).

Table 4.9. Pearson correlation coefficients and probabilities between mean sensory score for each sensory attribute and the concentration of SBF and SPI.

Attributes		SBF	SPI
Roasted	Coefficient	0.662*	-0.692*
	(P)	(0.019)	(0.013)
Soymilk-like	Coefficient	-0.634*	0.714**
	(P)	(0.027)	(0.009)
Cereal	Coefficient	-0.638*	0.711**
	(P)	(0.026)	(0.010)
Beefy	Coefficient	0.626*	-0.696*
	(P)	(0.030)	(0.012)
Yeasty	Coefficient	0.668*	-0.659*
	(P)	(0.017)	(0.020)

* and **, significant at $p \leq 0.05$ and $p \leq 0.01$, respectively (n=12; means of 12 samples).

Table 4.10. The mean intensity values of the 5 attributes for the 12 mixtures of SBF and SPI in descriptive sensory evaluation[1].

Roasted		Soymilk-like		Cereal		Beefy		Yeasty	
Sample	Mean score	Sample	Mean score	Sample	Mean score	Sample	Mean score	Sample	Mean score
S5H	1.93 a	S2H	1.85 a	S2H	1.73 a	S5	2.54 a	S5H	2.80 a
S5	2.66 a	S2	2.53 ab	S2	2.32 ab	S5H	2.69 a	S5	3.11 a
S3H	3.46 ab	S4H	2.88 ab	S4H	2.33 ab	S3H	3.73 a	S3H	3.20 a
S3	5.69 bc	S4	3.25 ab	S4	3.57 ab	S3	6.71 b	S3	4.17 ab
S6H	6.83 cd	S1	3.52 ab	S1	3.65 ab	S6H	7.19 b	S6	6.32 bc
S6	7.39 cde	S1H	4.02 ab	S1H	3.73 ab	S6	7.72 bc	S6H	6.97 cd
S1H	8.53 def	S6H	4.73 bc	S6H	4.69 bc	S1	8.47 bcd	S1	7.50 cd
S1	9.63 ef	S6	6.25 cd	S6	6.19 cd	S4H	9.92 cd	S4	7.97 cd
S4	10.23 fg	S3	6.73 cd	S3	6.84 cde	S4	9.93 cd	S1H	8.43 cd
S4H	10.63 fg	S3H	7.87 de	S3H	7.91 def	S1H	10.13 cd	S4H	8.43 cd
S2	10.82 fg	S5H	8.98 e	S5H	9.25 ef	S2H	10.78 d	S2	9.29 d
S2H	12.14 g	S5	9.98 e	S5	9.99 f	S2	10.88 d	S2H	9.41 d

[1]Mean scores for each attribute within a column with different letters are significantly different ($p \leq 0.05$) using Duncan's multiple comparison test (n=12; 6 panelists with 2 replications). Scores are listed in ascending order. Refer to Table 4.3 for the sample code.

Table 4.11. The 15 selected indicator peaks for the simulated beef flavour.

Indicator peak	RT	Peak identification (ID No.)[1]	Detection frequency[2] (%)	Pearson correlation coefficient for beefy attribute
IP1	1.717	3-Methyl furan (P1)	50	0.710*
IP2	7.832	2-Acetyl furan (P7)	75	0.658*
IP3	12.046	delta-3-Carene (P17)	88	0.872***
IP4	15.772	2-Ethyl-3,6-dimethyl pyrazine (P24)	88	0.601*
IP5	16.133	Unknown	100	0.852***
IP6	16.864	Unknown	100	0.827**
IP7	18.199	Unknown	75	0.605*
IP8	19.744	2,3-Diethyl-5-methylpyrazine (P30)	50	0.590*
IP9	22.062	Decanal (P35)	75	0.715**
IP10	24.103	2-Isoamyl-6-methylparazine (P38)	50	0.599*
IP11	25.374	Unknown	75	0.598*
IP12	25.703	Unknown	75	0.794**
IP13	28.204	delta-Elemene (P44)	50	0.931***
IP14	30.291	beta-Cubebene (P49)	63	0.850***
IP15	35.680	Calamenene (P71)	75	0.883***

[1]Peak numbers (P#) and tentative identification by GC-MS analysis as reported previously (Moon et al., 2006).
[2]Frequency of detection by 8 panelists at a sniffing port in GC-O as reported previously (Moon et al., 2006).
*, **, and ***, significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

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CHAPTER 5. ASSESSMENT OF ADDED INGREDIENT EFFECT ON THE INTERACTION OF SIMULATED BEEF FLAVOUR AND SOY PROTEIN ISOLATE BY GAS CHROMATOGRAPHY AND SPECTROSCOPIC TECHNIQUES[4]

5.1. Introduction

Soy protein has gained increasing attention as a promising protein source with various health benefits in addition to functional properties. Due to the problematic retention of indigenous off-flavour, which has been attributed primarily to lipid oxidation products arising from the action of lipoxygenases present in soybean, much research has been conducted to investigate the interaction of soy protein with model compounds including aldehydes, ketones and alcohols. In addition to investigating indigenous, undesirable off-flavour related compounds of soy products, research is required to elucidate the binding and release of intentionally added, desirable flavour components by soy protein. For example, this type of interaction is important in formulation of soy-based products with simulated meat flavours for the vegetarian market (Malcolmson and McDaniel, 1987).

Interaction of flavour compounds with soy proteins in model systems has been reported (Damodaran and Kinsella, 1981a and 1981b; Gremli, 1974). The types and degree of interaction between flavours and soy protein are dependent on characteristics of the flavour compounds in addition to the environment of the components. Wilson (1985) proposed that hydrogen bonding was involved in flavour binding to soy proteins in non-aqueous systems. Damodaran and Kinsella (1981a) reported that the interaction of carbonyls with soy proteins in the native state was due to hydrophobic forces, and that the binding affinity for 2-nonanone was increased by partial denaturation of soy protein, which might enhance the hydrophobicity of the binding site via reorganization of the subunits. Gremli (1974) reported that aldehydes, especially unsaturated compounds, reacted more strongly with the proteins than ketones while alcohols did not interact with soy protein. No binding affinity of carboxylic acid (Beyeler and Solms, 1974) or hexane

[4] A version of this chapter will be submitted for publication. Moon, S. -Y., and Li-Chan, E. C. Y. Assessment of added ingredient effect on the interaction of simulated beef flavour and soy protein isolate by gas chromatography and spectroscopic techniques. *The Journal of the American Oil Chemists' Society.*

(Thissen, 1982) with soy protein was reported. Beyeler and Solms (1974) indicated that binding properties of model flavour compounds including ketones and aldehydes with soy protein isolate (SPI) were rather independent of pH and temperature, while binding of vanillin with soy protein was decreased with increasing temperature (Li et al., 2000). Moreover, Zhou and Cadwallader (2006) reported that hydrocarbons showed weak interaction with SPI whereas alcohol compounds strongly interacted with SPI, suggesting that hydrogen bonding was involved. Ester, ketone and aldehyde compounds interacted with SPI with similar forces and Zhou and Cadwallader (2006) proposed that hydrogen bonding, dipole forces, and van der Waals dispersion forces might be involved. Some conflicting data in terms of binding properties of flavour compounds to soy proteins were found in the literature, which may be attributed to the differences in methods used, origin and degree of denaturation of soy protein material during preparation and the experimental conditions applied in addition to different composition of buffer, which could affect the interaction between the protein and flavour compounds (O'Keefe et al., 1991).

Most of the studies conducted to date have used model systems of single ingredients or selected volatile model compounds such as series of aldehydes, ketones, alcohols, or alkanes. Although valuable thermodynamic information such as binding affinity or number of binding sites was acquired from the results of these studies, the knowledge may not be directly applicable to the real food system, in which the flavour ingredients usually contain intricate combinations of various subclasses of compounds. Soy protein could also bind with certain desirable flavour compounds. Depending on the nature and the strength of the binding, each aroma compound could be released at a different rate from SPI, which could have an impact on flavour suppression or alteration of flavour profiles in the final food products due to changes in the odour balance. Therefore, for the development of soy products with acceptable flavour quality it is imperative to elucidate the nature of the interactions of soy proteins with the mixture of compounds present in the actual flavour ingredients.

Recently, research has been conducted to elucidate the impact of SPI on aroma characteristics of a commercially produced simulated beef flavour (SBF) ingredient. By using headspace solid phase micro-extraction coupled with gas chromatography (GC), GC-mass spectrometry (GC-MS)

and GC-olfactometry (GC-O) analysis, the odour-active components were recognized, and several high aroma impact compounds from the volatile compounds in SBF were identified (Moon et al., 2006). Descriptive analysis (DA) and GC analysis were conducted to assess the aroma characteristics of SBF in mixtures with SPI, and the volatile compounds in SBF that were related to a "beefy" note were identified. Several indicator peaks were selected to represent the beefy aroma notes. Addition of SPI resulted in decreasing of "beefy", "roasted", and "yeasty" aroma notes in the samples (Chapter 4 of this thesis).

In this study, the interaction between SPI and SBF was examined by addition of compounds intended to produce changes in the potential forces which may affect SPI conformation. Specifically, alteration of several types of interaction was carried out by adding a third component in order to explore the effect of conformational changes of SPI on its flavour holding capacity, particularly with regard to beef attribute related aroma compounds in SBF. Four components, i.e. glucosamine, sucrose, ascorbic acid, and polyethylene glycol, were selected based on their functional characteristics as food ingredients or food additives and potential to alter hydrogen bonding, hydrophobic interactions, and disulfide bonds in SPI. Sucrose (β-D-fructofuranosyl-α-D-glucopyranoside) is a disaccharide containing poly-hydroxyl groups, while glucosamine (2-amino-2-deoxy-D-glucose) is an amino sugar with amino as well as hydroxyl groups. Both sucrose and glucosamine can serve as hydrogen donors for hydrogen bonding through the hydroxyl group (-OH) and/or amino group ($-NH_2$). Polyethylene glycol is a polymer of ethylene oxide which may affect hydrophobicity of proteins through the repeating hydrophobic monomeric unit ($-CH_2-CH_2-$). It can also act as a hydrogen bond donor but more likely serves as a hydrogen acceptor, due to the –OH group and unshared electron pairs on the oxygen atom, respectively. Ascorbic acid is an organic acid with antioxidant properties. It is easily oxidized to dehydroascorbic acid, which is relatively stable. The application of ascorbic acid as a reducing agent in biological systems was reported (Kashiba-Iwatsuki et al., 1997). Ascorbic acid may also induce disulfide-sulfhydryl interchange reactions through cleavage and reformation of disulfide bonds (Dong and Hoseney, 1995). Moreover, it is known that reducing agents such as cysteine, ascorbic acid, β-mercaptoethanol, and dithiothreitol reduce disulfide cross-links and hence modify the conformation of proteins (Cheftel et al., 1985). Therefore, ascorbic acid could affect the status of disulfide bonds or sulfhydryl groups of SPI, which may lead to conformational

changes in the protein. In addition to the effect of each ingredient, the combined effect of ascorbic acid and polyethylene glycol on SPI was also investigated in this study to examine possible additive or synergistic effects since the reduction of disulfide bonds may enhance conformational changes induced by hydrophobic interactions, which generally tend to be located on interior part of proteins.

Among parameters affecting protein structures, hydrophobicity was reported to be extensively related to the functional properties of proteins (Nakai, 1983). Therefore a fluorescence probe method using 6-propionyl-2(N,N-dimethyl-amino)naphthalene (PRODAN) was conducted to determine surface hydrophobicity of SPI affected by the ingredients, by measuring changes in fluorescence upon binding of the probe to accessible hydrophobic regions of the protein in aqueous solution. Compared to the anionic fluorescent probes such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and cis-parinaric acid (CPA), PRODAN is a neutral probe, which could minimize possible contribution of electrostatic interaction between fluorescent probe and protein (Alizadeh-Pasdar and Li-Chan, 2000). In addition, conformational changes of SPI by addition of the ingredients were assessed by FT-Raman spectroscopy, which has been reported as a useful tool to investigate in situ protein structural changes under various conditions relevant to processing (Li-Chan, 1996).

Therefore, the objectives of the study in Chapter 5 were (a) to investigate the changes detected by GC on the flavour holding behavior of the SPI in terms of odour-active compounds contributing to beefy notes in SBF as affected by the ingredients such as glucosamine, sucrose, ascorbic acid, and polyethylene glycol, (b) to monitor changes in the protein structure in SPI induced by the ingredients by means of disulfide and sulfhydryl group measurement, fluorescence probe and FT-Raman spectroscopy to understand the effect of each ingredient on the holding capacities of beef volatile flavour components in samples containing SPI and (c) to identify sensory impact on the mixture of SBF and SPI treated ingredient. The results from this study may provide a foundation for better understanding of the interaction between SPI and SBF.

5.2. Experimental methods

5.2.1. Materials

Commercially available simulated beef flavour (SBF; Mastertaste, Arlington Heights, IL) and soy protein isolate (SPI; Solae, St. Louis, MO) were used, as described in Section 2.2.1 and Section 4.2.1, respectively. Sucrose, ascorbic acid (min. 99 %), glucosamine (min. 99 %), and polyethylene glycol (average Mw. 8,000) were purchased from Sigma Chemical Co. (St. Louis, MO) and spectral-grade methanol was from Fisher Scientific (Fairlawn, NJ). The fluorescent probe, 6-propionyl-2-(dimethylamino) naphthalene (PRODAN), and Ellman's reagent, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). The solid phase assembly holder, 50/30 μm stableflex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 15 mL capacity GC sample vials and polypropylene hole cap with PTFE/silicone septa were purchased from Supelco (Sigma-Aldrich Canada, Oakville, ON).

5.2.2. Differential scanning calorimetry (DSC)

The thermal behavior of SPI was investigated by a multi-cell differential scanning calorimeter thermal analyzer (model 4207 MC-DSC, Calorimetry Science Corp). Three concentrations of SPI (0.2 %, 5 %, and 10 %) were prepared in pH 7.5 phosphate buffer (3.88 mM NaH_2PO_4, 15.5 mM Na_2HPO_4). Approximately 0.05 g of each sample was placed in Hastelloy C ampoules, which were sealed then weighed accurately. A sealed empty cell was used as a reference. For DSC analysis, each sample was held at 25 °C and equilibration time was set at 600 seconds. Heating scan was conducted from 25 °C to 105 °C at a rate of 1 °C/min for each sample.

5.2.3. Gas chromatography

For headspace solid phase microextraction (HS-SPME), 100 mg of each ingredient i.e. glucosamine, sucrose, polyethylene glycol, or ascorbic acid, was first mixed with 250 mg SPI in a 15 mL capacity GC sampling vial with a magnetic stirring bar. Following addition of 5 g buffer (0.05 M Tris-HCl buffer pH 7.4), the vial was incubated at either room temperature (RT; 23 °C) or 60 °C for 20 minutes to compare potential changes due to heat treatment. To investigate the effects of a mixture of polyethylene glycol and ascorbic acid, the sample was first incubated with

100 mg of ascorbic acid for 20 minutes at RT or 60 °C, and then 100 mg of polyethylene glycol was added and the mixture was incubated for another 20 minutes at the same temperature before adsorption.

SBF (500 mg) was added to the vial containing the incubated SPI and ingredients, and the vial was tightly capped with a polypropylene hole cap with a PTFE/silicone septum. As a control, a sample containing SPI and SBF was prepared without any additional ingredients and analyzed under the same condition. A sample with only SBF (no SPI) was also analyzed. Stirring with a magnetic stirring bar was consistently applied.

To extract headspace volatile compounds, adsorption was performed with 50/30 μm DVB/CAR/PDMS SPME fibre exposed to the headspace above the sample solution for 60 minutes at 60 °C in a thermostat controlled water bath (± 2 °C). Analysis of the samples adsorbed at RT was also conducted to observe changes in volatile components induced by adding ingredients to SPI in the absence of heat. Analysis of volatile components was performed by gas chromatography – flame ionization detector as previously described by Moon and Li-Chan (2004). The effect of each ingredient at each temperature condition was analyzed in triplicate with 3 independently prepared samples and the results were presented as the mean of the triplicate values. In addition, SBF alone without either SPI or ingredients as well as SBF with SPI but no ingredients were also analyzed in triplicate; the average coefficients of variation for individual peak areas of these triplicates were 10.1 % and 10.6 %, respectively. The area of each individual peak in each sample was expressed as relative peak area (%) based on the peak area of SBF (100 %) for comparison purpose.

5.2.4. Sulfhydryl and disulfide content

SPI (250 mg) and ingredient (100 mg of glucosamine, sucrose, polyethylene glycol, or ascorbic acid) were placed in a 15 mL sample vial together with 10 mL pH 8.0 buffer (85 mM Tris, 100 mM glycine, 4 mM EDTA, 10 mg/mL SDS). The samples were heated in a water bath at 60 °C for 20 minutes with stirring. For the sample mixture of ascorbic acid and polyethylene glycol, SPI was first incubated at 60 °C for 20 minutes with ascorbic acid only, prior to addition of

polyethylene glycol and further incubation for another 20 minutes at 60 °C. Ingredient blank samples were prepared as above but without any SPI.

The content of total sulfhydryl groups (SH) was determined with Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid or DTNB) (Ellman, 1959) by the method of Beveridge et al. (1974) as modified by Chung et al. (2005). Freshly prepared Ellman's reagent solution (200 µL of 4 mg/ml) was added to each tube containing 3 mL of sample and 3 mL of the pH 8.0 buffer. The tubes were covered with aluminum foil and incubated in an oven at 40 °C for 15 minutes, then centrifuged at 14950 *g* for 30 minutes at 10 °C Absorbance at 412 nm of each sample was measured on a Shimadzu UV1700 UV/visible spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD). Net A_{412} of sample was calculated as:

Net A_{412} = A_{412} of sample with Ellman's reagent – protein blank – ingredient blank,

where protein blank contained only protein samples without Ellman's reagent and ingredient blank contained the ingredient in the buffer with the reagent but no protein. The sulfhydryl content was calculated based on an extinction coefficient of 13,600 $M^{-1}cm^{-1}$ (Ellman, 1959), as shown in the following equation:

$$\mu\text{mole SH/g protein} = 73.53 \times \text{net } A_{412}\,(D/C) \qquad \text{[equation 11]}$$

where D was the protein concentration in mg/mL and C was a dilution factor. Results were expressed as the mean of duplicate analyses.

Determination of total sulfhydryl and disulfide content (SH+SS) in the samples was conducted by the method of Thannhauser et al. (1984), with incubation in the dark as suggested by Damodaran (1985). The 2-nitro-5-thiosulfobenzoate (NTSB) stock solution was prepared by dissolving 100 mg of DTNB in 10 mL of 1 M sodium sulfite and adjusting to pH 7.5; oxygen was bubbled through the solution held in a water bath at 38 °C or 2 hours until the bright red colour turned into a pale-yellow colour. The NTSB stock solution was divided into 400 µL aliquots and stored frozen at –18 °C until used for analysis.

The NTSB stock solution was diluted in a ratio of 1 to 100 with a freshly made solution at pH 9.5 containing 2.0 M guanidine thiocyanate, 0.2 M Tris, 100 mM sodium sulfite and 3 mM EDTA to make NTSB assay solution. 200 µL of protein sample in pH 8.0 buffer described above was

added to 3.0 mL of the NTSB assay solution. After incubating in the dark for 30 minutes followed by centrifugation at 14950 *g* for 30 minutes at 10 °C, the absorbance at 412 nm was recorded against a blank containing 3 mL of NTSB assay solution and 200 µL of dd-water. The content of (SH+SS) was calculated as described above in equation 11. The content of disulfide (SS) groups was determined by subtracting the content of total SH from the content of (SH+SS). Results were expressed as the mean of duplicate analyses.

5.2.5. Surface hydrophobicity

Surface hydrophobicity of SPI samples was determined by the hydrophobic fluorescent probe method using PRODAN as described by Alizadeh-Pasdar and Li-Chan (2000). PRODAN stock solution was prepared by dissolving 3.2 mg PRODAN in 10 mL spectral-grade methanol; 1 mL aliquots in 1.5 mL Eppendorf tubes wrapped with aluminum foil were stored at –18 °C for future use. The concentration of the PRODAN stock solution was spectrophotometrically determined at 360 nm as 1.8×10^{-3} M using a molar absorption coefficient ε_{360} of 1.8×10^4 $M^{-1}cm^{-1}$ (Alizadeh-Pasdar and Li-Chan, 2000). SPI stock solution was prepared to contain 1 % (w/v) SPI in Tris buffer (0.05 M, pH 7.4) with 0.02 % sodium azide. To prepare ingredient treated stock solutions, 10 mL SPI stock solution was mixed with the ingredient (40 mg of glucosamine, sucrose, polyethylene glycol, or ascorbic acid). Ingredient blank samples were prepared using buffer without any SPI. The samples were heated at 60 °C for 20 minutes. Additional heating at 60 °C for 20 minutes was employed after adding polyethylene glycol for the sample mixture of ascorbic acid and polyethylene glycol. After cooling down the samples in cold water for 15 minutes, serial dilution was performed to obtain 5 SPI concentrations ranging from 0.010 % to 0.0025 %.

For measurement of surface hydrophobicity, 10 µL PRODAN was added to 4 mL of each SPI dilution and vortexed. After incubating 15 minutes in the dark, the relative fluorescence intensity (RFI) of samples was measured by a Shimadzu RF-540 (Shimadzu Corp., Kyoto, Japan) spectrofluorometer. The excitation/emission wavelengths were 365 nm/465 nm and excitation/emission slits were set at 5 nm/5 nm. All procedures dealing with PRODAN were conducted in a dark room due to its light sensitive nature and the analyses were carried out in triplicate. Standardization was performed to correct for day-to-day fluctuations of the instrument by measuring the RFI of 10 µL of PRODAN in 4 mL methanol and adjusting to a standard value

of 50. RFI was also measured for blanks, which contained SPI and/or ingredients but no PRODAN. Net RFI was obtained by subtracting the RFI of the blank sample without PRODAN from the RFI of the sample with PRODAN. The initial slope (S_0) of net RFI versus SPI concentration was calculated by linear regression analysis and reported as surface hydrophobicity of the sample.

5.2.6. FT-Raman spectroscopy

Samples containing SPI with or without ingredient were prepared as described in Section 5.2.2. (GC analysis) and then freeze-dried due to the poor signal to noise ratio of the spectra of the aqueous samples observed in preliminary experiments. The freeze-dried samples and original (not freeze-dried) SPI powder were placed in NMR tubes and FT-Raman analyses were conducted using a Nexus 670 FT-Raman spectrometer equipped with CaF_2 beam splitter and Ge detector (Thermo Nicolet Corp., Madison, WI). The analyses for each sample were performed under the following conditions: laser power, 0.5 watt; scan number, 512; spectral resolution, 4 cm^{-1}. The spectrum of polystyrene standard was used to adjust sample position for maximal signal intensity to verify the system before analyzing samples. The spectrum of each ingredient was also analyzed and subtracted from the sample spectrum. Spectra of samples were obtained by OMNIC Version 6.0a (Thermo Nicolet Corp., Madison, WI). Spectral data processing including baseline correction, subtraction of ingredient spectra and normalization to the intensity of phenylalanine peak at 1003 cm^{-1} was performed using GRAMS/AI Version 7.02 (Thermo Galactic, Galactic Industries Corp., Salem, NH). Assignments of the bands in the Raman spectra to the specific vibrational modes of amino acid chain or polypeptide backbone were based on the literature (Howell and Li-Chan, 1996; Li-Chan, 1996). Secondary structure analysis based on the amide I region (Williams and Dunker, 1981) using the Raman Spectral Analysis Package (RSAP) program (version 2.1) of Przybycien and Bailey (1989) was conducted with ingredient subtracted Raman spectral data. FT-Raman spectroscopic analyses were conducted in duplicate; coefficients of variation of peak intensities for duplicate spectra were 1 % or less.

5.2.7. Sensory evaluation

Five panelists from the previous study (Chapter 4 in this thesis) and two additional panelists recruited from graduate students in the Food Science program at UBC and from staff at a food

company producing meat substitute products in Vancouver were invited to participate in descriptive sensory evaluation. To remind the panelists of the 5 sensory attributes selected during the previous training session, a 20 minute-training session was held using a hand-out with the previously defined attributes (Table 4.2). For the training session, three different samples (SBF only, SBF with SPI, and SBF with ascorbic acid treated SPI) were prepared. The sample incubation, serving, evaluating, and discussion methods as well as sensory evaluation sheet were as described in Chapter 4 of this thesis.

For the main descriptive sensory analysis, 3 different samples were prepared using the procedure described in section 5.2.3., as follows: 250 mg SPI in 5 g dd-water with 150 mg SBF but no ingredient added (SPIF), 250 mg ascorbic acid treated SPI in 5 g dd-water with 150 mg SBF (AF), and 250 mg ascorbic acid and polyethylene glycol treated SPI in 5 g dd-water with 150 mg SBF (APF). The 3 samples (coded with 3-digit random numbers) and a reference (250 mg SPI in 5 g dd-water with 150 mg SBF) were covered with aluminum foil and held at 60 °C for 20 minutes before serving. Refer to Section 4.2.2.2 of this thesis for detailed description of the sensory procedure.

5.2.8. Statistical analysis

General linear model of analysis of variance (ANOVA) was performed using Minitab software (version 13.30, Minitab Inc. PA) to determine different ingredient effects, and Fisher's least significant difference (LSD) test was performed to compare samples at the 95 % confidence level. For FT-Raman spectral data, the mean values from duplicate analyses were reported. To verify significant difference among samples, lower and upper 95 % confidence limits were calculated based on the assumption of 5 % coefficient of variation for the FT-Raman analysis (Badii and Howell, 2003).

5.3. Results

5.3.1. Differential scanning calorimetry

The thermal behaviour of the SPI was assessed using DSC between 25 °C and 105 °C at a rate of 1 °C/min. The thermograms of SPI at three different concentrations (0.2 %, 5 %, and 10 % SPI in phosphate buffer, pH 7.5 are shown in Figure 5.1. Generally, native proteins show endothermic changes associated with the rupture of hydrogen bonds or exothermic changes upon weakening of hydrophobic interactions and aggregation of proteins by thermally induced denaturation (Li-Chan and Ma, 2002). The absence of endothermic or exothermic peaks in the thermograms from any of the heating scans of the SPI used in this study, suggests that the proteins in the SPI were already considerably denatured most likely due to the treatment during the processing of SPI.

5.3.2. Gas chromatography

Changes in areas of peaks related to beefy characteristic in SBF in the presence of SPI, as influenced by addition of the ingredients, were investigated by HS-SPME GC method. The relative % area of individual peak in each sample using HS-SPME adsorption at room temperature and at 60 °C are presented in Table 5.1 and Table 5.2. In these tables, only peaks, which were significantly ($p \leq 0.05$) positively correlated with beefy note in sensory evaluation (Chapter 4 of this thesis), were shown and compared.

The areas of 8, 1, 4, 12, 13 peaks were increased by RT adsorption or 60 °C adsorption in samples with glucosamine, sucrose, polyethylene glycol, ascorbic acid, and ascorbic acid with polyethylene glycol, respectively. Although incubation of the mixtures at RT followed by adsorption at RT captured much fewer volatile compounds in the headspace of the samples, it is interesting to note that glucosamine and ascorbic acid alone or with polyethylene glycol seemed to have an effect under these conditions in terms of alleviating the beefy related compounds holding property of SPI. However, the effect of glucosamine was not shown under the condition of 60 °C incubation or 60 °C adsorption.

Generally, addition of ascorbic acid either by itself or with polyethylene glycol significantly affected the flavour holding property of SPI. Under the condition of RT adsorption (Table 5.1),

ascorbic acid significantly increased areas of 4 peaks (29.971, 30.729, 31.271, and 33.306 minutes) when added on its own, but it increased areas of 6 peaks (29.971, 30.729, 31.271, 31.881, and 33.306 minutes) when added with polyethylene glycol. Under 60 °C adsorption (Table 5.2), the areas of 8 peaks (1.734, 12.132, 13.915, 20.608, 25.794, 26.954, 30.377, and 31.317 minutes) were significantly increased by addition of ascorbic acid only while the areas of 8 peaks (1.734, 12.132, 13.915, 20.608, 30.377, and 31.317 minutes) were increased by ascorbic acid with polyethylene glycol. A number of the peaks which were increased by ascorbic acid, such as peaks with retention time of 29.971, 30.729, 31.981 and 33.306 minutes in Table 5.1 and 12.132, 30.377, and 31.317 minutes in Table 5.2, were highly positively related to the beefy note from SBF ($p \leq 0.001$). In addition, some of these peaks (e.g. with retention time at 1.734, 12.132, 25.794, and 30.377 minutes) were associated with high detection frequency (greater than 50 %) in GC-O and may therefore be expected to have an impact on perceived aroma profile in the mixture. Fifteen peaks had been selected as indicator peaks in Chapter 4 based on their potential contribution to beefy note in the mixture. Among the 15 peaks, the areas of 2, 4 and 4 peaks were significantly increased in samples containing polyethylene glycol, ascorbic acid, and ascorbic acid with polyethylene glycol, respectively (Table 5.2).

5.3.3. Sulfhydryl and disulfide content

The contents of total SH and disulfide in each sample are shown in Table 5.3. The results should be considered with caution as high protein blanks were observed due to the turbidity of the protein in the testing buffer for these assays, even after being centrifuged at 14950 *g* for 30 minutes. The total SH content of SPI was 0.6 μmole/g protein. No or only small differences in the content of total SH were observed by addition of glucosamine, sucrose, and polyethylene glycol to SPI, while considerable difference resulted in the presence of ascorbic acid, or ascorbic acid with polyethylene glycol. In addition, the content of SS was determined as 36.7 μmole/g protein and a significant decrease of disulfide content was observed in samples containing ascorbic acid and ascorbic acid with polyethylene glycol.

5.3.4. Surface hydrophobicity

The surface hydrophobicity of SPI and ingredient added SPI determined by the neutral fluorescent probe, PRODAN, are shown in Table 5.4. The S_0 of SPI was determined to be

145 $\%^{-1}$ and was not affected by addition of glucosamine or sucrose. However S_0 was significantly increased by addition of polyethylene glycol or ascorbic acid as shown in Table 5.4. The increase in S_0 was amplified in the sample treated by the combination of ascorbic acid and polyethylene glycol implying additive effects of ascorbic acid and polyethylene glycol in exposing surface hydrophobic groups in SPI.

5.3.5. FT-Raman spectroscopy

A typical FT-Raman spectrum of SPI (freeze-dried powder from 5 % w/w of SPI solution in 0.05 M Tris-HCl buffer pH 7.4) is shown in Figure 5.2. Effects of additional ingredients on the FT-Raman spectrum are depicted in Figure 5.3. The tentative assignments of the major bands are listed in Table 5.5.

Comparison of the tryptophan band near 760 cm^{-1} in each sample spectrum shows a significant decrease in peak intensity in the sample containing ascorbic acid with polyethylene glycol compared to SPI, as shown in Table 5.6. Further changes in aromatic amino acid residues may be noted in the intensity ratio of the doublet bands at 850 and 830 cm^{-1}, which are assigned to tyrosine residues. There were significant decreases in the tyrosine doublet ratio for SPI in the presence of ascorbic acid and ascorbic acid with polyethylene glycol compared to SPI alone (Table 5.6). The microenvironment of aliphatic amino acid residues was examined by Raman bands at 1450 cm^{-1} ($C-H_2$ bending), 1465 cm^{-1} (C-H bending) or 2935 cm^{-1} (C-H stretching). Although there were no significant differences in the intensities at 2935 cm^{-1}, a significant increase in the intensity at 1450 cm^{-1} was observed in samples containing polyethylene glycol and ascorbic acid with polyethylene glycol. The SS-stretching band near 510-550 cm^{-1} clearly indicated an effect of addition of ingredients on the disulfide bonds, as shown in Table 5.6. Significant increase in intensity at 540 cm^{-1} was shown in the sucrose added sample while significantly decreased intensity at 537 and 539 cm^{-1} was observed in samples containing ascorbic acid and ascorbic acid with polyethylene glycol, respectively.

Among several distinct vibrational modes of the –CO-NH- amide or peptide bond, the amide I and III bands are the most useful to determine the secondary structure of proteins (Li-Chan, 1996). In this study, compositions of the secondary structure for SPI and SPI with added

ingredient were analyzed by least squares analysis of the Amide I band. The secondary structure compositions of the original (not freeze-dried) SPI powder (NOFD) and freeze-dried SPI (SPI) were very similar, as shown in Table 5.7. There was no significant difference in the fraction of α-helix, β-reverse turn and unordered structure, while a slightly increased proportion of β-sheet was observed in SPI after freeze-drying.

The added ingredients led to considerable changes in the secondary structure of SPI. When SPI was treated with glucosamine, the proportion of β-sheet increased while total unordered structure decreased. Sucrose seemed to have the least effect on changing the secondary structure of SPI, with the only difference being a decrease in unordered structure. Addition of polyethylene glycol caused an increase in the proportion of α-helix and a decrease in β-sheet compared to SPI. The greatest change in terms of propensity of secondary structure of SPI was detected by addition of ascorbic acid, observed in both the amide I (1650-1685 cm^{-1}) and amide III (1235-1305 cm^{-1}) bands (Figure 5.3). Table 5.7 shows that the α-helix and unordered structure were significantly increased by more than 5 % and 12 %, respectively, while β-reverse turn was drastically decreased by about 17 % when SPI was treated with ascorbic acid. Similar changes occurred in SPI treated by ascorbic acid with polyethylene glycol, but to a lesser extent. Considerable differences in the unordered structure were found between SPI and ingredient added SPI. The fraction of unordered structure was significantly increased in the samples containing ascorbic acid or ascorbic acid with polyethylene glycol, and considerable decrease was observed in samples containing sucrose or glucosamine, while no significant difference was shown in samples containing polyethylene glycol.

5.3.6. Sensory evaluation

The mean sensory scores of 7 panelists for the 5 sensory attributes and the results of Fisher's LSD tests are shown in Table 5.8. The comparison of the 5 attributes for the 3 samples, (i.e. SPI without any ingredient added (SPIF), SPI containing ascorbic acid (AF) and SPI containing ascorbic acid with polyethylene glycol (APF)) is illustrated in Figure 5.4.

The SPI containing ascorbic acid with polyethylene glycol showed significantly higher score for roasted attribute and significantly lower scores for soymilk-like and cereal attributes compared to

SPI without any ingredient. A higher score for beefy attribute and lower score for yeasty attribute were also noted for SPI containing ascorbic acid with polyethylene glycol, but these differences were not statistically significant ($p > 0.05$). In general the intensities of SPI with ascorbic acid for all the sensory attributes except yeasty note were located between those of SPI without ingredient and of SPI containing ascorbic acid with polyethylene glycol.

5.4. Discussion

5.4.1. Changes in peak area by the ingredients in GC analysis

The effect of each added ingredient on beefy flavour retention in SPI with regard to changes in beefy characteristics was investigated using GC-FID. Through preliminary work, the ratio of SPI to ingredient was adjusted from 50:1 to 2.5:1. This ratio was required in order to detect changes in the area of individual peaks in the GC chromatogram, beyond the signal noise and above the area reject limit value of 1000. Although this ratio was rather high by comparison to the real food system, the results could provide useful information to monitor the effects of different types of ingredients, which could affect binding property of SPI with flavour compounds. The flavour holding capacity should be distinguished from flavour binding property, where investigation of binding affinity and number of binding sites on SPI with individual flavour component (ligand) are focused.

The ability of SPI to suppress release of beefy related volatile compounds in the headspace, as indicated by the GC peak areas, was noticeably reduced in SPI containing glucosamine at RT incubation and RT adsorption condition, as shown in Table 5.1. However, the effect of glucosamine disappeared under the condition of 60 °C incubation or 60 °C adsorption. The observed result may be attributed to the changes in hydrogen bonding interactions of the SPI affected by the hydrogen in the amino group of glucosamine. The energy of hydrogen bond was reported as 8-40 kJ/mol and it is readily weakened by heating while hydrophobic interaction is enhanced (Cheftel et al., 1985). Ionic interactions between SPI and glucosamine were not considered to contribute significantly in this study where Tris buffer at pH 7.4 was used, since these forces would only be expected to play an important role in the pH range between 4.6 and

pH 6.9, i.e. between the pI of SPI and pKa of glucosamine where SPI would be negatively charged and glucosamine would be positively charged.

Moreover, addition of ascorbic acid alone or with polyethylene glycol showed a potential to increase the areas of beef attribute related flavour compounds and some of the peaks were significantly recovered toward the level found in SBF (Table 5.1 and Table 5.2). Although these results cannot provide information on whether the increase of the peak areas was due to alleviating the binding constants between the flavour compounds and SPI, decreasing the number of binding sites for the flavour compounds on SPI, or reducing binding affinity due to possible changes in the flavour compounds by the ingredients, it clearly demonstrated that among the ingredients added, ascorbic acid had the biggest effect on flavour holding behaviour, in terms of decreasing suppression of beef attribute related aroma components by SPI in the mixture. Therefore, it can be postulated that addition of ascorbic acid alone or with polyethylene glycol could increase the beefy aroma characteristic of the sample mixture at the usual serving temperature (60 °C), although it might result in concomitant distorted beefy profile due to the unbalanced increase among beefy related aroma compounds.

5.4.2. Changes in sulfhydryl and disulfide content by the ingredients

The increased areas of GC peaks related to the beefy note seem to be closely related to the changes in protein structure of SPI. The analysis of SH and SS content demonstrated a significant increase in SH content and decrease in SS content by the addition of ascorbic acid alone or with polyethylene glycol (Table 5.3). The increased content of total SH in ascorbic acid added samples might arise from cleavage of disulfide bonds in the samples with added ascorbic acid, a potent reducing agent. Therefore, it can be postulated that addition of ascorbic acid to SPI may lead to reduction of the disulfide linkage leading to possible changes in the conformation of SPI. Differences in total SH+SS among samples were observed in this study. The decreases of total SH+SS in SPI containing ascorbic acid or SPI containing ascorbic acid with polyethylene glycol may be partly attributed to possible involvement of the thiol groups in other bonds upon reduction of the disulfide bonds by ascorbic acid.

A wide range of SH+SS contents of soy proteins have been reported. Boatright and Hettiarachchy (1995) reported that total SH content of SPI was 8.3 μmole/g protein and it was increased to 10.1 μmole/g protein after adding the antioxidant Tenox 22, while the total SH + SS content of 52.9 μmole/g protein was decreased to 43.9 μmole/g protein by antioxidant addition. Total SH contents of 0.29 and 5.4 μmole/g protein were observed in SPI in buffer at pH 8 and pH 3, respectively, and these values were decreased with high pressure treatment between 400 and 600 MPa (Puppo et al., 2004). The average values of surface, internal, and total SH contents of glycinin from five soybean cultivars were reported to be 0.6, 1.3, and 1.9 mole SH/mole glycinin, respectively (Nakamura et al., 1984), or 1.9, 4.1, 5.9 μmole SH/g glycinin, respectively. Determination of 15 freeze-dried preparations of glycinin showed 0.6 – 2.2 mole SH/ mole glycinin with an average of 1.4 mole SH/mole glycinin, which is equivalent to 1.9 – 6.9 μmole/g glycinin (Wolf, 1993) and the SH content increased by treatment with reducing agents such as 2-mercaptoethanol, dithiothreitol, or sodium borohydride.

In the present study, the SH content in SPI was 0.6 μmole/g protein, which is less than values reported in the literature for glycinin. The discrepancy may be partly due to the presence of β-conglycinin with glycinin in the ratio of about 1:1 in SPI (Martins and Netto, 2006). Both α′ and α subunits were reported to include low levels of cysteine and methionine while β subunit did not contain any methionine. In comparison each acid polypeptide in glycinin is linked with the basic polypeptide through a disulfide bond (Liu, 1997). Moreover, partial denaturation of the SPI during processing, as shown in Figure 5.1 and discussed in Section 5.3.1, may affect the total SH content of the SPI used in this study. In addition, the turbidity observed in the protein blanks could imply potential inaccessibility of interior SH that could not be accessed by Ellman's reagent due to the reported cross-link formation of unnatural covalent bonds during processing such as lysinoalanine in SPI (Wu et al., 1999), or due to SS bonds that would limit unfolding and exposure of interior SH in the SPI, even in the presence of SDS.

5.4.3. Changes in surface hydrophobicity by the ingredients

Surface hydrophobicity was also affected by adding ingredients into SPI as shown in Table 5.4. Although results of S_0 have been published using anionic fluorescence probes such as ANS and

CPA, to the author's knowledge, this thesis is the first to report S_0 of SPI using the neutral uncharged probe, PRODAN, which measures surface hydrophobicity based on the binding between probe and protein primarily from the hydrophobic interaction with minimal interference from electrostatic interaction.

While no significant difference was detected by addition of sucrose or glucosamine to SPI, significant increases in surface hydrophobicity were observed when polyethylene glycol, ascorbic acid, or the combination of the two ingredients were added to SPI. Sucrose is a disaccharide with eight hydroxyl groups and glucosamine is an amino sugar including four hydroxyl groups with an amino group. Polyols, which are ingredients with poly hydroxyl groups, have been reported to contribute to the stabilization of the structure of protein molecules (Gekko et al., 1999). Various concentrations of glycerol and sorbitol enhanced thermal stability of soy protein, β-conglycinin and glycinin in aqueous solution. Addition of polyols was assumed to strengthen intra-molecular hydrophobic interaction due to the preferential solvent interaction with the protein molecules. According to the mechanism described by Gekko and Timasheff (1981), polyols such as glucosamine and sucrose in this study are fundamentally hydrophilic compounds, which can occupy a part of the solvation sheath around SPI with concomitant stabilization of solvent structure. Polyols were reported to show a stabilization effect on hydrophobic interaction of protein molecules more effectively compared to peptide-peptide hydrogen bonds (Gekko, 1981).

In contrast, surface hydrophobicity of SPI with polyethylene glycol was significantly increased. Mild denaturing treatment by polyethylene glycol may lead to increasing surface hydrophobicity on SPI. Polyethylene glycol is highly soluble in water but includes a repeating hydrophobic region ($-CH_2-CH_2-$) per monomer unit, which could evoke relocation of the hydrophobic side chains of non-polar amino acid residues in SPI. It can be hypothesized that addition of ascorbic acid increased the surface hydrophobicity through structural changes in SPI accompanying the reduction of disulfide bonds to sulfhydryl groups (Table 5.3), and the increasing proportion of unordered structure as analyzed by FT-Raman spectroscopy (Table 5.7). The breakage of disulfide bonds in SPI could enable buried amino acid residues with non-polar side chains to become exposed to the surface of the protein molecule, thus increasing the binding affinity or number of binding sites for the hydrophobic fluorescence probes such as PRODAN. The

denaturing effect by polyethylene glycol seemed to be enhanced by reduction of disulfide bond by ascorbic acid, resulting in considerable increase of surface hydrophobicity in SPI treated with both ascorbic acid and polyethylene glycol.

5.4.4. Changes in FT-Raman spectra by the ingredients

Addition of ingredients also affected the FT-Raman spectra of SPI. The most significant changes were observed in SPI containing ascorbic acid with polyethylene glycol, as shown in Table 5.6, implying the presence of additive effect between those two ingredients. Compared to SPI, the FT-Raman spectrum of SPI containing ascorbic acid with polyethylene glycol showed decreased intensity at 760 cm^{-1} indicating decrease of "buriedness" or hydrophobic microenvironment of the tryptophan indole ring (Li-Chan, 1996). The decreased intensity suggested that when the ascorbic acid and then polyethylene glycol was added to SPI, the tryptophan residues became exposed from a buried, hydrophobic interior to a more polar microenvironment. A decrease in the intensity ratio of the 850 and 830 cm^{-1} doublet could indicate intermolecular interactions of the tyrosine residues as a strong hydrogen donor or increased buriedness around the residues (Howell and Li-Chan, 1996). The observed decrease in the ratio of the doublet in the samples containing ascorbic acid alone or with polyethylene glycol might suggest the possibility of interaction between tyrosine residues and polyethylene glycol, which could serve as a hydrogen bond acceptor. Increase of the intensity at 1450 cm^{-1} in the samples including polyethylene glycol alone or with ascorbic acid indicate decreased interior hydrophobic interactions due to increasing polarity of the environment around hydrocarbon chains, which implies unfolding of proteins in those samples. Changes in intensity of the SS stretching band (510-550 cm^{-1}) could be explained by possible formation or stabilization of disulfide bonds in SPI by addition of sucrose, in contrast to disulfide bond breakage in samples containing ascorbic acid or ascorbic acid with polyethylene glycol, probably due to reduction by ascorbic acid, which is in good agreement with the results of SH and SS determination as discussed in the previous section (5.3.3 of this thesis). In addition, the Raman wavenumber shift of the SS-stretching band suggests conformational changes in disulfide bonds, from a *gauche-gauche-trans* conformation in SPI to a *trans-gauche-trans* conformation when the ingredient was added to SPI. However, no peaks were found in the region of 2550-2558 cm^{-1}, which could give information on S-H stretching, probably due to the low concentration of sulfhydryl groups in these samples as shown by analysis with Ellman's reagent.

Analysis of the Amide I band of the FT-Raman spectrum indicated that the SPI in this study contained 14.4 % α-helix, 55.2 % β-sheet, 19.0 % β-turns, and 11.5 % unordered structures, and was not changed by freeze-drying. Rickert et al. (2004) reported that a SPI produced by pilot-plant-scale process contained 47.6 % of glycinin and 47.2 % of β-conglycinin. Based on its amino acid sequence, the composition of secondary structure of glycinin was predicted to contain 25 % α-helix, 25 % β-sheet, 42 % turns, and 8 % unordered structures (Argos et al., 1985). Abbott et al. (1996) investigated the secondary structure of unheated glycinin using infrared spectroscopy having 25 % α-helix, 33 % β-sheet, 31 % turns, and 12 % unordered structures in aqueous buffer and found that glycinin in solution had the same secondary structure as glycinin in hydrated solids. The observed secondary structure composition of SPI shown in Table 5.7, i.e. higher β-sheet and lower α-helix than the reported values for SPI in the literatures, is consistent with the results from DSC indicating denaturation of the SPI used in this study. Increase in the β-sheet at the expense of existing α-helix has been reported to be associated with denaturation and/or aggregation of proteins (Herald and Smith, 1992; Przybycien and Bailey, 1991; Puppo et al., 2004).

The secondary structure composition of SPI was changed by the addition of ingredients, as shown in Table 5.7. The decrease of unordered structure by addition of glucosamine and sucrose might be explained by the reported roles of sugars, which have been used as a protein stabilizer in food industry, with the hydrating ability through the poly-hydroxyl groups. Increased unordered structure in the samples containing ascorbic acid alone or with polyethylene glycol might be related to their increased surface hydrophobicities (section 5.3.4) along with the postulated disulfide breakage discussed in section 5.3.3.

Generally, various functional properties of globular proteins depend on their physico-chemical properties, which are primarily governed by structural and conformational attributes (Hettiarachchy and Kalapathy, 1997). Denaturation has been known to be important in gelation property of SPI, and has been regarded as a necessary step for gel formation (Renkema et al., 2002). As a result of thermal denaturation during gelation, hydrophobic areas buried in the native conformation are exposed to surrounding solvent. This change in partially unfolded protein

creates an aggregation process due to the imbalance between attractive and repulsive forces of molecules (Berli et al., 1999). Similarly, denaturation or changes in molecular structure induced by added ingredients must have played an important role in changing the flavour holding behavior of SPI. In the present study, the flavour holding property of SPI was most affected by structural changes of SPI through disulfide bond breakage in addition to hydrophobic interaction. Breakage in disulfide bond might be necessary to facilitate conformational changes in SPI.

5.4.5. Descriptive sensory evaluation

According to the results from GC analysis, the areas of beef attribute related peaks increased by addition of ascorbic acid alone or with polyethylene glycol. Furthermore, some of these peaks were associated with high detection frequency in GC-O analysis, which might imply improvement of aroma perception of SBF in mixtures of SPI and SBF. Therefore, descriptive sensory analysis was conducted with the 3 samples, which were SPIF, AF and APF as described in section 5.2.6. The sample of SBF with SPI containing ascorbic acid with polyethylene glycol showed considerable changes in the sensory scores of all the attributes although the changes were not statistically significant ($p > 0.05$) in beefy and yeasty notes. The sample of SBF with SPI containing ascorbic acid showed a similar trend with the sample containing those two ingredients but it was not statistically significant.

Since 5 out of the 7 panelists in this study also participated in the previous study (Chapter 4 of this thesis) and the same sensory evaluation method including evaluation sheet, reference sample, and procedure for sample preparation, serving and evaluation was used, this result was compared to the sensory result from Chapter 4 of this thesis. The sample coded "SPIF" in this study has the same composition as sample "S3" in Chapter 4 shown in Figure 4.1, Figure 4.2 and Table 4.10, consisting of 250 mg SPI in 5 g dd-water with 150 mg SBF (medium SPI with low SBF). By comparison of S3 in Figure 4.1 with SPIF in Figure 5.4, all the attributes except yeasty note were similarly assessed by the panelists. Different perception in yeast note was probably due to poor consensus of the definition for yeasty note.

The result of PCA of all the samples including the 12 samples evaluated in Chapter 4 and the 3 samples assessed in this chapter is shown in Figure 5.5. Roasted and beefy attributes were

demonstrated to be strongest in the sample APF. It is interesting to note that the characteristic of APF was evaluated to be similar to sample S1, which contains the same amount of SBF but without any SPI or additional ingredient.

Table 5.9 compares the mean intensity values of the 5 attributes for the 3 samples (SPIF, AF, and APF) with the results from Table 4.10. The intensity of the sample APF for the each of the attributes for roasted, soymilk-like, cereal, and beefy, was similar to the intensity of sample S1, which contains SBF but no SPI. The results clearly demonstrated that sample APF fell into the same group as S1 with no significant difference in roasted, soymilk-like, cereal, and beefy attributes, indicating that the roasted and beefy characteristics were recovered while soymilk-like and cereal notes were suppressed to a certain extent by adding ascorbic acid to SPI before mixing with SBF, and the effect was enhanced by treating with ascorbic acid and polyethylene glycol in SPI.

Overall, it could be postulated that adding ascorbic acid and/or polyethylene glycol in SPI prior to interacting with SBF resulted in an increased release of flavour compounds involving the beefy characteristic. The lack of a significant difference in beefy note may probably be attributed to the complexity of "beef" aroma, which can not be constructed easily, and possible deformation of beefy profile due to the unbalanced increase among beefy related aroma compounds. Nevertheless, although significant difference was not found in beefy note among the samples under these conditions, beefy characteristic in sample APF was expressed by enhancement of roasted note and alleviation of soymilk-like and cereal notes in the mixture of SBF and SPI. The results therefore could be considered successful in terms of recovering some of the beef characteristic and suppressing the soy characteristics, but also indicate the need for further research.

5.5. Conclusion

GC analysis of SBF in the presence of SPI alone and SPI with ingredients demonstrated that the mixture of SBF and SPI containing ascorbic acid alone or ascorbic acid with polyethylene glycol contributed to increasing peak area of individual beef attribute related volatile compounds in GC

chromatogram. According to the results of sulfhydryl and disulfide groups, surface hydrophobicity, and FT-Raman spectroscopy, it can be postulated that while glucosamine and sucrose had little effect, the addition of ascorbic acid especially in conjunction with polyethylene glycol triggered conformational changes in SPI structure mainly through disulfide bond reduction, resulting in increased surface hydrophobicity and unordered structure on SPI, which in turn affected improvement of SBF perception in the mixture of SBF and SPI. The results of hydrophobicity measurement and FT-Raman analysis indicated that the denaturing effect was likely to be enhanced by the combined treatment of ascorbic acid and polyethylene glycol. Polyethylene glycol induced interior hydrophobic side chains in SPI to be exposed, resulting in unfolding of the SPI, in other words, relocation of the non-polar amino acid residues of SPI induced by changes in hydrophobic interaction with the hydrophobic repeating unit of polyethylene glycol, which can be enhanced by means of modification of disulfide bond by ascorbic acid. The reduction of disulfide bonds, increased surface hydrophobicity and increased unordered structure in SPI, along with increased GC peak areas of indicator peaks in the SPI containing ascorbic acid alone or with polyethylene glycol, were found to be associated with an increase in the perceived beef characteristic attributes in descriptive analysis. These results provide the basis for further research to elucidate strategies maximizing perception of beefy aroma in soy products.

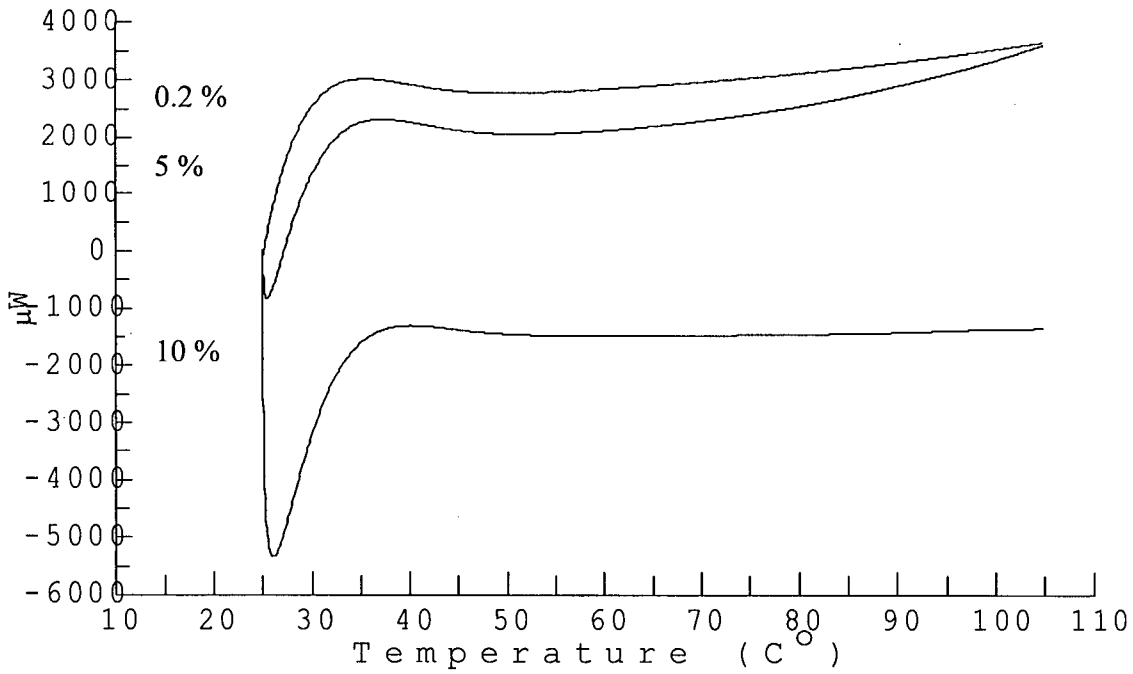

Figure 5.1. Differential scanning calorimetric (DSC) thermograms of SPI (0.2 %, 5 %, and 10 % in pH 7.5 phosphate buffer).

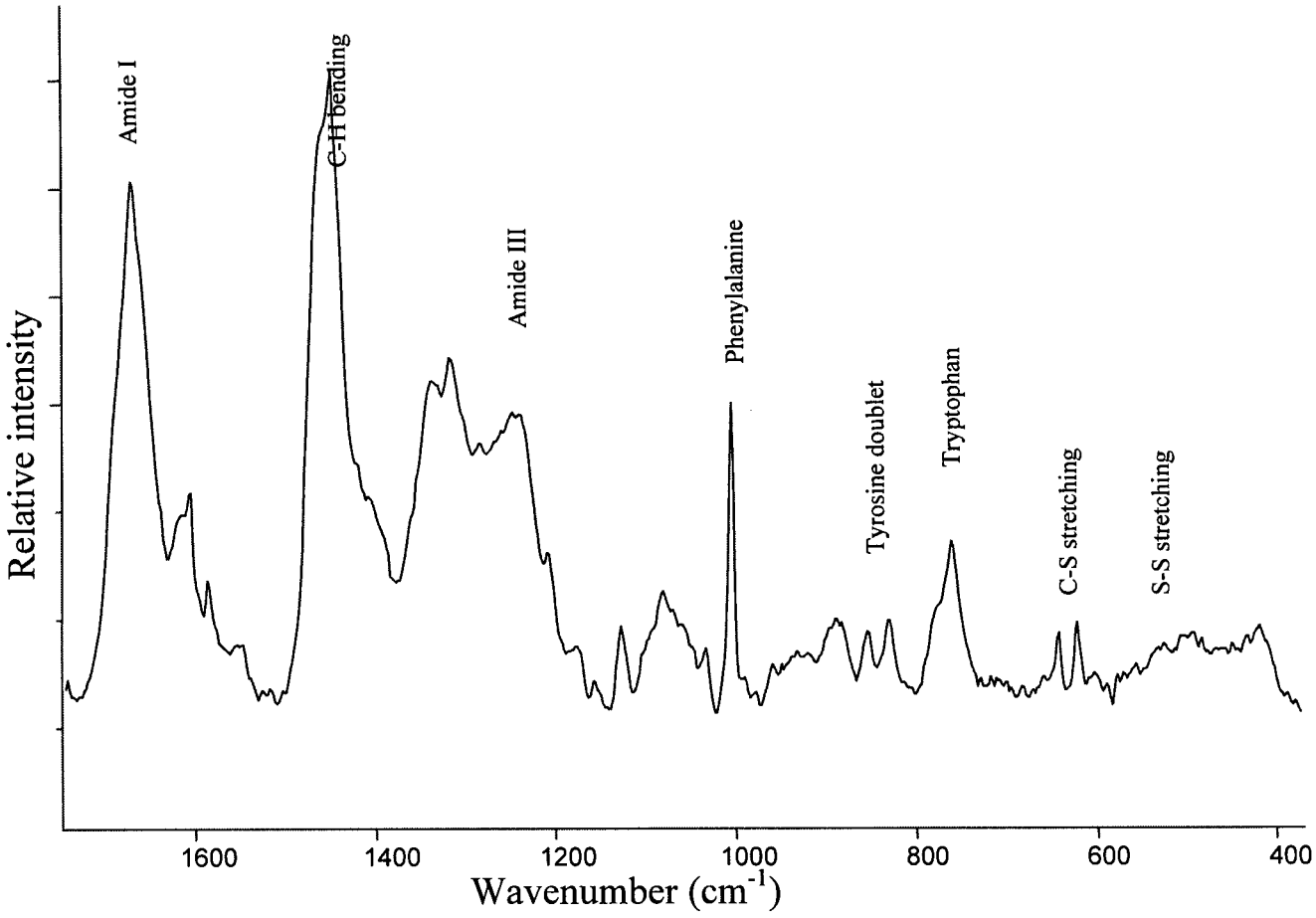

Figure 5.2. FT-Raman spectrum (400-1700 cm^{-1}) of SPI powder obtained by freeze-drying SPI solution (5 % w/w in 0.05 M Tris-HCl buffer pH 7.4).

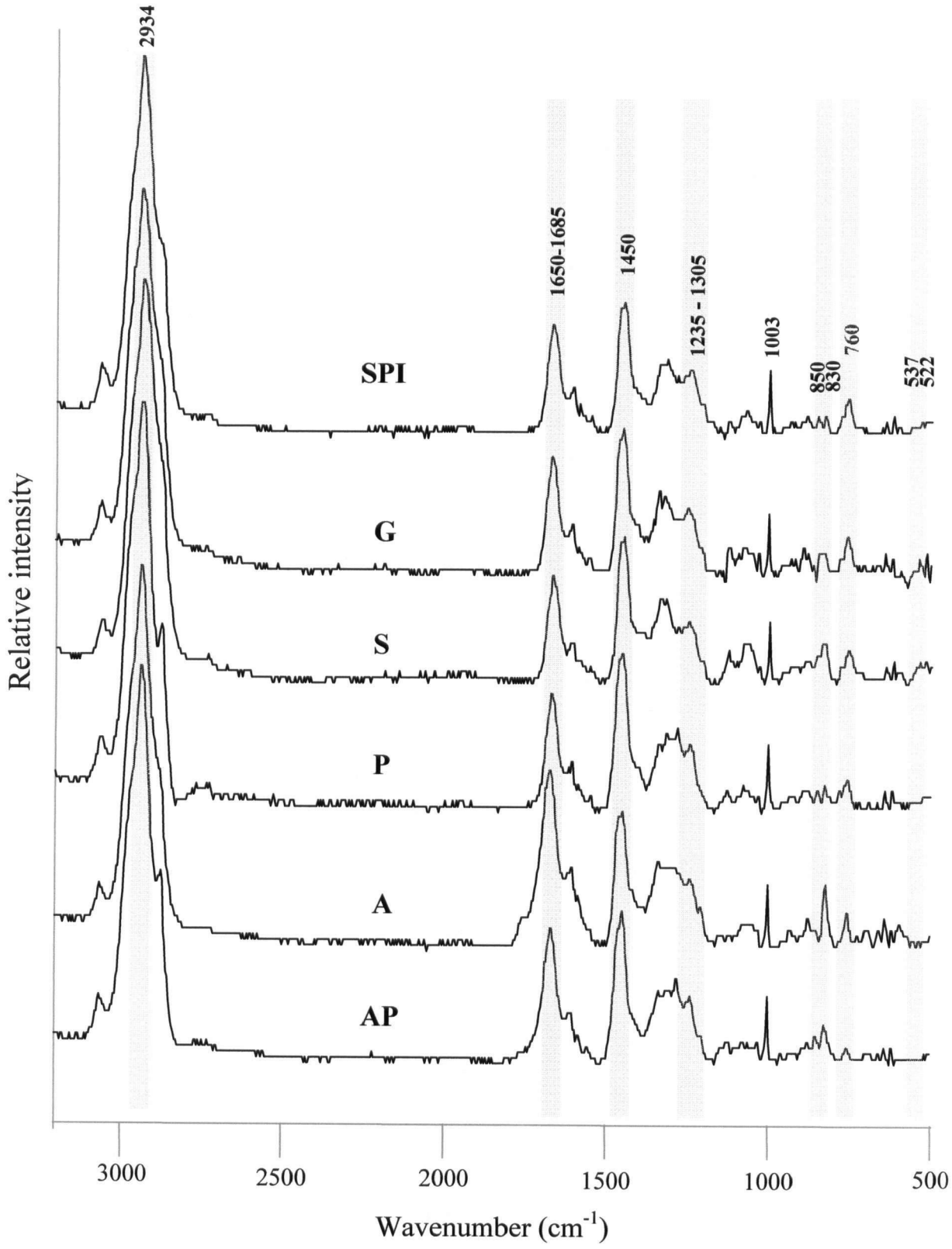

Figure 5.3. FT-Raman spectra of freeze-dried SPI and SPI treated with various ingredients (baseline corrected, ingredient spectrum subtracted, and normalized to the intensity of phenylalanine peak at 1003 cm^{-1}). G : glucosamine; S : sucrose; P : polyethylene glycol; A : ascorbic acid; AP : ascorbic acid with polyethylene glycol.

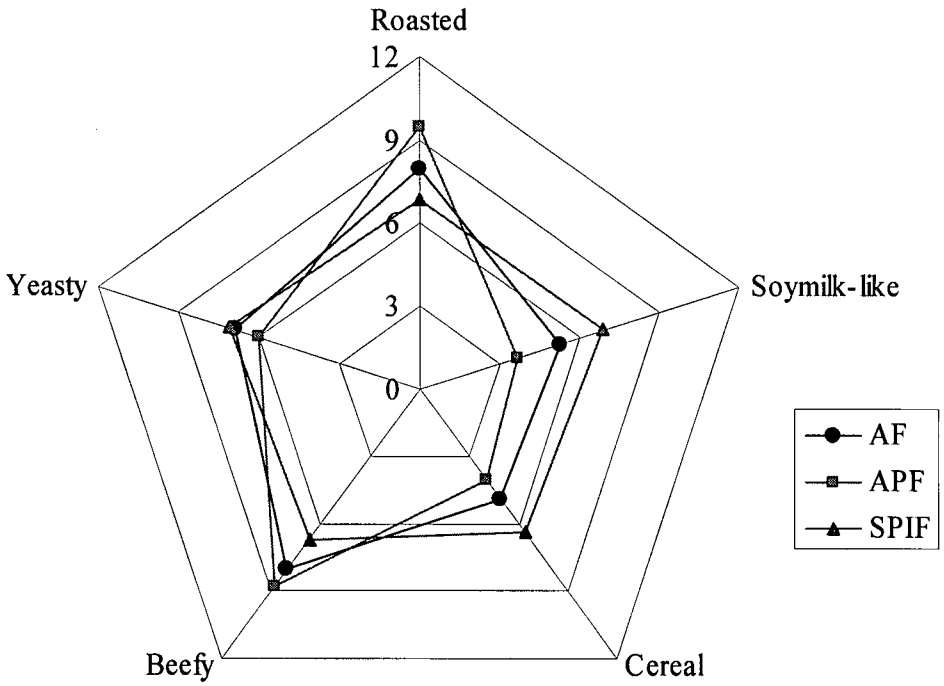

Figure 5.4. Cobweb diagram of the sensory scores from the descriptive analysis of simulated beef flavour in the presence of soy protein isolate (SPIF), SPI containing ascorbic acid (AF) and SPI containing ascorbic acid with polyethylene glycol (APF) (n=14; 7 panelists with 2 replications).

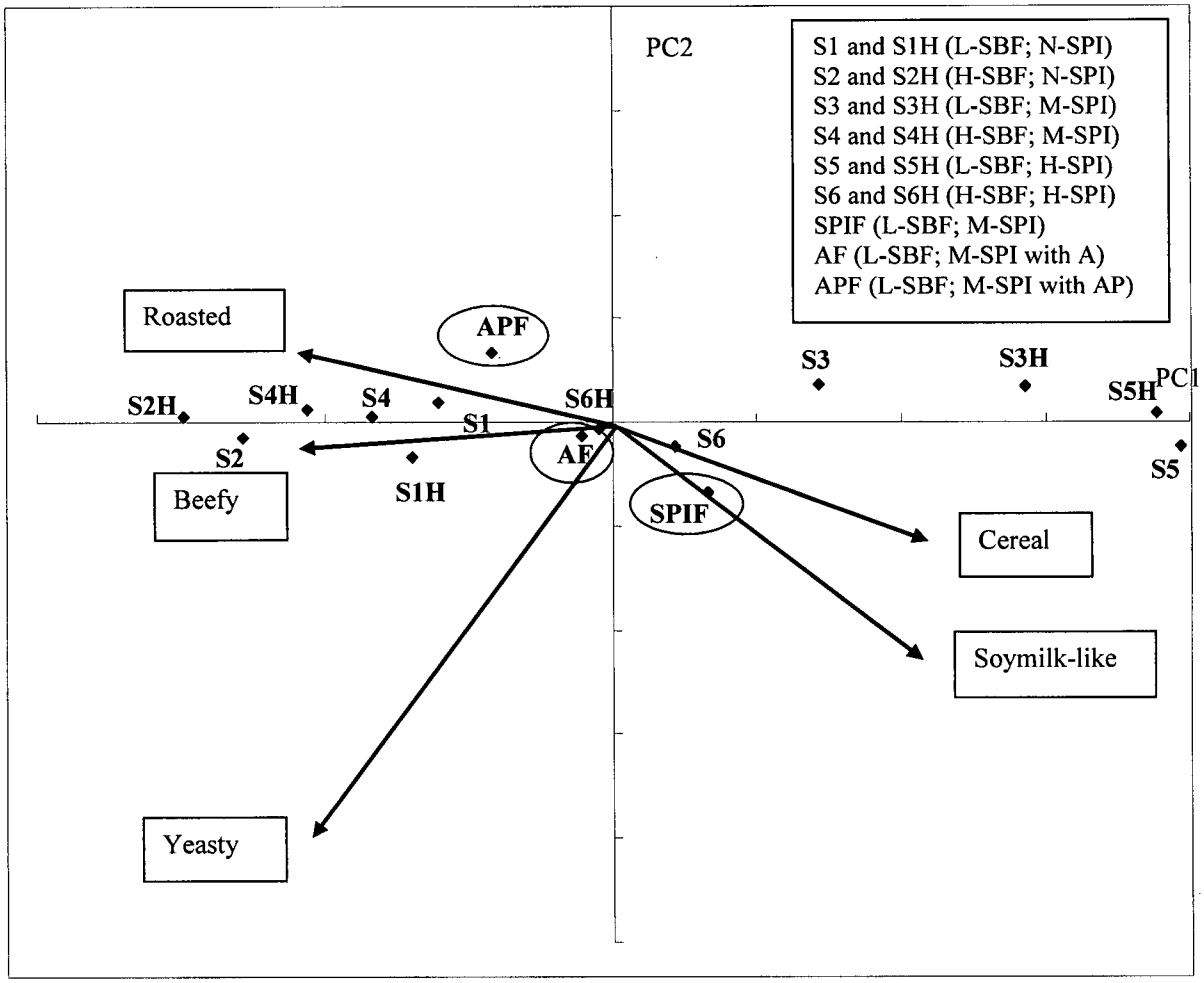

Figure 5.5. PC loadings and scores of the sensory attributes and the sample mixtures by principal component analysis; PC1 versus PC2.

L-SBF and H-SBF represent low (150 mg) and high (500 mg) dose of simulated beef flavour while N-SPI, M-SPI, and H-SPI symbolize no (0 mg), medium (250 mg), and high (500 mg) amount of soy protein isolate in the sample. Refer to Table 4.3 and Figure 5.4 for the sample codes.

Table 5.1. Effect of added ingredient in SBF-SPI mixture incubated at RT or 60 °C on the peak areas of volatile compounds captured by HS-SPME under the adsorption condition at RT[1, 2].

IP #[3]	Retention time[4]	SBF		RT incubation – RT adsorption % Area proportion based on SBF						60 °C incubation – RT adsorption % Area proportion based on SBF						Peak characteristics		
		Area	%	SPIF	GF	SF	PF	AF	APF	SPIF	GF	SF	PF	AF	APF	Tentative peak identification	DF[5] (%)	Beefy[6]
IP1	1.734	11512	100	71	94	68	60	88	48	42	42	40	32	55	56	3-Methyl furan	50	0.710*
	13.271	2514	100	19	15	0	0	0	0	17	0	0	0	0	0	Limonene		0.713**
IP13	28.288	13837	100	42	**72**	50	50	63	64	50	45	42	34	57	57	delta-Elemene	50	0.931***
	29.122	933	100	129	**387**	159	104	58	133	0	0	0	0	0	0	Unknown		0.713**
	29.971	15016	100	48	**73**	57	58	**71**	**74**	49	45	42	35	59	59	Unknown		0.928***
	30.729	1897	100	0	**57**	**56**	42	**75**	**77**	22	39	19	21	**71**	**67**	Unknown		0.929***
	31.271	4513	100	57	89	65	74	**288**	**184**	66	64	55	43	**110**	78	Unknown		0.638*
	31.530	2735	100	24	83	29	50	70	66	49	53	0	26	87	78	Unknown		0.750**
	31.881	113227	100	49	**78**	58	61	63	**76**	54	52	46	41	67	68	Unknown		0.924***
	32.231	3743	100	53	79	59	61	70	77	61	52	53	38	73	75	Unknown		0.879***
	33.166	3793	100	50	**75**	54	61	68	72	58	46	50	37	67	70	Unknown		0.826**
	33.306	6149	100	50	**79**	58	63	**73**	**83**	56	55	49	45	71	72	Unknown		0.894***
	34.691	828	100	0	52	0	0	50	106	0	0	0	0	0	0	Unknown		0.881***
	35.365	1501	100	57	80	0	0	26	57	0	0	0	0	0	29	Unknown		0.823**
IP15	35.759	417	100	0	105	0	0	98	220	0	0	0	0	85	90	Calamenene	75	0.883***
	35.887	1624	100	0	40	32	215	58	**393**	81	181	77	147	82	**296**	Unknown		0.685*
	36.322	5318	100	46	81	55	60	68	82	51	48	44	43	66	67	Unknown		0.822**

SPIF : SBF in the presence of SPI; GF, SF, PF, AF, and APF : SBF with SPI containing glucosamine, sucrose, polyethylene glycol, ascorbic acid, and ascorbic acid with polyethylene glycol, respectively.

[1] Bold numbers indicate significant ($p \leq 0.05$) difference from SPI in peak area of GC chromatogram by Fisher's LSD test.

[2] Peak area and % area are the average values from 3 replicate GC analyses.

[3] Number of indicator peak as selected from the previous study (Chapter 4 of this thesis).

[4] Retention time in minutes on GC chromatogram of this study.

[5] Detection frequency (%) – percentages of panel from a total of 8 panels perceived the aroma compound in GC-olfactometry.

[6] Pearson correlation coefficient for beefy notes in descriptive sensory analysis performed in Chapter 4 of this thesis.

Table 5.2. Effect of added ingredient in SBF-SPI mixture incubated at RT or 60 °C on the peak areas of volatile compounds captured by HS-SPME under the adsorption condition at 60 °C[1,2]. (Refer to Table 5.1 for sample codes)

IP #[3]	Retention time[4]	SBF		RT incubation–60 °C adsorption % Area proportion based on SBF						60°C incubation–60°C adsorption % Area proportion based on SBF						Peak characteristics		
		Area	%	SPIF	GF	SF	PF	AF	APF	SPIF	GF	SF	PF	AF	APF	Tentative peak identification	DF[5] (%)	Beefy[6]
IP1	1.734	6953	100	106	91	113	94	**163**	**158**	80	83	85	78	**131**	**146**	3-Methyl furan	50	0.710*
IP2	7.893	5537	100	122	83	141	89	83	74	77	62	75	65	59	63	2-Acetyl furan	75	0.658*
IP3	12.132	3360	100	66	58	67	54	62	49	20	31	32	17	**43**	**45**	delta-3-Carene	88	0.872***
	13.271	1929	100	24	0	30	42	0	0	18	0	0	0	0	20	Limonene		0.713**
	13.915	1352	100	33	34	39	31	**240**	**174**	0	**82**	25	0	**133**	**148**	Unknown		0.648*
	15.014	6302	100	84	63	62	27	89	73	67	73	68	67	60	67	Unknown		0.773**
IP4	15.877	1761	100	54	51	88	80	87	47	64	50	53	52	48	51	2-Ethyl-3,6-dimethylpyrazine	88	0.601*
IP5	16.247	2543	100	49	23	68	75	82	49	45	35	36	32	43	49	Unknown	100	0.852***
IP6	16.953	1935	100	20	0	49	39	38	0	20	18	0	0	18	18	Unknown	100	0.827**
IP8	19.917	2881	100	0	23	35	28	62	0	46	0	53	0	0	37	2,3-Diethyl-5-methylpyrazine	50	0.590*
	20.608	3328	100	47	44	61	59	**99**	**81**	49	46	45	48	**75**	71	4-Terpeneol		0.678*
IP9	22.151	3951	100	57	52	59	58	77	64	54	55	50	47	64	**75**	Decanal	75	0.715**
IP10	24.232	468	100	0	0	0	0	0	0	0	0	0	0	0	0	2-Isoamyl-6-methylpyrazine	50	0.599*
IP12	25.794	8033	100	41	47	46	**51**	**50**	40	38	42	45	50	71	43	Unknown	75	0.794**
	26.319	3792	100	19	18	40	41	38	34	0	0	17	0	16	19	Unknown		0.684*
	26.954	2156	100	0	19	21	**99**	**63**	19	24	24	0	0	0	43	Unknown		0.752**
	27.841	3710	100	16	35	38	56	22	95	40	22	35	21	0	0	Unknown		0.887***
IP13	28.288	19545	100	48	36	55	55	62	42	50	42	47	42	46	46	delta-Elemene	50	0.931***
	28.830	8259	100	44	41	53	61	35	28	43	41	47	50	25	26	Unknown		0.678*
	29.122	7556	100	131	130	130	128	158	109	116	149	117	151	115	123	Unknown		0.713**
	29.971	25200	100	46	39	52	53	62	54	51	45	49	44	50	53	Unknown		0.928***
	30.220	1260	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.617*
IP14	30.377	3031	100	16	14	49	**50**	**62**	**60**	40	17	15	19	36	36	beta-Cubebene	63	0.850***
	30.615	1639	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.645*
	30.729	9478	100	34	29	42	39	46	42	40	37	40	35	37	43	Unknown		0.929***
	30.950	721	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.868***
	31.271	1849	100	88	0	106	102	0	0	34	0	46	41	0	0	Unknown		0.638*
	31.317	11730	100	54	71	60	66	**158**	**131**	69	77	67	57	94	**104**	Unknown		0.914***
	31.530	4843	100	44	50	39	56	98	76	50	74	72	82	74	73	Unknown		0.750**

IP #[3]	Retention time[4]	SBF		RT incubation–60 °C adsorption % Area proportion based on SBF						60°C incubation–60°C adsorption % Area proportion based on SBF						Peak characteristics		
		Area	%	SPIF	GF	SF	PF	AF	APF	SPIF	GF	SF	PF	AF	APF	Tentative peak identification	DF[5] (%)	Beefy[6]
	31.881	262026	100	50	43	57	57	55	50	55	47	54	49	48	53	Unknown		0.924***
	32.231	7117	100	41	25	43	44	53	47	42	38	40	33	54	48	Unknown		0.879***
	32.679	4244	100	42	29	48	44	30	30	42	39	40	36	40	48	Unknown		0.863***
	33.166	6537	100	43	38	44	45	56	53	51	45	45	38	56	57	Unknown		0.826**
	33.306	24572	100	50	45	57	57	55	51	54	48	53	49	48	52	Unknown		0.894***
	33.620	4026	100	9	13	12	25	25	14	29	9	11	0	24	34	Unknown		0.779**
	33.813	4478	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.689*
	34.211	7156	100	31	17	31	49	26	30	32	26	33	37	15	24	Unknown		0.775**
	34.346	11082	100	30	26	31	35	31	28	36	32	35	32	22	26	Unknown		0.787**
	34.481	2104	100	0	0	0	0	0	0	0	0	62	47	0	0	Unknown		0.600*
	34.691	9569	100	40	34	44	46	45	43	43	38	29	26	39	41	Unknown		0.881***
	34.871	4778	100	33	9	11	10	11	9	20	18	18	19	8	0	Unknown		0.781**
	35.097	9752	100	27	35	43	45	40	38	38	36	38	36	38	42	Unknown		0.872***
	35.365	15997	100	34	31	34	37	37	36	36	33	33	31	36	40	Unknown		0.823**
IP15	35.759	16917	100	39	35	41	38	47	39	38	37	39	32	41	41	Calamenene	75	0.883***
	35.887	20361	100	39	42	40	**404**	55	**407**	43	48	40	140	46	**379**	Unknown		0.685*
	36.322	49502	100	36	33	40	40	46	43	38	35	37	34	41	43	Unknown		0.822**
	36.646	11216	100	37	26	40	40	30	28	41	38	41	37	29	32	Unknown		0.780**
	37.033	3176	100	0	61	26	54	64	54	59	27	69	23	41	43	Unknown		0.800**
	37.347	12581	100	32	33	38	40	38	30	38	33	39	37	27	31	Unknown		0.778**
	37.674	2119	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.815**
	38.001	12714	100	45	39	47	47	31	26	38	31	51	53	35	37	Unknown		0.746**
	38.425	6531	100	44	36	43	48	42	41	40	43	46	42	37	33	Unknown		0.634*
	38.658	13153	100	40	36	43	52	39	38	39	36	44	44	34	30	Unknown		0.767**

[1] Bold numbers indicate significant ($p \leq 0.05$) difference from SPI in peak area of GC chromatogram by Fisher's LSD test.

[2] Peak area and % area are the average values from 3 replicate GC analyses.

[3] Number of indicator peak as selected from the previous study (Chapter 4 of this thesis). IP7 and IP11 were not detected in this study due to the peak area being small than the applied area limit of 1000.

[4] Retention time in minutes on GC chromatogram of this study.

[5] Detection frequency (%) = percentage of 8 panelists who perceived the aroma compound in GC-olfactometry.

[6] Pearson correlation coefficient for beefy notes in descriptive sensory analysis performed in Chapter 4 of this thesis.

Table 5.3. Sulfhydryl groups and disulfide bonds in SPI and SPI treated with various ingredients[1].

Sample[2]	Total sulfhydryl group (μmole SH/g protein)	Disulfide bond (μmole SS/g protein)
SPI	0.6 [b]	36.7 [b]
G	0.6 [b]	36.8 [b]
S	0.8 [c]	38.6 [b]
P	0.5 [a]	36.8 [b]
A	2.0 [d]	31.0 [a]
AP	2.4 [e]	30.8 [a]

[1]Mean values (n=2) with different superscripts (a-e) within a column are significantly ($p \leq 0.05$) different.

[2]G : glucosamine; S : sucrose; P : polyethylene glycol; A : ascorbic acid; AP : ascorbic acid with polyethylene glycol.

Table 5.4. Surface hydrophobicity (S_0) of SPI and SPI treated with various ingredients.

Sample[1]	Surface hydrophobicity[2] ($\%^{-1}$)
SPI	145 ± 4^a
G	146 ± 2^a
S	150 ± 2^a
P	170 ± 9^b
A	171 ± 6^b
AP	198 ± 24^c

[1]G : glucosamine; S : sucrose; P : polyethylene glycol; A : ascorbic acid; AP : ascorbic acid with polyethylene glycol.

[2]Samples with different superscripts (a-c) are significantly ($p \leq 0.05$) different in surface hydrophobicity (S_0). Values shown are mean ± standard deviation (n=3).

Table 5.5. Tentative assignment of major bands in the FT-Raman spectrum of SPI and SPI treated with various ingredients (Adapted from Li-Chan, 1996).

Wavenumber region ($\pm$ 2 cm^{-1})	Tentative assignment
510, 522, 539	S-S stretching of cystine
760	Tryptophan indole ring
850/830	Tyrosine ring
1003	Phenylalanine ring
1450	C-H bending of aliphatic residues
1655 $\pm$ 5	Amide I C=O stretch, N-H wag (α-helix)
1670 $\pm$ 3	Amide I C=O stretch, N-H wag (anti-parallel β-sheet)
1665 $\pm$ 3	Amide I C=O stretch, N-H wag (solvated disordered structure)
1685	Amide I C=O stretch, N-H wag (non-hydrogen bonded disordered structure)
2550-2580	S-H stretching of cysteine
2800-3000	C-H stretching of aliphatic residues

Table 5.6. Normalized intensity values at selected regions of the FT-Raman spectra of SPI and SPI treated with various ingredients.

Band assignment	Mean of normalized peak intensity[1,2] [wavenumber (cm^{-1})]					
	SPI	G	S	P	A	AP
SS of cystine (Gauche-gauche-trans)	0.55[b] [522]	-	-	-	-	-
(Trans-gauche-trans)	-	0.60[b] [537]	**0.64**[c] [540]	0.52[b] [541]	**0.48**[a] [537]	**0.48**[a] [539]
Tryptophan indole ring	0.74[b] [760]	0.80[b] [759]	0.76[b] [759]	0.74[b] [759]	0.76[b] [759]	**0.58**[a] [759]
Tyrosine doublet ratio	0.97[b] [850/830]	0.99[b] [850/830]	0.89[b] [850/830]	0.98[b] [850/830]	**0.60**[a] [850/830]	**0.85**[a] [850/830]
Aliphatic residues C-H bending	1.62[a] [1448]	1.71[a] [1450]	1.76[a] [1450]	**1.80**[b] [1449]	1.64[a] [1449]	**1.81**[b] [1449]
Aliphatic residues C-H stretching	3.79[a] [2934]	3.79[a] [2935]	3.99[a] [2932]	4.10[a] [2933]	3.75[a] [2934]	4.06[a] [2932]

[1]Mean values are shown from duplicate analyses. Samples with different superscripts (a-c) within a row are significantly ($p \leq 0.05$) different. The numbers in bold letter indicate intensity difference ($p \leq 0.05$) in Raman intensity of ingredient added SPI from SPI.
[2]G : glucosamine; S : sucrose; P : polyethylene glycol; A : ascorbic acid; AP : ascorbic acid with polyethylene glycol.

Table 5.7. Composition of secondary structure in SPI and SPI treated with various ingredients[1,2].

Structure	NOFD (%)	SPI (%)	G (%)	S (%)	P (%)	A (%)	AP (%)
Total α-helix	14.4^{a}	13.6^{a}	13.1^{a}	15.0^{ab}	16.5^{bc}	18.9^{d}	17.5^{cd}
Total β-sheet	55.2^{ab}	57.2^{c}	59.6^{d}	57.2^{c}	55.1^{a}	56.8^{bc}	56.9^{c}
β-reverse turn	19.0^{cd}	18.6^{cd}	20.0^{d}	19.8^{d}	17.0^{c}	1.4^{a}	7.6^{b}
Unordered structure	11.5^{b}	10.6^{b}	7.3^{a}	8.0^{a}	11.4^{b}	22.9^{d}	18.0^{c}
Structure total	100	100	100	100	100	100	100

[1]Mean values are shown from duplicate analyses. Samples with different superscripts (a-d) within a row are significantly ($p \leq 0.05$) different.

[2]NOFD : original SPI (not freeze-dried); SPI : freeze-dried SPI without any ingredient;
G : glucosamine; S : sucrose; P : polyethylene glycol; A : ascorbic acid; AP : ascorbic acid with polyethylene glycol.

Table 5.8. Results of Fisher's least significant difference test on mean sensory scores of each sample for 5 attributes.

	Mean score[1]		
	SPIF[2]	AF[3]	APF[4]
Roasted	6.81 [a]	7.94 [ab]	9.49 [b]
Soymilk-like	6.88 [b]	5.34 [ab]	3.67 [a]
Cereal	6.41 [b]	4.85 [ab]	4.08 [a]
Beefy	6.71 [a]	8.03 [a]	8.79 [a]
Yeasty	7.09 [a]	6.93 [a]	6.02 [a]

[1]Mean scores for each attribute within a row with different letters are significantly different ($p \leq 0.05$) using Fisher's least significant difference test (n=14; 7 panelists with 2 replications).

[2] Mixture containing SBF and SPI but without any ingredient

[3] Mixture containing SBF and SPI treated with ascorbic acid

[4] Mixture containing SBF and SPI treated with ascorbic acid and polyethylene glycol

Table 5.9. Location of the mean intensity values of the 5 attributes for the 3 samples in comparison with the samples in Table 4.10[1].

Roasted		Soymilk-like		Cereal		Beefy		Yeasty	
Sample	Mean score	Sample	Mean score	Sample	Mean score	Sample	Mean score	Sample	Mean score
S5H	1.93 a	S2H	1.85 a	S2H	1.73 a	S5	2.54 a	S5H	2.80 a
S5	2.66 a	S2	2.53 ab	S2	2.32 ab	S5H	2.69 a	S5	3.11 a
S3H	3.46 ab	S4H	2.88 ab	S4H	2.33 ab	S3H	3.73 a	S3H	3.20 a
S3	5.69 bc	S4	3.25 ab	S4	3.57 ab	**SPIF**	**6.71** -	S3	4.17 ab
SPIF	**6.81** -	S1	3.52 ab	S1	3.65 ab	S3	6.71 b	**APF**	**6.02** -
S6H	6.83 cd	**APF**	**3.67** -	S1H	3.73 ab	S6H	7.19 b	S6	6.32 bc
S6	7.39 cde	S1H	4.02 ab	**APF**	**4.08** -	S6	7.72 bc	**AF**	**6.93** -
AF	**7.94** -	S6H	4.73 bc	S6H	4.69 bc	**AF**	**8.03** -	S6H	6.97 cd
S1H	8.53 def	**AF**	**5.35** -	**AF**	**4.58** -	S1	8.47 bcd	**SPIF**	**7.09** -
APF	**9.49** -	S6	6.25 cd	S6	6.19 cd	**APF**	**8.79** -	S1	7.50 cd
S1	9.63 ef	S3	6.73 cd	**SPIF**	**6.41** -	S4H	9.92 cd	S4	7.97 cd
S4	10.23 fg	**SPIF**	**6.88** -	S3	6.84 cde	S4	9.93 cd	S1H	8.43 cd
S4H	10.63 fg	S3H	7.87 de	S3H	7.91 def	S1H	10.13 cd	S4H	8.43 cd
S2	10.82 fg	S5H	8.98 e	S5H	9.25 ef	S2H	10.78 d	S2	9.29 d
S2H	12.14 g	S5	9.98 e	S5	9.99 f	S2	10.88 d	S2H	9.41 d

[1]Mean scores for each attribute within a column with different letters are significantly different ($p \leq 0.05$) using Duncan's multiple comparison test (n=12; 6 panelists with 2 replications), which was performed for the 12 samples in Chapter 4 of this thesis. Scores are listed in ascending order. Refer to Table 4.3 and Table 5.8 for the sample code.

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CHAPTER 6. CONCLUSION

6.1. Overall conclusions as related to the proposed hypotheses

A reliable method for analysis of the SBF and mixtures of SPI and SBF using HS-SPME coupled with GC-FID was established. The study in Chapter 2 demonstrated a successful application of fractional factorial experimental design based on Taguchi's orthogonal array to efficiently screen significant factors for HS-SPME method for GC analysis of volatile components in SBF. The establishment of this reproducible and representative method for analysis of volatile compounds to monitor changes in the headspace above samples containing SBF provided a consistent analytical technique throughout the thesis.

Identification of volatile compounds and determination of odour-active components in SBF was investigated by using GC-FID, GC-MS and GC-O in Chapter 3. The results revealed an intricate blend of aroma compounds from several sub-classes in the headspace of SBF. Moreover, GC-O by detection frequency method was very valuable to quantify the main odour-active compounds which contribute to the aroma profile of SBF. Several sulfur- and nitrogen-containing compounds as well as various terpenoids were proposed to be of essential importance for the flavour profile of the SBF. Although one of the most powerful odour-active compounds identified in SBF was 2-methyl-3-furanthiol, which has been reported to possess meat-like flavour, it was not selected as one of the indicator peaks to represent beefy note in SBF due to its low concentration compared to other volatile compounds. Instead, furans and nitrogen containing compounds such as 3-methyl furan, 2-acetyl furan, 2-ethyl-3,6-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, and 2-isoamyl-6-methylpyrazine were selected as indicator peaks to monitor the release of beef attribute related aroma compounds by SPI under various conditions. It was also demonstrated that volatile compounds in SBF included less sulfur- and nitrogen-containing compounds but many more alicyclic hydrocarbons including a great number of terpenoids than boiled or roasted beef. This difference might contribute to differences in aroma characteristics of SBF and the real beef system.

The results presented in Chapter 4 showed that descriptive analysis along with GC analysis was

useful to monitor and describe changes in aroma characteristics of SBF upon increasing SPI content in the sample mixture. Moreover, well trained panelists could be of great importance in precisely analyzing the aroma profile of samples, which can not be achieved with any other analytical instrument. The results in Chapter 4 strongly demonstrated that "roasted", "beefy", and "yeasty" notes were highly positively correlated with SBF concentration in the SBF and SPI sample mixtures, and the beefy related notes were severely suppressed by increasing SPI content. Using PCA, differences in aroma profile of the mixtures with various combinations of SBF and SPI were effectively expressed. Moreover, increased "yeasty" notes in heated samples compared to non heat treated samples were detected by using PC3. Finally, selection of indicator peaks was accomplished to represent "beefy" characteristics in the sample satisfying two criteria set by the analysis of GC-FID and GC-O. All 15 selected indicator peaks were odour-active compounds in GC-O analysis, and at the same time were significantly positively correlated with "beefy" characteristics analyzed by DA, therefore the indicator peaks could monitor the changes in aroma perception by retention of beef flavour in samples containing SPI due to SPI-SBF interactions.

Research results from Chapter 5 clearly showed that interaction of SPI with odour-active compounds in SBF was affected by addition of different ingredients. Addition of ascorbic acid led to reduction of disulfide bonds in SPI, increase in surface hydrophobicity, and relocation of interior hydrophobic side chain residues in SPI to the less hydrophobic exterior. The denaturing effect was enhanced by the combined treatment of ascorbic acid and polyethylene glycol. Conformational changes were induced by cleavage of the disulfide bond by a reducing agent, ascorbic acid, and by the interactions between hydrophobic side chains of aromatic and aliphatic amino acid residues and the repeating hydrophobic regions of polyethylene glycol. Possible intermolecular hydrogen bonding between tyrosine residues and polyethylene glycol was also proposed. In turn, the resultant structural changes in SPI seemed to affect the flavour holding capacity of SPI. Results of GC analysis showed that indicator peak areas of beef attribute related volatile compounds were increased by addition of ascorbic acid alone or with polyethylene glycol, and that these were also expressed by enhancement of roasted note and diminished soymilk-like and cereal notes in the mixture of SBF and ingredients added SPI. Therefore, it could be postulated that suppression of beef flavour in SBF due to binding of odour-active components with SPI could be improved by addition of ascorbic acid especially in conjunction with

polyethylene glycol, resulting in maximizing of SBF perception in the mixture of SBF and SPI.

6.2. Significance of this thesis research to the field of study

McGorrin (1996) classified the nature of interactions between volatile aroma compounds and non-flavour food matrix components into three types, i.e., binding, partitioning, and releasing. Considerable research has been conducted and reported in the literature on the binding of volatiles to proteins using model systems with individual chemicals or a series of chemicals, including lipid oxidation products responsible for off-flavour of soy product. Although these studies have provided useful information on binding properties such as binding constants and number of binding sites of each volatile compound by determination of thermodynamic parameters related to binding, their application to the food system is limited.

A significant aspect of the present research described in this thesis is that the interaction between SPI and SBF was investigated from the flavour "releasing" point of view, focusing on the "availability of flavour compounds from the bulk food into the gas phase for sensory perception" based on flavour holding capacity of SPI, rather than flavour "binding", explained as "retention or absorption of volatile compounds onto non-volatile substrates" (McGorrin, 1996).

In the present study, commercially available SBF and SPI were used, rather than a model system with individual volatile compounds. Therefore, the results may be more practical and could easily be transferred to food processing applications. Furthermore, the results from this study could form a constructive basis to formulate strategies to enhance the beefy aroma perception in soy protein based products.

This thesis also provided information on the identity of volatile compounds in SBF and, through the comparison of the components in SBF with cooked beef such as roasted or boiled beef, similarities and differences between the simulated beef flavouring and authentic beef were discussed. Moreover, odour-active components contributing to the aroma profile of SBF were documented using GC-O. Knowledge regarding identification of volatile compounds and odour-active components in SBF may provide the basis to develop effective flavouring products to

simulate beef flavour by the flavour industry.

In addition, selected indicator peaks should be useful in the future to monitor interaction between SBF and SPI in terms of beefy flavour releasing under various environmental conditions. The use of indicator peaks may reduce the number of sensory evaluations, which are generally time- and labour-consuming work and cannot be replaced easily. Furthermore, the procedure to select the indicator peaks may be applied to other food systems during food product development or processing.

6.3. Suggestions for future research

There is no doubt that flavour is one of the most important factors for consumers to select food products. Research over the last several decades on flavour and food component interactions in either model system or food matrix has shown that food constituents such as proteins, polysaccharides, and lipids interact with flavour compounds (Chevance and Farmer, 1998; Mottram, 1998; Hau et al., 1996). Since attenuation of even a single odour-active component may have a remarkable effect on flavour perception, the flavour-binding behavior of food components must be considered in the development of food products. In this thesis interactions of SPI in terms of flavour holding behavior toward volatile compounds in SBF were examined. However, in a real food system, proteins comprise merely one of the many components in a food product. Therefore, flavour interactions between flavours and lipids, carbohydrates, proteins, or complex food components as a function of different pH, temperature, humidity and pressure should be investigated.

When a food product is consumed, the aroma of the food is perceived in two different steps. Food flavour is first perceived by olfactory epithelia in our nose while a food is being served. The other route involves perception of aroma by releasing of the aroma compounds from food while the food is masticated with saliva at body temperature in our mouth. Volatile compounds should be transported from the saliva phase to the air phase in the mouth, and then to the olfactory receptors in the nose, which is affected by the rate of breathing, swallowing and salivation (Taylor, 2002). Therefore understanding of aroma perception relating to odour

compounds release would be imperative to improve flavour quality in food products. Non-volatile compounds that contribute to the taste of the SBF and perception of taste of the flavour in gustatory receptors of taste buds would be another area to be elucidated, which can be explored using high performance liquid chromatography.

In addition, variance in composition of SPI and SBF should be considered. Zhou and Cadwallader (2006) investigated the binding properties of three SPIs from different origins and reported that those SPIs showed similar flavour binding patterns although absolute flavour binding capacities were not the same. More research results on the effects of variability of SPI and SBF from different origins or processing methods should be gathered. As indicated in Chapter 5 of this thesis, the SPI used in this study was already denatured. It would be worth performing research using SPIs with different degrees of denaturation.

Some of the odour impact compounds relating to beefy note in SBF were sulfur containing compounds. Given the knowledge that most sulfur containing compounds have low thresholds, i.e., high aroma impact, further investigations that focus specifically on changes of the sulfur containing compounds under various conditions would be valuable. Although GC-FID is very sensitive for most volatile compounds, usually the concentration of sulfur containing compounds in food ingredients are extremely low. Gas chromatography coupled with flame photometric detector (FPD), which is a specific detector for sulfur- or phosphorus-bearing compounds (Patterson et al., 1978; Yao et al., 2001) may be a complementary technique in investigations focused on sulfur containing compounds.

6.4. References

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APPENDIX

Appendix I. Correlation matrix of mean value of the peak area in GC chromatogram and the mean scores of the 5 sensory attributes[1].

RT	Peak #[2]	Roasted	Soymilk-like	Cereal	Beefy	Yeasty
1.717	P1	0.734 **	-0.742 **	-0.756 **	0.710 *	0.757 ***
4.144		-0.735 **	0.740 **	0.746 **	-0.702 *	-0.824 **
4.188		-0.811 **	0.819 **	0.824 **	-0.793 **	-0.883 ***
6.212	P5	0.529	-0.502	-0.500	0.451	0.498
7.832	P7	0.701 *	-0.670 *	-0.673 *	0.658 *	0.711 *
9.833	P11	-0.283	0.301	0.312	-0.240	-0.389
10.250		0.435	-0.429	-0.436	0.433	0.463
11.605		-0.557	0.523	0.514	-0.555	-0.573
12.046	P17	0.864 ***	-0.877 ***	-0.858 ***	0.872 ***	0.797 **
12.428		-0.167	0.221	0.209	-0.080	-0.191
13.205	P20	0.736 **	-0.728 **	-0.707 *	0.713 **	0.739 **
13.822		0.615 *	-0.634 *	-0.633 *	0.648 *	0.633 *
14.726		-0.144	0.193	0.201	-0.125	-0.236
14.881		0.804 **	-0.787 **	-0.787 **	0.773 **	0.811 **
15.014		0.745 **	-0.696 *	-0.696 *	0.696 *	0.721 **
15.772	P24	0.686 *	-0.596 *	-0.598 *	0.601 *	0.664 *
16.133	(P81)[3]	0.895 ***	-0.874 ***	-0.873 ***	0.852 ***	0.914 ***
16.526		-0.636 *	0.754 **	0.754 **	-0.645 *	-0.663 *
16.864		0.887 ***	-0.834 **	-0.844 **	0.827 **	0.847 **
17.064	P27	0.460	-0.474	-0.452	0.495	0.387
17.338		0.544	-0.561	-0.596 *	0.526	0.581 *
17.570		0.568	-0.619 *	-0.643 *	0.519	0.613 *
17.930		0.426	-0.394	-0.388	0.329	0.371
18.199	(P84)[3]	0.631 *	-0.635 *	-0.633 *	0.605 *	0.657 *
18.740		0.340	-0.224	-0.217	0.321	0.269
19.673		0.553	-0.565	-0.595 *	0.486	0.560
19.774	P30	0.580 *	-0.570	-0.558	0.590 *	0.491
20.198		0.038	-0.155	-0.176	0.068	0.149
20.517	P32	0.569	-0.623 *	-0.642 *	0.678 *	0.692 *
20.770		0.624 *	-0.617 *	-0.606 *	0.545	0.605 *
21.196	P33	0.601 *	-0.581 *	-0.591 *	0.544	0.589 *
21.488		0.442	-0.339	-0.344	0.443	0.395
22.062	P35	0.694 *	-0.709 *	-0.702 *	0.715 **	0.664 *
23.145		-0.254	0.318	0.322	-0.225	-0.377
23.380		0.643 *	-0.624 *	-0.654 *	0.594 *	0.634 *
24.103	P38	0.671 *	-0.634 *	-0.646 *	0.599 *	0.646 *
24.682		0.004	0.001	0.026	0.058	-0.076
25.034		0.599 *	-0.574	-0.564	0.537	0.572
25.374		0.596 *	-0.598 *	-0.594 *	0.598 *	0.593 *
25.703	(P87)[3]	0.805 **	-0.798 **	-0.803 **	0.794 **	0.823 **
25.948		0.722 **	-0.707 *	-0.730 **	0.697 *	0.733 **

RT	Peak #[2]	Roasted	Soymilk-like	Cereal	Beefy	Yeasty
26.223		0.710 *	-0.634 *	-0.640 *	0.684 *	0.646 *
26.707		0.542	-0.526	-0.527	0.496	0.539
26.866		0.747 **	-0.764 **	-0.759 **	0.752 **	0.757 **
26.983		0.397	-0.403	-0.397	0.310	0.383
27.154		-0.563	0.475	0.476	-0.574	-0.550
27.209	P43	-0.355	0.442	0.443	-0.327	-0.469
27.742		0.875 ***	-0.897 ***	-0.900 ***	0.887 ***	0.902 ***
27.968		0.515	-0.504	-0.507	0.487	0.526
28.204	P44	0.899 ***	-0.879 ***	-0.881 ***	0.931 ***	0.928 ***
28.322		-0.396	0.267	0.255	-0.388	-0.281
28.746	P45	0.715 **	-0.648 *	-0.626 *	0.678 *	0.681 *
29.038		0.756 **	-0.685 *	-0.688 *	0.713 **	0.738 **
29.384		0.068	-0.061	-0.039	0.127	-0.008
29.519		0.556	-0.545	-0.543	0.509	0.555
29.767		0.375	-0.239	-0.238	0.337	0.360
29.888	P48	0.904 ***	-0.885 ***	-0.887 ***	0.928 ***	0.934 ***
30.140		0.626 *	-0.633 *	-0.633 *	0.617 *	0.666 *
30.291	P49	0.879 ***	-0.890 ***	-0.895 ***	0.850 ***	0.924 ***
30.525		0.723 **	-0.702 *	-0.678 *	0.645 *	0.675 *
30.648	P50	0.914 ***	-0.905 ***	-0.906 ***	0.929 ***	0.937 ***
30.849		0.890 ***	-0.866 ***	-0.871 ***	0.868 ***	0.896 ***
31.162		0.674 *	-0.679 *	-0.677 *	0.638 *	0.684 *
31.238		0.881 ***	-0.894 ***	-0.911 ***	0.914 ***	0.953 ***
31.438		0.736 **	-0.753 **	-0.753 **	0.750 **	0.762 **
31.813	P54	0.896 ***	-0.886 ***	-0.888 ***	0.924 ***	0.931 ***
32.148		0.867 ***	-0.868 ***	-0.870 ***	0.879 ***	0.900 ***
32.438		0.624 *	-0.630 *	-0.632 *	0.618 *	0.666 *
32.598		0.859 ***	-0.857 ***	-0.857 ***	0.863 ***	0.896 ***
33.083		0.813 **	-0.828 **	-0.828 **	0.826 **	0.854 ***
33.225	P58	0.875 ***	-0.872 ***	-0.873 ***	0.894 ***	0.908 ***
33.540		0.778 **	-0.772 **	-0.771 **	0.779 **	0.817 **
33.727		0.717 **	-0.705 *	-0.694 *	0.689 *	0.727 **
33.935		-0.704 *	0.812 **	0.818 **	-0.715 **	-0.726 **
34.130		0.782 **	-0.770 **	-0.766 **	0.775 **	0.811 **
34.261		0.779 **	-0.780 **	-0.780 **	0.787 **	0.817 **
34.403		0.585 *	-0.589 *	-0.593 *	0.600 *	0.632 *
34.610	P67	0.868 ***	-0.858 ***	-0.859 ***	0.881 ***	0.899 ***
34.780		0.792 **	-0.764 **	-0.762 **	0.781 **	0.816 **
35.011		0.858 ***	-0.864 ***	-0.864 ***	0.872 ***	0.899 ***
35.172		0.214	-0.258	-0.278	0.353	0.359
35.283		0.859 ***	-0.859 ***	-0.855 ***	0.823 **	0.868 ***
35.562		0.544	-0.510	-0.512	0.503	0.541
35.680	P71	0.869 ***	-0.866 ***	-0.870 ***	0.883 ***	0.905 ***
35.816		0.675 *	-0.735 **	-0.742 **	0.685 *	0.748 **
36.093		-0.458	0.496	0.494	-0.456	-0.495
36.242	P72	0.810 **	-0.813 **	-0.816 **	0.822 **	0.853 ***

RT	Peak #[2]	Roasted	Soymilk-like	Cereal	Beefy	Yeasty
36.565		0.792 **	-0.780 **	-0.774 **	0.780 **	0.813 **
36.755		0.533	-0.519	-0.521	0.495	0.537
36.948		0.789 **	-0.793 **	-0.798 **	0.800 **	0.842 **
37.071		-0.267	0.254	0.248	-0.332	-0.232
37.263	P75	0.793 **	-0.787 **	-0.789 **	0.778 **	0.819 **
37.582		0.805 **	-0.816 **	-0.809 **	0.815 **	0.803 **
37.921		0.757 **	-0.755 **	-0.759 **	0.746 **	0.790 **
38.081		0.153	-0.167	-0.167	0.169	0.251
38.125		0.253	-0.264	-0.265	0.276	0.315
38.342	P78	0.668 *	-0.653 *	-0.658 *	0.634 *	0.715 **
38.548		0.774 **	-0.756 **	-0.756 **	0.767 **	0.788 **
38.906		-0.610 *	0.613 *	0.605 *	-0.632 *	-0.570
39.030		0.674 *	-0.679 *	-0.675 *	0.672 *	0.713 **
39.148		-0.600 *	0.604 *	0.596 *	-0.618 *	-0.575
39.281	P79	-0.670 *	0.782 **	0.783 **	-0.694 *	-0.647 *
39.462		-0.642 *	0.625 *	0.618 *	-0.650 *	-0.620 *
39.758		-0.524	0.484	0.477	-0.518	-0.430
39.865		-0.719 **	0.693 *	0.674 *	-0.703 *	-0.669 *
40.126		-0.729 **	0.653 *	0.647 *	-0.753 **	-0.653 *
40.261		0.160	-0.280	-0.272	0.217	0.236
40.403		0.721 **	-0.709 *	-0.706 *	0.710 *	0.753 **
40.512		-0.502	0.527	0.505	-0.535	-0.441
40.666		0.362	-0.387	-0.389	0.371	0.419
40.887		-0.800 **	0.774 **	0.767 **	-0.801 **	-0.755 **
40.927		0.616 *	-0.647 *	-0.653 *	0.626 *	0.699 *

[1]*, **, and ***, significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

[2]The peaks were numbered based on GC-MS identification of volatile components in SBF (Moon et al., 2006). Peaks in this table without peak numbers were either not identified by GC-MS or were components derived from SPI.

[3]The peak numbers P81, P84, and P87 were assigned to these peaks due to high aroma value (over 75 % detection frequency) in GC-olfactometry analysis, although they were not identified by GC-MS analysis (Moon et al., 2006).