

**Effects of Micronization, Ethanol Washing, and Enzymatic Hydrolysis Processing
Alone or in Combination on Trypsin Inhibitors, Lipoxygenase Activities and Selected
“Beany” Flavour Related Compounds in Soybean Flour**

By

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Abstract

Soybean production and consumption has increased in recent decades. However, trypsin inhibitor activity and “beany” flavour are two drawbacks limiting the utilization of soybean. In the present study, micronization, ethanol washing, and enzymatic hydrolysis (alone or in combination) were used to treat soybean. Micronization at 100 °C and 135 °C decreased the activity of both trypsin inhibitors (53% and 80% respectively), and lipoxxygenase (51% and 99%, respectively). Ethanol increased the trypsin inhibitor activity while alcalase hydrolysis decreased its activity. Different treatment combinations affected trypsin inhibitor activity, with micronization having a major influence. “Beany” flavour related volatiles (hexanal, (E)-2-hexenal, 1-hexanol, heptanal, (E)-2-octenal, (E)-2-nonenal, (E,E)-2,4-nonadienal, 2,4-decadienal, (E,E)-2,4-decadienal, 1-octen-3-ol, 2-pentylfuran and 3-octen-2-one) were significantly decreased with micronization. Ethanol effects varied with different volatiles. Soybean micronized at 135°C and washed with 65% ethanol was recommended for soybean processing due to its low trypsin inhibitor activity and low “beany” related volatile content.

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TABLE OF CONTENTS

ABSTRACT.....	I
ACKNOWLEDGMENTS	II
LIST OF FIGURES	VIII
LIST OF TABLES	XI
1 Introduction.....	1
2 Literature Review	4
2.1 Soybeans	4
2.1.1 Soybean nutrition.....	4
2.1.2 Soybean production	5
2.1.3 Soybean products.....	6
2.2 Trypsin Inhibitors	6
2.2.1 Kuntiz trypsin inhibitor (KIT)	6
2.2.2 Bowman-Birk trypsin inhibitor (BBTI)	7
2.2.3 Heat stability of trypsin inhibitor.....	8
2.2.4 Health implications of trypsin inhibitors	9
2.3 Control of Trypsin Inhibitors.....	10
2.3.1 Trypsin inhibitors and micronization.....	10
2.3.2 Trypsin inhibitor and ethanol washing	12
2.3.3 Trypsin inhibitor and alcalase hydrolysis treatment	15

2.4	Beany Flavour.....	17
2.4.1	Key volatiles responsible for beany flavour	17
2.4.2	Sensory studies on beany flavour	18
2.4.3	Soybean Lipoxygenase (LOX)	20
2.5	Control of Beany Flavour	23
2.5.1	Lipoxygenase, beany flavour and micronization	23
2.5.2	Off flavour and organic solvent washing treatment.....	24
3	Material and Methods	28
3.1	Tempering.....	28
3.2	Micronization Treatment	28
3.3	Dehulling, Grinding and Defatting	29
3.3.1	Dehulling and grinding	29
3.3.2	Defatting soybean with hexane	29
3.4	Ethanol Treatment.....	30
3.5	Enzyme Hydrolysis.....	30
3.6	Proximate Analysis of Soybean Treated with Different Processing.....	32
3.6.1	Moisture content determination	32
3.6.2	Protein content determination	32
3.6.3	Crude fat content determination.....	32
3.7	Study 1: Effects of Micronization, Ethanol Washing and Enzyme Hydrolysis on Trypsin Inhibitor and Lipoxygenase Activities in Soybean Flour	33
3.7.1	Degree of hydrolysis determination.....	33

3.7.2	Differential Scanning Calorimetry (DSC) analysis	35
3.7.3	Trypsin inhibitor activity determination	36
3.7.4	Lipoxygenase activity of soybean flour determination.....	39
3.8	Study 2: Effects of Micronization, Defatting and Ethanol Treatments on Volatile Compounds in Soybean Flour	40
3.8.1	Simultaneous distillation extraction (SDE) of soybean volatiles by Likens-Nickerson apparatus.....	40
3.8.2	Analysis of volatiles by Gas Chromatograph–Mass Spectrometer (GC-MS)	44
3.8.3	Identification and semi-quantification of volatiles	44
3.9	Statistical Analysis.....	48
4	Results and Discussion	49
4.1	Proximate Analysis of Soybean Treated with Different Processing.....	49
4.1.1	Proximate analysis on full fat soybean flour.....	49
4.1.2	Yield of the soybean flour after ethanol washing	51
4.1.3	Effects of processing on moisture content in defatted soybean flour	52
4.1.4	Effects of different processing on protein content in defatted soybean flour	58
4.1.5	Crude fat content determination among treated soybean flours	62
4.2	Study 1: Effects of Micronization, Ethanol Washing and Enzyme Hydrolysis on Trypsin Inhibitor and Lipoxygenase Activities in Soybean Flour	64
4.2.1	Degree of hydrolysis determination on alcalase treated soybean flour.....	64
4.2.2	Protein structural analysis on the soybean flour using Differential Scanning Calorimetry (DSC) analysis	67

4.2.3	Effects of micronization, ethanol washing, and enzyme hydrolysis on trypsin inhibitors in soybean flour	73
4.2.4	Effects of micronization, defatting, ethanol washing and enzymatic hydrolysis on soybean Lipoxygenase (LOX) activity	87
4.3	Study 2: Effects of Micronization, Defatting, and Ethanol Washing on Soybean Flour Volatiles (related to soybean “beany” flavour)	91
4.3.1	Soybean flour volatile analysis	91
4.3.2	Effect of micronization, defatting, and ethanol washing on volatiles generated by lipoxygenase oxidation of unsaturated fatty acid in soybean flour	99
4.3.3	Effect of micronization, defatting, and ethanol washing on volatiles generated by singlet oxygen induced unsaturated fatty acid oxidation in soybean flour	118
4.3.4	General discussion on effects of micronization, defatting and ethanol washing on “beany” flavour related volatiles	124
5	Conclusions.....	126
6	Applications and Recommendations.....	129
	REFERENCES	130
	Appendix 1 Amount of nitrogen from the free amino groups in 1 gram of soybean sample	146
	Appendix 2 Analysis of variance table for moisture content using SAS with a factorial design	146
	Appendix 3 Analysis of variance table for protein content using SAS with a factorial design	146

Appendix 4 Analysis of variance table for degree of hydrolysis (DH%) by using SAS with a factorial design	147
Appendix 5 Analysis of variance table for Differential Scanning Calorimetry (DSC) data by using SAS with a factorial design	147
Appendix 6 Analysis of variance table for trypsin inhibitor activity by using SAS with a factorial design	148
Appendix 7 Mass spectrum of selected volatile compounds related to “beany” flavour ..	149
Appendix 8 Effects of different processing on concentration (ppm) of “beany” flavour related volatile compounds in soybean flour	162

LIST OF FIGURES

Figure 2.1 Soybean productions (tonnes) in Canada from 2003 to 2013. Source: Food and Agricultural Organization (2015)	5
Figure 2.2 Primary structure of soybean Kuntiz trypsin inhibitor (A) and secondary structure model of Kuntiz trypsin inhibitor (B) from soybean. Adapted from Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz (2013) (open access).....	7
Figure 2.3 Primary structure of soybean Bowman-Birk trypsin inhibitor (A) and secondary structure model of Bowman-Birk trypsin inhibitor (B) from soybean. Adapted from Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz (2013) (open access).....	8
Figure 2.4 Lipxygenase catalyzed oxygenation of linoleic acid to produce 13-hydroperoxy- cis- 9, trans-11- octadecadienoic acid (13-HPOD) Source: Robinson et al. (1995).....	21
Figure 2.5 Possible degradation products arising from the action of hydroperoxide lyases and isomerases. Source: Robinson et al. (1995).	22
Figure 3.1 Reaction of TNBS with amino acid groups. Adapted from Adler-Nissen (1979) with open access.	35
Figure 3.2 Simultaneous Distillation Extraction (SDE) system for soybean flour volatile extraction by using Likens and Nickerson's apparatus.....	43
Figure 3.3 GC-MS graph for alkane standards. Different alkanes were as indicated.....	47
Figure 4.1 Moisture content (%) for defatted soybean flour with different treatments	54
Figure 4.2 Protein content (%) for defatted soybean flour with different treatments	59
Figure 4.3 Crude fat content (dry basis) for soybean flour treated with different treatments	63

Figure 4.4 Degree of hydrolysis (DH) % for enzymatic hydrolysis on alcalase hydrolyzed soybean flour.....	66
Figure 4.5 DSC thermogram of the defatting soybean flour, 65% ethanol treated & 85% ethanol treated soybean flour	70
Figure 4.6 Effects of ethanol treatment and hydrolysis on trypsin inhibitor activity (dry basis) in unmiconized soybean flour, and soybean flour that was micronized at 100°C and 135°C	74
Figure 4.7 Effects of the micronization on lipoxygenase activity in defatted soybean flour.....	89
Figure 4.8 A chromatogram of volatile compounds extracted from original full-fat soybean	93
Figure 4.9 Effects of micronization, defatting, and ethanol treatment on hexanal in soybean flour.....	99
Figure 4.10 Effects of micronization, defatting, and ethanol treatment on (E)-2-hexenal in soybean flour.....	103
Figure 4.11 Effects of micronization, defatting, and ethanol treatment on 1-hexanol in soybean flour.....	105
Figure 4.12 Effects of micronization, defatting, and ethanol treatment on heptanal in soybean flour.....	107
Figure 4.13 Effects of micronization, defatting, and ethanol treatment on (E)-2-octenal in soybean flour.....	110
Figure 4.14 Effects of micronization, defatting, and ethanol treatment on (E)-2-nonenal in soybean flour.....	112

Figure 4.15 Effects of micronization, defatting, and ethanol treatment on (E, E)-2-4-nonadienal in soybean flour	114
Figure 4.16 Effects of micronization, defatting, and ethanol treatment on 2-4-decadienal in soybean flour.....	115
Figure 4.17 Effects of micronization, defatting, and ethanol treatment on (E, E)-2-4-decadienal in soybean flour	117
Figure 4.18 Effects of micronization, defatting, and ethanol treatment on 1-Octen-3-ol in soybean flour.....	119
Figure 4.19 Effects of micronization, defatting, and ethanol treatment on 2-pentylfuran in soybean flour.....	121
Figure 4.20 Effects of micronization, defatting, and ethanol treatment on 3-octen-2-one in soybean flour.....	123

LIST OF TABLES

Table 3.1 Retention time (RT) and linear retention indices values of n-alkane standards (C8-C20 10 µg/mL)	46
Table 4.1 Proximate analysis of the original and micronized soybean flour	49
Table 4.2 Soybean flour recovery rate (%) after ethanol washing	52
Table 4.3 Effects of the micronization, ethanol washing, and alcalase hydrolysis on denaturation temperature (°C) and enthalpy (J/g) for glycinin and β -conglycinin in soybean storage protein.....	71
Table 4.4 Effects of the micronization, defatting processing on lipoxygenase activity in soybean flour.....	89
Table 4.5 Summary of possible compounds extracted from original full-fat soybean	94
Table 4.6 List of compounds and their sensor attributions in soybean and soybean products as has been reported in published literature	96
Table 4.7 Selected volatiles correlated to soybean beany flavour and their attributes	97

1 Introduction

Soybean is a well-established agricultural product due to its very unique chemical composition. Compared to the other legume species, it has the highest protein content (~40%) and the quality of soybean protein is equivalent to animal protein; it has the second highest oil content (~20%) after peanut (~48%) (Liu, 1997). Soybeans are also rich in oligosaccharides, dietary fibre, phosphides, phytochemicals (mainly isoflavones) and minerals (Liu, 1997; Mateos-Aparicio et al., 2008). Soybean has been used in human foods in a variety of forms including cheese, drinks, miso, tempeh, tofu, salami, and vegetarian meat substitutes. Soybean protein has also been used as infant formulas and soy ingredients include, flours, protein isolates and concentrates, and textured fibres (Friedman and Brandon, 2001).

Soy products have been linked to the prevention of cancer, osteoporosis, renal disease and heart disease in human. The health benefits of soybean products are mainly due to its bioactive phytochemicals such as isoflavones, phytic acids, and phytosterols (Isanga and Zhang, 2008). Among those bioactive phytochemicals, the functions of isoflavones (majorly genistein and daidzein) have been well studied. Isoflavones had been reported to reduce the risk of breast cancer, prostate cancer and colon cancer possibly due to their inhibition effects on cancer cells (Isanga and Zhang, 2008; Mateos-Aparicio et al., 2008). Besides, health claim about soybean protein stated that eating at least 25 grams soybean protein per day may reduce the risk of heart disease (U.S. Food and Drug Administration, 2013). Therefore, there is an increasing interest in studying soybean as a potential functional food.

Under the appropriate proportion, consuming soybean may have beneficial effects on health. In many cases, the bioactive compounds which are responsible for the health effects, are considered as anti-nutrients by the same interaction mechanism (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013). However, the level of these bioactive compounds at which the beneficial and harmful effects occur is hard to evaluate because their physiological effects in different people are different. In soybean, the anti-nutritional factors are mainly phytic acids, protease inhibitors, isoflavones, lectins, oligosaccharides and tannins (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013).

Despite the health effects associated with consumption of soybean products, there are some limitations in utilizing soybean as an ingredient; the beany flavour is one of the biggest issues related to the acceptability of soybean products. Most soybean products have a beany flavour. To some consumers, this flavour is objectionable even if the level of these flavour volatiles is very low. For example, the threshold for detecting hexanal, a major component for “greeny” flavour in soybean products, is 5.87 ppm while for E,E-2,4-decadienal, it is only 0.47 ppm (Brewer and Vega, 1995). Products which contain soybean often have objectionable flavour compared with the same product without soybean. Ivanovski, Seetharaman and Duizer (2012) indicated that there was a significantly stronger beany flavour in bread made using 12-20% soy protein substitution level compared to an all-wheat control bread based on the sensory evaluation by trained panels (Ivanovski, Seetharaman and Duizer, 2012).

Lipoxygenase had been highly correlated to the beany flavour generation in soybean products due to its role in the oxidation of unsaturated fatty acid resulting in the

generation of odour compounds in soybean. Lipoxygenase-deficient soybean species produced lower off-flavour than normal soybean (Boué et al., 2005).

High levels of stable trypsin inhibitors in soybean are another drawback limiting the use of soybean. Despite suggestions that trypsin inhibitors can act as bioactive phytochemicals, high amount of trypsin inhibitors can cause protein mal-absorption. Based on animal studies, rats fed soybean extracts with trypsin inhibitors intact showed lower growth performance than the rats fed with the trypsin inhibitor-free soybean extracts; forty percent of depression in growth was attributed to the trypsin inhibitors (Kakade, Hoffa and Liener, 1973). Apart from causing growth inhibition, trypsin inhibitors can induce pancreatic hypertrophy and pancreatic adenoma due to their anti-tryptic and anti-chymotryptic activities (Friedman and Brandon, 2001).

The objective of this study was to investigate the effects of different treatments of soybeans flours on trypsin inhibitors and lipoxygenase activities as well as on flavour compounds which might be related to the “beany” flavours in soybean products. The three treatments considered were micronization, ethanol washing or alcalase hydrolysis individually or in combination. Beany flavour evaluation was based on extraction of volatiles from soybean flour using pentane as solvent with simultaneous distillation method. The volatile compounds profile was analyzed by using gas chromatography in cooperated with mass spectrometry.

2 Literature Review

2.1 Soybeans

2.1.1 Soybean nutrition

Soybean is one of the most important and nutritious foods in the world. It is the major part of the Asian diet and has been gaining popularity in Western countries as well. Soybean seeds are the most important vegetable source of the edible oils and high quality proteins.

Soybean contains about 20% lipid content and the majority of the lipids are located in the soybean cotyledon (Johnson et al., 2008). The predominant fatty acid is linoleic acid (18:2), whilst the other important fatty acids are oleic (18:1), palmitic (16:0), linolenic (18:3) and stearic acid (18:0) (Johnson et al., 2008; Liu, 1997). Soybean oil is an excellent source of essential fatty acids, which provide calories, and fat-soluble vitamins. The high levels of linoleic acid in soybean could help lower cholesterol by reducing blood lipid levels. In addition, it also contains 8% linolenic acid, which is an omega-3 fatty acid that assists in lowering the risk of heart disease. However, the high levels of unsaturated fatty acid in soybean oil makes it susceptible to oxidation, which can lead to the development of beany flavours (Liu, 1997).

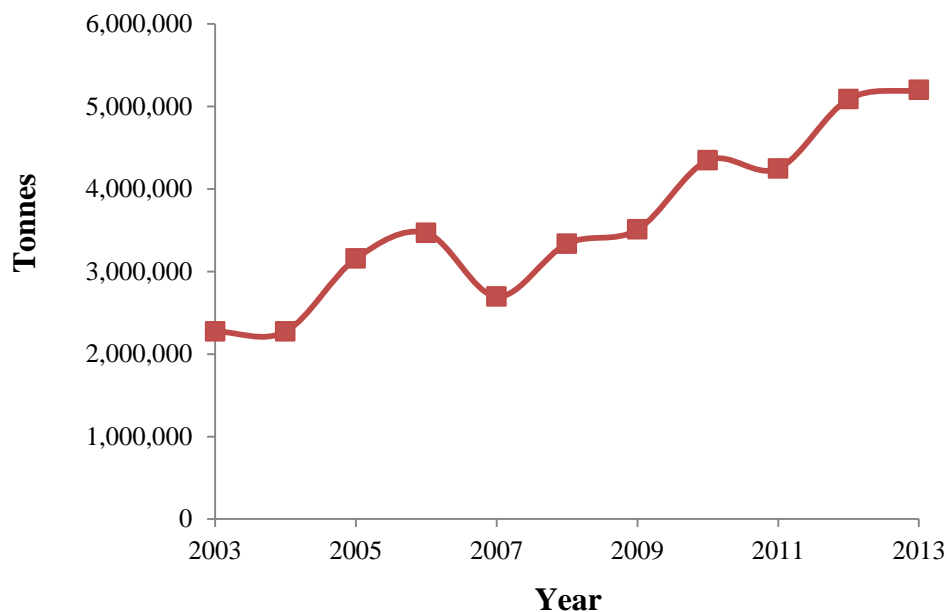
Soybean protein is the most commercially available, predominant and inexpensive source of high nutritional quality vegetable protein in the world. Proteins make up the largest part of the soybean seed (~40%). Soybean protein contains all eight essential amino acids and has been shown to be equal to the protein quality of milk, meat, egg and be easily digested by humans (Riaz, 2005). Unfortunately, soybean is used largely as

animal feed, with only small portions used for human consumption (Liu, 1997). However, soybean and soybean protein products are becoming more and more acceptable by the world because of their health benefits.

2.1.2 Soybean production

Soybean production uses 6% of the world's arable land and has expanded much faster than other major grains and oilseeds (Johnson et al., 2008). In Canada, soybean production has increased from 2,273,300 tonnes to 5,198,400 tonnes from 2003 to 2013 (Figure 2.1). Compared with 2003, over twice the amount of soybean was produced in 2013 indicating an increasing demand for soybean. Ontario and Manitoba are the two largest soybean producing provinces accounting for 60% and 21% of soybean production (FAO, 2015).

Figure 2.1 Soybean productions (tonnes) in Canada from 2003 to 2013. Source: Food and Agricultural Organization (2015)



2.1.3 Soybean products

China has been consuming soybean products for more than 5000 years. Therefore soybean has been transformed into different various forms such as soybean milk, tofu and soy sauce. There are two classes of traditional soybean product: nonfermented and fermented soybean. Tofu, soymilk, and soybean are the most popular nonfermented soybean products whereas soy sauce, miso, tempeh and natto are the most popular fermented soybean products (Liu, 1997). However, soybean oil could be extracted for use as edible oil and the defatted soybean flour can be further processed into different types of soybean protein products including defatted soybean flour, soybean protein concentrate and soybean protein isolates (Liu, 1997). Soybean could also be used as dairy analogs, such as soy ice cream, and meat analogs.

2.2 Trypsin Inhibitors

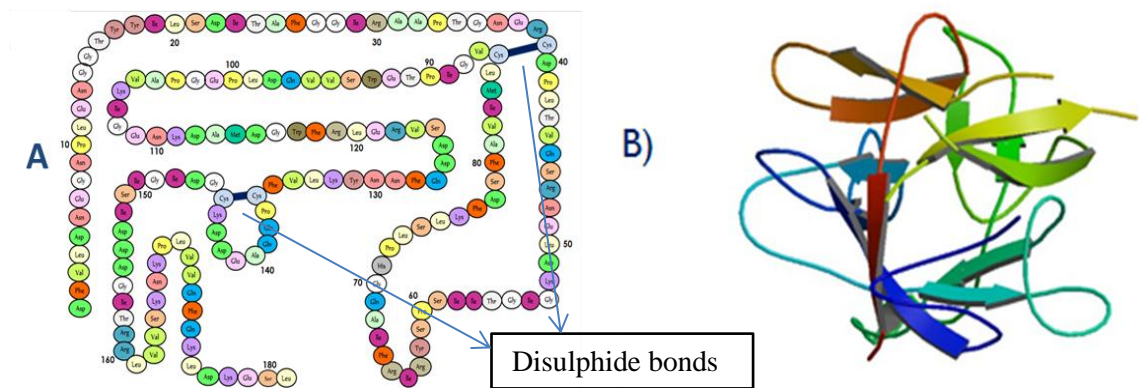
Protease inhibitors, low molecular weight proteins with unusual amino acid composition, play important roles in the plant kingdom; they may act as regulator of protease, as storage proteins, or as protective agents against insects and microorganisms (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013; Žilić, Bozović and Šukalović, 2012). Soybean contains several types of trypsin inhibitors (TI), but the two well-characterized trypsin inhibitors are Kuntiz trypsin inhibitor (KTI) and Bowman-Birk trypsin inhibitor (BBTI). Those two TIs are responsible for the majority of bioactive proteins in the 2S albumin soybean protein fraction (Žilić, Bozović and Šukalović, 2012).

2.2.1 Kuntiz trypsin inhibitor (KIT)

The KTI (one head inhibitor) has a molecular weight of approximately 21.5 kDa with 181 amino acid residues including two disulphide bonds and can directly inhibit

trypsin (Figure 2.2). KTI is a competitive inhibitor which binds to the active sites of trypsin in the same way the substrate of trypsin does. As results of this binding reaction, peptide bonds between amino acids at the reactive sites in the trypsin inhibitor and trypsin are hydrolysed. After hydrolysis, the trypsin inhibitor maintains its original conformation due the disulphide bond (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013).

Figure 2.2 Primary structure of soybean Kuntiz trypsin inhibitor (A) and secondary structure model of Kuntiz trypsin inhibitor (B) from soybean. Adapted from Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz (2013) (open access)



2.2.2 Bowman-Birk trypsin inhibitor (BBTI)

The BBTI (two head inhibitor) has a molecular weight of about 8 kDa with 71 amino acids including seven disulphide bridges (Figure 2.3). The disulphide bonds make the BBTI stable to heat, acids and bases. BBTI is also a competitive inhibitor which can simultaneously or independently inactivate two enzymes such as trypsin (Lys16-Ser17) / trypsin (Leu44-Ser45) or trypsin (Lys16-Ser17) /chymotrypsin (Leu44-Ser45) (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013; Qi, Song and Chi, 2005; Sarwar Gilani, Wu Xiao and Cockell, 2012).

2.2.4 Health implications of trypsin inhibitors

TIs are commonly recognized as important anti-nutritional factors and believed to reduce protein digestion in human and animals. Kakade, Hoffa and Liener (1973) observed that rats fed with soy extracts from unprocessed soybeans with TI intact showed lower growth performance than rats fed with soy extracts without TI (Kakade, Hoffa and Liener, 1973). TIs form inactive complexes with trypsin or chymotrypsin which reduce the amount of available digestive enzymes such as trypsin, thus making absorption of products of proteolysis, including amino acids, very difficult. In addition, the TI-enzyme complexes which are rich in sulfur amino acids cannot be utilized and will be excreted through the feces (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013). Due to the increased secretion of proteases such as trypsin and chymotrypsin more sulfur-containing amino acids are required. Therefore, these amino acids are diverted from the synthesis of body tissue protein to the synthesis of proteases which are subsequently lost in the faeces (Friedman and Brandon, 2001). However, only about 40% of the growth depression produced by soybeans has been attributed to TI. This observation indicated that TI is not the sole factor responsible for growth depression in human body. Other factor could be the resistance of unheated soybean protein to tryptic attack which causes problem in digesting soybean protein (Kakade, Hoffa and Liener, 1973).

In addition to protein mal-absorption, the increased synthesis and secretion of proteases (e.g., trypsin, chymotrypsin and elastase) due to TI may cause enlargement of the pancreas in susceptible animals. In long-term rat-feeding trials with soy extract, pancreatic hypertrophy and hyperplasia diseases were observed in rats (Friedman and Brandon, 2001). The mechanism for TIs induced pancreatic diseased is not entirely clear.

A theory involving negative feedback secretion has been proposed, such that, when the level of pancreatic enzymes is reduced to a certain point, the endocrine cells of duodenal mucosa release a hormone which leads the pancreas to synthesize more proteases (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013). TIs do not express adverse effects only. BBTIs were proven to have the ability to act as a powerful cancer preventive agent at extremely low levels. However, the mechanism is still obscure (Friedman and Brandon, 2001; Kennedy, 1993).

The adverse effects of TIs continue to be a concern. Currently, there are no regulatory upper safe limits for dietary TI, and, as a result, there is no guarantee that commercial soybean products have been processed to a point where the effects of TI cannot be seen. For example, TI in some infant formula was retained up to a level of 28% of the original trypsin inhibitor activity (Sarwar Gilani, Wu Xiao and Cockell, 2012). Similarly, several commercial soya beverages have been reported to maintain up to 70% TIs of that in raw soybean (Sarwar Gilani, Wu Xiao and Cockell, 2012). Liener (1986) expressed a concern about consumption of TI content in the range from 5 to 20% as having a negative effect on human health. Ensuring a low TI activity is essential to health, particularly for infants or young children who need protein but are allergic to cow's milk and rely on soybean formula or soybean milk as the main source of protein for growth (Yuan et al., 2008).

2.3 Control of Trypsin Inhibitors

2.3.1 Trypsin inhibitors and micronization

Although the nutritional effects of TI on humans remain controversial, it is generally desirable to inactivate the TIs in soybean foods. As TIs are proteins, they can

be inactivated to varying degree by heat processing including steam treatment, extrusion, dry roasting, infrared radiation (micronization), and autoclaving (Sarwar Gilani, Wu Xiao and Cockell, 2012; Žilić, Bozović and Šukalović, 2012). Among these thermal treatments, infrared radiation is an innovative cooking technique. Sharma (2009) explained the mechanism of heating by infrared radiation is as follows. When radiation energy is absorbed by food, interaction of polar molecules with the electric component of the electromagnetic radiation causes the molecules to change orientation. As a result, heat is generated by inter-molecular friction. Infrared radiation processing can be referred to as a type of “high temperature short time” method of processing due to its high efficiency in achieving high temperatures. For example, soybean can reach a temperature of 110 °C in 90 s using infrared radiation. Other commercial methods often require at least 15- 30 min to reach this temperature depending on the size of the material being heated. With short time infrared processing, nutrients such as vitamins and minerals in the food are maximally conserved. Moreover, compared with other thermal treatments, infrared radiation processing has many benefits such as instant heating, reduced operational cost, and clean and safe processing (Sharma, 2009).

Due to these benefits, more and more studies are focusing on the effects of micronization. Temperature, moisture content, and treatment duration are three main factors which affect the TI inhibition during the micronization (Žilić, Bozović and Šukalović, 2012). In a study investigating the effect of the physical treatments on anti-nutritional factors in legumes seeds, seeds were tempered to 24% moisture and micronized to a temperature of 90 °C for 2.5 min for cowpea and pea, and 3.0 min for kidney bean (Khattab and Arntfield, 2009). Micronization was shown to cause an 88% -

94% reduction in TI activity. It is worthwhile noting that all other heat treatments (boiling, roasting, microwave cooking and autoclaving) used in this study resulted in total inactivation of TI. However, the possible reason for this is that the temperature used for micronization was not enough to totally destroy the TI in seeds and higher temperatures were obtained with the other heat treatments (Khattab and Arntfield, 2009). Recently Žilić, Bozović and Šukalović (2012) conducted an experiment studying the effects of thermal processing on TI in bioactive soybean proteins. Micronization was performed at 100, 125, 140, and 150 °C for 1.5 min. Although the temperatures were higher than in the previous studies, the residual TI was more than 40% of the initial value for all micronization treatments. The possible reason for that was the low moisture content of the soybean seed during processing. The authors implied the moisture content in the soybean played a very important role in TI degradation and inactivation (Žilić, Bozović and Šukalović, 2012). The two studies discussed above both confirmed that the TIs in legumes have a high thermal stability which cannot always be eliminated with heat processing. The optimum conditions for processing (in particular with micronization) soybean to totally eliminate TI while maintaining functional properties of the soybean protein have not yet been determined.

2.3.2 Trypsin inhibitor and ethanol washing

In addition to destroying TI activity by thermal treatment, alcohol washing has proven to be effective in reducing TI activity. As an alternative to hexane extraction of soybean oil, alcohol extraction has been shown to efficiently remove off flavours (Rackis, Sessa and Honig, 1979) and improve the color and protein functionality (Ashraf, 1986). Tolman (1995) investigated the effect of hot aqueous ethanol extraction on anti-

nutritional factors in raw pea and isolated pea protein. Three ethanol concentrations (55%, 65%, and 75% ethanol in water) were selected to extract the flour at temperatures of 50°C, 65°C, or 80°C for 2.5 h. Results showed that an alcohol concentration of 55% with a temperature of 80 °C gave the lowest residual level of TI activity. With increasing alcohol concentration or decreasing treatment temperature, the ability to inactivate TI decreased. The author also observed that low ethanol concentrations with high temperatures induced the highest values for weight loss (lower yield) and protein loss (Tolman, 1995). The possible mechanism responsible for reducing the TI activity by alcohol extraction was the denaturation of protein (Fukushima, 1969). Normally the hydrophilic areas are oriented towards the exterior and hydrophobic areas are oriented towards the interior of protein. With the strong hydrogen bond-forming capability, water can disrupt the hydrophilic regions of the protein, but has little impact on the inner hydrophobic regions. A solvent such as ethanol, which can modify the hydrophobic regions due to its hydrophilic and hydrophobic properties, is required to induce protein denaturation without using high temperatures. At high ethanol concentrations, the role of water in breaking the outside hydrophilic regions of protein molecule is weak. This prevents ethanol from gaining access to the hydrophobic regions and less denaturation of protein will occur (Fukushima, 1969).

In addition to denaturing the TI, the obvious drawback associated with low ethanol concentration extraction is the denaturation of the storage proteins, resulting in a change in their functionality. Different from the mechanism of denaturing TI with low concentration ethanol treatment, another possible mechanism stated that TI could be extracted by using different solvents. In general, KTI can be isolated by extracting with

water and precipitating it with ethanol. In contrast to the alcohol- insoluble KTI, isolation of BBTI involves extracting soybean flour with alcohol and precipitating it with acetone (Liu, 1997). Therefore, treating soybean with high ethanol concentration may not effectively decrease TI activity, but it may extract the BBTI and simultaneously maintain better protein properties. Liu and Markakis (1991) extracted the TI from soybean with varying ethanol concentrations (from 0 to 95%) for 1.5 h, and the results indicated that extraction of KTI with ethanol concentrations less than 20% had a similar effect to extraction with water alone and none of the BBTI fraction was extracted. With increasing ethanol concentrations, the KTI in the extract decreased and at 70% ethanol concentration it reached a value of zero. In contrast, the BBTI content in the extract increased as ethanol concentration was increased to 85%. In other words, extraction of soybean with ethanol concentrations less than 20% contained only KTI in the extracts; extraction with ethanol concentrations higher than 70% contained only BBTI in the extracts; and extraction with ethanol concentrations between 20-70% contained both. Extracts from ethanol treatment with concentrations higher than 85% showed decreased TI activity. This unusual phenomenon was, in part, attributed to turbidity in the extracts which interfered with the inhibitor assay (Liu and Markakis, 1991).

Overall, extraction with ethanol concentrations >55% helped maintain protein quality (Tolman, 1995) and ethanol concentrations between 20%- 70% partially removed both KI and BBTI from the soybean protein (Liu and Markakis, 1991). Therefore, the optimal ethanol extraction condition which produces a significant reduction in TI activity with minimal damage of protein should be between 55%- 70%. Tolman (1995) proposed that a treatment with aqueous ethanol concentration of 65% at a temperature of 65 °C

may be desirable for pea protein. To our knowledge, no studies have been published on effect of ethanol concentration of 65% at 65 °C on soybean. Therefore, further studies are required.

2.3.3 Trypsin inhibitor and alcalase hydrolysis treatment

2.3.3.1 *Enzymatic hydrolysis of protein*

Soybean has gained more and more popularity not only in the Asian continent but in other parts of the world. Its popularity is associated with the health benefits such as preventing the cardiovascular disease and cancer. These health benefits are due to the presence of biologically active compounds in soybean including isoflavones, saponins, as well as bioactive peptides (Dia et al., 2012). The bioactive peptides can be produced by enzymatic hydrolysis or fermentation. For instance, peptides with biological activities from milk protein can be produced by enzymatic hydrolysis with digestive enzymes or fermentation of milk with specific starter culture (Korhonen and Pihlanto, 2003). Korhonen and Pihlanto (2003) also stated enzymatic hydrolysis of protein is the most common way to produce bioactive peptides. It can improve the nutrition and functional properties of the protein, which might allow a broader range of utilization of the protein. In addition, the hydrolysates derived from the enzymatic treatments have various physiological activities such as antimicrobial, immunomodulatory, antihypertensive, antioxidant, and mineral binding (Zhu, Zhou and Qian, 2006). Some research suggested that the enzymatic hydrolysis can also reduce the anti-nutritional factors. The most prominent anti-nutritional factors are trypsin inhibitors, accounting for 40% growth depression, and lectins, which are responsible for 50% of growth inhibition (Liener, 1994). Ma and Wang (2010) indicated that a single enzyme did not hydrolyze lectins, but

lectins could be inactivated when an enzymatic hydrolysis was combined with heat treatments. Since lectins and trypsin inhibitors are both proteins, and lectins can be inactivated by hydrolysis, there is a possibility that trypsin inhibitor activity can also be reduced by enzymatic treatments.

2.3.3.2 *Trypsin inhibitors and alcalase*

“Alcalase”, also known as subtilisin, is a proteolytic enzyme produced from a selective strain of *Bacillus licheniformis*. Alcalase belongs to the serine proteases family and is widely used as an additive in protein digestion (Chen et al., 1992). It is a non-specific protease, which can attack the peptide bond through a serine residue at its active site. The active site in the serine protease contains the catalytic triad consisting of three amino acids: serine (Ser 221), histidine (His 64), and aspartic acid (Asp 32) (Chen et al., 1992).

The use of alcalase to reduce TI, a very important anti-nutritional factor in soybean protein, has been studied. Dia et al. (2012) investigated the effects of germination, alcalase hydrolysis and their combinations on the content of KTI and BBTI in soybean. Germination was carried out for 18 h and 72 h at 25 °C. Alcalase hydrolysis of the soybean was performed at pH 8.0 at 50 °C for 0 h, 1 h, 2 h, and 3h. After 3 h of alcalase hydrolysis, there were significant reductions in both KTI (77.1%) and BBTI (82.2%). However, the highest reduction in KTI (97.8%) and BBTI (96.9%) was induced by the combination of germination (18 h) and hydrolysis (3 h). One benefit of using enzymatic hydrolysis is that no heat or lower heat treatments than the traditional processes are involved, and as a result, protein functionality was preserved. Therefore, the use of

enzymatic hydrolysis to inactivate trypsin inhibitor is worthy of further investigation (Dia et al., 2012).

2.4 Beany Flavour

2.4.1 Key volatiles responsible for beany flavour

Despite the benefits associated with soybean consumption, many soybean products have an undesirable aroma and flavour, described as “beany”, which limits the acceptability of the products by many consumers in western countries (Vara-Ubol, Chambers and Chambers, 2004; Wansink and Park, 2002). In America, the flavour of soybean has been described as an off-flavour. For example, foods containing soy tend to give a negative impression due to the beany flavour (Wansink and Park, 2002). Therefore, for better utilization of soybean products, it is desirable to identify compounds responsible for the beany flavour and eliminate them.

Unfortunately, the compounds responsible for the beany flavour have not been well identified and characterized. A variety of the volatile compounds such as alcohols, aldehydes, ketones, pyrazines, and furans, have been reported as potential contributors of the beany flavour in soy products (Vara-Ubol, Chambers and Chambers, 2004) and different compounds have been associated with the beany flavour in different soybean products. For example, Arai, Koyanagi and Fujimaki (1967) isolated several alcohols from the volatile flavour components in the raw soybeans. Among those alcohols, compounds such as isopentanol, hexanol, and heptanol, were believed to be important for giving the beany like flavour to soybean (Arai, Koyanagi and Fujimaki, 1967). In defatted soybean flour, the key compounds responsible for the beany odours were ethyl vinyl ketone and 2-pentyl furan. A second important group of volatiles in these products

included pentanol, hexanol, 1-octen-3-ol, and hexanal (Hsieh, Huang and Chang, 1982). Chang et al. (1966) reported that 2-pentyl furan was the major compound responsible for the beany flavour in oxidized soybean oil. Other volatile compounds related with green beany flavour were hexanal, cis-3-hexenal, and trans-3-hexenal while in soybean protein isolates, the beany flavour is believed to be contributed by dimethyl trisulphide, trans,trans-2,4-decadienal, 2-pentyl pyridine, trans-2,4-nonadienal, hexanal, acetophenone, and 1-octen-3-one (Boatright and Lei, 1999). Zhang, et al. (2012) believed that hexanal, hexanol, 2-pentylfuran, and trans, trans-2,4-nonadienal were the major volatile compounds responsible for beany flavour in the soymilk (Zhang et al., 2012). Wang et al. (1997) showed that, among the alcohols and aldehydes, hexanol and hexanal were the predominant compounds that caused beany flavour in various soybean products including soybean isolate, defatted soy flour, and soy milk. Clearly controversial results on volatile compounds for beany flavour have been reported (Wang et al., 1997). Some of the chemicals that have been identified as beany by some authors may not be considered beany by others. For instance, 2-octenal was associated with beany flavour but it was also considered as a fatty aroma; 1-octen-3-one was considered as beany but it was also described as musty or mushroom-like (Vara-Ubol, Chambers and Chambers, 2004).

2.4.2 Sensory studies on beany flavour

It is hard to identify the beany flavour associated with soybeans because there are no clear sensory standards for beany flavour. Vara-Ubol, Chambers and Chambers (2004) have done a systematic evaluation on the sensory properties of the chemical compounds which were potentially related to beany aroma. There were two stages in their

experiments. In the first stage, fourteen different bean samples were evaluated by five highly trained descriptive panellists to identify and define the sensory attributes contributing to beany flavour. In the second stage, nineteen different chemical compounds which were potentially associated with beany flavour were examined at seven different concentrations. Results indicated that chemicals that had beany characteristics were 1-octen-3-ol, pentanol, 3-methyl-1-butanol, 2,4-heptadienal, acetophenone, 1-octen-3-one, and 3-isopropyl-2-methoxypyrazine (Vara-Ubol, Chambers and Chambers, 2004). Most of these chemicals exhibited beany characteristics at low concentration (1 or 10 ppm). At higher concentrations, most of these compounds totally changed and were no longer described as beany.

Chemical compounds such as hexanol, hexanal, and 2-pentyl furan, which were cited most frequently in the literature as contributing to beany flavour were not found to be beany when smelled as single compounds. Other chemicals like pentanal and trans-2-octenal were not classified as beany in this experiment but may have some characteristics related to beany flavour. Vara-Ubol, Chambers and Chambers 2004 proposed that these compounds might produce “beaniness” when combined with other chemicals. Bott and Chambers (2006) conducted an experiment on the sensory characteristics of combinations of chemicals potentially associated with beany flavour. The beany volatile compounds and non-beany volatile compounds identified in the previous research by Vara-Ubol, Chambers and Chambers (2004) were investigated in combination to determine whether an overall beany characteristic was present. The combinations were comprised of one beany chemical and one non-beany chemical, or two beany chemicals, or two non-beany chemicals. Results showed that the combination of 1-octen-3-one, a beany chemical, and

hexanal, a non-beany chemical resulted in the highest overall beany intensity of all combinations (Bott and Chambers, 2006). Addition of hexanal to other non-beany chemicals such as trans-2-nonenal, trans-2-hexenal and, trans-2, 4-decadienal produced an overall beany flavour which had not appeared in either of the individual compounds. Hexanal was the most abundant volatile compounds found in soybean volatile fraction and while it did not contribute any beany flavour by itself, combining it with other volatile compounds maintained or increased the sensation of beaniness (Vara-Ubol, Chambers and Chambers, 2004).

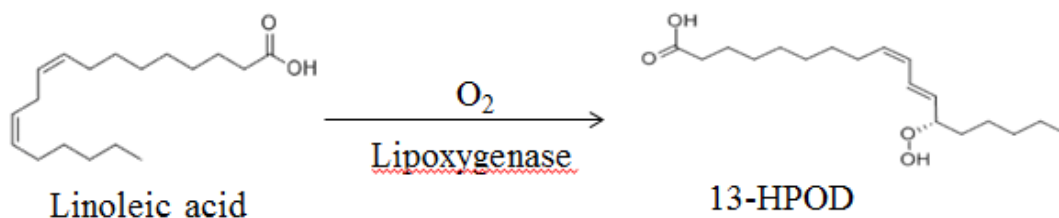
2.4.3 Soybean Lipoxygenase (LOX)

Lipoxygenases (LOX) are a class of iron-containing dioxygenases, which are able to catalyze the oxidation of lipids containing cis-cis-pentadiene structure. Lipoxygenases and their substrates are widely present in nature such as in plants (especially soybean), and animals (Shibata et al., 1988; Shibata et al., 1987).

Soybean seeds contain at least four distinct isomers of LOX, L-1, L-2, L-3a, and L-3b. All four isomers are proteins with molecular weight of approximately 100,000 and contain one atom of tightly bound non-heme iron per molecule (Shibata et al., 1987). L-1 is the best characterized enzyme among the isozymes. It has proven to be more heat stable, and prefers anionic substrates (linoleic and linolenic acids). L-2 and L-3 are less heat stable, and prefer esterified substrates (Liu, 1997). The isomers also differ in their product regiospecificity: L-1 prefers the 13 position as the site for hydroperoxidation, whereas L-2 and L-3 choose either position 9 or 13 when they catalyze linoleate (Liu, 1997).

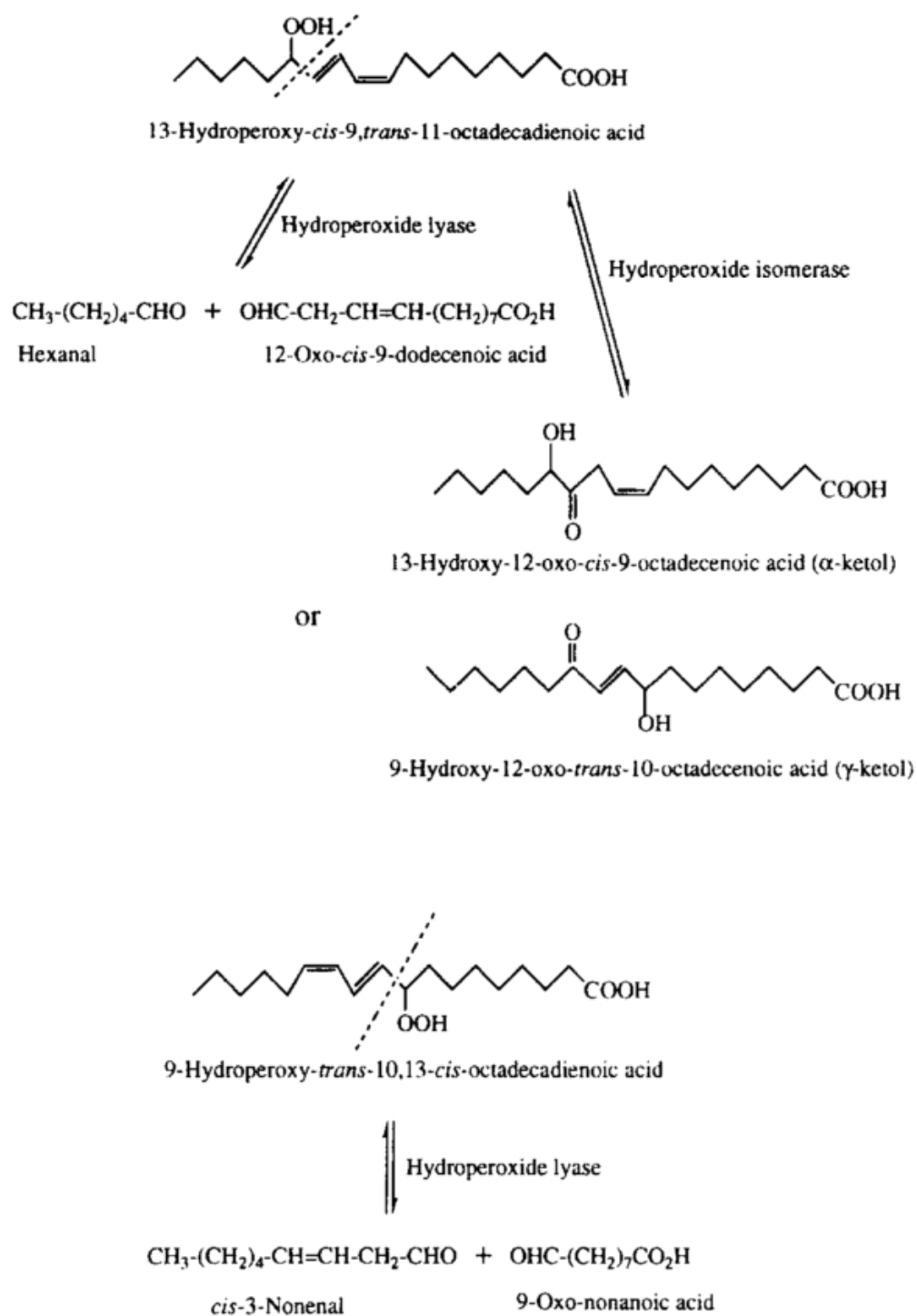
The reason LOXs in soybean are of particular interest is LOXs have been implicated as the major culprit for undesirable flavours in soybean related foods (Liu, 1997). Soybean is well known as the richest source of LOXs (Liu, 1997). It is also an oil seed with oil content of about 20%. Moreover, the majority of fatty acids in soybean are unsaturated fatty acids, with linoleic acid (C18:2) accounting for more than 50% of total fatty acids (Liu, 1997). During milling of soybean, LOXs gain access to their substrates (unsaturated fatty acids). In the presence of oxygen, LOXs catalyze the oxidation of the unsaturated fatty acid producing hydroperoxides, which are the primary products (Figure 2.4). In this oxidation process, there are three steps: first LOXs are activated; then, a proton from the methylene group in linoleic acid is removed; and oxygen is inserted into the substrate forming the hydroperoxide (13-HPOD) (Robinson et al., 1995).

Figure 2.4 Lipoxygenase catalyzed oxygenation of linoleic acid to produce 13-hydroperoxy- cis- 9, trans-11- octadecadienoic acid (13-HPOD) Source: Robinson et al. (1995).



The initial oxidation products from linoleic acid can be further degraded into a variety of short chain products by the enzyme hydroperoxide lyase or isomerase (Figure 2.5) (Robinson et al., 1995). Those volatile compounds are aldehydes, ketones and alcohols, many of which are positively related to the beany flavour of soybean products.

Figure 2.5 Possible degradation products arising from the action of hydroperoxide lyases and isomerases. Source: Robinson et al. (1995).



2.5 Control of Beany Flavour

2.5.1 Lipxygenase, beany flavour and micronization

Among all the volatile compounds which are related to the off-flavour of soybean, hexanal is the most frequently studied. Hexanal is a product of lipid oxidation and it has been positively correlated with the lipxygenase (LOX) content in soybean (Yuan and Chang, 2007). LOX is a group of iron-containing dioxygenase that can catalyse the oxidation of certain unsaturated fatty acids, producing hydroperoxides. These hydroperoxides further degrade to various alcohols, aldehydes and ketones (Žilić, Bozović and Šukalović, 2012). Many of these compounds are considered to be off-flavours. Lv et al. (2011) revealed that hot water blanching and grinding at temperatures above 80 °C for 2-6 min reduced LOX activity by up to 57%. Simultaneously, the flavour of soymilk was improved (Lv et al., 2011). Zhang et al. (2012) combined grinding with the ultrahigh temperature (UHT) processing on soybean to inhibit the formation of off-flavours or to remove the objectionable soy odour after its formation. In this work, 99% of LOX was inactivated after hot grinding (80.5°C) and the hexanal content was significantly reduced to an undetectable level mainly due to the inactivation of the LOX (Zhang et al., 2012). Yuan et al. (2008) conducted a very similar experiment on removing the beany flavour from soymilk by using UHT. They also observed that after UHT processing there was no hexanal left in the soymilk. The absence of hexanal in the soymilk was attributed to the total inactivation of LOX (Yuan et al., 2008).

Micronization is a “high temperature short time” process (Sharma, 2009) which shared some similarities with UHT in terms of high temperature and short time although micronization is used for solids while UHT is used for liquids. While a similar effect to

that obtained with UHT on reducing the off flavours might be expected with micronization, the different starting materials might be an issue. Studies looking at the effects of micronization on the LOX and off-flavours are rare. Žilić, Bozović and Šukalović (2012) studied the thermal inactivation of soybean bioactive proteins including the TI and LOX using micronization; the effect on the TI was discussed previously. Results indicated that micronizing the soybean at 125 °C for 90 s resulted in a 95% reduction in LOX activity. A total inactivation was achieved when the soybean was micronized at 150°C. The off-flavour profile was not investigated in their experiment (Žilić, Bozović and Šukalović, 2012). Wanasundara et al. (2012) investigated the effect of micronization on off-flavours in lentils and chickpeas. The samples were tempered to 15% and 23% moisture and treated by micronization at various temperatures (115, 130, 150, or 165 °C). Results showed that micronization at temperatures higher than 130 °C significantly reduced the key compounds associated with beany aroma and flavour in both lentils and chickpeas (Wanasundara et al., 2012). There is lack of information about the effects of the micronization on beany flavour of soybeans. More studies are needed in this field.

2.5.2 Off flavour and organic solvent washing treatment

For decades, food scientists and soybean processors have put in a lot of effort to find an efficient way to control the off-flavour of the soybean protein based products while maintaining high quality products. To improve the flavour of soybean products, two principles are commonly used: a) effectively removing the pre-existing flavour in the soybean, and b) preventing and/or removing the off flavour derived from lipid oxidation (Rackis et al., 1972).

2.5.2.1 *Defatting by hexane*

It is believed that the off flavour compounds are derived from oxidation of polyunsaturated fatty acids. Commercially, the soybean flour is prepared by hexane extraction to remove oil. However, after extraction of 97% of the lipids, the beany, bitter, astringent flavours remained in the resulting soybean flour (Sessa and Rackis, 1977). Even with 99.8% oil removed from the full fat flakes, objectionable flavours remained (Rackis et al., 1970). Even following protein isolation, 2 to 4 % phospholipid like materials, which could contribute to the beany flavour, remained (Eldridge et al., 1963). Clearly the use of hexane during oil extraction did little to address the beany flavour found in soybean protein products.

2.5.2.2 *Ethanol washing*

Lipid-like materials were not extracted by water, dialysis or ammonium sulfate precipitation, but could be extracted using alcohol (Eldridge et al., 1963). Rackis et al. (1979) also indicated that bound lipids were strongly associated with protein and the extraction efficiency depended on the ability of the solvent to disrupt hydrogen bonds. Hydrogen bond-breaking solvents such as alcohol are more effective in removing the residual lipids and flavour constituents from defatted soy flours, soy flakes, and soy protein isolates (Eldridge et al., 1963; Rackis, Sessa and Honig, 1979; Sessa and Rackis, 1977). More specifically, in a study looking at the aqueous alcohol treatments of defatted soybean flours and flakes, three alcohols (ethanol, methanol, and isopropyl) at various concentrations were evaluated. The results showed that the soybean flour extracted with 70% ethanol at 30 °C had the highest score in both flavour and odour among all alcohol

treatments (Baker, Mustakas and Warner, 1979). This indicated ethanol washing might have high potential for improving the flavour of the soybean based products.

Hua et al. (2005) investigated the properties of the soybean protein isolate prepared from aqueous alcohol washed soy flakes. Defatted soy flakes were treated with 85% aqueous ethanol at 30 °C for 30 min and this was followed by water extraction to produce soy protein isolates (ASPI). The functional properties and flavour profile of ASPI were compared to CSPI (commercial soy protein isolate). Fourteen different volatile flavour compounds were found in CSPI, but only 5 of these volatiles were found in ASPI. Furthermore, the total peak areas, which represented the off-flavour volatiles of CSPI, were 10 times higher than those of ASPI. All of these volatiles were reported to be products of LOX catalyzed oxidation of unsaturated fatty acids. The authors explained that the aqueous alcohol washing removed residual lipids in soy flake which otherwise would have acted as substrates for LOX oxidation during the water extraction of the soybean protein (Hua et al., 2005).

Emulsifying and foaming stability, which are important functionality attributes of the soybean flake, were also improved by the alcohol washing (Hua et al., 2005). Recently, a similar study was conducted by Wu et al. (2011) on the flavour volatiles and functional properties of soy protein products. Five different methods were used to treat the soybean flour or to extract the soy protein. The protein extracted from the flour which was treated with ethanol washing (65%) and hydrothermal cooking (50 °C) (EWHT) showed better emulsifying ability and color compared with other treatment methods such as acid precipitated soy protein (AP), oil-body associated proteins (OBA), oil-body and lipid removed proteins (OBA-less), and countercurrent extracted proteins (CE). In term

of the flavour profiles, EWHT showed the least total off-flavour level among all methods. Organoleptic evaluation showed that EWHT had the highest flavour score of 6.24 and odour score of 5.88, which meant that the flavour of EWHT was better than any of the other soy protein products. Results from the flavour volatile analysis and the organoleptic evaluation both implied that alcohol washing could be an effective way to produce soybean protein products with reduced off-flavours (Wu et al., 2011). A variety of ethanol treatment conditions such as 70 % ethanol at 30 °C (Baker, Mustakas and Warner, 1979), 85% ethanol concentration at 30 °C (Hua et al., 2005), and 65% ethanol concentration at 50 °C (Wu et al., 2011) have been evaluated. All of those ethanol treatment conditions were reported to significantly reduce off-flavours, but there are no studies that have clarified the optimum condition for ethanol treatment to modify soybean flavour.

3 Material and Methods

3.1 Tempering

Soybean seeds (Glycine max, PR70207RR, a variety with high protein) were grown locally (Winnipeg, MB. Canada). Soybean seeds were tempered to 13% moisture prior to micronization. Moisture content was determined (details of moisture content determination are given below) before seeds were tempered. The amount of moisture needed to reach 13% was calculated as follows:

$$\text{Weight of H}_2\text{O} = \frac{\text{weight (soybeans)} \times [\% \text{H}_2\text{O (target)} - \% \text{H}_2\text{O (original)}]}{100 - \% \text{H}_2\text{O (target)}}$$

As the original seed contained 7% water, 0.42 kg of water was added to each 6 kg batch of soybean to increase the soybean moisture content to 13%. Soybean seeds and water were then mixed by rolling overnight using a cement mixer (Red Lion, Canada); the mixer was covered all the time and held at room temperature. After tempering, the moisture content of the tempered seeds was evaluated and was found to be only 11.85%. The lower moisture content in the tempered soybean seeds might be due to the evaporation of moisture during prolonged mixing.

3.2 Micronization Treatment

The micronization process was carried on the whole soybean seeds. Since the soybean seeds had been tempered, the higher moisture content allowed the infra-red radiation to better penetrate the soybean seeds and denature the soybean proteins. A pilot scale micronizer (Micronizing Company, Suffolk, UK) composed of a ceramic infrared

gas burner and a vibrating belt was used. To achieve a desired soybean temperature, the slope of the conveyer in micronizer was adjusted to an appropriate angle. A longer time of exposure to infra-red radiation led to a higher temperature on the surface of the soybean seeds. Decreasing the slope of the conveyer gave a longer time for seeds to pass through the micronizer. Therefore, a higher surface temperature was achieved. The desired surface temperatures in this study were chosen as 100 °C ($\pm 5^{\circ}\text{C}$) and 135°C ($\pm 5^{\circ}\text{C}$) which were monitored using a hand held infrared thermometer (Cole-Palmer Instruments Corporation, USA).

3.3 Dehulling, Grinding and Defatting

3.3.1 Dehulling and grinding

Soybean seeds were dehulled using a mill (SATAKE Corporation, Japan) to produce soybean hulls and cotyledons. The cotyledons were ground (Fred Stein Laboratory Grinder, USA) to pass through a 32 mesh sieve (500 μm , Cetyler, Canada). The ground soybean was stored at -40°C for later use.

3.3.2 Defatting soybean with hexane

As an oilseed, soybean was originally grown in western countries for oil extraction. Industrially, the oil content was reduced below 3% using hexane extraction (Sessa and Rackis, 1977). To mimic industrial processing and produce a product with about 3% fat, different extraction methods were tested. Ten g of ground soybean were extracted using 25 mL hexane ($\geq 95\%$, Fisher Scientific, USA) for 3 min and 5 min. The defatted material was then air dried in a fume hood at room temperature for 24 h to evaporate residual hexane. The resulting fat content was determined using a Soxhlet extractor (Lab-line Instruments, Inc., USA) with 16 h hexane extraction. Neither of the

extraction times gave the desired defatting effect; the residual fat content for 3 min extraction flour and 5 min extraction were 12.5% and 14.3% respectively. However, 3 min extraction had a better effect on removing fat from soybean than 5 min extraction. Therefore, 10 g of ground soybean and 25 mL hexane were again mixed and shaken for 3 min using a wrist action shaker (Burrell Scientific. PA. USA). Hexane extracts and flour were separated by centrifuging at 600 X g at 25 °C for 1 min (Sorvall RC 6 Plus, Thermo Fisher Scientific Inc., USA). After removal of the hexane extract, 25 mL fresh hexane was re-added. The extraction was repeated twice. After three extractions with fresh hexane, the residual fat content was below 3% (~2.6%). Therefore, this multiple extraction approach was selected as the extraction method for all soybean samples.

3.4 Ethanol Treatment

Ethanol at concentrations of 65% and 85% were selected to treat the soybean flour. Ten g of defatted flour were placed in a 100 mL flask. Fifty mL of either 65% or 85% ethanol were added. After stirring for 30 min, the ethanol sample mixture was filtered using No.1 Whatman paper with a vacuum filtration system (GE Healthcare UK Limited, UK). The material on the filter paper was put in the fume hood at room temperature for 24 h to totally evaporate the ethanol. The weight of the dried treated flour was also monitored for calculating the recovery rate of flour.

$$\text{Recovery rate (\%)} = \frac{\text{Weight of Residual flour}}{\text{Weight of initial flour}} \times 100$$

3.5 Enzyme Hydrolysis

To make an 8% soybean flour suspension, 8 g of flour was added to 92 mL of distilled water in a 250 mL flask. After stirring for 30 min, the mixture was pre-warmed

to 50 °C in a water bath (Fisher Scientific, USA) for 20 min. The pH of the soybean suspension was adjusted to 8 with 0.1N NaOH before adding 0.625 mL of alcalase (P4860, Batch #: 056K1213, Sigma-Aldrich, USA). Hydrolysis was allowed to continue for either 0.5 or 3 h and the pH was readjusted to 8 every 10 min. The enzymatic hydrolysis of the protein was stopped by immersing the flask into a 95 °C water bath for 10 min as this high temperature deactivates alcalase. The hydrolysate mixture was cooled in an ice bath for 10 min and put into a freezer (-18°C) prior to freeze drying. Samples were freeze dried (Genesis Freeze dryer, VirTis, SP Industries, NY. USA) and ground using a pestle and mortar, and stored at -18°C for later analysis.

In preliminary work, enzymatic hydrolysis times of 0, 0.5, 1 h, 2h, and 3h were evaluated in relation to the extent of hydrolysis obtained. The enzymatic hydrolysis procedure was the same as described above. The only difference was that after cooling in the ice bath the hydrolysates were centrifuged at 14000 x g at 10 °C for 30 min (Thermo-Fisher Scientific, USA). The supernatant from the mixture was then recovered using a Pasteur pipette and filtered through No.4 Whatman filter paper (GE Healthcare UK Limited, UK). The recovered supernatant was freeze dried before determining the degree of hydrolysis. Degree of hydrolysis was defined as the proportion of cleaved peptide bonds in protein hydrolysate. Degrees of hydrolysis of the soybean protein were 5.9%, 6.8%, 8.2% and 9.2% for 0.5, 1, 2, and 3 h hydrolysis times, respectively. To get different enzymatic hydrolysis effects, hydrolysis times of 0.5 and 3 h were selected as treatments for soybean.

3.6 Proximate Analysis of Soybean Treated with Different Processing

3.6.1 Moisture content determination

Moisture content determination followed the AACC (2014) method (44-15.02). Aluminium moisture dishes were dried in an air oven (Stabil-Therm, Blue M Electric Company, Illinois, USA) at 135 °C for 30 min. Dried dishes were quickly transferred into a desiccator. After cooling for 20 min, the dish weight was recorded. Approximately 2 g of sample were accurately weighed and added to the pre-weighed dish and dried in the air oven at 100 °C for 16 h. The dried sample was quickly transferred into a desiccator. The dish and sample were cooled and weighed. Moisture determinations were carried out in triplicate. Calculation of moisture content used the following equation:

$$\% \text{ moisture} = \frac{\text{weight of sample} - (\text{weight of dried sample and dish} - \text{weight of dried dish})}{\text{weight of sample}} \times 100$$

3.6.2 Protein content determination

Protein content determination was done based on nitrogen combustion (TruSpec[®], LECO Corporation, USA) using approximately 10-20 mg of soybean flour. EDTA and rice flour were used as references to verify satisfactory system performance. The protein content of the soybean samples was then calculated using the following equation:

$$\text{Protein content (\%)} = \text{nitrogen content (\%)} \times \text{conversion factor}$$

The nitrogen-protein conversion factor for soybean is 5.7 (Sosulski and Holt, 1980). Protein determinations were done in duplicate.

3.6.3 Crude fat content determination

Crude fat content was determined using the Soxhlet apparatus (Lab-line Instruments, Inc., USA). A 250 mL flat bottom flask containing 3-5 boiling chips was

pre-dried in an air-oven at 125 °C for 30 min, and transferred into a desiccator to cool prior to weighing. Approximately 3 g of sample were accurately weighed into an extraction thimble. A wad of glass wool was put over the sample to prevent the sample loss. The thimble was then put in the Soxhlet unit. Hexane (160 mL) was added to the weighed 250 mL flat bottom flask. Samples were extracted for 16 h. After the extraction, hexane in the flask was evaporated using a heating mantle in the fume hood leaving the crude fat extracted from the sample in the flask. To ensure complete evaporation of the hexane, flasks with fat were placed in the air-oven at 100 °C for 1 h. After cooling to room temperature in the desiccator, flasks with fat were weighed. Calculation for crude fat content was as follows:

$$\% \text{ crude fat} = \frac{(\text{weight of crude fat \& flask} - \text{weight of flask})}{\text{weight of sample}} \times 100$$

The fat content determination for each sample was conducted in triplicates.

3.7 Study 1: Effects of Micronization, Ethanol Washing and Enzyme Hydrolysis on Trypsin Inhibitor and Lipoygenase Activities in Soybean Flour

3.7.1 Degree of hydrolysis determination

The degree of hydrolysis of samples was determined by using the 2, 4, 6-trinitrobenzene sulfuric acid (TNBS) method (Adler-Nissen, 1979). A 0.2125M sodium phosphate buffer at pH 8.2 was prepared using two buffers; one was 0.2125 M sodium phosphate monobasic (Fisher Scientific, Canada) and the other was 0.2125 M sodium phosphate dibasic (Fisher Scientific, Canada). The monobasic buffer was then added to the bibasic buffer until pH 8.2 was achieved. One percent (w/v) sodium dodecyl sulfate (Sigma-Aldrich, Canada) in 0.2125 M sodium phosphate buffer was prepared for

dissolving the sample and standards. TNBS was prepared immediately prior to being used by diluting 5% TNBS (Sigma-Aldrich, Canada) in a 50 mL volumetric flask with sodium phosphate buffer in the dark. The solution was kept dark by securely placing aluminium foil around the volumetric flask and working in low light conditions.

Freeze dried hydrolysate (2.5 mg) was weighed into a 2 mL micro-centrifuge tube. One mL of the 1% sodium dodecyl sulphate was added to dissolve the sample. A standard curve was made using D-lysine (Sigma-Aldrich, Canada) at the following concentrations: 0 mM, 0.5 mM, 1.0 mM, 1.5mM, 2.0mM and 2.5 mM. A 250 μ L aliquot of each sample and standard solution was transferred to labelled clean glass test tubes. To each test tube, 2 mL of 0.2125 M sodium phosphate buffer (pH 8.2) was added. All test tubes were pre-warmed in a water bath (Isotemp, Fisher Scientific, USA) at 50 °C for 10 min. To each test tube, 2 mL of 0.1% TNBS was added. Addition of TNBS to different samples was staggered by 20 s so that a consistent time for the interaction to occur was attained. The mixture was incubated for 1 h in a water bath at 50 °C. During incubation, the TNBS solution reacts with primary amine groups producing an orange-coloured derivative, which can be measured by ultraviolet-visible (UV-Vis) spectrophotometry (Figure 3.1). Immediately following the 60 min incubation, 4 mL of 0.1 N HCl (Fisher Scientific, Canada) were added to each test tube to stop the reaction. The samples and standards were allowed to cool for 20 min prior to measuring the absorbance at 340 nm using a spectrophotometer (Ultrospec 1100 Pro, AP. USA). Deionized water was used as the blank. The degree of hydrolysis was conducted in triplicate and calculated as follows:

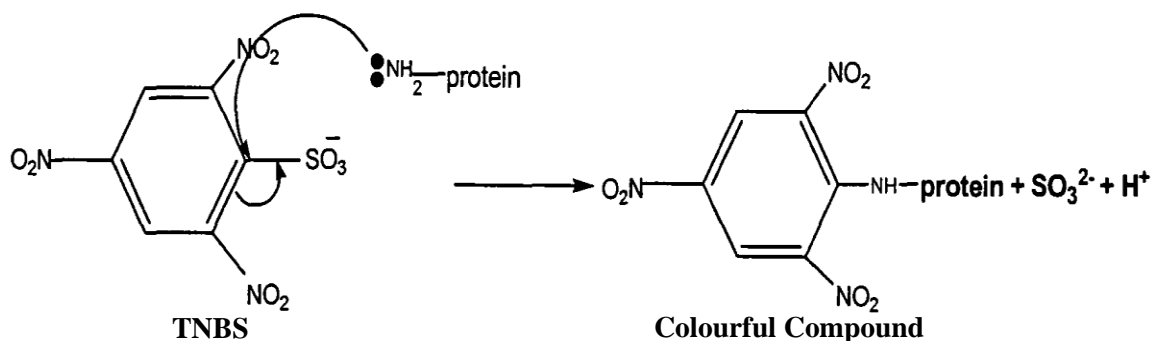
$$\text{Degree of hydrolysis (\%)} = \frac{AN_2 - AN_1}{N_{pb}}$$

AN₁ is the amino nitrogen content before hydrolysis in mg/g

AN₂ is the amino nitrogen content after hydrolysis in mg/g

N_{pb} is the nitrogen content of peptide bonds in mg/g; 175.4 mg/g for soybean proteins

Figure 3.1 Reaction of TNBS with amino acid groups. Adapted from Adler-Nissen (1979) with open access.



3.7.2 Differential Scanning Calorimetry (DSC) analysis

The structure of the soybean storage proteins (glycinin and β-conglycinin) was studied using Differential Scanning Calorimetry (DSC Q200, TA Instruments Inc. DE USA). Two mg of soybean flour were weighed into an aluminium Tzero pan and hermetically sealed. An empty aluminium DSC pan with lid was used as the reference.

The DSC was programed using TA Explorer software. Both sample pan and reference pan were heated from 40 °C to 120 °C at a heating rate of 10 °C/min. The difference between sample heat flow and reference heat flow was recorded. The DSC results were analyzed using the TA Universal Analysis program. The denaturation temperature (°C) and enthalpy (J/g) data for two different storage proteins were analyzed from the graph following manual assignment of a baseline. DSC analysis for soybean sample was conducted in duplicate.

3.7.3 Trypsin inhibitor activity determination

Trypsin inhibitor activity determination followed the AACC (1999) method (22-40.01) with some modifications. Tris buffer (0.05 M, pH 8.2) was made by dissolving 6.05 g trishydroxymethyl aminomethane (Fisher Scientific, USA) and 2.94 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (Fisher Scientist, USA) in 1 L distilled water. Forty mg Na-Benzoyl-D, L-arginine 4-nitroanilide hydrochloride (BAPA) (Sigma-Aldrich, USA) was dissolved in 1 mL of dimethyl sulfoxide (Fisher Scientific, USA) and diluted to 100 mL with Tris buffer (0.05M, pH 8.2). BAPA was the substrate for the enzymatic reaction and was pre-warmed (Isotemp, Fisher Scientific, USA) at 37 °C. A trypsin solution containing 4 mg of trypsin (from bovine pancreas) (Sigma-Aldrich, USA) in 200 mL 0.001 M HCl was used in the assay.

Soybean flour was first ground to pass through a 100 mesh sieve (150 μm , Cetyler, Canada) and extracted with 0.01N NaOH (Fisher Scientific, USA). Soybean flour (0.1g) was weighed into a 50 mL capped centrifuge tube (Oak Ridge PPCO Centrifuge Tube, USA) and extracted with 25 mL 0.01 N NaOH using a wrist action shaker (Burrell, PA USA) for 1.5 h. After extraction, the sample was centrifuged for 20 min at $14,000 \times g$ at 4 °C (Sorvall RC 6 Plus, Thermo Fisher Scientific Inc., NC. USA). The supernatant was transferred into a 20 mL glass bottle with cap for later use.

There are two stages in the trypsin inhibitor determination assay. The first part involved determination of a dilution factor for each sample. Soybean suspensions should be diluted to the point where 1 mL produces 40-60% trypsin inhibition. The pH of supernatant was adjusted to 9.0 (± 0.5) first. Several dilution factors were examined including 1:0, 1:1, 1:2, 1:4, and 1:10 (suspension/distilled water) for each sample. One

mL of diluted suspension was added into a test tube and the total volume was adjusted to 2 mL with distilled water. An extra sample test tube only containing 2 mL of distilled water was also prepared as a reference. A second set of test tubes was prepared in the same way for each sample; for these sample blanks, the trypsin was not added until after the acetic acid so that there would be no trypsin activity. Two mL of trypsin solution were pipetted into one test tube for each sample (not the sample blank) and mixed using a vortex mixer (Vortex-Genie Mixer, Scientific Industries Inc., USA). All test tubes, including sample blanks, were warmed in a 37 °C water bath (Isotemp, Fisher Scientific, USA) for 15 min before adding 5 mL substrate solution (BAPA). The trypsin-substrate reaction time was controlled to be exactly 10 min. To stop the reaction 1 mL of 30% acetic acid solution was added. Two mL of trypsin solution were now added to the sample blanks so that the overall composition and volume were the same. Solutions were then filtered through Whatman No. 2 paper (GE Healthcare UK Limited, UK). Absorbance of the samples and sample blanks were measured by using an UV/visible spectrophotometer (Ultrospec 1100 Pro, AP, USA) at 410 nm wavelength. Distilled water was used to zero the spectrophotometer. Calculation of TIU/ml for each sample was calculated as follows:

$$\text{TIU\%/mL} = \frac{\text{Abs } S_0 - \text{ABS } S_1}{\text{Abs } S_0} \div V_s \times 100$$

Abs S_0 : increase of absorbance for sample reference: Abs S_0 = Abs sample reference-

Abs reference blank test tube (trypsin added after acetic acid)

Abs S_1 : increase of absorbance for soybean trypsin inhibitor sample: Abs S_1 = Abs

sample - Abs blank test tube (trypsin added after acetic acid)

V_s : volume of extracts added (1 mL)

Once the correct dilution to give 40-60% trypsin inhibition for the soybean extracts had been determined, the TIA determination was replicated for all samples. To ensure the 40-60% inhibition range was met, different portions (0, 0.6, 1.0, 1.4, and 1.8 mL) of diluted suspension were pipetted into test tubes. Distilled water was added to make up 2 mL total volume. The analysis of TIA was as described above. One trypsin unit is defined as increase of 0.01 absorbance unit at 410 nm. Trypsin inhibitor activity was defined as the number of trypsin units inhibited (TIU) per mg of sample and expressed as TIU/mg. The calculation for TIU activity was as follows:

$$\text{TIU/mL} = (\text{Abs } S_0 - \text{Abs } S_1) / 0.01 \div V_s$$

$$\text{TIU/mg} = \text{TIU/mL} \times 25\text{mL} \times \text{Dilution factor} / S_{\text{weight}}$$

Abs S_0 : increase of absorbance for sample reference

Abs S_1 : increase of absorbance for soybean trypsin inhibitor sample

V_s : volume of extract (mL) added into test tube

Dilution factor: dilution value selected in the first part of experiment

25mL: total volume of the soybean trypsin inhibitor extracts

S_{weight} : weight of soybean flour

The analyses of the trypsin inhibitor activities were conducted in triplicate.

3.7.4 Lipoxygenase activity of soybean flour determination

Lipoxygenase activity in soybean flour was detected according to the method developed by Chang and McCurdy (1985) with some modifications. Sodium phosphate buffers (0.2 M) at pH 6.6 and pH 6.4 were prepared. Crude lipoxygenase was extracted from the soybean flour by shaking 0.1 g of sample with 20 mL pH 6.6 sodium phosphate buffer at 4 °C using a wrist action shaker (Burrell, PA USA) for 60 min. Extracts were separated by centrifugation for 30 min at 12,000 x g (Sorvall RC 6 Plus, Thermo Fisher Scientific Inc., USA). The supernatant was filtered through Whatman No.4 paper (GE Healthcare UK Limited, UK) into a 25 mL glass container. The extract was stored at 4 °C for later analysis. The substrate was prepared by mixing 100 µL of linoleic acid (Fisher Scientific, USA) and 100 µL of Tween 20 (polyoxyethylene sorbitan monolaurate) (Fisher Scientific, USA) and slowly adding 5 mL of 0.1 N potassium hydroxide (Fisher Scientific, USA) until the mixture became clear. The substrate was further diluted to 25 mL using pH 6.4 sodium phosphate buffer and stored at 4 °C.

Using a quartz cuvette, 0.3 mL substrate solution was first added and followed by 2.7 mL pH 6.4 sodium phosphate buffer. The cuvette was covered with parafilm and shaken to achieve a good mixture. Crude lipoxygenase extract (0.5 µL) was then added to the cuvette and shaken 3 times. A blank containing only 0.3 mL substrate and 2.7 mL buffer was also prepared. Absorbances of samples and blanks were measured every 30 s for 3 min at 234 nm using a UV/Visible spectrophotometer with a reaction kinetic program (Ultrospec 4300 Pro, AP, USA). One unit of lipoxygenase activity was defined as an increase of 0.1 in absorbance per min. A linear curve was plotted by plotting the

absorbance difference between sample and blank against time. An equation was used to determine the lipoxygenase activity:

$$y = kx + b$$

y: absorbance

k: constant value

x: time

b: the Y intercept

LOX activity could be calculated by using constant $k/0.1$. LOX determination was done in triplicate for each sample.

3.8 Study 2: Effects of Micronization, Defatting and Ethanol Treatments on Volatile Compounds in Soybean Flour

3.8.1 Simultaneous distillation extraction (SDE) of soybean volatiles by Likens-Nickerson apparatus

The simultaneous distillation extraction apparatus was created in 1964 when Likens and Nickerson designed it for analysis of hop oil (Likens and Nickerson, 1964). In the current experiment, a similar apparatus was used (Figure 3.2). The apparatus is composed of a cold finger, a condenser jacket, and two distillation arms of different heights. The cold finger is connected to the condenser jacket. Chilled water was produced by mixing ice and distilled water, which was continuously pumped into the system. This water first entered the bottom of the condenser jacket and then went to the cold finger through the connecting flexible rubber tube. The water returned to the pump through the top of the cold finger. The chilled water system was checked during the extraction to ensure there was sufficient ice to maintain the low temperature needed at the cold

finger surface. The water bath was prepared before volatile extraction and maintained between 58-62 °C all the time.

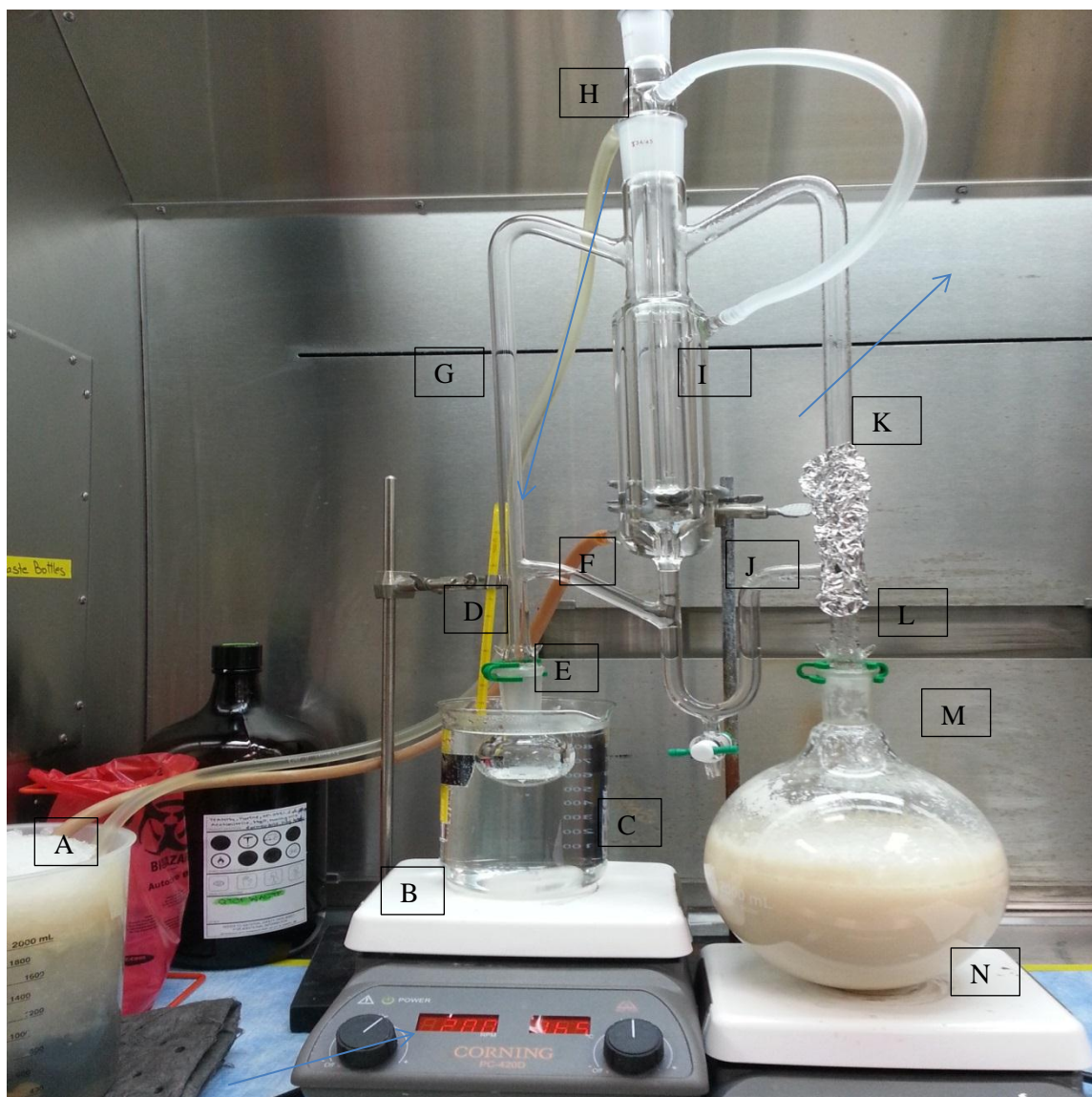
Fifty g of soybean flour and 50 g of sodium chloride (Sigma-Aldrich, USA) were weighed and mixed in a 600 mL beaker. The mixture was dissolved in 1000 mL of distilled water and stirred with a glass rod to break any clumps. If the clumps did not break easily, a magnetic stirrer was necessary. The solution was then transferred into a 2 L round bottom flask (Fisher Scientific, Canada) with a magnetic stirrer. An internal standard (1, 2 di-chlorobenzene (Sigma-Aldrich, USA) at 1 mg/mL in methanol) was prepared. The internal standard (100 µL) was added to the soybean flour solution before SDE extraction. The flask was connected to the extractor. At the same time 27 mL of pentane (Sigma-Aldrich, USA) and 3 mL of di-ethyl ether (Sigma-Aldrich, USA) were added to a 50 mL round bottom flask and connected with the solvent arm on the left side of the apparatus. Twelve mL of distilled water and 3 mL of pentane (Sigma-Aldrich, USA) were added through the top of the apparatus. Water remained in the bottom of the apparatus because it had a higher density while pentane stayed on top of the water. The cold finger was connected to the condenser jacket and the chilled water pumped through the system.

When the soybean mixture started boiling, a lot of foam was generated. The heating rate had to be adjusted until equilibrium occurred between generating foam and breaking foam. The temperature of the sample arm slowly increased due to the steam generated. After seeing the first drop of condensate from the sample arm, the solvent flask was immersed in the 58-62 °C water bath and immediately it started boiling. The temperature of the water bath, the function of the cooling system, and generation of foam in the 2L flask were constantly monitored during the 2 h extraction (3 h for full fat soybean). The volatile compounds were steam distilled and extracted by the pentane vapour. Both sample steam and solvent vapour condensed on the surface of the cold finger and dropped to the bottom of the apparatus where the extraction occurred. Condensed

solvent and water were separated in the bottom part of apparatus based on density differences. Much of the solvent and water went back to the 50 mL receiving flask and 2L flask respectively.

After 2h (defatted soybean flour) or 3 h (full fat soybean flour) of volatile extraction, the sample heater was turned off. The water bath then was removed from the solvent flask. After cooling for 10 min, the solvent from the apparatus was collected in the solvent receiving flask, stoppered and stored at 20°C overnight. After freezing, ice crystals in the sample flask were easily removed. To ensure complete dryness of the solvent, 2-3 g of anhydrous sodium sulphate (Sigma-Aldrich, Germany) was added to the volatile extract and left for 30 min concentrating the volatiles. To increase the concentration of the volatile to improve resolution in the GC-MS determination, the extract was concentrated to 1 mL using a nitrogen evaporator (Organomation Associates Inc., USA). The concentrated volatile extracts were transferred into a 2 mL crimped cap vial (Agilent, Canada), sealed and stored at -20 °C prior to GC-MS analysis. Volatile extractions were done in triplicate for each sample.

Figure 3.2 Simultaneous Distillation Extraction (SDE) system for soybean flour volatile extraction by using Likens and Nickerson's apparatus



A: chilled water with pump

B: water bath heater with stir function

C: 2 L beaker with warm water

D: thermometer to control temperature between 58-62 °C

E: 50 mL round bottom solvent receiving flask

F: solvent distillate return arm

G: solvent arm

H: cold finger

I: condenser jacket

J: condensed water return arm

K: sample arm

L: keck clip for ground joint 24/40 mm

M: 2 L round bottom sample flask

N: sample heater with stir function

3.8.2 Analysis of volatiles by Gas Chromatograph–Mass Spectrometer (GC-MS)

GC-MS analysis of soybean volatile extracts was performed using a 450 Gas Chromatograph (Agilent Technologies, Walnut Creek, USA) with 240MS/4000 Mass Spectrometry (Agilent Technologies, Walnut Creek, USA) with a VF-5ms low bleed/MS fused-silica capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m× 0.25mm, Varian). A blank containing only pentane (Sigma-Aldrich, USA) was used. An aliquot of 1 µL sample of pentane was injected using an auto-sampler in a split less mode at 250 °C and a 3 min filament delay was selected for each to protect the mass spectrophotometer filament from burning the solvent. The temperature of the oven in the GC was programmed to hold at 40 °C for 5 min and then increase to 220 °C at the rate of 4 °C/min. Helium was used as the carrier gas at a rate of 1 mL/min. The ion source was operated in electron ionization (EI) mode with 70 eV voltages. The MS scanned from 50 to 700 m/z in a full scan mode with a scan average of 4 micro-scans (1.75 s/scan). Sample extracts were analyzed in duplicates by using the same setting as the blank.

3.8.3 Identification and semi-quantification of volatiles

Volatiles seen in the GC-MS chromatograph were analyzed by using the MS Workstation software. Peaks in the graph were identified by matching their mass spectra and ion distribution with those of authentic compounds, which had been previously analyzed and reported in the National Institute of Standard and Technology (NIST) library (MS Search Version 2.0). To be more confident about the identification of the compounds in the GC-MS graph, linear retention indices (LRI) (Schindler et al., 2012) for each compound were calculated and compared to the LRI previously reported in the NIST library. An n-alkane standard containing homogenous alkanes from C8 to C20 (40 µg/mL) (Sigma-Aldrich, USA) in hexane was diluted by adding 50 µL standard into 150

μL of pentane, and run in the GC-MS using the same condition as the sample extract. Following identification based on the GC-MS graph of the alkanes (Figure 3.3), the retention times were analyzed using the MS Workstation software. Alkane retention times and linear retention indices are shown in Table 3.1. To make sure that the retention time for both alkane standard and volatiles in the mixture did not change, alkane standard and sample extracts mixture were checked for each treatment. LRI calculation for volatiles was as follows:

$$LRI = \frac{RT_{volatile} - RT_{alkane\ n-1}}{RT_{alkane\ n} - RT_{alkane\ n-1}} \times 100 + RTI_{alkane\ n-1}$$

LRI value for the volatile was also compared with LRI reported with a similar column from the LRI library at the University of Reading (University of Reading, 2014). To separate noise from compounds, which may have very similar retention times, the core ion was selected from the ion distribution for each volatile. The peak for the core ion was isolated and analyzed for peak area. Semi-quantification for each volatile compound was calculated by comparing the area for the sample core ion and internal standard (1, 2 di-chlorobenzene) core ion peak area. The calculation was as shown:

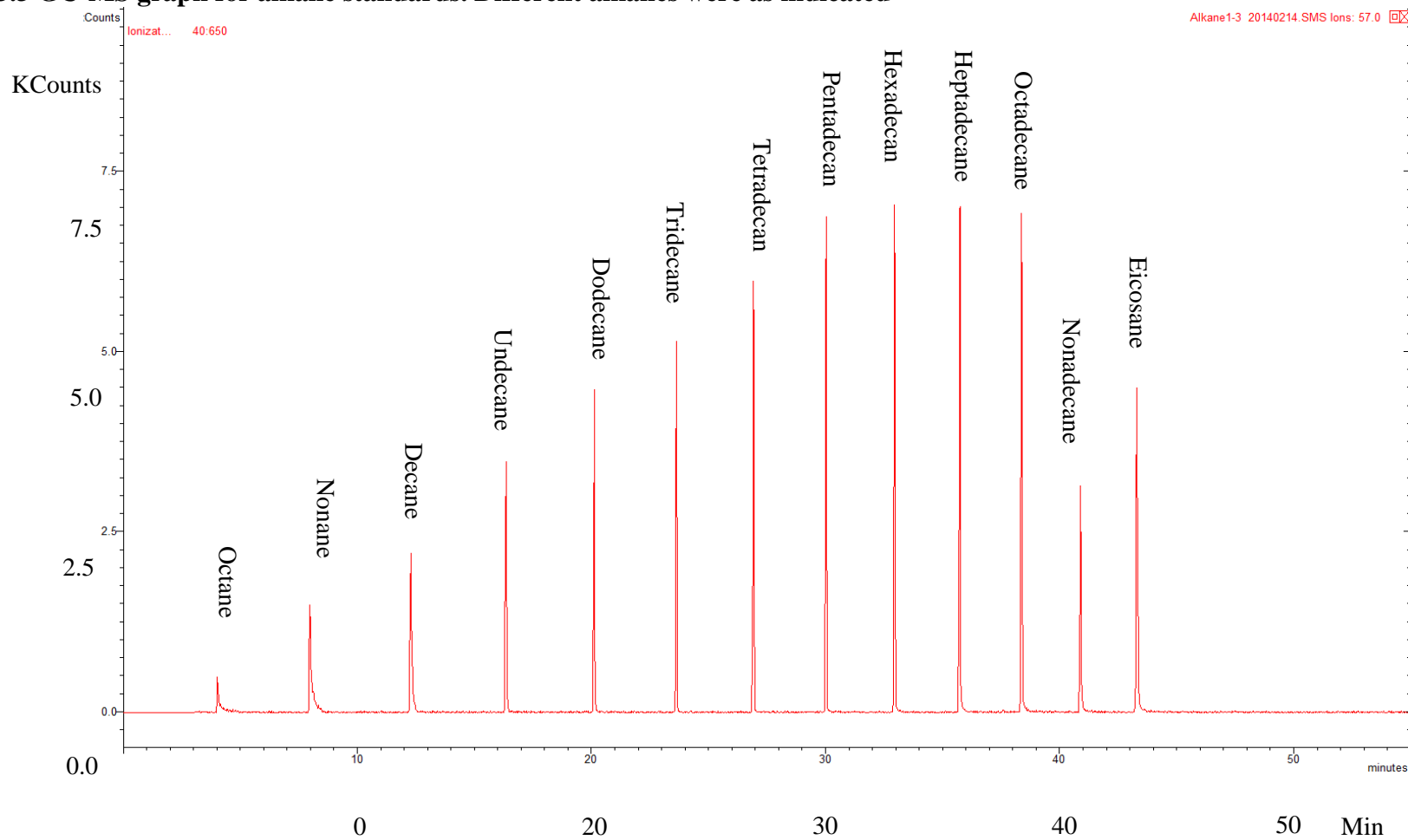
$$\text{Semi- quantification of target volatile} = \frac{\text{Target Peak Area}}{\text{Standard Peak Area}}$$

Semi-quantification provided a rough indication of the amount of volatile in the sample. This may not represent the exact concentrations of volatiles in soybean.

Table 3.1 Retention time (RT) and linear retention indices values of n-alkane standards (C8-C20 10 µg/mL)

Compounds	RT (min)	LRI
Octane	6.32	800
Nonane	10.56	900
Decane	14.93	1000
Undecane	19.04	1100
Dodecane	22.87	1200
Tridecane	26.44	1300
Tetradecane	29.79	1400
Pentadecane	32.96	1500
Hexadecane	35.93	1600
Heptadecane	38.77	1700
Octadecane	41.46	1800
Nonadecane	44.02	1900
Eicosane	46.47	2000

Figure 3.3 GC-MS graph for alkane standards. Different alkanes were as indicated



3.9 Statistical Analysis

Statistical analyses were performed with SAS (Version 9.3) using PROC GLM with a factorial design followed by a least significant difference test of significance. The probability (p) value of significant difference was selected as $p < 0.05$.

4 Results and Discussion

4.1 Proximate Analysis of Soybean Treated with Different Processing

4.1.1 Proximate analysis on full fat soybean flour

Soybean seeds were first micronized at the different temperatures (100°C and 135°C). Soybean seeds then underwent a milling process to produce soybean flour. The proximate parameters such as moisture, protein and fat content for full fat soybean flours were analyzed before the defatting process (Table 4.1).

Table 4.1 Proximate analysis of the original and micronized soybean flour

Soybean flour	Moisture content (%)	Protein content (%)*	Fat content (%)*
FFM0	11.9 ± 1.10 ^a	35.8 ± 0.33 ^a	27.63 ± 1.5 ^b
FFM100	9.02 ± 0.02 ^b	35.8 ± 0.19 ^a	26.3 ± 0.10 ^b
FFM135	4.38 ± 0.08 ^c	35.3 ± 0.06 ^a	29.0 ± 1.1 ^a

FFM0/FFM100/FFM135: full fat unmiconized/ full fat micronized at 100°C / full fat micronized at 135°C

Same letter between the different rows in the same column means not significantly different (P<0.05)

Results are expressed as the mean ± standard deviation

* : Protein and fat content as were calculated on a dry basis

Moisture content determination was analysed by the AACC method (44-15.02). The moisture content for tempered soybean (FFM0) was 11.85% (Table 4.1). Although, the goal of the tempering process was to achieve 13% moisture content in soybean seeds, loss of moisture content was due to the evaporation of moisture during the tempering and milling processes. In the tempering process, the water added could not be totally absorbed by soybean seeds. On the other hand, the milling process generated a little heat (temperature increased to ~35°C after milling soybean flour) which could further decrease the moisture content in the soybean flour. After micronizing soybean at 100°C and 135°C, the moisture content significantly dropped to 9.02% and 4.38%, respectively

due to the increase in the temperature of the soybean. In a study conducted by Wiriyampaiwong, Soponronnarit and Prachayawarakorn (2004), micronizing full fat soybean at 120°C for 15 min caused a soybean moisture content decrease from 11.6% to 7%. The average moisture content (7%) in their micronized samples was in the same range as the present study (4%-9%) because the temperature they used (120°C) was between the micronization temperatures used in the current study (100°C and 135°C).

Protein content determination was done using a combustion method. The method consisted of combusting soybean flour in a high temperature (~900°C) chamber in the presence of oxygen. Carbon dioxide, water and nitrogen were released. Carbon dioxide and water were absorbed using a special column. The remaining nitrogen content was determined. The protein content for soybean flour was then calculated based on the nitrogen content by using a nitrogen-to-protein conversion factor of 5.7 (Mosse, 1990). The protein content for the raw soybean was 35.8% (dry basis, Table 4.1). Results matched those reported by Wolf et al. (1982) which ranged from 34%-40% and by Marega Filho et al. (2001) which ranged from 35.7%-41.8%. Micronization did not have a significant effect on the protein content of the full fat soybean in the present study. The protein content of soybean flour was based on the nitrogen content which was not expected to be affected by the heating process. However, effects of the micronization processing on amino acids composition of protein have been reported (Khattab, Arntfield and Nyachoti, 2009).

Crude fat was extracted from soybean flour using hexane with the Soxhlet apparatus. The fat extracted from soybean was observed to be yellow in colour and the colour was darker for higher fat contents. The Soxhlet method was based on the

assumption that after 16h solvent extraction, all fat from the soybean sample was extracted. The original fat content in the soybean was 27.6% (dry basis, Table 4.1). Fat content in the present study was higher than the data reported by Marega Filho et al. (2001) where fat content ranged from 16.6%-20.4%. This was likely due to the different soybean cultivars and growing environment used. The present soybean was harvested locally in Manitoba, while the soybean seed in the work of Marega Filho et al. (2001) was from Brazil. Micronizing the full fat soybean at 100°C had no significant effects on fat content; however, soybean micronized at 135°C had a higher fat content (about 4.8% increase compared to the original raw soybean flour). In a study by Dominguez, Nunez and Lema (1995), oil from the soybean was extracted by enzymatically-assisted hexane extraction, and the resulting soybean flour was found to have 8-10% more extractable oil than the untreated soybean flour (Dominguez, Nunez and Lema, 1995). This was due to the enzymatic treatment breaking down the cell wall structure enhancing the liberation of oil (Dominguez, Nunez and Lema, 1995). In the present study, the higher extractable oil from soybean micronized 135°C could be due to changes in the soybean storage protein and carbohydrate structures at this temperature causing an increase in the amount of extractable oil.

4.1.2 Yield of the soybean flour after ethanol washing

Following the defatting process, the soybean flour was subjected to an ethanol wash (1:5 v/v sample/solvent). The solvent containing the soluble matrix from soybean flour was then removed by vacuum filtration. Residual ethanol was evaporated at room temperature for 24h. The yields of the soybean flour are shown in Table 4.2. Micronization at 100°C did not significantly affect the soybean flour recovery when

compared with unmicronized soybean flour treated with 65% or 85% ethanol. However, the highest soybean flour recovery was obtained with 85% ethanol washing of soybean flour micronized at 135°C. Micronization at 135°C significantly increased the soybean flour recovery compared with unmicronized soybean flour after being treated at both 65% and 85% ethanol. Micronization at this high temperature denatured the soybean protein making it less soluble in the ethanol mixture (Bellido et al., 2006; Zheng et al., 1998). The ethanol treatment at 85% achieved significantly higher soybean flour recoveries than the 65% ethanol treatment at all micronization levels. It has been reported that 60% ethanol selectively removes carbohydrate, fatty acids and phosphatides (Johnson and Lusas, 1983). Removal of these compounds by the more polar solvent (65% ethanol) would account for the lower yields obtained.

Table 4.2 Soybean flour recovery rate (%) after ethanol washing

Soybean flour	Soybean flour recovery rate (%)
M0A65	83.2 ± 0.20 ^d
M0A85	90.7 ± 0.08 ^b
M100A65	84.4 ± 0.45 ^d
M100A85	90.9 ± 0.07 ^b
M135A65	86.5 ± 0.49 ^c
M135A85	93.6 ± 0.83 ^a

M0/M100/M135: unmicronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration.

Same letter between the column means not significantly different (P<0.05)

Results are expressed as the mean ± standard deviation

4.1.3 Effects of processing on moisture content in defatted soybean flour

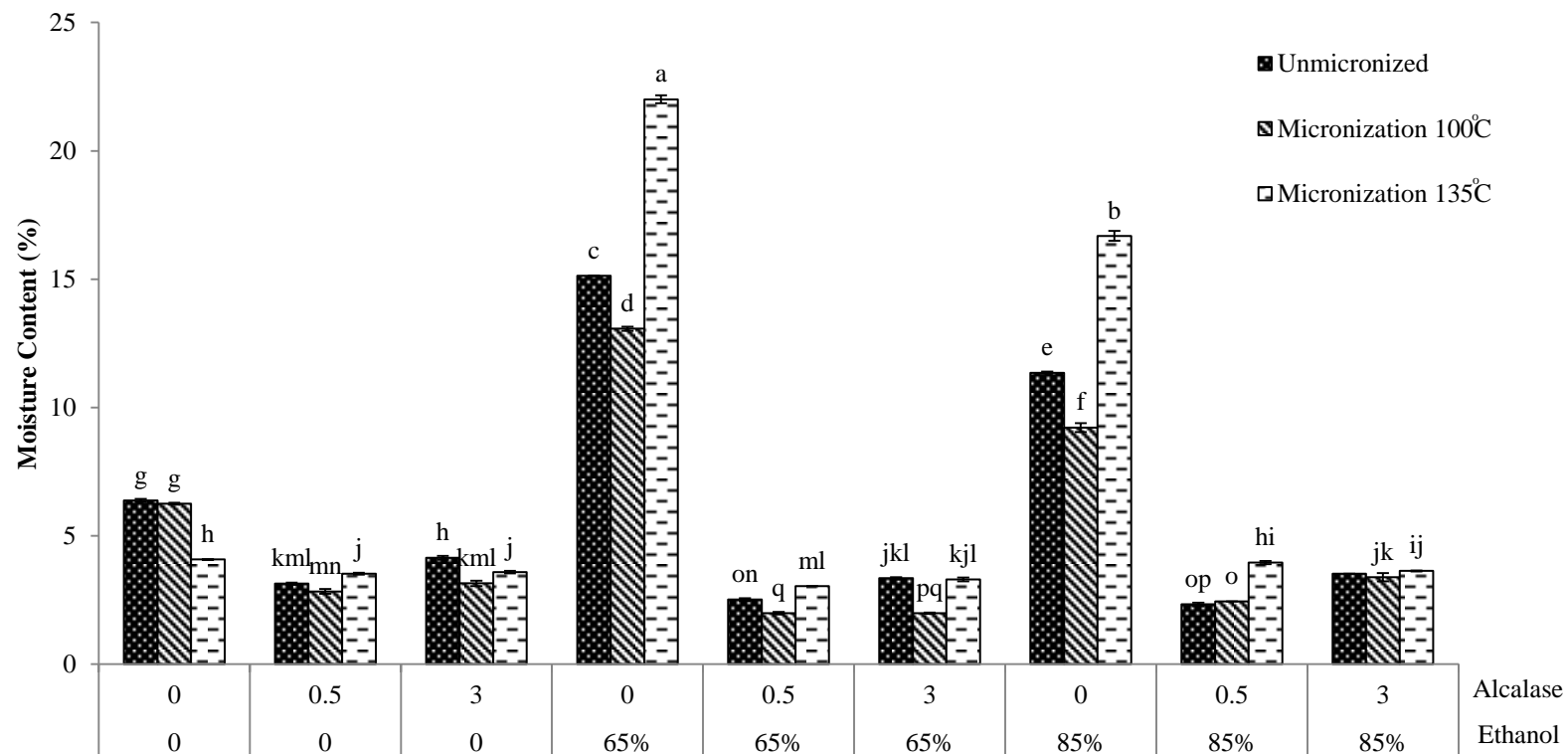
Moisture contents from the defatted soybean flour that had been micronized, treated with ethanol and hydrolyzed with alcalase were measured. Data analysis indicated a three-way interaction between these factors (Appendix 2). Data are summarized in Figure 4.1. As the objective of this work was to consider both individual factors as well

as factors in combination, micronization, ethanol and alcalase will be considered in the absence of the other two factors before examining the interaction. As most previous work has focused on single factors, this will also make it possible to compare individual results to the literature.

4.1.3.1 *Effect of the micronization on moisture content*

The effect of micronization alone can be obtained by considering only the data for 0 ethanol and 0 alcalase. Micronizing soybean at 100°C (M100A0AL0) had no significant effect on moisture content compared with the unmiconized soybean (M0A0AL0) (Figure 4.1). However, micronizing soybean at 135°C (M135A0AL0) significantly reduced the moisture content compared with both unmiconized soybean and soybean micronized at 100°C. This was because micronizing at 135°C evaporated more moisture out from the soybean. Wiriyaumpaiwong, Soponronnarit and Prachayawarakorn (2004) stated that the water mobility could be accelerated by micronization. In their study, after micronizing soybean at 121°C for 15 min, the moisture content in soybean was reduced by 40% deduction. This was a greater reduction than was achieved by micronizing at 135°C (~34% deduction) in present study. This may be due to the longer micronization time used in the work of Wiriyaumpaiwong, Soponronnarit and Prachayawarakorn (2004).

Figure 4.1 Moisture content (%) for defatted soybean flour with different treatments



Results are expressed as the mean \pm standard deviation and same letter means not significantly different ($P < 0.05$)
 Alcalase treatments were for 0, 0.5h or 3h

4.1.3.2 *Effect of the ethanol treatment on moisture content*

Ethanol effects alone were evaluated by comparing data for unmicronized soybeans with no alcalase treatment (Figure 4.1). Defatted soybean flours treated with ethanol had significantly higher moisture contents, with significantly higher % moisture for 65% ethanol (M0A65AL0 - 15.1%) compared to 85% ethanol (M0A85AL0 - 11.35%). Similar results for the 85% ethanol treatment were observed in the study conducted by Hua et al. (2005) where the moisture content of soy flakes washed with 85% ethanol was 11.12% which was higher than the defatted soybean flour (10.49%). The higher moisture content for the 65% ethanol treatment was due to the higher water content in the 65% ethanol solvent. After the ethanol treatment, higher amounts of water remained in the 65% ethanol treated soybean than 85% ethanol treated flour.

4.1.3.3 *Effect of the alcalase treatment on moisture content*

The effect of alcalase alone on moisture content was investigated by examining unmicronized samples that had no ethanol treatment (Figure 4.1). Alcalase treated samples had significantly lower moisture contents. Following the alcalase treatment, freeze-drying was used to remove remaining water. As a result, the moisture content in the alcalase treated-freeze dried soybean flour was reduced to 3– 4%, which was lower than the un-hydrolyzed soybean flour. After the freeze-drying, only bound moisture remained in the sample. Significantly higher moisture content was seen in the samples that were hydrolyzed for 3h in comparison to the 0.5h hydrolysis time. This indicated that with increasing the hydrolysis time, more free water was bound to the soybean hydrolysate.

4.1.3.4 *Effect of combining micronization, ethanol and alcalase processes on moisture content*

When examining the interactions between micronization, ethanol and alcalase treatments, the strong effects of alcalase and ethanol on decreasing and increasing moisture content, respectively, dominate the results. Nevertheless some interactions were observed (Figure 4.1).

While micronization at 135°C decreased the moisture content when no other factors were involved, for soybeans which were hydrolyzed by alcalase for 0.5h and micronized at 135°C (M135A0AL0.5) the moisture content was significantly higher than the comparable unmiconized sample (M0A0Al0.5). In contrast, soybeans which were hydrolyzed by alcalase for 3h, micronizing at 135°C (M135A0AL3) significantly decreased the moisture content in soybean hydrolysate compared with the unmiconized sample (M0A0Al3). Nevertheless the moisture content for both hydrolysis times at 135°C micronization were not significantly different reflecting the role of freeze-drying following hydrolysis in determining the final moisture content. For the hydrolysed soybean, micronization at 100°C resulted in decreased moisture content compared to the unmiconized hydrolyzed samples, indicating a synergistic effect between alcalase and micronization, as micronization at this temperature alone did not affect moisture.

For soybean treated with 65% or 85% ethanol washing, micronizing at 100°C (M100A65AL0 or M100A85AL0) significantly decreased the moisture content compared to unmiconized soybean flour (M0A65AL0 or M0A85AL0), while micronizing at 135°C (M135A65AL0 or M135A85AL0) significantly increased the moisture content. The increase in moisture content due to the ethanol treatment that was noted for ethanol alone

was also seen for flours that had been pre-micronized at 100°C or 135°C. This was due to the extra water from the ethanol treatment remaining in the soybean flour.

The increased moisture content due to ethanol treatment was not seen when combined with 0.5h or 3h hydrolysis (Figure 4.1). Any increase in moisture content due to residual moisture from solvent was evidently removed in the freeze-drying process. This result also confirmed that with increasing hydrolysis time, more free water was bound with the soybean hydrolysate.

Overall the effects of micronization, ethanol and alcalase were strongly influenced by the effects of individual factors, in particular the alcalase treatment. The freeze-drying step that followed this treatment significantly reduced the moisture level regardless of the increased moisture content resulting from the ethanol treatment.

For soybean treated with 65% and 85% ethanol and hydrolyzed with alcalase for 0.5h or 3h, micronizing at 135°C (Figure 4.1) generally resulted in significantly higher residual moisture than soybean micronized at 100°C, suggesting the higher micronization temperature increased the amount of bound water remaining after freeze-drying. The exception to this was the sample 85% ethanol treatment with the 3 h hydrolysis (M135A85AL3) which was not significantly different from samples at lower micronization temperatures.

For soybean pre-micronized at the 100°C and 135°C (Table 4.1) and treated with 0.5h or 3h hydrolysis, ethanol treatment at 85% significantly increased the moisture content compared with the ethanol treatment at 65% (except for M135A85AL3). This is in contrast to the effect of ethanol alone and suggests that the effects of heat and ethanol

on protein structure change the level of bound water and hence the moisture level following the freeze-drying step that was required following the alcalase treatment.

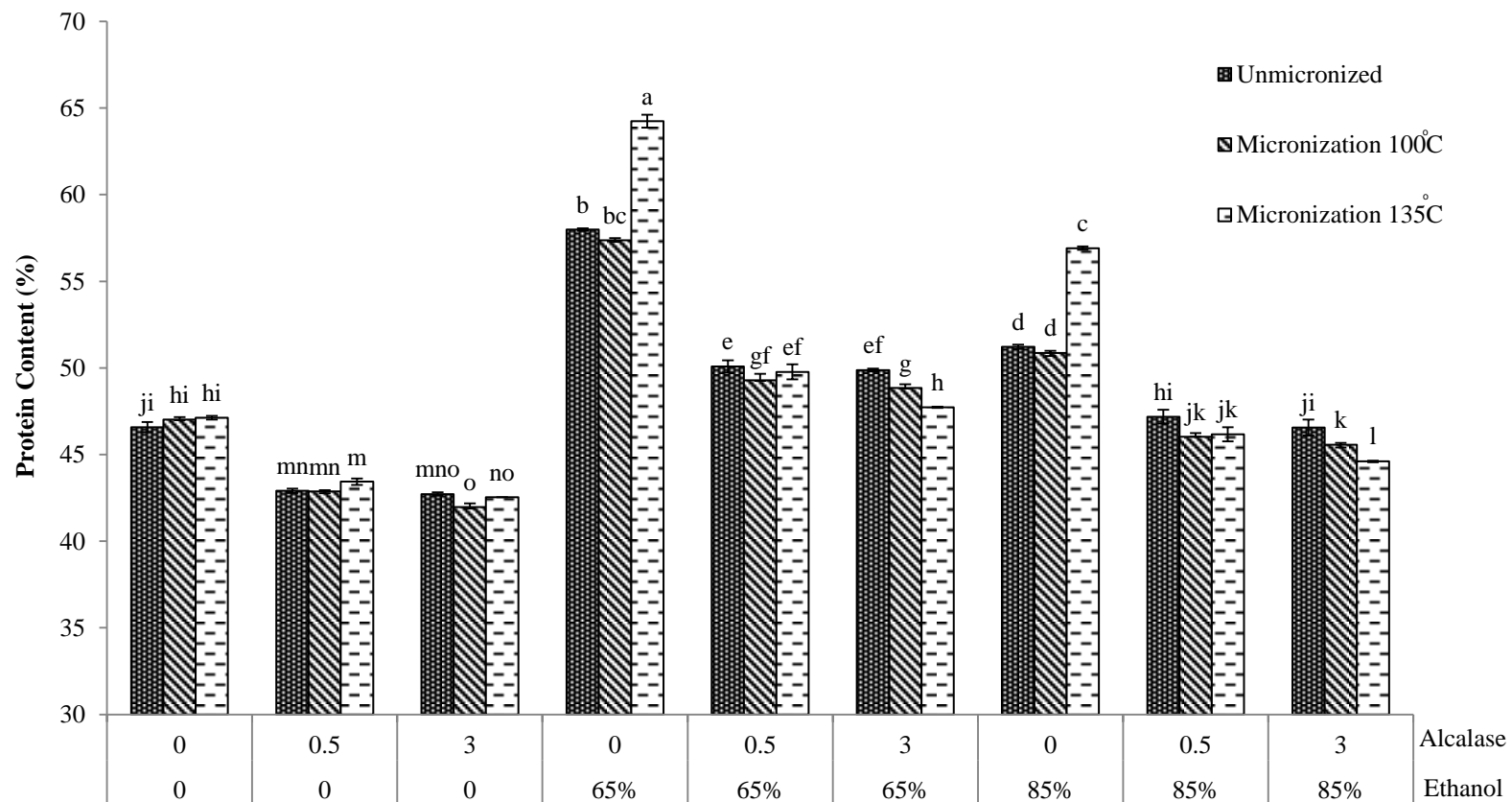
4.1.4 Effects of different processing on protein content in defatted soybean flour

The protein content for full fat soybean flour was 35.8% (Table 4.1) which falls in the range of 30-50% that has previously been reported for soybean protein content (Johnson et al., 2008). After defatting, the protein content in the defatted soybean (46.6%) was higher than the original soybean flour (Figure 4.2). This was due to removal of fat thereby concentrating the protein in the defatted flour. The influences of micronization, ethanol and alcalase on protein contents are summarized in Figure 4.2 and as was the case with moisture, a three-way interaction between these factors was present upon statistical analysis (Appendix 3). As a result, to consider both individual factors as well as factors in combination, micronization, ethanol and alcalase effects will be considered in the absence of the other two factors before examining the interaction.

4.1.4.1 Effect of the micronization alone on protein content

Micronization at 100°C or 135°C with no other treatments did not significantly change the protein content in comparison to the original defatted flour (Figure 4.2). It can be concluded that micronization did not change the protein content in the defatted soybean.

Figure 4.2 Protein content (%) for defatted soybean flour with different treatments



Protein content (dry basis) are expressed as the mean \pm standard deviation and same letter means not significantly different ($P < 0.05$)
 Alcalase treatments were for 0, 0.5h or 3h

4.1.4.2 *Effect of the ethanol treatment alone on protein content*

After the defatting process, defatted soybean flour was further treated with the ethanol washing at 65% and 85% concentration. These treatments in the absence of the micronization and alcalase treatments significantly increased the protein content to 58.0% and 51.2% respectively. A similar observation was made by (Hua et al., 2005) where 85% ethanol treated soy flakes contained 70.9% protein, which was higher than defatted soybean flake (57.36%). The ethanol treatment removed additional fat and other ethanol soluble materials from the defatted soybean flour, further concentrating the protein. The sample treated with 65% ethanol achieved significantly higher protein content than the 85% ethanol treated flour. This indicates that the 65% ethanol solution had stronger capacity to remove the ethanol soluble materials from soybean than 85% ethanol solution. This was also supported by the soybean flour recovery (Table 4.2) where defatted soybean flour treated with 65% ethanol showed a significantly lower flour recovery rate (83.24%) than 85% ethanol (90.67%).

4.1.4.3 *Effect of the alcalase hydrolysis alone on protein content*

Alcalase hydrolysis for 0.5h and 3h in the absence of other treatments (Figure 4.2), followed by freeze drying, significantly reduced the protein content compared to the non-hydrolyzed soybean flour with no ethanol treatment or micronization. The reason for this was not investigated in this study. Also there was no significant difference in protein content between alcalase hydrolysis at 0.5h and 3h for defatted soybean flour for which the only treatment was with alcalase.

4.1.4.4 Effect of combining micronization, ethanol and alcalase processes on protein content

Micronization alone had no significant effect on the protein content in soybean flour. The same was true for the soybeans treated with 65% or 85% ethanol washing, and micronized at 100°C when compared to un-micronized soybean flour treated with ethanol (Figure 4.2). However, micronization at 135°C significantly increased the protein content of both ethanol treated samples (Figure 4.2). The increase in protein content as a result of the ethanol treatment alone was also seen for soybean flour which was pre-micronized at 100°C or 135°C or when the sample had been hydrolyzed for 0.5 or 3h. This was due to removal of the soluble material from the soybean by the ethanol treatment. The higher protein content with the 65% ethanol treatment for all micronization and alcalase treatments in comparison to the 85% ethanol treatment again supports the recovery data in Table 4.2 where lower yield was noted for the 65% ethanol treatments, due to removal of non-protein material.

As was seen for the micronization treatment alone, for soybeans which were hydrolyzed by alcalase for 0.5h and 3h, micronization had no significant effect on protein content (Figure 4.2). The decrease in protein as a result of the alcalase treatment that was noted for alcalase alone was also noted for the soybeans that had been micronized at 100°C or 135°C.

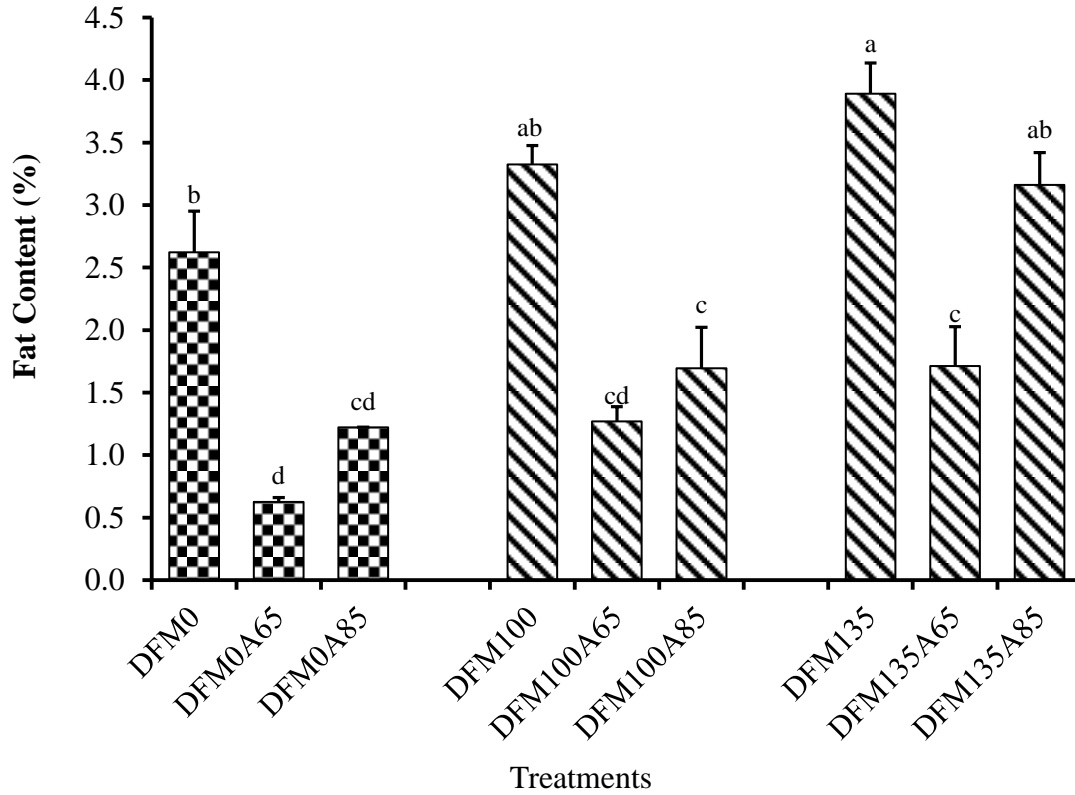
Overall, for soybeans pre-micronized at the 100°C or 135°C (Figure 4.2) and treated with 65% or 85% ethanol, the effects of the alcalase treatment were similar to the effects of alcalase treatment alone. Hydrolyzed samples had a significantly lower protein content. For soybeans treated with 65% and 85% ethanol washing, and micronized at

100°C, there was no significant difference in the protein content due to hydrolysis time (Figure 4.2); however, for samples micronized at 135°C a significantly lower protein content was seen after 3h alcalase treatment compared to the 0.5h alcalase treatment (Figure 4.2). For all treatments, the ethanol treatment at 65% significantly increased the protein content compared with the ethanol treatment at 85% due to the ethanol removal of the soybean matrix concentrating the protein content. Protein contents were most affected by the ethanol treatments.

4.1.5 Crude fat content determination among treated soybean flours

The original soybean had a fat content of around 27.6% (FFM0) which is higher than the value (20%) reported by Liu (1997). Micronizing the full fat soybean at 135°C (FFM135) significantly increased the extractable fat content in soybean as seen in Table 4.1. Micronizing soybean seeds at 135°C may have denatured the soybean protein and destroyed the soybean wall structure which led to the release of the bound lipid from the protein. Therefore, the extractable lipid from 135°C micronized soybean increased. Micronization at 100°C did not result in a significant increase in fat content. As expected, defatting soybean with hexane three times significantly reduced the fat content in soybean flour. About 90% of the fat was removed, only 2.62% fat remained in the defatted flour (DFM0) (Figure 4.3). This is expected as hexane is known to be an efficient solvent for oil extraction in the soybean oil extraction industry (Liu, 1997).

Figure 4.3 Crude fat content (dry basis) for soybean flour treated with different treatments



Same letters between different columns means not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Defatted soybean was further treated with ethanol. It was expected that the residual lipid in defatted soybean flour would be removed. The fat analysis results met this expectation. Ethanol treatments at 65% and 85% significantly reduced the fat content compared with the untreated soybean flour, except for the sample micronized at 135°C and treated with 85% ethanol (DFM135A85). There was no significant difference between the fat contents for the 65% and 85% ethanol treatments for soybean flour that was unmiconized, or was micronized at 100°C.

The fat content in research done by Hua et al., (2005), however, was only 0.12% when the defatted soybean flour was treated with 85% ethanol; this was lower than that in the present study (1.22% residual fat for DFM0A85). The difference for the fat content may be due to the different fat content determination method used: fat was extracted using chloroform and methanol at a ratio of 2:1 in their research while the pure hexane solvent was used in current research. Oil extraction time for their research was not mentioned while in the present research, a 16h fat extraction was used.

4.2 Study 1: Effects of Micronization, Ethanol Washing and Enzyme Hydrolysis on Trypsin Inhibitor and Lipoxygenase Activities in Soybean Flour

4.2.1 Degree of hydrolysis determination on alcalase treated soybean flour

The degree of hydrolysis (DH %) is very important to evaluate the progress of protein hydrolysis. The DH is expressed as the percentage of hydrolyzed peptide bonds. It is based on the assumption that a free amino group is released every time a peptide bond is hydrolyzed. The DH for hydrolyzed samples was calculated based on the ratio of the amino group released to the total amino groups in the soybean protein. Therefore for each hydrolyzed sample, the original number of amino groups in the corresponding un-hydrolyzed sample was used as a control. Although different un-hydrolyzed samples had slightly different free amino group content (Appendix 1), in this present study the degree of hydrolysis for an un-hydrolyzed sample was treated as zero. Before looking at the interactions seen (Appendix 4) for the DH% data, the effect of alcalase alone will be examined in the absence of the other two factors.

4.2.1.1 *Effect of the alcalase hydrolysis time on DH (%)*

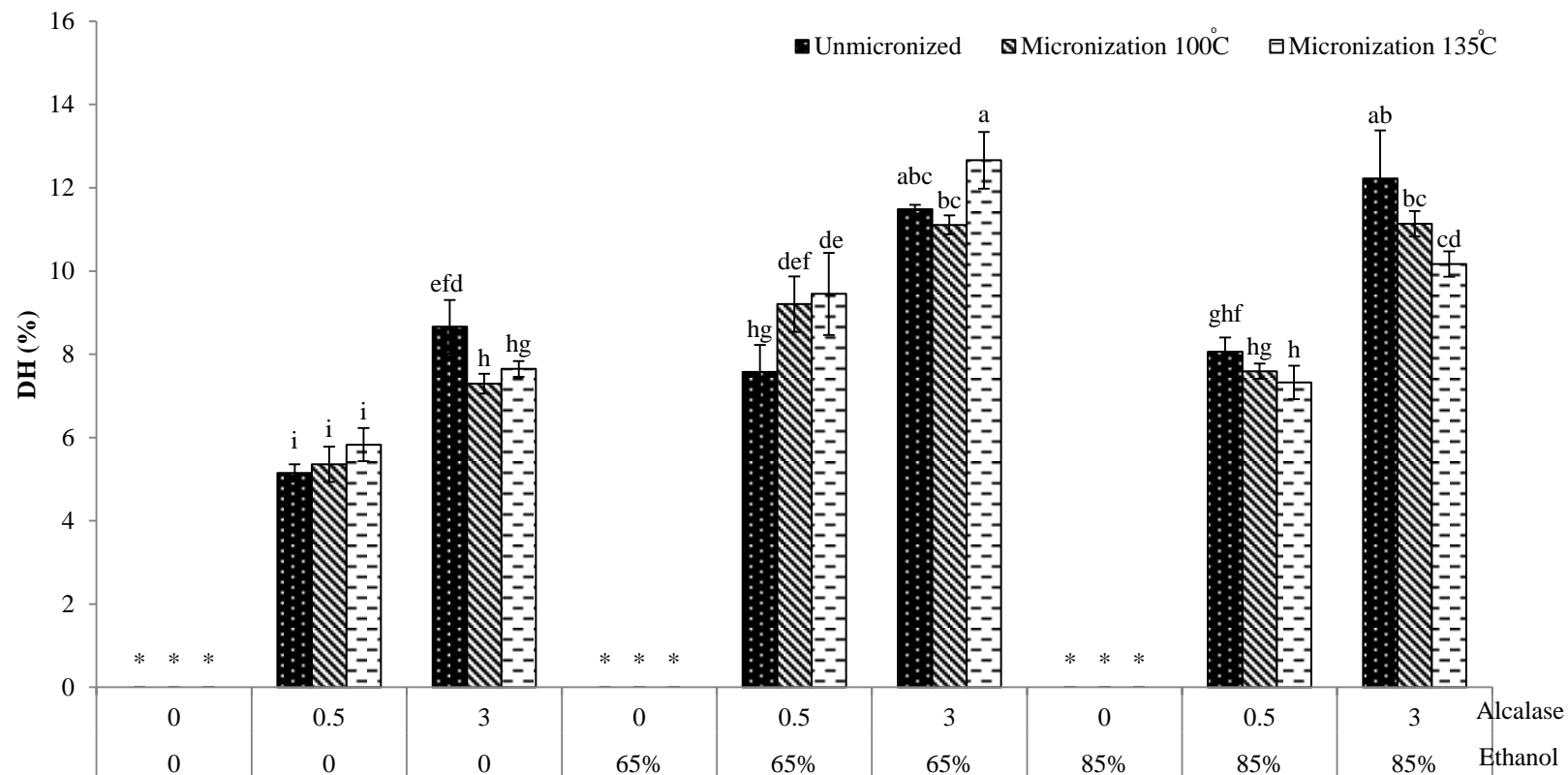
The DH% results for the hydrolyzed samples are summarized in Figure 4.4. When examining those samples for which there is no micronization or ethanol treatment, 5% of total peptides in soybean flour were released after 0.5h of alcalase hydrolysis (M0A0AL0.5). Alcalase hydrolysis for 3h (M0A0AL3) significantly increased the percentage of total amino groups released to 8.66%. In similar research conducted by hydrolyzing defatted soybean flour with alcalase, treatment times of 0.5h and 3h gave DH (%) values of ~4% and ~9% (Lee, Lee and Lee, 2001), close to the results obtained in the current project. In a similar experiment conducted by Hreckova, Rusnakova and Zemanovic (2002), even though the hydrolysis results were expressed as amino acid concentration (mg/ml), the trend of increased amino group concentration from 0h to 3h was similar to the current research.

4.2.1.2 *Influence of micronization and ethanol treatment on alcalase hydrolysis and the resulting DH (%)*

Although micronization should denature the protein making it more open to proteinase attack, there were no significant differences in the DH (%) for the samples pre-micronized at 100°C and 135°C for either 0.5h or 3h. It would appear that changes in the protein during micronization were not required for enzymatic hydrolysis.

Soybean which was treated with either 65% ethanol or 85% ethanol without micronization achieved significantly higher DH (%) than the corresponding un-treated flours after 0.5h and 3h alcalase hydrolysis (Figure 4.4). This was due to ethanol-water denaturation of the soybean protein, disrupting the internal structure of the protein and increasing the susceptibility of protein to proteinase attack (Fukushima, 1969).

Figure 4.4 Degree of hydrolysis (DH) % for enzymatic hydrolysis on alcalase hydrolyzed soybean flour.



Same letter means not significantly different ($P < 0.05$)

Alcalase treatments were for 0, 0.5h or 3h

*Control: blank (treated as 0 DH %) for the corresponding hydrolysis sample for calculating degree of hydrolysis

For soybean samples treated with 65% ethanol and 0.5h hydrolysis, micronizing at 100°C and 135°C significantly increased the DH (%) compared to the corresponding unmiconized sample (Figure 4.4); there was no significant difference due to the micronization temperature. For soybean sample treated with the 65% ethanol treatment and 3h alcalase hydrolysis, micronizing at 100°C and 135°C had no significant effect on DH (%) compared with the unmiconized samples (Figure 4.4); however, the DH (%) values were all higher than the comparable samples with no ethanol treatments. In contrast, when the 85% ethanol treatment was combined with the 0.5h hydrolysis, micronization had no effect on the DH (%), and with the 3h hydrolysis, the DH (%) were actually lower for the micronized samples; values for the DH (%) after 3h, however, were all higher than after 0.5h of hydrolysis. Clearly the removal of additional lipid influences the effect of micronization on hydrolysis time.

4.2.2 Protein structural analysis on the soybean flour using Differential Scanning Calorimetry (DSC) analysis

Differential scanning calorimetry (DSC) is a thermo-analytical technique which monitors the difference between the amount of heat required to increase the temperature at the same rate for a sample and a reference. By using DSC, important parameters of protein such as denaturation temperature (°C) and enthalpy (J/g), which are indicators of the structure of the protein can be studied.

The two endothermic peaks generated during a typical DSC analysis are shown in Figure 4.5. The first peak represents β -conglycinin (7S) globulin and the second peak represents glycinin (11S) globulin (Ahmed et al., 2009). Thermograms are shown for untreated samples (M0A0AL0) and samples treated only with 65% (M0A65AL0) and 85% ethanol (M0A85AL0). Thermograms for samples with varying levels of

micronization were similar (graphs not shown). Hydrolyzed samples showed no endotherms. The β -conglycinin denaturation transition for defatted soybean flour (M0A0AL0) was observed as a very small peak at 79.4°C with an onset temperature of 73.9°C. The heat of denaturation was only 0.435 J/g of sample. For glycinin in untreated defatted soybean protein, a larger peak was observed with a denaturation temperature at 100.0°C, an onset temperature of 90.4°C and an enthalpy of denaturation of 2.04 J/g. The denaturation temperatures for β -conglycinin (79.4°C) and glycinin (100.0°C) in the present research were found to be higher than those in the research by Mo, Sun and Wang (2004) who reported denaturation temperature for β -conglycinin and glycinin of only 73.4°C and 89.7°C respectively. The enthalpies of denaturation for the storage proteins were higher in Mo, Sun and Wang (2004)'s research at 7.25 J/g for β -conglycinin and 12.1 J/g for glycinin. The differences could be due to the different samples used in two studies. In the present research, soybean flour was used to study the protein thermal denaturation properties while isolated β -conglycinin and glycinin were used in the research of Mo, Sun and Wang (2004). It is possible that other compounds in the flours suppress the enthalpy of denaturation.

4.2.2.1 *Effect of alcalase hydrolysis on the soybean protein structure*

Alcalase hydrolyzed soybean flour showed a straight line in the DSC thermogram, indicating that the enzymatic attack totally destroyed the structure of the soybean protein. As a result, no denaturation temperature and enthalpy for alcalase treated sample have been included in Table 4.3 (designated Nd). Similar results were obtained by Molina Ortiz and Wagner (2002) where total enthalpies of denaturation of both β -conglycinin and glycinin were found to be ~0 J/g for the protein extracted from thermally treated and

enzymatic hydrolyzed soybean flour. However, for the protein isolates extracted from native soybean flour, only glycinin was totally denatured; a small peak of β -conglycinin with a low enthalpy (~ 0.8 cal/g) was observed (Molina Ortiz and Wagner, 2002). In the present results, no peaks were observed for any of the hydrolyzed samples, possibly because soybean flour was used for DSC analysis in the current study while soybean protein isolates were used by Molina Ortiz and Wagner (2002). In addition, alcalase was selected to hydrolyze soybean flour in the present study whereas bromelain was used by Molina Ortiz and Wagner (2002). As was done in previous sections, individual effects (in the absence of the other two) will be discussed prior to looking at the interactions in the statistical outputs (Appendix 5).

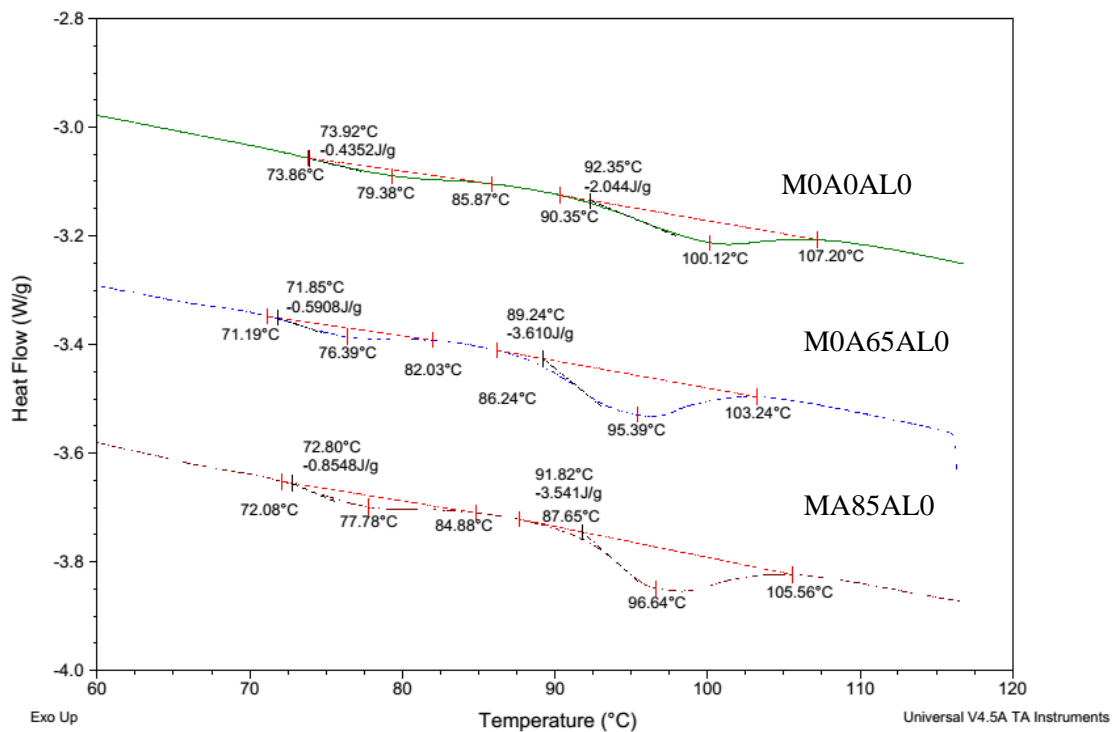
4.2.2.2 Effect of micronization on the soybean protein structure

Micronizing at 135°C had a significant effect on the structure of β -conglycinin in soybean protein (Table 4.3) in that soybean which was micronized at 135°C (M135A0AL0) only showed a single endothermic peak (11S) in the DSC thermogram indicating β -conglycinin was totally denatured. The denaturation temperature and enthalpy of denaturation for β -conglycinin micronized at 100°C (M100A0AL0) were significantly reduced in comparison to data from the un-micronized sample (M0A0AL0), indicating β -conglycinin was partially denatured after micronizing at 100°C.

For the larger storage protein in soybean, glycinin, micronizing at 100°C and 135°C significantly decreased the denaturation temperature, indicating that after micronization at 100°C (M100A0AL0) and 135°C (M135A0AL0), glycinin was less stable and the energy needed to totally denature micronized soybean protein decreased. There was no significant difference between the denaturation temperature for

micronization at 100°C (M100A0AL0) and 135°C (M135A0Al0). The enthalpy of denaturation for soybean which was micronized at 135°C was significantly lower than the untreated defatted soybean flour (M0A0AL0) confirming that glycinin was partially denatured.

Figure 4.5 DSC thermogram of the defatting soybean flour, 65% ethanol treated & 85% ethanol treated soybean flour



Denaturation temperature (°C) and enthalpy (J/g) are as indicated. M0: unmicronized defatted soybean flour. M0A65: 65% ethanol treated soybean flour. M0A85: 85% ethanol treated soybean flour

Table 4.3 Effects of the micronization, ethanol washing, and alcalase hydrolysis on denaturation temperature (°C) and enthalpy (J/g) for glycinin and β -conglycinin in soybean storage protein

Storage protein	β -conglycinin		glycinin	
	DT (°C)	Enthalpy(J/g)	DT (°C)	Enthalpy(J/g)
M0A0AL0	78.19 \pm 1.26 ^a	0.58 \pm 0.05 ^b	99.73 \pm 1.96 ^a	2.09 \pm 0.04 ^c
M0A65 AL0	76.45 \pm 0.56 ^b	0.60 \pm 0.01 ^b	95.10 \pm 0.47 ^d	3.45 \pm 0.14 ^a
M0A85 AL0	76.89 \pm 0.78 ^b	0.85 \pm 0.07 ^a	95.93 \pm 0.67 ^{cd}	3.32 \pm 0.23 ^{ab}
M100A0AL0	76.74 \pm 0.62 ^b	0.41 \pm 0.08 ^c	97.28 \pm 0.77 ^{bc}	3.12 \pm 0.15 ^b
M100A65 AL0	76.80 \pm 0.90 ^b	0.34 \pm 0.02 ^d	95.06 \pm 0.89 ^d	3.22 \pm 0.30 ^{ab}
M100A85 AL0	77.34 \pm 0.55 ^{ab}	0.42 \pm 0.01 ^c	96.11 \pm 0.53 ^{cd}	3.36 \pm 0.23 ^{ab}
M135A0AL0	Nd	Nd	97.88 \pm 0.65 ^b	1.08 \pm 0.04 ^d
M135A65AL0	Nd	Nd	95.48 \pm 0.21 ^d	1.37 \pm 0.11 ^d
M135A85AL0	Nd	Nd	95.99 \pm 0.00 ^{cd}	1.38 \pm 0.06 ^d

Results are expressed as the mean \pm standard deviation and the same letter in the same column means no significant difference (P<0.05).

Nd: not detected

M0/M100/M135: Non-micronized / micronization at 100 or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration. AL0: no alcalase treatment

Data for alcalase treatments of 0.5 and 3 h are not included as all curves were straight lines and no values could be obtained.

4.2.2.3 *Effect of ethanol treatment on the soybean protein structure*

Soybean flour treated with both 65% (M0AL65AL0) and 85% (M0A85AL0) ethanol showed significantly lower denaturation temperatures for β -conglycinin and glycinin compared to the untreated defatted soybean flour (M0A0AL0) indicating the stability of β -conglycinin and glycinin were partially reduced. However, ethanol treatments at 65% and 85% ethanol did not significantly reduce the enthalpy for denaturing β -conglycinin. In contrast, both ethanol treatments significantly increased the energy needed to denature glycinin in comparison to the untreated sample. This phenomenon was not expected because ethanol was expected to influence hydrophobic interaction involving glycinin thereby reducing the energy needed to do it (Brandts and Hunt, 1967). The lower protein stability noted by the lower denaturation temperature values did not lead to a reduction in the denaturation enthalpy in the present research.

4.2.2.4 *Effect of micronization and ethanol interactions on the soybean protein structure*

For β -conglycinin, with the ethanol treatments (65% or 85%), micronization at 100°C (M100A65AL0 or M100A85AL0) had no significant effects on denaturation temperature in comparison to the corresponding unmiconized sample (M0A65AL0 or M0A85AL0). However, the enthalpies of denaturation were significantly reduced. Micronizing soybean at 135°C in combination with the ethanol treatments (65% or 85%) totally denatured the β -conglycinin as indicated by a lack of peak in the DSC thermograph (Table 4.3). With micronization at 100°C, the ethanol treatments did not have any significant effects on denaturation temperature or enthalpy in comparison to the micronized sample without the alcohol treatment (M100A0AL0). Although micronization in the absence of ethanol and ethanol in the absence of micronization partially denatured the β -conglycinin (based on the denaturation temperature results), the combination of these treatments did not appear to affect β -conglycinin structure.

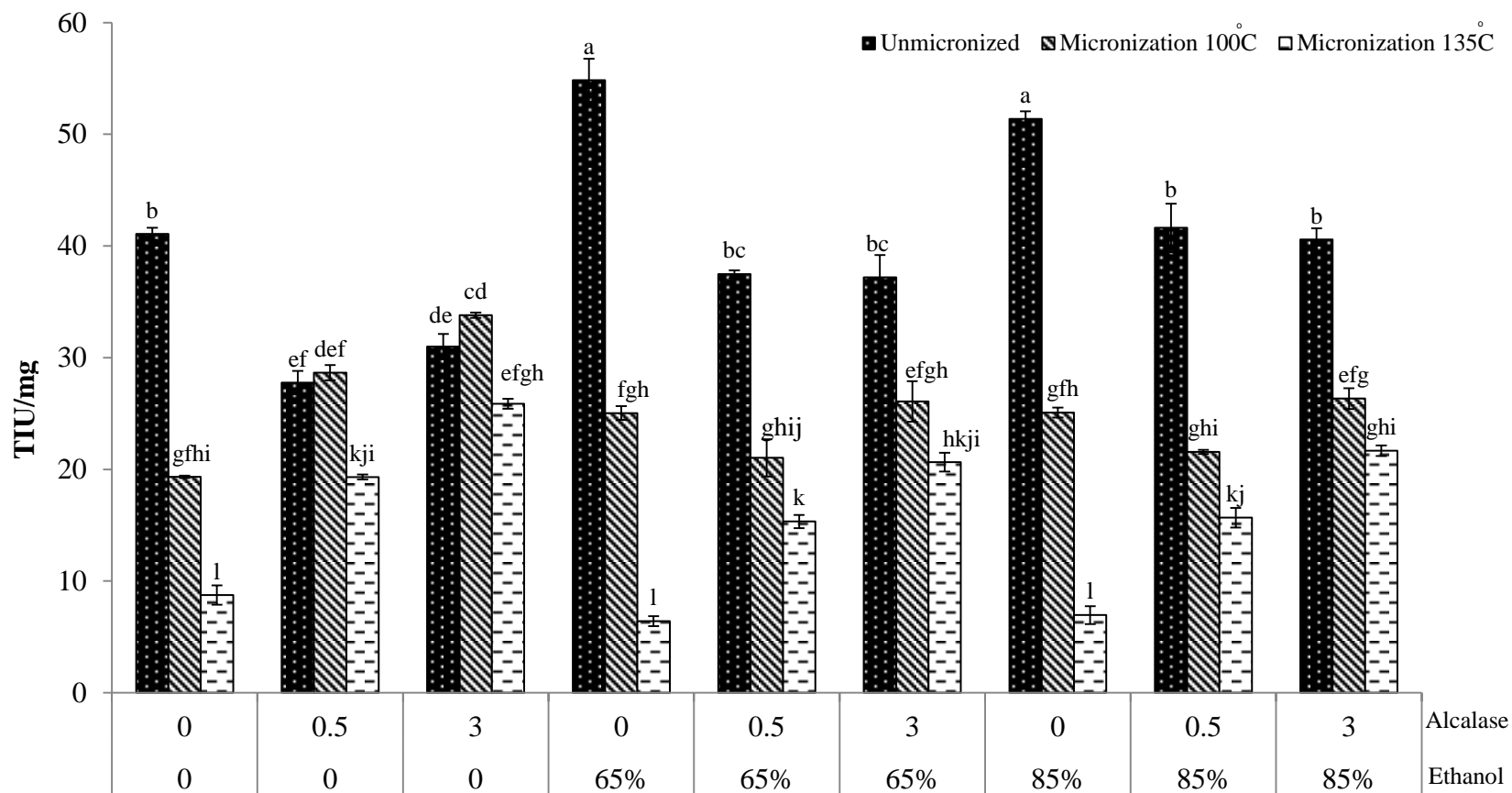
For glycinin, with the ethanol treatments (65% or 85%), micronizing at 100°C (M100A65AL0 or M100A85AL0) had no significant effects on denaturation temperature and enthalpy in comparison to those in the unmiconized samples (M0A65AL0 or M0A85AL0). However, micronization at 135°C with ethanol treatments (M135A65AL0 or M135A85AL0) significantly decreased the enthalpy of denaturation for glycinin indicating that the combination of micronization at 135°C with ethanol treatment partially denatured the glycinin. When micronizing at 100°C or 135°C, the ethanol treatments had no significant effects on enthalpy when compared to samples for which there had been no ethanol treatment (M100A0AL0 or M135A0AL0) with the exception of the denaturation

temperatures for M100A65AL0 which decreased. Clearly the micronization of the seeds had more of an impact on protein structure than the ethanol washings when both treatments were applied.

4.2.3 Effects of micronization, ethanol washing, and enzyme hydrolysis on trypsin inhibitors in soybean flour

Trypsin inhibitors are serine protease inhibitors that decrease the biological activity of trypsin. Trypsin is an enzyme involved in breaking down proteins as part of digestion in humans and other animals. High amounts of trypsin inhibitors can cause protein mal-absorption. Ideally, trypsin inhibitors should be inactivated in ready to eat foods. To achieve this goal, different processes with varying conditions were selected to treat soybean. The trypsin inhibitor activity was then determined in the soybean flour by using AACC (1999) method (22-40.01) where one trypsin activity unit (TU) is defined as an increase of 0.01 absorbance units at 410 nm. Therefore, one inhibitor activity unit (TIA) was defined as one trypsin activity inhibited under the experiment conditions. Data are summarized in Figure 4.6. The objective of this work was to consider both individual factors as well as factors in combination. Also the statistical analysis (Appendix 6) indicated a three-way interaction between those factors. Therefore, micronization, ethanol washing and alcalase hydrolysis will be considered in the absence of the other two factors before examining their interactions.

Figure 4.6 Effects of ethanol treatment and hydrolysis on trypsin inhibitor activity (dry basis) in unmicronized soybean flour, and soybean flour that was micronized at 100°C and 135°C



Columns with the same letter are not significantly different ($P < 0.05$)

4.2.3.1 *Effects of micronization processing alone on trypsin inhibitor in soybean flour*

Micronization is an infrared heating process which can be classified as “high temperature short time (HTST)” processing. Infrared radiation has wavelengths between visible and microwave and is highly efficient in achieving high temperatures in materials (Sharma, 2009). Micronization can be applied to cereals and legumes to produce products with better flavour and functionality.

Many researchers have investigated the effects of the micronization on trypsin inhibitors in legumes and their products (Hutton and Foxcroft, 1975; Khattab and Arntfield, 2009; Koroteeva et al., 2007; Kouzeh-Kanani et al., 1983; Yalcin and Basman, 2015; Žilić, Bozović and Hadži-Tašković Šukalović, 2012). The effects of the micronization heat treatment in the absence of the other two factors (ethanol washing and alcalase hydrolysis) on trypsin inhibitor activity in soybean from the current research can be seen in Figure 4.6. Trypsin inhibitor activity in the defatted untreated soybean flour was 41.1 TIU/mg. When the soybean seeds were micronized to surface temperatures of 100°C and 135°C prior to grinding and defatting, 53% and 80% reductions in trypsin inhibitor activity, respectively, were achieved. The absorbed energy from micronization was responsible for disrupting interactions contributing to the structural integrity of the trypsin inhibitors which led to their denaturation. However, micronization at the higher temperature investigated (135°C) was unable to totally eliminate trypsin inhibitor activity, confirming the high thermal stability of soybean trypsin inhibitors. Compared with results achieved by Žilić, Bozović and Hadži-Tašković Šukalović (2012), micronization in the present experiment was more effective in reducing trypsin inhibitors at both 100°C and

135°C; only 6% and 34% trypsin inhibitor activity reductions for micronization at 100°C and 140°C were observed in their study. The improved trypsin inhibitor elimination by micronization in the current study may be due to the tempering process used to treat the soybean seeds before micronization, something that wasn't done by Žilić, Bozović and Hadži-Tašković Šukalović's study (2012). High moisture content benefits greater denaturation of trypsin inhibitors. This point was also confirmed by Yalcin and Basman (2015) who showed that micronization of a soaked soybean sample had a significantly lower trypsin inhibitor activity compared to an unsoaked sample. They also looked at the effects of the infrared power (814 W, 1003 W, 1208 W, and 1342 W) and micronization time (10 min and 15 min) individually or in combination on the trypsin inhibitor activity in two different soybean varieties. The combination of soaking for 30 min (~40% moisture content) with a 1342 W infrared micronization treatment for 15 min resulted in more than 95% inactivation of trypsin inhibitors (Yalcin and Basman, 2015), better trypsin inhibitor inhibition than in the present research. Many factors could contribute to this difference. The higher moisture content and longer micronization time used by Yalcin and Basman (2015) may have enhanced the effects of the micronization on reducing trypsin inhibitor activity in soybean. However, it is worth noting that the initial inhibitor activity for the soybeans in the study by Yalcin and Basman (2015) was very high (more than 88 TIU/mg) and this could also affect the efficiency of the micronization process (Žilić, Bozović and Hadži-Tašković Šukalović, 2012).

Other crops have also been investigated in relation to the effect of micronization on trypsin inhibitors. Khattab and Arntfield (2009) examined the micronization effects on

other legume seeds such as cowpeas, kidney beans and peas. According to their results, 88% - 95% of the trypsin inhibitor were inactivated which were greater reductions than in the present research. The reason for this difference may be the higher moisture (24%) and longer processing time (2.5 min) in the research by Khattab and Arntfield (2009). It is clear from both the literature and the present research that micronization is an efficient process for reducing trypsin inhibitor content. In the current work, micronization at 135°C had a greater effect than at 100°C, although some burned or dark colour soybean were found in seeds micronized at 135°C. None of the research described above (Khattab and Arntfield, 2009; Yalcin and Basman, 2015; Žilić, Bozović and Hadži-Tašković Šukalović, 2012) achieved a 100% trypsin inhibitor activity reduction, confirming that trypsin inhibitor is a somewhat heat resistant proteinase. However, when compared to the previous studies (Khattab and Arntfield, 2009; Yalcin and Basman, 2015; Žilić, Bozović and Hadži-Tašković Šukalović, 2012), a higher moisture content and longer micronization time or higher temperature could have further reduced the trypsin inhibitor activity.

4.2.3.2 Effects of ethanol washing alone on trypsin inhibitor in soybean flour

Ethanol washing has been shown to have a high potential for improving the flavour, colour and functionality of the soy protein products (Hua et al., 2005). In addition, many researchers have investigated the effect of ethanol on the trypsin inhibitors (Liu and Markakis, 1991; Tasneem and Subramanian, 1990; Tolman, 1995).

Effects of the ethanol washing on the trypsin inhibitor activity in unmiconized and unhydrolyzed soybean flour are shown in Figure 4.6. After treatment of the soybean

flour with 65% and 85% ethanol, trypsin inhibitor activity significantly increased from 41.1 TIU/mg to 54.8 TIU/mg and 51.4 TIU/mg, respectively. There was no significant difference between the effects of 65% ethanol treatment and 85% ethanol treatment on trypsin inhibitor activity. The increase in trypsin inhibitor after treatment with ethanol could be explained by the tendency of ethanol to leach out low molecular weight proteins and other soluble material which concentrated the trypsin inhibitor in the extracted soybean meal. The results were in agreement with those of Tasneem and Subramanian (1990) who treated guar meal by ethanol washing. The trypsin inhibitor activity was higher in the ethanol extracted meals (9.0 TIU/mg of protein) than in the defatted guar meal (7.6 TIU/mg of protein). The authors explained that, based on the molecular size of the inhibitor (the trypsin inhibitor for guar meal was described as a macromolecule), it could not be leached using an 80% ethanol solution. In contrast, soybean trypsin inhibitors have relatively low molecular weights: 21.5 kDa for Kuntiz trypsin inhibitor and 8 kDa for Bowman-Birk trypsin inhibitor (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013). The Bowman-Birk trypsin inhibitor, in particular, should be extracted by using 60% ethanol solution (Sessa and Wolf, 2001). Research by Liu and Markakis (1991) confirmed that ethanol concentration between 20-70% extracted both Kuntiz and Bowman-Birk trypsin inhibitors. Therefore, in present research, at least part of the trypsin inhibitor was expected to be removed from soybean flour due to the ethanol treatments; a decrease in the amount of the trypsin inhibitor in the sample was expected. However the extent to which other soluble matrix were leached appeared to be more important and as a result, an increase of trypsin inhibitor activity was obtained. The present results were in contrast with results achieved by Tolman (1995) who studied the

hot aqueous ethanol treatment effects on pea flour where trypsin inhibitor activities significantly decreased (30% reduction) after pea flour was treated with 65% ethanol at 50°C for 2.5 h. A ten percent trypsin inhibitor reduction was achieved after treatment with 75% ethanol at 50°C and with an increase in the temperature to 80°C, 90% of the trypsin inhibitor was removed (Tolman, 1995). Instead of treating soybean flour with ethanol at room temperature as in present study, higher temperatures, such as 80°C, may have been able to achieve a higher trypsin inhibitor reduction. In addition, a longer extraction time and/or a lower ethanol concentration may also have reduced trypsin inhibitors. Fifty percent ethanol was shown to promote maximum denaturation of soybean protein (Fukushima, 1969) and the range of 20% - 70% ethanol was recommended for the extraction the trypsin inhibitor from soybean (Liu and Markakis, 1991).

4.2.3.3 *Effects of alcalase hydrolysis alone on trypsin inhibitors in soybean hydrolysates*

Enzymatic hydrolysis has been used for modification of the nutritional value and functional properties such as solubility, foaming, emulsifying and gelation (Spellman et al., 2003), as well as improvement in the appearance and flavour of the food protein (Lee, Lee and Lee, 2001). However, to our knowledge, only a few studies have investigated the effects of the enzymatic hydrolysis on trypsin inhibitor activity in soybean (Dia et al., 2012; Yang, Chen and Huang, 2001).

In the present study, the defatted soybean flour (without micronization and ethanol treatment) was treated by alcalase hydrolysis for either 0.5 h or 3 h. The degree of

the hydrolysis (DH (%)) was measured to determine the effectiveness of the hydrolysis. The trypsin inhibitor activity for un-hydrolyzed or hydrolyzed soybean flours is shown in Figure 4.6. Alcalase hydrolysis of the defatted soybean flour led to significant reductions in the trypsin inhibitor activity; reductions of 24.6% (3 h of hydrolysis) and 32.4% (0.5 h of hydrolysis) were obtained. Trypsin inhibitor activity in samples hydrolyzed for 3 h were not significantly different in comparison to those hydrolyzed for 0.5 h. The present results were similar to the results from Dia et al. (2012) who studied the effects of germination and alcalase hydrolysis individually or in combination on anti-nutritional factors in Brazilian soybeans. Hydrolysis times of 1 h, 2 h or 3 h significantly reduced both the Kuntiz trypsin inhibitor and the Bowman-Birk trypsin inhibitor content in the soybean protein. There was no significant difference in the level of trypsin inhibitors between alcalase hydrolysis for 1 h and 2 h. However, inhibitor activity after 3 h of hydrolysis was significantly reduced (77% reduction of the Kuntiz trypsin inhibitor) compared with 1 h of hydrolysis (Dia et al., 2012). The differences between the results from their study and the present study were likely due to differences in experiment conditions and method of analysis. In the Dia et al. (2002) study, 1.2 mL 0.1 HCL was used to stop the enzymatic hydrolysis while the 10 min in a 95°C water bath was used to inactivate the alcalase activity in the present study. In addition, in the Dia et al. (2002) study, the Kuntiz trypsin inhibitor and Bowman-Birk trypsin inhibitor content were measured in the protein hydrolysates which were separated from the precipitates by centrifugation, whereas in the present study, the trypsin inhibitor activities were determined in the hydrolyzed soybean flour.

Another group of the researchers also used alcalase hydrolysis to treat defatted soybean. After 1 h of alcalase hydrolysis, approximately 10% of the trypsin inhibitor activity was removed (Yang, Chen and Huang, 2001) which was less than trypsin inhibitor activity reduction achieved in the present research (32% reduction for 0.5h hydrolysis). However, it is interesting to note that there was a sharp decrease in the trypsin inhibitor activity between 3 h and 4 h hydrolysis and no further changes were noted beyond the 4 h hydrolysis time (Yang, Chen and Huang, 2001). Yang, Chen, and Huang (2001) found that most of the Kuntiz trypsin inhibitor was inactivated while Bowman-Birk trypsin inhibitor maintained its bioactivity. This was likely due to more disulphide bonds (5 more than in the Kuntiz inhibitor) which were critical for the function of the Bowman-Birk inhibitor (Roychaudhuri et al., 2003; Yang, Chen and Huang, 2001).

None of the previous research effectively explained the mechanism by which enzymatic hydrolysis decreased the trypsin inhibitor activity. Generally, to have bioactive effects, trypsin inhibitors need to maintain their structural integrity. Denaturation of inhibitors by heat or by ethanol treatment could lead to the inactivation of their activities as reported by many researchers (Cabrera-Orozco, Jiménez-Martínez and Dávila-Ortiz, 2013; Dia et al., 2012; Liu and Markakis, 1991; Tolman, 1995; Yalcin and Basman, 2015; Žilić, Bozović and Hadži-Tašković Šukalović, 2012). Alcalase hydrolysis is known to degrade the protein in soybean flour resulting in the release of low molecular peptide chains (Dia et al., 2012; Korhonen and Pihlanto, 2003). A longer time of hydrolysis results in the release of a larger amount of the peptide chains (Dia et al., 2012), a result

which agreed with our degree of hydrolysis data (Figure 4.4). In addition, the DSC data from the present research also demonstrated denaturation of the soybean protein due to hydrolysis (Table 4.3). Although, these results do not provide direct evidence proving that trypsin inhibitor was denatured or hydrolyzed by alcalase, there is high possibility that the trypsin inhibitor was degraded along with the storage protein resulting in destruction of the trypsin inhibitor activity in the soybean flour.

In the present study, micronization alone had achieved a higher trypsin inhibitor reduction (53% at 100°C and 80% at 135°C) than the alcalase hydrolysis alone (24.6% for 3h hydrolysis and 32.42% for 0.5 h hydrolysis). Inactivation of the trypsin inhibitors by heating processes (microwave, boiling, roasting, autoclaving) has been most often attributed to the partial denaturation of the trypsin inhibitor (Barać and Stanojević, 2005; Khattab and Arntfield, 2009; Yoshida and Kajimoto, 1988). However, alcalase was also proven as an efficient approach to reduce trypsin inhibitor activity (Dia et al., 2012). In addition, based on the DSC results in the present study, alcalase was more effective than micronization in denaturing the soybean protein (Table 4.3). As a result it is unlikely that inactivation of trypsin inhibitor due to alcalase hydrolysis can be simply explained by denaturation of the trypsin inhibitors. Other factors such as aggregation of the denatured peptides and existence of other food matrix (carbohydrate and lipids) may also need to be taken into consideration.

4.2.3.4 *Effects of micronization, ethanol washing, and alcalase hydrolysis in combination on trypsin inhibitor in soybean flour*

While the effects of the micronization (Hutton and Foxcroft, 1975; Khattab and Arntfield, 2009; Koroteeva et al., 2007; Kouzeh-Kanani et al., 1983; Yalcin and Basman, 2015; Žilić, Bozović and Hadži-Tašković Šukalović, 2012) and ethanol processes (Liu and Markakis, 1991; Tasneem and Subramanian, 1990; Tolman, 1995) on trypsin inhibitor activity have been well studied and effects of alcalase hydrolysis on trypsin inhibitor activity in soybean have also been reported (Dia et al., 2012), the combination of these processing effects on the trypsin inhibitor activity has not yet been studied.

Although the ethanol treatment did not reduce trypsin inhibitor activity in soybean flour in the present study, ethanol washed soybean flour has been proven to be superior in colour, foaming and gelling properties (Hua et al., 2005). More importantly, ethanol washing has resulted in improved flavour and odour for soybean flour by removing the lipid-like materials (Eldridge et al., 1963; Rackis, Sessa and Honig, 1979; Sessa and Rackis, 1977). Therefore, it is important to include the ethanol treatment along with other treatments when considering the overall implications for changes in trypsin inhibitor activity as was done in this study. Data have been summarized in Figure 4.6.

As discussed previously, micronization significantly decreased the trypsin inhibitor activity because of the denaturation of the trypsin inhibitor by heat. In contrast, ethanol washed soybean flour showed significantly higher trypsin inhibitor levels mainly due to a concentrating effect on trypsin inhibitor by removing the ethanol soluble materials. When ethanol at either 65% or 85% was used to treat soybean flour that had

been micronized at both 100°C and 135°C the concentrating effect seen with the ethanol treatment of the unmiconized sample was no longer evident; there were no significant changes in trypsin inhibitor activity in comparison to the micronized samples. Partial denaturation of the soybean protein caused by micronization (Tolman, 1995) may have increased the susceptibility of the trypsin inhibitor to ethanol denaturation. On the other hand, the soybean recovery rate increased (Table 4.2) when comparing ethanol treated micronized soybean flour (M100A65, M100A85 and M135A65, M135A85) with similarly treated unmiconized soybean flour (M0A65, M0A85), suggesting less ethanol soluble matrix was removed from micronized soybean flour during the ethanol treatments. Thus, the micronization heat treatment was the dominant factor responsible for the reduction in trypsin inhibitor activity.

In contrast, the effects of treating the flour with ethanol prior to alcalase hydrolysis was still evident for those samples which had not been micronized (Figure 4.6). With hydrolysis times of both 0.5 and 3 h, the level of trypsin inhibitors was higher in the ethanol treated samples compared to no ethanol treatment at comparable hydrolysis times, with no ethanol, and the amount of ethanol used did not significantly affect the results. This is not surprising as the ethanol treatment preceded alcalase hydrolysis and the concentrating effect noted above would likely occur. In addition, the influence of alcalase hydrolysis on the ethanol treated unmiconized samples was similar to that seen with alcalase alone; hydrolysis decreased the level of trypsin inhibitor activity with the time of hydrolysis not being a factor. To increase the utilization of soybean flour with respect to functionality, colour and odour, the combination of ethanol treatment and alcalase

hydrolysis may be preferred (Lee, Lee and Lee, 2001). Based on the present study, if an ethanol treatment was needed to improve properties such as colour for flour that had not been subjected to heat, doing it in combination with an alcalase treatment would help limit the level of trypsin inhibitor activity in the final product. In the current study, the combination of ethanol and alcalase produced materials in which the level of trypsin inhibitor activity was unchanged in comparison to the untreated soybean seeds. In the soybean oil extraction industry, enzymatic hydrolysis could be used as an additional treatment to ethanol extraction to prevent the increase of trypsin inhibitor in the soybean meal.

It has been suggested that the partial denaturation of the soybean proteins as a result of micronization would increase their susceptibility to enzymatic hydrolysis (Tolman, 1995). Therefore, alcalase hydrolysis following the micronization treatment was expected to further decrease trypsin inhibitor activity. However, in the present study micronized samples without ethanol treatment, the level of trypsin inhibitors increased as a result of the alcalase hydrolysis. With an increase in the hydrolysis time from 0.5 h to 3 h, the trypsin inhibitor activity also increased such that after 3 h of alcalase hydrolysis, the trypsin inhibitor activity in soybean that had been pre-treated only by micronization at 100°C and 135°C, significantly increased by 75% and 196%, respectively (Figure 4.6). Another interesting phenomenon was that after 3h of alcalase hydrolysis of micronized soybean at both 100°C and 135°C, the levels of trypsin inhibitor activity were comparable to those in the un-micronized soybean that had been hydrolyzed for 3 h. This indicated that alcalase hydrolysis for 3 h resulted in similar levels of trypsin inhibitor

activity regardless of the micronization effect. According to the previous studies (Barać and Stanojević, 2005; Khattab and Arntfield, 2009; Yoshida and Kajimoto, 1988), heat processing denatured the trypsin inhibitor. Roychaudhuri et al. (2003) also stated that the bioactivity of a protein was dependent on the maintenance of its native conformation. This was not always evident when heat processing was combined with alcalase hydrolysis possibly because of the greater effect of alcalase on protein structure. More studies are needed to better understand the principles by which alcalase hydrolysis influences trypsin inhibitors in soybean.

Micronization also influenced the way in which the alcalase and ethanol treatments interacted (Figure 4.6). For samples micronized at 100°C and then treated with 65% or 85% ethanol, further alcalase treatments (0.5 h or 3 h) did not significantly affect the trypsin inhibitor activity compared to the corresponding un-hydrolyzed sample. In addition, there was also no significant difference in trypsin inhibitor activity for the 0.5 and 3 h hydrolysis times. However, for samples micronized at 135°C with a 65% ethanol treatment, the trypsin inhibitor activity significantly increased during alcalase hydrolysis but there were no significant differences between these two hydrolysis times. Micronizing at 135°C with the 85% ethanol treatment also resulted in increased trypsin inhibitor activity as a result of alcalase hydrolysis; in this case, however, the level of trypsin inhibitor was significantly higher after 3 h hydrolysis in comparison to the 0.5h hydrolysis.

For all ethanol treated (65% or 85%) samples, with and without hydrolysis, the effect of micronization dominated the changes in trypsin inhibitor levels; for these

conditions, all levels of trypsin inhibitor activity were significantly lower than in the corresponding non-micronized samples. With the exception of the soybean flour treated with 85% ethanol and hydrolyzed for 3 h, the higher micronization temperature resulted in a greater decrease in trypsin inhibitor activity; values with 85% ethanol and 3h hydrolysis were not significantly different for the two micronization temperatures. In addition, the reduction in trypsin inhibitor activity for hydrolyzed samples due to micronization was greater when the ethanol treatment was included. Regardless of whether the flour had been subjected to micronization or ethanol treatment, hydrolysis with alcalase resulted in higher levels of trypsin inhibitor activity. Minimal levels of trypsin inhibitors, a desirable attribute when using soybean flour, were achieved by micronization at 135°C with no hydrolysis treatment; similar results were obtained with and without the ethanol treatment.

4.2.4 Effects of micronization, defatting, ethanol washing and enzymatic hydrolysis on soybean Lipoxygenase (LOX) activity

The lipoxygenase in soybean is of particular interest because lipoxygenase has been implicated as a major culprit contributing to the undesirable flavours in soybean based foods (Liu, 1997). Lipoxygenase catalyses the oxidation of the unsaturated fatty acid producing hydroperoxides which could further decompose to a series of compounds related to the beany flavour. In the present study, crude lipoxygenase activity was detected using emulsified linoleic acid as a substrate. One unit of lipoxygenase activity was defined as an increase of 0.1 in absorbance per min.

The results of the lipoxygenase activity analysis in defatted soybean are shown as Figure 4.7. Compared with the full fat soybean (Table 4.4), lipoxygenase activity was

much higher in the defatted soybean flour. This phenomenon has not been reported previously and the reason for the difference between the lipoxygenase activity in defatted and full fat soybean was unclear. The lipoxygenase activity in the defatted unmiconized soybean flour (DFM0) was 136.9 LOX/mg which was higher than the value (85.9 LOX/mg) reported by Sosulski and Gadan (1988) who used the same method for analysis. However, micronization at 100°C and 135°C achieved 51.4% (for DFM100) and 99.69% (for DFM135) reductions compared with DFM0. For the ethanol treated or alcalase hydrolyzed soybean flour, there was no lipoxygenase activity detected.

The present results of decreasing LOX by micronization were similar to what was reported by Žilić, Bozović and Hadži-Tašković Šukalović (2012) where micronizing the soybean at 100°C and 125°C for 90 s resulted in 51.3% and 95% reduction in LOX activity. Total inactivation of LOX activity was achieved when the soybeans were micronized at 150°C (Žilić, Bozović and Hadži-Tašković Šukalović, 2012). Der (2010) also reported that > 99.9% of lipoxygenase activity was inhibited after micronizing lentils at 130-135°C. All results from the present research and from the previously published literature indicated that micronization was a very efficient processing technique for inactivation of lipoxygenase activity (Der, 2010; Žilić, Bozović and Hadži-Tašković Šukalović, 2012). The present results also confirmed that the lipoxygenase was more heat labile than trypsin inhibitor in soybean flour as has been reported previously (Baker and Mustakas, 1973; Žilić, Bozović and Hadži-Tašković Šukalović, 2012)

Table 4.4 Effects of the micronization, defatting processing on lipoxygenase activity in soybean flour

Soybean flour	LOX/mg
FFM0	3.78 ± 1.03^a
FFM100	1.33 ± 0.13^b
FFM135	1.48 ± 0.069^b

Results are expressed as mean \pm standard deviation

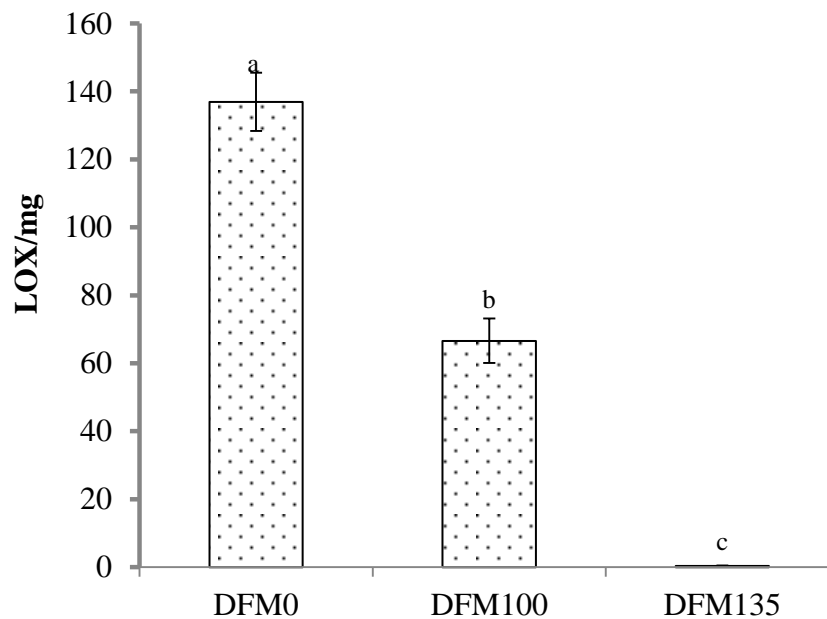
Same letter means not significantly different ($P < 0.05$)

FF: Full fat. M0/M100/M135: Unmicronized or micronization at 100°C or 135°C

Lipoxygenase activities was used as reference for that in the defatted soybean flour

Statistical analysis was run separately for full fat data and for defatted soybean data

Figure 4.7 Effects of the micronization on lipoxygenase activity in defatted soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

DF: Defatted. M0/M100/M135: Unmicronized or micronization at 100°C or 135°C

Data for ethanol treatment and alcalase hydrolysis are not include as no values obtained for LOX activity

Ethanol washing of defatted soybean flour resulted in a total inactivation of lipoxygenase activity. According to Sessa and Rackis (1977), 99% of lipoxygenase

activity in raw legumes was destroyed by ethanol treatment and ethanol treatment was not only used to extract flavour and lipids but also to destroy the enzymes which generate undesirable flavours. Different results were obtained by Borhan and Snyder (1979) who indicated that washing whole soybean with 80% ethanol for 2-6 h did not efficiently destroy the lipoxygenase activity. The difference may be due to the soybean materials used; defatted soybean flour was used in current study while whole soybeans were used by Borhan and Snyder (1979). The authors explained that the concentrated ethanol could not efficiently penetrate the soybean seed to denature lipoxygenase (Borhan and Snyder, 1979).

To our knowledge, researchers have rarely studied the effects of alcalase hydrolysis on lipoxygenase activity in soybean. In the present study, where the degree of hydrolysis ranged from 5.15% to 12.7% for the soybean hydrolysates (Figure 4.4), there was no lipoxygenase activity in these soybean hydrolysates. The results are in agreement with the research by Qi, Hettiarachchy and Kalapathy (1997) who investigated soybean protein isolate that had been hydrolyzed by pancreatin. Based on SDS-PAGE electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis) results, they concluded that the lipoxygenase fraction decreased as the degree of hydrolysis increased and was absent once 11% hydrolysis had been attained (Qi, Hettiarachchy and Kalapathy, 1997). Although different hydrolysis proteinases and lipoxygenase determination methods were used in comparison to the present study, a similar conclusion can be reached. Lipoxygenase could be completely destroyed by enzymatic hydrolysis.

4.3 Study 2: Effects of Micronization, Defatting, and Ethanol Washing on Soybean Flour Volatiles (related to soybean “beany” flavour)

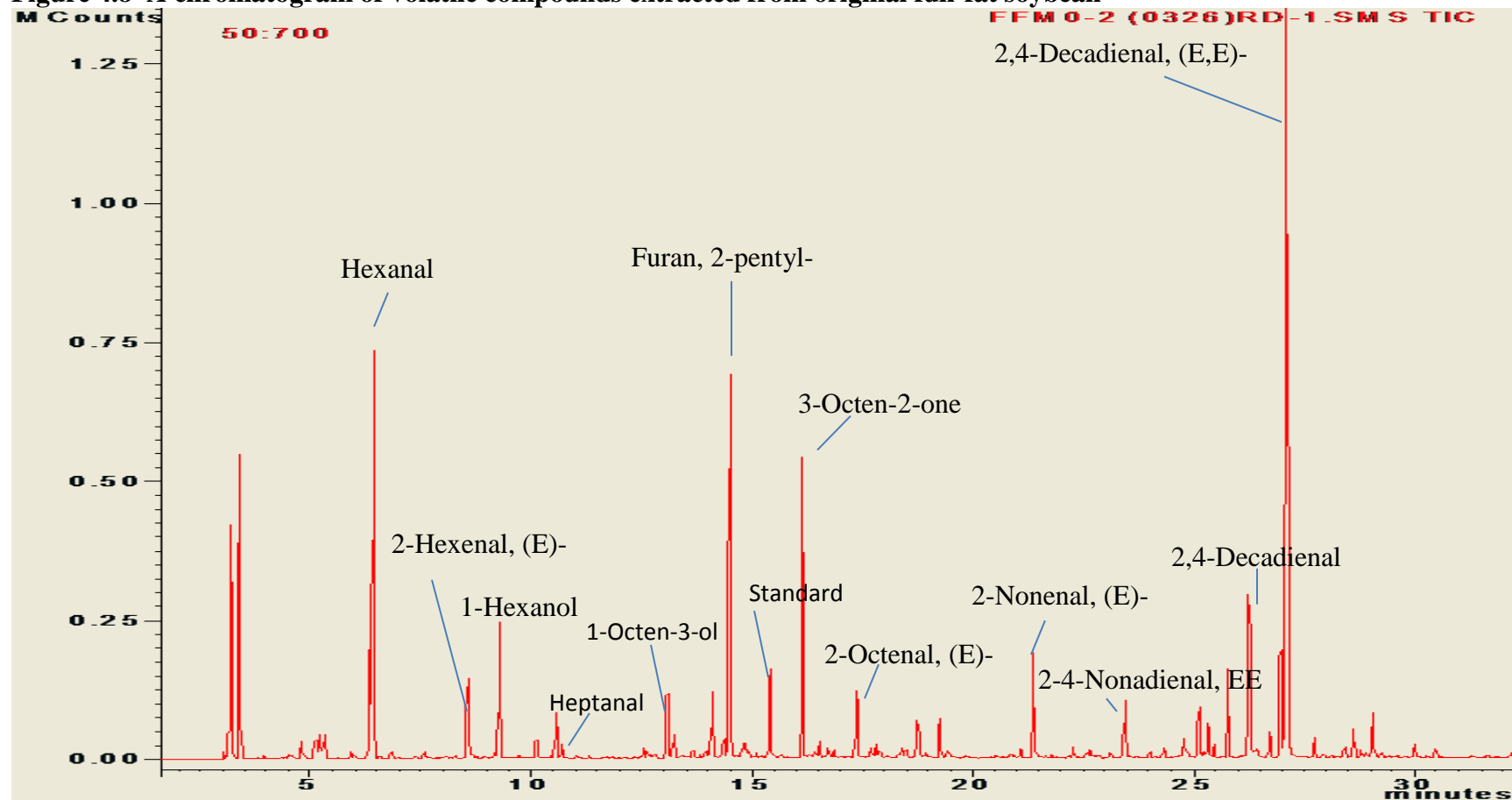
4.3.1 Soybean flour volatile analysis

In the present study, volatiles from soybean flour were extracted with pentane by using simultaneous distillation extraction (SDE) apparatus. Concentrated volatile extracts were analyzed by gas chromatograph-mass spectrometer (GC-MS) with a VF-5ms low bleed/MS fused-silica capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m× 0.25mm, Varian). Identification of volatile compounds was done by comparing the ion distribution and linear retention indices (LRI), calculated based on the LRI of alkane standards, with resources in NIST Library and literature. A semi-quantification technique based on the ratio of the compound main ion peak area to the internal standard (1,2-dichlorobenzene) main ion peak area was used to approximate the concentration of the volatiles in soybean flour.

As shown in Figure 4.8 and Table 4.5, 38 compounds were positively identified from the soybean flour extract including 6 alcohols, 6 ethers, 6 ketones, 14 aldehydes, and some diverse functional groups including phenols and carboxylic acids. Twelve of the compounds from the volatile extracts profile which were previously reported as “beany” flavour related compounds in literature were selected (Table 4.6) for further evaluation. The sensory attributes of these 12 selected compounds and their LRI values which were reported on the University of Reading website are as shown in Table 4.7. The mass spectra distribution for each compound in comparison with the mass spectra distribution of the known compounds reported in the NIST library are included in

Appendix 7. The volatile analysis results in the present research matched those obtained by Arai, Koyanagi and Fujimaki (1967), where a series of alcohols, aldehydes, and acids were also found. They identified the volatile compounds isopentanol, n-hexanol, and n-heptanol as those which were considered to give green bean-like odour (Arai, Koyanagi and Fujimaki, 1967). In the present research, of these three compounds, only n-hexanol was found. The compound ethyl vinyl ketone, which was reported as an important contributor to the green beany flavour by Mattick and Hand (1969), was not found in the present research either. This difference may have been due to the different volatile extraction methods used. Head space technique was used to deliver the volatile into the GC in Mattick and Hand's study (1969) while in the present study, simultaneous distillation extraction (SDE) was used.

Figure 4.8 A chromatogram of volatile compounds extracted from original full-fat soybean



12 selected “beany” flavour related volatiles are as shown

Standard: 1,2-dichlorobenzene

Table 4.5 Summary of possible compounds extracted from original full-fat soybean

Class	*Possible Compounds	Retention time (minutes)	LRI Calculated	NIST LRI
Ether	2-Ethylfuran	3.39	<800	676
	2-Propylfuran	5.93	<800	782
	2-pentylfuran	14.59	990	977
	(E)-2-(2-pentenyl)furan	14.85	998	984
	Spiro-1-(cyclohex-2-ene)- 2'-(5'-	40.20	1705	1772
	oxabicyclo[2.1.0]pentane), 1',4',2,6,6-pentamethyl- Phthalic acid, 6-ethyl-3- octyl butyl ester	45.30	1952	2505
Aldehyde	Hexanal	6.38	802	806
	2-Hexenal, (E)-	8.54	853	822
	Heptanal	10.68	903	882
	2-Heptenal, (E)-	13.08	958	942
	2-Octenal, (E)-	17.37	1059	1034
	4-Nonenal, (E)-	18.93	1098	1112
	2-Nonenal, (Z)-	20.85	1147	1125
	2,6-Nonadienal, (E,Z)-	21.08	1153	1125
	2-Nonenal, (E)-	21.36	1161	1133
	1,3-Cyclohexadiene-1- carboxaldehyde, 2,6,6- trimethyl-	22.79	1198	1173
	2-4-Nonadienal, EE	23.44	1216	1188
	2,4-Decadienal	26.24	1294	1270
	2,4-Decadienal, (E,E)-	27.08	1319	1291
	3,5-di-tert-Butyl-4- hydroxybenzaldehyde	40.19	1753	1772
Alcohol	1,4-Pentadien-3-ol	4.80	<800	661
	1-Hexanol	9.26	869	852
	1-Octen-3-ol	14.10	981	963
	3-Nonen-1-ol, (Z)-	19.25	1106	1134
	2-Cyclohexen-1-ol, 1- butyl-	25.32	1269	1211
	4,4,6-Trimethyl-cyclohex- 2-en-1-ol	27.719	1338	1085
Ketone	3-Octen-2-one	16.53	1039	1015
	Butan-2-one, 4-[pyrrolidin- 2-one-5-yl]-	24.01	1232	1258
	2-Buten-1-one, 1-(2,6,6-	29.07	1379	1361

	trimethyl-1,3-cyclohexadien-1-yl)-, (E)-4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	29.99	1406	1460
	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	31.67	1459	1143
	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	44.00	1899	1929
Phenol	2-Methoxy-4-vinylphenol	26.71	1308	1282
	Phenol, 2,4-bis(1,1-dimethylethyl)-	33.07	1504	1539
Carboxylic acids	Hexanoic acid	13.92	977	973
	Benzoic acid, 4-ethoxy-, ethyl	33.62	1522	1521
	n-Hexadecanoic acid	45.42	1957	1942
N-containing	Pyridine	4.577	<800	695

*possible compounds are identified by comparing target's ion distribution (not shown here) and LRI calculated with that as reported in NIST Library.

Table 4.6 List of compounds and their sensory attributions in soybean and soybean products as reported in published literature

Food Source	*Volatile compounds	Attributes	References
Raw soybean	Isopentanol ^{ab} n-Hexanol ^{ab} n-Heptanol ^{ab} Ethyl vinyl ketone ^c	Green beany-like	a. Arai, Koyanagi and Fujimaki (1967); (Hsieh, Huang and Chang, 1982) b. Fujimaki et al. (1965) c. Mattick and Hand (1969)
Defatted soybean flakes	Ethyl vinyl ketone ^a 2-Pentulfuran ^a Pentanol ^a Hexanol ^a 1-Octen-3ol ^a Hexanal ^{ab}	Beany, grassy and green odour	a. Hsieh, Huang and Chang (1982) b. Fujimaki et al. (1965)
Soybean protein isolates	Dimethyl trisulphide ^a Trans-trans-2-4-decadienal ^a 2-Pentylfuran ^{ac} Trans-trans-2,4-nonadinal ^a Hexanal ^{ac} (Samoto et al., 1998) Acetophenone ^a 1-Octen-3-one ^b 1-Penten-3-ol ^c (E)-2-Hexenal ^c 1-Pentanol ^c Hexanol ^c Nonanal ^c 1-Octen-3-ol ^c	Beany odour	a. Boatright and Lei, (1999) b. Wu et al. (2011) c. Samoto et al. (1998)
Soybean milk	Hexanal ^{abc} Hexanol ^{abc} 2-Hexanal ^{ab} 1-Octen-3-ol ^{ab} Ethyl vinyl ketone ^a 2-Pentylfuran ^{ac} Trans-2-nonenal ^{bc} Trans-2,trans-4-decadienal ^{bc} 1-Octen-3-one ^c (E,E)-2,4-Nonadienal ^{ac}	Green beany flavour	a. Wilkens and Lin (1970) b. Yuan and Chang (2007) c. Zhang et al. (2012)
Soybean oil	2-Pentylfuran ^{ab} Hexanal ^{ab} Cis-3-hexenal ^{ab} Trans-3-hexenal ^{ab} Ethyl vinyl ketone ^b	Beany, grass flavour	a. Chang et al. (1966) b. Sessa and Rackis (1977)

*volatiles with same letter in the same row means they are reported in the same previously published literature

Table 4.7 Selected volatiles correlated to soybean beany flavour and their attributes

Volatile compounds	Attributes	Class	Retention time (min)	LRI (Calculated)	University of Reading LRI*	Main ion selected*
Hexanal	Green grass	Aldehyde	6.387	802	795-802	67
2-Hexenal, (E)-	Green	Aldehyde	8.547	853	848-862	83
1-Hexanol		Alcohol	9.264	869	865	56
Heptanal	Green fatty oily	Aldehyde	10.686	903	896-902	55
1-Octen-3-ol	Mushroom grass fruity	Alcohol	14.097	981	978-988	57
Furan, 2-pentyl-	Fruity green pungent	Furan (Ether)	14.486	990	989-995	81
3-Octen-2-one	Spicy fatty green fruit	Ketone	16.532	1039	1040	55
2-Octenal, (E)-	Fatty green nutty	Aldehyde	17.367	1059	1059-1062	55
2-Nonenal, (E)-	Fatty green paper	Aldehyde	21.367	1161	1160-1162	55
2-4-Nonadienal, (E,E)-	Fatty oily	Aldehyde	23.442	1216	993-1222	81
2,4-Decadienal		Aldehyde	26.242	1294	1295	81
2,4-Decadienal, (E,E)-	Fatty oily wax	Aldehyde	27.081	1319	1295-1322	81

*University of Reading LRI: Previously reported LRI for each volatile compound from <http://www.odour.org.uk/lriindex.html> as compared to LRI (calculated) was used for confirming the identity of volatile compounds

*Main ion: the core ion selected from the mass spectrum of each volatile compounds for semi-quantification

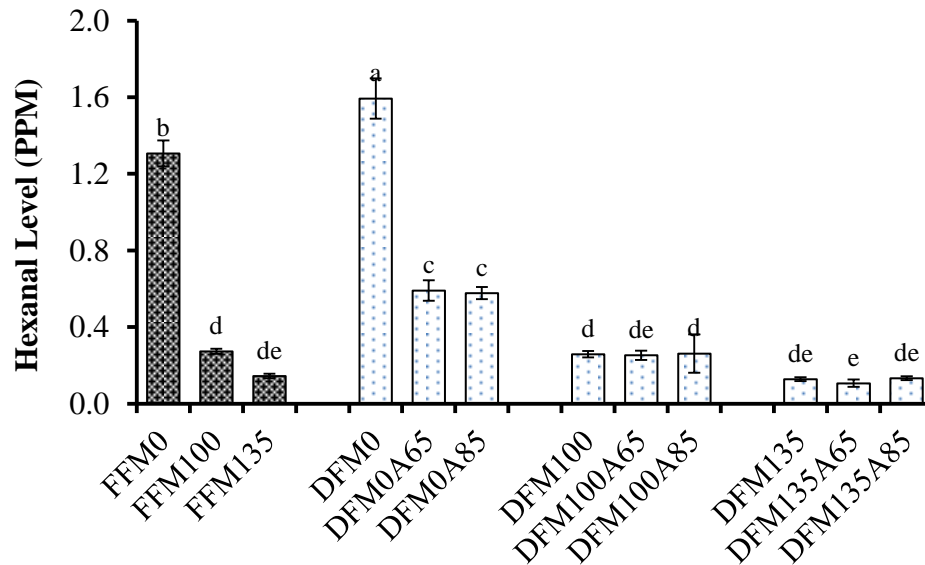
As shown in Table 4.5, the majority of previously published research focused on identification of the volatile compounds in the soybean and soybean products such as soybean protein, soybean milk and soybean oil. Few of them looked at the effects of the thermal treatments on the profile of volatiles in soybean milk (Lv et al., 2011; Zhang et al., 2012). None of the previously published research investigated the effects of micronization on the profile of soybean volatiles. In one study, soybean protein products isolated from ethanol treated soybean flasks revealed better odour scores than products that were treated with ethanol (Baker, Mustakas and Warner, 1979; Hua et al., 2005; Rackis, Sessa and Honig, 1979; Samoto et al., 1998; Wu et al., 2011). Effects of micronization and ethanol treatments in combination on volatiles in soybean flour has not been reported previously. In the current study, soybean samples treated with micronization and ethanol individually or in combination were used to investigate the volatiles in soybean. The effects of enzymatic hydrolysis on “beany” related volatiles in soybean were not investigated because there was no evidence to suggest enzymatic hydrolysis was reducing beany flavour volatiles. In contrast, Fujimaki et al. (1968) revealed that 12 individual proteolytic enzymes increased beany flavour in soybean protein hydrolyses significantly. In some cases, the intensity of bitterness was also increased due to generation of low molecular peptides. As the aim was to reduce the beany flavour, this did not seem to be a viable option. A summary table showing all the levels of selected “beany” related volatile compounds from soybean which were treated with different processing methods were compared with those from original soybean flour (Appendix 8). Differences will be discussed for individual beany volatiles.

4.3.2 Effect of micronization, defatting, and ethanol washing on volatiles generated by lipoxygenase oxidation of unsaturated fatty acid in soybean flour

4.3.2.1 Effect of micronization, defatting, and ethanol washing, on hexanal in soybean flour

Hexanal exists in soybean and soybean products such as soybean flakes, soybean protein isolates, soybean milk and soybean oil (Boatright and Lei, 1999; Fujimaki et al., 1965; Hsieh, Huang and Chang, 1982; Samoto et al., 1998; Wilkens and Lin, 1970; Zhang et al., 2012). Hexanal is a product of the lipid oxidation of linoleic acid and it has been positively correlated with the lipoxygenase content in soybean (Lumen et al., 1978; Matsui et al., 2003; Yuan et al., 2008). It was determined to be the major volatile compound, and was described as a compound with a low threshold (0.1 ppm) responsible for the “green-beany” flavour in soybean (Larsen, Poll and Olsen, 1992; Wilkens and Lin, 1970).

Figure 4.9 Effects of micronization, defatting, and ethanol treatment on hexanal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Unmicronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Effects of micronization, defatting and ethanol treatment on hexanal are shown in Figure 4.9. In the absence of the ethanol treatment, micronization was an efficient process for reducing hexanal content. Reductions in hexanal concentration of 79.1% and 88.9% were achieved when soybean was micronized at 100°C (FFM100) and 135°C (FFM135), respectively, compared to the unmiconized soybean (FFMO). This decreased hexanal level was likely due to the significant reduction in lipoxygenase activity as a result of micronization which meant less hexanal was produced during soybean processing (dehulling and milling). To the best of our knowledge, no study previously investigated the effects of micronization on soybean flour volatiles. However, Lv et al. (2011) revealed that hot water blanching and grinding at temperatures above 80°C for 2-6 min achieved ~98% reduction in the hexanal level as a result of inactivation of the lipoxygenase. Similar results were also found by Zhang et al. (2012) who investigated the grinding and heating effects on volatiles in soymilk and found a significant reduction (83% - 97% reduction) of hexanal was achieved by hot grinding and cooking (Zhang et al., 2012). Shariati-Levari (2013) investigated micronization effects on volatiles in chickpea and lentil flours. About 40% of the hexanal was reduced by micronizing chickpeas at 130°C but no reduction of hexanal was found by micronizing green lentil at 130°C (Shariati-Levari, 2013). Difference in the micronization effects in the present study (88.9% reduction at 135°C) compared to those of Shariati-Levari (2013) include the moisture content (tempering vs no tempering, respectively) during micronization and type of legume (soybean vs pea and lentil, respectively) processed. The effects of micronization on the physicochemical properties have been shown to depend on the seeds, moisture content, micronization temperature and time (Sharma, 2009).

Interestingly, the hexanal concentration significantly increased (22%) during defatting with hexane (DFM0). However, the defatting process did not have an effect on hexanal for samples which had been micronized (DFM100 compared to FFM100 and DFM135 compared to FFM135). Defatting combined with micronization significantly reduced the hexanal level in DFM100 and DFM135 in comparison to the full fat, unm micronized sample (FFM0). The reduction in hexanal levels was most likely due to inactivation of lipoxygenase by micronization.

The ethanol treatments of the defatted soybean (DFM0A65 or DFM0A85) significantly reduced (~63% reduction) the level of hexanal in comparison to that level in the sample that was only defatted (DFM0). This may be attributed to total inactivation of lipoxygenase activity in ethanol treated soybean as compared to the high lipoxygenase activity in DFM0. Unlike the ethanol washing effects on DFM0, the level of the hexanal remained unchanged when defatted micronized soybeans (DFM100 and DFM135) were treated with either 65% ethanol or 85% ethanol. However, the lowest hexanal concentration (0.107 ppm) was achieved by soybean that underwent micronization at 135°C, and were defatted and treated with ethanol at 65% (DFM135A65). The present results are in agreement with the results of Hua et al. (2005) who investigated the volatiles in soybean protein isolate prepared from the aqueous alcohol washed soy flakes. The peak area for hexanal from soybean protein isolates from flour treated with 85% ethanol was 10 times lower than that from soybean protein isolates from non-ethanol treated defatted flour (Hua et al., 2005). Similar results were also found by Wu et al. (2011). The protein isolates prepared from defatted soybean flour treated with 65%

ethanol at 50°C resulted in a 66% decrease in hexanal content in comparison with protein isolated from raw defatted flour. The improvement in the flavour of soybean isolated from ethanol treated flour was attributed to the decrease in the hexanal content (Hua et al., 2005; Wu et al., 2011).

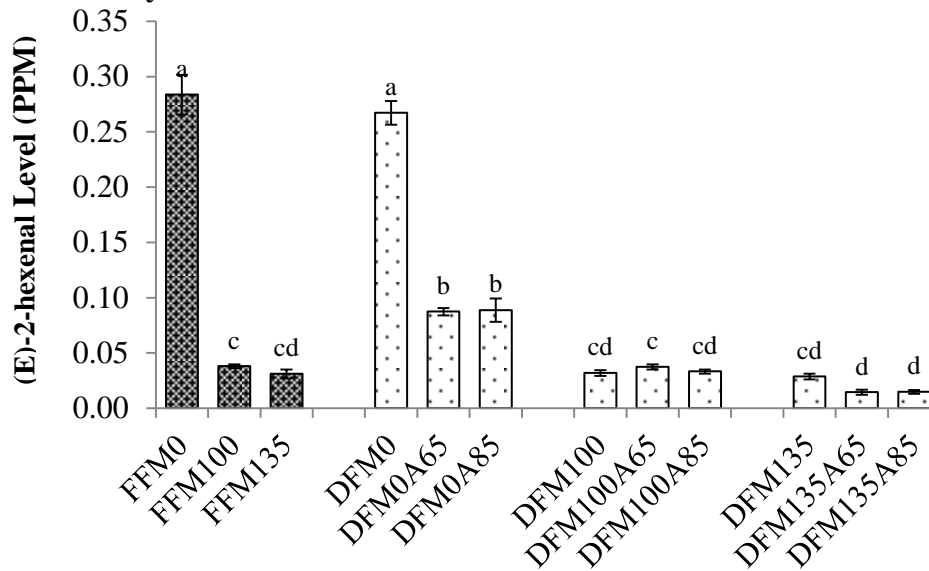
4.3.2.2 *Effect of micronization, defatting, ethanol washing, on (E)-2-hexenal in soybean flour*

Despite the fact that both (E)-2-hexenal and hexanal are from the oxidation of polyunsaturated fatty acids and have the same detection threshold (0.1 ppm) (Larsen, Poll and Olsen, 1992; Lumen et al., 1978; Samoto et al., 1998), (E)-2-hexenal has not been as well studied as hexanal. Only a few studies investigated (E)-2-hexenal as a “beany” flavour contributor in soybean (Samoto et al., 1998). (E)-2-hexenal was not considered as a “beany” aroma because it did not exhibit any “beany” characteristic when tested as a single aroma at any concentration (Vara-Ubol, Chambers and Chambers, 2004). It was instead described as a “green/grass, cherry, almond” aroma (Vara-Ubol, Chambers and Chambers, 2004). However, it has been reported that (E)-2-hexenal together with non “beany” compounds such as (E)-2-octenal could exhibit a “beany” odour (Bott and Chambers, 2006).

Effects of micronization, defatting and ethanol treatment on (E)-2-hexenal are shown in Figure 4.10. The concentration of (E)-2-hexenal (0.248 ppm) was low compared with hexanal (1.3 ppm) in FFM0. However, the effects of the micronization were very similar. Micronization was an efficient process for reducing (E)-2-hexenal content with 86.6% and 89.1% reductions of (E)-2-hexenal concentration for FFM100

and FFM135, respectively, compared to that from the full fat non-micronized sample (FFMO). As was the case with hexanal this was likely due to the significant reduction in lipoxygenase activity by micronization. However, there was no significant difference between soybeans micronized at two different temperatures.

Figure 4.10 Effects of micronization, defatting, and ethanol treatment on (E)-2-hexenal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Unmicronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Despite the defatting process not affecting the level of (E)-2-hexenal (Figure 4.10), ethanol treatment at 65% and 85% of defatted unmicronized soybean flour (DFMO) significantly decreased the levels of (E)-2-hexenal (~68% reduction). This may be due to the inactivation of lipoxygenase activity and removal of residual lipid by the ethanol treatment. It has been reported that removal of the oil-body-associated protein and polar lipids from the soybean protein isolate results in complete removal of (E)-2-hexenal (Samoto et al., 1998). As for soybean flour micronized at 100°C or 135°C (DFM100 and

DFM135), further ethanol treatments did not change the (E)-2-hexenal level. All treatments except FFM0 and DFM0 reduced the level of (E)-2-hexenal to below its threshold level (0.1 ppm).

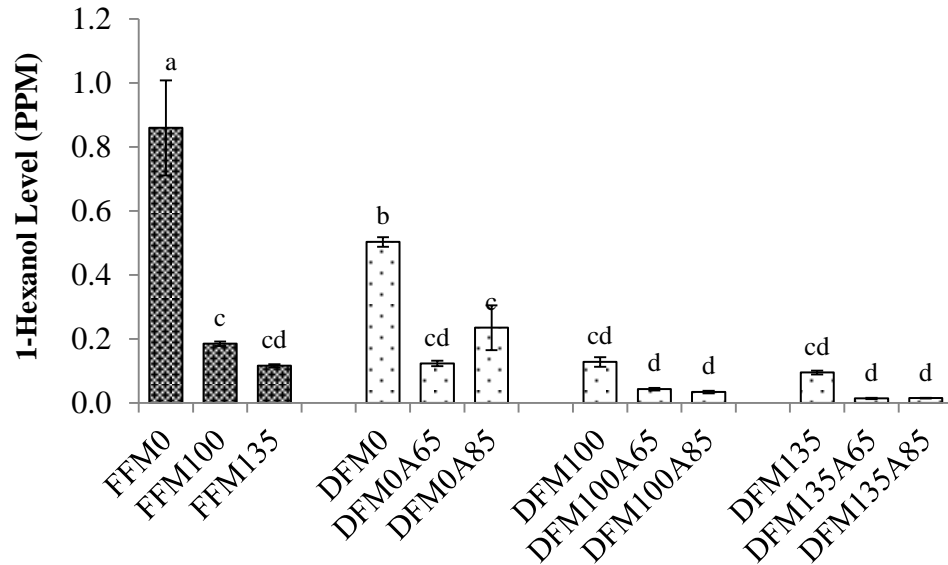
4.3.2.3 *Effect of micronization, defatting, and ethanol washing, on 1-hexanol in soybean flour*

Hexanol is a secondary product derived from the oxidation of linoleic acid (Lumen et al., 1978; Zhang et al., 2012). Hexanol is considered as a contributor to the off flavour in most soybean products such as soybean, soybean flakes, soybean protein and soybean milk (Arai, Koyanagi and Fujimaki, 1967; Fujimaki et al., 1965; Wilkens and Lin, 1970; Yuan and Chang, 2007; Zhang et al., 2012).

Effects of micronization, defatting and ethanol treatments on hexanol are shown in Figure 4.11. After micronizing full fat soybean seeds at 100°C and 135°C, a significant reduction in hexanol was observed (79.33% and 86.50% reductions, respectively). Results indicated that micronization was an efficient processing for reducing hexanol. The same conclusion was reached by Shariati-Levari (2013), who found 31.12% and 73.75% less hexanol due to micronization of chickpea at 130°C and 150°C, respectively. However, a 92.9% reduction in hexanol was achieved by micronizing lentils at 150° (Shariati-Levari., 2013). Current results showed no significant difference in hexanol levels for soybeans micronized at 100°C and 135°C. Higher surface temperatures such as 150°C for micronization may be required to achieve a better reduction of hexanol. However, as can be seen in Figure 4.11, the highest concentration of hexanol (0.895 ppm)

among the treated soybean samples was lower than its detection threshold (2.5 ppm) reported by Belitz, Grosch and Schieberle (2009).

Figure 4.11 Effects of micronization, defatting, and ethanol treatment on 1-hexanol in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Unmicronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Unlike hexanal and (E)-2-hexenal, defatting soybean flour (DFM0) significantly reduced the hexanol level by 58.56%. It is most likely that hexanol was removed by hexane. However, the defatting process did not affect the level of hexanol in micronized samples (DFM100 and DFM135 in comparison to FFM100 and FFM135 respectively).

Ethanol treatment of defatted unmicronized soybean flour (DFM0) also significantly decreased the level of the hexanol. This may due to the removal of residual lipid and hexanol during the ethanol washing. Samoto et al. (1998) reported that after

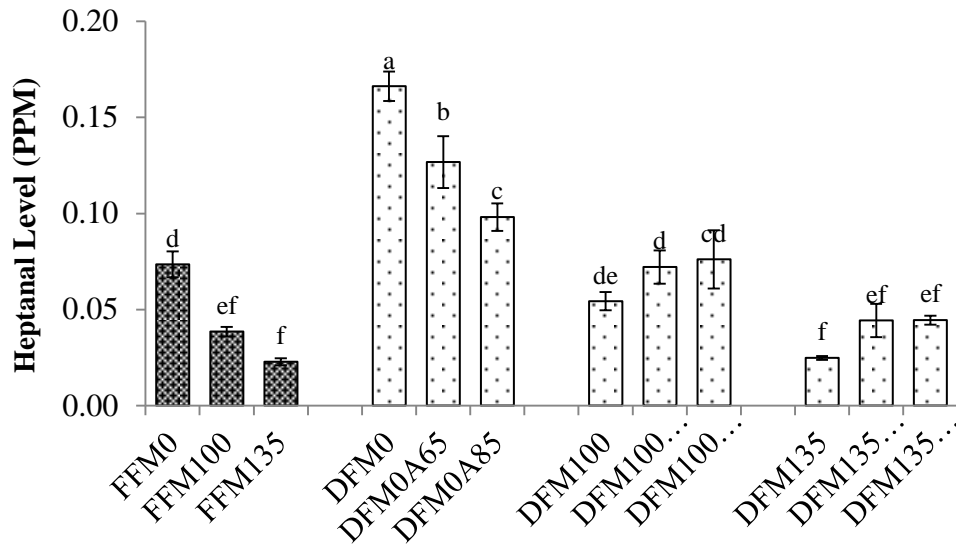
removing the oil-body-associated protein and polar lipids from the soybean protein isolate, hexanol was completely removed. Total removal of the hexanol was not achieved in the present study possibly due to association of hexanol with the denatured soybean protein through hydrophobic interactions (Maga, 1973; Sessa and Rackis, 1977). Further ethanol treatment of defatted soybean flour (DFM100 and DFM135) did not change the level of hexanol.

4.3.2.4 *Effect of micronization, defatting, and ethanol washing on heptanal in soybean flour*

Like hexanal, heptanal is produced by enzymatic or auto-oxidation of polyunsaturated fatty acids in food legumes and is considered as a “grass-beany” flavour contributor (Blagden and Gilliland, 2005; Sessa and Rackis, 1977; Shariati-Levari, 2013; Wsowicz et al., 2004). The odour associated with heptanal has also been described as “green leaf, fatty” and has a very low threshold of 31 ppb (Roh et al., 2006).

After micronizing full fat soybean seeds at 100°C and 135°C, significant reductions in heptanal content of 46.6% (FFM100) and 64.5% (FFM135) were observed (Figure 4.12). Heptanal levels for the two micronization temperatures were not significantly different. Shariati-Levari (2013) also found a significant decrease (37%) in heptanal after micronizing chickpea at 130°C and 150°C in comparison to the raw chickpea. The reduced heptanal level was positively related to decreased lipoxygenase activity. This is not surprising because heptanal is formed from linoleic acid by catalytic action of the enzyme lipoxygenase (Wilkins and Lin, 1970).

Figure 4.12 Effects of micronization, defatting, and ethanol treatment on heptanal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Effects of the defatting process turned out to be very interesting. After defatting, the concentration of heptanal increased from 0.039 ppm (FFM0) to 0.166 ppm (DFM0). However, there was no significant change in the heptanal as a result of defatting the micronized samples (DFM100 and DFM135). Again results reflected the lipoxygenase results in the present study in that with the decrease of the lipoxygenase activity from DFM0 to DFM100 to DFM135 (Figure 4.7), the level of the heptanal decreased accordingly. These results confirmed that the generation of heptanal was largely due to lipoxygenase activity in soybean (Zhang et al., 2012).

Only 23.5% and 33.6% of the heptanal were removed after washing the defatted soybean flour with 65% and 85% ethanol. Even after the 85% ethanol washing, the level

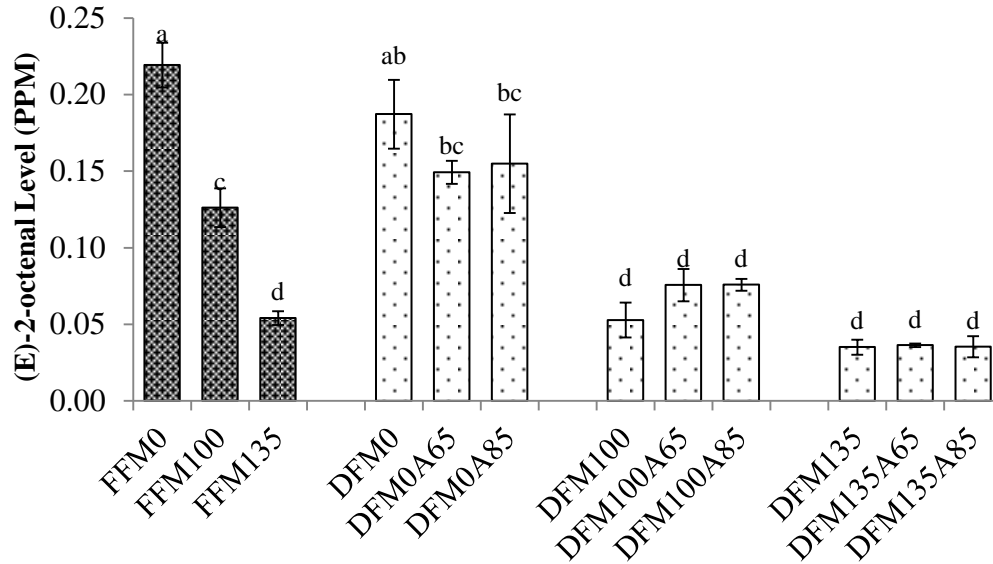
of the heptanal was still significantly higher than in the untreated full fat soybean (FFM0) (Figure 4.12). Treatment of the micronized defatted samples with ethanol had no effect on heptanal levels. However the levels of heptanal in the ethanol treated samples that had been micronized at 100°C (DFM100A65 and DFM100A85) were higher than that in the fat free micronized sample (FFM100). When micronized at 135°C, the ethanol treatments (DFM135A65 and DFM135A85) did not change the level of the heptanal level in comparison to the full fat sample micronized at this temperature (FFM135). Hua et al. (2005) and Wu et al. (2011) also investigated the volatile profile of the soybean protein isolate prepared from the ethanol washed defatted soybean flake. Neither of them found heptanal in their volatiles. This may be due to the different volatile extraction method used where headspace solid-phase micro-extraction (HS-SPME) was used in studies by Hua et al. (2005) and Wu et al. (2011) while simultaneous distillation extraction (SDE) was used in the present study. SDE has been proven to be 10 times more sensitive in detecting the heptanal compared with HS-SPME due to the exhaustive extraction of volatile compounds in SDE (Madruga et al., 2009). To our knowledge, there is no previous research studying the volatile profile in defatted soybean flour by using SDE. The reason for the inability of ethanol to remove heptanal from the micronized soybeans is unclear; however, it was possible that heptanal combines with the denatured soybean protein which makes it hard to remove by ethanol washing.

4.3.2.5 *Effect of micronization, defatting and ethanol washing on (E)-2-octenal in soybean flour*

(E)-2-Octenal has been described as a “musty, brown spicy” aroma that does not exhibit beany characteristic when examined alone; it has a low detection threshold of 0.003 ppm (Hoffmann, 1962; Maga, 1981; Vara-Ubol, Chambers and Chambers, 2004). However, when it was combined with some other “beany” compounds such as 1-octane-3-one or 3-methyl-1-butanol it intensified the “beany” attribute of those compounds (Bott and Chambers, 2006). (E)-2-Octenal was reported as the secondary product from lipoxygenase induced oxidation of linoleic acid by Choe and Min (2006) and Kobayashi et al. (1995).

As for (E)-2-octenal level in the full fat soybean flour, micronization significantly reduced its level from 0.219 ppm (FFM0) to 0.126 ppm (FFM100) and 0.054 ppm (FFM135) for micronization temperatures of 100°C and 135°C, respectively (Figure 4.13). The defatting process did not affect the level of (E)-2-octenal of the unmicronized flour (DFM0) or the flour that was micronized to 135°C (DFM135). The (E)-2-Octenal level significantly decreased after defatting the soybean flour micronized at 100°C (FFM100). However, micronized defatted samples (DFM100 and DFM135) had significantly reduced (E)-2-octenal levels compared to the defatted sample that was not micronized (DFM0). This could be attributed to inactivation of the lipoxygenase activity by micronization processing.

Figure 4.13 Effects of micronization, defatting, and ethanol treatment on (E)-2-octenal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Despite the fact that the defatting process did not reduce the (E)-2-octenal level, further ethanol treatment on defatted soybean (M0) significantly decreased the (E)-2-octenal level as compared with FFM0. However the removal of (E)-2-octenal by ethanol washing was not considered as efficient. Ethanol treatment of DFM100 and DFM135 did not have a significant effect on (E)-2-octenal concentration. This could be due to the association of (E)-2-octenal with the denatured soybean protein through hydrophobic interactions which would minimize the effects of ethanol removal of (E)-2-octenal (Sessa and Rackis, 1977).

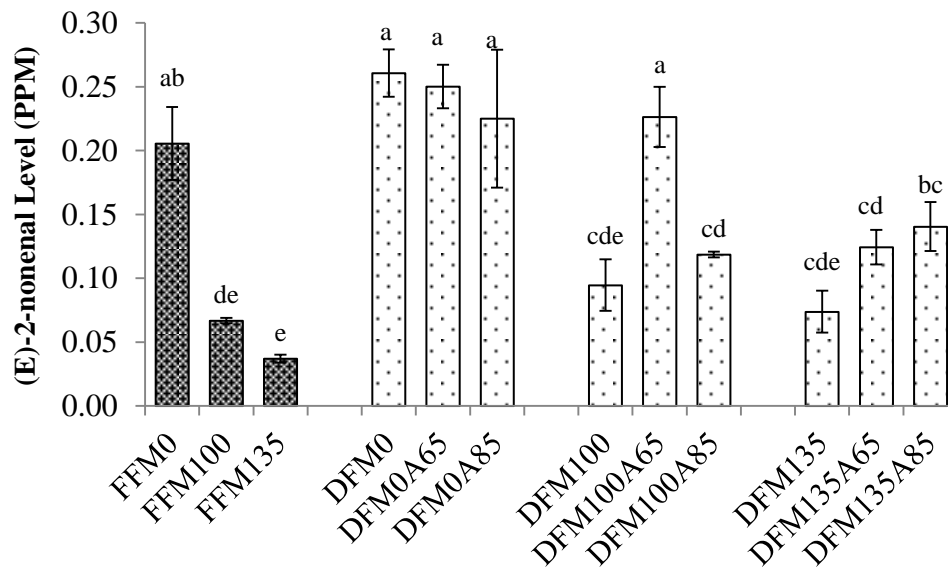
4.3.2.6 *Effect of micronization, defatting, and ethanol washing on (E)-2-nonenal in soybean flour*

(E)-2-Nonenal has been reported to have a cooked carrot odour with a very low threshold of 0.08 ppb (Zhang et al., 2012). However, in the study by Vara-Ubol, Chambers and Chambers (2004), it was described as a “green-grass, musty, and chemical like” odour. In addition, when combining (E)-2-nonenal with other non beany volatiles such as hexanal it has been shown to induce a beany odour (Bott and Chambers, 2006). Yuan and Chang (2007) and Matsui et al. (2003) suggested lipoxygenase was involved in the formation of (E)-2-nonenal.

Micronization significantly reduced the level of the (E)-2-nonenal concentration in full fat soybean. After micronizing soybean seeds at 100°C and 135°C, 67.5% (FFM100) and 82.0% (FFM135) reductions of (E)-2-nonenal, respectively, were achieved; there was no significant difference between levels in FFM100 and FFM135 (Figure 4.14). Zhang et al. (2012) revealed that hot grinding produced low levels of (E)-2-nonenal in soy milk compared to normal grinding and concluded that inactivation of lipoxygenase activity could partly restrict the formation of (E)-2-nonenal.

Defatting did not change the level of the (E)-2-nonenal level in the defatted soybean flour. Also, ethanol treatments (65% and 85%) did not change the level of (E)-2-Nonenal as compared to their defatted soybean flour with no ethanol treatment (DFM0, DFM100 and DFM135). The one exception was for the sample micronized at 100°C and treated with 65% ethanol (DFM100A65) where an increase (E)-2-nonenal level was noted.

Figure 4.14 Effects of micronization, defatting, and ethanol treatment on (E)-2-nonenal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

4.3.2.7 *Effect of micronization, defatting, and ethanol washing on (E, E)-2-4- nonadienal in soybean flour*

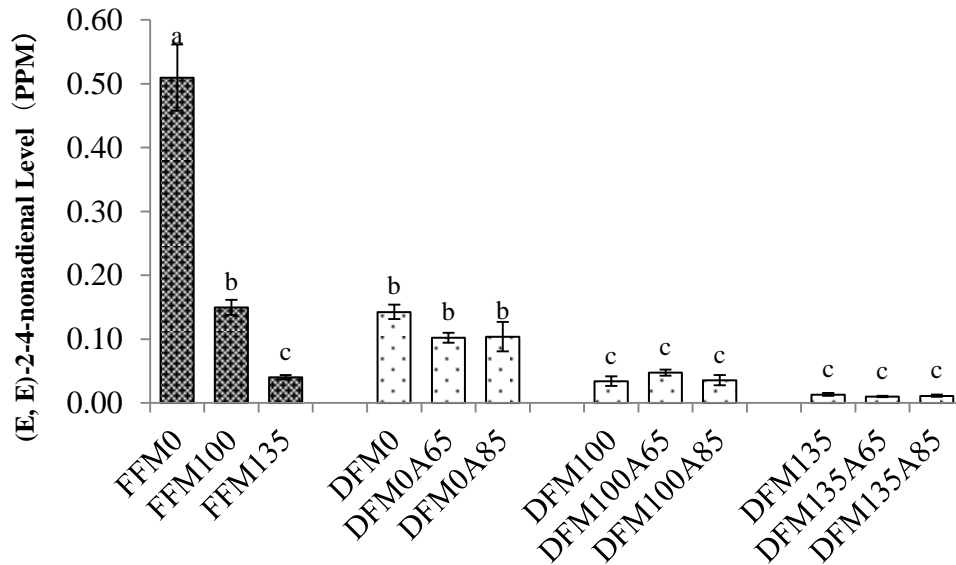
(E, E)-2-4-Nonadienal has been reported to have a beany note with a very low threshold of 0.09 ppb (Zhang et al., 2012). However, in the study by Vara-Ubol, Chambers and Chambers (2004), it had been described as a “heated oil, green-grass, sweaty, and chemical like” odour. Similar to (E)-2-Nonenal, combining (E, E)-2-4-nonadienal with other non beany volatiles such as hexanal or beany volatiles such as 1-octan-3-one could induce a beany odour or enhance the beany flavour attribute of that compound (Bott and Chambers, 2006). Zhang et al. (2012) and Kobayashi et al. (1995)

implied that lipoxygenase activity played a very important role in the formation of (E, E)-2-4-nonadienal.

Micronization significantly decreased the level of (E, E)-2-4-nonadienal in the full fat soybean (FF0), with a greater reduction for micronization at 135°C (FFM135) than at 100°C (FFM100) (Figure 4.15). Defatting with the solvent hexane significantly reduced the level of (E, E)-2-4-nonadienal in the non-micronized soybean (DFM0) and soybean micronized at 100°C (DFM100). However, defatting did not change the level of (E, E)-2-4-nonadienal following micronization at 135°C (DFM135). Both micronization treatments (DFM100 and DFM135) achieved a significantly lower (E, E)-2-4-nonadienal concentrations than the defatted unmicronized material (DFM0). The reduction in the (E, E)-2-4-nonadienal reflected the lower lipoxygenase activity in the micronized soybean samples. The current results are in agreement with those of Zhang et al. (2012) who revealed that hot grinding decreased lipoxygenase activity resulting in a lower (E, E)-2-4-nonadienal level in comparison to soybean milk prepared with normal grinding.

Ethanol treatments at 65% and 85% had no effect on (E, E)-2-4-nonadienal level for all levels of micronization. The low levels of (E, E)-2-4-nonadienal for samples that had been micronized and treated with ethanol may due to interactions between the volatile and denatured soybean.

Figure 4.15 Effects of micronization, defatting, and ethanol treatment on (E, E)-2-4-nonadienal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

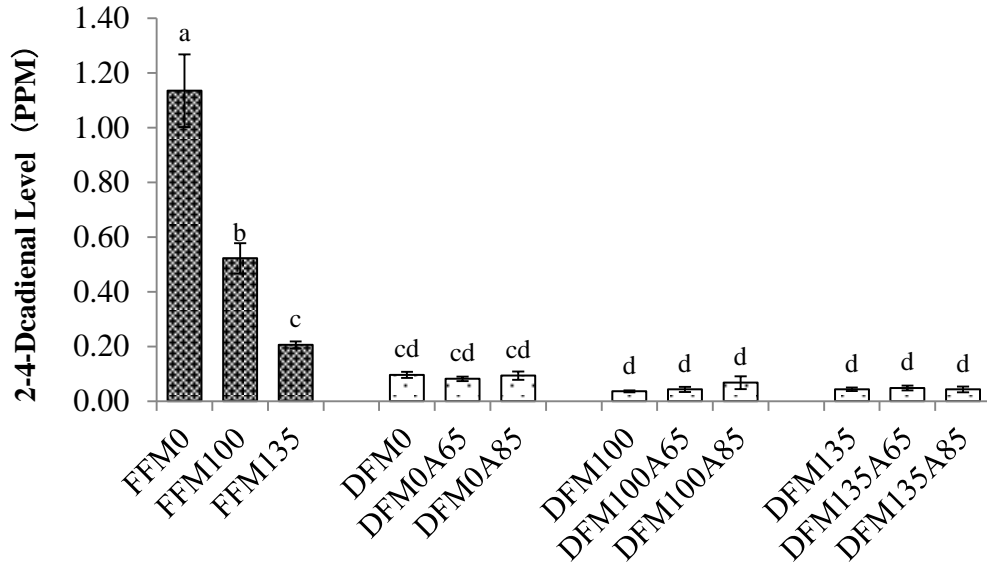
4.3.2.8 *Effect of micronization, defatting, and ethanol washing on 2-4- decadienal in soybean flour*

2-4-Decadienal has been reported as a “beany” odour contributor in the research by Shariati-Levari (2013) and Sessa and Rackis (1977). Like heptanal, hexanal, and 2-octanal, 2-4-decadienal is believed to be a product of the catalytic action of lipoxygenase on linoleic acid (Wilkins and Lin, 1970).

Micronization significantly decreased the level of 2-4-decadienal in full fat soybean (FFM0), with lower levels at the higher micronization temperature (FFM135) (Figure 4.16). Shariati-Levari (2013) also found a significant 70% decrease in 2-4-decadienal after micronization of chickpea at 150°C in comparison to raw chickpea. The

inhibition of the 2-4-decadienal was positively related to the decrease in lipoxygenase activity as would be expected since 2-4-decadienal is formed by the catalytic action of the enzyme lipoxygenase on linoleic acid (Wilkins and Lin, 1970).

Figure 4.16 Effects of micronization, defatting, and ethanol treatment on 2-4-decadienal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Defatting significantly reduced the level of (E, E)-2-4decadienal for both non-micronized and micronized samples (FFM0, FFM100 and FFM135). The results indicated that the 2-4-decadienal was effectively removed by hexane. Further treatments with ethanol (65% and 85%) did not reveal any additional changes in 2-4-decadienal level; the concentration of 2-4-decadienal in the ethanol treated samples remained very low compared to the full fat soybean (FFM0). The residual 2-4-decadienal may have interacted with the soybean protein making it difficult to remove.

4.3.2.9 *Effect of micronization, defatting, and ethanol washing on (E, E)-2-4-decadienal in soybean flour*

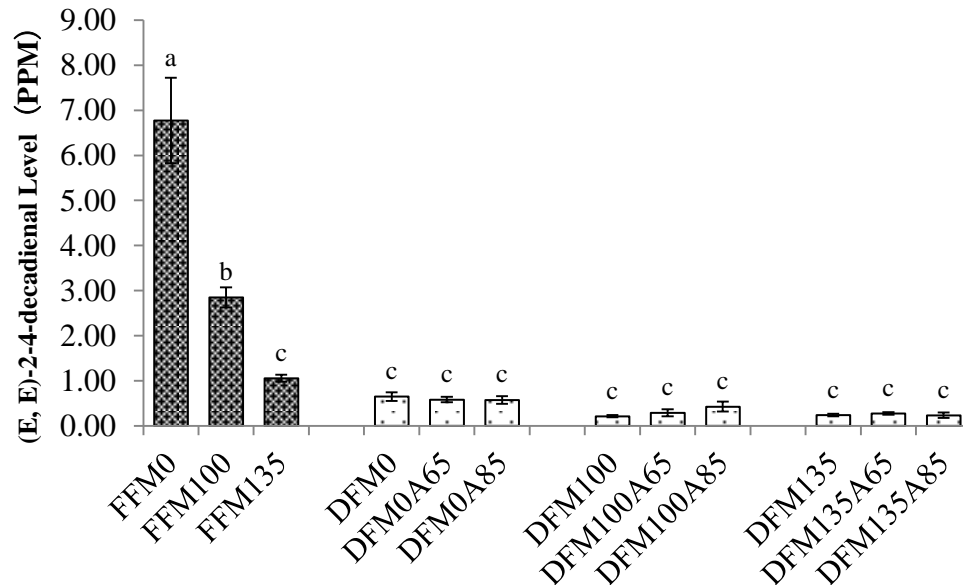
(E,E)-2-4-decadienal has been described as a fried fatty odour with a detection threshold of 180 ppb (Belitz, Grosch and Schieberle, 2009). Vara-Ubol, Chambers and Chambers (2004) reported it as being a “heated oil, floral” odour without any beany characteristic; however, when combined with (E)-2-octenal, another non beany volatile, the mixture was reported to induce a strong beany odour (Bott and Chambers (2006). Although Frankel, Neff and Selke (1981) concluded that (E,E)-2-4-decadienal could be derived from autoxidation and photosensitized oxidation of linoleic acid, lipoxygenase may also play a role in the formation of (E,E)-2-4-decadienal (Kobayashi et al., 1995; Yuan and Chang, 2007; Zhang et al., 2012).

Among all of the “beany” flavour related volatiles detected in soybean samples, (E, E)-2-4-decadienal was present at the highest concentration (6.77 ppm in the full fat sample) (Figure 4.17). However, micronization at 100°C or 135°C significantly reduced this level to 2.85 ppm (FFM100) and 1.05 ppm (FFM135), respectively. This agreed with the results of Zhang et al. (2012) who demonstrated that hot grinding decreased the lipoxygenase activity resulting in an absence of (E,E)-2-4-decadienal in comparison to soybean milk prepared by normal grinding.

Defatting significantly reduced the level of (E, E)-2-4-decadienal for both the unmicronized sample (FFM0) and the sample micronized at 100°C (FFM100). This is likely due to the removal of (E, E)-2-4-decadienal by hexane; however, defatting did not

change the level of (E, E)-2-4- decadienal in DFM135, possibly because the value was already low.

Figure 4.17 Effects of micronization, defatting, and ethanol treatment on (E, E)-2-4-decadienal in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Further removal of fat with the ethanol (65% and 85%) had no effect on (E, E)-2-4-decadienal levels for micronized and non-micronized samples. For all defatted samples, the concentration of (E, E)-2-4-decadienal in the ethanol treated sample remained very low compared to untreated full fat sample (FFM0). This residual (E, E)-2-4- decadienal may have interacted with soybean protein making it difficult to be completely removed.

4.3.3 Effect of micronization, defatting, and ethanol washing on volatiles generated by singlet oxygen induced unsaturated fatty acid oxidation in soybean flour

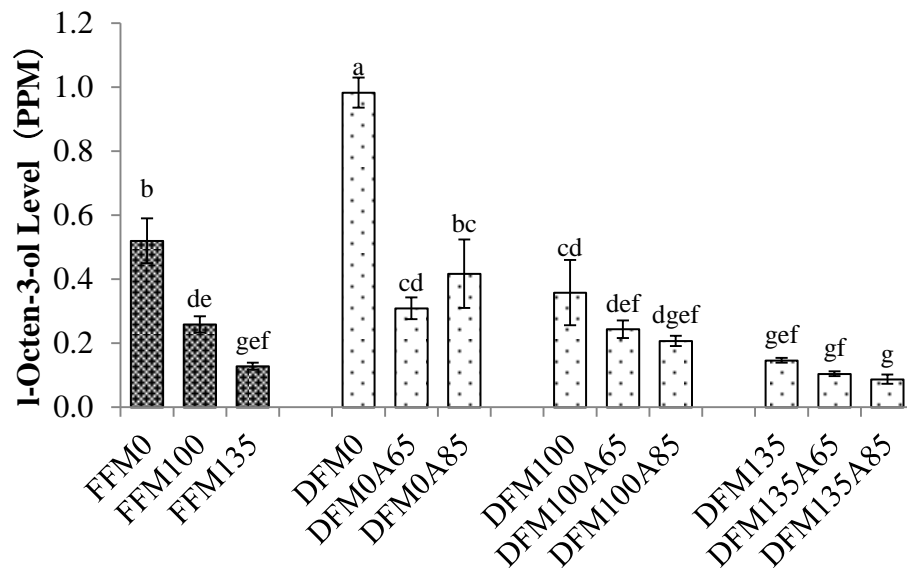
4.3.3.1 *Effect of micronization, defatting, and ethanol washing, on 1-octen-3-ol in soybean flour*

1-Octen-ol has a mushroom odour and an extremely low threshold of 0.005 ppm (Buttery, 1989). Vara-Ubol, Chambers and Chambers (2004) reported that 1-Octen-3-ol exhibited a beany characteristic at concentrations between 100-1000 ppm. Lipxygenase-deficient soybean varieties have similar levels of 1-octen-3-ol as normal soybean varieties (Yuan and Chang, 2007) indicating that its production may be something other than the lipxygenase-activated oxidation. It has been suggested that 1-octen-3-ol was derived from 10-hydroperoxide which was formed by the reaction of linoleic acid with singlet oxygen in the presence of light (Kobayashi et al., 1995; Matsui et al., 2003; Zhang et al., 2012).

Micronizing the full fat soybean (FFM0) at 100°C (FFM100) and 135° (FFM135) reduced the level of the 1-octen-3-ol from 0.520 ppm to 0.259 ppm and 0.128 ppm, respectively (Figure 4.18). These levels are still very high when compared with the 0.005 ppm threshold, even in soybean micronized at 135°C.

Defatting of the FFM0 significantly increased the level of 1-octen-3-ol in the untreated sample (DFM0). However, there was no significant defatting effect for the micronized samples (FFM100 and FFM135).

Figure 4.18 Effects of micronization, defatting, and ethanol treatment on 1-Octen-3-ol in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

The ethanol treatment significantly reduced the level of the 1-octen-3-ol in the unmicronized defatted samples (DFM0M65 and DFM0A85), but there was no significant effect due to ethanol concentration. Ethanol treatment of samples micronized at 100°C and 135°C did not change the level of 1-octen-3-ol. It is possible the generation of 1-octen-3-ol in the presence of light (Matsui et al., 2003) during the ethanol treatment process, may have offset the removal of 1-octen-3-ol level by ethanol.

4.3.3.2 *Effect of micronization, defatting, and ethanol washing, on 2-pentylfuran in soybean flour*

In conjunction with hexanol and hexanal, 2-pentylfuran has been one of the most frequently cited contributors to “beany” aroma in soybean (Arai, Koyanagi and Fujimaki,

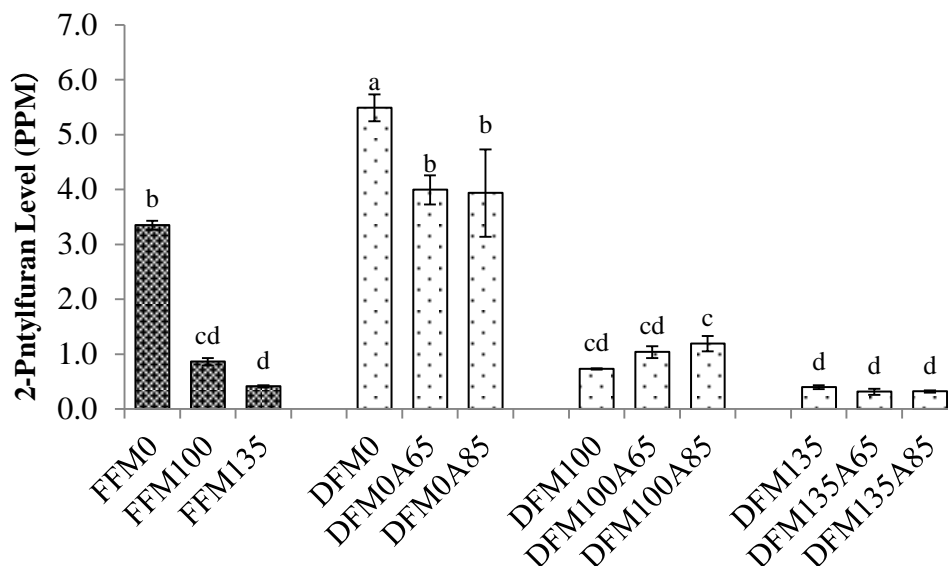
1967; Chang et al., 1966; Wilkens and Lin, 1970). Vara-Ubol, Chambers and Chambers (2004) described 2-pentylfuran as “mushroom –like” rather than “beany” because it did not exhibit any beany characteristic when examined on its own. Krishnamurthy et al. (1967) determined the flavour threshold for 2-pentylfuran to be 1 ppm and they believed that the formation of the 2-pentylfuran resulted from the decomposition of hydroperoxides produced from linoleic acid. Lee et al. (2003) also stated that 2-pentylfuran could be generated by singlet oxygen action on linoleic acid.

The initial 2-pentylfuran concentration in the full fat soybean (FFM0) was high (3.350 ppm) compared to the other volatile aromas studied. After micronizing soybean at 100°C and 135°C, the 2-pentylfuran level decreased to 0.846 ppm (FFM100) and 0.414 ppm (FFM135) respectively (Figure 4.19). These results indicated that micronization was very efficient at reducing the 2-pentylfuran concentration. Shariati-Levari (2013) found a significant decrease (41.4%) in 2-pentylfuran after micronization of chickpea at 130°C and 150°C. In addition, Zhang et al. (2012) reported that hot grinding at 80°C for 3 min significantly reduced 2-pentylfuran formation compared with normal ground soy milk. The inhibition of the lipoxygenase could partially prevent the formation of 2-pentylfuran (Shariati-Levari, 2013; Zhang et al., 2012).

Defatting further increased the concentration of 2-pentylfuran (63.89%) as compared with FFM0. There was no significant effect of defatting on FFM100 and FFM135, the micronized samples. This could be explained by the long-time exposure of the soybean flour under light during the defatting process which could promote the

singlet oxygen reaction with linoleic acid thereby generating more 2-pentylfuran in soybean flour.

Figure 4.19 Effects of micronization, defatting, and ethanol treatment on 2-pentylfuran in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Ethanol treatment of the defatted sample (DFM0) had the opposite effect to defatting such that the 2-pentylfuran levels in the unmicronized samples (DFM0A65 and DFM0A85) was reduced to a level comparable to what was seen for the full fat sample (FFM0). However, ethanol treatment did not affect the level of 2-pentylfuran in the micronized samples (DFM100 and DFM135). Any removal of the 2-pentylfuran due to the ethanol treatment may have been offset by the generation of singlet oxygen during ethanol extraction which reacted with the lipid in soybean generating more 2-pentylfuran. This theory is plausible as 0.6%-3% fat remained in the ethanol treated soybean (Figure

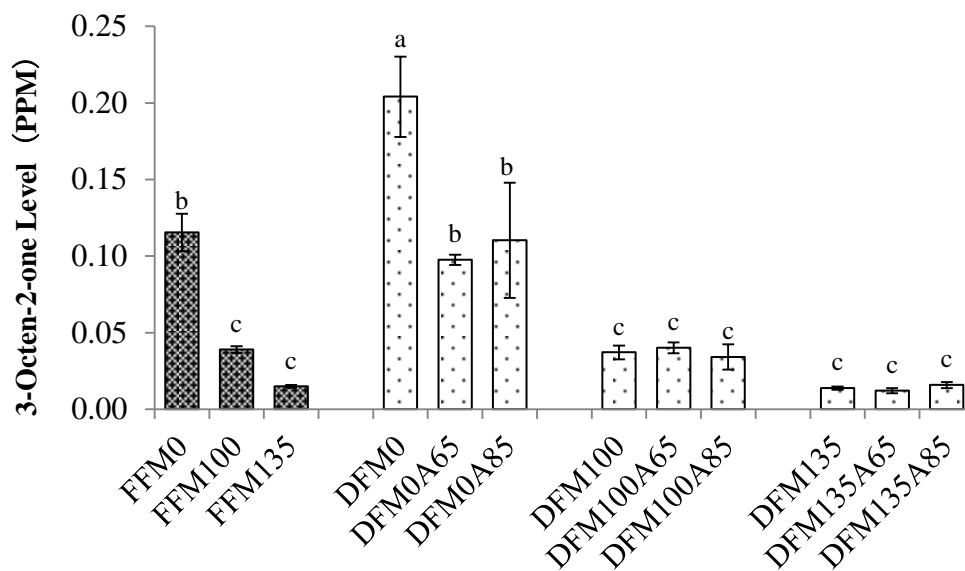
4.3). The extraction of the soybean flavour has been closely associated with the removal of highly unextractable lipids (Sessa, Honig and Rackis, 1969).

4.3.3.3 *Effect of micronization, defatting, and ethanol washing on 3-octen-2-one in soybean flour*

3-Octen-2-one is a ketone that has been found in different soybean products such as soybean milk, soybean protein and soybean lecithin (Achouri, Boye and Zamani, 2006; Lei and Boatright, 2001; Stephan and Steinhart, 1999). 3-octen-2-one has been described as having a “nutty, fruity” odour with a detection threshold of 0.25 ppm (Stephan and Steinhart, 1999). The combination of (E,E)-2-4-nonadienal, 1-octen-3-one and 2-pentylfuran have been reported to produce an unidentified sulfurous odour (Lei and Boatright, 2001). Yang et al. (2007) demonstrated that 3-octen-2-one could be produced by riboflavin-photosensitization of linoleic acid.

The levels 3-octen-2-one in soybean flour were all below the reported threshold level (Figure 4.20). Micronization significantly decreased the levels of the 3-octen-2-one concentration in the full fat soybean (FFM100 and FFM135 as compared with FFM0). However, there was no significant difference between 3-octen-2-one level for the two micronization temperatures (FFM100 and FFM135). Defatting untreated soybean significantly increased the concentration of 3-octen-2-one (DFM0). Micronization of the soybean prior to defatting (DFM100 and DFM135) reduced the level of 3-octen-2-one compared to the defatted untreated sample (DFM0) and the values were comparable to the full fat soybean (FFM100 and FFM135). This indicated that micronization was able to partially prevent 3-octen-2-one formation.

Figure 4.20 Effects of micronization, defatting, and ethanol treatment on 3-octen-2-one in soybean flour



Columns with the same letter are not significantly different ($P < 0.05$)

FF: Full fat. DF: Defatted. M0/M100/M135: Non-micronized or micronization at 100°C or 135°C. A65/A85: Ethanol treatment at 65% or 85% concentration

Despite the increase in the 3-octen-2-one concentration upon defatting (DFM0), further ethanol treatment reduced the level of 3-octen-2-one such that it was same level as in the full fat sample (FFM0). However, there was no significant difference between levels of 3-octen-2-one for the two ethanol treatments (DFM0A65 and DFM0A85). The ethanol treatment of the micronized samples (DFM100 and DFM135) did not change the level of 3-octen-2-one (Figure 4.20). Hua et al. (2005) achieved a total inhibition of 3-octen-2-one formation in soybean protein isolate by treating the flour with ethanol. The fact that the ethanol treatments (DFM0A65 and DFM0A85) did not completely remove the 3-octen-2-one in the current study may be a result of the generation of singlet oxygen during ethanol extraction which generated more 3-octen-2-one.

4.3.4 General discussion on effects of micronization, defatting and ethanol washing on “beany” flavour related volatiles

The twelve selected beany flavour related volatiles from soybean flour volatile profile fit into two categories: lipoxygenase induced oxidation and singlet oxygen related oxidation of unsaturated fatty acid. Micronizing full fat soybean at 100°C and 135°C generally decreased the content of volatiles derived from lipoxygenase oxidation of unsaturated fatty acid such as hexanal, (E)- 2-hexenal, 1-hexanol, heptanal, (E)-2-octenal, (E)-2-nonenal, (E,E)-2, 4-nonadienal, 2,4-decadienal, and (E,E)-2,4-decadienal in comparison to original soybean flour. This may due to inactivation of the lipoxygenase activity by the micronization process. It is worth noting that the level of the short-chain volatiles such hexanal, (E)- 2-hexenal, and 1-hexanol did not differ between micronizing full fat soybean at 100°C and 135°C while medium chain volatiles such as (E)-2-octenal, (E)-2-nonenal, (E,E)-2, 4-nonadienal, 2,4-decadienal, and (E,E)-2,4-decadienal had significant lower levels at the higher micronization temperature (135°C). Higher micronization temperatures could enhance the generation of the singlet oxygen which could react with the double bond in the medium chain aldehydes forming hydroperoxides (Min and Boff, 2002). Those hydroperoxides could be further degraded. On the other hand, micronizing full fat soybean significantly decreased the level of the singlet oxygen derived volatiles such as 1-octen-3-ol, 2-pentylfuran and 3-octen-2-one. Possibly micronization at high temperature degraded the chlorophyll and riboflavin which are excellent singlet oxygen sensitizers (Butnariu et al., 2013). Thus, 1-octen-3-ol, 2-pentylfuran and 3-octen-2-one level decreased after micronizing soybean.

In general, the defatting process had a significant effect on reducing the medium chain volatiles, especially (E,E)-2, 4-nonadienal, 2,4-decadienal, and (E,E)-2,4-decadienal because the hexane can dissolve low polarity compounds (Burke, 1984). In contrast, ethanol treatment significantly reduced compounds from lipoxygenase derived hydroperoxides such as hexanal, (E)- 2-hexenal, and 1-hexanol due to the polarity of those compounds. Similar effects of ethanol

treatment were seen in singlet oxygen derived compounds such as 1-octen-3-ol, 2-pentylfuran and 3-octen-2-one due to removal of the extra lipids or degradation of singlet oxygen sensitizers by ethanol washing (Jadwiga, 2006). Ethanol washed soybean flours have been reported to have superior flavours (Rackis, Sessa and Honig, 1979). The current research indicated that the reduction of hexanal, (E)-2-hexenal, 1-hexanol 1-octen-3-ol, 2-pentylfuran and 3-octen-2-one by ethanol washing might have contributed to this improvement.

For defatted soybean flour, micronization on its own had better effects on reducing the level of volatiles in comparison with ethanol washing on its own. Micronization and ethanol treatments in combination did not significantly change the levels of volatiles selected associated with beany flavour when compared to micronization on its own. For defatted soybean flour with or without ethanol treatment, micronization at 100°C and 135°C had similar effects on the level of volatiles. Overall, micronization of soybean at 100°C or 135°C with or without ethanol treatment would be recommended if only the beany flavour associated volatiles in soybean flour needed to be considered.

5 Conclusions

This study was designed to investigate the effects of different treatments (micronization, ethanol washing or alcalase hydrolysis individually or in combination) of soybeans flours on trypsin inhibitors and lipoxygenase activities as well as on flavour compounds which may be related to the “beany” flavours in soybean products.

Proximate analysis data on soybean flour indicated that moisture, fat, and protein content in the soybean flour changed with different processing treatments. In general, alcalase hydrolysis on soybean flour treatment decreased the moisture and protein contents as compared to the corresponding unhydrolyzed soybean flour regardless of whether the soybean was pretreated with micronization and ethanol treatment individually or in combination. In contrast, ethanol treatment increased the moisture and protein content as compared to unmicronized or micronized defatted soybean flour. Among all the treatments, defatted soybean micronized at 135°C with ethanol treatment at 65% concentration had the highest protein content which is generally beneficial from a nutritional point of view. On the other hand, ethanol treatment especially at 65% concentration on either micronized or unmicronized defatted soybean flour significantly reduced the crude fat content which could prevent the generation of undesirable flavours.

In general, micronization alone decreased the trypsin inhibitor activity; however the effects of the inhibition of trypsin inhibitor by micronization increased when combined with ethanol treatments, especially at a concentration of 65%. Trypsin inhibitor results were more complicated when alcalase hydrolysis was involved. Alcalase hydrolysis individually or in combination with ethanol treatments, at either 65% or 85%,

in the absence of micronization decreased trypsin inhibitor activity in soybean flour. In contrast, trypsin inhibitor activity increased due to the alcalase hydrolysis when combined with micronization at 135°C. The mechanism by which this occurred has not been fully delineated. However, the lowest trypsin inhibitor level was obtained from soybean micronized at 135°C with or without any ethanol treatments (65% and 85%). However, the lowest residual trypsin inhibitor activity (6.4 TIU/mg) in the present study was still relatively high compared to other food products.

Total inhibition of lipoxygenase activity was achieved by individual treatments such as ethanol treatment (65% and 85%) or alcalase hydrolysis (0.5 h and 3 h) or by any treatment combinations. Micronization itself or in combination significantly decreased the lipoxygenase activity which could inhibit the production of beany flavours related to volatiles from lipoxygenase induced oxidation of fatty acid.

Soybean volatiles extracted by simultaneous distillation extraction method were analyzed by running in a gas chromatograph in collaboration with mass spectrometry. Twelve of 38 positively identified compounds were considered as beany flavour related volatiles: hexanal, 2-hexenal, 1-hexanol, heptanal, 1-octen-3-ol, 2-pentylfuran, 3-octen-2-one, (E)-2-octenal, (E,E)-2, 4-nonadienal, 2,4-decadienal, and (E,E)-2,4-decadienal. Among them, (E,E)-2,4-decadienal was the predominant volatile in the full fat soybean flour regardless of micronization treatment. However, after defatting, 2-pentylfuran became the predominant volatile. Micronization decreased levels of both lipoxygenase derived volatiles such as hexanal, (E)- 2-hexenal, 1-hexanol, heptanal, (E)-2-octenal, (E)-2-nonenal, (E,E)-2, 4-nonadienal, 2,4-decadienal, and (E,E)-2,4-decadienal and singlet

related volatiles such as 1-octen-3-ol, 2-pentylfuran and 3-octen-2-one, all of which could contribute to the “beany” flavour in the micronized soybean flour. Ethanol was not as effective in reducing “beany” flavour related volatiles as micronization alone. As expected, micronization and ethanol washing treatment in combination also reduced the volatile compounds. Based on the current research, micronization is a necessary pre-treatment to control “beany” flavour generation in soybean seeds and is therefore recommended.

6 Applications and Recommendations

In general, soybean flour with a low level of the trypsin inhibitor and less beany flavour is more desirable for consumer consumption especially in western countries. Among all the treatments examined in this study, defatted soybean micronized at 135°C with an ethanol treatment at a concentration of 65% had higher protein content, lower trypsin inhibitor activity as well as a lower level of selected “beany” flavour related volatiles and thereby could be recommended for soybean processing. However, the drawback of this processing was the brown colour generated by micronization of seeds at this temperature. Future research should focus on methods for preventing the brown colour generated during micronization. Higher moisture content during micronization might be worth considering. The current research concluded that alcalase hydrolysis alone decreased trypsin inhibitor activity in the hydrolysed soybean flour, but when combined with micronization at 135°C, an increase of the trypsin inhibitor activity resulted. Sodium dodecyl sulphate –polyacrylamide gel electrophoresis (SDS-PAGE) could be used to study the change in the trypsin inhibitor when treated with micronization at 135°C and alcalase hydrolysis (0.5h and 3h). This will enhance the understanding of the mechanism by which trypsin inhibitor activity is affected during enzymatic hydrolysis. Although, the decrease of volatile compounds related to “beany” flavour is credited for better sensory score in soybean products and is generally accepted by researchers, future research needs to confirm the volatile results in the present research actually reflect changes in the sensory characteristics of soybean flour treated with different processes. Consumer preference tests and tests by sensory evaluation panels are recommended.

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Appendices

Appendix 1 Amount of nitrogen from the free amino groups in 1 gram of soybean sample

Soybean sample	N in free amino group (mg)
M0	4.33±0.11 ^b
M0A65	6.01±0.24 ^a
M0A85	5.55±0.14 ^a
M100	4.09±0.30 ^b
M100A65	6.32±0.40 ^a
M100A85	6.00±0.23 ^a
M135	2.07±0.29 ^c
M135A65	4.15±0.37 ^b
M135A85	3.69±0.04 ^b

M0/100/135: Unmicronized or micronized at 100°C/135°C. A65/85: 65% /85% ethanol washing

Columns with same letter means not significantly different (P<0.05)

Appendix 2 Analysis of variance table for moisture content using SAS with a factorial design

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	42.9	21.4	3062	<.0001
Solvent	2	98.9	49.5	7054	<.0001
Enzyme	2	864	432	61614	<.0001
Heat*Solvent	4	34.1	8.52	1215	<.0001
Solvent*Enzyme	4	284	71.2	10160	<.0001
Heat*Enzyme	4	34.3	8.59	1224	<.0001
Heat*Solvent*Enzyme	8	50.5	6.32	901	<.0001

Appendix 3 Analysis of variance table for protein content using SAS with a factorial design

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Block	1	0.390	0.390	4.31	0.0478
Heat	2	0.260	0.130	1.43	0.2565
Solvent	2	351	175	1927	<.0001
Enzyme	2	66.9	33.4	366	<.0001
Heat*Solvent	4	3.52	0.880	9.66	<.0001
Solvent*Enzyme	4	4.11	1.030	11.3	<.0001
Heat*Enzyme	4	13.2	3.29	36.1	<.0001

Appendix 4 Analysis of variance table for degree of hydrolysis (DH%) by using SAS with a factorial design

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	0.450	0.22	1.27	0.2891
Solvent	2	84.8	42.38	240	<.0001
Enzyme	2	1505	752.91	4268	<.0001
Heat*Solvent	4	8.89	2.22	12.6	<.0001
Solvent*Enzyme	4	45.1	11.27	63.9	<.0001
Heat*Enzyme	4	5.50	1.38	7.80	<.0001
Heat*Solvent*Enzyme	8	6.28	0.78	4.45	0.0003

Appendix 5 Analysis of variance table for Differential Scanning Calorimetry (DSC) data by using SAS with a factorial design

a. Denaturation temperature for glycinin

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	0.900	0.450	1.83	0.1695
Solvent	2	15.4	7.70	31.4	<.0001
Enzyme	2	167642	83821	341331	<.0001
Heat*Solvent	4	2.46	0.620	2.51	0.0525
Solvent*Enzyme	4	30.8	7.70	31.4	<.0001
Heat*Enzyme	4	1.80	0.450	1.83	0.1356
Heat*Solvent*Enzyme	8	4.93	0.610	2.51	0.0214

b. Enthalpy for glycinin

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	6.72	3.36	359.	<.0001
Solvent	2	0.690	0.340	36.9	<.0001
Enzyme	2	111	55.6	5941	<.0001
Heat*Solvent	4	0.530	0.130	14.2	<.0001
Solvent*Enzyme	4	1.38	0.340	36.9	<.0001
Heat*Enzyme	4	13.4	3.36	359	<.0001
Heat*Solvent*Enzyme	8	1.06	0.130	14.2	<.0001

c. Denaturation temperature for β -conglycinin

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	11879	5939	40099	<.0001
Solvent	2	0.470	0.240	1.60	0.2109
Enzyme	2	47517	23758	160399	<.0001
Heat*Solvent	4	1.38	0.350	2.33	0.0672
Solvent*Enzyme	4	0.950	0.240	1.60	0.1872
Heat*Enzyme	4	23758	5939	40099	<.0001
Heat*Solvent*Enzyme	8	2.77	0.340	2.33	0.0313

d. Enthalpy for β -conglycinin

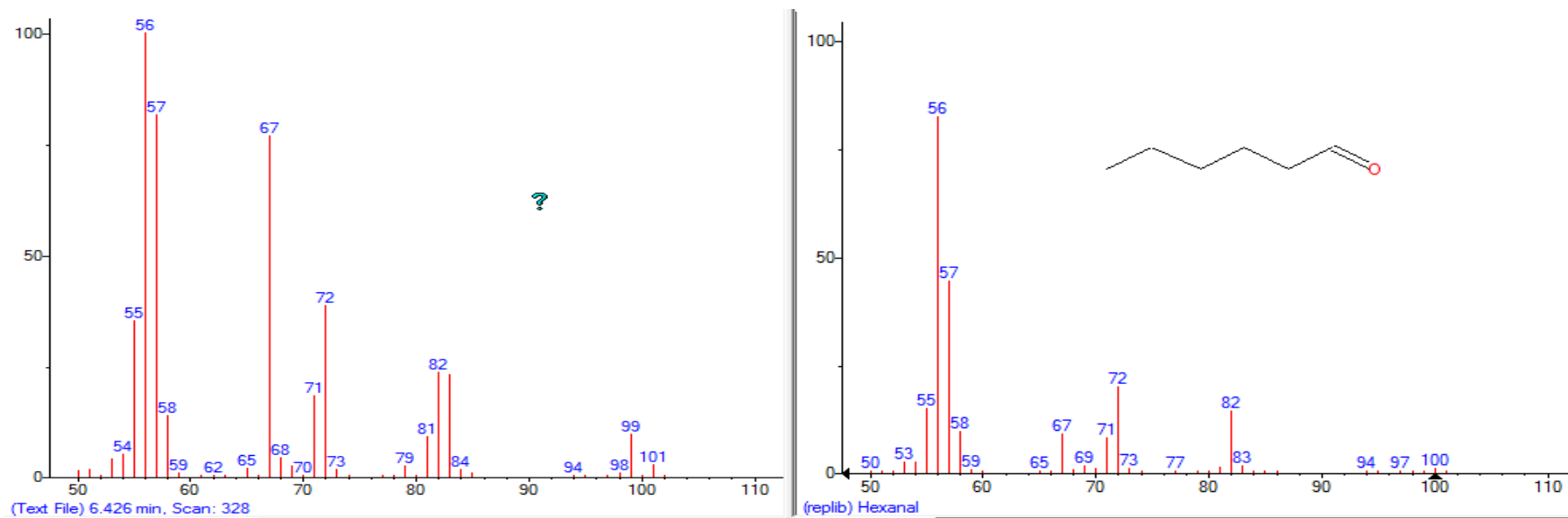
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	0.690	0.350	673	<.0001
Solvent	2	0.0200	0.0100	19.9	<.0001
Enzyme	2	2.28	1.14	2214	<.0001
Heat*Solvent	4	0.0290	0.00720	13.9	<.0001
Solvent*Enzyme	4	0.0410	0.0100	19.9	<.0001
Heat*Enzyme	4	1.39	0.350	673	<.0001
Heat*Solvent*Enzyme	8	0.0580	0.00720	13.9	<.0001

Appendix 6 Analysis of variance table for trypsin inhibitor activity by using SAS with a factorial design

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Heat	2	8373	4186	3902	<.0001
Solvent	2	38.3	19.1	17.8	<.0001
Enzyme	2	211	105	98.5	<.0001
Heat*Solvent	4	776	194	180	<.0001
Solvent*Enzyme	4	163	40.8	38.0	<.0001
Heat*Enzyme	4	2077	519	484	<.0001
Heat*Solvent*Enzyme	8	145	18.1	16.9	<.0001

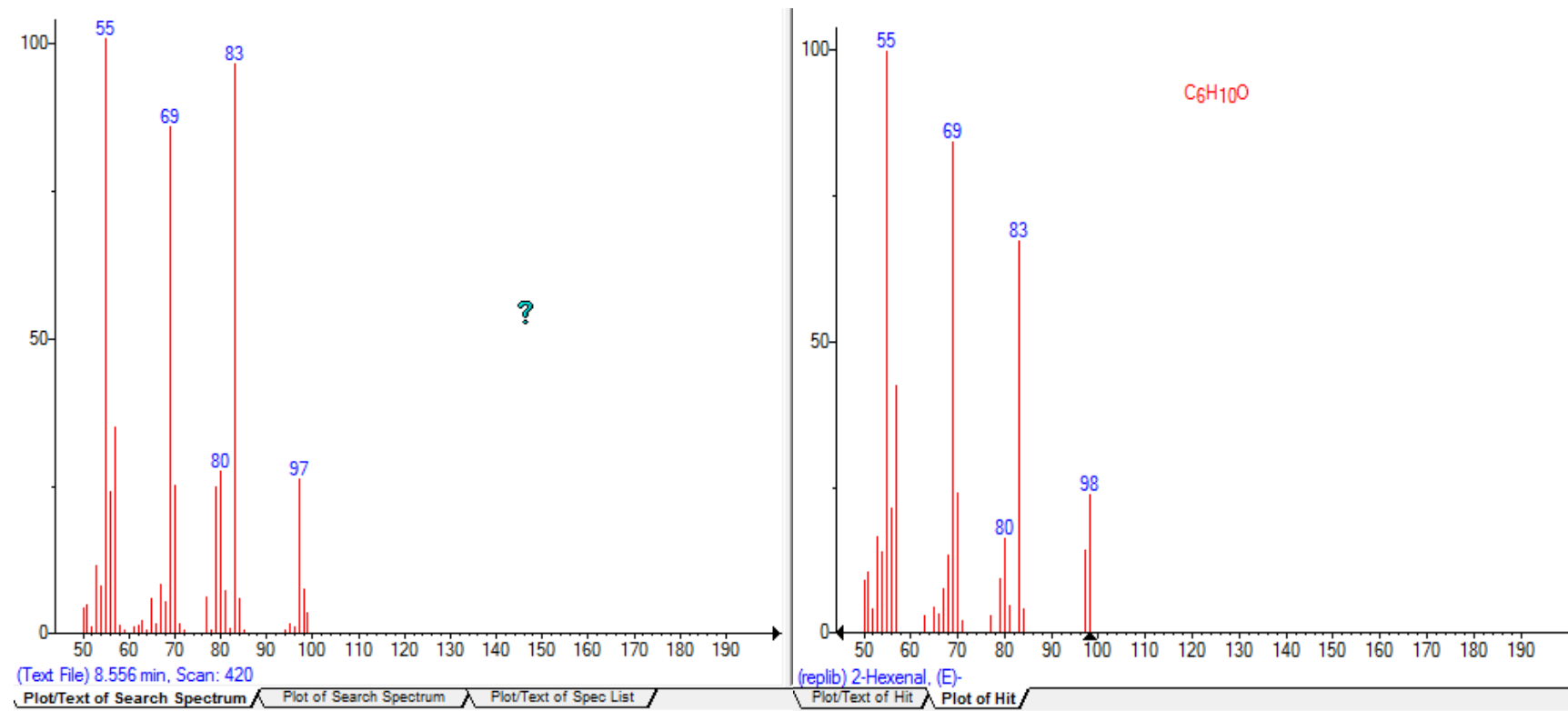
Appendix 7 Mass spectrum of selected volatile compounds related to “beany” flavour

Hexanal



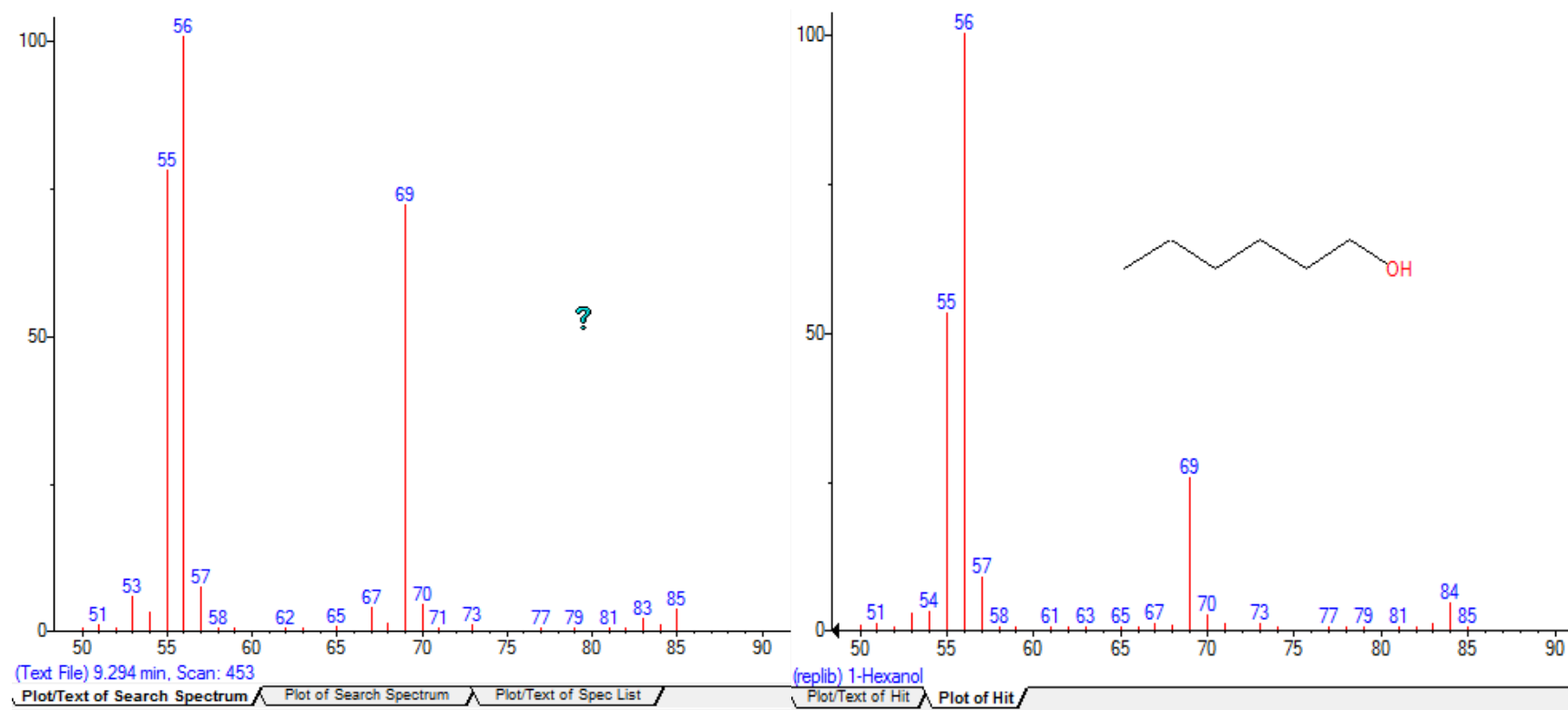
Spectrum 1: Mass spectrum is obtained at 6.426 min in non-micronized soybean flour volatile extracts

2-Hexenal, (E)



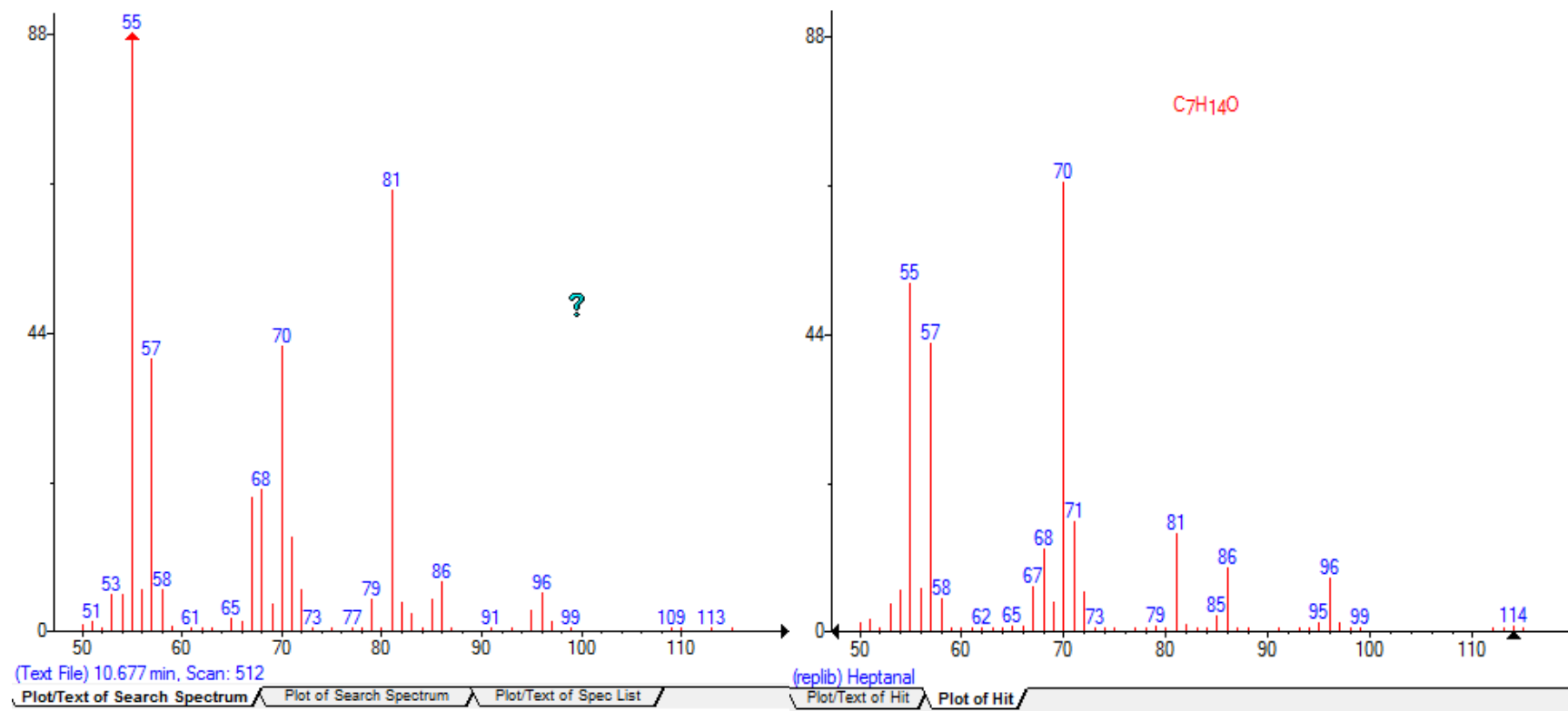
Spectrum 2: Mass spectrum is obtained at 8.556 min in non-micronized soybean flour volatile extracts

1-Hexanol



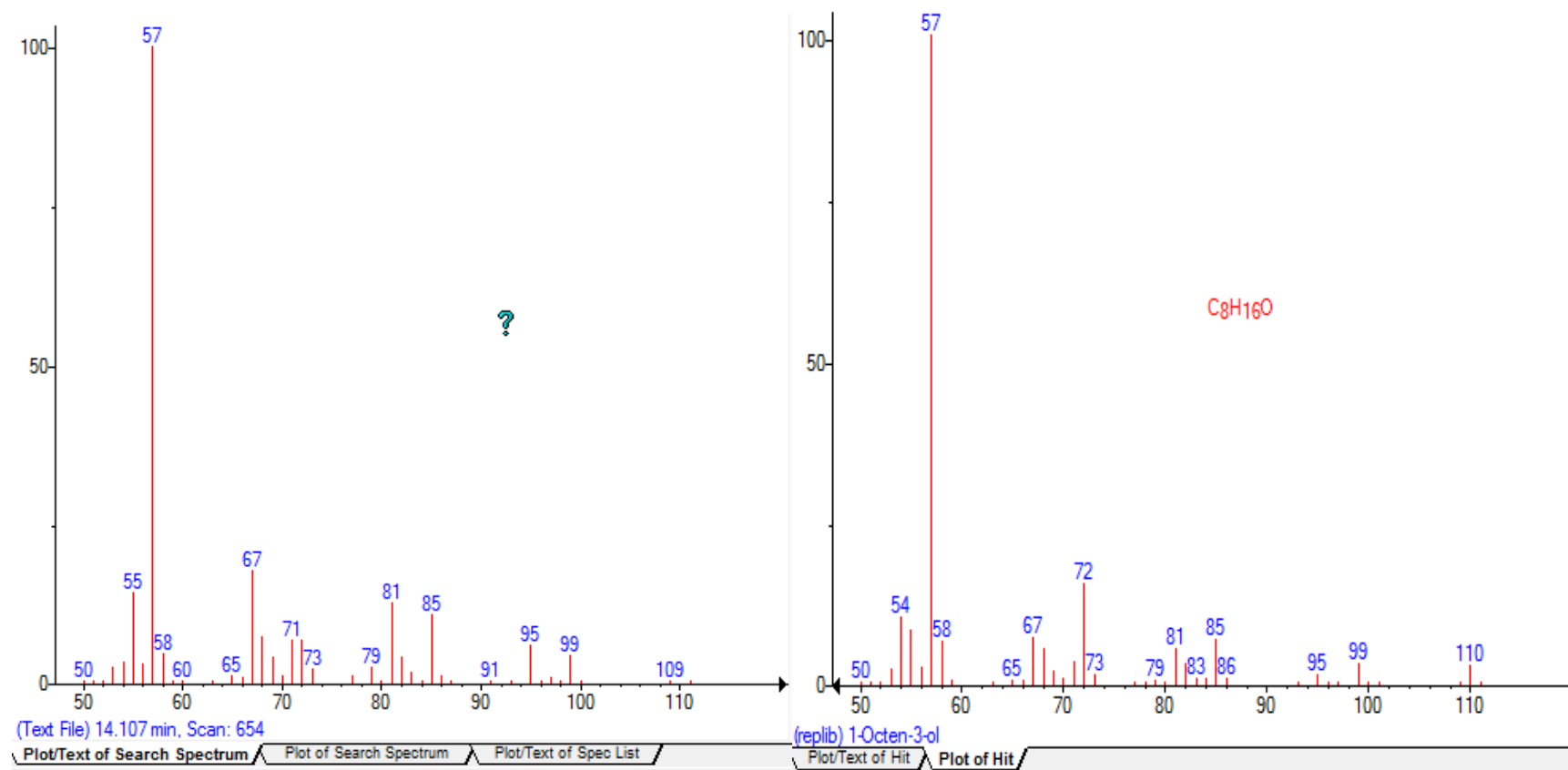
Spectrum 3: Mass spectrum is obtained at 9.294 min in non-micronized soybean flour volatile extracts

Heptanal



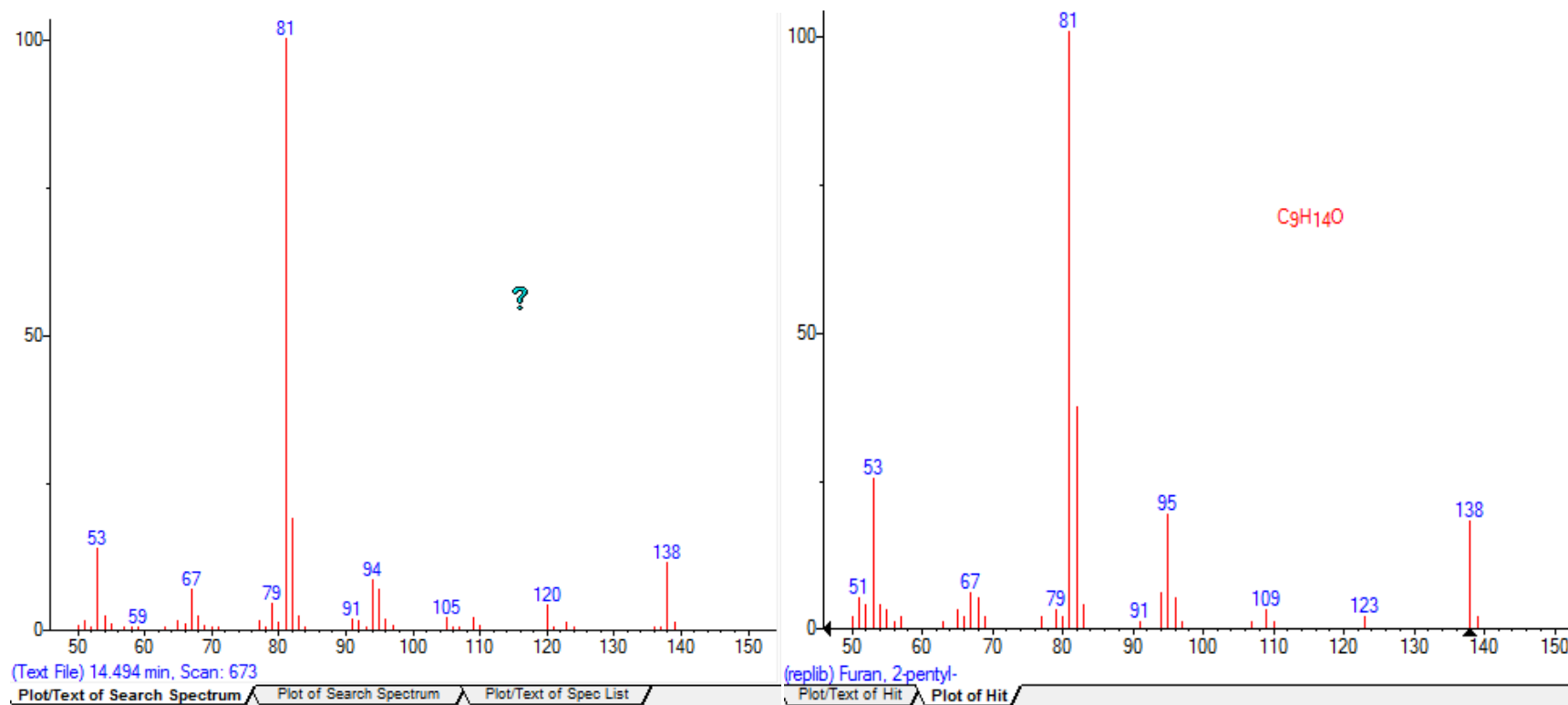
Spectrum 4: Mass spectrum is obtained at 10.677 min in non-micronized soybean flour volatile extracts

1-Octen-3-ol



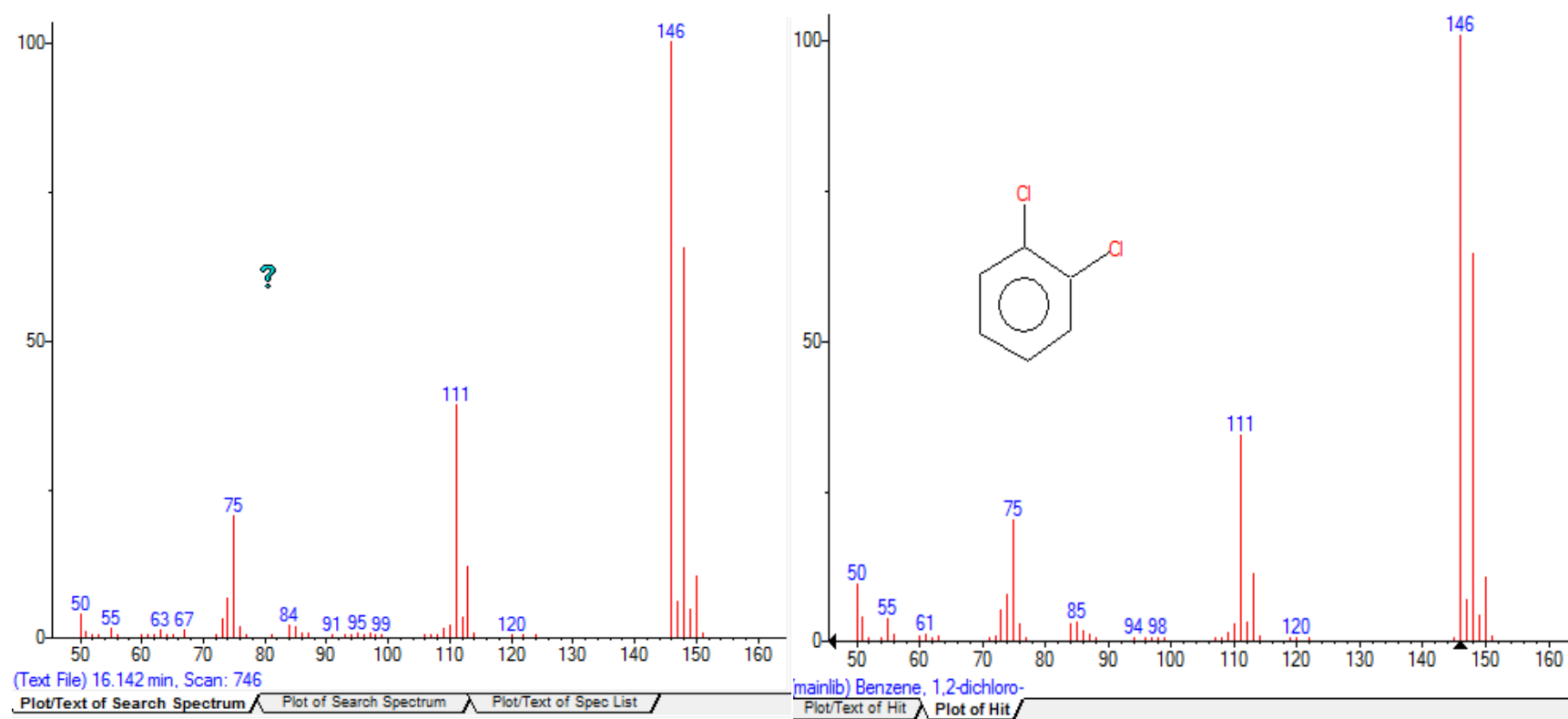
Spectrum 5: Mass spectrum is obtained at 14.107 min in non-micronized soybean flour volatile extracts

Furan, 2-pentyl-



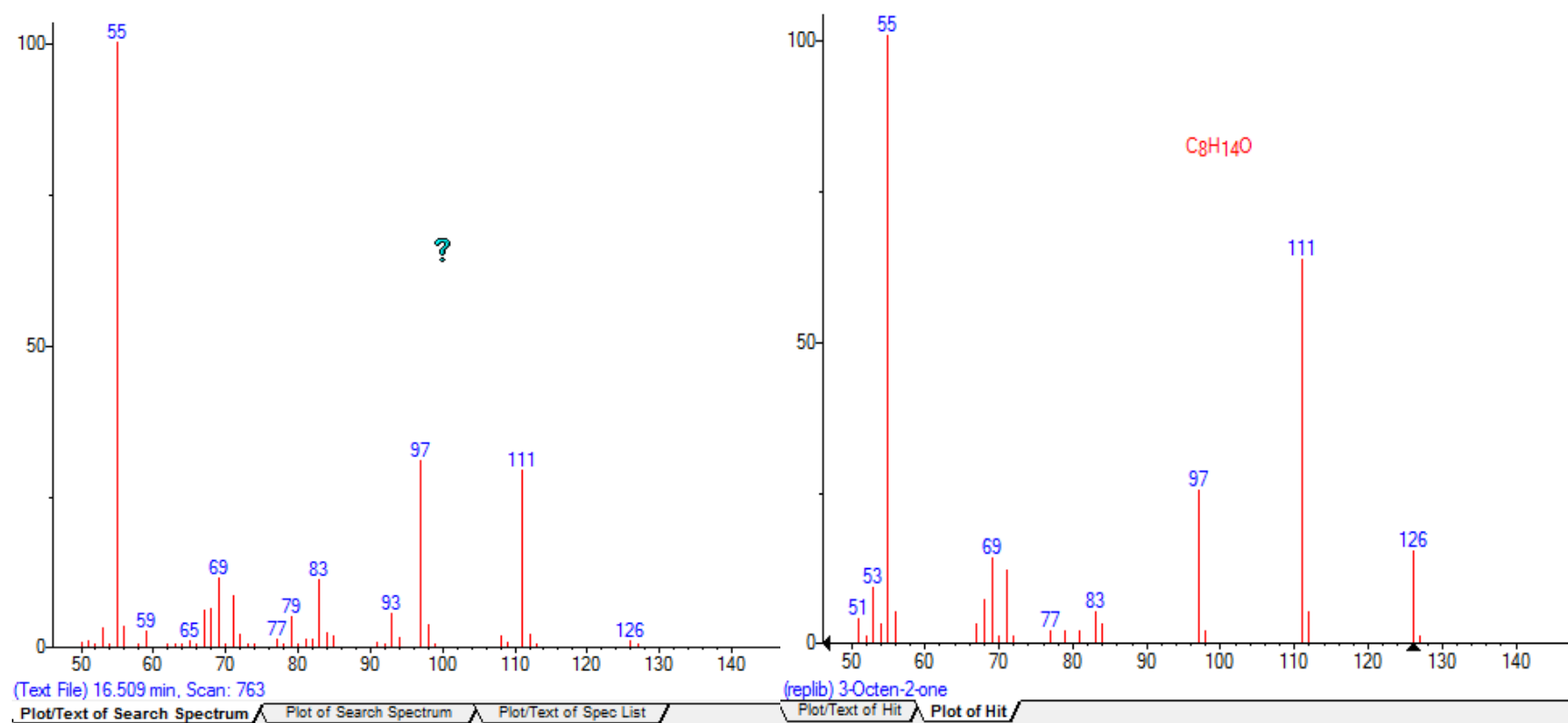
Spectrum 6: Mass spectrum is obtained at 14.494 min in non-micronized soybean flour volatile extracts

1,2-Dichloro-benzene



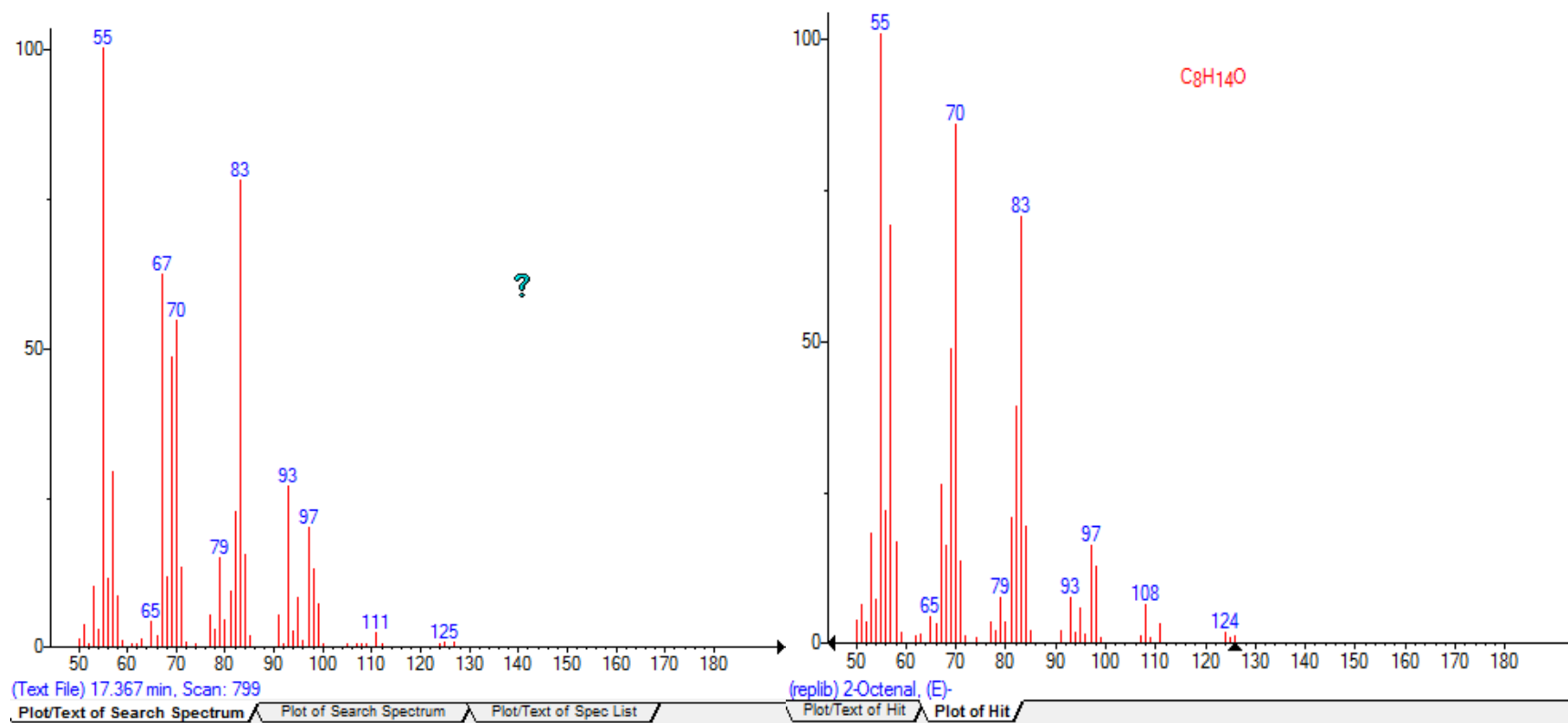
Spectrum 7: Mass spectrum is obtained at 16.142 min in non-micronized soybean flour volatile extracts (Internal Standard)

3-Octen-2-one



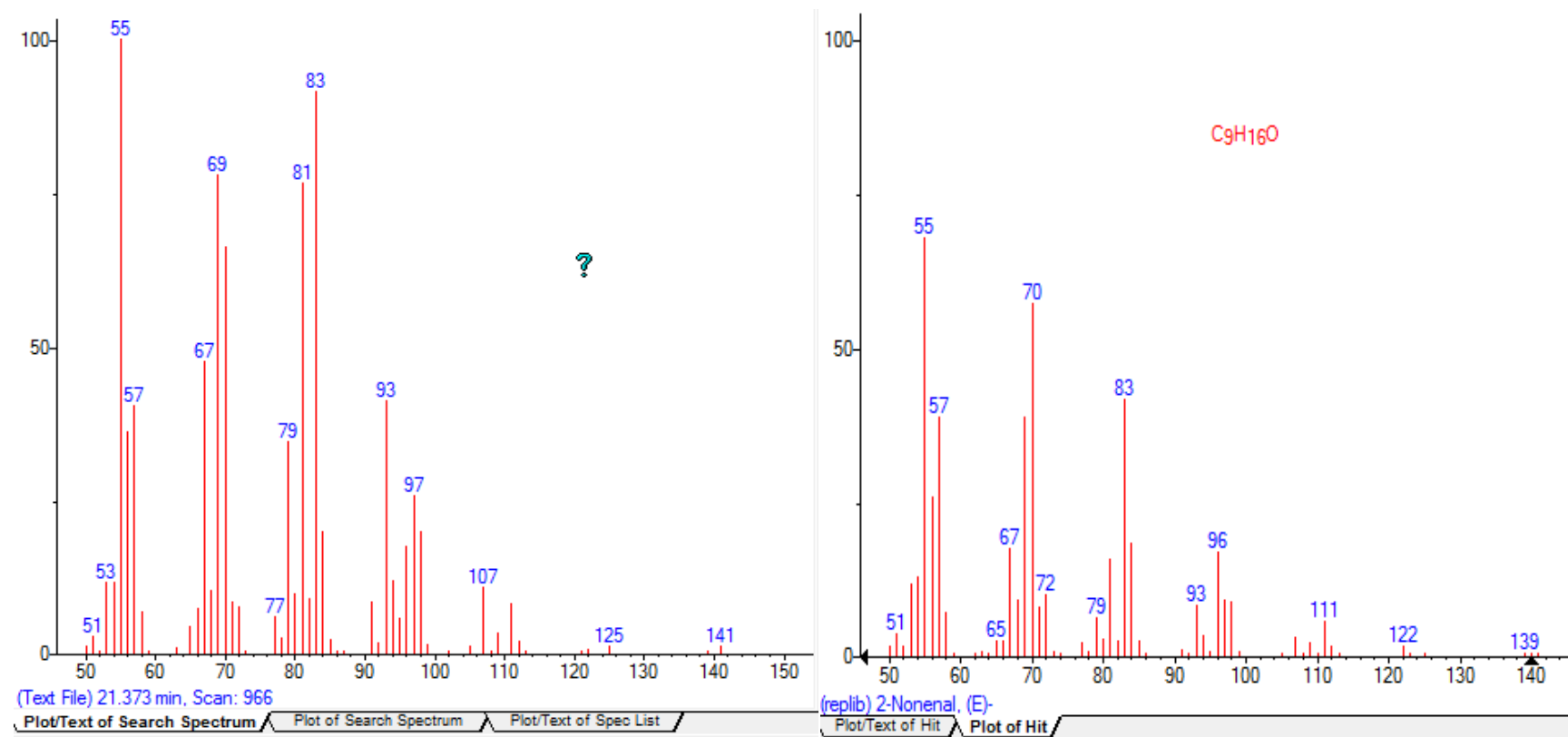
Spectrum 8: Mass spectrum is obtained at 16.509 min in non-micronized soybean flour volatile extracts

2-Octenal, (E)-



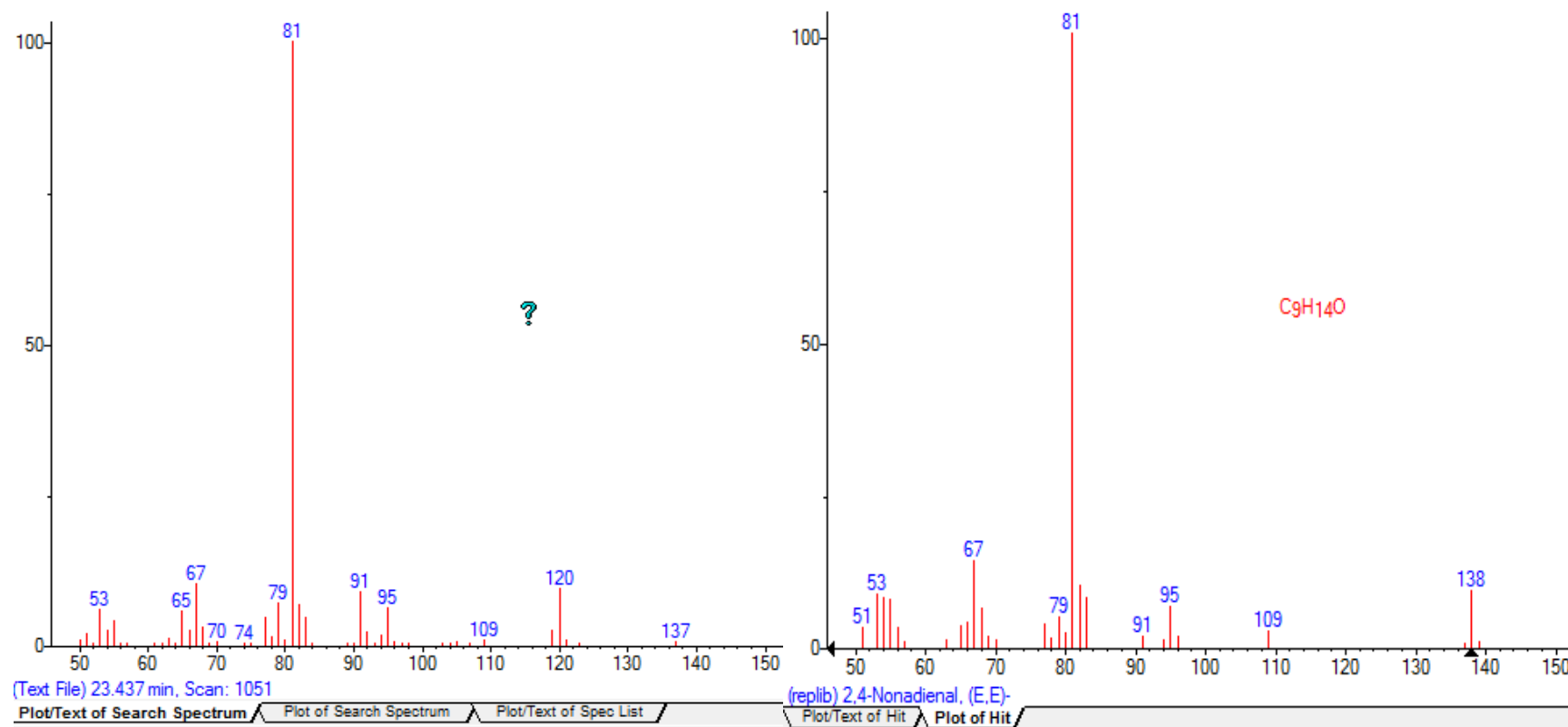
Spectrum 9: Mass spectrum is obtained at 17.367 min in non-micronized soybean flour volatile extracts

2-Nonenal, (E)-



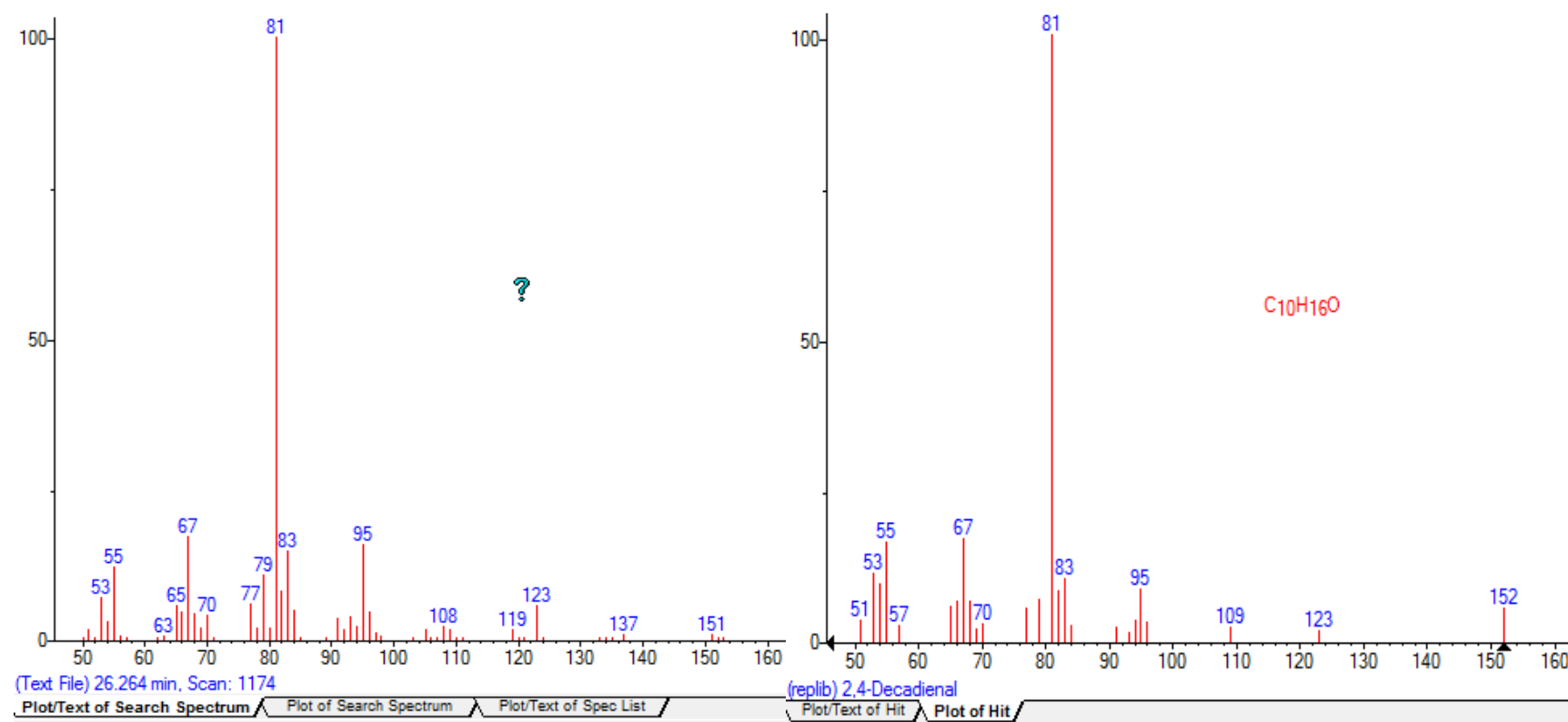
Spectrum 10: Mass spectrum is obtained at 21.373 min in non-micronized soybean flour volatile extracts

2,4-Nonadienal, (E,E)-



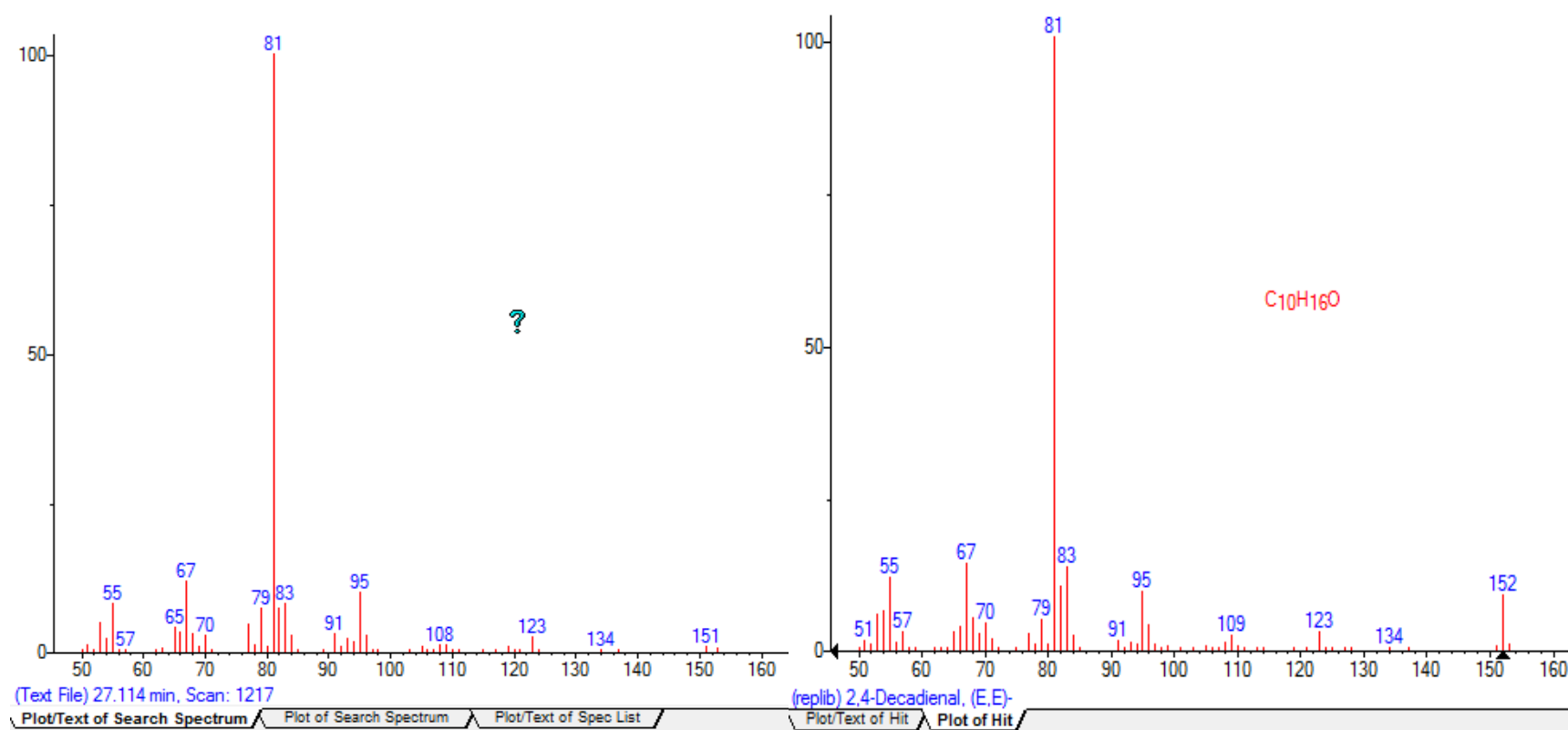
Spectrum 11: Mass spectrum is obtained at 23.437 min in non-micronized soybean flour volatile extracts

2,4-Decadienal



Spectrum 12: Mass spectrum is obtained at 26.264 min in non-micronized soybean flour volatile extracts

2,4-Decadienal, (E,E)-



Spectrum 13: Mass spectrum is obtained at 27.114 min in non-micronized soybean flour volatile extracts

Appendix 8 Effects of different processing on concentration (ppm) of “beany” flavour related volatile compounds in soybean flour

Volatile compounds	Hexanal	2-Hexenal, (E)-	1-Hexanol	Heptanal	1-Octen-3-ol	Furan, 2-pentyl-	3-Octen-2-one	2-Octenal, (E)-	2-Nonenal, (E)-	2-4-Nonadienal, (EE)-	2,4-Decadienal	2,4-Decadienal, (E,E)-
FFMO	1.31 ^{bc} (0.067)	0.284 ^{ad} (0.018)	0.859 ^{acd} (0.14)	0.073 ^{db} (0.0068)	0.520 ^{bcd} (0.07)	3.350 ^{bb} (0.081)	0.115 ^{bd} (0.012)	0.219 ^{ad} (0.015)	0.206 ^{abd} (0.029)	0.510 ^{acd} (0.052)	1.135 ^{ac} (0.13)	6.774 ^{ba} (0.95)
FFM100	0.273 ^{de} (0.014)	0.038 ^{ce} (0.002)	0.185 ^{cde} (0.007)	0.039 ^{efe} (0.002)	0.259 ^{de} (0.026)	0.864 ^{cd} (0.067)	0.039 ^{cd} (0.002)	0.126 ^{cde} (0.013)	0.067 ^{de} (0.002)	0.150 ^{bde} (0.012)	0.523 ^{bc} (0.055)	2.849 ^{ba} (0.226)
FFM135	0.145 ^{de} (0.012)	0.031 ^{cd} (0.004)	0.116 ^{cd} (0.005)	0.023 ^f (0.002)	0.128 ^{gef} (0.011)	0.414 ^{db} (0.021)	0.015 ^{cf} (0.001)	0.054 ^{def} (0.004)	0.037 ^{ef} (0.003)	0.040 ^{cf} (0.003)	0.206 ^{cc} (0.013)	1.054 ^{ca} (0.080)
DFM0A0	1.594 ^{ab} (0.105)	0.267 ^{ae} (0.011)	0.503 ^{bde} (0.015)	0.166 ^{af} (0.008)	0.983 ^{ac} (0.047)	5.490 ^{aa} (0.246)	0.204 ^{af} (0.026)	0.187 ^{abf} (0.022)	0.261 ^{ae} (0.018)	0.143 ^{cd} (0.011)	0.096 ^{cd} (0.011)	0.648 ^{cd} (0.098)
DFM0A65	0.591 ^{cb} (0.105)	0.087 ^{bc} (0.011)	0.123 ^{cd} (0.015)	0.127 ^{bc} (0.008)	0.310 ^{cd} (0.047)	3.992 ^{ba} (0.246)	0.098 ^{bc} (0.026)	0.149 ^{bc} (0.022)	0.250 ^{ac} (0.018)	0.102 ^{cd} (0.011)	0.082 ^{cd} (0.011)	0.582 ^{cb} (0.098)
DFM0A85	0.577 ^{cb} (0.031)	0.089 ^{bb} (0.011)	0.236 ^{cb} (0.070)	0.098 ^{cb} (0.007)	0.417 ^{bc} (0.107)	3.935 ^{ba} (0.798)	0.110 ^{bb} (0.038)	0.155 ^{bc} (0.032)	0.225 ^{ab} (0.054)	0.104 ^{cd} (0.023)	0.093 ^{cd} (0.015)	0.573 ^{cb} (0.084)
DFM100A0	0.258 ^{dc} (0.017)	0.032 ^{cd} (0.003)	0.128 ^{cd} (0.015)	0.054 ^{de} (0.005)	0.358 ^{cd} (0.102)	0.729 ^{cd} (0.013)	0.037 ^{ce} (0.005)	0.053 ^{de} (0.011)	0.095 ^{cde} (0.020)	0.034 ^{de} (0.008)	0.036 ^{de} (0.004)	0.213 ^{cd} (0.023)
DFM100A65	0.253 ^{de} (0.024)	0.037 ^{cc} (0.002)	0.044 ^{dc} (0.004)	0.072 ^{dc} (0.009)	0.244 ^{def} (0.027)	1.039 ^{cd} (0.108)	0.040 ^c (0.004)	0.076 ^{dc} (0.011)	0.226 ^{ab} (0.024)	0.047 ^{dc} (0.005)	0.043 ^{dc} (0.009)	0.289 ^{cb} (0.076)
DFM100A85	0.262 ^d (0.100)	0.033 ^{cd} (0.002)	0.035 ^d (0.004)	0.076 ^{cd} (0.015)	0.207 ^d (0.016)	1.191 ^{ca} (0.142)	0.034 ^{cd} (0.008)	0.076 ^d (0.004)	0.119 ^{cde} (0.002)	0.036 ^d (0.008)	0.068 ^d (0.023)	0.426 ^{cb} (0.113)
DFM135A0	0.128 ^{de} (0.009)	0.029 ^{cd} (0.003)	0.095 ^{cd} (0.006)	0.025 ^{fg} (0.001)	0.146 ^{def} (0.007)	0.396 ^{da} (0.036)	0.014 ^c (0.001)	0.035 ^d (0.005)	0.074 ^{cd} (0.016)	0.013 ^d (0.002)	0.044 ^d (0.007)	0.237 ^{cb} (0.027)
DFM135A65	0.107 ^{eb} (0.019)	0.014 ^{de} (0.002)	0.015 ^{de} (0.002)	0.044 ^{ed} (0.009)	0.105 ^g (0.007)	0.315 ^{da} (0.055)	0.012 ^{cd} (0.002)	0.036 ^{de} (0.001)	0.124 ^{cd} (0.014)	0.010 ^{de} (0.001)	0.049 ^d (0.009)	0.273 ^{ca} (0.029)
DFM135A85	0.132 ^{de} (0.010)	0.015 ^{de} (0.002)	0.015 ^{de} (0.001)	0.044 ^{ed} (0.002)	0.088 ^g (0.014)	0.322 ^{da} (0.021)	0.016 ^{ce} (0.002)	0.035 ^d (0.007)	0.140 ^{cd} (0.019)	0.011 ^{de} (0.002)	0.043 ^d (0.010)	0.234 ^{cb} (0.060)

FF/DF: Full fat/ defatted. M0/100/135: Unmicronized or micronized at 100°C/135°C. A0/65/85: no ethanol treated or 65% /85% ethanol washing

Values in the bracket means standard deviation

Columns with same low case letter means not significantly different (P<0.05)

Rows with same upper case letter means not significantly different (P<0.05)