CANOLA PROTEINS AND GLUCOSINOLATES: EFFECT OF PROTEIN ISOLATION AND POST-ISOLATION PROCESSING

BY

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

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Department of Food Science
University of Manitoba
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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DEDICATION

To my family, for their love and continual encouragement during this challenging journey of my life, and for teaching me to be strong in any circumstances.

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There are endless lists of people whose encouragement and assistance have made my life meaningful and brighter. There are several special significant people who have made the completion of this thesis possible. I have developed great admiration for Dr. S. Arntfield who was a key person in guiding my thesis direction, as well as, providing advice, inspiration, support, and patient during the course of this research project. Special thanks are also extended to committee members of Dr. A.W. Hydamaka and Dr. B.A. Slominski for their guidance and assistance. An on-going technical assistance, support and friendship were generously provided by Aniko Bernatsky and Jim Rogers. The invaluable contributions of these individuals will always be appreciated.

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ABSTRACT

Knowledge of the behavior of problematic factors, including the glucosinolates and their degradation products, is important if increased utilization of canola protein in human food is to be realized. Using a series of samples representing various stages in the protein micellar mass (PMM) process, the goals of this work were to determine how effective this process is in recovering protein and removing glucosinolates from low-temperature heated canola meal. Furthermore, the effects of post-isolation processing on the residual glucosinolates and glucose, and how these influence isolate color were examined.

In addition to the standard gas chromatography (GC) technique, the thiocyanate (SCN^-) ion determination and a diabetic test kit technique were used and evaluated as methods for assessing the glucosinolate levels in canola protein isolates. These latter two are based on the measurements of free SCN^- ions and the glucose released upon the glucosinolate decomposition, respectively. Our results showed a good agreement (R^2 = 0.93) between the GC method and the estimate of total glucosinolates from the diabetic test kit technique although an overestimation of glucose was observed possibly due to the presence of free glucose from other sources in the canola samples.

Ultrafiltration was an important stage in recovering canola protein, as well as reducing the glucosinolates. The Vivaflow 200 tangential ultrafiltration system was significantly better than the Amicon stirred cell system in achieving both functions, although removal of glucosinolate was not as great. Poor protein recovery (<12%) from canola meal has suggested that the PMM process is a selective technique for isolating only 12S salt-soluble globulins.

Processing conditions involving alkaline, high heat and low salt concentration significantly promoted the indole glucosinolates to decompose and release free SCN^- ions. Results on glucose level of protein isolates suggest a mixture of monosaccharides, including glucose, was readily formed when exposed to alkaline pH at low temperatures whereas the conversion from mono- to disaccharides took place in an acidic environment. A significant inverse relationship between lightness (Hunter color L* values) and glucose content, and a direct relationship between yellowness (b* values) and glucose content in the isolate could be traced to an undesirable Maillard reaction. The prevention of the glucosinolate decomposition and darker color caused by the Maillard reaction can be obtained by keeping the pH and temperature low.

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1.0 INTRODUCTION

Currently food scientists and the food industry have recognized canola as a potential source of food-grade plant protein. However, problem of underutilizing canola protein from the large volume of canola meal by-product must be overcome so that the protein can be consumed by humans thus increasing the profitability of the oilseed in food, and any other related industries. Despite the fact that plant breeders have successfully reduced the total aliphatic glucosinolates to no more than 30 μmole per gram of canola meal in canola varieties (Eskin and McDonald, 2000), glucosinolates are still one of the problematic factors present in canola. They are considered as antinutritional factors since some of their degraded products have significant goitrogenic and toxic effects in animals (Mithen *et al.*, 2000). Thus, the use of canola protein in humans has been prevented by these undesirable antinutritional factors. One protein isolation method, known as the protein micellar mass (PMM) process, has been developed to produce food-grade high quality canola protein. Employing only canola meal, salt and water, the PMM isolation method is claimed to be a mild and selective technique to isolate native protein with low levels of antinutritional factors (Burgess, 1991).

The three main stages of the PMM procedures, extraction, ultrafiltration, and dilution and precipitation, were examined to understand mass balance of protein and glucosinolates in the isolation process. NaCl, claimed as the most frequently used solubilizing agent (Burgess, 1991; Ismond and Welsh, 1992; Léger and Arntfield, 1993; and Murray, 2001), was used to extract canola protein. Following the extraction stage, the salt-extracted canola protein was concentrated and purified by ultrafiltration.

Inclusion of an ultrafiltration system into the PMM procedure has proven to be efficient in removing glucosinolates with minimal loss of proteins (Tzeng *et al.*, 1990a). Two types of ultrafiltraition systems, including stirred ultrafiltration cell and tangential flow ultrafiltration, were compared. By reducing the ionic strength of precipitation environment with the optimum dilution factor of one to six (Burgess, 1991), precipitation of the purified salt-extracted canola protein was the final stage of the PMM procedure. It is hoped that the PMM isolation process can result in higher canola protein recovery with minimal recovery of glucosinolates. Therefore, the first objective of this work was to demonstrate a mass balance on canola protein and glucosinolates during the PMM procedure. Evaluation of protein and glucosinolates as value-determining compounds are of particularly significance. This study also focused on qualification and quantification of total and individual glucosinolates, including the aliphatic and aromatic glucosinolates.

Glucosinolates in canola products can be determined through direct determination or indirect measurements of glucosinolate degradation products (Szmigielska *et al.*, 2000). Gas chromatography (GC) was selected as the direct method to determine the nature and levels of individual glucosinolates in the canola samples. The indirect methods measure the enzymatically or chemically released products, such as thiocyanate, isothiocyanate, nitrile, sulfate, and glucose, to estimate the glucosinolate levels (Szmigielska *et al.*, 2000). Applications of new methodologies such as using diabetic test kits are currently being evaluated to estimate the total glucosinolate level in canola (Vescio *et al.*, 2001). Accordingly, part of the objectives in this work was to evaluate the use of a diabetic test kit, as well as the thiocyanate (SCN^-) ion determination used by Campbell and Slominski (1990) to assess the extent of thermal

degradation of indole glucosinolates in canola seed and meal, as methods for monitoring the release of glucose and glucosinolate degradation products, respectively, by comparing to the standard GC technique.

Attempts have been made to apply a number of processing treatments, such as roasting, cooking and fermentation (Mahajan *et al.*, 1999), as a means to enhance the value of protein isolates in food systems in order to increase the acceptability of these proteins. However, the quality of protein isolates, including their physical and chemical attributes, may change upon the influence of processing conditions. Processing treatments can lead to great variation in glucosinolate degradation (Campbell and Cansfield, 1983; Sørensen, 1990), and degradation products are believed to exert a greater variety of antinutritional and toxic effects than the intact glucosinolates (Sørensen, 1990). Chemical changes of soluble sugars are another important chemical attribute of protein isolates to be evaluated upon processing as their presence is also claimed to affect the quality of plant proteins. Similar to glucosinolates and sugars, color, an important aspect of food acceptance, of protein isolates may change when subjected to processing treatments. As a result, another objective of this work was to examine the effects of processing factors on glucosinolate decomposition, change of sugar levels and color of canola protein isolates. Different pH, heat and salt concentration were used to demonstrate the behavior of canola glucosinolates and glucose in isolates, as well as isolate color, in potential food systems. In all, knowledge of protein and glucosinolates as value-determining components throughout the PMM procedures, and the possible glucosinolate decomposition and change of isolate color upon different processing conditions are important in order to achieve an optimum production of food-grade high quality canola protein isolates.

2.0 LITERACTURE REVIEW

2.1 Canola and Its Proteins

Canola, known in many countries as rapeseed, refers to cultivars of an oilseed crop that has a long history of use in Europe and Asia (Shahidi, 1990). As it is a cool season crop, canola has become one of the most valuable agricultural commodities in countries such as Canada, China and some European countries. Canada is the leading exporter of canola seed in the world, exporting an average volume of 3.4 million tons annually over the period 1997-2003, but is involved in less than 10% of the value-added processing in the world (Canola Council of Canada, 2003). Therefore, canola remains a potential source in Canada's food industry for value-added processing.

Canola is conventionally processed into oil and defatted canola meal by employing an extraction process. Oil is the main product where it is used as vegetable oil or a functional ingredient for the food industry. However, the meal, a major by-product that is used primarily for animal feed, plays an important role in determining the economic value of canola. Thus, the application and value of defatted canola meal has been considered for many years.

Interest in processing canola proteins for human consumption has increased due to the fact that production volume of the defatted canola meal is large and it contains a large amount of protein. The protein content of canola meal varies with variety and environment, but the average protein content is 40% (Shahidi, 1990). In addition, a continuing shift in consumer preference to plant protein over protein from animal sources has increased the potential commercial value of canola protein. Therefore, it

would seem that there is huge potential for application of this valuable source of protein in food.

2.1.1. Protein Isolation

Canola protein, more specify the 12S globulin, was first isolated by Bhatty and coworkers (1968) by extracting the defatted rapeseed meal with a NaCl solution, followed by a dialysis-precipitation against water and finally purifying the protein with chromatographic separation. Due to the fact that the 12S globulin is the major storage protein in canola seed, study of the isolation and subsequent characterization of canola proteins has focused primarily on the 12S globulin. Subsequently, numerous processes for isolating the 12S globulins have been developed and widely documented (Naczk and Shahidi, 1989; Tzeng *et al.*, 1990b; Chen and Rohani, 1992; Ismond and Welsh, 1992; Xu and Diosady, 1994; Klockeman *et al.*, 1997; Murray, 2001; Xu and Diosady, 2002). One of the current methodologies is the protein micellar mass (PMM) method that employs only canola meal, table salt and water. It is claimed to be a mild and selective technique to isolate native protein with low levels of antinutritional factors (Burgess, 1991). It should be noted that work in this laboratory demonstrated the use of PMM method to isolate canola protein, and is, therefore, be the main isolation method discussed in this chapter.

2.1.1.1 Protein Micelle Phenomena

Although the PMM process appears simple, the related chemical interactions are complex (Murray, 2001). In 1981, Murray and his co-workers demonstrated the

formation of a viscous gelatinous mass of protein called PMM from fababean. They attributed the formation of these mass to non-covalent interactions between the constituent proteins. This approach involved the extraction of the protein in a high salt environment followed by the formation of insoluble PMM through a rapid reduction of the ionic strength of their surrounding environment. As proteins are amphiphilic molecules, they would self-associate to form microscopically large protein spheres, known as micelles, and hence the name was used. This approach has since been applied to the isolation of canola proteins (Burgess, 1991; Ismond and Welsh, 1992).

In a polar aqueous environment, hydrophobic interactions, which are non-covalent protein-protein interactions, play an important role in micelle formation. Hydrophobic interactions arise from an energetically unfavourable relationship between water and the non-polar residues of a protein (Nakai and Li-Chan, 1988). In order to reduce the entropic changes in the system, the protein is forced to fold into micelles with the non-polar residues coalesced together inside and the polar residue on the outside. Burgess (1991) has further concluded that the hydrophobic interactions are the non-covalent driving forces for the formation of these protein micelles.

Several studies have concluded that the formation of micelles is strongly influenced by the initial amount and type of protein residue, pH and ionic strength of the surrounding milieu, and the dilution factor used (Murray *et al.*, 1981; Ismond, 1984; Welsh, 1988; Burgess, 1991). In general, proteins with high numbers of hydrophobic amino acid residues have good micelle forming capacity. A comparison of several oilseeds proteins is shown in Table 1, where the 12S globulin from canola has the highest average hydrophobicity ($H\emptyset_{ave}$) and non-polar side chain frequency (NPS), as well as the lowest polarity ratio (P). As a consequence, the 12S canola globulin is more

hydrophobic, and therefore has greater potential to form micelle compared to other oilseed proteins.

Table 1. Hydrophobicity values of various 12S plant globulin

Source of 12S plant globulin	Average hydrophobicity, $H\emptyset_{ave}$ (cal/residue)	Non-polar side chain frequencies, NPS	Polarity ratio, P
Canola	1041	0.36	1.01
Sunflower	951	0.31	1.21
Soybean	944	0.30	1.35

Adapted from Schwenke *et al.*, 1981.

2.1.1.2 Protein Extraction, Recovery and Purification

As mentioned earlier, this discussion will focus on the PMM method of isolating canola protein, and therefore, special consideration will be given to the protein extraction using salt, and precipitation upon dilution, as well as recovery or purification process of the extracted protein. There have been a number of methods used to produce canola protein employing extreme treatments involving acids and alkalis. For example, Tzeng *et al.* (1990b), Chen and Rohani (1992), Xu and Diosady (1994), Klockeman *et al.* (1997), and Aluko and McIntosh (2001) had isolated canola protein by alkaline extraction. The choice of alkali has become a preferred route for number of researchers due to the fact that higher protein is yielded as higher solubility of canola protein is associated with higher pH values. Acidic precipitation has often been used to recover the extracted 12S globulin (Gillsberg and Turnell, 1976a; 1976b; Gillsberg, 1978). These acidic and alkaline chemicals may reduce the nutritional value and functional properties of the protein and also provide environmental challenges for clean-up and disposal

(Murray, 2001). Therefore, PMM method is an alternative route to produce high-quality canola protein in an environmental-friendly way.

A number of salt solutions have been used to extract protein by manipulating surface hydrophobicity of protein in micelle formation (Burgess, 1991). NaCl is a moderately stabilizing salt that produces a desirably strong micelle response (Burgess, 1991). Also, as it is an inexpensive, readily available and a recognized food ingredient, NaCl is used most frequently as a solubilizing agent in canola protein isolation (Burgess, 1991; Ismond and Welsh, 1992; Léger and Arntfield, 1993; and Murray, 2001). As a result, NaCl was selected as the milieu to extract canola protein for this study. However, Klockeman *et al.* (1997) have reported that only 51% of the canola protein was soluble in 5% w/v NaCl compared to 91% in 0.4% NaOH. This shows that canola protein is less soluble in salt solutions than in alkaline solutions.

Protein recovery has become an obstacle in achieving optimum production yield of canola protein isolates. According to Chen and Rohani (1992), the recovery of canola meal protein by precipitation at pH 3.7 averaged only 53%. Maximum precipitation has been reported to occur at a range of pH values including pH 4.0 (Aluko and McIntosh, 2001), pH 3.7 (Chen and Rohani, 1992) or pH 3.5 (Klockeman *et al.*, 1997). Alternately, while precipitating in this pH range, the addition of sodium hexameta phosphate (SHMP) (Chen and Rohani, 1992) or $CaCl_2$ (Tzeng *et al.*, 1990b) during protein isolation was found to improve the recovery of canola protein. As a result, it is hoped that the inclusion of the dilution technique for salt-extracted canola protein (Burgess, 1991; Ismond and Welsh, 1992; Léger and Arntfield, 1993; Murray, 2001) by reducing the ionic strength of precipitation environment can result in higher canola protein recovery.

According to Burgess (1991), extraction of the 12S globulin from defatted canola meal was optimal in 0.5 M NaCl at pH 6.0. Formation of the micelle did not occur at extreme pH conditions, pH 4 or 9, due to the strong repulsive forces created as a result of an increase in net surface charges. Following the extraction step, the amount of aqueous solvent used to dilute the high salt protein extract in order to bury the hydrophobic residues of the protein is also critical for micelle formation. As a result, Burgess (1991) also suggested that the micelle response is optimal at a dilution factor of 1:10 if the pH of the protein milieu was at pH 6. Thus, it has been concluded that hydrophobic interactions with a good balance of hydrophobic-hydrophilic force are critical for micelle formation.

After the protein is extracted, it is necessary to concentrate and purify canola protein prior to the precipitation step in order to allow the protein to interact and form micelles during precipitation (Ismond and Welsh, 1992; Murray, 2001). Ultrafiltration is an excellent purification process based on membranes with macromolecule-sized openings to allow permeation of water and small, low-molecular-weight dissolved components, while retaining large molecules (Tzeng *et al.*, 1990a). Therefore, it is an ideal process to concentrate the protein by separating low-molecular-weight contaminants. Incorporation of an ultrafiltration system into the protein isolation processes has proven to be effective in removing glucosinolates, with minimal loss of proteins (Tzeng *et al.*, 1990a). Ultrafiltration, in combination with a drying process of the supernatant following precipitation of canola proteins, has also been used to recover high levels of soluble protein representing the 2S canola protein in the supernatant (Tzeng *et al.*, 1990a; 1990b; Rubin *et al.*, 1990).

In addition to removing glucosinolates from canola protein isolates, there are other specific compounds that researchers try to reduce or eliminate as well. These include fiber, protease inhibitors, phytic acid and phenolics. Dehulling seems to be the most effective way to minimize excess fiber problem in most of the oilseed proteins. However, mechanical dehulling does not provide a good separation on small canola seeds (Niewiadomski, 1990) as the hull tends to adhere to the endosperm (Thakor *et al.*, 1995). Besides, dehulling is reported to adversely affect oil recovery (McCurdy, 1992), and, therefore, inclusion of the dehulling process is rarely applied in the canola seed industry. In most cases, protease inhibitors that cause reduced pancreatic activity and growth inhibition (Shahidi, 1997) are inactivated upon heating prior to consumption (Ceciliani *et al.*, 1994). However, Ceciliani *et al.* (1994) have reported that heating alone may not completely eliminate thermally stable protease inhibitors and, thus, their presence should be monitored during the preparation of any oilseed protein products. Phytic acid is capable of interfering with the availability of important divalent cations, such as zinc, iron, copper, and manganese, and as a result, high phytic acid content may decrease the bioavailability of essential minerals (Thompson, 1990). Early research has shown that minimal solubility of phytic acid is at the pH values of 10-12 (Gillsberg and Turnell, 1976a). Therefore, by extracting the protein from the canola meal at a pH value greater than 10, the formation of phytate, the salts of phytic acid, in the canola protein can be reduced due to the fact that the number of positively charged groups on the protein is decreased. However, despite the fact that PMM method isolates canola protein at a neutral pH, protein purification, such as ultrafiltration, seems to be the preferred route to reduce phytate levels in canola protein. Phenolic compounds in canola have become more of an obstacle in relation to their impacts on the flavour, color and mineral

availability in canola protein products (Shahidi, 1997). Not only effective in removing both intact glucosinolates and their breakdown products (Shahidi *et al.*, 1990a; Pecháček *et al.*, 2000), the use of a methanol-ammonia-water treatment has been also shown to be effective in significantly reducing the levels of phenolic compounds in oilseed protein isolates (Naczk and Shahidi, 1989; Wanasundara and Shahidi, 1994a; 1994b). More recently, Xu and Diosady (2002) has successfully used a treatment with Na_2SO_4 and polyvinyl pyrolidone (PVP) to decrease the levels of phenolic compounds to produce high-quality canola protein isolates. In all, it should be noted, however, that the glucosinolates will be the only antinutritional or toxic compounds to be addressed more in details and as they will be evaluated in relation to the quality of canola protein in this study.

The PMM method has been used to isolate canola protein experimentally for years (Burgess, 1991; Ismond and Welsh, 1992; Han, 1994). Efforts to modify the PMM method are still being undertaken to maximize the recovery of protein from canola while efficiently removing the undesirable compounds, including phytic acid, phenolic compounds and glucosinolates. While many researchers recognize the nutritional quality of canola protein, the potentially toxic or antinutritional factors must be addressed.

2.1.2 The Canola Protein

The canola protein consists of mainly salt-soluble globulins and water-soluble albumins (Norton, 1989). The albumins are the main metabolically active proteins. Representing 20% of total protein in the seed, they are responsible for the biosynthesis and degradation of globulins. On the other hand, as mentioned before, the globulins,

which are utilized as nitrogen reserves during germination, are considered to be the major storage proteins in the seed. In general, the globulin protein fractions from plant sources possess four components, named by their sedimentation coefficients, 1.7S, 7S, 12S and 15S or 17S (Bhatty *et al.*, 1968; McKenzie and Blakely, 1972). However, according to Norton (1989), the 7S fraction is rarely distributed in *Brassica* species, including canola. Also, Prakash and Narasinga Rao (1986) further confirmed that the 15S or 17S fraction is a result of polymerization of the 1.7S, 7S, or 12S rather than being inherently present in the seed. Although the 1.7S protein fraction accounts for 20% of the total protein in canola seeds, it is difficult to recover during the processing of canola concentrates and isolates (Bhatty *et al.*, 1968; Schwenke *et al.*, 1983). This is because the 1.7S fraction is a low molecular weight (12,000 – 18,000) protein containing two disulfide-linked polypeptides chain with an isoelectric point of 9.5 – 13.0 (Ericson *et al.*, 1986). Therefore, the 1.7S fraction is soluble in low ionic strength aqueous media, independently related to the pH, and is often discarded along with the supernatant during protein precipitation. As a consequence, the 12S fraction, which accounts for about 60% of the total seed protein, remains as the main protein fraction in the protein concentrate or isolate (Bhatty *et al.*, 1968). Therefore, as described earlier, production of canola protein is primarily based on the isolation of the 12S globulin.

Other components of the protein fractions have, also, been reported. Only 87% of the nitrogen is present in the form of protein. The rest is present in the form of a non-protein nitrogen fraction (Niewiadomski, 1990), which includes water soluble peptides and free amino acids. Myrosinase-binding protein is another example of a non-storage protein that exists in canola protein fraction (Falk *et al.*, 1995; Rask *et al.*, 2000). These proteins, in conjunction with glucosinolate-myrosinase system in canola, may have some

impact on the properties and level of isolated storage protein, and, therefore, should not be forgotten.

2.1.2.1 Properties of The 12S Canola Globulins

The 12S globulin from canola is a large, neutral, oligomeric protein with a molecular weight of 300,000 (Schwenke *et al.*, 1983). To form a spherical or globular structure, it is composed of six subunit pairs of polypeptide chains arranged as a trigonal antiprism. Each subunit pair consists of two symmetrical domains with a channel or cavity in the centre. The presence of these subunits was further confirmed by work of Léger (1992) with the use of SDS-PAGE.

Clearly, the subunit profile helps explain the capability of the 12S globulin to dissociate into sub-fractions (Schwenke *et al.*, 1983). Figure 1 illustrates the dissociation profile of 12S globulin, which depends on the pH, ionic strength and the presence of dissociating agents. The dissociation between the native 12S fraction and the 7S fraction is reversible and depends on the ionic strength. In contrast, the dissociation from 7S to 2-3S is irreversible under extreme conditions of pHs or in the presence of dissociating agents, such as urea. Also, these dissociation profiles revealed the 12S globulin was stabilized by noncovalent interactions, particularly entropically driven hydrophobic interactions, rather than by covalent disulphide bonds (Prakash and Narasinga Rao, 1986).

Uniquely, canola protein has a neutral isoelectric point, which is around pH 7 (Meith *et al.*, 1983a; 1983b) compared to pH 4.5 – 5.0 as is the case for other oilseed proteins (Prakash and Narasinga Rao, 1986). This difference has a large impact on

protein solubility where pH 4.0 and pH 8.0 have been reported to be the minimum solubility points for canola protein compared to a single minimal solubility around pH 4.5 for other oilseed proteins.

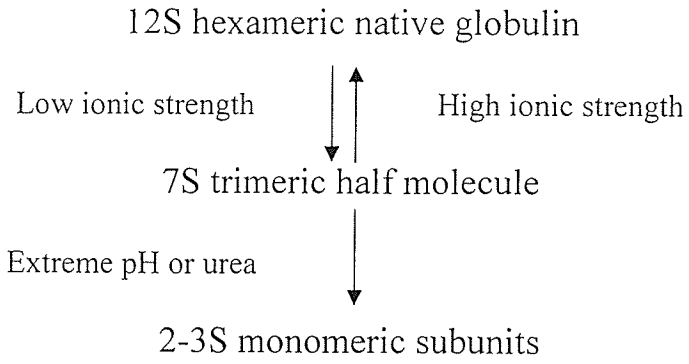


Figure 1. Dissociation mechanism of 12S canola globulin. (Schwenke *et al.*, 1983)

2.1.2.2 Nutritional and Functional Aspects of Canola Protein

The quality of protein in canola, assessed by its nutritional value, compares favourably with that of soybean. Table 2 shows canola protein having a well balanced amino acid profile with relatively higher level of the sulfur-containing amino acids, cysteine and methionine (Eskin and McDonald, 2000). Also, in terms of the protein efficiency ratio (PER), canola protein was estimated to have PER of 3.29, which is higher than that of casein (3.13) and soy protein (1.60) (Friedman, 1996). Therefore, the quality of canola protein has been demonstrated to approach that of soy protein, and PER of canola protein was far superior to soy protein.

Other than nutritional value, functional utility of any protein ingredient in food is a more important attribute in determining the commercial value of protein in the food industry. The application of proteins as functional food ingredients has increased

Table 2. Proximate analysis and amino acid composition of canola and soybean meals

	Canola meal		Soybean meal	
	As fed, %	In protein, %	As fed, %	In protein, %
Proximate analysis				
Moisture	8.0		11.0	
Crude fiber	12.6		7.3	
Ether extract	3.4		0.8	
Protein (N x 6.25)	39.0		45.2	
Amino acid composition				
Alanine	1.73	4.65	1.95	4.31
Arginine	2.32	6.15	3.03	6.71
Aspartic acid	3.03	8.16	5.27	11.66
Cystine	1.05	2.80	0.71	1.61
Glutamic acid	6.46	17.38	8.43	18.65
Glycine	1.92	5.18	2.06	4.55
Histidine	1.26	3.34	1.12	2.48
Isoleucine	1.66	4.38	1.82	4.03
Leucine	2.68	7.07	3.36	7.44
Lysine	2.21	5.95	2.82	6.24
Methionine	0.76	2.03	0.70	1.59
Phenylalanine	1.52	4.04	2.13	4.72
Proline	2.48	6.67	2.27	5.03
Serine	1.69	4.55	2.38	5.27
Threonine	1.68	4.52	1.74	3.85
Tryptophan	0.44	1.19	0.52	1.15
Tyrosine	1.14	3.02	1.33	2.95
Valine	2.14	5.68	1.89	4.18

Adapted from Eskin and McDonald, 2000.

dramatically in recent years. Like other proteins, canola protein possesses a variety of functionally properties. These include foaming, whipping, gel-forming abilities, emulsification capacity, and water- or fat-binding properties, which make canola protein a potentially key ingredient in many processed food products (Murray, 2001). For example, the use of canola protein as fat mimics can supply the functional roles of the fat influencing texture, flavor, water binding, foaming and emulsification properties in the reduced fat or fat free food categories.

While soy protein is being actively used, a number of studies have been undertaken to modify the canola protein to improve its functional properties to make it better suited for use in the human diet. For instance, Han (1994) found that canola protein with mild acid treatment for four h exhibited the highest solubility at pH 7.0, whereas 20 min of chymotrypsin enzymatic treatment caused the canola protein to have the highest solubility at pH 4.5. This result demonstrates that enzymatically modified protein can potentially be used as a functional ingredient in fruit juice.

2.2 The Glucosinolates-Myrosinase System

Many food scientists believe that soy meal has more economic value than canola meal (Klockman, 1996), because the value of canola meal is reduced by the presence of glucosinolates that can render it unpalatable and lead to toxic problems or digestive disorders in some livestock. This fact currently makes the application of the canola protein in food limiting. This is due to the fact that glucosinolates and their hydrolysis products may be extracted from canola meal and end up in the canola protein through the isolation process. Therefore, knowledge of the structure and mechanism of glucosinolate-

myrosinase system is critical in developing a method to remove the glucosinolates when producing high quality canola protein for use in feed and food.

The glucosinolate-myrosinase system includes sulphur-containing secondary metabolites called glucosinolates, degradative enzymes known as myrosinases and a variety of cofactors (Bones and Rossiter, 1996). Upon autolysis, following the mechanical damage, infection or insect attack, the stored glucosinolates are exposed to myrosinase and degraded into a wide range of degradation products. This complex glucosinolate-myrosinase system has affected the value of most members of the *Cruciferae*, including cabbage, broccoli, Brussels sprouts, canola and several types of condiments like horseradish and mustard. The presence of this system is a major problem in canola.

2.2.1 The Glucosinolate-Myrosinase System in Canola

Glucosinolates consists of a β-thioglucose moiety, a sulphonated oxime moiety and a highly variable side chains derived from amino acids (Figure 2). There are more than one hundred different side chain structures, which can be aliphatic, aromatic or heterocyclic (indolyl). Table 3 lists the common glucosinolates that are present in canola meal. Of these, 4-hydroxyglucobrassicin is reported to be the most quantitatively dominating glucosinolate in nearly all of the known double-low canola seeds (Sang and Truscott, 1984; Slominski and Campbell, 1987; Bjergegaard and Sørensen, 1996). However, it is also one of the more unstable glucosinolates, and easily oxidizes and decomposes in alkaline condition.

As they are strongly acidic compounds, all glucosinolates can only be handled as salts (Olsen and Sørensen, 1981). As a result, they are strongly hydrophilic owing to the salt and thioglucoside structure, and relatively unstable at extreme pH conditions. For instance, degradation of some of the glucosinolates would be promoted if the compounds are kept in alkaline solution for prolonged periods at elevated temperatures.

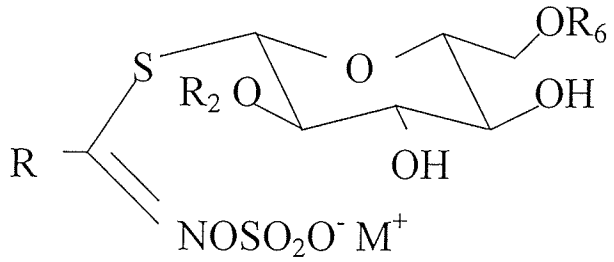


Figure 2. General structure of a glucosinolate molecule. R denotes the variable side chains; M^+ denotes cation. (Mithen, 2001)

Table 3. Side chain and trivial names for major glucosinolates[1] present in canola meal

Types of glucosinolate	Side chain of glucosinolate	Trivial name
Aliphatic	Prop-2-enyl- or allyl-	Sinigrin[2]
	But-3-enyl-	Gluconapin
	Pent-4-enyl-	Glucobrassicianapin
	2-Hydroxy-but-3-enyl-	Progoitrin
	2-Hydroxy-pent-4-enyl-	Gluconapoleiferin
Aromatic	*p*-Hydroxy-benzyl-	Glucosinapin[2]
Heterocyclic	Indol-3-ylmethyl- or indole	Glucobrassicin
	4-Hydroxyindol-3-ylmethyl- or 4-Hydroxyl indole	4-Hydroxylglucobrassicin

Adapted from Mawson *et al.*, 1993.

[1] Other minor glucosinolates include glucoraphanin, glucoalyssin, gluconasturtiin and neoglucobrassicin.

[2] Sinigrin and glucosinapin may be present in commercial meal due to weed seed contamination.

Glucosinolates are found in all parts of the cruciferous plants, but particularly in the seeds (Mithen *et al.*, 2000), and they can exhibit large variation throughout the entire food production chain. Their levels differ not only by tissue type, but also by physiological stage, plant health and nutrition. Therefore, there is a broad range of glucosinolate levels among the varieties, and even in the same variety of canola. Genetic and environmental factors determine their quantity in the original *Cruciferae* (Dekker *et al.,* 2000). However, the overall variation of glucosinolate levels also depends on the extent and nature of the processing, as well as packaging and storage conditions, of cruciferous foods.

Myrosinases, multiple forms of thioglucoside glycohydrolase E.C. 3.2.3.1, are found to accompany one or more glucosinolates in *Cruciferae* (Bones & Rossiter, 1996). Different isomeric forms exhibit different levels of glycosylation. All of these isomers are involved in catalyzing the hydrolysis of glucosinolates into the various products found in *Cruciferae*. Thus, it is important to consider the properties of myrosinase in relation to glucosinolate-myrosinase system.

Studies have speculated that myrosinases are located in the cytoplasm while glucosinolates are situated in the vacuole of specialized myrosin cells scattered throughout the plant tissue (Lűthy and Matile, 1984). Therefore, hydrolysis only occurs when tissue damage brings glucosinolates and myrosinases into contact. However, it should be kept in mind that no direct correlation between myrosinase activity and glucosinolate content has been found (Bones, 1990). Also, it has been found that some of the myrosinase isomers are present in complexes together with other proteins, such as myrosinase-binding proteins (Rask *et al.*, 2000). Eriksson *et al.* (2002) have further

confirmed that myrosinase-binding proteins are necessary for myrosinase complex formation in oilseed extracts, but do not affect glucosinolate degradation profiles and myrosinase localization.

In addition, it has been found that the myrosinase activity is potentially affected by factors such as pH, temperature, pressure and the presence of ascorbic acid (Ludikhuyze *et al.*, 2000). Thus it is necessary to control myrosinase activity with regards to the quality and safety of *Cruciferae* by changing their extrinsic factors such as pH and temperature.

In the presence of water, myrosinase catalyzes the hydrolytic cleavage of the thioglucosidic bond, giving D-glucose and an unstable aglycone known as thiohydroximate-*O*-sulphonate (Figure 3). The aglycone will rearrange non-enzymatically to produce a mixture of products. Possible breakdown products, such as thiocyanate, isothiocyanate and nitrile, result depending on such factors as types of glucosinolates, pH or the availability of cofactors in the *Cruciferae*.

In general, mixtures of products are formed. The formation of isothiocyanate is favored at neutral or high pH by means of the Lossen rearrangement (Ciska and Kozlowska, 1998). The Lossen rearrangement, however, would be blocked at low pH or in the presence of ferrous ions so that the formation of nitrile is promoted under these conditions. Epithionitriles can be produced when the epithiospecifier protein, a small protein of molecular weight 30 to 40 kDa, associated with ferrous ions is present (Bones and Rossiter, 1996). Only allyl-, benzyl-, and 4-methylthiobutyl- of the naturally occurring glucosinolates undergo enzymatic degradation to produce thiocyanate. All of these major breakdown products may spontaneously further degrade into a larger variety

of products. An example of this is the formation of goitrin, which results from spontaneous cyclization of 3-butenyl isothiocyanate (Duncan, 1991).

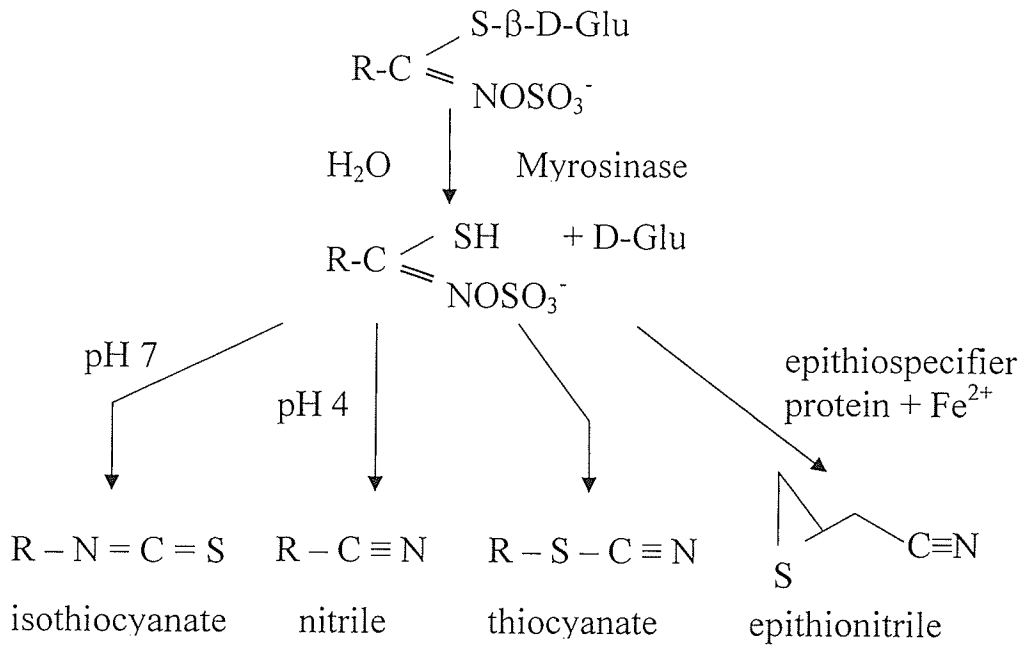


Figure 3. Myrosinase catalyzed degradation of glucosinolate to give isothiocyanate, nitrile, thiocyanate and epithionitrile. (Bones and Rossiter, 1996)

2.2.2 The Antinutritional and Toxicological Effects of Glucosinolates

Due to the fact that most of the breakdown products are more harmful than the intact glucosinolates, glucosinolate degradation during processing must be avoided (Sørensen, 1990). Many researchers agree that glucosinolate breakdown products exert a variety of antinutritional and toxic effects in higher animals (Mithen *et al.*, 2000). These adverse effects have limited the use of *Cruciferae* in human consumption. This has also restricted the utilization of canola meal in feedstuffs for livestock.

The adverse effects on thyroid metabolism have been the most thoroughly studied (Heaney and Fenwick, 1995). Although the mechanisms of actions are different, both goitrin and thiocyanate, which are degraded from glucosinolates with an aliphatic side-chain containing beta-hydroxyl group, are goitrogenic (Mithen *et al.*, 2000). Goitrin inhibits thyroid functions by interfering the incorporation of iodine in the reactions leading to the formation of thyroid hormones, including thyroxine and diiodotyrosine. On the other hand, thiocyanates are iodine competitors. For both, the growth rate and feed intake of higher animals are reduced. Apart from their goitrogenic properties, glucosinolate breakdown products may also induce other less specific toxic effects (Mithen *et al.*, 2000). Nitrile, for example, is considered toxic due to their biotransformation to free cyanides which can damage the kidney and livers (Duncan, 1991).

For years, plant breeding strategies have concentrated on reducing the glucosinolate contents of canola in order to meet increasingly stringent requirements from government regulatory institution, as well as the processing industry (Bones and Rossiter, 1996). To date, in Canadian oilseed industry, canola is a term referred as seed, oil, and meal from *Brassica napus* and *Brassica rapa* plants that contain no more than 30 μmole of total aliphatic glucosinolates per gram of oil free meal (Eskin and McDonald, 2000). Therefore, it is still a challenge for scientists or technologists to determine the role of glucosinolate-myrosinase system in today's oilseed production chain. In this particular case, armed with the knowledge on the complexity of glucosinolate-myrosinase system, it is hoped that food-grade high quality of canola proteins can be developed.

2.3 Method of Glucosinolates Determination

There is wide range of methods for glucosinolate determination, notably in canola, as glucosinolates have received more attention from analysts than any other constituent. However, method of analysis can affect the reported content of glucosinolates and serious problems with detection and quantitative determinations of some glucosinolates have been reported (Sørensen, 1990). Thus, there have been recent developments and improvements in methodology in order to develop a more simple, rapid, and reliable method for determining the glucosinolates.

Glucosinolates in canola can be determined through direct determination or indirect measurement of glucosinolate hydrolysis products (Szmigielska *et al.*, 2000). Gas chromatography (GC) and high-pressure liquid chromatography (HPLC) are the typical direct methods to determine the levels of individual glucosinolates. In indirect measurements, enzymatically or chemically released products such as thiocyanate, isothiocyanate, nitrile, sulfate and glucose are measured and used to estimate the total glucosinolate levels.

2.3.1 Chromatography

Analytical methods for direct determination of glucosinolates have been described elsewhere in the literature (Heaney and Fenwick, 1982; Daun and McGregor, 1983; Slominski and Campbell, 1987; Campbell and Slominski, 1990; Szmigielska *et al.*, 2000; Chevolleau *et al.*, 2002). As mentioned earlier, two typical direct methods are GC and HPLC. Due to the fact that HPLC is widely held and is able to provide successful applications, it is now an Official Method of the European Community, as well as the

approved method of the Canadian Grain Commission (Mawson *et al.*, 1993). However, as the GC was readily available for this laboratory work, it was selected as the direct method to determine the nature and levels of individual glucosinolates present in the canola samples.

In the early literature, GC was claimed to be unsuitable for the analysis of indole glucosinolates due to the fact that 4-hydroxyglucobrassicin, the major indole glucosinolate in canola, was unstable and poorly determined by GC (Daun and McGregor, 1983). There is now evidence showing that accurate quantification of all glucosinolates, including indole glucosinolates, present in canola is possible by using temperature programmed GC with proper control of extraction and isolation procedures (Slominski and Campbell, 1987). The modifications are based on the principle of determining the trimethylsilyl (TMS) derivatives of desulpho-glucosinolates that was first introduced by Underhill and Kirkland (1971). Over the years, the method of quantifying the desulpho-glucosinolates directly has gained wide acceptance because they tend to be more accurate and precise.

In a typical procedure, the sample preparation required before chromatographic analysis is complex. In defatted canola meal, glucosinolates are generally extracted using heat treatment or addition of methanol, which, at the same time, inactivates myrosinase enzyme in the meal (Szmigielska *et al.*, 2000). According to Slominski and Campbell (1987), substantial thermal degradation of indole glucosinolates has resulted from the wet heat treatment at 95 ^{o}C. As opposed to wet heat treatment, dry heat treatment had little or no effect on any of the glucosinolates. In this regard, it was recommended that 15 min dry heat treatment followed by three min wet heat treatment is required to determine effectively all of the glucosinolates. In addition, the selection of

an internal standard is essential to determine the effectiveness of glucosinolate analysis. In commercial canola meals, benzyl glucosinolate, known as glucotropaeolin, is a more appropriate internal standard than allyl glucosinolate (also known as sinigrin) for glucosinolate analysis (Daun and McGregor, 1983; Slominski and Campbell, 1987). This is because the possible presence of sinigrin, together with glucosinalbin, in the canola meal samples is probably the result of contamination with other cruciferous weed seeds. The level of contamination has been shown to vary, depending on the origin of the canola meal (Slominski and Campbell, 1987).

Following the extraction step, the crude glucosinolate extracts are purified on a traditional ion exchange column. DEAE-Sephadex A-25 suspension in a mini column is used as an anion exchange column. Desulphation is carried out overnight in the DEAE-Sephadex A-25 column with the addition of sulphatase in order to isolate desulpho-glucosinolates. However, it has been found that commercial sulphatase contains enzymatic activities other than glucosinolate sulphatase activity, and this may result in additional peaks in the GC chromatograms of desulpho-glucosinolates (Sørensen, 1990). Therefore, additional purification steps of commercial sulphatase are required prior to applying to the DEAE-Sephadex A-25 column. After standing overnight at room temperature, desulpho-glucosinolates are eluted with water or other selected solvents. Sufficient elution volumes are required to prevent the problems of underestimating the desulpho-glucosinolates.

TMS derivatization has to take place before the purified extracts can be injected directly into the GC. This is critical because it enables the samples to be vaporized and carried by the mobile gas phase (the carrier gas) through the column. Furthermore, Slominski and Campbell (1987) have confirmed the conclusion made by Heaney and

Fenwick (1982) that the use of high derivatization temperatures, as well as the use of temperature-programmed chromatography, is essential to separate and identify all of the glucosinolates in canola, including the indole glucosinolates.

In all, GC method coupled with several modifications has been shown to be adequate for the identification and quantification on the individual glucosinolates in canola seed and meal. Both GC and HPLC are efficient, reliable and accurate, but the sample preparation for these methods is time-consuming. Therefore, there has been considerable pressure from the canola industry for quicker methods to determine the total glucosinolate levels in order to discriminate between the quality of canola seeds or meals. Thiocyanate (SCN^-) ion determination and diabetic test kit techniques are two examples of indirect determination on glucosinolates.

2.3.2 Thiocyanate Ion Determination

The indole glucosinolates, notably 4-hydroxyglucobrassicin prevalent in canola, give rise to free thiocyanate (SCN^-) ions with a release of alcohol known as indolemethanol in the presence of myrosinase upon mechanical damage on plants' cells (Fenwick *et al.*, 1989). In addition to enzymatically hydrolysis, the formation of free SCN^- ions is possibly followed by a thermal degradation of indole glucosinolates (Slominski and Campbell, 1987; 1989).

The instability of indolemethanol in aqueous solution, where it converts to aldehydes, diindolylmethanes or ascorbigen (Slominski and Campbell, 1989), makes determination impossible. Therefore, determination of SCN^-, instead of the indolemethanol, is used to assess the degradation of indole glucosinolates (Johnston and

Jones, 1966; Srivastava and Hill, 1975, McGregor, 1978, Slominski and Campbell, 1987; 1989). For years, free SCN^- ion has been reported as a major hydrolysis product of indole glucosinolates.

Campbell and Slominski (1989) used this technique to assess the extent of thermal degradation of indole glucosinolates present in canola seed and meal during the heat treatment, which was used to inactivate myrosinase in the sample preparation for glucosinolate analysis using GC. The free SCN^- ions found in samples of seed and commercial canola meal is believed to be released from thermally decomposed indole glucosinolates. The desolventization step of commercial meal preparation may also release the free SCN^- ions from glucosinalbin. Thus, the SCN^- ion determination could be an adequate routine tool to assess the decomposition of indole glucosinolates upon processing.

Basically, the SCN^- ion determination technique is a simple colorimetric method by which the concentration of free SCN^- ions is determined by treating the sample in trichloroacetic acid (TCA) with a solution of $Fe(NO_3)_3$ (Johnston and Jones, 1966). Samples containing free SCN^- ions will react with addition of excess $Fe(NO_3)_3$ in HNO_3 to give raise the red ferric thiocyanate ($Fe(SCN)_3$). However, the $Fe(NO_3)_3$ will also react with the phenolic compounds in the canola samples and produce a yellowish coloration that interferes with the direct measurement of $Fe(SCN)_3$ concentration. This can be overcome by preparing duplicate aliquots of each sample solution and adding a little $HgCl_2$ to one of them to destroy the $Fe(SCN)_3$ and thus serve as a blank reading. The colorimetric estimation of SCN^- is based on the difference in absorptions between the sample solution and the blank. The absorption difference is proportional to the SCN^-

concentration in the sample. This technique determines only the free SCN^- ions released from decomposed indole glucosinolates.

However, the SCN^- ion determination can be combined with a myrosinase treatment to estimate the total amount of indole glucosinolates present in the sample. This is evident by the good agreement for the determination of indole glucosinolates between GC method and SCN^- ion determination technique with myrosinase treatment obtained by Slominski and Campbell (1987). In the presence of the myrosinase enzyme, bound SCN^- is enzymatically released from intact indole glucosinolate. As a result, SCN^- colorimetric technique determines the absorption of total SCN^-, the sum of free SCN- ions and previously bound SCN^-, and estimates the corresponding indole glucosinolates in the samples.

2.3.3 Diabetic Test Kit Technique

Measurement of the glucose released on hydrolysis of glucosinolates is another indirect method which indicates only the total amount of glucosinolates in a sample. As mentioned earlier, glucose with other breakdown products results from the glucosinolate-myrosinase system upon the mechanical damage of cells of plant materials.

In this regard, there are many methods based on the estimation of released glucose from glucosinolates followed by hydrolysis with myrosinase (Smith and Dacombe, 1987; Heaney *et al.*, 1988). However, the use of myrosinase is claimed to limit the speed of these methods (Mawson *et al.*, 1993). Brzezinski and Mendelewski (1984) have, therefore, avoided the use of myrosinase, and estimated the glucose released by hydrolysis of glucosinolates with H_2SO_4. Similarly, the use of H_2SO_4 allows

the released glucose to react with phenolic compounds to produce chromophores. In addition, carefully controlled use of glucose test tapes (Smith and Donald, 1988) and enzyme biosensors (Koshy *et al.*, 1988) are reported to be able to provide adequate and quicker means for screening breeders' lines. All of these methods are considered as sensitive but less specific than others like the SCN^- ion determination (Mawson *et al.*, 1993). Consequently, further developments and improvements of these methods are necessary. One issue that must be addressed is the problem of the inhibition of myrosinase by contaminants.

A recent study using diabetic test kits to estimate the level of glucosinolates (Vescio *et al.*, 2001) is encouraging and it is expected that this technique will meet the requirement of oilseed breeders. Diabetic test kits technique is a rapid, easy-to-use and inexpensive method for glucosinolate determination in oilseeds, such as canola and mustard. These diabetic test kits can be either glucose sensitive reagent strips or modern glucometer, which are used to determine glucose level in urine or in blood, respectively (Vescio *et al.*, 2001). Similar to other methods of glucose measurement, glucosinolate estimation using diabetic test kits is done by determining the glucose released from glucosinolate when samples are hydrolyzed in the presence of water (one mole of glucosinolate releases one mole of glucose), due to endogenous myrosinase.

The active agents, including glucose oxidase and peroxidase, in the glucose sensitive reagent strips will react with a chromophore to change the color of the strips (Vescio *et al.*, 2001). Bayer Diastic strips is one example of strips that can give a reasonable semi-quantitative estimation for glucosinolate level but does not give a stable and noticeable color change in the range of 8 μmol/g to 20 μmol/g (Vescio *et al.*, 2001).

Furthermore, due to the fact that its reactive components are separated from the surface of the strips by plastic, it produces non-uniform color that makes it difficult to read. The color, also, becomes darker if the exposure time is longer than the ideal time of one min after the strip is tested. As a result, the use of reagent strips would be recommended for plant breeding selection only when the number of samples is very large.

On the other hand, the glucometer estimates the glucose based on a glucose sensor electrode (Vescio *et al.*, 2001). Although it costs more than strips, it provides more stable readings and a wider range of glucosinolate levels from 10 to 30 μmol/g. A good agreement was obtained for the determination of total glucosinolate levels by glucometer and by glucose release method or NIR spectrometer method (Vescio *et al.*, 2001). Occasional errors in reading, however, might happen due to seed particles blocking the capillary of glucometer. These errors can be reduced by having three or more replicates. Therefore, it is expected that total glucosinolate levels of canola samples could be easily and rapidly estimated with the use of diabetic test kit and the results will be comparable to the results from the GC method used in this laboratory work.

2.4 Color and Canola Protein

Even if the canola proteins are approved as safe-to-consume food grade products, the overall consumer acceptability is also determined by the appeal of the products to the consumers. Therefore, protein isolation is only the first step towards the successful expansion of the market for canola protein. One of the most important aspects of food acceptance is the color of the food product. Color is an interpretation by the brain of the

light signal coming from an object (Francis, 2003), and, thus, it is considered as a major physical attribute of a food product.

The dark color of canola meal, or the protein isolated from canola meal, is reported to be related to the presence of phenolic compounds in canola meal or protein (Rubino *et al.*, 1996; Xu and Diosady, 2000; 2002). According to Xu and Diosady (2002), removal of phenolic compounds has been confirmed to improve the color, as well as the taste, of canola protein isolates. This is mainly because, on oxidation, quinones are formed from phenolic compounds and these readily react with proteins to cause dark green or brown colors in protein solutions, and the color remains in the protein isolates after the proteins are precipitated at their isoelectric points (Sosulski, 1979). However, limited research is found in the literature relating to color changes in protein isolates upon post-isolation processing. It is proposed that decomposition of glucosinolates may be linked to possible color changes in canola protein isolates upon processing.

One way to describe color is to express it in terms of the coordinates of a color solid (Francis, 2003). The L* a* b* system, where L = lightness or darkness, +a = redness, -a = greenness, +b = yellowness, -b = blueness, is popular as a research or quality control tool to determine color in food products. This is because coordinates of L*, a*, and b* are easy to visualize and more uniform throughout the color space (Francis, 2003). Although the data are empirical, it is useful to characterize or control the color of the products. As a result, the L* a* b* coordinates were used to examine the color of canola protein in the present work.

2.5 Processing of Canola Protein

Efforts have been made to increase the acceptability of canola protein isolates so that protein isolates are safe to consume and ready to be used as a food ingredient. The behaviour of the protein when subjected to a number of processing treatments must be considered. Processing treatments, including roasting, puffing, pressure cooking, microwave cooking, germination and fermentation, have been evaluated as a means to improve functional properties of protein in canola meal (Mahajan *et al.*, 1999). Accordingly, these treatments may be the promising approaches to enhance the behavior of protein isolates in food system, and, thus, increases the acceptability of these proteins.

Unfortunately, not much work has been done to determine the influence of processing conditions on canola protein isolates when they, rather than canola meals, are subjected to a variety of processes. The quality of protein isolates, including its physical and chemical attributes, may be changed by these treatments. The study was, therefore, conducted to demonstrate the post-isolation processing effects on two such attributes, glucosinolate decompositions and color changes in isolates.

As described earlier, the glucosinolate content varies widely, not only due to differences between species, cultivars and environmental factors, but various types of processing effects can also affect the reported content. For instance, extraction of oil from canola leads to a variation of glucosinolate degradation ranging from 40 to 60% of the total (Campbell and Cansfield, 1983), and in some cases 4-hydroxyglucobrassicin is totally destroyed (Sørensen, 1990). Thermal decomposition that takes place during desolventizing and toasting of the extracted meal are of particular importance in

contributing to this reduction, by which nitriles and thiocyanate ions are believed to be the major decomposition products (Campbell and Cansfield, 1983).

It is reported that these processing conditions may denature myrosinase, stimulate the enzymatic breakdown of glucosinolates, and inactivate the enzymatic cofactors (Dekker *et al.*, 2000). More importantly, processing, particularly thermal processing such as blanching and cooking, allows non-enzymatic heat degradation of glucosinolates and breakdown products (Dekker *et al.*, 2000). One example would be the production of isothiocyanates and nitriles non-enzymatically from glucosinolates at higher temperature (Uppström, 1995). As a result, while the properties of protein isolates are being improved, processing effects may also be critical in terms of safety. It is important to limit the content of glucosinolates and to restrict their ability to degrade into other toxic breakdown products. In addition to glucosinolates, glucose levels of canola protein isolates could be altered as a result of various processing conditions.

Similar to glucosinolates and also glucose, the color of canola protein isolates may change upon processing. As mentioned earlier, the dark green or brown color of canola protein isolates is usually attributed to the presence of phenolic compounds under alkaline processing conditions. Accordingly, attempt to remove phenolic compounds during protein isolation procedures have been made (Rubino *et al.*, 1996; Xu and Diosady, 2000; 2002). In addition, heating of protein in the presence of active carbonyls, such as carbohydrates, can result in pronounced changes in the color of protein products, often as a result of the Maillard reaction or Strecker degradation (Fennema, 1986). In all, type and condition of the processing may play a crucial role in food quality, safety and characteristics of protein isolates. Undesirable changes do happen during the processing

in some situations, but, it is hoped that the advantages of processing on protein isolates are sufficient to outweigh the disadvantages.

3.0 MATERIALS AND METHODS

3.1 Canola Protein Extraction Method

Two series of samples representing various stages of canola protein isolation were collected from PMM methodology procedures (Figure 4) as adapted from Ismond and Welsh (1992), where the raw defatted canola meals (AL018; low temperature treatment during meal preparation) were obtained from BMW Canada located in Winnipeg, Manitoba. The samples collected included: I, canola meal; II, debris; III, first supernatant; IV, ultrafiltrated filtrate; V, ultrafiltrated retentate; VI, second supernatant; and VII, PMM. The ultrafiltration step for the first series was done by using an Amicon stirred ultrafiltration cell unit under a pressure of 60-70 psi through a XM100A Diaflo® ultrafiltration membrane (10,000 molecular weight cut-off). A tangential flow module of Vivaflow 200 ultrafiltration unit (Vivascience) under a pressure of 36 psi (in equivalent to 2.5 bars) was used in ultrafiltration step of the second series of samples through a 30,000 molecular weight cut-off of Regenerated Cellulose membrane. These two series of samples were compared in terms of recovery of protein and glucosinolates.

A 1:10 ratio of meal samples to NaCl solution was stirred to extract protein from raw defatted canola meal (I). Due to the difference of hold-up volume for samples in the two types of ultrafiltration units, 25 g of meal were mixed for one h in 250 mL of 0.5 M NaCl in the first series; whereas samples of meal weighing 100 g were mixed in 1 L of

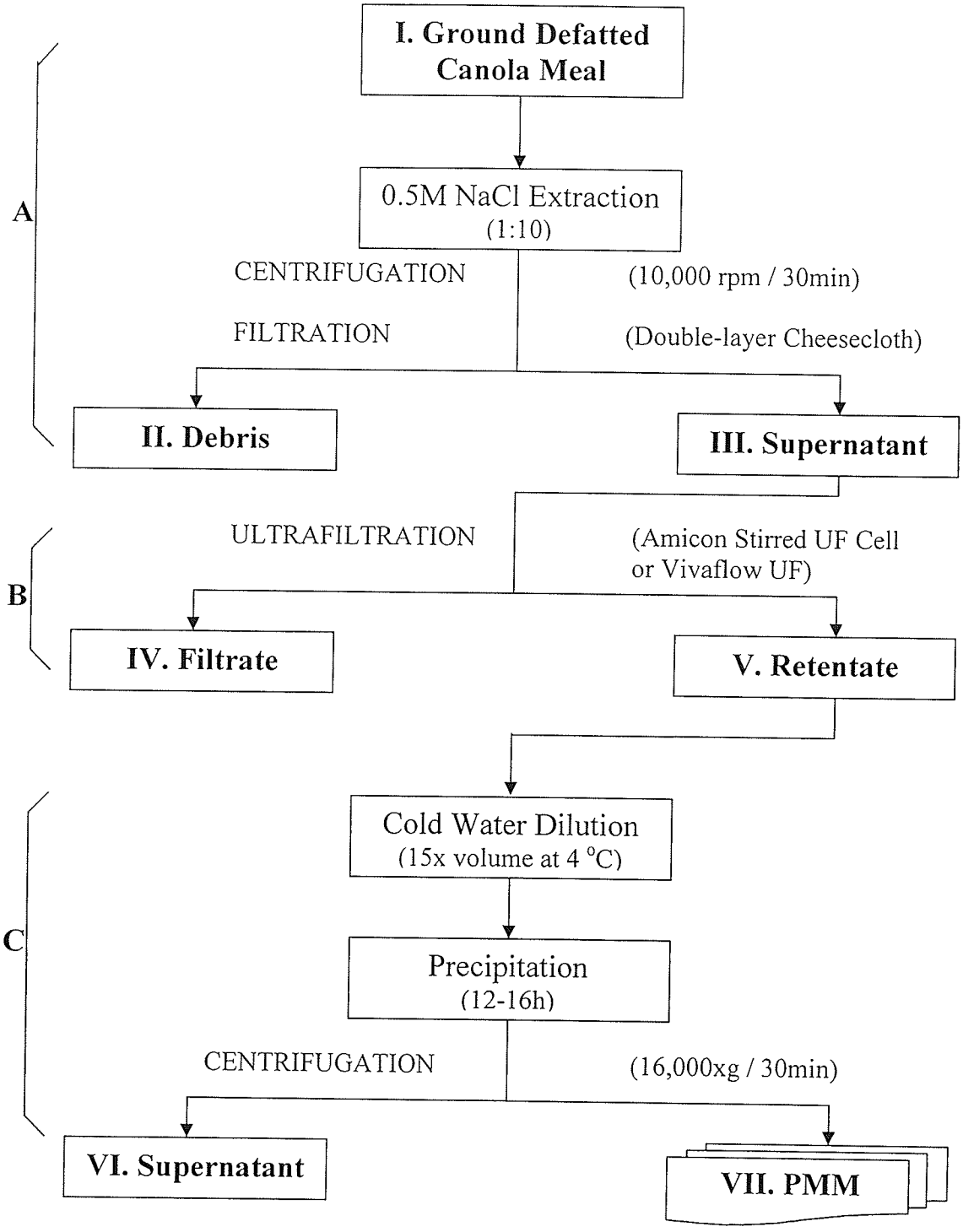


Figure 4. A schematic diagram showing the isolation of canola protein using the PMM process through A) extraction, B) ultrafiltration, and C) dilution and precipitation stages. Two series of samples (I, II, III, IV, V, VI, and VII) were collected for further analysis.

0.5 M NaCl for two h in order to prepare the second series of samples. Samples were then centrifuged using Sorvall Refrigerated Centrifuges, Model RC2-B and Model RC-3 for series one and two respectively, at a speed of 10,000 rpm for 30 min. This separated the seed and hull debris fraction (II) from the first supernatant (III) containing the solubilized protein. To remove any further debris, the supernatant was filtered through double-layered pre-moistened cheesecloth. Then, the supernatants of two series were concentrated through the corresponding ultrafiltration units. The volume of the supernatant was reduced at least four times to obtain retentate (V) containing concentrated, solubilized protein and the filtrate (IV).

The retentate was diluted to 15 times its volume with refrigerated distilled water and left overnight at 4-8°C to allow insoluble protein micelles to precipitate on the bottom of the dilution vessel. Approximately 16 h later, the protein micelles were collected by means of centrifugation using the same centrifuge that was used at the earlier step. After centrifuging at 10,000 rpm for 30 min, the decanted supernatant (VI) and the extracted protein (VII) were collected. All collected samples were frozen at – 40°C and freeze-dried (Freeze Mobile 6, Unitop 600L; Virtis).

3.2 Design and Method for Processing Commercial Protein Isolates

A two-level, three-factor full factorial design using pHs between 4 to 10, salt concentrations of 0.1 M to 1.0 M, and heating effects of 50°C to 100°C was created using Design Expert® Software (Stat-Ease Inc., MN) in order to examine the effects of three processing factors on glucosinolate decomposition and isolate color of canola protein isolates (CPI). The starting material of CPI (sample no. 040001) was obtained

from BMW Canada located in Winnipeg, Manitoba. A total of 18 samples, including duplicate samples, were produced (Appendix 1).

To prepare the samples, 10 g of CPI was mixed with 50 mL of NaCl solution at the required concentration and the pH was adjusted using either 1 M of NaOH or 1 M of HCl. Samples were left for at least 30 min, their pHs were monitored and was readjusted if necessary. This is because a short period of time is required for protein to be stabilized in acidic or basic conditions. Then, the samples were immersed in an oil bath to reach their required heating temperatures and maintained at that required temperature for four min. After heating, samples were freeze-dried and stored for further analysis.

3.3 Determination of Protein Content

Protein determination was performed on a micro-Kjeldahl distillation apparatus, Kjeltee System 1002 Distilling Unit (Tecator, Sweden), in accordance with the Kjeldahl methodology outlined in the AOAC Official Methods of Analysis (1975) with slight modifications. Total nitrogen is measured to obtain an estimate of protein content in a sample. In the micro-kjeldahl method, the sample is digested in acid by which nitrogen in the form of ammonia is recovered by distillation and measured by titration.

About 100 mg accurately weighed pre-dried samples of canola meal or canola protein extract were wrapped in cigarette papers and transferred to a 100 mL digestion flask. After placing the flasks on a heated digestion rack for one h, the samples were completely digested with 3 mL of concentrated H_2SO_4 in the presence of catalysts made up of K_2SO_4, TiO_2 and $CuSO_4$. A blank sample was prepared containing everything except the canola meal or protein samples. The flasks were then removed from the

digestion rack to cool down the samples. To each flask, 10 mL of distillated water was added to dissolve the remaining solids in the samples. Each of the digestion flasks was connected to the steam distillation apparatus while an Erlenmeyer flask containing 10 mL of H_3BO_3 was placed underneath the condenser in distillation apparatus. Once 8-10 mL of $NaOH/Na_2S_2O_3$ mixture of alkali was dispensed, the Erlenmeyer flask was lifted up in order to allow the condenser tip to extend below the level of H_3BO_3. The Erlenmeyer flask was removed from the distillation apparatus after about 50 mL of H_3BO_3 with distillate were collected. In the micro-kjeldahl method, the collected distillate was directly titrated from clear green color to light pink with standardized HCl acid solution at a concentration of 0.0294 N.

Percentage of protein was obtained from the percentage of total organic nitrogen present in the samples based on the following calculation:

$$\%\,\mathrm{Pr}otein = \frac{[(S-B)xNx14.007]x100}{mg\_Sample} x5.80$$

where S = mL of HCl for the sample
B = mL of HCl for the blank
N = Normality of HCl
5.80 is the Kjeldahl conversion factor for oilseeds such as canola.

3.4 Analysis of Glucosinolates Using Gas Chromatography

The type and level of glucosinolates were determined by gas-liquid chromatography as desulpho-trimethylsilyl (TMS) derivatives in accordance to the modified method described by Slominski (1995) which is derived from Slominski and Campbell (1987). Due to the fact that different chemical structures of various

Table 4. Relative response factor (RRF) of major glucosinolates in canola

Glucosinolates	RRF
Prop-2-enyl-	1.06
But-3-enyl-	1.02
Pent-4-enyl-	0.98
2-Hydroxy-but-3-enyl-	0.91
2-Hydroxy-pent-4-enyl-	0.88
p-Hydroxy-benzyl-	0.91
Indol-3-ylmethyl-	0.94
4-Hydroxyindol-3-ylmethyl-	0.90

Adapted from Slominski, 1995

glucosinolates affect the flame ionization detector response, updated relative response factors (RRF) calculated by Slominski (1995) from the ratios of TMS carbon number for benzyl glucosinolate, an internal standard, and the respective glucosinolate TMS carbon number were used. Variations in the level of the different glucosinolates were accounted for by the use of these RRFs, which are shown in Table 4.

In this study, gas chromatographic analysis of glucosinolates was carried out using a Varian Aerograph Model 3700 gas chromatograph equipped with a flame ionization detector and a Hewlett Packard Integrator model 3390A (Mississauga, ON). A glass column (1.2 m x 2 mm i.d.; Supelco, Bellefonte, PA), which was packed tightly with liquid phase of 2% OV-7 on an inert solid support of diatomaceous earth (Chromosorb W®,AW-DMDCS, 100-200 mesh) coated with a thin film of liquid, was used with He as the carrier gas flowing at a rate of 20 mL/min. The oven temperature

was kept at 185°C for 4 min, and then increased at 3°C/min to 275°C. Temperatures for injection port and detector were 280 and 300°C respectively.

A summary of the function of a GC is included as careful control of all conditions is necessary in this assay. An inert carrier gas (in this case, He), also known as mobile phase, flows continuously through the injection port, the column, and the detector; while at the same time, air and N_2 flow into detector to create the flame used in the flame ionization detector. Once the sample is injected into the heated injection port, it is vaporized and carried into the column. The sample partitions between the mobile and stationary phases and is separated into individual components based on relative solubility in the liquid phase and relative vapor pressure (McNair and Miller, 1998). Once the carrier gas and sample reach the detector, the detector measures the quantity of the sample and passes the signal to the integrator to generate a chromatogram. Several important components or procedures would be discussed in greater details.

i. Carrier gas & flow control: It is important that He be of high purity because impurities such as oxygen and water can destroy the stationary phase in the column (McNair and Miller, 1998). The measurement and control of the He is also critical in terms of ensuring the production of reproducible chromatograms. In this study, comparison of retention times was used for identification of individual glucosinolates. Therefore, a constant and reproducible flow rate was essential in ensuring the retention time could be reproduced.

A soap-bubble flowmeter was used to measure flow rate of the He. It is a modified pipette through which the carrier gas flows. By squeezing a rubber bulb at the bottom of pipette, a soap solution was raised into the path of the flowing gas. After several bubbles, one bubble was accurately timed by a stopwatch as it traveled through a

defined volume and the flow rate was calculated in mL/min. The flow rate of He was kept at 20 mL/min.

ii. Column conditioning: Proper column preparation is necessary for ensuring separation of compounds in a GC. Column conditioning is an essential step to achieve a more accurate GC analysis by removing unwanted or excess impurities in a new column. To prevent contamination of the detector, the column was not connected to the detector during conditioning. The column was then purged for 30 min at the established flow rates of approximately 20mL/min and at ambient temperature. Following the initial conditioning for 30 min, the oven temperature was programmed to increase at 2°C/min to approximately 20°C above the maximum expected analysis temperature of 275°C. It must be noted that the oven temperature should not exceed the maximum temperature of the liquid phase of OV-7 (350°C). The column was held overnight at this temperature. After approximately 16 h, the column was cooled to ambient temperature and the carrier gas was then turned off. The column was ready for use and was installed properly to both injector and detector ports. It should be also noted that the column should be conditioned regularly to avoid excess impurities.

iii. DEAE-Sephadex A-25 column: Purification of the glucosinolate extract for GC sampling was improved with the aid of a DEAE-Sephadex A-25 ion exchange column. A DEAE-Sephadex A-25 suspension was prepared from the powder (Sigma A-25-120, St. Louis, MO) by stirring in distilled water. The Sephadex material was then soaked for one h in distilled water with a volume equal to twice that of the resin.

Mini columns were prepared by shortening and flame polishing Pasteur pipettes and adding plugs of glass wool at the bottom. The DEAE-Sephadex A-25 suspension was transferred to each column until it reached a height of 8 mm from the upper line of

glass wool, and was then washed with distilled water. The DEAE-Sephadex A-25 columns immersed in water were kept in the refrigerator.

Once the sample had passed through the DEAE-Sephadex A-25 column, it was required to regenerate the column to ensure that it was in pyridine acetate form. One mL of distilled water, followed with 1 mL of 0.5 M NaOH, was passed through the column. After washing the column three to four times with 1 mL of distilled water, 1 mL of 0.5 M pyridine-acetate was passed through. Following three to four times washings with 1 mL distilled water, the DEAE-Sephadex A-25 column was regenerated and ready for use.

iv. Sample preparation: Ground samples weighing 100 mg were deposited in small Erlenmeyer flasks. Two mL of methanol, 1 mL of each internal standards (IS; 0.5 μmol/mL) of benzyl glucosinolate (glucotropaeolin; Raneylab, AG Canada, SK) and allyl glucosinolate (sinigrin monohydrate from horseradish; Sigma S-1647, St. Louis, MO), and 0.1 mL of 0.6 M lead-barium acetate were added to the samples and shaken for three h. This extraction step was modified by Slominski (1995) in order to more efficiently extract the glucosinolates and inactivate the myrosinase, while adding the internal standards. After this time period, the mixture was centrifuged at a speed of 3,000 rpm for 10 min. One mL of the resulting supernatant was applied to a DEAE-Sephadex A-25 column (pyridine acetate form), which was then washed with 1 mL of 67% methanol, 1 mL of distilled water and 1 mL of 0.02 M pyridine acetate. 0.05 mL of purified sulphatase solution (from sulphatase EC 3.1.6.1, type H-1; Sigma S-9626, St. Louis, MO) was added to the column and the contents were allowed to stand overnight at ambient temperature.

The following day, desulphoglucosinolates were eluted with four washings of 0.6 mL of 60% methanol and combined in a single vial. The contents of the vial were dried

using a stream of N_2 at 60°C. After drying, 200 μL of premix containing acetone, BSA (N, O-bis-trimethyl-silyl-acetamide; Supleco 33037-U, Bellefonte, PA), TMCS (trimethyl-cholorosilance; Supleco 3-3014, Bellefonte, PA) and 1-methylimidasole (Sigma M-8878, St. Louis, MO) in a ratio of 4:2:0.2:0.1 v/v was added to the vial, which was capped immediately with Teflon-lined caps and let sit for 30 min. These derivatized desulphoglucosinolate samples were then ready to be analyzed by GC. Based on preliminary testing, 12 μL of the samples produced reproducible peaks on a chromatogram. Therefore, 12 μL of the derivatized desulphoglucosinolate samples were separated using the Varian Aerograph Model 3700 gas chromatograph.

v. Sampling & injection port: In this study, our samples were prepared in a liquid form so that they were ready to be vaporized once they were injected into the heated injection port. Syringes are popular methods for injection of liquids. A 25-microliter liquid syringe (RN needle; Hamilton Co., Reno, NV) was used to inject 12 μL of sample in this study. It is important to exclude all air before filling the syringe with liquid. To inject the samples, the needle was inserted rapidly through a self-sealing septum (Thermogreen™ LB-2, Supleco, Bellefonte, PA) and pushed as far into the injection port as possible. After the plunger was depressed, the needle was withdrawn as rapidly as possible while the plunger depressed. Note that unskillful injection often ended up with poor resolution or non-reproducible chromatograms. In addition, the syringe must be cleaned after each injection to avoid extra peaks in chromatogram. This was done by repeatedly washing with methanol.

Septa are self-sealing and made of high-temperature stable polymeric silicone (McNair and Miller, 1998). A complete syringe injection was accomplished only if the

septum was in good condition. Therefore, the septum was changed regularly to avoid leaking of the samples and carrier gas.

vi. Programmed temperature: Effective programming of the temperature in a GC is a very effective method for optimizing a GC analysis. This can be accomplished by increasing the column temperature during a GC run resulting in a better separation of compounds with wide boiling points. Higher temperatures also improve detection limits, peak shape and precision, especially for late eluting peaks (McNair and Miller, 1998). Following a number of preliminary tests, it has found that by keeping the oven temperature at 185°C for four min, then increasing it at 3°C/min to 275°C gave the best resolutions of the peaks, although the total analysis time was about 32 min. The initial oven temperature was set lower than that used by Slominski and Campbell (1987); the main reason for doing this was to get rid of the interfering peaks eluting at an early retention time.

vii. Detector: In this study, the GC was equipped with a flame ionization detector. After approximately 70 runs, the flame in the detector went out regularly making it impossible to complete the analyses. Surprisingly, the detector was found to be blocked by layers of white powder, which are believed to be by-products of derivatization agents from the samples. As a result, it was necessary to check and clean the detector on a regular basis.

viii. Integrator: A Hewlett Packard Integrator (model 3390A) was used to produce both the chromatogram and a report for quantitative analysis. It was set to calculate the area percent rather than height percent of each peak in the quantitative report. Based on preliminary runs, an alternative factor for peak height was set at 2^1 so

that the smallest peaks of interest were large enough to be readily visible in the chromatograms.

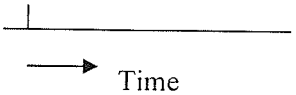
ix. Calculation: Levels of individual glucosinolate in the samples was calculated based on the report obtained from integrator and the following formula:

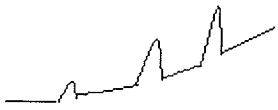
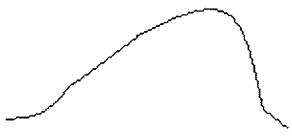
$$\text{Amt Z} = \frac{\text{Amt IS x Area Z x RRF x DF}}{\text{Area IS x Sample weight (g)}}$$

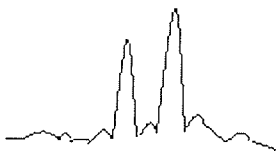
where Amt Z = μmol/g of glucosinolate Z in the sample
Area Z = Peak area of glucosinolate Z in the chromatogram
Amt IS = Amount (0.5 μmol/mL) of benzyl glucosinolate in the sample
Area IS = Peak area of benzyl glucosinolate in the chromatogram
RRF = Relative response factor of glucosinolate Z (refer to Table 4)
DF = Dilution factor

x. Trouble shooting: Table 5 has been inserted to show various chromatograms with different peak shapes that were encountered during GC analysis in this study. The time axis runs from left to right.

Table 5. Troubleshooting on GC system in this study

Symptom	Possible Cause	Checks
1. No peaks  	a. No carrier gas flow.	a. Turn carrier gas flow on. Replace gas tank if empty.
	b. Injector septum leaking.	b. Replace septum.
	c. Column connections loose.	c. Check leaks, tighten column connection. If ferrule crakes, replace one.
	d. Syringe leaking.	d. Replace the syringe.
	e. Flame out.	e. Check the detector. Clean the white residues that blocks the detector, and light the flame again.

Symptom	Possible cause	Checks
2. Smaller peak with normal retention time 	a. Insufficient sample size.	a. Increase sample size, check syringe to see if leaks.
	b. Poor sample injection skill.	b. Inject sample rapidly, review proper sample injection techniques.
	c. Syringe or septum leaking.	c. Replace syringe or septum.
	d. Carrier gas leaking.	d. Tighten the loose connection, replace ferrule if necessary.
	e. Attenuation is too high.	e. Reduce attenuation value in integrator.
	f. Sample is not well-prepared.	f. Prepare the sample again.
	g. Detector response low.	g. Check and clean the detector.
3. Raising baseline when temperature programming. 	a. Adsorption of impurities in column.	a. Condition the column overnight.
4. Peak split. 	a. Poor sample injection skill.	a. Inject sample rapidly, review proper sample injection techniques.
	b. Adsorption of impurities in column.	b. Condition the column overnight.
	c. Poor sample preparation.	c. Prepare the sample again.
5. Broad peak 	a. Sample size is too large.	a. Reduce injection volume.
	b. Adsorption of impurities in column.	b. Condition the column overnight.
	c. Peak width is too large.	c. Reduce peak width value in integrator.

Symptom	Possible cause	Checks
6. High background signal (noise) 	a. Carrier gas flow too high.	a. Reduce carrier gas flow rate.
	b. Air/N_2 flow too high or too low.	b. Adjust flow rate.
	c. Dirty injector.	c. Clean injector port and replace septum.
	d. Carrier gas flow leak.	d. Locate leak and correct.
	e. Peak width is too small.	e. Increase peak width value in integrator so that it can distinguish peaks from noise or each other.
	f. Threshold value is too large.	f. Set a threshold value above noise which integrator recognizes peaks.

3.5 Analysis of Glucosinolates Using Thiocyanate Ion Determination

The method of Slominski and Campbell (1989) was used for the analysis of thiocyanate ion in canola samples. The concentration of thiocyanate ion was determined colorimetrically by Ultrospec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech) in which potassium thiocyanate (KSCN) was used to prepare a standard curve.

(i) Free thiocyanate ion: 10 mL of distilled water was added to 0.5 g of each sample. After extraction for 30 min, 5 mL of 20% trichloroacetic acid (TCA) was added to precipitate the protein from the mixtures. The sample mixtures were then centrifuged using a Sorvall Refrigerated Centrifuge, Model RC2-B at 3,000 rpm for 10 min, and triplicate 3 mL aliquots of supernatant were mixed with 3 mL of 0.4 M $Fe(NO_3)_3$ in 1 N HNO_3. Two drops of 5% $HgCl_2$ solution were added to one vial to represent the blank sample. Readings were taken for all tubes within 1-2 min at 460nm.

(ii) Total thiocyanate ion: Use of exogenous myrosinase enzyme provided a tool to determine the thiocyanate ion released upon incubation of the samples. 0.5 g of samples was treated with 7 mL of distilled water and 3 mL of myrosinase solution (10 mg/mL, obtained from Department of Animal Science, University of Manitoba, Winnipeg, MB). Following incubation for two h, 5 mL of 20% TCA was added and total thiocyanate ion contents were determined as described for free thiocyanate ion determination.

(iii) Bound thiocyanate ion: The content of bound thiocyanate ion that was released upon incubation of the samples with the myrosinase enzyme was obtained by subtracting free thiocyanate ion content from that of total thiocyanate ion content.

3.6 Analysis of Glucosinolates Using Diabetic Test Kit Technique

The total amount of glucosinolates in a sample was determined using a diabetic test kit technique in which the glucose is released by endogenous myrosinase enzyme and estimated based on a glucose sensor electrode. The method was adapted from Vescio *et al.* (2001). At least duplicate analyses were carried out on all samples.

As the sample size was limited, 0.2 g of ground samples was used for this measurement. The samples were weighed into 25 mL Erlenmeyer flasks and mixed with 1.25 mL of distilled water with constant stirring on a magnetic stirrer. A preliminary test was conducted to determine the optimum time to detect the amount of glucose released from the samples. Result showed that 3-4 min after the water was added was the optimum detection time (Appendix 2). Therefore, after 3-4 minutes from when the water was added, a portion of the sample mixture was transferred to Pasteur pipette to obtain a

small drop of liquid. Liquid was then taken into the glucometer by capillary action through a glucose sensor electrode strip (One Touch® Ultra, Lifescan) and the glucose concentration was read after 30 s. A modern glucometer named One Touch® Ultra Blood Glucose Meter (I^{n}Dup™ Brand) was selected to be used in determining the glucose concentration of the samples in the range of 1.1-33.3 mmol/L. The glucose sensor electrode strip had to be changed after each sample reading.

3.7 Color Determination

The color of the samples was measured using a handheld Minolta Spectrophotometer CM-525i with a measurement area of 25 mm for measuring the spectral reflectance of small objects such as CPI. It is measured over the wavelength ranging from 400 to 700 nm, and a double-beam feedback system automatically eliminates for the effects of variation in the illumination from the pulsed xenon arc lamp.

Before the actual measurement, a pre-flash was automatically performed to determine the suitable intensity of light for measurements according to the reflectance of the specimen surface. When a measurement cycle is started, the xenon lamp fires at low intensity such that the light reflected from the specimen surface is used to determine the maximum spectral reflectance of the specimen surface, as well as the wavelength at which this occurs. In this way, only the necessary amount of light is used for measurements, thus improving the results and reducing the power consumption. High-reflectance specimens are measured using a small amount of light and vice versa. As the measurement cycle continues, the xenon lamp was fired again to take the actual measurement, with the intensity of the light adjusted based on the pre-flash results. Light

reflected from the specimen surface at an angle of 8° to the normal entered the optical fiber cable and was transmitted to spectral sensor. The spectral sensor consisted of two sections, named as the double-beam feedback system. One receives the light reflected by the specimen surface and the other section monitors the light inside the integrating sphere. In this way, it can compensate for slight variations in the illumination by calculation. The spectral sensor converted the received light into electrical currents in which are proportional to the intensity of light. These electrical currents were then converted to analog voltages and further to digital signals. Once the digital signals were received by the CPU, they were converted into standard Hunter L^*, a^*, b^* scale. For each measurement, approximately 1-2 g of samples were used.

3.8 Statistical Analysis

A t-test was used to compare the differences between results using the SAS® system (SAS Institute, Inc., Cary, NC) and at least duplicate analyses. The SAS® system was also used to determine correlation and simple linear regression to investigate the relationship between two variables. For those results processed using the full factorial design, the statistical analysis (particularly analysis of variance, ANOVA) was done by using Design Expert ® Software (Stat-Ease Inc., MN).

4.0 RESULTS AND DISCUSSION

4.1 Preliminary Analysis

Determinations of moisture, protein and total glucosinolate contents were performed on the starting commercial canola meal and the commercial PMM protein isolate. Not only to confirm their contents, these analyses were also used in later discussion to evaluate how the components of these starting materials changed as a result of the effect of the PMM procedure or post-isolation processing.

The results presented in Table 6 indicate that the canola meal contained 12.23% moisture, 39.43% protein and 38.44 μmol/g of total glucosinolates; while moisture and protein content in the commercial PMM isolates were 9.01% and 91.16%. Higher moisture contents than those supplied with the materials were observed for both the canola meal and commercial PMM isolates. The meal may absorb water from its surrounding environment causing the moisture content to increase from 9.73% (value determined by BMW Canola) to 12.23% before the initiation of this research project. However, canola meal moisture levels vary in the literature. Burgess (1991) reported value of 9.95% while Han (1994) reported 7.40% for moisture content in the meal. Han (1994) also reported lower value of moisture content (7.3%) in the PMM isolates compared to this study.

A protein content of approximately 40% (N x 5.8) in canola meal was confirmed. As mentioned earlier, the protein content of canola meal ranges between 11-42% (Mieth *et al.*, 1983b), and varies with variety and environment. Thus the meal used for the present work is superior in protein content than many varieties and is an excellent source

Table 6. Moisture, protein and glucosinolate content[1] of canola meal and PMM

Component	Moisture[2], % dry weight basis	Protein[3], % dry weight basis	Glucosinolate[4], μmol/g dry weight basis
Canola Meal	12.23 ± 0.06	39.43 ± 0.04	38.44 ± 0.44
PMM	9.01 ± 0.01	91.16 ± 0.08	nd[5]

[1] Each value represents a mean of two determinations.
[2] Oven method, 130°C for one h
[3] Micro-Kjeldhal % protein = N% x 5.8
[4] Total glucosinolates (dry weight basis) was determined by GC analysis.
[5] 'nd' refers as not detected.

as a starting material. In addition, this value was comparable to the results reported by BMW Canola (39.14 ± 0.64%; Leco % protein = N x 6.25) although the method, as well as the nitrogen conversion factor, that was used were different. Furthermore, higher precision was obtained by AOAC micro-kjeldhal method used in the present work. The commercial PMM isolate was shown to contain a protein content of 91.16%. This demonstrates the PMM protein isolation method concentrated the protein content by at least two times and produced potentially high quality PMM protein isolates. Furthermore, it appeared that the glucosinolates were eliminated by the PMM procedure from the level of 38.44 μmol/g in meal to below a detectable level in the commercial PMM isolates.

Table 6 has definitely provided a clear picture of how effective the PMM protein isolation method is in isolating canola protein while, at the same time, removing the antinutritional glucosinolates. The following section will cover more detail of the analysis on each stage of the PMM canola protein isolation procedure.

4.2 Analysis of Mass Balance on Canola Protein Isolation

In the first study, a mass balance on the isolation of canola protein using the PMM process was examined. Both the protein and glucosinolates were determined at each of the three main stages of the PMM procedure: extraction, ultrafiltration, and dilution/ precipitation stages. As a result, seven types of samples, including starting material of canola meal (I), followed by debris (II), first supernatant (III), filtrate (IV), retentate (V), second supernatant (VI) and finally the PMM (VII), were screened in two series (Refer to Figure 4). One series was produced using the Amicon stirred ultrafiltration cell unit while the Vivaflow 200 tangential flow ultrafiltration unit produced the other series.

4.2.1 Quantification of Protein

The effects of each stage of the PMM procedure on percent protein were examined. The protein contents found in each sample at various stages of the PMM process are detailed in Figure 5. The PMM process has successfully isolated 90% and 93% protein through the stirred ultrafiltration cell unit and Vivaflow 200 tangential flow ultrafiltration unit, respectively, from canola meal containing approximately 40% of protein. This laboratory scale of the PMM process has shown to be effective in isolating high purity of PMM protein isolates compared to protein (91.16%) found in commercial PMM (Table 6).

No significant differences were observed between protein percentages for two series of the samples prior to the ultrafiltration stage. This is reasonable as the same

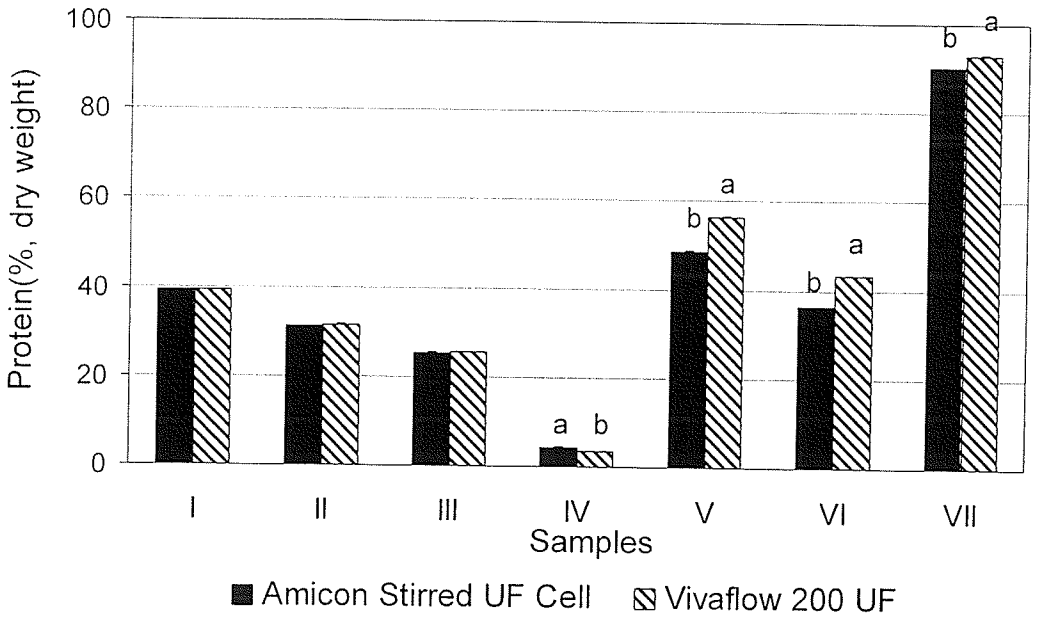


Figure 5. Comparison of protein content throughout the PMM isolation procedure using two types of ultrafiltration systems (I, canola meal; II, debris; III, first supernatant; IV, filtrate; V, retentate; VI, second supernatant; VII, PMM). [a, b] Values within a sample stage having different letters are significantly different ($p < 0.05$)

extraction procedure and conditions were used in the two series of the samples although the amount of meal used in the second series, the one which involved tangential flow ultrafiltration, was increased from 25 g to 100 g. This increase was due to the larger volume capacity of the Vivaflow 200 tangential flow ultrafiltration unit. In general, the second series of the samples gave more desirable results in terms of protein purity than the first. The use of the Vivaflow 200 ultrafiltration unit allowed less permeation of protein to the filtrate (IV), while retaining more protein in retentate (V). This resulted in more solubilized protein in the concentrate and thus more insoluble PMM protein produced in the final diluting and precipitating stage of the PMM procedure. However, as shown in Figure 5, protein losses can be obtained from the amount of protein remaining in debris (II) from the initial extraction and the supernatant (VI) decanted

from the final precipitate (VII). Modifications to the PMM procedure should be investigated such that these proteins can be more readily recovered, increasing the feasibility of the PMM process. Protein recovery is also an important factor in determining the commercial value of the PMM protein isolates. A discussion on the issues of protein recovery at each stage in the PMM procedure follows.

The PMM method has been used as a simple and selective technique, which specifically isolates 12S globulin from canola meal, for years (Burgess, 1991; Ismond and Welsh, 1992; Han, 1994). Yet the PMM protein isolates have been limiting in terms of protein recovery. Extreme treatments involving alkalis (Tzeng *et al.*, 1990b; Chen and Rohani, 1992; Xu and Diosady, 1994; Klockeman *et al.*, 1997; Aluko and McIntosh, 2001) and acids (Gillsberg and Turnell, 1976a; 1976b; Gillsberg, 1978) have become preferred routes to produce canola protein with higher protein recovery.

4.2.1.1 Protein Extraction

In the extraction stage, canola meal was introduced into a salt environment (0.5 M NaCl) followed by centrifugation. Salt was required to solubilize globulin proteins that are otherwise insoluble in water such that nonspecific electrostatic interactions are maximized. Salt reacts with counter charged groups on the proteins promoting a salting-in effect. As a result, formation of a double layer of ionic groups decreases the favorable interaction between proteins and thereby proteins from meal were extracted. The goal of this stage was to solubilize as much protein from the meal as possible. However, as shown in Table 7, only 31.39% of the canola protein was recovered in the extraction stage, while the rest of the protein remained mostly in the decanted debris

Table 7. Recovery of canola protein[1] at the extraction stage of the PMM procedure from commercial defatted canola meal

Sample	% Protein recovery
Debris	68.26 ± 0.13
First supernatant	31.39 ± 0.01
Loss	0.36 ± 0.12

[1] Recovery of canola protein is expressed in percentage based on dry weight of samples. Results are means of duplicate.

with minimal loss of protein during handling of the samples. This protein recovery value was lower than the result (47.03%) obtained from the study which identified 0.5 M NaCl as the optimum value for protein extractability of canola meal (Burgess, 1991).

Other than the salt concentration, the poor protein recovery at the extraction stage of the PMM procedure could be attributed to the effect of meal to solvent ratio, extraction pH and time on protein extractability from the meal. However, Burgess (1991) has demonstrated that there was no significant difference between meal to solvent ratio of 1:10 and 1:20 on protein extractabilities. In addition, the typical pH (5.9-6.1) of the unadjusted mixture of salt and meal falls in a pH range of 5.5 to 6.5, which was found to have no significant effect on protein recoveries during the extraction stage of the PMM procedure (Welsh, 1988). Thus, meal to solvent ratio and extraction pH were not considered to be critical factors causing poor protein recovery. While considering the extraction time, Burgess (1991) also concluded that the four h extraction using 5 g of meal produced only slightly more solubilized protein than the two h extraction with the same weight of the meal. In the present work, larger sample sizes, 25 and 100 g of meal, extracting for one and two h, respectively, were used. Insufficient mixing time of meal

with NaCl for these larger samples is a possibility. Alternatively, the treatment to which the proteins have been subjected during meal production, for example heat, may affect protein solubility.

In addition, according to Burgess (1991), the overall poor protein recovery could be partially attributed to handling loss prior to filtration. Some of the soft pellet remained disperse within the supernatant after centrifugation and therefore filtration through cheesecloth was required. Burgess (1991) suggested that centrifugation speeds higher than 10,000 rpm are needed to settle the debris firmly at the bottom so that use of cheesecloth for further filtration could be avoided. Table 7, however, shows only 0.36% of protein loss during sample handling even though the samples were handled at the speed of 10,000 rpm and filtered through double-layered cheesecloth. Therefore, a higher speed of centrifugation would have minimal benefit in increasing protein recovery in the PMM process.

4.2.1.2 Ultrafiltration

Figures 6 and 7 show the protein recovery and flux when the supernatant containing the solubilized protein was subjected to the ultrafiltration stage of the PMM process. At this stage, ultrafiltration uses pressure to continuously force the supernatant through a membrane with a specific pore size and to collect retentate of interest, where most of the canola proteins were concentrated and purified. Protein recovery in the retentate was in the range of 74-79% while 8-15% of canola protein ended up in the filtrate, with 10-13% protein lost due to handling of the samples. According to Tzeng *et al.* (1990), protein nitrogen had a rejection coefficient close to unity, which implies that

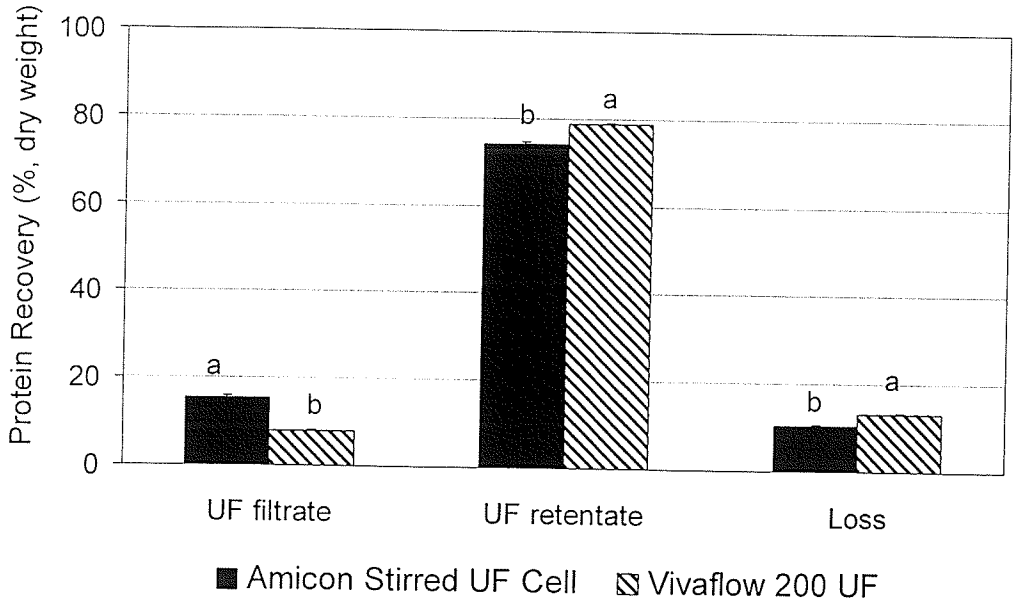


Figure 6. Recovery of canola protein at ultrafiltration stage of the PMM procedure with first supernatant as starting materials compared in two different ultrafiltration systems. [a, b] Values within a sample stage having different letters are significantly different ($p < 0.05$)

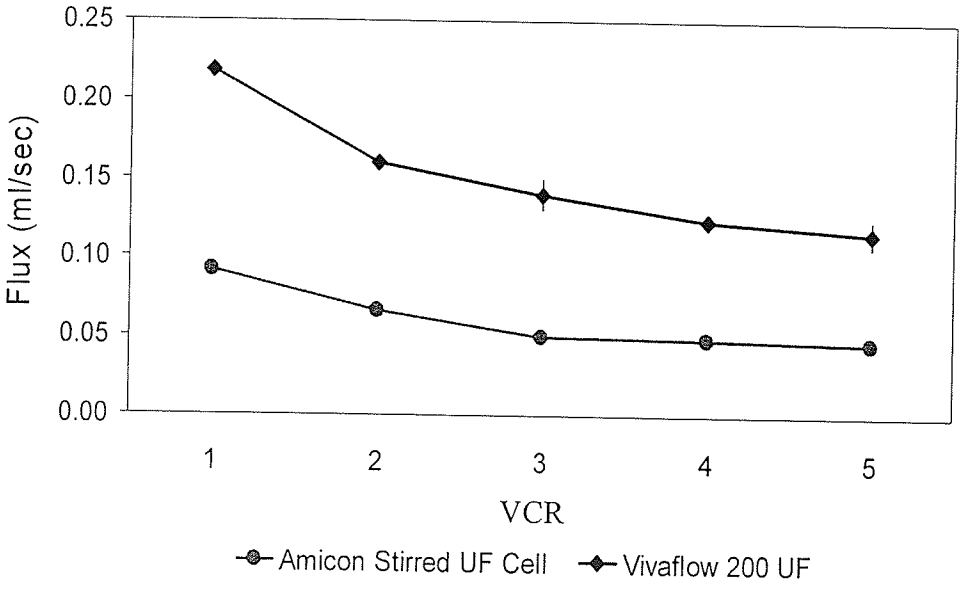


Figure 7. Volume concentration ratio (VCR) effects on the flux of isolating canola protein based on two different ultrafiltration systems

most of the protein remained in the retentate and only some non-protein nitrogen was lost to the filtrate stream. The presence of non-protein nitrogen, a complex fraction which contains free amino acids, peptides, and other nitrogen-containing compounds such as melanoid compounds (Bhatty and Finlayson, 1973), has been reported to result from the heat treatment during canola meal preparation, where dissociation of protein occurs (Tzeng *et al.*, 1990). These compounds, with molecular weight less than the molecular weight cut-off (MWCO) of the membrane, would pass freely through the membrane into the filtrate.

In addition to using the Amicon stirred ultrafiltration cell unit cited in the literature (Ismond and Welsh, 1992), the Vivaflow 200, a tangential flow ultrafiltration unit, was evaluated during the second stage of the PMM process to compare its effectiveness in concentrating the protein. The concentration factor, or degree to which the original volume of the supernatant was reduced, was kept at about four times for both units to allow for a comparison. The MWCO of the membranes for the two ultrafiltration units were different with the Amicon stirred ultrafiltration cell and Vivaflow 200 tangential flow ultrafiltration units using 10,000 and 30,000 MWCO membranes, respectively. Figure 6 indicates that the 30,000 MWCO membrane in the Vivaflow 200 unit was more effective in retaining canola protein in retentate (78.78% of first supernatant) with less canola protein passing through into the filtrate (8.06% of first supernatant). Compared to the Vivaflow 200, the Amicon stirred ultrafiltration cell unit with 10,000 MWCO membrane recovered significantly less canola protein in the retentate (74.18% of first supernatant) and passed more protein in the filtrate (15.37% of first supernatant). While one might expect to have a greater loss of protein with a higher MWCO, that was not the case. By comparing different MWCO of ultrafiltration

membranes, Diosday *et al.* (1984) reported that higher MWCOs, but not higher than 50,000, could improve the ultrafiltration recovery. Due to a certain degree of concentration polarization, higher MWCO membrane resulted in good retention of proteins compared to those with lower MWCO (Diosday *et al.*, 1984). However, ultrafiltration membranes with MWCO higher than 50,000 lose large amounts of proteins as smaller dissociated products from 12S globulin would not be recovered at this stage. These findings show the significance of membrane pore size on protein recovery.

The design of the filtration units could also contribute to the different results in protein recovery. Tangential flow and recirculation process are the design features in the Vivaflow 200 unit that minimize concentration polarization and help bring retained components back to the sample reservoir. These design features enhance the process of recovering of the proteins. Concentration polarization, a phenomenon where an impermeable gel occurs, could reduce the flux through the membrane (Tzeng *et al.*, 1990). This occurs because some of the globular canola protein may remain on the surface of the membrane blocking the pore size and thereby reducing the apparent MWCO. This problem was also minimized in the Amicon stirred ultrafiltration cell unit by introducing a magnetic stirrer. Not only does this reduce concentration polarization, but the magnetic stirrer also allows more rapid and effective filtration. In the present work, the Amicon ultrafiltration unit produced lower protein recovery and was also less efficient. It took approximately 6-7 h to concentrate the supernatant by four times with 60-70 psi pressure whereas the Vivaflow 200 unit achieved a greater than four times concentration in one h at 36 psi. In the Amicon ultrafiltration unit, proteins were forced to pass into the filtrate in a single direction as the driving force was relatively high. On

the other hand, the lower fluid pressure created by the Vivaflow 200 unit gently recirculated the samples and reduced contact between the components and the membrane to minimize concentration polarization. The efficiency of these two ultrafiltration units can be demonstrated by comparing the membrane flux at which the rate of a portion of the sample passed through the membrane (Figure 7). As the volume concentration ratio (VCR) increased, the flux of both ultrafiltration units declined due to concentration polarization and the associated change in the MWCO of membranes. Overall, the Amicon ultrafiltration unit had a relatively lower flux, and reduced protein recovery and is not recommended for use in the isolation of canola protein using the PMM procedure.

The Vivaflow 200 ultrafiltration unit allowed larger volumes of supernatant to be concentrated in a shorter time period but significantly greater handling losses occurred compared to the Amicon ultrafiltration unit. This is mainly due to the hold up volume in the unit.

4.2.1.3 Dilution and Precipitation

In the final stage of the PMM procedure, the solubilized, concentrated proteins are diluted by introducing them into a large volume of cold water causing the protein to precipitate. Under such an environment, hydrophobic interactions between the proteins are promoted, which in turn leads to the formation of microscopic micelles. This is the key step in isolating protein in the PMM procedure. However, Figure 8 demonstrates that poor protein recovery was achieved in the dilution and precipitation stage. Almost half of the protein from retentate (45-50%) remained in the second supernatant,

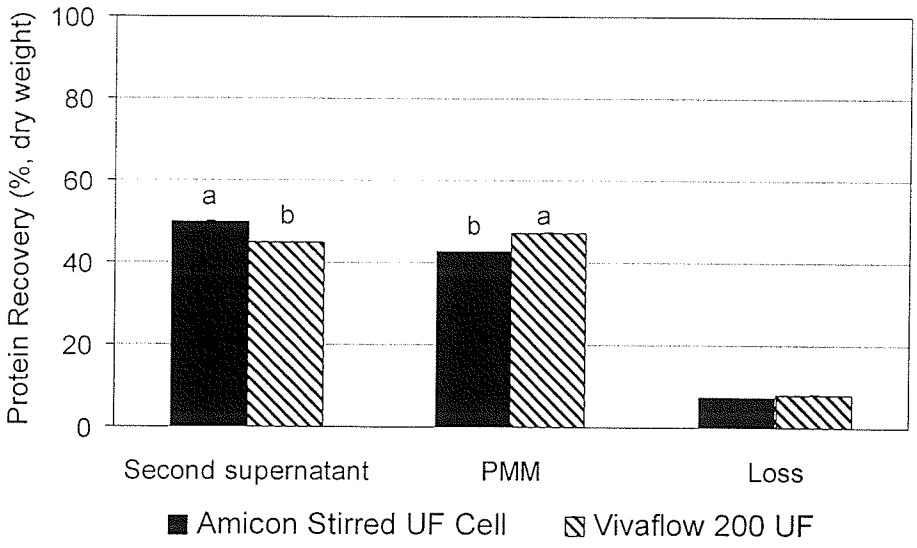


Figure 8. Recovery of canola protein at the dilution and precipitation stage of the PMM procedure with ultrafiltrated retentate as starting materials compared in two different ultrafiltration systems. [a, b] Values within a sample stage having different letters are significantly different ($p < 0.05$)

which was decanted in the PMM procedure. Only 43-47% of the protein from retentate ended up in the PMM protein isolate with approximately 8% protein lost. This low recovery is probably due to the PMM process being a selective technique which specifically isolates the 12S globulin protein. Hence, a high level of lower molecular weight protein fractions may be decanted along with the supernatant. These water-soluble protein fractions represents mainly the 1.7S canola protein fraction, which has been shown to be difficult to recover during the processing of canola concentrates or isolates (Bhatty *et al.*, 1968; Schwenke *et al.*, 1983). Techniques, particularly the use of further ultrafiltration with different MWCO membranes and drying of the supernatant (Tzeng *et al.*, 1990; Rubin *et al.*, 1990), have been used to recover this canola protein

fraction. If a similar approach was taken, the overall recovery of protein could be increased and the value of the PMM process improved.

Another possible reason for such a poor recovery is related to the driving force for hydrophobic interactions responsible for micelle formation, namely the dilution factor. The protein to water dilution factor must be sufficient to induce hydrophobic interactions for micelle formation, but not too high to dilute the protein concentration to a point where subsequent interactions between protein molecules are inhibited by the distance between them (Burgess, 1991). According to Burgess (1991), although dilutions for optimum micelle response appeared to be a function of pH, the micelle response generally tends to weaken as the proportion of water increases, representing the effect of reduced protein concentration. As stated earlier, the optimum micelle response was achieved with a dilution factor of 1:10 (protein: water) if the milieu of protein was at pH 6 and the protein concentration was high enough (Burgess, 1991). In this study, the retentates containing approximately 26% and 30% protein (on a wet basis) from the first and second series of the samples, respectively, was diluted to 15 times its volume in accordance to the literature (Ismond and Welsh, 1992). This dilution may have been too high resulting in instability of the protein-forming-micelle system which in turn permitted the protein to remain in solution. To increase protein recovery in the PMM protein isolates, lower dilution factor may be required, particularly at a protein milieu of pH 6.

Figure 8 also illustrates the effect of the different ultrafiltration units on protein recovery in the dilution and precipitation stage in the PMM procedure. Interestingly, the trend for protein distribution in the mass balance of third stage of the PMM procedure is similar to the second stage of the PMM procedure. The second series of the samples

using the Vivaflow 200 ultrafiltration unit have resulted in significantly greater protein recovery in the PMM protein isolates and less in the decanted supernatant compared to the first series of the samples prepared with the Amicon ultrafiltration unit. It is possible that the higher protein concentration in the sample concentrated in the Vivaflow 200 unit made it less sensitive to the effect of the high dilution ratio, resulting in increased micelle formation and protein recovery. As a result, a better hydrophobic-hydrophilic balance, claimed as a critical factor for strong micelle formation and subsequent interaction by Burgess (1991), was created. Consequently, double layer of ionic groups around the proteins would collapse upon dilution and leads to an increasing favorable hydrophobic interactions between proteins where proteins bury their hydrophobic sites to reduce the entropy of the system (Nakai and Li-Chan, 1988). Then, the canola protein would self-associate to form microscopically viscous and sticky micelles, the PMM protein isolates.

4.2.1.4 Protein Mass Balance with Two Ultrafiltration Systems

The PMM process appears to be straightforward; however, the effects of environmental manipulation on proteins at each stage of the PMM procedure and the resulting chemical interactions are complex, as has shown in earlier discussions. Further evidence is provided by the overall mass balance on canola protein during the PMM protein isolation procedure which is outlined in Figures 9 and 10, for the Amicon stirred cell and Vivaflow 200 unit, respectively. Although the present work was based on optimal isolation conditions cited in past literature (Burgess, 1991), the overall protein recoveries were less than ideal for the PMM process. Figures 9 and 10 indicate that

9.95% and 11.68% of the protein were recovered from canola meal in the Amicon stirred cell and Vivaflow 200 units, respectively. These protein recoveries are considered low compared to the same PMM process applied on fababean (Arntfield *et al.*, 1985) and alternative isolation methods using acidic precipitation on canola meal (Chen and Rohani, 1992) where the protein recoveries were 42.5% and 53% respectively. However, this modified PMM process has slightly increased protein recoveries when compared to the original PMM procedure where Welsh (1988) recovered less than 5% of the protein in canola meal. The modifications in comparison to what was used by Welsh (1988) include increasing the NaCl concentration and pH from 0.1 M, pH 5.5 to 0.5 M NaCl, pH 6.0. In addition, the increase in sample size from 25 g for the Amicon stirred cell to 100 g for the Vivaflow 200 ultrafiltration unit may also have affected protein recoveries. The conditions to extract the canola protein may influence the micelle response at later stages of the PMM procedure. It was evident that changing the extraction conditions to 0.5 M NaCl and pH 6.0 increased protein extraction thus facilitating better micelle formation and interaction resulting in increased protein recoveries (Burgess, 1991).

The choice of pH can influence the micelle response. As the pI (isoelectric point) of the 12S canola globulin is about 7 (Meith *et al.*, 1983a; 1983b), protein tends to have a slightly positive charge at pH 6.0, which in turn is considered as an important factor to give the critical hydrophilic – hydrophobic balance required for strong micelle responses (Burgess, 1991). The overall net positive charge also offers potential for the binding of Cl^-. With an increase in NaCl concentration to 0.5 M, the Cl^- binding to the protein may increase and thereby minimize the electrostatic forces on the micelle surfaces. In other words, the Cl^- binding would induce hydrophobic interactions by forcing hydrophobic amino acid residues to orientate towards the interior and bury themselves inside the

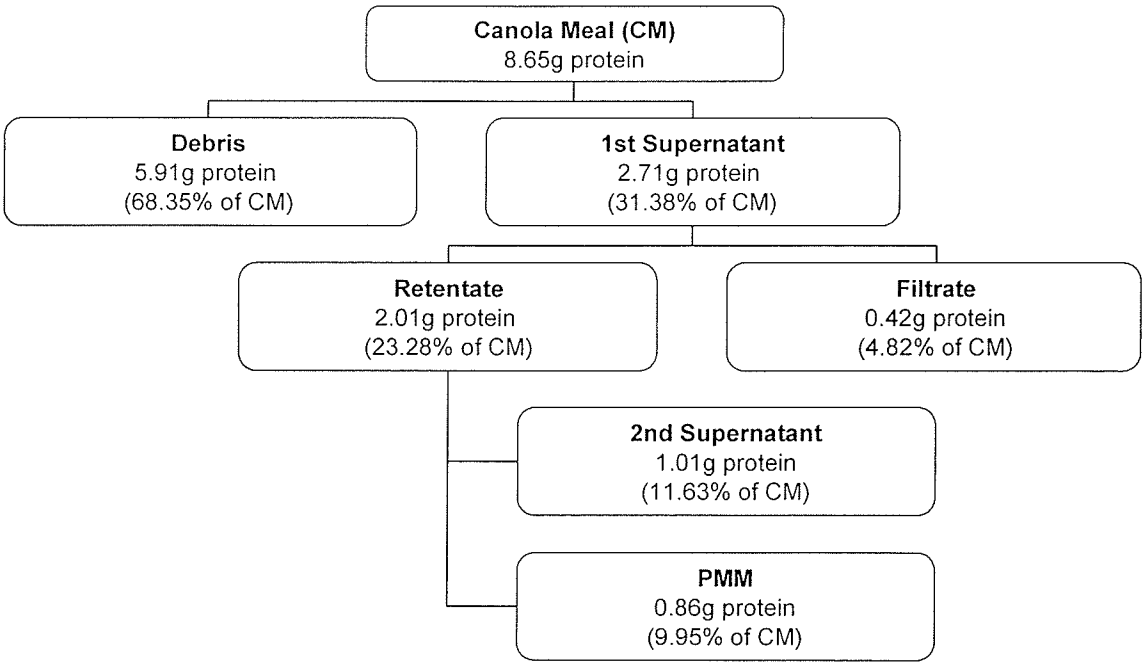


Figure 9. A mass balance diagram showing the canola protein recovery[1] at the different stages of the PMM process with the Amicon stirred cell unit as the ultrafiltration system. [1] Debris + Filtrate + 2nd Supernatant + PMM + Loss = 100% protein recovery; therefore, unaccounted protein loss is for about 0.45g or 5.25% of CM.

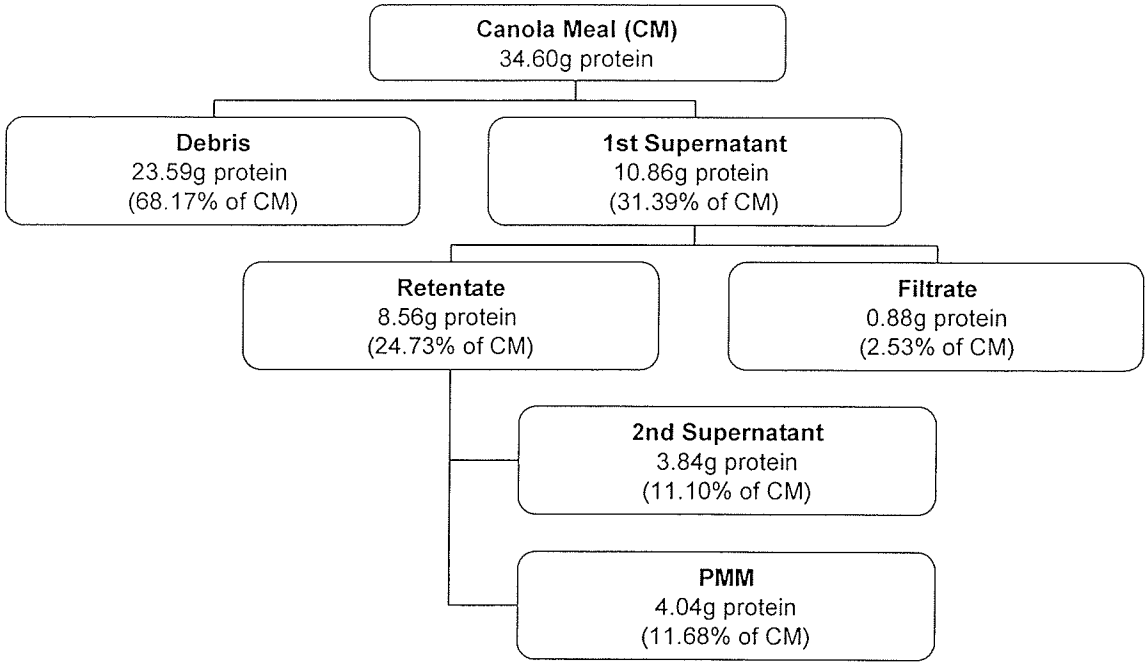


Figure 10. A mass balance diagram showing canola protein recovery[1] at the different stages of the PMM process with the Vivaflow 200 unit as the ultrafiltration system.
[1] Debris + Filtrate + 2nd Supernatant + PMM + Loss = 100% protein recovery; therefore, unaccounted protein loss is for about 2.25g or 6.52% of CM.

proteins. At lower salt concentrations, like 0.1 M NaCl, pre-dilution precipitation has been reported (Burgess, 1991), and hydrophobic residue orientation has been inhibited resulting in protein recoveries of less than 5%.

Figures 9 and 10 also show that large amount of proteins were discarded with the debris (average of 68.26%) and second supernatant (average of 11.37%). Therefore, to improve the PMM process, these two stages need to be improved to obtain higher protein recoveries. Improvements in the extraction stage are of particular importance. This is because, as stated earlier, initial protein solubility is critical in micelle formation. The dilution stage should also be improved; however, only the 12S globulin with a high level of hydrophobic amino acid residues are able to form strong micelles and be recovered in the PMM protein isolates. Some literatures suggest that the use of ultrafiltration followed by drying process could recover those decanted proteins (average of 11.37%) (Tzeng *et al.*, 1990; Rubin *et al.*, 1990). It should be noted, however, that the canola proteins which were unable to form micelle are mostly the 1.7S protein fraction and other dissociated forms of 12S globulin rather than the 12S canola globulin.

4.2.1.4 Comments on Two Ultrafiltration Systems

The use of the Vivaflow 200 ultrafiltration unit has shown to be more advantageous than the Amicon stirred ultrafiltration unit for number of reasons. More protein was recovered in the retentate (Figures 9 and 10) in a shorter time while at the same time the larger sample volume and lower pressure were required, resulting in a more efficient production of higher purity protein isolates (Figure 5). In addition, the

yield of the PMM protein isolates attained with the use of the Vivaflow 200 ultrafiltration unit was significantly higher than that obtained from the Amicon stirred cell unit (Figure 11). These benefits indicate that the Vivaflow 200 is the preferred ultrafiltration unit.

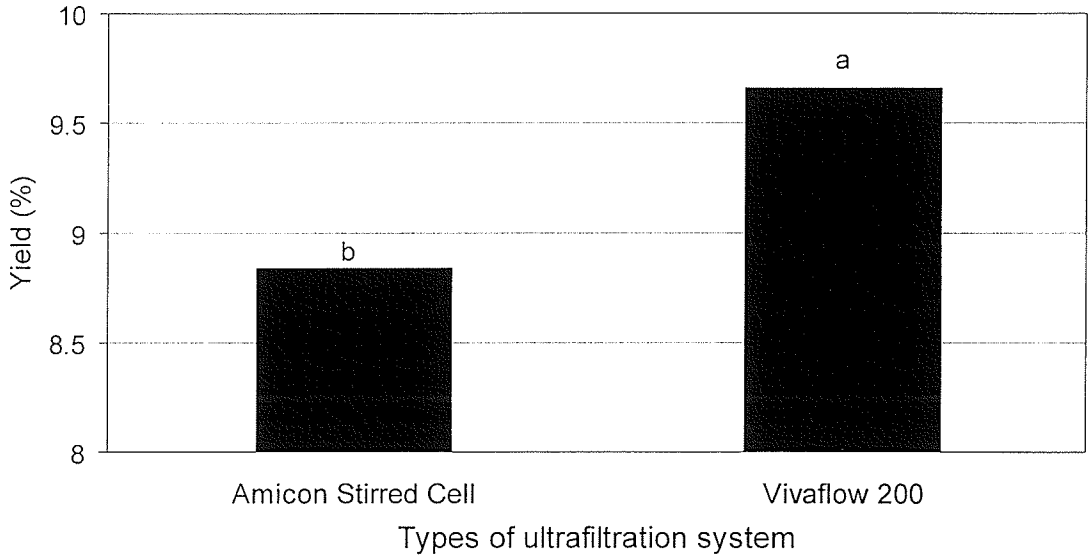


Figure 11. Comparison of the production yield of the PMM canola protein isolates based on the wet basis between the uses of the Amicon stirred ultrafiltration cell and Vivaflow 200 ultrafiltration in the PMM process. [a, b] Values in different ultrafiltration treatments having different letters are significantly different ($p < 0.05$)

4.2.2 Analysis of Glucosinolates

The use of canola protein isolates has been limited by the presence of undesirable glucosinolates as antinutritional or toxic factors. Thus, one objective of the current study was to assess the mass balance of canola glucosinolates during the PMM protein isolation procedure. Individual and total glucosinolate content of all samples were determined by GC as well as the analyses of the released glucose and the indole glucosinolate degradation products. There is a wide range of methods to determine

glucosinolates quantitatively; however, many of them suffer from lack of stringent conditions to ensure reproducible results. For comparison with the standard GC technique, the thiocyanate ion (SCN^-) determination and diabetic test kits technique, both of which claim to be simpler and more rapid methods, were used to determine the glucosinolate contents of these samples.

4.2.2.1 Standard Gas Chromatography Technique

4.2.2.1.1 Fate of Glucosinolates During Protein Isolation

Examples of gas chromatograms of TMS desulpho-glucosinolates from samples obtained using the Amicon stirred ultrafiltration cell are shown in Appendix 4. All of the glucosinolates were well separated with the modified chromatographic conditions. The glucosinolates identified by the peaks on the chromatograms include allyl- (sinigrin), but-3-enyl- (gluconapin), pent-4-enyl- (glucobrassicanapin), 2-hydroxy-but-3-enyl- (progoitrin), 2-hydroxy-pent-4-enyl- (gluconapoleiferin), *p*-hydroxybenzyl- (glucosinalbin), 3-indolyl-methyl- (glucobrassicin, also known as indole), and 4-hydroxy-3-indolylmethy- (4-hydroxyglucobrassicin, also known as 4-hydroxyl indole) glucosinolates plus an internal standard of benzyl-glucosinolate (glucotropaeolin). It should be noted that all of these glucosinolates were detected as intact molecules.

The fate of these glucosinolates from two ultrafiltration systems at various stages of the PMM procedure are detailed in Tables 8 and 9. The results show that aliphatic glucosinolates were predominant throughout most of the isolation for both systems. Generally, the total aliphatic content of glucosinolates did not exceed 30 μmol/g, the

value used to define canola. The exception was for the filtrate samples in both systems. However, this was not a concern as this product was discarded from the system. The final PMM protein isolates for the Amicon and Vivaflow systems had only 3.83 or 4.53 μmol of total aliphatic glucosinolate per gram, respectively. Of all these aliphatic glucosinolates, gluconapin and progoitrin together constitute more than half of the total content of glucosinolates in the samples. The results also indicate that aromatic/heterocyclic glucosinolates represent a significant proportion of the total glucosinolate content in the samples with 4-hydroxyglucobrassicin as the predominant glucosinolate. This supports previous work indicating that 4-hydroxyglucobrassicin is the major indole glucosinolate in canola samples (Daun and McGregor, 1983; Slominski and Campbell, 1987). Like in other studies (Slominski and Campbell, 1987; Campbell and Slominski, 1990), it appears that these samples were contaminated by admixtures of weed seeds such as commercial mustard, wild mustard and stinkweed (Campbell and Slominski, 1990) where sinigrin and glucosinalbin were present.

The results show that the PMM process has successfully eliminated the total content of glucosinolates by 81% and 71%, respectively, in the final protein from the ultrafiltration systems. Maximum removal of the glucosinolates from canola meal took place at the dilution and precipitation stage. In this final stage of the PMM procedure, hydrophobic interactions were promoted upon sudden dilution leading to precipitation of the PMM protein isolates. Under such conditions, most of the glucosinolates remained soluble and were, therefore, excluded from the protein isolates. However, the percent eliminated was lower than what was obtained by Ismond and Welsh (1992) where an average of 92.4% of the glucosinolates in the protein isolate were eliminated using extraction media of 0.01 M and 0.1 M of NaCl. The glucosinolate content of the starting

Table 8. Total and individual glucosinolates (μmol/g) present in the different stages of the PMM process with the Amicon stirred cell unit as the ultrafiltration system. (I, canola meal; II, debris, III, first supernatant; IV, filtrate; V, retentate; VI, second supernatant; VII, PMM; and 'nd' refers to not detected.)

Glucosinolate	Extraction			Ultrafiltration		Dilution & Precipitation	
	I	II	III	IV	V	VI	VII
Aliphatic							
Sinigrin	0.23 [a]	0.35	0.12	nd	0.31	0.13	nd [b]
Gluconapin	11.35 [a]	5.58	10.97	11.57	10.85	10.73	1.66 [b]
Glucobrassicanapin	2.64 [a]	0.65	2.07	1.41	3.02	1.63	0.19 [b]
Progoitrin	13.04 [a]	8.80	14.07	18.70	12.54	15.59	1.58 [b]
Gluconapoleiferin	0.69 [a]	0.18	0.49	0.29	0.61	0.34	0.40 [b]
Subtotal:	*27.95 [c]*	*15.21 [c]*	*27.60 [c]*	*31.97 [c]*	*27.33 [c]*	*28.42 [c]*	*3.83*
Aromatic/Heterocyclic							
Glucosinalbin	0.86 [a]	0.15	1.55	0.85	1.23	0.75	0.04 [b]
Glucobrassicin	0.63	0.12	1.34	0.63	0.83	0.38	0.12
4-OH-Glucobrassicin	9.00 [a]	4.51	15.80	20.80	10.87	11.50	3.42 [b]
Subtotal:	*10.49 [d]*	*4.78 [d]*	*18.69 [d]*	*22.28 [d]*	*12.93 [d]*	*12.63 [d]*	*3.58*
Overall total:	**38.44**	**20.34**	**46.41**	**54.25**	**40.26**	**41.05 [E]**	**7.41 [E]**

[a, b] For comparison of canola meal (I) and PMM isolate (VII), values for the same individual glucosinolate having different letters are significantly different ($p < 0.05$)

[c, d] For aliphatic and aromatic glucosinolate subtotals, values in a same treatments having different letters are significantly different ($p < 0.05$)

[E, F] For different ultrafiltration systems, total values in a same sample having different letters are significantly different ($p < 0.05$) (Compared to Table 9)

Table 9. Total and individual glucosinolates (μmol/g) present in the different stages of the PMM process with the Vivaflow 200 unit as the ultrafiltration system. (I, canola meal; IV, filtrate; V, retentate; VI, second supernatant; VII, PMM and 'nd' refers to not detected.)

Glucosinolate	Extraction			Ultrafiltration		Dilution & Precipitation	
	I	II	III	IV	V	VI	VII
Aliphatic							
Sinigrin	0.23	0.41	0.17	0.36	nd	0.31	0.56
Gluconapin	11.35 [a]	5.75	9.98	11.61	7.99	10.80	2.36 [b]
Glucobrassicanapin	2.64 [a]	0.75	1.91	2.09	1.35	2.75	0.23 [b]
Progoitrin	13.04 [a]	8.45	13.78	17.08	11.63	12.93	1.38 [b]
Gluconapoleiferin	0.69 [a]	0.19	0.39	0.85	0.48	0.61	nd [b]
Subtotal:	*27.95* [c]	*15.55* [c]	*26.23* [c]	*31.99* [c]	*21.45*	*27.40*	*4.53* [c]
Aromatic/Heterocyclic							
Glucosinalbin	0.86	0.21	1.24	1.62	1.19	2.74	0.07
Glucobrassicin	0.63	0.09	1.42	1.84	1.41	3.07	0.15
4-OH-Glucobrassicin	9.00	5.11	16.28	18.41	16.54	16.18	6.43
Subtotal:	*10.49* [d]	*5.41* [d]	*18.94* [d]	*21.87* [d]	*19.14*	*21.99*	*6.65* [d]
Overall total:	**38.44**	**20.96**	**46.41**	**50.93**	**40.59**	**49.39** [F]	**11.18** [F]

[a, b] For comparison of canola meal (I) and PMM isolate (VII), values for the same individual glucosinolate having different letters are significantly different *($p < 0.05$)*

[c, d] For aliphatic and aromatic glucosinolate subtotals, values in a same treatments having different letters are significantly different *($p < 0.05$)*

[E, F] For different ultrafiltration systems, total values in a same sample having different letters are significantly different *($p < 0.05$)* (Compared to Table 8)

canola meal in their study was only 8.10μmol/g, compared to the 38.44 μmol/g for the low-temperature heated canola meal used in this study. The different heat treatments used may contribute to this initial difference in glucosinolates. High temperatures during heating, also called cooking, coalesce small oil droplets to larger ones, adjust the moisture content of the seed before the solvent extraction, and coagulate proteins for easier separation (Unger, 1990). The heat also serves an important function in reducing enzymatic activity. Of particular interest in the canola seed is the myrosinase which can hydrolyze the glucosinolates. High temperatures may also breakdown the glucosinolates into various degradation products resulting in a meal with lower glucosinolate levels. A study, however, claimed that most of the degradation products are more harmful than the intact glucosinolates (Sørensen, 1990). Even though a heating process may control myrosinase activity, a small portion of glucosinolates, together with the possible breakdown products, may carry into the canola products such as protein isolates, limiting their use in food grade protein formulations. It was hoped that with the use of the PMM process to produce canola protein isolates from low-temperature heated canola meal, the goal of maximizing protein recoveries while efficiently removing the intact glucosinolates with minimal degradation of these glucosinolates could be achieved.

4.2.2.1.2 Comparison of Two Ultrafiltration Systems

By comparing data from Tables 8 and 9, it was found that significant differences in the total glucosinolate contents were detected between the two ultrafiltration systems only at the dilution and precipitation stage. As well, there were significant differences between the aliphatic and aromatic/heterocyclic glucosinolates remaining in the samples

within samples for each ultrafiltration system, except for the PMM isolates with the Amicon stirred cell unit, and retentate and second supernatant with the Vivaflow 200 unit. Significant differences for individual glucosinolate content were detected between the starting canola meal and the final PMM isolates prepared with the Amicon stirred cell unit, except for one aromatic/heterocyclic glucosinolate, named glucobrassicin. Similar results for aliphatic glucosinolates were obtained with the Vivaflow 200 unit; however, no significant differences existed for the aromatic/heterocyclic glucosinolates between the canola meal and the PMM isolates.

When considering the overall effect of the ultrafiltration systems on glucosinolates, greater glucosinolate removal was achieved in the PMM isolates with the Amicon stirred cell ultrafiltration system and that almost every single glucosinolate, except for glucobrassicin, was significantly reduced with a total value of only 7.42 μmol/g. Despite the fact that both of the ultrafiltration systems have lowered the aliphatic glucosinolates, the results indicated that the Vivaflow 200 ultrafiltration system did not significantly reduce the aromatic/heterocyclic glucosinolates and left a significantly greater residual of glucosinolates (11.19 μmol/g) in the PMM protein isolate. This was somewhat unexpected as the Vivaflow 200 unit, with a 30,000 MWCO, should be more effective in separating the lower molecular weight glucosinolates (Lönnerdal *et al.*, 1977), than of the Amicon stirred cell unit with only a 10,000 MWCO membrane. As the Vivaflow 200 unit retained more of the glucosinolates (Fraction V), especially the aromatic/heterocyclic glucosinolates, the PMM isolates would, therefore, contain more of these glucosinolates. Further discussions on glucosinolate recovery will provide more insight into the effectiveness of the PMM process on removing the glucosinolates from protein isolates.

4.2.2.1.3 Effect of Extraction

The relatively similar recoveries of aliphatic, aromatic/heterocyclic, and total glucosinolates between the samples for both series prior to the ultrafiltration stage in the PMM procedure indicates that no significant different of their recoveries occurred in the extraction stage (Figure 12). This was because of similar extraction conditions, except larger sample size and longer extraction time with the Vivaflow 200 unit, were used. Minor differences in glucosinolates recoveries at this stage would be expected due to variability in the sample handling.

In the extraction stage, there was a relatively constant level of aliphatic, aromatic/heterocyclic, and total glucosinolates in the debris (II) (Figure 12; Table 10). By contrast, the recovery of aromatic/heterocyclic glucosinolates tended to be significantly higher than aliphatic glucosinolates recovery in the first supernatant (III) for both systems resulting in higher total glucosinolate recovery in the first supernatant (Table 10). Since glucosinolates tend to be hydrophilic in the presence of salt (Sørensen, 1990), this result may be a reflection of the fact that aromatic/heterocyclic glucosinolates, in particular the 4-hydroxyglucobrassicin, were more readily soluble with the extraction conditions used.

4.2.2.1.4 Effect of Ultrafiltration

Ultrafiltration is an important stage in the PMM process in that it allows the separation of the impurities with low molecular weight, including glucosinolates, from the high molecular weight desirable canola proteins. This is, in fact, an added benefit for ultrafiltration over concentrating systems. From Figure 12, it is obvious that the Amicon

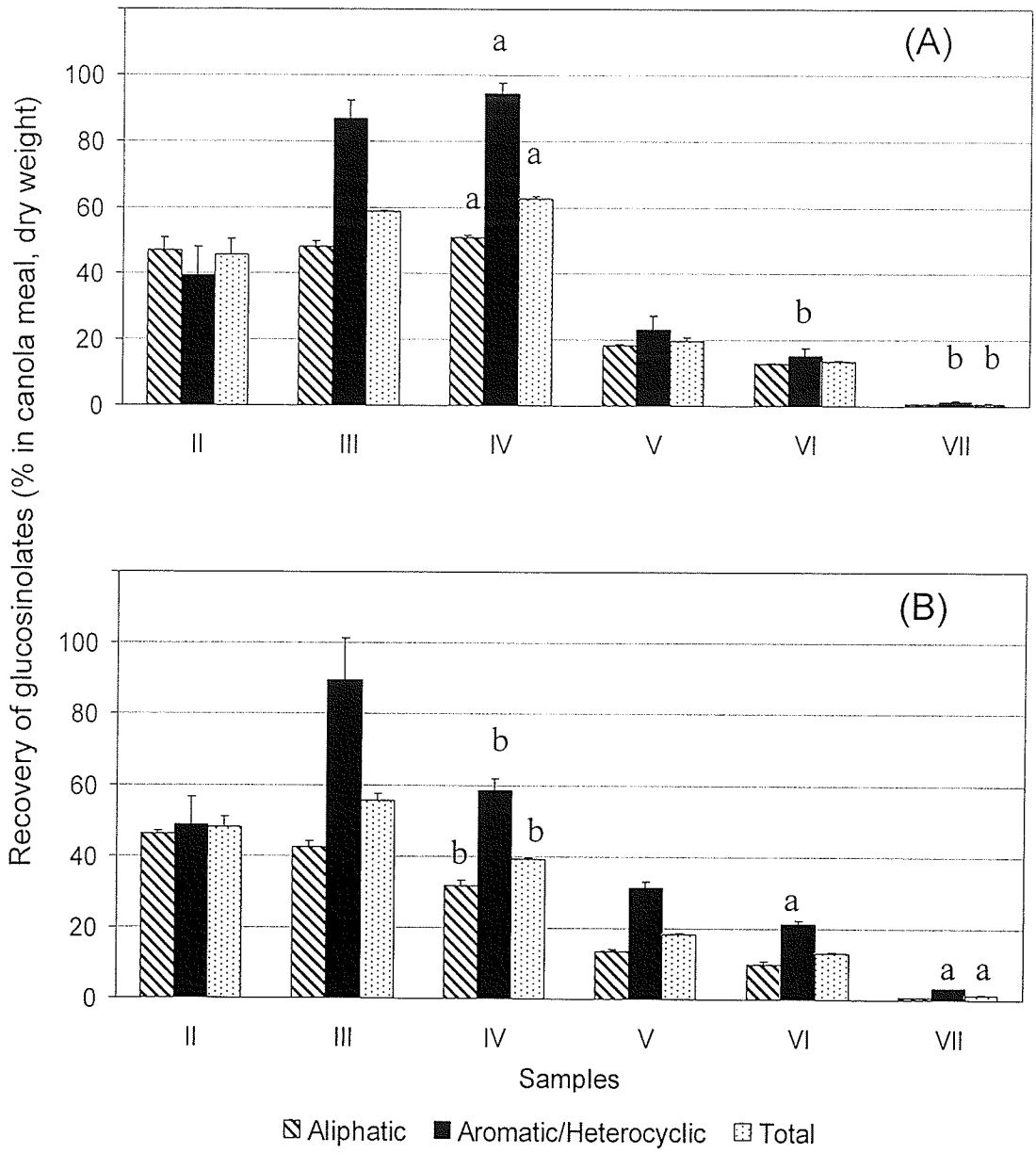


Figure 12. Recovery of intact glucosinolates using GC at various stages of the PMM process with two ultrafiltration systems: Amicon stirred ultrafiltration cell (A) and Vivaflow 200 ultrafiltration (B). (II, debris; III, first supernatant; IV, filtrate; V, retentate; VI, second supernatant; VII, PMM ; and total recovery = II + IV +VI + VII).
[a , b] For different ultrafiltrations, values in a same sample treatment and glucosinolate category having different letters are significantly different ($p < 0.05$)

stirred ultrafiltration cell unit (A) allowed significantly more glucosinolates, both aliphatic and aromatic/heterocyclic, from first supernatant (III) to pass through the membrane into the filtrate (IV), and left less glucosinolates in the retentate (V), which in turn lowered the glucosinolate content in samples at later stages of the PMM procedure. While a similar trend was seen for the Vivaflow 200 unit, there were lower levels of glucosinolates removed in the filtrate (IV) compared to the Amicon stirred cell unit. For the aromatic/heterocyclic glucosinolates this eventually contributed to significantly higher recoveries of these glucosinolates in both the second supernatant (VI) and PMM protein isolates (VII).

These differences were even more evident when looking at the recovery of glucosinolates in the ultrafiltration stage as a function of the glucosinolate content of the first supernatant (Table 10). For the Amicon stirred ultrafiltration cell unit (A; 10,000 MWCO), a significantly lower recovery of aromatic/heterocyclic glucosinolates was observed in the retentate compared to aliphatic glucosinolates; while for the Vivaflow 200 ultrafiltration unit (B; 30,000 MWCO) there was no significant differences in the percentages of glucosinolates recovery for both aliphatic and aromatic/heterocyclic glucosinolates in retentate. This shows that the effect of MWCO on elimination of the glucosinolates was opposite to the statement of Diosady *et al.* (1984) that removal of impurities, including glucosinolates, was best with the higher MWCO membrane. The reason for this difference is not clear, but it may be related to the different flow behavior of the samples for these ultrafiltration systems. The continuous flow for the Vivaflow 200 system appeared to minimize the selective removal of aromatic/heterocyclic glucosinolates. While the Vivaflow 200 unit improves protein recovery, it has also increased the recovery of undesirable glucosinolates, resulting in higher aromatic/

Table 10. Recovery of aliphatic, aromatic/heterocyclic, and total glucosinolates (GC methodology) in the three main stages of the PMM procedure using the Amicon stirred ultrafiltration cell (A) and Vivaflow 200 ultrafiltration (B) systems

PMM stages	Samples	Recovery of glucosinolate (%)		
		Aliphatic	Aromatic/ Heterocyclic	Total
(A) Amicon stirred ultrafiltration cell				
Extraction (% of canola meal)				
	Debris	47.13	39.46	45.82
	First supernatant	48.11 [b]	86.61 [a]	58.83
	Loss	4.76	*	*
Ultrafiltration (% of first supernatant)				
	Filtrate	105.54	108.61	106.50
	Retentate	38.01 [a]	26.86 [b]	33.68
	Loss	*	*	*
Dilution & Precipitation (% of retentate)				
	Second supernatant	69.88	65.20	68.04
	PMM	3.24 [b]	6.34 [a]	4.22
	Loss	26.87	28.46	27.74
(B) Vivaflow 200 ultrafiltration				
Extraction (% of canola meal)				
	Debris	46.18	48.65	47.96
	First supernatant	42.50 [b]	89.71 [a]	55.60
	Loss	*	*	*
Ultrafiltration (% of first supernatant)				
	Filtrate	74.74	65.21	70.70
	Retentate	31.19	35.13	32.80
	Loss	*	*	*
Dilution & Precipitation (% of retentate)				
	Second supernatant	73.81	67.15	70.85
	PMM	5.30 [b]	9.95 [a]	7.89
	Loss	20.89	22.91	21.25

'* ' refers to overestimation of glucosinolates.

[a, b] For aliphatic and aromatic/heterocyclic glucosinolates, values in a sample and ultrafiltration treatments having different letters are significantly different ($p < 0.05$)

heterocyclic glucosinolate levels in both the second supernatant and the PMM protein isolates (Table 10).

4.2.2.1.5 Effect of Dilution and Precipitation

In the final stage of the PMM procedure, a relatively large percentage of the glucosinolates was recovered in the decanted second supernatant (VI) as most remained soluble upon dilution and were separated from the PMM protein isolates for both systems (Figure 12). The significantly higher recoveries of aromatic/heterocyclic glucosinolates in both of the second supernatant (VI) and PMM protein isolate (VII) from the Vivaflow 200 unit (B) is probably a reflection of the higher level of glucosinolate from the previous stage of the PMM process. Again, these results were confirmed by looking at the recovery of glucosinolates in the dilution and precipitation stage as a function of the glucosinolates in the retentate (Table 10). There were significantly higher percentages of the aromatic/heterocyclic glucosinolates compared to aliphatic's recovered in the PMM protein isolates in this stage for both systems. The difference was probably due to the interaction between the micelles and glucosinolates occurring upon the formation of the hydrophobic interaction. It is also possible that the improved micelle forming capacity for the sample from the Vivaflow 200 unit promoted hydrophobic interactions between the proteins and the amount moiety of the glucosinolates, resulting in a higher level of entrapment of the aromatic/heterocyclic glucosinolates in the protein isolate.

4.2.2.1.6 Comments on Gas Chromatography Analysis

It should be noted that overestimations of the intact glucosinolates were, generally, observed when looking either at total glucosinolate recovery in the PMM process (Figure 12) or the recovery at each of the three main stages (Table 10). On the other hand, the percentages of the glucosinolates in the last stage of the PMM process for both systems indicated some were not accounted for (Table 10). This might due to the problem of sample handling or due to the decomposition of the glucosinolates since GC is capable of determining the intact glucosinolates only. It is possible that hydrolysis of glucosinolates by myrosinase contributed to the losses in recovery of the intact glucosinolates. As the source material was a low-temperature heated meal, myrosinase may still be active. Youngs and Wetter (1967) demonstrated that hydrolysis of glucosinolates by myrosinase could be achieved if the moisture content exceeds 10%. In the dilution and precipitation stage of the PMM procedure, the starting materials of retentate was diluted to 15 times its volume with water in order to induce the hydrophobic interactions which in turn lead to the formation of micelles. In this regard, the marked losses in recovery of the intact glucosinolates were probably a consequence of glucosinolate decomposition by myrosinase, indicating that the myrosinase may remain active up the last stage of the PMM process. This may be of concern for the PMM canola protein isolates in the current study in terms of their use in food grade protein formulations.

4.2.2.2 Thiocyanate Ion Determination

Although the concentration and recovery of glucosinolates were examined previously by GC, it was not clear whether breakdown products from the glucosinolate-myrosinase system were extracted in the PMM process. The breakdown products are considered more harmful than the intact glucosinolates. To address this issue, the thiocyanate (SCN^-) ion determination was carried out, not only to indirectly determine the indole glucosinolates, but also to examine this concern associated with complexity of the glucosinolate-myrosinase system in the canola samples.

Earlier results have shown that the heterocyclic, or indole glucosinolates, including glucobrassicin and 4-hydroxyglucobrassicin, were well separated by GC and represented a relatively high proportion of the glucosinolates in most samples collected from the mass balance of the PMM process. These are the major glucosinolates which contribute to the formation of an instable 3-indolemethanol with the release of free SCN^- ions upon enzymatically hydrolysis or thermal degradation (Slominski and Campbell, 1989). The free SCN^- ion can also be released by glucosinalbin in the desolventization step of meal preparation (Campbell and Slominski, 1990). Production of free SCN^- ions was used as an indication of the degradation of glucosinolatespresent in the canola samples.

The effects of each stage of the PMM procedure on degradation of the indole glucosinolates were examined only in the samples from the series using Vivaflow 200 unit based on the fact that there was a relatively higher concentration of indole glucosinolates were present in most of these samples. Results are shown in Figure 13 and are expressed in μmol/g and percentage of the total at each stage. It should be noted

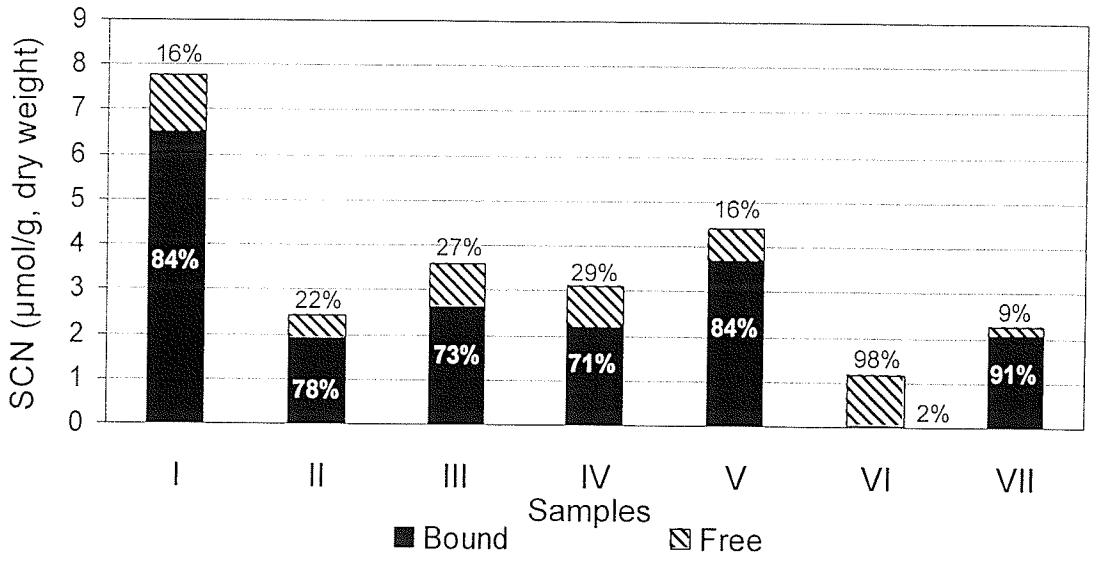


Figure 13. Use of thiocyanate (SCN^-) ion analysis[1] to determine concentration of indole glucosinolates[2] in protein samples collected from various stages of the PMM canola protein isolation process using the Vivaflow 200 ultrafiltration system (I, canola meal; II, debris; III, first supernatant; IV, filtrate; V, retentate; VI, second supernatant; VII, PMM).

[1] The sum of free and bound SCN^- equals to the total SCN^-.

[2] One µmol/g dry weight of SCN^- corresponds to one µmol/g of indole glucosinolate.

that the sum of free and bound SCN^- is equaled to the total SCN^- present in the samples. It is assumed that the release of the bound SCN^- upon treatment of the samples with myrosinase is an indication of the presence of intact indole glucosinolates.

Bound SCN^- was predominant in all samples, except for second supernatant (VI), (Figure 13). The bound SCN^- are the estimated proportions corresponding to the intact indole glucosinolates, which were analyzed by GC. However, in the present work, consistently lower values for the SCN^- ion determination were obtained compared to the GC method, as a possible consequence of the problem of overestimating the glucosinolates by GC. For example, based on bound SCN^-, canola meal (I) and PMM protein isolates (VII) had a content of 6.50 and 2.06 µmol/g, respectively, of indole

glucosinolates, which were much lower than the determined intact indole glucosinolates by GC of 9.63 and 6.58 μmol/g, respectively (Table 8). Therefore, the estimation of intact indole glucosinolates based on bound SCN^- followed with myrosinase treatment are not in agreements with the GC method (R^2=0.560). It appears that the SCN^- ion determination has failed to meet the goal of indirectly determining the intact indole glucosinolates of canola samples in the current study. However, this method still provides a tool to assess the extent of glucosinolate decomposition, particularly the unstable indole glucosinolates that may arise during the PMM process.

Smaller quantities of free SCN^- ions were found in all samples, except second supernatant (VI) (Figure 13), indicating that some decomposition of indole glucosinolates had occured. These were, in part, a consequence of the mild heat treatment used during the preparation of this canola meal and the subsequent hydrolysis at various stages in the PMM process. For instance, only 1.28 μmol/g (or 16% of total SCN^-) of free SCN^- ions was found in the canola meal (I) prior to the PMM process. Similarly, the PMM isolates (VII), produced from low-heated canola meal in the present work, had much lower free SCN^- ions with a content of only 0.21 μmol/g (or 9% of total SCN^-) compared to a commercial canola protein isolate (CPI), which had 0.99 μmol/g of free SCN^- ions (or 92% of the total SCN^- fraction). The commercial CPI was isolated from commercial canola meal that was heated to a high temperature to inactivate the myrosinase. The heat treatment however caused the substantial thermal degradation of indole glucosinolates and, therefore, promoted the release of free SCN^- ions (Slominski and Campbell, 1987; 1989; Campbell and Slominski, 1990). Differences in the heat treatment employed during meal preparation can result in different levels of free SCN^- ions in isolated canola protein.

Throughout the process, the ratio of bound to free SCN^- ions remained large, except for second supernatant (VI) which was almost 98% free SCN^- ions. Since the indole glucosinolate is one of the more unstable glucosinolates, in that is easily oxidized (Sørensen, 1990), these results indicate that the PMM process is a mild and gentle technique for isolating canola protein isolates with minimal glucosinolate degradation. However, additional discussions are required to further prove this hypothesis by looking at individual stages of the PMM process.

4.2.2.2.1 Effect of Extraction

In the extraction stage, it seems that first supernatant (III) contained more of the total SCN^-, both bound and free SCN^- ions, than the debris (Figure 13). This is because, as concluded earlier, the indole glucosinolates, from which the bound SCN^- is derived, are more readily soluble in water and therefore preferentially extracted in the first supernatant. In addition, in this aqueous environment, more of the free SCN^- ions may be released by hydrolysis from indole glucosinolates. By examining the recovery of SCN^-, it is clear that all of the SCN^- in both of the debris and first supernatant were comparable while leaving high percentage of SCN^- loss during handling of the samples (Figure 13; 14). More than 50% of the bound SCN^- was lost during this extraction stage. Other than problem of sample handling, one possible reason for such a large loss of the bound SCN^- in these samples may be due to the decomposition of indole glucosinolates by endogenous myrosinase. This, however, is unlikely since approximately 30% of free

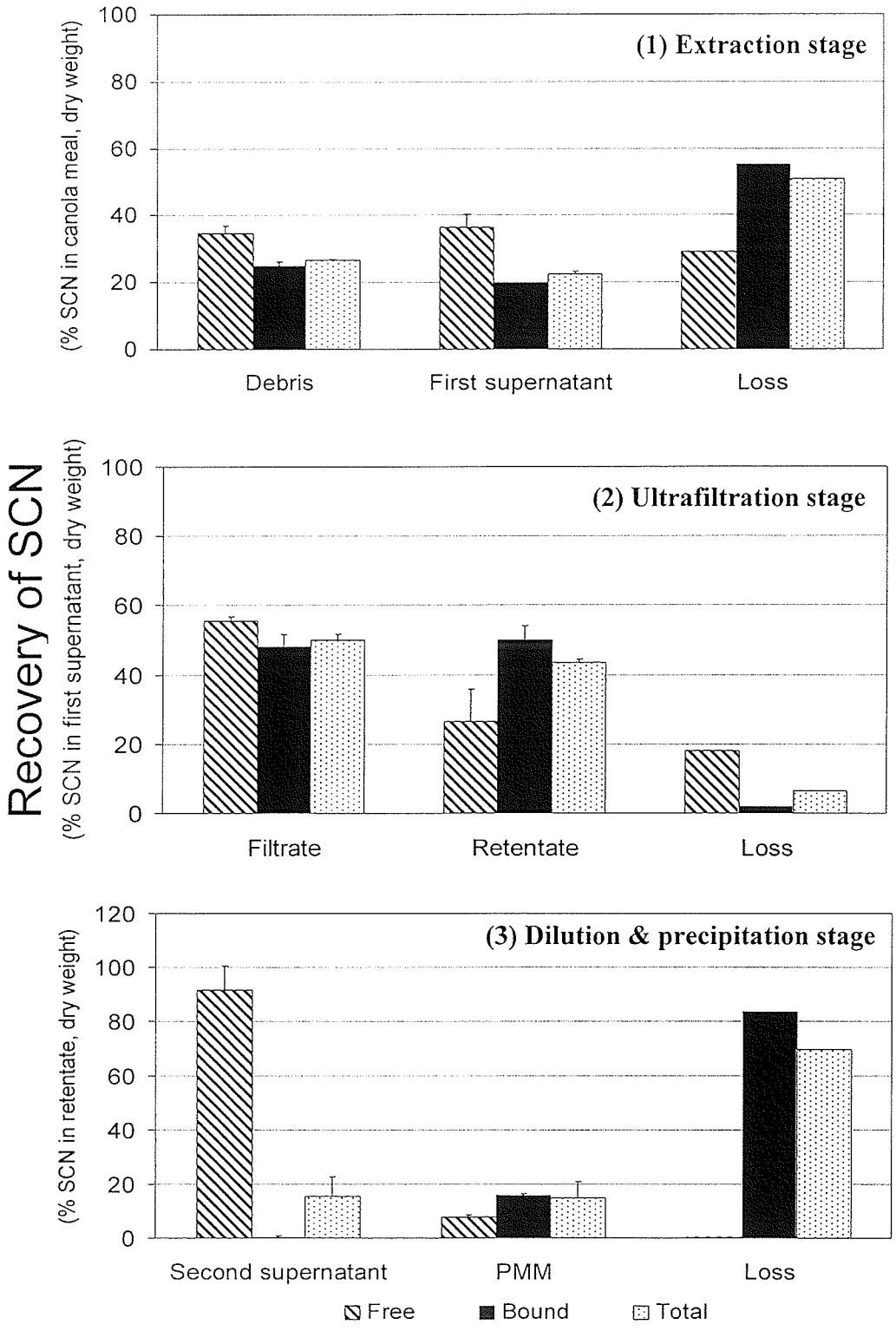


Figure 14. Recovery of free, bound, and total thiocyanate (SCN^-) from the SCN^- ion determination in the three main stages of the PMM procedures using the Vivaflow 200 ultrafiltration system

SCN^- ions (therefore about 50% in total SCN^-) was also lost. The reason for this high loss is not clear and requires further study.

4.2.2.2.2 Effect of Ultrafiltration

The data presented in Figure 13 also shows the effects of ultrafiltration on the concentration of SCN^- between filtrate (IV) and retentate (V). Ultrafiltration concentrated not only the proteins, but also the bound SCN^- (3.71 μmol/g or 84% of total SCN^-) corresponding to indole glucosinolates from first supernatant (III), which consisted of only 2.65 μmol/g (or 73% of total SCN^-), into the retentate (V). This is comparable to the earlier results, where the Vivaflow 200 ultrafiltration system did not significantly reduce the aromatic/heterocyclic glucosinolates as determined by GC. Although the Vivaflow 200 ultrafiltration system did not remove the indole glucosinolates, it allowed approximately 56% of free SCN^- ions to pass through the membrane into the filtrate, resulting in less free SCN^- ion (about 26%) in retentate (Figure 14). Therefore, ultrafiltration has potential to remove degradation products smaller than the membrane's MWCO from the retentate. However, it should be noted that some free SCN^- ions were still recovered in retentate.

4.2.2.2.3 Effect of Dilution and Precipitation

The dilution required for the formation of the micelles was shown to affect the levels of SCN^-, in particular the free SCN^- ions (Figure 13). A comparison of second supernatant and PMM isolates in term of SCN^- recovery indicates that the second

supernatant recovered almost 92% of free SCN^- ion from retentate (Figure 14). This shows that the dilution and precipitation stage of the PMM procedure performs an added benefit other than promoting the formation of the micelles, which is potentially removing glucosinolate degradation products, particularly the free SCN^- ion resulting from decomposition of the indole glucosinolates. It appears that more bound SCN^- was recovered in the PMM isolates than in the supernatant. Once again, this was probably due to the interaction between the micelles and glucosinolates through the hydrophobic interactions.

However, a high percentage of bound SCN^-, and therefore total SCN^-, was not recovered in this stage (Figure 14). Although the SCN^- ion determination fails to correspond to intact indole glucosinolates, this information can be taken as an indication of further decomposition of indole glucosinolates. In this regard, the results appear to support the evidence from the GC method which indicated there was glucosinolate decomposition in the last stage of the PMM process. Other than possible problem of sample handling, the marked loss of the indole glucosinolates was also probably due to the hydrolysis of glucosinolates by endogenous myrosinase when the moisture content exceeded 10% (Youngs and Wetter, 1967). Furthermore, the fact that approximately 83% of bound SCN^- was lost with no overestimation of free SCN^- ion (Figure 14) indicates that degradation products other than free SCN^- ions may be formed in the dilution and precipitation stage. Approximately 50% of other degradation products during the preparation of commercial meal were also reported to be something other than free SCN^- ion (Slominski and Campbell, 1987). These degradation products may have negative implications in the human diet due to their potential antinutritional and toxic effects and, therefore, require further study.

4.2.2.2.4 Evaluation of the Thiocyanate Ion Determination

The SCN^- ion determination has potential to asses the extent of the indole glucosinolate decomposition during the PMM process. The related chemical interactions between proteins and SCN^- however complicate the analysis of the effects of each stage of the PMM process on potential decomposition of glucosinolates. The overall mass balance on the total SCN^- in canola protein products during the PMM protein isolation procedure is outlined in Figure 15. During the extraction stage slightly more total SCN^- goes into the debris than the first supernatant. However, at this stage (Figure 14), a relatively high percentage of total SCN^- lost in the extraction stage for unknown reasons. Furthermore, the Vivaflow 200 ultrafiltration system successfully recovered more of the total SCN^- into the unwanted filtrate (11.25% of canola meal) than in retentate (9.81% of canola meal). As stated earlier, this is due to removal of the degradation products more than the intact indole glucosinolates. However, it was the dilution and precipitation stage that resulted in the greatest change in the glucosinolate decomposition, leading to only 1.52% and 1.45% of total SCN^- in the canola meal recovered in second supernatant and PMM isolates, respectively. This fact is also in agreement with the previous GC method stating that the greatest extent of glucosinolate decomposition occurred in the last stage of the PMM process.

The PMM process to isolate canola proteins from low-heated (or high-glucosinolate) canola meal consisting of active myrosinase has resulted in a large amount of glucosinolate decomposition. The PMM isolate is shown to recover only 1.45% of total SCN^- from canola meal; while about 59% of total SCN^- from canola meal was not accounted for. Further study is, thus, required to determine the type and level of

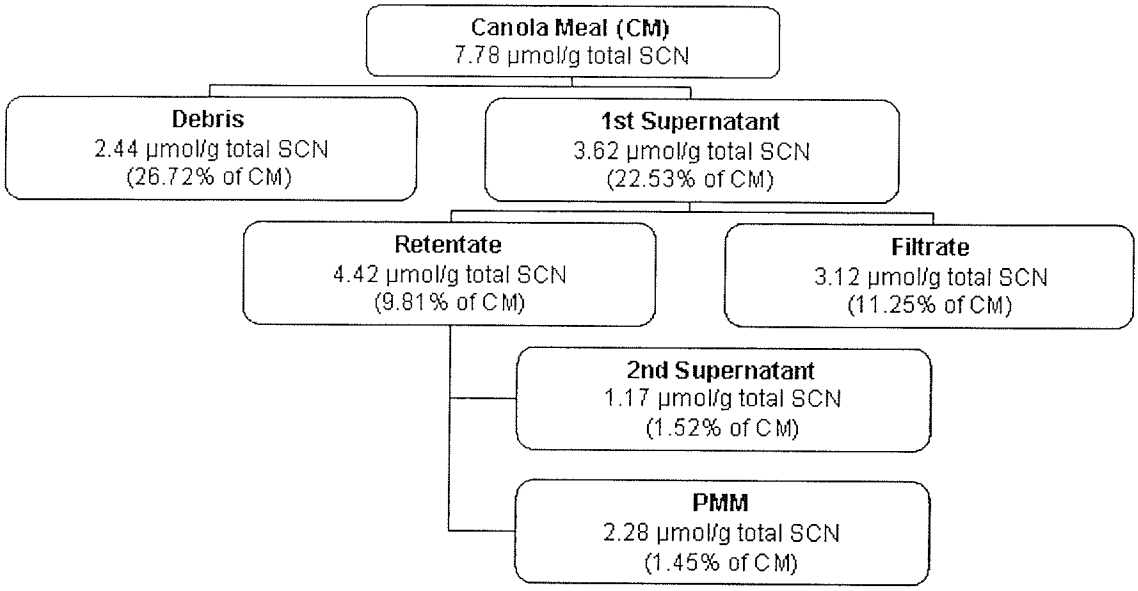


Figure 15. A mass balance diagram showing the recovery of total thiocyanate (SCN^-) [1] at the different stages of the PMM process with the Vivaflow 200 unit as the ultrafiltration system. [1] Debris + Filtrate + 2^{nd} Supernatant + PMM + Loss = 100% recovery; therefore, loss of total SCN^- ion is unaccounted for about 59% of CM.

these other degradation products, because this may be of concern for the PMM canola protein isolates in terms of their use in the human diet.

In the present work, it can be concluded that the SCN^- ion determination is one of many indirect methods that suffers from a lack of stringent conditions to ensure quantitative hydrolysis to measure compounds, in this case the SCN^-. Also, the fact that indole glucosinolates may yield the free SCN^- ions during analysis makes the SCN^- ion determination not well suited to the determination of total indole glucosinolates. There is another reason making this method inappropriate for estimating the bound SCN^- to indirectly determining the corresponding indole glucosinolates, and that is the heat treatments to which canola meals are exposed. In this study, the low heated canola meals had higher glucosinolate content than commercial canola meals which were treated with high temperatures. Without the high temperature treatment, myrosinase remained active even up to the last stage of the PMM process, as indicated from previous results by the GC method. This may have caused hydrolysis of glucosinolates by myrosinase during the analysis when water was added to the samples and extracted for 30 min. As a result, it can be concluded that some of the free SCN^- ions may have originated from the breakdown of glucosinolates during the analysis, that the results did not reflect the true picture of glucosinolate decomposition during the PMM process.

4.2.2.3 Diabetic Test Kit Technique

In recent years, simple and rapid methods for determining glucosinolates have received more attention. As the SCN^- ion determination had limited value in this study, an alternative method was investigated. Vescio *et al.* (2001) successfully estimated the

glucosinolates in canola and mustard seeds using another indirect method called the diabetic test kit technique, and provided evidence of good agreement between data from the diabetic test kit and data obtained using a NIR spectrometer. Thus, it is hoped that the application of the diabetic test kit technique will achieve the goal of accurately measuring glucosinolate levels in this study. This method is based on the measurement of the glucose released following hydrolysis of glucosinolates by endogenous enzyme.

This technique is simple and rapid. Water is added to the sample to initiate and optimize the endogenous myrosinase activities for catalyzing the hydrolysis of glucosinolates. The glucosinolates breakdown at this point and the glucose is released and measured by the One Touch ® Blood Glucose Meter. The use of endogenous myrosinase within the glucosinolate-myrosinase system contributes to the speed of this technique. Speed was a motivating factor in the development of the technique (Brzezinski and Mendelewski, 1984; Mawson *et al.*, 1993). The fact that the endogenous myrosinase in the samples remained active throughout the PMM process made this an appropriate technique to use. Unlike the canola meal used in the present work, the commercial canola meal tested had little or no myrosinase activity. The application of the traditional process to produce oil and meal from canola seeds using high temperatures decreases the activity of myrosinase by over 80% (Mawson *et al.*, 1995). Therefore, the diabetic test kit technique is not an appropriate rapid method to determine glucosinolates in those commercial canola meals or their related products.

The objectives of this section was not only to evaluate the use of diabetic test kits as a method for monitoring the total glucosinolate levels, but also to determine the mass balance of canola glucosinolates during the PMM process. Figure 16 shows the estimated total glucosinolate levels from the two ultrafiltration systems for samples

representing various stages of the PMM process. Starting with the canola meal, the diabetic test kit technique estimated a value of approximately 42 μmol total glucosinolate per gram of canola meal in both series. This compares well to the results obtained with GC of about 39 μmol/g. This demonstrates that this technique has the potential to indirectly determine total glucosinolate content in canola samples based on their glucose equivalents. The glucosinolate level in the debris was estimated to be about 22 μmol/g again comparing well with 21 μmol/g obtained by GC. However, the ability of this technique to produce results similar to those from the GC was not achieved for the rest of the samples. The total glucosinolate contents estimated from the diabetic test kit were consistently higher than those obtained by the GC method. It seems that the diabetic test kit technique works best with products that are originally in a dry state, including canola and mustard seed that were determined by Vescio *et al.* (2001), and canola meal and debris in the present work.

One explanation is the fact that the glucose meter detected not only the glucose released from the glucosinolate-myrosinase system, but also other glucose in the samples. According to Shahidi *et al.* (1990b), canola and mustard meal contain 3.93-5.73% sucrose, 0.27-0.62% raffinose, and 0.83-1.61% stachyose as the major soluble sugars, while glucose and fructose were present in trace amounts. It should be noted, however, that the types and amount of these soluble sugars varied, depending on their origin and the chemical analysis method employed (Naczk and Shahidi, 1990). Although the free glucose was present in trace amounts, glucose may also have been released by hydrolysis of other oligosaccharides in the presence of water.

As a result, the glucose levels of the samples were, in part, related to their conditions present during the three stages of the PMM process. In the extraction stage,

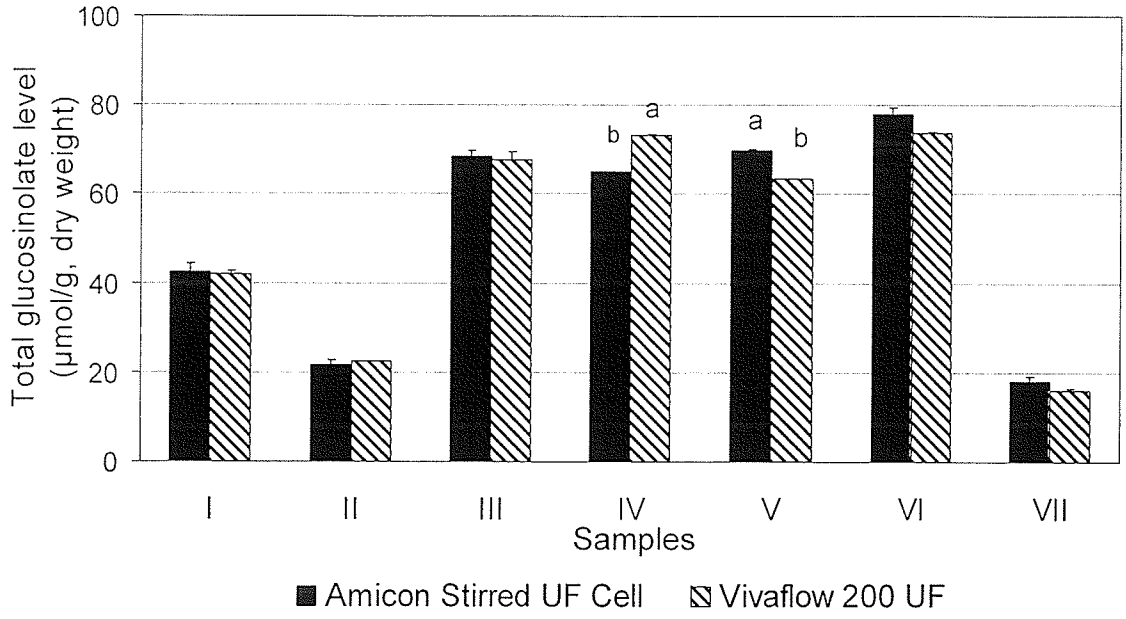


Figure 16. Use of diabetic test kit to estimate the total glucosinolate levels in protein samples collected from various stages of the PMM canola protein isolation procedure with Amicon stirred cell unit and Vivaflow 200 unit as the ultrafiltration systems (I, canola meal; II, debris; III, first supernatant; IV, filtrate; V, retentate; VI, second supernatant; VII, PMM). [a, b] For different ultrafiltrations, values in a same sample treatment having different letters are significantly different ($p < 0.05$)

these sugars were readily soluble in the first supernatant, and some oligosaccharide hydrolysis may occur to release more free glucose. This investigation of glucose was further supported by the fact that about 78% of the glucose was recovered in the first supernatant while an average of 45% was recovered in the debris for both series (Table 11). This increased the readings of the glucose meter, resulting in the first supernatant containing approximately 68 µmol/g of glucose (average for both series; Figure 16). As there were only 42 µmol/g of total glucosinolates in the first supernatant according to the GC results, about 22 µmol/g of glucose existed as non-glucosinolate glucose. This high glucose profile remained in the samples at subsequent stages of the PMM process

although minor differences due to the ultrafiltration systems were noted. In the last stage of the PMM process, the readily soluble glucose remained in the second supernatant leaving lower amounts in the PMM isolates. Comparable results have been reported by Aman and Gilberg (1977) where their canola protein isolates contained 86.0-88.5% less neutral sugars than those originally present in the meal. However, the PMM isolates in the present work still possess high amounts of glucose other than those released from the glucosinolate-myrosinase system as the values from the diabetic test kit technique were again higher than what was obtained using the GC.

Despite the fact that the diabetic test kit technique has, generally, overestimated the total glucosinolates in aqueous samples, our results showed a good agreement, particularly for samples prepared with the Vivaflow 200 ultrafiltration system (R^2=0.9307), between the GC method and the estimate of total glucosinolate contents from glucose released following the hydrolysis of glucosinolates by endogenous myrosinase (Appendix 5). The diabetic test kit technique is a non-specific indirect method which measures glucose regardless of where it comes from. It appears that the glucose meter used in this study detects the glucose only if it exists in a free form. This is supported by the fact that the canola meal and debris had comparable total glucosinolate contents to the results obtained by GC. The glucose meter was capable of determining only the free glucose, either the monosaccharide glucose itself or the glucose released from the glucosinolate-myrosinase system, but not the glucose bound to other molecules in the system. To use the diabetic test kit to monitor the release of the glucose on hydrolysis of glucosinolates in order to estimate the total concentration of glucosinolates in a sample, allowance must be made for the contribution of other non-

Table 11. Recovery of glucose using diabetic test kit technique at each stage of the PMM procedures using the Amicon stirred ultrafiltration cell (A) and the Vivaflow 200 ultrafiltration (B) systems

PMM stages	Samples	Glucose , % dry weight basis	
		Amicon stirred UF cell (A)	Vivaflow 200 UF (B)
Extraction (% of canola meal)			
	Debris	44.57 ± 0.05[1]	45.78 ± 0.97
	First supernatant	78.53 ± 4.89	78.32 ± 0.39
	Loss	* [2]	*
Ultrafiltration (% of first supernatant)			
	Filtrate	86.46 ± 1.79 [a]	62.67 ± 1.33 [b]
	Retentate	39.53 ± 1.02 [a]	33.44 ± 0.87 [b]
	Loss	*	3.89 ± 0.30 [b]
Dilution & precipitation (% of retentate)			
	Second supernatant	74.64 ± 1.73 [a]	67.88 ± 0.33 [b]
	PMM	5.91 ± 0.32 [b]	7.27 ± 0.24 [a]
	Loss	19.45 ± 1.41 [b]	24.85 ± 0.56 [a]

[1] Recovery of glucose is expressed in percentage based on the dry weight basis of the samples. Results are means of triplicate.

[2] ' * " refers to overestimation of glucose.

[a , b] For different ultrafiltrations, values in a same sample treatment having different letters are significantly different ($p < 0.05$)

glucosinolate glucose. In the current study, the results, however, can still be used to estimate the total glucosinolates in the samples.

Using this technique (Figure 16), the only significant differences in the glucose values representing the total glucosinolate content were observed during the ultrafiltration stage. Ultrafiltration allows the separation of the low and high molecular weight components, with carbohydrates such as the soluble sugars, ending up in the filtrate. The results show that the Vivaflow 200 ultrafiltration unit, with higher MWCO (30,000), allowed significantly more glucose from first supernatant through the membrane into the filtrate with significantly lower amounts of glucose in the retentate compared to the Amicon stirred ultrafiltration cell unit (10,000 MWCO). These results are in agreement with those of Diosady *et al.* (1984) where the removal of impurities, including glucose, was best with the higher MWCO membrane. Further support for this difference can be seen in the recovery of glucose during the ultrafiltration stage based on the glucose content in the first supernatant (Table 11). A significantly lower percentage of glucose was recovered in retentate by the Vivaflow 200 unit. Considering the better elimination effect of the Vivaflow 200 unit on impurities, it is surprising the glucose in the filtrate from the Vivaflow 200 unit was also lower. An apparent loss of glucose suggests there is a problem in sample handling perhaps related to the fact that small portions of the filtrate were left in the tubing of the unit itself.

In the dilution and precipitation stage, the second supernatant produced with the Vivaflow 200 ultrafiltration unit was shown to recover a significantly lower percentage of the glucose (Table 11). However, even with the lower recovery, almost 68% of the soluble glucose was still recovered in second supernatant. By contrast, the PMM isolates from the Vivaflow 200 unit recovered significantly higher percentage of glucose than

with the Amicon stirred cell unit. Higher total glucosinolate content, which was determined by GC in these PMM isolates, is also responsible for such a higher recovery of the glucose. It is also that possible interactions between the proteins and constituents including glucose contribute to this value. As concluded earlier, the proteins from the Amicon ultrafiltration unit were less likely to form a micelle based on the fact that lower protein was recovered. Thus, the PMM isolates from the Vivaflow 200 unit contained more proteins, and therefore higher levels of soluble sugar, and thus significantly higher recovery of glucose in the isolate. It is also possible that micelles in these PMM isolates interact with glucose, rendering them inaccessible to the glucose meter.

The diabetic test kit technique results in overestimation of glucose in the canola samples, particularly those originally in liquid form. This is demonstrated in Table 12 which shows the overall glucose recovery in the mass balance of the PMM process in the two ultrafiltration systems. As these values show total glucose recovery, glucose was recovered in descending order, for the terminal products as follows: the filtrate, then debris followed by second supernatant and finally the PMM isolates. This is, in fact, the same order as was obtained by the GC method.

Although overestimation occurs as a consequence of the determination of non-glucosinolate glucose, there is still a strong inference that the diabetic test kit was an appropriate method to estimate the total glucosinolate content of these canola samples. As both glucosinolate and glucose share the same property of being more hydrophilic, Table 12 indicates that glucosinolates were more readily soluble in water, and therefore most of glucose indicating the total glucosinolates were recovered in first supernatant (78.32-78.53%), filtrate (49.09-67.90%) and second supernatant (23.17-17.18%) in each of the corresponding stages, leaving only 1.83-1.90% of the glucose in the final product.

Table 12. Recovery of glucose[1] using diabetic test kit technique in the three main stages of the PMM procedure with canola meal as starting materials using two ultrafiltration systems: (A) Amicon stirred ultrafiltration cell and (B) Vivaflow 200 ultrafiltration cell

PMM stages	Samples	Glucose , % of canola meal	
		Amicon stirred UF cell (A)	Vivaflow 200 UF (B)
Extraction			
	Debris	44.57 ± 0.05[2]	45.78 ± 0.97
	First supernatant	78.53 ± 4.89	78.32 ± 0.39
Ultrafiltration			
	Filtrate	67.90 ± 2.83 [a]	49.09 ± 0.80 [b]
	Retentate	31.04 ± 1.13 [a]	26.19 ± 0.55 [b]
Dilution & precipitation			
	Second supernatant	23.17 ± 1.39 [a]	17.78 ± 0.29 [b]
	PMM	1.83 ± 0.03	1.90 ± 0.02
TOTAL RECOVERY[3]:		137.47 ± 4.23	114.55 ± 2.03

[1] One μmol/g dry weight of glucose is corresponding to one μmol/g of total glucosinolates.

[2] Recovery of glucose is expressed in percentage based on the dry weight basis of the samples. Results are means of triplicate analysis.

[3] Debris + Filtrate + 2nd Supernatant + PMM + Loss = 100% recovery; however, no loss but overestimation in this case.

[a , b] For different ultrafiltrations, values in a same sample treatment having different letters are significantly different ($p < 0.05$)

4.3 Processing Effects on Commercial Canola Protein Isolates

Processing can alter a protein's native structure, as well as that of other components including the glucosinolates and glucose. For instance, heating has been shown to inactive proteinase inhibitors, which normally complex with proteinases and render them inactivate (Ceciliani *et al.*, 1994). This results in reduced pancreatic activity and growth inhibition (Shahidi, 1997). By understanding the effects of processing conditions on proteins and associated components, we are able to preserve the desirable protein functional properties, and eliminate unwanted materials. This study was undertaken to assess the extent of glucosinolate decomposition, sugar hydrolysis and color change in a commercial canola protein isolate (CPI) at various processing conditions. Three processing effects were evaluated including pH (pH 4 -10), salt concentration (0.1 – 1.0 M) and heat (50 – 100°C). The experimental design was a duplicated two level full factorial design with center point.

Previous glucosinolate analyses conducted during the PMM preparation indicated a considerable amount of glucosinolates and myrosinase activity was still present in the final protein isolate. Although the glucosinolate level for the commercial CPI (Table 6) was below the level detectable by the GC, breakdown products may still play a role in limiting the use of commercial CPI in the human diet. Therefore, it is hoped that the current work could provide some valuable information for future application of these proteins in food grade protein formulations.

4.3.1 Decomposition of Glucosinolates

In general, processes such as oil extraction often result in undesirable breakdown of glucosinolates (Sørensen, 1990). The extent of breakdown can vary greatly, usually from 30% to 70% of the total amount, and in some cases, such as 4-hydroxyglucobrassicin, total destruction can occur (Daun, 1986). Therefore, it is important to quantify the change in the level of glucosinolates in commercial CPI at different post-isolation processing conditions.

Based on the SCN^- ion determination, the original commercial CPI contained 0.991, 0.090 and 1.081 μmol/g of free, bound and total SCN^-, respectively. As noted earlier, this indicates that only 0.090 μmol/g of intact indole glucosinolate was present in the commercial CPI while more than 90% was thermally degraded to the free SCN^- ions probably due to the high heat treatments reached during the preparation of the commercial canola meal. The data indicating the effects of the varying processing conditions on glucosinolate decomposition are shown in Table 13. Ranging from 0.673 to 1.073 μmol/g, the total SCN^- levels for the protein isolates treated with different combinations of the processing conditions tended to be lower than that of the original commercial CPI. In addition, it was observed that free SCN^- ions were the predominant form of SCN^- in all samples, and CPI at center points of three processing factors (salt concentration of 0.55 M, pH 7 and 75°C) produced all three forms of SCN^- in the middle range of the results obtained from the rest of the samples.

The Analysis of Variance (ANOVA) revealed that the processing effects are highly significant ($p < 0.001$) so that the model can describe the effects of temperature, pH and salt on the free and bound SCN^- in the CPI samples (Appendix 6; 7). It should be

Table 13. Effect of processing conditions on the extent of glucosinolate decomposition of commercial CPI[1] based on their SCN^- profiles, including free, bound and total SCN^- in terms of μmol/g of dry weight basis ± SD. Results are means of duplicate.

pH	Heating Temp (°C)	Salt Concentration (M)								
		0.1			1.0			0.55		
		Free	Bound	Total	Free	Bound	Total	Free	Bound	Total
4	50	0.575 ± 0.02	0.315± 0.01	0.890± 0.06	0.520± 0.01	0.200± 0.02	0.720± 0.02			
	100	0.766± 0.11	0.069± 0.04	0.835± 0.01	0.457± 0.06	0.231± 0.08	0.688± 0.02			
10	50	0.641± 0.01	0.223± 0.02	0.864± 0.02	0.583± 0.05	0.090± 0.01	0.673± 0.02			
	100	1.055± 0.06	0.018± 0.02	1.073± 0.03	0.713± 0.02	0.069± 0.03	0.781± 0.02			
7	75							0.569± 0.01	0.159± 0.01	0.728± 0.02

[1] Free, bound and total SCN^- of the original commercial CPI were 0.991, 0.090 and 1.081 μmol/g on the dry weight basis, respectively.

noted that the processing effects on the total SCN^- are not discussed in the current study although all main effects and interactions between pH and heat, and between pH and salt concentration were significant. The reason for limiting the discussion to the free and bound SCN^- is that they provide more valuable information regarding the effects of processing on commercial CPI.

As the presence of free SCN^- ions infers substantial decomposition of glucosinolates, all three processing factors had highly significant effects on glucosinolate decompositions of CPI (Appendix 6). In addition, there were highly significant interactions between heat and pH and between heat and salt concentration. These interactions can be seen in Figure 17.

Not surprisingly, high pH has been shown to degrade the indole glucosinolates, particularly the predominant 4-hydroxylglucobrassicin. The thioglucoside structure of glucosinolates makes them relatively unstable at extreme pH values (Olsen and Sørensen, 1981). This is particularly true for the 4-hydroxylglucobrassicin in alkaline solutions. In general, the indole glucosinolates produce isothiocyanates, which are unstable at neutral and alkaline conditions, and continually breakdown to produce an alcohol and free SCN^- ions (Fenwick *et al.*, 1989). This explains why CPI treated with NaOH (pH 10) had significantly more indole glucosinolate degradation, resulting in the release of indolemethanol and free SCN^- ions. As nitrile formation is favored at acidic conditions (Fenwick *et al.*, 1989; Bones and Rossiter, 1996; Ciska and Kozlowska, 1998), less glucosinolate degradation to free SCN^- ions was seen by lower levels of free SCN^- ions released at pH 4.0.

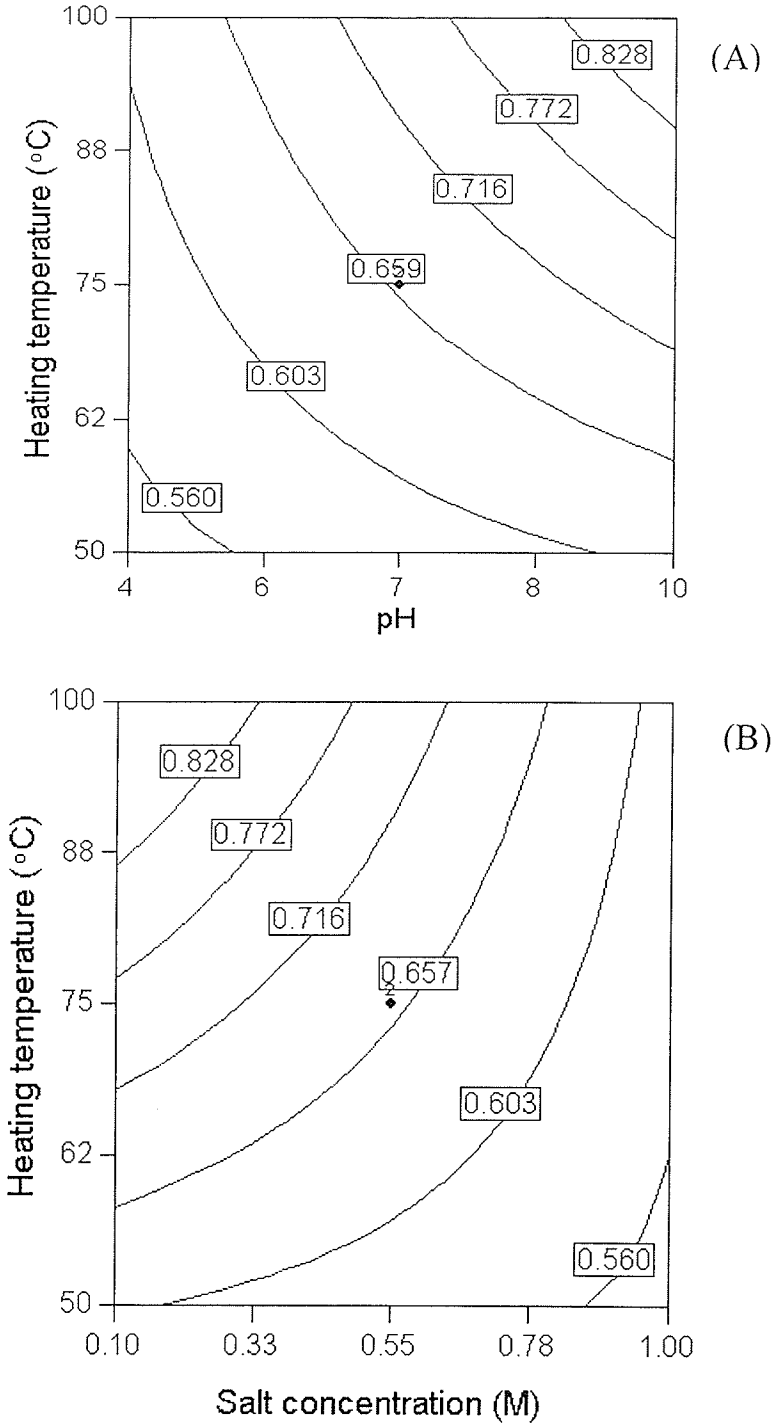


Figure 17. Contour plots showing the interaction effects between pH and heat at 0.55 M salt concentration (A), and salt concentration and heat at pH 7.0 (B) on free SCN^- ions of CPI

Indole glucosinolates have been shown to undergo thermal decomposition both during the heat treatment required for myrosinase inactivation and during cooking procedures (Slominski and Campbell, 1987; 1989; Campbell and Slominski, 1990). The presence of water is critical to this type of decomposition. This explains the significant thermal degradation of indole glucosinolates as indicated by an increase in free SCN^- ions at higher temperatures. It should be also noted that isothiocyanates and nitriles can also be produced at higher temperature (Uppström, 1995).

The combination of high pH and high temperature, therefore, had the greatest impact on the release of free SCN^- ions as demonstrated in Figure 17A. It is also evident that CPI at pH 10 was much more sensitive to temperature (a small increase in temperature resulted in a noticeable increase in the level of free SCN^-) than the CPI at pH 4. By contrast, the low pH, high temperature combination limited the release of SCN^- ions. When either pH or temperature was low, a large change in the other was required to promote the release of free SCN^- ion, as noted by the separation between the contour lines along the x and y axes (Figure 17A).

The influence of salt concentration on glucosinolate decomposition can be seen in the contour plot of the salt by temperature interaction (Figure 17B). Higher salt concentrations inhibit the release of the free SCN^- ions. As there is no literature available on the effect of salt concentration on glucosinolate decomposition, the reason for this is unclear. As stated earlier, all glucosinolates can be treated as salts because they are strongly acidic compounds (Olsen and Sørensen, 1981). Therefore, increasing salt concentration means increasing the ionic strength, allowing for the added salts to compete for binding sites on the glucosinolates. In such a highly ionic environment, there may be fewer tendencies for degradation of the glucosinolate structure. At lower

salt concentration, on the other hand, the binding of other factors, such as metallic ions, to the glucosinolates may promote their breakdown, and release of free SCN^- ions. Alternately, the change in salt concentration may affect the myrosinase activity, which catalyzes the glucosinolate decomposition. High salt levels will compete with the ability of myrosinase to interact with water and thus, prevent them from catalyzing glucosinolate decomposition Overall, high pH, high temperature and low salt concentration promoted the decomposition of glucosinolates and as evidenced by the release of free SCN^- ions.

The bound SCN^- represents the intact indole glucosinolates in the samples. Only pH and heat treatments had highly significant impacts on bound SCN^- of CPI (Appendix 7); however there were highly significant interactions between pH and salt concentration and between heat and salt concentration. In addition there was a significant 3-way interaction among pH, heat and salt. The influences of interactions between pH and salt concentration and between salt concentration and heat on bound SCN^- of CPI are shown in Figure 18. Unlike the effects seen for free SCN^- ions, both low pH and low temperature during processing retained more of the intact indole glucosinolates. The fact that salt was involved in significant interactions supports the idea that a combination effect of these processing factors acts in a manner that masks the effect of salt concentration alone on bound SCN^- in CPI.

The influence of pH on the intact indole glucosinolates (bound SCN^-) was most noticeable at high salt concentrations; however, regardless of salt concentration, higher levels of intact glucosinolates were seen at lower pH values (Figure 18A). This complements the data obtained for the free SCN^- ions, indicating that there was less degradation at the lower pH. The effect of temperature on bound SCN^- also

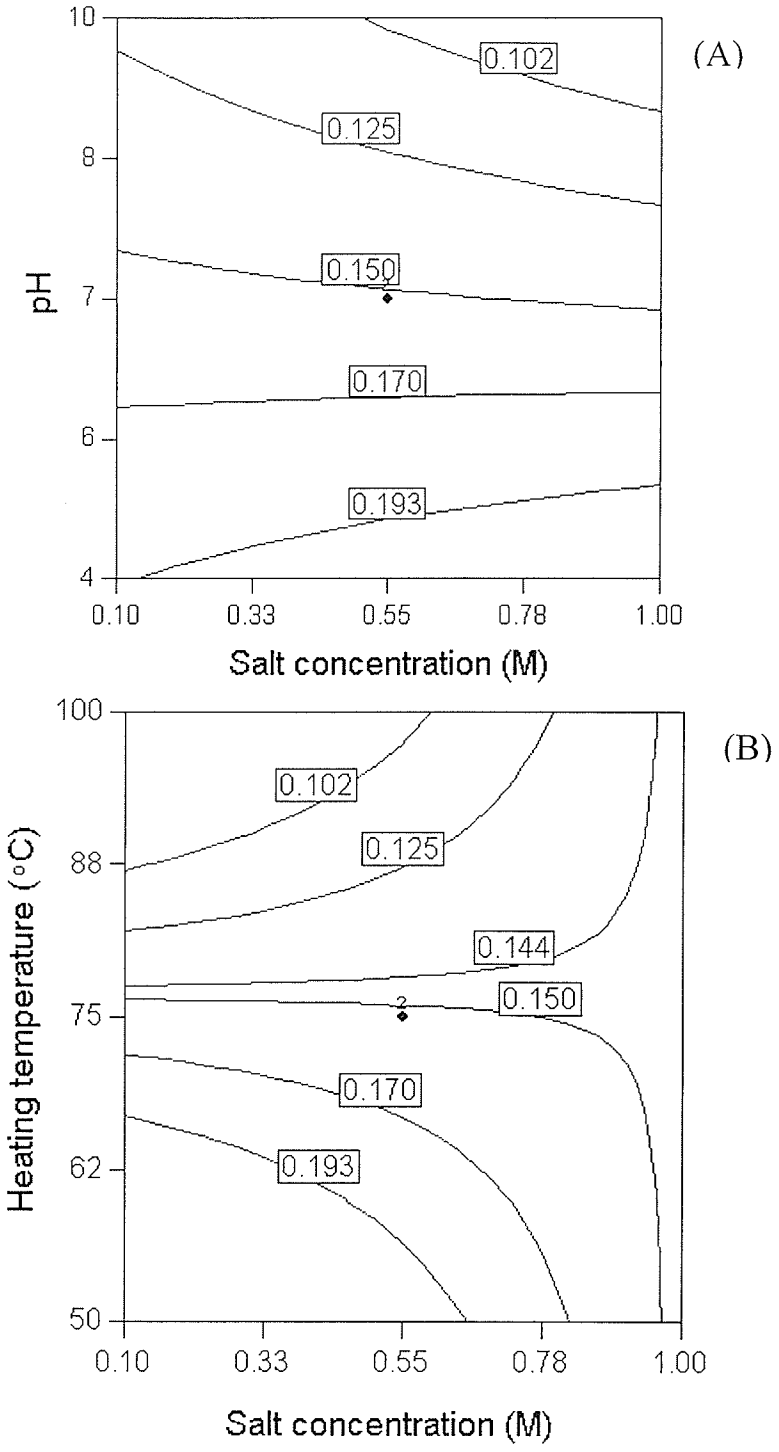


Figure 18. Contour plots showing the interaction effects between salt concentration and pH at 75 °C (A), and salt concentration and heat at pH 7.0 (B) on bound SCN^- of CPI

supports the free SCN^- ions data in that low temperatures had higher levels of intact indole glucosinolates, and therefore less degradation. The influence of salt on this effect was such that the ability of heat to degrade the glucosinolates was greater at lower salt concentrations (Figure 18B). Higher salt concentrations may have stabilized the glucosinolates against degradation. The influence of salt was also temperature dependent, with higher levels of salt producing more degradation (lower levels of bound SCN^-) at temperatures below 75°C, but low levels of salt promoting degradation at temperatures above 75°C.

An interesting aspect of these results is that, for the processing conditions, bound SCN^- values were generally higher and free SCN^- ions were generally lower than those of the original commercial CPI. This was most evident at pH 4 with the heating at 50°C at both salt concentrations. The decrease in free SCN^- ions suggests that these conditions may have promoted the binding of the free SCN^- ions with protein, thereby increasing the bound SCN^- in the samples. This was particularly true when CPI was processed at the conditions where protein solubility was low. For instance, pH 4 had a large effect on increasing the bound SCN^- of CPI. As minimum solubility for canola protein occurs at pH 4 and 8 (Prakash and Narasinga Rao, 1986), free SCN^- ions may be binding with the proteins at pH 4. This observation, however, may be affected by the presence of free SCN^- ions. This is because large quantities of free SCN^- ions may increase the ionic strength to a point that protein solubility is affected.

It is important that canola protein glucosinolates, in particular the prevalent indole glucosinolates, can be preserved in the innocuous intact form to minimize their degradation into more harmful products. As the SCN^- ion determination results indicated, to diminish the indole glucosinolate decomposition, it is best to process the CPI in

conditions where pH is low, or a combination of high salt concentration and high heating temperature can be obtained. However, it should be remembered that the free SCN^- ion that is bound to proteins may dissociate during cooking or chewing and exhibit antinutritional and toxic effects when they are consumed in large quantities.

4.3.2 Change in Glucose Level

The soluble carbohydrates, including glucose, fructose, galactose, sucrose, raffinose and stachyose, constitutes approximately 48% of the carbohydrate fraction in canola meal, while sucrose is the major soluble sugar present (Siddiqui *et al.*, 1973). The oligosaccharides, raffinose and stachyose, are generally claimed to affect nutritional value and quality of food products. For instance, food containing raffinose or stachyose could produce an excessive amount of gas causing flatulence in animals, as well as humans (Fleming, 1981). This is caused by the anaerobic fermentation in the large intestine as the enzyme α-galactosidase is lacking in the human digestive system. In addition, the presence of reducing sugars, such as glucose, and protein will ultimately influence the color of the food products. In this study, glucose measurements were used as a tool to follow the degradation of glucosinolates, but the influence of these endogenous sugars must not be overlooked when interpreting the results obtained.

Processing effects were examined with the use of the diabetic test kit technique to detect glucose in CPI (Table 14). It appears that differences between the observed glucose levels for different salt concentration were largest at the high pH and low heating temperature, while a middle level of glucose was observed at the mild

Table 14. Effect of processing conditions on the glucose content of commercial CPI[1] expressing in terms of μmol/g of dry weight basis ± SD. Results are means of triplicate.

pH	Heating Temp (°C)	Salt Concentration (M)		
		0.1	1.0	0.55
4	50	9.27 ± 0.14	7.69 ± 0.27	
	100	< 6.88	7.75 ± 0.18	
10	50	19.34 ± 0.50	16.73 ± 0.50	
	100	7.19 ± 0.44	7.46 ± 0.59	
7	75			10.63 ± 0.88

[1] The original commercial CPI contained 11.2 μmol/g of glucose in dry weight basis.

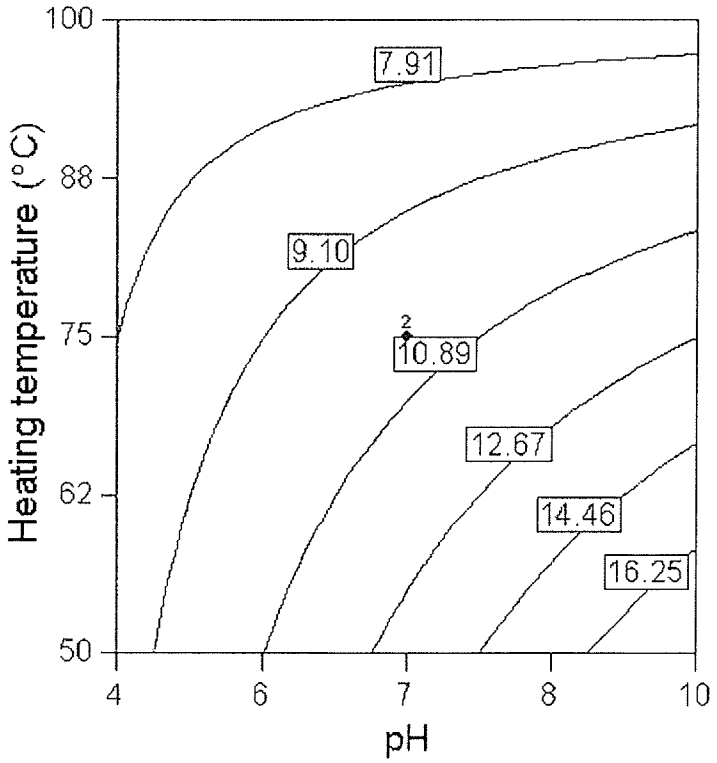


Figure 19. Contour plots showing the interaction effects between pH and heat at 0.55 M salt concentration on glucose of CPI

processing treatments (centre points of all conditions). Based on the ANOVA test (Appendix 8), only pH, heat and a pH by heat interaction had significant effects on the level of glucose detected. Salt concentration had no apparent effect on glucose content of CPI. From the perspective of the total glucose level determined by the diabetic test kit technique, there are three possible explanations for the changes due to processing treatments compared to the original CPI.

One explanation relates to the original objective of using this technique to determine the glucose released upon glucosinolate decomposition. Certainly, at low temperatures (≤75°C), pH had a large effect on elevating glucose levels of CPI (Table 14; Figure 19) as glucosinolates readily breakdown at higher pH values. Myrosinase also plays an important role in the transformation of glucosinolates into their degraded products, with the release of glucose. It is well documented that the optimal activity for myrosinase takes places at pH 4.5-8.0 (Mieth *et al.*, 1983b) and at 50-70°C with adequate moisture (exceeds 13%) (Young and Wetter, 1967), while ascorbic acid operates as a stimulator (Mieth *et al.*, 1983b). This activity would explain the high glucose level for CPI treated with mild pH and temperature (pH 7 and 75°C) (Table 14, Figure 19). The thermal inactivation of myrosinase apparently controlled glucosinolate decomposition, as glucose levels were lower at higher temperatures (Figure 19). At high temperatures, the role of glucosinolate decomposition on glucose levels was minimal even though the thermally glucosinolate decompositions may be stimulated. The inactivation of myrosinase at these temperatures may have had a larger impact on limiting glucose release suggesting that this enzyme activity and the effect of heat on enzyme activity contributed more to glucosinolate decomposition than a direct heating effect.

A second explanation for the increased levels of glucose could possibly be related to the hydrolysis of sucrose, the predominant soluble sugar in canola meal, at levels ranging from 5.93 to 7.58% (Shahidi *et al.*, 1990b). Sucrose is hydrolyzed as the glycosidic bond holding glucose and fructose together is cleaved by the enzyme glycosidase to form equal quantities of D-glucose and D-fructose (DeMan, 1985a). This enzymatic hydrolysis is facilitated by an acidic medium and the presence of water. In addition, raffinose and stachyose, which are present in lower amounts in canola, can also yield glucose due to hydrolysis. However, sugars in solution may undergo a number of other reactions depending on their stability (DeMan, 1985a). The most obvious example is the mutarotation of glucose where the equilibrium between structural isomers pyranose in α- and β-forms occurs as a result of the dissolved glucose forming an enediol such as fructose and mannose (DeMan, 1985a). In addition, dimerization and polymerization of reducing sugars may also take place. All of these reactions are greatly affected by the surrounding pH. Overall, a mixture of sugars, including glucose, fructose and mannose, will be formed if the glucose in solution is placed at an alkaline pH whereas the formation of disaccharides such as maltose and gentiobiose takes place when high concentrations of sugar are left at an acidic pH at ambient temperature (DeMan, 1985a). Soluble sugars may undergo caramelization reactions, one type of non-enzymatic browning reaction which occurs in the absence of amino groups (DeMan, 1985b), when the CPI, which contains a considerable amount of sugar, is subjected to extensive heating. Considering caramelization requires a temperature above melting point of sugars (> 100°C) (Eskin, 1990), the occurrence of the caramelization in the treated CPI is, therefore, minimal in this study as the highest heating treatment on CPI was only 100°C for four min. The effect of temperatures on the glucose level of CPI is

further complicated by the fact that high temperature heating can inactivate the enzyme glycosidase, which is responsible for the hydrolysis of sucrose. As the diabetic test kit technique detects sugar only in free glucose form, the higher glucose levels observed for CPI in alkaline conditions at low temperatures may be related to changes in the natural sugars. However, these reactions can be somehow complex when looking at combined processing effects.

Finally, the interactions between protein and sugars may also influence the glucose detected in the CPI. Seed proteins including canola protein can undergo conformation change and interaction with other compounds when subjected to variations in pH and temperature (Meith *et al.*, 1983). Not surprisingly, lower glucose levels can be traced to a Maillard reaction occurring between proteins and reducing sugars such as glucose. The Maillard reaction is a non-enzymatic browning reaction involving the α-amino groups of amino acids and the carbonyl groups of reducing sugars, resulting in the formation of brown nitrogenous polymers called melanoidins (DeMan, 1985b; Eskin, 1990). It has been reported that increasing temperature and pH favor these browning reactions. Therefore, the lower glucose level observed when treating CPI with higher temperatures (Figure 19) may be a result of the Maillard reaction. The Maillard reaction has been reported to increase with the temperatures (DeMan, 1985b; Eskin, 1990) although the reaction can also occur at temperature as low as 2°C (Ledl and Schleicher, 1990). In model systems, it has been reported that the Maillard reaction rate increases 2-3 times for each 10°C rise in temperature (DeMan, 1985b). The involvement of glucose in the Maillard reaction will profoundly affect the color of CPI, as will be discussed later.

Despite the fact that most glucosinolates degraded and released glucose at an alkaline pH, the contribution of glucosinolate decomposition to the increased glucose

levels in CPI at higher pH may be minimal as only small amounts of intact glucosinolates existed in commercial CPI. As stated earlier, glucosinolates were below the detectable level by the GC method, and levels based on the determination of bound SCN^- were only 0.090 μmol/g. The Maillard reaction is favored under alkaline conditions as amino acids are in the basic form and can rapidly react with reducing sugar like glucose (DeMan, 1985b; Eskin, 1990). If this reaction was prevalent with the CPI, lower levels of glucose would be produced at alkaline conditions as more of the available glucose would participate in the Maillard reaction forming melanoidins. In this study, however, higher glucose levels were seen at higher pH values (and lower temperatures). The most likely scenario is that the mutarotation or enolization of the glucose outweighed the Maillard reaction based on the effect of pH on the glucose in the CPI. This is mainly because the occurrence of both mutarotation and enolization of glucose has been reported to be reversible and are greatly favored by alkaline pH (DeMan, 1985a). Furthermore, dimerization and polymerization of glucose, which preferably takes place at acidic pH values, may contribute to the lower glucose levels at lower pH values. It is clear that the detectable glucose in CPI is greatly influenced by the chemical reactions of glucose alone.

4.3.3 Change in Color

In addition to chemical changes, sensory properties such as color, texture and flavor can also vary due to processing conditions. Color changes are particularly widespread during food storage and processing. In the case of CPI, it is clear that changes in color can be influenced by pH, salt concentration and temperature. Therefore,

it is appropriate to discuss color changes in CPI resulting from the post-isolation processing as this will impact food acceptance.

The colors of CPI from different processing conditions were measured and are presented in Table 15. The L* values in Table 15 were measurements of sample lightness, with 0 being black and 100 black. The redness was read by a* values from varying between +100 (red) and -80 (green), whereas the b* values indicated sample color changed from yellow (+100) to blue (-80). The L*, a* and b* values of the original commercial CPI were 37.09, 0.17 and 16.92, respectively, representing a light-tan color for the CPI. These values were, in fact, lower than those for canola protein isolates produced from combined treatments of alkaline extraction, isoelectric precipitation, ultrafiltration followed by diafiltration, and drying (L*= 69.3, a* = 0.8 and b*= 17.5) (Xu and Diosady, 2002). Accordingly, the canola protein isolates of Xu and Diosady (2002) were described as having an off-white color. Certainly the higher L* value would indicate a lighter color was obtained for the latter isolate, but the higher a* and b* values indicates the yellow and red pigments are also more intense than for the sample used in this study. However, both off-white and light-tan colors for CPI are acceptable in terms of customer satisfaction. When producing canola protein isolates for incorporation into human food formulation, changes in color must be monitored.

It was confirmed by the ANOVA test that pH and salt concentration had significant effects on lightness, redness and yellowness of CPI, expressed by the L*, a* and b* values, respectively, while temperature had significant effects on only the lightness and yellowness of CPI (Appendix 9; 10; 11). No significant interactions were observed.

Table 15. Effect of processing conditions on the color change of commercial CPI[1] based on the L* a* b* system. Results are means of duplicate ± SD.

pH	Heating Temp (°C)	Salt Concentration (M)								
		0.1			1.0			0.55		
		L*	a*	b*	L*	a*	b*	L*	a*	b*
4	50	32.74 ± 0.61	0.91 ± 0.23	15.44 ± 0.56	36.92 ± 0.70	-0.13 ± 0.40	12.13 ± 0.41			
	100	37.03 ± 0.57	0.37 ± 0.22	10.99 ± 0.78	41.95 ± 0.56	-0.37 ± 0.22	8.96 ± 0.51			
10	50	28.88 ± 0.61	-0.51 ± 0.57	19.03 ± 0.24	34.52 ± 0.63	-1.85 ± 0.25	14.82 ± 0.61			
	100	34.43 ± 0.48	-0.81 ± 0.52	11.61 ± 0.38	33.60 ± 0.68	-0.76 ± 0.16	11.45 ± 0.43			
7	75							29.94 ± 0.47	0.71 ± 0.16	15.09 ± 0.01

[1] The L*, a* and b* values of the original commercial CPI were 37.09, 0.17 and 16.92, respectively.

As shown in Table 15, the CPI became lighter (higher L*) with high temperatures, high salt concentrations and low pH treatments. Treatments at low pH and low salt concentrations resulted in higher a* values, giving an undesirable brownness to the CPI. It also seems that the effects of processing on b* (yellow) were opposite to those for the L* values in that higher b* values for the CPI were obtained with treatments of high pH, low temperatures and low salt concentrations. However, the high b* values (> 15) resulted in CPI that appeared to have a bright yellow color, which is not desirable. Thus the combination of high L* values and low b* values for CPI is preferred.

Many studies have reported that the phenolic compounds are major causes of the darker color often associated with canola protein isolates. This conclusion was based on the fact that those protein isolates containing lowered phenolic contents were discovered to be lighter in color (Sosulski, 1979; Ismond and Welsh, 1992; Xu and Diosady, 2000; 2002). As canola possesses about 10 times the phenolic contents of soybean, the phenolic compounds are difficult to completely eliminate from canola protein isolates (Xu and Diosady, 2000; 2002). Under alkaline conditions, both enzymatic and non-enzymatic oxidation of phenolic compounds result in the formation of quinones, which in turn react with proteins (Xu and Diosady, 2000; 2002). As a result, covalently bonded phenolic-protein complexes can be formed causing dark green or brown colors in protein isolates (Ismond and Welsh, 1992; Xu and Diosady, 2000; 2002).

Based on the effects of processing on the lightness (L* value) of CPI, the dark color of CPI in the present study may result from their phenolic contents. This is because lower L* (darker) values were obtained at high pH, low salt concentrations and low temperatures. Lower salt concentrations would open up the chain of proteins and

promote stronger covalent reactions between the SH-containing amino acids such as cysteine and the quinones that were derived from phenolic compounds under alkaline conditions (Xu and Diosady, 2002). High salt concentrations, on the other hand, promote interactions between proteins, decreasing the tendency to form the phenolic-protein complexes. Such complex formation is also minimized when the temperatures were too high resulting in protein denaturation and aggregation, again masking the reaction site. However, these processing conditions did not follow similar patterns for the two other color coordinates, a* and b*, suggesting that other possible reactions may occur upon post-isolation processing of CPI. Therefore, the relationship between residual glucosinolates and glucose has also been evaluated in this study.

4.3.3.1 Correlations Among Glucosinolates, Glucose and Color of Canola Protein Isolates

Correlations among SCN^- in three forms, including free, bound and total SCN^-, free glucose and color of commercial CPI are shown in Table 16. A significant inverse relationship ($p<0.05$) between lightness (L*) and yellowness (b*) in CPI supported the earlier statement such that processing had opposite effects on L* and b* values. To achieve an acceptable product color for CPI, it would be possible to monitor either of these two coordinates. In addition, the free and bound SCN^- were inversely correlated ($p<0.05$) to each other. As the free SCN^- ions were predominant in CPI, it is not surprisingly to find out that there is also a high correlation between free and total SCN^-.

Table 16. Pearson correlation coefficients' test for the color (L, a, b), glucose and SCN^- (free, bound, total) profiles in commercial CPI (N = 18; Prob > |r| under H0: Rho=0)

	L	a	b	Glucose	Free SCN^-	Bound SCN^-	Total SCN^-
L	1.00000	-0.17911[1] 0.4770[2]	-0.85040 **<.0001**	-0.53503 **0.0221**	-0.17661 0.4833	-0.04452 0.8608	-0.29600 0.2330
a		1.00000	0.06826 0.7878	-0.40542 **0.0951**	-0.18006 0.4746	0.47657 **0.0456**	0.17835 0.4789
b			1.00000	0.80919 **<.0001**	-0.11497 0.6496	0.34084 0.1663	0.14009 0.5793
Glucose				1.00000	-0.22978 0.3590	0.18167 0.4706	-0.18289 0.4676
Free SCN^-					1.00000	-0.71865 **0.0008**	0.78845 **0.0001**
Total SCN^-						1.00000	-0.14630 0.5624
Bound SCN^-							1.000000

[1] The first line in each row contains the correlation coefficients.
[2] The number beneath each coefficient is the significance of the correlation coefficient (in terms of whether it is significantly different from zero).

It is clear that glucose is highly correlated to both the L* and b* values, although, as expected, the correlation with L* value is an inverse relationship. As well, glucose shows some correlation ($p<0.10$) with the a* value of CPI. The correlation between glucose and CPI color can be easily traced to the Maillard browning reaction between an amino group of proteins and the carbonyl group of reducing sugars, such as glucose, during processing. It should be noted that the development of color from the Maillard reaction in the present work is undesirable. As little work has been done on color development for sugar-protein models in relation to processing conditions, these results are valuable.

In addition to the brown polymers or copolymers of melanoidins that form at the final stage of the Maillard reaction, other low molecular weight colored compounds may also results from the Maillard reaction (Ames and Nursten, 1989). These compounds, which typically comprise two to four linked rings, are yellow in color and are produced as intermediates compounds during the Maillard reaction (Ames, 1992). As glucose was shown to be highly, positively correlated to b* (yellowness) values, it is possible that, in addition to the phenolic-protein complexes, these colored compounds contribute to the color seen for the CPI samples in the present work. The inverse correlation between glucose and the L* value for CPI, however, does not seem to relate to the Maillard reaction. Although the inverse correlation between glucose and the a* value of CPI is weaker, it tends to support the conclusion that lower molecular weight colored compounds, rather than melanoidins, contribute to the color of CPI. If melanoidin production was favored, more glucose would produce the brown color associated with melanoidins resulting in higher a* values and a direct correlation. As heating treatments were short, the complex, multistage Maillard reaction may not have had time to form the

brown polymers or copolymers of melanoidins. Apart from resulting color change, the Maillard reaction is also responsible for nutritional losses by reducing essential amino acids, particularly lysine, as well as aroma development in foods. (DeMan, 1985b; Eskin, 1990; Ames, 1992). Thus, the control of the Maillard reaction is of great interest and importance in utilizing high quality food-grade of canola protein isolates.

In addition to the glucose, bound SCN^- representing the indole glucosinolates was also correlated with the a* (redness) value of CPI ($p<0.05$), with higher a* values associated with higher levels of bound SCN^-. The reason for such results is not clear. It may be due to the metal ions, particularly Fe^{2+}, that can bind to the anionic site of the intact glucosinolates (Figure 2), contributing the redness/brownness of CPI. If these ions are present, they may also bind with free SCN^- ions and increase the a* values. As there was no correlation between a* value and free SCN^- ions, this was unlikely in the present work. Therefore, further study on this relationship is required. While the decomposition of the glucosinolates under processing conditions is a concern, there is only minimal evidence linking this process to CPI color at this time. On the other hand, the color of CPI appears to be strongly influenced by its phenolic and glucose contents. While many researchers have emphasized the need to remove the phenolic compounds, work should be also done to control the Maillard reaction during the processing of canola protein isolates.

5.0 CONCLUSIONS

Considering that the PMM procedure is a simple and environmental friendly process, selective isolation of the 12S globular proteins has made the overall protein recoveries from low-temperature heated canola meal a challenge. In terms of protein quality, the PMM process has proven to be a success as high quality canola protein isolates (>90% protein) were produced with more than 70% reduction in glucosinolates.

As increasing the protein recovery is desirable, there are improvements required, particularly in the extraction, and the dilution and precipitation stages of the PMM process. Conditions of extracting canola protein need to be improved such that lower amounts of the protein and more of the glucosinolates are discharged along with the debris. Incorporation of further ultrafiltration, followed with drying, on the second supernatant could be used to recover the water-soluble 1.7S canola protein fraction that was decanted in the final stage of the PMM process. If such an approach was taken, careful attention to membrane selection must be considered based on the fact that high levels of glucosinolates were readily concentrated in the retentate from the initial ultrafiltration, and therefore present at high levels in these supernatants.

The use of the tangential flow module of the Vivaflow 200 unit with 30,000 MWCO was the preferred ultrafiltration system over the Amicon stirred cell unit in terms of its effectiveness and efficiency in recovering canola protein. Although this ultrafiltration unit has been shown to retain significantly more of the indole glucosinolates, ultrafiltration is, overall, an effective stage for producing canola protein low in glucosinolates.

To reduce the potential toxic effect of the glucosinolate degradation products, low-temperature heated canola meal was not recommended for use as starting materials of the PMM process as myrosinase may still be active to further decompose the relatively high levels of glucosinolates that were evident in this canola meal. Further research should be focused on the adaptation of the conditions of the PMM process to optimize the protein recovery with maximal reduction of glucosinolates.

Of all three methods evaluated, the diabetic test kit technique was the simplest and most rapid one for determining glucosinolates based on the measurement of the glucose released. It is well suited for the present research as this technique is only applicable to those samples in which the endogenous myrosinase is still active and able to hydrolyze the glucosinolates and release the free glucose. While glucose from other sources may be picked up by the diabetic test kit causing an overestimation of glucosinolates, the findings from this research suggest the total glucosinolate levels of canola samples could be easily and rapidly estimated with the use of this technique since the results were correlated to those from GC analysis. Despite that fact that the SCN^- ion determination was used as an indication of the decomposition of indole glucosinolate, it failed to reflect the true picture in the present research. This is probably because some of the free SCN^- ions may have originated from the glucosinolate decomposition during the analysis, given that myrosinase was shown to remain active up to the last stage of the PMM process. Therefore, it is suggested to modify the SCN^- ion determination by adding 20% TCA before the samples and water are mixed together. By doing so, myrosinase would inactive and prevent the hydrolysis of indole glucosinolate in releasing free SCN^- ions, which would in turn affect the accuracy of this method.

Indole glucosinolates, which were found in relatively large amounts in canola protein isolates, and some of their myrosinase-catalyzed transformation products have been recently shown to exert anticarcinogenic properties (Shahidi, 1997). Establishing a desirable quality of PMM canola protein isolates is, therefore, a controversial issue, depending on types of glucosinolates, possible decomposition by myrosinase from other source or even intestinal bacteria, different processing conditions, metabolism of individual, as well as the amount of protein consumed. Further understanding of the behavior of these compounds associated with canola protein isolates is needed so that future canola protein isolation processes can be carefully manipulated in such a way that they optimize the quality of canola proteins produced.

To maximize the application of these proteins in food grade protein formulations, the processing effects on the glucosinolate decomposition, sugar hydrolysis and color change in commercial CPI were studied under carefully defined pure systems. This information can be used as a guide for further study on complex food systems, considering the interaction of proteins with other compounds in food systems are always more complex than in a pure system. Based on the SCN^- ion determination, all three of the factors examined influenced glucosinolate decomposition. High pH and high heat were particularly effective in promoting glucosinolate decomposition in CPI. While only pH, heat and a pH by heat interaction exerted significant effects on the glucose determination with the diabetic test kit technique, chemical reactions among the sugars themselves may have greatly influenced the glucose levels detected in CPI. The color of CPI (L* and b* values) was shown to be significantly affected by the glucose. An interaction between glucose and the protein may have lead to the Maillard reaction, thus

causing the color change in CPI. Future work should be done to confirm the impact of the Maillard reaction on the quality of the canola protein.

In conclusion, this thesis successfully provides guidelines to improve canola protein recovery in the PMM process with maximal removal of glucosinolates. The use of SCN^- ion determination and diabetic test kit technique to monitor the value-determining compounds in canola protein has been evaluated, with the diabetic test kit technique being more effective. The processing effects on these compounds, as well as the color of the protein, have been assessed and low pH was best for controlling the reactions associated with undesirable color changes.

LITERATURE CITED

Aluko, R.E. and McIntosh, T. 2001. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. J. Sci. Food Agric. 81:391-396.

Aman, P. and Gilberg, L. 1977. Preparation of rapeseed protein isolates: a study of the distribution of carbohydrates in the preparation of rapeseed isolates. J. Food Sci. 42(1): 114-116.

Ames, J.M. 1992. The Maillard reaction. Pages 99-153 in: *Biochemistry of Food Proteins*. Hudson, B.J.F. ed. Elsevier Applied Science Pulishers Ltd, London, U.K.

Ames, J.M. and Nursten, H.E. 1989. Recent advances in the chemistry of colored compounds formed during the Maillard reaction. Pages 8-14 in: *Trends in Food Science*. Lien, W.S. and Foo, C.W. eds. Singapore Institute of Food Science and Technology, Singapore.

AOAC. 1975. Official Methods of Analysis 12th edition, Association of Official Analytical Chemists, Washington, D.C.

Arntfield, S.D., Ismond, M.A.H. and Murray, E.D. 1985. The fate of antinutritional factors using the preparation of a fababean protein isolate using a micellization technique. Can. Inst. Food Sci. Technol. J. 18:137-143.

Bhatty, R.S. and Finlayson, A.J. 1973. Extraction of non-protein nitrogen from oilseed meals with different solvents. Cereal Chem. 50:329.

Bhatty, R.S., McKenzie, S.L. and Finlayson, A.J. 1968. The proteins of rapeseed (*Brassica napus L.)* soluble in salt solutions. Can. J. Biochem. 46:1191-1197.

Bjergegaard, C. and Sørensen, H. 1996. Antinutritional compounds in feed and food. Presented at the Proceedings of 4th International Feed Production Conference, Piacenza Facolta Di Agraria, Italy. 105-135 pp.

Bones, A. 1990. Distribution of β–thioglucosidase activity in intact plants, cell and tissue cultures and regenerant plants of *Brassica napus* L. Exp Bot . 41:737-744.

Bones, A.M., and Rossiter, J.T. 1996. The myrosinase-glucosinolate system, its organization and biochemistry. Physiol. Plant. 97:194-208.

Brzenzinski, W. and Mendelewski, P. 1984. Determination of total glucosinolate content in rapeseed meal with thymol reagent. Z.Pflanzenzucht. J. Plant Breed. 93(3):177-183.

Burel, C., Boujard, T., Kaushik, S.J., Boeuf, G., Van Der Geyten, Mol, K.A., Kuhn, E.R., Quinsac, A, Krouti, M. and Ribailleir, D. 2000. Potential of plant-protein source as fish meal substitutes in diets for turbot (*Psetta maxima*): growth, nutrient utilization and thyroid status. Aquaculture 188:363-382.

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

Campbell, L.D. and Cansfield, P.E. 1983. Influence of seed processing on intact glucosinolates and implications regarding the nutritive quality of canola meal. Pages 50-54 in: *Research on Canola Seed, Oil and Meal, Seventh Progress Report*, McGregor, E.E. ed. Canola Council of Canada, Winnipeg, MB.

Campbell, L.D. and Slominski, B.A. 1990. Extent of thermal decomposition of indole glucosinolates during the processing of canola seed. J. Amer. Oil Chem. Soc. 67(2):73-75.

Canola Council of Canada, URL: http://www.canola-council.org. Accessed date: 04 October 2003.

Ceciliani, F., Bortolotti, F., Manegatti, E., Ronchi, S., Ascenzi, P. and Palmieri, S. 1994. Purification, inhibitory properties, amino acid sequence and identification of the reactive site of a new serine proteinase inhibitor from oil-rape (*Brassica napus*) seed. FEBS Letters 342:221-224.

Chen, M. and Rohani, S. 1992. Recovery of canola meal proteins by precipitation. Biotechnol Bioeng. 40:63-68.

Chevolleau, S., Debrauwer, L., Boyer, G. and Tulliez, J. 2002. Isolation and structure elucidation of a new thermal breakdown product of glucobrassicin, the parent indole glucosinolate. J. Agric. Food Chem. 50(18):5185-5190.

Ciska, E. and Kozlowska, H. 1998. Glucosinolates of cruciferous vegetables. Pol.J.Food Nutr. Sci. 7/48: 5-22.

Daun, J.K. 1986. Glucosinolate levels in western Canadian rapeseed and canola. J. Am. Oil. Chem. Soc. 63(5):639-643.

Daun, J.K. and McGregor, D.I. 1983. Glucosinolate analysis of rapeseed (canola), Agriculture Canada, Winnipeg, MB. 1-32 pp.

Dekker, M., Verkerk, R. and Jongen, W.M.F. 2000. Predictive modeling of health aspects in the food production chain: A case study on glucosinolates in cabbage. Trends In Food Science & Technology 11:174-181.

DeMan, J.M. 1985a. Carbohydrates. Pages 135-170 in: *Principles of Food Chemistry*. DeMan, J.M.ed. The AVI Publishing Company, Inc., Westport.

DeMan, J.M. 1985b. Proteins. Pages 86-134 in. *Principles of Food Chemistry*. DeMan, J.M.ed. The AVI Publishing Company, Inc., Westport.

Diosady, L.L., Tzeng, Y. and Rubin, L.J. 1984. Preparation of rapeseed protein concentrates and isolates using ultrafiltration. J. Food Sci. 49:768-777.

Duncan, A.J. 1991. Glucosinolates. Pages 126-147 in: *Toxic Substance in Crop Plants*. Felix Ð Mello, J.P., Duffs Carol, M. and Duffus John, H. eds. The Royal Society of Chemistry, Thomas Graham House, Science Park, Cambridge.

Ericson, M.L., Rödin, J., Lenman, M., Glimelius, K., Josefsson, L. and Rask, L. 1986. Structure of the rapeseed 1.7S storage protein, napin, and its precursor. J. Biol. Chem. 261(31):14576-14581.

Eriksson, S., Andréasson, E., Ekbom, B., Granér, G., Pontoppidan, B., Taipalensuu, J., Zhang, J., Rask, L. and Meijer, J. 2002. Complex formation of myrosinase isoenzymes in oilseed rape seed are dependent on the presence of myrosinase-binding proteins. Plant Physiol. 129:1592-1599.

Eskin, N.A.M. 1990. Biochemistry of food processing: Browning reactions in foods. Pages 239-296 in: *Biochemistry of Foods 2nd edition*. Eskin, N.A.M. ed. Academic Press, California.

Eskin, N.A.M. and McDonald, B.E. 2000. Canola Oil. Pages 248-262 in: *Wiley Encyclopedia of Food Science and Technology 2nd edition, Vol.1*. Francis, F. J. ed. John Wiley & Sons, Inc., NY.

Falk, A., Taipalensuu, J. Ek B, Lenman, M. and Rask, L. 1995. Characterization of rapeseed myrosinase-binding protein. Planta. 195:387-395.

Fennema, O. 1986. Chemical changes in food quality as influenced by the type of processing. Pages 1-21 in: *Role of Chemistry in The Quality of Processed Food*. Fennema, O.R., Zhang, W-H. and Lii, C-Y. eds. Food & Nutrition Press, Inc., Westport, Ct.

Fenwick, G. R., Heaney, R.K. and Mawson, R. 1989. Glucosinolates. Pages 1-41 in: *Toxicants of Plant Origin, Vol. II: Glycosides*. Cheeke, P.R. ed. CRC Press, Boca Raton, Florida, CA.

Fleming, S.E. 1981. A study of relationships between flatus potential and carbohydrate distribution in legume seeds. J. Food Sci. 46:794-798.

Francis, F. J. 2003. Color analysis. Pages 529-541 in: *Food Analysis 3rd edition*. Nielsen, S.S. ed. Kluwer Academic / Plenum Publishers, NY.

Friedman, M. 1996. Nutritional value of proteins from different food sources: A review. J. Agric. Food Chem. 44: 6-29.

Gillsberg, L. 1978. Influence of electrolytes on the solubility of rapeseed protein isolates. J. Food Sci. 43:1219-1228.

Gillsberg, L. and Turnell, B. 1976a. Preparation of rapeseed protein isolates: Dissolution and precipitation behaviors of rapeseed proteins. J. Food Sci. 41:1063-1069.

Gillsberg, L. and Turnell, B. 1976b. Preparation of rapeseed protein isolates. Precipitation of rapeseed proteins in the presence of polyanions. J. Food Sci. 41:1070-1075.

Han, A.H.L. 1994. Characterization and functional properties of canola protein hydrolysates. M. Sc Thesis, University of Manitoba, Winnipeg, MB.

Heaney, R.K. and Fenwick, G.R. 1982. The analysis of glucosinolates in *Brassica* species using gas chromatography: Direct determination of the thiocyanate ion precursors, glucobrassicin and neoglucobrassicin. J. Sci. Food Agric. 33:68-70.

Heaney, R.K. and Fenwick. G.R. 1995. Natural toxins and protective factors in *brassica* species, including rapeseed. Nat Toxins. 3:233-237.

Heaney, R.K., Spinks, E.A. and Fenwick, G.R. 1988. Improved method for the determination of the total glucosinolate content of rapeseed by determination of enzymically released glucose. Analyst. 113:1515-1517.

Ismond, M.A.H. 1984. The role of noncovalent forces in food protein interactions- a study system using legume storage protein vicilin. Ph. D. Thesis, University of Manitoba, Winnipeg, MB.

Ismond, M.A.H. and Welsh, W.D. 1992. Application of new methodology to canola protein isolation. Food Chem. 45:125-127.

Johnston, T.D. and Jones, D.I.H. 1966. Variations in the thiocyanate content of kale varieties. J.Sci. Food Agric. 17:70-71.

Klockeman, D.M. 1996. Isolation, characterization and enzyme hydrolysis of canola meal protein. Ph. D Thesis, University of Georgia, Athens, Georgia.

Klockeman, D.M., Toledo, R. and Sims, K.A. 1997. Isolation and characterization of defatted canola meal protein. J. Agric. Food Chem. 45:3867-3870.

Koshy, A., Bennetto, H.P., Delaney, G.M., Macleod, A.J. and Mason, J.R. 1988. An enzyme biosensor for rapid assay of glucosinolates. Analytical Letters 21(12):2177-2194.

Ledl, F. and Schleicher, E. 1990. New aspects of the Maillard reaction in foods and in the human body. Angew. Chem. Int. Ed. Engl., 29:565-594.

Léger, L. W. 1992. Thermally induce gelation of the 12S canola globulin. M. Sc. Thesis, University of Manitoba, Winnipeg, MB.

Léger, L. W. and Arntfield, S.D. 1993. Thermal gelation of 12S canola globulin. J. Amer Oil Chem. Soc. 70:853-861.

Lönnerdal, B, Gillberg, L. and Törnell, B. 1977. Preparation of rapeseed protein isolates: A study of rapeseed protein isolates by molecular sieve chromatography. J. Food. Sci. 42:75.

Ludikhuyze, L., Rodrigo, L. and Hendrickx, M. 2000. The activity of myrosinase from Broccoli (*Brassica oleracea* L. cv. Italica): Influence of intrinsic and extrinsic factors. J. Food Prot. 63(3): 400-403.

Lűthy, B. and Matile, Ph. 1984. The mustard oil bomb: Rectified analysis of the subcellular organization of the myrosinase system. Biochem. Physiol. Pfanz. 170:5-12.

Mahajan, A., Bhardwaj, S. and Dua, S. 1999. Traditional processing treatments as a promising approach to enhance the functional properties of rapeseed (*Brassica campestris* var. *toria*) and sesame seed (*Sesamum indicum*) meals. J. Agric. Food Chem. 47:3093-3098.

Mawson, R., Heaney, R.K., Piskula, M. and Kozlowska, H. 1993. Rapeseed meal glucosinolates and their antinutritional effects. Part 1. Rapeseed production and chemistry of glucosinolates. Die Nahrung 37(2):131-140.

Mawson, R., Heaney, R.K., Zduńczyk, Z. and Kozlowska, H. 1995. Rapeseed meal glucosinolates and their antinutritional effects. Part 7. Processing. Die Nahrung 39 (1):32-41.

McCurdy, S.M. 1992. Infrared processing of dry peas, canola, and canola screenings. J. Food Sci. 57:941-944.

McGregor, D.I. 1978. Thiocyanate ion, a hydrolysis product of glucosinolates from rape and mustard seed. Can. J. Plant Sci. 58:795-800.

McKenzie, S.L., and Blakely, J.A. 1972. Purification and characterization of seed globulins from *Brassica juncea, B. nigra and B. hirta.* Can. J. Botany. 50:1825-1834.

McNair, H.M. and Miller, J.M. 1998. Instrument overview. Pages 14-28 in: *Basic Gas Chromatography.* McNair, H.M. and Miller, J.M. eds. John Wiley & Sons, Inc, NY.

Mieth, G., Bruckner, J., Kroll, J. and Pohl, J. 1983a. Rapeseed: Constituents and protein products. Part II. Preparation and properties of protein-enriched products. Die Nahrung 27(8): 759-801.

Mieth, G., Schwenke, K., Raab, B. and Bruckner, J. 1983b. Rapeseed: Constituents and protein products. Part I. Composition and properties of proteins and glucosinolates. Die Nahrung 27(7): 675-697.

Mithen, R. 2001 Glucosinolates: Biochemistry, genetics and biological activity. Plant Growth Regulation 34:91-103.

Mithen, R.F., Dekker, M., Verkerk, R. and Rabot, S. 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. J Sci Food Agric. 80:967-984.

Murray, E.D. 2001. Isolating protein from canola. Oils & Fats International 17(3): 19-21.

Murray, E.D., Myers, C.D., Barker, L.D. and Maurice, T.J. 1981. Functional attributes of proteins: A noncovalent approach to processing and utilizing plant proteins. Pages 158-176 in: *Utilization of Protein Resources*, Stanley, D.W., Murray, E.D. and Lees, D.H. eds. Food and Nutritional Press, Inc., Westport, Ct.

Naczk, M. and Shahidi, E. 1989. The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola. Food Chem. 31:159-164.

Naczk, M. and Shahidi, E. 1990. Carbohydrate of canola and rapeseed. Pages 211-220 in: *Canola and Rapeseed.* Shahidi, F. ed. Van Nostrand Reinhold, NY.

Nakai, S. and Li-Chan, E. 1988. *Hydrophobic Interactions in Food Systems.* CRC Press Inc., Boca Raton, Florida, CA.

Niewiadomski, H. 1990. *Rapeseed Chemistry and Technology*, Elsevier, NY.

Norton, G. 1989. Nature and biosynthesis of storage proteins. Pages 165-191 in: *Oil Crops of the World: Their Breeding and Utilization.* Röbbelen, G., Downey, R.K. and Ashri, A. eds. McGraw-Hill Publishing Co., NY.

Olsen, O. and Sørensen, H. 1981. Recent advances in the analysis of glucosinolates. J. Am. Oil Chem. Soc. 58:857-865.

Pecháček, R., Velíšek, J. and Davídek, J. 2000. Decomposition of sinigrin by methanol/ammonia/water treatment in model systems and mustard (*Brassica nigra* L.) seed meal. Eur Food Res Technol. 210:196-201.

Prakash, V. and Narasinga Rao, M.S. 1986. Physicochemical properties of oilseed proteins. CRC Crit. Rev. Biochem. 20:265-363.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J. 2000. Myrosinase: Gene family evolution and herbivore defense in *Brassicacea*. Plant Mol Biol. 42:93-113.

Rubin, L.J., Diosady, L.L. and Tzeng, Y-M. 1990. Ultrafiltration in rapeseed processing. Pages 307-330 in: *Canola and Rapeseed.* Shahidi, F. ed., Van Nostrand Reinhold, NY.

Rubino, M.I., Arntfield, S.D., Nadon, C.A. and Bernatsky, A. 1996. Phenolic protein interactions in relation to the gelation properties of canola protein. Food Res. Int. 29:653-659.

Sang, J.P. and Truscott, R.J.W. 1984. Liquid chromatographic determination of glucosinolates in rapeseed as desulfoglucosinolates. J. Assoc. Off. Anal. Chem. 67:829-833.

Schwenke, K.D., Raab, B. and Damaschun, G. 1983. The structure of the 12S globulin from rapessed (*Brassica napus L.*). Die Nahrung 27(2):165-175.

Schwenke, K.D., Raab, B., Linow, K-J, Pähtz, W. and Uhlig, J. 1981. Isolation of the 12S globulin from rapeseed (*Brassica napus* L.) and characterization as 'neutral' protein. On seed proteins. Part 13. Die Nahrung 25:271-280.

Shahidi, F. 1990. Rapeseed and canola: Global production and distribution. Pages 3-13 in: *Canola and Rapeseed.* Shahidi, F. ed. Van Nostrand Reinhold, NY.

Shahidi, F. 1997. Beneficial health effect and drawbacks of antinutrients and phytochemicals in foods. Pages 1-9 in: *Antinutrients and Phytochemicals in Foods.* Shahidi, F. ed. Amer Chem Soc (Symposium Series 662), Washington D.C.

Shahidi, F., Gabon, J.E., Rubin, L.J. and Naczk, M. 1990a. Effect of methanol-ammonia-water treatment of the fate of glucosinolates, J. Agric. Food Chem. 38:251-255.

Shahidi, F., Naczk, M. and Myhara, R.M. 1990b. Effect of processing on the soluble sugars of *Brassica* seeds. J. Food Sci. 55:1470-1471.

Siddiqui, I.R., Wood, P.J. and Khanzada, G. 1973. Low molecular weight carbohydrates from rapeseed (*B. campestris*) meal. J.Sci. Food Agric. 24(1):427-435.

Slominski, B.A. 1995. Gas chromatography of trimethylsilyl derivatives of glucosinolates, Animal Science Department, University of Manitoba, Winnipeg, MB.

Slominski, B.A. and Campbell, L.D. 1987. Gas chromatography determination of indole glucosinolate: A re-examination. J.Sci. Food Agric. 40:131-143.

Slominski, B.A. and Campbell, L.D. 1989. Formation of indole glucosinolate breakdown products in autolyzed, steamed, and cooked *Brassica* vegetables. J. Agric. Food Chem. 37(5):1297-1302.

Smith, C.A. and Dacombe, C. 1987. Rapid method for determining total glucosinolates in rapeseed by measurement of enzymatically released glucose. J.Sci. Food Agric. 38(2):141-150.

Smith, D.B. and Donald, C.N. 1988. The measurement of glucosinolates in oilseed rape by glucose release. Plant Varieties and Seeds 1(2):121-130.

Sørensen, H., 1990. Glucosinolate: Structure-Properties-Function. Pages 149-172 in: *Canola and Rapeseed.* Shahidi, F. ed. Van Nostrand Reinhold, NY.

Sosulski, F.W. 1979. Organoleptic and nutritional effects of phenolic compounds on oilseed protein products: A review. J. Am. Oil Chem. Soc. 56:711-714.

Srivastava, V.K. and Hill, D.C. 1975. Thiocyanate ion formation in rapeseed meals. Can. J. Biochem. 53:630-633.

Szmigielska, A.M., Schoenau, J. J. and Levers, V. 2000. Determination of glucosinolates in canola seeds using anion exchange membrane extraction combined with the high-pressure liquid chromatography detection. J. Agric. Food Chem. 48: 4487-4491.

Thakor, N.J., Sokhansanj, S., McGregor, I. and McCurdy, S. 1995. Dehulling of canola by hydrothermal treatments. J. Amer Oil Chem. Soc. 72:597-602.

Thompson, L.U. 1990. Phytates in Canola/Rapeseed. Pages 173-192 in: *Canola and Rapeseed.* Shahidi, F. ed. Van Nostrand Reinhold, NY.

Tzeng, Y-M, Diosady, L.L., Chen, B-K and Rubin, L.J. 1990a. Ultrafiltration rejection coefficient of canola meal components. Can. Inst. Food Sci. Technol. J. 23(2/3): 131-136.

Tzeng, Y-M, Diosady, L.L., Rubin, L.J. 1990b. Production of canola protein materials by alkaline extraction, precipitation and membrane processing. J. Food Sci. 55:1147-1151,1156.

Underhill, E.W. and Kirkland, D.F. 1971. Gas chromatography of trimethylsilyl derivatives of glucosinolates. J. Chromatogr. 57:47-54.

Unger, E.H. 1990.Commercial processing of canola and rapeseed: Crushing and oil extraction. Pages 235-249 in: *Canola and Rapeseed.* Shahidi, F. ed. Van Nostrand Reinhold, NY.

Uppström, B. 1995. Seed chemistry. Pages 217-242 in: *Brassica Oilseed: Production and Utilization*. Kimber, D. S. and McGregor, D.I. eds. CAB International, Wallingford, UK.

Vescio, K., Scott, C. and Daun, J. 2001. Rapids methods for glucosinolate determination in canola and mustard using diabetic test kits. Canadian Grain Commission, Winnipeg, MB.

Wanasundara, J.P.D. and Shahidi, F. 1994a. Alkanol-ammonia-water/hexane extraction of flaxseed, Food Chem. 49:39-44.

Wanasundara, J.P.D. and Shahidi, F. 1994b. Functional properties and amino-acid composition of solvent-extracted flaxseed meals, Food Chem. 49:45-51.

Welsh, W.D. 1988. Application of new methodology to canola protein isolation. M. Sc. Thesis, University of Manitoba, Winnipeg, MB.

Xu, L. and Diosady, L.L. 1994. The production of Chinese rapeseed protein isolates by membrane processing. J. Amer Oil Chem. Soc. 71:935-939.

Xu, L. and Diosady, L.L. 2000. Interactions between canola proteins and phenolic compounds in aqueous media. Food Res. Int. 33:725-731.

Xu, L. and Diosady, L.L. 2002. Removal pf phenolic compounds in the production of high-quality canola protein isolates. Food Res. Int. 35:23-30.

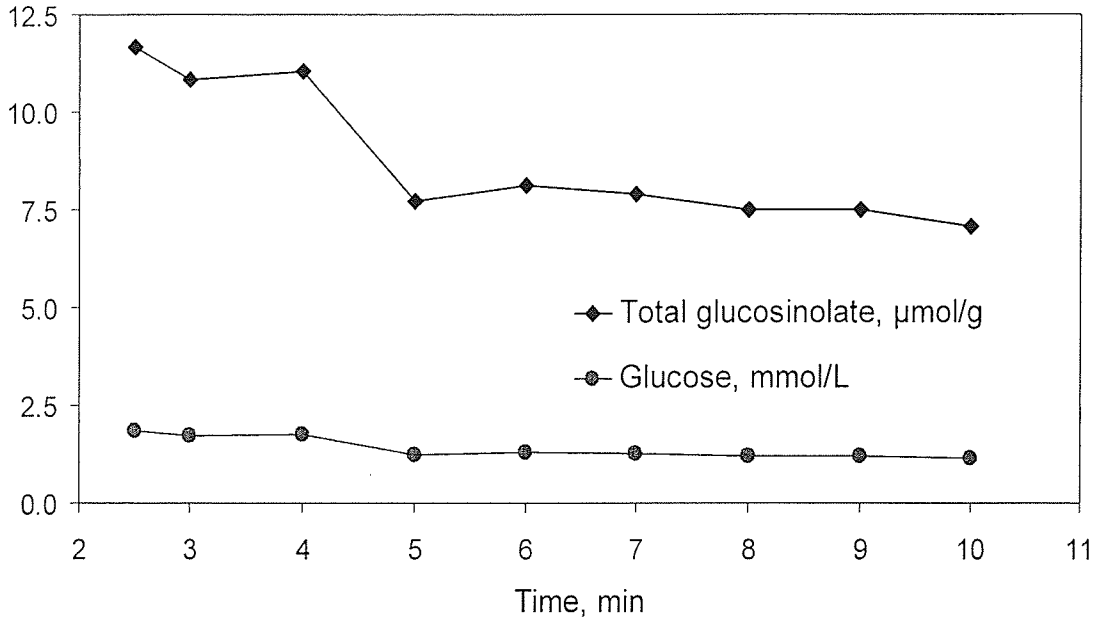
Youngs, C. G. and Wetter, L. R. 1967. Microdetermination of the major individual isothiocyanates and oxazolidinethiones in rapeseed. J. Am. Oil Chem. Soc. 44:551-555.

APPENDICES

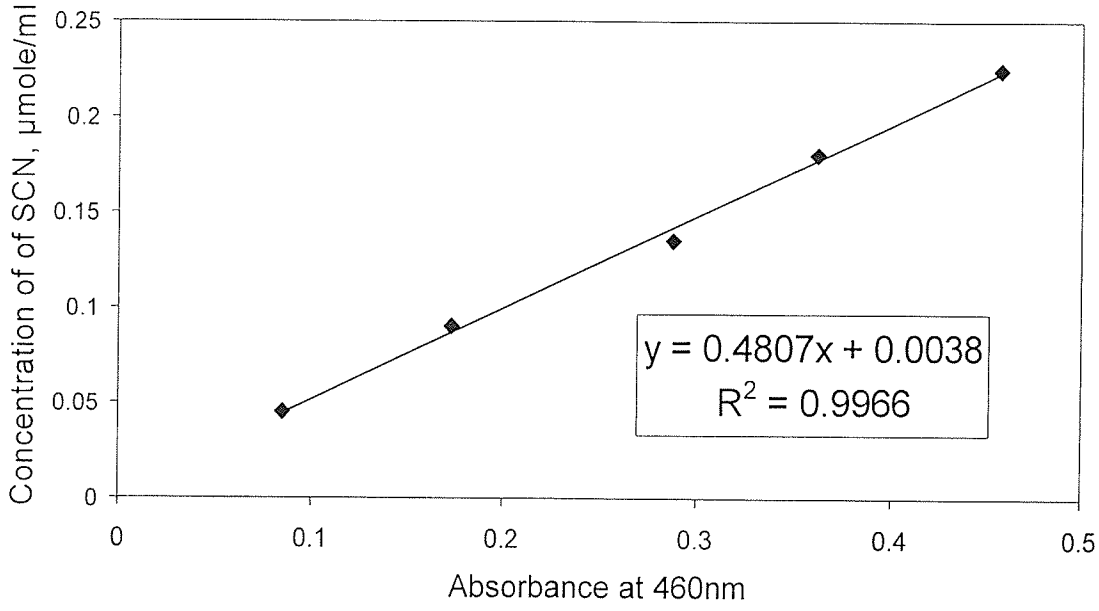
APPENDIX 1. A two-level, three-factor full factorial design with pHs between 4 to 10, salt concentrations of 0.1 M to 1.0 M, and heating effects of 50°C to 100°C on canola protein isolates (CPI) by Design Expert® Software

Std	Run	Block	Factor A:pH	Factor B:Salt Conc. M	Factor C:Heat deg. C
1	8	Block 1	4	0.10	50
2	11	Block 1	4	0.10	50
3	9	Block 1	10	0.10	50
4	13	Block 1	10	0.10	50
5	4	Block 1	4	1.00	50
6	14	Block 1	4	1.00	50
7	3	Block 1	10	1.00	50
8	18	Block 1	10	1.00	50
9	10	Block 1	4	0.10	100
10	2	Block 1	4	0.10	100
11	12	Block 1	10	0.10	100
12	1	Block 1	10	0.10	100
13	17	Block 1	4	1.00	100
14	6	Block 1	4	1.00	100
15	7	Block 1	10	1.00	100
16	16	Block 1	10	1.00	100
17	5	Block 1	7	0.55	75
18	15	Block 1	7	0.55	75

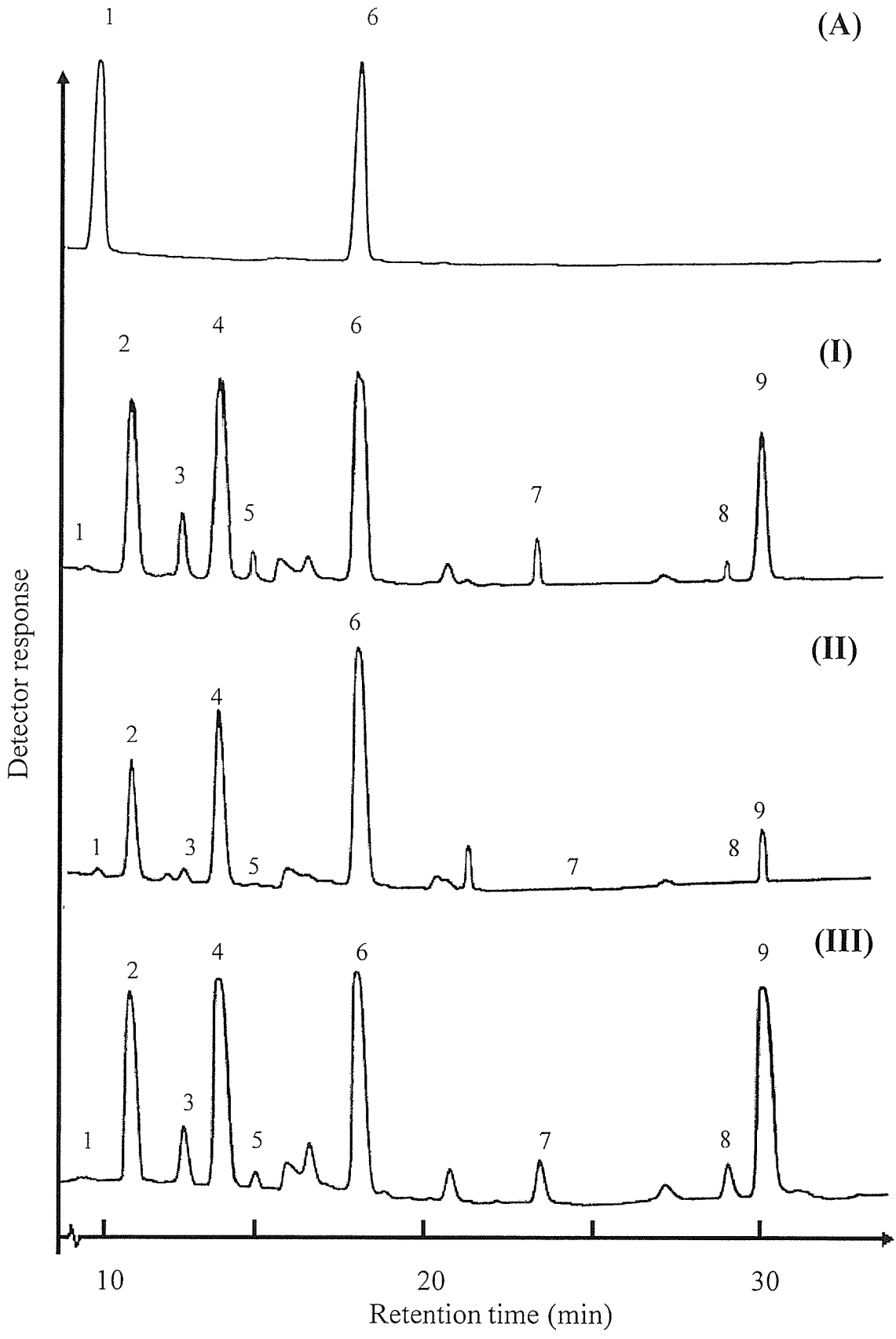
APPENDIX 2. Effect of time on the development of glucometer readings on glucose concentrations and estimated total glucosinolate levels from three samples of canola protein isolates (CPI)

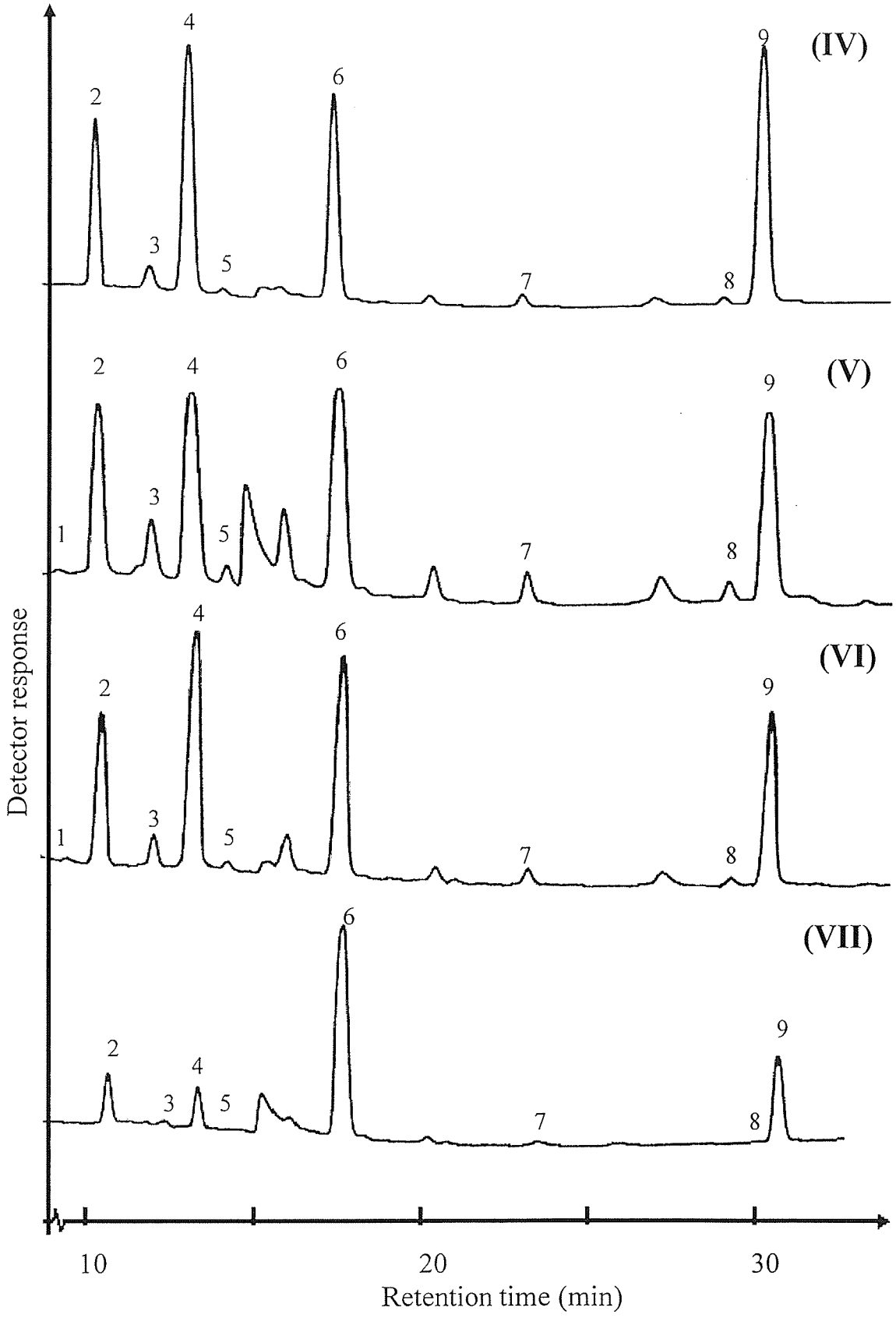


APPENDIX 3. A potassium thiocyanate (KSCN) calibration curve for thiocyanate (SCN^-) ions determination of the canola samples at 460 nm



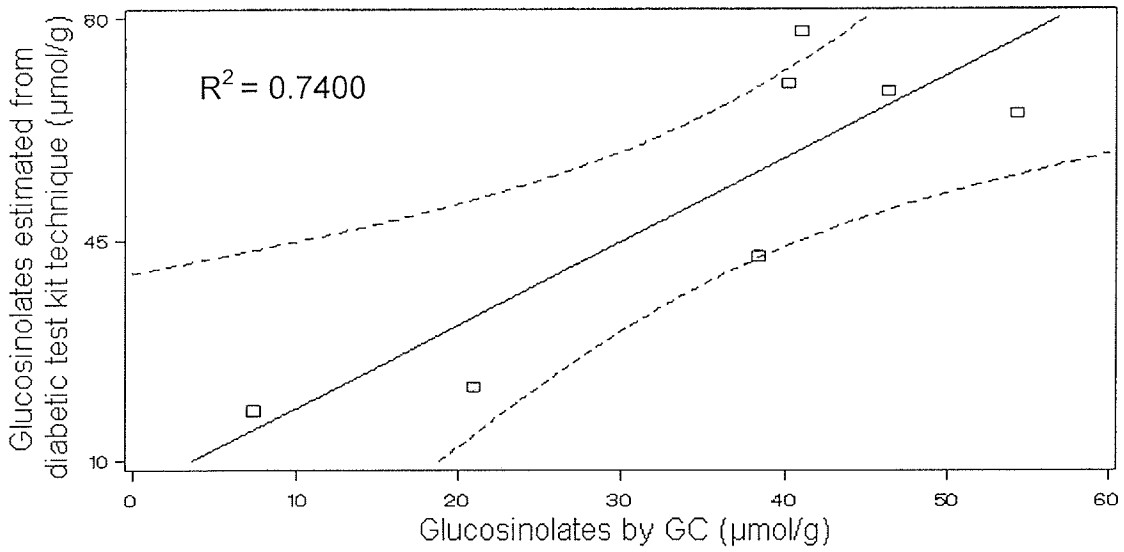
APPENDIX 4. Gas chromatograms showing trimethylsilyl (TMS) derivatives of desulpho-glucosinolates from (I) canola meal, (II) debris, (III) first supernatant, (IV) filtrate, (V) retentate, (VI) second supernatant, and (VII) PMM collected from the PMM isolation procedure followed with the Amicon stirred ultrafiltration cell system compared to (A) internal & external standard solutions. Peak numbers are identified as 1. allyl- (sinigrin); 2. but-3-enyl- (gluconapin); 3. pent-4-enyl- (glucobrassicanapin); 4. 2-hydroxy-but-3-enyl- (progoitrin); 5. 2-hydroxy-pent-4-enyl- (gluconapoleiferin); 6. benzyl- (glucotropaeolin); 7. *p*-hydroxybenzyl- (glucosinalbin); 8. 3-indolyl-methyl- (glucobrassicin); and 9. 4-hydroxy-3-indolylmethy- (4-hydroxyglucobrassicin) glucosinolates.


(A)
1
6
(I)
1
2
3
4
5
6
7
8
9
(II)
1
2
3
4
5
6
7
8
9
(III)
1
2
3
4
5
6
7
8
9
Detector response
10
20
30
Retention time (min)

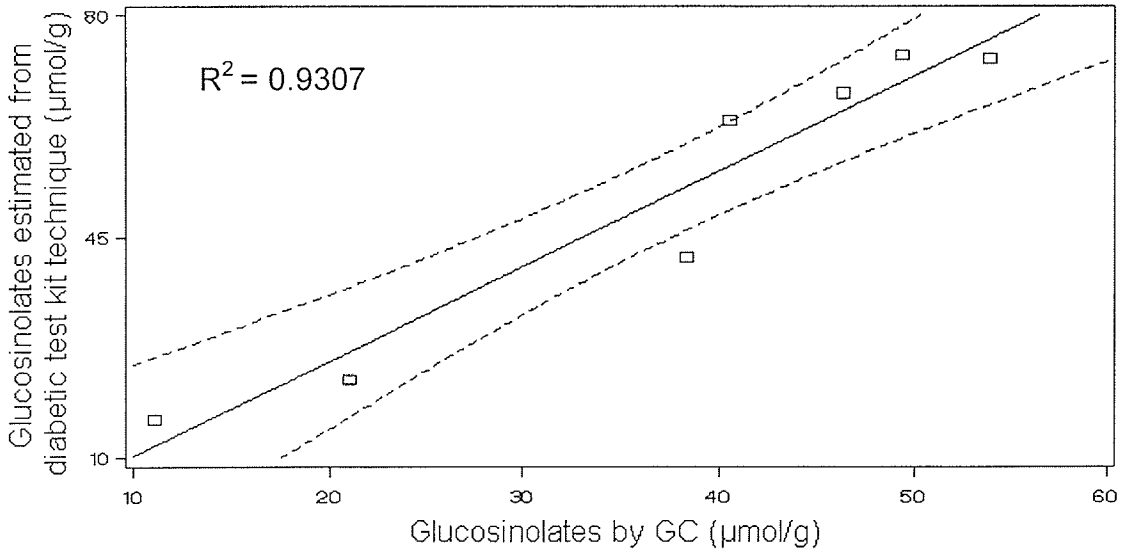

(IV)
(V)
(VI)
(VII)
1
2
3
4
5
6
7
8
9
Detector response
10
20
30
Retention time (min)

APPENDIX 5. Scatter plots of the relationship between the total glucosinolate levels estimated by the diabetic test kit technique and obtained by the GC method for canola samples collected from the PMM process followed with two ultrafiltration systems: (A) the Amicon stirred ultrafiltration cell unit and (B) the Vivaflow 200 ultrafiltration unit. Solid line and dashed line depict the prediction line and 95% confidence limits, respectively.

(A) Amicon stirred ultrafiltration cell



(B) Vivaflow 200 ultrafiltration



APPENDIX 6. The Analysis of Variance (ANOVA) test of the processing effects on free SCN^- ions of commercial CPI

Source	Degree of Difference (DF)	Sum of Squares (SS)	Mean Square (MS)	F Value	Prob > F
Model	5	0.49	0.098	406.28	<0.0001
Curvature	1	0.016	0.016	66.71	<0.0001
Residual	11	0.0026	0.00024		
Lack of Fit	*2*	*0.00055*	*0.00027*	*1.18*	*0.3.511*
Pure Error	*9*	*0.0021*	*0.00023*		
Corrected Total	17	0.51			

Factors	DF	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|
Intercept	1	0.66		
A-pH	1	0.084	22.08	<0.0001
B-Salt conc.	1	-0.096	-25.03	<0.0001
C-Heat	1	0.084	22.01	<0.0001
AC	1	0.052	13.63	<0.0001
BC	1	-0.067	-17.69	<0.0001
Center point	1	-0.095	-8.30	<0.0001

APPENDIX 7. The ANOVA test of the processing effects on bound SCN^- of CPI

Source	Degree of Difference (DF)	Sum of Squares (SS)	Mean Square (MS)	F Value	Prob > F
Model	5	0.15	0.030	95.28	<0.0001
Curvature	1	0.000083	0.000083	0.26	0.6198
Residual	11	0.00348	0.00032		
Lack of Fit	*2*	*0.00035*	*0.00018*	*0.50*	*0.6225*
Pure Error	*9*	*0.00313*	*0.00035*		
Corrected Total	17	0.15			

Factors	DF	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|
Intercept	1	0.15		
A-pH	1	-0.052	-11.65	<0.0001
C-Heat	1	-0.055	-12.41	<0.0001
AB	1	-0.016	-3.64	0.0039
BC	1	0.057	12.91	<0.0001
ABC	1	-0.012	-2.60	0.0247
Center point	1	0.007	0.51	0.6198

APPENDIX 8. The ANOVA test of the processing effects on the released glucose of CPI

Source	Degree of Difference (DF)	Sum of Squares (SS)	Mean Square (MS)	F Value	Prob > F
Model	3	323.53	107.84	115.86	<0.0001
Curvature	1	0.20	0.20	0.22	0.6495
Residual	13	12.10	0.93		
Lack of Fit	*4*	*10.17*	*2.54*	*11.83*	*0.0012*
Pure Error	*9*	*1.93*	*0.21*		
Corrected Total	17	335.84			

Factors	DF	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|
Intercept	1	10.29		
A-pH	1	2.39	9.91	<0.0001
C-Heat	1	-2.97	-12.31	<0.0001
AC	1	-2.39	-9.89	<0.0001
Center point	1	0.34	0.47	0.6495

APPENDIX 9. The ANOVA test of the processing effects on the L* values (lightness) of CPI

Source	Degree of Difference (DF)	Sum of Squares (SS)	Mean Square (MS)	F Value	Prob > F
Model	3	170.96	56.99	16.18	<0.0001
Curvature	1	45.71	45.71	12.98	0.0032
Residual	13	45.77	3.52		
Lack of Fit	*4*	*31.32*	*7.83*	*4.88*	*0.0228*
Pure Error	*9*	*4.45*	*1.61*		
Corrected Total	17	262.44			

Factors	DF	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|
Intercept	1	35.01		
A-pH	1	-2.15	-4.58	0.0005
B-Salt conc.	1	1.74	3.70	0.0027
C- Heat	1	1.74	3.72	0.0026
Center point	1	-5.07	-3.60	0.0032

APPENDIX 10. The ANOVA test of the processing effects on the a* values (redness) of CPI

Source	Degree of Difference (DF)	Sum of Squares (SS)	Mean Square (MS)	F Value	Prob > F
Model	2	7.84	3.92	20.67	<0.0001
Curvature	1	2.16	2.16	11.40	0.0045
Residual	14	2.65	0.19		
Lack of Fit	*5*	*1.67*	*0.33*	*3.05*	*0.0697*
Pure Error	*9*	*0.99*	*0.11*		
Corrected Total	17	12.66			

Factors	DF	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|
Intercept	1	-0.39		
A-pH	1	-0.59	-5.39	<0.0001
B-Salt conc.	1	-0.38	-3.51	0.0034
Center point	1	1.10	3.38	0.0045

APPENDIX 11. The ANOVA test of the processing effects on the b* values (yellowness) of CPI

Source	Degree of Difference (DF)	Sum of Squares (SS)	Mean Square (MS)	F Value	Prob > F
Model	3	130.39	43.46	33.62	<0.0001
Curvature	1	7.39	7.39	5.72	0.0326
Residual	13	16.80	1.29		
Lack of Fit	*4*	*11.77*	*2.94*	5.25	*0.0184*
Pure Error	*9*	*154.59*	*0.56*		
Corrected Total	17				

Factors	DF	Coefficient Estimate	t for H_0 Coeff=0	Prob > |t|
Intercept	1	13.05		
A-pH	1	1.17	4.13	0.0012
B-Salt conc.	1	-1.21	-4.27	0.0009
C- Heat	1	-2.30	-8.10	<0.0001
Center point	1	2.04	2.39	0.0326