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APPLICATION OF NEW METHODOLOGY TO CANOLA PROTEIN ISOLATION

by

WILLIAM WELSH

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CANOLA PROTEIN ISOLATION

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WILLIAM WELSH

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

New methodology has been developed to isolate protein fractions from canola meal with almost complete elimination of the antinutritional factors. Modifications of a unique protein micellar mass (PMM) procedure developed initially for pulse storage proteins has resulted in extracts of approximately 83% protein. One of the modifications has involved the choice of an extraction buffer; selected through a screening process whereby the efficiencies of the buffers in removing antinutritional factors and preserving protein nativity have been graphically plotted and ranked, hence allowing the most efficient buffer to be chosen and used in the PMM process. Average extract phytic acid and glucosinolate levels have been reduced to 24 and 7% of the original meal, respectively. In addition, the average phenolic compounds reduction in the extract has been found to be approximately 15% of the raw meal. Enthalpy of denaturation values (ΔH) as high as 9.67 J/g with thermal denaturation (Td) temperatures of approximately 88-89°C reflect the mildness of the PMM approach. Analysis of the protein structure using SDS-PAGE and HPLC/gel filtration revealed four constituent polypeptide chains of the 12S storage globulin. Commercialization of this process may impact the economics of canola production; the value of the protein component would increase if it were suitable for human consumption.

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1.0 INTRODUCTION

Canola is the second largest cash crop in Canada. To date, it holds a very high value for the nation in terms of vegetable oil production and exports. Canada is one of the largest exporters of canola oil in the world. The oil is employed in the food industry while the commercial meal is used as animal feed despite a well-rounded amino acid composition in the 25% of the seed that is considered proteinaceous. As well, canola does not contain the enzyme lipoxygenase that promotes oxidative rancidity in other cereals such as soybeans. Despite these positive attributes, the protein present in the defatted commercial meal is destined mainly for animal feed, and Canada continues to import soybean flour for its food grade protein from the United States.

The primary reason for the underutilization of the protein is related to the problematic antinutritional components associated frequently with the canola protein fraction. Although reduced through selective breeding, the antinutritional factors must be reduced further or even removed in order for the protein to become food grade or suitable for human consumption. These antinutritional factors include the glucosinolates, phytic acid, and the phenolic compounds.

Mere presence of the glucosinolates themselves do not constitute a toxicity problem. Rather, it is the products of enzymatically-mediated glucosinolate hydrolysis that may cause goitre and other related goitrogenic effects.

Phytic acid is found in various concentrations in all oilseeds and constitutes another antinutritional factor. Divalent minerals such as zinc, copper, iron, and calcium are readily bound by the phytic acid to form phytate complexes. This activity results in decreased bioavailability of these minerals.

A third problematic component involved with the canola proteins concerns the presence of phenolic compounds which cause the brown colour in the seed. The phenolic compounds, most notably sinapine, also contribute to the bitter taste of the seed. As well, it has been reported that the phenolic compounds interfere with protein digestibility.

The objective of this project was to extract the 12S storage globulin protein from commercially defatted canola meal so as to preserve the protein's native state while removing the antinutritional factors.

The extraction methodology used was based on the protien micellar mass (PMM) process developed by Murray et al. (1981). This represents a novel approach to the extraction of protein as the general methodology of acid solubilization/alkali precipitation of proteins is not used. This latter method risks a certain degree of protein

denaturation whereas the PMM process has been shown to preserve protein nativity.

The effects of the extraction process on the structure of the 12S globulin were also studied to determine the molecular weights of the subunits that comprise the larger globulin structure. For this, SDS-PAGE, and HPLC/gel filtration modes of analyses were used.

2.0 LITERATURE REVIEW

2.1.1 The Canola/Rapeseed Protein

In recent years there has been considerable interest shown in the study and processing of canola seed proteins for use in foods for human consumption. Knowledge of the composition and properties of the components of canola and their alterations by different processing methods is essential for obtaining an optimal system of treatments for the production of protein flours, concentrates and isolates.

The seed proteins are largely located in the protein bodies of the cotyledon (Mieth et al., 1983) and can constitute up to 20-25% of the seed in some varieties (Mieth et al., 1983). Other reports have claimed the percentage protein in rapeseed to be over 30% (Nagano and Okamoto, 1976). In terms of amino acid content the proteins in rapeseed are considered to be well-rounded, containing lysine and methionine which may be somewhat deficient in some other cereals and pulses. As an example, Table 2.1 lists the amino acid composition of several varieties of rapeseed.

The canola proteins consist of differing fractions, namely the globulins, albumins and the alkali-soluble proteins (Mieth et al., 1983). These fractions may be separated and defined according to their respective tendencies towards solubility and precipitability (Mieth et al., 1983; Finlayson, 1969; Finlayson, 1976). As well, they

Table 2.1 Amino Acid Analysis of the 12S Globulin from Rapeseed*

Amino Acid	B. *napus*	B. *campestris*
Aspartic acid	0.703 ± 0.02	0.782 ± 0.03
Threonine	0.344 ± 0.02	0.782 ± 0.03
Serine	0.388 ± 0.03	0.376 ± 0.03
Glutamic acid	1.260 ± 0.05	1.510 ± 0.10
Proline	0.435 ± 0.01	0.434 ± 0.03
Glycine	0.675 ± 0.01	0.770 ± 0.03
Alanine	0.476 ± 0.01	0.502 ± 0.05
Valine	0.435 ± 0.03	0.456 ± 0.03
Cysteine-Cystine	0.040 ± 0.01	0.145 ± 0.02
Methionine	0.108 ± 0.01	0.088 ± 0.03
Isoleucine	0.332 ± 0.02	0.330 ± 0.03
Leucine	0.585 ± 0.02	0.612 ± 0.02
Tyrosine	0.156 ± 0.01	0.151 ± 0.02
Phenylalanine	0.266 ± 0.02	0.310 ± 0.01
Lysine	0.266 ± 0.02	0.220 ± 0.01
Histidine	0.124 ± 0.02	0.120 ± 0.01
Arginine	0.352 ± 0.05	0.421 ± 0.02
Tryptophan	0.050	0.050

* millimoles per gram protein (17% N)

(Goding *et al*., 1970)

may be separated and characterized by differing molar masses and levels of basicity, ultracentrifugal, chromatographical and electrophoretical behavior (Mieth et al., 1983).

Investigations of the canola proteins have focussed primarily on the isolation and subsequent characterization of the 12S globulin. The 12S globulin is the main storage protein in the seed of the Brassica species (Mieth et al., 1983; Bhatty et al., 1968; Finlayson et al., 1969). It is generally recognized that Bhatty et al. (1968) were the first to isolate the 12S globulin from Brassica napus var. Nugget rapeseed (Mieth et al., 1983). Bhatty and his co-workers isolated the protein via extraction in a 10% sodium chloride solution followed by a dialysis-precipitation against water and finally purifying the protein by gel-filtration on Sephadex-G100 (Mieth et al., 1983; Bhatty et al., 1968; Schwenke et al., 1983). A 12S globulin was isolated from two species of rapeseed (B. napus L. and B. campestris L. by Goding et al. (1970) by first extracting the protein from the rapeseed in a 0.1M borate buffer/1.0M NaCl solution. This extraction was then followed by high-speed centrifugation and subsequent ultra-centrifugation. Purification of the protein was achieved on a gel filtration column using a Sephadex G-100 column. (Goding et al., 1970; Mieth et al., 1983). Other methodologies for 12S globulin separation from rapeseed have been numerous and widely documented, including the use of fractionation precipitation

and dissolution by ammonium sulphate for protein isolation (Simard and Boulet, 1978) plus combined gel and ionic-exchange and chromatographical purification of the 12S globulin (Schwenke et al.; 1981, Schwenke et al., 1983; Mieth et al., 1983).

A variation of Bhatty's isolation process (Bhatty et al., 1968) was used by Schwenke et al. (1981) whereby the dialysis (as described in Bhatty's methodology) was carried out with 5% NaCl instead of wter. The precipitated protein was found to be heterogeneous and contained large quantities of low molecular weight 1.7S proteins. (Schwenke et al., 1981; Mieth et al., 1983). This fraction represents the main portion of the isoelectrically nonprecipitable albumin. Schwenke et al. (1981) have found that twice repeated gel-chromatographic purification by Sephadex G-200 and ion-exchange chromatography by means of DEAE-Sephadex A-50 produces a homogeneous 12S protein (Mieth et al., 1983; Schwenke et al., 1981; Schwenke et al., 1983).

Some controversy arose concerning the molar mass of the 12S globulin fraction. Gill and Tung (1976) determined the molecular weight of the globulin to be in the range of 120,000 to 134,000 g/mol while Simard and Boulet (1978) estimated a higher molecular weight of 163,000 g/mol. Nagano and Okamoto (1976), using gel chromatography, uncovered more convoluted results. In their analysis, they found that the globulins from rapeseed consisted of three

components: α, β, and γ factors. The α and β components correlated to weights of 23,000 and 162,000, and the sedimentation constants seemed to be 1S and 12S respectively. It was determined that part of the globulin molecule was assumed to be composed of several sub-unit components which were bound by disulphide bonds. Electrophoretic results showed that the α and γ components consisted of the same subunit (13,000 M.W.) while the β component was composed of two kinds of subunit structures (32,000 M.W. and 19,000 M.W.) (Mieth et al., 1983; Nagano and Okamoto, 1976). It was also shown, by gel filtration studies, that the α and γ components were stable against pH change and heat treatment in contrast with the β component, which was coagulated by the addition of acid (pH 4.0), or alkali (pH 11.5) and by heating at 80°C (Nagano and Okamoto, 1976). It was found, using gel chromatography studies, that coagulation was manifested by unfolding of the protein under these conditions.

Any contradictions concerning the molecular weight of the rapeseed 12S globulin have been addressed and subsequently clarified by Schwenke et al. (1983). Schwenke and his co-workers have determined the molecular weight of the 12S globulin to actually be 300,000 $\pm$ 10,000 g/mol (Mieth et al., 1983; Schwenke et al., 1983; Schwenke et al., 1980). In determining the molecular weight of the globulin itself, Schwenke et al. (1980; 1983) had to allow for the

tendency of the protein to both associate and dissociate into its respective subunits. The weight of 300,000 ± 10,000 g/mol is in agreement with published molar masses for other 11/12S globulin proteins from plant sources (Schwenke, 1975, as cited in Mieth, 1983).

The 12S globulin is an oligomeric protein consisting of six subunits (hence the 12S globulin is hexameric in its un-dissociated native state), each containing smaller polypeptide chain units (Schwenke et al., 1983; Mieth et al., 1983; Gwiazda and Schwenke, 1984). Table 2.2 illustrates the physico-chemical properties and structure of the 12S rapeseed globulin. Dissociation of the globulin's quaternary structure is stepwise, and depends upon the protein's environment (Schwenke et al., 1983; Mieth et al., 1983). Figure 2.1 shows the behavior of the 12S globulin in low and high ionic conditions as well as in conditions of extreme pH values (or the presence of denaturing urea levels) (Mieth et al., 1983; Schwenke et al., 1983). As well, the dissociation proceeds quite similarly to that with other 11/12S proteins (Schwenke, 1975 as cited in Mieth et al., 1983; Schwenke et al., 1982).

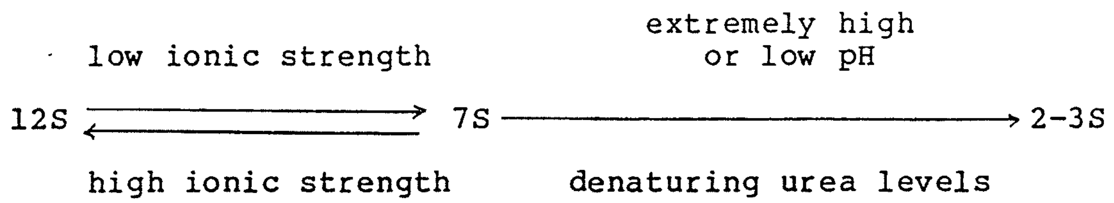
As may be observed from Figure 2.1, the dissociation of the 12S globulin to the 7S trimeric intermediate is reversible and depends upon the ionic strength of the environment involved (Schwenke et al., 1982; Mieth et al., 1983). In conditions of pH extremes (<pH 3.0) or sufficient

Table 2.2 Physico-Chemical Properties and Structures of 12S Globulin From Rapeseed

Property	Value
Isoelectric point	7.2
Coefficient of sedimentation (S^{o}_{20}, W [10^{-13}s])	12.7
Coefficient of diffusion (D^{o}_{20}, W [$10^{-7}m^{2}S^{-1}$])	3.8
Stokes radius	5.7
Partial specific volume V[ml/g]	0.729
Molar mass [g/mol]	300,000
Molar mass of polypeptide chains [g/mol]	18,500 21,100 26,800 31,200
Molecular form dimension [nm[	oblate rotations ellipsoid 11.3 x 11.3 x 9.2 (electron microscopy) 10.5 x 10.5 x 9.2 (small angle scattering)
Quaternary structure	trigonal antiprism
Number of subunits	6
Number of polypeptide chains	12
Secondary structure [%]	- helical structure:11 - β -sheet structure:31 aperiodic:58

(Mieth *et al.*, 1983)

Figure 2.1 **Dissociation of the 12S Globulin from Rapeseed**
(Mieth *et al.*, 1983, Schwenke *et al.*, 1982)


low ionic strength
extremely high
or low pH
12S
7S
2-3S
high ionic strength
denaturing urea levels

concentrations of urea (4M) the globulin further dissociates into its substituent 2-3S monomeric subunit components (Mieth et al., 1983; Schwenke et al., 1983. The fate of the 12S globulin in acidic pH extremes is illustrated in Figure 2.2. This dissociation is irreversible hence the subunits do not re-associate to form either of the 7S half-molecule or the 12S native globulin (Bhatty et al., 1968; Schwenke et al., 1982). The main reason for the irreversibility of the reaction in acid/alkaline extremes or in the presence of strong dissociating agents (i.e. urea) is due to protein denaturation (Schwenke et al., 1983).

Investigations of the smaller subunits of the 12S seed globulin have shown that the 3S subunit component is not a true monomeric unit but is rather a composite basic and acidic polypeptide chain that is bridged by disulphide bonds (Schwenke et al., 1983; Derbyshire et al., 1976). According to Schwenke et al. (1983), the 3S component possesses a molecular weight of approximately 50,000 hence representing an intermediate unit. According to the same researchers, the 3S component is heterogenous and is readily formed via recombination of the dissociated polypeptide chains (Schwenke et al., 1983). Simard et al., (1979) treated the 12S globulin with 8M urea to dissociate the globulin into the smaller constituent subunits and, using gel electrophoresis, found only one band with a molecular weight of 53,000. Treatment of the globulin with sodium dodecyl

Figure 2.2 **Fate of the 12S Globulin in pH Extremes**
(Kishore and Rao, 1984)

$$\underset{(pH\ 5.0)}{12S} \xrightarrow[H^+]{\text{up to pH 3.0}} \begin{matrix}\text{dissociation}\\\text{and}\\\text{denaturation}\end{matrix} \xrightarrow[H^+]{\text{below pH 3.0}} \begin{matrix}\text{refolding}\\\text{and}\\\text{aggregation}\end{matrix}$$

sulphate followed with 2-mercaptoethanol resulted in the detection of four polypeptide chain fractions with molecular weights of 18,000, 20,000, 28,000 and 30,000 (Simard et al., 1979). Similar results were obtained by Schwenke et al. (1983) with the weights of the polypeptide chains listed as 18,500 ± 800, 21,000 ± 500, 26,800 ± 900 and 31,200 ± 1600 (Schwenke et al., 1983). Schwenke's analysis confirmed the findings of Rao and Rao (1979) who showed that the 12S protein from mustard, which is botanically related to rapeseed, undergoes an association, dissociation and denaturation when exposed to different concentrations of sodium dodecyl sulphate (Rao and Rao, 1979). These researchers used the techniques of ultracentrifugation, gel filtration, gel electrophoresis, viscometry, ultraviolet difference spectra and fluorescence spectra to determine the 12S dissociation (Rao and Rao, 1979; Schwenke et al., 1983). The dissociation of the globulin into subunits is evidence of the 12S globular protein's existence as a true oligomer (Mieth et al., 1983). Table 2.3 illustrates the polypeptide chains of the smallest subunits of the 12S globulin.

It has been found, via X-ray scattering studies, that each monomeric subunit contains two domains and that one domain in each subunit occupies a smaller volume than the other (Plietz et al., as cited in Schwenke et al. 1983; Cleemann and Kratky, 1969, as cited in Schwenke et al., 1983). The polypeptide patterns of the globulin when

Table 2.3 Polypeptide Chains of the 12S Rapeseed Globulin After Denaturation and Polyacrylamide Gel Electrophoresis in the Presence of SDS/2-mercaptoethanol.

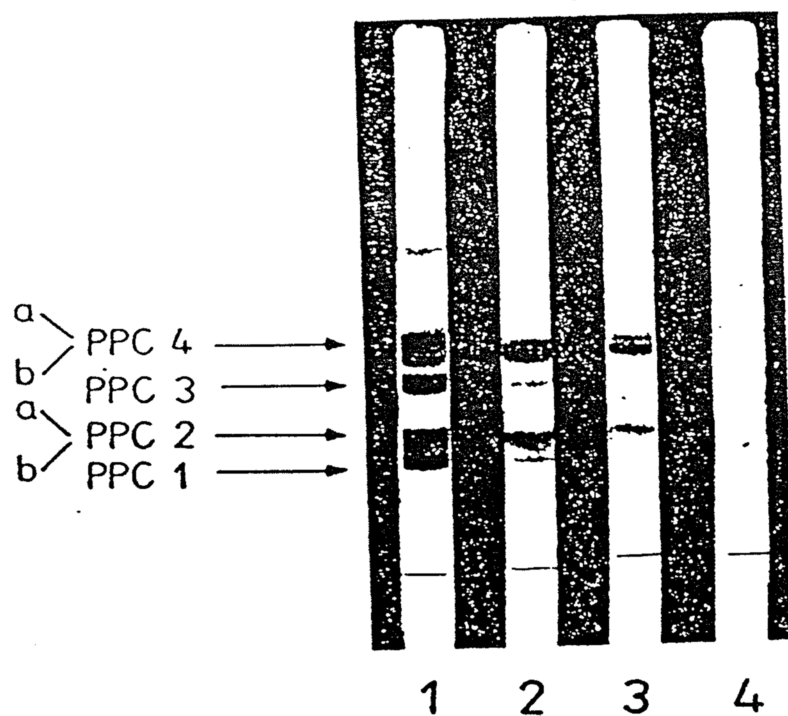
Polypeptide Chain	Molecular Weight ± Standard Deviation
PPC1	18,500 ± 800
PPC2	21,100 ± 500
PPC3	26,800 ± 900
PPC4	31,200 ± 1600

(Schwenke *et al.*, 1983)

analyzed by SDS-polyacrylamide gel electrophoresis is shown in Figure 2.3 (Gwiazda and Schwenke, 1984). Schwenke et al. (1983) state that each of the smaller units (PPC1 and PPC2) combines with the larger PPC3 and/or PPC4 chains to form one monomeric subunit of the globular structure consisting of a molecular weight of approximately 50,000. Hence, as is pointed out by Schwenke, a completely associated protein would possess a molecular weight of approximately 293,000 which is in agreement with the hydrodynamically determined value of 300,000 ± 10,000 (Schwenke et al., 1983).

The 12S globulin was originally categorized as a glycoprotein (Goding et al., 1970). However, as Mieth et al. (1983) state, at 3.6 residues per molecule protein, the sugar content is somewhat low. Mieth et al. (1983) have identified the sugar residues as galactosamine that is linked to the protein subfraction. While Goding et al. (1970) and Schwenke et al. (1981) have determined the 12S globular sugar content to be that of 0.5%, other researchers have determined the sugar content to be closer to 12.9% carbohydrate (Gill and Tung, 1978). Mieth et al. (1983) accounts for the higher carbohydrate content by stating that it may be linked to a Maillard reaction between protein and the existing carbohydrate component in the rapeseed that might have occurred during industrial meal processing (Mieth et al., 1983).

Figure 2.3 Polypeptide Patterns of Rapeseed Globulin Remaining in Solution After 5 Min. in 0.5M Phosphate Buffer at Different Temperatures. (Gwiazda and Schwenke, 1984)



1-75°C 2-85°C 3-87.5°C 4-90°C

2.2.1 Phytic Acid in Canola and Rapeseed

The relationship between phytic acid and proteins has been studied extensively in recent years. Phytates, the mixed calcium and magnesium salt of phytic acid, comprise roughly one to two percent by weight of many cereals and oilseeds and may comprise as much as between three and six percent for particular varieties (Cheryan, 1980). The greatest amount of phytate is to be found in cereals, legumes, and nuts (Harland and Harland, 1980). The typical phytic acid content of defatted rapeseed stands at 3.69% (% dry basis) (Erdman, 1979). The phytic acid content of various cereals and oilseeds is illustrated in Table 2.4. Phytates are considered to be the predominant form in which phosphates and inositol are stored in the plant (Miller et al., 1986; Erdman, 1979). While phosphorus is an important nutrient and mostly occurs in the phytate form in legumes and oilseeds, it has been widely documented that the presence of phytate is of serious concern due to its ability to bind divalent minerals such as Zn, Cu, Ca, Fe, Mn, Mo and Co (Miller et al., 1986; Maga, 1982; Bjorck et al., 1987; Navert et al., 1985; Nolan et al., 1987). Once bound, these minerals become unavailable as nutrients. As well, it has been shown that phytic acid may also interact with protein, hence preventing enzymic digestion (O'Dell et al., 1976; Singh et al., 1982; Cheryan, 1980).

Table 2.4 Typical Phytic Acid Contents (% dry basis) of Some Cereals and Oilseeds

Sample	Phytic Acid
Corn	0.89
Wheat, soft	1.13
Rice, brown	0.89
Soybeans	1.4
Peanut meal, defatted	1.7
Sesame meal, defatted	5.18
Rapeseed meal, defatted	3.69
Cottonseed flour, glandless	4.8
Lima beans	2.52
Navy Beans	1.78
Barley	0.99
Oats	0.77
Coconuts	2.38

(Cheryan, 1980)

Phytic acid presence in food systems has not assumed a completely negative role. As previously mentioned, phytate in plants is the main storage form of phosphorus (Martens, 1982; Nahaptian and Bassiri, 1975). As well, during potato maturation, starch-bound phosphorus is quantitated in the form of phytic acid, perhaps in order to prevent excessively high levels of inorganic phosphorus (Martens, 1982). This has also been argued by other researchers (Cheryan, 1980; Asada *et al.*, 1968). As well, it has been suggested that phytic acid plays an inhibitory/mycological role by preventing or restricting the availability of zinc to aflatoxin-producing molds (Martens, 1982; Cheryan, 1980; Gupta *et al.*, 1975).

To a certain extent, phytic acid plays a somewhat positive role as a regulator of phosporus in the maturation and ripening stages of potatoes and rice to name only a few examples. However, due to its interactions with minerals and/or proteins, the subsequential adverse nutritional effects render phytic acid as an undesirable factor in a food system.

2.2.2 Structure and Chemistry of Phytic Acid

A considerable amount of disagreement has developed over the actual structure of phytic acid. Much of the disagreement has encompassed the various isomeric forms of

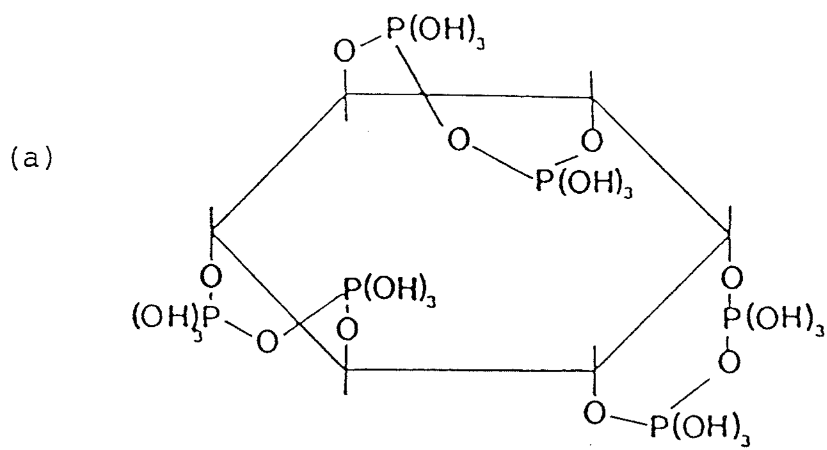
the phosphate groups and their respective cross-linkages within the molecule. More specifically, the controversies have centered around the various isomeric forms of the hydroxyl groups on the phytic acid structure (Martens, 1982; Erdman and Forbes, 1977).

Two structures have been suggested as being the correct conformation of phytic acid (Figure 2.4). While it is generally accepted that Anderson's structure is probably the correct one, it was not without a certain amount of conflict with that structure proposed by Neuberg (Cheryan, 1980). Neuberg's structure was distinguished by three P-O-P linkages between pairs of adjacent phosphate groups (Figure 2.4). Research involving a variety of experimental techniques has been submitted to support one or the other phytic acid structure.

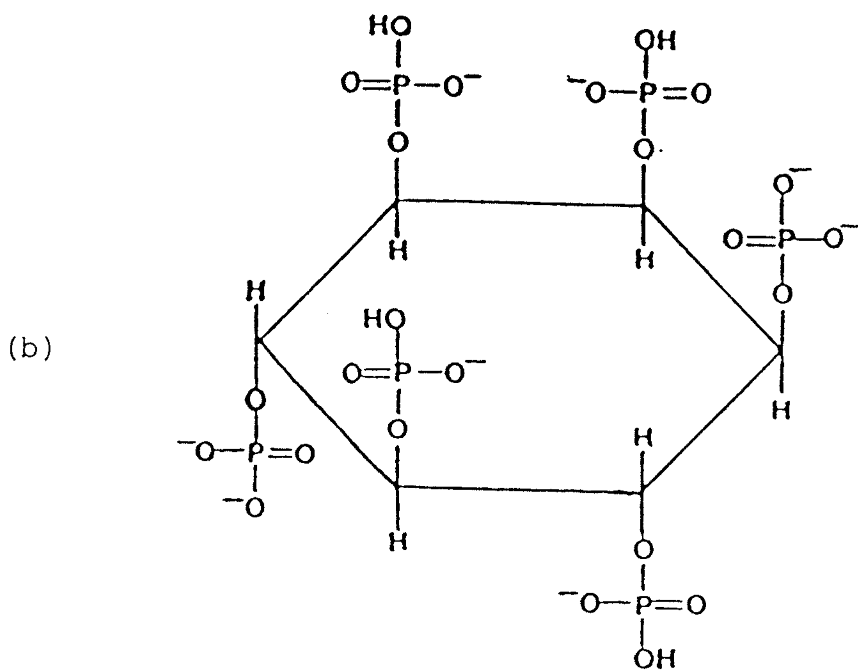
It has been suggested that both structures may indeed exist in equilibrium with one another and that the Anderson structure may, in fact, be a degradation product of the Neuberg structure. The reason for this theory lies in the fact that the two phytic acid structures differ by three water molecules (Brown _et al_., 1961; Cheryan 1980). It is generally proposed that because the Anderson structure of phytic acid best explains or accounts for many of the physiochemical and nutritional interactions, it is the structure of choice in current literature (Cheryan 1980).

Figure 2.4 Suggested Structures of Phytic Acid
a) Neuberg Form, b) Anderson Form
(Cheryan, 1980)

(a)



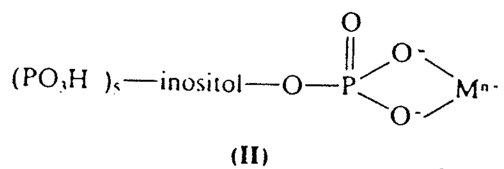
(b)

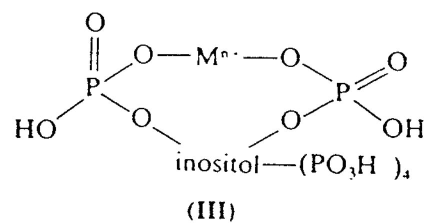


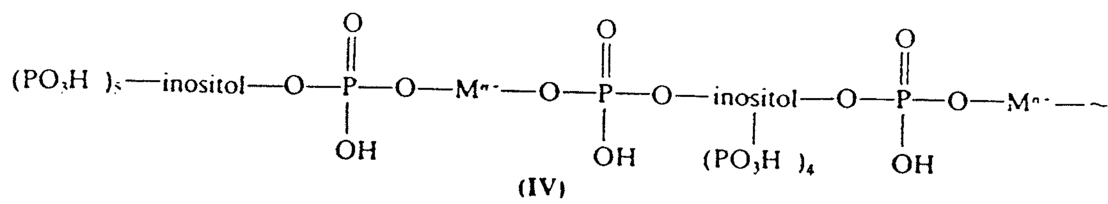
Using the Anderson structure of phytic acid as a basis, the correct IUPAC name for phytic acid is myoinositol-1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate) (IUPAC-IUB, 1968 as cited in Cheryan, 1980). From Figure 2.4 it can be seen that the Anderson structure carries an overall negative charge. This negative charge accounts for the strong binding capacity that phytic acid displays with the previously mentioned cationic species. This is especially true in physiological pH conditions whereby phytic acid is thoroughly ionized and is capable of interacting quite strongly with proteins and the metal ions (Nolan et al., 1987). A wide range of phytate-metal complexes is possible (Figure 2.5). For example, the phosphate groups may chelate to form II, a 4-membered ring complex (Anderson, 1977). Conversely, two or more phosphate groups from the same or differing phytate ions may chelate one metal cation to give III and the polymeric bridge IV. Also, a bridge V between the two metal cations may be formed by means of a phosphate groups (Jones et al., 1977 as cited in Nolan et al., 1987). As Nolan points out, however, there is little structural information available on these complexes and more research is required, perhaps employing X-ray crystallography.

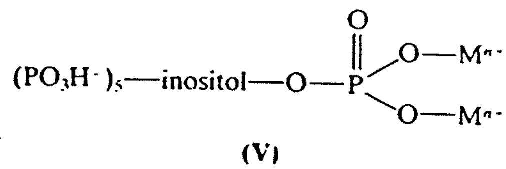
Interactions between the proteins and phytic acid are thought to be ionic at low pH while mediated by cations (i.e. cationic minerals and metals), through the formation

Figure 2.5 **Possible Phytate-Metal Complexes**
(Nolan *et al.*, 1987)


(PO3H)5—inositol—O—P
O
O-
O-
Mn+
(II)


O
P
HO
O—Mn+—O
O
P
O
OH
O
O
inositol—(PO3H)4
(III)


(PO3H)5—inositol—O—P—O—Mn+—O—P—O—inositol—O—P—O—Mn+—~
O
OH
(PO3H)4
(IV)


(PO3H-)5—inositol—O—P
O
O—Mn+
O—Mn+
(V)

of phytate-cation-protein complexes at high pH (Nolan *et al.*, 1987). In turn, the interactions lead to reduced protein solubility and a distortion of their more solubility-dependent characteristics such as the protein's behavior in aqueous environments, dispersibility in water as well as their foaming and emulsifying abilities (Nolan *et al.*, 1987).

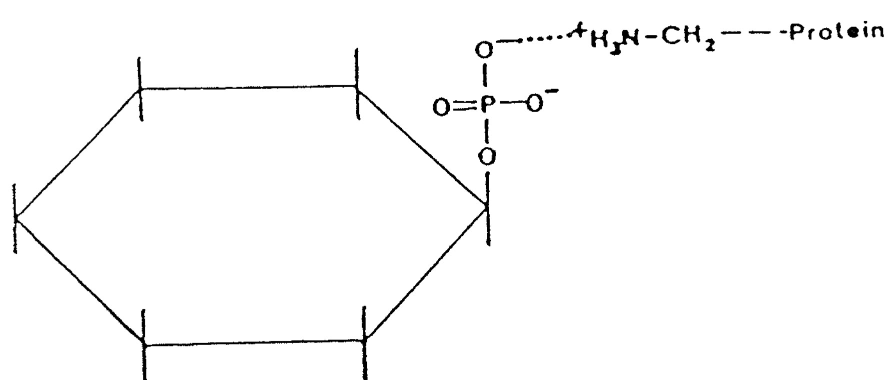
The strong interaction between proteins, salts and other components along with phytic acid make interpolation of such studies quite difficult (Cheryan, 1980). It has been reported that the solubility of phytic acid somewhat parallels the solubility behavior of proteins in soybeans and rapeseed (Saio *et al.*, 1967; Saio *et al.*, 1968; Gillberg and Tornell, 1976; Cheryan, 1980). As Cheryan (1980) has pointed out, the solubility tendencies of phytic acid are different in the presence of protein as opposed to the absence of protein. This, Cheryan states, is proof of protein-phytate interaction.

Mechanisms of protein-phytate interaction have been proposed at three pH regions (<pH 5, pH 5-7, >pH7) (Okubo *et al.*, 1975; Omosaiye and Cheryan, 1979; Cheryan, 1980). These three regions are classified as low, intermediate, and high pH regions respectively and are considered to have significantly different effects on the protein-phytate complexes and the way the complexes are formed.

In regions of low pH (i.e. below the isoelectric point of the protein), the formation of the protein-phytate complex is regarded as the formation of a unionized salt. According to Fox et al. (1957 as cited in Cheryan, 1980) the protein acts as the positively-charged cation while phytic acid provides the anion. The resulting complex is illustrated in Figure 2.6.

In environments of intermediate or alkaline pH, the nature and structure of the protein-phytate interaction appears to be somewhat uncertain (Cheryan, 1980). In regions above the isoelectric point of the protein both the protein and phytic acid possess a negative charge. However, according to Cheryan, available data indicate some type of protein-phytate complex existing in the mildly alkaline region. Jackman and Black (1951), as cited in Cheryan, 1980 state that phytates should be insoluble at alkaline pH, however experiments have shown phytate to be soluble in this region, suggesting phytate interaction (deRham and Jost, 1979; Fontaine et al., 1946). Steinhardt and Reynolds (1969 as cited in Cheryan, 1980) stated that anionic binding to proteins with a net negative charge is not unknown. Moreover, they suggest, that it is possible that a direct salt-like linkage between phytic acid and the α $-NH_2$ terminal groups and the ε $-NH_2$ group of lysine occurs. The salt linkage is facilitated by the protonation of the α $-NH_2$ terminal groups and the ε $-NH_2$ of lysine, hence providing

Figure 2.6 **Possible Structure of Phytic Acid-Protein Complex at Low pH**
(Cheryan, 1980)


O
$^{+}H_3N-CH_2$ Protein
O=P—O^{-}
O

the cations for phytic acid-protein linkages. Other researchers have not met with considerable success in establishing evidence of a direct protein-phytate complex at any pH above 4.9 (Okubo et al., 1976). Others have not been able to correlate the basic amino acid content and extent of phytate binding (O'Dell and DeBoland, 1976). It has also been found that some contradictions have arisen concerning the role of the basic amino acids, their protonated states, and the effects of phytic acid binding (Cheryan, 1980).

The behavior of phytate at alkaline pH appears to be strongly influenced by a salt linkage (Cheryan, 1980). This theory of phytate-protein complex behavior has been reinforced by the binding of calcium to soybean protein as affected by phytic acid (Saio et al., 1968). Saio et al. (1967) observed that multiple molecules of calcium and phytic acid may be bound by a singular protein molecule and that bound calcium increases as phytic acid added to the protein increases (Saio et al., 1968).

As the pH increases above the intermediate pH range (i.e. > pH 6), the stability of the ternary protein-cation-phytate increases. Two principle mechanisms have been proposed to explain the status of the phytate complex at these higher pH levels (Figures 2.7 and 2.8) (Omosaiye and Cheryan, 1979). The second equation in Figure 2.7 corresponds to the structure of the complex illustrated in Figure 2.8. The complex is most stable at pH 10. Beyond

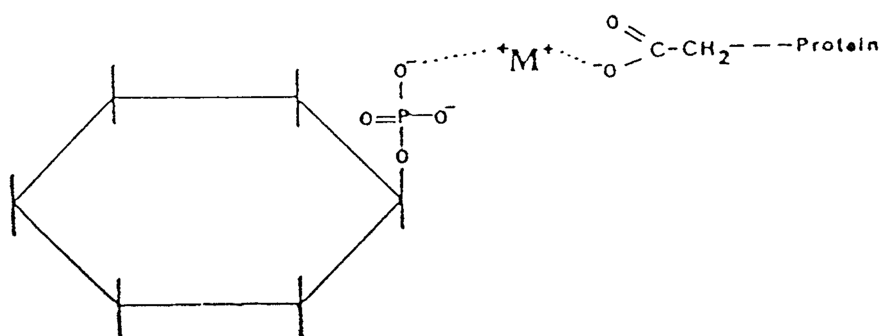
Figure 2.7 Proposed Mechanisms for Phytate Complex at High pH
(Cheryan, 1980)

a) Cation & Phytic Acid $\rightleftharpoons$ (Cation-Phytic Acid)

b) Protein & Cation & Phytic Acid $\rightleftharpoons$ (Protein-Cation-Phytic Acid)

Figure 2.8 **Possible Structure of the Phytate-Protein Ternary Complex at Alkaline pH***
(Cheryan, 1980)

* M indicates multivalent cation


O
O=P—O⁻
O
⁺M⁺
⁻O
C-CH2
Protein

pH 10, the mineral-phytate-protein complex is disrupted and the phytic acid becomes insoluble (Cheryan 1980).

While references have been made, thus far, to the negative effect that phytic acid has on mineral bioavailability and absorption, it has also been known to have a negative effect on protein availability (Carnovale et al., 1988). It has been shown that complex formation of phytic acid with proteins obstructs the enzymatic breakdown of the proteins (O'Dell and deBoland, 1976). As well, the effects of phytic acid and polyphenols on both starch digestion and fibre degradation have been documented (Bjorck and Nyman, 1987).

Using several cultivars of faba bean and pea, Carnovale et al., (1988) have researched the effects of phytic acid-protein interactions on in vitro protein digestibility. These researchers support the theory that phytic acid is bound to the protein component of the seed, quite possibly to the protein bodies. Multienzymic methodology of Hsu et al., (1977) and modified by Satterlee et al (1979, both cited in Carnovale et al., 1988) was used to study protein digestibility. Gauging various levels of endogenous and exogenous phytic acid, the researchers established a negative correlation between phytic acid content and protein digestibility. The total negative effect on protein digestibility by phytic acid may be difficult to ascertain due to the presence of other factors that also negatively

impact protein digestibility (Carnovale et al., 1988). Citing a previous work (Carnovale et al., 1983), these other factors include antitryptic factors, tannins, and fibre remnants.

The role of other factors combining with phytic acid has been studied. Bjorck and Nyman (1987) have studied the effects of phytic acid and polyphenols on starch digestion and fiber degradation. In this particular experiment, the impact of phytic acid on starch digestion and fiber degradation has been negligible at best with the polyphenolics (i.e. tannins) inhibiting starch digestion and fibre degradation.

The effects of phytic acid on the functional properties of proteins has been studied. In comparing rapeseed protein isolates at different phytate levels, it has been shown that the low-phytate products possess better emulsifying properties than the high-phytate protein products (Dev and Mukherjee, 1986). As well, the level of phytic acid barely affects the foaming properties of the rapeseed protein products. Phytic acid affects only the emulsifying capacity and emulsion stability (Dev and Mukherjee, 1986; Blaicher et al., 1983). Similar results were obtained with soy-protein isolates (Naczk et al., 1986).

Various attempts to counter the antinutritional effects of phytic acid by supplementing the diet with an excess of zinc have been undertaken (Jones, 1979). Other attempts to

counter phytic acid by effecting its removal have also been undertaken. Complete removal of phytic acid is difficult if not outwardly impossible via physical processes (Carnovale _et al._, 1988). These researchers found that phytic acid reductions greater than 60% were possible through solubilization but a larger phytic acid reduction was offset by low protein recovery. Greater success has been achieved by other researchers with the use of a phytase enzyme removing up to 88% of the phytic acid present in rapeseed (Serraino and Thompson, 1984).

Currently, there is specific concentration on the production and manufacture of low-phytic acid rapeseed protein concentrates (Schwenke _et al._, 1985). In this approach, the rapeseed meal is mixed with an edible polyanionic precipitating agent yielding the formation of an insoluble protein precipitate. The mixture is then extracted at a pH giving minimal solubility of the protein while promoting the maximum solubility of phytic acid. The resulting protein concentrates contain low phytic acid levels. As well, this literature states that protein losses are low and the process is free from thermal denaturation (Schwenke _et al._, 1985 as cited in Food Science and Technology Abstracts _18_(3) 1986). However, no mention of the protein's resolubilization characteristics are discussed in the literature.

2.3.1 Phenolic Compounds in Canola and Rapeseed

Phenolic acids and their many derivatives are commonly occurring compounds in plants. These acids are largely responsible for the deterioration in the taste, odour, and colour of prepared protein concentrates and other food products (Kozlowska et al., 1983). As well as unfavorable organoleptic changes, oxidized phenolic compounds may bind with lysine or methionine which are two essential amino acids. Once bound into a phenolic-amino acid complex, these essential amino acids are no longer able to become assimilated in the digestive tract (Kozlowska et al., 1983, Davies et al., 1968).

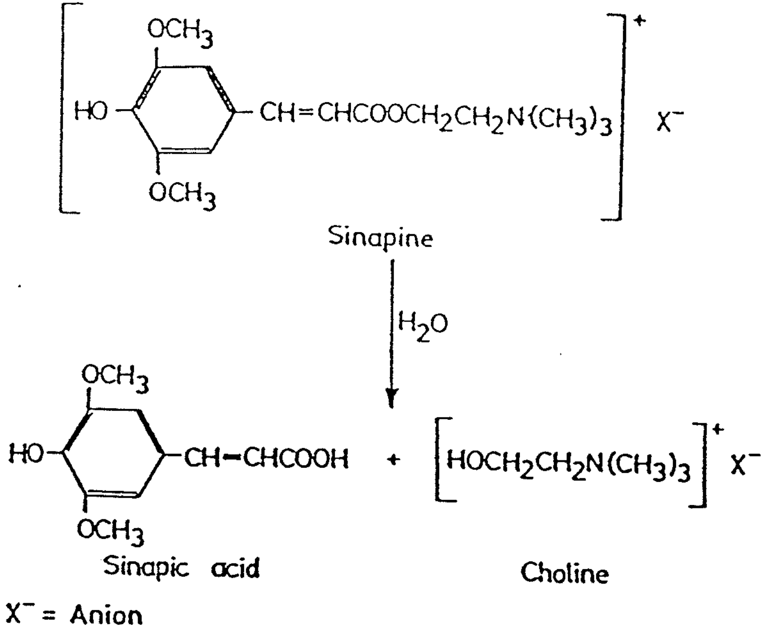
The phenolics have also been shown to negatively impact the functional properties of rapeseed protein (Ponnampalam et al., 1987). It is thought that during the production of rapeseed protein flours and concentrates, quinone oxidation products of polyphenols may covalently link with the sulfhydryl group of cysteine, the ε -amino group of lysine, and the α -terminal amino groups of proteins reducing protein digestibility, functionality, stability and possibly altering sensory properties (Ponnampalam et al., 1987; Loomis, 1974). Phenolic compounds (including polyphenolic compounds) may also react noncovalently with proteins through hydrogen bonding, ionic and hydrophobic interactions

(Ponnampalam *et al.*, 1987; Loomis, 1974; Oh *et al.*, 1980; Arntfield *et al.*, 1985).

It has been found that rapeseed contains a wide variety of phenolic acids (Kozlowska *et al.*, 1983; Kozlowska *et al.*, 1975; Dabrowski and Sosulski, 1984). Sinapine, the choline ester of sinapic acid, constitutes 80-99% of the total amount of esterified phenolic compounds in rapeseed (Kozlowska *et al.*, 1983; Ponnampalam *et al.*, 1987; Krygier *et al.*, 1982). Sinapine and its hydrolytic cleavage properties are illustrated in Figure 2.9. Other phenolic compounds include p-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, p-coumaric and ferulic acids (Kozlowska *et al.*, 1983). Sinapine has become the best studied of the phenolics not only because of its role as the predominant phenolic compound in rapeseed but of its early association with glucosinolates. Where the major glucosinolates of rapeseed have been isolated in the form of their potassium salts, the p-hydroxphenyl glucosinolate of white mustard has been isolated as the sinapine salt (Appelqvist and Ohlson, 1972).

The dominance of sinapic acid in rapeseed as the major phenolic acid accounts for most of the bitter taste and astringency of the seed (Appelqvist and Ohlson, 1972; Ponnampalam *et al.*, 1987). Previously, it had been thought the presence of both the phenolics and polyphenolics did not represent any toxic or antinutritional threat besides poor

Figure 2.9 Sinapine and its Hydrolytic Cleavage Products (Appelqvist and Ohlson, 1972)


OCH3
HO
CH=CHCOOCH2CH2N(CH3)3
X⁻
OCH3
Sinapine
H2O
OCH3
HO
CH=CHCOOH
+
HOCH2CH2N(CH3)3
X⁻
OCH3
Sinapic acid
Choline
X⁻ = Anion

palatability due to the bitter taste of the meal (Appelqvist and Ohlson, 1972; Clandinin, 1961; Austin and Wolff, 1968). Some recent studies have focused on the protein-phenolic interactions that may take place in rapeseed meal (Hurrell et al., 1982; Oh and Hoff, 1987; Ford and Hewitt, 1979). It was found by Ford and Hewitt (1979) that tannins may reduce protein digestibility and possibly the bioavailability of other nutrients. Many theories have been developed as to the nature of the tannin (phenolic) - protein interaction as mentioned previously (hydrogen bonding between the hydroxyl groups of tannins and the carbonyl groups of the protein peptides, ionic interactions, hydrophobic interactions). However, it appears that more recent evidence suggests that hydrophobic interactions could be the most vital influence in tannin-protein complex formation (Oh and Hoff, 1987; Oh et al., 1980). It was found that complex precipitation of the protein with the tannins is a two-step reaction in which the initial stage involves the binding of the tannin molecules to the protein. It was reported that the number of methylene groups in the amino acid side chain corresponds positively with the magnitude of hydrophobic interaction (Oh et al., 1980). The second step in the interaction involves the aggregation of the tannin-protein complexes into larger units. These units then precipitate (Oh and Hoff, 1987). The amount of precipitation depends on the ability of the cross-linking tannin-protein bonds in overcoming any

electrostatic forces of repulsion that may exist between the two entities.

Oh and Hoff (1987) suggest that the repulsive forces should be minimal at the isoelectric point of the protein and therefore, it will be at this point where the tannin-protein interaction/precipitation will be at its maximum. Haggerman and Butler (1978) reported that, in fact, maximum tannin-protein precipitation occurred within one pH unit of the isoelectric point. This led to the establishment of a "critical pH" in complex formation which was usually observed within 0.5 pH unit from the isoelectric point (Oh and Hoff, 1987). At pH levels above the isoelectric point of the protein, Oh and Hoff (1987) observed a marked decrease in phenolic-protein complex formation.

Other adverse effects of phenolic-protein interactions have been widely documented. It has been reported that the nutritional quality of the proteins, and in particular lysine, are adversely impacted as a result of the interactions between protein and the quinone oxidation products of plant polyphenols (which include all phenolic acids, flavonoids, and tannins)(Davies et al., 1978; Hurrell et al., 1982). As well, it has been shown that methionine and tryptophan can react with and be oxidized by the quinones. It also appears that the sulfhydryl groups of cysteine and the ε -amino groups of lysine as well as the

α-terminal amino groups can combine most readily with the quinones (Hurrell et al., 1982).

The effects of the amino acids' association and oxidation by quinone on actual protein quality was confirmed by Horigome and Kandatsu (1968). These researchers reacted a casein solution with polyphenolics and reported that the resulting dark-coloured protein possessed lower biological values (BV), protein digestibilities and fluoridinitrobenzene (FDNB) - reactive lysine values than the untreated casein control. Hurrell et al. (1982) has confirmed that polyphenol browning reactions (i.e. Maillard browning) reduces bioavailable lysine, and that these reactions include enzymic browning and phenolic-protein reactions under alkaline conditions. It also appears that both the enzymic browning and phenolic-protein reactions are O_2-dependent which, as Hurrell et al. (1982) pointed out, could be of importance during the preparation of protein concentrates and/or flours.

Phenolics have been shown to interfere with the digestion of starch as illustrated by the ability of tannic acid to complex with α-amylase, amyloglucosidase/maltase and starch, itself (Bjorck and Nyman, 1987). It has been demonstrated that the binding of polyphenols has been associated with a decrease in starch digestibility (in vitro) and that different starches vary in their ability to bind both tannic acid and catechin (Deshpande and Salunkhe,

1982). Catechin was also shown, by the mentioned researchers, to bind to α-amylase and amyloglucosidase enzymes but to a lesser extent. Deshpande and Salunkhe (1982) point out that, in fact, the main digestive enzymes concerned with starch digestion in the gastrointestinal tract were inhibited by tannic acid in their experiment. However, as they also pointed out, the experiment represents in vitro findings. In vivo experiments on rats by Griffiths and Moseley (1980), as cited in Bjorck and Nyam, (1987) show reduced starch digestion patterns when fed a field bean meal containing a high polyphenolics content.

Nutritional studies comparing tannin-containing and tannin-free faba bean (Vicia faba) meal effects on young chicks have focused on energy, protein, and starch digestibility (Lacassagne et al., 1988). In this study, it was found that tannin-free protein was more digestible (82.6%) than tannin-containing protein (68.2%). However, unlike the study mentioned previously, Lacassagne et al. (1988) found that starch from the tannin-containing cultivars was more digestible (84.5%) than starch from the tannin-free cultivar (75.1%). Citing a previous work of Guillaume (1978), Lacassagne et al. (1988) feels that the discrepancy could be attributed to the possibility that the measurement of glucose by the glucosoxidase method used by Guillaume (1978) is not suitable for tannin-containing cultivars. Still citing Guillaume (1978), Lacassagne et al.

(1988) found that the energy values (measured in Apparent Metabolisable Energy values of MJoules/kg DM) of the faba bean seeds to be lower in tannin-free cultivars than in tannin-containing cultivars.

2.4.1 Glucosinolates in Canola and Rapeseed

The glucosinolate antinutritional factors appear to be the most limiting factor for the use of canola proteins for human consumption. The glucosinolates (thioglucosides, mustard oil glucosides, etc.) and their decomposition products represent one of the antinutritional barriers to be overcome (Hill, 1979 as cited in Sang et al., 1984). Some of these decomposition products include isothiocyanates, oxazolidinethiones, and cyano compounds (Holmes, 1980). These breakdown products can cause goitre and can obstruct normal growth and reproductive capacities.

Glucosinolates may be found in all green plants as well as foods of the Cruciferae family (Mieth et al., 1983). The nature and level of the glucosinolates differ in different plant species. The seed, depending on plant species, may contain up to 5% glucosinolates while the leaf tissue, again depending on species, may contain only 0.1% glucosinolates on a fresh weight basis (Sang et al., 1984).

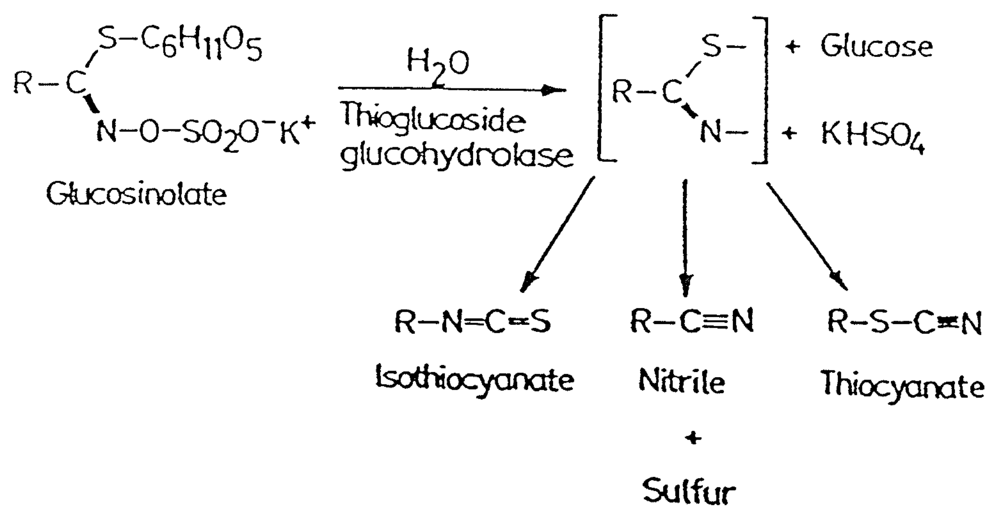
It has been found that, in terms of glucosinolate location within the rapeseed plant, there are significant

differences between the seed, leaf, and root with respect to both amounts and derived types of glucosinolates present (Sang et al., 1984). It is generally agreed that the glucosinolates are found in the parenchymal tissue in rapeseed (Mieth et al., 1983; Appelqvist and Ohlson, 1972). Various studies have shown the concentration of glucosinolates to be highest in the seed itself, slightly declining with maturity (Kondo et al., 1985; Palmer et al., 1987; McGregor, 1988).

The glucosinolates are derivatives of thiohydroxamic acids, and differ from one another by virtue of the structure of their respective aglucons or organic radicals. As well, they may differ in terms of the frequency of their presence and the properties of their decomposition (hydrolysis) products (Mieth et al., 1983, Olsen et al., 1981). It has been found and widely documented that the enzyme myrosinase (Thioglucosidase: EC 3.2.3.1.) is vital for the undesirable properties of rapeseed meal, should existing moisture levels be sufficient (Sang et al., 1984, Appelqvist and Ohlson, 1972, Belzile et al., 1963). The basic enzymatic hydrolysis of glucosinolates to their breakdown products is given in Figure 2.10.

Different cultivars of the Brassica species possess slightly different hydrolysis/decomposition products of glucosinolates in varying quantities. For example, in Brassica napus, the main hydrolysis product is

Figure 2.10 The General Structures of Glucosinolates and Products Formed by Enzymatic Hydrolysis. (Appelqvist and Ohlson, 1972)



S–C6H11O5
R–C
N–O–SO2O⁻K⁺
Glucosinolate
H2O
Thioglucoside
glucohydrolase
S–
R–C
N–
+ Glucose
+ KHSO4
R–N=C=S
Isothiocyanate
R–C≡N
Nitrile
+
Sulfur
R–S–C≡N
Thiocyanate

(R)-2-hydroxy-3-butenylglucosinolate (progoitrin) that is predominant in the order of 60-70% (Mieth *et al.*, 1983). Other products, such as 3-butenylglucosinolate (gluconapin) and 4-pentylglucosinolate (glucobrassicanapin) are each present in the order of approximately 15-20% (Mieth *et al.*, 1983). For the *Brassica campestris* variety, progoitrin constitutes only 20% of the hydrolysis products while gluconapin and glucobrassicanapin each make up 35-45% of the hydrolysis products. Furthermore, *Brassica napus* contains relatively more butenyl-glucosinolate than pentenyl-glucosinolate while in *Brassica campestris*, their amounts are almost equal (Mieth *et al.*, 1983; Downey *et al.*, 1969; Van Etten, 1981; Youngs and Wetter, 1967). Other *Brassica* varieties (i.e. *B. nigra*, *B. hirta*) contain only allyl-glucosinolates (sinigrin) (Mieth *et al.*, 1983; Olsen and Sorensen, 1980). Standard varieties of *Brassica napus* contain, on the average, more glucosinolate (4-8%) than *Brassica campestris* (3-6%) (Mieth *et al.*, 1983). It has also been found that new cultures of rapeseed differ from standard varieties in terms of the types of glucosinolates present. For example, some of these new cultures have been found to contain large amounts of p-hydroxybenzyl-glucosinolate (sinalbin) and indolyl-methyl-glucosinolate (Olsen and Sorensen, 1980). There may be room for caution, however, as it has been determined that degradation products of glucosinolates can, depending on the type of experimental

conditions used in extractions, undergo subtle changes. These changes may impact the type and relative amount of the volatile glucosinolate compounds produced in the plant (Kondo et al., 1985).

2.4.2 The Antinutritional Effects of Glucosinolates

As mentioned previously, the products of myrosinase decomposition of the glucosinolates have been shown to promote harmful side-effects when metabolized (Mieth et al., 1983; Appelqvist and Ohlson, 1972). The main deleterious effects of the glucosinolates are their disruptive activities upon basic metabolism by negatively influencing iodine metabolism (Nagano and Okamoto, 1976 as cited in Mieth et al., 1983). Appelqvist and Ohlson (1972) citing a 1941 experiment by Kennedy and Purves, reported that Brassica seed fed to laboratory rats resulted in enlarged thyroids in the rats. As well, it was stated that this effect could not be reversed or wholly counteracted by feeding the rats iodine. Hypophysectomization of the thyroid glands and subsequent tests determined that rapeseed digestion causes thyroid enlargement by the interference with thyroxin synthesis (Appelqvist and Ohlson, 1972). Enlargement of the thyroid gland is related to the stimulation of the anterior pituitary gland to produce thyroid-stimulating hormone (TSH) which acts upon the

thyroid glands hence promoting hypertrophy and hyperplasia (Appelqvist and Ohlson, 1972). Matsumoto et al. (1969, as cited in Mieth et al., 1983) reported that oxazolidinethione decomposition product of glucosinolates interferes with the secretion of the thyroid hormone into the blood from the thyroid gland. Appelqvist and Ohlson (1972) report that more than five percent rapeseed meal fed to growing chicks caused thyroid hypertrophy and depressed growth.

Astwood et al. (1949) isolated a goitrogenic component from rapeseed (and other Brassica species) and determined it to be 5-vinyl-2-oxazolidinethione, one of the decomposition products of glucosinolates. The thiocyanate ion that may be split off from the p-hydroxylbenzyl isothiocyanate is also goitrogenic. VanderLann and VanderLann (1947, as cited in Appelqvist and Ohlson, 1972) found that this glucosinolate decomposition product competes with the iodine uptake in the thyroid gland but this may be counteracted by supplementing the diet with iodine.

Isothiocyanates have been responsible for palatability problems due to their strong pungent tastes and odours. In a personal communication between J.M. Bell and L.A. Appelqvist (Appelqvist and Ohlson, 1972), the former intimated that ingestion of high concentrations of the isothiocyanates damage the skin and mucous membranes, and, that by performing this activity, there is injury to the alimentary canal that may result in weight-gain depression.

Furthermore, Bell also found that the reduction in weight-gain of mice that have been fed rapeseed meal is approximately correlated with the sum of contents of glucosinolates yielding both oxazolidinethiones and isothiocyanates (Appelqvist and Ohlson, 1972). Other studies have confirmed the goitrogenic impacts of the isothiocyanates (Mieth _et al_., 1983; Van Etten _et al_., 1969; Appelqvist and Ohlson, 1972).

It is thought that glucosinolates serve as the basis for a hydrolytic splitting by the microbially-produced enzymes in the gastrointestinal tract of mammals. Here, there is support for the formation of nitriles in the gut system (Mieth _et al_., 1983). The formation of nitriles from glucosinolates occurs at lower pH levels, and is accelerated greatly by the presence of the ferrous ion (Uda _et al_., 1986a; Van Etten _et al_., 1966; Gill and MacLeod, 1980). It has been recently demonstrated that the presence of some thiol compounds accelerate the formation of nitriles from glucosinolates in the presence of ferrous ions to an even greater extent (Uda _et al_., 1986a; Uda _et al_., 1986b).

The nitriles are considered harmful, therefore undesirable. Rats fed crambe meals that contained either active enzymes or autolysis products containing nitriles showed signs of enlargement of the liver and kidneys (Van Etten, 1969). The enlargement was coupled with the appearance of small lesions in both organs. Diets of meal

containing only nitriles resulted in the deaths of the rats (Appelqvist and Ohlson, 1972; Van Etten, 1969). It has been suggested by the Canadian government (1967) that nitriles formed from the glucosinolates of rapeseed behave similarly (Appelqvist and Ohlson, 1972).

In 1975, low-glucosinolate rapeseed was introduced into Canadian production. The average level of glucosinolates has declined from 80 μmol/g to 25 μmol/g in the 1985 Canadian crop (Daun, 1986). Since 1983, over 90% of the rapeseed planted in Western Canada has been consistently of canola quality (<30 μmol/g glucosinolates) and has contained as low as 20 μmol/g glucosinolates (Daun, 1986).

There have been many efforts made to reduce or remove glucosinolates from rapeseed and canola meal. Goering et al. (1960as cited in Appelqvist and Ohlson, 1972) has approached the problem of glucosinolates removal from rapeseed via autolysis and distillation. This technique calls for water to be added to the meal in order to optimize myrosinase activity. The glucosinolates split at this stage and the steam volatile isothiocyanates are removed by distillation. Bell and Belzile (1965, as cited in Appelqvist and Ohlson, 1972) applied autoclaving and steam stripping to remove glucosinolates from rapeseed. While indeed successful in removing the glucosinolates, the commercial meal was rendered useless as a protein source due to almost complete protein denaturation. Bell and Belzile

theorized that the inadequacy of the denatured meal was due to lysine destruction. They were, however, unable to prove this.

Combinations of ammonia and heat have been shown to destroy the glucosinolates effectively (Kirk et al., 1966). However, the resulting glucosinolate meal has exhibited palatability problems for cattle. Glucosinolates have been destroyed by cooking the meal, under pressure, with sodium carbonate. This methodology has met with some success (Mustakas et al., 1968). Youngs and Perlin (1967) found that the addition of ferrous sulphate promoted the glucosinolates to decompose to yield nitriles. This treatment was followed by a steam treatment to remove the nitriles. It was found that the nitriles were not completely removed (Appelqvist and Ohlson, 1972). Because the toxicity of the nitriles is higher than that of the isothiocyanates or oxazolidinethiones, this approach can only be considered relatively unsuccessful (Youngs and Perrin, 1967; Appelqvist and Ohlson, 1972).

In another approach, Belzile and Bell (1966) have removed more than 80% of the glucosinolates using a mild buffer system. As well, applications of ethanol-extraction of the glucosinolates from rapeseed meal have been undertaken (Appelqvist and Munck, unpublished, as cited in Appelqvist and Ohlson, 1972; van Megen, 1983). Using an 80% ethanol extraction media, the glucosinolate-reduced/removed

rivals casein in terms of weight gain (Appelqvist and Ohlson, 1972). It has been found that the history of the meal is important with respect to glucosinolate extractability from the meal (van Megen, 1983). Using an aqueous 75% ethanol medium, van Megen has been able to remove 99.5% of the glucosinolates. With the exception of Bell and Belzile (1965, as cited in Appelqvist and Ohlson, 1972), very few of the above-mentioned researchers alluded to the effects of their respective methodologies on the nativity of the rapeseed protein.

Recently, methanol-ammonia-water extraction solutions have been used to remove glucosinolates from canola meal (Diosady *et al*., 1987). This method has met with considerable success as glucosinolates have been reduced to below detectable levels while the polyphenols have been reduced by 80% (Diosady *et al*., 1987).

2.5.1 Canola/Rapeseed Protein Processing

The number of processes developed for the extraction of canola/rapeseed proteins are highly numerous. A vast array of both simple and complex schematics have been developed to accomplish this goal. Due to the magnitude of publications on this topic, only a comparatively few processes can be outlined concerning protein concentrates (70-90% protein) and isolates (>90% protein) from the canola/rape oilseed. Processing parameters and methodologies for the preparation of flours (50-70% protein) will not be considered here.

2.5.2 The Canola/Rapeseed Protein Concentrates

Much of the work surrounding the protein extraction from canola/rapeseed has originated with methodologies developed for protein extraction from soybeans. In these processes, some underlying principles have been established in order to immobilize the major protein components in the defatted meal while extracting the water-soluble components (Youngs, 1985). These general approaches have involved heat denaturation of the proteins, isoelectric aqueous extraction as well as aqueous alcohol extraction (Youngs, 1985). The situation is rather complicated with canola/rapeseed, however, due to the presence of antinutritional glucosinolates and myrosinase enzyme which hydrolyzes the

glucosinolates to toxic aglucones. It has been found that immersing the rapeseed in boiling water for 1.5 to 3 minutes is an effective method for inactivating the myrosinase (Eapen et al., 1968). In another experiment, seeds have been boiled in water for 1.5 to 3 minutes, passed through a plate grinder, and extracted three times with hot water (80°C). This method removed over 99% of the glucosinolates, however, it also removed 26% of the protein and proved to be very damaging to the total protein in the seed (Youngs, 1985; Eapen et al., 1969).

Sosulski et al. (1972) proposed a diffusion extraction of intact seeds. They first inactivated the myrosinase enzyme by immersing the seeds in boiling water for 3 minutes. The seeds were then extracted five times (one hour/extraction) with 60°C 0.01N sodium hydroxide at a 1:20 seed to solvent ratio. This method reduced the glucosinolates to trace levels but also lost in excess of 15% of the solids including 20% of the seed nitrogen content (Youngs, 1985; Sosulski et al., 1972).

A process developed by Tape et al. (1972, as cited in Youngs, 1985) started with the inactivation of myrosinase enzyme in boiling water. The seeds are then sheared in order to increase the meal surface area prior to a countercurrent aqueous extraction. The detoxified seed material is then dried and defatted. After the oil extraction stage, the desolventized meal is air classified

into protein rich and protein poor fractions. This process is referred to as the FRI-68 process. A modification of the FRI-68 process was undertaken to provide for dehulling, myrosinase inactivation in boiling water, aqueous extraction, drying and defatting (Jones, 1979 as cited in Youngs, 1985). Figures 2.11 and 2.12 illustrate both the FRI-68 and its modification (FRI-71) processes.

These processes involve the use of partial heat denaturation of the protein as well as protein extraction from meals to contain protein losses during glucosinolate removal. There have been some studies involving extraction at the protein's isoelectric point to reduce protein solubility. Cervenkova et al. (1983) suggest that extraction should proceed at pH 4-5 in order to maximize glucosinolate and phytate removal while minimize potential protein loss (Cervenkova et al., 1983; Youngs, 1985).

It would appear that the use of alcohol solutions to extract proteins has been widely employed (Youngs, 1985). Ethanolic sodium hydroxide has been used for diffusion extraction of the glucosinolates which has the advantages of inhibiting myrosinase activity and inhibiting protein losses. However, this method reduced protein solubility due to partial denaturation of the protein (Bhatty and Sosulski, 1972).

Berot and Briffand (1983) compared aqueous methanol, ethanol and isopropanol at 60% v/v each for the extraction

Figure 2.11 **The FRI-68 Process**
(Youngs, 1985)

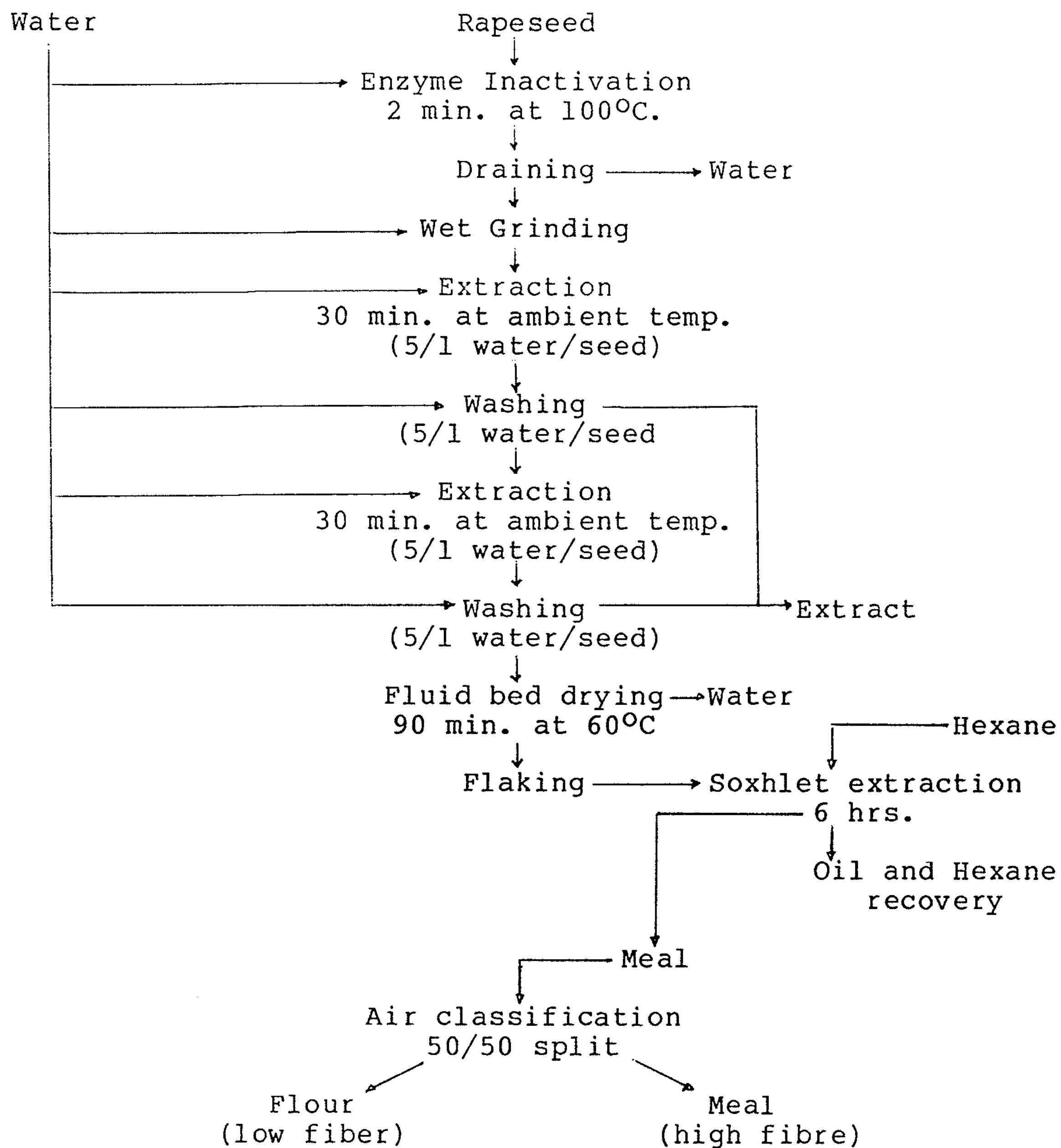
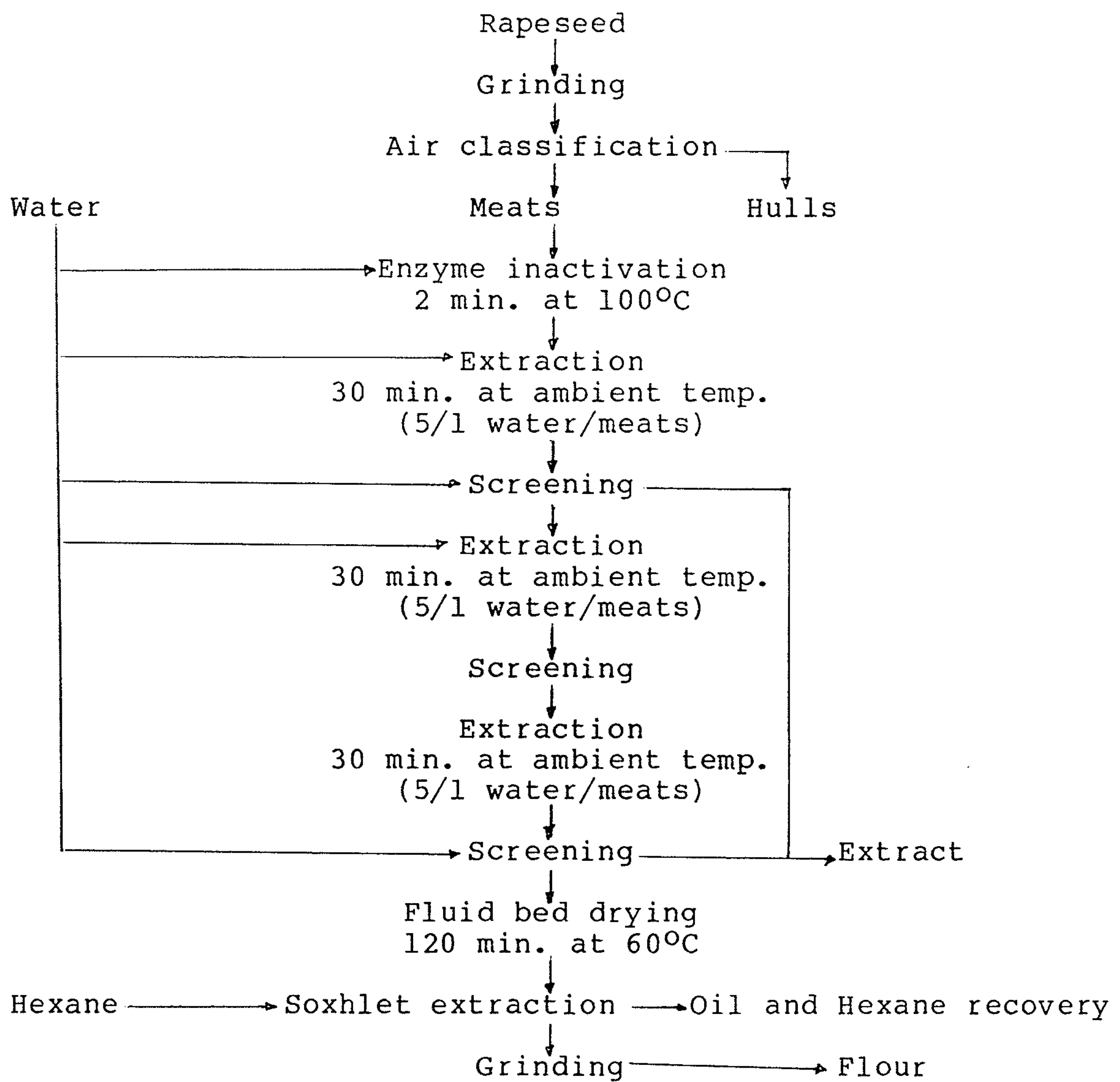

Water
Rapeseed
Enzyme Inactivation
2 min. at 100°C.
Draining
Water
Wet Grinding
Extraction
30 min. at ambient temp.
(5/1 water/seed)
Washing
(5/1 water/seed
Extraction
30 min. at ambient temp.
(5/1 water/seed)
Washing
(5/1 water/seed)
Extract
Fluid bed drying
Water
90 min. at 60°C
Hexane
Flaking
Soxhlet extraction
6 hrs.
Oil and Hexane
recovery
Meal
Air classification
50/50 split
Flour
(low fiber)
Meal
(high fibre)

Figure 2.12 **The FRI-71 Process**
(Youngs, 1985)


Rapeseed
Grinding
Air classification
Water
Meats
Hulls
Enzyme inactivation
2 min. at 100°C
Extraction
30 min. at ambient temp.
(5/1 water/meats)
Screening
Extraction
30 min. at ambient temp.
(5/1 water/meats)
Screening
Extraction
30 min. at ambient temp.
(5/1 water/meats)
Screening
Extract
Fluid bed drying
120 min. at 60°C
Hexane
Soxhlet extraction
Oil and Hexane recovery
Grinding
Flour

of antinutritional factors from rapeseed flour. They found that the different alcohols did not produce significant differences in their abilities to extract proteins. The nitrogen yields were approximately 90%. It was found, however, that less than 1% of the total glucosinolates remained in the concentrate with ethanol and isopropanol whereas 25% of the glucosinolates remained with methanol, hence making it a less suitable solvent. The authors report that all of the concentrates were bland flavored and white coloured.

Mixtures of water and alcohol have been used to effect protein extraction. It was found that a 75% ethanol/25% water solution proved to be effective in removing the glucosinolates from rapeseed meal but it was also found that the history of the meal affected the ease of extraction (Van Megen, 1983). Diosady et al., (1985, as cited in Youngs, 1985) found that the addition of ammonia to aqueous alcohol solutions increased the efficiency of extraction.

2.5.3 The Canola/Rapeseed Protein Isolates

As with the rapeseed protein concentrates, traditional procedures have been developed for protein isolates from soybeans that have been used in rapeseed isolates. These procedures have involved the extraction of the rapeseed protein by first solubilizing the protein in alkali,

followed by acid precipitation. Sosulski and Bakal (1969) reported that, by using dilute alkali, only 80-85% of the nitrogen from rapeseed could be extracted (hence, a concentrate and not an isolate) and that 25-30% of the nitrogen remained in the extraction media after protein precipitation at pH 4.5. As well, phenol-protein interactions were evident, as the colour of the "isolate" was dark brown and its taste was bitter. Other studies have confirmed the findings of Sosulski and Bakal (1969) (Korolczuk and Rutkowski, 1971).

Gillberg and Tornell (1976a; 1976b) found that the presence of phytic acid strongly influenced protein recovery. They found that adding sodium phytate to a protein extract prepared at pH 11 increased precipitation yield from 35 to 75%. Gillberg and Tornell (1976b) pointed out that the addition of sodium phytate is not an acceptable agent and proceeded to study and report the use of other, more acceptable precipitating agents that do not themselves possess antinutritional characteristics. Some of these agents included acidic polymers, hexametaphosphate, carboxymethyl-cellulose and polygalacturonic acid (Gillberg and Tornell, 1976b). It was reported that these precipitating agents all produced an increase in the amount of precipitate.

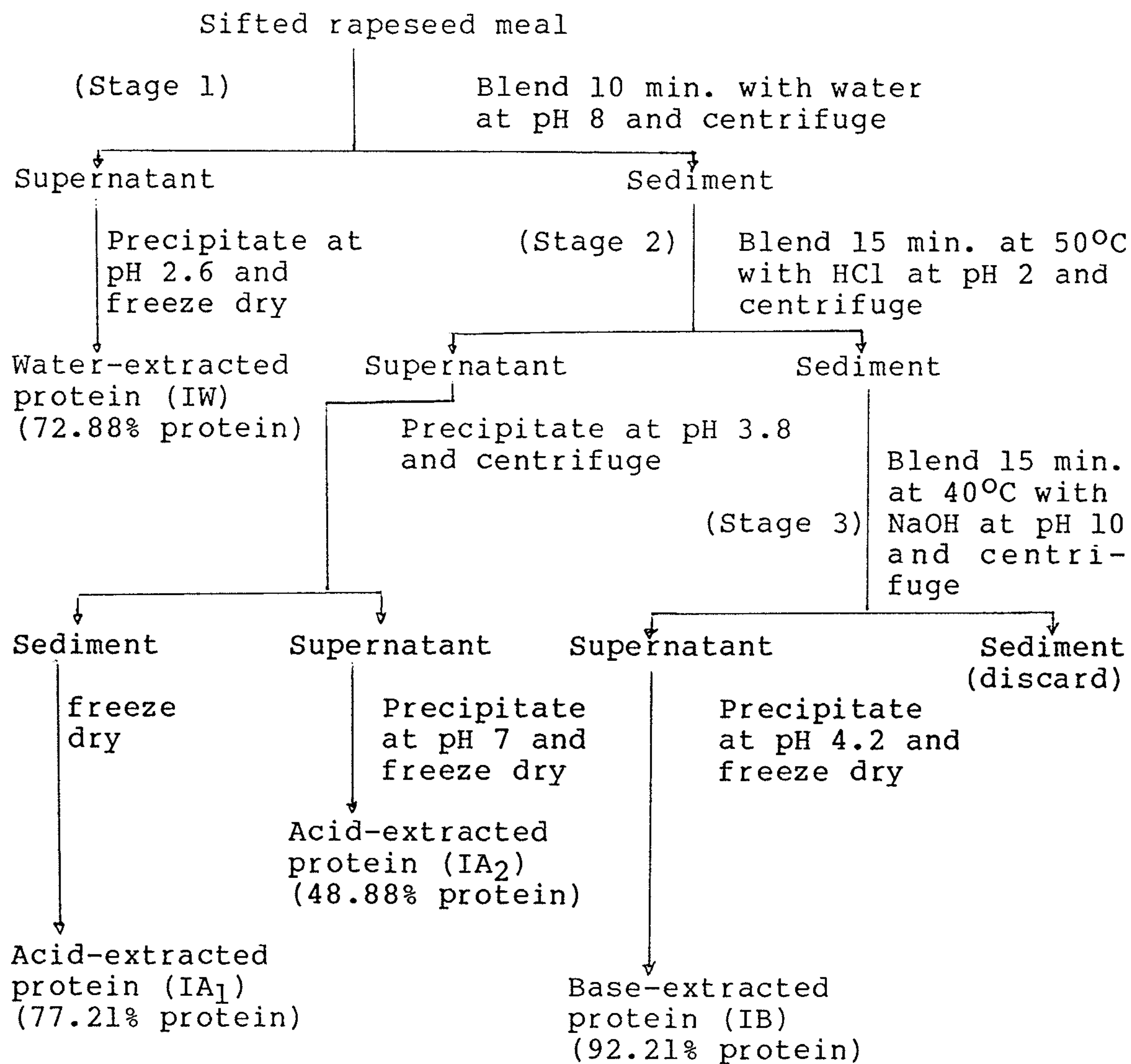
Thompson *et al*. (1976) performed a double extraction on rapeseed flour with 2% sodium hexametaphosphate at an

adjusted pH of 7.0. The flour to solvent ratio for the first extraction was 1:10 while the ratio for the second extraction was 1:6. The entire mixture was diluted with an equal volume of water at pH 2.5. The resulting protein precipitate was then washed and spray-dried. It was found that the nitrogen content in the protein extract was 53% and the colour of the product was lighter than those extracts obtained with alkaline extraction. As well, the glucosinolates were reduced to non-detectable levels (Thompson et al., 1976).

Extractions of protein from rapeseed meals have also been carried out with sodium chloride solutions (Siy and Talbot, 1932 as cited in Youngs, 1985, Finlayson et al., 1976; Girault, 1973). In these extractions, the nitrogen solubilization values have varied from 40% to 81% (Youngs, 1985). The major disadvantage arising from this approach is that even at the highest extraction rates of 67-81%, only 22.8% to 27.7% of the seed nitrogen is recoverable via isoelectric precipitation at pH 3.0 (Youngs, 1985).

A complex scheme for the isolation of rapeseed protein was developed by Keshavarz et al. (1977). The schematic approach is presented in Figure 2.13. It was found that protein content for the water-extracted protein was 72.88% while the protein content of the two acid-extracted precipitates (IA_1 and IA_2) were 77.21% and 48.88% respectively. The base-extracted precipitate (IB) was found

Figure 2.13 **Flow Diagram of the Modified Three-Stage Extraction Procedure**
(Kodagoda *et al.*, 1977)

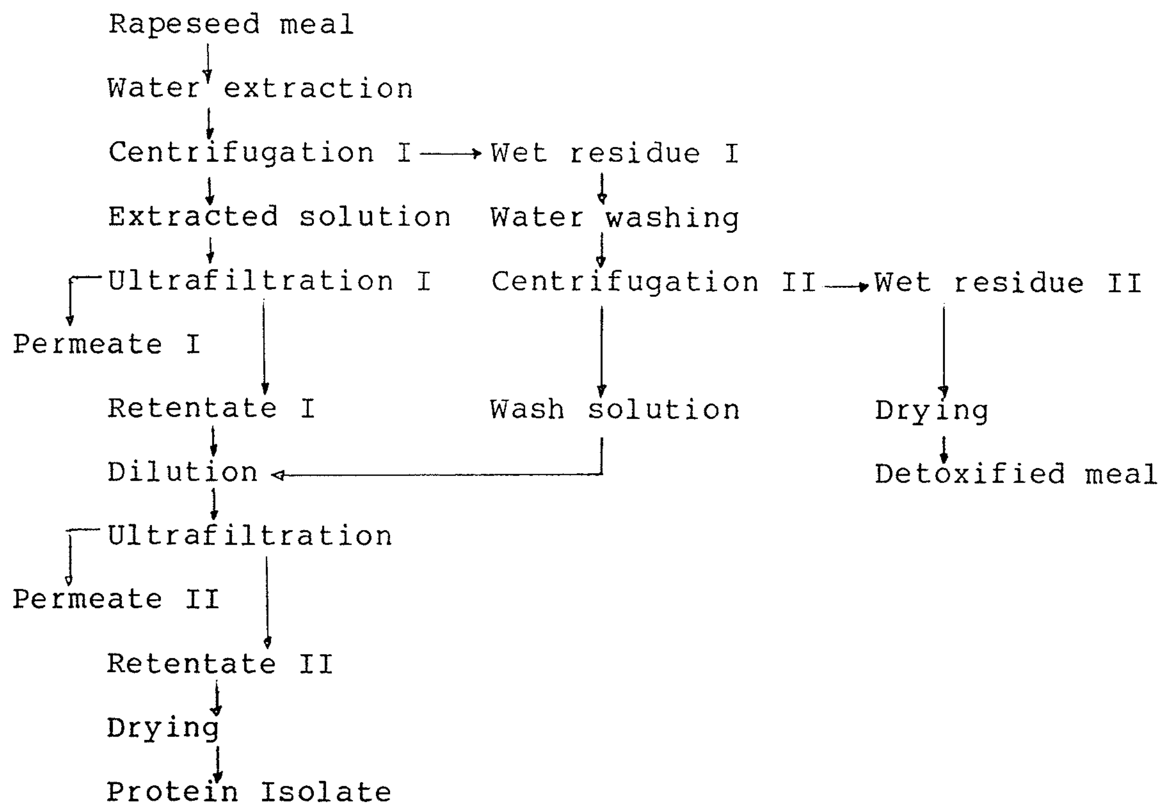

Sifted rapeseed meal
(Stage 1)
Blend 10 min. with water at pH 8 and centrifuge
Supernatant
Sediment
Precipitate at pH 2.6 and freeze dry
(Stage 2)
Blend 15 min. at 50°C with HCl at pH 2 and centrifuge
Water-extracted protein (IW) (72.88% protein)
Supernatant
Sediment
Precipitate at pH 3.8 and centrifuge
Blend 15 min. at 40°C with NaOH at pH 10 and centrifuge
(Stage 3)
Sediment
Supernatant
Supernatant
Sediment (discard)
freeze dry
Precipitate at pH 7 and freeze dry
Precipitate at pH 4.2 and freeze dry
Acid-extracted protein (IA2) (48.88% protein)
Acid-extracted protein (IA1) (77.21% protein)
Base-extracted protein (IB) (92.21% protein)

to have the highest protein content at 92.21%. This precipitate was also shown to possess the most powerful emulsifying capacity and emulsion stability of all the extracts obtained.

A Canadian patent has described the extraction of rapeseed protein by subjecting the meal to sodium chloride solution at 0.1M strength and at a pH range of 5.5 to 8.5 (Canadian Patent Application 372874). It was found that extraction at pH values below 5.5 make extraction of the protein difficult to achieve. The authors report, also, that protein extraction at >pH 8.5 result in protein denaturation. After extraction, the mixture is diluted by at least twice its volume with water at a temperature of 0°C to about 14°C. The patent refers to the subsequent formation of rapeseed protein particles as the isolate formation. The protein may then be washed to remove excess salt.

A novel process for the preparation of rapeseed protein isolates has been developed using ultrafiltration (Diosady et al., 1984). The schematic is presented in Figure 2.14. In this process, the meal is leached with water in a two-stage application and the dissolved proteins are recovered in a two-stage ultrafiltration process. The first water extract is ultrafiltered and the resulting retentate is diluted with the second extract. The mixture is then subjected to ultrafiltration again. For this approach, the

Figure 2.14 **Flow Diagram for the Production of Rapeseed Protein Isolates Using Ultrafiltration**
(Diosady *et al.*, 1984)


Rapeseed meal
Water extraction
Centrifugation I
Wet residue I
Extracted solution
Water washing
Ultrafiltration I
Centrifugation II
Wet residue II
Permeate I
Retentate I
Wash solution
Drying
Dilution
Detoxified meal
Ultrafiltration
Permeate II
Retentate II
Drying
Protein Isolate

authors have used four ultrafiltration membranes, with molecular weight cut-offs of 5,000, 10,000, 50,000, and 100,000 daltons. They have found that the 50,000 MWCO filter removes the impurities and glucosinolates and does not suffer extensive protein losses as is the case with the 100,000 MWCO filter. The authors report a protein content of 80.4% in the isolate collected via this process (Diosady et al., 1984; Youngs, 1985). This value actually designates the protein extract as a concentrate rather than an isolate.

Most of the work on rapeseed protein isolates has occurred at the laboratory bench scale. While thermal processes are potentially damaging to the protein, which in turn result in a functionally-poor protein, these same thermal processes are important for myrosinase inactivation and oil extraction (Mieth et al., 1983). Attempts have been made to upscale protein extraction and recovery systems. The German Federal Ministry of Research and Technology supported the construction of a pilot plant to process mild defatting of the rapeseed and subsequent extraction of the rapeseed protein (Elstner and Stein, 1982, as cited in Youngs, 1985). The extract was 80-85% protein and was of a yellowish-green appearance. Although making an excellent feed-stuff, the extract did not meet human consumption requirements.

It would appear from most of literature available, some of which have been cited here, that the presence of high

levels of phenolics, phytates as well as glucosinolates and the precipitation of the protein call for conflicting conditions to affect the removal of the former group while preserving the nativity and solubility of the latter. As Youngs (1985) points out, although there have been reports of high quality product, quite often in low yield, many of the methods involve economically unattractive processing steps.

2.6.1 Functional Aspects of Canola Protein

Functional properties are defined as the intrinsic physico-chemical characteristics which affect the behavior of protein in food systems during processing, manufacturing, storage, and preparation (Kinsella, 1979). Functional properties may be gauged in terms of protein measurement, nitrogen solubility, water and fat absorption, oil emulsification, foam volume, foam stability, dispersion viscosity, gelation, colour, and flavour (Youngs, 1985). One of the main problems in this area of concern is the fact that functional properties of a particular protein are dependent on the sources of that protein and even more so dependent on the type of processing and temperatures used (Youngs, 1985). Harsh conditions of extraction have been known to adversely affect the functionality of a protein and its performance in a food system. Mild conditions of protein extraction are therefore desirable as protein denaturation is minimized hence preserving a large degree of functionality.

Sosulski _et_ _al_. (1976) reported somewhat mediocre results for the canola isolate. Decreased nitrogen solubilities and poor gelation characteristics in comparison to a soy isolate were the major findings. The foaming and whippability capacities of the canola isolate seemed to be similar to the soy isolate. Similar results have been

reported by other researchers (Gwiazda and Rutkowski, 1983 as cited in Youngs, 1985; Thompson et al., 1982b). Gwiadza and Rutkowski also reported lower water absorption, emulsifying capacity and nitrogen solubilities. Thompson et al. (1982b) found that the rapeseed protein concentrate that had been extracted with 2% hexametaphosphate possessed good nitrogen solubility at > pH 7.0, good fat absorption and emulsification properties. As well, the extract was shown to possess good whipping capacities. When used as a meat extender in wieners, it was found that the rapeseed protein concentrate increased emulsion stability and protein, but lowered the fat content of the wieners. The cooking yield of the wieners supplemented with the rapeseed protein concentrate was less than the soybean-supplemented wieners. As well, firmness of the wieners was lower and the sensory evaluation reports were not encouraging (Thompson et al., 1982b). The results for the use of the protein extracts as meat extenders in meat patties were more positive. Thompson reported increased cooking yields, shrinkage reduction, and tenderization in the meat patties supplemented with the rapeseed protein concentrate. In these studies, those products supplemented with soy isolate protein displayed more acceptable flavour qualities. It was also found by Thompson and her coworkers that meringue made from 9% rapeseed concentrate dispersion lacked the stability and related quality characteristics of those from egg white

meringue. However, when the rapeseed concentrate was mixed with egg white on a ratio of 1:1, the mixture produced a meringue of considerably higher quality approaching that of pure egg white meringue. The only exception was the slightly darker colour and off-flavour in the meringue. Overall, Thompson et al. (1982b) found that the rapeseed protein concentrate did not perform any worse than the soy isolates and that, in fact, most of the effects in food systems were favourable. However, the researchers state that more research is needed to address the problems of colour and flavour.

The functional properties of rapeseed protein isolates and concentrates have been studied through the use of these isolates and concentrates in bread-baking. Kodagoda et al. (1973) found that a 5% replacement of wheat flour with the isolates and concentrates decreased loaf volume by 10-15% and 20% respectively. This volume decrease was corrected by the addition of 0.5% Atmul 124 emulsifier. The emulsifier was found to increase the loaf volume by 10-15% for those loaves prepared with the water and HCl-extracted isolates over those loaves prepared with the all-wheat control. Addition of the Atmul 124 emulsifier to the NaOH-extracted concentrate loaves was found to increase loaf volume by 8% over the all-wheat controls. Kodagoda et al. (1973) found that the HCl-extracted isolate provided a 10% larger specific volume than egg-white meringue in whipping tests.

Whipping tests with the isolates and concentrates found that 3% replacement of egg white protein by the extracts resulted in reduced specific volume in comparison with an all-egg white control. It was reported in this publication that all rapeseed protein fractions (both concentrates and isolates) improved foam stability, especially the water-extracted isolates which showed no sign of foam deterioration or drip for 1.5 hours.

This same study reported that in terms of emulsification capacity, the water-extracted isolates were ranked as the best. The water-extracted isolates emulsified 45ml of corn oil/100mg protein as opposed to 35ml of corn oil/100mg protein for the other fractions. As well, emulsions consisting of the HCl-extracted protein displayed a high degree of stability: 200 min. compared to 3 min. for the control (Kodagoda et al., 1973). These researchers did not observe any significant correlation between the solubility of rapeseed protein and its functional properties.

Efforts to increase the solubility and water-holding capacity of canola/rapeseed protein have been undertaken by Jones and Tung (1983, as cited in Youngs, 1985). These researchers found that the addition of trypsin increased the solubility of the canola/rapeseed isolates and concentrates, and that linoleate treatment increased the water-holding

capacity of the canola/rapeseed isolate but decreased that of the other two concentrates.

It is not unreasonable to seriously consider the role of canola/rapeseed isolates or concentrates in foods where soy protein is being actively used and tested. The meat industry is presently engaging in research areas devoted to the reduction of water activity in meat products as well as extending beef bullock restructured steaks with protein hydrolysates (Vallejo-Cordoba et al., 1986; Miller et al., 1986; Ensor et al., 1987).

2.6.2 Nutritional Aspects of Canola Protein

Rapeseed protein has a nutritional value better than casein and approaching that of beef (Jones, personal communication). As was discussed in an earlier section on the canola/rapeseed protein, this protein has a well-balanced amino acid content, especially with respect to both lysine and the sulfur-containing amino acids. In terms of protein quality, as gauged by protein efficiency ratio (PER) the concentrates and isolates have consistently been shown to be superior to soy protein concentrates (Jones and Holme, 1979 as cited in Youngs, 1985; Olson et al., 1978). Some of the protein efficiency values found for rapeseed protein extracts when mixed with other food sources are shown in Tables 2.5 and 2.6. The concentrates have also been shown

Table 2.5 **Relationships Between Amino Acid Composition and the Feed Intake, Protein Digestibility and Protein Utilization of Rapeseed-based Diets in Rats**

Protein Source in Diet	Amino Acid Composition of Diet Lys. (% of rate requirements)	Met+Cys	Thr	Try	Chem. Score	Av. Daily Feed g	Av. Daily Gain g	Feed Gain Ratio g/g	Prot. Digestibility %	PER unadjusted	PER adjust. to Casein =2.50
Casein	88	63[1]	75	171	63	11.35	33.3	3.42	93	2.97	2.50
Rapeseed Concentrate	66	55	73	65	55	12.07	3.39	3.47	80	3.07	2.58
Rapeseed & Wheat	44	50	59	65	44	11.15	2.45	4.56	86	2.10	1.76
Rapeseed & Field Pea	76	47	71	65	47	11.79	3.04	3.89	79	2.69	2.26
Rapeseed & Beef	75	52	70	67	52	12.87	4.12	3.13	87	3.21	2.70

[1] First limiting amino acid(s) is underlined.

(Ohlson *et al.*, 1978)

Table 2.6 Nutritional quality of Rapeseed Protein Prepared from Different Rapeseed Varieties

Rapeseed Variety	Protein Efficiency Ratio (PER) (Casein = 2.5)
Echo (*B. campestris*)	2.5
Span (*B. campestris*)	2.5
Bronowski (*B. napus*)	2.8
Oro (*B. napus*)	2.6
Tower (*B. napus*)	2.6

(Jones, personal communication)

to be equivalent or better than methionine-supplemented casein (Youngs, 1985). McDonald et al. (1978a) found that true digestibility (TD), biological value (BV) and net protein utilization (NPU) were all higher than those values from soy flour and that neither the phenolics or phytate adversely impacted the rapeseed protein utilization by rats. It was also found that ground meat mixtures supplemented with rapeseed protein concentrate yielded higher BV and NPU values than the all-meat control. Soy-supplemented meats did not yield values as high (McDonald et al., 1978b, as cited in Youngs, 1985).

Further nutritional improvement of the rapeseed protein has been made through the addition of methionine and tyrosine - two limiting amino acids in rapeseed (Delisle et al., 1987). It was reported by Delisle that the nutritive value and digestibility of rapeseed protein concentrates were not improved by methionine supplementation. However, it was reported that these qualities could be enhanced through the addition of tyrosine. Supplementing water-extracted rapeseed proteins with 0.30% methione was found to increase the PER from 2.83 (water-extracted protein) to 3.35 (methionine supplemented protein) (Bruckner and Mieth, 1984). The nutritional ratios of rapeseed protein in comparison with those values calculated for other foods considered to be a high source of protein are shown in Table 2.7.

Table 2.7 Comparative Nutritional Value of Rapeseed Protein with other Food Proteins

Diet	PER	NPR	RPER	RNPR
Casein & Methionine	4.04	5.30	100	100
Egg White Solids	3.71	5.08	91	95
Minced Beef	3.36	4.83	83	91
Rapeseed Protein Concentrate	3.29	4.59	81	87
Casein	3.13	4.55	78	86

PER: Protein Efficiency Ratio
RPER: Relative PER
NPR: Net Protein Ratio
RNPR: Relative NPR

(Jones, personal communication)

The role of the antinutritional factors for most of the isolates and concentrates has been minimal at best. It has been reported that mineral bioavailability is not affected by phytic acid present in the rapeseed concentrate (Liu *et al*., 1982). With the phytic acid levels reduced to minimal levels and the glucosinolates largely removed or non-detectable, it would seem that the use of rapeseed protein isolates and concentrates could have a potential application in the future.

3.0 MATERIALS & METHODS

3.1.1 Protein Extraction Method

Raw defatted canola meal was obtained from the POS corporation in Saskatoon, Saskatchewan. The canola protein was subsequently extracted from the meal using methodology derived from the protein micellar mass (PMM) procedure as outlined by Murray _et al._, (1981). Samples of meal weighing 25gm were mixed for one hour in a buffer (250ml) of 0.1M NaCl and 0.1M Na_2HPO_4 adjusted to pH 5.5 with phosphoric acid. After one hour, the mixture was centrifuged using a Sorvall Refrigerated Centrifuge, Model RC2-B at 16,300xg for 30 minutes in order to remove the seed and hull debris. The supernatant containing the solubulized protein was filtered through double-layered pre-moistened cheesecloth to remove any further debris. The supernatant was then concentrated through a 10^4 molecular weight cut-off PM10 filter in an Amicon ultrafiltration unit (UF) device operating under a pressure of 60-70 psi. The volume of supernatant was subsequently concentrated from approximately 200ml to approximately 50ml.

The solubilized protein concentrate was diluted by 15 times its volume with distilled water at a temperature of 4^oC and left overnight (approximately 16 hours) in cold storage (4^oC). During this time, insoluble protein micelles

settled on the bottom of the dilution vessel. The protein micelles were collected the following day by means of centrifugation of the micellar suspension at 16,300xg for 30 minutes. The supernatant was discarded. The extracted protein was then frozen and freeze-dried for 72 hours.

3.2.1 Modifications to the Protein Extraction Method

3.2.1 A. Variation of Extraction Media Ionic Strength and pH Levels.

In order to maximize the positive response variables (i.e. % protein extracted, preservation of the protein native state) and minimize the presence of the negative response variables (i.e. maximum removal of phytic acid, glucosinolates, and the total phenolic compounds), the protein was extracted from the canola meal under six different conditions. These conditions are outlined in Table 3.1 and were chosen to screen both a pH and ionic strength range that would largely accomplish the removal of the antinutritional factors while not inflicting any undue damage on the protein. Those conditions best accomplishing these goals were used in the PMM-variation extraction procedure.

Table 3.1 Buffers Used in Protein Extraction Screening Procedure*

Buffer Composition	pH
0.1M NaCl/0.1M Na_2HPO_4	5.5
0.01M NaCl/0.01M Na_2HPO_4	5.5
0.1M NaCl/0.1M Na_2HPO_4	6.0
0.01M NaCl/0.01M Na_2HPO_4	6.0
0.1M NaCl/0.1M Na_2HPO_4	6.5
0.01M NaCl/0.01M Na_2HPO_4	6.5

*All buffers were adjusted to the designated pH with phosphoric acid. All buffers were prepared fresh daily.

3.3.1 Determination of Protein

Preliminary analysis of the canola protein extract for percent nitrogen content and percent protein was carried out in accordance with the Kjeldahl methodology outlined in the AOAC Official Methods of Analysis (1975) on a conventional microkjeldahl distillation apparatus.

Samples of raw canola meal and protein extract weighing between 10-15mg were wrapped in cigarette papers and deposited in digestion flasks. Cigarette papers were used as blanks. To each flask, 1.9 $\pm$ 0.1gm K_2SO_4, 40 $\pm$ 10mg HgO and 2.0 $\pm$ 0.1 ml concentrated H_2SO_4 were added. The flasks were then placed on a heated digestion rack for one hour to complete the digestion process.

After the digestion was complete, the flasks were removed from the digestion rack and the digested contents were transferred to microkjeldahl distillation flasks. Some rinsing with 1-2ml aliquots of distilled water was required to facilitate the transfer. The distillation flasks were then attached to the microkjeldahl distillation apparatus. Erlenmeyer flasks containing 5ml saturated H_3BO_3 were placed underneath the condenser with the condenser tip extending below the level of the boric acid in the erlenmeyer flask.

About 8-10ml of NaOH - $Na_2S_2O_3$ mixture were added to the distillation flask by means of the top of the still. About 15ml of distillate were collected in the erlenmeyer

flask before the flask was removed from the distillation apparatus. Once removed, the collected distillate was diluted to 50ml and then titrated from a clear light green colour to a grey end point with standardized 0.02N HCl acid solution.

The calculations used for estimating the % protein present in the 15mg samples were as follows:

[(ml.HCl - ml.blank x normality x 14.007 x 100) x 5.80]/ mg sample

where 5.80 is the kjeldahl multiplication factor for oilseeds such as canola.

In conducting the screening process to choose the best conditions under which to extract the canola protein, kjeldahl determinations, however, were performed on a Kjeltac Auto 1030 Analyzer (Tecator, Sweden).

Samples of raw canola meal and canola protein were weighed out in the order of 50mg ± 10mg into a digestion flask. To each flask, a Kjeltab S 3.5 catalytic tablet (Tecator, United Kingdom) containing 3.5g K_2SO_4 and 0.0035g Se was added. Concentrated H_2SO_4 (8ml) was then added into each flask. The flasks were then set into a Tecator digestor block for one hour to facilitate sample digestion. The contents of the flask were then titrated on the Kjeltec Auto 1030 Analyzer with 0.1N H_2SO_4. The results were calculated similarly to those Kjeldahl percentage protein

values determined on the microkjeldahl distillation apparatus.

3.4.1 Determination of Phytic Acid

Phytic acid content analysis on both the raw canola meal and canola protein extracts was carried out in accordance with the methodology outlined by Latta and Eskin (1980). The phytic acid content analysis was performed in three basic stages - extraction of the sample, column chromatography, and the calorimetric test.

3.4.1 A. Sample Extraction

A sample of defatted canola meal weighing 0.5g was stirred for one hour in 10ml 2.4% HCl. The mixture was then centrifuged at 12,000 x g for 10 minutes using the Sorvall Refrigerated Centrifuge model RC2-B. Five to 10ml of the clear supernatant were removed and placed in cold storage until actual phytic acid analysis.

3.4.1 B. Column Chromatography

A glass column measuring 0.7cm x 27cm was packed first with glass wool and then with 0.5g of analytical grade AG 1-X8 200-400 mesh anion exchange resin (chloride form, Biorad). The column was then rinsed with 15ml of 5% HCl followed by 20ml of deionized water.

The sample was diluted 5:25 with distilled water and 10ml of the diluted sample was pipetted onto the column. After the sample had been allowed to pass through the resin, 15ml of 0.1M NaCl was pipetted onto the column. The eluant was then discarded. Removal of the phytate anion from the column was effected by rinsing the column with 15ml of 0.7M NaCl and collecting the resultant eluant (containing the phytate anion) in a 25ml volumetric flask. The collected eluant was then diluted to 25ml with distilled water.

3.4.1 C. Colorimetric Determination

Three millilitres of blank (water), phytic acid standards (10, 20, 30, and 40 μg/ml), and samples were pipetted into separate 15ml conical centrifuge tubes. Exactly 1ml of Wade reagent (0.15g $FeCL_3 \cdot 6H_2O$) = 1.50g sulphosalicylic acid in 500ml, Fisher Labs) was added to the tubes and vortexed. The tubes were then centrifuged for 10 minutes at full speed (6000rpm) on a Sorvall GLC-1 benchtop centrifuge. Absorbance of the samples and standards were read at 500nm on an LKB ultrospec II spectrophotometer after zeroing with a water blank. Linear regression analysis was performed on the absorbance data with the interpolated results being expressed as % phytic acid.

3.5.1 Determination of Phenolic Compounds

The methodology used to measure the phenolic compounds present in both the canola meal and extracted protein was a variation of the method proposed by Dorrell (1976) with Lowry reagent being substituted for Folin-Denis reagent as suggested by Schandrel (1970) in the methodology of Swain and Hillis (1959). The analyses were performed in two stages - the sample extraction and phenolics extraction.

3.5.1 A. Sample Extraction

Samples of both canola meal and protein extract weighing 50mg were refluxed in 12.5ml of 80% ethanol at pH 4.0 for 30 minutes. If availability permitted, 100mg sample sizes in 25ml of 80% ethanol were used. After refluxing, the samples were allowed to cool and then centrifuged on a Sorvall GLC-1 benchtop centrifuge for 5 minutes at 2000 rpm. The supernatant was then adjusted to a volume of 25ml with distilled water.

3.5.1 B. Phenolic Compounds Extraction

An aliquot of supernatant (0.5ml) was diluted to 7ml in a 10ml graduated test tube with distilled water. The tube was mixed for approximately 2 to 3 seconds after which 0.5ml of Lowry reagent was added to the solution. The tube was mixed again briefly and allowed to sit for 3 minutes after which 1ml of saturated sodium carbonate solution was

added. The entire solution was then made up to a 10ml volume with distilled water and allowed to sit for one hour at room temperature. After one hour had passed, the absorbances of the samples and tannic acid standards (50, 100, 150, and 200 g/ml) were read at 750nm on the Hewlett-Packard Diode Array Spectrophotometer using a blank-reference of distilled water.

3.6.1 Analysis of the Glucosinolates Using Gas Chromatography

The glucosinolates in the canola protein were analyzed according to the method described by Slominski and Campbell (1987). Samples of raw meal and protein weighing 100mg were deposited in test tubes and placed in a boiling water bath for 10 minutes. After this time period, 1ml aliquots of hot (95°C) water were added to each tube and the contents mixed and heat-treated for an additional 3 minutes. This heat treatment/extraction procedure was standard and used to extract the glucosinolates and inactivate the myrosinose enzyme.

After cooling, 1ml of internal standard solution was added to each tube. The internal standard solution consisted of either 1 mol/ml allyl glucosinolate (Aldrich, Montreal) or 1μmol/ml benzyl glucosinolate (Canola Council of Canada, Winnipeg). A 1:1 (v/v) mixture of 0.5M barium acetate and 0.5M lead acetate was made with 125μl of this mixture being added to the actual sample. The entire

subsequent mixture was centrifuged at 2000 x g for 10 minutes. The supernatant (0.5ml) was then applied to a DEAE Sephadex A-25 column (pyridine acetate form, 8 x 5 mm i.d., Sigma, St. Louis, MO, USA) which was then washed with 1ml of 0.02M pyridine acetate solution (Pierce, Rockford, IL, USA). Purified sulphatase solution (50 μl) (from aryl sulphatase, type H-1, Sigma) was added to the column. The contents were then allowed to stand overnight at room temperature. Desulphoglucosinolates were eluted with water (4 x 0.5ml) into a 2ml sealed-cap vial. The procedure involved the use of 2ml of water for the elution step.

The contents of each vial were subjected to nitrogen drying at 60°C. After drying, 100 μl pyridine, 50 μl MSTFA (N-methyl-N-TMS-trifluoroacetamide), and 10 μl TMCS (trimethyl-chlorosilane) (Pierce) were added to each vial, which was then capped, mixed and heated at 120°C for 20 minutes. The desulphoglucosinolates were derivatised at this point and separated using a Varian Vista 6000 gas chromatograph equipped with a flame ionisation detector and a Vista 402 computer. Separation of the desulphoglucosinolates was carried out in a 1.2m x 2mm i.d. glass column packed with 2% OV-7 on Chromosorb W, AW-DMCS (100-200 mesh) with helium gas at a flow rate of 40ml/min. Oven temperature was kept constant at 200°C for 4 minutes, then increased at 5°C/minute to a temperature of 275°C. The temperatures of the injection port and detector were 280 and

300°C, respectively. The relative response factors (RRF) were calculated from the ratios of trimethylsilylated (TMS) internal standard carbon number and the respective glucosinolate TMS carbon number.

3.7.1 Analysis of Thermal Properties of Canola Protein

An analysis of the degree of denaturation of the extracted protein was performed by studying the thermal properties of the protein using differential scanning calorimetry (DSC). A DuPont 9900 computer thermal analyzer with a 910 differential scanning calorimeter cell base was used.

A slurry of canola protein and distilled water was prepared having approximately 40% (w/w) total solids. From this slurry, samples of 10-15mg were weighted into DuPont aluminum pans, coated on the interior with an inert polymer. All weight determinations were assessed with a Mettler AE163 analytical balance. Following weighing, the pans were then hermetically sealed.

A reference (empty) sealed pan and a sample pan were placed in a nitrogen-pressurized DSC cell using a silicone heat sink compound (Dow Corning) for better thermal conductivity. The cell was operated at 200 psi while the protein sample was subjected to a heating rate of 10°C/min. over a range of 10°C to 120°C.

The thermal properties of the protein were graphically represented by a thermal curve, plotted on a DuPont Instruments pen-plotter. The DSC Standard Data Analysis Program (Version 1.1C) was used to collect and analyze the data. The results were expressed in terms of the maximum heat flow into the protein (the thermal denaturation temperature, Td in degrees Celsius) as well as the enthalpy of denaturation (the $\triangle$H value expressed in Joules/g of sample).

3.8.1 Analysis of Canola Protein Using SDS-PAGE

The physical and structural subunit characteristics of the extracted canola protein were studied by measuring the various responses of the protein in an applied electrical field. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method described by Ismond (1984) in which an LKB Bromma 2117 Multiphor apparatus (LKB, Sweden) with an LKB Bromma 2103 Constant Power Supply system were used. The system was cooled using a Haake CI/Haake G liquid-coolant system. All gels contained 7.5% polyacryamide. The gel composition is outlined in Table 3.2.

Protein samples (approximately 2mg/ml) in sample buffer were prepared and incubated at 37°C for two hours. The sample buffer composition is outlined in Table 3.3. Ten μl

of the sample mixture were pipetted into the slots on the horizontal acrylamide gel which had been pre-electrophoresed for 30 minutes at 150 mA. Immediately after sample injection, the power supply was adjusted to 10mA for 10 minutes and then adjusted to 195mA for 4.5 hours.

The procedure used for staining the gel is outlined in Table 3.4. Gels stained in Coomassie Blue were photographed after 1 to 2 days of destaining. Molecular weight standards for SDS-PAGE were obtained from Sigma Chemical Co. and are listed in Table 3.5.

Table 3.2 Composition of Polyacrylamide Gel for SDS-PAGE

Solution	Volume of solution for a final acrylamide concentration of 7.5%
Distilled water	7.5ml
Buffer stock solution*	33.0ml
Acrylamide solution*	22.2ml
Ammonium persulphate*	3.2ml
N, N, N^1, N^1 tetramethyl ethylene diamine (TEMED)	0.1ml
Final volume	66.0ml

*For composition of each solution, see Table 3.3

Table 3.3 **Chemicals Required for SDS-PAGE**

Chemical	Composition	Procedure
1. Phosphate Buffer	39.0 $NaH_2PO_4 \cdot H_2O$ 102.3g Na_2H_4 10.0g sodium dodecyl sulphate (SDS)	Adjust volume to 3000 ml. Bring pH to 7.1 with 0.2M NaOH. Make up to 5000ml with distilled water.
2. Sample Buffer	5ml phosphate stock buffer 1ml 2-mercaptoethanol 1g SDS 0.015g bromophenol blue	Make up to 100ml with distilled water.
3. Electrode Buffer	--	1 part stock buffer plus 1 part distilled water.
4. Acrylamide Solution	2.2g acrylamide (Eastman Kodak Co., Enzyme Grade) 0.6g biscarylamide	Make up to 100ml with distilled water. Store in dark bottle at 4°C.
5. Ammonium Persulphate	150mg ammonium persulphate	Make up to 10ml with distilled water. Prepared fresh daily.
6. Detergent Solution	--	Add 0.1ml Triton-X100 to 100ml distilled water.
7. Fixing Solution	57.0 trichloro-acetic acid, 17.0g sulphosalicyclic acid, 150ml methanol, 350ml distilled water	Add to acids to the mixture of methanol and distilled water

Table 3.3 (Cont'd)

Chemical	Composition	Procedure
8. Staining Solution	1.25g Coomassie Brilliant Blue R-250 227ml methanol 227ml distilled water 46ml glacial acetic acid	Dissolve the dye in the solution of methanol and distilled water. Add acetic acid. Filter through a Whatman #1 filter. Store in a dark bottle.
9. Destaining Solution	1500ml ethanol 500ml acetic acid	Make up to 5000ml with distilled water
10. Preserving Solution	300ml ethanol 100ml acetic acid 100ml glycerol	Make up to 1000ml with distilled water.

Table 3.4 Staining Procedure for SDS-PAGE using Coomassie Blue

Solution	Time
Fixing Solution	1 hour
Staining Solution	2 hours
Destaining Solution	18-24 hours*
Preserving Solution	45 min. - 1 hour

*Solution is changed frequently in this time period.

Table 3.5 Molecular Weights of Protein Standards used for SDS-PAGE

Protein	Molecular Weight
Lysozyme	14,300
Trypsinogen	24,000
Pepsin	34,700
β-lactoglobulin	36,000 (total)
	18,400 (per subunit)
Ovalbumin	45,000
Bovine Serum Albumin (BSA)	66,000

The relative mobility (R.M.) of the proteins was calculated from the following relationship:

$$R.M. = \frac{\text{Distance of protein migration}}{\text{gel length after drying}} \times \frac{\text{gel length after fixing}}{\text{distance of dye migration}}$$

A calibration curve for the relationship between relative mobility of the molecular weight standards and log molecular weight was established by standard linear regression analysis. (Appendix A) The molecular weights of the canola protein subunits were then calculated from the linear regression equation.

3.9.1 Analysis of the Canola Protein Using Gel Filtration/High Performance Liquid Chromatography (HPLC)

The extracted canola protein was assessed for its purity by using the principles of size-exclusion in a gel filtration/HPLC application to separate and quantitate any contaminating entities accompanying the protein.

Protein samples were prepared for injection into the HPLC by solubilizing in a solution of 0.01M Na_2SO_4 in 0.02M NaH_2PO_4 at pH 6.8 at a concentration of 2mg protein/ml. This solution was also the mobile phase used in a Waters HPLC system. The stationary phase consisted of a Brownlee Aquapore OH300 SEC (size exclusion) gel filtration column with dimensions of 25cm in length and 4.6 mm in inner

diameter. The standards were obtained from Biorad and outlined in Table 3.8.

Sample protein (20 µl) was injected into the entry port of the HPLC with the column flow rate set at 2ml/min. A Waters U6K injector with a 2 ml sample loop was used in conjunction with a Waters Alodel 6000A pump. Responses were measured on a Waters Model 440 ultraviolet fixed wavelength detector set at 280nm. The results were integrated and plotted on a Varian/Vista series CDS 401 computer.

Table 3.6 Protein Standards used for Gel Filtration/ HPLC Analysis

Protein	Molecular Weight
thyroglobulin	670,000
gamma-globulin	158,000
ovalbumin	44,000
myroglobulin	17,000

(Biorad Laboratories)

4.0 RESULTS

4.1.1 Results of Extraction and Selection of Screening Procedure

Extraction of the protein from canola was carried out under the six conditions described previously. The protein was extracted from 25g samples of defatted canola meal. The effects of the six extraction conditions on the dry weights (g) and subsequent % extraction of the brown-coloured protein pellet from the raw meal are described in Table 4.1

It appears from Table 4.1 that those conditions with the greater saline content provided greater bench-top weight yields in contrast to those extraction conditions containing less salt (i.e. 0.01M NaCl in 0.01M Na_2HPO_4 buffer)

Statistical analysis revealed that interactions between the effects of pH and the effects of ionic strength were not significant at the 0.05 level. No significant differences at the 0.05 level were found to exist between the different pH levels and their effects on the extractions. Significant differences (at the 0.05 level) were found to exist between the two ionic strengths used in the extraction process (Appendix B.3)

The six extraction conditions represented a screening procedure to be used in further extractions of canola protein. The choice of one of the six extraction conditions to remove the protein from canola meal was based on the ability of that extraction buffer to meet those parameters considered optimal in terms of the desired protein qualities listed in Table 4.2.

Table 4.1 **Effect of Extraction Conditions on Dry Weight Pellet Yield from Canola Meal**[1]

	pH 5.5		pH 6.0		pH 6.5	
	0.01M[2]	0.1M[3]	0.01M[2]	0.1M[3]	0.01M[2]	0.01M[3]
average yield (g)	0.382±0.15	0.962±0.14	0.470±0.05	0.964±0.15	0.364±0.11	1.072±0.18
% extraction[4]	1.53±0.62[a]	3.85±0.57[b]	1.96±0.20[a]	3.86±0.62[b]	1.46±0.41[a]	4.29±0.73[b]

[1] Each extraction was carried out using 25g of raw meal
[2] All buffers were prepared with 0.01M NaCl in 0.01M Na_2NPO_4
[3] All buffers were prepared with 0.1M NaCl in 0.1M Na_2NPO_4
[4] The dry weight of the extracted pellet when calculated in terms of the 25g starting material is referred to as % extraction.

Values followed by the same letter are not significantly different at the 0.05 level (Appendix B.1-B.3)

Table 4.2 Goals of the Extraction Buffer

1) Permit optimal protein extraction (i.e. large % protein content) (KJELDAHL)*

2) Permit high thermal denaturation (Td) value.

3) Permit large enthalpy of denaturation ($\triangle$H).

4) Provide greatest removal of glucosinolates (GLUCOSIN), phytates (PHYTICS), and phenolic compounds (PHENOLICS)

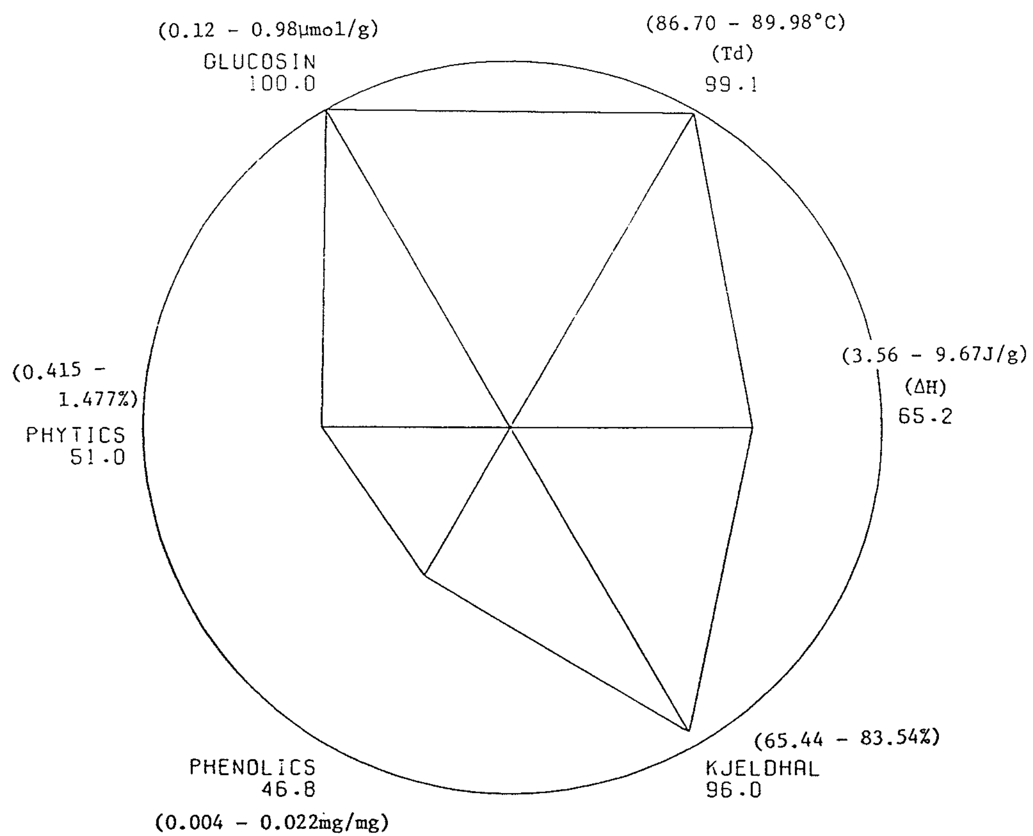
* Higher-cased words in parentheses denote response variables constituting the G-PROC stars of comparison of extraction buffers (Figures 4.1-4.6).

Graphical representations in the form of G-PROC stars of comparison were used to choose that extraction buffer that best met the criteria outlined in Table 4.2. Each star possesses six spokes, with each spoke representing one of the six response variables (i.e. Kjeldahl, Td, H etc.) is reflected by an extended spoke. That is to say, the longer the spoke, the greater the optimization. Similarly, longer spokes for that side of the stars constituting the negative response variables (i.e. glucosinolates, phytic acid, phenolic compounds) reflect greater removal of those undesirable components from the protein. Ideally, the larger the star appears, the more optimally are all of the goals met (Table 4.2).

G-PROC stars were constructed for all extraction conditions (Figs. 4.1 to 4.6). The effect of the individual extraction buffers on each response variable is ranked as is denoted by a number ranging between zero and 100 appearing above the spoke. That buffer of the six extraction conditions that best optimizes a particular response variable is assigned a value of 100. The other buffers are ranked in accordance with respect to that particular response variable on the same scale, using the buffer that is ranked 100 as the standard.

The buffer prepared with 0.1M NaCl in a Na_2HPO_4 adjusted to pH 5.5 was chosen as the extraction condition that best achieved the above-mentioned parameters

Figure 4.1 Standardized Mean Response Analysis for Canola Protein Extracted with 0.01M NaCl at pH 5.5. Means are expressed as percent of optimum response. "Glucosin", "Phytics", and "Phenolics" measure removal.



(0.12 - 0.98µmol/g)
GLUCOSIN
100.0
(86.70 - 89.98°C)
(Td)
99.1
(0.415 -
1.477%)
PHYTICS
51.0
(3.56 - 9.67J/g)
(ΔH)
65.2
(65.44 - 83.54%)
KJELDHAL
96.0
PHENOLICS
46.8
(0.004 - 0.022mg/mg)

Figure 4.2 Standardized Mean Response Analysis for Canola Protein Extracted with 0.10M NaCl at pH 5.5. Means are expressed as percent of optimum response. "Glucosin", "Phytics", and "Phenolics" measure removal.

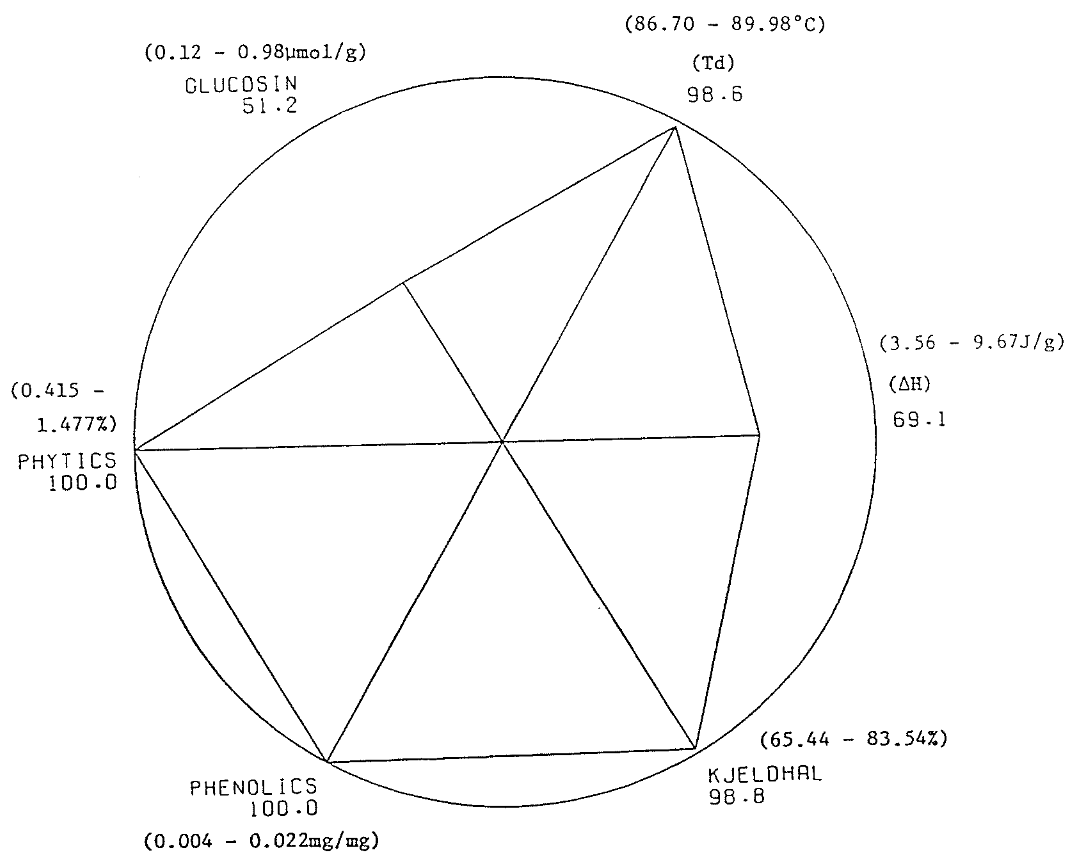

(86.70 - 89.98°C)
(Td)
98.6
(0.12 - 0.98μmol/g)
GLUCOSIN
51.2
(3.56 - 9.67J/g)
(ΔH)
69.1
(0.415 -
1.477%)
PHYTICS
100.0
(65.44 - 83.54%)
KJELDHAL
98.8
PHENOLICS
100.0
(0.004 - 0.022mg/mg)

Figure 4.3 Standardized Mean Response Analysis for Canola Protein Extracted with 0.01M NaCl at pH 6.0. Means are expressed as percent of optimum response. "Glucosin", "Phytics", and "Phenolics" measure removal.

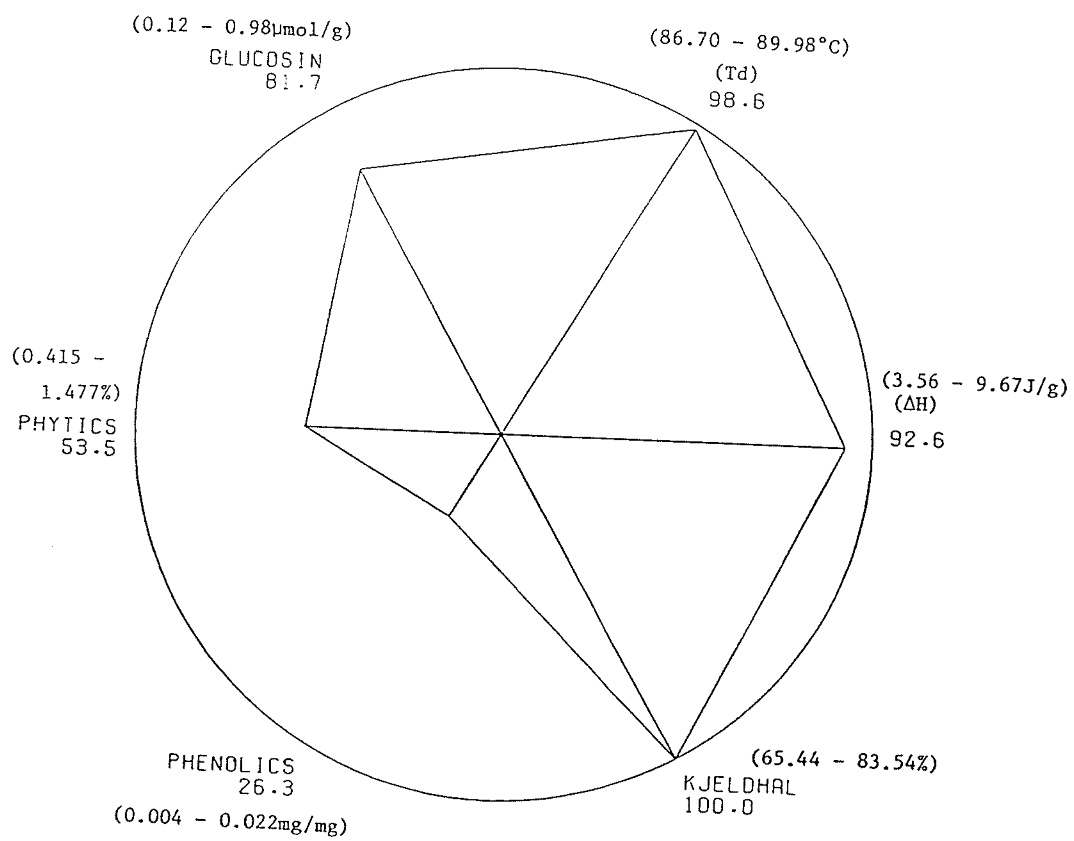
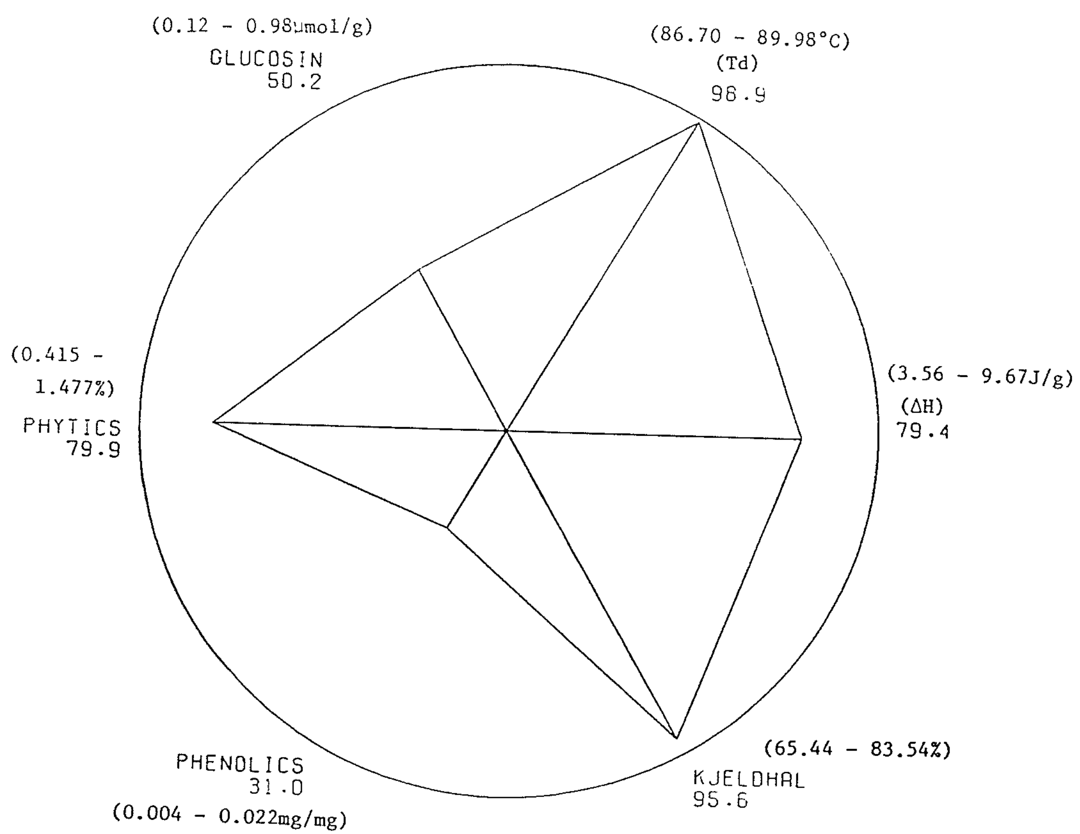

(0.12 - 0.98µmol/g)
GLUCOSIN
81.7
(86.70 - 89.98°C)
(Td)
98.6
(0.415 -
1.477%)
PHYTICS
53.5
(3.56 - 9.67J/g)
(ΔH)
92.6
PHENOLICS
26.3
(0.004 - 0.022mg/mg)
(65.44 - 83.54%)
KJELDHAL
100.0

Figure 4.4 Standardized Mean Response Analysis for Canola Protein Extracted with 0.10M NaCl at pH 6.0. Means are expressed as percent of optimum response. "Glucosin", "Phytics", and "Phenolics" measure removal.



(0.12 - 0.98µmol/g)
GLUCOSIN
50.2
(86.70 - 89.98°C)
(Td)
98.9
(0.415 -
1.477%)
PHYTICS
79.9
(3.56 - 9.67J/g)
(ΔH)
79.4
PHENOLICS
31.0
(0.004 - 0.022mg/mg)
(65.44 - 83.54%)
KJELDHAL
95.6

Figure 4.5 Standardized Mean Response Analysis for Canola Protein Extracted with 0.01M NaCl at pH 6.5. Means are expressed as percent of optimum response. "Glucosin", "Phytics", and "Phenolics" measure removal.

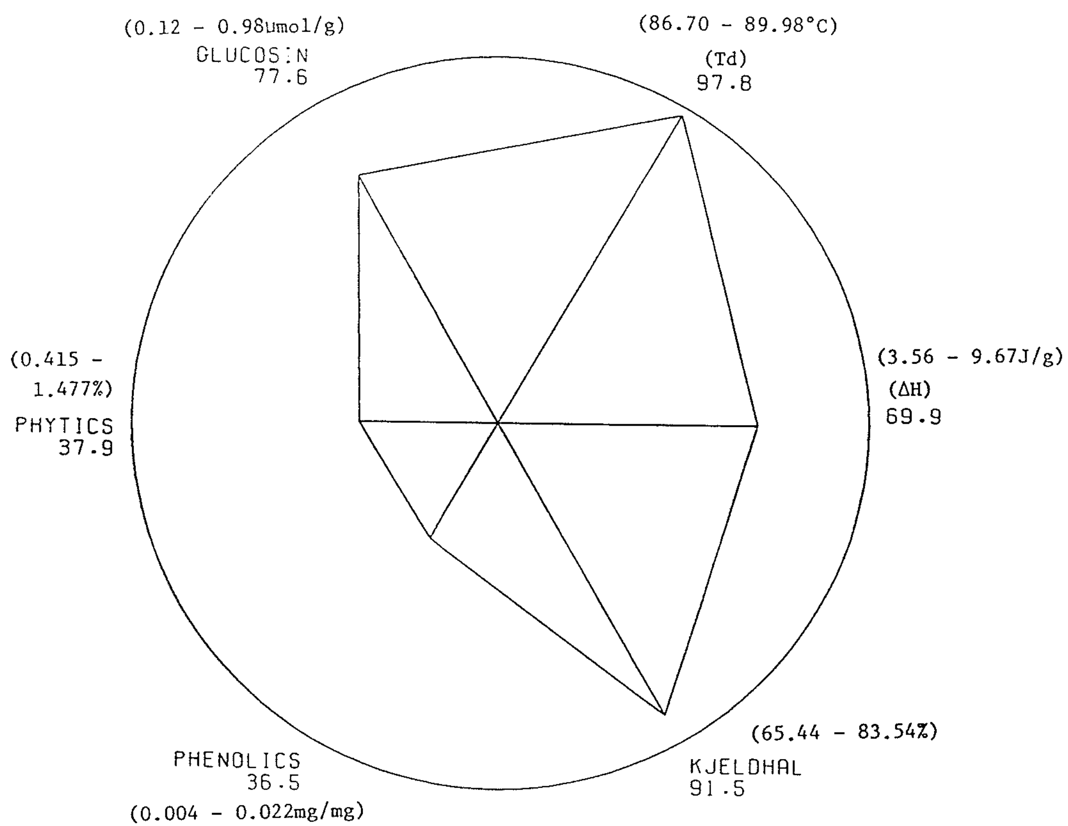
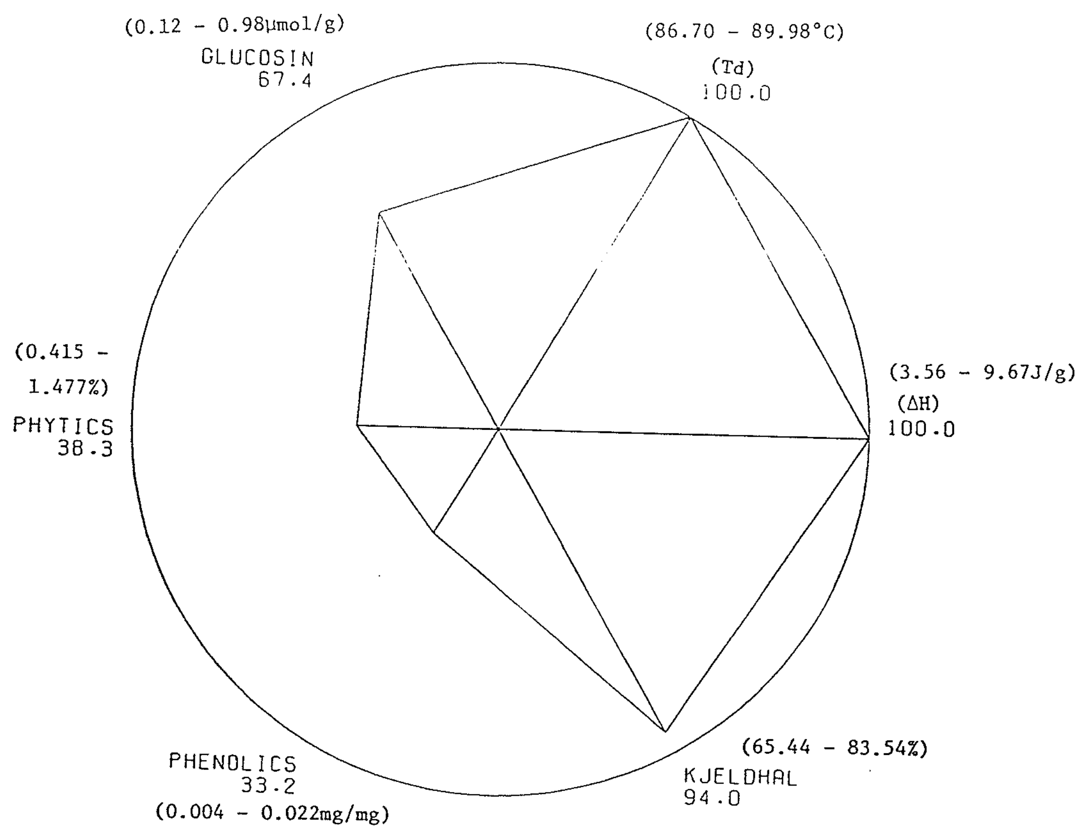

(0.12 - 0.98µmol/g)
GLUCOSIN
77.6
(86.70 - 89.98°C)
(Td)
97.8
(0.415 -
1.477%)
PHYTICS
37.9
(3.56 - 9.67J/g)
(ΔH)
69.9
PHENOLICS
36.5
(0.004 - 0.022mg/mg)
(65.44 - 83.54%)
KJELDHAL
91.5

Figure 4.6 Standardized Mean Response Analysis for Canola Protein Extracted with 0.10M NaCl at pH 6.5. Means are expressed as percent of optimum response. "Glucosin", "Phytics", and "Phenolics" measure removal.


(0.12 - 0.98μmol/g)
GLUCOSIN
67.4
(86.70 - 89.98°C)
(Td)
100.0
(0.415 -
1.477%)
PHYTICS
38.3
(3.56 - 9.67J/g)
(ΔH)
100.0
PHENOLICS
33.2
(0.004 - 0.022mg/mg)
(65.44 - 83.54%)
KJELDHAL
94.0

(Figure 4.5). This buffer was rated as the overall best of the six conditions that removed phytates and phenolic compounds. As well, it was 98.8% as effective as that buffer prepared with 0.01M NaCl in a Na_2HPO_4 buffer and adjusted to pH 6.0 in the extraction of protein. It appears as though the buffer of choice rated poorly in terms of glucosinolate removal in comparison to the other extraction buffers. It is to be noted that the entire range of glucosinolate removal over all six extraction conditions must be considered. The ranges of removal and optimization are included in each figure. The use of the comparison stars program was not intended to replace statistical analyses of the data. Application of the stars program was intended to augment the statistical analyses in order to select an optimal buffer for the extraction process.

4.2.1 Protein Quantitation Using Kjeldahl Analysis

The effects of the six extraction conditions on percentage protein yield were examined. The levels of protein yield found with the various conditions are detailed in Table 4.3. No significant differences were found between the effects of the six extraction conditions on the protein yield. From the analysis of variance data (Appendix B.4), it was found that there were no significant pH - ionic strength interactions existing at the 0.05 level (Appendix B.4 and B.5). As well, there were no significant

Table 4.3 Effects of the Extraction Conditions on the Protein Yields[1] from Commercial Canola Meal[2] Using Kjeldahl Analysis[3].

pH	0.01M NaCl[3]	0.1M NaCl[4]
5.5	78.53±4.20[a]	80.78±0.79[a]
6.0	81.83±2.16[a]	78.20±2.21[a]
6.5	74.88±8.87[a]	76.94±3.46[a]

[1] All values are expressed as % protein
[2] Raw canola meal was found to have a protein content of 34.50±4.15% protein.
[3] All buffers were prepared using a 0.01M Na_2HPO_4 buffer.
[4] All buffers were prepared using a 0.1M Na_2HPO_4 buffer.

Values followed by the same letter are not significantly different ($P \leq 0.05$)

differences found between the effects of ionic strength on the final % protein values. However, it was found that significant differences existed between the effects of pH at the 0.05 level (Appendix B.6).

4.3.1 Analysis of Phytic Acid

The effects of the six extraction conditions on the removal of phytic acid from the sample meal ($2.00\pm0.06\%$) were studied and are presented in Table 4.4. From the analysis of variance data (Appendix B.8), it was found that there existed a 99.9% probability of significant differences existing between the extraction treatment (the combined pH-ionic strength effects). This is borne out by the data in Scheffe's Text (Appendix B.9) whereby significant differences indeed existed (at the 0.05 level) among all treatments except between protein samples extracted at pH 5.5 at 0.01M NaCl and those samples extracted at pH 6.0 at 0.01M NaCl.

The greatest removal of phytic acid from the protein was found to be effected with the buffered extraction medium adjusted to pH 5.5 and possessing an ionic strength of 0.1M NaCl. The overall mean value of phytic acid in the protein extracted under these conditions was found to be $0.489\pm0.21\%$ phytic acid remaining in the extract. This result was found to be significantly different (at the 0.05 level) from those phytic acid values calculated for the other conditions. One

Table 4.4 Effects of the Extraction Conditions on the Phytic Acid Content[1] in Commercial Canola Meal[2] Protein

pH	0.01M NaCl[3]	0.1M NaCl[4]
5.5	0.96±0.04[a]	0.49±0.21[c]
6.0	0.92±0.03[a]	0.61±0.02[d]
6.5	1.29±0.23[b]	1.28±0.01[e]

[1] All values are expressed as % phytic acid remaining in the protein extract.
[2] Canola meal was found to contain 2.00±0.06% phytic acid
[3] All buffers were prepared using a 0.01M NaCl in 0.01M Na_2HPO_4
[4] All buffers were prepared with 0.1M NaCl in 0.1M Na_2HPO_4

Values followed by the same letter are not significantly different ($P \leq 0.05$)

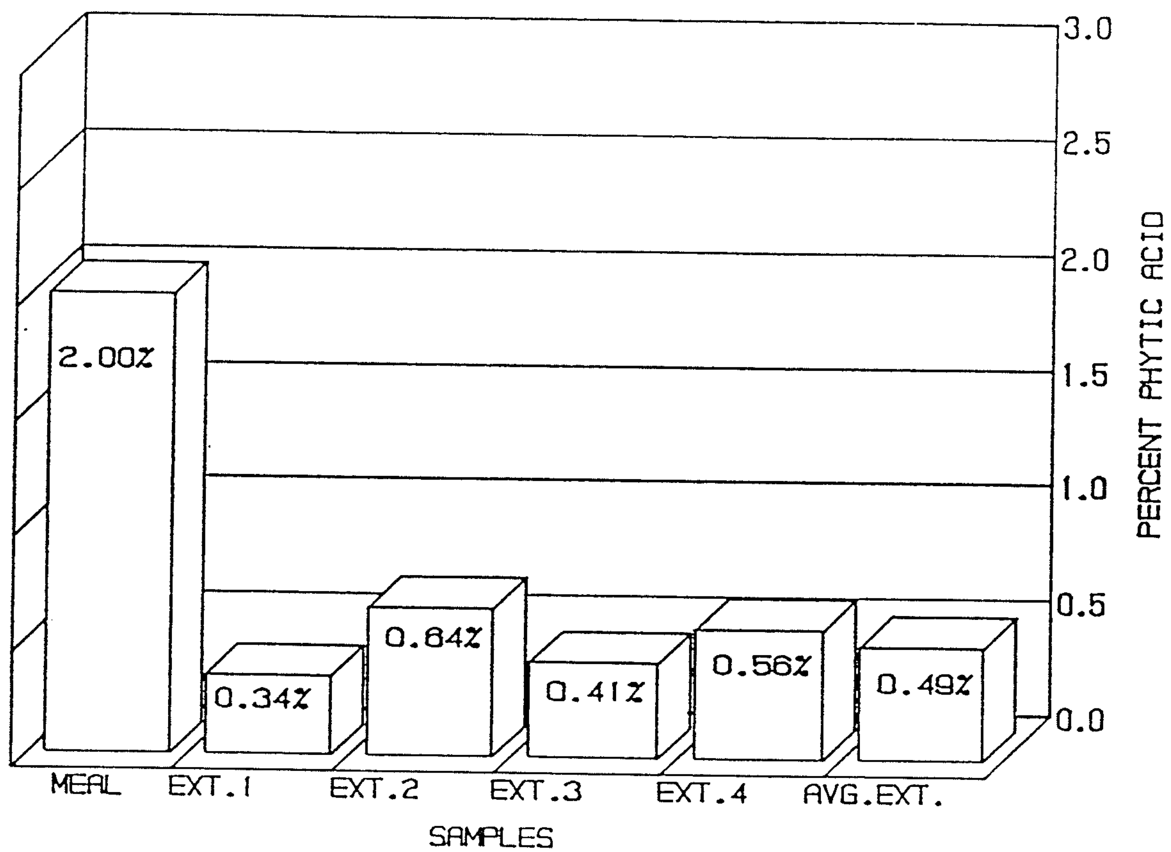
extract in this series was found to contain as low as 0.342% phytic acid remaining resulting in an 83% phytate removal.

The poorest removal of phytic acid was found in that protein extracted from canola with the extraction medium adjusted to pH 6.5 and possessing an ionic strength of 0.01M NaCl. The overall mean value was found to be 1.292±0.230% phytic acid remaining in the extracted protein. This represents a 35% reduction of phytic acid from the original canola meal which contained 2.00±0.06% phytic acid. One extract in this series displayed a mere 27% removal of phytic acid resulting in a phytate content of 1.455% in the protein.

Considering the effects of pH alone, a Scheffe's test comparison (Appendix B.10) found that all effects of pH were significantly different from one another at the 0.05 level. As well, the effects of ionic strength effects were found to be significantly different at the 0.5 level (Appendix B.11)

In considering the extraction of protein using a 0.1M NaCl in 0.1M Na_2HPO_4 buffer system, phytic acid content in the meal was found to be reduced by an average of 75.5% in the extraction process. This finding is illustrated in Figure 4.7.

Figure 4.7 Reduction of Phytic Acid in Canola Meal Protein Using 0.1M NaCl/0.1M Na_2HPO_4 at pH 5.5 (opposite page)


3.0
2.5
2.0
1.5
1.0
0.5
0.0
PERCENT PHYTIC ACID
2.00%
0.34%
0.64%
0.41%
0.56%
0.49%
MEAL
EXT.1
EXT.2
EXT.3
EXT.4
AVG.EXT.
SAMPLES

4.4.1 Analysis of the Glucosinolates

The effects of the six extraction conditions on glucosinolate removal from the extracted canola protein were examined. The glucosinolate levels remaining in the extracted protein are presented in Table 4.5. It was found in a Scheffe's test (Appendix B.13) that there existed no significant differences between the combined pH and ionic strength treatments on the glucosinolate removal from the protein. As well, no significant differences were detected between the effects of pH on the glucosinolates removal. However, significant differences were detected between the two ionic strengths used in terms of their effects on the glucosinolate removal (Appendix B.15).

When considering the combined overall pH and ionic strength effects on glucosinolate removal, it is obvious that a narrow range of glucosinolate removal exists between the treatments and that no one particular extraction condition treatment can be rated as being clearly superior to the others. Using the extraction buffer prepared with o.1M NaCl and 0.1M Na_2HPO_4 at pH 5.5, it was found that glucosinolates were reduced by 92.5% of those glucosinolates found in the meal (Figure 4.8).

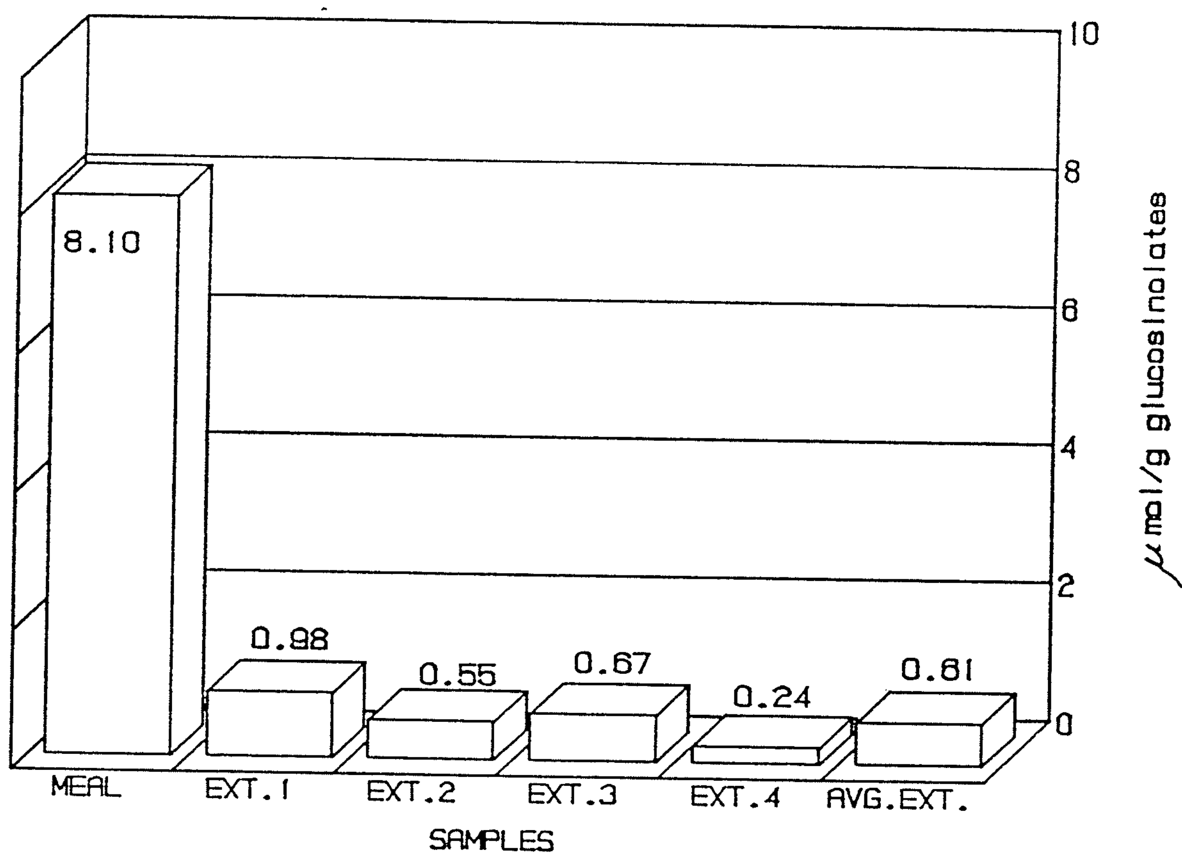
Table 4.5 Effects of the Extraction Conditions on the Glucosinolate Content[1] in Commercial Canola Meal[2] Protein

pH	0.01M NaCl[3]	0.1M NaCl[4]
5.5	0.31 ± 0.05^{a}	0.61 ± 0.07^{a}
6.0	0.38_0.06^{a}	0.62 ± 0.04^{a}
6.5	0.40 ± 0.08^{a}	0.46 ± 0.02^{a}

[1] All values are expressed as μmol glucosinolates/g protein
[2] Raw canola has been found to contain 8.10 ± 0.04 μmol glucosinolates/g raw meal.
[3] All buffers were prepared using a 0.01M Na_2HPO_4 buffer
[4] All buffers were prepared using a 0.1M Na_2HPO_4 buffer

Values followed by the same letter are not significantly different ($P\leq0.05$)

Figure 4.8 Reduction of Glucosinolates in Canola Meal Using 0.1M NaCl/0.1M Na_2HPO_4 at pH 5.5



8.10
0.98
0.55
0.67
0.24
0.61
10
8
6
4
2
0
μ mol/g glucosinolates
MEAL
EXT.1
EXT.2
EXT.3
EXT.4
AVG.EXT.
SAMPLES

4.5.1 Analysis of Phenolic Compounds

The effects of the six extraction conditions on the removal of phenolic compounds from the canola protein were studied and are outlined in Table 4.6.

Raw defatted canola was found to contain 0.034±0.010 mg phenolic compounds/mg meal. Statistical analysis revealed that significant differences existed between some of the treatments (Appendix B.16 and B.17). Greatest removal of the phenolic compounds was achieved by extracting the protein with the 0.1M $NaCl/Na_2HPO_4$ buffer at pH 5.5 resulting in an average of 0.005±0.0013 mg phenolic compounds remaining per mg protein. This results in an 85.3% reduction in total phenolics (Figure 4.9). The poorest removal of phenolic compounds was determined with protein samples extracted with the 0.01M $NaCl/Na_2HPO_4$ buffer adjusted to pH 6.0. In this extraction, 0.019±0.001 mg phenolic compounds/mg protein remained in the extract resulting in a 44.4% reduction of total phenolic compounds.

Considering the effects of pH alone, on the removal, it was found that significant differences existed between all pH comparisons (Appendix B.18). As well, significant differences existed between the two ionic strengths used in the extractions (Appendix B.19).

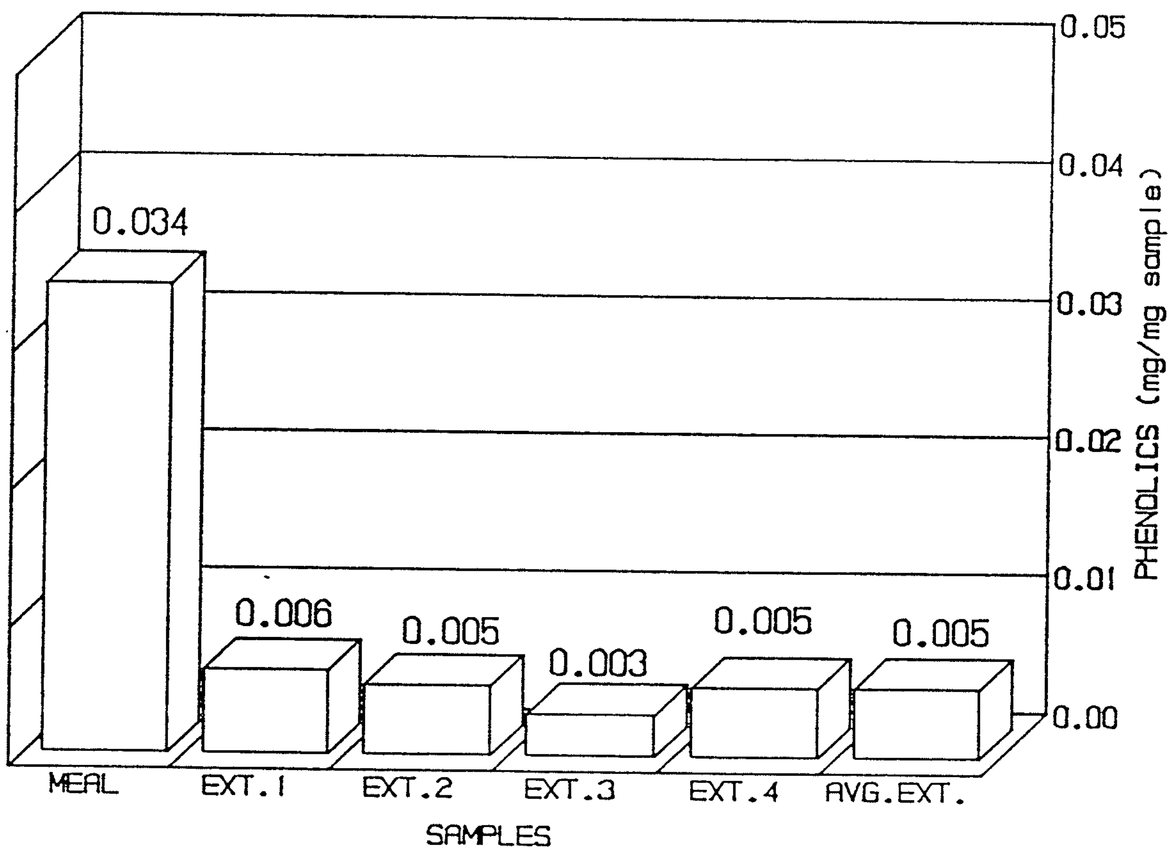
Table 4.6 Effects of the Extraction Conditions on the Phenolic Compounds[1] in Canola Protein[2]

pH	0.01M NaCl[3]	0.1M NaCl[4]
5.5	0.011±0.0017[a]	0.005±0.0013[c]
6.0	0.019±0.0013[b]	0.016±0.0008[b]
6.5	0.013±0.000[b]	0.015±0.0027[b]

[1] All values are expressed as mg phenolics/mg sample
[2] Raw canola has been found to contain 0.034±0.01 mg phenolics/mg meal
[3] All buffers were prepared using a 0.01M Na_2HPO_4 buffer
[4] All buffers were prepared using a 0.1M Na_2HPO_4 buffer

Values followed by the same letter are not significantly different ($P \leq 0.05$)

Figure 4.9 Reduction of Phenolic Compounds in Canola Meal Protein Using 0.1M NaCl/0.1M Na_2HPO_4 at pH 5.5


0.034
0.006
0.005
0.003
0.005
0.005
0.05
0.04
0.03
0.02
0.01
0.00
PHENOLICS (mg/mg sample)
MEAL
EXT.1
EXT.2
EXT.3
EXT.4
AVG.EXT.
SAMPLES

4.6.1 Analysis of the Thermal Properties of the Protein

4.6.2 Thermal Denaturation Temperature (Td)

The relative denaturation of the protein was examined by observing the effects of the six extraction conditions on the thermal properties of the protein. From these data, it is possible to determine the levels of denaturation, if any, inflicted on the protein from the various treatments. More specifically, any evident denaturation is reflected by lower levels of heat flow into the protein sample.

The effects of the six extraction conditions are outlined in Table 4.6 with the analysis of variance data found in Appendix B.20. It was found that there were significant differences between the combined pH/ionic strength effects at the 99.62% level. However, when the Scheffe's test was applied to the data, it was found that only one pairwise comparison between the two media at pH 6.5 at 0.1M NaCl and pH 6.5 at 0.01M NaCl displayed significant differences at the 5% level. No significant differences were detected between the effects of pH or between the effects of the two ionic strengths used in the extractions (Appendix B.22 and B.23).

Despite a narrow range of temperatures over all conditions, the extraction medium found to best preserve a native structure (albeit not significantly overall) was that buffer adjusted to pH 6.5 at 0.1M NaCl. That buffer considered the poorest in preserving protein nativity (i.e.

Table 4.6 Effects of the Extraction Conditions on the Thermal Denaturation Temperature (Td in °C)[1] of the Canola Protein.

pH	0.01M NaCl[2]	0.1M NaCl[3]
5.5	88.31±0.03	87.87±0.99
6.0	87.92±0.25	88.21±0.65
6.5	87.18±0.49*	89.15±0.50*

[1] All values are expressed as thermal denaturation temperatures (Td) in °C.
[2] All buffers were prepared using a 0.01M Na_2HPO_4 buffer
[3] All buffers were prepared using a 0.1M Na_2HPO_4 buffer

Values followed by an asterisk are significantly different from one another at the 0.05 level.

* Scheffe's test on the combined effects of pH and ionic strength indicate that the 0.1M NaCl/0.1M Na_2HPO_4 at pH 6.5 treatment is not significantly different ($P \leq 0.05$) from the other treatments with the exception of 0.01M NaCl/0.01M Na_2HPO_4 at pH 6.5 (Appendix B.21)

lowest thermal denaturation temperature) was that prepared at pH 6.5 at 0.01M NaCl.

4.6.3 Enthalpy of Denaturation (△H)

The△H specifically describes the actual heat flow into the protein in the denaturation process. The greater the heat flow, the greater the state of nativity is known to exist in the protein before the heat treatment.

The effect of the various extraction treatments on the enthalpy of denaturation of the protein is detailed in Table 4.7. The analysis of variance is outlined in Appendix B.24. It was found that there were significant differences at the 98.05% level, however, a Scheffe's test did not reveal any significant differences between the effects of the various extraction buffers on the enthalpy of denaturation (Appendix B.25). That is, no significant differences between the combined pH/ionic strength treatments were detected in a Scheffe's test. As well, no significant differences were detected between the effects of pH alone, on the enthalpy of denaturation values. Significant differences were, however, detected at the 0.05 level between the ionic strengths used in terms of the enthalpy of denaturation values (Appendix B.27).

The highest recorded △H for this project was found to be 9.67 Joules/g protein for a sample extracted at pH 6.5 at 0.1M NaCl. The lowest △H ever found in this project was

Table 4.7 Effects of the Extraction Conditions on the Enthalpy of Denaturation (ΔH in J/g)[1] of the Canola Protein

pH	0.01M NaCl[2]	0.1M NaCl[3]
5.5	5.30 ± 0.33^a	5.69 ± 0.13^a
6.0	7.64 ± 0.20^a	6.54 ± 0.41^a
6.5	5.75 ± 1.69^a	8.23 ± 1.28^a

[1] All values are expressed are in Joules/g protein
[2] All buffers were prepared using a 0.01M Na_2HPO_4 buffer
[3] All buffers were prepared using a 0.1M Na_2HPO_4 buffer

Values followed by the same letter are not significantly different at the 0.05 level.

for a protein extracted at pH 6.5 at 0.01M NaCl and was calculated to be 3.56 Joules/g protein.

The entire range of enthalpy of denaturation values tabulated illustrates relatively high ΔH values, hence testifying to the mildness of the extraction process on the protein.

Graphically, the plotted thermal curves illustrating the heat flow into the protein and maximum temperature (^{o}C) bore similar trends throughout the entire screening series (Figures 4.10 through 4.15 illustrate this trend).

Figure 4.10 Thermal Curve of Canola Protein Extracted at 0.01M NaCl at pH 5.5.

Sample: CANOLA PROTEIN 5501-1-2
Size: 14.50 mg
Method: PROT-P
Comment: pH 5.5 0.01 M NaCL

DSC

File: CPE.02
Operator: B.WELSH
Run date: 11/02/87 22:31

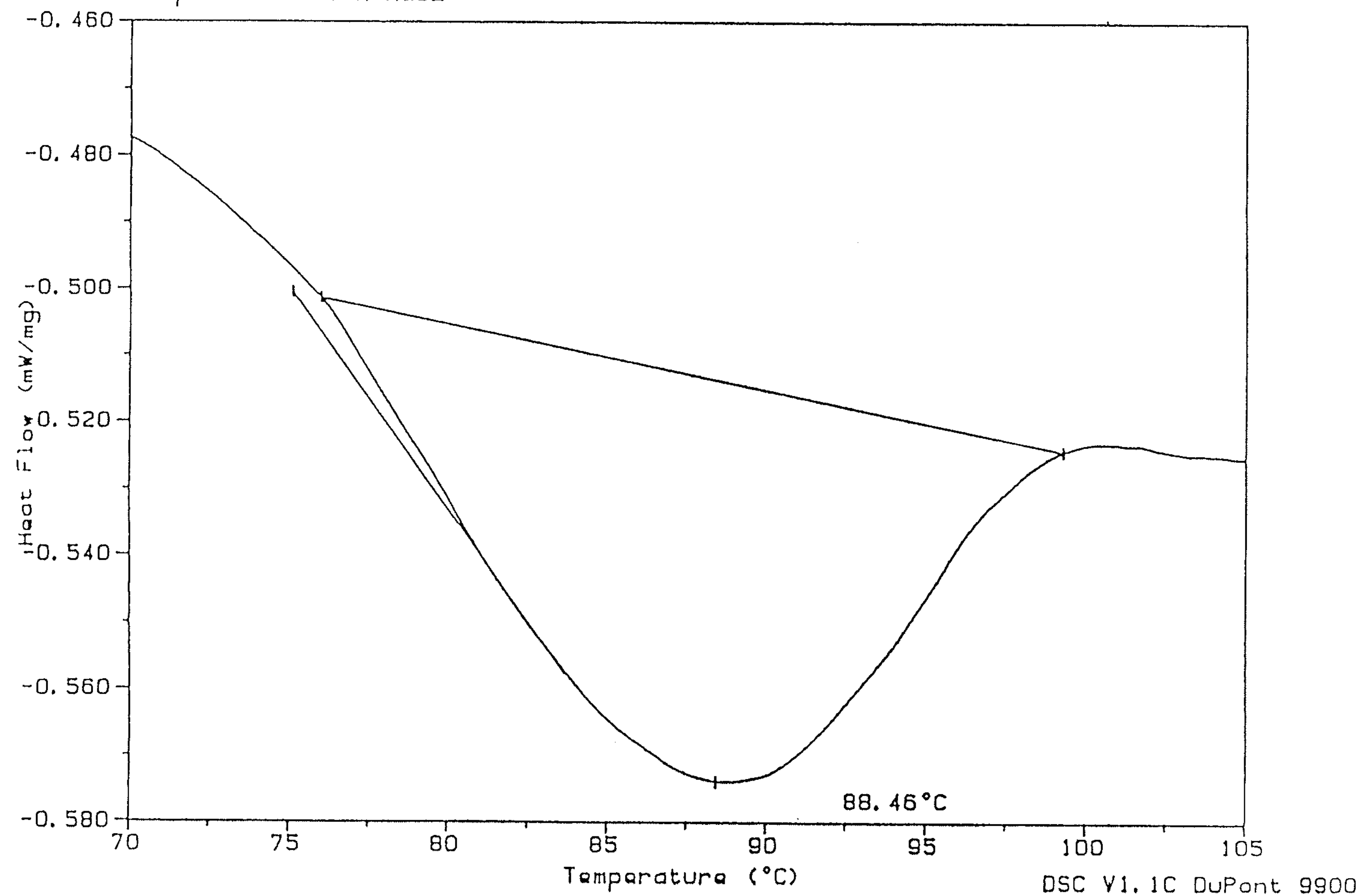


Figure 4.11 Thermal Curve of Canola Protein Extracted at 0.10M NaCl at pH 5.5.

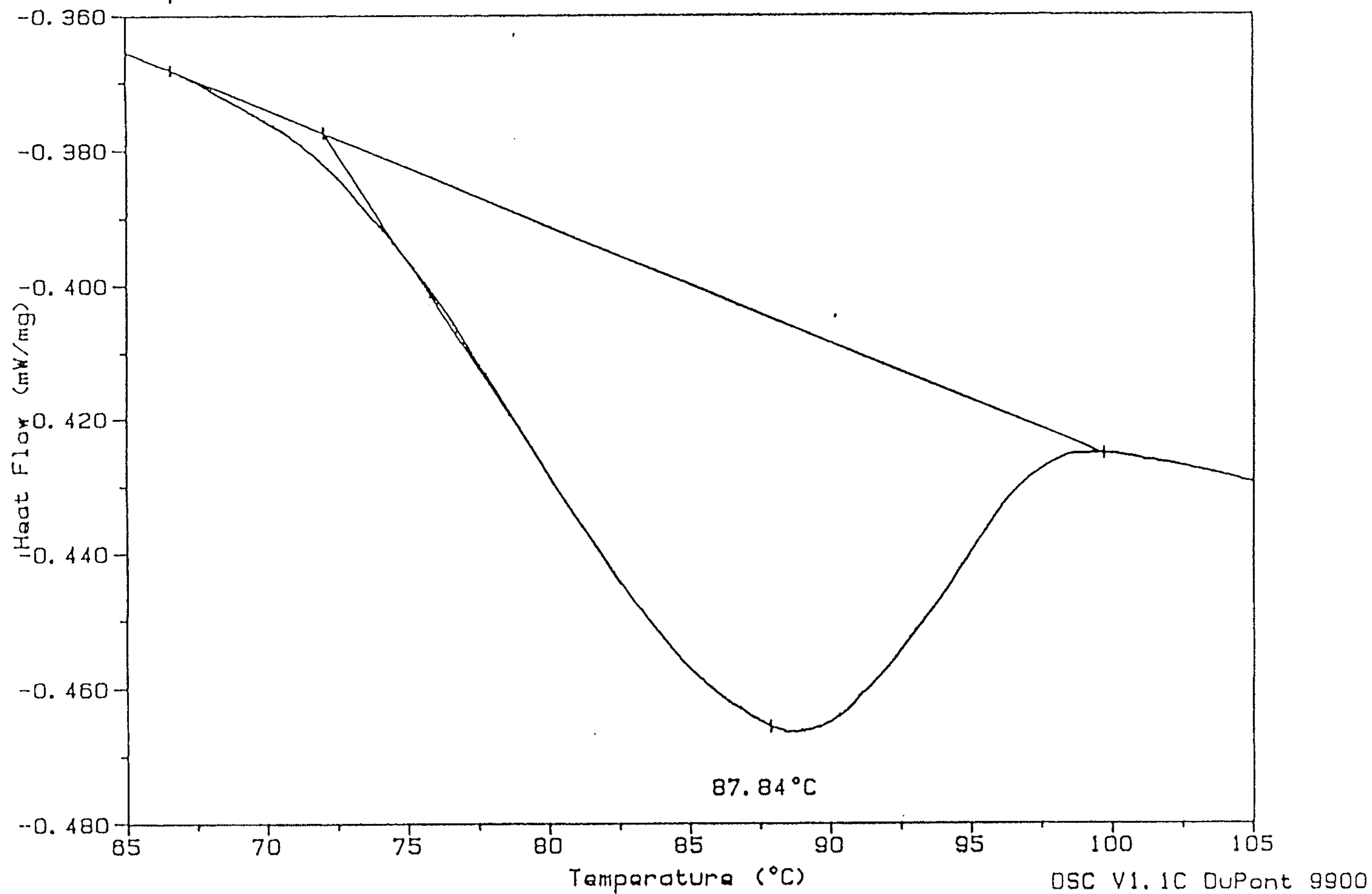

Sample: CANOLA PROTEIN 551-3-2
Size: 12.54 mg
Method: PROT-P
Comment: pH 5.5 0.1M NaCl
DSC
File: CPE.16
Operator: B.WELSH
Run date: 11/03/87 17:50
-0.360
-0.380
-0.400
-0.420
-0.440
-0.460
-0.480
Heat Flow (mW/mg)
87.84°C
65
70
75
80
85
90
95
100
105
Temperature (°C)
DSC V1.1C DuPont 9900

Figure 4.12 Thermal Curve of Canola Protein Extracted at 0.01M NaCl at pH 6.0.

Sample: CANOLA PROTEIN 6001-3-1
Size: 11.92 mg
Method: PROT-P
Comment: pH 6.0 0.01M NaCl

DSC

File: CPE.21
Operator: B.WELSH
Run date: 11/03/87 21:31

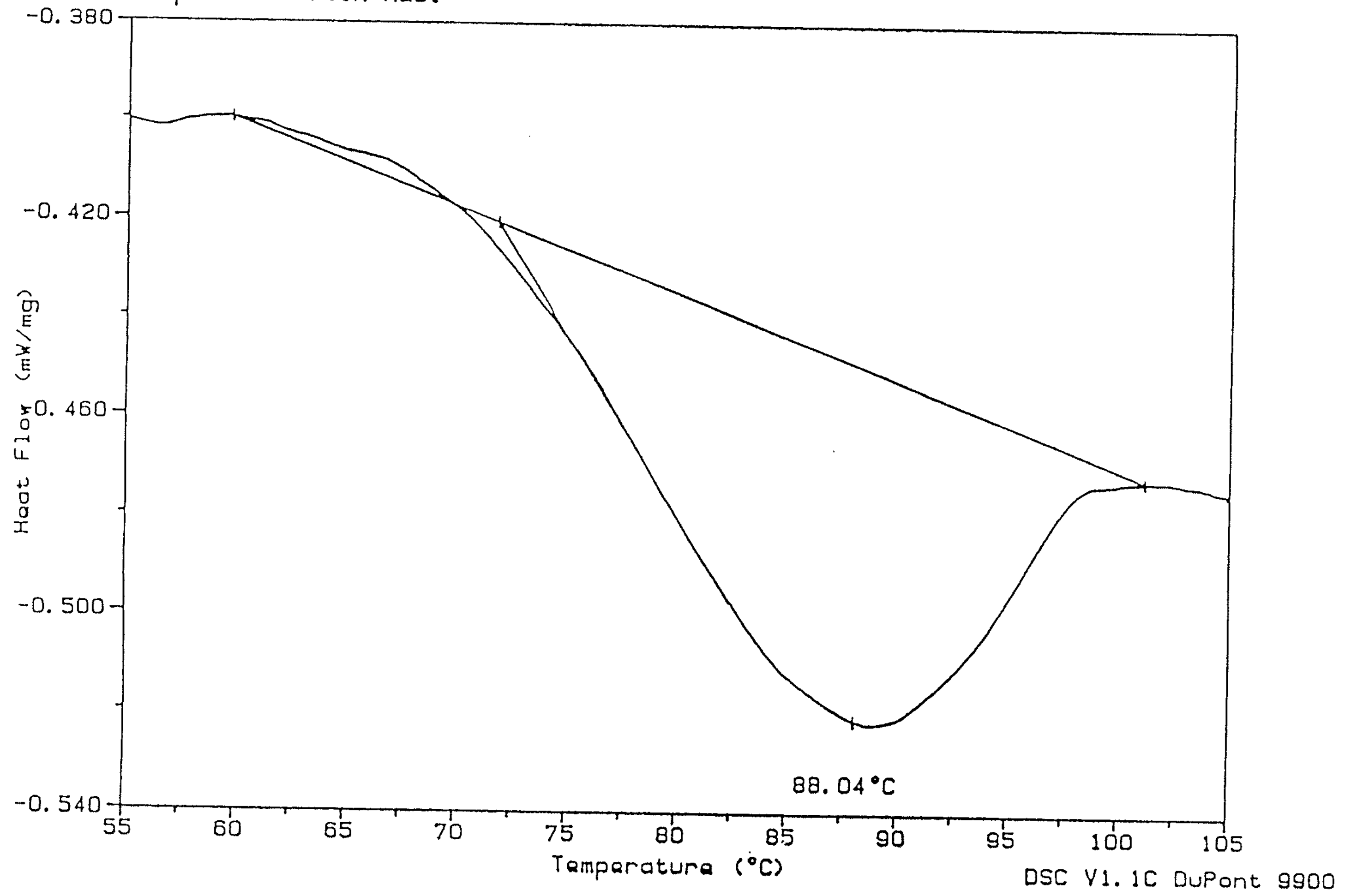
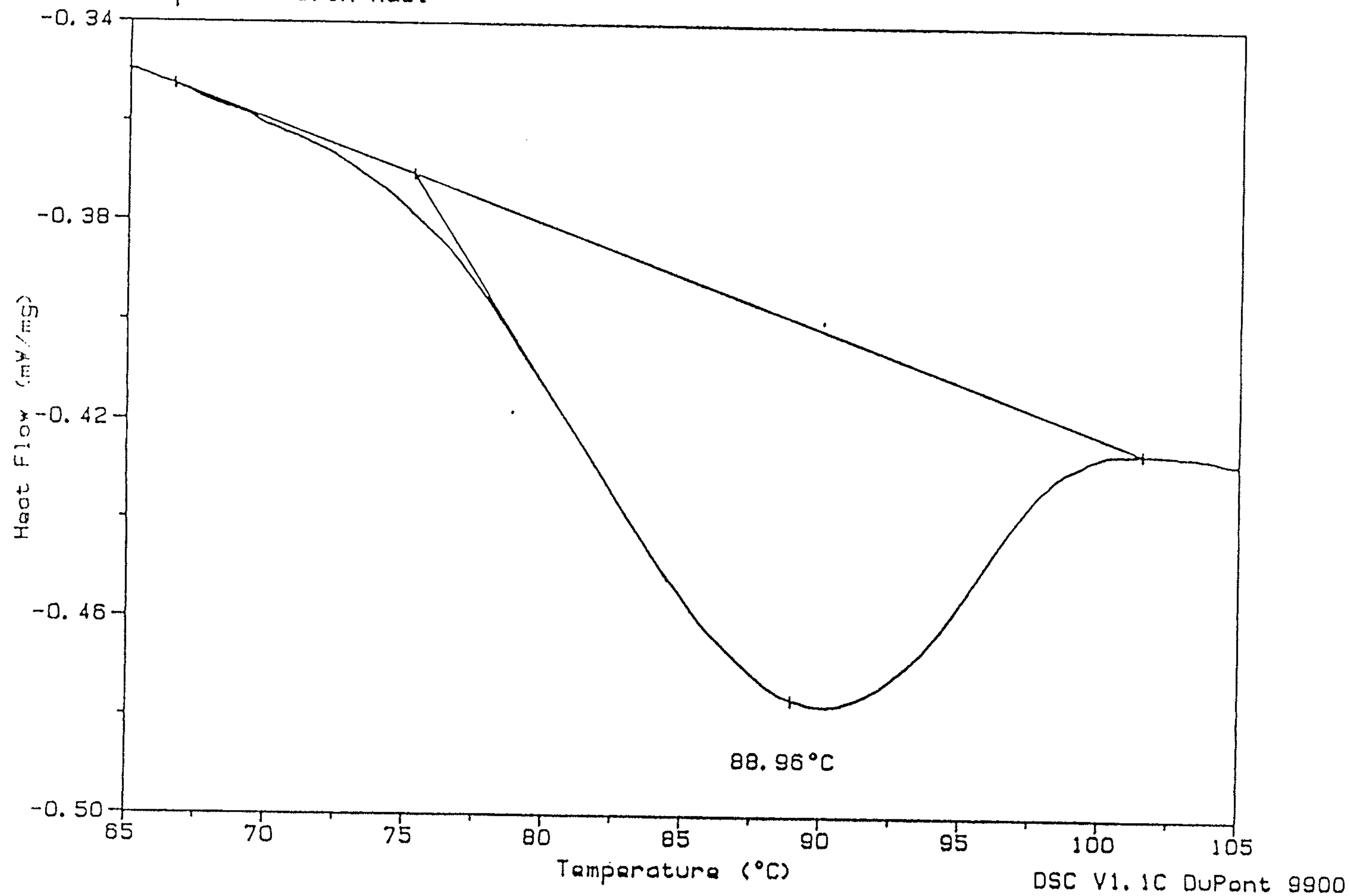
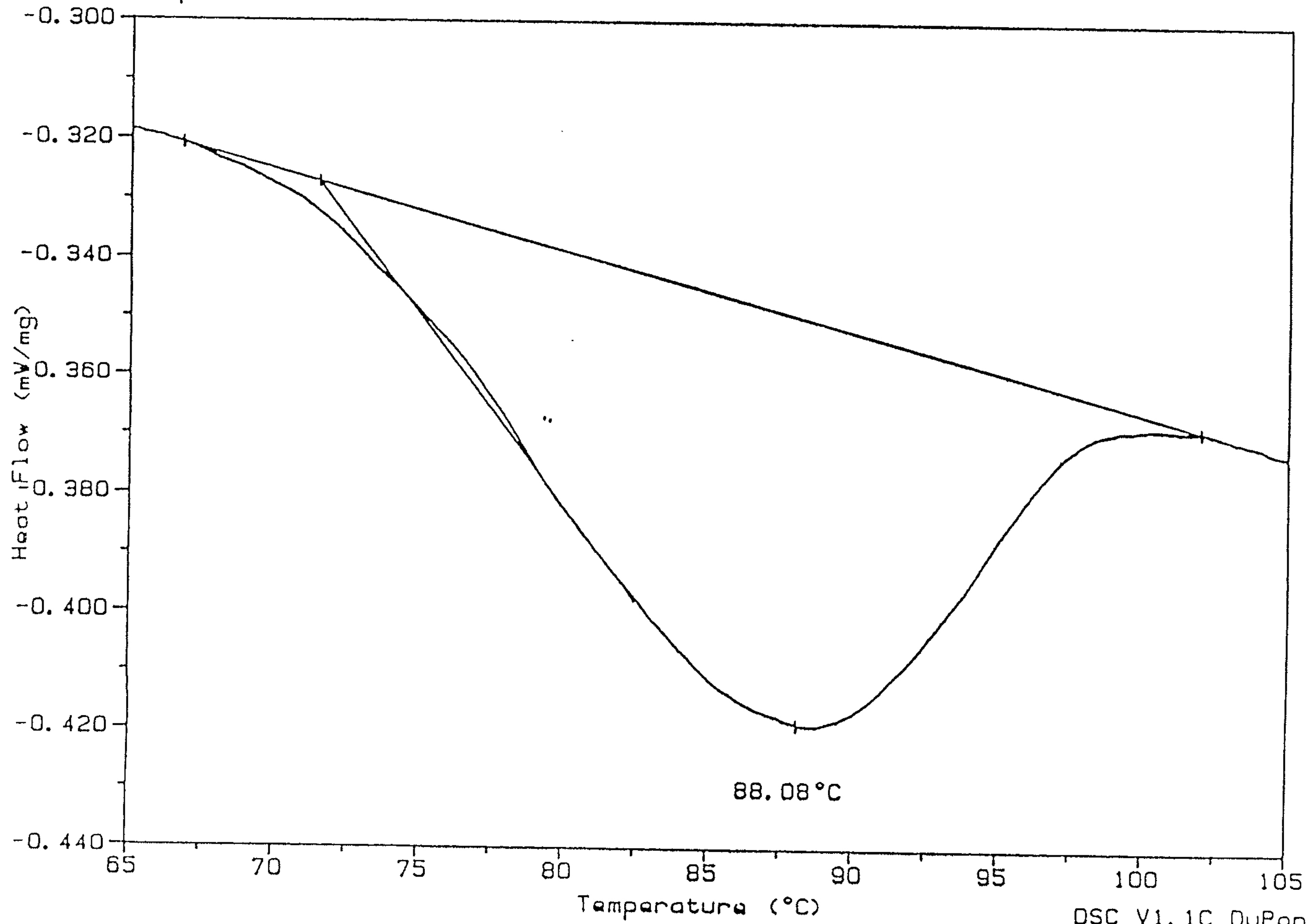


Figure 4.13 Thermal Curve of Canola Protein Extracted at 0.10M NaCl at pH 6.0.



Sample: CANOLA PROTEIN 601-2-2
Size: 11.17 mg
Method: PROT-P
Comment: pH 6.0 0.1M NaCl
DSC
File: CPE.26
Operator: B.WELSH
Run date: 11/04/87 00:36
-0.34
-0.38
-0.42
-0.46
-0.50
Heat Flow (mW/mg)
88.96°C
65
70
75
80
85
90
95
100
105
Temperature (°C)
DSC V1.1C DuPont 9900

Figure 4.14 Thermal Curve of Canola Protein Extracted at 0.01M NaCl at pH 6.5.



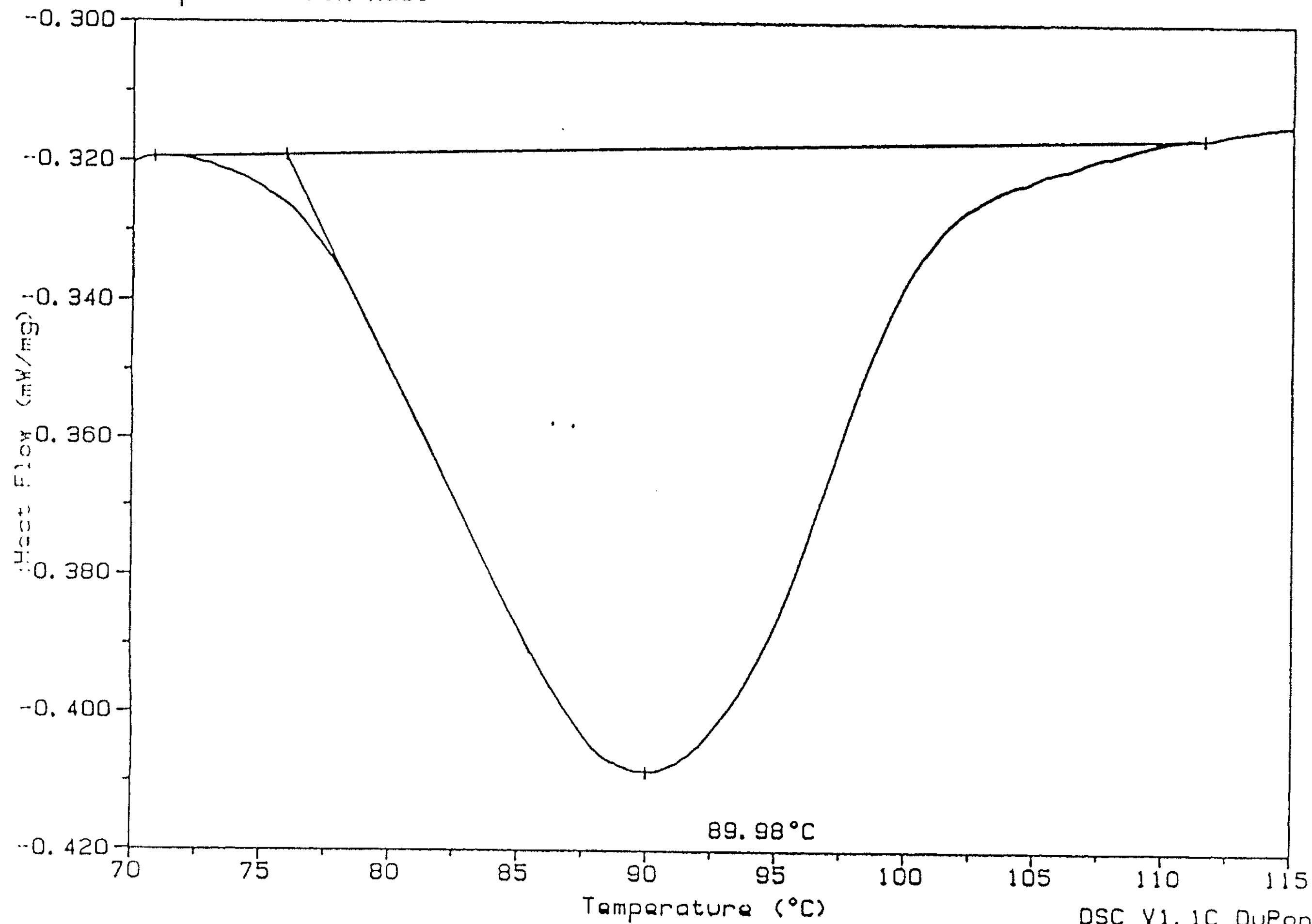
Sample: CANOLA PROTEIN 8501-2-1
Size: 10.03 mg
Method: PROT-P
Comment: pH 6.5 0.01M NaCl
DSC
File: CPE.31
Operator: B. WELSH
Run date: 11/04/87 10:06
-0.300
-0.320
-0.340
-0.360
-0.380
-0.400
-0.420
-0.440
Heat Flow (mV/mg)
88.08°C
65
70
75
80
85
90
95
100
105
Temperature (°C)
DSC V1.1C DuPont 9900

Figure 4.15 Thermal Curve of Canola Protein Extracted at 0.10M NaCl at pH 6.5.

Sample: CANOLA PROTEIN 651-1-2
Size: 12.96 mg
Method: PROT-P
Comment: pH 6.5 0.1M NaCl

DSC

File: CPE.36
Operator: B. WELSH
Run date: 11/04/87 21:38



DSC V1.1C DuPont 9900

4.7.1 Protein Analysis Using SDS-PAGE

Canola protein extracts from each of the six extraction conditions were studied in order to determine any effect the conditions might have on the protein subunit molecular weights. Using the standards and procedures previously outlined, the samples were applied to the gel and the subunits subsequently identified and calculated for their respective molecular weights.

It appeared that no significant differences existed between the extraction conditions with respect to the subunit molecular weights calculated for the proteins. Each protein separated into 4 bands on the gel (Figure 4.16) denoting 4 subunits. Table 4.8 lists the overall average weights for each subunit.

Table 4.8 Determination of Subunit Molecular Weights of Canola Protein Extract Using SDS-Page[1]

	pH 5.5		pH 6.0		pH 6.5	
	0.01M[2] /	0.1M[3]	0.01M[2] /	0.1M[3]	0.01M[2] /	0.1M[3]
gel band 1	48642 ±487	47863 ±0	47862 ±1	48699 ±558	47874 ±9	48978 ±0
gel band 2	30426 ±674	30567 ±1218	31787 ±439	30388 ±1053	31462 ±328	31623 ±0
gel band 3	28415 ±957	27870 ±829	28187 ±530	27714 ±960	28219 ±27	28512 ±379
gel band 4	18788 ±642	18310 ±635	19282 ±573	18409 ±245	19925 ±35	18947 ±217

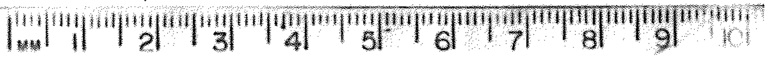
[1] Weights are expressed in daltons
[2] All buffers were prepared with 0.01M NaCl in 0.01M Na_2HPO_4
[3] All buffers were prepared with 0.1M NaCl in 0.1M Na_2NPO_4

Figure 4.16 **SAS-PAGE Analysis* of Canola Proteins Extracted Under the Following Conditions:**

2. 0.01M NaCl at pH 5.5
3. 0.10M NaCl at pH 5.5
4. 0.01M NaCl at pH 6.0
5. 0.10M NaCl at pH 6.0
6. 0.01M NaCl at pH 6.5
7. 0.10M NaCl at pH 6.5

* 1. Standards (lysozyme, trypsinogen, pepsin, β-lactoglobulin, ovalbumin, bovine serum albumin)

1
3
2
5
4
1
7
6
3
1
2
5
4
7
1
6
3
2
5



4.8.1 Analysis of the Canola Protein Using High Performance Liquid Chromatography/Gel Filtration

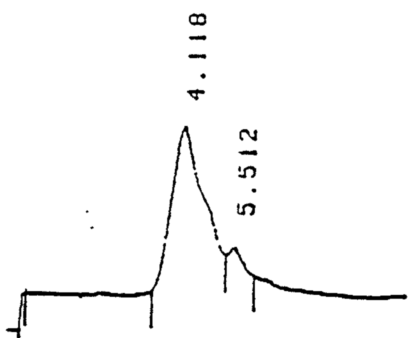
The purity of the protein was studied in order to determine the effects of the extraction processes on the protein molecular weight. Analysis of the protein using HPLC/gel filtration methodology as outlined previously was curtailed due to complications arising with the protein strongly binding to the size exclusion column used on the Waters HPLC. Washing the column of the protein proved to be most difficult.

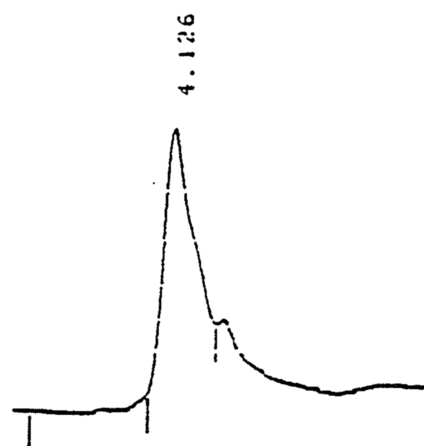
Samples representing two of the six extraction conditions were analyzed before suspension of the analysis. Protein molecular weights were calculated in accordance to protein elution profiles from the column (Figure 4.17 and Table 4.9).

Elution profiles consisted of a predominantly single peak followed by a smaller peak which was identified in one sample run. In the other elution profiles, this smaller shoulder-type peak was not assigned an elution time by the integrator, presumably due to its either existing as part of the larger, singular elution curve or the relatively minute amount of protein it represents.

The determined molecular weights were not equivalent to the theoretical weight of the 12S protein calculated at 294 000 to 300 000 daltons (Meith _et al._, 1983). The results suggested a dissociation of the proteins into their subunit

Figure 4.17 Calculation of Molecular Weights Based on Gel Filtration/HPLC Data





peak 1 (4.118 min)
- 43652 mw

peak 2 (5.512 min)
- 5495

peak 1 (4.126 min)
- 42658

Table 4.9 Analysis and Determination of Canola Protein Molecular Weight Using HPLC/Gel Filtration

Extraction Condition	Elution Time(s)	Molecular Weight
0.1M NaCl at pH 5.5	5.118 min. 5.512 min.	43652 daltons 5495 daltons
0.1M NaCl at pH 5.5	4.126 min.	42658 daltons
0.1M NaCl at pH 6.5	3.887 min.	60256 daltons
0.1M NaCl at pH 6.5	3.912 min.	58884 daltons

components. This was perhaps due to the buffer used to solubilize and prepare the canola protein for HPLC/gel filtration analysis.

5.0 DISCUSSION

5.1.1 The Protein Extraction Process

The methodology used to extract the protein from the raw canola meal was based on the protein micellar mass (PMM) method devised by Murray et al. (1981) as outlined previously.

The production of a solubilized protein according to the PMM procedure represented the classical salting-in process used to solubilize proteins. In this process, the use of salt solutions have been used to introduce non-specific electrostatic repulsions between the amino acids in order to solubilize the globulin which is otherwise insoluble in water. The use of salt hydrates the protein by creating similarly charged regions around the protein, hence inducing molecular stresses whereby hydrophobic interactions between hydrophobic amino acid residues are suppressed. Ordinarily, in an aqueous environment, these residues hydrophobically interact with one another, allowing the protein to retain its insoluble state. The similarly charged regions on the protein result in forces of electrostatic repulsion between the residues hence the molecular stresses on the protein which, in turn caused the solubilization of the protein.

The effects of pH have also been used to solubilize proteins. It is known that a protein will assume a net

positive or negative charge if the pH of the environment surrounding the protein is altered to an extreme acid or alkali condition respectively (Murray _et al_., 1978). The protein's assumption of a negative or positive charge results from the ionization of the protein's reactive groups in the extreme pH conditions. Subsequently, severe molecular stresses may occur within the protein structure characterized by the forces of repulsion existing between similarly charged reactive groups on the protein. These repulsions aid in protein solubilization by suppressing hydrophobic interactions between the residues (and other proteins) by not allowing the molecules to come sufficiently close together (Murray _et al_., 1981).

The protein is precipitated by the addition of cold distilled water. The precipitation represents the minimizing of the forces of electrostatic repulsion by the ensuing larger forces of hydrophobic interactions between the hydrophobic amino acid residues. In other words, the addition of water reduces the salt concentration that electrostatically sustains the protein in a soluble state. The increased presence of water permits hydrophobic interactions between hydrophobic residues (i.e. water avoidance phenomena) resulting in the formation of microscopically discreet spheres that will bind to a protein-binding dye such as Ponceau 2R. This proteinaceous spherical structure has been referred to as a micelle while

the "salting-in/hydrophobic out" process has been called the protein micellar mass (PMM) process (Murray et al., 1981).

The variation of the PMM procedure developed for the extraction of the canola protein followed the same above-mentioned principles developed for the extraction of protein from other plant sources (Murray et al., 1981; Arntfield et al., 1985; Ismond et al., 1986b).

The extractions were carried out at 0.1M and 0.01M NaCl over pH 5.5, 6.0, and 6.5. The purpose of this particular approach was to screen that particular ionic strength/pH condition that optimized protein extraction and antinutritional removal while minimizing denaturation of the protein.

The results of the varying pH and saline combinations are outlined in Table 4.1. In this table, the greater protein yields are evidenced from those extracts produced using 0.1M NaCl in the phosphate buffer. This result testifies to the increased "salting-in" of the protein using higher levels of NaCl. The effects of pH did not seem to contribute greatly to extraction yields. The possibility exists, however, that the effects of pH could have been masked by the changes in ionic strength or the combination effects of ionic strength and pH.

Kjeldahl analysis was conducted to measure the amount of protein in the extracted samples.

Statistical analysis of the effects of the pH and ionic strength combinations revealed that no significant differences existed between the six conditions in terms of combined pH/ionic strength effect on % protein in the extracts. As well, it was found that no significant differences existed between the two ionic strength conditions on % protein extracted when considered separately. However, it was found that significant differences existed between the effects of pH on % protein in the extracts. The comparisons of pH effects are found in Appendix B.6.

The protein extracts produced at pH 6.0 at 0.01m NaCl were found to have produced the highest % protein of the six conditions as illustrated by the standardized mean response comparison stars (Figures 4.1-4.6). However, as previously mentioned, no significant differences were found to exist between the pH/ionic strength combinations on the % protein. While the singular effects of pH on % protein content in the extracts have displayed some significant differences, to ascertain as to which singular pH (or singular ionic strength buffer) is best suited to optimize a particular (or several) response variable is difficult due to possible masking effects on, for example, the % protein extracted, by the combined pH and ionic strength conditions. In order to determine the singular effects of pH and ionic strength, rather than the combined pH and ionic strength effects on

the response variables, pH-ionic strength interactions must be statistically considered. If the condition interactions are not significantly different from one another, the singular pH and ionic strength effects may be considered to determine significant differences between pH levels and significant differences between ionic strengths used.

In terms of pH, there were no significant differences between pH 5.5 and pH 6.0 effects on % protein in the extract. The increase in acidity represents the assumption of an increased positive charge on the protein, therefore increasing molecular repulsion and consequently solubilization of the protein.

5.2.1 A Removal of Antinutritional Factors -Phytic Acid

The PMM process has been shown to remove phytic acid from plant protein (Murray et al., 1981; Arntfield et al., 1985). As mentioned previously, three different phytate-protein relationships have been determined depending on the pH of the environment (Cheryan, 1980; Arntfield et al., 1985). Electrostatic interaction between the phytic acid and protein exists at pH environments below the isoelectric point of the protein. Above the isoelectric point but below pH 11, a ternary complex exists whereby the phytate and protein are linked through a divalent cation such as Zn^{2+} or Ca^{2+}. The stability of the complex increases with increasing pH up to pH 10. Above pH 11, there does not seem

to be any evidence of interactions existing between phytic acid and protein.

Extraction of the canola protein at pH 5.5 using a 0.1M NaCl/0.1M Na_2HPO_4 buffer resulted in a 75.5% removal of phytic acid (Figure 4.7). Of the six extraction conditions used, this medium was found to have been the most effective at removing the phytic acid from the meal protein. This correlates with the findings of other researchers where phytate removal from canola/rapeseed protein has been best effected at around pH 5 (Cervenkova et al., 1983; Youngs, 1985).

The isoelectric point of the 12S storage globulin protein is 7.2. Subjecting the meal to an extraction environment of pH 5.5 would classify the phytate-protein relationship as electrostatic in nature. It would appear that the extraction medium prepared at pH 5.5 with 0.1M NaCl/0.1M Na_2HPO_4 has disrupted the electrostatic interactions between the protein and phytate the greatest of the six extraction buffers used. As well, the extraction environment adjusted to pH 5.5 was adequate in terms of minimizing direct electrostatic interactions between the protein and phytate and was not sufficiently alkaline to promote the formation of a ternary complex. Therefore the bonding of the phytate to the protein was weakened. Upon dilution with cold water, the driving forces of hydrophobic interaction were strong enough to disrupt the phytate-

protein linkages. In a manner similar to previous extractions of protein from fababeans (Arntfield et al., 1985), the phytic acid remained soluble while the protein precipitated, hence, effecting phytate removal.

5.2.2 B Removal of Antinutritional Factors - Phenolic Compounds

As mentioned previously, the presence of phenolic compounds in proteins may result in protein-phenolic interactions that are undesirable due to a bitter taste being imparted to the protein. As well, the presence of phenolic compounds, particularly tannins, may reduce protein digestibility and possibly the bioactivity of other nutrients (Ford and Hewitt, 1979).

The mechanisms of interaction may involve hydrogen bonding, ionic bonding, and following oxidation to o-quinones, covalent bonding. As well, hydrophobic interactions between phenolic compounds and proteins are of significance. It has been shown that hydrophobic interactions are mainly responsible for this association (Arntfield et al., 1985; Oh et al., 1980; Oh and Hoff, 1987).

In the PMM process, many of the phenolic compounds associated with the non-proteinaceous components (hulls and other seed remnants) are removed after the first centrifugation step. Findings by Arntfield et al., (1985) have concluded that the use of relatively mild extraction

conditions would not be sufficient to overcome hydrogen bonding associations between phenolic compounds and protein. This is reinforced by a 96.5% reduction of phenolic compounds in fababean protein isolates (possessing 3.5% phenolic compounds of those present in the starting material) using the PMM process (Arntfield et al., 1985). Arntfield suggests that the presence of salt (in the extraction medium) could reduce potential hydrophobic associations by bonding to the proteins and creating an electrostatic double layer. This results in an electrostatic repulsion phenomenon which would inhibit hydrophobic interactions between the phenolic compounds and the protein. Given this situation, the phenolic compounds in the extract (undiluted) would be soluble rather than protein-bound. Upon dilution of the solubilized protein, the impending protein-protein hydrophobic interactions are favoured over any possible protein-phenolic interactions, hence protein precipitation. This "hydrophobic favoritism" is perhaps, as Arntfield suggests, due to the close proximity of the hydrophobic side chains on the proteins. In isolating fababean protein, it was found that phenolic compounds had been reduced to less than 0.2% of that in the original starting material.

The extraction buffer that best removed the phenolic compounds from the canola protein was that buffer prepared with 0.1M NaCl/0.01M Na_2HPO_4 at pH 5.5. This buffer was

significantly different (at the 0.05 level) from the remaining five extraction buffers (Table 4.6) and was found to reduce the total phenolic compounds in the protein extract by 85.3% upon dilution with cold distilled water (Figure 4.9). Exclusion of phenols during the PMM process supports the theory that hydrophobic associations are mainly responsible for the interactions between proteins and phenolic compounds (Oh et al., 1980).

It was apparent that some phenolic compounds were not removed from the protein as the freeze-dried extract was a light brown colour in appearance. This may suggest that a somewhat stronger form of protein-phenolic compound association may also exist besides the more predominantly hydrophobic associations. Such an association may be due to hydrogen bonding; an interaction not readily disrupted in the mentioned relatively mild extraction conditions.

5.2.3 C Removal of Antinutritional Factors - Glucosinolates

The glucosinolates are distributed diffusely in the parenchymal tissues of the canola seed (Appelqvist and Ohlson, 1972). The glucosinolates are readily soluble in water with some of the glucosinolates, most notably the oxazolidinethiones, being actively hydrophilic (Appelqvist and Ohlson, 1972).

Maximum glucosinolate removal from the canola protein was effected by that extraction buffer prepared with 0.01M

NaCl/0.01 Na_2HPO_4 at pH 5.5 (Figure 4.1) With this buffer 3.8% glucosinolates remained in the extract (i.e. 96.2% removal). However, in considering the fact that none of the extraction media were significantly different (at the 0.05 level), it may be implied that all of the buffers lowered the glucosinolates to a great extent (Table 4.5). That buffer prepared with 0.1M NaCl/0.1M Na_2HPO_4 at pH 5.5 was found, through the PMM process, to have reduced the glucosinolate content by 92.5% (Figure 4.8), leaving a residual glucosinolate content of 0.61 μmol glucosinolates g^{-1} protein extract.

Dilution of the solubilized protein (in the form of a high salt protein extract or HSPE) results in hydrophobic interactions between the proteins which in turn accounts for the precipitation of the protein. In this environment, the glucosinolates are readily soluble in the water used to promote hydrophobic interaction. The glucosinolates are therefore separated from the protein.

5.3.1 Protein Nativity

Changes in secondary, tertiary or quaternary structure of a protein molecule are referred to as denaturation. In plant or food proteins, these changes stand to adversely affect the functionality or performance and therefore their applicability in food systems. Heat, organic solvents, acid, alkali, detergents, urea and guanidine hydrochloride

are some of the factors that may be responsible for denaturation of the protein (Arntfield and Murray, 1981).

As mentioned previously, extraction of the protein from rapeseed and canola has been conducted by other researchers using acid solubilization and alkali precipitation of the protein. Often, this has resulted in a protein that has not been satisfactorily functional in a food system. This would imply that the extraction methodologies used in these experiments have sufficiently denatured the protein thus preventing its effectiveness in a food system.

The use of differential scanning cabrimetry (DSC) to study the thermal properties of protein has been used extensively in the past (Arntfield and Murray, 1981). Degrees of protein denaturation may be plotted by the thermal curve (endotherm) generated by heat flow into the protein (Figures 4.10 to 4.15). Analysis by DSC can also detect differences in proteins from the same plant source that have undergone different treatments or extraction conditions. The lack of a discernable endotherm for an extracted protein indicates decreased heat flow into the protein. This suggests that denaturation of the protein has occurred. The sensitivity of the protein to denaturing effects of extraction is quantitated through the temperature of denaturation (Td) and the enthalpy of denaturation (ΔH) values. Increased ΔH values denote lack of protein

denaturation hence suggesting suitable functionality of the protein in a food system.

The thermal curves detailed in figures 4.10 to 4.15 represent the thermal transitions of the canola protein that has been extracted under the six buffers mentioned previously. The thermal denaturation temperatures (Td) ranged from 87.87^{o}C to 89.15^{o}C, thus illustrating a narrow Td range over the six conditions used to extract the protein from the meal. This temperature range is detailed in Table 4.6.

It appears from Table 4.7 that the nativity of the canola protein has not been damaged by any of the six extraction conditions. The buffers are not significantly different from one another in terms of the enthalpy of denaturation values calculated for protein extracted with each buffer. The relatively large ΔH values testify to the mildness of the PMM process on the native state of the extracted protein. This finding is essentially very similar to the findings of other researchers where proteins from other plant sources such as soybeans and fababeans have been isolated using the PMM process (Arntfield and Murray, 1981). In these extractions, DSC analysis revealed the preservation of the native state for the isolated protein.

5.4.1 Protein Structure and Purity

The subunit structure of the extracted canola protein was studied using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In the presence of the detergent SDS, the noncovalently linked subunits of the proteins dissociate into individual subunits. The subunits undergo denaturation, changing from an ordered three-dimensional form to a random coil configuration. In the denaturing environment, each protein possesses a rod-like shape. The use of SDS also eliminates the factor of charge difference which may be used to separate proteins. It accomplishes this by binding to each protein molecule which introduces one negative charge per bound molecule of SDS on the protein. Thus, at neutral pH, the total charge of the protein-SDS complex is largely dependent on the charge of the SDS molecules. The charge per unit mass is constant, therefore, the electrophoretic mobility of the complex is dependent mostly on the molecular weight of the protein. Because the factor of charge difference has already been eliminated at this point, the subunits are separated on the basis of size through the sieving effect in the gel matrix rather than on the basis of charge differential. In addition to the SDS detergent, mercaptoethanol is used to disrupt any covalent disulphide linkages between the subunits.

From Figure 4.16 and Table 4.8, it may be seen that four protein bands have been separated. Over the six extraction conditions there were no differences in the molecular weights calculated for each of the bands. This is especially true when the standard deviation ranges are considered. The bands formed corresponded with those values calculated for the constituent polypeptide chains of the protein by other researchers (Tables 2.2 and 2.3) (Mieth *et al.*, 1983; Schwenke *et al.*, 1983).

The purity of the protein was studied by high performance liquid chromatography/gel filtration analysis as well as isoelectric focusing. In preparing the protein for these analyses, it was found that the protein was not very soluble in the buffers intended to resolubilize the protein. It was initially thought that a resolubilization was difficult due to some partial denaturation of the protein from the freeze-drying process. However, it was found that resolubilization of a non-freeze dried protein was just as difficult. This could have been due to very strong electrostatic interactions within the protein that may occur after the extraction stage, thus resisting resolubilization.

Because of the harsh conditions used to partly resolubilize the protein, the HPLC/gel filtration revealed proteins of 40,000-60,000 daltons with a smaller protein "shoulder" denoting a protein of approximately 5,500 daltons (Figure 4.17 and Table 4.9). This is not the expected 12S

weight of approximately 300,000 daltons. This suggests the possibility of protein dissociation in preparing the sample for HPLC/gel filtration analyses. Analyses were curtailed, as it was found that the protein in question was binding excessively to the size exclusion column which made the washing of the protein from the column extremely difficult.

Analysis of the protein with isoelectric focusing (IEF) for purity assessment was unsuccessful. In this situation, the protein was applied to the PAG plate in solubilized form. When the current began to flow throughout the gel, ions keeping the protein in the soluble state migrated away from the protein, allowing the protein to precipitate on the PAG plate, preventing its migration towards its corresponding isoelectric point (pI) on the surface of the plate. In this light, analysis of the protein using IEF was discontinued.

6.0 CONCLUSIONS AND RECOMMENDATIONS

It has been determined that extraction of the protein from canola meal using the PMM approach at 0.1M NaCl at pH 5.5 provides a medium in which the protein is extracted gently. As well, this medium was found to have best removed the compounds and the phytate from the protein of the six extraction buffers used. Having removed the protein using this mild approach, some avenues of direction may be taken to further develop this protein for the food industry.

Although the antinutritional factors have been largely reduced and in some cases, almost entirely removed, complete removal of these factors should be addresed. Such a study might involve the consideration of possibly more than one type of binding force existing between an antinutritional factor and the protein. Such considerations may provide explanations for the existence of residual antinutritional factors in the protein extract. This approach may be especially relevant to the residual phytate and phenolic compounds content in the protein. A phytate-free, white flour-like protein extract, similar in appearance to wheat flour, would provide greater versatility to the food industry. Production of such a flour could involve the use of a phytase enzyme in conjunction with an activated carbon filtration treatment of the solubilized protein at the appropriate stage of the PMM process.

Various food industries, in particular those industries involved in the manufacture of baby foods are currently using protein from other plant sources in their products. This specialized area of the industry is one of the largest targets for canola protein application. For this reason, studies concerning the canola protein's performance in a food system should be considered through both sensory/nutrition and rheological modes of analyses.

The increased popularity of canola oil in the United States promises a positive outlook in the future for the canola crop in Canada. Development and subsequent large-scale production of canola protein would provide a protein that is native and possesses a well-rounded amino acid composition for use in the Canadian food industry. This protein would also compete strongly with imported soybean protein in the industry.

The development of the protein and the existing polysaccharides from defatted canola meal would increase the overall value of the canola crop in both the Canadian and American markets, in addition to the production of canola oil.

The canola crop has reportedly displayed a certain degree of hardiness in drought-oriented conditions. This characteristic, coupled with the emergence of other nations as wheat producers indicate a strong potential for the uses of the products of the canola crop.

Appendix A: Linear Regression Analysis of SDS-PAGE Data

1) Calculation of Relative Mobility (Rf).

Rf =

$$\frac{\text{distance of protein migration}}{\text{distance of dye migration}} \times \frac{\text{length before drying}}{\text{length after preserving}}$$

(all distances and lengths in mm)

2) Standards

Standard	Molecular weight (daltons)	Log_{10} Molecular Weight	Rf value
BSA	66 000	4.82	0.31
Ovalbumin	45 000	4.65	0.51
Pepsin	34 700	4.54	0.61
Trypsinogen	24 000	4.38	0.74
- Lactoglobulin	18 400	4.27	0.92
Lysozyme	14 000	4.16	1.03

Appendix A: Linear Regression Analysis of SDS-PAGE Data (continued)

3) Linear Regression Analysis

Relative mobilities (Rf) of standards were plotted against the logarithm of the standard molecular weights on a Texas Instruments Programmable 59 calculator with a Texas Instruments PC-100A integrator/printer to obtain a standard curve. The following equation was calculated:

y-intercept = 5.493
slope = -1.075
correlation coefficient = -0.996.
$y = -1.075\ x + 5.493$
$x = (5.493 - y)/1.075$

Logarithms of sample weights are calculated by substitution of sample Rf values for y in equation. Sample weights (in daltons) are calculated by determining the antilogarithm of x.

Appendix B.1 Analysis of Variance of Protein Micellar Mass (PMM) Extraction Process

Treatments	df	SS	MS	F
Model	5	42.772	8.554	28.26
Error	24	7.265	0.303	PR>F
Corrected Total	29	50.037		0.0001
pH/Ionic Strength Pairwise Comparison	5	42.772	8.554	28.26**
pH 2	0.279			0.46**
Ionic Strength	1	41.395		136.74*
pH-Ionic Strength Interactions	2	1.098		1.81**

* significant at 0.05 level

** not significant at 0.05 level

Appendix B.2 Scheffe's Test for Pellet Extraction from Canola Meal (Effects of pH)

(Means with the same letter are not significantly different at the 0.05 level)

Scheffe Grouping	Mean	N	pH
A	2.9080	10	6.0
A	2.8720	10	6.5
A	2.6880	10	5.5

Appendix B.3 Scheffe's Test for Pellet Extraction from Canola Meal (Effects of Ionic Strength)

(Means with the same letter are not significantly different at the 0.05 level)

Scheffe Grouping	Mean	N	Ionic Strength
A	3.9973	15	0.1M
B	1.6480	15	0.01M

Appendix B.4 Analysis of Variance of Kjeldahl Analysis

Treatments	df	SS	MS	F
Model	5	0.988	0.197	8.10
Error	4	0.098	0.024	PR>F
Corrected Total	9	1.086		0.0322
pH	2	0.722		14.78*
Ionic Strength	1	0.155		6.34**
pH-Ionic Strength Interactions	2	0.105		2.16**

* significant at 0.05 level

** not significant at 0.05 level

Appendix B.5 Scheffe's Test for Kjeldahl Analysis (Combined pH/ionic strength effects)

Comparisons significant at 0.05 Level are indicated by "***")

pH/ionic strength (M) comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.0/0.01-5.5/0.10	-9.707	0.998	11.704
6.0/0.01-5.5/0.01	-7.400	3.306	14.011
6.0/0.01-6.0/0.10	-7.070	3.635	14.341
6.0/0.01-6.5/0.10	-6.671	4.892	16.455
6.0/0.01-6.5/0.01	-3.753	6.952	17.658
5.5/0.10-5.5/0.01	-8.398	2.308	13.013
5.5/0.10-6.0/0.10	-8.068	2.637	13.342
5.5/0.10-6.5/0.10	-7.670	3.894	15.457
5.5/0.10-6.5/0.01	-4.751	5.954	16.660
5.5/0.01-6.0/0.10	-10.376	0.329	11.035
5.5/0.01-6.5/0.10	-9.977	1.586	13.149
5.5/0.01-6.5/0.01	-7.059	3.647	14.352
6.0/0.10-6.5/0.10	-10.307	1.257	12.820
6.0/0.10-6.5/0.01	-7.388	3.317	14.023
6.5/0.10-6.5/0.01	-9.503	2.060	13.624

Appendix B.6 Scheffe's Test for Kjeldahl Analysis (Effects of pH)

(Comparisons significant at the 0.05 level are indicated by "***)"

pH comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.5 - 6.0	0.0169	0.5211	1.0253***
6.5 - 5.5	0.1490	0.5607	0.9724***
6.0 - 5.5	-0.4646	0.0396	0.5438

Appendix B.7 Scheffe's Test for Kjeldahl Analysis (Effects of Ionic Strength)

(Means with the same letter are not significantly different at the 0.05 level)

Scheffe Grouping	Mean	N	Ionic Strength
A	1.08350	5	0.01M
A	0.82939	5	0.10M

Appendix B.8 Analysis of Variance of Phytates

Treatments	df	SS	MS	F
Model	5	2.964	0.592	460.87
Error	16	0.021		PR>F
Corrected Total	21	2.984		0.0001
pH-Ionic Strength pairwise comparison	5	2.964		460.87*
pH	2	1.914		743.91*
Ionic Strength	1	0.749		582.14*
pH-Ionic Strength Interactions	2	0.212		82.27*

* significant at 0.05 level

Appendix B.9 **Multiple Extraction Condition Effect Comparison on Phytic Acid Content in Canola Meal Protein[a] (Scheffe's Test)**

pH/Ionic Strength (M) comparison	simultaneous lower-confid-ence limit	difference between means	simultaneous upper confid-ence limit	
5.5/0.01-6.0/0.01	-0.02809	0.06767	0.16344	
5.5/0.01-6.0/0.10	0.27488	0.37065	0.46642	***
5.5/0.01-5.5/0.10	0.54518	0.64095	0.73672	***
6.0/0.01-6.0/0.10	0.20721	0.30297	0.39874	***
6.0/0.01-5.5/0.10	0.47751	0.57327	0.66904	***
6.0/0.10-5.5/0.10	0.17453	0.27030	0.36607	***
6.5/0.01-6.5/0.10	0.06258	0.17317	0.28375	***
6.5/0.01-5.5/0.01	0.36840	0.47184	0.57528	***
6.5/0.01-6.0/0.01	0.43607	0.53952	0.64296	***
6.5/0.01-6.0/0.10	0.73905	0.34249	0.94593	***
6.5/0.01-5.5/0.10	1.00935	1.11279	1.21623	***
6.5/0.10-5.5/0.01	0.19523	0.29867	0.40212	***
6.5/0.10-6.0/0.01	0.26291	0.36635	0.46979	***
6.5/0.10-6.0/0.10	0.56588	0.66932	0.77277	***
6.5/0.10-5.5/0.10	0.83613	0.93962	1.04307	***

a) comparisons significant at the 5% level are indicated by ***

Appendix B.10 Scheffe's Test for Phytate Analysis (Effects of pH)

(Comparisons significant at the 0.05 level are indicated by "***")

pH comparison	simultaneous lower-confid-ence limit	difference between means	simultaneous upper confid-ence limit	
6.5 - 6.0	0.55221	0.60442	0.65663	***
6.5 - 5.5	0.65352	0.70573	0.75793	***
6.0 - 5.5	0.05297	0.10131	0.14965	***

Appendix B.11 Scheffe's Test for Phytate Analysis (Effects of Ionic Strength)

(Means with the same letter are not significantly different at the 0.05 level)

Scheffe Grouping	Mean	N	Ionic Strength
A	1.08710	11	0.01M
A	0.69663	11	0.10M

Appendix B.12 Analysis of Variance of Analysis of Glucosinolates

Treatments	df	SS	MS	F	
Model	5	0.319	0.064	1.90	
Error	17	0.570	0.034	PR>F	
Corrected	22	0.889			
pH-Ionic Strength pairwise comparison	5	0.319		1.90	**
pH	2	0.018		0.27	**
Ionic Strength	1	0.226		6.75	*
pH-Ionic Strength Interactions	2	0.055		0.82	**

* significant at 0.05 level

** not significant at 0.05 level

Appendix B.13 Scheffe's Test for the Analysis of Glucosinolates (Combined Effects of pH and Ionic Strength)

(Comparisons significant at the 0.05 level are indicated by "***")

pH/Ionic Strength (M) comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.0/0.10-5.5/0.10	-.4727	0.0125	0.4977
6.0/0.10-6.5/0.10	-0.3649	0.1592	0.6833
6.0/0.10-6.5/0.01	-0.2652	0.2200	0.7052
6.0/0.10-6.0/0.01	-0.2452	0.2400	0.7252
6.0/0.10-5.5/0.01	-0.1752	0.3100	0.7952
5.5/0.10-6.5/0.10	-0.3774	0.1467	0.6708
5.5/0.10-6.5/0.01	-0.2777	0.2075	0.6927
5.5/0.10-6.0/0.01	-0.2577	0.2275	0.7127
5.5/0.10-5.5/0.01	-0.1877	0.2975	0.7827
6.5/0.10-6.5/0.01	-0.4633	0.0608	0.5849
6.5/0.10-6.0/0.01	-0.4433	0.0808	0.6049
6.5/0.10-5.5/0.01	-0.3733	0.1508	0.6749
6.5/0.01-6.0/0.01	-0.4652	0.0200	0.5052
6.5/0.01-5.5/0.01	0.3952	0.0900	0.5752
6.0/0.01-5.5/0.01	-0.4152	0.0700	0.5552

Appendix B.14 Scheffe's Test for the Analysis of Glucosinolates (Effects of pH)

(Comparisons significant at the 0.05 level are indicated by "***")

pH comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.0 - 5.5	-0.20408	0.04125	0.28658
6.0 - 6.5	-0.18001	0.07393	0.32787
5.5 - 6.5	-0.22126	0.03268	0.28662

Appendix B.15 Scheffe's Test for the Analysis of Glucosinolates (Effects of Ionic Strength)

(Means with the same letter are not significantly different at the 0.05 level)

Scheffe Grouping	Mean	N	Ionic Strength
A	0.57455	11	0.10M
B	0.36583	12	0.10M

Appendix B.16 Analysis of Variance of Analysis of Phenolic Compounds

Treatments	df	SS	MS	F
Model	5	0.00045424	0.000090	46.15
Error	25	0.00002953	0.00000197	PR>F
Corrected Total	20	0.00043377		0.0001
pH-Ionic Strength pairwise comparison	5	0.00045424		46.15 *
pH	2	0.00034614		87.91 *
Ionic Strength	1	0.00002363		12.00 *
pH-Ionic Strength Interactions	2	0.00004405		11.19 *

* significant at 0.05 level

Appendix B.17 Scheffe's Test for the Analysis of Phenolic Compounds (Effects of Combined pH and Ionic Strength)

(Comparisons significant at the 0.05 level are indicated by "***")

pH/Ionic Strength (M) comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit	
6.0/0.01-6.0/0.10	-0.0011955	0.0025833	0.0063621	
6.0/0.01-6.5/0.01	-0.0003871	0.0036944	0.0077760	
6.0/0.01-6.5/0.01	0.0013907	0.0054722	0.0095538	***
6.0/0.01-5.5/0.01	0.0040018	0.0080833	0.0121649	***
6.0/0.01-5.5/0.10	0.0099295	0.0137083	0.0174871	***
6.0/0.10-6.5/0.10	-0.0029704	0.0011111	0.0051927	
6.0/0.10-6.5/0.01	-0.0011927	0.0028889	0.0069704	
6.0/0.01-5.5/0.01	0.0014184	0.0055000	0.0095816	***
6.0/0.10-5.5/0.10	0.0073462	0.0111250	0.0149038	***
6.5/0.10-6.5/0.01	-0.0025856	0.0017778	0.0061411	
6.5/0.10-5.5/0.01	0.0000255	0.0043889	0.0087523	***
6.5/0.10-5.5/0.10	0.0059323	0.0100139	0.0140954	***
6.5/0.01-5.5/0.01	-0.0017523	0.0026111	0.0069745	
6.5/0.01-5.5/0.10	0.0041546	0.0082361	0.0123177	***
5.5/0.01-5.5/0.10	0.0015434	0.0056250	0.0097066	***

Appendix B.18 Scheffe's Test for Analysis of Phenolic Compounds (Effects of pH)

(Comparisons significant at the 0.05 level are indicated by "***")

pH comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit	
6.0 - 5.5	0.0012353	0.0032917	0.0053481	***
6.0 - 6.5	0.0080353	0.0100060	0.0119766	***
6.5 - 5.5	0.0045959	0.0067143	0.0088327	***

Appendix B.19 Scheffe's Test for the Analysis of Phenolic Compounds (Effects of Ionic Strength)

(Means with the same letter are not significantly different at the 0.05 level)

Scheffe Grouping	Mean	N	Ionic Strength
A	0.0145167	10	0.01M
B	0.016515	11	0.10M

Appendix B.20 Analysis of Variance of Thermal Denaturation Temperature (Td) (A) (pH/Ionic Strength Pairwise Comparison)

Treatments	df	SS	MS	F
pH-Ionic Strength pairwise comparison	5	6.257	1.252	6.91 *
Error	11	1.991	0.181	PR>F
Corrected Total	16	8.249		0.0038

* significant at 0.05 level

Analysis of Variance of Thermal Denaturation Temperature (Td)(B)

Treatments	df	SS	MS	F
Model	5	126.041	25.208	1.55
Error	17	277.342	16.314	PR>F
Corrected Total	22	403.383		0.2285
pH	2	74.858		2.29 **
Ionic Strength	1	0.339		0.02 **
pH-Ionic Strength Interaction	2	44.221		1.36 **

* not significant at 0.05 level

Appendix B.21 Scheffe's Test for Analysis of Thermal Denaturation (Combined Effects of pH and Ionic Strength)

(Comparisons significant at the 0.05 level are indicated by "***")

pH/Ionic Strength (M) comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit	
6.5/0.10-5.5/0.01	-0.7270	0.8275	2.3820	
6.5/0.10-6.0/0.10	-0.4521	0.9383	2.3288	
6.5/0.10-6.0/0.01	-0.1538	1.2367	2.6271	
6.5/0.10-5.5/0.10	-0.1088	1.2817	2.6721	
6.5/0.10-6.5/0.01	0.5812	1.9717	3.3621	***
5.5/0.01-6.0/0.10	-1.4437	0.1108	1.6654	
5.5/0.01-6.0/0.01	-1.1454	0.4092	1.9637	
5.5/0.01-5.5/0.10	-1.1004	0.4542	2.0087	
5.5/0.01-6.5/0.01	-0.4104	1.1442	2.6987	
6.0/0.01-6.0/0.01	-1.0921	0.2983	1.6888	
6.0/0.10-5.5/0.10	-1.0471	0.3433	1.7338	
6.0/0.10-6.5/0.01	-0.3571	1.0333	2.4238	
6.0/0.01-5.5/0.10	-1.3454	0.0450	1.4354	
6.0/0.01-6.5/0.01	-0.6554	0.7350	2.1254	
5.5/0.10-6.5/0.01	-0.7004	0.6900	2.0804	

Appendix B.22 Scheffe's Test for Analysis of Thermal Denaturation (Effects of pH)

(Comparisons significant at the 0.05 level are indicated by "***")

pH comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.0 - 5.5	-5.078	0.334	5.747
6.0 - 6.5	-1.351	4.252	9.854
6.5 - 5.5	-1.685	3.917	9.520

Appendix B.23 Scheffe's Test for the Analysis of Thermal Denaturation (Effects of Ionic Strength)

(Means with the same letter are not significantly different)

Scheffe Grouping	Mean	N	Ionic Strength
A	78.813	11	0.10M
A	78.413	11	0.01M

Appendix B.24 Analysis of Variance for Enthalpy of Denaturation (A) (pH/Ionic Strength Pairwise Comparisons)

Treatments	df	SS	MS	F
pH-Ionic Strength pairwise comparison	5	19.077	3.815	4.37 *
Error	11	9.602	0.873	PR>F
Corrected Total	16	28.678		0.0195

* significant at 0.05 level

Analysis of Variance for Enthalpy of Denaturation (B)

Treatments	df	SS	MS	F
Model	5	6.257	1.252	6.91
Error	11	1.991	0.181	PR>F
Corrected Total	16	8.249		0.0038
pH	2	0.032		0.09 **
Ionic Strength	1	1.522		8.41 *
pH-Ionic Strength Interaction	2	4.265		11.78 *

* significant at 0.05 level
** not significant at 0.05 level

Appendix B.25 Scheffe's Test for Analysis of Thermal Denaturation (Combined Effects of pH and Ionic Strength)

(Comparisons significant at the 0.05 level are indicated by "***")

pH/Ionic Strength (M) comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.5/0.10-6.0/0.01	-2.4615	0.5917	3.6448
6.5/0.10-6.0/0.10	-1.3615	1.6917	4.7448
6.5/0.10-6.5/0.01	-0.5748	2.4783	5.5315
6.5/0.10-5.5/0.10	-0.5098	2.5433	5.5965
6.5/0.10-5.5/0.01	-0.5189	2.8947	6.3082
6.0/0.01-6.0/0.10	-1.9532	1.1000	5.1532
6.0/0.01-6.5/0.01	-1.1665	1.8867	4.9398
6.0/0.01-5.5/0.10	-1.1015	1.9517	5.0048
6.0/0.01-5.5/0.01	-1.1106	2.3030	5.7166
6.0/0.10-6.5/0.01	-2.2665	0.7867	3.8398
6.0/0.10-5.5/0.10	-2.2015	0.8517	3.9048
6.0/0.10-5.5/0.01	-2.2106	1.2030	4.6166
6.5/0.01-5.5/0.10	-2.9882	0.0650	3.1182
6.5/0.01-5.5/0.01	-2.9972	0.4163	3.8299
5.5/0.10-5.5/0.01	-3.0622	0.3513	3.7649

Appendix B.26 Scheffe's Test for Analysis of Enthalpy of Denaturation (Effects of pH)

(Comparisons significant at the 0.05 level are indicated by "***")

pH comparison	simultaneous lower-confidence limit	difference between means	simultaneous upper confidence limit
6.0 - 6.0	-0.5916	0.1017	0.7949
6.5 - 5.5	-0.6129	0.1142	0.8413
6.0 - 5.5	-0.7146	0.0125	0.7396

Appendix B.27 Scheffe's Test for the Analysis Enthalpy of Denaturation (Effects of Ionic Strength)

(Means with the same letter are not significantly different)

Scheffe Grouping	Mean	N	Ionic Strength
A	88.4100	9	0.10M
B	87.7400	8	0.01M

Appendix C Linear Regression Analysis of Phytate Analysis

1) Standards.

Sodium Phytate Standards (μg/ml)	Initial Absorbance (500nm)	Final Absorbance
0	0.560	0.000
10	0.466	0.094
20	0.386	0.174
30	0.303	0.257
40	0.230	0.330
60	0.088	0.472

2) Linear Regression Analysis.

The standard curve was obtained by plotting the final absorbances against the standard concentrations to calculate the following equation:

y-intercept = 0.006
slope = 0.008
correlation coefficient = 0.999

$$y = 0.006 + 0.008x$$

$$x = (-0.006 + 4)/0.008$$

Sample concentrations are calculated by substituting y for sample absorbances (at 500nm). The sample concentrations are divided by 50 to account for dilutions throughout the analysis to express the values as percent phytate content.

Appendix D G-Proc Program Used to Calculate and Plot Comparison Stars.

```
//  JOB ',,T=10,I=20'
//    EXEC SAS,OPTIONS='NODATE,NONUMBER,LINESIZE=78'
//SYSIN DD *
TITLE 'PHENOLICS';
DATA PHENOLIC;
  INPUT PH $ MOL $ SER REP PHENOL;
CARDS;

;

DATA PHYTICS;
  INPUT PH $ MOL $ SER REP PHYTIC;
CARDS;

;

DATA KJELDHAL;
  INPUT PH $ MOL $ SER REP KJELD;
CARDS;

;
DATA DSCMAX;
  INPUT PH $ MOL $ SER REP DSCMAX;
CARDS;

;

DATA DELTAH;
  INPUT PH $ MOL $ SER REP DELTAH;
CARDS;

;

DATA COMBINED;
    LENGTH PHMOL $ 6.;
  TITLE1 'ANALYSIS OF CANOLA DATA';
  MERGE PHENOLIC PHYTICS KJELDHAL DSCMAX DELTAH GLUCOSIN;
  BY PH MOL SER REP;
  PHMOL = TRIM(LEFT(PH))||'-'||TRIM(LEFT(MOL));
  LABEL PH='TREATMENT PH'
        MOL='MOLARITY TREATMENT'
        SER='SERIES NUMBER'
        REP='SERIES REPLICATION'
        PHENOLIC = 'PHENOLICS'
        PHYTIC='PHYTICS'
        KJELD='KJELDHAL'
        DSCMAX='DSCMAX'
        DELTAH='DELTAH'
        GLUCOSIN='GLUCOSIN';

PROC SORT DATA=COMBINED;
  BY PH MOL;

PROC MEANS DATA=COMBINED NOPRINT;
  BY PH MOL;
  VAR PHENOL--GLUCOSIN;
  OUTPUT OUT=MEANS MEAN=PHENOL PHYTIC KJELD DSCMAX DELTAH GLUCOSIN;

PROC MEANS DATA=MEANS NOPRINT;
  VAR PHENOL--GLUCOSIN;
  OUTPUT OUT=STANDARD MIN=MIN1-MIN6 MAX=MAX1-MAX6 RANGE=R1-R6;

DATA JOINT;
  SET MEANS;
  IF _N_=1 THEN SET STANDARD;

  SPHENOL = 100*MIN1/PHENOL;
  SPHYTIC = 100*MIN2/PHYTIC;
  SKJELD = 100*KJELD/MAX3;
  SDSCMAX = 100*DSCMAX/MAX4;
  SDELTAH = 100*DELTAH/MAX5;
  SGLUCOSI = 100*MIN6/GLUCOSIN;

PROC TRANSPOSE DATA=JOINT OUT=TRANS1 PREFIX=RAW;
```

```
  BY PH MOL;
  VAR PHENOL--GLUCOSIN;

PROC TRANSPOSE DATA=JOINT OUT=TRANS2 PREFIX=MEAN;
  BY PH MOL;
  VAR SPHENOL--SGLUCOSI;

DATA TRANSPOS;
  MERGE TRANS2 TRANS1;
  BY PH MOL;
  IF PH='55' THEN PH='5.5';
    ELSE IF PH='60' THEN PH='6.0';
    ELSE PH='6.5';
  IF MOL='01' THEN MOL='0.01 M';
    ELSE MOL='0.10 M';
  IF _NAME_='DELTAH' THEN VARIABLE=1;
  IF _NAME_='DSCMAX' THEN VARIABLE=2;
  IF _NAME_='GLUCOSIN' THEN VARIABLE=3;
  IF _NAME_='PHYTIC' THEN VARIABLE=4;
  IF _NAME_='PHENOL' THEN VARIABLE=5;
  IF _NAME_='KJELD' THEN VARIABLE=6;

  LABEL MEAN1='PERCENT OF OPTIMUM RESPONSE'
        RAW1='RAW MEAN*RESPONSE';
  RENAME MEAN1=RESPONSE
         RAW1=RAW
         _NAME_=MEASURE;

  DROP _LABEL_;

  FOOTNOTE1 'MEANS ARE EXPRESSED AS PERCENT OF OPTIMUM RESPONSE';
  FOOTNOTE2 'GLUCOSIN, PHYTICS AND PHENOLICS MEASURE REMOVAL';

PROC FORMAT;
  VALUE NUMFORM 1='DELTAH'
                2='DSCMAX'
                3='GLUCOSIN'
                4='PHYTICS'
                5='PHENOLICS'
                6='KJELDHAL';

PROC PRINT DATA=TRANSPOS SPLIT='*';
  TITLE2 'DATA AS PLOTTED IN STARS';
  BY PH MOL NOTSORTED;
  VAR VARIABLE RAW RESPONSE;
  FORMAT VARIABLE NUMFORM.;

GOPTIONS DEVICE=XEROX COLORS=(BL);

PROC GCHART DATA=TRANSPOS;
  BY PH MOL NOTSORTED;
  STAR VARIABLE / SUMVAR=RESPONSE STARMIN=0 STARMAX=100 DISCRETE;
  TITLE2 'STANDARDIZED MEAN RESPONSE';
  FORMAT VARIABLE NUMFORM.;
```

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