DETOXIFICATION OF RAPESEED PROTEIN ISOLATES BY AN ACTIVATED CARBON TREATMENT

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ABSTRACT

Rapeseed protein isolate from pH 10 NaOH extraction was analyzed by gas chromatography (isothiocyanates) and UV absorption (goitrin) (Youngs and Wetter, 1967) and found to contain glucosinolates at levels equivalent to 0.75 mg 3-butenyl isothiocyanate, 0.57 mg 4-pentenyl isothiocyanate, and 0.51 mg oxazolidinethione (goitrin) per g isolate.

A two-stage process was developed to decrease the levels of these toxins. Isolate slurry was incubated at pH 7.2 with crude myrosinase extracted from white mustard seed (to convert glucosinolates to isothiocyanates and goitrin), adjusted to pH 10, and passed through a granular activated carbon column. Subsequent analysis revealed only 0.006 mg 4-pentenyl isothiocyanate per g isolate. Goitrin was not detectable. Infrared analysis confirmed that the column was also partially effective in nitrile removal.

To eliminate the need for myrosinase purification, the process was modified to include ground white mustard seed addition directly to rapeseed meal slurry. After incubation, the protein was extracted, purified by isoelectric precipitation, re-dissolved, and treated by the activated carbon column. This modification was included in the "recommended detoxification procedure".

Subsequent experiments on protein extracts prepared and carbon treated at pH's from 3 to 12, inclusive, revealed that all treatments in the range of pH 3 to 10 were at least 93% effective in isothiocyanate removal. A lower efficiency was observed above pH 10.

Storage tests (24 hours) on aglycone containing protein solutions showed increased loss of isothiocyanates with increasing pH from 5 to 10. This could be due to their interaction with protein (Bjorkman, 1973).

The column completely removed chromatographically purified glucosinolates from aqueous solution. However, the results could not be duplicated for solutions containing rapeseed protein. Glucosinolate content was determined by trimethylsilation and gas chromatography (modified method of Underhill and Kirkland, 1971).

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CHAPTER I

LITERATURE REVIEW

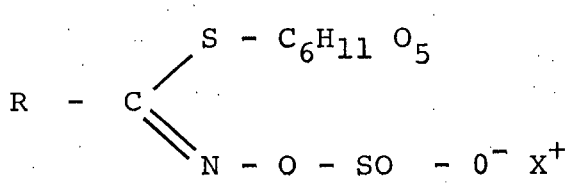
A. INTRODUCTION

1. Natural Occurrence of Glucosinolates

The presence of water-soluble glucosides (glucosinolates; thioglucosides) capable of undergoing enzymatic hydrolysis to oil-soluble isothiocyanates (mustard oils; aglycones), hydrogen sulfate, and D-glucose in certain families of plants has drawn the attention of scientists since the early 1800's, although medicinal use of these compounds dates back to a much earlier period of time.

Almost all members of the family Cruciferae investigated so far have contained at least one thioglucoside (Kjaer, 1960; Daxenbichler et al., 1964). Other families producing these compounds are Capparidaceae, Moringaceae, and Resedaceae -- all belonging to the order Rhoeadales. Occasionally thioglucosides have been found in plants outside this order.

The general chemical structure of a thioglucoside or glucosinolate established by Ettlinger and Lundeen in 1956 is illustrated in Figure I-1 below:



X^+ is a cation, usually potassium

FIGURE I-1 Glucosinolate Structure

2. Glucosinolates in Rapeseed

Rapeseed (family Cruciferae) contains the predominant glucosinolates progoitrin, gluconapin, and glucobrassicanapin with low concentrations of glucoiberin, sinalbin, and gluconasturtiin (Kjaer and Boe Jensen, 1956; Rutkowski, 1971). Levels at which these compounds occur in individual plants (0.5% to 6.4% moisture and fat free basis) reported by Lo and Hill, 1971) vary with environment and species (Wetter, 1955; Wetter and Craig, 1959; Youngs and Wetter, 1967; Josefsson and Appelqvist, 1968). Canadian grown rapeseed (Brassica napus and Brassica campestris) contains approximately 40% oil (Downey, 1965), 24% protein (Anderson and Sabry, 1970), and 10% moisture (Eapen et al., 1968). The remainder is made up of carbohydrates and cellulose (Wetter, 1965).

Most glucosinolates, or rather their hydrolytic products are goitrogenic in nature (Astwood et al., 1949; Belzile et al., 1963; Rutkowski, 1971; Josefsson and Munck, 1972) and therefore limit the use of rapeseed meal currently being produced as a by-product of the oil extraction process (Bell and Belzile, 1965). Research aimed at the development of glucosinolate-free strains of rapeseed is progressing (Downey et al., 1967).

Structures of "R-components" and respective hydrolytic products of rapeseed glucosinolates are shown in Table I-1.

TABLE 1-1

Glucosinolates in Rapeseed

		Glucosinolate	Aglycone Product
Name	Chemical Structure (R)*	Name	
3-butenyl	$CH_2 = CHCH_2CH_2 -$	Gluconapin	Isothiocyanate (Volatile)
4-pentenyl	$CH_2 = CHCH_2CH_2CH_2 -$	Glucobrassicanapin	Isothiocyanate (Volatile)
2-hydroxy-3-butenyl	$CH_2 = CHCHCH_2 -$ OH	Progoitrin	5-vinyl-2-Oxazolidinethione (goitrin) (non-Volatile) or 1-cyano-2-hydroxy-3-butene (non-Volatile)
3-methylsulphinylpropyl	$CH_3SCH_2CH_2CH_2 -$ O	Glucoiberin	Isothiocyanate (non-Volatile)
2-phenylethyl	$C_6H_5-CH_2CH_2 -$	Gluconasturtiin	Isothiocyanate (Volatile)
p-hydroxybenzyl	$HOC_6H_4CH_2 -$	Sinalbin	Isothiocyanate (Volatile)

* See Figure 1-1

B. CHEMISTRY OF GLUCOSINOLATES

1. Characterization and Isolation of Glucosinolates and their Aglycones

Early attempts at separation of the steam distillable isothiocyanates produced on enzymatic degradation of glucosinolates included fractional distillation (Schmalfuss, 1938; Schwarze, 1949; Ettlinger and Hodgkins, 1955; Kjaer and Boe Jensen, 1956) and distribution between partially miscible solvents (Schmid and Karrer, 1948a,b). A combination of isothiocyanate (Kjaer et al., 1953), thiourea (ammonia treated isothiocyanate) (Kjaer and Rubinstein, 1953), and glucosinolate (Gmelin, 1954; Schultz and Gmelin, 1952 ; Schultz and Wagner, 1956; Wagner, 1956) paper chromatographic techniques resulted in the separation and characterization of several of the parent glucosides and their aglycones. Over fifty glucosinolates have now been isolated in some form and several more identified (VanEtten, et al., 1969).

The first reported chemical synthesis of a naturally occurring glucosinolate was that of Ettlinger and Lundeen (1957). Their product, glucotropaeolin, is the major glucosinolate of Tropaeolum majus L.

2. Enzymatic Hydrolysis of Glucosinolates

The general hydrolysis reaction of glucosinolates by the enzyme myrosinase (thioglucoside (glucosinolate) glucohydrolase; EC 3.2.3.1) involves first an enzymatic

cleavage of the glucose and sulfate moieties followed by a Lossen rearrangement (Ettlinger and Lundeen, 1957) to produce the isothiocyanate as illustrated in Figure I-2 below:

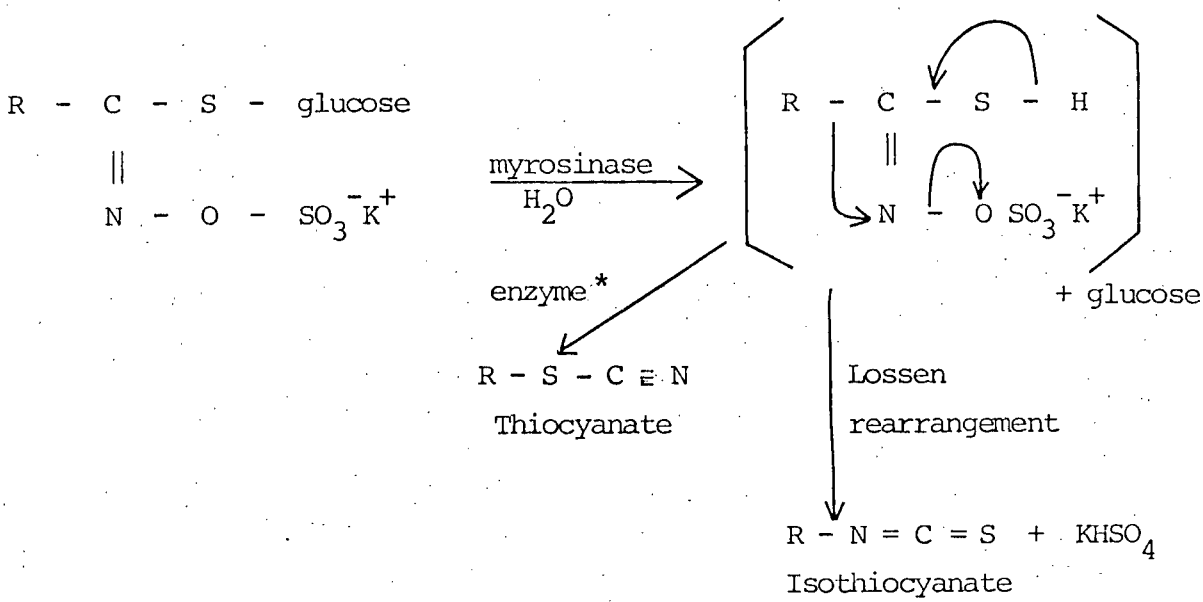
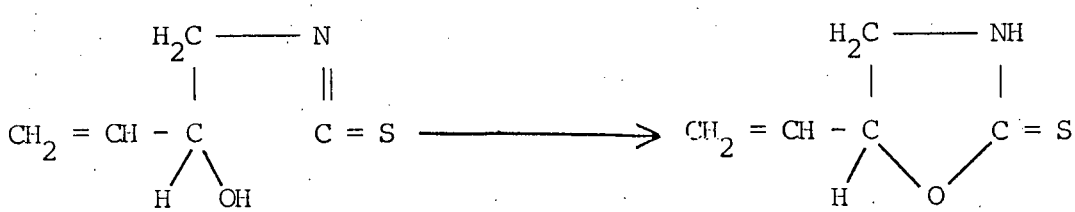


FIGURE 1-2 Enzymatic Degradation of Glucosinolates

* Alternate pathway in special cases

If a B-hydroxyl group is present in "R" of the enzymatically produced isothiocyanate, a cyclization immediately occurs, producing the oxazolidinethione. This reaction is illustrated in Figure I-3 for 2-hydroxy-3-butenyl glucosinolate (progoitrin) isolated from rutabaga seeds by Greer in 1956:



2-hydroxy-3-butenyl Isothiocyanate

5-vinyl-2-oxazolidinethione (goitrin)

FIGURE 1-3 Formation of Goitrin

The aglycone, goitrin, was isolated from yellow turnip by Astwood et al., in 1949 and the structure proved by Ettlinger in 1950 by chemical synthesis.

Other hydrolysis mechanisms have been investigated (Kjaer, 1960). Some plants contain enzymes capable of producing thiocyanates instead of isothiocyanates and goitrins (Gmelin and Virtanen, 1959a,b). Daxenbichler et al. (1966) and VanEtten, et al. (1966, 1969) studied formation of the nitrile 1-cyano-2-hydroxy-3-butene (Figure I-4) as an alternate product to goitrin in the enzymatic degradation of progoitrin and concluded that the respective amounts of each produced depended on temperature, pH, and source of myrosinase.

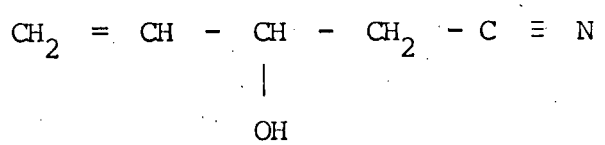


FIGURE 1-4 1- cyano-2-hydroxy-3-butene

Investigations on crambe seed meal (Crambe abyssinica) by VanEtten, et al. (1966) showed that preferential enzymatic formation of goitrin occurred with increased temperature and pH, dilution of the meal with water, dry heating the meal, and storage of the seed under ambient conditions. Daxenbichler et al. (1966) reported that at decreasing pH's from pH 7 to 3, nitrile formation was

increased during hydrolysis of progoitrin by myrosinase derived from white mustard seed (Sinapis alba).

3. Preparative Isolation of Glucosinolates

Attempts at separation of glucosinolates on a preparative scale have generally met with limited success. Initial separations were performed by paper (Schultz and Gmelin, 1952; Schultz and Wagner, 1956; Kjaer and Boe Jensen, 1956) and thin layer (Wagner et al., 1965; Matsuo, 1970) chromatographic procedures.

Chromatographic column techniques have resulted in only partial separation and then only from plants extremely abundant in one particular glucosinolate. Greer isolated progoitrin from rutabaga seed (alumina; 1956) and Brassica seed (anion exchange on Amberlite IR-4B in the Cl^- form; 1962). Bachelard and Trikojus (1963) used cellulose and alumina columns to identify glucosides present in pasture weeds. Bjorkman (1972) employed DEAE Sephadex A-25 anion exchange chromatography for preliminary separation of rapeseed glucosinolates and affinity chromatography (arginine coupled Sephadex G-10) for further purification.

In 1970, Aleksiejczyk et al. were moderately successful in isolating these compounds by high voltage paper electrophoresis. However, complete separation of glucosinolates from rapeseed has never been satisfactorily achieved on a preparative scale.

4. Quantitative Analysis of Glucosinolates and Aglycones

Quantitation of glucosinolates has usually been dependent on analysis of released products of enzymatic hydrolysis (Youngs and Wetter, 1967). Total thioglucoside content has been determined by sulfur titration (VanEtten, et al., 1965) or glucose analysis (Schultz and Gmelin, 1954) after hydrolysis. Glucose determination by the anthrone procedure following sulfuric acid cleavage of thioglucosides (Gmelin, 1954; Schultz and Gmelin, 1954) has met with limited success due to the presence of other carbohydrates in plant material (Kjaer, 1960).

Volatile isothiocyantes have been determined by argentimetric methods (Wetter, 1955). After production of thioureas by steam distillation into aqueous ammonia, silver nitrate was added to facilitate decomposition of the product to a mono-substituted carbodiimide and insoluble silver sulfide. The unreacted silver was then determined by the Volhard method using potassium thiocyanate.

Thioureas (Kjaer et al., 1953; Moll, 1963; Appelqvist and Josefsson, 1965, 1967) and goitrin (Astwood et al., 1949; Wetter, 1957) have been quantitated by their absorbance in the ultra-violet region. Szewczuk et al. (1969a) used the goitrin catalysis of iodine reduction by azide ion as a base for a chemical method of goitrin determination where excess iodine was titrated with sodium

arsenite.

Recently, gas chromatography of isothiocyanates and other volatile hydrolysis products has been accomplished (Jart, 1961; Kirk et al., 1964; Youngs and Wetter, 1967). Daxenbichler et al. (1970) applied this method to the determination of goitrins and nitriles.

In 1971, Underhill and Kirkland devised a procedure for glucosinolate analysis without myrosinase hydrolysis by employing trimethylsilation and gas chromatography of the corresponding derivatives. However, this method was limited by the unavailability of pure standards.

5. Interaction of Aglycones with Rapeseed Proteins

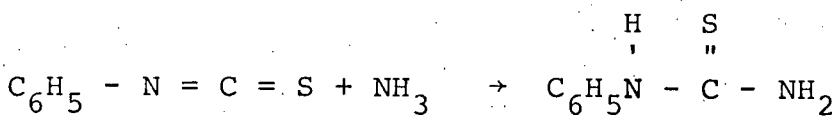
In 1973 Bjorkman reported the occurrence of an isothiocyanate-protein reaction at pH values higher than 6. By incubation of ^{35}S labelled gluconapin and glucoalyssin with rapeseed meal at 40°C for 1 hour and subsequent fractionation by Sephadex G-50 and G-200 chromatography, it was determined that 36% of the amount of added isothiocyanate was incorporated by the protein at pH 9. Incorporation was pH dependent, increased linearly with amount of isothiocyanate added, and did not increase with a longer reaction time. From the Sephadex elution patterns, it was

found that the radioactive isothiocyanates reacted mainly with low molecular weight basic proteins (isoelectric points approximately 11) rich in lysine. Only a very small amount of goitrin was associated with the protein and this interaction was independent of pH. Association of intact glucosinolates with rapeseed proteins was not detected.

An isothiocyanate is used in protein sequencing procedures (Edman, 1970). In this method, phenyl isothiocyanate couples with the free amino group of the N terminal amino acid of a peptide at basic pH to produce the phenyl thiocarbamyl- amino acid. This derivative is subsequently cleaved from the rest of the peptide by an aqueous acid treatment at elevated temperature thereby producing the thiazolinone, which in turn can be converted to the more stable 3-phenyl-2-thiohydantoin-amino acid. This reaction and similar reactions involving isothiocyanates are illustrated in Figure I-5.

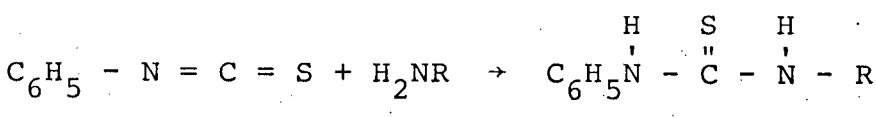
The isothiocyanate-protein reaction reported by Bjorkman (1973) must be the formation of the thiocarbamyl-amino acid and the effect of ammonia on the reaction can be explained by the formation of the substituted thiourea (see Figure I-5).

1. With ammonia (Noller, 1965)



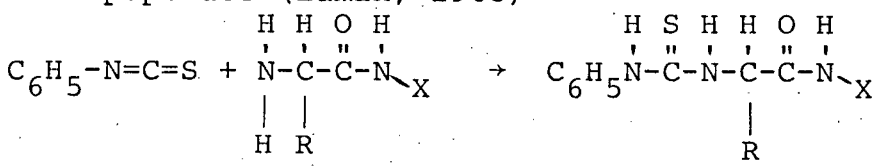
phenyl thiourea

2. With 1° and 2° amines (Noller, page 349, 1965)

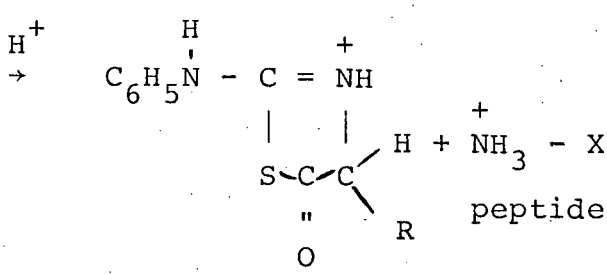


substituted thiourea

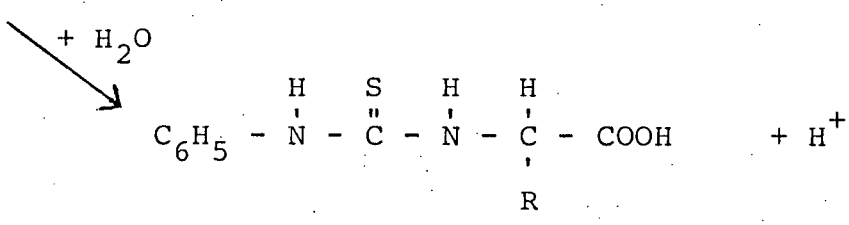
3. With peptides (Edman, 1965)



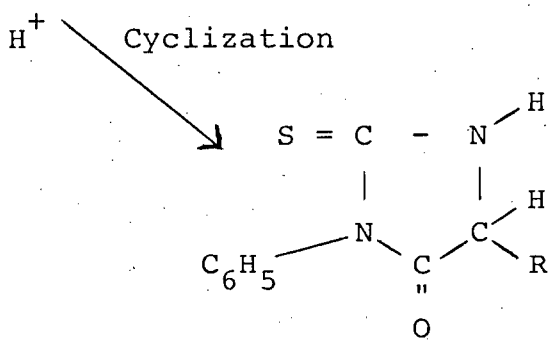
phenylthiocarbamyl derivative



2-anilino-5-thiazolinone derivative (ammonium form)



phenylthiocarbamyl acid



phenylthiohydantoin amino acid

FIGURE 1-5 Reactions of Phenyl Isothiocyanate

Konigsberg (Edman, 1970) reported a possible reaction of isothiocyanate with sulfhydryl groups of proteins, particularly cysteine. However, this was not observed by Edman (1970).

C. THE MYROSINASE ENZYME SYSTEM

Myrosinase (thioglucoside (glucosinolate) glucohydrolase EC 3.2.3.1) responsible for the hydrolysis of glucosinolates to aglycones has been found in plants (Kjaer, 1960; VanEtten, et al., 1969; Vaughan et al., 1968; MacGibbon and Allison (1970), bacteria (Oginsky et al., 1965) and mammals (Goodman et al., 1959).

Bjorkman and Janson (1972) characterized the myrosinase system of white mustard seed and demonstrated the existence of a number of isoenzymes. Lonnerdal and Janson (1973) isolated the main component of this enzyme from Brassica napus L. and reported that the myrosinase content of rapeseed was only one fifteenth that of white mustard seed (Sinapis alba).

Myrosinase is active over the pH range of 3 to 11 (VanEtten, et al., 1966), although maximum release of isothiocyanates and oxazolidinethiones occurs at pH 6-9 and pH 7-9, respectively (Appelqvist and Josefsson, 1967). This observed decrease in goitrin production below pH 7 could be due to nitrile formation (Daxenbichler et al., 1966).

Maximum hydrolysis rate was observed at 70°C. Addition of ascorbic acid to the system increased myrosinase activity by a factor of 2.9 and 3.8 for isothiocyanate and goitrin release, respectively (Appelqvist and Josefsson, 1967).

Ascorbate activation of myrosinase was originally reported by Nagashima and Uchiyama (1959a) who claimed that the cleavage rate of sinigrin was increased by 260 percent on addition of 0.001 M L-ascorbate. According to Henderson and McEwen (1972), maximum acceleration was achieved between pH 5.5 and 6.5 (citric acid-sodium phosphate buffer). The enzyme was also found to be inhibited by sulphydryl blocking agents (Nagashima and Uchiyama, 1959b).

Mode of enzyme preparation affects ascorbate activation (Ettlinger et al., 1961). Addition of 0.002 M L-ascorbate to a standard preparation of myrosinase and to whole mustard seed enhanced the rate of hydrolysis of sinigrin by a factor of four (Nagashima and Uchiyama, 1959a; Schwimmer, 1960) and eighty respectively, while a modified method of enzyme preparation increased the rate by 400 (Ettlinger et al., 1961).

D. TOXICITY OF GLUCOSINOLATES AND AGLYCONES

When glucoside containing seeds are crushed, the myrosinase system is activated, decomposing biologically inactive thioglucosides to goitrogenic vinyloxazolidine-thiones, nitriles, isothiocyanates, and thiocyanates (Rutkowski, 1971).

Vinyloxazolidinethione, the most active compound of the group, acts by blocking the irreversible mechanism involved with the organic binding of iodine in the thyroid, thereby suppressing thyroxine synthesis (Rutkowski, 1971). The resulting imbalance stimulates secretion of excessive amounts of thyrotropine by the hypophysis which in turn causes increased thyroid growth (Clandinin and Bayly, 1960). This effect cannot be alleviated by iodine supplementation (Rutkowski, 1971), although after prolonged feeding of vinyloxazolidinethione containing meal to chickens, physiological equilibrium was apparently achieved with increased thyroid size (Clandinin, 1965). Nitriles have been shown to be about 8 times as toxic as goitrin, acting primarily on the liver and kidneys (VanEtten, et al., 1969). The mechanism of their action is not clear since reproducibility of results has not always been possible (Rutkowski, 1971).

Isothiocyanate activity which can be eliminated by dietary iodine supplementation (Rutkowski, 1971) consists of blocking iodine uptake by the thyroid gland and liberating the iodine already accumulated there (Fertman and Curtis, 1951; Gmelin and Virtanen, 1960). Thiocyanates exhibit only mild goitrogenic properties (Rutkowski, 1971).

Monogastric mammals are the most susceptible to the detrimental effects of aglycones. Negative effects observed in swine have been in maturation, reproduction,

and lactation (Bowland, 1965; Bell, 1965; Rutkowski, 1971). Poultry exhibit a depression period in development in which thyroid size increases rapidly. After this initial adjustment, near normal growth is generally observed (Clandinin et al., 1959; Clandinin and Bayly, 1960; Clandinin, 1965).

While ruminants seemed almost unaffected by goitrogens (Apslund and McElroy, 1961; Whiting, 1965), vinyloxazolidinethione was thought to be transmitted to the milk from these animals (Clements and Wishart, 1956 ; Virtanen et al., 1958; Clandinin and Bayly, 1960). However, ingestion of milk from Brassica fed cows revealed no disturbance in the accumulation of radioactive iodine uptake in the human thyroid gland (Virtanen et al., 1959; Virtanen, 1961). In similar experiments Hoppe et al., (1971) reported the presence of thiocyanides which affected cheese ripening. Such compounds were considered undesirable constituents in milk (Rutkowski, 1971).

E. PRODUCTION OF GLUCOSINOLATE-FREE RAPESEED MEALS, CONCENTRATES, AND ISOLATES.

Meal and flour derived from rapeseed is an excellent source of protein, approximately 40 and 55%, respectively (Lo and Hill, 1971; Tape et al., 1970). However the presence of "goitrogenic compounds" (glucosinolates and aglycones) in rapeseed limits its potential at the present time (Bell, 1955, 1957; Bell and Belzile, 1965).

Detoxification procedures reported in the literature can be divided into five major categories (Rutkowski,

1970) and will be presented in that manner here.

1. Removal of volatile substances by heating without prior breakdown of glucosinolates.

The most direct solution to the problem is steaming or toasting. Bell and Belzile (1965) reported that during steaming, the amount of pressure had a marked effect on the rate of disappearance of isothiocyanate and goitrin producing compounds. In one case, after 2 hours of treatment, 90% and 75% of these respective compounds had been eliminated although a corresponding decrease in protein quality was also observed. Jakubowski et al., (1970) reported that superheated steam treatment for over 3 hours completely eliminated goitrogenic compounds.

In Poland, industry has adopted a toasting process to reduce glucosinolate content by 75%. However, a 50% decrease in toxin content by this method is accompanied by a corresponding decrease of about one-third in soluble protein (Rutkowski, 1970).

2. Direct chemical degradation and microbial destruction of glucosinolates.

A direct ammoniation process resulting in the transformation of glucosinolates to thiourea

derivatives has been described by Chanet (1970) (see Figure I-5). The process has not been widely accepted (Rutkowski, 1970) and is currently under investigation (Bell et al., 1970).

Bell et al., (1970) patented a process for the degradation of glucosinolates to their corresponding steam volatile nitriles by treatment with copper and iron salts. However, the nitrile of goitrin, 1-cyano-2-hydroxy-3-butene, was not volatile and the process was therefore limited to meals of low progoitrin content. The reaction of iron salts with glucosinolates has been investigated by Austin et al., 1968.

Dilute sulfuric acid degradation of glucosinolates was proposed by Szewczuk et al. (1969b, 1970). However, the process resulted in considerable degradation of lysine.

In a microbial approach described by Starton (1970) it was found that after a fermentation period of 85 hours at 37°C with the culture Geotrichum candidum, complete degradation of glucosinolates in rapeseed meal and elimination of their products had occurred along with a 90% solubilization of the available protein. Final pH of the process was pH 4.0.

Faruga et al. (1973) found that silage made from steamed potatoes and rapeseed meal was free of goitrogenic properties.

3. Hydrolysis of glucosinolates and removal of products by distillation.

Preliminary degradation of glucosinolates to aglycones followed by steam distillation was proposed by Schwarze (1949), Andre (1955), and Goering (1961). Unfortunately, goitrin remained in the meal (Bell and Belzile, 1965). A similar process was outlined by Mustakas et al. (1962, 1963) for the detoxification of mustard seed (Brassica juncea) which is known to contain only isothiocyanate producing glucosides. A similar process to be effective for rapeseed would require too much heat and would therefore be economically unfeasible (Rutkowski, 1970).

4. Deactivation of the myrosinase enzyme system.

If myrosinase were inactivated before the decomposition of glucosinolates to aglycones, the meal would not be considered toxic since glucosinolates are not goitrogenic themselves. However, the presence of enzymes in the gastrointestinal tract (Greer, 1962), capable of degrading glucosinolates to their aglycones and the ability of

E. coli and A. aerogenes to produce these same enzymes (Bell, 1955), limits the usefulness of this approach.

Currently, myrosinase inactivation is considered beneficial during production of rapeseed oil and meal to minimize sulfur contamination of the oil from glucosinolate hydrolysis at the crushing stage (Bell, et al., 1963).

Although dry heating was inefficient in myrosinase inactivation (Bell and Belzile, 1965), complete success was obtained with a wet-heat process consisting of a 2 or 3 minute treatment at 100°C (Eapen et al., 1968). This method has been applied by other researchers as a preliminary step in their detoxification procedures, (e.g., Sosulski, et al., 1972).

5. Extraction of glucosinolates and hydrolysis products.

Although simple extraction of rapeseed meal by aqueous and ethanolic solutions increased feeding value (Allen and Dow, 1952; Bell, 1957), hot water extraction resulted in a 20% loss of solids (Bell and Belzile, 1965).

Tookey et al. (1965) produced a biologically improved meal from Crambe abyssinica by allowing myrosinase hydrolysis to proceed and subsequently

extracting with 88 to 98% acetone (aqueous).

Eapen et al. (1968, 1969) published a feasibility study for the detoxification and dehulling of rapeseeds involving preliminary inactivation of myrosinase, wet grinding, and three 30 minute hot or cold water extractions. The detoxified residue was dried, flaked, defatted, and air classified into two products with 33 and 60% protein respectively. When the process was applied to meal, a 27 to 39% loss of protein occurred to the aqueous phase compared to 14 to 26% with ground seed.

Ballester et al. (1970) found that double water extraction (14 hours + 1 hour) at room temperature reduced oxaxolidinethione and isothiocyanate producing compunds by 84 and 77%, respectively, and increased biological quality of the meal.

The method of Eapen et al. (1969) was modified by Tape et al. (1970) by the incorporation of two thirty minute extractions at ambient temperature and a filter wash (vibratory screen) to produce rapeseed flour and meal containing 50% and 30% protein respectively, with only traces of glucosinolates.

Eklund et al. (1971) obtained 3 lipid-rich rapeseed protein concentrates (approximately 48% protein, 31% fat) by a 28% ethanol, M NaCl

extraction and centrifugation. Although 67% of the oxazolidinethiones were liberated during the initial extraction, the final product contained only 5% of the originally present goitrogens.

The major disadvantages of the process were the high loss of material to the solvent (30% and 50% of the original fat and protein) and the high lipid content of the final product.

In 1972, Ballester et al. shortened their original 15 hour batch extraction procedure (Ballester et al., 1970) to a 2 hour continuous process capable of removing 100% and 97% of the isothiocyanate and oxazolidinethione producing glucosides. However, feeding trials of the resulting meal indicated the presence of other potentially harmful factors in rapeseed meal. High concentrations of tannins have been reported (Durkee, 1971; Yapar and Clandinin, 1972).

Owen and Chichester (1971) produced a protein isolate containing 84% protein and only 0 to 3% of the originally present oxazolidinethione producing glucosinolates from rapeseed meal by 6% NaCl extraction, removal of supernatant, four water washes, addition of calcium carbonate (to increase curd strength and accelerate precipitation), and

adjustment to pH 5. The supernatant was siphoned off and precipitated protein centrifuged out. When the method was duplicated by Girault (1973), an 18% yield was obtained.

A similar procedure (Lo and Hill, 1970) employing 10% NaCl extraction of Brassica napus meal, filtration, and a 3-day dialysis, yielded a product containing 61 to 64% protein (approximately 75% of the original meal nitrogen) and 30% of the original glucosinolate content.

A unique approach to the problem was taken by Sosulski et al. (1972) who used diffusion extraction of whole rapeseeds at pH's from 1.1 to 12.3. Five or six 1 hour extractions with 0.01 N NaOH at 60°C proved most efficient. However, unless the seeds were boiled before treatment, a high sulfur content resulted in the oil. Bhatty and Sosulski (1972) modified the method by using 0.01 N NaOH in 50% ethanol for 4 two hour extractions at 70°C. Only low levels of thioglucosides were detected in the product. The major disadvantage of the process was reduced protein solubility of the meal because of partial denaturation.

Kozlowska et al. (1972) duplicated five previously reported glucosinolate extraction pro-

cedures: aqueous extraction of meal (Ballester, et al. 1970); enzyme inactivation and aqueous extraction of crushed seeds (Tape et al., 1970); diffusion extraction (0.01 N NaOH) of intact seeds and diffusion extraction (0.01 NaOH) of intact seeds with preliminary enzyme inactivation (Sosulski et al., 1972); and diffusion extraction (50% v/v ethanolic NaOH adjusted to pH 12) of intact seeds (Bhatty and Sosulski, 1972).

The aqueous extraction (Ballester et al., 1970) was the most effective in glucosinolate removal. However, as in the method of Tape et al. (1970) almost one-third of the meal proteins were lost to the solvent. The ethanolic process (Bhatty and Sosulski, 1972), although preventing myrosinase action and most completely detoxifying rapeseed samples tested, required long extraction times and resulted in denaturation of meal proteins.

Girault (1973) outlined optimum conditions for extraction and precipitation of proteins from rapeseed meals. Most effective extraction was obtained with 10% NaCl or 0.1 N NaOH and maximum precipitation at pH 3 for NaCl and pH 6.5 for NaOH extraction. Sodium hydroxide extracted 20% more of the total nitrogen than sodium chloride,

and did not seem to have any detrimental effect on extracted proteins. The actual yield obtained with the 0.1 N NaOH method was 30%. Decrease of thioglucoside content was not investigated.

F. OBJECTIVE OF THIS STUDY

Kodagoda et al. (1973a) produced acid, neutral, and base soluble protein isolates (average 91% protein content) from rapeseed flour prepared by Tape et al. (1970). However, the process was not considered industrially attractive since traces of glucosinolates were still detectable in the final product (personal communication) and dependence on rapeseed flour as a raw material was economically unfavourable.

The object of this study was to develop a method of preparation of glucosinolate-free rapeseed protein (either concentrate or isolate) similar to that of Kodagoda et al. (1973a) directly from commercial rapeseed meal. The process was to be adaptable to other rapeseed protein extraction procedures such as those of Girault (1973) to yield a completely detoxified protein product acceptable for human consumption.

The proposed method of detoxification entailed preliminary glucosinolate hydrolysis and subsequent removal of aglycone products by activated carbon treatment of the protein solution. Processes involving activated carbon adsorption currently in use by the food industry are for the elimination of contaminating pigments (from beer, wine, sugar, oils and fats, water) and odoriferous compounds (from brandy, wine,

beer, water) characterized by a high degree of resonance, usually due to pi-unsaturation and the presence of electronegative groupings sometimes containing nitrogen and sulfur atoms (Mantell, 1928, 1968; Smisek and Cerny, 1970). Since aglycones seemed to be in this category, prospects of success seemed favorable.

CHAPTER II

DETOXIFICATION OF pH 10 SOLUBLE RAPESEED PROTEIN

A. INTRODUCTION

Preliminary experiments confirmed that the mustard odor of enzymatically treated rapeseed meal decreased after carbon treatment indicating that there was indeed partial or total elimination of aglycones by carbon adsorption. Therefore, an experiment was designed to determine three critical factors: first, the efficiency of myrosinase conversion of glucosinolates to isothiocyanates and 5-vinyl-2-oxazolidinethione (goitrin) in rapeseed meal; second, the actual decrease in concentration of liberated aglycones by carbon treatment; and third, the adsorption capacity of the carbon available. Success of the proposed process would result in the production of aglycone-free rapeseed protein suitable for human consumption.

Industrial adoption of such a process, however, would depend on the toxicity of protein-incorporated isothiocyanates reported by Bjorkman (1973) during the course of this study (see section IB5). Although these products would likely remain attached to the integral structure of the protein at the preparation stage, they would be released during digestion. The most toxic aglycone, goitrin, would be unaffected by the interaction (section IB5).

The pH 10 soluble rapeseed protein, similar to that prepared by Kodagoda et al. (1973a), was chosen for the first detoxification trial since a high proportion of rapeseed protein should be soluble at that pH, and success with this fraction would therefore allow extension of the process to most rapeseed proteins. pH 7.2 was chosen for myrosinase treatment to maximize isothiocyanate and goitrin production (Appelqvist and Josefsson, 1967), eliminate the formation of nitriles which would occur at lower pH (Daxenbichler et al., 1966), and minimize isothiocyanate protein interaction (Bjorkman, 1973). Because the possibility of nitrile production could not be overlooked, an experiment was designed to study nitrile adsorption by activated carbon. For this study, myrosinase treatment was performed at pH 3.3. For use in all experiments described in Chapter II, a semi-pure form of the enzyme myrosinase was prepared from white mustard seed.

Carbon column capacity was not determined under actual experimental conditions used in the process; i.e., in the presence of protein. Instead, an aqueous solution of pure isothiocyanate was used. The entire experiment will be fully described in the next section.

B. METHODS

1. Preparation of Base Soluble Protein Fractions

a. Protein extract

As outlined in the flow chart of Figure 2-1, commercial rapeseed meal (Brassica campestris var. Span) was obtained from Canbra Foods Ltd., Lethbridge, Alberta; defatted by petroleum ether extraction; ground in a Wiley Mill with a 20-mesh screen; and sieved through a 60-mesh screen to yield a product termed "meal flour". Preliminary investigation of the meal indicated complete lack of myrosinase activity.

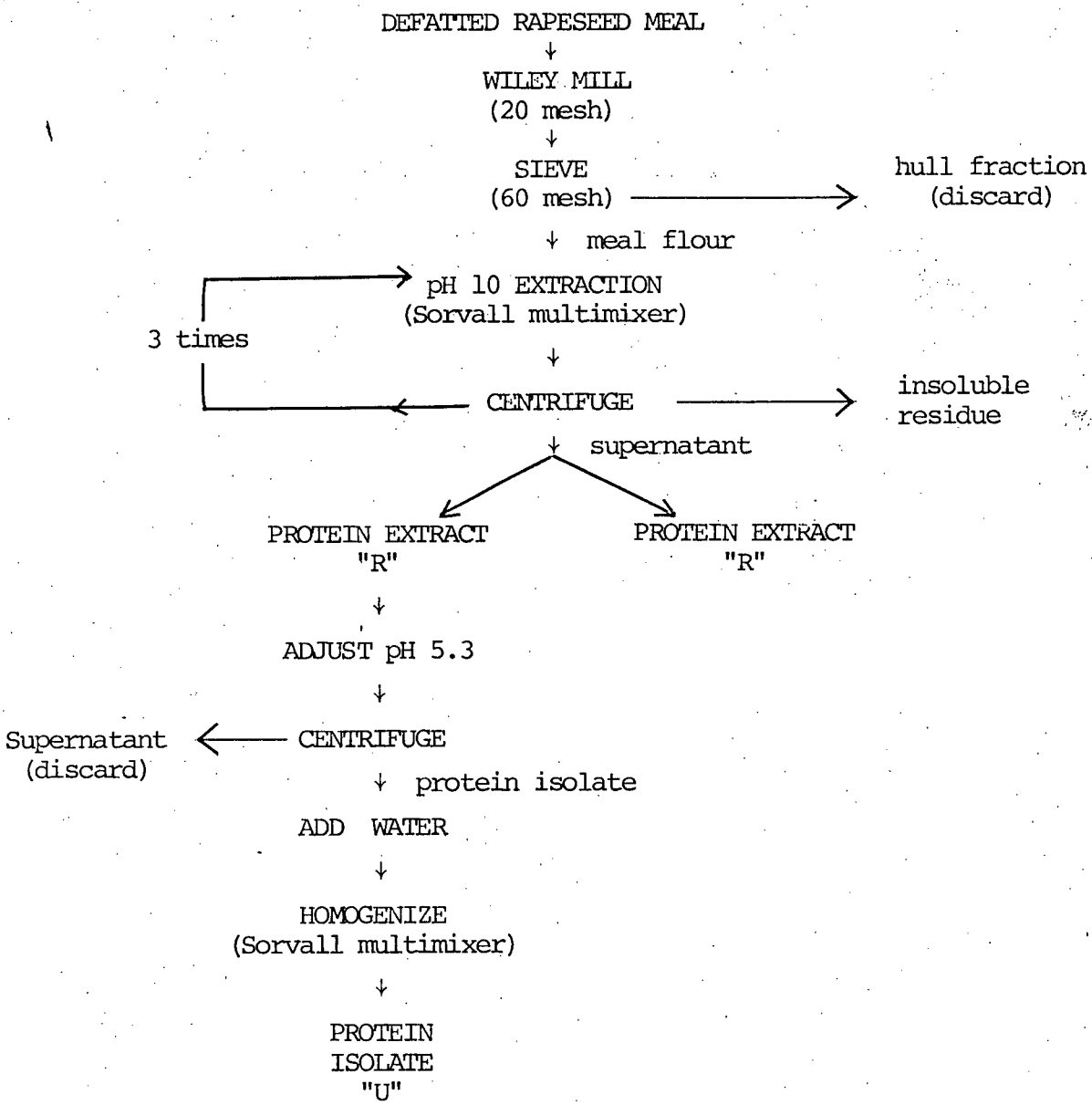
NaOH (0.1 N, 600 ml) was added to 100 gm of meal flour; the mixture was blended in the Sorvall Multimixer at maximum setting for 3 minutes; pH was adjusted to 10 with N NaOH by autotitration (Radiometer); and the mixture was centrifuged. The solids fraction was collected and the extraction repeated twice at pH 10 with 600 ml of NaOH solution. The pooled supernatant (1700 ml collected) was filtered and labelled protein extract "R". The insoluble hull-rich solids residue was lyophilized pending further study.

b. Protein isolate

Protein extract "R" (1.1 litres) was adjusted to pH 5.3 to isoelectrically precipitate the base soluble fraction (Kodagoda et al., 1973a) and centrifuged (Figure 2-1). The supernatant was decanted; sedimented protein isolate was collected and suspended in 200 ml of water; and the mixture

FIGURE 2-1

Preparation of Rapeseed Protein Fractions



was blended for 1 minute in the multimixer at maximum setting to produce a finely divided protein suspension or slurry which was later adjusted to a total volume of 600 mls. Centrifuged protein isolate was not washed after the decantation step to minimize protein loss. The slurry was labelled protein isolate "U".

2. Myrosinase Preparation

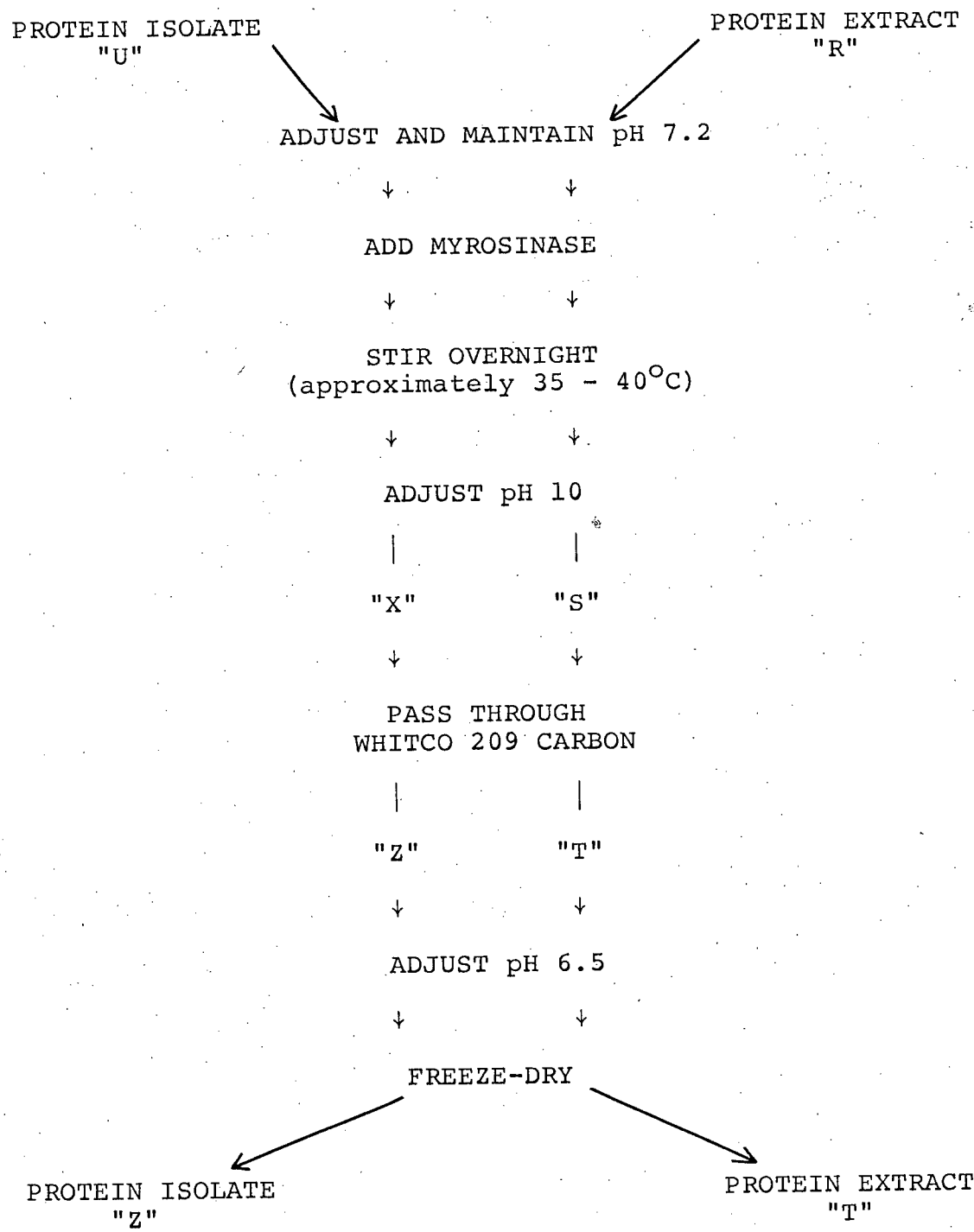
A crude form of the enzyme, myrosinase, was prepared according to the method used by Appelqvist and Josefsson (1967). White mustard seed, obtained from the Canadian Grain Commission, Vancouver, was ground on the Wiley Mill (20-mesh); blended with three volumes of cold water (4°C) held at that temperature for 1 hour; and then centrifuged. The supernatant was filtered into an equal volume of ice cold 90% ethanol to precipitate the enzyme; centrifuged; collected on a buchner funnel; washed with cold 70% ethanol; and dissolved in a volume of water equal to that of the weight of the original mustard. This aqueous solution was further purified by centrifugation and filtration. The filtrate was freeze-dried to yield the crude myrosinase powder which was subsequently stored frozen in a desiccator.

3. Production of Aglycones

At this stage, protein extract "R" and protein isolate "U" were treated identically as shown in Figure 2-2. The protein solution was adjusted to pH 7.2, 50 to 100 mg of crude myrosinase powder was added, and the solution was

FIGURE 2-2

TREATMENT OF RAPESEED PROTEIN FRACTIONS



stirred and maintained at pH 7.2 by auto titration (0.5 N NaOH) for a period of approximately 12 hours at 35-40°C. During this period, glucosinolates were converted to isothiocyanates, goitrin, bisulfate, and glucose by the action of myrosinase (see Section IB2). Protein solutions were labelled "S" (from "R") and "X" (from "U").

A sample analogous to "R" was treated overnight with myrosinase at pH 3.3 to serve as a "nitrile-rich" sample for the nitrile adsorption experiment. At this pH, instead of complete conversion of 2-hydroxy-3-butenyl isothiocyanate to 5-vinyl-2-oxazolidinethione (goitrin), a significant amount of the nitrile, 1-cyano-2-hydroxy-3- butene, is formed (Daxenbichler et al., 1966).

4. Carbon Column Treatment

Grade 209, Whitco granular activated carbon, 18 x 40 mesh, was obtained, courtesy of the Whitco Chemical Company, New York, N.Y. The carbon was slurried in water to remove fines and adjusted to the pH of maximum solubility of the protein solution being treated. In this experiment, pH 10 was used. The carbon was packed with water in a 2 x 20 cm glass column.

As shown in the flow chart of Figure 2-2, the protein solutions, "S" and "X", were solubilized at pH 10 and passed through the column at a flow rate of approximately 15 to 20 ml per minute. Constant flow rate was not strictly maintained.

Effluents were labelled "T" (from "S") and "Z" (from "X").

The "nitrile-rich" sample was also passed through the column at pH 10.

5. Analyses

a. Isothiocyanates

Isothiocyanate content of protein samples was determined by gas chromatographic analysis (Youngs and Wetter, 1967) with n-butyl isothiocyanate as internal standard. Peak areas were determined by the height-width method.

Response factors for ethyl (C_2) and allyl (C_3) isothiocyanates relative to n-butyl (C_4) isothiocyanate agreed with the theoretical values of 2 and 1.33 respectively. Since detector response can be assumed to be independent of unsaturation in the case of flame ionization detectors (from the above observation and as reported by Youngs and Wetter, 1967), response factors of 1.00 and 0.80 were used for 3-butenyl (C_4) and 4-pentenyl (C_5) isothiocyanates, respectively relative to n-butyl isothiocyanate.

Procedure: For samples already treated with myrosinase ("S, T, X, Z"), 2 or 3 ml aliquots of protein solution were withdrawn into glass stoppered test tubes immediately after preparation and shaken with 3 to 5 ml of methylene chloride containing internal standard n-butyl isothiocyanate. The methylene chloride phase was withdrawn for gas chromatographic analysis.

Samples containing glucosinolates (e.g. "R,U") were

treated as above except that after methylene chloride addition 1 ml of 0.1N sodium citrate-phosphate buffer pH 7.0 was added followed by about 5 mg of myrosinase. The reaction vessels were shaken periodically during a span of at least 3 hours before aliquots of the methylene chloride phase could be withdrawn for subsequent analysis. Operating conditions for the gas chromatograph are given in Table 2-1.

b. Glucosinolates

Glucosinolate content of protein fractions was determined by trimethylsilation and gas chromatography (Underhill and Kirkland, 1971) with trehalose as internal standard. Only the predominantly occurring glucosinolates: gluconapin, glucobrassicanapin, and progoitrin were quantitated. Peak areas were determined by height-width measurement.

Glucosinolate peaks on the gas chromatograms were identified by first preparing a stock solution of glucosinolates from rapeseed meal by 80% methanol extraction, and then partially separating them by column chromatography using first, DEAE Sephadex A-25 followed by affinity chromatography on arginine coupled Sephadex G-10 according to the method of Bjorkman (1972). Resolution of glucosinolates by the Sephadex columns was determined by silation and gas chromatographic analysis of effluent fractions. From the elution patterns, three samples labelled "Q_1, Q_2 and Q_3", were chosen. Each sample contained predominantly one glucosinolate

TABLE 2-1

Gas Chromatographic Operating Conditions

Instrument Microtech MT-220

Analysis	Isothiocyanates	Glucosinolates
Carrier gas	Nitrogen	Nitrogen
Flow rate (exit)	45-50 ml/min	45-50 ml/min
Column dimensions	1/4 in. X 6 feet stainless steel	1/4 in. X 6 feet stainless steel
Solid support	Chromosorb W A/W DMCS	Chromosorb W 80/100 mesh
Liquid phase	20% FFAP	5% OV-1
Temperatures:		
Inlet	175 C	250 C
Detector	200 C	275 C
Column	130 C	220 C

and was contaminated by only low levels of others. Myrosinase was added to the samples and released aglycones were identified and quantitated by gas chromatography (isothiocyanates; section IIB5a), and ultra-violet Spectroscopy (goitrin; section IIB5c). In the analysis, "Q_1" and "Q_2" were pooled since they contained gluconapin and glucobrassicanapin, both releasing only isothiocyanates on enzyme hydrolysis (Table I-I). Ideally, it had been hoped that a 100% separation of glucosinolates could be obtained in the three "Q" fractions, thereby allowing quantitation of glucosinolates in a chemical manner without the use of the myrosinase enzyme system. One such method would have been chemical degradation of glucosinolates by sulphuric acid followed by glucose determination by the anthrone procedure (Gmelin, 1954). However, since time did not permit further work on isolation, quantitation of glucosinolate content of the standard solutions was dependent on efficiency of the enzyme system (see section IC).

The obtained data was used in the calculation of response factors for the individual glucosinolates with respect to the internal standard, trehalose. These were compared to response factors derived under actual experimental conditions; i.e., from parallel analyses of glucosinolates and released aglycones in protein solutions. For example, molar progoitrin content of "R" was equivalent to molar goitrin content of "S". Similarly, protein solutions "U" and "X" were compared.

Equivalent isothiocyanate content of "R" and "U" could not be determined from respective analyses of "S" and "X" since significant evaporation of isothiocyanates had occurred during overnight myrosinase hydrolysis (section IIB3). There was also the possibility of protein-isothiocyanate interaction (Bjorkman, 1973) which would further decrease detectable isothiocyanate content. Therefore, in this analysis, myrosinase was added to aliquots of "R" and "U" as described later in this section.

Analyses were performed on freeze-dried protein fractions "R" and "U" as well as on individual aliquots of protein solutions to ensure accuracy of determined response factors.

Procedure: Internal standard, trehalose was added to 2 or 3 ml aliquots of neutralized protein in ground glass stoppered test tubes. Since in the final gas chromatogram, a low broad interfering peak was present in the vicinity of the trehalose peak, it was necessary to use a concentration of trehalose which would produce a peak of larger size than that of the glucosinolate peaks, even to the point of requiring a change of attenuation. By this procedure, error from the interference peak was minimized.

After addition of the internal standard, the solutions were mixed thoroughly and evaporated to dryness under a stream of nitrogen. Remaining traces of moisture were removed by

vacuum desiccation.

For analysis of dry samples, approximately 5 to 10 mg of myrosinase-free sample was accurately weighed into a ground glass stoppered test tube followed by 2 to 3 ml of diluted trehalose standard. The mixture was then treated as the "protein solution" already described.

The dried samples were silated by addition of 1 to 2 ml of anhydrous pyridine, 0.5 ml hexamethyl disilazane, and 0.25 ml chlorotrimethylsilane. The reaction vessels were immediately stoppered and placed in a 97°C oil bath overnight. After this reaction period, pyridine and excess silation reagents were removed by vacuum distillation at room temperature; 1 to 2 ml of iso-octane was added to take up silated products; the sample was filtered; and gas chromatographic analysis was performed on the filtrate. Injection size varied between 2 to 7 microlitres.

Operating conditions for the gas chromatograph are given in Table 2-1. Quantitation of glucosinolates was achieved by the following formula:

millimoles glucosinolate in sample =

$$\frac{\text{Millimoles trehalose added} \times \text{response factor} \times \text{area of glucosinolate peak}}{\text{(area of trehalose peak)}}$$

c. Goitrin

Goitrin content of protein samples was determined after freeze-drying, by ultra-violet analysis according to a modified method of Youngs and Wetter (1967). Log molar absorptivity coefficient for goitrin was assumed to be 4.25 as reported by Tookey et al. (1965).

Since samples being analyzed ("S, T, X, Z") had already been treated with myrosinase at pH 7.2 during preparation, no further treatment was necessary (see section II5Cb).

Procedure: Approximately 20 to 40 mg of sample was weighed into a glass stoppered test tube followed by 3 drops of water. When the sample was thoroughly wetted, 1 ml of methylene chloride was added to the test tube and the contents mixed on the Vortex mixer for 1 minute. When the methylene chloride phase cleared, 0.5 ml was withdrawn and mixed with 5 ml of absolute ethanol, the mixture was allowed to stand for 3 hours, and absorbance was determined in the range of 200 to 275 nm on the Unicam Ultraviolet Spectrophotometer SP 1800 against a blank of 0.5 ml methylene chloride in 5 ml ethanol.

Absorbance was calculated by drawing a baseline to the curve (Figure 2-3) and measuring the net absorbance at the maximum (243-245 nm).

d. Nitriles

The change in nitrile content due to carbon treatment

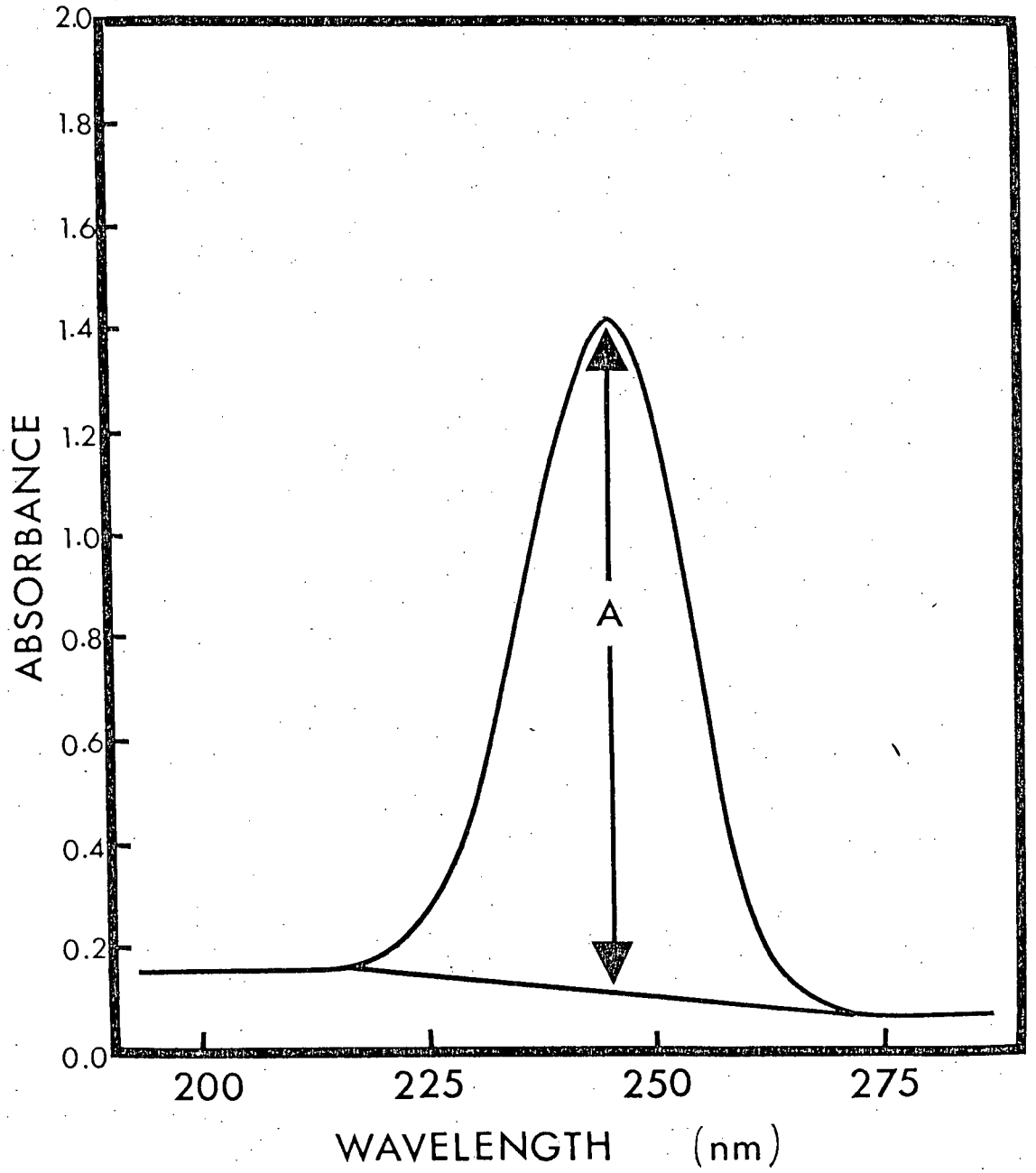


FIGURE 2-3 Ultraviolet Spectrum of Goitrin

of the "nitrile-rich" sample (preparation described in section IIB3) was determined by measurement of the nitrile peak height at 2257 cm^{-1} (4.43 μm) (Daxenbichler et al., 1966) obtained from the infra-red spectra run before and after treatment. The instrument used was the Perkin Elmer Model 475 Grating Spectrophomometer equipped with 0.5 mm sodium chloride cells.

Procedure: Samples before and after treatment were collected, isoelectrically precipitated, and centrifuged. Aliquots of supernatant (50 to 100 ml) were withdrawn and extracted 3 times with 3 volumes of chloroform. The extract was dried over sodium sulfate, filtered, evaporated to dryness under a stream of nitrogen and vacuum dessicated. The oil residue was dissolved in 1 ml of chloroform for infra-red analysis. Chloroform was used in the reference cell.

6. Carbon Column Capacity

Dry activated carbon (Whitco 209, 3 g), was packed in a 1 x 7 cm glass column as described in section IIB4. An aqueous solution of allyl isothiocyanate (approximately 50 mg per litre) was passed through the column at a rate averaging 12 ml per minute. Aglycone was detected in the effluent by measurement of absorbance in the range of 200 to 250 nm on the Unicam Ultra Violet Spectrophotometer. Although allyl isothiocyanate is not predominant in rapeseed, it was chosen as the aglycone for this experiment due to its availability

and its chemical structure. The presence of an olefinic double bond in this compound should make its adsorption characteristics somewhat analogous to those of the naturally occurring aglycones, 3-butenyl, and 4-pentenyl isothiocyanates.

Although the carbon capacity may be affected by protein or other substances present in rapeseed, the value obtained from the described procedure should still provide some indication of the true capacity of the carbon.

C. RESULTS AND DISCUSSION

1. Efficiency of the Process

a. Isothiocyanate and goitrin content

A typical gas chromatogram of isothiocyanates released from rapeseed meal is shown in Figure 2-4. Corresponding r.f. values of these compounds with respect to n-butyl isothiocyanate appear in Table 2-2 below. The standard was eluted approximately 8 1/2 minutes after injection.

TABLE 2-2

Gas Chromatographic r.f. values of Isothiocyanates with respect to n-butyl Isothiocyanates

Aglycone	r.f. ± S.D.*
3-butenyl isothiocyanate	1.319 ± 0.003
4-pentenyl isothiocyanate	1.911 ± 0.009
n-butyl isothiocyanate (standard)	1.000

* S.D. calculated from formula given in Table 2-3.

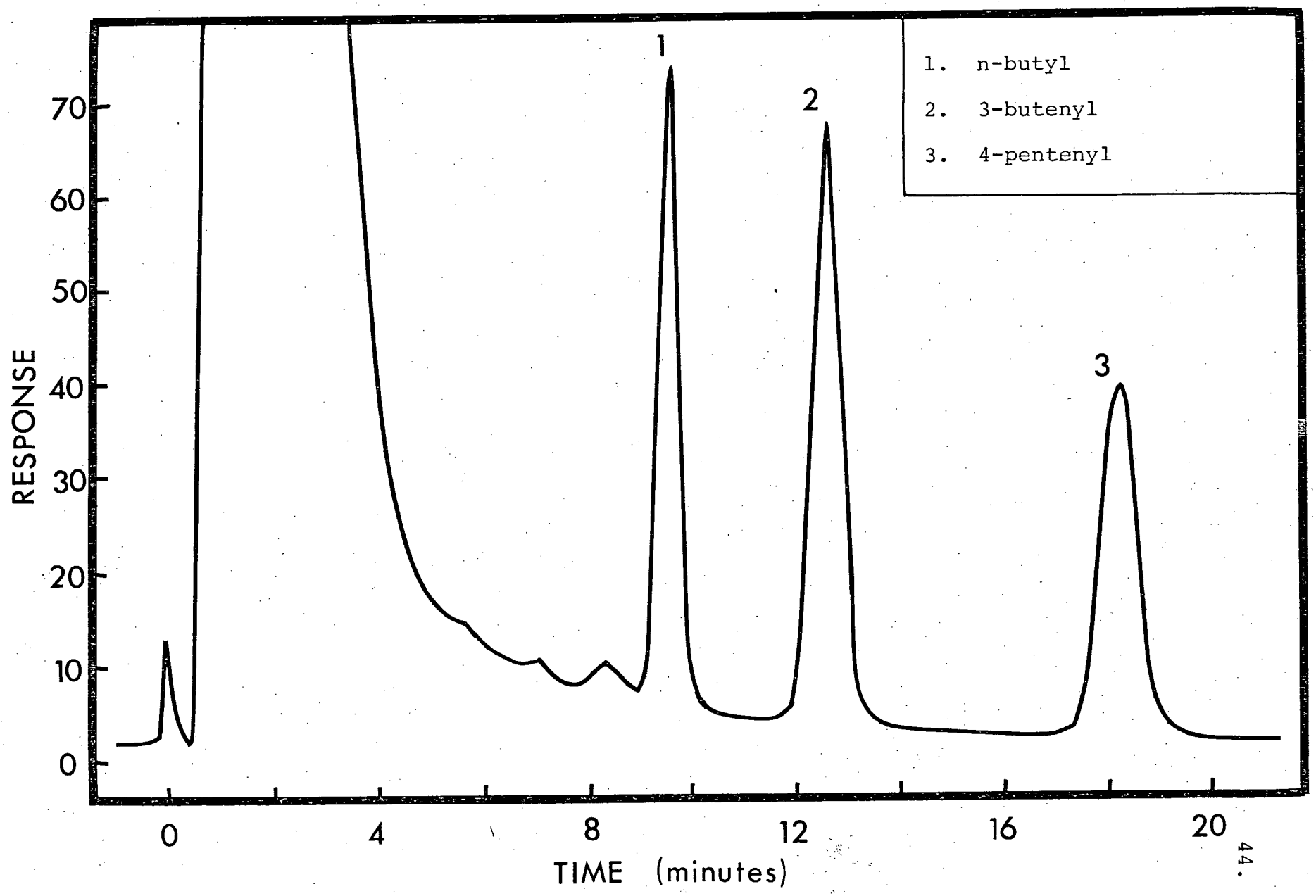


FIGURE 2-4. Gas Chromatogram of Isothiocyanates from Rapeseed Meal

Sampling of protein solutions was performed as soon as possible after preparation of minimize loss of isothiocyanates by evaporation. Triplicate or quadruplicate analyses of two samples were normally sufficient to provide acceptable results.

Aglycone content in mg per gram dry weight of protein fractions (see Figure 2-1, 2-2 or Appendix 1) at different stages of preparation appears in Table 2-3. Both median and average values with standard deviations are presented. Average values were used for calculation of "Total Aglycone Content" and for comparison purposes.

Aglycone levels were found to be near the detection limit in some fractions. For example, in protein extract "R" neither 3-butenyl nor 4-pentenyl isothiocyanate were detectable before myrosinase treatment. After treatment in the presence of methylene chloride to extract liberated isothiocyanates, a value of 16.84 mg aglycone per gram dry weight was obtained. This figure appears in Table 2-3 opposite "R+ myrosinase". The value for goitrin, 6.37 mg/ml included in this total was derived from the analysis of goitrin in fraction "S". Since "S" was prepared from "R" by the addition of myrosinase at pH 7.2, the molar goitrin content of both was assumed to be identical. Similarly, the value for goitrin of "U+ myrosinase" was derived from the analysis of goitrin in "X".

The discrepancy in isothiocyanate content between protein extracts "R+ myrosinase" and "S" and protein isolates

TABLE 2-3

Aglycone Content of Protein Fractions (mg/g)†

	3-butenyl Isothiocyanate		4-pentenyl Isothiocyanate		5-vinyl-2-oxazolidinethione (goitrin)		Total
	Median	Average	Median	Average	Median	Average	Average
R	N.D.	N.D.	N.D.	N.D.	0.07	0.07 ± 0.01	0.07
S	1.30	1.33 ± 0.10	1.49	1.52 ± 0.07	6.37	6.37 ± 0.04	9.22
T	0.034	0.036 ± 0.008	0.023	0.025 ± 0.003	0.350	0.356 ± 0.007	0.417
X	0.156	0.161 ± 0.012	0.116	0.118 ± 0.020	0.502	0.506 ± 0.015	0.785
Z*	N.D.	N.D.	0.006	0.006 ± 0.0002	N.D.	N.D.	0.006
R + myrosinase	6.68	6.68 ± 0.097	3.75	3.79 ± 0.099	6.37††	6.37†† ± 0.04	16.84
U + myrosinase	0.744	0.753 ± 0.02	0.578	0.573 ± 0.038	0.502††	0.506†† ± 0.015	1.83
Flour **		0.22		0.21		0.15	0.58

$$SD = \left(\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n - 1} \right)^{1/2}$$

* Concentrations at detection limit

** Flour prepared by Tape et al., 1970. Equivalent Aglycone content.

N.D. Not detected

† Median and average ± S.D.

†† Assumed equal to respèctive "S" and "X" values.

"U+ myrosinase" and "X" can be attributed to two factors: evaporation of isothiocyanates from "S" and "X" during their preparation which entailed overnight stirring at pH 7.2 in the presence of myrosinase; and to protein-isothiocyanate interaction (see section IB5 and IlA).

The final product of the process, "Z", contained only trace amounts of free aglycones. Goitrin and 3-butenyl isothiocyanate concentrations were below the detection limit of the methods employed in the analyses, and only 0.006 mg 4-pentenyl isothiocyanate per gram of protein material was detectable.

For comparison purposes, rapeseed flour prepared by the Food Research Institute (Ottawa) according to the method of Tape et al. (1970) was analyzed by gas chromatography of glucosinolates (section IIB5b) and the results converted to an equivalent aglycone content. The value from Table 2-3 is 0.58 mg aglycones per gram of flour.

A complete comparison of aglycone content among all fractions is given in Table 2-4. Values in the table below the dotted line were derived by dividing detected aglycone content of protein fractions in column "a" (left column of Table 2-4) by detected aglycone content of protein fractions in row "b" (top row of Table 2-4) and multiplying by 100 to convert to percent. For example, the value of 10.9% appearing opposite "U+ myrosinase" and under "R+ myrosinase" was calculated by dividing 1.83 mg/gm by 16.84 mg/gm and

TABLE 2-4

Comparison of Aglycone Content Among Protein Fractions Expressed as Percent Change* (between "a" and "b")

a \ b	R + myrosinase	S	U + myrosinase	X	T	Z**
R + myrosinase		45.2	89.1	95.3	97.5	99.96
S	54.8		80.2	91.5	95.5	99.94
U + myrosinase	10.9	19.8		57.1	77.2	99.67
X	4.7	8.5	42.9		46.9	99.24
T	2.5	4.5	22.8	53.1		98.56
Z**	0.04	0.06	0.33	0.76	1.44	

* Values in this table calculated by:

1. Below dotted line = $(\frac{a}{b})$ X 100
2. Above dotted line = $(100 - \frac{a}{b})$ X 100

** Concentrations at detection limit.

multiplying by 100. These numbers (1.83 and 16.84) were obtained from the "Total Aglycone Content" column of Table 2-3 opposite the respective protein fractions. The ratio, 10.9% can be interpreted as the percent of original aglycone remaining after isoelectric precipitation of protein extract "R" to produce protein isolate "U". In this case, the figure actually represents "equivalent aglycone change" since the aglycones are in the glucosinolate form during the preparation of "U".

The fraction of original isothiocyanates and goitrin remaining after column treatment can be determined by comparing "S" and "T" or "X" and "Z". The values from Table 2-4 are 4.5% and 0.76% respectively and represent the efficiency of the carbon column step.

The potential efficiency of the entire process can be determined by comparing "Z" to "R+ myrosinase". The value from the lower half of the table is 0.04%, which means that in this case, the entire process of isoelectric precipitation followed by myrosinase addition and finally carbon treatment was 99.96% effective in aglycone removal. This value includes evaporation and possible protein-isothiocyanate reaction products. A similar comparison eliminating this error could be made by comparing "S" to "Z". From the table, the process evaluated in this manner is 99.94% effective. These figures can also be derived from the top of Table 2-4 by reading the value opposite "R+ myrosinase" and under "Z".

The top half of Table 2-4 represents percent removal of aglycones between any two fractions and was calculated by the formula 100%-b/a.

Comparing "R" and "U" using the top of Table 2-4, 89.1% of the aglycones are lost in the isoelectric precipitation step. Also from the table, column treatment of "S" and "X" resulted in 95.5% and 99.2% aglycone removal, respectively. Column treated protein extract "T" was not isoelectrically precipitated to produce an isolate analogous to "U". Although such a step would have reduced aglycone content considerably, it is not possible to accurately predict the actual reduction which can be expected since aglycones have different solubility properties than those of glucosinolates. Carbon adsorption of protein-isothiocyanate reaction products mentioned in section IIA should also be investigated.

b. Glucosinolate content

A typical gas chromatogram of silated glucosinolates derived from rapeseed meal appears in Figure 2-5. Corresponding r.f. values for the silated compounds with respect to silated standard trehalose appear in Table 2-5. It was found that on the OV-1 column, internal standard hexacosane, used by Underhill and Kirkland (1971), was inadequately resolved from glucosinolates and could therefore not be used in the available system. After only limited success with a range of other compounds, trehalose was found to be most acceptable as an internal standard, even though

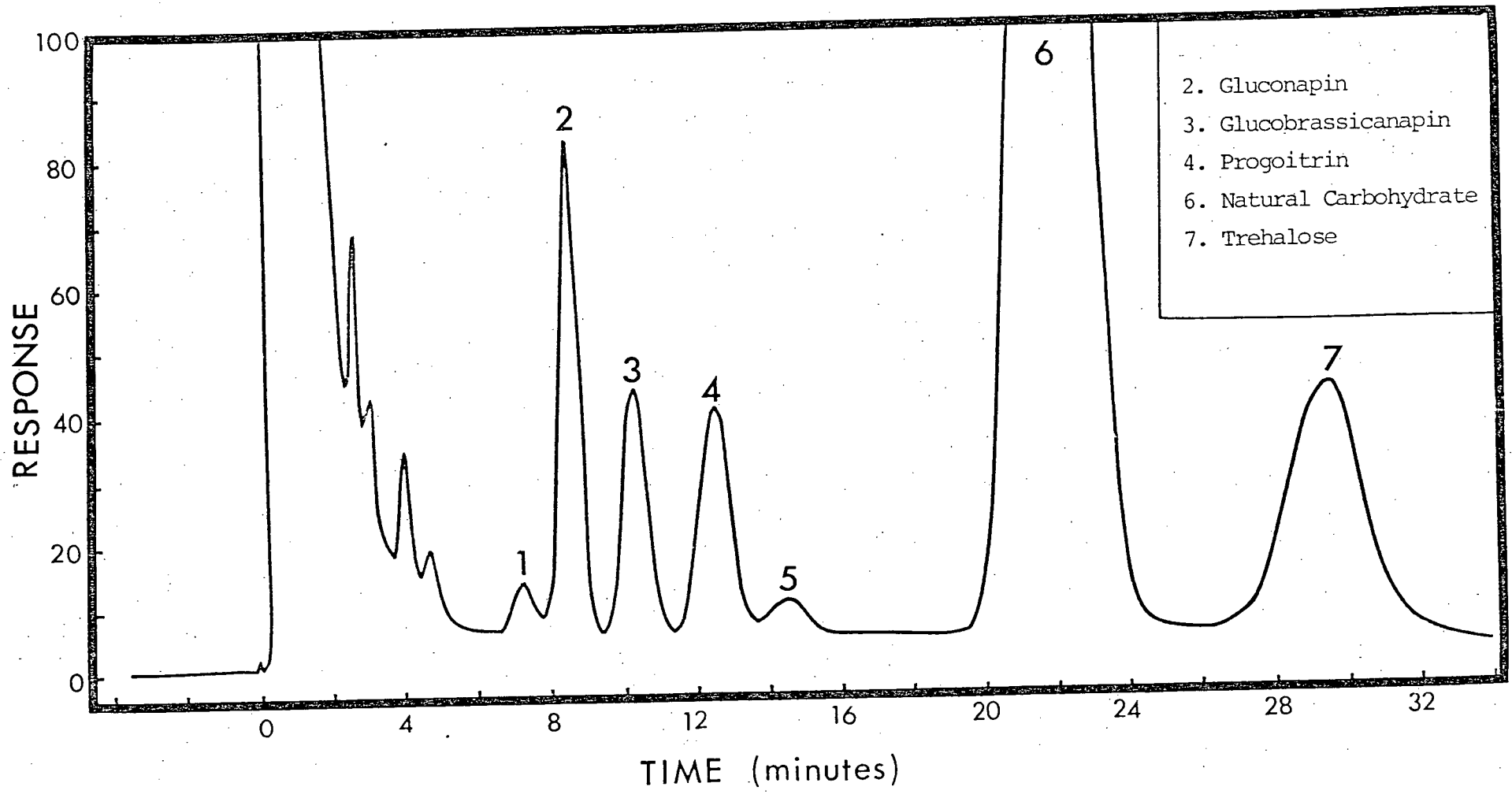


FIGURE 2-5 Gas Chromatogram of Glucosinolates from Rapeseed Meal

there was a slight interference problem as described in section IIB5b. Only peaks 2, 3, 4, and 7, corresponding to the compounds 3-butenyl glucosinolate (gluconapin), 4-pentenyl glucosinolate (glucobrassicanapin), 2-hydroxy-3-butenyl glucosinolate (progoitrin), and trehalose were determined quantitatively. The standard was eluted approximately 30 minutes after injection.

TABLE 2-5

Gas Chromatographic r.f. Values of Silated Glucosinolates with respect to Silated Trehalose

Glucosinolate	r.f. ± S.D.*
Gluconapin	0.2961 ± 0.0015
Glucobrassicanapin	0.3568 ± 0.0010
Progoitrin	0.4298 ± 0.0009
Trehalose (standard)	1.0000

* S.D. calculated from formula given in Table 2-3

From Figure 2-5, peaks 1 and 5 were glucosinolates as determined by their susceptibility to myrosinase hydrolysis, but were not present in high enough concentrations to facilitate isolation by the employed separation techniques. Peak 6 was a naturally occurring carbohydrate of rapeseed meal which, from its position in the gas chromatogram, was assumed to be a disaccharide. The concentration of this

compound was very low in protein isolate fractions.

Experimentally derived response factors of glucosinolates with respect to trehalose appear in Table 2-6. Comparison of glucoinolate peak areas (relative to the trehalose standard peak area) with enzymatically released isothiocyanates and goitrin in aliquots of protein solutions resulted in some variation of determined response factors. Accuracy was limited by number of steps required in preparation of samples for analysis (sections IIB5a, b and c), nature of the protein solution, and dependence on the enzyme system (section IIB5b). Samples analyzed for response factor determination appear to the left of Table 2-6.

Unfortunately, the concentration of standard trehalose used in "$Q_1 + Q_2$" and "Q_3" (section IIB5b) was too low to eliminate error caused by the presence of interfering compounds, the peaks of which appeared at positions in the gas chromatogram corresponding to that of trehalose. Additional "Q" samples were not available for further analyses incorporating higher concentrations of trehalose to eliminate the problem. Therefore, it was decided to use average response factors derived from analyses on "R" and "U" since these represented results obtained under typical experimental conditions.

Gas chromatograms of "$Q_1 + Q_2$" and "Q_3" appearing in Figures 2-6 and 2-7 demonstrate the degree of separation of glucosinolates achieved by the Sephadex column separation

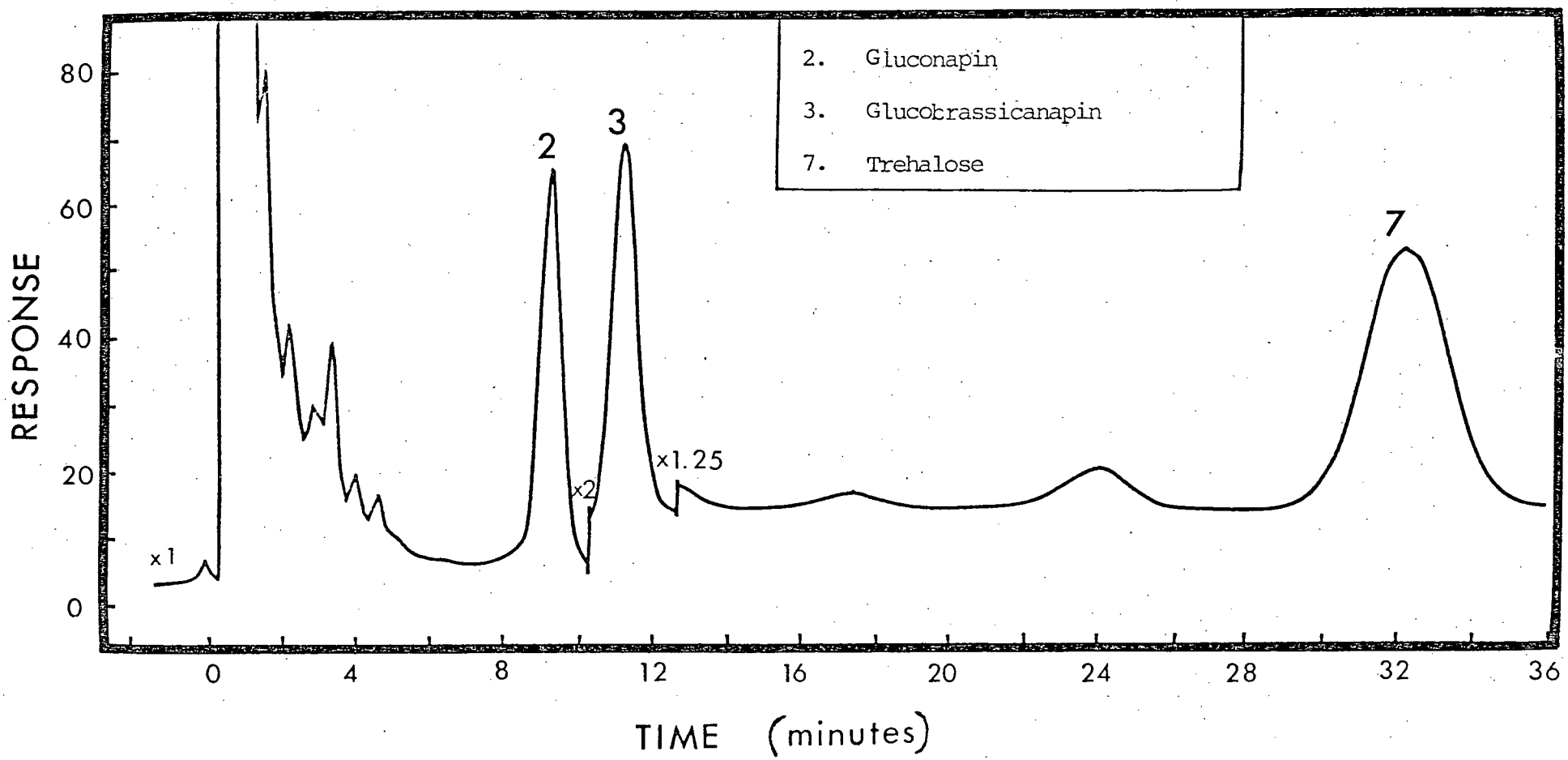


FIGURE 2-6. Gas Chromatogram of Fraction "$Q_1 + Q_2$". (Attenuation notation (e.g. X 2) signifies further increase in sensitivity).

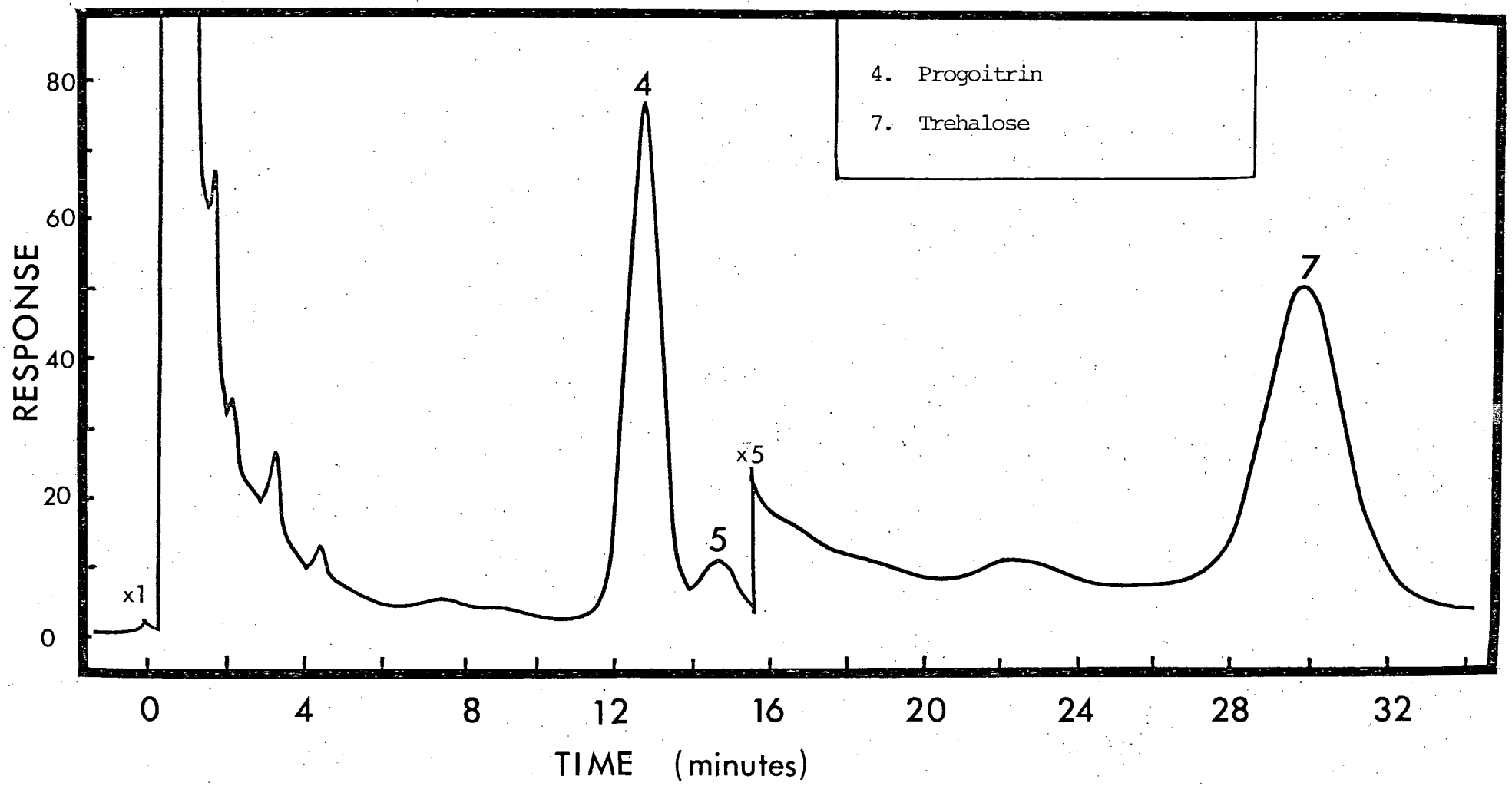


FIGURE 2-7. Gas Chromatogram of Fraction "Q_3". (Attenuation notation (e.g. X 5) signifies further increase in sensitivity.

technique described in section IIB5b.

TABLE 2-6

Gas Chromatographic Molar Response Factors for Silated Glucosinolates with respect to Silated Trehalose

Source	3-butenyl Glucosinolate (Gluconapin)	4-pentenyl Glucosinolate (Glucobrassicanapin)	2-hydroxy-3-butenyl Glucosinolate (Progoitrin)
"R"	1.54	1.32	2.00
"U"	1.48	1.37	2.01
"$Q_1 + Q_2$"	1.6	1.4	-
"Q_3"	-	-	1.7
Accepted	1.51	1.34	2.00

Glucosinolate content of protein extract "R", protein isolate "U", protein extract "S", and rapeseed flour prepared by the Food Research Institute, Ottawa, by the method of Tape et al. (1970), appears in Table 2-7. Values of the glucosinolate content of "U" and "R" were derived from the conversion of molar values of released aglycones listed in Table 2-4.

Protein extract "S" contained trace amounts of gluconapin (gas chromatogram) immune to further myrosinase hydrolysis. It was assumed that this residual level was due

TABLE 2-7

GLUCOSINOLATE CONTENT OF PROTEIN FRACTIONS (mg/g) †

	Gluconapin (3-butenyl)	Glucobrassicanapin (4-pentenyl)	Progoitrin (2-hydroxy-3-butenyl)	Total
R*	24.3	12.7	21.2	58.2
U*	2.7	1.9	1.7	6.3
S**	Trace	N.D.	N.D.	Trace
Meal Flour	10.1 ± 0.10	5.6 ± 0.07	9.1 ± 0.18	24.8
Extracted Residue	0.2 ± 0.01	0.2 ± 0.06	0.2 ± 0.01	0.6
Flour (Tape et al., 1970)	0.8 ± 0.06	0.7 ± 0.07	0.5 ± 0.03	2.0

† As potassium salts average ± S.D.
S.D. calculated from formula, Table 2-3.

* From aglycone content (Table 2-3).

** Not degradable by myrosinase

N.D. Not detected

to the presence of the desulfonated form of the glucosinolate reported by Underhill and Kirkland (1971) which cannot be converted to its respective aglycone by myrosinase hydrolysis. Glucosinolates of all other fractions were susceptible to myrosinase treatment. The result of this analysis therefore proved the myrosinase treatment of "R", described in section IIB3, to yield protein extract "S", to be essentially complete.

The hull-rich residue remaining after protein extraction contained only 0.60 mg/g total glucosinolates compared to 58.2 in protein extract "R". Meal flour, from which "R" was derived, contained 24.8 mg/g glucosinolates. This value is approximately half that of "R", indicating that there was a 2-fold concentration of glucosinolates during the preparation of "R". This figure is dependent on the extent of extraction of glucosinolates from the meal flour (almost 100%; see value for extracted residue, Table 2-7), and the total yield of extracted material from the meal, (approximately 45-50% of the meal flour). The greater the yield of protein material in any process, the lower will be the concentration effect on the glucosinolate content of the extract.

Detoxified rapeseed flour prepared by the Food Research Institute, Ottawa, (Tape et al., 1970) had a glucosinolate content approximately 1/3 that of untreated protein isolate "U".

c. Nitrile content

Infra-red spectra of chloroform extracts of "nitrile-rich" protein extracts (preparation described

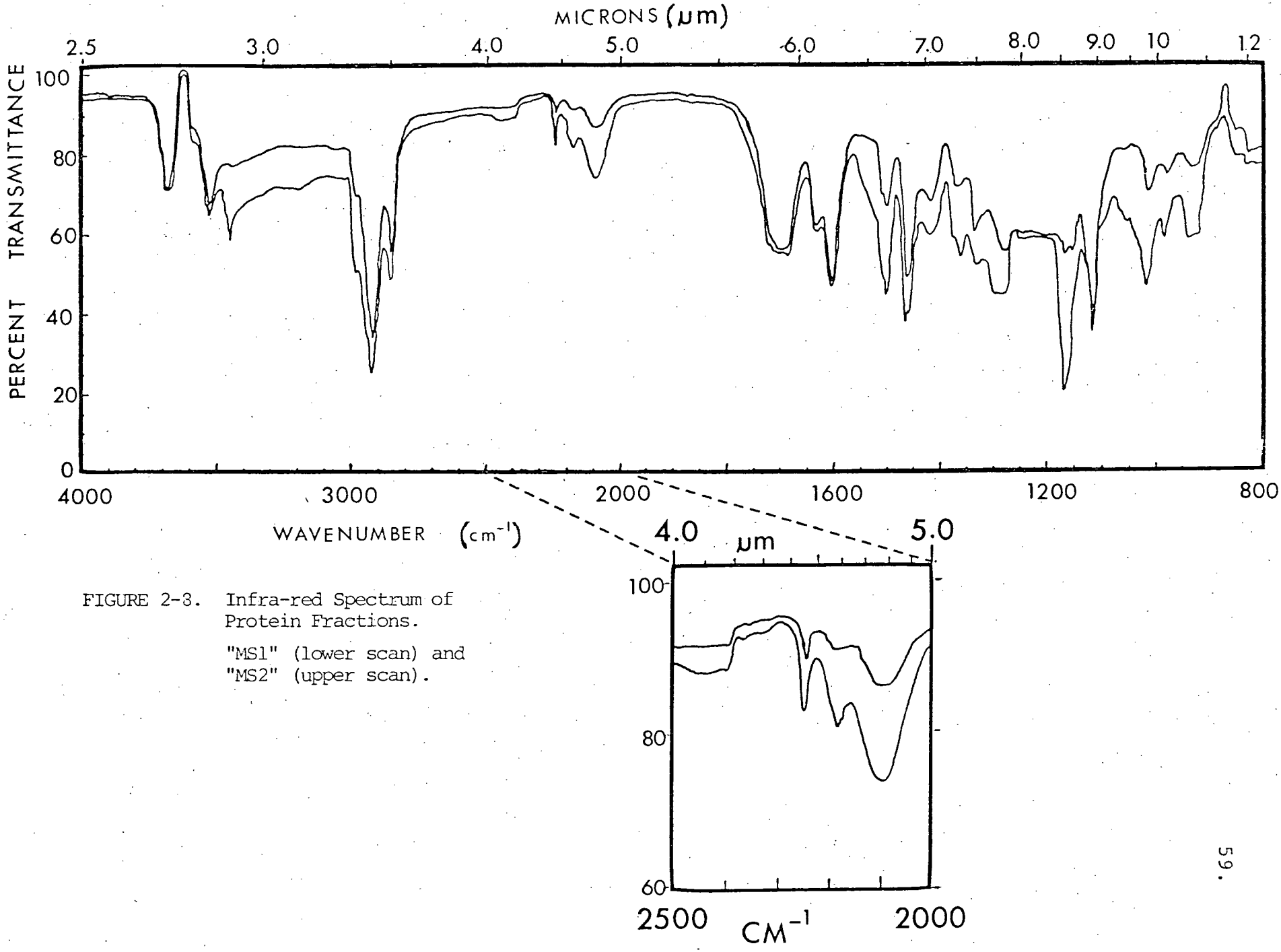


FIGURE 2-3. Infra-red Spectrum of Protein Fractions.

"MS1" (lower scan) and "MS2" (upper scan).

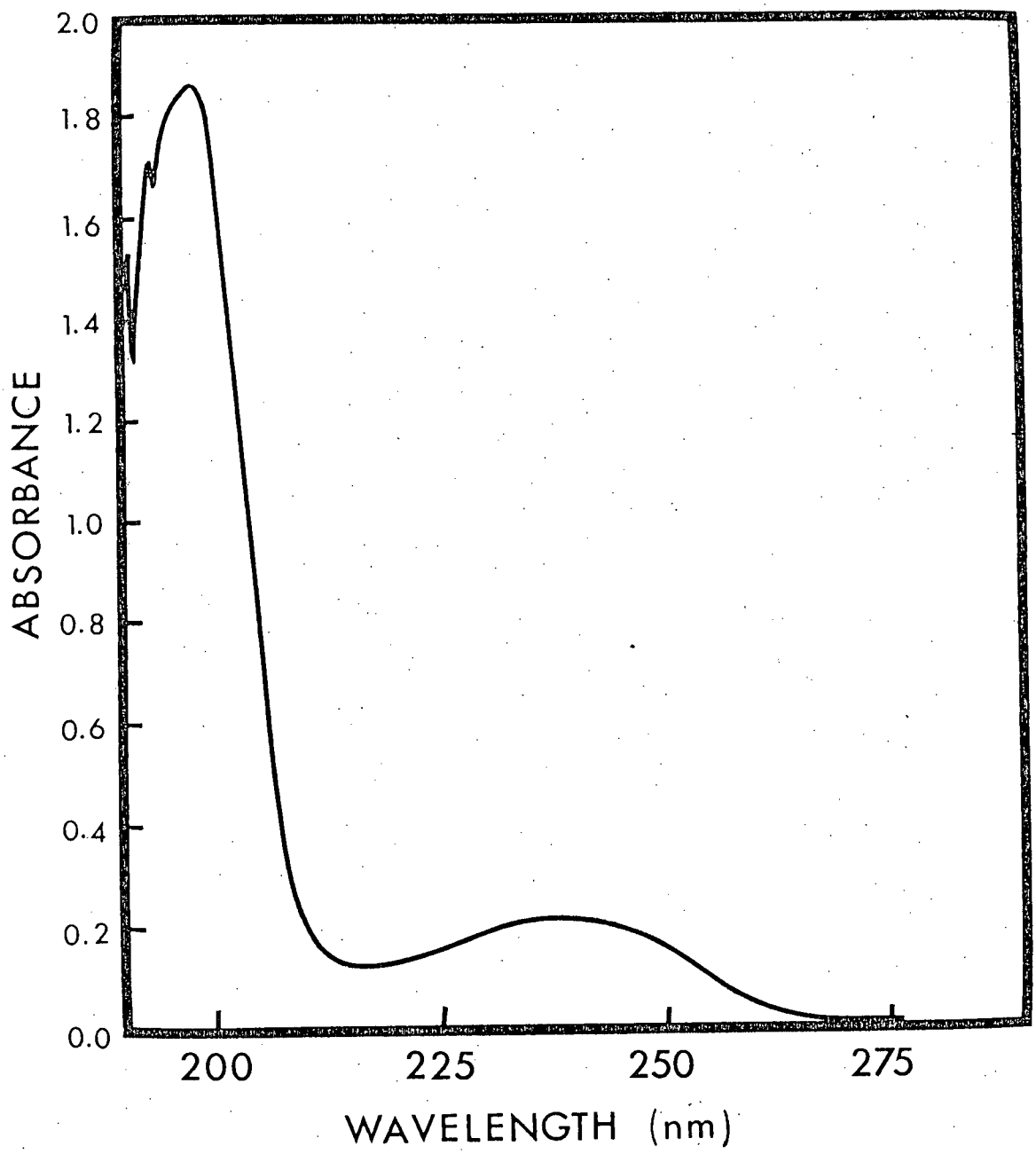


FIGURE 2-9. Ultra-violet Spectrum of Allyl Isothiocyanate

section IIB3) before and after carbon column treatment appear in Figure 2-8. The size of the weak nitrile peak at 4.43μm characteristic of the C≡N stretch was used to determine change of nitrile content due to carbon column treatment. From the decreased height of this peak, it was assumed that partial nitrile adsorption by the carbon had occurred. Peak height decreased by approximately 40%.

Intense peaks at 6.63μm and 8.57μm characteristic of goitrin disappeared or decreased markedly after treatment indicating that goitrin was indeed adsorbed by the column.

The degree of purity of the extract would not justify the formulation of further conclusions from the obtained spectra.

2. Adsorption Capacity of the Carbon

The obtained ultra-violet spectrum of allyl isothiocyanate appears in Figure 2-9. Carbon capacity was determined to be approximately 380 to 400 mg allyl isothiocyanate per gram of carbon. Using "R+ myrosinase" and U+ myrosinase" aglycone values, the carbon should be capable of detoxifying approximately 20 g of protein extract or 200 g of isolate per gram of carbon.

D. CONCLUSIONS AND GENERAL DISCUSSION

The following conclusions can be drawn from the results:

a. The myrosinase conversion of glucosinolates to isothiocyanates and goitrin was essentially complete

except for the de-sulfo analogues of glucosinolates which are not considered harmful due to their immunity to myrosinase hydrolysis.

b. Since only partial carbon adsorption of nitriles occurred, nitrile formation in the detoxification process should be avoided by incubation (VanEtten et al., 1966) and maintenance of neutral or basic pH during hydrolysis (Daxenbichler et al., 1966).

c. Free isothiocyanates and goitrin were adsorbed by the carbon column with greater than 95% and 99% efficiency for protein extract and protein isolate solutions respectively at pH 10. Presumably, isoelectric precipitation of the carbon treated protein extract "T" would lower aglycone content to a level comparable to carbon treated protein isolate "U" since isoelectric precipitation was found to decrease glucosinolate levels by 89% in the above reported experiment. Treatment of isolate compared to extract treatment would have the advantage of increasing carbon lifetime due to the lower aglycone content of the isolate compared to that of the extract.

d. The levels of free aglycones in the finished product "Z" were very low, 0.006 mg 4-pentenyl isothiocyanate per gram dry weight. Since this level was near the

detection limit of the methods used for analysis, there is a possibility that trace amounts of 3-butenyl isothiocyanate and goitrin were still present. However, these levels should be considered safe for human consumption since according to Downey et al. (1967), 3-butenyl isothiocyanate and goitrin occur naturally in cabbage (Brassica oleracea) at levels of 0 to 3 and 0 to 7 mg/g respectively. These compounds are also found in other commonly ingested foods, (VanEtten, et al., 1969).

e. The by-product of the process, the extracted residue, contained only 0.6 mg/g glucosinolates and would therefore be an excellent material for incorporation into livestock feed providing the material was sufficiently digestable. The composition of this material should be further investigated.

f. Toxicity of isothiocyanate-protein interaction products (section IB5) should be investigated and their formation further minimized by decreasing myrosinase hydrolysis time. This could be achieved by an increase in temperature (Appelqvist and Josefsson, 1967) and possible addition of ascorbate (section IC). Duration of high pH treatments should also be strictly controlled.

g. In addition to the removal of aglycones, the carbon treatment removed natural rapeseed odor present in the protein extract and isolates. Palatability was also improved. However, no change in protein color (light brown to brown-green) was observed during carbon treatment.

CHAPTER III

MODIFICATION OF THE RAPESEED PROTEIN DETOXIFICATION PROCEDURE

A. INTRODUCTION

Although the process described in Chapter II was successful, a number of economic "shortcuts" were deemed possible. Proposed changes were:

1. Direct incorporation of mustard seed as a source of myrosinase to eliminate the need for enzyme extraction and purification.
2. Application of carbon treatment at various pH's to allow extension of the carbon detoxification process to all rapeseed protein isolates and decrease or eliminate isothiocyanate-protein interaction (section IB5).
3. Possible direct application of glucosinolate solutions to activated carbon, thereby completely eliminating the need for myrosinase treatment.

Experiments were designed to investigate the above possibilities. As well, the pH experiment included a study of isothiocyanate stability at various pH's.

A final "recommended detoxification process" was elucidated defining conditions for maximum detoxification of rapeseed protein isolates.

B. METHODS

1. Mustard as Source of Myrosinase

Although it would be possible to use intact rapeseed

as a source of myrosinase, it was decided to use mustard seed because of its higher enzyme activity (Lonnerdal and Janson, 1973).

Procedure: Rapeseed meal flour prepared as in section IIBla was slurried in ten volumes of water and blended for 3 minutes in the Sorvall Multimixer at maximum setting. The mixture was adjusted to pH 7.2 and freshly ground undefatted white mustard seed, 1% by weight of meal flour was added. pH 7.2 was maintained overnight (35-40^{o}C) by stirring and autotitration with N NaOH. The slurried meal was then extracted at pH 10, isoelectrically precipitated at pH 5.3 to yield a protein isolate ("MS1") analogous to "U" or "X" of Chapter II, dissolved at pH 11, and treated by the activated carbon procedure described in Section IIB4.

Glucosinolate content of "MS1" was determined by gas chromatography (section IIB5b) to evaluate extent of myrosinase hydrolysis.

Isothiocyanate analyses (section IIB5a) were performed before and after column treatment to determine efficiency of carbon adsorption at pH 11. Treated "MS1" was labelled "MS2". The hull-rich extracted residue was also analyzed for isothiocyanate content.

2. Effect of pH on Carbon Adsorption of Isothiocyanates and on Isothiocyanate Stability.

Procedure: Commercial rapeseed meal was slurried

with ground white mustard seed and incubated overnight at pH 7.2 and 35-40°C, in the manner described in section IIIB1 above. The meal slurry was divided into 5 equal portions which were subsequently extracted at pH 12, 10, 7, 5, or 3 to yield characteristic protein extracts for each of the pH's. The extracts corresponded in nature to "R" or "S" prepared in section IIB1.

Each extract was then carbon treated (section IIB4) at its respective pH. Sampling before and after column treatment was performed as follows:

a. 3 ml protein solution + 5 drops pH 5.0, N sodium citrate buffer + 5 ml methylene chloride*.

b. Same as "a" but without citrate buffer.

c. Unbuffered protein solution (no carbon treatment), stored in cold room in stoppered Erlenmeyer flask.

Sample sets "a" and "b" were analyzed for isothiocyanate content (section IIB51) immediately after preparation and again 24 hours later. Set "c" was sampled 24 hours after preparation and isothiocyanate content determined in a manner analogous to the method used for set "b".

The experiment was performed only once in its entirety and must therefore be considered preliminary. Goitrin levels were not investigated.

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* Methylene chloride containing standard n-butyl isothiocyanate

3. Carbon Adsorption of Glucosinolates

a. Isolation of glucosinolates

A stock solution of glucosinolates was prepared according to the method of Greer (1962) from rapeseed meal by employing 75% acetone extraction of rapeseed meal. The extract was concentrated by flash evaporation and subsequently applied to a column of Amerlite IR-4B in the chloride form. After organic material was eluted from the column by exhaustive water washing, glucosinolates were eluted with 0.1 N NaCl. The effluent was evaporated to dryness on the flash evaporator and taken up in a small volume of ethanol. This solution served as the glucosinolate stock solution.

b. Preparation of protein isolate

Base soluble rapeseed protein isolate equivalent to "U" was prepared as in section IIB1b. This protein solution was further isoelectrically precipitated, centrifuged and redissolved in distilled water. The entire procedure was repeated a total of five times to remove all traces of glucosinolates from the protein which served as the "protein isolate fraction" for the experiment to follow.

c. Application to carbon column

Diluted glucosinolate stock solutions at pH 3, 7, and 10 were applied to carbon columns of corresponding pH's as described in section IIB4 and change in glucosinolate content determined by gas chromatography (section IIB5b).

Protein isolate was added to diluted glucosinolate stock solution and the mixture was applied to carbon columns at pH 10 and 7. The pH 7 sample was in the form of a protein slurry since the protein was only slightly soluble at this pH. Glucosinolate content was determined as described above.

A basic protein extract similar to "R" (section IIB1a) and whey from the pH 5.3 isoelectric precipitation of that extract were also applied to the carbon column at pH 10 and 5.3, respectively to determine efficiency of carbon adsorption of glucosinolates from native rapeseed protein solutions.

C. RESULTS AND DISCUSSION

1. Mustard

Glucosinolate analysis of "MS1" (see section IIIB1 or Appendix) revealed no detectable myrosinase degradable glucosinolate content, indicating that ground mustard seed was an effective source of myrosinase for the process. An investigation of the minimum level of mustard or minimum time required for complete glucosinolate degradation was not carried out since these factors would depend on the quality of mustard seed used.

Free 3-butenyl and 4-pentenyl isothiocyanates were present in "MS2" at 20.6% and 12.6% of their respective levels in "MS1" indicating that carbon treatment at pH 11 was less efficient than the pH 10 experiment described in Chapter II. Besides inefficiency of carbon adsorption of

isothiocyanates at this high pH, deterioration of protein quality could also occur. Therefore, pH 11 cannot be recommended for the detoxification process.

The insoluble residue remaining after extraction contained 3-butenyl and 4-pentenyl isothiocyanates at levels of 0.04 and 0.085 mg/g dry weight respectively. This material would therefore be suitable for incorporation into animal feeds providing it was palatable and digestable.

2. Effect of pH

a. Isothiocyanate adsorption

As shown in the graph of Figure 3-1, the column was greater than 93% effective in removal of isothiocyanates from rapeseed protein extracts at pH values below 10. pH 3 treatment was greater than 98% effective in total isothiocyanate removal. However, at pH 12, 27% and 46% of the original 3-butenyl and 4-pentenyl isothiocyanates remained in the column effluent.

Although the above results must be considered tentative due to lack of replication and the fact that a change of flow rate or temperature would affect reproducibility of the results, the observation that the efficiency of the column is greater than 93% over the range of pH 3 to pH 10 must be considered significant. The low isothiocyanate removal at pH 12 is credible since the values obtained in the previously described experiment for the pH 11 treatment of protein isolate agree closely (Figure 3-1). However, pH 10 carbon treatment of protein extract "S" to yield "T" (section IIB4) resulted in

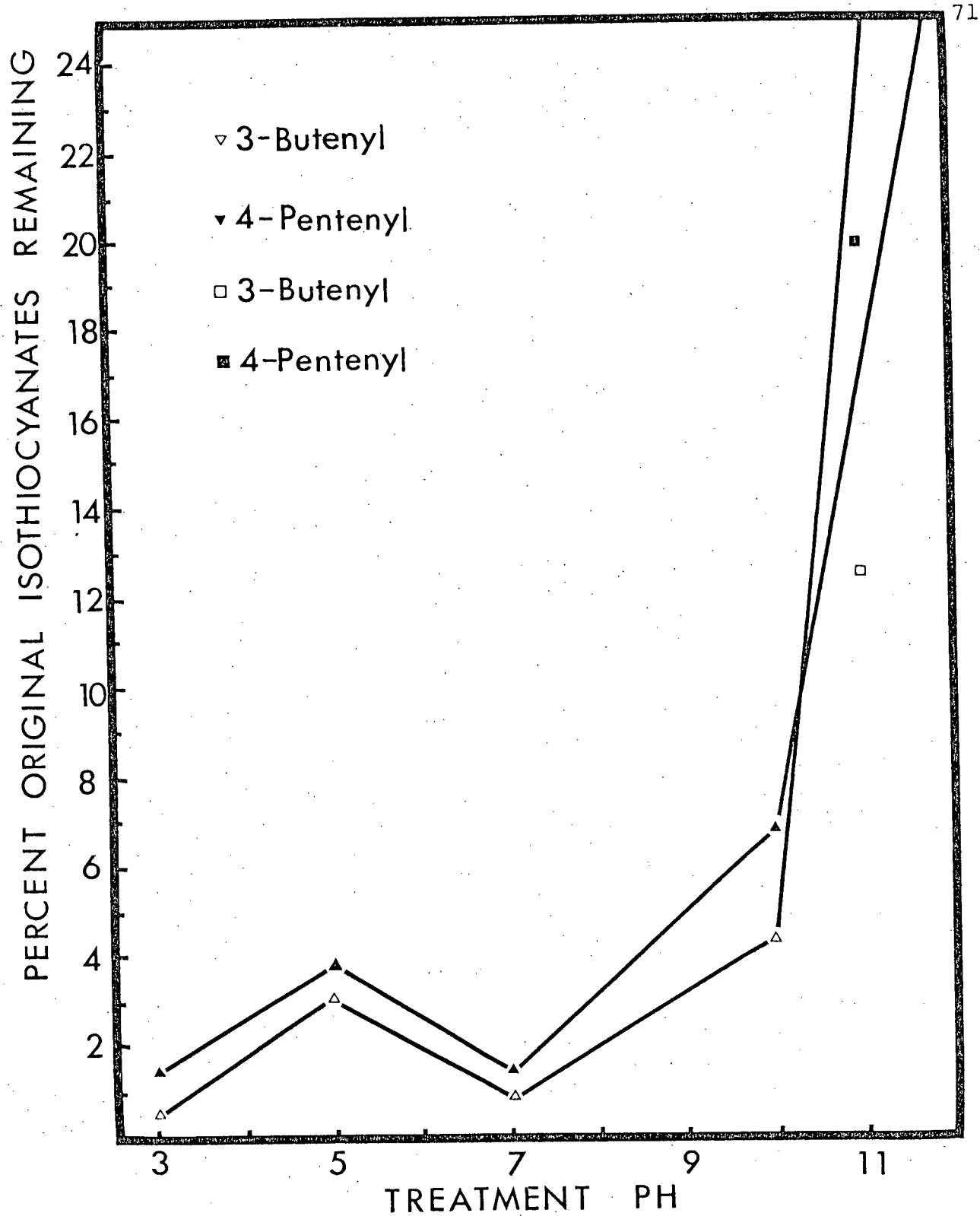


FIGURE 3-1. Effect of pH on Carbon Adsorption of Isothiocyanates

□■ From MS2 protein isolate

only 2.7% and 1.6% residual 3-butenyl and 4-pentenyl isothiocyanates in "T" compared to the original level in "S". These values are low compared to 4.4% and 6.8% obtained in the present study.

Since the curve rises steeply after pH 10, this region is probably extremely sensitive to operating parameters such as ionic strength of the solution, flow rate, temperature and other unknown factors and would therefore produce slightly variable results. A commercial process operating at this pH would have to be carefully controlled.

b. Isothiocyanate stability

No change in isothiocyanate content of individual samples was observed after 24 hours if the samples were stored over methylene chloride as in sets "a" and "b". Also, complete agreement was obtained for isothiocyanate content of corresponding samples of "a" and "b" indicating that in the tested cases, buffering had no effect on isothiocyanate extractability if extraction was performed immediately.

However, sample set "c" stored in the cold room and extracted after 24 hours, showed a decrease in isothiocyanate content from that of "a" and "b". The decrease was a function of pH as shown in graph of Figure 3-2. Decrease in detectable content of both 3-butenyl and 4-pentenyl isothiocyanates was linear between pH 5 and pH 10, confirming the findings of Bjorkmann (1973) who reported the isothiocyanate-protein interaction to be linearly correlated with pH in the range of pH 6 to pH 10. Highest remaining isothiocyanate content, i.e., region of highest isothiocyanate stability was pH 5.

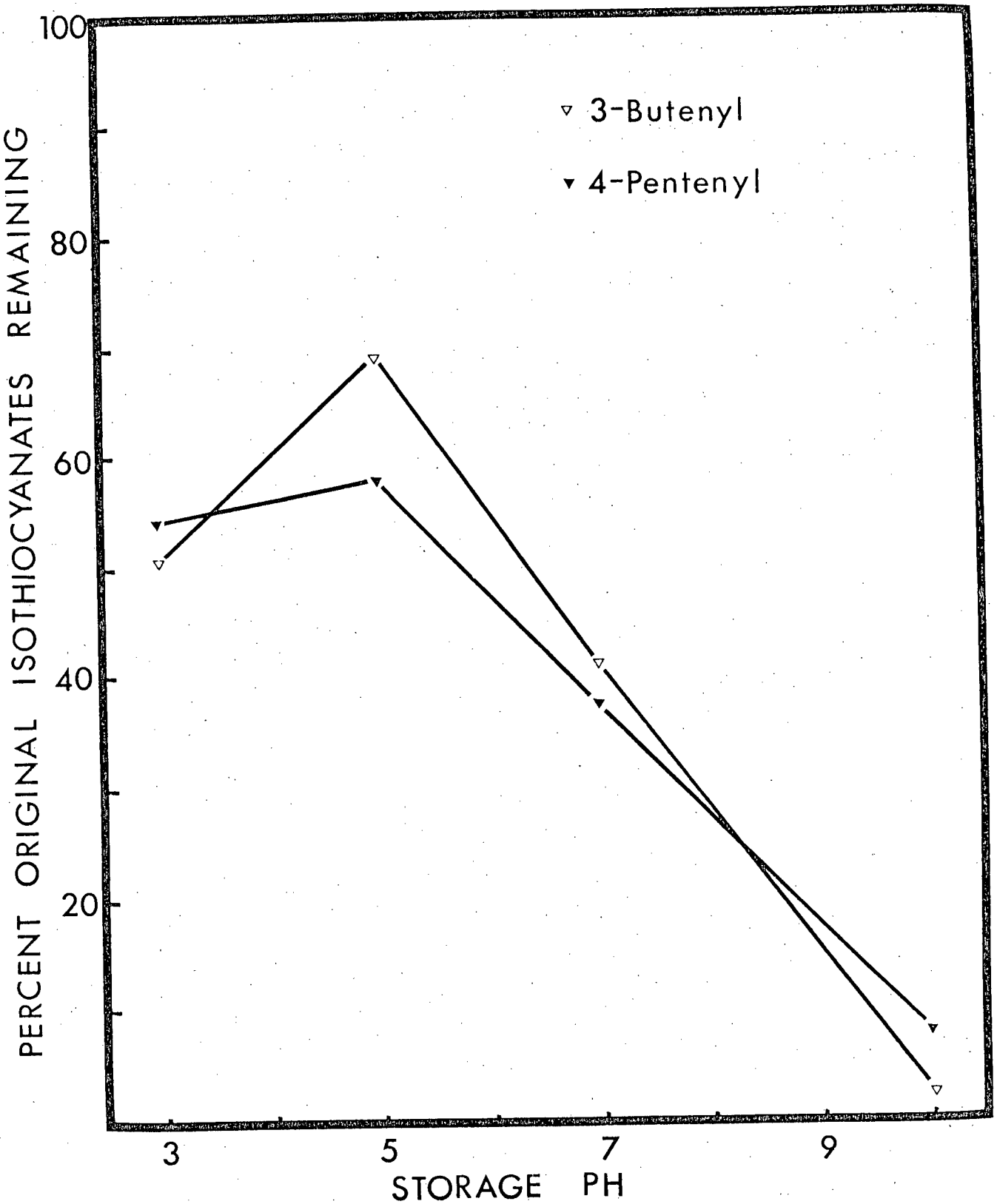


FIGURE 3-2. Decrease of Free Isothiocyanate Content After 24 hours Storage at 5°C.

Although much of the isothiocyanate loss can be attributed to evaporation, the isothiocyanate-protein interaction would be expected to proceed more completely at high pH where there is increased accessibility of sulphydryl, ε-amino, and terminal α-amino groups of the rapeseed protein (Bjorkmann, 1973). In this experiment the extent of interaction or loss of isothiocyanates was determined by comparing samples of "a" and "b" to samples "c" stored in the cold room for 24 hours. However, samples "a" and "b" had been prepared for approximately 1 hour before methylene chloride extraction was performed and it is not therefore known to what extent the interaction had proceeded in these solutions before sampling was complete. Recognizing this fact, and comparing it to the observation that after 24 hours storage, only 5% of the original free isothiocyanate content was detectable in the pH 10 sample of "c" due to assumed interaction, it may be speculated that an industrial process operating at pH 10 and requiring a total time interval of less than 1 hour would be relatively free from isothiocyanate-protein interaction product formation.

The presence of ammonium salts in the meal would compete with the isothiocyanate-protein interaction at high pH where free ammonia would be liberated from the salts to form thioureas with the isothiocyanates (section IB5). The entire problem of complex formation can be minimized by decreasing the myrosinase incubation time and minimizing the duration of high pH treatments. Such modifications would be

possible in high speed industrial situations. More work remains to be done in this area.

The fact that any pH in the range of pH 3 to 10 was feasible for isothiocyanate removal from protein isolates by the carbon column permits the extension of the process to all soluble rapeseed isolates without the pH 10 solubilization of all fractions previously advocated. Effect of pH on carbon adsorption of goitrin must be investigated.

As shown in the experiments in Chapter II, no improvement in protein color was observed at any pH. Presumably the adsorption process would be more efficient at higher temperatures.

3. Carbon Adsorption of Glucosinolates

The results of carbon column treatment of glucosinolate solutions is shown in Table 3-1. Stock glucosinolate solution was completely adsorbed by the column at pH 10, and 7 while pH 3 column treatment only partially decreased glucosinolate content. When protein was added to the system, the column had absolutely no effect on glucosinolate content in any of the tested cases. The only logical explanation for this phenomenon is that some type of interaction exists between protein and glucosinolate thereby interfering with carbon adsorption. However, Bjorkmann (1973) reported no interaction between protein and glucosinolate. In the above reported experiment, the glucosinolate interacting protein

TABLE 3-1

Effect of Carbon Treatment on Solutions Containing Glucosinolates

SOLUTION	DECREASE OF GLUCOSINOLATE CONTENT		
	None	Partial	Complete
Stock Glucosinolate solution at:			
pH 10			*
pH 7			*
pH 3		*	
Stock Glucosinolate solution & protein isolate at:			
pH 10	*		
pH 7	*		
Rapeseed protein extract at:			
pH 10	*		
Rapeseed protein whey at:			
pH 5.3	*		

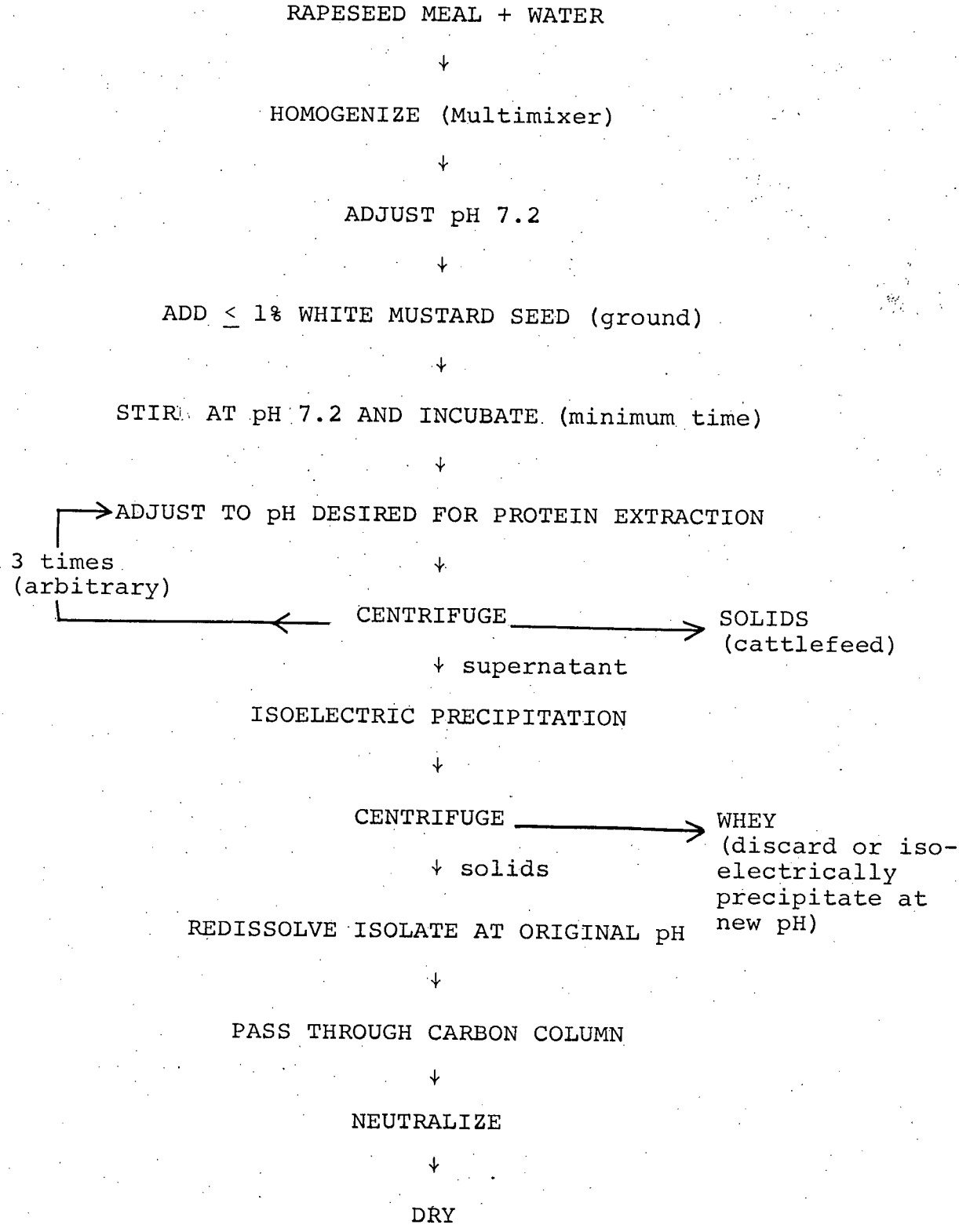
or substance is present in both the pH 5.3 isoelectrically precipitated base soluble protein fraction and in the whey from this protein, since carbon treatment failed to decrease glucosinolate content in either case (Table 3-1). Until some way is found to overcome this association, activated carbon treatment cannot be applied directly to rapeseed protein solutions without prior degradation of glucosinolates to their respective aglycones by myrosinase treatment.

D. RECOMMENDED DETOXIFICATION PROCEDURE

A flow chart for the recommended detoxification process appears in Figure 3-3. Two products are produced: detoxified protein isolate; and, a residue fraction suitable for animal feed. The only loss of protein during isolate preparation occurs from incomplete isoelectric precipitation. This loss could be decreased by subsequent isoelectric treatment of the whey at another pH or application of ultra filtration methods. The recovered protein could be detoxified by the outlined procedure as well. At no time in the process is it recommended that the isolate be washed, although incorporation of this step would further decrease aglycone content. A potential processor requiring pure protein isolate could add this step at the expense of protein yield. An isolate treated in this manner would have more well defined and reproducible functional properties due to its increased homogeneity (e.g. Kodagoda et al., 1973b).

FIGURE 3-3

RECOMMENDED PROCEDURE FOR DETOXIFICATION OF RAPESEED PROTEIN ISOLATES



Production of detoxified rapeseed protein concentrate (not isoelectrically precipitated) is possible with this process. However, this product would be high in ash and organic impurities which would affect functional properties. According to Yapar and Clandinin (1965), rapeseed meal contains high levels of tannins. This factor would have to be considered in the production of protein concentrate.

Actual values such as extraction volumes, amount of mustard seed added, duration of myrosinase treatment, and method of drying the protein isolate will have to be determined for a particular industrial situation and cannot be meaningfully defined here. Level of mustard seed and hydrolysis time required will depend on enzyme activity of the available seed, temperature and pH of hydrolysis, and presence of activators (ascorbate; section IC) or inhibitors (SH blocking) (Nagashima and Uchiyama, 1959b). If whole mustard seed is not used, mode of preparation will have to be considered in determination of optimum hydrolysis time (section IC). Removal of lipid from the ground seed may also be advisable.

Resolubilization of protein isolate after isoelectric precipitation of extract should be carried out in as small a volume of water as possible to facilitate the drying step at the end. With proper design, it may be possible to treat

protein isolates in a homogenized protein slurry without complete solubilization. This would eliminate isothiocyanate-protein interaction in base soluble fractions.

Effects of varying flow rate, temperature, and size of column were not determined. Decreasing flow rate, raising temperature, and increasing column length should increase efficiency of carbon adsorption, although detoxification efficiencies of 100% were never achieved in the described experiments on protein solutions, regardless of conditions used.

The observed residual aglycone content may be due to a weak protein-aglycone interaction. Since such an association would likely be an equilibrium, incorporation of a holding step for re-equilibration of the system after carbon treatment and a second treatment could be considered. However, the low levels of aglycones present in the product from the porposed process and the added expense of adding another step would not warrant such a modification.

A possible future incorporation into this detoxification process would be the use of a system in which myrosinase was bound to a solid support. Such an advance would allow the process to be carried out in a continuous automated system.

E. CONCLUSION

A relatively successful detoxification process for rapeseed protein extracts and isolates soluble in the

range of pH 3 to pH 7 has been devised. Extension of the process to a higher pH range depends on toxicity of isothiocyanate-protein reaction products and on the ability of a potential processor to provide a high speed extraction system to minimize duration of high pH treatments. Besides the above mentioned factors, industrial adoption of the proposed process will depend on the following factors:

1. Economics:
   - cost of raw materials
   - cost of energy, etc.
2. Demand for product:
   - improvement of color of the product
   - well defined functional properties -- possibly unique to the product
   - acceptance by food processors into traditional products
   - acceptance by the public.

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APPENDIX 1

KEY TO PROTEIN FRACTIONS

R	pH 10 protein extract
S	Myrosinase treated protein extract (R)
T	pH 10 carbon treated protein extract (S)
U	pH 5.3 isoelectrically precipitated and purified protein isolate from pH 10 protein extract (R)
X	Myrosinase treated protein isolate (U)
Z	pH 10 carbon treated protein isolate (X)
MS1	Protein isolate (similar to X) prepared from rapeseed meal slurry incubated with ground mustard seed
MS2	pH 11 carbon treated protein isolate (MS1).