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RAPESEED PROCESSING

by

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ABSTRACT

A research investigation was undertaken to develop a detoxification method for improving the nutritional value of Tower rapeseed meal, by removing the volatile butenyl - (BIT) and pentenyl - (PIT) isothiocyanates resulting from the enzymatic hydrolysis of the meal glucosinolates. The method consists of the following steps:

1) dehulling the seed, thereby reducing the meal's content of indigestible fibers from a potential 25% to about 4%;
2) extracting the oil from the dehulled seed (meat);
3) adding water to the defatted meat (meal) at a water-to-meal (W/M) ratio of 150 ml: 1 g meal (dry basis) to hydrolyze the glucosinolates,
4) freezing the wet meal;
5) removing the isothiocyanates (ITC) during the freeze-drying of the meal.

The method reduced the ITC in the dried meal by 0.34 mg/g meal (dry basis) from the original level of 1.52 mg/g. The use of the high ratio of 150:1 prevented the formation of the toxic nitriles and minimized the reaction of the meal protein with the isothiocyanates to form the potentially toxic dithiocarbamates and substituted thioureas.

Furthermore, there was no loss in meal components from the detoxification method used. The use of freeze-drying also prevented the formation of objectionable color changes, thus allowing the meal to retain its original yellow color. However, economic analysis indicated that if a plant were to use this method for the detoxification of rapeseed, it would not be profitable enough.

For the development of this detoxification method, modelling and optimization techniques were used. Solutions to the problems encountered with this approach are discussed in the text.

During this study, the gas-liquid-chromatography (GLC) method for the analysis of volatile ITC, developed by Youngs and Wetter (235), was modified. The major features of the modification was the use of the endogenous myrosinase enzyme to hydrolyze the seed glucosinolates and the use of a high W/M ratio of 150:1. The modified method had a reasonably low coefficient of variation of 4.5%. Mass spectroscopy was used to identify the ITC eluted from the GLC column. The coelutants with the BIT and PIT were also identified by their mass spectra.

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[1] Currently with Sun Life of Canada.

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CHAPTER 1

INTRODUCTION

One group of substances present in rapeseed meal is glucosinolates. Upon hydrolysis, glucosinolates yield, among other hydrolysis products, the following potentially toxic (130) aglucons: isothiocyanates (ITC), nitriles, oxazolidinethiones (OZT), and thiocyanates (13,220,225). It should be noted that elemental sulfur, glucose, and bisulfate are always produced as a result of the formation of nitriles. The type of glucosinolate, and conditions of the hydrolysis, determine the amount and type of the aglucon formed. Glucosinolates per se do not seem to be toxic (13,29,167).

Unlike glucosinolates, aglucons are soluble in both oil and hexane. Therefore, if these aglucons were present in the seed during extraction of the oil, they would contaminate the extract with sulfur compounds, leading to the poisoning of the hydrogenation catalysts used in the seed oil industry (47,130).

Due to the presence of glucosinolates, feeding rapeseed meal to a variety of animal species and to human beings could adversely affect the adrenal cortex, pituitary, kidneys, and liver, causing thyroid adenoma, and in some cases, death. The effect on ruminant animals has been found to be less than that on non-ruminants (14,18,220) and it varies according to the type of glucosinolate (228). It has also been observed that the animals' growth rate, the number of litters they have, and the amount of milk they produce are depressed (169,172). Moreover, the milk produced could be contaminated with potentially toxic aglucons, or with thiocyanate ion which could pass to the animals' offspring (169,220) and/or humans (220,225) feeding on the milk. In addition, these compounds may poison milk bacteria and thus prevent the transformation of milk into other useful products such as cheese (169).

In most current seed-defatting processes, the whole seed is heat-treated at temperatures in the range of 80-90°C to maximize oil yields (14). The aim of this thermal treatment is to deactivate the enzyme myrosinase which is responsible for the hydrolysis of glucosinolates, thus leaving these glucosinolates intact in the meal (56). However, studies have shown that heat treatment does not

completely deactivate the enzyme, and hydrolysis of glucosinolates may still occur (57,87). Also, the enzyme may be reintroduced by ingestion with other food, or by its synthesis by bacteria in the intestinal tract of the host species (56,220). In addition, glucosinolates per se could be toxic even though not accompanied in the diet by active myrosinase (119,120,121).

The need to utilize rapeseed meal, and in particular its valuable protein component, arises from: (a) the world shortage of food and (b) the fact that raising domestic animals for food represents an inefficient use of food resources, as it takes about five pounds of cereal to produce one pound of animal meat (173). However, the presence of glucosinolates in rapeseed meal hinders its full utilization for nutritional purposes. Also, the thermal treatment used during industrial rapeseed defatting is a protein denaturing process which results in a browning of the meal; this reduces the meal's nutritional value (90) and its acceptability as a food. In addition, the meal contains high levels of indigestible fibers which originate mainly from the seed hulls (25). Furthermore, the solubility of the meal protein in water is low; thus, it is impractical to separate the protein from the carbohydrates and fibers to produce protein isolates or concentrates

(116). A solution to this problem is to solubilize the meal protein by enzymatic degradation, but this procedure produces protein with less than optimum functionality[1] (191).

Rapeseed grows in as little as 100 frost-free days (116). It can then be grown satisfactorily in countries, such as Canada, that do not have suitable agricultural or environmental conditions for growing other oil seeds. Canada is the world's largest producer (3.8 tonnes in 1979) and exporter of the seed (11,12,28,116), and rapeseed crushing is one of Canada's fastest growing industries. At present, most of the world's rapeseed meal production is destined for animal feeds.

The amino acid score[2] and protein efficiency ratio - weight gain per protein consumed - of rapeseed protein are higher than most vegetable proteins, including soybean (60). Rapeseed thus appears to offer the best protein for

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[1] Protein functionality is defined as its water and fat absorption, oil emulsification, whippability, foam stability, solubility, pH, color, viscosity and gelation characteristics (191).

[2] Is based on the assumption that the nutritive value is that of its constituent amino acid. The score is established by comparing the amino acid composition of a protein to that of a standard.

supplementing human diets. Rapeseed protein is also characterized by a relatively high content of lysine which is the first limiting essential animo acid[3] in cereals and other vegetable proteins (169,171). Furthermore, high nutritional value and satisfactory functional properties of rapeseed protein concentrates are known (63). These properties all justify intensive examination of rapeseed's possible use in human nutrition.

Plant breeders have succeeded in developing rapeseed varieties called double-zero, which not only have a low level of glucosinolates in the seed, but also a low content of erucic acid in the oil (11,28,203). This is advantageous, since oils fed to animals containing high erucic acid levels have produced retarded growth and undesirable physiological and histopathological changes in the heart (cardiac lesions) and in the skeletal muscles (88).

The first double-zero cultivar was developed in Canada and licensed in 1974 (29). This was a Brassica napus species and the variety was called "Tower" (29). The

---

[3] Essential amino acids are those whose absence could cause some physiological abnormalities. Generally they cannot be biologically synthesized inside the human body from other amino acids.

name "canola"[4] was adopted in 1979 to apply in Canada to all double-zero cultivars (29). Nutritional studies carried out on Tower have produced various conclusions. It was found that Tower meal may completely (39,84,155,156, 185) or partially (4,40,71,109,111,134,155,156,204,211) replace soybeam in animal feed without any adverse effects on the animals. However, other studies have found that Tower meal significantly reduced growth and feed conversion efficiency as compared to soybean (3,92,108,141). Furthermore, replacing soybean with Tower meal in cow rations resulted in an increased thiocyanate concentration in the milk (108,110). Also, it was observed that weaning pigs fed 10 to 20% Tower rapeseed in their diet developed hyperplasia and hypertrophy of follicullar epithelia (29,141). These results indicate that the low glucosinolate rapeseed varieties are not yet suitable for human consumption (224).

Other approaches to detoxifying rapeseed meal utilized physical, chemical, and biological methods. The purpose of these approaches has been to develop methods to remove glucosinolates from rapeseed meal and render it fit for human consumption. Ideally, protein produced from a

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[4] Since this Thesis deals in many parts with varieties other than canola, the name rapeseed will be used throughout.

successful rapeseed detoxificiation method should retain its biological value and functionality, and not be denatured (more details on protein denaturation are in Appendix 3). This method should also reduce the meal's indigestible fiber content, and the protein produced should be free from objectionable colors which would limit its potential uses (189). These conditions should be met without sacrificing the quality or yield of the oil extracted from the seed.

The considerable number of previous investigations aimed at detoxifying rapeseed failed to meet some or all of the conditions stated above. Indeed, the difficulties encountered in attempting to detoxify rapeseed meal have led some experts to conclude that, in practice, it is impossible to remove the glucosinolates and their aglucons (170).

The investigation described in this Thesis was undertaken to modify and improve existing rapeseed processing technology. The aim was to explore new techniques for alleviating problems associated with the rapeseed industry without significantly disturbing its traditions of maximum yield of good quality oil.

1.1 RESEARCH OBJECTIVES AND OUTLINE

The aim of this research investigation was the laboratory development of a procedure that could improve the nutritional value of rapeseed meal by removing the potentially toxic glucosinolates or their aglucons.

It should be noted that because this development was a laboratory study, the emphasis was on the evolution rather than the economics of a potential industrial application. However, whenever possible economic apsects were considered.

In carrying out this development, the following points were considered:

(1) Since the seed oil is the most valuable component, a maximum quantity of it should be extracted. Also, the sulfur content of the oil should be at a low level. Thus it was decided that in the preparation of the meal, the oil would be extracted until its residual amount in the meal was within the industrially acceptable range of 0.5 to 2% (14). Since aglucons are soluble in oil and hexane, while glucosinolates are not, the moisture content of the seed

to be defatted was kept below 8% to minimize the formation of aglucons from the hydrolysis of the glucosinolates (88). This would keep the sulfur level in the oil below the industrially acceptable level of 10 ppm (14). It should be noted that since the research was a laboratory development, oil was extracted only by a solvent (hexane) and no pre-pressing was used. This all-solvent defatting procedure is commonly used in laboratory investigations (70,74).

(2) Denaturing of the protein in the detoxified meal should be kept to a minimum. This aspect was considered not only because denaturation reduces the quality of the protein, but also because it limits further processing of the protein to, for example, produce protein isolates (75,116). A proposed solution was to modify the oil extraction conditions by using lower temperatures (75). Thus it was decided, in the ensuing development, to delete the thermal treatment of the seed prior to extracting the oil.

(3) The color of the detoxified meal should not change from its original yellow to dark colors. This is

because it would be difficult to incorporate such dark colored meals into food (189). To achieve this, aqueous treatment conditions that do not change the meal's color were used. Also, the aqueously treated meals were dried by freeze-drying.

(4) The detoxified meal produced should have a low content of indigestible fibers. Since these fibers are mainly from the seed hulls, a dehulling step was included in the preparation of the meal used in this investigation. To prepare the seed for dehulling, the seed was only cracked and not ground. Thus the intact hulls could be separated from the meat by air classification. This method of preparation also serves to keep the seed's tissue mostly unruptured, preventing the contact between the glucosinolates and the myrosinase. This would result in minimizing the hydrolysis of the glucosinolates before the defatting step, thus keeping the amount of sulfur in the extracted oil at a low level. Also for the same reason, the seed's moisture was kept below 8%.

(5) The reaction between the ITC and the protein should be kept at a minimum since the resulting products are toxic.

(6) The develpment of the research strategy should take into account the fact that the aglucons produced from the meal glucosinolates determine the toxic effect of the meal. For example, the estimated toxicity (LD_{50}) of the VOT is 126 to 1415 mg/kg body weight, while for the nitriles that could be formed instead, it is 159 to 240 mg/kg body weight. Thus the strategy followed was to inhibit or minimize the formation of the nitriles.

(7) To reduce the loss in meal components during detoxification by aqueous extraction, the meal was treated at minimum solubility conditions. For the same reason, the meal was not ground. In addition to simplifying the preparation procedure, non-ground meal might be advantageous for some commercial uses.

(8) To provide a means of monitoring the aglucons during the development of the detoxification method, the literature was searched for appropriate analytical techniques.

The development research concentrated on the major volatile ITC present in the meal, namely the BIT and the PIT.

(9) A starting point for the development research was the study of the detoxification of the meal by aqueous extraction. Conclusions reached were subsequently used to plan the processing strategies.

CHAPTER 2

A REVIEW OF DETOXIFICATION METHODS OF RAPESEED

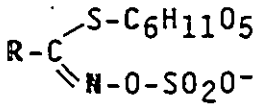
Over the last two decades; the demand for vegetable oils as food for humans has brought about a significant rise in the production of rapeseed. However, an increase in rapeseed oil production has not led to an increase in oil alone. In every case, the major by-product is the meal which is mainly composed of carbohydrates and protein. The need for these by-products (in particular, their more valuable protein content) to overcome the world's food shortage has attracted the attention of scientists and nutritionists to rapeseed.

There is, however, a major factor limiting the use of protein-rich rapeseed meal as food: the presence of toxic glucosinolates. For the past decade or so, a great number of detoxification methods have been investigated. To gain

insight into these methods, the properties of glucosinolates and their hydrolysates are explained in the following section.

2.1 Glucosinolates and their Hydrolysis

Glucosinolates, earlier called thioglucosides, are found in rapeseed as well as in all the species of its family the Cruciferae. Although distributed throughout the plant, glucosinolates occur in highest concentration in the mature seed (220). To date, there are about 90 identified glucosinolates occurring naturally in plants (194).
The chemical formula of the glucosinolates is:



All glucosinolates generally share the basic skeleton and differ only in the side chain R- (67). The glucosinolates reported in the literature as being present in Tower rapeseed are allyl-(178), 3-butenyl-* and 4-pentenyl-(131,145,178), 2-phenylethyl- and 4-hydroxybenzyl-* (145), 3-indolylmethyl- and 4-hydroxy-3-indolylmethyl-* (178), 2-hydroxy-3-butenyl-* (131,145,178) and 2-hydroxy-4-pentenyl- (145,178).

---

* Present in relatively large amounts.

All natural glucosinolates studied thus far are associated with an enzyme that hydrolyzes them. According to the rules of the International Union of Biochemistry, this enzyme is called thioglucoside glucohydrolase E.C.3.2.3.1 (194,225). For simplicity its trivial name, myrosinase, will be used in this text. The myrosinase enzyme is a glycoprotein which exists in multiple forms called isoenzymes. These forms differ slightly in net electrical charge, molecular weight, and carbohydrate content (37,82). It was indicated that the myrosinase could not completely hydrolyze glucosinolates from seeds other than its own (37,143).

In the presence of water in the crushed seed or in the meal, myrosinase acts on glucosinolates to hydrolyze the glucose residue (169,198). The sulfate part is also lost from the molecule, and what remains (the unstable aglucon) undergoes intramolecular rearrangement. This gives rise to the isothiocyanates (ITC) after Lossen-type rearrangement, to thiocyanates and nitriles, as well as to glucose, bisulfate, and sulfur (15,99,220). The formation of glucose and bisulfate, is quantitative and independent of the conditions under which the hydrolysis occurs (220). If the R-group of the ITC contains a beta-hydroxyl group such as the 2-hydroxy-3-butenyl isothiocyanate, it

spontaneously rearranges itself and cyclizes to 5-vinyl-2-thio-oxazolidone (VOT), commonly called goitrin (13,14). The enzymatic hydrolysis of the glucosinolates is presented in Figure 2.1.a and nitrile formation is detailed in Appendix 18. It has been reported that the hydrolysis of rapeseed glucosinolates does not give rise to organic thiocyanates (44,67). This was also mentioned specifically for the Brassica napus rapeseed (225), of which Tower rapeseed is a member. However, the thiocyanate ion is a product of the hydrolysis of the rapeseed glucosinolates (67,126,145,199,220,225). McGregor (126) indicted that the glucosinolates precursors of this ion form about 38% of the total glucosinolates in Tower rapeseed.

It has been found that the precursors of the thiocyanate ion are 3-indolylmethyl glucosinolates and its N-methoxy-derivative (126,145,199). The ion is also formed from 4-hydroxybenzyl glucosinolate (126,145). Chemical equations detailing the pathways of the formation of thiocyanate ion are presented in Appendix 18.

2.2 Factors Affecting the Hydrolysis of Glucosinolates

Hydrolysis conditions that affect the pathway of the aglucon rearrangement, and which are of concern to this

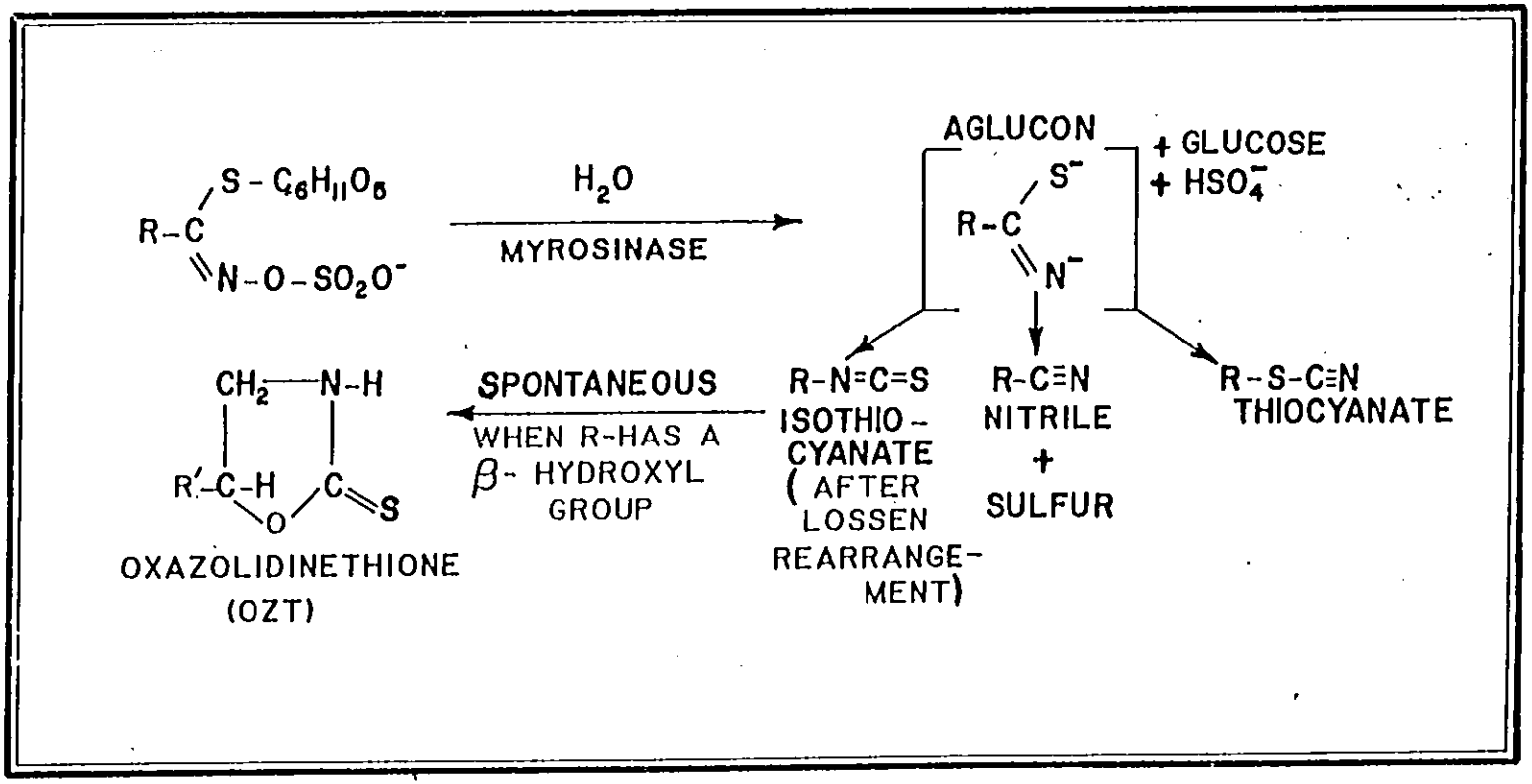


Figure. 2.1.a ENZYMATIC HYDROLYSIS OF GLUCOSINOLATES

work, are water-to-seed (or meal) ratio, hydrolysis time, temperature, pH, and the preheating of the seed. These conditions determine the type and quantity of the hydrolysis products. The only effect of the conditions discussed is on the formation of the competing hydrolysis products, namely the nitriles and the ITC. As the formation of OZT in relation to nitriles follows the same pattern as the ITC (16,99), information on the OZT discussed here is therefore valid for ITC. This is because, as can be seen in Figure 2.a, the OZT is an isothiocyanate whose R-group has a beta-hydroxyl group. This isothiocyanate cyclizes spontaneously to form the OZT. Information on the effects of the hydrolysis conditions on the formation of nitriles and ITC is presented below. This information will be referred to throughout this Thesis.

a) Effect of Water-to-Meal (W/M) Ratio

It was reported that the amount of vinyl-OZT (VOT) formed from the autolysis (hydrolysis with meal's endogenous myrosinase) of the progoitrin (common name of the glucosinolate precursor of VOT) increases with the increase in the W/M ratio (222,225). However, the rate of this formation is reduced with the increase in the ratio. For example, VanEtten et al. (225) reported that for unheated

Brassica napus rapeseed meal, an increase in W/M ratio from about 7:1 to 40:1 increases the formation of VOT from about 2.5% to 20% of the progoitrin. However, doubling the amount of the VOT formed from 20% to 40% of the progoitrin requires an increase in the W/M ratio to 320:1. The autolysis was carried out at a pH in the range of 4.8 to 5.6, and at 25 to 27°C. Also, the water was mixed with the meal by spatula for a few minutes (Dr. M.E. Daxenbichler, personal communication), and the mixture was allowed to stand for 1 h without agitation before determining the hydrolysates. It is of interest to note that the continuous agitation during the autolysis step to keep the meal dispersed had no effect on the results (199). For this reason, the effects of stirring were not investigated in the work of this Thesis.

b) Effect of Time

The formation of the hydrolysis products was also reported to increase with time (16,153,222). It was found that when rapeseed meal, whose myrosinase was thermally deactivated, was treated with a W/M ratio of 100:1 at 30°C and pH7, and in the presence of an exogenous myrosinase, the ITC and VOT reached their final levels in less than 60 min (16). Also, the rate of VOT formation is slower than

that of ITC (6). It was not known if this is due to a slower release of 2-hydroxy-3-butenyl isothiocyanates from progoitrin than from the non-hydroxylated ones, or due to the time required for the cyclization of the hydroxylated isothiocyanates to form VOT (16).

From another work by VanEtten et al. (222), relating the autolysis of glucosinolates of Crambe abyssinica meal (in Cruciferae family, as rapeseed) to the formation of VOT and nitriles, it was concluded that the autolysis rate is faster when the hydrolysates formed are essentially nitriles, than when the VOT constitutes a substantial proportion of the hydrolysates.

c) Effect of Temperature

Appelqvist and Josefsson (16) investigated the effect of temperature on the formation of ITC and VOT from the hydrolysis of the glucosinolates of Matador rapeseed meal. The hydrolysis conditions in their study were a W/M ratio of 100:1, pH7, and a hydrolysis time of 5 min. The meal's endogenous myrosinase was thermally deactivated and replaced by 15 mg of myrosinase prepared from white mustard meal.

The results of the investigation indicated that the maximum amounts of ITC and VOT were formed at 70°C. This maximum was considered 100% formation for each of ITC and VOT. The magnitude of these maxima can be appreciated when compared with the amounts formed at 20°C, where only about 15% ITC and 10% VOT were formed. Furthermore, at 80°C, the amounts of ITC and VOT formed were 40 and 60%, respectively. The decrease in the amount of ITC and VOT formed above 70°C was attributed by Appleqvist and Josefsson (16) to the rapid deactivation of the myrosinase at such tempertures. This phenomenon was also reported by Bjorkman and Lonnerdal (37) in their work on the properties of myrosinase.

Similar conditions were reported by VanEtten et al. (220,222,225) in connection with the autolysis under the same conditions as in (a) above, of the Cambe abyssinica meal's glucosinolates. They reported that at room temperature, VOT was not formed from the autolysis. However, its formation, in significant amounts, started at 45°C and reached a maximum at 68°C. The increase in the amount of VOT formed was accompanied by a concomitant decrease in the formation of the nitriles (222). This could be explained by the presence of a compound called epithiospecifier protein (for details see Appendix 18) or other compounds

that direct the hydrolysis of glucosinolates to form nitriles being deactivated, since they are heat labile (127,216).

It was also found that in the range of 4 to 65°C, 98% of the meal glucosinolates was hydrolyzed (222). However, at 75°C, only 51% was hydrolyzed because of the thermal deactivation of the myrosinase (222).

d) Effect of pH

In a study carried out by Paik et al. (153) on the autolysis of the glucosinolates of Tower rapeseed meal, it was found that acidic conditions (pH4) favored the formation of nitriles. For example, for a Tower meal which they denoted "A", at pH4 the amount of nitriles formed was 1.54 mg/g meal while the amount of VOT was 0.45 mg/g meal. However, at pH7 the nitriles formed were reduced to 1.34 mg/g meal and the VOT increased to 0.58 mg/g meal. The conditions of autolysis for this work by Paik et al. were W/M ratio of 15:1, a time of 1 h, and a temperature of 37°C. The meal was ground and buffer solutions were used to control the pH in the range of 4 to 7.

In studies by VanEtten et al. (220,222,225) on two Crambe abyssinica meals, a similar trend was observed. An

exception was that for one of the meals studied, the trend was reversed in the pH range of 4 to 5.

Appleqvist and Josefsson (16) studied the effect of the pH range of 4 to 10 on the autolysis of the glucosinolates of Matador rapeseed. The results indicate that in this range, the amount of ITC and VOT formed increases continuously to a maximum at pH7. However, beyond pH7, the amount of ITC and VOT formed decreases gradually. No information was given on the formation of the nitriles. However, it could be inferred from the work by Daxenbichler et al. (51) on Crambe abyssinica, where the maximum amount of VOT was also obtained at pH7, that at this pH the amount of nitriles formed were minimum.

It is of interest to note that Daxenbichler et al. (51) also mentioned that the glucosinolates were hydrolyzed only to 5% at pHs 2.3 and 12 on standing for 24 h. The transformation of the glucosinolates to carboxylic acids in the acidic medium, or to amino acids and/or other degradation products in the alkaline medium (194), might be an explanation for this result.

Effect of Preheating the Seed

It has been observed that heating the seed or the meal prior to hydrolyzing its glucosinolates favors the formation of ITC (16,99) and VOT (154,220,222,225) and reduces the amount of nitriles formed (127). This could be due to the destruction of the ESP which directs the hydrolysis of glucosinolates to form nitriles (127,216). If mild heating is used (dry heating for periods of a few hours at 90-120°C), the meal's myrosinase is not completely destroyed and could autolyze the glucosinolates (57,220, 222, 225). However, when heating is carried out by immersing the seed or meal in boiling water, even for a few minutes, its myrosinase is completely deactiviated (56). To hydrolyze the glucosinolates of a seed or a meal treated this way, the addition of exogenous myrosinases is needed to substitute for the deactivated enzyme (16,153). These exogenous enzymes were prepared from other seeds such as white mustard (Sinapis alba) (16,99). An example of the effect of this heat treatment was seen in the work of Paik et al. (153), where the autolysis of the glucosinolates of Tower rapeseed meal "A" yielded between 0.45 to 0.58 mg/g meal of VOT and between 1.34 to 2.68 mg/g meal of nitriles. However, when the meal was autoclaved at 110°C for 15 min, hydrolysis with an exogenous myrosinase produced a higher

amount of VOT of 1.66 mg/g meal (153). At the same time, the amount of nitriles formed was reduced to only 0.1 mg/g meal (153).

2.3 Properties of Glucosinolates and the Hydrolysates

As shown in Figure 2.1.b[1], isothiocyanates react with amino and sulfydryl groups of the low molecular weight basic proteins (isoelectric point is about pH 11) to form substituted thioureas and dithiocarbamates by causing a change in the physico-chemical properties of the protein (36,79).

The reaction proceeds as follows (112):

$$\underset{\text{(ITC)}}{R\text{-}N{=}C{=}S} + \underset{\text{(Protein)}}{H_2N\text{-}R} \longrightarrow \underset{\text{(ITC - Protein)}}{R\text{-}NH\text{-}\overset{\overset{S}{\|}}{C}\text{-}NH\text{-}R}$$

The reaction products, which will be referred to as ITC protein, are potentially harmful to humans and animals, as they could cause a neoplastic effect and might be carcinogenic (115). They may also induce agranulocytosis and thrombopenia in human blood. Their single toxicity

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[1] Only the hydrolysates of interest to this part of the Thesis are shown in this figure.

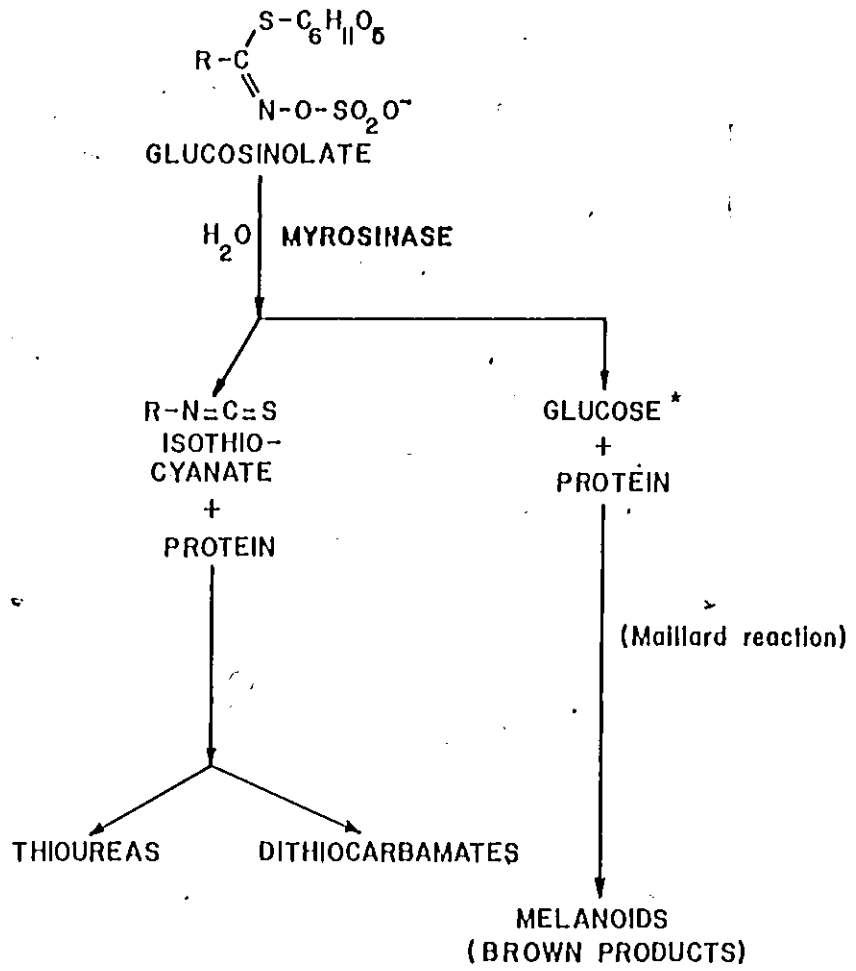

Figure. 2.1.b REACTION OF PROTEIN WITH ISOTHIOCYANATES AND GLUCOSE.

(* Could be present from other sources such as meal carbohydrates)

dose appears, however, to be low (76). Based on the work of Edman (58) and Goksoyr (79), Appelqvist and Josefsson (15), this reaction between ITC and rapeseed protein takes place at pH above 5. However, an investigation by Bjorkman (36) showed that this reaction is not completely inhibited in an acidic medium.

Bjorkman (36) also found that neither glucosinolates nor VOT appear to react with protein. However, he found that small but significant amounts of them were associated with the high molecular weight protein in the void fraction. These were independent of pH and contact time, and probably resulted from non-specific absorption rather than from chemical bonding (36).

Glucosinolates are not oil soluble (105), but they are easily dissolved in water, ethanol, methanol (123), and acetone (1). However, they are more soluble in an aqueous solution of these solvents than in the pure form (1,217). ITC and VOT are soluble in ethyl ether and chloroform. Steam-distillable ITC are oil and hexane soluble, while non-volatile ITC are less soluble in both liquids. OZT is relatively water soluble (14). ITC, OZT, and nitriles dissolve readily in methylene chloride (235).

The toxicity of nitriles (formed from hydroxy butenyl glucosinolates) is higher than that of VOT (formed from the same glucosinolates) (170,225). This can be seen by comparing their estimated LD_{50} for the former of 159-240 and that for the latter of 1260-1415 mg/kg body weight (225). ITC have a considerably lower goitrogenicity than OZT, while the thiocyanates have an even weaker goitrogenicity than ITC (170). It has been reported that unhydrolyzed glucosinolates do not seem to be toxic (13,29,167).

The toxicity of the nitriles is reduced upon drying the meal at 50°C, presumably as a result of their polymerization (225), or possibly due to their volatilization (31,43).

The other hydrolytic products (glucose and bisulfate) are not toxic and present no problem to animals. However, glucose undergoes the Maillard reaction with the meal protein to produce dark colored melanoids (66,170), thus reducing the nutritional value and acceptability of the meal. The formation of the melanoids (66,170) is shown in Figure 2.1b and explained in detail in Appendix 4. The reaction is favored by the presence of a moderate water content and prolonged contact with it when: pH is greater than the isoelectric point of the protein, at high temperature, and in concentrated solutions (66).

2.4 Methods of Detoxification

2.4.1 Enzyme Deactivation

The justification of this approach is that since the glucosinolates do not seem to be toxic, a meal which has had the enzyme (myrosinase) completely destroyed should not be toxic (14). In present commercial practices, a method of thermal deactivation of myrosinase is used to obtain a meal which is free of hydrolysis products of the glucosinolates. The treatment temperature is usually in the range of 80 to 90°C with a moisture content of 6 to 10% (14,56,142).

It was found that this thermal treatment might not be effective and that the enzyme could retain a residual activity (57). However, higher temperatures are not a desirable solution because they deteriorate the protein quality by causing extensive denaturation to it, thus resulting in losses of the amino acids, especially the basic ones (14,56,169). Also, if the temperature used exceeds 100°C, a chemical breakdown of glucosinolates may occur, with the possible formation of oil-soluble sulfur-containing products that could cause difficulty in the hydrogenation of the oil (142). If the moisture content of the seed is high and the temperature rise is slow, gluco-

sinolates might be hydrolyzed before the myrosinase is deactivated (142). In feeding trials, it was found that rapeseed meal containing intact glucosinolates whose myrosinase was deactivated produced toxic symptoms in the test animals (119,120,121).

The deactivation of the myrosinase by microwave (65,132,133), or infra-red (65) heating of the rapeseed was investigated. Results of these investigations indicate that the treated seeds still caused goitrogenicity in the test animals (65,132). Furthermore, it was found that microwave heat could also cause scorching of the meal and give it a disagreeable odor (133). This is due to the high intensity of the microwave heating and the difficulty in controlling it.

Hare et al. (80) deactivated the enzyme by treating the whole seed with at least one of the following organic acids: propionic, acetic, and butyric. This method was claimed to be effective in improving the nutritional value of the seed.

Gamma irradiation was found to deactivate the myrosinase but did not destroy glucosinolates (113). It was also found that irradiation could adversely affect the seed's oil and animo acid content.

Although myrosinase deactivation (in wet or dry medium) improved the nutritional quality of the meal, it was found that the enzyme deactivation is incomplete and its residual activity (57) could hydrolyze the glucosinolates. It was also found that the enzyme could be reintroduced by its ingestion with food or its synthesis by bacteria in the intestinal tract of the host species, and act on the intact glucosinolates (14,56,57,64,84,169,220, 225). Furthermore, high temperature treatment, especially in the presence of water, denatures the protein and reduces its solubility (75,87).

This treatment also causes a decrease in the content of available lysine (90,91), as well as in protein solubility (73). Moreover, although the hot water treatment deactivates the myrosinase, it could produce some sulfur-containing enzymatic degradation products. Some of these products are oil- and hexane-soluble, and subsequently contaminate the oil with sulfur during the defatting step, while the remaining glucosinolates stay in the meal. This sulfur in the oil could poison the oil hydrogenation catalysts (87,142). The thermal treatment could also contaminate the oil by seed components such as phospho.ipids and chlorophyll (87). Thus the purification and refining of the oil becomes difficult (87).

Srivastava and Hill (200) investigated the wet deactivation of Tower rapeseed by dipping the seed in boiling water for 3 min. The seed was then dried at a temperature below 50°C in a forced-air oven. They confirmed that Tower meal is improved by heat treatment. However, they concluded that the reason for its improvement is not clearly known and suggested that heat labile compounds influencing feed intake might be involved (200).

2.4.2 Removal or Destruction of Glucosinolates or their Hydrolysates

a) Steam Stripping and Distillation

These methods are based on the removal of the volatile hydrolysates by distillation or steam stripping (43,170,230). For example, the meal glucosinolates were autolyzed and isothiocyanates were removed by distillation (14). In a study by Ballester et al. (26), steam was directly passed through the rapeseed meal at a temperature of 100°C and at a pressure of about 1.5 atm. for 3 h. They also investigated heating the meal at 60°C under the same conditions of pressure and time. It was found that indirect steaming reduced the ITC content by almost 50% (of its original level), but it only slightly decreased the VOT level. In contrast, direct steaming considerably reduced

the levels of both ITC and OZT by about 80 and 65%, respectively. This reduction in ITC might have been partly due to the reaction between the ITC and the meal protein (15,36) to form toxic compounds (76,115). Also, the autolysis of glucosinolates during steaming might have resulted in the formation of the non-volatile nitriles in lieu of the VOT (225). These nitriles are much more toxic than the VOT (169,225).

When meal glucosinolates were catalytically hydrolyzed at temperatures of 90 to 130°C, they yielded organic nitriles rather than the ITC or VOT formed by enzymatic hydrolysis (31,43). The nitriles corresponding to the ITC are usually more volatile than the ITC, and these are readily steam-stripped from the meal. The hydroxy nitriles corresponding to the OZT are only slightly more volatile by steam distillation than the non-volatile OZT (31,57). The bulk of this material, which has a toxicity of about 8 times that of the VOT (225), remains in the meal, resulting in an end product that is more toxic than the original material (57). It was observed in these methods that there was a gradual deterioration in the quality of the meal protein as the time of steam-stripping was extended (57,179,230).

b) Treatment with Ammonia or Sodium Carbonate

Treatment with ammonia consists of injecting the meal with anhydrous or aqueous ammonia up to the maximum absorption level (14,43,94). The excess ammonia is then removed by steaming (43). This treatment destroys 50% of the meal glucosinolates (14,68,94). However, there have been palatability problems on feeding ammonia-treated meal to cattle (14).

Treatment of the crushed seed or meal by cooking with sodium carbonate under pressure also reduced the amount of glucosinolates (14,30,180). However, the reaction products formed remain to be determined (14). The palatability of the meal treated by this method was better than that of untreated or ammonia-treated meal. However, it was still not competely satisfactory (14,180). Furthermore, the cooking temperature resulted in a marked reduction in the lysine, methionine and cystine content, and in the digestibility of the protein (30,180).

c) Treatment with Sulfuric Acid

This method consists of detoxifying rapeseed by heating it with 15% H_2SO_4 at temperatures close to 100°C for long periods of time (138,206). It was claimed that

glucosinolates, VOT, and ITC were destroyed by this treatment (138,206). However, Zgainski and Krurzewski (236) found that the addition of H_2SO_4 did not reduce the content of the toxic components. Furthermore, the heating of rapeseed under the acidic conditions of this method could cause protein denaturation and considerable destruction of the lysine (206).

d) Isolation and Concentration of Rapeseed Protein

The successful production of soybean protein isolates and concentrates directed attention to the possible application of those techniques to rapeseed. Although the preparation of soya protein presents no serious technical difficulties, this is not the case for rapeseed (17).

Generally, there are four major steps in the preparation of a rapeseed protein concentrate or isolate: deactivation of the enzyme myrosinase, leaching for the glucosinolates, dehulling, and oil extraction. The order of these steps is irrelevant and some of them could be omitted in a given procedure (8).

(i) Isolates

Soybean isolates are commonly prepared by extracting the protein from the meal with water, alkali, sodium chloride or polyphosphate, followed by precipitation with or without heat at an acidic pH or by some other means (8,17,70,117,213,214,215). However, rapeseed contains two major protein groups (34,69,75,77,165); one is acidic and the other is basic (34,69,77), as opposed to one major acidic group in soybean (186,187). The acidic protein group of rapeseed is of a high molecular weight that dissociates into subunits at acid pH values. The basic group is of low molecular weight (34,69,77). The presence of these two groups could explain the reason why rapeseed protein could not be easily extracted in satisfactory quantities (189). Direct precipitation by acid of alkaline rapeseed protein extracts, as in the soybean technology, generates a low yield of protein isolate (73,122,189). For example, a low yield of rapeseed protein isolate (about 30% of the original in the seed) was obtained from industrial rapeseed meal by traditional soybean technology (75). To increase the yield of the extraction of the rapeseed meal protein, Kodagoda et al. (101) used a three-stage extraction at neutral, pH2, and pH10. The protein that

remained in the solution could be precipitated with great difficulty by trichloroacetic acid (75). This difficulty in precipitation is due to the fact that rapeseed proteins, in contrast to most other proteins, are relatively soluble in 5% trichloroacetic acid (122). However, El Nockrashy et al. (63) increased the yield of the isolated proteins to over 90% of the meal protein, by precipitating the extracted proteins at pHs corresponding to the isoelectric points of the two major groups that compose the extracted protein.

Other investigators have achieved high recoveries of the extracted proteins by adding an anionic polymer to the extract prior to acidification (6,73,74,122). Inositol hexaphosphoric acid (phytic acid) has also been used, but the yield was only 75% (73). Furthermore, the presence of the phytates is likely to adversely affect the nutritional value of the isolates by reducing the availability of minerals for biological needs (73,129,184). The addition of these polymers and phytic acid increased the dry matter content of the isolates; they also reduced the protein solubility at moderate pHs, thus limiting the usage of these isolates (73,74,191).

Another approach involved the co-precipitation of rapeseed protein and cheese whey using acid and heat

treatment. However, the co-precipitates had a poor functional property compared to that of soy protein isolates (212). This could be attributed to protein denaturation by the high temperature (95°C) involved in the preparation.

In other investigations, meal protein was extracted with a saline solution followed either by dialysis for three days and then freeze-drying (118), or by isoelectric precipitation of the extract (75,160,161,162). In the work of Owen et al. (151,152) the precipitate was repeatedly washed and the curd was then spray-dried. After freeze-drying, the products showed a considerably greater ash content, up to 33%, and a lower content of glucosinolate that did the starting meal (101). The precipitation method resulted in a loss of about 60% of the extracted protein, and its protein isolate yield was only 18% of the total protein in the meal (151,152).

Eklund et al. (2,59,61) extracted rapeseed with a salino-alcoholic solution followed by centrifugation at low temperatures. The precipitate, which contained between 36-41% of the original protein, was actually a lipoprotein concentrate consisting of 48% protein and 31% fat. About 50% of the initial seed nitrogen and about 30% of the seed

oil were lost to the aqueous ethanol phase obtained after centrifugation of the extract. The oil extracted from the seed residue, and that separated from the aqueous extracts, were contaminated with sulfur at the high levels of 200 and 580 ppm, respectively [acceptable level is below 10 ppm (14)]. Feeding tests with rats showed that these lipoprotein concentrates had some toxic effects, such as the enlargement of the thyroid gland and liver (62).

Rapeseed protein isolates usually have an objectionable dark color (13,191). Thus, some procedures include a color-removal step. This could be accomplished by treating the isolates with organic solvents (63,213,214), hydrogen peroxide (96), thiosulfate (214) and sulfite salts or sulfur dioxide (87). However, the organic solvents used are flammable and require special handling and expensive equipment. Hydrogen perioxide causes a significant loss in sulfur containing amino acids (7). The sulfites are not suitable for the production of food preparations because they are toxic, and they adversely affect the organoleptic properties of the isolate (160,162).

(ii) Concentrates

In analogy to soybean technology, the rapeseed protein concentrate may be defined as the product prepared

from high-quality clean seed by removing most of the hull, oil, and water-soluble non-protein components (8).

In Sweden, a pilot-plant has been in operation since 1972 using a process developed by A.B. Karlshamns Oljefabriker and Alfa-Laval AB to produce a rapeseed protein concentrate (8,9). A flow sheet (obtained from S.-E. Kroon of Alpha-Laval AB) outlining this process is shown in Figure 2.2. In Canada, the Food Research Institute of Agriculture Canada devised a similar method (17,56,57,207). Northern Alberta Rapeseed Processors Co-Op (Sexsmith, Alberta) planned to exploit the Swedish method with an output of 5 000 t/yr of concentrates containing 65% protein (86). For economic reasons, however, this plan did not proceed.

The Swedish procedure (8,9) consists of cracking the seed to loosen the hull, followed by a sieve classification into different sizes to facilitate the separation of the hull and meat. The myrosinase is then deactivated by dipping the meat in boiling water for 3 to 10 min. Then, the glucosinolates are removed by counter-current water leaching. The leached meat is dried and then the oil is extracted from it. The final step in the preparation is to mill the remaining meal into a flour with an oil content of

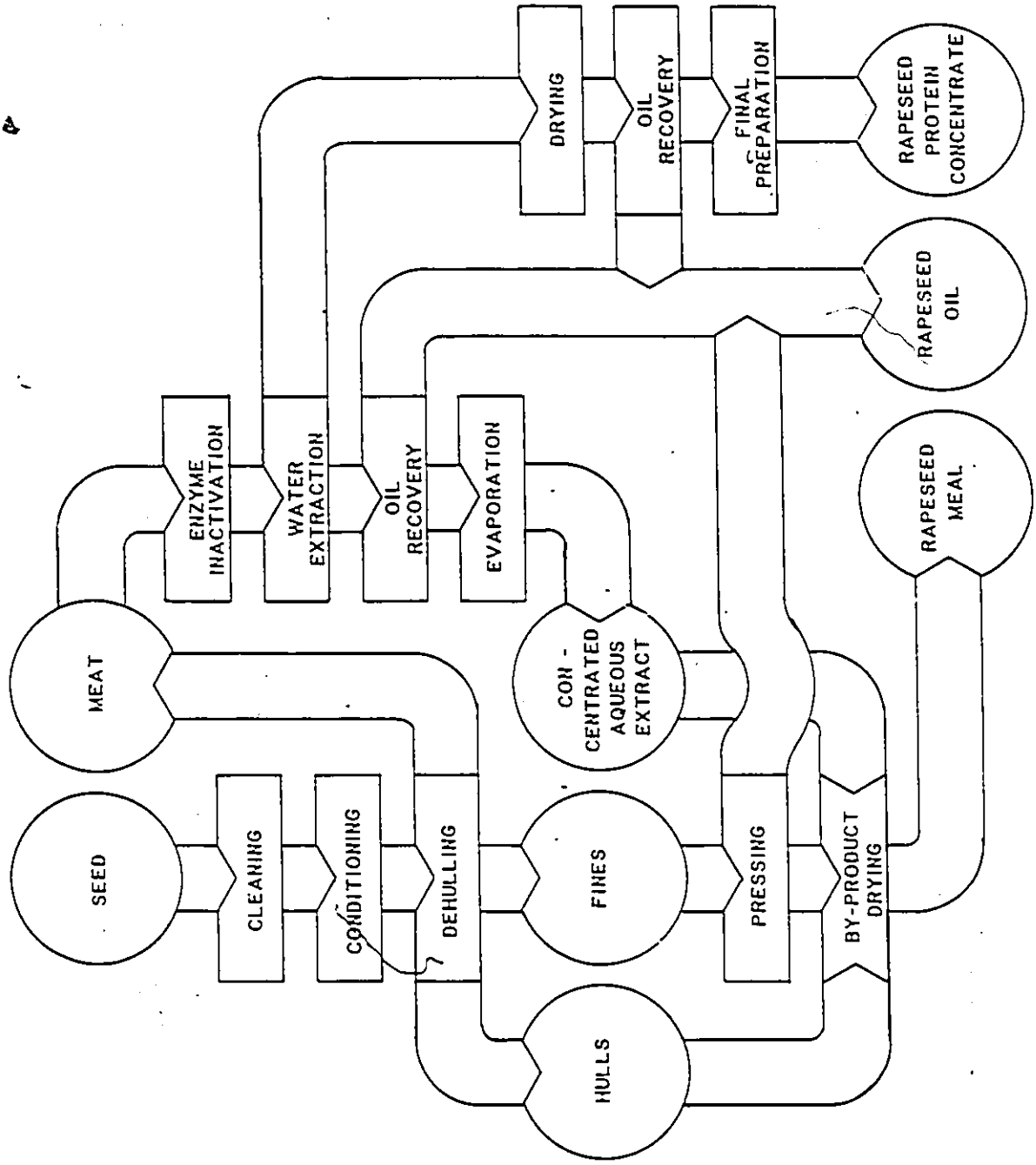

Figure. 2.2 FLOW SHEET OF THE SWEDISH PROCEDURE FOR THE DETOXIFICATION OF RAPESEED MEAL.

1-3%. The product is called rapeseed protein concentrate (RPC). The leach water from the process is not sewered but rather evaporated; the remaining solids are then added to the hulls and fines. The mixture is called feed meal. The yield of fractions and the glucosinolate content of the products of this process are shown in Table 2.1. Information in this table is extracted from the work of Anjou and Ohlson (10) which was carried out on a rapeseed containing 4% glucosinolates.

TABLE 2.1

Yield and Glucosinolate Content of Different Fractions of the Swedish Method

FRACTION	YIELD %	GLUCOSINOLATE CONTENT %
RPC	25	0.03
Oil	37	0.00
Feed Meal	38	6.00

e) Aqueous Extraction

Ballaster et al. (25,26,27) attempted, by extraction with water, to detoxify rapeseed meal that was not thermally

treated to deactivate its myrosinase. They found that the extracted meal contained 0.02% VOT and no detectable ITC. However, they observed that rats fed this treated meal suffered thyroid gland abnormality. They suggested that other toxic compound(s) may have been present in rapeseed meal which was/were not extracted.

Attempts have been made to remove glucosinolates from whole or ground rapeseed after the deactivation of the myrosinase by boiling water. Eapen et al. (56,57) and Tape et al. (207) claimed they had achieved complete removal of glucosinolates from rapeseed meals with water extraction. This process, however, resulted in up to 40% loss in the meal protein. Diosady et al. (55) in their investigation to detoxify rapeseed meals with water leaching, used ultrafiltration to recover the protein from the extracted water. The loss in the meal protein was thus reduced to as low as 1.3%

f) Diffusion Extraction

To overcome the losses reported above in Tape's method, Sosulski et al. (193) investigated the method of diffusion extraction of glucosinolates from intact (whole) rapeseed. The diffusion extraction method is based on the principle that low molecular weight glucosinolates will

diffuse through seed membranes which retain large molecules, such as proteins and oils (35,193).

Boiling the seed before diffusion extraction (105) or using ethanolic sodium hydroxide (35) or hot alkaline solution (106) were effective in controlling the myrosinase activity and maintained the sulfur content at less than 10 ppm. It was also found that dehulling the seed before the diffusion extraction allowed the use of a short extraction time and a low solvent-to-seed ratio (106).

In the diffusion extraction method, the loss of extracted solids was about 15% and the loss of nitrogen was less than 10%, most of which was non-protein nitrogen (35). However, results of feeding tests carried out on rats indicate that meals treated by this method have poor nutritional value (184).

g) Solvent Extraction

It has been shown that glucosinolates could be extracted from rapeseed and crambe seed meals with organic solvents. The preparation of the rapeseed used in most of these investigations includes the deactivation of myrosinase with boiling water (1). The important solvents studied are aqueous solutions of methanol (33,104,222),

ethanol (33,104,138,228), propanol (33), isopropanol (33, 87,224) isobutanol (33), and acetone (64,92,99,217). It was found that aqueous acetone is the most effective solvent for the removal of glucosinolates from rapeseed meal or meat (1,15).

Diosady et al. (54) found that the addition of ammonia to lower aliphatic alkanols improved their efficiency in the removal of glucosinolates from rapeseed meals. Also, the efficiency of removal increased with the increase in the water content of the ammoniacal alkanols and with the decrease in the number of carbon atoms (i.e., with an increase in solvent polarity, thus methanol is the most effective). An example of the improvement is that for a laboratory-prepared meal, 10% ammonia in methanol containing 5% water removed glucosinolates to trace levels. In the absence of ammonia, only 56% of the glucosinolates was removed.

Diosady et al. (53) and Naczk et al. (137) investigated the anhydrous ammonia-methanol as a polar phase in the two-phase solvent extraction system for glucosinolates removal from rapeseed, while Rubin et al. (168) added water to the polar phase mentioned above. In these three investigations the non-polar phase of the extraction systems was hexane. Results of these investigations

indicate that the two-phase solvent extraction system is effective in reducing the glucosinolates content of the meal produced. The functional properties of these meals indicate that they could be used as binders and extenders for meat (136).

h) Miscellaneous Methods

The treatment of rapeseed with hydrogen peroxide was investigated as a detoxification method (7). The treatment was found to be effective in reducing the glucosinolate content. However, the production of oxidized sulfur containing amino acids, in particular methionine sulfone, considerbly reduced the nutritional value of the protein (7).

Nakaya (139) investigated the destruction of the toxic VOT present in rapeseed meal by gamma irradiation. The treatment succeeded in reducing the level of VOT and also resulted in a loss of the available lysine in the meal. The extent of these changes was similar to that of rapeseed meal which had been autoclaved.

The detoxification of rapeseed by fermentation was also investigated (43,163,201,202). This method resulted

in a reduction in the VOT and improvement in the protein quality and quantity of the meal. However, the process is time consuming with the fermentation step requiring about 50 h (201).

The use of activated carbon to detoxify rapeseed isolates by removing the glucosinolates hydrolysates was studied (234). However, the method was not satisfactory in removing nitriles (234). The ITC might also have reacted with the meal protein[2] (15,36,234), thus giving a pseudo-detoxification.

2.5 Discussion

It is clear that existing detoxification methods do not completely remove the toxic compounds from the meal; they are not adaptable to commercial practice; and they have not solved the problems of excessive protein loss and reduced quality.

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[2] In a letter addressed to the author, Dr. S. Nakai, co-developer of the activated carbon method, agreed on the possibility of this reaction. A copy of this letter is presented in Appendix 16.

To overcome protein losses, it was necessary to study the solubility of Tower rapeseed protein as a function of temperature and pH, in order to determine both the intended minimum solubility points and the conditions that do not drastically change the meal's color. The data obtained were used to indicate the meal detoxification conditions that would reduce the protein losses and avoid drastic changes in the meal's color. Also, in all stages of the investigation, conditions that could cause a deterioration in the quality of the protein were avoided.

The author doubted the accuracy of the reported residual toxicities. This doubt arose from two facts that have been overlooked: 1) the reaction between the ITC and protein would give a low value for ITC content, 2) the aglucon determination methods used to evaluate the detoxification procedures might not be sufficiently accurate. For example, some analysis methods are based on a deactivation of the rapeseed and a replacement with mustard seed myrosinase. This approach does not take into account the fact that rapeseed glucosinolates could only be completely hydrolyzed to their aglucons by their native myrosinases (37,145). Thus, these methods might underestimate the amount of aglucons present (145). A case in point is the method for producing a rapeseed protein concentrate

developed by Alfa-Laval AB and AB Karlshamns Oljefabriker (8,9,10), which has a potential for industrial application. However, it is important to note that this method (results of which are presented in Table 2.1) is deficient in the balance of the glucosinolates. When taking a material balance around the glucosinolates, in 100 kg seed, the following could be found:

Glucosinolates in the seed = 4.00 kg

Glucosinolates in the feed meal = $\frac{38 \times 6}{100}$ = 2.28 kg

Glucosinolates in RPC = $\frac{25 \times 0.03}{100} \simeq$ 0

Glucosinolates in the oil = 0

Total glucosinolates in the products = 2.28 kg

Difference in glucosinolates balance = 4.00-2.28 = 1.72 kg

(N.B. The extracts containing glucosinolates and meal components are not sewered but rather dried to form the feed meal).

This difference of 1.72 kg, which represents about 43% of the initial glucosinolates in the meal, is not accounted for. This discrepancy provides a strong incentive for studying the methods of analysis of glucosinolates and developing a more accurate one. This study of the methods of analysis is discussed in Chapter 3.

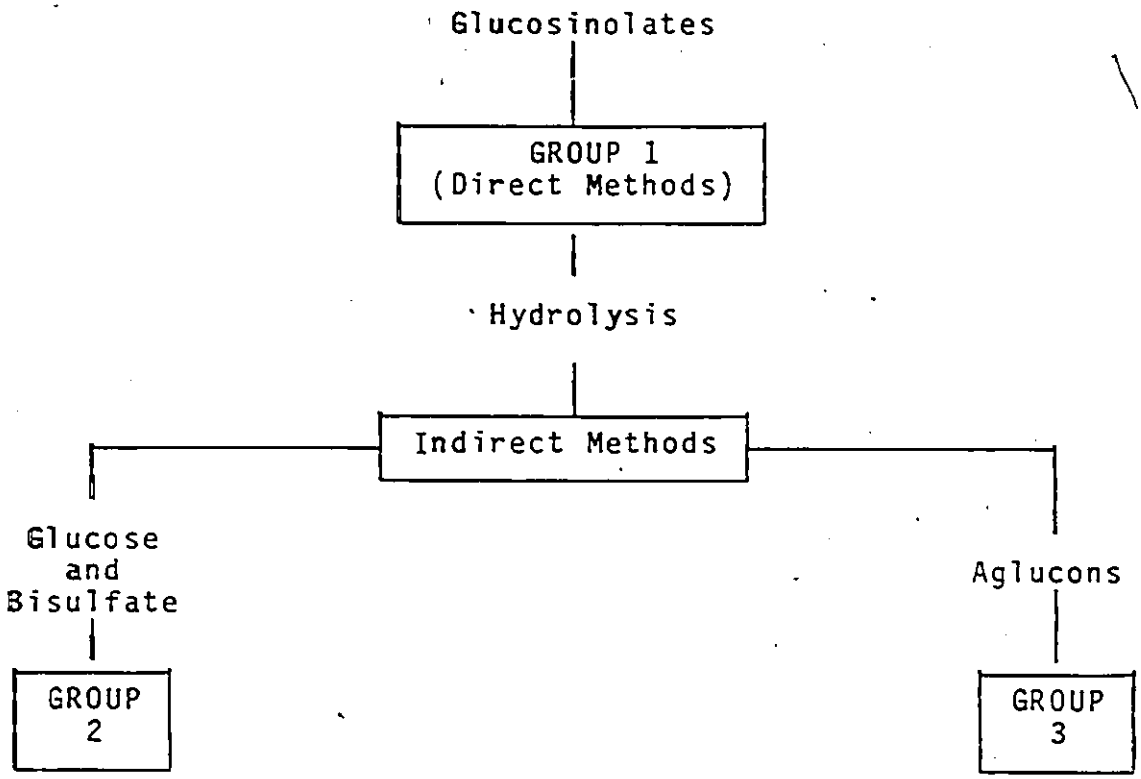
CHAPTER 3

METHODS FOR DETERMINING GLUCOSINOLATES

Rapeseed breeding, detoxification, and the use of rapeseed as food necessitated the search for a rapid and sensitive method for determining the amounts of glucosinolates and their hydrolysates present in the seed or its products. Figures 2.1 (a and b) show the hydrolysis of the glucosinolates by the action of the enzyme myrosinase.

Since glucosinolates are not of the same type in all species and since the resultant hydrolysates differ with treatment condition, numerous methods of glucosinolate analysis have been devised. A modification for a widely used method, carried out by the author, is presented in this chapter.

Methods of analysis of glucosinolates can be divided into the three groups shown in the following diagram:



Typical procedures of these methods are described in the following sections.

3.1 Direct Determiniation

3.1.1 Trimethylsilyl Derivative (TMS)

In this method, the myrosinase of the defatted seed is thermally deactivated. The seed glucosinolates are then

extracted with water containing singirin as an internal standard. The extract is then eluted on a DEAE-sephadex A-25. The eluted glucosinolates are converted to their corresponding trimethylsilyl derivatives (TMS) by reacting with methylsilylhexafluorobutyramide (MSHFBA). These derivatives are then measured by gas liquid chromatography (GLC) (158,208,209,210). The method is useful for a low glucosinolate content of less than 6 mg/g defatted meal.

The indolyl glucosinolates cannot be determined by the TMS procedure described above. However, the desulfation of the extracted glucosinolates, followed by the derivatization of the formed desulfoglucosinolates, allows the determination of the indolyl glucosinolates by the GLC (81). The desulfation step is usually carried out on the DEAE-sephadex A-25 column by arylsulfatase (Sigma, H-1) (81,190).

Olsen et al. (145) mentioned that a possible source of error in this method is that the glucosinolates are hydrolyzed during their preparation procedure. Furthermore, the indolyl glucosinolates (such as 3-indolylmethyl glucosinolates and 4-hydroxybenzyl glucosinolates) could be absorbed on the columns used, resulting in their elution being delayed (145). Also, due to the instability of the

indolyl glucosinolates, the accuracy of their determination by this method is in doubt (48).

3.1.2 Desulfoglucosinolate Derivative (175,177,178)

The desulfated glucosinolates are prepared as mentioned above. However, the main difference is that in this procedure, desulfoglucosinolates are not derivatized to form their corresponding TMS compounds but, rather, remain in a hydrophilic state. The desulfoglucosinolates formed are determined by high pressure liquid chromatography. It seems that the major advantage of this procedure is that both indolyl and non-indolyl glucosinolates can be directly compared on one chromatogram. However, as in the TMS method, there is uncertainty about the quantitation of the indolyl glucosinolates (178). Although this procedure is more time consuming and complicated than the TMS method, it has a lower variability (38).

3.1.3 Sulfur Balance (119)

According to this method, the total amount of sulfur in the seed comes from the glucosinolates and amino acids containing sulfur, such as cystine and methionine. Therefore, by determining the total sulfur content of the seed

and that of the amino acids, the glucosinolate content is obtained from the following formula:

$$\% \text{ Glucosinolates} = \frac{(S_{\text{total}} - S_{\text{amino acid}}) \times (\text{M. wt. glucosinolate})}{2\,(\text{Atomic wt. of sulfur}) \times (\text{Sample weight})} \times 100$$

Another procedure is based on the same approach in which the enzyme is deactivated in boiling water and then the glucosinolates are extracted by hot water. From the weight of the meal before and after extraction; and the total sulfur values, the weight of the sulfur extracted by hot water can be calculated and substituted into the following equation:

$$\% \text{ Glucosinolates} = \frac{(\text{Wt. of extracted sulfur})(\text{M. wt. glucosinolates})}{2\,(\text{Atomic weight of sulfur})\,(\text{Sample weight})} \times 100$$

This approach requires a knowledge of the molecular weight of the glucosinolates, thus necessitating the determination of their quantity and type. It is obvious that if these are unknown, the method is of no use. Further dis-

advantages are that, it is time consuming, requires a knowledge of the number of sulfur atoms per mole of glucosinolates, and the need for large samples. Furthermore, the presence of sulfur compounds other than amino acids or glucosinolates, is a source of error.

Another approach is that of using X-ray fluorecence to determine the sulfur content of rapeseed (181). Then the glucosinolate content is determined from its correlation with the sulfur. The method based on this approach is fast and requires only small samples. However, establishing the correlation requires the use of rapeseed with known glucosinolate content, and the detection limit of the glucosinolates is relatively high at about 0.9 mg/g meal (181). In addition, the method does not identify the type of glucosinolates present in the seed.

3.1.4 Silver Complexing (119)

This method is based on the reaction of silver with glucosinolates in an excess solution of $AgNO_3$. The unreacted Ag^+ is then determined volumetrically by the Volhard method. The meal enzymes are thermally deactivated before extracting the glucosinolates with hot water. The

determination could become difficult if color bodies are present. In addition, the stability of glucosinolates in boiling water is not known and may differ according to the type of glucosinolate. The presence of water-soluble sulfur compounds, other than glucosinolates, that could react with Ag^+ is also a source of error.

3.2 Indirect Determination in Terms of Sulfate Ion and Glucose

The common principle of this group of methods is that glucose and inorganic sulfates result from all known glucosinolate hydrolysis reactions.

3.2.1 Sulfate Ion

Glucosinolates are hydrolyzed, then the meal is filtered and washed several times to extract the sulfate ion which is determined gravimetrically as barium sulfate. Coprecipitation of carbonate or protein matter could give a high glucosinolate content (98,125). In addition, this method cannot be used in studies where the treatment of the meal is done by H_2SO_4 or its salts.

3.2.2 Glucose

Glucose is determined colorimetrically after specific enzymatic oxidations with readily available reagents containing a glucose oxidase, a peroxidase, and a chromogen (128,230,188,176). The method is subject to error, however, because of the presence of colored materials and interfering substances.

Glucosinolate determination by glucose analysis could give erroneous results because of the possible presence of glucose in the meal, other than that resulting from the hydrolysis of the glucosinolates (125). VanEtten et al. (221) alleviated this problem by purifying the glucosinolates prior to hydrolysis on an anion exchange resin. The accuracy of the determination in their procedure was 94.8%±7.3% and the coefficient of variation was 4.6%.

Another approach for the determination of hydrolyzed glucose is to form glucose trimethylsilyl ethers (TMS) and then measure these ethers with GLC (147).

3.3 Indirect Determination in Terms of Aglucons

In this section, the determination of the organic thiocyanates will not be discussed since they are not formed from the hydrolysis of the glucosinolates present in rapeseed.

As for the thiocyanate ions which could be formed during hydrolysis of the glucosinolates in Tower rapeseed, one method for determining them is by measuring the optical density of their red complex compounds formed with ferric nitrate in the presence of nitric acid (126). However, as the thiocyanate ion was not of concern in this study, it was not addressed further.

3.3.1 Reduction of Iodine by Azide Ion (for OZT and ITC)

This method is based on the catalyzing effect of OZT and substituted thioureas, formed by the reaction of ITC with ethanolic methylamine, on the reduction of iodine by azide ion. The iodine consumed is proportional to the amount of OZT and ITC. The iodine in this method is titrated by sodium arsenite (107,205).

3.3.2 Argentimetric Method (for ITC) (231)

In this method, the glucosinolates are hydrolyzed, and the formed ITC are separated from the meal by steam distillation. The ITC are then reacted with ammonia, forming a substituted thiourea which is subsequently decomposed in an excess ammoniacal silver nitrate with the formation of insoluble silver sulfide and monosubstituted carbodiimide. The unreacted silver is measured volumetrically by the Volhard method.

3.3.3 Infra-Red Method (for Nitriles) (51)

In this method, the nitriles present in the meal are extracted with ether or methylene chloride. The extract is dried over Na_2SO_4, filtered, and carefully evaporated to dryness. The residue is then dissolved in spectro quality chloroform. The net absorption from the compound is obtained by subtracting the minimum absorption near 4.5 μm from peak absorption which occurs at this wave length. From this result, and by knowing the specific absorptivity, the amount of nitriles could be estimated. However, the method is subject to error if other C≡N group-containing compounds are present (51). Another source of error is the loss of the volatile nitrile compounds during the evaporating step.

In the present investigation, this method was tested and details are in Appendix 19.

3.3.4 Ultra-Violet Method (for OZT and ITC) (16,233,235)

This method is based on the specific ultra-violet (UV) absorbance of the substituted thioureas and OZT, the first of which is formed by reacting isothiocyanates with ammonia in ethyl alcohol.

The enzyme myrosinase of the meals to be tested is deactivated by dry heating overnight at 110°C, or by heating for a short period of time in a phosphate-citrate buffer solution (pH 7.0). The treated meal is then incubated with an exogenous enzyme prepared from white or yellow mustard seed to hydrolyze the seed glucosinolates.

The meal-aqueous emulsion is then broken by centrifugation and the aqueous solution is separated. OZT and ITC could be extracted from this aqueous solution by a non-polar organic solvent such as methylene choloride, and then determined spectrophotometrically (16,233,235). Another approach is to separate the volatile ITC from the aqueous solution by steam distillation or selective solvent extractions (232). Both OZT and ITC are then individually

measured. Also, methylene chloride could be added to the seed-aqueous solution mixture at the beginning of the hydrolysis to extract ITC and OZT as they are formed (233,235).

The UV method is simple and fast (70). However, Mullin (135) found that ITC could react with the ethyl alcohol used forming thiocarbamates, which might cause an error in the determination. Also, traces of protein in the solvent extract could interfere with the measurements (50). Furthermore, it was mentioned that the method produced erroneous results for seeds that contain low levels of glucosinolates (38).

The UV method of Wetter and Youngs (233), which was the industry standard for several years, was tried in this investigation, but it was not suitable. Details are presented in Appendix 8.

3.3.5 Chromatographic Methods (for all Aglucons)

Gas liquid chromatography (GLC) has been used for the determination of toxic hydrolysates (aglucons). The high sensitivity of the flame ionization detectors (FID) makes GLC analysis a suitable method for the quantitative microdetermination of these aglucons (235).

An example of this method for the determination of ITC is that of Youngs and Wetter (235). In this method, the meal's endogenous myrosinase is deactivated thermally. Then the seed glucosinolates are hydrolyzed by a prepared exogencus enzyme. As previously mentioned, this treatment is meant to prevent the formation of nitriles. The released ITC, as the result of the hydrolysis by the exogenous enzyme, is extracted from the meal slurry by a methylene chloride solution containing butyl isothiocyanates as an internal standard. A minute amount of the extract is injected into the column of the gas chromatograph (GC).

The column used in the work of Youngs and Wetter (235) was of stainless steel and had an inside diameter of 4.5 mm. The column was packed with 20% FFAP on 60/80 mesh Anakrom ABS.

Daxenbichler et al. (49,50) have developed a procedure for the determination of aglucons, OZT and nitriles. In their procedure, these aglucons are extracted from meal slurries by methylene chloride. The extract solvent is then reduced in volume, and a known amount of methyl palmitate, as an internal standard, is added to it. A small portion of this solvent containing methyl palmitate is

injected into a temperature programmed GC. Two glass columns were connected to the GC and results obtained on a column were checked or complemented on the other column. The first column was packed with 1% EGSS-X on 100/120 mesh Gas-Chrom Q. The packing of the second column was comprised of 3% Apiezon L on 80/100 mesh Gas-Chrom Q. The stationary phases (EGSS-X and Apiezon L) used were sufficiently different in polarity to provide adequate differences in elution times, as a means for component identification when compared with standard mixtures (223).

This procedure is time consuming; it takes about 90 min to complete an injection before the GC starts to cool down (49). The use of high pressure liquid chromatography (HPLC) overcomes this problem and reduces the time needed to about 5 min. However, it only determines the ITC and OZT (131,135) and does not determine the nitriles (70).

3.3.6 Modification of the Analytical Procedure for ITC (166)

A method for the determination of ITC in the meal was needed for the investigation reported in this Thesis. Preliminary examinations of the methods of analysis dis-

cussed above in this Chapter indicated that the UV and the GLC methods might be suitable. In the final analysis, the Youngs and Wetter GLC method (235) (called here Youngs' method) was chosen over the UV method. The reason for this choice is detailed in Appendix 8.

This GLC method provides an indirect[1] determination of the glucosinolates. As mentioned in Chapter 3.3.5, this method includes a thermal deactivation step for the endogenous enzyme myrosinase (235). This is done by heating the meal at 100°C for 15 min with a pH7 buffer solution, or at 110°C in a forced air oven for 16 h. It was mentioned that the heat treatment would ensure the hydrolysis of the seed glucosinolates quantitatively to ITC (16,99) and OZT (153,220,222,225). However, it is felt that this heat treatment could also cause the aglucons present in the meal (due to the planned seed treatment) to react with meal protein (15), to volatilize (154), and to polymerize (225). In addition, the meal myrosinase is specific to its native glucosinolates (37); therefore, its inactivation and replacement by an exogenous enzyme could result in an incomplete hydrolysis of the glucosinolates

[1] Since each molecule of the glucosinolates, under the appropriate conditions, is hydrolyzed to one molecule of isothiocyanate, the glucosinolate content can be indirectly determined as their ITC.

(143,198). This could result in an underestimation of the amount of aglucon present in the seed (144,198). It can be concluded that, although this thermal enzyme deactivation prevents the formation of nitriles, it could be a source of error.

Also, the incubation of the meal with the mustard enzyme at such a low W/M ratio of 5:1, could cause the formation of a substantial amount of nitriles (23,222,225). It is also thought that the addition of the hydrolyzing solution before the extracting solvent (methylene chloride) would allow the formed ITC to react with the meal protein.

Before using Youngs' method in this investigation, it was decided to alleviate these possible sources of error. To facilitate the discussion, the work carried out to modify Youngs' method is summarized in Tables 3.1.a and 3.1.b. The former illustrates the hydrolysis of the glucosinolates at pH 4.5 and the latter in the pH range of 6 to 7. It should be noted that at pH7 the formation of ITC is maximum, but beyond this pH it declines (16). In all the experiments presented in these tables, the meal glucosinolates were hydrolyzed for 2 h at 23°C. Youngs and Wetter (235) and Schwimmer (182) indicated that after a hydrolysis time of 1½ h, no change in the amount of

aglucons formed was observed. Thus, as in Youngs and Wetter (235), a 2 h hydrolysis time was used in this work.

In the experiments carried out following Youngs' method, an exogenous myrosinase was used to hydrolyze the meals's glucosinolates. This enzyme was prepared from yellow mustard (Sinapis alba) and furnished by Dr. J. Jones of Agriculture Canada.

TABLE 3.1 DETERMINATION OF ITC

(a) pH 4.5

	BIT mg/g meal[3]	PIT mg/g meal[3]	ITC[2] mg/g meal[3]	S.D. mg/g meal[3]
Following Youngs' method	0.02	0	0.02	0.0036(3)
VMAP (A3)	0.20	0.02	0.22	0.0042(4)
VMAP (A5)	0.25	0.02	0.27	0.0053(3)
MAP (A5)	0.32	0.01	0.33	0.0048(6)

MAP: Modification of the Analysis Procedure
A : Hydrolysis at pH 4.5

Number beside A is W/M ratio
In MAP : Methylene chloride is added to meal, then the water.
In VMAP: Water is added to the meal, followed by methylene chloride.

[2] ITC: = BIT + PIT

[3] All through the Thesis results are reported on dry basis, unless otherwise mentioned.

TABLE 3.1 DETERMINATION OF ITC

(b) pH 6 - 7

	BIT mg/g meal[3]	PIT mg/g meal[3]	ITC[2] mg/g meal[3]	S.D. mg/g meal[3]
Following Youngs' method	0.31	0	0.31	0.0427(3)
MAP (N5)	0.40	0.01	0.41	0.0085(3)
MAP (N10)	0.49	0.01	0.50	0.0059(5)
MAP (N150)	1.40	0.12	1.52	0.0682(3)
Variation in MAP (N150)	1.17	0	1.17	0.0120(3)

MAP: Modification of the Analysis Procedure

N : Hydrolysis at pH 6 to 7

Number beside N is W/M ratio

In MAP : Methylene chloride is added to meal, then the water.

In VMAP: Water is added to the meal followed by methylene chloride.

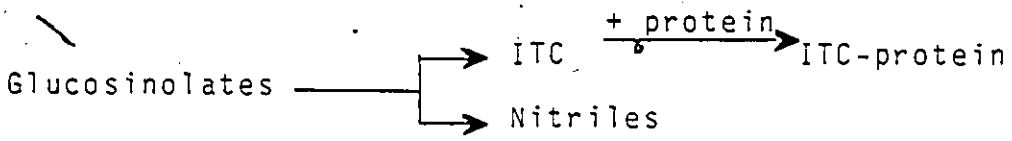
---

[2] ITC: = BIT + RIT

[3] All through the Thesis it is on dry basis, unless otherwise mentioned.

In examining the tabulated results, it was essential to establish a criterion for judging them. Thus, examination of Figure 2.1.a and subsequent discussions show that the aglucons of interest, yielded by the enzymatic hydrolysis of the rapeseed meal glucosinolates, are the nitriles and the isothiocyanates (OZT are cyclized isolthiocyanates). Also, Figure 2.1.b shows that the ITC could react with the meal protein.

This could be outlined as follows:



As mentioned in Chapter 2, the formation of the nitriles is competing with that of the isothiocyanates. Therefore, the amount of isothiocyanates formed could be underestimated by the incomplete hydrolysis of the glucosinolates and/or by the formation of the nitriles. Furthermore, these isothiocyanates could react with the rapeseed protein. During the analysis of the isothiocyanates, the reacted portion could not be determined since it is bound to the protein.

In view of this discussion, the aim of the isothiocyanates analysis methods is threefold: to completely hydrolyze the glucosinolates to only the isothiocyanates; to prevent their reaction with the seed protein; and to measure them quantitatively. The validity of the analytical procedure is to be judged on the basis of, and on the assumption that, the best procedure would show the highest ITC content in a given sample. This criterion was also used by Appelqvist and Joseffson (15), Appelqvist (16), Youngs and Wetter (235) to find out, while developing procedures for the determination of ITC and OZT, the suitable pH for the hydrolysis of rapeseed glucosinolates. Furthermore, Srivastava and Hill (198) used the same approach to conclude that in aglucon analysis procedures, the use of exogenous myrosinase would underestimate the amount of aglucons produced from the hydrolysis of the meal glucosinolates.

Wetter and Youngs (233) have used another approach to judge an analysis procedure. This approach involves the addition of known amounts of glucosinolates samples to meals. These glucosinolates are then determined by the procedure to be judged, and the results are compared with the known amounts added. This approach was not used in this study because it was felt that the native glucosino-

lates embedded in the meal's structure might behave differently from the added glucosinolates.

Initial experiments were made, following Youngs' method, to test the effect of using pH 4.5 instead of 7 on the formation of ITC by hydrolysis at a W/M ration of 3:1. By applying the criterion discussed above, it was concluded that the hydrolysis of the glucosinolates at pH7, which yielded 0.31 mg/g meal ITC, was better than hydrolysis at pH 4.5, which produced only 0.02 mg ITC/g meal. This indicates that in Youngs' method it is correct to hydrolyze the glucosinolates at the neutral pH. However, it was decided to investigate the planned modification at pH 4.5, since part of the work that is reported in Chapter 5 was carried out at this pH.

The next modification[4] introduced was the use of the meal's endogenous enzymes, which is presumably not significantly altered by the meal's preparation procedure, to hydrolyze the glucosinolates. This modification resulted in an increase in the amount of ITC determined to 0.22 mg/g meal from 0.02 for Youngs' method, under the same experimental conditions.

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[4] Only one modification as tested at a time.

An increase in the W/M ratio from 3:1 VMAP (A3) to 5:1 VMAP (A5) was then investigated. This resulted in an increase in the amount of ITC determined to 0.27 mg/g meal from 0.22 mg/g meal.

The next modification studied was the order of addition. Thus, comparing the amount of ITC determined by VMAP (A5) of 0.27 mg/g meal to that of MAP (A5) of 0.33 mg/g meal, indicated that the order of addition followed in MAP is favorable in the determination of the ITC of rapeseed meal.

A comparison between MAP (N5) and MAP (A5) was conducted to find out if, in spite of the incorporated modification, the analysis at pH 6 to 7 still gives better results than at pH 4.5. This proved to be the case because the amount of ITC determined in the former at 0.41 mg/g meal was higher than that for the latter of 0.33 mg/g meal. In line with the conclusions reached during the study of the analysis at pH 4.5, the increase of the W/M ratio increased the amount of ITC determined. This could be seen when examining MAP (N5), MAP (N10), and MAP (N150)[5], where the ITC determined were 0.33, 0.50, and 1.42 mg/g meal,

[5] Will be called MAP only.

respectively. The W/M ratio was not increased beyond 150:1, because this ratio was the highest used in the work reported in Chapter 5.

An investigation on the effect of the order of addition of chemicals in the hydrolysis pH range of 6 to 7, by comparing MAP to VMAP (N150) of determined ITC of 1.17 mg/g meal, confirms that the change in order of addition of MAP improved in the analytical method.

In conclusion, the modifications effected in this study improved upon Youngs' ITC determination method. The modified method is simple, fast, and reasonably accurate (having a coefficient of variation of 4.5%). However, the method is not suitable for the determination of glucosinolates such as the indolyls whose ITC are unstable at a neutral pH (details are in Appendix 18). The method also cannot be applied to meals whose myrosinase enzyme has been deactivated.

The detailed procedure of MAP is as follows:

Procedure

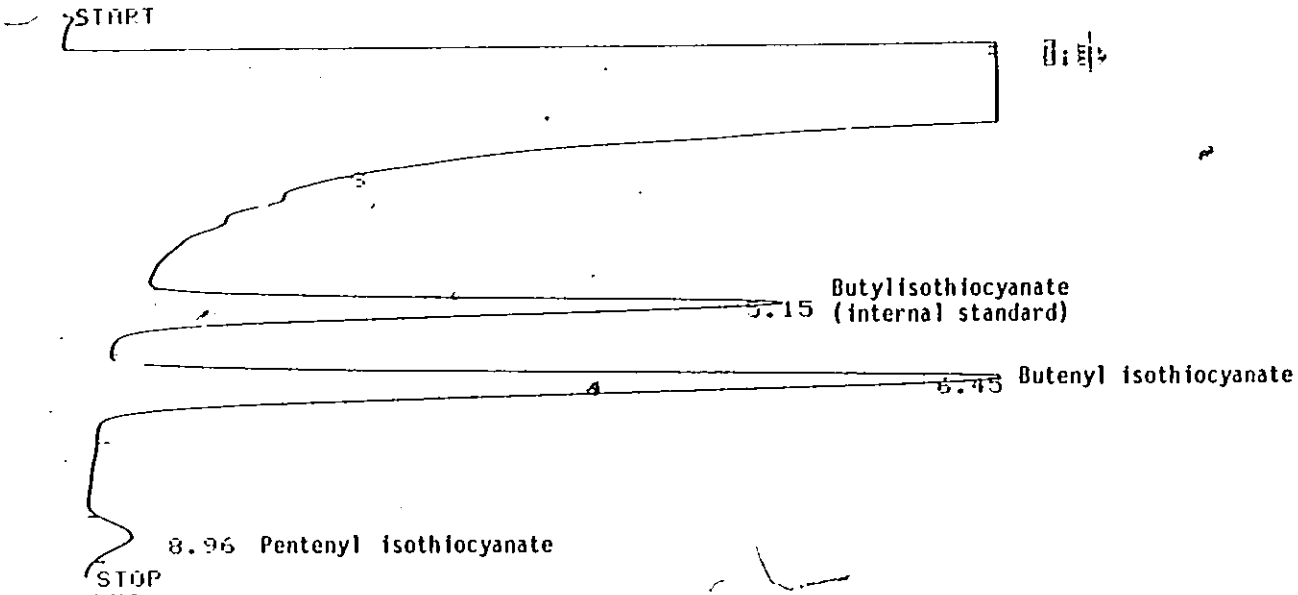
One hundred mg of the meal and a 6 mm glass bead were placed in a 20 ml culture tube. Two ml of methylene

chloride at 23°C were also added to the tube. The tube was capped with a Teflon-lined screw cap and the contents mixed in a vertical position without rotation for 10 min. The methylene chloride contained an internal standard of 40 mg/l butyl isothiocyanate. Fifteen ml of water were added to the tube which was then shaken for 2 h in a rotary shaker immersed in a 23°C water bath. In this procedure, the solvent was added before the water, not only to extract any existing aglucons if present, but also to prevent the ITC resulting from the hydrolysis of the meal glucosinolates from reacting with the meal protein. After shaking, the emulsion in the tube was broken by centrifugation at 2500 rpm. The bottom layer (methylene chloride) of the solution was removed with a long-needled syringe and the extract kept in a 4 ml vial with a polyethylene snap cap. One μL of this extract was injected by a microsyringe into a stainless steel column (outside and inside diameters 6.0 and 4.5 mm, respectively), packed with 20% FFAP on 60/80 mesh Anakrom ABS, connected to a 5830 Hewlett-Packard GC unit. Part of this unit is an 1885DA GC area integrator. The column was purchased from Chromatographic Specialties of Brockville, Ontario. Helium was used as the carrier gas at a flow rate of 32 ml/min. The temperatures of the injection port, oven, and flame ionization detector (FID) were 250, 180, and 300°C, respectively. The attenuation

used in this gas chromatograph was 1/18 and the slope sensitivity was 0.5. The area rejection was nil, thus all peaks were integrated. The integrator chart speed was 1 cm/min.

A typical gas chromatogram of the MAP ITC analysis is shown in Figure 3.1. The two unkown peaks appearing in this figure were identified by mass spectrometry to be for butenyl- and pentenyl-isothiocyanate. The discussion of the spectra and of co-eluted compounds during the chromatography are detailed in Appendix 15.

As can be seen in Figure 3.1, the retention time (RT) for the peaks of butyl- (the internal standard), butenyl- and pentenyl-isothiocyanates were 5.15, 6.45, and 8.96 min, respectively. The integrated areas in millivolts corresponding to these peaks were 28880, 49950, and 2647, respectively. The amounts of the individual butenyl- and pentenyl-isothiocyanates were calculated as outlined in the work of Youngs et al. (235). The calculation procedure is presented in detail in Appendix 13.



hp 5830A
AREA %

RT	AREA	AREA %
0.69	85840	0.038
0.81	314000	0.140
0.89	223400000	99.785
5.15	28880	0.013
6.45	49950	0.022
8.96	2647	0.001

XF: 1.0000 E+ 0

Figure. 3.1 GAS CHROMATOGRAM OF THE ISOTHIOCYANATES.

CHAPTER 4

SOLUBILITY OF MEAL PROTEIN IN AQUEOUS SOLUTIONS

4.1 Introduction

This investigation was carried out to determine the solubility of rapeseed meal protein in aqueous solutions. Particular attention was paid to changes in the meal's color and to conditions of minimum solubility. Information on the minimum solubility was needed in order to develop a meal detoxification process by aqueous extraction, while minimizing protein loss. The meal used was not ground to affect a low protein solubility level in aqueous solutions.

In the following detoxification study, monitoring the changes in the meal's color was done to avoid processing conditions giving drastic changes in the meal's color. This is important since a change in the meal's color (from its original yellow to dark brown or green)

would seriously limit the potential incorporation of the meal into most foods (189).

The solubility study was also necessary since relevant data in the literature were obtained for rapeseed meals other than Tower. Also, the preparation methods and specifications of these meals were different than those required for the present work. For example, Quinn and Jones (164) used the Echo variety, while Korolczuk and Rutkowski (102) did not mention the variety used in their work. In both procedures the seed was ground.

The purpose of this investigation was to study the effect of pH at different temperature levels on the solubility of Tower meal protein. Thus the pH, varying between 1 to 13 and temperatures set at 15, 25, 35, 45, and 55°C (±0.5%) were the factors studied. This upper temperature limit was chosen because it was felt that at higher temperatures, excessive protein solubility would occur (102).

A high W/M (wet basis) ratio of 50:1 was used in this investigation. Since the meal absorbs water, this high ratio was needed to minimize the relative amount of this absorbed water to the ratio used.

Initially, buffer solutions were used to maintain a constant pH (33,63). However, it was found that the pH drifted from its initial value. This could have been because the carboxylic and amino acid groups in the meal protein exercised a strong effect on the solution. Also, the buffer solutions, with their varying compositions, might not have established a fair base for comparing the protein solubility, since the solubility is affected by the ions present in the solution. Furthermore, the buffer compositions might have affected the activity[1] of the myrosinase (82), which would have intefered with the planned detoxification of the meal. Since buffer solutions do not offer a stable pH in the presence of the meal, and since there are problems associated with their use, it was decided to use solutions of either sulfuric acid or sodium hydroxide instead. (Distilled water was used in the preparation of all solutions throughout this Thesis to achieve controlled conditions.) Solutions of either NaOH or H_2SO_4 (HCl in some cases) were also used in similar investigations (73,102,164,186,187). This alleviated the problems associated with the use of buffers, except for the drifting of the pH from its initial value.

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[1] Defined as: mole glucosinolates hydrolyzed/mole enzyme. min.

Preliminary experiments were carried out at the W/M ratio of 50:1 and at the 5 temperature levels mentioned. During these experiments, the solutions were stirred magnetically and the pH was constantly monitored for 1 h. It was noticed during the monitoring that in all cases, the pH was stabilized in less than 20 min. After that, the pH remained virtually unchanged for the remainder of the hour. Thus, it was felt that the use of a 1 h extraction time would ensure that the system had reached a constant pH. This same extraction time was used by Smith et al. (187) while in other investigations only 30 min were used (102,164,186). In the work of Gillberg and Tornell (73), the meal was also extracted for 30 min after the pH was stabilized which, for samples of a 2.5 g size, was within 2.5 min[2].

As conducted in other investigations (73,102,164, 186,187), the solubility of the protein was determined as dissolved nitrogen.

[2] The time in the work of Gillberg and Tornell (73) is lower than the 20 min found in this investigation, possibly because they ground the meal, which might have allowed its components a faster exposure to the solution.

4.2 Experimental Procedure

The meal used in this experimental procedure was prepared from rapeseed (Brassica napus var. Tower) which was cracked, dehulled, and defatted. (Details are presented in Appendix 1.)

Fifty ml of an aqueous solution with a known pH (initial pH) was added to 1.0 g of meal (wet basis) in a 125 ml Erlynmeyer flask. The flask was then stoppered with a rubber bung and the mixture was shaken for 1 h in a constant-temperature water bath. After this shaking period, the pH of the solution was measured and denoted as the final pH. As discussed above, after the shaking period of 1 h, the solution would have reached an equilibrium. Thus, this final pH was considered the pH at which the protein solubility had occurred. This approach has also been used in similar investigations (102,164).

The supernatant was subsequently decanted and centrifuged for 5 to 6 min at 2500 rpm. Ten ml of the clear supernatant was digested according to the standard Kjeldahl method (141) (details are presented in Appendix 5). The digested supernatant was then transferred

quantitatively to a 1 L volumetric flask and distilled water was added to the mark.

An Orion Ammonia Electrode, model 95-10, which is capable of detecting dissolved ammonia in aqueous solutions over the concentration range of 1 to 10^{-4} M, was used to measure the ammonia of the digested supernatant. This method is called by the manufacturer "The Known Addition Method" because a solution of a known ammonia concentration is added to that to be measured (149). A more detailed description of the function of the electrode is given in Appendix 2.

An aliquot of 20 ml of the dilute digested sample was then transferred to a 150 ml beaker. Two ml of an alkaline reagent (10M NaOH + 2M NaI) and 80 ml of distilled water were added to the aliquot. The NaOH in this regent releases the ammonia from its sulfate salt by raising the pH to over 11. The NaI replaced the ammonia in its complex with the mercury catalyst, thus freeing the ammonia to enable its measurement by the electrode. To avoid loss of the ammonia to the air during the addition of the alkaline reagent and the distilled water, the contents of the beaker were always cooled, from the outside, to about 25°C. The solution was magnetically stirred at a low velocity without

forming a vortex, which could have resulted in the escape of the ammonia from the solution (149). About 1 cm of the ammonia electrode, which was connected to an Accumet 420 digital pH/ion meter, was quickly immersed in the solution. Then, after 1 min, which is an adequate time for the electrode to stabilize (149), an initial reading (E_1) was taken from the expanded millivolt scale of the meter. Ten ml of a standard NH_4Cl solution (20 ppm as nitrogen) was added and after another minute, a second reading (E_2) was recorded. By subtracting the former reading from the latter, a value of ΔE was obtained. A Table in the instruction manual of the electrode (copy shown in Appendix 2) relates ΔE to a value Q, which is the ratio of the concentration of the nitrogen in the aliquot to that of the added standard solution. By multiplying Q by 500[3], the amount of dissolved nitrogen in the solution is obtained.

This procedure for the determination of the dissolved nitrogen in the supernatant was also used for the determination of the meal's nitrogen content. The accuracy

[3] Result of multiplication of 100 (factor in the supplier's manual) to convert values of Q to mg and 5 (ratio of the volume of the solutions 50 ml used to dissolve the protein to the volume of the digested supervatant (10 ml).

of the procedure followed was tested and the coefficient of the variation was found to be 1.1%. Details are in Appendix 7.

At the beginning and end of each series of measurements, the precision of the electrode was checked by determining the nitrogen content of a digested known amount of the amino acid glycine, which contains 18.66% nitrogen. If the coefficient of variation (C.V.) for the check measurements of a series was not less than 2%, the results of the series were discarded. The measurement procedure was examined until the reason for the high C.V. was found and alleviated.

A blank was prepared by digesting about 1 g of solid dextrose. The nitrogen content of the blank was too small to be measurable, therefore showing that there was no need to purify the chemicals used or to compensate for their nitrogen content.

4.3 Results and Discussion

The experimental results and the conditions under which they were obtained are presented in Table 4.1 (located in Appendix 6) and shown in Figure 4.1. It should be noted that the pH in Figure 4.1 is the final pH.

By comparing the initial and final pHs reported in Table 4.1, it can be seen that the meal exerted a buffering action. For example, at 15°C and an initial pH of 2.00, the final pH was 4.26. Also, at 25°C, for the initial pH of 10.78, the final pH was 6.5.

It was not possible to extensively duplicate experiments at the same final pH. This is because the desired final pH level could not be reached with precision by adjusting the initial pH. For example, at 15°C and very close initial pHs of 0.55 and 0.56, the final pHs were substantially different at 0.48 and 0.70, respectively. Furthermore, at 25°C and at different initial pHs of 4.98, 5.69, and 5.82, final pHs were almost identical at 6.16 for the first and 6.17 for the latter two. However, an illustration of the accuracy of the results can be seen when the dissolved nitrogen of these three experiments are com-

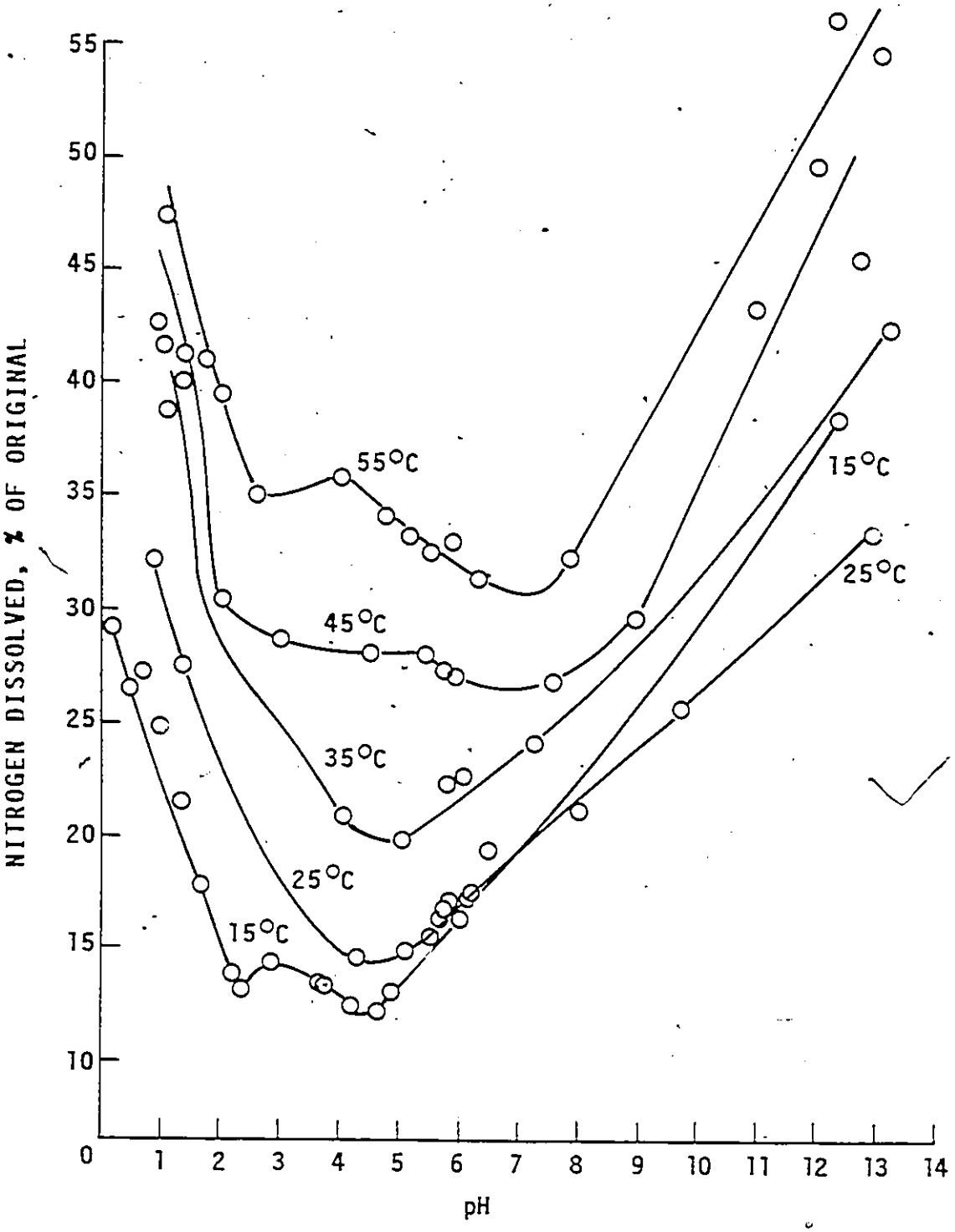

Figure. 4.1 SOLUBILITY OF TOWER RAPESEED PROTEIN (as nitrogen) IN AQUEOUS SOLUTIONS.

pared. Their dissolved nitrogen of 17.2%, 17.4%, and 17.4%, respectively, indicated that the process is reasonably accurate.

From Figure 4.1, it could be seen that at 15°C in the pH range[4] of 2.24 to 4.92, the nitrogen solubility was low at only between 12.2 and 13.5%. In this range, it could also be seen that the changes in the pH had little effect on the levels of the dissolved nitrogen. A similar nitrogen solubility trend was also observed at 45 and 55°C, in wide pH ranges of 3.06 to 8.98 and 2.68 to 7.90, respectively. As for the temperatures of 25 and 35°C, the pH ranges were narrow at 4.3 to 6.17 and 4.13 to 6.10. This narrowness is most likely due to a lack of sufficient data to establish the full range. As shown in Table 4.1, no color change of the meal occurred at, or near, the low nitrogen solubility regions. In other regions, the color of the meal generally changed from its original yellow to gray and to dark green, in the direction of increasing acidity and alkalinity, respectively. Similar, though more pronounced, color changes were also observed in the extract. Thompson et al. (213) observed similar color behavior in their work on rapeseed protein isolates.

[4] Boundary of a pH range is formed by the experimental data.

The minimum nitrogen solubility points were identified from the constructed lines of Figure 4.1. The levels of these minima and the conditions of their occurrence are presented in Table 4.2. As can be seen in this table, the level of the minimum nitrogen solubility and the corresponding pH increase with the increase in temperature. This could also be noticed in Figure 4.2 which is extracted from the work of Korolczuk and Rutkowski (102)[5]. In similar

TABLE 4.2

MINIMUM SOLUBILITY OF TOWER RAPESEED PROTEIN IN AQUEOUS SOLUTIONS

Temperature °C	pH	Minimum Solubility (% of original)
15	4.4	11.8
25	4.5	14.2
35	4.8	19.0
45	7.0	26.2
55	7.2	30.2

[5] The meal used was prepared by defatting the seed with a solvent. The meal produced was air-dried and then dried under vacuum. Conditions of the study were: W/M ratio of 20:1, duration of extraction of 30 min, and temperature levels of 20, 40, 60, 80 and 100°C. After cooling, the samples were centrifuged at 2000 rpm for 20 min. The nitrogen in the solution was determined by the Kjeldahl method. Their results were reported as contours of dissolved nitrogen (in % of original in meal) as a function of pH and temperature.

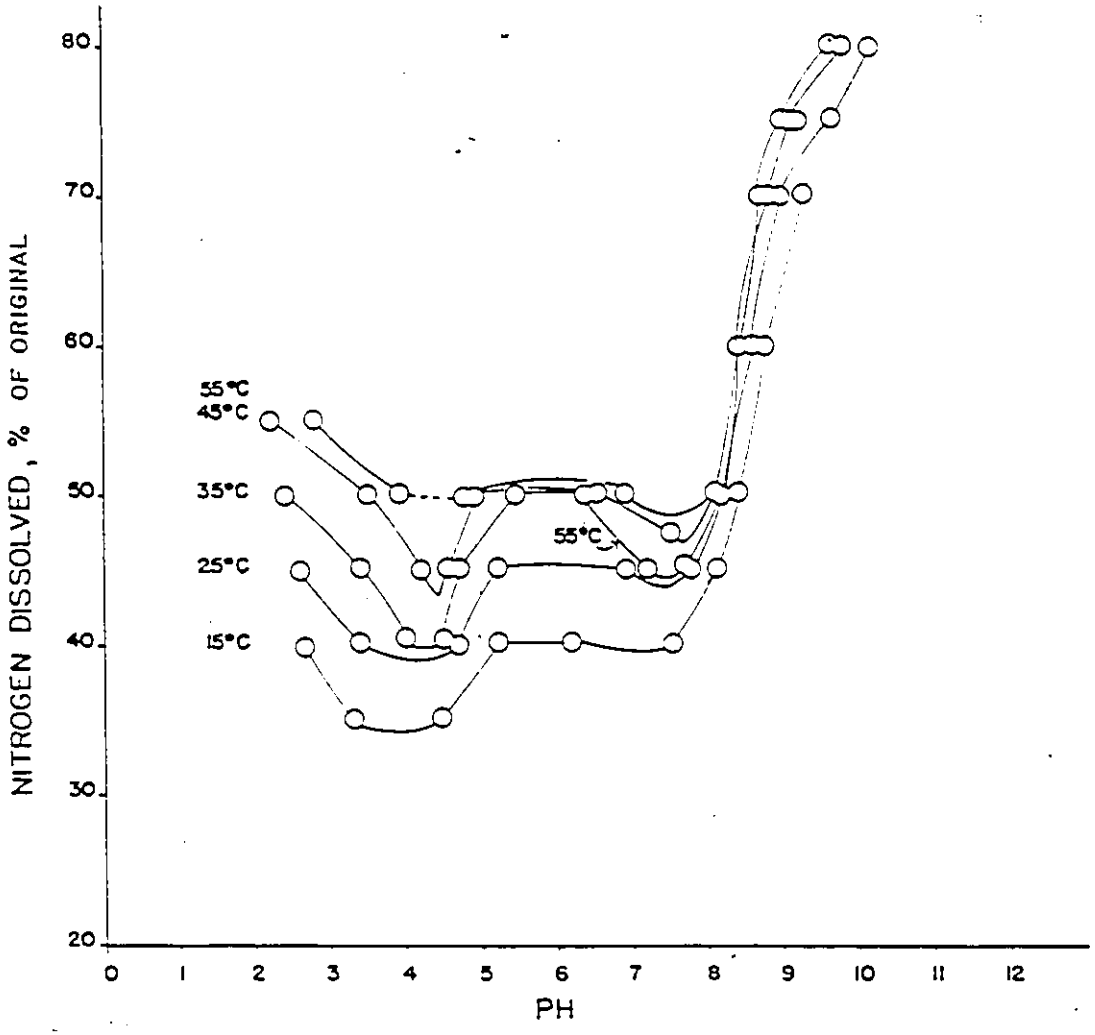

Figure. 4.2 SOLUBILITY OF RAPESEED PROTEIN; FROM THE WORK OF KOROLCZUK et. al.

investigations, it was found that the minimum solubility of the protein of soybean meal at a temperature between 24 and 26°C occurs between a pH of 3.5 and 4.5 (186,187). For radish (187) and Crambe abyssinica (224) meals (both, like rapeseed, in the Cruciferae family), the corresponding minima were between pH of 3.8 and 4.3, and pH of 4 and 4.2, respectively. The temperature for the former study was 25°C, while that for the latter was not mentioned. The reported value in the work of Sosulski and Sarwar (192) for rapeseed at room temperature was in the pH range of 4.4 to 4.7, while that obtained in the investigation of Quinn and Jones (164) on the rapeseed Echo variety was in the pH range of 3.7 to 4.6 (temperature was not mentioned). From Table 4.2, it could be seen that Tower rapeseed, under similar conditions, has a pH value of 4.5, which is in agreement with the values cited above.

In all of the five temperature levels studied, the amount of dissolved nitrogen increases substantially in going to both alkaline and acidic extreme pHs. For example, at 15°C and pHs of 0.19 and 12.46, the nitrogen dissolution was 29.5 and 38.8% of that in the meal, respectively. It can also be observed in Figure 4.1 that the nitrogen solubility generally increases with temperature.

The increase in the protein solubility at the alkaline pH values might have been the result of reduced protein-phytate interactions. As the pH increases from the pH of minimum nitrogen solubility, there is a tendency for these interactions to be reduced. This is a result of the competition (5,73,144) between carboxylic groups (of aspartate and glutamate residues) in the protein and phytate anion for the basic groups of lysine (ϵ-animo groups), histidine (imidazole groups), and arginine (guanidino group) residues of the protein. At the very high pH values ($pH > 11$), the protein-phytate interactions are minimal. This might be related to the relatively high nitrogen solubility at the higher pH values.

As reported in the literature (63,73,102,164,165, 225), Figure 4.1 may show an indication of two isoelectric points. For example, they could be seen at 55°C around the pHs of 2.68 and about 7.0. This phenomenon could be explained by the presence of two major protein groups in rapeseed, one acidic and the other basic (22,66,75,77,165).

The solubility levels of this investigation, shown in Figure 4.1, were compared with those in Figure 4.2 of Korolczuk and Rutkowski (102). It can be seen from the comparison at the same pH level that, in spite of the use

of a higher W/M ratio of 50:1 in this investigation than that of 20:1 used by Korolczuk and Rutkowski (102), the nitrogen solubility level in the former is lower than the latter. For example, at 25°C and pH of 4.5, the nitrogen solubility in this investigation was 14.2%, while that of Koroluczuk and Rutkowski (102) was about 41.0%. A possible explanation might be that not grinding the meal in the present investigation reduced its nitrogen solubility level.

In summary, this investigation has resulted in the determination of the experimental conditions for minimum protein solubility. With these conditions, it was found that the meal did not turn to an objectionable dark color. This information was needed for carrying out the subsequent detoxification study.

CHAPTER 5

DEVELOPMENT OF A DETOXIFICATION METHOD FOR RAPESEED MEAL

In undertaking the research investigation to detoxify Tower meal, the extraction of glucosinolates and their aglucons by acidic solutions (called the **Aqueous Extraction Method**) was investigated first. Conclusions reached in this investigation were used to plan a second phase of the development, called the **Acidic Medium Method.** The meal in the second method was treated essentially in the same manner as in the Aqueous Extraction Method. The main difference is the deletion, in the Acidic Medium Method, of the filtration step. Information obtained from the Acidic Medium Method was then used to formulate a research plan for a third and last phase of development. In this last phase, the meal was treated in the same way as in the **Acidic Medium Method,** except that the pH used was between 6 and 7; hence it is called the **Neutral Medium Method.**

The guidelines followed in this investigation were that all the processing steps used were to be mild, so that the denaturation of the meal protein and changes in its color would be minimized. Also, the fibre content of the meal was reduced. Details of these guidelines can be found in Section 1.1 under the heading "Research Objectives and Outline".

The procedure for preparing the meal is explained in detail in Appendix 1 and outlined in the flow sheet in Figure 5.1. This figure also contains a plan of the Aqueous Extraction Method. The major features of this flow sheet are that the myrosinase was not thermally deactivated because the heat treatment causes protein denaturation. Also, under certain circumstances this treatment could contaminte the seed oil with sulfur compounds. The drawbacks of the deactivation treatment have been discussed in more detail in Chapter 2, where it was noted that hydrolysis of the glucosinolates before the defatting of the meal could contaminate the oil with sulfur. The moisture content of the seed to be dehulled was kept below 8%. This low moisture level inhibited (or kept at a minimum) any hydrolysis of the glucosinolates during dehulling.

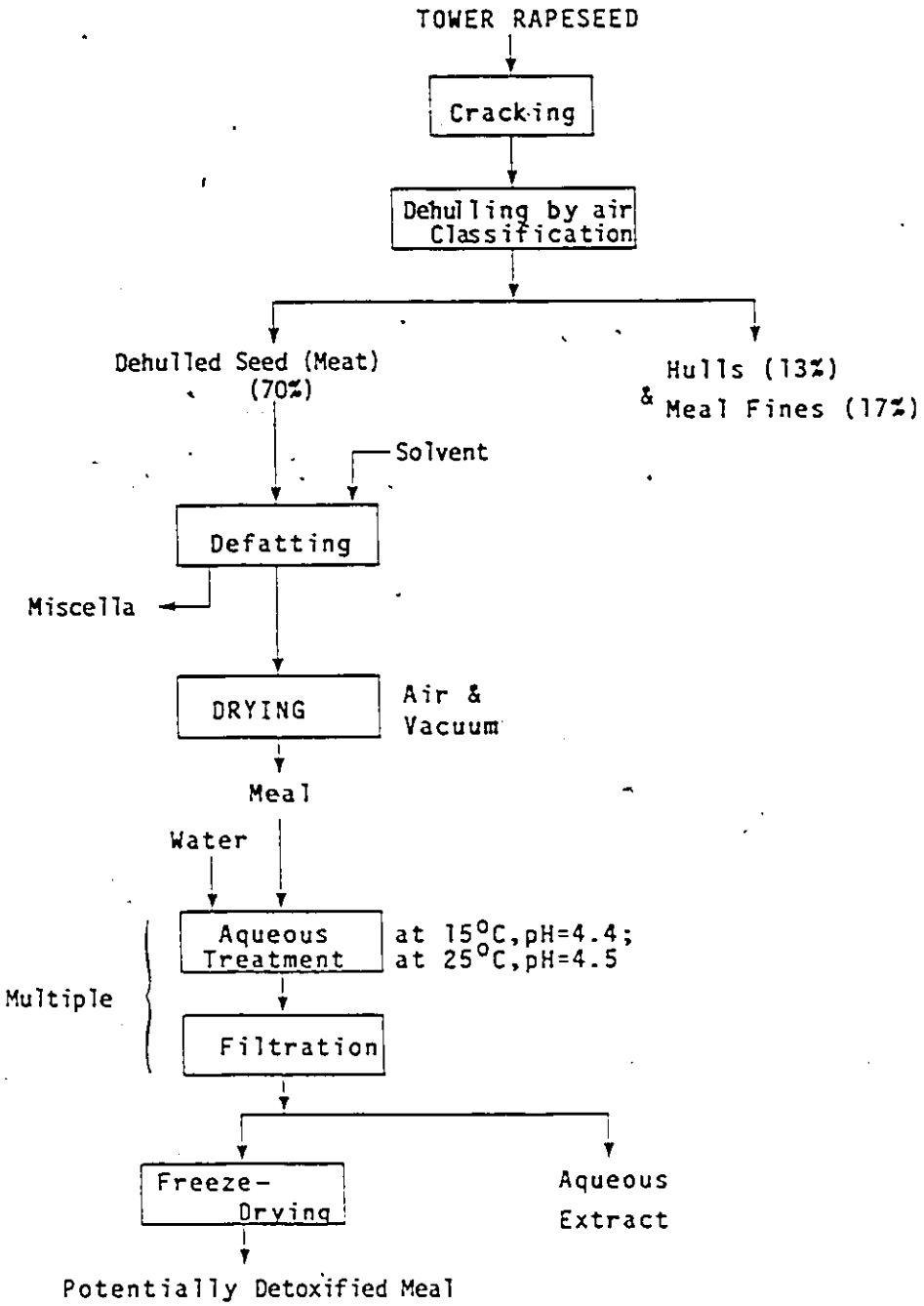

Figure.5.1 FLOW SHEET* of MEAL PREPARATION and INITIAL PLAN for DETOXIFICATION DEVELOPMENT.

* This procedure differs from that outlined in Figure 2.2 in extracting the meal under conditions of minimum protein solubility and deleting the enzyme deactivation step.

The dehulling step resulted in two fractions: the meat, which was about 70% of the seed, and the other 30% of the seed, which contained the hulls (13%) and meat fines (17%). The oil was extracted from the meat with food grade hexane in a Soxhlet extractor for 24 h[1]. The reason for this long duration is discussed in Appendix 1. In principle, the fines (in the hull and fines fraction) could have been defatted in the same way. The residual oil in the meal was found to be about 0.55% of the meal. This compares well with the range of 0.5 to 2% residual oil common for industrially produced meals (14). The extracted oil contained less than 10 ppm sulfur[2]. This is the same order of magnitude for oils produced industrially (14).

The dehulling step reduced the indigestible fiber content (mainly from hulls) of the meal from about 25% (if seed were not dehulled) to about 4%, as determined at Agriculture Canada. Calculation of the fibre content for the meal containing hulls is detailed in Table A.1. This reduction in the fiber content could be considered to be an improvement in the nutritional value of the meal.

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[1] Dr. D.J. Jones of Agriculture Canada determined in experiments that the required oil extraction time, in the Soxhlet used, for the Tower meat is between 23 and 31 h. Thus the 24 h extraction was adopted.

[2] Determined at Svaloff Research Station, Svaloff, Sweden through Dr. J. Jones of Agriculture Canada, by a modified Schöniger Combustion method (14).

The rate of formation of melanoids, which would give the meal an objectionable dark color, is high at low moisture contents and high temperatures (69). Freeze-drying was thus used to control their formation (97). Also, meal treatment conditions used during the development were chosen, in light of the study on the solubility of protein (presented in Chapter 4), to avoid inducing the formation of objectionable colors in the meal.

5.1 Aqueous Extraction Method

In this method, as shown in Figure 5.1, the meal was aqueously treated at the experimentally determined conditions (described in Chapter 4) for minimum solubility of protein at 15°C and 25°C, and corresponding pH values of 4.4 and 4.5, respectively. In these acidic conditions, the reaction between the ITC (resulting from hydrolysis of glucosinolates during the aqueous treatment) and protein to form dithiocarbamates and substituted thioureas would be minimal (36). The use of three W/M ratios[3] (3:1, 4:1, and 5:1) for aqueous treatment was investigated in this

[3] It should be noted that the intentional use of a much higher ratio of 50:1 in the solubility study was meant to single out the effects of pH on the solubility of protein (details are in Chapter 4). Moreover, the use of these three low ratios was only a starting point and, as will be seen later, the W/M ratios were increased.

method. The aqueous treatment time used was in the range of ½ to 5 h. This range was also adopted by Eapen et al. (57) in their study on the detoxification of rapeseed meal. After aqueously treating the meal, the resulting slurry was filtered.

The aim was to maximize both the detoxification and the amount of remaining protein and solids (defined as the treated meal after its freeze-drying). This strategy involved successive extractions, each of which was optimized until the meal was completely detoxified.

It was also planned to recover the protein from the combined filtrates by step-wise precipitation, centrifugation or membrane filtration. It should be noted that this was a tentative plan that could be changed at any stage of the experiment, according to the results obtained.

5.1.1 Experimental Procedure

The apparatus used for this detoxification study is shown in Figure 5.2. The extraction step was carried out in a cylindrical plexiglass extractor having a 3 mm thick flat bottom. Both its inside diameter and its height were 63 mm and wall thickness was 2 mm. In the preliminary

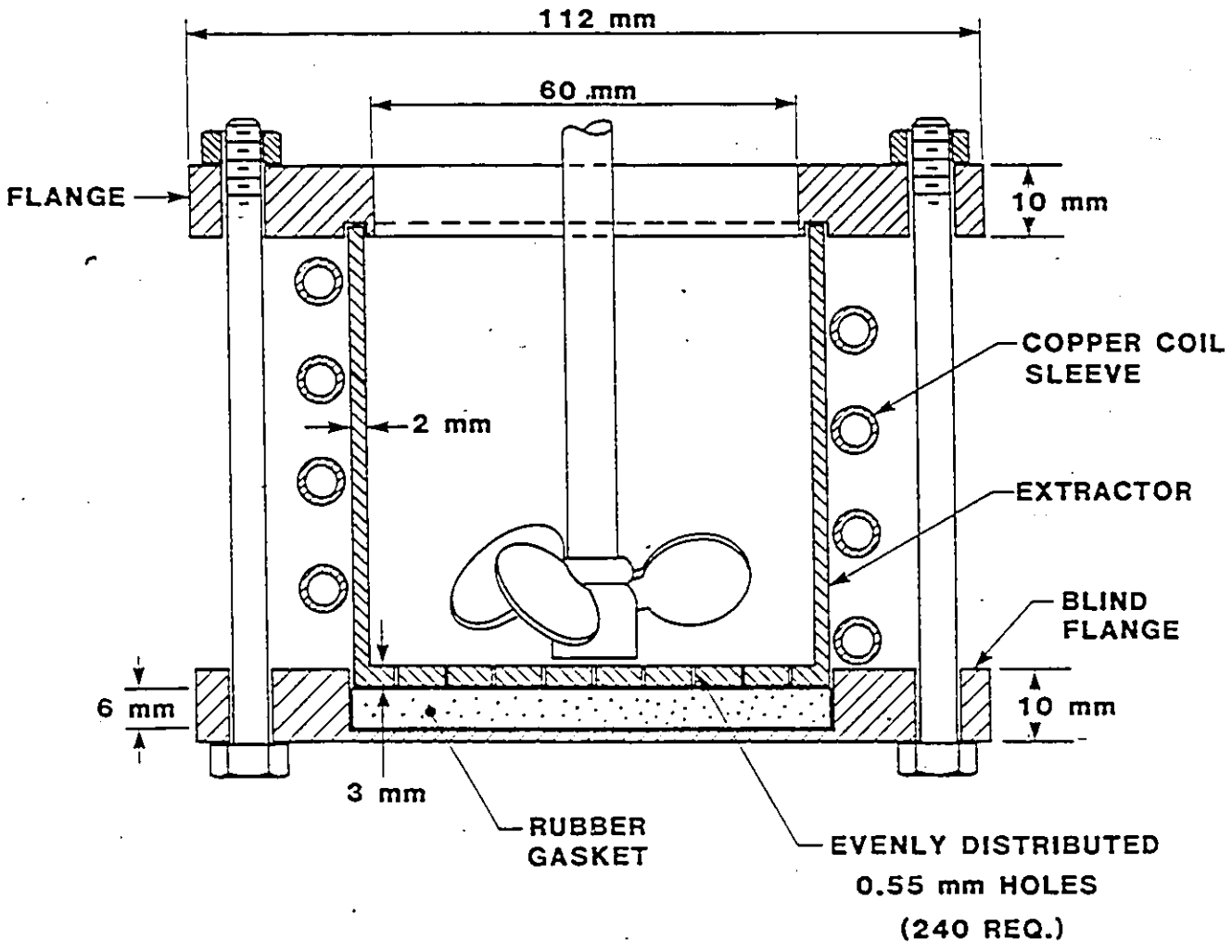


Figure. 5.2 EXTRACTION APPARATUS

aqueous extraction experiments carried out in a glass beaker, the aqueously treated meal did not filter quickly in a buchner through filter paper (even with the use of a vacuum) due to pore plugging. To overcome this problem, 240 holes with a diameter of 0.55 mm were drilled in the bottom of the extractor, which was used as a filter disc for the meal slurry. It should be noted that filtering rapeseed meal slurries through relatively large pore openings is considered an acceptable technique and has been widely used (1,57,105,118). For example, in the work of Afzalpukar et al. (1) the opening size of the filter holes was 0.84 mm. The appropriateness of a 0.55 mm hole size for this work is discussed in Section 5.1.2. During the aqueous treatment period, the bottom holes were tightly closed by a rubber gasket 6 mm thick and 69 mm in diameter. The gasket was seated in a groove of the same diameter in a blind flange 112 mm in diameter. Another flange, also with an outside diameter of 112 mm and an opening of 60 mm, was put on the top of the extractor. The lip of the extractor was placed in a 2 mm groove (same thickness as the extrator's wall) around the edge of the flange opening. The blind flange was firmly pressed against the extractor by screwing nuts to four bolts passing through both flanges.

A coiled sleeve of copper tubing was wound around the extractor, through which water of a constant temperature was passed to attain the required temperature in the meal slurry, in a range of ±0.5°C (as measured by a calibrated mercury thermometer inserted in the slurry). The extraction apparatus was held firmly in place by a clamp.

The contents (meal and aqueous solution) were mixed with a Teflon covered triple propeller stirrer that measured 50 mm in diameter and 10 mm in height. The propeller blades were angled at 45°. The speed of the stirring was approximately 300 rpm, as measured by a tachometer. The stem of the stirrer was passed through a positioning bearing to maintain the motion in the centre of the extractor.

The mixing of the meal slurry was considered adequate on the basis of visual observation of the movement of the meal and because the change in pH readings was monotonic and not drifting erratically, even after the acid or alkali were added to the slurry. In addition, the agitation times used (up to 5 h) resulted in a thorough mixing. Furthermore, the mercury thermometer and the pH electrode were positioned in the extractor to act as baffles, thus further improving the mixing of the meal slurries. To simplify Figure 5.2, the thermometer and the electrode

are not shown in it. The mixing was also judged to be adequate from observing the dispersion of the red color of methyl orange in the slurries of experiments carried out solely for this purpose. In this regard, it is of interest to mention that agitation had no effect on the amount of each aglucon produced from the hydrolysis of the meal glucosinolates (224).

Ten g of meal were weighed in the extractor. Then the extractor was mounted to the flanges described above and the apparatus was fastened. To bring the pH to the required level of 4.4 or 4.5, between 15 and 35 ml of dilute sulfuric acid was gradually added to the meal in the extractor. The amount of acid depended on the W/M ratio and time used in the extraction experiment. The pH was measured by a pH electrode connected to an Accumet 320 analogue pH meter. The pH of the slurry was maintained at the desired level by adding either relatively concentrated sulfuric acid or sodium hydroxide. The acid and hydroxide were added to the slurry, through the opening of the apparatus, from burettes controlled magnetically by a Luft 77 (on/off) controller. The feedback signal for the controller was received from the pH meter. The concentration, amount, and rate of the initial addition of the acid were adjusted so that 90% of the desired ratio was

added in about 10 to 15 min. The remaining 10% allowed (without exceeding the desired W/M ratio) the control of the pH by the addition of acid or alkali (up to extraction time of 4 h, usually only the addition of acid was needed). The full amount of the desired ratio was reached at least 30 min before the end of the experiment. In the case of the ½ h treatment, the desired ratio was reached at least 10 min before the end. It was felt that this addition procedure was the best possible compromise[4] between the need to control the pH of the solution and the required final W/M ratio. It should be noted that if the execution of an experiment did not satisfy this compromise, the experiment was discarded.

At the end of the treatment time, the nuts were unscrewed. The extractor was removed from the seating blind flange and the meal slurry was then filtered through

[4] A search of the literature for similar work indicated that a compromise had to be used that was satisfactory for the purpose. For example, VanEtten et al. (224) followed a similar procedure as the one under discussion in this chapter. The only difference was that HCl was used instead of H_2SO_4. Furthermore, Appelqvist and Joseffson (16) used high concentrations of buffer solutions at the required W/M ratio. Thus, during the experiment, the pH was continuously adjusted with HCl or NaOH, which might have changed the ratio. In addition, Paik et al. (153) added buffer solutions at the desired ratio to their meal. In some cases, this resulted in the final pH of the meal slurry being different from what it was intended to be.

the bottom holes of the extractor with the aid of a water vacuum pump. After filtering, the extractor was placed in a deep-freezer at -50°C until its contents were frozen. Then it was freeze-dried at a pressure of less than 10 μm for 24 h on a heat rack placed in the drying chamber of a Virtis 10-100 freeze-dryer. The temperature of the condenser of the freeze-dryer was -60°C. The temperature of the rack was maintained in order to give a temperature of 28°C in the meal after complete sublimation of the ice. To prevent material losses during drying, the extractor was tightly covered with microporous plastic sheeting. After drying, the remaining solids (defined as the extracted freeze-dried meal) were weighed and their percentage of the 10 g meal used was calculated. The results are presented in Table 5.1 and Figure 5.3 ("a" and "b" denote 15 and 25°C, respectively). The dried meal was kept under a nitrogen atmosphere in tightly capped glass jars and preserved in a refrigerator.

The remaining nitrogen content expressed in mg/g solids was determined by the Ammonia Ion Electrode method after the Kjeldahl digestion (141) as described in Chapter 4. The remaining nitrogen, in percentage of the original content of 82.5 mg/g meal, was calculated and is presented in Table 5.1. In the literature (146, for

TABLE 5.1

EFFECT OF EXTRACTION TEMPERATURE, W/M RATIO AND EXTRACTION TIME ON MEAL SOLIDS, NITROGEN AND ISOTHIOCYANATES

W/M (ml:g)	Extraction Time (h)	Remaining solids (% of orig.)	Remaining nitrogen++ (mg/g solids)	Remaining Protein# (% of orig)	BIT* (mg/g solids)	PIT** (mg/g solids)	ITC+ (mg/g solids)
			15°C				
3:1	½	78.48	91.0	86.5	.0295	.0045	.0370
3:1	1	77.50	89.7	84.2	.0259	.0035	.0294
3:1	2	77.00	87.8	81.9	.0168	.0023	.0191
3:1	3	77.20	86.5	80.9	.0193	.0024	.0217
3:1	4	79.24	91.0	87.4	-	-	-
3:1	4	79.18	91.0	87.2	.0116	.0023	.0139
3:1	5	78.00	88.4	83.5	-	-	-
3:1	5	76.14	87.1	80.3	.0121	.0026	.0147
4:1	½	70.53	91.0	77.8	.0340	.0069	.0379
4:1	1	68.02	92.4	76.1	.0220	.0038	.0258
4:1	2	66.15	92.4	74.0	.0181	.0037	.0218
4:1	3	64.68	88.4	69.3	.0153	.0044	.0197
4:1	4	65.80	88.4	70.5	.0115	.0029	.0144
4:1	5	60.94	86.5	63.9	.0106	.0022	.0128

* : Butenyl Isothiocyanate

** : Pentenyl Isothiocyanate

\+ : ITC = BIT + PIT

\# : $= \frac{\text{Remaining Nitrogen (mg/g solids)} \times \% \text{ Remaining Solids}}{\text{Nitrogen meal (82.5 mg/g)}}$

++ : Values higher than the original 82.5 mg/g meal indicate that the protein has been concentrated in the freeze-dried meal solids. This might be because the meal protein, with its high molecular weight and pH maintained at the minimum protein solubility conditions, dissolved less than the other meal components.

TABLE 5.1 (cont'd)

W/M (ml:g)	Extraction Time (h)	Remaining solids (% of orig.)	Remaining nitrogen++ (mg/g solids)	Remaining Protein# (% of orig)	BIT* (mg/g solids)	PIT** (mg/g solids)	ITC+ (mg/g solids)
			15°C				
5:1	½	65.50	90.7	72.0	.0395	-	.0395
5:1	1	64.68	88.0	68.9	.0387	.0054	.0441
5:1	2	60.96	87.3	64.5	.0327	.0017	.0344
5:1	3	60.33	82.8	60.5	.0212	.0038	.0250
5:1	4	61.14	85.3	63.2	.0212	.0016	.0228
5:1	5	60.82	81.5	60.0	.0162	.0016	.0178

* : Butenyl Isothiocyanate

** : Pentenyl Isothiocyanate

\+ : ITC = BIT + PIT

\# : $= \frac{\text{Remaining Nitrogen (mg/g solids)} \times \text{\% Remaining Solids}}{\text{Nitrogen meal (82.5 mg/g)}}$

++ : Values higher than the original 82.5 mg/g meal indicate that the protein has been concentrated in the freeze-dried meal solids. This might be because the meal protein, with its high molecular weight and pH maintained at the minimum protein solubility conditions, dissolved less than the other meal components.

TABLE 5.1 (cont'd)

W/M (ml:g)	Extraction Time (h)	Remaining solids (% of orig.)	Remaining nitrogen++ (mg/g solids)	Remaining Protein# (% of orig)	BIT* (mg/g solids)	PIT** (mg/g solids)	ITC+ (mg/g solids)
			25°C				
3:1	½	80.27	89.7	87.2	.0301	.0041	.0342
3:1	1	79.00	89.7	85.9	.0259	.0020	.0279
3:1	2	79.50	88.4	85.1	.0079	.0013	.0092
3:1	3	80.90	87.1	85.4	.0073	0	.0073
3:1	4	81.00	87.1	85.5	.0061	0	.0061
3:1	5	82.40	85.5	85.4	.0036	0	.0036
4:1	½	77.50	99.7	93.6	.0445	.0058	.0503
4:1	1	75.10	98.2	89.3	.0235	.0034	.0269
4:1	2	74.00	91.0	81.6	.0135	.0011	.0146
4:1	3	74.40	89.7	80.8	.0107	.0013	.0120
4:1	4	74.20	89.7	80.6	.0075	0	.0075
4:1	5	73.40	91.0	80.9	.0048	0	.0048

* : Butenyl Isothiocyanate

** : Pentenyl Isothiocyanate

+ : ITC = BIT + PIT

: $= \frac{\text{Remaining Nitrogen (mg/g solids)} \times \text{\% Remaining Solids}}{\text{Nitrogen meal (82.5 mg/g)}}$

++ : Values higher than the original 82.5 mg/g meal indicate that the protein has been concentrated in the freeze-dried meal solids. This might be because the meal protein, with its high molecular weight and pH maintained at the minimum protein solubility conditions, dissolved less than the other meal components.

TABLE 5.1 (concluded)

W/M (ml:g)	Extraction Time (h)	Remaining solids (% of orig.)	Remaining nitrogen++ (mg/g solids)	Remaining Protein# (% of orig)	BIT* (mg/g solids)	PIT** (mg/g solids)	ITC+ (mg/g solids)
			25°C				
5:1	½	77.67	95.2	89.6	.0342	.0021	.0363
5:1	1	69.94	95.2	80.7	.0205	.0044	.0249
5:1	2	64.49	92.4	72.2	.0208	.0023	.0231
5:1	3	62.44	88.4	66.9	.0079	0	.0079
5:1	4	62.29	89.3	67.4	.0059	.0004	.0063
5:1	5	60.85	88.4	65.2	.0050	.0012	.0062

* : Butenyl Isothiocyanate

** : Pentenyl Isothiocyanate

\+ : ITC = BIT + PIT

\# : $= \frac{\text{Remaining Nitrogen (mg/g solids)} \times \% \text{ Remaining Solids}}{\text{Nitrogen meal (82.5 mg/g)}}$

++ : Values higher than the original 82.5 mg/g meal indicate that the protein has been concentrated in the freeze-dried meal solids. This might be because the meal protein, with its high molecular weight and pH maintained at the minimum protein solubility conditions, dissolved less than the other meal components.

Figure. 5.3.a. MEAL SOLIDS REMAINING AFTER EXTRACTION (at 15°C) AND FREEZE DRYING.

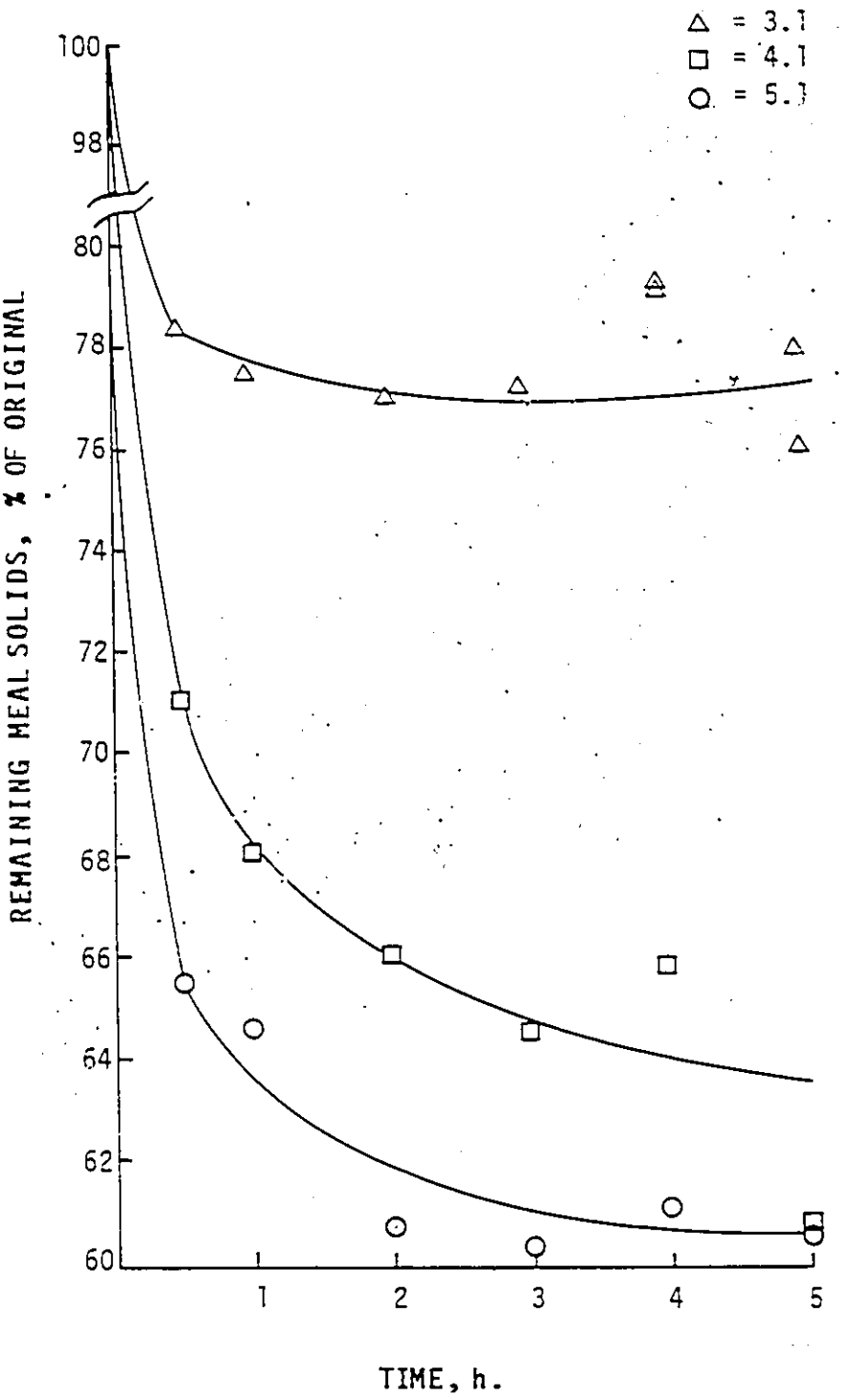
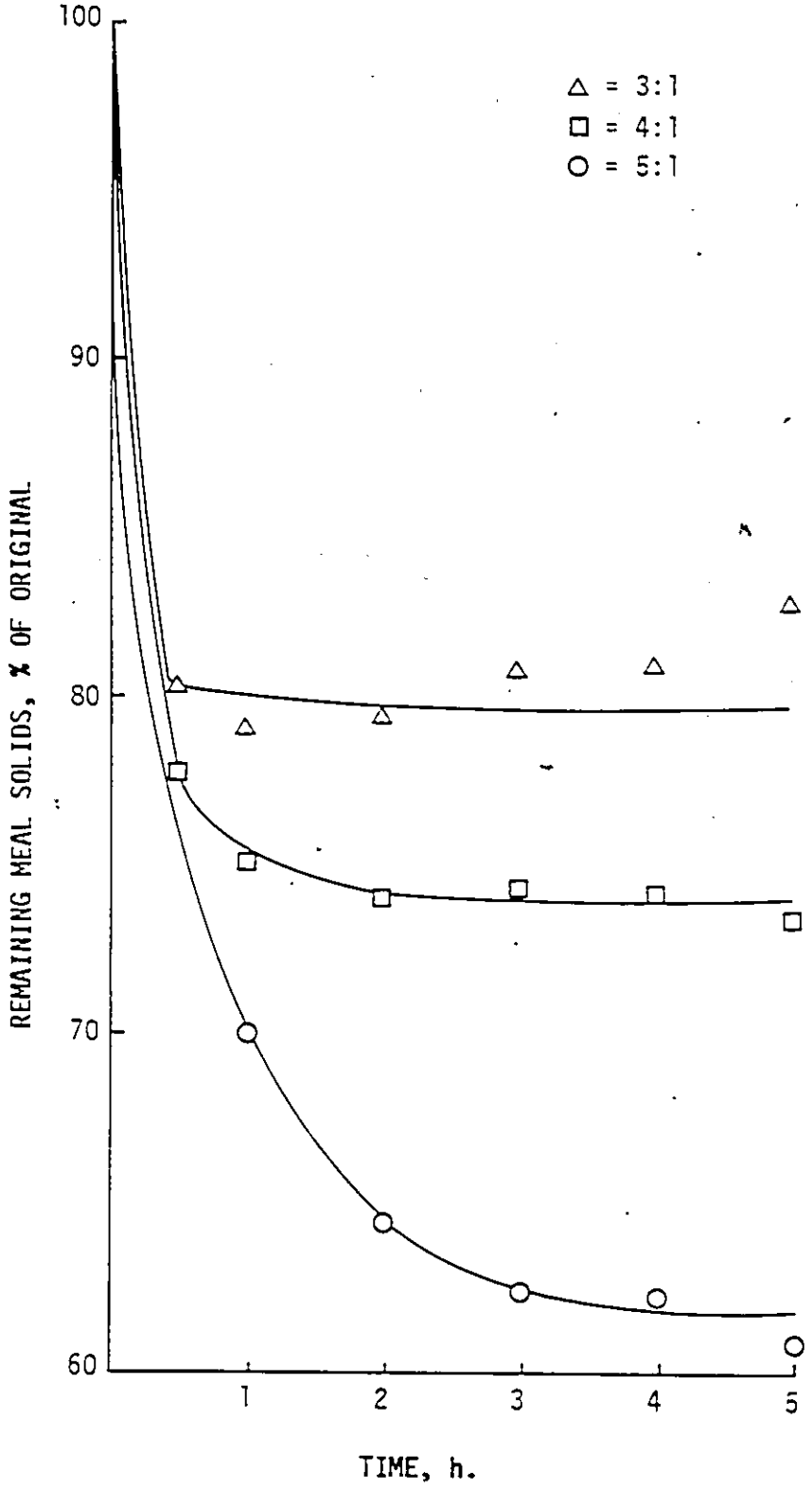


Figure. 5.3.b. MEAL SOLIDS REMAINING AFTER EXTRACTION (at 25°C) AND FREEZE RDYING.



example), this percentage is considered to be equal to the percentage of the remaining crude protein.[5]

Both the absolute amount and the percentage of the nitrogen are presented in Table 5.1. However, only the values of the percentage of protein remaining, as a function of the treatment time, are depicted in Figure 5.4 ("a" and "b").

The total ITC content of the meal solids was determined as mg/g by the modified Youngs method (MAP) (details in Chapter 3). Results are presented in Table 5.1 and shown in Figure 5.5 ("a" and "b"). It should be noted that only the BIT and PIT were measured as being of interest to this study (for details see Research Objectives and Outline in Chapter 1) and that the determined ITC content includes the amount of the unhydrolyzed glucosinolates (determined indirectly as ITC).

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[5] Crude protein, in mg = nitrogen content, in mg/g meal X a factor of 6.25. This factor (100/16) is obtained from the theoretical relation of each 100 g protein containing 16 g nitrogen. In this Chapter, however, only the term protein will be used with the understanding that it is actually crude protein.

Figure. 5.4.a REMAINING PROTEIN AFTER EXTRACTION (at 15°C) AND FREEZE DRYING.

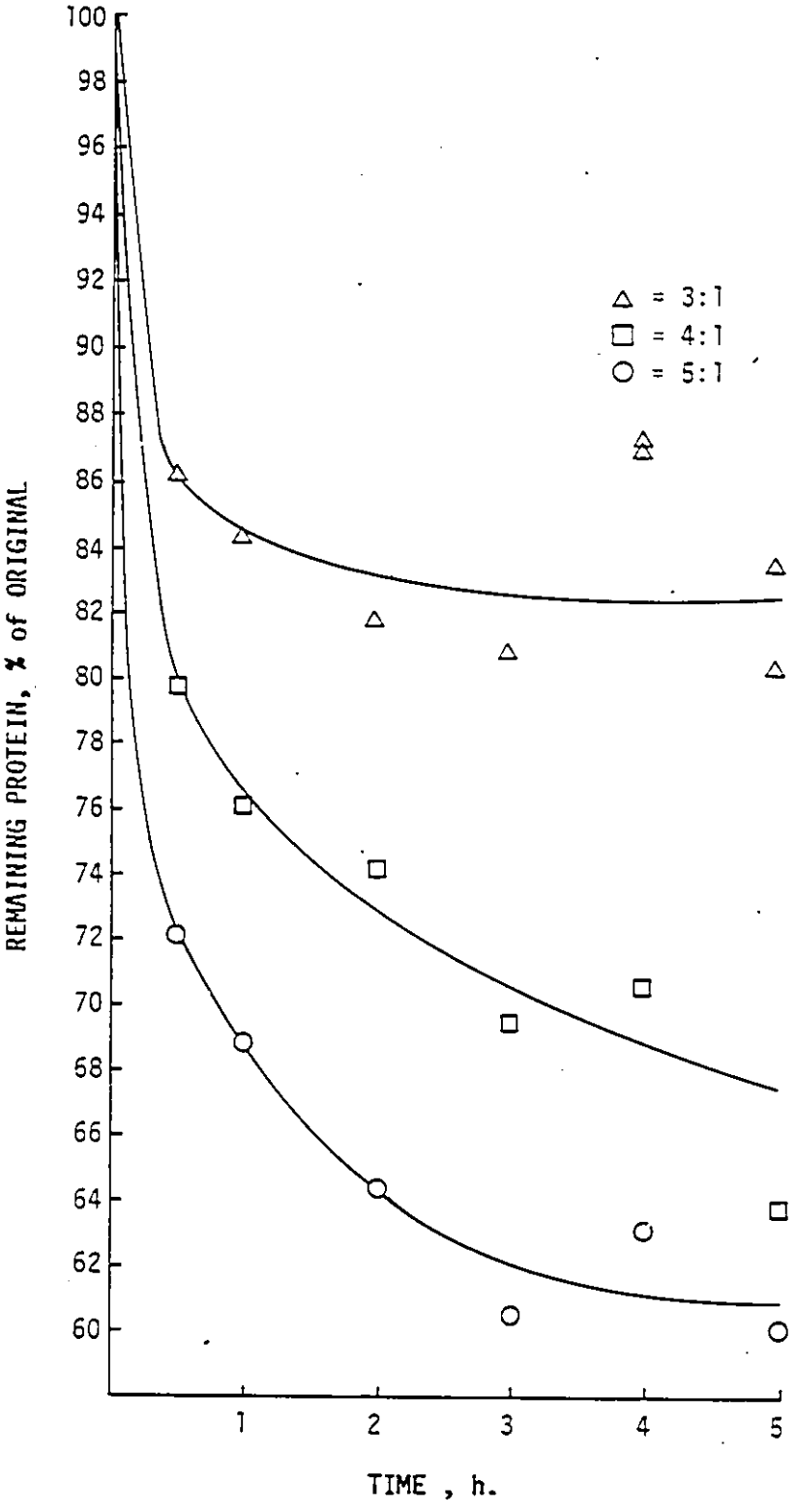


Figure. 5.4.b REMAINING PROTEIN AFTER EXTRACTION (AT 25 C) and FREEZE-DRYING.

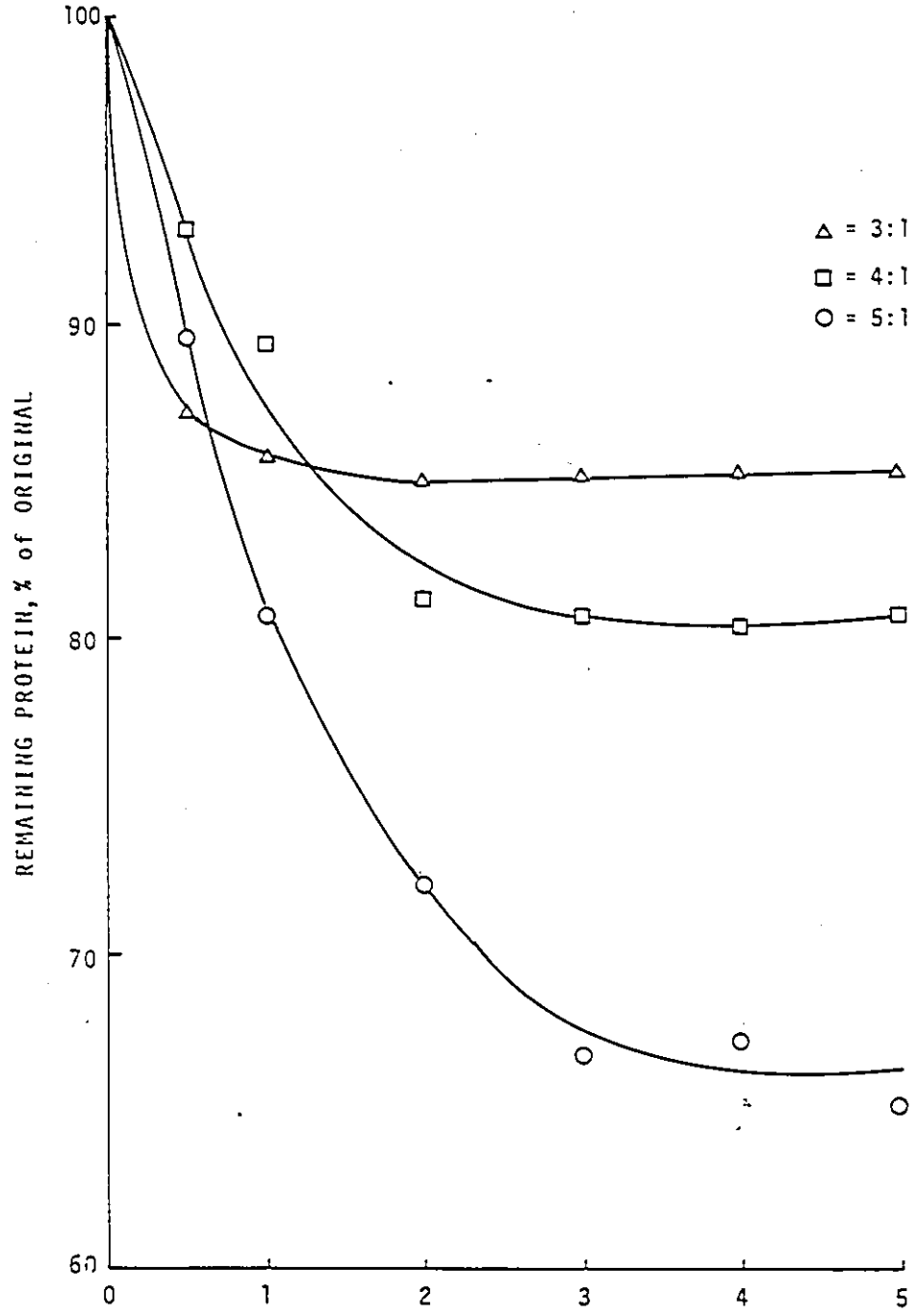


Figure. 5.5.a. TOTAL ITC in MEAL AFTER EXTRACTION (at 15°C) AND FREEZE-DRYING.

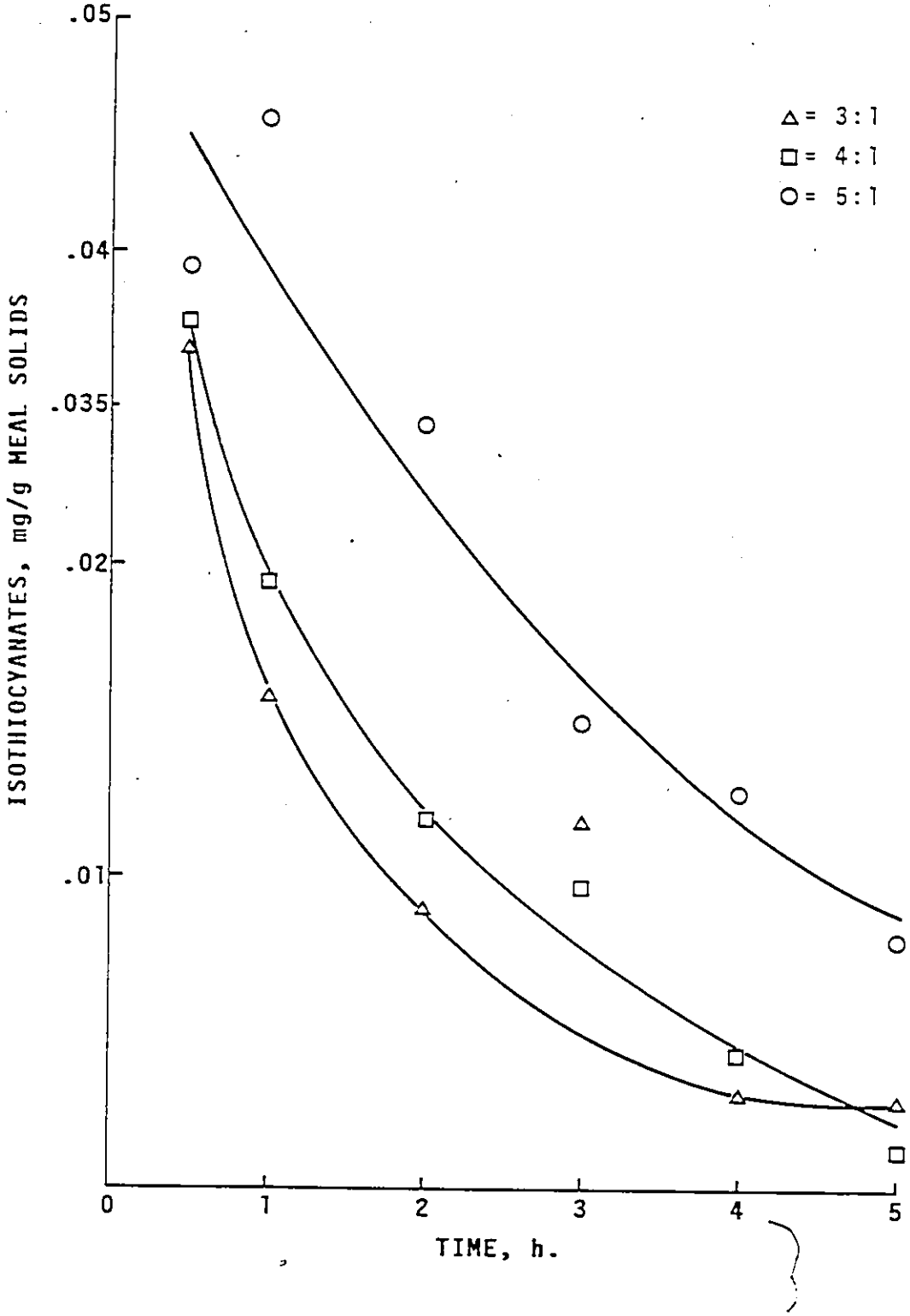
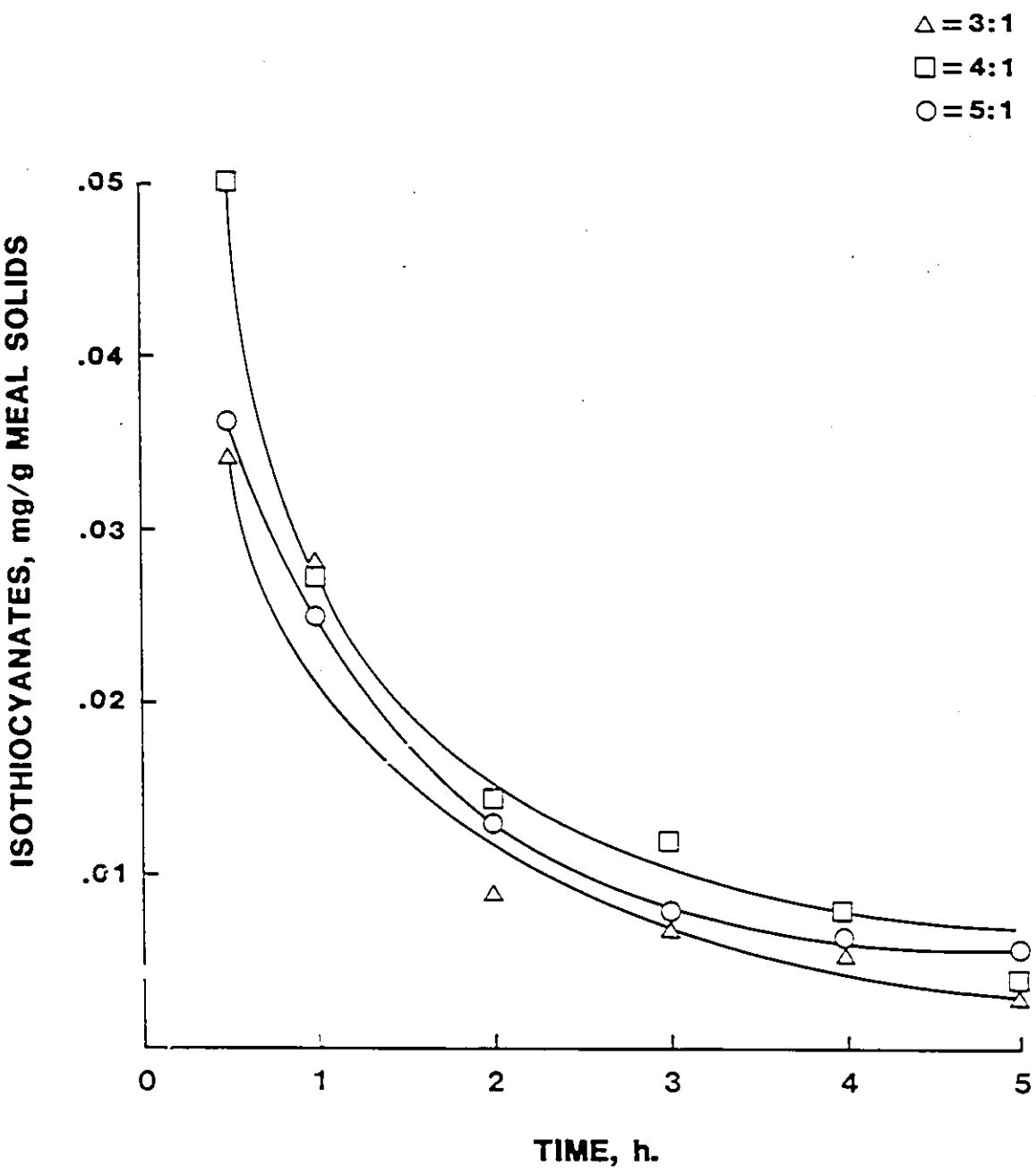


Figure. 5.5.b TOTAL ITC in MEAL AFTER EXTRACTION (at 25 °C) AND FREEZE-DRYING.



5.1.2 Results and Discussion

It was found that filtering the meal slurry through the 0.55 mm bottom holes of the extractor was appropriate for the purpose. The duration of the filtration time was short (less than 5 min). Also, the amount of meal fines that passed through was negligible.

The effects of the extraction conditions on the remaining meal solds, remaining meal protein, and ITC in the meal solids are discussed below.

i) **The Meal Solids**

From Figure 5.3 ("a" and "b") it can be seen that the remaining meal solids follow the usual mass transfer pattern and are being reduced exponentially with time. Also, the increase in the W/M ratio increased the solubility of the meal components, thus resulting in a smaller quantity of remaining solids. However, the remaining meal solids for experiments, which were aqueously extracted at 15°C, were always lower than those extracted at 25°C. For example, for a 5 h extraction time, the W/M ratio of 3:1 at 15°C was 76.14%, as compared to 82.40% for 25°C. This unusual behaviour is due to the fact that the meal at the extraction temperature of 15°C absorbed less water than at

25°C. This means that there was more free (not bound to meal) water that can be filtered at the former temperature than at the latter. For example, for a duration of extraction of 5 h, a W/M ratio of 3:1, and at 15°C, there were 7 ml of free water, while at 25°C there were only 3 ml. Thus, the greater amount of free water at the former temperature than at the latter would result in more meal components being dissolved.

ii) **The Meal Protein**

As in the remaining solids pattern, the amount of remaining protein (shown in Figure 5.4, "a" and "b"), for both temperatures and for all W/M ratios, was reduced exponentially with time. Also, at the same temperature, the remaining protein is reduced with the W/M ratio. For the same W/M ratio, the remaining protein is higher at the aqueous extraction temperature of 25°C than at 15°C.

iii) **The Isothiocyanates**

It is noticeable in Figure 5.5 ("a" and "b") and Tables 5.1 that the ITC content of the meal is reduced exponentially. Also, the ITC content of the meal at 25°C was less than that at 15°C for the same W/M ratio and time. Furthermore, it was expected that the increase in the W/M

ratio would reduce the ITC remaining in the meal solids; however, this was not the case.

The effect of temperature and W/M ratio on the ITC content in the meal solids implies that the ITC might have been reacting with the meal protein and that the increase in the W/M ratio diluted the reactants, thus reducing the rate of reaction. To assess this possibility, the data was examined for an order of reaction and for the effect of temperature on its rate. The first-order kinetics was investigated first. The ITC content of the meal versus time was plotted (by the least square technique) on a semi-log graph, as shown in Figure 5.6 for the ratio 4:1, as an example. It can be seen from this figure that the points fit a straight line. This indicates that the kinetics involved could be a first-order type: It was also found from the figure that for the increase in the treatment temperature by 10°C (from 15 to 25°C), the rate of decrease of ITC in the meal almost doubled (from 33 X 10^{-4} to 51 X 10^{-4} mg/g meal solids). These two observations point to the possibility that the reduction in the amount of ITC in the meal is due to a chemical reaction.

Furthermore, at 25°C for the W/M ratio of 5:1 and duration of treatment ½ h, the ITC content in meal solids

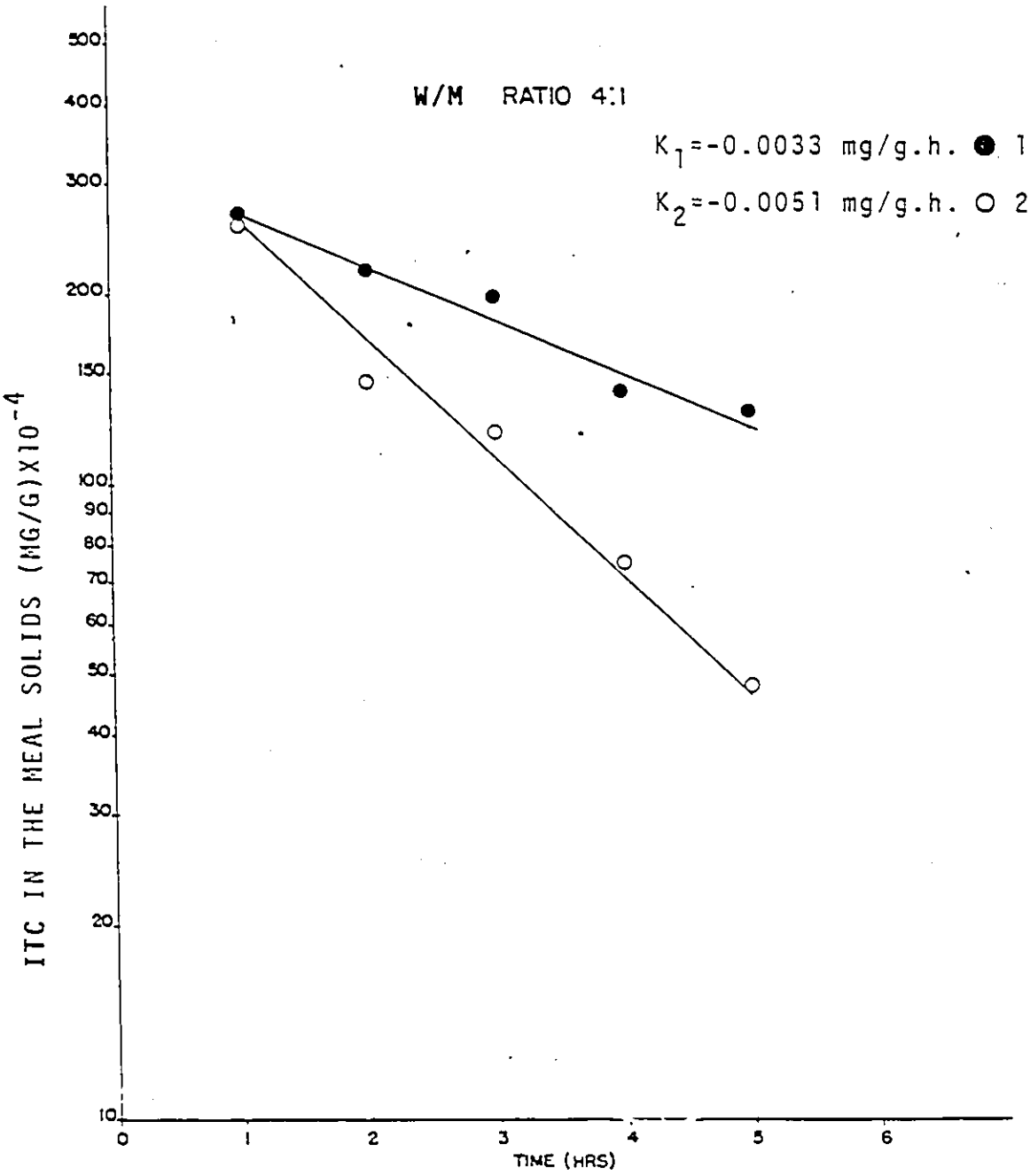


Figure. 5.6 ISOTHIOCYANATES IN THE MEAL AFTER EXTRACTION FOR WATER-TO-MEAL RATIO OF 4:1

was low at only 0.0363 mg/g, as compared to 0.3326 mg/g ITC determined by analysis to have been produced in the meal at the same ratio and temperature.

Moreover, comparing the ITC content in the freeze-dried filtrates and meal solids did not show the concentration gradient expected for the mass transfer processes. For example, at 15°C and a W/M ratio of 5:1, the ITC content of the meal solids was 0.0344 mg/g and that for the freeze-dried filtrate was 0.0416 mg/g.

A further investigation was carried out to clarify the nature of this process. Meal solids obtained at 25°C, with a time of 5 h and a W/M ratio of 3:1, were analyzed by Dr. M.E. Daxenbichler for other aglucons. It was found that the meal solids contained 0.7 mg/g VOT (goitrin), 0.18 mg/g epithiobutane (threo and erythreo), and 0.14 mg/g 1-cyano-2-hydroxy-3-butene (see Appendix 17 for details). The numerical sum of these amounts approximates the initial progoitrin content in the meal determined as VOT. Hence, the decrease in the ITC content is most likely not due to its removal by water extraction. These results and observations indicate the possibility that, contrary to appearance, the water extraction process did not actually remove the ITC. There was also the possbility that as the ITC was

volatile; it could have been removed during the freeze-drying process.

To determine the reasons for this unexpected behaviour, 10 g of the meal was treated in a beaker (same inside diameter as that of the extractor) with acidic solution having the same pH of 4.5 as the Aqueous Extraction Method, and a W/M of 3:1. Two samples were taken simultaneously from the meal slurry, between ½ to 5 h. The ITC content was determined for one of the samples removed from the slurry and the other one was freeze-dried at 28°C for 24 h before the determination was made. The results are presented in the first set of data in Table 5.2 and shown in Figure 5.7. It can be seen that the total ITC content of the meal was substantially reduced, thus indicating a possible reaction between the ITC and the protein of the meal, which is in agreement with the work of Bjorkman (37). It could also be seen in Figure 5.7 that, for the same duration time, the ITC in the dried samples is less than that in the slurry samples. This indicates that the ITC evaporated during the freeze-drying process. A similar observation was recently reported by Paik et al. (154) on the reduction of the nitriles in the meal after freeze-drying.

TABLE 5.2

EFFECT TO WATER-TO-MEAL RATIO, HYDROLYSIS TIME, AND EVAPORATION TEMPERATURE ON THE REMOVAL OF THE ISOTHIOCYANATES

W/M Ratio	Time (h)	Drying Temp. (°C)	BIT*(mg/g solids)		PIT**(mg/g solids)		ITC(mg/g solids)		ITC # Re-moved
			Slurry	Dry	Slurry	Dry	Slurry	Dry	
3:1	½	28	.0619	.0571	.0055	.0009	.0674	.0580	.0094
3:1	1	28	.0460	.0323	.0028	.0018	.0488	.0341	.0147
3:1	2	28	.0351	.0143	.0038	.0038	.0389	.0181	.0208
3:1	3	28	.0150	.0065	.0040	0	.0190	.0065	.0125
3:1	4	28	.0137	.0064	.0027	0	.0164	.0064	.0100
3:1	5	28	.0113	.0032	.0007	0	.0120	.0032	.0088

* : Butenyl isothiocyanate
** : Pentenyl isothiocyanate
\+ : ITC = BIT + PIT
\# : = $ITC_{slurry} - ITC_{dry}$

TABLE 5.2 (cont'd)

W/M Ratio	Time (h)	Drying Temp. (°C)	BIT*(mg/g solids)		PIT**(mg/g solids)		ITC+ (mg/g solids)		ITC # Removed
			Slurry	Dry	Slurry	Dry	Slurry	Dry	
3:1	¼	55	.2049	.0561	.0031	.0014	.2080	.0575	.1513
3:1	½	55	.0619	.0256	.0055	0	.0674	.0256	.0418
3:1	1	55	.0460	.0104	.0028	0	.0488	.0104	.0384
3:1	2	55	.0351	0	.0038	0	.0389	0	.0389
3:1	3	55	.0150	0	.0040	0	.0190	0	.0190
3:1	4	55	.0137	0	.0027	0	.0164	0	.0164
3:1	5	55	.0113	0	.0007	0	.0120	0	.0120
4:1	¼	55	.0913	.0246	.0043	.0046	.1956	.0292	.1664
4:1	½	55	.1370	.0164	.0040	0	.1410	.0164	.1246
4:1	1	55	.0839	.0077	.0043	0	.0882	.0077	.0805
4:1	2	55	.0088	0	0	0	.0088	0	.0088
4:1	3	55	.0061	0	0	0	.0061	0	.0061
4:1	4	55	.0049	0	0	0	.0049	0	.0049
4:1	5	55	.0027	0	0	0	.0027	0	.0027

* : Butenyl isothiocyanate
** : Pentenyl isothiocyanate
\+ : ITC = BIT + PIT
\# : = $ITC_{slurry} - ITC_{dry}$

TABLE 5.2 (cont'd)

W/M Ratio	Time (h)	Drying Temp. (°C)	BIT*(mg/g solids)		PIT**(mg/g solids)		ITC+ (mg/g solids)		ITC # Removed
			Slurry	Dry	Slurry	Dry	Slurry	Dry	
5:1	¼	55	.2147	.0603	.0043	.0033	.2190	.0636	.1554
5:1	½	55	.2129	.0141	.0031	0	.2160	.0141	.2019
5:1	1	55	.0903	.0066	.0037	0	.0940	.0066	.0874
5:1	2	55	.0261	.0047	.0043	0	.0304	0	.0304
5:1	3	55	.0044	0	.0008	0	.0051	0	.0051
5:1	4	55	.0027	0	.0009	0	.0036	0	.0036
5:1	5	55	.0035	0	0	0	.0035	0	.0035
10:1	¼	55	.2636	.0481	.0072	0	.2703	.0431	.2227
10:1	1	55	.1423	.0323	0	0	.1423	.0323	.1100
10:1	2	55	.1177	.0207	0	0	.1177	.0707	.0970
10:1	3	55	.0578	.0168	0	0	.0575	.0168	.0410

* : Butenyl isothiocyanate
** : Pentenyl isothiocyanate
\+ : ITC = BIT + PIT
\# : = $ITC_{slurry} - ITC_{dry}$

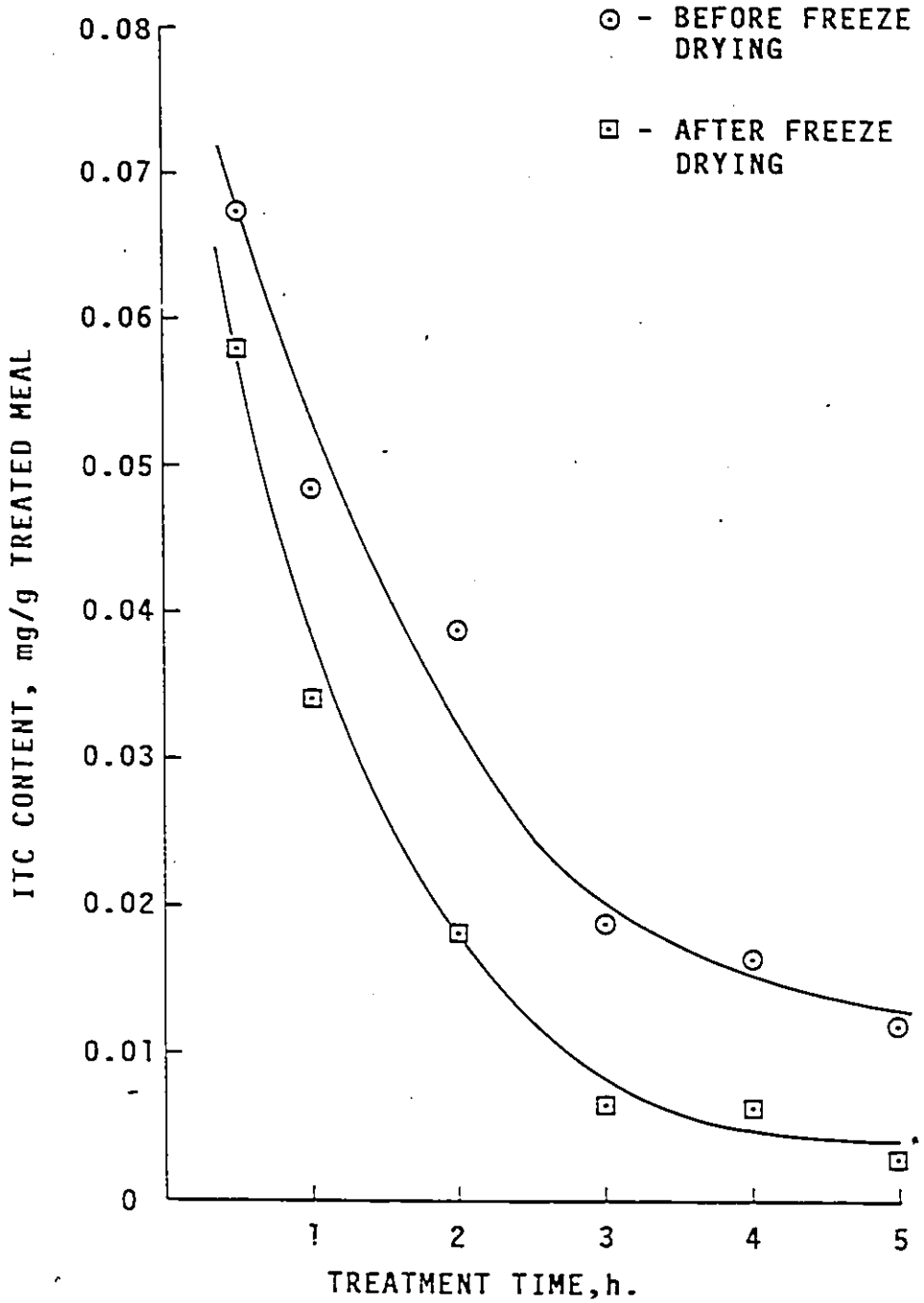


Figure. 5.7. ITC in MEAL SLURRY BEFORE AND AFTER FREEZE DRYING at 28°C

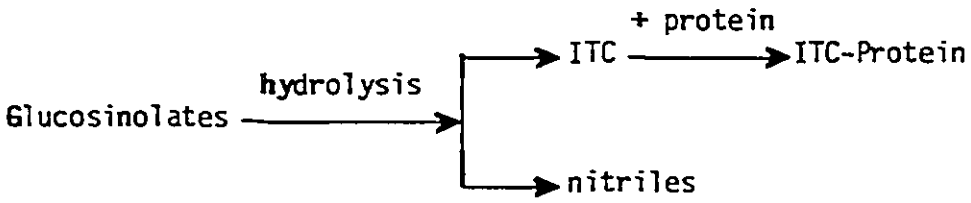
The results of this investigation (as shown in Table 5.2 and in Figure 5.7) suggest that the ITC reduction is mainly due to its reaction with the protein, such that a small amount (0.0088 to 0.0208 mg/g solids) evaporates during the freeze-drying.

It should be noted that although the study of detoxification by aqueous extraction did not progress as planned, it was useful in pointing out that another detoxification method could be developed by inhibiting the reaction between ITC meal and protein, and then evaporating the free ITC during freeze-drying. Also, since the study of the Aqueous Extraction Method was abandoned, the planned protein recovery from the extract was not investigated.

5.2 Acidic Medium Method

The acidic medium method is based on the conclusions reached from the aqueous extraction method, that ITC could be volatilized during the freeze-drying of the meal slurry. To make use of this conclusion, it was decided to subject the meal to an aqueous treatment to hydrolyze its glucosinolates and then, **without filtration,** freeze-dry it.

To facilitate explaining the strategy followed to achieve a maximum ITC removal, the chemical reactions involved are simplified as follows:



From the start of the hydrolysis, the concentration of the glucosinolates decreases exponentially, while that of ITC rises up to a maximum and then decreases. However, the concentration of ITC-Protein increases continuously (114). Simultaneously, the nitriles' formation is competing with that of ITC. The goal was to freeze the meal slurry, in preparation for its freeze-drying, at the time corresponding to the presence of a maximum amount of free ITC (not reacted with the meal protein). A determination of these free ITC is explained below in Section 5.2.1. The strategy was to bring the molecular concentration of the ITC as close as possible to that of the glucosinolates originally present in the meal by inhibiting the reaction between the ITC and the protein, and by reducing the amount of nitriles formed. As mentioned in Chapter 2, the increase in the W/M ratio would reduce the

amount of nitriles formed and instead form ITC and OZT. Also, this increase would reduce the reaction between the ITC and the protein by diluting the reactants. This reaction could be further reduced by hydrolyzing the meal in an acidic medium. However, it is also known, as mentioned in Chapter 2, that the acidic conditions would increase the formation of nitriles. A compromise should then be sought.

5.2.1 Experimental Procedure

Ten grams of the meal in a 250 ml beaker were treated with W/M ratios of 3:1, 4:1, 5:1, and 10:1 at pH 4.5 and at 25°C. The beaker chosen had the same inside diameter as the extractor used in Section 5.1 (63 mm) in order to keep the same experimental conditions. For the same reason, the mixing of the solution with the meal and the duration of this mixing were the same as in Section 5.1, except that a time of ¼ h was added. This time was added because, as can be seen in Table 5.2 for W/M ratio of 3:1, the ITC content of the meal slurry after a treatment time of ½ h was already low at only 0.0674 mg/g solids (aqueously treated freeze-dried meal). This indicated that a large amount of ITC formed had reacted with the protein. Thus, it was felt that at a lower time, more free (not reacted with the meal

protein). ITC may have been present in the slurry. Also, for the ratio 10:1, only times of ¼, ½, ¾ and 1 h were used. This adjustment in the conditions for the ratio of 10:1 was done because information from the other ratios indicated that experiments with times over 1 h do not give high ITC evaporation. The addition of the aqueous solution, and the control of the temperature and pH, were done in the same way as in the aqueous extraction procedure described in Section 5.1.

For each W/M ratio, a sample of the meal slurry was drawn at each treatment time. A part of the sample was frozen in the deep-freezer at -50°C. Also from the sample, a portion of the slurry containing 0.5 g meal was weighed into a 20 ml culture tube. Two ml of methylene chloride, which contained an internal standard of 40 mg/l butyl isothiocyanate, and a 6 mm glass bead were added to the contents of the tube. The tube was then shaken for 2 h in a rotary shaker. The shaker was immersed in a water bath with temperature controlled at 25°C (±0.5°C). After the 2 h shaking period, the methylene chloride was separated by centrifugation at 2500 rpm. The ITC content of the meal slurry was then determined from the methylene chloride by GLC and denoted ITC_{slurry}.

The frozen sample was freeze-dried at a pressure of less than 10 μm for 24 h. The heat rack was controlled to maintain a temperature of 55°C (this being a common practice in industrial freeze-drying) in the meal after the complete sublimation of ice. The temperature was increased to 55°C from the 28°C used in Section 2.1, in aiming to remove more ITC and to evaporate the nitriles (154) expected to be formed at this acidic condition. Another advantage of this choice is that the toxicity of the meal solids is reduced by drying them at 50°C (225). To 0.5 g freeze-dried meal solids placed in a 20 ml culture tube, 2 ml of methylene chloride, containing the internal standard, and a 6 mm glass bead were added. After mixing the contents for 5 min, distilled water was added to make the original W/M ratio of the slurry before being dried. The tube was shaken for 2 h at 25°C. The tube was then processed as above and the ITC of the meal solids were determined and denoted ITC_{dry}. The use of the original ratio for the determination of ITC_{dry} served to keep all the parameters in the determination of ITC_{slurry} and ITC_{dry} the same.

It should be noted that the ITC_{slurry} is composed of free ITC present at the time of taking the sample from the meal slurry, and of ITC resulting from the subsequent

hydrolysis of glucosinolates, if present. The ITC_{dry} is composed of free ITC that were not removed and the unhydrolyzed glucosinolates, which are not volatile, if present. Thus the evaporated ITC could be calculated from the difference between the ITC_{slurry} and the ITC_{dry}. Results obtained are tabulated in Table 5.2 and depicted in Figures 5.8 and 5.9. For purposes of comparison, the results obtained in Section 5.1 (for meals treated at a W/M ratio of 3:1 and freeze-dried at 28°C) were included in these figures.

A single experiment was done with 10 g of meal, using a W/M ratio of 10:1 and a time of 15 min, in order to compare the amount of ITC collected in the freeze-dryer's condenser with that removed by freeze-drying (as obtained by calculation from ITC_{slurry}- ITC_{dry}). The freeze-dryer and its condenser were washed several times with aqueous alcohol and methylene chloride until they were free of ITC or any other aglucons, as tested by the GLC, and then dried. After drying the frozen meal slurry, the refrigeration to the condenser stopped and the frozen condensate was left to melt. Ten ml of aliquot of this condensate was put into a 20 ml culture tube, and a 6 mm glass bead was added into the tube. Two ml of methylene chloride containing the internal standard buytl isothiocyanates was added

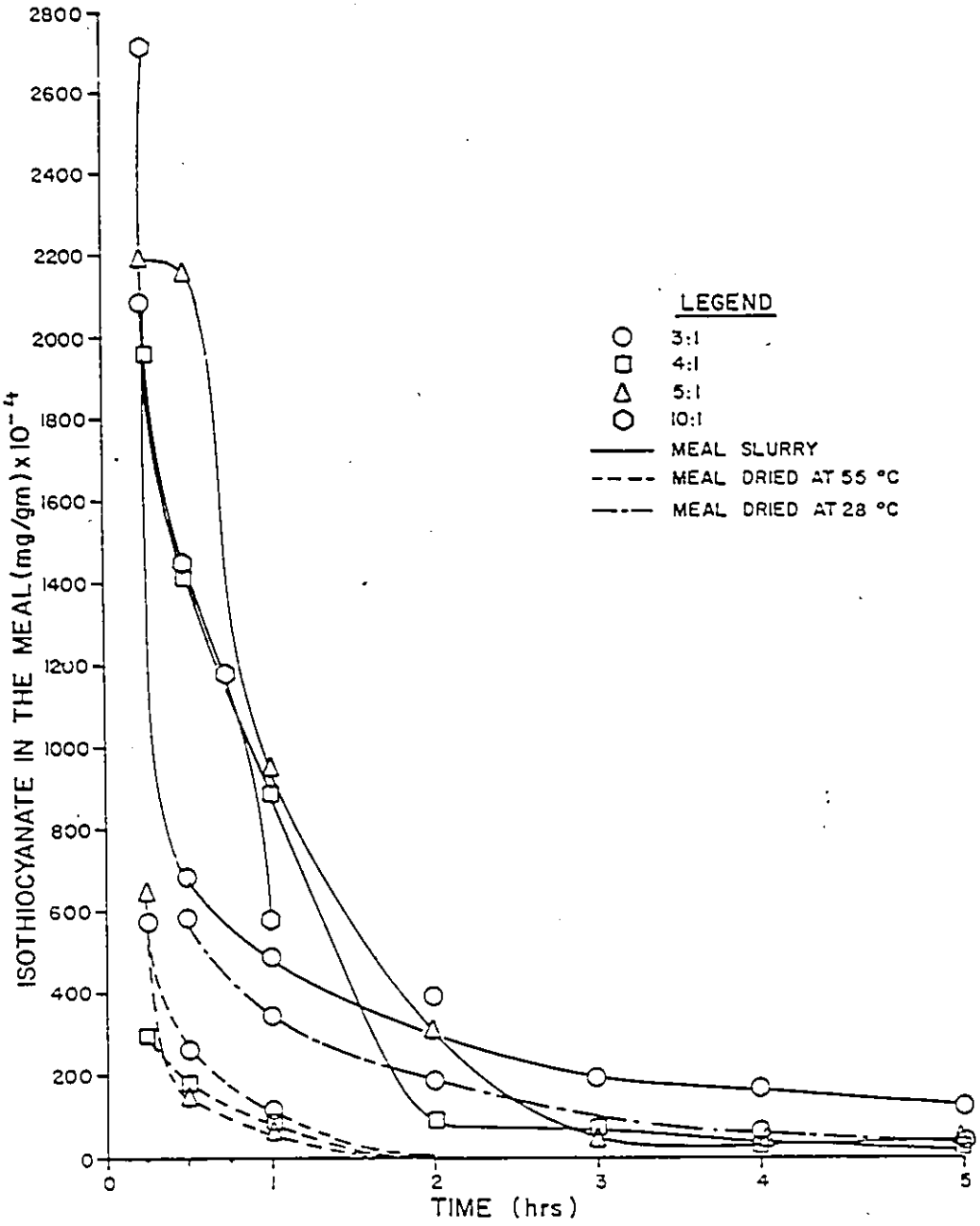


Figure. 5.8 ISOTHIOCYANATES IN MEALS TREATED BY THE ACIDIC METHOD.

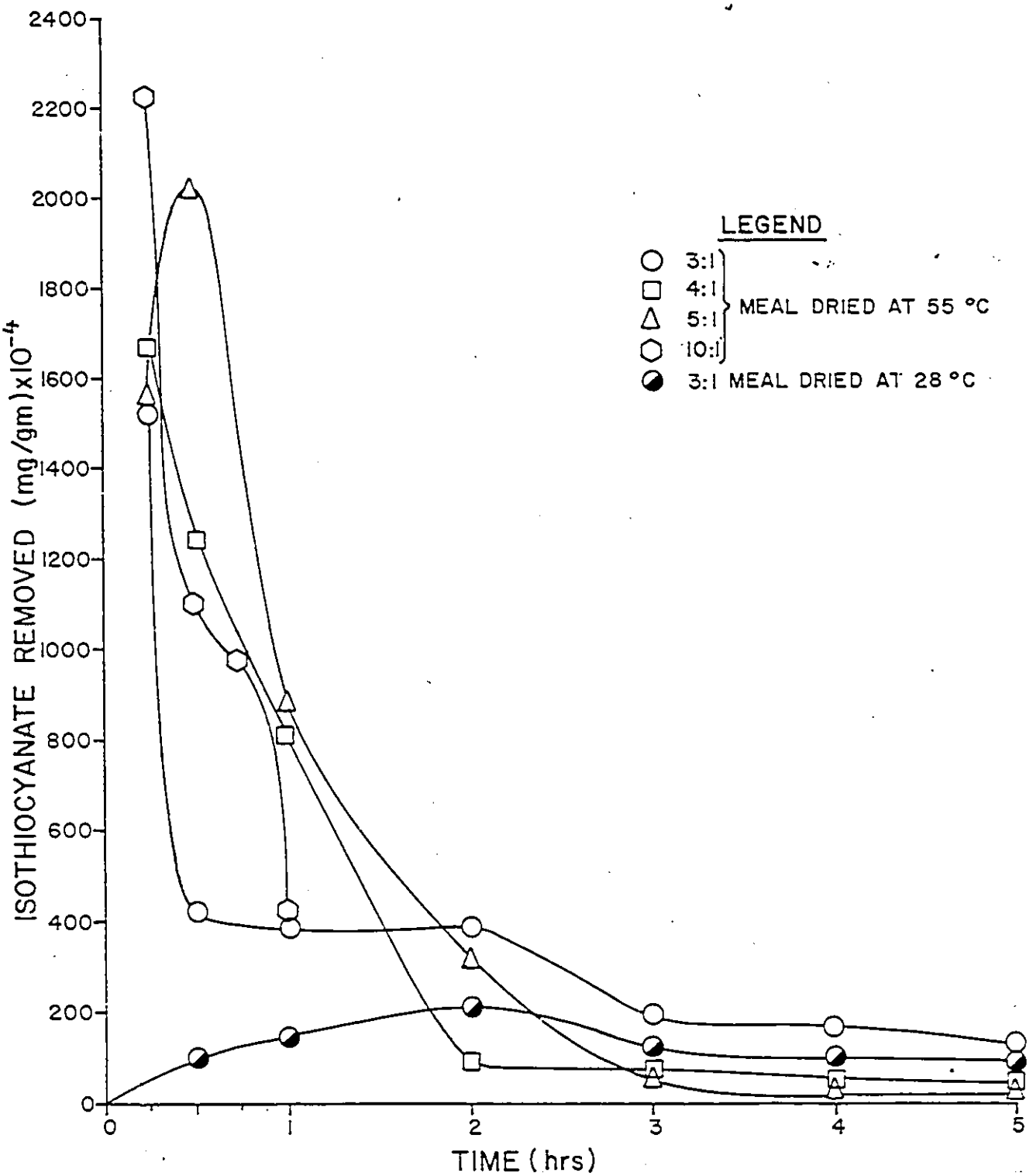


Figure. 5.9 ISOTHIOCYANATES REMOVED BY THE ACIDIC METHOD

to the aliquot. The contents of the tube were shaken in a rotary shaker for 30 min to extract the ITC in the 10 ml distillate by the methylene chloride. Afterwards, the methylene choride was separated from the distillate by centrifugation at 2500 rpm. The ITC content of the extract was determined by GLC.

The VOT and the nitriles content of the dried meal from this experiment were determined by the method of Daxenbichler et al. (49,50).

5.2.2 Results and Discussion

As shown in Figure 5.8, results obtained at the W/M ratio of 3:1 indicate that the meals dried at 55°C contained much less ITC than those dried at 28°C. For example, at the ½ h treatment time, the ITC remaining in the meal solids after the drying at 55°C was 0.0256 mg/g, and for the 28°C was 0.0580 mg/g. Thus, an increase in the drying temperature from 28°C to 55°C resulted in an improved removal rate of ITC. The ITC content of samples of the meal solids taken after 2 h and dried at 55°C was negligible. This is explained by the fact that it took 1½ h to completely hydrolyze the glucosinolates at 25°C (182). Consequently there were no intact glucosinolates in

the meal to give ITC upon determination. Also, the ITC released up to 1½ h was either evaporated in the drying process or reacted with the protein.

The results in Figure 5.9 thus indicate that increasing the W/M ratio at a treatment period of 15 min increases the amount of the ITC evaporated. After this time, the amount evaporated decreases considerably. For example, for the ratio 10:1, the ITC removed was 0.2227 mg/g solids at the time of ¼ h. However, at the time of ½ h, it was reduced by about 50% to 0.1100 mg/g solids. There was, however, an exception for the ratio of 5:1, where the amount evaporated attained a maximum of 0.2019 mg/g solids at ½ h.

In the experiment done to compare the quantity of ITC evaporated with that collected in the condenser of the freeze-dryer, it was found that the total amount of the ITC in the condensate was about 2 mg/g. This is almost equal to the amount of ITC evaporated during the freeze-drying of the 10 g meal 2.227 mg/g meal solids. Therefore, the amount of ITC evaporated could be calculted from the difference between the ITC_{slurry} and the ITC_{dry}.

The VOT and nitriles content of the dried meal, from the experiment done on 10 g meal with a W/M ratio of 10:1, was 0.76 mg/g solids and 0.34 mg/g, respectively. Increasing the heating temperature during the drying step to 70°C from 55°C did not result in further reductions in the amounts of nitriles. The presence of nitriles in the dried meal solids was a drawback for the Acidic Medium Method because of their high toxicity. It was hoped that at the drying temperature of 55°C, under pressure of 10 μm for 24 h, the nitriles might be removed. The results, however, indicated otherwise. Furthermore, since more nitriles were expected to form at 15°C than at 25°C, the investigation was not extended to the former temperature.

From the understanding acquired of the properties of glucosinolates and their hydrolysates, and the potential of freeze-drying for the removal of ITC, the work to develop the new method was carried out bearing in mind that the formation of the nitriles should be eliminated during the detoxification of the meal.

5.3 Neutral Medium Method

In this method it was planned, not only to inhibit the reaction between the ITC and the protein, but also, as

mentioned in Section 5.2.2, to completely eliminate the formation of nitriles. The nitriles are formed during the hydrolysis of glucosinolates instead of the ITC (16,99) and VOT (225). Therefore, a reduction in the formation of the nitriles would enhance the detoxification of the meal, particularly, if the hydrolysis is properly controlled to increase the maximum amount of the free ITC (not bound to protein) in the meal which could be removed by freeze-drying.

It was previously mentioned in Section 2.2 that nitrile formation is reduced with pH, temperature, and W/M ratio. In addition, the hydrolysis of the glucosinolates, and the reaction between the ITC and the meal protein, increases with time.

As also mentioned in Section 2.2, one way to suppress the formation of nitriles is to heat the meal at 100 to 120°C prior to its aqueous treatment. However, as thermal treatment of the seed denatures the meal protein, the use of high temperatures was excluded from this investigation. It was decided to use W/M ratio, time, and temperature as the variables for the hydrolysis of the meal glucosinolates prior to freeze-drying. The pH, however,

was kept constant in the range of 6 to 7, which is that of the meal in distilled water.

5.3.1 Experimental Procedure

A preliminary experiment was carried out on 1.0 g of meal in a culture tube, provided with a Teflon-lined cap, to determine if pH 6 to 7 of the neutral medium method could lead to a nitrile-free meal. The temperature of hydrolysis, the time, and the W/M ratio were 25°C, 30 min and 10:1, respectively. The hydrolysis time of 30 min was chosen because it was found that in the acidic medium method at 25°C, a hydrolysis of about 80-90% of the glucosinolates was attained. A 6 mm glass bead was added to the slurry and the tube was shaken in a rotary shaker. After shaking for 30 min, 2 ml of methylene chloride was added, and the shaking continued for another hour. The emulsion was broken after centrifugation at 2500 rpm and the total nitrile content in the methylene chloride layer was determined by Daxenbichler et al.'s method (49,50).

The nitrile content was found to be low at 0.02 mg/g as compared to 0.34 mg/g for the acid method. Furthermore, the amount of ITC was determined by a GLC for another

sample of 1 g meal under the same conditions, and its amount in the mixture was found to be only about 0.07 mg/g. These results indicate the possibility of suppressing the formation of the nitriles by hydrolyzing the glucosinolates at the pH range of 6 to 7, and also the need to optimize the operating conditions to increase the amount of the free ITC.

To determine the effects of the three variables, and the optimum operating conditions on the ITC removal by freeze-drying, a set of 18 runs was performed according to a modified central composite design of Box-Wilson as represented in Figure 5.10 (25,85,157). The three variables (W/M ratio, time, and temperature) were coded and represented by x_1, x_2, x_3, respectively. The coding was obtained from the following equations:

$$x_1 = \frac{\text{Water-to-meal ratio (ml:g)} - 12}{4}$$

$$x_2 = \frac{\text{Time (min)} - 30}{12.5}$$

$$x_3 = \frac{\text{Temperature (°C)} - 15}{5}$$

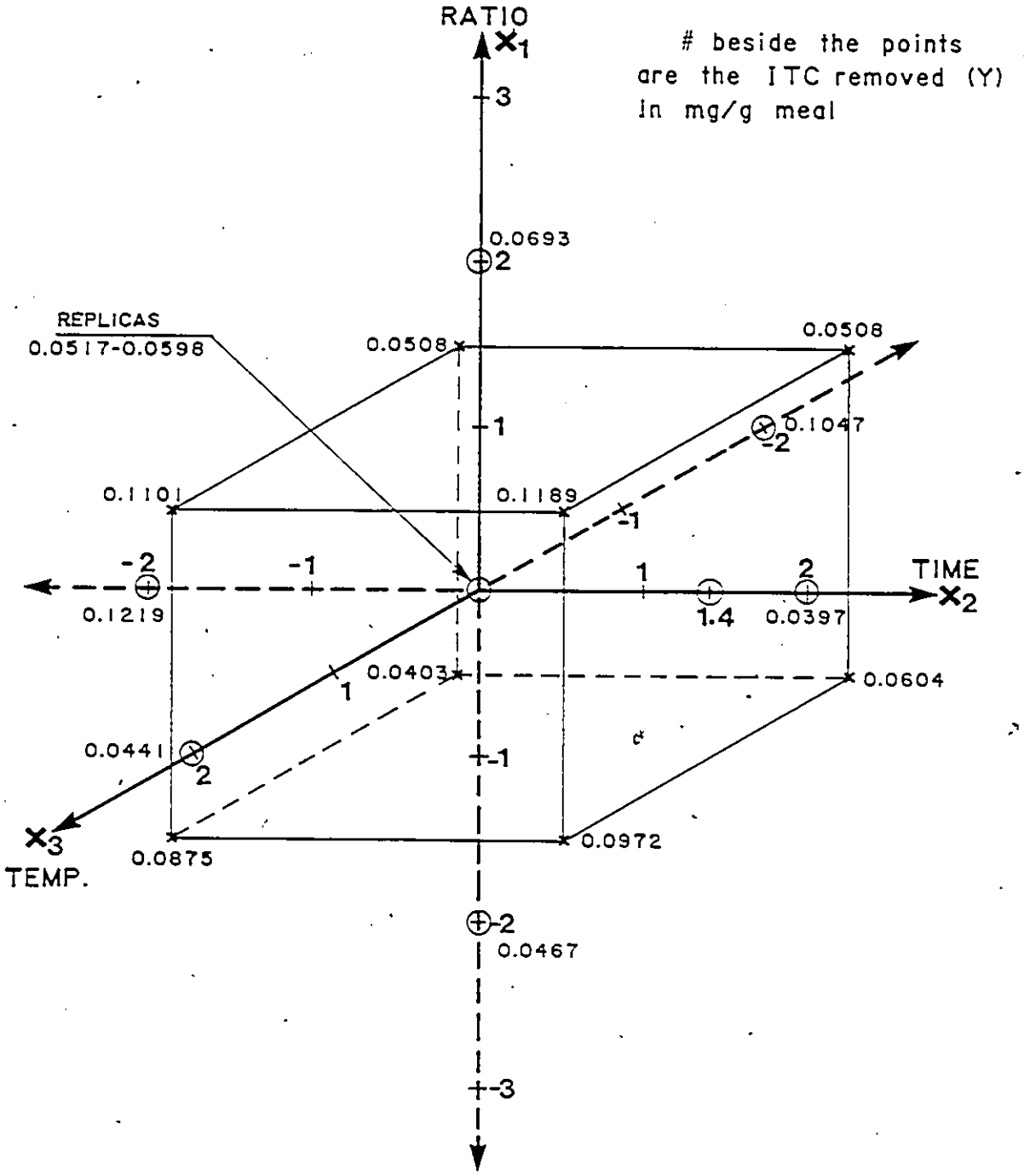


Figure. 5.10 THE MODIFIED CENTRAL COMPOSITE DESIGN AND THE ITC REMOVED (mg/g meal) AT OPERATING LEVELS

These equations were derived as follows:

$$\text{Coded value} = \frac{\text{Actual value} - \frac{1}{2}(\text{highest value} + \text{lowest})}{\frac{1}{2}(\text{highest value} - \text{lowest})}$$

The ranges of the variables studied were as follows:

Water-to-meal ratio;	from	4:1	to	20:1
Time (min) ;	from	5	to	55
Temperature (°C);	from	5	to	25

In preliminary experiments, a maximum time of 95 min was used at a W/M ratio of 12 and at temperatures of 5 and 25°C. The amount of ITC evaporated was found to be low at 0.0299 mg/g meal and 0.0093, respectively. Thus, it was decided to reduce the maximum time to 55 min. Complete results of the runs at times of 95 min are presented in the first two lines of Table 5.4.

The operating levels in coded form are listed in Table 5.3 (located in Appendix 14). The experimental runs were randomized and only one run was executed per day to avoid introducing systematic errors and bias in the results.

TABLE 5.4

MAXIMIZATION OF ITC EVAPORATION BY OPTIMIZING OPERATING LEVELS

W/M Ratio	Time (min)	Treatment Temp.(°C)	BIT*(mg/g meal)		PIT**(mg/g meal)		ITC+(mg/g meal)		ITC# Re-moved
			Slurry	Dried	Slurry	Dried	Slurry	Dried	
12:1	95	25	.0166	.0073	-	-	.0166	.0073	.0093
12:1	95	5	.0553	.0254	-	-	.0553	.0254	.0299
12:1	30	25	.1471	.1030	-	-	.1471	.1030	.0441
12:1	30	5	.1608	.0561	-	-	.1608	.0567	.1047
12:1	55	15	.1001	.0604	-	-	.1001	.0604	.0397
12:1	5	15	.3494	.2396	.0121	-	.3615	.2396	.1219
20:1	30	15	.1399	.0706	-	-	.1399	.0706	.0693
4:1	30	15	.1213	.0706	-	-	.1213	.0746	.0467
12:1	30	15	.1302	.0785	-	-	.1302	.0785	.0517
12:1	30	15	.1293	.0714			.1294	.0714	.0580
12:1	30	15	.1363	.0800	-	-	.1363	.0800	.0563
12:1	30	15	.1363	.0765	-	-	.1363	.0765	.0598

* : Butenyl isothiocyanate

** : Pentenyl isothiocyanate

\+ : BIT + PIT

\# : $ITC_{slurry} - ITC_{dry}$

N.B.: ITC produced from hydrolyzing meal glucosinolates in neutral medium, as shown in Table 3.1 for MAP N 150, is 1.52 mg/g meal.

TABLE 5.4 (cont'd)

W/M Ratio	Time (min)	Treatment Temp.(°C)	BIT*(mg/g meal)		PIT**(mg/g meal)		ITC+(mg/g meal)		ITC# Re-moved
			Slurry	Dried	Slurry	Dried	Slurry	Dried	
16:1	47½	20	.1282	.0774	-	-	.1282	.0774	.0508
16:1	47½	10	.1124	.0616	-	-	.1124	.0616	.0508
16:1	17½	20	.1901	.0800	-	-	.1901	.0800	.1101
16:1	17½	10	.2217	.1035	-	-	.2217	.1028	.1189
8:1	47½	20	.1249	.0846	-	-	.1249	.0846	.0403
8:1	47½	10	.1026	.0422	-	-	.1026	.0422	.0604
8:1	17½	20	.1708	.0833	-	-	.1708	.0833	.0875
8:1	17½	10	.1952	.0980	-	-	.1708	.0833	.0875
20:1	5	5	.2641	.2565	.0103	.0085	.2744	.2650	.0094
20:1	10	5	.2479	.2052	.0091	-	.2570	.2052	.0518

* : Butenyl isothiocyanate

** : Pentenyl isothiocyanate

\+ : BIT + PIT

\# : $ITC_{slurry} - ITC_{dry}$

N.B.: ITC produced from hydrolyzing meal glucosinolates in neutral medium, as shown in Table 3.1 for MAP N 150, is 1.52 mg/g meal.

TABLE 5.4 (cont'd)

W/M Ratio	Time (min)	Treatment Temp.(°C)	BIT*(mg/g meal)		PIT**(mg/g meal)		ITC+(mg/g meal)		ITC# Re-moved
			Slurry	Dried	Slurry	Dried	Slurry	Dried	
36:1	5	17.0	.4264	.2963	.0158	.0075	.4422	.3038	.1384
36:1	10	17.0	.3827	.1835	.0116	-	.3943	.1835	.2108
36:1	15	17.0	.2927	.1231	-	-	.2927	.1231	.1696
100:1	15	17.0	.3190	.1203	-	-	.3190	.1203	.1937
100:1	20	17.0	.2901	.1200	-	-	.2901	.1200	.1701
100:1	8.83	20	.4054	.2182	.0106	-	.4160	.2182	.1978
100:1	10	20	.3440	.1691	.0123	-	.3563	.1691	.1872
100:1	15	20	.3059	.1004	.0075	-	.3134	.1004	.2130

* : Butenyl isothiocyanate
** : Pentenyl isothiocyanate
\+ : BIT + PIT
\# : $ITC_{slurry} - ITC_{dry}$
N.B.: ITC produced from hydrolyzing meal glucosinolates in neutral medium, as shown in Table 3.1 for MAP, N 150, is 1.52 mg/g meal.

TABLE 5.4 (concluded)

W/M Ratio	Time (min)	Treatment Temp.(°C)	BIT*(mg/g meal)		PIT**(mg/g meal)		ITC+(mg/g meal)		ITC# Re-moved
			Slurry	Dried	Slurry	Dried	Slurry	Dried	
150:1	3.88	25	.2794	.0716	.0066	-	.2860	.0716	.2144
150:1	7	25	.3835	.1517	.0129	-	.3964	.1517	.1447
150:1	7	23	.4921	.1828	.0163	-	.5084	.1828	.3256
150:1	7	20	.4751	.2700	.0180	-	.4931	.2700	.2231
150:1	10	20	.4178	.2002	-	-	.4178	.2002	.2176
150:1	5	23	.5100	.3201	.0163	-	.5263	.3201	.2052
150:1	8	23	.4627	.2111	.0200	-	.4827	.2111	.2716
150:1	7.5	23	.5737	.2547	.0206	-	.5943	.2547	.3396
150:1	8	23	.5529	.2887	.0167	-	.5696	.2827	.2809
150:1	7.5	23	.5857	.2687	.0195	-	.6052	.2687	.3366
150:1	6.5	23	.6109	.3656	.0224	-	.6333	.3656	.2677

* : Butenyl isothiocyanate

** : Pentenyl isothiocyanate

\+ : BIT + PIT

\# : $ITC_{slurry} - ITC_{dry}$

N.B.: ITC produced from hydrolyzing meal glucosinolates in neutral medium, as shown in Table 3.1 for MAP N 150, is 1.52 mg/g meal.

For every run, 0.5 g[6] of meal and a 6 mm glass bead were placed in each of the three 20 ml culture tubes. The tubes and their contents were then brought to the temperature of the run by placing them in a water bath that was maintained at that temperature for 10 min. Then, distilled water at the same temperature was added to the contents of the tubes at the operating W/M ratio. Afterwards, the tubes were shaken in a rotary shaker immersed in the water bath. After shaking for the desired time, 2 ml of methylene chloride, at the same temperature, were added to one of the tubes. The shaking was then continued for 4 h more. A shaking time of 4 h was adopted instead of the 2 h used in the Modified Analytical Procedure (Section 3.3.6) and in the literature (235). The 4 h period has been experimentally determined as the required time to completely hydrolyze the meal glucosinolates at the lowest operating 5°C (details in Appendix 9). At this temperature, the rate of hydrolysis of the glucosinolates is the slowest of the experimental design. To have a good basis for comparison, the 4 h time was adopted for all runs. After this shaking period, the methylene chloride layer was separated from the aqueous solution by centrifugation at 2500 rpm; it was then

[6] In runs where W/M ratios were 36:1 or more, the meal's weight used was reduced in order to accomodate these high ratios in the same 20 ml culture tubes.

removed by a long-needle syringe and transferred to a 4 ml vial. The ITC in the extract was determined by the GLC method and denoted ITC_{slurry}.

The contents of the other two tubes were poured into a beaker, placed in a deep-freezer at a temperature of -50°C and left there until frozen. They were then freeze-dried at 55°C, as mentioned in Section 5.2.

One-half g (or the same weight used initially) of the dried meal was placed in a culture tube similar to those originally used in the runs. A 6 mm glass bead and 2 ml of methylene chloride were added to the tube. The contents were shaken in the rotary shaker for 10 min. Then distilled water was added to the contents of the tubes at the same W/M ratio as the original run. Shaking was then continued for 4 h. The methylene chloride and the water used had the same temperature as the run. The rotary shaker was immersed in the water bath. As for the slurry, the methylene chloride layer was separated by centrifugation and its ITC content was determined by GLC and denoted ITC_{dried}.

The removed ITC, denoted Y, is the difference between ITC_{slurry} and the ITC_{dried}. Replicated runs at the center of the design had a standard deviation of 0.0035 mg/g meal solids at an average ITC removal of 0.0565 mg/g solids, which indicated that the experimental results were well reproduced.

5.3.2 Results and Discussion

The results are presented in Table 5.4 and Figures 5.10-5.16. The operating conditions in Figures 5.14-5.16 are not coded to facilitte comparison. The resulting ITC removed (Y) and its corresponding operating levels were reduced by a multiple linear regression analysis to the linear second order polynomial model (85) of the form:

$$Y = B_0+B_1X_1+B_2X_2+B_3X_3+B_{12}X_1X_2+B_{13}X_1X_3+B_{23}X_2X_3 +B_{11}X^2_1+B_{22}X^2_2+B_{33}X^2_3$$

where the Bs are empirical coefficients.

The model suggested above did not give a satisfactory fit for the results of the 18 runs. This was explained from the results obtained for the acidic medium

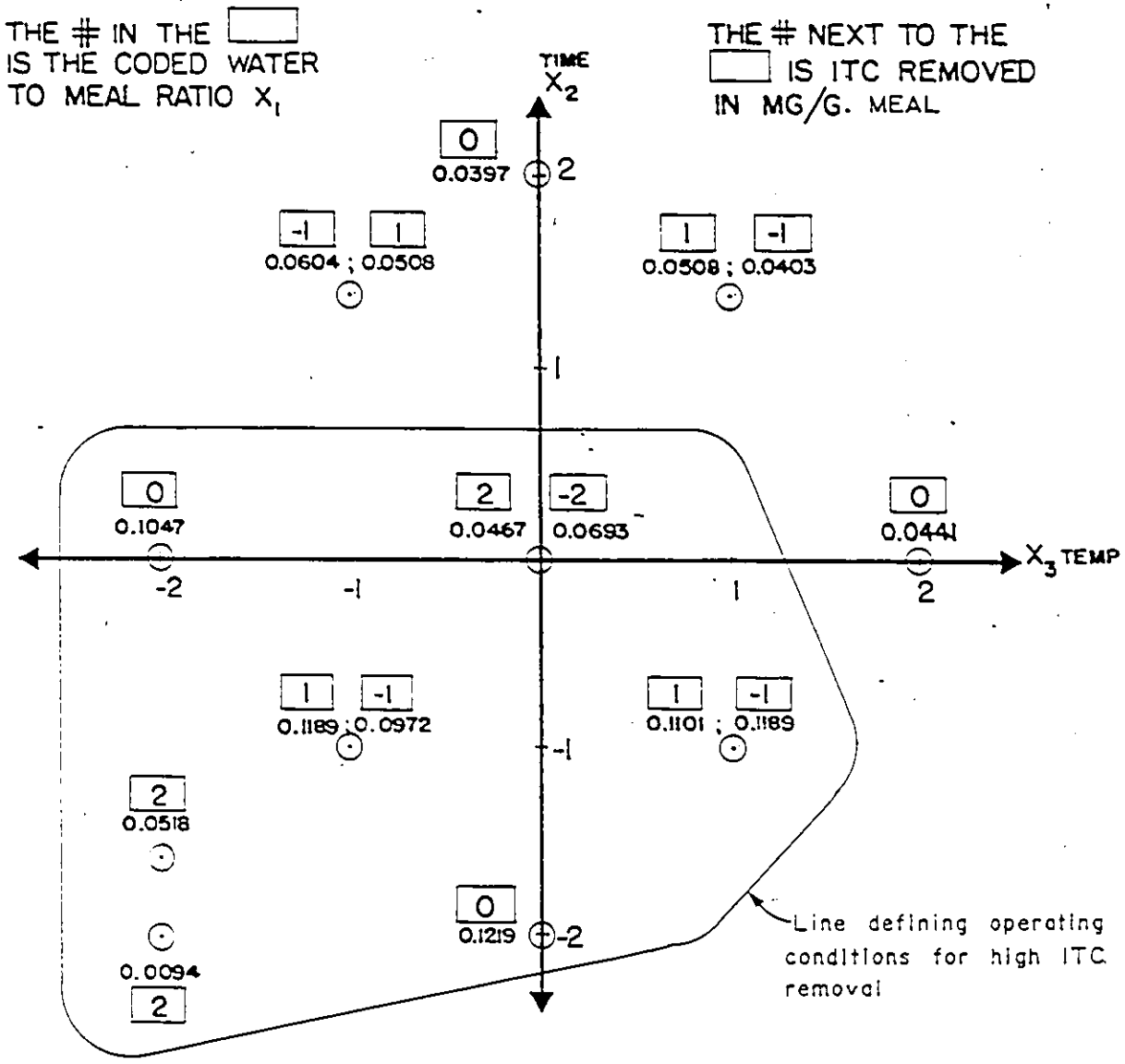


Figure 5.11: ISOTHIOCYANATES REMOVED (mg/g meal) AT OPERATING LEVELS, PRESENTED AS TIME VS. TEMPERATURE

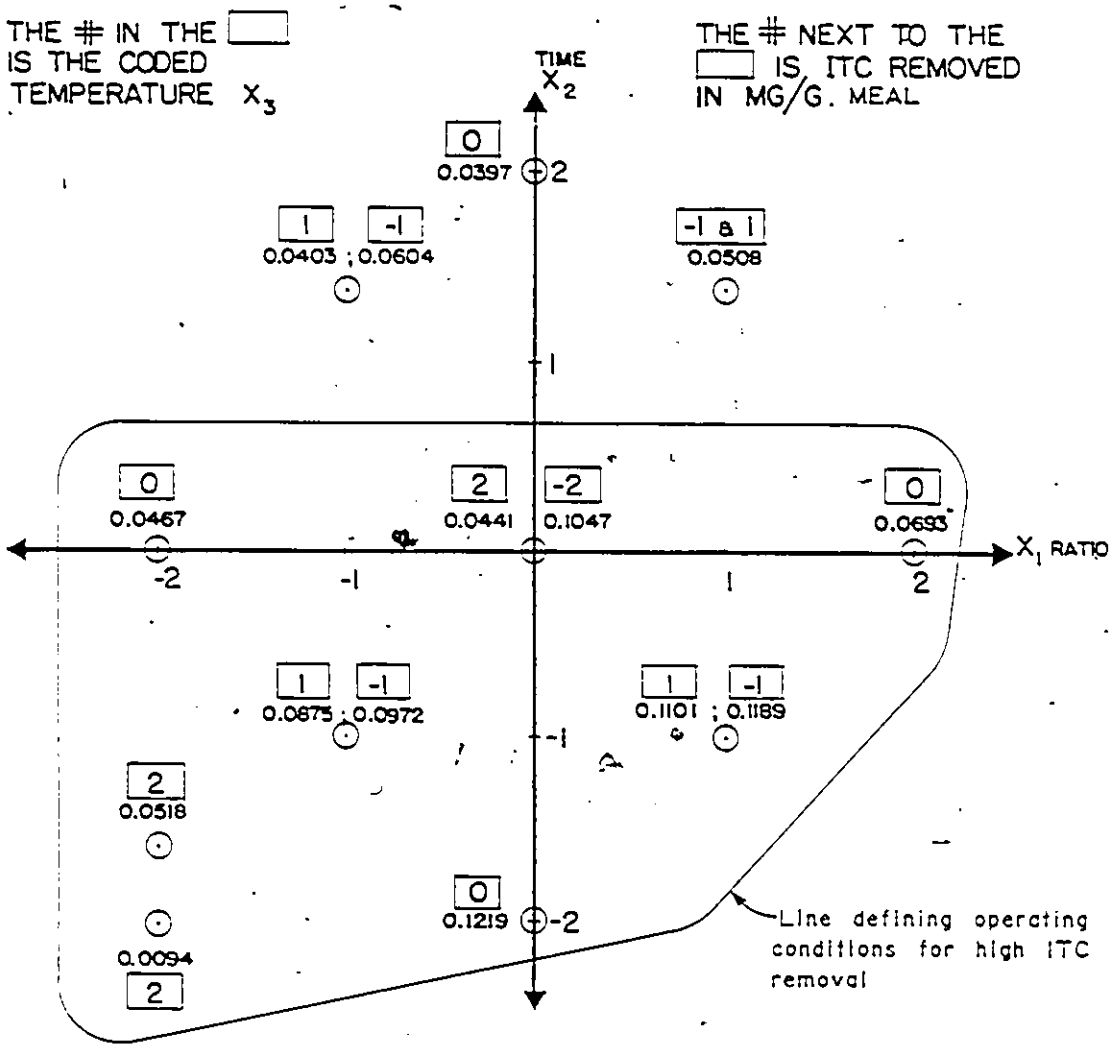


Figure. 5.12: ISOTHIOCYANATES REMOVED (mg/g meal) AT OPERATING LEVELS, PRESENTED AS TIME VS. WATER-TO-MEAL RATIO

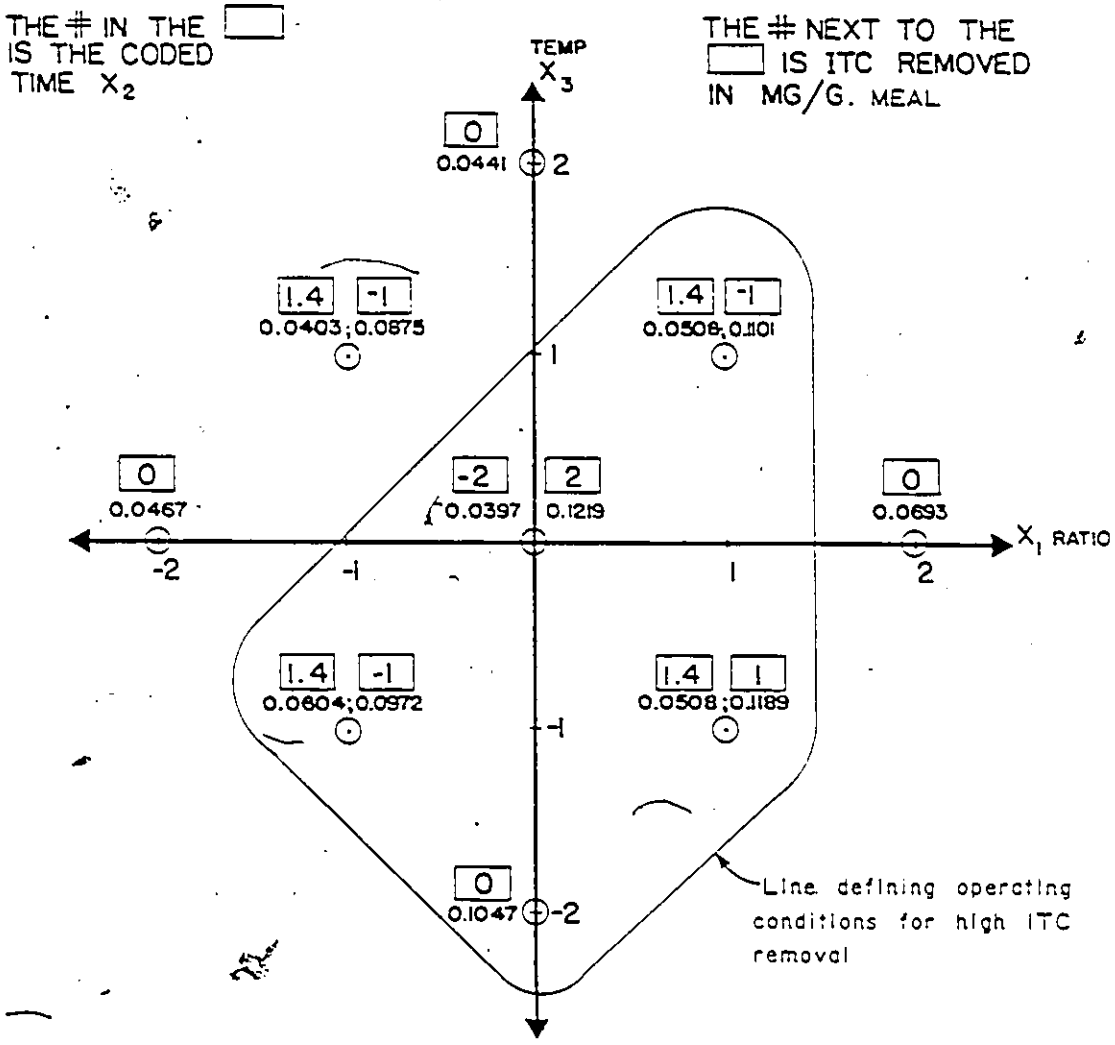


Figure. 5.13 ISOTHIOCYANATES REMOVED (mg/g meal) AT OPERATING LEVELS, PRESENTED AS TEMPERATURE VS. WATER-TO-MEAL RATIO

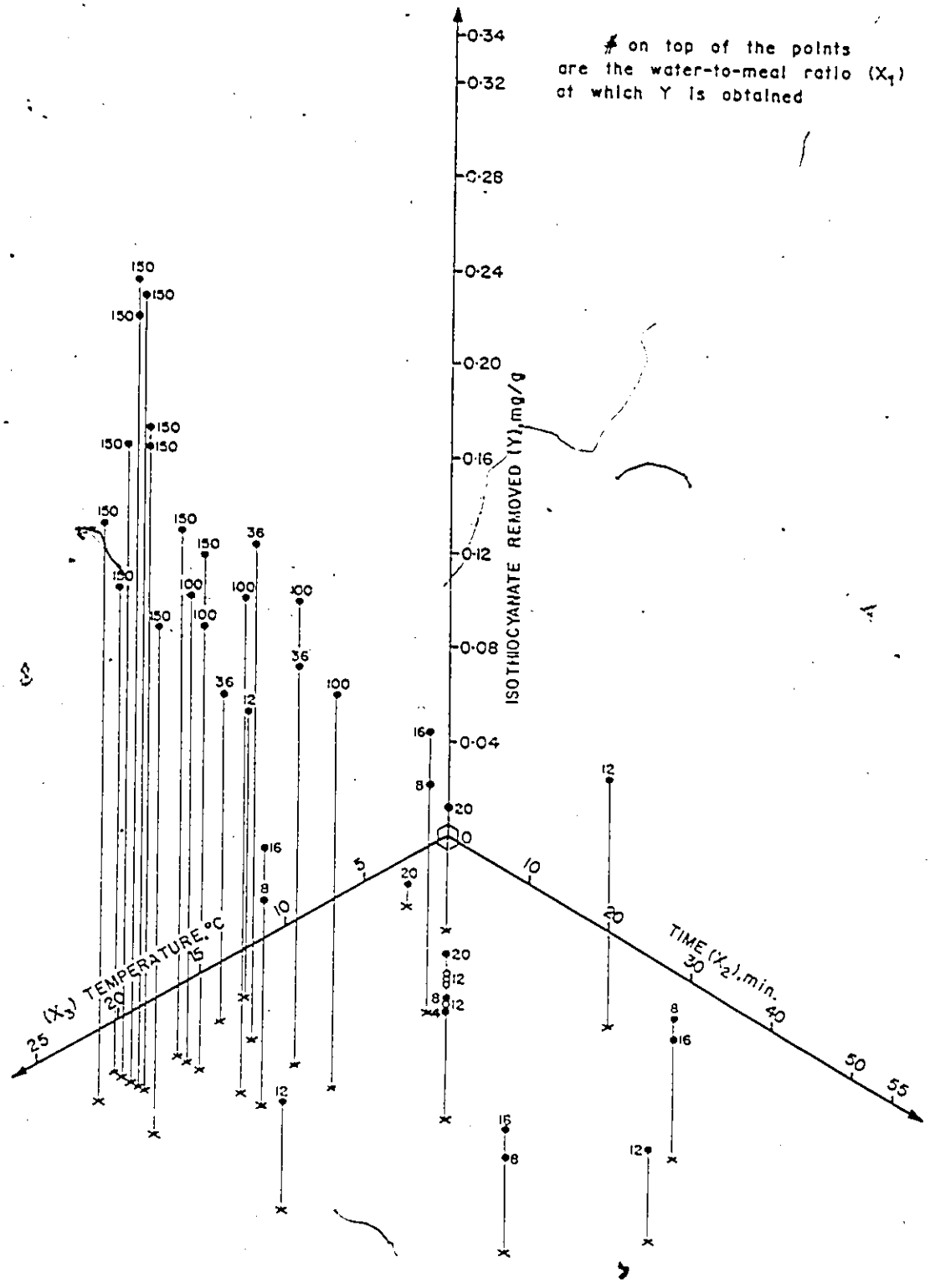


Figure. 5.14. ISOTHIOCYANATES REMOVED BY THE NEUTRAL METHOD FOR ALL RUNS.

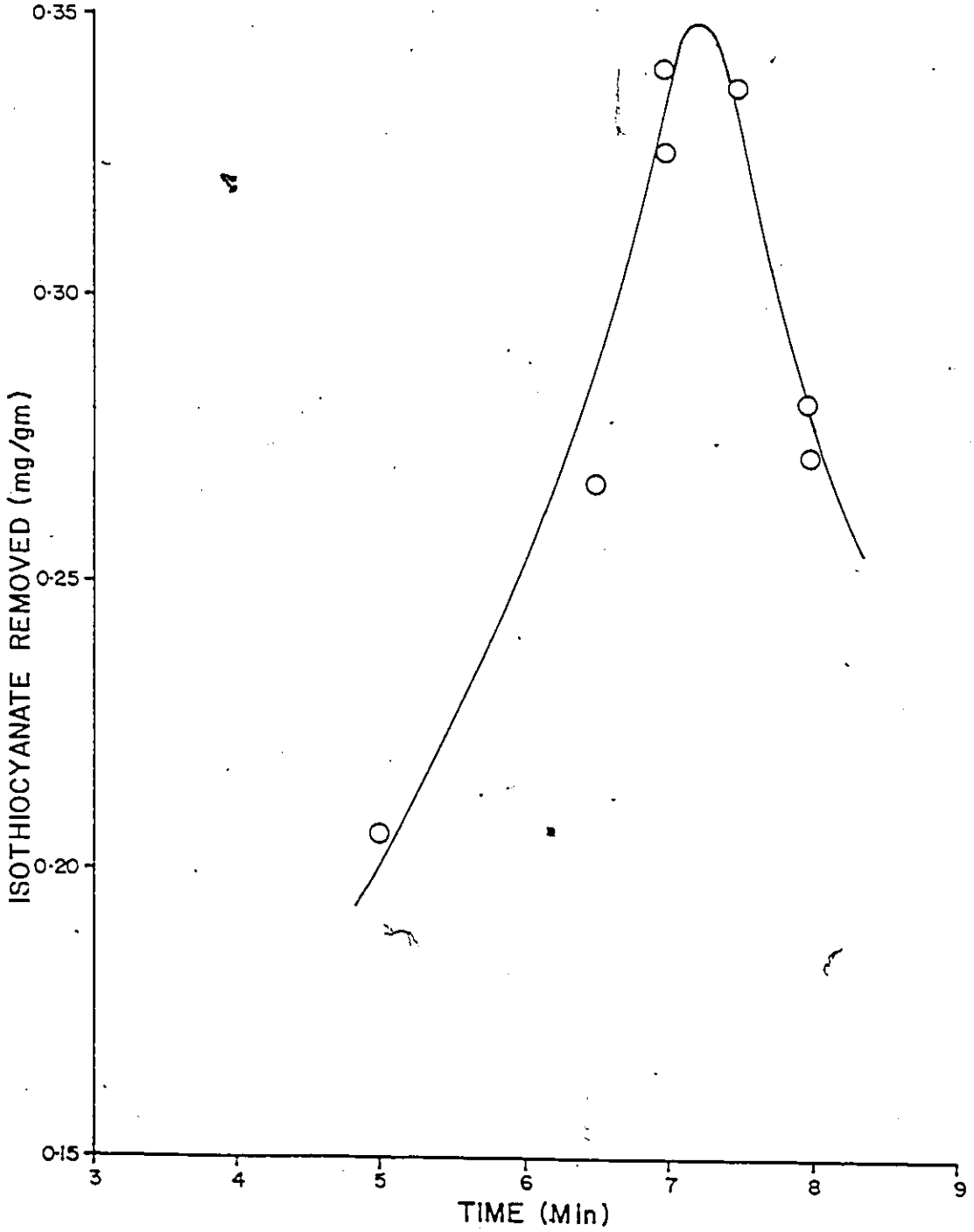


Figure. 5.15 ISOTHIOCYANATES REMOVED VS. TIME, AT TEMPERATURE OF 23°C AND WATER-TO-MEAL RATIO OF 15·1

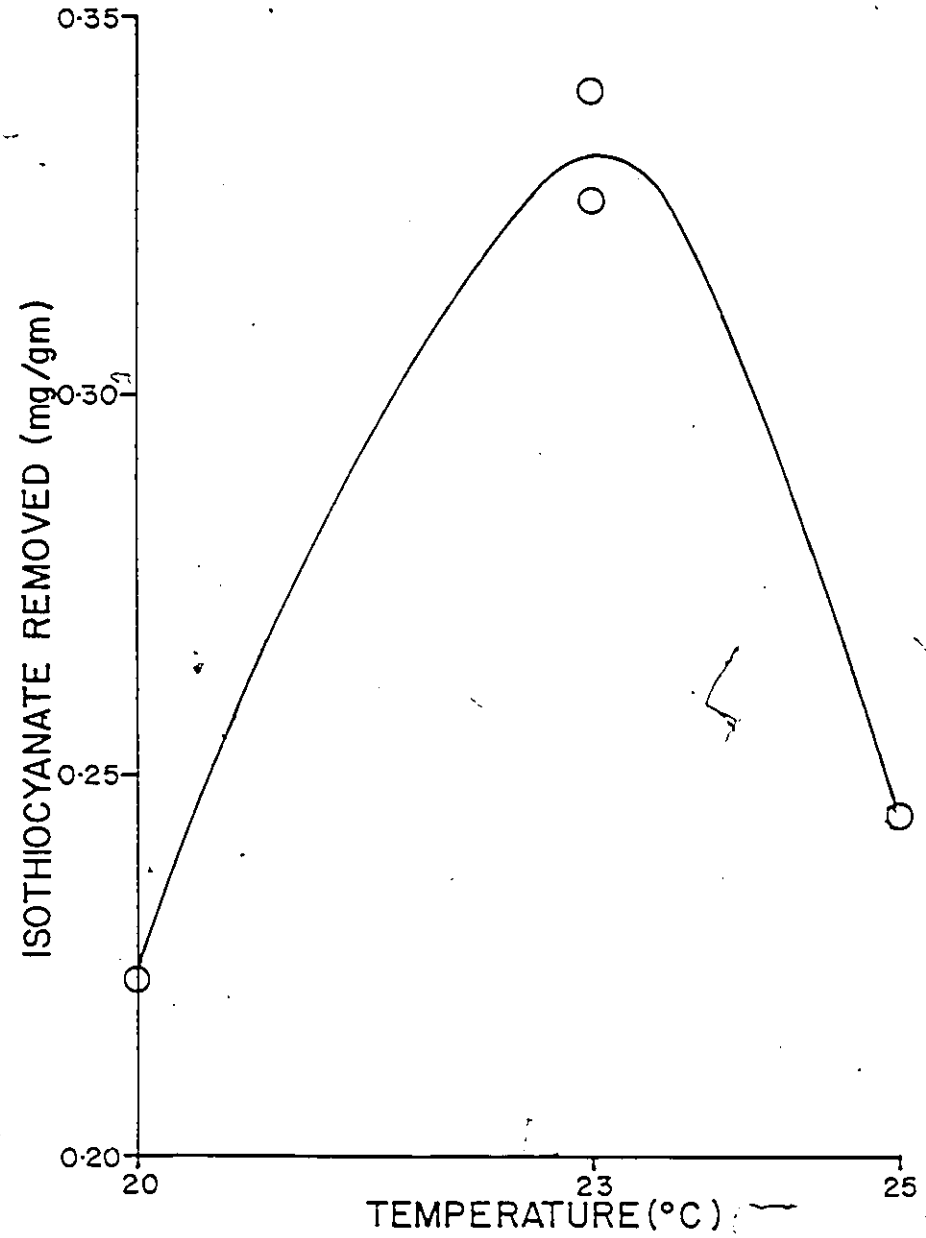


Figure. 5.16 ISOTHIOCYANATES REMOVED VS. TEMPERATURE, AT TIME OF 7 Minutes AND WATER-TO-MEAL RATIO OF 150.1

method temperature of 25°C in Figure 5.9. Considering the fact that the curves should pass through the origin, it is clear that the curves for ITC removal for the drying temperature of 55°C take a Gaussian shape skewed to the left and a great drop at 15 to 30 min. These curves were obtained in an acidic medium where the reaction between the ITC and the protein was inhibited. However, in the present case, the pH was higher at a range of 6 to 7, which would result in a more pronounced skewness and a greater drop in ITC removal than at pH 4.5. After this sharp drop, the curve would have a much lower slope.

In view of this information, Figures 5.10-5.13 were reexamined. This examination revealed that the high Y's are clustered about the center of the operating conditions and the low Y's are at the outskirts. These two levels of y could be the reason for the poor fit of the model by all the results. Since the aim was to maximize Y (the ITC removed), the runs that had a low level of Y were eliminated. These eliminated runs were the five long operating times of 47½ and 55 min, and the ones with an operating temperature of 25°C, thus making 12 the total points remaining to be fitted.

Three more experiments were done at operating conditions which would help to shape the region of the maximum. These points were at the following operating levels:

X_1 Water-to-Meal Ratio	X_2 Time (min)	X_3 Temperature °C
8:1	30	15
20:1	5	5
20:1	10	5

The results obtained are represented in Table 5.4.

By adding these 3 new points to those 12, and fitting the total 15 points to the model, the model obtained was very satisfactory in representing the points. Adequacy tests mentioned in the literature (25,85) confirmed its appropriateness. The fitted model for Y (the ITC removed) is:

$$Y = 0.0568+0.0057X_1-0.0902X_2-0.0528X_3$$
$$-0.0055X_1X_2-0.0481X_2X_3-0.0289X^2_2-0.0144X^2_3$$

The Xs could have had negative or positive values. From the model it can be seen that Y increases by increasing X_1 and decreasing X_2 and X_3, which is noticeable in Figure 5.14.

It can be seen in this model that the amount of ITC removed is very dependent on time X_2 and temperature X_3; that is to say Y is sensitive to small changes in X_2 and X_3, and it was found that the coefficient of the former is almost double that of the latter, for example:

	X_2	X_3	X^2_2	X^2_3
The coefficients	0.09	0.05	0.029	0.014

However, the coefficients for X_1 are much smaller than those for X_2 and X_3, which indicates that a large change in X_1 is needed to affect Y.

The model was then transformed to the canonical form to locate the centre of the surface (stationary point) and then deterimine new operating conditions which would give a higher Y (ITC removal). This mathematical treatment is provided in the literature (25,85). The canonical transformation and the knowledge of the physical processes involved were used to determine these operating conditions. For example, the transformation predicted a very high Y at zero time. However, a substantial hydrolysis would not take place before 5 min, which was chosen as the lower limit for time X_2. Furthermore, the transformation predicted a very high operating temperature and a corres-

ponding below zero time, which is not feasible. These problems could occur when working with an empirical model, as the model represents the behavior of the system in a specific region and would not necessarily hold when extrapolated outside it.

The new operating conditions chosen (which were reached by model predictions and the physical knowledge of the system) are: W/M ratio of 36:1, time of 5 min, and temperature of 17°C. They produced a **Y** of 0.1384 mg/g solids. Although this value was larger than those obtained before, there was the possibility that a 5 min duration was not adequate to substantially hydrolyze the glucosinolates. Therefore, two runs were performed at times of 10 and 15 min for the same W/M ratio of 36:1 and temperature of 17°C. The expectation proved to be valid, and **Y** was 0.2108 for the former and 0.1696 mg/g solids for the latter. This also proved that the ITC formation and reaction with the meal protein were of a Gaussian type.

The process of maximizing the amount of Y (ITC removed) continued sequentially as explained above, until a maximum **Y** of 0.2447 mg/g solids occurred at a W/M ratio of 150:1, time of 7 min, and temperature of 25°C.

The model fitted at this stage suggested an increase in the W/M ratio to increase Y. However, this was not possible without sacrificing the accuracy and reliability of the results, because the weight of the meal sample would have had to be reduced to 0.05 g or less. Such a small sample size is required since the volume of the tubes used in this study was only about 20 ml. To avoid introducing a change in the experimental conditions, a larger tube size was not used.

Another approach was, therefore, taken which required an assessment of the results obtained, particularly at the ratio of 150:1. At this ratio, temperature of 20°C, and time of 7 min, a Y of 0.2231 mg/g solids was obtained, which is comparable with the one previously mentioned at the temperature of 25°C.

It was expected that the system under investigation should be Gaussian; however, these two Ys were almost at the same level. To have the Gaussian shape, there is a possibility that a Y higher than both of these occurs in between 20-25°C and time of 7 min. Also, since Y at 25°C was higher than that at 20°C, if a higher Y exists, it would probably be closer to 25°C than to 20°C. Therefore, a run was conducted at a temperature of 23°C, a W/M ratio

of 150:1 and a time of 7 min. The resulting Y was 0.3396 mg/g solids which is the highest obtained and indicates that the approach taken was correct. To confirm the result, the run was repeated and a Y of 0.3366 mg/g solids was produced. These results are compiled in Table 5.4. The relation between the ITC removed as a function of temperature is shown in Figure 5.16, where the large increase is noticeable.

For the same W/M ratio of 150:1 and temperature of 23°C, runs were carrid out at duration times of 5, 6.6, 7.5, and 8 min, with the latter being repeated. The results are shown in Figure 5.15, where the great increase is very noticeable. This figure also clearly illustrates the sensitive dependency of Y (ITC removed) on time. When comparing Figures 5.15 and 5.16, it is obvious that the system is more sensitive to time than to temperature, a fact which was previously proven by the obtained model. The available results were not sufficient to fit a model which would describe the system and include the sharp peak that the response elicited.

The occurrence of this sharp peak may be explained by the fact that the ITC formation rate is higher than the

ITC reaction rate with protein up to a time of 7 min. At that point, the two become equal; beyond the 7 min, the trend reverses.

Summary

In the Neutral Medium Method, the amount of ITC removed by freeze-drying was 0.34 mg/g solids. The operating conditions at which the meal glucosinolates were hydrolyzed prior to the freeze-drying are W/M ratio of 150:1, a time of 7 min, and a temperature of 23°C. The freeze-dried meal treated at these conditions was free of nitriles and retained its original yellow color.

5.3.3 Economics Study:

It was decided to study the economics of a plant using the Neutral Medium Method to produce dehulled detoxified rapeseed meal (DDM). The basis and assumptions of this economic study were made under the best possible conditions to enhance the economics of the process. The market consumption of the DDM would probably be a few thousand tonnes (t) per year. Thus, it was assumed that the plant would process 24 t/day.

Since producing the DDM results in oil, and hulls and fines fraction, the plant would be attached to an existing rapeseed crusher for the convenience of selling these products to the crusher.

It is also assumed that after further research, as recommended in Chapter 7, it would be possible to remove water from the meal slurry by decantation before the freezing step. Also, the decantation would reduce the W/M ratio from 150:1 to 2.5:1. The meal losses by the decantation was taken to be negligeable. In addition, the duration of the freeze-drying would be reduced from 24 to 12 h.

Details of this economic study, its mass balance, and energy requirement are presented in Appendix 20, which also includes a Process Flow Sheet (Figure A.20.1).

The following is a summary of the economic study and the basis and assumptions used to calculate it:

Basis:

1. Rapeseed and products from:

- Dehulling of 1 t rapeseed gives 0.7 t of meat and 0.3 t of hulls and meat fines fraction. This fraction is composed of 43% hulls and 57% meat fines.

- Defatting of the 0.7 t of meat gives 0.35 t of meal and 0.35 t of oil.

- The 0.35 t of meal produces 0.35 t of DDM.

- Industrially produced meal (IPM) contains 23% of hulls.

Prices of rapeseed and products from the detoxication process are presented below:

Material	Price $/t	Remarks
- rapeseed	225	contains 13% of hulls
- unrefined oil	3 500	to be sold to the crushing plant
- hulls & fines fraction	147	priced for its meat fines content in proportion to that of rapeseed - to be sold to crushing plant
- IPM	144	
- DDM	250	price assumed higher than IPM by 23% for having no hulls and by an additional 25% for being less toxic

(N.B. Most sources who supplied the prices and information used in this economic analysis asked to remain anonymous. This is to keep the economics of their companies and crushing plants confidential.)

2. Other Materials:

- Hexane is recovered from miscella in solvent recovery units. However, the amount of hexane lost to recycling is 1.5 kg/t of the meal produced. The price of hexane is $0.50/kg.

- Water is needed at a ratio of 150 t/t meal (52.5 t/h). An additional 5% is assumed to be needed for other purposes. Thus total water needed is 55.13 t/h (463 050 t/yr). The price of water taken as $0.10/t.

3. Utilities:

- Steam was used to supply heat to evaporate the frozen water of the meal slurry, to melt the ice accumulated on the freeze-dryer condensers, to condition the seed, and to recover the oil.

- Electric power.

4. Operating Period and Labour:

- For maximum profitability, it was decided that the plant would operate for three 8 h shifts/day for 350 days/year.

- It was estimated that 5 operators are needed per shift.

5. Equipment:

Types, number of units, and cost of equipment needed are presented below in Table 5.5. As a safety measure, the capacity of all equipment was chosen to be 10% more than that needed. For the same reason, the number of units were chosen to be not less than two. Because of the long residence time of 12 h, the freeze-drying units would be operated in a staggered manner.

TABLE 5.5

Details of Equipment for DDM Plant

Equipment	No. of Units	Total Capacity, t/h	1987 Price $000
1. Seed Conditioning Unit	2	1.1	52
2. Seed Cracking Mill	2	1.1	29
3. Dehulling Unit	2	1.1	33
4. Solvent Extractor & Solvent Recovery	2	3.08	2 700
5. Desolventizer Toaster	2	0.97	800
6. Mixer	2	58.3	100
7. Decanter	2	58.3	400
8. Freezing-Belt	2	0.97	500
9. Cold Room	2	0.97	500
10. Freeze-Dryer	8	0.97	16 000
Sub Total - Equipment Price			21 114

Taking cost of installation, contingencies, instrumentations, and auxiliaries (boilers, motors, etc.) as 42% of equipment price, then total cost of equipment installed = $30 000 000. Usually, higher factors are used to cover such items. In this case, however, 42% was considered sufficient since the cost of a freeze-dryer is exhorbitant.

6. Building:

The building to house the plant was calculated to cost approximately $2 000 000. This figure was arrived at on the basis of a 40 000 ft^2 plant costing $50.00/$ft^2$.

7. Miscellaneous:

Information on other direct costs, such as packaging and depreciation, is presented in Appendix 21.

Economic Analysis:

The economic analysis is presented in detail in Appendix 21 and it was calculated with a proprietory finance model. Results of this analysis indicate that for a $250/t DDM, the 10-year-income year discount cash flow (DCF)* return is only 2.12%. In spite of deliberately enhancing the economics of the process, the analysis shows that is not profitable enough. It also indicates that operating profits before taxes are low at $2 091 800.

From these results, it can be concluded that producing DDM by the Neutral Medium Method is not profitable enough. This conclusion remains the same even if the price of DDM is increased to $1 000/t. In this case, the DCF and operating profits are still unacceptable at 8.08% and $4 134 200, respectively.

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* DCF: The discount rate at which the present value of a project's cash inflows equals that of outflows.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The rapeseed meal used in this study was obtained from the Tower variety after dehulling and defatting. The dehulling step reduced the fiber content in the meal to about 4% from what would otherwise be about 25%. The solubility of the meal protein in aqueous solutions was studied with a W/M ratio of 50:1. A pH range of 1 to 13 was used at five different temperatures from 15 to 55°C at 10°C intervals. The minimum protein solubilities at these temperatures (as percentages of the original protein content) were 11.8, 14.2, 19.0, 26.2, and 30.2, occurring at pHs 4.4, 4.5, 4.8, 7.0, and 7.2, respectively.

The meal retained its original yellow color at, and in the vicinity of, these conditions for minimum protein solubility. The results of this solubility study were needed for the subsequent investigation on the removal of isothiocynates (ITC).

In the attempt to remove the ITC, three methods were investigated:

(i) Aqueous extraction method

(ii) Acidic medium method

(iii) Neutral medium method

Aqueous Extraction Method

The experiments carried out in this method were aimed at aqueously extracting the aglucons resulting from the autolytic hydrolysis of the meal's glucosinolates. To minimize the loss of the meal's protein, the extraction was done at pHs of 4.4 and 4.5, which corresponded to the minimum protein solubility conditions at the utilzed temperatures of 15 and 25°C. Other extraction conditions used were W/M ratios of 3:1, 4:1, and 5:1, and treatment times ranging from ½ h to 5 h. The extracted meals were then freeze-dried at a final temperature inside the meal solids of 28°C. The ITC content of all freeze-dried meal solids was low, largely because of the reaction of the ITC formed from the hydrolysis of the meal glucosinolates with the meal protein. However, it was noticed in experiments carried out at a W/M ratio of 3:1 and a temperature of 25°C, that a small amount of ITC (between 0.0088 and 0.0208 mg/g meal solids) was removed during the freeze-drying step.

Acidic Medium Method

Since the extraction in the previous method did not contribute to the removal of the ITC, it was omitted from the further development of a detoxification method. It was decided to further investigate the removal of the ITC by freeze-drying. This investigation was carried out at a temperature of 25°C and a pH 4.5. To lessen the reaction between the ITC and the protein, the range of W/M ratio and treatment time was extended beyond that of the previous method to include a 10:1 ratio and a 15 min time. To increase the amount of ITC removed, the freeze-drying was done at 55°C. This ratio and treatment time gave the maximum ITC removal of 0.2227 mg/g meal for this method. However, the hydrolysates in the acidic medium resulted in the formation of 0.35 mg/g of non-volatile nitriles. These nitriles were formed instead of the volatile isothiocyanates and vinyl oxazolidinethione (VOT). The VOT, like the nitriles, is not volatile. However, its toxicity is about 1/8 that of the nitriles. Since it was expected that the level of the nitriles formed would be even higher for the aqueous treatment temperature of 15°C, this temperature was not investigated.

In this method, there was no loss in the meal components as determined gravimetically.

Neutral Medium Method

Since the formation of the nitriles from the hydrolysis of the meal glucosinolates is reduced at a high pH, it was decided in this method to proceed with the aqueous treatment in a neutral medium. W/M ratio, time, and temperature were the parameters studied in this investigation. These parameters were chosen because the increase in W/M ratio and temperature (up to 70°C) would reduce the formation of the nitriles. However, the reaction between the ITC formed and the protein also increases with temperature. Thus, an optimization of these three parameters was needed to suppress the formation of the nitriles, and the reaction between the ITC and the protein. This resulted in maximizing the amount of the free ITC (not reacted with the meal protein), which could then be removed by freeze-drying. To determine the conditions for the maximum removal of the free ITC, the experiments were planned in a modified central composite statistical design and optimized by modelling according to the Box-Wilson procedure. The initial range of the three parameters studied were a W/M ratio of 4:1 to 20:1, a treatment time of 5 to 55 min, and a temperature of 5 to 25°C. The meal slurry was freeze-dried at 55°C. A maximum ITC removal was obtained by sequentially optimizing the results.

A maximum ITC removal of about 0.34 mg/g meal occurred at a treatment time of 7.5 min, a W/M ratio of 150:1, and a temperature of 23°C. The nitrile content in the meal treated under these conditions was not detectable. This maximum ITC removal was found to lie on a sharp peak, which is sensitive to changes in temperature and treatment time. It was noted that the meal solids resulting from these treatment conditions retained their original yellow color.

Modification of Analytical Procedure

In this research study, the widely used Youngs' method for the analysis (by GLC) of volatile ITC in rapeseed meals was modified. The major features of the modification were the use of the endogenous myrosinase to hydrolyze the meal glucosinolates, and the use of a high W/M ratio of 150:1. The modified method (MAP) was found to be reasonably accurate, having a coefficient of variation of 4.48%. However, since the use of the endogenous myrosinase is an essential modification, the modified method is applicable only to meals whose myrosinase is not deactivated.

Significant Contributions

The significant contribution of this work is the opening up of a new approach for detoxifying rapeseed meal. This new approach involves the use of freeze-drying to remove the toxic volatile components. Also, the knowledge gained and the conclusions reached in this investigation could be extended to other seeds or research fields.

As an essential part of this work, Youngs' method for the analysis of the isothiocyanates content of rapeseed meal was modified to simplify the procedure and provide a better measure of the isothiocyanates level.

CHAPTER 7

RECOMMENDATIONS FOR FUTURE RESEARCH STUDIES

Completion of the current study suggested the following areas of research which could be worthwhile pursuing:

1. Carrying out a kinetic study on the formation of the aglucons from the hydrolysis of the meal glucosinolates, in conjunction with the reaction of the ITC with the meal protein. This would also involve the search for an inhibitor for the reaction mentioned above, thus allowing more available free ITC to be removed by freeze-drying.

2. Results of this kinetic study might lead to a reduced W/M ratio to be used for hydrolyzing the glucosinolates. Also, methods such as decantation to remove water from the meal slurry prior to its

freezing (in preparation for freeze-drying), could be explored.

3. Examining the application of the modified analysis method (MAP) to rapeseed varieties other than Tower.

4. Developing an analytical method that could simultaneously determine all the aglucons produced from the hydrolysis of the glucosinolates of rapeseed meal. Such a method could be helpful for the kinetic study suggested above.

5. Searching for other uses for the removal of, or separation of, materials by freeze-drying.

CHAPTER 8

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APPENDIX 1

PREPARATION OF RAPESEED MEAL

Stefanson and Kondra (203) have developed the rapeseed Brassica napus "Tower" variety. This is representative of the so-called "double-zero" varieties which have a low content of erucic acid and glucosinolates. The former is part of the oil and the latter exist in the rapeseed meal (cotyledon and hypocotyl) (220). Both erucic acid (14) and glucosinolates (165,220) are potentially harmful to animals and human beings.

Tower rapeseed is composed of about 16% indigestible fibers. These fibers come mainly from the hulls (cuticles) which constitute roughly 13% of the meal. Therefore, a dehulling step was essential to reduce the fiber content. The dehulling was done by Dr. J. Jones at the Food Research Institute (FRI), Agriculture Canada. It should be mentioned here that this preparation procedure is essentially that developed at the FRI by Jones and Holme (88). Thus, prior to the dehulling step, the seed was conditioned to a moisture content below 8%. At this low moisture level the potential of the hydrolysis of the glucosinolates during the preparation is substantially reduced.

The dehulling step consisted of coarsely cracking the seed in a 20 cm Bauer Disc Mill fitted with splined discs and a variable speed drive. The speed of the rotating disc and the space between the stationary and rotating discs were adjusted so that of all the seed was fragmented into coarse particles without pulverisation or a build-up of powdered seed on the discs (17,87). The seed fragmentation, without grinding, prevented the hydrolysis of the glucosinolates (161), thus eliminating the inclusion of sulfur in the oil (87).

The cracked seed was separated by air classification, capable of separating 5 kg at a time, into two fractions: a hull-free fraction which is called meat (70% of the seed) and hulls plus meat fines (30%). By selective control of the air flow through the classifier, which operated in a fluidized bed fashion, the hulls plus fines were carried over in the air stream into a cyclone and collected. The dehulled seed, being denser, remained in the fluid bed bucket and was recovered. The air flow was supplied to the classifier by an industrial exhaust fan which could produce 14 000 l/min at 200 mm static pressure when powered by a 1 H.P. electric motor. A variable speed belt drive provided speeds up to 4000 rpm. The classifier and its operation are described in the work of Hergert (83).

After removing the hulls (which constitute about 13% of the seed), the resulting meat contained only about 2% fibers. The meat was defatted in a 10 L Soxhlet extractor with food-grade hexane until the hexane passing through the seed mass and draining into the boiler was colorless and contained no oil. The duration of the extraction was about 24 h. The reason for this long extraction time was that the filling of the Soxhlet's tube until the syphoning started was slow and took up to 2 h. This slowness was related to the capacity of the heating mantle used. The ratio of hexane-to-meat, just before the start of the syphoning, was between 1:1 and 2:1. The defatted meat (which is called meal) was drained of hexane and the meal was spread in shallow trays. As carried out by Finlayson et al. (70), the meal was then air-dried for three days in a fume hood to remove the hexane vapour. Afterwards, the remaining solvent was completely removed by vacuum. The pressure inside the container was below 10 mm Hg and was applied for 1 h at room temperature. The meal was kept in plastic containers which were flushed with nitrogen before being tightly closed. The containers were then placed in a refrigerator containing a drying agent to keep them at a low humidity inside the refrigerator. The moisture content of the meal was always kept below 8%, as determined by periodic checks. The meal was prepared under these mild

conditions to prevent any denaturation of the meal protein (189). The defatting and drying steps were carried out by the author. Proximate analysis of the meal was done at Agriculture Canada by the standard methods of the Association of Official Analytical Chemists (133) and the results are presented in Table A.1.

TABLE A.1

PROXIMATE ANALYSIS OF TOWER RAPESEED MEAL

	As received (%)	Dry basis (%)
Water content	7.75	---
Fat	0.55	0.60
Nitrogen*	7.61	8.25
Protein (N_2 x 6.25)	47.55	51.54
Fiber**	4.34	4.71
Ash	9.39	10.18
Carbohydrate (by difference)	30.42	32.97

* Done by the author. Details are in Appendix 6.
** Seed contains about 15% fiber (major part is from hulls) and about 42% oil (dehulled meat contains about 50% oil). Thus, without dehulling the meal would have contained about 25% fibers.

APPENDIX 2

AMMONIA SPECIFIC ION ELECTRODE (149,150,165)

The ammonia electrode used in this work was a selective gas-detecting electrode which senses the level of dissolved ammonia in aqueous solutions. Ammonia concentrations can be read from calibration curves, or directly on a specific ion meter. They can also be determined by a procedure called the "Known Addition Method" using tables, or by Gran's Plot Paper (special graph paper developed by Orion, the electrode manufacturer).

The electrode can be used to measure ammonia in aqueous solutions, ammonium ion (after converting it to ammonia by adding a base), nitrate (after reduction to ammonia), and organic nitrogen (after Kjeldahl digestion of the sample). Sample color and turbidity do not affect measurements and samples need not be distilled. Anions, cations, and dissolved species do not interfere.

The "Known Addition Procedure" allows samples having variable ionic strength, and containing complexing agents, to be measured. The procedure consists of adding a known

amount of the species being measured to a known value of the sample and observing the resulting change in electric potential. The original sample concentration can be computed as previously mentioned from tables or special graphs. Concentration can also be read directly from the known increment scale of a specific ion meter.

Theory of Operation (149,150)

The ammonia electrode uses an hydrophobic gas-permeable membrane to separate the sample solution from the electrode internal solution, which is a high concentration of ammonium chloride. The ammonia of the sample solution diffuses through the membrane until the partial pressure of the ammonia, which is proportional to its concentration, is the same on both sides of the membrane.

Ammonia diffused through the membrane reacts reversibly with the water of the filling solution, according to the following chemical equation:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \qquad (1)$$

The equilibrium constant K_1 of this equation is:

$$K_1 = \frac{[NH_4^+]\ [OH^-]}{[NH_3]} \qquad (2)$$

The ammonium ion concentration is considered constant because of its presence in high levels in the internal solution. Accordingly, equation (2) becomes:

$$K_2 = \frac{[OH^-]}{[NH_3]} \text{ or } OH^- = K_2[NH_3]\ ; \qquad (3)$$

where K_2 is a constant.

As the potential of the electrode-sensing element is Nernstian with respect to the hydroxidion level, which is directly proportional to the ammonia concentration, the electrode total potential E_1 therefore is:

$$E_1 = E_o + S \log C_1 \qquad (4)$$

where E_o = the portion of the total potential due to references and internal solutions.

S = 2.3 RT/nF;
R (the gas constant) = 1.987 cal/(g mole)(°K);
T is the temperature in degrees Kelvin;
n is the charge of the ion (including the sign);
C_1 is concentration of the ammonia being measured;
F (Faraday's constant) = 96487 coulombs.

When the solution with a known concentration is added, it changes the total concentration to C_2. The subsequent total potential of the electrode becomes E_2, which is defined as:

$$E_2 = E_o + S \log (C_1 + C_2) \qquad (5)$$

The potential difference, ΔE, is obtained by subtracting equation (4) from equation (5) and thus:

$$\Delta E = E_2 - E_1 = S \log \frac{C_2}{C_1} \qquad (6)$$

Rearranging equation (6) yields:

$$\frac{C_1}{C_2} = 10^{-\Delta E/S} = Q \qquad (7)$$

In the instruction manual of the ammonia electrode, Orion Research Inc. values of ΔE versus corresponding Q were calculated from equation (7). This table, a copy of which is reproduced below, is used in the "Known Addition Method" to find out the concentration of the ammonia (C_1) of the solution being measured.

values of Q vs. ΔE at 25°C and with a 58 mv electrode slope

ΔE	Q	ΔE	Q	ΔE	Q	ΔE	Q	ΔE	Q
-5.0	0.297	9.0	0.178	16.0	0.0952	24.0	0.0556	32.0	0.0354
5.1	0.293	9.1	0.176	16.2	0.0938	24.2	0.0549	32.2	0.0351
5.2	0.288	9.2	0.174	16.4	0.0924	24.4	0.0543	32.4	0.0347
5.3	0.284	9.3	0.173	16.6	0.0910	24.6	0.0536	32.6	0.0343
5.4	0.280	9.4	0.171	16.8	0.0897	24.8	0.0530	32.8	0.0340
5.5	0.276	9.5	0.169	17.0	0.0884	25.0	0.0523	33.0	0.0336
5.6	0.272	9.6	0.167	17.2	0.0871	25.2	0.0517	33.2	0.0333
5.7	0.268	9.7	0.165	17.4	0.0858	25.4	0.0511	33.4	0.0329
5.8	0.264	9.8	0.164	17.6	0.0846	25.6	0.0505	33.6	0.0326
5.9	0.260	9.9	0.162	17.8	0.0834	25.8	0.0499	33.8	0.0536
6.0	0.257	10.0	0.160	18.0	0.0822	26.0	0.0494	34.0	0.0319
6.1	0.253	10.2	0.157	18.2	0.0811	26.2	0.0488	34.2	0.0316
6.2	0.250	10.4	0.154	18.4	0.0799	26.4	0.0482	34.4	0.0313
6.3	0.247	10.6	0.151	18.6	0.0788	26.6	0.0477	34.6	0.0310
6.4	0.243	10.8	0.148	18.8	0.0777	26.8	0.0471	34.8	0.0307
6.5	0.240	11.0	0.145	19.0	0.0767	27.0	0.0466	35.0	0.0304
6.6	0.237	11.2	0.143	19.2	0.0756	27.2	0.0461	36.0	0.0289
6.7	0.234	11.4	0.140	19.4	0.0746	27.4	0.0456	37.0	0.0275
6.8	0.231	11.6	0.137	19.6	0.0736	27.6	0.0450	38.0	0.0261
6.9	0.228	11.8	0.135	19.8	0.0726	27.8	0.0445	39.0	0.0249
7.0	0.225	12.0	0.133	20.0	0.0716	28.0	0.0440	40.0	0.0237
7.1	0.222	12.2	0.130	20.2	0.0707	28.2	0.0435	41.0	0.0226
7.2	0.219	12.4	0.128	20.4	0.0698	28.4	0.0431	42.0	0.0216
7.3	0.217	12.6	0.126	20.6	0.0689	28.6	0.0421	43.0	0.0206
7.4	0.214	12.8	0.123	20.8	0.0680	28.8	0.0421	44.0	0.0196
7.5	0.212	13.0	0.121	21.0	0.0671	29.0	0.0417	45.0	0.0187
7.6	0.209	13.2	0.119	21.2	0.0662	29.2	0.0412	46.0	0.0179
7.7	0.207	13.4	0.117	21.4	0.0654	29.4	0.0408	47.0	0.0171
7.8	0.204	13.6	0.115	21.6	0.0645	29.6	0.0403	48.0	0.0163
7.9	0.202	13.8	0.113	21.8	0.0637	29.8	0.0399	49.0	0.0156
8.0	0.199	14.0	0.112	22.0	0.0629	30.0	0.0394	50.0	0.0149
8.1	0.197	14.2	0.110	22.2	0.0621	30.2	0.0390	51.0	0.0143
8.2	0.195	14.4	0.108	22.4	0.0613	30.4	0.0386	52.0	0.0137
8.3	0.193	14.6	0.106	22.6	0.0606	30.6	0.0382	53.0	0.0131
8.4	0.190	14.8	0.105	22.8	0.0598	30.8	0.0378	54.0	0.0125
8.5	0.188	15.0	0.103	23.0	0.0591	31.0	0.0374	55.0	0.0120
8.6	0.186	15.2	0.1013	23.2	0.0584	31.2	0.0370	56.0	0.0115
8.7	0.184	15.4	0.0997	23.4	0.0576	31.4	0.0366	57.0	0.0110
8.8	0.182	15.6	0.0982	23.6	0.0569	31.6	0.0362	58.0	0.0105
8.9	0.180	15.8	0.0967	23.8	0.0563	31.8	0.0358	59.0	0.0101

NOTE: Known addition tables for other temperatures and other electrode slopes are available from the Technical Services Group at Orion Research.

APPENDIX 3

PROTEIN DENATURATION (69)

Following a treatment so mild[1] that ordinary chemical compounds remain unaltered, proteins undergo profound changes in many specific properties. These changes in specific properties, characterizing the identity of proteins, are collectively called "denaturation".

Changes in protein properties after denaturation are:

1) Peptide bonds of the protein are more readily available for hydrolysis by proteolytic enzymes;
2) Solubility is decreased;
3) Enzymic activity, if originally present, is decreased or lost;
4) Crystalization of the protein is no longer possible;
5) Optical rotation of the protein solution is increased;
6) Intrinsic viscosity is increased.

The increase in intrinsic vicosity would suggest that the protein molecule had unfolded and had become more asymmetrical. This would expose more hydrophobic residues and would decrease the solubility of the protein.

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[1] Does not cause breaking of the protein's peptide bonds.

The sensitivity of a protein to denaturation is determined by the ease with which denaturing agents disrupt the bonds that are essential to the maintenance of a three-dimensional structure. Since proteins differ widely in structure and in amino acid composition, their sensitivities to denaturing agents, and the types of alterations they undergo, could be expected to vary. This means that comparisons of the extents of their denaturation are difficult.

Denaturation can be defined as any modification of the secondary, tertiary, or quaternary structure of the protein molecule, excluding breakage of the covalent bonds. Denaturation is therefore a process by which hydrogen bonds, hydrophobic interactions, and salt linkages are broken and protein is unfolded. In some instances the protein molecules completely unfold and assume a random coil-type structure. Proteins in random coil structures could more readily form aggregates. Although proteins are difficult to study in their native state, it is generally accepted that native proteins are those prepared by the mildest extraction methods possible.

Denaturation under different conditions does not lead to the same changes in a given protein molecule. For example, the denaturation of a protein by heat results in

changes different from those produced by dilute acid or alkali.

Denaturing agents may be divided into two classes, physical and chemical. Heat is the most important physical agent. The rate and extent of protein denaturation is highly dependent on temperature. Whereas for most chemical reactions, the rate doubles for each 10°C rise in temperature, with protein denaturation the rate increases about 600 fold for every 10°C rise in temperature. The effects of denaturing agents can therefore be greatly reduced by working at low temperatures.

The water content of partially dried protein preparations has a great effect on the rate of heat denaturation. Ionic strength, pH, and the type of ions present in the solution also affect the susceptibility of proteins to heat denaturation. Most proteins undergo denaturation when heated over 50 to 60°C.

The pH of the medium has a profound effect on the denaturation of proteins. Most proteins are stable within a fairly narrow pH range, and exposure to pH values outside this range often causes denaturation. Moving away from the pH of optimum stability increases repulsive forces within

the protein molecule. These changes in ionic forces favor the unfolding of the molecule structure. If the resulting structural changes are not too extreme, the protein often can resume its native structure when returned to the stable pH range. Organic solvents, such as acetone and alcohol, also cause denaturation of proteins, although their effects are reduced at low temperatures.

APPENDIX 4

MAILLARD REACTION (69)

The Maillard reaction is actually a complex group of many reactions, its main feature being that the amino acids, peptides, and proteins condense with sugars and act as a catalyst for the reaction steps and form the dark brown colored melanoids. Condensation occurs as the food is heated and/or dehydrated.

Many different types of basic amino compounds can condense with the acyclic carbonyl forms of sugars, amino acids, peptides, and basic amino groups of proteins when the pH of the adjacent medium rises above the isoelectric point of the amino compound. An exposed amino group of lysine residue in proteins reacts like a separate amine.

Some melanoids are soluble in water and aqueous alcohol, while others are not. Their compositions and molecular weight vary according to the reactants and reacting conditions. Usually, they show reductone reducing power, even after dialysis, and antioxidant activity toward linoleic acid in strongly browned foods and dark beers.

The chemistry of the Maillard reaction of sugars with proteins is the least understood of all types of browning reactions. Presumably the sugar condenses, enolizes and is dehydrated, but how it forms strong bonds with protein remains to be determined.

Studies involving the heating of purified proteins in the presence of reducing sugars have shown that percent losses of lysine from globulin proteins are greater than that from gluten proteins (containing much less lysine).

The inhibition of Maillard browning reactions is best accomplished by keeping the pH below the isoelectric points of the amino acids, peptides, and proteins; by reducing the concentration of the reactants (for example, by diluting with water); and by keeping temperatures as low as possible during storage and processing such as freeze-drying (97). As the Maillard reactions vary more sharply in rate with changing temperatures than does the drying rate, the least amount of melanoids are formed for a given amount of water removal when freeze-drying is used (97). Freeze-drying also inhibits the formation of the melanoids because of the relatively rapid transition from a fully hydrated condition to a low moisture (97).

APPENDIX 5

KJELDAHL METHOD FOR DETERMINATION OF NITROGEN CONTENT OF MEAL SAMPLES

Ten millilitres[1] of the dissolved protein supernatant were mixed with 0.7 g of HgO, 15 g of powdered K_2SO_4, and 25 ml concentrated H_2SO_4 in a 500 ml Kjeldahl flask. A small funnel was put on the flask's mouth to act as a condenser. The HgO and the K_2SO_4 have a catalytic effect on the digestion of the meal components by H_2SO_4, thus reducing the time required for the digestion. The flask was then heated to boiling in an inclined position, on a Kjeldahl heating rack, until the contents became clear. After the clearing of the solution, heating was continued for at least 30 min. The concentrated sulfuric acid converts the nitrogen of the protein into ammonia which is fixed by the excess acid as ammonium sulphate. The flask was cooled and about 200 ml of distilled H_2O were added. The contents were further cooled to about 25°C and transferred quantitatively to a 1 L volumetric flask. Distilled water was then added to the 1 L mark.

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[1] or a known weight of the meal or meal solids.

APPENDIX 6

Table 4.1

EFFECT OF TEMPERATURE AND pH ON PROTEIN SOLUBILITY AND COLOR OF MEAL

pH		Temp °C	Dissolved Nitrogen **			Color * of the meal after shaking
Initial	Final		mg/g Meal	SD* mg/g meal	% of original+	
0.12	0.19	15	22.5	(2) 0.28	29.5	yellowish green
0.55	0.48	15	20.4	(2) 0.11	26.8	yellowish green
0.56	0.70	15	20.8	(3) 0.63	27.4	yellowish green
1.26	0.98	15	19.1	(2) 0.00	25.1	pale yellow
1.20	1.38	15	16.6	(2) 0.21	21.8	light grey
1.34	1.71	15	13.6	(2) 0.00	17.9	pale grey
1.63	2.24	15	10.5	(2) 0.03	13.8	pale grey
1.74	2.35	15	10.1	(3) 0.36	13.3	yellow
1.85	2.88	15	11.0	(2) 0.03	14.4	yellow
1.91	3.63	15	10.3	(3) 0.85	13.5	yellow

TABLE 4.1 (cont'd)

pH		Temp °C	Dissolved Nitrogen **			Color * of the meal after shaking
Initial	Final		mg/g Meal	SD* mg/g meal	% of original+	
1.95	3.72	15	10.3	(3) 0.45	13.5	yellow
2.00	4.26	15	9.6	(2) 0.08	12.6	yellow
2.24	4.63	15	9.3	(3) 0.16	12.2	yellow
2.53	4.92	15	10.1	(2) 0.11	13.3	yellow
3.14	5.61	15	11.9	(2) 0.08	15.6	yellow
3.78	5.84	15	12.7	(2) 0.00	16.7	yellow
4.25	5.97	15	13.0	(2) 0.24	17.1	yellow
6.02	6.09	15	12.6	(3) 0.19	16.5	yellow
10.90	8.08	15	16.3	(2) 0.11	21.4	yellow
11.66	10.74	15	24.9	(2) 0.00	32.7	pale green
12.43	12.46	15	29.5	(4) 0.84	38.8	greenish yellow

TABLE 4.1 (cont'd)

pH		Temp °C	Dissolved Nitrogen **			Color * of the meal after shaking
Initial	Final		mg/g Meal	SD* mg/g meal	% of original+	
0.78	0.91	25	24.7	(4) 0.53	32.4	grey
1.17	1.38	25	21.1	(3) 0.23	27.8	grey
1.96	4.30	25	11.1	(3) 0.19	14.6	yellow
2.32	5.11	25	11.3	(2) 0.11	14.8	yellow
2.73	5.72	25	12.4	(3) 0.06	16.3	yellow
3.66	6.10	25	13.1	(2) 0.00	17.2	yellow
4.98	6.16	25	13.1	(3) 0.18	17.2	yellow
5.69	6.17	25	13.2	(2) 0.04	17.4	yellow
5.82	6.17	25	13.2	(3) 0.10	17.4	yellow
10.78	6.50	25	14.8	(2) 0.07	19.4	yellow
11.83	9.80	25	19.7	(2) 0.77	25.8	light green
13.02	12.97	25	25.5	(2) 0.14	33.5	dark green

TABLE 4.1 (cont'd)

pH		Temp °C	Dissolved Nitrogen **			Color * of the meal after shaking
Initial	Final		mg/g Meal	SD* mg/g meal	% of original+	
1.30	1.13	35	29.7	(2) 0.00	39.0	grey
1.00	1.38	35	30.6	(2) 0.25	40.2	pale grey
2.00	4.13	35	15.9	(2) 0.07	20.9	yellow
2.20	5.12	35	15.2	(2) 0.00	19.9	pale yellow
3.02	5.87	35	17.1	(2) 0.14	22.5	pale green
5.50	6.10	35	17.3	(2) 0.00	22.8	pale green
11.50	7.31	35	18.5	(2) 0.00	24.3	yellow
13.05	13.30	35	32.5	(3) 0.61	42.7	brownish green

TABLE 4.1 (cont'd)

pH		Temp °C	Dissolved Nitrogen **			Color * of the meal after shaking
Initial	Final		mg/g Meal	SD* mg/ meal	% of original+	
0.98	0.98	45	32.7	(2) 0.14	42.9	reddish yellow
0.99	1.08	45	31.8	(2) 0.28	41.8	reddish yellow
1.23	1.39	45	31.8	(2) 0.28	41.8	grey
1.43	1.74	45	31.4	(2) 0.14	41.3	grey
1.72	2.09	45	23.3	(2) 0.00	30.6	grey
1.78	3.06	45	22.0	(5) 0.62	28.9	yellow
2.22	4.55	45	21.5	(3) 0.23	28.3	yellow
2.74	5.47	45	21.5	(4) 0.29	28.3	yellow
3.20	5.77	45	21.0	(2) 0.18	27.6	yellow
5.84	5.95	45	20.8	(2) 0.18	27.4	yellow
11.65	7.65	45	20.6	(2) 0.11	27.0	greenish yellow
11.90	8.98	45	33.1	(2) 0.11	43.5	green
12.20	11.05	45	33.1	(2) 0.14	43.5	brownish green
12.53	12.13	45	38.0	(2) 0.39	50.0	brownish green
12.98	12.82	45	34.9	(2) 0.00	45.8	brownish green

TABLE 4.1 (concluded)

pH		Temp °C	Dissolved Nitrogen **			Color * of the meal after shaking
Initial	Final		mg/g Meal	SD* mg/g meal	% of original+	
0.95	1.05	55	36.3	(2) 0.00	47.7	grey
1.52	2.07	55	30.3	(3) 0.32	39.8	grey
1.66	2.68	55	26.8	(3) 0.34	35.2	yellow
1.92	4.10	55	27.4	(2) 0.25	36.0	yellow
2.13	4.85	55	26.1	(2) 0.25	34.3	yellow
2.33	5.18	55	25.5	(2) 0.00	33.5	yellow
2.63	5.56	55	24.9	(2) 0.00	32.7	yellow
5.71	5.95	55	25.5	(2) 0.11	33.5	yellow
11.20	6.36	55	24.1	(3) 0.35	31.6	yellow
11.71	7.90	55	24.7	(2) 0.11	32.4	greenish yellow
12.60	12.30	55	42.3	(2) 0.21	55.5	grey
13.20	13.17	55	41.1	(3) 0.96	54.0	dark green

* Original color of the meal is yellow
** Original nitrogen content is 76.10 mg meal (wet basis)

$$+ = \frac{\text{Dissolved nitrogen (mg/g meal)}}{\text{Original nitrogen content (mg/g meal)}} \times 100$$

Example:
At a temperature of 55°C and a final pH of 5.18, the dissolved nitrogen was 25.55 mg/g meal. Thus:

$$\text{Dissolved nitrogen, \% of original} = \frac{25.55}{76.19} \times 100 = 33.51\%$$

#SD of electrode measurements. The weight of the meal is on wet basis.

APPENDIX 7

NITROGEN CONTENT OF MEAL

Meal Weight mg	ΔE mV	Q	Nitrogen content meal, mg*	Nitrogen content meal, %**
0.17651	-11.8	0.135	13.5	7.65
0.22755	-9.4	0.171	17.1	7.52
0.40666	-4.7	0.312	31.2	7.67

* Q X 100. The 100 is a factor obtained from the ammonia electrode manual.

** Wet basis

The mean of the nitrogen content in the meal is 7.61% and the coefficient of variation is 1.1%.

APPENDIX 8

DETERMINATION OF TOTAL GLUCOSINOLATES (AS ITC & OZT) BY THE ULTRA-VIOLET SPECTROPHOTOMETERY

The procedure followed in this study for the determination of the glucosinates as their hydrolysates, ITC and 02T, is described in the literature (16,232,233,235). Briefly, in this procedure, 100 mg of the heated meal was weighed into a 4 ml screw-cap culture tube containing a 6 mm glass bead. Two ml of methylene chloride, followed by 15 ml of distilled water was added to the tube. The contents of the vial were mixed with the aid of a 6 mm glass bead in a rotary shaker for 2 h at 23°C. Afterwards, the emulsion was broken by centrifugation at 2500 rpm. Fifty μL of the methylene chloride extract was added to 3 ml of 20% (by volume) ammoniacal ethanol in a culture tube. The ammonia is used to convert the ITC to their thioureas. The tube was capped and the mixture was then heated for 2 h at 50°C in a water bath. After cooling, the ultraviolet optical density (OD) of the solution was determined by scanning it in a double beam UV spectrophotometer. The spectrophotometer used was Bausch & Lomb's model, Spectronic 210. The setting of the spectrophotometer was 0.1 volts full scale, and the ABS range from 0 to 2. The spectrophotometer was connected

to a Fisher chart recorder, Recordall Series 5000. The speed of the chart was 5 cm/min. The maximum absorbance of both the OZT and the thioureas is in the opt. al density of 245 nanometers (nm). The ITC themselves do not exhibit an appreciable optical density at 245 nm. Thus, it is possible to measure only the amount of OZT, if the ethanol used did not contain ammonia. By subtracting this value for OZT from that of ITC and OZT together, it is possible to obtain the amount of ITC.

The UV procedure was abandoned in favor of the modified Youngs' GLC method developed during this investigation (see Chapter 3.3.6). This was done because of low concentrations of the ITC and OZT to be measured and because the presence of other compounds interfered with the measurements. An example of this encountered interference is shown in Figure A.8, where an interfering peak, almost as intense as that to be measured, appears at the wave length of 225 nm. The interfering substances in the UV spectroscopic measurements might have been the seed protein (50). It is of interest to mention that Blake and Marianchuck (38) indicated that the UV spectroscopy is not suitable for rapeseeds that contain low levels of glucosinolates, such as the Tower variety used in this investigation.

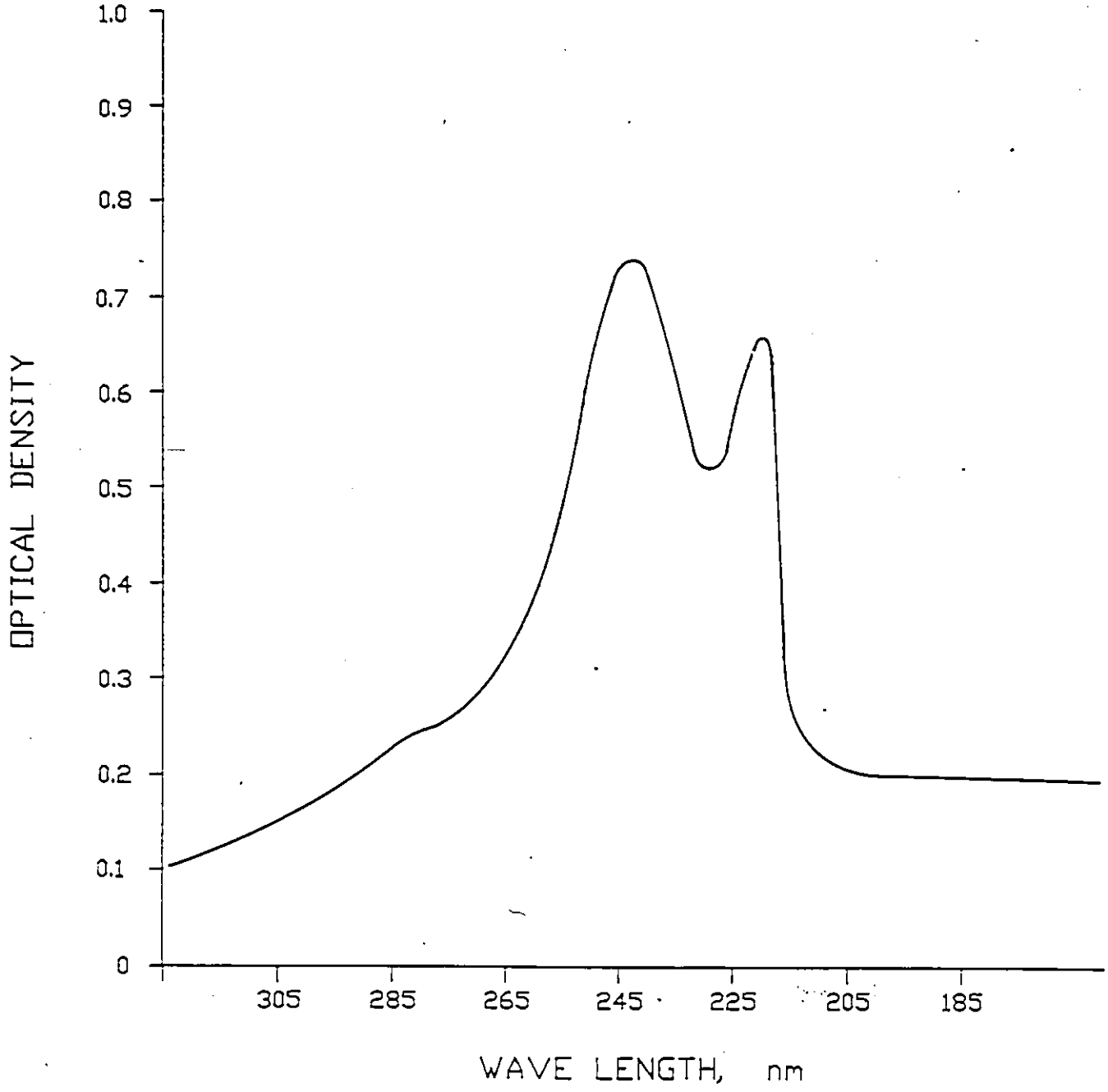


Figure A.8 Ultra Violet SPECTROGRAM of ITC (as thioureas) and OZT

APPENDIX 9

FORMATION OF ITC FROM HYDROLYSIS OF GLUCOSINOLATES OF RAPESEED AT 5°C AND 15°C

The formation of the ITC was used as an indicator of the progress of the hydrolysis of the glucosinolates. The range studied was between ½ and 5 h. The hydrolysis W/M ratio was 12:1, as that for the runs carried out a 5 and 15°C in the experimental design presented in Chapter 5.3. Thus, 0.5 g of meal, 2 ml of methylene chloride, and a 6 mm glass bead were placed in each of five culture tubes. The temperature of the added methylene chloride was the same as that for the experiment to be carried out. The tubes were then shaken for 10 min in a rotary shaker immersed in a water bath. The temperature of the water bath was controlled to the temperature of the run.

Afterwards, 6 ml of distilled water, also at a temperature of 5°C, were added to each tube. The shaking was then continued. At each time interval, a tube was removed and then centrifuged at 2500 rpm. The methylene chloride extract was separated from the aqueous solution as a result of the centrifugation. The methylene chloride

extract layer was removed from the tube by a long-needled syringe and transferred to a 4 ml vial. The ITC content of the extract was determined by the GLC method (15). Details of this method are in Chapter 3.

The results for 5°C are presented in Table A.9.1; results for 15°C in Table A.9.2 and depicted in Figure A.9.

TABLE A.9.1 FORMATION OF ITC FROM HYDROLYSIS OF GLUCOSINOLATES AT 5°C

TIME h	BIT mg/g meal	PIT mg/g meal	ITC* mg/g meal	SD mg/g meal
½	0.0983	0.0049	0.1032 (3)	3.2×10^{-4}
1	0.1944	0.0097	0.2041 (4)	4.1×10^{-3}
2	0.3054	0.0150	0.3204 (4)	4.7×10^{-3}
3	0.3378	0.0168	0.3546 (2)	1.9×10^{-3}
4	0.3558	0.0186	0.3744 (2)	5.6×10^{-4}
5	0.3480	0.0193	0.3673 (2)	7.8×10^{-4}

* ITC = BIT + PIT

TABLE A.9.2 FORMATION OF ITC FROM HYDROLYSIS OF GLUCOSINOLATES AT 15°C

TIME h	BIT mg/g meal*	PIT mg/g meal*	ITC** mg/g meal*	SD mg/g meal*
½	0.1727	0.0081	0.1808 (3)	3.10×10^{-3}
1	0.2849	0.0139	0.2988 (2)	0.42×10^{-3}
1½	0.3527	0.0171	0.3698 (2)	0.45×10^{-3}
2	0.3861	0.0189	0.4050 (2)	0.56×10^{-3}
2½	0.3925	0.0195	0.4120 (2)	0.99×10^{-3}
3	0.3975	0.0207	0.4182 (2)	4.81×10^{-3}

* Dry weight

** ITC = BIT + PIT

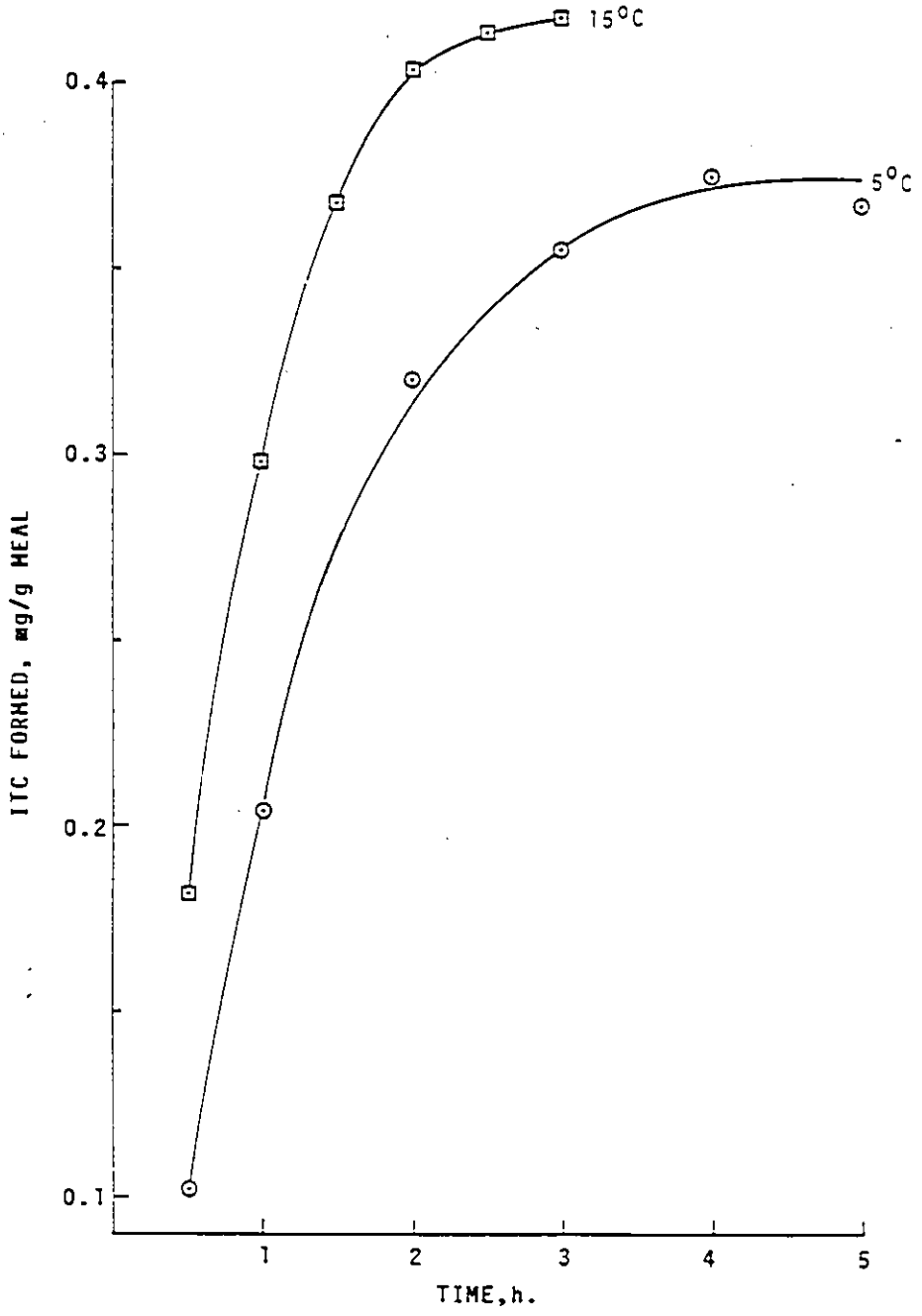
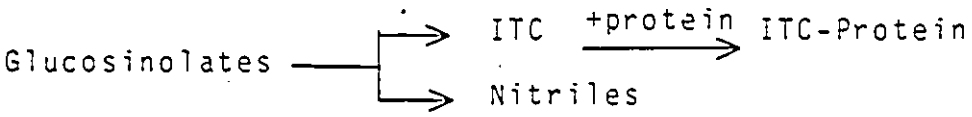


Figure. A.9 FORMATION OF ITC FROM HYDROLYSIS OF GLUCOSINOLATES AT 5°C and 15°C, AS FUNCTION OF TIME.

APPENDIX 10

PRELIMINARY INVESTIGATION ON THE EFFECT OF WATER-TO-MEAL RATIO

As previously mentioned, the two groups of aglucons of concern in this study, which are formed from the enzymatic hydrolysis of the glucosinolates, are the nitriles and the ITC (OZT are cyclized ITC). Also, the ITC react wnth the meal protein. This hydrolysis and the ITC reaction with the protein could be represented by the following simplified chemical equation:



The strategy needed in the research work presented in Chapter 5.3 (Neutral Meduim Method), was to maximize the amount of the ITC present in the meal. That is to say, the hydrolysis of the glucosinolates would give a high yield of ITC with the amount of the formed nitriles and the ITC-protein being minimal.

The literature search indicated that during the hydrolysis of the glucosinolates, an increase in the W/M ratio would increase the amount of the ITC formed (222, 225). Also, as can be seen in the above equation, the nitriles are competing reaction products with the ITC. Thus, this increase in the amount of the ITC would reduce the amount of the nitriles formed.

Furthermore, it was expected that the increase in the W/M ratio would reduce the rate of the reaction between the ITC and the protein. This is because of the concentration of the reactants is reduced by the high ratio.

EXPERIMENTAL:

A preliminary investigation was undertaken to determine whether the increase in the W/M ratio could achieve the desired ITC maximization. Hence, the effect of increasing the W/M ratio to 20:1 from 10:1 on the amount of the free ITC (not bound to the meal's protein) in the meal was studied. The investigation was carried out in duplicate, at a temperature of 25°C and at hydrolysis times in the range of 5-25 min. Experiments were not carried out below 5 min because it was felt that longer periods were

needed for the hydrolyzing water to diffuse into the seed and for the initiation period of the hydrolysis of the glucosinolates.

The upper limit of 25 min was adopted, because (as was found in Chapter 5.2) it allows a substantial hydrolysis of the glucosinolates. For example, from Table 5.2, at a W/M ratio of 5:1 and a time of 15 min, about 80% of the hydrolysis was completed. Furthermore, at the same ratio and at the time of 30 min, the hydrolysis progressed to about 95%.

The experiments were carried out in culture tubes. One half gram of meal (wet basis) was weighed into each of the tubes. A 6 mm glass bead and the desired amount of distilled water, at a temperature of 25°C, were added to the meals. The tubes were quickly sealed with their Teflon lined caps and shaken by a rotary shaker which was immersed in a water bath maintained at 25°C.

The shaking was maintained for the time during which part of the formed ITC would have reacted with the meal protein. Then 2 ml of methylene chloride, containing 40 mg/L butylisothiocyonates as a marker, was added to the tube to extract the formed ITC and to prevent it from further reacting with the meal protein. Shaking was

continued for up to a total time of 2 h (timed from the moment of the addition of water).

Afterwards, the methylene chloride was separated from the liquid mixture by centrifugation at 2500 rpm. The methylene chloride layer in each tube was removed with a long-needled syringe and placed in a 4 ml vial which was then capped with polyethylene caps.

The ITC content extracted by the methylene chloride was determined by injecting 1 μL of the solvent into a gas chromatograph (GC). The conditors of the GC were the same as those used in the modified analysis presented in Chapter 3.3.6.

The experiments were repeated and the duplicated results are presented below in Table A.10.a for the ratio 10:1, and A.10.b for the ratio 20:1. The amount of ITC in these two tables is presented in Figure A.10. This figure also includes the amount of ITC at time 0 for the ratio 10:1. This is the amount of ITC in the meal as determined in Appendix 13 for the ratio 10:1.

TABLE A.10

FREE ITC AS A FUNCTION OF HYDROLYSIS TIME AT THE TEMPERATURE OF 25°C

(a)

W/M Ratio 10:1

TIME min	BIT mg/g meal		PIT mg/g meal		ITC** mg/g meal		W^+ mg/g meal	W^2	ITC mg/m meal
	D_1*	D_2	D_1	D_2	D_1	D_2			
5	0.3137	0.3174	0	0.0068	0.3137	0.3242	0.0105	1.102×10^{-4}	0.3189
10	0.1951	0.1993	0	0	0.1951	0.1993	0.0042	1.764×10^{-5}	0.1972
15	0.1216	0.1227	0	0	0.1216	0.1227	0.0011	1.210×10^{-6}	0.1222
20	0.1045	0.1023	0	0	0.1045	0.1023	0.0022	4.840×10^{-6}	0.1034
25	0.0792	0.0809	0	0	0.0792	0.0809	0.0017	2.890×10^{-6}	0.0800

Standard deviation (SD)++ = 3.698×10^{-3} mg/g meal.

---

* D_n is the duplicate number n

** ITC = BIT + PIT

\+ $W = |D_1 - D_2|$ ITC

++ Calculated from the formula:

$$SD = \sqrt{\frac{\Sigma W^2}{2K}} \qquad (148)$$

K is the number of measured samples (5 in this case).

TABLE A.10

FREE ITC AS A FUNCTION OF HYDROLYSIS TIME AT THE TEMPERATURE OF 25°C

(b)

W/M Ratio 20:1

TIME min	BIT mg/g meal		PIT mg/g meal		ITC** mg/g meal		W^+ mg/g meal	W^2	ITC mg/m meal
	D_1*	D_2	D_1	D_2	D_1	D_2			
5	0.3320	0.3141	0.0058	0.0083	0.3278	0.3224	0.0054	2.916×10^{-5}	0.3251
7.5	0.2799	-	0	0	0.2799	-	-	-	-
10	0.2271	0.2205	0	0	0.2271	0.2205	0.0066	4.356×10^{-5}	0.2238
15	0.1506	0.1472	0	0	0.1508	0.1472	0.0034	1.155×10^{-5}	0.1489
20	0.1060	0.1099	0	0	0.1060	0.1099	0.0039	1.521×10^{-5}	0.1079

Standard deviation $(SD)^+ = 3.526 \times 10^{-3}$ mg/g meal.

---

* D_n is the duplicate number n

** ITC = BIT + PIT

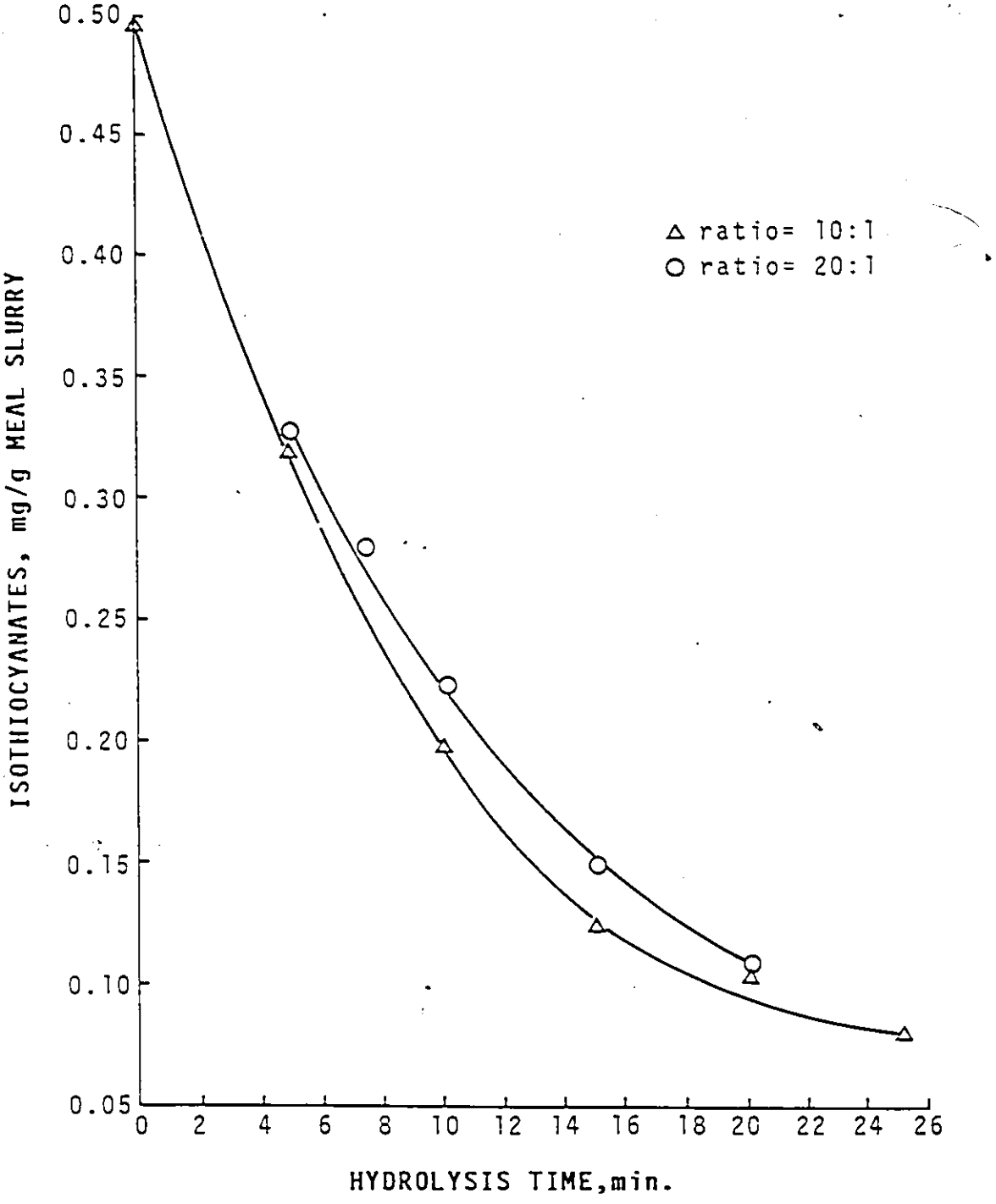
\+ $W = |D_1 - D_2|$ of ITC

++ Calculated from the formula:

$$SD = \sqrt{\frac{\Sigma W^2}{2K}} \qquad (148)$$

Where K = the number of measured samples (4 in this case).

Figure. A-10. AMOUNT OF ITC in MEAL SLURRY v.s.HYDROLYSIS TIME.



From the tables and figure, it can be noticed that for the hydrolysis times of 10 and 15 min, the amount of ITC for the W/M ratio of 20:1 is higher than for the ratio 10:1. The same can also be noticed for the hydrolysis times of 5 and 20 min. However, the difference is not significant, possibly because the difference between the two ratios was not large enough to have a substantial effect (217,225). In spite of this, these preliminary investigations point out that it might be possible to increase the amount of free ITC in the meal by increasing the W/M ratio.

APPENDIX 11

DESCRIPTION OF THE DEEP-FREEZER

The deep-freezer used in this study was co-designed by the author and Mr. J. Gasperetti of the University of Ottawa. The freezer was subsequently manufactured at the same university by Messrs. J. Gasperetti, D. Roy, A. Bonaldo and D. Lefebvre.

The freezer is schematically shown in Figure A.11. As can be seen in this figure, the freezer was set up on a cart having two shelves. These shelves were made of wood with dimensions of 76 x 56 x 1.5 cm. The bottom and top shelves were at a distance of 20 cm and 64 cm from the ground, respectively. A 1/4 hp compressor unit powered by 115 volts AC electricity (made by Tecumseh, Model CAQT 1211K) was placed on the bottom shelf. The refrigeration gas used for this compressor was Freon 12.

The deep-freezer (a 2.5 cm thick stainless steel chamber) was placed on the top shelf. The inside dimensions of the chamber were 56 x 29 x 1.5 cm. The chamber was insulated on the sides and on the bottom with 2.5 cm styrofoam. The removable top cover of the chamber was of

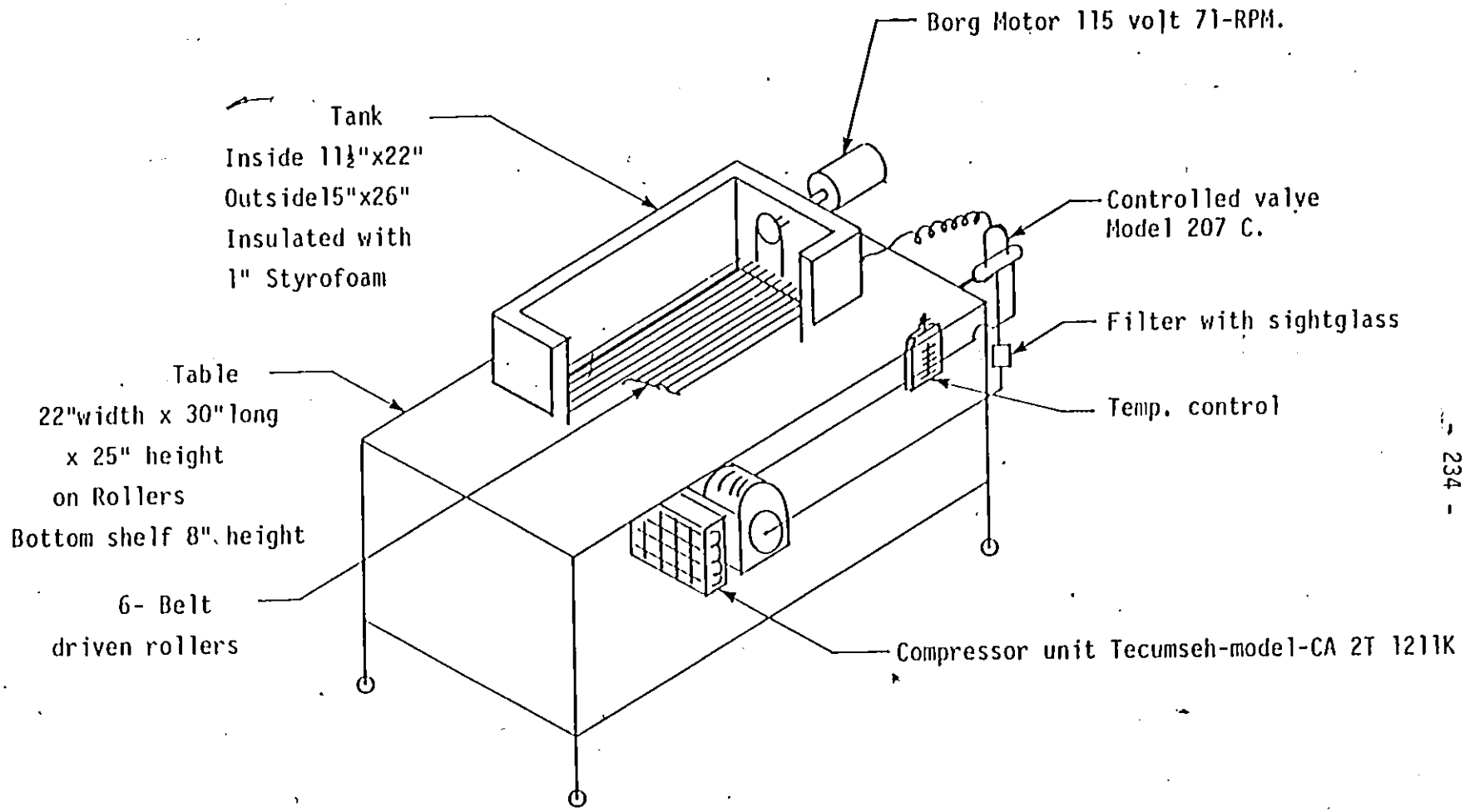


Figure. A.11. SCHEMATIC DIAGRAM OF THE DEEP-FREEZER.

double-walled plexiglass. On the floor of the chamber, there were six bronze rollers on a bronze bearing support. The rollers were 3 cm in diameter and 50 cm in length. The rollers were driven at a speed of 71 rpm, via belts, by a Borg motor powered by 115 volts AC electricity. This roller arrangement was to be used in freezing materials in bottles. The bottles would be laid horizontally on the turning rolls. The frozen material would thus form on a large surface and facilitate the evaporation of its water. The roller arrangement also served to support a double-surface plexiglass tray. On this tray, containers other than bottles could be placed. Culture tubes and vials could be held by this tray by securing them in holes in the top surface of the tray. The solvent medium in the chamber was methyl alcohol.

The temperature of the solvent could be brought down to -50°C by circulating Freon gas cooled in the compressor through a copper serpentine immersed in the solvent. Prior to passing through the serpentine, the Freon gas went into a filter with a sight glass and then to a control valve. The valve controlled the flow of the Freon gas to bring the temperature of the solvent to the setting on the temperature controller.

A thermocouple immersed in the solvent gave a feed-back control signal to the valve. It was possible, by this control system, to adjust the temperature of the solvent to the desired temperatures down to -50°C.

This deep-freezer operated reasonably well during this study.

APPENDIX 12

AMINO ACID ANALYSIS OF TOWER RAPESEED MEAL

The amino acid analysis of the meal protein was carried out according to the method of Spackman et al. (196). Thus, two duplicate meal samples of 10 to 15 mg each were weighed into 13 x 100 mm test tubes. The samples were then mixed in the tubes with 1 ml of constantly boiling hydrochloric acid. Four drops of 5% phenol were added to the mixture to prevent the oxidation of the amino acids during the acid hycholysis of the protein. The contents of the tubes were then frozen by placing the tubes in liquid air. Oxygen in the tubes was removed by flushing them three times with nitrogen. Afterwards, the tubes were sealed and then heated in a rotary oven at a temperature of 110° C. After 22 h of heating, the tubes were opened and placed in a desiccator. The acid in the tubes was evaporated by applying vacuum to the desiccator. The contents of the tubes were dissolved in 5.0 ml of 0.2 N sodium citrate buffer (pH 2.2) and transfered to 5 ml volumetric flasks.

The buffer contained norleucine at a concentration of 0.0787 g/L. The norleucine acted as an internal standard for the meal's amino acid determination. The sample solutions were filtered and stored frozen in a deep-

freezer. Prior to analysis, the samples were melted and diluted. The dilution was needed to give good readings on the analyser's integrator.

One-half ml of each of the filtered sample solutions was injected into a Beckman Model 120 °C Amino Acid Analyzer. The amino acids were separated and eluted in the Analyzer on a single jacketed column. The dimensions of the column were 460 x 6 mm (inside diameter). The column was filled to a height of 339 mm with Amenex A-9 cation exchange resin beads size 11 ± 0.5μ manufactured by Beckman Instruments. The amino acids were eluted with a successive series of three buffers A, B, and C (compositions and specifications are in Table A-12.a). The flow rates of the three buffers were the same at 34 ml/h. The temperature of the buffers was controlled at 55°C by passing hot water through the column's jacket.

Buffer A was used first until the elution time of 85 min, after which it was replaced by buffer B. This time for buffer A allowed the following amino acids to elute: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, and cystine. Buffer B eluted methionine, isoleucine, leucine, norleucine, tyrosine, and

TABLE A-12.a BUFFERS' COMPOSITION

Buffer	A	B	C
Na citrate, g	78.44	78.44	138.28
NaCl, g	-	-	549.08
Phenol, g	4.0	4.0	4.0
Isopropanol, ml	80.0	80.0	160.0
Thiodiglycol, ml	10.0	10.0	-
Benzyl Alcohol, ml	-	40.0	-
Caprylic Acid, ml	0.4	0.4	0.4
Constant Boiling HCl, ml	110.0	50.0	100.0
pH	3.10	4.00	5.32
Na Concentration, N	0.2	0.2	1.40

Buffers were prepared by dissolving the compounds in the order given and made up to 4 L.

phenylalanine. Eleven minutes before the change from buffer B to buffer C, which occurred at an elution time of 120 min, the temperture of the water passing through the jacket was raised to 60°C from the initial 55° C. The temperature was increased to facilitate the separation of tyrosine from phenylalanine. The change from buffer B to buffer C occurred at an elution time of 131 min. The 60°C column temperature was also maintained during the elution, with buffer C, of the remaining amino acids lysine, histidine, and arginine.

After the elution of the last amino acid at a time of 209 min, the flow of buffer C was stopped and the column was regenerated with 0.2N NaOH solution which contains 0.25% EDTA for 4 min.

The column was then re-equilibrated by reducing its temperature back to 55°C and by passing buffer A for 41 min before the subsequent analysis. The total time of analysis (which includes a 20 min delay period) and regeneration was 276 min.

The concentration of each eluted amino acid was determined colorimetrically. The color was developed by reacting the amino acids with ninhydrin in a nanobore

reaction coil submerged in a boiling water bath. The intensity of the colors formed is directly related to the amount of the amino acids. The ninhydrin used was dissolved in a solution of sodium acetate in ethylene glycol monomethylether. Stannous chloride as an antioxidant was added to keep the ninhydrine in a reduced state, which is its reactive form. The flow rate of ninhydrin was 15 ml/h.

The intensity of the color formed was continuously measured, at 570 and 440 nm wavelength, with a single low-volume cuvette (pathlength 6+6 mm). The reference base line was produced by the integration. The colorimetric readings were recorded by a Honeywell chart recorder Model Electronic 16.

The amount of each eluted amino acid was integrated by a Beckman 125 integrator (model Systems AA Autolab). The integrator was programmed to have a delay period of 20 min from the start of the elution with buffer A.

A standard mixture of amino acids (supplied by Chromatographic Specialties of Brockville, Ont.) was used to calibrate the results of the analysis procedure.

Calculation of amount of each amino acids.

Amount of amino acid in nmole/mg meal =

$$\frac{\text{Integrated value (nmoles)}}{\text{Injected sample volume (ml)}}$$

$$\times \frac{\text{Total sample volume (ml) x dilution factor}}{\text{Weight of hydrolyzed sample (mg)}}$$

$$\times \frac{\text{Expected integrated value for norleucine (nmoles)}}{\text{Observed integrated value for norleucine (nmoles)}}$$

The last term of this equation is a correction factor obtained from the norleucine internal standard.

The values in this equation common to both duplicate analyses are the volume of the injected sample (0.5 ml), the total sample volume (5 ml), and the expected integrated value for norleucine (50 nm). The other values specific for each duplicate will be mentioned later while presenting their amino acid content.

The results of this duplicate amino acid analysis are presented in Table A-12.b.

Table A-12.b RESULTS OF AMINO ACID ANALYSIS OF TOWER RAPESEED MEAL

	Duplicate No.1	Duplicate No.2
Sample Weight, mg	12.84	18.0
Norleucine Correct Factor	$\frac{50.00 \text{ nmoles}}{65.94 \text{ nmoles}} = 0.7583$	$\frac{50.00 \text{ nmoles}}{53.21 \text{ nmoles}} = 0.9397$
Dilution Factor	5.24	5.25

Amino Acid	Elution Time, min	Integrated value, nmole	Amino acid, nmole/mg meal	Elution Time, min	Integrated value, nmole	Amino acid, nmole/mg meal	Average amino acid, nmole/mg meal
Aspartic Acid	34.8	5.75	25.63	33.4	6.27	24.76	25.19
Threonine	39.4	1.71	7.62	38.0	1.50	5.94	6.77
Serine	41.4	3.50	15.62	40.0	3.22	12.71	14.17
Glutamic Acid	48.5	10.58	47.15	48.7	9.50	37.49	42.31
Proline	52.5	1.45	6.46	52.5	1.27	5.00	5.74
Glycine	67.0	6.65	29.65	65.8	7.62	30.08	29.87
Alanine	72.1	5.28	23.54	70.8	6.25	24.66	24.12
Valine	92.7	4.29	19.10	91.4	5.00	19.73	19.42

Table A-12.b (cont'd)

	Duplicate No.1	Duplicate No.2
Sample Weight, mg	12.84	18.0
Norleucine Correct Factor	$\frac{50.00 \text{ nmoles}}{65.94 \text{ nmoles}} = 0.7583$	$\frac{50.00 \text{ nmoles}}{53.21 \text{ nmoles}} = 0.9397$
Dilution Factor	5.24	5.25

Amino Acid	Elution Time, min	Integrated value, nmole	Amino acid, nmole/mg meal	Elution Time, min	Integrated value, nmole	Amino acid, nmole/mg meal	Average amino acid, nmole/mg meal
Cystine	100.4	0.98	4.37	100.2	0.95	3.76	4.07
Methionine	103.2	1.16	5.18	102.9	1.39	5.48	5.34
Isoleucine	108.9	2.86	12.74	108.7	3.19	12.60	12.67
Leucine	111.7	5.17	23.04	111.5	5.98	23.60	23.32
Tyrosine	121.0	1.73	7.704	120.7	1.91	7.55	7.63
Phenylalanine	124.3	2.38	10.57	124.0	2.69	10.63	10.61
Lysine	171.5	4.02	17.90	170.8	4.05	16.01	16.96
Histidine	174.2	1.42	6.35	173.9	0.71	2.81	4.58
Arginine	193.2	3.85	17.15	193.1	4.27	16.87	17.02
Total			279.79			259.68	269.77

APPENDIX 13

CALCULATION OF THE AMOUNT OF INDIVIDUAL ISOTHIOCYANATES OBTAINED FROM THE GAS CHROMATOGRAM

The amount of each individual isothiocyanate was calculated from the following expression:

$$\text{Isothiocyanate content (mg/g meal)} = \frac{A_2}{A_1} \times \frac{N_1}{N_2} \times C \times V \times \frac{1}{M}$$

where:

A is the integrated area of the isothiocyanate's peaks (mv)

N is the number of carbon atoms in the alkyl part of the isothiocyanates (4 for butyl- and butenyl- and 5 for pentenyl-)

C is the concentration of the butyl isothiocyanate in the internal standard (0.04 mg/ml)

V is the volume of methylene chloride (2 ml)

M is the weight of the meal (0.1 g)

the subscripts 1 and 2 denote internal standard and sample, respectively.

A sample calculation is presented below for the results obtained in Figure 3.1.

$$\text{amount of BIT, mg/g meal} = \frac{49950}{28880} \times \frac{4}{4} \times 0.04 \times 2 \times \frac{1}{0.1} = 1.3837$$

$$\text{amount of PIT, mg/g meal} = \frac{2647}{28880} \times \frac{4}{5} \times 0.04 \times 2 \times \frac{1}{0.1} = 0.0587$$

These results, in addition to two other repeats, are tabulated below.

	BIT (mg/g meal)	PIT (mg/g meal)	ITC* (mg/g meal)
Replica #1	1.3837	0.0587	1.4424
Replica #2	1.4196	0.1503	1.5699
Replica #3	1.4102	0.1378	1.5480
		Sum =	4.5603
		Mean (x) =	1.5201

Standard deviation (SD) = 0.0682 mg/g meal

* ITC = BIT + PIT

The determination of formed ITC from the hydrolysis of the meal's glucosinolates was also examined for the W/M ratio of 10:1. The procedure followed was the same as that for the ratio of 150:1, except the amount of meal used was 0.5 g instead of 0.1 g. The results are tabulated below.

	BIT (mg/g meal)	PIT (mg/g meal)	ITC* (X) (mg/g meal)
Replica #1	0.4763	0.0220	0.4983
Replica #2	0.4816	0.0215	0.5031
Replica #3	0.4905	0	0.4905
Replica #4	0.4915	0.0144	0.5059
Replica #5	0.4972	0	0.4972
		Sum =	2.4950
		Mean (x) =	0.4990

Standard deviation (S.D.) = 5.93×10^{-3} mg/g meal.

It can be seen from these two tables that the SD of the ITC determination is reasonably good. This indicates that the modified analysis method is reasonably accurate.

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* ITC = BIT + PIT

APPENDIX 14

Table 5.3

OPERATING LEVELS OF WATER-TO-MEAL RATIO, TIME, AND TEMPERATURE IN CODED FORM

	X_1 (Water-to-Meal Ratio)	X_2 (Time, min)	X_3 (Temp. °C)
Factorial Points	1	1.4	1
	1	1.4	-1
	1	-1	1
	1	-1	-1
	-1	1.4	1
	-1	1.4	-1
	-1	-1	1
	-1	-1	-1
Axial Points	2	0	0
	-2	0	0
	0	2	0
	0	-2	0
	0	0	2
	0	0	-2
Center Points (Replicas)	0	0	0
	0	0	0
	0	0	0
	0	0	0

APPENDIX 15

MASS SPECTROSCOPY OF THE ISOTHIOCYANATES

The ITC resulting from the hydrolysis of the glucosinolates of three different Tower rapeseed samples were prepared essentially in the same manner as MAP (described in Chapter 3.3.6). Briefly, the preparation conditions were a W/M ratio of 150:1, a time of 2 h, and a temperature of 23°C. The methylene chloride used to extract the ITC formed did not contain the internal standard butyl isothiocyanates because it was not needed. The methylene chloride extract, while being cooled, was reduced to between 5 and 10% of its volume under a nitrogen current.

The mass spectrometer used was a VG Analytical 7070E with a PDP8a data system. The ion source was 70 ev and its temperature was 200°C. The accelerating voltage was 6 Kv. The gas chromatograph used was a DANI 3800. The temperature of the injection port was 250°C. The injection was done directly into a 6 ft glass column with an internal diameter of 2 mm. The column was filled with 20% FFAP on 60/80 mesh chrom W/HP. Five μL of the concentrated methylene chloride extract was injected into the column. The initial temperature of the oven was 100°C and was

maintained for 2 min. The temperature was increased in increments of 10°C/min to 230°C; the oven was then cooled down to the initial temperature. A blank mass spectrum was obtained and background signals were accounted for when reporting the identified compounds. It should be noted that the identification of the compounds under discussion are tentative.

Identification of the But-3-enyl Isothiocyanate (Molecular Weight 113)

The mass spectra obtained for the gas chromatographic peak that was suspected to be but-3-enyl isothiocyanate had a molecular weight of 113. The mass spectra for this peak and the percent height (relative abundance) values of these spectra are presented in Figures and Tables A.15.1, A.15.2, and A.15.3, respectively. The available information in the literature about the mass spectra of but-3-enyl isothiocyanates (41,72,100,131) were used in the analysis of these figures and tables. From the mass spectra obtained, it can be concluded that the dominant cleavage is an allylic one, giving a base peak at m/e* 72 corresponding to the ion $CH_2=N^+=C=S$; an allylic ion at m/e 41 is also visible (relative abundance 4-6%). The

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* mass/charge

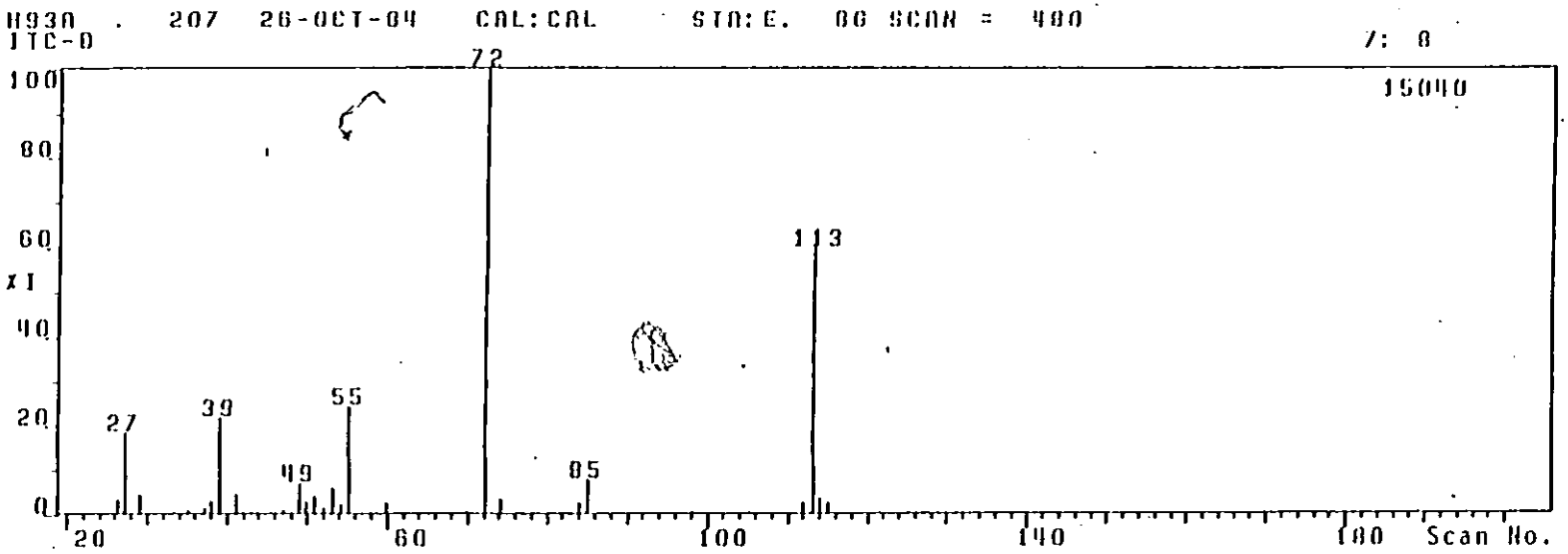


Figure. A.15.1 MASS SPECTRUM OF BUT-3-ENYL ISOTHIOCYANATE (Sample 1)

TABLE A.15.1

Mass Spectrum of But-3-enyl Isothiocyanate (Sample 1)

H93A.207 RT 0: 7: 8 26-OCT-84 TIC=799 AUTO GAIN=1000
CAL BASE INT.=15040 B/G SCAN=480 STATUS:1E
ITC-0

PEAK NO.	MASS	%HT. MOD.
2	26.3	3.12
3	27.2	18.24
5	29.0	4.00
12	35.1	0.63
14	37.1	1.03
15	38.0	2.73
16	39.0	21.53
19	41.0	4.26
25	47.1	0.82
27	48.9	6.57
28	49.9	2.49
29	50.9	3.78
30	52.0	1.03
31	53.1	5.55
32	54.1	1.91
33	55.2	24.14
39	59.9	2.21
49	69.9	0.45
52	72.0	100.00
54	74.1	2.90
60	84.0	2.05
61	85.1	7.33
66	98.0	0.50
70	112.0	2.40
71	113.1	59.73
72	114.1	3.51
73	115.1	2.36

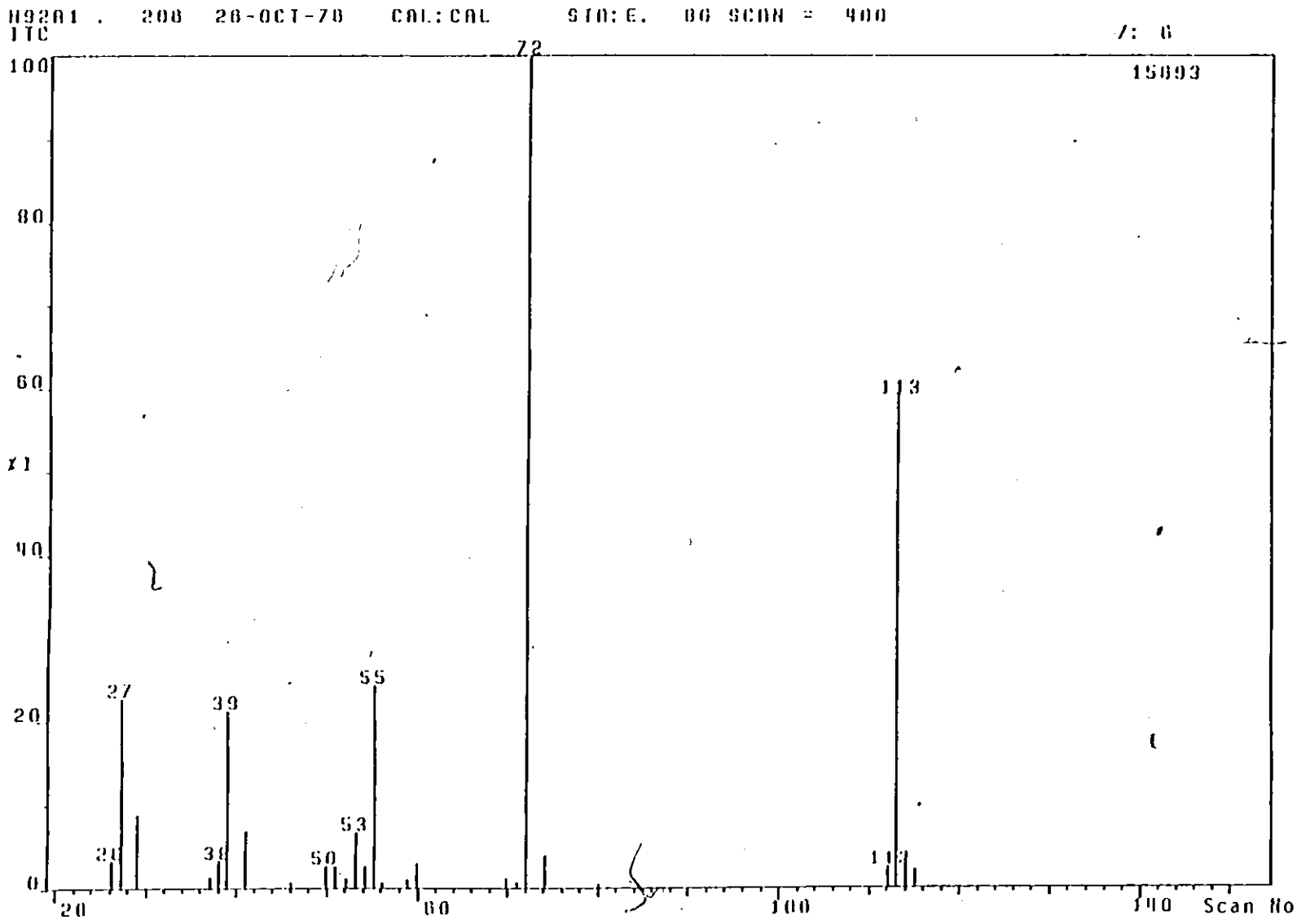


Figure. A.15.2 MASS SPECTRUM OF BUT-3-ENYL ISOTHIOCYANATE (Sample 2)

TABLE A.15.2

Mass Spectrum of But-3-enyl Isothiocyanate (Sample 2)

H92A1.206 RT 0: 7: 6 26-OCT-76 TIC=741 AUTO GAIN=100
CAL BASE INT.=15693 B/G SCAN=400 STATUS:1E
ITC

PEAK NO.	MASS	%HT. MOD.
2	26.3	3.26
3	27.2	22.63
5	29.0	8.74
12	37.1	1.22
13	38.0	3.21
14	39.0	21.19
17	40.9	6.84
22	46.1	0.67
26	49.9	2.51
27	51.0	2.57
28	52.0	1.18
29	53.1	6.54
30	54.1	2.64
31	55.1	24.14
32	56.1	0.66
36	58.9	0.96
37	59.9	2.94
46	69.9	1.12
47	71.0	0.66
48	72.0	99.99
50	74.1	3.94
54	80.0	0.48
58	85.1	7.74
65	112.0	2.40
66	113.1	59.01
67	114.1	3.82
68	115.1	2.15

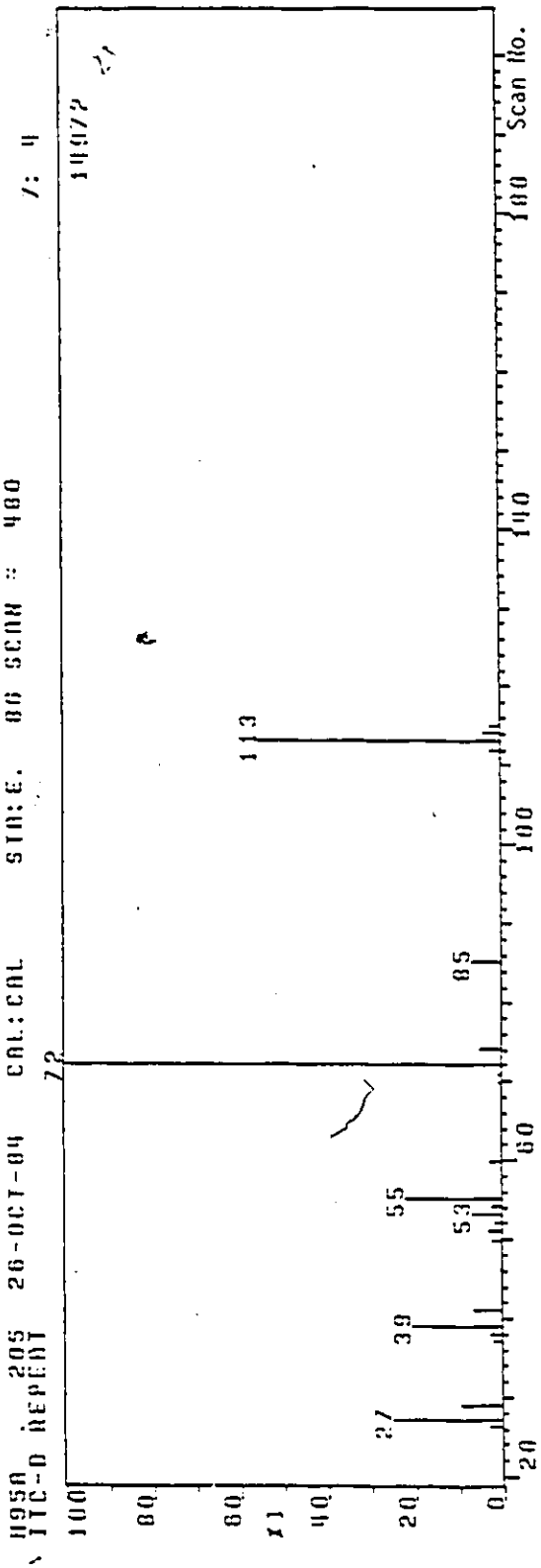


Figure. A.15.3. MASS SPECTRUM OF BUT-3-ENYL ISOTHIOCYANATE (Sample 3)

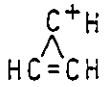
TABLE A.15.3

Mass Spectrum of But-3-enyl Isothiocyanate (Sample 3)

H95A.205 RT 0: 7: 4 26-OCT-84 TIC=710 AUTO GAIN=100
CAL BASE INT.=14972 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.
2	26.3	2.97
3	27.2	24.75
5	29.0	9.44
13	37.1	1.82
14	38.0	2.73
15	39.0	20.38
18	40.9	6.31
23	46.1	0.46
27	49.9	2.07
28	51.0	2.92
29	52.0	1.40
30	53.1	6.59
31	54.1	2.21
32	55.1	22.01
36	58.9	0.42
37	59.9	2.66
47	69.9	0.89
48	71.0	0.79
49	72.0	100.00
51	74.1	4.68
59	85.1	6.61
65	112.0	2.21
66	113.1	55.01
67	114.1	3.73
68	115.1	2.05

parent ion (M+), m/e 113 in agreement with the molecular formula $C_5H_7NS^+$, was the second most abundant species formed (rel. abund. 55-60%). Other important fragments having relative abundances of about 20% gave signals at m/e 27, 39, and 55, and are probably the vinyl ion ($CH_2=C^+H$), the cyclopropenyl ion



and the ion $CH_2=CH-C^+H-CH_3$, respectivly. The latter is presumably formed from the primary fragment $CH_2=CH-CH_2-C^+H_2$ via a 1,2 hydride shift to give a more stable allylic species. Others which were less abundant (relative abundance less than 10%) can be assigned the formulae of an ethyl ion ($CH_3-C^+H_2$, m/e 29), a butadienyl ion ($CH_2=CH-CH=C^+H$) and a vinyl isothiocyanate ion ($CH_2=CH-N=C=S^+$, m/e 85).

Therefore, based on this analysis, it is evident that the mass spectrum in discussion corresponds to the compound but-3-enyl isothiocyanate.

Identification of the Pent-4-enyl Isothiocyanates

The mass spectra of the pent-4-enyl isothiocyanates of three samples are shown in Figures and Tables

A.15.4, A.15.5 and A.15.6. In these Figures and Tables, it can be seen that the most abundant ionized fragment is the allyl ion (CH_2=CH-C^+H_2, m/e 41). Additional ions identified are the M^+ (m/e 127 relative abundance 21.97 to 39.16) and the vinyl isothiocyanate ion (CH_2=CH-N=C=S^+, m/e 85 relative abundance 15.18 to 31.61). These identified ionized fragments are characteristic of pent-4-enyl isothiocyanate (100). Other major ionized fragments appeared in the mass spectra of the three samples, which confirm the identity of the compound under study to the pent-4-enyl isothiocyanates tabulated below:\

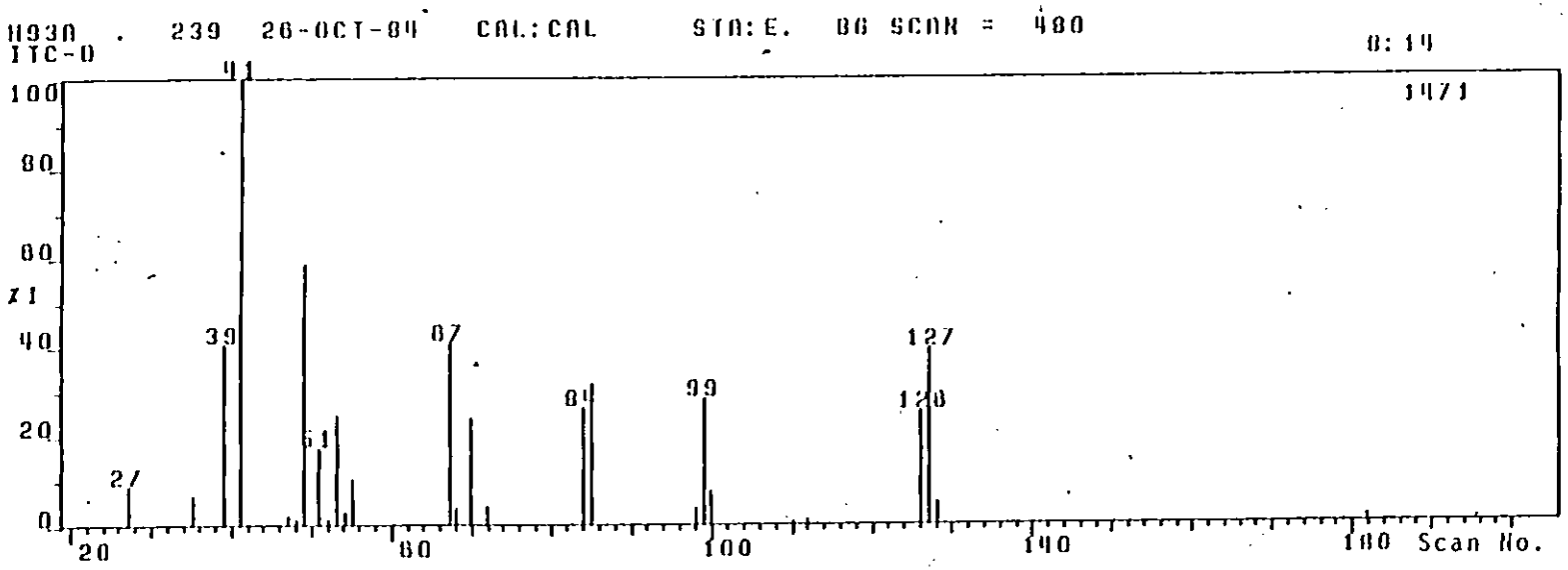


Figure. A.15.4 MASS SPECTRUM OF PENT-4-ENYL ISOTHIOCYANATE (Sample 1)

TABLE A.15.4

Mass Spectrum of Pent-4-enyl Isothiocyanate (Sample 1)

H93A.239 RT 0: 8:14 26-OCT-84 TIC=267 AUTO GAIN=1000
CAL BASE INT.=1471 B/G SCAN=480 STATUS:1E
ITC-D

PEAK NO.	MASS	%HT. MOD.
2	27.2	8.36
11	35.1	6.12
16	39.0	40.38
20	41.0	100.00
27	47.0	1.84
28	48.0	1.02
29	48.9	58.46
31	50.9	17.13
32	52.0	1.09
33	53.1	24.47
34	54.1	2.99
35	55.2	10.06
46	67.1	40.72
47	68.0	3.54
49	69.9	23.59
52	72.0	4.01
63	84.0	26.10
64	85.0	31.61
71	98.0	3.87
72	99.0	28.08
73	99.9	7.34
78	110.0	0.88
79	112.0	0.95
82	126.0	25.02
83	127.0	39.16
84	128.0	4.76
87	179.0	0.54
88	181.8	1.43

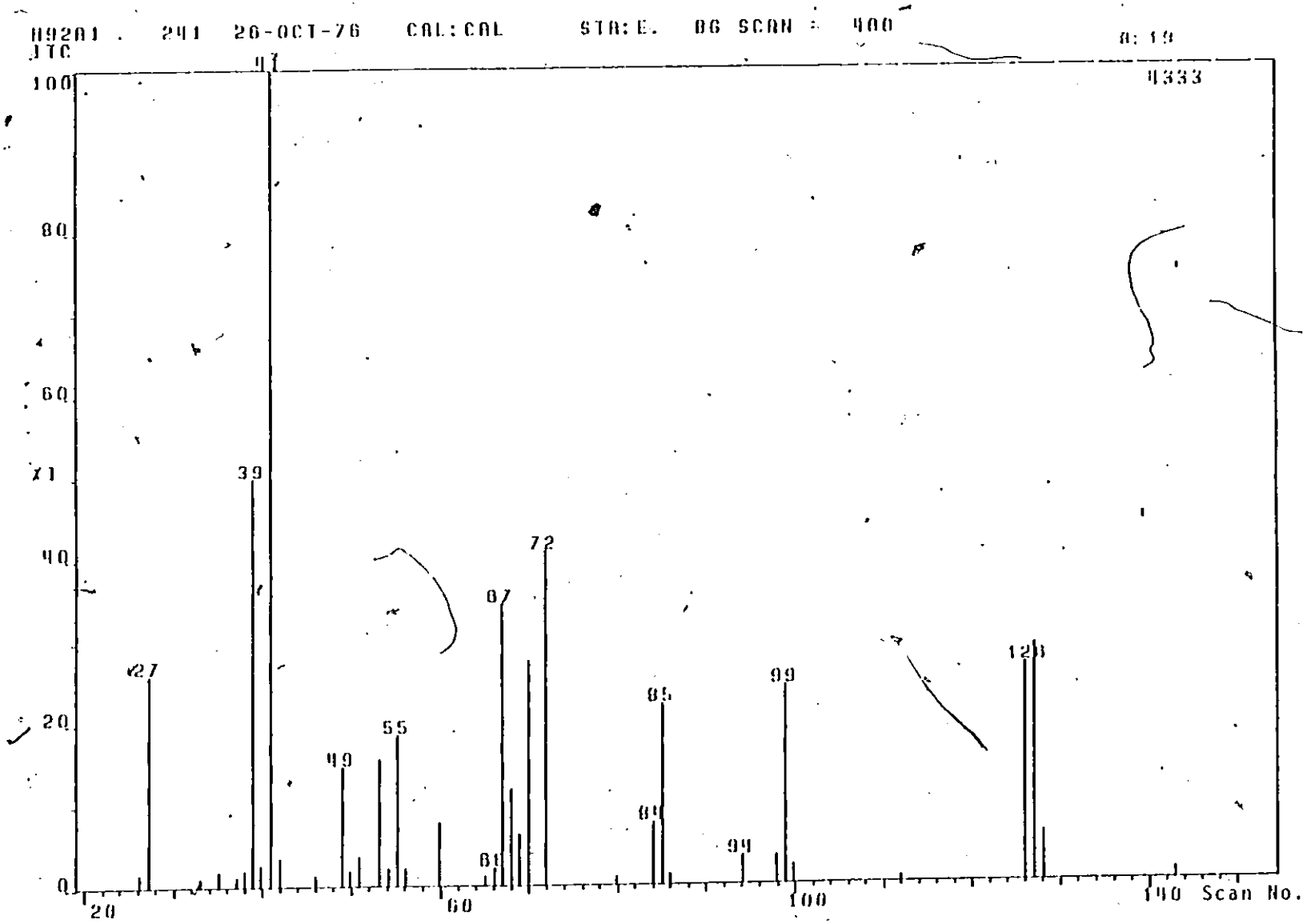


Figure. A.15.5 MASS SPECTRUM OF PENT-4-ENYL ISOTHIOCYANATE (Sample 2)

TABLE A.15.5

Mass Spectrum of Pent-4-enyl Isothiocyanate (Sample 2)

H92A1.241 RT 0: 8:19 26-OCT-76 TIC=468 AUTO GAIN=1000
CAL BASE INT.=4333 B/G SCAN=400 STATUS:1E
ITC

PEAK NO.	MASS	%HT. MOD.
1	26.3	1.55
2	27.2	25.87
9	33.0	0.90
11	35.1	1.85
13	37.1	0.48
14	37.1	1.13
15	38.0	1.92
17	39.0	49.80
18	39.8	2.58
20	41.0	99.98
21	42.0	3.44
25	46.1	1.22
28	48.9	14.45
29	49.9	1.73
30	50.9	3.55
32	53.1	15.49
33	54.1	2.15
34	55.2	18.42
36	56.1	2.03
41	59.9	7.69
45	65.1	1.13
46	66.1	2.05
47	67.1	34.43
48	68.0	11.82
50	69.0	6.30
51	69.9	27.37
54	72.0	40.73
60	79.9	0.99
65	84.0	7.52
66	85.0	22.16
68	86.0	1.27
73	94.1	3.32
76	98.0	3.44
77	99.0	24.23
78	99.9	2.22
84	112.0	0.62
89	126.0	26.54
90	127.0	29.06
91	128.0	5.98
93	143.0	1.34

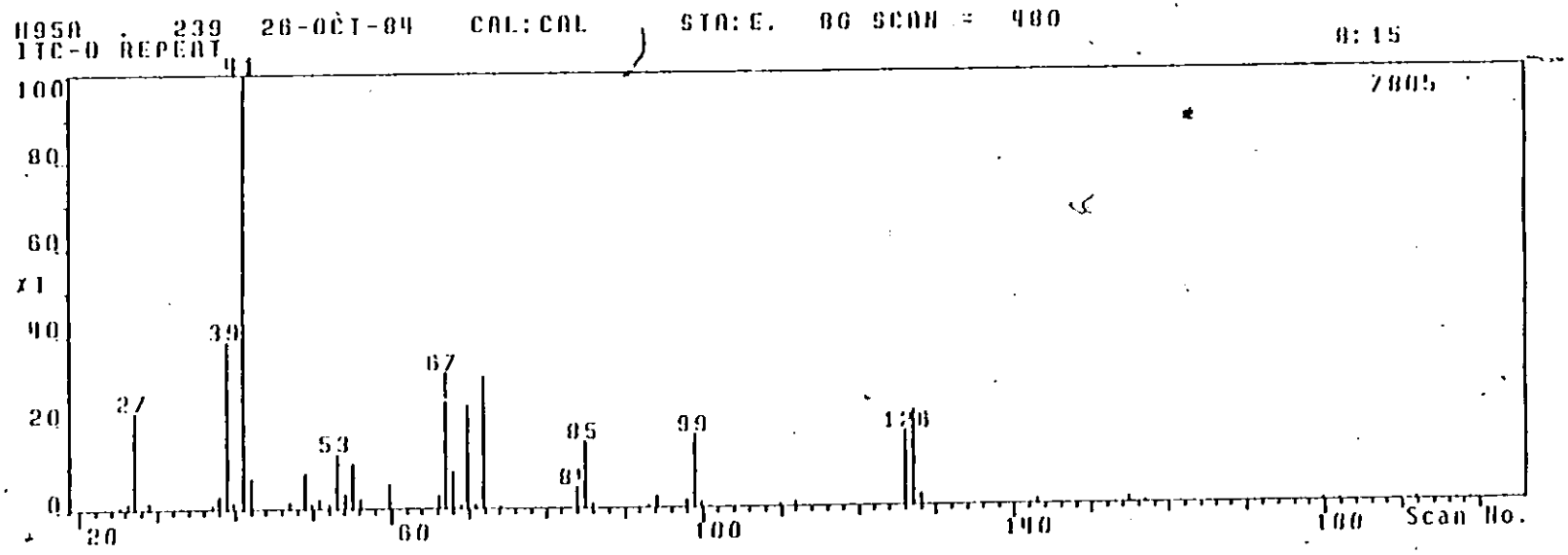


Figure. A.15.6 MASS SPECTRUM OF PENT-4-ENYL ISOTHIOCYANATE (Sample 3)

TABLE A.15.6

Mass Spectrum of Pent-4-enyl Isothiocyanate (Sample 3)

H95A.239 RT 0: 8:15 26-OCT-84 TIC=665 AUTO GAIN=1000
CAL BASE INT.=7805 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
1	25.3	0.56	49	69.0	1.01
2	26.3	1.59	50	69.9	23.69
3	27.2	22.05	51	70.9	1.18
6	29.0	1.45	52	72.0	30.34
10	33.0	0.41	54	74.0	0.70
14	37.1	1.27	61	84.0	4.65
15	38.0	2.83	62	85.0	15.18
17	39.0	38.69	63	86.0	0.94
18	39.8	1.45	67	93.1	0.72
20	41.0	99.99	68	94.1	2.54
21	42.0	7.05	72	98.0	1.99
26	47.1	1.31	74	99.0	16.76
28	48.9	8.16	75	99.9	1.40
29	49.9	0.97	81	110.0	0.74
30	50.9	2.08	82	112.0	1.11
31	52.0	0.96	87	126.0	17.03
32	53.1	12.53	89	127.0	21.97
33	54.1	3.16	90	128.0	2.52
34	55.2	10.45	92	143.0	1.11
35	56.1	2.04	95	154.9	1.35
40	59.9	5.50	96	156.9	0.92
46	66.1	2.92	97	179.8	6.60
47	67.1	31.01	98	181.9	0.78
48	68.0	8.70	99	191.8	0.67

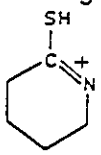
Ionized Fragments of Pent-4-enyl Isothicyanates

m/e	Chemical formula
127	$CH_2=CH-CH_2-CH_2-CH_2-N=C=S$ (M^+)
126	$CH_2=CH-CH_2-CH_2-CH=N^+=C=S$ $(M-1^+)$
99	$CH_2=CH-CH_2-N=C=S^+$
85	$CH_2=CH-N=C=S^+$
84	$C^+H=CH-N=C=S$
72	$C^+H_2-N=C=S$
67	$CH_2=CH-\overset{+}{C}H-CH=CH_2$
55	$CH_2=CH-\overset{+}{C}H-CH_3$
53	$CH_2=CH-\overset{+}{C}H=CH^+$
41	$CH_2=CH-CH_2^+$
39	$HC=CH$ with C^+H (Cyclopropenyl ion)
27	$CH_2=C^+H$ (vinyl ion)

Two mass spectra had a M^+ of m/e 161 (shown in Tables and Figures A.15.7 and A.15.8 and A.15.9) and m/e 175 (shown in Figures and Tables A.15.10 and A.15.11), respectively. A search of the literature indicated that these spectra might have been 4-methylthiobutyl isothiocyanate (CH_3-S-$[CH_2]_4$-N=C=S, mole wt 161) and 5-methylthiopentyl isothiocyanate (CH_3-S-$[CH_2]_5$-N=C=S, mole wt 175).

Identification of 4-methylthiobutyl Isothiocyanate (Mole Wt 161)

The identification of the 4-methylthiobutyl isothiocyanate was done by comparing the mass spectra shown in Figures and Tables A.15.8, A.15.12, and A.15.13 with those reported in the literature (21,42,44,45,72,100). The identified ionized fragments for this isothiocyanate are:

m/e	ion
161	M^+
61 (base peak)	CH_3-S-$^+CH_2$
115	
72	$CH_2=N^+=C=S$
55	$C_4H_7^+$
41	$C_3H_5^+$
27	HCN^+

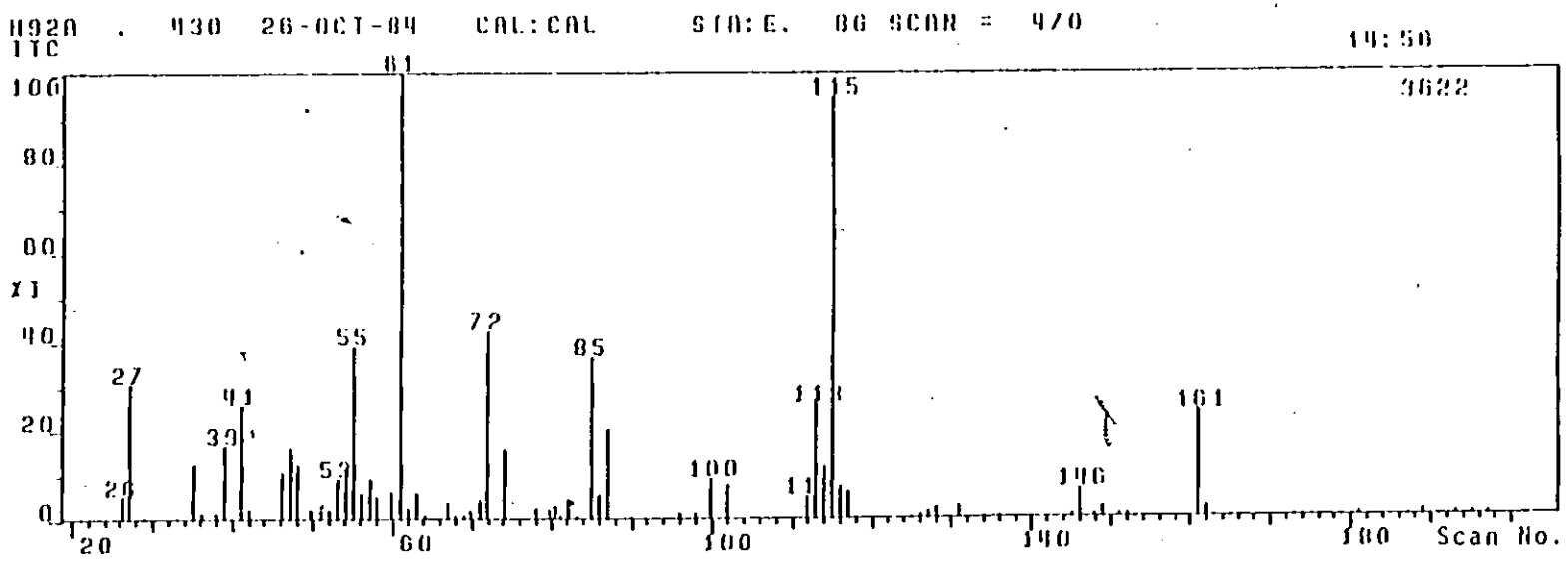


Figure A-15.7 Mass Spectrum of 4- Methylthiobutyl Isothiocyanate (Sample 1)

TABLE A.15.7

Mass Spectrum of 4-Methylthiobutyl Isothiocyanate
(Sample 1)

H92A.430 RT 0:14:56 26-OCT-84 TIC=882 AUTO GAIN=1000
CAL BASE INT.=3622 B/G SCAN=470 STATUS:IE
ITC

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
3	26.3	4.64	64	81.0	0.77
4	27.2	30.48	65	82.0	4.14
6	29.0	0.47	66	83.1	0.50
12	33.1	0.50	68	85.0	36.39
14	35.1	12.23	69	86.0	5.25
15	36.1	1.16	70	87.0	20.02
17	38.0	1.27	74	89.9	0.44
18	39.0	16.23	79	96.1	1.16
21	41.0	25.65	82	98.0	1.27
22	42.0	2.29	84	99.9	9.06
28	46.1	10.44	86	102.0	7.59
29	47.1	15.76	93	112.0	4.89
30	48.0	12.09	95	113.0	25.54
32	49.9	1.71	96	114.0	11.49
33	51.0	1.99	97	115.1	94.40
34	52.0	1.91	98	116.0	7.21
35	53.1	8.78	99	117.0	5.74
36	54.1	10.60	105	126.0	0.83
37	55.2	38.85	106	127.0	1.44
38	56.1	5.58	107	128.0	2.26
39	57.1	8.97	109	131.0	2.46
40	58.1	4.89	112	134.1	0.44
42	59.9	5.77	114	136.1	0.47
43	61.0	100.00	117	145.0	0.64
44	62.0	2.32	118	146.0	6.32
45	63.1	5.41	120	148.0	0.86
47	64.1	0.83	121	149.0	2.32
49	67.1	3.29	122	151.0	0.72
50	68.1	0.77	123	152.0	0.58
51	69.0	0.86	124	161.0	23.52
52	69.9	1.77	125	162.0	2.10
53	71.0	4.00	131	180.9	0.66
54	72.0	42.35	132	189.0	1.21
56	74.1	15.49	134	193.0	0.66
60	78.0	2.37	135	195.0	0.83
62	79.9	1.88	136	197.0	0.72
63	80.4	2.76			

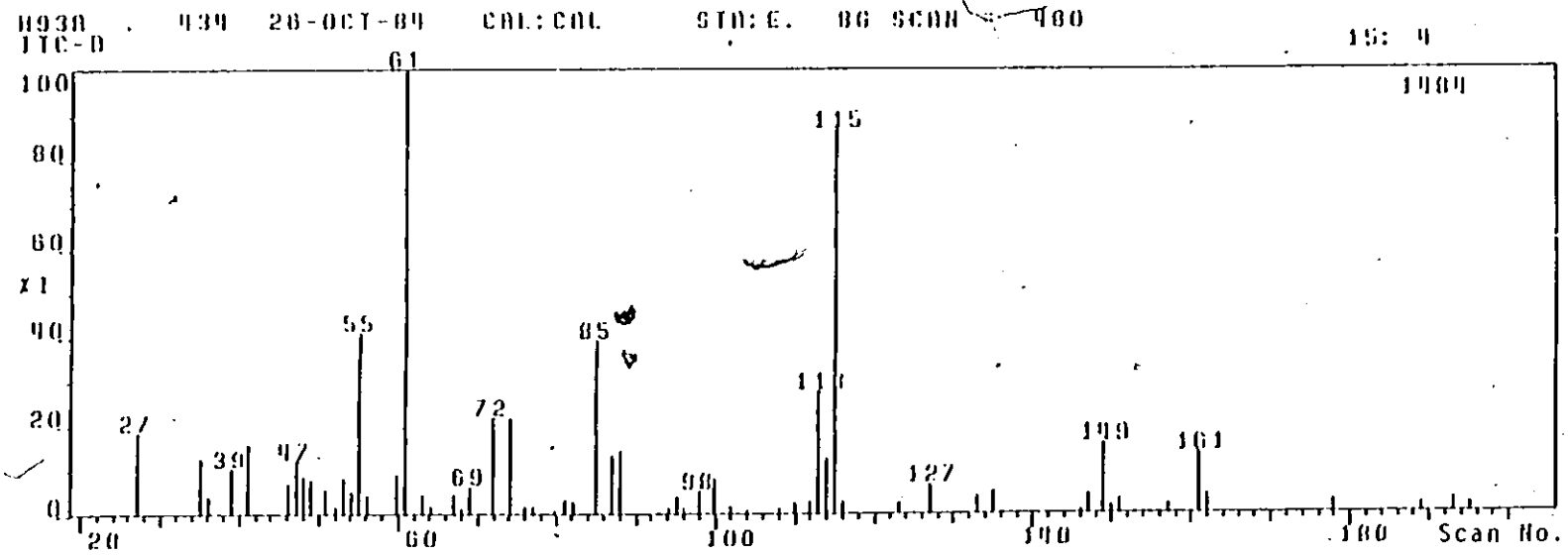


Figure. A.15.8. MASS SPECTRUM OF 4-METHYLTHIOBUTYL ISOTHIOCYANATE (Sample 2)

TABLE A.15.8

Mass Spectrum of 4-Methylthiobutyl Isothiocyanate (Sample 2)

H93A.434 RT 0:19: 4 26-OCT-84 TIC=620 AUTO GAIN=1000
CAL BASE INT.=1484 B/G SCAN=480 STATUS:1E
ITC-D

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
3	27.2	18.06	74	94.1	1.21
12	35.1	12.33	75	95.1	3.71
13	36.1	3.64	76	96.0	1.15
17	39.0	10.11	80	98.0	4.99
20	41.0	15.63	81	99.0	0.47
25	46.1	6.54	82	99.9	7.88
26	47.1	11.73	84	102.0	1.62
27	48.0	8.22	86	104.0	0.74
28	48.8	7.55	88	108.0	1.08
30	50.9	5.19	90	109.9	2.16
31	52.0	1.42	91	110.0	1.89
32	53.1	7.61	92	112.0	2.49
33	54.1	4.85	93	113.0	27.49
34	55.2	40.90	94	114.0	12.26
35	56.1	4.18	95	115.1	86.46
39	59.9	8.63	96	116.0	2.56
40	61.0	100.00	104	123.1	2.29
42	63.1	4.11	105	127.0	6.33
43	64.0	1.55	110	133.1	3.64
47	67.1	4.04	112	135.1	4.78
48	68.0	1.21	116	146.3	0.67
49	69.0	5.93	117	147.0	4.25
52	72.0	21.56	119	149.0	15.70
54	74.0	21.56	120	150.0	1.55
56	76.1	1.55	121	151.0	2.90
57	77.0	1.62	122	157.1	1.82
60	79.9	0.88	123	161.0	13.27
62	81.0	2.83	124	162.0	4.25
63	82.0	2.56	130	178.0	2.43
66	85.0	38.75	131	189.0	2.02
68	87.0	13.07	134	193.0	3.03
69	88.0	14.02	135	195.1	1.68

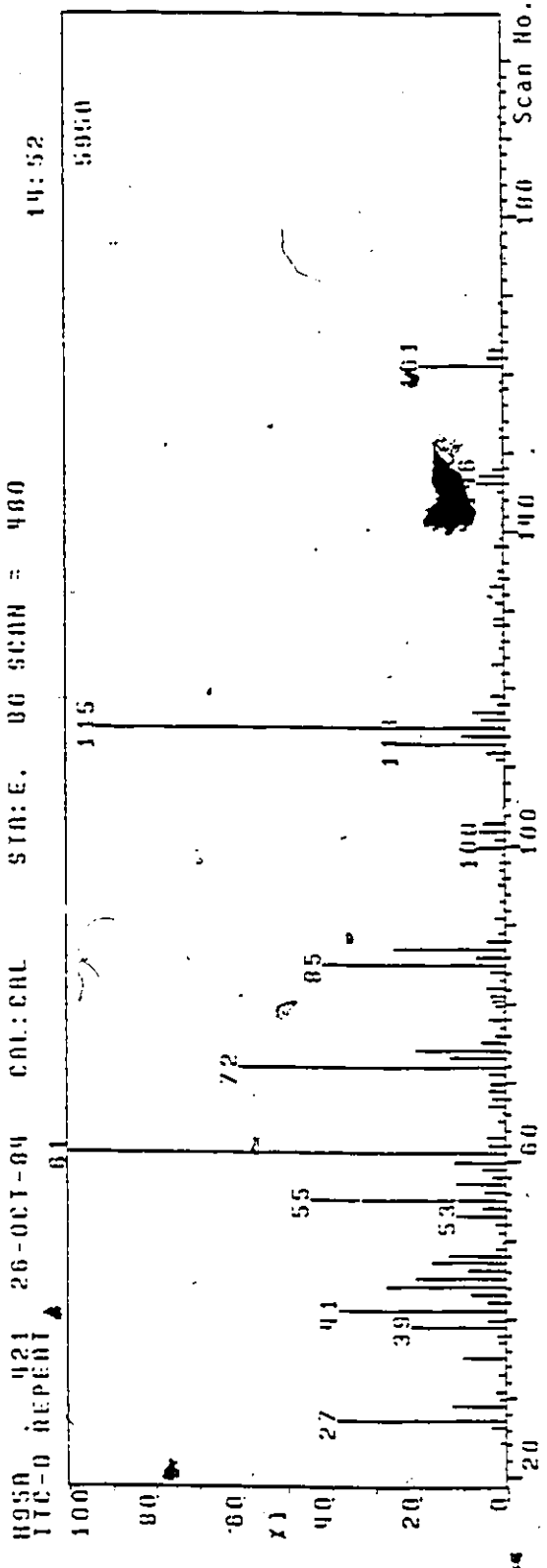


Figure: A.15.9 MASS SPECTRUM OF 4-METHYLTHIOBUTYL ISOTHIOCYANATE (Sample 3)

TABLE A.15.9

Mass Spectrum of 4-Methylthiobutyl Isothiocyanate
(Sample 3)

H95A.421 RT 0:14:52 26-OCT-84 TIC=1275 AUTO GAIN=1000
CAL BASE INT.=5950 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
1	24.3	0.96	51	68.1	2.99
3	26.3	3.51	52	69.0	3.56
4	27.2	38.62	53	69.9	3.39
6	29.0	6.18	54	71.0	3.56
7	29.0	12.10	55	72.0	60.24
8	29.8	1.31	56	73.1	12.74
9	30.9	2.22	57	74.1	20.55
11	33.0	0.86	58	75.1	5.39
12	33.1	1.33	59	76.1	2.13
14	35.1	9.78	60	77.1	1.14
16	37.1	1.60	61	78.0	3.60
17	38.0	1.98	62	79.0	0.69
18	39.0	21.53	63	79.9	2.25
19	39.8	4.24	64	80.4	2.79
21	41.0	38.18	65	81.0	3.34
22	42.0	4.10	66	82.0	4.12
23	43.1	7.63	67	83.1	0.99
24	43.1	2.55	68	84.0	3.23
25	44.1	26.87	69	85.0	41.48
27	45.2	20.39	70	86.0	6.55
28	46.1	8.49	71	87.0	25.01
29	47.1	16.59	72	88.0	4.10
30	48.0	13.06	73	89.0	1.36
31	49.0	2.20	75	91.0	2.12
32	49.9	0.79	76	92.0	0.40
33	51.0	1.60	77	93.1	1.06
34	52.0	2.30	79	95.1	1.65
35	53.1	10.94	80	96.1	0.74
36	54.1	5.16	81	97.1	1.09
37	55.2	44.39	82	98.0	1.14
38	56.1	4.66	83	99.0	0.82
39	57.1	10.96	84	99.9	5.88
40	58.0	2.96	85	101.0	2.07
41	59.0	5.13	86	102.0	5.55
42	59.9	11.41	87	103.0	4.66
43	61.0	99.97	89	105.0	0.42
44	62.0	3.83	91	108.0	0.47
45	63.1	4.25	94	111.0	1.71
48	65.1	1.38	95	112.0	3.93
49	66.1	0.87	96	113.0	23.87
50	67.1	3.83	97	114.0	9.66

TABLE A.15.9 (cont'd)

Mass Spectrum of 4-Methylthiobutyl Isothiocyanate
(Sample 3)

H95A.421 RT 0:14:52 26-OCT-84 TIC=1275 AUTO GAIN=1000
CAL BASE INT.=5950 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.
98	115.0	92.91
99	116.0	5.33
100	117.0	6.94
101	118.0	1.31
102	119.0	3.01
105	122.0	0.67
106	123.1	2.44
108	125.1	1.14
109	126.0	0.82
110	127.0	0.54
111	128.0	2.30
112	129.0	2.27
114	131.0	1.92
115	132.0	0.49
116	133.0	2.96
117	134.0	0.96
118	135.0	1.13
121	138.0	1.68
123	139.0	0.79
125	141.0	0.44
126	143.0	0.40
127	145.0	1.06
128	146.0	5.85
129	147.0	5.21
130	148.0	2.18
131	148.9	0.54
133	152.0	0.59
134	153.1	0.84
136	157.9	0.69
137	158.9	0.59
138	160.9	18.71
139	161.9	3.21
140	163.0	2.87
141	164.0	0.55
142	165.1	0.69
144	169.0	0.72
145	169.1	0.62
151	189.0	0.42
152	189.9	0.44
154	192.9	0.86
156	197.0	0.91

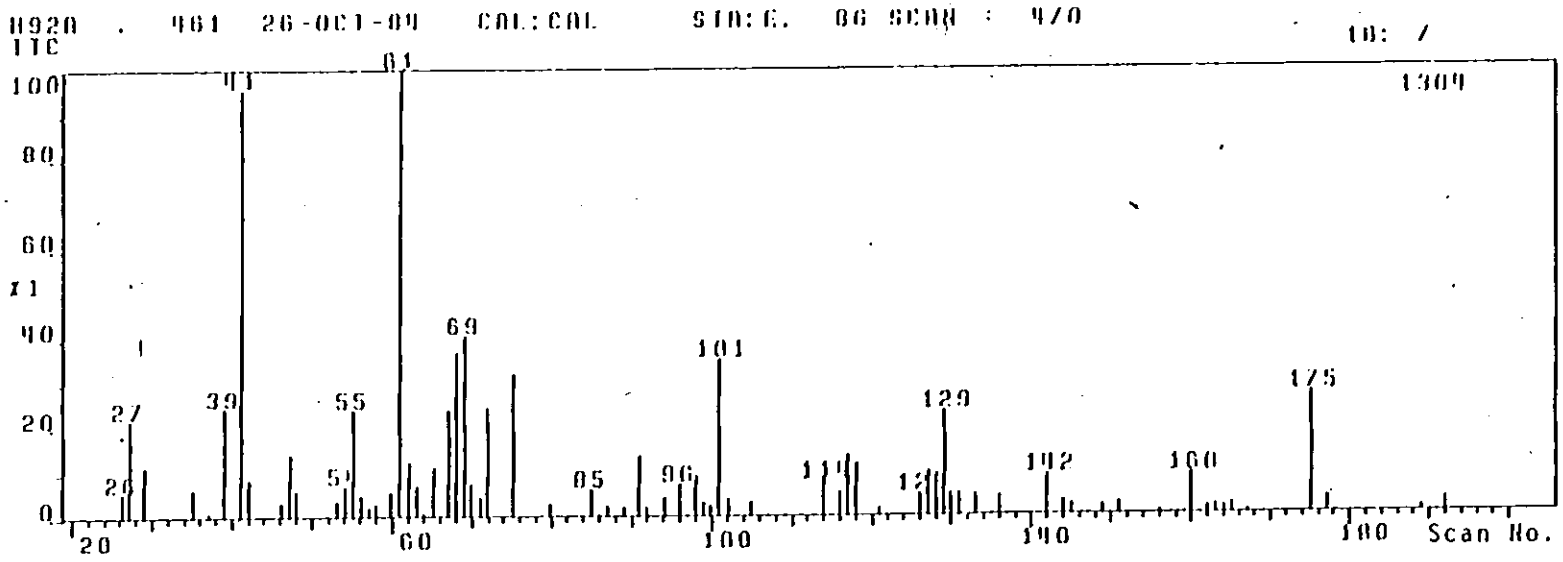


Figure. A.15.10 MASS SPECTRUM OF 5-METHYLTHIOPENTYL ISOTHIOCYANATE (Sample 2)

TABLE A.15.10

Mass Spectrum of 5-Methylthiopentyl Isothiocyanate (Sample 2)

H92A.461 RT 0:16: 7 26-OCT-84 TIC=694 AUTO GAIN=1000
CAL BASE INT.=1304 B/G SCAN=470 STATUS:1E
ITC

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
2	26.3	5.06	79	96.1	7.21
3	27.2	21.55	81	98.0	9.05
5	29.0	11.20	82	99.0	2.91
11	35.1	5.75	83	99.9	2.30
13	37.1	0.77	84	101.0	35.20
17	39.0	24.16	85	102.0	3.83
20	40.9	95.71	88	105.0	2.84
21	42.0	8.21	95	114.1	7.75
25	46.1	3.14	97	116.1	5.37
26	47.1	13.88	98	117.0	13.50
27	48.0	5.60	99	118.0	11.35
32	53.1	3.22	102	121.0	1.30
33	54.1	6.52	104	126.0	4.98
34	55.2	23.85	105	127.0	9.66
35	56.1	4.29	106	128.0	9.36
36	57.1	1.84	107	129.0	23.24
37	58.1	2.68	108	129.9	4.83
39	59.9	5.14	109	131.0	4.91
40	61.0	100.00	111	133.1	4.52
41	62.0	11.73	113	136.0	4.06
42	63.1	6.75	116	142.0	8.74
43	64.0	0.84	117	144.1	2.84
45	65.1	10.81	118	145.1	2.15
47	67.1	23.77	122	149.0	1.92
48	68.0	36.50	123	151.0	2.76
49	69.0	40.49	124	156.0	0.77
51	69.9	6.98	125	159.0	0.46
52	71.0	3.91	126	159.9	8.74
53	72.0	24.23	128	162.0	1.38
57	75.1	31.98	129	163.0	1.69
62	79.9	2.53	130	164.0	1.46
68	85.1	5.75	131	165.0	2.38
69	86.0	0.46	132	167.0	0.69
70	87.1	2.22	133	175.0	27.15
72	89.0	1.84	135	177.0	3.22
74	91.0	13.19	136	178.0	0.54
75	92.0	1.84	139	189.0	1.00
77	94.1	4.14	141	192.0	3.07

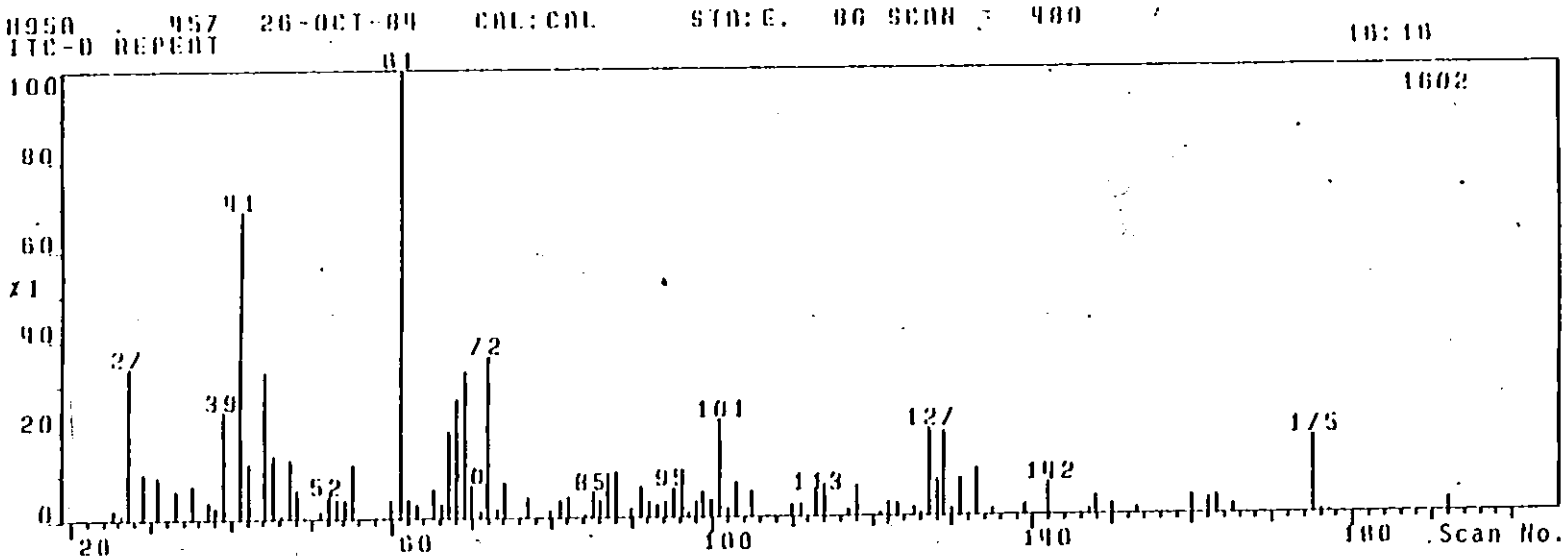


Figure. A.15.11 MASS SPECTRUM OF 5-METHYLTHIOPENTYL ISOTHIOCYANATE (Sample 3)

TABLE A.15.11

Mass Spectrum of 5-Methylthiopentyl Isothiocyanate (Sample 3)

H95A.457 RT 0:16:16 26-OCT-84 TIC=582 AUTO GAIN=1000
CAL BASE INT.=1602 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
1	25.3	2.06	56	77.0	4.37
2	26.3	1.06	59	79.9	1.50
3	27.2	33.58	60	81.0	3.68
5	29.0	10.05	61	82.0	4.37
7	30.9	9.24	63	84.0	0.56
9	33.0	0.94	64	85.1	5.62
10	33.1	6.37	65	86.0	3.68
12	35.1	7.37	66	87.0	9.55
14	37.1	3.56	67	88.0	9.93
15	38.0	2.75	69	89.9	1.87
16	39.0	23.72	70	91.0	6.68
19	41.0	68.41	71	92.0	3.25
20	42.0	12.05	72	93.1	2.50
22	44.1	32.77	73	94.1	3.43
23	45.1	14.04	74	95.1	6.12
24	46.1	0.75	75	96.1	10.17
25	47.1	12.98	77	97.1	1.25
26	48.0	6.24	78	98.0	3.31
27	49.0	0.50	79	98.9	5.68
29	51.0	1.44	81	99.9	3.62
30	52.0	4.68	82	101.0	21.47
31	53.1	4.06	83	102.0	1.75
32	54.1	4.18	84	103.0	7.55
33	55.2	11.67	86	105.0	5.49
37	59.0	0.50	88	110.0	2.62
38	59.9	4.18	89	111.0	2.56
39	61.0	100.00	90	113.0	5.18
40	62.0	3.93	91	114.0	6.93
41	63.1	2.93	94	117.0	1.31
44	65.1	6.43	95	118.0	6.55
45	66.1	3.06	98	121.0	0.62
46	67.1	19.10	99	122.0	2.81
47	68.1	26.28	100	123.1	2.62
48	69.0	32.46	102	125.1	1.75
49	69.9	7.12	103	126.0	0.81
50	71.0	1.50	105	127.0	19.10
51	72.0	35.96	106	128.0	8.11
52	73.1	2.00	107	129.0	18.41
53	74.0	7.87	108	130.0	1.62

TABLE A.15.11 (cont'd)

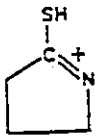
Mass Spectrum of 5-Methylthiopentyl Isothiocyanate (Sample 3)

H95A.457 RT 0:16:16 26-OCT-84 TIC=582 AUTO GAIN=1000
CAL BASE INT.=1602 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.
109	131.0	8.24
110	132.0	0.44
111	133.1	10.30
112	135.0	1.62
114	139.0	2.00
115	139.1	2.25
116	142.0	6.93
119	147.0	1.06
120	148.0	4.00
122	149.9	2.25
123	153.0	1.50
124	159.9	4.06
126	162.0	3.43
127	163.0	3.93
128	165.1	1.75
129	175.0	16.85
130	176.0	0.62
131	177.0	0.44
133	192.0	3.12

Identification of 5-methylthiopentyl Isothiocyanate

Similarly, the 5-methylthiopentyl isothiocyanate was identified from the literature (100). The ionized fragments of this isothiocyanate shown in Figures and Tables A.15.10, A.15.10, and A:15.11 are:

m/e	ion
175	M^+
61 (base peak)	$CH_3\text{-}S\text{-}C^+H_2$
72	$CH_2{=}N^+{=}C{=}S$
69	$C_3H_5^+$
27	HCN^+
101	
41	C_3H_5
129	$^+CH_2\text{-}(CH_2)_4\text{-}N{=}C{=}S$

Identification of 2-phenylethyl Isothiocyanate (Molecular Weight 163)

As the mass spectra of two samples of the compound (which are presented and shown in Figures and Tables A.15.12 and A.15.13) have an M^+ of 163, the spectra was suspected to be for 2-phenylethyl isothiocyanate. The spectrum was then identified to be for this isothiocyanate by comparing it with the spectra available in the literature (42,72,100,140,197). The ionized fragments composing the spectra of Figures A.15.12 and A.15.13 are:

m/e	ion
163	M^+
91 (base peak)	 (troyplium ion)
105	$C_6H_5.C_2H_4^+$
77	$C_6H_5^+$
72	$CH_2=N^+=C=S$

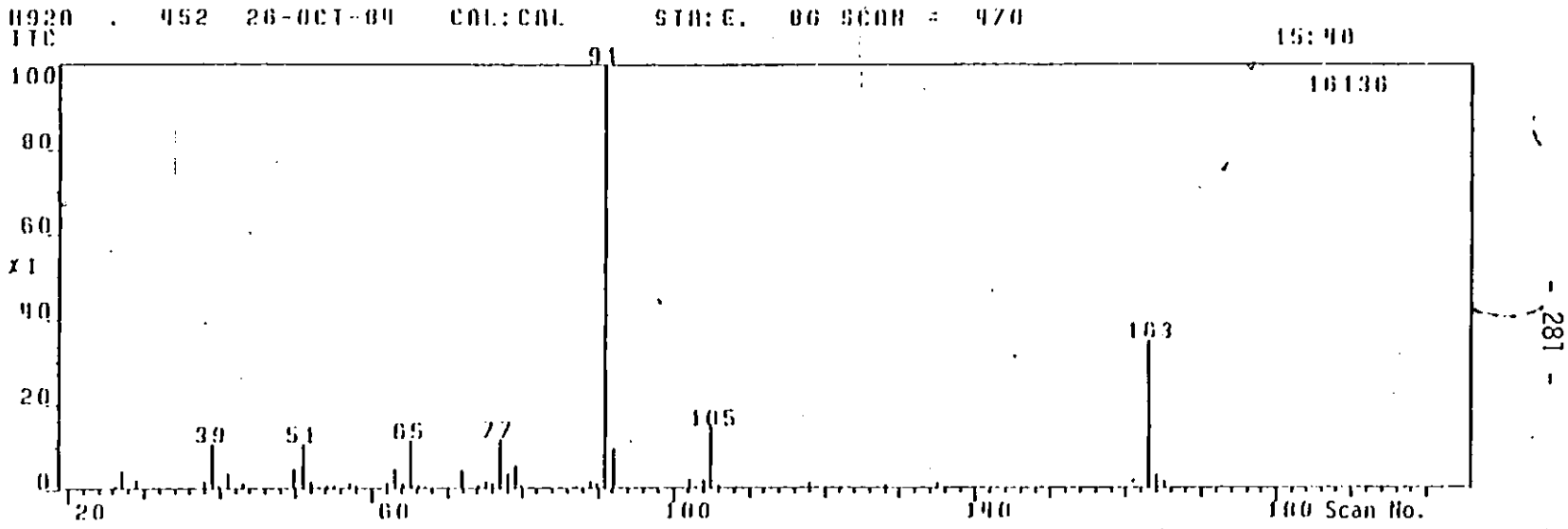


Figure. A.15.12 MASS SPECTRUM OF 2-PHENYLETHYL ISOTHIOCYANATE (Sample 2)

TABLE A.15.12

Mass Spectrum of 2-Phenylethyl Isothiocyanate
(Sample 2)

H92A.452 RT 0:15:46 26-OCT-84 TIC=1232 AUTO GAIN=1000
CAL BASE INT.=16136 B/G SCAN=470 STATUS:IE
ITC

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
3	26.3	0.90	57	75.1	1.50
4	27.2	4.09	58	76.1	0.94
.6	29.0	2.00	59	77.1	11.32
16	38.0	1.53	60	78.0	3.32
17	39.0	10.49	61	79.0	5.18
18	39.8	0.77	62	79.9	0.63
20	40.9	3.33	69	87.0	0.45
22	43.1	1.29	71	89.0	1.52
30	49.9	4.31	72	89.9	1.06
31	51.0	10.23	74	91.0	100.00
32	52.0	1.52	75	92.0	9.23
33	53.1	0.53	87	102.0	1.80
34	54.1	0.63	88	103.0	0.50
35	55.2	0.87	89	104.1	1.82
38	57.1	1.02	90	105.1	14.06
39	58.1	0.60	91	106.1	0.86
43	62.0	1.24	102	118.0	0.98
44	63.1	4.54	110	128.0	0.63
45	64.1	1.16	115	133.1	0.45
46	65.1	11.28	117	135.0	0.97
47	66.1	0.88	124	149.0	0.53
49	67.1	0.48	129	163.0	34.28
54	72.0	4.20	130	164.0	2.89
56	74.1	0.89	131	165.0	1.34

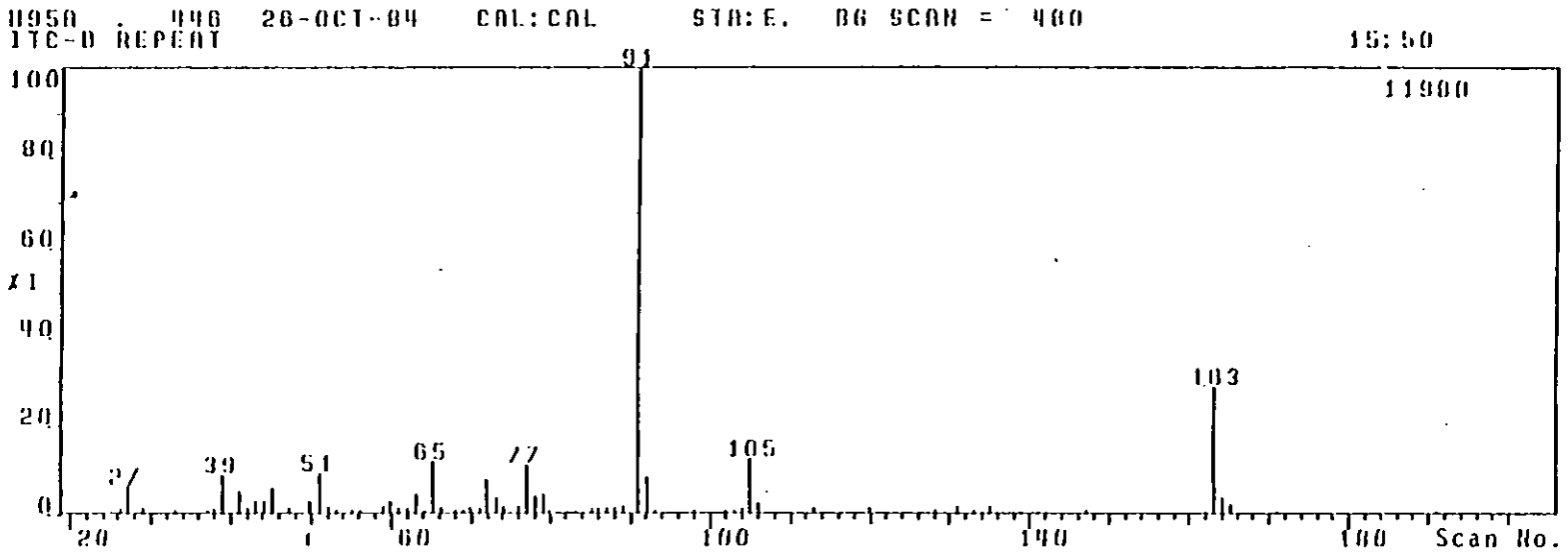


Figure. A.15.13 MASS SPECTRUM OF 2-PHENYLETHYL ISOTHIOCYANATE (Sample 3)

TABLE A.15.13

Mass Spectrum of 2-Phenylethyl Isothiocyanate
(Sample 3)

H95A.446 RT 0:15:50 26-OCT-84 TIC=959 AUTO GAIN=1000
CAL BASE INT.=11980 B/G SCAN=480 STATUS:1E
ITC-D REPEAT

PEAK NO.	MASS	%HT. MOD.	PEAK NO.	MASS	%HT. MOD.
1	26.3	1.00	54	77.0	10.66
2	27.2	6.09	55	78.0	3.60
4	29.0	1.28	56	79.0	4.15
8	33.1	0.75	57	79.9	0.84
11	37.1	0.73	60	82.0	0.46
12	38.0	1.08	61	83.1	0.51
13	39.0	8.51	64	85.1	1.04
16	41.0	4.68	65	86.0	0.95
17	42.0	1.22	66	87.0	1.06
18	43.1	2.45	67	88.0	1.06
19	44.1	2.67	68	89.0	1.34
20	45.1	5.62	70	91.0	100.00
23	47.1	0.94	71	92.0	8.18
26	49.9	2.50	72	93.0	0.90
27	51.0	8.79	77	98.0	0.58
28	52.0	1.45	80	102.0	0.76
29	53.1	0.78	81	103.0	0.62
31	55.2	0.81	82	104.1	1.16
32	56.1	0.63	83	105.1	12.20
35	59.0	1.60	84	106.1	2.13
36	59.9	2.75	89	113.0	1.24
37	61.0	1.14	96	119.9	1.25
38	62.0	1.06	105	128.0	0.74
39	63.1	4.09	108	131.0	1.54
41	64.1	0.83	110	133.0	0.91
42	65.1	11.41	111	134.0	0.47
43	66.1	1.20	112	135.0	1.39
45	68.0	0.64	116	141.1	0.44
46	69.0	0.80	120	147.0	0.78
47	69.9	0.94	129	162.0	0.42
48	71.0	1.14	130	163.0	28.51
49	72.0	7.50	131	164.0	3.20
50	73.0	3.29	132	165.0	1.93
51	74.0	1.53	133	170.9	0.45
53	76.0	1.49	139	191.0	0.42

Co-elutants

In the GLC for this mass spectrum study, the last identified three isothiocyanates (5-methylthiopentyl-, 4-methylthiobutyl-, and 2-phenylethyl-) were co-elutants with BIT and PIT. The glucosinolate precursors to these co-elutants are present in Tower rapeseed in relatively small quantities. Thus, the isothiocyanates formed from them were in such small amounts that they did not appear in the ITC determinations covered in this Thesis. However, the increase in the concentration of their co-elutants, in the methylene chloride extract by its evaporation to 5 to 10% of its original volume, led to their appearance in this GC/MS study.

APPENDIX-16

LETTER FROM PROFESSOR S. NAKAI

THE UNIVERSITY OF BRITISH COLUMBIA
2075 WESBROOK MALL
VANCOUVER, B.C., CANADA
V6T 1W5

DEPARTMENT OF FOOD SCIENCE

July 3, 1979

Mr. M. N. Radwan
Research Associate
Chemical Engineering Department
University of Ottawa
Ottawa, Ontario K1N 9B4

Dear Mr. Radwan:

I was pleased to have had a chance to talk to you at the CIFST Annual Conference.

I completely agree with you about the possibility of interactions of isothiocyanates and rapeseed protein which might affect the detoxification effect. We emphasized this importance in our paper (Woyewoda and Nakai, 1978, CIFSTJ 11: 107). I feel that the determinations of SH and ε-amino groups may be required to check the possible interaction in the treated protein whenever the detoxification effect of rapeseed protein is studied.

Please write again to me or Mr. Woyewoda at the Department of Fisheries and Environment Technology Branch, Halifax, N.S. B3J 2R3 if you have any further questions on our publication.

Sincerely,

S. Nakai

S. Nakai
Professor

SN/jch

APPENDIX-17

LETTER FROM DR. M.E. DAXEMBICHLER

AGRICULTURAL RESEARCH SERVICE
NORTH CENTRAL REGION

OF UNITED STATES
DEPARTMENT OF
AGRICULTURE

NORTHERN REGIONAL RESEARCH CENTER
1815 NORTH UNIVERSITY STREET
PEORIA, ILLINOIS 61604

August 5, 1977

AIRMAIL

Dr. M. N. Radwan
Department of Chemical Engineering
Faculty of Science and Engineering
University of Ottawa
Ottawa, Ontario K1N 6N5
Canada

Dear Dr. Radwan:

Mr. VanEtten and I have now completed a rather cursory examination of the processed rapeseed meal that you sent to me. We are currently very much over extended in our work scheduling and could not spend any more time on the analyses. Single determinations for intact glucosinolates or for the cyano and epithio compounds and/or goitrin that may be derived from progoitrin, if present as such in the meal, gave the following results. The measurement of intact glucosinolates indicated only trace or none remained. The other determination for breakdown products present in the meal did, however, show these components present. Readily recognized on two columns (by GLC) were:

1-cyano-2-hydroxy-3-butene (0.14 mg/g)
1-cyano-2-hydroxy-3,4-epithiobutane (threo isomer 0.09 mg/g)
1-cyano-2-hydroxy-3,4-epithiobutane (erythro isomer 0.09 mg/g)
5-vinyloxazolidine-2-thione (goitrin 0.7 mg/g)

Other peaks were seen in the GLC chromatogram that have been derived from other glucosinolates. I hope these data will be of value to your research. My opinion is that the meal still contains compounds that are toxic.

Sincerely,

M. E. Daxenbichler

M. E. Daxenbichler, Research Chemist
Natural Toxicants Research
Horticultural and Special Crops Laboratory

APPENDIX-18

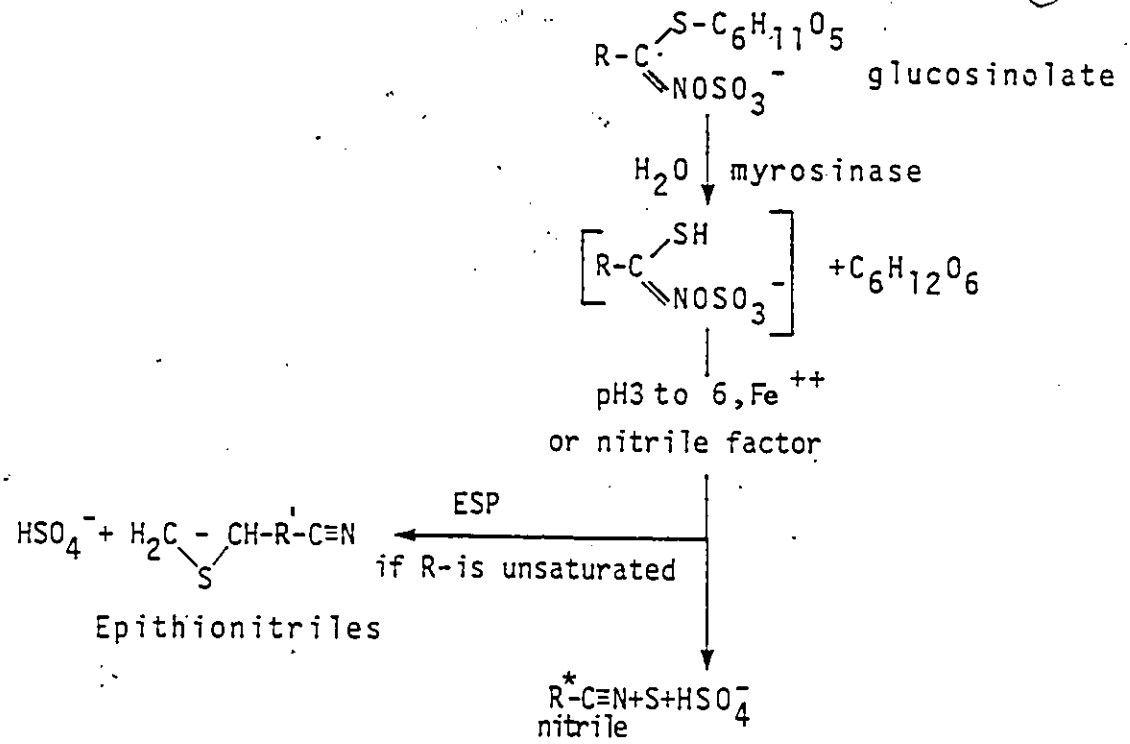
NITRILES AND THIOCYANATE ION FROM HYDROLYSIS OF GLUCOSINOLATES

1. Nitriles:

Tookey (216) has isolated a heat-labile protein or possibly glycoprotein that directs the hydrolysis of the glucosinolates to form the epithionitriles. This protein is termed epithiospecifier protein (ESP) and by itself, in absence of myrosinase, cannot hydrolyze the glucosinolates (216). However, when combined with myrosinase it influences the hydrolysis of the alkenyl glucosinolates, such as allyl glucosinolates to yield 1-cyano-2,3-epithiopropane (127,216,220). It was shown that both ferrous ion and ESP are required for the epithionitrile formation (124,216, 220). The ferrous ion could come from endogenous iron in the seed (216). However, the addition of ferrous salts to rapeseed meals while hydrolyzing their glucosinolates not only increases the formation of the epithionitriles but also all other nitriles (31,124,216,218). It was indicated that ferrous ion is not involved in the enzymatic liberation of glucose from glucosinolates but affects the subsequent degradation to aglucons (219). It was also

indicated that this effect is a pH dependent (219). In the absence of myrosinase, ferrous ions act upon the glucosinolates to yield thionamides, in addition to the nitriles (19,20).

As reported in the literature (52,71,99,127,159, 216,218,220), the nitriles are formed from the hydrolysis of the glucosinalates according to the chemical equations outlined below. It should be noted that these equations only represent the compounds of concern.

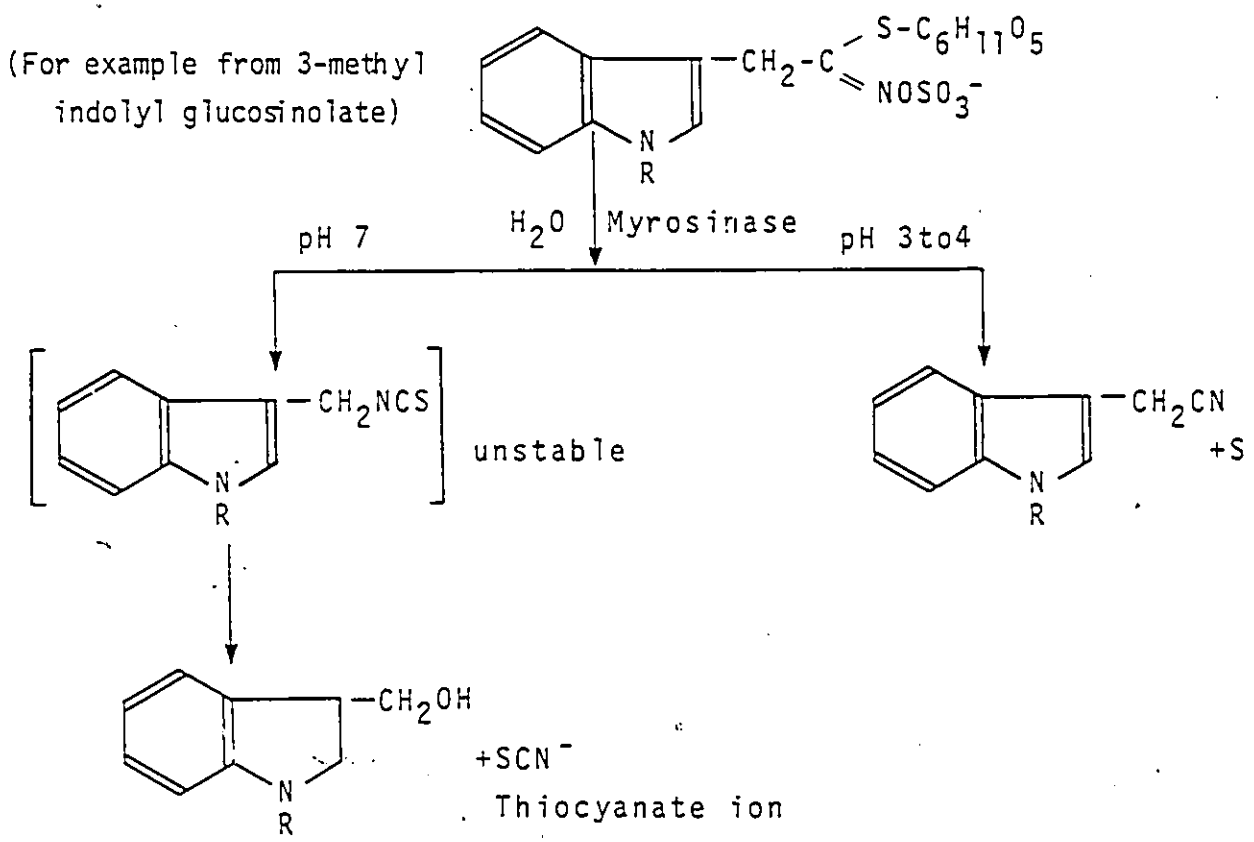


*R-is CH_2=CH-R'-

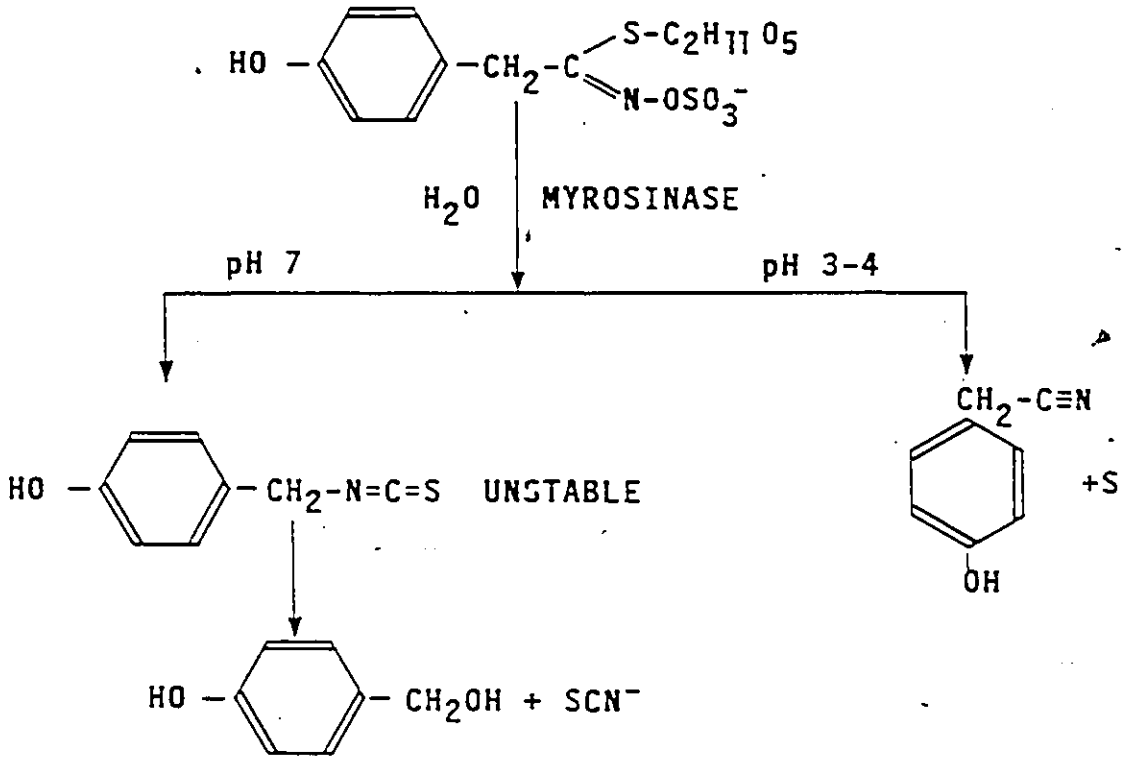
2. Thiocyanate ion:

It has been postulated (70) that the thiocyanate ion is formed from the hydrolysis of glucosinolates according to the following chemical equations:

a) From indolyl glucosinolates (16,220,225,229)



b) From 4-hydroxybenzyl glucosinolate (93,127,220)



APPENDIX 19

INFRA-RED METHOD FOR DETERMINATION OF NITRILES

During the search for an appropriate method for the determination of the nitriles resulting from the hydrolysis of the meal glucosinolates, the infra-red method was tested. In this method the C≡N group of the nitriles is detected at a wavelength of about 4.5 μm. Briefly, the procedure followed in testing was that 3 ml of water, 1 g of meal, and a 6 mm glass bead were placed in a culture tube. The contents of the tube were mixed in a rotary shaker for 1½ h. Then 2 ml of methylene chloride was added to the tube and the shaking continued for an additional ½ h. Afterwards, the emulsion was broken in a centrifuge at 2500 rpm. The methylene chloride (bottom) layer was removed by a long-needled syringe and dried over anhydrous Na_2SO_4. One ml of the dried methylene chloride was evaporated to dryness over KBr powder. From a repeat of the procedure, the 1 ml of the dried methylene chloride was placed in a 4 ml vial and evaporated to dryness. Then it was redissolved in ½ ml of chloroform. The evaporation in both cases was done under a nitrogen atmosphere and the samples were cooled.

The samples were scanned in a Perkin-Elmer Infra-red Spectrophometer model 735B. The samples dissolved in chloroform were prepared for scanning by placing them in a capillary film between two AgCl pellets. Typical examples of the scans of the sample, both dried over KBr and dissolved in chloroform, are presented in Figures A.19.1 and A.19.2, respectively. The IR spectra of the chloroform used is shown in Figure A.19.3. After testing this method, it was decided to abandon it because the possibility remained that the compounds being measured could evaporate during the drying step. Also, the method did not identify the compounds being measured. In addition, the presence of other groups such as the N=C=S (174) and the $-NH_2$ of the protein amino acids (46) could have interfered with the measurements. This could be seen in Figure A.19.1 where if* the peak at a wavelength of 4.6 m were for C=N group, the peak at 4.8 m interfered with it. However, as shown in Figure A.12.2, for the measurements of the samples dissolved in chloroform, the peak at 4.6 m did not appear. This was because the concentration of this sample was lower than the instrument's limit of detection.

*It is not certain, because of the presence of a mixture of components, that this peak is for the C=N group.

For these reasons it was decided to use the GLC method developed by Daxenbichler et al. (49,50) and discussed in Chapter 3.3.5. This chromatographic method, in addition to separating the aglucons individually, also determined the VOT.

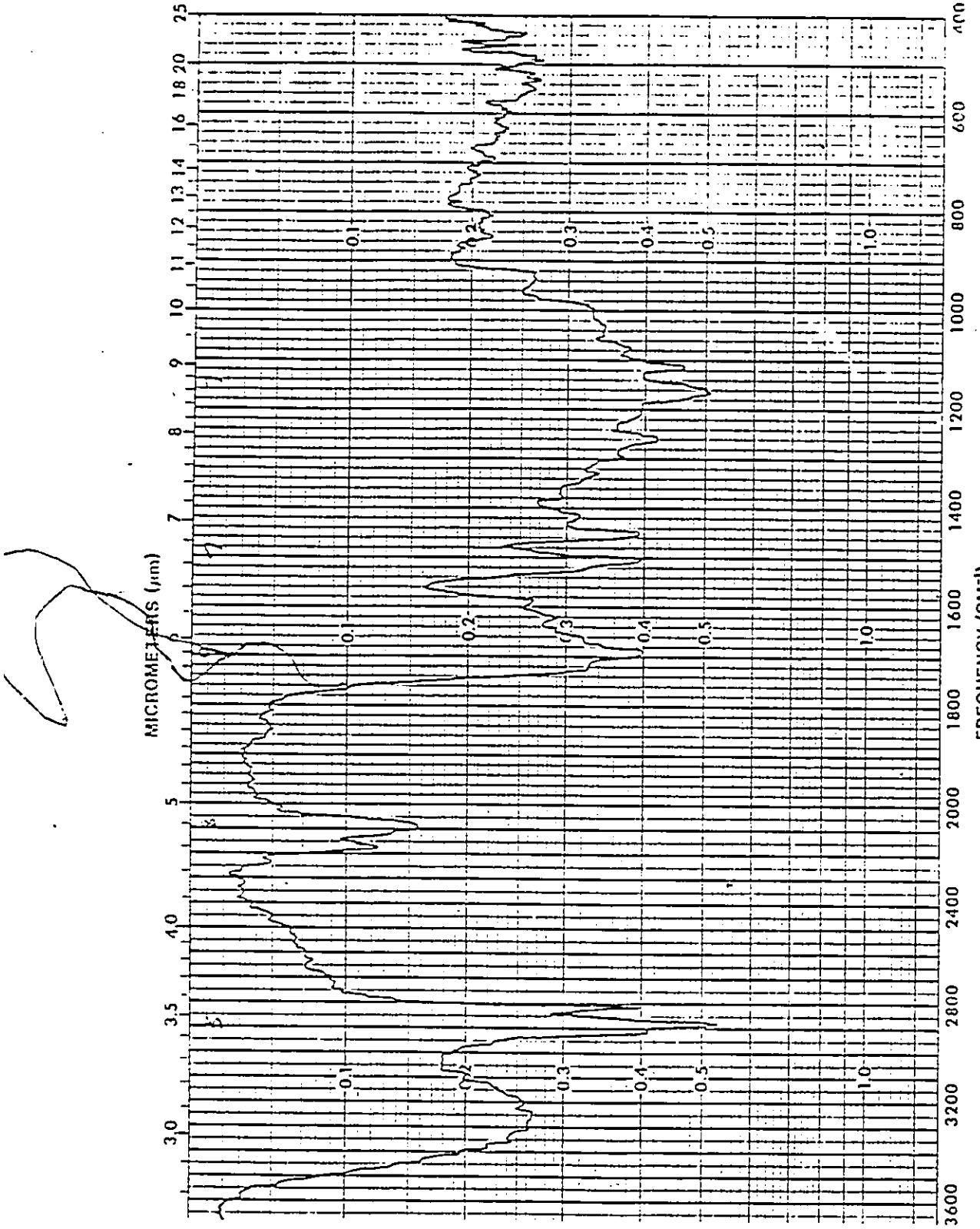


Figure. A.19.1 IMFRA-RED SPECTRUM OF AGLUCONS FILM ON KBr

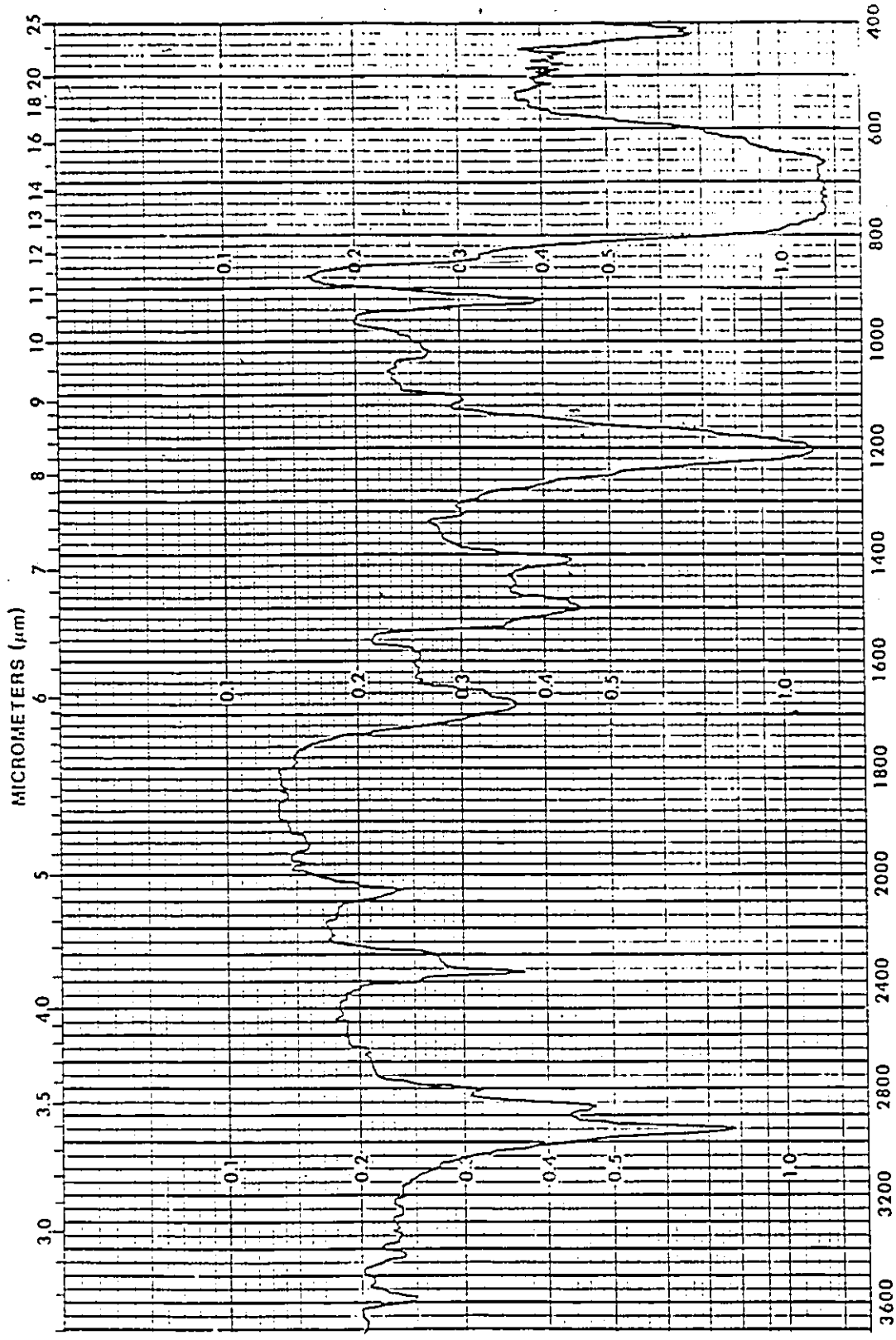


Figure. A.19.2 INFRA-RED SPECTRUM OF AGLUCONS IN CHLOROFORM

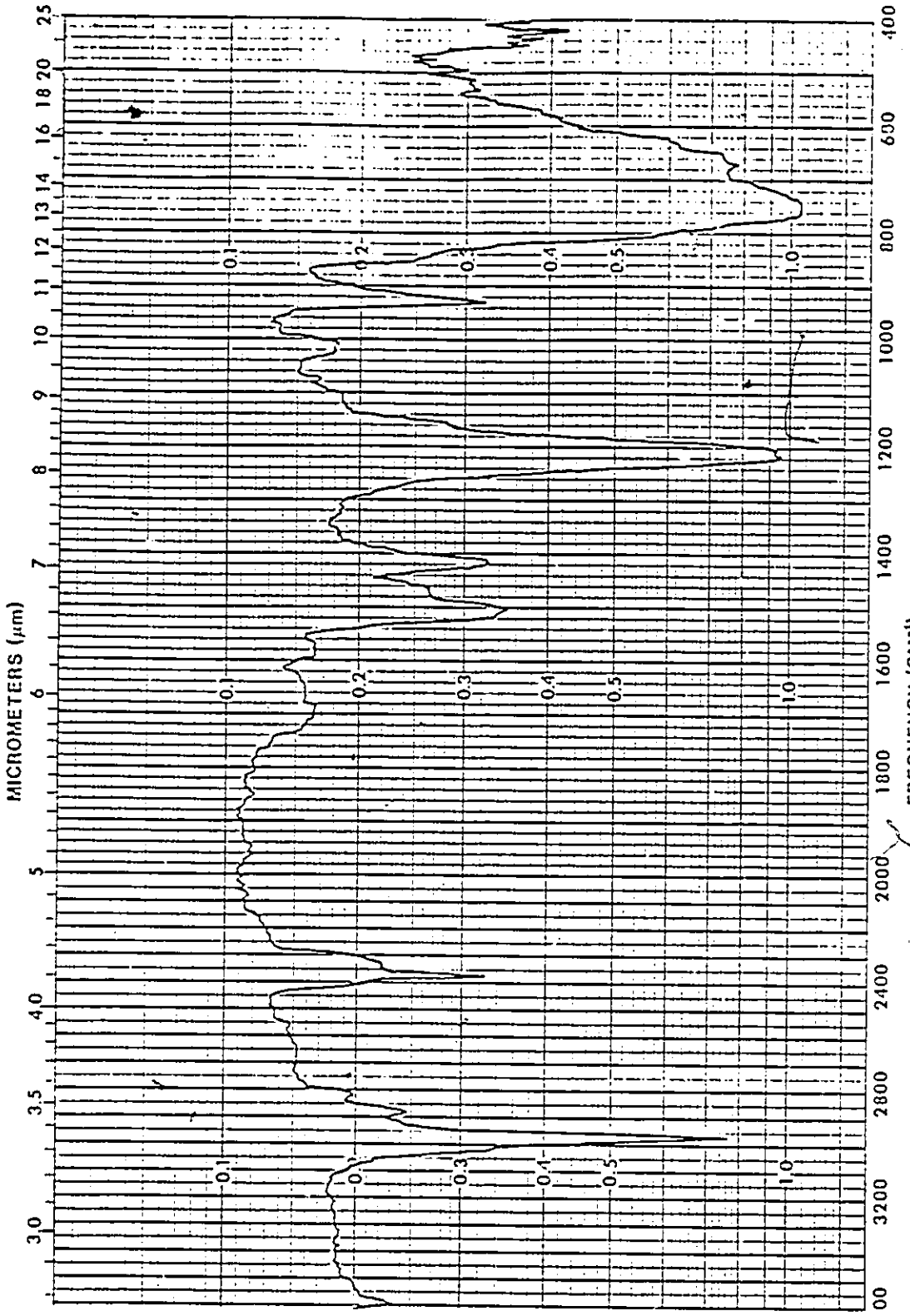


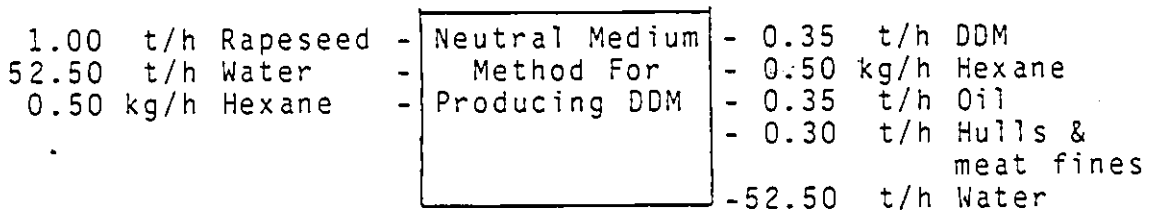
Figure. A.19.3 INFRA-RED SPECTRUM OF CHLOROFORM

APPENDIX 20

MASS BALANCE AND ENERGY REQUIREMENT FOR THE NEUTRAL MEDIUM METHOD

1. MASS BALANCE:

The mass flow rates in the Neutral Medium Method of the materials used and the products are summarized in the following diagram:



CALCULATION OF FLOW RATES:

- Rapeseed assumed 1 t/h.

- Its dehulling will result in 0.7 t/h meat and 0.3 t/h (hulls & meat fines)

- Defatting of the meat will result in 0.35 t/h oil and 0.35 t/h meal.

- Hexane lost with meal is at a rate of 1.50 kg/t meal. Thus the replacement of the lost hexane will be at a rate of 0.5 kg/h.

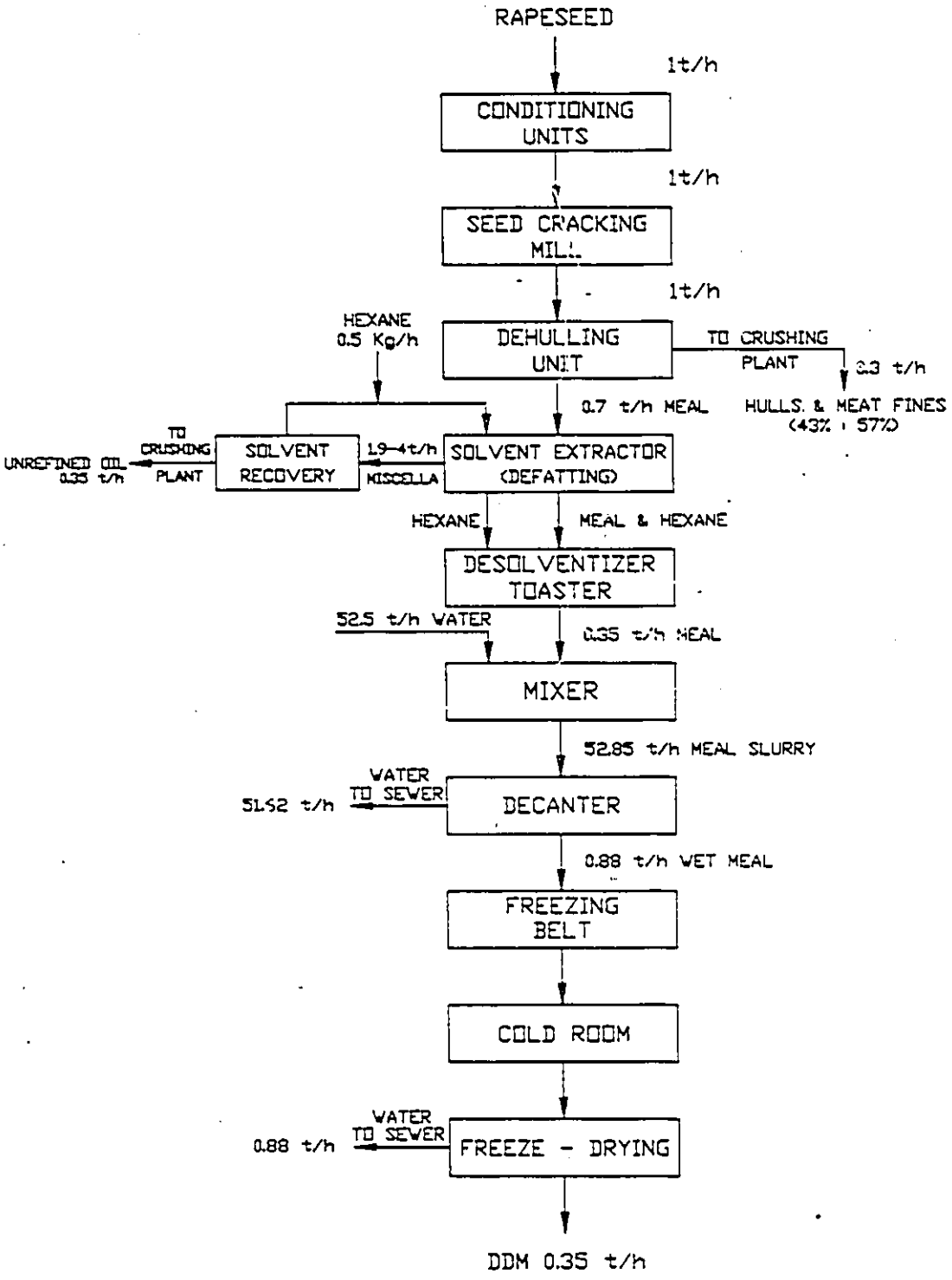
- Water to be used at a ratio of 150:1 meal (t/t). Taking the density of the water at 1 g/ml, then the flow rate of water will be 52.5 t/h. Taking an additional 5% for other purposes, the total amount of water needed is 157.5 t/t DDM.

- After decanting the water it was assumed that there is no loss in the meal with decanted water. Also that after decantation, W/M ratio is reduced to 2.5:1.

- Thus the flow rate of the decanted water to be sewered is calculated to be 51.62 t/h. The 0.88 t/h water in the wet meal will be removed by freeze drying. Thus the total water to be sewered will equal the 52.5 t/h added to the meal. In this calculation, the 8% moisture in the meal (i.e., only 0.028 t/h) was considered negligeable.

- The rate of producting the DDM is equal to that for the meal of 0.35 t/h.

Fig. A20.1: PROCESS FLOW SHEET.



2. ENERGY REQUIREMENT:

- For oil extraction:
  0.35 t steam/h (1 t/t DDM)

- For the Freeze-Drying Process (180.a):
  -- Steam: 3.8 t/t water evaporated (9.5 t/t DDM)
  -- Fuel oil (for cooling compressors): 0.7 t/t water evaporated (1.75 t/t DDM)
  -- Electricity: 700 kWh/t water evaporated (1 750 kWh/t DDM)

An additional energy estimated at 5 % of the above will be needed for other purposes.

Thus total energy needed per 1 000 t DDM will require:

Steam: (9.5 + 1.0) x 1 000 x 1.05 = 11 025 t

Fuel oil: 1.75 x 1 000 x 1.05 = 1 837.5 t

Electricity: 1 750 x 1 000 x 1.05 = 1 837 500 kWh

APPENDIX 21

DETAILS OF ECONOMIC ANALYSIS

a) FOR PRICE OF DDM OF $250.00/t
DEHULLED DETOXIFIED RAPESEED MEAL

TABLE I

ESTIMATED MANUFACTURING COST PER YEAR

PRODUCTS	PRODUCTION LEVEL (/000 t) ANNUAL (8400 HR/YR)
DEHULLED&DET MEAL	2.9
UNREFINED OIL	2.9
HULLS & FINES	2.5
TOTAL	8.4

DIRECT COSTS		$/YEAR	$/t NO.1	PC NS
RAW MATERIALS		1894096.	644.250	17.0
STEAM		213929.	72.765	1.9
ELECTRIC POWER		202585.	68.906	1.8
OIL		1188495.	404.250	10.7
PROCESS WATER		46305.	15.750	.4
PACKAGING		30752.	10.460	.3
SUPPLIES (5% OPC MAINT.)		80500.	27.381	.7
VEHICULE FUEL		10000.	3.401	.1
LABOUR				
DIRECT (12.00 $/HR)	504000.			
FRINGE (27.0 PC)	136080.			
SUBTOTAL	640080.	640080.	217.714	5.8
TOTAL DIRECT COSTS		4306742.	1464.878	38.8

PERIOD COSTS			
SUPER./QUAL. CONT./FACT. ADM. (25.00 PC DL)	126000.	42.857	1.1
MAINT./MAT. AND LAB. (5.00 PC FC)	1610000.	547.619	14.5
INSUR. AND TAX. (1.50 PC FC)	483000.	164.286	4.3
TOTAL PERIOD COSTS	2219000.	754.762	20.0
TOTAL MANUFACTURING COSTS	6525742.	2219.640	58.7
BULD. AND EQUI. DEPRECIATION	1937500.	659.013	17.4

TABLE II

ESTIMATED OPERATING PROFIT IN /000 $

GROSS SALE VALUES	/000 t	/000 $	$/t NO.1	PC NS
DEHULLED&DET MEAL	2.9	735.0	250.000	6.6
UNREFINED OIL	2.9	10290.0	3499.999	92.6
HULLS & FINES	2.5	370.4	147.000	3.3
TOTAL	8.4	11395.4	3875.999	102.6
SALES DEDUCTIONS (2.5PC GS)		284.9	96.900	2.6
NET SALES		11110.6	3779.098	100.0
TOTAL DIRECT COSTS		4306.7	1464.878	38.8
DIRECT MARGIN		6803.8	2314.220	61.2
PERIOD MANUFACTURING COSTS		2219.0	754.762	20.0
DEPRECIATION		1937.5	659.013	17.4
MANUFACTURING PROFIT		2647.3	900.445	23.8
GENERAL EXPENSES (5.0PC NS)		555.5	188.955	5.0
OPERATING PROFIT		2091.8	711.490	18.8
OPERATING PROFIT + DEPR.		4029.3	1370.503	36.3

TOTAL INVESTMENT	/000$	/000$
FIXED CAPITAL		32200.0
WORKING CAPITAL (20.0PC NS)		2222.1
RESEARCH COSTS		.0
TAXABLE GRANT OR ITC		1852.6
START UP		200.0
TOTAL		32769.5

TABLE III

CALCULATION OF CASH IN FLOWS AND PRESENT VALUES

YEAR	C.C.A. BUILDING /000 $	C.C.A. EQUIP. /000 $	OTHER EXPENSES /000 $	COLS 1+2+3 /000 $	CASH FLOW AFTER 42.0 % TAX: PROF BEFORE TAX + DEPR. /000 $	(5)*.420 /000 $	(6)*.580 /000 $	TOTAL /000 $	PV FACTOR AT	PRESENT VALUE
1	50.0	7520.0	.0	7570.0	.0	3179.4	.0	3179.4	.989	3143.3
2	97.5	15036.0	-1123.3	14010.2	905.1	5884.3	525.0	6409.3	.968	6204.2
3	92.6	7528.8	- 529.3	7092.1	2779.6	2978.7	1612.2	4590.9	.948	4351.1
4	88.0	23.0	.0	111.0	4029.3	46.6	2337.0	2383.6	.928	2211.9
5	83.6	23.4	.0	102.0	4029.3	42.9	2337.0	2379.8	.909	2162.3
6	79.4	14.7	.0	94.2	4029.3	39.5	2337.0	2376.5	.890	2114.2
7	75.4	11.8	.0	57.2	4029.3	36.6	2337.0	2373.6	.871	2067.5
8	71.7	9.4	.0	81.1	4029.3	34.1	2337.0	2371.0	.853	2022.1
9	68.1	7.5	.0	75.6	4029.3	31.8	2337.0	2368.8	.835	1977.9
10	64.7	6.0	.0	70.7	4029.3	29.7	2337.0	2366.7	.818	1934.9
11	61.4	4.8	.0	66.3	4029.3	27.8	2337.0	2334.8	.800	1893.0
12	58.4	3.9	.0	62.2	.0	26.1	.0	2913.0	.734	2283.0
TOTAL	890.8	30184.5	-1652.6	29422.8	35919.0	12357.6	20833.0	36077.4		32365.2

N.B. THE TOTAL CASH FLOW FOR YEAR 12 INCLUDES TERMINAL ALLOWANCE (TAX DEPRECIATED VALUE/NON TAXABLE) OF 1109.2 THOUSAND DOLLARS AND 80 PCT OF WORKING CAPITAL, I.E., 1777.7 THOUSAND DOLLARS

(1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11)

TABLE IV

LIST OF ANNUAL SUMS OF CAPITAL DISBURSEMENTS AND OTHER EXPENSES AND CALCULATION OF PRESENT VALUES

YEAR	AMOUNT /000 $	AT 2.1 PC	VALUE
1D	32200.0	.989	31834.7
2D	-12.2	.968	-11.9
3D	137.3	.948	130.2
4D	444.4	.928	412.4
5D	.0	.909	.0
6D	.0	.890	.0
7D	.0	.871	.0
8D	.0	.853	.0
9D	.0	.835	.0
10D	.0	.818	.0
11D	.0	.800	.0
TOTAL			32365.4

TABLE V

INCREMENTAL SOURCE AND USE OF FUNDS /000 $

YEAR	1	2	3	4	5	6	7	8	9	10
SOURCES										
NET SALES	.0	5555.3	8888.4	11110.6	11110.6	11110.6	11110.6	11110.6	11110.6	11110.6
LESS ALL CASH EXPENSES	.0	4650.1	6108.8	7081.3	7081.3	7081.3	7081.3	7081.3	7081.3	7081.3
OP PROF BEF DEPR & TAX	.0	905.1	2779.6	4029.3	4029.3	4029.3	4029.3	4029.3	4029.3	4029.3
INC.TAX.PAY.(MINUS MEANS TAX CR)	-3179.4	-5504.1	-1811.2	1645.7	1649.4	1652.8	1655.7	1658.2	1660.5	1662.6
TOTAL CASH INFLOW AFTER TAXES	3179.4	6409.3	4590.9	2383.6	2379.8	2376.5	2373.6	2371.0	2368.8	2366.7
USE OF FUNDS										
RESEARCH	.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
TAXABLE GRANT OR ITC	.0	1323.3	529.3	.0	.0	.0	.0	.0	.0	.0
FIXED CAPITAL										
- EQUIPMENT	30200.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
- BUILDING	2000.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
- SUBTOTAL	32200.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
WORKING CAPITAL INCREMENT	.0	1111.1	666.6	444.4	.0	.0	.0	.0	.0	.0
OTHER EXPENSES	.0	200.0	.0	.0	.0	.0	.0	.0	.0	.0
TOTAL CASH OUTFLOW	32200.0	-12.2	137.3	444.4	.0	.0	.0	.0	.0	.0
NET FUNDS GENERATED										
- ANNUAL	-29020.6	6421.5	4453.5	1939.2	2379.8	2376.5	2373.6	2371.0	2368.8	2366.7
- CUMULATIVE	-29020.6	-22599.1	-18145.5	-16206.4	-13826.5	-11450.0	-9076.4	-6705.3	-4336.6	-1969.9

TABLE V (CONT'D)

INCREMENTAL SOURCE AND USE OF FUNDS /000 $

DISCOUNTED CASH FLOW RETURN (PC/10 INCOME YEARS)		AFTER TAX PAYBACK (YEARS)	
START OF PROJECT	2.12	START OF PROJECT	10.83
START OF PLANT CONSTRUCTION	2.12	START OF PLANT CONSTRUCTION	10.83

DCFI IS GREATER THAN 100 PC

BREAK EVEN POINT			
PC OF FULL CAPACITY	100.00	TURNOVER RATIO	.339
OR			
GROSS SELLING PRICE OF	$/t		
PRODUCT NO. 1	250.000		
PRODUCT NO. 2	3500.000		
PRODUCT NO. 3	147.000		

TABLE VI

PRODUCT NAME	PRODUCTION M t/YR	G.S.PRICE $/t	PACKAGING $/t
1 DEHULLED&DET MEAL	2.94	250.000	10.460
2 UNREFINED OIL	2.94	3500.000	.000
3 HULLS & FINES	2.52	147.000	.000

RAW MATERIAL	(1) CONSUMPTION t/t No.1	(2) COMPONENT COST $/t	(3) TOTAL $/YEAR /000	(4) UNIT COST
1 RAPESEED	2.8600	225.000	1891.9	643.50
2 HEXANE	.0015	500.000	2.2	.75
			1894.1	644.25

RESEARCH COSTS ($/000)

YEAR	COST
1	0.
2	0.
3	0.
4	0.
5	0.
6	0.
7	0.
8	0.
9	0.
10	0.

TAXABLE GRANT OR INVESTMENT TAX CREDIT ($/000)

YEAR	AMOUNT
2	1323.
3	529.
5	0.
6	0.
7	0.
8	0.
9	0.
10	0.
11	0.
12	0.

FIXED CAPITAL ($/000)

	TOTAL COST	C.C.A. (PC)	DEPR. (YRS)	1st YEAR DISBURSEMENT
BUILDING	2000.	5.	40	2000.D
EQUIPMENT	30000.	50.SL	16	30000.D
EQUIPMENT	200.	20.DB	16	200.D

OTHER EXPENSES ($/000)

	YEAR	COST/YEAR
START UP	2D	200.

TABLE VII

DISCOUNTED CASH FLOW

YEAR	1	2	3	4	5	6
DISCOUNTED CASH FLOW RETURN (PC)						
START OF PROJECT	.00	.00	.00	.00	.00	.00
START OF PLANT CONSTRUCTION	.00	.00	.00	.00	.00	.00
TOTAL CASH INFLOW AFTER TAXES	3179.4	6409.3	4590.9	2383.6	2379.8	2376.5
NET PROFIT AFTER TAX	1241.9	4471.8	2653.4	446.1	442.3	439.0
NET OPERATING ASSETS (W.C.+TAX. DEP. F.A.)	24630.0	10607.6	3652.8	3986.1	3884.1	3790.0
NET RETURN ON NET OPERATING ASSETS (PC)	5.04	42.16	72.64	11.19	11.39	11.58
PC OF NET SALES						
A) DIRECT MARGIN	.00	61.24	61.24	61.24	61.24	61.24
B) OPERATING PROFIT	.00	-18.58	9.47	18.83	18.83	18.83

TABLE VII (CONT'D)

DISCOUNTED CASH FLOW

YEAR	7	8	9	10	11	12
DISCOUNTED CASH FLOW RETURN (PC)						
START OF PROJECT	.00	.00	.00	.00	.29	2.12
START OF PLANT CONSTRUCTION	.00	.00	.00	.00	.29	2.12
TOTAL CASH INFLOW AFTER TAXES	2373.6	2371.0	2368.8	2366.7	2364.8	2913.0
NET PROFIT AFTER TAX	436.1	433.5	431.3	429.2	427.3	975.5
NET OPERATING ASSETS (W.C.+TAX. DEP. F.A.)	3702.70	3621.6	3546.0	3475.3	3409.0	3346.7
NET RETURN ON NET OPERATING ASSETS (PC)	11.78	11.97	12.16	12.35	12.54	29.15
PC OF NET SALES						
A) DIRECT MARGIN	61.24	61.24	61.24	61.24	61.24	.00
B) OPERATING PROFIT	18.83	18.83	18.83	18.83	18.83	.00

b) FOR PRICE OF DDM OF $1 000.00/t DEHULLED DETOXIFIED RAPESEED MEAL

TABLE I

ESTIMATED MANUFACTURING COST PER YEAR

PRODUCTS	PRODUCTION LEVEL (/000 t) ANNUAL (8400 HR/YR)
DEHULLED&DET MEAL	2.9
UNREFINED OIL	2.9
HULLS & FINES	2.5
TOTAL	8.4

DIRECT COSTS		$/YEAR	$/t NO.1	PC NS
RAW MATERIALS		1894096.	644.250	14.3
STEAM		213929.	72.765	1.6
ELECTRIC POWER		202585.	68.906	1.5
OIL		1188495.	404.250	9.0
PROCESS WATER		46305.	15.750	.3
PACKAGING		30752.	10.460	.2
SUPPLIES (5.0PC MAINT.)		80500.	27.381	.6
VEHICULE FUEL		10000.	3.401	.1
LABOUR				
DIRECT (12.00 $/HR)	504000.			
FRINGE (27.0 PC)	136080.			
SUBTOTAL	640080.	640080.	217.714	4.8
TOTAL DIRECT COSTS		4306742.	1464.878	32.5
PERIOD COSTS				
SUPER./QUAL. CONT./FACT. ADM. (25.00 PC DL)		126000.	42.857	1.0
MAINT./MAT. AND LAB. (5.00 PC FC)		1610000.	547.619	12.1
INSUR. AND TAX. (1.50 PC FC)		483000.	164.286	3.6
TOTAL PERIOD COSTS		2219000.	754.762	16.7
TOTAL MANUFACTURING COSTS		6525742.	2219.640	49.2
BULD. AND EQUI. DEPRECIATION		1937500.	659.013	14.6

TABLE II

ESTIMATED OPERATING PROFIT IN /000 $

GROSS SALE VALUES	/000 t	/000 $	$/t NO.1	PC NS
DEHULLED&DET MEAL	2.9	2940.0	1000.000	22.2
UNREFINED OIL	2.9	10290.0	3499.999	77.6
HULLS & FINES	2.5	370.4	147.000	2.8
TOTAL	8.4	13600.4	4626.000	102.6
SALES DEDUCTIONS (2.5PC GS)		340.0	115.650	2.6
NET SALES		13260.4	4510.348	100.0
TOTAL DIRECT COSTS		4306.7	1464.878	32.5
DIRECT MARGIN		8953.7	3045.470	67.5
PERIOD MANUFACTURING COSTS		2219.0	754.762	16.7
DEPRECIATION		1937.5	659.013	14.6
MANUFACTURING PROFIT		4797.2	1631.695	36.2
GENERAL EXPENSES (5.0PC NS)		663.0	225.517	5.0
OPERATING PROFIT		4134.2	1406.178	31.2
OPERATING PROFIT + DEPR.		6071.7	2065.191	45.8

TOTAL INVESTMENT	/000$	/000$
FIXED CAPITAL		32200.0
WORKING CAPITAL (20.0PC NS)		2652.1
RESEARCH COSTS		.0
TAXABLE GRANT OR ITC		1852.6
START UP		200.0
TOTAL		33199.5

TABLE III

CALCULATION OF CASH IN FLOWS AND PRESENT VALUES

	C.C.A.				CASH FLOW AFTER 42.0 % TAX					
YEAR	BUILDING /000 $	EQUIP. /000 $	OTHER EXPENSES /000 $	COLS 1+2+3 /000 $	PROF BEFORE TAX + DEPR. /000 $	(5)*.420 /000 $	(6)*.580 /000 $	TOTAL /000 $	PV FACTOR AT	PRESENT VALUE
1	50.0	7520.0	.0	7570.0	.0	3179.4	.0	3179.4	.958	3048.7
2	97.5	15036.0	-1123.3	14010.2	1926.3	5884.3	1117.3	7001.6	.885	6198.3
3	92.6	7528.8	- 529.3	7092.1	4413.5	2978.7	2559.8	5538.5	.818	4529.8
4	88.0	23.0	.0	111.0	6071.7	46.6	3521.6	3568.2	.756	2696.1
5	83.6	18.4	.0	102.0	6071.7	42.9	3521.6	3564.4	.698	2488.1
6	79.4	14.7	.0	94.2	6071.7	39.5	3521.6	3561.1	.645	2296.5
7	75.4	11.8	.0	87.2	6071.7	36.6	3521.6	3558.2	.596	2119.9
8	71.7	9.4	.0	81.1	6071.7	34.1	3521.6	3555.6	.550	1957.0
9	68.1	7.5	.0	75.6	6071.7	31.8	3521.6	3553.3	.508	1806.8
10	64.7	6.0	.0	70.7	6071.7	29.7	3521.6	3551.3	.470	1668.3
11	61.4	4.8	.0	66.3	6071.7	27.8	3521.6	3549.4	.434	1540.4
12	58.4	3.9	.0	62.2	.0	26.1	.0	3257.0	.401	1305.9
TOTAL	890.8	30184.5	-1652.6	29422.8	54913.2	12357.6	31849.7	47438.0		31653.7

N.B. THE TOTAL CASH FLOW FOR YEAR 12 INCLUDES TERMINAL ALLOWANCE (TAX DEPRECIATED VALUE/NON TAXABLE) OF 1109.2 THOUSAND DOLLARS AND 80 PCT OF WORKING CAPITAL, I.E., 2121.7 THOUSAND DOLLARS

(1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11)

TABLE IV

LIST OF ANNUAL SUMS OF CAPITAL DISBURSEMENTS AND OTHER EXPENSES AND CALCULATION OF PRESENT VALUES

YEAR	AMOUNT /000 $	AT 6.1 PC	VALUE
1D	32200.0	.958	30855.6
2D	202.7	.885	179.5
3D	266.3	.818	217.8
4D	530.4	.756	400.8
5D	.0	.698	.0
6D	.0	.645	.0
7D	.0	.596	.0
8D	.0	.550	.0
9D	.0	.508	.0
10D	.0	.470	.0
11D	.0	.434	.0
TOTAL			31653.7

TABLE V

INCREMENTAL SOURCE AND USE OF FUNDS /000 $

YEAR	1	2	3	4	5	6	7	8	9	10
SOURCES										
NET SALES	.0	6630.2	10608.3	13260.4	13260.4	13260.4	13260.4	13260.4	13260.4	13260.4
LESS ALL CASH EXPENSES	.0	4703.9	6194.8	7188.8	7188.8	7188.8	7188.8	7188.8	7188.8	7188.8
OP PROF BEF DEPR & TAX	.0	1926.3	4413.5	6071.7	6071.7	6071.7	6071.7	6071.7	6071.7	6071.7
INC.TAX.PAY.(MINUS MEANS TAX CR)	-3179.4	-5075.2	-1125.0	2503.5	2507.2	2510.6	2513.5	2516.0	2518.3	2520.4
TOTAL CASH INFLOW AFTER TAXES	3179.4	7001.6	5538.5	3568.2	3564.4	3561.1	3558.2	3555.6	3553.3	3551.3
USE OF FUNDS										
RESEARCH	.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
TAXABLE GRANT OR ITC	.0	1323.3	529.3	.0	.0	.0	.0	.0	.0	.0
FIXED CAPITAL										
- EQUIPMENT	30200.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
- BUILDING	2000.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
- SUBTOTAL	32200.0	.0	.0	.0	.0	.0	.0	.0	.0	.0
WORKING CAPITAL INCREMENT	.0	1326.0	795.6	530.4	.0	.0	.0	.0	.0	.0
OTHER EXPENSES	.0	200.0	.0	.0	.0	.0	.0	.0	.0	.0
TOTAL CASH OUTFLOW	32200.0	202.7	266.3	530.4	.0	.0	.0	.0	.0	.0
NET FUNDS GENERATED										
- ANNUAL	-29020.6	6798.8	5272.2	3037.8	3564.4	3561.1	3558.2	3555.6	3553.3	3551.3
- CUMULATIVE	-29020.6	-22221.8	-16949.6	-13911.8	-10347.4	-6786.3	-3228.1	327.6	3880.9	7432.2

TABLE V (CONT'D)

INCREMENTAL SOURCE AND USE OF FUNDS /000 $

DISCOUNTED CASH FLOW RETURN (PC/10 INCOME YEARS)		AFTER TAX PAYBACK (YEARS)	
START OF PROJECT	8.08	START OF PROJECT	7.91
START OF PLANT CONSTRUCTION	8.08	START OF PLANT CONSTRUCTION	7.91

DCFI IS GREATER THAN 100 PC

BREAK EVEN POINT			
PC OF FULL CAPACITY	100.00	TURNOVER RATIO	.399
OR			
GROSS SELLING PRICE OF	$/t		
PRODUCT NO. 1	1000.000		
PRODUCT NO. 2	3500.000		
PRODUCT NO. 3	147.000		

TABLE VI

PRODUCT NAME	PRODUCTION M t/YR	G.S.PRICE $/t	PACKAGING $/t
1 DEHULLED&DET MEAL	2.94	1000.000	10.460
2 UNREFINED OIL	2.94	3500.000	.000
3 HULLS & FINES	2.52	147.000	.000

RAW MATERIAL	(1) CONSUMPTION t/t No.1	(2) COMPONENT COST $/t	(3) TOTAL $/YEAR /000	(4) UNIT COST
1 RAPESEED	2.8600	225.000	1891.9	643.50
2 HEXANE	.0015	500.000	2.2	.75
			1894.1	644.25

RESEARCH COSTS ($/000)

YEAR	COST
1	0.
2	0.
3	0.
4	0.
5	0.
6	0.
7	0.
8	0.
9	0.
10	0.

TAXABLE GRANT OR INVESTMENT TAX CREDIT ($/000)

YEAR	AMOUNT
2	1323.
3	529.
5	0.
6	0.
7	0.
8	0.
9	0.
10	0.
11	0.
12	0.

FIXED CAPITAL ($/000)

	TOTAL COST	C.C.A. (PC)	DEPR. (YRS)	1st YEAR DISBURSEMENT
BUILDING	2000.	5.	40	2000.D
EQUIPMENT	30000.	50.SL	16	30000.D
EQUIPMENT	200.	20.DB	16	200.D

OTHER EXPENSES ($/000)

	YEAR	COST/YEAR
START UP	2D	200.

TABLE VII

DISCOUNTED CASH FLOW

YEAR	1	2	3	4	5	6
DISCOUNTED CASH FLOW RETURN (PC)						
START OF PROJECT	.00	.00	.00	.00	.00	.00
START OF PLANT CONSTRUCTION	.00	.00	.00	.00	.00	.00
TOTAL CASH INFLOW AFTER TAXES	3179.4	7001.6	5530.5	3560.2	3564.4	3561.1
NET PROFIT AFTER TAX	1241.9	5064.1	3601.0	1630.7	1626.9	1623.6
NET OPERATING ASSETS (W.C.+TAX. DEP. F.A.)	24630.0	10822.5	3996.7	4416.1	4314.1	4219.9
NET RETURN ON NET OPERATING ASSETS (PC)	5.04	46.79	90.10	36.93	37.71	38.47
PC OF NET SALES						
A) DIRECT MARGIN	.00	67.52	67.52	67.52	67.52	67.52
B) OPERATING PROFIT	.00	-.17	23.34	31.18	31.18	31.18

TABLE VII (CONT'D)

DISCOUNTED CASH FLOW

YEAR	7	8	9	10	11	12
DISCOUNTED CASH FLOW RETURN (PC)						
START OF PROJECT	.00	.32	3.20	5.33	6.93	8.08
START OF PLANT CONSTRUCTION	.00	.32	3.20	5.33	6.93	8.08
TOTAL CASH INFLOW AFTER TAXES	3558.2	3555.6	3553.3	3551.3	3549.4	3257.0
NET PROFIT AFTER TAX	1620.7	1618.1	1615.8	1613.8	1611.9	1319.5
NET OPERATING ASSETS (W.C.+TAX. DEP. F.A.)	4132.7	4051.6	3976.0	3905.2	3838.9	3776.7
NET RETURN ON NET. OPERATING ASSETS (PC)	39.22	39.94	40.64	41.32	41.99	34.94
PC OF NET SALES						
A) DIRECT MARGIN	67.52	67.52	67.52	67.52	67.52	.00
B) OPERATING PROFIT.	31.18	31.18	31.18	31.18	31.18	.00