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The quantitation of glucosinolates in rapeseed

John Thomas Tholen
University of Wollongong

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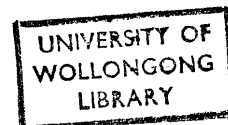
**THE QUANTITATION OF GLUCOSINOLATES
IN RAPESEED.**

**A thesis submitted in partial fulfilment of the
requirement for the award of the degree**

of

MASTER OF SCIENCE (Honours)

from



THE UNIVERSITY OF WOLLONGONG

by

JOHN THOMAS THOLEN

**DEPARTMENT OF CHEMISTRY
JANUARY 1991**

For my wife, Ae-Soon Tholen.

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SUMMARY

The aim of this project was to develop in the first instance, a method for determination of the total glucosinolate (GSL) content in rapeseed, which could readily be adopted by a non-specialist chemical laboratory. Chapter 2 of this thesis describes a rapid yet simple assay which employs a reflectance measurement. This method is capable of being used by plant breeders and non technical personnel at collection points of silos.

Clinistix*, an enzyme-linked assay, was selected from a range of glucose test strips, since these were found to provide the greatest sensitivity and reproducibility.

The hydrolysis of the GSLs in rapeseed by endogenous myrosinase to liberate glucose, was achieved at pH 9.0, within 5 minutes. The liberation of glucose from sources other than GSLs was observed only at lower pH values. The average free glucose in rapeseed was 3 μ mole glucose per g seed (Table 4).

Inhibitors of the Clinistix enzymes were readily removed with activated charcoal, which also played a role in lowering the pH of the final extract which needed to be approximately pH 5 to ensure a clear colourless supernatant.

A reflectometer was developed, TruBluGlu meter, which incorporated a commercial AMES readhead assembly. This meter provided a direct digital display of the GSL content with function keys giving step by step instruction.

The values for GSL content of four rapeseed varieties were determined by reflectance and compared with those determined by the thymol method. The close correlation between the two methods suggests that the reflectance method can give accurate GSL values over the range normally found in rapeseed samples (5 to 30 μ mole/g seed).

During the development of the reflectance method, it was necessary to compare the results with a standard reference method. The thymol method was employed, however modifications to the method of Brzezinski and Mendelewski, were found to be necessary for reproducible results.

The results given in Chapter 3 of this thesis detail the experiments aimed at optimising the conditions for the determination of GSLs in rapeseed (*Brassica napus L*) using the colourimetric thymol procedure. Glucose could not be substituted for sinigrin when preparing a standard curve for GSL determination unless a correction factor was employed, since these two chemicals did not give equivalent colour yields per mole at 100 °C. The value of the correction factor was found to depend on the concentration of sulfuric acid. It was found necessary to increase the amount of thymol present to a final value of 0.23 % to ensure complete reaction of GSLs.

The modified thymol method for GSL determination has been found to provide reproducible results using either seed or meal of rapeseed.

HPLC determination of individual DS GSLs has been examined in Chapter 4. The on-column desulfation of GSLs was found to lead to a significant under estimation of GSLs in rapeseed, which could be attributed to glycosidase enzymes present in the crude sulfatase. The sulfatase purified by gel permeation gave constant GSL recovery, however the purification scheme using a published ion-exchange method demonstrated a gradual loss in DS GSL recovery with time. The elution profile of protein and enzyme activities from gel permeation displayed a baseline separation of the aryl sulfatase from the β -glucosidase and β -glucuronidase activities.

Further purification of the aryl sulfatase using ion-exchange and Con A-Sepharose gave a 50 fold increase in the specific activity with 61 % recovery.

Gel electrophoresis of the aryl sulfatase showed a native molecular weight of approximately 100,000. SDS PAGE revealed a subunit molecular weight of approximately 32,000, indicating a possible trimer. The aryl sulfatase when also examined by isoelectric focussing, demonstrated a broad range of pIs from 4.2 to 5.6. An SDS PAGE gel was blotted onto a PVDF membrane and the aryl sulfatase N terminal sequence determined as : Ala Glu Asp Pro Pro Lys Val Ile Leu Pro Glu Val Phe Glu.

Kinetic studies on aryl sulfatase revealed optimum activity at pH 5.5. At pH 5.8 aryl sulfatase was inhibited by the substrate sinigrin, with no detectable activity at 1.0 mM. Substrate inhibition was also observed with glucobrassicin at concentrations greater than 1.0 mM.

A scheme for the rapid analysis of DS GSLs is proposed in chapter 4, which utilises initial purification of the GSLs on DEAE Sephadex A-25 followed by elution with potassium sulfate. An aliquot of the eluate is mixed with purified aryl sulfatase, before being analysed by HPLC.

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ABBREVIATIONS

Act.	: Activated
BM	: Boehringer-Mannheim
Con A	: Concanavalin A
DEAE	: diethylaminoethyl
DS GSL	: desulfated glucosinolate
EDTA	: ethylenediaminetetraacetic acid
GC	: Gas chromatography
GSL	: Glucosinolate
GLB	: glucobrassicin
HPLC	: High Performance Liquid Chromatography
oNPGlu	: o-nitrophenyl- β - D-glucopyranoside
oNPGal	: o-nitrophenyl- β - D-galactopyranoside
PAGE	: Polyacrylamide gel electrophoresis.
pI	: Isoelectric point
PIT	: Phenylisothiocyanate
pNCS	: p-nitrocatechol sulfate
PVDF	: Polyvinylidene difluoride
R.P.	: reversed phase
SD	: standard deviation
SDS	: Sodium dodecyl sulfate
TBG	: TruBluGlu
Tris	: tris (hydroxymethyl) amino methane
uv	: Ultraviolet
μ mole	: micromole

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The Cruciferae and several other plant families are known to possess a group of secondary metabolites known as glucosinolates (GSLs).¹

The Cruciferae family is of particular interest because it comprises a number of agriculturally important crops, such as cole crops (*Brassica oleracea*), condiments (*B. nigra*; *B. juncea*), oilseeds for example rapeseed (*B. campestris* ; *B. napus*) and forage crops.

Approximately 100 β -D-thioglucosides have been identified with about twenty occurring in the Brassica genus alone. The concentration of GSLs within the parenchymatous tissues of plants varies depending on both the cultivar and the part of the plant.^{2,3} Environmental conditions also have a major impact on the levels of these secondary metabolites.^{4,5}

Associated with the GSLs are a group of β -thioglucosidase (3.2.3.1) enzymes.^{6,7} The compartmentalisation of the endogenous myrosinases within specialised cells (idioblasts), is characteristic of all tissues of isothiocyanate producing plants and provides a mechanism by which the production of the toxic glucosinolate breakdown products may be controlled. During cellular disruption these enzymes catalyze the initial hydrolysis of the GSLs, giving rise to aglucones (some of which are unstable), sulfate and glucose.^{8,9} Further breakdown of an unstable aglycone occurs spontaneously, giving rise to isothiocyanates, thiocyanates or nitriles depending on the conditions and the type of GSLs present.^{10,11} The characteristic pungent volatile flavour of *Brassica* crops originates from the aglucone products (isothiocyanates, nitriles, thiocyanates, and

oxazolidine- 2-thiones).

Numerous studies have demonstrated that consumption of these chemicals in food material can result in a variety of physiological and biochemical effects.¹² Additional investigations have revealed the involvement of GSLs and their breakdown products as feeding attractants for some insects and as feeding deterrents for others.¹³

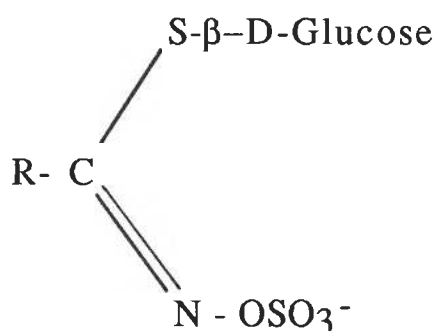
The occurrence of GSLs in the medium supporting the roots of some plants containing GSLs¹⁴ and the release of volatiles from the leaves¹⁵ have ramifications with regard to allelopathy. A number of plants have been shown to exhibit allelopathic activity, whereby chemicals released by the roots or leaves have some affect, either harmful or beneficial on neighbouring plants and animals.^{16,17} These properties of GSLs and associated breakdown products will be discussed in more detail in following sections.

As a result of the adverse effects caused by GSLs and associated breakdown products, investigations directed at reducing GSL content in potentially valuable feed sources is of major importance.^{18,19}

Rapeseed (*Brassica napus*), an oil seed, is of commercial importance as a source of edible cooking oil. The tissue after oil extraction (seed meal) contains high quality protein, being rich in essential amino acids. Hence rapeseed devoid of GSLs would have considerable commercial value. Plant breeders in recent years have reduced the GSL content in low GSL rapeseed samples, from more than 100 to less than 30 $\mu\text{mol/g}$ seed. This improvement has been achieved in part by the development of increasingly sensitive and accurate methods of GSL analysis.

1.2 CHEMISTRY OF GLUCOSINOLATES AND BREAKDOWN PRODUCTS.

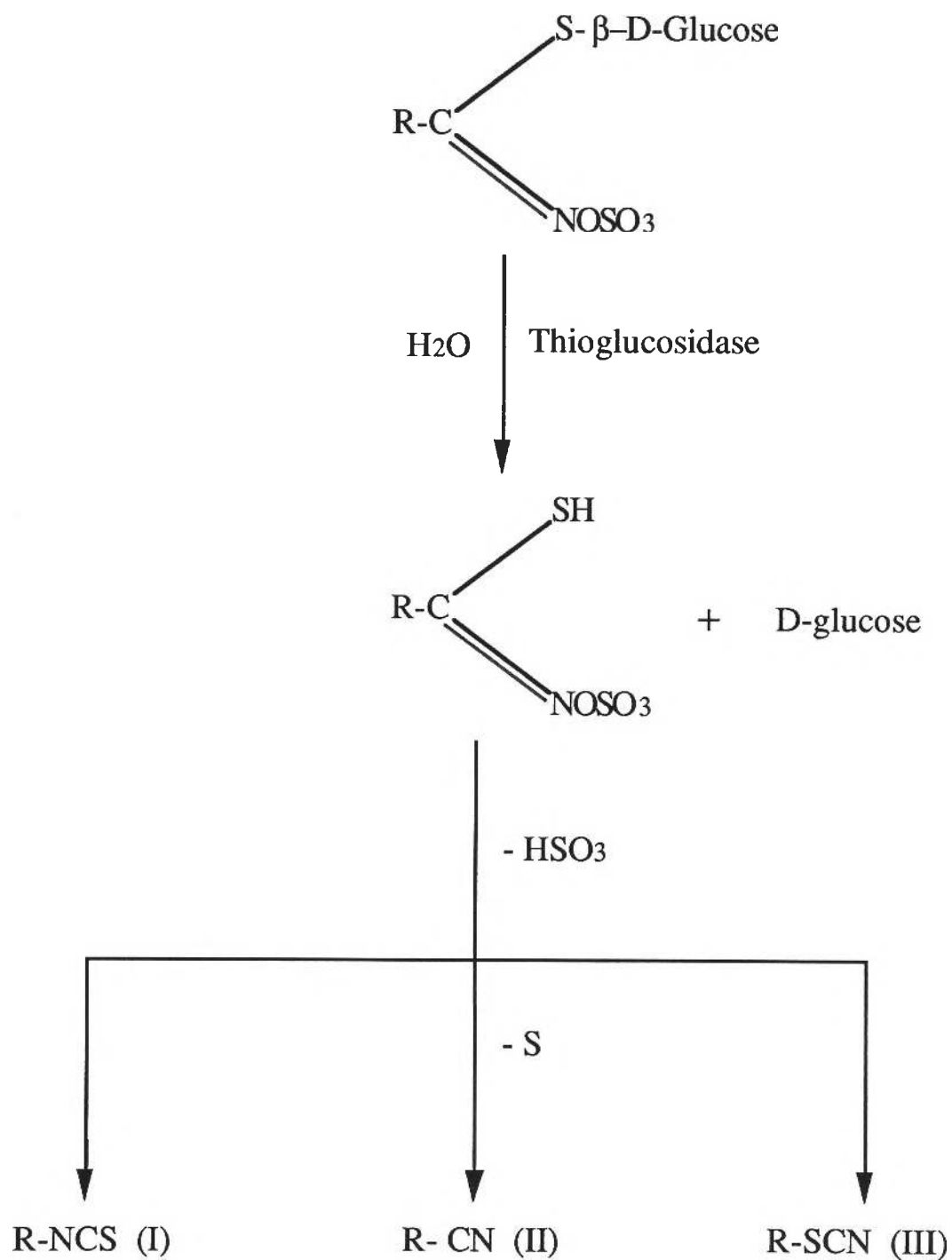
The general structure of GSLs was established in 1956 by Ettlinger and Lundeen (shown below).²⁰ The R moiety can vary from simple alkyl groups to more complex indolyl structures. GSLs, such as sinapic acid esters have also been identified in cruciferous seeds.²¹



The formation of the unstable aglucone by myrosinase activity is spontaneously followed by a Lossen rearrangement (Scheme 1) which can produce the corresponding isothiocyanate.¹ Indolyl GSLs however, give rise to thiocyanates, with the indole migrating to the sulfur rather than the nitrogen (III)²².

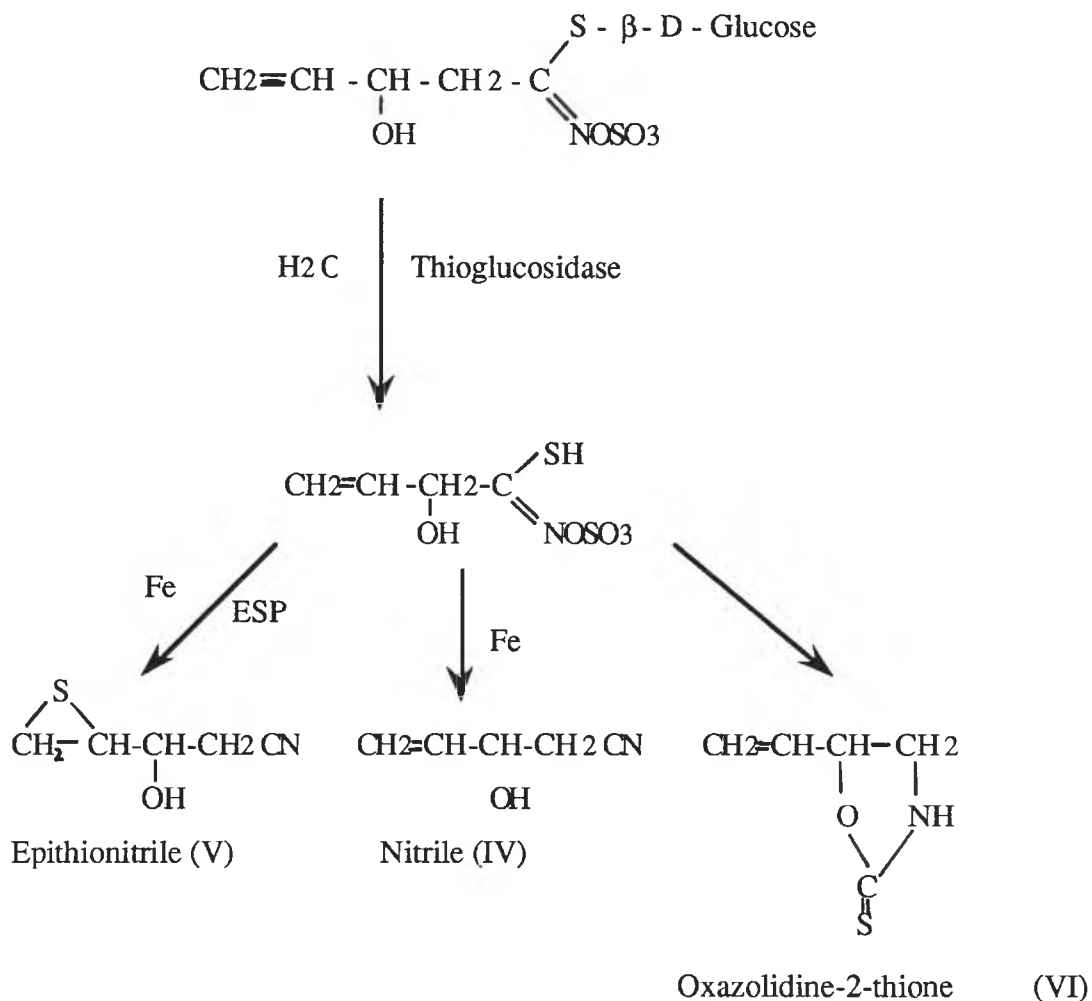
In some plants nitrile (II), hydroxynitriles (IV) and epithionitrile (V) derivatives may result^{23,24,25} (Scheme 1 and 2).

When ferrous ion and acidic conditions occur hydroxynitriles have been found to predominate.^{26,27}



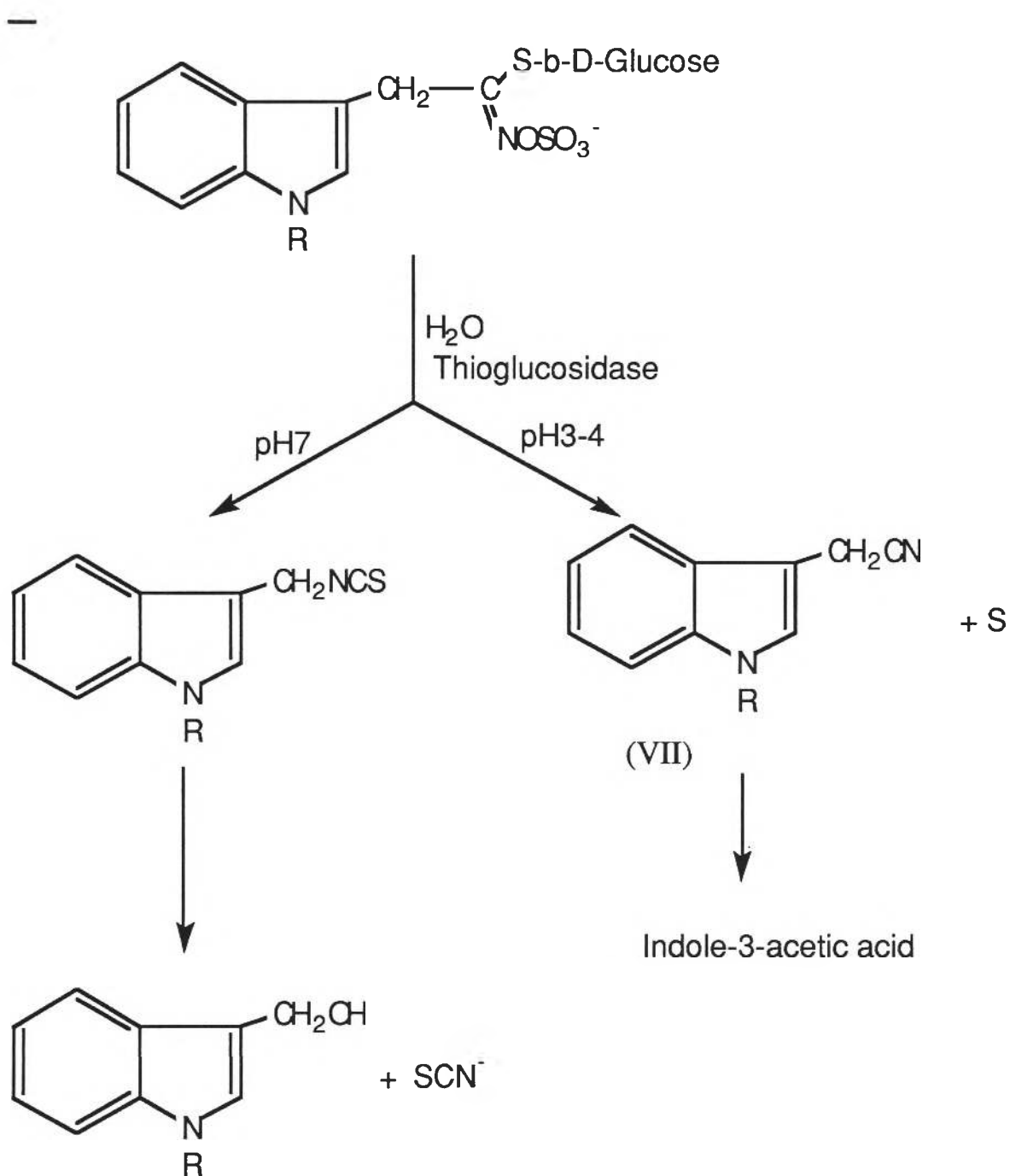
Scheme 1. Products of myrosinase hydrolysis of GSLs.¹²

GSLs such as progoitrin possess a β - hydroxyl group (Scheme below), which after degradation to an isothiocyanate undergo spontaneous cyclization to the oxazolidine-2-thione (VI), especially under high pH conditions.



Scheme 2. The products of myrosinase hydrolysis of progoitrin :
(2-hydroxy-3- butenyl glucosinolate) ¹²

Indolyl GSLs which may represent as much as 50 % of the total GSL in rapeseed give rise to unstable isothiocyanates, particularly at higher pH. Under acid conditions however, indolyl-3-acetonitrile(VII) and elemental sulfur are produced during breakdown (Scheme 3). The formation of indolyl-3-acetonitrile *in vivo* may be a precursor for indole-3-acetic acid, a potent auxin.



R = H or OCH₃

Scheme 3. The products of Myrosinase hydrolysis of Indole GSL.¹²

1.3. UNDESIRABLE PROPERTIES OF GLUCOSINOLATES

The inclusion of GSLs in the feeds of livestock or poultry has been demonstrated to affect the flavour of the food products derived from those animals. One reported case involved the contamination of feed with a small quantity of stinkweed (50 g), which led to the production of onion flavoured milk.²⁸ In another report, poultry and eggs developed a fishy taint after chickens were given rapeseed meal. One GSL : progoitrin (2-hydroxy-3-butenyl-glucosinolate) ingested by poultry was hydrolysed by bacterial thioglucosidase in the gastrointestinal tract. The corresponding GSL breakdown product (-)5-vinylloxazolidine-2-thione was found to be a potent inhibitor of trimethylamine oxidase. Inhibition of this liver enzyme then allowed the accumulation of trimethylamine responsible for the fishy taint.²⁹ The (-)5-vinyl-oxazolidine-2-thione also interfered with thyroxine synthesis leading to hypothyroidism (thyroid enlargement).^{30,76}

Other GSL breakdown products such as 2-propenylisothiocyanate,³¹ 3-methylsulfonyl- and propylisothiocyanate³² are also goitrogenic. The indolyl GSLs which produce thiocyanate ion are goitrogenic only when there is iodine deficiency. This occurs because the thiocyanate ion has the same ionic radius as iodine and is able to compete with iodine for uptake into the thyroid.³³

Numerous studies involving feeding experiments have also shown considerable enlargement of adrenal gland, kidney and liver.¹² The inclusion of rapeseed meal in poultry diet was also associated with liver hemorrhage (Liverhemorrhagic syndrome) which is believed to be caused by hydroxynitriles and intact GSLs.³⁴ Significant weight losses have also been reported, with epithiobutane being responsible for significant weight loss and lesions

to the liver, pancreas and kidney.

The GSL breakdown product 2-propenylisothiocyanate has been reported to exhibit a number of effects such as inhibition of protein synthesis, interference with sugar metabolism, elevation of hepatic plasma levels in rat, reduction in blood coagulation times and the reduction in serum vitamin A and β -carotene A in liver. The GSL breakdown product phenylisothiocyanate has also been shown to be cytotoxic.¹²

1.4 ALLELOPATHIC ASPECTS OF GLUCOSINOLATES.

Chemical interactions between plants was first reported in 1832 by M.A.P. de Candolle.³⁵

Many allelochemicals produced by plants interact with soil microbes. Such interactions may cause degradation of the allelochemical into simpler or more toxic forms. The myriad of chemicals that form, may act directly or indirectly on other species giving rise to a complex network of interactions, that may be harmful or beneficial.¹³

There have been numerous reports concerning the activity of plant secondary metabolites such as GSLs, and their interaction with insects, fungi, mammals and other plant species.^{12,36,38}

1.4.1 PHYTOTOXICITY

Plants of the mustard family contain powerful volatile inhibitors of germination and growth. The GSL degradation products allyl-isothiocyanate and β -phenylisothiocyanate, for example, are potent inhibitors of seed germination.³⁶

The possible routes by which the allelochemicals may be released from plant material are depicted in Figure 5. Chemicals can originate from living leaves as volatiles or leachates or from roots through exudation or sloughing off dead tissues or from leaf litter on the soil surface.³⁶

In the annual grasslands of California, *Brassica nigra* (black mustard) an introduced species, has become established in almost pure strands. This appears to be the result of allelopathic mechanisms where water soluble toxins are leached from the dead

stalks and leaf material. The alleochemicals so far implicated are the GSLs : allylisothiocyanate and 2-phenylethylisothiocyanate.³⁶

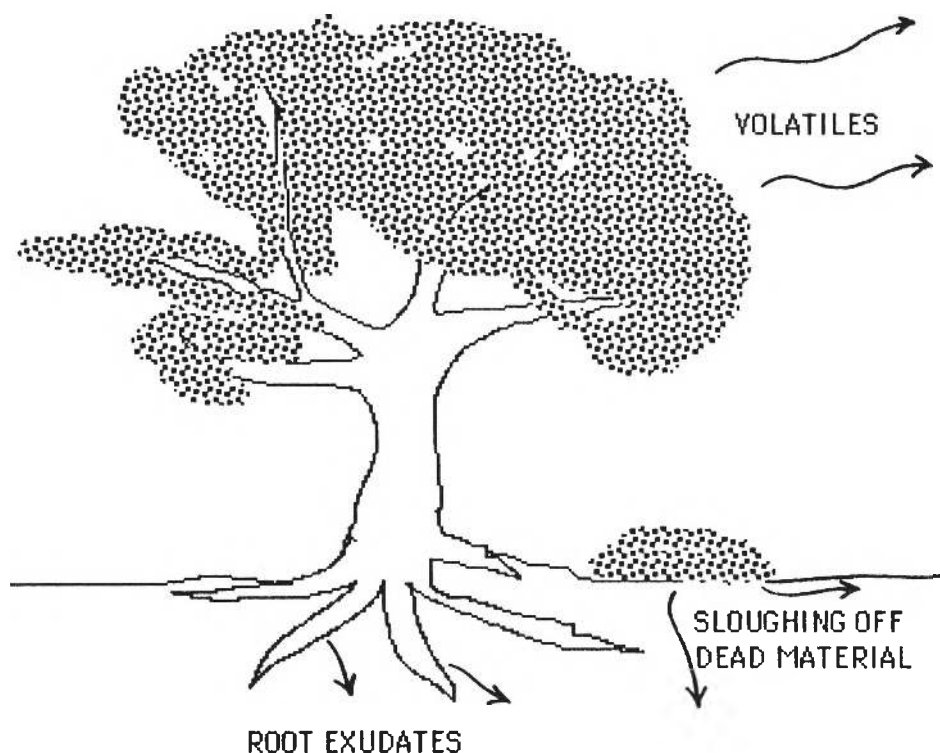


Figure 1. Chemical release pathways.

Evanari (1949) showed that the germinating seeds of *B. nigra* were also able to inhibit germination of wheat *in vitro*.³⁷

The cultivation of a commercially important dye plant called woad (*Isatis tinctoria*) during the Middle Ages in England, was subject to licensing because of the reported loss of soil fertility after cultivation. The phytotoxic effect of woad may be derived from the indolyl GSLs which accumulate in the leaves and roots of this herb. During tissue culture it was reported that intact GSLs from woad were released into the medium.¹⁴ The intact GSL 3-indolyl-

methylGSL and its more active degradation product 3-indolyl-acetonitrile (at 100 mM) inhibited growth of wheat and clover, however at still lower concentrations a growth enhancing effect was reported, which is a typical response elicited by an auxin.

1.4.2 PLANT-INSECT INTERACTIONS.

The feeding behaviour of phytophagous insects has been investigated more widely. Selection or avoidance of potential host plants by such insects is guided by a complex combination of physical and chemical stimuli. The chemical factors involved are generally divided into three phases namely orientation, feeding and oviposition.

Many insects, such as flea beetles (*Phyllotreta cruciferae* and *P. striolata*), cabbage root fly (*Phorbia floralis*), aphids, vegetable weevil (*Listroderes obliquus*), cabbage butterflies (*Pieris rapae*) and the diamondback moth (*Plutella maculipennis*), are attracted to plants containing GSLs.⁴³ Several insects such as the cabbage looper, armyworms and aphids are major pests on Cruciferae. The adult cabbage maggot (*Hylemya brassicae*) is attracted to the volatile aglucone 2-propenylisothiocyanate, however the presence of the parent intact GSL stimulates oviposition.³⁸ The 2-propenyl-, *p*-hydroxybenzyl- and benzyl GSLs are effective in stimulating oviposition.¹²

Cruciferous weed species *Camelina sativa* or *Coronopus didymus* lack volatile GSLs and do not attract or stimulate oviposition. Those plants with a greater degree of breakdown products appear to have a higher rate and degree of egg deposition.³⁹

Many Cruciferous plants contain the GSL sinigrin which during hydrolysis forms the aglucone allylisothiocyanate. Both the intact and volatile aglucone have been shown to be involved in the host selection and behaviour of several insects.⁴⁰ The presence of specific chemicals may offer protection from one insect pest, however the same toxins may increase herbivory by other insects.

In the case of the Cabbage white butterfly (*Pieris brassicae* and *P. rapae*) mustard oil accumulated in the larvae.⁴⁰ The *Pieris* species having co-evolved with a Brassica, have a mechanism for avoiding the toxicity of the aglucone. Traynier (1979), has observed the recognition of GSLs by cabbage butterflies through receptors located in their feet.⁴¹

Insects in at least six orders sequester a variety of plant natural products⁴² including alkaloids,⁴³ cardenolides,⁴⁴ mustard oils⁴¹ and cannabinoids⁴⁵ and it was therefore not surprising that sinigrin and mustard oils have been identified in the pupae of the large cabbage white butterfly and the small cabbage white butterfly. It is assumed that these have been sequestered by the larvae from its food plant and stored. Recent data has revealed a high oviposition in the cabbage white butterfly in response to indole GSLs.⁴⁶

With improved methods for isolating the GSLs a reinvestigation of *Pieris brassicae* and *P. rapae* may be warranted.

1.5. QUANTITATION

Methods for measuring GSLs in rapeseed meal can be categorised into two main approaches :

1. Those methods requiring the reaction of the intact GSL or one of its breakdown products with a chromogenic substance which in turn can be measured spectrophotometrically and
2. Methods involving the use of chromatographic equipment such as gas chromatography (GC) or High Performance Liquid Chromatography (HPLC).

Many methods for the isolation, separation and quantitation of total GSLs in seeds and meal of rapeseed have been published.⁴⁷⁻⁵⁰ The early methods such as gravimetric assay of inorganic sulfate⁴⁷ or the combined spectroscopic determination of oxazolidinethione coupled with titration of the volatile isothiocyanates after steam distillation were both time consuming and labour-intensive.⁴⁸ These methods also lacked sensitivity and precision, but more seriously, inaccuracy resulted from measurement of only the volatile isothiocyanate ions as a measure of total GSL content. More recent methods of GSL measurement have enabled improved sensitivity and accuracy. The following sections discuss this in greater detail.

1.5.1 **COLORIMETRIC DETERMINATION OF GLUCOSINOLATES**

These methods are concerned only with the total GSL content in the rapeseed sample and offer no information about the relative amounts of individual GSLs.

Such methods are best suited for centres concerned with screening rapeseed samples for acceptable total GSL levels.

The use of non-specific complexation of the intact GSL or the enzymic measurement of the glucose resulting from GSL hydrolysis, has been in use for many years. These methods will be treated as two groups, those involved in colorimetric measurement of a GSL hydrolysis product (a) and those that measured the intact GSL by complexation (b).

a) Enzymic Glucose Determination

The indirect quantitation of GSLs after myrosinase hydrolysis, involved GC determination of the liberated glucose.⁴⁹ The use of GC for quantitating glucose could be substituted for more sensitive enzyme linked assays,⁵⁰ which utilise either hexokinase + ATP/glucose-6-phosphate dehydrogenase + NADP (e.g. Glucose-UV-Test)⁵¹ or by the glucose oxidase/peroxidase-system (e.g. Glukotest, Boehringer).⁵² These methods are all prone to interferences by chemicals in the extract, which are coloured or by inhibition of colour development with the glucose reagent. The latter can result in under estimation of the GSL levels.

A major advance in GSL analysis occurred when Thies (1979) described the use of ion-exchange chromatography as a means of concentrating and purifying the GSLs from plant tissue.⁵³ The use of microcolumns packed with DEAE Sephadex A-25 provided not only a means of concentrating the GSLs, but at the same time many of the interfering substances as well as free glucose were eliminated by washing prior to hydrolysis and elution. On-column hydrolysis of the GSLs by addition of myrosinase

liberated glucose which was then measured enzymically.⁵⁴ The main disadvantage was the number of steps involved. Beginning with intact seed, the oil must be removed and the seed enzymes inactivated before GSL extraction. The intact GSLs are isolated by ion-exchange chromatography and hydrolysed with myrosinase before eluting the liberated glucose. Such methods are laborious and not ideally suited for relatively unskilled personnel attempting batch wise screening of GSL content.

The use of Reflectometry for measurement of the glucose colour reaction was first reported by Thies (1987). The glucose liberated after myrosinase hydrolysis was measured using glucose test paper impregnated with glucose oxidase/peroxidase enzymes and a chromogen. Interfering compounds were removed by filtration of the rapeseed extract through a charcoal filter unit.⁵⁵

The application of glucose test strips offered a fast and simple means of quantitating the glucose. The method however still required the boiling of seed to inactivate seed enzymes prior to maceration and the addition of myrosinase. The added difficulties of accurately applying the sample solution to the paper strip and the possible failure of the charcoal filter was also apparent. This method also lacked sensitivity, being suited for the higher GSL containing varieties (up to 100 μ mole GSL/g seed).

More recent developments utilising reflectance, involved the selective hydrolysis of GSLs in the seed by the endogenous myrosinase. The liberated glucose was measured by a glucose test strip and read on a calibrated meter for the direct GSL content.⁵⁶

The latter method provides a rapid and sensitive (0.0 to 1.0 mM) measure of GSL content in seed, which is suitable for use in grain handling terminals and for plant breeders. This methodology is discussed in detail in Chapter 2.

b) Colorimetric Measure of Intact Glucosinolates.

A method developed by Thies⁴⁸ and further elaborated by Moller et al.(1984), depended on the ability of extracted intact GSLs to form complexes with palladium salts.⁵⁷ The results obtained suggested that this was suitable for distinguishing a high, medium or low GSL level in rapeseed. The removal of interfering compounds such as phenolics, using charcoal did not improve the sensitivity and the method was only suited to measuring levels above 50 μ mole GSL/g meal.⁴⁸ The variable quantitation of GSLs was also dependent on the type of structure present, for example DS GSLs, aglucones, thiocyanate and isothiocyanates would give varying colour development intensity.⁵⁸

Another colorimetric method utilised thymol reagent and was originally applied by Shettlar and Masters for the purpose of measuring carbohydrates in biological material.⁵⁹ The thymol reaction involved the reduction of glucose in the presence of sulfuric acid to its dehydrated 5-hydroxymethylfurfural, which reacted with thymol to give a red coloured complex.⁶⁰

The application of the thymol reaction for quantitation of GSLs was first reported by Olsen and Sorensen (1980).⁶¹ Later a method published by Brzezinski and Mendelewski (1984)⁶² utilised a microcolumn of DEAE Sephadex A-25 for initial purification of the GSLs prior to thymol reaction. This methodology however suffered from poor reproducibility and accuracy. A modified thymol method by Tholen and Truscott (1989) was designed to minimise the difficulties applicable to the previous methodologies.⁶³ The thymol method is a preferred method for total GSL estimation since it had several advantages over current methods :

1. No enzymic preparation was required, eliminating enzyme purification and testing of enzymatic activity or purity.
2. No expensive reagents or standards were required, keeping the cost of analysis low.
3. Since only part of the eluate was used for the thymol reaction, the remainder could be analysed for individual GSLs.
4. The elimination of lengthy digestion or incubation time made the analysis rapid; one person could carry out at least 200 determinations per day.
5. The high sensitivity made it possible to use small sample sizes.
6. Columns were easily regenerated and reusable.
7. The elution of intact glucosinolate from the ion exchanger with potassium sulfate, ensured that novel GSLs which contain negatively charged side chains, such as 1-sulfoglucosinolate could also be quantitated.

1.5.2. Total Glucosinolate Determination by Gas Chromatography or High Performance Liquid Chromatography.

The quantitation of GSLs in rapeseed has become increasingly important with the growing knowledge of the various biochemical activities of individual GSLs. It has also become apparent from measuring individual GSLs, that initial plant breeding directed at reducing total GSL content did not at least initially lower the potent indolyl GSLs. Hence the need exists to be able to accurately investigate the GSL profile of rapeseed samples.

a) Analysis of GSLs by Gas Chromatography (GC)

The measurement of volatile GSL degradation products by GC replaced the early steam distillation and isothiocyanate titration method.^{64,65} GC was then widely used in obtaining profiles of GSLs from a variety of plant tissues. However, a basic deficiency of the method was that not all GSLs form volatile isothiocyanates on hydrolysis. This is true for sinalbin and indole GSLs, which can represent as much as 50% of the total GSL content.⁶⁶

A significant improvement in the quantitation of individual GSLs occurred when Thies⁶⁷ used the enzyme aryl sulfatase (EC 3.1.6.1) from the snail *Helix pomatia* for desulfation of GSLs on a Sephadex DEAE A-25 minicolumn. Desulfated GSLs were separated by GC as their per-trimethylsilyl derivatives.^{61,68-70}

Two disadvantages of this method were the inability to accurately measure methylsulfinylpropyl GSL (glucoiberin) which gave rise to multiple peaks during GC analysis and losses in recovery of the indoleGSL: 4-hydroxyglucobrassicin and cinnamoyl GSLs.

b) Analysis of Glucosinolates by High Performance Liquid Chromatography (HPLC)

Two approaches have been reported for quantitation of individual GSLs by HPLC, involving either the intact GSL or the desulfated structures. The use of anion exchange columns allowed the purification of the intact GSLs which could then be eluted with pyridine acetate or desulfated on-column prior to HPLC analysis. The use of HPLC in the analysis of individual GSLs held a number of advantages over GC analysis for example no derivatisation or degradation during analysis.²

The quantitation of intact GSLs by HPLC was reported by Helboe (1980), using reversed phase ion-pair chromatography.⁷¹ The poor resolution for the common GSLs such as 3-butenyl and 2-phenylethyl GSLs meant more than one gradient system was required for complete separations. An added difficulty was the reduced HPLC efficiency, which lead to peak broadening. Carefully controlled pH conditions in conjunction with a column oven for temperature control were also necessary to obtain reproducible retention times.

The use of DS GSLs however, allowed complete separations to be obtained on C18 R.P. HPLC within 30 minutes. Given complete separations and a suitable internal standard, such as benzyl GSL or oNPGal, it is possible to obtain response factors for the individual GSLs.⁷² The peak fractions can also be quantitated with the thymol reagent, which can accurately detect micromole amounts of glucose liberated from the DS - or intact GSL.⁵² The combination of accurate response factors with rapid analysis time using C18 reversed phase HPLC enabled a more detailed screening protocol, whereby selected individual GSLs may be screened by plant breeders.

On-column desulfation followed by separation on reversed phase HPLC conveyed a number of advantages over GC analysis or HPLC analysis of the intact GSLs, for example: the complete resolution of the four indoleGSLs : 3-indolylmethyl GSL (gluco-brassicin), 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin and 1-methoxyglucobrassicin (neoglucobrassicin), with little or no interferences. Also both indole and non-indole GSLs can be desulfated and analysed simultaneously with little or no degradation by HPLC. The sulfoxide-containing DS GSLs chromatograph as a single peak as do the indole GSLs.

Some problems, however, exist in regard to the desulfation, which affects both GC and HPLC analysis systems. Apart from being time consuming due to overnight desulfation,^{72,73} those GSLs with additional acidic groups such as 1-sulfoglucobrassicin from *Isatis tinctoria*, can not be analysed with methods such as this, since the desulfated GSL will not be eluted due to the additional charge on the side chain. More serious is the loss of 4-hydroxy-3-indolylmethyl GSL during the desulfation on-column, since this is a major GSL in rapeseed. Such losses may also extend to the other GSLs which may be degraded by contaminant enzymes such as glucosidases or galactosidases. To prevent such a loss in GSLs during the desulfation step, Sang and Truscott (1984) performed the desulfation at pH 8 thereby preventing possible glycosidase action toward the GSLs during desulfation.⁷² Although the aryl sulfatase remained active at pH 8, the rate of desulfation was reduced. The autooxidation of 4-hydroxyglucobrassicin was increased as a result of high pH desulfation. This however was counteracted with the addition of the antioxidant mercaptoethanol, which lead to a two fold increase in recovery. The question still arises as to whether full recovery of 4-hydroxyglucobrassicin has been achieved. The unpleasant smell of mercaptoethanol and the increased desulfation time at higher pH hindered the acceptance of this methodology.

Thus, ideally, a method of purification of aryl-sulfate sulfohydrolase (aryl sulfatase EC 3.1.6.1.) was needed in order to achieve quantitative recovery of all GSLs during desulfation without the need for high pH or the inclusion of antioxidants. A purified aryl sulfatase would not only improve the recoveries but could increase the overall rate of analysis without adding excessive amount of protein.

The purification of aryl sulfatase from the gut of *Helix*

pomatia, has been attempted in two instances, involving ethanol fractionation and ion-exchange chromatography.^{73,74} Although these gave a three fold increase in specific activity it was not indicated whether any degradative enzymes were removed.

The desulfation of GSLs without preliminary purification on DEAE Sephadex A-25 has been attempted and although a rapid profile of individual GSLs using R.P. HPLC was obtained, there were difficulties regarding the co-elution of other plant metabolites and the need to include background subtraction.⁷⁵ The added protein and pigments loaded onto the HPLC system would not be expected to be totally retained by the pre-column leading to irregular retention times and reduced life of the analytical column.

CHAPTER 2

QUANTITATION OF GLUCOSINOLATES BY REFLECTANCE

2.1 INTRODUCTION

The numerous reports concerning the toxic affects associated with GSLs resulted in a reduction in the use of feeds containing such chemicals.^{12,76} Rapeseed meals, however, represent a source of high quality protein. Hence in 1970 the breeding of rapeseed varieties with low GSL levels was initiated.⁷⁷

In recent years breeders have reduced the total GSL content from more than 100 $\mu\text{mol/g}$ to less than 30 $\mu\text{mol/g}$ seed and to continue this process, a relatively accurate and precise method of analysis is required, without the prerequisite of expensive equipment.

Glucose liberated from GSLs after myrosinase hydrolysis, can be measured using an enzyme system such as glucose oxidase/peroxidase.⁷⁸ Although 200 analyses a day was reported possible,⁵¹ the methodology and equipment required were not suitable for use by plant breeders or unskilled personnel at grain terminals.

The use of test paper (Glucotest) impregnated with the glucose oxidase/peroxidase enzymes allowed rapid screening of high and low GSL containing rapeseeds by visual estimation.⁷⁹ Later developments incorporated the use of reflectometry to quantify the test strip reaction. A method using glucose test paper with a reflectance meter was developed by Thies (1985).⁵⁵ This provided a relatively inexpensive means of measuring GSL content in the region 0 to 100 $\mu\text{mole/g}$ seed. The main disadvantage however, was the number of steps required for sample preparation

and the need to impregnate the glucose test paper with an exact volume of sample prior to reading the reflectance.

Another method was developed by Feibig (1987), which utilised the BM reflectance meter-Reflolux and blood glucose test strips.⁸⁰

Recent developments using enzymatic measurement of liberated glucose are based on the diffusion of GSLs from seed overnight followed by hydrolysis with myrosinase. Interferences are not removed prior to colour reaction and although measurements are made on a microtiter plate reader, the accuracy is poor.⁸¹

The aim of the work presented in this chapter was to develop a method for measuring total GSL content which could provide a rapid answer with sufficient accuracy and precision to be suitable for plant breeding. In addition to speed, simplicity and sensitivity, the method should be inexpensive, requiring relatively simple reagents and equipment, and should not be technically demanding.

2.2 EXPERIMENTAL

2.2.1 MATERIALS

A standard glucose solution (5.56 mM); trizma acid and base; chlorohexidine diacetate phenylisothiocyanate and glycine were purchased from Sigma Chemical Company. Activated charcoal powder (sulfuric acid treated, Cat. No.33032), tri-sodium citrate and citric acid were obtained from BDH chemicals. Myrosinase was supplied by Biocatalyst, Treforest Industrial Estate, Pontypridd, Mid-Glamorgan, U.K.

Hydrochloric acid, chloroform, ethanol and petroleum ether were purchased from Ajax Chemical Co. Rapeseed seeds were supplied by Pacific Seeds Ltd. Toowomba Aust. Clinistix (Cat. No. 2847), Hydridstix and the Ames 2 Glucometer Readhead assembly were supplied by Miles laboratory Australia Pty. Ltd, Mulgrave, Victoria, Australia. BM hypoglycemic glucose test strips were obtained from Boehringer- Mannheim and Tes-Tape from Lily Pty Ltd.

Clark glucose test strips and the reflectance meter : digital 4000, were supplied by Australian Biotransducers Pty Ltd.

Reflectance meter; the TruBluGlu meter was produced in conjunction with Systrix Pty Ltd.

Ultra Turrax TP 18/10 with 8n shaft from Janke and Kunkel.

Polypropylene centrifuge tubes (10 ml graduated) were purchased from Bio-rad. Filter papers (No. 41) were supplied by Whatman.

A Coffee mill " Krups 50" was used.

2.2.2 METHODS

2.2.2.1 Myrosinase Extraction and Purification

Myrosinase was obtained by a modification of the method of Appelquist and Joseffson.⁴⁷

White mustard seed (500 g) was ground in a coffee grinder to a fine powder and transferred to a 5 litre conical flask. The white mustard powder was then extracted with 2 x 1500 ml petroleum ether and the mixture passed through Whatman No.1 filter paper with suction. The vacuum was maintained for 30 minutes to dry the meal before regrinding. The oil-free meal was mixed with 1200 ml water at 4 °C for 1 hour and the insoluble material removed by centrifugation at 1000 x g for 15 minutes. The supernatant was fractionated with a equal volume of 90 % ice-cold ethanol and the precipitate collected by centrifuging at 15,000 x g for 15 minutes. The pellet was then resuspended in 70% ethanol and the precipitate collected by centrifugation at 2,000 x g for 30 minutes. The precipitate was dissolved in 400 ml water, centrifuged at 1000 x g for 15 minutes and passed through a sintered glass buchner filter funnel. The solution was then dialysed against 1 % NaCl followed by 0.5 % NaCl and finally water prior to lyophilisation.

2.2.2.2 Preparation of Buffers.

Glycine-sodium hydroxide buffer (50 mM , pH 9.0) was prepared by mixing 25 ml 0.2 M glycine (15.01 g/l) with 9.45 ml 0.2 M sodium hydroxide and diluting to 100 ml with water.

Citric acid-sodium citrate buffer pH 5.0 was prepared by mixing 35 ml of 0.1 M citric acid (21.01 g/l citric acid monohydrate) with 65ml of 0.1 M trisodium citrate (29.41 g/l trisodium citrate dihydrate).

Sodium acetate-acetic acid buffer pH 5.0 solution was prepared by combining 70 ml of 0.1 M acetate solution (27.22 g/l sodium acetate trihydrate) with 30 ml of 0.1 M acetic acid.

Phthalate buffer (0.1 M, pH 5.0) was prepared by combining 50 ml of potassium hydrogen phthalate (20.42 g/l) with 22.6 ml of 0.1 M sodium hydroxide and diluting to 100 ml.

Tris buffer ("Trizma") was prepared according to the Sigma Technical Bulletin No. 106B.

Sodium carbonate-sodium bicarbonate buffer, pH 9.0 was prepared by mixing 10 ml of 0.1 M sodium carbonate.10 H₂O (28.6 g/l) with 90 ml of 0.1M sodium bicarbonate (8.40 g/l).

2.2.2.3 BM-Hypoglycemic Test Strips.

Standard glucose solutions were prepared by diluting a stock 5.56 mM standard glucose solution (Sigma). Each concentration (0 to 2.0 mM) was applied to a BM-hypoglycemic test strip using a pipette and after 30 seconds, the excess moisture was removed by a gentle wipe using tissue paper. The strip was then placed in the reflectometer and measured. The measurements were repeated allowing a 60 second reaction period. Reflectances determined using the Digital 4000 reflectometer were plotted against glucose concentration.

2.2.2.4 Tes-Tape Glucose Test Paper.

Tes-tape rolls were cut into 1.25 cm strips and reacted with 10 mL of a standard glucose solution. Using forceps, the glucose test strip was placed across the light aperture and the reflectance measured after 1 and 2 minutes.

2.2.2.5 Preparation of Oil-free Meals.

Seeds of rapeseed (~50 g) were placed in a cotton bag and lowered into a boiling water bath for 15 minutes. The seed was then allowed to dry before being macerated in a Krupps coffee grinder and defatted with petroleum ether (40-60 °C) in a soxhlet apparatus for 20 hours. Defatted meal was then allowed to dry before being ground to a powder.

2.2.2.6 Intact Rapeseed and Rapeseed Meal Extraction

Rapeseed (400 mg) was weighed into a 10 ml plastic centrifuge tube and immersed into a boiling water bath. Boiling water (2 ml) was added to the seed and heated in boiling water for 15 minutes before macerating the seed using a Ultra Turrax homogeniser. The shaft of the homogeniser was washed with two 1.0 ml volumes of distilled water and combined with the homogenate.

Rapeseed meal (200 mg) was treated in a similar manner as the seed, however Ultra Turrax homogenisation was not required.

The extracts were then centrifuged at 1000 x g for 5 minutes to give a clear yellow extract containing the intact GSLs.

2.2.2.7 Glucose Standards Spiked with Rapeseed Meal Extract.

Standard glucose solutions : 0.125, 0.25, 0.375, 0.5, 0.75 and 1.0 mM were prepared from 5.56 mM glucose by aliquoting 45, 90, 135, 180, 270 and 360 μ l into separate vials. A 1.0 ml aliquot of the rapeseed meal extract, prepared according to the Method Section 2.2.2.5 and extracted according to Method Section 2.2.2.6. was added to each vial. The final volume was adjusted to 2.0 ml and the protein precipitated by adding 20 μ l of 10 % chlorohexidine diacetate. Activated charcoal (25 mg) was added and the mixture vortexed and centrifuged at 1000 x g for 5 minutes resulting in a clear and colourless supernatant. The glucose was measured using a glucose test strip and reflectometer.

2.2.2.8 Meter Calibration (Digital 4000 and TruBluGlu).

The zero was set by immersion of a Clinistix strip in water and shaking off excess moisture, the strip was then placed in the meter and the reflectance measured and adjusted to read zero. Another test strip was then dipped into a 1.0 mM glucose solution pH 5.0, and after 5 seconds the excess moisture removed by shaking. After a 2 minute colour development time the Clinistix colour intensity was measured and the gain control adjusted. The calibration was then repeated. The Digital 4000 meter with a zero and gain control was set to give the maximum difference in reading between the zero (distilled water) and 1.0 mM glucose reflectance values. Figures 1 to 6 were obtained from a Digital 4000 lacking a zero and gain controls.

2.2.2.9 Time Required for Optimum Colour Development in Clinistix.

Glucose solutions were prepared by dilution of the 5.56 mM standard glucose solution with water to give 0.1, 0.5 and 1.0 mM glucose. The Clinistix strips were immersed for 5 seconds and the excess liquid shaken off. At time intervals 0.25, 1.0, 2.0, 2.5, 3.0 and 5.0 minutes the colour intensity of the Clinistix strips was measured using the Digital 4000 reflectometer.

2.2.2.10 Clinistix Colour Stability

The Clinistix strips were dipped in a 1.0 mM glucose solution and allowed to develop for 2 minutes and then placed either in the dark or under a 40 watt lamp approximately 20 cm distance. For time intervals up to 15 minutes the rate of colour fading was measured.

2.2.2.11 Effect of Temperature on Colour Intensity in Clinistix.

Standard glucose solutions were obtained according to Method Section 2.2.2.18 and the TruBluGlu meter calibrated as in Method Section 2.2.2.8. The standards were chilled to 6 °C and Clinistix strips immersed into the standards were maintained at 6 °C for 2, 4 and 6 minute development times. After each incubation period the reflectances were measured and plotted against glucose concentration.

2.2.2.12 Time Course of Sinigrin Hydrolysis.

1.0 ml aliquots of sinigrin (1.0 mM) at pH 7 were added to vials containing 50 μ l and 100 μ l of myrosinase (mg/2.5 ml) at 25 °C. At 5 minute intervals over 30 minutes the reaction was stopped with the addition of 50 μ l chlorohexidine diacetate (10 % w/v in EtOH). The white cloudy solution was centrifuged (1000 x g, 5 minutes) to give a clear and colourless solution. The measurement of the liberated glucose was performed by dipping the Clinistix strip (5 seconds) into the final sample and shaking off excess moisture. After 2 minutes the colour intensity was measured using the reflectance meter, Digital 4000.

2.2.2.13 Reflectance Standard Curve using Sinigrin.

The reflectance standard curve for GSL determination was obtained using the GSL, sinigrin. A series of sinigrin solutions (1.0 ml) were prepared (0 to 2 mM) and hydrolysed by addition of myrosinase (5 units). The liberated glucose was measured after 3 hours by Clinistix strip and measuring the colour development after 2 minutes with the Digital 4000 reflectometer. These measurements were repeated after protein precipitation by 50 μ l of chlorohexidine diacetate (10 % w/v methanol). Any cloudiness was removed by centrifugation (1000 x g, 5 minutes), prior to glucose measurement.

2.2.2.14 Role of Activated Charcoal in Clinistix Colour Intensity.

The meals of two commercial rapeseed samples (Marnoo and Cowra 503) were extracted (200 mg) into 6.0 ml each of boiling water

2.2.2.12 Time Course of Sinigrin Hydrolysis.

1.0 ml aliquots of sinigrin (1.0 mM) at pH 7 were added to vials containing 50 μ l and 100 μ l of myrosinase (mg/2.5 ml) at 25 °C. At 5 minute intervals over 30 minutes the reaction was stopped with the addition of 50 μ l chlorohexidine diacetate (10 % w/v in EtOH). The white cloudy solution was centrifuged (1000 x g, 5 minutes) to give a clear and colourless solution. The measurement of the liberated glucose was performed by dipping the Clinistix strip (5 seconds) into the final sample and shaking off excess moisture. After 2 minutes the colour intensity was measured using the reflectance meter, Digital 4000.

2.2.2.13 Reflectance Standard Curve using Sinigrin.

The reflectance standard curve for GSL determination was obtained using the GSL, sinigrin. A series of sinigrin solutions (1.0 ml) were prepared (0 to 2 mM) and hydrolysed by addition of myrosinase (5 units). The liberated glucose was measured after 3 hours by Clinistix strip and measuring the colour development after 2 minutes with the Digital 4000 reflectometer. These measurements were repeated after protein precipitation by 50 μ l of chlorohexidine diacetate (10 % w/v methanol). Any cloudiness was removed by centrifugation (1000 x g, 5 minutes), prior to glucose measurement.

2.2.2.14 Role of Activated Charcoal in Clinistix Colour Intensity.

The meals of two commercial rapeseed samples (Marnoo and Cowra 503) were extracted (200 mg) into 6.0 ml each of boiling water

according to Method Section 2.2.2.5. Five extracts of each sample was prepared and treated as follows: The extracts were hydrolysed by the addition of 50 units of myrosinase (given one unit will hydrolyse 1 μ mole GSL per hour). After 1 hour the extracts were treated with 50 μ l of 10 % chlorohexidine diacetate followed by varying amounts of activated charcoal : 0, 25, 50, 100 and 250 mg. The resulting mixture was vortexed and centrifuged for 10 minutes at 1000 x g. The extracts were then measured for glucose using Clinistix strip with the reflectance meter, Digital 4000.

2.2.2.15 Glucosinolate Determination by the Addition of Myrosinase.

Rapeseed (200 mg) or meal (100 mg) were extracted into 4.0 ml of boiling water, according to Methods Section 2.2.2.6. The extracts were centrifuged and each supernatant decanted into a 10 ml graduated tube containing 50 units of myrosinase. After 1 hour the volumes were adjusted to 6.0 ml and treated with 50 μ l of 10 % chlorohexidine diacetate followed by 250 mg of activated charcoal.

The sample was centrifuged at 1000 x g for 10 minutes to give a clear and colourless extract. The supernatant was removed using a pasteur pipette and applied to the Clinistix strip. The GSL content was obtained by measuring the reflectance of the Clinistix colour yield against a standard curve prepared according to Methods Section 2.2.2.13.

2.2.2.16 Interference of Clinistix Colour Development by Glucosinolate Breakdown Products.

Four rapeseed samples were prepared as described in Methods Section 2.2.2.15. The GSL concentration was determined by measuring Clinistix reflectance and conversion to $\mu\text{mole GSL/g seed}$ using a standard curve prepared according to Methods Section 2.2.2.13. An aliquot of Phenylisothiocyanate (PIT) was added to each extract to give a 1.0 mM concentration and its GSL content re-determined.

2.2.2.17 Time Course of Glucosinolate Hydrolysis.

Two rapeseed meals from the seed samples (Marnoo and Cowra 503) were extracted (200 mg in 10 ml sodium acetate buffer pH 5.0) at 100 °C for 10 minutes. After cooling to room temperature, the extract was centrifuged to give a clear, yellow-coloured supernatant. Myrosinase (50 units) was mixed with the supernatant and at set time intervals, a 1.0 ml aliquot was removed and the reaction stopped with the addition of 50 μl of 10% chlorohexidine diacetate. Activated charcoal (50 mg) was then added to each sample and the mixture vortexed. A clear and colourless supernatant was obtained after centrifugation at 1000 x g for 5 minutes. The liberated glucose was measured using Clinistix test strip and Digital 4000 meter.

2.2.2.18 Standard Curve Construction For GSL Determinations.

Preparation of curve A:

Standard curve A was prepared by aliquoting 27, 45, 90, 135 and 180 μl of standard glucose (5.56 mM) into separate vials containing 166 μl of 0.1 M citric acid/sodium citrate buffer pH 5.0. The total volume in each was adjusted to 1.0 ml with distilled water, giving 0.15, 0.25, 0.50, 0.75 and 1.00 mM glucose.

Preparation of curve B :

Rapeseed (400 mg) was extracted according to Methods Section 2.2.2.6. Standard glucose (5.56 mM) was aliquoted into vials as described for curve A. Each standard was mixed with 500 μl of rapeseed meal extract followed by 166 μl of 0.1 M citric acid/sodium citrate buffer pH 5.0. The final volume was adjusted to 1.0 ml with distilled water. Activated charcoal (25 mg) was added and the mixture vortexed and centrifuged at 1000 x g for 10 minutes. The clear and colourless supernatant was measured for glucose using Clinistix strips.

2.2.2.19 Free Glucose Determination

The seed (200 mg) of several rapeseed samples was extracted as described in Methods Section 2.2.2.5. The extracts were mixed with 50 μl chlorohexidine diacetate (10 % w/v EtOH) and 250 mg of activated charcoal. The resulting mixture was adjusted to pH 5 and centrifuged at 1000 x g for 10 minutes. A clear and colourless supernatant was measured for free glucose using the Clinistix strip and Digital 4000. The reflectance was read against the standard curve obtained according to Methods Section 2.2.2.15 (a).

2.2.2.20 Determination of HCl (1 M) required for pH Adjustment.

Intact rapeseed (200 mg) was combined with 6.0 ml of 25 mM Tris buffer at either pH 8 or 9 in separate centrifuge tubes. After maceration the pH was measured and the volume of 1 M HCl necessary to lower the pH to between 5 to 6 was determined using a pH meter.

2.2.2.21 Glucosinolate Hydrolysis by the Endogenous Myrosinase.

A rapeseed extract for each time interval was prepared by weighing 200 mg of rapeseed (Cowra 503) and extracting without boiling into 6 ml of 50 mM glycine buffer at pH 9. At each time interval the rapeseed extract was treated with 50 μ l of 10 % chlorohexidine diacetate followed by 200 mg of activated charcoal. The pH was then adjusted to pH 6 by adding 800 μ l of 1 M HCl before centrifugation at 1000 x g for 10 minutes. The clear and colourless extract was measured for liberated glucose using Clinistix strip and Digital 4000 reflectometer. The time course was then repeated by performing the extraction at pH 5.0 with 50 mM Tris-HCl buffer and treating the sample as before.

2.2.2.22 Selection of pH 5.0 Buffer for Final pH Adjustment.

The following stock solutions of buffers were prepared : 0.1 M citrate/citric acid pH 5.0 ; 0.2 M sodium acetate/acetic acid pH 5.0 ; 0.1 M potassium hydrogen phthalate/NaOH pH 5.0 (see Methods Section 2.2.2.2.). A equal volume of standard glucose solution was combined with the stock buffer giving a final glucose concentration

of 0, 0.25, 0.50 and 1.0 mM in each buffer with final concentrations : citrate buffer (50 mM) and acetate buffer (100 mM) and potassium hydrogen phthalate buffer (50 mM).

2.2.2.23 Intact Rapeseed Analysis.

Air-dried rapeseed (200 mg) was weighed into a 10 ml centrifuge tube. 3.0 ml of 50 mM glycine-NaOH buffer (pH 9.0) was added and the mixture homogenised thoroughly (15 seconds).

The ultra turrax shaft was rinsed with 2 x 1.0 ml aliquots of buffer solution dispensed through the top hole in the shaft. The suspension was gently mixed and incubated at room temperature. After 10 minutes, 1.0 ml of chloroform was added and the tube sealed and mixed thoroughly by shaking. The protein was precipitated by adding 50 μ l of 10 % chlorohexidine diacetate in methanol. The final pH was adjusted to pH 5.0 by adding 1.0 ml of 100 mM citric acid/sodium citrate, followed by a scoop of activated charcoal (0.25 g). The mixture was shaken and then centrifuged at 1000 x g for 2 minutes. A clear colourless supernatant must be obtained. A Clinistix strip was dipped and the reflectance measured as explained in Methods Section 2.2.2.8.

2.3 RESULTS

2.3.1 REFLECTANCE METHOD AND DEVELOPMENT

2.3.1.1 Selection of a Suitable Glucose Test Strip.

The Digital 4000 (reflectance meter) was selected initially for screening a range of commercially available glucose test strips.

It was found that 200 mg of seed (30 μ mole GSL/g seed containing approximately 10% moisture or approximately 60 μ mole GSL/g meal) could be extracted efficiently into 6.0 ml of buffer, which corresponds to approximately 1.0 mM GSL. Those glucose test strips capable of distinguishing glucose levels ranging from 0 to 1.0 mM glucose were screened for sensitivity. The results of BM hypoglycemic strips used in conjunction with the Digital 4000 meter are shown in Figure 2.

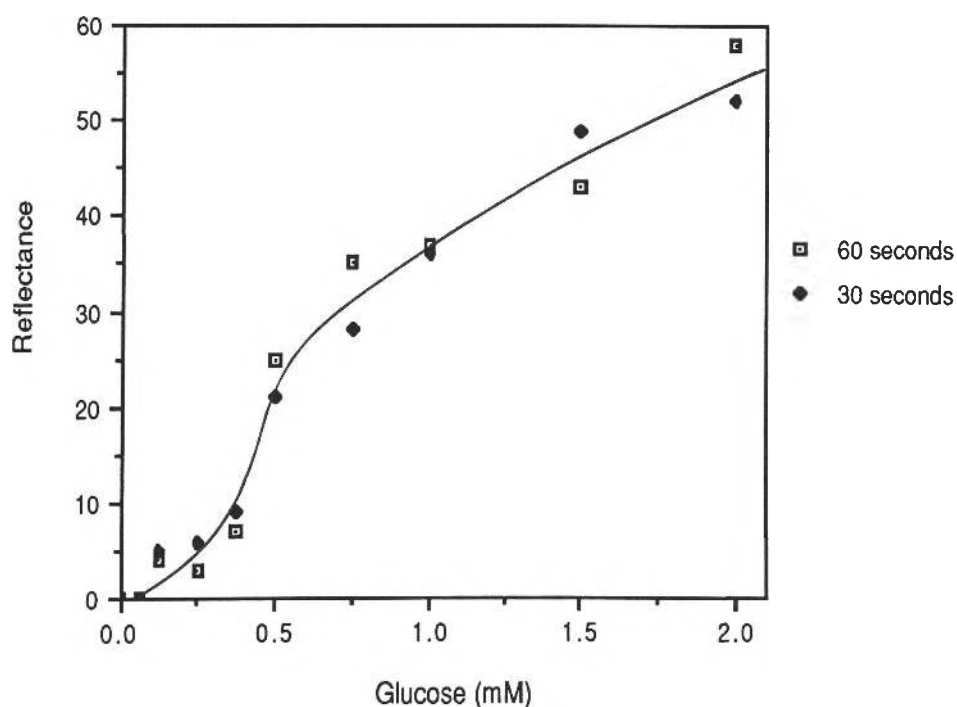


Figure 2. The meter was calibrated (see Methods Section 2.2.2.8). Standard glucose solutions were applied to BM hypoglycemic test strips and measurements taken according to Method Section 2.2.2.3.

A 60 second reaction time was found to give a slightly increased colour intensity, however in both trials the strip's reaction to glucose was not measurable accurately at concentrations less than 0.5 mM. At glucose concentrations 0.5 to 2.0 mM a colour change in the test strip was detected (Figure 2).

Clark glucose test strips, also designed for blood glucose analysis, were found to give a similar response to that obtained using the BM-hypoglycemic test strips.

Tes-Tape unlike other glucose test strips, is a roll of glucose test paper and not a pad on the end of a plastic strip. It is designed for visual estimation of glucose in urine and gives an indication of high, medium or low glucose, by comparison with a colour chart matching the yellow to green glucose colour response.

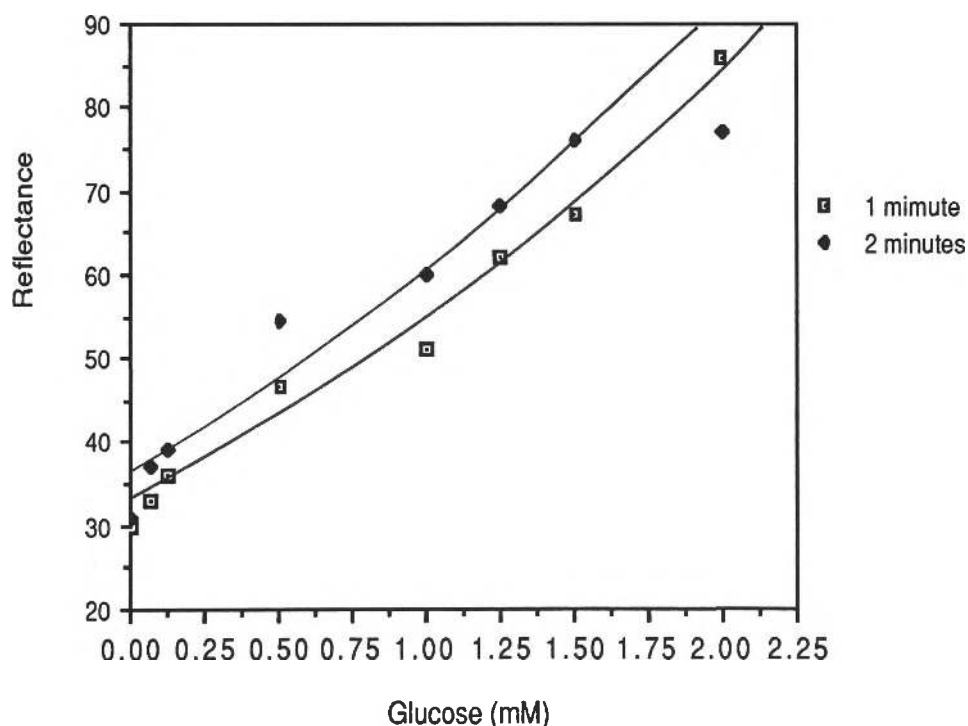


Figure 4. A 10 μ l aliquot of each standard glucose solution was applied to the strips of Tes-Tape. The colour development was measured using the Digital 4000 reflectance meter according to Method Section 2.2.2.4. No zero or gain controls are fitted.

A consistent change in reflectance for glucose levels 0 to 2.0 mM was obtained (Figure 4). A more intense colour development was observed at 2 minutes, followed by a gradual fading. Glucose at 0.063 mM was detectable, however the sensitivity overall still appeared poor.

Measurements taken at one minute appeared to be complete for low glucose concentrations however, at the higher glucose levels a 2 minute colour development time is necessary for optimum colour intensities.

To check the response of Tes-tape to glucose in a rapeseed extract the standard glucose solutions used in Figure 4 were re-measured with the addition of a rapeseed extract.

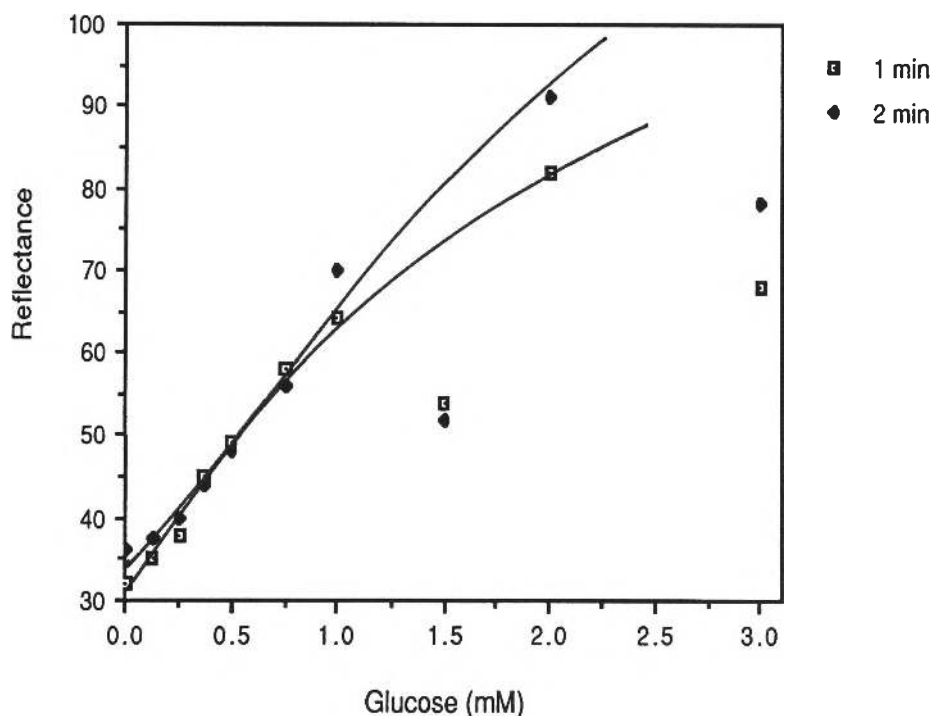


Figure 5. Standard glucose solutions were spiked with rapeseed extract to produce a standard curve comparable with rapeseed samples as described in Methods Section 2.2.2.7. The reflectance for each concentration was measured at 1 and 2 minute intervals using Tes-Tape.

Reflectance values for the glucose standards 1.5 and 3.0 mM did not fall on the apparent straight generated by the other values (Figure 5). This apparent inaccuracy was attributed to the white cloudy appearance of these sample solutions. The Tes-Tape gave a measurable response to the glucose concentration range 0.25 to 2.0 mM, which is seen as a colour change from yellow to dark green.

Clinistix strips like Tes-Tape, are marketed for visual glucose estimation in urine and are designed to provide an indication of low, medium or high glucose levels in the region 14 to 28 mM. The application of Clinistix strips for the estimation of glucose solutions spiked with rapeseed meal extract was found to give good sensitivity in the region 0.375 to 1.0 mM glucose (Figure 6), with a 70 % change in the metered reflectance units response for the concentration range 0 to 1.0 mM glucose.

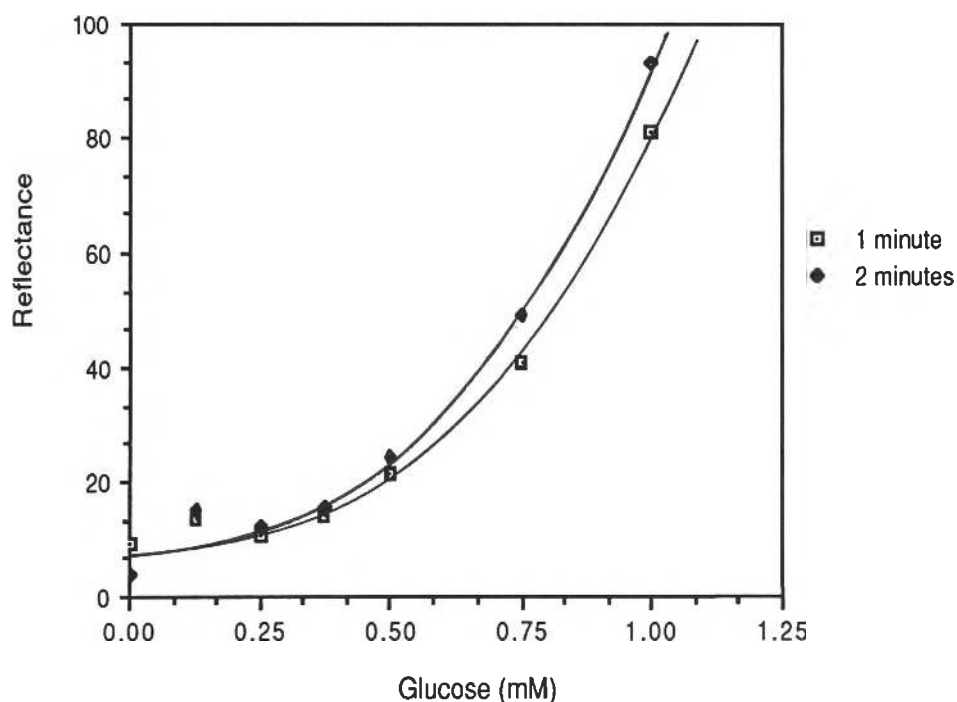


Figure 6. Clinistix strips were immersed into glucose standards spiked with rapeseed extract as described in Methods Section 2.2.2.7. The colour development was measured at 1 and 2 minutes. This Digital 4000 lacked zero and gain controls.

Although the reflectance measurement using the Digital 4000 was not linear, glucose levels down to 0.25 mM could be measured. The Clinistix strip colour intensity after 1 minute (Figure 6) appears satisfactory for the lower glucose levels, however an additional 1 minute colour development time resulted in an appreciable increase in colour intensity at the higher glucose concentrations. The Tes-Tape responses as shown in Figure 5, gave a more linear response, however the sensitivity was not adequate. Hence, in order to optimise the detection of the Clinistix strip and Tes-Tape, the Digital 4000 was fitted with both zero (base line) and gain (slope) controls. Shown in Figure 7, the optimum meter setting for the Tes-Tape colour change enabled detection of low glucose levels, however the sensitivity was unchanged with glucose concentrations from 0.065 to 1.0 mM giving a 30 % change in reflectance reading.

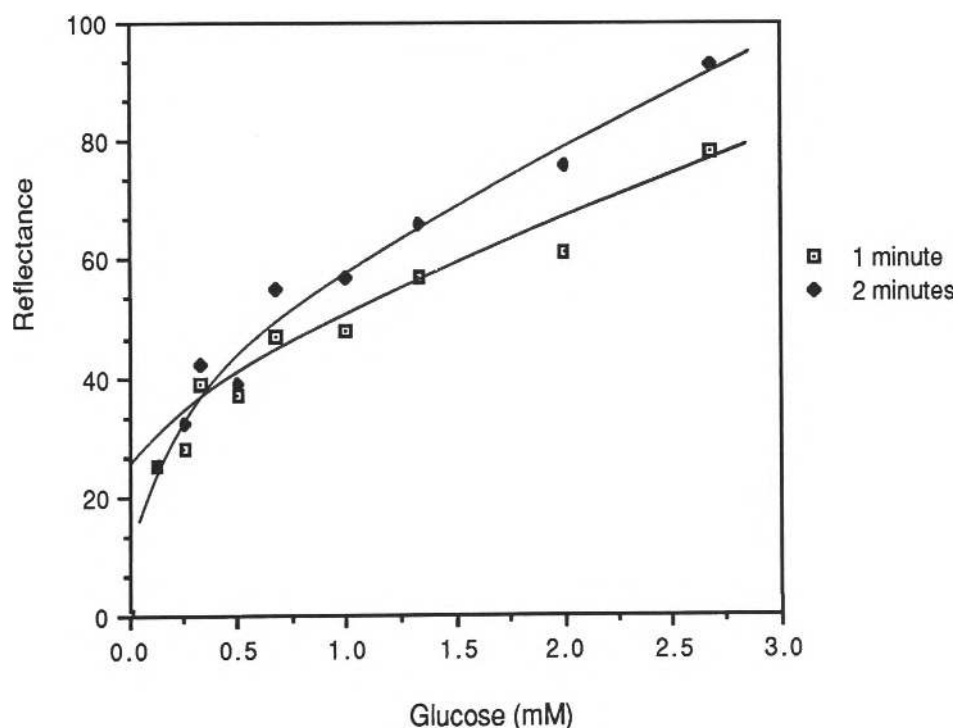


Figure 7. The use of Tes-Tape in the Digital 4000 with the zero and gain controls set to give optimum sensitivity in the range 0 to 1.0 mM glucose. Calibration was according to Methods Section 2.2.2.8.

Although improved detection of slight colour changes has been achieved (Figure 6), the overall sensitivity has not been improved. Ideally, a full scale reflectance change would correspond to a 0 to 1.0 mM glucose concentration.

The use of Clinistix strips with adjustment of the zero and gain controls is shown in Figure 8. Optimisation of the meter response to the colour formed by 0 mM and 1.0 mM glucose resulted in a 60 % change in the reflectance reading.

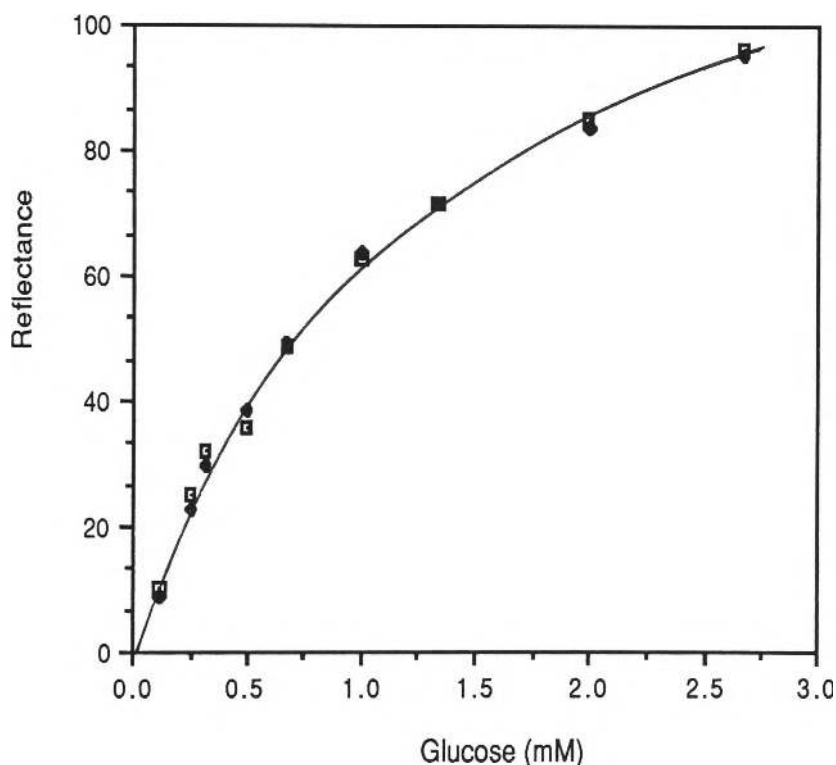


Figure 8. The reflectometer (Digital 4000) optimisation of clinistix measurement by adjustment of zero and gain controls. The meter was set for optimum sensitivity for low glucose levels according to Methods Section 2.2.2.8. □ : Clinistix strips with 9 μ l volume of standard glucose solution applied to the test pad using a micropipette. ◆ : Clinistix strips dipped into the glucose solution and excess moisture shaken off. Both strips were read after 2 minutes.

A consistent response is seen for the glucose standards, with an increasing sensitivity at the lower levels. The consistency in the reflectance values obtained for the Clinistix glucose test strip (Figure 8) was attributed to the absorbent nature of the test pad which retains the pigment without it leaching out.

A plastic strip for supporting and handling the pad enables ease of use and assists in a more reproducible application of the sample to the pad and in the positioning of the test pad in the meter.

2.3.1.2 Optimisation of Clinistix Colour Development

Clinistix colour development was investigated to determine the optimum time required for glucose levels 0 to 1.0 mM to react and produce the most intense colour. The volume of liquid absorbed by the Clinistix strip was found to be 9 μ l. As depicted in Figure 8, the dip time required for optimum colour intensity was approximately 5 seconds. Where the Clinistix strip is dipped for less than 3 seconds or for periods longer than 10 seconds a lower colour yield resulted.

It was observed that the change in colour intensity in response to glucose during and after development, occurred at a rate sufficient to give a significant error if the colour was not measured at a specified time. Hence the time for optimum colour intensity needed to be determined.

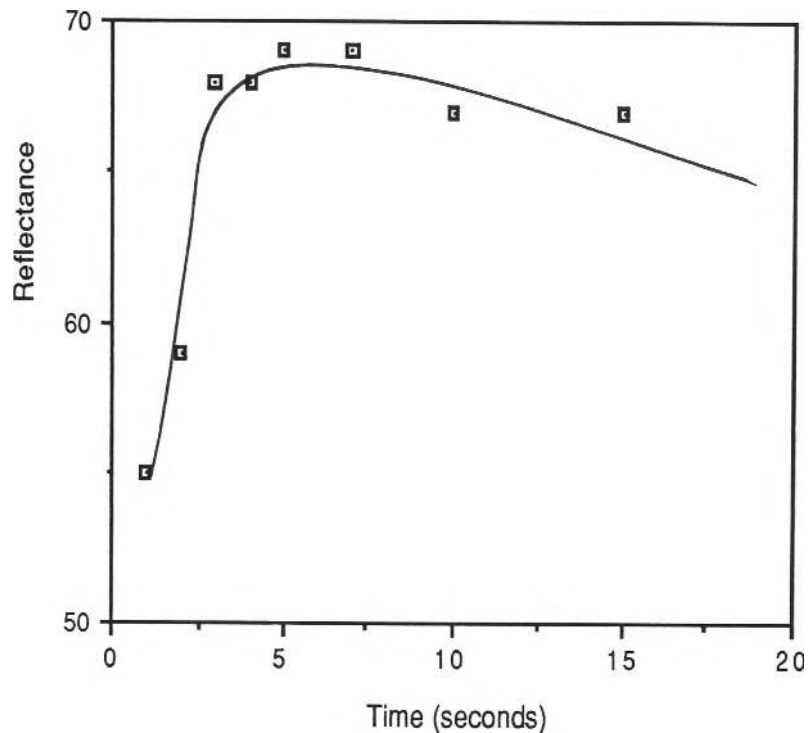


Figure 9. Determination of optimum time for immersion of Clinistix in glucose solution. A 1.0 mM standard glucose solution was measured using clinistix strips and the Digital 4000. The test strips were inserted into the glucose solution for various time periods, removed and excess moisture shaken off. The colour intensities in each trial was measured after 2 minutes.

Using the optimum time required for the absorption of solution into the Clinistix pad (Figure 9), the time required for complete reaction of glucose was also investigated, at both high and low glucose concentrations.

The results in Figure 10 demonstrate that the optimum colour development for a 5 second dip time was 2 minutes. Reflectance readings after a 3 minute development time could still be made but with reduced sensitivity. To investigate the factors responsible for the fading of the Clinistix strip, the colour development was monitored in the dark and under illumination.

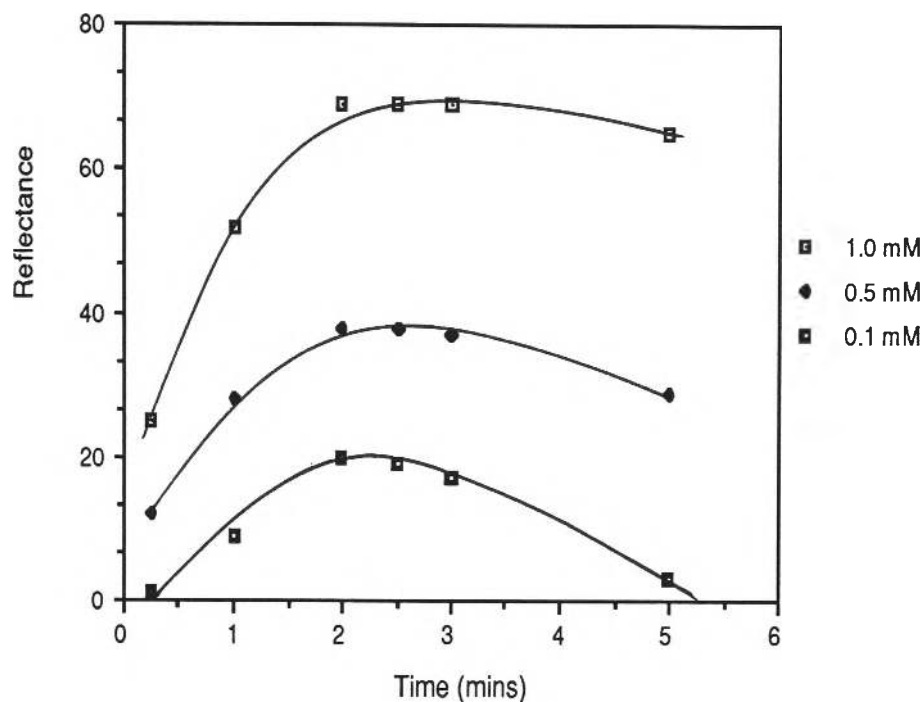


Figure 10. The Clinistix strips were dipped into 0.1, 0.5 and 1.0 mM glucose solutions and the colour development monitored by the Digital 4000 reflectance meter (Methods Section 2.2.2.9).

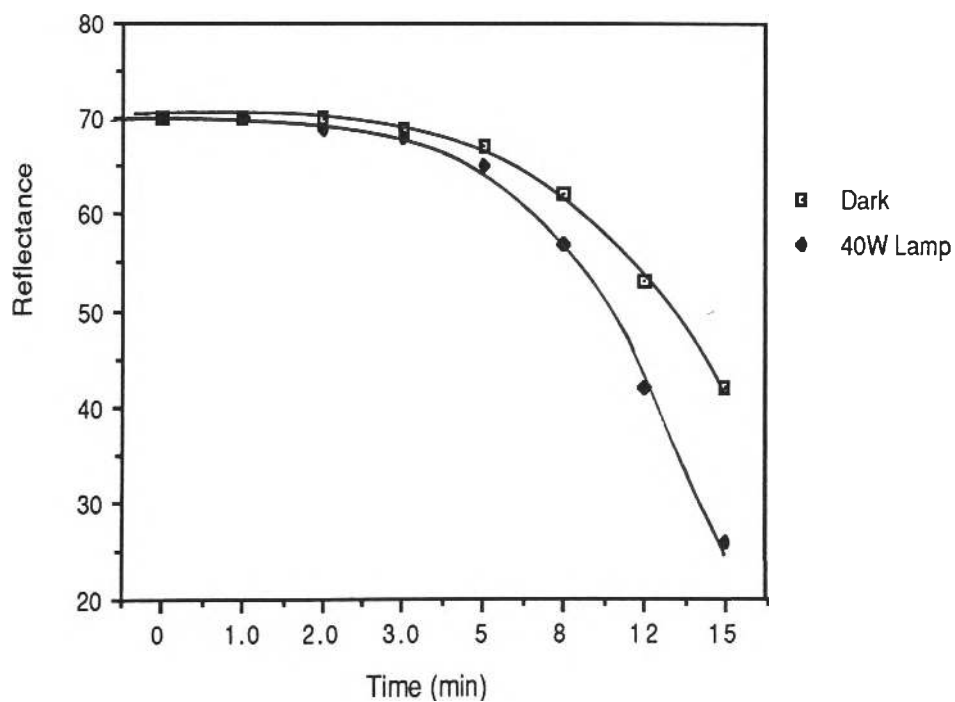


Figure 11. The stability of Clinistix strip colour to light was determined using 1.0 mM glucose according to the Methods Section 2.2.2.10.

The effect of light on the stability of the Clinistix colour is shown in Figure 10. Exposure of the strip to intense light during development was seen to reduce the colour yield at a greater rate than that which would normally occur.

Provided the Clinistix colour intensity was measured within 3 minutes of dipping in sample solution, measurements could be obtained before detectable fading.

2.3.1.3 Reflectance Measurement of a Hydrolysed Standard GSL.

As a trial for determination of GSL content in rapeseed, a standard GSL (sinigrin) was incubated with commercial myrosinase and the liberated glucose measured with Clinistix strips and reflectance meter : Digital 4000.

The time course of glucose release (Figure 12) demonstrated that changes in glucose concentration, following hydrolysis of sinigrin was feasible in the presense of GSL and GSL hydrolysis products.

The activity of the myrosinase purified from white mustard (*Sinapis alba*) according to Methods Section 2.2.2.1 was also determined for the substrate : sinigrin.

The time course of sinigrin hydrolysis by myrosinase (Figure 12) seemed to plateau at 25 minutes. Hence, assuming complete hydrolysis, the activity of the myrosinase was determined as approximately 50 units/mg, given the definition 1 unit/ μ mole sinigrin hydrolysed/hour.

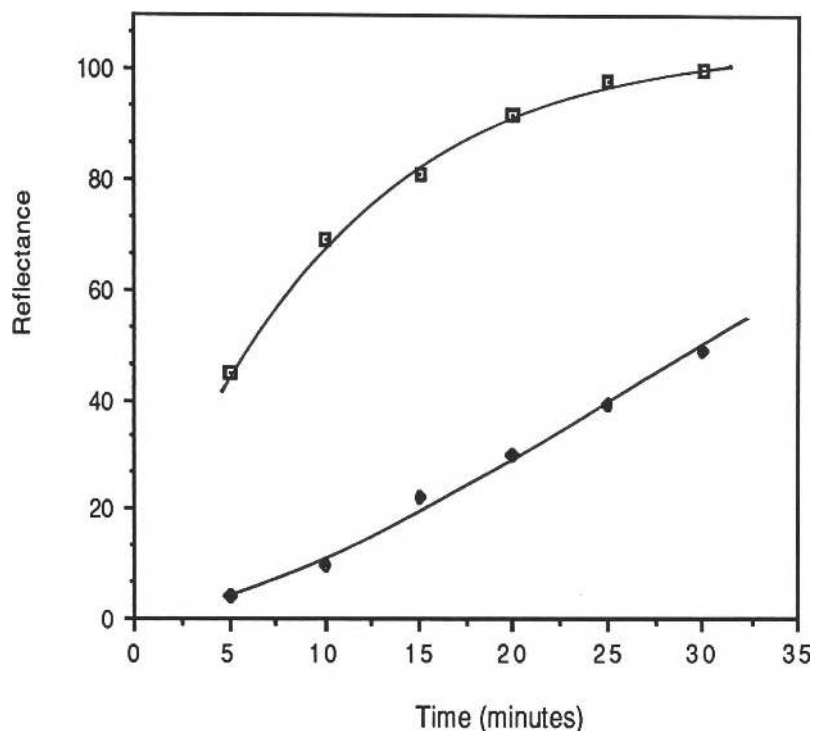


Figure 12. Sinigrin hydrolysis by myrosinase was monitored by measuring the liberated glucose with Clinistix strips and Digital 4000, according to the Methods Section 2.2.2.12. Vials containing 1.0 mM sinigrin (1.0 ml) were prepared and incubated with \square : 100 μ l of myrosinase (2 mg/5 ml) and \blacklozenge : 50 μ l of myrosinase.

A standard curve was prepared using an intact GSL (sinigrin). Sinigrin was incubated with myrosinase and the released glucose measured for each sinigrin concentration (shown in Figure 13).

The addition of myrosinase for the hydrolysis of standard sinigrin solutions (Methods Section 2.2.2.13) was observed to produce a pale white cloudy mixture. Centrifugation at 1000 x g for 15 minutes did not clarify the solution and measurements of the free glucose using Clinistix strips and the Digital 4000 gave lower than expected readings. The removal of the cloudiness was achieved with the addition of a protein precipitating reagent (chlorohexidine diacetate), which after centrifugation at 1000 x g for 5 minutes

resulted in a clear and colourless solution. Measurements of glucose after removal of protein gave readings more like the standard glucose curve shown in Figure 8.

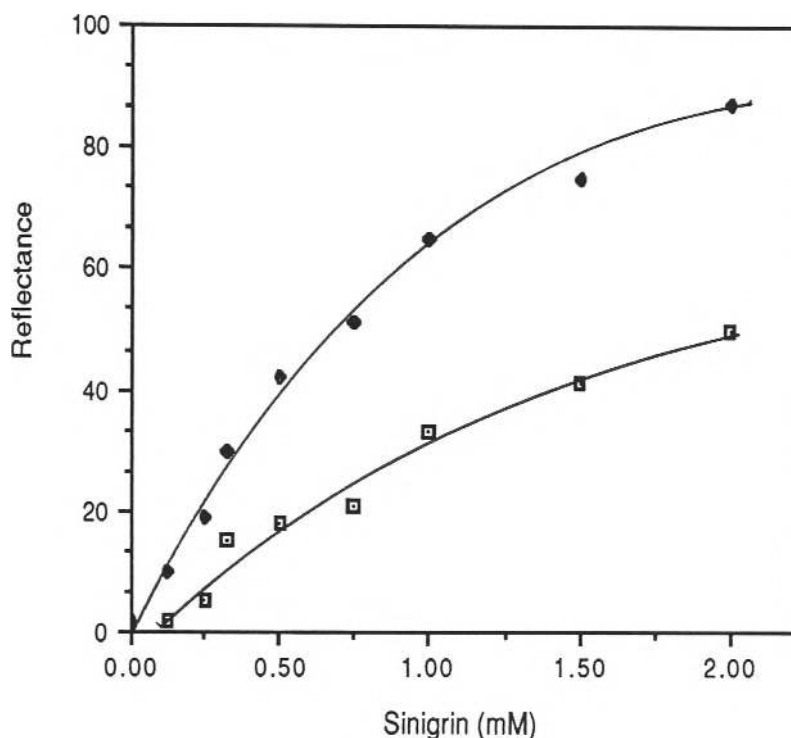


Figure 13. A standard curve using sinigrin hydrolysed by myrosinase, was prepared according to Methods Section 2.2.2.13. The liberated glucose was measured by reflectance using Clinistix and the Digital 4000. ◻ : without removal of protein and ♦ : is the standard curve produced when protein is removed.

2.3.1.4 Reflectance Measurement of a Rapeseed Extract.

a) Role of Activated Charcoal

A rapeseed extract was prepared according to Methods Section 2.2.2.6 and 2.2.2.15, but the amount of activated charcoal added was varied for each trial.

The results shown in Table 1 demonstrated the inhibition of the Clinistix strip colour development and the need to add at least 50 mg activated charcoal for consistent Clinistix colour development.

Table 1. The Effect Of Activated Charcoal On Colour Development

Amount of Act. Charcoal (mg)	Reflectance	
	Marnoo	Cowra 503
0	8	4
25	58	22
50	64	35
100	66	38
250	64	35

Table 1. Two commercial rapeseed meal samples were extracted and prepared for glucose measurement according to Methods Section 2.2.2.14.

b) Effect of GSL Breakdown Products on Clinistix Enzymes.

The reported instances of reduced enzyme activities in the presence of GSL breakdown products prompted the investigation into the possible interference of Clinistix enzyme activities in the presence of GSL breakdown products. The GSL levels of four rapeseed samples were measured according to Methods Section 2.2.2.15. The measurements were repeated after the addition of the GSL breakdown product.

Table 2. GSL Determination by Reflectance in the Presence of Phenylisothiocyanate. (PIT)

Rapeseed Sample	GSL μ mole/g seed	
	No PIT	With PIT
BLN 270	6	6
RX 9	12	14
BLN 241	15	13
Thomo 42	16	15
Jumbuck	31	33

Table 2. GSL determination in five commercial rapeseed samples was performed according to the Methods Section 2.2.2.16. The measurements were then repeated in the presence of 1 mM PIT.

The reflectance values were compared to a standard curve prepared using glucose spiked with extract to give an equivalent matrix as the seed sample(see method section 2.2.2.18 (b)).

Phenylisothiocyanate (PIT) was added to the extracts giving a final concentration of 1 mM. The addition of Phenylisothiocyanate (PIT) shown in Table 2 indicates no apparent inhibition of the Clinistix strip colour yield.

c) Removal of Oil from Rapeseed Extract.

The high oil content of rapeseed (approximately 50 % w/w) formed an emulsion during seed maceration, which after centrifugation resulted in a oil layer over the sample solution. This presented a

physical interference to the reading of the Clinistix strips in the Digital 4000.

The removal of the oil from the aqueous extract was achieved by chloroform extraction prior to Clinistix measurement. Chloroform was chosen since it was more dense than water and thus was thought that it may remove the oil and form a layer beneath the GSL-containing solution. A volume of 1 ml chloroform per 200 mg seed was sufficient for the oil extraction after seed maceration. The resulting solution was observed to be free of the oily layer and after centrifugation a clear extract solution resulted.

d) Time Course of GSL Hydrolysis with Addition of Myrosinase.

A time course of GSL hydrolysis was performed with the addition of myrosinase, according to Methods Section 2.2.2.17.

The glucose liberated was monitored with Clinistix strip, to determine the time required for the GSLs to be completely hydrolysed (Figure 14). A plateau in the glucose level with time was found after 10 minutes for the rapeseed sample Marnoo (20 $\mu\text{mol/g}$ seed using HPLC) whereas the Cowra 503 sample was apparently complete within 6 minutes (7 $\mu\text{mol/g}$ seed by HPLC).

Given a constant content of liberated glucose with time from approximately 8 minutes onwards, it was evident that a measure of glucose corresponding to the GSL content could be made.

The reflectance value could then be converted to the amount of GSL by using the standard curve prepared in Methods Section 2.2.2.15.

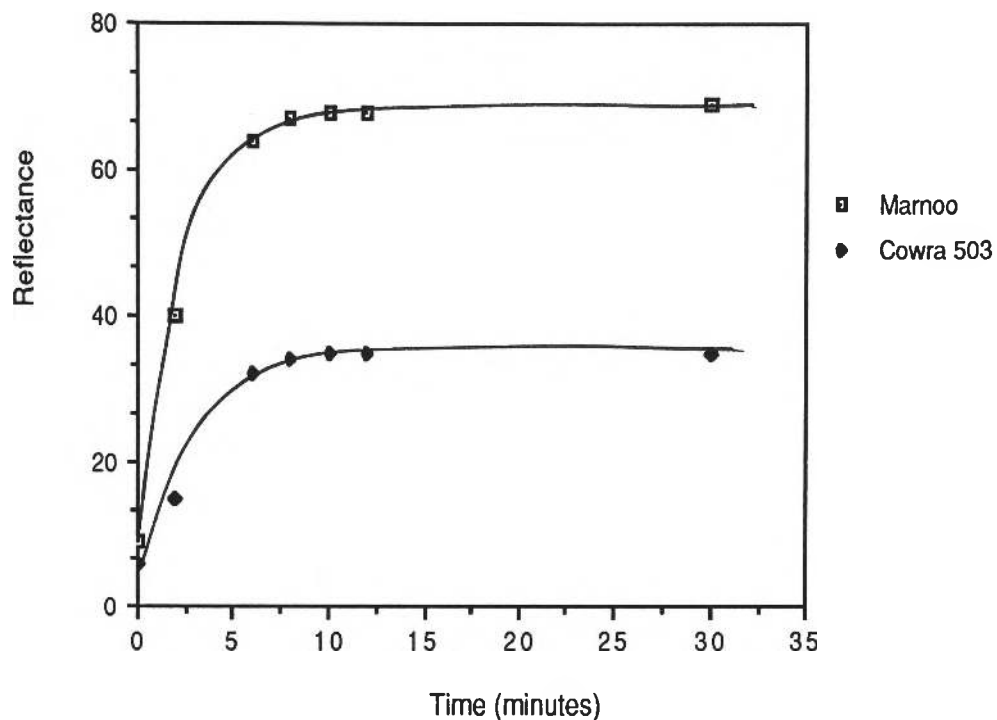


Figure 14. A time course of rapeseed GSL hydrolysis using purified myrosinase was performed on two rapeseed samples. The procedure is given in the Methods Section 2.2.2.17.

e) Adjustment of Meter Response

Adjustment of intensity of the light source used for reflectance measurement was found to maximise the sensitivity of the detector for the Clinistix strip colour change. Thus it was found that a reduction in the aperture or meter lamp voltage resulted in a marked improvement in the detection of lower GSL concentrations. The standard curve shown in Figure 15 demonstrated almost full reflectance range for glucose concentrations 0 to 1.0 mM. A 1.0 mM glucose solution, representing a high GSL concentration in rapeseed (30 $\mu\text{mole/g}$ seed), is seen to correspond to about 90 % of the scale. This also gives better measurement of low GSL levels where smaller differences in colour yield, need to be accurately measured.

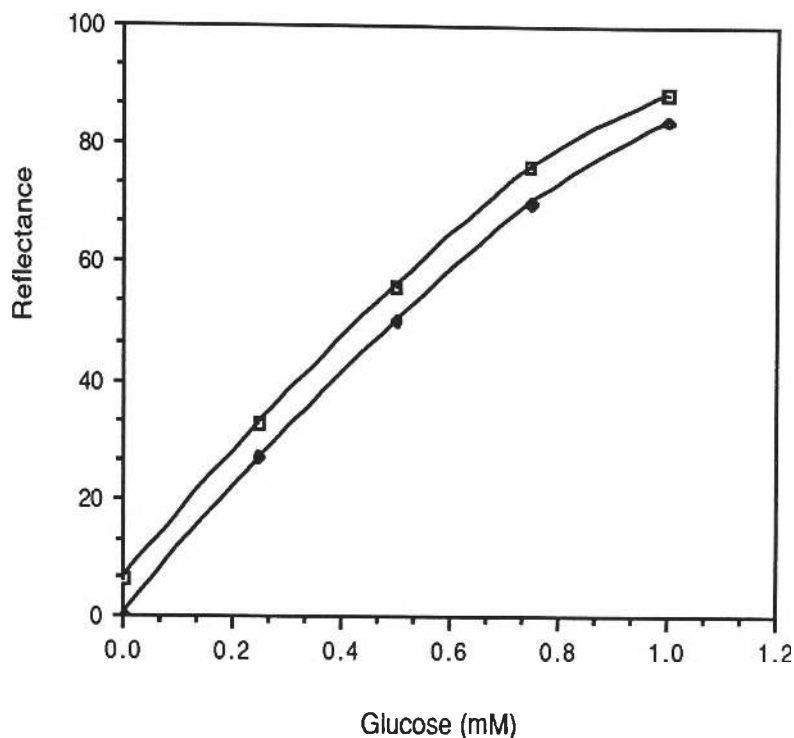


Figure 15. Dependence of reflectance values on the glucose concentration in the presence (□) and absence (♦) of rapeseed extract. Standard curves were prepared as described in the Methods Section 2.2.2.18.

Where a high GSL content (greater than 30 $\mu\text{mole/g}$ seed) needs to be measured, and the glucose concentration exceeds the meter range, the extract can be diluted or less seed used during extraction.

f) GSL Determination by Addition of Purified Myrosinase

Several rapeseed meal extracts were prepared and the GSLs hydrolysed by the addition of purified myrosinase (Methods Section 2.2.2.15). The reflectance values obtained were converted to total GSL content by reference to the standard curve B prepared in Methods Section 2.2.2.18. To evaluate the accuracy of the reflectance methodology, the estimations were compared with those obtained using the glucose oxidase/ peroxidase method.

Table 3 GSL Determinations for Method Comparison.

Rapeseed Sample	Total GSL Content : $\mu\text{mole/g Meal}$	
	Glucose Oxidase/ Peroxidase Method	Reflectance
BLN 270	9	12
RX 9	20	26
BLN241	23	30
Tatyoon	41	44
Jumbuck	59	62
Thomo 47	13	16
Thomo 42	22	28

Mean of duplicate GSL determinations are given for all methods.

Several rapeseed samples were analysed by two methods utilising the glucose oxidase/ peroxidase system for glucose determination.

The glucose oxidase/peroxidase method however, uses the chromophore 4-aminoantipyrine. The Reflectance method is described in Methods Section 2.2.2.15. The Reflectance method utilised intact rapeseed which was converted to $\mu\text{mole/g meal}$ based on approximate oil content of 50 %.

g) Free Glucose in the Rapeseed.

Since the reflectance method estimates GSLs by conversion to glucose it is important that endogenous free glucose levels are low and reasonably consistent between samples. Measurement of free glucose present in rapeseed was necessary to evaluate the consistency in the quantity of free glucose present in various rapeseed samples. The average free glucose content of several rapeseed samples was shown to be 6 $\mu\text{mol/g}$ meal which is approximately 3 $\mu\text{mol/g}$ seed.

Table 4. Determination Of Free Glucose in Rapeseed Seed.

Rapeseed Sample	Reflectance	Free Glucose $\mu\text{mole/g}$ meal
Global	12	8
BLN 270	13	8
RX 9	14	8
BLN 241	8	5
Robinson	7	4
Thomo 42	10	7
Tatyoon	11	7

Several rapeseed samples were measured for free glucose using the Clinistix strip method. The reflectance values were converted to glucose content using the standard curve shown in Figure 13 according to Methods Section 2.2.2.13

It is possible to correct the standard curve for a particular variety by spiking standard glucose solutions with a volume of boiled rapeseed extract to give a concentration equivalent to that used in the normal procedure. If necessary, a separate measurement of free glucose content in rapeseed or meal can be performed as described in Methods Section 2.2.2.19.

2.3.2 SIMPLIFICATION OF THE REFLECTANCE METHOD

1. Conditions For Selective Hydrolysis By Endogenous Myrosinase

In order to simplify the reflectance methodology and reduce costs, an investigation was undertaken to determine whether endogenous myrosinase present in seed samples could be employed in the assay method.

Experiments herein were directed at optimising the conditions for utilising the endogenous myrosinase in rapeseed to hydrolyse the GSLs during extraction.

The use of endogenous myrosinase in rapeseed to hydrolyse the GSLs required the elimination of the activities of other seed enzymes such as glucosidases which are capable of liberating glucose from conjugates or starch reserves. The following experiments were used to determine the conditions that would specifically allow the myrosinase enzyme to remain active.

Myrosinase shows activity over a broad pH range. The extraction of rapeseed was therefore performed at pH 8.5-9.0, thus hopefully suppressing other enzyme activities, as it has been shown that glucosidase enzymes are inactive at pH values greater than 8.⁸²

2. pH Adjustment of Rapeseed Extract

The initial pH of Tris buffer (25 mM) was varied to determine the appropriate extraction solution that would give an initial extract at $\text{pH} > 8$. Re-adjustment of the pH after selective hydrolysis by myrosinase was necessary for optimal Clinistix strip response and the amount of HCl required to give a final approximate pH 6 extract was determined.

Table 5. Determination of the Buffer and Acid Strength Necessary For pH Adjustment of Rapeseed Extract.

	Initial pH	HCl (1M)	Final pH
50 mM Tris pH 9	8.50	300 μl	7.78
	8.55	500 μl	7.24
	8.35	700 μl	6.06
	8.60	800 μl	5.50
50mM Tris pH 8	7.45	50 μl	7.06
	7.41	100 μl	6.83
	7.45	150 μl	5.50

Data in Table 5 was prepared by extracting rapeseed (see Methods Section 2.2.2.6.) into Tris buffer at pH 8 and 9. The amount of HCl (1 M) needed to lower the pH after hydrolysis by the endogenous myrosinase was determined using a pH meter, according to Methods Section 2.2.2.20.

The results demonstrated that a 50 mM pH 9 Tris buffer was able to maintain the pH > 8. Re-adjustment of extract to pH 6, prior to the Clinistix test, was found to require the addition of 700 μ L of 1 M HCl.

3. Conditions Affecting Solution Clarity.

The accuracy of the reflectance measurements are dependent on the clarity of the sample solution, hence the final extract solution needed to be clear and colourless. The results using Tris buffer are shown in Table 6.

Table 6. Determination of the Conditions Resulting in Clear Extract.

	Additives	Final Appearance
Extract 1.	1. 25mM tris buffer pH 9 2. 50 μ l chlorohexidine diacetate (10% w/v EtOH) 3. Add 500mg Act. charcoal 4. Centrifuge 1000 x g 10 min	Oily surface, aqueous solution slightly cloudy white.
Extract 2.	1. 25mM tris buffer 2. 50 μ l chlorohexidine diacetate (10%w/v EtOH) 3. 500mg Act. charcoal 4. Add 1.0ml chloroform 5. Centrifuge 1000 x g 10 min	Oily surface removed. Aqueous solution slightly cloudy white.

Table 6. A rapeseed extract (Marnoo) was extracted at pH 9 and treated with chlorohexidine diacetate. The visual changes in the extract with addition of the reagents was noted.

The final solution after protein precipitation, oil extraction, colour removal with charcoal and adjustment to pH 6.5 was cloudy white in appearance.

a) Sodium Bicarbonate Extraction

A 25 mM sodium bicarbonate extraction buffer was found to be sufficient to keep the extract at pH 8. Addition of activated charcoal (250 mg) lowered the pH but the sample extract remained turbid and green coloured (Table 7).

The use of Sodium carbonate/bicarbonate extraction buffer gave a similar result to that found with Tris buffer (Table 6).

Table 7. Observations Using Sodium Carbonate/Bicarbonate Extraction Buffer.

Buffer (mM)	Extract pH	Colour of mixture	Addition of Activated Charcoal	
			pH	Appearance
50	9.00	dark yellow	7.90	green and cloudy
50	8.02	dark yellow	7.75	green and cloudy
25	8.84	dark yellow	6.90	green and less cloudy

Table 7. Rapeseed was extracted into Na_2CO_3 / NaHCO_3 buffer and the pH measured. After protein precipitation using chlorohexidine diacetate, activated charcoal (250 mg) was mixed with the sample before centrifugation at $1000 \times g$ for 15 minutes. The appearance of the supernatant was noted.

The results in Table 7 indicate some improvement in the clarity of the extract when the 25 mM buffer was used which coincides with a lower pH. The green colouration was not removed or reduced with the addition of activated charcoal.

b) Role of Activated Charcoal in pH Adjustment.

The effect of activated charcoal was determined according to Methods Section 2.2.2.14 and the results are shown in Table 8 below. The observed changes in the sample extract with the addition of activated charcoal (sulfuric acid washed) coincided with lowering of the pH.

Table 8. Effect of Activated Charcoal on Rapeseed Extract.

Activated Charcoal (mg)	pH	Observation
50	7.5	green and fairly clear
200	6.9	faint green and slightly cloudy
300	6.8	faint green and clear

The results indicated that at a clear extract could be obtained at lower pH in the presence of activated charcoal. The green colouration however was not entirely removed even with large quantities of activated charcoal (300 mg).

c) Sodium Carbonate Buffer Extraction of Rapeseed with pH Final Adjustment.

The results shown below (Table 9) demonstrated a combined effect of pH lowering with HCl in the presence of activated charcoal which produced a clear and colourless extract.

Table 9. Effect of Lowering pH in the Presence of Activated Charcoal

Rapeseed Sample	pH of Extract	pH adjusted Extract	Extract Appearance
BLN 270	8.36	6.90	Yellow and cloudy supernatant
CRX 9	8.35	6.12	Clear and colourless
Thomo 42	8.30	6.00	
Jumbuck	8.40	6.00	

Table 9. The results were obtained by extracting the rapeseed into 50 mM sodium bicarbonate buffer at pH 9. After 10 minutes the proteins were precipitated and the pH was lowered by adding 700 μ l of 1 M HCl. Activated charcoal (250 mg) was added and the mixture vortexed and centrifuged. The reflectance values were converted to μ mole/g seed by using the standard curve of reflectance versus GSL concentration shown in Figure 14.

The results shown in Table 9 clearly demonstrated the need to lower the final pH of the sample solution down to approximately pH 6 in conjunction with activated charcoal. The use of bicarbonate buffer however, was not suitable since gas evolution after pH adjustment to

pH 6 caused particulate matter to disperse after centrifugation. Gas evolution on the Clinistix pad was also observed along with the formation of white salt. These effects would definitely cause errors in the reflectance reading and therefore another buffer, glycine was tested.

d) Rapeseed Extraction with Glycine Extraction Buffer.

The results (Table 10) suggest a 50 mM Glycine buffer at pH 9 was necessary to maintain the seed extract at pH >8.

Table 10. Rapeseed Extraction Appearance and pH after Glycine Buffer Extraction.

Glycine Buffer	pH after seed maceration	Appearance of Final Extract
10mM pH9.0	6.5	Clear and colourless pH 5.0
25mM pH8.5	6.5	Clear and colourless pH 5.0
35mM pH8.5	8.0	Slight white cloudiness pH 6.8
50mM pH8.5	8.2	Slight cloudiness and light green colouration pH 7.2 Addition of extra 50mg charcoal gave colourless extract, cloudy pH 6.7
50mM pH9.0	8.5	Slightly cloudy at pH 7 Clear and colourless after adjusting to pH 4.5 with HCL

Table 10. The seeds of a rapeseed sample (Cowra 503) were extracted using varying buffer strengths. The extract was mixed with activated charcoal (250 mg) and 1.0 ml chloroform before centrifugation. No final pH adjustment was used and the clarity of the extract and the pH were recorded.

It can be seen from the above results that a clear and colourless extract was obtained when the final pH was 5.

Extractions using a 50 mM glycine/NaOH buffer were able to maintain the initial pH at 8 to 9, however after hydrolysis, the activated charcoal alone did not cause an appreciable drop in the final pH. These extracts were cloudy and green coloured. It appeared from these results that extractions using 50 mM glycine pH 8.0 buffered the rapeseed extract at 8.0, however the addition of a suitable acid would be required to give a final clear and colourless extract for glucose determination using reflectance.

- e) Rapeseed Extraction with Glycine/NaOH buffer with Final pH Adjustment Prior to GSL Determination.

Analysis of six rapeseed samples using 50 mM glycine buffer (pH 9) gave extracts with pH > 8 (Table 11). After protein precipitation, oil extraction with chloroform and addition of activated charcoal (250 mg), 1M HCl was used to lower the pH. A final pH 4 to 5 was found from previous results (Tables 9 and 10) to give a final clear and colourless extract. The final extract was centrifuged (1000 x g, 10 minutes) and measured for liberated glucose.

The results shown in Table 11 demonstrated a good correlation between the values obtained using a colorimetric glucose measurement and those by GSL hydrolysis using endogenous myrosinase (see Figure 16).

Table 11. Comparison of pH adjusted Reflectance measurement with Thymol method.

Variety	pH initial after maceration	pH final	μ mole GSL/g meal	
			Reflectance	Thymol
BLN 270	8.31	4.00	12 14	12 13
CRX 9	8.14	4.20	23 24	26 29
BLN 241	8.25	4.18	32 31	30 28
Thomo 47	8.30	4.52	13 15	16 17
Tatyoan	8.58	4.21	47 45	44 44
Jumbuck	8.50	4.63	64 61	62 61

Table 11. Rapeseed was extracted and prepared for GSL measurement according to the Methods Section 2.2.2.21. Duplicate total GSL estimates are listed with those obtained using a colorimetric method (thymol method).

In summary the extraction of rapeseed using a 50 mM Glycine/NaOH buffer pH 9.0 was found to give satisfactory extraction with final pH values of the extracts being above pH 8. A final clear and colourless extract was obtained in all the trials by employing pH adjustment with HCl.

f) Selection of a Buffer for pH Adjustment of Final Extract

The need to lower the pH with addition of activated charcoal after protein precipitation mandated the use of an appropriate buffer to control the final pH more precisely.

Clinistix strips function by use of enzymes, whose activity in

turn, are dependent on pH. Hence it was desirable to obtain a consistent pH of the final extract in order to have reproducible colour development. The results in Table 11 demonstrated the need for the addition of a suitable buffer to maintain the final pH.

A group of buffers were therefore examined to determine their compatibility with the Clinistix enzymes and colour development (Table 12).

Table 12. Selection of Buffer for Final pH Adjustment.

Glucose in mM	Reflectances at pH 5.0				
	dist. water	Citrate	Acetate	KH phthalate	std. Glucose
0	0	2	0	< 0	< 0
	1	1	< 0	0	0
0.25	29	29	29	20	25
	27	30	26	22	28
0.50	55	54	47	38	52
	53	56	46	33	53
1.00	80	81	75	66	87
	80	83	75	64	85

Table 12. Glucose standards are prepared and spiked with a buffer at pH 5 according to the Methods Section 2.2.2.22. Standard glucose; a 1.0 mM glucose solution (Sigma) and buffer was used to calibrate the meter. The column headed 'Distilled water' represents a glucose solution prepared by dissolving powdered glucose (BDH) and adjusting to pH 5 with HCl.

The use of potassium hydrogen phthalate caused suppression of the reflectance values. A suppression in the reflectance value was also apparent when acetate buffer was used. A similar reflectance response to a glucose solution (BDH) adjusted to pH 5 with HCl was found, suggesting benzoic acid preservative does not interfere with the Clinistix strip. The use of 50 mM sodium citrate was found to give the least reduction in Clinistix colour development.

4. Glucosinolate Hydrolysis by the Endogenous Myrosinase.

The enzymic liberation of glucose was monitored at both pH 8.5 and 5 (Methods Section 2.2.2.21). The results shown in Figure 16 demonstrated that a steady increase in glucose was detected with the Clinistix strip. The extraction using Glycine buffer pH 8.5 resulted in a fixed amount of glucose being released within 5 minutes. Since the amount of glucose liberated at pH 8.5 is equivalent to the estimated GSL content as determined by Glucose oxidase/peroxidase method (BLN 270 11 $\mu\text{mol/g}$ seed), it appeared that only myrosinase among other glucose releasing enzymes maintained a detectable level of activity at pH 8.5. The measurement of glucose produced at pH 8.5 was continued for 5 hours to confirm that no other enzyme activities were gradually liberating glucose.

The extraction of the rapeseed at pH 5.0 (Figure 16, curve a) demonstrated an initially rapid liberation of glucose followed by a slow increase in glucose concentration. Approximately twice the amount of glucose was detected after 5 minutes compared with extraction at pH 8.5.

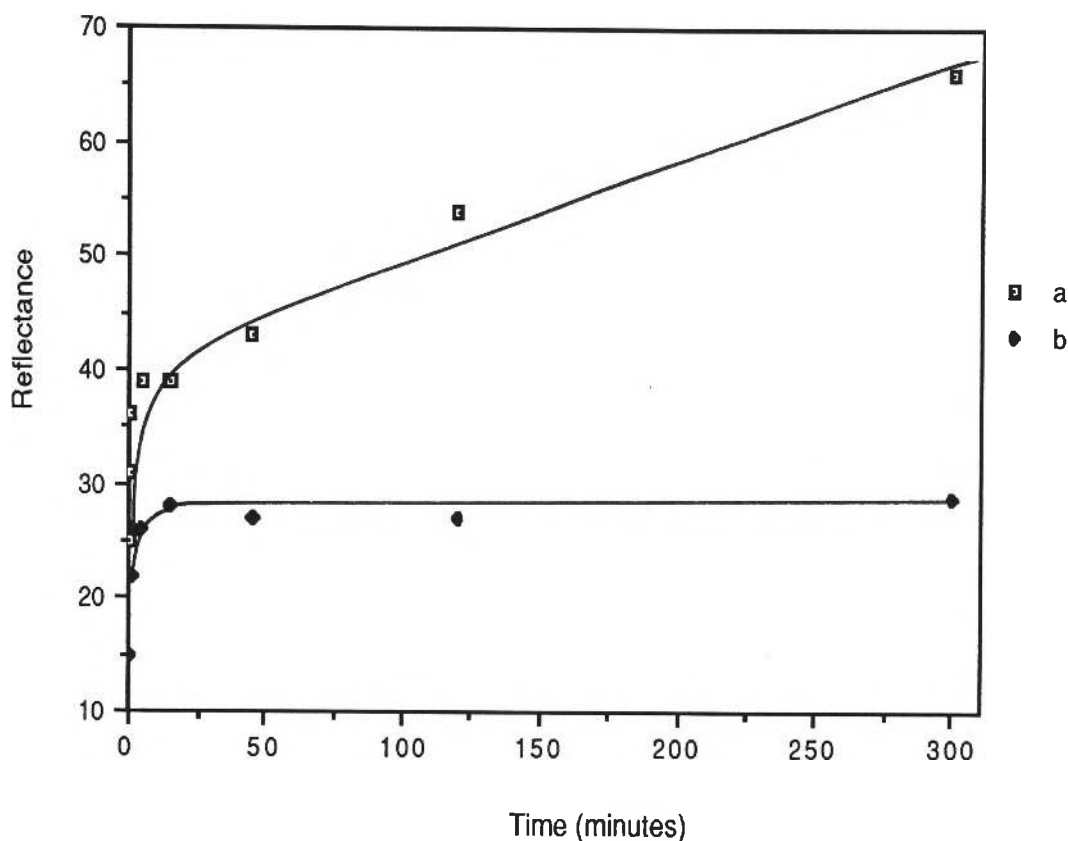


Figure 16. Time course reaction of glucose release. (a) : Extraction using pH 5 Glycine buffer. (b) : Extraction using Glycine buffer pH 8.5. Glucose was measured by Clinistix strips and Digital 4000 reflectance meter and plotted against time (minutes), according to the Methods Section 2.2.2.21.

5. Quantitation of GSLs in Rapeseed by Reflectance Using Endogenous Myrosinase for GSL Hydrolysis.

The results shown in Table 13 demonstrated that the endogenous myrosinase hydrolysis of GSLs, coupled with the Clinistix strips and reflectance meter could enable the estimation of total GSL in rapeseed.

Table 13. Reflectance Measurement of GSLs using Endogenous Myrosinase

Variety	Glucosinolate $\mu\text{mole/g}$ seed		
	Test 1	Test 2	Test 3
BLN 270	4	8	5
RX 9	13	15	13
BLN 241	16	14	14
Tatyoan	24	24	21
Jumbuck	32	31	31
Thomo 47	9	8	8
Thomo 42	15	16	13

Table 13. Several commercial samples of rapeseed were analysed in duplicate on individual days according to the methods section 2.2.2.23. The reflectance values were performed in duplicate and the average recorded.

2.3.3 DEVELOPMENT OF REFLECTANCE METER FOR GSL MEASUREMENT IN RAPESEED.

The use of reflectance to monitor GSL levels in rapeseed has therefore the potential to meet the main requirements of a cheap and accurate means of screening for low levels of GSL (less than 30 $\mu\text{mol/g}$ seed). However this could be useful only if a meter was available to properly read the Clinistix strip without the need for

careful positioning of the strip across the light aperture such as that required for the Digital 4000. The programming of the meter to give an instant digital display of the GSL concentration was also considered desirable.

1 Strip Positioning within Meter.

The careful positioning of the Clinistix pad over the light aperture of any reflectance meter was found to be necessary for accurate results to be obtained. The possibility of stray light or misalignment must be eliminated by having a strip holder and strip design which is compatible. It was found that the read head assembly used in the AMES 2 blood glucose meter appeared ideal for this purpose.

The Clinistix strip design was altered by having the Clinistix test pad mounted onto a plastic strip designed specifically for the read head. These strips, called "Hybridstix", allowed rapid and correct positioning of the Clinistix pad over the aperture.

The removable strip holder from the AMES 2 meter also provided easy removal for cleaning.

2 Meter Assembly.

A portable meter was developed (Figure 17) which incorporated an LCD display, read head, rechargeable batteries, multiple push button function, calibration control and update eproms.

After construction, a series of glucose standards were prepared and their reflectance values were measured and displayed as a glucose concentration according to the programmed standard curve.

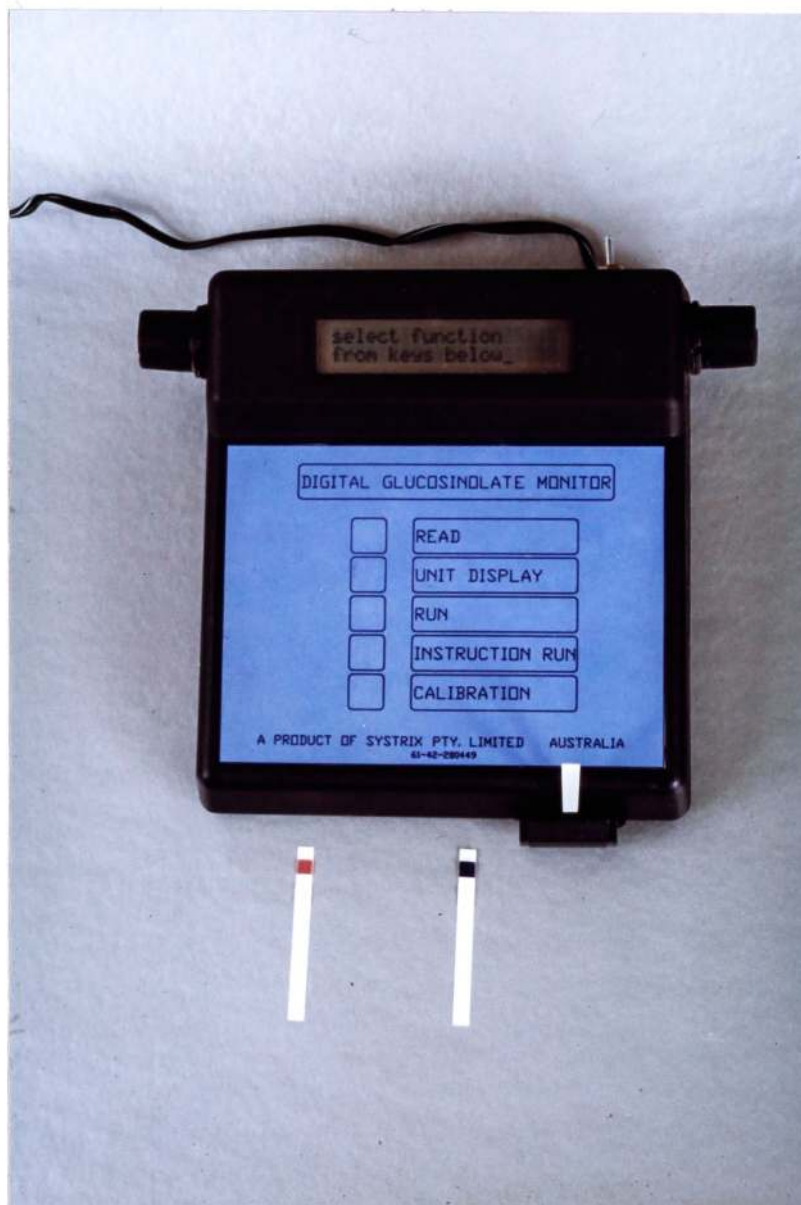


Figure 17. TruBluGlu reflectometer.

The results shown in Figure 18, demonstrate low sensitivity at glucose concentrations less than 0.25 mM. Further adjustment of the gain and zero controls for optimum sensitivity for low glucose concentrations resulted in a sigmoidal response with loss of sensitivity at both low and high glucose concentrations and adjustment of zero and gain controls did not improve the response as shown in Figure 18.

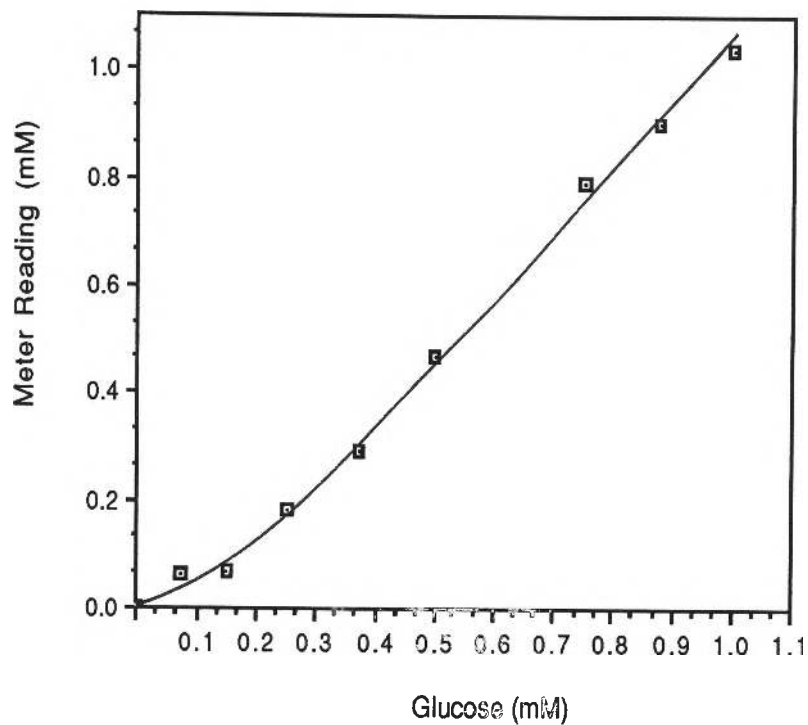


Figure 18. The meter was calibrated as in Methods Section 2.2.2.8. A series of glucose standards were prepared and their concentration determined according to their reflectances using Hybridstix and the programmed TruBluGlu meter according to Methods Section 2.2.2.18.

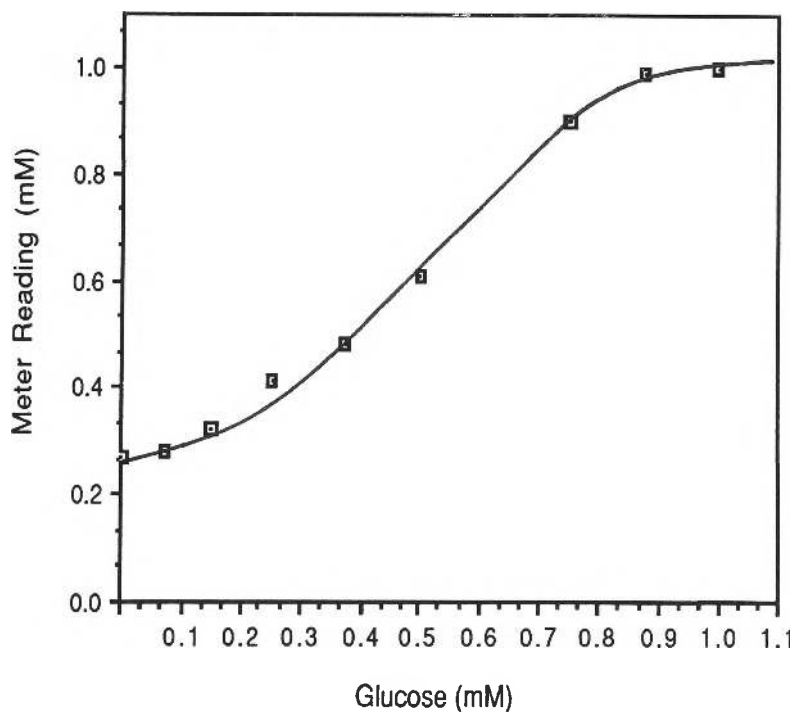


Figure 19 The glucose standards were measured as in Figure18. The lamp voltage was 3.0 volts.

A similar response had occurred with the Digital 4000, but improved detection in the lower glucose levels was achieved by reducing the size of the aperture. This was not a commercially desirable adjustment since this would involve altering the design of a pre-existing readhead component such that the light apertures in different meters were identical. As an alternative adjustment it was found that the lamp voltage could be lowered from 3.0 to 2.5 volts, thereby reducing the quantity of reflected light reaching the detector. The calibration curve shown in Figure 19 thus became linear and allowed detection of the Clinistix colour change in the concentration range 0 to 1.0 mM glucose.

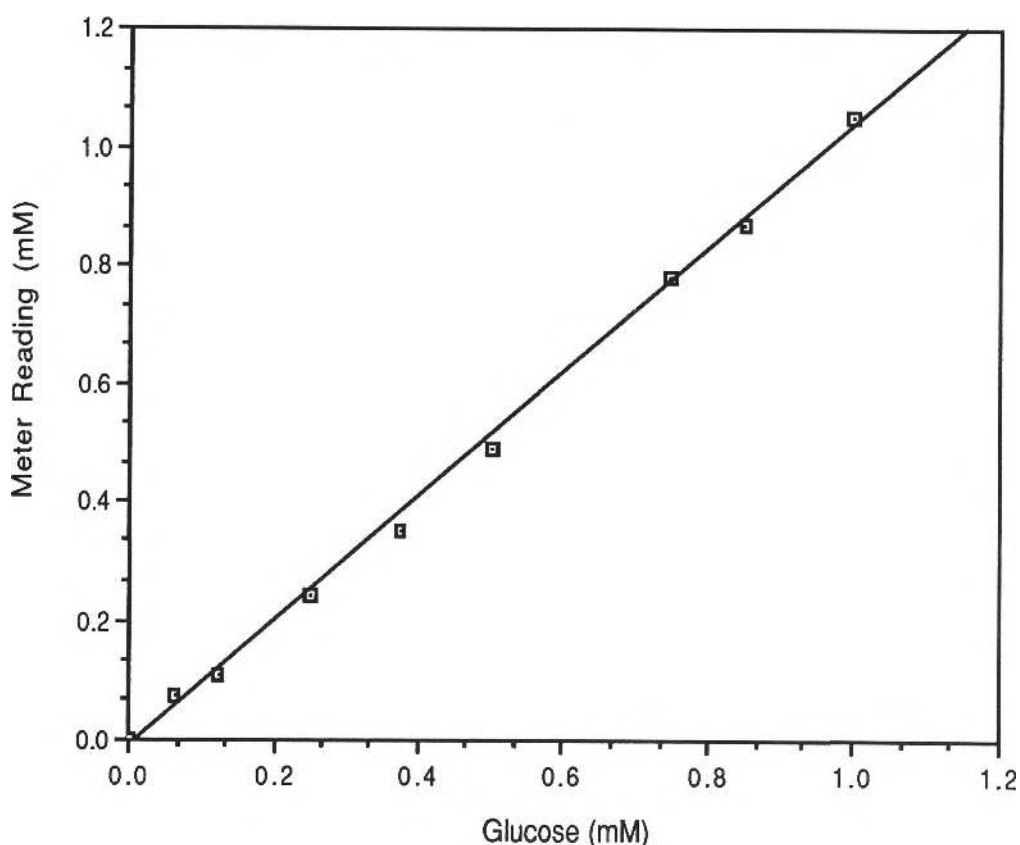


Figure 20. Adjustment of the lamp voltage from 3 to 2.5 volts. Glucose standards prepared as in Methods Section 2.2.2.18 were measured for free glucose using Hybridstix and TruBluGlu reflectometer.

3. Field Trial : Effect of Temperature on Clinistix Colour Development.

Field trials of the TruBluGlu reflectometer with "Hybridstix" were performed at the Oilseed Research Institute in Wuhan, Peoples Republic of China in 1987. These tests revealed the need to provide compensation for low temperature conditions.

As shown in Figure 21, the colour development time needed to be doubled when operating temperature was lowered to 6 °C.

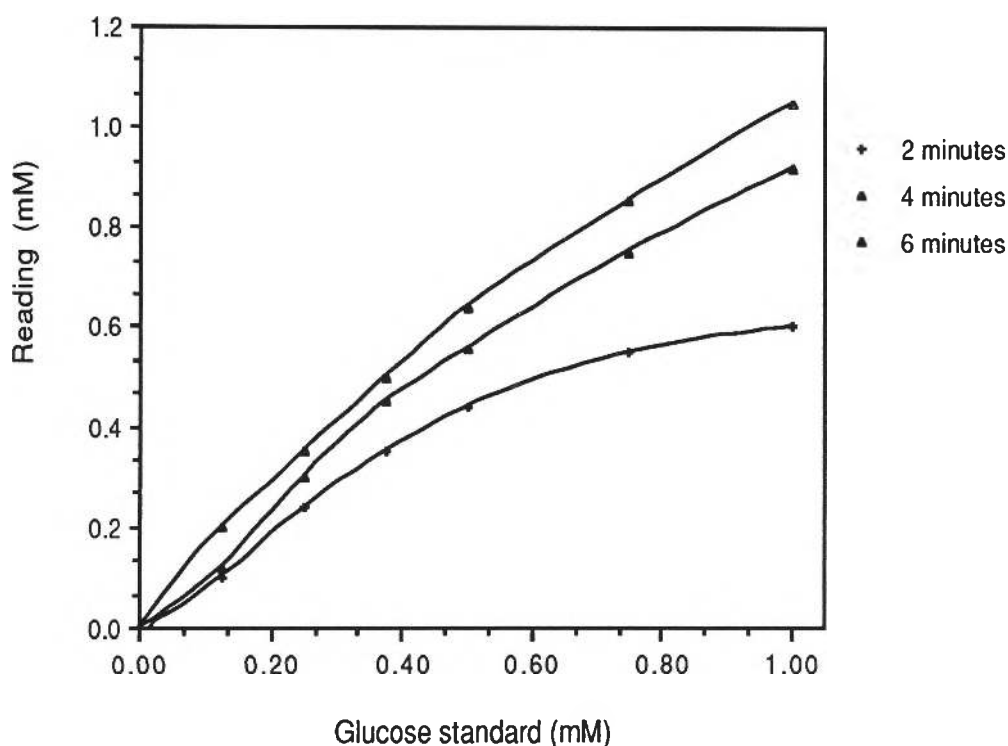


Figure 21. Standard glucose solutions at 6 °C were measured using Hybridstix according to Methods Section 2.2.2.11. The colour development of the Hybridstix was monitored at 6 °C over 2, 4 and 6 minutes. The standard curves for colour intensity of the Hybridstix at these times is shown.

The use of this reflectance methodology in low temperature conditions ($\sim 6\text{ }^{\circ}\text{C}$) was initially found to give low estimates of GSL content in rapeseed. It was found that at low laboratory temperatures ($6\text{ }^{\circ}\text{C}$) a 4 minute development time was required for correct values to be obtained. The results shown in Figure 20 also demonstrated that with longer development times at $6\text{ }^{\circ}\text{C}$, a more intense colour development could be obtained with the Hybridstix

To determine whether adjustment of the sample solution temperature was sufficient to allow normal Clinistix strip colour development, the glucose measurements were performed on standard glucose solutions at $23\text{ }^{\circ}\text{C}$ and at $6\text{ }^{\circ}\text{C}$.

The results shown in Figure 22 indicated that the temperature of the extract or glucose solution alone was not a factor in affecting the colour reaction.

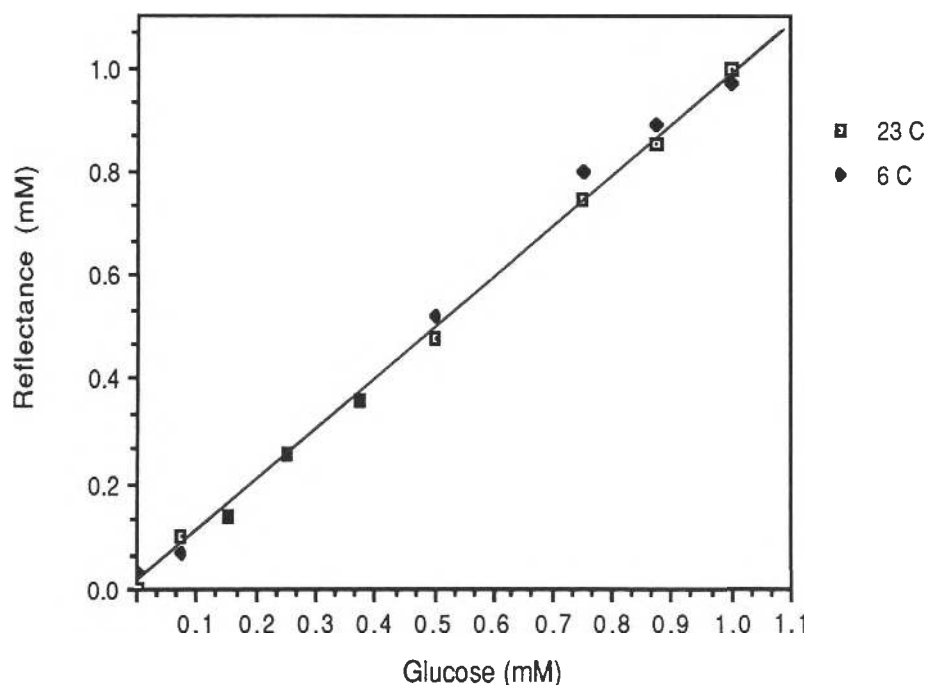


Figure 22 A glucose standard curve was prepared using Hybridstix dipped into standard solutions at $6\text{ }^{\circ}\text{C}$ and $23\text{ }^{\circ}\text{C}$. The colour development was at $23\text{ }^{\circ}\text{C}$. ◆ : The standard solutions were cooled to $6\text{ }^{\circ}\text{C}$. □ : Standard solutions at $23\text{ }^{\circ}\text{C}$.

These results suggested that the temperature at which colour development occurred was the prime factor causing variation in the rate of colour development in the Hybridstix.

To examine, in more detail the effect of temperature on the Hybridstix, glucose standards were measured at various temperatures and the colour developments monitored.

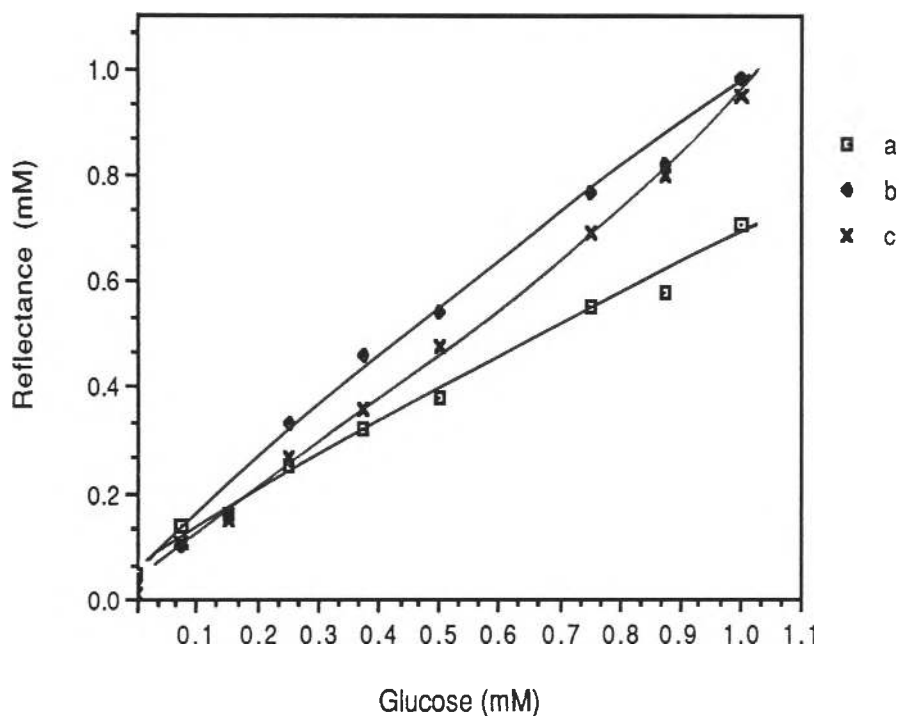


Figure 23. Hybridstix and TruBluGlu meter were used to measure standard glucose solutions, the Hybridstix colour development was performed at (a) 6 °C (b) 13 °C (c) 25 °C and measured after 2 minutes.

The results shown in Figure 23 suggested a significant change in the rate of colour development in the Hybridstix, particularly when the temperature fell below 13 °C. The variation in rate of colour development as a function of temperature was compensated for by increasing the amount of gain adjustment. This meant that although

a 1.0 mM glucose gave only 70 % the expected colour intensity after 2 minutes at 13 °C, there was sufficient zero and gain adjustment to set this intensity to read 1.0 mM (100 %). Hence the TruBluGlu reflectometer calibration (Methods Section 2.2.2.8) has sufficient range to compensate for significant temperature variations.

The reflectance readings were repeated for four standard glucose solutions to determine whether a significant loss in precision occurred with the low temperature Hybridstix reaction.

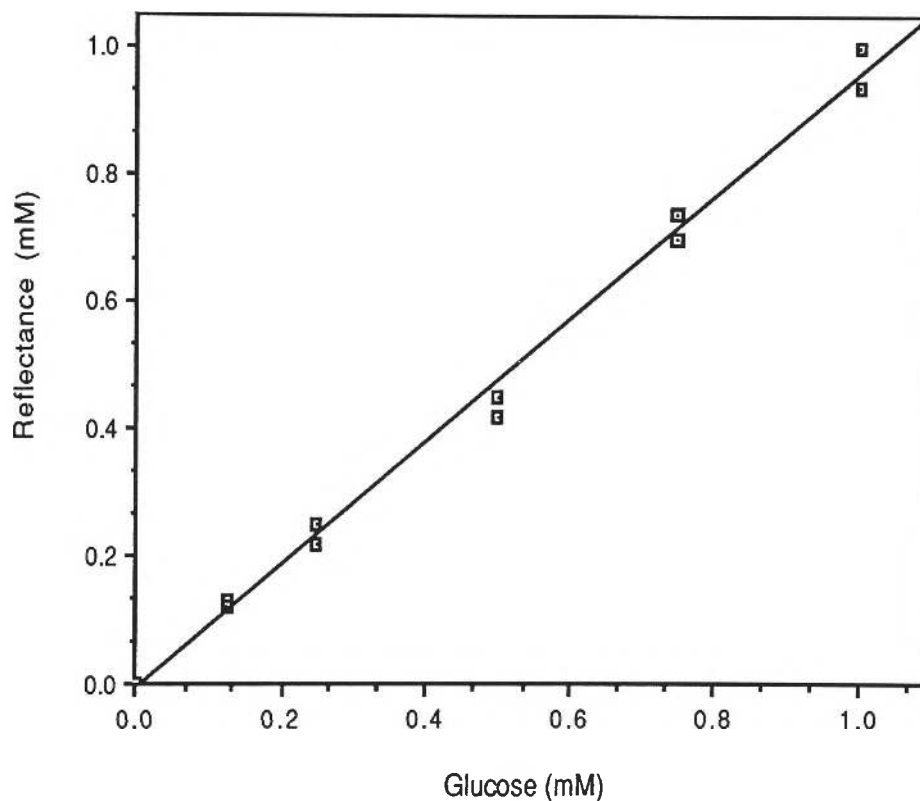


Figure 24. Standard glucose solutions were measured in duplicate at 6 °C, using Hybridstix and TruBluGlu reflectometer.

The results shown in Figure 24, demonstrated that under low operating temperatures, no significant loss in precision was observed and since there was good correlation (0.994) between meter measurement and the standard glucose concentration, it is apparent that accurate results could be obtained at low temperature

using this method. Alternatively a standard curve could be constructed using glucose solutions at the ambient temperature of the laboratory.

2.4 DISCUSSION

Plant breeders require relatively inexpensive and sensitive methods for the large-scale screening of rapeseed samples.

Complexiometric analysis using tetrachloropalladate appeared unsuitable since it lacks specificity.⁴⁸ A thymol method was shown to be very sensitive⁶² however difficulties were experienced regarding the reproducibility of measurements. The use of 80% sulfuric acid for thymol reaction required extra care during the 30 minute incubation in boiling water. Hence this method was not widely used for GSL measurements by breeders.

The measurement of glucose as an indirect estimation of GSL content has been reported in numerous studies involving colorimetric methods.^{50,57,61} Commercial myrosinase from white mustard (*B. hirta*) can be used to hydrolyse the GSLs to give stoichiometric amounts of glucose. Use of glucose oxidase/ peroxidase or hexokinase/ATP with glucose-6-phosphate dehydrogenase /NADP⁺ systems in conjunction with a chromophore, enables glucose quantitation. The major drawback with such methods is the number of steps, such as the need for DEAE Sephadex A-25 for GSL purification or the use of enzymes for GSL hydrolysis and glucose measurement. The hydrolysis of intact GSLs with the addition of commercial myrosinase is also time consuming, requiring up to 2 hours for completion.⁵⁴ The enzymic glucose determination also required as much as 30 minutes for completion.⁵¹ Although as many as 200 samples could be screened daily, the method required constant attention by skilled personnel.

The reagents were also relatively expensive and a spectrophotometer was required.

Attempts to simplify this methodology by omitting the use of ion exchange columns were not successful due to the presence of interfering compounds.⁸³

A critical step applicable to all methods to date concerns the initial quantitative extraction of the intact GSLs. The presence of endogenous myrosinases required initial heat treatment of seed by immersion into boiling water, prior to extraction of the GSLs. Alternatively, the seed was dried before maceration, before dissolving into boiling water for 1 minute.⁸⁴ The latter method is not recommended since complete myrosinase inactivation has been shown to require heating at 100° C for at least 15 minutes.⁸⁵

The use of reflectometers for GSL measurement was first described by Thies (1985).⁵⁵ The reflectance method had several drawbacks, such as the need for air dried rapeseed, the use of commercial myrosinase, the lack of pH control and the tedious application of the sample to a paper tape. More recent methods involve simply macerating the seed in the presence of myrosinase without terminating enzyme activity with a protein precipitant.⁸¹ Such methods would give considerable over-estimation of the GSL content since enzymically released glucose from other sources would be measured. It is seen in Figure 16 that the presence of rapeseed enzyme activities can quickly liberate significant amounts of glucose from sources other than GSLs. However this would not be a problem provided the GSLs are purified on ion exchange chromatography.

The use of reflectometry nevertheless has the potential for providing a more economical and efficient means of screening rapeseed samples by plant breeders.

Given the need to select Canola varieties (less than 30 $\mu\text{mole GSL/g}$ seed), it was desirable to extract 200 mg seed (approximately 10 seeds) into a 6.0 ml volume allowing efficient extraction and a final concentration of 1.0 mM GSL, at 30 $\mu\text{mole/g}$ seed. The selection of a suitable glucose test strip was therefore dependent on its sensitivity.

Feibig and Kallweit utilised the blood glucose test strips Haemo-Glukotest 20-800R which are dedicated for use with the Reflolux 2 reflectometer from Boehringer-Mannheim.⁸⁰ Despite the expense associated with blood glucose test strips (approximately 20 times the cost of urine glucose test strips), the published results suggested that only a estimation of low, medium or high GSL content was possible due to the insensitivity of the test strip and/or meter used. The use of meters and strips designed for diabetics are not ideally suited for other applications such as GSL determination, due to the pre-programming of the meter and the need to enter a bar code which calibrates the meter for the measure of glucose in blood samples. For these reasons the reflectance meter Digital 4000 was used, since it gave a direct digital display of reflected light as a inverse function, hence as less light is reflected, a higher reading would be given. When Clinistix reacts with glucose, the higher the glucose the darker the Clinistix strip pad and the higher the reading. The later models also allowed manual calibration with zero and gain controls.

The results obtained using urine glucose test strips (Tes-tape and Clinistix) are shown in Figures 3 to 7. These showed good sensitivity, which was consistent with Thies's application of Glucotest paper. Clinistix strips, like other glucose test strips or papers, are impregnated with enzymes specific for glucose. The typical reaction that occurs in response to glucose is shown below.



The Clinistix strips in conjunction with the TruBluGlu meter were capable of distinguishing slight changes in glucose concentration (0.1 mM). This was also applicable at glucose concentrations in the region of 0.25 mM. Alternative glucose test strips, for example, Haemo-Glukotest 20-800 used by Feibig and Kallweit, give a white to blue colour change.⁸⁰ Such strips, when applied to standard glucose concentrations (0 to 1.0 mM) failed to give a measurable reflectance.

Further complications were experienced during removal of excess moisture. Where test strips or papers are immersed or applied to sample solution, the excess moisture needed to be removed prior to reflectance measurement. A gentle wipe to remove excess moisture often leads to a reduction in colour intensity due to dye leaching. It was found that Clinistix could be simply shaken once, after sample application, to remove excess moisture. This also avoided possible abrasion of the pad surface, since irregularities in the surface caused by wiping or blotting may vary the reflectance. The blood glucose strips used in other reflectance methods are especially prone to dye leaching. With removal of excess moisture considerable amounts of dye were removed, adding further to reduce sensitivity and reproducibility. It was found that Clinistix strips immersed in sample solution for 5

seconds allowed for optimum colour intensity (Figure 9). These strips unlike other test papers or strips, have a absorbent pad (0.5 mm thick) and absorb approximately 9 μ L of solution. Dye leakage from the Clinistix test strip during dipping was evident by a yellowing of the sample solution after repeated testing.

Although Clinistix are designed for visual estimation of glucose concentrations in the range 10 to 20 mM in urine, it was found that glucose standards could be detected in the range 0 to 2.0 mM (shown in Figure 8).

Using Clinistix strips, (unlike Tes-Tape or the Glucotest paper used by Thies⁵⁵) the pad was fixed to a plastic strip allowing better handling. The advantage is seen with immersion of the pad into a solution and removal of excess moisture by shaking. The critical positioning of the pad across the aperture was also simplified. The use of Glucotest paper required cutting test paper to length and manipulating the paper into an aliquot of sample solution before placing it across the light aperture with tweezers.

The instability of glucose test papers and strips after colour development was also encountered by Thies.⁵⁵ Some reduction in the rate of fading by shading the strip from direct light is supported by the results in Figure 11. The fading of the strip colour intensity however is not a serious problem provided the measurements are made at the specified time of 2 minutes after sample application.

Although Clinistix strips are designed only for the estimation of glucose in urine, different batches were found to give consistent colour development for glucose concentrations 0.0 to 1.0 mM. In order to obtain accurate glucose measurements the removal of pigments, phenolics and protein from the extract was found to be necessary. Thies used charcoal filters, which were not always

effective, but for reasons of cost, powdered charcoal was chosen in this work.⁵⁵ It was shown that 50 mg of powdered activated charcoal is required for removal of inhibitors (see Table 1) and as much as 250 mg of activated charcoal could be added without detectable adsorption of glucose.

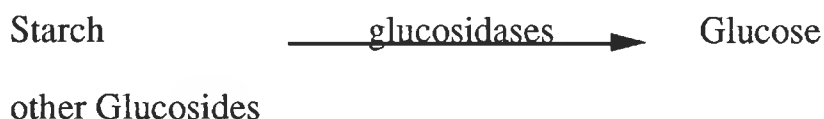
A difficulty associated with powdered charcoal was the adsorption of floating particles onto the test strip pad causing variable reflectance measurements. This could be remedied by either using a pasteur pipette to transfer some sample solution to the pad or by passing some solution through a filter paper.

With the selection of a suitable glucose test strip the reflectance meter and methodology were optimised to allow rapid and simple GSL determinations.

Rapeseed, being typical of all GSL containing plants, contain myrosinase type enzymes, which are essential for degrading the GSL to its more toxic forms.⁸⁶ Myrosinase has been reported to exhibit an activity over a broad pH range with considerable activity remaining at pH 9 (Figure 16). Given that glucosidases and β -galactosidase have no detectable activity at pH 8 (see Chapter 4, Figure 31) it was conceivable that plant material extracted in a pH 9 buffer would exhibit only myrosinase activity. Hence the amount of glucose liberated under such conditions may be directly proportional to the GSL content. It was also necessary however, to examine the free glucose already present in the seed to prevent an apparent over estimation of GSL content. The glucose detected was found to be fairly constant between rapeseed samples, at approximately 3 μ mole/g seed (shown Table 4). The increased colour intensity attributed to the free glucose could be corrected for during construction of the standard curve. Questionable values could

be checked by boiling the seed prior to extraction and measuring the level of glucose, as described in Methods Section 2.2.2.19. A significant amount of free glucose could result in cases where the seed has hydrated or begun to germinate.

The results shown in Figure 16 demonstrated the influence of pH on the glucose content. During extraction at pH 5 the liberation of glucose with time is thought to be the result of reactions such as that shown below.



The use of 50 mM glycine buffer at pH 9 for rapeseed extraction was found to give a final extract with pH 8-9 (Table 10). Under these conditions a constant amount of glucose liberation equivalent to the expected GSL concentration was observed (Figure 16). Hence the myrosinase retained sufficient activity at this pH to hydrolyse the GSLs.

A lowering of the pH after protein precipitation was considered necessary for two reasons, firstly it facilitated the removal of interfering compounds with activated charcoal (Tables 8-10) and secondly, it brought the solution pH into the region required for optimum enzymic measurement of glucose with the glucose oxidase/ peroxidase system (Clinistix strips). Since the Clinistix strips are enzymic, the rate of reaction is pH dependent and it was important to ensure the final pH of the solution to be measured was identical to that of the standards. This was achieved by screening a group of buffers for compatibility and effectiveness at giving the desired final pH 5, which was shown to be necessary

for obtaining a clear and colourless extract. Sodium citrate buffer (final concentration: 50 mM) was selected, since no major alteration in Clinistix strip colour development was observed (Table 12).

Having optimised the sample methodology to allow detection of low levels of glucose in a rapeseed sample using Clinistix strips (Method Section 2.2.2.23), it was necessary to ensure a simple and correct measurement of the colour intensity which was also achieved.

The development of a meter designed specifically for GSL quantitation was considered to be a necessary adjunct to the methodology for GSL extraction and hydrolysis described previously. A protocol for extraction and selective hydrolysis of intact GSLs in rapeseed, for example by plant breeders or personnel at receival depots, can provide meaningful results only if the liberated glucose can subsequently be measured by simple means. For this reason it was considered desirable that a portable meter should incorporate a digital display and the strip holder should be as free as possible of operator error when positioning the strip.

Although the read head and strip holder were designed for blood glucose strips, a modified Clinistix pad was specifically manufactured with the design of a blood glucose strip. These strips were referred to as Hybridstix and were found to be fit securely across the light aperture of the Ames read head without needing special care. The meter was provided with a digital reading capable also of giving method instruction. An EPROM was programmed by measuring standard solutions every 0.1 mM and inputting the corresponding reflectance. A zero and gain control allowed the EPROM to be adjusted so that fine tuning could be achieved. Pressure buttons ensured no foreign material entered the meter while being used in a non laboratory area. Option buttons allow the

operator to select the units of measurement such as Reflectance, glucose (mM) or GSL ($\mu\text{mol/g}$ seed). A programmed method giving a step by step display of the method for sample preparation or for use of the Hybridstix was also provided.

The meter has dimensions 20 x 25 cm with rechargeable batteries, allowing portability. The readhead assembly has a detachable strip holder allowing easy removal if cleaning is required.

The reflectance meter (TruBluGlu meter) used in conjunction with 'Hybridstix' and the methodology described in the Methods Section 2.2.2.23, enables relatively accurate screening of rapeseed samples as judged by the comparison of results with the Glucose oxidase/peroxidase system (Table 3).

This methodology developed primarily for rapid and economical screening of rapeseed samples has been demonstrated to provide a sensitive and reproducible (Table 13) measure of the total GSL content. A comparison with results obtained using the thymol method, showed good correlation for several rapeseed varieties (Table 15).

In summary, some advantages offered by this reflectance method are :

1. No need for expensive reagents or equipment.
2. No enzymes are required for GSL hydrolysis.
3. Interfering compounds are removed with activated charcoal eliminating the need for DEAE Sephadex A-25 purification step.
4. Rapid sample preparation, no oil extraction or seed drying is required.
5. The meter is compact and portable; rechargeable batteries allow 24 hour run time.

6. A digital display of the GSL content with recall memory of method instructions.
7. The Hybridstix require simple dipping in the sample solution and placement into the meter.
8. The Hybridstix strips are inexpensive.
9. The meter can be calibrated for fine tuning, taking account of the operating temperature if necessary
10. GSL levels between 0 and 30 $\mu\text{mole/g}$ seed are readily measured requiring only 200 mg of seed (approx. 10 seeds). High GSL containing varieties can simply be diluted.
11. The extracts can be stored for hours without detectable change (Figure 16).

Disadvantages.

1. The Hybridstix strips are unstable after colour development and tend to fade appreciably after a 3 minute development time.
2. The Hybridstix have a expiry date (generally two years from date of manufacture), and once the bottle has been opened, the strips deteriorate at a faster rate.
3. The presence of free glucose can cause inaccuracy however this can be checked by firstly boiling the seed and measuring the free glucose. This has not been found to be a problem, however seeds stored under conditions of high humidity may begin to germinate without this being apparent.
4. Fine particles of activated charcoal may need to be removed by filtering prior to dipping the Hybridstix, or the sample solution may need to be applied to the strip with a pasteur pipette.

CHAPTER 3

THE THYMOL METHOD FOR GLUCOSINOLATE DETERMINATION

3.1 INTRODUCTION

A method for the determination of total glucosinolate (GSL) content in rapeseed meal using thymol was first reported by Brzezinski and Mendelewski(1984).⁶² It has the advantages of being technically straightforward and inexpensive when compared with the HPLC method or GC/trimethylsilyl procedure.

In conjunction with the development of a new reflectance method for measurement of total GSL content in rapeseed, a reference procedure was needed with which to compare the reflectance results. This chapter outlines experimental results which led to modification of the Brzezinski and Mendelewski (1984) thymol method for routine analysis of GSL content in rapeseed.

3.2 EXPERIMENTAL

3.2.1 MATERIALS

Sinigrin monohydrate, glucocheirolin, glucoerucin and standard glucose solution (5.56 mM) were purchased from the Sigma Chemical Company, St Louis, Mo 63178 USA. DEAE Sephadex A-25 was purchased from Pharmacia, Uppsala Sweden. Thymol A.R. grade was purchased from Merck, Darmstadt F.R.G. 78 % sulfuric acid was prepared by combining 800 ml of concentrated A.R. sulfuric acid with 200 ml of water (actual 78.4 %). Potassium sulfate solution, (0.3 M) was prepared by dissolving 26.14 g of K_2SO_4 in 500 ml water. Plastic disposable cuvettes were purchased from Mallinckrodt, Clayton, Vic, Australia; Glass test tubes 20 x 150 mm were purchased from Lab Supply, Marrickville, N.S.W. Australia; Econo columns and plastic clamps were obtained from Bio-Rad, Richmond, Ca., USA. The ultra turrax (Junke and Kunkle) with 8n shaft were purchased from John Morris Pty. Ltd. Chatswood, N.S.W. Australia.

3.2.2. METHODS

3.2.2.1. Preparation of Oil Free Rapeseed Meal.

Seed of rapeseed (50 g) was placed in a cotton bag and immersed into a boiling water bath for 15 minutes. The seed was then allowed to dry before being macerated in a Krupps 52 coffee grinder and defatted with petroleum ether (40 to 60 °C) in a soxhlet apparatus for 20 hours. Defatted meal was then allowed to dry before being ground to a powder.

3.2.2.2. Standard Curve

A 0.5 ml aliquot of sinigrin solution (0.05 to 0.50 mM) was added to a clean dry test tube, followed by 0.1 ml of 6 % thymol in ethanol and 2.0 ml 78 % H₂SO₄. Each tube was then covered with aluminium foil and the contents mixed using a vortex mixer, before heating in a boiling water bath. After 45 minutes the tubes were removed and allowed to cool to room temperature (or under running water) and vortexed. The absorbance of the solution was then read at 505 nm using a plastic disposable cuvette.

3.2.2.3 Preparation of Carboxymethylthioglucose.

Thioglucose (0.30 g) was slowly added to a 1.2 molar excess of iodoacetic acid (in 2.0 ml with ammonium bicarbonate, pH 8).

After reacting in the dark for approximately 4 hours, the mixture was applied to Sephadex G-10 and eluted with water. The peak fraction (corresponding to MWt. approximately 260) was collected and lyophilised.

Carboxymethylthioglucose (0.0138 g) was then dissolved in 100 ml of water to give a 0.5 mM solution.

3.2.2.4 Absorption Spectra of Thymol Complexes.

Glucose, sinigrin, glucocheirolin, glucoerucin and carboxymethylthioglucose were all prepared as 0.5 mM solutions. Aliquots (0.5 ml) of each were placed in 10 ml glass test tubes and mixed with 100 ml thymol solution (6 % w/v ethanol), followed by 2.0 ml of 78 % sulfuric acid. After incubation at 100 °C for 45 minutes the solution was vortexed and allowed to cool prior to recording the uv-visible spectra (300-700 nm, Shimadzu UV-265) of the thymol complex.

3.2.2.5 Thymol Reaction in the Presence of Benzoic Acid.

Samples of sinigrin stock solution (0.5 mM) were aliquotted (0.25 ml) into five 10 ml test tubes. The following aliquots of benzoic acid (50 mg/10 ml ethanol) : 0, 50, 100, 200, 250 µl, were then added to each sample and the final volume adjusted to 0.5 ml with ethanol before adding thymol reagent and treating according to Methods Section 3.2.2.10.

3.2.2.6 Influence of Thymol Concentration on Colour Yield.

Samples of sinigrin standard solutions (0.50 mM and 0.25 mM) were aliquotted (0.5 ml) into test tubes and treated with 100 µl of the following thymol solutions : 0, 0.5, 1.0, 2.0, 4.0, 6.0, 10.0 % (w/v ethanol). The thymol reactions were performed in duplicate as described in Methods Section 3.2.2.10.

3.2.2.7 Extraction of Rapeseed Meal

Three commercial rapeseed samples (*Brassica napus* L. cv. Cowra 503, Cowra RX9 and Bunyip) were macerated and defatted as described in Methods Section 3.2.2.1. Each meal (200 mg) was weighed into a 10 ml graduated centrifuge tube and incubated for 5 minutes in a boiling water bath with 3.0 ml of hot distilled water. The samples were then vortexed to ensure complete extraction before being centrifuged (2000 x g, 5 minutes). The supernatants were each decanted into a graduated tube and the meals re-extracted with 2.0 ml of hot water. The supernatants were centrifuged again (2000 x g, 5 minutes) and the supernatants combined with the first. The combined extracts were mixed with 0.5 ml of a solution containing 0.5 M each of lead and barium acetate and the total volume adjusted to 6.0 ml. The solution was then centrifuged at 600 x g for 5 minutes.

3.2.2.8 Extraction of Intact Rapeseed

Seeds of three rapeseed samples (400 mg per centrifuge tube) were placed into a boiling water bath with 3.0 ml of hot distilled water for a period of 10 minutes before being homogenised while hot. The residual extract was washed from the homogenizer shaft with water (2 x 1.0 ml) into the centrifuge tube and then returned to the boiling water bath for a further 5 minutes. The extract was mixed with a solution containing 0.5 M each of barium and lead acetate (0.5 ml), and centrifuged. The clear supernatant was made to 6.0 ml with distilled water.

3.2.2.9 Purification of Intact Glucosinolates

An aliquot of the supernatants (1.0 ml) obtained in Methods Section 3.2.2.7 or 3.2.2.8 was applied to a DEAE Sephadex A-25 (10 mg) column equilibrated in water. The columns were then washed with two 1.0 ml aliquots of 30 % formic acid followed by two 1.0 ml aliquots of water (or until no more coloured eluate was observed). The intact glucosinolates were then eluted with three 500 μ l aliquots of 0.3 M potassium sulfate into a 5.0 ml volumetric flask and made to volume with water.

3.2.2.10 Thymol Reaction

Following the elution of the intact GSLs, as described in Method Section 3.2.2.9, a 0.5 ml aliquot of the column eluate was transferred to a test tube. A 100 μ l aliquot of 6 % thymol solution was added followed by 2.0 ml of 78 % sulfuric acid. The solution was vortexed and incubated in a boiling water bath for 45 minutes, before being cooled and the colour intensity measured at 505 nm.

3.3 RESULTS

3.3.1 Determination of Thymol Concentration

The influence of thymol concentration on colour development is shown in Figure 25. A 6 % (w/v) stock solution was found necessary to ensure complete colour development. This concentration was used in all subsequent studies.

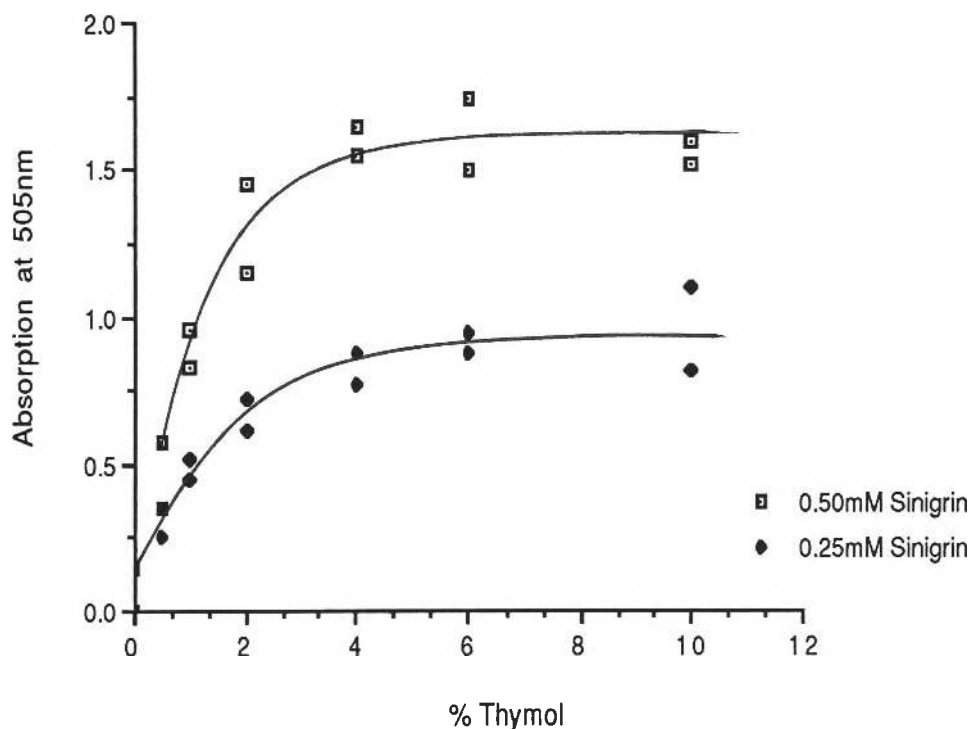


Figure 25. Two sinigrin solutions (0.5 and 0.25 mM) were incubated with increasing concentrations of thymol, according to Methods Section 3.2.2.6. The absorption at 505 nm was measured and plotted against % thymol concentration.

The use of 6 % thymol gives a final concentration of 0.23 % w/v thymol in the reaction solution, which is double the concentration used in other procedures.^{62,87} The higher concentration of sinigrin used in Figure 25 is equivalent to 2.5 μ mole GSL on column or

75 μ mole GSL per gram of meal, when prepared according to the Methods Section 3.2.2.7 and 3.2.2.8

3.3.2. Standard Curves Using Glucose and Sinigrin

The standard curves obtained from glucose and sinigrin are shown below in Figure 26. The regression coefficient in the case of sinigrin was 0.240 and that for glucose was 0.304.

The different linear regression coefficients obtained for the standard curves in Figure 26 are reflected in their intensities of the absorption peaks at 505 nm, shown in Figure 27.

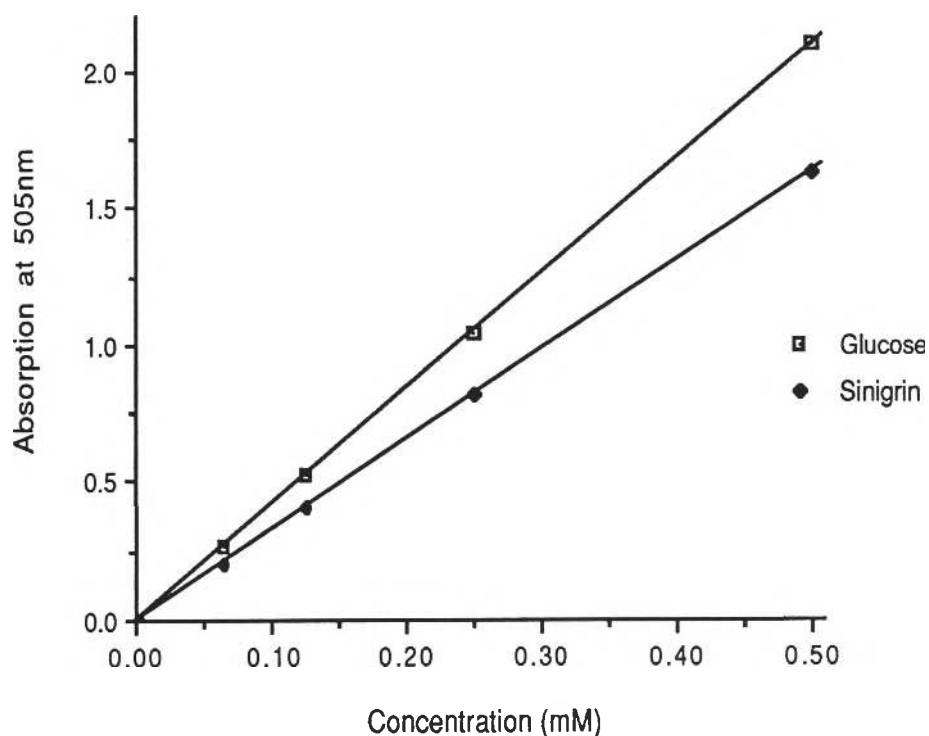


Figure 26. The standard curves obtained from glucose and sinigrin reaction with thymol, according to Method Section 3.2.2.2.

3.3.3. Absorption Spectra of Thymol Complex

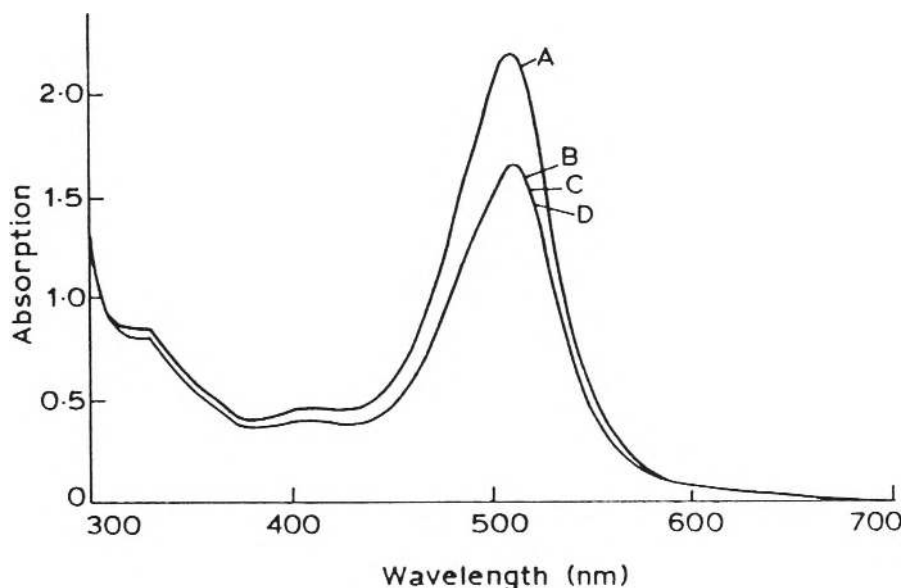


Figure 27. Glucose (a), carboxymethylthioglucose (b) and three GSLs : sinigrin (c), glucocheirolin (d) and glucoerucin (e), were incubated with thymol and their u.v.-visible spectra recorded according to the Methods Section 3.2.2.4.

Only a small difference (1 nm) in the wavelengths of maximum absorbance was observed but the colour yields of the glucose and glucosinolate complexes differed by a factor of 0.8. Three GSLs were examined, sinigrin (allylglucosinolate), glucocheirolin (3-methylsulfonylpropyl glucosinolate), glucoerucin (4-methylthio-butyl glucosinolate) to ensure that sinigrin gave a colour yield typical of GSLs. The carboxymethylated thioglucose, being a thioglucose derivative as are the GSLs, was also examined for thymol reaction colour intensity.

3.3.4. Affect of Benzoic Acid on Thymol Reaction

Benzoic acid (1 %) a preservative used in the standard glucose solution was examined, to see if this acid could interfere with the thymol reaction.

Table 14. Affect of Benzoic Acid on Thymol Reaction

Vial	Benzoic Acid (mM)	Sinigrin (mM)	Absorbance 505 nm
1	0	0.25	1.05
2	0.04	0.25	1.07
3	0.08	0.25	1.01
4	0.16	0.25	0.97
5	0.20	0.25	1.10

The thymol reaction was performed in the presense of benzoic acid according to Methods Section 3.2.2.5.

The results shown in Table 14 demonstrated that the preservative benzoic acid, does not interfere with the absorbance values obtained during thymol reaction using the standard glucose solution.

3.3.5. Thermogravimetric Moisture Analysis of Sinigrin

Thermogravimetric analysis was used to ensure that the sinigrin concentrations prepared for the standard curve were accurate. A sample of sinigrin (20 mg) was subjected to a temperature gradient starting at room temperature and increasing to 200 °C over

30 minutes and the weight of sample measured continually. The results obtained indicated a moisture content equivalent to one mole of water for every mole of sinigrin, which was consistent with the information supplied by the manufacturer.

3.3.6. Time Course of Colour Development at Set Temperatures

The time course of colour development using glucose and sinigrin with thymol reagent was monitored at various incubation temperatures as shown in Figures 28a, 28b, and 28c.

At all temperatures tested, the rate of colour formation for sinigrin was slower than that for glucose (see Figure 28a, 28b and 28c).

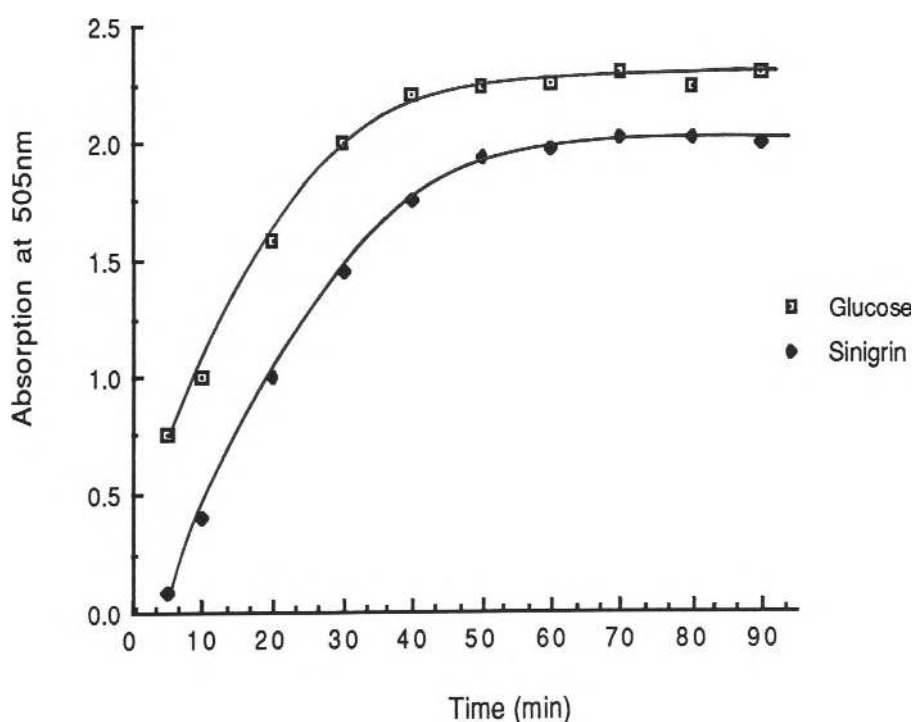


Figure 28 a : Thymol reaction at 88 °C. see Methods Section 3.2.2.10.

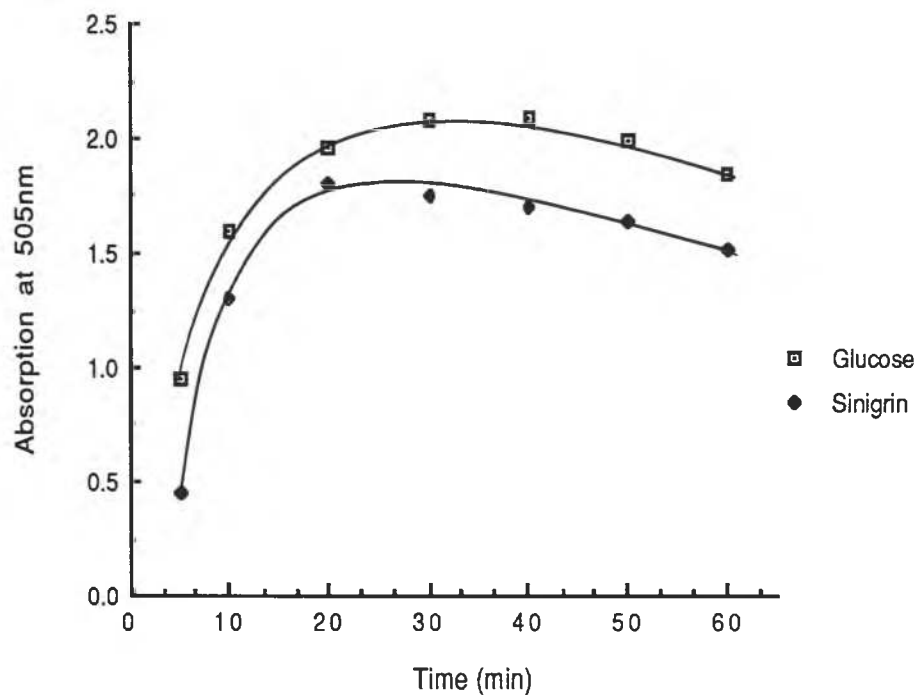


Figure 28 b : Thymol reaction at 95 °C, according to Methods Section 2.2.2.10

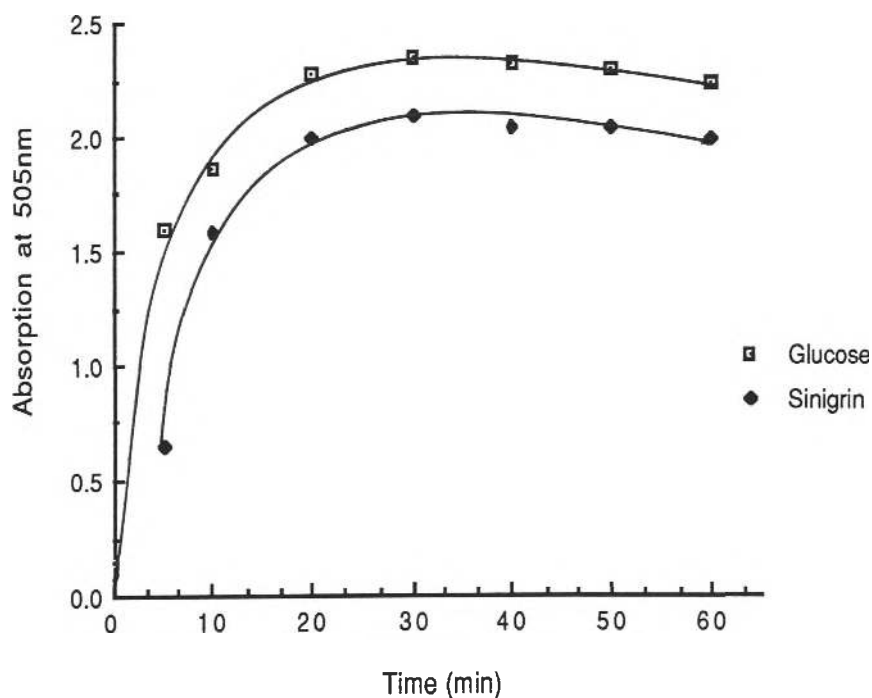


Figure 28 c : Thymol reaction at 100 °C (Methods Section 3.2.2.10).

The colour yield ratio for the two compounds became close to unity when the temperature was lowered from 100 °C to 88 °C, however, lengthy incubation times were necessary.

At 100 °C, thermal decomposition of both the sinigrin and glucose thymol complexes was observed. The decrease in absorbance appeared to begin sooner in the case of sinigrin (Figure 28 c). The ratio of absorbance at 505 nm for sinigrin and glucose, however remained relatively constant from 40-60 minutes at 100 °C.

In the laboratory, the use of a boiling water bath represents the simplest and cheapest method for performing incubations at elevated temperatures.

3.3.7. Influence of Sulfuric Acid on Colour Development

The influence of sulfuric acid concentration on the colour development of the thymol complexes with glucose and sinigrin is depicted in Figure 29. Both the absolute absorbance values and the ratio of colour yield for sinigrin and glucose were found to depend on the concentration of sulfuric acid used in the incubation.

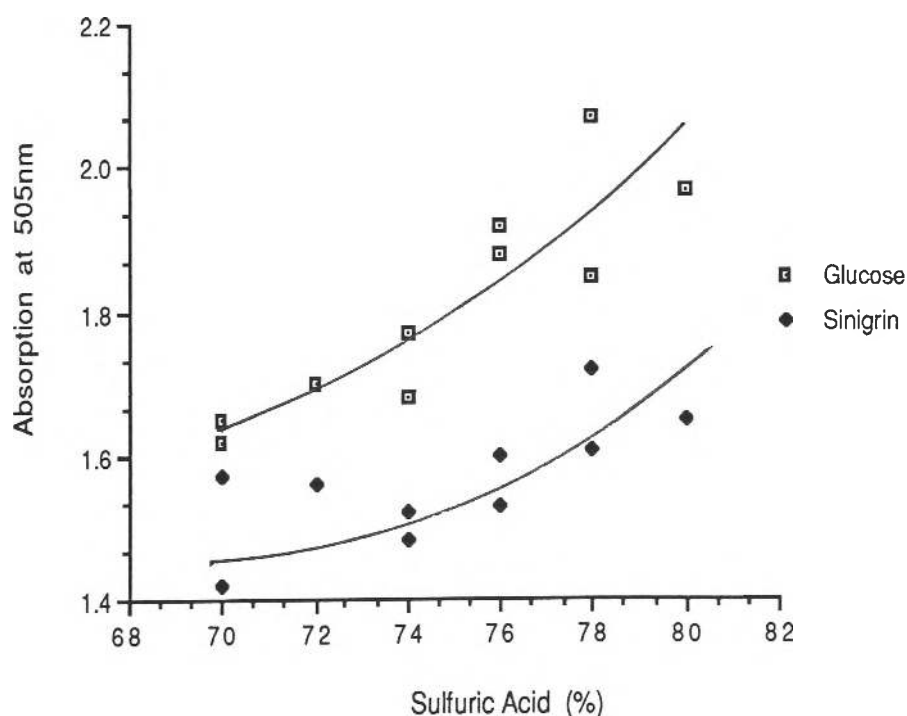


Figure 29. The thymol reaction was performed according to Methods Section 3.2.2.10, with varying concentration of sulfuric acid.

On the basis of these results it is strongly recommended that sinigrin be used for preparation of the standard curve. If sinigrin is not readily available, GSL values can be estimated by using a correction factor. In our hands a factor of 0.78 - 0.80 has been achieved consistently using a reaction time of 45 minutes and a sulfuric acid concentration of 78 %. However, if this method is employed, particular attention must be paid to the concentration of the sulfuric acid since the correction factor does alter with sulfuric acid concentration and sulfuric acid solutions are well known for their hygroscopic properties. Hence water absorption will ultimately lead to dilution of the sulfuric acid

Since the absolute absorbance value for sinigrin also depends on the concentration of H_2SO_4 (Figure 29), it is recommended that a standard curve should be included with each GSL determination. This becomes especially significant when stock solutions of H_2SO_4 are used for extended periods of time.

The use of plastic disposable cuvettes has greatly reduced the risks to health and materials associated with the repetitive transfer of concentrated H_2SO_4 solutions which also contain thymol. These cuvettes can be rinsed thoroughly with water and reused.

3.3.8 Determination of Glucosinolates in Rapeseed.

In order to determine the reproducibility of the modified thymol procedure, three rapeseed varieties were measured for their total GSL content using the protocol in Methods Section 3.2.2.9 and 3.2.2.10 (see Table 15). The values obtained are presented as the mean of four experiments using rapeseed and the corresponding rapeseed meal for each variety.

Table 15 Total GSL Content in Rapeseed by Thymol Method

Rapeseed Sample	MEAL		SEED	
	Mean $\mu\text{mol/g}$	S.D.	Meal * $\mu\text{mol/g}$	S.D.
C503	13.0	1.0	13.0	0.3
CRX9	26.0	1.9	25.5	1.1
Bunyip	62.0	1.8	62.1	1.2

*Expressed per gram meal

Conversion of $\mu\text{mol/g}$ seed to $\mu\text{mol/g}$ meal was achieved by determining the oil and moisture content in the seed. The results demonstrate excellent reproducibility and good correlation between rapeseed and rapeseed meal using the revised protocol.

In the range 0.2 - 2.5 μmol sinigrin added to the DEAE Sephadex A-25 column, the recovery was 97 \pm 1%.

Thus, this modified method for GSL determination has been found to provide reproducible results using either seed or meal of rapeseed. The thymol method now appears to have the potential to be used as a reference method for the measurement of total GSL content against which other methods of GSL quantitation can be compared.

3.3.8 Comparison of Total GSL Determinations by Four Methods

A comparison of the thymol method with three other methods of GSL determination has been performed using several varieties of rapeseed.

Table 16. Total GSL determination in Several Commercial Rapeseed Samples using four methods of Analysis.

Rapeseed Sample	Total Glucosinolate Determination (umole/g meal)			
	HPLC*	Glucose oxidase/ peroxidase #	Reflectance	Thymol
BLN 270	7.3	9.0	12	10
RX 9	21	20	24	26
BLN 241	23	23	29	30
Tatyoan	46	41	48	45
Jumbuck	59	59	64	62
Thomo 47	14	14	14	15
Thomo 42	23	22	20	26

Mean of duplicate determinations are given for each method.

#HPLC results were provided by J.P. Sang, State Chemical Laboratory, 5 MacCarther Pl., East Melbourne.

*Glucose oxidase/peroxidase method for GSL analyses, was provided by R. Mailer, Wagga Agricultural Research Institute, Wagga Wagga, N.S.W

3.4 DISCUSSION

When the thymol procedure was first published in 1984 ⁶² it appeared to offer a simple and direct method for the measurement of total GSL levels in plant tissues. A particularly attractive feature of the technique was that it employed no enzymic hydrolysis step, but instead involved reaction of the intact GSLs, purified by ion-exchange chromatography, with the thymol reagent. In practice however, a number of laboratories have experienced difficulties with the method. These problems included lack of reproducibility, apparent interferences, difficulties in repetitive readings of concentrated sulfuric acid solutions using glass cuvettes and relatively lower GSL values in those rapeseed varieties with high contents of GSLs.

The apparent underestimation of GSL content in rapeseed varieties which contain high levels of GSL may have two possible origins. Firstly, in previously published protocols (e.g. ⁶² and ⁸⁷), the final concentration of thymol has been 0.11 - 0.12 %. However, the result of this study on the influence of thymol concentration on colour development has demonstrated that a 6 % stock solution was necessary to ensure complete colour development (shown in Figure 25). This concentration was used in all subsequent studies. The 6 % stock solution gives a final concentration of 0.23 % thymol in the reaction tube, being double that used in previously mentioned (0.11 - 0.12 %).^{62,88} The results in Figure 25 indicate that at the lower thymol concentration (0.11 - 0.12 %; corresponding to 3 % on x axis) there is incomplete reaction with sinigrin. This is especially noticeable in samples containing high levels of GSLs. In Figure 25 the top curve corresponds to rapeseed with 75 μmol GSLs/g meal and the bottom curve to 37 $\mu\text{mol/g}$ meal.

In addition, since the lower concentration of thymol is not within the plateau region of the curve, an increase in variability would be expected. These problems, as shown in Figure 25, have been overcome by using a two-fold higher concentration of thymol.

Secondly, serious errors can result from the use of glucose for the production of standard curves for GSL measurement.^{62,88} In contrast to the results of Brzezinski and Mendelewski(1984) it was not found that glucose and sinigrin give equivalent colour yields per mole at 100 °C (see Figure 26).⁶² The difference in intensity becomes especially apparent at higher GSL concentrations (Figure 26). Furthermore the colour intensity obtained using 3-methylsulfonylpropyl and 4-methylthiobutyl GSLs was the same as that obtained with sinigrin (Figure 27). This supports the preference for sinigrin as a standard, as it appears to behave similarly to other GSLs in terms of colour response per mole of GSL.

CHAPTER 4

PREPARATION OF DESULFOGLUCOSINOLATES FOR HPLC ANALYSIS USING ARYL SULFOHYDROLASE (EC 3.1.6.5) Type H-1 FROM THE MOLLUSC, *Helix pomatia*

4.1 INTRODUCTION

The quantitation of glucosinolates (GSL) in rapeseed provides a indicator of the nutritional value of the feed and many methods have been developed for obtaining a rapid total estimation of the GSLs. This information allows plant breeders to select low GSL-containing rapeseed cultivars. On the other hand some reports concerning the various beneficial properties derived from the intact GSL, or their breakdown products suggest the value of maintaining particular GSLs in crops.^{12,89}

Therefore a knowledge of the GSL profiles of Brassica vegetables in conjunction with modern plant breeding techniques may enable selective breeding for individual GSLs, for example retaining those responsible for flavour and reducing those which attract insect pests.

Gas chromatographic (GC) analysis of GSLs via the volatile isothiocyanate breakdown products, was widely used due to its sensitivity and availability of the equipment needed.⁹⁰ However it has become apparent that this method is not ideal, since the indolyl GSLs do not decompose to volatile isothiocyanates and the hydroxy-alkenylGSLs, for example progoitrin, give rise to oxazolidinethiones. The latter were measured spectrophotometrically as thiourea derivatives.^{91,48}

Using the enzyme myrosinase to hydrolyse the intact GSLs has also its inherent complications, in that the enzyme may cause spontaneous autolysis which can lead to a range of different

products.⁹²

The spectrophotometric determination of total GSL content by the palladate method suffered from inaccuracy due to the presence of plant pigments or phenolic chemicals.⁴⁸ To overcome this problem, Thies utilised DEAE Sephadex A-25 as a GSL purification step and later adopted this step for the preparation of DS GSLs using aryl sulfatase from *Helix pomatia*. The GSLs, while retained on ion exchange resin were incubated with aryl sulfatase resulting in cleavage of the sulfate group. DS GSLs were then eluted with water, lyophilised and analysed as their volatile trimethylsilyl derivatives.⁶⁸ This method gave increased sensitivity and eliminated sulfate ions which gave rise to sulfuric acid during derivatisation. Some problems have been encountered as a result of 3-methylsulfinylpropylGSL giving rise to multiple products during derivatisation. Further difficulties have been associated with the analysis of the indolyl GSLs, which require careful optimisation of the derivatisation conditions.⁷⁰ The thermal degradation of GSLs on GC column was observed to give rise to nitriles and isothiocyanates depending on the temperature.⁹³

Analysis of GSLs by HPLC was found to provide many advantages over the traditional GC methods, such as not requiring a derivatisation step and being capable of measuring all GSLs found in rapeseed. The analysis of intact GSLs by HPLC involves pyridine acetate elution of the GSLs from DEAE Sephadex A-25 and then chromatography using reversed phase ion-pair HPLC.⁷¹

The poor resolution of some GSLs meant that more than one separation scheme was required. Further complications were associated with the need for carefully controlled pH and temperature conditions for reproducible HPLC retention times.

DS GSLs however were found to be ideally suited to reversed phase HPLC without the need for ion-pair reagents. A

gradient elution gave complete separation of all GSLs in rapeseed within 30 minutes.⁸⁸

Recovery of 4-hydroxyindole GSL however was a major problem affecting all the methods to date. This has been attributed to its susceptibility to oxidation.⁹⁴ Improved recoveries have been achieved by inclusion of antioxidants during the desulfation step, even if uncertainty remains as to the percentage recovery.⁷²

The use of on-column desulfation prior to analysis by HPLC is a relatively slow procedure requiring overnight incubation. This period of desulfation may lead to losses of unstable GSLs. Other losses have been reported during the desulfation step and are believed to originate from the use of sulfatase contaminated with glucosidase enzymes. For this reason the desulfation protocol according to Sang and Truscott⁷² was performed at high pH, since glycosidases are inactive at pH values greater than 8.⁸² This method required the use of an antioxidant during high pH desulfation, to retard the rate of autooxidation of 4-hydroxyindoleGSL, which increases with increasing pH.⁷² The high pH however, also reduced the aryl sulfatase activity requiring either more enzyme or longer incubation periods for desulfation.

A protocol for desulfation of GSLs and their determination by HPLC within 1 hour was reported by Quinsac and Ribailier (1984).⁷⁵ The method however did not use a selective isolation of the GSLs or removal of protein before HPLC separation. The use of a guard column was considered sufficient to enable a number of analysis to be performed without damaging the analytical column. The need for background correction was necessary however, to compensate for co-eluting non GSL compounds. The method was prone to inaccuracy since some GSLs were lost in the impurity peaks. The need for background absorption corrections was advised,

even though this would at least double the analysis times, involving more reagents and reduced life of the analytical column. The injection of phenolics, proteins and pigments would also be expected to cause considerable noise and loss of efficiency of the column chromatography.

The aim of this project therefore was to develop a simple method for preparing DS GSLs for HPLC analysis without the difficulties associated with the methods discussed previously.

4.2 EXPERIMENTAL

4.2.1 MATERIALS

Aryl sulfatase type H-1 powder from *Helix pomatia* (S 9626), β -glucosidase (Emulsin) from sweet almond, 4-nitrocatechol sulfate, phenolphthalein- β -D-glucoside, sinigrin monohydrate, o-nitrophenyl- β -D-glucopyranoside, o-nitrophenyl- β -D-galactopyranoside, 6-amino-n-hexanoic acid 2-mercaptoethanol, methyl α -D-mannopyranoside, pre-stained molecular weight standards and coomassie brilliant blue R-250 were all purchased from Sigma Chemical Co., St Louis, MO, U.S. Rapeseed seed was obtained from Pacific Seeds Ltd. Toowoomba QLD. Sepharose CL-6B, Sephadex G-10, DEAE Sephadex A-25, DEAE Sephadex A-50, Concanavalin A-Sepharose, Phast gel homogenous for native electrophoresis, Phast gel isoelectric focussing gels, pharmalyte carrier ampholytes, pH 3-9, 2.5-5. The low molecular weight marker proteins contained 64 μ g phosphorylase b (PH), 83 μ g bovine serum albumin (BS), 147 μ g ovalbumin (OV), 83 μ g carbonic anhydrase (CB), 80 μ g soybean trypsin inhibitor (TI) and 121 μ g β -lactalbumin (LB). Isoelectric focussing marker proteins (broad pI calibration kit) A vial of the pI calibration kit contained 20-50 μ g of each pI marker protein amyloglucosidase (AG), soybean trypsin inhibitor (TI), β -lactoglobulin (LG), bovine carbonic anhydrase (CB), human carbonic anhydrase (CH), horse myoglobin (MY), lentil lectin (LL) and trypsinogen (TY), were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

Ultrogel AcA 34 was obtained from The Australian Chromatography Company, Sydney.

Arylamide, N,N'-methylenebisacrylamide, SDS, TEMED, ammonium persulfate and Immobilon PVDF membranes were purchased from

Bio-Rad Laboratories P/L North Ryde NSW 2113.

Quinol, sodium azide, potassium sulfate, urea, HPLC grade acetonitrile, ethanol and methanol were purchased from BDH, Kilsyth, Vic., Australia.

Centricon-10 ultrafilters were supplied by Amicon Scientific Australia, Fawkner, Vic., Australia.

Spherisorb C-18 RP (250 x 4 mm) analytical HPLC column was supplied by ICI Australia, Dingley, Vic., Australia. All water was obtained from a Milli Q system, a C18 RP Radial Pak compression cartridge (140 x 10 mm) were supplied by Millipore Pty Ltd, Mars Rd, Lane Cove, Sydney.

All other reagents were A.R grade.

Buffers

Sodium acetate-acetic acid buffer solution (0.01 M) for gel permeation was prepared by weighing 0.820 g anhydrous sodium acetate and dissolving in 1 litre of distilled water and adjusting to pH 5.5 with glacial acetic acid and monitoring the pH with a pH meter.

50 mM Tris buffer, pH 6 at 25 °C, was prepared by mixing Trizma base and Trizma HCl (Sigma Chemical Co., St Louis, MO) according to Sigma Technical Bulletin No. 106B. The stock buffer was diluted to 18 mM before use.

Ethylenediamine acetic acid buffer (pH 5.8) was prepared from ethylenediamine solution (33 mM) and acetic acid (33 mM).

4.2.2 METHODS

4.2.2.1 Protein Determinations

- a. Biuret method,⁹⁵ with bovine serum albumin as standard.

Reagent : 1.5 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 6 g of sodium potassium tartrate were added to 500 ml of water. After mixing thoroughly, 300 ml of 10 % (w/v) sodium hydroxide solution (carbonate free) was added followed by 1 g of potassium iodide and then made up to 1 litre with water. The solution was stored in a plastic bottle.

Method : 4.0 ml of Biuret reagent and 1.0 ml of protein solution, containing 1 to 10 mg of protein, were mixed and allowed to stand at room temperature for 30 minutes. The absorbance at 550 nm was then determined. The protein concentration was obtained by comparison with 1 to 10mg of BSA.

- b. Bradford Dye binding method⁹⁶

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95 % ethanol and mixed with 85 % (w/v) phosphoric acid (100 ml). The solution was diluted to 1L with distilled water and gravity filtered through Whatman No 1 filter paper.

Micro Assay : Emulsin was used for the standard curve, since this glycoprotein was likely to give a similar reaction as those proteins being measured. The concentration of emulsin for use in Bradford method was first determined using the Biuret procedure. The standard curve was made by serial dilution of the stock protein standard giving 0, 2, 5 and 10 $\mu\text{g}/100 \mu\text{l}$. The Bradford reagent (1.0 ml) was mixed and the readings recorded at 595 nm after 30 minutes. The sample fractions were suitably diluted and measured as with the standards.

4.2.2.2 Enzyme Activities

a) Aryl sulfatase

The sulfatase was assayed by measuring the production of nitrocatechol from 4-nitrocatechol sulfate according to A.B. Roy (1953).⁹⁷ The protein precipitation step using 2% phosphotungstic acid in 0.1 N HCl was omitted.⁹⁸

Alkaline quinol reagent, was prepared immediately prior to use by adding 5 ml of a 4 % solution of quinol in 0.1 N HCl to 100 ml of 2.5 M NaOH containing 5 % Na₂SO₃.7H₂O.

The reaction mixture consisted of 0.4 ml of 2.5 mM 4-nitrocatechol sulfate at pH 4.5 (HCl) and 0.2 ml of 0.5 M sodium acetate buffer at pH 5, pre-incubated at 37 °C. The aryl sulfatase preparation was then added, mixed and incubated for 30 minutes at 37 °C.

The reaction was stopped by the addition of 3.0 ml 0.1 M HCl and a 3.0 ml aliquot removed and mixed with 5.0 ml alkaline quinol reagent. An intense red complex signified sulfatase activity and was measured at 515 nm. ($\epsilon_{515 \text{ nm}} = 12\,600 \text{ litre.mol}^{-1} \text{ cm}^{-1}$).

One unit of enzyme activity was defined as the amount liberating 1 μmol of nitrocatechol/min under the above conditions. The specific activity was expressed as units per mg of protein.

b) β -glucosidase.

The aqueous enzyme (10 μl) was incubated with 1.0 ml of 5 mM o-nitrophenyl- β -D-glucopyranoside (oNPGlu) in 0.2 M Na₂HPO₄-0.1 M Citric acid buffer pH 5.4. The tubes were incubated at 25 °C for 4 hours and the absorbance read at 425 nm. The absorbance was

converted to concentration given an absorbance of 0.5 is equivalent to 5 μ mole of o-nitrophenol (1.0 ml), at 425 nm. ⁹⁹

c) β -glucuronidase.

The activity was assayed by measuring the production of phenolphthalein which gives an intense pink coloration in alkaline solution.

Phenolphthalein glucuronide (1.5 mM) in 0.2 M phosphate buffer pH 6.8 (1.0 ml) was incubated for 1 hour at 25 °C with 50 μ l aliquots of fractions obtained from gel permeation chromatography.

The reaction was stopped by adding 5 ml of 0.4 M glycine buffer pH 10.4. The absorbance was measured at 550 nm.¹⁰⁰

4.2.2.3(a) Preparation of the Aryl Sulfatase

All operations were carried out at 10 °C unless otherwise mentioned. A uv-visible monitor (280 nm) with flow cell was used to record the protein elution at each stage. The concentration of protein was determined using the Bradford dye binding method 4.2.2.1 (b).

Stage 1. Gel permeation: Sepharose Cl-6B and Ultrogel AcA 34

Crude sulfatase powder type H-1 (200 mg) was dissolved in 2.0 ml of 10 mM sodium acetate buffer (pH 5.5) and centrifuged at 1000 x g for 5 minutes. The supernatant was then applied to a column (2.5 x 80 cm) of either Sepharose CL-6B or Ultrogel AcA 34 and equilibrated with 10 mM sodium acetate buffer (pH 5.5),

containing 20 mM EDTA and 0.02 % azide. The proteins were eluted with equilibration buffer at a flow rate 0.08 ml/minute and fractions were collected every 25 minutes (2.0 ml fractions).

The elution profile is shown in Figure 37a and 37b. The pooled fractions containing the aryl sulfatase activity were concentrated to 5 ml by ultrafiltration using a Diaflo PM-10 membrane with an Amicon apparatus (Amicon corp., Lexington, MA, U.S.A). The aryl sulfatase fraction was then loaded onto a column of Sephadex G-25 (30 x 2.5 cm) and eluted with 20 mM Tris/HCl pH 7.6. Fractions (5.0 ml) were collected, and those containing the aryl sulfatase activity pooled.

Stage 2. Ion exchange Chromatography: DEAE Sephadex A-50

The pooled sulfatase fraction from gel permeation(30 mg protein) was chromatographed on a column (20 x 0.8 cm) of DEAE Sephadex A-50 equilibrated in 20 mM Tris/HCl buffer (pH 7.6). The column was washed with 80 ml of the equilibration buffer at 0.25 ml/minute, before eluting with a continuous salt gradient from 0 to 0.2 M NaCl in the same buffer (Figure 39). Fractions were collected every 4.0 ml and those containing sulfatase activity were pooled and dialysed for 24 hours against 20 mM Tris/HCl buffer pH 7.4 containing 150 mM NaCl /1 mM $MnCl_2$ /1 mM CaCl.

Stage 3. Group Specific Adsorbents : Concanavalin A-Sepharose Chromatography.

A column (10 x 0.6cm) was packed with pre-swollen Con A-Sepharose and equilibrated in 20 mM Tris/HCl buffer at pH 7.4 containing 150 mM NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ at room

temperature (20-25 °C).

Fractions containing aryl sulfatase activity in the equilibrium buffer were loaded onto the Con A-Sepharose column at 0.25 ml/ min. The column was washed with equilibration buffer until unretained material was eluted (about 50 ml). The proteins retained by Con A-Sepharose were eluted with 0.6 M methyl α -D-mannoside in equilibration buffer (Figure 40) and 2.5 ml fractions collected.

Fractions containing aryl sulfatase activity were pooled and dialysed against 2 litres of 10 mM sodium acetate buffer pH 5.0. After 15 hours the sulfatase solution was concentrated to 5 ml by ultra-filtration using a Diaflow PM-10 membrane.

b) Sulfatase Purification Protocol by Heaney and Fenwick⁷⁴

Sulfatase type H-1 (70 mg) was dissolved in water (3 ml) and diluted with an equal volume of ethanol. After centrifugation, the precipitate was discarded and the supernatant treated with ethanol (9.0 ml). The mixture was again centrifuged, the supernatant discarded, and the precipitate dissolved in water (2 ml). The aqueous solution was passed through a column of DEAE Sephadex A-25 (20 mg, acetate form) and then through a column of SP Sephadex C-25 (sodium form). The eluate from the second column was stored at -20 °C and thawed immediately before use.

4.2.2.4 Electrophoresis

a) Phast System electrophoresis

Phast Gel homogenous 7.5 % PAGE was used for non-denatured protein samples. The separation conditions were according to the Separation Technique Manual.

Phast system gels were scanned with a laser densitometer (LKB).

b) Discontinuous Polyacrylamide-gel electrophoresis (PAGE) was performed as described by Laemmli (1970).¹⁰¹

For native PAGE, SDS was omitted from the gel and sample buffer. SDS PAGE sample buffer containing 25 % (w/v) urea, was prepared according to Sigma Technical Bulletin Number MWS-877PSB,

The stacking gel was 5 % Acrylamide/ 0.11 % NNN'N'-tetramethylene-diamine pH 6.5. A gradient separation (8 to 25 %) was performed in 0.375 M Tris/HCl buffer at pH 8.8. The electrophoresis employed constant current 25 mA for the stacking gel and 38 mA for the separation gel.

Samples were dissolved in sample buffer and centrifuged in a Beckman microfuge E (15,850 x g, 5 minutes) prior to electrophoresis.

c) Preparative Isoelectric focussing

Aryl sulfatase purified by gel permeation, ion-exchange and Con A-Sepharose was subsequently loaded onto a Rotofor containing Pharmalyte 3-9. A constant power of 25 watts was supplied for 5 hours before harvesting the fractions. Individual fractions were measured for pH and those containing the sulfatase activity were re-focussed with Pharmalyte 2.5-5. The fractions were again measured for pH and the fractions containing aryl sulfatase lyophilised and stored at -60 °C .

d) Western Blotting.

SDS-PAGE gels were electroblotted using a TRANS-UNIT semidry blotter according to the method of Kyhse-Andersen.¹⁰² However, PVDF membranes were used in place of nitrocellulose and 4-aminocaproic acid buffer was used in place of Tris/Glycine buffer. A constant 500 mA current was used with a 25 watt power limit.

e) Protein Sequencing.

The PVDF membrane containing the aryl sulfatase band was stained with Coomassie Brilliant Blue R-250, the band excised and analysed by a gas phase amino acid sequencer, Applied Biosystems Model 470A equipped with a modified cartridge, with on-line PTH amino acid analyser : Applied Biosystem Model 120A HPLC system.

The amino acid sequencing was performed by Dr. A. Gooley, Macquarie University, Sydney.

4.2.2.5 Electrophoresis Staining.

a) Coomassie Brilliant Blue R-250 Staining

Polyacrylamide gels were stained with 0.1 % Coomassie Brilliant Blue R-250 in fixative (40 % Methanol, 10 % acetic acid) solution.

The gels were destained with 40 % methanol/ 10 % acetic acid.

b) Silver Staining.

The staining was performed by immersing the gel in 30 % ethanol/ 10 % acetic acid overnight, followed by washing with distilled water to remove excess acid. The gel was then treated with Bio-Rad silver nitrate solution for 1 hour. The silver solution was then decanted and the gel washed with distilled water for 2 minutes before developing with the Bio-Rad developer.

4.2.2.6 Sulfatase Detection on Native Gel

The crude sulfatase was run on a Phast System Native 7.5 % PAGE according to Methods Section 4.2.2.4 (a). After electrophoretic separation the gel was cut into two, one half was stained with Coomassie Brilliant Blue R250 and scanned by a laser densitometer.

The remaining half of the gel was cut into slivers at 1 mm intervals and the pieces placed into an Eppendorf tube containing 5 mM 4-nitrocatechol sulfate (250 μ l) in sodium acetate buffer pH 5 and incubated at 27 °C for 6 hours. The 4-nitrocatechol produced was monitored at 407 nm.

4.2.2.7 HPLC Analysis of Glucosinolates in Rapeseed.

a). HPLC System.

A Waters HPLC solvent gradient delivery system fitted with Rheodyne injector, was coupled to a Knauer variable wavelength detector. A wavelength of 226 nm was used for all HPLC separations. Chromatograms were recorded and peak areas and retention times were measured using a Shimadzu C-R6A chromatopac integrator.

A Waters Z-module (Millipore-Waters Assoc., Inc., Milford, MA) fitted with a 100 x 8 mm Radial-Pak ODS reversed phase C18 (5 mm particle size) cartridge was used. The elution scheme at 1.5 ml min^{-1} involved 1 minute water followed by a linear gradient over 20 minutes to 20 % acetonitrile which remained constant for 5 minutes before re-equilibration. The separations were performed at ambient temperature. An analytical column (250 x 4 mm) 5 μ m ODS 2 Spherisorb HPLC column was also used at a flow rate of 1.5 ml/min. The solvent program consisted of 100 % water for 1 minute; a

gradient of 0-12 % acetonitrile over 30 minutes and a constant 12 % for a further 20 minutes. The typical DS GSL profile is shown in Figure 32a,b, using a Radial Pak cartridge.

b) Protein Removal

Samples taken for HPLC analysis were placed into a Centricon-10 ultrafiltration unit and centrifuged at 1000 x g for 10 minutes. The filtrate, free of protein, was then injected onto the HPLC.

c) Oil Removal from Rapeseed.

Seeds of rapeseed (*Brassica napus* L. cv. Cowra) were boiled for 10 minutes to inactivate endogenous myrosinase. The pulverised seed was then defatted with diethyl ether by soxhlet extraction for 4 hours. The seed material was re-ground in a Krupps coffee grinder and re-extracted as before. Oil free meal was spread on paper and air dried before storing in paper bags.

d) Extraction of Rapeseed Meal.

Defatted rapeseed meal of Cowra RX 9 (5 g) was extracted with 20 ml of boiling water for 5 minutes. The slurry was centrifuged (1000 x g, 10 minutes) and the supernatant decanted. The precipitate was re-extracted with hot water and the protein in the combined extracts precipitated with 0.5 M of both lead and barium acetate (5 ml). The volume was adjusted to 50 ml and centrifuged at 1000 x g for 10 minutes. A 10 ml aliquot was used for the isolation of the GSLs.

e) Isolation of Intact Glucosinolates (GSLs)

i) Pyridine Acetate Elution

A column (8 x 1 cm) of DEAE Sephadex A-25 (1 g) was converted to the acetate form by washing with 0.5 M ammonium acetate (12 ml) and then equilibrated in water. A GSL extract (10 ml), prepared according to Methods Section 4.2.2.7 (d) (i), was diluted to 30 ml with water and loaded onto the column. Unretained material was eluted with 0.06 M pyridine acetate (3 x 1 ml) followed by GSL elution with 0.75 M pyridine acetate (40 ml). The pyridine acetate was removed under vacuum and the yellow oil containing the GSLs dissolved in 2.0 ml of water. A stock solution was prepared by dissolving 1.0 ml of this intact GSL solution in 5 ml of 20 mM Tris buffer at pH 6.0.

ii) Potassium Sulfate Elution

A 5 ml aliquot of the GSL extract was diluted to 15 ml and loaded onto a DEAE Sephadex A-25 column (4 x 1 cm). The column was washed with 0.06 M pyridine acetate (3 x 1 ml) followed by water. The intact GSLs were then eluted with 0.3 M potassium sulfate (10 ml). The 10 ml solution of intact GSLs was divided into two 5 ml portions and adjusted to pH 6 using 1 M HCl. These were then desulfated either with crude aryl sulfatase or aryl sulfatase purified by gel permeation.

f) Desulfation Time Course of GSLs Eluted with Pyridine Acetate.

A rapeseed extract (*Brassica napus cv L. Cowra* sample RX 9) prepared according to Methods Section 4.2.2.7 (e)(i) was used to prepare a 5 ml stock solution containing approximately 2 μ mole GSL/ml. The desulfation using crude aryl sulfatase (1 mg) was performed in 20 mM Tris buffer, pH 6 at ambient temperature.

At set time intervals (15 mins, 30 mins, 2, 12 and 20 hours) a 250 μ l aliquot was passed through a Centricon 10 ultrafilter and a 50 μ l aliquot of the filtrate analysed by HPLC with the detector set to 0.5 AUFS.

The peak areas were converted to μ mole GSL/ g seed using the thymol method (Methods Section 3.2.2.10) and plotted against time.

g) Quantitation of DS GSL Peak Area by Thymol Method.

The conversion of the peak area of an individual DS GSL to μ mole involved the collection of a DS GSL as it was eluted from the HPLC. Individual DS GSLs were then lyophilised and reacted with thymol reagent according to the thymol procedure in Chapter 3.2.2.10. Given the amount of DS GSL determined by the thymol method corresponding to a particular peak area, a response factor was calculated. The amount of DS GSL/ g seed was then plotted against time (hours).

h) Desulfation Time Course using GSLs Eluted with Potassium Sulfate.

Two aryl sulfatase preparations : crude aryl sulfatase and aryl sulfatase purified by gel permeation were used to desulfate two identical batches of intact GSLs.

Each GSL portion (5 ml, Methods Section 4.2.2.7 (e) (ii)) received equivalent amounts of arylsulfatase activity (100 units), given 10 units will produce approximately 1 μ mole DS GSL per hour. At selected time intervals a 250 μ l aliquot was removed from each portion and passed through a Centricon 10 ultrafilter. An aliquot of the filtrate (50 μ l) was mixed with 100 μ l of the internal standard (0.05 mM oNPGal) and 150 μ l injected onto R.P. HPLC. The response factors listed in the following section were used to quantitate the peak areas.

i) Isolation of GSLs from Brussels sprout Leaf.

Leaf material was extracted according to Sang, J.P., et al. (1984).² In brief, 50 g of leaf tissue frozen by immersion in liquid nitrogen was pulverised and 20 g weighed before being added slowly to 100 ml boiling methanol. After 3 minutes the suspension was filtered through gauze and the tissue re-extracted as before with 100 ml of 75 % (v/v) methanol. The combined filtrates were rotary evaporated to remove methanol and the aqueous solution transferred quantitatively to a volumetric flask containing 5 ml of 0.5 M barium and lead acetate solution. The volume was made to 50 ml with water and the solution centrifuged (1000 x g, 10 minutes). An aliquot (5 ml) was removed and diluted to a final acetate concentration of 0.025 M. The DS GSL were then purified according to Method section 4.2.2.7 e (i).

j) Quantitation of DS GSL Peak Area with Response Factors.

Liquid chromatography retention times and response factors used for quantitation of DS GSLs, as reported by Sang and Truscott, 1984.⁷²

Table 17. DS GSLs retention times and corresponding response factors.

Glucosinolate	Retention Times (mins)	Response Factors
Progoitrin	4.10	9.23
Napoleiferin	8.47	9.34
Gluconapin	10.49	9.87
Sinalbin	13.18	4.68
4-hydroxyglucobrassicin	17.23	2.44
Glucobrassicinapin	19.53	9.87
Glucoerucin	27.43	9.23
Glucobrassicin	28.25	2.62

Table 17. The time courses of desulfation using the intact GSLs eluted with potassium sulfate (0.3 M), were obtained by determining the relative peak areas of the DS GSL's relative to the internal standard oNPGal at 226 nm. These were then converted to μ mole of GSL by using the response factors calculated by Sang and Truscott 1984.⁷²

At 226 nm, $(\text{Area DS GSL}/\text{Area oNPGal}) \times \text{response factor} = \mu\text{mole DS GSL}$.

k) HPLC Analysis of Rapeseed Meal

200 mg of oil free rapeseed meal was weighed into a 10 ml graduated centrifuge tube and heated in a boiling water bath for 1 minute. Boiling water (4 ml) was added to the sample and the extract mixed with vortex mixer. The caps were loosened and the tubes heated in boiling water for 10 minutes, before centrifuging at 1000 x g for 10 minutes. A 2.0 ml aliquot was mixed with 0.5 ml of protein precipitating reagent containing 0.5 M lead and 0.5 M barium acetate and then centrifuged at 1000 x g for 10 minutes. A 2.0 ml aliquot of the supernatant was diluted to 10 ml and loaded onto a DEAE Sephadex A-25 column (100 mg) and washed with 0.5 ml formic acid (30 %) followed by two 1.0 ml aliquots of water. The retained GSLs were then eluted with 0.3 M potassium sulfate (2 ml) into a 5.0 ml volumetric flask and the volume adjusted to the mark. An aliquot (1.0 ml) was mixed with purified aryl sulfatase (20 units) in sodium acetate buffer pH 5 and incubated at room temperature for 2 hours. The DS GSLs were then passed through a Centricon 10 ultrafilter and 150 μ l of the filtrate mixed with internal standard solution (50 μ l) and injected onto reversed phase C18 HPLC. The DS GSLs were separated as described in Methods Section 4.2.2.7 (a).

4.2.2.8 Determination of Optimum pH for Aryl Sulfatase Activity

The effect of pH on reaction rate was determined by incubating 1.0 ml of the substrate (p-nitrocatechol sulfate; 0.125 mM) in buffer, with 50 μ l aqueous sulfatase at ambient temperature. A decrease in absorbance at the selected wavelength occurred with desulfation of the 4-nitrocatechol sulfate.

Table 18. Maximum absorptivity of 4-nitrocatechol sulfate at various pH.

Buffer	pH	Absorbance maximum	Absorbance
0.2M KCl/ HCl	2.0	316.4	2.011
	3.0	317.0	2.062
0.2M Sodium Phosphate	4.0	317.6	2.046
	5.0	316.2	2.012
	6.0	316.8	1.703
	7.0	408.2	2.381
	8.0	403.2	2.600
0.2M Na ₂ HPO ₄ / NaOH	9.0	407.6	2.588

Table 18. The waveleth of maximum absorptivity of 4-nitrocatechol sulfate at a particular pH was determined by recording the spectrum of the substrate in the buffer being used.

These figures were used to calculate molar absorptivities at each wavelength. pH 2 to 5 was $\epsilon_{317 \text{ nm}} = 16,400 \text{ Lmol}^{-1}\text{cm}^{-1}$, pH 6 was $\epsilon_{317 \text{ nm}} = 13,600 \text{ Lmol}^{-1}\text{cm}^{-1}$ and for pH 7 to 9 $\epsilon_{407 \text{ nm}} = 20,000 \text{ Lmol}^{-1}\text{cm}^{-1}$

From the above data the rate of desulfation at a particular pH was measured on a Shimadzu uv-265 spectrophotometer. The decrease in absorbance with time was recorded and converted to amount of substrate (μmole) desulfated. Activity (μmole p-nitrocatechol sulfate desulfated per hour) was plotted against pH.

4.2.2.9 DS GSL incubation with sulfatase Purified by Ion Exchange Chromatography.

DS GSLs (approximately 15 μ mole) were isolated from a rapeseed meal extract. Each extract was incubated for 12 hours with 250, 50 or 25 μ l of aryl sulfatase (purified by ion exchange) at pH 6 and 25 $^{\circ}$ C. The enzyme preparation was 9.6 mg protein/ml (10 units/ml) aryl sulfatase activity, where 1 unit = 1 μ mol p-nitrocatechol sulfated per hour). The peak area of the individual DS GSL were measured relative to the internal standard: oNPGal with HPLC.

4.2.2.10 Incubation of Intact GSL with Emulsin

Aliquots containing 10 μ mole of the intact GSL (sinigrin) were loaded onto DEAE Sephadex A-25 ion exchange columns (100mg). Each column was then incubated with β -glucosidase (emulsin) : 0; 0.008; 0.05 or 0.15 units in 0.1 M sodium acetate buffer pH 5.0 were added (1 unit liberates 1 μ mole o-nitrophenol from the substrate oNPGlu per hour). After a 12 hour incubation at ambient temperature the liberated glucose from the intact GSL was measured by eluting the column with water and reacting the eluate with thymol reagent according to the Chapter 3, Methods Section 3.2.2.10

4.2.2.11 Incubation of DS GSL with Emulsin

Emulsin (5 units; 1 unit will hydrolyse 1 μ mole oNPGlu per hour) a β -glucosidase enzyme type II from sweet almond (EC 3.2.1.21), was incubated with 1.0 mM DS sinigrin in 0.01 M phosphate buffer (5.0 ml) at pH 6. At various times a 50 μ l sample was chromatographed by reversed phase HPLC with the internal standard of sulfanilic acid.

4.2.2.12 Incubation of the β -glucosidase type Activity from Crude Sulfatase with Standard DS GSLs.

Fractions 70 to 75 (Figure 37a) from gel permeation containing β -glucosidase activity, were pooled and subsequently incubated with standard DS GSLs. The activity of the β -glucosidase used was varied by the volume added viz., 0, 200, 400 and 600 μ l, (1 unit/100 μ l given 1 unit hydrolyses μ mole oNPGlu/hour) and the pH adjusted to 5.6 using 0.05 M citric acid-disodium orthophosphate buffer (400 μ l). The final volume of each vial was adjusted to 2.0 ml with water. The reaction was analysed after 20 hours by passing 0.5 ml through a Centricon 10 and injecting 250 μ l on the HPLC. The profile was obtained using an analytical HPLC column (250 mm x 4 mm) packed with 5 micron Spherisorb C18.

4.2.2.13 Sulfatase Activity in the Presence of Sulfate Ion.

The possible inhibition of aryl sulfatase activity by the presence of sulfate ion was investigated using purified aryl sulfatase from gel permeation. Aliquots of 2.5 mM 4-nitrocatechol sulfate in water (1.0 ml) were mixed with potassium sulfate to give final sulfate ion concentrations of 0, 0.05, 0.10, 0.20, 0.35 and 0.75 M. A 20 μ l aliquot of aryl sulfatase (0.034 mg) purified by gel permeation was added and the mixture incubated at room temperature for 15 minutes. The reaction was stopped with 2.0 ml HCl (0.1 M) followed by alkaline quinol reagent. The absorbances were measured at 515 nm.

4.2.2.14 Determination of Kinetics/Properties of Aryl Sulfatase for GSL Desulfation.

a) Desulfation of Sinigrin.

Varying concentrations of sinigrin (0 to 1.0 mM) in 33 mM ethylene diamine/acetic acid buffer at pH 5.8 (2.0 ml) were desulfated with 50 μ l of aqueous aryl sulfatase. The rate of desulfation was monitored at 228 nm. The initial velocity was determined and plotted against the sinigrin concentration. This is a modification of the method of Robbelen (1987).⁸⁴

b) Desulfation of an Indole GSL.

The purified sulfatase from gel permeation (fractions 156-165, Figures 37a) was used for desulfation of an indole GSL. Aqueous aryl sulfatase (25 μ l) was added to glucobrassicin (0.5, 1.0, 2.0 mM) in sodium acetate buffer pH 5.

After 5 and 10 minutes incubation times, the reaction was stopped by passing the solution through a Centricon 10 ultrafilter. The amount of DS glucobrassicin was quantified by injecting 50 μ l into the HPLC and determining the peak area at 226 nm. The rate of desulfation of the indole GSL was calculated by converting the change in peak area to μ mole DS GSL produced/ hour and plotting this against the concentration of glucobrassicin (mM).

4.3 RESULTS

4.3.1 pH VERSUS ACTIVITY

The optimum rate of desulfation of 4-nitrocatechol sulfate in 0.1 M phosphate buffer was obtained at approximately pH 5.5. It was also apparent that the aryl sulfatase exhibits activity over a broad pH range of 2 to 9.5.

As shown in Figure 30 below, the on-column desulfation of GSLs can be achieved at pH 8, but the rate was reduced by approximately 80 % compared with optimum activity at pH 5.

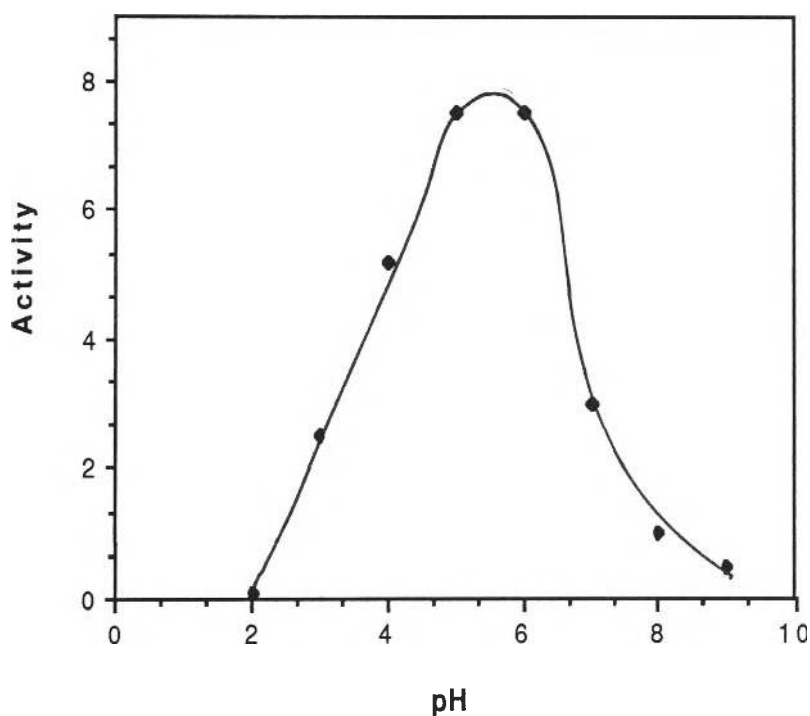


Figure 30. Variation with pH of the rate of hydrolysis of 4-nitrocatechol sulfate by the aryl sulfatase of *H. pomatia*. The rate of desulfation was measured as a decrease in absorbance with time according to Methods Section 4.2.2.8

The data in Figure 31 (below) indicate that glycosidase enzymes occurring in the gut of the edible snail (*Helix pomatia*) and in the commercial aryl sulfatase type H-1 from Sigma, are likely to have activities over the pH range 2.5 to 7 with little or no detectable activity above pH 8 (Figure 31).

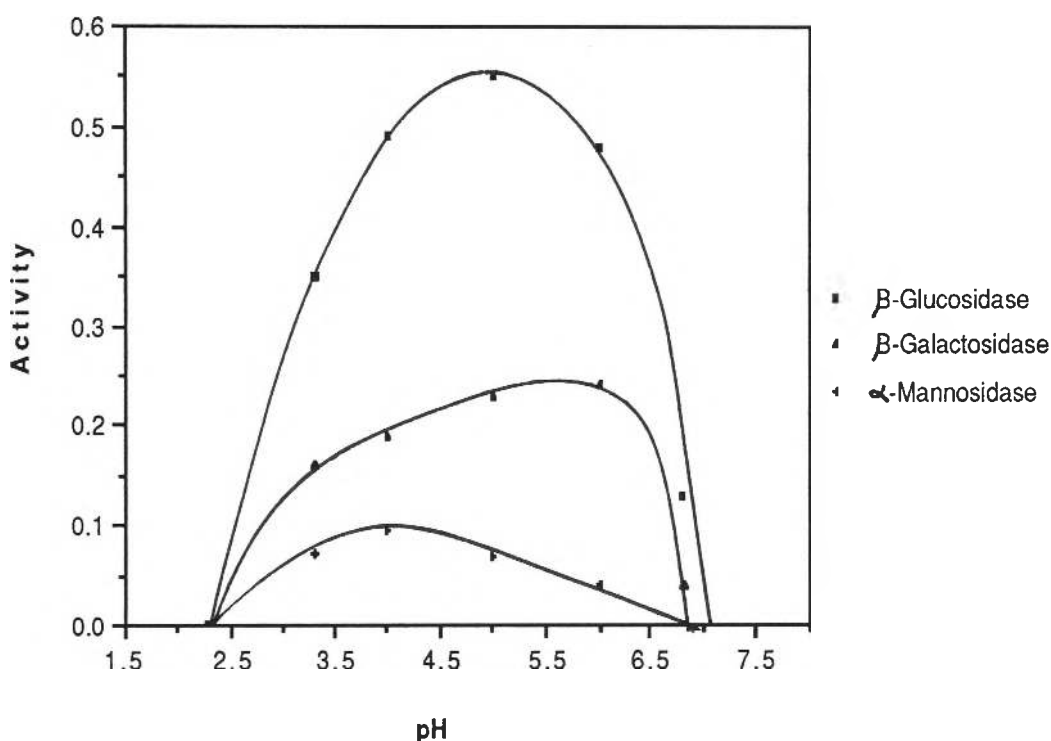


Figure 31. The pH versus activity profile of glycosidase type enzymes found in snail gut. Reproduced from "The Carbohydrates" Ward Pigman (1957).⁸²

The results shown in Figures 30 and 31 demonstrate the potential of performing the desulfation of GSLs at pH above 7.5, whereby the high pH prevents the possible degradation of the GSLs by glycosidase-like enzymes as suggested previously by Sang and Truscott (1984).⁷²

4.3.2 TIME COURSE WITH CRUDE ARYL SULFATASE

If glycosidase enzymes are indeed responsible for losses in GSL content during desulfation, a decrease in DS GSL with time of desulfation should be observed by HPLC. A time course study was therefore used to determine whether a reduction in recovery of the DS GSL with time occurred during the desulfation process.

GSLs from rapeseed were purified in their intact form as described in the Methods Section 4.2.2.7 (e),(i). These intact GSLs were incubated directly with the crude aryl sulfatase at pH 6 and at certain time intervals aliquots were removed and analysed by HPLC.

The typical reversed phase HPLC profiles of DS GSLs obtained from rapeseed, cultivar "Bunyip" and Brussels Sprout leaf are shown in Figure 32a and 32b. A gradient elution on a reversed phase C 18 Radial pak cartridge allowed complete separation of all GSLs from plant tissue within 30 minutes (Figure 32a) according to Methods Section 4.2.2.7(a).

Quantitation in μmole from the peak area of each DS GSL was achieved by collecting the peak fractions which eluted from the reversed phase HPLC column and reacting the DS GSLs with thymol reagent according to Methods Section 4.2.2.7 (g). This provided a direct means of relating the peak area at 226 nm with the amount of a particular GSL. This was especially useful when no internal standard is available. The results shown below are the response factors used for conversion of the peak areas to μmole GSL.

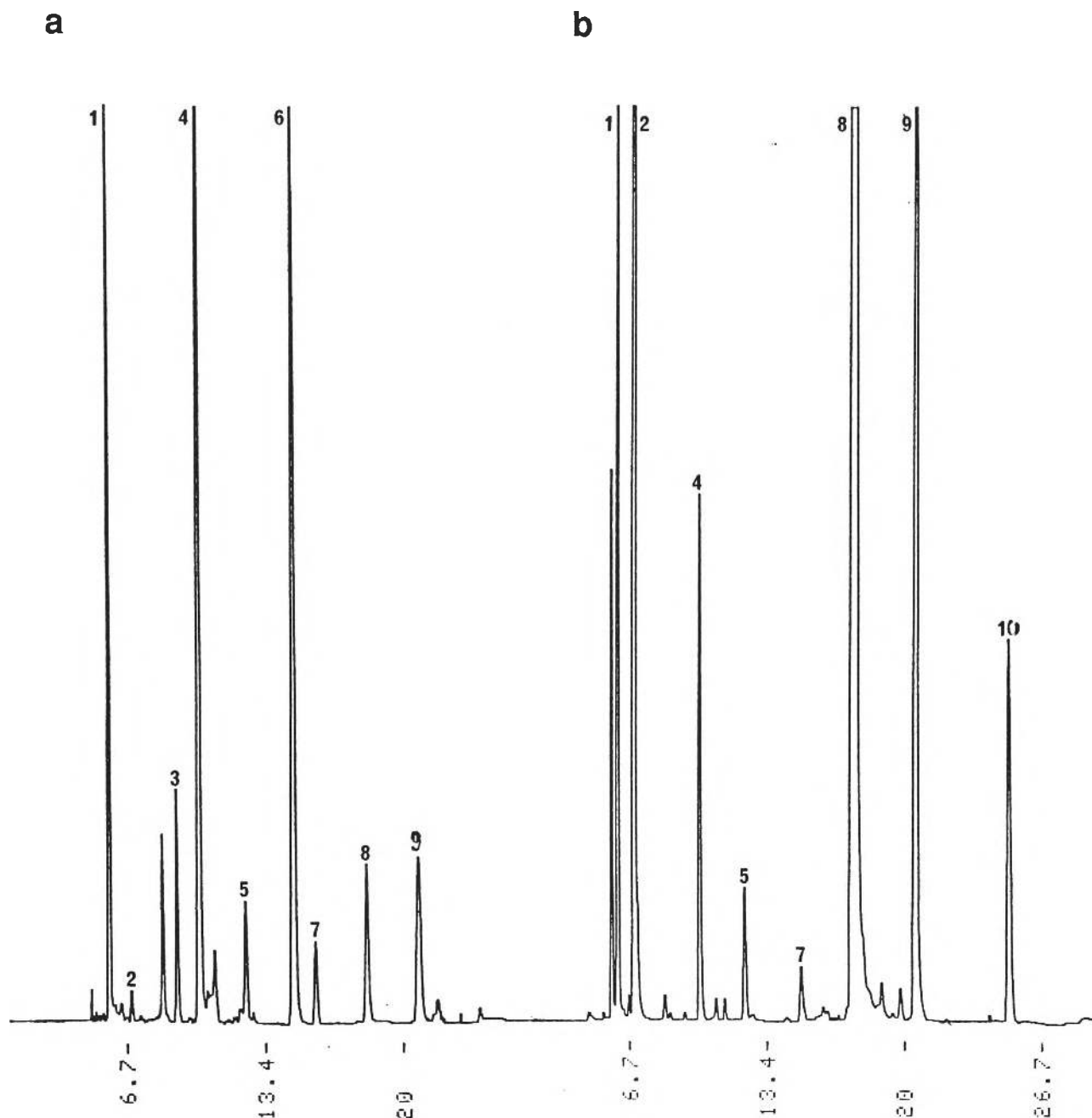


Figure 32a,b

HPLC profiles of DS GSLs from Rapeseed(a) and Brussels Sprout Leaf(b).

1. Progoitrin 2. Sinigrin 3. Napoleiferin 4. Gluconapin 5. 4-Hydroxyglucobrassicin 6. Glucobrassicinapin 7. Glucoerucin 8. Glucobrassicin 9. 4-methoxyglucobrassicin 10. Neoglucobrassicin

Given the time course conditions in Methods Section 4.2.2.7(f), it was estimated that the production of DS GSL would peak at approximately 2 hours. However the time course was monitored over

the following 20 hours to determine whether a decline in the recovery of the DS GSL was detectable.

Table 19. Liquid Chromatography Retention Times and Conversion Factors for Quantitation of the DS GSLs.

Glucosinolate	Retention Times (mins)	Conversion Factors
Progoitrin	4.10	9.07×10^{-8}
Napoleiferin	8.47	3.53×10^{-8}
Gluconapin	10.49	5.99×10^{-8}
Glucosinalbin	13.18	1.76×10^{-7}
4-hydroxyglucobrassicin	17.23	2.42×10^{-8}
Glucobrassicinapin	19.53	1.56×10^{-7}
Glucoerucin	27.43	2.14×10^{-7}
Glucobrassicin	28.25	1.88×10^{-7}

$\mu\text{mole GSL} = \text{Peak area} \times \text{conversion factor.}$

uv-visible detector range set to 64 and the attenuation 10.

The results shown in Figure 33 demonstrate a rapid production of DS GSLs which peak at approximately 2 hours. After this time a gradual decline in recovery can be observed over the 20 hours.

A rapid rate of desulfation may be attributed to the desulfation reaction taking place in the absence of ion exchange resin, since similar amounts of aryl sulfatase activity usually required overnight incubation to liberate an equivalent amount of DS GSL.

In addition, the time course reaction was performed in 20 mM Tris buffer at pH 6. The presence of Tris has been reported to activate the aryl sulfatase enzyme.¹⁰³

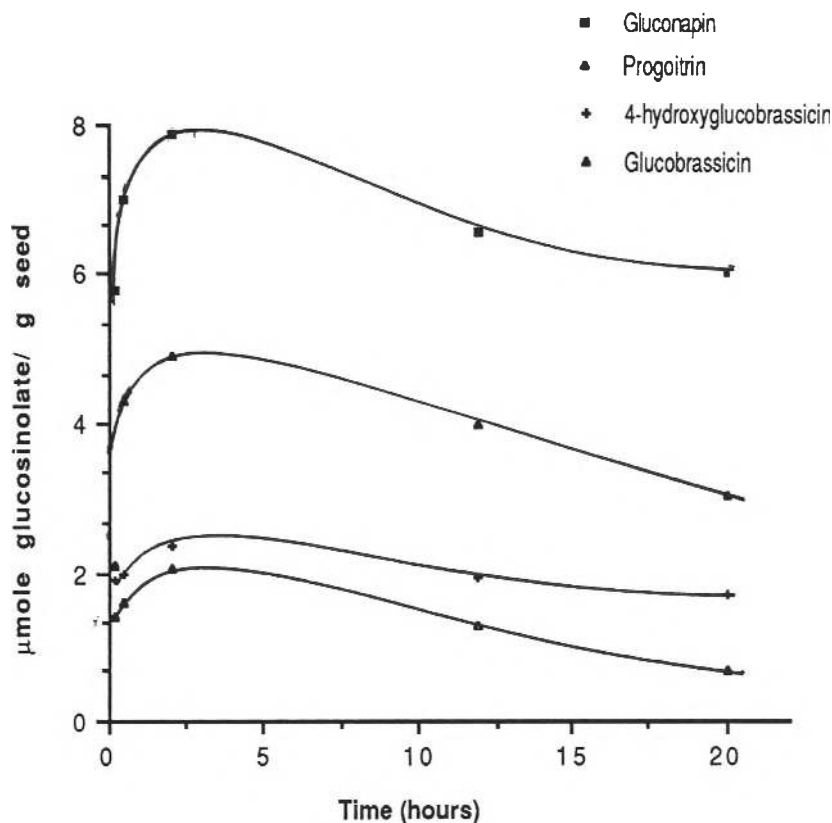


Figure 33. Time course of hydrolysis of GSLs with crude aryl sulfatase at pH 6.0. See Methods Section : 4.2.2.7 (f).

The gradual reduction in the recovery of the DS GSLs during the incubation with crude aryl sulfatase (Figure 33) confirmed the need to either purify the aryl sulfatase or to perform the desulfation according to the protocol reported by Sang and Truscott.⁷²

4.3.3. GSL RECOVERY IN THE PRESENCE OF ARYL SULFATASE PURIFIED BY ION-EXCHANGE CHROMATOGRAPHY.

A protocol for the purification of aryl sulfatase for GSL analysis has been reported by Heaney and Fenwick (1980).⁷⁰ This purification scheme involved ethanol fractionation followed by passing the crude sulfatase supernatant through sequential anion and cation exchange resins (Methods Section 4.2.2.3 b). The resulting enzyme had approximately 2.5-fold increase in specific activity.

A time course study was undertaken to determine whether the reduced recovery of DS GSLs observed in Figure 33 could be prevented by using sulfatase purified by ion exchange chromatography according to Heaney and Fenwick (1980).

As Table 20 shows, a gradual loss in DS GSLs was observed with the addition of aqueous sulfatase purified by ion-exchange chromatography.

The degree of DS GSL loss increased with the quantity of aqueous enzyme added. This preliminary experiment suggested that some component within the aryl sulfatase is capable of degrading the DS GSL and that the use of ion exchange chromatography to purify the aryl sulfatase did not appear to remove the cause of GSL loss.

Table 20 DS GSLs Incubated with Sulfatase Purified by Ion Exchange Chromatography.

Glucosinolate	% Relative Areas		
	25 ul	50 ul	250 ul
Progoitrin	62.20	38.76	20.75
Sinigrin	1.43	1.74	N.D.
Gluconapin	25.60	18.59	11.59
4-Hydroxyglucobrassicin	52.16	47.77	25.06
Glucobrassicinapin	4.87	3.71	2.44
Glucobrassicin	11.02	8.56	5.93

N.D. : not detected.

The amount of DS GSL present after 12 hours incubation with aryl sulfatase was determined by HPLC according to Methods Section 4.2.2.9. Relative peak areas were obtained by dividing the peak area of DS GSLs by the peak area of the internal standard oNPGal. Note: A 50 μ l aliquot of the aqueous aryl sulfatase was equivalent to a 0.2 % crude aryl sulfatase preparation normally used for on-column desulfation.⁶⁶

4.3.4 REACTION OF A GSL WITH EMULSIN
(a β -glucosidase derived from Sweet Almond).

4.3.4.1 Intact GSL and Emulsin

To investigate the possibility that β -glucosidases can act on the thio- β -D-glucose portion of GSLs, a commercially available β -glucosidase from sweet almond (Emulsin) was incubated with an intact GSL (Sinigrin).

Table 21 Sinigrin Conversion to Glucose during Incubation with Emulsin

Units of Emulsin Activity Added	Glucose (μ mole)
0.000	N.D
0.008	0.02
0.050	0.03
0.150	N.D.

Sinigrin (intact GSL) while retained on ion exchange resin was incubated with increasing amounts of emulsin. After 12 hours the column was washed with water and measured for free glucose using the thymol method. (see Methods Section 4.2.2.10) N.D : Not Detected.

The results (Table 21) indicated that little or no detectable glucose was liberated by the cleavage of the β -linkage. The addition of 0.15

units of β -glucosidase activity used in this experiment was sufficient to liberate 10 μ mole of glucose from oNPGlu during the same 12 hour incubation period. The slight absorbance in some samples was attributed to the presence of protein in the eluate. The results were confirmed using the Clinistix reflectance method (see Chapter 2) which also gave no colour reaction.

4.3.4.2 Desulfoglucosinolate incubated with Emulsin.

Although the intact GSLs did not appear to be degraded by emulsin it was found that the desulfated form of the GSL was susceptible to hydrolysis (Figure 34).

The rate of degradation of the desulfated GSL (DS sinigrin) by emulsin was monitored with HPLC and the peak area was determined relative to an internal standard, sulfanilic acid.

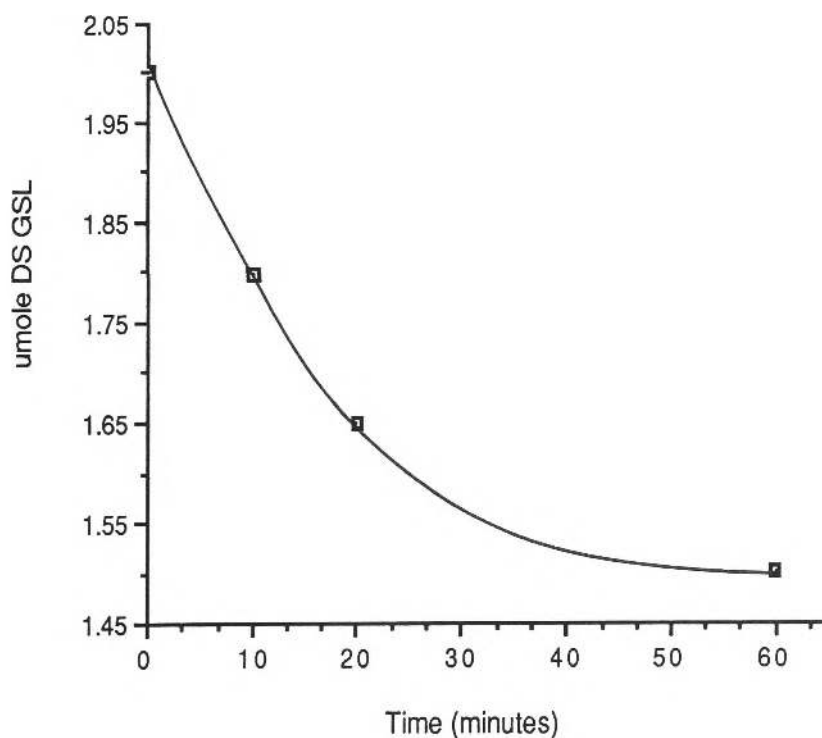


Figure 34 The effect of emulsin on DS GSLs was determined by measuring the recovery of the DS GSL, sinigrin (1 mM) after

incubation with emulsin. The DS GSL was chromatographed on HPLC and the peak area divided by the peak area of the internal standard, sulfanilic acid (Methods Section 4.2.2.11).

It was found that the rate at which emulsin acted on the DS GSL was approximately three times slower than with the substrate oNPGlu. The results show that a loss of 0.58 μ mole DS sinigrin (20%) occurred in the first hour. Hence these results demonstrated that enzymic breakdown of DS GSL can occur in the presence of β -glucosidase type activity.

4.3.5 BATCH TO BATCH VARIATION IN ARYL SULFATASE AND β -GLUCOSIDASE ACTIVITIES IN COMMERCIAL CRUDE SULFATASE

Four different batches of crude sulfatase from *Helix pomatia* were measured for the content of both aryl sulfatase and β -glucosidase activities. Table 22 shows the sulfatase and β -glucosidase activities in four separate commercial batches of both crude sulfatase and the corresponding activities after ion exchange chromatography. Activity is listed in units each of which is equal to one μ mole of substrate hydrolysed per hour. The substrate 4-nitrocatechol sulfate was used aryl sulfatase and oNPGlu for β -glucosidase activities. A significant variation in the absolute enzyme activities from batch to batch of crude aryl sulfatase preparation from Sigma Chemicals is apparent (Table 22). However the proportion of β -glucosidase activity to aryl sulfatase activity is fairly consistent. The significant levels of β -glucosidase activity, which remain after ion exchange purification shown in Table 20 may explain the gradual degradation of the DS GSL during the desulfation step.

Table 22. Sulfatase and β -glucosidase activities of various batches of commercially available sulfatase type H-1 (Sigma) before and after purification according to Heaney and Fenwick⁷⁴ (Methods Section 4.2.2.3 (b)).

Batches	Enzymes	Activity (units/mg)	
		Crude	Partially Purified @
Batch 1	Sulfatase	40	94.7
	β -glucosidase	0.8	1.3
Batch 2	Sulfatase	55	128
	β -glucosidase	0.9	2.3
Batch 3 *	Sulfatase	9.3	18
	β -glucosidase	0.3	0.9
Batch 4	Sulfatase	22	52
	β -glucosidase	0.7	1.5

* This batch was delayed for many months in transit during summer. @ Refers to aryl sulfatase purified according to Heaney and Fenwick⁷⁴

Assuming that the β -glucosidase activity in crude aryl sulfatase from snail gut is capable of hydrolysing DS GSLs as was shown for the almond enzyme, then for quantitative GSL recoveries a purification scheme should be directed at removing such contaminants. Since the published ion exchange procedure failed to remove the β -glucosidase-like enzyme (Table 22) it was necessary to apply a new strategy for the purification of aryl sulfatase from *Helix pomatia*.

4.3.6 PURIFICATION OF ARYL SULFATASE

1. Native PAGE of Crude Aryl Sulfatase

As a first step in the purification of commercial aryl sulfatase type H-1, the crude enzyme was examined by native polyacrylamide gel electrophoresis (PAGE). The laser densitometer scan revealed (Figure 35) that a range of polypeptides is present in the commercial aryl sulfatase powder.

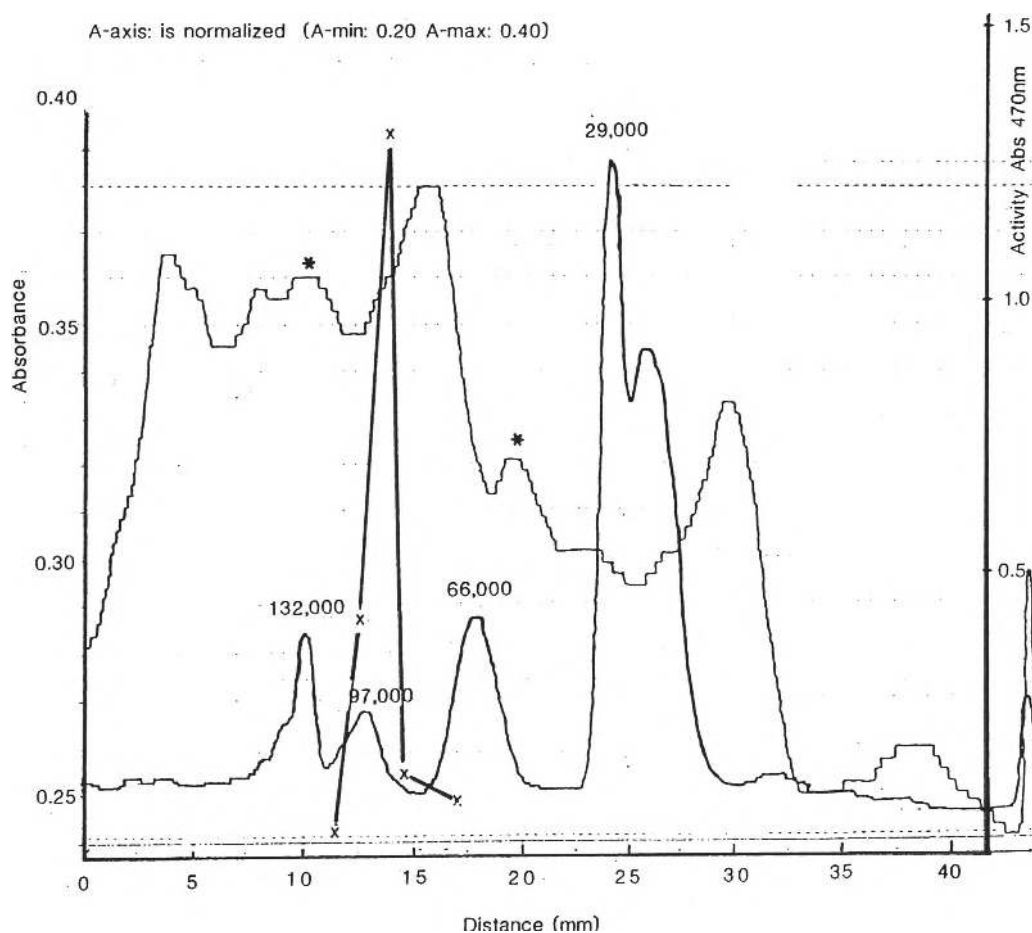


Figure 35. Crude aryl sulfatase was electrophoresed on a homogenous 7.5 % PAGE using the Phast system as described in the Method Section 4.2.2.4.(a). The aryl sulfatase activity (x-x), was determined according to the Methods Section : 4.2.2.6

The native protein standards bovine serum albumin : 132,000 (dimer), 67,000 (monomer), phosphorylase b : 97,000, and carbonic anhydrase 29,000, were run under identical conditions as the crude aryl sulfatase.

The relative position of the aryl sulfatase to the standards was determined by a positive reaction toward the substrate pNCS (shown in Figure 35 : x-x). These results suggested a native molecular weight of the order of 100,000. Incubation of the gel with the substrate oNPGlu revealed the location of the β -glucosidases. A yellow colouration at two positions suggested molecular weights of the order of 130,000 and 40,000 (Shown as * in Figure 35).

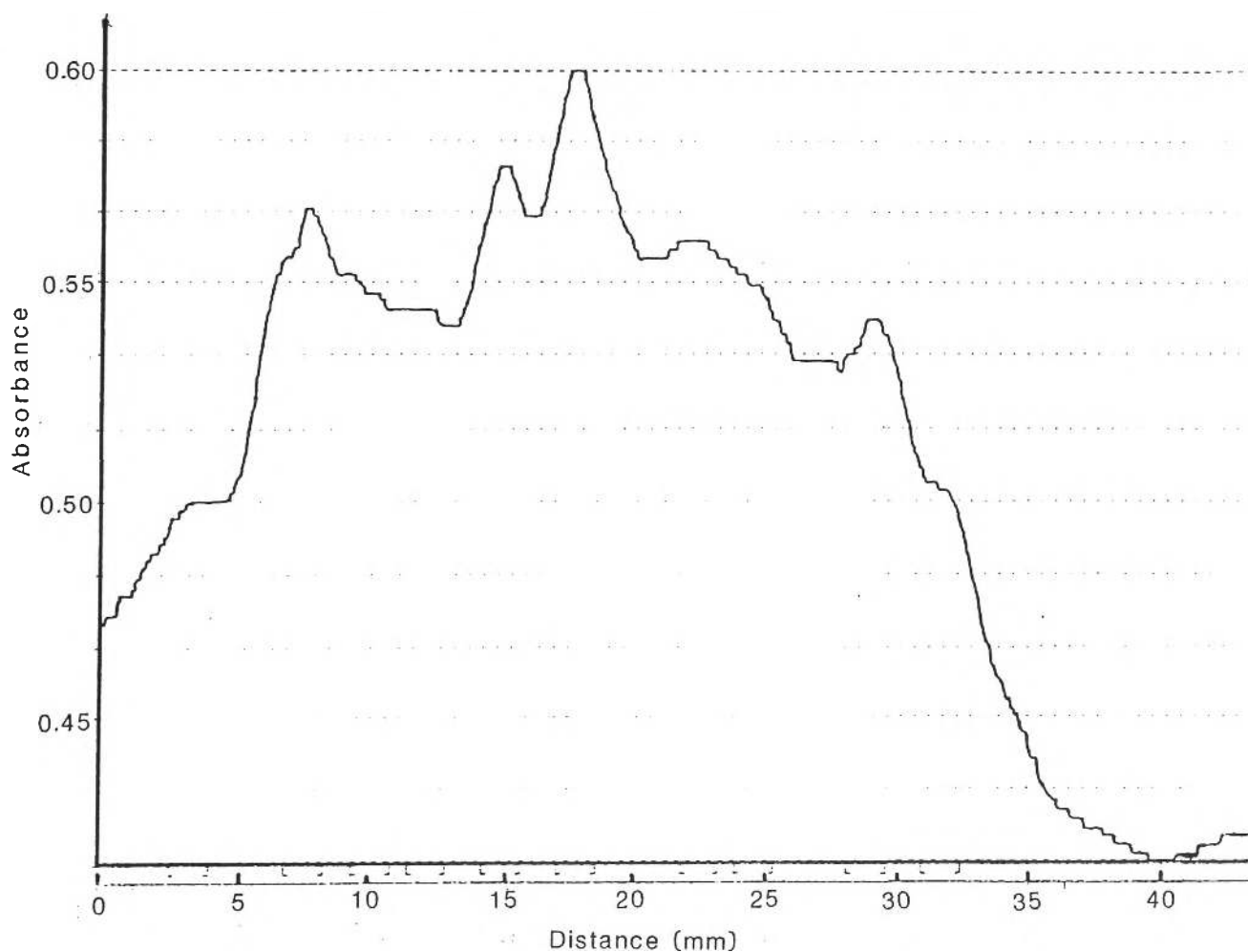


Figure 36 : The densitometer scan of the electrophoresis profile of aryl sulfatase purified by ion exchange according to Methods Section 4.2.2.3 (b).

2. Native PAGE of Aryl Sulfatase Purified by Ion Exchange Chromatography according to Heaney and Fenwick (1980).

The protein components of the aryl sulfatase purified by a published ion exchange chromatography procedure were also examined.

Although a 2.5 fold increase in specific activity of aryl sulfatase was obtained (Table 22) the β -glucosidase activity co-purified giving a similar increase in specific activity. The densitometer scan of the electrophoresis profile (Figure 36) reveals a range of proteins similar to that obtained for the crude aryl sulfatase (Figure 35).

4.3.6.3 Purification of Aryl Sulfatase by Gel Permeation

Although aryl sulfatase did not appear to be purified adequately by the ion exchange chromatography it could be seen from the electrophoresis profile that the aryl sulfatase had an apparent molecular weight significantly different from enzymes reactive toward β -glucosides. It therefore seemed feasible to examine a purification scheme based initially on gel permeation. Since the native molecular size appeared to be of the order of 100,000 a suitable gel permeation packing was used to give optimum separation in this molecular weight range.

The gel permeation profile of crude aryl sulfatase on a Sepharose CL-6B column (Figure 37a), showed a major protein peak which contained the aryl sulfatase activity. Eluting before and after aryl sulfatase were protein fractions containing activity toward β -glucosides. The molecular weights of these proteins were approximately 130,000 and 40,000. The latter eluting protein was omitted from the results since it was not reproduced using Ultrogel AcA 34. This could be attributed to dissociation of the " β -glucosidase-like" protein giving an active subunit in some environments. The gel permeation material; Ultrogel AcA 34, was employed to determine whether the resolution between the aryl sulfatase and the glucosidase activity could be improved. The results using Ultrogel AcA 34 are shown in Figure 37(b). The base-line separation between the sulfatase and glucosidase activity was similar to that obtained for Sepharose CL-6B. Analysis of β -glucuronidase using the substrate phenolphthalein glucuronide revealed considerable activity, which eluted as two peaks one of which overlapped the β -glucosidase activity. A slight misalignment of these overlapping activity peaks suggested that these are two different enzymes.

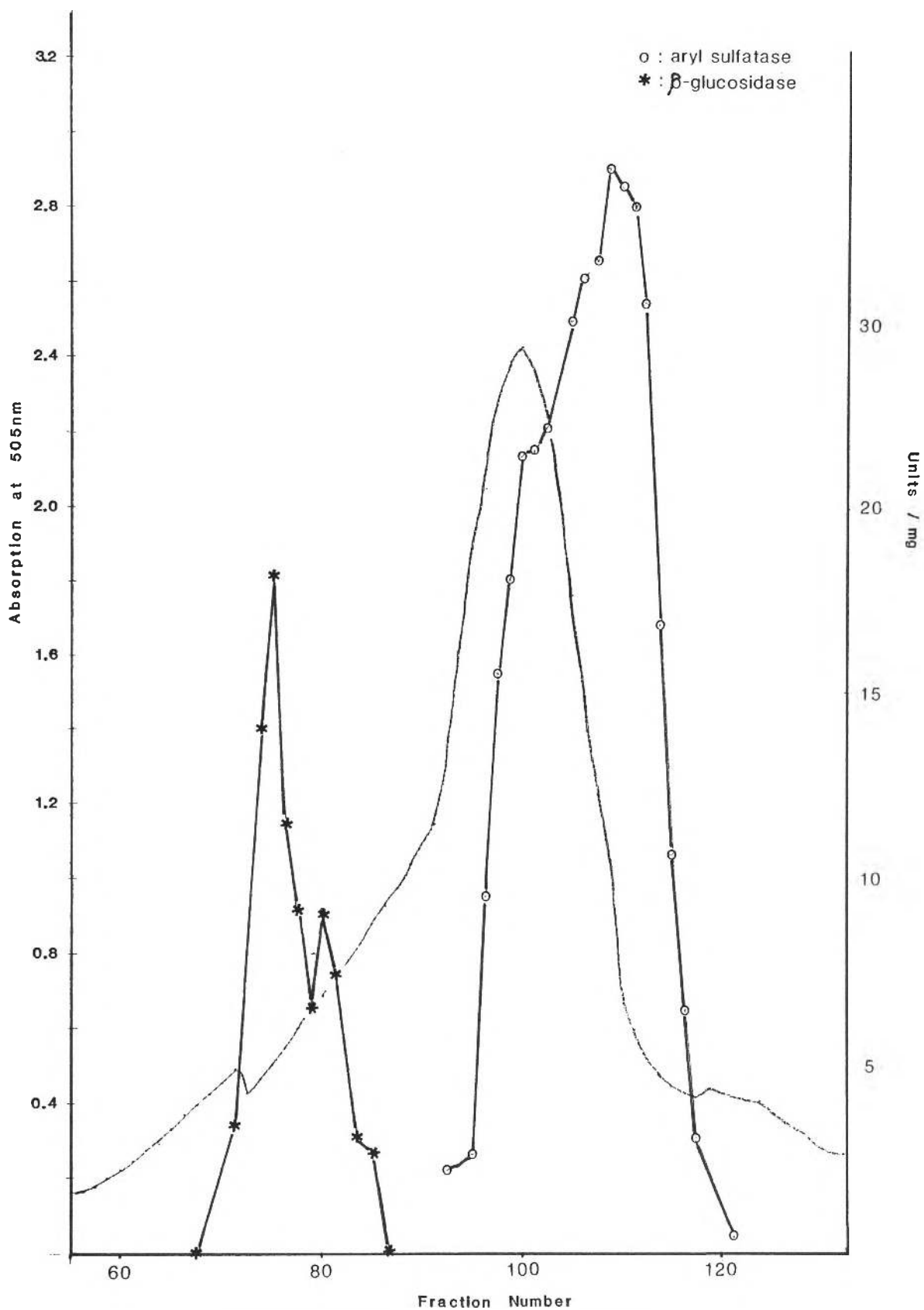


Figure 37 (a) Gel permeation chromatography of crude aryl sulfatase on Sepharose Cl-6B. (Methods Section 4.2.2.3(a).

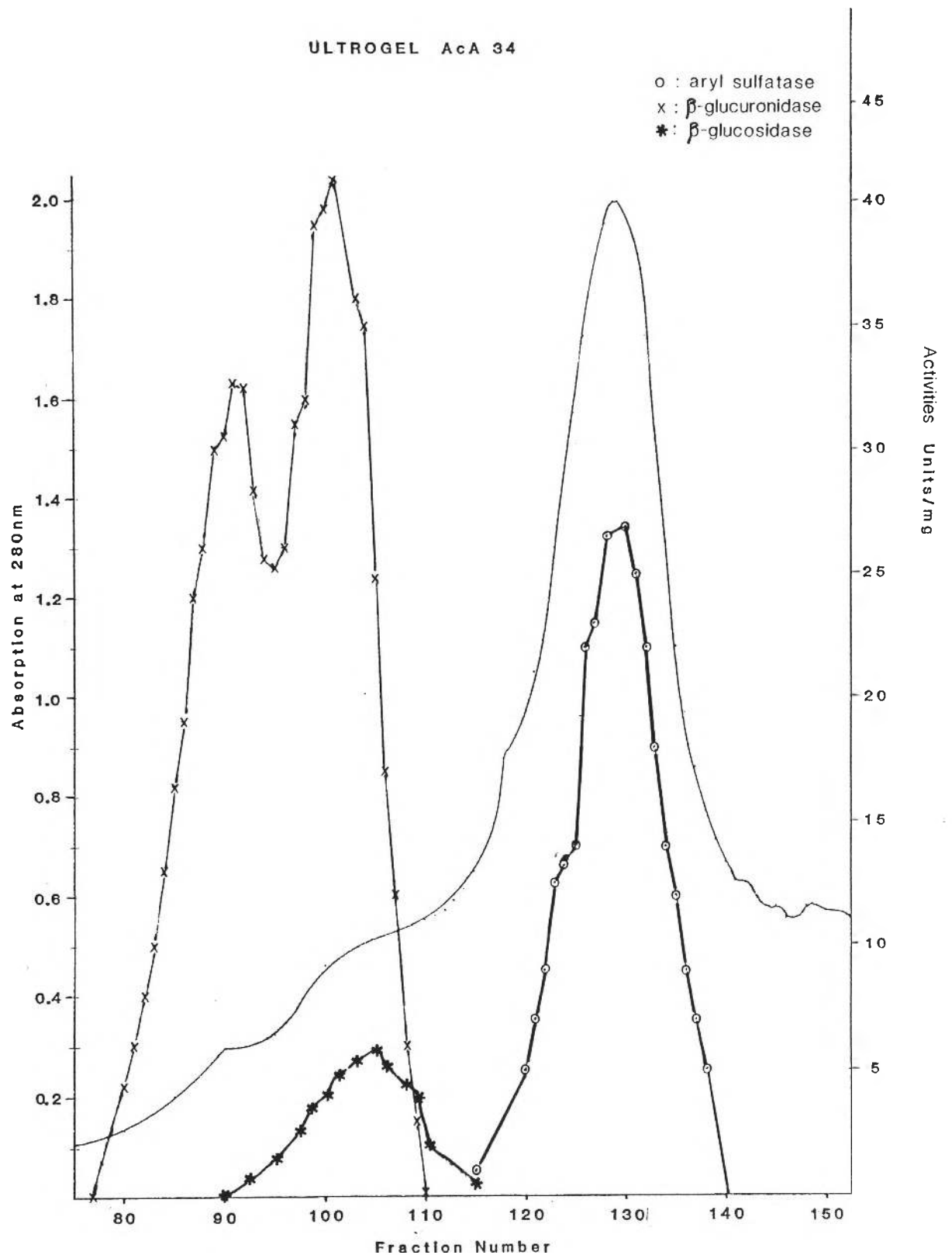


Figure 37(b). Gel permeation chromatography of crude aryl sulfatase on Ultrogel AcA 34. (Methods Section 4.2.2.3(b)).

4.3.6.4 Native PAGE of aryl sulfatase from gel permeation

A Phast electrophoretic examination of the early part of the peak containing the aryl sulfatase activity from the Sepharose CL-6B elution (fractions 95-110) was performed. The results of Native PAGE (Figure 38) show a significant improvement in the purity of the aryl sulfatase as a result of this one chromatographic step. Although the aryl sulfatase enzyme is not pure (Figure 38) the contaminant β -glucosidase activity (Figure 35) appears to have been removed.

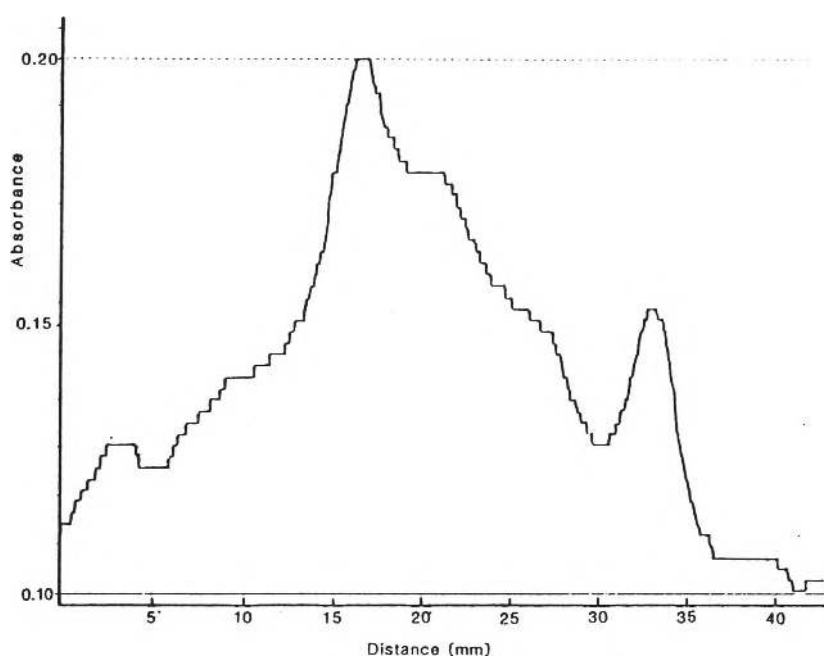


Figure 38. Gel scan of Native PAGE of gel permeation fraction

The pooled fraction 95-110 from the Sepharose CL-6B was found to contain 84 % of the original enzyme activity applied to the gel. The specific activity has been improved by a factor of 3. The pooled aryl sulfatase fractions 101 to 108 have a specific activity 5 times the original activity. Having removed the enzymes believed to be responsible for the reduced recovery of the GSLs during desulfation, further purification of the aryl sulfatase was sought to allow investigation of the physical and kinetic properties of the enzyme.

4.3.6.5 Aryl Sulfatase Elution from Ion Exchange Chromatography.

The pooled sulfatase fractions from gel permeation on Ultrogel AcA 34 (Figure 37 (b); Fractions 120 to 140) were concentrated and desalted on Sephadex G-10 according to Methods Section 4.2.2.3(a), and then applied to a column of DEAE Sephadex A-50 (Figure 39). An increase in the specific activity of the aryl sulfatase was achieved however, examination of the peak fractions on SDS-PAGE revealed the presence of at least five other major components. (Data not shown)

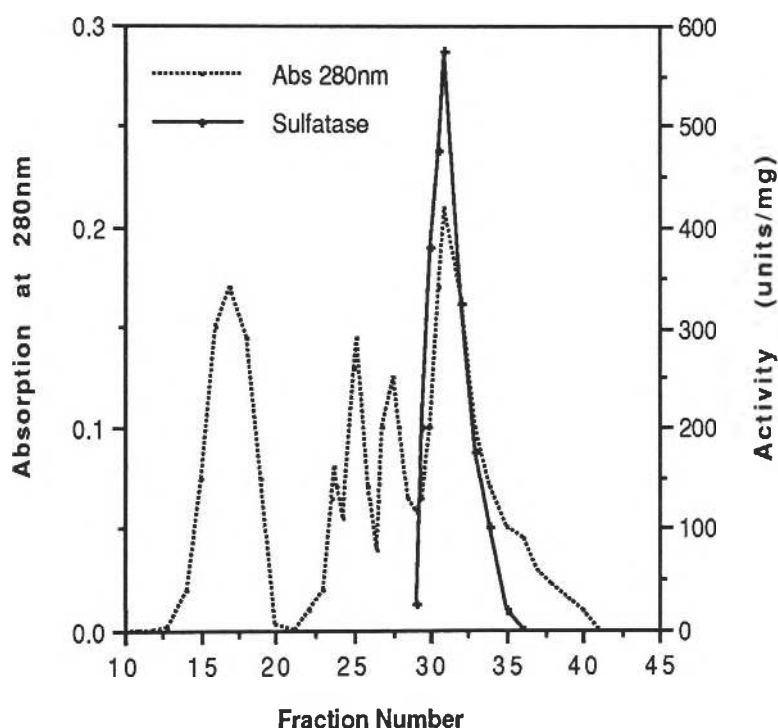


Figure 39 : The pooled sulfatase activity from gel permeation was loaded onto DEAE Sephadex A-50. Aryl sulfatase was retained and eluted using a 0 to 0.2 M NaCl gradient. The elution occurred at about 0.1 M salt. See Methods Section 4.2.2.3(a) (stage 2).

4.3.6.6 Con A-Sepharose Purification of Aryl Sulfatase purified by

Gel Permeation and Ion-Exchange Chromatography.

It has been reported that aryl sulfatases from most other sources such as sheep brain, ox liver and rabbit kidney^{104,105,106} are glycoproteins. Concanavalin A-Sepharose (Con A-Sepharose) was therefore employed in the purification of the aryl sulfatase from *Helix pomatia*.

The pooled aryl sulfatase fractions from 28 to 35 (Figure 39) from DEAE Sephadex A-25 ion-exchange chromatography were dialysed against distilled water and then concentrated by ultrafiltration to approximately 5 mls.

The retention of the aryl sulfatase from *H. pomatia* on the Con A-Sepharose column and elution by methyl α -D-mannoside indicated the presence of carbohydrate moieties in this aryl sulfatase (Figure 40).

An electrophoretic examination of the pooled aryl sulfatase fractions obtained from Con A-Sepharose by SDS-PAGE revealed three protein bands.

At this stage approximately 60 % recovery had been achieved with a 50 fold increase in specific activity. A summary of the purification scheme (Table 23) reveals approximately 3 fold increase in specific activity for each purification stage.

The use of Ultrogel AcA 34 and DEAE Sephadex A-25 each gave 80 % recovery of the aryl sulfatase activity. Con A-Sepharose gave a 5 fold increase in specific activity and approximately 97 % recovery of aryl sulfatase activity.

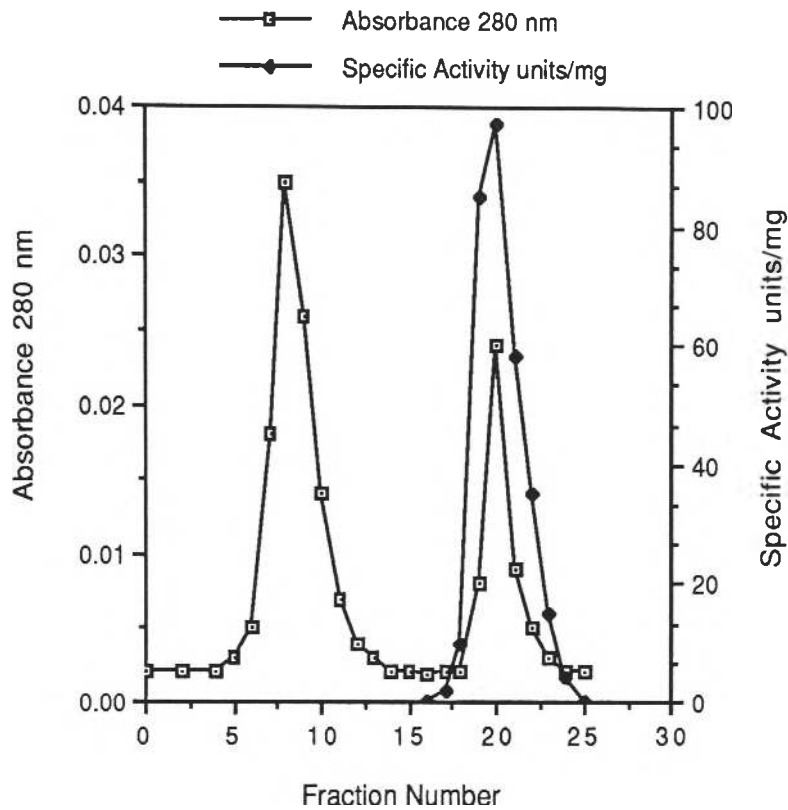


Figure 40. Con A-Sepharose elution of pooled aryl sulfatase fractions from Ion-exchange chromatography (Methods Section 4.2.2.3(a) stage 3)

Table 23 Summary of Aryl Sulfatase Recoveries and Specific Activity for each Purification Step.

Purification Stage	Total Act. (units)	Total Protein (mg)	Specific Act. (units/mg)	Yield (%)
Crude powder from snail gut	2,200	100	22	100
Ultrogel AcA 34	1,850	31.0	59.4	84
DEAE Sephadex A-50	1389	7.2	196.5	63
Concanavalin A-Sepharose	1347	1.2	1,122	61

4.3.8. INVESTIGATION OF ALTERNATIVE PURIFICATION SCHEMES

Since Con A-Sepharose chromatography was simple, gave good recovery and a 5 fold increase in specific activity, it was investigated as a means of directly preparing crude aryl sulfatase free of β -glucosidase activities.

4.3.7.1. Concanavalin A-Sepharose Purification of Crude Sulfatase

To determine whether Con A-Sepharose can be used in place of gel permeation as a means of obtaining purified sulfatase in one step for subsequent use in preparing DS GSLs, it was necessary to see if those contaminant enzymes causing breakdown of DS GSLs were also glycoproteins. The protein elution profile shown in Figure 41 was measured for sulfatase activity and β -glucosidase type activity: it was apparent that the contaminant enzymes were also retained and co-eluted with the aryl sulfatase.

The results shown in Table 24 demonstrated that almost 50 % of the protein (22 mg) was retained with only a 10 % loss in recovery of aryl sulfatase activity.

The activity profiles however demonstrated that Con A-Sepharose was not useful as a single step purification method, since those enzymes suspected of causing reduced DS GSL recovery were found to co-elute with the aryl sulfatase.

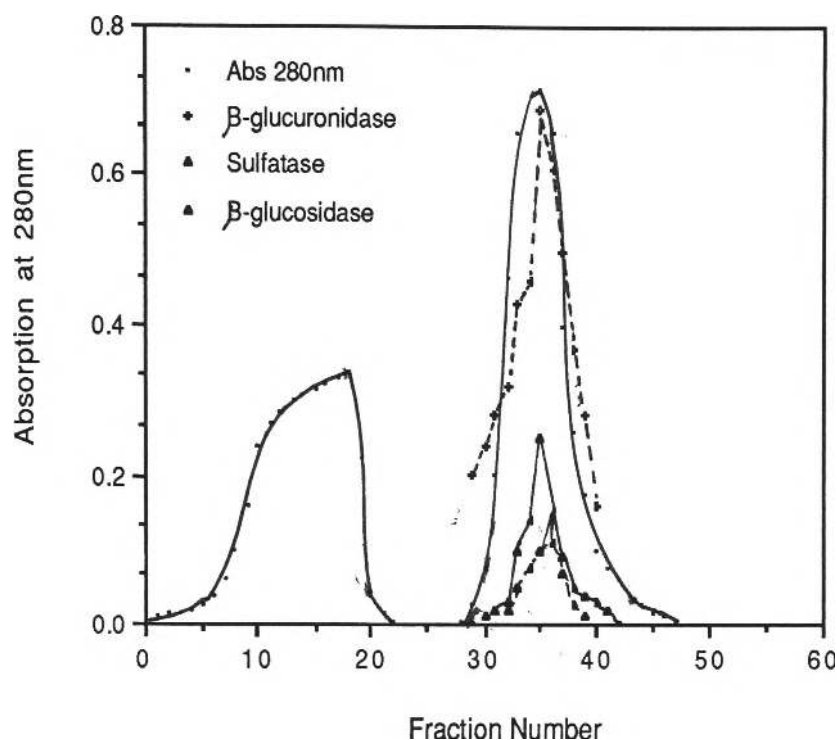


Figure 41 : The crude aryl sulfatase (50 mg) was dialysed for 24 hours against the equilibration buffer before loading onto Con A-Sepharose. (Methods Section 4.2.2.3 (a) stage 3). The elution of protein was monitored at 280 nm.

Table 24. Con A-Sepharose Purification of Crude Aryl Sulfatase

Enzyme purification Stage	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg)	Yield %
Crude Powder	1,100	50	22	100
Con A-Sepharose	996	22.32	45	90.5

The purification of crude aryl sulfatase using Con A-Sepharose is summarised in Table 24. The aryl sulfatase activity was determined according to Methods Section 4.2.2.2(a).

4.3.8.2 Con A-Sepharose after Gel Permeation.

The fractions isolated by gel permeation (Figure 37 (b) fractions 120 to 140) were chromatographed on Con A-Sepharose. The elution profile was similar to that shown in Figure 41. A considerable amount of protein was not retained and this purification step gave a 5-fold increase in specific activity with approximately 2 % loss in aryl sulfatase recovery.

Table 25 Recovery and purification of Aryl sulfatase.

Enzyme purification Stage	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg)	Yield %
Crude Powder	2,200	100	22.0	100
Ultrogel AcA 34	1,850	31	59.4	84
Con A-Sepharose	1,804	6.4	279.5	82

A summary of the purification is shown in Table 25. The recoveries are given for each stage of purification. The specific activity was found to increase from 59.4 units/mg to 279.5 units/mg protein a five fold increase in aryl sulfatase activity.

4.3.9. INCUBATION OF THE POOLED β -GLUCOSIDASE ACTIVITY FRACTION FROM GEL PERMEATION WITH DS GSLs.

To verify the results showing the presence of enzymes responsible for the reduced recovery during desulfation, the fractions (90-115) from gel permeation corresponding to the β -glucosidase activities were incubated with standard DS GSLs.

Table 26. Relative Peak Area Recoveries of DS GSLs after Incubation with the β -glucosidase from *H. pomatia*.

DS GSLs	% Relative Peak Area		
	Blank	400ul	600ul
Sinigrin	156	139	132
Gluconapin	107	84	72
Glucosinalbin	53	54	41
4-hydroxyindoleGLB	89	79	74
Glucobrassicinapin	94	81	71
NeoGLB	91	77	64

Relative peak areas were measured against the internal standard oNPGlu. The standard GSLs were measured according to Methods Section 4.2.2.12

After the addition of 400 ml and 600 ml of the pooled β -glucosidase activity from gel permeation it was observed that a significant reduction in peak area of the DS GSLs occurred. Hence an enzyme responsible for reduced recovery of DS GSLs has been separated

from the crude aryl sulfatase by gel permeation.

Examination of the pooled AcA 34 fractions causing breakdown of the DS GSL on PAGE suggested there were a number of proteins with apparent molecular weights in the region of 130,000.

4.3.10. TIME COURSE OF DESULFATION USING PURIFIED ARYL SULFATASE FROM GEL PERMEATION.

A time course reaction employing rapeseed GSLs, as discussed in an earlier section (see Methods Section 4.2.2.7 (h)), was used to confirm the removal of the contaminant glucosidase enzymes by gel permeation. As previously shown in table 22 the enzymes reacting with the substrate oNPGlu appeared to be responsible for the observed loss of GSLs during desulfation. However, an enzyme which is not active on oNPGlu but active on GSLs may still be present in the aryl sulfatase. To determine whether higher recoveries of the GSLs could be achieved using purified aryl sulfatase (free of glucosidases), a time course study was performed. Two identical stock solutions of intact GSL from rapeseed were incubated with either crude aryl sulfatase or the purified aryl sulfatase.

The GSLs incubated with the crude aryl sulfatase gave a peak recovery below that obtained using the purified aryl sulfatase. The amount of DS GSLs using the crude aryl sulfatase declines over the following 25 hours. This finding was consistent with that previously observed in Figure 33.

In both trials of crude and purified aryl sulfatase using 100 units of aryl sulfatase (10 units = 1 μ mole GSL per hour), the conversion of the GSL to DS GSL appear to be completed by 2 hours as judged by the plateau in the time course study (Figure 42).

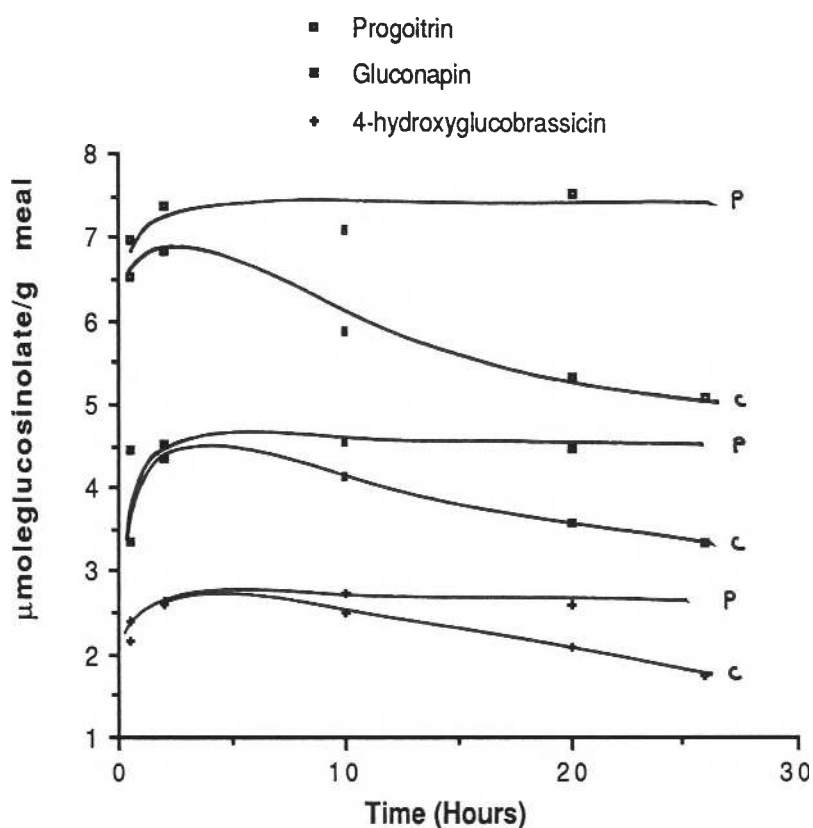


Figure 42. Time course of digestion of two 5.0 ml stock solutions of intact GSLs (1 mM) with 100 units of crude aryl sulfatase and 100 units of sulfatase purified by gel permeation. The GSLs were hydrolysed in 20 mM Tris buffer pH 6 at 28^o C. Method Section 4.2.2.7 (h). p = Purified aryl sulfatase, c = crude aryl sulfatase.

The DS GSL recovery with time using purified aryl sulfatase in the time course study however, demonstrated a plateau phase which remained approximately constant over 20 hours.

4.3.11 GSL RECOVERY FROM CENTRICON-10

On completion of the desulfation step, the DS GSLs were passed through a protein filter with a molecular weight cut off of 10,000 (Centricon 10). This served two functions, the more important was the protection of the analytical HPLC column. Although a guard column is adequate for this purpose some protein may pass through and accumulate on the C18 analytical column and the precolumn causing poor DS GSL resolution and peak shape. The other potential benefit was the recovery of the purified aryl sulfatase enzyme which could then be washed and reused, providing any residual DS GSLs have been removed. This however was not tested.

To ensure that the larger more hydrophobic GSLs are not retained by the Centricon 10, desulfoglucobrassicin was passed repeatedly through a Centricon and the filtrate re-injected onto HPLC as described in the Methods Section 4.2.2.7 (b)

Table 27 DS GSL Recovery from Centricon 10

Eluate Load	Amount of GSL recovered (mM)	
	Sinigrin	Glucobrassicin
1	2.07	2.00
2	1.98	2.10
3	2.21	2.12
4	2.11	2.03

Two standard GSLs, sinigrin and glucobrassicin were passed through a Centricon 10 ultrafiltration and a 50 μ l aliquot injected onto

C18 HPLC. The remainder of the filtrate was reloaded onto another Centricon and on each occasion an aliquot injected on HPLC. The peak areas were measured and the concentration of GSL in the filtrate shown in Table 27.

The HPLC peak areas demonstrated that the DS GSL can be passed through a Centricon 10 without significant losses.

Given a purification protocol for crude aryl sulfatase type H-1, it was desirable to further investigate the properties of this enzyme for the purpose of optimisation in preparing DS GSLs for HPLC analysis.

4.3.12 DETERMINATION OF THE PHYSICAL PROPERTIES OF ARYL SULFATASE.

4.3.12.1 Isoelectric Focussing

The aryl sulfatase prepared according to Methods Section 4.2.2.3(a) was further purified by preparative isoelectric focussing using a Rotofor according to Methods Section 4.2.2.4 (c).

A broad range pI ampholyte was used firstly, followed by a narrow pI 2.5 to 5.0 ampholyte for the second fractionation.

The results after the second focussing step are shown below in Figure 43 which suggest the aryl sulfatase has a pI ranging from 3.9 to 4.8.

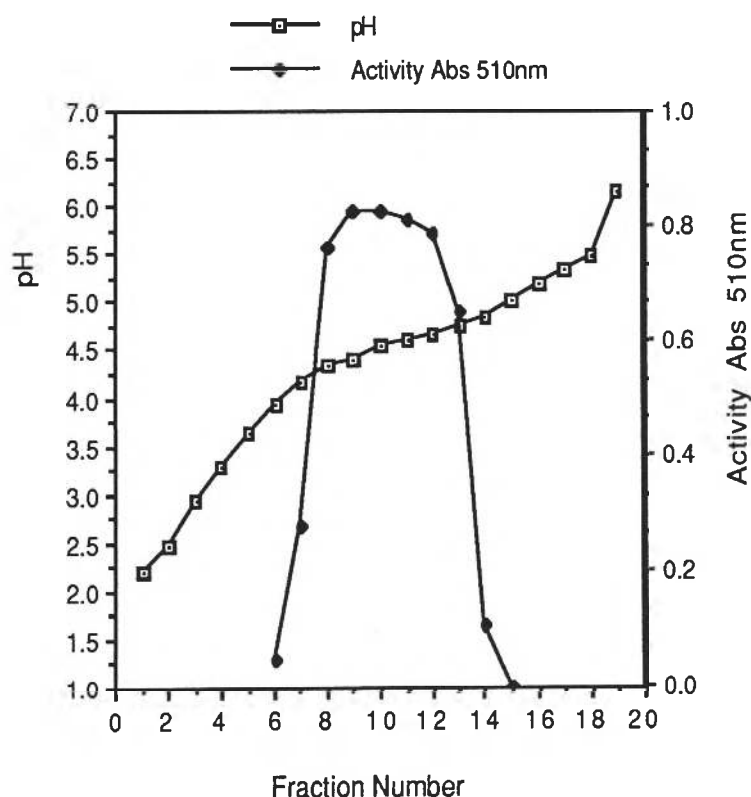


Figure 43. Preparative IEF using a Rotofor according to Methods Section 4.2.2.4 (c). Fractions collected after focussing were measured using a pH probe. The aryl sulfatase was detected according to Methods Section 4.2.2.2 (a).

4.3.12.2 Native and Subunit Molecular Weights.

The aryl sulfatase purified according to Methods Section 4.2.2.3(a) followed by preparative isoelectric focussing according to Methods Section 4.2.2.4 (c) was run on Native PAGE and then stained with substrate as a positive confirmation of its location, according to Methods Section 4.2.2.6. A Native PAGE of aryl sulfatase run with the standards bovine serum albumin (BSA) and soybean trypsin inhibitor (TI) was silver stained according to Methods Section 4.2.2.5 (b). The results shown below in Figure 44a demonstrate a single band with an apparent molecular weight of approximately 90,000 which was confirmed as aryl sulfatase subsequently by substrate staining.

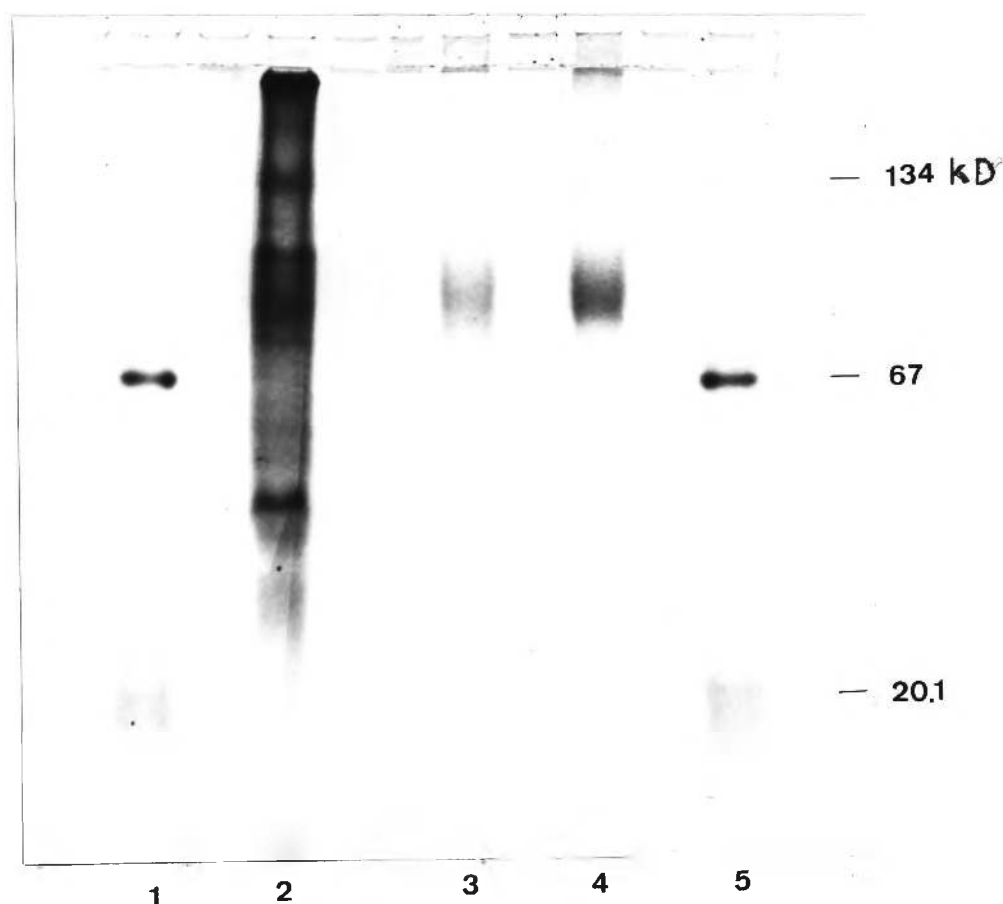


Figure 44a Native PAGE of purified aryl sulfatase.

Lanes 1 and 5 : MWt. markers. lane 2 : Crude aryl sulfatase. lanes 3 and 4 : purified aryl sulfatase.

The aryl sulfatase is seen to migrate as a broad band. Aryl sulfatase fractions of varying pI were found to give the same molecular weight estimations. The aryl sulfatase run on SDS PAGE according to Methods Section 4.2.2.4 (b) demonstrated a subunit molecular weight of the order of 32,000. This suggests that the aryl sulfatase from *H. pomatia* is possibly a trimer.

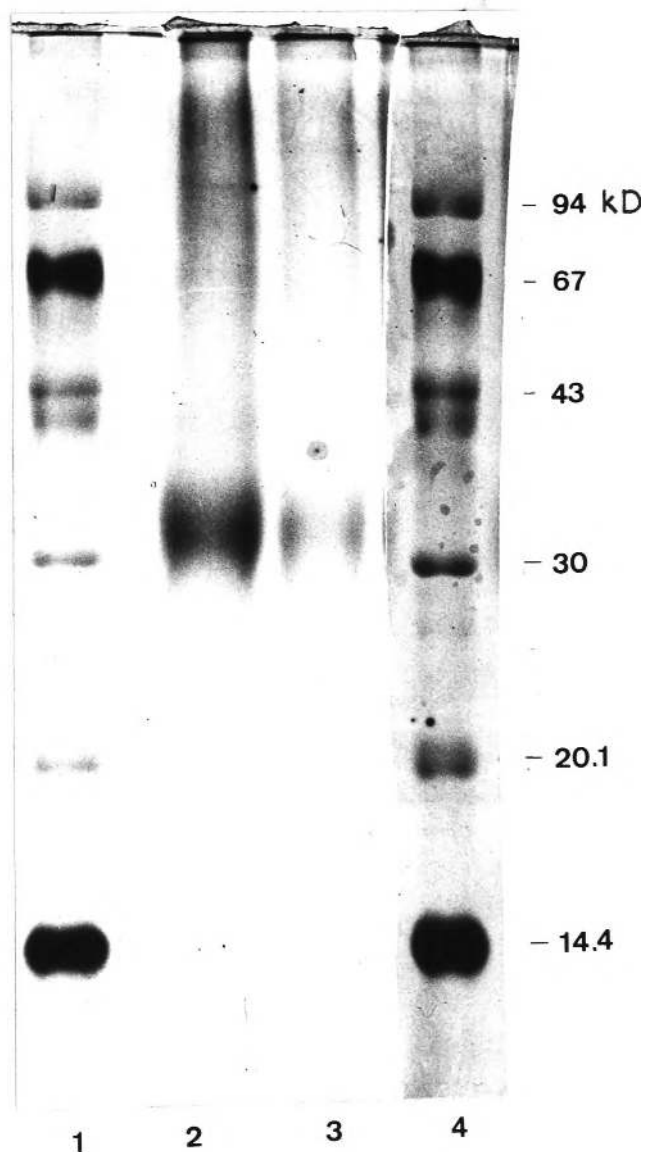


Figure 44 (b) An SDS PAGE of pure sulfatase prepared according to Methods Section 4.2.2.4 (b). Lanes 1 and 4 are low molecular weight markers, lanes 2 and 3 are aryl sulfatase 1 and 2 respectively.

4.3.12.3 Sequencing of Aryl Sulfatase

The purified aryl sulfatase run on SDS PAGE was subsequently blotted onto a PVDF membrane and the band corresponding to aryl sulfatase excised for amino acid sequencing according to Methods Section 4.2.2.4 (d and e). The first 14 amino acids sequenced from the terminal end were as follows : Ala Glu Asp Pro Pro Lys Val Ile Leu Pro Glu Val Phe Glu.

The 14 amino acids sequence was not matched with aryl sulfatases from other sources and the closest match was with glutamine synthetase. The relatively high amount of proline is consistent with the mammalian aryl sulfatases.¹⁰⁷

4.3.13 KINETIC STUDIES ON ARYL SULFATASE

4.3.13.1 Effect of Sulfate Ions on Activity of Aryl sulfatase from *H. pomatia*.

The elution of intact GSLs derived from plant material from DEAE Sephadex A-25 using 0.3 M potassium sulfate was shown in Chapter 3 to give good recovery. Since on-column desulfation proceeds slowly, it was considered desirable to attempt to elute the intact GSLs and perform the desulfation in solution, without the possibility of a charged matrix restricting enzyme-substrate interaction. The presence of sulfate or phosphate ions has been reported to be strongly inhibitory for aryl sulfatase enzyme of the edible snail.⁶⁷

To examine whether the sulfatase activity would be appreciably affected by 0.3 M potassium sulfate, a series of activity assays in the presence of increasing sulfate were conducted (Table

28). The results suggest that aryl sulfatase was not markedly inhibited by 0.3 M potassium sulfate, since no significant sulfatase inhibition occurred even in the presence of sulfate concentrations as high as 0.75 M (Table 28). This finding is consistent with those reported for other type 1 aryl sulfatase enzymes.¹⁰⁵

Table 28. Sulfatase Activity in the Presence of Sulfate ion
Methods Section 4.2.2.13

Potassium Sulfate (mM)	Rate of Hydrolysis umole/mg/min
0	66.5
0.05	66.9
0.10	67.5
0.20	67.0
0.35	66.6
0.75	65.3

4.3.13.2 Sulfatase Activity in the Presence of GSL Substrates.

To determine the rate at which desulfation will occur for a particular rapeseed extract it is important to know the K_m value of the aryl sulfatase toward both aliphatic and indole GSLs, since variable concentrations of the GSL substrate may appreciably affect the rate of desulfation. Given an estimate of the total GSL content, the plant or seed extract can be diluted if necessary to a concentration that will give an optimum rate of desulfation.

a) Sinigrin Incubation with Aryl Sulfatase.

The rates of desulfation was determined using the aliphatic GSL: sinigrin at varying concentrations.

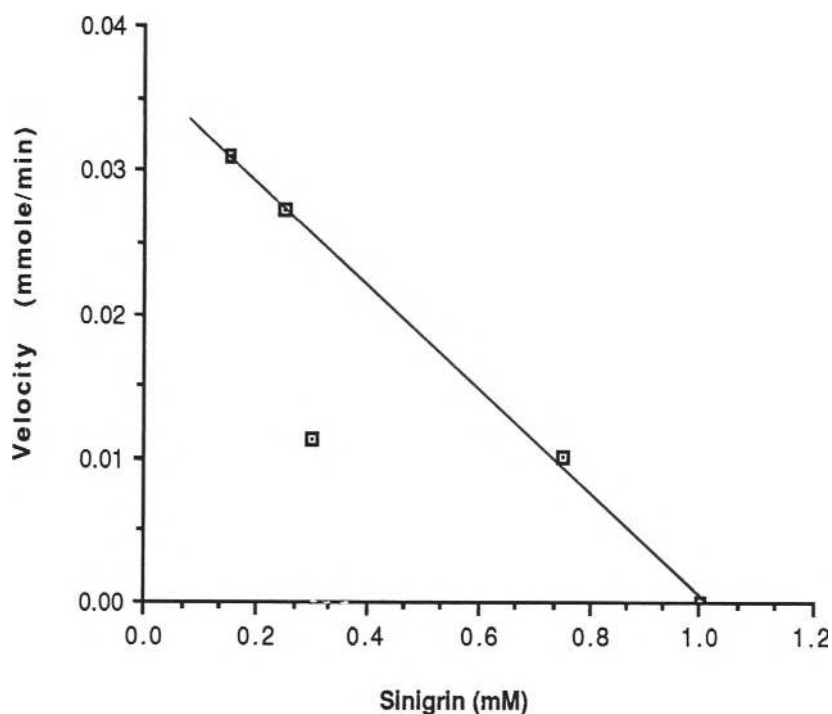


Figure 45. The rate of desulfosinigrin formation was determined spectrophotometrically in 33 mM ethylene diamine/acetic acid buffer at pH 5.8 according to Methods Section 4.2.2.14 (a).

A plot of velocity versus substrate concentration (Figure 45) reveals that aryl sulfatase appears to act as a homotropic enzyme in which the aliphatic GSL serves as an inhibitory or negative modulator. This is consistent with other reports concerning the inhibition of aryl sulfatase activity with increasing substrate concentration.¹⁰⁹ However it has also been reported that the degree of substrate inhibition is pH dependent whereby changes in activity was attributed to a change in pH optimum in association with substrate concentration.¹⁰⁹

b) Glucobrassicin Incubation with Aryl Sulfatase

The rate of desulfation using the substrate glucobrassicin appeared to occur considerably more slowly than that of aliphatic GSLs. This is consistent with the observed desulfation rates of GSLs in rapeseed extracts. An optimum rate of desulfation was reached at approximately 1.0 mM glucobrassicin at pH 5. A concentration above 2.0 mM was found to inhibit aryl sulfatase activity.

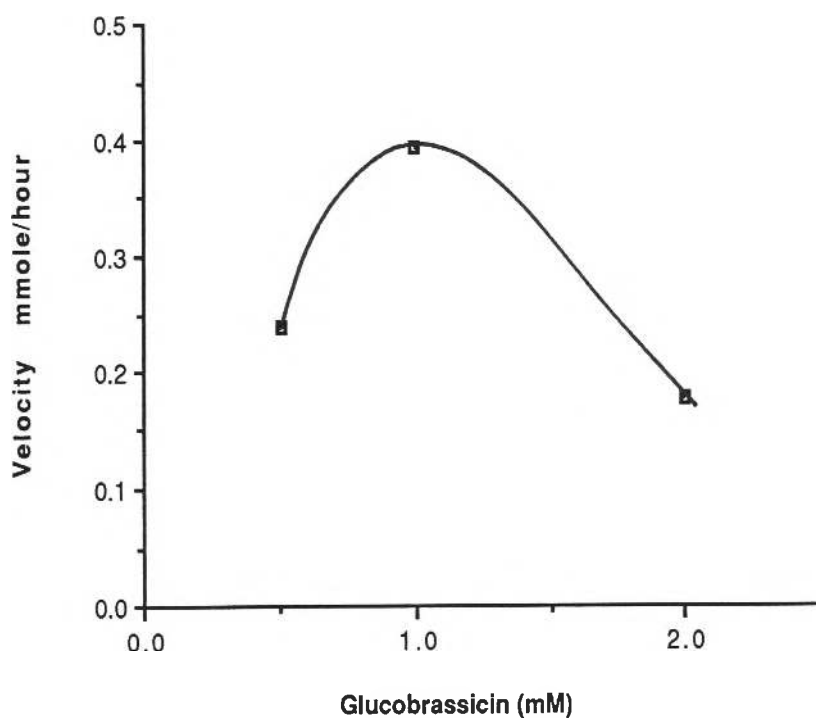


Figure 46. The rate of desulfation by aryl sulfatase in 20 mM acetate buffer at pH 5.0 was measured by the rate of formation of DS glucobrassicin using HPLC at 226 nm. The conditions are given in Method Section 4.2.2.14 (b).

4.4 DISCUSSION

The use of aryl sulfatase from *H. pomatia* for on-column desulfation of GSLs was first reported by Theis 1979.⁶⁷ A recent protocol utilising on-column desulfation prior to HPLC analysis using reversed phase C18 chromatography was reported by Sang and Truscott (1984).⁶⁶ This methodology involved on-column desulfation in 20mM Tris buffer at pH 8.0. The high pH desulfation was employed to prevent contaminant glycosidase enzymes in the crude aryl sulfatase from acting on the GSLs.

The need to heat treat the DS GSLs after desulfation was reported by McGregor in order to inhibit the degradation of the internal standard oNPGal by the glycosidase present in the crude aryl sulfatase.⁵²

It has been shown that some glycosidases become inactive at pH values above 7.5 (Figure 2). Aryl sulfatase however maintained approximately 15 % of its optimal activity (pH 5.5) in 0.1M phosphate buffer at pH 8.0. It has been reported that the aryl sulfatase from *Aerobacter aerogenes* strain 9621 retains 40 % of its activity in 0.5 M Tris-chloride pH 10.¹⁰⁸ This activity assay was however performed in Tris buffer which has been shown to activate aryl sulfatase giving an increased apparent K_m and V_{max} .¹⁰⁹

High pH desulfation using aryl sulfatase from *H. pomatia* resulted not only in slower rates of desulfation therefore requiring more enzyme, but more seriously in the loss of 4-hydroxyindoleGLB. This GSL will be underestimated due to oxidation particularly at high pH. The use of an antioxidant mercaptoethanol, gave a substantial improvement in the recovery of the indole GSL.⁷² However the use of mercaptoethanol was not a desirable solution for most laboratories considering the pervasive smell of this reagent.

The time course study shown in Figure 33 demonstrated the need to inhibit the activities of contaminant enzymes toward GSLs during the desulfation step, assuming that these were responsible for the observed decrease in recoveries.

A report by Buchner (1987) demonstrated variable recovery of the DS GSLs depending on the presence of buffer. The time course of desulfation was performed in water pH 4.7 or sodium acetate buffer pH 4.5 at room temperature. Although a plateau was obtained in both studies, the yields of individual DS GSLs in water were variable, being approximately 20 to 30 % less than that obtained with 20 mM sodium acetate buffer pH 4.5.¹¹⁰ This may possibly be attributed to the inhibition of suspected glycosidase enzymes by the acetate ion.⁹⁹ Further results demonstrated variable yields depending on the temperature of desulfation. Hence the existence of glycosidase enzymes in aryl sulfatase may explain the variable yields observed during desulfation.

To confirm the possibility that β -glucosidase-like enzymes may act on the β -D-thioglucose moiety of GSLs, emulsin a β -glucosidase from sweet almond was incubated with both intact and DS GSLs.

The results shown in Table 21 and Figure 34 suggested that only the DS GSL was susceptible to hydrolysis. Emulsin was reported to behave similarly to the β -glucosidase from *H. pomatia*.⁸²

The rate of decline of DS GSL concentrations in the time course study (Figure 33) was seen to be approximately equal for all DS GSLs which was also consistent with a report that the snail β -glucosidase is not greatly influenced by variations in the aglycone structure.⁸²

The amount of β -glucosidase activity measured in the crude aryl sulfatase from *H. pomatia* was determined in four samples (Table 22) and found to be in sufficient amounts to cause the

observed losses during desulfation of the GSLs.

A purification protocol published for the crude sulfatase by Heaney and Fenwick was based on ion-exchange chromatography.⁷⁰ Although a 2.5 fold increase in the sulfatase specific activity (Table 22) was obtained, the incubation of this aryl sulfatase preparation with DS GSLs resulted in reduced peak areas of the DS GSLs depending on the amount of enzyme added (Table 20). This demonstrates that glycosidase enzymes are not removed from the aryl sulfatase by the ion exchange chromatography method. This can result in the loss of DS GSLs. Attempts by Sang and Truscott to purify the aryl sulfatase by charge differences such as DEAE Sepharose or Chromatofocusing at pH 4-6 also failed to improve the recovery of the GSLs during desulfation.⁷²

Gel permeation on Sepharose CL-6B or Ultrogel AcA 34 gave good separation of the aryl sulfatase and β -glucosidase activities. The β -glucuronidase activities were also well resolved from the aryl sulfatase, which was consistent with reports by Wynn (1966)¹¹¹ and Yamaguchi(1982)¹¹², in which mammalian aryl sulfatase and β -glucuronidase were resolved by gel permeation. The pooled aryl sulfatase fractions from Ultrogel AcA 34 was found to have a three fold increase in the specific activity with 84 % recovery. Further purification by DEAE Sephadex A-50 (Figure 39) gave an approximately four fold increase in specific activity, but 20 % loss in the total aryl sulfatase occurred (Table 23).

The separation of sulfatases 1 and 2 can be achieved using ion exchange chromatography. However, it was reported by Roy and Williams (1989) that the enzyme preparation needs to be first dissolved in 10 mM Tris-HCl, pH 7.4 with 75 mM NaCl in order to separate the two forms.¹⁰⁹ A NaCl gradient then elutes aryl sulfatase 1 at 0.1 M NaCl and aryl sulfatase 2 at 0.4 M NaCl. Aryl

sulfatases 1 and 2 were found to be indistinguishable in terms of their kinetic properties.¹⁰⁹

The binding site of Concanavalin A-Sepharose is specific for α -D-mannosyl, α -D-glucosyl and sterically similar residues. Aryl sulfatase from *H. pomatia* was found to be retained by Con A-Sepharose providing a simple and efficient purification step (Figure 40 and Table 23). For the purpose of preparing DS GSLs for analysis on HPLC, alternative purification schemes were investigated.

The use of Con A-Sepharose as a one step purification of the crude sulfatase was not successful due to the binding and co-elution of the β -glucosidase enzyme with aryl sulfatase.

The use of Con A-Sepharose after gel permeation gave a 10 fold increase in the specific activity with only 2 % loss of activity (Table 25). If it is desirable to decrease the amount of protein added for the purpose of using aryl sulfatase for preparing DS GSLs, gel permeation followed by Con A-Sepharose chromatography was found to be advantageous.

To confirm the separation of aryl sulfatase from the glycosidase enzymes acting on DS GSLs, studies relating to the recovery of DS GSLs were performed.

The time course desulfation study performed in 20 mM Tris buffer pH 6 (Figure 42) demonstrated that desulfation of GSLs was completed by 2 hours and further aliquots of the DS GSLs taken over the following 20 hours showed no apparent decline in DS GSL recovery. Some minor decrease in the 4-hydroxyindoleGLB was apparent after 5 hours, but the use of aryl sulfatase preparations with higher specific activity could enable complete desulfation within 30 minutes, thereby minimising the time for analysis and loss of those GSLs susceptible to oxidation.

The inhibition of aryl sulfatase by sulfate ions reported by

Thies, W.⁶⁷ was not observed in these experiments (Table 33). As much as 0.75 M potassium sulfate was found not to cause any appreciable loss in aryl sulfatase activity from *H. pomatia*. This result is consistent with the classification of the aryl sulfatase from *H. pomatia* as a type 1 aryl sulfatase. These enzymes are typically insensitive to sulfate ion. For example, studies on aryl sulfatases have shown that *Aerobacter oryzae* produces an aryl sulfatase which is also not strongly inhibited by sulfate.¹⁰⁷ Similarly the aryl sulfatase from *A. aerogenes* is not inhibited by sulfate, however a phosphate concentration of 10 mM gave 55 % inhibition using nitrophenylsulfate as substrate.¹⁰⁸

A recent report by Roy 1989, has demonstrated that the inhibition of aryl sulfatase by sulfate is pH dependent however, sulfite was found to be a potent inhibitor.¹⁰⁹

Potassium sulfate elution of GSLs, as described in the Thymol procedure (chapter 3), prior to desulfation should lead to improved enzyme-substrate interaction by removing the need for on-column desulfation thus giving an increased rate of desulfation. Another advantage is the elution of novel negatively charged DS GSLs such as 1-sulfoGLB which may not be eluted using on-column desulfation. In addition there is potential for the concurrent analysis of the eluate by thymol for total GSL determination which can be correlated with the HPLC technique.

At pH 5.5 and increasing sinigrin concentration it was found that the rate of desulfation decreased with no detectable desulfation by aryl sulfatase observed at 1 mM sinigrin. With increasing concentrations of glucobrassicin, the rate of reaction firstly increases to a maximum at 1 mM, and then declines to 25 % of the maximum at 2 mM (Figure 46). Moreover, the rate of desulfation was markedly less than that for sinigrin at all substrate concentrations. It

should be noted, however, that different buffers were used in these assays. Substrate inhibition has also been reported by Dodgson (1954) when aryl sulfatase A from *A. metalcaligenes* was incubated with increasing concentrations of 4-nitrocatechol sulfate.⁹⁷ The aryl sulfatase A from ox liver was also reported to be inhibited by concentrations of 4-nitrocatechol sulfate higher than 3.0 mM.¹⁰³ More recent reports by Roy (1989) have demonstrated that substrate inhibition of aryl sulfatase from *H. pomatia* is pH dependent.¹⁰⁹

Given the protein purification protocol it is possible to purify large amounts of crude aryl sulfatase (250 mg) in a single step by gel permeation and although it has been reported that carbohydrases are capable of attacking Sephadex supports, acrylamide-agarose gel permeation columns have been found to be durable. This allows relatively simple and repetitive purifications of aryl sulfatase to be achieved. Although further purification of the aryl sulfatase is not necessary the use of Con A-Sepharose provides enzyme preparations of higher specific activity. With 200mg of crude sulfatase, the total activity remaining after gel permeation and Con A-Sepharose was approximately 1,800 units. Since 1 unit will desulfate approximately 0.5 μ mole GSL per minute, this is sufficient aryl sulfatase activity to analyse approximately 600 samples.

A rapeseed meal (60 μ mole GSL/g meal) prepared according to Methods Section 4.2.2.7 (j), will yield approximately 1 μ mole of GSL in a 1.0 ml aliquot. Desulfation with 1 unit (or 20 μ g) of aryl sulfatase from gel permeation, is estimated to be completed by 15 minutes at pH 5. This rapid rate of sample preparation and desulfation should allow good recovery of unstable GSLs, for example, 4-hydroxyglucobrassicin.

A summary of the proposed method for HPLC analysis of the GSLs in plant tissue is shown below.

Plant Tissue

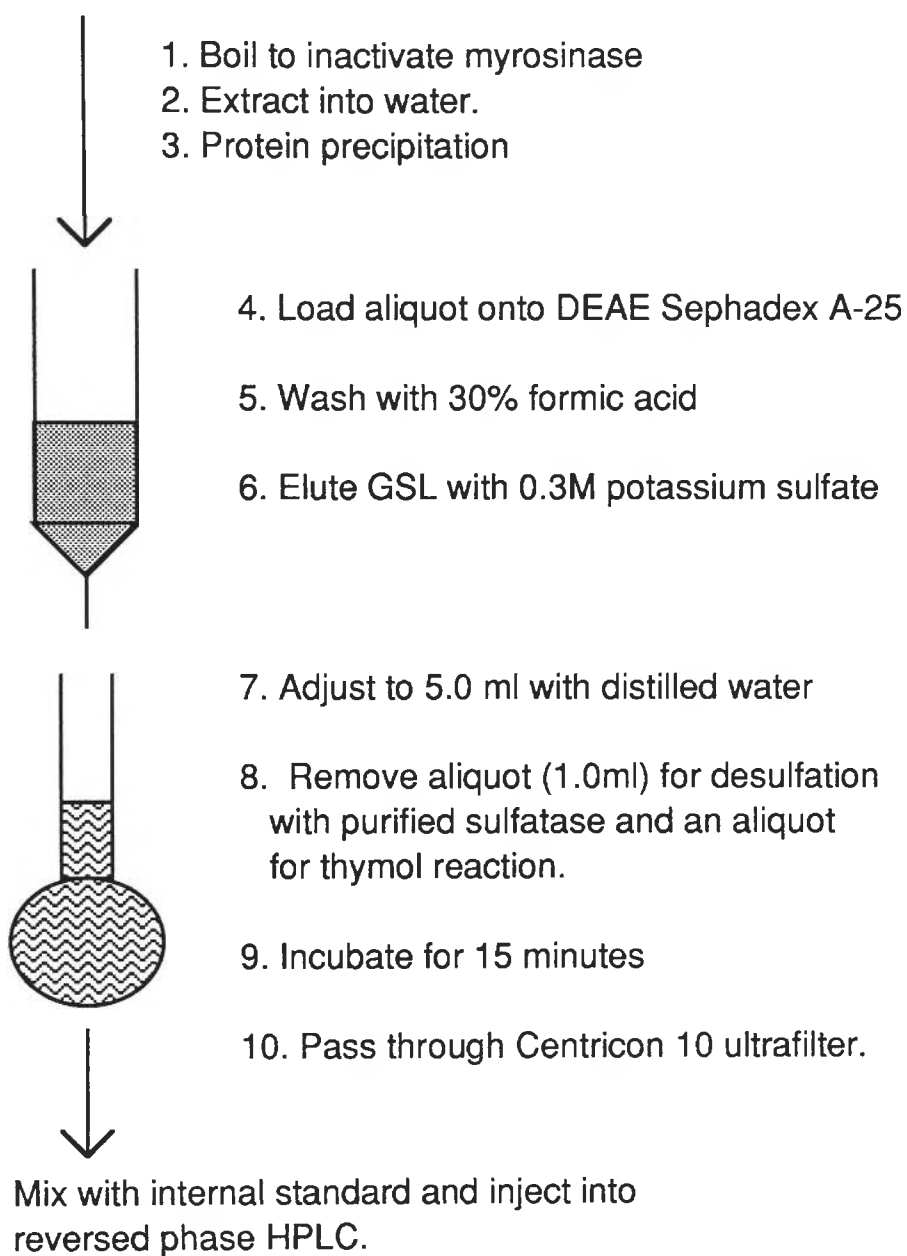


Figure 46. Strategy for the preparation of DS GSL for reversed phase HPLC analysis.

This methodology should enable efficient determination of GSL profiles in all cruciferous seeds and could be applied with modification of initial extraction conditions to other plant tissue samples. Previous methods have involved overnight on-column desulfation or have required elution of the intact GSLs with pyridine acetate prior to desulfation.

Errors in the quantitation of the unstable 4-hydroxyglucobrassicin will be minimised by performing the desulfation at low pH (pH 6 instead of pH 8), which both improves the rate of desulfation and reduces oxidation which is more pronounced at higher pH.

The other advantage of this methodology is the elution of novel GSLs which possess an additional acid group not cleaved by aryl sulfatase. For example, the analysis of 1-sulfoglucobrassicin may not be possible using on-column desulfation, since the additional sulfate group would prevent elution. Elution by potassium sulfate, however, ensures 97 % of the GSLs are recovered.

Given the elution of the intact GSLs with potassium sulfate, an aliquot could be removed for estimation of the total GSL content using the thymol method.

Overall, this strategy can provide a fast, simple and accurate measure of individual GSLs in rapeseed meals and other GSL-containing plant tissues.

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