ANALYTICAL AND MICROPREPARATIVE HPLC COMBINED WITH SPECTROSCOPIC AND ENZYMATIC METHODS FOR GENERATING A GLUCOSINOLATE LIBRARY

By

LINO GERARDO ARGUELLO

Bachelor of Science

National Autonomous University of Nicaragua

1992

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1998

OKLAHOMA STATE UNIVERSITY

ANALYTICAL AND MICROPREPARATIVE HPLC
COMBINED WITH SPECTROSCOPIC AND
ENZYMATIC METHODS FOR GENERATING
A GLUCOSINOLATE LIBRARY

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Ziad El Rassi, for his supervision, constructive guidance, inspiration, and friendship. My sincere appreciation extends to my other committee members Dr. Horacio A. Mottola and Dr. K. Darrell Berlin for their support and valuable suggestions. I would like to thank Dr. Feng Qiu for his help in the NMR experiments and Dr. Dilip K. Sensharma for his help in the MS experiments.

Also, I would like to express my gratitude to the Fulbright organization for providing me with a graduate study fellowship that has allowed me to obtain this degree.

My special appreciation goes to my wife, Brenda Luz Blanco de Arguello, for her precious help in my research, her strong encouragement at times of difficulty, love and understanding throughout this whole process.

I would like to dedicate this small portion of my life's work to my parents, Jorge Arguello and Isidra Salgado. Thank you for the love, support and encouragement that you have given me over the years. I will always be thankful for all the good values that you have instilled in me.

Finally, I would like to thank my friend Dr. William Lawson for having spent time in teaching me English and chemistry.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

NOMENCLATURE

FAB	Fast atom bombardment
GC	Gas chromatography
GS	Glucosinolate
HPLC	High performance liquid chromatography
HVE	High voltage electrophoresis
LC	liquid chromatography
LSIMS	liquid secondary ion mass spectrometric
MS	Mass spectrometry
ODS	octadecylsilica
PC	Paper chromatography
PGC	Porous graphitized carbon columns
RPC	Reversed phase chromatography
TLC	Thin layer chromatography
SAX	Strong anion exchange

CHAPTER I

OBJECTIVES, BACKGROUND AND RATIONALE OF THE RESEARCH

Introduction and Objectives

Glucosinolates are thioglucosides whose structures vary mainly by the nature of their R groups (see Fig. 1 and Table 1). The R group can be alkyl, alkenyl, arylalkyl methylthioalkyl, methylsulfinylalkyl, methylsulfonylalkyl, or indolylalkyl (see Table 1). The great diversity of the R group leads to a wide variation in the polarity of these natural products.

Glucosinolates are found in every species of the plant family Cruciferae, specially in Brassica. During food preparation or ingestion, glucosinolates are easily broken down, leading to compounds with detrimental characteristics (1). The nature and level of glucosinolates vary from plant-to-plant and also from a part to another part in the same plant (2).

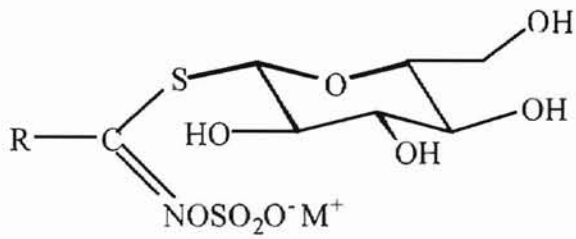
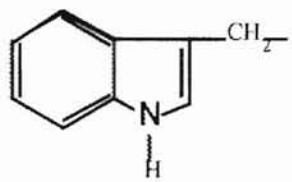


Figure 1. General structure of glucosinolate. R = side chain, M^+ = cation, usually K^+.

The problem concerning glucosinolates in food are related to the total amount, to the type of glucosinolates present, and to the products produced by autolysis or other degradation processes (3) (see page 3).

Table I. Some natural glucosinolates and their corresponding R groups.

Trivial name	Chemical mame[a]	Structure of R group
Glucocapparin	Methyl-GS	CH_3
Sinigrin	Allyl-GS	CH_2=CH-CH_2
Glucoibervirin	3-Methylthiopropyl-GS	CH_3-S-$(CH_2)_3$
Glucoiberin	3-Methylsulfinylpropyl-GS	CH_3-SO-$(CH_2)_3$
Gluconapin	3-Butenyl-GS	CH_2=CH-$(CH_2)_2$
Glucoerucin	4-Methylthiobutil-GS	CH_3-S-$(CH_2)_4$
Glucoraphanin	4-Methylsulfinylbutyl-GS	CH_3-SO-$(CH_2)_4$
Glucoerysolin	4-Methylsulfonylbutyl-GS	CH_3-SO_2-$(CH_2)_4$
Progoitrin	2(R)-Hydroxy-3-butenyl-GS	CH_2=CH-$CHOHCH_2$
Glucoberteroin	5-Methylthiopentyl-GS	CH_3-S-$(CH_2)_5$
Glucoalyssin	5-Methylsulfinylpentyl-GS	CH_3-SO-$(CH_2)_5$
Glucotropaeolin	Benzyl-GS	$C_6H_5CH_2$
Gluconasturtiin	2-Phenylethyl-GS	$C_6H_5CH_2CH_2$
Sinalbin	*p*-Hydroxybenzyl-GS	*p*-$HOC_6H_4CH_2$
Glucobrassin	Indol-3-ylmethyl	

Glucosinolates have some negative as well as beneficial health effects. Some of the negative health effects can be summarized as follows: glucosinolates are relatively nontoxic, but their hydrolysis products, formed by myrosinase (thioglucosidase), adversely affect animal growth, reproduction, and performance and cause goiter and abnormalities in internal organs of animals (4). In addition, the glucosinolate degradation products are involved in off-flavor, antinutritive, and toxic effects associated with too high concentrations of these compounds in food and feed (5). Thus, the qualitative and quantitative determination of glucosinolates and their break down products occurring in the Brassica species are important. On the other hand, the degradation products of glucosinolates by myrosinase are important species because they have been found to decrease the growth of yeast and microorganisms (6). Another benefit of glucosinolates is that some isothiocyanates degradation products from glucosinolates are known to be phase II anticarcinogens (7). Indoles, which are the degradation products of some glucosinolates, have been demonstrated to be inhibitors of either dimethylbenzanthracene-induced mammary cancer in the rat or benzo(α)pyrene-induced cancer of the forestomach in mice (8).

The majority of the studies involving the negative and beneficial effects of glucosinolates, as far as the human and animal health are concerned, have dealt with a limited number of glucosinolates (9). This is due in part to the lack of efficient isolation methods to generate quantities of pure glucosinolate standards which can be used to study the toxicological effects of individual as well as of total glucosinolates and their degradation products.

Thus, the aim of this research is to develop HPLC methods for the isolation of intact glucosinolates from real world samples (i.e., generation of a library of glucosinolates) at the micropreparative level (i.e., a few hundred μg/day). Although preparative HPLC at the laboratory scale (>1g/day) is not simply using larger columns and higher flow rates than in micropreparative HPLC, we expect that the micropreparative HPLC methods described herein will be beneficial for the development of scaled-up preparative separation processes. Concurrent to this objective is the development of analytical HPLC methods for the determination of glucosinolates and their degradation products in real plant extracts. The third objective of this study is to better understand the chromatographic behavior of glucosinolates and their degradation products under various elution conditions. A fourth objective is concerned with evaluating spectral as well as enzymatic and chemical tools in the identification of isolated glucosinolates. These objectives were pursued during the present investigation, and the results are presented and discussed in Chapters II and III. While Chapter II deals with the micropreparative and analytical aspects, Chapter III describes the methods used for the identification of isolated glucosinolates. As a representative Crucifarae plant, white cabbage was selected for the study because it is readily available from local grocery outlets, is consumed worldwide by man in million of tons, and is a good source for glucosinolates (10, 11).

Background and Rationale

Chemistry of Glucosinolates

All glucosinolate-containing plants seem to contains β–thioglucosidase (myrosinase) (12). When the plant is crushed, the glucosinolates are hydrolyzed by the liberated enzymes, and during the autolysis process, a type rearrangement may occur with the formation of isothiocyanates. With some cruciferous plants, autolysis leads to other products such as thiocyanates, nitriles, or oxazolidinethiones, depending on the pH value of the reaction medium (4,12). Also, glucosinolates can be hydrolyzed by chemical reactions. In strong acid solutions, glucosinolates are hydrolyzed to the corresponding carboxylic acids, and in strong alkaline solutions some glucosinolates are transformed to aminoacids in a Neber type rearrangement (3).

Glucosinolates in contact with the enzyme myrosinase (thioglucosidase) undergo a detachment of glucose, producing an aglucon (II) (see Fig. 2). Under neutral conditions, the aglucon undergoes a Lossen-type of molecular rearrangement yielding an isothiocyanate (III). On the other hand, myrosinase hydrolysis under weakly acidic conditions or in the presence of ferrous ions produces nitriles (IV) and elemental sulphur (13-15). The formation of the thiocyanate (V) from glucosinolates needs two enzymes (at least) namely a myrosinase, and an aglucone-rearranging enzyme (16). Therefore, the thiocyanate degradation product is not observed when the isolated glucosinolate is treated with only exogenous myrosinase. Isothiocyanates carrying a hydroxyl group in the β–position (VI) (see Fig. 3) spontaneously cyclize to yield substituted oxazolidene-2-thiones (VII) (11-13). At relatively low pH, the products of the myrosinase hydrolysis of

the glucosinolate progoitrins are a mixture of 1-cyano-2-hydroxy-3-butene and 1-cyano-2-hydroxy-3,4-epithiobutene (VIII and IX) (see Fig. 3).

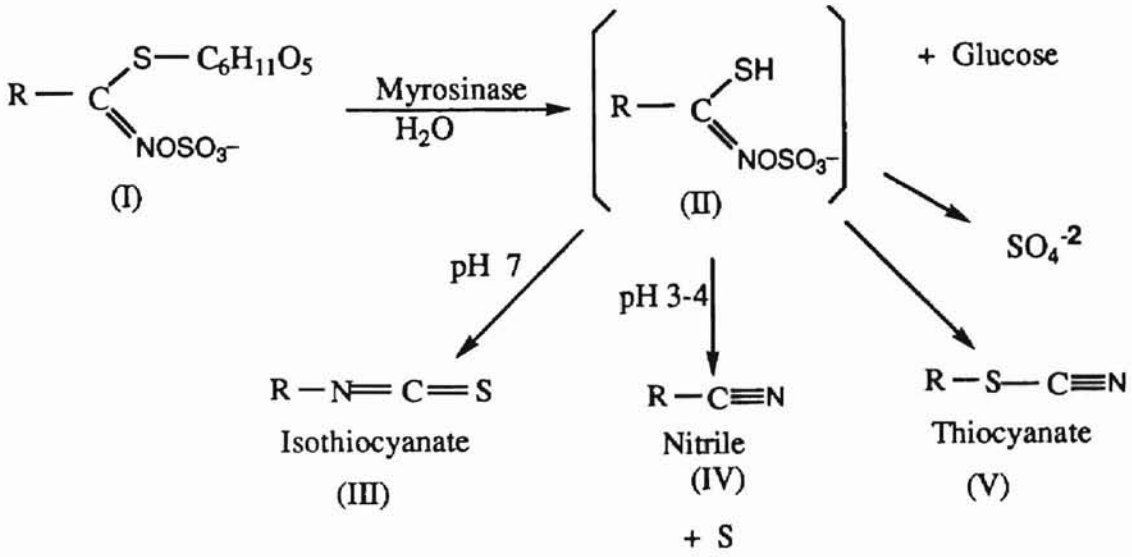


Figure 2. Enzymatic hydrolysis of glucosinolates by myrosinase (15).

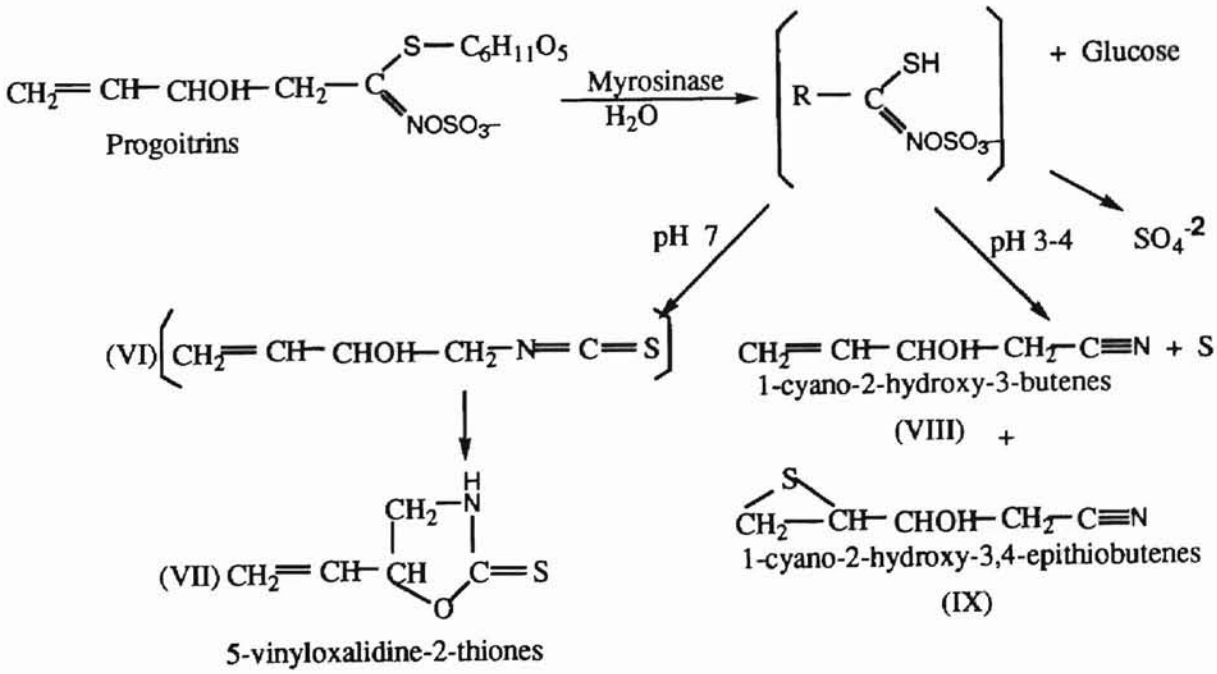


Figure 3. Enzymatic hydrolysis of progoitrins by myrosinase (15).

If the R group of the glucosinolate is an indol, e.g., glucobrassicin, the product of the enzymatic hydrolysis with myrosinase at relatively low pH is indole-3-acetonitrile (IAN), while at neutral pH the alcohol indole-3-carbinol (I3C) is produced first, and then this alcohol is further converted to produce 3,3' –diindolyl-methane (I33') (see Fig. 4) (17). Consequently, to analyze the indolyl glucosinolates via their degradation products, it is convenient to carry out the reaction at pH 3-4 in order to have only one degradation product. In this way the formation of a mixture of several products can be avoided.

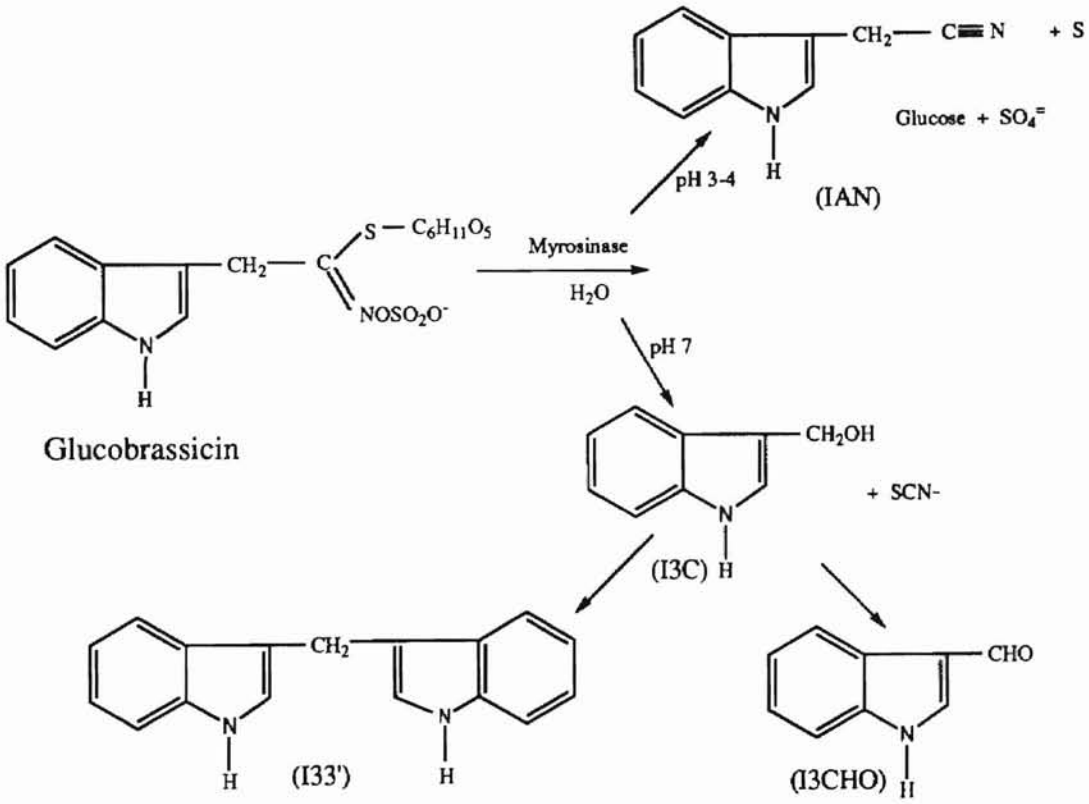


Figure 4. Autolytic product of glucobrassicin (GB) (17). The pH-dependent, enzymatic hydrolysis of GB yields either the acetonitrile (IAN) or the alcohol (I3C). The resulting I3C is unstable in aqueous solutions and undergoes self-condensation to a dimer (I33') or oxidation to the aldehyde (I3CHO).

In addition to the most common glucosinolates generally represented in Fig. 1, other glucosinolates with acylated thioglucose and/or glycosylated R-group have recently been reported (18). Typical examples include glucosinolates with either sinapoyl or isoferuloyl residues substituted at either C2 or C6 or at both C2 and C6 of the thioglucose residue. Other substitutions on the R group include sulfonation of the N group in the indolyl glucosinolates. The degradation products of these compounds by myrosinase digestion or by chemical methods are not yet known. This is due to the lack of reliable preparative techniques to isolate sufficient amounts of the substituted glucosinolates so that more accurate studies on the chemistry of these glucosinolates can be performed. Usually, the substituted glucosinolates are not detectable in methods based on glucosinolate degradation products including the well established desulfoglucosinolate technique (19).

An important chemical degradative process for glucosinolates is that of acid hydrolysis (16). It has been shown that glucosinolates produce carboxylic acids, glucose, hydroxylamine, and hydrogen sulfate upon treatment with strong acid (16) as follows:

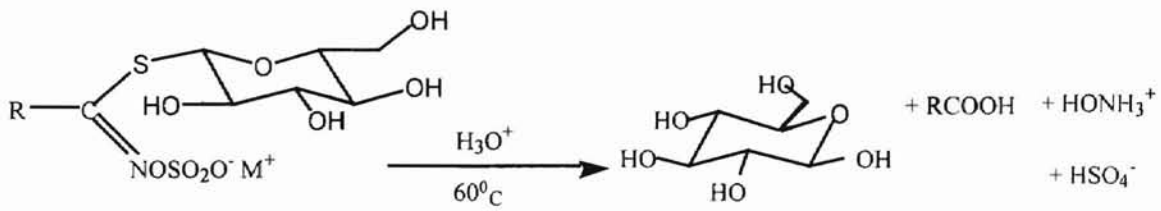


Figure 5. Acid hydrolysis of glucosinolates.

Isolation of Glucosinolates

Despite major advances in preparative HPLC (20) in both instrumentation and column technology, low pressure liquid chromatography (i.e., conventional LC) has been the major technique for the isolation of glucosinolates. Conventional LC is a technique characterized by intensive labor, long separation times and sometimes poor reproducibility. The following discussion summarizes the conventional LC methods used for the preparative isolation of glucosinolates.

Before the early seventies, HPLC was not developed, and low pressure LC was the only approach available for the isolation of glucosinolates. In fact, Gmelin (21) separated individual glucosinolates present in a filtered methanol extract by passing the extract first on a basic anion exchange column or anionotropic aluminum oxide column, operating at low pressure, followed by a purification step involving paper chromatography (PC) and using a mixture of *n*-butanol to acetic acid to water (4:1:5 or 4:1:3) as the mobile phase. In another approach, glucosinolates were isolated from a crude extract of rapeseed by conventional liquid chromatography using a DEAE-Sephadex A-25 column or arginine coupled to sephadex G–10 column (12). Glucosinolates from the methanol extract of cabbage were isolated by using an anion exchange resin Dowex^{R} 1-X2 column (22). In another application, sample clean-up was performed by low pressure ion-exhange chromatography to remove carbohydrates and other impurities before derivatization (23).

In the period extending from the late seventies until the present, low pressure LC has still been the primary technique for the isolation of glucosinolates despite the wide spread use of HPLC in many areas of the life sciences. In fact, Olsen and Sorensen (3),

isolated glucosinolates by first passing the extract of glucosinolates on a column of Amberlite IR-120 (ion-exchange) of dimensions 2.5 x 90 cm. The collected fractions in which the glucosinolates were present were then concentrated. The concentrated fractions were passed on through another low pressure column, namely Aceteola-cellulose AcO^-, of dimensions 2.5 x 80 cm. These two columns yielded the glucosinolates in a few fractions without the separation of the individual components. The fractions that contained all the glucosinolates were first dried and then purified by preparative high-voltage electrophoresis using a solution of pH 1.9 composed of a mixture of acetic acid-formic acid-water at the ratio of 4:1:45. This was followed by preparative paper chromatography. This methodology required various steps and, consequently, was time consuming, wasted large amounts of materials, and was not easy to carry out. In addition to low throughput (i.e., amount of purified material/unit time), the authors worked at an extreme pH which could hydrolyze the glucosinolates. Bjerg and Sorensen (24) used an ion–exchange material DEAE-Sephadex A-25 packed in mini-columns (plastic pipette tips with plugs of glass wool at the bottom) to purify the crude extracts of glucosinolates and then analyzed the glucosinolates by HPLC. Again, this method was performed at a relatively extreme pH (e.g., collect the sample in a beaker which have 0.1 ml of 1.5 *M* HCl) in which hydrolysis of glucosinolates can occur. Similar to the work in reference 3, the workers did not separate the individual glucosinolates and thus it is difficult to know when the glucosinolates were eluted from the column. Hu and coworkers (25) isolated the 2-hydroxyethyl glucosinolate by passing the concentrated extract through a column of acidic alumina, which was first washed with water and then eluted with 5% aqueous K_2SO_4. Thereafter, the collected glucosinolate fraction was applied to another column of

G-10 Sephadex (ion-exchange). The column was eluted with water and the fractions were monitored by TLC. The fractions containing the glucosinolates were combined and applied to a column of C-18 silica and eluted under flash chromatographic conditions with 5% aqueous K_2SO_4. The product was dried and recrystallized. This procedure required the use of 3 different columns, TLC to monitor the fraction content, and finally recrystallization. Recently, Sakushima and coworkers (26) isolated sinapinyl but-3-enylglucosinolate by applying the concentrated methanolic extract on a column of Sephadex L H-20 (50g) and MCl gel (100g). The column was eluted with water, and the collected fractions were monitored by TLC. Fractions containing the desired product were purified on another column of Sephadex L H-20 (50 cm x 2.5 cm I.D.)

To the best of our knowledge, the only work that described the use of HPLC in the isolation of glucosinolates has been that of Prestera et al. (27) which just appeared in the literature. The glucosinolates were isolated by reversed-phase ion-pair chromatography. They used tetraoctylammonium bromide, and tetramethylammonium bromide as counter ions. They were able to remove the counter ion by exchange with ammonium chloride and ammonium sulfate, followed by solvent extraction with chloroform and methanol. In this procedure, the extraction required several steps in order to obtain the glucosinolate in the ammonium salt form. They used ^{1}H NMR spectrometry and mass spectral analysis of ammonium salts by negative-ion fast atom bombardment (FAB) to determine the *m/z* of the $[M - H]^-$ ion.

Analysis of Glucosinolates

Traditionally, the determination of glucosinolates has been performed by PC (28), TLC (26, 29), gas chromatography (GC), GC-MS (30, 31), and high voltage electrophoresis (HVE) (18, 29), and more recently by HPLC (32, 33-38) and HPLC-MS (13, 39). Due to the polar nature of glucosinolates, it is not possible to analyze intact glucosinolates directly by GC. In GC usually glucosinolates are first converted to desulfoglucosinolates by sulfatase action and then derivatized with a silylating reagent to yield trimethylsialylated desulfoglucosinolates (3, 40). Although GC and GC-MS analyses of trimethylsialylated desulfoglucosinolates are very reliable approaches, some problems still exist concerning the quantitation of methylthio-, methylsulfinyl-, or methylsulfonyl-alkyl-glucosinolates. However, GC-MS is a useful technique for the identification of some glucosinolates via their R-isothiocyanate degradation products. Thin layer chromatography and PC provide only qualitative information, and it is not possible to determine the exact amount of glucosinolates. However, PC and TLC techniques are rapid approaches for monitoring glucosinolate isolation in low pressure chromatography.

In the following section, an overview of the use of HPLC in the analysis of glucosinolates and their degradation products is provided. For the clarity of discussion, a short overview and some other specific rationales are given in Chapter II.

Elfakir et al. (5) worked with strong anion exchange (SAX) columns packed with silica having surface-bound quaternary ammonium groups [$-N(CH_3)_3^+$]. They studied the behavior of 5 standard glucosinolates (progoitrin, sinigrin, gluconapin, glucotropaeolin, and glucobrassicin). They also suggested that the glucosinolates are

retained on a silica-based anion exchanger by a combination of two retention mechanisms. The main mechanism involved is ion-exchange, while the second one is hydrophobic interaction which assists the ion-exchange process and improves the separation. They also found that the addition of an organic modifier in the mobile phase decreased retention and selectivity. In their work, they pointed out that the determination of the total glucosinolate content in a natural mixture on a silica-based anion exchanger was not satisfactory because sufficient selectivities were not observed.

In another report from the same research group (41), the separation of some standard glucosinolates was studied on styrene-divinylbenzene copolymeric column anion exchanger under isocratic elution conditions. The eluent used was an alkane sulfonate at different concentrations. The glucosinolates were eluted in the order of decreasing polarity as in reversed phase chromatography (RPC). They also found that increasing the concentration of the alkane sulfonate, or carbon number of the alkyl group in the alkane sulfonate anion, decreased the analysis time. Due to the strong nonpolar nature of styrene-divinylbenzene-based stationary phases, the alkyl sulfonates compete with the solutes for the hydrophobic binding sites on the surface of the stationary phase. The alkyl sulfonate also compete with the solutes for the anion exchange binding sites. The net result of both phenomena is a decrease in the retention time of glucosinolates.

Using the same 5 standards as in (5), Elfakir et al. (42) studied the chromatographic behavior of the standard glucosinolates on a polymethacrylate copolymer anion exchange, and compared the results with those obtained on a silica anion exchange and a polystyrene divinylbenzene copolymeric anion exchange columns using isocratic elution. Higher retention and better selectivities were obtained on the

polymethacrylate anion exchanger than on the silica anion exchanger. The importance of the reversed phase interactions with respect to ion exchange interactions in the glucosinolates retention mechanism was reinforced on a polymethacrylate-based column in comparison with a silica-based column and lessened in comparison with a silica-based exchanger.

Recently, Elfakir and Dreux (43) evaluated a porous graphitized column for the qualitative analysis of glucosinolates and desulfoglucosinolates by HPLC. They used 5 standards of glucosinolates (as those in Ref. 5) and achieved separation using 0.1% (v/v) aqueous trifluoroacetic acid-acetonitrile (85:15 v/v) as the eluent. The intact glucosinolates and desulfated glucosinolates were eluted roughly in the order of decreasing polarity as in RPC. They suggested that the anionic charge of the intact glucosinolates involved a complementary electronic interaction with the electron density of the graphite surface and the presence of an electronic modifier, such as trifluoroacetic acid, in the aqueous organic eluent was indispensable to elute these compounds.

More recently, Zrybko et al. (13) developed an HPLC method to separate and quantify glucosinolates in mustard and winter cress plants using RPC with ion-pairing reagents to modify the mobile phase and enhance retention and selectivity. They confirmed the identity of glucosinolates by using negative ion electrospray mass spectrometry and photodiode-array detection. They used formic acid (F) and triethylamine (TEA) as a volatile buffer medium which is required for HPLC-electrospray mass spectrometry. The formic acid and triethylamine produce triethylammonium formate (TEAF) which interacts with the glucosinolates in the aqueous phase of the system. Once bound, the glucosinolate-TEAF complex has an

increased affinity for non-polar, organic stationary phase resulting in an increase in retention. This ion-pairing reagent volatilizes during HPLC-MS analysis and therefore does not clog the LC-MS interface probe. The column used was Phenomenex 5 μm ODS 25cm x 4.6 mm I. D. The mobile phase used was water (A) and methanol (B), both containing the ion-pairing reagent consisting of 0.15% (v/v) triethylamine and 18% (v/v) formic acid. The mobile phase was run isocratically at 100% A for 10 min and then switched to a linear gradient elution from 100% A to 100% B over 60 min at a flow-rate of 1.0 ml/min. This method required at least 70 min, which is a relatively long time, and then required at least another 10 min for column equilibration. The chromatograms in the presence of the ion-pairing agent exhibited broad peaks, and some of them were not well resolved.

Quinsac and Ribaillier (1) introduced an HPLC method for the analysis of desulfated derivatives of rapeseed glucosinolates under isocratic elution conditions with different CN-bonded stationary phases. The mobile phase used was water and water-acetonitrile (95-5% v/v). They found lower efficiencies with pure water in comparison with a water-acetonitrile (95-5% v/v) mixture. In this method, 9 standard desulfoglucosinolates were separated using a Lichrospher CN column of 25 cm x 4 mm I.D. The retention time of these standards was less than 10 min so that in a real sample this method will not work because of the matrix interferences which usually have short retention times.

In another report concerning the HPLC of desulfoglucosinolates, Ishida and coworkers (44) used liquid chromatography/mass spectrometry (LC-MS) to combine the outstanding separation ability of HPLC with the wide qualitative ability of mass

spectrometry. LC-MS can directly separate and identify the compounds which can not be readily analyzed by GC-MS due to the sensitivity to high temperature, nonvolatility, and derivatization requirement. HPLC was used with atmospheric pressure chemical ionization mass spectrometry (APCI/MS) and achieved the separation of desulfoglucosinolates on an ODS column using a gradient elution at increasing acetonitrile in water. The authors confirmed the total amount of glucosinolates obtained by HPLC with the palladium-glucosinolate complex method (44). This study did not confirm the structures of glucosinolates by NMR.

Sang and coworkers (2) separated and collected by HPLC the desulfoglucosinolates from cabbage extract and others plants. They used reversed-phase ODS column under linear gradient elution conditions from 0 to 12% (v/v) acetonitrile in water in 30 min. The desulfoglucosinolates were then identified after derivatization by GC-MS. They found that the principal glucosinolates in the leaf of cabbage were 3-indolylmethyl-glucosinolate (glucobrassicin) and 4-methoxy-3-indolylmethyl-glucosinolate (4-methoxyglucobrassicin), with 2-hydroxybut-3-enyl-glucosinolate (progoitrin) present in smaller amounts. In addition, they reported the presence of allyl glucosinolate (sinigrin), 4-methylsulfinylbutyl glucosinolate (glucoraphanin), and 4-hydroxy-3-indolylmethyl glucosinolate (4-hydroxyglucobrassicin) in low amounts.

Individual glucosinolates can be determined via the aglucone degradation products (e.g., nitriles, isothiocyanates) obtained after myrosinase digestion of glucosinolates. The degradation products are reflective of the parent glucosinolates (4, 17, 34, 45). In these works, RPC has been the chief HPLC technique for the separation of the various degradation products. Indeed, the autolysis products of the indolylmethyl

glucosinolate, including indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3'-diindolyl-methane (I33') (17), were separated on an Ultrasphere ODS column of dimensions 25 cm x 4.6 mm packed with 5 μm particles. The indoles were eluted and separated using a linear gradient elution from 5 to 80% (v/v) acetonitrile in 5 m*M* sodium phosphate dibasic (pH 7.0) over a period of 60 min at a flow-rate of 1.0 ml/min.

Furthermore, the individual isothocyanates and oxazolidinethione in myrosinase digests of rapeseed and rapeseed meal were determined simultaneously by RPC (4, 34, 45). The identity of peaks corresponding to these compounds was confirmed by MS.

Conclusions

It is clear from the above review of pertinent literature concerning the isolation and determination of glucosinolates in real world samples, that there is still an ample room for improvement in HPLC of glucosinolates. The present dissertation addresses this need by evaluating two different HPLC methods, namely reversed phase chromatography and anion exchange chromatography (Chapter II) in the analytical and micropreparative HPLC of glucosinolates. Furthermore, to establish a glucosinolate library from a real plant extract, spectroscopic and enzymatic approaches were combined with HPLC.

CHAPTER II

ANALYTICAL AND MICROPREPARATIVE HPLC OF INTACT GLUCOSINOLATES

Introduction

High performance liquid chromatography has found an increasing use in the separation of glucosinolates. For instance, reversed phase chromatography (RPC) using octadecylsilica (ODS) stationary phases was shown to be useful for the separation of intact glucosinolates (2, 13, 33-36). However, for some of the more polar glucosinolates, their separation by RPC required the addition of an ion-pairing agent to enhance their retention and in turn resolution (27, 37). Ion-pair RPC is not suitable for micropreparative work and, in addition, requires a time-consuming equilibration of the column and offers poor reproducibility. To overcome these impediments encountered with RPC of intact glucosinolates, several researchers separated glucosinolates as desulfoglucosinolates by treating the glucosinolates with sulfatase (33, 35, 38, 44). However, most of the work dealt with synthetic standards, and, moreover, desulfoglucosinolates were not sufficient solutes for many further studies on the separated analytes. Recently, porous graphitized carbon columns (PGC) (43) were introduced to the separation of glucosinolates. Due to the fact that PGC column provided strong hydrophobic and electronic interaction, peaks eluted were very broad.

On the other hand, the potentials of high performance anion exchange chromatography have been elucidated by a few brief studies (5, 41, 42) dealing exclusively with synthetic standards. To the best of our knowledge, the isolation of glucosinolates in a "real world" sample by high performance anion exchange chromatography has not yet been reported.

There is a strong need for preparative methods by HPLC for establishing libraries of pure glucosionolates from plant extracts. In fact, there are only two commercially available glucosinolate standards, namely sinigrin (allyl glucosinolate) and gluconasturtin (phenethyl-glucosinolate). While sinigrin from Sigma is reasonably priced ($1.60/10 mg) gluconasturtin from LKT laboratories is priced 77 times higher than sinigrin, i.e., $124/10 mg. This fact provides another rationale for the development of micropreparative HPLC methods that will find general use in the isolation of glucosinolates in any given plant extract. This chapter addresses this need by describing the various steps involved in the micropreparative isolation of glucosinolats from "real world" samples. We are expecting that the methodology will be readily transposed to large scale purification using larger columns and higher flow rates to prepare gram quantities of pure glucosinolates.

Experimental

Materials

HPLC-grade methanol, reagent-grade sodium phosphate monobasic and dibasic, barium chloride, and sodium chloride were purchased from Fisher (Fair Lawn, NJ, USA). Sinigrin monohydrate, and thioglucosidase (myrosinase) 859 units/g were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate was from J.T. Baker (Phillipsburg NJ, USA).

Apparatus

The liquid chromatograph was assembled from an LDC-Milton Roy (Riviera Beach, FL, USA), Model CM4000 solvent delivery pump with a variable wavelength detector Model Spectromonitor 3100, a Rheodyne (Cotati, CA, USA) Model 7125 sampling valve with a 100-µl sample loop, and a C-R5A Chromatopac integrator from Shimadzu (Columbia, MD, USA). The detection wavelength was set at 228 nm for the glucosinolates and 210 nm for their degradation products.

A column of dimensions 25 cm x 6.2 mm I.D., packed with Zorbax ODS spherical silica having 5 µm mean particle diameter, 60 Å mean pore diameter and 350 m^2 /g specific surface area, and a column of dimensions 25 cm x 4.6 mm packed with Zorbax SAX of 5 µm mean particle diameter, 70 Å mean pore diameter, and 300 m^2 /g specific surface, area were obtained from Dupont Co. (Wilmington, DE, USA). Another column of dimensions 15 cm x 4.6 mm I.D., packed with Microsorb-MV C18 spherical silica of 5 µm mean particle diameter, 100 Å mean pore diameter, and 190 m^2 /g specific surface area, was purchased from Rainin (Emerylle, CA, USA).

A fraction collector, Model 1200 PUP from ISCO, Inc. (Lincoln, NE, USA) was used in micropreparative HPLC. A coffee and spice grinder from Regal ware (Kewaskum, WI, USA) was used to grind the cabbage. A speed Vac Model SC110, refrigerator vapor trap Model RVT4104, and a Valupump Model VLP120 were from Savant (Holbrook, NY, USA).

Extraction

Typically, 50 g of cabbage were first frozen in liquid nitrogen, and then ground in a coffee grinder. To this fine material was added 100 ml of boiling pure methanol. Thereafter, the mixture was heated until reaching the temperature of 70 °C. The temperature was maintained for an additional 2 to 3 min. The resulting methanolic mixture was filtered under vacuum using a water aspiration. Finally, the filtrate was dried in a speed vac.

Sample Clean-up

The sample clean-up involved the following steps. The dried extract of cabbage (approximately 1 g) obtained as described above was dissolved in 5 ml of water. To this solution, was added 0.5 ml of $BaCl_2$ saturated solution. Thereafter, the mixture was centrifuged for 9 min, and the solid was discarded. The resulting solution was dried in a speed vac. The solid material obtained after solvent evaporation was dissolved in 3 ml of methanol, and the mixture was centrifuged for 5 min. The supernate was then concentrated by speed vac to approximately 2 ml. To this concentrate was added 2 ml of ethyl acetate while mixing until a precipitate was formed. The mixture was centrifuged for 5 min, and the precipitate was collected. This precipitate was treated again with 1 ml of ethyl acetate while mixing, and the mixture was centrifuged for 5 min. After discarding the liquid, the precipitate (enriched with glucosinolates) was dried by a speed vac.

Digestion of Glucosinolate by Myrosinase

The dry extract from white cabbage, approximately 0.5 g, was dissolved in 1 ml of a buffer solution of 50 m*M* sodium phosphate dibasic, pH 7.0. Approximately, 1 mg of myrosinase was added to this solution. Then the solution was stirred for more than 3 hours. For the "cleaned-up" extract, the reaction required more time (approximately 12 hours).

Results and Discussion

Chromatographic Behavior of Glucosinolates

Reversed Phase Chromatography (RPC). RPC employing nonpolar stationary phases, e.g., ODS, and hydroorganic mobile phases (e.g., buffered aqueous-methanol) was introduced to the analysis and purification of intact glucosinolates. Due to the hydrophilic nature of glucosinolates (see Fig. 1 in Chapter I), and consequently their relatively weak interactions with the RPC column, a mobile phase of 50 mM sodium phosphate monobasic, pH 3.2, was used. These conditions were selected to ensure that the glucosinolates would be retarded sufficiently and in turn be separated from each other as well as from the sample interferences. Higher ionic strength promotes stronger hydrophobic interaction and in turn higher retention (46). The wavelength used in the study of the glucosinolates was 228 nm because the glucosinolates give maximum absorptivity around this wavelength.

A column of dimensions 15 cm x 4.6 mm I.D., packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, was first used for the RPC of

glucosinolates. The glucosinolates from white cabbage were injected onto the column which was developed with a linear gradient elution from 0 to 60% (v/v) methanol in 50 mM sodium phosphate monobasic, pH 3.2. Under these conditions, and given the fact that the Microsorb-MV C18 has a relatively low surface coverage with octadecyl ligands (%C = 12% w/w), the strongly polar glucosinolates were not sufficiently retained and eluted at the beginning of the chromatogram with no resolution from sample interferences (see Fig. 6 A). This is true for peak # 1 which almost coeluted with the sample interferences. To allow a better resolution of the glucosinolates under investigation, a different column of dimension 25 cm x 6.2 mm I.D., packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter and 20% w/w carbon load, was used. Fig. 6B shows the chromatogram of the glucosinolates extracted from white cabbage. As can be seen in Fig. 6B, at least 6 glucosinolates can be separated and detected, assuming complete resolution of the glucosinolates components. However, peak # 5 was better resolved from some interferences on the Microsorb C18 column.

Comparing Fig. 6A to Fig. 6B revealed that the 5 μm Zorbax ODS had higher retentivity toward early eluting peaks (# 1 - 4) than the Microsorb-MV C18 column but exhibited similar retention toward peaks # 5 and 6 which eluted at a higher % organic in the gradient. This may be primarily due to a higher carbon load of the Zorbax ODS column. It should be noted that Zorbax ODS column exhibited a larger plate number N = 19913 as compared with N = 9380 for Microsorb-MV C18. Generally, it is necessary to have a column of high carbon loading as well as of high plate count for increasing the retention and resolution of the more polar glucosinolates.

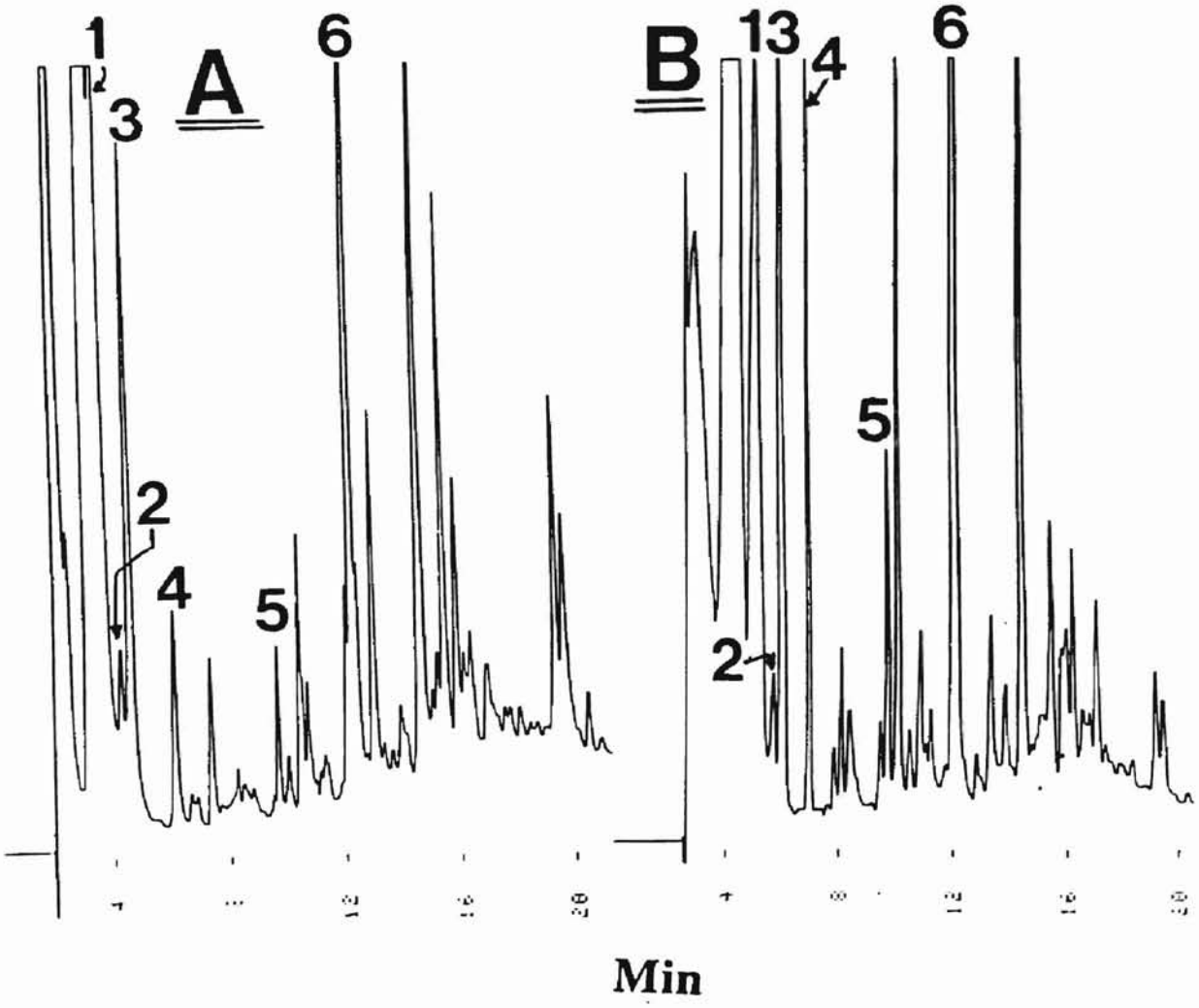


Figure 6. Chromatograms of the white cabbage extract by RPC. Columns, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å mean pore diameter in (A) and 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter in (B). Flow rates, 1 ml/min in (A) and 1.8 ml/min in (B). Consecutive linear gradients from 0 to 60% (v/v) methanol and from 60 to 80% (v/v) methanol in 17 and 3 min, respectively, in 50 m*M* sodium phosphate monobasic, pH 3.2. This is followed by a 3 min isocratic elution at 80% (v/v) of methanol in 50 m*M* sodium phosphate monobasic, pH 3.2. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4.

Strong Anion Exchange (SAX). Due to the fact that the glucosinolates have SO_3^- groups in their structures, a strong anion exchange column (SAX) should be suitable for their separation. The negatively charged glucosinolates can interact strongly with the quaternary ammonium groups on the surface of the stationary phase in the SAX column.

First, it was necessary to determine the best conditions for the separation of glucosinolates from white cabbage with the SAX column. In this regard, the first step was to investigate the effect of the pH of the mobile phase. A column of dimensions 25 cm x 4.6 mm, packed with Zorbax SAX of 5 μm mean particle diameter, was used. The glucosinolates extracted from white cabbage were injected onto the column which was developed with a linear gradient elution from 0 to 1.0 *M* NaCl in 20 m*M* sodium phosphate monobasic (all the sodium phosphate buffers were titrated with phosphoric acid or sodium hydroxide to the desired pH) at pH 3.0, 6.0, and 7.0 and in 20 m*M* sodium acetate at pH 4.0 and 5.0. The best pH for optimum resolution was found to be 3.0 as shown in the chromatogram in Fig. 7A. Improvement of the separation was achieved by changing the composition of the gradient former (i.e., mobile phase B) to 0.50 *M* NaCl in 20 m*M* sodium phosphate at pH 3.2. This allowed better separation to occur (see chromatogram in Fig. 7B). Going from pH 3.0 to pH 3.2 (a difference of 0.2 unit pH) and decreasing the ionic strength from 1.0 to 0.50 *M* NaCl resulted in a significant improvement in the overall separation which translated into 5 resolved peaks of glucosinolates as opposed to 4 resolved peaks at pH 3.0 and using a linear gradient from 0 to 1.0 *M* NaCl. One important behavior that was observed upon increasing pH was that the retention decreased, and at pH 7.0 all the components of the mixture eluted faster with little or no separation as shown in Fig. 8A. In fact, only 2 peaks can be identified as

glucosinolates. At pH 7.0, the net positive charge density of the stationary phase is lowered due to the ionization of the unreacted silanols. This generates repulsive forces between the negative groups of the stationary phase and the negatively charged glucosinolates causing lower retention. To describe the effect of pH on glucosinolate retention on the SAX column, a standard glucosinolate, namely sinigrin, was injected over a wide range of pH. The results are plotted in terms of adjusted retention volume versus pH in Fig. 9. As can be seen in Fig. 9, the adjusted retention volume, V'_R, decreases monotonically in the pH range 3.0–7.0.

Determining the Glucosinolates Peaks in the Cabbage Extract Via Myrosinase

In order to distinguish the peaks of the glucosinolates from interference peaks present in the white cabbage extract, the extract was treated with myrosinase (thioglucosidase), and the chromatogram was compared to that of the intact extract. This information was used for expediting subsequent analysis by auxiliary techniques (e.g., NMR, MS) and also to evaluate column resolution in the determination of glucosinolates. The use of myrosinase allowed us to assess which peaks were glucosinolates and to estimate approximately the number of glucosinolates present in our samples, see Fig. 10. In this approach, the extract of cabbage in a buffer solution of 50 m*M* sodium phosphate monobasic, pH 6.0, was treated with myrosinase for more than 3 hours. Then, the myrosinase treated cabbage extract was injected onto the HPLC column, and the resulting chromatogram was compared to the chromatogram obtained with the intact extract. Comparing the 2 chromatograms (see Fig. 10A and 10B) with and without myrosinase, it is possible to identify the glucosinolates.

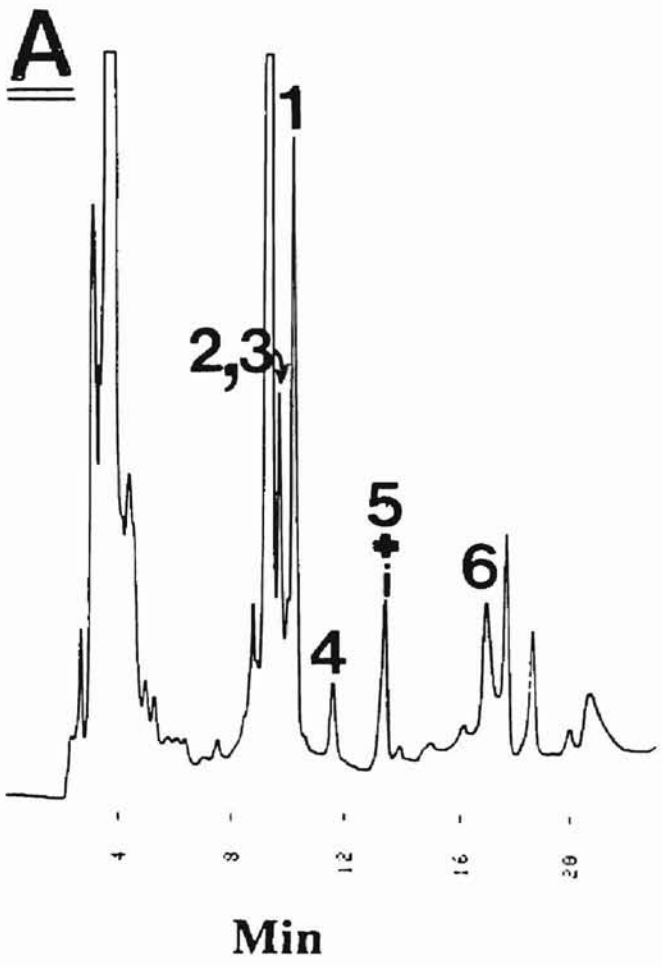


Figure 7A. Chromatogram of the white cabbage extract by SAX. Column, 25 cm x 4.6 mm, packed with Zorbax SAX of 5 μm mean particle diameter, 70 Å mean pore diameter. Flow rate, 1 ml/min. 25 min linear gradient from 0 to 1.0 *M* NaCl in 20 m*M* sodium phosphate monobasic, pH 3.0, followed by 1 min isocratic elution with the gradient former. i, impurity; UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 3.

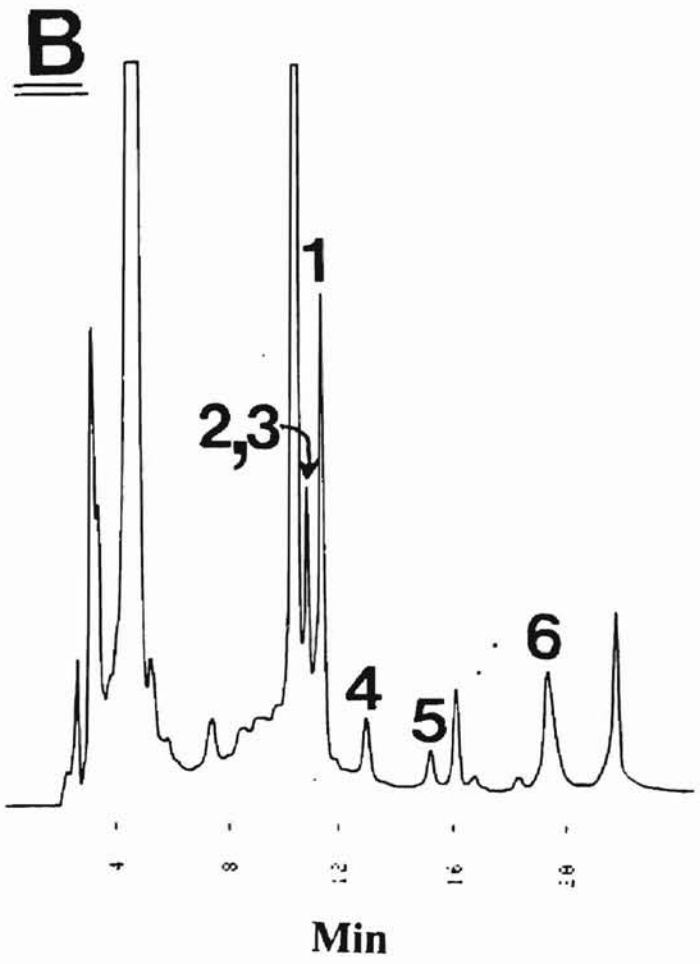


Figure 7B. Chromatogram of the white cabbage extract by SAX. Column, 25 cm x 4.6 mm packed with Zorbax SAX of 5 μm mean particle diameter, 70 Å mean pore diameter. Flow rate, 1 ml/min. 20 min linear gradient from 0 to 0.50 *M* NaCl in 20 m*M* sodium phosphate monobasic, pH 3.2, followed by 1 min isocratic elution with the gradient former. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 3.

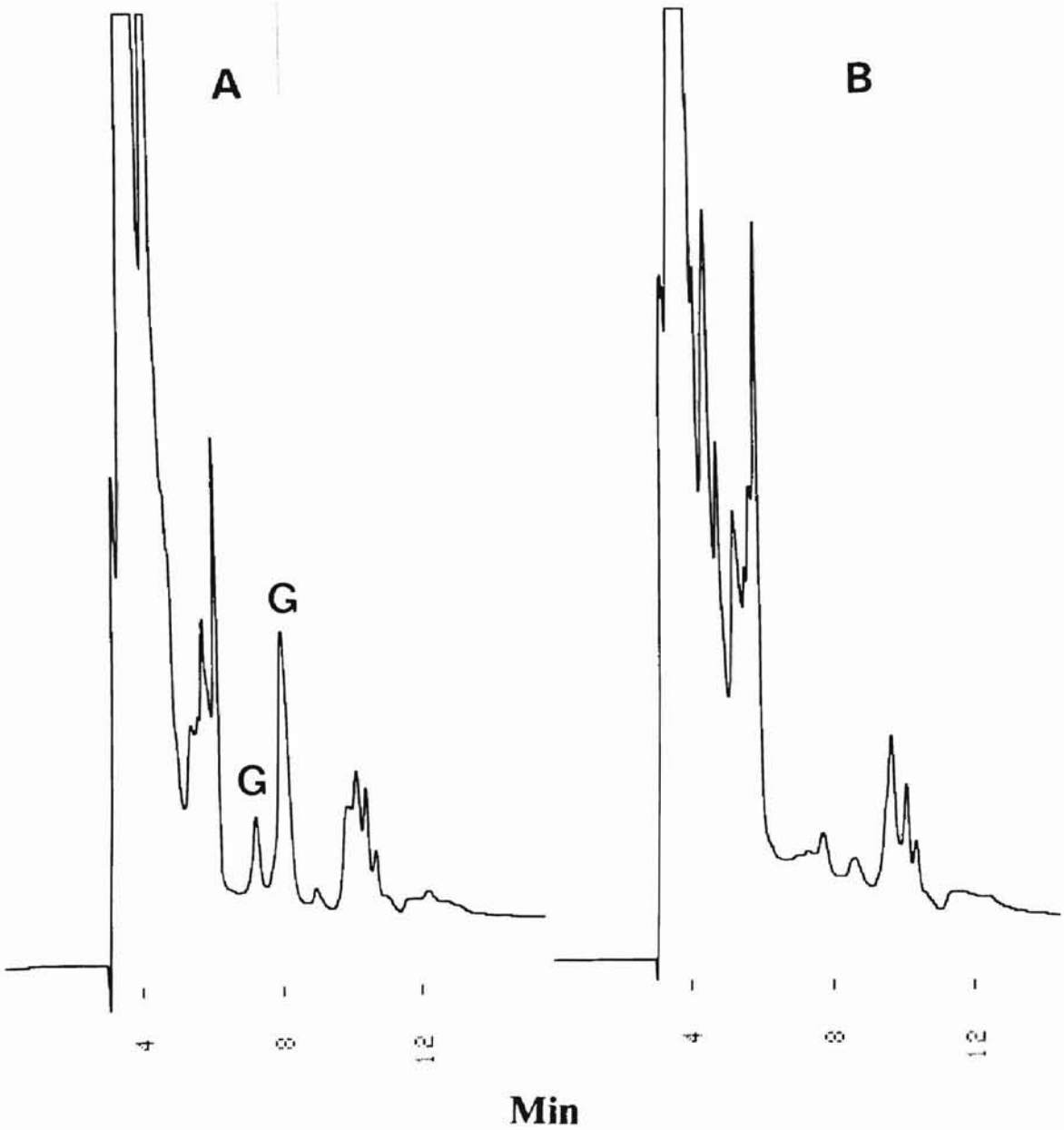


Figure 8. Chromatograms obtained on a SAX column of the white cabbage extract in (A) and white cabbage extract treated with myrosinase in (B). Column, 25 cm x 4.6 mm packed with Zorbax SAX of 5 μm mean particle diameter, 70 Å mean pore diameter. Flow rate, 1 ml/min. 25 min linear gradient from 0 to 1.0 M NaCl in 20 mM sodium phosphate, pH 7.0, followed by 1 min isocratic elution with the gradient former. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 3. G, glucosinolates.

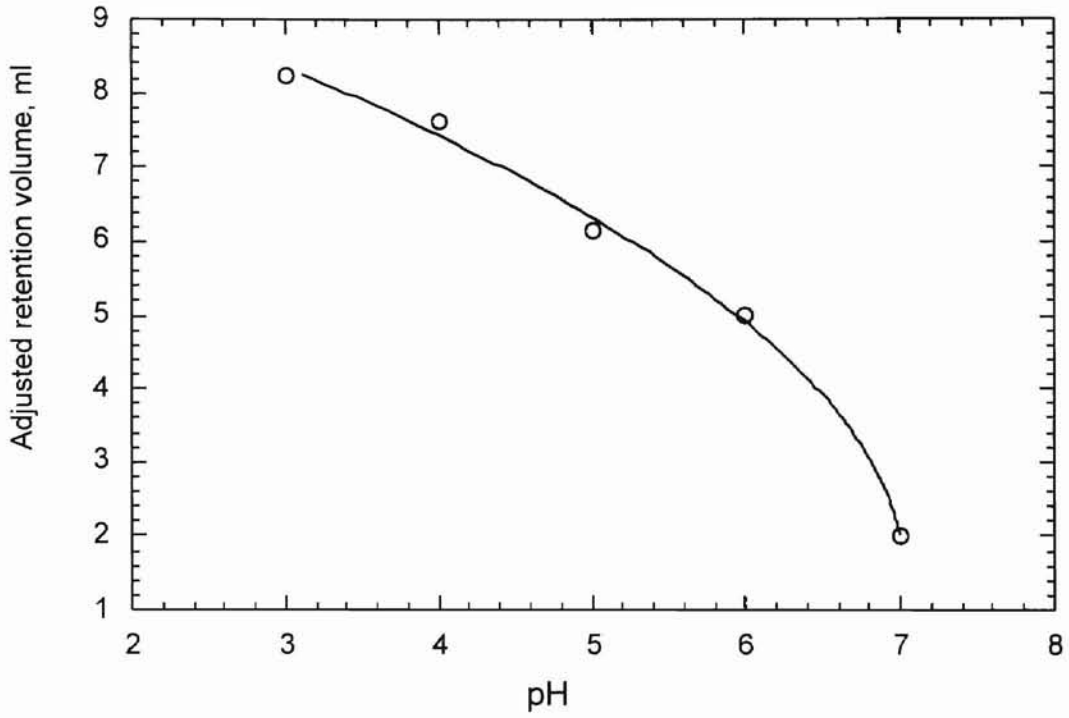


Figure 9. Plot of adjusted retention volume V'_R (ml) of sinigrin at different pH values obtained on a SAX column. Column, 25 cm x 4.6 mm packed with Zorbax SAX of 5 μm mean particle diameter. Flow rate, 1 ml/min. 25 min. Linear gradient from 0 to 1 *M* NaCl in 20 m*M* sodium phosphate, pH 3.0, 6.0, and 7.0 or sodium acetate at pH 4.0 and 5.0, followed by 1 min isocratic elution with the gradient former. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation 3.

In the case of RPC (see Fig. 10), it can be seen that there are at least 6 glucosinolates present in the cabbage extract because there are 6 peaks that disappeared, and an approximate equal number of new peaks appeared in the chromatogram of the injected white cabbage extract that was treated with myrosinase. In the case of SAX, it was not possible to obtain the separation of the 6 glucosinonates, and only 5 of the 6 glucosinolates were separated. In addition, the first 2 peaks were poorly resolved as shown in Fig. 11A and 11B. As can be seen, better separation for the glucosinolates was achieved by RPC than by SAX.

In order to develop a better understanding of the retention behavior of glucosinolates on SAX and RPC, the different peaks separated on the RPC column were collected and injected into the SAX column. Thus, we know the order of elution of the glucosinolates in the SAX system. Peaks #2 and #3 coeluted first followed by peaks #1, 4, 5 and 6 in that order, see Fig. 11. The numbering used in Fig. 11 is that corresponding to the peak numbering with the RPC column. The slight change in the elution order when going from an RPC to a SAX column indicates the implication of a reversed phase mechanism in SAX. This is expected since the stationary phase used in SAX is a composite material containing both quaternary ammonium groups and nonpolar organic functions.

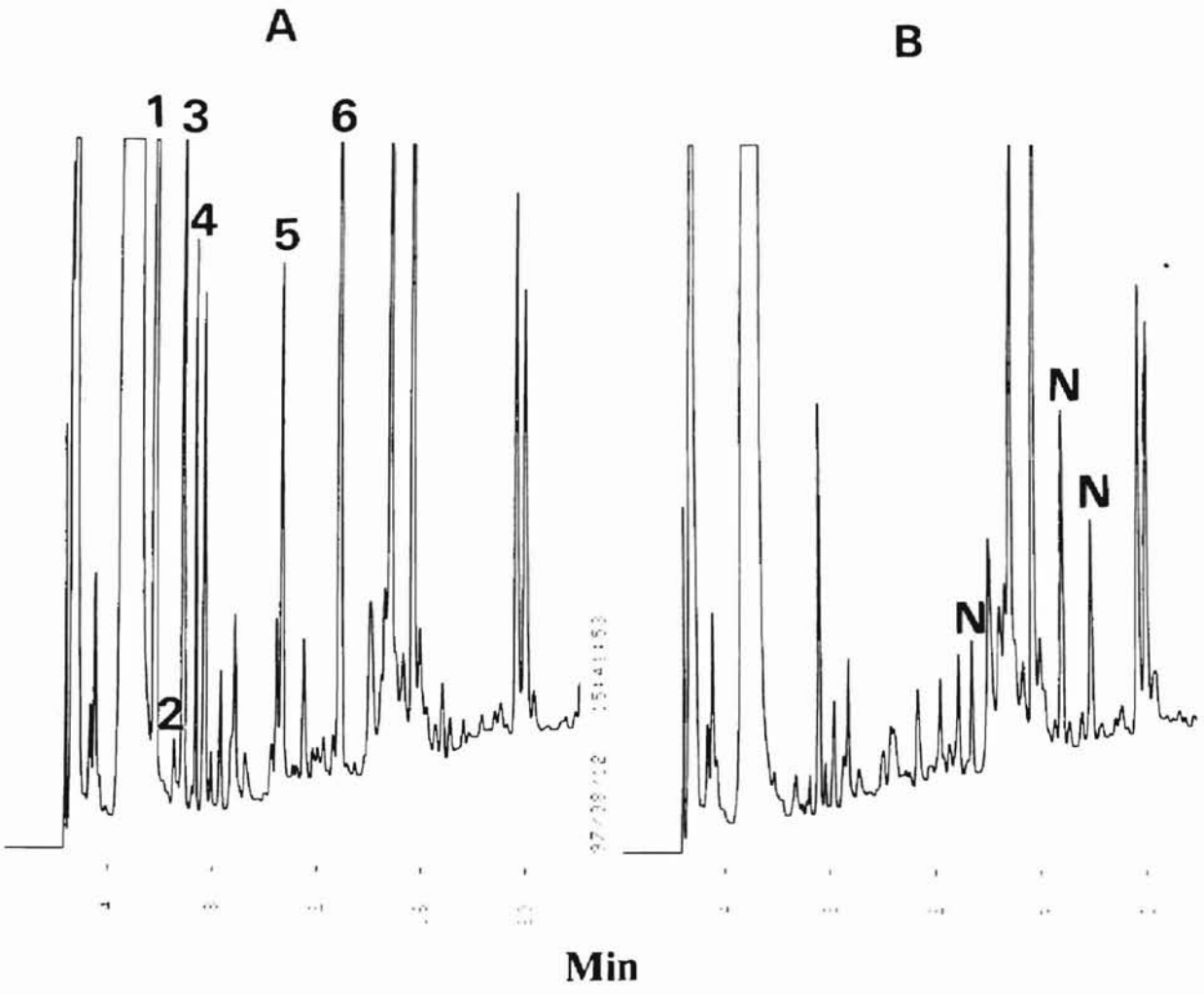


Figure 10. Chromatograms obtained on an RPC column of the intact white cabbage extract in (A) and myrosinase treated extract in (B). Column, 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter. Flow rate, 1.8 ml/min. Consecutive linear gradients from 0 to 60% (v/v) methanol and from 60 to 80% (v/v) methanol in 17 and 3 min, respectively, in 50 m*M* sodium phosphate monobasic, pH 3.2. This was followed by 3 min isocratic elution at 80% (v/v) methanol in 50 m*M* sodium phosphate, pH3.2. UV detection, 228 nm; detector sensitivity, 0.5 AUFS; integrator attenuation, 4. N, new peaks.

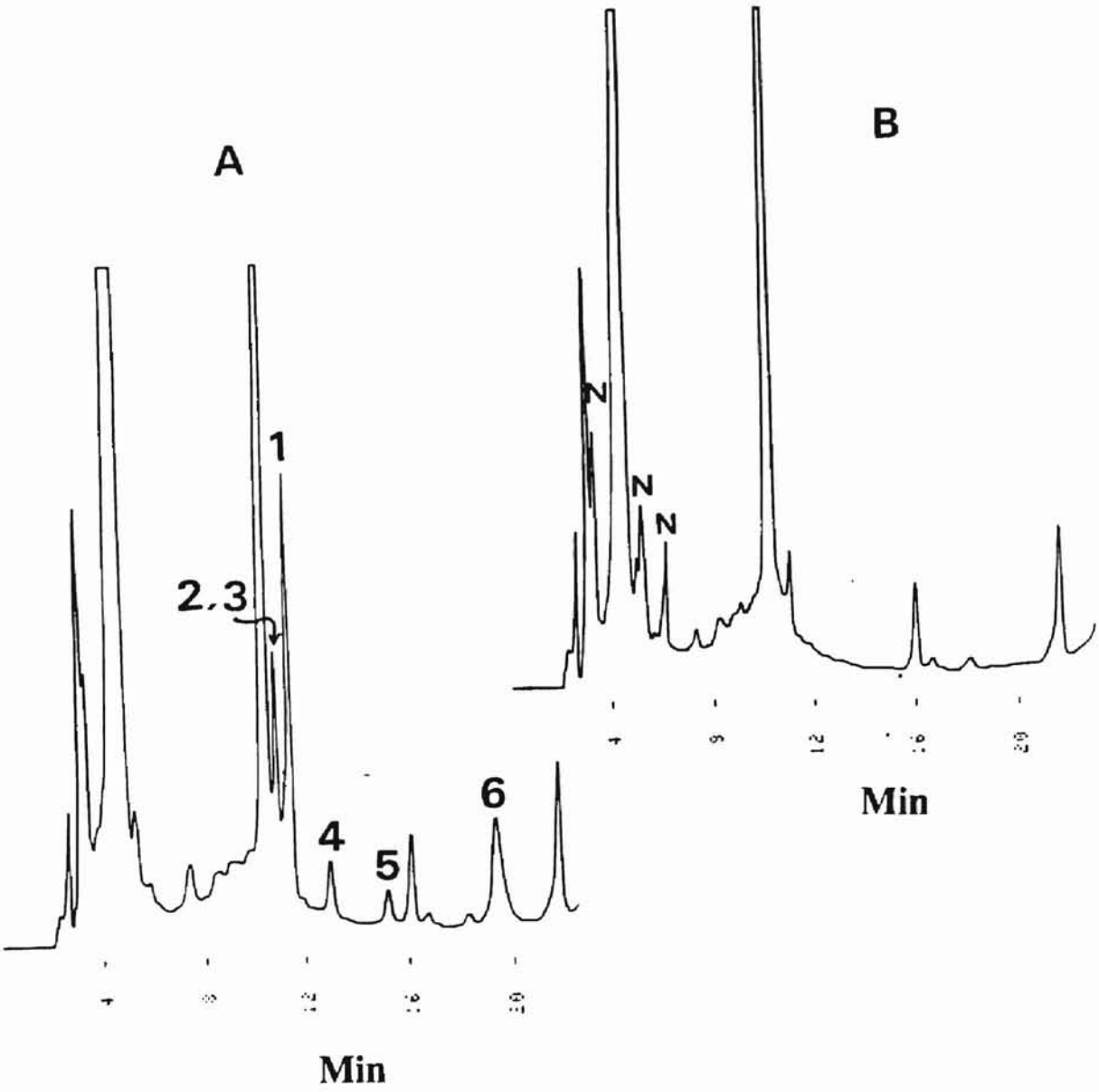


Figure 11. Chromatograms obtained on a SAX column of the intact cabbage extract in (A) and cabbage extract treated with myrosinase in (B). Column, 25 cm x 4.6 mm packed with Zorbax SAX of 5 μm mean particle diameter, 70 Å pore diameter. Flow rate, 1 ml/min. 20 min linear gradient from 0 to 0.5 *M* NaCl in 20 m*M* sodium phosphate, pH 3.2 followed by 1 min isocratic elution with the gradient former. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 3. N, new peaks.

Micropreparative HPLC of Glucosinolates

Stages Involved in Preparative HPLC of Glucosinolates from Plant Extracts. Usually, natural product extracts are complex mixtures of multiple components, a fact that renders the isolation of the components of interest from a plant extract to be rather difficult. A preparative separation method for glucosinolates purification involves various steps (see Fig. 12) where preparative HPLC is only one step in the purification of difficult samples. Solving the preparative separation problem starts with generating information on the origin of the glucosinolate sample (e.g., number of glucosinolates, number of interferences, etc.). First, different extraction and sample clean-up are required (e.g., filtration, centrifugation, solvent extraction, crystallization, etc.) depending on the origin of the glucosinolates whether from seeds, stems, leaves, or roots (22). It is also critical to know the number of individual glucosinolates as well as the total amount of glucosinolates in the extract, so that a preparative separation can be performed efficiently. This can usually be done by running analytical HPLC (see above) and high performance capillary electrophoresis (HPCE) analysis to determine the number of glucosinolates as well as the total amount of glucosinolates (47). Thereafter, the separation is first established on analytical scale to maximize the selectivity factor (α) and optimize resolution. Scaling-up of the analytical separation involves using larger columns and higher flow rates. The collected fractions are checked for purity by several approaches such as HPLC under different conditions (see later), capillary electrochromatography (CEC) or HPCE. Pure fractions are then combined and solvent is evaporated. Impure fractions will be rechromatographed under different conditions using either the same or different stationary phase (see later).

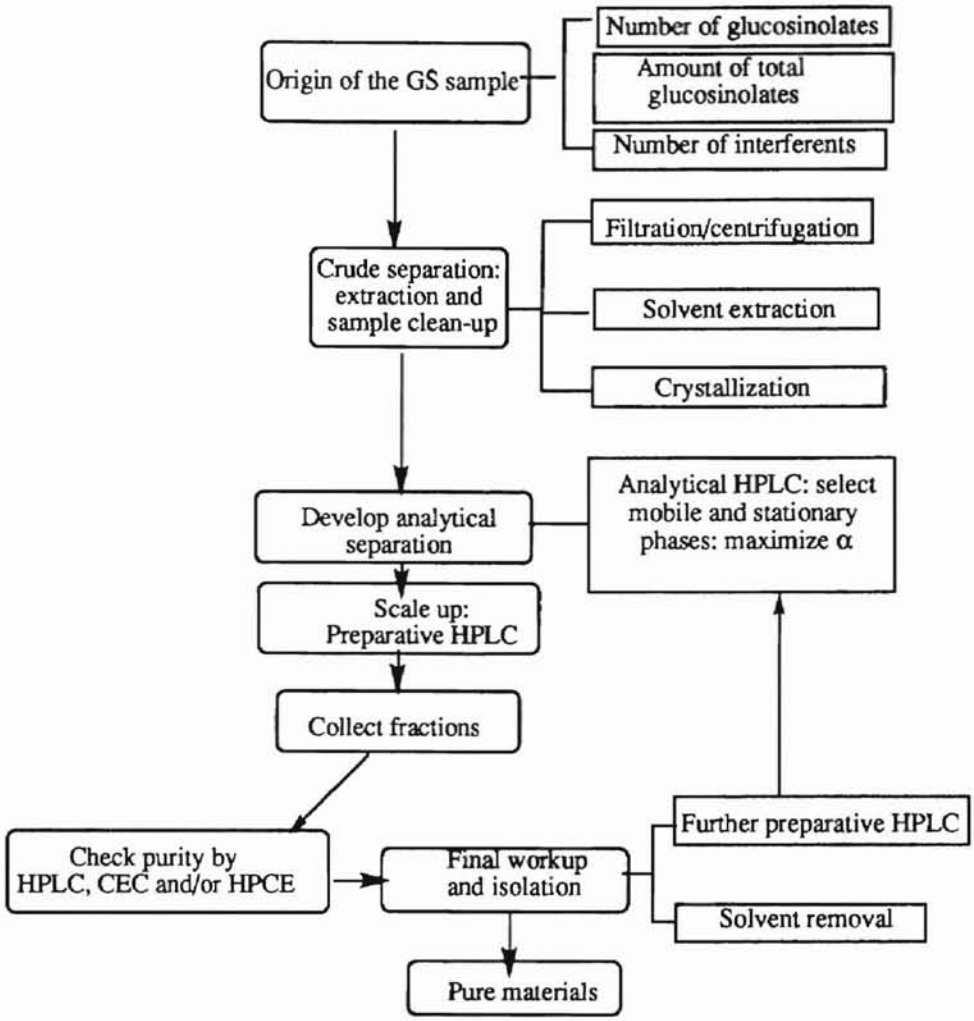


Figure 12. Stages involved in preparative HPLC of glucosinolates in plant extracts.

Reversed Phase Chromatography. Due to instrumental limitations, we were not able to conduct preparative HPLC of glucosinolates. Because we have HPLC instruments which can handle only analytical to micropreparative flow rates, we decided to do micropreparative HPLC of the glucosinolates extracted from white cabbage. The main idea for obtaining pure glucosinolates is to describe an HPLC method that can find general use for preparing a glucosinolate library or libraries from a given plant or plants. Other goals were (i) to evaluate spectroscopic tools (e.g., NMR, MS) in the identification of glucosinolates, (ii) to introduce enzymatic means for expediting the identification of the nature of glucosinolates, and (iii) to study the chromatographic behavior of glucosinolates in various modes of HPLC. Having this knowledge will allow a scale-up for a macropreparative separation, thus facilitating technology transfer.

Because the column used was only a micropreparative column of dimensions 25 cm x 6.2 mm I.D., it was necessary to perform several runs in order to collect sufficient amounts of pure glucosinolates. With this column, the maximum amount that can be injected was about 100 µl of glucosinolates at approximately 10^{-2} *M*. For the analysis by NMR of the HPLC isolated glucosinolates, a relatively high concentration (i.e., 0.3-1.0 mg/0.6 ml D_2O), and high purity samples were required. On the other hand, for the analysis by MS, a high purity of the sample was needed. This demanded some care in the preparation of the isolated glucosinolates to avoid contamination and degradation of the sample.

The procedure used consisted of the following. First, a clean-up of the extract as described in the experimental section was performed, and then a sample was injected onto the RPC column using a 17 min linear gradient elution from 0 to 60% (v/v)

methanol in 50 m*M* sodium phosphate monobasic, pH 3.2. The different peaks which correspond to the glucosinolates were isolated and neutralized to pH 6.0 to 7.0 using buffer of sodium phosphate, pH 8.0. Thereafter, the sample was dried by speed vac and kept in the freezer for further purification. It is important to neutralize the fraction to avoid the hydrolysis of glucosinolates in the drying process. In addition, the drying of the sample was performed in a brown glass to protect the sample from light.

It is important to clean-up first the extract from cabbage to remove some impurities which may reduce the life time of the column and most importantly makes the preparative separation difficult to realize. Fig. 13 shows the different chromatograms before and after the clean-up of the extract from white cabbage and also the extract that have been treated with myrosinase. Comparing the different chromatograms in Fig. 13, reveals the importance of sample clean-up which minimize the size and number of impurities, thus facilitating the isolation of pure glucosinolates from this complex extract.

Further Purification by SAX. After collecting the different peaks from the micropreparative RPC column, it was necessary to perform further purification. Peaks #1 and #3 were passed on a SAX column using a 20 min linear gradient elution from 0 to 0.50 *M* NaCl in 20 m*M* sodium phosphate at pH 6.0, see Fig. 14A and B. With this procedure, the glucosinolates were cleaned from some impurities that remained after the separation by RPC. The collected fractions were concentrated by speed vac. Although use of the SAX column was for analytical purposes, the fact that we isolated only one glucosinolate from one or two impurities has allowed to overload the column and recover the purified glucosinolate in quantitative yields.

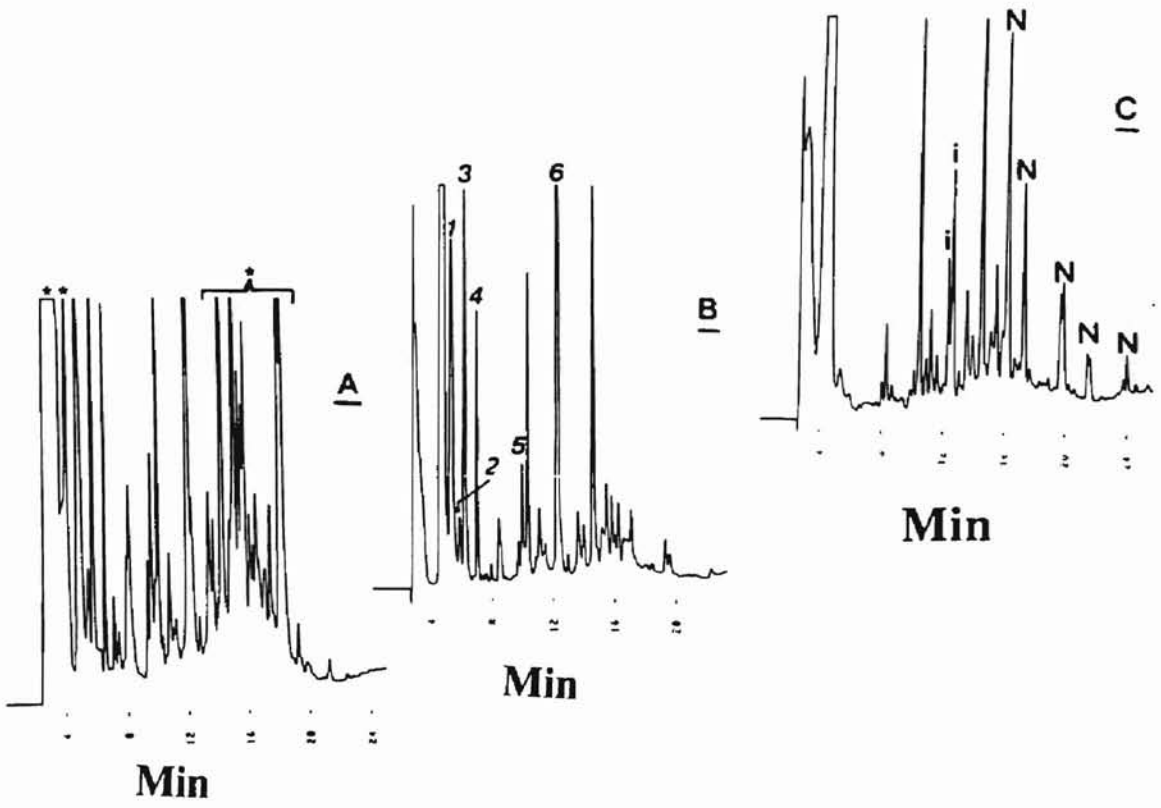


Figure 13. Chromatograms obtained by RPC of the intact extract from white cabbage without clean-up (A), intact extract from white cabbage after the clean-up (B) and cleaned-up extract from white cabbage treated with myrosinase (C). Column, 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter. Flow rate, 1.8 ml/min. Consecutive linear gradients from 0 to 60% (v/v) methanol and from 60 to 80% (v/v) methanol in 17 and 3 min, respectively, in 50 m*M* sodium phosphate monobasic, pH 3.2. This is followed by 3 min isocratic elution at 80% (v/v) methanol in 50 m*M* sodium phosphate, pH 3.2. UV detection, 228 nm; detector sensitivity, 0.5 AUFS; integrator attenuation, 4. *, interferents; I, impurity peaks; N, new peaks.

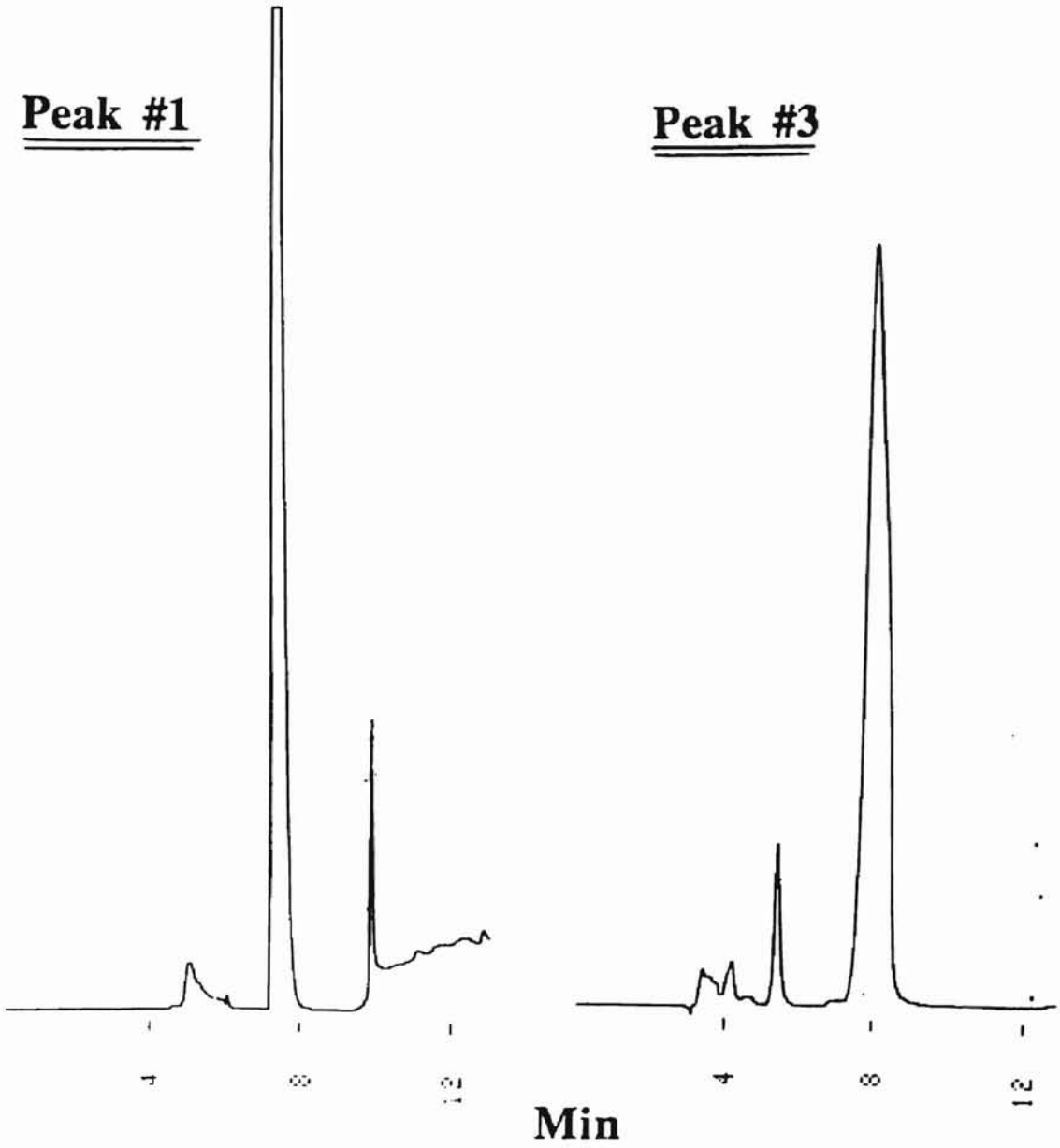


Figure 14. Micropreparative purification of peaks #1 and #3 on the SAX column. Column, 25 cm x 4.6 mm packed with Zorbax SAX of 5 μm mean particle diameter, 70 Å mean pore diameter. Flow rate, 1 ml/min. 15 min linear gradient from 0 to 0.5 *M* NaCl in 20 m*M* sodium phosphate monobasic, pH 6.0, followed by 1 min isocratic elution with the gradient former. UV detection, 228 nm; detector sensitivity, 0.5 AUFS; integrator attenuation, 4.

The last step of the purification of glucosinolates #1 and #3 involved removing the salt that remained in the collected fractions according to the following procedure. This involved passing the sample onto the RPC column using the following scheme. After isocratic elution for 3 min with 10 m*M* sodium phosphate monobasic, pH 6.0, a stepwise elution was performed for 9 min using 5.0 m*M* sodium phosphate monobasic at 80 % (v/v) methanol. This was followed by returning to the equilibrating mobile phase for 5 min before repeating the same cycle again. Under these conditions, in the first 3 min the salt leaves the column, and the glucosinolates are eluted between the retention times of 3.5 and 5.5 min depending on the number of the GS. These final HPLC fractions were dried by speed vac and were ready to analyze by NMR.

To obtain an interpretable NMR spectrum, it was necessary to remove most of the salts before submitting the sample for analysis by NMR. For the MS analysis of the collected peak, it was necessary to remove almost all the phosphate that remained in the isolated HPLC fraction. This was done by dissolving the dry glucosinolates with a small amount of methanol. The glucosinolates are soluble in methanol, and the phosphate is almost insoluble.

Further Purification by RPC. Peaks #4, 5 and 6, collected from the RPC column using the conditions stated in Fig. 8 were further purified by changing the conditions of the mobile phase while keeping the RPC column the same (see Fig. 15). The new conditions consisted of a 17 min linear gradient elution from 0 to 80% (v/v) methanol and from 10 to 5 m*M* sodium phosphate, pH 6. It can be seen that the amount of sodium phosphate in the purified fraction is reduced as the percent of the second mobile phase of

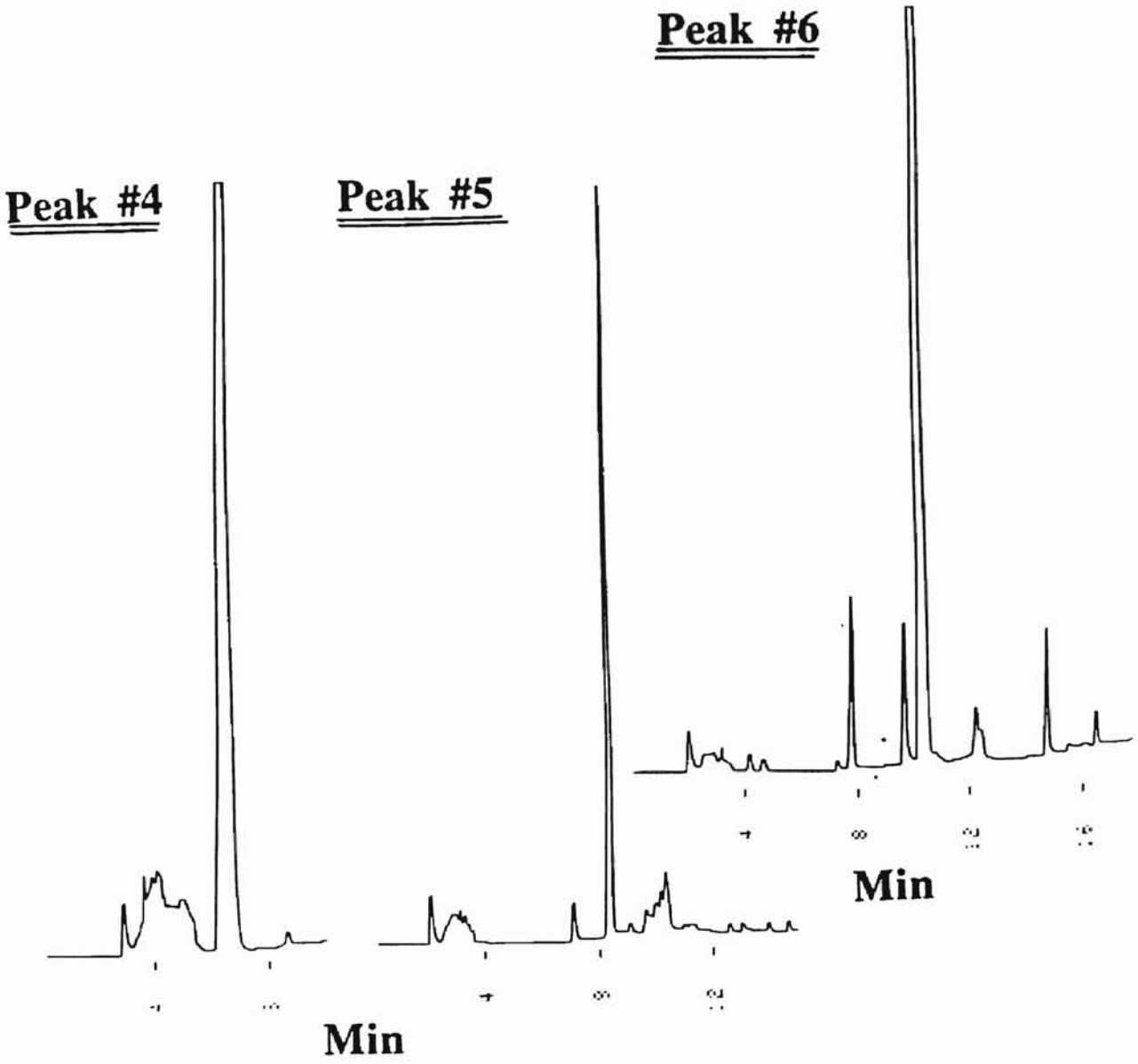


Figure 15. Micropreparative purification of peaks #4, 5 and 6, on the RPC column under different conditions. Column, 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter. Flow rate, 1.8 ml/min. A 17 min linear gradients from 0% methanol in 10 m*M* sodium phosphate monobasic to 80% methanol in 5 m*M* sodium phosphate monobasic, pH 6.0. UV detection, 228 nm; detector sensitivity, 0.5 AUFS; integrator attenuation, 4.

low sodium phosphate content is increased during the gradient. This resulted in low amounts of sodium phosphate monobasic, and therefore no further purification was required for these glucosinolates. Trying to decrease further the amount of sodium phosphate in the second mobile phase led to a decrease in resolution from impurity peaks. Fortunately, these procedures work only for the glucosinolates #4, 5 and 6 because they have longer retention times. For the glucosionolates #1 and 3, it was not possible to use this last procedure because the retention times were short under this condition and did not allow the separation to take place. For these reasons, it was necessary to purify the glucosinolates #1 and 3 by SAX. Although this required one more step than the glucosinolates #4, 5 and 6, glucosinolates #1 and 3 were obtained very pure. Again, and as with the SAX column, since only one glucosinolate was to be further purified from one or two impurities, the column could be overloaded with sample without sacrificing separation efficiency and resolution.

As will be discussed in Chapter III, NMR analysis of peak #5 resulted in a crowded spectrum which was not easy to interpret, indicating the presence of impurities or more than one glucosinolate in peak #5. In a separate experiment, an aliquot of peak #5 was treated with myrosinase and injected on the RPC column, Fig. 16. As can be seen in Fig. 16 the glucosinolate peak disappeared totally indicating that this fraction is free from non-glucosinolate impurities. To determine the cause of crowding in the NMR spectrum, we then investigated other RPC conditions. As can be seen in Fig. 17, fraction #5 from an RPC column yielded at least three peaks (one major and two minor) that were partially resolved.

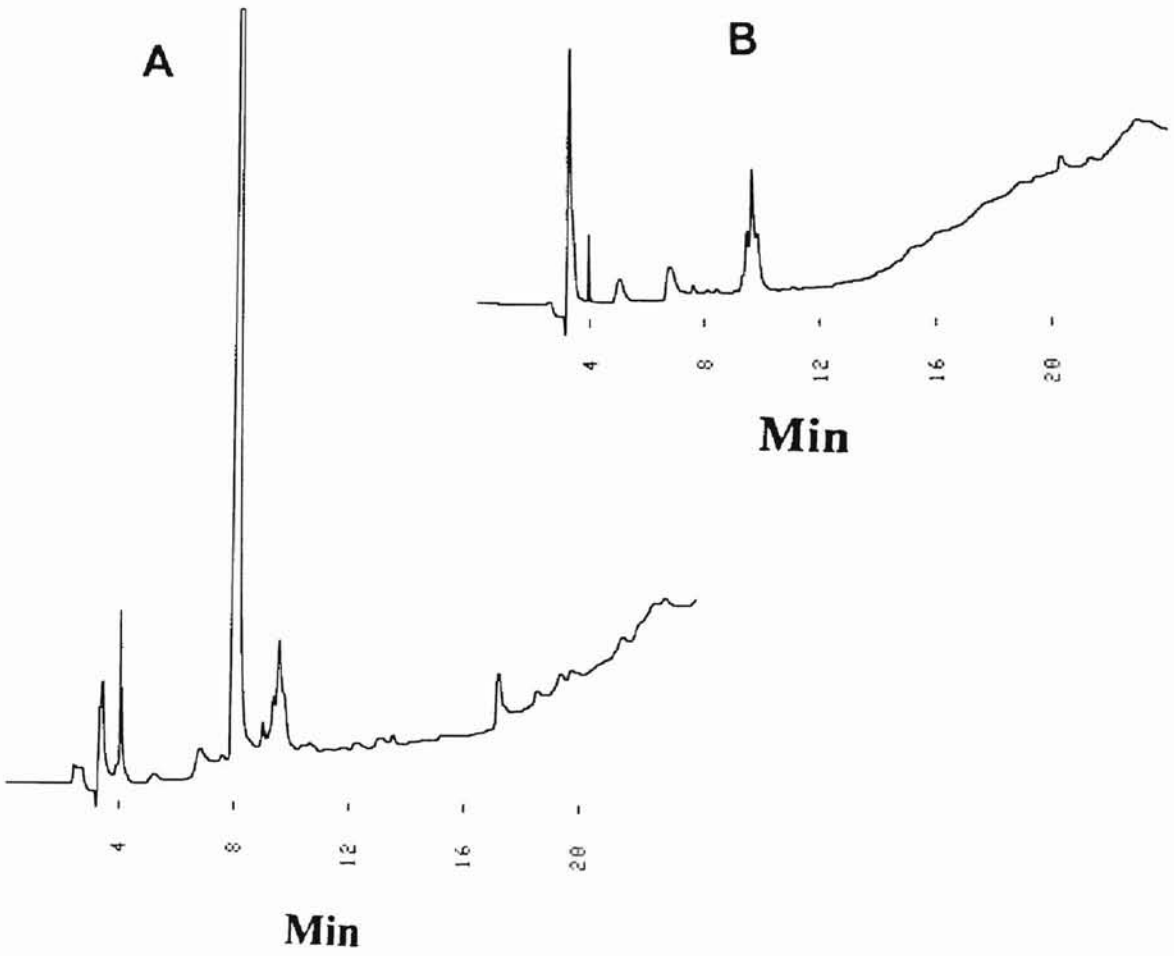


Figure 16. Micropreparative purification of peak, 5 on the RPC column under different conditions. Intact the glucosinolate #5 in (A) and myrosinase treated in (B). Column, 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter. Flow rate, 1.8 ml/min. A 17 min linear gradients from 0% methanol in 10 m*M* sodium phosphate monobasic to 80% methanol in 5 m*M* sodium phosphate monobasic pH 6.0. UV detection, 228 nm; detector sensitivity, 0.5 AUFS; integrator attenuation, 4.

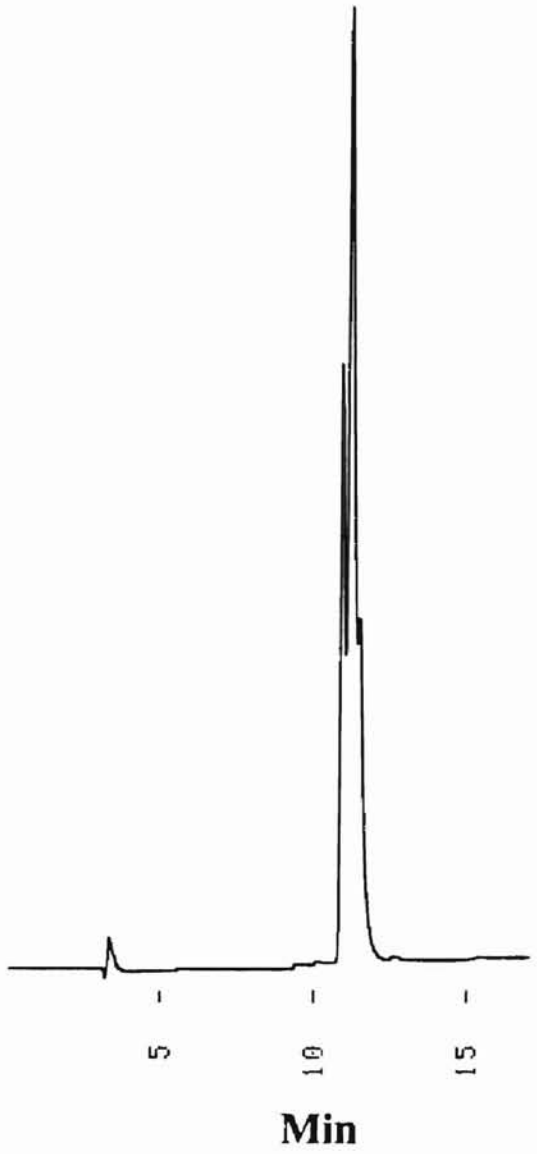


Figure 17. Chromatograms obtained on an RPC column of intact glucosinolate #5. Column, 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter. Flow rate, 1.8 ml/min. Consecutive linear gradients from 0 to 40% (v/v) in 17, in 50 m*M* sodium phosphate monobasic, pH 3.0. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4.

Conclusions

We have shown that the isolation by HPLC of a library of glucosinolate standards from a given plant extract required the combination of two different retention mechanisms, such as ion exchange and reversed-phase chromatography. Hydrophilic glucosinolates, which elute at the beginning of gradient from an RPC column, are better purified by rechromatography on a SAX column, while more hydrophobic glucosinolates that elute later in the gradient elution on an RPC column can be further purified by rechromatographing the collected fractions on the same RPC column using different elution conditions. Analytical HPLC is an important step in the overall microprepatative HPLC in terms of obtaining information on the number of interferences and glucosinolates in the extract as well as in terms of monitoring sample clean-up and in distinguishing glucosinolates peaks from interference peaks. We are expecting that the methodologies established in this study can find general use in the isolation and purification of other libraries of glucosinolates from other kind of plant extracts.

CHAPTER III

IDENTIFICATION OF GLUCOSINOLATES IN CABBAGE EXTRACTS BY MEANS OF SPECTROSCOPIC, ENZYMATIC AND CHEMICAL METHODS

Introduction

Ancillary tools and techniques (e.g., NMR, MS, chemical, and enzymatic hydrolysis) are usually used either alternatively or in concert to provide the exact structure of the isolated components of interest by micropreparative or preparative HPLC. This chapter is to provide a comprehensive methodology for the identification of the glucosinolates separated by HPLC. This methodology includes spectral identification by NMR, MS and GC-MS analyses as well as by chemical and enzymatic approaches. Although it has been used to a limited extent, NMR is a powerful tool for the determination of glucosinolates (25-27, 48, 49). With the development of high field NMR instruments (e.g., 400 and 600 MHz), the structure of glucosinolates can be determined more accurately (26, 27) than with low fields instruments because the ^{1}H NMR spectra of glucosinolates are complex and difficult to resolve at low fields (50, 51). Thus, with high field NMR instruments, a 1D ^{1}H NMR spectrum can be readily obtained for any HPLC fraction, which then can facilitate the screening of the HPLC fractions. For HPLC fractions containing glucosinolates for which no structures have been reported

or the ^{1}H NMR spectrum is not easily interpreted, ^{13}C and 2D NMR experiments will reveal the fine structural features of the glucosinolate of interest.

To further provide structural confirmation, spectral data from MS can be used. Mass spectrometry has been proved to be a valuable technique in the analysis of glucosinolates (13, 27, 40, 52-55). The various ionization methods, e.g., chemical ionization (54, 55) and fast atom bombardment (or LSIMS) (27, 52, 53), that are available in a typical mass spectrometer can be utilized to confirm the identity of glucosinolates. For example: (a) negative ion LSIMS technique can be used to obtain important information on the molecular ion $(M\text{-cation})^{-}$ (53); (b) positive ion ammonia chemical ionization gives information on a class of diagnostically important ions (53) such as $[R\text{-}CN : NH_4]^{+}$. With knowledge of the accurate mass of this ion, it is possible to identify the R group, and consequently the glucosinolate under investigation. Since we had the opportunity to do NMR measurements, negative ion LSIMS provided the molecular mass needed to confirm the structure of the isolated glucosinolate. As will be discussed later in this chapter, GC-MS was very suitable for assessing the structures of glucosinolates in the HPLC fraction when this fraction contained more than one glucosinolate component, e.g., peak #5 (Refer to Chapter II). As discussed in chapter I, GC-MS is a valuable technique for determining the structure of the R group (30, 31) via the isothiocyanate degradation products resulting from myrosinase digestion of glucosinolates.

To further characterize and confirm the observations obtained in NMR and MS analyses, enzymatic means were examined. The enzyme myrosinase, which is known to degrade glucosinolates to isothiocyanates or nitriles depending on the pH (14-16), is a

useful tool when combined with HPLC measurements. This approach, which is referred to as the enzymatic peak shift, allows with the use of standards of the isothiocyanates or nitriles to identify the R group, and consequently the glucosinolate under investigation.

Experimental

Chemicals and Materials

Benzylcyanide (99%), hydrocinnamonitrile (99%), (4-methoxyphenyl)acetonitrile (97%), allylcyanide (98%), 4-hydroxybenzylcyanide (98%), 4-methoxyphenylisothiocyanate (98%), benzylisothiocyanate (98%), phenethylisothiocyanate (99%), allylisothiocyanate (95%), vinylacetic acid (97%), 4-hydroxyphenylacetic acid (98%), 4-methoxyphenylacetic acid (98%), 3-methoxyphenylacetic acid (99%), hydrocinnamic acid (99%), *m*-hydroxyphenylacetic acid (99%), propylbenzene (98%), butylbenzene (99%), ethylbenzene (99%) and deuterium oxide were purchased from Aldrich (Milwaukee, NJ, USA). Indole 3-acetic acid was obtanined from Sigma (St. Louis, MO, USA). Phenylacetic acid was from Eastman organic (Rochester, NY, USA). L-Sulforaphane (96%) was from LKT Labs, Inc. (St. Paul, MI, USA). Methylene chloride was from Fisher Scientific (Fair Lawn, NJ, USA).

HPLC Instrument and Columns

The HPLC instrument and columns used are the same as in Chapter II.

NMR Instrument and Procedures

The dry HPLC fraction was dissolved in 0.6 ml deuterium oxide (99.9% D), and the solution was introduced into a 5 mm NMR tube. All spectra were recorded on a Varian Unity INOVA 600 spectrometer with a 5 mm Nalorac pfg, triple-resonance probe operating at an ^{1}H frequency of 598.75 MHz. Also, Double Quantum Filter Correlation SpectroscopY (DQFCOSY) was carried out on one of the glucosinolate containing the HPLC fraction to confirm the structure of the glucosinolate. The observed ^{1}H chemical shifts are reported with respect to the D_2O signal, which is at 4.706 ppm.

MS Instrument and Technique

The instrument used for the mass spectrometric analysis of glucosinolate samples was a VG analytical Model ZAB-2SE magnetic sector, double focusing mass spectrometer. Negative ion liquid secondary ion mass spectrometry (LSIMS) analyses was used for glucosinolates. The energy of the bombarding cesium ions was set at 20 keV. Glycerol was used as supporting matrix for the sample. Samples were dissolved in methanol before mixing with glycerol matrix.

GC-MS Instrument and Technique

The GC-MS instrument used was a GCD System G 1800A from Hewlett Packard (Palo Alto, CA USA), equipped with a column HP-5 crosslinked 5% PH ME silicone of dimension 30 m x 0.25 mm I.D. x 1.0 μm film thickness. The inlet temperature was set at 250 °C and that of the detector at 280 °C. A linear gradient of temperature from 60 °C to 280 °C in 21 min, following by keeping this temperature for 4 min. Helium was used

as carrier gas at a flow rate, 1 ml/min. The solvent used to dissolve the sample was methylene chloride.

Acid Hydrolysis Procedure

The acid hydrolysis of glucosinolates was done via a slightly different way than previously reported (29). The cabbage extract (0.5g) was dissolved in 2 ml of 3 *M* HCl. Then, it was heated at 60 °C for 1 hour.

Results and Discussion

Spectral Identification by NMR and MS

The structural determinations of the glucosinolates isolated by HPLC were first performed using a Varian INOVA 600 MHz instrument. With the advent of high fields NMR instruments more accurate determination of the structures of the glucosinolates can be obtained (26, 27) on relatively small amounts of samples in a reasonable time period.

To further confirm the structure of glucosinolates, the HPLC fractions were submitted for MS analysis. A negative ion liquid secondary ion mass spectrometry (LSIMS) mode was used because this ionization mode gives the molecular weight of the glucosinolate anions $(M - cation)^-$. In other words, $(M - cation)^-$ is the most abundant anion (27, 52, 53) in the negative ion mode. Also, the sensitivity in the negative ion spectra was about two orders of magnitude higher than in the positive ion mass spectra. In the positive ion LSIMS, the glucosinolate undergoes several fragmentation giving rise to a complex spectrum (53). Although in the positive LSIMS, each glucosinolate will

give a particular fingerprint, this mode may become useful once a negative LSIMS was performed.

The glucosinolate that was readily confirmed was the # 3 HPLC fraction which corresponds to the trivial name sinigrin (Fig. 18). This was facilitated by the fact that we have a standard for sinigrin, and both the standard and the sample yielded the same NMR spectrum (see Fig. 19). With only few exceptions (see chapter 1), in all glucosinolates, the β-D-glucose have some characteristic peaks, including the β-1 with a chemical shift of 4.96 ppm and a J = 10.09 Hz (doublet), the proton in the positions 2 and 3 which have a chemical shift of 3.36 ppm (multiplet), the protons in position 4 and 5, which have a chemical shift of 3.45 ppm (multiplet), and the protons in position 6 (a, b) which have two peaks doublet of doublet (dd) at 3.6 and 3.8 ppm because they are next to the chiral center (they are not equivalent). These peaks are in agreement with previously reported NMR analysis (26, 49). For sinigrin, the protons in position 8 have a multiplet at 3.45 ppm. The proton in the position 9 (=CH-) has a multiplet at 5.94 ppm. The protons in the position 10 (CH_2=) have two doublets at 5.2 ppm due to the two protons that are not equivalent. By making use of the negative ion LSIMS it was possible to confirm the molecular weight of the glucosinolate (sinigrin) which was $(M–cation)^-$ = 358 g/mol (see Fig. 20).

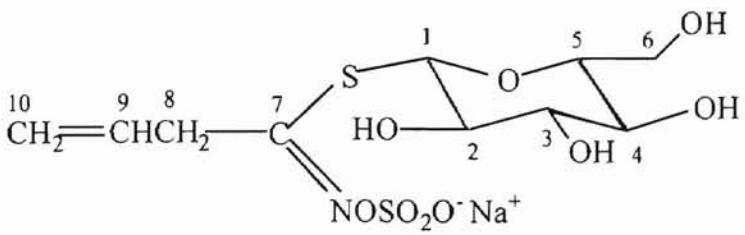


Figure 18. Structure of the glucosinolate #3, which is sinigrin.

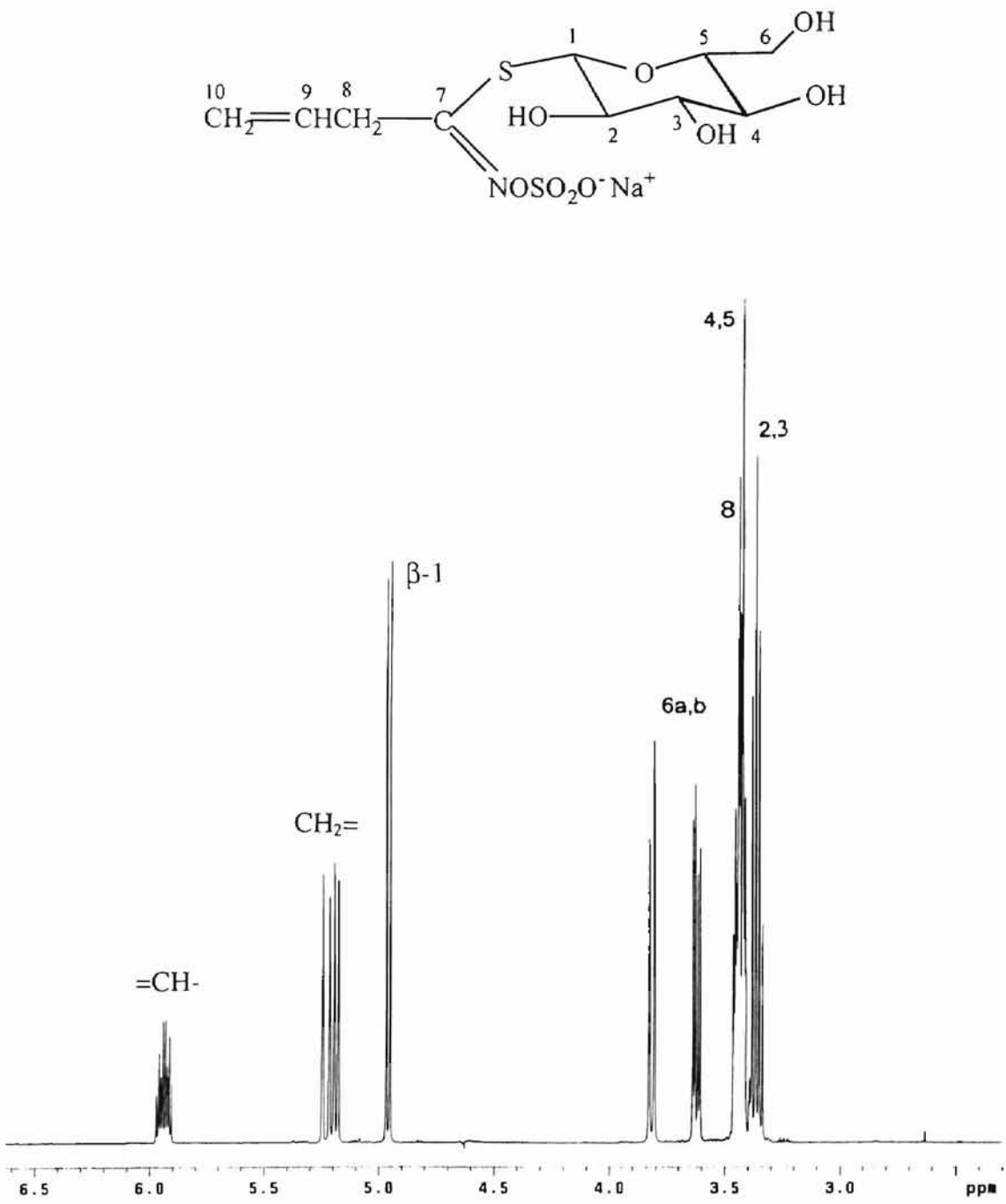


Figure 19. 600 MHz ^{1}H NMR spectrum of HPLC fraction # 3.

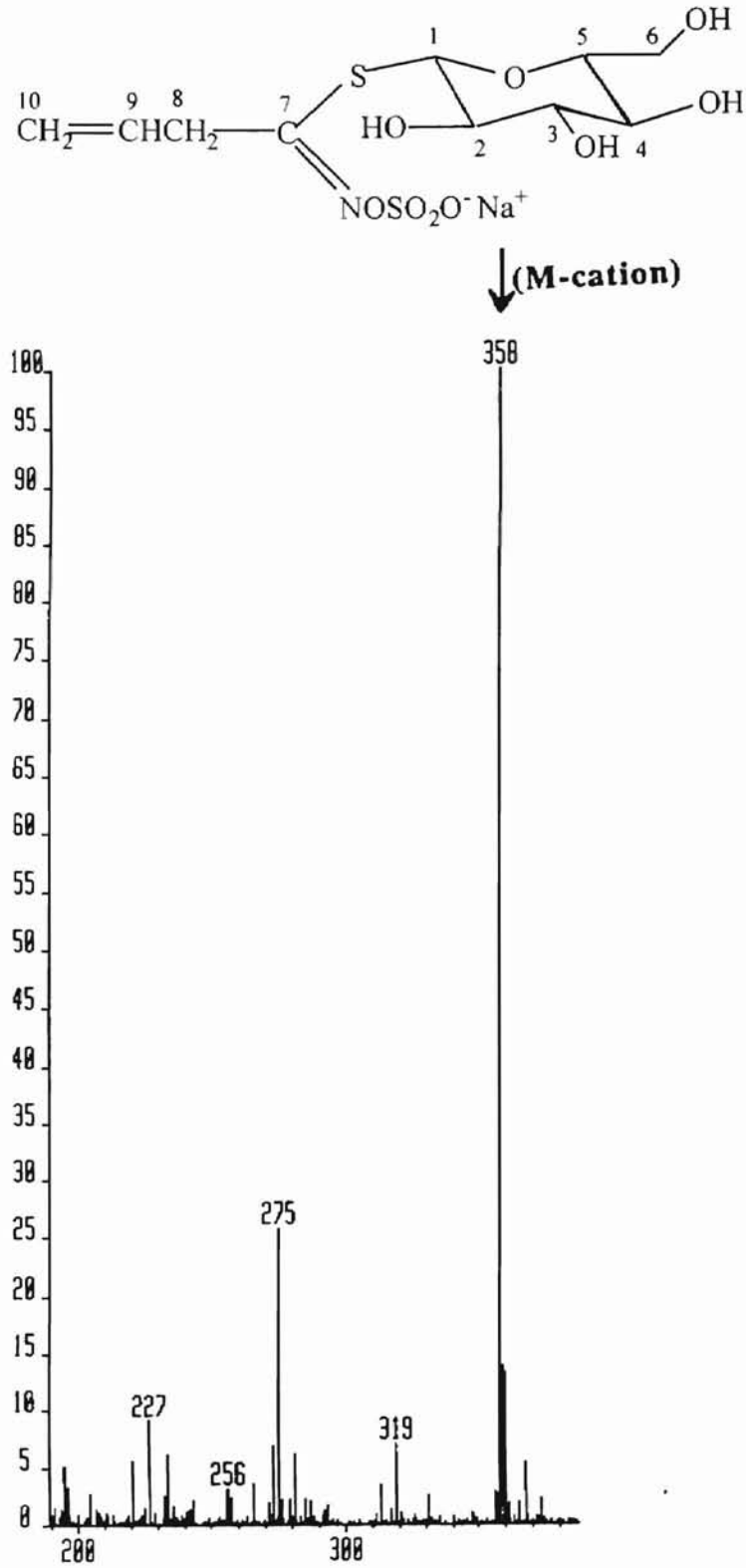


Figure 20. Negative ion liquid secondary ion mass spectrometry of HPLC fraction # 3. Cs^+ ion gun (20 keV) was used to ionize the sample deposited on a probe tip.

As for sinigrin (i.e., HPLC fraction #3), the spectrum of the glucosinolate in the HPLC fraction # 1 has as above all the peaks characteristics of β-D-glucose. The R group was identified to be $CH_3SOCH_2CH_2CH_2$- (see Fig. 21 and 22). The protons in the position 8 have a multiplet of a chemical shift of 2.85 ppm, while the protons in the position 9 have a multiplet of a chemical shift of 2.1 ppm. The protons in the position 10 have a multiplet of a chemical shift of 2.95 ppm. The protons in position 11 have a singlet at 2.61 ppm.

From 2D-DQFCOSY (see Fig. 23), it is possible to see that the peaks at 2.1 ppm (multiplet) correlated to signals at 2.85 and 2.95 ppm which confirms that the CH_2 is in position 9, and also the $-CH_2CH_2CH_2$- grouping. The singlet at 2.61 ppm does not have correlation with other protons. This glucosinolate corresponds to the semisystematic name of 3-methylsulfinylpropyl glucosinolate and the trivial name of glucoiberin (see Fig. 21). By making use of the negative ion LSIMS it was possible to confirm the molecular weight of the glucosinolate which was $(M\text{-cation})^- = 422$ g/mol (see Fig. 24).

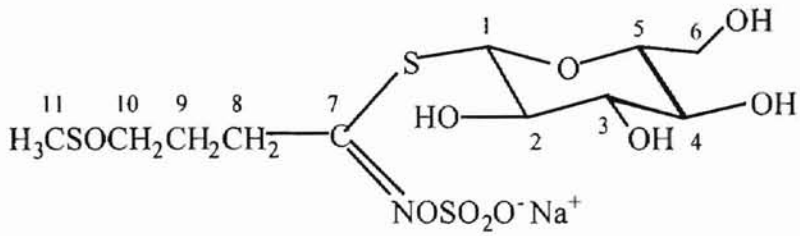


Figure 21. Structure of the glucosinolate #1, which is glucoiberin.

The glucosinolate in the HPLC fraction # 4 has the same peaks for the β-D-glucose and the R group is $CH_3SOCH_2CH_2CH_2CH_2$- (see Fig. 25). The protons in the position 12 (CH_3SO-) have a singlet at 2.6 ppm while the protons in the position 8 have a triplet at 2.7 ppm. The protons in the position 9 and 10 have a multiplet at 0.75 ppm. The protons in the position 11 have a multiplet at 2.85 ppm. The structure of this

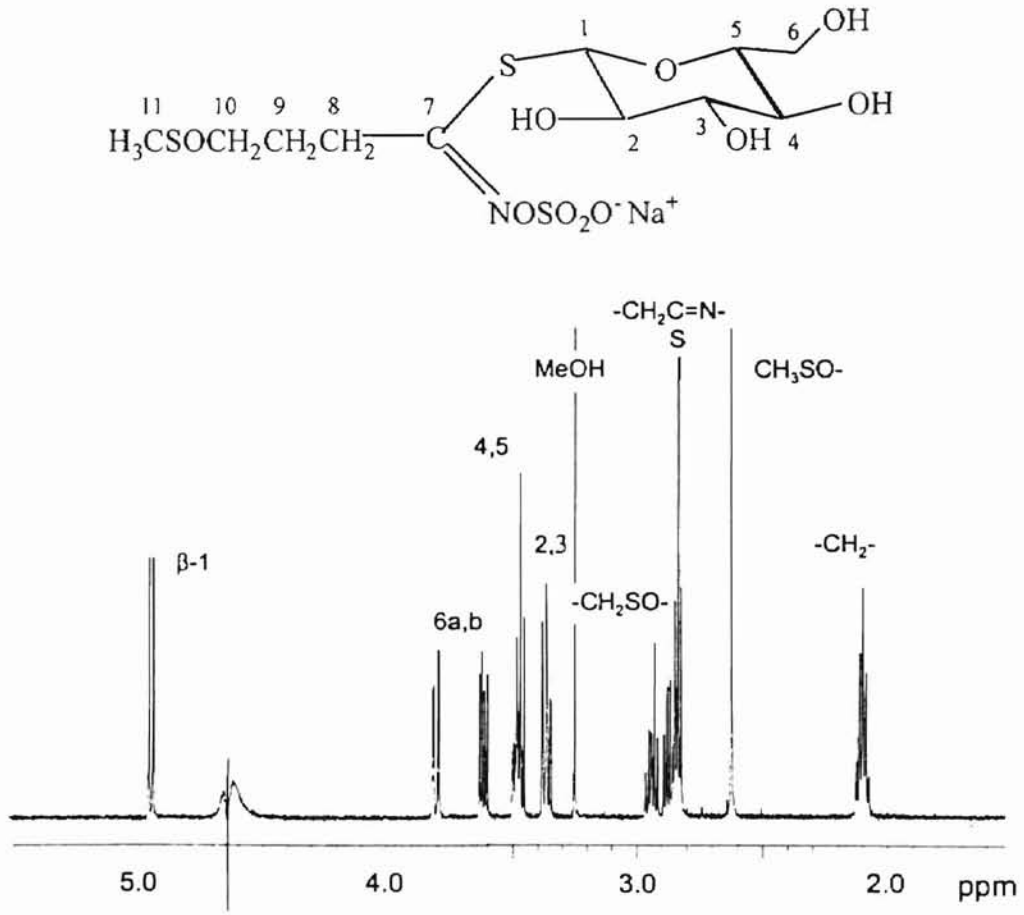


Figure 22. 600 MHz ^{1}H NMR spectrum of HPLC fraction #1.

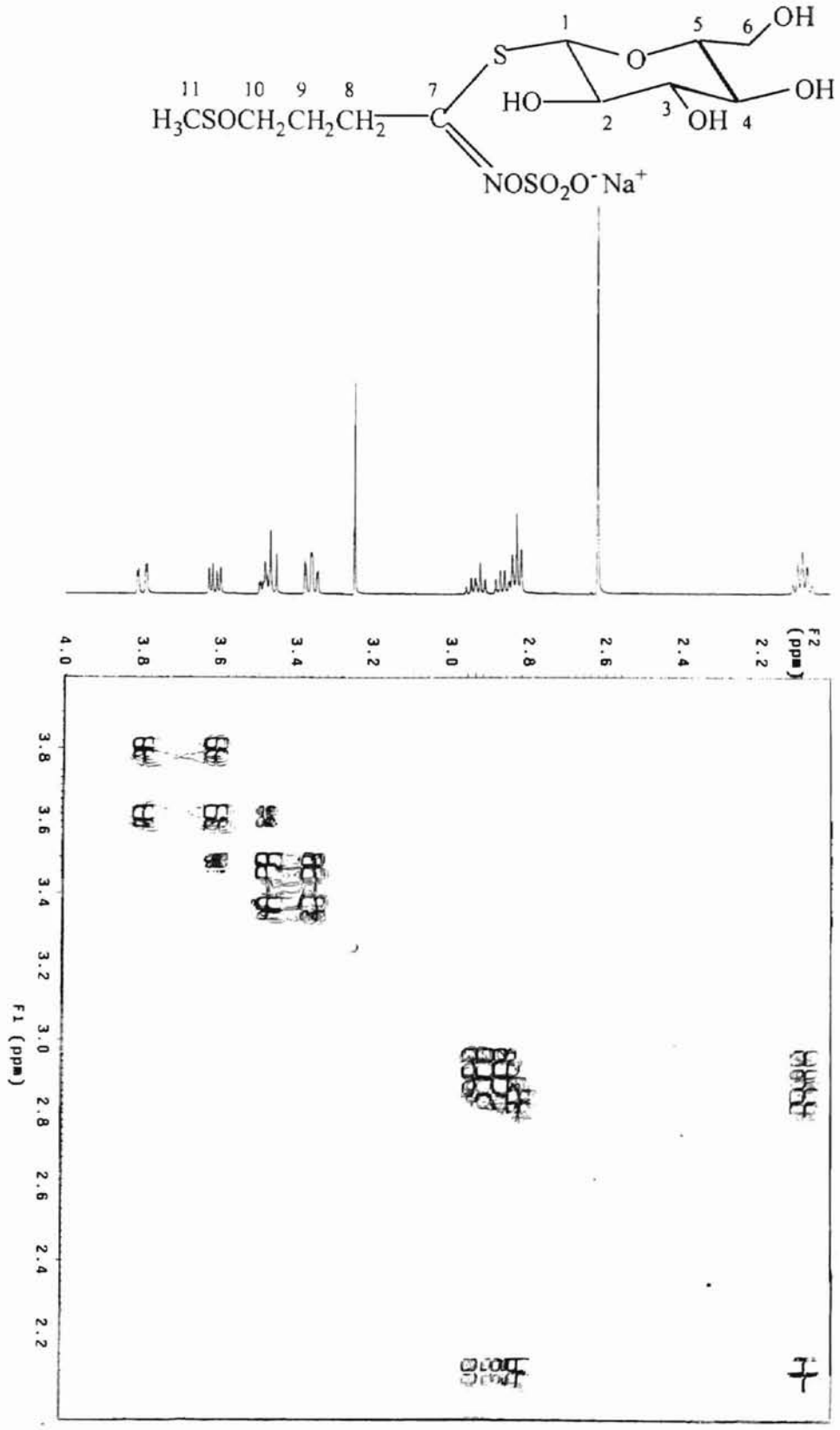


Figure 23. 2D-DQFCOSY spectrum of HPLC fraction #1.

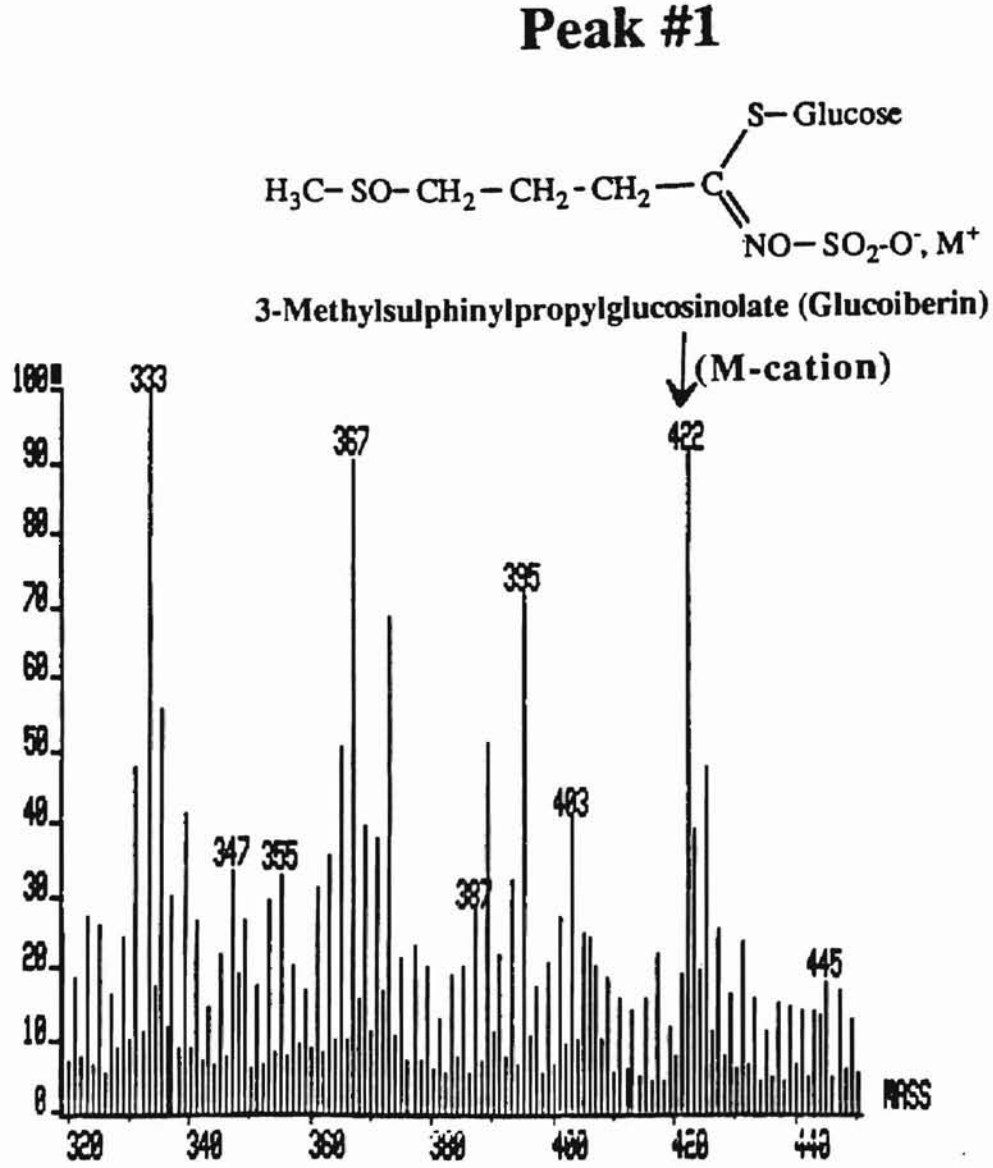


Figure 24. Negative ion liquid secondary ion mass spectrometry of glucosinolate peak #1. Cs^+ ion gun (20 keV) was used to ionize the sample deposited on a probe tip.

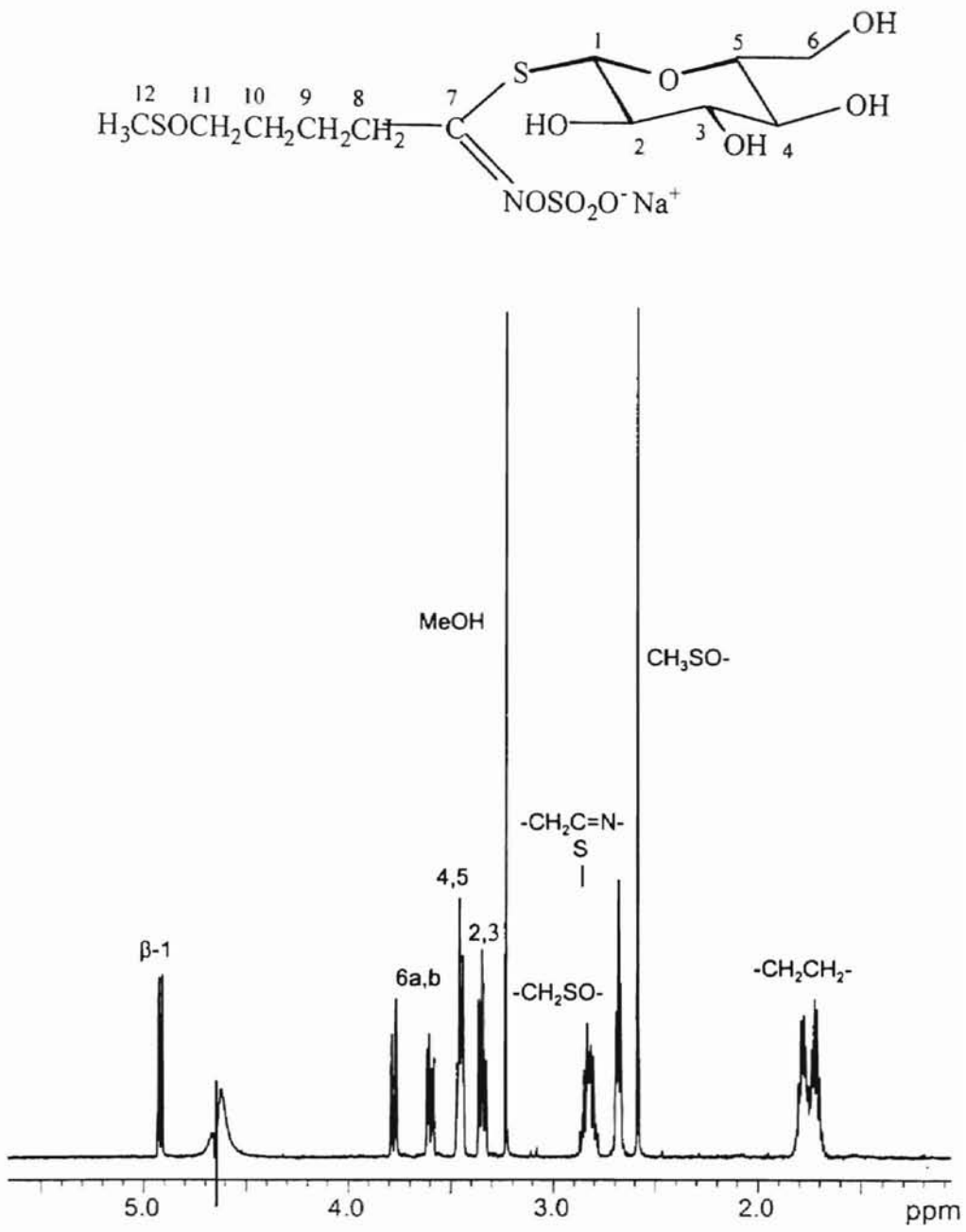


Figure 25. 600 MHz ^{1}H NMR spectrum of HPLC fraction #4.

glucosinolate corresponds to the semisystematic name of 4-methylsulfinylbutyl glucosinoltate and the trivial name of glucoraphanin (see Fig. 26). As it can be seen, the difference in structure between the glucosinolates # 1 and 4 is the presence of one additional CH_2 in the glucosinolate # 4. The spectra are similar with the difference in the peaks at 0.75 ppm. In addition, the data correlate well with those reported for this. By making use of the negative ion LSIMS it was possible to confirm the molecular weight of the glucosinolate which was $(M\text{-cation})^- = 436$ g/mol, see Fig. 27.

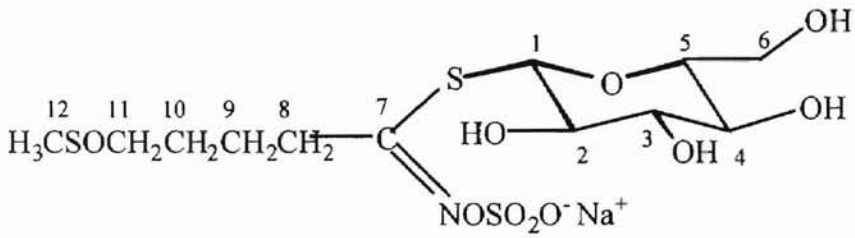


Figure 26. Structure of glucosinolate #4, which is glucoraphanin.

For the glucosinolate in the HPLC fraction # 6, the R group is an indol (see Fig. 28). The proton β-1 was at 4.85 ppm (doublet). The protons in the position 6 (a, b) occurred at 3.45 ppm. The proton in the position 4 was at 3.25 ppm (triplet). The proton in the position 2 has a 3.18 ppm (triplet). The proton in the position 3 occurred at 3.10 ppm (triplet). The proton in the position 5 was at 2.83 ppm (multiplet). For the R group, the protons in the position 8 ($-CH_2-$) have two doublet of 4.05 and 4.10 ppm. The proton in position 2' appeared at 7.25 ppm (singlet). The proton in the position 6' has a 7.45 ppm (doublet). The proton in the position 7' has a 7.2 ppm (triplet). The proton in the position 8' has a 7.1 ppm (triplet). The proton in the position 9' has a 7.65 ppm (doublet). The structure of glucosinolate in the HPLC fraction # 6 was found to be the one that corresponds to the semisystematic name indol-3-ylmethylglucosinolate and the trivial

name of glucobrassicin (see Fig. 29). In addition, the data agree with those reported for this structure in reference (27). By making use of negative ion LSIMS, it was possible to confirm the molecular weight of the glucosinolate which was (M-cation)$^-$ = 447 g/mol, see Fig. 30.

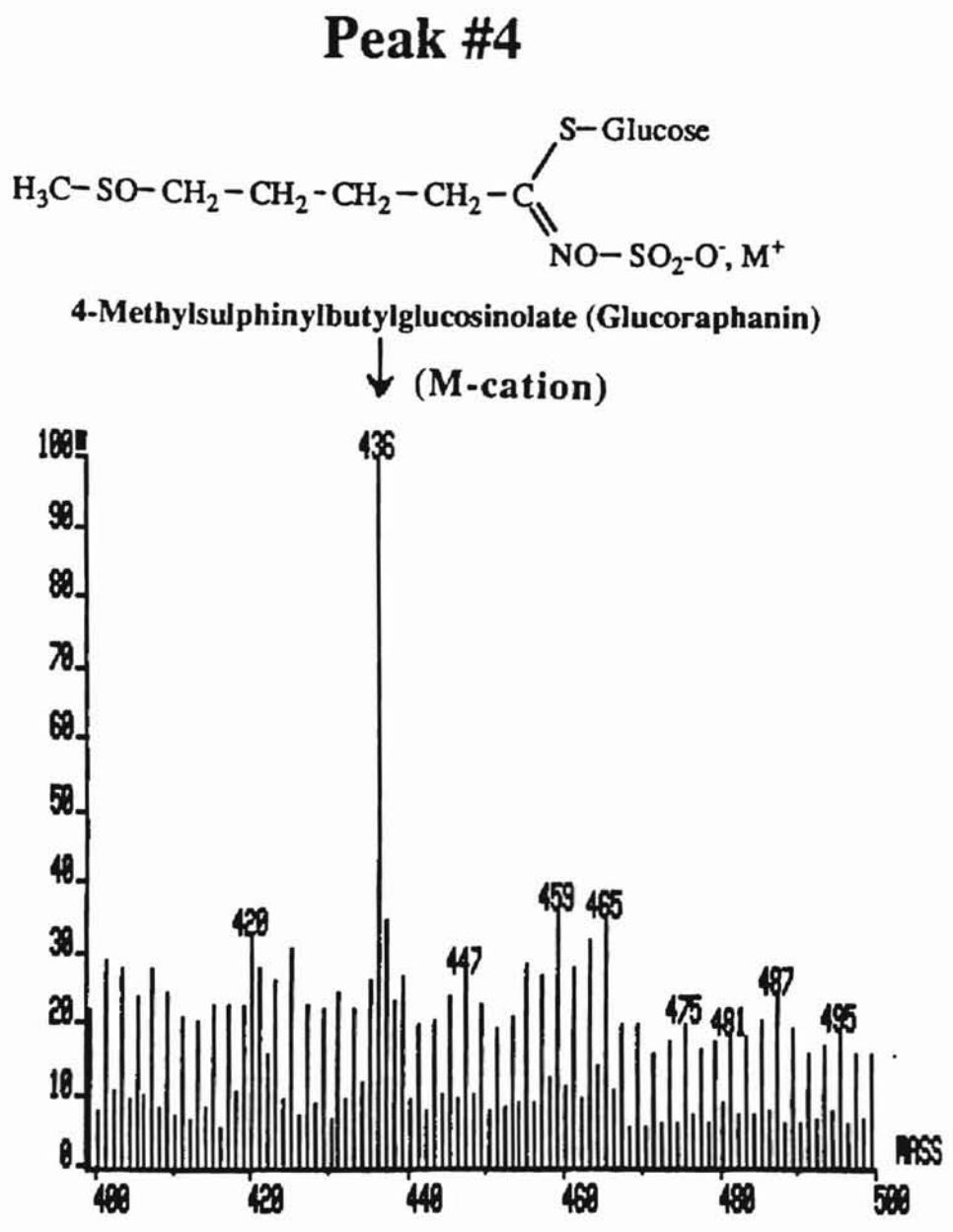


Figure 27. Negative ion liquid secondary ion mass spectrometry of glucosinolate peak #4. Cs^+ ion gun (20 keV) was used to ionize the sample deposited on a probe tip.

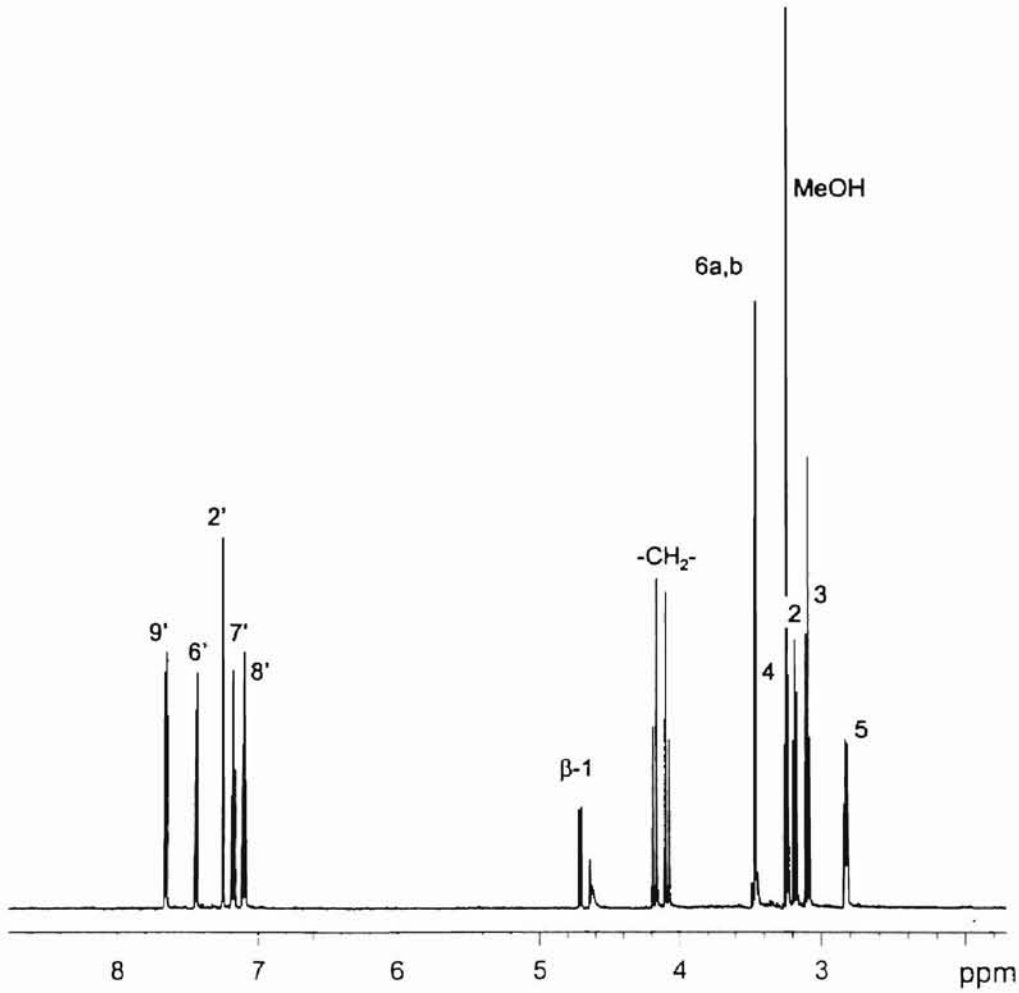


Figure 28. 600 MHz ^{1}H NMR spectrum of HPLC fraction #6. For structure see Fig. 29.

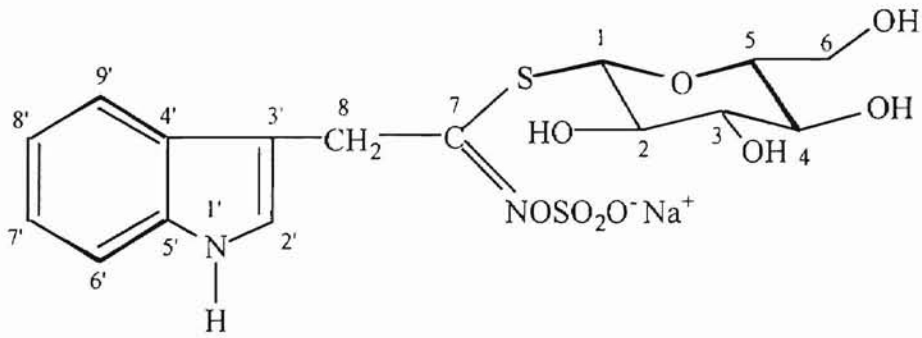


Figure 29. Structure of the glucosinolate #6, which is glucobrassicin

Peak #6

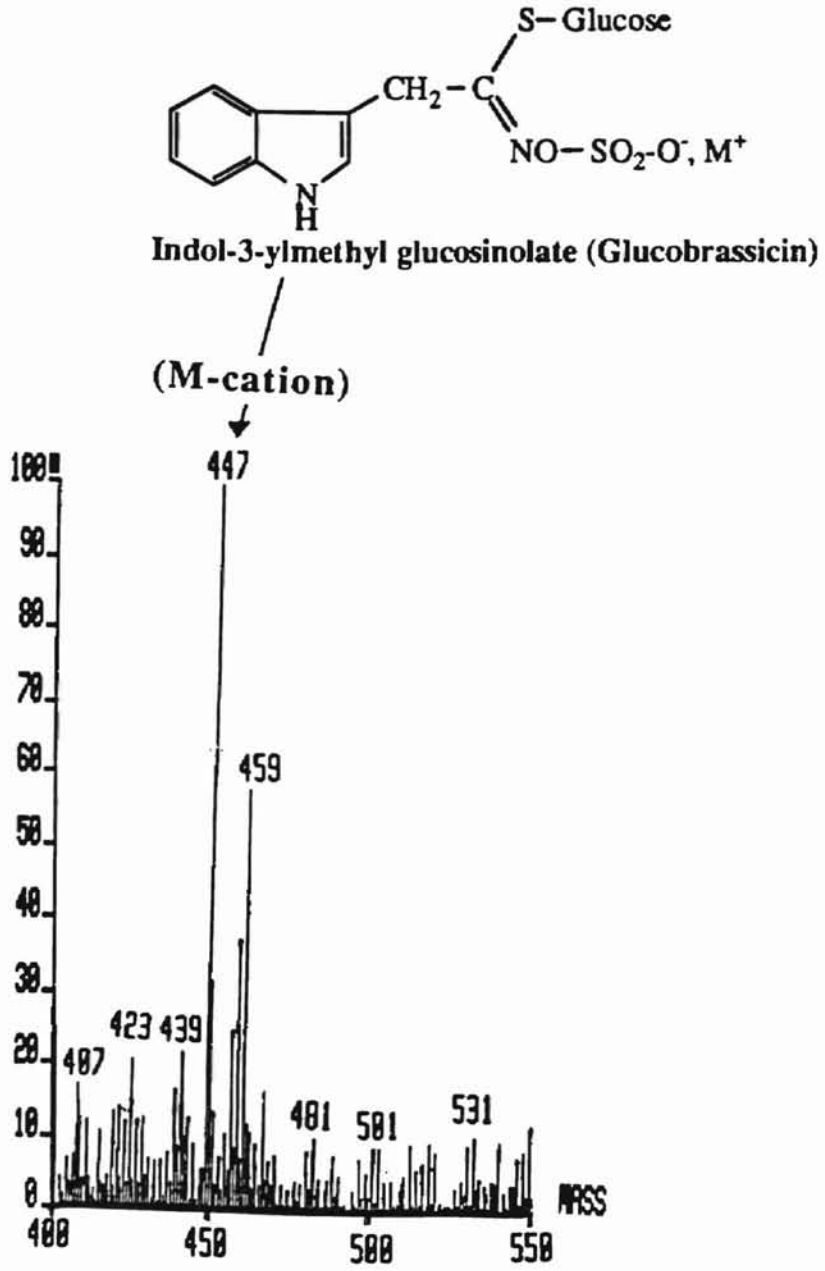
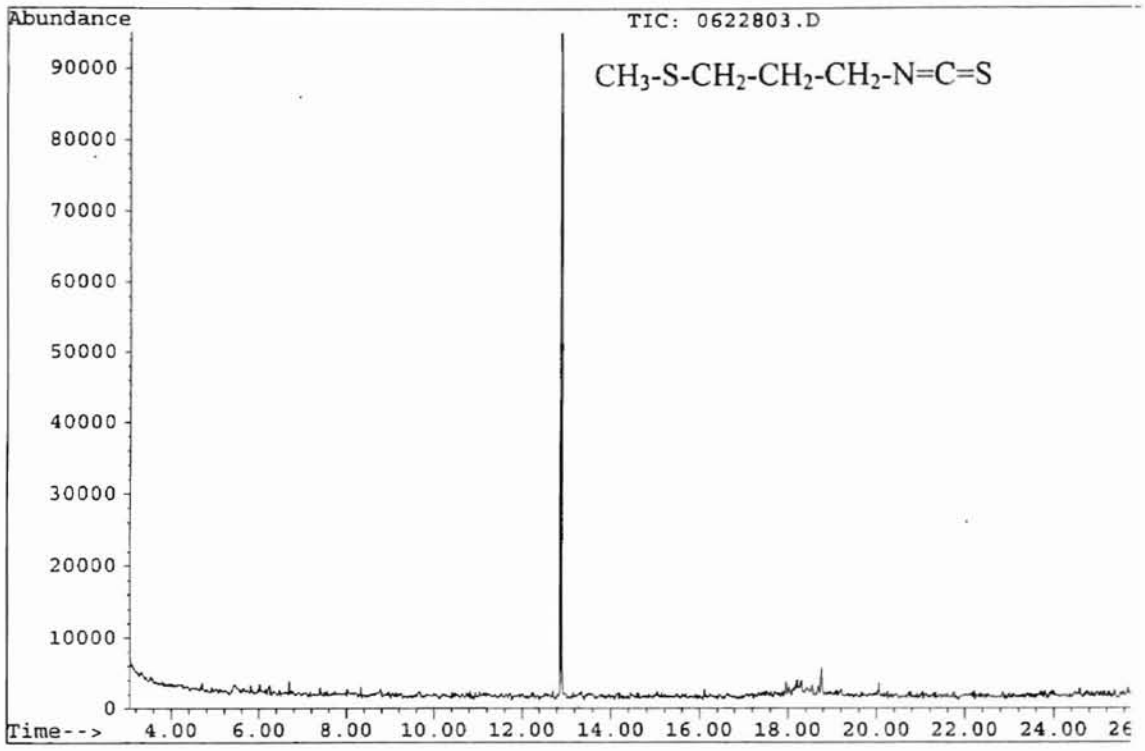


Figure 30. Negative ion liquid secondary ion mass spectrometry of glucosinolate peak #6. Cs^+ ion gun (20 keV) was used to ionize the sample deposited on a probe tip.

As mentioned earlier (see Chapter II), peak #5 required a different scheme for identification which involved the use of GC-MS. As can be seen in Fig. 16 and 17, fraction #5 does not contain non-glucosinolates impurities but does contain more than one glucosinolate (see Fig. 17). These glucosinolates seem to have R groups with no strong chromophoric centers, because it was not possible to detect the degradation products after myrosinase digestion (see Fig. 16) by HPLC with a UV detection at 210 nm. As seen in Fig. 6 (Chapter II), peak #5 is minor with respect to other glucosinolates in the sample. In addition, RPC does not yield high resolution separation of the components in fraction #5 (see Fig. 17) to guarantee collection of a pure fraction under the conditions used. Under these circumstances, peak #5 was collected without any sample interferences (see Fig. 15) and digested with myrosinase at pH 7.0 to yield degradation products that are reflective of the parent glucosinolates. These degradation products were first extracted with CH_2Cl_2 and then analyzed by GC-MS. Fig.31 depict a typical GC chromatogram showing one peak at 13.63 min. This peak was identified as 3-methylthiopropylisothiocyanate having the structure: CH_3-S-CH_2-CH_2-CH_2-N=C=S. The corresponding glucosinolate is 3-methylthiopropyl-GS with the trivial name of Glucoibervirin. The mass spectrum showed the peak at 147 which is the MW of the 3-methylthiopropylisothiocyanate. In addition, we have the peak at 101 which is the fragment produced of propane-1-isothiocyanate. The fragment at 72 is due to –CH_2-N=C=S. The fragment at 61 is due to CH_3-S-CH_2-. It was not possible to have the structure of the other two components of the fraction #5 because they did not show peaks in GC.



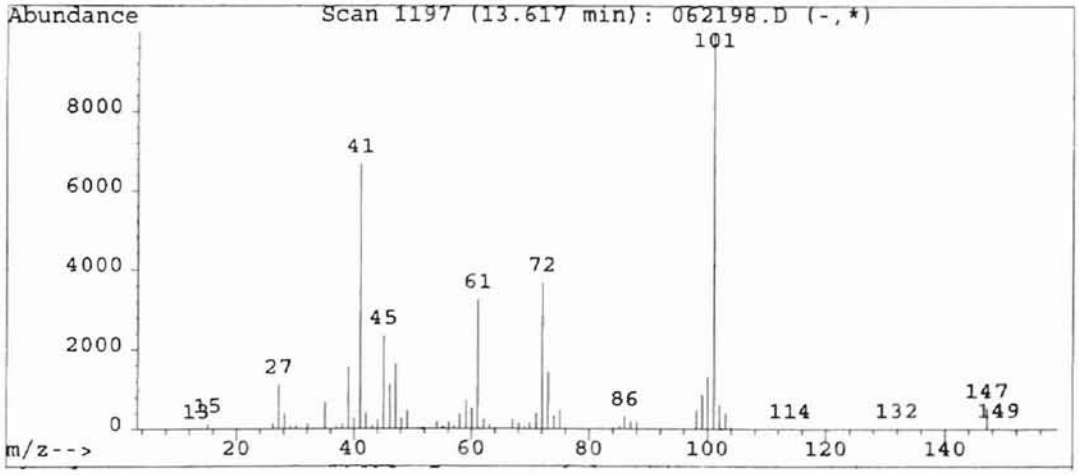


Figure 31. MS spectrum of peak #5. GC-Ms, column HP-5 crosslinked 5% PH ME silicone of dimension 30 m x 0.25 mm I.D. x 1.0 μm film thickness. Inlet temp. 250 °C, detector temp. 280 °C. Linear gradient of temperature from 60 °C to 280 °C in 21 min followed by keeping this temperature for 4 min. Flow rate, 1 ml/min.

Finally, peak #2 (RPC) was a very minor glucosinolate component, and therefore no effort was made to identify it within the frame work of this dissertation which involved micropreparative HPLC that is not very suitable for generating enough material of a minor component to perform spectral identification.

Identification of Glucosinolates Via Their Aglycone Group (R Group) Resulting from Chemical or Enzymatic Degradation of Glucosinolates

Glucosinolates are known to breakdown into fragments that are reflective of the parent glucosionlates via acid hydrolysis, enzymatic degradation, etc. (29, 56), see reactions in Chapter I. In this study, we investigated the degradation in the presence of acid (Fig. 5), and myrosinase (Fig. 2).

Reversed Phase Chromatography of the Carboxylic Acid Degradation Products of Glucosinolates Via Acid Hydrolysis. Initially, we studied the conditions under which we could separate some standards of carboxylic acids that were the acid degradation products of some representative glucosinolates. The idea was to identify the glucosinolates via their acid hydrolysis products. The standards used in the study was carried out were vinyl acetic acid, 4-pentenoic acid, phenylacetic acid, hydrocinamic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-methoxyphenylacetic acid, 4-methoxyphenylacetic acid and 3-indolacetic acid. To find the best conditions for the separation of these standards, the pH of the mobile phase was varied in the range 3-7. Using a column of dimensions 10 cm x 4.6 mm I.D. packed with Microsorb-MV C18

spherical silica of 5 μm mean particle diameter and a 16 min linear gradient elution from 0 to 80% (v/v) methanol in 10 m*M* sodium phosphate, pH 3.0, 6.0, and 7.0 or sodium acetate at pH 4.0 and 5.0, it was found that the best pH for the separation was between pH 6 and 7 (see Fig. 32). In Fig. 32, it can be seen that between pH 6 and 7 the separation is better as the difference in adjusted retention volume is larger. By using a longer column of 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, using a pH 6.5, and keeping the gradient profile the same, base line resolution of all of the studied acids was achieved (see Fig. 33).

When we tried the acid hydrolysis on the white cabbage extract, the extract underwent a non specific transformation, including both the glucosinolates and the matrix interferences. This led to a fingerprint that was not easy to interpret (see Fig. 34). When the standard sinigrin was treated with acid during 20 min of reaction, two products where obtained. One of them corresponds to vinylacetic acid, and the other peak is not known (see Fig. 35). Due to the nonspecificity of the acid hydrolysis and the generation of several products, we decided not to continue with this method.

Reversed Phase Chromatography of Isothiocyanates. By making use of a column of dimensions 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter and under linear gradient elution from 16% to 90% methanol in 17 min (see Fig. 36), it was possible to separate the following standards: allyl isothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate and p-methoxyphenol isothiocyanate. Under these conditions, a white cabbage extract which was previously

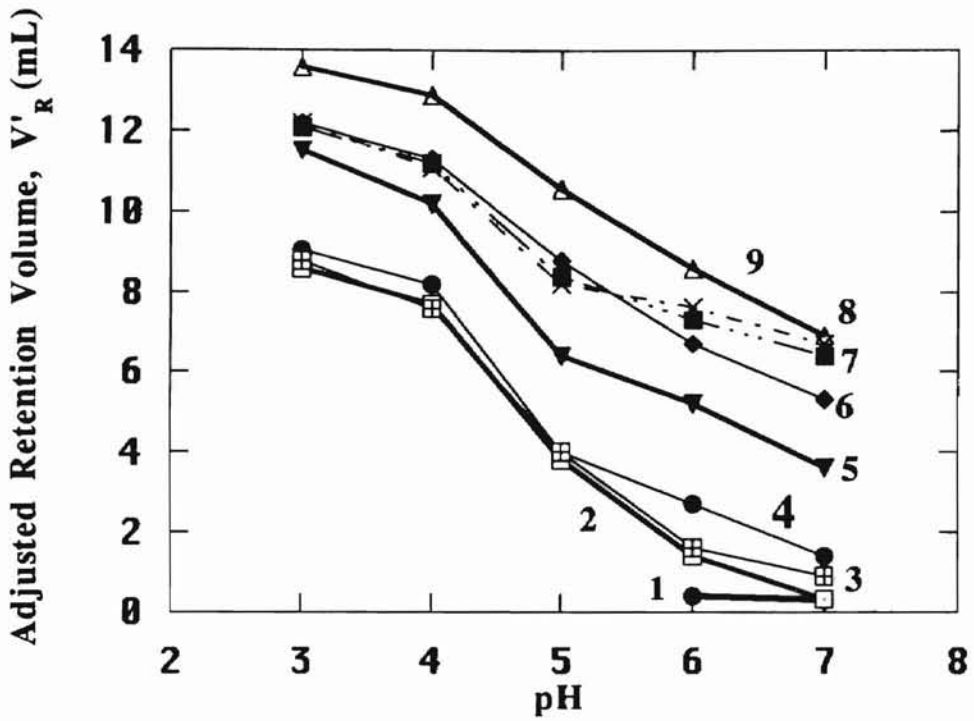


Figure 32. Plots of adjusted retention volume, V'_R (mL), of the standard acid hydrolysis products of glucosinolates versus pH of the mobile phase using an RPC column. Column, 10 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å mean pore diameter. Flow rate, 1 ml/min. A 16 min linear gradient elution from 0 to 80% (v/v) methanol in 10 m*M* sodium phosphate, pH 3.0, 6.0, and 7.0 or sodium acetate at pH 4.0 and 5.0. UV detection, 210 nm; detector sensitivity, 0.2 AUFS; recorder imput, 10 mV. Solutes: 1, vinylacetic acid; 2, 4- petenoic acid; 3, 4-hydroxyphenylacetic acid; 4, 3-hydroxyphenylacetic acid; 5, phenylacetic acid; 6, 3-indolacetic acid; 7, 4-methoxyphenylacetic acid; 8, 3-methoxyphenylacetic acid; 9, hydrocinamic acid.

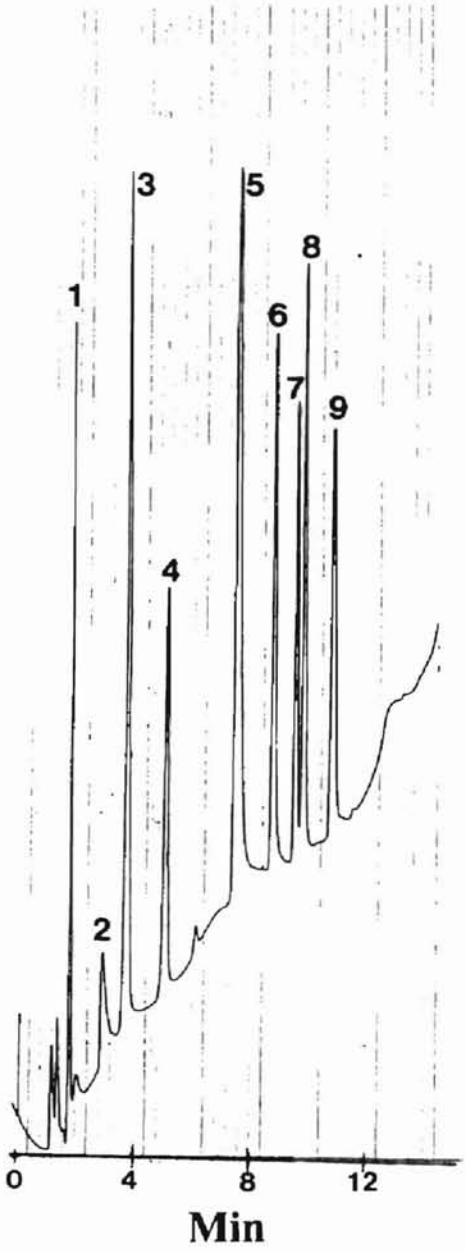


Figure 33. Chromatogram of the standards acid hydrolysis products of glucosinolates. Column, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å mean pore diameter. Flow rate, 1 ml/min. A 16 min linear gradient elution from 0 to 80% methanol in 10 m*M* sodium phosphate, pH 6.5. UV detection, 210 nm; detector sensitivity, 0.1 AUFS; recorder imput, 10 mV. Solutes: 1, vinylacetic acid; 2, 4-petenoic acid; 3, 4-hydroxyphenylacetic acid; 4, 3-hydroxyphenylacetic acid; 5, phenylacetic acid; 6, 3-indolacetic acid; 7, 4-methoxyphenylacetic acid; 8, 3-methoxyphenylacetic acid; 9, hydrocinnamic acid.

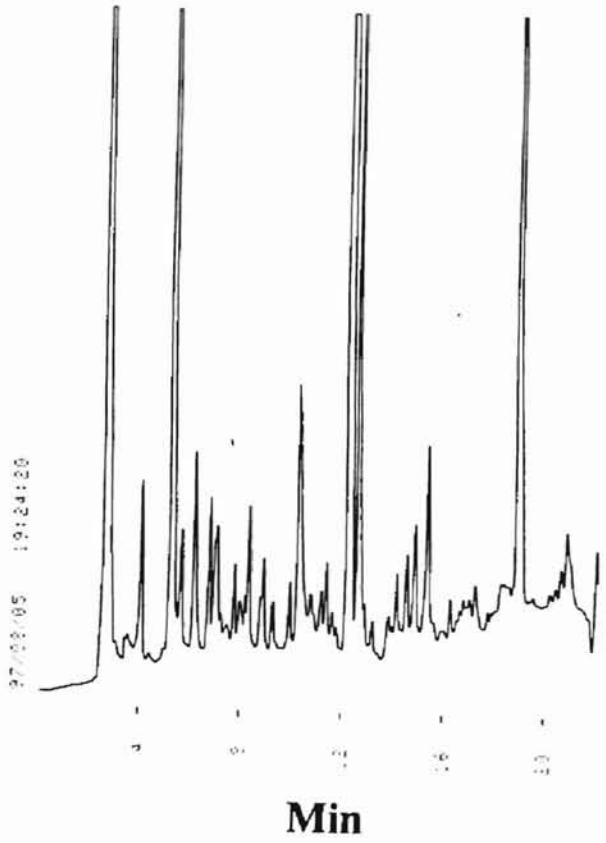


Figure 34. Chromatogram of the white cabbage extract after acid hydrolysis. Column, 25 cm x 6.2 mm I.D. packed with Zorbax ODS spherical silica of 5 μm mean particle diameter, 60 Å mean pore diameter. Flow rate, 1.8 ml/min. Consecutive linear gradients from 0 to 60% (v/v) methanol and from 60 to 80% (v/v) methanol in 17 and 3 min, respectively, in 50 m*M* sodium phosphate monobasic, pH 3.2. This was followed by 3 min isocratic elution at 80% (v/v) methanol in 50 m*M* sodium phosphate monobasic, pH 3.2. UV detection, 228 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4.

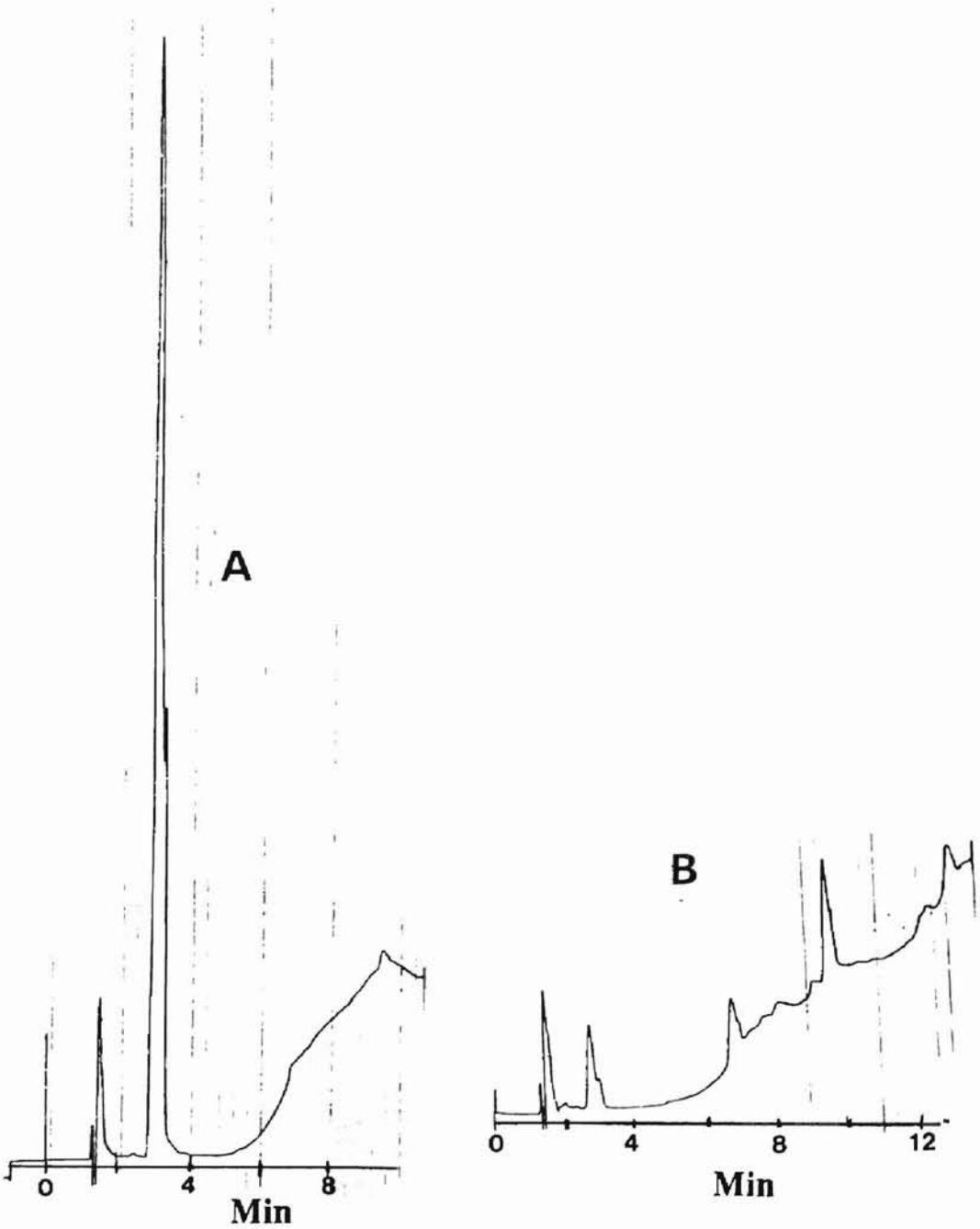


Figure 35. Chromatogram of the standard sinigrin before (A) and after 20 min of acid hydrolysis (B). Column, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å mean pore diameter. Flow rate, 1 ml/min. A 16 min linear gradient elution from 0 to 80% methanol in 10 m*M* sodium phosphate monobasic, pH 3.0. UV detection, 210 nm; detector sensitivity, 0.1 AUFS; recorder imput, 10 mV.

treated with myrosinase was injected on the HPLC column (see Fig. 37).

Having the standards allyl isothiocyanate and L-sulforaphane which are the products of the enzymatic hydrolysis at pH 7.0 of the sinigrin glucosinolate (HPLC fraction #3) and glucoraphanin glucosinolate (HPLC fraction # 4) permitted the identification of the content of fraction #3 and #4 by the enzymatic peak shift approach. The glucosinolate #3 (sinigrin) and the glucosinolate #4 (glucorophanin) were injected before and after treatment with myrosinase using a 17 min linear gradient from 0 to 80% (v/v) methanol in 50 mM sodium phosphate pH 3.2 (see Figs 38 and 39). The retention times of the products of the enzymatic hydrolysis coincided with those of the standard isothiocyanates, thus confirming that the glucosinolates #3 and #4 are sinigrin and glucoraphanin. The retention time of sinigrin was 3.9 min, and after the treatment with myrosinase a new peak appeared at a retention time of 19 min which is the same retention time at which the standard allyl isothiocyanate eluted from the RPC column (see Fig. 38). For the glucoraphanin the retention time was 5.2 min, and after the treatment with myrosinase the degradation product eluted at 15 min which is the same retention time as that of the standard L-sulforaphane (see Fig. 39).

Reversed Phase Chromatography of Nitriles. By making use of an RPC column of dimensions 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and under linear gradient elution from 16% to 90% (v/v) methanol in 17 min, it was found that nitriles have shorter retention times than the isothiocyanates. The standard nitriles were separated together with the standard isothiocyanates and they were allyl cyanide, benzyl cyanide, hydrocinnamonitrile,

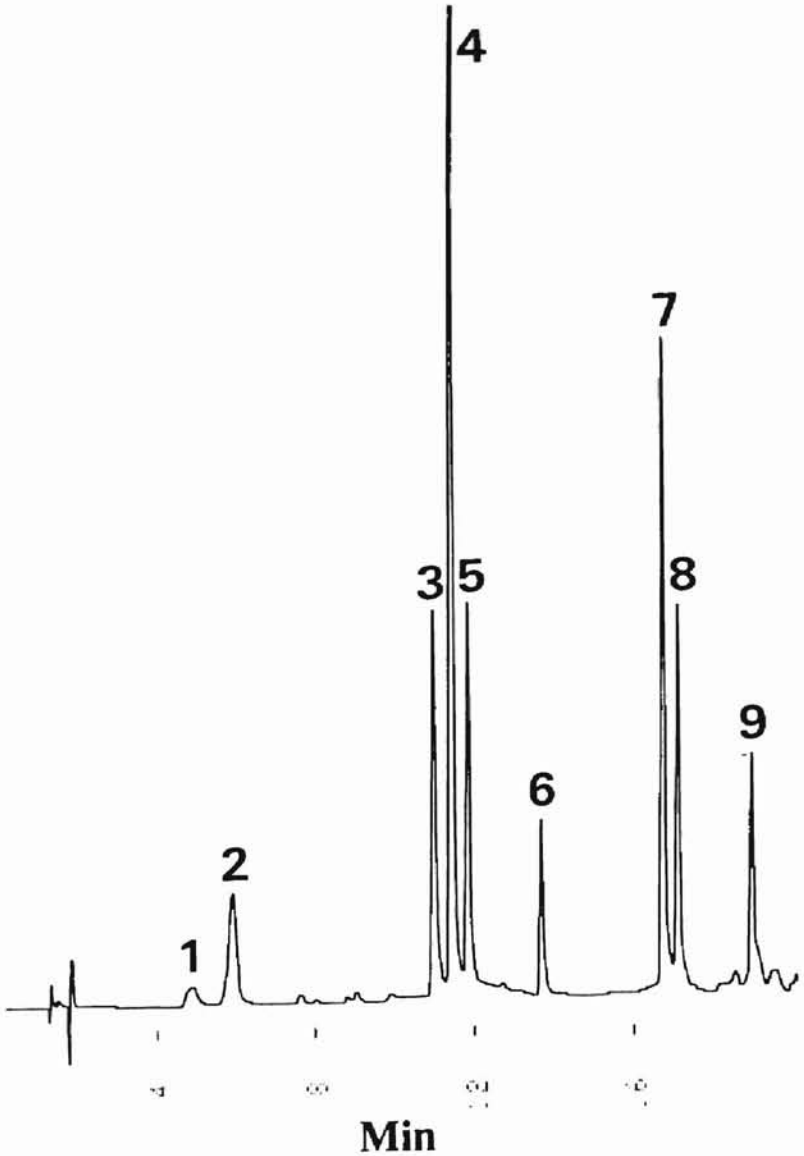


Figure 36. Chromatogram of the standards nitriles and isothiocyanates degradation products of some glucosinolates obtained on an RPC column. Column, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å mean pore diameter. Flow rate, 1 ml/min. A 17 min linear gradient elution from 16% to 90% (v/v) methanol. UV detection, 210 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4. Solutes: 1, allyl cyanide; 2, p-hydroxybenzyl cyanide; 3, benzyl cyanide; 4, p-methoxylphenylacetonitrile; 5, hydrocinnamonitrile; 6, allyl isothiocyanate; 7, benzyl isothiocyanate; 8, phenethyl isothiocyante; 9, p-methoxyphenol isothiocyanate.

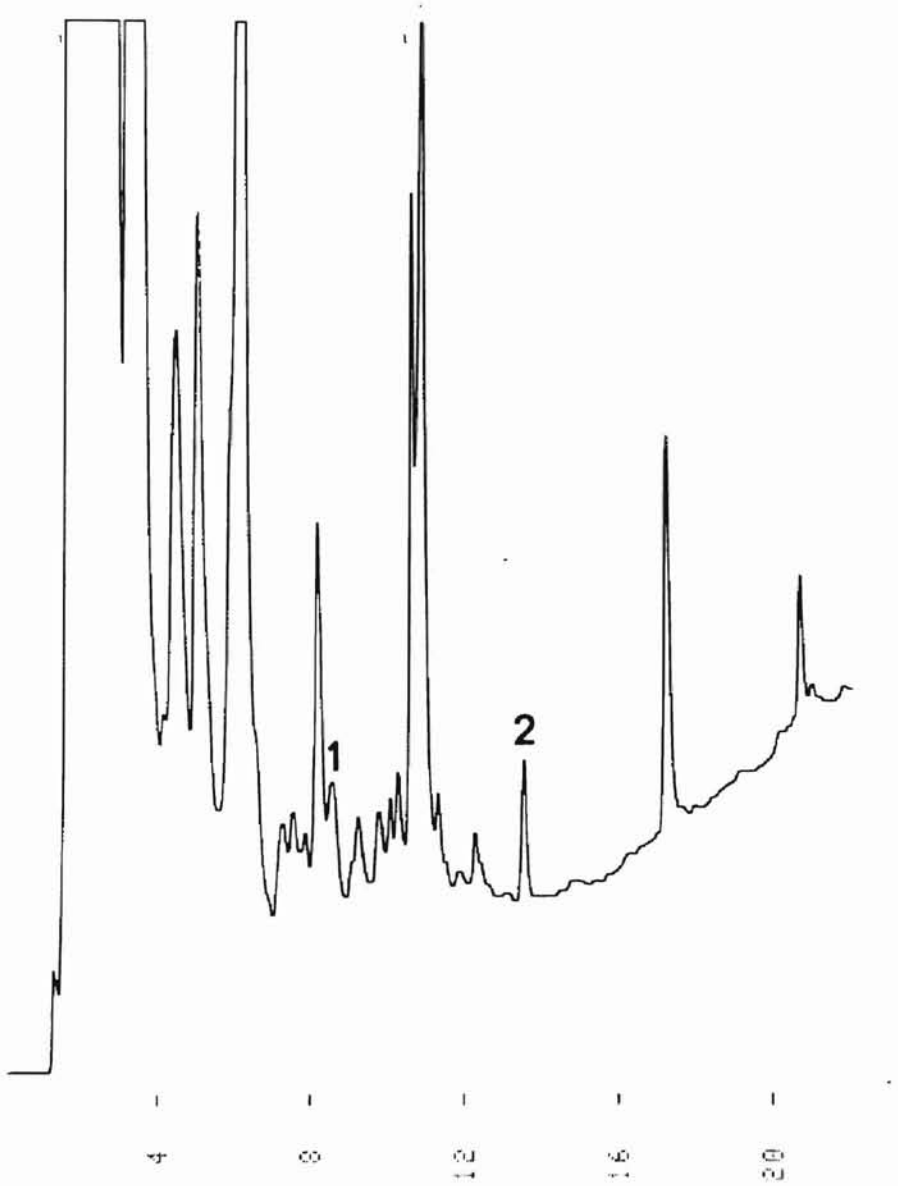


Figure 37. Typical Chromatogram obtained on an RPC column for the white cabbage extract treated with myrosinase. Column, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å pore diameter. Flow rate, 1 ml/min. A 17 min linear gradient elution from 16% to 90% (v/v) methanol. UV detection, 210 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4. Solutes: 1, L-sulforaphane; 2, allyl isothiocyanate.

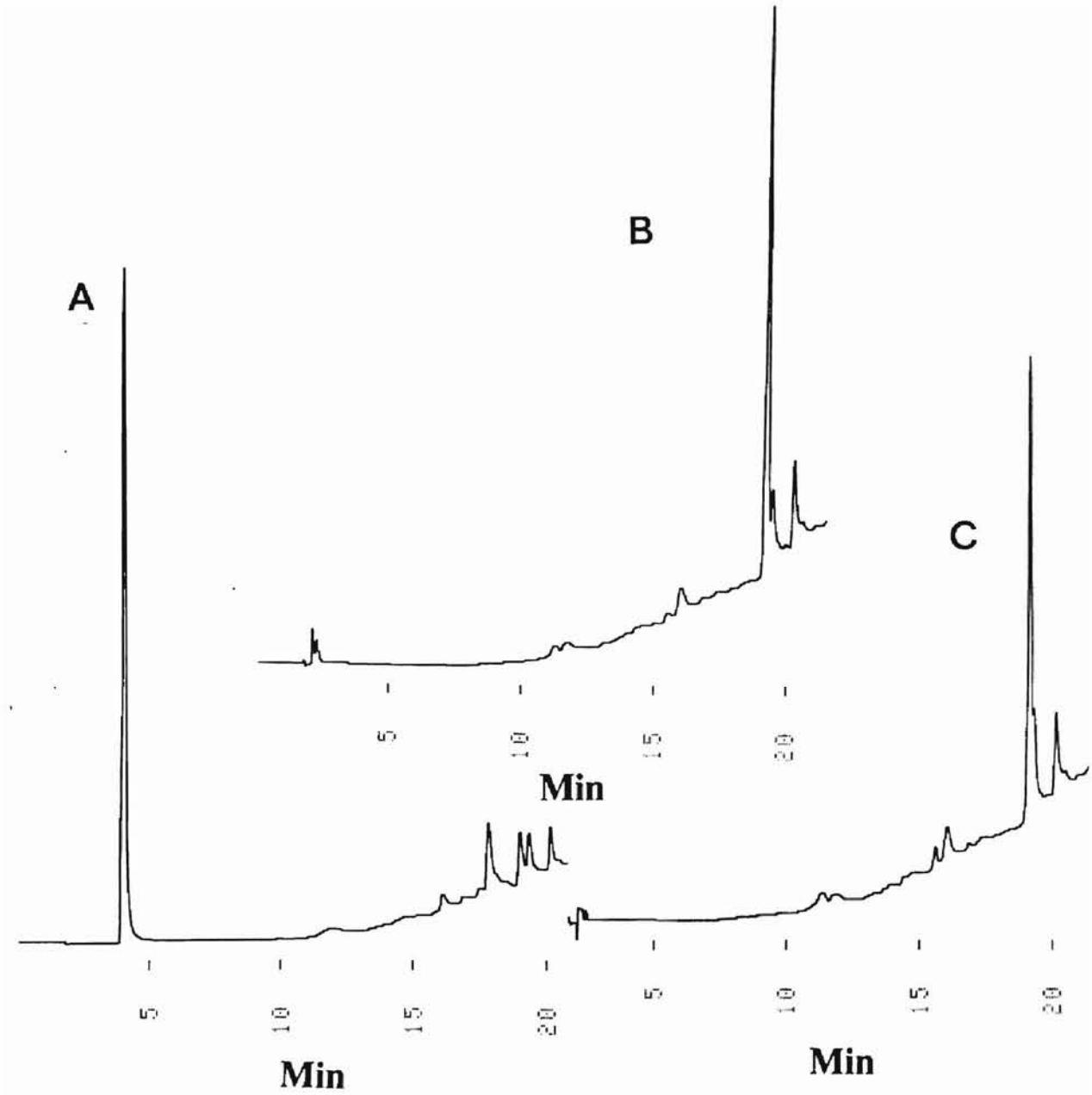


Figure 38. Chromatograms of the intact HPLC fraction #3 in (A), HPLC fraction #3 treated with myrosinase in (B) and standard allyl isothiocyanate in (C). Column, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter, and 100 Å mean pore diameter. Flow rate, 1 ml/min. A 17 min linear gradient from 0 to 80% (v/v) methanol in 50 mM sodium phosphate pH 3.2. UV detection, 210 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4.

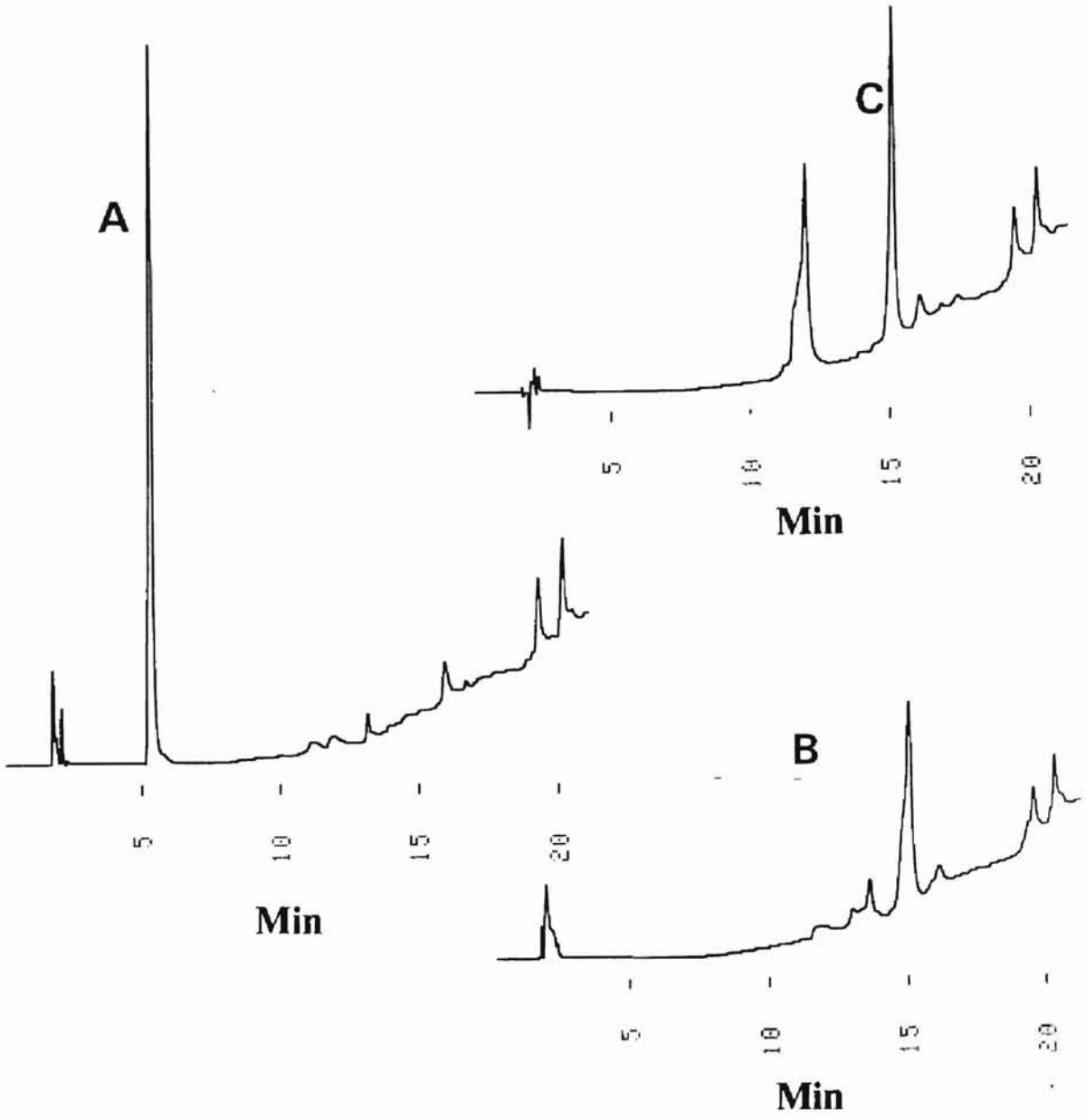


Figure 39. Chromatograms of intact HPLC fraction #4 in (A), HPLC fraction #4 treated with myrosinase in (B) and standard L-sulforaphane in (C). Column, 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 µm mean particle diameter, and 100 Å mean pore diameter. Flow rate, 1 ml/min. A 17 min linear gradient from 0 to 80% (v/v) methanol in 50 mM sodium phosphate pH 3.2. UV detection, 210 nm; detector sensitivity, 0.2 AUFS; integrator attenuation, 4.

p-hydroxybenzyl cyanide, and p-methoxylphenylacetonitrile (see Fig. 36). In the white cabbage extract treated with myrosinase (see Fig. 37), it was possible to observe the presence of isothiocyanates because the reaction was carried out at pH 7.0. If the reaction was carried out at pH lower than 7.0, the products would be a mixture of isothiocyanates and nitriles which are difficult to identify and quantify. The complication arises from the matrix interferences that migrate at lower retention time where nitriles are eluted from the RPC column. Therefore, it is more convenient for the identification of glucosinolates via myrosinase treatment to work at pH 7.0 to identify the reflective isothiocyanate degradation products. When there is complication is better to isolate the glucosinolate and digest the glucosinolate at pH 7.0 and at pH 3-4 to have the isothiocyanate products at pH 7.0 and the nitriles at low pH and confirm the structure with the standards of these degradation products.

Quantification of Glucosinolates by Using the Enzymatic Degradation Products

The quantification was carried out with the standard L-sulforaphane and allyl isothiocyanate. Benzyl isothiocyanate was used as the internal standard (I.S.). Figures 40 and 41 shows the calibration curves for the two standards which are the products of the enzymatic hydrolysis with myrosinase at pH 7.0. These standard curves were constructed from RPC analyses using a column of dimensions 15 cm x 4.6 mm I.D. packed with Microsorb-MV C18 spherical silica of 5 μm mean particle diameter and a 17 min linear gradient elution from 16% to 90% (v/v) methanol followed by 5 min isocratic elution with the gradient former. Under these conditions, the allyl isothiocyanate has a retention time of 13.4 min and the L-sulforaphane has a retention time of 8.4 min.

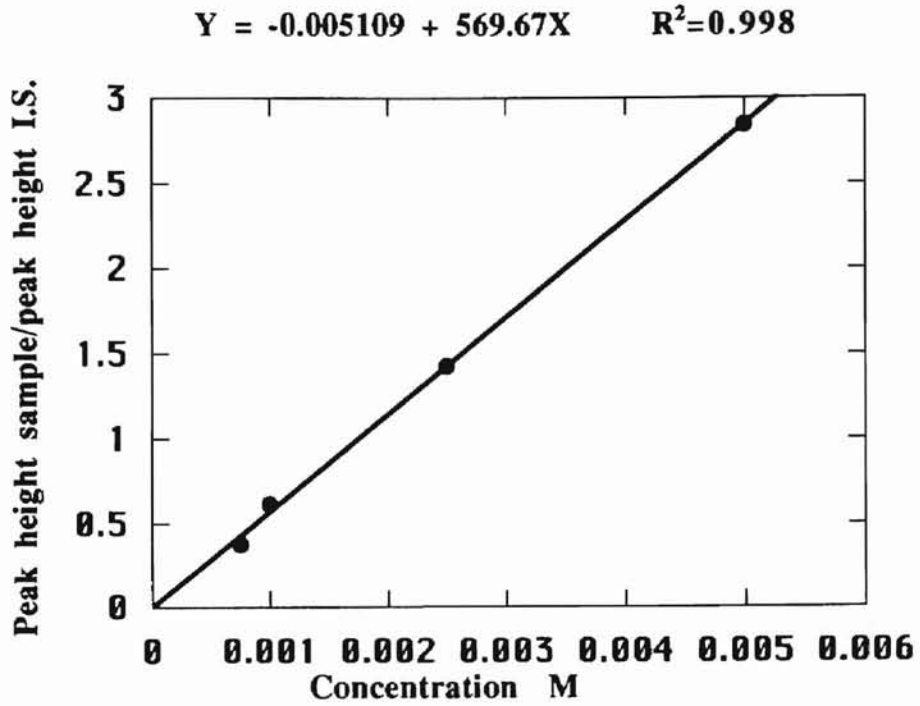


Figure 40. Calibration curve of L-sulforaphane. The conditions are the same as Fig. 37.

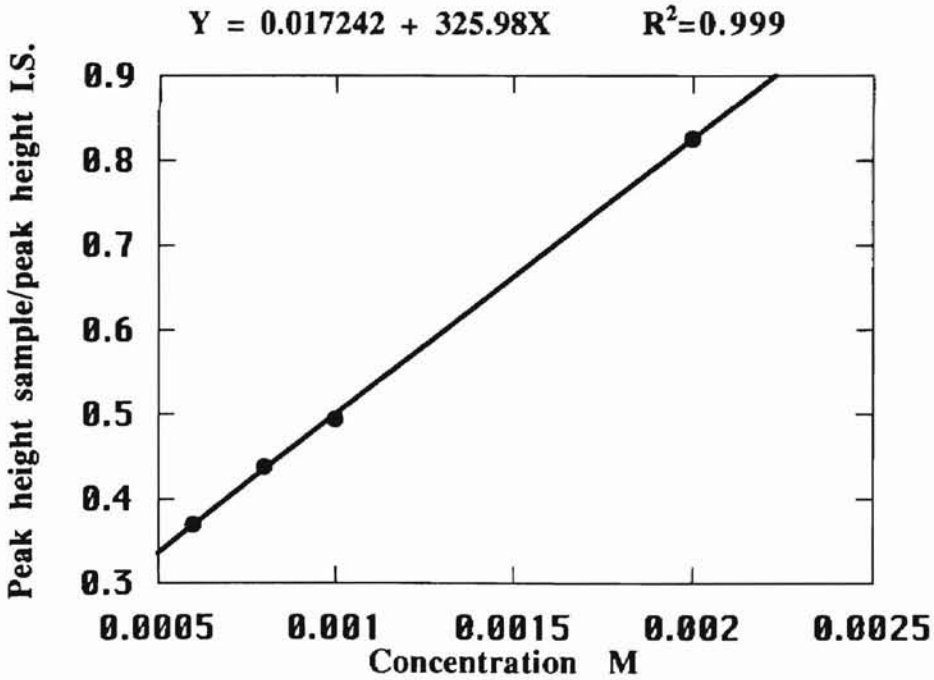


Figure 41. Calibration curve of allyl isothiocyanate. The conditions are the same as Fig. 37.

The concentration of sinigrin was found to be 75.15 μg/g and for the glucoraphanin 78.2 μg/g of fresh cabbage. These data agree with previously reported literature data on cabbage content of sinigrin and glucoraphanin (10, 11). It is believed that the quantification of glucoraphanin was not very reliable because it was not possible to separate the L-sulforaphane completely from the other products of degradation and matrix interferences. Hence, the amount reported for glucoraphanin is an approximation.

Conclusions

By making use of the high resolution NMR and MS techniques, the structures of the glucosinolates corresponding to the peaks # 1, 3, 4, 5 and 6 of the RPC column were confirmed. Also, the conditions for the identification of the degradation products via myrosinase digestion were demonstrated. The identification via myrosinase has one advantage over the spectroscopic method in that it is possible to confirm the structure of the glucosinolate with low amounts of sample and do not require an expensive instrument such as NMR and MS. The disadvantage of identification via myrosinase is that it requires the standard of the degradation product. The acid hydrolysis products derived from glucosinolates was shown to be not a good way for the identification of glucosinolates in real plant extracts.

References

1. A. Quinsac, D. Ribaillier, C. Elfakir, M. Lafosse, and M. Dreux, *A new approach to study of glucosinolates by isocratic liquid chromatography. Part I. Rapid determination of desulfated derivatives of rapeseed glucosinolates.* J. Assoc. Off. Anal. Chem. 1991, 74: 932-939

2. J.P. Sang, I.R. Minchinton, P.K. Johnstone, and R.J.W. Truscott, *Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede*. Can. J. Plant Sci. 1984, 64: 77-93.

3. O. Olsen and H. Sorensen, *Sinalbin and other glucosinolates in seed of double low rape species and brassica napus CV. Bronowski.* J. Agric. Food Chem. 1980, 28: 43-48.

4. B. Matthaus and H.J. Fiebig, *Simultaneous determination of isothiocyanates, indoles, and oxazolidinethiones in myrosinase digests of rapeseeds and rapeseed meal by HPLC.* J. Agric. Food Chem. 1996, 44: 3894-3899.

5. C. Elfakir, M. Lafosse, and M. Dreux, *Retention of some glucosinolates in anion exchange chromatography. Part I: Qualitative study on a silica-trialkylammonium exchanger*. Chromatographia, 1993, 37: 313-318.

6. P.J. Delaquis and G. Mazza, *Antimicrobial properties of isothiocyanates in food preservation.* Food Technol. 1995 11: 73-84.

7. C.A. Bradfield and L.F. Bjeldanes, *High-performance liquid chromatographic analysis of anticarcinogenic indoles in Brassica oleracea*. J. Agric. Food Chem. 1987, 35: 46-49.

8. L.W. Wattenberg and W.D. Loub, *Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles*. Cancer Res. 1978, 38: 1410-1413.

9. C.H. Van Etten and H.L. Tookey, *Glucosinolates*, in Handbook of Naturally Occurring Food Toxicants, M. J. Recheigh, Editor, 1983, CRC Press: Boca Raton, FL, pp. 15-30.

10. C.H. VanEtten, M.E. Daxenbichler, H.L. Tookey, W.F. Kwolek, P.H. Williams and O.C. Yoder, *Glucosinolates*: Potential toxicants in cabbage cultivars. J. Am. Soc. Hortic. Sci., 1980, 105, 1710.

11. K. Sones, R.K. Heaney and G.R. Fenwick, *The glucosinolate content of UK vegetables-cabbage (Brassica Oleracea), swede (B. napus) and turnip (B. campestris).* Food Additives Contam. 1984, 1, 289.

12. R. Bjorkman, *Preparative isolation and ^{35}S-labelling of glucosinolates from rapeseed (Brassica napus L.),* Act Chem. Scand. 1972, 26: 1111-1116.

13. C.L. Zrybko, E.K. Fukuda, and R.T. Rosen, *Determination of glucosinolates in domestic and wild mustard by high performance liquid chromatography with confirmation by electrospray and photodiode-array detection.* J. Chromatogr. A, 1997, 767: 43-52.

14. G.R. Fenwick, R.K. Heaney and R. Mawson. *Glucosinolates.* Toxicant of Plants Origin Vol. II P.R. Chem. (ed) CRC Press: Boca Raton, 1989, pp. 1-41.

15. S. Tsunoda, K. Hinata and C. Gomez-campo. *Brassica Crops and Willd Allies. Biology and Breeding,* Japan Scientific Societies Press: Tokyo, 1980, 285-299.

16. G.A. Rosenthal and D.H. Janzen. *Herbivores Their Interaction with Secondary Plant Metabolites.* Academic Press, Inc.: 1979, pp. 471-500.

17. C.A. Bradfiel and L.F. Bjeldanes, *High – Performance Liquid Chromatographic Analysis of Anticarcinogenic Indoles in Brassica oleracea.* J. Agric. Food Chem. 1987, 35: 46-49.

18. H. Sorensen, *Glucosinolates: Structure-properties-function, in canola and rapeseed, production, chemistry, nutrition and processing technology*, F. Shahidi, Editor, 1990. Van Nostrand Reinhold: New York. pp. 149-172.

19. B. Bjerg, and H. Sorensen, *Quantitative analysis of glucosinolates in oilseed rape based on HPLC of desulfoglucosinolates and HPLC of intact glucosinolates, in world crops: Production, utilization, description, glucosinolates in rapeseeds:* Analytical aspects, ed. J. P. Wathelet. Dordrecht: Martinus Nijhoff. 1987, 13: 125-150.

20. P.D. McDonald and B.A. Bidlnignmeyer, *Strategies for successful preparative liquid chromatography*, B.A. Bidlnigmeyer, Editor, 1987, Elsevier: Ansterdam, pp. 1-103.

21. R. Gmelin, *Chemieder S-haltigen brassica-inhaltstoffe und ihre beziehungen zuden brassica-faktoren, in naturally occurring goitrogens and thyroid function*, Podoba, J. and Langer, P., Eds., Veda: Bratislava, 1964, pp. 17-34.

22. C. H. Van Etten, M. E. Daxenbichler, P. H. Willians, and W. F. Kwolek, *Glucosinolates and derived products in cruciferous vegetables*, Agric. Food Chem., 1976, 24: 452-455.

23. W. Thies, *Detection and utilization of a glucosinolate sulphohydrolase in the edible sanil, helix pomatia.* Naturwissenschaften, 1979, 66: 364-365.

24. B. Bjerg, and H. Sorensen, *Isolation of intact glucosinolates by column chromatography and determination of their purity, in World Crops: Production, Utilization, Description, Glucosinolates in rapeseeds: Analytical aspects*, ed. J. P. Wathelet. Dordrecht: Martinus Nijhoff. 1987, 13: 59-75.

25. Z. Hu, J. A. Lewis, A. B. Hanley and G. R. Fenwick. *2-Hydroxyethyl glucosinolate from capparis masaikai of Chinese origin*, Phytochemistry, 1989, 28: 1252-1254.

26. A. Sakushima, M. Coskun and T. Maoka. *Sinapinyl but-3-enylglucosinolate from Boreava orientalis*. Phytochemistry, 1995, 40: 483-485.

27. T. Prestera, J.W. Fahey, W.D Holtzclw, C. Abeygunawardana, J.L. Kachinski and P. Talalay, *Comprehensive chromatographic and spectroscopic methods for the separation and identification of intact glucosinolate*. Anal. Biochem., 1996, 239: 168-179.

28. E. Josefssn, *Distribution of thioglucosides in different parts of brassica plants.* Phytochemistry, 1967, 6: 1617-1627.

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

30. B.W. Chistensen, Kjaer, and J. Ogaard Madsen, *Mass-spectrometry characteristics of some pertrimethyl-sialylated desulfoglucosinolates*. Tetrahedron, 1982, 38: 353-359.

31. A.J. Macleod, G. Macleod, and G. Reader, *Evidence for the occurrence of butyl-and isobutylglucosinolates in seeds of Brasssica Oleracea. Phytochemistry*, 1989, 28: 1405-1407.

32. C. Elfakir, M. Lafosse, M.C. Viaud, and M. Dreux, *New artificial standards for the HPLC analysis of natural glucosinolates*. J. High Resolut. Chromatogr., 1992, 15: 392-398.

33. B. Bjorkvist and A. Hase, *Separation and determination of intact glucosinolates in rapeseed by high-performance liquid chromatography*. J. Chromatogr., 1988, 435: 501-507.

34. A. Quinsac, D. Riballier, P. Rollin, and M. Dreux, *Analysis of 5-vinyl-1,3-oxazolidene-2-thione by liquid chromatography*. J. AOAC Int., 1992, 75: 529-536.

35. H.J. Fiebig and M. Jorden, *HPLC of desulfoglucosinolates*. J. High Resolut. Chromatogr., 1990, 13: 377-379.

36. H.J. Fiebig, *Desulphation of glucosinolates-potential pitfalls of the HPLC method for double zero rapeseed.* Fat Sci. Techol., 1991, 93: 264-270

37. P. Helboe, O. Olsen, and H. Sorensen, *Separation of Glucosionolates by High-Performance Liquid Chromatography.* J. Chromatogr., 1980, 197: 199-205.

38. I. Michinton, J. Sang, D. Burke, and R.J.W. Truscott, *Separation of desulfoglucosinolates by reversed-phase high-performance liquid chromatography.* J. Chromatogr., 1982, 247: 141-148.

39. J.L. Wolfender, M. Maillard, and K. Hostettmann, *Themospray liquid chromatogrophy-mass spectrometry in phytochemical analysis.* Phytochem. Anal., 1994, 5: 153-182.

40. G.J. Show, D. Andrzejewski, J.A.G. Roach, and J.A. Sphon, *Separation and identification of glucosinolates from Brassica vegetables using high-performance capillary gas chromatography (GC)-positive-ion chemical ionization mass spectrometry (PICIMS) and GC-PICIMS/MS.* J. Agric. Food Chem. 1989, 37: 372-378.

41. C. Elfakir, J.P. Mercier, and M. Dreux, *Retention of some glucosinolates in anion exchange chromatography. Part II: Qualitative study on a styrene-divinylbenzene copolymeric anion exchanger.* Chromatographia, 1994, 38: 585-590.

42. C. Elfakir, S. Xitra, M. Dreux, *Comparison of a styrene-divinylbencene copolymeric anion exchanger and a poymethacrylate copolymeric anion exchanger for glucosinolate sepatation.* J. Liq. Chrom. & Rel. Technol., 1997, 20: 907-921.

43. C. Elfakir and M. Dreux, *Simultaneous analysis of intact and desulfated glucosinolates with a porous graphitized carbon column.* J. Chromatogr. A, 1996, 727: 71-92.

44. M. Ishida, I. Chiba, Y. Okuyama, Y. Takahata, and N. Kaizuma, *Separation and identification of desulfoglucosinolates in Japanese rapeseed by LC/APCI-MS.* JARQ, 1997, 31: 73-80.

45. P.N. Maheshwari, D.W. Stanley, J.I. Gray and F.R. Van de Voort, *An HPLC method for the simultaneous quantitation of individual isothiocyanates and oxazolidinethione in myrosinase digests of rapeseed meal*, J. Am. Oil Chem. Soc. 1979, 56: 837-841.

46. Wayne R. Melander and Csaba Horvath "Reversed-Phase Chromatography" in *High-performance Liquid Chromatography. Advances and Perspectives.* Volume 2 Academic Press, Inc.: New York, 1980, pp. 114-303.

47. A. Karcher, H.A. Melouk and Z. El Rassi, *High performance liquid phase separation of glucosides. III. Determination of total glucosinolates in cabbage via the enzymatically released glucose*. Anal. Biochem. Submitted for publication.

48. M. Visentin, A. Tava, R. Iori, and S. Palmieri, *Isolation and identification of trans-4-(methylthio)-3-butenyl glucosinolate from radish roots (Raphanus sativus L.)*. J. Agric. Food Chem., 1992, 40: 1687-1691.

49. I.J. Cox, A.B. Hanley, P. Belton and R. Fenwick. *N.m.r. spectra (^{1}H, ^{13}C) of glucosinolates*. Carbohyd. Res. 1984, 132: 323-329.

50. M.C. Elliott and B.B. Stowe, *Indole compounds related to auxins and goitrogens of woad (Isatis tinctoria L.)*. Plant Physiol. 1971, 47: 366-372.

51. O. Olsen and H. Sorensen, *Isolation of glucosinolates and the identification of o-(α-L-Rhamnopyranosyloxy) benzylglucosinolate from reseda odorata*, Phytochemistry, 1979, 18: 1547-1552.

52. G. Bojesen and E. Larsen, *Characterization of five glucosinolates by fast atom bombardment mass spectrometry and collision activation of [M – HJ*, Biol. Mass Spectrum. 1991, 20: 286-288.

53. G.R. Fenwick, J. Eagles and R. Self, *The fast atom bombardment mass spectra of glucosinolates and glucosinolate mixtures*. Orga. Mass spectrom., 1982, 17: 544-546.

54. J.B. Westmore and M.M. Alauddin, *Ammonia chemical ionization mass spectrometry*. Mass Spectrom. Rev., 1986, 5: 381-465.

55. J. Eagles, G.R. Fenwick, R. Gmilin, and D. Rakow, *The chemical ionization mass spectra of glucosinolates (mustard oil glycosides) and desulphoglucosinolates. A useful aid for structural analysis*. Biomed. Mass Spectrom., 1981, 8: 265-269.

56. E.W. Underhill, *Glucosinolates, in Secondary Plant Products*, E. A. Bell and B.V. Chrlowood, Editors. 1980, Springer-Verlag: Berlin pp. 493-511.

VITA

Lino Gerardo Arguello

Candidate for the Degree of

Master of Science

Thesis: ANALYTICAL AND MICROPREPARATIVE HPLC COMBINED WITH SPECTROSCOPIC AND ENZYMATIC METHODS FOR GENERATING A GLUCOSINOLATE LIBRARY

Major Field: Chemistry

Biographial:

Personal Data: Born in Leon, Nicaragua, On October 23, 1964, the son of Jorge Arguello and Isidra Salgado.

Education: Graduated from Tecnico La Salle High School, Leon, Nicaragua in 1985; receive Bachelor of Science degree in Chemistry from the National Autonomous University of Nicaragua in September 1992. Completed the requirements for the Master of Science degree with a major in Analytical Chemistry at Oklahoma Sate University in July, 1998.

Professional Experience: Analyst chemist, employed by LAQUISA, Nicaragua, 1993-1995; Technician in the Applied Research Laboratory, employed by National University of Engineering, Nicaragua, 1991-1993.

Professional Memberships: Chemical Society of Nicaragua.