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Comprehensive Chromatographic and Spectroscopic Methods for the Separation and Identification of Intact Glucosinolates

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Much effort has been devoted to developing methods for the efficient isolation and identification of glucosinolates. Existing methods for separation involve ion exchange, GLC, and HPLC (mostly after chemical modification by enzymatic sulfate removal and/or silylation). We demonstrate a simple and direct strategy for analyzing the glucosinolate content of plant extracts, made possible by a new combination of widely available techniques: (a) reverse-phase paired-ion chromatography (PIC) of plant extracts, (b) hydrolysis of glucosinolates by myrosinase and quantitation of resulting isothiocyanates by cyclocondensation with 1,2-benzenedithiol; (c) a novel method for replacing the PIC counterions by ammonium ions, permitting direct bioassay, mass, and ^{1}H NMR spectrometry; (d) mass spectrometric analysis of ammonium salts by negative-ion fast atom bombardment (FAB) to determine *m/z* of the $[M - H]^-$ ion, and by chemical ionization (CI) in ammonia to obtain accurate masses of characteristic fragment ions, principally $[R—CN:NH_4]^+$, $[R—CH{=}NOH:H]^+$ and $[R—CH{=}NOH:NH_4]^+$; and (e) high-resolution ^{1}H NMR spectroscopy of intact glucosinolates. FAB and CI mass spectra, as well as high-resolution ^{1}H NMR spectra were obtained for a variety of glucosinolate standards. The results provide guidance for the isolation and characterization of unknown glucosinolates from plants. These combined procedures were applied to a sample of broccoli (cultivar SAGA), in order to resolve and identify its major glucosinolates: 4-methylsulfinylbutyl glucosinolate (glucoraphanin) and 4-methylthiobutyl glucosinolate (glucoerucin). Thus, this analytical strategy provides a powerful technique for identifying and quantitating glucosinolates in plant extracts without resorting to derivatization. © 1996 Academic Press, Inc.

This paper describes a sequential strategy for the chromatographic separation, identification, and quantitation of glucosinolates (GS)[2] from crude plant extracts. Glucosinolates are naturally occurring and often abundant constituents of the Cruciferae plant family that are formed biosynthetically from amino acids. They are β-thioglucoside *N*-hydroxysulfates in which the aglycone (or R group) may contain alkyl, alkenyl, aromatic, or indole groups. Often the alkyl or alkenyl glucosinolates also contain sulfur atoms in various oxidation states (S, SO, or SO_2). When plants are injured, chopped, or chewed, the coexisting but normally physically segregated myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) is released and catalyzes the hydrolysis of glucosinolates to intermediates, which undergo a Lossen rearrangement to produce isothiocyanates, hydrogen sulfate, and glucose or, less commonly, other breakdown products (reviews 1–5).

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[2] Abbreviations used: ACN, acetonitrile; CI, chemical ionization; DCI, direct chemical ionization; EI, electron impact ionization; FAB, fast atom bombardment; GS, glucosinolate; HPLC, high-performance liquid chromatography; 1D, one-dimensional; myrosinase, thioglucoside glucohydrolase (EC 3.2.3.1); NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PFK, perfluorokerosene; PIC, paired-ion chromatography; TDA, tetradecylammonium ion; TDAB, tetradecylammonium bromide; TMA, tetramethylammonium ion; TMAB, tetramethylammonium bromide; TOA, tetraoctylammonium ion; TOAB, tetraoctylammonium bromide.

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$$R{-}C\begin{matrix} \diagup S{-}C_6H_{11}O_5 \\ \diagdown\!\!\diagdown N{-}OSO_2O^- \end{matrix} + H_2O \xrightarrow{\text{Myrosinase}} R{-}N{=}C{=}S + HSO_3O^- + \text{D-glucose}$$

About 100 glucosinolates have been isolated from edible plants (1, 3, 6). Predominant glucosinolates include sinigrin (allyl-GS) in mustards and horseradish, 4-methylthio-3-butenyl-GS (dehydroerucin) in radish, and phenethyl-GS (gluconasturtiin) in watercress. Whereas certain genera of crucifers such as *Arabidopsis* may contain as many as 23 different glucosinolates (7), most *Brassica oleracea* varieties (e.g., vegetables such as broccoli, cauliflower, cabbage, and brussels sprouts) contain no more than 5 or 6 major glucosinolates including indole and aromatic glucosinolates (8). The glucosinolates of *Brassica napus,* a major oilseed crop, include indole glucosinolates as well as butenyl-GS, pentenyl-GS, and their hydroxylated analogs (1, 9). Glucosinolates are distributed throughout the plant body, although they tend to accumulate in the seeds. For example, rapeseed may contain over 80 μmol of glucosinolates per gram of seed (10).

Estimates of average per person daily intake of glucosinolates range from about 16 mg in Canada (11) to 30 mg in the United Kingdom (12) and 112 mg in Japan (13). Knowledge of the chemical types and quantities of glucosinolates in plants consumed by humans and animals is important, since both toxic and beneficial effects have been attributed to these substances (1). Thus rapeseed breeders have been involved in major efforts to reduce glucosinolate content of these plants since some glucosinolates have goitrogenic and toxic (antinutritional) effects. Other glucosinolates and their isothiocyanate degradation products have been shown to exert powerful effects in reducing the incidence of cancer in experimental animals (see review 14). One major mechanism underlying these chemoprotective effects of isothiocyanates and glucosinolates is the induction, by enhanced transcription, of the activities of Phase 2 detoxication enzymes (e.g., glutathione transferases, NAD(P)H:quinone reductase, glucuronosyltransferases, epoxide hydrolase) in animal cells (15). Our laboratory has had a long-standing interest in the chemoprotective effects of phytochemicals that modulate carcinogen metabolism, particularly those that raise Phase 2 enzymes, and in the possibility that the widely recognized cancer-lowering effects of fruit and vegetable consumption (16–18) may be in part attributable to these actions of glucosinolates and isothiocyanates. For example, sulforaphane, isolated from broccoli, is the most potent naturally occurring inducer of Phase 2 enzymes, and blocks mammary cancer development in rats treated with 7,12-dimethylbenzanthracene (19, 20).

Since certain glucosinolates and isothiocyanates are chemoprotective while others are toxic or goitrogenic, the availability of rapid and reliable methods for the identification and quantitation of individual glucosinolates in vegetables and food products is of considerable importance. The efficient separation of glucosinolates is difficult because these molecules are highly charged (the sulfate group with a pK_a value of about −9 is ionized under nearly all circumstances), yet separations depend upon sometimes subtle differences in the nonpolar side chains (R).

Methods for the separation and analysis of glucosinolates have been reviewed recently by Heaney and Fenwick (21), by Betz and Fox (5), and in a monograph on this subject (22). Separations were originally performed by gas–liquid chromatography of trimethylsilylated derivatives of glucosinolates from which the sulfate group had been removed (23). This method was further refined by using a sulfohydrolase to remove the sulfate group before derivatization. However, certain glucosinolates yielded multiple products upon derivatization (21, 24). These desulfation methods were adapted for use with HPLC by Minchinton *et al.* (25), Spinks *et al.* (26), and Sang and Truscott (27), but were still subject to difficulties in interpretation because of concerns over the effects of pH, time, and enzyme concentration on desulfation products. Helboe *et al.* (28) and more recently Betz and Fox (5) have utilized paired-ion chromatography (PIC) in the presence of hydrophobic anions with reverse-phase HPLC for separation of intact glucosinolates. This system represents an important, but apparently underutilized, advance in separation methods for glucosinolates because it permits recovery of intact glucosinolates under neutral conditions that do not promote decomposition and that permit quantitative recovery of glucosinolate salts. Unfortunately, the fractions obtained by PIC contain large excesses of highly hydrophobic counter ions which interfere with various subsequent analyses, i.e., (a) mass spectrometry (because of lack of volatility), (b) NMR spectroscopy (because of addition of potentially interfering resonances), and (c) toxicity of these counter ions to enzymes and bioassay systems.

By replacing the TOA or TDA counter ions with the ammonium ion as described in the present study, it was possible to hydrolyze the glucosinolates by treatment with myrosinase, to quantitate the liberated isothiocyanate aglycones by cyclocondensation with 1,2-benzenedithiol and UV measurement of the product (29), and to bioassay the isothiocyanates for inducer potency for Phase 2 enzymes in murine hepatoma cells (30, 31). None of these steps would have been possible with the desulfation procedures or the currently available PIC techniques (5, 28).

TABLE 1

Names, Structures, and Retention Times of 14 Glucosinolates on Reverse-Phase Paired-Ion Chromatography with either Tetraocytlammonium (TOA) or Tetradecylammonium (TDA) as Counter Ions

Compound No.	Name of glucosinolate: Chemical	Name of glucosinolate: Common	R group ($R-C(S\text{-glucose})=NOSO_2O^-$)	Chromatography retention time (min): TOA	TDA
1	3-Methylsulfinylpropyl	Glucoiberin	$CH_3SOCH_2CH_2CH_2-$	2.50	3.59
2	4-Methylsulfinylbutyl	Glucoraphanin	$CH_3SOCH_2CH_2CH_2CH_2-$	2.63	3.60
3	2-Hydroxyethyl		$HOCH_2CH_2-$	2.73	4.27
4	3-Methylsulfonylpropyl	Glucocheirolin	$CH_3SO_2CH_2CH_2CH_2-$	3.12	5.92
5	2-Hydroxybut-3-enyl	Progoitrin	$CH_2{=}CHCH(OH)CH_2-$	3.19	6.18
6	Allyl	Sinigrin	$CH_2{=}CH-CH_2-$	3.76	7.36
7	4-Hydroxybenzyl	Glucosinalbin	$HO-C_6H_4-CH_2-$	4.07	8.01
8	But-3-enyl	Gluconapin	$CH_2{=}CHCH_2CH_2-$	4.60	9.20
9	4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	[4-hydroxyindol-3-yl]$-CH_2-$ (OH, N–H)	4.66	10.96
10	Pent-4-enyl	Glucobrassicanapin	$CH_2{=}CHCH_2CH_2CH_2-$	5.20	11.82
11	4-Methylthiobutyl	Glucoerucin	$CH_3SCH_2CH_2CH_2CH_2-$	5.25	11.95
12	Benzyl	Glucotropaeolin	$C_6H_5-CH_2-$	5.48	12.88
13	Indol-3-ylmethyl	Glucobrassicin	[indol-3-yl]$-CH_2-$ (N–H)	5.93	15.39
14	1-Methoxyindol-3-ylmethyl	Neoglucobrassicin	[indol-3-yl]$-CH_2-$ (N–OCH_3)	8.22	22.34

Note. The chromatography was carried out on a μBondapak C_{18} reverse-phase column (250 × 4.6 mm) with isocratic elution with 50% ACN/50% water, containing 5 mM TOAB or TDAB. Compounds are numbered in ascending order of their retention times. All retention times were determined using the same solvent mixture on the same day. All glucosinolates were injected individually in 60 μl or less, 10–30 μg per injection; flow rate was 3 ml/min, absorbance was monitored at 235 nm. Retention times are reported as time elapsed from injection to the peak maximum.

The identity of the 14 glucosinolate standards (Table 1) used in the present study was confirmed by sequential: (a) negative-ion fast atom bombardment (FAB) mass spectrometry to obtain the mass of the quasimolecular or $[M-H]^-$ ion; (b) ammonia chemical ionization (CI) mass spectrometry with accurate mass determination of certain diagnostically important fragment ions such as $[R-CN{:}NH_4]^+$; and (c) high-resolution NMR spectroscopy. Both the CI and EI (electron ionization) mass spectra of some glucosinolates and of desulfoglucosinolates have been reported by Fenwick and collaborators (32, 33). FAB mass spectra have been obtained on a limited number of glucosinolates (34, 35). Introduction of a novel procedure for replacing the counter ions used in the PIC separation protocol by ammonium ions has made it possible to analyze the samples directly by FAB and CI mass spectrometry. Although these mass spectra will usually lead to the identification of the glucosinolate anions, high-resolution NMR

spectra provide structural confirmation and unequivocal identification in rare cases where ambiguity exists.

MATERIALS AND METHODS

Chemicals

2-Hydroxyethyl-GS, pent-4-enyl-GS (glucobrassicanapin), indol-3-ylmethyl-GS (glucobrassicin), 1-methoxyindol-3-ylmethyl-GS (neoglucobrassicin), 4-hydroxyindol-3-yl-GS (4-hydroxyglucobrassicin), 4-methylthiobutyl-GS (glucoerucin), 3-methylsulfonylpropyl-GS (glucocheirolin), 3-methylsulfinylpropyl-GS (glucoiberin), and 4-hydroxybenzyl-GS (glucosinalbin) were gifts from Robert K. Heaney (Institute of Food Research, Norwich, UK). Benzyl-GS (glucotropaeolin) and but-3-enyl-GS (gluconapin) were gifts from D. Ian McGregor (Agriculture Canada Research Station, Saskatoon, Saskatchewan, Canada). 4-Methylsulfinylbutyl-GS (glucoraphanin) was purified in our laboratory from broccoli (cultivar SAGA). Tetraoctylammonium bromide (TOAB), tetramethylammonium bromide (TMAB), and sinigrin (allyl-GS) were obtained from Aldrich (Milwaukee, WI). Tetradecylammonium bromide (TDAB) was purchased from Sigma (St. Louis, MO). All other chemicals and solvents were obtained from Sigma, Aldrich, or J. T. Baker (Phillipsburg, NJ).

Paired-Ion Chromatography

Analysis and purification of both plant extracts and glucosinolate samples was performed by HPLC on an analytical μBondapak C_{18} reverse-phase column (250 × 4.6 mm) (Waters, Milford, MA) coupled to a 5-μm LiChrospher 100 RP-18 guard column (EM Separations, Gibbstown, NJ). Columns were extensively equilibrated with a minimum of 100 column void volumes of solvents. The HPLC system was equipped with a Waters Model 996 Photodiode Array Detector, and a Waters WISP automated injector. Data were processed with Waters Millennium software. Solvents were filtered and degassed for a minimum of 30 min.

Mass Spectrometry

Chemical ionization/electron impact ionization (CI/EI) mass spectrometry was performed on a VG70-S mass spectrometer (Manchester, England). Ammonia (electronic grade, purity 99.99% min) was used for positive ion desorption CI. Methanol (30 μl, HPLC grade) was added to dissolve the sample. A 5-μl aliquot was placed in small portions on the filament of the DCI (direct chemical ionization) probe; evaporation of the solvent left a thin film of the sample on the filament. The DCI probe was then positioned inside the source. Source temperature was set at 180°C, electron energy at 150 eV, emission current at 0.5 mA, and instrument resolution at 1000 for nominal spectra. Ammonia was introduced into the ion block such that the source housing pressure was maintained at $2-3 \times 10^{-5}$ mbar. The DCI probe was then heated independently (0.2–0.8 A) to desorb the sample thermally and generate the ions for production of mass spectra "in beam."

Precise ion masses were obtained via peak matching with perfluorokerosene (PFK) as the internal standard at a resolution of 10,000. A partial CI technique was employed in peak matching PFK to sample peaks in ammonia CI.

The presence of any radical cations produced under high-resolution CI conditions was confirmed by terminating introduction of ammonia and observing the persistence of the suspected peak.

FAB mass spectrometry was performed in the negative ion mode with a cesium iodide gun on a Kratos Concept ^{1}H mass spectrometer (Manchester, England). Triethanolamine or monothioglycerol was used as the matrix.

NMR Spectroscopy

Proton NMR spectra were obtained in D_2O at 600 MHz on a Varian Unity Plus 600 NMR spectrometer at 32°C. Ammonium salts of glucosinolates were dissolved in D_2O and dried in a vacuum centrifuge. They were then dissolved in 625 μl of D_2O at a concentration of 1 to 10 mM and one-dimensional (1D) proton spectra were obtained in high-quality NMR tubes. The observed ^{1}H chemical shifts are reported with respect to the HOD signal, which is at 4.706 ppm downfield from external sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4 (TSP) in D_2O (0.0 ppm) at 32°C. One-dimensional difference decoupling spectra were obtained with low power irradiation during the data acquisition interval.

Preparation and Myrosinase Treatment of Plant Extracts

B. oleracea var. *italica* (broccoli) plants grown in a plant growth chamber at 25°C (day), 20°C (night), with 16 h light daily were harvested by plunging in boiling water at a ratio of 47 mg dry wt/ml water. They were maintained at 100°C for 3 min after the water returned to a rolling boil, and then homogenized in their infusion broth for 2 min by using a Brinkman Polytron Homogenizer (Westbury, NY) at $\frac{1}{2}$ maximum power. The homogenate was centrifuged at 3000*g* for 15 min and the supernatant fluid was filtered through a 0.22-μm Millex-GV filter (Millipore, Bedford, MA) and stored at −80°C until further use.

Myrosinase was purified from daikon (*Raphanus sativus*) to a specific activity of 200 units/mg protein by a modification of the method of Palmieri and coworkers (36). Myrosinase was added to the thawed,

filtered homogenate at 26.5 units/g plant dry wt. Complete hydrolysis of glucosinolates occurred in less than 10 min (1 unit = the amount required to hydrolyze 1 μmol sinigrin/min). This mixture was incubated for 2 h at 37°C, after which all glucosinolates had disappeared, and then stored frozen or used directly for PIC.

RESULTS AND DISCUSSION

Paired-Ion Chromatography of Glucosinolate Mixtures

Separation of individual glucosinolates is difficult because these molecules are highly charged and water-soluble; resolutions depend on the properties of the less polar side chains. Many less than ideal chromatographic methods have been developed for the isolation and separation of glucosinolates (see review 22). A structural feature common to all glucosinolates is the presence of strongly acidic sulfate groups. Ion-pair formation between ammonium ions and sulfate groups is extremely favorable. Thus, when counter ions such as tetraoctylammonium (TOA), tetradecylammonium (TDA), or tetramethylammonium (TMA) are paired with sulfates, the negative charge of the sulfate group is effectively masked and the ion pair behaves like a hydrophobic molecule which can be separated by reverse-phase chromatography. Such methods have been described for glucosinolates (5, 28).

In order to develop methods for the rapid and accurate identification of glucosinolates in plant extracts, 14 glucosinolate standards, representing the major side-chain families of glucosinolates occurring in plants, were subjected to PIC. The solvent conditions of Helboe *et al.* (28) [70% methanol: 30% H_2O with 5 mM TOAB and 20 mM sodium phosphate, pH 7.0] were used initially, but better resolution and greater overall speed were achieved by simple modifications [50% ACN:50% H_2O with 5 mM TOAB or TDAB]. Helboe and co-workers (28) included phosphate buffer in order to control pH and thus prevent any degradation of glucosinolates (which are unstable at both pH extremes). However, omitting the phosphate buffer improved resolution and simplified subsequent purification techniques. The apparent pH of 50% ACN containing 5 mM TOAB or TDAB was ca. 8.0. The pH of the injected solutions was kept in the range of 6.0 to 8.0. Although the solvent system has only minimal buffering capacity, degradation of glucosinolates was not observed. All 14 glucosinolates were chromatographed in this system as a mixture, individually, and in various combinations. Table 1 lists the glucosinolates, their structures, and retention times, with either TOA or TDA as counter ions. Although the order of retention times was identical with either TOA or TDA as the counter ion, TDA

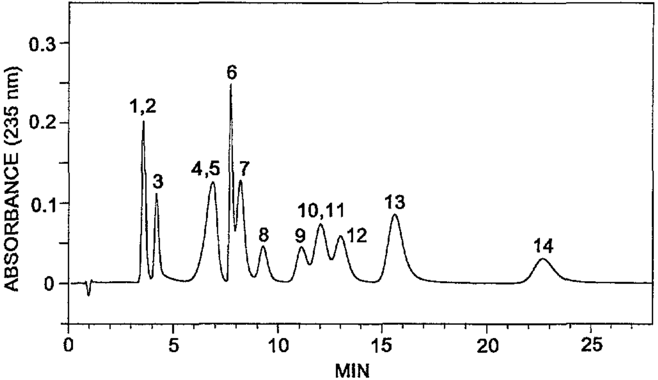


FIG. 1. Paired-ion chromatography of 14 glucosinolates. An aqueous solution (100 μl) containing 10–30 μg of each glucosinolate listed in Table 1 was injected onto a C_{18} reverse-phase HPLC column and developed isocratically with 50% ACN:50% water containing 5 mM TDAB at a flow rate of 3 ml/min. Absorbance was monitored at 235 nm. The peaks were identified by injection of individual glucosinolates, and are numbered according to their glucosinolate content (Table 1).

retention times were longer and provided better separations of these glucosinolates.

The mixture of 14 glucosinolates was resolved into 11 separate peaks when TDA was the counter ion (Fig. 1). Not all compounds were separated under these conditions. Two compounds (**1, 2**), which differ by only a single methylene group, were not well separated. In contrast, compounds **6** and **8** which also differ by only one methylene group, were very well separated. Compounds **10** and **11,** differing by the presence of a thioether in **11** in place of an olefin in **10,** were not resolved. Improved resolutions, however, were obtained by the use of other counter ions (see below).

When TOA was used as the counter ion, only 10 peaks were resolved, and the resolution was significantly worse than with TDA. The resolution obtained at a flow rate of 3 ml/min was equivalent to that at 1.5 ml/min (data not shown). If the column is not equilibrated thoroughly with a minimum of 100 column void volumes of solvent, inconsistent results are obtained. Slight variations in the solvent ratio also result in significant differences in retention times. More rapid elution of glucosinolates is obtained by increasing the percentage of ACN used, but resolution is generally decreased. If the proportion of ACN is reduced to less than 50%, the retention and separation times are increased, but the TOAB and TDAB (both only sparingly soluble in H_2O) tend to precipitate.

When retention times of separately injected glucosinolates were compared with either TOA or TDA as counter ion, there was a linear correlation between retention times in the two solvent systems ($r^2 = 0.979$). Thus, if a glucosinolate has a retention time of x when chromatographed with TAO, its retention time (y) in

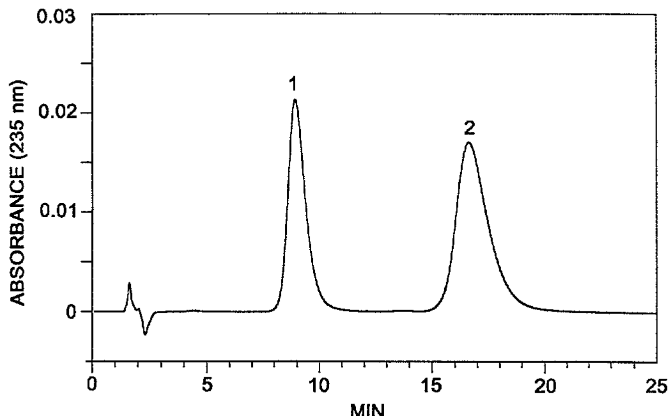


FIG. 2. Paired-ion chromatography of glucoiberin (**1**) and glucoraphanin (**2**). An aqueous solution (88.0 μl) containing 4.0 μg of each glucosinolate was injected onto a C_{18} reverse-phase HPLC column and developed isocratically with water containing 5 mM TMAB at a flow rate of 2 ml/min. Absorbance was monitored at 235 nm. The peaks were identified by injection of individual glucosinolates and are numbered according to their glucosinolate reference number (Table 1).

the presence of TDA is increased, and can be predicted by the relationship: $y = 3.24x - 4.64$. Since this analysis included all the major chemical types of glucosinolates, it seems reasonable to expect that this relationship will hold for most glucosinolates.

Separation of glucosinolates **1** (glucoiberin) and **2** (glucoraphanin) was of particular interest to us because these compounds coexist in some plant extracts and are not separated by PIC in the presence of TOA or TDA counter ions. These glucosinolates were completely separated on the same column in the presence of 5 mM TMAB in water (Fig. 2); the elution times were 8.94 and 16.6 min for **1** and **2,** respectively.

Purification of Standard Glucosinolates by Paired-Ion Chromatography

When glucosinolates are chromatographed by PIC and peaks are collected, these fractions contain an excess of the counter ion. Indeed, a given 1-ml fraction contains as much as 5 μmol of the contaminating hydrophobic counter ion, which makes both mass spectrometry and NMR analyses difficult. In addition, chemical or bioassay of Phase 2 enzyme induction potential of these glucosinolates (after myrosinase treatment) (29–31) is impossible because low concentrations of the highly hydrophobic contaminating counter ions are very toxic to the cultured cells used in the bioassay and inhibit myrosinase activity. Therefore, it is imperative to remove the hydrophobic counter ions from the glucosinolates before further chemical or biological analyses.

In order to solve this problem we developed simple techniques to remove the excess counter ion and to exchange the TOA or TDA ions (with which the glucosinolates are paired following PIC) for ammonium ions, which pose none of the aforementioned problems and promote volatility for mass spectrometry. In this procedure, glucosinolate peaks are collected from PIC and then evaporated in a vacuum centrifuge. In a typical experiment about 1 mg (ca. 2.5 μmol) of glucosinolate was collected. Chloroform (0.5–1.0 ml) was added to the dried material (a white silky powder), followed by 500 μl of 20 mM ammonium chloride. After thorough mixing, the aqueous phase was separated and reextracted twice with chloroform. The aqueous phase contained all the glucosinolate, but no appreciable quantity of TOA or TDA, as demonstrated by PIC analysis of the dried chloroform fractions, and NMR analysis of the aqueous fraction. Although this extraction procedure was utilized for milligram quantities of glucosinolates, it proved less satisfactory for microgram quantities because of the large excesses of ammonium chloride which interfere with the mass spectroscopic analysis. Consequently, a modified procedure was developed in which the 500 μl of ammonium chloride was replaced by 250 μl of 2–3 mM ammonium sulfate adjusted with NH_4OH to pH 7.0. After the three chloroform extractions, the aqueous phase is removed and dried. The residue is resuspended in 300 μl of methanol, and the insoluble ammonium sulfate is removed by centrifugation. The methanol phase, containing the glucosinolate is then dried. This procedure removed essentially all of the excess ammonium sulfate.

Technique for Purification of Glucosinolates from a Crude Plant Extract

Crude aqueous plant extracts were characterized directly and their glucosinolate content was quantitated by the following sequential procedure: direct injection of crude aqueous extracts onto the PIC system, collection of the appropriate peaks, conversion of glucosinolates to their ammonium salts, removal of excess counter ion, and direct spectroscopic analyses of the ammonium salts.

Crude plant extracts may give rise to UV-absorbing PIC peaks that are not glucosinolates. Therefore, we first compared PIC (TOA as counter ion) of a crude aqueous extract of broccoli with an extract that had been treated with myrosinase as described under Materials and Methods (Fig. 3). Three prominent peaks [A, B, C] with elution times of 2.91, 5.36, and 6.03 min, respectively, were present in the crude extract and were eliminated upon treatment with myrosinase. This provided presumptive evidence that these peaks are indeed glucosinolates, and thus we subjected them to the following procedures.

The peaks were purified by PIC and converted to

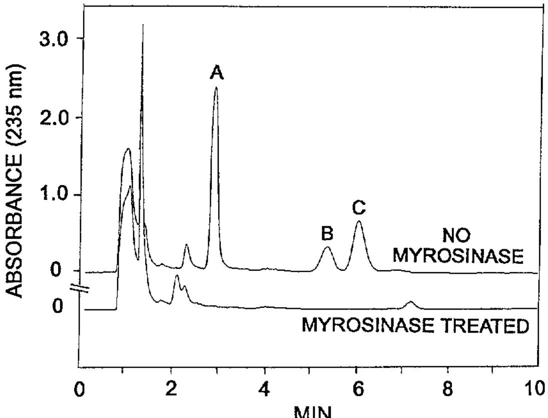


FIG. 3. Paired-ion chromatography of an aqueous extract of SAGA broccoli. The effects of myrosinase treatment are shown. The extracts (either untreated or treated with myrosinase) were injected onto a reverse-phase HPLC column, equilibrated, and isocratically eluted with 50% ACN:50% water containing 5 mM TOAB. Aliquots (300 μl) of each extract were diluted to 1 ml with water and a 600-μl portion was injected onto the PIC system. The flow rate was 3 ml/min. The absorbance was monitored at 235 nm. Three major myrosinase-sensitive peaks (A, B, and C) were eluted from the column.

ammonium salts by extraction with chloroform in the presence of 20 mM ammonium chloride as described above. A portion of these ammonium salts was then treated with myrosinase followed by cyclocondensation with 1,2-benzenedithiol (29) to provide a quantitative measure of isothiocyanate (the main product of myrosinase treatment of glucosinolates) content. Only the myrosinase hydrolysates of peaks A and C produced significant quantities of isothiocyanates. Peak B was not further characterized since it was apparently not an alkyl, aryl, or arylalkyl glucosinolate. It is likely that peak B was either a hydroxyalkyl-GS or an indole-GS, which could have been degraded by myrosinase and then undergone further conversions to products that were not isothiocyanates (1), and thus did not react with 1,2-benzenedithiol. Ultraviolet spectra of peaks A and C were characteristic of glucosinolates (λ_{max} at 224 nm). Assuming a molar absorbance coefficient of ca. 7000 M^{-1} cm^{-1} at this wavelength, the amounts of glucosinolates in peaks A and C were in agreement with those determined by the cyclocondensation assay after myrosinase hydrolysis.

The ammonium salts of peaks A and C were then characterized by three mass spectroscopic techniques: (a) negative ion FAB on a thioglycerol matrix provided values of *m/z* 436 (peak A) and *m/z* 420 (peak C) for the negative $[M—H]^-$ ions; (b) CI mass spectrometry in the presence of ammonia provided two principal fragments for each peak. For peak A, the principal fragment masses were *m/z* 146 ($[RCN:H]^+$) and *m/z* 163 ($[RCN:NH_4]^+$ or $[RCH{=}NOH]^+$) (see Table 2). For peak C, the fragment masses were *m/z* 130 ($[RCN:H]^+$) and *m/z* 147 ($[RCN:NH_4]^+$ or $[RCH{=}NOH]^+$). See discussion below, Table 2 and Eagles *et al.* (33). The masses of the $[M—H]^-$ peaks and those of the fragments provide strong evidence that peak A is 4-methylsulfinylbutyl-GS (glucoraphanin, compound **2**), and peak C is the closely related 4-methylthiobutyl-GS (glucoerucin, compound **11**) (Table 1). Consequently, these identifications could be made, without resorting to accurate mass determinations.

Proton NMR spectra of peaks A and C (Fig. 4) unequivocally confirmed the identities of these compounds as glucoraphanin and glucoerucin which have been isolated from other broccoli cultivars (8). As expected (see below) the glucose protons were well separated from R-group protons in both spectra and showed very similar chemical shifts. Coupling constants indicated the β-anomeric configuration for the glucose residues ($J_{1,2}$ = 9.6 Hz). Two overlapping triplets arising from H2 and H4 as well as a multiplet due to exact overlap of H3 and H5 protons were observed. The resonances arising from the R groups show clear correlations to their covalent structure. As expected, the major differences in ^{1}H chemical shifts appear in the methylene groups near the sulfur, and are indicative of the oxidation state of the sulfur atom. For example, chemical shifts of methyl and methylene protons flanking the sulfur atom in glucoerucin (2.117 and 2.607 ppm), glucoraphanin (2.713 and 2.956 ppm), and glucocheirolin (3.133 and 3.999 ppm) (Table 3) appear to correlate with S, SO, and SO_2 functionalities, respectively. In addition, methylene protons attached to S and SO_2 groups appear as a degenerate triplet (J = 7 Hz), whereas two methylene protons attached to SO show unequal chemical shifts in both glucoraphanin (broad multiplet centered at 2.96 ppm) and glucoiberin (multiplets at 3.052 and 2.982 ppm). These unequal chemical shifts arise from the chirality of the SO group.

Mass Spectra of Glucosinolates

FAB mass spectra. Various forms of FAB mass spectrometry (sometimes in combination with more complex instrumentation) have been used successfully for the determination of the masses of the molecular ions of a number of glucosinolates (34, 35, 37). When the ammonium salts of standard glucosinolates were subjected to negative ion FAB in the presence of a triethanolamine matrix (monothioglycerol in the case of **13**), the appropriate $[M—H]^-$ ions were observed without difficulty (Table 2). The ion masses for 13 glucosinolates varied from *m/z* 358 (sinigrin) to *m/z* 477 (neoglucobrassicin). Since many of the known glucosinolates have different masses, negative ion FAB is an important diagnostic tool for obtaining initial information on the identity of isolated glucosinolates.

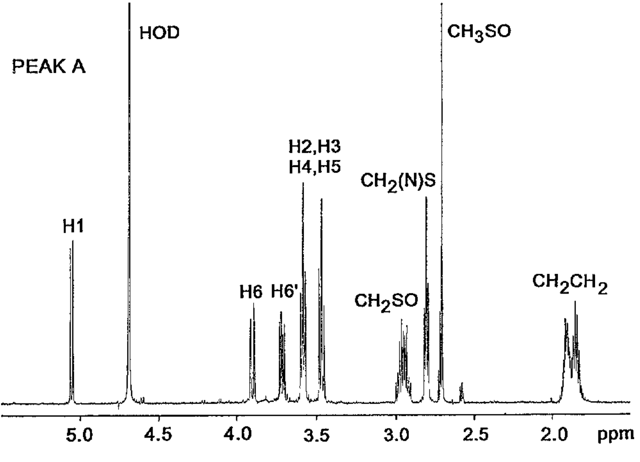
TABLE 2

Negative Ion FAB and Ammonia CI Mass Spectra of Glucosinolates: Accurate Mass Determinations

No	Glucosinolate	FAB $(M-H)^-$	Nominal masses of Principal CI Peaks	R fragments						Glucose		Other fragments	
				$[R-CN]^{\cdot +}$	$[R-CN \cdot H]^+$	$[R-CH=NOH]^{\cdot +}$	$[R-CN.NH_4]^+$	$[R-CH=NOH.H]^+$	$[R-CH=NOH:NH_4]^+$	180 *180.0872*	214 *214 0749*		
1	Glucoiberin	**422**	132, 149	—	**132.0480**	—	**149.0746**	—	—	**180.0875**			
				131.0405	*132.0483*	*149.0511*	*149 0749*	*150 0589*	*167.0854*				
2	Glucoraphanin	**436**	146; 163	—	**146.0640**	—	**163.0906**	—	—	**180.0870**	**214.0747**		
				145.0561	*146.0640*	*163.0667*	*163.0905*	*164.0745*	*181.1011*				
3	2-Hydroxyethylglucosinolate	**362**	90, 107	—	**72.0450**	—	—	**90.0555**	**107.0823**	**180.0875**	**214.0754**		
				71.0371	*72.0449*	*89 0477*	*89 0715*	*90.0555*	*107.0821*				
4	Glucocheirolin	**438**	165; 183	—	—	—	**165.0699**	**166.0539**	**183.0807**		**214.0754**		
				147 0354	*148.0432*	*165.0460*	*165.0698*	*166.0538*	*183 0803*				
5	Progoitrin	**388**	115; 116; 133	—	**98.0606**	—	**115.0872**	**116.0713**	**133.0974**				
				97.0528	*98 0606*	*115 0633*	*115 0871*	*116.0712*	*133.0977*				
6	Sinigrin	**358**	85; 86; 103	—	—	—	**85.0766**	**86.0604**	**103.0869**	**180.0877**			
				67.0422	*68.0500*	*85.0528*	*85.0766*	*86.0606*	*103 0871*				
7	Glucosinalbin	**424**	107; 151; 152	—	—	**151.0633**	**151.0875**	**152.0712**	**169.0980**	**180.0873**		**107.0498**	HO—⟨ring⟩—$CH_2^{\oplus}$
				133 0528	*134.0606*	*151.0633*	*151.0871*	*152 0712*	*169.0977*			*107.0497*	
8	Gluconapin	**372**	99, 100; 117	—	—	**99.0670**	**99.0923**	**100.0762**	**117.1029**				
				81.0578	*82.0657*	*99.0684*	*99.0922*	*100.0762*	*117 1028*				
10	Glucobrassicanapin	**386**	114; 131	—	**96.0814**	—	**113.1077**	**114.0919**	**131.1186**	**180.0867**	**214.0745**		
				95 0735	*96.0813*	*113 0841*	*113 1079*	*114.0919*	*131.1184*				
11	Glucoerucin	**420**	147, 148	—	**130.0688**	**147.0721**	**147.0953**	**148.0796**	**165.1065**				
				129.0612	*130 0690*	*147 0718*	*147 0956*	*148.0796*	*165.1062*				
12	Glucotropaeolin	**408**	135; 136; 153	—	—	**135.0682**	**135.0924**	**136.0764**	**153.1027**	**180.0868**	**214.0749**	**91.0550**	⟨ring⟩—$CH_2^{\oplus}$
				117.0578	*118 0657*	*135.0684*	*135 0922*	*136.0762*	*153.1028*			91 0548	
13	Glucobrassicin[a]	**447**	156; 174	**156.0690**	**157.0762**	—	**174.1029**	—	—	**180.0875**		**130.0654**	indole (N–H)–$CH_2^{\oplus}$
				156 0687	*157.0766*	*174.0793*	*174.1031*	*175.0871*	*192.1137*			*130 0657*	
14	Neoglucobrassicin	**477**	187; 205	—	**187.0872**	—	**204.1135**	**205.0975**	—	**180.0870**		**160.0763**	indole ($N-OCH_3$)–$CH_2^{\oplus}$
				186 0793	*187 0871*	*204.0899*	*204 1137*	*205.0977*	*222 1243*			*160 0762*	

Note. **Bold**, measured masses; *italic*, calculated masses; —, ion not detected.

[a] FAB was performed with monothioglycerol as matrix for this compound because triethanolamine has a prominent matrix peak at 446 amu.



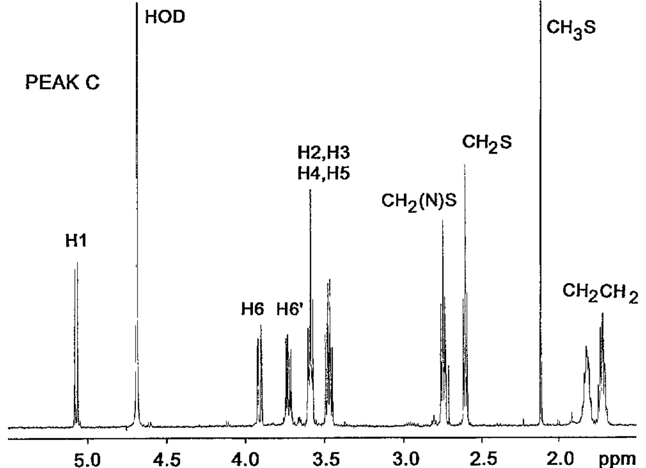


FIG. 4. The 600-MHz NMR spectra of peaks A and C obtained by PIC from aqueous broccoli extracts (see Fig. 3). Spectra were obtained in D_2O as described under Materials and Methods. Note that the chirality of SO (sulfoxide) in peak A induces a multiplet for CH_2SO, not observed for the CH_2S group (peak C). Peak A is glucoraphanin (**2** in Table 1) and peak C is glucoerucin (**11** in Table 1).

Ammonia CI mass spectra. Most glucosinolates gave rise to prominent ions at nominal masses of *m/z* 180 and *m/z* 214 (Table 2). Both of these fragments are derived from the β-thioglucoside moieties, as previously reported (33). We have confirmed the correctness of the proposed assignments of these ions by accurate mass measurements:

$$R{-}C(=N{-}O{-}SO_2{-}O^-){-}\beta{-}S{-}C_6H_{11}O_5 \quad \begin{cases} +NH_4^+ + H = (m/z\ 214) \\ +NH_3 = (m/z\ 180) \end{cases}$$

Thus the observed range of masses of *m/z* 180.0867–180.0877 (9 compounds) is in excellent agreement with the calculated mass of *m/z* 180.0872 for $[C_6H_{11}O_5 + NH_3]^+$. Furthermore, the range of masses of *m/z* 214.0745–214.0754 (5 compounds) corresponds to $[C_6H_{11}O_5 + S + NH_3 + 2H]^+$ or $[C_6H_{11}O_5SH + NH_4]^+$ (calcd *m/z* 214.0749).

Certain radical cations (see a and c below) have been proposed as diagnostic fragments generated under CI conditions (33). For ammonia CI, some of these fragments cannot easily be distinguished from ammonia addition or other processes leading to the formation of fragments such as b, d, e, and f:

$[R{-}CN]^{\cdot+}$	$[R{-}CN{:}H]^+$	$[R{-}CH{=}NOH]^{\cdot+}$	$[R{-}CN{:}NH_4]^+$
(R + 26)	(R + 27)	(R + 44)	(R + 44)
a	b	c	d

$[R{-}CH{=}NOH{:}H]^+$	$[R{-}CH{=}NOH{:}NH_4]^+$
(R + 45)	(R + 62)
e	f

Hence the diagnostically useful ion $[RCN + 18]^+$ may be either c or d (38). Nevertheless, accurate mass determinations on fragment ions show conclusively that contrary to previous suggestions (38) for the majority of glucosinolates the radical cations a and c are not the most frequently observed or most abundant ion species (Table 2). The most commonly observed ion was d (12/13 compounds), followed by ions e (10/13 compounds) and f (9/13 compounds). In contrast, of the 13 glucosinolates analyzed, radical cation c was observed for only 4 and a for only 1 compound. These findings are at variance with those of Eagles *et al.* (33) and may be attributable to the somewhat different technique (direct desorption CI) used in our studies. Consequently, once the $[M{-}H]^-$ ion has been identified by negative-ion FAB mass spectrometry, an early fruitful strategy for identifying an unknown glucosinolate will involve search for ion d $[R{-}CN{:}NH_4]^+$ which should have the nominal mass of $(M + NH_4 - 273)$. An accurate mass determination of this diagnostic ion should then lead to definitive information on the identity of the R group. For reasons that are unclear we did not observe any fragment ions on ammonia CI of compound **9.**

Table 2 also shows that diagnostic aromatic and indole fragments were observed in ammonia CI of certain glucosinolates, therefore providing additional confirmation of the structure of the R group.

High-Resolution Proton NMR Spectra of Glucosinolates

It is clear from the discussion above that the identification of an isolated glucosinolate is straightforward by the use of mass and NMR spectroscopy. In order to establish a data base of NMR assignments, all glucosi-

TABLE 3

^{1}H NMR Chemical Shift Assignments (ppm) of Glucosinolates at 600 MHz in D_2O

Compound No.	R Group							Glucose 1	2	3	4	5	6	6′
3						DO—CH_2	—CH_2—							
						3.948	2.969	5.078	3.476	3.573	3.476	3.587	3.915	3.727
1			CH_3—SO	—CH_2—	—CH_2—	—CH_2—								
			3.052											
			2.734	2.982	2.211	2.945		5.066	3.477	3.578	3.474	3.593	3.916	3.727
4			CH_3—SO_2	—CH_2—	—CH_2—	—CH_2—								
			3.133	3.999	2.282	2.957		5.068	3.486[b]	3.576	3.474[b]	3.589	3.916	3.731
2			CH_3—SO	—CH_2—	—CH_2—	—CH_2—	—CH_2—							
			2.713	2.956	1.915	1.853	2.811	5.043	3.472	3.575	3.472	3.57	3.910	3.722
11			CH_3—S	—CH_2—	—CH_2—	—CH_2—	—CH_2—							
			2.117	2.607	1.727	1.827	2.765	5.059	3.479[b]	3.575	3.469[b]	3.578	3.910	3.726
6			CH_2=	=CH—	—CH_2—									
			5.335											
			5.292	6.042	3.525			5.062	3.454[b]	3.543	3.470[b]	3.553	3.919	3.726
8			CH_2=	=CH—	—CH_2—	—CH_2—								
			5.174											
			5.101	5.945	2.490	2.830		5.037	3.497[b]	3.570	3.472[b]	3.563	3.904	3.721
5			CH_2=	=CH—	—CHOD—	—CH_2—								
			5.356			2.990								
			5.253	5.981	4.657	2.917		5.195	3.476	3.574	3.484	3.573	3.902	3.726
10			CH_2=	=CH—	—CH_2—	—CH_2—	—CH_2—							
			5 141											
			5.076	5.932	2.186	1.827	2.725	5.107	3.465[b]	3.562	3.481[b]	3.553	3.896	3.726
7				Hζ	Hε	Hδ	—CH_2—							
				—	7.290	6.937	4.070	4.74	3.34[a]	3.34[a]	3.423	3.252	3.694	3.661
12				Hζ	Hε	Hδ	—CH_2—							
				7.43[a]	7.43[a]	7.43[a]	4.162	4.71	3.33[a]	3.33[a]	3.415	3.268	3.691	3.646
13		Hη	$Hζ_3$	$Hζ_2$	Hε	Hδ	—CH_2—							
						4.296								
		7.299	7.222	7.558	7.781	7.371	4.208	4.828	3.303	3.204	3.360	2.951	3.58	3.58
14	CH_3O						4.259							
	4.127	7.390	7.259	7.601	7.786	7.552	4.187	4.823	3.324	3.219	3.371	3.005	3.52	3.57
9							4.522							
		7.09[a]	7.09[a]	6.571	—	7.189	4.378	4.919	3.315	3.200	3.407	2.940	3.579	3.507

[a] Strong coupling.
[b] Assignment can be interchanged.

nolate samples listed in Table 1 were subjected to high-resolution NMR analysis.

The proton chemical shifts of glucosinolates are summarized in Table 3. Proton resonance assignments were initially based on covalent structure of the R groups as well as on the analysis of the coupling patterns in the 1D proton NMR spectra. With the exception of 2-hydroxyethyl glucosinolate (**3**), the compounds in Table 1 could be assigned to four groups on the basis of their R-group structure. One member from each

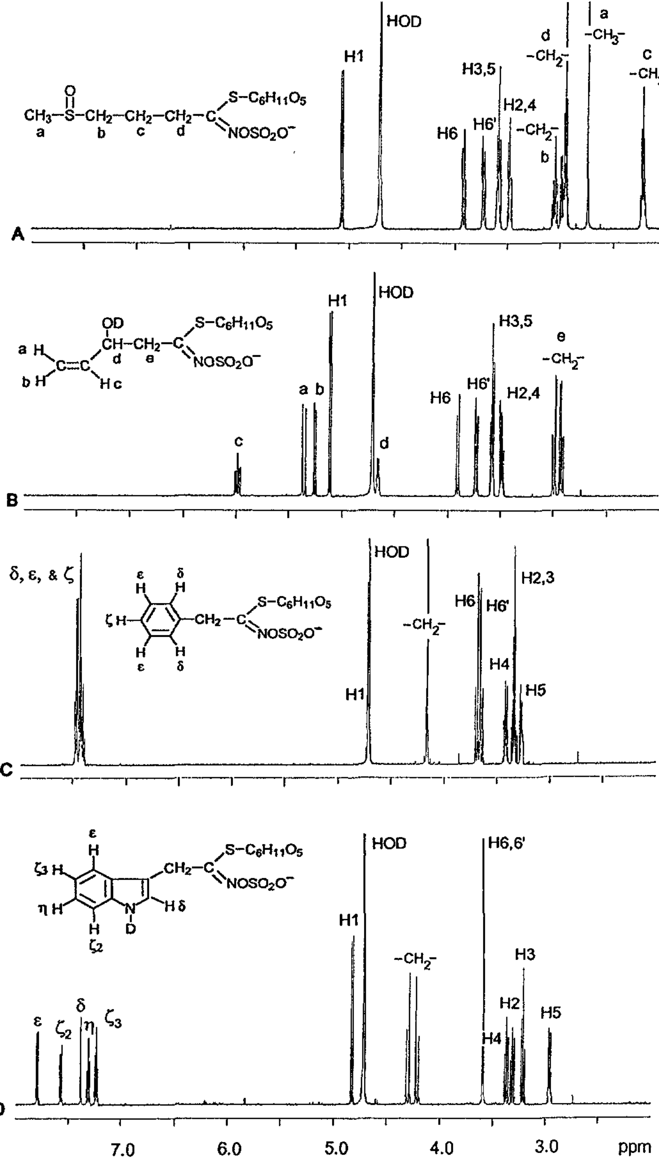


FIG. 5. The 600-MHz NMR spectra in D_2O of: (A) glucoiberin (**1** in Table 1); (B) progoitrin (**5**); (C) glucotropaeolin (**12**); and (D) glucobrassicin (**13**). Note that the resonance patterns for the β-thioglucoside moiety of the aliphatic glucosinolates (A and B) and those of the aromatic glucosinolates (C and D) are markedly different, presumably because of ring currents arising from the stacking of the aromatic and glucose rings.

group was then selected and assignments were subsequently confirmed by the 1D difference decoupling method. Assignments of the remaining members of each class were made by chemical shift analogy. Figure 5 shows the 1D spectra of four representative glucosinolate samples in D_2O at 32°C.

The chemical shifts for the glucose protons in glucosinolates with aliphatic R groups show similar values (Table 3) and appeared to be independent of the structure of the R group (Figs. 5A and 5B). Glucosinolate samples with aromatic R-groups (Figs. 5C and 5D), on the other hand, showed significant changes in chemical shifts of glucose protons. Although the extent of these shifts and the patterns are different for phenyl (**7** and **12**) and indole (**9, 13,** and **14**) derivatives, their sugar protons showed upfield shifts, presumably due to ring current effects.

Figure 5C shows the proton NMR spectrum of glucotropaeolin (**12**) for which strong coupling and overlap complicate the analysis. Anomeric proton (H1) shows distorted multiplet structure due to strong coupling of H2 and H3 and partially overlaps with the HOD signal. Figure 5D shows the proton spectrum of glucobrassicin (**13**) where all the protons gave well-resolved multiplets except for H6 and H6′ of glucose. The indole protons were assigned by the analysis of the coupling pattern as well as NOEs between CH_2 and Hε as well as Hδ. NOESY also showed weak interactions between H1 of glucose to CH_2 and Hδ, as well as H3, to Hε of the indole ring (data not shown). Qualitative interpretation of these NOE connectivities implies that stacking interactions occur between the hydrophobic faces of the glucose ring and indole ring. To our knowledge, this is the first observation of such interactions of sugar molecules with aromatic rings in solution.

CONCLUSIONS

In this study we have described a general sequential strategy for the direct isolation, identification, and quantitation of intact glucosinolates from crude plant extracts. This procedure entails reverse-phase paired-ion chromatography of the hydrophobic tetraalkyl ammonium salts of the glucosinolates in the presence of an excess of these counterions, conversion of these glucosinolate salts to their ammonium salts, direct FAB and CI mass spectroscopic analysis, and finally ^{1}H NMR spectroscopy. Enzymatic hydrolysis with myrosinase is used to confirm the presence of glucosinolates by their conversion to isothiocyanates, which are quantitated by cyclocondensation with 1,2-benzenedithiol. The procedure is relatively rapid, requires no highly specialized equipment, and obviates the need for derivatization and/or removal of the sulfate group from the glucosinolates.

Not all the above-mentioned spectroscopic techniques are required to establish the identity of most glucosinolates. A survey of about 80 known glucosinolates reveals that about one-half can be unequivocally identified by their unique nominal $[M-H]^-$ ion masses obtained by negative-ion FAB. For some glucosinolates, however, this is not possible. For instance, there are three naturally occurring glucosinolates consistent with a nominal $[M-H]^-$ value of *m/z* 450: (a) 4-methylsulfonyl-3-butenyl-GS (accurate mass *m/z* 450.019,655), (b) 4-phenylbutyl-GS (*m/z* 450.089,253), and (c) 5-methylsulfinylpentyl-GS (*m/z* 450.056,240).

On accurate mass determination of fragment ions (most commonly $[R—CN:NH_4]^+$) these three possible glucosinolates can be distinguished. For some isomeric glucosinolates, however, knowledge of the accurate fragment masses cannot unequivocally establish the structure. For instance, 2-methyl-2-propenyl-GS and but-3-enyl-GS have identical $[M—H]^-$ with *m/z* 372.042,303, and consequently, final identification must be made by high-resolution NMR spectroscopy. Thus, the proposed sequential strategy should be widely applicable to the analysis of glucosinolates from plants.

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