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GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF GLUCOSINOLATES*

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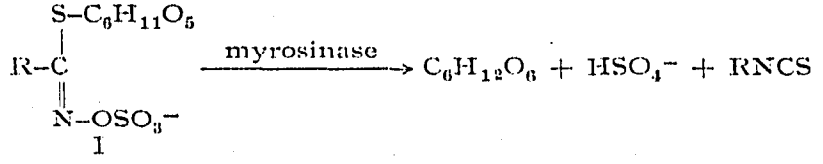
SUMMARY

A method has been developed for the qualitative assay of glucosinolates by gas chromatography. Silylation of eleven glucosinolates resulted in the formation of the trimethylsilyl derivatives of the corresponding desulfoglucosinolates and all could be completely separated using two silicone columns. The trimethylsilyl derivatives of four glucosinolates extracted from rapeseed meal were well resolved on a single column and preliminary results of their quantitative determination compared favorably with results obtained by an independent assay.

INTRODUCTION

More than fifty glucosinolates or mustard oil glucosides are known to occur in higher plants (for review see ref. 1) and all are anions of the general structure (I).



They are characterized further by their ability to be hydrolyzed by the enzyme myrosinase (EC 3.2.3.1) to yield D-glucose, sulfate and an isothiocyanate aglycone (mustard oil); if the released aglycone possesses an hydroxyl on the β-carbon cyclization occurs and a substituted 2-oxazolidinethione forms. Generally, glucosinolates are difficult to crystallize and the identity of the products formed upon myrosinase hydrolysis is often an important starting point in their structural analysis. In addition, quantitative analytical methods are based entirely upon analysis of one or more of the enzymic fission products (*cf.* refs. 2–5 and references contained therein); no methods have been reported for quantitative analysis of mixtures of non-hydrolyzed glucosinolates.

Paper chromatographic (PC) methods introduced by SCHULTZ *et al.*[6,7] have been used extensively for separation and identification of glucosinolates present in plant extracts. WAGNER *et al.*[8] and MATSUO[9] have described a number of solvent systems

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which have been employed for glucosinolate separation. Gas–liquid chromatography (GLC) of allylglucosinolate (sinigrin) following treatment with hexamethyldisilazane (HMDS) and trimethylchlorosilazane (TMCS) in pyridine has been reported[10]. However, three components were eluted from the column, none of which were identified. Since GLC offers the potential advantages of greater resolution and sensitive quantitation of the glucosinolates directly, we have examined the possibility of utilizing this technique as a supplement to PC and thin-layer chromatography (TLC).

In this communication we report the GLC separation of the trimethylsilyl (TMS) derivatives of eleven glucosinolates, the identification of the eluted material, and the results of some preliminary investigations on quantitation of glucosinolates in rapeseed meals.

EXPERIMENTAL

Glucosinolates

p-Hydroxybenzylglucosinolate and methylglucosinolate were obtained from Calbiochem. Allylglucosinolate, 3-butenylglucosinolate and 2-hydroxy-3-butenylglucosinolate were gifts from Dr. L. R. WETTER of this laboratory. Other glucosinolates were isolated[11] from appropriate plant sources. Desulfoallylglucosinolate was a gift from Mr. M. D. CHISHOLM of this laboratory. The remaining desulfoglucosinolates were synthesized according to reported procedures[11,12].

Gas chromatography

A Hewlett-Packard model 5754 gas chromatograph equipped with dual flame ionization and thermal conductivity detectors was used. Injector and detector temperatures were maintained at 270°. Flow rates were 30 ml/min for helium (carrier gas), 20 ml/min for hydrogen and about 250 ml/min for air. Stainless steel columns (6 ft. × 1/8 in. O.D.), one packed with 1.5% SE-52 on 80–100 mesh, acid-washed, DMCS-treated Chromosorb G, the other with 1.5% OV-225 on the same solid support, were operated isothermally at 230° and 225°, respectively. Peak areas were determined using an Infotronics model CRS-104 digital integrator. Compounds were collected by condensation in 1/8 in. O.D. glass tubes inserted in the thermal conductivity detector exit port.

Trimethylsilylation

Preparative scale. The glucosinolate or desulfoglucosinolate (100 mg) in anhydrous pyridine (5 ml) was treated with 3 ml HMDS and 1.5 ml TMCS in a 100 ml round bottom flask, tightly sealed with a clamped ground glass stopper, at 105° overnight. The excess reagents were removed *in vacuo*. The residue was taken into chloroform and after filtration was either distilled under vacuum (0.05 mm) at 145–160° or fractionated by chromatography on the SE-52 column.

Analytical scale. Desulfoglucosinolates and glucosinolates (0.1–5 mg) in 1 ml of anhydrous pyridine were treated with 0.1 ml HMDS and 0.05 ml TMCS in 5 ml vials sealed with teflon-lined screw caps at 105° overnight, unless indicated otherwise.

Glucosinolate extraction from rapeseed meal

Boiling water (2 ml) was added to ground and defatted seed meal (100 mg) in a 15 ml centrifuge tube which had previously been placed in a boiling water bath. The

tube was capped and the heated extraction continued for 1.5 h. The mixture was filtered through a cotton plug and a 0.4 ml aliquot was transferred to a 5 ml vial. A second 0.4 ml aliquot was removed and treated with myrosinase for 3 h when glucosinolate hydrolysis was required. Most of the water was evaporated from the aliquot under a stream of warm air and remaining traces of moisture removed *in vacuo* at 60°. The residue was silylated as described above (*Analytical scale*).

Infrared spectroscopy

IR spectra of TMS derivatives of glucosinolates and desulfoglucosinolates were determined using a Perkin Elmer model 257 grating IR spectrophotometer equipped with a beam condenser and attenuator and using silver chloride microcells with 0.01 mm path length (Beckman "Extrocells").

Nuclear magnetic resonance spectroscopy

NMR spectra of TMS derivatives of glucosinolates and desulfoglucosinolates in $CDCl_3$ were recorded using a Varian HA-100 high resolution spectrometer. Tetramethylsilane and formic acid were used as internal and external standards, respectively.

Thin-layer and paper chromatography

Chromatograms were developed on Silica Gel F_{254} using *n*-butanol–*n*-propanol–acetic acid–water (3:1:1:1)[8] and on Whatman No. 1 paper using *n*-butanol–acetic acid–water (4:1:1.8). TMS derivatives of glucosinolates and their desulfo analogues, isolated after GLC or distillation, were hydrolyzed in 60% methanol under reflux for 2 h before application to the chromatograms. Alkaline silver nitrate[13] was used to detect the spots on paper chromatograms. Spots on thin-layer chromatograms were visualized as UV absorbing areas on a fluorescent background. R_F values for allylglucosinolate, benzylglucosinolate and 2-phenylethylglucosinolate on paper chromatograms were 0.11, 0.29, and 0.48; whereas the respective desulfoglucosinolates had R_F values of 0.48, 0.65 and 0.68. TLC of these glucosinolates gave R_F values of 0.46, 0.59 and 0.62; and 0.75, 0.81 and 0.83 for the respective desulfoglucosinolates.

RESULTS

Preliminary investigation indicated that derivatization of a glucosinolate at elevated temperatures overnight resulted in the elution of a single symmetrical peak from an SE-52 column. The extent of derivatization of benzylglucosinolate was determined from the flame detector response factor obtained using a distilled sample of the silylated glucosinolate and *n*-hexacosane (internal standard). When the reaction mixture was heated at 105° overnight, derivatization was 80% complete, whereas lower temperatures and shorter periods of time resulted in considerably lower yields of product and the appearance of multiple peaks on the chromatogram. Similar results with allylglucosinolate confirmed that derivatization at 105° overnight gave a single TMS derivative in reproducible yield and suggested that quantitation was feasible.

In these preliminary studies it was observed that the retention times of the TMS derivatives of allylglucosinolate, benzylglucosinolate and 2-phenylethylglucosinolate were the same as the retention times of the TMS derivatives of their corresponding

desulfoglucosinolates. Desulfation of glucosinolates during derivatization with HMDS and TMCS in the presence of pyridine could be expected, since ETTLINGER AND DATEO[14] have found that alcoholysis of the pyridinium salt of a glucosinolate leads to its desulfation and the formation of an alkyl sulfate. The fact that glucosinolates do indeed undergo desulfation during derivatization was established by demonstrating that the TMS derivative of a glucosinolate is identical to the TMS derivative of its corresponding desulfoglucosinolate.

Identification of the glucosinolate and desulfoglucosinolate derivatives was carried out as follows: the TMS derivatives (50–100 mg) of allylglucosinolate, benzylglucosinolate and 2-phenylethylglucosinolate and their desulfo analogues were prepared in duplicate. One sample of each duplicate was recovered by distillation under reduced pressure and the other collected from the gas chromatograph. Both the IR and NMR spectra of each silylated glucosinolate and its desulfo analogue obtained by GLC and by distillation were identical. Integration of the NMR proton signals led to the conclusion that each derivative possessed five TMS groups. Integration of the NMR spectrum of the TMS derivative of 2-hydroxy-2-phenylethylglucosinolate confirmed the presence of six TMS groups, as expected. Aliquots of each of the TMS derivatives collected from the gas chromatograph were refluxed in 60 % methanol to remove the TMS groups and the hydrolysis products were subjected to PC and TLC. In all cases the R_F values of the hydrolysis products, whether from the collected TMS glucosinolates or the collected TMS desulfoglucosinolates, corresponded with the R_F values of the corresponding desulfoglucosinolates and not with those of the original glucosinolates.

The KOVATS indices[15] and retention times of TMS derivatives of eleven glucosinolates on a 1.5% SE-52 column are listed in Table I. Figs. 1A, 1B, 2A and 2B are tracings of gas chromatograms obta ned under similar experimental conditions. Good resolution of the mixture of eight glucosinolates (Fig. 1A) was observed except for TMS-2-phenylethylglucosinolate (peak 6) and TMS-2-hydroxy-2-phenylethylglucosinolate (peak 7). This partially resolved pair could be completely separated by chro-

TABLE I

GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF GLUCOSINOLATES[a]

A 6 ft. × 1/8 in. O.D. column packed with 1.5 % SE-52 on acid-washed, DMCS-treated Chromosorb G, 80/100 mesh, was employed. Instrument conditions are given in the text.

Glucosinolate TMS derivative	*Peak No.*[a]	*Kovats index*	*Relative retention time*
Methyl	1	2315	0.30
Allyl	2	2355	0.34
3-Butenyl	3	2410	0.41
4-Pentenyl	9	2482	0.52
2-Hydroxy-3-butenyl	10	2528	0.59
2-Hydroxy-2-methylpropyl	4	2551	0.63
Benzyl	5	2700	1.00
2-Phenylethyl	6	2820	1.43
2-Hydroxy-2-phenylethyl	7	2847	1.50
p-Hydroxybenzyl	8	2942	2.10

[a] Numbers correspond to glucosinolate peaks in Figs. 1 and 2.

matography on a column packed with 1.5 % OV-225 on Chromosorb G at 225°; a retention time of 0.73 for TMS-2-hydroxy-2-phenylethylglucosinolate relative to TMS-2-phenylethylglucosinolate (eluted in approximately 9 min) was observed. The TMS derivatives of allylglucosinolate and those of a purified extract of rapeseed glucosinolates were used to obtaind the tracing 1B. From the retention times of glucosinolate standards, peaks 2, 3 and 10 were established as the TMS derivatives of allylglucosinolate, 3-butenylglucosinolate and 2-hydroxy-3-butenylglucosinolate, respectively. 4-Pentenylglucosinolate and its 2-hydroxy analogue were known to be present

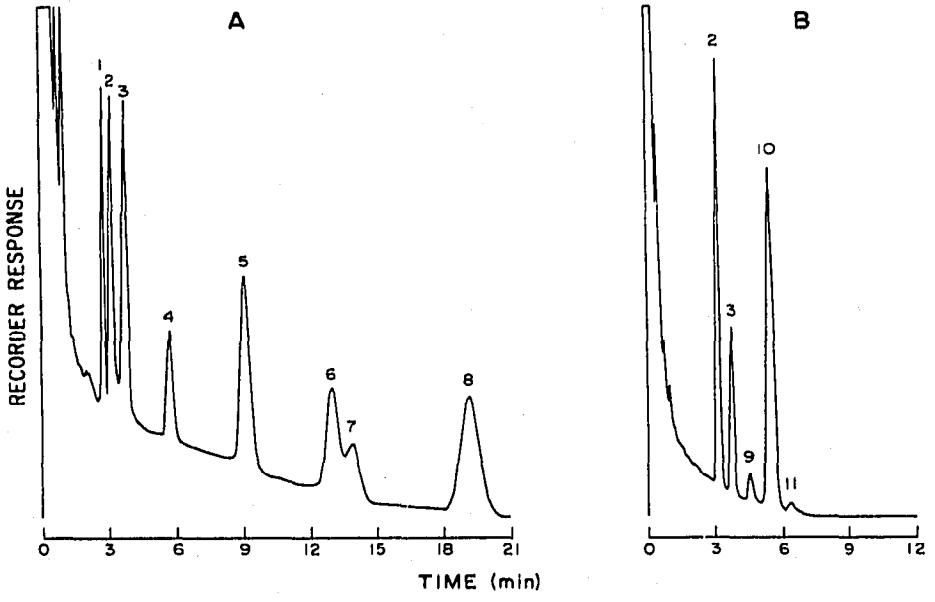

Fig. 1. Gas–liquid chromatogram of TMS derivatives of glucosinolates. (A) Synthetic mixture of 8 glucosinolates. (B) Mixture of allylglucosinolate and purified extract of rapeseed glucosinolates. Peak numbers of glucosinolates refer to those listed in Table I.

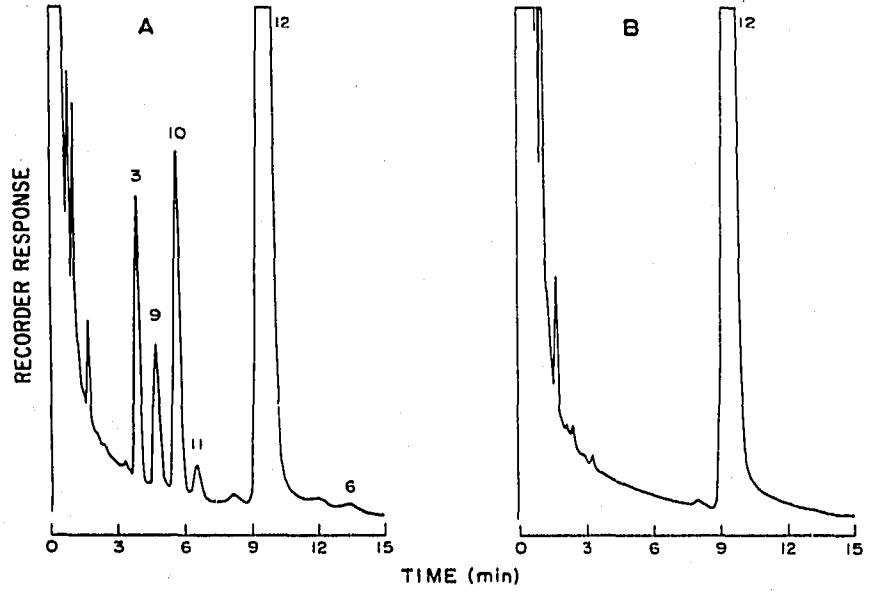

Fig. 2. (A) Gas–liquid chromatogram of TMS derivatives of glucosinolates extracted from rapeseed meal. (B) Myrosinase added to extract prior to derivatization and GLC.

in the glucosinolate mixture. We have established peak 9 to be derived from TMS-4-pentenylglucosinolate since a plot of log retention time against carbon number was linear for peaks 2, 3 and 9, indicating an homologous series. The minor peak 11, which was not present when the glucosinolate mixture was treated with myrosinase prior to derivatization, is believed to be TMS-2-hydroxy-4-pentenylglucosinolate.

Figs. 2A and 2B are chromatographic tracings obtained after derivatization of two aliquots from a rapeseed meal extract. No myrosinase activity was present in aliquot 2A whereas myrosinase was added to aliquot 2B to hydrolyze the glucosinolates prior to derivatization. Enzymic hydrolysis would thus remove glucosinolates from aliquot 2B, and provide a GLC tracing (following the usual derivatization procedure) which would represent an analytical blank. A comparison of Figs. 2A and 2B confirms that no interfering compounds were eluted from the column (2B) at retention times corresponding to the elution of the TMS glucosinolates (2A). The TMS-glucose peak resulting from derivatization of liberated glucose following glucosinolate hydrolysis is eluted with the solvent and thus is not evident in the tracing (2B). Peak 12 has the same retention time as octa-O-(trimethylsilyl)-sucrose. Other oil-free meal extracts including *Crambe abyssinica, Brassica juncea* and *B. campestris* variety yellow sarson, when treated in a similar manner showed the presence of the known glucosinolates in the myrosinase free aliquot. Only minor, if any, amounts of interfering substances were eluted from the myrosinase treated aliquot at retention times corresponding to the TMS glucosinolates.

The amount of glucosinolates in a number of rapeseed meal samples was determined by the method of YOUNGS AND WETTER[5] and by GLC analysis of the TMS glucosinolate derivatives (see Table II). The method of YOUNGS AND WETTER is based on combined GLC and UV assays of the released glucosinolate aglycones; the total amount of 2-hydroxy-3-butenylglucosinolate and 2-hydroxy-4-pentenylglucosinolate is determined by UV assay of their aglycones and calculated in terms of the former glucosinolate. In calculating the relative mole composition of the glucosinolates present in the silylated samples, and lacking pure standards of these glucosinolates, we have assumed that the extent of derivatization would be the same for each of the four glucosinolates assayed and that their mole composition would be proportional to the

TABLE II

RELATIVE GLUCOSINOLATE COMPOSITION OF RAPESEED MEALS

Meal sample	*Mole % glucosinolate composition determined by*						
	Method of Youngs and Wetter			*GLC assay of TMS derivatives*			
	3-Butenyl	*4-Pentenyl*	*2-Hydroxy-3-butenyl*	*3-Butenyl*	*4-Pentenyl*	*2-Hydroxy-3-butenyl*	*2-Hydroxy-4-pentenyl*
1	31.1 ± 0.14[a]	7.6 ± 0.06	61.3 ± 0.18	26.9 ± 0.11	4.5 ± 0.08	67.3 ± 0.18	1.3 ± 0.04
2	31.9 ± 0.12	43.8 ± 0.24	24.3 ± 0.21	31.4 ± 0.10	40.4 ± 0.18	24.7 ± 0.16	3.5 ± 0.08
3	33.2	19.3	47.5	30.5	16.8	50.7	2.1
4	64.7	29.0	6.3	65.0	28.9	6.1	Trace
5	32.2	38.5	29.3	30.7	41.2	23.0	5.1
6	26.7	6.3	67.0	27.1	4.4	67.3	1.2

[a] Standard error of the mean obtained from 5 replicate analyses.

peak areas divided by the number of carbon atoms present in the glucosinolate TMS derivative.

The rapeseed meals selected for analysis were chosen because of the presence of varying proportions of the four glucosinolates. It is apparent from the standard errors that good reproducibility was obtained in both the derivatization and the subsequent GLC analysis. In general, the overall composition of the meals determined by TMS glucosinolate analysis was similar to that obtained using the method of YOUNGS AND WETTER. However, the two methods of analysis did not give identical results. We believe that further experiments to establish correction factors may increase the accuracy of the quantitative assay employing TMS derivatives.

DISCUSSION

Our results demonstrate that direct analysis of glucosinolates in plant extracts is possible by gas chromatography. However, it must be noted that a glucosinolate cannot be distinguished from its desulfo analogue by GLC after silylation. Caution must be exercised in interpretation of GC data obtained using plant extracts since desulfoglucosinolates may occur in the same plant as the glucosinolates. Such an occurrence is in fact probable on the basis of studies on glucosinolate biosynthesis[11] where it has been shown that desulfobenzylglucosinolate is a precursor of benzylglucosinolate in *Tropaeolum majus*. To ascertain whether a particular peak on a chromatogram was derived from a glucosinolate or not, one need only treat another aliquot of the plant extract with myrosinase prior to derivatization. Disappearance of the peak in the myrosinase treated aliquot is indicative of a glucosinolate. However, a peak due to a desulfoglucosinolate would not disappear since ETTLINGER AND LUNDEEN[16] and we (unpublished data) have observed that desulfoglucosinolates are not hydrolyzed by the enzyme. At the attenuation of the electrometer signal required for analysis of the TMS derivatives of the rapeseed glucosinolates reported here, we were not able to detect the presence of desulfoglucosinolates in the enzyme treated controls.

The large number of glucosinolates differing by a single methylene carbon is an unusual feature of this group of plant products and GLC offers one of the most effective methods of separating such homologues. In this communication, for example, we report the separation of five glucosinolates representing three different homologous series (compounds 2, 3 and 9; 10 and 11; 5 and 6). In a recent study on glucosinolate biosynthesis, as yet unpublished, we have successfully separated and quantitated with high sensitivity the TMS derivatives of ^{14}C-labeled 2-phenylethylglucosinolate and 2-hydroxy-2-phenylethylglucosinolate.

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