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SEPARATION OF DESULPHOGLUCOSINOLATES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of glucosinolates as their corresponding desulphoglucosinolates by high-performance liquid chromatography is reported. Reversed-phase chromatography on a C_{18} column using gradient elution with acetonitrile gave good separation of glucosinolates normally found in *Brassica* species. The procedure has the advantage that no buffer solutions or ion-pairing reagents are required, thus fractions can be collected in a pure form suitable for identification by mass spectroscopy or in milligram quantities for use as primary standards.

INTRODUCTION

The separation and quantification of glucosinolates in Cruciferae has received much attention in recent years[1,2]. The impetus for these studies is the major importance of the nature and levels of glucosinolates in new varieties of oil-seed crops being bred throughout the world. Older varieties of rape-seed contained relatively high levels of glucosinolates which restricted the amount of meal that could be included in animal diets[3], however, the new "Double low" varieties of rapeseed (*Brassica napus* and *Brassica campestris*) produce both high grade oil for human consumption and a protein meal with nutritional qualities rivalling that of soy bean.

Procedures for estimating glucosinolates as their enzyme hydrolysis products (*e.g.*, isothiocyanates), have been in use for many years[4,5]. This approach, however, has the disadvantage that not all glucosinolates can be measured[6]. Glucosinolates can also be estimated after isolation using ion-exchange chromatography and separation of the trimethylsilyl (TMS) derivatives using gas chromatography (GC)[7–9]. This lengthy procedure enables good resolution of the individual glucosinolates.

Intact glucosinolates, purified by ion-exchange methods, have also recently been separated using reversed-phase high-performance liquid chromatography (HPLC)[10] with a solvent system consisting of phosphate buffer and tetraheptylammonium bromide as ion-pairing agent.

A major advance in glucosinolate analysis came with the introduction by Thies[11,12] of on-column desulphation of glucosinolates using the enzyme aryl sul-

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phatase. This simple procedure is becoming widely used since it allows one readily to obtain a fraction containing a mixture of almost pure desulphoglucosinolates. This fraction can then be dried, silylated and the derivatives separated by GC.

This paper presents a new procedure for the separation of desulphoglucosinolates using reversed-phase HPLC. The method is rapid and fractions can easily be collected in a form suitable for mass spectroscopy or as primary standards for both HPLC or GC quantitative procedures.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and methanol were purchased from Ajax Chemicals (Sydney, Australia). Aryl sulphatase, Type H-1 was purchased from Sigma (St. Louis, MO, U.S.A.). Pyridine and TriSil concentrate were obtained from Pierce (Rockford, IL, U.S.A.). Supelcoport (100–120 mesh) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from Supelco (Bellefonte, PA, U.S.A.). DEAE-Sephadex A-25 was from Pharmacia (Uppsala, Sweden).

Glucoiberin, glucocheirolin, glucoerucin, glucocapparin, glucotropaeolin and glucosinalbin were purchased from Carl Roth (Karlsruhe, G.F.R.). Sinigrin was purchased from Serva (Heidelberg, G.F.R.).

Preparation of meal

Myrosinase activity in rape-seed, mustard and cabbage seed samples was destroyed by heating the seeds in a water-bath at 100°C for 3 min. Seeds were then defatted using the homogeniser of a Fosslet 15300 oil determination apparatus. Approximately 6 g of seed were placed in the extraction chamber and extracted with dichloromethane (10 ml) for 20 sec. The pulverised sample was then filtered, washed with hexane to remove residual oil, then air dried.

Isolation of desulphoglucosinolates

Defatted meal (200 mg) was weighed into a centrifuge tube and placed in a water-bath at 100°C for 3 min. Boiling water (4 ml) was then added to the preheated sample and after mixing, was allowed to stand for a further 5 min at 100°C. After cooling and centrifugation, the protein in the supernatant was precipitated by adding a solution (0.5 ml) containing both lead acetate and barium acetate (0.5 *M*). After centrifugation, the clear solution was passed through a 100-mg column of DEAE-Sephadex A-25 which had been washed with 0.5 *M* pyridine acetate and was equilibrated in water. The column was washed with 0.02 *M* pyridine acetate (3 ml) followed by water (2 ml). Aryl sulphatase (0.5 ml, 0.2%) was run into the column and allowed to stand overnight. The desulphoglucosinolates were eluted with water (2 ml), and the eluate freeze dried. The residue was taken up in water (0.2 ml) and filtered through a 0.45-μm Acrodisc (Gelman Sciences), prior to HPLC.

High-performance liquid chromatography

A Spectra-Physics SP8000 single pump, ternary gradient instrument, fitted with a Valco injector, was coupled to a Spectromonitor 111 (Laboratory Data Control) variable-wavelength ultraviolet detector. A wavelength setting of 227.5 nm was

used for all HPLC separations. Chromatograms were recorded and peak areas and retention times measured by means of a SP-4000 chromatography data system. The chromatograph was operated in the constant flow mode at 1.0 ml/min., with an oven temperature of 35°C. A 250 × 4.6 mm Zorbax ODS column (5–6 μm) having 24,500 theoretical plates was purchased from DuPont. Glass distilled water was filtered through a 0.45-μm filter (Millipore). The solvent program consisted of 100% water for 10 min; a gradient of 0 to 12% acetonitrile over the next 30 min and constant 12% acetonitrile for a further 25 min. For collection, 100 μl of the concentrated desulphoglucosinolate solution (corresponding to a 200-mg sample) was injected. HPLC profiles were obtained by injecting 20 μl (corresponding to 2-mg sample) at 0.2 a.u.f.s.

Gas chromatography

Separated fractions from HPLC runs were freeze-dried, taken up in the minimum volume of water, transferred to 1-ml Reacti-Vials and re-dried. The dried desulphoglucosinolates were silylated (120°C, 20 min) using 50 μl of pyridine–BSTFA–TriSil (100:20:2, v/v/v). The separation of glucosinolate TMS derivatives was performed on a Packard 419 gas chromatograph using a 1.2 m × 2 mm glass column packed with 2% OV-7 on Supelcoport (100–120 mesh). The column packing was prepared using the fluidised bed technique[13]. For GC, a nitrogen flow-rate of 20 ml min^{-1} was used and the temperature programmed from 200 to 280°C at 5°C min^{-1}.

Mass spectrometry (MS)

Silylated desulphoglucosinolates were separated on a Jaeggi SE54 WCOT (20 m × 0.32 mm) glass capillary column temperature programmed from 200 to 280°C at 4°C min^{-1}. The column was linked to a Hewlett-Packard 5985 A mass spectrometer through an open coupling. Electron impact mass spectra were obtained using an emission current of 300 mA at 70 V. Anhydrous NH_3 at an ion source pressure of 0.4 Torr was used as chemical ionisation reagent gas with an emission current of 300 mA at 240 V. In both instances all heated zones (GC injector, interface, ion source) were at 250°C.

RESULTS AND DISCUSSION

The elution profile of seven standard glucosinolates (Table I) as the desulpho derivatives is depicted in Fig. 1. As can be seen, peaks are generally well separated with only two sulphur-containing glucosinolates, glucoiberin and glucocheirolin, being incompletely resolved. Sensitivity based on absorption at 227.5 nm is easily sufficient for the determination of profiles in low glucosinolate rape-seed varieties. For the chromatogram in Fig. 1, approximately 4 μg of each glucosinolate derivative were injected onto the column with the detector set at 0.2 a.u.f.s. As expected, under reversed-phase conditions, the desulphoglucosinolates are eluted roughly in order of decreasing polarity. This serves as an aid in the identification of unknown glucosinolates. Provisional identification of glucosinolates for which standards were not commercially available was obtained by collecting the HPLC peaks of interest, drying and then forming the silyl derivatives. Determination of the relative GC retention time of the derivative on OV-7 by comparison with those published by Heaney and Fenwick[2] combined with a knowledge of the glucosinolates known to be present in certain

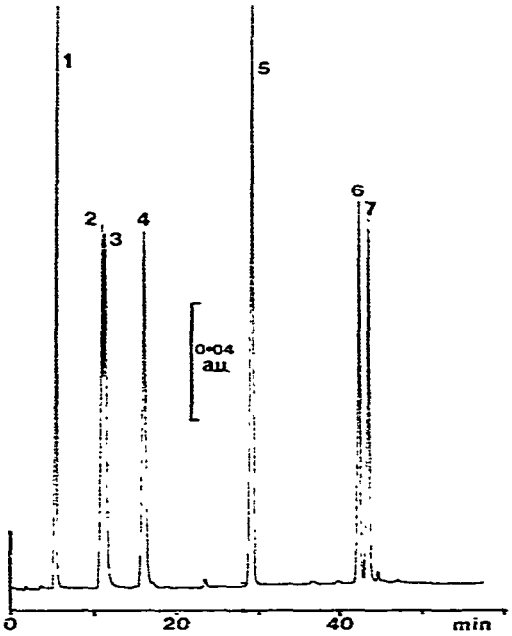
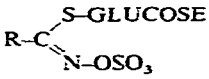


Fig. 1. HPLC elution pattern of desulphoglucosinolates derived from commercially available glucosinolates. Peak numbers refer to the glucosinolates listed in Table I. Chromatographic conditions are described in the text. Detection wavelength: 227.5 nm.

TABLE I

STRUCTURES OF GLUCOSINOLATES



Compound	*R group*	*Name of corresponding glucosinolate*	*Trivial name*
1	CH_3–	Methylglucosinolate	Glucocapparin
2	CH_3–SO–CH_2–CH_2–CH_2–	3-Methylsulfinylpropylglucosinolate	Glucoiberin
3	CH_3–SO_2–CH_2–CH_2–CH_2–	3-Methylsulfonylpropylglucosinolate	Glucocheirolin
4	CH_2=CH–CH_2–	Allylglucosinolate	Sinigrin
5	*p*-$HOC_6H_4CH_2$–	*p*-Hydroxybenzylglucosinolate	Glucosinalbin
6	$C_6H_5CH_2$–	Benzylglucosinolate	Glucotropaeolin
7	CH_3–S–CH_2–CH_2–CH_2–CH_2–	4-Methylthiobutylglucosinolate	Glucoerucin
8	CH_2=CH–CH(OH)–CH_2–	2-Hydroxybut-3-enylglucosinolate	Progoitrin
9	CH_2=CH–CH_2–CH(OH)–CH_2–	2-Hydroxypent-4-enylglucosinolate	Napoleiferin
10	CH_2=CH–CH_2–CH_2–	But-3-enylglucosinolate	Gluconapin
12	CH_2=CH–CH_2–CH_2–CH_2–	Pent-4-enylglucosinolate	Glucobrassicanapin
13	Indole-3-CH_2–	Indol-3-ylmethylglucosinolate	Glucobrassicin
14	C_6H_5–CH_2–CH_2–	2-Phenylethylglucosinolate	Gluconasturtiin
15	CH_3–S–CH_2–CH_2–CH_2–	3-Methylthiopropylglucosinolate	Glucoibervirin

Brassica species enabled a tentative identification to be made. The identity of the compounds was confirmed using a combination of electron impact and ammonia chemical ionization mass spectrometry.

Fig. 2 shows the HPLC elution pattern of desulphoglucosinolates obtained from an extract of rape-seed to which benzylglucosinolate had been added as internal standard. Using the water–acetonitrile solvent program, good separation of glucotropaeolin (R = benzyl) and glucobrassicanapin (R = pentenyl) was achieved whereas these compounds were not separated if methanol replaced acetonitrile (data not shown). Pentenylglucosinolate is a major component of rape-seed and therefore separation of these two glucosinolates is important because benzylglucosinolate is often chosen as an internal standard in the analysis of rape-seed.

A very different glucosinolate profile was obtained by chromatographing an extract of cabbage seed and Fig. 3 shows that good resolution was again obtained. This was confirmed using GC–MS of collected peaks. This observation suggests that the HPLC method could be used for the quantitive determination of individual glucosinolates in plant tissues.

Retention times appear to be influenced to some extent by the age of the HPLC column. Chromatograms shown in Figs. 1–4 were produced with a flow-rate of 1.0 ml min^{-1}, however column flow-rates of less than 0.5 ml min^{-1} should enable better resolution of the earlier eluting peaks such as those found in cabbage seed.

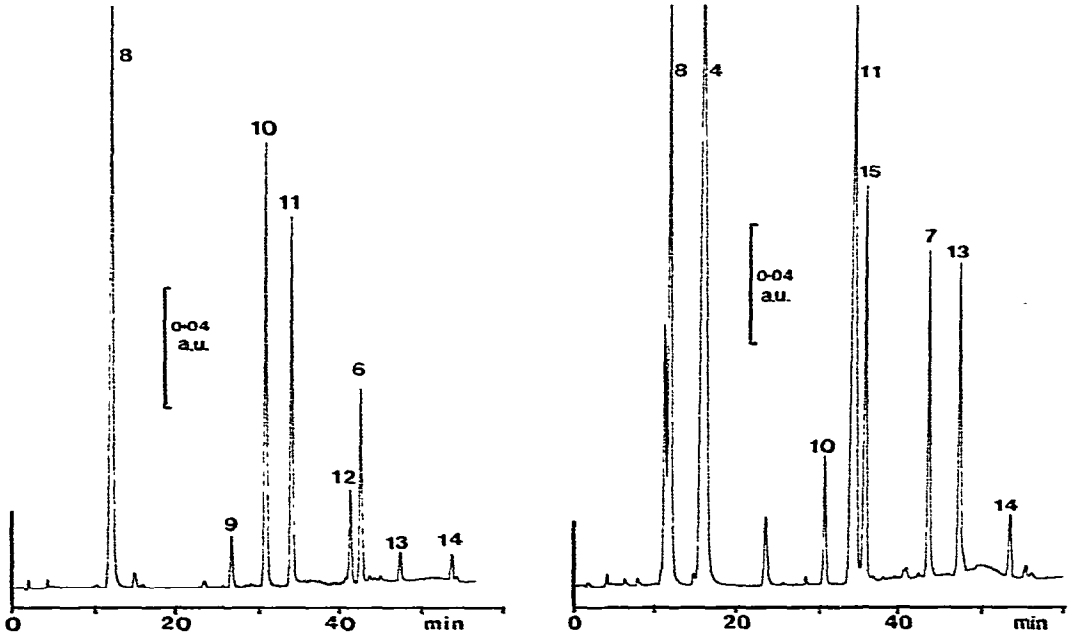


Fig. 2. HPLC elution profile of desulphoglucosinolates obtained from an extract of seeds of *B. napus* L. cv. Midas. Benzylglucosinolate was added as an internal standard prior to desulphation. Peak numbers refer to glucosinolates listed in Table I. Chromatographic conditions are described in the text.

Fig. 3. HPLC elution profile of desulphoglucosinolates isolated from cabbage seed (*B. oleraceae* cv. Golden Acre).

Fig. 4. HPLC elution profile of desulphoglucosinolates isolated from mustard seed (*Sinapis alba*). The peak number refers to the glucosinolate listed in Table I. Chromatographic conditions are described in the text.

Injections of plant extracts equivalent to 200 mg of sample provided ample quantities of separated desulphoglucosinolates for examination by GC and GC–MS as the silyl derivatives. Collected desulphoglucosinolates were also examined without derivatisation using direct insertion mass spectrometry with ammonia as reagent gas. The results obtained were comparable to those detailed by Eagles *et al.*[14].

The HPLC method seems to be ideally suited to the preparation of desulphoglucosinolate standards for use in quantitative glucosinolate profiling. For the isolation of specific desulphoglucosinolates, plant tissues can be chosen which are known to contain high levels of the glucosinolates of interest. An example of this is depicted in Fig. 4 which shows the glucosinolate profile obtained from mustard seed. We have used this procedure to purify milligram quantities of desulphoglucosinalbin and the method can be readily scaled up by utilising preparative or semi-preparative HPLC columns.

Of interest in this study is the peak (No. 11) in the chromatograms of rape-seed and cabbage seed having a retention time slightly longer than desulphogluconapin. This peak was consistently observed in the extracts from these species as well as other Cruciferae. Rerunning the rape-seed sample with the HPLC UV detector set at 280 nm gave three prominent peaks, one corresponding to the peak in question and the other two to glucobrassicin and neoglucobrassicin. The structure of this unidentified glucosinolate is at present under investigation.

The HPLC method has an advantage over GC for glucosinolate analysis because drying and derivatisation are unnecessary since the solution of desulphoglucosinolates eluted with water from the Sephadex A-25 column can be injected directly

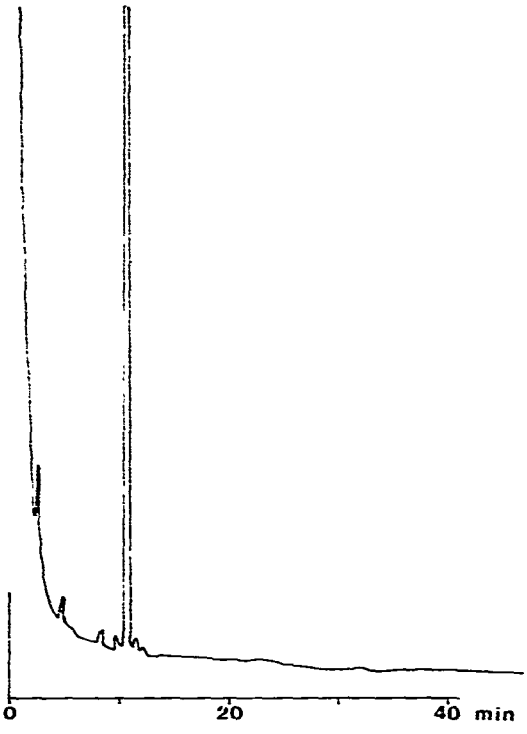


Fig. 5. GC trace of the silylated desulphoglucosinolate corresponding to peak 9 in Fig. 2. The HPLC peak was collected, dried and silylated. The identity of the component as 2-hydroxypent-4-enylglucosinolate was confirmed by GC–MS. Conditions for GC on 2% OV-7 are described in the text.

into the chromatograph. Because practically all impurities are removed during the "on column" desulphation procedure, fractions collected from HPLC can be expected to be of high purity. This supposition seems to be borne out by the appearance of GC traces obtained from collected HPLC peaks (*e.g.*, Fig. 5).

CONCLUSIONS

The HPLC procedure for the separation of desulphoglucosinolates described in the paper provides a simple means for obtaining information on the glucosinolate profiles in different plants and is a valuable adjunct to the ion-pairing HPLC procedure already published for intact glucosinolates[10].

Due to the remarkable specificity of the desulphoglucosinolate isolation procedure, and the resolving power of HPLC, it appears to be relatively easy to obtain a variety of pure desulphoglucosinolate standards using this HPLC method. These standards can then be used for either preparing calibration curves for accurate quantitation using GC methods or for quantitative analysis by HPLC. Since the basis of separation is very different from that of GC, the HPLC method may enable separation of glucosinolates which cannot be resolved by conventional GC techniques. In addition, since the HPLC procedure does not employ buffer ions or ion-pairing reagents, collected fractions can be dried directly and used for structure confirmation or

elucidation by mass spectrometry. Solvent consumption is also very economical as mobile phase mixtures contain high percentages of water.

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