Journal of Chromatography, 197 (1980) 199–205
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,946

SEPARATION OF GLUCOSINOLATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received April 24th, 1980; revised manuscript received May 13th, 1980)

SUMMARY

The separation of a series of glucosinolates by reversed-phase ion-pair liquid chromatography is reported. A rapid and adequate separation was achieved on a column of Nucleosil 5 C_{18}, with 0.01 *M* phosphate buffer at pH 7.0–methanol (3:7) containing 0.005 *M* tetraheptylammonium bromide as mobile phase. The modifier concentration, the nature of the counter-ion and the pH greatly influence the separation of the glucosinolates from each other and from non-ionic impurities. The isolation of the total glucosinolate fraction was performed by a newly developed ion-exchange chromatographic method. By combination with the separation method described here, acidic and basic conditions (which would lead to decomposition of the glucosinolates) are completely avoided. The proposed method is briefly discussed in relation to methods previously used for the isolation, identification and quantitative determination of glucosinolates.

INTRODUCTION

Several different methods for the isolation, separation and quantitative determination of components of the complex mixtures of naturally occurring glucosinolates have been reported[1–3]. All are based on the determination of one or more of the enzymatic hydrolysis products or of derivatives of the glucosinolates, but detection and determination of some of the glucosinolates are unsatisfactory[4].

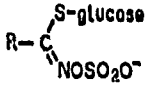
Recently, a method was developed for the quantitative isolation of intact glucosinolates by ion-exchange chromatography[5]. This method, followed by gas chromatography (GC) of the trimethylsilylated derivatives of the glucosinolates, offers a reliable procedure for the qualitative and quantitative analysis of complex glucosinolate mixtures[6,7].

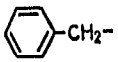
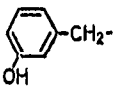
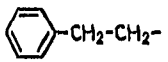
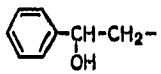
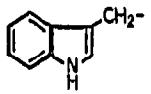
The determination of polar compounds such as glucosinolates by high-

0021-9673/80/0000–0000/$02.25

TABLE I

CHEMICAL STRUCTURES OF THE GLUCOSINOLATES



Compound No.	*Nature of group R*	*Value of k'**	*Name of glucosinolate*	*Trivial name of the salt*
1	CH_3–	3.3	Methylglucosinolate	Glucocapparin
2	CH_2=CH–CH_2–	4.2	Allylglucosinolate	Sinigrin
3	CH_2=CH–CH_2–CH_2–	5.0	But-3-enylglucosinolate	Gluconapin
4	CH_2=CH–CH_2–CH_2–CH_2–	5.5	Pent-4-enylglucosinolate	Glucobrassicanapin
5	CH_2=CH–CH(OH)–CH_2–	3.4	2-Hydroxybut-3-enylglucosinolate	Progoitrin
6	CH_2=CH–CH_2–CH(OH)–CH_2–	3.7	2-Hydroxypent-4-enylglucosinolate	Napoleiferin
7	CH_3–SO–CH_2–CH_2–CH_2–	2.1	3-Methylsulfinylpropylglucosinolate	Glucoiberin
8	CH_3–SO–CH_2–CH_2–CH_2–CH_2	–	4-Methylsulfinylbutylglucosinolate	Glucoraphanin
9	CH_3–SO–CH_2–CH_2–CH_2–CH_2–CH_2–	–	5-Methylsulfinylpentylglucosinolate	Glucoalyssin
10	CH_3–SO_2–CH_2–CH_2–CH_2–	2.9	3-Methylsulfonylpropylglucosinolate	Glucocheirolin
11		6.7	Benzylglucosinolate	Glucotropaeolin
12		6.4	*m*-Hydroxybenzylglucosinolate	Glucolepigramin
13		5.0	*p*-Hydroxybenzylglucosinolate	Sinalbin
14		9.8	Phenethylglucosinolate	Gluconasturtiin
15		6.2	2-Hydroxy-2-phenylethylglucosinolate	Glucobarbarin
16		9.8	Indol-3-ylmethylglucosinolate	Glucobrassicin

* Experimental conditions as in Fig. 5.

performance liquid chromatography (HPLC) seems obvious. However, the lack of suitable methods for separation of the glucosinolate fraction from other plant constituents prior to HPLC has prevented the use of this technique. This problem has now been overcome by use of the above-mentioned ion-exchange method. The glucosinolates are obtained as their pyridinium salts contaminated only by small amounts of other plant constituents.

The application of reversed-phase ion-pair liquid chromatography as a convenient alternative to ion-exchange chromatography for the separation of ionic compounds was demonstrated in 1975[8], and its advantages in separating mixtures of ionic and non-ionic compounds have also been shown[9].

This paper presents a reversed-phase ion-pair liquid chromatographic method, which, in combination with the new isolation procedure for the total glucosinolate fraction, allows fast and reliable determination of individual glucosinolates. Until now, HPLC has been used only for the determination of glucosinolate-degradation products[10–14], and this paper is the first report of an HPLC method for determining intact glucosinolates.

EXPERIMENTAL

Chemicals

Tetraalkylammonium bromides were obtained from Fluka (Buchs, Switzerland); all other reagents were of analytical grade from E. Merck (Darmstadt, G.F.R.).

Compounds investigated

Table I lists the glucosinolates studied; they were isolated from natural sources, purified and identified as previously described[5,6]. The total glucosinolate fraction from 2 g of seeds of *Brassica napus* L. cv. Tower was isolated as described elsewhere[7].

Chromatography

The liquid chromatograph used consisted of an Altex Model 110 solvent metering pump, a Pye-Unicam LC-UV spectrophotometer detector, and a Rheodyne Model 7120 injection valve. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder, and retention times and peak areas were measured by means of a Hewlett-Packard Model 3353A laboratory data system.

Preliminary experiments were performed on a column (12 cm × 4.65 mm I.D.) (Knauer, Berlin, G.F.R.) packed with Nucleosil 5 C_{18} (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.). For the analytical separations, two columns in series, each packed with the same material, were used; both columns were packed as described earlier[15]. The efficiency of the columns, expressed as the number of theoretical plates (N) measured for naphthalene when eluted by 90% methanol in water (capacity factor $k' = 1.0$) at a linear solvent velocity of 1.5 mm sec^{-1}, was 5500 for the single column and 12,900 for the two columns in series.

The mobile phase was 0.01 M phosphate buffer (pH 7.0) modified with 60% or 70% of methanol. Different tetraalkylammonium bromides at a concentration of 0.005 M were used as sources of counterions.

Pyridinium salts of glucosinolates were dissolved in water to give 0.01–0.04% solutions and 10-μl samples were injected on to the column.

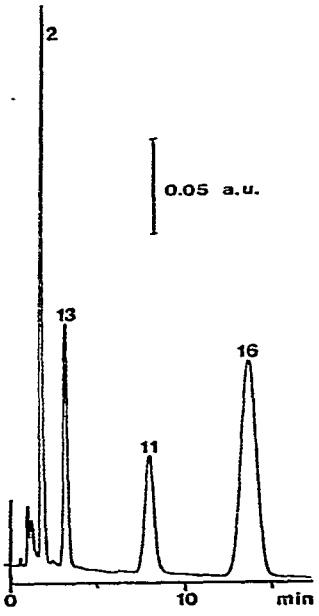
RESULTS AND DISCUSSION

For the elaboration of the HPLC method, solutions of the glucosinolates identified by previous methods[5–7] were used alone and/or in mixtures. Reversed-phase chromatography on Nucleosil 5 C_{18} was investigated. Since glucosinolates are unstable in both acidic and basic solutions, a phosphate buffer (0.01 *M*; pH 7.0) modified with methanol was used as mobile phase.

Detection was at 235 nm, at which wavelength the contribution to the absorbance from the glucosinolate group (Table I) is almost at its maximum, whereas the contribution from interfering aromatic R groups will be of limited magnitude, leading to similar absorptivity values for all glucosinolates.

A reasonable separation of a mixture of the compounds 2, 11, 13 and 16 (Table I) was achieved with a modifier concentration of 5%, but peak shapes were not optimal (Fig. 1). Further, non-ionic impurities originating from the plant material, as well as pyridine originating from the glucosinolate pyridinium salts, were completely retained on the column. This accumulation would in time alter the characteristics of the column.

A change of the modifier concentration to 60% produced rapid elution of pyridine and non-ionic impurities, and, by adding tetraalkylammonium bromides as sources of counter-ions, retention of the glucosinolates was achieved (Fig. 2). As



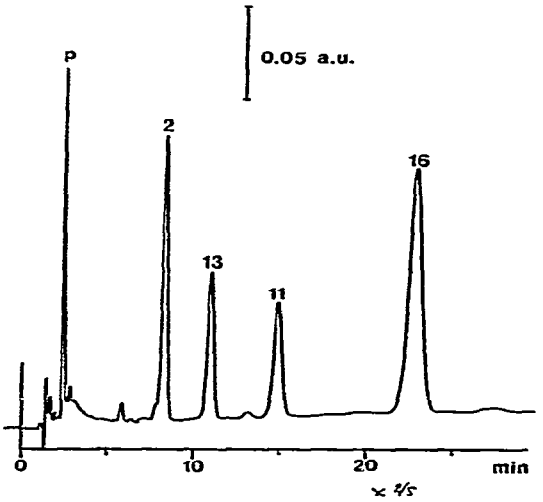


Fig. 1. Chromatogram of glucosinolates 2, 11, 13 and 16 (see Table I) on a column (12 cm × 4.6 mm) of Nucleosil 5 C_{18}. Mobile phase: phosphate buffer (0.01 *M*; pH 7.0)–methanol (95:5). Solvent velocity: 1.5 mm/sec. Detection wavelength: 235 nm.

Fig. 2. Chromatogram of glucosinolates 2, 11, 13 and 16 and pyridine (p). Mobile phase: phosphate buffer (0.01 *M*; pH 7.0)–methanol (4:6) containing tetraheptylammonium bromide (0.005 *M*); other conditions as in Fig. 1.

stated by Sood[16], three factors are important in controlling retention in reversed-phase ion-pair chromatography, *viz.*, pH, counter-ion concentration and nature of the counter-ion.

As mentioned above, a pH of *ca.* 7 is required to ensure stability of the glucosinolates. At this pH, the glucosinolates are totally ionized, owing to the low pK_a value of the sulphate group, and have a negative charge[6].

As earlier reported for the ion-pair separation of amines, with alkanesulphonates as counter-ions[17], a counter-ion concentration of 0.005 *M* was also chosen for the separations described here.

The influence of the nature of the counter-ion is demonstrated in Fig. 3. An increase in the carbon number of the tetraalkylammonium ions, and thereby in their lipophilic properties, caused an increase in the retention of the ion-pairs, but did not affect the retention of pyridine. Fig. 3 shows that the separation of the glucosinolates was optimal within a reasonable time when tetraheptylammonium bromide was used as a source of counter-ions. However, further investigations showed that, when handling more complex mixtures of glucosinolates (see below), the selectivity of the chromatographic system was improved by changing the modifier concentration to 70% and using tetraoctylammonium bromide (Fig. 4).

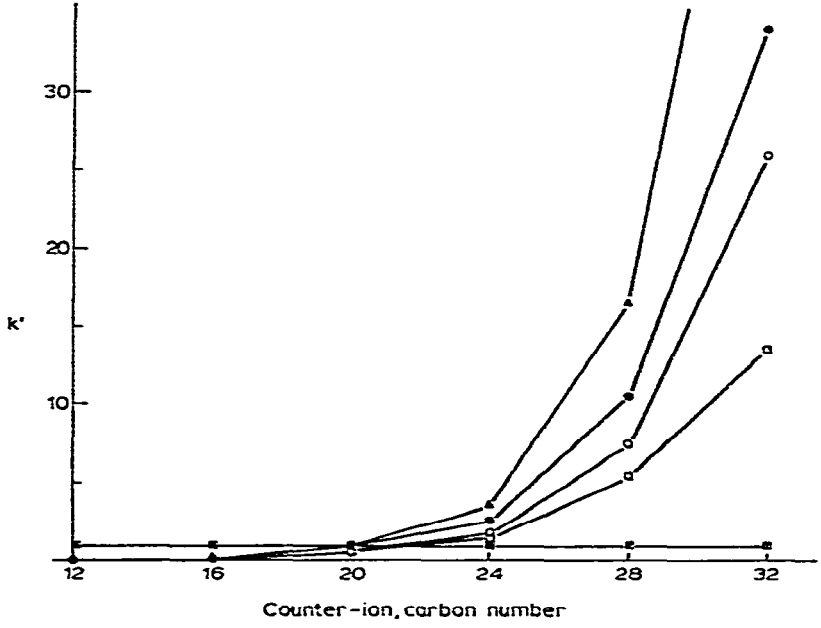


Fig. 3. Influence of nature of counter-ion on retention of the compounds studied (see Table I); ■ = pyridine; □ = 2; ● = 11; ○ = 13; ▲ = 16. Chromatographic conditions: single column, phosphate buffer (0.01 *M*; pH 7.0)–methanol (4:6) containing different tetraalkylammonium bromides (0.005 *M*).

Fig. 5 shows the chromatogram of an artificial mixture of glucosinolates isolated from different plants[6,7] and used as reference compounds; a few of these compounds were not separated. The trend in the separation was similar to that of paper

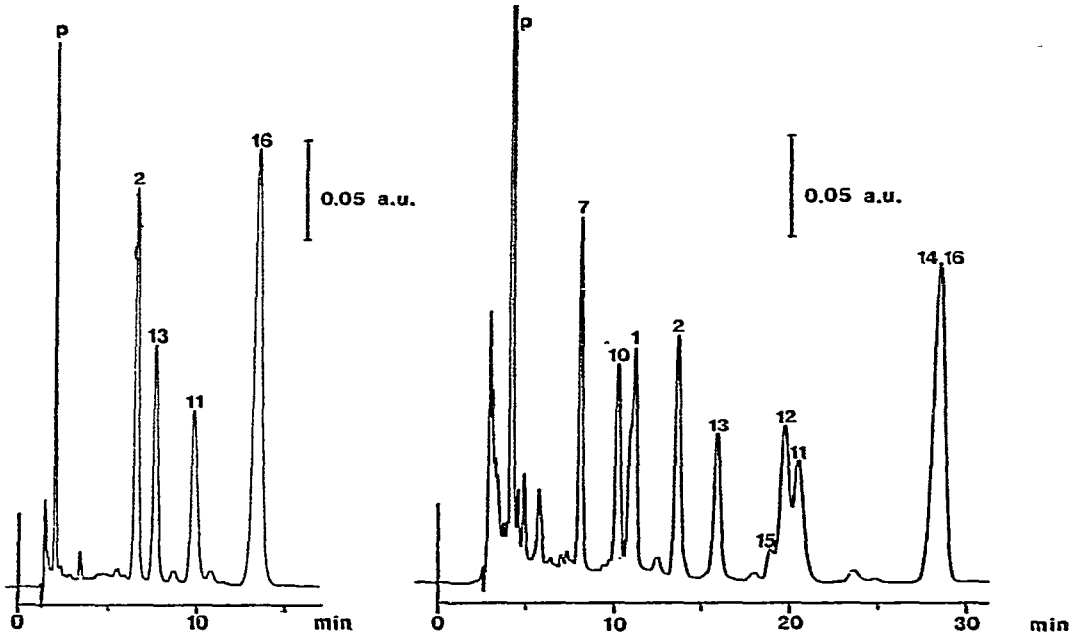


Fig. 4. Chromatogram of glucosinolates 2, 11, 13 and 16 and pyridine (p). Mobile phase: phosphate buffer (0.01 *M*; pH 7.0)–methanol (3:7) containing tetraoctylammonium bromide (0.005 *M*); other conditions as in Fig. 1.

Fig. 5. Chromatogram of artificial mixture of glucosinolates (1–4 μg of each) on a column (24 cm × 4.6 mm) of Nucleosil 5 C_{18}; conditions as in Fig. 4. The value of k' for pyridine (p) is 0.6; k' values for the other compounds are shown in Table I.

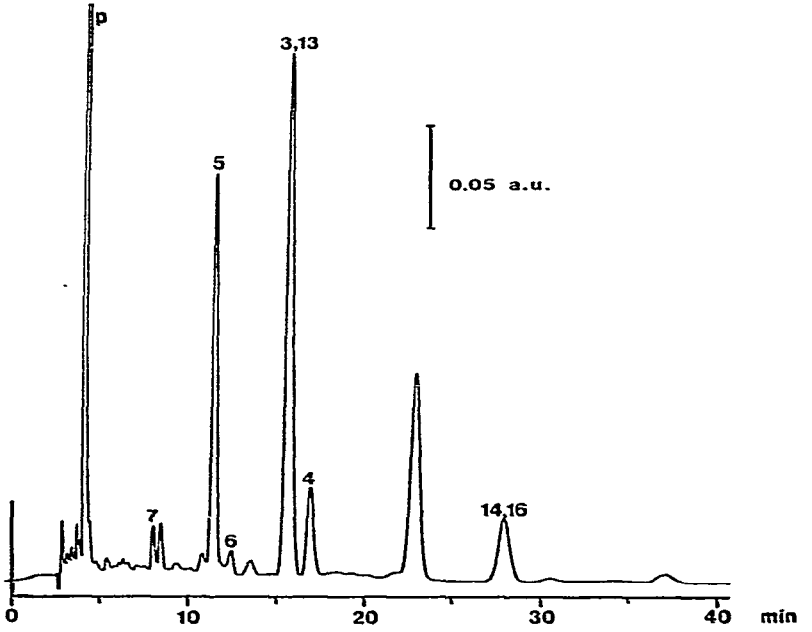


Fig. 6. Chromatogram of glucosinolates isolated from seeds of *B. napus* L. cv. Tower; conditions as in Fig. 5.

chromatography (PC)[5,6], but the efficiency of the HPLC separations was far superior to that of PC.

Fig. 6 shows the separation of the mixture of glucosinolates isolated from seeds of *B. napus* L. cv. Tower. The seeds were from the same lot as used before, and the results were in accordance with those previously reported[7]. The dominant peak, with a retention time (t_R) of 4.1 min, is due to pyridine. The major peaks in the HPLC chromatogram correspond to the compounds 5 (t_R = 11.5 min), 3 + 13 (t_R = 15.7 min), 4 (t_R = 16.9 min) and 14 + 16 (t_R = 27.9 min). As indicated in the chromatogram, some of the remaining peaks might be due to methylsulphinylglucosinolates (*e.g.*, compounds 7, 8, and 9), but the identity of these and some other unidentified glucosinolates in Tower seeds[7] has not yet been unequivocally established.

CONCLUSION

The HPLC method described permits the rapid separation and quantitative determination of intact individual glucosinolates under gentle conditions, and is therefore an important supplement to the GC method, in which trimethylsilylated desulfoglucosinolates are quantitatively determined[7]. Both the GC and the HPLC method avoid problems associated with determination of the many products of enzymatic degradation of glucosinolates.

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