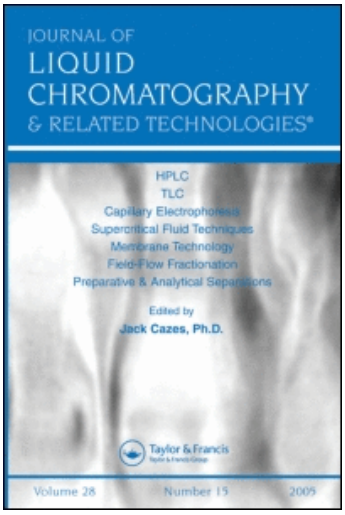

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

New Principles of Ion-Exchange Techniques Suitable to Sample Preparation and Group Separation of Natural Products Prior to Liquid Chromatography

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To cite this Article Bjerg, Birthe , Olsen, Ole , Rasmussen, Kim Wedel and Sørensen, Hilmer(1984) 'New Principles of Ion-Exchange Techniques Suitable to Sample Preparation and Group Separation of Natural Products Prior to Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 7: 4, 691 — 707
To link to this Article: DOI: 10.1080/01483918408073996
URL: http://dx.doi.org/10.1080/01483918408073996

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(4), 691-707 (1984)

NEW PRINCIPLES OF ION-EXCHANGE TECHNIQUES SUITABLE TO SAMPLE PREPARATION AND GROUP SEPARATION OF NATURAL PRODUCTS PRIOR TO LIQUID CHROMATOGRAPHY

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ABSTRACT

A Gentle method of group separation of low molecular weight hydrophilic natural products is reported. The method is based on separation of the compounds according to their net charge at different pH values using different types of ion-exchange columns connected in series. Precolumns retaining interfering compounds are used in some cases. Elution of the compounds retained on the columns is performed by use of volatile eluents. The elution principle for two of the ion-exchangers in question is removal of the charges on the column materials while for the third column the positive net charge on the compounds retained is removed. Thereby, the total amount of ions retained on the different columns is released and eluted into small volumes, which after evaporation leaves the ions as well defined salts. The method is experimentally simple and efficient to separation of natural products into groups suitable to direct use in sensitive methods of analysis as e.g. high-performance liquid chromatography and gas chromatography. Combinations of these column chromatographic methods have been adpated for micro or semimicro determinations of naturally occurring compounds, e.g., aromatic choline esters, amines, amino acids and esters of phenolic carboxylic acids. The methods seem to be general practicable for group separation of low molecular weight hydrophilic compounds.

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INTRODUCTION

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are methods of analysis which possess high resolution when used for well defined mixtures of low molecular weight compounds. Reversed-phase ion-pair HPLC has been described as a rapid and simple quantitative method of analysis for intact individual glucosinolates (1). Furthermore, HPLC appears to be an efficient supplement to GC and other chromatographic methods in studies of glucosinolates (2) and several other groups of natural products including amino acids and pyrimidines (3,4), phenolic carboxylic acids (5) as well as derivatives thereof such as neutral and acidic esters (6), amides (7) and choline esters (8). The easy access to these methods of analysis makes it desirable with group separation of the complex mixtures of natural products present in all extracts from living cells prior to chromatographic investigations and quantitative determinations. Otherwise, reliable interpretations of the results are complicated (2) and especially the HPLC column materials are rapidly contaminated and destroyed.

Anion-exchange chromatography based on a new elution principle has been described as a suitable method for the quantitative isolation of unstable acidic compounds such as glucosinolates (9,10). This ion-exchange principle has recently been adapted to a new cation-exchange chromatographic technique which allows quantitative isolation of phenolic choline esters under gentle conditions (11).

The present communication describes further developments of these techniques to comprise a semimicro-one-step group separation of different types of natural products using the columns connected in series.

The intention is not a review of the numerous papers which describes traditional ion-exchange methods. It is a

presentation of a group separation procedure based on a new way of using ion-exchange materials in special combinations and comprising new elution principles. Thereby, the problems with purification of unstable hydrophilic natural products as well as problems with unacceptable recoveries of especially aromatic compounds from the columns are reduced. Application of the new isolation procedure in combination with HPLC or other chromatographic methods allows efficient,simple and reproducible semimicrodeterminations of individual intact glucosinolates, phenolic choline esters and amines.The separation of amino acids into groups of basic, neutral and acidic amino acids as well as a convenient alternative separation method for investigation of other ionic and hydrophilic natural products are briefly described.

EXPERIMENTAL

Plant materials

Seeds of Sinapis alba L. cv. Trico (white mustard), of Brassica oleraceae cv. Ditmarsken and of Vicia faba L. were obtained from Trifolium Silo A/S, DK-2630 Tåstrup, Denmark. Seeds of Reseda luteola were collected from plants growing in their natural habitat at Faxe, Denmark.

Chemicals and materials

Tetraalkylammonium bromides and sodium heptanesulphonic acid monohydrate were obtained from Fluka (Bucks, Switzerland); all other reagents were of analytical grade from E. Merck (Darmstadt, G.F.R.). Only deionized water was used and the HPLC solvents were filtered under vacuum through a 0.5 µm Millipore FH type filter or 0.45 µm Millipore HA type filter and degassed before use. The column material CM-Sephadex C-25 was obtained from Pharmacia (Sweden); Dowex 50 w x 8 was obtained from Fluka (Bucks,

Switzerland); Servacel Ecteola 23 was obtained from Serva (Heidelberg, G.F.R.); Bondapak C_{18} 75 µm was obtained from Waters Associates (Roskilde, Denmark). Fintips 61 for microliter pipettes, range 200-1000 µl code 940 1070, Labsystem OY were obtained from Pulttitie 9 (Helsinki, Finland).

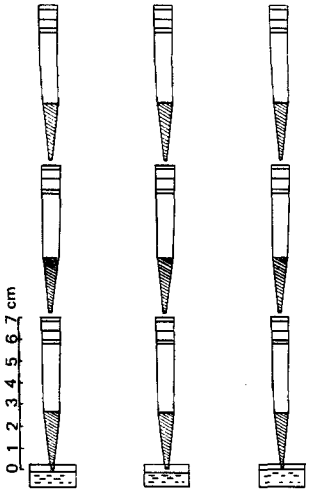
Crude extracts

Preparations of crude extracts were performed according to a previously described method (11) but slightly modified and adapted to the semimicro method now presented. The plant materials were homogenised three times in boiling methanol-water (7:3) with an Ultra Turrax homogeniser, using 3 x 12 ml to samples of 2 g lyophilised plant material, 3 x 8 ml to 0.5 g seed samples, 3 x 5 ml to 0.1 g samples. The homogenates were centrifuged (3000 x g, 10 min, 0°C) and the combined supernatants were concentrated to about 1 ml and centrifuged again at the same conditions. The supernatant was used as the crude extract.

Combined ion-exchange columns and group separations

The column materials were regenerated in relatively large columns as shown elsewhere (10). CM-Sephadex C-25 and Dowex 50 w x 8, 200-400 mesh were treated with 1 M HCl (10 x column volume) and washed to neutral pH with water. Ecteola-cellulose was treated with 2 M acetic acid (10 x column volume) and washed to neutral pH with water. Bondapak C_{18} was activated with methanol and washed with water prior to use.

The columns employed for the crude extracts from 2 g samples were pasteur pipettes with a small plug of glass wool as bottom and filled with the appropriate column materials to a height of 7 cm. In the case of extracts from 0.1 g and 0.5 g samples the columns were Fintips with a small plug of glass wool as bottom and filled to a height of 2-3 cm with the appropriate column materials (Fig. 1).



(A) COLUMNS: weakly acidic cation-exchangers.

$-OCH_2COO^-$, H^+

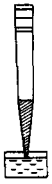
(B) COLUMNS: strongly acidic cation-exchangers.

$-SO_2O^-$, H^+

(C) COLUMNS: weakly basic anion-exchangers.

$-(OCH_2CH_2)_3{}^+NH$, AcO^-

H_2O effluents from the columns.



(A) COLUMN:

$-OCH_2COO^-$, B^+

B^+ = metal ions, alkaloids, amines, basic amino acids.

Elution of retained cations by 1 M CH_3COOH. The eluent protonates the functional groups of the ion-exchanger to $-OCH_2COOH$, thereby releasing the retained cations.

(B) COLUMN:

$-SO_2O^-$, $H_3{}^+N-\underset{\displaystyle R}{\overset{\displaystyle COOH}{\underset{|}{\overset{|}{C}}}}-H$

R = side chain in neutral and acidic amino acids

Elution of retained neutral and acidic amino acids by 1 M pyridine. The eluent deprotonates the carboxyl groups of the amino acids to $RCH^+NH_3COO^-$, thereby releasing the retained compounds.

(C) COLUMN:

$-(OCH_2CH_2)_3{}^+NH$, A^-

A^- = carboxylates, phosphates, sulphonates, sulphates.

Elution of retained anions by 1 M pyridine. The eluent deprotonates the functional groups of the ion-exchanger to $-(OCH_2CH_2)_3N$:, thereby releasing the retained anions

FIGURE 1. Columns fit up for group separation, distribution of different types of natural products on the columns after flushing with water and elution principles used for the three different types of ion-exchange columns.

The columns were fit up vertically on a glass plate with stick tape, and at least three columns were connected in series above each other. From top to bottom the columns were: (A) CM-Sephadex C-25 (H^+); (B) Dowex 50 w x 8, 200-400 mesh (H^+); (C) Ecteola-cellulose (AcO^-) (Fig. 1).

The crude extract was applied to the (A) column and the columns were allowed to drain. The tube which contained the crude extract was washed with water (3 x 0.5 ml). These solutions were also applied to the (A) column before washing with water commenced (approximately 10 x column volume). The effluent passing through the columns was collected. Thereafter, the columns were disconnected and the (A) column was eluted with 2 M acetic acid-methanol (1:1) whereas both the (B) and (C) columns were eluted with 1 M pyridine. If necessary, the eluates were concentrated to a volume appropriate for further investigations.

Chromatography

The liquid chromatograph used consisted of two Waters M-6000 A pumps, a Waters M-450 variable wavelength absorbance detector, a Waters M-720 system controler and a Rheodyn Model 7125 injection valve with a 20 µl loop. Chromatograms were recorded on a Kipp and Zonen Model BD-41 recorder. The experiments were performed on 120 x 4.6 mm or 250 x 4.6 mm I.D. columns (Knauer, Berlin, G.F.R.) packed by the dilute slurry technique. The experimental details used for HPLC analysis of the different types of compounds are described in the legend to the respective figures.

Details concerning paper chromatography (PC), thin layer chromatography (TLC), high voltage electrophoresis (HVE) and GC of choline esters, amines, amino acids, carboxylic acids, glucosinolates and carbohydrates have been described elsewhere (2,9-15.

RESULTS AND DISCUSSION

Consideration of the principles underlying the method of group separation is necessary for elaboration and use of the described technique (Fig. 1). Compounds with positive net charge at pH 3-5 are retained on the (A) column in the serie which is a weakly acidic cation-exchanger, e.g., choline esters, amines, alkaloids, basic amino acids, and metal ions. Compounds such as neutral and acidic amino acids, including the large group of naturally occurring glutamyl and aspartyl peptides (12), as well as some purine and pyrimidine derivatives (vide infra) do not have a positive net charge at the pH in the (A) column. In strongly acidic solutions, however, they are protonated and therefore retained on the (B) column which is a strongly acidic cation-exchanger. Compounds passing through the first two columns but with protolytic active groups which results in a negative net charge at pH 6-8 are retained on the (C) column in the serie which is a weakly basic anion-exchanger, e.g., carboxylates, phosphates, sulphonates and sulphates including glucosinolates. Compounds which do not obtain a net charge at the pH in any of the applied ion-exchange columns are flushed through the three columns with water if strong adsorptions to the materials in the columns are not involved. In such special cases a fourth Bondapak column has been used as a pre-column.

Elution of the ions retained on the (A), (B) and (C) columns is performed with weakly acidic and weakly basic volatile eluents, respectively, (Fig. 1) leaving the eluted compounds as salts after evaporation of the eluates to dryness. Thereby, volatile amines retained on the (A) column and volatile acids retained on the (C) column do not escape during evaporation of the eluents. The principles underlying the elution of

compounds from these two columns is removing of the charges on the column materials, as shown in Fig. 1, whereas elution of compounds from the (B) column, e.g. neutral and acidic amino acids, is obtained by removal of their positive net charge by use of 1M pyridine. This weak and volatile base is removed by evaporation leaving the amino acids as zwitterions. Elution of compounds retained on the pre-column is performed by use of methanol. These elution principles make it possible to obtain quantitative elution applying only some few ml of eluent, and excesses of eluents can be removed by evaporation at gentle conditions, e.g., by lyophilization.

The ion-exchange materials used in the (A)- and (C) columns have polymeric carbohydrates as supports which minimize unwanted adsorption due to hydrophobic interactions, which otherwise are a problem with other types of ion-exchange materials with e.g. polyvinylbenzene as support. Recoveries of aromatic choline esters from the small (A) columns (Fig. 1) using the described elution technique are in fact close to quantitative. Several types of low molecular weight aromatic and phenolic compounds without positive net charge are retained on the (B) column, most likely caused by adsorption or hydrophobic interaction. However, these types of compounds can be retained on the described Bondapak C 18 pre-column introduced in the serie of columns before the (B) column. Alternatively, the (B) column has been omitted from the serie allowing these compounds to pass through the (C) column, including the neutral amino acids; anions, including the acidic amino acids are in this case retained on the (C) column. Recoveries of vicine and the amino acids retained on the (B) column (vide infra) are close to 100% when the small columns and the described elution techniques are used, except for dopa as some oxidation of this compound occurs. Also

the elution technique used to release the compounds retained on the (C) column appeared to result in quantitative recoveries for the investigated compounds (vide infra).

Amino acids in the eluate from the (B) column are separated into groups of acidic and neutral amino acids, respectively, by renewed use of the (C) column. Neutral amino acids are flushed through this column with water whereas acidic amino acids are retained on the column and released again with pyridine as described above for the (C) column.

Separation and quantitative determination of the individual compounds in each group are then performed by selected methods of analysis, e.g. HPLC (vide infra), PC, TLC, HVE and/or amino acid analyser (13). Irrespective of choice of analytical method, the results obtained are much improved when the described method of group separation is included.

Figure 2 shows the chromatograms obtained by HPLC analysis of the total amine and choline ester fraction isolated from S. alba seeds (0.1 g). The eluate from the (A) column was dissolved in water (5 ml) and a sample of this solution (20 µl) was injected. The results reveal that reversed-phase ion-pair HPLC (8) in combination with the group separation method is an efficient analytical procedure for phenolic choline esters. It is possible to make some discriminations between the individual compounds by use of different detection wavelengths. Alkaline conditions are avoided during all isolation steps which is essential for quantitative isolation of these esters (11) as well as for other alkaline labile phenolic cations, e.g. anthocyanins, retained on the (A) column. The elution principle makes it furthermore to an efficient method of isolation of amines and basic amino acids.

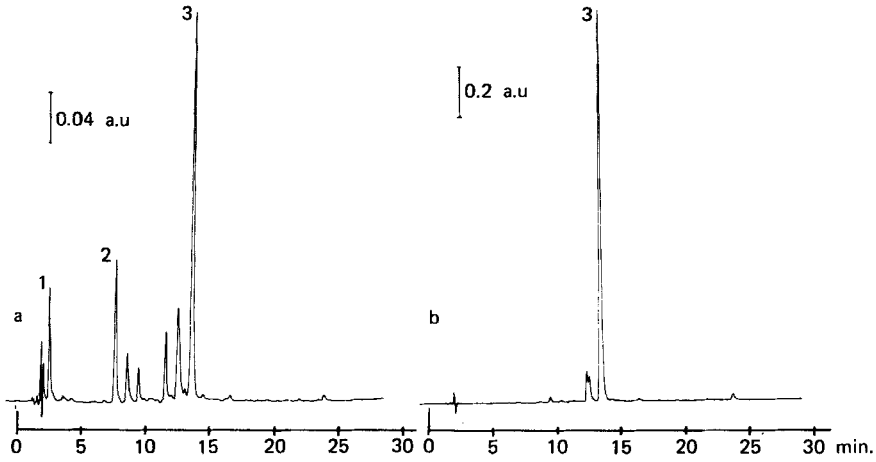


FIGURE 2. HPLC chromatograms of the eluate from column (A) obtained by group separation of the compounds in an extract from 0.1 g of seeds of Sinapis alba cv. Trico. Support: Nucleosil 5 C_8, 120 x 4.6 mm. Mobile phase: a linear gradient of solvent A - solvent B (20:80) to (70:30) for 30 min, flow 1.0 ml/min.
Solvent A: 0.02 M phosphate buffer, 0.02 M dibutylamine and 0.02 M sodium heptanesulfonic acid (pH 2.0) modified with 50% acetonitril. Solvent B: 0.01 M phosphate buffer, 0.01 M dibutylamine and 0.01 M sodium heptanesulfonic acid (pH 2.0). Recorder speed 5 mm/min. Detection wavelength; (a) 280 nm, (b) 313 nm. Peaks: 1 = 4-hydroxybenzylamine; 2 = 4-hydroxybenzoylcholine; 3 = 3,5-dimethoxy-4-hydroxy-cinnamoylcholine (sinapine) other peaks are unknown compounds discussed elsewhere (8,11).

Figure 3 shows the chromatogram from HPLC analysis of the (B) column eluate obtained by group separation of an extract from 0.5 g Reseda luteola seeds. The eluate was dissolved in water (5 ml) and a sample (20 µl) thereof was injected. Figure 3 comprises also a standard chromatogram of authentic reference compounds, including the acidic aromatic amino acids previously

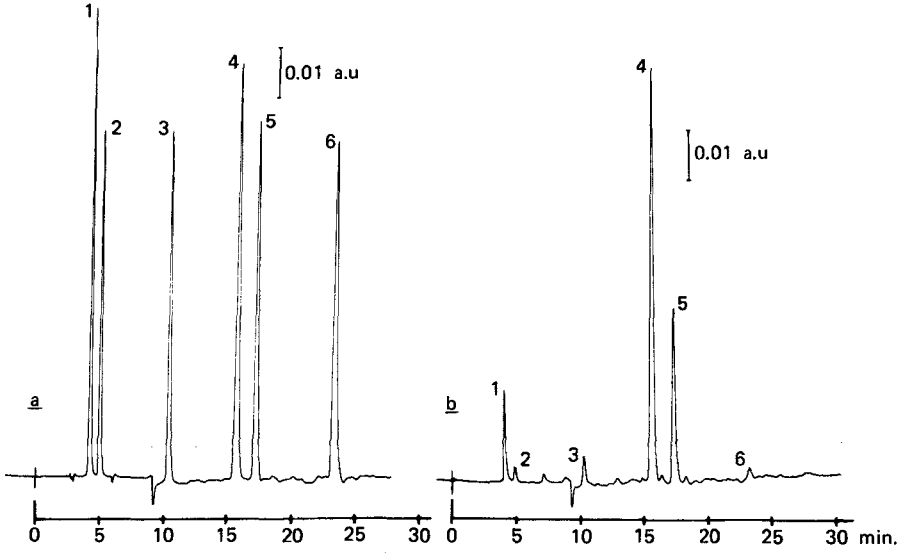


FIGURE 3. HPLC chromatograms of the eluate from column (B) obtained by group separation of the compounds in an extract from 0.5 g of seeds of Reseda luteola. Support: Nucleosil 5 C_{18} (250 x 4.6 mm). Mobile phase: a linear gradient of solvent B (0:100) to (50:50) for 25 min, flow 1.0 ml/min. Solvent A: 0.0125 M sodium formiate buffer (pH 3.5) modified with 25% acetonitrile. Solvent B: 0.01 M sodium formiate buffer (pH 3.5). Recorder speed 5 mm/min. Detection wavelength 280 nm. Peaks: 1 = 3-carboxy-4-hydroxyphenylglycine; 2 = 3-carboxyphenylglycine; 3 = tyrosine; 4 = 3-carboxytyrosine; 5 = 3-carboxyphenylalanine; 6 = tryptophane. Chromatogram a) is an artificial mixture of authentic reference compounds; chromatogram b) is the mixture of amino acids isolated from R. luteola.

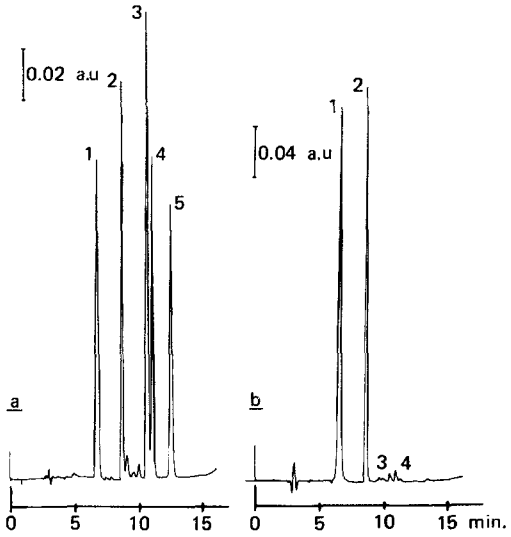


FIGURE 4. HPLC chromatograms of aromatic amino acids and pyrimidine glucosides isolated from Vicia faba; a) an artificial mixture of authentic reference compounds; b) an extract from V. faba seeds.
Support: Nucleosil 5 C_{18}, 250 x 4.6 mm. Mobile phase: a linear gradient of solvent A - solvent B (1:99) to (99:1) for 10 min maintaining this final conditions for additional 5 min, flow 1 ml/min.
Solvent A: 0.0125 M phosphate buffer (pH 2.0) modified with 25% methanol. Solvent B: 0.01 M phosphate buffer (pH 2.0). Recorder speed 5 mm/min. Detection wavelength 280 nm. Peaks: 1 = 2,6-diamino-5-(β-B-glucopyranosyloxy)-4-pyrimidinone (Vicine); 2 = 6-amino-2-hydroxy-5-(β-D-glucopyranosyloxy)-4-pyrimidinone (Convicine); 3 = 3,4-dihydroxyphenylalanine (Dopa); 4 = 4-hydroxy-3-(β-D-glucopyranosyloxy)phenylalanine (Dopa-glucoside); 5 = tyrosine.

isolated from R. luteola (16). Separation of these acidic amino acids by the described HPLC method of analysis is in accordance with their pKa_2 values and depends on careful adjustment of pH in the solvents used as mobile phase. Subsequent group separation of the compounds in the (B) column eluate by reuse of the (C) column, revealed that the neutral amino acids flushed through the column with water while the acidic amino acids were retained on the column and finally eluted therefrom with M pyridine. Slightly modified HPLC conditions appeared to be more efficient to separation of mixtures containing some other aromatic amino acids and pyrimidine derivatives (Fig. 4). Tyrosine, Dopa and Dopa-glucosid co-occur with vicine and convicine as quantitative dominating constituents of Vicia faba (17) and other legumes. Vicine and the amino acids are retained on the (B) column whereas convicine appears in the water effluent in accordance with pKa values for the aromatic amino groups. Owing to the high concentrations of vicine and convicine in V. faba seeds and the efficient UV-absorption of these compounds (17) it is possible to perform HPLC analysis direct on deproteinised extracts from these seeds without further purification (3,4) (Fig. 4). However, this is not the case when extracts with lower concentrations of these compounds and higher concentrations of interfering constituents are to be analysed e.g., investigations of other legumes, especially green parts, food and feed, contents in the digestive tracts of animals, urine and blood.

The eluate from the (C) column contains acidic compounds as carboxylic acids, glucosinolates and phosphates. When seed extracts from glucosinolate containing plants are investigated for individual intact glucosinolates (2) it is possible to use the eluate from the (C) column directly for HPLC analysis (1,10) without ad-

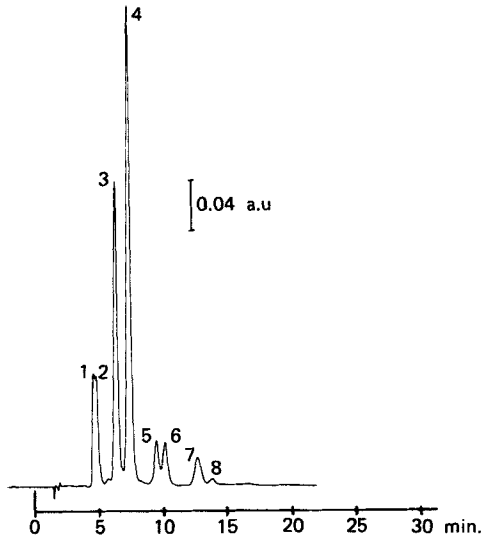


FIGURE 5. HPLC chromatogram of the eluate from column (C) obtained by group separation of the compounds in an extracts from 0.1 g of seeds of Brassica oleracea cv. Ditmarsken.
Support: Nucleosil 5 C_{18}, 120 x 4.6 mm. Mobile phase: 0.01 M phosphate buffer (pH 7.0) modified with 60% methanol containing 0.005 M tetraheptylammonium bromide, flow 1.0 ml/min. Recorder speed 5 mm/min. Detection wavelength 235 nm. Peaks: 1 = 3-methylsulfinylpropylglucosinolate; 2 = 4-methylsulfinylbutylglucosinolate; 3 = 2-hydroxybut-3-enylglucosinolate; 4 = allylglucosinolate; 5 = but-3-enylglucosinolate; 6 = 3-methylthiopropylglucosinolate; 7 = 4-methylthiobutylglucosinolate; 8 = pent-4-enylglucosinolate.

ditional purification (Fig. 5). However, to avoid serious problems caused by interfering compounds, pre-columns are needed prior to HPLC analysis of extracts obtained from blood and contents in the digestive tract of animals (18) as well as from seedlings and green parts of some plants with a particular low glucosinolate content e.g. double low rape varieties.

CONCLUSIONS

The combined column chromatography method now described is experimentally simple, fast, cheap and efficient for semimicro group separation of hydrophilic natural products. The principle implies separation of the compounds according to their net charges at different pH values using gentle conditions. Neutral to weakly acidic conditions are used throughout the isolation of compounds retained on the (A) column. This is especially important for the quantitative isolation of volatile amines and cations which are unstable in alkaline solutions e.g. choline esters, anthocyanins and several other phenolic compounds. Protons released from the cation-exchangers are immediately neutralised on the weakly alkaline anion-exchanger - the (C) column. Thereby, long time extreme pH values are avoided in the solutions containing compounds to be retained on this column. This is important for quantitative isolation of e.g. glucosinolates. The groups of compounds isolated are thus purified and obtained as well defined salts suitable to further qualitative and/or quantitative investigations of the individual compounds by use of sensitive methods of analysis. Problems caused by contamination of columns and detection systems in e.g. HPLC and GC instruments are efficiently reduced. Resolution and reliability in interpretation of results obtained by

these and other chromatographic methods are much improved. The method show off to advantage when large number of samples have to be examined for different types of hydrophilic low molecular weight constituents owing to the easy way of arranging several series of columns side by side on glass plates.

ACKNOWLEDGEMENTS

Support from the Danish Agricultural and Veterinary Research Council, from EEC and from DANIDA, the Danish Council on Development Research, is gratefully acknowledged.

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