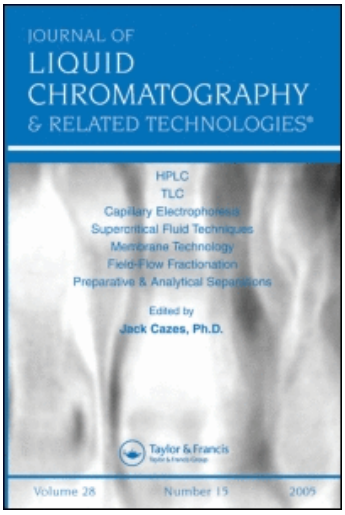

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COMPARISON OF A STYRENE-DIVINYLBENZENE COPOLYMERIC ANION EXCHANGER AND A POLYMETHACRYLATE COPOLYMERIC ANION EXCHANGER FOR GLUCOSINOLATE SEPARATION

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ABSTRACT

Studies on gradient elution were undertaken on a Hamilton PRP-X100 column. Satisfactory analysis was produced by using a concentration gradient in which the eluent ion concentration was increased over the run. To conclude the overall investigation of glucosinolate separation by anion exchange chromatography, a polymethacrylate copolymeric anion exchanger was evaluated and compared with a silica anion exchanger and a polystyrene-divinylbenzene copolymeric anion exchanger for an isocratic analysis of glucosinolates. Different commonly used inorganic and organic anions as well as alkanesulfonate anions and borate-gluconate mixtures were tested as aqueous mobile phase.

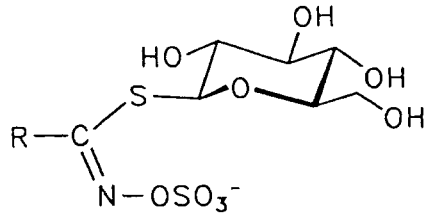

Figure 1. General structure of glucosinolates.

INTRODUCTION

Glucosinolates are thioglucosides which are naturally occurring constituents of Brassica vegetables. These compounds are important because of their potential toxicity and because epidemiology and other evidence indicate that some of them may inhibit some carcinogenic processes when consumed as part of the normal diet.[1-3] Accordingly, on the one hand, the identification and quantization of these compounds and on the other hand, obtaining purified glucosinolates in relatively large amounts as materials for nutrition and physiological investigations have become important. Glucosinolates are natural anionic compounds frequently isolated from plants as potassium salts or sometimes, as salts of sinapine (the choline ester of sinapic acid). Their general structure is shown in Figure 1.

Recently, we have proved that the glucosinolates can be analyzed by anion exchange chromatography.[4,5] All glucosinolates carry the same anionic sulfate group, but the great diversity of the R group leads to wide variations in the polarity and the hydrophilic and lipophilic characters of these natural compounds. In anion exchange chromatography, glucosinolates were eluted principally in order of decreasing polarity (alkenyl, arylalkyl then indolylglucosinolates), as in ion-pair chromatography.[6-8] On a silica-based ion exchanger, the ion exchange mechanism is predominant[4] and consequently no sufficient selectivities have been observed on this type of support between glucosinolates carrying the aglycone R group which have similar hydrophobicities. Contrariwise, the low capacity polymeric ion exchanger provides both adsorption and ion exchange sites with which the organic analyte ions may interact.[9-11] On a styrene-divinylbenzene (PS-DVB) copolymeric anion exchanger very strong reverse phase interactions between the glucosinolates, specially the indolylglucosinolates, and the polymeric backbone are noticed[5] and so, it was necessary to use an alkanesulfonate with a chain length of at least six carbons as the eluent anion in the mobile phase to elute

glucosinolates on this type of support. A good resolution for the analysis of many natural mixtures can be obtained with this type of anion exchanger, but with a long and unsatisfactory time of analysis for indolylglucosinolates in an isocratic elution mode.

In this paper to complete our previous study, a PS-DVB polymer-based column was evaluated under a gradient elution mode to optimize the conditions, specially the analysis time, for the separation of various glucosinolates greatly differing in substituent nature. The main aim of our overall study on the chromatographic behavior of glucosinolates as organic anions was the search for high performance liquid-solid extraction systems to obtain very pure individual intact glucosinolates from different natural products. Owing to the wide range of polarities of these compounds to be analyzed and/ or purified, there is a real need for several simple, reliable, complementary and alternative LC methods. So, in this paper the third type of support commonly used in anion exchange chromatography, a polymethacrylate copolymeric anion exchanger, which was a relatively more hydrophilic type polymer backbone than PS-DVB was also investigated, as a possible additional column material for the isocratic analysis of glucosinolates by anion exchange chromatography.

MATERIALS

Table 1 lists the glucosinolates studied. Sinigrin and glucotropaeolin were obtained from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) respectively. Gluconapin was purchased from Dr. A. Quinsac (Centre Technique Interprofessionnel des Oléagineux Métropolitains, Ardon, France). Progoitrin was extracted and isolated from rapeseed in our laboratory. Glucobrassicin was synthesized in our laboratory.[12] Eluent constituents were purchased as follows: distilled water from the Cooperative Pharmaceutique Française (Melun, France); acetonitrile (ACN), RS for preparative HPLC, from Carlo Erba (Milan, Italy); sodium acetate, potassium sulfate, sodium citrate, boric acid, sodium tetraborate decahydrate, sodium gluconate and glycerol from Prolabo (Paris, France); 1-butanesulfonic acid sodium salt, 1-hexanesulfonic acid sodium salt and 1-octanesulfonic acid sodium salt from Eastman Kodak (Rochester, NY, USA). All reagents were of analytical grade. Two compositions of borate-gluconate eluents were tested and prepared from a concentrate A containing 18 g/L boric acid, 25 g/L sodium tetraborate decahydrate, 16 g/L sodium gluconate and 250 mL/L glycerol. The borate-gluconate 1 was prepared by diluting 10 mL of concentrate A with 60 mL of acetonitrile and 430 mL of water and the borate-gluconate 2 was prepared by diluting 1 mL of concentrate A with 499 mL of water.

Table 1

Natural Glucosinolates Studies

Systematic Name of R Group	Trivial Name	Symbol
2-Propenyl	Sinigrin	SIN
3-Butenyl	Gluconapin	GNA
(2R)-2-Hydroxy-3-butenyl	Progoitrin	PRO
Benzyl	Glucotropaeolin	GTL
Indol-3-ylmethyl	Glucobrassicin	GBS

The liquid chromatographic apparatus consisted of a Varian (Palo Alto, CA, USA) Model 2010 solvent-delivery pump or a Bruker gradient pump (Merck, Darmstadt, Germany), a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20 μL sample loop and a Varian Model 2550 UV spectrophotometric detector or a Vydac (Wescan Instruments, Santa Clara, CA, USA) Model 213 A conductivity detector.

Separations were performed on a 100 x 4.1 mm I.D. column packed with 10 μm Hamilton PRP-X100 (Bonaduz, Switzerland) and on a 100 x 4.6 mm column packed with 10 μm Shimadzu Shim-pack IC-A1 (Kyoto, Japan).

Data were processed with a Shimadzu (Kyoto, Japan) CR 6A integrator/recorder. Experiments were carried out at room temperature and the detection wavelength was 229 nm.

To determine the system void volume, V_0, 20 μL of water were injected in each system. Each glucosinolate was injected alone and in admixture in the chromatographic mixtures.

The retention time of each compound was determined in each eluent by three consecutive determinations. The concentration of the standards were ca. 100 ppm in aqueous solution.

RESULTS AND DISCUSSION

Linear Gradient Elution on a PS-DVB Copolymeric Anion Exchanger

As we have demonstrated,[5] the isocratic analysis of glucosinolates on a Hamilton PRP-X100 column with hexylsulfonate eluent ion in water-acetonitrile mixture as mobile phase could be used as a complement to the current ion pairing method. However, when the natural mixture is composed of various glucosinolates greatly differing in substituent nature, a long and unsactifactory analysis time was observed (about 30 min at flow rate 2 mL/min to elute GBS) with this method. The judicious choice of a mobile phase gradient should avoid this disadvantage.

The strong elution strength of alkanesulfonate eluent ion can be explained by reverse phase interactions between the hydrophobic alkylchain and the polystyrene divinylbenzene polymeric backbone. So, with a view to developing, as far as possible, a gradient elution mode with this type of mobile phase, for a given concentration of hexylsulfonate in the eluent, we have investigated the amount of adsorbed hexylsulfonate on the stationary phase and the rate of attainment of equilibrium for the chromatographic system. Using a conductivity detector it was possible to monitor the effluent conductivity versus time. Whatever the nature of the solvent (water, acetonitrile or water/acetonitrile mixture) used to pre-wet the polymeric support, the amount of alkanesulfonate adsorbed was very low (10 µmole for a hexylsulfonate solution 5 mM in water/acetonitrile) and after eluent percolation of 2 mL the value of the effluent conductivity was equal to that of the eluent before percolation. Increasing the hexylsulfonate concentration or increasing the acetonitrile percentage in the mobile phase lead to no significant variation in the adsorbed amount. The total desorption was also very fast. Consequently, when the eluent composition was modified, returning to the inital conditions was easy and very quick.

These results are different from those classically observed in ion pair chromatography. The amount of adsorbed alkanesulfonate on n-alkyl bonded silica is noticeably more important than on the Hamilton PRP-X100 column and increases with an increase in the alkanesulfonate concentration or a decrease in the acetonitrile percentage in the mobile phase.[15] The strong reverse phase interactions between the alkanesulfonate eluent ion and the polymeric matrix were not a problem to carry out an elution gradient. The re-equilibration time needed between gradient runs should be short.

To optimize the conditions for the gradient mode analysis, it was necessary to seek the best column efficiency, to decrease the strong adsorption of the indolylglucosinolates onto the polymeric backbone without losing a good selectivity between each alkenylglucosinolate. The choice of acetonitrile rather than methanol or tetrahydrofuran as organic modifier in the mobile phase provided the best efficiency for all glucosinolates. Two approaches to gradient separation were investigated.

The first approach involved increasing the amount of the organic modifier (acetonitrile), thereby decreasing the retention time of glucosinolates that are retained predominantly by reverse phase such as arylglucosinolate GTL or indolylglucosinolate GBS. This method proved to be of limited utility. UV detection at 229 nm was used for the glucosinolate analysis. At this wavelength, all acetonitrile variation during the gradient run leads to variations in UV absorbance consequently increasing noticeably the background drift. Moreover system peaks and poor system reproducibility were observed.

The second approach was to utilize a concentration gradient in which the concentration of the hexylsulfonate eluent ion in water-acetonitrile (90:10) solution was increased over the run and satisfactory gradients were produced by this method. Figure 2 depicts the elution pattern of five glucosinolates (PRO, SIN, GNA, GTL, GBS) with an hexylsulfonate concentration gradient. The mobile phase gradient was obtained as following: hexylsulfonate 5 mM in water/acetonitrile (90:10) to hexylsulfonate 50 mM in water/acetonitrile (90:10) in 5 min then this composition was maintained for a further 10 min. A satisfactory analysis time for GBS (about 12 min at flow rate 1.5 mL/min) and a good separation of PRO, SIN, GNA are observed. Excellent retention time reproducibility was noted.

Isocratic Elution on a Polymethacrylate Copolymeric Anion Exchanger

The third type of support commonly used in anion exchange chromatography, a polymethacrylate copolymeric anion exchanger was then evaluated as a possible additional column material for the isocratic analysis of glucosinolates. For this study, five natural glucosinolates were selected on the basis of the large diversity of their aglycone part, which can be alkenyl: SIN and GNA, hydroxyalkenyl: PRO, arylalkyl: GTL or indolylalkyl: GBS. The great diversity of the R groups leads to wide variations in the polarity and the hydrophilic and lipophilic characters of these natural compounds. A chromatographic study of GTL and GBS compared to that of GNA should display the possible separation of glucosinolates in accordance with the three

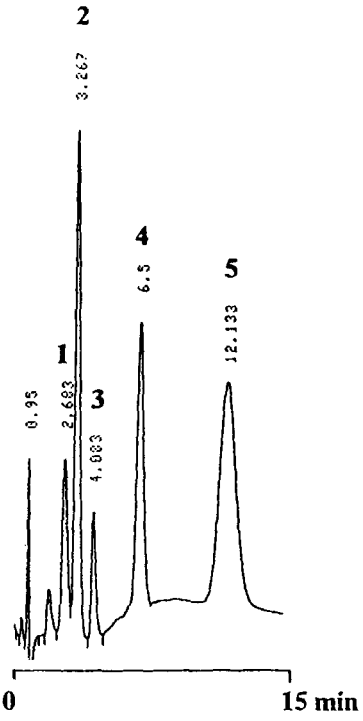

Figure 2. Chromatogram of five glucosinolates with an eluent ion concentration gradient. Column Hamilton PRP-X100 (100 x 4.1 mm I.D.), flow rate 1.5 mL/min and detection UV at 229 nm. Mobile phase gradient: hexylsulfonate 5mM in water/acetonitrile (90:10) to hexylsulfonate 50 mM in water/acetonitrile (90:10) in 5 min then standing this eluent composition to 15 min and returning to hexylsulfonate 5 mM to 17 min.
Elution order: (1) Progoitrin, (2) Sinigrin, (3) Gluconapin, (4) Glucotropaeolin, (5) Glucobrassicin

important subclasses: alkenyl, arylalkyl or indolylalkyl. SIN and GNA are two organic anions which differ only by one methylene group on their aglycone side chain R. By comparing the behaviour of SIN and GNA in different chromatographic systems, it was possible to evaluate and compare the ability of these systems to separate two compounds with a similar polarity. For the rapeseed glucosinolate analysis, the resolution of the PRO-GNA pair is particularly interesting owing to the importance of the ratio of these two major glucosinolates in this cruciferae family.

Table 2

Capacity Factors (k') and Relative Retention (α^a) of Some Glucosinolates on a Shim-pack IC-A1 (100 x 4.6 mm I.D.) Column With Different Anion Eluents at the same Concentration

	Capacity Factor					Relative Retention[a]	
Eluent Ion 2.5mM	k'_{PRO}	k'_{SIN}	k'_{GNA}	k'_{GTL}	k'_{GBS}	$\alpha_{PRO,GNA}$	$\alpha_{PRO,GBS}$
CH_3COO^-	8.11	10.40	*	*	*		
SO_4^{2-}	1.72	2.53	3.87	12.52	36.8	2.25	21.4
citrate, pH=8	0.79	1.08	1.40	3.81	11.21	1.8	14.2
$C_4H_9SO_3^-$	3.37	4.60	5.69	14.66	48.95	1.7	14.5
$C_6H_{13}SO_3^-$	2.67	3.39	4.12	9.54	28.11	1.5	10.5
$C_8H_{17}SO_3^-$	0.75	0.92	1.07	2.26	6.4	1.4	8.5

* compound with $k' > 20$.

[a] $\alpha_{1,2} = \frac{k'_2}{k'_1}$

Our study was mainly carried out on a Shimadzu Shim-pack IC-A1 polymethacrylate support incorporating a quaternary ammonium base as a functional group. Table 2 gives the capacity factors, k', for the five selected natural glucosinolates using different eluent anions. All eluent ionic strengths were obtained with the same 2.5 mM concentration of salt.

The choice of acetonitrile rather than methanol or tetrahydrofuran provided the best efficiency for all glucosinolates. As in anion exchange chromatography with a silica trialkylammonium exchanger[4] or a PS-DVB copolymeric anion exchanger,[5] the glucosinolates were eluted principally in order of decreasing polarity: alkenyl (PRO, SIN, GNA), arylalkyl (GTL) then indolylglucosinolates (GBS).

More retention and more selectivity have been observed on a Shim-pack IC-A1 column than on a silica anion exchanger for the glucosinolate analysis: Table 2 shows that increasing the length of the aglycone R part by one methylene unit in the case of the two alkenylglucosinolates SIN and GNA is sufficient to bring about a retention variation between these compounds.

Whatever the nature of the eluent ion, this separation has not been observed on a silica anion exchanger.[4] With commonly used monovalent eluent anion (as CH_3COO^- or NO_3^-), it is possible to elute arylalkyl or indolylglucosinolates on a silica anion exchanger[4] but not on a polymethacrylate anion exchanger (Table 2).

Less retention and less selectivity have been observed on a Shim-pack IC-A1 column than on a PS-DVB copolymeric anion exchanger: the elution strength of a divalent eluent anion such as SO_4^{2-} is sufficient to elute the more lipophilic compounds, GTL and GBS on a Shim-pack IC-A1 column but on a Hamilton PRP-X100 column, only the more hydrophilic compound PRO can be eluted under these conditions.

As expected in anion exchange chromatography, Table 2 shows that the elution strength of the different eluent ions is increased in the following order: monovalent eluent ion (CH_3COO^-) then divalent eluent ion (SO_4^{2-}) and then trivalent eluent ion (citrate pH 8). These results are in good agreement with those obtained on a Partisil SAX column and this supports the idea that normal ion exchange was occurring predominantly between the eluent ion and the organic eluted anion in spite of the very low ion exchange capacity of the Shim-pack IC-A1 column (50μeq/g). By increasing the concentration of the sulfate eluent (2.5 mM to 5 mM), the elution strength increases. The sulfate eluent ion at 5 mM concentration enables elution of all glucosinolates within a reasonable analysis time, typically twenty one minutes for GBS with a flow of 1 mL/min, but under these conditions the alkenylglucosinolates are rapidly eluted and are thus poorly separated out.

Since the ion exchange capacity of the Shim-pack IC-A1 column is very low, increasing the concentration of the eluent ion increases rapidly the background level and involves a loss of efficiency. Changing the nature of the eluent anion rather than increasing the concentration of the eluent anion would be better to decrease the glucosinolate retention on a polymethacrylate anion exchanger.

With the eluent anions commonly used in anion exchange chromatography (CH_3COO^-, SO_4^{2-}, citrate) the Shim-pack IC-A1 column produces poor chromatographic performance for glucosinolate analysis. Peaks are badly tailed and the number of theoretical plates are low even for solutes which are not much retained as PRO, SIN and GNA. This is illustrated in Figure 3 which shows the separation resulting on the Shim-pack IC-A1 column when sulfate was used as eluent. However it is interesting to note that the relative retention between PRO and GNA, the two major glucosinolates in rapeseed, is sufficient to consider that the simple chromatographic system

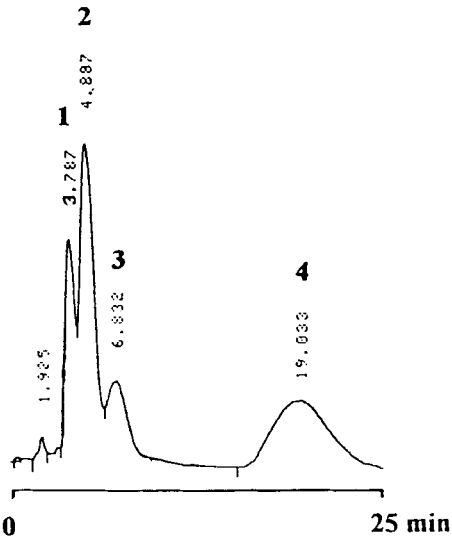

Figure 3. Chromatogram of standard mixture of glucosinolates. Column Shim-pack IC-A1 (100 x 4.6 mm I.D.), flow rate 1 mL/min and detection UV at 229 nm. Eluent: aqueous sulfate solution at 2.5 mM concentration.
Elution order : see Figure 2.

consisting of a Shim-pack IC-A1 column and an aqueous mobile phase containing SO_4^{2-} at low concentration (2.5 mM) is suitable for isolating pure individual progoitrin and gluconapin from natural mixtures. With this system, the concentration of the inorganic eluent anion is about 100-fold lower than the one used with a Hamilton PRP-X100 column.

We then tested the elution strength of borate-gluconate 1 as eluent. This eluent containing boric acid, sodium tetraborate and sodium gluconate acid with acetonitrile and glycerol is the standard eluent composition used with most polymethacrylate-based columns because of its low background conductance and relatively strong elution power.[13,14] Some researchers have suggested that the addition of acetonitrile in this eluent facilitates phase transfer and produces sharper peaks and shorter retention times for the analysis of polarizable anions such as nitrate and bromide.[13,14] With the chromatographic system made up of a Shim-pack IC-A1 column and the borate-gluconate 1 as mobile phase, the alkenylglucosinolates were eluted in the void volume and a very low retention for GTL and GBS was observed. Borate-gluconate 2 was then prepared by

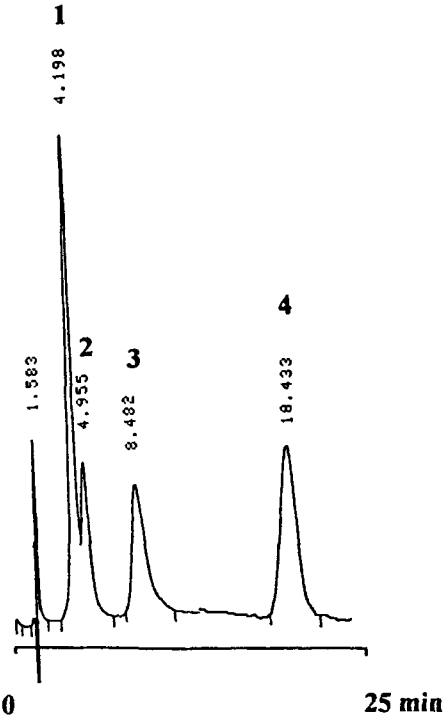

Figure 4. Chromatogram of standard mixture of glucosinolates. Column Shim-pack IC-A1 (100 x 4.6 mm I.D.), flow rate 1 mL/min and detection UV at 229 nm. Eluent borate-gluconate 2 prepared by diluting 1 mL of a concentrate containing 18 g/L boric acid, 25 g/L sodium tetraborate decahydrate, 16 g/L sodium gluconate and 250 mL/L glycerol with 499 mL of water.
Elution order: (1) Progoitrin, (2) Gluconapin, (3) Glucotropaeolin, (4) Glucobrassicin.

diluting concentrate A 500-fold with water and was then tested as eluent in the column. As can be seen in Figure 4, it is possible to separate a mixture of four glucosinolates (PRO, GNA, GTL, GBS) with this eluent and with a better efficiency than with a sulfate eluent ion (Fig. 3). However, the elution strength of borate-gluconate 2 was too strong and consequently a poor separation of alkenylglucosinolates was observed. Lower concentrations of borate-gluconate mixture should permit greater retention of alkenylglucosinolates but the concentration required was so low that deformation of the peak shape was observed as a result of column overloading when glucosinolates were injected.

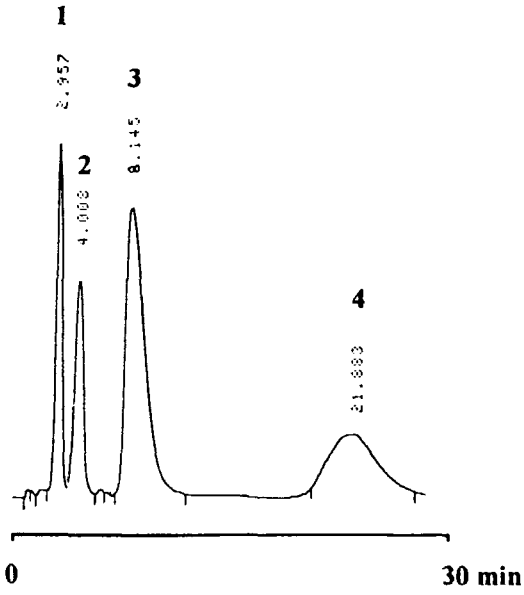

Figure 5. Chromatogram of standard mixture of glucosinolates. Column Shim-pack IC-A1 (100 x 4.6 mm I.D.), flow rate 1.5 ml.min^{-1} and detection UV at 229 nm. Eluent: aqueous hexylsulfonate solution at 2.5 mM concentration.
Elution order: see Figure 4.

In our previous paper,[5] we showed that the use of alkanesulfonate anions with a chain length of at least six carbons, as eluent anions, decreases noticeably the strong reverse phase interactions between the more lipophilic glucosinolates and the PS-DVB polymeric backbone. So, the elution strength of some alkanesulfonate anions on the polymethacrylate support was then tested.

Table 2 gives the capacity factors k' for the five glucosinolates using butylsulfonate, hexylsulfonate or octylsulfonate as eluent anion and Figure 5 depicts the elution pattern of a standard mixture of four glucosinolates (PRO, GNA, GTL, GBS) with an aqueous hexylsulfonate eluent ion at 2.5 mM concentration. It is now possible with these monovalent organic eluent anions in aqueous medium to elute all the glucosinolate subclasses. The separation efficiency was similar to that observed with sulfate anion eluent (Fig.3 and 5).

For a given eluent concentration, as the carbon number, n, of the alkanesulfonate eluent ion increases (n = 4, 6 or 8), the glucosinolate retention decreases (Table 2). The same results were observed for glucosinolate analysis on a Hamilton PRP-X100 column.[5] As expected,[11] solvophobic interaction increased with the carbon number, so the octylsulfonate anion was held more strongly at the ion exchange site than the butylsulfonate anion and thus the ion exchange equilibrium constant of glucosinolates was reduced with increasing carbon number in the eluent ion. The greater elution strength of these alkanesulfonate homologues compared with that of a monovalent organic eluent anion such as acetate can be explained by interactions between the hydrophobic alkyl chain and the polymethacrylate matrix. These interactions are not possible with acetate anions or inorganic anions.

The alkenylglucosinolates are eluted more rapidly with the divalent sulfate eluent than with the monovalent hexylsulfonate eluent whilst a shorter analysis time was observed for the indolylglucosinolates with the hexylsulfonate eluent than with the divalent eluent. These results confirm that the adsorption of the organic analyte ions onto the polymeric backbone was all the stronger as the compound includes a much more apolar aglycone component. On a polymethacrylate anion exchanger, the contribution of the reverse phase interactions with respect to the ion exchange interactions was more reinforced in the indolylglucosinolate than in the alkenylglucosinolate retention mechanism.

Table 2 clearly shows that the separation of the PRO/GNA and PRO/GBS pairs were related to the eluent ion used. The values of the relative retention α for these two pairs decreased as the carbon number n of the alkanesulfonate increased, whereas minor variations for these α values were observed on a PS-DVB polymer-based column. On a polymethacrylate-based column, the nature of the alkane residue of alkanesulfonate is an important parameter not only for decreasing the capacity factors as shown on a PS-DVB polymer-based column but also for increasing the selectivity between two glucosinolates.

For the qualitative analysis of glucosinolate mixtures including different subclasses, the chromatographic system made up of a Shim-pack IC-A1 column and an aqueous mobile phase containing 2.5 mM hexylsulfonate as eluent ion, under isocratic elution mode (Fig. 5) was a convenient chromatographic system which can be used in place of the chromatographic system made up of a column Partisil SAX and an aqueous mobile phase containing dihydrogenocitrate 0.5 mM as eluent ion.[4] The analysis time of the more retained compound (GBS) was similar in these two systems (about twenty minutes) and a better separation for the PRO/GNA and PRO/GBS pairs was observed on the polymethacrylate-based column.

CONCLUSION

For glucosinolate analysis, more retention and better selectivities were observed on a polymethacrylate anion exchanger than on a silica anion exchanger. The importance of the reverse phase interactions with respect to the ion exchange interactions in the glucosinolate retention mechanism was reinforced on a polymethacrylate-based column in comparison with a silica-based column and lessened in comparison with a PS-DVB copolymeric anion exchanger. Satisfactory separation between each glucosinolate subclass was observed in about 20 min on a Shim-pack IC-A1 column with an aqueous hexylsulfonate solution or an aqueous borate-gluconate mixture as mobile phase in an isocratic mode. The chromatographic system, made up of a column Shim-pack IC-A1 and an aqueous mobile phase containing SO_4^{2-} is suitable to isolating pure individual alkenylglucosinolates. To decrease the stong adsorption of the indolylglucosinolates onto the PS-DVB polymeric backbone without losing a good selectivity between each alkenylglucosinolate, an eluent ion concentration gradient on a Hamilton PRP-X100 column could be used. A good separation and a satisfactory analysis time for natural mixtures composed of various glucosinolates greatly differing in substituent nature could be obtained under these conditions.

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