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HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF ORGANIC ACIDS

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SUMMARY

The advantages of a partially quaternized diethylaminoethyl derivative of Spheron™/Separon HEMA™ in comparison with classical microporous polystyrene anion exchangers for the rapid anion-exchange chromatography of organic acids are demonstrated. An investigation of the effects of the composition and pH of the mobile phase, of the column temperature and of the flow-rate resulted in two optimized procedures. The first is suitable for the separation of some organic acids frequently occurring in agricultural and food samples of plant origin. These organic acids are eluted in about 20 min at 60°C using 0.6 *M* sodium sulphate as the eluent. The second method employing a more dilute mobile phase (0.1 *M* sodium sulphate) is proposed for the analysis of oligo(galacturonic acids) in pectin hydrolysates.

INTRODUCTION

Organic acids in biological materials may be analyzed by various separation techniques, applicability of which to routine analytical practice is determined by the simplicity and rapidity of the determination, including the sample preparation and accessibility of the instrumentation. Gas chromatography[1,2] does not meet the first requirement because of the complicated preparation of samples. On the other hand, the very rapid and ingenious isotachophoresis[3] needs rather specialized and complex equipment.

Both of the above requirements may presumably be met by using high-per-

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formance liquid chromatography (HPLC). The first attempts to realize a fully automated liquid chromatography of these compounds employed complicated instruments, which were in principle analogous to amino acid analyzers. The analyzer designed by Kesner and Muntwyller[4] and improved by others[5–9] has been utilized for the separation of organic acids by partition chromatography on silica impregnated with sulphuric acid. The eluted acids were detected photometrically after mixing the column effluent with an acid–base indicator. This method is highly sensitive and has a high resolving capacity, but is time-consuming (analyses usually took several hours) and requires a complicated regeneration of columns[9]. The mobile phases used in this procedure are not compatible with the usual detectors for HPLC (differential refractometers and UV photometers operating in the region around 200 nm).

The first experiments with automated ion-exchange chromatography of organic acids employed detection systems based on post-column derivatization[10–12]. Subsequently, the introduction of detectors which do not cause extensive spreading of peaks enabled the reduction of the analysis times in ion-exchange chromatography on anion exchangers[13,14] and in ion-exclusion chromatography on cation exchangers[15,16] to those times common in HPLC. The shortcoming of all these methods still consists in the application of classical swelling microreticular ion exchangers, the volume of which strongly depends on the mobile phase composition and on the flow-through velocity in the column. In addition to this, microporous ion exchangers are not the most suitable from the standpoint of chromatography kinetics (slow diffusion in the swollen polymer).

It has been demonstrated, that these shortcomings of classical materials for ion-exchange chromatography may be eliminated in the chromatography of biopolymers[17] and saccharides[18,19] by using semi-rigid macroporous ion exchangers based on 2-hydroxyethyl methacrylate copolymers Spheron™/Separon HEMA™. In this paper we have studied the use of partially quaternized DEAE-Spheron in the rapid ion-exchange chromatography of organic acids.

EXPERIMENTAL

Instrumentation

A simple apparatus for medium-pressure chromatography was assembled from a piston micropump MC-300 (Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia), a septum sampler LCI-20 (Laboratory Instrument Works, Prague, Czechoslovakia), thermostatted stainless-steel chromatographic columns (25 × 0.6 cm) and a variable-wavelength UV detector UVM-4 (Development Workshops of Czechoslovak Academy of Sciences). Chromatograms were recorded with a line recorder TZ 4003 (Laboratory Instrument Works) and an automatic integrator (Minigrator; Spectra-Physics, Santa Clara, CA, U.S.A.).

Solid phases for chromatographic columns

A partially quaternized diethylaminoethyl derivative[20] was synthesized from the hydroxyethyl methacrylate copolymer with spherical microparticles supplied by Lachema (Brno, Czechoslovakia) under the trade-name Spheron 40 and by Laboratory Instrument Works under the trade-name Separon HEMA-40. Unlike in previous work[18,19], a more extensively cross-linked starting material was used (exclusion limit for dextran standards *ca.* 40,000). The mean particle size used for ionogenic

substitution was 14.3 ± 2.3 μm. The raw product after introduction of ionogenic groups was purified by gradual washing with a 50-fold excess of 1 *M* lithium hydroxide, 1 *M* sodium chloride, 1 *M* hydrochloric acid, 1 *M* sodium sulphate and 1 *M* sulphuric acid. Each elution was followed by extraction with distilled water, methanol, distilled water, acetone and again with distilled water. In this way, the removal of compounds absorbing in the UV region led to a material with a completely stable baseline in chromatographic applications. The fully quaternized strongly basic anion exchanger with a polystyrene matrix, Ostion LG AT 0800 (mean particle size 10.2 μm), produced by Corporation for Chemistry and Metallurgy, Ústí nad Labem, Czechoslovakia, was used as a reference. The exchanger was converted into the sulphate form with 1 *M* sulphuric acid.

Packing of columns

The microreticular anion exchanger Ostion LG AT 0800 was packed as a degassed 50% suspension in 1 *M* sodium sulphate under a pressure of no more than 3 MPa using the micropump MC-300 (Method A). The same procedure was initially used for packing columns with DEAE-Spheron™. Alternatively, this phase was packed as a degassed 50% suspension in 35% aqueous ammonium sulphate solution by means of the pulseless linear pump HPP 4001 (Laboratory Instrument Works) under a pressure of 8 MPa (Method B).

Preparation of mobile phase

Aqueous solutions of sodium sulphate of various concentrations were degassed under a vacuum.

Preparation of standards of organic acids

Homologous oligo(D-galacturonic acids), polymerization degree 2–9, were isolated by gel chromatography on Sephadex G-25 Fine[21] from the enzymatic hydrolysate of sodium pectate, produced in a column reactor with endo-D-galacturonase immobilized on poly(ethylene terephthalate)[22]. The purity of preparations was checked by thin-layer chromatography (TLC) on silica[23]. The degree of polymerization was determined from the ratio of the content of reducing groups to the content of carboxylic groups, as well as by means of a plot of log $R_F/(1 - R_F)$ *vs.* degree of polymerization[24], using D-galactopyranuronic acid (Fluka, Buchs, Switzerland) as a standard.

Other organic acids were commercial preparations from Lachema and Reanal (Budapest, Hungary). Stock solutions of concentration 0.5000 g per 10 ml were prepared from these compounds in distilled water; standards of the poorly soluble fumaric acid and adipic acid were prepared in lower concentrations. These standards were stored in a frozen state and used for preparation of standard mixtures by dilution in distilled water before chromatography.

RESULTS AND DISCUSSION

The effects of the following experimental conditions on the retention and resolution of organic acids and on the efficiency of the column were investigated:

(1) Type of anion exchanger used as the column packing.

(2) The packing method.
(3) Column temperature in the range 30–60°C.
(4) Concentration of sodium sulphate in the mobile phase, which was varied in five steps in the range 0.332–1.0 *M*.
(5) The pH value of the eluent, varied in the range pH 2–8.
(6) The mobile phase flow-rate, varied in the range 18–91 ml/h.

Optimization of the separation of organic acids

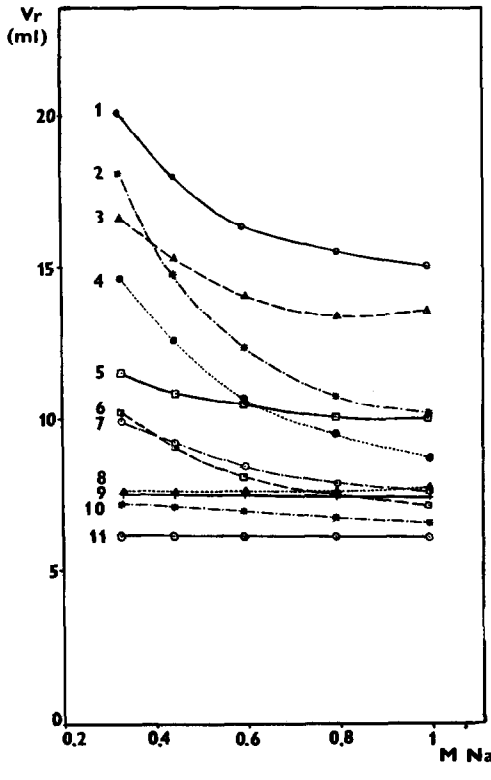
A column packed with the macroreticular anion exchanger DEAE-Spheron by Method A exhibited lower efficiency (920–1900 plates) than the column packed with the polystyrene anion exchanger Ostion LG AT 0800 (1200–7400 plates depending on the type of acid). On the other hand, the column packed with DEAE-Spheron by Method B had a higher efficiency (in the range 2400–6100 plates) for seven of the twelve tested organic acids than the column packed with the classical ion exchanger of smaller particle diameter. The kinetic advantages of the separation on Spheron are clearly visible from a comparison of the reduced heights of a theoretical plate (2.9–7.4 on DEAE-Spheron, 3.4–19.6 on Ostion LG AT 0800). The efficiencies of the columns were compared at 60°C and at a flow-rate of 68.6 ml/h (*i.e.*, linear flow velocity 4.05 cm/min) using 1 *M* sodium sulphate as the mobile phase. The efficiencies were not compared for malonic acid and citric acid which gave asymmetric flat peaks on the Ostion column.

The comparison on the retention times of organic acids on both column packings under identical conditions revealed that all tested compounds, except malonic acid, exhibited greater retention on the anion exchanger Ostion LG AS 0800 than on the macroporous anion exchanger based on hydroxyethyl methacrylate copolymer. These differences were clear-cut, at a significance level of 95% with glycolic, lactic, succinic, itaconic, α-ketoglutaric, fumaric, aconitic, oxalic and tartaric acids. The last five compounds exhibited especially marked differences in retention; their retention volumes on the Ostion exchanger were 160–280% of the values observed on the column packed with DEAE-Spheron.

It follows from the above results that, from the standpoint of kinetics and equilibria, the macroreticular anion exchanger based on hydroxyethyl methacrylate copolymer is better suited to the rapid liquid chromatography of organic acids than the classical microporous polystyrene anion exchanger. Similar conclusions were made from a previous comparison of both types of ion exchangers in the chromatography of borate complexes of saccharides[18,19].

Investigations of the effect of sulphate-anion concentration on the retention of organic acids are illustrated in Fig. 1. The dependence of the retention volumes of lactic, acetic and pyrrolidonecarboxylic acids on the concentration of sodium sulphate in the eluent has not been demonstrated, but it is clearly evident for other compounds (significance level 99%). Important adsorption interactions with the hydroxyethyl methacrylate copolymer may be assumed from the fact that the monocarboxylic acids differ in their retention volumes, although the dependence of their retention on the concentration of exchanger gegenion in the mobile phase is small.

Evidence in support of this assumption is provided by the dependence of the retention volumes (V_r of acids on the concentration of mobile phase, plotted according to V_r *vs.* $1/[SO_4^{2-}]^{a/b}$, where a is the charge of the ion-exchanger gegenion and b is the



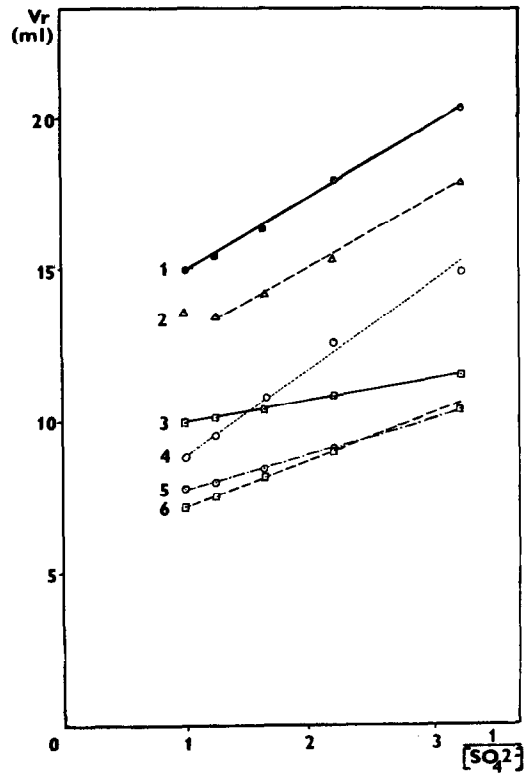


Fig. 1. Dependence of the retention volumes of organic acids on the concentration of sodium sulphate in mobile phase. Chromatographic conditions: column of DEAE-Spheron 40 (SO_4^{2-}), 250 × 6 mm; temperature 60°C. Curves: 1 = α-ketoglutaric acid; 2 = citric acid; 3 = malonic acid; 4 = oxalic acid; 5 = succinic acid; 6 = tartaric acid; 7 = malic acid; 8 = acetic acid; 9 = lactic acid; 10 = glycolic acid; 11 = pyrrolidonecarboxylic acid.

Fig. 2. Linearized dependence of the retention volumes of dicarboxylic organic acids on the concentration of sodium sulphate in the mobile phase. Chromatographic conditions as in Fig. 1. Curves: 1 = α-ketoglutaric acid; 2 = malonic acid; 3 = oxalic acid; 4 = succinic acid; 5 = tartaric acid; 6 = malic acid.

charge of the separated ion. This relationship is given in Fig. 2 for dicarboxylic acids, where virtually total dissociation of both carboxyls may be expected in the solution of neutral sodium sulphate. The variance between the values of V_r extrapolated to infinitely large concentration of ion-exchanger gegenion in the mobile phase may also be explained by adsorption effects.

The strong dependence of retention volume on the sulphate molarity in the eluent is particularly evident for the trifunctional citric acid, whose position in a chromatogram with respect to other compounds can easily be influenced in this way, and for oxalic acid which is adsorbed only to a very small extent (see Fig. 2).

The column temperature (Fig. 3) has a significant effect on the retention of various pairs of organic acids and its increase from 30 to 60°C leads to an inversion in the selectivity for the pairs α-ketoglutaric acid–itaconic acid and oxalic acid–citric acid. The dependence of retention of fumaric acid on the column temperature ob-

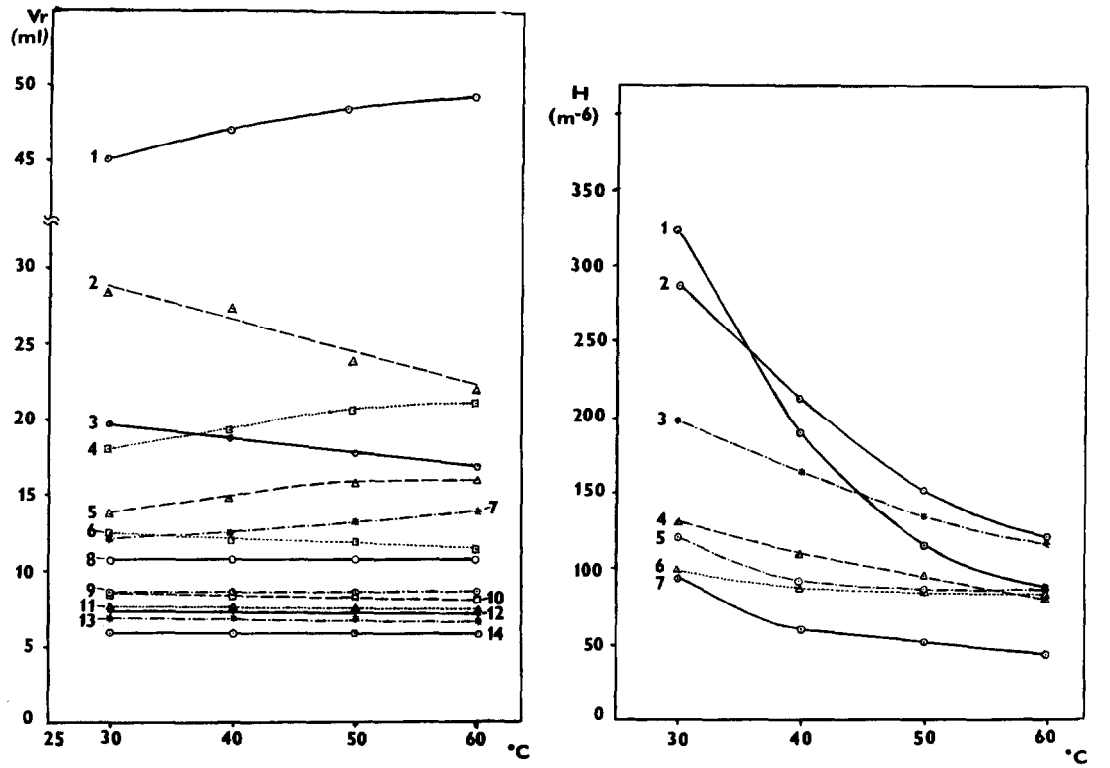


Fig. 3. Dependence of the retention volumes of organic acids on column temperature. Chromatographic conditions: column of DEAE-Spheron 40 (SO_4^{2-}), 250 × 6 mm; eluent 0.6 *M* sodium sulphate. Curves: 1 = aconitic acid; 2 = fumaric acid; 3 = α-ketoglutaric acid; 4 = itaconic acid; 5 = malonic acid; 6 = oxalic acid; 7 = citric acid; 8 = succinic acid; 9 = malic acid; 10 = tartaric acid; 11 = acetic acid; 12 = lactic acid; 13 = glycolic acid; 14 = pyrrolidonecarboxylic acid.

Fig. 4. Dependence of the plate height (*H*) on column temperature for selected organic acids. Chromatographic conditions: column of DEAE Spheron 40 (SO_4^{2-}), 250 × 6 mm; eluent 0.6 *M* sodium sulphate, flow-rate 65.6 ml/h. Curves: 1 = α-ketoglutaric acid; 2 = aconitic acid; 3 = citric acid; 4 = fumaric acid; 5 = malic acid; 6 = acetic acid; 7 = glycolic acid.

served in our work is in accord with the results of Bengtsson and Samuelson[11] obtained with a polystyrene anion exchanger, however the temperature dependence of the retention of aconitic acid and citric acid observed by us differs from that in the previous work. An increase in temperature results in an increase in column efficiency (Fig. 4), therefore the highest column temperature (60°C) was chosen for routine analyses.

The effect of mobile phase flow-rate on the efficiency of a column packed with DEAE-Spheron is illustrated in Figs. 5 and 6 for selected organic acids. It should be noted that the kinetics of chromatography on ion-exchange derivatives of Spheron has not yet been studied in detail, and therefore we have used for the interpretation of experimental data the linear model for the dependence of plate height on flow-rate employed previously[25] for the separation of amino acids on polystyrene cation exchangers. The experimental data were treated by the least-squares methods.

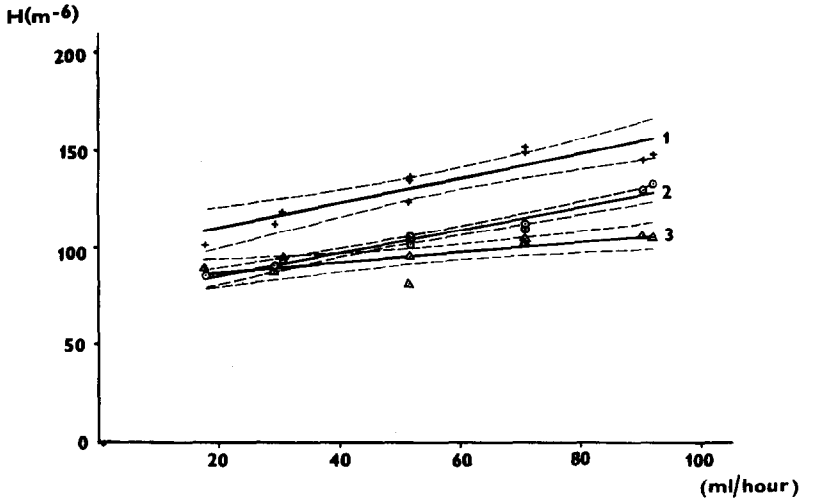


Fig. 5. Dependence of the plate height on the mobile phase flow-rate for selected organic acids. Chromatographic conditions as in Fig. 3; column temperature 60°C. Curves: 1 = malic acid; 2 = fumaric acid; 3 = acetic acid. Dashed lines indicate intervals of reliability at the significance level of 95%.

The dependence of retention of organic acids on pH was studied only qualitatively because it was not possible for practical reasons to work with solutions containing free sulphuric acid. A particularly pronounced decrease in retention was found with most organic acids in the region of pH 3, *i.e.*, in the region of the pK_1 of most acids. However, the lack of experimental data does not allow the interpretation of the retention volume dependences on pH as the analogues of titration curves. The folowing sequence of retention was observed at pH 2: pyrrolidonecarboxylic acid, glycolic,

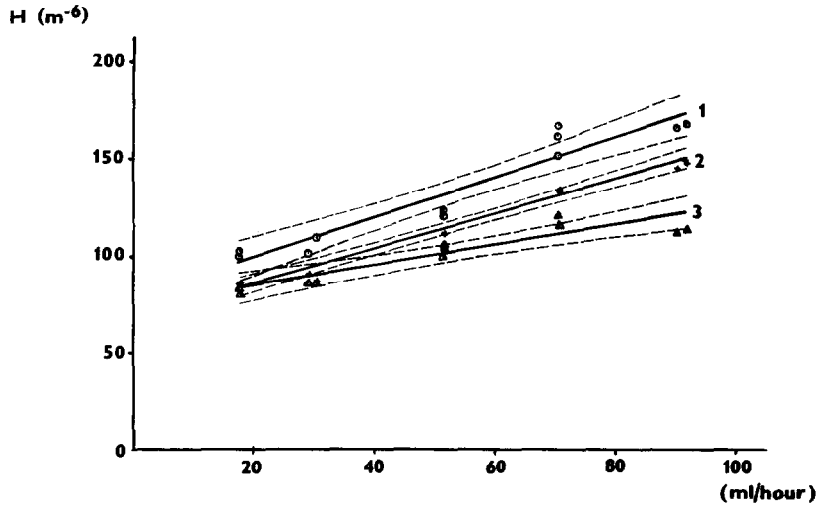


Fig. 6. Dependence of the plate height on the mobile phase flow-rate for selected organic acids. Chromatographic conditions as in Fig. 5. Curves: 1 = citric acid; 2 = α-ketoglutaric acid; 3 = pyrrolidonecarboxylic acid. Dashed lines as in Fig. 5.

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR THE SEPARATION OF ORGANIC ACIDS IN MATERIALS OF PLANT ORIGIN, AND OF OLIGO(GALACTURONIC ACIDS) IN PECTIN HYDROLYSATE

Eluent	*0.6 M Sodium sulphate (organic acids) or 0.1 M sodium sulphate (oligo(galacturonic acids))*
Column packing	DEAE Spheron 40 (14.3 ± 2.3 μm)
Ionic form	SO_4^{2-}
Column size (mm)	250 × 6
Column temperature (°C)	60
Eluent flow-rate (ml/min)	1.0–1.1
UV detection (nm)	210–220

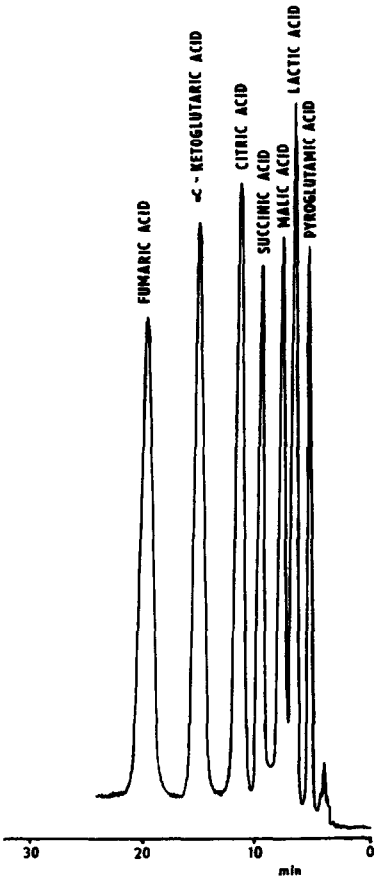


Fig. 7. Separation of a standard mixture of organic acids. Chromatographic conditions as in Table I. Mobile phase flow-rate 66.3 ml/h; pressure drop 1.9–2.6 MPa; UV detection at 215 nm. Sample injected: 30 μl of a solution containing 45 μg pyroglutamic acid, 225 μg lactic acid, 180 μg malic acid, 225 μg succinic acid, 300 μg citric acid, 30 μg α-ketoglutaric acid and 2.25 μg fumaric acid.

TABLE II

RETENTION VOLUMES OF ORGANIC ACIDS ELUTED WITH 0.6 *M* SODIUM SULPHATE UNDER CONDITIONS LISTED IN TABLE I

No.	*Compound*	*Retention volume, V_r (ml)*
1	Galacturonic acid	5.1
2	Glucuronic acid	5.3
3	Pyrrolidonecarboxylic acid	6.0
4	Glycolic acid	6.7
5	Lactic acid	7.1
6	Levulinic acid	7.3
7	Acetic acid	7.6
8	Tartaric acid	8.2
9	Malic acid	8.5
10	Glutaric acid	10.3
11	Succinic acid	10.8
12	Oxalic acid	11.4
13	Adipic acid	11.9
14	Citric acid	13.7
15	Malonic acid	15.7
16	α-Ketoglutaric acid	16.7
17	Itaconic acid	20.7
18	Maleic acid	21.9
19	Fumaric acid	22.1
20	Aconitic acid	50.2

lactic, tartaric, acetic, malic, citric, succinic, malonic, α-ketoglutaric, itaconic, oxalic, fumaric and aconitic acids.

The conditions given in Table I are proposed for practical analytical applications on the separation of organic acids on DEAE-Spheron in the sulphate form. Fig. 7 presents an example of a separation of a standard mixture of organic acids on the macroreticular anion exchanger under these conditions with 0.6 *M* sodium sulphate as mobile phase. The calibration mixture containing pyrrolidonecarboxylic, lactic, succinic, citric, α-ketoglutaric and fumaric acids was completely separated within 20 min (Table II).

TABLE III

RETENTION VOLUMES OF OLIGO(GALACTURONIC ACIDS) ELUTED WITH 0.1 *M* SODIUM SULPHATE UNDER CONDITIONS LISTED IN TABLE I

Polymerization degree (DP)	*Retention volume, V_r (ml)*
1	5.7
2	5.9
3	6.5
4	7.9
5	9.8
6	13.5
7	22.1
8	37.7

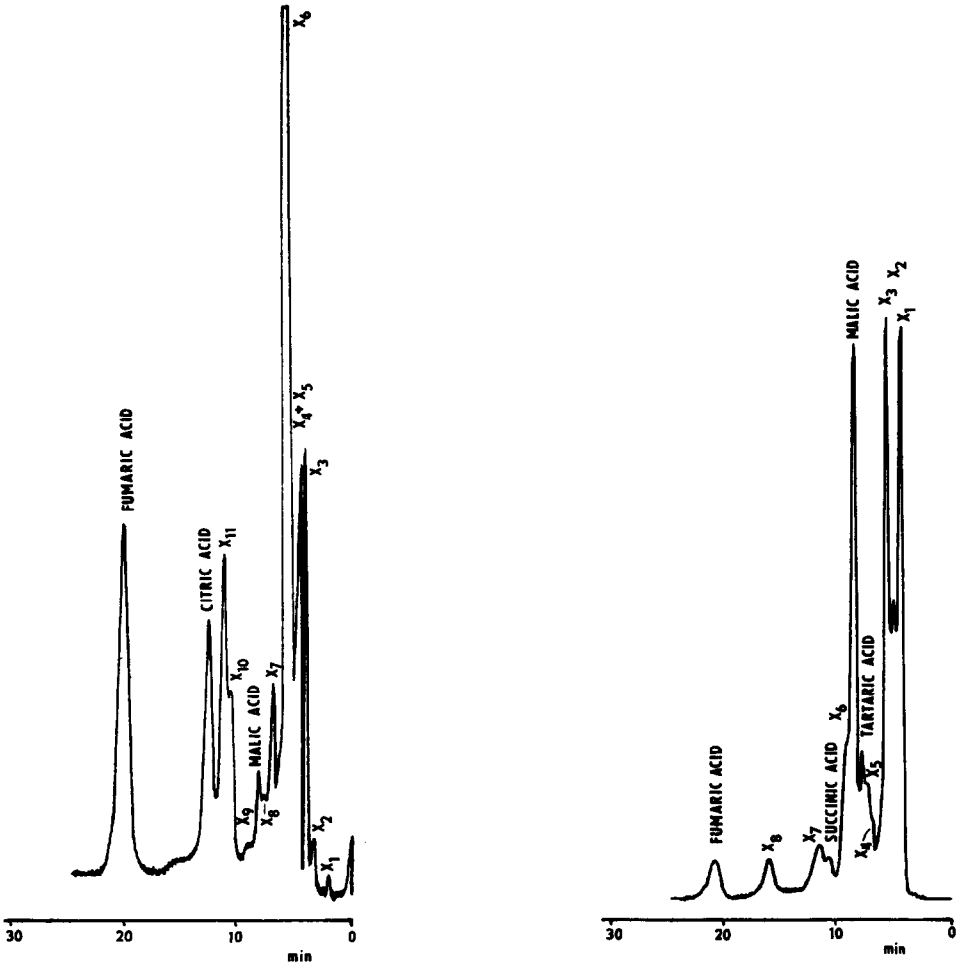


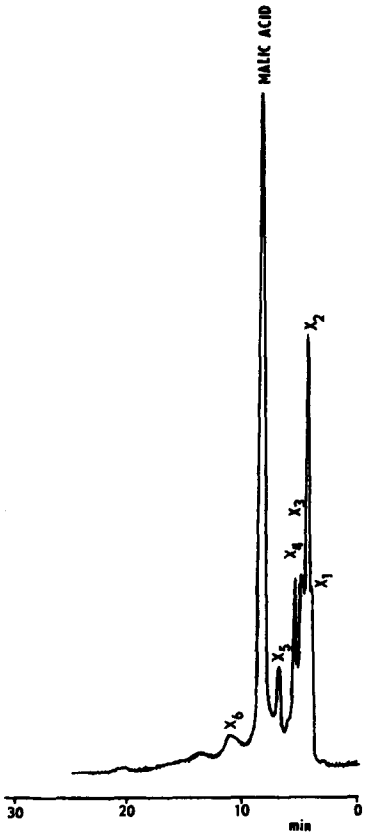
Fig. 8. Separation of organic acids in crude beet juice. Chromatographic conditions as in Fig. 7. Sample injected: 100 μl of solution refined through a cation exchanger in hydrogen-ion cycle. X_1–X_{11} = unidentified peaks.

Fig. 9. Separation of organic acids in white wine. Chromatographic conditions as in Fig. 7. Sample injected: 30 μl of wine. X_1–X_8 = unidentified peaks.

The separation of oligo(galacturonic acids) (Table III), occurring in enzymatic hydrolysates of pectin, required less concentrated mobile phases. We failed to distinguish the monomer and dimer of galacturonic acid even under the conditions in Table I, *i.e.*, 0.1 *M* sodium sulphate as eluent. Differentiation of other oligo(galacturonic acids) was fair, peaks corresponding to degrees of polymerization greater than 3 being resolved down to the baseline.

Practical applications

Examples of practical application of the described procedure for the separation



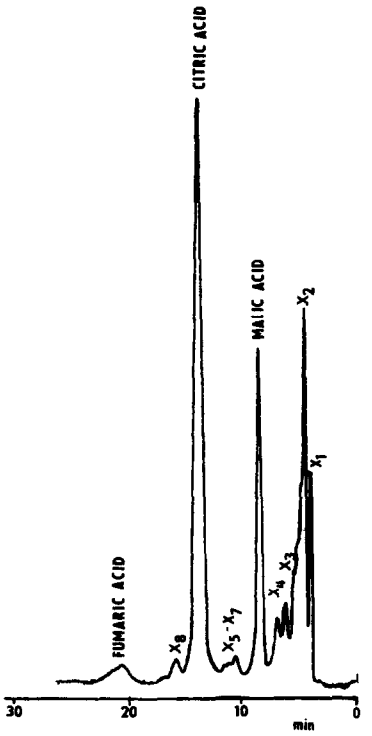


Fig. 10. Separation of organic acids in cider. Chromatographic conditions as in Fig. 7. Sample injected: 30 μl of cider. X_1–X_6 = unidentified peaks.

Fig. 11. Separation of organic acids in squash. Chromatographic conditions as in Fig. 7. Sample injected: 100 μl of squash freed from carbon dioxide by acidifying. X_1–X_8 = unidentified peaks.

of organic acids in samples of food products are shown in Figs. 8–11. The chromatogram of beet juice (Fig. 8) contains, in addition to the identified peaks of malic, fumaric and citric acids, a number of peaks of unidentified components. The sample was prerefined on the cation exchanger in hydrogen ion cycle prior to injection into the column. The samples of white wine (Fig. 9) and cider (Fig. 10) were injected without preliminary treatment. For both materials the dominant peak was of malic acid. The chromatogram of squash (trade-mark Liberta) (Fig. 11), which was freed from carbon dioxide before injection into the column, shows the peaks of malic, citric and fumaric acids.

The described method was also successfully used for the analysis of alcoholic extracts of sugar-beet. However, its resolution power was not sufficient to distinguish organic acids in sugar-beet molasses, except the major component, lactic acid.

In a test of applicability to the quantitative analysis of agricultural and food samples, anion-exchange chromatography on DEAE-Spheron 40 was compared with

ion-exclusion chromatography on a cation exchanger[15,16,26] and with reversed-phase chromatography on silica C_{18} (refs. 27 and 28). All three methods gave identical results for determination of higher contents of organic acids in a not too complicated system. In the determination of organic acids present at low concentrations, differences between the tested procedures were found in some cases. Thus, anion-exchange chromatography gave lower (and obviously more correct) values in the determination of malic acid in beet juice (Fig. 8) than the reference methods.

The described procedure resulted in much shorter analysis times in comparison with earlier separations of organic acids on microreticular anion exchangers[10–13]. In order to attain retention times similar to those on the macroporous Spheron DEAE derivative it was necessary to operate at pressures about five times higher when using microreticular anion exchangers[14]. Consequently, simpler equipment for medium-pressure liquid chromatography is quite sufficient for fast separation of organic acids on DEAE-Spheron.

The sulphate form of the anion exchanger was chosen on the basis of results obtained by Bengtsson and Samuelson[11], which revealed that this form of ion exchanger reduces tailing of peaks and enables the attainment of reasonable retention volumes. With respect to the chosen method of detection, the application of DEAE-Spheron in the form of a salt of some organic acid was not considered. The disadvantage of the method described, from the point of view of analysis of some alimentary and agricultural samples, consists in the insufficient resolution of lactic and acetic acids, which could not be significantly improved by changing the composition of eluent or column temperature. However, the reference polystyrene anion exchanger exhibited even worse selectivity for this pair of acids. The problem can be solved by enhancing the efficiency of the column either by its extension or using a packing with finer particles.

In contrast with the separation of organic acids by the reversed-phase method on silica C_{18}, the quality of separation of organic acids on DEAE-Spheron does not depend on the polarity of the injected sample (application of the sample in ethanol instead of water on silica C_{18} completely changes the course of elution). Compared with the method of ion-exchange chromatography on a cation exchanger (ion exclusion)[15,16,26], the anion-exchange chromatography does not require removal of interfering cations from the sample.

However, all three mentioned HPLC procedures may advantageously be integrated in the analysis of materials containing a more complex spectrum of organic acids, if the mentioned shortcomings of reversed-phase and ion-exclusion chromatography are eliminated by a suitable adjustment of the sample.

The eventual deactivation of a column packed with DEAE-Spheron, manifested by a shift of the position of the peak of citric acid relative to other peaks, can be reversed only by emptying the packing from the column and regeneration with 1 *M* sulphuric acid or, even better, by the ion exchanger treatment cycle as described in Experimental.

A modification of the method for separation of oligo(galacturonic acids) can be used for investigation of the enzymatic hydrolysis of pectin as the methods of ion-exchange chromatography currently in use[29,30] are rather time-consuming.

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