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Determination of monomeric sugar and carboxylic acids by ion-exclusion chromatography

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Abstract

The influence of the temperature and the eluent (proton) concentration on the retention and separation of thirty organic compounds, including sugar acids, lactones, hydroxy acids, mono acids and dicarboxylic acids, on the Merck cation- exchange column Polyspher OA-HY was studied by applying ion-exchange chromatographic methods and HPLC instrumentation. The different responses of the various groups of compounds to changes of the chromatographic parameters were used to select the optimized separation conditions. A combination of two parameter sets (I: temperature of 45°C, sulphuric acid eluent concentration of 0.01 *N*, flow-rate of 0.5 ml min^{-1}; II: 10°C, 0.1 *N* H_2SO_4, same flow-rate) was found to enhance the chromatographic versatility and substance identification. Five-point linear calibrations were conducted under both conditions, and the respective relative standard deviations, mean percentage errors and detection limits were determined.

1. Introduction

Many scientific disciplines are in need of analytical methods for the accurate, sensitive and fast determination of sugar acids in various types of samples. Subject areas involved in sugar acid analysis are for instance phytochemistry, phytopathology, clinical chemistry, microbiology, and food and beverage engineering. New areas of application are coming up in the field of environmental technologies. Organic residues, derived from agriculture and food engineering, are under investigation for their potential to serve as sources of chelating agents able to remove heavy metals from polluted materials. Sugar acids are known to be effective chelators under alkaline pH conditions [1,2]. They are easily obtainable from the oxidation of carbohydrate-rich residues (molasses, potato peel sludge, wine yeast, whey powder) by nitric acid [3].

The different sample types reflect the various research topics leading to the analysis of sugar acids. For instance, under investigation are liquors from sugar processing [4–6], culture media of bacteria [7,8], fermentation broths [9], plant materials [10,11], beverages [11–13], degradation products of acidic polysaccharides [7,11], products of catalytic sugar oxidation [14,15] and alkaline sugar degradation [16].

Besides colorimetric analysis, enzymatic determination, thin-layer and gas–liquid chromatography, several techniques of liquid chromatography have gained importance in sugar acid analysis.

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Whereas the application of anion-exchange resins under various pressure conditions was dominant in the sixties and seventies (a brief overview is given in Ref. [7]), an increasing preference for ion-exclusion techniques started with the availability of small-size, pressure-durable, pH-stable polymer-based materials. Frequently, the ion-exclusion chromatographic separation of organic acids is performed on strong cation exchangers such as sulphonated polystyrene–divinylbenzene (PSDVB) polymers. The Donnan exclusion effect, caused by the ionic repulsion between the negatively charged resin and the more or less negatively charged analytes, is the main separation principle. Under separation conditions allowing the analytes to deprotonate to a certain degree, the pK_a values of the acids and the proton concentration in the mobile phase are deciding factors for the separation process. Further influences on the partition behaviour of organic acids can be derived from size-exclusion effects, hydrophobic (reversed-phase) interactions and Van der Waals forces. In general, the influence of these secondary factors on the separation process increases with decreasing water solubilities, increasing pK_a values, molecular sizes and polarizabilities of the analytes and increasing proton concentration of the mobile phase. Because size-exclusion effects and Van der Waals forces contribute to the chromatographic behaviour of sugar acids, it can be expected that the exchange capacity of a certain resin and the physical (resin structure, degree of cross-linking) and chemical (presence of further polarizable functional groups) properties of the ion exchanger affect the chromatographic performance.

Ion-exclusion chromatographic separations of sugar acids have been conducted with Aminex or BioRad HPX-87H columns mainly, using sulphuric acid as eluent and UV or RI detection [4–7,9,10,17]. The HPX-87H column (300 × 7.8 mm I.D.) is packed with 9-μm spherical, sulphonated PSDVB beads with 8% cross-linking, providing an ion-exchange capacity of 1.7 mmol g^{-1}.

The Merck (Darmstadt, Germany) Polyspher OA-HY cation-exchange column, identical to the Interaction (Mountain View, CA, USA) ORH-801 column according to the information of the company, contains a similar bed material. The ion exchanger (Merck 46-67A resin, identical to the Interaction IC 8101-8) consists of 8-μm spherical, sulphonated PSDVB beads with 8% cross-linking. The ion-exchange capacity is not noted [18]. Although this column is widely used for organic acid analysis [19], no investigations have been published on the applicability and performance of the column in sugar acid determination. We have therefore made some efforts to close this information gap and to extend the already existing experience in sugar acid analysis. Besides questions of the retention behaviour of these compounds, aspects of obtainable determination sensitivity and accurate quantification are in the foreground of the study. Because the practical aspects of this study are orientated towards the analysis of products formed during the acidic oxidation of carbohydrate-rich residues, several aliphatic mono- and polyfunctional carboxylic acids were additionally included in the investigation.

2. Experimental

2.1. Materials

All materials were of puriss. or p.A. quality (purity >99%) unless stated otherwise. Some lactones were studied both in their original chemical forms and as acids after saponification with equivalent amounts of diluted sodium hydroxide (Merck, p.A.) solution (pH kept between 8.0 and 9.0 until complete saponification had occurred).

Glycolic acid, L-threonic acid (hemi-Ca salt, >97%), D-glyceric acid (hemi-Ca salt, monohydrate, >98%), D-erythrono-1,4-lactone, D-ribono-1,4-lactone, D-galactono-1,4-lactone (purum), malonic acid, glutaric acid (98%), L-lactic acid (40% solution in water, purum), *n*-propionic acid, 5-keto-D-gluconic acid (K salt, monohydrate), sorbic acid (>98%), D-quinic acid (<98%), 2-keto-glutaric acid and adipic

acid were purchased from Fluka (Buchs, Switzerland). Oxalic acid dihydrate, glyoxylic acid monohydrate (97%), D-gluconic acid (Na salt), D-galacturonic acid (monohydrate), succinic acid, formic acid, acetic acid and *n*-butyric acid were obtained from Merck. Other chemicals and their sources were D-glucaric (or -saccharic) acid, K salt; 2-keto-D-gluconic acid (hemi-Ca salt, monohydrate), L-mannono-1,4-lactone, D-mannurono-6,3-lactone and D-gulono-1,4-lactone from Sigma (St. Louis, MO, USA) and D-galactaric acid (>98%) and glucuronic acid (Na salt, monohydrate) from Roth (Karlsruhe, Germany).

All aqueous solutions and dilutions were prepared with ultrapure Milli-Q water (Millipore, Eschborn, Germany). Calibration standards were prepared by mixing aliquots of aqueous stock solutions and subsequent dilution to the desired concentration by addition of 0.01 *N* sulphuric acid (Merck, Titrisol).

2.2. Apparatus

The liquid chromatograph consisted of a Gynkotek (Germering, Germany) 600-200 dual piston high-pressure pump with a Gynkotek 250-B ternary gradient former and an Erma ERC-3520 eluent degasser unit, a Rheodyne (Cotati, CA, USA) 8125 injector for manual and automatic injection, fitted with a 20-μl sample loop, a Gynkotek Gina 50 autosampler and an SPD-10AV Shimadzu (Duisburg, Germany) dual-beam UV–VIS detector (detection wavelength set to 210 nm). Normal detection sensitivity was 0.01 a.u.f.s. except in the determination of substance detection limits (0.002 a.u.f.s.). The separations were carried out on a sulphonated polystyrene–divinylbenzene-based Merck Polyspher OA-HY cation-exchange column (300 × 6.5 mm I.D.), packed with Merck 46–67 Å resin (identical with Interaction IC 8101-8 resin, 8 μm particle size, 8% cross-linking) and combined with a guard column (20 × 3.0 mm I.D.) containing the same resin. The columns were enclosed in a thermostat (Industrial Electronics). Sulphuric acid in various concentrations served as eluent.

Data collection and processing was handled by the Gynkosoft (Gynkotek) chromatography data system.

3. Results

3.1. Determination of capacity factors

It is well known that temperature and proton (eluent) concentration are deciding factors in the retention behaviour of organic acids in ion-exclusion chromatography. Therefore, the influence of these factors on the retention times was studied extensively. Capacity factors related to the separation system (combination of guard and separation column) were calculated on the basis of the observed retention times and void times (eluent peak). The results are combined in Figs. 1–6.

The effect of temperature on the chromatographic behaviour of the organic acids is illustrated in Figs. 1–3.

In general, the retention of the sugar acids increases with increasing temperature (Fig. 1). Strong effects are provoked in the case of D-galactaric, D-gluconic and L-threonic acids, whereas only slight changes are recognizable in the case of D-glucaric and D-galacturonic acids. The retention of D-glucuronic and D-galacturonic acids increases almost regularly with increasing temperature. The retention of D-galactaric and L-threonic acids is particularly enhanced by a rise in temperature from 55 to 65°C.

In contrast to the retention of sugar acids, the retention of lactones decreases with increasing temperature (Figs. 1 and 2). This effect is the more pronounced as the molecular masses of the aldono-lactones decrease. Slight variations of the reaction of the hexono-lactones on changes of the separation temperature are noticeable: the difference between the k' values at 10 and 65°C is 0.01 for D-galactono-1,4-lactone and 0.08 for D-mannono-1,4-lactone. The response of the D-mannurono-6,3-lactone is stronger: the decrease of its capacity factor is 0.26 units within the same temperature range.

Short-chain hydroxy and keto acids do not

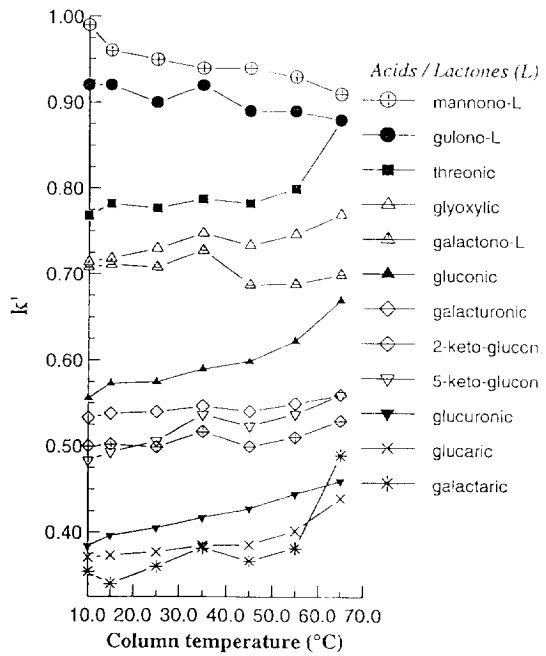

Fig. 1. Effect of temperature on capacity factors of sugar acids and hexono-1,4-lactones.

show a uniform retention behaviour (Figs. 1 and 2). The retention of D-glyceric and D-quinic acids seems to be independent of temperature within the range tested, whereas the retention times of L-lactic and glyoxylic acids increase with increasing temperature. The retention time of glycolic acid is slightly shortened.

The decline of the retention of mono- and dicarboxylic acids with increasing temperature is a function of the alkyl chain length generally (Fig. 3). Increasing carbon numbers of the compounds increase the difference between the capacity factors at low and high temperatures. The retention times of the dicarboxylic acids decrease almost linearly with increasing temperature within the observed value range. The retention of the monocarboxylic acids remains approximately constant between 10 and 35°C. The introduction of an α-keto group into glutaric acid eliminates the temperature influence totally.

The differences between the k'-values of the acids at 10 and 65°C are as follows: formic 0.09, acetic 0.11, *n*-propionic 0.19, *n*-butyric 0.40,

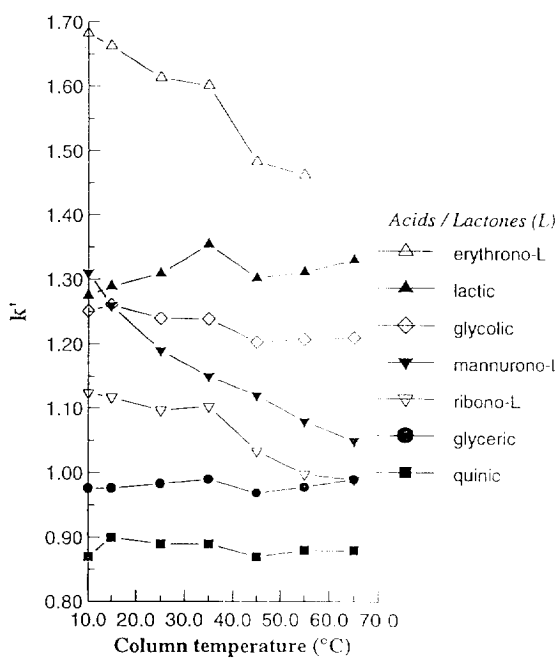

Fig. 2. Effect of temperature on capacity factors of lactones and hydroxy acids.

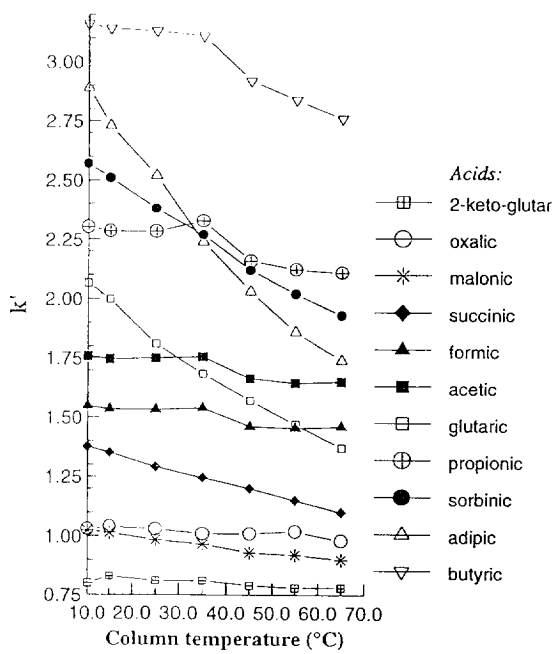

Fig. 3. Effect of temperature on capacity factors of mono- and dicarboxylic acids.

oxalic 0.05, malonic 0.12, succinic 0.28, glutaric 0.70 and adipic 1.15.

The influence of the eluent (i.e. proton) concentration on the separation process is depicted in Figs. 4–6.

According to the low pK_a values of oxalic, malonic, 2-keto-D-gluconic and 2-keto-glutaric acids, their capacity factors are shifted to greater values by increasing the proton concentration. For these compounds, the pH influence is more important for the definition of their retention times than the temperature conditions are. The effects of the two chromatographic parameters are roughly balanced in the case of sorbic acid. Practical consequences are inherent, for instance for the improvement of the resolution between 2-keto-D-gluconic acid and 5-keto-D-gluconic acid.

Except the keto acids, the sugar acids show slight or moderate responses on the variation of the sulphuric acid concentration (Fig. 4). The retention factors increase with increasing sulphuric acid concentration from 0.001 to 0.02 *N*

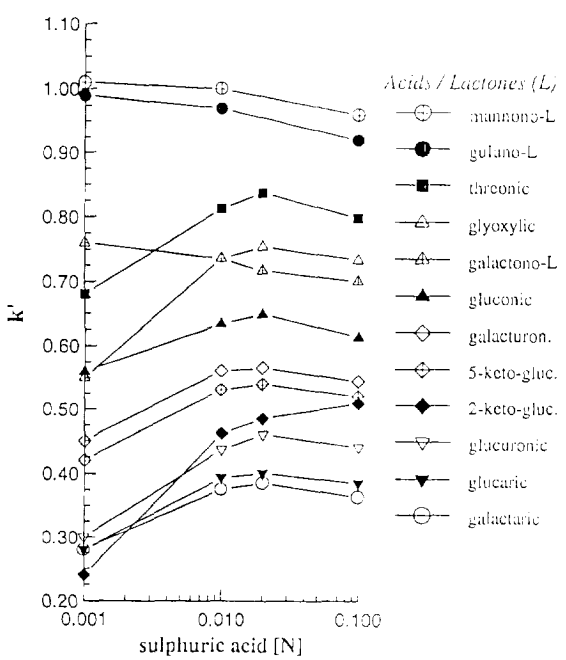

Fig. 4. Effect of sulphuric acid concentration on capacity factors of sugar acids and hexono-1,4-lactones.

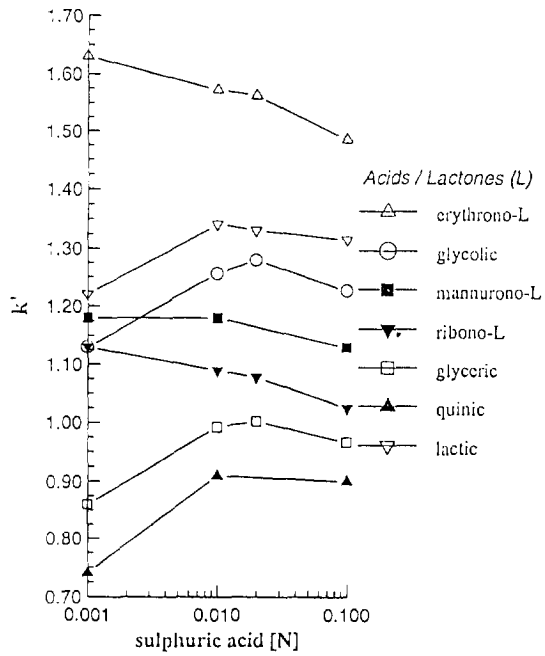

Fig. 5. Effect of sulphuric acid concentration on capacity factors of lactones and hydroxy acids.

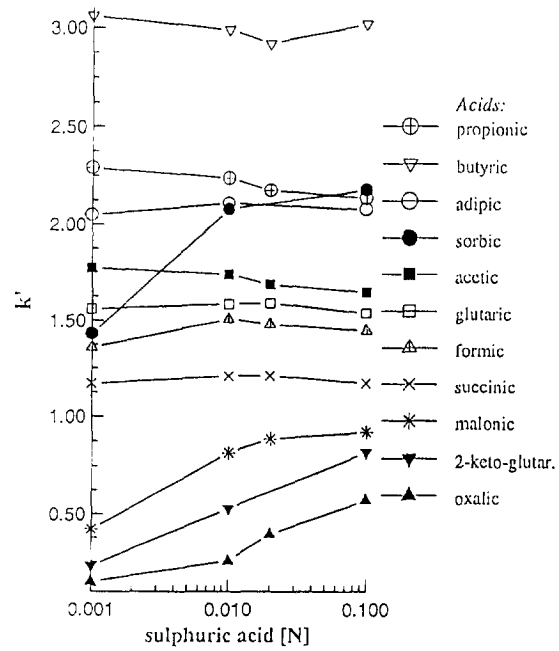

Fig. 6. Effect of sulphuric acid concentration on capacity factors of mono- and dicarboxylic acids.

and tend to decrease slightly with a further elevation of the H_2SO_4 concentration. The retention of the lactones decreases with increasing sulphuric acid concentration. The dimension of this effect is correlated with the molecular size of the lactones in the same way as it is described for the influence of the temperature on their chromatographic properties.

A change of the eluent flow-rate does not alter the capacity factors significantly. A certain influence is noticeable for glutaric acid. Nevertheless, in some cases the improvement of the peak separation obtainable by lowering the flow-rate surpasses the adverse effects of peak broadening and peak-height reduction. Examples are the separation of the substance combinations L-threonic acid–malonic acid and D-erythrono-1,4-lactone–glutaric acid.

3.2. Selection of chromatographic standard conditions

Reflecting the chromatographic behaviour of the compounds tested, it seemed favourable to select two distinct separation conditions, which differ in their separation performances for various groups of analytes. Further, the combination of the results received from different chromatographic conditions should improve the chemical identification of analytes. Separation condition I (45°C, 0.01 *N* H_2SO_4, 0.5 ml min^{-1} flow-rate) intends to achieve a relatively increased retention of sugar acids together with a shortage of the retention of dicarboxylic acids and 2-keto-D-gluconic acid. In contrast to that, separation condition II (10°C, 0.1 N H_2SO_4, 0.5 ml min^{-1} flow-rate) aims at characteristically altered (higher) retention of dicarboxylic acids, higher retention of 2-keto-D-gluconic acid and consequently increased resolution of 2-keto- and 5-keto-D-gluconic acid. Furthermore the chemical stabilities of sugar acids should be increased to prevent the formation of lactones during chromatographic analysis.

The separation performances of these analytical conditions are shown in Figs. 7 and 8. The substance combinations are chosen to demonstrate separation capabilities for complex mixtures. Although compromises regarding the resolution were accepted, the resolutions obtained are adequate for substance characterizations by their retention times and for quantification provided that the concentrations of the substance pairs 2-keto-D-gluconic acid–5-keto-D-gluconic

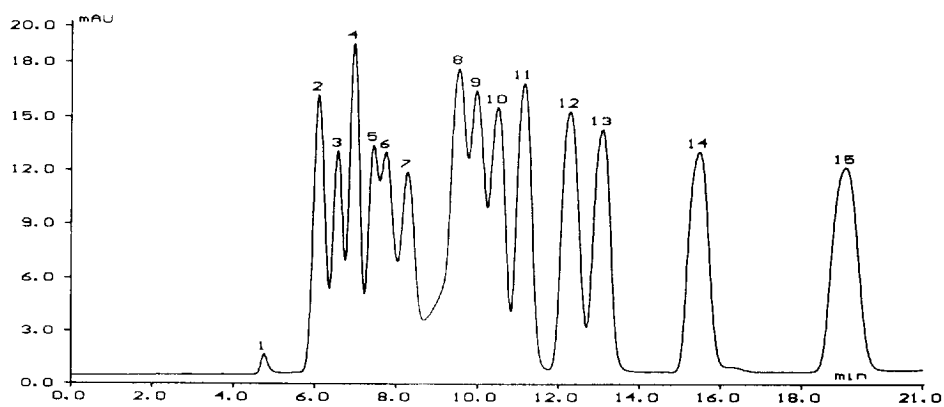

Fig. 7. Separation of sugar acids, lactones and aliphatic acids at high column temperature and low sulphuric acid concentration (conditions I). Conditions: Merck OA-HY column plus guard column; temperature, 45°C; flow-rate, 0.5 ml/min; eluent, 0.01 *N* H_2SO_4; UV detection at 210 nm and 0.01 a.u.f.s. Peaks (concentrations in mmol/l): 1 = void peak; 2 = oxalic acid (0.05); 3 = D-galactaric acid (0.2); 4 = glucuronic acid (0.5); 5 = D-galacturonic acid (0.5); 6 = gluconic acid (0.5); 7 = D-galactono-1,4-lactone (0.5); 8 = D-glyceric acid (0.5); 9 = D-ribono-1,4-lactone (2.0); 10-succinic acid (1.0); 11 = latic acid (1.0); 12 = glutaric acid (1.0); 13 = acetic acid (2.0); 14 = propionic acid (2.0); 15 = *n*-butyric acid (2.0).

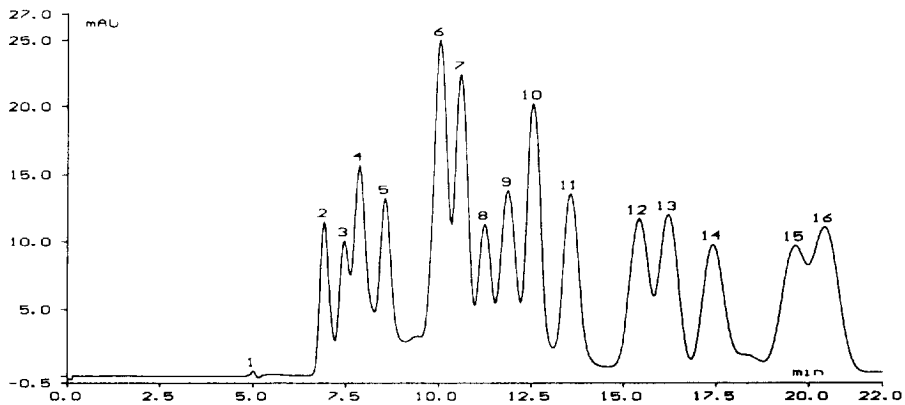

Fig. 8. Separation of sugar acids, lactones and aliphatic acids at low column temperature and high sulphuric acid concentration (conditions II). Conditions: Merck OA-HY column plus guard column; temperature, 10°C; flow-rate, 0.5 ml/min; eluent, 0.1 *N* H_2SO_4; UV detection at 210 nm and 0.01 a.u.f.s. Peaks (concentrations in mmol/l): 1 = void peak; 2 = D-glucaric acid (0.5); 3 = 5-keto-D-gluconic acid (0.5); 4 = D-galacturonic acid (0.5); 5 = glyoxylic acid (1.0); 6-malonic acid (1.0); 7 = D-ribono-1,4-lactone (2.0); 8 = glycolic acid (1.0); 9 = succinic acid (1.0); 10 = formic acid (2.0); 11 = acetic acid (2.0); 12 = glutaric acid (1.0); 13 = propionic acid (2.0); 14 = sorbic acid (not specified); 15 = adipic acid (1.0); 16 = butyric acid (2.0).

acid and D-glyceric acid–D-ribono-1,4-lactone (condition I) are of the same order of magnitude.

Hexaric, hexuronic and hexonic acids have capacity factors between 0.35 and 0.60 under chromatographic conditions I. The capacity factors of the corresponding lactones range from 0.70 to 1.10. The narrow retention ranges for sugar acids and lactones with identical carbon numbers have two consequences. First, the separation of compounds, which differ in their configuration only, is very difficult if not impossible at all. Second, the separation of mono- and dicarboxylic acids, having capacity factors greater than 1.2, can be achieved in one run together with sugar acids.

Oxalic acid, well separated from the void volume, has the shortest retention time and does not provide complications for the separation of dicarboxylic sugar acids.

Omitting the galactose-derived acids the chromatographic conditions I allow the separation of glucose-derived sugar acids together with further sugar acids and lactones (D-galactono-1,4-lactone, L-mannono-1,4-lactone, D-mannurono-6,3-lactone, D-erythrono-1,4-lactone) and other organic acids.

The chromatographic separation at conditions II delivers complementary informations, useful for the identification of dicarboxylic acids especially.

The chromatographic conditions II achieve better results for following substance combinations, which were not or poorly separated at 45°C and 0.01 *N* H_2SO_4: D-glucuronic–2(5)-keto-D-gluconic acid (absence of D-glucaric acid); separation of malonic acid from L-threonic acid, glyoxylic acid, D-galactono-1,4-lactone and D-quinic acid, separation between D-ribono-1,4-lactone, glycolic acid and succinic acid as well as between formic acid, D-erythrono-1,4-lactone and glutaric acid (absence of acetic acid). Additionally, separation of adipic and sorbic acid is possible.

3.3. Calibration

Specific calibration standards were prepared for both separation conditions. Standard I (condition I) contains oxalic, D-glucaric, 2-keto-D-gluconic, 5-keto-D-gluconic, D-gluconic, L-threonic, D-glyceric, succinic, L-lactic, glutaric, acetic, *n*-propionic and *n*-butyric acids together with D-ribono-1,4-lactone (fourteen compounds). Standard II (conditions II, eight compounds) comprises D-gluconic, malonic, succinic, glycolic,

formic, glutaric and adipic acids together with D-ribono-1,4-lactone. The calibrations were performed by analysis of five concentration levels of the standard mixture, each injected twice. The ratio of the maximum/minimum level of each substance was 50:1. With respect to the different detection sensitivities of the compounds, four concentration ranges were chosen to obtain approximately equal peak areas for each compound. Calibration functions were calculated using curve fitting by means of linear regression analysis. Moreover, relative standard deviations and percentage mean errors were determined for the highest and lowest calibration level on the database of five subsequent standard injections. Detection limits for single substances within given substance combinations were deduced from signal-to-noise ratios obtained by analysis of further dilutions of the minimum standard.

Calibration parameters and some statistical data are listed in Tables 1 and 2.

Despite the partially poor resolution, all compounds were calibrated resulting in coefficients of linear regression of the data points >0.999. The correspondence between the calibration functions of D-glucaric and D-gluconic acid at conditions I and the good agreement between the standardization of D-gluconic acid at both conditions demonstrate the consistency of the calibration procedures. The determination of the R.S.D. values (lowest level, conditions I) yielded a more homogeneous data set with values ranging typically from 4 to 8%. The mean R.S.D. of the six sugar acids and lactones (7.5%) is somewhat higher than that of the eight other acids (4.9%). Regarding the R.S.D. values, very precise results can be obtained for 5-keto-D-gluconic acid, propionic acid and butyric acid even at low concentrations.

The R.S.D. values deduced from the analysis of the concentrated standard I ranged from 0.2 to 2.57. Again the mean of the R.S.D. values of sugar acids and lactones (1.33) is slightly higher than that of the other acids (0.85%). The specific detector response for 50 μmol l^{-1} substance concentrations ranged from 5.05 to 0.2 detection units with typical response ranges of 0.5–0.7 units for C_6 sugar acids, 0.35–0.4 units for methyl group-containing dicarboxylic acids and 0.15–0.2 units for monocarboxylic acids. The low detection sensitivity for D-ribono-1,4-lactone is consistent with the evaluation of the peak areas of other lactones obtained during the determination of their capacity factors.

Limit concentrations for substance identification at given substance combinations were determined instead of the calculation of detection limits for single-compound injections. This procedure achieves data with higher practical use.

Fig. 9 shows a separation of the minimum calibration standard for conditions II. The maximum peak height is about 0.5 ma.u.f.s. The good signal-to-noise ratio is obvious.

4. Discussion

Reflecting the general behaviour of organic acids including sugar acids on ion-exclusion columns, the Polyspher OA-HY column shows almost the same separation properties as described earlier [20,21] for other ion-exclusion chromatography columns. Some of the main effects are as follows:

- Increase of the capacity factors of mono- and dicarboxylic acids with increasing carbon number due to an increase of the pK_1 values and increasing hydrophobic interactions, resulting in a favoured adsorption on the gel beads.

- Decrease of the capacity factors with increasing polarity of the functional molecular groups, expressed by the elution order oxalic < glyoxylic < glycolic < acetic acid. This order is valid even under strong acidic elution conditions. Therefore, the parallel increase of the pK_1 values cannot be the reason for this effect. The sugar acids fit in this rule, as it can be deduced from their k' factor sequence: glucaric < glucuronic < ketogluconic < gluconic acid.

- Higher retention times of the neutral lactones compared with the free acid forms [15].

- The increase of the retention times of lactones (D-galactono- < D-ribono- < D-erythrono-1,4-lactone) and aldonic acids, represented by the formula CH_2OH-$(CHOH)_n$-COOH (i.e. D-gluconic < L-threonic < D-glyceric < glycolic

Table 1
Parameters of linear calibration at separation conditions I

Acid lactone	Retention time (min)	Concentration range (μmol l^{-1})	Intercept (μA.U. min)	Slope ($\times 10^{-3}$)	Detector response[a] (mA.U. min)	Relative molar response[b]	R.S.D., high level[c] (%)	R.S.D., low level[c] (%)	Detection limit[d] (μmol l^{-1})
Oxalic	6.14	1–50	47	102	5.05	9.35	1.01	4.97	0.2
D-Glucaric	6.65	10–500	30	10	0.52	0.96	2.57	12.13	2.0
2-Keto-D-gluconic	6.95	10–500	16	12	0.60	1.11	0.22	6.86	1.0
5-Keto-D-gluconic	7.33	10–500	0	14	0.71	1.31	1.09	3.78	1.0
Gluconic	7.75	10–500	27	10	0.54	1	1.04	8.01	1.0
L-Threonic	8.62	10–500	67	16	0.87	1.64	1.53	6.83	0.5
D-Glyceric	9.54	10–500	36	20	1.02	1.89	0.95	2.43	2.0
Succinic	10.51	20–1000	16	6	0.35	0.65	1.49	6.07	4.0
L-Lactic	11.15	20–1000	15	7	0.37	0.69	1.22	6.82	1.5
Glutaric	12.28	20–1000	7	7	0.38	0.70	0.94	7.71	1.0
D-Ribono-1,4-lactone	9.97	40–2000	39	3	0.16	0.30	1.55	5.58	10.0
Acetic	13.08	40–2000	1	3	0.16	0.30	0.74	7.09	2.0
Propionic	15.47	40–2000	2	3	0.31	0.26	0.26	3,82	4.0
Butyric	19.07	40–2000	1	4	0.21	0.39	0,2	0,41	4.0

Merck OA-HY guard and separation column, column temperature 45°C, 0.01 *N* sulphuric acid/0.5 ml min^{-1} flow-rate, five injection levels, two injections per level.
[a] Calculated for 50 μM concentrations.
[b] Relative to gluconic acid.
[c] Five manual injections.
[d] Limit concentration for substance identification at given substance combination coefficient of linear regression >0.999 for all calibration functions.

Table 2
Parameters of linear calibration at separation conditions II

Acid/ lactone	Retention time (min)	Concentration range (μmol l^{-1})	Intercept (μA U. min)	Slope ($\times 10^{-3}$)	Detector response[a] (mAU min)	Relative molar response[b]	R.S.D., high level[c] (%)	R.S.D., low level[c] (%)	Detection limit[d] (μmol l^{-1})
Gluconic	7.88	10–500	17	11	0.55	1	2.31	10.16	1.0
Malonic	10.09	20–1000	24	7	0.38	0.69	1.23	11.24	5.0
Glycolic	11.25	20–1000	8	4	0.21	0.38	0.79	12.01	5.0
Succinic	11.87	20–1000	6	6	0.30	0.55	1.22	6.06	4.0
Glutaric	15.41	20–1000	6	7	0.36	0.65	0.74	5.44	4.0
Adipic	19.61	20–1000	14	8	0.39	0.71	0.04	6.66	10.0
D-Ribono-1,4-lactone	10.60	40–2000	28	4	0.24	0.44	1.05	6.51	10.0
Formic	12.57	40–2000	7	4	0.20	0.36	1.23	3.94	6.0

Merck OA-HY guard and separation column, column temperature 10°C, 0.1 *N* sulphuric acid, 0.5 ml min^{-1} flow-rate, five calibration levels, two injections per level.
[a] Calculated for 50 μmol l^{-1} concentrations.
[b] Relative to gluconic acid.
[c] Five manual injections.
[d] Limit concentration for substance identification at given substance combination coefficient of linear regression >0.999 for all calibration functions.

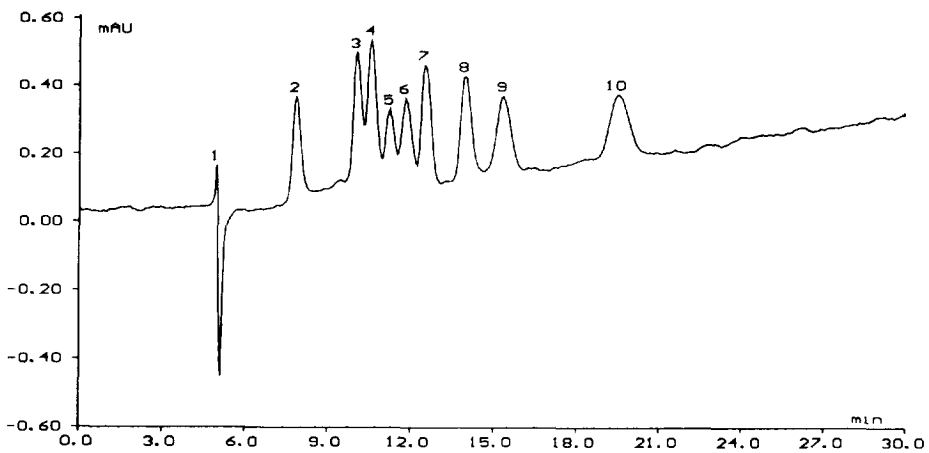

Fig. 9. Chromatogram of the minimum calibration level standard, obtained under conditions II. Conditions: Merck OA-HY column plus guard column; temperature, 10°C; flow-rate, 0.5 ml/min; eluent, 0.1 *N* H_2SO_4; UV detection at 210 nm and 0.01 a.u.f.s. Peaks (concentrations in μmol/l): 1 = void peak; 2 = gluconic acid (10.0); 3 = malonic acid (20.0); 4 = D-ribono-1,4-lactone (40.0); 5 = glycolic acid (20.0); 6 = succinic acid (20.0); 7 = formic acid (40.0); 8 = unknown; 9 = glutaric acid (20.0); 10 = adipic acid (20.0).

acid) with decreasing carbon numbers leads to the assumption that size-exclusion effects take part in the separation of the compounds. This hypothesis was formulated earlier [22] to explain the respective elution order obtained by ion-exchange chromatography of organic acids.

The differences in the effect of temperature on the retention behaviour of the compounds reflect differences in their chemical properties and retention mechanism. The increase of the capacity factors of sugar acids with increasing temperature is affected mainly by the partial formation of their lactones. A temperature-dependent increase of the retention times of some sugar acids following the order 2-keto-D-gluconic acid < 5-keto-D-gluconic < D-glucuronic acid < D-gluconic acid is also described by Hicks et al. [7]. They explain this with substance-specific degrees of lactonization under the chromatographic conditions provided. Dicarboxylic sugar acids seem to be more stable against lactonization than uronic and monocarboxylic acids.

The reduction of the capacity factors of most of the other compounds may be interpreted as follows:

- The balance of the lactone–acid equilibrium is favoured by an increase of the column temperature. The short retention times of the lactones are connected with the degree of their conversion into the acid form and the absolute retention time difference of the pure acid and lactone form under the given chromatographic conditions.

- The pK_a values decrease with increasing temperature causing an intensified repulsion by the Donnan membrane.

- As far as the adsorption of organic compounds onto the polymer material, caused by hydrophobic interactions, is an exothermic process (release of heat of adsorption), the free adsorption enthalpy is diminished by a rise of the temperature. Because the adsorption strength is a function of the number of active molecular units (i.e. methylene groups), which act in the adsorption process, the decline of the retention time depends on the carbon chain length. Based on the same reaction principles the increase of the substance retention with increasing carbon chain length of homologous aliphatic mono- and dicarboxylic acids at constant temperature and the decrease of their retention times with increasing molecular mass at increasing temperature are complementary effects.

- As a minor effect, the swelling of the column material under the influence of higher temperatures may reduce the pore volume and pore size,

resulting in a lower molecular size limit for entering the pores of the stationary phase.

Considering the influence of the proton concentration on the retention behaviour of the acids, the interpretations of Kihara et al. [20] were largely confirmed. For the dicarboxylic acids for instance, an important increase of the retention with an increase of the sulphuric acid concentration was found for those compounds only (oxalic, malonic and 2-keto-glutaric acid), which are partially dissociated at the lowest eluent concentration (pH of about 3). The achieved enhancements of the substance-specific retentions following the orders glyoxylic > glycolic >> acetic acid and D-glyceric > lactic >> *n*-propionic acid reflect the supporting influences of the additional functional groups on the dissociation of the carboxyl group. At pH 3, the pK_1 value may be the dominant factor for the separation of sugar acids with similar sizes and identical carbon numbers. This could be a reasonable explanation for the observed elution order of the glucose-derived acids at lowest sulphuric acid concentration: 2-keto-D-gluconic < D-glucaric acid < 5-keto-D-gluconic acid < D-gluconic acid. As a consequence of the repression of the acid dissociation at high eluent concentrations, the differences between the retention times of the sugar acids were reduced and size-exclusion effects seem to gain a higher importance for the separation process.

Due to the absence of an ionizable functional group, lactones do not respond to an increase of the proton concentration or respond with a small decrease of their retention times (D-ribono-1,4-lactone, D-erythrono-1,4-lactone and D-gulono-1,4-lactone). The latter effect may be attributed to a slightly diminished stability of the lactones against conversion into their acidic forms under these chromatographic conditions.

The systematic variation of main separation parameters confirmed that the capacity ranges are narrow for the detection of sugar acids and lactones, which are very similar in their molecular sizes and chemical properties. The capacity ranges for the tested sugar acids are 0.2–0.85 and 0.7–1.65 for lactones. These results are comparable with those of other authors. Hicks et al. [7] reported for the separation of sugar acids and lactones on BioRad-HPX-87-H (0.6 ml min^{-1} flow-rate, 35°C, 0.009 *N* sulphuric acid) approximately the same relative retention times (relative to D-glucuronic acid) as we found for 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-galacturonic acid, D-gluconic acid, D-galactono-1,4-lactone, D-mannono-1,4-lactone, D-ribono-1,4-lactone and D-mannurono-6,3-lactone. It is assumed that the capacity factors are nearly identical as well. Further correspondences of the separation characteristics of the OA-HY column with the HPX-87-H column are noticeable for some mono and hydroxy acids. De Bruijn et al. [16] determined capacity factors for formic, acetic, glycolic, L-lactic and D-glyceric acids (conditions: 0.6 ml min^{-1} flow-rate, 0.01 *N* H_2SO_4, 60°C). We compared their values with our data, given in parentheses, for 0.5 ml min^{-1} flow-rate, 0.01 *N* H_2SO_4, 45°C: glyceric acid 1.01 (0.99), glycolic acid 1.19 (1.26), lactic acid 1.28 (1.34), formic acid 1.42 (1.51) and acetic acid 1.64 (1.74). Considering the differences of the chromatographic conditions, the correspondence of the data is substantial.

5. Conclusions

As a consequence of a detailed investigation of the chromatographic charateristics of the Merck HY-OA ion-exclusion column, two separation conditions were chosen, which use the different responses of sugar acids, lactones and dicarboxylic acids to changes of temperature and proton concentration for their separation and identification. Optimized separation conditions for some of those substance combinations, which can not be separated by one of the both methods, can be deduced from the plots of capacity factors. Sensible substance detection and linear calibration functions that are valid for greater concentration ranges as well as acceptable reproducibilities of single measurements were achieved with both methods. Furthermore, far-reaching similarities between the separation properties of Aminex/BioRad HPX-87H and

Merck OA-HY column were found by comparison of experimental with literature data.

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