AUTOMATED ION EXCHANGE CHROMATOGRAPHY OF ORGANIC ACIDS IN ACETATE MEDIA

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A method for the automated analysis of complex mixtures of uronic acids by anion exchange chromatography in sodium acetate and acetic acid was described in a previous paper[1]. This work has now been extended to include a large number of other acids such as aldonic and saccharinic acids, and aliphatic keto acids. The main purpose was to find working conditions suitable for practical analytical work in various branches of carbohydrate chemistry. Correlations between the elution behavior and changes in the eluant composition were also studied. The possibility of predicting the positions on the chromatograms from independent physico-chemical data is still very limited but some predictions can be made which give some guidance in the identification of unknown mixtures.

MATERIALS

D-Threonic and D-lyxonic acid were prepared according to HARDEGGER *et al.*[2,3] and purified by ion exchange chromatography. Their identity was verified by mass spectrometry using the trimethylsilyl ethers of their 1,4-lactones[4].

The methylated aldonic acids were kindly supplied by Dr. MICHAEL H. B. HAYES, Birmingham and β-isosaccharinic acid by Dr. OLOF THEANDER, Stockholm. The other acids were prepared in connection with previously published work[5–7] or obtained from commercial sources.

PROCEDURE

All experiments were performed with a jacketed column, 6 × 1350 mm, containing a strongly basic resin (Dowex 1 X-8) fractionated to give a particle diameter of 26–32 μ. The resin was in the acetate form and conditioned with the eluant to be used in the subsequent elutions. The eluant was boiled continuously under reflux before it was fed into the column, whose temperature was kept at 30.0° by circulating thermostated water in the jacket.

If lactones were present, these were saponified by means of an Autotitrator by keeping the solution at pH 8.0 for 5 h before the sample (1–4 mg) was applied to the column. The flow rate during the elution was 2.8–7.7 $ml \cdot cm^{-2} \cdot min^{-1}$.

The analysis system was the same as described earlier[1]. By means of a peristaltic pump the eluate was divided into two streams; one part was analysed by the carbazole method and the other by the chromic acid method. The color developed in the two channels was recorded simultaneously.

All acids were recorded in the chromic acid channel. Uronic acids and 5-keto-gluconic acid gave strongly positive reactions with carbazole. Aldobionic, lactic, and glyoxylic acids were also recorded in the carbazole channel, but the color intensity was lower. A further lowering in color intensity was observed with glyceric, β-metasaccharinic and α-isosaccharinic acids, and only a very slight color was recorded with α-metasaccharinic and glycolic acids. The other acids were not recorded in the carbazole channel.

ELUTION WITH SODIUM ACETATE

Several of the acids studied in this work have previously been subjected to anion exchange chromatography in sodium acetate without added acetic acid. In a recent paper[1], it was shown that interconversions among certain uronic acids could occur at the high pH prevailing in this medium, and for this reason the pH was lowered to 5.9 by addition of acetic acid. All elutions reported in this section were, therefore, made with sodium acetate (0.08*M*) solution with acetic acid added to obtain pH 5.9. Under these conditions the hydroxy acids can be considered as virtually completely ionized and, as expected, this addition had only a slight or negligible influence upon their elution behavior. The peak elution volumes of various species were determined in a large number of runs with single acids, and checked in runs with mixtures of two and more acids. From these values the volume distribution coefficients (D_v) were calculated as usual[8]. The results are given in Table I.

As mentioned in previous papers[5,6,9], there is a general trend among hydroxy acids such that those containing a larger number of hydroxyl groups appear before those with a lower number. As can be seen from Table I this rule holds true with most of the acids within the aldonic acid series, represented by the formula $CH_2OH \cdot (CHOH)_n \cdot COOH$. One exception is mannonic acid which, although a hexonic acid, appears among the pentonic acids. The second exception, D-*glycero*-L-*manno*-heptonic acid has a distribution coefficient which is higher than that of some of the hexonic acids. The other 16 acids with n-values varying between 0 and 5 follow the rule. Similarly the aldobionic acids which contain a larger number of hydroxyl groups than any of the other acids investigated, have their expected position ahead of the heptonic acids.

With all these strongly polar anions the non-ionic interactions with the styrene–divinylbenzene matrix must be small, and can explain neither the exceptions mentioned above, nor the fact that there exist individual differences among the diastereomers. The rule mentioned above is explained by the assumption that the hydrated ionic volumes have a predominant influence upon the uptake of the anions, those with a smaller hydrated volume being held more firmly by the ion exchanger. Admittedly the hydrated ionic volume is not a well-defined quantity and in rigorous thermodynamic treatments of ion exchange equilibria its use is avoided (*cf.* ref. 8).

With inorganic cation exchangers[10] and with cation exchange resins[11,12], this rule has been found to hold true (for instance in exchanges of alkali metals) whereas with anion exchange systems studied earlier, specific interactions between various anions and the resin seem to be a major factor[13]. The correlation observed with aldonic acids indicates, however, that for this type of anion the specific interactions are less important and that the ionic size is the predominant factor which determines the

TABLE I

VOLUME DISTRIBUTION COEFFICIENTS (D_v) IN 0.50 M ACETIC ACID AND IN 0.08 M SODIUM ACETATE (pH 5.90)

	D_v		$K_{HB} \times 10^4$
	In acetic acid	*In Na acetate*	
Aldonic acids:			
Glycolic	18.6	14.8	1.6
Glyceric	20.2	11.9	2.2
D-Erythronic	18.8	10.4	2.3
D-Threonic	19.5	10.6	2.4
D-Arabinonic	14.2	8.89	2.0
D-Lyxonic	19.5	10.3	2.4
D-Ribonic	9.17	9.24	1.2
D-Xylonic	15.7	8.19	2.5
D-Galactonic	11.3	7.51	1.9
D-Gluconic	12.5	7.21	2.2
D-Gulonic	13.5	7.70	2.3
D-Mannonic	17.5	9.49	2.4
D-Talonic	6.25	7.37	1.0
D-*glycero*-L-*manno*-Heptonic	14.2	7.70	2.4
D-*glycero*-D-*gulo*-Heptonic	10.8	6.63	2.1
6-Deoxy-D-mannonic	19.3	10.9	2.3
Aldobionic acids:			
Cellobionic	5.86	3.71	2.0
Lactobionic	5.09	3.15	2.1
Maltobionic	7.35	3.69	2.6
Melibionic	4.62	2.64	2.2
Methylated aldonic acids:			
2,3,5-Tri-O-methyl-D-galactonic	5.38	2.64	2.6
3,5,6-Tri-O-methyl-D-gluconic	7.12	3.90	2.4
2,3,4,6-Tetra-O-methyl-D-gluconic	4.76	2.18	2.9
Saccharinic acids:			
2-Hydroxypropionic (lactic)	15.1	13.8	1.4
D-*threo*-2,3-Dihydroxybutyric	16.9	12.0	1.8
2,4-Dihydroxybutyric	14.6	11.7	1.6
3,4-Dihydroxybutyric	3.39	9.31	0.4
2-Methyl-2,3-dihydroxypropionic	14.4	11.1	1.6
D-*threo*-2,4,5-Trihydroxyvaleric	11.8	9.17	1.6
α-D-Glucoisosaccharinic	6.09	6.06	1.3
β-D-Glucoisosaccharinic	14.8	6.46	3.0
α-D-Glucosaccharinic	5.41	6.72	1.0
α-D-Glucometasaccharinic	6.84	7.16	1.2
β-D-Glucometasaccharinic	9.56	7.59	1.6
Uronic acids:			
D-Galacturonic	21.4	8.40	5.4
D-Glucuronic	44.1	11.7	5.3
L-Guluronic	24.0	10.7	2.9
L-Iduronic	29.9	12.7	3.1
D-Mannuronic	36.5	12.9	3.8
Aldehydo and keto acids:			
Glyoxylic	65.6	20.8	4.3
Levulinic	3.64	13.2	0.4
2-Keto-D-gluconic	95.0	13.7	11.4
5-Keto-D-gluconic	38.8	13.9	3.7
Heterocyclic acids:			
2-Furoic	>60	>60	

distribution coefficient. The two exceptions to the rule can be explained by the assumption that because of intramolecular hydrogen bonding the hydrated volumes of the corresponding anions are smaller than those of their diastereomers.

With 6-deoxymannonic acid it can be assumed that interactions between the methyl group and the resin should contribute to an increased distribution factor. Similarly the hydrated ionic volume would be expected to be less than that of the hexonic acids. Taken together, these two effects explain why this acid is held more strongly than the hexonic acids. The same factors explain why 2-hydroxypropionic (lactic) acid appears later than 2,3-dihydroxypropionic (glyceric) acid, and 2,3-dihydroxybutyric acid is eluted after the tetronic acids.

These factors offer a satisfactory explanation for the change in position when the hydroxyl group on the last carbon atom in an aldonic acid is substituted by hydrogen. However, the positions of the unbranched 3-deoxyhexonic (metasaccharinic), the 3-deoxypentonic (D-*threo*-2,4,5-trihydroxyvaleric) and the 2-deoxytetronic (3,4-dihydroxybutyric) acids do not fit into this scheme.

Introduction of O-methyl groups into hexonic acids results in a large decrease in the D_v-values. This change was expected since previous investigations showed that methylated uronic acids appear before the non-methylated species[9]. A probable explanation is that increased hydrated ionic volumes are largely responsible for this effect and that with these compounds the non-ionic interaction forces with the resin are small.

Substitution of a hydroxyl group for an aldehyde or keto group results in a large increase in the D_v-value. The results are in agreement with those earlier observed with pyruvic acid[14]. Specific interaction forces between the anions and the resin seem to be operative in these systems.

The hexuronic and saccharinic acids have been studied previously[9,6] and the D_v-values are included mainly for a comparison with the results obtained by acetic acid elution. 2-Furoic acid which has also been studied previously[15] is included as an example where interaction forces with the resin matrix have a predominant influence upon the elution both in sodium acetate and acetic acid.

The influence of the sodium acetate concentration (0.04–0.09 M) upon the D_v-values was studied with some of the acids (talonic, galactonic, lactobionic, lactic, glycolic). Plots of log D_v against —log [acetate] gave straight lines with slopes very close to unity (*cf.* ref. 8). The results are in agreement with those reported previously[5,6,14]. The tabulated D_v-values can therefore be used for predicting the peak positions on the chromatogram by using this correlation.

From a practical point of view it is noteworthy that with all the species studied previously, the order of elution found in the present work was in agreement with that reported earlier, indicating that the variations in properties of the resin batches did not affect this order. After the column had been used for a long time a drop in its effective capacity was observed (about 5 % per year) with a corresponding drop in the D_v-values, but the order of elution remained unchanged. Recalibration with a standard mixture of acids allows corrected D_v-values to be obtained. The same method can be applied to estimate the D_v-values in columns with other batches of resin but even though no reversal in the order of elution was observed in our experiments it should be remembered that significant variations in the positions can occur[14].

A more rapid loss in effective capacity often occurs when natural products *e.g.*

hydrolyzates of wood, are being analyzed. This is explained by retention of solutes held irreversibly by the resin. In the applications studied in this laboratory the order of elution was found to be unchanged in such cases.

Anions whose distribution coefficients differ by about 10 % or more can be well separated from each other on the column used in this work. It can be seen from Table I that the differences are less with several acids. The method is very useful for a preliminary group separation of complicated mixtures and for analyses of solutions containing for instance acids of varying molecular weight. With diastereomers it is a valuable complement to separations in other media.

ELUTION WITH SODIUM ACETATE–ACETIC ACID SOLUTIONS

When a weak organic acid (HB) is eluted with a solution containing both sodium acetate and acetic acid a decrease in pH at constant acetate concentration will result in a decreased D_v-value. The weaker the acid the greater is the influence of the hydrogen ion concentration. If it be assumed that the sorption of HB (in its non-dissociated form) can be neglected, the following correlation should account for the influence of the molar concentrations of acetate ions [A] and hydrogen ions [H] upon the volume distribution coefficient (*cf.* ref. 8):

$$D_v = k_{A,B}\rho_r \frac{K_{HB}}{K_{HB} + [H]} \frac{[A]_r}{[A]}$$

where K_{HB} is the stoichiometric dissociation constant (molarity scale), $[A]_r$ the acetate concentration in the resin phase, $k_{A,B}$ the selectivity coefficient and ρ_r the mass of dry resin per cm^3 of the column.

If [A] is high compared to the concentrations of other anions and the variations in [A] and [H] limited to an interval within which the ratios between the activity coefficients of the anions in both phases remain unchanged $[A]_r$, $k_{A,B}$ and ρ_r will be constant. The equation can thus be written:

$$\frac{1}{D_v[A]} = C_1 \frac{[H]}{K_{HB}} + C_1$$

where C_1 is a constant.

A plot of $1/D_v[A]$ against [H] should thus give a straight line. Based upon elution experiments at four different acetate concentrations and varying hydrogen ion concentrations plots of this type were made for some of the organic acids included in this investigation. The concentrations of acetate and hydrogen ions in the solutions were calculated using the stoichiometric (molal) dissociation constants of acetic acid in potassium chloride solutions determined by HARNED AND HICKEY[16].

The results obtained with talonic, galactonic, 6-deoxymannonic, and glycolic acids are reproduced in Fig. 1. It is seen that there is a straight-line relationship with all these acids which indicates that within the concentration range covered by these experiments the assumptions upon which the equation is based are valid. At the lowest hydrogen ion concentration the experimental results obtained at varying acetate concentrations coincided completely, which is a consequence of the correlation

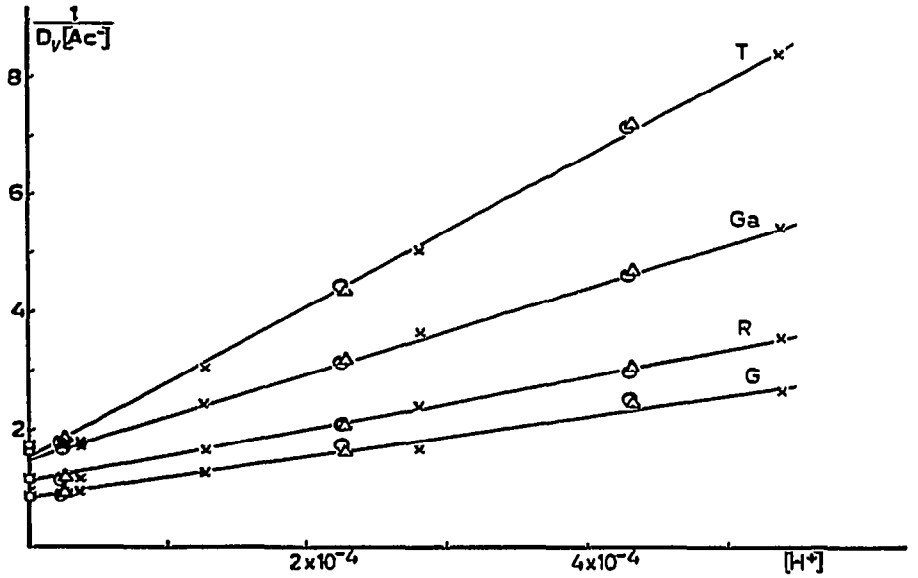

Fig. 1. Relationship between $1/D_v[Ac^-]$ and $[H^+]$ for various hydroxy acids at varying ionic strength: (⊙) 0.04; (△) 0.06; (□) 0.08; (×) 0.09. T = Talonic ($1.3 \cdot 10^{-4}$); Ga = galactonic ($2.0 \cdot 10^{-4}$); R = 6-deoxymannonic ($2.2 \cdot 10^{-4}$); G = glycolic ($2.4 \cdot 10^{-4}$); lactic ($2.1 \cdot 10^{-4}$).

mentioned in the previous paragraph. At higher hydrogen ion concentrations there is some scattering but no systematic influence of the acetate concentration can be traced. It is obvious that the correlation discussed above is quite useful for predicting D_v-values by interpolation from experimental results.

From these plots and from analogous determinations made with lactic acid the apparent (stoichiometric) dissociation constants were calculated. The results are given within parenthesis in Fig. 1. The determinations are not accurate enough to permit any influence of ionic strength upon the dissociation constants to be detected.

Finally it should be stressed that according to the equation given above the most favorable separation factors (ratio between the distribution coefficients of two species in the same medium) are obtained either in sodium acetate or in pure acetic acid. From a practical point of view mixtures of sodium acetate and acetic acid can have some advantages, for instance when only incomplete resolution of species with great differences in acid strength is achieved in sodium acetate. In such separations the elution can be speeded up (lower peak elution volumes) if a mixture of acetic acid and sodium acetate is used as eluant instead of pure acetic acid. A typical example is the separation of 2-ketogluconic and 5-ketogluconic acids which cannot be separated in sodium acetate and are eluted very slowly in acetic acid (*cf.* Table I).

ELUTION WITH ACETIC ACID

The elution of four hydroxy acids was studied over a large range of acetic acid concentrations. In Fig. 2 the D_v values are plotted in the same way as in Fig. 1. The two first determinations (at low hydrogen ion concentrations) were made in the presence of sodium acetate (*cf.* Fig. 1). It is seen that curved lines are obtained, which means that the correlation discussed in the previous paragraph cannot be applied for quan itative calculations in pure acetic acid. Several factors may contribute to this deviation from a straight-line relationship. In practical chromatographic work the

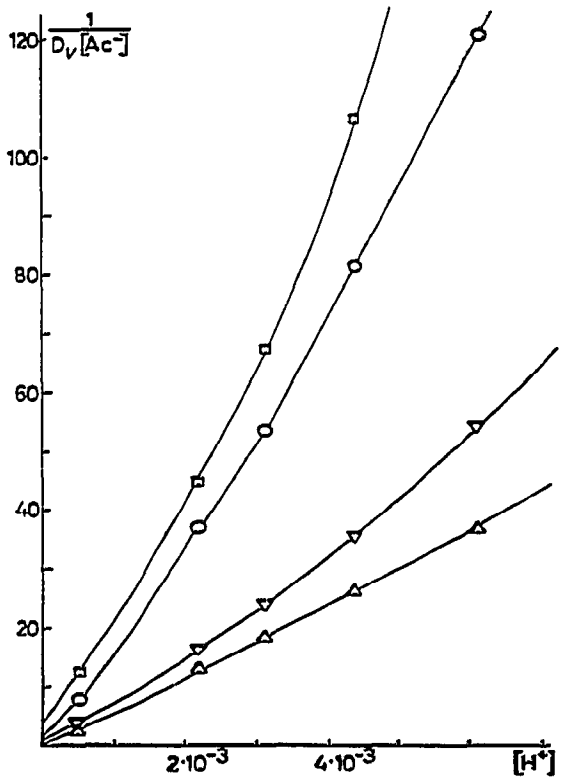

Fig. 2. Relationship between $1/D_v[Ac^-]$ and $[H^+]$ for various hydroxy acids in acetic acid. (□) Lactobionic; (○) talonic; (▽) arabinonic; (△) glycolic; $[H^+] = [Ac^-]$ were obtained by interpolation from data reported by OWEN[17] and recalculated to molar concentrations.

correlation can be used for rough calculations of the changes in peak positions with changes in eluant concentration. Similarly, a calculation of the D_v-value in acetic acid based only upon the D_v-value in sodium acetate and a literature value for the dissociation constant of the acid can give some guidance but the results from such calculations must be treated with great caution.

The volume distribution coefficients determined in 0.5M acetic acid are given in Table I together with the K_{HB}-values calculated from the D_v-values determined in this medium and in sodium acetate by using the equation given above. It should be emphasized that the K_{HB}-values should not be considered as accurate values for the dissociation constants. These K_{HB}-values differ somewhat from the apparent dissociation constants calculated from runs referred to in Fig. 1. Few reliable literature data are available for comparison. With ions which exhibit large specific interaction forces with the resin it is likely that the activity coefficient ratio inside the resin phase is more affected by changes of the medium than with those species for which these forces are small. The K_{HB}-values should therefore reflect the true acid strength better for the polyhydroxy acids than, for instance, for the keto acids. From a practical point of view the values can be used to predict roughly the elution volume at other concentrations of acetic acid in the eluant.

With diastereomers it is probable that the approximations introduced in the calculations will give about the same errors in all calculations and that for this reason the relative magnitude of the K_{HB}-values will be fairly accurate. The observation that ribonic and talonic acids appeared much earlier than their diastereomers can for

instance be explained by the assumption that these acids are much weaker than their diastereomers. This was confirmed in a separate study of their neutralization curves. Similarly, the observation that α-D-glucoisosaccharinic acid in acid medium appeared much earlier than the β-isomer indicates large differences in acid strength of these acids. Sufficient amounts of these acids were not available to permit an independent determination of their dissociation constants.

A large number of factors such as hydrogen bonding, resonance, inductive and steric effects affect the dissociation constants of the acids, and at present it is only possible to make reliable predictions of their chromatographic behavior from their structure in simple cases. From the well-known fact that aliphatic hydroxy acids with a hydroxyl group in the 2-position are stronger than those substituted at carbon atom 3 it can be predicted that 2,4-dihydroxybutyric acid should be held more strongly than 3,4-dihydroxybutyric acid. Likewise it should be expected that the hexuronic acids which are known to be stronger than aldonic acids should appear later than the hexonic acids. The experimental results agree with these predictions.

As far as the individual behavior of various non-cyclic stereoisomers such as aldonic acids is concerned, it should be expected that the positions of the hydroxyl groups on carbon atoms 2, 3 and 4 should have a greater influence than those in positions more distant from the carboxyl group. From Table I it is seen that the pentonic acids appear in the following order: ribonic, arabinonic, xylonic, lyxonic. Among the hexonic acids studied, talonic acid which has a *ribo*-configuration appears much earlier than the other acids belonging to this group. Galactonic acid, which is the second hexonic acid, has an *arabino*-configuration. Gluconic acid has a *xylo*-configuration whereas gulonic, mannonic and rhamnonic acids, which exhibit the highest distribution coefficients, have a *lyxo*-configuration. It is evident that both with pentonic and the hexonic acids studied the following correlation exists between the configuration and the order of elution: *ribo* < *arabino* < *xylo* < *lyxo*. These results indicate that the position on the chromatogram can give information about the structure of certain hydroxy acids and give indications of interest for their identification even if authentic samples are lacking and the dissociation constants are unknown.

Formally, the correlation given above also holds true for the uronic acids studied provided that these are represented by the open-chain Fischer formulae and that the positions α, β and γ from the carboxyl group are considered. Since uronic acids have a ring structure the formal validity of the above correlation can only be coincidence.

From a practical point of view it is important that the separation factors of most isomeric acids are more favorable in acetic acid than in sodium acetate. In many practical analyses acetic acid is, therefore, a more useful eluant than sodium acetate. Since different structural factors determine the elution behavior in these two media their use in successive separation stages permits resolution of complex mixtures of various organic acids.

A typical chromatogram from a run in acetic acid of a complex mixture is reproduced in Fig. 3. It can be seen that with the mixture of 12 hydroxy acids tested there is only serious overlapping of the bands corresponding to glycolic and lyxonic acids. However, these acids can be well separated in sodium acetate, and if only an approximate quantitative estimation is required the results from the run in acetic acid can be used for this purpose.

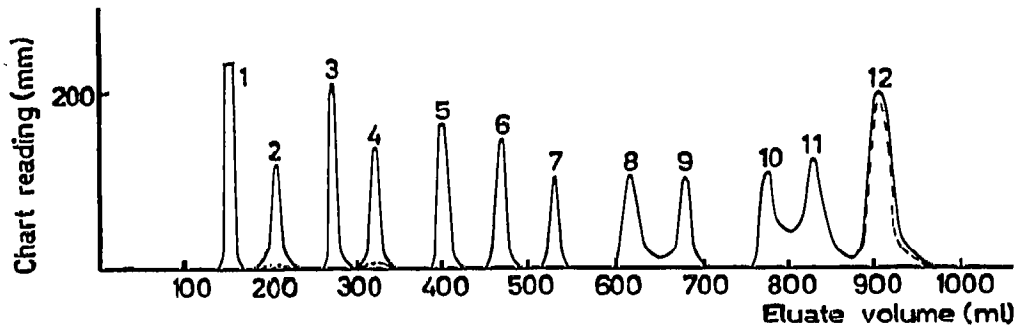

Fig. 3. Separation of 3,4-dihydroxybutyric (1); melibionic (2); D-talonic (3); maltobionic (4); D-ribonic (5); D-*glycero*-D-*gulo*-heptonic (6); D-gluconic (7); 2-methyl-2,3-dihydroxypropionic (8); D-xylonic (9); glycolic (10); D-lyxonic (11); and D-galacturonic acid (12). Carbazole method: broken line; dichromate method: full line. Eluant: 0.50 *M* acetic acid; flow rate: 5.2 ml·cm^{-2}·min^{-1}.

The results of a study of the influence of the flow rate are given in Fig. 4. It is seen that the peak elution volumes are independent of the flow rate whereas a broadening of the elution curves occurred at the higher flow rate. Talonic and maltobionic acids, which were separated completely at the lower flow rate showed some overlapping at the higher rate. Glycolic and 6-deoxymannonic acids were chosen as an example of a pair of acids which gave serious overlapping already at the lower rate.

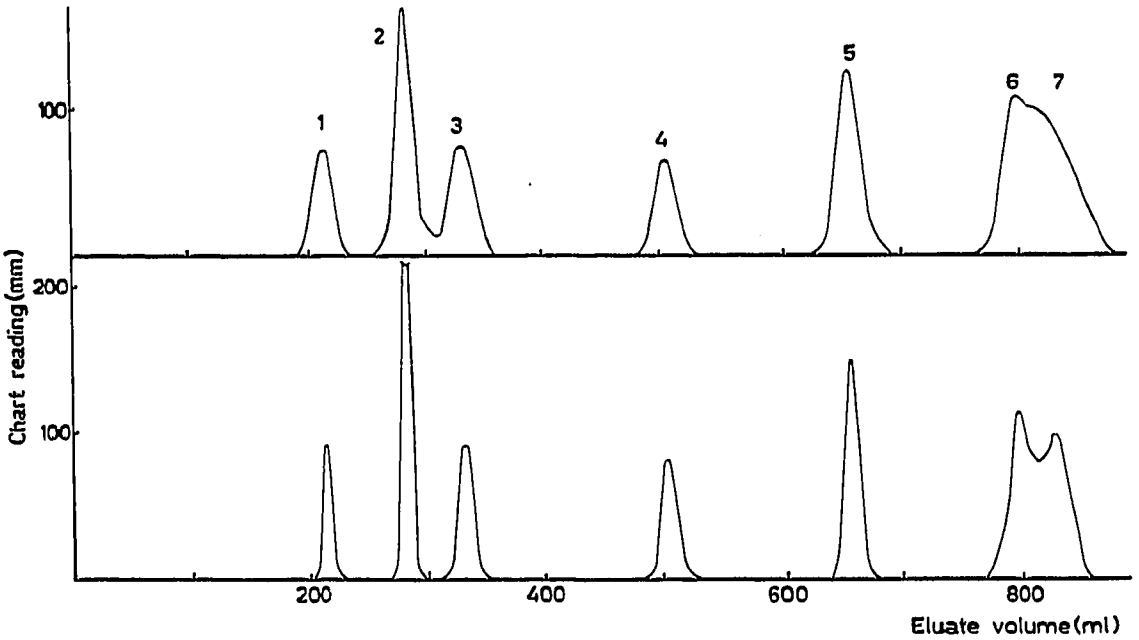

Fig. 4. Separation of lactobionic (1); D-talonic (2); maltobionic (3); D-galactonic (4); lactic (5); glycolic (6); and 6-deoxymannonic acid (7) at the flow rates 7.7 ml·cm^{-2}·min^{-1} (upper chromatogram) and 2.8 ml·cm^{-2}·min^{-1} (lower chromatogram). Eluant: 0.50 *M* acetic acid.

At the higher flow rate it is still possible to establish that two different acids are present but a quantitative determination cannot be made. With acids whose separation factors are large, excellent separations were achieved at the highest flow rate. In the run represented by the upper chromatogram the elution was completed within 7

hours. Since the runs can be carried out overnight without any attention it is preferable to use a somewhat lower flow rate in analyses of complex mixtures of unknown acids.

ACKNOWLEDGEMENT

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SUMMARY

The chromatographic separation of 44 organic acids, mainly hydroxy acids, on anion exchange resins has been studied using sodium acetate and acetic acid as eluants. By a combination of both media most species can be separated and determined automatically.

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