THE SEQUENCE OF ELUTION OF PLANT ORGANIC ACIDS FROM SILICA GEL CHROMATOGRAPHIC COLUMNS

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INTRODUCTION

In a programme of work on the effect of potassium nutrition on the organic acids of leaves, a knowledge of the sequence of elution of the common plant acids proved valuable in identification of acids separated by partition chromatography. The sequence of elution of these and related acids (total 39), by gradient elution chromatography on silica gel, is presented together with a discussion on the effect of structural relationships on the sequence.

Work on the partition chromatography of acids has been comprehensively reviewed to 1956 by LEDERER AND LEDERER[1]. Based on paper chromatographic data, HOWE[2] demonstrated relationships between the R_M values of about 100 acids and certain features of their molecular structure, *e.g.*, the number of carboxyl and other substituent groups, and indicated that this information should prove useful in identification of unknown compounds.

EXPERIMENTAL

Silica gel chromatography of the acids was carried out by the gradient elution method of WAGER AND ISHERWOOD[3], essentially as modified by BLUNDSTONE AND DICKINSON[4]. In the present work, reservoir 1 contained initially *n*-butanol–chloroform, 50:50 (v/v) (400 ml) and reservoir 2 contained chloroform (340 ml). The eluate was initially pure chloroform and its *n*-butanol content was progressively increased to 35 % (v/v). The acids (0.25–1 mequiv. each) were chromatographed in groups of 2–8 components, chosen to minimise overlapping of peaks. The volume of the fraction collector (syphon device) was 2.6 ml. The volume of chloroform eluted from the column during addition of the sample was 10 ml (≡ 4 fractions) and this was combined with the first fraction for titration. The rate of transfer of mixed solvent from reservoir 1 to reservoir 2 was about 30 drops per min from a capillary tube of 2 mm I.D., 7 mm O.D., but maximum resolution in the early stages of a separation was obtained when this rate was halved. Two hundred fractions were collected during 8 h.

The column was packed with Mallinckrodt silicic acid (100 mesh powder, A.R.). As preliminary treatment, it was washed several times by decantation with water to remove the finer particles, washed once with 6 *N* hydrochloric acid and several times with distilled water to remove excess acid and finally washed with ethanol and with

acetone and dried overnight in an oven at 100°*. Chloroform (Hopkin & Williams Ltd., G.P.R. "A" grade) was treated with excess of anhydrous sodium carbonate to remove any traces of acid and filtered. Ethanol, 2 % (v/v), present as a preservative was not removed before use. *n*-Butanol (Hopkin & Williams Ltd., G.P.R. grade) was the other component of the solvent mixture. The acids were the purest samples available commercially in the U.K.

Non-volatile acids were identified by thin-layer chromatography on Silica Gel G (Merck) with *n*-butyl formate–90 % formic acid–water (7:2:1, v/v) as mobile phase[5] and the observations were confirmed by spot tests[6].

TABLE I

SEQUENCE OF ELUTION OF ACIDS FROM SILICA GEL COLUMN

The acids were eluted with chloroform containing a progressively increasing proportion of *n*-butanol. The stationary phase was 0.5 *N* sulphuric acid. Fraction volume: 2.6 ml.

Acid	*Elution range (fraction numbers)*	*Peak maximum (fraction number)*	*Acid*	*Elution range (fraction numbers)*	*Peak maximum (fraction number)*
n-Butyric	1–10	2	5-Pyrrolidone-2-carboxylic	65– 80	69
n-Valeric	1– 9	2	Glyoxylic	53– 81	70
Isobutyric	1–12	3	Diglycollic	64– 76	70
Propionic	5–19	8	Oxalacetic	67– 79	73
Acetic	22–29	25	Oxalic	76– 95	81
Mesaconic	24–30	27	Tricarballylic	80– 93	88
Pyruvic	28–38	32	Glycollic	84– 98	92
Adipic	28–38	32	Nitric	87–147	93
Formic	31–38	34	*cis*-Aconitic	97–108	103
Glutaric	32–37	34	DL-Malic	103–120	111
Citraconic	34–45	39	Citric	134–153	141
Itaconic	39–47	42	DL-Glyceric	152–170	160
Maleic	38–50	45	DL-Isocitric	176–195	183
Fumaric	38–52	45	Sulphuric	178–192	181
Thymol blue indicator	47–53	50		193–230	198
			Shikimic	175–206	198
Succinic	57–63	60	D(+)-Tartaric	200–233	211
Lactic	57–63	60[a]	Phosphoric	> 239	—
α-Ketoglutaric	54–67	62	L-Glutamic		
trans-Aconitic	66–72	69	Quinic	> 252	—
Malonic	64–75	69	L-Aspartic		

[a] From ref. 4.

RESULTS

The sequence of elution of 39 acids is given in Table I in terms of the first and last fraction numbers in which the acids appeared and the fraction number at peak maximum for each. These fraction numbers were used for the sake of simplicity but may be converted to the more fundamental effluent volumes (ml) by multiplying by the factor 2.6 and addition of the column "hold-up" volume (10 ml). In the gradient elution method, the rate of transfer of the mixed solvent from reservoir 1 to reservoir 2

* A commercial product of controlled particle size is now available as Mallinckrodt silicic acid, SilicAR CC-4, 100–200 mesh.

is a variable which cannot be precisely controlled; this leads to a lack of reproducibility of the order of ± 5 fractions from the stage of elution of the volatile acids onwards. The emergence of the indicator (thymol blue) about 10 fractions earlier than the succinic acid peak maximum (prominent in the chromatograms of many plant extracts) provided a convenient marker. Recovery of acids was normally better than 95 %.

DISCUSSION

The following generalisations were made on the effect of the structural relationships of the acids on their sequence of elution as described above (Table I).

Fatty acids, $H(CH_2)_nCOOH$

The sequence was inversely related to n in fatty acids of the type $H(CH_2)_n$-COOH, where n = 0–4. There was a smooth relationship between n and the fraction number (F) at peak maximum (graph not reproduced here). Mean ΔF per CH_2 group in the series formic to n-butyric acids was —11.

Monocarboxylic acids with zero to four hydrophilic groups

The relationship between the number of polar groups (other than the carboxyl group) (a) in the molecule and the fraction number at peak maximum (F) is summarised in Table II. There was a linear relationship between (a) and (F). From the graph (not reproduced here), ΔF for first OH group was 55 and ΔF for second OH group was 80.

TABLE II

MONOCARBOXYLIC ACIDS—RELATIONSHIP BETWEEN NUMBER OF POLAR GROUPS AND ELUTION

Number of polar groups (a)	*Number of carbon atoms*	*Acid*	*Peak maximum (fraction number)* (F)	
0	2	Acetic	25	
1	3	Lactic	60	Mean 66
1	5	Pyrrolidonecarboxylic	69	
1	2	Glyoxylic	70	
1	2	Glycollic	92	
2	3	DL-Glyceric	160	
3	7	Shikimic	198	
4	7	Quinic	> 252	

Saturated dicarboxylic acids

The effect of chain length (n) in dicarboxylic acids of the type $HOOC(CH_2)_n$-COOH, in the range n = 0–4 is shown in Table III. Mean ΔF per CH_2 group in this series was —12, substantially the same as ΔF in the fatty acids. The introduction of an ether linkage, as in diglycollic acid, increased the hydrophilic character of the substance (F = 70) as compared with succinic acid (F = 60).

Cis- and trans-isomers

It proved impossible to separate maleic and fumaric acids on the silica gel

column under the conditions described above but their methyl homologues (citraconic and mesaconic acids) were readily separated, particularly when the *n*-butanol content of the mixed solvent was slowly increased (*F* mesaconic = 37; *F* citraconic = 57). *cis*- and *trans*-aconitic acids were widely separated. In each of these cases the *trans*-acid was eluted before the corresponding *cis*-isomer.

TABLE III

SATURATED DICARBOXYLIC ACIDS—RELATIONSHIP BETWEEN CHAIN LENGTH (*n*) IN HOOC·$(CH_2)_n$·COOH AND ELUTION

Chain length (*n*)	*Acid*	*Peak maximum* (*fraction number*) (*F*)
0	Oxalic	81
1	Malonic	69
2	Succinic	60
3	Glutaric	34
4	Adipic	32

Tricarboxylic acids

In citric and isocitric acids, the pronounced effect of the shift of the hydroxyl group, from the β- to the α-position, in increasing the hydrophilic property is noteworthy. ΔF for the hydroxyl group in the above acids is 53 (β) and 95 (α) as compared with 51 (first OH) and 100 (second OH) in the dicarboxylic acids and 55 (first OH) and 80 (second OH) in the monocarboxylic series, (see above). In the dicarboxylic acids, introduction of a double bond in the main chain increased hydrophobicity but *cis*-aconitic acid behaved in the contrary manner and was eluted later than tricarballylic acid.

Oxalacetic and α-ketoglutaric acids

Introduction of a methylene group into the main chain increased the hydrophobicity of oxalacetic acid and ΔF was —11. As compared with the parent acid, introduction of an α-keto-group increased hydrophilic properties but the extent of the change varied considerably in the three examples studied. The variation may be due, at least in part, to the presence of the corresponding enol-forms of the acids.

Propionic ⟶ pyruvic acid, ΔF (CO) was 24

Glutaric ⟶ α-ketoglutaric acid, ΔF (CO) was 28

Succinic ⟶ oxalacetic acid, ΔF (CO) was 13

Number of carboxyl groups

In the unsubstituted polycarboxylic acid series (acetic, succinic, tricarballylic) and the corresponding monohydroxyl compounds (glycollic, malic and isocitric acids) the addition of a —CH_2COOH group led to a marked increase of hydrophilic properties in the acids but the differences in the ΔF values are not regular. Addition of a carboxyl group in the keto-acid series (pyruvic and oxalacetic) had a similar effect.

Nitrate is present in relatively large amounts in many plants, particularly when

grown in sand culture. The acid was eluted from silica gel columns as an asymmetrical peak with a steep leading edge and a marked tailing edge and the peak therefore overlapped several others including the important malic and citric acid peaks. This occurred independently of column loading in the range 0.25–1 mequiv. of nitrate. The quenching acid, sulphuric, gave rather indefinite twin peaks, one of which may have been due to the acid sodium salt. Phosphoric (*cf.* ref. 7) and quinic acids and the amino acids, L-aspartic and L-glutamic were not eluted before fraction 239. As observed previously under similar though not identical conditions[7,8] the acids eluted early in the sequence gave sharp, symmetrical peaks (*aiguilles*) whereas those eluted late were less steep and tended to tail slightly. Thus, in a separation of α-ketoglutaric and D(+)tartaric acids (1 mequiv. each), the former gave a symmetrical peak in which the height/base ratio was 15.5 whereas the corresponding ratio for the latter was 1.8. With certain exceptions, the sequence of elution described above was similar to the order of increase of partition coefficients of organic acids between water and ethyl ether as reported by DERMER AND DERMER[9]. It is of interest that whereas the partition coefficients of L- and DL-malic and D- and *meso*-tartaric acids, respectively, were different, there are no reports of separation of these optical isomers on silica gel columns. BULEN *et al.*[8] drew attention to the effect of development solvent composition on the sequence of elution of acetic, fumaric, pyruvic and glutaric acids. Other publications relevant to the present work are as follows. Automatic determination of acids on a microscale (0.05–3 μequiv. per acid) was described by KESNER AND MUNTWYLER[10]. Thirteen acids were eluted from silica gel with chloroform–*tert.*-amyl alcohol mixtures and the sequence was, in general, similar to that found in the present work and by others with chloroform–butanol mixtures as the development solvent. An equation for calculating the concentration of solvent delivered by a mixing device similar to that used in this work has been reported[11].

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SUMMARY

The sequence of emergence of 39 plant and related acids from silica gel chromatographic columns by gradient elution with chloroform–*n*-butanol mixtures is described. The interference of nitrate in the determination of several acids and, most importantly, malic and citric is reported. The effect of structural relationships such as chain length, number of carboxyl, hydroxyl or keto groups and *cis* and *trans* isomerisation on the volume of solvent required for elution is discussed.

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