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IMPROVEMENTS IN THE AUTOMATIC DETERMINATION OF ORGANIC ACIDS FROM PLANT TISSUES BY LIQUID–LIQUID CHROMATOGRAPHY ON A SILICA GEL COLUMN

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SUMMARY

Improvements are described in the automatic determination of organic acids from plant tissues by liquid–liquid chromatography. Narrower and more symmetrical peaks are obtained by the use of smaller particles of silicic acid with a more homogenous granulometry (Bio-Sil A; 20–44 μm). A great improvement in reproducibility has been attained by using a new method for regenerating the column, and this also obviates the packing of a new column for each analysis. Further improvements result from the use of high pressure, stainless-steel column, dry column packing and an injection port.

INTRODUCTION

Since organic acids are metabolites that play a central role in the anabolism and catabolism of such complex organic molecules as carbohydrates, lipids, amino acids and proteins, their accurate determination is of prime importance in metabolic studies, especially with tracer methods. At present, liquid–liquid chromatography, based on the partition of acids between a stationary water phase and a moving organic phase of increasing polarity, is the only method that gives quantitatively reproducible results without denaturation of the acids. We have already made improvements in the method[1]; these include:

(1) Better resolution, obtained by decreasing the number of acids in the extract being analysed. This was done by fractionation of the acids into two groups (mono-

carboxylic acids, and dicarboxylic together with tricarboxylic acids) with the aid of an anion-exchange resin.

(2) Improvement in the efficiency of the column, and in the resolution, by use of a series of mixtures of chloroform–cyclohexane (50:50, v/v) progressively enriched with *tert.*-amyl alcohol.

(3) Automation of the elution and detection systems, which makes the method less tedious and more reproducible.

Although these improvements resulted in better separation, the duration of an analysis was doubled by the pre-fractionation process, and small losses of some acids occurred. In order to prevent this, we have attempted to separate most of the acids on a single column; we have further refined the technique by utilising recent improvements in high-speed liquid–liquid chromatography[2,3].

A further disadvantage of the liquid–liquid chromatography of organic acids, compared with ion-exchange chromatography, is the necessity of packing a new column for each analysis; this results in less reproducible chromatograms and constitutes a real and important problem. To overcome this, we have devised a method of regenerating the column so that it can be used repeatedly.

METHOD

Preparation of the silicic acid column

Type of silicic acid. Bio-Sil A, 20–44 μm (Bio-Rad Labs., Richmond, Calif., U.S.A.), was used as support for the stationary phase. It is a porous support (pore diameter < 100 Å) with a medium specific surface area of 200 m^2/g, especially activated for partition chromatography by the method of Ramsey and Patterson[4].

Preparation of silicic acid. A 5.5-g portion of Bio-Sil A is heated at 110° for 48 h, then cooled to 22° and transferred to a 50-ml erlenmeyer flask. Aliquots of 0.5 ml of 0.1 *N* sulphuric acid are progressively added until the total volume is 3 ml; after each addition of acid, the mixture is stirred with a glass rod in order to obtain a homogeneous powder that does not stick to the walls of the flask. The silicic acid thus prepared is saturated to 60% of its dry weight with the stationary phase. This high degree of impregnation improves the partition rate of the organic acids and also the stability and capacity of the column. However, too high a degree of impregnation must be avoided, as this can lead to the formation of a thick film of stationary liquid outside the layer of silicic acid particles; this induces aggregation of the particles and renders homogeneous packing of the column difficult.

Column packing. Silicic acid prepared as described above is poured, in several portions, into the stainless-steel column (600 × 4.6 mm I.D.) by using a small funnel. The column is fitted at the bottom with an LDV (low dead volume) reducing union ($^1/_4$ in. to $^1/_{16}$ in.) plus a filter of porous stainless steel (pore diameter 2 μm). After adding each portion of the silicic acid, the column should be tapped gently and rotated in order to obtain maximum density of filling. When the packing has well settled the top section is fitted with a reducing union to permit connection with a solvent pump. A mixture of chloroform–cyclohexane (50:50, v/v) is then passed through the column at a pressure higher than that used during normal operation; this is to settle the column completely. After this treatment, it is sometimes necessary to add a small amount of saturated silicic acid to the stationary phase to complete the packing of the column.

Introduction of the sample. The ethanol–water[1] crude extract, which contains approximately 50 μequiv., is dried by a stream of compressed air at room temperature (22°), and the residue is suspended in 200 μl of 10 *N* sulphuric acid; this suspension is directly injected on to the top of a pre-column (100 × 4.6 mm I.D.) filled with 0.8 g of silicic acid prepared in the same way as for the main column. This pre-column has to be refilled after each analysis, as it retains certain impurities present in the plant extract.

Chromatography of the organic acids

The basic scheme of the elution and detection system is illustrated in Fig. 1. Resistance to organic solvents is achieved by using stainless steel or PTFE fittings and tubing.

Elution. A series of mixtures of chloroform–cyclohexane (50:50, v/v) progressively enriched with *tert.*-amyl alcohol[1] is used to elute the organic acids. Each of the mixtures can be selected automatically by means of a six-way rotary valve and is supplied at a constant flow-rate (120 ml/h) to the top of the column by a Dosapro Milton-Roy pump. The eluents have the following volumes and contents of *tert.*-amyl alcohol.

Mixture I (240 ml): 4.5% of *tert.*-amyl alcohol.
Mixture II (240 ml): 8% of the alcohol.
Mixture III (280 ml): 11% of the alcohol.
Mixture IV (180 ml): 25% of the alcohol.
Mixture V (180 ml): 32% of the alcohol.
Mixture VI (80 ml): 40% of the alcohol.

Before use, each mixture is saturated in the stationary phase and degassed ultrasonically.

Detection and analysis of acids. First, the effluent from the column is passed through a UV detector (Isco, U.A. 5), which continually monitors the absorbance at 254 and 313 nm (see ref. 5) of the phenolic and unsaturated aliphatic acids present in the material being analysed. The effluent is then mixed with a solution of sodium 2-nitrophenolate (200 mg of sodium 2-nitrophenolate in 100 ml of ethanol). This indicator[6] is delivered, at the bottom of the column and at a constant flow-rate (60 ml/h), by means of another Dosapro pump. In this treatment, the sodium salts of the acids and an equivalent amount of free 2-nitrophenol are formed; the absorbance of the remaining sodium 2-nitrophenolate is measured at 435 nm (Isco, U.A. 5).

In this way, it is possible to detect 0.05–10 μequiv. of each acid. The amounts of phenolic and aliphatic acids present are found by reference to standards.

Column regeneration

Until now, columns have been repacked after each analysis. This is time-consuming, tedious and wasteful, and the columns cannot be reproduced with exactly the same characteristics. For this reason, we have tried with success, to regenerate the columns after each analysis. To do this it is necessary to remove all acidity and all *tert.*-amyl alcohol from the column and to re-establish completely the water–acid stationary phase. This can be done by passing through the column, successively, 150 ml of chloroform–cyclohexane (50:50, v/v), 150 ml of 0.1 *N* sulphuric acid in 50% ethanol and 150 ml of chloroform–cyclohexane (50:50, v/v).

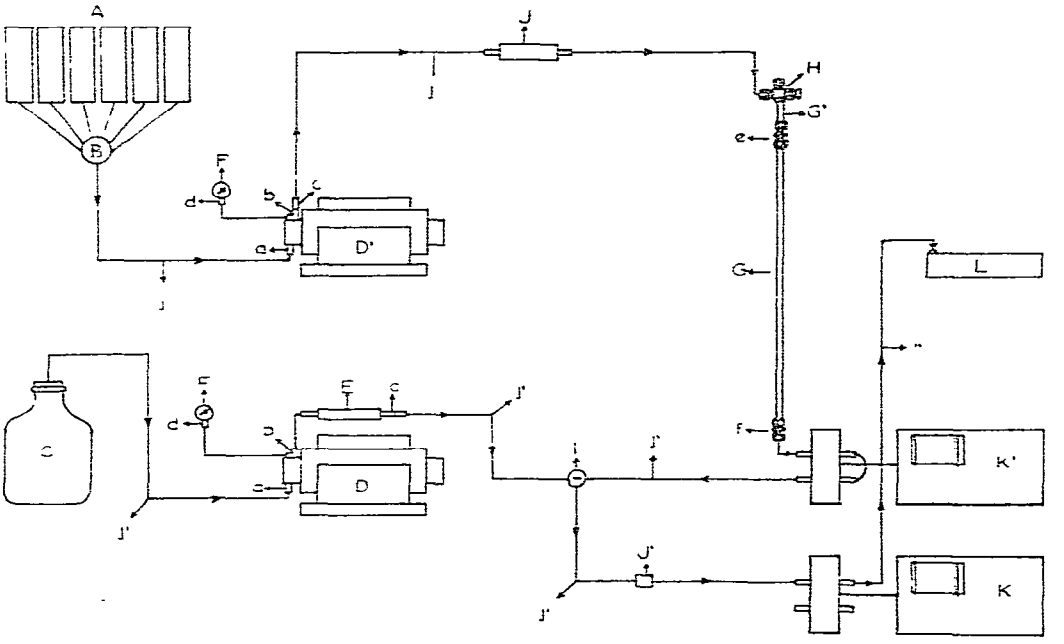

Fig. 1. Organic acid analyzer. A, brown-glass bottles for solvents that have been de-gassed and are blanketed with nitrogen; B, 6-position "cheminert" valve (Chromatronix, Berkeley, Calif., U.S.A.), Model R. 60.31. V. 6. A; C, brown-glass bottle for indicator (2-nitrophenol, sodium salt), initially de-gassed and blanketed with nitrogen; D and D', pumps (Dosapro minipump, Milton-Roy, Pont-St-Pierre, France), Type 196/33, maximum capacity 155 ml/h, maximum pressure 250 bars; E, 2C check and relief valve, 25 p.s.i. (Nupro, Cleveland, Ohio, U.S.A.); F, pressure gauges, type "manomètres alarme acier", Φ 52, 1/8 Briggs, 200 kg/cm^2 (Blondelle, Paris, France); G, chromatography column, stainless-steel tubing (600 × 4.6 mm I.D., 6.35 mm O.D.) (Reeve Angel, Clifton, N.J., U.S.A.), with nuts, 1/4 in. (LP 173; Reeve Angel), and ferrules 1/4 in. (LP 203; Reeve Angel); G', pre-column, stainless-steel tubing (100 × 4.6 mm I.D., 6.35 mm O.D.) (Reeve Angel), with LP 173 nuts and LP 203 ferrules; H, injection port, Model LLB (1/4 in. to 1/6 in. (LA 102; Reeve Angel), with septa (micro-sep. F 153; Reeve Angel); I, mixing chamber; J, pulse damper; J', pneumatic regulators; K, absorbance monitor with peak separator (ISCO, Lincoln, Nebr., U.S.A., Model U.A.5), double-beam UV/visible optical unit, 435 nm (Model 1140), Type 6, standard 250-μl 10-mm flow cells and multiplex expander (Model 1132); K', absorbance monitor (ISCO, Model UA 5), optical unit 254 and 313 nm (Type 6); L, fraction collector (ISCO, Model 328) with flow interrupter valve drop former connection); a, 200-2-2 male elbow, 1/8 in. to 1/8 in. (Swagelok, Cleveland, Ohio, U.S.A.); b, 200-3 TMT male-run tee, 1/8 in. to 1/8 in. (Swagelok); c, 201-6-1 reducing union, 1/8 in. to 1/16 in. (Swagelok); d, 200-8-2 female elbow, 1/8 in. to 1/8 in. (Swagelok); e, LP 008 union, tubing-to-tubing, 1/4 in. to 1/4 in. with 1/4-in. stainless-steel frit (retention size value 2 μm), LA 225 (Reeve Angel); f, LP 304 reducer low-dead-volume reducing union, 1/4 in. to 1/16 in., with 1/4-in. stainless-steel frit (LA 225); j, stainless-steel tubing (O.D. 1/16 in.; j', PTFE tubing (1/16 in. O.D.).

By using this method, it is possible to regenerate the column 10–30 times without affecting the reproducibility of results.

RESULTS

Fig. 2A illustrates the separation of an artificial mixture of organic acids by our technique; complete separation is attained in 10 h. Here follows a list of the acids

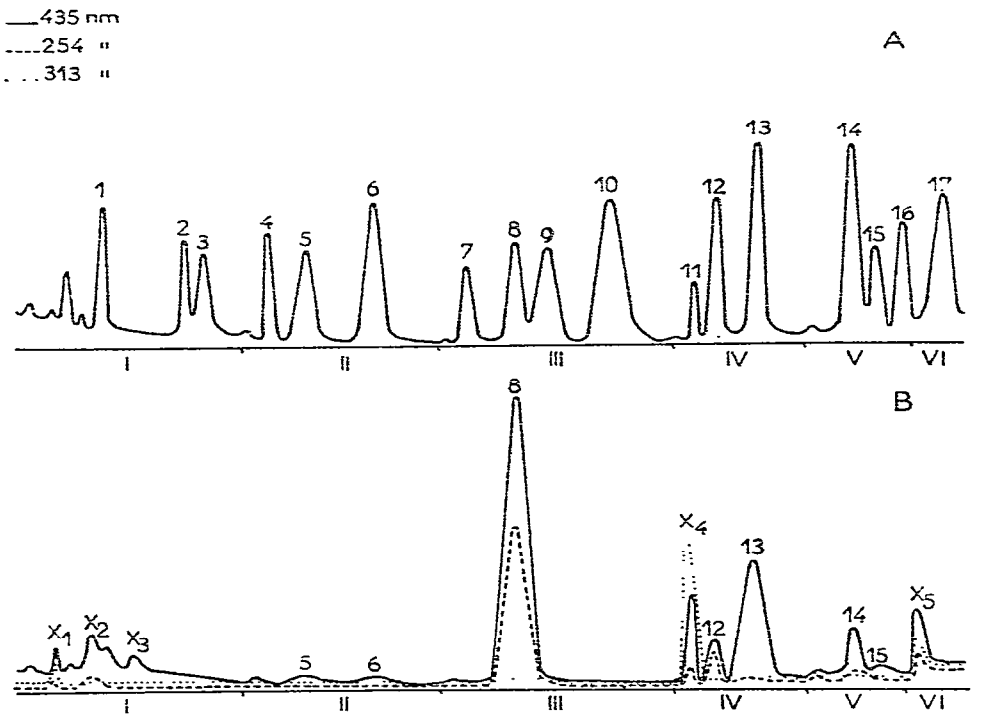

Fig. 2. Elution patterns of some organic acids. (A) Standard mixture: 1 = mesaconic; 2 = pyruvic; 3 = fumaric; 4 = glutaric; 5 = lactic and 3-hydroxybutyric; 6 = succinic; 7 = malonic; 8 = *trans*-aconitic; 9 = glycolic and pyrrolidonecarboxylic; 10 = oxalic; 11 = citramalic; 12 = *cis*-aconitic; 13 = malic; 14 = citric; 15 = isocitric; 16 = glyceric; 17 = tartaric. (B) Acids from a crude extract of maize leaves: X_1, X_2, X_3 = aconitic esters; 5 = lactic and 3-hydroxybutyric; 6 = succinic; 8 = *trans*-aconitic; X_4 = chlorogenic; 12 = *cis*-aconitic; 13 = malic; 14 = citric; 15 = isocitric; X_5 = unknown acid.

that can be separated, together with their retention times (in minutes): mesaconic, 46 ± 0.5; pyruvic, 94 ± 1; fumaric, 108 ± 1; glutaric, 148 ± 2; lactic and 3-hydroxybutyric, 170 ± 1; succinic, 214 ± 2; malonic, 270 ± 2; *trans*-aconitic, 300 ± 3; glycolic and pyrrolidonecarboxylic, 320 ± 4; oxalic, 360 ± 6; citramalic, 410 ± 4; *cis*-aconitic, 424 ± 5; malic, 448 ± 5; citric, 506 ± 5; isocitric, 520 ± 8; glyceric, 538 ± 7; tartaric, 560 ± 9. 3-Hydroxybutyric and lactic acids, and pyrrolidonecarboxylic and glycolic acids, are not completely separated; in order to separate them, further chromatography on another silica gel column is necessary, with chloroform–*tert*.-butyl alcohol as eluent[1].

Fig. 2B shows the results of an analysis of an ethanol–water crude extract of maize leaves; separation is very satisfactory without pre-fractionation, and small amounts (0.05 μequiv.) of acids can be detected.

Table I shows the experimental data for mesaconic and fumaric acids chromatographed under the following conditions: column, 600 × 4.6 mm I.D.; hydratation of the silica gel, 60% (w/w); concentration of *tert*.-amyl alcohol in mobile phase, 5%; flow-rate of mobile phase, 120 ml/h; particle diameter of silicic acid,

TABLE I

EXPERIMENTAL DATA FOR MESACONIC AND FUMARIC ACIDS

Parameter	*Mallinckrodt silicic acid*		*Bio-Sil A*	
	Mesaconic acid	*Fumaric acid*	*Mesaconic acid*	*Fumaric acid*
Retention time, sec	2850	6610	2760	6480
Peak width, sec	1080	2370	580	1260
No. of theoretical plates	111	125	362	423
HETP, mm	5.4	4.8	1.7	1.4
Resolution	2.18		4.04	

150 μm (for the Mallinckrodt product) or 20–44 μm for the Bio-Sil A. It can be seen that a better HETP, better efficiency, better N (number of theoretical plates), (height equivalent to a theoretical plate) and better resolution values are obtained by using the fine particle size range and homogeneous granulometry of Bio-Sil A.

Finally, it should be noted that the method of regenerating the column *in situ* offers significant advantages for separating the organic acids of biological samples. The procedure obviates the necessity of repacking the column after each analysis and gives retention times constant to within $\pm$ 2%, as can be seen from the above data. In comparison, retention times obtained with a new column for each analysis are constant only to within $\pm$ 3–10%. As regards the quantitative evaluation of each acid, our results are in agreement with those of Kesner and Muntwyler[6] and confirm the high reproducibility claimed by those workers.

CONCLUSION

We have improved liquid–liquid chromatography in two main respects: (1) by devising a method for regenerating the column *in situ*, which dispenses with packing a new column for each analysis and provides highly reproducible results; and (2) by obtaining better efficiency from the column and better resolution of the acids. This comes from the use of silicic acid of smaller particle size and permits separation of most of the acids on a single column, so rendering unnecessary the pre-separation proposed in our previous paper.

Further improvements result from the use of such techniques as high pressure, a stainless-steel column, dry column packing, and an injection port.

The method described provides superior resolving power for a wide range of plant extracts and permits greater reproducibility in their analysis. At present, this method is the only one known to us that can separate the main acids from biological tissues without denaturation.

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