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Note

Further studies of practical high-speed liquid chromatographic separations of tricarboxylic acid cycle organic acids and carbohydrates

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In a previous paper[1], we reported the possibility of the high-speed liquid chromatographic (HSLC) separation of tricarboxylic acid (TCA) cycle organic acids and carbohydrates. In this paper, we describe studies of the practical separation of these compounds with a high-resolution, highly sensitive detection method that does not involve decomposition and that is very useful for chemical analysis. We compared adsorption chromatography with ion-exchange chromatography for the separation of these organic acids, and also examined the possibility of carrying out high-speed permeation chromatography of carbohydrates by using Merckogel PGM-2000.

EXPERIMENTAL

The liquid chromatograph was assembled from custom-made and commercial parts. The variable UV detector was a Cecil 212 (Cecil, Cambridge, Great Britain), and the FID was a Pye Unicam LCM-2 (Pye Unicam, Cambridge, Great Britain). Solvents were pumped with a Milton Roy Model 196-31 (Milton Roy, Philadelphia, Pa., U.S.A.) or a Waters 6000 pump with a Waters 660 flow programmer (Waters Ass., Milford, Mass., U.S.A.).

Special-grade TCA cycle organic acids were used, comprising succinic, fumaric, glycolic, glutaric, malic, α-ketoglutaric, *cis*-aconitic, *trans*-aconitic, citric, tartaric, pyruvic, acetic and formic acids, dissolved in tetrahydrofuran (THF).

General-purpose reagent grade carbohydrates were used, comprising rhamnose, xylose, glucose, galactose, sucrose, ribose, fructose and mannose, dissolved in water.

For the separation of the organic acids, we used a polystyrene gel in the carboxymethyl form (LS-140, Toyo-Soda Co. Ltd., Shinnanyo, Yamaguchi, Japan), Corasil C_{18} (Waters) and Yana-Pak SILS (Yanagimoto Co. Ltd., Kyoto, Japan) as supports, and Corasil AX (Waters), Vydac HC-SCX and Merckogel PGM-2000 for the separation of carbohydrates.

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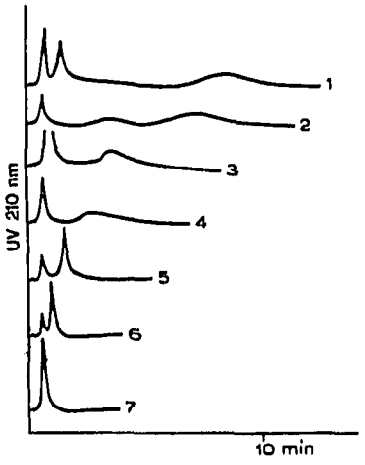
RESULTS AND DISCUSSION

Separation of TCA cycle organic acids

First, we used Corasil C_{18} as a support instead of Corasil II[1], as the reproducibility of chromatograms obtained on the latter were unsatisfactory owing to the influence of water. Chromatograms on Corasil C_{18} are shown in Fig. 1. These chromatograms were reproducible but the resolution was poor. We then prepared LS-140 as a support because its carbonyl group should form a hydrogen bond with a hydrogen atom of the acids. The elution curves obtained on LS-140 are shown in Fig. 2. We used flow programme in order to reduce the retention times. These chromatograms show that special-grade organic acids contain some impurities.



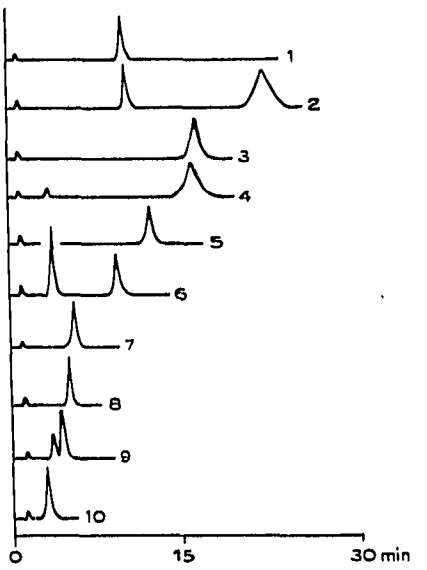


Fig. 1. Elution curves for organic acids on Corasil C_{18}. Eluent, *n*-hexane–THF–*tert.*-butanol (100:3:2); column, 50 cm × 2.1 mm I.D., stainless steel; flow-rate, 1.0 ml/min (25 kg/cm²). 1 = *cis*-aconitate, 2 = citrate, 3 = L-malate, 4 = α-ketoglutarate, 5 = glycolate, 6 = succinate, 7 = fumarate.

Fig. 2. Elution curves for organic acids on LS-140 (8 μm). Eluent, *n*-hexane–THF–*tert.*-butanol (100:5:5); column, 25 cm × 2.5 mm I.D., glass; flow-rate, 1.5–3.0 ml/min (20 min, 56 kg/cm²). 1 = *trans*-aconitate, 2 = *cis*-aconitate, 3 = citrate, 4 = tartrate, 5 = α-ketoglutarate, 6 = L-malate, 7 = glycolate, 8 = glutarate, 9 = succinate, 10 = fumarate.

We also used dimethylsilanized silica (5 μm, porous silica) as a stable adsorbent and the chromatograms obtained are shown in Fig. 3. This chromatography gave a high resolution, but the necessity for high pressures made the experiments difficult.

From the comparison of these supports, LS-140 was found to be the best adsorbent for practical liquid chromatography. Separations of mixed samples on LS-140 are shown in Figs. 4 and 5.

In this system, practical detection limits were about 2 μg for succinic acid and 5 μg for α-ketoglutaric acid.

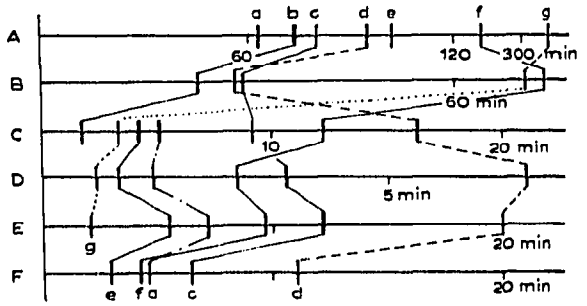
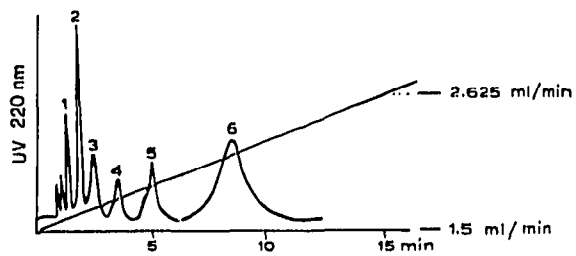


Fig. 3. Elution times of TCA cycle organic acids under different conditions. Elution conditions (column, eluent, flow-rate, column temperature): A[3], anion-exchange resin 8% (17–20 μm), 2 m × 3 mm I.D., 0.2 *N* HCl, 0.32 ml/min, 60°; B[4], Aminex A-25, 90 cm × 7 mm I.D. (with pre-column 3.6 cm × 7 mm I.D.), 1 *N* sodium formate, 1.0–2.5 ml/min, 70° (pre-column 25°); C, LS-140 (8 μm), 25 cm × 2.5 mm I.D., glass, *n*-hexane–THF–*tert.*-butanol (100:5:5), 1.5–3.0 ml/min, room temp. (56 kg/cm²); D, Corasil C_{18}, 50 cm × 2.1 mm I.D., *n*-hexane–THF–*tert.*-butanol (100:3:2), 1.0 ml/min, room temp. (25 kg/cm²); E[1], Corasil II, 1 m × 2.1 mm I.D., *n*-hexane–isopropanol (100:3), 1.12 ml/min, room temp.; F, YanaPak SIL, 25 cm × 2.1 mm I.D., *n*-hexane–THF–*tert.*-butanol (100:2:2), 1.5 ml/min (320 kg/cm²). Sample: a, glycolate; b, acetate; c, malate; d, citrate; e, succinate; f, α-ketoglutarate; g, fumarate.

Retention times of TCA cycle organic acids in various systems are summarized in Fig. 3. The difference between ion-exchange chromatography and adsorption chromatography can be seen in Fig. 3; while the elution order of one group (systems A and B) is different from that of another group (systems C, D, E and F), in the second group the elution order depends on the numbers of carboxylic groups that can form a hydrogen bond between its hydrogen atom and an oxygen atom of the support. On the other hand, the elution order of the first group would depend upon dissociation constants, and therefore this type of chromatography is classified ion-exchange chromatography. In Fig. 3, LS-140 with *n*-hexane–THF–*tert.*-butanol is the most useful system. Three practical separations were examined and the elution curves are shown in Fig. 6. The samples were concentrated about ten-fold, diluted ten-fold with THF and then injected on to the column.



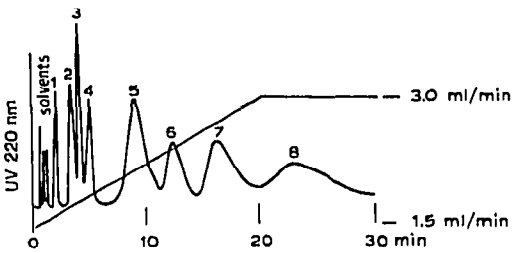


Fig. 4. Elution curve for organic acids on LS-140 (8 μm). Eluent, *n*-hexane–THF–*tert.*-butanol (100:5:5); column, 25 cm × 2.5 mm I.D., glass; flow-rate, 1.5–3.0 ml/min (56 kg/cm²). 1 = ethyl acetate, 2 = acetate, 3 = formate, 4 = fumarate, 5 = unknown, 6 = pyruvate.

Fig. 5. Elution curve for organic acids on LS-140 (8 μm). Eluent, *n*-hexane–THF–*tert.*-butanol (100:5:5); column, 25 cm × 2.5 mm I.D., glass; flow-rate, 1.5–3.0 ml/min (20 min, 56 kg/cm²). 1 = acetate, 2 = fumarate, 3 = succinate, 4 = glycolate, 5 = L-malate, 6 = α-ketoglutarate, 7 = citrate, 8 = *cis*-aconitate.

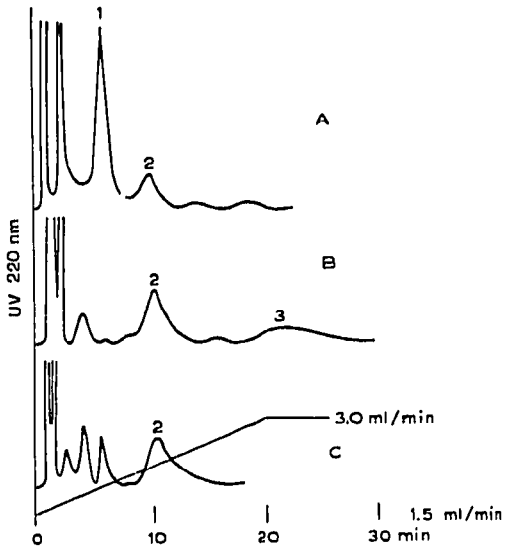


Fig. 6. Elution curves for some samples on LS-140 (8 μm). Eluent, *n*-hexane–THF–*tert.*-butanol (100:5:5); column, 25 cm × 2.5 mm I.D., glass; flow-rate, 1.5–3.0 ml/min (20 min, 56 kg/cm²). 1 = Glycolate, 2 = L-malate, 3 = citrate. Samples: A, soy; B, sauce; C, apple juice.

Separation of carbohydrates

We used Corasil AX instead of an anion-exchange resin[2], and selected acetone–ethanol–water as the eluent instead of ethanol–water[2]. A chromatogram of five monosaccharides is shown in Fig. 7. In this system, the detection limit of xylose is about 5 μg, but the separation of galactose from glucose was difficult, so Vydac HC-SCX cation exchanger was used as the support. In the latter system (Fig. 8), the separation of galactose from glucose was satisfactory, but other separations were poor. Therefore, both systems have to be used in the practical analysis of carbohydrates. An example of the practical separation of carbohydrates on Corasil AX is shown in Fig. 9.

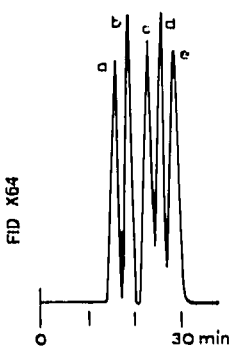
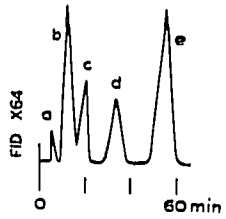


Fig. 7. Elution curve for carbohydrates on Corasil AX. Eluent, acetone–ethanol–water (20:2:1); column, 2 m × 2.1 mm I.D., stainless steel; flow-rate, 16 ml/h (60 kg/cm²). (a) Rhamnose; (b) xylose; (c) fructose; (d) mannose; (e) galactose (glucose).



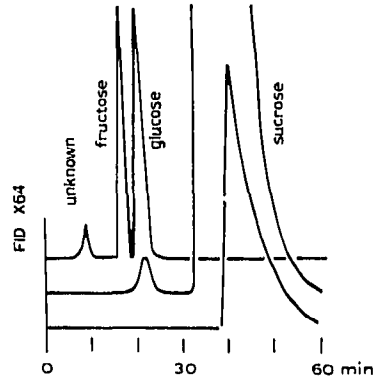
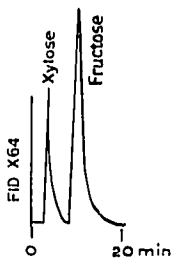


Fig. 8. Elution curve for carbohydrates on Vydac HC-SCX. Eluent, acetone–ethanol–water (20:2:1); column, 50 cm × 2.1 mm I.D., stainless steel; flow-rate, 8 ml/h. (a) Unknown; (b) rhamnose, (c) xylose, (d) glucose (ribose, fructose, mannose), (e) galactose.

Fig. 9. Elution curve for carbohydrates on Corasil AX. Eluent, acetone–ethanol–water (20:2:1); column, 2 m × 2.1 mm I.D., stainless steel; flow-rate, 20 ml/h (80 kg/cm²).

The separation of xylose and fructose was possible on silica (Fig. 10). Comparison of the chromatograms obtained on silica with those obtained on ion exchangers show that both types of chromatography depend on the same adsorption mechanism, and are not classified as ion-exchange chromatography.

The possibility of applying high-speed permeation chromatography to carbohydrates was also examined by using Merckogel PGM-2000, and chromatograms of corn syrup are shown in Fig. 11. This gel is not as hard as the new types of polystyrene



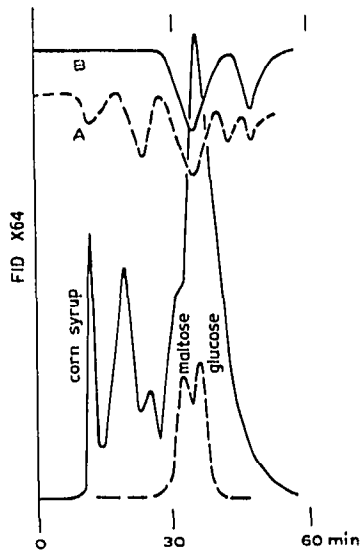


Fig. 10. Elution curve for carbohydrates on silica. Eluent, isopropanol–acetone (1:3); column, 50 cm × 2.1 mm I.D., stainless steel; flow-rate, 32 ml/h.

Fig. 11. Elution curves for carbohydrates on Merck PGM-2000. Eluent, water; column, 50 cm × 8 mm I.D., stainless steel; flow-rate, 24 ml/h (45 kg/cm²). A, hydrolysis once; B, hydrolysis twice.

gel, but is not soft like Sephadex, and therefore marketing of hydrophilic solid supports remains a problem.

CONCLUSION

High-speed, highly sensitive liquid chromatography of TCA cycle organic acids and carbohydrates without decomposition has been carried out by an adsorption technique depending upon hydrogen bonding. TCA cycle organic acids are separated with LS-140 as support and *n*-hexane–tetrahydrofuran–*tert.*-butanol as eluent with UV detection at 220 nm, and carbohydrates are separated with chemically bonded anion and cation exchangers with acetone–ethanol–water as eluent and flame ionization detection. The compounds were detected at the microgram level without decomposition.

ACKNOWLEDGEMENTS

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