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IDENTIFICATION OF HYDROXY ACIDS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

In an aqueous solution of lactone-forming acids, several forms of the acids exist, *e.g.* lactone forms and open-chain forms. The different forms of such acids were separated as trimethylsilyl (TMS) derivatives by gas–liquid chromatography (GLC) on OV-17. At pH 10 the lactone-forming acids used in this investigation were entirely converted in the open-chain form, and under these conditions each acid gave one peak by GLC of the corresponding TMS derivative. Acids derived from carbohydrates were reduced in mixtures with other acids to their corresponding alditols and separated as acetates by GLC on OV-225.

The following acids can be separated by the above-mentioned methods: citric, erythronic, D-galacturonic, D-glucaric, DL-isocitric, DL-malic, D-ribonic, and *meso*-tartaric acids.

INTRODUCTION

A mixture of acids derived from plants may contain different types of lactone-forming hydroxy acids: lactone-forming acids with few hydroxy groups, *e.g.* isocitric acid[1], or acids of carbohydrate derivation, *e.g.* D-glucaric[2], D-galactaric[3] and D-galacturonic acid[4].

Gas–liquid chromatography (GLC) of the open-chain trimethylsilyl (TMS) derivatives is a convenient method for the separation and identification of lactone-forming acids[5,6]. It has been shown that silylation of the lactonized acids, followed by GLC, often gives multiple peaks on the gas chromatogram[7].

Co-chromatography with reference substances is necessary to ensure a reliable identification. Most acids of carbohydrate derivation are difficult to obtain, but a number of alditols are readily available. The configuration of the acids can be confirmed by reduction to the corresponding alditols which, after derivatization, can be analysed by GLC[8,9]. The differences between the mass spectra of the diastereomeric acids of carbohydrate derivation are due to differences in the relative intensities of their peaks[10,11]. Thus identification of such acids by use of gas chromatography–mass spectrometry (GC–MS) depends on the availability of reference compounds.

In an investigation of lactone-forming acids in succulent plants[12] the identifi-

cation of such acids was greatly influenced by the methods for separation of the acid mixtures, and the availability of reference substances. The results presented here show that different methods of derivatization, combined with different conditions for the GLC analysis, give information that is of great importance for the identification of different types of hydroxy acid in mixtures, especially when reference acids are not available. All methods for GLC separation described in this paper can be applied to GC–MS analysis. Two common plant acids, malic and citric acid, were included in the investigation, which was carried out with pure acids.

EXPERIMENTAL

Chemicals

The following substances were derivatizated and chromatographed: citric acid (Merck, Darmstadt, G.F.R.), erythronolactone (supplied by Dr. F. Wold, University of Illinois, Urbana, Ill., U.S.A.), D-galacturonic acid monohydrate (Schuchardt, München, G.F.R.), DL-isocitric acid lactone (Type III, Sigma, St. Louis, Mo., U.S.A.), DL-malic acid (BDH, Poole, Great Britain), D-ribonolactone (Sigma), D-glucaric acid 1,4-lactone (Sigma) and *meso*-tartaric acid monohydrate (Fluka, Buchs, Switzerland).

The silylation was performed with trimethylchlorosilane (pure, Koch-Light, Colnbrook, Great Britain) and 1,1,1,3,3,3-hexamethyldisilazane (Merck) and pyridine (Merck) which, before use, was distilled and kept over pellets of sodium hydroxide (Elektrokemiska Aktiebolaget, Bohus, Sweden).

The chemicals used for the preparation of the alcohol acetates were: Dowex 50W-X8 (H^+), 20–50 mesh (Fluka), pretreated and kept in anhydrous methanol (Merck)[13], sodium borohydride (for synthesis, Merck–Schuchardt), The acetic acid, acetic anhydride and chloroform were all from Merck and of analytical reagent grade.

Apparatus

A Varian 1400 gas chromatograph with a flame-ionization detector was used. Of the two columns applied, one column (glass coil, 3 m × 2 mm I.D.) was filled with 10% OV-17 on Gas-Chrom Q (80–100 mesh), the other (glass coil, 2 m × 2 mm I.D.) with 3% OV-225 on Varaport 30 (100–120 mesh). The OV-17, the Gas-Chrom Q and the Varaport 30 were purchased from Supelco (Bellefonte, Pa., U.S.A.), while the OV-225 was from Applied Science Labs. (State College, Pa., U.S.A.). The detector temperature was 300° for the OV-17 column and 280° for the OV-225 column. The injector temperature was 250°, and the carrier gas (nitrogen) was used at a flow-rate of 40 ml/min. The temperature was programmed from 120° at a rate of 1°/min (OV-17), and from 150° at a rate of 2°/min (OV-225).

The retention times were recorded by an Autolab minigrator (Spectra Physics) and the chromatograms printed out by an OmniScribe recorder (Houston Instruments).

Acid mixture

Samples of 5 mg of each acid were dissolved in water (40 ml) and kept at room temperature for 3 days. Aliquots of 10 ml of this solution were used for derivatization.

Sodium salts

The acid (10 mg) was dissolved in water (10 ml) and sodium hydroxide (0.1 *N*) added until pH 10 was reached[5]. The neutralized solution was heated for 30 min at 60° and the procedure repeated until pH remained at 10. The acid mixture was treated in the same way.

TMS derivatives

The TMS derivatives were prepared directly from each reference acid, from the dried acid mixture, from the sodium salt of each acid and from the sodium salts prepared from the acid mixture. The silylation reagent was the same as that used by Raunhardt *et al.*[7].

Reduction of the acids

Each acid (10 mg) and the dried mixture were separately esterified with methanol and Dowex 50W-X8 (H^+)[2]. The methyl esters, dissolved in water (10 ml), were reduced by adding sodium borohydride (10 mg) twice, at an interval of 2 h.

After a total reduction time of 20 h, the excess of borohydride was neutralized with acetic acid (8 *M*) and removed by repeated evaporation following addition of acetic acid (0.8 *M* in methanol). Finally the alcohols were dissolved in anhydrous methanol and evaporated to dryness.

Acetylation of the reduced acids

To the dried alcohols acetic anhydride (1 ml) was added and the mixture heated for 1 h at 100°. After removal of excess acetic anhydride by evaporation, the acetates were dissolved in water (2 ml) and extracted with chloroform (2 ml). Prior to GLC analysis the acetates were dissolved in methanol.

RESULTS AND DISCUSSION

The GC retention data of the substances, after different methods of derivatization, are listed in Table I. The retention times were always measured relative to the corresponding derivative of the *meso*-tartaric acid. Typical chromatograms of the different derivatives of the acid mixture are shown in Figs. 1—3.

TMS derivatives

The lactone-forming acids usually exist in aqueous solution as an equilibrium between the different forms. In most cases, evaporation and desiccation of a solution of a lactone-forming acid does not lead to complete lactonization of the acid, hence several peaks appear on the gas chromatogram (Fig. 1). For all of the lactone-forming acids used in this investigation, adjustment to pH 10 was sufficient to convert the acid entirely into the open-chain form (Fig. 2). Under the conditions used two peaks were obtained for galacturonic acid, originating from the α- and β-anomeric forms.

The retention time for the open-chain TMS derivative is shorter than for the corresponding derivative of the same acid in lactone form. Acids that cannot form lactones always give peaks with the same retention times. A comparison between the gas chromatogram of the TMS derivatives directly prepared from an unknown acid mixture and the chromatogram of the TMS derivatives prepared from the sodium

TABLE I

RETENTION TIMES OF THE DIFFERENT ACID DERIVATIVES RELATIVE TO THE CORRESPONDING DERIVATIVES OF *MESO*-TARTARIC ACID

For the chromatographic conditions, see under *Apparatus*.

Substances	*TMS derivatives of the substances chromatographed on the OV-17 column*	*Acetates prepared of the reduced substances chromatographed on the OV 225 column*
Citric acid	1.83	2.13
Erythronic acid	0.78	1.00
Erythronolactone	0.86	1.00
α-Galacturonic acid	2.26	2.31
β-Galacturonic acid	2.39	2.31
Isocitric acid	1.90	2.13
Isocitric acid lactone	1.94	2.13
Malic acid	0.78	0.48
Ribonic acid	1.37	2.01
Ribonolactone	1.45	2.01
Glucaric acid	2.19	3.22
Glucaric acid 1,4-lactone	2.33	3.22
meso-Tartaric acid	1.00	1.00

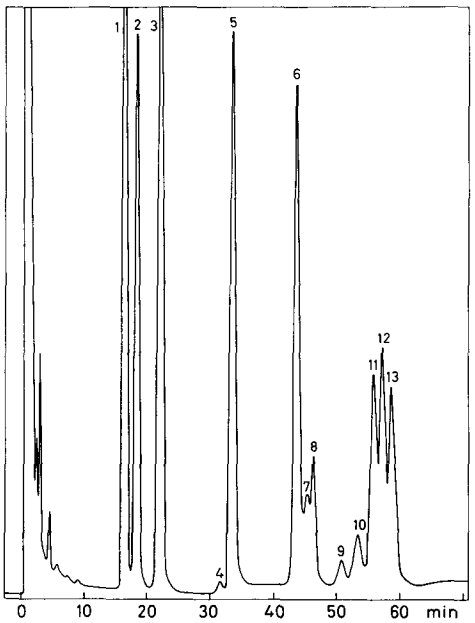

Fig. 1. Chromatogram of the TMS derivatives of the acid mixture. Column: OV-17. Peaks: 1, malic acid; 2, erythronolactone; 3, *meso*-tartaric acid; 4, ribonolactone; 5, ribonic acid; 6, citric acid; 7, isocitric acid; 8, isocitric acid lactone; 9, unknown; 10, glucaric acid; 11, α-galacturonic acid; 12, glucaric acid 1,4-lactone; 13, β-galacturonic acid.

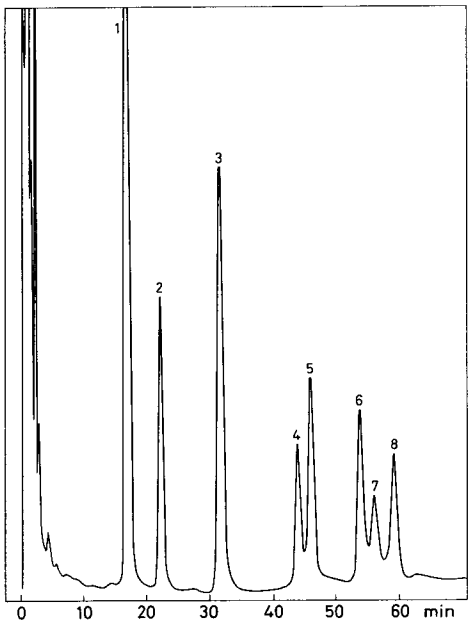

Fig. 2. Chromatogram of the TMS derivatives prepared from the sodium salts of the acid mixture. Column: OV-17. Peaks: 1, erythronic acid, malic acid; 2, *meso*-tartaric acid; 3, ribonic acid; 4, citric acid; 5, isocitric acid; 6, glucaric acid; 7, α-galacturonic acid; 8, β-galacturonic acid.

salts of the same mixture, provides an important basis for identification of the individual acids (Figs. 1 and 2). In this way not only could the lactone-forming acids be located on the chromatograms, but also the number of the different lactones could be evaluated.

The mass spectra of the TMS derivatives gave additional information. For the acids derived from carbohydrates, the type of acid (-uronic, -onic, -aric) and the number of carbon atoms were readily established[10,11,14].

Acetates of the reduced acids

The configurations of the acids derived from carbohydrates were confirmed by GLC of the acetates of the reduced acids. Many alditols, not commercially available, can easily be prepared by reduction of the corresponding aldoses and can be used as reference compounds.

The acetate of the reduced malic acid gave a single peak whereas the corresponding derivatives of citric and isocitric acid gave a number of small peaks together with the major ones (Fig. 3). The two last-mentioned acids were not separated under the chromatographic conditions used. As expected, erythronolactone and *meso*-tartaric acid gave peaks with the same retention time. Like the TMS derivatives, the acetates can be identified by means of GC–MS.

These results show that the acids derived from carbohydrates can be reduced to alditols and chromatographed as acetates in the presence of normally occurring plant acids.

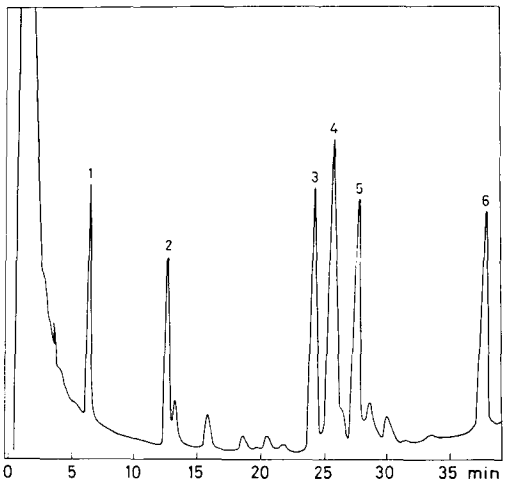

Fig. 3. Chromatogram of the acetates of the reduced acid mixture. Column: OV-225. Peaks: 1, malic acid; 2, *meso*-tartaric acid (= erythritol), erythronic acid (= erythritol); 3, ribonic acid (= ribitol); 4, citric acid, isocitric acid; 5, galacturonic acid (= galactitol); 6, glucaric acid (= glucitol). Most unnumbered peaks are due to citric and isocitric acid.

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