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GAS–LIQUID CHROMATOGRAPHIC METHOD FOR SEPARATION OF ORGANIC ACIDS AND ITS APPLICATION TO PINE NEEDLE EXTRACTS

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SUMMARY

A gas–liquid chromatographic method for the separation of a mixture of twenty organic acids is described. A column packed with Gas-Chrom Q (100–120 mesh) coated with 4% XE-60 was employed. Both TCA* cycle acids as well as a number of important non-TCA cycle acids were separated satisfactorily with this column. Pine needle organic acids were separated and identified with this column. Quinic and shikimic acids were found to be the major constituents of pine needle organic acids accounting for over 60% of the total peak area.

INTRODUCTION

This study was initiated to develop biochemical techniques to assess the impact of air pollutants on the organic acid metabolism in forest vegetation, particularly in pine needle tissues. It is known that the organic acid composition of conifers[1,2] is different from that of common agricultural species. This prompted us to search for the most suitable and reliable method for the analysis of organic acids in pine needle tissues.

Gas–liquid chromatography (GLC) is the most versatile, reliable and simple method to accomplish such an analysis. During the past two decades various GLC methods for the separation and quantification of naturally occurring organic acids, particularly the members of the TCA* cycle, have been described[3–5]. Normally, the organic acids are first converted into either methyl esters or trimethylsilyl (TMS) derivatives to make them volatile under chromatographic conditions. Starting with either of the derivatives, one can easily separate and quantify the organic acids in a biological preparation. Since conifers contain large amounts of shikimic and quinic acids[1,2], we decided to study a number of columns with a view to separating TCA cycle and non-TCA cycle acids on a single column. While our work was in progress, Phillips and Jennings[6] reported that they encountered difficulties in separating by GLC the TMS derivatives of shikimic, citric, and isocitric acids with either SE-30 or SE-52 columns. They reported[6] a successful separation of citric, isocitric, and shikimic acids on a 2% QF-1 column, but *cis*-aconitic acid could not be separated from citric

* TCA = tricarboxylic acid.

acid with this column. We describe in this paper a method for satisfactory separation of 20 organic acids (TCA and non-TCA cycle acids) of plant origin, including the above four, without an apparent compromise in resolving ability.

EXPERIMENTAL

Reagents

The organic acids were purchased from a number of suppliers including Sigma (St. Louis, Mo., U.S.A.), Aldrich (Milwaukee, Wisc., U.S.A.), and K & K Rare and Fine Chemicals (Plainview, N.Y., U.S.A.). Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), N,O-bis(trimethylsilyl)acetamide (BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and anhydrous pyridine were obtained from Pierce (Rockford, Ill., U.S.A.). Pyridine was distilled and stored over KOH pellets.

Plant material

Jack pine (*Pinus banksiana* Lamb.) seedlings were grown in styroblock trays as described previously[7]. The seedlings were grown at 22–24° under 10,500-lx light source and 18-h photoperiod. Needles from 6-month-old seedlings were utilized for extraction of organic acids.

Derivatization of organic acids

Each anhydrous organic acid standard (0.1 mmole) was dissolved in 5 ml of anhydrous pyridine and kept in a desiccator in a refrigerator. An exception was isocitric acid (trisodium salt), which was dissolved in 5 ml of water. An aliquot of the isocitric acid solution was transferred to the reacti-vial and evaporated to dryness under a stream of dry N_2 in a 60° oil bath. The residue was further dried by azeotropic removal of moisture with CH_2Cl_2. For derivatization, dried isocitric acid residue or an appropriate aliquot of the organic acid stock solution was mixed with anhydrous pyridine, HMDS, and TMCS in a reacti-vial. Regardless of the number of organic acids to be derivatized, the ratio among pyridine, HMDS, and TMCS was always maintained at 4:2:1, respectively. After the addition of the reagents into the reacti-vial, the screw cap with the PTFE septum was put on tightly, and the vial was shaken for 30 min on a wrist-action shaker. The mixture was then placed into an oil bath at 60° for another 30 min. The precipitate that formed during the reaction was allowed to settle in the reacti-vial cone for 30 min, after which an appropriate aliquot of the clear solution was injected into the GLC column.

Two other silylating agents, BSA and BSTFA, were also tried for derivatizing the organic acids, but the results were not reproducible from one day to the next. We obtained more consistent results with a mixture of HMDS and TMCS.

When the oxo-acids, α-oxoglutaric and oxaloacetic, were derivatized without the addition of hydroxylamine hydrochloride, they produced multiple peaks. To obtain single peaks, 3 mg of hydroxylamine hydrochloride was added to the vial before adding HMDS and TMCS, as recommended by Horii *et al.*[3].

Extraction of organic acids from pine needles

About 5 g of fresh pine needles were cut into small segments and dropped into a flask containing boiling 95% ethanol. After the ethanol boiled for 5 min, the flask

was allowed to cool down, and the liquid was filtered under suction. The needle tissue was homogenized for 5 min in 50 ml of 60% ethanol and filtered through a büchner funnel, and the residue was washed with 60% ethanol. The pigments were removed from the combined 60% and 95% ethanol extracts with light petroleum (b.p. 35–60°). After the removal of pigments, the extract was evaporated to dryness with a rotary evaporator. The residue was redissolved in 10 ml water and clarified by centrifugation. The phenolic compounds were removed from the supernatant with insoluble polyvinylpyrrolidone, which was purified according to McFarlane and Vader[8]. The phenol-free extract was filtered through a 0.8-μm Millipore filter to obtain a clear solution. This solution was passed first through a Dowex 50W-X8 (H^+; 20–50 mesh) cation-exchange column and then a Dowex 1-X8 (formate, 20–50 mesh) anion-exchange column. After we had washed the anion-exchange resin with water to remove neutral compounds, the organic acids were eluted with 2 *N* formic acid. The eluate was evaporated to dryness, and the residue was redissolved in 5 ml water. An appropriate aliquot of this solution was transferred into a reacti-vial and evaporated to dryness. To the residue was added 2 nmole of glutaric acid as an internal standard (I.S.), 0.4 ml anhydrous pyridine, 0.2 ml HMDS, and 0.1 ml of TMCS, and the derivatization was carried out as described before.

Gas–liquid chromatography

Samples were analyzed in a Hewlett-Packard, model 5830A gas chromatograph equipped with dual-flame ionization detectors and multi-function digital processor. Either 183-cm or 366-cm glass column (2 mm I.D.) was used to separate the organic acids.

A total of five liquid phases were tested for the separation of organic acids. These were 10% OV-11 on Chromosorb W, 110–120 mesh; 3% OV-3 on Chromosorb W, 80–100 mesh; 3.5% OV-1 on Gas-Chrom Q, 80–100 mesh; 10% silicone DC-560 on Chromosorb W, 80–100 mesh; 4% XE-60 on Gas-Chrom Q, 100–120 mesh. The column length for these analyses was 183 cm.

The XE-60 column (366 cm) was conditioned at 250° for 3 days with a nitrogen flow-rate of 25 ml/min to avoid column bleeding. The chromatographic conditions were as follows: temperature program from 105 to 220° at 4°/min after an initial hold for 5 min at 105°. The injector and detector temperatures were set at 265°. The nitrogen carrier gas flow-rate was set at 25 ml/min and flame hydrogen and air flow-rates were set at 60 and 240 ml/min, respectively.

RESULTS AND DISCUSSION

A mixture of eight or ten organic acids containing TCA cycle acids as well as such non-TCA cycle acids, as tartaric, shikimic, quinic, *p*-coumaric, ferulic and caffeic acids were analyzed on OV-11 OV-3, OV-1 and DC-560 columns. The OV-11 column was found to be completely unsuitable, whereas separation characteristics of OV-3 and OV-1 columns were comparatively much better. On OV-3 column, all but shikimic and citric acids were separated reasonably well. Whereas, on an OV-1 column, there was distinct lack of separation between shikimic and citric acids and between *p*-coumaric and quinic acids. In view of our inability to separate shikimic and citric acids on either OV-3 (*cf.* SE-52[6,9,10]) or OV-1 (*cf.* SE-30[11–14]) column, the

citric acid peak in the published gas chromatograms[11–16] could very well have been a combined signal from shikimic and citric acids. Reinvestigation would be required to shed light into this question. The DC-560 column proves useful when only TCA cycle acids[17] or non-TCA cycle acids[18] are to be separated, but not a mixture of the two because shikimic and citric acids appear together.

The problem of non-resolution between citric and shikimic acids on a SE-52 column was pointed out first by Boland and Garner[9], but they overcame the problem by using a second column of 3% XE-60. Recently, Phillips and Jennings[6] were faced with a similar problem in separating isocitric, citric and shikimic acids on a SE-52 column. In the SE-52 column all three acids eluted together as one peak. By employing a 2% QF-1 column, Phillips and Jennings were successful in separating citric and isocitric acids in the samples that did not contain shikimic acid. However, they were unable to separate citric and *cis*-aconitic acids. In view of the problem of non-separation of citric acid from shikimic acid and to a lesser degree the separation of aconitic acid from isocitric acid, advantage was taken of relatively less polar characteristics of shikimic acid and *cis*-aconitic acid compared to those of citric and isocitric acids, respectively, and a column was packed with 4% XE-60, which is much more polar than other columns described above.

The results in Fig. 1 show a very reasonable separation of a mixture of 20 organic acids, including the four problem acids namely shikimic, citric, isocitric and aconitic acids. An attempt to separate *cis*- and *trans*-aconitic acids from a mixture was, however, unsuccessful; both eluted at the same time. Boland and Garner[9] used an XE-60 column to separate shikimic acid from citric acid but they were unable to recognize the versatility of XE-60 liquid phase as demonstrated in this paper. All the acids that they separated on an SE-52 column could be separated even better on an XE-60 column. We were able to separate 20 different organic acids without any major compromise (Fig. 1 and Table I). The XE-60 column was found to suit our needs and was, therefore, used to separate the organic acids from pine needle extracts.

Separation of pine needle organic acids

The results obtained with the silicone XE-60 column are shown in Fig. 2 and

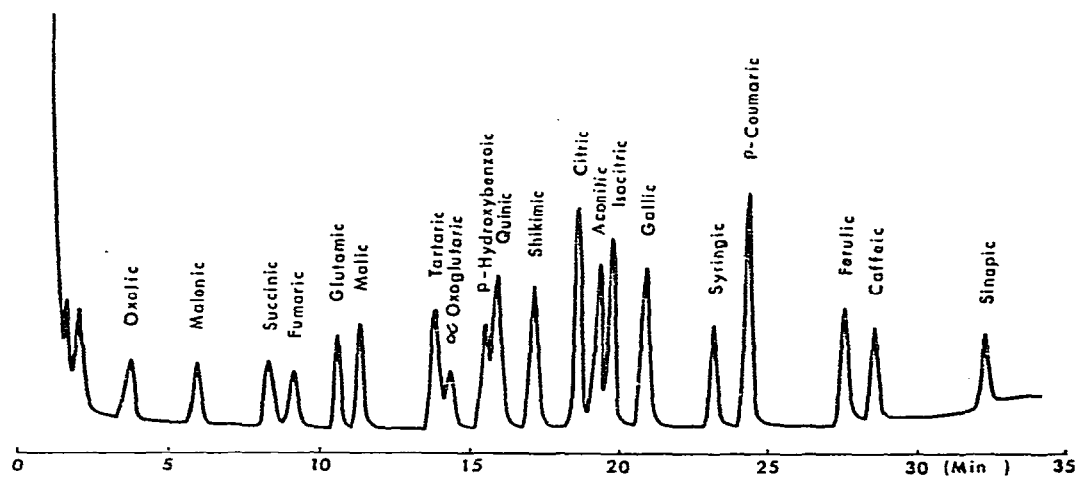

Fig. 1. Chromatogram of TMS derivatives of 20 organic acids on the XE-60 column. Chromatographic conditions are described in the text.

TABLE I

RETENTION TIME AND RELATIVE MOLAR RESPONSE OF A MIXTURE OF 20 ORGANIC ACIDS ON THE XE-60 COLUMN

Organic acid	*Retention time (min)*	*Relative molar response*
Oxalic	4.53	0.38
Malonic	6.19	0.90
Succinic	8.61	1.07
Fumaric	9.45	0.94
Glutaric (I.S.)	10.93	1.00
Malic	11.67	1.27
Tartaric	14.19	1.73
α-Oxoglutaric	14.71	0.86
p-Hydroxybenzoic	15.88	1.27
Quinic	16.28	2.44
Shikimic	17.52	2.05
Citric	18.99	1.44
Aconitic	19.57	1.13
Isocitric	20.04	1.60
Gallic	21.24	2.20
Syringic	23.57	1.27
p-coumaric	24.71	1.40
Ferulic	27.95	1.68
Caffeic	28.96	1.22
Sinapic	32.74	0.87

Table II. The pine needle extract contained three major components with retention times of 16.28, 17.53 and 20.70 min that accounted for about 80% of the total acids. The peak at 16.28 min was identified as quinic acids by enrichment with standard quinic acid and also the combined GLC–mass spectral analysis. Using the same techniques, the peak at 17.53 min was identified as shikimic acid. The peak at 20.70 min could not be identified. As well as these three major peaks, pine needles also contained trace amounts of malic and syringic acids. Other smaller peaks were not identified. The presence of the above acids, along with tartaric, malonic and fumaric acids, in the needles of *Pseudotsuga menziensii*, *Picea abies*, and *Larix decidua* has

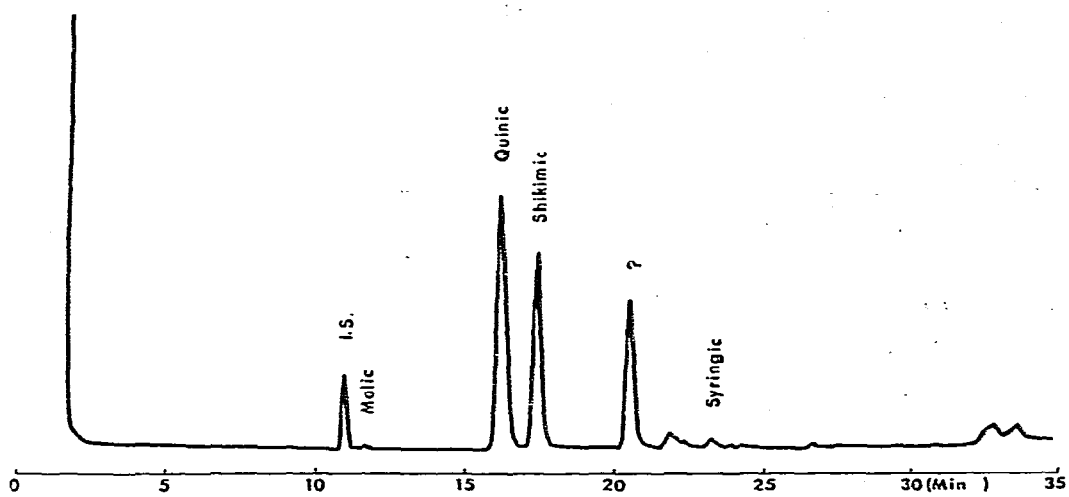

Fig. 2. Chromatogram of TMS derivatives of organic acids in pine needle tissues. 0.5 μl of the derivatives was injected. Chromatographic conditions were the same as described for Fig. 1.

TABLE II

RETENTION TIME, IDENTITY, AND AMOUNT OF ORGANIC ACIDS IN PINE NEEDLE EXTRACTS

Identity	*Retention time (min)*	*Amount (μmole/g dry wt.)*
Malic	11.67	1.32
Quinic	16.28	184.73
Shikimic	17.53	148.77
?	20.69	—
Syringic	23.50	8.02

been reported by Oechssler[19]. Neish[20] has reported the presence of shikimic acid in the leafy twigs of *Picea pungens* Engelm. and *Picea glauca* (Moench) Voss. Shikimic, malic, citric and quinic acids were also found in *Pinus sylvestris* needles by Muir *et al.*[21]. Most recently, Ishizaki and Hasegawa[22] have reported the occurrence of oxalic, citric, malic, quinic, shikimic, and succinic acids in *Pinus thunbergii* Parl. and *P. densiflora* Sieb. et Zucc.

The silicone XE-60 column that has been shown to be capable of separating 20 different TCA and non-TCA cycle organic acids is currently being utilized to study the effect of SO_2 on organic acid metabolism in pine seedlings.

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REFERENCES

1 S. Hattori, S. Yoshida and M. Hasegawa, *Physiol. Plant*, 7 (1954) 283.
2 M. Hasegawa, S. Yoshida and T. Nakagawa, *Kagaky (Science)*, 24 (1954) 421.
3 Z. Horii, M. Makita and Y. Tamura, *Chem. Ind. (London)*, 34 (1965) 1494.
4 M. G. Horning, E. A. Boucher, A. M. Moss and E. C. Horning, *Anal. Lett.*, 1 (1968) 713.
5 M. H. Harmon and H. W. Doelle, *J. Chromatogr.*, 42 (1969) 157.
6 R. D. Phillips and D. H. Jennings, *New Phytol.*, 77 (1976) 333.
7 S. S. Malhotra, *New Phytol.*, 76 (1976) 239.
8 W. D. McFarlane and M. J. Vader, *J. Inst. Brew.*, 68 (1962) 254.
9 R. L. Boland and G. B. Garner, *J. Agr. Food Chem.*, 21 (1973) 661.
10 Z. Horii, M. Makita, I. Takeda and Y. Ohnishi, *Chem. Pharm. Bull.*, 13 (1965) 636.
11 R. L. Brunette, R. L. Schoenman and G. E. Martin, *J. Ass. Offic. Anal. Chem.*, 50 (1967) 329.
12 E. Fernandez-Flores, D. A. Kline and A. P. Johnson, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 17.
13 W. Weissberger, T. E. Kavanagh and P. G. Keeney, *J. Food Sci.*, 36 (1971) 877.
14 G. E. Martin, J. A. Schmit and R. L. Schoenman, *J. Ass. Offic. Anal., Chem.*, 48 (1965) 962.
15 S. J. Jadhav and Wm. Andrew, *Can. Inst. Food Sci. Technol. J.*, 10 (1977) 13.
16 R. F. Coward and P. Smith, *J. Chromatogr.*, 45 (1969) 230.
17 S. K. Sarkar and S. S. Malhotra, unpublished results.
18 S. Sarkar and C. T. Phan, *Plant Sci. Lett.*, 2 (1974) 41.
19 G. Oechssler, *Z. Pflanzenphysiol.*, 59 (1968) 213.
20 A. C. Neish, *Can. J. Bot.*, (1958) 649.
21 J. W. Muir, R. I. Morrison, C. J. Bown and J. Logan, *Soil Sci.*, 15 (1964) 220.
22 A. Ishizaki and M. Hasegawa, *Bull. Fac. Agric. Tamagawa Univ.*, 14 (1976) 40.