COMMUNICATIONS

Improvements for Gas Chromatographic Analysis of Organic Acids in Plant Tissue

Improvements are reported in gas–liquid chromatography which reduce analysis time and improve separation of plant organic acids. A 4-ft U tube column packed with 9.5% OV-3 liquid phase on Chromosorb W-HP yielded good separation of a standard organic acid mixture containing oxalic, malonic, succinic, fumaric, malic, aconitic, tartaric, shikimic, citric, and quinic acid. Temperature programming from 150 to 230° increased acid separation and reduced analysis time to about 7 min. Regisil + 1% TMCS (*N,o*-bis-trimethylsilyl fluoracetimide plus 1% trimethyl chlorosilane) was found to be superior to BSA (*N,o*-bis-trimethylsilyl acetimide) for silylating organic acids from plant material.

The use of gas–liquid chromatography for the analysis of plant organic acids has been reported (Atkins and Canvin, 1971; Clark, 1969; Fernandez-Flores *et al.*, 1970; Rumsey *et al.*, 1967). The application of glc to organic acid analysis is dependent upon derivatization of the nonvolatile acids to render them volatile. One method has been to form the methyl derivatives (Atkins and Canvin, 1971; Rumsey *et al.*, 1967). However, this is a time-consuming procedure. The use of silylating reagents to form the trimethylsilyl derivative is a more rapid and convenient method of derivatizing organic acids.

Initial attempts to derivatize organic acids using BSA (*N,o*-bis-trimethylsilyl acetimide) (Regis Chemical Co.) as the silylating reagent (Clark, 1969) were not successful. Aconitic acid appeared to be easily "desilylated" so that if the reaction mixture was not injected shortly after mixing, the response per unit weight of acid injected decreased.

Many plants also contain appreciable amounts of the cyclic acids, shikimic and quinic acid, which are not easily silylated (Pierce, 1968). Silylation attempts with BSA produced a primary shikimic acid peak with a relative retention time of 1.28 and a secondary peak, corresponding to the partially silylated product, at relative retention time 1.43. This secondary peak had a relative retention time so close to that of quinic acid that they could not be separated. In a similar manner, quinic acid also yielded two peaks, using BSA, at relative retention times of 1.42 and 1.57, respectively. The percent silylation could be increased by heating the reaction mixture and increasing the reaction time after mixing. Unfortunately, these procedures accelerated the loss of response for aconitic acid in the sample.

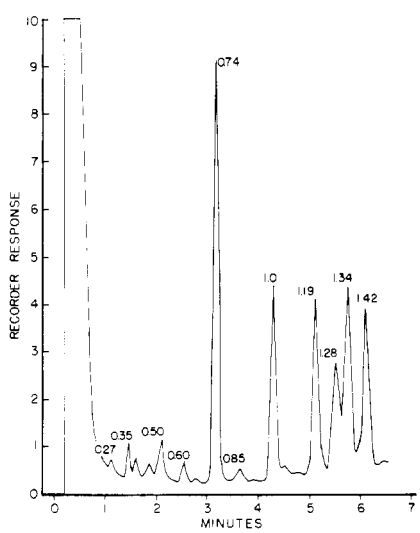

Figure 1. Chromatogram of organic acids from orchard grass. The number above each peak indicates the relative retention time of the acid relative to tartaric acid. The acids are identified as follows: 0.27, oxalic; 0.35, malonic; 0.50, succinic; 0.74, malic; 1.0, tartaric (internal std); 1.19, aconitic; 1.28, shikimic; 1.34, citric; 1.42, quinic; all others unknown.

This paper reports improvements in methodology which have greatly increased the speed of analysis and overcome difficulties experienced with previously published methods on glc organic acid analysis. These improvements make the glc method even more advantageous for the analysis of large numbers of samples such as might be encountered in screening tests or fertility experiments involving multi-treatments and replications.

EXPERIMENTAL SECTION

Extraction and Preparation of Plant Material. Leaf tissue from orchard grass (*Dactylis glomerata* L.) and several other cool season forages was the primary plant material used in this study. Freshly collected samples were freeze dried and ground through a 40-mesh screen. One gram of plant tissue plus 2 ml of 0.125% tartaric acid solution (internal standard) was homogenized at high speed for 4 min in 45 ml of 80% ethanol using a Virtis homogenizer. The residue was collected on a filter paper in a Buchner funnel using vacuum to speed filtering. The residue was thoroughly rinsed first with 50% ethanol and then with water until a total of approximately 200 ml of filtrate had been collected. The residue was discarded and the filtrate was evaporated to dryness under an air stream on a 50° water bath. The dried filtrate was suspended in approximately 25 ml of hot water and centrifuged at 10,000

Table I. Retention Times and Relative Retention Times of the Organic Acid Standard Mixture

Acid	Retention time, min	Relative retention time
Oxalic	1.17	0.27
Malonic	1.53	0.35
Succinic	2.12	0.49
Fumaric	2.28	0.53
Malic	2.24	0.74
Tartaric	4.38	1.00
Aconitic	5.21	1.19
Shikimic	5.67	1.30
Citric	5.84	1.34
Quinic	6.19	1.42

Table II. Effect of Silylating Reagent and Time of Silylation Reaction on gc Response

Silylation reaction time[a]	Response[b] (×1000)						
	Succinic	Malic	Tartaric	Aconitic	Shikimic	Citric	Quinic
Regisil + 1% TMCS							
0	11.3	52.9	20.0	41.2	35.7	33.3	40.5
3 min	11.2	52.1	20.0	40.9	34.2	33.7	44.9
10 min	11.2	52.2	20.0	39.5	33.3	33.7	46.5
30 min	11.2	51.9	20.0	36.8	32.6	33.0	45.6
BSA							
0	11.1	51.7	20.0	40.0	Did not separate		41.7
3 min	11.0	50.9	20.0	34.4	14.0	40.0	48.9
10 min	11.6	52.5	20.0	20.9	18.3	37.9	50.0
30 min	11.7	52.1	20.0	6.9	22.4	36.1	46.2

[a] Silylation time represents the time delay from mixing sample with silylating reagent to injection into gas chromatograph; 0 equals immediate injection after mixing. [b] The response for each acid in a run is adjusted to correspond to a response of 20,000 units for the internal standard. This corrects differences in response due to minor variations in volume of sample injected.

× *g* to clarify the solution. The solution was passed through a cation exchange resin (AG50-X4, 200–400 mesh, H^+ form) and an anion exchange resin (AGI-X4, 200–400 mesh formate form). Both resin columns were about 8 cm high and 1 cm in diameter. The anion column was eluted with 25 ml of 20% formic acid, followed by 25 ml of 50% formic acid and then rinsed with water. The combined anion elutant solution was evaporated to dryness under an air stream on a 50° water bath. The sample was suspended in 25 ml of hot water. A 10-ml aliquot was taken to dryness and suspended in 1.5 ml of reagent-grade pyridine. A residue sometimes remained in the pyridine–sample solution but this did not appear to affect the analysis.

Determination. Gas chromatographic analyses were carried out on a Hewlett-Packard Model 402 equipped with a flame ionization detector. Retention times and peak areas were measured with a Hewlett-Packard Model 3373B electronic integrator. The helium carrier gas flow rate was 60 ml/min and the air and hydrogen flow rates were 240 and 30 ml/min, respectively. The flash heater temperature was 250°. Detector temperature was 275°. The column was a glass 4 ft × ¼ in. i.d. U tube packed with 9.5% OV-3 on 80–100 mesh Chromosorb W-HP.

Silylation was accomplished by mixing 0.05 ml of the sample organic acids in pyridine with 0.1 ml of *N,o*-bis-trimethylsilyl fluoracetimide plus 1% trimethyl chlorosilane, Regisil + 1% TMCS (Regis Chemical Co.). After a 3-min reaction time at room temperature, a 2–3 μl sample was injected into the chromatograph. The oven temperature was immediately programmed from 150° at injection to 230° at a rate of 20°/min. Total time for the analysis was 6–7 min, after which the oven was cooled and allowed to equilibrate at 150° for several minutes before the next injection.

The silylation reaction was carried out in a small (10 × 75 mm) disposable test tube which was covered with plastic film to reduce the contact of moisture in the air with the silylating reagent. Regisil is very labile and reacts instantaneously with water, thus reducing its activity. An approximate 80-fold excess of silylating reagent to sample was used in these experiments to ensure maximum silylating activity, even with possible traces of water introduced into the sample from the air, glassware, or pyridine.

The internal standardization method was used to convert peak areas to percent organic acid in the original sample. Tartaric acid was used as the internal standard for the forage samples used in these experiments. This acid would not be a suitable internal standard for certain other plant species or fruits, such as grapes, which naturally contain significant quantities of this acid.

RESULTS AND DISCUSSION

A standard organic acid solution containing oxalic, malonic, succinic, fumaric, malic, *trans*-aconitic, tartaric, shikimic, citric, and quinic acid was injected twice daily to standardize the system. *trans*-Aconitic acid was used in the standard mixture, although the glc analysis could not separate the cis and trans isomers. A linear relationship was obtained between peak area and weight of acid inject-

Table III. Repeatability of Extraction, Purification, and Gas Chromatography of Organic Acids from an Orchard Grass Sample

Subsample no.	Injection no.	Percent organic acid (dry wt basis)					
		Succinic	Malic	Aconitic	Shikimic	Citric	Quinic
1	1	0.05	0.57	0.40	0.22	0.40	0.27
	2	0.05	0.57	0.41	0.21	0.40	0.28
	3	0.05	0.56	0.43	0.21	0.40	0.29
2	1	0.05	0.55	0.48	0.20	0.43	0.31
	2	0.05	0.50	0.44	0.18	0.40	0.28
	3	0.05	0.55	0.48	0.19	0.43	0.29
3	1	0.06	0.56	0.44	0.22	0.43	0.32
	2	0.06	0.57	0.40	0.21	0.40	0.28
	3	0.06	0.57	0.40	0.22	0.41	0.28
$\bar{X}$		0.05	0.55	0.43	0.21	0.41	0.29
SE subsample		0.006	0.019	0.029	0.014	0.010	0.008
SE gc analysis		0.000	0.010	0.012	0.004	0.008	0.010

ed up to about 7 μg of any acid injected. The retention times and relative retention times of the standard organic acids are listed in Table I.

A comparison of the two silylating reagents used in this study, Regisil + 1% TMCS and BSA, is shown in Table II. The response of aconitic acid diminished only 17% after 30 min reaction time with Regisil + 1% TMCS, whereas the response of aconitic acid using BSA decreased 89% in the same period of time. The silylation of both shikimic and quinic acid was essentially complete after 3 min reaction time with Regisil + 1% TMCS. In fact, there was a slight loss of response of shikimic acid with time, as was found with aconitic acid, indicating the need to use a constant reaction time for all standards and samples. The incomplete silylation of shikimic and quinic acid with BSA is very evident. Response of shikimic acid was still increasing after 30 min, while the response of quinic acid was very inconsistent due to its incomplete silylation, plus interference from the partially silylated shikimic acid peak. Quantification of these two acids, and in particular quinic acid, was nearly impossible.

The separation of silylated derivatives of shikimic and citric acid is difficult, especially when the concentration of shikimic acid is very low compared to citric acid. This is often the case, since many plant species are known to contain large amounts of citric acid. Nonseparation of these acids results in shikimic acid eluting as a shoulder on the citric acid peak, thus complicating quantification of both acids. A series of liquid phases, OV-1, OV-3, and OV-7, were tested with regard to overall separation and speed of analysis. A 9.5% coating of OV-3 was found to give the best separation of the standard organic acids when used with the programming conditions stated previously. Injection at 150° allowed separation of the smaller 2, 3, and 4 carbon acids, while the rapid rate of temperature programming increased the resolution and consequently the separation of shikimic and citric acid, and also reduced analysis time. While these operating parameters resulted in good separation with this chromatograph, other operators using different instruments may have to vary these conditions somewhat in order to obtain optimum separation.

A representative chromatogram illustrating the separation of organic acids in orchard grass is presented in Figure 1. Identification of the acid was made using the relative retention times of the standard acids listed in Table I. The results of multiple gc analysis on each of three orchard grass subsamples are presented in Table III. Malonic and oxalic acid are not included in Table III, since they were only present in trace quantities (<0.01%) in this sample. Error attributable to the gc analysis is very low. Repeatability of this procedure as indicated by the standard error values among subsamples is also quite good. This precision coupled with the speed of analysis represents an improvement in glc analysis of plant organic acids and should be beneficial to many laboratories involved in analytical determination of organic acids.

LITERATURE CITED

Atkins, C. A., Canvin, D. T., *Can. J. Biochem.* **49**, 949 (1971).
Clark, R. B., *Crop Sci.* **9**, 340 (1969).
Fernandez-Flores, E., Kline, D. A., Johnson, A. R., *J. Ass. Offic. Anal. Chem.* **53**, 17 (1970).
Pierce, A. E., "Silylation of Organic Compounds," Pierce Chemical Company, Rockford, Ill., 1968, pp 161–162.
Rumsey, T. S., Noller, C. H., Rhykerd, C. L., Burns, J. C., *J. Dairy Sci.* **50**, 214 (1967).

Allan L. Barta*
Charles A. Osmond

Agronomy Department
Ohio Agricultural Research and Development Center
Wooster, Ohio 44691

Received for review August 17, 1972. Accepted November 17, 1972.

Enzymatic Hydrolysis of Fatty Acids in Orange Juice Phospholipids

Palmitic acid was found to be located primarily at the 1 position and linoleic acid was at the 2 position of phosphatidyl choline and phosphatidyl ethanolamine in orange juice. Lipase or phospholipase hydrolysis of lipids during storage of commercial orange juice is questioned.

A recent publication by Nagy and Nordby (1970) has described changes during storage in the fatty acid content of chilled orange juice. These authors postulated that action by phospholipases on phospholipids in the juice caused significant changes in the proportions of palmitate (16:0) and oleate (18:1) remaining after storage. Also, Vandercook *et al.* (1970) have listed the quantities of the major phospholipids in some citrus juices.

Studies in our laboratory (Braddock, 1972) have described the distribution of the major fatty acids of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) from orange juice. The purpose of this communication is to present evidence which indicates that lipolytic enzymes were not primarily responsible for phospholipid hydrolysis in stored orange juice, as suggested by Nagy and Nordby (1970).

EXPERIMENTAL SECTION

Experimental procedures have been described in detail in a previous publication (Braddock, 1972). Essentially, these methods entail purification of the phospholipids from orange juice and gas chromatographic analyses of fatty acid methyl esters from PC, PE, and their respective lyso derivatives.

Table I. Fatty Acid Composition of Valencia Orange Juice Phospholipids and of Fatty Acids in the 2 Position

	% glc peak area[a]					
	16:0	16:1	18:0	18:1	18:2	18:3[b]
PE						
Phospholipid	24	4	2	19	42	9
2 position	3	0	0	23	62	12
PC						
Phospholipid	22	5	2	26	34	9
2 position	2	0	0	31	57	10

[a] Average of nine different juice samples. [b] Notation signifying carbon chain length:number of double bonds.