Gas-Liquid Chromatography of Organic Acids in Citrus Tissues

Alegre Sasson, Yair Erner,[1] and Shaul P. Monselise*

Organic acids of orange peel and juice were quantitatively determined by gas–liquid chromatography (GLC) with and without purification by eluting through Dowex exchange resin columns (6.0 and 13.25 N formic acid) or by fractionation with petroleum ether, at pH 3.6 or 7.5. Satisfactory recovery and resolution were usually obtained without purification. Under our conditions elution with 6.0 N formic acid gave low yields. Succinic*, adipic*, malic, citric, malonic, and oxalic acids were detected both in juice and peel (flavedo). In addition, juice contained traces of α-ketoglutaric, aconitic, and isocitric acids, while flavedo contained lactic* and benzoic* acids. Acids marked by asterisks had not yet been reported in flavedo tissues; α-ketoglutaric acid had not yet been reported in orange juice.

Organic acids of citrus peel have been studied by chromatography on silicic acid (Clements, 1964) or by collecting successive eluates from Dowex exchange resin columns (Rasmussen, 1964). Citrus juices have also been studied by this method (Rasmussen, 1964), by paper chromatography (Ting and Vines, 1966), and by thin-layer chromatography (Primo et al., 1963). In all these works quantitative data were obtained by titration against NaOH. Qualitative separation of organic acids in orange juice has also been carried out by gas–liquid chromatography (GLC) of methylated acids (Primo et al., 1963; Gepshtain and Lifshitz, 1970) and of Me_3Si derivatives (Fernandez-Flores et al., 1970). More recently a quantitative method to determine pure organic acids by GLC has been worked out by Hautala and Weaver (1969).

Suitable ways have now been developed (see also Erner et al., 1975) to use the above GLC techniques with citrus fruit tissue extracts and juice and are presented in the following.

EXPERIMENTAL SECTION

Sample Preparation. Ethanol (40 ml) and 70% extracts of 10 g of colored (flavedo) or 10 g of white (albedo) Shamouti orange peel tissues were evaporated to dryness in a Buechi rotavapor at 45 °C, redissolved in 5 ml of BF_3–methanol complex (14%), and left overnight at 25 °C. The contents of the methylation flask were transferred, the flask was washed with 4 ml of ammonium sulfate (33%), and the washings and 2 ml of chloroform were added to methylated extracts and thoroughly mixed. Afterward samples were centrifuged at 20 000*g* for 20 min. The chloroform fraction was then separated with a Pasteur pipet and used for injection in GLC.

Alternatively, 40-ml ethanol extracts (obtained from 10 g, fresh weight) were poured on top of columns (10 × 120 mm) of Dowex 1-X10 anion exchange resin (200–400 mesh) in the formate form and eluted with large volumes of 6 N formic acid (Davis et al., 1965). Eluate was evaporated to dryness and methylated as above.

Extracts were also partitioned twice with petroleum ether (40–60 °C) at their original pH value (6.1) or after having been brought to pH 7.5. The petroleum ether fraction was discarded and the ethanolic residue partitioned against chloroform. The ethanolic water residue was evaporated and methylated as above.

One milliliter of Shamouti orange juice was evaporated to dryness and either methylated as described above (except for the fact that 2 ml of methylating agent was used) or eluted through Dowex 1 columns before methylation. Fractionation as described above was also carried out at pH 3.6 or 7.5.

GLC Conditions. A Packard Model 7400 gas chromatograph with flame ionization detector was fitted with a glass column (1880 × 2mm i.d.) packed with 5% diethylene glycol succinate, 100/120 mesh, acid-washed, DMCS-treated, high-performance Chromosorb W. Carrier gases were N_2 at 30 cm^3/min, H_2 at 30 cm^3/min, and air at 350 cm^3/min; temperature programming: 3 min at 60 °C, then 20 °C/min up to 185 °C, final hold 10 min. Injector and detector temperatures were 195 and 220 °C, respectively. About 1 μl was injected, and the exact volume was recorded. The acids were identified by comparison of retention times with standard acids. For quantitative determinations peak heights of identified and standard acids were compared.

RESULTS AND DISCUSSION

Efficiency of Methylation. Figure 1 shows percentages of recovery after juice methylation with different amounts of BF_3–methanol complex, allowing reaction to continue overnight at 25 °C. Each acid has a different pattern of reaction to increasing amounts of the methylating agent. The reasons for this behavior are not known; all of them, however, show satisfactory recovery values at the 2-ml level. Therefore, this amount has been chosen for further work with juice.

A similar experiment (not reported here) was carried out with flavedo extracts finding in this case optimal recovery when methylation was performed with 5 ml of BF_3–methanol complex (see sample preparation section).

Table I shows recovery with different times and temperatures of methylation by the BF_3–methanol complex. When the methylating agent was allowed to react for 30 min only, at 25 °C, recovery of all acids was relatively poor in comparison with the standard method, especially so (4.2%) with citric acid, which is present in control, in very high amounts (1270 mg/100 ml). If, however, reaction proceeded for the first 10 min at 65 °C it was enhanced and recovery was much higher, in certain cases even above values of the standard procedure. The use of 2 ml, 25 °C overnight, should be recommended since citric and malic acids are the main components of juice and their full recovery is important, while succinic acid is a minor one.

When diazomethane was tried as a methylating agent at 2 ml (20 min), 5 ml (20 min) or 5 ml (overnight), all at 25 °C, recovery of juice acids was much reduced in all cases. Only adipic acid recovery was about 70%, while recoveries of succinic, malic, and citric acids were about 30, 18, and 7%, respectively. Differences between diazomethane treatments were negligible. Diazomethane has

Faculty of Agriculture, Department of Horticulture, Hebrew University of Jerusalem, Rehovot, Israel.

[1]Present address: Volcani Agricultural Research Center, Beit Dagan, Israel.

Table I. Effects of Reaction Time and Temperature during Methylation on Recovery of Different Organic Acids from 1 ml of Orange Juice (Averages of Two Replicate Runs)

Methylation conditions	Units	Succinic acid	Adipic acid	Unknown	Malic acid	Citric acid
2 ml of BF_3, overnight at 25 °C	mg/100 ml of juice	8.66	4.76	723[a]	82.6	1271.1
	mequiv/100 ml of juice	0.14	0.06		1.23	19.87
	%[b]	35.5	100.0	42.2	88.2	100.0
2 ml of BF_3, 30 min at 25 °C	%[b]	3.0	49.1	tr[c]	68.7	4.2
2 ml of BF_3, 10 min at 65 °C + 20 min at 25 °C	%[b]	100.0	76.6	100.0	100.0	57.0

[a] Values in centimeters peak height per 100 ml of juice. [b] Percentage of maximum recovery (recovery in percent of highest absolute values found for this acid in this table). [c] Traces.

Table II. Comparison of Purification Methods for Organic Acids from Flavedo Tissue of Shamouti Orange Peel (Ethanol Extract of 10 g Fresh Weight (FW); Averages of Two Replicate Runs)

Purification method	Units	Lactic acid (1)[a]	Oxalic acid (2)	Malonic acid (3)	Succinic acid (4)	Benzoic acid (5)	Adipic acid (6)	Malic acid (7)	Citric acid (8)
Crude extracts	mg/100 g FW	4.73	5.32	125.22	0.96	1.92	4.09	72.35	4.15
	SE of the mean, %[b]	4.5B	32.9A	6.5B	16.4B		13.7B	10.2B	24.0AB
	mequiv/100 g FW	0.053	0.11	2.40	0.016	0.016	0.056	1.08	0.065
	%[c]	100.0	49.7	92.1	100.0	100.0	100.0	72.2	100.0
Dowex, 6 N formic acid	%[c]	20.1	14.2	66.9	40.6	tr[d]	7.3	47.4	32.5
Dowex, 13.2 N formic acid	%[c]	65.1	100.0	100.0	93.7	94.2	2.9	100.0	45.5
Fractionation, pH 6.1	%[c]	20.9	28.7	85.6	95.8	72.4	50.6	47.9	100.0
Fractionation, pH 7.5	%[c]	tr[d]	50.0	86.0	90.6	69.2	75.7	47.4	100.0

[a] Numbers in parentheses refer to peak numbers in Figure 2. [b] Half of difference between two replicate runs in percent of mean value, average of 8-12 pairs of observations. [c] Percentage of maximum recovery, see footnote *b* to Table I. [d] Traces.

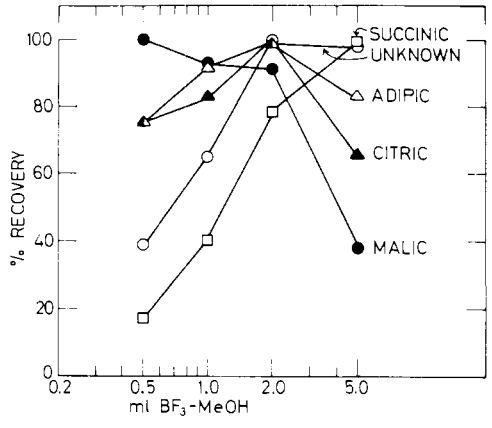

Figure 1. Effects of amounts of BF_3-methanol complex on recovery of different organic acids from orange juice.

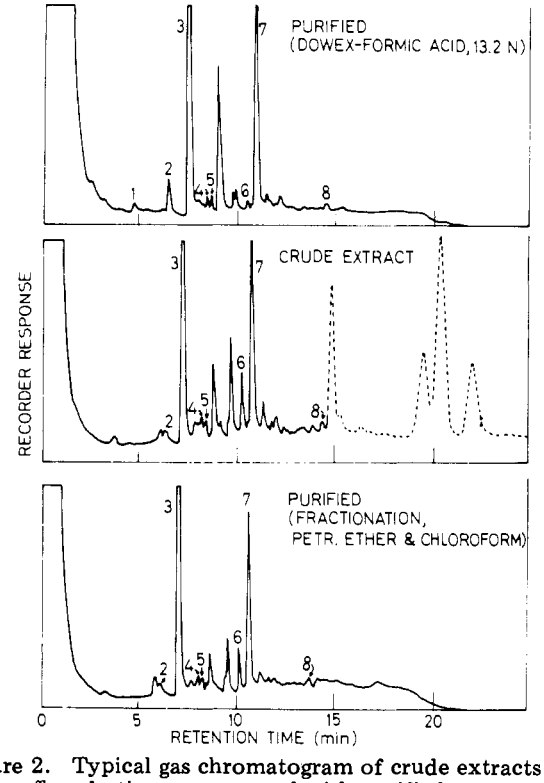

Figure 2. Typical gas chromatogram of crude extracts of orange flavedo tissues compared with purified extracts (Dowex 1-X10 anion exchange eluted with 13.25 N formic acid and fractionation with petroleum ether and chloroform, as described in the text). Numbers identifying organic acids are identical with those of Table II. Dotted peaks represent nonpolar materials found in the crude extract.

been used by Gepshtain and Lifshitz (1970) and Primo et al. (1963) for their qualitative GLC identifications in orange juice.

Efficiency of Purification. Table II shows results of different purification methods as compared with the crude extract of flavedo tissue (composite sample of 15 fruits, taken at the equator). Eight different organic acids could be quantitatively determined (Figure 2). Four of them have been detected usually in the past, while lactic, succinic, benzoic, and adipic acids had not yet been detected, to the best of our knowledge, in citrus peel. Succinic acid, however, was found in lime fruitlets and in the juice of frost damaged oranges (Ting and Attaway, 1971).

In our sample (taken from Shamouti oranges at an advanced stage of maturity) malonic (Clements, 1964) and malic acids predominate, while others are present in much smaller amounts. It is evident that in most cases highest amounts are detected in the crude extract. Elution by 6 N formic acid from columns, which is one of the usual

Table III. Comparison of Purification Methods for Organic Acids in Shamouti Orange Juice (1 ml of Juice; Averages of Two Replicate Runs)

Purification method	Units	Succinic acid	Adipic acid	Unknown	Malic acid	Citric acid
Unpurified juice	mg/100 ml of juice	22.25	5.47	937[a]	75.77	949.5
	mequiv/100 ml of juice	0.37	0.07		1.13	14.83
	%[b]	100.0	82.2	100.0	100.0	100.0
Dowex, 6 N formic acid	%[b]	18.4	65.9	14.8	76.1	38.8
Dowex, 13.2 N formic acid	%[b]	14.2	68.6	3.9	67.0	68.7
Fractionation, pH 3.6	%[b]	83.8	100.0	78.3	77.6	64.0
Fractionation, pH 7.5	%[b]	73.9	92.4	80.0	89.7	84.2

[a] Values in centimeters peak height per 100 ml of juice. [b] Percentage of maximum recovery, see footnote *b* to Table I.

purification methods (Gepshtain and Lifshitz, 1970; Rasmussen, 1964), mostly yields a reduced percentage of recovery, ranging between traces and 67% only of crude extract. Elution by 13.25 N formic acid yields a much higher percentage of recovery (except for adipic acid). It should be noted that in the case of malonic, malic, and especially oxalic acids, yields are higher than in crude extracts.

Petroleum ether fractionation at both pH values always yielded lower values than either crude extracts or 13.25 N formic acid elution (except for adipic acid).

Flavedo tissues contain large amounts of aromatic compounds which might have interfered with organic acid chromatography. The above results show that purification is actually not needed and crude extracts are more reliable than extracts purified according to the above methods, and especially by column chromatography followed by 6 N formic acid elution. Purification only helps to eliminate peaks of nonpolar materials with considerably longer retention time than organic acids (see dotted line in Figure 2).

Similar conclusions can also be drawn from Table III. Here natural orange juice was compared with purified juice. In this case only five main acids could be quantitated. Citric acid predominates as expected, while others are present in much smaller amounts. In most cases natural juice yields maximum amounts, while both formic acid elution methods attain much lower values. In the case of juice, petroleum ether purification yielded values ranging between 64 and 100% of contents in natural juice. There seems to be no point in purifying juice before GLC.

In addition to the five acids above, a large sample (100 ml) of juice, purified through the Dowex 1 column, eluted by 13.25 N formic acid, and methylated with 5 ml of BF_3–methanol complex, yielded additional peaks identifiable as oxalic, malonic (both also found in flavedo, see Table I), as well as α-ketoglutaric, aconitic, and isocitric acids. α-Ketoglutaric acid had never been found in juice, as far as we know, while the others have already been found by others (Sanchez et al., 1964). Identification of all these acids in peel and juice has been confirmed by GLC on 10% DC 560 phenyl methyl silicon, 80/100 mesh Chromosorb AW, under the same experimental conditions. On both columns used the above-mentioned acids showed retention times identical with respective standards.

Experiments with albedo tissues showed that also in this case crude extracts can be successfully analyzed without previous purification. The same acids as those reported in flavedo could be detected, while their absolute content was apparently lower except for citric acid which was higher than in flavedo.

We may conclude that satisfactory quantitative results can be obtained with peel and juice by GLC using the BF_3 complex as a methylating agent and without purification by conventional methods.

LITERATURE CITED

Clements, R. L., *J. Food Sci.* **49**, 276 (1964).
Davis, C., Hartley, R. D., Lawson, G. L., *J. Chromatogr.* **18**, 47 (1965).
Erner, Y., Reuveni, O., Goldschmidt, E. E., *Plant Physiol.*, **56**, 279 (1975).
Fernandez-Flores, E., Kline, E. D., Johnson, A. R., *J. Assoc. Off. Anal. Chem.* **53**, 17 (1970).
Gepshtain, A., Lifshitz, A., *Food Sci. Technol.* **3**, 115 (1970).
Hautala, E., Weaver, M. L., *Anal. Biochem.* **30**, 32 (1969).
Primo, E., Sanchez, J., Alberola, J., *Rev. Agroquim. Tecnol. Aliment.* **3**, 349 (1963).
Rasmussen, G. K., *Proc. Am. Soc. Hort. Sci.* **84**, 181 (1964).
Sanchez, J., Alberola, J., Garcia, I., *Rev. Agroquim. Tecnol. Aliment.* **4**, 371 (1964).
Ting, S. V., Attaway, J. A., "The Biochemistry of Fruits and Their Products", Vol. 2, Hulme, A. C., Ed., Academic Press, New York, N.Y., 1971, p 107.
Ting, S. V., Vines, H. M., *Proc. Am. Soc. Hort. Sci.* **88**, 291 (1966).

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