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Organic Acids in the Juice of Acid Lemon and Japanese Acid Citrus by Gas Chromatography

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Acetate, glycolate, butyrate, oxalate, malonate, succinate, fumarate, glyoxylate, malate, tartarate, cis-aconitatc and citrate were detected in the juice of Hanayu (*Citrus hanaju* Hort. ex Shirai), Daidai (*C. aurantium* Linn. var. Cynthifera Y. Tanaka), Kabosu (*C. sphaerocarpa* Hort. ex Tanaka), 'Lisbon lemon (*C. limon* Burm. f. Lisbon) and Yuzu (*C. junos* Sieb. ex Tanaka) with compositions and contents varied according to sampling years and species. Citrate and malate were predominant, accounting for more than 90% and 3-9% of the total detected acids, respectively. The other acids presented in tracts, accounting totally for roughly less than 0.5%.

INTRODUCTION

A number of chromatographic methods have been employed for determining organic acids (OAs) in citrus extracts. Based on the nature of mobile phase, they basically can be grouped into liquid (LC) or gas chromatography (GC). High performance liquid chromatography (HPLC) is recently the most frequently used method of LC due to its simplicity and quickness. Its capability, however, is very limited. Volatile OAs are not detected, and only a very few major non-volatile OAs are able to be detected in a single injection by HPLC. As OAs are involved in various processes in plant metabolisms, some OAs present in low concentrations are difficult to be detected by HPLC. From a physiological point of view, however, some acids in spite of their very low concentrations are very important. In GC methods, both volatile and non-volatile OAs present in a low concentration can be detected.

A considerable number of OAs has been detected in the juice of the so-called table citrus (Clements, 1964; Matsumoto and Shiraishi, 1977; Monselise and Galily, 1979; Nisperos-Carriedo et al., 1992; Sawamura et *al.*, 1983; Shiraishi et *al.*, 1991). Reports on organic acid compositions and contents in acid citrus are, however, hardly found especially those dealing with acid citrus other than acid lemon. In those which are available, the results varied due in part to the capability of the method employed (Osajima et al., 1971; Yamaki, 1989).

The present paper reports a simultaneous gas chromatographic determination on the compositions and contents of organic acids in the juice of acid lemon and some Japanese acid citrus.

MATERIALS AND METHODS

The fruits investigated were those of Hanayu (Citrus *hanaju*), Daidai (C. aurantium), Kabosu (C. *sphaerocarpa*), 'Lisbon' lemon (C. *limon*), and Yuzu (C.

junos). Ten fruits randomly sampled around the canopies in 1992 and 1993 were peeled, and their juice was extracted with a hand-pressed juicer and centrifuged (2,500 rpm for 20 minutes). One hundred ml of the supernatant juice was used for analysis.

Sample preparation for gas chromatography

Organic acid determination was conducted according to a modified method based on the butylester method of Yamashita *et al.* (1973) and Matsumoto and Shiraishi (1977) as follows. After extraction of OAs with a consecutively connected column series of Amberlite IR-120 cation exchange resin (1.6 cm I. D. × 4 cm) and CG-4B anion exchange resin (1.6 cm I. D. × 2 cm), and washing with about 80 ml 2 N NH_4OH and 40 ml water, the solution was vaccum-evaporated with a rotary evaporator and water bath at 40°C until red color due to added phenolphthalein was just disappeared. The solution was then repassed through a column of IR-120, and its total acidity was determined with titration using 0.1 N NaOH and an indicator of phenolphthalein. A volume of the solution containing 100 mg citrate was again vaccum-evaporated with a rotary evaporator at 60°C; comparing to the data of total acidity, the volume of juice actually taken was later used for the calculation of organic acid content. After the volume decreased to about 5 ml, it was transferred to another flask and again vaccum-evaporated to dryness at 60°C.

Esterification of organic acids in the dried material was conducted as in the methods of Yamashita *et al.* (1973) and Matsumoto and Shiraishi (1977), but without filtration on the esterified solution. As an internal standard, 0.6% n-dodecane (dissolved in n-hexane) was used. Ten µl of the sample was then chromatographed.

Standard preparation for gas chromatography

Table 1. Retention times of organic acid standards?.

Organic acids	Retention time (minute)”
Formate	2.00 ± 0.04
Acetate	2.45 ± 0.02
Propionate	5.15 ± 0.05
Glycolate	8.62 ± 0.04
Butyrate	8.94 ± 0.03
Lactate	9.89 ± 0.07
Oxalate	23.40 ± 0.07
Malonate	25.97 ± 0.07
Succinate	29.76 ± 0.06
Fumarate	30.49 ± 0.02
Glyoxylate	31.35 ± 0.02
Malate	32.19 ± 0.07
Tartarate	35.14 ± 0.17
Cis-aconitate	45.05 ± 0.50
Citrate	46.70 ± 0.07

‘A gas chromatograph Shimadzu GC-8A run under conditions: Primary pressure was 6 kg/cm^2, carrier gas (nitrogen) was 1 kg/cm^2, hydrogen and air pressure were 0.5 kg/cm’. Sample (10 µl) was injected at 55°C for 5 min., then programmed for 4°C/min up to 230°C. Total running time was set for 50 min. 0.6% n-dodecane was the internal standard, its retention time was 17.28 ± 0.07.

“Mean values of standard from 13 injections in 13 different days.
Mean ± S. D.

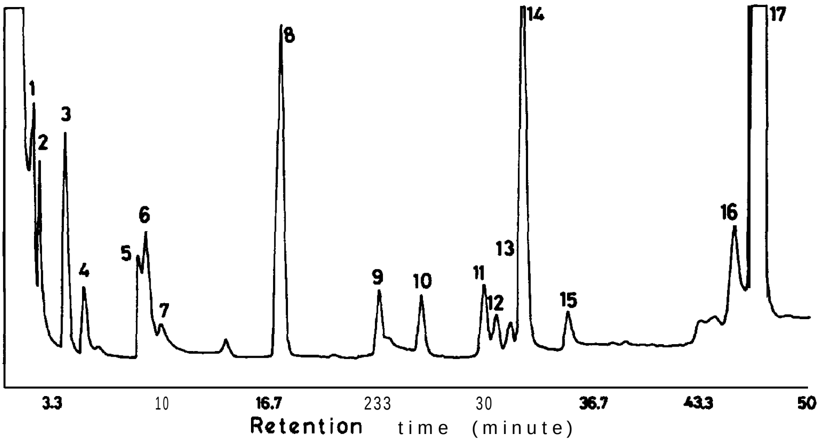

Fig. 1. Gas chromatogram of butyl derivatives of organic acid standard.
1. Formate, 2. Acetate, 3. Butyl ester, 4. Propionate, 5. Glycolate, 6. Butyrate, 7. Lactate, 8. n-dodecane, 9. Oxalate, 10. Malonate, 11. Succinate, 12. Fumarate, 13. Glyoxylate, 14. Malate, 15. Tartarate, 16. cis-Aconitate, 17. Citrate.

Organic acids standards used were shown in Table 1. The preparations were done as in the sample preparation, without titration but neutralization with 0.1 N NaOH. After their peaks were individually identified, they were mixed into a single standard (Fig. 1). One ml of 0.6% n-dodecane was quickly weighed in a stoppered flask and used as an internal standard.

Gas chromutographic determination of organic acids

A gas chromatograph Shimadzu GC-8A equipped with an FID (Flame Ionization Detector) was run under conditions as follows. A glass column (1 m X 3 mm I. D.) filled with 20% Silicone DC 560 on Chromosorb W (AW-DMCS) of 60-80 mesh was used. The primary pressure was 6 kg/cm^2, the carrier gas (nitrogen) pressure was 1 kg/cm”, hydrogen and air pressures were 0.5 kg/cm’. The sample (10 μl) was injected at an initial temperature of 55°C, let it run for 5 minutes, then programmed for 4°C/min, up to a final temperature of 230°C. During our preliminary study, the temperature rate of 6°C/min (see Yamashita et al., 1973; Matsumoto and Shiraishi, 1977) seemed to be too fast especially for those present not in a single, separate peak or close to each other such as succinate, fumarate, glyoxylate and cis-aconitate. Therefore, 4°C/min was operated. In addition, the rate produced a rather smooth base line (Fig. 1), especially that after tartarate, which likely gave a more accurate quatification. The total running time was set for 50 minutes.

The eluting compounds were detected based on their retention times and plotted with a Shimadzu C-R3A Chromatopac set to the speed chart of 6 mm/min and the detected minimum area of 10. The organic acid concentrations were automatically calculated by a build-in program available based on the actual weight of n-dodecane as an internal standard, and divided by the actual volume of juice used after the titration. The results were expressed as me/100 ml.

RESULTS AND DISCUSSION

In a gas chromatographical determination of organic acids, simplicity and time consumed for extraction and isolation become a real concern even though the method offers more organic acids to be detected, especially those present in traces but possessing physiological importance. In the present method, by a direct extraction of the juice, changing temperature from 40°C to 60°C during evaporation, and without filtration after esterification (Matsumoto and Shiraishi, 1977; Yamashita et al., 1973), time consumed seemed likely to much decrease and it was more simple.

Table 1 shows the retention times of the organic acids. The retention time of each organic acid, except that of cis-aconitate, varied only slightly. When the standard was being developed, without the introduction of citrate, the peak of isocitrate actually could be detected, appeared after the peak of cis-aconitate (Fig. 2), came very slightly after the peak of citrate. Under a gas liquid chromatographic determination, Sasson and Monselise (1977) also detected the presence of isocitrate after the peaks of aconitate and citrate. However, it was then covered by the peak of 80 mg of citrate. As the amount of citrate used is believed to fall within the range of citrate found in acid citrus, and isocitrate usually presents in a trace, under the condition of the analysis, the present of isocitrate's peak was disregarded.

The organic acid compositions and contents of the acid citrus on two sampling years of 1992 and 1993 are presented in Table 2. Of 15 organic acids used in the standard, 12 organic acids were detected to be present in the acid citrus studied in which its composition and content, varied greatly according to sampling years and species. Citrate and malate were predominant, accounting for more than 90% and 3-9% of the total detected acids, respectively. Daidai had significantly less malate than the other citrus. The other acids presented in traces, accounting totally for roughly less than 0.5%, except in Kabosu of 1993. Of minor acids detected, butyrate, oxalate, malonate, succinate, fumarate, tartarate and cis-aconitate were detected to be present in all acid citrus studied (Table 2). Glyoxylate was not detected in Daidai. Acetate was detected only in Kabosu

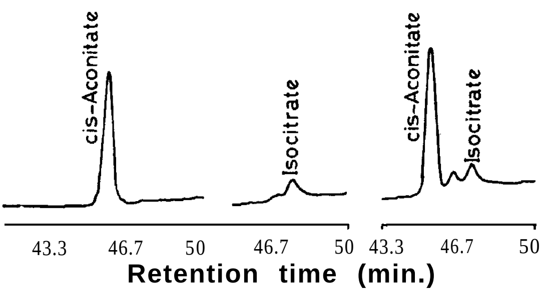

Fig. 2. Gas chromatograms of cis-aconitate and isocitrate standards.

Table 2. Organic acid composition and contents in the juice of Hanayu, Daidai, Kabosu, 'Lisbon' lemon, and Yuzu, 1992 and 1993'.

Organic acid	Hanayu		Daidai		Kabosu		Lisbon		Yuzu	
	1992	1993	1992	1993	1992	1993	1992	1993	1992	1993
	me/100 ml		me/100 ml		me/100 ml		me/l 00 ml		me/100 ml	
Formate	-	-	-							
Acetate						0.0441		**0.0396**		
Glycolate	-			0.0849						0.0192
Butyrate	0.0518	0.0804	0.0769	0.0760	0.0406	0.0774	0.0538	0.1008	0.0493	0.0763
Oxalate	0.0399	0.1423	0.1163	0.1117	0.0480	0.0934	0.0581	0.1703	0.0388	0.1884
Malonate	0.0071	0.0202	0.0089	0.0340	0.0099	0.0452	0.0065	0.0086	0.0105	0.0203
Succinate	0.0359	0.1971	-	0.0500	0.0257	0.0543	0.0135	0.0500	0.0251	0.0640
Fumarate	0.0262		0.4273	0.0203	0.0324		0.0193	0.0183	0.0255	
Glyoxylate	0.0100	-			0.0303		0.0208	-	0.0221	
Malate	4.5765	10.0549	2.9458	**3.8089**	4.6311	7.4170	4.4018	9.3923	4.9843	6.1846
Tartarate	0.0145	-		0.0352	0.0045	0.6895		0.0522	0.0056	0.0436
Cis-aconitate	0.0006		0.0027	0.0099	0.0003	0.0909	0.0003	0.0003	0.0007	0.0015
Citrate	57.8685	101.7758	109.8974	100.3118	46.4069	85.1667	93.2349	126.5177	62.3871	115.9711
Total	62.6265	112.2707	113.4753	104.5427	51.2297	93.6785	67.8090	136.3501	67.5490	122.5740
Minors(%)'	0.30	0.39	0.56	0.40	0.37	1.17	0.25	0.32	0.26	0.34
Malate(%)	7.31	8.96	2.60	3.64	9.04	7.92	6.49	6.89	7.38	5.05
Citrate(%)	92.39	90.65	96.84	95.96	90.59	90.91	93.26	92.79	92.36	94.61

'A gas chromatograph Shimadzu GC-8A run under conditions as mentioned in Table 1

Sum of organic acids other than malate and citrate, as percent of total.

and Yuzu and glycolate was only in Daidai and Yuzu.

Except in Daidai, the organic acid content of 1992 was significantly lower than that of 1993. The total organic acid content was found to be as low as 51.23 me/100 ml in Kabosu of 1992 and as high as 136.35 me/100 ml in Lisbon of 1993. Working with a method of silica gel chromatography, Osajima et *al.* (1971) detected acetate, formate, oxalate, glycolate, malate, citrate, peaks consisted of pyruvate and fumarate, of succinate and lactate, and of *α* -ketoglutarate, aconitate and malonate to be present in the juice of Yuzu and Daidai. Yamaki (1989) employing the same method as used by Osajima et al. (1971) found only oxalate, malate, citrate, peaks consisted of lactate, succinate, -ketoglutarate, and of glutarate, fumarate and formate in 'Lisbon' lemon, Yuzu, Hanayu and Kabosu. He found also acetate in 'Lisbon' and Yuzu. The total content detected by Osajima *et al.* (1971) and Yamaki (1989) fall quite well within the range of our data of 1992-1993. The citrate and malate contents, precipitated by lead acetate, of 'Lisbon' lemon obtained by Sinclair and Eny (1945) also fall within our data of 1992-1993, whereas those of 'Eureka' lemon detected by Clements (1964) were lower than our 'Lisbon'.

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