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https://doi.org/10.5109/24090

出版情報：九州大学大学院農学研究院紀要. 40 (1/2), pp.29-37, 1995-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 40 (1・2), 29-37 (1995)

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

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(Received June 15, 1995)

Whereas the flavedo and albedo of Hanayu (*Citrus hanaju* Hort. ex Shirai), Daidai (C. *aurantium* Linn. var. Cyathifera Y. Tanaka), 'Lisbon' lemon (C. *limon* Burm. f. Lisbon), and the albedo of Kabosu (*C. sphaerocarpa* Hort. ex Tanaka) and Yuzu (C. *junos* Sieb. ex Tanaka) contained a very low concentration of citrate, citrate was found to significantly predominate in the flavedo of Kabosu (79.96 µme/g or 65.37% of total) and Yuzu (44.26 µme/g or 59.72% of total). Except in the flavedo of Kabosu and Yuzu, malate was the predominating acid in flavedo of Hanayu, Daidai and Lisbon. Organic acids other than malate and citrate found in appreciable amounts were tartarate, oxalate and malonate, followed by acetate and butyrate. Succinate, fumarate and glyoxylate were found in much lesser amounts. Comparing to citrate and malate, other acids both in total and individual contents clearly should not be considered as traces. An unidentified organic acid eluted at about 39.76 minutes was consistently detected in both flavedo and albedo, being the highest in the flavedo of Hanayu (64.60% of the internal standard's peak area) and the lowest in the albedo of Kabosu (0.97%)

INTRODUCTION

The most significant advantage of gas chromatographic (GC) methods over liquid chromatographic (LC) methods such as high performance liquid chromatography (HPLC) in determining organic acids (OAs) in citrus extracts is that GC can detect both volatile and non-volatile OAs present in low concentrations. Its high sensitivity is needed because OAs are present in a much lower concentration in citrus peel sap than in citrus juice.

Reports on OA composition and content in the juice of the so-called table citrus are accumulated (Clements, 1964a and b; Monselise and Galily, 1979; Rasmussen, 1964; Sasson and Monselise, 1977; Shiraishi *et al.*, 1991a and b). Whereas those dealing with acid citrus are fewer (Clements, 1964a; Di Giacomo and Calvarano, 1972 in Sinclair, 1984; Sinclair and Eny, 1945 and 1947), none has reported the OA composition and content in the flavedo and albedo of Japanese acid citrus although they are grown and, in addition to acid lemon (C. *limon*), consumed for particular purposes in Japan. The present lack of information on the OA composition and content in the peel of Japanese acid citrus is very unfortunate because upon squeezing or slicing for dishes, OAs are also squeezed out together with essential oils. Differences in the OA composition and content in the peel saps may then affect flavor.

The present paper reports a gas chromatographic determination on organic acid composition and content in the flavedo and albedo of acid lemon and some Japanese acid citrus.

MATERIALS AND METHODS

The fruits used were those of Hanayu *(Citrus hanaju),* Daidai (C. *aurantium),* Kabosu (C. *sphaerocarpa),* 'Lisbon' lemon (C. *limon),* and Yuzu (C. *junos).* Flavedo and albedo of 10 fruits sampled in 1993 were separately peeled and chopped. As many as 50 g (fresh weight) of each was added with a little water enough to get a slurry homogenate after homogenizing. The water used was distilled-deionized one. The homogenate was centrifuged (2,500 rpm for 20 minutes) and the supernatant was brought to a given volume in a volumetric flask by adding water.

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. Ten ml of the supernatant was pipetted, passed through a consecutively connected column series of Amberlite IR-120 cation exchange resin (1.6 cm I. D. x 4 cm) and CG-4B anion exchange resin (1.6 cm I. D. X 2 cm) at about 2 ml/min, and washed well with water. The water used was destilled-deionized one. Organic acids trapped in the CG-4B was then washed with about 80 ml 2 N NH_4OH and about 40 ml water. One drop of phenolphthalein was added into the collected solution. The solution was vaccum-evaporated with a rotary evaporator and water bath at 40°C until red color was just disappeared. The solution was repassed through the same column of IR-120, washed well with water untill about 200 ml solution was obtained (by doing so, the acids remaining in the column were practically negligible. Therefore, it was valid to assume that the acid content in the obtained solution was the same as in the original solution. This was important as the peel weight contained in the 10 ml of the original was later used for the calculation of organic acid content). The obtained solution was then neutralized with 0.1 N NaOH, and again vaccum-evaporated with a rotary evaporator at 60°C. After the volume decreased to about 5 ml, it was transfered to another flask and again vaccum-evaporated to dryness at 60°C.

Two ml of butanol, 0.1 ml of cone. H_2SO_4 and about 0.5 g of anhydrous Na_2SO_4 were added to the dried material, and then the mixture was mild-heated for 30 min on a mantle heater. After esterification, about 5 ml of n-hexane was added, shaken well, and a small amount of water was added. The n-hexane layer was transferred into a 25-ml volumetric flask containing 1 ml of 0.6% n-dodecane (dissolved in n-hexane) as an internal standard and the contents were made to the volume by repeating the isolation with n-hexane. About 0.5 g of anhydrous Na_2CO_3 was added to this n-hexane solution, shaken well, and the solution was transferred to a sample tube. Ten µl of the sample was then chromatographed.

Standard preparation for gas chromatography

Organic acid standards used were as shown in Table 1. The preparations were done as in the sample preparation. After their peaks were individually identified, they were mixed into a single standard (Fig. 1). As an internal standard, 1 ml of 0.6% n-dodecane was quickly weighed in a stoppered flask. All transferring and preparation steps after the introduction of n-dodecane were done as quickly and carefully as possible to minimize losses.

Table 1. Retention times of organic acid standards[a].

Organic acids	Retention time (minute)[b]
Acetate	1.94 ± 0.04
Propionate	3.96 ± 1.10
Butyrate	7.57 ± 0.13
Lactate	8.64 ± 0.16
Oxalate	22.46 ± 0.01
Malonate	25.07 ± 0.02
Succinate	28.87 ± 0.01
Fumarate	29.59 ± 0.01
Glyoxylate	30.42 ± 0.02
Malate	31.26 ± 0.03
Tartarate	34.26 ± 0.10
Citrate	45.70 ± 0.05

[a] 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^2. Sample (10 µl) was injected at 60°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 16.20 ± 0.02.

[b] Mean values and standard deviations of standard from 4 injections.

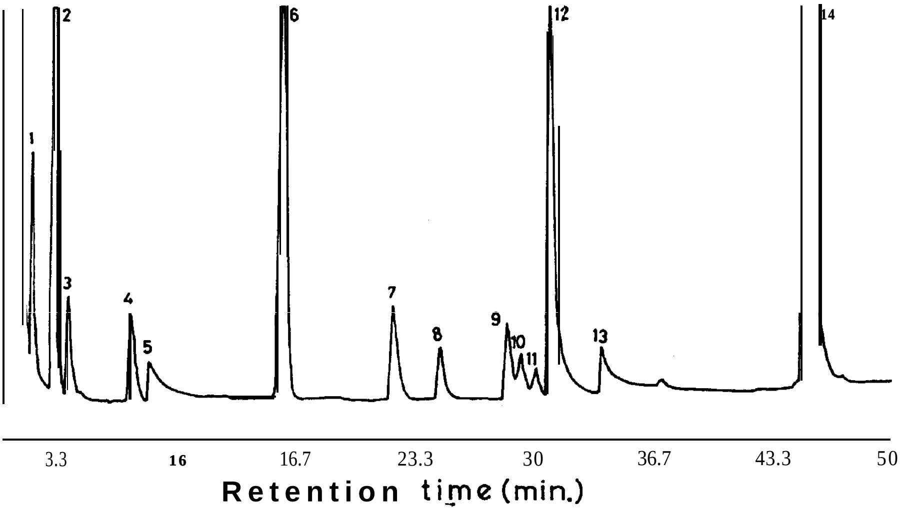

Fig. 1. Gas chromatogram of butyl derivatives of organic acid standards. 1. Acetate, 2. Butyl ester, 3 Propionate, 4. Butyrate, 5. Lactate, 6. n-dodecane, 7. Oxalate, 8. Malonate, 9. Succinate, 10. Fumarate, 11. Glyoxylate, 12. Malate, 13. Tartarate, 14. Citrate.

Gas chromatographic 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’, 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 60°C, let it run for 5 minutes, then programmed for 4°C/min, up to a final temperature of 230°C.

The total running time was set for 50 minutes. The FID control was set to the range of 10^{2} and the attenuation of 16 (or 4 mV/full scale). 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 peel weight contained in the 10 ml of the original supernatant. The results were expressed as µme/g fresh weight.

RESULTS

During analysis in late Spring of 1994, in an unair-conditioned room when the outside air temperature was about 34°C, an initial temperature of 55°C was unable to be set. Therefore, it was set to 60°C instead. Such a high initial temperature resulted in decreasing capability for detecting peaks eluted close each other such as formate and glycolate.

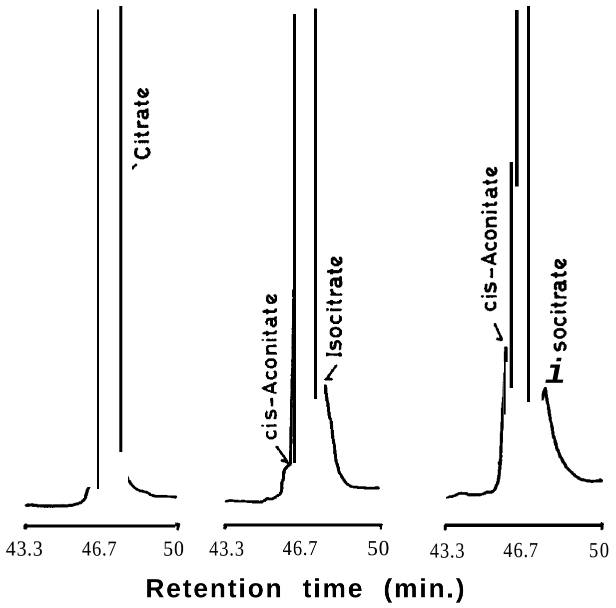

Fig. 2. Gas chromatograms of cis-aconitate, citrate and isocitrate standards.

Table 2. Organic acid composition and contents[a] in the flavedo and albedo of Hanayu, Daidai, Kabosu, 'Lisbon' lemon and Yuzu.

Organic	Hanayu		Daidai		Kabosu		Lisbon		Yuzu	
acid	Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo	Flavedo	Albedo
	(μme/g fresh weight)		(μme/g fresh weight)		(μme/g fresh weight)		(μme/g fresh weight)		(μme/g fresh weight)	
Acetate	**1.72**	**0.64**	**02.02**	**1.89**	**0.74**	**0.03**	**1.41**	**2.01**	**0.69**	**1.20**
Butyrate	0.86	0.70	1.26	0.74	0.82	0.77	0.78	0.74	0.81	1.45
Lactate					0.02	-	-	-	0.007	-
Oxalate	3.48	1.10	5.90	4.38	3.00	0.58	2.46	3.64	4.04	1.74
Malonate	0.19	1.01	3.93	1.04	3.84	1.01	4.56	1.01	7.69	1.44
Succinate	0.23	-	0.22	0.04	0.58	-	0.13	-	0.43	-
Fumarate	0.75	0.34	0.23	0.11	0.23	-	0.17	0.21	0.15	0.05
Glyoxylate			1.13	0.38	0.72		0.43	0.63	0.72	-
Malate	8.83	6.62	16.84	0.23	25.90	0.52	12.43	0.25	7.73	0.84
Tartarate	4.73	1.31	4.54	2.99	6.54	2.33	11.56	1.01	7.58	1.23
Unidentified[b]	(64.60%)	(13.91%)	(1.66%)	(2.48%)	(10.81%)	(0.97%)	(3.32%)	(10.74%)	(15.60%)	(5.95%)
Citrate	4.38	1.30	2.28	0.90	79.96	1.42	0.78	0.92	44.26	4.36
Total	25.17	13.02	38.35	12.70	122.33	6.66	34.71	10.42	74.11	12.31
Others*	47.52	39.17	50.14	91.10	13.46	70.87	61.94	88.77	29.85	57.76
Malate[c]	35.08	50.85	43.91	1.81	21.17	7.81	35.81	2.40	10.43	6.82
Citrate[c]	17.40	9.98	6.39	7.09	65.37	21.32	2.25	8.83	59.72	35.42

[a] A gas chromatograph Shimadzu GC-8A run under conditions as mentioned in Table 1.
[b] A peak eluted at a retention time of about 39.76 minutes, as per cent of peak area of the internal standard. [c] As per cent of total detected organic acids.

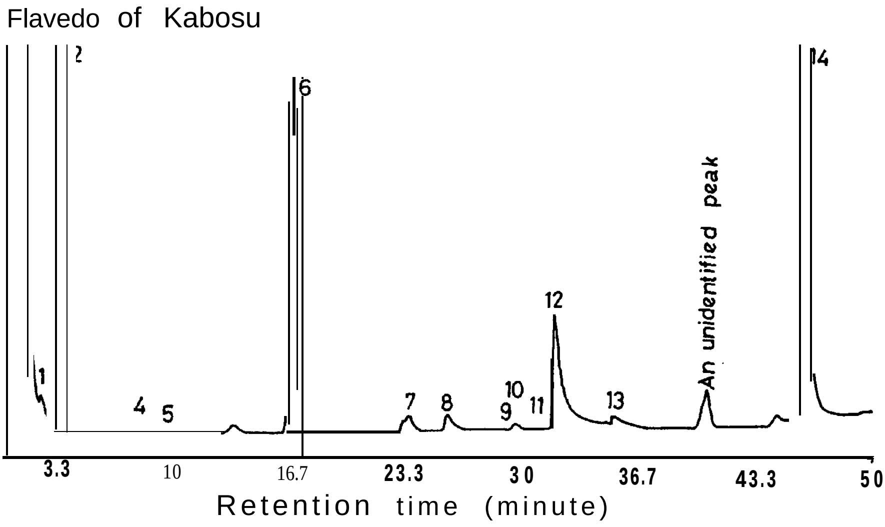

Fig. 3. Gas chromatogram of organic acids in flavedo of Kabosu. Numbers of peaks are as shown in Fig. 1.

Therefore, they were excluded from the standard. Still, with such a high initial temperature, most of organic acid standards were able to be eluted (Fig. 1) with their retention times shown in Table 1. Cis-aconitate and isocitrate, due to too close to citrate (Fig. 2), were also excluded.

Table 2 shows organic acids detected in flavedo and albedo of the acid citrus. Of 12 organic acids, acetate, butyrate, oxalate, malonate, succinate, fumarate, malate, tartarate and citrate were all detected in the flavedo of the acid citrus studied. Lactate was detected in a trace only in the flavedo of Kabosu and Yuzu, no glyoxylate was detected in both flavedo and albedo of Hanayu, and propionate was never found in both flavedo and albedo of the citrus studied. Flavedo contained more organic acids than albedo.

The content of individual organic acid, however, varied. Among acid citrus studied, citrate was found to be a predominating organic acid only in the flavedo of Kabosu (79.96 µme/g or 65.37% of total, Table 2 and Fig. 3) and Yuzu (44.26 µme/g or 59.72% of total). Except in Kabosu and Yuzu, malate predominated in the flavedo. Comparing to citrate and malate, other acids both in total and individual contents clearly should not be considered as traces. Total content of organic acid of flavedo was higher than that of albedo. An unidentified organic acid eluted at about 39.76 minutes was consistently detected in both flavedo and albedo, being the highest in the flavedo of Hanayu (64.60% of the internal standard's peak area) and the lowest in the albedo of Kabosu (0.97%) (Table 2 and Fig. 4).

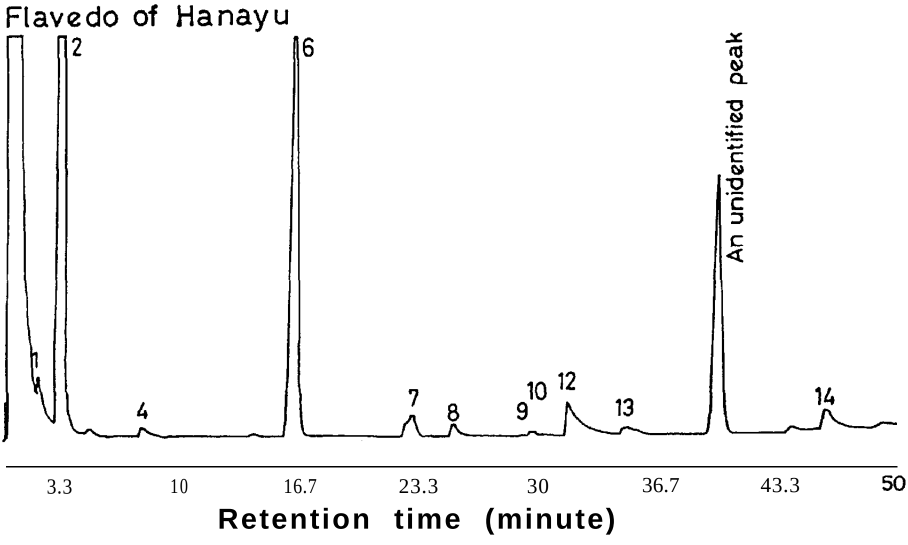

Fig. 4. Gas chromatogram of organic acids in flavedo of Hanayu. Numbers of peaks are as shown in Fig. 1.

DISCUSSION

Our data show that flavedo generally contained more organic acids both in composition and concentration. The total organic acid content in the flavedo ranged from as low as 25.17 µme/g FW in Hanayu and as high as 122.33 µme/g FW in Kabosu. The total acid content obtained in this study was much lower than total acidity of Yamaki (1989) determined by titration. However, comparison between data obtained by GC and by titration should be done with undertanding that the two methods employed different conditions. Firstly, titratable acidity is calculated from the quantity of base to reach the pH decided for the end point of the assay. Under this condition, organic acids and a part of phosphoric acid and phenols are involved in the final result (Ulrich, 1970). Secondly, comparing to citrus juice, peel of citrus fruits contains a comparatively smaller amount of organic acids and their salts. This system consequently makes citrus peel sap not highly buffered (Sinclair and Eny, 1947). As a result, dilution on the peel extract will affect considerably to the results obtained by titration, especially in determining total acidity. Therefore, total acidity obtained by titration is frequently much higher than that obtained by chomatographic methods (see Yamaki, 1989; and Sinclair and Eny, 1947). Moreover, differences in cultural practices and climatic condition are ones of many factors contributing to variations. They all have to be considered.

Except in Palestine sweet lime in which malate predominates in the juice (Clements, 1964a), citrus juice is characterized by a predominance of citrate, and acids other than citrate and malate present in extremely low concentrations. Citrus peel, however, behaves differently. Citrate is usually found in a low concentration in the peel of wide citrus species and varieties (Clements, 1964a and b; Rasmussen, 1964; Sinclair and Eny, 1945 and 1947; Sasson and Monselise, 1977; Monselise and Galily, 1979; Di Giacomo and Calvarano, 1972 in Clements, 1984; Shiraishi et al., 1991a and b). In the case of citrate in peel, in the present study, whereas the flavedo and albedo of Hanayu, Daidai, ‘Lisbon’ lemon, and the albedo of Kabosu and Yuzu followed a typical phenomena of other citrus, citrate was found to significantly predominate in the flavedo of Kabosu (65.37% of total detected acids, Table 2 and Fig. 3) and Yuzu (59.72%) (Table 2). Unfortunately, whereas the phenomena of organic acid accumulation in citrus vesicles has been studied by Bogin and Wallace (1966) by comparing sweet and sour lemon fruits, there is no study directed toward understanding the organic accumulation in citrus peel. Hence, the citric acid accumulation found in the flavedo of Kabosu and Yuzu awaits further explanation.

Except in the flavedo of Kabosu and Yuzu, malate was the predominating acid in that of Hanayu, Daidai and Lisbon (Table 2). In the present study, organic acids other than malate and citrate found in appreciable amounts were tartarate, oxalate and malonate, followed by acetate and butyrate. Succinate, fumarate and glyoxylate were found in much lesser amounts. The variabilities might partly due to variations in the degree of maturity as also noted in grapefruit by Monselise and Galily (1979). Comparison between flavedo and albedo acids to other published reports was unable to be done due to lack of information on acid citrus other than acid lemon, differences in methods employed and, even if data of some organic acids are available, no discrimination between flavedo and albedo (Sinclair and Eny, 1945 and 1947; Di Giacomo and Calvarano, 1972 in Sinclair, 1984). Our data of total organic acid content in the flavedo of some Japanese acid citrus investigated, however, fall within the range of as low total organic acid (as a total of citrate, malate, and oxalate) as 36 µme/g in the peel of lemon and as high as 81 µme/g in the peel of ‘Navel’ orange (Sinclair and Eny, 1947).

The exceptional phenomena of citric acid accumulation in the flavedo of Kabosu and Yuzu we observed is both physiologically and nutritionally interesting. Up to the present knowledge of the accumulation of citric acid in citrus, there are numerous evidence that citrus fruit is capable of synthesizing citric acid (Sinclair, 1984). Moreover, Bean and Todd (1960) have indicated that each tissue of citrus fruit is individually capable of fixing a significant amount of activity through dark reactions with CO_2. The results we found indicated that an exceptionally high percentage of citric acid in the flavedo of Kabosu and Yuzu, different from the common accumulation of citric acid in the peel of various citrus species (Sinclair, 1984), further supported the present knowledge of citric acid accumulation; the accumulation is not a simple matter of translocation from one tissue to another. Citric acid in lemon fruit is believed to be formed from reducing sugar and pentosans by enzymatic reaction (Sinclair, 1984). In addition, various aromatic and other natural products can be synthesized from reducing sugar through the formation of quinic and shikimic acids as intermediates (Sprinson, 1960). In other words, the biosyntheses of citric acid and aromatic products in the flavedo may compete for the same substrate. Moreover, there is a synergistic effect between α-tocopherol and ascorbic acid or citric acid as antioxidants (Sinclair, 1984). In studying the effect of SO_2 on orange oil oxidation, Beard et *al. (1972)* found that the presence of citric acid reduced the rate of oxygen absorption by orange oil. Therefore, differentiation in the pattern of citrate accumulation we observed is noteworthy as it will directly affect taste and finally flavor (Pfaffmann, 1958). By further studying seasonal changes in acid accumulation in flavedo, a confirmation may be possible whether high citrate concentrations found in the flavedo of Kabosu and Yuzu are just fluctuations in only a given moment or reflections from a different pattern of their acid accumulation.

An elution of an unidentified acid at a retention time of about 39.76 minutes should recieve attention as it presented in an appreciable amount in the flavedo of Hanayu (Table 2 and Fig. 4). It was very unlikely to be α-ketoglutaric, pyruvic, shikimic or quinic acids as they were unable to be eluted (Matsumoto and Shiraishi, 1977).

ACKNOWLEDGMENT

The authors wish to thank Dr. Akira Wakana of the Fruit Trees Experiment Station of Kyushu University, Sasaguri, Fukuoka for the fruits used in this study.

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