AcademicPres

Available online at www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not. Bot. Hort. Agrobot. Cluj 38 (1) 2010, 44-48

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

HPLC Organic Acid Analysis in Different Citrus Juices under Reversed Phase Conditions

Violeta NOUR[1)], Ion TRANDAFIR[2)], Mira Elena IONICA[1)]

[1)] *University of Craiova, Faculty of Horticulture, 13 A. I. Cuza Str., Craiova, Dolj, Romania; vionour@central.ucv.ro*

[2)] *University of Craiova, Faculty of Chemistry, 107 Calea Bucuresti Str., Craiova, Dolj, Romania; nelutrandafir@yahoo.com*

Abstract

A reversed phase HPLC method for separation and quantification of organic acids (oxalic, citric, tartaric, malic, ascorbic and lactic acids) in fruit juices was developed. The chromatographic separation was performed with a Surveyor Thermo Electron system at 10°C by using a potassium dihydrogen orthophosphate buffer (pH 2.8) as mobile phase, an Hypersil Gold aQ Analytical Column and diode array detection at λ=254 nm for ascorbic acid and λ=214 nm for the other organic acids. Organic acid profiles of ten species of *Citrus*: sweet orange, minneola, clementine, mandarin orange, pomelo, lemon, lime, sweetie, white and pink grapefruit were established. Species significantly affect the organic acid distribution of citrus fruit juices. In all citrus juices, the most abundant organic acid was citric acid, ranging from 6.88 to 73.93 g/l. Citrus juices are good sources of ascorbic acid (0.215-0.718 g/l). The average ascorbic acid was the highest in lemon juice followed by sweet orange juice, sweetie and white grapefruit.

Keywords: organic acids, simultaneous RP HPLC, diode array detection, citrus species

Introduction

The identification and quantitative analysis of major organic acids in fruits is considered very important for food and beverage technology and quality evaluation (Hasib *et al.*, 2002). Organic acids are a useful index of authenticity in fruit products, since they have lower susceptibility to change during processing and storage than other components of fruits (Camara *et al.*, 1994). Accurate knowledge of organic acid levels (and ratios) might be useful for determining the percentage juice and also for detecting misbranding and/or adulteration in fruit juices, since each fruit has a unique pattern of organic acids. The organic acid composition of fruits is also of interest due to its impact on the sensory properties. Even though they are minor components, in combination with sugars, they are important attributes of the sensorial quality of raw and processed fruits.

Citrus fruits, one of the important fruit crop groups, are consumed mostly as fresh or as juice because of their nutritional value and special flavor. Consumption of citrus juice is found to be beneficial in preventing coronary diseases and chronic asthma (Abd-Ghafar *et al.*, 2010). Citrus fruit extracts are also found to have antioxidant, anti-inflammatory, anti-tumor, anti-fungal and blood clot inhibition activities (Abeysinghe *et al.*, 2007). These health benefits of citrus fruit have mainly been attributed to the presence of bioactive compounds, such as ferrulic acid, hydrocinnamic acid, cyanidin glucoside, hisperidine, vitamin C, carotenoid and naringin content (Abeysinghe *et al.*, 2007; Xu *et al.*, 2008).

Citrus fruits are classified as acid fruits, since their soluble solids are composed mainly of organic acids and sugars, which are used as the main index of maturity and one of the major analytical measures of flavor quality. The main acids of citrus fruits are citric and malic acids with trace amounts of tartaric, benzoic, oxalic and succinic acids reported (Karadeniz, 2004).

Organic acid accumulation in the vacuole of cells of citrus fruits is a developmentally regulated process, the degree and timing of which varies greatly among species and varieties and is highly susceptible to agroclimate (Canel *et al.*, 1995).

Besides analytical methods involving colorimetric reaction and enzyme assay, chromatographic techniques allow simultaneous analysis of most of the organic acids. In this field, high performance liquid chromatography (HPLC) is one of more promising and more used techniques, either by direct determination or by the analysis of derivatized products. Most of procedures developed until now for food and beverage analysis utilize either reverse phase partition chromatography (Czajkowska and Jaroniec, 1997; Fransson and Ragnarsson, 1998) or ion exchange chromatography (Casella and Gatta, 2001; Guillén *et al.*, 1998; Linget *et al.*, 1998) with a refractive index (RI), UV spectrophotometric, conductimetric or electrochemical detection (Buchberger, 2000).

The separation of organic acids with liquid chromatography and their quantitative determinations are extremely

difficult because there is no difference between their structural similarities and spectral characteristics. Besides, pKa values of most of the organic acids are rather similar and this situation limits the usage of pH for chromatographic separation (Aktas *et al.*, 2005).

The present study was designed, on one side, to determine the organic acid distribution of citrus juices prepared from different species of citrus fruits: sweet orange, minneola, clementine, mandarin orange, pomelo, lemon, lime, sweetie, white and pink grapefruit and on the other side, to separate, identify and quantify major organic acids in natural citrus juices using HPLC with photodiode array detection (DAD), which will identify compounds not only with their retention times but also with their individual spectra.

Materials and methods

Fresh fruits of sweet orange (*C. sinensis*), minneola (*C. tangelo*), clementine (*C. clementina*), mandarin orange *(C. reticulata),* pomelo *(C. maxima),* lemon *(C. limonum),* lime *(C. aurantifolia*) and grapefruit (*C. paradisi*) of sweetie, white and pink hue varieties of commercial ripened stages were purchased from the local markets. Healthy fruits were selected randomly for uniformity of shape and color.

Preparation of juice sample

The citrus fruit juice was extracted by cutting the fruit in half and careful hand-squeezing to obtain the juice. The juice was passed through a strainer to remove pulp and seeds. The freshly squeezed juice was centrifuged at 3000 g for 10 min and the supernatant was diluted 1:50 for citric acid determination and 1:5 for the other acids. The dilutions were membrane filtered (0.45 μm) before injection. Two samples were analyzed in duplicate.

Chemicals

Citric acid, malic acid, tartaric acid, oxalic acid, ascorbic acid and lactic acid were purchased from Merck and Sigma-Aldrich. Acetonitrile, potassium dihydrogen orthophosphate and phosphoric acid were of analytical purity or for chromatographic use. The water used was ultrapure, Basic TWF.

Organic acid standards

A mixed standard stock solution was prepared containing 1000 mg/l citric acid, 2000 mg/l malic acid, 300 mg/l oxalic and ascorbic acid respectively, 700 mg/l tartaric acid and 400 mg/l lactic acid. The stock solution and the corresponding dilutions was made in ultrapure water and stored in dark places between the experiments, at low temperature (+4°C).

Analysis by HPLC

The organic acids in the sample test solution were separated by reversed phase chromatography on a 250 mm×4.6 mm i.d., 5 μm particle Hypersil Gold aQ Analytical Column, of which were detected by absorbance and quantified with external calibration graphs. For the simultaneous detection of the six analytes, the detector was set at λ=254 nm for ascorbic acid and λ=214 nm for the other organic acids. This setting was chosen since ascorbic acid has its maximum optical absorbance close to 254 nm.

The HPLC analysis was performed with a Surveyor Thermo Electron system comprising a vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP autosampler and diode array detector with 5 cm flow cell. Integration, data storage and processing were performed by Chrom Quest 4.2 software.

The determinations were made in isocratic conditions, at 10°C, using a mobile phase made of 50 mM phosphate solution (dissolve 6.8 g potassium dihydrogen phosphate in 900 ml water; the pH value should be adjusted to pH =2.8 with phosphoric acid and then filled to 1000 ml with water) filtered through a polyamide membrane (0.2 μm) and degassed in a vacuum. The flow rate of the mobile phase was 0.7 ml/min for all the chromatographic separations. The separation column was balanced with mobile phase until the baseline was stabilized. Sample injections were made at this point. The volume injected was 5 μl for either prepared sample or standard solution.

Results and discussion

In Fig. 1 the separation of the six organic acids in the standard mixture solution was given.

Under the applied method, the optimization of chromatographic conditions (i.e., the pH and flow rate of the

Tab. 1. Retention times, concentration ranges of lineal response, equations of the calibration graphs and correlation coefficients for standard organic acids

Organic acids	λ, nm	RT (min)	Response factor	Concentration range (mg/l)	Correlation coeffiicient r^2	Detection limit (mg/l)
Oxalic acid	214	4.122	7.6732 e-005	0.2-300	0.9998	0.1
Tartaric acid	214	4.834	3.2152 e-004	1-700	0.9993	0.5
Malic acid	214	6.186	6.6382 e-004	20-2000	0.9992	10
Lactic acid	214	6.890	1.2860 e-003	40-400	0.9992	20
Ascorbic acid	254	7.353	1.2981 e-005	1-300	0.9990	0.5
Citric acid	214	12.538	5.5751 e-004	16-1000	0.9998	8.0

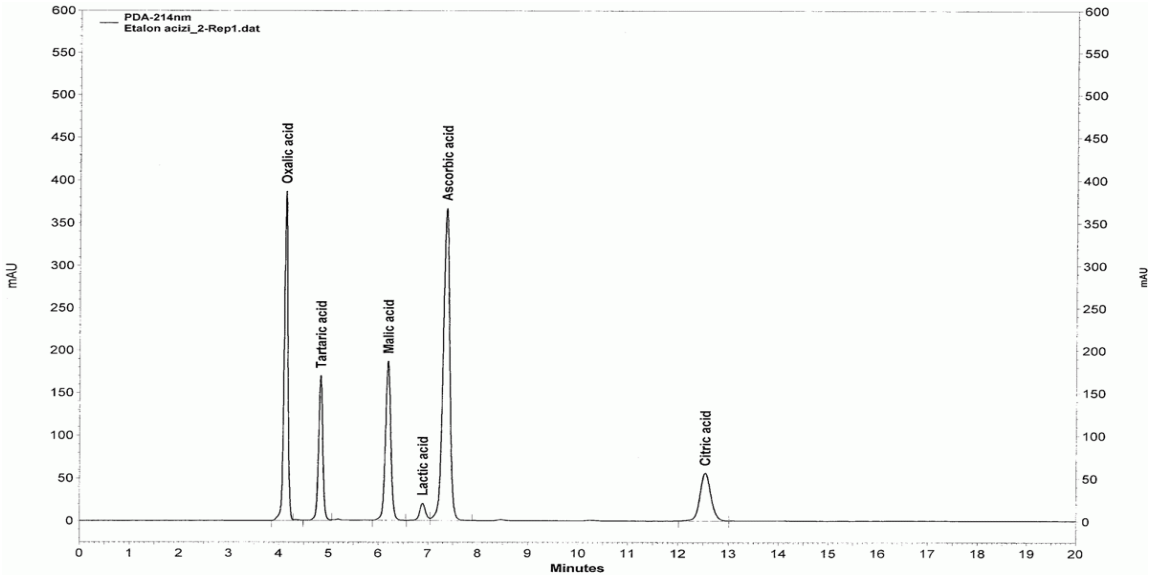

Fig. 1. Chromatogram of the standard mixture of oxalic, tartaric, malic, lactic, ascorbic and citric acids

mobile phase, the absorption wavelength and temperature of column) that affect the separation degree and peak shape of organic acids was obtained.

The linearity of the method was evaluated according to area response. Selected wavelengths for the analytes, detection, retention times, concentration ranges of linear response, response factor and correlation coefficients for standard organic acids were summarised in Tab. 1. Although the response factor varied within acids, the calibration graphs were linear over these concentration ranges with correlation coefficients higher than 0.999. Therefore the external standard method was used for the quantitative analysis.

The reproducibility of test peak area and retention time was tested with the help of Chrom Quest software, which calculated the relative standard deviations (RSD) for the retention time of the analytes for all levels of the calibration graphs and for a peak area at each calibration level. Precision of areas was <2% RSD while precision of retention times was <0.5% RSD.

The relative standard deviations (RSD) for the retention time were between 0.047% for oxalic acid and 0.209% for citric acid therefore, in standard solutions, the developed HPLC method provided stable retention times. The RSD values for peak areas were between 0.028% and 1.530% indicating the stability of the method in terms of peak area.

The detection limit (LOD) could be defined as the smallest peak detected with a signal height three times that of the baseline, while the limit of quantification (LOQ) referred to the lowest level of analyte which could be determined with an acceptable degree of confidence. In the

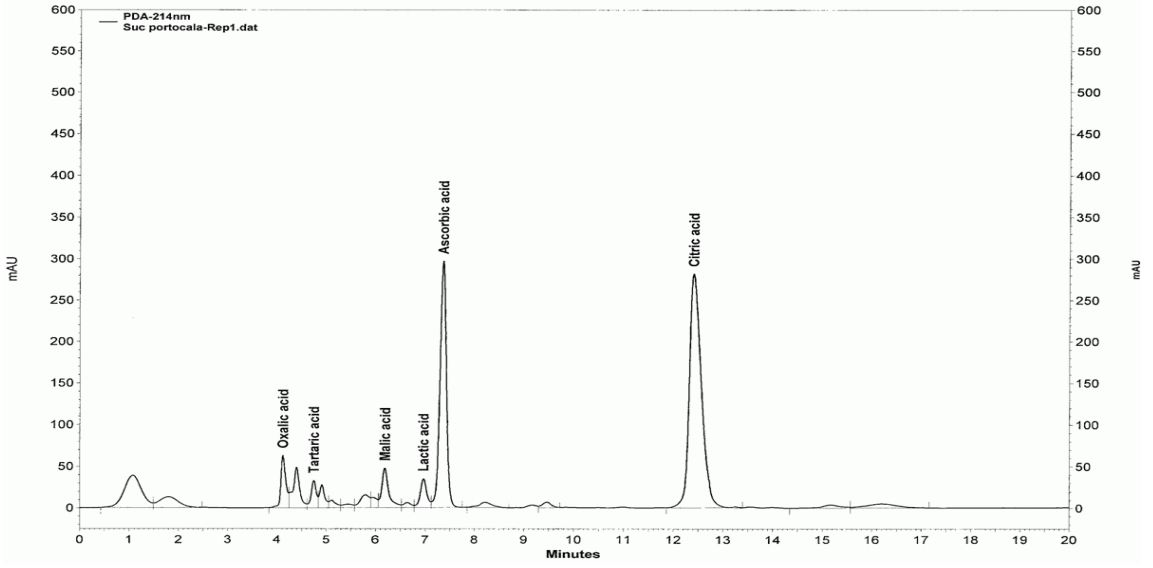

Fig. 2. Chromatogram of sweet orange juice

Tab. 2. Organic acid contents of citrus fruits juices

Species	Oxalic (g/l)	Tartaric (g/l)	Malic (g/l)	Lactic (g/l)	Citric (g/l)	Ascorbic (g/l)
Sweet orange	0.109	0.336	1.516	1.857	13.918	0.636
Minneola	0.156	0.376	1.564	2.016	19.858	0.215
Clementine	0.049	0.141	1.367	0.821	11.921	0.340
Mandarin orange	0.088	0.214	1.775	1.229	12.735	0.515
Pomelo	0.268	0.237	0.871	-	12.998	0.419
Lemon	0.094	0.073	1.465	1.545	73.936	0.718
Lime	0.110	0.012	5.183	0.915	61.497	0.354
Sweetie	0.064	0.263	1.071	2.679	6.887	0.622
White grapefruit	0.117	0.169	0.089	0.641	23.053	0.580
Pink grapefruit	0.143	0.115	1.819	0.595	21.907	0.463

A mean of two samples analyzed in duplicate.

present work, detection limits were estimated according to the hypothesis that a peak, to be detected, should have a signal-to-noise ratio >3. The detection limits for the separated organic acids are presented in Tab. 1.

Precision was tested on six replicated analyses of independent preparations of sweet orange juice. The RSD values ranged from 0.154 to 3.18% indicating that the method was precise with a high degree of repeatability, especially for citric, ascorbic, lactic and oxalic acids (RSD <2%). The recovery of organic acids from citrus juices ranged from 95.8 and 102.1% confirming the accuracy of the separation and analysis conditions.

The developed method was applied to establish the organic acid profiles of ten commercially-available species of citrus fruits and the samples were eluted from the system within 20 minutes.

Fig. 2 illustrates the chromatographic separation of organic acids from sweet orange juice. The comparison of retention times and absorption spectra of samples with the standards were used to identify the organic acids present in citrus juices.

The amounts of each organic acid found in citrus juices were shown in Tab. 2. It was clear that the impact of species was significant on organic acid distribution of citrus fruit juices. As indicated by previous researchers (Cunha *et al.*, 2002; Karadeniz, 2004), citric acid is the major organic acid found in citrus juices (6.88-73.93 g/l), followed by malic acid and lactic acid. In general, oxalic, tartaric and ascorbic acids were present in minor quantities in citrus juices. For sweet orange, minneola, lemon, sweetie and white grapefruit juices, lactic was found to be in higher concentrations than malic acid. The malic acid content of lime (5.183 g/l) was significantly higher then that of the other citrus juices (0.871-1.819 g/l) while the tartaric acid content was the lowest (0.012 g/l). Higher amounts of tartaric acid were found in minneola (0.376 g/l), sweet orange (0.336 g/l) and sweetie (0.263 g/l). In pomelo, the amount of lactic acid was under the limit of quantification. Ascorbic acid ranged between 0.215 g/l and 0.718 g/l.

Average ascorbic acid was highest in lemon juice and followed by sweet orange juice (0.636 g/l), sweetie (0.622 g/l) and white grapefruit (0.580 g/l).

Conclusions

This work is a contribution to the development of a rapid and precise HPLC procedure for quantitative determination of organic acids in fruit juices under reverse phase conditions. Oxalic, tartaric, malic, lactic, citric and ascorbic acids have been determined simultaneously and eluted from the column within 20 minutes. Considering the easiness and conciseness of sample preparation, the proposed analytical procedure could be considered as an efficient, accurate and rapid method of organic acid determination.

The method could successfully used to quantify organic acids in natural and commercial citrus juices. Citric acid was the main acid in all citrus juices followed either by malic or lactic acid varying within citrus fruit species.

It was observed that the organic acids present in citrus juices were species, cultivar and horticultural practice dependent and could be considered as an active parameter for authenticity determination.

References

Abeysinghe, D. C., X. Li, C. D. Sun, W. S. Zhang, C. H. Zhou and K. S. Chen (2007). Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. Food Chem. 104:1338-1344.

Abd-Ghafar, M. F., K. N. Prasad, K. K. Weng and A. Ismail (2010). Flavonoid, hesperidine, total phenolic contents and antioxidant activities from *Citrus* species. Afr. J. Biotechnol. 9(3):326-330.

Aktas, A. H., S. Şen, M. Yılmazer and E. Cubuk (2005). Determination of carboxylic acids in apple juice by RP HPLC, Iran. J. Chem. Chem. Eng. 24(1):1-6.

Buchberger, W. W. (2000). Detection techniques in ion analysis: what are our choices?. J. Chromatogr. A 884(1-2):3-22.

Camara, M. M., C. Diez, M. E. Torija and M. P. Cano (1994). HPLC determination of organic acids in pineapple juices and nectars. Eur. Food Res. Technol. 198:52-56.

Canel, C., J. N. Bailey-Serres and M. L. Roose (1995). Pummelo fruit transcript homologous to ripening-induced genes. Plant Physiol. 108:1323-1324.

Casella, I. G. and M. Gatta (2001). Determination of electroactive organic acids by anion-exchange chromatography using a copper modified electrode. J. Chromatogr. A 912(2):223-233.

Cunha, S. C., J. O. Fernandes and I. M. L. V. O. Ferriera (2002). HPLC/UV determination of organic acids in fruit juices

and nectars. Eur. Food Res. Technol. 214:67-71.

Czajkowska, T. and M. Jaroniec (1997). Selectivity of alkylamide bonded-phases with respect to organic acids under reversed-phase conditions. J. Chromatogr. A 762(1-2):147-58.

Fransson, B. and U. Ragnarsson (1998). Separation of enantiomers of α- hydroxy acids by reversed-phase liquid chromatography after derivatization with 1-(9-fluorenyl) ethyl chloroformate. J. Chromatogr. A 827(1):31-36.

Guillén, D. A., C. G. Barroso, L. Zorro, V. Carrascal and J. A. Pérez-Bustamante (1998). Organic acids analysis in "Brandy de Jerez" by ion-exclusion chromatography, "post-column" buffering and conductimetric detection. Analusis 26(4):186-189.

Hasib, A., A. Jaouad, M. Mahrouz and M. Khouili (2002). HPLC determination of organic acids in Moroccan apricot. Cienc. Tecnol. Aliment. 3:207-211.

Karadeniz, F. (2004). Main organic acid distribution of authentic citrus juices in Turkey. Turk. J. Agric. For. 28:267-271.

Linget, C., C. Netter, D. Heems and E. Vérette (1998). On-line dialysis with HPLC for the automated preparation and analysis of amino acids, sugars and organic acids in grape juice and wines. Analusis 26(1):35-39.

Xu, G., D. Liu, J. Chen, X. Ye, Y. Ma and J. Shi (2008). Juice components and antioxidant capacity of citrus varieties cultivated in China. Food Chem. 106:545-551.