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Development and validation of a capillary electrophoresis method for direct measurement of isocitric, citric, tartaric and malic acids as adulteration markers in orange juice

L. Saavedra, A. García, C. Barbas*

Facultad de CC Experimentales y Técnicas, Universidad San Pablo-CEU, Urbanización Montepríncipe, Ctra. Boadilla del Monte km 5,3, 28668 Madrid, Spain

Abstract

Fruit juices each have very distinct organic acids profiles that can be used as fingerprints for establishing authenticity. A method has been developed, optimised and validated for measuring by capillary electrophoresis citric, isocitric, malic and tartaric acids as authenticity markers in orange juices, without any sample treatment other than dilution and filtration. Final conditions were phosphate buffer 200 m*M*, pH 7.50, −14 kV as applied potential, and 57 cm length neutral capillary. Detection was direct UV at 200 nm. Different kinds and marks of orange juice, chosen from the great variety existent in the market, were analysed and clear differences could be found between them and just pressed orange juice.

Keywords: Validation; Friut juices; Food analysis; Organic acids

1. Introduction

Adulteration of fruit juices and concentrates is an ongoing problem that has received wide publicity in the news media. Furthermore, recent health problems related with food industry have increased general concern. Common methods of adulteration either alone or in combination include addition of water, pulp wash, cheaper juices, colourants, and other undeclared additives, often to mimic the composition profiles of pure juices [2,12,17]. Because of the diversity in adulteration techniques, a matrix of text methods may be necessary [16,18] and between them organic acids measurement is always considered [12,19]. Fruit juices each have very distinct organic acids profiles that can be used as fingerprints for establishing authenticity. HPLC methods that compare ratios of the major acid components have been developed [1,6,12,21]; however, this is of limited value in most cases because the adulterators can add synthetic organic acids so that the ratios are consistent with those found in a pure juice. Some minor acids analysis is a more powerful technique [23], since it is not economically feasible to adjust the levels of all acid components, many of which are expensive [9].

Grapefruit added to orange juice can be detected by tartaric acid. Isocitrate, existing in natural orange juices in small but constant amounts, has been used as an adulteration indicator [17,18]. On the other hand, while citric acid levels in fresh fruit decrease during maturation, malic acid levels, a minor component, remain constant [18].

Several HPLC methods [19,20] have substituted previous ones employing gas chromatography which

*Corresponding author. Fax: +34-91-351-0475.
E-mail address: cbarbas@ceu.es (C. Barbas)

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needed extraction and derivatization steps, but generally they do not completely achieve the intended separations [12].

Capillary electrophoresis (CE) has proved to be a good choice for investigation of samples in aqueous media, since usually no more than a simple dilution of samples is needed. Supporting this statement are the studies of Kenney [11] who reported the separation of some organic acids with interest in a variety of food matrices with indirect detection employing a commercial pack. Huang et al. [10] reported the separation of a six-component mixture of monocarboxilic acids from formate to hexanoate with conductivity detection. Devêvre et al. [7] presented the separation of 14 organic acids with indirect UV detection using a commercial modifier; Turcat et al. [24] reported the monitorization of six organic acids in snow and rain water and Shirao et al. [22] reported the determination of organic acids in urine. Our research group has developed several methods of separation of organic acids in diverse matrices [3,4,8] with direct UV detection at 200 nm where the absorbance has a maximum. This low wavelength can be employed when working with non UV-absorbing aqueous buffers.

In the present paper a method has been developed, optimised and validated for determining the four acids in natural or commercial orange juices by capillary electrophoresis with direct UV detection and no ample treatment other than dilution and filtration. The method has been applied to different samples and the results are discussed.

2. Materials and methods

2.1. Instrumentation

The separation was performed on a capillary electrophoresis P/ACE (Beckman, Palo Alto, CA, USA) with UV detection at 200 nm. The injection was by pressure for 5 s. The neutral capillary was polyacrilamide coated (Beckman, Madrid, Spain) 57 cm long and 50 μm internal diameter and was operated at −14 kV potential. The electrolyte used was optimised during method development by varying the pH and concentration of a sodium phosphate buffer.

2.2. Chemicals

Standards were obtained from Sigma (St. Louis, MO, USA). Phosphoric acid 85% was from Merck (Darmstadt, Germany), sodium hydroxide from Panreac (Madrid, Spain) and organic solvents from Scharlau (Barcelona, Spain).

2.3. Samples

Samples used in the development and validation of the method were obtained from one of the many marks available in the market. Samples were diluted in a 1:1 ratio with Milli-Q water (Millipore Ibérica, Madrid, Spain) before filtration for the analysis. Filtration was made with acetate filters (MSI, Minetonka, MN, USA) of 0.45 μm of pore size.

2.4. Optimisation of the separation

Buffers used in the optimisation were prepared from phosphoric acid, 200 m*M*, and NaOH in order to increase the corresponding pH. Different pH values were assayed from pH 3 to pH 8 increasing 0.5 pH points each time. Changes in the potential were also assayed in those pH ranges exhibiting better separation. Different capillary lengths were also assayed in order to avoid some interferences observed in the separation.

Final conditions were: phosphate buffer 200 m*M*, pH 7.50, −14 kV as potential source, and 57 cm×50 μm I.D. as capillary dimensions.

2.5. Validation

A stock solution containing the four acids was prepared 50.1157 g/l for citric acid, 0.5003 g/l for isocitric acid, 8.073 g/l for tartaric acid and 8.05 g/l for malic acid, in Milli-Q quality water, and stored at −20°C.

Linearity of response for standards was tested assaying by triplicate five levels of concentrations, around 10 to over 300% of the medium concentration hoped in samples that is 5.1 g/l for citric, 0.05 g/l for isocitric, 0.8 g/l for malic and 0.8 g/l for tataric acids.

Linearity of response for samples was tested in the

same way but replacing half part of the water with orange juice.

Recovery was estimated comparing the values obtained in the linearity of the orange juice calibration, with the standards linearity, taking into account the orange juice concentrations, which had been previously analyzed.

Within-day precision was tested both to check the constancy of instrumental response to a given analyte and the concentration and migration time repetitiveness, since the latter is a key parameter for peak assignment. For this purpose, the assay was performed with six solutions of standards and ten solutions of samples in the same day, in the medium concentration of the calibration curve for all the compounds. For intermediate precision six standards and ten samples were analyzed in different days.

Limits of detection were calculated following IUPAC recommendations $[(a + 3\ S_B)/b]$ [15] for chromatographic methods, where S_B = blank standard deviation, a = intercept and b = slope. Three standards in a low concentration were analysed by triplicate and the standard deviation of the zero value was calculated by extrapolation. This value was interpolated in the corresponding equation.

2.6. Measurement of samples

Different kinds and marks of orange juice chosen from the great variety existent in the market were analysed. Juices without pulp were directly measured after only 1:1 (v/v) dilution with water and filtration. Juices with pulp were previously centrifuged, 2000 *g* for 10 min, in order to eliminate the pulp; then, they were analysed in the same way as the others juices.

All the samples were injected by duplicate and were processed simultaneously with standards corresponding to the medium point of the calibration graph.

3. Results and discussion

Previous studies in our laboratory with short chain organic acids [3,8] had shown that neutral capillaries yielded superior reproducibility in retention times and more steady baseline than fused-silica capillaries, so this type was employed. The electrolyte buffer is one of the most important and flexible variables in capillary zone electrophoresis. The pH, the concentration, the type of buffer and the presence of additives or modifiers can all significantly influence the selectivity, the efficiency and the speed of separation [13].

At pH lower than 6.0 the analytes present different ionisation degrees, they have two to three ionisation constants with pK_a values 3.1; 4.8; 6.4 for citric acid, 3.29; 4.71; 6.40 for isocitric acid, 3.4; 5.11 for malic acid and 2.98; 4.34 for tartaric acid [14], and the effective mobility for some of them are very similar which results in poor resolution. At pH equal to 6.0 or higher a good resolution was shown in standards, but when samples were run an interference appeared in the isocitrate peak, probably due to an isomer of malic acid which was separated at pH 7.50. At this pH all these acids are in the higher ionisation degree and elution order corresponds with their charge to mass ratio, except for citric acid. This might possibly be due to steric factors related with polar interactions that give it a more expanded configuration in citric acid, and elution is actually performed by charge to size ratio. Addition of small amounts of methanol as modifier (5–10%) did not produce enough resolution, and increasing analysis time. Lowering the applied potential and increasing potential makes current intensity values too high. With respect to capillary length, standards are completely resolved with a 37 cm length capillary being able to run the analysis in less time, but samples need a 57 cm length capillary for resolution of interferences, so this size was chosen.

Fig. 1 shows an electropherogram of standards in final conditions corresponding to the medium point of the calibration graph. Fig. 2 shows an electropherogram of three different samples, one of them containing tartaric acid, another a diluted commercial sample and the third just pressed orange juice.

3.1. Validation

As shown in Table 1, standards fit the linear model ($r > 0.99$) for the four organic acids and no bias was found. Samples also showed a good linearity, with correlation coefficients over 0.99. A small bias can be observed in malic and tartaric acids. Although it has statistical significance, it has no

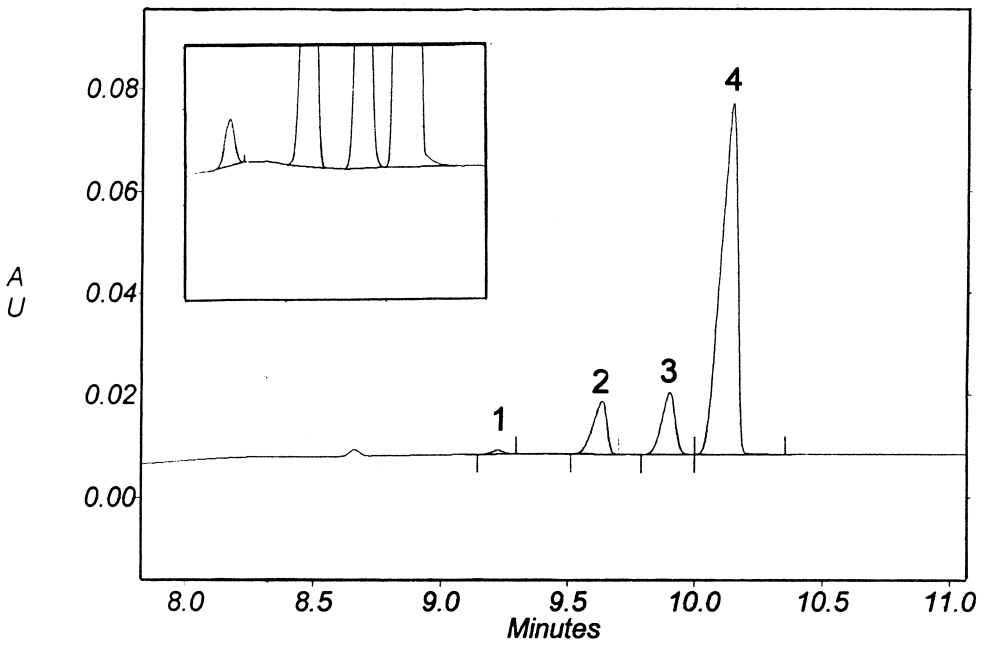

Fig. 1. Electropherogram of standards of isocitric, malic, tartaric and citric acids. For conditions see the text.

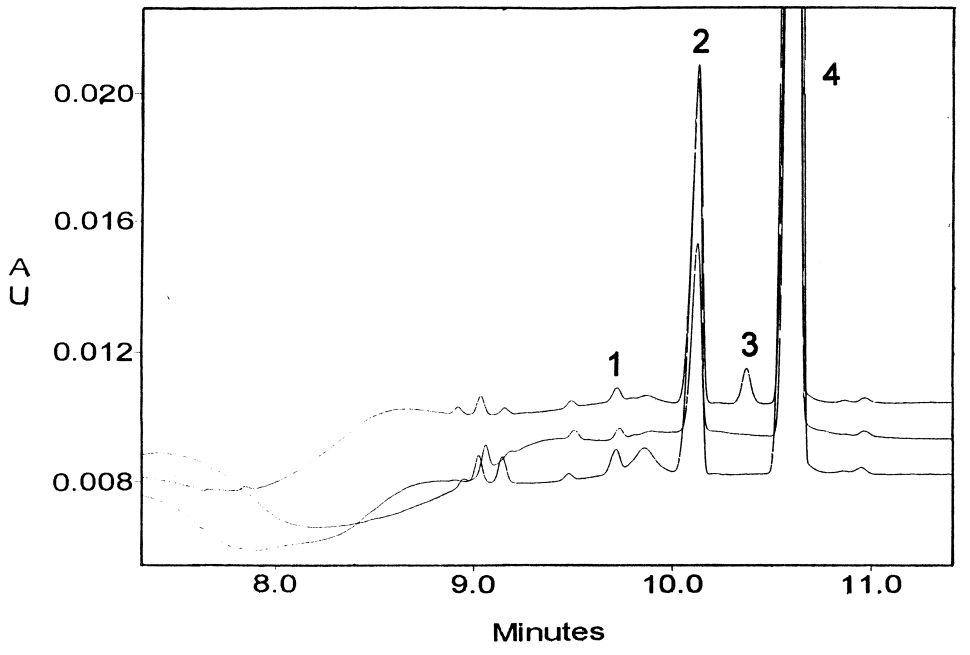

Fig. 2. Electropherogram of two commercial orange juices with different degrees of adulteration, as compared with just pressed orange juice. For conditions see the text.

Table 1
Main validation parameters

		Isocitric	Malic	Tartaric	Citric
Standards linearity	Intercept	143±197	−725±2279	−769±2502	5284±15821
	Slope	39 009±2112	41 441±1700	48 069±1861	51 797±1893
	r	0.997	0.997	0.998	0.998
	Range (g/l)	0.018–0.18	0.080–2.820	0.080–2.830	0.500–17.54
Sample linearity	Intercept	−30±36	−2984±505	−1507±228	−13 122±4873
	Slope	44 083±807	46 199±562	53 781±353	58 536±916
	r	0.9994	0.9997	0.9999	0.9995
Accuracy	Standards	103	103	103	97
% recovery	RSD (%)	11	7	7	14
	Samples	100	102	104	102
	RSD (%)	10	4	6	6
Standards intra-assay precision	Mean (g/l)	0.05	0.81	0.81	5.01
($n=12$)	RSD (%)	3.5	3.8	3.8	3.3
	Mean (t_M)	9.18	9.59	9.85	10.09
	RSD (%)	0.7	0.8	0.7	0.8
Samples intra-assay precision	Mean (g/l)	0.04	0.74	0.42	4.23
($n=9$)	RSD (%)	4	1.3	1.9	2
	Mean (t_M)	9.14	9.54	9.79	10.03
	RSD (%)	0.6	0.5	0.5	0.5
Standards inter-assay precision	Mean (g/l)	0.05	0.81	0.80	5.01
($n=24$)	RSD (%)	7.6	4.4	3.8	3.2
	Mean (t_M)	9.19	9.60	9.86	10.10
	RSD (%)	0.5	0.5	0.6	0.6
Samples inter-assay precision	Mean (g/l)	0.03	0.75	0.41	4.18
($n=44$)	RSD (%)	6.7	4.4	6.8	4.9
	Mean (t_M)	9.2	9.60	9.85	10.10
	RSD (%)	0.5	0.5	0.6	0.6
Limit of detection (g/l)		0.005	0.009	0.002	0.002

practical consequences as can be seen in the recoveries that are near 100% in the whole range for both, and it could be justified with the accurate fit of points to the regression line that makes the limits of confidence very narrow. For accuracy, recoveries ranged from 97 to 104% and they did not statistically differ from 100%.

Running 12 runs per day of standards and nine of samples, daily RSD in concentrations are low enough to consider the method acceptable (3.3–4.0%).

Intermediate precision evaluated in different days and operators with a total of 24 runs for standards and 44 for samples provided RSD values lightly superior to intra-assay precision (3.2–7.6%), as could be expected. Detection limits are 0.005 g/l for isocitrate, 0.009 g/l for malate, 0.002 g/l for tartrate and 0.002 g/l for citrate. Mean values described in bibliography [18] as normal for these acids are: 0.02–0.2 g/l for isocitrate, 0.9–1.4 g/l for malate, tartrate ought to be absent and 5–20 g/l for citrate, so it would be applicable for measuring all kind of samples.

3.2. Orange juice measurement

Results for commercial and natural samples are shown in Table 2 and values found in the literature for just pressed juices are collected in Table 3. As can be observed, our values for the content in the three acids in the natural juice are fully in agreement with those found in literature for Spanish or Mediterranean Basin oranges. Moreover, one of the commercial samples (H), announced as coming directly from oranges without any concentration or dilution, gives the same values. Some of the juices (A, D and E) have different quantities of grape juice added as can be seen by the tartaric acid, when it is not mentioned

Table 2
Isocitric, malic, tartaric and citric acids in orange juices by capillary electrophoresis (ND: non detectable)

	Isocitric (mg/l)	Malic (g/l)	Tartaric (mg/l)	Citric (g/l)
Sample A	44.7	1.6	134	7.0
Sample B	42.7	1.9	ND	8.2
Sample C	54.4	1.6	ND	8.5
Sample D	42.1	1.6	46	7.2
Sample E	45.7	1.3	11	7.4
Sample F	39.2	0.8	ND	6.5
Sample G	46.7	2.1	ND	7.7
Sample H	83.8	1.7	ND	10.7
Sample I	44.3	2.0	ND	7.6
Natural juice (just pressed)	83.7	2.0	ND	11.2

Table 3
Mean values±confidence intervals for citric, isocitric and malic acids in orange juices of different origins ($P<0.05\%$).

Origin	Citric (g/l)	Isocitric (mg/l)	Malic (g/l)	Ref.
Spain	12.6±2	100±20	1.8±0.3	[5]
Spain	10.9±0.5	115±5		[2]
California	9.6	76.7	0.9	[17]
Mediterranean basin	11.5±1.1	162±32	1.2±0.3	[19]
USA and Cuba	8.4±1.2	94±15	1.6±0.2	[19]
Others South America	11.4±1.5	112±16	1.7±0.1	[19]

in the label and the remaining samples are more diluted than natural juice.

4. Conclusion

The developed method is rapid, simple and reliable for assessing a certain kind of fraud in orange juices, using isocitrate, citrate, malate and tartrate as markers.

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