Rapid Determination of Nonaromatic Organic Acids in Honey by Capillary Zone Electrophoresis with Direct Ultraviolet Detection

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A rapid capillary zone electrophoresis (CZE) method with direct ultraviolet (UV) detection has been set up and developed to determine the most important nonaromatic organic acids in honey with a really simple treatment of the sample. The determination of oxalic, formic, malic, succinic, pyruvic, acetic, lactic, citric, and gluconic acids has been carried out in 4 min. The electrolyte composition was phosphate as the carrier buffer (7.5 mM NaH_2PO_4 and 2.5 mM Na_2HPO_4), 2.5 mM tetradecyl-trimethylammonium hydroxide (TTAOH) as electroosmotic flow modifier, and 0.24 mM $CaCl_2$ as selectivity modifier, with the pH adjusted at 6.40 constant value. The running voltage was −25 kV at a thermostated temperature of 25 °C. The injections were performed in hydrodynamic mode (30 s), and the detection mode was UV direct at 185 nm. Validation parameters of the method as detection and quantification limits, linearity, precision (repeatability and reproducibility), and recovery were also studied. The advantages related to the technique such as simplicity, short analysis times, and low consumption of chemicals as well as the good validation parameters obtained for this method permit it to be considered as adequate for routine analysis in honey.

INTRODUCTION

Organic acids make very important contributions to honey properties. Despite being in small quantities (<0.5%), organic acids contribute to antibacterial and antioxidant activities. They can be used as fermentation indicators, and some of them are currently used for the treatment of varroa infestation. Organic acids have also been deployed to discriminate among honeys according to their botanical and/or geographical origins. Organic acids are related to honey color and flavor and to physical and chemical properties, such as pH, acidity, and electrical conductivity. The current literature related to this significance of nonaromatic organic acids in honey was reviewed previously (*1*). Furthermore, an organic acids profile could be a better parameter than free acid to determine honey spoilage. Not more than 50 mequiv/kg of free acid is allowed for honey in general, and not more than 80 mequiv/kg of free acid is allowed for baker's honey (*2*). Free acid is difficult to determine, due to the fact that there is no agreement about the most proper procedure to analyze this parameter (*3*–*5*) and the different

methods give different results. In addition, honeys from humid climates contain xerotolerant yeasts that increase free acid with no honey damage (*6*, *7*), so that, nowadays, parameters other than free acid, but related to it, should be sought to properly determine honey spoilage. In this respect we think that an organic acids profile could be more appropriate.

Organic acids have been traditionally analyzed in honey by different techniques such as enzymatic methods (*8*–*23*), gas chromatographic methods (*24*–*29*), and liquid chromatographic methods (*20*, *30*–*41*).

In general, the advantages of enzymatic methods are sensitivity, specificity (that allows the determination of D/L organic acid forms), and simple instrumentation. However, only one organic acid can be determined with these methods. Therefore, if we need know the honey's organic acid profile, other techniques such as chromatography are more suitable. However, chromatographic procedures applied to honey organic acids are tedious and time-consuming. With regard to chromatographic methods, gas chromatography requires a derivatization process because most organic acids are nonvolatile. Nevertheless, there are identification studies of aromatic organic acids, which are components of honey flavor (*42*–*46*). Finally, liquid chromatography has been widely used to determine organic acids in

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1542 *J. Agric. Food Chem.,* Vol. 54, No. 5, 2006

Mato et al.

honey because of its sensitivity and reproducibility. The separation and quantification of the acids have been usually carried out by high-resolution liquid chromatography (HPLC) and by ion chromatography (IC). The matrix effects, mainly sugar interferences, can lead to problems in conventional HPLC and/or IC organic acid analysis. Therefore, honey pretreatment techniques (for example, a solid-phase extraction procedure) and/ or several columns connected in series were used to improve the organic acid determination and to eliminate these matrix interferences (*32*, *34*, *37*, *47*).

Capillary zone electrophoresis (CZE) is currently acquiring considerable importance as a technique for the separation of low molecular weight organic acids due to its good resolution, automation, simplicity, short analysis times, low consumption of chemicals, and reduced sample preparation (*48*, *49*). Nowadays many methods have been developed to determine organic acids in a wide range of foodstuffs, such as beer (*50*), brandy and other spirits (*51*, *52*), coffee (*53*), fruit juices (*54*−*56*), milk and dairy products (*57*, *58*), musts and wines (*52*, *59*−*63*), and sugar refinery products (*64*). Surprisingly, only two papers applied capillary electrophoresis previously for the identification of organic acids in honey. Boden et al. (*65*) developed a CZE method to allow only the identification (but not the quantification) of citric acid in honey, together with inorganic anions (chloride and phosphate). Recently, Navarrete et al. (*66*) applied a CZE method for the determination of organic acids in honey. In that method, only oxalic, fumaric, maleic, and malic acids were quantified in 14 min. Therefore, several important organic acids, such as gluconic (a predominant acid in honey), citric (useful for distinguishing between floral, honeydew, and sugarfed honeys), acetic (which may indicate fermentation), and formic and lactic acids (used for the treatment of varroa infestation), were not determined in honey.

The aim of this work has been to research the capillary electrophoresis technique for the development of a rapid and simple method for the simultaneous determination of the most important nonaromatic acids in honey without the handicap of sugar interferences.

MATERIALS AND METHODS

Chemicals. All analytical standard-grade organic acids were obtained from Sigma Chemical Co. (St. Louis, MO) as their sodium or potassium salts, but glutaric acid was supplied by Merck (Darmstadt, Germany). Stock standard solutions were obtained by dissolution of salts of the acids in Milli-Q water (10 g/L), and they were stored at 4 °C for 1 month. The Milli-Q water was purified by passage through a Compact Milli-RO and Milli-Q water system from Millipore (Milford, MA). Working standard solutions were prepared daily by dilution with Milli-Q water.

Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogen phosphate (Na_2HPO_4), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), hydrochloric acid fuming (37%), and sodium hydroxide pellets were of analytical reagent grade and supplied by Merck. Tetradecyltrimethylammonium hydroxide (TTAOH), commercial name OFM-OH, was supplied by Waters (Milford, MA). The electrolyte was filtered through 0.45 μm Phenomenex AFO-0504 nylon membrane filters (Phenomenex, CA) and must be prepared fresh daily. The samples were filtered through 0.5 μm PTFE membrane filters (MFS, CA).

Apparatus. Separation was carried out on a Waters capillary ion analyzer (CIA system, 1.3 version) equipped with a negative power supply and a fixed-wavelength UV−vis detector with a mercury lamp (Waters Chromatography, Milford, MA). Fused-silica capillaries Waters Accusep Part 250-05, with a length of 60 cm and a 75 μm internal diameter, were used. Electropherograms were collected and plotted by a Millennium 2010 v. 2.15 data acquisition system with specific option CIA for capillary electrophoresis (Waters Chromatography).

A Crison micropH 2002 pH-meter (Crison Instruments S.A., Alella, Barcelona, Spain) and a Selecta Agimatic-S magnetic stirrer (Selecta, Abrera, Barcelona, Spain) were also used.

Electrophoretic Procedures. *Capillary Column Conditioning.* Prior to first use, a new capillary was pretreated with the following sequence: Milli-Q water (10 min), 1 M NaOH (10 min), 0.01 M NaOH (10 min), Milli-Q water (30 min), and running electrolyte (90 min). Prior to daily use, the capillary was conditioned with 0.01 M NaOH for 10 min, followed by Milli-Q water for 30 min and carrier electrolyte for 90 min. Before each run, the capillary was flushed with running electrolyte for 2 min. After all analyses of the day, the capillary was also washed with 0.01 M NaOH (10 min) and Milli-Q water (30 min).

Separation Conditions. Sample injection was carried out in a hydrodynamic mode by elevating the sample at 10 cm for 30 s. The running voltage was −25 kV at a thermostated temperature of 25 °C. The detection mode was UV direct, and the wavelength was 185 nm. The electrolyte composition was phosphate as the carrier buffer (7.5 mM NaH_2PO_4 and 2.5 mM Na_2HPO_4), 2.5 mM TTAOH as electroosmotic flow modifier, and 0.24 mM $CaCl_2$ as selectivity modifier; the pH was adjusted at 6.40 constant value. All standards and honey samples were injected in triplicate.

Samples and Sample Treatment. The work was carried out on 10 samples from Galicia (northwestern Spain). The Galician samples were labeled "Producto Galego de Calidade-Mel de Galicia", which is a Certified Brand of Origin (CBO) (*67*), and were provided by the Galician Association of Beekeepers. The samples were stored in darkness at room temperature (between 15 and 25 °C) until the analysis. The botanical origin of the samples was determined according to the procedure described in ref *68*. After that, the sediment in the honeys was treated and dyed using the method reported in ref *69*. Three samples were *Castanea sativa* Miller honeys, three samples were *Eucalyptus* sp. honeys, and four samples were multifloral honeys.

A 2.50 g amount of honey was dissolved in 75 mL of Milli-Q water. The pH was adjusted to ∼10.50 using 0.1 M NaOH, and the mixture was stirred for 10 min using a magnetic stirrer at room temperature. This procedure is carried out to guarantee the total hydrolysis of D-glucono-δ-lactone to D-gluconic acid (*15*) and to improve the reproducibility of the organic acid migration times. The pH was then adjusted to ∼5.00 using 0.1 M HCl. The mixture was transferred with Milli-Q water to a 100 mL volumetric flask, filled to the mark, and stirred. After filtration through a 0.5 μm PTFE membrane filter, the obtained solutions were directly injected in the capillary electrophoresis device. Sometimes, a 2.50 g amount of honey (in 100 mL) was in excess and peak broadening appeared. For these samples with high content of organic acids, the adequate amount of honey was 1 g in 100 mL, so a 1:2.5 dilution of initial honey solution was necessary. A relationship between the content of organic acids and color was found, so we could use the official AOAC method for the color (*3*) to introduce the adequate amount of honey. If honey had a color included in an amber or light amber category, it was probably necessary to use 1 g/100 mL.

RESULTS AND DISCUSSION

Optimization of Electrophoretic Conditions. First, a mixture of 16 standards of organic acids (oxalic, formic, tartaric, malic, succinic, maleic, glutaric, pyruvic, acetic, lactic, citric, butyric, benzoic, sorbic, ascorbic, and gluconic acids) was separated and identified according to this electrophoretic method (**Figure 1**). This solution of 16 standards of organic acids was tested to optimize the electrophoretic conditions.

Influence of Buffer pH. The influence of buffer pH on migration times is used to adjust the selectivity of organic acids. In addition to affecting solute charge, changing the pH also causes a change in electroosmotic flow (EOF) (*70*). The influence of buffer pH (6.00−7.60) on the separation was investigated. As we can see in **Figure 2**, if the buffer pH increased, the absolute migration times of the organic acids decreased until pH 7.20.

Determination of Nonaromatic Organic Acids in Honey

J. Agric. Food Chem., Vol. 54, No. 5, 2006   **1543**
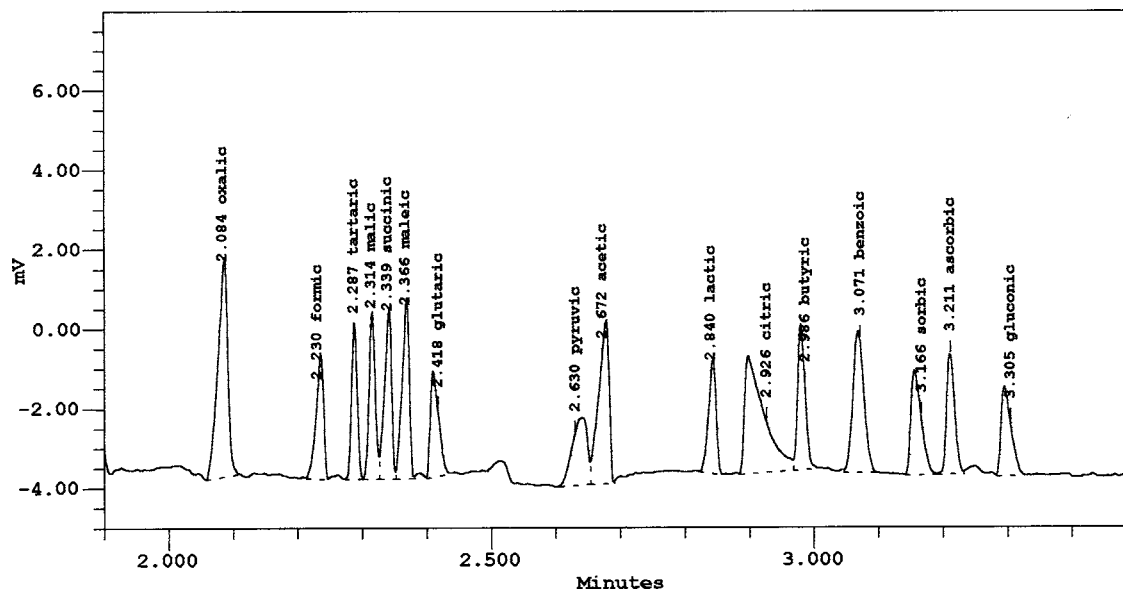

Figure 1. Electropherogram of a solution consisting of a mixture of standards of the organic acids separated by the proposed CZE method (glutaric acid was added as reference acid to calculate the relative migration times of the honey organic acids). Concentrations of the acids were as follows: oxalic, formic, tartaric, malic, succinic, maleic, glutaric, pyruvic, acetic, lactic, butyric, and sorbic acids (5 ppm); citric acid (12.5 ppm); benzoic acid (1 ppm); and ascorbic and gluconic acids (10 ppm). Conditions: phosphate buffer (7.5 mM NaH$_2$PO$_4$ and 2.5 mM Na$_2$HPO$_4$), 2.5 mM TTAOH, 0.24 mM Ca^{2+}, pH 6.40, −25 kV, 25 °C, hydrodynamic injection (30 s), and direct UV detection (185 nm).
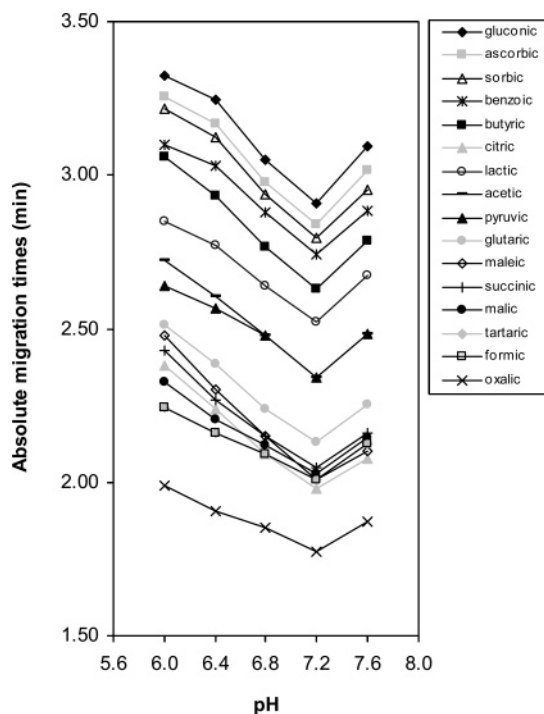

Figure 2. Effect of buffer pH on absolute migration times of organic acids [phosphate buffer (7.5 mM NaH$_2$PO$_4$ and 2.5 mM Na$_2$HPO$_4$), 2.5 mM TTAOH, 0 mM Ca^{2+}, −25 kV, 25 °C, hydrodynamic injection (30 s), and direct UV detection at 185 nm].

The direct pH of the electrolyte was ~6.80. At this pH, overlapping between tartaric and formic acids, between succinic and maleic acids, and between pyruvic and acetic acids was found. The best separation was at pH 6.40. In this case, coelution between tartaric and formic acids was found. Furthermore, citric acid was a broad peak and its tail overlapped succinic acid. On the basis of the literature, these overlappings were resolved by the addition of some additives in the electrolyte (*64*). Therefore, in this study, the pH of 6.40 was finally chosen for performing the separation.

Influence of the Concentration of Electroosmotic Flow Modifier. Two conditions were necessary to carry out the analysis of anions. First, the polarity of the power supply (negative polarity) was reversed to switch the cathode to the injection end and, second, a cationic surfactant was added to the electrolyte to modify the EOF. The EOF modifier used to perform the separation was TTAOH.

To check the influence of the concentration of EOF modifier, several assays were made in the range of concentration from 2.0 to 4.0 mM. As is shown in **Figure 3**, the absolute migration times of the organic acids decreased while the concentration of TTAOH increased.

If the concentration of TTAOH was >2.5 mM, overlapping would appear between benzoic, sorbic, ascorbic, and gluconic acids. When the concentration of TTAOH was <2.5 mM, a negative peak appeared between maleic and glutaric acids, besides a poor resolution between butyric and benzoic acids. For these reasons, 2.5 mM TTAOH was chosen as the optimum EOF modifier concentration.

The variation of the concentration of TTAOH had a small effect on the selectivity of the separation of organic acids. Even by modifying the concentration of TTAOH, we were not able to solve the coelution of formic and tartaric acids.

Influence of the Concentration of Ca^{2+} Ions. Some organic acids have very similar structures or the same electrophoretic behaviors, and their mobilities are not sufficiently different to allow a good resolution. Several authors reported that the selectivity and/or resolution could be improved by the addition of organic solvents or salts because these substances modify electroosmotic flow (*48*, *71*, *72*). Organic solvents usually reduced EOF due to the increase of electrolyte viscosity (*72*). On the basis of literature data, several organic solvents were used to increase separation selectivity, for example, acetonitrile (*73*), methanol (*56*), or polyethyleneglycol (*74*). The relative mobility of organic acids can be also influenced by changing their charge state through selective complexation (*64*, *75*, *76*). Calcium salts (*64*, *75*, *77*), copper salts (*76*, *78*), magnesium

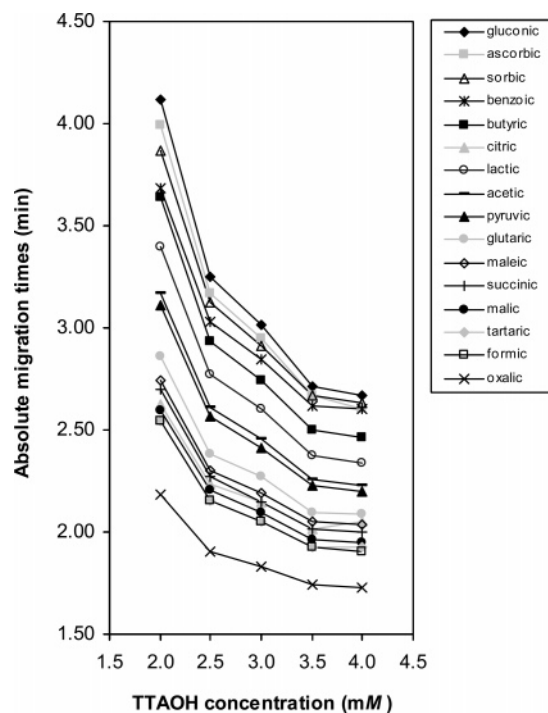**1544** *J. Agric. Food Chem.,* Vol. 54, No. 5, 2006

Mato et al.

Figure 3. Effect of concentration of tetradecyltrimethylammonium hydroxide (TTAOH) on absolute migration times of organic acids [phosphate buffer (7.5 mM NaH_2PO_4 and 2.5 mM Na_2HPO_4), pH 6.40, 0 mM Ca^{2+}, −25 kV, 25 °C, hydrodynamic injection (30 s), and direct UV detection at 185 nm].

Figure 4. Effect of concentration of Ca^{2+} on absolute migration times of organic acids. Other electrophoretic conditions were the same as in **Figure 1**.

salts (*76*), nickel salts (*76*), and a mixture of calcium and magnesium salts (*52*) were used.

In our case, the modifications on buffer pH and on TTAOH concentration were not enough to solve the comigration of formic and tartaric acids and the overlapping between citric and succinic acids. The addition of calcium salts to the buffers has been previously reported for the separation of formic and tartaric acids, and with the Ca^{2+} present, citrate is strongly retarded (*64*). Therefore, calcium salt was chosen for performing the separation.

The influence of concentration of Ca^{2+} (0.00−1.00 mM) on the separation was investigated. As is shown in **Figure 4**, the absolute migration times of the organic acids increased when the concentration of Ca^{2+} increased. Oxalic, tartaric, malic, and citric acids were the organic acids that suffered the most important changes in the elution order. The migration times of these di- or tricarboxylic acids are affected more than those of the monocarboxylic acids. This is explained by their larger stability constants, which cause a tangible retardation that leads to prolonged migration times with increased Ca^{2+} concentrations (*77*). Oxalic, tartaric, and citric acids are also well-known as complexing agents for divalent cations (*79*). Optimum separation with minimal analysis time was obtained at 0.20 mM Ca^{2+}, but citric and lactic acids were overlapped. If the calcium concentration increased (0.40 mM), a comigration between malic and succinic acids occurred and citric acid overlapped with benzoic acid. To resolve the citric−lactic overlapping, slight variations of Ca^{2+} between 0.20 and 0.30 mM were studied. Therefore, the optimum Ca^{2+} concentration was fixed at 0.24 mM.

Validation of the Electrophoretic Method. When this proposed method was applied to honey samples, the identification and quantification of oxalic, formic, malic, succinic, pyruvic, acetic, lactic, citric, and gluconic acids in honey were carried out in 4 min. Tartaric and maleic acids were not detected
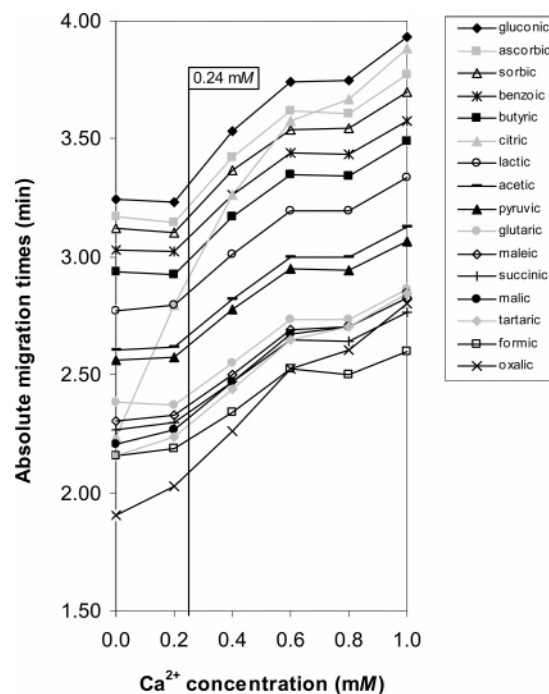
in any analyzed honeys. Butyric, benzoic, sorbic, and ascorbic acids were not correctly identified because they appeared in an area of the electropherogram with a lot of interferences, so those organic acid responses could be overlapped with other substances. The importance of these acids, which were not correctly identified, is not very high in honey, but it is possible to use this method to determine them in other samples.

To compare the validation results of the proposed method with the results obtained in enzymatic, chromatographic, and electrophoretic methods previously employed by other authors, a summary of these analytical methods is included in **Tables 1** and **2**.

Migration Times. Fluctuations in the absolute migration times of solutes are one of the major reasons for the lack of reproducibility in CZE (*80*). To minimize this problem, the relative migration times were used (*48, 81−83*). In this method, the relative migration times of the organic acids were calculated as the ratio between their migration times and the glutaric acid migration time used as reference compound. The glutaric acid was previously used as internal standard in HPLC studies (*34*).

Table 3 shows the absolute and relative migration times of organic acids analyzed in honey. The precision of relative migration times was better than the precision of absolute migration times. When relative migration times are used to identify organic acids, the variations produced by the different conductivities of the samples, by the analyte concentration, or by small fluctuations in electrolyte composition and pH, temperature, or applied voltage could be minimized.

Detection and Quantification Limits. The detection limit (LOD) was calculated as $s_b + 3s$, where s_b is the average signal of 10 blank injections (absolute area value of each organic acid migration time ± 2%) and s the standard deviation. The quantification limit (LOQ) was calculated as $s_b + 10s$, where s_b is the average signal of 10 blank injections and s the standard deviation (*84*).

Table 4 shows detection and quantification limits of organic acids analyzed in honey. The detection limits ranged from 0.4

Table 1. Summary of the Validation Parameters of the Enzymatic Methods Previously Employed by Other Authors for the Quantification of Organic Acids in Honey

organic acid	LOD (mg/kg)	LOQ (mg/kg)	precision (%)	recovery (%) (RSD%)	reaction time (min)	ref
formic	0.3			96.0 (6.5)	25	*9*
D- and L- lactic	0.3			96.0 (6) (D-lactic acid)	65	*10*
				83.0 (13) (L-lactic acid)		
formic	5–10				25	*11*
citric			≤1.72	92.0 (6.8)	10	*12*
formic			≤4.09	97.6 (1.69)	25	*13*
formic and D- and L-lactic	11 (formic acid)				25	*14*
	16 (lactic acid)				65	
total D-gluconic	200		≤0.30	99.8 (0.40)	35	*15*
oxalic	3				52	*16*
citric	23		≤1.6	98.0–100.9	10	*17*
L-malic	19		≤3.5	100 (3.5)	13	*18*
oxalic	0.80	2.66		90.8 (1.0)	52	*19, 20*
formic and oxalic	3–5		≤4.4	93 (4) (formic acid)	25	*21*
			≤18.4	84 (24) (oxalic acid)	52	
total D-gluconic	200		≤3.93	97.9 (1.12)	35	*23*

Table 2. Summary of the Validation Parameters of the Chromatographic and Electrophoretic Methods Previously Employed by Other Authors for the Quantification of Organic Acids in Honey

technique	organic acid	LOD (mg/kg)	LOQ (mg/kg)	precision[a] (%)	recovery (RSD%)	analysis time (min)	other organic acids analyzed	ref
HPLC	gluconic, pyruvic, malic, citric, succinic	0.7–2			96.0–103.3	30	fumaric	*30, 32*
IC	formic	1.4				5		*33*
HPLC	citric, pyruvic, gluconic, malic, succinic, formic	0.377–8.678				30	galacturonic, citramalic, quinic, fumaric	*34*
							succinic,	
HPLC	oxalic, lactic, formic	1.92–12.74	6.38–42.44	1.3–4.3	90.84–95.04	12		*20*
IC	oxalic	1.547	5.158	4.05	92.72	35		*35*
HPLC	gluconic				98	5		*36*
HPLC	malic, citric, succinic	1.44–7.57	2.72–10.93	repeat.: 0.27–3.20 reprod.: 2.59–4.86	62.9–99.4	15	maleic, fumaric	*37, 38*
HPLC	oxalic, citric, pyruvic, D-gluconic, formic	0.5–97.3	1.8–325	repeat.: 0.6–5.5 reprod.: 0.8–7.2		60	D-glucuronic, galacturonic, glutaric	*39, 40* reprod.: 0.8–7.2
HPLC	gluconic, oxalic, pyruvic, malic, citric, succinic	0.084–8.47	0.21–11.60	repeat.: 1.36–3.07 reprod.: 3.21–4.25	78.30–99.60	15	fumaric, isobutyric, butyric	*41*
CZE	oxalic, malic	1.80–4.80	6.30–16.03		98.32–99.54	14	fumaric, maleic	*66*

[a] repeat, repeatability; reprod, reproducibility.

Table 3. Absolute and Relative Migration Times of Organic Acids in Honey ($n = 3$)

organic acid	absolute migration time (min) mean ± SD	RSD (%)	relative migration time mean ± SD	RSD (%)
oxalic	2.31 ± 0.04	1.8	0.85 ± <0.01	0.5
formic	2.49 ± 0.05	1.9	0.91 ± <0.01	0.2
malic	2.59 ± 0.05	2.0	0.95 ± <0.01	0.2
succinic	2.62 ± 0.05	1.9	0.96 ± <0.01	0.2
pyruvic	3.01 ± 0.07	2.4	1.10 ± <0.01	0.4
acetic	3.04 ± 0.07	2.4	1.12 ± <0.01	0.4
lactic	3.26 ± 0.09	2.6	1.20 ± <0.01	0.6
citric	3.39 ± 0.09	2.6	1.24 ± <0.01	0.7
gluconic	3.88 ± 0.12	3.1	1.42 ± 0.02	1.1

Table 4. Detection and Quantification Limits and Parameters and Correlation Coefficients (*r*) of Calibration Plots for the Organic Acids in Honey[a]

organic acid	LOD (mg/kg)	LOQ (mg/kg)	calibration plots a	b	R
oxalic	0.4	12	26.7	502	0.9999
formic	2.1	23	10.7	212	0.9996
malic	2.0	21	10.8	201	0.9997
succinic	2.0	12	13.4	294	0.9998
pyruvic	7.0	39	9.8	335	0.9996
acetic	11	34	17.9	233	0.9999
lactic	4.2	26	9.6	204	0.9999
citric	9.2	28	15.3	160	0.9996
gluconic	38	78	5.3	56	0.9999

[a] Calibration plots are expressed as regression lines ($y = ax + b$), where *y* is the peak area and *x* is the amount of organic acid in mg/kg of honey. The calibration test was repeated three times.

mg/kg (oxalic acid) to 38 mg/kg (gluconic acid), and the quantification limits ranged from 12 mg/kg (oxalic and succinic acids) to 78 mg/kg (gluconic acid). Other acids such as tartaric and maleic acids were not detected in any analyzed honeys. Nevertheless, if both acids were found in honeys in a higher amount than the detection and/or quantification limits, they could be identified and/or quantified by using this method. Detection and quantification limits were 15 and 53 mg/kg for tartaric acid and 2.4 and 6.8 mg/kg for maleic acid.

Calibration Curves. The quantification of organic acids was carried out by using an external standard calibration. Calibration curves were determined for seven different concentrations of a mixture of organic acid standard solutions. Each calibration point was injected in triplicate. Calibration graphs for each compound were obtained daily by plotting peak area against concentration and applying the least-squares method. In CZE, peak areas are linearly related to sample concentration over a broader range than peak heights. For this reason peak areas are used as the basis for quantitative analysis (*70*). Calibration plots are expressed as regression lines ($y = ax + b$), where *y* is the peak

Table 5. Repeatability and Reproducibility of the Proposed Method for Determination of Organic Acids in Honey Samples

organic acid	sample 1 mean[a]	sample 1 RSD (%)	sample 2 mean[a]	sample 2 RSD (%)	sample 3 mean[a]	sample 3 RSD (%)
		(A) Repeatability (*n* = 5)				
oxalic	14.0 ± 0.6	4.3	42.3 ± 1.1	2.6	113.9 ± 2.1	1.8
formic	63.2 ± 2.9	4.6	120.1 ± 3.2	2.7	908.4 ± 9.9	1.1
malic	NQ[b]		105.3 ± 2.8	2.7	539.1 ± 6.4	1.2
succinic	ND[c]		37.1 ± 1.7	4.6	59.8 ± 2.2	3.7
pyruvic	ND		ND		ND	
acetic	62.4 ± 1.7	2.7	86.6 ± 1.0	1.2	323.4 ± 3.1	1.0
lactic	NQ		180.4 ± 3.3	1.8	302.9 ± 0.6	0.2
citric	48.5 ± 1.2	2.5	109.1 ± 0.9	0.8	329.2 ± 0.9	0.3
gluconic	3.73 ± 0.03	0.8	8.35 ± 0.04	0.5	12.92 ± 0.04	0.3
		(B) Reproducibility (*n* = 3)				
oxalic	16.0 ± 1.6	10.0	40.4 ± 2.4	5.9	116.0 ± 3.8	3.3
formic	59.1 ± 3.5	5.9	124.2 ± 5.3	4.3	905.6 ± 27.3	3.0
malic	NQ		108.1 ± 1.0	0.9	552.7 ± 14.0	2.5
succinic	ND		36.0 ± 2.5	6.9	61.7 ± 1.9	3.1
pyruvic	ND		ND		ND	
acetic	64.8 ± 3.8	5.9	89.1 ± 2.5	2.8	323.9 ± 1.5	0.5
lactic	NQ		179.7 ± 8.1	4.5	314.2 ± 12.5	4.0
citric	46.3 ± 3.8	8.2	107.0 ± 2.6	2.4	351.8 ± 24.4	6.9
gluconic	3.65 ± 0.04	1.10	8.20 ± 0.13	1.59	12.77 ± 0.19	1.52

[a] Mean concentrations of all organic acids are quoted as mg/kg units ± standard deviation except for gluconic acid, which is quoted as g/kg units. [b] Not quantifiable. [c] Not detectable.

Table 6. Study of the Recovery of the Proposed Method To Determine Organic Acids in Honey

organic acid	sample A mean (%) ± SD	sample A RSD (%)	sample B mean (%) ± SD	sample B RSD (%)
oxalic	98.2 ± 0.5	0.5	99.6 ± 2.1	2.1
formic	103.2 ± 3.2	3.1	103.0 ± 0.5	0.5
malic	99.0 ± 5.4	5.4	100.7 ± 1.5	1.5
succinic	96.6 ± 4.5	4.6	100.8 ± 3.1	3.0
pyruvic	98.2 ± 6.8	6.9	96.4 ± 5.8	6.0
acetic	104.6 ± 4.8	4.6	100.0 ± 5.1	5.1
lactic	97.5 ± 2.6	2.7	101.2 ± 2.8	2.8
citric	100.5 ± 5.1	5.1	89.4 ± 10.1	11.3
gluconic	100.2 ± 1.2	1.2	100.5 ± 2.8	2.8

area and *x* the amount of organic acid in milligrams per kilogram of honey. **Table 4** lists the parameters and correlation coefficients of the calibration plots.

Each plot was linear over a wide interval from the quantification limit to at least 2400 mg/kg for oxalic and formic acids, 1600 mg/kg for malic and succinic acids, 800 mg/kg for pyruvic acid, 4000 mg/kg for acetic, lactic, and citric acids, and 7200 mg/kg for gluconic acid. These quantities were calculated when the amount of honey was 2.5 g/100 mL. If the organic acid content was high, the amount of honey was 1 g/100 mL and the previous maximum quantities must be increased by the dilution factor of 2.5.

Precision. The precision study comprised repeatability and reproducibility studies. These were developed in three different honeys that contained low (sample 1), medium (sample 2), and high (sample 3) organic acid levels.

The repeatability was established by injecting the honey samples five times. The obtained results are showed in **Table 5A**. The relative standard deviation (RSDs) of the repeatability ranged between 0.2 and 4.6%.

The reproducibility was determined by analyzing each honey sample on three different days over ~1 month. **Table 5B** shows the obtained reproducibility results. The RSDs of the reproducibility ranged between 0.5 and 10.0%.

Recovery. The recovery study was carried out on two honey samples with low organic acid content. It was established by adding three increasing amounts of an organic acid standard mixture to a half amount of honey (1.25 g). **Table 6** summarizes the recovery results for the organic acids analyzed in both samples, as mean, standard deviation (SD), and RSD% parameters. As indicated in this table, the mean recoveries of proposed method for both honey samples ranged between 89.4 and 104.6%.

As can be seen in **Tables 1** and **2**, the proposed CZE method has validation results similar to those of other techniques when validation parameters are compared. Furthermore, the proposed method quantifies a greater number of acids (including the predominant in honey, gluconic acid) and is more rapid than the CZE method proposed by Navarrete et al. (*66*).

Organic Acid Content of Analyzed Honeys. The proposed method was applied to determine the organic acid contents of 10 honey samples (**Table 7**). Data found for formic acid were corrected for mean recovery values because 100% was not inside the mean ± confidence interval. **Table 7** shows an important variability in the composition of the honeys' organic acids. The values found are within the range of values previously described in the literature by using enzymatic or chromatographic methods
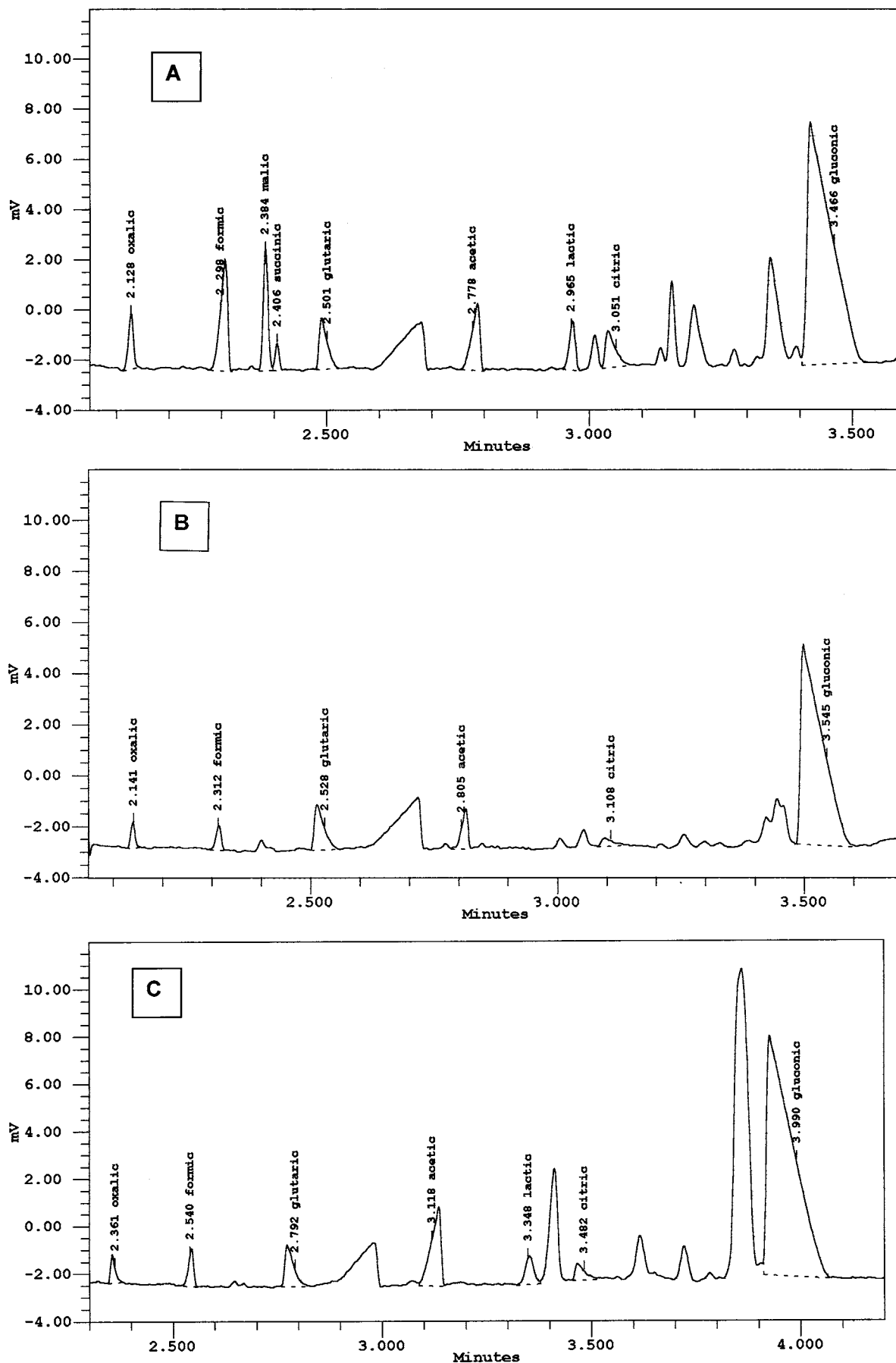
Table 7. Content of Organic Acid in Honeys from Different Botanical Origins

honey botanical origin	oxalic acid (mg/kg)	formic acid (mg/kg)	malic acid (mg/kg)	succinic acid (mg/kg)	pyruvic acid (mg/kg)	acetic acid (mg/kg)	lactic acid (mg/kg)	citric acid (mg/kg)	gluconic acid (g/kg)	total analyzed acids (g/kg)
C. sativa Miller	109	278	486	187	ND[a]	130	579	351	8.0	10.1
C. sativa Miller	114	908	539	60	ND	323	303	329	12.9	15.5
C. sativa Miller	82	889	206	29	ND	336	154	158	14.4	16.3
Eucalyptus sp.	14	63	NQ[b]	ND	ND	62	NQ	49	3.7	3.9
Eucalyptus sp.	NQ	61	ND	ND	ND	NQ	78	48	6.7	6.9
Eucalyptus sp.	NQ	64	ND	ND	39	325	29	ND	8.4	8.9
multifloral	45	131	127	26	ND	97	166	140	5.0	5.7
multifloral	18	131	75	17	ND	39	115	133	11.8	12.3
multifloral	NQ	46	ND	ND	ND	46	34	71	11.9	12.1
multifloral	23	93	21	ND	ND	98	632	91	12.3	13.3
mean	40.4	266.4	145.5	31.9		145.7	208.9	137.0	9.52	10.49
SD	45	340	205	58		131	227	118	3.6	4.1
V_{min}	NQ	46	ND	ND	ND	NQ	NQ	ND	3.7	3.9
V_{max}	114	908	539	187	39	336	632	351	14.4	16.3

[a] Not detectable. [b] Not quantifiable.

Figure 5. Electropherograms of organic acids analyzed in a *C. sativa* Miller honey (**A**), in a *Eucalyptus* sp. honey (**B**), and in a multifloral honey (**C**) according to the proposed CZE method (glutaric acid was added as reference acid to calculate the relative migration times of the honey organic acids). Electrophoretic conditions were the same as in **Figure 1**.

(8−23, 30−41), but obviously they depend on the floral and geographical origins of the honey.

Acetic acid was previously identified in honey (27, 85) but it was not quantified. To the authors' knowledge, this is the first time that this acid has been quantified in honey. The quantification of acetic acid can be used as a fermentation indicator. Gluconic acid was quantified in all honeys, whereas pyruvic acid was found in only one *Eucalyptus* sp. honey (39 mg/kg). The gluconic acid content represents between 79 and 98% of the total content of analyzed acids, so it is possible to suggest the study of it as a useful parameter to determine total acid. Apart from gluconic acid, other acids found in major concentration in analyzed honeys were formic and lactic acids.

Figure 5 shows electropherograms of a *C. sativa* Miller honey (**A**), a *Eucalyptus* sp. honey (**B**), and a multifloral honey (**C**). The highest contents of organic acids were found in *C. sativa* Miller honeys and the lowest contents in *Eucalyptus* sp. honeys. The content of the organic acids of multifloral honey was between the contents of *C. sativa* Miller honeys and *Eucalyptus* sp. honeys.

Conclusions. A reliable, rapid, and simple capillary zone electrophoresis method with direct UV detection has been developed to determine the most important nonaromatic organic acids in honey. The identification and quantification of nine acids (oxalic, formic, malic, succinic, pyruvic, acetic, lactic, citric, and gluconic acids) have been carried out in 4 min with a simple pretreatment of the sample. This is the first time that the predominant acid (gluconic acid) and the minoritary acids (formic, succinic, pyruvic, acetic, lactic, and citric acids) have been determined in honey by using a CZE method. The relative migration times of organic acids were selected as identification criteria. The proposed CZE method is reproducible, linear, precise, accurate, and low-cost for routine analysis of organic acids in honey or even in other foods with a high sugar content.

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1550 *J. Agric. Food Chem.,* Vol. 54, No. 5, 2006

Mato et al.

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