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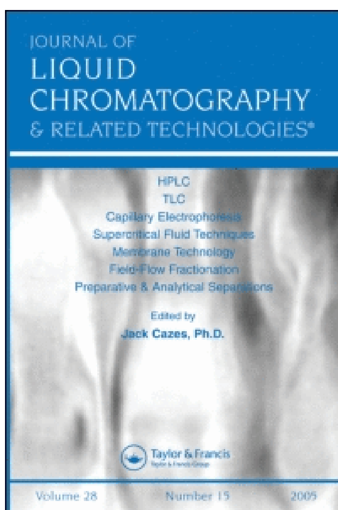

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Online publication date: 26 March 2003

To cite this Article Nozal, Ma. J. , Bernal, J. L. , Diego, J. C. , Gómez, L. A. and Higes, M.(2003) 'HPLC Determination of Low Molecular Weight Organic Acids in Honey with Series-Coupled Ion-Exclusion Columns', Journal of Liquid Chromatography & Related Technologies, 26: 8, 1231 — 1253

To link to this Article: DOI: 10.1081/JLC-120020107

URL: http://dx.doi.org/10.1081/JLC-120020107

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 8, pp. 1231–1253, 2003

HPLC Determination of Low Molecular Weight Organic Acids in Honey with Series-Coupled Ion-Exclusion Columns

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ABSTRACT

A high performance liquid chromatographic method for determination of low molecular weight organic acids (oxalic, *d*-glucuronic, citric, galacturonic, propionic, pyruvic, malic, citramalic, quinic, *d*-gluconic, lactic, formic, glutaric, fumaric, succinic, and butyric) in honey, honeydew, and some anatomic structures of honeybees is reported. In honey and honeydew analysis a dilution and filtration of the sample is enough. Honeybee samples need to be centrifuged and filtered before analysis. The best chromatographic separation of the acids is achieved by using four ion-exclusion columns connected in series, employing water with an 0.1%

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DOI: 10.1081/JLC-120020107 1082-6076 (Print); 1520-572X (Online)
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(v/v) of *o*-phosphoric acid as a mobile phase. Oxalic acid analysis can be carried out using only two columns. The detection is made at 210 nm. The procedure is applied satisfactorily to a variety of matrices.

Key Words: Honey; Organic acids; Ion-exclusion chromatography.

INTRODUCTION

Determination of low molecular weight organic acids in food and beverages has an increasing interest, not only because they are important contributors to taste, but also because they can serve as indicators of quality and authenticity.[1–3] Acids in honey are strongly related to botanical source and also to changes due to storage and aging conditions,[4,5] so, their evaluation can be used to distinguish the origin and as a reference pattern to study the evolution and alteration of the product.[6,7] Moreover, apiculture is nowadays trying to change the use of some acaricides by natural products, among them some organic acids such as formic, lactic, and mainly oxalic are being used to control some beehive diseases. In such cases, attention must be paid to avoid an increase to their proportion in honey, which would cause possible risks to the consumer, or damage to the bees. For this reason, it is necessary to use analytical methodology that allows the determination of many acids, including oxalic.

Oxalic acid is not usually included in the application of conventional reversed liquid chromatography to food and beverage analysis, because its retention and separation is particularly challenging, due to its high polarity.[7,8] For this reason several alternatives had been proposed, especially ion-chromatography with different detection devices[9–12] and, nowadays, capillary electrophoresis.[3,13–15] Other options are the use of combined systems with different columns[16] or its determination as anions, usually employing ion-exclusion chromatography with conductimetric detection.[17,18] At this moment, we think that ion exclusion chromatography is a good alternative because it provides, in honey samples, rapid information about the most important acids including oxalic.

Although ion exclusion chromatography is usually considered unsatisfactory for acid determination because of its poor peak resolution and because of the fact that oxalic acid co-elutes in the dead volume, in this work we have solved the problem by coupling several columns in series. The sensitivity and simplicity of the procedure also makes it possible to obtain information about the acid profile in some anatomic structures of bees, which can be helpful to elucidate the origin of some long term toxic effects[19] observed in bees after applying the oxalic acid to control the *Varroa* mite.

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EXPERIMENTAL

Reagents and Chemicals

Analytical standard-grade (oxalic, *d*-glucuronic, citric, galacturonic, propionic, pyruvic, malic, citramalic, quinic, *d*-gluconic, lactic, formic, glutaric, fumaric, succinic, acetic, and butyric acid) were obtained from Sigma Aldrich Química (Madrid, Spain). Water was purified by passage through a Compact Milli-RO and Milli-Q water system from Millipore (Bedford, MA). Sulfuric acid, *o*-phosphoric acid, and all other chemicals used were of analytical-reagent grade and supplied by Merck (Darmstad, Germany). All solutions used were filtered through a 0.45 μm membrane filter from Millipore to remove any impurity.

Apparatus and Experimental Conditions

The chromatographic set-up consisted of a PV-1580 pump with degasser module, a LG-1580-04 gradient module, and an AS-1555 autosampler for 50 samples with a variable injection loop of 5–100 μL, all from Jasco Analitica Spain (Madrid, Spain). A UV–ViS Diode Array Detector UV6000, in addition to a UV6000 LP Chromatography Data System, from TSP Spectra System (San José, CA) was also used.

An ultrasonic bath, a vibromatic stirrer, and a centrifuge were all from Selecta (Barcelona, Spain). A vortex mixer from Fisher Scientific (Pittsburgh, PA) and a 5810 R refrigerated centrifuge from Eppendorf (Hamburg, Germany) were also used.

The columns used in the experimentation were: An Aqua® 5 μm, 250 × 4.6 mm and a Rezex® 8 μm, 300 × 7.8 mm, both from Phenomenex (Torrance, CA), and four IC-Pak® ion-exclusion columns of 300 × 7.8 mm, 7 μm, 50 A, from Waters Assoc. (Milford, MA). The column temperature was kept using a Peltier Column Thermostat A-2103 from Thermotechnic Products GmbH (Langenzersdorf, Austria).

The chromatographic separation of the acids was achieved by using water with 0.1% (v/v) *o*-phosphoric acid as mobile phase, at a flow rate of 1 mL/min.

Samples were injected by means of the autosampler using a fixed volume of 10 μL and detected at 210 nm.

Standard Solutions and Calibration

Stock solutions of acids of 1 g/L were always made in the mobile phase and stored at 4°C. Mixtures of different concentrations were made from those solutions.

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Calibration graphs were obtained using 10 different concentrations of the mixed standard solutions. All samples were prepared and injected in triplicate.

Samples

To check the applicability of the method, it was tested on several honey samples all collected in the same geographical area and belonging to different botanical origins, which were confirmed by standard pollen analysis.

The method has also been applied to trace analysis of the acids in some anatomic structures of bees. For this purpose, the bees were gassed with a current of carbon dioxide and dissected.

Sample Treatment

Prior to HPLC analysis, honey samples were subjected to a 1 g : 10 mL dilution, adding, firstly, 0.2 mL of 2 M sulfuric acid and completing the volume with the aqueous solution of 0.1% in *o*-phosphoric acid.

Honeybee samples were obtained from 10 adult bees, placed in an eppendorf microvial, adding 50 μL of water, and kept in the refrigerator at 4°C. Before their analysis, they were taken out until they reached room temperature. After centrifuging to 10,000*g* and pouring them through a 0.45 μm filter, they were injected in the chromatographic system.

RESULTS AND DISCUSSION

Optimization of the Separation

In order to maximize the resolution, different experimental parameters influencing the retention (stationary phase, mobile phase, temperature) were always optimized by varying one parameter at a time, and keeping all the others constant. The study began using two columns coupled in series, afterwards a third column was added and finally four columns were coupled. The variation of the retention time of the acids according to the number of columns used can be observed in Fig. 1. The results shown below belong to the four column system. The other coupling will be mentioned only in case of a remarkable difference.

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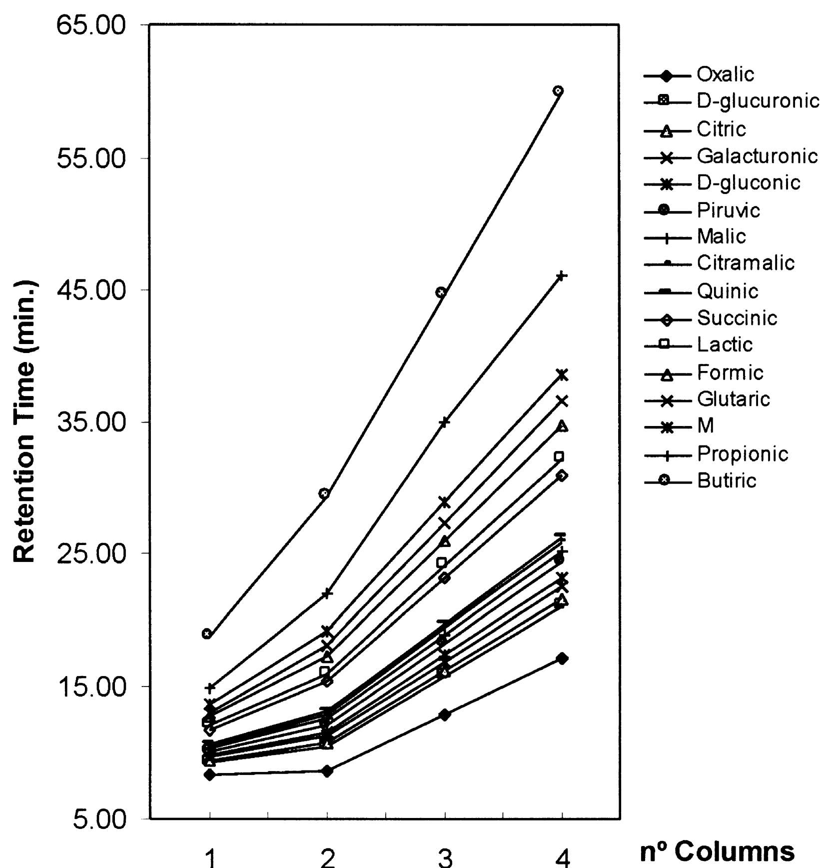

Figure 1. Influence of the number of ion exclusion columns on the retention time of the acids.

Stationary Phase

As has been indicated, our intention was initially to find a method to determine oxalic acid, and in order to get it we tried several columns, typically used to separate organic acids in different matrices. Firstly, a C_{18} column, Aqua was selected. Several mobile phases such as ammonium dihydrogen phosphate, sulfuric acid, and phosphoric acid, at different concentrations and pH were tested, but good separation among the front, other acids, and the oxalic acid was not obtained. Nevertheless, *o*-phosphoric acid showed the best results. In another attempt, and taking our previous experience in organic acid

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profile study into account, we assayed the coupling of that column with another, the Rezex column, satisfactorily used in other works.[20] With this system, we also found several interferent effects while applying it to real samples, due to the presence of other acids.

Finally, we decided to try ion-exclusion columns and aqueous *o*-phosphoric acid as a mobile phase. The use of only one column did not allow a good separation of oxalic acid; this problem can be solved by coupling two similar columns in series, although the other acids considered in this study still eluted very overlapped.

Influence of the Amount of *o*-Phosphoric Acid in the Mobile Phase

Studying the influence of the *o*-phosphoric acid concentration on solute retention, it was observed that concentrations higher or lower than 0.1% led to overlapping among several acids, so finally an 0.1% (v/v) of *o*-phosphoric acid was selected.

Influence of Column Temperature

In order to know the effect of temperature on the separation, the columns were set from 20 to 60°C, at intervals of 10°C, using an aqueous mobile phase containing a 0.1% (v/v) of *o*-phosphoric acid. As can be expected, higher temperatures implied a decrease in the retention times, particularly for the most strongly retained compounds. A temperature of 40°C was selected, because with this temperature most of the acids considered eluted individually.

Taking the above results into account, an aqueous phase of 0.1% (v/v) of *o*-phosphoric acid and a working temperature of 40°C were selected. Under these conditions, an overlapping (fumaric-acetic, peak named **m**, confirmed by spectral analysis), still appeared. The chromatogram of a standard mixture of the acids considered is shown in Fig. 2.

Retention times were highly reproducible among chromatograms; the variation coefficients obtained for the mixture of standards (250 mg/L each) in six consecutive runs ranged from 2.25×10^{-2} for pyruvic to 9.55×10^{-2} for quinic.

Method Validation

Calibration

The standard addition methodology was used to carry out the calibration. The sets of data obtained in two calibration experiments, one with standard

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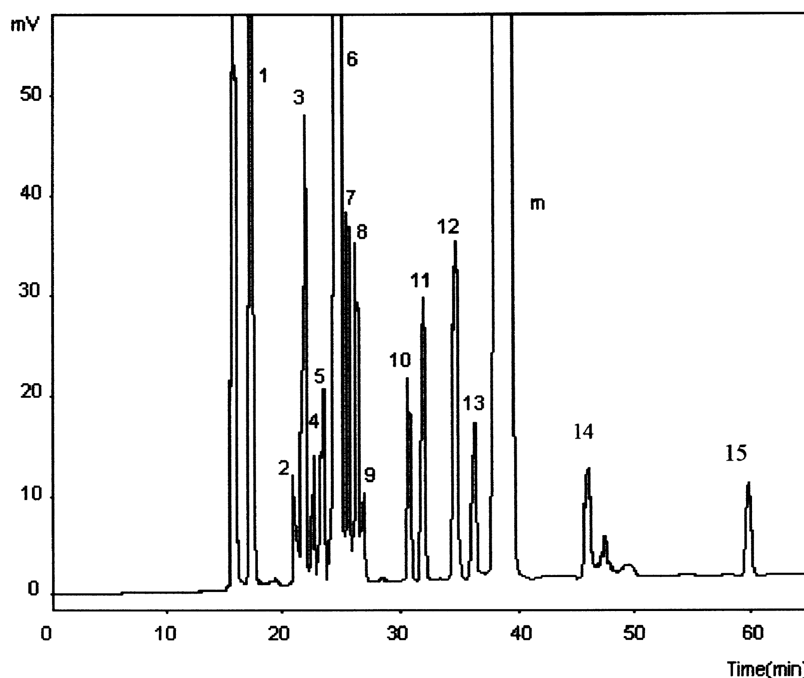

Figure 2. Chromatogram obtained using four columns coupled in series for a mixture of standards (250 μg/mL in each). Peak identification numbers as in Table 2.

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solutions (namely: standard calibration, SC), and the other one with standard additions (AC) were used for each acid. The accuracy of the analytical results was tested by comparing both the analytes content in the different calibrations and the recoveries, calculated by dividing the found net content by that used in each addition.

The SC experiment was run with triplicate injections of the standard solutions, and from the responses vs. concentrations, the linearity ranges were deduced.

Only one injection of each solution was made in the other calibration procedure. The numerical values of the parameters of these calibrations are shown in Table 1. The conclusion obtained is that the determination of acids in honey can be carried out directly by the SC method. The analytical characteristics of the proposed SC method are summarized in Table 2.

The detection and determination limits were calculated experimentally as a signal-to-noise ratio of 3 and 10, respectively.

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Table 1. Features of the proposed HPLC method for the determination of acids.

Acid		r^2 (%)	S_{xy}	S_a	S_b	Calibration curve
Oxalic	SC	99.99	2.16×10^4	8.87×10^3	1.29×10^2	$y = 1.28 \times 10^4 + 4.51 \times 10^4 x$
	AC	99.88	3.73×10^4	1.75×10^3	5.31×10^2	$y = 2.62 \times 10^4 + 4.38 \times 10^4 x$
D-Glucuronic	SC	99.94	2.95×10^3	1.37×10^3	0.63×10^1	$y = 1.67 \times 10^3 + 5.94 \times 10^2 x$
	AC	99.80	5.66×10^2	2.72×10^3	1.87×10^1	$y = 3.57 \times 10^2 + 5.95 \times 10^2 x$
Citric	SC	99.99	4.88×10^3	2.27×10^3	1.06×10^1	$y = 1.18 \times 10^4 + 3.08 \times 10^3 x$
	AC	99.60	4.40×10^3	2.05×10^3	5.64×10^1	$y = 7.71 \times 10^4 + 2.88 \times 10^3 x$
Galacturonic	SC	99.98	1.91×10^3	9.69×10^2	0.42×10^1	$y = 7.91 \times 10^3 + 8.00 \times 10^2 x$
	AC	99.40	2.56×10^3	4.28×10^3	7.11×10^1	$y = 8.18 \times 10^3 + 8.43 \times 10^2 x$
D-Gluconic	SC	99.87	2.05×10^3	2.1×10^3	1.15×10^1	$y = 0.97 \times 10^3 + 5.87 \times 10^2 x$
	AC	99.07	3.5×10^3	3.5×10^3	2.0×10^1	$y = 1.35 \times 10^3 + 6.05 \times 10^2 x$
Pyruvic	SC	99.94	5.64×10^4	3.56×10^4	2.37×10^2	$y = 6.99 \times 10^4 + 2.69 \times 10^4 x$
	AC	99.80	2.80×10^4	1.13×10^4	4.04×10^2	$y = -1.13 \times 10^4 + 2.57 \times 10^4 x$
Malic	SC	99.98	1.44×10^3	1.20×10^3	0.54×10^1	$y = 2.86 \times 10^3 + 1.31 \times 10^3 x$
	AC	99.20	2.64×10^4	2.21×10^4	7.88×10^1	$y = 2.21 \times 10^4 + 1.29 \times 10^3 x$

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Citramalic	SC	99.90	6.75×10^3	4.75×10^3	1.76×10^1	$y = 1.86 \times 10^3 + 7.43 \times 10^2 x$
	AC	99.96	6.69×10^2	8.19×10^4	0.99×10^1	$y = 3.19 \times 10^2 + 7.65 \times 10^2 x$
Quinic	SC	99.98	2.01×10^2	9.07×10^2	0.39×10^1	$y = 9.64 \times 10^2 + 9.69 \times 10^1 x$
	AC	99.60	1.13×10^3	1.39×10^3	0.41×10^1	$y = 9.65 \times 10^2 + 1.02 \times 10^2 x$
Succinic	SC	99.92	5.56×10^3	2.26×10^3	1.12×10^1	$y = 1.17 \times 10^3 + 1.92 \times 10^3 x$
	AC	99.88	9.63×10^3	4.47×10^3	8.16×10^1	$y = 1.16 \times 10^3 + 1.75 \times 10^3 x$
Lactic	SC	99.98	4.83×10^3	5.74×10^3	2.48×10^1	$y = 3.93 \times 10^4 + 2.51 \times 10^3 x$
	AC	99.80	8.08×10^3	8.52×10^3	1.44×10^2	$y = -2.75 \times 10^4 + 2.41 \times 10^3 x$
Formic	SC	99.96	1.07×10^4	4.16×10^3	1.36×10^1	$y = 5.78 \times 10^3 + 2.25 \times 10^3 x$
	AC	99.20	4.14×10^3	2.39×10^3	8.25×10^1	$y = 4.90 \times 10^3 + 2.43 \times 10^3 x$
Glutaric	SC	99.96	8.86×10^3	4.63×10^3	2.01×10^1	$y = 8.21 \times 10^3 + 2.12 \times 10^3 x$
	AC	99.40	3.93×10^3	1.85×10^3	4.87×10^1	$y = 4.45 \times 10^4 + 2.01 \times 10^3 x$
Propionic	SC	99.81	2.76×10^3	1.28×10^3	0.63×10^1	$y = 7.44 \times 10^3 + 5.75 \times 10^3 x$
	AC	99.05	3.16×10^3	2.45×10^3	1.66×10^1	$y = 7.35 \times 10^3 + 5.72 \times 10^3 x$
Butyric	SC	99.99	9.67×10^3	5.05×10^3	2.03×10^1	$y = 1.20 \times 10^3 + 1.83 \times 10^3 x$
	AC	99.40	3.98×10^3	4.17×10^3	6.84×10	$y = -7.06 \times 10^3 + 1.69 \times 10^3 x$

Notes: SC, standard calibration; AC, standard addition calibration; y, absorbance (peak area); x, concentration in $mg\,L^{-1}$.

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Table 2. Characteristics of the analytical method derived from the standard calibration data set.

Acid	Precision (RSD%)	Detection limit (mg/L)	Determination limit (mg/L)	Linearity range (mg/L)	Linearity (%)
1. Oxalic	1.66	0.05	0.18	0.20–500	99.99
2. *D*-Glucuronic	1.54	0.78	2.59	3.00–500	99.94
3. Citric	1.87	0.16	0.56	0.60–500	99.99
4. Galacturonic	2.01	1.70	5.70	6.00–500	99.98
5. *D*-Gluconic	2.05	9.73	32.5	10.00–500	99.87
6. Pyruvic	1.39	0.07	0.26	0.30–500	99.94
7. Malic	0.83	0.67	2.23	2.50–500	99.98
8. Citramalic	0.88	1.91	6.36	7.50–500	99.90
9. Quinic	1.34	21.75	72.5	75.00–500	99.98
10. Succinic	1.98	7.51	25.3	30.00–500	99.92
11. Lactic	1.55	0.51	1.70	2.00–500	99.98
12. Formic	0.68	1.17	3.91	4.00–500	99.96
13. Glutaric	1.32	1.07	3.58	4.00–500	99.96
14. Propionic	1.33	20.14	66.83	70.0–500	99.81
15. Butyric	1.74	1.66	5.54	6.00–500	99.99

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Selectivity

In Fig. 3, some chromatograms obtained applying the procedure to unifloral and honeydew samples are shown. Those belonging to some parts of the honeybees appear in Fig. 4. As can be observed in both Figures, it is possible to determine the acids without interference from the matrix. The main problem arises with the mutual interference among acetic and fumaric. Nevertheless, it can be appreciated that in the *Thymus vulgaris* honey chromatogram, the peak **m** does not appear, so it means that in this botanical origin none of these acids are found.

Accuracy

The method was applied both to standards prepared separately, and to a multifloral honey sample prepared in quintuplicate. The results obtained are shown in Tables 3 and 5.

Precision

The method was also applied, under optimal working conditions, both to five aliquots of the same standard and to five aliquots of the same honey,

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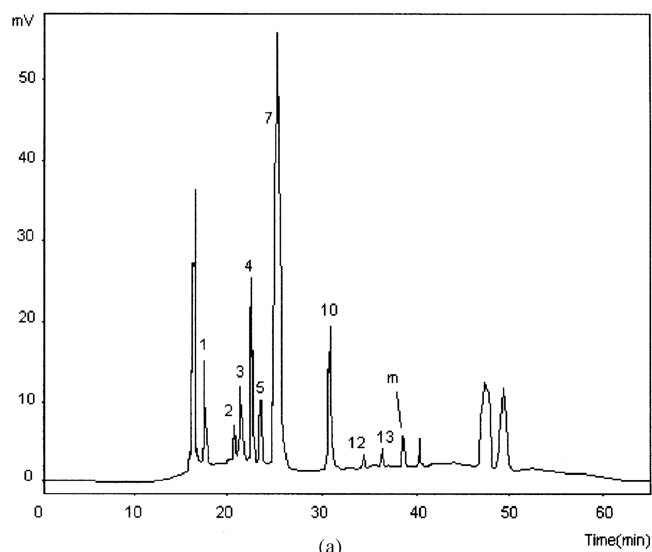

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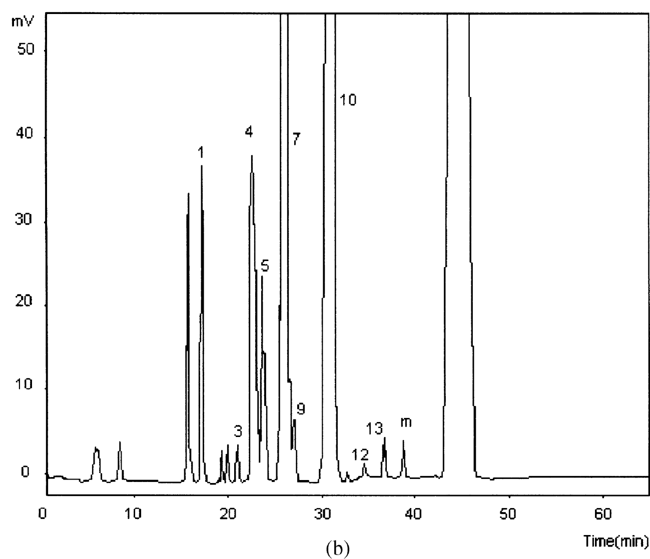

(b)

Figure 3. Chromatograms obtained, using four columns coupled in series, from honey samples. (a) *Multifloral* honey sample, (b) *Calluna vulgaris* honey, (c) *Quercus ilex* honeydew, (d) *Thymus vulgaris* honey. Peak identification numbers as in Table 2.

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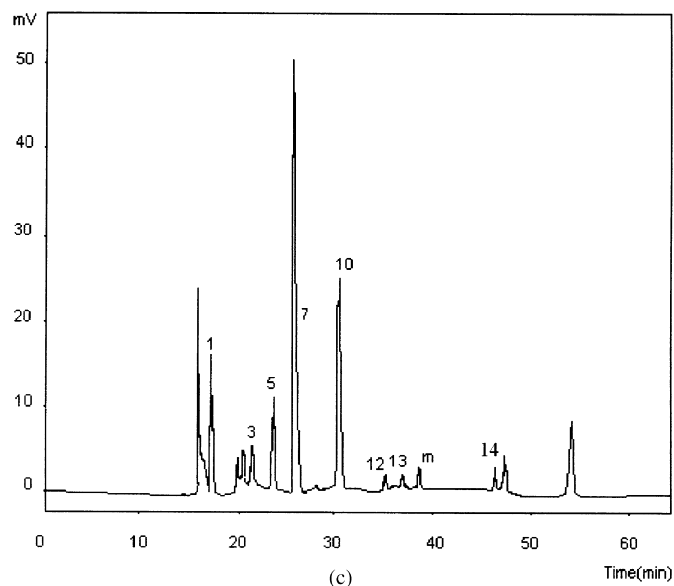

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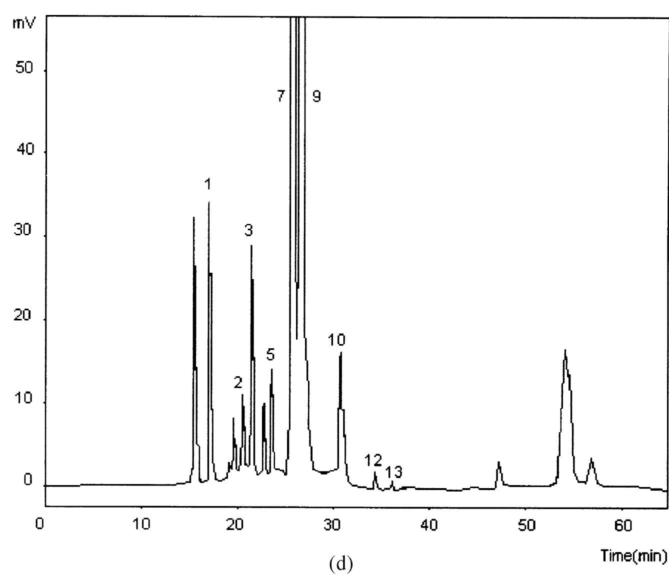

(d)

Figure 3. Continued.

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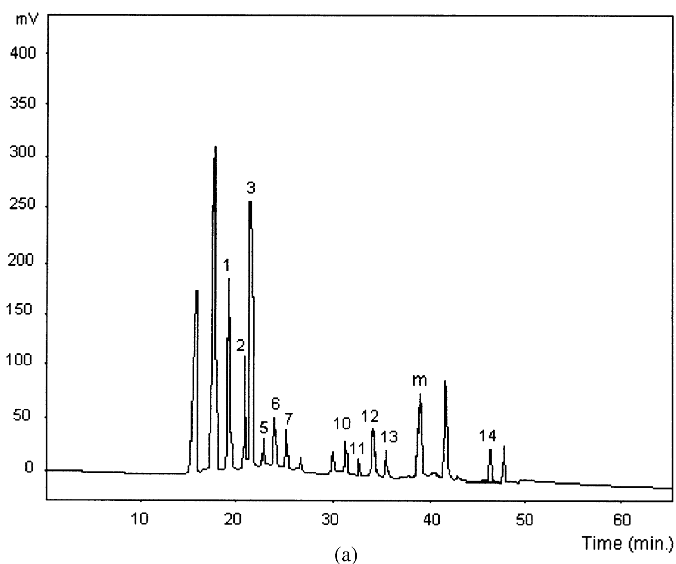

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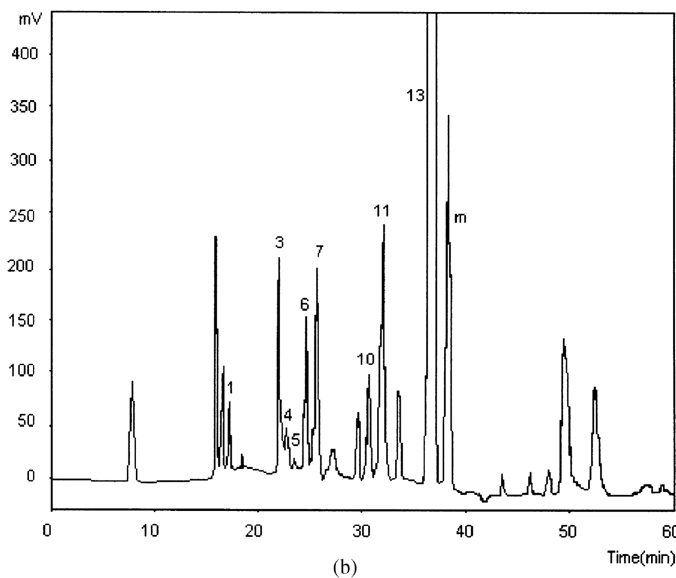

(b)

Figure 4. Chromatograms obtained, using four columns coupled in series, from some anatomic structures of honeybees. (a) Haemolymph honeybee sample, (b) Rectum, (c) Digestive tube, (d) Malpighian tube. Peak identification numbers as in Table 2.

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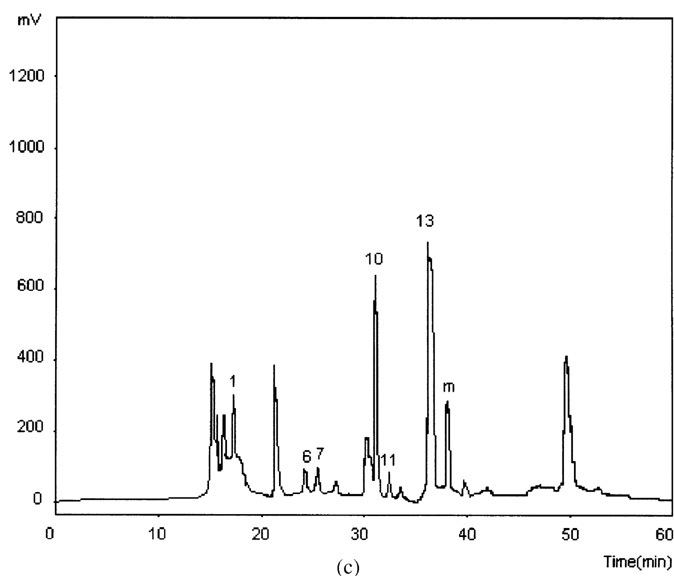
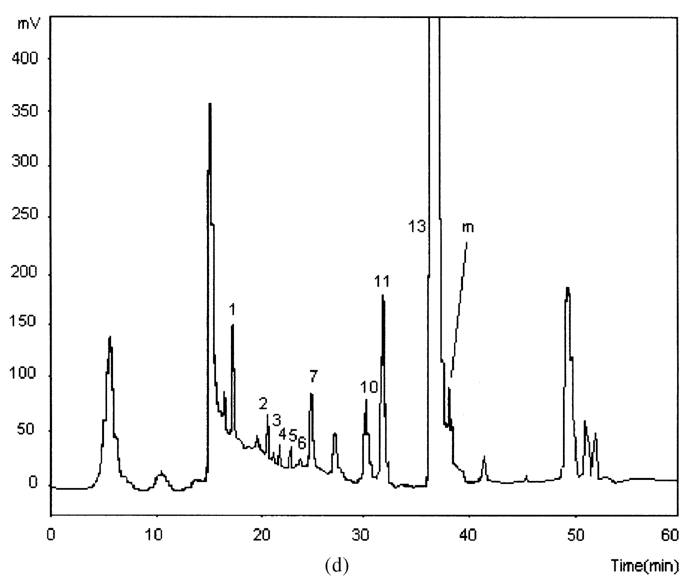


(c)



(d)

Figure 4. Continued.

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Table 3. Accuracy of the method obtained on a multifloral honey (sample preparation repeated five times).

	Oxalic	*D*-Glucuronic	Citric	Galacturonic	*D*-Gluconic	Pyruvic	Formic	Glutaric
MM1 (mg/K)	72.7	52.5	253.6	322.9	2510.0	4.6	<LC	<LC
MM2 (mg/K)	73.4	45.8	239.7	296.1	2529.4	5.4	<LC	<LC
MM3 (mg/K)	74.0	49.4	246.7	304.6	2568.9	4.9	<LC	<LC
MM4 (mg/K)	73.1	48.8	248.5	317.2	2583.9	4.9	<LC	<LC
MM5 (mg/K)	74.0	44.1	249.3	336.3	2531.1	4.5	<LC	<LC
Average (mg/K)	73.4	48.1	247.6	315.4	2544.7	4.9		
σ_{n-1}	0.6	3.3	5.1	15.7	30.6	0.4		
CV (%)	0.8	6.8	2.0	5.0	1.2	7.2		

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Table 4. Precision obtained by injecting a multifloral honey sample five times.

	Oxalic	*D*-Glucuronic	Citric	Galacturonic	*D*-Gluconic	Pyruvic	Formic	Glutaric
MMa (mg/K)	71.6	45.8	245.0	289.5	2445.5	4.9	<LC	<LC
MMb (mg/K)	71.2	43.2	249.4	315.9	2397.6	4.8	<LC	<LC
MMc (mg/K)	71.7	46.4	248.7	295.1	2450.6	5.0	<LC	<LC
MMd (mg/K)	72.2	44.5	240.5	298.6	2445.4	5.4	<LC	<LC
MMe (mg/K)	72.2	43.9	243.8	305.5	2444.2	5.4	<LC	<LC
Average (mg/K)	71.8	44.8	245.5	300.9	2436.6	5.1		
σ_{n-1}	0.4	1.3	3.7	10.2	22.0	0.3		
CV (%)	0.6	3.0	1.5	3.4	0.9	5.5		

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Table 5. Accuracy of the method for standards prepared five times.

	Theor. Conc.[a] (mg/L)	Standard 1 (mg/L)	Standard 2 (mg/L)	Standard 3 (mg/L)	Standard 4 (mg/L)	Standard 5 (mg/L)	Average	σ_{n-1}	CV (%)
Oxalic	90.0	85.5	89.3	90.8	91.0	89.6	89.2	2.2	2.5
D-glucuronic	85.0	85.6	87.5	84.3	87.6	89.5	86.9	2.0	2.3
Citric	85.0	81.5	86.3	87.6	84.9	86.0	85.3	2.3	2.7
Galacturonic	80.0	73.4	78.7	78.2	79.4	82.0	78.3	3.1	4.0
D-gluconic	125.0	121.8	125.9	128.7	120.4	118.4	123.0	4.2	3.4
Pyruvic	75.0	68.4	73.5	75.5	74.8	77.4	73.9	3.4	4.6
Malic	75.0	72.4	78.7	75.3	77.0	78.9	76.5	2.7	3.5
Citramalic	45.0	48.0	41.7	40.9	45.6	45.8	44.4	3.0	6.8
Quinic	90.0	85.5	89.3	90.8	91.0	89.6	89.2	2.2	2.5
Succinic	85.0	85.6	87.5	84.3	87.6	89.5	86.9	2.0	2.3
Lactic	85.0	81.5	86.3	87.6	84.9	86.0	85.3	2.3	2.7
Formic	80.0	73.4	78.7	78.2	79.4	82.0	78.3	3.1	4.0
Glutaric	125.0	121.8	125.9	128.7	120.4	118.4	123.0	4.2	3.4
M	75.0	68.4	73.5	75.5	74.8	77.4	73.9	3.4	4.6
Propionic	106.4	104.5	97.6	98.8	95.3	106.4	100.5	4.7	4.7
Butyric	75.0	72.4	78.7	75.3	77.0	78.9	76.5	2.7	3.5

[a]Standard theoretical concentration (mg/L).

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Table 6. Precision obtained by injecting each standard five times.

	Theor. Conc.[a] (mg/L)	Standard a (mg/L)	Standard b (mg/L)	Standard c (mg/L)	Standard d (mg/L)	Standard e (mg/L)	Average	σ_{n-1}	CV (%)
Oxalic	90.0	90.3	91.3	92.3	89.4	89.1	90.5	1.3	1.5
D-glucuronic	85.0	85.6	86.4	84.3	85.2	84.9	85.3	0.8	0.9
Citric	85.0	85.9	83.9	84.3	86.3	85.6	85.2	1.0	1.2
Galacturonic	80.0	73.6	73.9	74.4	75.8	76.3	74.8	1.2	1.6
D-gluconic	125.0	123.7	129.8	131.5	127.4	122.8	127.0	3.8	3.0
Pyruvic	75.0	71.0	76.5	72.8	77.3	76.4	74.8	2.7	3.7
Malic	75.0	76.4	73.4	76.2	77.2	74.5	75.5	1.5	2.0
Citramalic	45.0	44.0	46.8	43.9	47.2	43.8	45.1	1.7	3.8
Quinic	90.0	80.4	78.3	81.5	80.9	79.3	80.1	1.3	1.6
Succinic	85.0	87.4	89.7	81.2	83.4	84.8	85.3	3.3	3.9
Lactic	85.0	80.7	77.5	83.4	80.8	79.5	80.4	2.1	2.7
Formic	80.0	80.7	85.5	79.7	81.7	78.5	81.2	2.7	3.3
Glutaric	90.0	86.8	91.2	92.7	88.9	92.5	90.4	2.5	2.8
M	225.0	219.1	230.7	227.9	221.4	228.3	225.5	5.0	2.2
Propionic	100.0	105.3	97.5	98.3	102.4	101.4	101.0	3.2	3.1
Butyric	75.0	70.6	78.5	71.5	72.8	76.8	74.0	3.4	4.6

[a]Standard theoretical concentration (mg/L).

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Table 7. Interval and concentration average values obtained in the samples analyzed.

Botanical origin		Oxalic	*D*-Glucuronic	Citric	Galacturonic	*D*-Gluconic
Multifloral(6)	Interval (μg/g)	19.3–133.1		46–372	114–403	2788–4258
	Con. Average (μg/g)	46.2		133	280	3522
Ling(6) (*Calluna vulgaris*)	Interval (μg/g)	104.2–336.0		325–1138	91.7–1217	4942–6892
	Con. Average (μg/g)	189.2		650	446.3	5695
Heather(6) (*Erica sp*)	Interval (μg/g)	85.5–168.4		353–1481	174–725	5477–7016
	Con. Average (μg/g)	128.5		644	445	5943
Lavender(6) (*Lavandula latifolia*)	Interval (μg/g)	11.3–46.4		47–270		2466–4953
	Con. Average (μg/g)	24.9		108		3762
Thyme(5) (*Thymus vulgaris*)	Interval (μg/g)	41.6–114.6	223–659	53–221		2796–4360
	Con. Average (μg/g)	71.2	176	195		3863
Rosemary(4) (*Rosmarinus officinalis*)	Interval (μg/g)	15.3–23.2		12–46		1319–2216
	Con. Average (μg/g)	21.0		23		1733
Oak(2) (*Quercus robur*)	Interval (μg/g)	50.1–58.2	13.7–17.1	183–371		5200–5893
	Con. Average (μg/g)	54.1	15.4	277		5547
French lavender(2) (*Lavandula stoechas*)	Interval (μg/g)	20.9–22.4		29.5–33.8		2683–5544
	Con. Average (μg/g)	21.6		31.7		4114
Honeydew(2)	Interval (μg/g)	52.3–73.1		161–277		3366–4043
	Con. Average (μg/g)	62.7		219		3705

(*continued*)

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Table 7. Continued.

Botanical origin		Quinic	Formic	Glutaric	Propionic
Multifloral(6)	Interval (μg/g)	869–6454		73–246	627–2141
	Con. Average (μg/g)	1596		103	995
Ling(6) (*Calluna vulgaris*)	Interval (μg/g)	1121–7365	63–567	271–554	576–1621
	Con. Average (μg/g)	2207	287	434	1259
Heather(6) (*Erica sp*)	Interval (μg/g)		57–225	164–259	440–886
	Con. Average (μg/g)		147	212	674
Lavender(6) (*Lavandula latifolia*)	Interval (μg/g)			254	618–1213
	Con. Average (μg/g)			143	871
Thyme(5) (*Thymus vulgaris*)	Interval (μg/g)		46–152	43–151	
	Con. Average (μg/g)		78	73	<LC
Rosemary(4) (*Rosmarinus officinalis*)	Interval (μg/g)			37–264	
	Con. Average (μg/g)			62.2	<LC
Oak(2) (*Quercus robur*)	Interval (μg/g)				295–379
	Con. Average (μg/g)				337
French lavender(2) (*Lavandula stoechas*)	Interval (μg/g)			128–140	
	Con. Average (μg/g)			134	<LC
Honeydew(2)	Interval (μg/g)			163–177	630–685
	Con. Average (μg/g)			170	657

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injecting each sample five times. The results obtained are shown in Tables 4 and 6. As can be appreciated from the results obtained for RSD, the proposed method is repetitive.

Application of the Proposed Method

The system using four columns was applied to the different samples related in this study. In Table 7 the results obtained in the study of the acid profile of some honey and honeydew samples are summarized. As can be observed, there are important differences among their botanical origins and the concentration ranges are also of different amplitude. The samples with the highest content are those belonging to *erica* genus and honeydew.

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CONCLUSIONS

The use of four ion-exclusion columns coupled in series allows the analysis of the acids: oxalic, *d*-glucuronic, citric, galacturonic, propionic, pyruvic, malic, citramalic, quinic, *d*-gluconic, lactic, formic, glutaric, fumaric, succinic, and butyric, in honey and anatomic parts of honeybees. To evaluate only the oxalic acid, two columns coupled in series is enough. In both cases, the mobile phase is an aqueous solution of 0.1% (v/v) *o*-phosphoric acid.

Low molecular weight organic acids are found in honey in different proportions according to the botanical origin considered.

ACKNOWLEDGMENT

Financial support of this Project (API98-002) by MAPA-INIA (Spain) is gratefully acknowledged.

REFERENCES

1. Miwa, H. High-performance liquid chromatographic determination of mono-, poly-, and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides. J. Chromatogr. A **2000**, *881*, 365–385.
2. Molnar-Perl, I. Role of chromatography in the analysis of sugars, carboxylic-acids and amino-acids in food. J. Chromatogr. A **2000**, *891*, 1–32.

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3. Klampfl, C.W.; Buchberger, W.; Haddad, P.R. Determination of organic-acids in food samples by capillary-zone-electrophoresis. J. Chromatogr. A **2000**, *881*, 357–364.
4. White, J.W., Jr. Honey. In *Advances in Food Research*; Academic Press: New York, 1978; Vol. 24, 288–375.
5. Singhal, R.S.; Kulkarni, P.R.; Dege, D.V. Honey. Quality criteria. In *Handbook of Indices of Food Quality and Authenticity*; Woodhead Pub.: Cambridge, 1997; 358–385.
6. Cherchi, A.; Espanedda, L.; Tuberoso, C.; Cabras, P. Solid-phase extraction and high-performance liquid-chromatographic determination of organic-acids in honey. J. Chromatogr. A **1994**, *669*, 59–64.
7. Suárez-Luque, S.; Mato, S.; Huidobro, J.F.; Simal-Lozano, J.; Sancho, M.T. Rapid determination of minority organic acids in honey by high-performance liquid chromatography. J. Chromatogr. A **2002**, *955*, 207–214.
8. Szmigielska, A.M.; Vanrees, K.C.J.; Cielinski, G.; Huang, P.M. Comparison of liquid and gas-chromatography for analysis of low-molecular-weight organic-acids in rhizosphere soil. Comm. Soil Sci. Plant Anal. **1997**, *28*, 99–111.
9. Yang, L.; Liu, L.; Olsen, B.A.; Nussbaum, M.A. The determination of oxalic-acid, oxamic acid, and oxamide in a drug substance by ion-exclusion chromatography. J. Pharm. Biomed. Anal. **2000**, *22*, 487–493.
10. Poels, I.; Schasfoort, R.B.M.; Picioreanu, S.; Frank, J.; Vandedem, G.W.K.; Vanderberg, A.; Nagels, L.J. An isfet-based anion sensor for the potentiometric detection of organic-acids in liquid-chromatography. Sensors and Actuators B (Chemical) **2000**, *67*, 294–299.
11. Johnson, S.K.; Houk, L.L.; Feng, J.R.; Johnson, D.C.; Houk, R.S. Determination of small carboxylic-acids by ion-exclusion chromatography with electrospray mass-spectrometry. Anal. Chim. Acta **1997**, *341*, 205–216.
12. Nozal, M.J.; Bernal, J.L.; Diego, J.C.; Gómez, L.A.; Ruiz, J.M.; Higes, M. Determination of oxalate, sulfate and nitrate in honey and honeydew by ion-chromatography. J. Chromatogr. A **2000**, *881*, 629–638.
13. Galli, V.; Olmo, N.; Barbas, C. Development and validation of a capillary electrophoresis method for the measurement of short-chain organic acids in natural rubber latex. J. Chromatogr. A **2000**, *894*, 135–144.
14. Fu, C.G.; Wang, L.X.; Fang, Y.Z. Determination of oxalic acid in urine by co-electroosmotic capillary electrophoresis with amperometric detection. Talanta **1999**, *50*, 953–958.
15. Yang, W.C.; Yu, A.M.; Dai, Y.Q.; Cheng, H.Y. Separation and determination of di- and tricarboxylic acids in fruits by capillary zone electrophoresis with amperometric detection. Anal. Chim. Acta **2000**, *415*, 75–81.

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16. Nozal, M.J.; Bernal, J.L.; Marinero, P.; Diego, J.C.; Frechilla, J.I.; Higes, M.; Llorente, J. J. Liq. Chrom. & Rel. Technol. **1998**, *21*, 3197–3214.
17. Masson, P. Influence of organic-solvents in the mobile-phase on the determination of carboxylic-acid and inorganic anions in grape juice by ion chromatography. J. Chromatogr. A **2000**, *881*, 387–394.
18. Guillén, D.A.; Barroso, C.G.; Zorro, L.; Carrascal, V.; Perez Bustamante, J.A. Organic-acids analysis in brandy de Jerez ion-exclusion chromatography, postcolumn buffering and conductimetric detection. Analusis **1998**, *26*, 186–189.
19. Higes, M.; Meana, A.; Suarez, M.; Llorente, J. Negative long-term effects on bee colonies treated with oxalic acid against Varroa jacobsoni Oud. Apidologie **1999**, *30*, 289–292.
20. Levart, A.; Gucek, M.; Pihlar, B.; Veber, M. Determination of organic-acids in air by capillary-electrophoresis and ion-exclusion chromatography. Chromatographia **2000**, *2*, S321–S324.

Received August 14, 2002
Accepted December 23, 2002
Manuscript 5936

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