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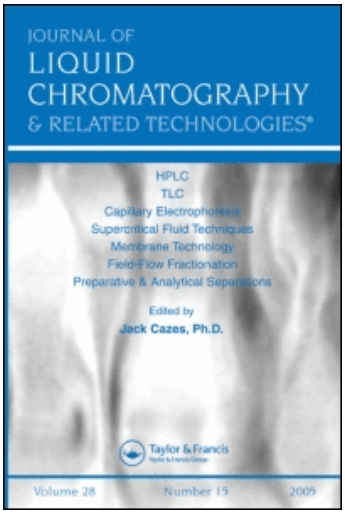

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High Performance Liquid Chromatographic Determination of Organic Acids in Honeys from Different Botanical Origin

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ORGANIC ACIDS IN HONEYS FROM DIFFERENT BOTANICAL ORIGIN

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ABSTRACT

A high performance liquid chromatographic method for the determination of organic acids in honeys, is reported. The components were removed from the matrix by solid-phase extraction with anion exchange cartridges and subsequently separated using two chromatographic systems. The chromatographic separation of the acids was achieved by means of an Organic Acids column, using sulfuric acid as mobile phase. To confirm the identification of the acids two Spherisorb ODS-1 S5 columns connected in series were also used, the mobile phase was ammonium dihydrogen phosphate pH=2.2. The compounds were detected with a UV-Vis detector (210nm).

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The proposed procedure has been applied to determine several organic acids (pyruvic, citric, galacturonic, gluconic, citramalic, glycolic, formic, acetic, butyric, tartaric, malonic, malic, quinic, fumaric, succinic, lactic and propionic) in honey samples from different botanical origin.

INTRODUCTION

Data on organic acids in foods are increasingly required by the food industry for quality control and they can also be used as indicators of deterioration due to the storage, aging, or even to measure the purity and authenticity.

In honey, it is well known that the organic acid fraction contributes to the flavor and also to the taste, however in the latter the sugar content does not allow to appreciate its contribution. The ripening from nectar to honey enhances the acidity; the enzymatic activity transforms mainly glucose into gluconic acid but it also originates another acids by a similar mechanism.

Other factors that also contribute to increase the acid content are the time between the nectar collection and the complete filling of the honeycomb and another enzymatic fermentative processes.[1]

The acid fraction represents about 0.3% of the total honey composition, in this fraction the gluconic, oxalic, citric, succinic, glutamic, formic, malic, and butyric are the most abundant acids.

There are differences between the acid content of monofloral honeys, with a pH average of 3.91 and the honeydew honeys with a pH average 4.45.[2] These differences are also found among the monofloral honeys with different botanical origin.

To obtain the organic acid profile and to determine the most abundant acids, enzymatic[3] or chromatographic[4-8] methods are usually chosen, mainly high performance liquid chromatography on ion-exchange[4] or reversed-phase columns;[5-8] but, in the literature the profile is often found without labelling the peaks or with the determination of only some of them.

In this work we tried to obtain the most complete and possible profile of several monofloral honeys and quantify all the peaks. To confirm the peak identity we propose the use of two different columns, and to isolate the acids we use a clean-up step using strong anion exchange cartridges. The procedure is applied to samples belonging to nine different botanical origins.

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EXPERIMENTAL

Reagents

Analytical standard-grade (pyruvic, citric, galacturonic, gluconic, citramalic, glycolic, formic, acetic, butyric, glutaric, tartaric, malonic, malic, quinic, fumaric, succinic, lactic, and propionic acid) were obtained from Sigma Aldrich Química (Madrid, Spain). The water was purified by passage through a Compact Milli-RO and Milli-Q water system from Millipore (Milford, MA, USA). Ammonium dihydrogen phosphate and all other chemicals used to prepare buffers were analytical-reagent grade and supplied by Merck (Darmstad, Germany). Sulfuric acid was purchased from Scharlau (Barcelona, Spain). All solutions used were filtered through an 0.45 mm membrane from Millipore to remove any impurity.

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Apparatus and Chromatographic Conditions

The chromatographic set-up used consisted of a CD4000 multi-solvent partitioning pump and an SM4000 variable-wavelength UV-Vis detector, all from LDC Analytical (Riviera Beach, FL, USA) in addition to a JCL6000 Chromatography Data System from Jones Chromatography (Littlenton, CO, USA). An ultrasonic bath, a vibromatic stirrer, a centrifuge, (all of them from Selecta, Spain) and a vortex mixer from Fisher Scientific (Pittsburgh, PA, USA) were also used.

The column used was a 30 x 0.78cm I.D. packed with 8 μm particles of Rezex Organic Acids from Phenomenex (Torrance, CA, USA). It was thermostated as required by using a Jones Chromatography oven from Jones Chromatography. To confirm the identification of the acids, two Spherisorb ODS-1 S5 (5 μm) columns (25cm x 0.46cm I.D.) (Phase Separations, Waddinxveen, Netherlands) connected in series were also used. A UV-Vis detector at 210 nm was used in both procedures.

Samples were injected by means of a Marathon autosampler from Spark Holland (Emmen, Netherlands) furnished with a fixed-volume (20 μL) loop.

Samples

In this work 57 samples of honey from 9 different botanical origin were analyzed, and they were collected from the same geographical area (Soria Province, Spain) during the years 1993-1996. Their botanical origin was determined by standard pollen analysis techniques and they were: *Onobrychis Sativa Lam* (4

samples), *Rosmarinus Officinalis* (2 samples), *Lavandula Latifolia* (12 samples), *Lavandula Estoechas* (3 samples), *Thymus spp* (4 samples), *Quercus spp* (10 samples), *Multifloral* (9 samples),.*Calluna Vulgaris* (11 samples), and *Erica spp* (2 samples).

Internal Standard Calibration

Stock solutions of organic acids at a concentration of 5 g/L were made in nanopure water. These solutions were used to prepare different standards by sequential dilution with nanopure water and stored at 4°C.

To assay the influence of the matrix, the analytes were firstly quantified in spiked syrups using the internal standard method. Calibration curves were done with 7 different concentrations of the mixed standard; all samples were prepared and injected in triplicate of a fixed volume of 20 μL in order to calculate coefficients of variation and the chromatographic reproducibility.

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Calibration curves were obtained by using the least-squares method. Peak-height ratios between organic acids and the glutaric acid were used to make the least-squares regression line. The concentration of organic acids in the samples was determined by interpolation from the graphs using the peak-height ratios obtained from unknown samples.

Extraction Clean-up

To isolate the acid fraction from the honey, a procedure involving solid-phase extraction on ion-exchange cartridges was chosen.

Different concentrations of the mixed standard were added to 1g of syrup of glucose and maltose with a known amount of internal standard (glutaric acid) in 10 mL of water. After stirring in a vortex mixer, the mixture was passed through an ionic exchange cartridge (Waters Accell Plus QMA), packed with 500 mg of solid phase that was pre-activated with ammonium dihydrogen phosphate 5.10^{-2}M. The organic acids were eluted with acid and injected into the chromatograph.

The influence of parameters potentially affecting the extraction process was studied in order to establish the optimal conditions for maximum recovery of organic acids with minimum extraction of potential interferents. Experiments were always performed in duplicate. Two groups of samples of the same weight were chosen, known amounts of organic acids were added to one group. One of these groups was used to obtain the chromatogram background and the other one to assess recovery.

Statistical Analysis

Data was subjected to analysis of variance and was performed using the Statistica for Windows Stat Soft Inc 1993.

RESULTS AND DISCUSSION

Chromatographic Conditions

In order to maximize the resolution, the different experimental parameters influencing the retention (stationary phase, pH, ionic strength, and flow rate of the mobile phase) were optimized by varying one parameter at a time, and keeping all the other ones constant.

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Rezex column

Initially, we used a 30 x 0.78cm ID Rezex Organic Acids column packed with particles of 8 μm, often used to separate organic acids, using sulfuric acid as mobile phase at a flow-rate of 0.5 mL/min, as the manufacturer suggested. Several overlappings could be observed. Trying to solve them we studied the influence of the mobile phase and the temperature.

Influence of mobile phase concentration

Studying the influence of the sulfuric acid concentration on solute retention, it was observed that the retention time increased as the acid concentration increased. Finally a 5.10^{-3} M was selected. This concentration allowed the quantification of the formic acid and also to distinguish the pyruvic acid from the front.

Influence of the column temperature

In order to know the effect of the temperature, the column was thermostated from 45 to 65°C at intervals of 5°C, and a mobile phase consisting of 5.10^{-3}M sulfuric acid was used. Figure 1 shows the retention times obtained as a function of the temperature. As it can be seen, increasing temperatures implied a decreasing in the retention times, particularly for the most strongly retained compounds.

A temperature of 55°C was selected because from this temperature both groups, lactic-fumaric and succinic-glycolic, began to separate themselves. This temperature is the same as the usual one recommended by the manufacturer in most of the applications.

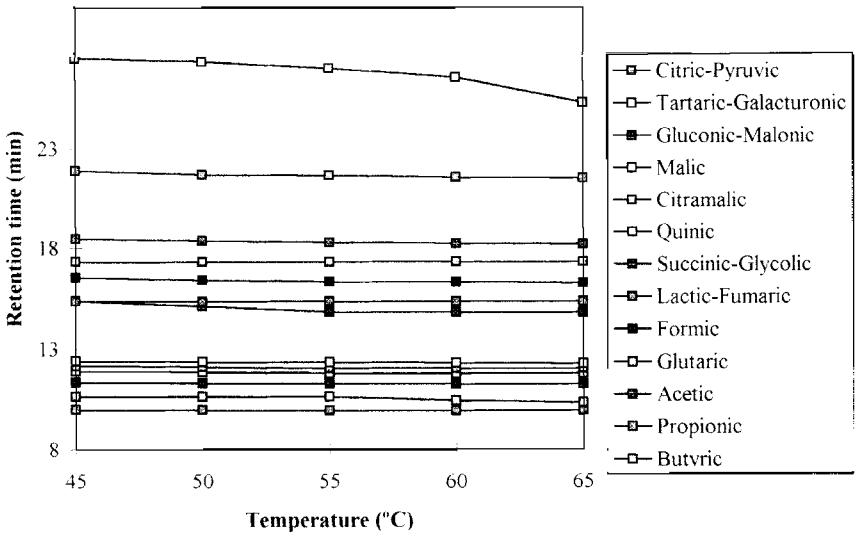

Figure 1. Influence of the column temperature on the retention time of the compounds, for the Rezex column. Mobile phase: sulfuric acid 5.10^{-3}M and 0.5 mL/min.

Taking into account the results obtained, a mobile phase of sulfuric acid 5.10^{-3} M and a working temperature of 55°C were selected. As it can also be observed several overlappings (citric-pyruvic, tartaric-galacturonic, gluconic-malonic, succinic-glycolic, and lactic-fumaric) still appear.

Reverse phase columns

Trying to solve some of those overlappings, we proved the alternative use of two Spherisorb ODS-1 S5 (5 μm) columns (25 cm x 0.46 cm I.D.) connected in series as it is usually recommended in the literature,[6] at a flow rate of 0.7 mL/min. We also studied the influence of the mobile phase and the temperature in order to select the best conditions.

Influence of pH

Taking into account the pKs of the different organic acid analyzed and the minimum pH recommended for the chromatographic column, we assayed the pH between 2.2 and 3.5, using 0.05 M solutions of sulfuric acid as mobile phase. It was observed that increasing the pH the retention times decreased.

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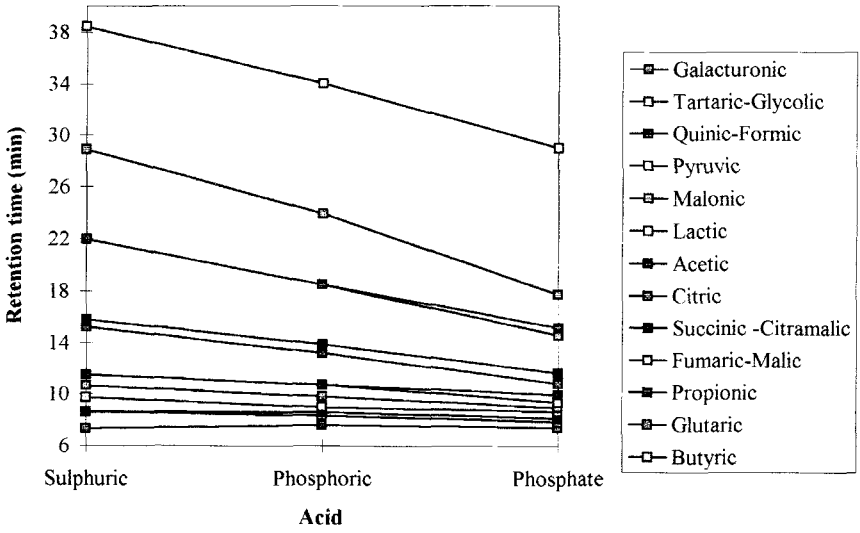

Figure 2. Variation in the retention time of the acids, on Spherisorb columns, according to the acid used in the mobile phase. Mobile phase: pH=2.2 and 0.7 mL/min.

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The pH had a marked effect mainly on the retention of pyruvic acid and galacturonic acid that coelutes with the front when the pH is higher than 2. Because of that and taking into account the inability of working at pH lower than 2.2, to avoid column deterioration, pH=2.2 was selected.

Column temperature

In order to determine the effect of temperature, the columns were thermostated from 25 to 60°C at intervals of 5°C. The same behavior as in Rezex column is repeated again; increasing temperatures implies a decreasing in the retention times, particularly for the most strongly retained compounds. Nevertheless a temperature of 25°C was selected because in this condition the separation of the citric acid was possible.

Mobile phase

In order to achieve better results we thought in the possibility of obtaining the pH 2.2 with a system different from sulfuric acid, so the phosphoric acid and the ammonium dihydrogen phosphate were tested. In Fig. 2 it can be seen that using phosphoric acid as mobile phase it is possible to separate into two groups the

Table 1

Retention Times of the Organic Acids in the Columns Used

A) Rezex Column			B) Spherisorb Columns		
Acid	**tr (min)**	**CV**	**Acid**	**tr (min)**	**CV**
1. Citric	9.92	0.13	5. Gluconic	---	---
2. Pyruvic	9.93	0.23	4. Galacturonic	7.40	0.11
3. Tartaric	10.60	0.21	3. Tartaric	7.83	0.04
4. Galacturonic	10.60	0.10	11. Glycolic	7.83	0.08
5. Gluconic	11.28	0.04	9. Guinic	8.12	0.01
6. Malonic	11.28	0.38	14. Formic	8.12	0.22
7. Malic	11.78	0.10	2. Pyruvic	8.62	0.11
8. Citramalic	12.03	0.16	6. Malonic	8,93	0.03
9. Quinic	12.33	0.08	12. Lactic	9.33	0.11
10. Succinic	14.80	0.13	15. Acetic	9.92	0.07
11. Glycolic	14.80	0.13	1. Citric	10.83	0.09
12. Lactic	15.33	0.05	8. Citramalic	11.63	0.14
13. Fumaric	15.33	0.15	10. Succinic	11.63	0.04
14. Formic	16.30	0.10	7. Malic	14.58	0.01
IS Glutaric	17.30	0.15	13. Fumaric	14.58	0.01
15. Acetic	18.27	0.09	16. Propionic	15.15	0.15
16. Proprionic	21.65	0.06	**IS Glutaric**	17.73	0.18
17. Butyric	26.97	0.01	17. Butyric	28.97	0.15

A) Organic acids column; mobile phase: sulfuric acid 5.10^{-3}M, 0.5 mL/min and 55°C. (B) Spherisorb columns; $(NH_4)H_2PO_4$ $5\cdot10^{-2}$M, pH 2.2, 0.7 mL/min

tartaric, glycolic, quinic, and formic acids, the same happens when the phosphate is used as mobile phase. Moreover in this latter situation it is also possible to separate the lactic and acetic acids, and the propionic acid from the group of fumaric and malic as well.

Taking into account those observations and also that the chromatogram time was reduced, we decided to select the phosphate as mobile phase, then we carried on studying the influence of the ionic strength varying the phosphate concentration between 5.10^{-3} and 10^{-1} M. The effect of the ionic strength on the retention time was slight, nevertheless it must be taken into account that when the ionic strength had lower values the peak width increased and the resolution was worse. After that, a mobile phase of ammonium dihydrogen phosphate 5.10^{-2}M, was selected. Under those conditions, some overlapping (tartaric-glycolic, quinic-formic, succinic-citramalic, and malic-fumaric) still appears.

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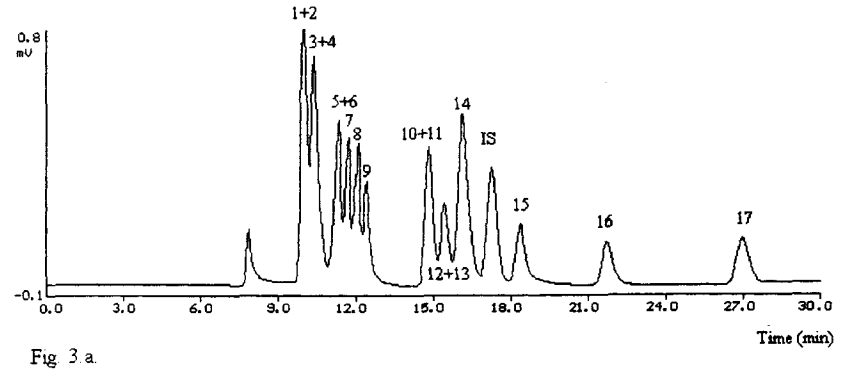

Fig. 3.a.

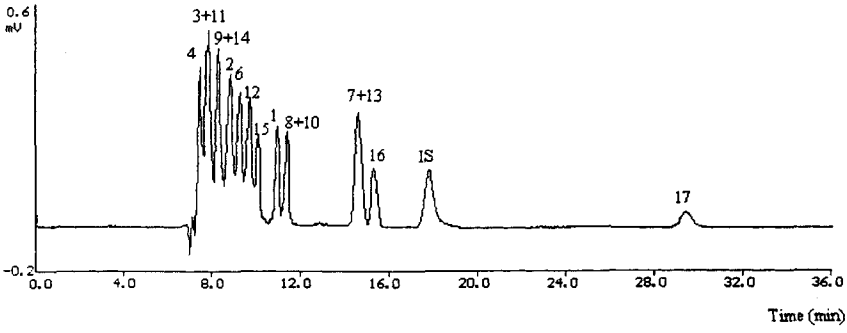

Figure 3. 5.10^{-3}M and 0.5 mL/min; 55°C. 3b. on two Spherisorb columns. Mobile phase: ammonium dihydrogen phosphate 5.10^{-2}M, pH=2.2 and 0.7 mL/min.**3.** Chromatogram obtained with a mixture of standards. 3.a. on the Rezex column. Mobile phase: sulfuric acid $5\cdot10^{-2}$M and 0.5 mL/min, 55°C. 3b. on 2 Spherisorb columns. Mobile phase: $(NH_4)H_2PO_4$ $5\cdot10^{-2}$M, pH 2.2, 0.7 mL/min.

Table 1 shows the retention times of the organic acids in both columns. It can be observed that the overlapping that appears are different, so this can be used to confirm the qualitative analysis and to facilitate the quantitative analysis. Figure 3 shows the chromatograms obtained for a mixture of standards on both columns. Retention times were highly reproducible between chromatograms.

The coefficients of variation obtained with the standards in 7 consecutive runs ranged from 0.01 to 0.38 on Rezex column and from 0.01 to 0.22 on two Spherisorb columns.

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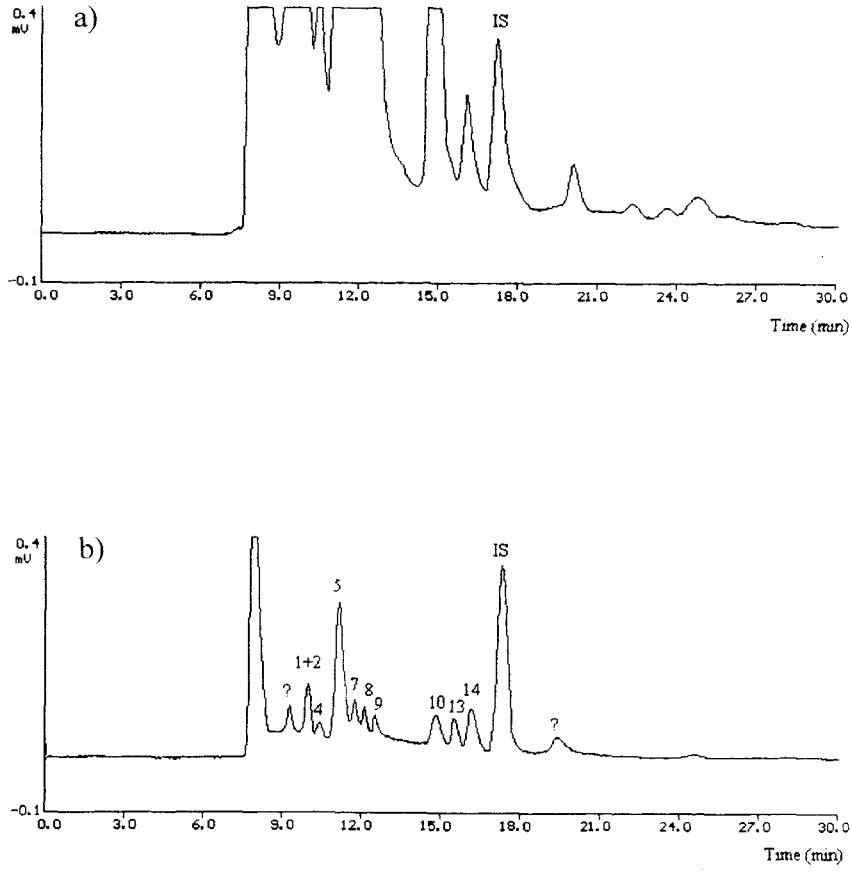

Figure 4. Chromatogram obtained with a multifloral honey on the rezex column: 4a. sample non treated, only diluted (1g in 10mL of nanopure water) 4b. sample submitted to the recommended procedure.

Extraction Clean-up

To analyze organic acid in honey it is necessary to include a clean-up step in order to avoid interferences that appear in great quantity (Figure 4.a). Because of the chemical nature of the acids, the solid phase extraction (SPE) on ionic exchange cartridges seemed to be a good alternative. To know the real possibilities of these cartridges, several experiments were done on a mixture of standards added on a glucose-maltose syrup (to simulate the matrix).

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Table 2

Average Recoveries Obtained for the Acids After Solid Phase Extraction

Acid	% R	CV
1. Citric	102.94	3.23
2. Pyruvic	96.50	5.93
3. Tartaric	94.52	4.40
4. Galacturonic	105.43	3.78
5. Gluconic	98.63	4.21
6. Malonic	83.84	3.98
7. Malic	92.99	5.62
8. Citramalic	103.17	6.67
9. Quinic	88.76	5.64
10. Sucinic	86.18	3.67
11. Glycolic	102.01	5.09
12. Lactic	85.98	2.54
13. Fumaric	83.94	1.51
14. Formic	99.26	6.03
15. Acetic	108.20	4.96
16. Propionic	102.08	5.96
17. Butyric	101.02	3.35
IS Glutaric	97.33	7.35

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Experimental variables whose effects on the extraction yield were examined included pH (8-11), phosphate volume to activate the cartridge (5-20mL), the nature (hydrochloric, phosphoric, sulfuric), the volume (1-3mL) and the concentration (0.1-0.5M) of the acid used to elute all the analytes.

To activate the cartridge a pH=9 was selected because the highest recovery percentages for almost all acids were obtained. With only 15mL of phosphate 0.05M all the acids were retained; higher volumes did not lead to high recoveries.

To elute the organic acids it could be observed that the final recovery increased in this order: hydrochloric acid, phosphoric acid, and sulfuric acid; so the sulfuric acid was chosen. Relating to the elution volume, it was observed that volumes less than 2mL do not allow the total recuperation of the analytes, and volumes higher than 2mL gave the same recovery, but diluting the sample unnecessarily. The concentrations of the eluting sulfuric acid assayed did not have a great influence on the recovery, so a value of 0.25M was selected; higher concentrations led to a bigger chromatographic front which was a problem to evaluate the least retained compounds.

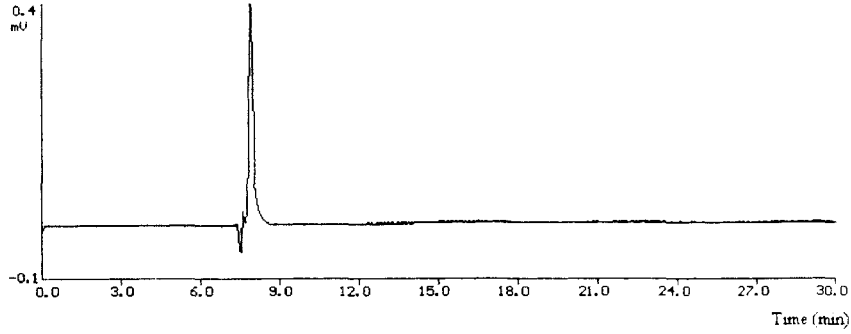

Figure 5. Chromatogram belonging to a none spiked syrup submitted to the procedure.

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From the experiences mentioned above the procedure selected was: the cartridge was activated with 15 mL of ammonium dihydrogen phosphate 5.10^{-2}M, pH=9, then a dilution of honey (1g in 10 mL of nanopure water) was passed through the cartridge, after that it was desiccated by passing N_2 for 10 minutes. The acids were eluted with 2 mL of sulfuric acid 0.25M and injected in the chromatograph.

Figure 4b shows the chromatogram obtained for the same honey as in Figure 4 after treating it with the mentioned procedure. It can be observed that all the interfering peaks have been eliminated completely.

The average recovery of the procedure applied on spiked syrups is shown in Table 2. Typical recoveries ranged from 84% for malonic acid to 108% for acetic acid in all spiked levels. The coefficients of variation were usually less than 7%.

As it can be seen in Figure 5, referring to an extraction on spiked syrup submitted to the procedure, there is no interference from the matrix, and it is possible to analyze acids in the diluted honey sample with high recovery.

Calibration Graphs

The calibration graphs for analytes obtained from the extraction of spiked samples were linear in a wide interval of concentrations from the limit of detection till at least 250 μg/g. The analytical characteristics (n=3) obtained in both chromatographic systems are given in Table 3 (limits of detection are s_b +3s, where s_b is the average signal of the blank and s the standard deviation).

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Table 3

Analytical Characteristics of the Procedure for Both Rezex and C_{18} Columns*

Acid	Concentration μg/mL	Slope		Origin		Regression		Detection Limit(μg.g)	
		Rezex	C_{18}	Rezex	C_{18}	Rezex	C_{18}	Rezex	C_{18}
1. Citric	2-250	2.994	3.046	-4.860	-11.231	0.99965	1.00000	4.332	1.053
2. Pyruvic	0.-25	19.324	65.112	4.810	-100.864	0.99976	0.99948	0.377	1.304
3. Tartaric	2-250	3.693	11.079	-2.636	-30.217	0.99988	0.99990	2.739	5.382
4. Galacturonic	2-250	0.862	10.008	-1.529	110.196	0.99962	0.99985	4.546	6.631
5. Gluconic	2-250	0.649	---	-0.570	---	0.99975	---	3.118	---
6. Malonic	2-250	2.288	5.759	-2.084	-53.474	0.99987	0.99999	3.209	1.706
7. Malic	2-250	1.956	5.903	-4.027	-40.791	0.99967	0.99998	4.492	2.217
8. Citramalic	2-250	1.892	3.230	-3.187	-32.954	0.99994	0.99995	2.040	3.836
9. Quinic	2-250	1.075	4.992	-2.920	-45.377	0.99985	0.99982	3.036	7.330
10. Succinic	2-250	0.949	1.757	-2.861	-15.130	0.99946	0.99999	5.891	2.026
11. Glycolic	2-250	1.185	6.017	-1.861	-42/318	0.99978	0.99998	4.269	2.295
12. Lactic	2-250	1.260	4.582	-2.807	-11.343	0.99955	1.00000	5.182	1.115
13. Fumaric	0.02-3	118.656	267.017	0.274	-44.674	0.99997	0.99998	0.012	0.066
14. Formic	1-100	4.849	9,922	-2.844	-95.154	0.99986	0.99983	1.226	8.678
15. Acetic	3-300	0.935	3.093	-6.463	-24.433	0.99959	0.99991	5.850	5.577
16. Propionic	3-300	0.776	0.712	-2.453	-7.875	0.99973	0.99935	5.545	17.728
17. Butyric	4-500	1.099	0.705	-8.308	-9.559	0.99945	0.99990	6.362	5.606

* (n=7).

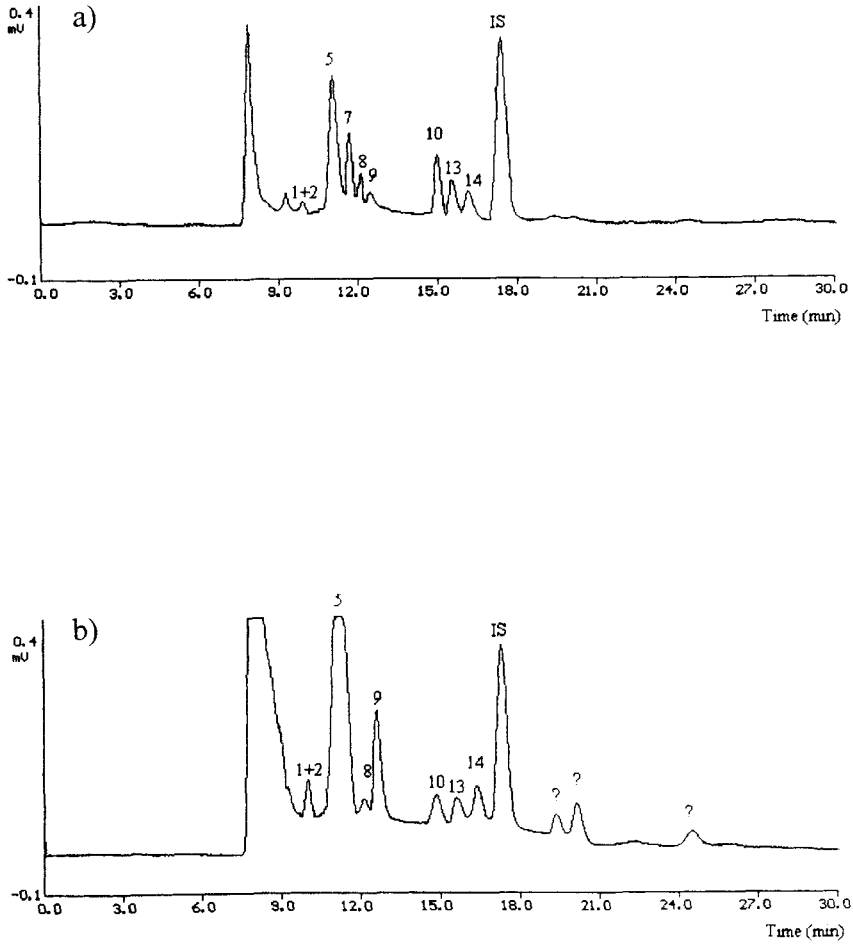

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Figure 6. Chromatogram showing honeys after applying the extraction clean-up procedure.: 6a.- *quercus spp* honey: 6*b*.- *erica spp* honey.

It can be observed that the obtained detection limits are lower using the Rezex column than using two C_{18} columns, except for citric, malonic, malic, succinic, glycolic, and lactic acids. Nevertheless, the sensitivity of the method is better when the C_{18} columns are used. This sensitivity is very high for the fumaric acid in both systems.

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Table 4

Analysis of Variance (ANOVA) for the Data of the Organic Acids in Honeys

Honey Botanical Origin	n Samples	Mean (g Acid/Kg Honey) Citric	Pyruvic	Galacturonic	Gluconic	Malic	Citramalic	Quinic	Succinic	Fumaric	Formic
Onobrychis sativa lam	4	0.096	0.005	0.000	1.600	0.000	0.029	0.000	0.128	0.001	0.098
Rosmarinus officinalis	2	0.049	0.019	0.000	2.437	0.000	0.047	0.051	0.095	0.002	0.075
Lavandula Latifolia	12	0.136	0.026	0.000	3.706	0.000	0.117	0.123	0.821	0.002	0.094
Lavandula Estoechas	3	0.181	0.017	0.000	4.053	0.000	0.027	0.000	0.451	0.003	0.168
Thymus spp	4	0.650	0.010	0.000	1.766	0.313	0.091	0.000	1.409	0.003	0.072
Quercus spp	10	0.191	0.002	0.000	2.199	0.897	0.244	0.015	1.785	0.004	0.030
Multifloral	9	0.404	0.019	0.052	2.678	0.321	0.236	0.171	1.078	0.002	0.094
Calluna Vulgaris	11	0.267	0.029	0.000	3.798	0.153	0.081	0.107	1.433	0.004	0.101
Erica spp	2	0.131	0.011	0.000	4.933	0.000	0.053	3.513	1.146	0.002	0.236
Fcal		2.568	3.900	0.616	4.060	3.425	0.748	18.042	1.266	1.577	1.748
p-Level		0021*	0.001*	0.760	0.001*	0.004*	0.649	0.001*	0.284	0.164	0.112

* Those variable were significant (significance level <0.05, Fcrit = 2.14).

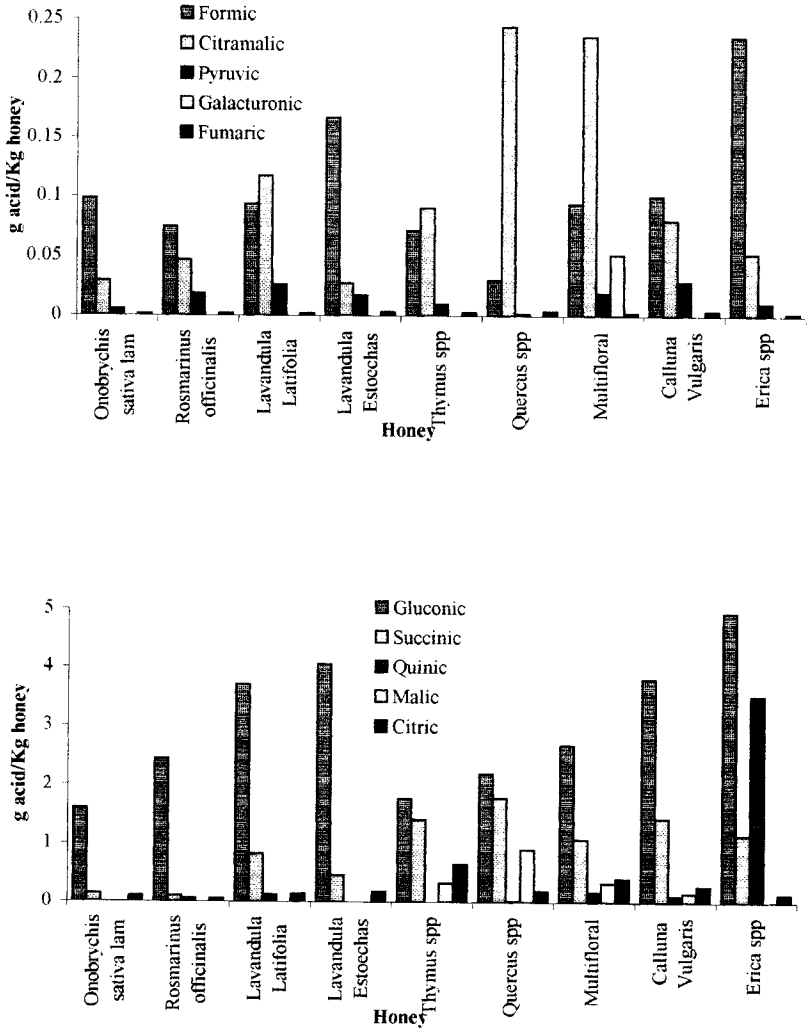

Figure 7. Average values of the acids obtained for each botanical origin.

Application to the Analysis of Honeys

When the selected procedure was applied to the 57 honey samples a noteworthy variability in the qualitative and quantitative acid content was found as it can be observed in Fig 4b and Figure 6, belonging to three typical honey samples *(multifloral, quercus* and *erica spp*).

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ANOVA was applied to the organic acid data trying to know the influence of the botanical origin, the results are shown in Table 4. The acids found in all samples were citric, pyruvic, gluconic, citramalic, succinic, fumaric, and formic, while quinic, malic, and the galacturonic acid appeared in only some of them. Obviously the most abundant acid was gluconic, while the least one was the fumaric .

The acids that can better define the botanical origin seem to be the citric, pyruvic, gluconic, malic, and quinic. So, as it can be observed in Figure 7, *Erica spp* honeys can be distinguished by their high content in quinic acid, *Quercus spp* by their low content in pyruvic acid and high content in malic and succinic acid, *Thymus spp* by their high content in citric acid, and *multifloral* honeys are the only ones where the galacturonic acid has been found.

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CONCLUSIONS

To obtain the overall profile of short chain organic acids in honey with conventional HPLC systems is a hard task, because some overlapping always appears and this can make difficult not only the qualitative analysis but also the quantitation. Using two different stationary phases where the retention is different, so it is possible to identify and determine a large number of organic acids may solve this problem.

Although the organic acids found in the honey samples are very similar they appear in very different proportions according to their botanical origin, so this could be used to characterise or identify the honey. For the analyzed honeys the citric, pyruvic, gluconic, malic, and quinic acids are the ones that best define the botanical origin.

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