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Journal of Chromatography A, 955 (2002) 207–214

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Rapid determination of minority organic acids in honey by high-performance liquid chromatography

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Received 12 December 2000; received in revised form 8 November 2001; accepted 3 December 2001

Abstract

A rapid high-performance liquid chromatographic method for the determination of organic acids in honey is reported. Malic, maleic, citric, succinic and fumaric acids were identified and quantified in 15 min. First time repeatibility, reproducibility and recoveries were determined out for these acids in honey samples. Maleic acid was also quantified for first time by a chromatographic method. The organic acids were removed from honey by using a solid-phase extraction procedure with anion-exchange cartridges. Previously, the solution of honey was adjusted to pH 10.50 with 0.1 *M* NaOH and stirred for 15 min at room temperature. Then, this solution was adjusted to pH 5.00 with 0.1 *M* H_2SO_4. This procedure was carried out to avoid interferences in the baseline. The chromatographic separation was achieved with only one Spherisorb ODS-2 S5 column thermostated at 25 °C. Metaphosphoric acid (pH 2.20) was used as mobile phase at a flow-rate of 0.7 ml/min. Organic acids were detected with a UV–vis detector (215 nm). The precision results showed that the relative standard deviations of the repeatability and reproducibility were ≤3.20% and ≤4.86%, respectively. The recoveries of the organic acids ranged from 62.9 to 99.4%. Under optimum conditions the detection limits ranged from 0.0064 to 7.57 mg/kg and the quantification limits ranged from 0.025 to 10.93 mg/kg.

Keywords: Honey; Organic acids

1. Introduction

Organic acids comprise a small proportion of honey (0.5%) and the total acidity can be used as an indicator of deterioration due to storage, aging or even to measure the purity and authenticity [1]. Organic acids are also components of the honey flavour [2]. The acidity of honey helps to preserve it against spoiling by microorganisms [3].

Nineteen organic acids have been identified in honey [4], which could be useful for characterising different honey types. Citric acid concentration is used as a reliable parameter for the differentiation of two main types of honey: floral and honeydew honey [5].

Some organic acids were determined by enzymatic methods. Citric and malic acids were quantified by an enzymatic test UV Boehringer-Mannheim [5–8].

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00248-0

Paper, column and ion-exchange chromatographic methods were used for identifying several organic acids [9].

With regard to high-performance liquid chromatography (HPLC) applied to organic acids analysis, two different methods were developed: the first one quantified five minority organic acids (pyruvic, malic, citric, succinic and fumaric) with two reversed-phase columns connected in series. The work was carried out on 48 samples. The separation time was 60 min [10]. The second method quantified nine minority organic acids and was carried out on 57 samples. A long and complex chromatographic procedure was needed. The time of separation was 30 min, similar to the method reported in [10]. Two chromatographic procedures were needed: the first one used two reversed-phase columns (Spherisorb ODS-1 S5) connected in series and only three organic acids (galacturonic, pyruvic and citric) were quantified. The second one needed one ion-exchange column (Rezex Organic Acids) and led the quantification of four acids (malic, citramalic, quinic and formic). Other two organic acids (succinic and fumaric), were quantified using both chromatographic methods [11].

The purpose of this paper was to develop a rapid HPLC method for the determination of some minority organic acids in honey using only one reversed-phase column.

2. Experimental

2.1. Materials and methods

2.1.1. Samples

The work was carried out on 50 samples from Galicia (northwestern Spain). The samples were harvested in 1997 and stored in darkness at room temperature until the analysis. The botanical origin of the samples was determined according to the procedure described in [12]. After that, the sediment in the honeys was treated and dyed using the method reported in [13]. Three samples were *Castanea sativa* honeys, 21 samples were *Eucalyptus* sp. honeys, 1 sample was *Trifolium L.* sp. honey and 25 samples were multifloral honeys.

2.1.2. Reagents and materials

Analytical standard-grade malic, maleic, citric, succinic and fumaric acid were obtained from Sigma (St. Louis, MO, USA). Stock standard solutions were prepared by dissolution of acids in Milli-Q water; 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, USA). Working standard solutions were prepared daily by dilution with Milli-Q water. Metaphosphoric acid, sulphuric acid and sodium hydroxide pellets were analytical-reagent grade and supplied by Merck (Darmstadt, Germany).

The samples were filtered through cellulose membrane filters Whatman (0.45 μm, Cat. No. 7000 0002, Whatman, Clifton, NJ, USA) and the solid-phase extraction (SPE) was achieved with a Waters Accell Plus QMA ion-exchange cartridge Part No. WAT020545 (Waters, Milford, MA, USA).

The eluent was filtered with membrane filters Phenomenex (0.45 μm, AFO-0504, CA, USA).

2.1.3. Apparatus

Chromatographic analyses were carried out using a Waters liquid chromatograph equipped with a Waters ILD on-line degasser, a Waters 600E pump, a Waters 717 plus autosampler and a Waters 996 diode-array UV detection system (Waters). The detector signals were recorded on a chromatography data system MILLENNIUM 32®. The column was a Spherisorb ODS-2 S5 (250×4.6 mm I.D., particle size 5 μm).

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

2.1.4. Assay procedure

2.1.4.1. Sample preparation

A 7.50-g amount of honey was dissolved in 75 ml of Milli-Q water. The pH was adjusted to approximately 10.50 using 0.1 *M* NaOH and the mixture was stirred for 15 min using a magnetic stirrer. The pH was then adjusted to approximately 5.00 using 0.1 *M* H_2SO_4. The mixture was transferred with Milli-Q water to a 100-ml volumetric flask, filled up to the mark, and stirred. A 10-ml volume of this

solution was filtered through a 0.45-μm cellulose acetate membrane. The SPE procedure involved an ion-exchange cartridge, which was activated with 10 ml of sodium hydroxide solution 0.1 *M* (percolation rate 3 ml/min). A 10-ml volume of honey solution was passed through at a flow-rate of 0.5 ml/min. The cartridge was washed with 10 ml of water (3 ml/min) and organic acids were eluted with 4 ml of sulphuric acid 0.1 *M* (0.5 ml/min). This solution was injected directly in the chromatograph.

2.1.4.2. Chromatographic conditions

All procedures were carried out isocratically using 4.5% metaphosphoric acid (pH 2.20), filtered through a 0.45-μm membrane, as the eluent at a flow-rate of 0.7 ml/min. This mobile phase must be prepared fresh daily. The column was thermostated at 25 °C. Injection volume was 20 μl and all standards and honey samples were injected in triplicate. The optimum wavelength for the simultaneous determination of the organic acids was 215 nm.

3. Results

The proposed method allowed the identification and quantification of malic, maleic, citric, succinic and fumaric acids in honey. These acids were identified by comparison of their retention times with those of standards and they were quantified by using an external standard calibration. Table 1 shows the retention times of organic acids.

3.1. Detection and quantification limits

The detection limit was calculated as $s_b + 3s$, where s_b is the average signal of ten blank injections and s the standard deviation. The quantification limit was calculated as $s_b + 10s$, where s_b is the average signal of ten blank injections and s the standard deviation [14]. Table 2 shows detection and quantification limits of organic acids analysed. The detection limits ranged from 0.0064 for fumaric acid to 7.57 mg/kg for succinic acid and the quantification limits ranged from 0.025 for fumaric acid to 10.93 mg/kg for succinic acid.

Table 1
Retention times of organic acids analysed

Organic acid	Retention time (min)±SD
Malic	5.81±0.01
Maleic	9.07±0.10
Citric	10.87±0.09
Succinic	11.81±0.10
Fumaric	13.61±0.10

Table 2
Detection and quantification limits of organic acids analysed

Organic acid	Detection limit (mg/kg)	Quantification limit (mg/kg)
Malic	1.55	2.93
Maleic	0.059	0.075
Citric	1.44	2.72
Succinic	7.57	10.93
Fumaric	0.0064	0.025

3.2. Calibration curves

Calibration curves were determined for seven different concentrations of a mixture of organic acids standard solutions. Each calibration sample was injected in triplicate. Calibration graphs for each compound were obtained by plotting concentration against peak height and applying the least squares method. Table 3 lists the parameters and correlation coefficients of the calibration plots. Each plot was linear over a wide interval from the detection limit to at least 400 mg/kg for malic and citric acid, 800 mg/kg for succinic acid, 5 mg/kg for maleic acid and 7.5 mg/kg for fumaric acid.

Table 3
Parameters and correlation coefficients (r) of calibration plots for organic acids analysed

Organic acid	a	b	r
Malic	19.79	45.79	1.0000
Maleic	1272	41.66	1.0000
Citric	19.65	0.5058	0.9999
Succinic	6.984	46.70	0.9994
Fumaric	1401	38.05	0.9998

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

3.3. Precision

The precision study was comprised of repeatability and reproducibility studies. These were developed in three different honeys which contained low, medium and high organic acids levels.

The repeatability was established by injecting the honey samples five times. The reproducibility was determined by analysing each sample of honey on 3 different days over about 1 month. Tables 4 and 5 show precision results. The relative standard deviations (RSDs) of the repeatability and the reproducibility are $\leq$3.20% and $\leq$4.86%, respectively. These results indicate that the present method can be used for quantitative analyses of organic acids in honey. It was not possible to compare the obtained results with consulted references [10,11] because these did not give precision data for analysis of honey samples.

Table 6
Standard solutions recoveries after solid-phase extraction procedure

Organic acid	Recovery (%) (mean±SD)	RSD (%)
Malic	101.8±0.18	0.18
Maleic	103.3±0.099	0.10
Citric	100.8±0.085	0.08
Succinic	99.2±0.34	0.34
Fumaric	103.4±1.43	1.38

3.4. Recovery

To establish the efficiency of the organic acids extraction, the procedure was applied to a mixture of standard solutions. The results are shown in Table 6.

This procedure was also performed on a mixture of organic acids added to honey. Table 7 shows the recoveries of these carboxylic acids after applying

Table 4
Repeatability of the proposed method for determination of organic acids in honey samples

Acid	Repeatability (n=5)					
	Sample 1		Sample 2		Sample 3	
	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)
Malic	35.5±1.9	2.29	109.4±2.9	2.67	274.1±8.8	3.20
Maleic	0.213±0.011	0.93	0.257±0.008	3.11	0.143±0.003	1.93
Citric	70.9±1.6	0.90	120.8±0.8	0.70	390.3±10.5	2.68
Succinic	23.44±0.04	0.27	31.2±0.22	0.71	152.9±3.1	2.02
Fumaric	0.130±0.008	1.21	1.011±0.007	0.68	6.88±0.24	2.94

Table 5
Reproducibility of the proposed method for determination of organic acids in honey samples

Acid	Reproducibility (n=3)					
	Sample 1		Sample 2		Sample 3	
	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)	Mean±SD (mg/kg)	RSD (%)
Malic	34.6±1.0	2.95	113.1±3.4	3.03	268.0±10.4	3.90
Maleic	0.203±0.008	3.72	0.252±0.011	4.40	0.147±0.004	2.57
Citric	69.5±3.2	4.68	122.3±3.2	2.59	378.4±12.0	3.18
Succinic	23.10±1.12	4.86	30.69±0.92	2.99	149.4±3.4	3.07
Fumaric	0.128±0.005	4.15	0.987±0.045	4.59	7.08±0.09	3.23

Table 7
Recovery of carboxylic acids added to honey after the solid-phase extraction procedure

Organic acid	Recovery (%) (mean±SD)	RSD (%)
Malic	62.9±4.4	7.0
Maleic	93.4±8.2	8.8
Citric	99.4±1.5	1.5
Succinic	75.0±5.0	6.7
Fumaric	94.4±4.6	4.9

the extraction procedure. It is not possible to compare our results those of other workers because it is the first time that this study has been carried out on honeys. In [10] and [11] the recovery results were carried out using standard solutions and syrup, respectively.

3.5. Organic acid content of honeys analysed

The organic acid contents of 50 honey samples are shown in Table 8. Data found for malic, maleic, succinic and fumaric acids were corrected for recovery values because 100% was not inside the mean±confidence interval. It is the first time that maleic acid has been determined and quantified. An important variability in the composition of the honeys' organic acids was found. This variability could be explained by the different origin of the honeys. The malic, citric, succinic and fumaric acids concentration were very high in *Castanea sativa* honeys and very low in *Eucalyptus* sp. honeys. The maleic acid concentration was high in multifloral honeys. Malic and succinic acids were not detectable in the *Trifolium L* sp. honey.

Fig. 1 shows chromatograms of a *Castanea sativa* honey and a multifloral honey.

Judging from the citric acid concentrations, there are no honeydew honeys in the samples analysed. These results have been confirmed by analysing the sediment [12].

4. Discussion

First of all, working with standard solutions, ten organic acids (gluconic, tartaric, formic, malic, lactic, acetic, maleic, citric, succinic and fumaric) were separated and identified by the present chromatographic method. Fig. 2 shows a chromatogram of these organic acids standard solutions. Although several problems appear when this method was applied to honey samples, malic, maleic, citric, succinic and fumaric acids were quantified in honey. Gluconic acid (predominant in honey at concentrations of g/kg), cannot be quantified because the anion-exchange cartridge was probably saturated. Formic, tartaric, lactic and acetic acids show very low and variable recovery values.

4.1. Chromatographic conditions

4.1.1. Influence of the column type

Three reversed-phase columns were used: a Nova-Pack C_{18} (150 mm×3.9 mm I.D., 4 μm) column; a Nova-Pack C_{18} (250 mm×4.6 mm I.D., 4 μm) column and a Spherisorb ODS2 S5 (250 mm×4.6

Table 8
Organic acids content of honeys of different botanical origin

Botanical origin of honey	No. of samples	Malic (mg/kg)			Maleic (mg/kg)			Citric (mg/kg)			Succinic (mg/kg)			Fumaric (mg/kg)		
		Mean	V_{min}	V_{max}	Mean	V_{min}	V_{max}	Mean	V_{min}	V_{max}	Mean	V_{min}	V_{max}	Mean	V_{min}	V_{max}
Castea sativa Miller	3	320	182	434	0.29	0.15	0.50	304	184	394	508	71	759	5.15	3.82	7.29
Eucalyptus sp.	21	70	13	123	0.29	0.12	0.52	38	20	60	49	12	101	0.39	0.04	0.70
Trifolium L, sp.	1	N.D.	N.D.	N.D.	0.14	0.14	0.14	78	78	78	N.D.	N.D.	N.D.	0.07	0.07	0.07
Multifloral	25	117	17	364	0.78	0.17	4.67	124	45	348	66	20	358	1.51	0.14	7.26
Total	50	110	13	434	0.54	0.12	4.67	99	20	394	88	12	759	1.23	0.04	7.29

Values from three replicate injections; N.D., not detectable.

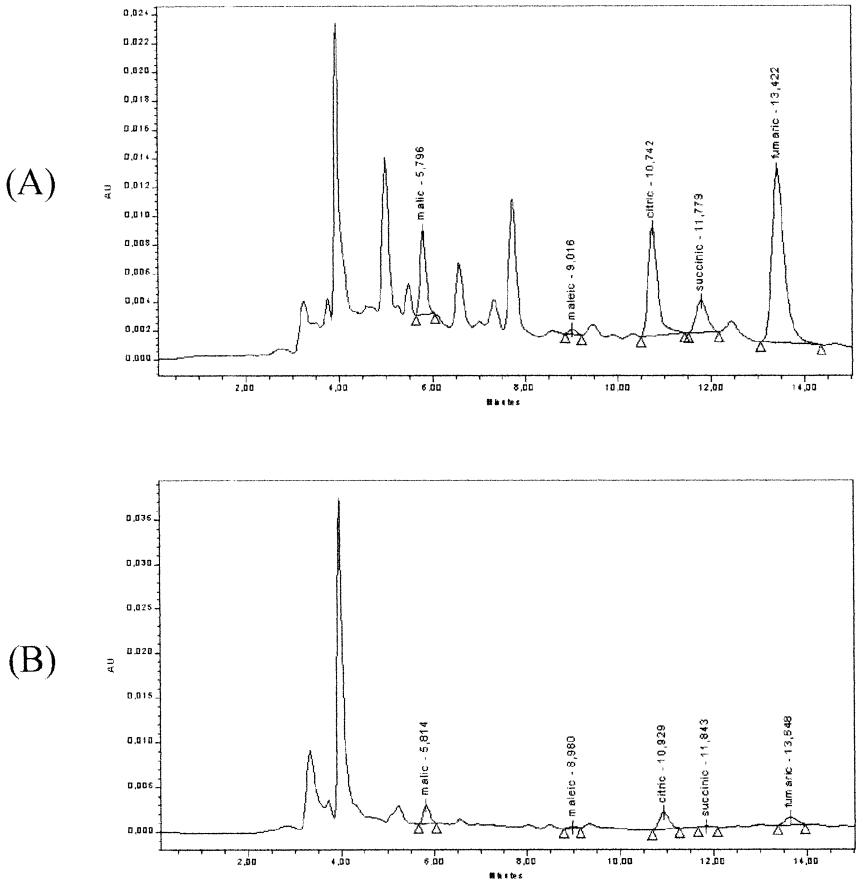

Fig. 1. Chromatograms of organic acids analysed in a *Castanea sativa* honey (A) and in a multifloral honey (B).

mm I.D., 5 μm) column. The Spherisorb ODS2 S5 column was selected because with the other two overlapping occurred.

4.1.2. Influence of the column temperature

The column was thermostated at several different temperatures. The best results were obtained at a temperature of 25 °C.

4.1.3. Influence of mobile phase

Solutions of sulphuric and metaphosphoric acids, recommended in the literature ([10,11] and [15–23]), were used. Metaphosphoric acid was selected because sulphuric acid produced interferences.

4.1.4. Influence of mobile phase pH and flow-rate

Several mobile phase flow-rates and pH values between 2.20 and 3.00 were tested using a solution of metaphosphoric acid and finally a flow-rate of 0.7 ml/min and pH 2.20 were selected.

4.2. Sample preparation

A 7.50-g amount of honey was used for the analyses. Higher amounts saturated the SPE cartridge. Centrifugation of the sample for 10 min at 3800 rpm was tried but the results did not improve. The pH of the sample solution was adjusted to 10.50 with 0.1 *M* NaOH for 15 min at room temperature, to hydrolyse lactones of organic acids [24] and avoid interferences in the baseline.

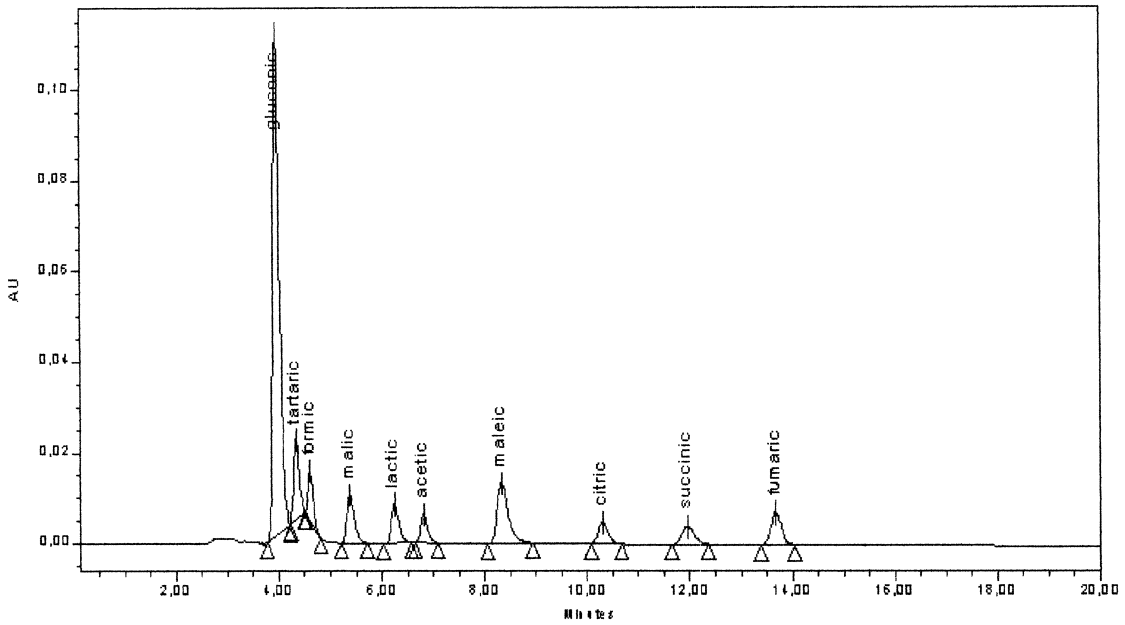

Fig. 2. Chromatogram of a mixture of standard solutions of the organic acids.

4.2.1. Solid-phase extraction procedure

The influence of parameters that potentially affected the extraction process was studied in order to establish the optimal conditions for maximum recovery of organic acids and minimum extraction of interferents.

A C_{18} and an ion-exchange cartridge were tested; the best recovery results were obtained with the ion-exchange cartridge. In order to choose the activation solution, methanol and 0.1 *M* NaOH were tried. The latter solution was selected because methanol generated separation interferences. To clean up the cartridge 10 ml of water Milli-Q was used. Higher volumes did not lead to higher recoveries. To elute the organic acids the best recoveries were obtained with sulphuric acid. To obtain reproducible results, it is very important that the percolation rate was constant.

The proposed method has the advantage of quantifying five minority organic acids (malic, maleic, citric, succinic and fumaric) with a sample preparation procedure similar to other authors ([10] and [11]) and a rapid chromatographic separation in 15 min using only one column.

5. Conclusions

The analysis of organic acids in honey with HPLC systems is a difficult task due to interferences.

The proposed method allows the quantification of malic, maleic, citric, succinic and fumaric acids in honey. To the author's knowledge this is the first time that maleic acid has been determined and quantified in honey.

The analysis is simple, rapid and does not require any complicated sample preparation and only one reversed-phase column is used for the chromatographic separation in less than 15 min.

Acknowledgements

We thank Professor Rafael Cela Torrijos of the Analytical Chemistry Department of the Chemistry Faculty and Juan Carlos García Monteagudo of the Chemistry–Physics Department of the Pharmacy Faculty both of University of Santiago de Compostela and Professor Jesús Simal Gándara of the

Science Faculty of University of Vigo for their helpful comments and for providing material.

We thank all of the beekeepers that provided the ''Producto Galego de Calidade-Mel de Galicia'' honey samples for this study.

We also especially thank the Consellería de Educación e Ordenación Universitaria of the Xunta de Galicia for grants that supported this study (Projects XUGA20308B96 and PGIDT99PXI20307B).

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