JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Capillary Zone Electrophoresis Method for the Determination of Inorganic Anions and Formic Acid in Honey

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A capillary zone electrophoresis method for the determination of inorganic anions and formic acid in honey samples was developed for the first time. The complete separation of chloride, nitrate, sulfate, phosphate, and formic acid was achieved with a simple electrolyte composed by 2 mM potassium dichromate as the carrier solution and background absorbance provider and 0.05 mM tetraethylenepentamine (TEPA) as electro-osmotic flow suppressor (pH 4.00). Injection was performed hydrostatically by elevating the sample at 10 cm for 10 s. The running voltage was −27 kV at 25 °C. Indirect UV absorption detection was achieved at 254 nm. The detection limit was in the range between 0.03 and 20 mg/kg, and the quantification limits ranged from 1.52 to 20.6 mg/kg. The calibration graphs were linear in the concentration range from the quantification limit to at least 2.5 g/kg for chloride, 0.25 g/kg for nitrate, 0.75 g/kg for sulfate, 1.50 g/kg for phosphate, and 0.75 g/kg for formic acid. Precision data in the honey samples analyzed showed repeatability and reproducibility relative standard deviations lower than 1.4 and 2.4% for migration time and lower than 1.8 and 4.3% for anion content, respectively. Recoveries of anions in honey samples analyzed ranged from 94.4 to 99.8%. Ten honey samples were analyzed to test the proposed method. Mean contents of 260.5, 3.93, 60.5, 139.4, and 209.3 mg/kg were found, respectively, for chloride, nitrate, sulfate, phosphate, and formic acid in analyzed honeys. These results agreed with literature data.

KEYWORDS: Anions; capillary zone electrophoresis; honey

INTRODUCTION

Honey is one of the most complex foodstuffs produced by nature and certainly the only sweetening agent that can be used by humans without processing (*1*). The mineral content of honey samples could give an indication of environmental pollution and herewith also an indication of the geographical origin of honey (*2*). Inorganic anions are related to conductivity of honeys, and this parameter is regulated in the quality control of honey samples. Formic acid is important due to its efficacy against *Varroa* infestation, similar to other widely used chemicals such as fluvalinate (*3*). However, formic acid is very volatile, and it is toxic when inhaled, so it is important to have analytical methodology available for its determination (*4*).

Some authors have previously determined anions in honey samples by different procedures. Ehrhardt and Liebig (*5*) analyzed some minerals by complexiomentry and gravimetry including chloride and phosphate, after desiccation of the samples. Chlorides were determined by potentiometric titration of a solution of honey by a mercurimetric volumetry (*6*) or with a silver nitrate solution (*7*). Phosphorus was analyzed by a vanadomolybdate colorimetry of phosphate (*6*, *7*). Sulfur determination consisted of a turbidimetry of barium sulfate (*6*, *7*). The simultaneous determination of anions in honey was carried out according to two procedures: ion chromatography (*8*−*11*) and inductively plasma coupled spectrometry (*12*−*16*). With regard to inductively plasma coupled spectrometry, all methods needed a previous treatment of the samples, such as digestion, mineralization, or calcination, thus making the determinations long and tedious. Ion chromatography seemed to be a good alternative for the determination of anions in honey samples, but one of the published methods needed a previous solid-phase extraction procedure on anionic cartridges (*10*) and the other two methods published were not validated (*9*, *11*). Besides, two of these methods (*10*, *11*) determined only nitrate and sulfate anions.

In relation to formic acid, several authors have developed enzymatic methods for its determination in honey samples (*17*−*21*). It was also determined by ionic chromatography with a

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10.1021/jf061536s CCC: $33.50
Published on Web 11/17/2006

conductivity detector (*22*), by HPLC (*23*−*25*), and by capillary zone electrophoresis (CZE) simultaneously with other organic acids (*26*).

Capillary zone electrophoresis is one of the more powerful separation techniques for the analysis of a wide variety of sample matrices. The advantages of ion analysis using this technique include resolution, speed, simplicity, and reduced sample preparation (*27*).

For these reasons, the aim of this work was the development of a CZE method for the simultaneous determination of the main inorganic anions in honey samples.

MATERIALS AND METHODS

Chemicals. Analytical standard grade chloride, nitrate, sulfate, phosphate, formic acid, potassium dichromate, and tetraethylenepentamine (TEPA) were obtained from Sigma. Stock standard solutions were obtained by dissolution of anions in purified water and stored at 4 °C. The water was purified by passage through an ultrapure water system Milli-Q Plus from Millipore.

Apparatus. Separation was carried out on a Waters capillary ion analyzer (CIA System, 1.3 version) equipped with a negative power supply and a UV detector with a 254 nm wavelength filter. Fused-silica capillaries of 75 μm i.d. and 60 cm in length were used. The distance from the point of injection to the window of on-column detection was 52.5 cm. Electropherograms were collected and plotted by the data acquisition system Millennium 2010 v. 2.15 with specific option CIA for capillary electrophoresis (Waters Associates, Inc., Milford, MA). 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. The electrolyte was filtered with membrane filters Phenomenex 0.45 μm, AFO-0504 (Phenomenex, CA).

Electrophoretic Procedures. Prior to use, new capillaries were treated with the following wash cycle: (i) 10 min with purified water, (ii) 10 min with 1 M NaOH, (iii) 10 min with 10 mM NaOH, (iv) 30 min with purified water, and (v) 60 min with the background electrolyte. Steps i and ii were omitted for daily conditioning of the capillary and, between injections, the capillary was washed for 2 min with running electrolyte.

After all analyses of the day, the capillary was also washed with 10 mM NaOH (10 min) and purified water (30 min).

Running buffer contained 2 mM potassium dichromate as the carrier buffer and background absorbance provider and 0.05 mM TEPA as flow modifier (pH 4.00). The buffer solution was freshly prepared and filtered through a 0.45 μm membrane.

Sample injection was carried out in a hydrodynamic mode by elevating the sample at 10 cm during 10 s. The separation run was at a constant voltage of −27 kV at 25 °C, and it is achieved in 12 min. Indirect UV detection was achieved at 254 nm.

Samples. This work was carried out on 10 samples from Galicia (northwestern Spain) labeled as "Indicación Xeográfica Protexida-Mel de Galicia". The samples were stored in darkness at room temperature until analysis. The botanical origin of the samples was determined after treating and dyeing the sediment (*28*, *29*). Three samples were *Castanea sativa* Miller honeys, four samples were *Eucalyptus* sp. honeys, and three samples were multifloral honeys.

Sample Treatment. For the electrophoretic analysis 1 g of honey was dissolved in 10 mL of purified water, filtered through a 0.45 μm membrane, and injected directly without any other sample treatment.

All standards and samples were injected in triplicate. All statistical analysis was made with Statgraphics (*30*).

RESULTS AND DISCUSSION

The proposed method allows the identification and quantification of chloride, nitrate, sulfate, and phosphate in honey samples. We can also determine formic acid simultaneously. These anions were identified by their migration times, and they were quantified by using an external standard calibration with peak area.

Table 1. Detection and Quantification Limits, Parameters, and Correlation Coefficients (R) of Calibration Plots of Analyzed Anions[a]

			calibration plots		
anion	LOD (mg/kg)	LOQ (mg/kg)	a	b	R
chloride	20.0	20.6	23.4	−446	0.9995
nitrate	0.03	1.52	21.3	31.3	0.9997
sulfate	1.3	3.0	23.3	11.2	0.9995
phosphate	0.5	5.0	58.7	184	0.9998
formic acid	2.5	6.6	38.9	4.2	0.9998

[a] Calibration plots are expressed as regression lines ($y = ax + b$), where y is the peak area and x is the amount in mg/kg.

Background Electrolyte. Dichromate and phthalate were tested as background electrolyte; dichromate was selected because the best results were obtained with this compound and, also, it is a good chromophore, widely used for anion determinations (*31*) and available for working at low pH.

The major problem for the separation of anions in honey is that the analytes are minor components in the presence of a large amount of organic compounds that can disturb the chemical equilibrium and affect the separation. One solution to this problem is converting all analytes into the anionic form and separating them under a suitable suppression of the electroosmotic flow (EOF) and setting the polarity of the driving high voltage in such a way that the anions will move into the column after injection in the opposite direction to the EOF, neutral compounds will not move, and cations will move away, thus not causing interference with the separation of the organic and inorganic anions. Alkyl amines were found to be able to suppress EOF at different degrees, TEPA being one of the most effective (*32*).

For these reasons TEPA was selected as EOF. This compound was previously used for the determination of organic and inorganic anions in Chinese traditional herbs (*32*). The effect of TEPA concentration was tested in the 0.05−0.25 mM range. We observed that the greater the concentration of TEPA was, the higher the migration times were and the poorer the baseline of the electropherogram was. Therefore, a 0.05 mM concentration of TEPA was selected.

Detection and Quantification Limits. The detection limit was calculated as $s_b + 3s$, where s_b is the average signal of 10 blank injections (absolute area value of each anion migration time ± 1%) and s the standard deviation. The quantification limit was calculated as $s_b + 10s$, where s_b is the average signal of 10 blank injections and s the standard deviation (*33*). **Table 1** shows detection and quantification limits of the analyzed anions. The detection limit was in the range between 0.03 and 20 mg/kg, and the quantification limits ranged from 1.52 to 20.6 mg/kg.

Calibration Curves. Calibration curves were determined for seven different concentrations of a mixture of anion standard solutions. Each calibration sample was injected in triplicate. Plotting peak area against concentration and applying the least-squares method, we obtained calibration graphs for each compound. Each plot was linear in a wide interval from quantification limit to at least 2.5 g/kg for chloride, 0.25 g/kg for nitrate, 0.75 g/kg for sulfate, 1.50 g/kg for phosphate, and 0.75 g/kg for formic acid, with correlation coefficients >0.9995. **Table 1** lists correlation coefficients and parameters of the calibration plots for the analyzed anions.

Table 2. Precision of the Proposed Method for the Determination of Anions in Honey Samples

	sample A		sample B	
anion	migration time	content	migration time	content
	Repeatability ($n = 5$)			
chloride	0.1	1.8	0.3	0.3
nitrate	0.1	1.6	0.3	0.8
sulfate	0.5	1.2	0.7	0.2
phosphate	0.3	1.4	0.8	0.3
formic acid	1.4	1.2	1.2	0.4
	Reproducibility ($n = 3$)			
chloride	0.5	3.5	0.4	0.3
nitrate	0.6	1.9	0.4	1.9
sulfate	1.4	1.5	1.3	0.3
phosphate	2.4	1.8	1.1	0.3
formic acid	1.9	4.3	0.5	0.4

Table 3. Recoveries of Analyzed Anions Obtained with the Method of Standard Additions and *p* Values from ANOVA Test for Comparison of the Slopes of Regression Lines Obtained When the Matrix Effect Was Studied

anion	recovery (%) ± SD	*p* value
chloride	94.4 ± 9.6	0.9935
nitrate	97.1 ± 3.1	0.2422
sulfate	98.8 ± 1.2	0.8692
phosphate	99.8 ± 0.4	0.1285
formic acid	97.4 ± 2.8	0.7003

Quantity of Sample and Hydrodynamic Injection Time. We did a study by modifying the dilution of the honey samples (from 1:10 to 1:40) and the hydrodynamic injection time (from 10 to 30 s), and the best response was obtained with the combination of dilution 1:10 and 10 s of injection time.

Precision. The precision study was established in two honey samples and included repeatability and reproducibility. The repeatability was established by injecting five times the same honey on the same day. The reproducibility analysis was determined by analyzing each sample of honey on three different days over about 1 month. **Table 2** shows data obtained (RSD %) in this study. Results were lower than 1.4% for migration time and 1.8% for anion content (milligrams per kilogram) in repeatability analysis and lower than 2.4% for migration time and 4.3% for anion content (milligrams per kilogram) in reproducibility analysis.

Recovery. We established the accuracy of the anion analysis in two honey samples by using the method of standard additions. Three different amounts of each anion standards were added to equal amounts of honey sample and then diluted to the same volume. Each assay was made with three different quantities of standard solutions. **Table 3** lists the percentage of recoveries obtained for each compound (mean of two samples studied ± standard deviation). Furthermore, to test whether there was a matrix effect, the recovery assay should be analyzed with different amounts of sample. If regression lines obtained from the comparison of recoveries were parallel, we could conclude that there was not a matrix effect. A honey sample at two different concentrations was analyzed, and parallel lines were tested with the analysis of the slopes of the regression lines with an ANOVA test. If the *p* value for the slopes was >0.10 **(Table 3)**, there were no statistically significant differences between the slopes for the different concentrations of sample at the 90% or higher confidence level. Therefore, there was not a matrix effect for the determination of inorganic anions and formic acid in honey samples with the proposed method.

Table 4. Content of Anions in Analyzed Honeys from Different Botanical Origins

		anion (mg/kg)				
sample	honey botanical origin	chloride	nitrate	sulfate	phosphate	formic acid
1	*Castanea sativa* Miller	106	2.4	75	195	199
2	*Castanea sativa* Miller	57	NQ[a]	152	127	506
3	*Castanea sativa* Miller	69	9.0	173	628	412
4	*Eucalyptus* sp.	490	4.8	8	21	50
5	*Eucalyptus* sp.	287	3.3	11	30	126
6	*Eucalyptus* sp.	311	2.8	17	31	128
7	*Eucalyptus* sp.	456	6.7	23	35	74
8	multifloral	282	2.9	51	129	203
9	multifloral	288	3.3	48	107	186
10	multifloral	259	2.6	47	91	209
mean		260.5	3.93	60.5	139.4	209.3
SD		148.1	2.3	57.9	180.8	144.2
V_{min}		57	NQ	8	21	50
V_{max}		490	9.0	173	628	506

[a] Not quantifiable.

Table 5. Anion Contents Obtained by Other Authors

	anion (mg/kg)				
ref	chloride	nitrate	sulfate	phosphate	formic acid
6[a]	262 (43–507)[b]		138 (12–243)	239 (31–653)	
7	303 (77–579)		40 (19–130)	119 (36–350)	
8[a]	833		114	702	
9	178 (27–634)	12 (2–101)	52 (10–262)	64 (13–208)	
10		1.7 (0.5–7.3)	86 (20–206)		
12[a]			146 (93–198)	287 (113–460)	
13[a]				234 (74–847)	
14[a]			87 (6–354)	310 (98–1220)	
15[a]			87 (57–129)	156 (80–294)	
16[a]				312 (157–473)	
17					216 (56–626)
19[a]					166 (41–428)
20					168 (34–340)
21					(17–284)
22					181 (5–1342)
23					108 (30–236)
24					58 (5–347)
25					180 (46–567)
26					266.4 (46–908)

[a] Data of these references have been converted (sulfur into sulfate and phosphorus into phosphate) in order to have comparable results. [b] Mean (V_{min}–V_{max}).

Content of Anions of Honeys Analyzed. Anion content of the studied honeys is shown in **Table 4**. A great variability in the quantitative composition of anions was found in honeys. In *Castanea sativa* Miller honeys, we found low levels of chloride and high levels of sulfate, phosphate, and formic acid. Conversely, *Eucalyptus* sp. honeys had high levels of chloride and the lowest levels of sulfate, phosphate, and formic acid. These results show that anions analysis could be potentially useful to characterize different honeys depending on their botanical origin. As can be seen in **Table 4**, chloride shows the highest levels and nitrate the lowest levels. These results are consistent with data obtained by other authors **(Table 5)**. **Figure 1** shows two electropherograms of two of these honeys, where the great variability found in the quantitative composition of anions in the analyzed honeys is evident.

In conclusion, the proposed method allows, for the first time, the separation and quantification of anions chloride, nitrate, sulfate, phosphate, and formic acid in honey samples by capillary electrophoresis. The optimized electrophoretic procedure is

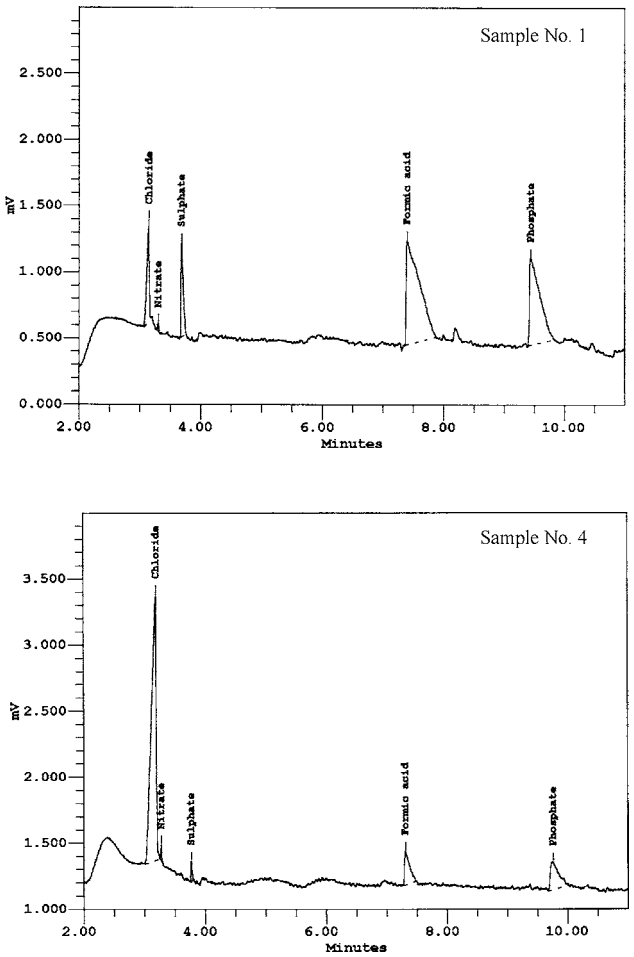

Figure 1. Electropherograms of analyzed anions by the proposed method in a *Castanea sativa* Miller honey (sample 1) and in a *Eucalyptus* sp. honey (sample 4).

simple and does not need any preparation of sample other than dilution and filtration. In addition, anions analysis has proved to be potentially useful for unifloral honey characterization.

ACKNOWLEDGMENT

We thank Professor Rafael Cela Torrijos of the Analytical Chemistry Department of the Chemistry Faculty and Professor Juan Carlos García Monteagudo of the Chemistry−Physics Department of the Pharmacy Faculty, both of the University of Santiago de Compostela, for helpful comments. We thank all of the beekeepers that provided the "Indicación Xeográfica Protexida-Mel de Galicia" honey samples for this study.

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Received for review June 1, 2006. Revised manuscript received October 8, 2006. Accepted October 9, 2006.

JF061536S