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Analysis of Phenolic Acids in Honeys of Different Floral Origin by Solid-phase Extraction and High-performance Liquid Chromatography

BURYA DIMITROVA[1], RENETA GEVRENOVA[2]* and ELKE ANKLAM[3]

[1] Department of Chemistry, Faculty of Pharmacy at the Medical University, 2 Dunav St, 1000 Sofia, Bulgaria
[2] Department of Pharmacognosy, Faculty of Pharmacy at the Medical University, 2 Dunav St, 1000 Sofia, Bulgaria
[3] European Commission, DG Joint Research Centre, Institute for Health and Consumer Protection, Food Products and Consumer Goods Unit, I-21020 Ispra (VA), Italy

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Abstract: The determination of 18 aromatic and arylaliphatic carboxylic acids in honey from different floral origin using solid-phase extraction (SPE) and reversed-phase high performance liquid chromatography (RP-HPLC) is reported. The behaviour of the solutes on SPE cartridges was predicted from preliminary calculations involving the pK_a constants of the carboxylic groups, the *n*-octanol:water partition coefficients and the distribution coefficients at different pH values of the conditioning and washing solvents. The proposed SPE isolation and pre-concentration of the acids was achieved on reversed-phase Bond Elut C_{18} cartridges using an acetonitrile:tetrahydrofuran (1:1, v/v) elution system. RP-HPLC separations were performed on a Spherisorb ODS-2 column using linear gradient elution with a mobile phase composed of 20 mm phosphate buffer (pH 2.92) and methanol, and with UV detection. The reported SPE and RP-HPLC methods were applied to the analysis of 49 authentic honey samples from various floral sources and the results indicate that they may serve with respect to the quantitative control of a number of phenolic acids in plant-derived foods and medicinal plants. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: RP-HPLC; SPE; aromatic and aryl-aliphatic carboxylic acids; partition coefficients; distribution coefficients; botanical origin.

INTRODUCTION

Aromatic and aryl-aliphatic carboxylic acids are a large group of phenolic compounds widely distributed in medicinal plants (Waksmundzka-Hajnos, 1998; Williamson *et al.*, 1999; Macheix *et al.*, 2005) and food products derived from plants (Midorikawa *et al.*, 2001; Fleuriet and Macheix, 2003; Robbins and Bean, 2004). Some phenolic acids (e.g. gallic, caffeic and ferulic acids) exert potential health-promoting effects by acting as antioxidant, anti-tumour, anti-mutagenic and anti-carcinogenic agents (Ogiwara *et al.*, 2002; Proteggente *et al.*, 2003; Macheix *et al.*, 2005).

Honey contains various aromatic and aryl-aliphatic carboxylic acids, mainly hydroxy- and methoxy-derivatives of benzoic and cinnamic acids, which are important ingredients contributing to the sensory qualities of the product and also possibly indicating the floral origin (Anklam, 1998). The concentrations of the acids in honey vary considerably and it is essential to analyse both the fundamental and the commercial aspects in order to evaluate the quality of the product. Acids are isolated from honey samples mainly by liquid–liquid extraction or column chromatography (Steeg and Montag, 1988; Amiot *et al.*, 1989; Sontag *et al.*, 1989; Jörg and Sontag, 1992; Ferreres *et al.*, 1996; Andrade *et al.*, 1997a, b). These methods suffer from some serious drawbacks (Hennion, 1999; Rodriguez *et al.*, 2000), which result in significant interference with further chromatographic determination. Solid-phase extraction (SPE) is a more appropriate and widely used clean-up technique, but little information is available concerning the SPE of honey constituents (Bogdanov, 1989; Chechi *et al.*, 1994; Del Nozal *et al.*, 2000; Suarez-Luque *et al.*, 2002) and no procedure for isolating phenolic acids from this matrix has been reported.

In this paper we report the results of a proposed SPE method for the isolation and pre-concentration of aromatic and aryl-aliphatic carboxylic acids from honey of different origins. By virtue of the complexity of the matrix and the presence of various polyphenols, the isolation of the acids was adjusted by predicting the behaviour of the analytes in the SPE system on the basis of pK_a constants of the carboxylic groups, the octanol:water partition coefficients (calculated log*P*, CLOGP) and the distribution coefficients (calculated log*D*, CLOGD) of the ionised analytes at different pH values (Hennion, 1999).

An improved RP-HPLC method involving gradient elution and UV detection for the simultaneous separation

* Correspondence to: R. Gevrenova, Department of Pharmacognosy, Faculty of Pharmacy, Medical University Sofia, Dunav St. 2, 1000 Sofia, Bulgaria. E-mail: rgevrenova@pharmfac.acad.bg

and quantification of 18 aromatic and aryl-aliphatic carboxylic acids in honey was applied following pretreatment of the samples by SPE. Using the analytical procedures proposed, 49 authentic honeys from various floral sources within member states of the European Union were analysed in order to control the quantity of phenolic acids.

EXPERIMENTAL

Reagents, standard solutions and honey samples. HPLC-gradient-grade methanol, acetonitrile, tetrahydrofuran and ethylacetate, and analytical-reagent grade chemicals (potassium dihydrogen phosphate, sodium hydroxide, and *ortho*-phosphoric, formic, acetic and hydrochloric acids), were purchased from Merck (Darmstadt, Germany). Double-distilled water was used. The standards of the studied acids and 4-hydroxycoumarin were of analytical grade and obtained from Fluka (Buchs, Switzerland), Sigma-Aldrich (Deisenhofen, Germany), Merck and Extrasynthese (Genay, France).

Stock solutions were prepared within the range 0.05–2.4 mg/mL by dissolving the standards in methanol. The concentration of each solution was selected according to the level of the acid expected in the honey samples. The solutions were stored in a refrigerator at –20°C and were stable for over 3 months. Working standard solutions were made daily by gradual dilution with methanol to the required concentration, which was based on the sensitivity of detection and the linearity range of the study.

The honey samples were received directly from several honey producers in Germany, Denmark, Italy, Spain, France, Netherlands, UK and Portugal and were stored at room temperature in the dark. The 49 samples of different botanical origin were selected from the very characteristic monofloral types, i.e. chestnut (*Castanea sativa* Miller), acacia (*Robinia pseudoacacia* L.), lime (*Tilia* sp.), eucalyptus (*Eucalyptus* sp.), lavender (*Lavandula* sp.), rapeseed (*Brassica napus* L.), sunflower (*Helianthus annuus* L.), rosemary (*Rosmarinus officinalis* L.), orange (*Citrus aurantium* L.), lemon (*Citrus limon* Burm), fior di sulla and *Echium plantagineum*. Information concerning the specific origin of the heather, bell heather and ling heather (*Erica* or *Calluna*) honey samples analysed was not available.

Calculation of pK_a values, octanol:water partition coefficients CLOGP and distribution coefficients CLOGD. pK_a values and octanol:water partition coefficients CLOGP were calculated according to the additivity method using the computer program ACD/Labs pK_a-/log*P* Calculator version 4.0 (Advanced Chemistry Development Inc., Toronto, Canada). The distribution coefficients CLOGD were calculated using the appropriate correction for ionisation.

SPE procedure of phenolic acids from honey. SPE was performed using a Vac Elut 10 vacuum manifold system (Varian, Harbor City, CA, USA). The prepared solution of each honey sample was homogenised in an ultrasonic bath for 3 min prior to loading onto a cartridge, and extraction was carried out according to the procedure shown in Table 1. The collected final

Table 1 Experimental conditions for the solid-phase extraction of honey samples

Parameters	Conditions	Notes
First stage		Bond Elut C_{18}, 3 mL, 500 mg cartridge
Conditioning sample	With 10 mL MeOH and 10 mL 1 mM NaOH at a flow-rate of 1 mL/min	
	Honey (2 g) dissolved in 10 mL 5 mM NaOH and adjusted to pH 8.0	More alkaline honey samples dissolved in 10 mL 1 mM NaOH and adjusted to pH 8.0.
Loading	At a flow-rate of 0.3 mL/min	Effluent contains the acidic fraction in deprotonated (A^-) form
Washing	With 6 mL 1 mM NaOH at a flow-rate of 1 mL/min	Washing portion is added to the effluent
Second stage		Bond Elut C_{18}, 3 mL (2 × 500 mg) cartridges
Conditioning sample	With 10 mL MeOH and 10 mL 10 mM HCl at a flow-rate of 1 mL/min	
	Effluent from the first stage adjusted to pH 1.5 with 4 M HCl and with 2 mL of 4-hydroxycoumarin (0.01 mg/mL) added	Acids are protonated (AH)
Loading	At a flow-rate of 0.3 mL/min	
Washing	With 8 mL 10 mM HCl at a flow-rate of 1 mL/min	
Drying	In a nitrogen stream for 10 min at room temperature	
Elution	With 1.5 mL acetonitrile:tetrahydrofuran (1:1) at a flow-rate of 0.3 mL/min	

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eluates of acetonitrile:tetrahydrofuran were evaporated under a gentle nitrogen steam and the residue was dissolved in 150 μL methanol:water (1:1) mixture; aliquots (20 μL) of the solution were injected into the HPLC system.

Chromatographic equipment and conditions. The chromatographic analyses were performed on a Varian (Walnut Creek, CA, USA) chromatographic system, which consisted of a tertiary pump model 9012, a Rheodyne injector with a 20 μL sample loop and a UV–vis detector model 9050. Acids were detected at 280, 310 and 220 nm according to the molar absorptivity of the solutes. A Varian Star Chromatography workstation running version 4.5 software was used to control the HPLC system and to collect the data. The separation was carried out with a Varian Spherisorb ODS-2 column (250 × 4.6 mm i.d.; 5 μm: Interchim, Montluçon, France), fitted with a Varian pre-column (30 × 4.6 mm i.d.) dry packed with Perisorb RP_{18} (30–40 μm: Merck) and periodically changed: both columns were maintained at room temperature. The mobile phase was composed of methanol and 20 mM potassium dihydrogen phosphate buffer (adjusted to pH 2.92 with *ortho*-phosphoric acid), which were filtered through Millipore (Watford, Ireland) 0.45 μm filters and degassed in an ultrasonic bath prior to use.

The SPE procedure was tested using C_{18} sorbents with different carbon loads and pore sizes, namely, Varian Bond Elut C_{18} (18.12% C, 60 Å, EC); Shandon (Runcorn, UK) C_{18} (5.75% C, 60 Å, EC) and Waters (Milford, MA, USA) Sep-Pak C_{18} (12% C, 125 Å, EC).

Precision assays and calibration. The instrument precision (injection repeatability) was determined by injecting (n = 10) standard mixtures of the acids at low and high concentration levels ranging, respectively, from 5 to 25 μg/mL and from 50 to 250 μg/mL. Reproducibility was tested over 10 working days by performing three injections per day of the same standard solutions (International Conference on Harmonisation, 1995). Instrument precision and reproducibility were expressed as relative standard deviation (RDS%) values. The precision of the retention times was evaluated from consecutive analytical runs (n = 10) of a mixture of standards by calculating mean values and their derived standard deviations.

Phenolic acids, with the benzoic acid carbon framework, present λ_{max} values in the 200–290 nm range, whilst cinnamic derivatives exhibit an additional broad absorbance band from 270 to 360 nm (Robbins and Bean, 2004). Data were collected at 280 nm since these gave the best signal-to-noise response for all of the acids simultaneously, and also assisted in distinguishing between hydroxycinnamic and benzoic acids. Calibration was performed at 280 nm using suitably diluted stock solutions of acids in the range 5–800 μg/mL and the calibration curves were constructed by plotting peak area vs analyte concentration. Benzoic, salicylic and aryl-alyphatic acids were calibrated at 220 nm, because of the higher molar absorptivity of the compounds at this wavelength. Three replicate injections of five or eight concentrations close to those that would be expected in honey samples were carried out, and the data obtained were subjected to statistical analysis (Statistikprogramm, Analytik-Software, Leer, Germany). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to ICH guidelines based on the standard deviation of the regression lines of specific calibration curves and their slope using analyte concentrations in the range of LOD and LOQ solutions (International Conference on Harmonisation, 1995).

Analytical recovery and accuracy studies. The recovery of analytes was evaluated by applying the entire SPE procedure to a control matrix of honey that had been spiked with a standard mixture of the acids; the samples were prepared at three concentration levels, low, medium and high, and were measured in triplicate. The utilised control honey matrix was subjected to the same extraction procedure, and the measured values were subtracted from those obtained for the non-spiked samples. The results were compared with the data of non-extracted acids that had not been subjected to sample pre-treatment. Using the data obtained, the mean recoveries and the standard deviations (SDs) were derived. The accuracy of the overall method was assessed analysing three different concentrations of analytes added to the control honey matrix and tested in triplicate. The obtained peak areas were corrected using the values recorded for the control matrix, and the amount of each compound was determined from the corresponding calibration curve. The accuracy of the method (expressed as a percentage) was calculated by dividing the deviation of the mean concentrations found from the nominal value by the nominal value of analytes (Causon, 1997).

RESULTS AND DISCUSSION

Choice of the SPE and chromatographic separation conditions

On account of the chemical complexity of the honey matrix the behaviour of the aromatic and aryl-aliphatic carboxylic acids on SPE supports was predicted by preliminary calculation of octanol:water partition coefficients CLOGP, the distribution coefficients CLOGD at different pH values and the pK_a constants of the carboxylic groups. The data obtained served to determine the retention and to improve the isolation of the acidic compounds by changing the pH of the pre-conditioning

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and washing systems. The proposed SPE procedure relies on two stages using a set of C_{18} cartridges preconditioned in different ways (Table 1). The calculated distribution coefficients CLOGD of the studied acids ranged from –1.91 to –3.89 at pH 8.0. These values reveal that the acidic fraction in the honey matrix is deprotonated (conjugated bases in A^- form) at the first stage of the extraction, since it was not retained on the reversed-phase support of the cartridge; the separation performed was mainly from the neutral phenolics and non-polar constituents. The calculated pKaCOOH values ranged from 3.01 to 4.57, therefore the collected acidic fraction was protonated before the second stage of the extraction by adjusting to pH 1.5 and the analytes were retained on the cartridges as neutral solutes (AH form). The following washing step was controlled to avoid a loss and insufficient recovery of the more polar compounds such as protocatechuic and gallic acids. The elution was carried out with a mixture of acetonitrile and tetrahydrofuran as a higher elutropic solvent, because of the essential difference in the polarity of the retained acids. The Bond Elut C_{18} cartridges, which have high carbon load and small pore size, were selected since both factors were responsible for the stronger retention and better recovery of the studied analytes.

The preliminary RP-HPLC experiments for the separation of the acids showed that certain pairs of analytes in the standard mixture, such as 3-hydroxybenzoic and vanillic, caffeic and syringic, *m*-coumaric and ferulic acids, could not be well resolved by currently used systems (Waksmundzka-Hajnos, 1998; Molnar-Perl, 2000; Robbins and Bean, 2004). A fairly good separation was obtained after adjustment of the linear gradient using a binary solvent system consisting of 20 mM phosphate buffer at pH 2.92 (solvent A) and methanol (solvent B) as mobile phase. The elution programme commenced with 3% B and attained 45% B at 55 min and 100% B at 65 min. By testing the most commonly used RPLC columns, a Spherisorb ODS-2 column was selected in order to enhance the separation efficiency of the critical peak pairs, especially that of m-coumaric and ferulic acids. Figure 1 shows the chromatograms obtained for the standard mixture of acids employing the selected conditions. The retention times showed acceptable reproducibility for the analytes of the standard mixture; the obtained SDs of the mean t_R values (n = 10) varied between 0.02 and 0.08 min.

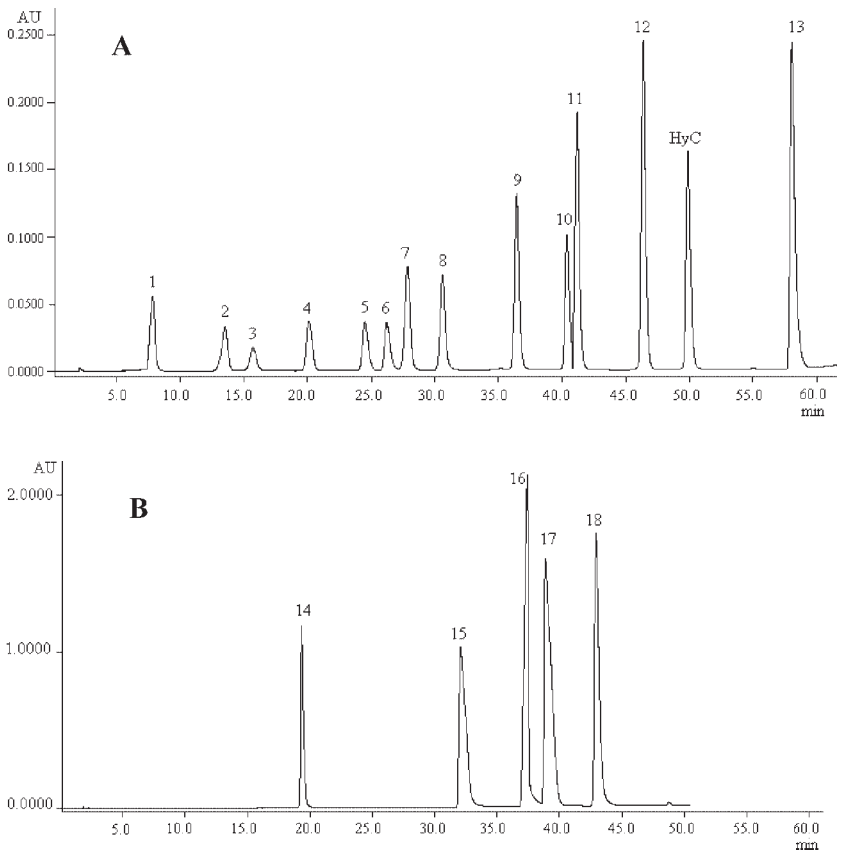

Figure 1 Chromatograms of a standard solution of acids detected at 280 nm (A) and 220 nm (B). Key to peak identities: **1**, gallic acid; **2**, protocatechuic acid; **3**, gentisic acid; **4**, 4-hydroxybenzoic acid; **5**, 3-hydroxybenzoic acid; **6**, vanillic acid; **7**, caffeic acid; **8**, syringic acid; **9,** *p*-coumaric acid; **10**, *m*-coumaric acid; **11**, ferulic acid; **12**, *o*-coumaric acid; **13**, *trans*-cinnamic acid; **14**, DL-*p*-hydroxyphenyllactic acid; **15**, DL-*β*-phenyllactic acid; **16**, salicylic acid; **17**, phenylacetic acid; **18**, benzoic acid; and **HyC**, 4-hydroxycoumarin. (For chromatographic protocol see the Experimental section.)

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Analytical performance

Under the analytical conditions proposed, the instrumental precision at low and higher concentration levels yielded RSDs values between 0.29% for phenylacetic acid and 1.24% for protocatechuic acid, and between 0.15% for salicylic acid and 1.03% for cinnamic acid, respectively. The reproducibility at these two concentration levels ranged from 1% for *o*-coumaric acid to 2.3% for gallic acid, and from 0.6% for syringic acid to 1.5% for 4-hydroxybenzoic acid, respectively. In the calibration experiments, all compounds showed acceptable linearity with correlation coefficients (r) higher than 0.999 within the range of concentrations investigated. Although the intercepts did not include the zero value, mainly due to the narrow limits of confidence, it does not presume any bias concerning the recoveries of the extreme values because of the good fit of the points to the regression lines.

The detection (LOD) and quantification (LOQ) limits determined would permit the quantivation of the acids at ppm levels, since the LODs and LOQs ranged from 0.3 to 9.2 ppm and from 1.0 to 30.8 ppm for *p*-coumaric and gentisic acids, respectively. The accuracy values varied between −13.8 to 8.4% indicating good agreement between the spiked and determined concentrations with respect to the complexity of the matrix studied. The mean recoveries (n = 3) of characteristic acids in the spiked control matrix ranged from 87.0 to 105.1% (Table 2). The precision of the overall analytical

Table 2 Accuracy, mean recovery and precision of the characteristic acids obtained from SPE of the spiked honey matrix

	Concentration (μg/mL)					
Compound	Added	Found ± SD[a]	RSD[b] (%)	Accuracy (%)	Recovery ± SD[a] (%)	RSD[b] (%)
Salicylic acid	5	4.8 ± 0.3	6.8	−5.0	95.1 ± 7.5	7.8
	50	43.5 ± 2.2	5.1	−13.0	87.0 ± 5.2	6.0
	150	135.0 ± 3.1	2.3	−10.0	90.0 ± 3.5	3.9
4-Hydroxybenzoic acid	25	27.1 ± 2.5	9.2	8.5	102.2 ± 9.0	8.8
	50	49.2 ± 3.3	6.7	−1.7	105.1 ± 7.0	6.7
	250	251.0 ± 17.6	7	0.4	100.3 ± 1.6	1.6
Vanillic acid	20	21.1 ± 1	4.6	5.6	93.3 ± 4.3	4.6
	40	37.5 ± 2.5	6.7	−6.3	98.4 ± 6.8	6.9
	200	196.4 ± 21.2	10.8	−1.8	98.3 ± 8.7	8.9
Syringic acid	10	10.8 ± 0.6	4.6	8.0	102.4 ± 5.9	5.8
	25	23.3 ± 1.5	6.3	−6.9	100.2 ± 7.2	7.2
	125	127.5 ± 3.2	2.5	2.0	101.3 ± 3.0	3.0
trans-Cinnamic acid	5	4.4 ± 0.1	2.9	−12.5	88.0 ± 2.3	2.6
	10	9.2 ± 0.6	6.3	−8.3	92.0 ± 5.7	6.2
	50	52.5 ± 3.1	5.9	5.0	104.9 ± 7.3	7
o-Coumaric acid	5	4.4 ± 0.2	4.5	−12.5	88.0 ± 1.9	2.2
	15	12.9 ± 0.9	6.9	−13.8	86.0 ± 5.9	6.9
	75	69.8 ± 8.8	12.6	−7.0	93.0 ± 11.3	12.2
p-Coumaric acid	5	4.7 ± 0.2	4.3	−6.5	94.0 ± 3.6	3.8
	20	17.7 ± 1.4	7.7	−11.5	88.5 ± 6.7	7.6
	80	70.4 ± 9.2	13.0	−12.0	88.0 ± 3.9	4.4
Ferulic acid	5	4.8 ± 0.3	5.5	−3.7	96.2 ± 5.3	5.3
	15	13.8 ± 0.8	5.9	−7.7	92.0 ± 5.7	6.2
	80	79.0 ± 11.5	11.5	−1.2	98.8 ± 11.7	11.8
DL-*β*-Phenyllactic acid	15	13.8 ± 1.1	8.2	−8.0	91.6 ± 4.9	5.3
	150	130.5 ± 2.2	1.7	−13.0	87.2 ± 1.4	1.6
	350	336.0 ± 4.4	1.3	−4.0	96.0 ± 1.2	1.3
DL-*p*-Hydroxyphenyllactic acid	5	5.2 ± 0.3	4.9	3.4	103.5 ± 5.0	4.8
	25	23.3 ± 3.3	14.4	−6.7	93.4 ± 11.0	11.8
	75	71.3 ± 3.0	4.3	−5.0	95.0 ± 4.0	4.2

[a] SD, standard deviation; [b] RSD, relative standard deviation.

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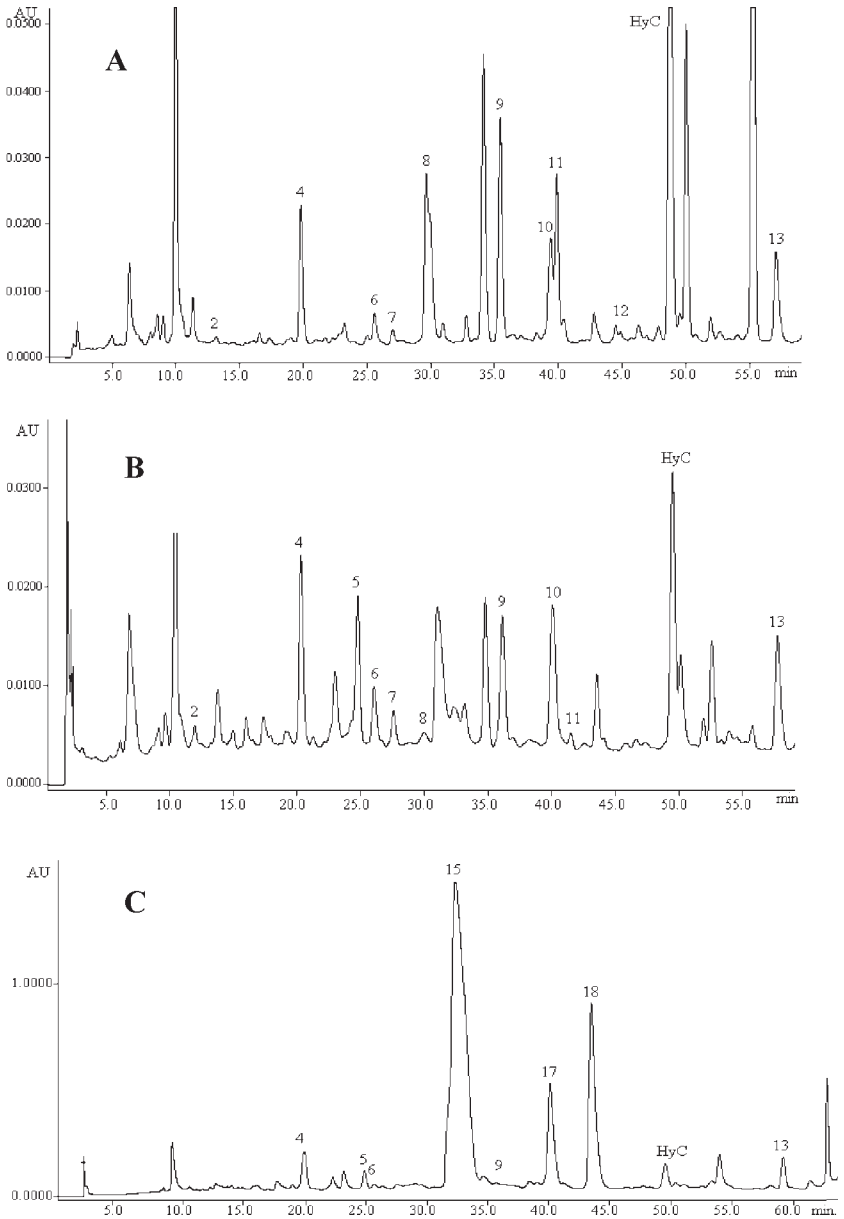

Figure 2 Chromatograms of (A) acacia honey detected at 280 nm; (B) eucalyptus honey detected at 280 nm; and (C) heather honey detected at 220 nm. Chromatographic conditions and peak assignments as in Fig. 1.

procedure was estimated by measuring the within-day repeatability; the relative standard deviations of the parallel (n = 3) results obtained are given in Table 2, and are in the range of 1.3 to 12.2%. Generally, the results varied for each acid, being predominantly higher for decreasing spiked concentrations. It should be noted that the results of the overall method are acceptable for complex matrices as honey and plant-derived foods (Huber 1997).

Application

The selected SPE and HPLC procedures were applied to analyse 49 authentic honey samples from various floral sources. The isolated acids were identified by comparing the retention times of chromatographic peaks of the analysed honey with those of the sample spiked with standards. Additional matching of the chromatograms and more reliable identification of the peaks were obtained by spiking the samples (Table 1) with an inert and suitably eluting compound (i.e. 4-hydroxycoumarin). Each sample was monitored at three different wavelengths (220, 280 and 310 nm, selected on the basis of the specific differences in UV absorption of the compounds assayed) in order to avoid interference from co-eluted analytes from the matrix. Figure 2 shows chromatograms of honey samples from different floral origin—heather, acacia, eucalyptus—in which the peaks of the acids are numbered to

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Table 3 Content (mg/kg) of aromatic and aryl-aliphatic carboxylic acids in honey samples

	Acacia ($n = 6$)			Lime ($n = 4$)			Chestnut ($n = 8$)			Heather ($n = 7$)			Bell heather ($n = 1$)	Ling heather ($n = 1$)	*Echium plantagineum* ($n = 1$)	Fior di Sulla ($n = 1$)
Acids	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max				
Benzoic acid									17.45	49.40	23.51	153.01	4.31	104.02	1.19	0.40
Salicylic acid							1.81	1.32	2.30			4.01				
3-Hydroxybenzoic acid				4.71	3.39	6.56			8.51							
4-Hydroxybenzoic acid	0.98	0.51	1.50				7.84	3.01	12.50	6.66	2.91	13.01	4.53	6.10		
Protocatechuic acid	0.61	0.12	1.41	2.18	0.54	3.68	5.76	3.59	8.37	2.78	0.57	6.01	4.10	2.61	0.16	0.92
Gallic acid										0.63	0.55	0.71				
Syringic acid	0.63	0.05	1.21			0.29			2.62			2.08				
Vanillic acid	0.61	0.17	1.32	1.19	1.01	1.49	3.46	0.92	5.61	2.30	0.67	4.10	1.29			
trans-Cinnamic acid	0.19	0.07	0.36			0.46	0.85	0.16	1.79	1.84	0.95	3.48	1.10	3.23	0.22	
o-Coumaric acid	0.05	0.03	0.08				1.46	0.48	3.50	0.89	0.25	2.30			0.02	
m-Coumaric acid	**1.41**	**0.70**	**2.11**				2.02	0.98	3.40							
p-Coumaric acid	0.22	0.12	1.01	1.41	1.19	1.87	2.03	0.17	5.48	1.87	0.62	4.01	0.35	3.39	0.32	0.05
Caffeic acid	0.40	0.15	0.72			1.57	5.44	3.10	9.13	2.97	0.97	6.11		1.82	0.67	0.19
Ferulic acid	0.53	0.26	0.98	0.94	0.33	1.48	6.00	0.47	12.62	1.03	0.49	1.99	2.12	1.58	0.40	0.09
Phenylacetic acid	3.64	0.59	8.50	29.77	7.47	75.72	71.17	2.21	193.02	176.61	60.89	318.20	13.73	225.47	8.30	10.52
L-β-Phenyllactic acid	9.71	3.32	24.41	26.41	15.06	37.28	47.95	0.64	86.71	820.38	378.3	1060.2	1.22	875.41	46.69	75.81
DL-*p*-Hydroxy-phenyllactic acid			0.23				6.00	0.88	7.50	8.76	6.27	10.70	5.20	5.63	1.01	0.71

correspond with those shown in the chromatograms of the standard mixtures [see Fig. 1(a) and (b)].

The results of the quantitative determinations of the acids in different types of honey are listed in Table 3. The data includes the mean, minimum and maximum values of the acid content in the honey samples studied (the analysis of each sample was replicated three times, i.e. from sample preparation to chromatographic analysis). It can be seen that the occurrence and distribution patterns varied significantly according to the floral origin of the honey. By comparing the obtained contents, the majority of the studied acids could be divided into three groups in accordance with the amounts present, i.e. benzoic and aryl-alyphatic acids; hydroxy- and methoxy-derivatives of benzoic acid; and cinnamic acid and its hydroxy- and methoxy-derivatives.

The first group of acids is characteristic of the heather and chestnut types of honey. The compounds with the highest concentrations in the heather samples were DL-β-phenyllactic acid, followed by phenylacetic and benzoic acids. The only heather sample showing a different profile was labelled 'bell heather'.

In general, all of the remaining floral types contained much lower concentrations of this group of acids. There were thus three concentration ranges for DL-β-phenyllactic acid: from 46.69 to 86.71 mg/kg in *Echium plantagineum* and chestnut honeys; from 15.06 to 40.52 mg/kg in lime and lavender samples; and from 3.32 to 10.49 mg/kg in acacia and rapeseed honeys. Variable amounts of phenylacetic acid were detected, but only the chestnut type contained relatively large amounts that were comparable with those found in heather samples (Table 3). The amounts of benzoic acid ranged between 0.25 and 6.50 mg/kg in orange and rapeseed honeys, and only one chestnut sample had 17.45 mg/kg. Derivatives of benzoic acid are also characteristic constituents of honey, with 4-hydroxybenzoic acid being considered as a helpful component for identifying the chestnut and heather honeys (Steeg and Montag, 1988; Jörg and Sontag, 1992). Both types of honey tend to exhibit higher contents of protocatechuic and vanillic acids relative to those found in eucalyptus, lime and sunflower samples. Salicylic acid was present in chestnut honey and in just one heather sample; 3-hydroxybenzoic acid was detected in chestnut, lime and eucalyptus type honey. Acacia and rosemary honeys presented low levels of this group of acids.

The data obtained for the cinnamic derivatives mostly indicated lower concentrations in comparison with other groups of acids. The highest levels of ferulic, *p*-coumaric and caffeic acids were present in chestnut and heather honeys, followed by sunflower, lime, eucalyptus and rapeseed.

Comparison with literature values is difficult since previous studies have employed different sample preparation and extraction conditions to those employed here. Except for *p*-coumaric and ferulic acids, the acid concentrations obtained for chestnut samples in the present study were larger than the reported values (in the range of 1, 3 and 4 mg/kg for 3-hydroxybenzoic, protocatechuic and salicylic acids, respectively; Jörg and Sontag, 1992). For the heather honey, the amounts of aromatic and aryl-aliphatic acids were generally higher than those previously reported (up to 90, 223 and 585 mg/kg for benzoic, phenylacetic and DL-β-phenyllactic acids, respectively; Steeg and Montag, 1988).

The levels of 4-hydroxybenzoic, ferulic, *o*-coumaric, and cinnamic acids were in agreement with previous

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Table 3 (*Continued*)

Lavender (n = 2)			Rosemary (n = 2)			Orange (n = 2)			Lemon	Rapeseed (n = 5)			Sunflower (n = 4)			Eucalyptus (n = 4)		
Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	(n = 1)	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
								0.25		3.66	1.30	6.50				0.86	0.27	2.00
		4.61																
																5.48	3.92	7.01
1.71	1.21	2.21			0.31	0.76	0.76	0.76	0.96	0.52	0.37	0.66	1.08	0.95	1.21	3.26	1.40	6.70
					0.53	1.73	1.68	1.77	5.19	0.16	0.07	0.30	1.23	0.63	2.41	4.09	0.71	8.70
6.31	1.35	11.27																1.40
0.64	0.32	0.95							0.37	0.54	0.10	1.02	0.72	0.46	1.30	1.18	0.45	1.56
										0.36	0.32	0.39				0.65	0.42	0.88
0.13	0.12	0.13			0.46			0.06	0.10	0.14	0.05	0.23	0.13	0.11	0.14	0.19	0.08	0.33
		0.09						0.07	0.06	0.42	0.01	0.83				0.09	0.06	0.15
																0.89	0.67	1.10
0.29	0.21	0.37	0.18	0.09	0.27	0.03	0.01	0.05	0.04	0.47	0.06	0.98	0.87	0.21	1.82	0.59	0.22	1.08
1.01	0.41	1.60	0.62	0.39	0.84					0.33	0.15	0.66	1.47	0.62	2.62	1.13	0.40	2.37
0.25	0.14	0.36	0.15	0.14	0.16	0.12	0.11	0.12		0.68	0.13	1.84	0.50	0.38	0.95	0.35	0.09	0.77
		6.50	3.24	2.87	3.61	6.11	4.39	7.83	3.71	4.16	1.01	7.10	8.03	6.30	9.91	8.30	4.28	11.90
		40.52	15.25	8.49	22.01	23.44	8.89	38.01	23.10			10.49				74.46	17.90	
																131.01		
		1.12														3.31	0.48	7.36

n = Number of the honey samples studied.

studies on Portuguese heather honey (in the range of 0.33, 0.54, 0.06 and 0.21 mg/100 g honey, respectively; Andrade *et al.*, 1997a); the content of *p*-coumaric acid was lower than that (up to 2.69 mg/100 g honey) reported by Andrade *et al.* (1997a). In addition, the presence of ellagic acid has been found to be a marker for the floral origin of *Erica* sp. honeys (Ferreres *et al.*, 1996; Andrade *et al.*, 1997a, b).

The content of the acids assayed in rapeseed was of the same order of magnitude as that given in the literature (in the range 0.5–2 mg/kg for benzoic and cinnamic acids derivatives and 5–6 mg/kg for benzoic acid; Steeg and Montag, 1988; Jörg and Sontag, 1992).

The content of gallic acid in lavender samples in our report was lower than that reported by Andrade *et al.* (1997a) (up to 237.20 mg/100 g honey).

The proposed SPE procedure coupled with RP-HPLC analysis enables the determination of phenolic acids in honey, with advantages in terms of sample preparation and the selective separation of compounds. The SPE method requires only a very small amount of sample, being potentially useful for the reliable isolation and pre-concentration of the acids studied in the complex honey matrix. In addition, both the SPE and RP-HPLC methods have the capability of being fully automated. By dividing the acids into the groups proposed according to their concentration levels, the quantitative data showed a good correlation, indicating that the aromatic and aryl-aliphatic carboxylic acids might be useful markers for the floral origin of certain honeys.

DL-β-phenyllactic acid, phenylacetic and benzoic acids are related to heather honey. 4-Hydroxybenzoic, DL-*p*-hydroxyphenyllactic, ferulic and phenylacetic acids could be used as markers for the floral origin of chesnut honey. Benzoic acid derivatives are specific for eucalyptus honey, whereas cinnamic acid derivatives can be used to identify acacia honey. Sunflower honey contains *p*-coumaric, ferulic and caffeic acids, whilst phenyllactic and *o*-coumaric acid are not present. Lime honey contains 3-hydroxybenzoic acid, and *o*-and *m*-coumaric acids are not detected. Lavender honey is characterised by gallic and caffeic acids. These SPE and HPLC procedures are suitable for determining the various acids in a range of plant and plant-derived food matrices.

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