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Solid-phase extraction and high-performance liquid chromatographic determination of organic acids in honey

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Abstract

A high-performance liquid chromatographic method is reported that allows the determination of organic acids in honey after sample purification by solid-phase extraction. The chromatographic separation was achieved with two Spherisorb ODS-1 S5 columns connected in series and sulphuric acid (pH 2.45) as the mobile phase. The average recoveries of the acids ranged from 89% to 104% and the detection limits from 0.002 to 3 ppm (w/w).

1. Introduction

The analytical composition and botanical origin are fundamental aspects of the evaluation of the quality of a honey. In order to characterize the typical components of this product, more and more specific investigations have been carried out. The glucidic fraction, which is quantitatively the most important, has been widely investigated, and also amino acids, vitamins, acids, polyphenols and the hydrocarbon fraction have been studied [1–5]. Owing to their low concentrations in honey and the need for sample purification, which is sometimes not easy, these compounds may be difficult to determine.

Papers on the organic acids in honey are scarce and mainly report enzymic or gas chromatographic methods. Enzymic methods allow the determination of about twenty acids [6], most of which are important from a biochemical point of view because they are part of the Krebs cycle or of similar enzymic pathways. Moreover, enzymic methods allow the determination of D- and L-isomers of some acids. Gas chromatographic methods especially allow the determination of previously derivatized aromatic and long-chain aliphatic acids [7–13]. No high-performance liquid chromatographic (HPLC) method for the determination of organic acids in honey has been reported, although several HPLC methods on organic acids in other foodstuffs have recently been published [14–18].

This investigation was concerned with the acidic part of honey. The method described allows a rapid and inexpensive sample clean-up using solid-phase extraction with strong anion-exchange (SAX) cartridges and the subsequent HPLC determination of organic acids.

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2. Experimental

2.1. Apparatus and chromatography

An Alltech (Deerfield, IL, USA) vacuum manifold with Bond-Elut SAX cartridges (500 mg, 2.8 ml) (Analytichem International, Harbor City, CA, USA) was used for sample purification.

A Kontron (Milan, Italy) liquid chromatograph consisting of a Model 325 pump, a Model 360 autosampler with a 100-μl loop and a Model 440 diode-array UV detector, was employed. The chromatograph was connected to a Model 3396 A reporting integrator (Hewlett-Packard, Avondale, PA, USA).

The chromatographic separations of the acids were achieved by means of two Spherisorb ODS-1 S5 (5 μm) columns (250 mm × 4.6 mm I.D.) (Phase Separations, Waddinxveen, Netherlands) connected in series, with an analogous guard column. The mobile phase was sulphuric acid (pH 2.45) at a flow-ate of 0.7 ml/min and was degassed with a Model ERC-3113 on-line degasser (Erma, Tokyo, Japan). The detector wavelength was set at 210 nm (0.1 AUFS), which was the optimum for the simultaneous determination of the acids. The chromatographic separation of some acids is shown in Fig. 1 as an example.

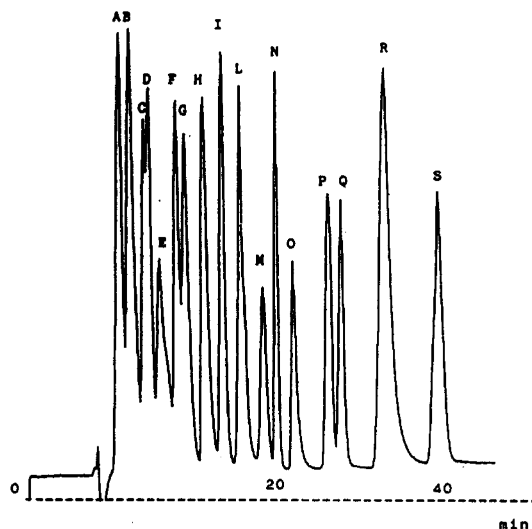

Fig. 1. Chromatography of a standard solution of organic acids. Columns, two ODS-1-S5 in series; mobile phase, sulphuric acid (pH 2.45); flow-rate, 0.7 ml/min; detection, UV at 210 nm. Peaks: A = galacturonic; B = gluconic; C = tartaric; D = pyruvic; E = quinic; F = malic; G = isocitric; H = lactic; I = acetic; L = α-hydroxybutyric; M = citric; N = succinic; O = dimethylglyceric; P = fumaric; Q = propionic; R = 2-oxopentanoic; S = glutaric acid.

2.2. Chemicals and materials

Acetic acid, sulphuric acid and sodium hydroxide were of analytical-reagent grade (Carlo Erba, Milan, Italy); water was deionized and filtered through a NANOpure apparatus (Barnstead Thermolyne, Dubuque, IA, USA) before use. The organic acids were of analytical-reagent grade (≥99.5%) (Aldrich, Carlo Erba and Merck, Milan, Italy). Stock standard solutions (0.1–10 g/l) were obtained by dissolution of acids in water and stored at 4°C; working standard solutions were prepared daily by dilution with the mobile phase. Unifloral source honey samples were kindly donated by local beekeepers and their botanical origin was checked by pollen analysis [19].

2.3. Extraction procedure

The organic acids were extracted from honey as follows. The cartridge was treated with 2 ml of 1 *M* sodium hydroxide solution (percolation rate 0.3 ml/min) and washed with 50 ml of water (3 ml/min), followed by 5 ml of acetic acid (pH 2.05) (0.5 ml/min) and finally 50 ml of acetic acid (pH 4.5) (3 ml/min). A solution of honey (0.5–1 g in *ca.* 10 ml of water) was then added and allowed to percolate at a flow-rate of 0.5 ml/min. The cartridge was washed with 30–40 ml of water (3 ml/min) and dried with a stream of compressed air at room temperature. The acids were eluted with 2 ml of 0.5 *M* sulphuric acid (0.3 ml/min). This solution was injected for HPLC analysis either directly or diluted with the mobile phase, depending on its total acidity.

The percolation rate of sodium hydroxide solution, acetic acid (pH 2.05) and sulphuric acid must be low because a high percolation rate has a negative effect on the acid recovery.

Each cartridge can be used several times (at

Table 1
Acid recoveries by solid-phase extraction

Acid	Level (ppm)	Recovery[a] (%)	Acid	Level (ppm)	Recovery[a] (%)
Aspartic	50	101.7 ± 0.6	α-Hydroxyglutaric	50	94.2 ± 0.1
	100	103.3 ± 0.5		100	95.7 ± 0.5
	200	99.8 ± 0.7		200	98.2 ± 0.4
Butyric	20	100.2 ± 0.4	Isocitric	50	95.2 ± 0.6
	50	99.6 ± 0.1		100	98.2 ± 2.1
	100	99.2 ± 0.5		200	89.6 ± 0.2
Citramalic	50	98.0 ± 2.0	α-Ketoglutaric	5	97.9 ± 3.2
	100	98.5 ± 0.2		10	97.7 ± 1.5
	200	99.1 ± 0.5		25	97.7 ± 1.5
Citric	25	100.2 ± 1.0	Lactic	25	99.1 ± 0.0
	50	97.3 ± 1.6		50	94.4 ± 0.0
	100	98.7 ± 0.0		100	95.7 ± 0.0
Dimethylglyceric	50	100.7 ± 0.9	Malic	25	101.2 ± 2.4
	100	99.3 ± 0.5		50	101.5 ± 2.8
	200	99.7 ± 0.3		100	99.8 ± 0.0
Formic	25	101.4 ± 1.7	Malonic	25	98.6 ± 0.6
	50	98.4 ± 1.4		50	100.3 ± 0.5
	100	99.4 ± 3.4		100	100.0 ± 0.5
Fumaric	0.1	98.8 ± 0.0	Methylmalonic	25	99.3 ± 0.3
	0.25	97.9 ± 0.4		50	97.3 ± 1.0
	0.5	99.0 ± 0.1		100	95.7 ± 0.1
Galacturonic	100	98.9 ± 2.2	2-Oxopentanoic	10	101.6 ± 0.6
	200	101.0 ± 0.1		30	96.3 ± 0.6
	300	100.1 ± 2.2		70	100.0 ± 1.1
Glucaric	50	104.1 ± 1.3	Propionic	50	99.0 ± 0.2
	100	102.0 ± 2.7		100	95.1 ± 0.4
	200	101.1 ± 1.9		200	96.0 ± 0.0
Gluconic	100	99.5 ± 1.0	Pyruvic	5	102.1 ± 0.9
	200	100.6 ± 0.1		10	96.6 ± 1.3
	300	103.3 ± 0.1		25	99.5 ± 0.8
Glutamic	25	103.0 ± 0.0	Quinic	50	99.6 ± 1.1
	50	101.4 ± 0.1		100	102.6 ± 0.7
	100	101.9 ± 0.1		200	101.5 ± 0.9
Glutaric	50	99.6 ± 1.2	Shikimic	2	101.3 ± 0.5
	100	99.1 ± 0.9		5	100.6 ± 0.0
	200	100.8 ± 0.1		10	103.2 ± 0.2
Glycolic	50	98.6 ± 0.2	Succinic	25	96.3 ± 0.2
	100	97.3 ± 0.6		50	96.0 ± 0.4
	200	99.1 ± 0.2		100	97.4 ± 0.1
Glyoxylic	50	104.1 ± 0.3	Tartaric	25	101.3 ± 4.1
	100	99.7 ± 0.1		50	97.0 ± 1.6
	200	101.8 ± 0.0		100	100.3 ± 0.3
2-Hydroxybutyric	50	98.8 ± 0.3			
	100	97.8 ± 1.0			
	200	99.2 ± 0.5			

[a] Mean values ± standard deviations for triplicate analyses from three replicates.

least five) if it is washed with 10 ml of methanol before a new extraction.

2.4. Recovery assays

Standard solutions of organic acids (0.1–300 ppm in water) and honey samples were employed. Honeys were fortified by adding 100 μl of aqueous standard solutions (0.5–200 ppm) of acids that were not present in their original composition, which was previously checked by HPLC. Standard solutions and fortified honey samples were processed according to the above-described procedure. Recovery assays showed analogous values for fortified honey samples and for standard solutions. The data from the recovery assays carried out on standard solutions of organic acids at three different concentrations for each compound are reported in Table 1.

3. Results and discussion

The described method allows the separation of a large number of organic acids. Many of these can be simultaneously determined with the mobile phase at pH 2.45. On varying the mobile phase pH, some acids whose peaks partially or completely overlap at pH 2.45 can be separated. In fact, the elution rate of organic acids changes considerably with any variation in the mobile phase acidity. The changes in the retention times (t_R) of some acids in relation to the variations of the mobile phase pH are reported in Table 2. It can be seen that polycarboxylic acids, such as citric and fumaric acid, are more sensitive than others to any change in the mobile phase pH.

Table 2
Retention times of some organic acids at different mobile phase pH values

Acid	Retention time (min)		
	pH 2.95	pH 2.45	pH 2.20
Gluconic	8.44	8.86	8.89
Formic	9.75	10.16	10.21
Malic	11.03	11.89	12.17
Shikimic	13.95	14.15	14.40
Acetic	14.54	14.60	14.61
Citric	16.11	17.96	21.30
Succinic	20.11	20.89	21.11
Fumaric	20.11	25.35	30.33
Propionic	28.90	28.90	28.98

Calibration graphs for each compound were constructed by plotting concentration against peak height. Good linearities were achieved in the range 0.1–300 ppm with correlation coefficients between 0.9976 and 0.9999. Under the optimum conditions, the detection limits [20] ranged from 0.002 to 3 ppm (Table 3).

As acetic acid was used for extraction, the determination of this and other acids (*e.g.*, ascorbic, *cis*-aconitic and 2-ketobutyric acid) with very close retention times (t_R between 14.4 and 15.0 min) was unachievable. Several attempts were made, both with inorganic (hydrochloric, metaphosphoric and sulphuric acid) and organic acids (propionic, butyric and citric acid) at various concentrations, but only acetic acid allowed satisfactory and repeatable recoveries without any interferences.

As a first step, 48 honey samples from different botanical sources were analysed according to the above-described method: twelve multifloral, fourteen strawberry-tree (*Arbutus unedo* L.), twelve asphodel (*Asphodelus microcarpus* Salzm. and Viv.) and ten red gum (*Eucalyptus camaldulensis* Dehmh.). A noteworthy variability in the qualitative and quantitative composition of the acidic part of the honeys was found. This could be explained by the different origins of the organic acids of honey. Some acids are derived from sugars by enzymes secreted by honeybees [21], some from the transformation of nectar into honey and others directly from the nectar sucked by honeybees. The indicated variability can be seen in Table 4, where the first results of this preliminary investigation are reported. The gluconic acid concentration was very high in strawberry-tree honey (about twice that in multifloral and red gum honeys), whereas it was much lower in asphodel honey. The latter proved to have the lowest organic acid content, as shown also in Fig. 2, where representative

Table 3
Retention times (t_R), absorbance maxima (λ_{max}), response factors (*RF*, referred to gluconic acid) and limits of detection of organic acids

Acid	t_R (min)	λ_{max} (nm)	*RF*	Limit of detection (ppm)
Glyoxylic	8.58	195	1.67	3.0
Galacturonic	8.64	195	0.93	3.0
Gluconic	8.86	195	1.00	2.0
Glucaric	8.86	195	1.11	2.0
Aspartic	9.11	195	1.65	1.6
Tartaric	9.34	195	3.08	1.0
Pyruvic	9.80	195	27.79	1.0
Glycolic	9.95	195	1.67	1.0
Formic	10.16	205	2.57	2.0
Glutamic	10.57	195	2.78	1.1
Quinic	10.76	195	0.74	3.0
α-Ketoglutaric	11.63	195	12.41	0.4
Malonic	11.74	195	2.23	0.4
Malic	11.89	195	1.82	1.0
Isocitric	12.42	195	0.96	2.3
Lactic	13.79	205	1.64	2.0
Shikimic	14.15	213	147.15	0.002
Acetic	14.60	204	1.41	1.0
α-Hydroxyglutaric	16.80	195	0.80	2.0
Citric	17.96	195	1.11	1.0
Citramalic	20.26	205	0.49	3.0
Succinic	20.89	202	1.03	0.7
Methylmalonic	21.19	195	2.27	1.6
2-Hydroxybutyric	21.98	210	0.54	3.0
Dimethylglyceric	22.69	195	0.60	3.0
Fumaric	25.35	209	199.77	0.004
Propionic	28.90	205	0.83	1.0
2-Oxopentanoic	34.79	195	2.25	0.5
Glutaric	41.88	205	0.75	1.0
Isobutyric	68.73	195	0.61	1.5
Butyric	72.18	205	0.46	2.0

Table 4
Mean concentrations of organic acids in honey samples

Honey	Acid					
	Gluconic (g/kg)	Pyruvic (mg/kg)	Malic (mg/kg)	Citric (mg/kg)	Succinic (mg/kg)	Fumaric (mg/kg)
Multifloral	6.9 ± 1.5	55.2 ± 1.4	135.4 ± 22.3	159.9 ± 47.7	33.4 ± 4.8	2.6 ± 1.3
Strawberry-tree	11.6 ± 2.2	45.8 ± 9.3	68.6 ± 18.9	64.0 ± 16.6	47.9 ± 2.1	1.3 ± 0.3
Asphodel	2.0 ± 0.7	8.9 ± 3.1	114.9 ± 15.6	64.2 ± 21.7	12.0 ± 5.2	0.5 ± 0.2
Red gum	6.1 ± 1.9	67.7 ± 8.0	144.9 ± 33.2	84.2 ± 22.1	41.1 ± 7.7	1.6 ± 0.4

Mean values ± standard deviations from 3 replicate analyses.

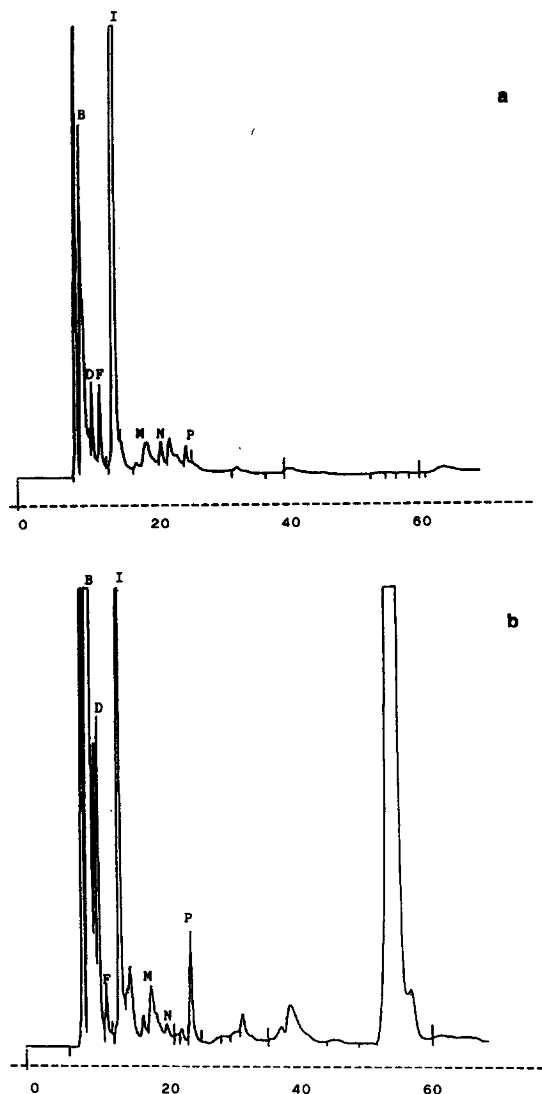

Fig. 2. Chromatography of organic acids in (a) asphodel and (b) strawberry-tree honeys. Peak identification and chromatographic conditions as in Fig. 1.

chromatograms of two typical Sardinian honeys (asphodel and strawberry-tree) are shown.

The described method allows the simultaneous determination of a number of organic acids, assisted by the fact that the problem of overlapping peaks can be overcome by varying the mobile phase pH. Further, the method could be used to prove the botanical origin of honey, in addition to physico-chemical, organoleptic and pollen analyses.

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