Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1047 (2004) 137–146
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Rapid and sensitive method for determining free amino acids in honey by gas chromatography with flame ionization or mass spectrometric detection☆

Mª.J. Nozal*, J.L. Bernal, M.L. Toribio, J.C. Diego, A. Ruiz

Department of Analytical Chemistry, Faculty of Sciences, University of Valladolid, Valladolid E-47005, Spain

Received 8 March 2004; received in revised form 26 June 2004; accepted 6 July 2004

Abstract

This paper describes a rapid, sensitive and specific method for determination of free amino acids in honey involving a new reaction of derivatization and gas chromatography (GC) with flame ionization (FID) and mass spectrometric (MS) detection. The method allows the determination of 22 free amino acids in honey samples in a short time: 8 and 5 min for GC–FID and GC–MS, respectively. Quantitation was performed using Norvaline as internal standard, with detection limits ranging between 0.112 and 1.795 mg/L by GC–FID and between 0.001 and 0.291 mg/L by GC–MS in the selected-ion monitoring mode. The method was validated and applied to a set of 74 honey samples belonging to four different botanical origins: eucaliptus, rosemary, orange and heather. The statistical treatment of data shows a correct classification of different origins over 90%.

Keywords: Honey; Food analysis; Principal component analysis; Amino acids

1. Introduction

Although the European Union food laws establish composition and quality parameters for honey, such figures have no relationship with the botanical or geographical origin of samples and so, they do not allow to characterize them.

Melisopalinology has been usually employed to get to know the botanical origin of honey, but nowadays, it is assumed that such procedure has severe drawbacks [1]. For this reason, several markers, including amino acid profile, and ratios deduced from physicochemical analysis are being proposed [2]. The origin of amino acids in honey is attributable both to animal and vegetal sources, although the main source is the pollen, so the amino acids profile of a honey could be characteristic of its botanical origin. To obtain that profile in several matrixes, different procedures have been proposed, in general based in the use of chromatographic techniques [3–8]. Such techniques frequently need a previous step of derivatization in order to enhance the sensitivity of the determination in high-performance liquid chromatography (HPLC) [1,4,6,9,10], or to increase the volatility of the analytes in gas chromatography (GC) [3,7,11,12], although it is also possible to perform a direct determination of underivatized amino acids [13]. To derivatize amino acids, several reagents have been proposed, either in precolumn or postcolumn modes. Ninhydrin has been widely used for postcolumn derivatization after separation by ion exchange and further UV detection [14–16]. Afterwards, other derivatizing reagents for precolumn mode, using a separation by reversed phase chromatography were proposed [17] among them, dansyl chloride [18,19], ortho-phtaldehyde (OPA) [16,18] which does not react with proline and cysteine; 9-fluorenylmethyl chloroformate (FMOC) with the problem of interferences from the excess of reagent or by-products of the

☆ Presented at the 3rd Meeting of the Spanish Association of Chromatography and Related Techniques and the European Workshop, 3rd Waste Water Cluster, Aguadulce (Almeria), 19–21 November 2003.

* Corresponding author. Fax: +34 983 423013.
E-mail address: mjdnozal@qa.uva.es (Mª.J. Nozal).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.07.013

reaction [20–23], phenyl isothiocyanate (PITC) [16,24,25], whose derivatives are difficult to obtain, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [26–30], whose hydrolysis product interferes in the determination, diethyl ethoxymethylenemalonate (EMMDE) [31–33], recently applied to wine and honey samples [9,34], and a modification of FMOC: 2-(9-carbazole)ethyl chloroformate (CEOC), used to determine amino acids and peptides in wood and beer [35]. Trying to solve some of the problems mentioned above, combinations of these reagents (OPA/FMOC-Cl, OPA/NBD, FMOC-Cl/ADAM) have also been suggested [4,36,37].

In relation to GC-based methods for amino acid analysis, all of them require a derivatization step to produce volatile adducts. The most commonly used procedure is that of Husek [38–40], a fast reaction in aqueous solution in which AAs react with a solution of ethylchloroformate (ECF), pyridine and ethanol [41] or trifluoroethanol [42]. Based on this reaction methods that involve the employment of an extracting-derivatization step together with gas chromatography mass spectrometry (GC–MS) for determination of amino acids in human urine were emerged [43]. Other chloroformate reagents, methyl chloroformate (MCF) and menthyl chloroformate (MenCF) have been used for the derivatization of seleno and sulphur amino acids [41]. *N*-methyl-*N*-tert-butyldimethylsilyl-trifluoroacetamide (MBDSTFA) in analysis of intracellular amino acids [44], and trimethylchlorosilane in analysis of non protein amino acids [45] have also been employed as reagents.

Recently, a method based in the use of a new reagents kit EZ:faast (Phenomenex) [46] has being applied satisfactorily to determine AAs in biological samples [47], allowing the determination of up to 50 amino acids and related compounds in times not longer than 15 min and with no interference of proteins, urea or other matrix constituents. Taking into account the frequent problems arisen with the use of the common derivatizing reagents mentioned above, the purpose of this work has been to adapt that methodology to amino acids determination in honey and to study the possibility that the results can be used in botanical origin characterization.

2. Experimental

2.1. Chemicals

Standards at a concentration of 200 mmol/L and reagents were supplied in the kit of reagents "EZ:faast GC–MS for free amino acid analysis" by Phenomenex (Torrance, CA, USA).

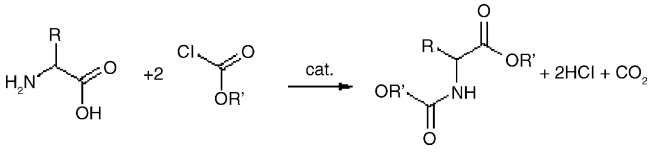


Fig. 1. Simplified diagram of the derivatization reaction.

2.2. Equipment

2.2.1. GC–flame ionisation detector (FID) conditions

An HP 5890 Series II gas chromatograph equipped with an HP 7673 autosampler and a flame ionization detector, all controlled by an HP 3365 Series II Chemstation from Hewlett-Packard (Avondale, PA, USA), were used.

A 10 m × 0.25 mm ZB-PAAC column from Phenomenex (Torrance, CA, USA) was used. The carrier gas (N_2) flow-rate was kept constant during the run at 1.6 mL/min (measured at 50 °C). Nitrogen (30 mL/min), hydrogen (35 mL/min) and synthetic air (350 mL/min) were used as auxiliary gases for

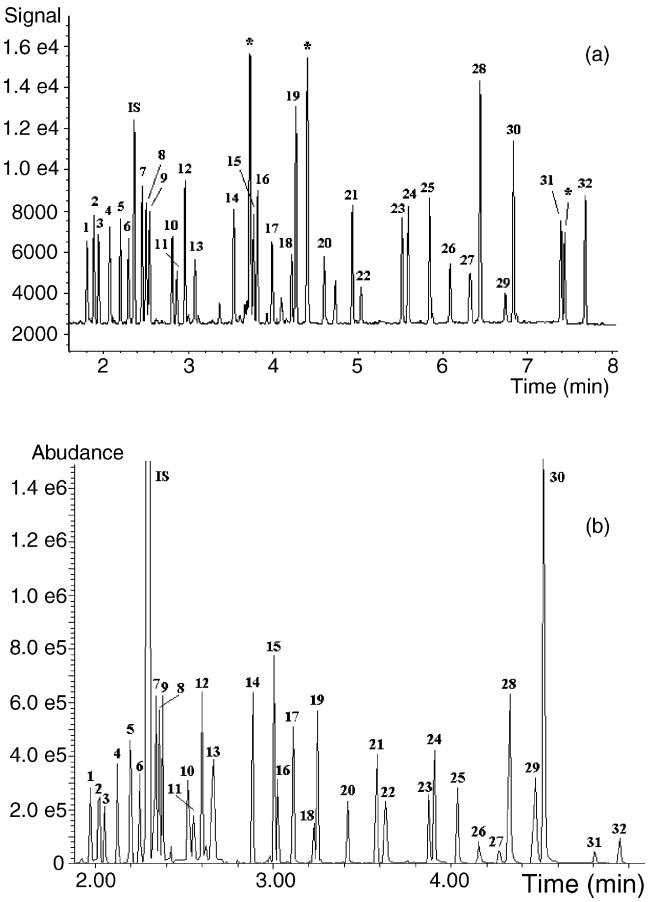


Fig. 2. (a) Chromatograms GC–FID of 100 nmol/mL and (b) GC–MS (SIM) of 40 nmol/mL of amino acid standard derivatives. Peaks: 1: Alanine (Ala); 2: Sarcosine (Sar); 3: Glycine (Gly); 4: α-Aminobutyric acid (ABA); 5: Valine (Val); 6: β-Aminoisobutyric acid; 7: Leucine (Leu); 8: allo-Isoleucine (aIle); 9: Isoleucine (Ile); 10: Threonine (Thr); 11: Serine (Ser); 12: Proline (Pro); 13: Asparagine (Asn); 14: Thiaproline (Tpr); 15: Aspartic acid (Asp); 16: Methionine (Met). 17: 4-Hydroxyproline (Hyp); 18: Glutamic acid (Glu); 19: Phenylalanine (Phe); 20: α-Aminoadipic acid (Aaa); 21: α-Aminopimelic acid (Apa); 22: Glutamine (Gln); 23: Ornithine (Orn); 24: Glycine-Proline (Gpr); 25: Lysine (Lys); 26: Histidine (His); 27: Hydroxylysine (Hly); 28: Tyrosine (Tyr); 29: Proline-Hydroxyproline (Php); 30: Tryptophan (Trp); 31: Cystathionine (Cth); 32: Cystine Cys–Cys). (I.S. Norvaline 200 nmol/mL.) *Unknown peaks by GC–FID.

the flame ionization detector. All gases were supplied by Carburos Metálicos (Barcelona, Spain).

The oven temperature program was as follows: initial temperature 110 °C, a 26 °C/min ramp to 320 °C, held for 1 min. The temperature of the injection port was 280 °C, while that of the detector was 320 °C. A 2 μL sample was injected in split mode (1:15, v/v).

2.2.2. GC–MS conditions

A Hewlett-Packard 6890 gas chromatograph (Little Falls Site, Wilmington, DE, USA) was directly coupled to a Hewlett-Packard 5973 mass spectrometer. The same column as in GC–FID was used, but changing the carrier gas and modifying the temperature program. The carrier gas (He) flow was kept constant at 1.5 mL/min. The oven temperature program was as follows, initial temperature 70 °C, a 20 °C/min ramp to 80 °C and then a 50 °C/min ramp to 320 °C, held for 1.7 min. The temperature of the injection port was 280 °C. The MS temperatures were as follows: ion source 240 °C, quadrupole 180 °C, and auxiliary 321 °C. The scan range was 45–450 (3.5 scans/s). Under these conditions a 2 μL sample was injected in splitless mode.

2.2.3. Additional equipment

An ultrasonic water bath and a vortex-mixer were obtained from Selecta (Barcelona, Spain). A model 5810R refrigerated centrifuge was supplied by Eppendorf (Hamburg, Germany). Micropipettes were obtained from Labmate (Poland). The rest of consumables including a microdispenser, syringes of 0.6 and 1.5 mL, sample preparation vials, autosampler vials with inserts, sorbent tips were included in the EZ:faast kit by Phenomenex.

2.3. Honey samples

Seventy-four honey samples were obtained directly from beekeeper associations and also on the Spanish market. From their melisopalinologic analysis and the label, it was assumed that their botanical origins were: 28 rosemary (*Rosmarinus officinalis* L.), 15 eucalyptus (*Eucalytus* spp.), 21 heather (*Ericaceae*, mainly *Erica* spp.) and 10 orange blossom (*Citrus* spp.) honey samples. The geographical origins of the honey samples were mainly from the Spanish regions of La Alcarria, Castilla y León, Galicia,Valencia, Extremadura and Aragón.

Raw honey samples were centrifugated at 16,000 × *g* and 4 °C to remove extraneous material and stored at 4 °C prior to the analysis.

2.4. Sample treatment

Fig. 1 shows the simplified diagram of derivation reaction for free AAs. The final procedure was as follows: 20 μL of a honey dilution with water (0.8 g/mL for GC–FID and 0.4 g/mL GC–MS) were pipetted into one glass vial and 200 μL of internal standard (norvaline at 200 μmol/L) were added. Then, a 40 μL resin packed-sorbent tip [46] was attached to a 1.5 mL syringe and the solution was slowly passed through the sorbent tip and collected in another vial, adding 200 μL of the washing solution. The solution was passed slowly through the same sorbent tip and into the syringe

Table 1
Mass fragment ions and their relative abundances of amino acids derivatives

Name	Fragment ions (% abundance)		
Ala	130 (100)	88 (11)	158 (3)
Sar	130 (100)	88 (23)	217 (5)
Gly	116 (100)	102 (24)	162 (8)
Aba	144 (100)	102 (13)	202 (1)
Val	158 (100)	116 (79)	72 (33)
Baib	116 (100)	143 (63)	130 (56)
IS	158 (100)	72 (31)	116 (15)
Leu	172 (100)	86 (20)	130 (8)
aIle	172 (100)	130 (77)	86 (18)
Ile	172 (100)	130 (68)	101 (18)
Thr	101 (100)	74 (34)	160 (13)
Ser	146 (100)	60 (81)	203 (25)
Pro	156 (100)	70 (50)	243 (1)
Asn	69 (100)	155 (88)	141 (20)
Tpr	88 (100)	174 (91)	147 (82)
Asp	216 (100)	130 (37)	88 (25)
Met	101 (100)	203 (93)	277 (20)
Hyp	172 (100)	86 (37)	68 (17)
Glu	230 (100)	170 (47)	305 (1)
Phe	148 (100)	190 (67)	206(51)
Aaa	98 (100)	244 (82)	144 (3)
Apa	198 (100)	258 (71)	286 (15)
Gln	84 (100)	187 (22)	142 (12)
Orn	156 (100)	70 (24)	286 (4)
Gpr	70 (100)	156 (24)	300 (2)
Lys	170 (100)	128 (21)	300 (4)
His	81 (100)	168 (67)	282 (58)
Hly	129 (100)	169 (46)	316 (5)
Tyr	107 (100)	206 (66)	308 (6)
Php	156 (100)	248 (8)	297 (4)
Trp	130 (100)	332 (8)	229 (5)
Cth	203 (100)	142 (94)	272 (52)
C-C	174 (100)	248 (87)	216 (51)

Table 2
Precision obtained in the determination of amino acids as a function of the amount of honey sample

Amino acid	R.S.D. (%)				
	1 g	2.5 g	3 g	4 g	5 g
Ala	4.89	3.85	3.69	4.62	6.71
Sar	6.32	4.15	4.89	4.00	7.04
Gly	8.18	7.25	7.21	6.84	8.32
Val	5.01	3.68	3.11	3.29	6.42
Leu	4.36	3.61	3.98	3.35	4.27
Ile	5.11	4.01	3.55	3.66	5.70
Thr	7.75	6.23	5.01	5.89	6.69
Pro	2.03	1.81	1.59	1.36	5.19
Asn	3.79	2.15	1.89	1.95	6.78
Glu	8.01	7.75	7.26	7.25	8.02
Phe	3.45	2.32	2.01	1.01	5.07
Gln	4.41	2.74	2.27	2.70	7.58
Lys	5.55	4.89	5.22	4.68	6.33
Tyr	5.03	4.89	4.88	4.63	6.41

Table 3
Concentration of amino acids (mg/L) obtained in GC–FID as a function of the volume of honey dilution (4 g/5 mL) used

Amino acid	*c* (mg/L)						
	Volume of honey solution (μL)						
	10	20	25	50	100	Mean	R.S.D. (%)
Ala	27.8	28.7	26.7	27.1	26.7	27.4	3.04
Sar	7.30	7.40	7.54	7.75	7.63	7.53	1.94
Gly	6.80	7.50	8.16	8.96	8.64	8.06	10.4
Val	30.7	28.3	29.3	23.9	27.6	28.0	9.05
Leu	12.2	13.2	15.2	13.6	15.6	14.0	10.2
Ile	21.4	21.1	23.7	19.7	22.5	21.7	6.94
Thr	2.10	2.10	1.82	1.74	1.89	1.97	10.0
Ser	35.8	42.4	39.8	41.0	36.5	39.1	7.33
Pro	517	518	518	470	491	503	4.30
Asn	121	123	121	98.6	113	115	8.79
Asp	70.7	75.8	76.3	44.6	38.9	61.3	29.4
Hyp	5.80	5.52	4.85	6.13	6.92	5.81	13.6
Glu	118	116	112	77.9	80.0	101	20.0
Phe	540	529	548	549	546	543	1.51
Gln	175	179	181	168	169	174	3.49
Orn	6.20	5.45	6.93	5.65	6.65	6.12	10.2
Lys	18.6	16.9	16.3	21.1	19.3	18.4	10.5
His	2.20	2.39	2.18	2.44	3.13	2.43	16.7
Tyr	10.5	11.8	13.4	13.0	13.7	12.5	10.5
Trp	2.10	1.9	2.52	1.93	2.38	2.14	13.7

Table 4
Parameters of the internal standard calibration curves: $y = a + b\,(c/c_{IS})$

Name	GC-FID			GC–MS (SIM)		
	b	*a*	r^2	*b*	*a*	r^2
Ala	0.992 ± 0.081	−0.008 ± 0.006	0.997	0.795 ± 0.013	0.018 ± 0.002	0.9993
Sar	1.157 ± 0.103	0.023 ± 0.010	0.999	0.935 ± 0.034	0.020 ± 0.006	0.998
Gly	1.160 ± 0.155	0.022 ± 0.010	0.999	0.792 ± 0.017	0.016 ± 0.003	0.999
Aba	0.990 ± 0.093	0.001 ± 0.001	0.9999	0.790 ± 0.013	0.021 ± 0.003	0.9992
Val	0.960 ± 0.059	0.012 ± 0.005	0.9999	1.097 ± 0.021	0.042 ± 0.005	0.999
Baib	0.908 ± 0.036	0.011 ± 0.005	0.9999	0.718 ± 0.013	0.017 ± 0.003	0.999
Leu	0.938 ± 0.104	0.015 ± 0.007	0.999	0.895 ± 0.029	0.036 ± 0.008	0.998
a-Ile	0.785 ± 0.029	0.003 ± 0.001	0.9999	0.936 ± 0.025	0.045 ± 0.007	0.998
Ile	0.825 ± 0.056	0.010 ± 0.005	0.9999	0.889 ± 0.097	0.022 ± 0.035	0.9992
Thr	0.690 ± 0.098	0.015 ± 0.007	0.996	0.640 ± 0.042	0.038 ± 0.015	0.992
Ser	0.566 ± 0.083	0.024 ± 0.011	0.991	0.416 ± 0.047	0.004 ± 0.005	0.993
Pro	1.237 ± 0.192	0.007 ± 0.003	0.998	1.330 ± 0.031	0.042 ± 0.008	0.999
Asn	0.630 ± 0.053	0.009 ± 0.004	0.997	1.041 ± 0.129	−0.008 ± 0.041	0.993
Tpr	1.045 ± 0.222	−0.038 ± 0.017	0.993	0.959 ± 0.037	0.044 ± 0.011	0.995
Asp	0.795 ± 0.096	0.022 ± 0.010	0.995	0.988 ± 0.011	0.051 ± 0.003	0.9996
Met	0.863 ± 0.093	0.009 ± 0.004	0.999	0.402 ± 0.015	0.019 ± 0.005	0.996
Hyp	0.704 ± 0.132	0.046 ± 0.021	0.992	0.856 ± 0.028	0.040 ± 0.008	0.997
Glu	0.424 ± 0.019	−0.004 ± 0.002	0.995	0.227 ± 0.008	0.010 ± 0.003	0.996
Phe	1.383 ± 0.251	0.015 ± 0.007	0.997	0.586 ± 0.012	0.041 ± 0.004	0.999
Aaa	0.365 ± 0.067	0.004 ± 0.002	0.995	0.361 ± 0.016	0.015 ± 0.006	0.994
Apa	0.864 ± 0.162	0.053 ± 0.024	0.992	0.565 ± 0.065	0.002 ± 0.025	0.991
Gln	0.435 ± 0.058	−0.004 ± 0.002	0.9999	0.464 ± 0.017	−0.008 ± 0.005	0.997
Orn	0.919 ± 0.127	−0.040 ± 0.018	0.999	0.696 ± 0.029	0.013 ± 0.004	0.999
GPR	0.909 ± 0.052	−0.069 ± 0.031	0.9999	0.576 ± 0.030	0.025 ± 0.003	0.9995
Lys	0.904 ± 0.133	−0.044 ± 0.019	0.995	0.656 ± 0.011	0.010 ± 0.004	0.9992
His	0.796 ± 0.138	0.019 ± 0.008	0.993	0.138 ± 0.008	0.011 ± 0.001	0.997
Hly	0.865 ± 0.147	−0.099 ± 0.044	0.993	0.174 ± 0.003	−0.003 ± 0.001	0.9994
Tyr	1.470 ± 0.092	0.011 ± 0.005	0.999	0.986 ± 0.017	0.088 ± 0.007	0.999
Php	0.508 ± 0.065	0.013 ± 0.006	0.998	N.L.	N.L.	N.L.
Trp	1.260 ± 0.208	−0.023 ± 0.010	0.996	1.567 ± 0.040	0.024 ± 0.019	0.999
Cth	0.964 ± 0.088	−0.028 ± 0.012	0.997	0.069 ± 0.004	−0.001 ± 0.001	0.995
C-C	0.738 ± 0.024	−0.007 ± 0.003	0.999	0.202 ± 0.009	−0.003 ± 0.002	0.9995

barrel. The liquid from the sorbent bed was drained by letting air through the sorbent tip. Afterwards, 200 μL of Eluting Medium were added and the sorbent was soaked in it, stopping when the liquid reached the filter plug in the sorbent tip. Liquid and sorbent particles were ejected out of the tip and into the vial. The addition of the eluting medium was repeated until all sorbent particles in the tip were expelled into the vial and then 50 μL of Reagent 4 were added, the liquid was emulsified in the vial with a vortex mixer in the touch mode for about 5–8 s. Reactions were allowed to proceed for 80 s, and then, the liquid was re-emulsified by vortexing again for about 8 s. At this moment, 100 μL of Reagent 5 were added to vial and mixed for about 6 s, after waiting for 1 min, the organic layer was transferred into a vial with insert, and evaporated slowly under a gentle stream of nitrogen. The residue was diluted with 100 μL of Reagent 6 and an aliquot of 2 μL was analysed by gas chromatography.

3. Results and discussion

3.1. Gas chromatography conditions

The GC–FID and GC–MS separations were carried out using a 10 m × 0.25 mm ZB-PAAC column (10 m × 0.25 mm) with the oven temperature programs detailed in the equipment section, which allowed us to obtain the highest separation efficiency. Fig. 2a shows the GC–FID chromatogram of a standard solution (each compound at 100 and 200 nmol/mL of IS). Nevertheless, when the same temperature program was used in the GC–MS system, a poor resolution for the least retained peaks was observed, so the thermal gradient was varied. In Fig. 2b it can be seen the GC–MS chromatogram in the selected-ion monitoring (SIM) mode with the new temperature program, belonging to the separation of a standard solution (each at 40 and 200 nmol/mL of IS). Table 1 shows the relative abundances and fragment ions observed for derivatized amino acids. As it can be observed in Fig. 2, under these conditions it is possible to achieve a good separation of the amino acids in 8 and 5 min by GC–FID and GC–MS, respectively, being feasible to determine 32 compounds.

3.2. Derivatization reaction

The procedure consists of a solid phase extraction clean-up, followed by a derivatization step using an organic phase with an alkyl chloroformate reagent, which react with both the carbonyl and the amino groups of the amino acids**,** forming derivatives stable at room temperature; finally a liquid/liquid extraction is carried out.

The influence of parameters potentially affecting the derivatization reaction was studied in order to establish the optimal conditions to obtain the maximum sensitivity. Firstly, the mass of sample was considered. For this purpose several quantities of honey (between 1 and 5 g) were diluted with water up to a final volume of 5 mL, then, 10 μL of the dilution were subjected to the derivatization procedure. In Table 2, the results obtained for some amino acids are listed. A sample amount of 4 g was selected for the analysis of the samples by GC–FID, because the variation coefficients were low enough, and a higher mass of honey gave higher variation coefficients and increased the viscosity of the mixture. But when the same derivatized sample was injected in the GC–MS system, which is more sensitive, a full overlapping between the signals of threonine and serine with proline and glutamic acid with phenylalanine was observed, this problem could be avoided by reducing the mass of honey to be diluted, so 2 g were selected. Taking into account that in honey the proportion of proline, phenylalanine and tyrosine are frequently higher, and considering the concentration

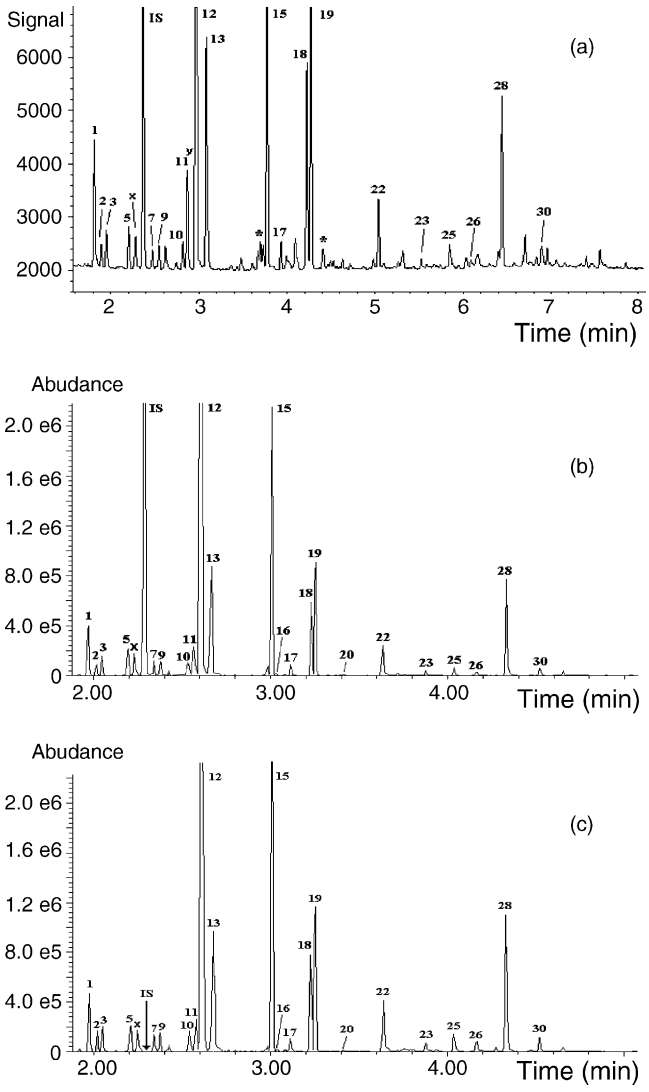


Fig. 3. Chromatograms obtained for a sample of heather honey: (a) 0.8 g/mL and 200 nmol/mL of internal standard GC–FID, (b) 0.4 g/mL and 200 nmol/mL of internal standard GC–MS (SIM), (c) the same as (b) but without addition of internal standard. Peaks as in Fig. 2. [x]β-Ala; [y]in honey, coelution of Serine + unknown peak.

Table 5
Reproducibility and accuracy obtained for a mixture of amino acids (40 nmol/mL in each) and for a honey sample (n = 5)

Name	GC–FID				GC–MS (SIM)			
	Standards (nmol/mL)		Honey sample (mg/L)		Standards (nmol/mL)		Honey sample (mg/L)	
	Recovery (%)	R.S.D. (%)	Mean	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Mean	R.S.D. (%)
Ala	112.32	3.47	13.6	6.59	102.58	4.10	12.0	5.22
Sar	111.20	4.19	3.9	5.31	109.93	5.16	3.6	7.24
Gly	105.32	3.56	4.0	5.68	100.61	4.94	4.5	5.66
Aba	94.32	4.02	–	–	111.74	4.33	–	–
Val	98.75	2.77	14.4	4.54	110.72	4.49	14.8	5.41
Baib	106.32	6.25	–	–	101.61	7.03	–	–
Leu	91.11	4.01	7.0	5.89	89.41	5.79	8.0	6.95
a-Ile	108.62	3.89	–	–	102.07	4.41	–	–
Ile	111.32	3.63	11.2	4.73	108.19	3.27	10.2	5.39
Thr	93.34	2.45	4.0	6.20	92.94	4.41	4.5	6.47
Ser	91.00	5.63	18.9	8.75	96.35	5.14	17.9	6.26
Pro	109.99	2.21	266.0	2.18	101.40	3.69	236.7	4.48
Asn	94.63	3.57	59.8	3.60	86.36	5.01	55.2	5.83
Tpr	114.66	3.32	–	–	122.20	3.46	–	–
Asp	99.69	4.04	36.3	4.16	93.78	5.59	32.7	6.51
Met	90.01	4.15	–	–	91.21	3.27	0.68	5.39
Hyp	87.69	2.39	2.3	7.51	94.16	3.82	2.5	7.94
Glu	92.25	2.59	47.0	5.71	91.13	3.28	51.3	6.45
Phe	89.95	2.64	275.8	3.81	89.93	3.79	247.5	5.91
Aaa	110.25	3.33	–	–	92.77	5.72	–	–
Apa	98.74	4.15	–	–	101.79	4.97	–	–
Gln	95.32	3.72	96.2	4.84	105.57	3.49	82.8	4.61
Orn	107.32	3.29	5.0	6.41	94.58	2.48	5.1	7.05
Gpr	124.01	2.89	–	–	115.93	5.13	–	–
Lys	104.33	6.94	7.6	8.06	82.55	7.13	7.8	9.25
His	98.14	3.64	0.8	4.74	116.74	5.07	1.0	7.19
Hly	91.88	2.87	–	–	108.88	7.76	–	–
Tyr	98.41	1.91	5.0	5.43	97.31	2.48	5.5	5.61
PHP	114.69	4.35	–	–	109.83	4.59	–	–
Trp	112.22	6.70	0.9	7.82	85.62	5.88	1.1	7.45
Cth	109.98	6.62	–	–	110.61	8.03	–	–
C-C	98.89	7.86	–	–	103.65	10.08	–	–

level to be derivatized, the volume of dilution to derivatize was varied between 10 and 100 μL. The results obtained are listed in Table 3, they are similar for GC–FID and GC–MS, finally a volume of 20 μL was selected, because with higher volumes, a decrease in the recovery for aspartic and glutamic acids was observed, and also because in chromatograms of honey samples with a high proline or phenylalanine content, the peak symmetry was worse.

In Fig. 3, the FID (a) and MS (b) chromatograms obtained derivatizing a heather honey sample are shown. Repeating the procedure for different samples belonging to the four botanical origins, it was observed that the norvaline peak never appeared and it did not overlap with other peaks, so this compound was selected as internal standard. In Fig. 3c, a chromatogram of the same sample, without any amount of internal standard added, is shown.

3.3. Method validation

3.3.1. Calibration curves and reproducibility

The calibration curves were obtained by plotting the peak area ratio between the derivatives of amino acids and that of norvaline (IS). In the range of concentration studied, from LOQ-30 mg/L for all amino acids (except for proline and phenylalanine LOQ-60 mg/L) in both methods a good linearity was obtained, as it can be observed in Table 4, excepting proline-hydroxyproline in GC–MS that was not adjusted to a lineal curve.

Reproducibility was evaluated by analysing five replicates of a mixture of standards (at 40 nmol/mL), and five replicates of a honey sample. The results are shown in Table 5.

3.3.2. Limits of detection (LOD) and quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by measuring the magnitude of the analytical background response, we deduced the LOD and LOQ values from the standard response, plus three and ten times the mean background response, respectively. The values obtained are listed in Table 6, they varied between 0.112 (Cys–Cys) and 1.795 mg/L (Gln) by GC–FID and between 0.001 (Aaa and Aba) and 0.291 mg/L (His) by GC–MS in the SIM mode.

Table 6
Retention times – LOD and LOQ by GC–FID and GC–MS in mode SIM expressed in mg/kg honey

Name	GC–FID			GC–MS (SIM)		
	Retention time (min)	*c* (mg/kg)		Retention time (min)	*c* (mg/kg)	
		LOD	LOQ		LOD	LOQ
Ala	1.79 ± 0.01	0.285	0.891	1.97 ± 0.01	0.049	0.149
Sar	1.87 ± 0.01	0.475	1.485	2.02 ± 0.01	0.061	0.186
Gly	1.93 ± 0.01	0.262	0.819	2.05 ± 0.01	0.205	0.626
Aba	2.07 ± 0.01	0.605	1.890	2.13 ± 0.01	0.003	0.005
Val	2.18 ± 0.01	0.144	0.451	2.20 ± 0.01	0.260	0.792
Baib	2.26 ± 0.01	0.360	1.125	2.25 ± 0.01	0.211	0.644
IS	2.35 ± 0.01	–	–	2.29 ± 0.01	–	–
Leu	2.45 ± 0.01	0.448	1.399	2.35 ± 0.01	0.108	0.328
a-Ile	2.50 ± 0.01	0.280	0.875	2.36 ± 0.01	0.077	0.234
Ile	2.53 ± 0.01	0.420	1.312	2.38 ± 0.01	0.076	0.233
Thr	2.80 ± 0.01	0.254	0.794	2.53 ± 0.01	0.013	0.041
Ser	2.85 ± 0.01	0.192	0.601	2.55 ± 0.01	0.064	0.215
Pro	2.95 ± 0.01	0.196	0.614	2.60 ± 0.01	0.118	0.360
Asn	3.06 ± 0.01	1.015	3.170	2.66 ± 0.01	0.108	0.330
Tpr	3.54 ± 0.01	0.268	0.839	2.89 ± 0.01	0.015	0.047
Asp	3.76 ± 0.01	0.497	1.553	3.01 ± 0.01	0.054	0.169
Met	3.80 ± 0.01	0.441	1.377	3.03 ± 0.01	0.021	0.061
Hyp	3.99 ± 0.01	0.559	1.748	3.12 ± 0.01	0.010	0.030
Glu	4.21 ± 0.01	0.157	0.490	3.24 ± 0.01	0.355	1.082
Phe	4.26 ± 0.01	0.211	0.661	3.26 ± 0.01	0.013	0.038
Aaa	4.61 ± 0.01	0.430	1.343	3.42 ± 0.01	0.003	0.011
Apa	4.94 ± 0.01	0.173	0.539	3.59 ± 0.01	0.012	0.037
Gln	5.02 ± 0.01	2.244	7.013	3.65 ± 0.01	0.193	0.589
Orn	5.52 ± 0.01	0.154	0.480	3.87 ± 0.01	0.052	0.164
Gpr	5.60 ± 0.01	0.509	1.590	3.91 ± 0.01	0.009	0.029
Lys	5.83 ± 0.01	0.144	0.450	4.05 ± 0.01	0.067	0.203
His	6.08 ± 0.01	0.744	2.327	4.16 ± 0.01	0.727	2.283
Hly	6.34 ± 0.01	0.519	1.622	4.27 ± 0.01	0.042	0.127
Tyr	6.43 ± 0.01	0.338	1.057	4.33 ± 0.01	0.135	0.412
Php	6.74 ± 0.01	0.436	1.361	4.49 ± 0.01	0.015	0.046
Trp	6.82 ± 0.01	0.073	0.228	4.52 ± 0.01	0.016	0.048
Cth	7.39 ± 0.01	0.142	0.445	4.82 ± 0.01	0.033	0.099
C-C	7.66 ± 0.01	0.140	0.437	4.96 ± 0.01	0.016	0.050

3.4. Application of the method and statistical analysis

The results obtained by applying the procedures to honey samples are summarized in Table 7. The main amino acids found for eucalyptus, rosemary and heather were proline, phenylalanine, tyrosine, glutamic and aspartic acids. For orange blossom were proline, asparagine, phenylalanine, glutamic acid, and lysine. In a first step, the values of concentrations of amino acids for each type of honey were compared by one-way analysis of variance (ANOVA). Principal component analysis was used to achieve a reduction of data dimension and allowed a primary evaluation of the similarities among the honey types analysed. Canonical and linear discriminant analysis were used to find the best combination of amino acids to characterise the four unifloral honey types. SPSS 10.0 (SPSS, 1999) and SAS 8.0 (SAS Institute, 2000) were designed for these tasks.

As it could be deduced from the application of ANOVA, differences among the group values (the four honey types) of arithmetical means were found significant ($P < 0.05$) for all the amino acids excepting Lys.

Principal component analysis (PCA) was applied to the AAs concentration data. Table 8 shows the component loadings matrix obtained for four components and the variance explained by each of them. The first principal component accounts for 55.9% of the variance, and the second component for 8.87%. The cumulative variance for two components is approximately 65% and with four principal components, it gets to 78%. It can also be observed, that the first principal component is basically a function of asparagine, aspartic acid, glutamic acid, threonine, glycine and serine. As the most important relative loadings in this component are positive ones, this can be interpreted as a general index of the size of each honey. Honeys with large values of the first component tend to indicate high values of these amino acids (see Fig. 4).

In Fig. 5a, botanical origins are exposed according to the two principal components. It can be seen that heather honeys have the highest scores in the first principal component.

Table 7
Distribution of amino acid concentration (mg/kg of honey) for the botanical origins considered

	Eucalyptus (n = 15)			Rosemary (n = 28)			Heather (n = 21)			Orange blossom (n = 10)		
	Mean	95% Confidence interval for mean		Mean	95% Confidence interval for mean		Mean	95% Confidence interval for mean		Mean	95% Confidence interval for mean	
		Lower bound	Upper bound		Lower bound	Upper bound		Lower bound	Upper bound		Lower bound	Upper bound
Ala	32.3	23.9	40.7	22.2	18.2	26.3	68.6	48.2	89.0	12.9	8.7	17.0
Sar	12.5	10.7	14.3	8.3	6.5	10.0	12.2	10.4	14.0	4.8	2.1	7.5
Gly	5.9	4.4	7.3	4.9	3.9	5.8	13.6	10.6	16.5	3.1	1.9	4.3
Val	19.5	14.3	24.7	11.4	9.1	13.6	28.7	22.4	35.1	7.4	4.3	10.5
Leu	20.0	13.0	27.0	6.3	3.1	9.6	14.5	10.1	18.9	3.3	1.0	5.7
He	12.3	8.4	16.1	9.0	7.6	10.4	17.7	13.2	22.3	4.8	3.4	6.1
Thr	11.2	7.4	14.9	8.3	6.6	10.1	25.5	19.3	31.8	6.0	4.0	8.1
Ser	17.2	12.2	22.2	11.7	9.3	14.2	38.5	31.5	45.6	12.8	1.4	24.1
Pro	370	339	401	290	264	317	467	420	515	243	212	273
Asn	31.3	13.6	49.1	20.1	13.5	26.7	93.9	64.4	123.4	26.8	18.6	35.0
Asp	37.6	20.5	54.7	25.2	16.6	33.8	126.2	100.6	151.8	17.4	11.5	23.2
Met	2.08	1.20	2.96	1.14	0.88	1.40	1.58	1.23	1.93	0.51	0.25	0.78
Hyp	6.4	5.2	7.6	2.5	1.9	3.0	10.6	8.8	12.4	2.1	1.4	2.7
Glu	50.9	32.6	69.3	28.1	18.0	38.1	191.9	139.5	244.4	16.9	8.2	25.6
Phe	215	121	308	114	64	163	281	188	374	36	18	53
Aaa	0.17	0.11	0.23	0.10	0.06	0.14	0.68	0.44	0.92	0.07	0.02	0.12
Gin	23.7	11.6	35.9	13.8	8.1	19.5	83.1	51.7	114.6	13.9	4.1	23.6
Om	1.58	1.11	2.06	1.20	0.92	1.48	9.64	5.62	13.66	1.57	0.93	2.21
Lys	28.3	22.3	34.3	22.5	17.3	27.6	25.9	19.3	32.6	17.8	9.7	26.0
His	14.4	12.5	16.4	16.1	14.3	17.8	14.4	12.2	16.7	11.6	9.6	13.5
Tyr	46.1	31.6	60.6	59.5	33.3	85.7	120.1	65.0	175.1	11.9	7.3	16.5
Trp	1.68	1.20	2.15	2.68	1.34	4.02	4.92	3.18	6.66	0.92	0.43	1.41

Table 8
Component loadings matrix obtained for the four factors and the variance explained by each of them

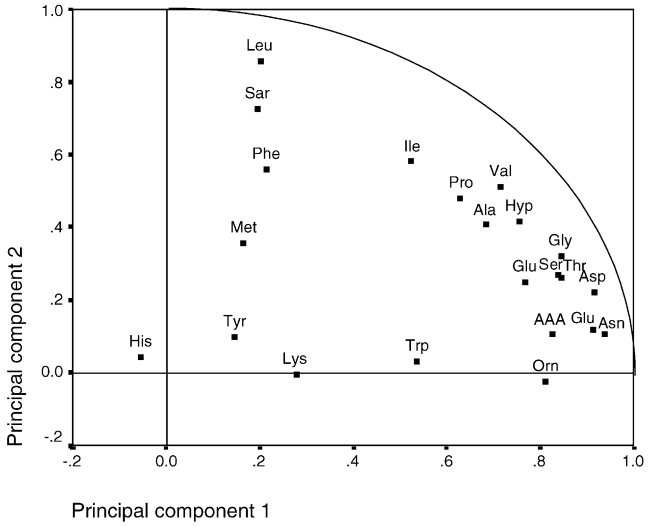
	Component			
	1	2	3	4
Ala	0.6837	0.4056	−0.1078	−0.0476
Sar	0.1954	0.7274	0.1151	0.2685
Gly	0.8435	0.3210	0.0460	0.2398
Val	0.7155	0.5100	0.1889	0.2385
Leu	0.2019	0.8572	0.0865	−0.1060
He	0.5253	0.5813	0.2524	0.3314
Thr	0.8436	0.2594	0.3006	0.1971
Ser	0.8399	0.2676	0.1103	0.1808
Pro	0.6295	0.4796	0.1118	0.3647
Asn	0.9384	0.1038	0.0843	0.0206
Asp	0.9149	0.2201	0.0310	0.2308
Met	0.1661	0.3572	0.6144	−0.0402
Hyp	0.7551	0.4169	−0.0263	0.2261
Glu	0.9121	0.1165	0.0235	0.1561
Phe	0.2143	0.5596	0.0793	0.5902
Aaa	0.8255	0.1041	0.3011	0.0189
Gin	0.7679	0.2476	0.2622	0.1252
Orn	0.8119	−0.0235	0.1343	0.3588
Lys	0.2779	−0.0063	0.8937	0.0292
His	−0.0532	0.0428	0.8180	0.1525
Tyr	0.1473	0.0974	0.0102	0.8365
Trp	0.5368	0.0302	0.2273	0.6088
Eigenvalues	12.30	1.95	1.69	1.27
% Variance	55.90	8.87	7.70	5.78
Cumulative % variance	55.90	64.77	72.47	78.25

For the second principal component, eucalyptus honeys have high scores because this kind of honey has high values in Leucine.

A canonical discriminant analysis was done from SPSS 10.0, trying to separate the four botanical origins studied in one step. The variables selected by stepwise method as the most discriminant were, in this order: Sar, Leu, Ile, Asn, Asp, Hyp, Phe, Aaa, Lys, His and Trp. They did not allow the succesfully separation among all the botanical origins, with a global percentage of honeys correctly classified up to 75%. The distribution of the canonical discriminant scores for all the honey samples is shown, on a scatter diagram, in Fig. 5b. Heather honeys appear separated of the rest of samples by the first canonical discriminant function, while eucalyptus honeys can be considered as an independent group of honeys by the second canonical discriminant function.

Orange and rosemary honeys are mixed, so classification into their own botanical origin by canonical discriminant functions did not yield good results.

A discriminant analysis in four steps from SAS 8.0 was made to improve the percentage of honey correctly classified. The percentages are between 87% for eucalyptus honey and 93% for rosemary. In the first step we were able to separate heather honey from the others, and the variables selected in a stepwise method were Asp, Hyp, Aaa, Asn, Lys and Ile. The percentage of samples correctly classified for this kind of honey reached 90%. In the second step, using



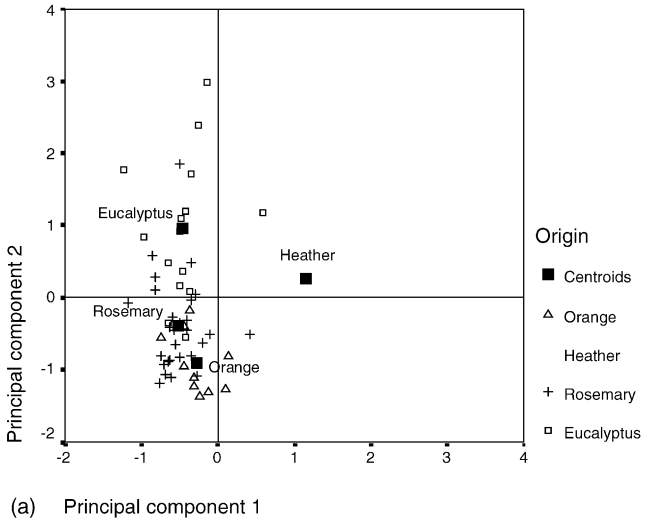


Fig. 4. Component loadings matrix representation of the two first principal components.

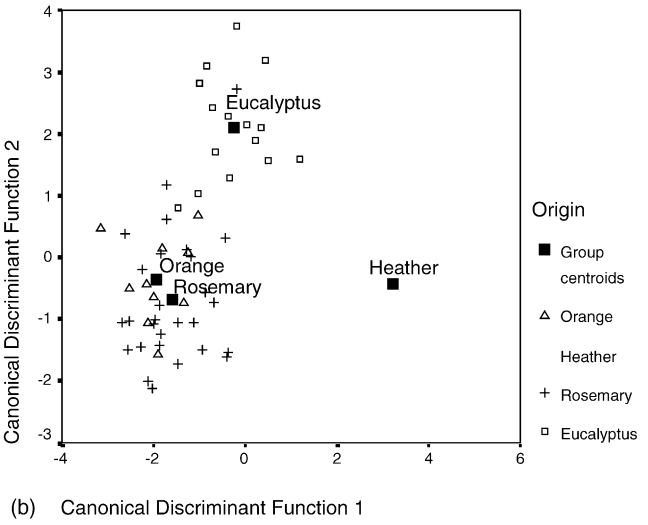


Fig. 5. (a) Two first component scores of honeys from the studied botanical origins and (b) canonical discriminant analysis of heather, eucalyptus, rosemary and orange honey, representing each sample on the plane formed by the two principal canonical variables.

the most powerful variables for the differentiation of eucalyptus honey, Hyp, Gly, Leu, Met, Trp and Sar, the percentage of success was 87%. By means of the third linear discriminant step we could separate rosemary from orange samples using only three amino acids, Ile, Orn and His obtaining 93% of the honey correctly classified after cross-validation.

As a final step, following cross-validation, 89% of the orange honey could be correctly classified, isolating it from a misclassified honey group composed of heather, eucalyptus and rosemary honeys which had not been classified according to their individual botanical origins. The amino acids employed at this step were Ala, Sar, Gly, Val, Leu, Ile, Thr, Ser, Pro, Asn, Asp, Met and Hyp.

We included this last step in the discriminant analysis scheme for isolating orange honey from this group of misclassified honeys with two proposes: first, for using this statistical treatment for all classes of honeys, such that, honey from an origin other than heather, eucalyptus, rosemary and orange could be characterised as misclassified; secondly, for improving the percentages of success when applying Bayes' theorem, under which we must consider all honeys not correctly classified according to their individual botanical origins. Thus, applying Bayes' theorem, we must consider that no honey from other groups has been classified as being classified heather and orange. As eucalyptus, only one rosemary honey has been classified and, as rosemary, one heather and one orange honey have been classified.

If the proposed discriminant scheme is applied to an unknown sample and is classified as heather or orange, 100% success will be obtained. Should this unknown sample be classified as eucalyptus or rosemary, the probabilities of success would then be 96 or 93%, respectively.

If the characteristics of an unknown honey, relative to its amino acid content, cannot be classified into a specific studied group, it will be classified as a misclassified honey.

4. Conclusions

The proposed method can be applied successfully to the analysis of amino acids in honey samples in a total time of 15 min (preparation of sample and chromatographic analysis included).

Linearity range, LOD and LOQ, reproducibility and accuracy are suitable for the quantification of amino acids in honey.

88% of studied honeys (65 out of 74) are correctly classified according to botanical origin with a discriminant analysis

in four steps and with the amino acids concentration as the variable employed.Acknowledgements

Financial support of this project (CAL01-066-C7-1) by the Spanish Ministry of Science and Technology (MC & T) is gratefully acknowledged.

References

[1] M.T. Iglesias, C. De Lorenzo, M. Polo, C. del, P.J. Martín-Álvarez, E. Pueyo, J. Agric. Food Chem. 52 (2004) 84.
[2] E. Anklam, Food Chem. 63 (4) (1998) 549.
[3] N. Muñoz, M. Ortigosa, P. Torre, J.M. Izco, Food Chem. 83 (2003) 329.
[4] D. Heems, G. Luck, C. Fraudeau, E. Vérette, J. Chromatogr. A 798 (1998) 9.
[5] A. Péter, D. Tourwè, M.E.M. Baunmann, M. Elsken, L. Goeyens, J. Liq. Chromatogr. Rel. Technol. 22 (1999) 1077.
[6] S.J.M. Mdachi, M.H.H. Nkunya, V.A. Nyigo, I.T. Urasa, Food Chem. 86 (2004) 179.
[7] B.M. Silva, S. Casal, P.B. Andrade, R.M. Seabra, M.B.P.P. Oliveira, M.A. Ferreira, J. Agric. Food Chem. 52 (2004) 1201.
[8] S.S. Yang, I. Smetena, Chromatographia 37 (1993) 593.
[9] I. Hermosín, R.M. Chicón, M.D. Cabezudo, Food Chem. 83 (2003) 263.
[10] J. Krischbaum, B. Luckas, W.D. Beinert, Deuts. Lebensm. Rundsch. 90 (1994) 224.
[11] B.M. Silva, S. Casal, P.B. Andrade, R.M. Seabra, M.B.P.P. Oliveira, M.A. Ferreira, Anal. Sci. 19 (2003) 1285.
[12] P. Husek, P. Simek, LC–GC 19 (2001) 986.
[13] K. Petritis, C. Elfakir, M. Dreux, J. Chromatogr. A 961 (2002) 9.
[14] S. Moore, D.H. Spackman, W.H. Stein, Anal. Chem. 30 (1958) 1185.
[15] H.F. Marshall, G.P. Shaffer, E. Conkerton, J. Anal. Biochem. 180 (1989) 264.
[16] B.H. Klein, J.W. Dudenhausen, J. Liq. Chromatogr. 18 (1995) 4007.
[17] P. Lehtonen, Am. J. Enol. Vitic. 47 (1996) 127.
[18] P. Fürst, L. Pollack, T.A. Graser, H. Godel, P. Stehle, J. Chromatogr. 449 (1990) 557.
[19] P. Martin, P.A. Suárez, C. Polo, M.D. Cabezudo, M.V. Dabrio, Anal. Bromatol. 32 (1980) 289.
[20] E.M. Sanders, C.S. Ough, Am. J. Enol. Vitic. 36 (1985) 43.
[21] S. Einarsson, J. Chromatogr. 348 (1985) 213.
[22] H. Brückner, M. Lüpke, J. Chromatogr. A 697 (1995) 295.
[23] J.R.E. Lewis, J.S. Morley, R.F. Venn, J. Chromatogr. 615 (1993) 37.
[24] B.A. Bidlingmeyer, S.A. Cohen, T.L. Tarvin, J. Chromatogr. 336 (1984) 93.
[25] S.A. Cohen, D. Strydom, J. Anal. Biochem. 174 (1988) 1.
[26] C. Van Wandelen, S.A. Cohen, J. Chromatogr. A 763 (1997) 11.
[27] J. Díaz, J.Ll. Lliberia, s.L. Comella, F. Broto-Puig, J. Chromatogr. A 719 (1996) 171.
[28] S.A. Cohen, K.M. De Antonis, J. Chromatogr. A 661 (1994) 25.
[29] H.J. Liu, J. Chromatogr. A 670 (1994) 59.
[30] V. Carnevillier, P. Schlich, J. Guerreau, C. Carpentier, M. Feuillat, Vitis 38 (1) (1999) 37.
[31] M. Alaiz, J.L. Navarro, J. Girón, E. Vioque, J. Chromatogr. 591 (1992) 181.
[32] J. Girón, M. Alaiz, E. Vioque, Anal. Biochem. 206 (1992) 155.
[33] A. Pirini, L.S. Conte, O. Francioso, G. Lecker, J. High Resolut. Chromatogr. 15 (1992) 165.
[34] R. Chicón, I. Hermosín, M.D. Cabezudo, Tecnol. Vino. 1 (2001) 95.
[35] J. You, Y. Shan, L. Zhen, L. Zhang, Y. Zhang, Anal. Biochem. 313 (2003) 17.
[36] I. Betnér, P. Földi, LC–GC 6 (1988) 832.
[37] F.A. Vazquezortiz, G. Caire, I. Higueraciapara, G. Hernández, J. Liq. Chromatogr. 18 (1995) 2059.
[38] P. Husek, J. Chromatogr. 552 (1991) 289.
[39] P. Husek, C.C. Sweeley, J. High Resolut. Chromatogr. 14 (1991) 751.
[40] P. Husek, FEBS Lett. 280 (1991) 354.
[41] C. Haberhauer-Troyer, G. Álvarez-Llamas, E. Zitting, P. Rodríguez-González, E. Rosenberg, A. Sanz-Medel, J. Chromatogr. A 1015 (2003) 1.
[42] P. Cao, M. Moini, J. Chromatogr. A 759 (1997) 111.
[43] A. Namera, M. Yashiki, M. Nishida, T. Kojima, J. Chromatogr. B 776 (2002) 49.
[44] C. Wittmann, M. Hans, E. Heinzle, Anal. Biochem. 307 (2002) 379.
[45] G.C. Kite, H. Ireland, Phytochemistry 59 (2002) 163.
[46] P. Husek, Phenomenex, Eur. Pat. 00301791.0-2204, priority CZ/04.03.99/CZ 769994, 3 March 1999.
[47] T. Farcas, J.E. Toulouee, M. Li, LC–CG Eur. (April 2002) 30.