FAST SPE EXTRACTION AND LC-ESI-MS-MS ANALYSIS OF FLAVONOIDS AND PHENOLIC ACIDS IN HONEY

Patrizio PULCINI, Francesco ALLEGRINI, Norma FESTUCCIA;
C.R.A - Istituto Sperimentale per la Zoologia Agraria, Sezione di Apicoltura, Via Leonida Rech, 36 00156 Roma (ITALY) – Tel. +390686895456 Fax +390686895463 e-mail: isza@apicoltura.org

Abstract
As generally recognized, flavonoids and phenolic acids are the most important components of honey related to its antioxidant power. A simple SPE extraction procedure was developed to acquire fractions for the determination in honey of antioxidants components, like phenolics, flavonoids and organic acids. An acidified honey solution is passed through a 500mg C18 and/or SDB polymeric SPE column to obtain a clear fraction containing phenolic acids and flavonoids. Then the filtrate is neutralised and passed through a SAX SPE column to obtain the fraction containing organic acids.
The procedure was validated analysing the extracts with HPLC-DAD methods to determine repeatability r and recovery %. Coupled reversed phase HPLC-Mass Spectrometry Detection with an ESI (Electro Spray Ionisation) interface in the negative ion mode was then used to analyse the phenolics and flavonoid containing fractions. Detection and Quantification limits are very low. The LC/ESI/MS/MS with full SCAN, DAUGHTER SCAN and MRM methods, permitted so far to identify and quantify, by mean of their specific *m/z* and fragmentation patterns, eight typical flavonoids and seven typical phenolic acids in 5 species of unifloral Italian honeys (*Robinia, Castanea, Eucalyptus, Helianthus, Erica arborea*).

Keywords: *honey, flavonoids, phenolic acids, organic acids ,SPE, LC-MS*

Introduction
The analysis of flavonoids, phenolic and organic acids in honey has been a very important item in recent years, especially to combine with other analyses for the attribution of floral origin and in the evaluation of the antioxidant power of honey. Flavonoids and phenolics can be generally distinguished for their origin, propolis, pollen or nectar, being the second ones more suitable as markers for the determination of botanical origin: the phenolics for the different content found in unifloral honeys (Amiot *et al*.1989, Ferreres *et al*.1996, Andrade *et al*. 1997), the flavonoids also for their typical HPLC profiles (Ferreres *et al*. 1994a, Soler *et al*. 1995, Tomas-Barberan *et al*. 2001). In some cases particular substances were proposed as markers, such as hesperetin for *Citrus* honey (Ferreres *et al*. 1993, 1994b), homogentisic acid for *Arbutus* honey (Cabras *et al*. 1999), while different contents of hydroxybenzoic, hydroxycinnamic, phenylacetic and other acids, flavones, flavanones, isoflavones and other polyphenols have been reported for the classification of honeys (Ferreres *et al*..1994a, Andrade *et al*. 1997, Tomas-Barberan *et al*. 2001). Generally, the extraction of the flavonoid containing fractions is carried out through chromatographic column methods and a purification step, using considerable amounts of honey and solvents, while the analyses were generally made by HPLC with UV-DAD detection (Tomas-Barberan *et al*. 2001, Ferreres *et al*. 1994c). Simpler SPE-SAX and HPLC procedures were used for organic acids (Cherchi *et al*. 1994, Del Nozal *et al*. 1998, Cossu *et al*. 2001, Suarez-Luque *et al*. 2002a, 2002b). Aims of this work were: a) to select, optimize and validate a simple extraction and clean-up procedure with SPE cartridges to obtain clear fractions of flavonoids and organic acids suitable for the analysis; b) to identify and quantify with low LOD, flavonoids and phenolics, using very sensitive LC-ESI-MS-MS methods. The investigated compounds were the flavonoids apigenin, kaempferol, naringenin, pynocembrin, chrisin, quercetin, hesperetin, myricetin and the phenolic acids ferulic, mandelic, vanillic, ellagic, gallic, homogentisic and p-coumaric. The method was applied to 5 unifloral honey samples: *Eucalyptus, Robinia, Castanea, Erica arborea* and *Helianthus*.

Materials and Methods

Reagents

Methanol, Acetonitrile and Water of HPLC and LC-MS grade from Sigma-Aldrich (34966, 34860, 34998, 34967, 34877, 39253) and Carlo Erba (412412, 412142); analytical grade sulphuric and hydrochloric acids, sodium hydroxide and chloride from Carlo Erba; standards of flavonoids, phenolics and organic acids from Sigma-Aldrich; SPE cartridges from Phenomenex: Strata SDB-L Polymer (100 µ, 260A) 500mg/3mL for flavonoids and phenolics; Strata SAX (55 µ, 70A) 500mg/3mL for Organic Acids.

SPE Procedure

Honey solution. 5g (± 0,01g) of honey were dissolved with 10 mL of HPLC grade Water, adjusted at pH=2 with HCL 1N, passed through the SDB-L cartridge, collected and neutralised to pH=7,0 with NaOH 1 N; then a volume of 5 mL was passed through the SAX cartridge.

SPE Conditioning: SDB-L : 3mL of Acetonitrile + 3 mL of Methanol + 3 mL of Water at 1mL/min
SAX : 3 mL of NaCl 1M + 3 mL of Water at 0,5 mL/min

SPE Washing: SDB-L : 3 mL of Water at pH=2 + 10 mL of Water at 1 mL/min.
SAX : 3+3 mL of Water at 0,5mL/min

SPE Eluting: SDB-L : 3 mL (1+1+1) of Methanol-Acetonitrile 2:1 at 1 mL/min
SAX : 3 mL (1+1+1) of Sulphuric acid 1 N at 0,5 ml/min

HPLC/LC- MS Solutions: SDB-L eluted was filtered through 0,45µ membrane filters ; SAX eluted was filtered through 0,45 µ membrane filters.

HPLC Analysis of Phenolics and Flavonoids: Varian Pro Star; Solvent Manager 230, DAD-UV 330, Autosampler 400. Column: Phenomenex Luna C18 (2) 5 µ 150x4,6 mm - Flow 1mL/min of Water-Formic Acid 19:1(Solvent A)/Methanol (Solvent B) - Loop 50 µL – Instantaneous gradient elution starting with 80:20 isocratic for 20 min up to 20:80 at 60 min. Detection UV- DAD. Chromatograms recorded at 290 and 340 nm.

HPLC Analysis of Organic Acids : Varian Pro Star : Separation Module 230, DAD-UV 330 and Autosampler 400. Two columns connected in series : Phenomenex Luna C18 (2) 5 µ 250x4,6 mm and Synergi 4 µ Hydro RP-80 250x4,6 mm - Flow 0,7 mL/min of Sulphuric Acid 0,002 N (pH=2,65) – Detection UV at 210 nm - Loop 100 µL -

SPE Extraction - Procedure validation for Organic Acids, Flavonoids and Phenolic Acids

Three selected unifloral honey samples were used: *Robinia*, *Castanea* and *Eucalyptus*. Single components analysed and fortified were malic, citric and succinic for the organic acids (OA) and ferulic acid, chrisin, pynocembrin and quercetin for the flavonoids-phenolics. Three repetitions for sample and six replicates were performed. Two fortification levels were carried out for flavonoids, Level 1 of 6 mg/Kg (R1), Level 2 of 12 mg/Kg (R2) and one level of 60 mg/Kg (R) for organic acids. Results are reported in Table I.

Table I. Mean values (**mg/Kg**), ***Repeteability r*** (**100*sd/Xm**) and ***Recovery*** (**%**) of the fortification levels ***R1, R2*** for flavonoids and ***R*** for OA**.** Data are means of ***n=6*** replicates.

	Robinia				***Castanea***				***Eucalyptus***			
	mean value	***r***	***R***		***mean value***	***r***	***R***		***mean value***	***r***	***R***	
			R1	***R2***			***R1***	***R2***			***R1***	***R2***
Malic acid	**44,9**	**4,5**	**90,6**		**169,7**	**4,5**	**94,7**		**28,5**	**5,7**	**91,1**	
Citric acid	**40,2**	**4,5**	**86,8**		**158,9**	**1,6**	**93,3**		**69,8**	**5,0**	**89,0**	
Succinic acid	**10,7**	**5,9**	**82,5**		**39,9**	**7,6**	**97,2**		**50,1**	**4,8**	**91,8**	
Ferulic acid	**1,0**	**10,2**	**99,1**	**100,1**	**2,6**	**7,0**	**99,9**	**94,9**	**2,5**	**4,0**	**97,4**	**98,5**
Pynocembrin	**5,7**	**7,2**	**99,4**	**95,3**	**2,3**	**6,8**	**90,4**	**98,6**	**4,7**	**5,8**	**100,9**	**98,4**
Chrisin	**1,1**	**11,0**	**93,3**	**97,6**	**1,3**	**8,6**	**91,3**	**87,8**	**1,2**	**10,6**	**88,4**	**88,7**
Quercetin	**2,3**	**6,9**	**95,4**	**96,6**	**1,8**	**6,2**	**93,1**	**90,3**	**2,7**	**2,2**	**99,0**	**93,4**

LC - ESI - MS - MS Analysis of Phenolics and Flavonoids: Waters Alliance 2695 Separation Module and 2695 Autosampler equipped with Waters 2487 Dual ? Absorbance Detector, Micromass quattro micro Triple Quadrupole Mass Spectrometer with Electrospray Ionisation (ESI) probe. Separation on X-Terra MS columns C18 5 µ 150x4,6 mm. Mobile Phase 0,1% Acetic acid in Water (Solvent A)/ 0,1% Acetic acid in Acetonitrile (Solvent B)- Flow 1,0 mL/min split with split ratio of 7:3 Instantaneous gradient elution starting with 90:10 up to 40:60 at 60 min. MS conditions: ESI - Ion Mode Negative – Capillary (kV) 3,20 - Cone (V) 30 in SCAN Mode – RF Lens 0,1. Resolution: LM 1 14,0 HM 1 14,0 LM 2 15,0 HM 2 15,0 - Ion Energy 1,0 – Source T 120°C – Desolvation T 350 °C.
The operating conditions are described in Table II.

Table II. LC-MS-MS characteristics of flavonoids and phenolic acids

Flavonoids	***Rt min.***	***MS m/z [M-H]-***	***MS/MS ions, m/z (rel. Int. %)***	***CV/CE* Cone Voltage / Collision Energy**	***LOD s/n = 3 (ppb)***
Apigenin	**31,1**	**269**	**151 (100), 117 (80)**	**-35/-15**	**3,4**
Chrisin	**40,0**	**253**	**107 (50), 63 (100), 253 (35)**	**-40/-30**	**1,3**
Hesperetin	**32,1**	**301**	**164 (90), 136 (100)**	**-40/-30**	**3,9**
Kaempferol	**31,9**	**285**	**93 (100), 285 (40)**	**-60/-55**	**13,9**
Myricetin	**19,6**	**317**	**317 (50), 151 (100), 137 (70)**	**-40/-25**	**42,8**
Naringenin	**30,8**	**271**	**119 (80), 151 (100), 271 (20)**	**-40/-30**	**3,3**
Pynocembrin	**41,2**	**255**	**107 (100), 65 (80), 255 (20)**	**-40/-25**	**2,3**
Quercetin	**27,7**	**301**	**151 (100), 121 (40), 301(20)**	**-35/-25**	**10,7**
Phenolics	**-------**	**-------**	**-------**	**-------**	**-------**
p-Coumaric	**13,4**	**163**	**119 (100), 163 (40)**	**-30/-15**	**27,1**
Ellagic	**14,2**	**301**	**145 (100), 129 (70), 301(35)**	**-40/-30**	**153,4**
Ferulic	**14,5**	**193**	**134 (100), 149 (30), 178 (55)**	**-30/-20**	**15,9**
Gallic	**2,4**	**169**	**169 (10), 125 (100)**	**-25/-15**	**26,8**
Homogentisic	**3,0**	**167**	**122 (100), 108 (35)**	**-35/-20**	**17,3**
Mandelic	**6,1**	**151**	**107 (100), 77 (40), 151(35)**	**-20/-15**	**122,0**

Vanillic	**7,9**	**167**	**91 (100), 123 (30)**	**-40/-20**	**153,7**

Results

The identification and quantification of the single components were carried out in compliance with the criteria defined for LC-MS-MS techniques in the Commission Decision 2002/657/CE, applying the Council Directive 96/23/CE. These compounds up to now are not indicated for MRPL limits. The MS-MS ion fragmentations and relative m/z ratios were defined and selected with standard infusion techniques, Daughter Scan and MRM modes using specific MRM optimization software, available from Waters Corporation.

Results are reported in Table II for the LC-MS-MS data, and in Table III for the contents found in the investigated honey samples. The ESI negative analysis is generally in agreement with data previously reported (Fang *et al*., 2002; Sanchez-Rabaneda *et al*., 2003), giving very appropriate results for flavonoids and a little less for phenolics, especially for ellagic, mandelic and vanillic acids, probably due to ionisation and/or desolvation difficulties of these molecules in the conditions used.

Table III. Mean contents (mg/Kg) of flavonoids and phenolic acids in honey. **(n=6; * n=3)**. ***loq*** = limit of quantification; ***lod*** = limit of detection.

Flavonoids	***Robinia***	***Castanea***	***Eucalyptus***	***Erica****	***Helianthus****
Apigenin	**0,19**	**0,06**	**0,08**	**0,04**	**0,22**
Chrisin	**1,56**	**0,91**	**1,06**	**0,22**	**0,73**
Hesperetin	**0,42**	**< loq**	**0,10**	**< loq**	**0,14**
Kaempferol	**0,38**	**0,13**	**0,35**	**0,20**	**1,67**
Myricetin	**< loq**	**< loq**	**0,34**	**1,60**	**0,81**
Naringenin	**0,82**	**< loq**	**0,38**	**0,05**	**0,16**
Pynocembrin	**6,00**	**2,52**	**5,46**	**0,78**	**1,91**
Quercetin	**2,95**	**1,56**	**2,26**	**0,24**	**1,31**
Phenolics	----------	----------	----------	----------	----------
p-Coumaric	**1,40**	**1,16**	**1,19**	**0,37**	**0,78**
Ellagic	**< loq**	**4,92**	**< lod**	**8,55**	**< lod**
Ferulic	**0,82**	**2,86**	**2,41**	**1,65**	**3,46**
Gallic	**< lod**	**< loq**	**< loq**	**< lod**	**< lod**
Homogentisic	**< loq**	**< loq**	**< lod**	**0,16**	**< loq**
Mandelic	**< lod**	**0,69**	**< loq**	**1,98**	**0,90**
Vanillic	**< loq**	**1,46**	**0,92**	**2,04**	**1,16**

Conclusions

The SPE procedure gives reproducible results and is shown to be suitable for the extraction of flavonoids and phenolics from honey.

The high recoveries obtained could indicate the use of SPE of flavonoids as a clean-up step. Tandem MS analysis of phenolics needs to be further studied, in order to improve its suitability, while it fits very well with flavonoid detection and quantification.

Data obtained are generally in agreement with the ones found so far for phenolics and flavonoids. A very interesting result is that hesperetin, previously indicated as a possible marker for *Citrus* honey (Ferreres et *al*. 1993, 1994b), was found, in different amounts, also in other honey types. Homogentisic acid, indicated as a possible marker for *Arbutus* honey (Cabras et *al.* 1999), was found also in honey from *Erica* (which belongs to the same botanical family as *Arbutus* (Ericaceae). These data indicate that the difference of the whole composition in flavonoids and phenolics might be more suitable than one single specific compound for the characterization of botanical origin. The results obtained should be further confirmed through the analysis of more extended samplings and other unifloral types.

References

[1] Amiot M.J., Aubert S.,Gonnet M., Tacchini M., (1989) Phenolic composition of honeys : preliminary study on identification and group quantification, Apidologie 20, 115-125

[2] Andrade P., Ferreres f., Amaral M.T., (1997) Analysis of honey phenolic acids by HPLC, its application to honey botanical characterization, J.Liquid Chromatogr. Rel. Technol. 20, 2281-2288

[3] Cabras P., Angioni A., Tuberoso C., Floris I.,Reniero F., Guillou C., Ghelli S., (1999) Homogentisic acid : A phenolic acid as marker of strawberry-tree (*Arbutus unedo*) honey, J.Agr.Food Chem., 47, 40674-4067

[4] Cherchi A., Spanedda L., Tuberoso C., Cabras P., (1994) Solid-phase extraction and high performance liquid chromatographic determination of organic acids in honey, J. of Chrom. A, 669, 59-64

[5] Cossu M., Alamanni M.C., Scano F., (2001) Uso del metodo SPE-HPLC per la ricerca di residui di acaricidi sintetici e naturali in una indagine sulla qualità dei mieli prodotti in Sardegna, Riv. Sci. dell' Alim., 30 (2) 125-132

[6] Del Nozal M.J., Bernal J.L., Marinero P., Diego J.C., Frechilla J.I., Higes M., Liorente J.,(1998) HPLC determination of organic acids in honeys from different botanical origin, J. Liq. Chrom. & Rel. Technol. 21(20), 3197-3214

[7] Fang N., Yu S., Prior R. L., (2002) LC/MS/MS Characterization of Phenolic Constituents in Dried Plums, J. of Agr. And Food Chem., 50, (12), 3579-3585

[8] Ferreres F.,Garcia –Viguera C., Tomas-Lorente F., Tomas-Barberan F.A.,(1993) Hesperetin : A marker of the floral origin of citrus honey; J. Sci. Food Agric., 61, 121-123

[9] Ferreres F., Andrade P., Tomas-Barberan F.A.,(1994a) Flavonoids from Portuguese heather honey, Z. Lebensm. Unters. Forsch. 1991, 32-37

[10] Ferreres F., Giner J.M., Tomas-Barberan F.A.,(1994b) A comparative study of hesperetin and methyl anthranilate as markers of the floral origin of citrus honey, J. Sci. Food Agric., 65, 371-372

[11] Ferreres F., Tomas-Barberan F.A., Soler C., Garcia-Viguera C., Ortiz A. and Tomas-Lorente F., (1994c) A simple extractive technique for honey flavonoid HPLC analysis, Apidologie 25 : 21-30

[12] Ferreres F., Andrade P., Gil M.I. and Tomas-Barberan FA, (1996) Floral nectar phenolics as biochemical markers for the botanical origin of heather honey, Z. Lebensm. Unters. Forsch. 202, 40-44

[13] Tomas-Barberan F.A., Martos I., Ferreres F., Radovic B.S., Anklam E., (2001) HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys, J. Sci. Food Agric. 81, 485-496

[14] Sanchez-Rabaneda F.,JaureguiO., Casals I., Andres-Lacueva C.,Izquierdo-Pulido M.,Lamuela-Raventos R. M., (2003) Liquid Chromatographic/Electrospray Ionisation Tandem Mass Spectrometric study of the phenolic composition of *cocoa* (Theobroma Cacao), Journal of Mass Spectrometry, 38 : 35-42

[15] Soler C., Gil MI, Garcia-Viguera C., Tomas-Barberan FA, (1995) Flavonoids patterns of French honeys with different floral origin, Apidologie 26 : 53-60

[16] Suarez-Luque S., Mato I., Huidobro H.F.,Simal-Lozano J., (2002a) Solid-phase extraction procedure to remove organic acids from honey, J. of Chrom. B, 770, 77-82

[17] Suarez-Luque S., Mato I., Huidobro J.F., Simal-Lozano J., Sancho M.T., (2002b) Rapid determination of minority organic acids in honey by high performance liquid chromatography, J. of Chrom. B 955, 207-214