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High-performance liquid chromatographic determination of methyl anthranilate, hydroxymethylfurfural and related compounds in honey☆

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Abstract

A high-performance liquid chromatographic method for determining 5-hydroxymethyl-2-furaldehyde (hydroxymethylfurfural), 2-furaldehyde (furfural), furan-2-carboxylic acid (2-furoic acid), furan-3-carboxylic acid (3-furoic acid), furan-3-carboxaldehyde (3-furaldehyde) and 2-aminobenzoic acid methyl ester (methyl anthranilate) in honey and honeydew samples is described. To prevent matrix interference and to isolate the compounds, a clean-up step which implies a solid-phase extraction on polymeric cartridges and an elution with 0.5 ml methanol is recommended. The compounds are separated on a reversed-phase column with a gradient of (A) 1% aqueous acetic acid–acetonitrile (97:3, v/v) and (B) acetonitrile–water (50:50, v/v), with UV detection at 250 nm. The method is applied to the analysis of samples from different botanical origin.

Keywords: Honey; Food analysis; Aldehydes; Hydroxymethylfurfural; Methyl anthranilate; Furaldehyde; Furoic acid; Furancarboxylic acid; Furancarboxaldehyde

1. Introduction

5-Hydroxymethyl-2-furaldehyde or hydroxymethylfurfural (HMF) is a cyclic aldehyde that is produced in the acidic decomposition of monosaccharides, so it appears naturally in all products where water coexists with monosaccharides in an acidic medium. Another way of natural HMF formation is through the condensation of carbohydrates that have free amine groups, according with the well-known Maillard reaction [1]. This produces an amino acid destruction together with the appearance of some anti-nutritive products, sometimes toxic ones. For this reason, attention has been focused on the study of the reaction products, including HMF and related compounds [2].

Honey is a natural sweetener where both situations arise, and because of that, there is a low HMF value in fresh honey and a high one in honey that has been heated, stored in non-adequate conditions, or adulterated with invert syrup. The European quality stan-

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dards allow a maximum of 4 mg/100 g of HMF, nevertheless honey produced in subtropical climates has high HMF values, exceeding the maximum standard. So, HMF is usually evaluated with respect to the legal requirements [3–6] and it is not used as a control of product origin [7], perhaps because other related compounds have not been considered.

The 2-aminobenzoic acid methyl ester or methyl anthranilate (MA) has been proposed as an indicator compound to distinguish citrus honey from other monofloral or multifloral non citrus ones [8], although MA also suffers changes in its concentration depending on different variables, which include storage conditions [9]. For this reason it is also interesting to determine it in fresh honey samples.

As HMF is a quality indicator in a large variety of foods, there are several methods proposed for its evaluation. In honey analysis, it is usually determined spectrophotometrically by the Winkler method [10] based on the reaction with *p*-toluidine–barbituric acid, or modifications of this method [11–13], and by the White procedure [14]. However, nowadays high-performance liquid chromatography (HPLC) is recommended [15–17] because it allows the simultaneous analysis of other compounds, such as methyl anthranilate [18], whereas gas chromatography requires a previous derivatization [19]. In the case of honey there are no references about the evaluation of certain compounds that could be related with the HMF content or with changes after honey harvesting: 2-furoic acid (2-OIC), furan-3-carboxylic acid (3-OIC) and furan-3-carboxaldehyde (3-AL).

Methyl anthranilate was initially determined in honey by spectrophotometry [8] but today HPLC is also preferred [18].

Taking into account the considerations above mentioned, the aim of this work has been to develop a HPLC method that allows not only the simultaneous determination of HMF and four related compounds, but also of MA, so, in the same chromatogram we would have two indicators of quality and some additional information about the presence of other compounds which could be considered, in conjunction with other physico–chemical parameters, as an aid to know honey origin instead of pollen analysis.

2. Experimental

2.1. General

2.1.1. Chemicals

Analytical standard-grade HMF, 2-AL, 2-OIC, 3-OIC, 3-AL and MA were obtained from Sigma–Aldrich (Madrid, Spain). Sulfuric acid and acetic acid were of analytical-reagent grade and supplied by Merck (Darmstadt, Germany). Acetonitrile and methanol of HPLC grade were purchased from Lab-Scan (Dublin, Ireland). Water was purified by using a Compact Milli-RO and Milli-Q water system from Millipore (Bedford, MA, USA). All solutions used were previously passed through a 0.45-μm membrane filter, from Millipore, to remove any impurities.

Oasis HLB solid-phase extraction cartridges of 30 mg were obtained from Waters (Milford, MA, USA).

2.1.2. Equipment

The chromatographic set-up used consisted of a PU-1580 pump, an LG-1580-04 quaternary gradient unit and an autosampler AS-1555 from Jasco (Tokyo, Japan). A Peltier column-thermostat A-2103 from Thermotechic Products (Langenzersdorf, Austria) was used. A diode array detector UV6000LP with PC1000 system software from Thermo Separation Products (Riviera Beach, FL, USA) was also used.

For sample treatment, an ultrasonic bath from Selecta (Barcelona, Spain), a vortex mixer from Fisher Scientific (Pittsburg, PA, USA) and a vacuum manifold from Waters were also used.

2.2. Column liquid chromatography

2.2.1. Column

The column used was a Luna 250×4.6 mm I.D. packed with 5 μm particles of C_{18} from Phenomenex (Torrance, CA, USA), operated at 30°C.

2.2.2. Mobile phase

To elute the six compounds, a binary gradient was used. Solution A was composed of 1% aqueous acetic acid–acetonitrile (97:3, v/v). Solution B was obtained by mixing acetonitrile with water (1:1,

v/v). The program used was: for the first 20 min, only A was used, then it was changed to A-B (30:70), through a fast linear gradient in 1 min this mixture was held along 19 min and afterwards, it was changed to B, through another linear gradient for 1 min, this composition was held for 5 min, finally a stabilisation step of 15 min with pure solvent A, was included.

Solution A can be also used in isocratic mode to separate HMF and the four related compounds.

The flow-rate was 1 ml/min and the injection volume 20 μl.

2.3. Detection

The spectra of the compounds show maxima at different wavelengths: HMF (284 nm), 2-OIC (253 nm), 3-OIC (239 nm), 2-AL (277 nm), 3-AL (224 nm) and MA (330 nm), all of them should be used to achieve the highest sensitivity, but, in case of not having diode array detection, 250 nm turns out to be very adequate to detect all the compounds. For this reason, this wavelength was preferred.

2.4. Standard calibration

Stock solutions of compounds of 1 g/l were made in water and stored in darkness at 4°C. Dilutions with 1 *M* sulfuric acid and mixtures of different concentrations were made from those solutions.

Calibration graphs were obtained using five mixtures with all the standards at different concentrations. Those solutions were passed through an Oasis HLB solid-phase extraction cartridge, which was pre-activated with 1 ml of methanol and 1 ml of an aqueous acetic acid solution at 0.5%. Then, the compounds were eluted with 0.5 ml of methanol and afterwards, injected into the chromatograph. All samples were prepared and injected in triplicate.

2.5. Samples

The method was applied to 40 samples of different botanical origin, 38 of them were collected from the same geographical area (Soria Province, Spain) in 1999, and the other two belong to commercial orange samples.

2.6. Sample treatment

Prior to HPLC analysis, samples were subjected to a solid-phase extraction on polymeric cartridges, in order to remove matrix compounds that might overlap the compounds of interest and to achieve a pre-concentration of the analytes.

The influence of parameters potentially affecting the extraction process was studied to establish the optimal conditions for maximum recovery.

3. Results and discussion

3.1. Chromatographic conditions

As the compounds have a very different polarity, first the mobile phase composition was optimised to achieve an adequate separation of HMF and the four related compounds, and later, the conditions were adapted so that MA separation could be carried out in the same chromatogram.

The retention was strongly influenced by the pH, which produced changes in the column selectivity, mainly in the case of more acidic compounds. For this reason, it was considered that one of the mobile phase components should be an acid, such as acetic acid. To select its percentage, we tested solutions in which the organic modifier, acetonitrile, was kept constant (5%), and the acetic acid proportion in water varied between 0.2 and 2% (higher percentages of acetic acid produced peak overlapping). The results obtained are summarised in Fig. 1. It can be seen that as the acetic acid proportion increases, a reduction, different for each compound, in the retention is produced. A proportion of 1% in acetic acid was selected.

In the same way, another test was done keeping the acetic acid (1%) constant and varying the acetonitrile proportion. As a consequence of this study, it could be deduced that acetonitrile percentages higher than 5% did not enhance the separation, whereas those proportions, lower than 3%, caused a high retention worsening the resolution, so 3% of acetonitrile was finally chosen. The mobile phase composition, named A, that allowed the separation was: 1% aqueous acid-acetonitrile (97:3).

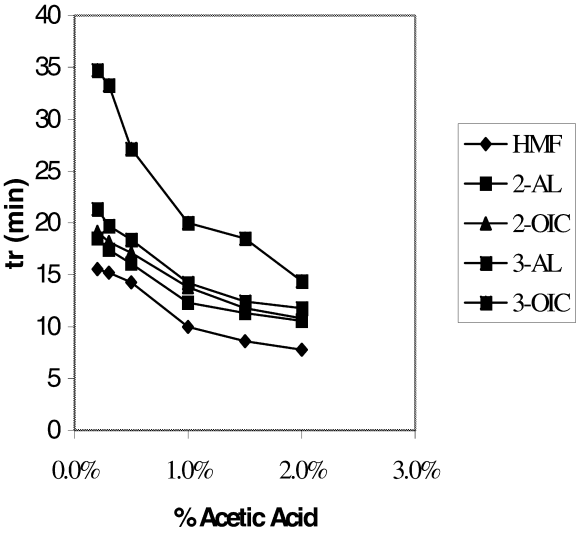

Fig. 1. Variation in the retention time of hydroxymethylfurfural and related compounds according to the percentage of acetic acid added to water in eluent A.

The influence of the temperature on the separation was as expected: an increase in temperature diminished the retention for all compounds (Fig. 2), so 30°C was considered the most adequate.

With this mobile phase, MA was strongly retained and the retention did not depend on the pH and could only be changed by increasing the modifier percentage. Testing with different proportions of acetonitrile, a composition of acetonitrile–water (50:50) (called B) was finally selected. The change between the two mobile phases was made through a mixture A–B (30:70). The separation achieved on a mixture of standards is shown in Fig. 3. The retention times were highly reproducible among chromatograms, and, the RSDs obtained for a mixture of standards in six consecutive runs, were lower than 1% for all the compounds.

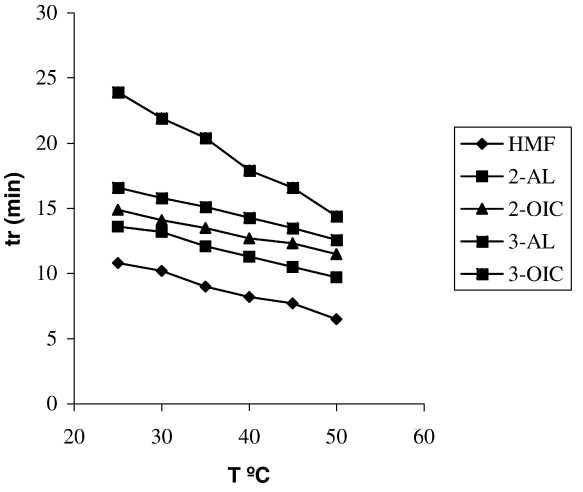

Fig. 2. Influence of the column temperature on the retention time of the compounds.

3.2. Extraction clean-up

Before the sample analysis, a clean-up step was considered necessary, because when a 2.5:10 (w/v) dilution of an orange honey in water sample was directly injected, a very dirty chromatogram was obtained (see Fig. 4a). Trying to remove those interferences and at the same time to pre-concentrate the analytes, several procedures based on liquid–liquid and solid-phase extraction were assayed. Experiments were always performed in duplicate. Two groups of samples of an orange honey, having the same mass (3 g), were used for the whole method development because it always has MA. Known amounts of the compounds (5 mg/l) were added to one group to assess the recovery, and the other one was used to obtain the background chromatogram.

Using the liquid–liquid extraction procedure, the highest recoveries were obtained with ethyl acetate, and all the compounds were solved. The influence of the different parameters affecting the extraction process, such as pH, volume of extractant, the use of mixtures of organic solvents, ionic strength and shaking time, was studied. The best results were obtained using 3 g of honey, diluted 1:10 with 0.6 *M* oxalic acid, 30 ml of ethyl acetate–ethanol (18:2, v/v) as extractant and 5 min of shaking time. The recoveries and RSDs ($n=5$) obtained under these

Table 1
Recoveries and RSDs obtained for the liquid–liquid extraction, applying the optimal conditions

Compound	Recovery (%)	RSD (%)
2-OIC	88.73	1.6
3-OIC	82.75	5.42
HMF	62.09	12.19
2-AL	51.7	24.02
3-AL	35.17	14.22

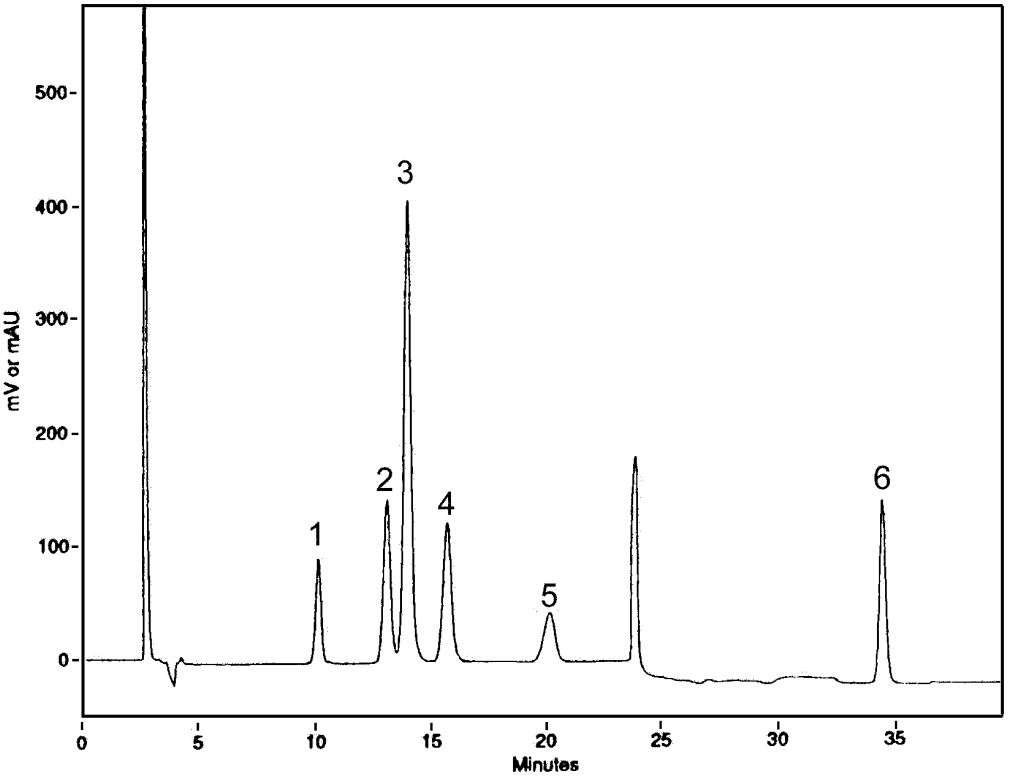

Fig. 3. Chromatogram obtained with a mixture of standards (5 mg/l each). Peaks: 1=HMF, 2=2-AL, 3=2-OIC, 4=3-AL, 5=3-OIC, 6=MA.

conditions are shown in Table 1. As it can be seen, the recoveries and specially the reproducibility are poor, except for the acidic compounds, so this procedure was rejected.

Solid-phase extraction on polymeric cartridges was selected, due to the fact that on this kind of sorbent, very different types of compounds (basic, acidic, neutral, polar, non-polar, etc.) can be retained so, regarding the nature of the compounds studied, it is the best choice. The best conditions to carry out the procedure were selected, taking always into account the idea of obtaining the highest recoveries and the lowest levels of interference.

The cartridge was activated with 1 ml of methanol

Table 2
Average recoveries obtained applying solid-phase extraction to a multifloral honey sample spiked at different levels

Amount added (mg/l)	Recovery (%)					
	HMF	2-AL	2-OIC	3-AL	3-OIC	MA
0.63	82.8	67.6	100.4	78.4	104.9	99.9
1.25	83.3	68.8	97.2	76.4	96.63	97.3
2.5	78.3	67.5	97.5	75.2	97.72	95.4
5	79.4	69.9	100.1	76.4	101.9	94.8
10	78.3	65.8	94.3	73.9	97.3	93.2
Average	80.4	67.9	97.9	76.1	99.7	96.1
RSD (%)	3.0	2.3	2.5	2.2	3.6	2.7

Table 3
Characteristics of the analytical method derived from the standard calibration data set

	HMF	2-AL	2-OIC	3-AL	3-OIC	MA
Precision (RSD, %)	2.47	2.18	2.02	4.35	2.02	2.24
Detection limit (mg/l)	0.04	0.03	0.01	0.04	0.08	0.02
Determination limit (mg/l)	0.13	0.10	0.03	0.14	0.25	0.07
Linearity range (mg/l)	0.13–100	0.10–50	0.03–50	0.14–50	0.25–50	0.07–50
Linearity (%)	99.98	99.91	99.99	99.99	99.98	99.98

and 1 ml of 0.5% (v/v) aqueous acetic acid. The selection of the acidic solution, instead of water, to activate the cartridge, was due to the fact that the acidic compounds were not quantitatively retained using water. As far as the nature of honey solvent is concerned, the best results were obtained using an acidic solution, being 1 *M* sulfuric acid the one which showed the highest recoveries and the cleanest chromatograms. Different honey masses ranging from 1 to 5 g, always dissolved in 10 ml of 1 *M* sulfuric acid, were assayed. Finally 2.5 g was selected as the most suitable. Moreover, it was observed that using 5 ml of the honey solution, the cartridge was not overloaded and 1 ml of water was enough to wash the cartridge and to eliminate interference. The quantitative elution of the compounds was achieved by using 0.5 ml of methanol.

Fig. 4b shows the chromatogram obtained for the same orange honey sample, after being treated with the mentioned procedure. As it can be observed the front has been reduced and now an adequate detection of the compounds is possible. Moreover the 3-OIC peak can be quantified.

The suitability of the method was examined by applying it to the same honey, aliquots spiked at different levels (between 0.6 and 10 mg/l for each compound).The results are summarised in Table 2. As be seen, typical recoveries ranged from 67.9% for 2-furaldehyde to 99.7% for furan-3-carboxylic acid.

3.3. Calibration

At 250 nm, the calibration graphs obtained for all the compounds, from standard solutions subjected to the whole procedure, were linear from the limit of quantitation to at least 50 mg/l. The analytical characteristics ($n=7$) of the proposed method are summarised in Table 3. The detection and determination limits were calculated experimentally at signal-to-noise ratios of 3 and 10, respectively.

Comparing the analyte content in the different calibration methods tested the accuracy of the analytical results: standard solutions (SCs) and standard additions (ACs). The results from SCs and ACs are not significantly different, so the method is accurate.

The average recoveries, from the AC method,

Table 4
Results of recovery assays (from the standard-addition calibration) to test accuracy

	Recovery (%)	Average recovery (%)
HMF (mg/l)		
2	93.42	94.53
4	94.37	
6	95.80	
2-AL (mg/l)		
2	95.50	95.28
4	96.32	
6	94.02	
2-OIC (mg/l)		
2	98.07	98.83
4	99.83	
6	98.60	
3-AL (mg/l)		
2	93.12	93.01
4	93.17	
6	92.74	
3-OIC (mg/l)		
2	98.90	99.36
4	101.67	
6	97.52	
MA (mg/l)		
2	94.92	94.98
4	95.83	
6	94.20	

An amount of 2.5 g honey is diluted to 10 ml in all cases.

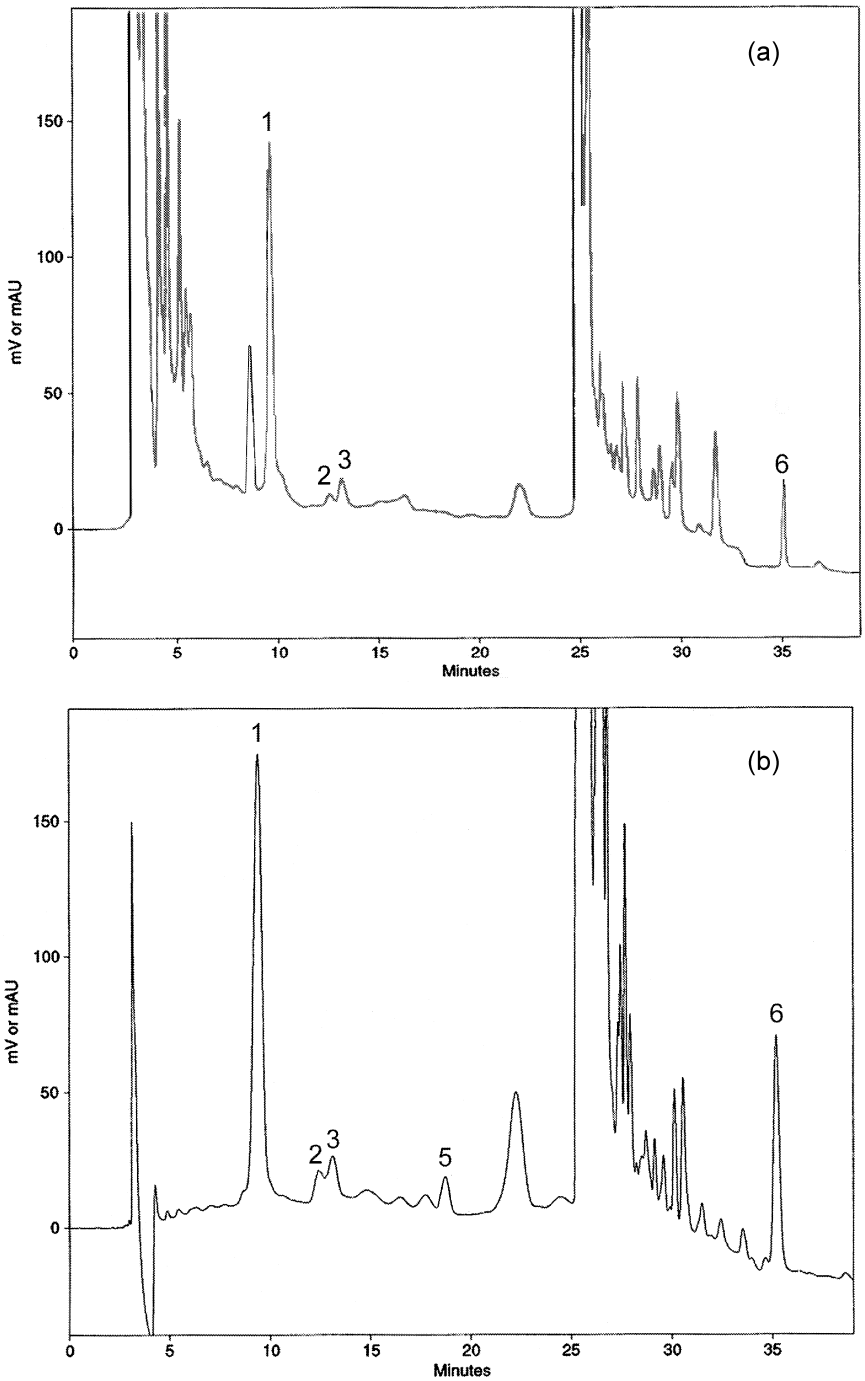

Fig. 4. (a) Chromatogram of a commercial orange honey sample diluted 2.5:10 (w/v) and directly injected; peaks: 1=HMF, 2=2-AL, 3=2-OIC, 6=MA. (b) Chromatogram of the same diluted honey after applying the proposed procedure; peaks: 1=HMF, 2=2-AL, 3=2-OIC, 5=3-OIC, 6=MA.

calculated dividing the content found by the one used for each addition, are shown in Table 4. These results confirm the accuracy of the method. So, the determination of the compounds in honeys and honeydews can be carried out directly by the SC method.

3.4. Analysis of honey samples

The selected procedure was applied to 38 honey and honeydew samples collected in the Province of Soria (Spain), in 1999, and to two commercial samples of orange honey. The results obtained are shown in Table 5, including the mean average and the minimum and maximum values found for each botanical origin. Logically, for those types of honey with very few samples (*Lavandula stoechas*, winter savory, acacia mill, *Onobrycis sativa* lam, evergreen oak) only the mean is indicated, and the results must be used only as a reference. For those samples in which the compounds are under the detection limit, values are not given. It can be observed that HMF content is very different according to the botanical origin, with highest values in orange ones and lowest in the biercol ones. In fact only orange honeys have MA. Regarding the other compounds, there is not a direct relation between them and the HMF content therefore they could be consider related to the botanical origin.

4. Conclusions

The proposed method allows the quantitation of 5-hydroxymethyl-2-furaldehyde, 2-furaldehyde, 2-furoic acid, furan-3-carboxylic acid, furan-3-carboxaldehyde and 2-aminobenzoic acid methyl ester in honey and honeydew samples. Applying it to several samples of different botanical origin it has been

Table 5
Mean average and interval (in parentheses) values, expressed in μg/g, obtained from the samples analysed

	HMF	2-AL	2-OIC	3-AL	3-OIC	MA
Multifloral [9]	3.44 (1.38–5.55)	0.06 (0.04–0.10)	0.55 (0.26–1.60)	2.36 (1.24–4.10)	0.35 (0.03–0.60)	–
Biercol [4] *Calluna vulgaris*	0.78 (0.01–2.24)	–	1.21 (0.09–2.32)	–	0.05 (0.02–0.07)	–
Heather [7] *Erica* sp.	2.06 (1.39–3.12)	–	0.93 (0.07–3.17)	–	0.15 (0.04–0.19)	–
Lavender [8] *Lavandula latifolia*	5.96 (2.16–13.00)	0.03 (0.01–0.05)	0.77 (0.18–2.14)	1.41 (0.16–4.85)	0.02 (0.02–0.03)	–
Thyme [5] *Thymus vulgaris*	1.80 (0.99–3.97)	0.03 (0.02–0.04)	0.32 (0.17–0.45)	2.02 (0.22–5.36)	0.93(0.91–0.95)	–
Sage [1] *Salvia* sp.	1.76	–	1.29	–	0.04	
Cantueso [1] *Lavandula stoechas*	8.58	0.10	0.14	–	–	–
Rosemary [1] *Rosmarinus officinalis*	6.05	–	0.04	–	–	–
Evergreen oak [1] *Quercus ilex*	4.91	–	–	0.65	0.06	–
Oak [1] *Quercus robur*	1.62	–	1.29	–	0.04	–
Orange blossom [2] *Citrus aurantium*	8.13 (7.83–8.43)	0.15 (0.14–0.16)	0.03 (0.03–0.04)	–	0.17 (0.06–0.27)	0.77 (0.43–1.11)

The figures in square brackets given by the botanical origin correspond to the number of samples analysed of each.

observed that there are important differences among the values obtained, which can be useful to know their origin better, logically in conjunction with other physicochemical parameters.

There is not a direct relation between the HMF values and those ones obtained for 2-AL, 3-AL, 2-OIC and 3-OIC, which do not appear in all the samples analysed.

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References

[1] A.J. Tomlinson, J.P. Landers, L.A.S. Lewis, S. Naylor, J. Chromatogr. 652 (1993) 171.
[2] S. Porreta, J. Chromatogr. 624 (1992) 211.
[3] S. Bogdanov, K. Rieder, M. Ruegg, Apidologie 18 (1987) 267.
[4] B. López, M.J. Latorre, M.I. Fernández, M.A. García, S. Garcïa, C. Herrero, Food Chem. 55 (1996) 281.
[5] C. Pérez Arquillue, P. Conchello, A. Arono, T. Juan, A. Herrero, Food Chem. 54 (1995) 167.
[6] M. Esti, G. Pamfili, E. Marconi, M.C. Trivisonno, Food Chem. 58 (1997) 125.
[7] R.S. Singhal, P.R. Kulkarni, D.V. Rege, in: Handbook of Indices of Food Quality and Authenticity, Woodhead, Cambridge, 1997, Chapter 7.
[8] J. Serra, Alimentaria 197 (1988) 37.
[9] J. Serra, F.V. Coll, J. Agric. Food Chem. 43 (1995) 2053.
[10] O. Winkler, Z. Lebensm. Unters. Forsch. 102 (1955) 160.
[11] D. Kanjahn, U. Jarms, H.G. Maier, Deutsche Lebens. Rundschau 92 (1996) 328.
[12] F. Salinas, A.E. Mansilla, J.J.B. Nevado, Fresenius J. Anal. Chem. 340 (1991) 250.
[13] O.O. Anam, R.K. Dart, Anal. Proc. 32 (1995) 515.
[14] J.W. White, J. Assoc. Off. Anal. Chem. 62 (1979) 515.
[15] E. Anklan, Food Chem. 63 (1998) 549.
[16] H.J. Kim, M. Richarson, J. Chromatogr. 593 (1992) 153.
[17] S. Bogdanov, P. Martin, C. Lüllmann, Apidologie Extra issue (1997) 23.
[18] P. Viñas, N. Campillo, M. Herrnández, M.E. Candela, Food Chem. 44 (1992) 67.
[19] K. Horvath, I. Molnar-Perl, Chromatographia 48 (1998) 120.