SIMULTANEOUS DETERMINATION OF BETAINES AND OTHER QUATERNARY AMMONIUM RELATED COMPOUNDS IN BEE POLLEN BY HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

The objective of this work was to develop a new method to determine nine betaines and other quaternary ammonium related compounds in bee pollen from different botanical origins; hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC-MS) was the technique employed. A quick and efficient sample treatment (average analyte recoveries were between 82% and 95% and no significant matrix effect on ionization was found), involving an extraction with an acetonitrile and water mixture (1:1, v/v), centrifugation, freezing with dry ice followed by dilution, was presented. Chromatographic analysis (15 min) was by means of a core-shell HILIC column and a mobile phase applied in gradient elution mode. The analytical characteristics of the method were evaluated and the data demonstrated that not only was it selective, but it also displayed a wide linearity range and good precision. Several bee pollen samples from different botanical origins were analyzed with the proposed methodology, and three betaines (betaine, choline and trigonelline) were detected in all of them in a wide range of concentrations (57-62236 mg/kg).

Keywords: Bee pollen; Betaines; Core-shell columns; Hydrophilic interaction liquid chromatography; Mass spectrometry; Sample treatment.

1. Introduction

Bee pollen is a natural product with numerous bioactive compounds (lipids, proteins, vitamins, amino-acids, minerals or phenolic compounds; [1,2]) that are beneficial to human health due to their nutritional and therapeutic properties (antioxidant, antimicrobial, antiviral, anti-inflammatory or antimutagenic; [2-7]). Its composition or more specifically that of its bioactive compounds, depends on several factors: not only the type of plant species from which it originates, but also the type of soil, climatic conditions, agricultural or apicultural practices, and even the way the product is treated during storage or processing prior to its commercialization [2,6,8,9].

In the last few years, literature data for determining bee pollen constituents has been mainly focused on the content of proteins, amino-acids, lipids and phenolic compounds [2], but no study has appeared so far on quantifying betaines and other quaternary ammonium related compounds in this matrix. These are quaternary ammonium compounds (see structures in Figure 1), which are widely distributed in the plant and animal kingdoms, produced by specific biosynthetic pathways involving the exhaustive nitrogen methylation of amino and imino-acids [10], or the enzymatic oxidation of choline [11]. They exert protective functions on plants in response to plant abiotic stresses [10,12]. Plants express characteristic patterns and levels of betaines according to their species, which implies that these compounds could be used as botanical biomarkers in order to specify the origin (botanical and geographical) of bee pollen. Indeed, determining origin is a particularly relevant issue for the beekeeping industry, especially if we consider that consumers' preference is influenced by this parameter [13], and that this could be employed to detect potential bee pollen fraud [14]. In addition, there is a growing interest in betaines due to their potential benefit to human health, as these compounds may also aid with digestion, heart health, liver function and detoxification [10-12,15,16]. However, it should also be mentioned that there is a potential hazard associated with high-frequency consumption of these compounds as some studies have shown that high dietary

levels of certain betaines, proline betaine and trigonelline, can be related with an increased risk of cardiovascular disease [12].

It must be remarked that although betaines have never been determined in bee pollen, the presence of glycine betaine in tomato pollen was assessed by ^{1}H-nuclear magnetic resonance in one not so recent study [17]. However, several studies have been carried out in which betaines were determined in food matrices, especially vegetables and fruits. Due to the physicochemical characteristics of these compounds, they have usually been determined by liquid chromatography coupled to several detectors (evaporative light scattering, UV, mass or tandem mass spectrometric (MS, MS/MS); see Supplementary Material, Table 1S; [10-12,15,16,18-27]), although in certain cases capillary electrophoresis [28], gas chromatography [29] and supercritical fluid chromatography [30] were also employed. Hydrophilic interaction liquid chromatography (HILIC) with different stationary phases has been the most chosen LC option when determining betaines in foods (see Supplementary Material, Table 1S), perhaps due to the high polarity of these compounds, although silica, C_{18}, C_8 and ion-exchange phases were also selected in some studies (see Supplementary Material, Table 1S). Solvent extraction has been predominant in the above-mentioned LC-based studies as sample treatments, involving different volumes of water, methanol, acetonitrile and dichloromethane (see Supplementary Material, Table 1S). Finally, as a result of the previously discussed bibliography, we decided to initially test a methodology using solvent extraction as the sample treatment followed by HILIC-MS evaluation.

Therefore, the purpose of the present study was to develop a specific analytical methodology to simultaneously determine nine betaines and related compounds in bee pollen using HILIC-MS; this substance was chosen since previously they had been mainly studied in other food matrices. Specific and efficient extraction and determination procedures were optimized, so as to ensure good recovery, minimizing the potential matrix effect, and fulfilling as far as possible the principles of

green analytical chemistry; this reduced not only costs but also the number of reagents used and the time employed [31]. Further aims of this work focused on validating the proposed method, as well as determining the betaine content in bee pollen samples from different botanical origins.

2. Materials and methods

2.1. Reagents and materials

Standards (betaine, Purity ≥ 99%; L- carnitine hydrochloride, Purity ≥ 98.0%; choline chloride, Purity ≥ 99.0%; trigonelline hydrochloride, Purity ≥ 98.5%; N,N-dimethylmyristylammonio acetate (myristyl betaine), Purity ≥ 97.0%; stachydrine hydrochloride (Proline betaine), Purity 97.0%; lauryldimethylammonio acetate (lauryl betaine), Purity ≥ 95.0%; N_6,N_6,N_6-trimethyllysine hydrochloride, Purity ≥ 97.0%; betonicine, Purity ≥ 98%; betaine-(trimethyl-d_9) hydrochloride (betaine-d_9; internal standard), isotopic purity 98.0%, ammonium acetate, formic and acetic acids were supplied by Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). An isotope labelled standard (betaine-d_9) was chosen as internal standard, since it has the same physical and chemical properties as the unlabelled analyte. LC grade methanol and acetonitrile were both obtained from Lab-Scan Ltd. (Dublin, Ireland), whilst ammonium was purchased from Scharlau Chemie S.A. (Barcelona, España). Syringe filters (17mm, Nylon 0.45 μm) were provided by Nalgene (Rochester, NY, USA), and ultrapure water was obtained from Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). An Eppendorf Centrifuge 5810R (Hamburg, Germany), a R, a Moulinette chopper device (Moulinex. Paris, France), IKA® Ultra-Turrax® T18 basic disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany), and a Vibromatic mechanical shaker, an ultrasound bath with heating, and a drying oven both from J.P. Selecta S.A. (Barcelona, Spain) were used for the sample treatment.

2.2. Standards

Individual standard stock (≈ 100 mg/L) solutions were prepared with ultrapure water and then further diluted with an acetonitrile and ultrapure water (1:1, v/v) mixture to prepare the working solutions. Reference standard in solvent (matrix-free) calibration curves (LOQ-see Table 1, 50, 100, 200, 400 and 1000 μg/L) were used to measure the bee pollen compounds as there was no significant matrix effect (see Subsection 3.3.3). However, matrix-matched calibration curves (LOQ, 25, 50, 100, 200 and 500 mg/kg) were also used for studying a potential matrix effect.

Blank bee pollen samples (1 g) were spiked with the analytes before (BF samples) or after (AF samples) sample treatment, along with 50 μg/L of the internal standard (betaine-d_9), to prepare the matrix-matched standards for evaluating the analytical characteristics of the method (see Subsection 3.3). These samples (AF and BF) were prepared with the same procedure described in our recent study [7]. It should be pointed out that all the bee pollen samples analyzed contained some endogenous betaines and related compounds (betaine, choline and trigonelline), although a much lower content was observed in the commercial bee pollen sample from multifloral origin; for this reason, the latter was used as blank for preparing the matrix-matched samples. Thus, in order to calculate the signal for the spiked bee pollen samples, the areas corresponding to endogenous levels had to be determined. These areas were subtracted from the total area obtained for the spiked samples, and the results were directly compared with the matrix-free solutions (matrix-effect evaluation); alternatively, the experimental concentrations were calculated and further compared with the theoretical ones (recovery-trueness studies). Each spiked sample for evaluating the analytical characteristics of the method and sample treatment studies was prepared with 100 mg of bee pollen samples spiked with three different concentrations of the compounds within the linear range: low- LOQ (see Table 1); medium- 50 mg/kg; and high- 500 mg/kg. The stock solutions were stored in glass containers in darkness at -20ºC; working and matrix-matched solutions were stored in glass containers and kept in the dark at 4ºC. All solutions remained stable for over two weeks.

2.3. Sample procurement and treatment

2.3.1. Samples

A total of twelve bee pollen samples were analyzed in the present study. One was obtained from a local market (Valladolid, Spain) and, according to the labelling was of multifloral origin (MF1), while eleven corbicular bee pollen samples were from experimental apiaries of the *Centro de Investigación Apícola y Agroambiental* (CIAPA; Marchamalo, Guadalajara, Spain) with homogeneous *Apis mellifera iberiensis* colonies. Their botanical origin was confirmed by palynological analysis, and corresponded to: rapeseed, Brassica t. (n = 3; BT1, BT2, BT3); maize, Zea mays (n = 2; MZ1, MZ2); sunflower, *Helianthus annuus* L. (n = 2; S1, S2); wild *Brassica* plants, Brassicaceae (n = 2; B1, B2); rock rose, Cistus t. (n = 1; C1); and radish, Raphanus t. (n = 1; R1).

2.3.2. Sample treatment

Bee pollen samples were individually mixed, ground and pooled for optimum sample homogeneity. Next, bee pollen was dried until the mass stabilized (humidity was between 9% and 12%), and subsequently it was stored in the dark at −20ºC until analysis. Then, 100 mg of bee pollen sample (dry weight, DW), the internal standard and 5 mL of an acetonitrile and water (1:1, v/v) mixture were transferred to a centrifuge tube. The mixture was shaken for 5 min in an Ultra-Turrax®, and then the centrifuge tube was placed in a polystyrene box filled with dry ice for 2 min. It was then centrifuged (10000 rpm, 5ºC) for 5 min, and 1 mL of the supernatant was collected, diluted (1:10, v/v) with an acetonitrile and water (1:1, v/v) mixture, and passed through a 0.45 µm nylon filter. Finally, a 3 µL aliquot was injected into the LC-MS system.

2.4. HILIC-MS method

An Agilent Technologies 1100 LC system (Palo Alto, CA, USA) composed of a vacuum degasser, a quaternary solvent pump, an autosampler with a column oven and a MS detector (single quadrupole) equipped with an electrospray ionization (ESI) source, was employed in all experiments. A Kinetex®

HILIC core-shell type column (50 × 2.1 mm, 2.6 µm, 100 Å; Phenomenex, Torrance, CA, USA) and a Kinetex® HILIC guard column (Phenomenex) were used in this study. The mobile phase was composed of acetonitrile (solvent A) and 0.1% (v/v) acetic acid in water (solvent B) applied in the following gradient mode: (i) 0 min (A–B, 88:12, v/v); (ii) 3 min (A–B, 88:12, v/v); (iii) 5 min (A–B, 55:45, v/v); (iv) 9 min (A–B, 55:45, v/v); (v) 11 min (A–B, 88:12, v/v); (vi) 15 min (A–B, 88:12, v/v). The final settings of the most relevant LC parameters are summarized in Table 2.

ESI in positive mode was selected to perform the experiments, both on the strength of preliminary tests and reports in the existing literature (see Supplementary Material, Table 1S). Flow injection analyses were conducted to choose the optimal ESI-MS parameters by infusion mode (5 µL/min) of matrix-free and matrix-matched standard solutions (see final settings in Table 2). Full-scan were obtained by scanning from *m/z* 50 to 350, and most of the compounds showed intense $[M+H]^+$ ions in their full-scan spectra (see Supplementary Material, Table 2S), which were employed for quantification (selected ion monitoring mode, SIM); meanwhile, two other ions with the highest signals were used for confirmation (see Supplementary Material, Table 2S).

3. Results and discussion

3.1. Optimization of the sample treatment

Water, methanol, acetonitrile and dichloromethane have generally been selected to extract these compounds in other food matrices (see Supplementary Material, Table 1S). We decided, however, to test only two (acetonitrile and water) and in combination (1:1, v/v), which had previously been chosen to prepare the working solutions since the use of chlorinated solvents should be avoided due to their toxicity, and because preliminary experiments showed that methanol did not provide good recovery rates for several of the analytes. To perform the experiments, 200 mg of blank bee pollen (BF samples; see Subsection 2.2) was weighed in a 50 mL centrifuge tube and 5 mL of solvent was added. The mixture was mechanically shaken for 5 min at 960 oscillations per min in either a Vibromatic

mechanical shaker or an Ultra-Turrax®, and then centrifuged for 5 min at 5ºC and 10000 rpm. Next, 1 mL of the supernatant was collected, diluted (1:1, v/v) with an acetonitrile and water (1:1, v/v) mixture, filtered and injected in the LC-MS system. These initial conditions were established after some preliminary tests based on a previous study [32] had been performed.

Once the results of these tests had been analyzed, we decided to select acetonitrile and water (1:1, v/v) as the solvent for extracting the analytes with an Ultra-Turrax® for two main reasons: i) higher recovery percentages (> 75%; see Supplementary Material, Table 3S); ii) cleaner chromatograms and better peak shapes. Following selection of the extraction solvent and Ultra-Turrax®, other steps of the sample treatment were also optimized. Firstly, various amounts of bee pollen (50-200 mg) were tested; 0.10 g was considered the optimal value as a result of sensitivity (offering the best signal to noise values) and recoveries. Next, different amounts of the extraction mixture (3-10 mL) and extraction and centrifugation times (3-10 min) were sequentially evaluated. Results indicated that 5 mL of the acetonitrile and water (1:1, v/v) mixture, 5 minutes of extraction, and 5 min of centrifugation, were good enough to obtain recovery percentages of over 80% for all the compounds (see Supplementary Material, Figure 1S). However, the influence of the matrix on the ionization was important for all the compounds studied (> 25%), and three of the analytes (betaine, choline and trigonelline) were present in the blank bee pollen sample at high concentrations, especially choline, which caused the saturation of the detector. Therefore, two modifications were introduced in the sample treatment to solve both problems. Firstly, a freezing step (cooling with dry ice for 2 min) was included before centrifuging, as this had been shown to be effective for minimizing matrix effect in bee pollen [33,34]. This is a simple method for lipid and protein removal from the extract requiring no reagent; meanwhile, the supernatant was diluted 1:10 (v/v) with an acetonitrile and water (1:1, v/v) mixture. A study of the results demonstrated that using these modifications significantly reduces matrix effect (< 15%) without affecting the recoveries (> 80%), whilst saturation of the detector is avoided.

The overall performance has demonstrated that the proposed treatment can be considered simple, efficient and relatively rapid. Good recovery values were obtained at all times (82%-95%; see Subsection 3.3.6 and Table 3); the matrix had no significant effect regarding the signals of the compounds (responses between 85% and 101%; see Subsection 3.3.3 and Table 3); and the overall procedure time was approximately 15 minutes. This sample treatment could be also considered as environment-friendly in terms of organic solvent consumption (7.5 mL), the absence of an evaporation phase, overall time and the number of steps and reagents required. Despite the good recovery values obtained with solvent extraction-based procedures for other food matrices (see Supplementary Material, Table 1S), these were longer and extra steps/instrumentation (solid-phase extraction or evaporation) or larger amounts of reagents were required in some cases. Meanwhile, the matrix effect was not studied/mentioned in most cases, although it was significant in two of the studies in which MS was used [15,20].

3.2. Optimization of the chromatographic conditions

Preliminary experiments were conducted by means of two HILIC columns (Luna® HILIC, 50 × 2.0 mm, 3 µm, 110 Å; Phenomenex; Kinetex® HILIC, 50 × 2.1 mm, 2.6 µm, 100 Å; Phenomenex), as HILIC was commonly employed for determining betaines in foods. These specific columns were selected due to their different characteristics. The Kinetex® HILIC column has a stationary phase formed by particles coated with porous material with a solid non-porous silica core, and it has been employed in previous publications in which betaines were determined in foods [15,24]. In the Luna® HILIC column, meanwhile, the silica surface is covered with cross-linked diol groups for polar selectivity in hydrophilic conditions of liquid chromatography [35].

Tests with matrix-free and matrix-matched samples standards (50 µg/kg) were carried out initially in an isocratic elution mode with modification of the mobile phase, which was a mixture of acetonitrile and ammonium formate (10 mM; see Supplementary Material, Table 1S). Results

showed that the analytes were poorly retained and co-eluted when the Luna® HILIC was used; consequently, the Kinetex® HILIC column was selected to continue with the optimization study. Next, the composition of the mobile phase was examined. Firstly, some preliminary tests in isocratic and gradient elution mode were carried out by injecting the above-mentioned standards. Three different aqueous components were initially selected (10 mM ammonium formate; 0.1% (v/v) formic acid; 0.1% (v/v) acetic acid). Only with acetic acid was it possible to separate most of the analytes whilst obtaining good peak symmetries and the shortest chromatographic run. The influence of the nature of the organic solvent (methanol and acetonitrile) was also investigated; acetonitrile was chosen, as longer analysis times were required when methanol was used, added to which the pressure in the system was too high. We also checked whether the addition of acetic acid to acetonitrile improved the ionization and peak shape of the analytes; no significant advantage was observed. Finally, 0.1% acetic acid in water and acetonitrile were selected as the mobile phase components. Several experiments were then conducted to test diverse mobile phase gradients, variable flow rates, temperatures and injection volumes (see Table 2). Under optimal chromatographic conditions (see Subsection 2.4 and Table 2), all compounds eluted in less than 11 minutes (see Figure 2) with an overall run time of 15 minutes. This, according to the existing literature, is not only the fastest LC-based proposal to date for analyzing two of more betaines in foods, but it also represents the largest number of betaines that have been simultaneously examined in any of these matrices with HILIC columns (see Supplementary Material, Table 1S).

3.3. Analytical characteristics of the method

3.3.1. Selectivity

Non-spiked samples together with spiked samples were injected onto the chromatographic system. No matrix interference was detected at analyte elution time, although three of the compounds studied (betaine, choline and trigonelline) were detected in all the samples (see Figures 2 and 3). In

addition, we observed a high degree of similarity between MS spectra in matrix-free and matrix-matched standards (see example in Figure 4).

3.3.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) were determined experimentally as, respectively, three and ten times the standard deviation of the intercept for the calibration curve (matrix-matched) divided by the slope. As may be observed in Table 1, the LOD and LOQ values were lower or equal than 6 mg/kg and 20 mg/kg, respectively. For the purposes of nutritional measurements and taking into account the concentrations found in all the samples, these values were considered satisfactory. They are similar in most cases to those presented in previous publications in which betaines were detected at higher concentrations in different food matrices, although in some cases lower LOQ values were obtained by using powerful MS/MS detectors (see Supplementary Material, Table 1S).

3.3.3. Matrix effect

This parameter was evaluated by comparing the responses (analyte peak areas) with standard in solvent (matrix-free) solutions and AF samples at three different concentration levels (low, medium and high). The analytes responses ranged from 85% to 101% (see Table 3), which implies that matrix did not affect the signals of the studied compounds. Moreover, the slopes from matrix-free and matrix-matched calibration curves were contrasted (see Table 1), and it was found that in all cases overlapping occurred at the confidence intervals of the slopes. Thus, it can be stated that matrix effect did not affect to the analytes´ ionization, which is a significant advantage of the proposed sample treatment in relation to some of previous proposals (see Supplementary Material, Table 1S).

3.3.4. Linearity

Calibration curves were constructed by plotting the signal on the *y*-axis (analyte peak area) against the analyte concentration on the *x*-axis. The graphs obtained in all the calibration curves were straight lines, with coefficient of the determination values (R^2) higher than 0.99 in all cases (see Table 1).

Linearity was verified by examining the deviation of back-calculation concentration from true concentration (< 20%; data not shown).

3.3.5. Precision

Precision experiments were performed concurrently by repeated sample analysis using BF samples on the same day (n = 6; intra-day precision), or over three consecutive days (n = 3; inter-day precision using day averages). Results, expressed as relative standard deviation (%RSD), was at all times lower than 6% (see Supplementary Material, Table 4S), which were better in all cases but one than those obtained in previous studies (see Supplementary Material, Table 1S).

3.3.6. Trueness

This was evaluated by the mean recoveries (as a measure of trueness), calculated by comparing the measured concentrations in BF samples and theoretical concentrations. Mean recoveries ranged from 82% to 95% with %RSD values lower or equal than 5% in all cases (see Table 3). Those values are comparable to those summarized in Table 1S (see Supplementary Material).

3.4. Application of the method

Several bee pollen samples (see Subsection 2.3) were analyzed, and three of the compounds under study (betaine, choline and trigonelline) were observed in all of them over a wide range of concentration (57-62236 mg/kg DW; see Table 4 and Figure 3). It should be noted that the concentrations in a few samples were outside the linear range, and consequently, in light of the sample and compounds, dilutions (1:50, and 1:150 v/v) were made with an acetonitrile and water (1:1, v/v) mixture for an accurate measurement to be obtained. As can be deduced from the results summarized in Table 4, the content of the compounds detected was different depending on the botanical origin of the bee pollen, showing the potential of these compounds as bee pollen´s biomarkers. According to these results, we may tentatively conclude that trigonelline was predominant in bee pollen from *Brassica* vegetables (Brassicaceae, Brassica t. and Raphanus t.) and rock rose (Cistus t.); betaine, meanwhile, was detected at the highest concentrations (> 54000

μg/kg), and it was also the most common compound in bee pollen from maize and sunflower. In addition, concentration of the three analytes was relatively similar for bee pollen of maize origin, and the lowest concentrations were found in the samples of multifloral origin (< 450 μg/kg).

Due to the non-existence of previous studies in the area of bee pollen, a comparison with our results is not possible, yet, according to the related bibliography, it can be stated that the concentrations were much higher than in other food matrices; for example, betaine has been detected in beet (*Beta vulgaris*) over a concentration range of 1900 and 5100 mg/kg [12], which until the present study was the highest rate of concentration reported for any betaine in food. Much lower concentrations of betaines have been observed in pasta (< 1000 mg/kg; [11]), cereal flours (< 620 mg/kg; [15]), or citrus fruit (< 550 mg/L; [22]). These, then, are significant results, as the presence of betaines and related compounds in bee pollen samples is an interesting finding hitherto unreported.

4. Conclusions

A rapid HILIC-MS methodology (< 35 min including sample treatment and chromatographic analysis) was proposed for simultaneously determining nine betaines and related compounds in bee pollen. A sample treatment based on a solvent extraction was optimized, and this has proven to be both fast and efficient, with the additional advantage that no significant matrix effect on ionization was found, and subsequently, matrix-free calibration curves were employed to perform the quantification . Separation was achieved in fifteen minutes by means of a core-shell technology based HILIC column; this is the fastest proposal for determining several betaines in any food matrix food with HILIC. Several bee pollen samples from different botanical origins were analyzed with the proposed methodology, and betaine, choline and trigonelline were detected in all of them over a wide range of concentration. Moreover, significant differences in these concentrations were observed in accordance with the origin of the bee pollen, suggesting the potential of these compounds as botanical biomarkers for this particular substance. Finally, these results could be useful as a starting point for new studies with a

greater number and diversity (botanical and geographical origins) of samples, for which a rapid, selective and efficient methodology such as that proposed in the present study would be required.

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Abbreviations

AF, samples spiked after sample treatment; **BF**, samples spiked before sample treatment; **DW,** dry weight; **ESI**, electrospray ionization; **HILIC,** hydrophilic interaction liquid chromatography; ***m/z***, mass-to-charge; **RSD,** relative standard deviation; **SIM,** selected ion monitoring.

Declaration of interest

None

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Figure captions

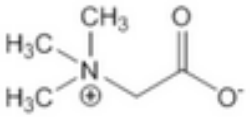
Figure 1.- Chemical structures of the studied compounds.

Figure 2.- Representative chromatograms (SIM in positive mode using the quantification ions; see Subsection 2.4 and Supplementary Material-Table 2S) obtained from a matrix-free standard mixture (50 μg/L of the internal standard (**5**, betaine-d_9) and 0.1 mg/L of: **1**, myristyl betaine; **2**, lauryl betaine; **3**, betonicine; **4**, betaine; **6**, trigonelline; **7**, proline betaine; **8**, L-carnitine; **9**, choline; **10**, trimethyllysine). The HILIC-MS conditions are summarized in subsection 2.4 and Table 2.

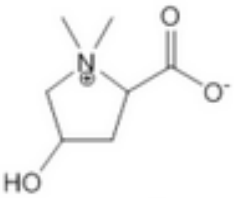
Figure 3.- Representative chromatogram (SIM in positive mode using the quantification ions; see Subsection 2.4 and Supplementary Material-Table 2S) obtained after injecting an extract from a bee pollen sample (#S1; see Table 4) with endogenous betaine (**4**), trigonelline (**6**) and choline (**9**) content, and 50 μg/L of the internal standard (betaine-d_9; **5**). The HILIC-MS conditions are summarized in subsection 2.4 and Table 2.

Figure 4.- Full scan ESI-MS spectra of betaine in (**A**) matrix free and (**B**) a bee pollen sample (#S1; see Table 4). The HILIC-MS conditions are summarized in subsection 2.4 and Table 2.

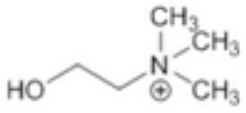
Figure 1



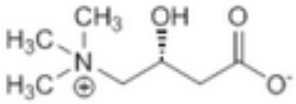
Betaine



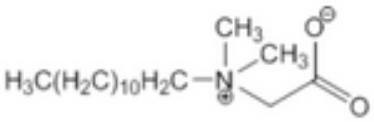
Betonicine



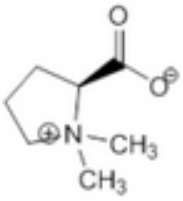
Choline



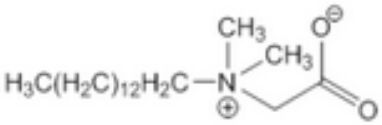
L-Carnitine



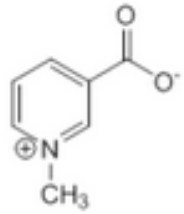
Lauryl betaine



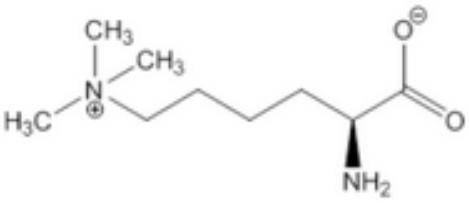
Proline betaine



Myristyl betaine



Trigonelline



Trimethyllysine

Figure 2

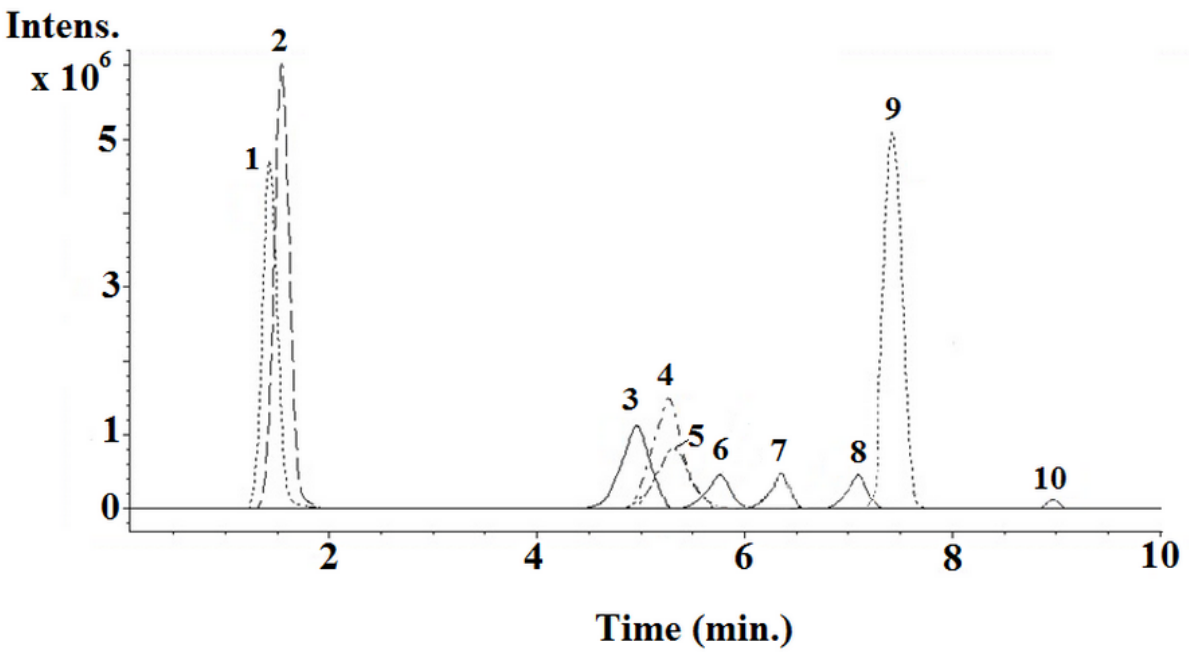


Figure 3

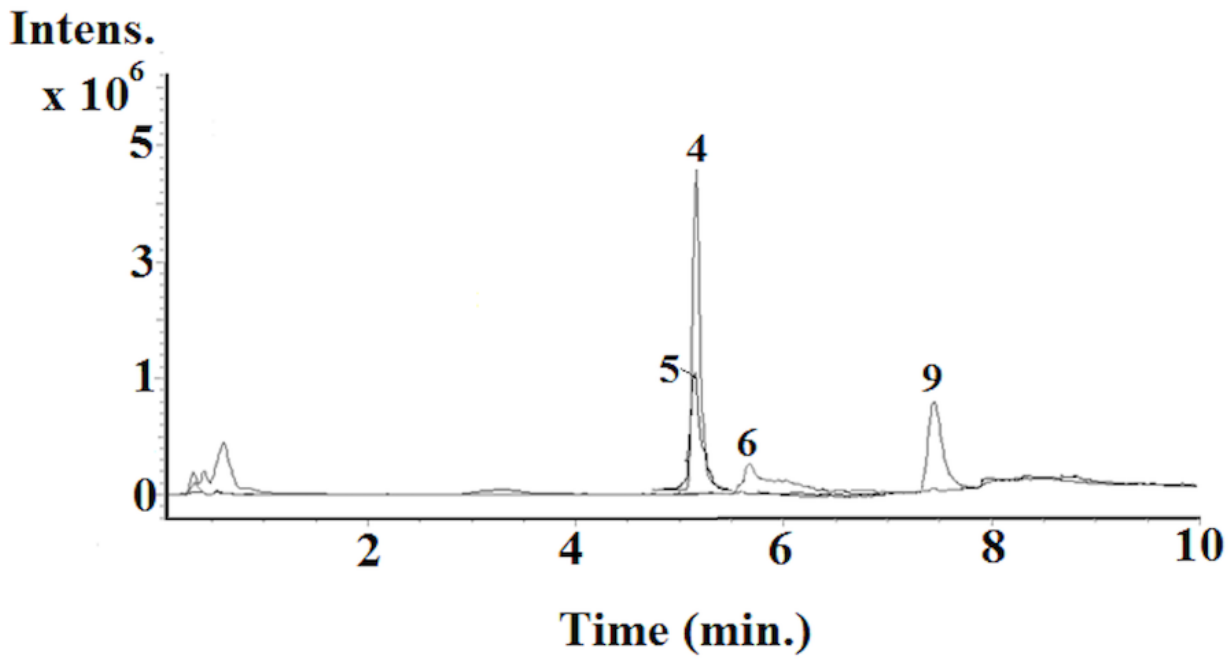


Figure 4

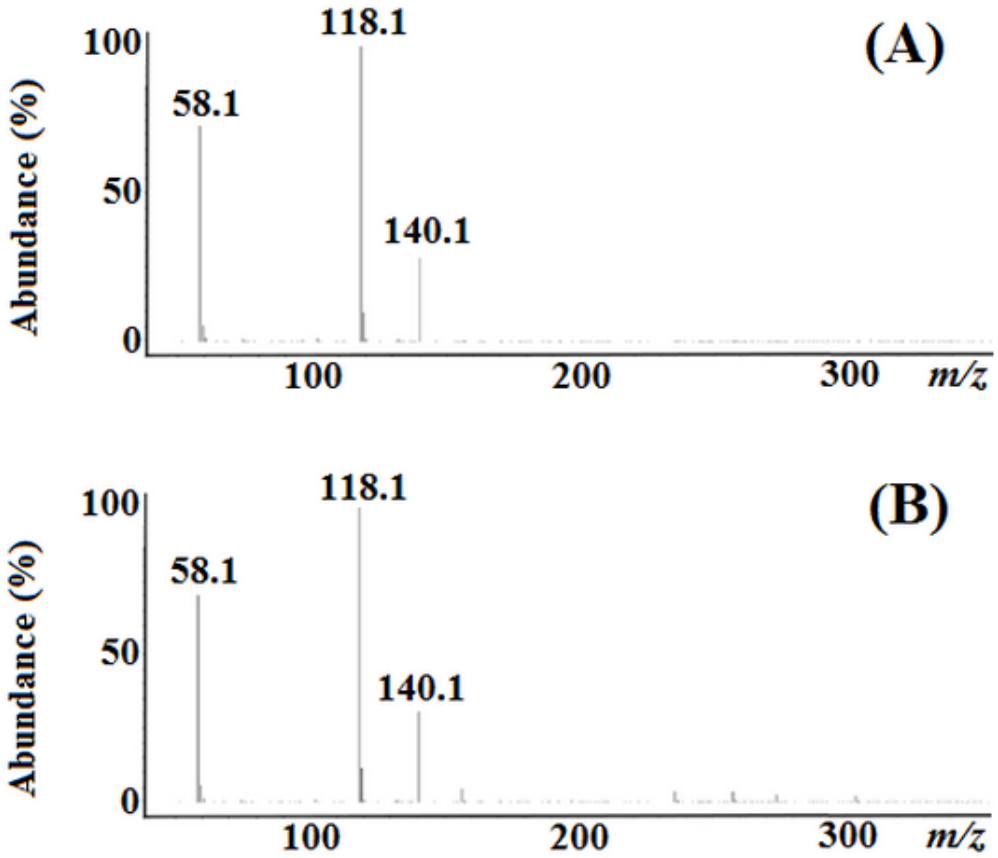

(A)
Abundance (%)
100
50
0
58.1
118.1
140.1
100
200
300
m/z
(B)
Abundance (%)
100
50
0
58.1
118.1
140.1
100
200
300
m/z

Table 1.- Calibration, linearity data, LOD and LOQ values.

Compound	Calibration curve[A]	Linear range[A]	Slope with confidence intervals	R^2	LOD (mg/kg)	LOQ (mg/kg)
Myristyl betaine	Matrix-free	LOQ-1000	21.8 ± 1.22	0.991	0.20	0.70
	Matrix-matched	LOQ-500	19.8 ± 1.09	0.992		
Lauryl betaine	Matrix-free	LOQ-1000	22.3 ± 0.691	0.992	0.20	0.60
	Matrix-matched	LOQ-500	21.9 ± 0.992	0.991		
Betonicine	Matrix-free	LOQ-1000	3.69 ± 0.279	0.998	1.2	4.0
	Matrix-matched	LOQ-500	3.25 ± 0.231	0.996		
Betaine	Matrix-free	LOQ-1000	3.37 ± 0.145	0.998	1.2	4.0
	Matrix-matched	LOQ-500	3.34 ± 0.112	0.998		
Trigonelline	Matrix-free	LOQ-1000	1.98 ± 0.0783	0.997	2.2	7.2
	Matrix-matched	LOQ-500	1.87 ± 0.0625	0.995		
Proline betaine	Matrix-free	LOQ-1000	2.15 ± 0.0934	0.997	2.0	6.7
	Matrix-matched	LOQ-500	2.02 ± 0.112	0.997		
L-Carnitine	Matrix-free	LOQ-1000	1.49 ± 0.0534	0.999	2.8	9.3
	Matrix-matched	LOQ-500	1.44 ± 0.0727	0.996		
Choline	Matrix-free	LOQ-1000	7.10 ± 0.283	0.998	0.60	2.0
	Matrix-matched	LOQ-500	6.79 ± 0.330	0.993		
Trimethyllysine	Matrix-free	LOQ-1000	0.74 ± 0.0321	0.998	6.0	20
	Matrix-matched	LOQ-500	0.68 ± 0.0841	0.994		

[A]Compounds´ concentrations were same in the matrix-free (μg/L) and matrix-matched samples (mg/kg) according to the proposed sample treatment and the unit conversion.

Table 2.- HILIC-ESI-MS parameters.

HILIC parameter	**Studied range**	**Final setting**
Flow-rate (mL/min)	0.3 – 0.7	0.5
Column temperature (ºC)	20 - 45	25
Injection volume (μL)	1 - 7	3
ESI-MS parameter	**Studied range**	**Final setting**
Capillary voltage (V)	2000 - 5000	2500
Drying gas (N_2) flow (L/min)	6 -12	9
Drying gas (N_2) temperature (ºC)	100 - 350	200
Fragmentor voltage (V)	50 - 350	140 and 290[T]
Nebulizer gas (N_2) pressure (psi)	10 - 60	40
Gain	5-20	5

[T]:Fragmentor voltage for trimethyllysine

Table 3.- Evaluation of the efficiency (recoveries) of the sample treatment and the matrix effect (comparison of responses). Data obtained as described in subsections 3.3.3 and 3.3.6.

	Evaluation of the sample treatment			Evaluation of the matrix effect		
	Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
Spiking level	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Myristyl betaine	93 ± 1	90 ± 2	88 ± 1	89 ± 3	88 ± 5	92 ± 4
Lauryl betaine	82 ± 5	83 ± 3	86 ± 2	96 ± 4	100 ± 3	97 ± 4
Betonicine	92 ± 2	90 ± 4	87 ± 3	90 ± 5	87 ± 3	85 ± 4
Betaine	88 ± 3	91 ± 4	89 ± 2	97 ± 4	101 ± 4	99 ± 5
Trigonelline	89 ± 2	93 ± 3	92 ± 3	94 ± 3	92 ± 3	95 ± 4
Proline betaine	83 ± 5	85 ± 3	87 ± 4	92 ± 2	98 ± 3	94 ± 5
L-Carnitine	85 ± 2	89 ± 3	92 ± 2	98 ± 3	95 ± 4	97 ± 5
Choline	93 ± 5	87 ± 4	89 ± 4	93 ± 3	98 ± 5	96 ± 3
Trimethyllysine	91 ± 4	94 ± 3	95 ± 2	94 ± 5	90 ± 3	91 ± 3

Low- LOQ (see Table 1); **Medium QC**-50 mg/kg; **High QC**-500 mg/kg.

Table 4.- Results (means of triplicate analyses (mg/kg; dry weight); %RSD < 6% in all cases)[A] of the investigation of bee pollen samples from different plant origins (multifloral, MF1; rapeseed, BT1, BT2, BT3; maize, MZ1, MZ2; sunflower, S1, S2; wild *Brassica* plants, B1, B2; rock rose, C1; radish, R1).

Sample	Betaine	Choline	Trigonelline
#B1	317	1104[D1]	15815[D1]
#B2	351	1682[D1]	13927[D1]
#BT1	1232[D1]	1422[D1]	18555[D1]
#BT2	1480[D1]	1299[D1]	14483[D1]
#BT3	1367[D1]	1158[D1]	16992[D1]
#C1	135	1854[D1]	5020[D1]
#MF1	57	350	124
#MZ1	32211[D2]	23836[D2]	15827[D2]
#MZ2	28113[D2]	25427[D2]	20792[D2]
#R1	349	1620[D1]	20974[D1]
#S1	54281[D2]	8845[D1]	411
#S2	62236[D2]	12493[D1]	1219[D1]

[A], all other compounds were below LOD in the analyzed samples.
[D1], requires a 1:50 (v/v) dilution with an acetonitrile and water (1:1, v/v) mixture.
[D2], requires a 1:150 (v/v) dilution with an acetonitrile and water (1:1, v/v) mixture.

Supplementary Material

SIMULTANEOUS DETERMINATION OF BETAINES AND OTHER QUATERNARY AMMONIUM RELATED COMPOUNDS IN BEE POLLEN BY HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Table 1S.-Comparison of the proposed LC-based method with previous proposals for determining betaines in food matrices.

Matrix (number of betaines)	Sample treatment (time)	Reagents (g, mL[O])	Matrix Effect[A]	Recoveries[A] (precision, %RSD)	LOQs	System (IM, SP, time)	Reference
Chestnut (3)	SE (> 60 min)	FA in water (NS)	NS	NS	NS	LC-MS/MS (ESI+, C_8, 18 min)	**[10]**
Cereals (1)	SE (> 15 min)	800 µL water	NS	NS	NS	LC-MS/MS (ESI+, Atlantis HILIC, 15.5 min)	**[11]**
Cereal flours (7)	SE (> 180 min)	FA in water (NS)	NS	NS	NS	LC-MS/MS (ESI+, C_8, 30 min)	**[12]**
Beta vulgaris (beet) (1)	SPE[LM] ASE + SPE[SM] (> 30 min)	10 mL of MeOH[LM], > 22 mL of MeOH[SM]	Yes	92%-94% (< 2%)[LM] 94%-97% (< 2%)[LM]	0.003 µg/mL	LC-MS/MS (ESI +, Kinetex HILIC, 9 min)	**[15]**
Fructus Lycii (1)	SE (> 60 min)	> 50 mL of water	No	99%-108% (< 26)	7.11 µg/mL	LC-ELSD (Atlantis HILIC, 30 min)	**[16]**
Cereals (1)	SE (> 20 min)	750 µL water + 750 µL MeOH	NS	82%-106% (< 15%)	< 0.060 mg/kg	LC-MS/MS (ESI+, HILIC (NS), 15 min)	**[18]**
Multiple foods (7)	SE (> 20 min)	Water + DCM (NS)	No	88%-99% (< 13%)	1 mg/kg (LOD)	LC-UV[D] (Alumina or NS, > 45 min)	**[19]**
Manila clam (1)	SE (≈ 20 min)	4 mL of ACN + 3 mL of DCM	Yes	97%-106% (< 7%)	2.5 mg/kg	UHPLC-MS/MS (ESI+, BEH HILIC, 4 min)	**[20]**

[A]: data related only to betaines; [D]: requires derivatization [O]: organic solvent; [LM]: liquid matrix; [SM]: solid matrix; **ACN,** acetonitrile; **ASE**, accelerated solvent extraction; **DCM,** dichloromethane; **ELSD**, evaporative light scattering detection; **ESI,** Electrospray ionization; **EV**, evaporation; **FA,** formic acid; **IM**, ionization mode; **MeOH**, methanol; **MS/MS**, tandem mass spectrometry; **NS**, not specified**; SD,** sample dilution; **SE**, solvent extraction; **SP,** stationary phase; **UHPLC**, ultra-high performance liquid chromatography.

Table 1S.- Continued.

Matrix (number of betaines)	Sample treatment (time)	Reagents (g, mL[O])	Matrix Effect[A]	Recoveries[A] (precision,%RSD)	LOQs (µg/Kg)[A]	System (IM, SP, time)	Ref.
Foods (1)	SE + EV (NS)	DCM, water and MeOH (< 2 mL)	NS	NS	NS	LC-MS/MS (ESI+, Silica, > 30 min)	**[21]**
Fruit juices (1)	SD + SPE (> 60 min)	Water or FA in water (NS)	NS	NS	NS	LC-MS/MS (ESI+, C_8, NS)	**[22]**
Bergamot (1)	SE (> 30 min)	Water (NS)	NS	NS	NS	LC-MS/MS (ESI+, C_8, 14 min)	**[23]**
Fructus Lycii (1)	SE (> 50 min)	7.5 mL of MeOH	No	94%-107% (< 13%)	10 µg/mL	LC-ELSD (Kinetex HILIC, 35 min)	**[24]**
Multiple foods (3)	SE (> 20 min)	Water + DCM (NS)	No	74%-113% (< 15%)	1 mg/kg (LOD)	LC-UV[D] (Alumina or NS, > 45 min)	**[25]**
Algae (2)	SE + EV (NS)	FA, water and MeOH (NS)	NS	83%-96% (< 6%)	0.0002-0.0004 µg/mL	LC-MS/MS (ESI+, SCX, 50 min)	**[26]**
Egg yolk (1)	SE + EV + SD (> 35 min)	Chloroform, water and MeOH (12 mL)	NS	101%-104% (< 11%)	NS	UPLC-MS/MS (ESI+, Ascentis Express HILIC, 20 min)	**[27]**
Bee pollen (7)	SE (≈ 15 min)	7.5 mL of ACN + 7.5 mL of water	No	82%-95% (< 6%)	0.7-5.0 mg/kg	LC-MS (ESI+, Kinetex HILIC, 15 min)	**Present study**

[A]: data related only to betaines; [D]: requires derivatization [O]: organic solvent; [LM]: liquid matrix; [SM]: solid matrix; **ACN,** acetonitrile; **ASE**, accelerated solvent extraction; **DCM,** dichloromethane; **ELSD**, evaporative light scattering detection; **ESI,** Electrospray ionization; **EV**, evaporation; **FA,** formic acid; **IM**, ionization mode; **MeOH**, methanol; **MS/MS**, tandem mass spectrometry; **NS**, not specified**; SD,** sample dilution; **SE**, solvent extraction; **SP,** stationary phase; **UHPLC**, ultra-high performance liquid chromatography.

Table 2S.- Quantification and confirmation ions.

Compound name	Molecular weight	Quantification ions	Confirmation ions
Myristyl betaine	299.5	300.0	230.1 322.0
Lauryl betaine	271.4	272.1	149.0 294.0
Betonicine	159.2	160.3	88.3 182.2
Betaine	117.1	118.1	58.1 140.1
Betaine-d_9	126.1	127.1	67.1 149.1
Trigonelline	137.1	138.1	94.1 160.0
Proline betaine	143.1	144.2	102.2 166.1
L-Carnitine	161.2	162.3	60.2 184.3
Choline	104.2	104.0	60.2 58.1
Trimethyllysine	188.2	189.0	130.1 211.0

Table 3S.- Recoveries (mean ± %RSD; n= 3) obtained after testing different extraction solvents with spiked blank bee pollen samples at medium concentration level (50 mg/kg).

Shaking device	Compound	Acetonitrile	Water	Acetonitrile:water (1:1, v/v)
Vibromatic	**Myristyl betaine**	22 ± 7	43 ± 6	40 ± 4
	Lauryl betaine	24 ± 7	44 ± 5	43 ± 6
	Betonicine	21 ± 5	40 ± 7	42 ± 6
	Betaine	19 ± 5	44 ± 6	39 ± 5
	Trigonelline	26 ± 6	49 ± 6	46 ± 4
	Proline betaine	17 ± 5	39 ± 5	36 ± 6
	L-Carnitine	24 ± 4	43 ± 4	42 ± 5
	Choline	19 ± 7	45 ± 4	40 ± 7
	Trimethyllysine	21 ± 6	40 ± 7	39 ± 6
Ultra-Turrax®	**Myristyl betaine**	36 ± 5	80 ± 4	92 ± 3
	Lauryl betaine	30 ± 7	78 ± 5	86 ± 3
	Betonicine	35 ± 5	81 ± 6	93 ± 4
	Betaine	33 ± 4	83 ± 6	92 ± 5
	Trigonelline	37 ± 6	86 ± 5	95 ± 5
	Proline betaine	29 ± 5	78 ± 5	87 ± 4
	L-Carnitine	30 ± 4	80 ± 4	91 ± 2
	Choline	28 ± 6	79 ± 6	88 ± 3
	Trimethyllysine	34 ± 6	82 ± 5	93 ± 4

Table 4S.- Summary of precision studies (%RSD).

	Intraday precision (%RSD)			Interday precision (%RSD)		
Spiking level	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Myristyl betaine	1	2	1	2	2	1
Lauryl betaine	3	3	2	4	3	3
Betonicine	2	3	3	4	4	3
Betaine	3	2	2	3	5	4
Trigonelline	2	3	2	3	2	3
Proline betaine	5	3	4	5	4	5
L-Carnitine	2	2	2	2	3	3
Choline	5	5	4	5	4	5
Trimethyllysine	2	4	3	4	5	4

Low- LOQ (see Table 1); **Medium-** 50 mg/kg; **High-** 500 mg/kg

Figure 1S.- Evaluation of the extraction efficiency (recoveries) obtained after testing different volumes of the acetonitrile and water (1:1, v/v) mixture and different shaking and centrifugation times with spiked BF samples at medium concentration level (50 mg/kg). Data represent the mean of three