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TECHNICAL NOTE

A confirmatory method for the determination of phenolic endocrine disruptors in honey using restricted-access material–liquid chromatography–tandem mass spectrometry

E. Rodríguez-Gonzalo · D. García-Gómez · R. Carabias-Martínez

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Abstract The present work describes the development and validation of an analytical method based on liquid chromatography (LC), coupled with tandem mass spectrometry (MS/MS) that allows the determination and confirmation of several endocrine-disrupting chemicals (EDCs) in honey. The EDCs studied were nine phenols of different nature: chlorophenols (2,4-dichlorophenol, 2,4,5-trichlorophenol, and pentachlorophenol), alkylphenols (4-*tert*-butylphenol, 4-*tert*-octylphenol, and 4-*n*-octylphenol) bisphenols (bisphenol-A and bisphenol-F), and 4-*tert*-butylbenzoic acid. The method incorporates a restricted-access material (RAM), coupled on-line to the LC-MS/MS system, which allows direct injection of the matrix into the RAM-LC-MS/MS system. The optimized method developed, RAM-LC-MS/MS, was applied to fortified honey samples, affording detection limits in the 0.6–7.2 ng g^{-1} range, calculated for a signal-to-noise ratio of 3. In addition, the method was validated as a quantitative confirmatory method according to European Union Decision 2002/657/EC. The validation criteria evaluated were linearity, repeatability, reproducibility, recovery, decision limits, detection capabilities, specificity, and ruggedness. Repeatability and within-laboratory reproducibility were evaluated at two concentration levels, being ±11% or below at 20 ng g^{-1}. The decision limits (CC_α) and detection capabilities (CC_β) were in the 1.7–12.6 and 2.8–21.6 ng g^{-1} range, respectively.

E. Rodríguez-Gonzalo (✉) · D. García-Gómez · R. Carabias-Martínez
Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Química, Universidad de Salamanca, 37008 Salamanca, Spain
e-mail: erg@usal.es

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Introduction

Honey is a healthy natural product with excellent nutritional properties. Since it is a product consumed worldwide, it is evident that human health should be taken into consideration in regards to its consumption. Owing to its sweetness, color, and different flavors, honey is often used as a sugar substitute, as is an ingredient, or used as a natural preservative in hundreds of manufactured foods. From the chemical point of view, honey is a complex mixture of sugars. Its composition depends strongly on the plant species from which the nectar or honey dew has been collected as well as on other factors such as environmental conditions and climate [1].

To ensure the safety and quality control of honey, it is necessary to perform the analysis of chemical contaminants in honey in order to assure that this natural product does not contain toxic residues in quantities that might imply a risk for consumers. Accordingly, sensitive, selective, fast, and inexpensive analytical methods for food analysis are continuously under development.

The analysis of hazardous compounds at trace level in complex matrices includes a procedure of sample preparation, which can cover analyte isolation, trace enrichment, and further cleanup to remove matrix interferents. The sample treatment techniques frequently employed for the isolation and enrichment of chemical contaminants from honey are liquid–liquid extraction (LLE) [2, 3], solid-phase extraction (SPE) [4–6], solid-phase microextraction (SPME) [7] and matrix solid-phase dispersion (MSPD)

[8]. Other analytical procedures reported involve supercritical fluid extraction (SFE) [9], ultrasound-assisted extraction (UAE) [10] or pressurized liquid extraction (PLE) [11].

Restricted-access materials (RAMs) are porous chromatographic supports specifically designed to allow solid–liquid extraction and the enrichment of small molecules, limiting the access of macromolecules to the interaction sites of a stationary phase bonded to their inner surface. Only small molecules are able to penetrate into the pores and interact with the solid sorbent on the inner surface, while large molecules are eluted with the clean-up solvent. RAMs were initially designed to remove proteins in the analysis of drugs in biological matrices [12–14], although they have also found applications in environmental analysis [15]. However, only a few studies have been reported regarding the applications of RAMs to sample treatment steps in food analysis [16].

The present study was aimed at developing a simple, fast, precise, accurate, and highly automated analytical approach for the identification and quantification of several EDCs in honey. The analytes assayed were: bisphenol A (BPA), bisphenol F (BPF), 4-*tert*-butylbenzoic acid (tBBA), the chlorophenols 2,4-dichlorophenol (DCPL), 2,4,5-trichlorophenol (TCPL) and pentachlorophenol (PCPL), 4-*tert*-butylphenol (tBPL), 4-*tert*-octylphenol (tOPL) and 4-*n*-octylphenol (nOPL). These compounds are used in industry or are produced as intermediates in the production of epoxy resins and polycarbonate plastics. These plastics are used in many food- and drink-packaging applications, whilst the resins are commonly used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. These compounds have been widely analyzed in environmental matrices such as water, sludge, and sediments [17, 18]. Food may be another important route of exposure to endocrine-disrupting compounds. Some EDCs could enter the food chain at several stages of its production and also via plastic packaging material, stretch films used for food packaging, and many foodstuff containers for oven or microwave cooking [19]. The presence of phenolic EDCs in different foods [20–24], among which honey is included [25, 26], has been reported by several authors, highlighting a possible risk for consumers.

The method proposed here is based on the use of a RAM coupled on-line to an LC-MS/MS system for the identification and quantification of nine EDCs in honey. The use of RAM material enables fast on-line clean-up of honey samples, efficiently eliminating matrix components and providing appropriate selectivity and sensitivity for the determination of these compounds at trace levels. The on-line configuration developed involves minimum sample treatment (a simple dilution and filtration step) and affords a shorter analysis time. The method was validated as a quantitative confirmatory method according to the EU Decision 2002/657/EC: linearity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limits, detection capabilities, specificity, and ruggedness were evaluated for the target compounds.

Experimental

Chemicals

Analytical standards of *bisphenol-F* (BPF), bis-(4-hydroxyphenyl)methane, CAS RN [620-92-8]; *bisphenol-A* (BPA), 2,2-bis(4-hydroxyphenyl)propane, CAS RN [80-05-7]; *tert-butylphenol* (tBPL), 4-(1,1-dimethylethyl)phenol, CAS RN [98-54-4]; *dichlorophenol* (DCPL), 2,4-dichlorophenol, CAS RN [120-83-2]; *trichlorophenol* (TCPL), 2,4,5-trichlorophenol, CAS RN [95-95-4] and *pentachlorophenol* (PCPL), 2,3,4,5,6-pentachlorophenol, CAS RN [87-86-5] were purchased from Dr. Ehrenstorfer (Augsburg, Germany). *tert-butylbenzoic acid* (tBBA), 4-(1,1-dimethylethyl)benzoic acid, CAS RN [98-73-7]; *n-octylphenol* (nOPL), 4-octylphenol CAS RN [1806-26-4] and *tert-octylphenol* (tOPL), 4-(1,1,3,3-tetramethylbutyl)-phenol, CAS RN [140-66-9] were obtained from Sigma-Aldrich (Steinheim, Germany).

The organic solvents acetonitrile (ACN) and methanol (MeOH) were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Navarra, Spain) Ultramatic water purification system.

All chemicals used for the preparation of the buffer and all other chemicals were of analytical reagent grade.

Instrumentation

HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany) equipped with a binary pump, an additional isocratic pump, a membrane degasser, an autosampler (equipped with a 1,500-μL capillary seat), a six-port valve, and a diode-array detector (DAD). The system was controlled by a HP ChemStation, which also performed data collection from the detectors and quantitative measurements. The RAMs used were LiChroCART 25-4LiChrospher alkyl-diol-silica (ADS, 25 μm, 25 mm×4 mm) RP18, RP8, and RP4 from Merck (Darmstadt, Germany). The analytical column was a 150×4.60 mm Luna PFP(2) packed with 3-μm particles (Phenomenex, Torrance, CA, USA).

The clean-up mobile phase, impelled by the isocratic pump, consisted of a 2.5 mmol L^{-1} formic acid/ammonium formate buffer (pH: 2.9) with 10% ACN. The separation mobile phase, impelled by the binary pump, consisted of a 1 mmol L^{-1} formic acid/ammonium formate buffer, pH: 3.4, (solvent A) and methanol (solvent B) gradient from

80% to 100% of B. The analytical column was thermostatted at 20 °C.

Mass spectrometry

The LC/MSD Trap XCT ion-trap mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray (ESI) source with a nebulizer spacer. The ESI settings were a capillary voltage of −3,500 V; a drying gas flow of 10 L min^{-1} at a temperature of 350 °C, and a nebulizer pressure of 50 psi. Optimization of the ionization and fragmentation parameters was achieved manually while injecting standard solutions of each analyte (5 μg mL^{-1}) with a syringe pump at a flow rate of 1 mL h^{-1}; these solutions were mixed with the mobile phase at 0.3 mL min^{-1} by means of a T piece. The trap parameters were set at a smart target of 50,000 to 100,000 and a maximum accumulation time of 200 ms at an *m/z* range from 60 to 400 u. A narrow isolation width of 4 u was selected. The optimized parameters and retention times for each analyte are listed in Table 1.

Honey samples

Samples of polyfloral honey commercially available from different commercial brands on the retail market were used in this work. All honey samples were first analyzed by the proposed method in order to check the natural occurrence of these compounds. No signals corresponding to the target analytes were found, and hence fortified honey samples were used.

Calibrant preparation and calibration procedure

Stock solutions of analytical calibrants were prepared by dissolving 12.5 mg of each analyte in 25 mL of acetonitrile (500 μg mL^{-1}). These stock solutions were stored at 4 °C in brown glass bottles. Matrix-matched calibrants, in the 10–200 ng g^{-1} range, were prepared daily by adding the appropriate amount of each stock solution to the honey samples. The fortified honey samples were capped and stored in the dark at room temperature for approximately 12 h to permit the interaction between the analytes and the matrix compounds. Finally, the fortified honey samples were diluted 1:5 (m/V) with UHQ water, shaken vigorously and filtered with a 5.0 μm nylon filter before analysis. Calibration curves were obtained by plotting the peak areas of the analytes versus concentration using matrix-matched standards.

Procedure for on-line sample preparation and separation

The experimental setup for RAM-LC-MS/MS was an on-line column switching configuration [27]. First, a predetermined volume of diluted honey was injected with the autosampler and the isocratic pump was immediately started to pump the clean-up mobile phase at 1 mL min^{-1} for 3 min with the valve in the "sample enrichment" position. The target compounds were withheld in the RAM, while the matrix components of the honey were washed to waste. At 3 min, the system setup was changed to the "sample elution" position and the separation gradient (binary pump) eluted the analytes at a flow rate of 0.65 mL min^{-1} in backflush mode to the analytical column, where they were separated and finally detected by the mass spectrometer. During this time, the isocratic pump changed the flow rate to 0.3 mL min^{-1} in order to save solvent until next injection. At 23 min, the separation ended and the gradient was returned to the initial conditions. The flow rate of the isocratic pump was changed to 1 mL min^{-1}. At 26 min, the valve was switched to the "sample enrichment"

Table 1 Ion-trap tandem mass spectrometer parameters optimized for the studied EDCs

Analyte[a]	t_R (min)	Identification transition	Confirmation transition	Fragmentation amplitude (V)	IPs[b]	Window
BPF	14.2	199→93	199→123	1.10	4	12–17.3 min
BPA	14.6	227→212	227→133	0.95	4	12–17.3 min
tBPL	15.7	149→133	–[c]	0.67	2.5	12–17.3 min
DCPL	15.9	161→125	163→125	0.68	3.5	12–17.3 min
tBBA	16.4	177	–[c]	–[c]	1	12–17.3 min
TCPL	17.6	195→159	197→161	0.95	5	17.3–25 min
tOPL	18.6	205→133	205→190	0.87	4	17.3–25 min
nOPL	20.1	205→106	–[c]	0.86	2.5	17.3–25 min
PCPL	21.4	265	267, 263[d]	–[c]	3	17.3–25 min

[a] *BPF* bisphenol-F, *BPA* bisphenol-A, *tBPL* 4-*tert*-butylphenol, *DCPL* 2,4-dichlorophenol, *tBBA* 4-*tert*-butylbenzoic acid, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *nOPL* 4-*n*-octylphenol, *PCPL* pentachlorophenol

[b] Identification Points (IPs) according to 2002/657/EC

[c] No satisfactory transition was found

[d] Chloride isotopes ions

mode and a 4-min post-run program was started, keeping the system under the initial conditions in order to equilibrate the analytical column for the next analysis.

Method validation

The method was validated, according to the pertinent legislation [28], by evaluating the following parameters:

- Specificity: by calculating ion suppressions and comparing patterns with matrix-matched samples.
- Determination of the statistical parameters and the quality of the linear regression using the confirmation transition. The matrix-matched calibrations were obtained in the 10–150 ng g^{-1} range. The decision limit (CC_α) and the detection capability (CC_β) were also calculated.
- Repeatability and within-laboratory reproducibility were determined as intraday and interday precisions by analyzing honey samples at two concentrations levels (20 and 100 ng g^{-1}).
- Recoveries were determined at two concentration levels (12.5 and 25 ng g^{-1}).
- Stability: by controlling the storage conditions of the analytes and stock solutions (see "Calibrant preparation and calibrant procedure" section), keeping them in the range in which stability is guaranteed by the manufacturer.

Results and discussion

The chromatographic separation of the analytes was optimized by searching for maximum compatibility with the detection performed with mass spectrometry. Thus, water/methanol mixtures with a high content in organic component were used as the mobile phase, facilitating the electrospray ionization process. Likewise, the presence of salts was reduced as much as possible, this aiming and increasing the ionizing efficiency of the analytes in the source [29]. Following these criteria, optimum chromatographic separation was observed in 19 min for the nine EDCs studied, applying a gradient of 80% to 100% of methanol, and using a 1.0 mmol L^{-1} formic acid/ammonium formate buffer, pH 3.4, as the aqueous component of the mobile phase, with a mobile phase flow rate of 0.65 mL min^{-1}.

Restricted-access materials: optimization of sample treatment using RAM-LC-DAD

The analysis of complex matrices using chromatographic methods usually requires one or several clean-up steps in order to remove the greatest amount possible of the compounds present in the matrix that could hinder analysis and shorten the useful lifetime of the chromatographic column.

RAMs have frequently been used in the clean-up steps of biological samples, but their use in the clean-up of food samples has not received much attention. In previous studies carried out by us, we observed that these materials may be highly suitable for the clean-up of samples with high contents of sugars. Accordingly, here we studied the possibilities offered by a restricted-access material of the ADS type as a sample treatment step for the analysis of phenolic EDCs in honey samples. For this study, an ADS-RAM coupled on-line to a LC-DAD system was used. Figure 1 shows the chromatograms for an unfortified honey sample injected directly into the LC-DAD system (Fig. 1a), together with those obtained upon injecting the same sample volume through the RAM-LC-DAD system: using ADS-RAMs of the C18 and C4 types (Fig. 1b–c). Regarding the direct injection, it is possible to observe a large number of very intense chromatographic peaks, corresponding to the matrix. The number and intensity of these peaks decreased considerably when the samples were injected through the RAM, which acted as an extraction and on-line clean-up step. The results obtained reveal the efficiency of the RAM in cleaning the components of the matrix in the honey, which is an important aspect in order to preserve the instrumental system and lengthen the life of the chromatographic column. The chromatograms obtained upon using the ADS-RAM of the C18 (Fig. 1b) and C4 (Fig. 1c) types for the same unfortified honey sample indicate that the level of clean-up is adequate in both cases, although the most favorable one was obtained with the ADS-RAM with the C4 stationary phase. It was thus decided to use the C4 RAM for later studies.

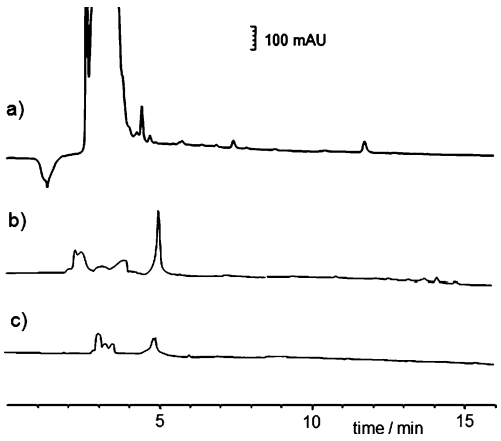

Fig. 1 LC-DAD chromatograms obtained for an unfortified honey sample injected **a** directly into the chromatographic column, **b** through the on-line RAM-LC-DAD using a C18 RAM, and **c** the same as in **b** using a C4 RAM. Injected volume, 100 μL, signal 214 nm

The next step consisted of optimizing the RAM washing solvent, i.e., the mobile phase impelled by the isocratic pump that acted as a washing solvent and at the same time transported the sample through the RAM. Study of the composition of this mobile phase (washing solvent) aimed at maximizing sample clean-up without negatively affecting the analyte recovery. There are two main factors that jointly affect these parameters: the percentage of organic phase in the washing solvent and the time that the washing step lasts. Increases in the organic component afforded a better cleaning of the interferents, although an excessive increase may lead to the elution of the analytes of interest, low recoveries being obtained. We therefore studied percentages of acetonitrile in the washing solvent between 0% and 25%, keeping a clean-up time of 3 min throughout the study. In each case, the recoveries of each analyte were determined by comparing the concentration obtained, by means of a calibration, with the real concentration at which the sample was originally fortified. The recoveries remained above 90% when a washing solvent with up to 10% acetonitrile was used. An increase in this percentage up to 25% led to a dramatic decrease in the recoveries for all the analytes studied. In light of the results, it was decided to use as a washing solvent, corresponding to the mobile phase impelled by the isocratic pump, 2.5 mmol L^{-1} formic acid/ammonium formate buffer (pH 2.9) with 10% of acetonitrile because this was the highest percentage of organic phase with which satisfactory recoveries were obtained.

With this composition for the washing solvent, a study was made of the effect of the RAM washing time; that is, the time during which it was circulating through the RAM once the sample had been injected. For washing times between 3 and 15 min, an increase in the washing time did not afford a significantly better clean-up but did elicit the corresponding increase in the analysis time. Accordingly, a time of 3 min was chosen as optimum since it afforded an adequate washing of the undesired components of the matrix.

Optimization of the on-line coupling RAM-LC-MS/MS

In the literature, it has been reported that RAMs admit the injection of large sample volumes [30]. Thus, the possibility of injecting large sample volumes into the RAM-LC-MS/MS system was studied in order to improve the sensitivity of the method. The sample volumes injected were in the 20–1,500-μL range. Figure 2 shows the results obtained for a honey sample fortified at 4 μg g^{-1}. For high injection volumes it is possible to observe detector saturation. Accordingly, an injection volume of 500 μL was chosen, considering this value as the highest volume that could be injected without saturating the MS/MS system.

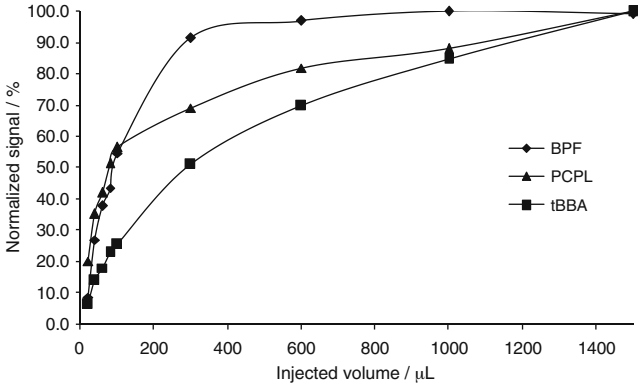

Fig. 2 Normalized (to the highest area) signals as a function of the volume of fortified honey injected according to the RAM-LC-MS/MS method developed. Fortified level: 4 μg g^{-1} for each analyte. *BPF* bisphenol-F, *PCPL* pentachlorophenol, *tBBA* 4-*tert*-butylbenzoic acid

Study of ion suppression

When ESI-MS is used as a method of detection, it is known that this ionization source (ESI) may undergo losses of sensitivity owing to the presence of other compounds that co-elute with the analytes of interest, a phenomenon known as ion suppression. Moreover, this effect may be more pronounced when large sample volumes are injected.

Here, we studied the possible effect of ion suppression on the signals of the analytes when 500 μL of honey sample was injected into the RAM-LC-MS/MS system. To accomplish this, the total ion chromatograms (TIC) were recorded (Fig. 3) for a standard aqueous sample of the analytes studied, fortified at 100 ng g^{-1} in UHQ water (Fig. 3a) and for a honey sample fortified at 100 ng g^{-1} injected using the proposed RAM-LC-MS/MS methodology (Fig. 3b). This honey sample was also directly injected into the LC-MS/MS system (Fig. 3c). It may be observed that the total signal of the analytes in the standard in UHQ water and in the honey injected through the RAM was similar both in shape and in intensity. However, when fortified honey was injected directly into the LC-MS/MS system, the signal of the analytes underwent a strong decrease owing to ion suppression. This points to the efficiency of the RAM in the cleaning of the matrix components.

Although upon injecting 500 μL of honey sample the RAM-LC-MS/MS configuration strongly reduced the ion suppression of the analytes, such suppression persisted. Thus, this matrix effect was calculated as ion suppression according to the method suggested by Feitosa-Felizzola et al. [31]. Table 3 shows the ion suppression (expressed as a percentage with respect to the signal obtained in UHQ water) for each of the analytes separately. The values of ion suppression varied between 4.6% for tBBA and 24% for tBPL.

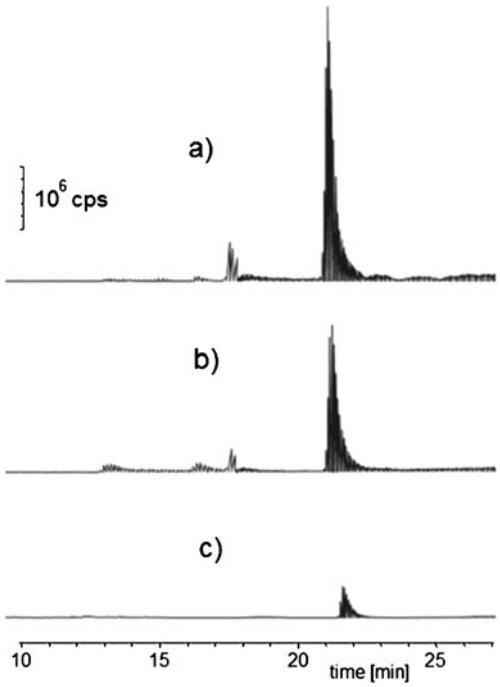

Fig. 3 Total ion chromatograms (*TICs*) obtained by the analysis of: **a**) a UHQ standard, **b**) a fortified honey sample injected according to the RAM-LC-MS/MS method developed, and **c**) a fortified honey sample injected directly into the LC-MS/MS system. Injected volume, 500 μL, fortified level, 100 ng g^{-1} for each analyte

The level of ion suppression undergone by the analytes did not seem to be related to their retention times. However, we did observe a correlation between ion suppression and the acidity constant (pK_a) of the analytes, i.e., with their tendency to lose a proton. The formation of ions in the electrospray is one of the most critical factors in liquid chromatography coupled to mass spectrometry. The efficiency of this step depends on the previous presence of the ions studied in liquid phase or on their later generation, through highly complex mechanisms, in the gas phase [29]. Accordingly, the pK_a of the compounds of interest is one of the most relevant factors affecting the efficiency of the formation of such ions. Table 2 shows the pK_a value for each of the EDCs studied, together with its corresponding ion suppression, calculated experimentally. The correlation between ion suppressions and pK_a (r^2=0.954) is coherent with the fact that all the compounds of interest were detected as anions arising from the loss of a proton: $[M-H]^-$. Since the effect of ion suppression leads to a loss of analytical signal, and hence sensitivity, this correlation could be extended to different analytical characteristics, such as the limit of detection.

Analytical characteristics of the RAM-LC-MS/MS method

Once the parameters of the RAM-LC-MS/MS method had been optimized, their analytical characteristics were studied. The MS/MS detection system was configured in multiple reaction monitoring (MRM) mode, analyzing the transition of identification for each of the analytes. The limits of detection (LODs), calculated as three times the signal–noise ratio, varied between 0.6 ng g^{-1} for PCPL and 7.2 ng g^{-1} for BPF. The limits of quantification (LOQs), calculated as ten times the signal–noise ratio, ranged between 2.0 ng g^{-1} for PCPL and 22.6 ng g^{-1} for BPA. Figure 4 shows the MS/MS chromatograms and total ion chromatogram (TIC) of the analytes studied, at a concentration level of 10 ng g^{-1}, in MRM mode, analyzing the transition of identification.

Validation of the RAM-LC-MS/MS method according to 2002/657/EC

In recent years, the Commission Decision of the 12 August 2002 implementing Council Directive 96/23/EC establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. This decision establishes common criteria for the interpretation of test results and introduces a procedure to progressively establish minimum required performance limits (MRPL) for analytical methods employed to detect substances for which no permitted limit (maximum limit) has been established. This, especially for substances whose use is not authorized or is specifically prohibited in the EU. Thus, through the 2002/657/EC Decision [28] the EU has established the characteristics that must be fulfilled by an analytical method for it to be required as a confirmatory quantitative method. The parameters to be evaluated are: the detection capacity (CC_β), the limit of decision (CC_α), recovery, precision, selectivity, and ruggedness.

Additionally, for confirmatory methods based on MS/MS detection, the 2002/657/EC Decision establishes a minimum of three points of identification (IPs) that must be presented for each analyte based on the number of mass and mass/mass signals found. The IPs found for the nine EDCs studied are shown in Table 1. Unfortunately, it was not possible to achieve the minimum number of identification points required by the EU [28] in the case of tBPL, tBBA, and nOPL; even when MS^3 experiments were performed for tBPL and nOPL, no additional fragment ions were observed. This is due to the poor fragmentation that these analytes present by collision-inducted dissociation (CID) in the ion trap. Accordingly, these analytes were removed from the validation process. For the other six target analytes (BPF, BPA, DCPL, TCPL, tOPL, and PCPL) IP values between 3 and 5 were observed. For PCPL, the three IPs corresponded to the three m/z ratios of the chloride isotope ions. A higher number of IPs can be obtained when it is possible to analyze two transitions for each analyte: a transition of identification

Table 2 Retention times, acidic constants and ion suppressions for the EDCs studied obtained by analyzing fortified honey samples

Analyte[a]	BPF	BPA	tBPL	DCPL	tBBA	TCPL	tOPL	nOPL	PCPL
t_r	14.2	14.6	15.7	15.9	16.4	17.6	18.6	20.1	20.4
pK_a	9.7	9.8	10.3	7.7	4.4	6.7	10.2	10.2	4.9
Ion supp. (%)	20	21	24	15	4.6	16	22	22	6.3

[a] *BPF* bisphenol-F, *BPA* bisphenol-A, *tBPL* 4-*tert*-butylphenol, *DCPL* 2,4-dichlorophenol, *tBBA* 4-*tert*-butylbenzoic acid, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *nOPL* 4-*n*-octylphenol, *PCPL* pentachlorophenol

and the other one for confirmation. To validate the method, the MS/MS detection system was configured in the Product Ion Scan mode. With this configuration, a complete spectrum of product ions was observed, with which it was possible to check the existence of both transitions and the correspondence of the ratios. For each analyte, the analytical signal was obtained by collecting an extracted ion chromatogram (EIC) of the product ion corresponding to the confirmation transition.

Table 3 shows the calibration characteristics for each analyte (intercept, slope, and r^2) corresponding to the confirmation transition and the validation parameters

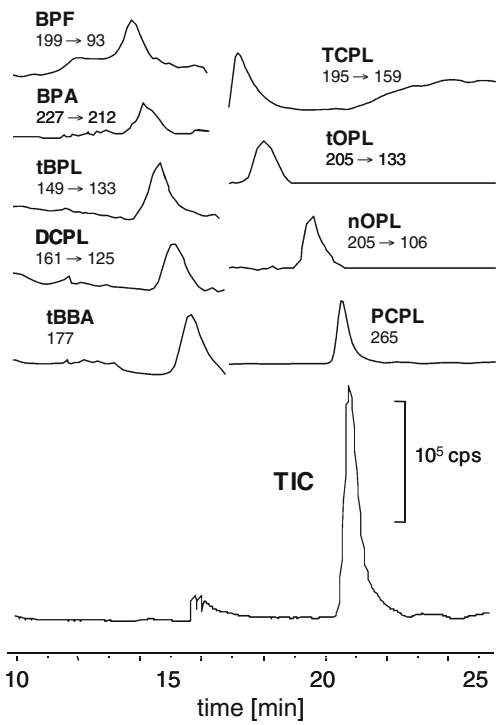

Fig. 4 MS/MS chromatograms and total ion chromatogram (*TIC*) of a fortified honey sample obtained using the developed RAM-LC-MS/MS method. Fortified level, 10 ng g^{-1} for each analyte. *BPF* bisphenol-F, *BPA* bisphenol-A, *tBPL* 4-*tert*-butylphenol, *DCPL* 2,4-dichlorophenol, *tBBA* 4-*tert*-butylbenzoic acid, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *nOPL* 4-*n*-octylphenol, *PCPL* pentachlorophenol

established by the 2002/657/EC Commission Decision. The limit of decision (CC_α) varied between 1.7 ng g^{-1} for PCPL and 12.6 ng g^{-1} for BPA and the values of the detection capabilities (CC_β) ranged between 2.8 ng g^{-1} for PCPL and 21.6 ng g^{-1} for BPA. The correlations between CC_α and CC_β versus the pK_a had r^2 values of 0.904 and 0.908, respectively.

Since no reference materials are available, recovery studies were carried out in order to check the accuracy of the proposed RAM-LC-MS/MS method. Polyfloral honey samples from different commercial brands that had not been used previously in the method were analyzed, none of the analytes studied being found in them at concentrations higher than the CC_α of the method. These samples were fortified at two levels, 12.5 and 25 ng g^{-1}. The signal obtained for each of the analytes was introduced into the corresponding calibration line. In the case of honey fortified at the lowest level of concentration, the recoveries varied between 88±16% for tOPL and 120±24% for BPA.

Repeatability and within-laboratory reproducibility were assessed as intraday and interday precision, respectively for the two concentration levels; 20 and 100 ng g^{-1}. The highest values of the relative standard deviation (RSD) varied in the 5.5–11% range for interday analysis at a level of 20 ng g^{-1}.

Finally, the specificity and ruggedness of the method were determined by the high level of selectivity characteristic of tandem mass spectrometry and by the different checks and calibrations to which the system had been subjected, respectively. At all times along the work, the pressure of the system was controlled; both that of the isocratic pump connected to the RAM column and that of the binary pump connected to the analytical column. The pressure control of both pumps, together with the background noise and the shape of the peaks obtained, allowed the proper functioning of the system to be checked. Moreover, a standard solution was injected each week with a view to controlling the intensity of the signals obtained in the MS/MS detector.

Along this work, only one RAM column was employed, supporting about 150 injections. This implies a total sample

Table 3 Statistic and performance characteristics, according to 2002/657/EC, of the proposed RAM-LC-MS/MS method obtained by analyzing fortified honey samples

Analyte[a]		BPF	BPA	DCPL	TCPL	tOPL	PCPL
Intercept (area units; au)		$(0.4\pm5.4)\times10^2$	$(-0.5\pm1.8)\times10^2$	$(0.0\pm3.0)\times10^2$	$(0.5\pm1.0)\times10^3$	$(1.7\pm2.2)\times10^3$	$(-0.6\pm6.0)\times10^5$
Slope (au; ng g^{-1})		4.70 ± 0.04	11 ± 0.2	$(2.8\pm0.2)\times10^2$	$(1.3\pm0.1)\times10^2$	$(0.7\pm0.1)\times10^2$	$(2.4\pm0.1)\times10^5$
r^2		0.998	0.997	0.999	0.999	0.998	1.000
CC_α[b] (ng g^{-1})		9.6	12.6	7.6	6.8	10.8	1.7
CC_β[c] (ng g^{-1})		16.6	21.6	12.9	11.5	18.4	2.8
Recovery[d] (%)	12.5 ng g^{-1}	112±16	120±24	104±16	112±16	88±16	96±8
	25.0 ng g^{-1}	100±12	96±8	108±8	104±8	98±8	104±4
RSD[e,f] Intraday (%)	20 ng g^{-1}	6.7	4.9	5.9	7.4	7.1	3.9
	100 ng g^{-1}	2.5	2.1	2.3	3.2	4.5	1.6
RSD[e,g] Interday (%)	20 ng g^{-1}	11	11	7.5	9.5	9.5	5.5
	100 ng g^{-1}	7.6	9.9	4.3	5.8	7.7	3.0

[a] Concentration range from 10 to 150 ng g^{-1} (five calibration points). *BPF* bisphenol-F, *BPA* bisphenol-A, *DCPL* 2,4-dichlorophenol, *TCPL* 2,4,5-trichlorophenol, *tOPL* 4-*tert*-octylphenol, *PCPL* pentachlorophenol

[b] Decision limit (CC_α) calculated from the confirmation transition in product ion scan mode. r^2 (CC_α vs. pK_a): 0.904

[c] Detection capability (CC_β) calculated from the confirmation transition in product ion scan mode. r^2 (CC_β vs. pK_a): 0.908

[d] Confidence intervals at 95% significance level

[e] *RSD* relative standard deviation

[f] Intraday precision (repeatability) was determined by eight injections

[g] Interday precision (within-laboratory reproducibility) was determined in three consecutive days (eight injections each day)

volume of approximately 80 mL (equivalent to 16 g of honey). It is unquestionable that the half-life of RAMs will depend on their use (number of injections). As an indication, replacement of the RAM column should be carried out when the pressure of the system is excessively high (>20%) with respect to the usual values or when the intensity of the signal and the shape of the peaks so indicate.

Conclusions

An automated method for the analysis of endocrine disruptors in honey samples, by using a configuration that incorporates a RAM material, has been developed. The results obtained showed that the use of restricted-access materials in the clean-up of honey samples with different origins is acceptable. The proposed on-line configuration allowed prior sample treatment to be minimized, this being reduced to a dilution and filtration step. Additionally, the RAM used (ADS-C4) proved to have a fairly long half-life (150 injections) and allowed the injection of at least 16 g of honey.

The method described, RAM-LC-MS/MS, is a sensitive, selective, and precise automated tool for the determination and confirmation of several different EDCs in a complex matrix such as honey. The ESI-MS/MS system showed acceptable sensitivity, which is related to the pK_a value of each of the analytes studied. In any case, it's acceptable sensitivity allowed limits of detection at the low nanogram per gram level to be obtained. For six of the nine analytes studied (BPF, BPA, DCPL, TCPL, tOPL, and PCPL) between three and five points of identification were found, such that for these compounds the proposed method has been validated as a quantitative confirmatory method in accordance with the 2002/657/EC Commission Decision.

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