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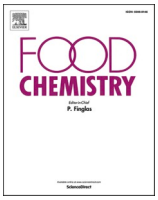

Development and validation of an analytical methodology based on solvent microextraction and UHPLC-MS/MS for determining bisphenols in honeys from different botanical origins

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ABSTRACT

A new analytical methodology was proposed to determine fourteen bisphenols in honeys from different botanical origins using ultra-high performance liquid chromatography-tandem mass spectrometry. A fast, efficient, environmentally-friendly and simple sample treatment (recoveries between 81% and 116%; matrix effect <20% for all studied compounds except for bisphenol E, F and S) was proposed, which involved a solvent microextraction with acetone and a small volume/amount of 1-hexanol. Chromatographic analysis (< 15 min) was performed in a Kinetex EVO C_{18} column under gradient elution mode. The method was validated in terms of selectivity, limits of detection (0.2–1.5 μg/kg) and quantification (0.5–4.7 μg/kg), linearity, matrix effect, trueness, and precision (relative standard deviation <17%). Finally, thirty honey samples were analyzed, revealing the presence of residues of nine bisphenols in some of them. However, quantification was possible only in two cases for bisphenol A, with a concentration of approximately 13 μg/kg.

1. Introduction

Bisphenols (BPs), which are organic compounds with two phenol functional groups, are used as additives to improve durability, flexibility and temperature resistance of plastics (Lestido-Cardama et al., 2021; Priovolos & Samanidou, 2023). The best-known is bisphenol A (BPA), a monomer used in the production of polycarbonate plastics and epoxy resins, which are commonly used in the manufacture of food packaging (Shaaban et al., 2022). BPA is a dangerous substance, recognized as an endocrine disruptor, which can cause serious problems at very low doses (Abraham & Chakraborty, 2019). Although the effects of endocrine disruptors on human health are not yet fully understood, their effects on women are especially serious. BPA has structural similarity to some hormones, especially those that control breast development (Martín-Gómez, Elmore, Valverde, Ares, & Bernal, 2024). Therefore, BPA exposure can lead to the proliferation of cancer cells in breast tissue (He et al., 2022; Zhao et al., 2021). Additionally, exposure during pregnancy can have adverse effects on the neural development of the fetus, increasing the risk of diabetes and heart disease (Aris, 2014). Moreover, exposure to BPA has been associated with an increased risk of spontaneous abortions (Liang et al., 2020). It should be mentioned that there is a wide range of BPA analogues, such as bisphenol AF (BPAF), bisphenol AP (BPAP), bisphenol B (BPB), bisphenol BP (BPBP), bisphenol C (BPC), bisphenol E (BPE), bisphenol F (BPF), bisphenol FL (BPFL), bisphenol M (BPM), bisphenol P (BPP), bisphenol PH (BPPH), bisphenol S (BPS), and bisphenol Z (BPZ), whose structures are shown in Fig. 1S (see Supplementary Material). These analogues are currently used as substitutes for BPA; however, they also pose a health risk due to their demonstrated genotoxicity and estrogenic activity, similar to that of BPA (Liao et al., 2012; Priovolos & Samanidou, 2023). The European Commission has established specific migration limits (SMLs) for BPA from varnishes or coatings into or onto food at 0.05 mg/kg of food, thereby prohibiting the

Abbreviations: AF, samples spiked after sample treatment; BAGI, blue applicability grade index; BF, samples spiked before sample treatment; BPA, bisphenol A; BPAF, bisphenol AF; BPAP, bisphenol AP; BPB, bisphenol B; BPBP, bisphenol BP; BPC, bisphenol C; BPE, bisphenol E; BPF, bisphenol F; BPFL, bisphenol FL; BPM, bisphenol M; BPP, bisphenol P; BPPH, bisphenol PH; BPs, bisphenols; BPS, bisphenol S; BPZ, bisphenol Z; DLLME, dispersive liquid-liquid microextraction; GC, gas chromatography; HPLC, high performance liquid chromatography; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; *m/z*, mass-to-charge; RSD, relative standard deviation; SE, solvent extraction; SML, specific migration limit; S/N, signal-to-noise; SCI, slope confidence intervals; SPE, solid phase extraction; UHPLC, ultra-high-performance liquid chromatography.

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use of BPA in articles intended for infants and young children (European Commission, 2018). However, as of now, no SMLs have been established for BPA analogues, except for BPS (0.05 mg/kg; European Commission, 2011).

This work focuses on the analysis of BPs in honey, which is a natural food made by bees from the nectar of flowers. Honey is one of the most complex natural foods, since it contains around 400 substances, such as carbohydrates, proteins, amino acids, phenolic compounds and vitamins, among others (Valverde, Ares, Stephen Elmore, & Bernal, 2022). All these compounds make the consumption of honey very beneficial for health. Indeed, honey has antioxidant, healing, anti-inflammatory, therapeutic, nutritional, antimicrobial and antidiabetic properties, making its consumption highly recommended (Notardonato et al., 2020a; Rana et al., 2018). Although BPs are not compounds that are found naturally in honey, it is possible for them to contaminate it, since honey bees interact with plants, air, soil, and water in close proximity to the hive. Therefore, if these matrices are contaminated with BPs, these compounds may be transferred to honey bees and, ultimately, find their way into hive products, including honey (Al Naggar et al., 2021). Moreover, like any food product, honey could potentially contain BPs, since these compounds may migrate from the packaging into the honey (Česen et al., 2016). Therefore, it is reasonable to propose the hypothesis that, given the potential of honey to present residues of BPs, there is a need for the development of specific and sensitive methodologies for their determination at low concentrations. In addition, the ubiquity of BPs in the laboratory underscores that special attention must be paid to minimizing this contamination in order to guarantee accurate analysis of the samples (Ballesteros-Gómez, Rubio, & Pérez-Bendito, 2009; Martín-Gómez et al., 2024).

Current methods for the analysis of BPs in honey are predominantly based on chromatographic techniques such as high-performance liquid chromatography (HPLC) or gas chromatography (GC; Martín-Gómez et al., 2024; see Supplementary Material, **Table 1S**). However, due to the low volatility of BPs owing to their high boiling points (> 200 °C), analysis of BPs by GC requires some derivatization steps involving alkylation, sylilation or acylation before chromatographic separation. These additional steps imply an increase in analysis time and, in addition, decrease the reproducibility of the method and introduce a possible source of contamination (Lestido-Cardama et al., 2021). HPLC can be coupled with fluorescence (FLD) and diode array (DAD) detectors, which offer advantages such as simplicity and low cost. However, to improve selectivity and sensitivity when analyzing BPs at low concentrations in a complex matrix, like honey, it is more convenient to use HPLC coupled to mass spectrometry (MS) and, specifically, tandem mass spectrometry (MS/MS; see Supplementary Material, **Table 1S**). Concerning the most commonly employed sample treatments to determine BPs in honey, an analysis of the existing literature (see Supplementary Material, **Table 1S**) indicates that solid phase extraction (SPE) with polymeric sorbents (Česen et al., 2016; Inoue, Murayama, Takeba, Yoshimura, & Nakazawa, 2003; Lo Turco et al., 2016; Zhou et al., 2019) and dispersive liquid-liquid microextraction (DLLME) alone or in combination with other techniques such as ultrasound (Khani, Khandaghi, Farajzadeh, Reza, & Mogaddam, 2021; Meng, Li, Wang, & Cao, 2022; Notardonato et al., 2020a, 2020b; Peñalver, Arroyo-Manzanares, Campillo, & Viñas, 2021) predominate. Other alternatives, like molecularly imprinted solid phase extraction employing a specifically prepared molecularly imprinted polymer with BPA as template and 4-vinylpyridine as the functional monomer (Herrero-Hernández, Carabias-Martínez, & Rodríguez-Gonzalo, 2009), a restricted-access material (alkyl-diol-silica) coupled on-line to a HPLC-MS/MS (Rodríguez-Gonzalo, García-Gómez, & Carabias-Martínez, 2010), or aqueous biphasic systems coupled with HPLC-FLD (Tian, Bai, & Xu, 2018), were also employed in some studies. It can also be deduced from the study of **Table 1S** (see Supplementary Material) that many of the proposed methods are not exclusive to either BPs or honey, and except for one work (Česen et al., 2016), more than four BPs have not been simultaneously investigated in honey. Furthermore, there is no evidence of an HPLC-MS/MS method in which the matrix effect was not relevant for any of the studied BPs, nor of a method in which its suitability for honeys from different origins has been tested, which is a relevant issue according to our experience (Fuente-Ballesteros et al., 2023).

Therefore, the main goal of the present study is to propose a method for determining simultaneously fourteen BPs (BPA, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPF, BPFL, BPM, BPP, BPPH, BPS, and BPZ) in honeys from three different botanical origins (multifloral, rosemary, and heather). An ultra-high-performance liquid chromatography (UHPLC)-MS/MS instrument was selected for the analysis, due to its potential for achieving better resolutions and sensitivities, as well as shorter running times, thereby implying reduced solvent consumption (Alarcón-Flores, Romero-González, Vidal, & Frenich, 2013). Another objective of this work is to propose an efficient, simple, cheap and fast sample treatment applicable to honeys from different origins. These conditions aim to ensure good recoveries, minimize the potential matrix effect, and adhere as closely as possible to the principles of green analytical chemistry, specifically by reducing time, cost, steps, and reagents, or avoiding derivatization procedures (Gałuszka, Migaszewski, & Namieśnik, 2013). To the best of our knowledge, this is the first study in which an analytical methodology has been proposed for determining BPs in different types of honey. Our study also aims to validate the proposed method in accordance with current European legislation (EURACHEM, 2014) and analyze experimental and commercial honey samples from the aforementioned origins.

2. Materials and methods

2.1. Reagents and materials

BP standards (BPA, BPA-d_{16}, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPF, BPFL, BPM, BPP, BPPH, BPS, BPZ; see structures in Supplementary Material, **Fig. 1S**), all of analytical-grade and with purity >98%, were purchased from Sigma-Aldrich Chemie Gbmh (Steinheim, Germany). All solvents and reagents were of chromatographic/analytical grade and obtained from VWR Prolabo Chemicals (Fontenay-sous-Bois, France; acetonitrile, hexane and methanol), Carlo Erba Reagents-SA (Milan, Italy; acetone, ammonia, ammonium acetate, ethyl acetate and tetrahydrofuran), and Sigma-Aldrich Chemie Gbmh (Steinheim, Germany; 1-hexanol, formic acid, magnesium sulfate, methyl tert-butyl ether, sodium chloride, and sodium sulfate). Ultrapure water was obtained using Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). A vortex mechanical mixer from Heidolph (Schwabach, Germany), a thermostated ultrasound bath, a drying oven, and a vibromatic mechanical shaker, all supplied by J.P. Selecta S.A. (Barcelona, Spain), a 5810 R refrigerated bench-top centrifuge from Eppendorf (Hamburg, Germany), a R-3 rotary evaporator from Buchi (Flawil, Switzerland), and Nylon syringe filters (17 mm, 0.45 μm; Nalgene, Rochester, NY) were employed for sample treatment. Bond Elut NH_2 (3 mL, 500 mg of sorbent; Agilent, CA, USA), Oasis HLB (3 mL, 60 mg; Waters, Milford, MA, USA), Strata X (3 mL or 6 mL, 100 or 200 mg; Phenomenex, Torrance, CA), Strata SCX (3 mL, 500 mg of sorbent; Phenomenex, Torrance, CA); Isolute HMN (5 mL, 3.5 g; Biotage Sweden AB, Uppsala, Sweden) cartridges, and a 10-port Visiprep vacuum manifold (Supelco, Bellefonte, PA), were used for the SPE extractions.

It should be highlighted that, due to the common presence of BPs in the laboratory environment, their potential presence in the background signal values of blanks must be controlled (Peñalver et al., 2021; Zhou et al., 2019). For example, the plastic consumables (pipettes and centrifuge tubes) and chemical reagents were newly used/opened, and the absence of BPs' residues was previously confirmed. Moreover, laboratory glassware was carefully and sequentially washed with ultrapure water, nitric acid, and ultrapure water again, followed by a final wash with a mixture of acetone and methanol (1:1, *v*/v) before being dried in an oven at around 100 °C for 60 min. Procedural blanks, in which

ultrapure water substituted honey, were run between sets of samples to monitor potential abnormal background values.

2.2. Standards

Standard stock (≈ 1000 mg/L) and working solutions of BPs, including the IS, were prepared in methanol. Honey samples, in which the absence of BPs had been previously confirmed using UHPLC-MS/MS (blank samples) were spiked with variable amounts of the analytes either before (BF samples) or after (AF samples) sample treatment (see Subsection 2.3) to prepare the standard in matrix extracts. The spiking of the samples was done similarly to Fuente-Ballesteros et al., 2023 (see Supplementary Material, **Table 2S**), and the internal standard (IS; BPA-d_{16}) was consistently added at the same concentration (0.1 mg/L). It should be mentioned that the IS was not added from the beginning of the sample treatment but at the reconstitution stage in the final method, as BPs were extracted in all cases with high efficiency and precision (see Subsection 3.3.6), and what was intended to compensate was the variability in the intensity of the MS/MS signals characteristic of this technique due to differences in ionization efficiency. These samples were used for validation (spiked samples (low, medium, and high) and calibration curves), as well as for sample treatment studies. It is important to note that three replicates from each origin, which were injected three times, were prepared for all the above-mentioned studies. Each spiked sample was prepared with 1 g of blank honey sample spiked with three different concentrations of the BPs within the linear range. These were as follows: low-LOQ (see **Table 1**); medium-50 μg/kg; high-250 μg/kg. The standard stock solutions were stored in glass containers in darkness at −20 °C, and working and standard matrix solutions were stored in glass containers and kept in the dark at +4 °C. Finally, it must be highlighted that no differences were observed in the stability of the deuterated IS (BPA-d_{16}) with respect to that of the rest of the BPs, since they remained stable in all cases for more than four weeks, and consequently the way of working with the IS was exactly the same as with the other BPs. To check this point, the working solutions were periodically injected with the mixture of all the BPs and the IS and it was checked if there were variations in the peak parameters (area, height, width) and analyte/IS peak area ratios.

2.3. Sample procurement and treatment

2.3.1. Samples

Honey samples (n = 30) were generously donated by beekeepers from different Spanish regions (Castilla y León, País Vasco) and the Center for Agroenvironmetal and Apicultural Investigation (CIAPA; Marchamalo, Guadalajara, Spain), or purchased from local markets (Valladolid, Spain; Helsinki, Finland; Tallin, Estonia). They were selected based on their origin (multifloral, rosemary, heather), and their different colors (white, light amber, and dark amber; see Supplementary Material, **Table 3S**). Moreover, the botanical origin of the CIAPA samples that were used as blank samples was confirmed by melissopalynological analysis and corresponded to rosemary (*Rosmarinus officinalis*; three samples); multifloral (three samples), and heather (*Erica spp*; three samples). It must be specified that in this work we have decided to select honey from three botanical origins (multifloral, rosemary, heather), to develop and validate the method for the following reasons: **i**) they have very different compositions and physicochemical characteristics. So, if the method is valid for these botanical origins, it would be probably valid for other types of honey. This allows increasing the relevance, validity, and interest of the proposed method; **ii**) these are three of the botanical origins that are most found in supermarkets and produced by beekeepers. This allows obtaining sufficient quantities and numbers of samples to carry out the development and validation of the methods. Meanwhile, a HI96785 honey color portable photometer (Hanna Instruments, Woonsocket, RI, USA) was used to determine the color of honey. For homogenization, each sample underwent individual stirring with a glass rod and was then stored in separate tubes in darkness at 4 °C until analysis. Three replicates (sub-samples) of each honey sample, injected in triplicate, were examined to determine the BP content.

2.3.2. Sample treatment

Briefly, 1.0 g of homogenized honey was weighed in a 50 mL centrifuge tube, after which 1 mL of ultrapure water was added, and the tube was shaken for 30 s in a vortex device. Next, 1.5 mL of acetone, 35 μL of 1-hexanol, and 500 mg of magnesium sulfate mixture were added to the tube and then put in an ultrasound bath for 5 min. After that, the mixture was centrifuged (4000 rpm; 5 °C) for 5 min. The upper phase (organic) was collected and evaporated to dryness at 60 °C in a rotary evaporator. Finally, the dry extract was reconstituted with 0.5 mL of an IS solution (0.1 mg/L) in methanol, and it was passed through a 0.45 μm nylon filter prior UHPLC-MS/MS analysis.

2.4. UHPLC-MS/MS parameters

UHPLC-MS/MS analyses were carried out using a UHPLC Sciex Exion system connected to a Sciex 6500+ triple quadrupole mass spectrometer from Sciex (Washington, DC, USA). The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in

Table 1
Calibration curve data, LOD, LOQ and SML values.

Compounds	Standard in solvent		Multifloral honey				Rosemary honey				Heather honey				SML
	SCI	R^2	SCI	R^2	LOD	LOQ	SCI	R^2	LOD	LOQ	SCI	R^2	LOD	LOQ	(μg/kg)
BPA	0.15 ± 0.03	0.992	0.14 ± 0.03	0.990	0.8	2.5	0.14 ± 0.03	0.990	1.0	3.0	0.14 ± 0.03	0.990	1.3	4.0	50
BPAF	1.85 ± 0.30	0.995	2.01 ± 0.30	0.992	0.2	0.5	2.45 ± 0.50	0.990	0.2	0.5	2.28 ± 0.20	0.994	0.3	0.8	NS
BPAP	1.06 ± 0.30	0.993	1.24 ± 0.20	0.991	0.2	0.5	1.14 ± 0.30	0.991	0.2	0.6	1.11 ± 0.10	0.991	0.2	0.7	NS
BPB	0.13 ± 0.20	0.991	0.15 ± 0.02	0.993	0.2	0.8	0.15 ± 0.03	0.992	0.3	1.0	0.14 ± 0.01	0.995	0.4	1.2	NS
BPBP	0.82 ± 0.20	0.993	0.86 ± 0.20	0.990	0.2	0.8	0.88 ± 0.20	0.990	0.2	0.8	0.87 ± 0.20	0.990	0.3	1.0	NS
BPC	0.01 ± 0.002	0.994	0.01 ± 0.002	0.995	0.8	2.8	0.01 ± 0.002	0.993	1.0	3.0	0.01 ± 0.002	0.995	1.4	4.4	NS
BPE	0.21 ± 0.30	0.992	0.13 ± 0.20	0.992	0.2	0.5	0.14 ± 0.20	0.991	0.2	0.6	0.14 ± 0.20	0.991	0.3	0.8	NS
BPF	0.08 ± 0.01	0.993	0.04 ± 0.01	0.990	0.3	1.1	0.04 ± 0.01	0.998	0.4	1.4	0.04 ± 0.01	0.999	0.6	2.0	NS
BPFL	1.68 ± 0.30	0.991	1.98 ± 0.30	0.992	0.2	0.5	1.95 ± 0.50	0.991	0.2	0.7	2.09 ± 0.40	0.991	0.3	1.0	NS
BPM	0.61 ± 0.20	0.990	0.76 ± 0.20	0.994	0.8	2.5	0.71 ± 0.20	0.997	1.1	3.4	0.73 ± 0.10	0.997	1.5	4.7	NS
BPP	0.47 ± 0.07	0.992	0.53 ± 0.06	0.996	0.3	0.9	0.52 ± 0.13	0.992	0.4	1.3	0.51 ± 0.08	0.992	0.5	1.7	NS
BPPH	0.27 ± 0.05	0.991	0.27 ± 0.09	0.990	0.2	0.8	0.32 ± 0.08	0.990	0.2	0.8	0.32 ± 0.06	0.992	0.3	1.0	NS
BPS	4.07 ± 0.50	0.995	1.76 ± 0.70	0.990	0.2	0.6	3.1 ± 0.2	0.999	0.2	0.6	1.48 ± 0.6	0.992	0.2	0.7	50
BPZ	0.38 ± 0.05	0.993	0.43 ± 0.07	0.992	0.2	0.5	0.47 ± 0.10	0.995	0.2	0.6	0.48 ± 0.06	0.995	0.3	0.8	NS

LOD, limit of detection; **LOQ**, limit of quantification; **NS**, not specified; **R^2**, determination coefficient; **SCI**, slope confident intervals ($\times 10^{-2}$); **SML**, specific migration limit.

negative mode. Chromatographic separation was accomplished using a reversed phase column, Kinetex EVO C_{18} (2.1 mm × 50 mm, 1.7 μm), protected with a Kinetex EVO C_{18} guard column, both from Phenomenex (Torrance, CA, USA). Mobile phase was composed of 10 mM ammonium acetate in ultrapure water (solvent A) and methanol (solvent B) at a flow rate of 0.3 mL/min in the following gradient mode: (i) 0.0 min (A–B, 50:50, *v*/v); (ii) 1.0 min (A–B, 50:50, v/v); (iii) 5.0 min (A–B, 15:85, v/v); (iv) 11.0 min (A–B, 15:85, v/v); (v) 13.0 min (A–B, 50:50, v/v); (vi) 15.0 min (A–B, 50:50, v/v). Injection volume and column temperature were set at 3 μL and 15 °C, respectively. With such conditions, the overall run time was 15 min (see Fig. 1). For mass spectrometry acquisition, multiple reaction monitoring (MRM) mode was employed. This mode recorded the transitions between the precursor ion and the most abundant product ions for each target analyte (see conditions and transitions in Table 2). Additionally, the ESI operational settings were as follows: capillary voltage, −4500 V; capillary temperature, 300 °C; ion source gas 1 and 2 pressure, 80 psi and 60 psi, respectively; curtain gas, 35 psi; collision gas, 9 psi. SciexOS software was employed for data acquisition and evaluation.

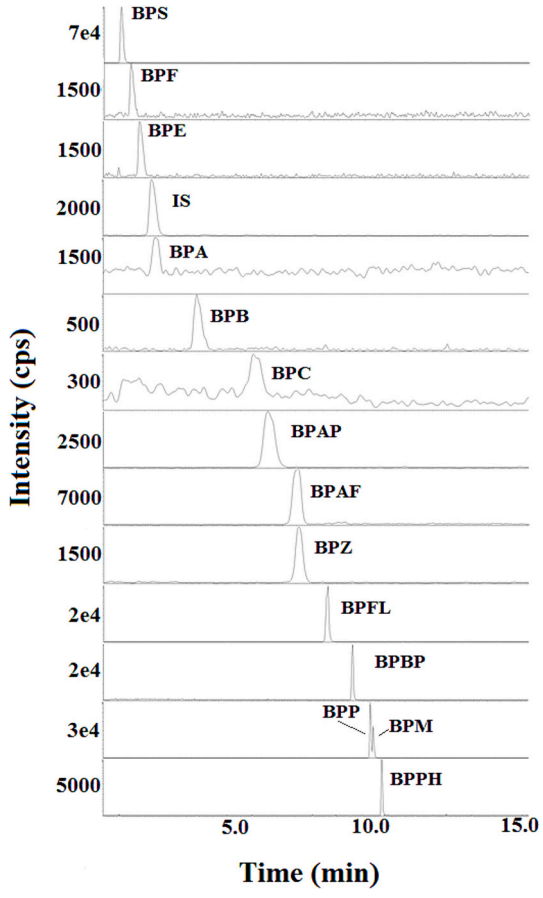

Fig. 1. Representative UHPLC-MS/MS chromatograms (MRM mode using the quantification transitions; see Table 2) obtained from a blank (analyte free) multifloral honey sample spiked with the selected BPs at 12.5 μg/kg). UHPLC-MS/MS conditions are summarized in Subsection 2.4 and Table 2. **BPA**, bisphenol A; **BPAF**, bisphenol AF; **BPAP**, bisphenol AP; **BPB**, bisphenol B; **BPBP**, bisphenol BP; **BPC**, bisphenol C; **BPE**, bisphenol E, **BPF**, bisphenol F; **BPFL**, bisphenol FL; **BPM**, bisphenol M; **BPP**, bisphenol P; **BPPH**, bisphenol PH; **BPS**, bisphenol S; **BPZ**, bisphenol Z; **IS**, BPA-d_{16} (100 μg/L).

3. Results and discussion

3.1. Optimization of the sample treatment

As mentioned in the introduction, the most used sample treatments when determining BPs in honey were SPE and DLLME (see Supplementary Material, **Table 1S**). However, considering our objective to propose a procedure that is simple, fast, and minimizes the consumption of reagents, we also explored extraction using supramolecular solvents (SUPRAS). SUPRAS are green water-immiscible solvents that are made up of aggregates of amphiphilic compounds, which are substances with a hydrophilic and a hydrophobic part. SUPRAS usually provide very high extraction yields in a short time, and, in addition, provide extracts clean enough to be analyzed directly without the need for additional cleaning steps (Ballesteros-Gómez, Ballesteros, & Rubio, 2024). Typically, SUPRAS are prepared by mixing amphiphilic compounds (such as long-chain alcohols) in appropriate proportions with water and a water-miscible solvent, which is usually tetrahydrofuran (THF). To promote the formation of these solvents, variations of temperature, pH, or the addition of an inorganic salt can be considered (Ballesteros-Gómez et al., 2024; Musarurwa & Tavengwa, 2021). The optimization process began with a multifloral honey (light amber) spiked at medium concentration (50 μg/kg; BPs free-blank, AF and BF samples; see Subsection 2.2). Firstly, we assessed the suitability of SPE for analyzing BPs in honey, employing a common procedure in almost all cases. This involved preconditioning the cartridges before passing the sample, which was usually a dilution with ultrapure water, followed by a washing step, drying time, and a final elution stage. A concentration step was then performed using rotary evaporation and, finally, reconstitution in 1 mL of the solution of the corresponding standards in methanol. During the SPE tests, various types of sorbents were tested, including polymeric, anionic, cationic and adsorption cartridges. The detailed results are provided in Table 3. The first tests (T1 − T4) were carried out exclusively with BPA in order to simplify the optimization procedure, as it is the most relevant BP. Polymeric SPE cartridges were initially checked, as they were predominantly employed for determining BPs in honey. Consequently, Oasis HLB (60 mg) cartridges were evaluated, and two different methods were tested for this cartridge (T1 and T2). Results were slightly better in terms of extraction efficiency and matrix effect for T2, in which methanol was selected as preconditioning and elution solvent. Thus, the method that used ethyl acetate was discarded. The same SPE conditions used for P2 were tested with the Strata X polymeric cartridge with two different amounts of sorbent, 100 mg (T3) and 200 mg (T4), and it was found that the recovery percentage with the 100 mg sorbent was much lower, although the matrix effect was slightly better than with 200 mg. Consequently, we decided to continue the SPE optimization with all the BPs employing the Oasis HLB (60 mg; T5) and Strata X (200 mg; T6) cartridges. Similar results were obtained for both cartridges. Oasis HLB showed recovery percentages >70% for eight BPs, while Strata X exhibited percentages higher than 70% for nine BPs. Furthermore, the matrix effect (suppression of ionization in all cases except for BPC) was very significant for all analytes. Thus, the use of polymeric cartridges was rejected due to low recoveries obtained for several of the BPs, and the strong matrix effect observed for all the compounds. Next, cartridges of anionic (Bond Elut NH_2, 500 mg; T7) and cationic (Strata SCX, 500 mg, T8) nature were tested, but in both cases much worse results were obtained in terms of recovery percentages, and on top of that, many of the BPs were not retained in them. As a final option, diatomaceous earth cartridges (Isolute HMN, 3.5 g) were tested. It was observed that hexane, a nonpolar solvent, was only able to elute three analytes from the cartridge (T9.1). However, when performing a second elution of the cartridge using methyl tert-butyl ether (T9.2), peaks corresponding to all analytes could be detected. Indeed, if a third elution with methyl tert-butyl ether (T9.3) was performed, peaks were also obtained for all analytes. Therefore, it was concluded that a more polar solvent was required to achieve a complete elution of the analytes. For this reason, it

Table 2
List of MRMs and mass spectrometry instrumental conditions for the target analytes and the internal standard.

Compound	Q_1	Q_3	Dwell time (ms)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
	227.0	210.9^{Q}	20	−60	−10	−24	−18
BPA	227.0	133.0^{C}	20	−40	−10	−34	−15
	227.0	117.0^{C}	20	−40	−10	−64	−13
	241.1	222.0^{Q}	20	−45	−10	−28	−11
IS (BPA-d_{16})	241.1	141.9^{C}	20	−45	−10	−40	−21
	241.1	97.9^{C}	20	−45	−10	−60	−5
	334.9	264.9^{Q}	20	−185	−10	−28	−18
BPAF	334.9	196.9^{C}	20	−40	−10	−52	−11
	334.9	176.8^{C}	20	−40	−10	−62	−11
	289.0	274.0^{Q}	20	−95	−10	−32	−27
BPAP	289.0	211.0^{C}	20	−80	−10	−36	−13
	289.0	195.1^{C}	20	−80	−10	−36	−11
	241.0	210.9^{Q}	20	−15	−6	−40	−8
BPB	241.0	224.8^{C}	20	−45	−10	−24	−21
	241.0	147.0^{C}	20	−45	−10	−34	−7
	351.0	273.0^{Q}	20	−60	−10	−36	−9
BPBP	351.0	283.1^{C}	20	−70	−10	−28	−9
	351.0	253.3^{C}	20	−70	−10	−36	−5
	255.1	147.0^{Q}	20	−30	−10	−15	−7
BPC	255.1	238.9^{C}	20	−55	−10	−28	−7
	255.1	223.0^{C}	20	−55	−10	−18	−13
	212.9	197.9^{Q}	20	−55	−10	−24	−17
BPE	212.9	118.8^{C}	20	−45	−10	−32	−13
	212.9	92.9^{C}	20	−45	−10	−30	−11
	198.9	104.9^{Q}	20	−80	−10	−30	−11
BPF	198.9	92.9^{C}	20	−60	−10	−30	−9
	198.9	77.0^{C}	20	−60	−10	−32	−9
	349.0	255.8^{Q}	20	−55	−10	−36	−31
BPFL	349.0	79.9^{C}	20	−55	−10	−78	−37
	349.0	281.2^{C}	20	−55	−10	−26	−17
	345.0	329.9^{Q}	12	−150	−10	−38	−21
BPM	345.0	251.6^{C}	12	−150	−10	−38	−21
	345.0	132.9^{C}	12	−150	−10	−48	−17
	345.0	133.0^{Q}	20	−30	−13	−60	−25
BPP	345.0	329.9^{C}	20	−135	−10	−38	−13
	345.0	315.0^{C}	20	−135	−10	−48	−9
	379.0	208.9^{Q}	12	−120	−10	−48	−17
BPPH	379.0	364.0^{C}	12	−120	−10	−34	−15
	379.0	192.9^{C}	12	−120	−10	−90	−11
	248.9	108.0^{Q}	20	−90	−10	−36	−10
BPS	248.9	92.0^{C}	20	−65	−10	−50	−11
	248.9	155.9^{C}	20	−65	−10	−30	−9
	267.0	173.0^{Q}	20	−60	−10	−36	−20
BPZ	267.0	224.0^{C}	20	−55	−10	−36	−31
	267.0	197.0^{C}	20	−55	−10	−36	−31

^{Q}quantification; ^{C}, confirmation; **CE**, collision energy; **CXP**, collision cell exit potential; **DP**, entry orifice potential; $\mathbf{Q_n}$, mass of pseudo-ion ``n''.

was decided to use ethyl acetate from the beginning of the procedure (T10.1), which allowed the detection of peaks for all BPs. However, low recoveries were observed for most of them, and when testing a second elution (P10.2), it was confirmed that the analytes were still retained in the cartridge. This finding implies the necessity to use larger volumes of solvent, which contravenes the principles of green analytical chemistry that we aim to follow in this work. Consequently, the use of SPE was definitively ruled out, and tests with modified DLLME and SUPRAS were performed. In order to try to optimize the solvent extraction, two procedures selected/adapted from the literature were used. One procedure (modified SUPRAS) was based on the addition of 1-hexanol and THF (Romera-García, Caballero-Casero, & Rubio, 2019), while the other involved a different approach in which 1-undecanol (1-hexanol in our study) and acetone were used (modified DLLME; Mahdavianpour et al., 2021). It should be mentioned that the function of 1-hexanol was not only to promote the extraction of BPs from honey in both sample treatments (Ballesteros-Gómez & Rubio, 2023; Habibi, Mohammadi, & Kamankesh, 2018), but also, to facilitate the separation of the phases in the case of the modified DLLME approach. In this last case, the extraction was achieved by the combined action of this alcohol together with acetone, which is also miscible with water, and consequently, in order to differentiate and collect the organic phase, 1-hexanol was needed. In this stage of the optimization procedure, different amounts of honey and shaking sources/instruments (vortex, vibromatic and ultrasound) were tested. The conditions and results of the most significant experiments are summarized in **Table 4**. It should be remarked that a multifloral honey (light amber) spiked at medium concentration (50 μg/kg; BPs free-blank, AF and BF samples; see Subsection 2.2) was selected to perform these experiments. As can be seen, the best overall performance (extraction recoveries between 85% and 110% for all BPs; matrix effect $< \pm$ 20% for all compounds except for BPE, BPF and BPS) was obtained in the experiment T15, in which 35 μL of 1-hexanol and 1500 μL of acetone were added to 1 g of honey dissolved in 1 mL of water. BPs were extracted with the help of an ultrasound for five minutes, and subsequently centrifuged at 4000 rpm for five minutes. The organic phase was collected and evaporated to dryness at 60 °C, and reconstituted with 0.5 mL of methanol. It should be mentioned that extraction recoveries were low for many of the BPs (< 70%) in several of the experiments (T13, T16 and T18), and the matrix effect was also significant for most BPs in many cases (T11, T12, T14, T16, T17 and T18). Consequently, it was decided to check the suitability of the selected sample treatment with multifloral honey samples spiked at the other two concentration levels (Low, LOQ; high, 250 μg/kg. The results showed that the extraction recoveries were good enough when spiking at high concentration (83%–113%), but they were slightly lower (< 80%) for some of the BPs at low concentration level (data not shown); meanwhile, matrix effect values were good for

Table 3
Evaluation of the extraction efficiency (recovery percentages) and the matrix effect when evaluating different SPE cartridges/sorbents and conditions (solvent and volumes) with spiked blank multifloral honey sample at medium concentration level (50 μg/kg). Data obtained as described in Subsections 2.2, 3.3. Results were obtained from three replicates that were injected in triplicate, and %RSD were lower than 17% in all cases.

Test (N°)	Cartridge (mg)	C	SL	W	DT (min)	E (min)	EE (min-max)	ME (min-max)
T1*	Oasis HLB (60)	5 mL (EA) + 5 mL (ACN) + 5 mL (UW)	3 g in 20 mL (UW)	10 mL (UW)	10	0.5 mL (ACN) + 3 mL (EA)	87% to 89%	−35 to −30
T2*	Oasis HLB (60)	6 mL (MeOH) + 6 mL (UW)	3 g in 20 mL (UW)	10 mL (UW)	10	6 mL (MeOH)	89% to 93%	−30 to −26
T3*	Strata X (100)	6 mL (MeOH) + 6 mL (UW)	3 g in 20 mL (UW)	10 mL (UW)	10	6 mL (MeOH)	74% to 78%	−38 to −35
T4*	Strata X (200)	6 mL (MeOH) + 6 mL (UW)	3 g in 20 mL (UW)	10 mL (UW)	10	6 mL (MeOH)	91% to 95%	−55 to −50
T5	Oasis HLB (60)	6 mL (MeOH) + 6 mL (UW)	3 g in 20 mL (UW)	10 mL (UW)	10	6 mL (MeOH)	40% to 113%	−72 to 200
T6	Strata X (200)	6 mL (MeOH) + 6 mL (UW)	3 g in 20 mL (UW)	10 mL (UW)	10	6 mL (MeOH)	44% to 91%	−85 to 230
T7	Bond Elut NH_2 (500)	5 mL (MeOH) + 5 mL (1% AA in UW)	4 g in 10 mL (2% NH_3 in UW)	4 mL (1%AA in UW)	5	10 mL (2% NH_3 in MeOH)	2% to 54%	−12 to 150
T8	Strata SCX (500)	6 mL (MeOH) + 6 mL (1% FA in UW)	5 g in 20 mL (0.1%FA in UW)	6 mL (0.1 M HCl in UW) + 6 mL (0.1 M HCl in MeOH)	10	6 mL (5% NH_3 in MeOH)	2% to 7%	10 to 160
T9.1	Isolute HMN (3500)	NE	3 g in 20 mL (UW)	NE	15	12 mL (HX)	3% to 40%	8 to 18
T9.2	Isolute HMN (3500)	NE	NE	NE	NE	12 mL (MTBE, 2^E)	17% to 99%	−67 to 228
T9.3	Isolute HMN (3500)	NE	NE	NE	NE	12 mL (MTBE, 3^E)	35% to 105%	−72 to 250
T10.1	Isolute HMN (3500)	NE	3 g in 20 mL (UW)	NE	15	12 mL (EA)	20% to 89%	−56 to 180
T10.2	Isolute HMN (3500)	NE	NE	NE	NE	12 mL (EA, 2^E)	42% to 98%	−65 to 240

*, tests only with BPA; **N^E**, number of elutions; **AA**, acetic acid; **ACN**, acetonitrile; **C**, conditioning; **DT**, drying time; **E**, elution; **EA**, ethyl acetate; **EE**, extraction efficiency; **FA**, formic acid; **HX**, hexane; **ME**, matrix effect; **MeOH**, methanol: **min**, minimum value; **max**, maximum value; **MTBE**, methyl *t*-butyl ether; **NE**, not employed; **SL**, sample loading; **UW**, ultrapure water; **W**, washing.

Table 4
Evaluation of the extraction efficiency (recovery percentages) and the matrix effect when evaluating different solvent extraction conditions (amount of sample, solvents, volumes, shaking devices and times) with spiked blank multifloral honey sample at medium concentration level (50 μg/kg). Data obtained as described in Subsections 2.2, 3.3. Results were obtained from three replicates that were injected in triplicate, and %RSD were lower than 17% in all cases.

Test (N°)	Honey (g)	Water (μL)	1-hexanol (μL)	THF (μL)	Acetone (μL)	V (min)	VB (min)	U (min)	C (min)	EE (min-max)	ME (min-max)
T11	0.3	600	200	500	NE	3	NE	NE	15	60% to 115%	−60 to 24
T12	0.3	600	200	NE	500	3	NE	NE	15	65% to 124%	−55 to 34
T13	0.3	600	100	250	NE	3	NE	NE	15	30% to 102%	−32 to 19
T14	0.5	600	200	500	NE	3	NE	NE	15	57% to 112%	−60 to 38
T15*	**1.0**	**1000**	**35**	**NE**	**1500**	**NE**	**NE**	**5**	**5**	**85% to 110%**	**−52 to 16**
T16	1.0	1000	35	1500	NE	NE	NE	5	5	50% to 114%	−46 to 28
T17	2.0	2000	200	NE	1350	NE	10	NE	5	75% to 150%	−64 to 30
T18	3.0	3000	100	NE	2800	NE	10	NE	5	20% to 78%	−70 to 40

*, best results; **C**, centrifuge; **EE**, extraction efficiency; **ME**, matrix effect; **min**, minimum value; **max**, maximum value; **NE**, not employed; **U**, ultrasound; **V**, vortex; **VB**, vibromatic.

all BPs at all concentration levels. Thus, it was decided to check if the addition of a salt could improve the extraction results. Therefore, 500 mg of sodium chloride, magnesium sulfate and ammonium sulfate were included in the proposed sample treatment. It was found that the addition of a salt improved not only the separation of the phases but also the extraction recoveries. Particularly, when using magnesium sulfate, extraction recoveries for all BPs ranged between 81% and 114%, while the matrix effect remained significant for the same three BPs (see Table 5). Finally, the selected sample treatment conditions were applied to honey samples from the other two botanical origins (rosemary, white; heather, dark amber) spiked at the three different concentration levels (see Table 5). The results were quite similar to those obtained for multifloral honey, as the extraction recoveries were good in all cases (81%–116%) and the matrix effect caused a significant signal suppression (> 20%) for BPE, BPF and BPS in all types of honey. Consequently, the conditions detailed above and summarized in Subsection 2.3.2 were deemed definitive.

To sum up, the proposed sample treatment can be considered as a promising alternative to previous proposals summarized in **Table 1S** (see Supplementary Material), since it is generally faster, simpler (with fewer stages), and involves little use of reagents (solvents, salts/sorbents), and is applicable to honeys from different botanical origins. Therefore, we may also conclude that the proposed method aligns more closely with the objectives of green analytical chemistry than most of those previously published. This is evident as the use of solvents and reagents is among the lowest (< 2.6 mL), it avoids the use of solvents

Table 5
Evaluation of the extraction efficiency (recovery percentages ± %RSD) of the sample treatment and the matrix effect (mean values ± %RSD). Data obtained as described in Sections 2.2, 3.3 and Table 4S, and the results were obtained from three replicates that were injected in triplicate.

Compounds	Multifloral honey						Rosemary honey						Heather honey					
	EE			ME			EE			ME			EE			ME		
	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL
BPA	104 ± 9	95 ± 12	97 ± 14	-11 ± 9	−14 ± 5	−7 ± 3	96 ± 4	97 ± 3	92 ± 5	−4 ± 3	−2 ± 1	−11 ± 6	102 ± 9	100 ± 8	98 ± 6	−9 ± 6	−12 ± 9	−8 ± 1
BPAF	94 ± 5	99 ± 11	102 ± 9	−1 ± 12	0 ± 3	9 ± 9	101 ± 8	103 ± 7	97 ± 5	14 ± 6	17 ± 10	15 ± 5	87 ± 14	93 ± 11	84 ± 10	1 ± 4	5 ± 2	1 ± 6
BPAP	104 ± 2	100 ± 9	103 ± 14	17 ± 10	15 ± 2	16 ± 6	95 ± 13	104 ± 8	97 ± 5	15 ± 4	18 ± 8	14 ± 5	91 ± 16	87 ± 12	82 ± 10	4 ± 2	−1 ± 4	9 ± 3
BPB	102 ± 5	107 ± 12	100 ± 15	11 ± 8	14 ± 4	12 ± 7	103 ± 8	101 ± 5	97 ± 4	6 ± 7	7 ± 5	10 ± 4	97 ± 10	96 ± 12	89 ± 5	−12 ± 14	−9 ± 14	−3 ± 11
BPBP	103 ± 13	105 ± 12	104 ± 16	16 ± 10	12 ± 7	15 ± 9	98 ± 3	103 ± 8	96 ± 5	15 ± 4	13 ± 9	18 ± 5	91 ± 8	90 ± 12	89 ± 10	4 ± 11	−2 ± 7	−7 ± 7
BPC	97 ± 15	94 ± 12	98 ± 13	8 ± 14	10 ± 8	13 ± 3	100 ± 3	93 ± 7	92 ± 4	8 ± 6	14 ± 7	9 ± 4	88 ± 8	87 ± 13	86 ± 5	13 ± 8	6 ± 4	9 ± 9
BPE	100 ± 7	95 ± 12	93 ± 9	−47 ± 9	−37 ± 5	−35 ± 7	89 ± 9	97 ± 4	90 ± 5	−41 ± 14	−39 ± 6	−40 ± 5	93 ± 11	94 ± 6	89 ± 10	−44 ± 15	−48 ± 9	−42 ± 10
BPF	86 ± 9	83 ± 15	89 ± 7	−44 ± 6	−45 ± 2	−47 ± 3	86 ± 3	84 ± 7	88 ± 7	−49 ± 1	−55 ± 2	−54 ± 7	87 ± 9	82 ± 13	81 ± 15	−60 ± 6	−55 ± 3	−53 ± 10
BPFL	103 ± 12	106 ± 13	104 ± 15	11 ± 9	16 ± 3	14 ± 8	98 ± 9	106 ± 4	97 ± 4	16 ± 4	15 ± 9	13 ± 4	92 ± 12	86 ± 15	93 ± 16	8 ± 12	4 ± 1	11 ± 7
BPM	102 ± 5	104 ± 4	108 ± 7	10 ± 9	4 ± 4	13 ± 11	114 ± 2	116 ± 5	106 ± 7	12 ± 7	17 ± 6	16 ± 7	96 ± 10	104 ± 9	98 ± 4	1 ± 7	3 ± 3	4 ± 2
BPP	98 ± 8	93 ± 11	102 ± 14	14 ± 7	6 ± 4	9 ± 9	104 ± 4	103 ± 9	96 ± 2	13 ± 7	−15 ± 9	16 ± 8	96 ± 3	92 ± 8	88 ± 7	−10 ± 9	−1 ± 4	−12 ± 7
BPPH	101 ± 9	94 ± 13	99 ± 11	9 ± 10	7 ± 8	4 ± 4	103 ± 9	104 ± 5	96 ± 7	12 ± 10	13 ± 6	17 ± 5	89 ± 16	97 ± 9	92 ± 15	−15 ± 7	−13 ± 1	−8 ± 3
BPS	90 ± 12	86 ± 8	82 ± 4	−48 ± 6	−52 ± 9	−57 ± 4	88 ± 14	85 ± 10	89 ± 7	−59 ± 12	−56 ± 8	−58 ± 9	94 ± 7	96 ± 16	93 ± 14	−78 ± 7	−80 ± 11	−76 ± 15
BPZ	105 ± 13	109 ± 15	103 ± 14	17 ± 7	12 ± 4	16 ± 6	95 ± 2	100 ± 4	97 ± 7	16 ± 7	14 ± 8	15 ± 5	88 ± 8	91 ± 10	86 ± 2	10 ± 10	5 ± 5	10 ± 8

EE, extraction efficiency; **ME**, matrix effect; **LL**, low level (LOQ, see Table 1); **ML**, medium level (50 μg/kg); **HL**, high level (250 μg/kg).

that are less environmentally friendly (such as acetonitrile, toluene, or benzene), it is one of the shortest of those proposed (≈ 15 min), and it is also one of the simplest; hence, it can be considered economical in comparison with other proposals. Moreover, recovery values were satisfactory for all the analytes studied, and, more importantly, the matrix effect was not significant for most of BPs (eleven of fourteen), which is an important improvement with regard to previous studies in which MS or MS/MS detectors were employed (see Supplementary Material, **Table 1S**), since in these works either this parameter was not studied or it was significant for all the BPs studied. However, it should be emphasized that the primary distinction and significant novelty in this context lies in the fact that it can be applied to honey from different botanical origins, which are quite varied in their composition. To the best of our knowledge, this is the first time that such an application has been proposed or demonstrated.

3.2. Optimization of the UHPLC-MS/MS conditions

Optimization experiments were conducted with Kinetex EVO C_{18} 100 Å (2.1 × 50 mm, 1.7 μm), as C_{18} columns were commonly employed for determining BPs in honey, and it was the stationary phase selected in the only work in which UHPLC was employed (Zhou et al., 2019). Kinetex EVO C_{18} uses a patented organo-silica grafting process that incorporates uniform, stabilizing ethane cross-linking. This design provides resistance to high pH attack while maintaining the mechanical strength of the core shell particle. The result is higher peak sensitivity and shorter overall analysis time (Phenomenex, 2024). Firstly, the MS/MS conditions were optimized by injecting individual standards of the BPs with a concentration of 100 μg/L and varying the analyzer parameters (QqQ); meanwhile, ESI in negative mode was always employed as it was predominantly selected in previous works (see Supplementary Material, **Table 1S**). The conditions that provided the best signals are listed in **Table 2**. With these conditions, it was possible to obtain the precursor ions and the products ions of the BPs, which are detailed in **Table 2**, and that will be used in the MRM mode to carry out the quantification and identification of the compounds. It should be mentioned that the initial focus of optimization was on BPA, due to its greater importance. Then, several tests were carried out to evaluate the separation and signal intensity of the studied BPs by using different mixtures and gradient elution modes of organic and aqueous solvents as mobile phase components. Methanol and acetonitrile were tested as organic solvents due to their predominance in previous studies. No significant differences in peak shape or signal intensity were observed when using one or the other (data not shown), but on contrary, the use of methanol was preferred as it solved the co-elution problem related to BPP and BPM. Those compounds are position isomers and it was not possible to separate them with acetonitrile, but when decreasing the strength of the mobile phase (using methanol instead of acetonitrile), apart from a slight increase in the retention time of all the analytes, both compounds were separated without affecting the separation of the rest of BPs. Likewise, the effect of pH on the signal obtained was verified, changing the nature of the aqueous solvent of the mobile phase (ultrapure water, pH ≈ 7.0; 0.1% (*v*/v) formic acid in ultrapure water, pH ≈ 2.7; 0.01% (v/v); ammonia in ultrapure water, pH ≈ 11.0; ammonium

acetate 10 mM in ultrapure water, pH ≈ 7). The latter (ammonium acetate 10 mM) was the one that solved the issue of low BPS retention, although the ionization of the BPs was slightly worse than with ammonia 0.01%. An additional test was performed with 20 mM (v/v) of ammonium acetate to evaluate whether ionization improved, but no significant differences were found. Finally, it was decided to continue with 10 mM ammonium acetate in the following tests. Once the mobile phase components were selected (methanol and ammonium acetate 10 mM), several experiments were then conducted to test diverse mobile phase gradients, variable flow rates, temperatures and injection volumes. The aim was to elute all the compounds rapidly whilst preventing as far as possible co-elution between them and with potential matrix components. Under optimal chromatographic conditions (see Subsection 2.4 and Table 2), all compounds eluted in <11 min (see Fig. 1), achieving an overall run time of 15 min. This, according to the existing literature, and considering the significant influence of matrix and the number of compounds on separation, not only establishes this method as the fastest proposal to date, whether GC or HPLC, for analyzing five or more BPs in honey, but also represents the method that has simultaneously examined the largest number of BPs in this matrix (see Supplementary Material, **Table 1S**).

3.3. Method validation

We performed method validation according to current legislation (EURACHEM, 2014). In addition, several of the main elements of uncertainty (Konieczka & Namieśnik, 2010) were considered when optimizing and validating this method (amount of sample used; recovery value of the analytical procedure; precision, and repeatability). Validation was performed with blank honeys, standards in the solvent, and standards in matrix extracts obtained according to the selected sample treatment (see Subsection 2.3.2). The specific procedures for determining the different validation parameters are summarized in **Table 4S** (see Supplementary Material).

3.3.1. Selectivity

Selectivity was evaluated by comparing the chromatograms and mass spectra of standards in solvents and blanks of honey from the different botanical origins. No matrix interferences were observed at the analytes` retention times (see Figs. 1 and **2S, Supplementary Material**). Moreover, we obtained similar mass spectra for the standards of BPs in solvents and in the matrix extracts (data not shown).

3.3.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) are summarized in Table 1. They ranged from 0.2 to 1.5 μg/kg and from 0.5 to 4.7 μg/kg, respectively. It should be mentioned that no significant differences in values were observed between honeys of different botanical origins/colors. Those values are below to the SMLs established by legislation (European Commission, 2011, 2018) and are comparable to the best values obtained in previous publications (see Supplementary Material, **Table 1S**). These results demonstrate the excellent sensitivity of the proposed method.

3.3.3. Matrix effect

To ascertain how the matrix influenced the MS signal of the studied compounds, we compared the detector responses (analyte peak area/IS area) of standards in matrix extracts (R_{matrix}; AF samples) and standards in solvents ($R_{solvent}$) of the different botanical origins spiked at three different concentrations (low-LOQ (see Table 1); medium-50 μg/kg; and high-250 μg/kg). This was calculated as follows: Matrix effect (%) = $[(R_{matrix}/R_{solvent})\ -1] \times 100$. Analyte responses at the three levels assayed ranged for most of the BPs between −16% of signal suppression to +18% of signal enhancement (see Table 5), except for three of the studied compounds (BPE, BPF and BPS), for which the signal suppression was quite significant in all types of honeys (> 30%). These results were confirmed by comparing the slope confidence intervals (SCIs) between standards in solvent and standards in matrix extracts. In all cases, there was an overlapping of SCIs, except for the three indicated BPs (see Table 1). Consequently, it can be concluded that the matrix did not significantly affect the BPs signals except for the three previously mentioned compounds.

3.3.4. Linearity/working range

Standard solvent calibration curves could be used to quantify BPs in all the honeys, except BPE, BPF and BPS that should be quantified with the standard in matrix calibration curves, due to the significant matrix effect observed. However, since the calibration and quantification were done for all the analytes simultaneously, it was necessary to work in the presence of matrix. Calibration curves ($n = 6$) were constructed by plotting the signal on the *y*-axis (analyte peak area/IS area) against analyte concentration on the *x*-axis. Concentration of the analytical curves varied between LOQ and 500 μg/L (LOQ (see Table 1), 25, 50, 100, 150, 250, and 500 μg/L), which corresponds to those between LOQ and 250 μg/kg. The graphs obtained in all the calibration curves were straight lines, with the coefficient of the determination values (R^2) higher than 0.99 in all cases (see Table 1). Moreover, the deviation of back-calculation concentration from true concentration was lower than 20% (data not shown).

3.3.5. Precision

We conducted concurrent experiments to assess precision, expressed as the relative standard deviation (% RSD). This was achieved through repeated sample analyses using BF samples spiked at three different concentration levels: low-LOQ (see Table 1); medium-50 μg/kg; and high-250 μg/kg. These experiments took place either on the same day (repeatability) or over three consecutive days (partial reproducibility). %RSD values were consistently lower than 17% in all cases (see Supplementary Material, **Table 5S**).

3.3.6. Trueness

Trueness was evaluated through recovery experiments by comparing the results (analyte peak area/IS area) between BF samples and AF samples, which were obtained from blank samples spiked at three different concentrations (low-LOQ (see Table 1); medium-50 μg/kg; and high-250 μg/kg). Mean recoveries for the BPs studied ranged in all cases from 81% to 116%, with %RSD values lower than 17% (see Table 5). These recovery values are similar to or even better than those obtained in previous studies (see Supplementary Material, **Table 1S**).

3.4. Assessment of the applicability of the method

The blue applicability grade index (BAGI) was applied in order to evaluate the practicality and applicability of the employed analytical methodology (Manousi, Wojnowski, Płotka-Wasylka, & Samanidou, 2023). BAGI is a novel and simple to use index that can efficiently assess the applicability of an analytical method. BAGI is complementary to the green assessment tools, and it revolves around the “blue” principles of White Analytical Chemistry, which are mainly related to practical aspects. BAGI considers ten main attributes, such as the type of analysis, the number of analytes that are simultaneously determined, the required instrumentation, or the automation degree, to produce a pictogram and a score that depicts the applicability of an analytical method in terms of practicality. A sequential blue color scale is used to represent the final score, with colors like dark blue, blue, light blue, and white, which represent high, medium, low, and no compliance with the method’s practical criteria, respectively. Moreover, according to BAGI guidelines, it is recommended that the total score to be higher than 60, so that the analytical method can be considered practical (Manousi et al., 2023). Therefore, to calculate the BAGI of the proposed method, its main attributes must be considered. It involved a quantitative, confirmatory, and multi-element analysis of fourteen BPs by UHPLC-MS/MS, with a

miniaturized sample preparation (microextraction) allowing for complete analysis of 2–4 samples per hour. In addition, common and commercially available reagents were used. The method involved a minimal sample amount (1.0 g of honey per sample), a preconcentration step, and a semi-automated analysis using an UHPLC autosampler. Taking all these factors into account and the guidelines proposed by Manousi et al. (2023), the method achieved a score of 65 (see **Fig. 2**), surpassing the 60-point threshold, and demonstrating its applicability.

3.5. Application of the method

The validated method was applied for determining potential BPs residues in 30 honeys from three different botanical origins and colors (see Subsection 2.3.1). Analyses were performed in triplicate and IS was added to all samples at the same concentration (0.1 mg/L). Among the fourteen BPs investigated, residues of nine (BPA, BPAF, BPAP, BPBP, BPFL, BPM, BPP, BPPH, and BPS) were detected in twelve of the samples (see **Fig. 3**). It should be specified that in most of the samples the residues of BPs were below the LOQs/LODs, and only BPA was quantified in two of the samples (C9, 12.5 μg/kg; C11, 12.9 μg/kg), with concentrations lower than the established SML (50 μg/kg; European Commission, 2018). Analysis of the results indicates that BPS was the most frequently detected BP, found in 40% of the samples (12 out of 30), followed by BPA and BPFL, each detected in 20% of the samples (6 out of 30). With regard to the origin of the samples, it can be concluded that the majority of positive BPs (91%, 40 out of 44) were detected in the commercial samples, with residues of BPs found in 56% of positive commercial samples (9 out of 16). In contrast, the number of detected BPs in samples from experimental apiaries was much lower (9%, 4 out of 44 positive BPs), with residues found in only 21% of the analyzed samples (3 out of 14). On the other hand, when considering the material of the honey packaging, residues have been found in both plastic and glass containers without distinction, although it is true that the majority of containers were glass.

Upon comparing the obtained results with those of previous works (see Supplementary Material, **Table 1S**), it can be deduced that the results regarding the BPS content of commercial honeys are similar to those reported in some studies (<LOQ-35 μg/kg; Inoue et al., 2003; Khani et al., 2021; Lo Turco et al., 2016), while in other cases the values are much higher (<LOQ-997 μg/kg; Çesen et al., 2016; Meng et al., 2022; Notardonato et al., 2020a, 2020b; Peñalver et al., 2021). It should also be mentioned that in most of the previous studies, only BPA was analyzed or found, consistent with the results in our study. As can be seen, in other studies, the concentration values were higher than the SML in many cases and varied significantly between samples. However, these studies were unable to establish a direct relationship between the BPs content and the botanical origin or the type of packaging, as has happened in our study.

Fig. 2. BAGI index pictogram of the proposed analytical method.

Finally, it should be emphasized that in this work, residues of nine different BPs have been determined, which in most cases they were found in concentrations lower than the LOQs, representing the largest number of BPs detected in honey to our knowledge. This underscores the necessity of developing specific and sensitive methodologies for determining these compounds in honey. This statement is supported by the fact that several BPs, especially BPA, have been previously detected in honeys from different countries at variable concentration levels (see Supplementary Material, **Table 1S**).

4. Conclusions

A new analytical method, based on solvent microextraction in combination with UHPLC-MS/MS, has been developed and validated for the determination of fourteen BPs in honeys from different botanical origins. The method features an efficient, simple, fast, economical, and environmentally-compatible sample treatment, involving solvent microextraction with acetone and 1-hexanol. With this procedure, not only have excellent recovery percentages been achieved for all the compounds, but the matrix effect has also been minimized for the majority of them (eleven out of fourteen BPs). In this regard, it must be highlighted that to our knowledge, this is the first time that honey from different origins has been taken into account in the development of the sample treatment to determine BPs. In addition, the UHPLC-MS/MS conditions have been specifically developed for this study and cannot be compared with previous works, since it is the first time that such a large number of BPs have been determined simultaneously in honey. The proposed method has been validated, and the results showed that the analytical performance of the method was similar or even better in most cases than previous proposals. The LODs and LOQs obtained were lower than the SMLs established for two of the studied compounds in honey (BPA and BPS), and comparable with the best published values. The proposed method was applied to analyze several commercial and experimental honey samples. Residues of nine BPs were detected in several of the samples at (> LOD), but only BPA was quantified in two of the samples, with a concentration lower than the established SML. Finally, these results justify the hypothesis included in the Introduction concerning the need to develop selective and sensitive methods for determining BPs in honey.

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CRediT authorship contribution statement

Beatriz Martín-Gómez: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation. **Silvia Valverde:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation. **José Bernal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization. **Ana María Ares:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors of this manuscript declare no conflict of interest.

Data availability

The datasets generated during the current study are included in this published article, or they are available from the corresponding author

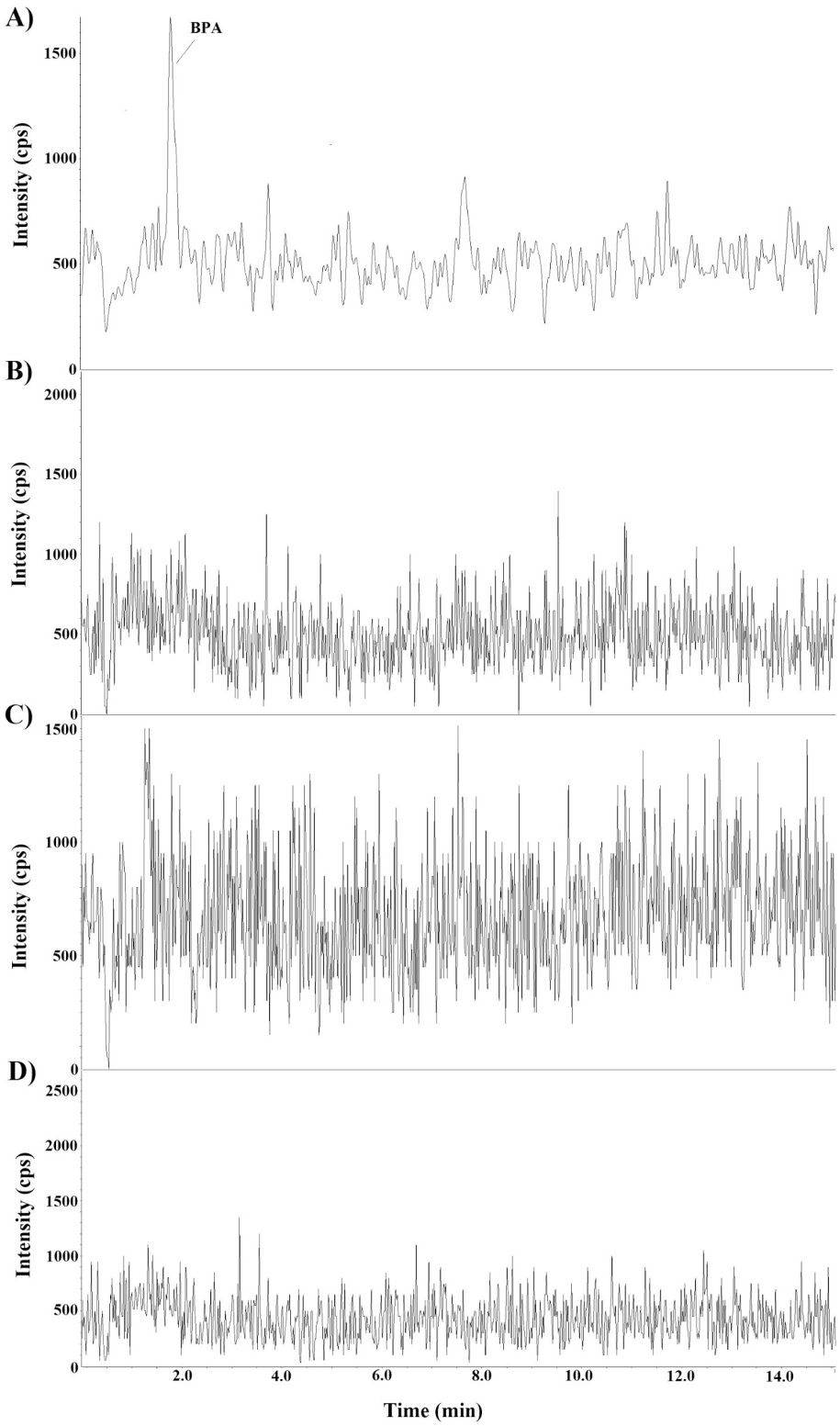

Fig. 3. Representative UHPLC-MS/MS chromatograms (MRM mode using the quantification transition; see Table 2) obtained from (**A**) honey sample with endogenous bisphenol A (BPA) content (C9; 12,5 μg/kg), (**B**) BPA-free multifloral honey sample (E6), (**C**) BPA-free rosemary honey sample (E3), and (**D**) BPA-free heather honey sample (E7). UHPLC-MS/MS conditions are summarized in Subsection 2.4 and Table 2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139358.

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