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Development and validation of a green analytical method for determining fourteen bisphenols in bee pollen by ultra-high-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

A new analytical method was developed and validated to determine fourteen bisphenols (A, B, C, E, F, M, P, S, Z, AF, AP, BP, FL, PH) in bee pollen using ultra-high-performance liquid chromatography-tandem mass spectrometry. Two different sample treatments were proposed and evaluated: one based on the QuEChERS (quick, easy, cheap, effective, rugged & safe) approach and the other utilizing microextraction with a supramolecular solvent (SUPRAS). In both cases, average analyte recovery ranged between 71 % and 114 %, and the matrix effect was between −45 % and +5 %, although it was not significant when using the QuEChERS-based method (<±20 %). The environmental impact of both sample treatments was assessed using different analytical metrics, with both procedures classified as environmentally friendly, though slightly better results were obtained for SUPRAS. The method was fully validated, showing that the QuEChERS approach had better overall performance, particularly regarding sensitivity and matrix effect. Consequently, the QuEChERS methodology was applied to determine bisphenols in thirty bee pollen samples from different Spanish regions. Residues of three bisphenols (M, P, and S) were detected, although only bisphenol S was quantified in several samples at low concentration levels (<7 μg kg^{-1}), which is below the established specific migration limit (SML; 50 μg kg^{-1}). However, regarding human health, the estimated daily intake, target hazard quotient, and hazard index assessed were higher than acceptable limits, suggesting a potential risk for human consumers.

1. Introduction

Bee pollen has proved to be a highly demanded food by society due to both its health-promoting effects (anticancer, anti-inflammatory and antioxidant) and its nutritional properties (Ares, Valverde, Bernal, Nozal, & Bernal, 2018). However, aside from being a functional food for human health, it is also essential for bee nutrition. Honeybees and beehive products, like bee pollen, have been shown to be bioindicators of numerous pollutants sources coming from agricultural and industrial activities such as pesticides, insecticides, and heavy metals (Cabrera de Oliveira, Queiroz, Pinto da Luz, Silveira Porto, & Rath, 2016; Fuente-Ballesteros et al., 2023; Valverde et al., 2018). In recent years, there has been a significant increase in plastic production, reaching approximately 400 million tons annually in 2022 (PlasticsEurope, 2023). Nevertheless, despite its usefulness and ubiquity, current patterns of plastic consumption and disposal are contributing to substantial pollution in the environment (Heidbreder, Bablok, Drews, & Menzel, 2019). One notable contributor to this pollution is the use of additives,

Abbreviations: AF, samples spiked after sample treatment; AGREE, analytical greenness calculator; BF, samples spiked before sample treatment; BP, bisphenol; 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; BW, body weight; CI, concentration intake; DAD, diode array detector; DI, dietary intake; EDI, estimated daily intake; EFSA, European Food Safety Authority; FLD, fluorescence detector; GAC, green analytical chemistry; GAPI, green analytical procedure index; HI, hazard index; HPLC, high-performance liquid chromatography; HQ, hazard quotient; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; *m/z*, mass-to-charge; MS, mass spectrometry; MS/MS, tandem mass spectrometry; QuEChERS, quick, easy, cheap, effective, rugged, and safe; R^2, determination coefficient; RfD, reference dose; RSD, relative standard deviation; SCI, slope confidence interval; SMLs, specific migration limits; SUPRAS, supramolecular solvents; t-TDI, temporary tolerable daily intake value; THQ, target hazard quotient; UHPLC, ultra-high-performance liquid chromatography; μ-SOA-MSPD, micro salting-out assisted matrix solid-phase dispersion.

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commonly known as plasticizers, aimed at enhancing the properties of plastics. One of the most employed plastic additives is bisphenol A (BPA), which belongs to the group of bisphenols that are phenolic compounds used both to produce polycarbonate and epoxy resins (Loganathan et al., 2023). BPA is considered as an endocrine disruptor with estrogenic activity even at low concentrations, meaning that BPA can bind estrogen receptors causing a disruption of hormonal activity, hence leading to negative health effects (Grignard, Lapenna, & Bremmer, 2012). However, BPA is currently being replaced by its less well-studied and regulated analogues. Some of them are bisphenol S (BPS), bisphenol F (BPF), bisphenol AP (BPAP), bisphenol AF (BPAF), bisphenol C (BPC), bisphenol E (BPE), bisphenol Z (BPZ), bisphenol B (BPB), bisfenol PH (BPPH), bisphenol BPFL (BPFL), bisphenol BP (BPBP), bisphenol P (BPP) and bisphenol M (BPM). The European Commission has established specific migration limits (SMLs) for bisphenol A (BPA) from varnishes or coatings into food at 0.05 mg kg^{-1} of food. Additionally, the use of BPA is prohibited in articles intended for infants and young children (European Commission, 2018). Currently, no SMLs have been set for BPA analogues, except for bisphenol S (BPS), which also has an SML of 0.05 mg kg^{-1} (European Commission, 2011). It is important to note that the existing legislation applies these SMLs uniformly across all types of food without differentiation. Given the high-fat content of bee pollen, there is a higher likelihood for these compounds to accumulate, increasing the potential exposure for consumers (Palsania, Singhal, Dar, & Kaushik, 2024).

To our knowledge, only one work dedicated to the analysis of BPs in pollen has been published (Zhang et al., 2021). In this work, an analytical method that consisted of a sample treatment based on micro salting-out assisted matrix solid-phase dispersion (μ-SOA-MSPD) followed by high-performance liquid chromatography (HPLC) was proposed for determining BPA and BPB in Chinese bee pollen samples. Current methods for determining BPs in food are predominantly based on chromatographic techniques such as HPLC or gas chromatography (GC), although HPLC is used more frequently than GC, as expected, to avoid the need for a derivatization step (Martín-Gómez, Elmore, Valverde, Ares, & Bernal, 2024). HPLC can be coupled with FLD and diode array detectors (DAD), 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 bee pollen, it is more convenient to use mass spectrometry (MS) and tandem mass spectrometry (MS/MS; Martínez-Gómez, Valverde, Bernal, & Ares, 2024) detectors. Regarding the stationary phase, C_{18}-based columns are commonly used for determining BPs in food (Martín-Gómez, Elmore, et al., 2024) including the only known study on this matrix (Zhang et al., 2021). In relation to the sample treatment and given the lack of specific approaches for determining BPs in bee pollen, except for the above-mentioned study, it was decided to evaluate the performance of some methodologies used in other fatty matrices (Luo et al., 2017), and the research group's experience to propose the most suitable procedure. One of these methods is QuEChERS (quick, easy, cheap, effective, rugged, and safe) due to its simplicity, speed, high sample throughput, and versatility in extracting both polar and non-polar compounds. Furthermore, it has exhibited satisfactory performance in extracting other contaminants in bee pollen (Fuente-Ballesteros, Augé, Bernal, & Ares, 2023). However, given the current trend in sample preparation techniques, which emphasizes simplification to reduce time, costs, and the number of reagents, the QuEChERS's performance was compared with that obtained using supramolecular solvents (SUPRAS). SUPRAS are considered as a promising alternative to conventional solvents/sample treatments, especially for its ability to significantly reduce phospholipid-based matrix effects (Salatti-Dorado, Caballero-Casero, Sicilia, Lunar, & Rubio, 2017).

The main goal of this study is to develop a method for the simultaneous determination of fourteen bisphenols (BPA, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPF, BPFL, BPM, BPP, BPPH, BPS, and BPZ) in bee pollen using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Additionally, the study aims to propose a sample treatment that is efficient, simple, cost-effective, and rapid. These conditions aim to ensure high recoveries, minimize potential matrix effects, and align with the principles of green analytical chemistry (Gałuszka, Migaszewski, & Namieśnik, 2013) by reducing time, cost, steps, and reagent use, and avoiding derivatization procedures. To achieve these goals, the study evaluates two sample treatment approaches: SUPRAS and QuEChERS. It also includes an assessment of the environmental impact of these treatments using green analytical metrics. To the best of our knowledge, this is the first study to develop and optimize a UHPLC-MS/MS method for analyzing many bisphenols in bee pollen. It represents the most comprehensive examination of bisphenols in this matrix to date. Further objectives include validating the method according to current European guidelines (EURACHEM, 2014), analyzing bee pollen samples from various Spanish regions, and evaluating potential risks to consumer health.

2. Materials and methods

2.1. Reagents and materials

BPs standards (BPA, BPA-d_{16}, BPAF, BPAP, BPB, BPBP, BPC, BPE, BPF, BPFL, BPM, BPP, BPPH, BPS, BPZ; see structures in Supplementary Material, Table 1S), all of analytical-grade and with purity greater than 98 %, were purchased from Sigma-Aldrich Chemie Gbmh (Steinheim, Germany). All solvents and reagents were of chromatographic/analytical grade and obtained from Scharlab S.L. (Barcelona, Spain) chloroform from Lab Scan Ltd. (Dublin, Ireland), acetone, methanol, ethanol, tetrahydrofuran and acetonitrile from Sigma-Aldrich Chemie Gbmh (Steinheim, Germany); 1-hexanol, 1-octanol, 1-dodecanol, formic acid, nitric acid, magnesium sulfate, sodium chloride, ammonium acetate from Panreac (Barcelona, Spain), primary secondary amine (PSA), graphitized carbon black (GCB), and C_{18} were provided by Supelco (Bellefonte, CA, USA). 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 vibromatic mechanical shaker, and a drying oven, all supplied by J.P. Selecta S.A. (Barcelona, Spain), IKA® Ultra-Turrax® T18 basic disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany), a 5810 R refrigerated bench-top centrifuge from Eppendorf (Hamburg, Germany), a R-3 rotary evaporator from Buchi (Flawil, Switzerland), and Nylon syringe filters (13 mm, 0.22 μm; Branchia, Barcelona, Spain) were employed for sample treatment.

To prevent cross-contamination since BPs are commonly present in reagents, materials, and laboratory equipment, a meticulous cleaning protocol was required (Martínez-Gómez et al., 2024). Initially, glassware underwent soaking in ultrapure water, followed by washing with 0.2 mol L^{-1} nitric acid and sonication for 20 min. Afterwards, the material was rinsed with the following solutions: **i**) ultrapure water, and the ultrasonic process was repeated; **ii**) it was cleaned with an acetone and methanol (1:1, v/v) mixture; **iii**) finally, it was dried at 100 °C for 1 h and covered with aluminum foil. All materials were previously tested before each analysis. Procedural blanks, in which ultrapure water substituted bee pollen, were run between sets of samples to monitor potential abnormal background values.

2.2. Standards

Individual standard stock solutions for each bisphenol and the internal standard (BPA-d_{16}) were prepared at a concentration of 1000 mg L^{-1} in methanol. These stock solutions were then combined and diluted with a 50:50 (v/v) mixture of deionized water and methanol to prepare an intermediate mixed solution and the working solutions. Blank (analyte-free; see Section 2.3.1) bee pollen samples, were either spiked before (BF samples) or after (AF samples) sample treatment with varying amounts of the BPs and 100 μg L^{-1} of the IS (BPA-d_{16}) to prepare the

matrix-matched standards. 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, which were injected three times, were prepared for all the above-mentioned studies. Each spiked sample was prepared by spiking bee pollen product with three different concentrations of BPs within the linear range. These concentrations were as follows: low–limit of quantification (LOQ) μg kg^{-1} (from 1.0 to 23 μg kg^{-1}; see Table 1); medium–50 μg kg^{-1}; high–250 μg kg^{-1}. The stock solutions and matrix-matched solutions were stored in opaque glass containers at − 20 °C and 4 °C, respectively. All solutions demonstrated stability for a period exceeding two weeks.

2.3. Sample procurement and treatment

2.3.1. Samples

Several bee pollen samples (n = 30) were selected according to their botanical origin, food packaging and composition. Half of the samples were kindly donated by the Center for Agroenvironmetal and Apicultural Investigation (CIAPA; Marchamalo, Guadalajara, Spain), and correspond to samples labeled with the letter E (E1-E15). The rest of the samples were acquired from commercial stores from different geographical areas of Spain labeled with the letter C (C1-C15). Their botanical origin was confirmed by palynological analysis at CIAPA (Ares et al., 2022; see Supplementary Material, Table 2S). All the samples were dried at 45 °C in an oven, individually ground in a mill, and subsequently stored in a vacuum desiccator before analysis. In the present study, all bee pollen samples were examined in triplicate and underwent a preliminary analysis by UHPLC–MS/MS to check for the presence of BPs. Once absence was confirmed in the samples (blank or analyte-free), different subsamples were generated and used to prepare matrix-matched standards for validation and sample treatment studies.

2.3.2. Sample treatment

2.3.2.1. SUPRAS. Briefly, 1 g ± 0.1 mg of ground and dried bee pollen was weighed into a 10 mL glass tube. Then, 4 mL of tetrahydrofuran, 1-hexanol and water (30:5:65, v/v/v) mixture, and 50 mg of ammonium acetate were added. The mixture was vigorously shaken for 30 s using a vortex device, and then centrifuged for 5 min (7000 *g*, 10 °C). The SUPRAS phase was collected using a syringe and evaporated to dryness under a nitrogen stream. The resulting dry residue was reconstituted with 500 μL of an internal standard (IS) solution (100 μg L^{-1}), and subsequently filtered through a nylon 0.22-μm filter into a vial for UHPLC-MS/MS analysis. Fig. 1A summarizes the steps of the selected sample treatment.

2.3.2.2. QuEChERS. Briefly, 1 g ± 0.1 mg of ground and dried bee pollen was weighed in a 10 mL glass centrifuge tube, and 8 mL of an acetonitrile and water (70:30, v/v) mixture, 150 mg of ammonium acetate and 150 mg of anhydrous magnesium sulfate were added. The mixture was mechanically shaken for 5 min (950 oscillations min^{-1}) in a vibromatic, and then centrifuged for 5 min (7000 *g*, 10 °C). The supernatant was collected and transferred to another glass tube containing 50 mg of PSA. The tube was shaken and centrifuged using the same conditions described above. Then, supernatant was collected evaporated to dryness at 60 °C in a rotary evaporator. Finally, the dry residue was reconstituted with 1 mL of IS solution (100 μg L^{-1}) and it was filtered through a nylon 0.22 μm filter into a vial for UHPLC-MS/MS analysis. Fig. 1B summarizes the steps of the selected sample treatment.

2.4. UHPLC-MS/MS conditions

Analyses were carried out using an UHPLC Sciex Exion system connected to a Sciex 6500 + triple-quadrupole mass spectrometer from Sciex (Washington, DC, USA) equipped with an electrospray ionization (ESI) source, which was used in negative mode. UHPLC-MS/MS conditions were optimized in a previous and recent work (Martínez-Gómez, Valverde, et al., 2024). 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^{-1} 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). Temperature and injection volume was set at 30 °C and 3 μL, respectively. With such conditions, the overall run time was 15 min (see Fig. 2). MS/MS conditions were optimized by flow injection analysis in infusion mode of each BP standard at 20 μL min^{-1}. 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. 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). Two transitions were selected for each target analyte to enhance the specificity and reliability of the analysis, and to minimize the risk of false positives. Dwell times were automatically chosen to ensure an adequate number of data points for each peak. SciexOS software was employed for data acquisition and evaluation

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

Compounds	Standard in solvent		SUPRAS				QuEChERS				SML
	SCI	R^2	SCI	R^2	LOD	LOQ	SCI	R^2	LOD	LOQ	(μg/kg)
BPA	0.16 ± 0.01	0.992	0.10 ± 0.01	0.995	6.0	19.0	0.16 ± 0.01	0.996	4.2	14.0	50
BPAF	1.90 ± 0.02	0.993	1.87 ± 0.03	0.997	3.0	9.5	1.93 ± 0.04	0.997	0.9	2.9	NS
BPAP	1.20 ± 0.34	0.997	1.08 ± 0.25	0.999	2.4	7.1	1.14 ± 0.30	0.999	0.3	1.0	NS
BPB	0.17 ± 0.03	0.994	0.15 ± 0.02	0.991	2.3	7.8	0.18 ± 0.02	0.993	1.4	4.8	NS
BPBP	0.82 ± 0.12	0.993	0.76 ± 0.10	0.996	2.6	7.0	0.80 ± 0.09	0.994	0.8	2.7	NS
BPC	0.01 ± 0.002	0.994	0.01 ± 0.001	0.997	6.8	22.4	0.01 ± 0.002	0.997	4.0	13.3	NS
BPE	0.25 ± 0.03	0.994	0.16 ± 0.02	0.992	1.9	6.3	0.21 ± 0.03	0.994	0.9	2.8	NS
BPF	0.05 ± 0.01	0.999	0.03 ± 0.01	0.991	4.5	15.0	0.04 ± 0.01	0.993	3.1	10.4	NS
BPFL	2.02 ± 0.15	0.999	1.92 ± 0.25	0.996	4.7	16.0	1.95 ± 0.31	0.992	2.2	7.3	NS
BPM	0.42 ± 0.01	0.999	0.27 ± 0.01	0.997	2.0	6.7	0.43 ± 0.01	0.999	1.2	3.9	NS
BPP	0.44 ± 0.07	0.995	0.43 ± 0.06	0.996	1.5	4.8	0.43 ± 0.10	0.997	0.8	2.8	NS
BPPH	0.37 ± 0.03	0.991	0.24 ± 0.02	0.993	1.9	6.2	0.33 ± 0.04	0.995	1.5	5.0	NS
BPS	5.10 ± 0.60	0.998	3.1 ± 0.30	0.997	0.6	2.1	4.50 ± 0.30	0.999	0.4	1.2	50
BPZ	0.38 ± 0.02	0.999	0.36 ± 0.02	0.996	4.0	13.0	0.37 ± 0.01	0.991	2.8	9.4	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.

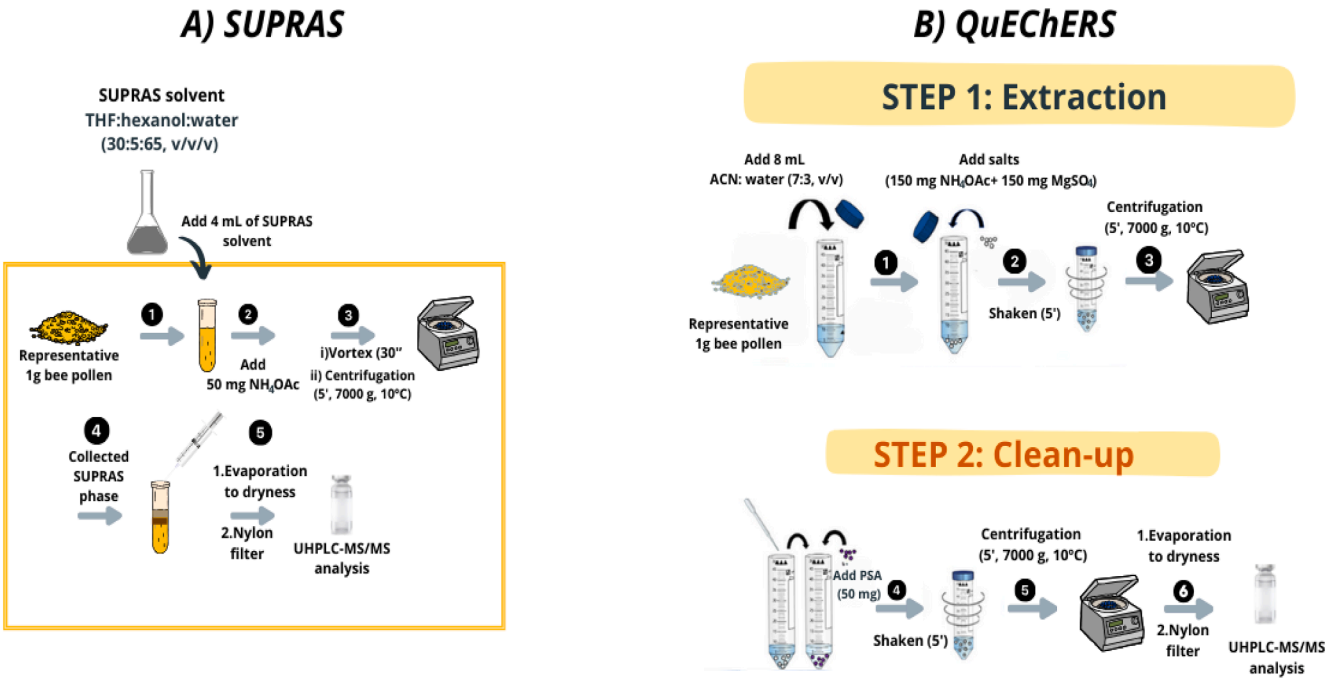


Fig. 1. Schemes of the proposed analytical procedures: (**A**) SUPRAS and (**B**) QuEChERS.

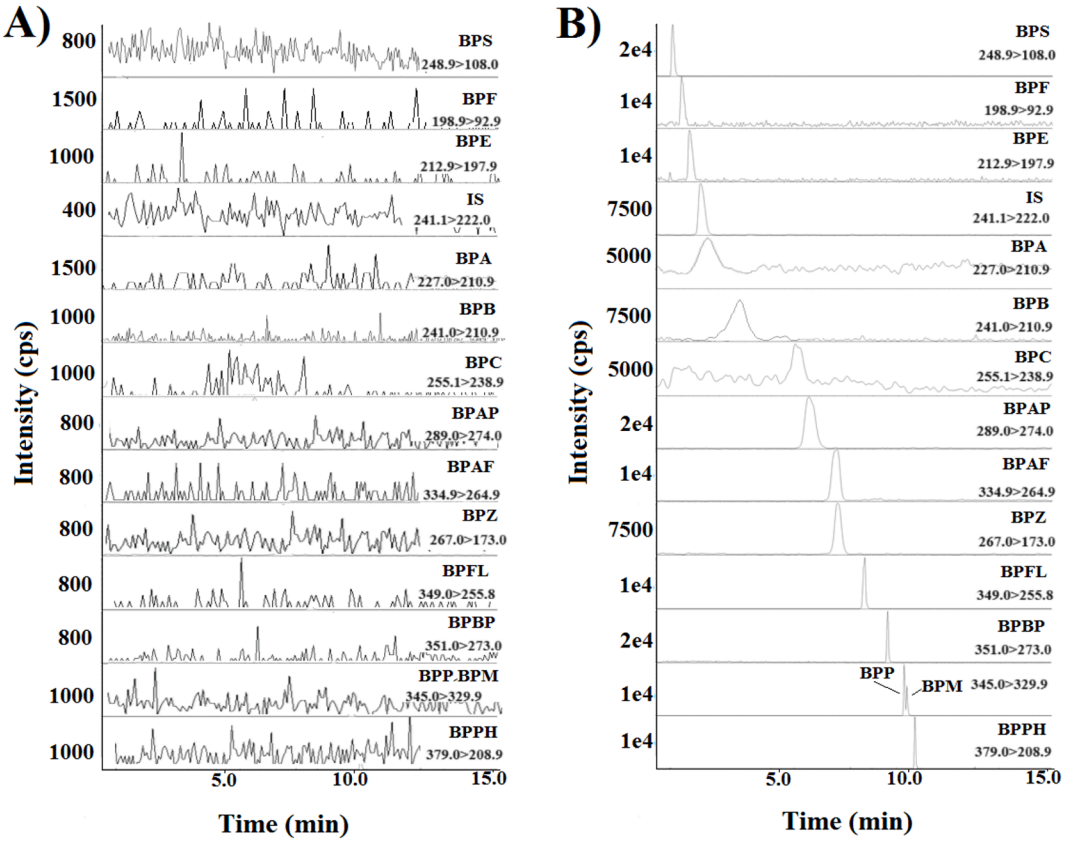


Fig. 2. Representative UHPLC-MS/MS chromatograms (MRM mode using the quantification transitions; see Table 2) obtained from (**A**) blank (analyte free) bee pollen sample, and (**B**) blank bee pollen sample spiked with the selected BPs at medium level (50 μg kg^{-1}) level. Bee pollen samples were treated with the proposed QuEChERS procedure. (Note: intensity of detected ions represented in cps, counts per second).

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

Compound	Q1	Q3	Dwell time (ms)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
BPA	227.0	210.9[Q]	20	−60	−10	−24	−18
	227.0	133.0[C]	20	−40	−10	−34	−15
	227.0	117.0[C]	20	−40	−10	−64	−13
IS (BPA-d_{16})	241.1	222.0[Q]	20	−45	−10	−28	−11
	241.1	141.9[C]	20	−45	−10	−40	−21
	241.1	97.9[C]	20	−45	−10	−60	−5
BPAF	334.9	264.9[Q]	20	−185	−10	−28	−18
	334.9	196.9[C]	20	−40	−10	−52	−11
	334.9	176.8[C]	20	−40	−10	−62	−11
BPAP	289.0	274.0[Q]	20	−95	−10	−32	−27
	289.0	211.0[C]	20	−80	−10	−36	−13
	289.0	195.1[C]	20	−80	−10	−36	−11
BPB	241.0	210.9[Q]	20	−15	−6	−40	−8
	241.0	224.8[C]	20	−45	−10	−24	−21
	241.0	147.0[C]	20	−45	−10	−34	−7
BPBP	351.0	273.0[Q]	20	−60	−10	−36	−9
	351.0	283.1[C]	20	−70	−10	−28	−9
	351.0	253.3[C]	20	−70	−10	−36	−5
BPC	255.1	238.9[Q]	20	−30	−10	−15	−7
	255.1	223.0[C]	20	−55	−10	−28	−7
	255.1	147.0[C]	20	−55	−10	−18	−13
BPE	212.9	197.9[Q]	20	−55	−10	−24	−17
	212.9	118.8[C]	20	−45	−10	−32	−13
	212.9	92.9[C]	20	−45	−10	−30	−11
BPF	198.9	92.9[Q]	20	−80	−10	−30	−11
	198.9	104.9[Q]	20	−60	−10	−30	−9
	198.9	77.0[C]	20	−60	−10	−32	−9
BPFL	349.0	255.8[Q]	20	−55	−10	−36	−31
	349.0	79.9[C]	20	−55	−10	−78	−37
	349.0	281.2[C]	20	−55	−10	−26	−17
BPM	345.0	329.9[Q]	12	−150	−10	−38	−21
	345.0	251.6[C]	12	−150	−10	−38	−21
	345.0	132.9[C]	12	−150	−10	−48	−17
BPP	345.0	329.9[Q]	20	−30	−13	−60	−25
	345.0	315.0[C]	20	−135	−10	−38	−13
	345.0	133.0[C]	20	−135	−10	−48	−9
BPPH	379.0	208.9[Q]	12	−120	−10	−48	−17
	379.0	364.0[C]	12	−120	−10	−34	−15
	379.0	192.9[C]	12	−120	−10	−90	−11
BPS	248.9	108.0[Q]	20	−90	−10	−36	−10
	248.9	92.0[C]	20	−65	−10	−50	−11
	248.9	155.9[C]	20	−65	−10	−30	−9
BPZ	267.0	173.0[Q]	20	−60	−10	−36	−20
	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; EP. entrance potential; Q_n. Mass of pseudo-ion "n".

3. Results and discussion

3.1. Optimization of sample treatment

3.1.1. SUPRAS

Firstly, the use of SUPRAS was examined, as it can be considered an interesting non-conventional solvent extraction to be used as an alternative to organic solvents, such as acetonitrile that was used in the only work where BPs have been determined in bee pollen (Zhang et al., 2021). SUPRAS offers advantages such as high enrichment factors, low solvent consumption, and rapid extraction. Furthermore, recent SUPRAS-based studies have been published reporting satisfactory results in terms of extraction efficiency and the influence of matrix effects when analyzing BPs and other plasticizers in other matrices (Ballesteros-Gómez, Ballesteros, & Rubio, 2024; Dueñas-Mas, Ballesteros-Gómez, & Rubio, 2022). The formation of SUPRAS involves two consecutive steps. Initially, three-dimensional aggregates, typically micelles or vesicles, spontaneously form through the self-assembly of amphiphilic compounds upon reaching a critical micellar/vesicular concentration. Subsequently, these aggregates suffer an expansion by the influence of a coacervating agent, such as a pH or temperature change, addition of a salt or a poor solvent for the amphiphile (Dueñas-Mas, de Dios-Pérez, Ballesteros-Gómez, & Rubio, 2023). This process results in the formation of three phases during extraction, the solid residue, the equilibrium solution and the creation of a new liquid phase enrichment denominated SUPRAS. SUPRAS solutions were previously synthesized from ternary mixtures of 10 mL containing amphiphile (5 % v/v), organic solvent (10–30 %, v/v) and water (60–85 %, v/v; Alabi, Caballero-Casero, & Rubio, 2014). Considering the proportions used in other studies, the initial SUPRAS volume was set at 2 mL and the sample amount was fixed at 50 mg. Then, the obtained solutions were shaken for 30 s in a vortex device, and centrifuged (5 min, 7000 *g*, 10 °C) to accelerate phase separation. Several amphiphiles substances (1-hexanol, 1-octanol and 1-decanol) and two organic solvents (aprotic-tetrahydrofuran; protic-ethanol) were tested, and ultra-pure water was selected as coacervating agent (see the synthesized SUPRAS solutions in Table 3S, Supplementary Material). In general, better results with higher recovery percentages were achieved using tetrahydrofuran as organic solvent (data not shown). It was also observed that a great dependence of the formation of the SUPRAS phase with the tetrahydrofuran and water ratio in the different solutions tested. Regarding the amphiphilic substance, a general trend was observed, as the hydrocarbon chain length increased, more turbid and dense phases were obtained. This phenomenon may be attributed to the co-extraction of lipids, as bee pollen could reach up to 15 % w/w of its content (Ares et al., 2018). The best results were achieved with 1-hexanol and tetrahydrofuran, where the formation of the SUPRAS phase was observed. This finding is in good concordance with those reported in other studies, predominating amphiphile-amphiphile interactions over amphiphile-solvent interactions (Caballero-Casero & Rubio, 2021). Once the composition of the SUPRAS phase was selected (THF 30 %, 1-hexanol 5 %, water 65 %; v/v), the next steps involved the selection of the SUPRAS volume (0.5–5.0 mL) and the amount of the sample (50–2000 mg). After performing several tests, 1000 mg and 4 mL of SUPRAS solvent were deemed as optimal values in terms of extraction recovery (55–70 % for all BPs), although a significant matrix effect (signal suppression) was observed for most compounds (data not shown). Then, to improve the coacervation process and thereby improve the extraction efficiency of BPs, the effect pH adjustment and salt addition were evaluated. Considering that the pKa values of the BPs are comprised between 9 and 11 (Chen et al., 2022), experiments were conducted at acidic pH values (2 and 3), but in both cases, it was observed that SUPRAS phase could not be clearly distinguished. Consequently, it was concluded that controlling pH did not improve the SUPRAS procedure. Thus, the effect of salt addition was subsequently investigated by using different salts (50 mg) like sodium acetate, sodium chloride and magnesium sulfate. The recovery efficiency increased in all cases, being ammonium acetate the salt that provided the highest recovery percentages (65–110 %) for all BPs with an acceptable matrix effect (see Supplementary Material, Fig. 1S). Next, the amount of ammonium acetate (25, 50, 100 y 150 mg) was optimized. It was observed that when the minimum amount of salt was used (25 mg), there was no improvement in the recovery efficiency. On the contrary, when higher quantities were tested (50–150 mg), quite similar results were obtained in terms of recovery percentages and matrix effect (data not shown). Therefore, the lowest quantity (50 mg) was selected to continue the optimization process. The influence of extraction (30–90 s) and centrifugation times (3–10 min) was also investigated. The results showed that the best recovery percentages (>70 %) were obtained with 30 s of vortex agitation, followed by 5 min of centrifugation. Finally, the SUPRAS phase (~80 μL) was collected with a syringe and evaporated to dryness under a nitrogen stream. Several methanol and water mixtures (50:50, 70:30, 80:20; 100:0 v/v) and volumes (100–1000 μL) were tested to dissolve the dry extract. It was observed that 500 μL of a 50:50 v/v mixture provided the best results in terms of extraction efficiency and matrix effect (data not shown).

3.1.2. QuEChERS

The first step of the optimization procedure was devoted to select the amount of bee pollen (0.5–2 g). Several tests were conducted to optimize the extraction process. Initially, it was determined that 1.0 g of bee pollen was the maximum amount needed to achieve optimal signal-to-noise ratios, which provided the best sensitivity (data not shown). Next, different solvent mixtures of acetonitrile and water (60:40, 70:30, 80:20, 90:10 v/v) were evaluated based on existing literature (Martín-Gómez, Elmore, et al., 2024). The best extraction efficiency was obtained with a mixture of acetonitrile and water 70:30 (v/v). Following this, different volumes (4–10 mL) of the selected solvent mixture were tested, with 8 mL being identified as the optimal volume. Additionally, the effect of various salts (sodium chloride, magnesium sulfate, ammonium acetate) on the partitioning step of the QuEChERS procedure was investigated. The highest recovery percentages (>70 %) were achieved when employing 150 mg of magnesium sulfate and 150 mg of ammonium acetate. Once the solvents and the salts were chosen, the influence of various extraction parameters was also examined. This involved the influence of agitation source (vibromatic, ultrasound and Ultra-Turrax®), extraction time (3–15 min), and centrifugation time (3–15 min), which were sequentially tested. Optimal extraction conditions were achieved with 5 min of mechanical agitation (vibromatic), and 5 min of centrifugation (7000 *g*, 10 °C). Then, the feasibility of a clean-up step to remove co-extracted interfering substances and minimize the matrix effect was evaluated. The supernatant was collected and transferred to a glass tube, in which PSA, C_{18}, GCB, and a mixture of them in combination with magnesium sulfate were added in different experiments with the aim of removing sugars, fatty acids (PSA) and non-polar compounds (C_{18}, GCB). Different amounts of sorbents were also tested (50–500 mg), and it was observed that 50 mg PSA provided the optimum efficiency in terms of minimizing matrix effect (<±20 %) without affecting the extraction efficiency (recovery percentages higher than 70 %; see Table 4S). The other evaluated sorbents induced a significant loss of some analytes, especially in the case of GCB. It should be mentioned that the shaking and centrifugation conditions for the clean-up step were the same as those used for the extraction stage. The supernatant was directly transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 60 °C. Different volumes (0.5–2.0 mL) of methanol and water (90:10, 80:20, 70:30, 60:40, 50:50, v/v) mixtures were tested to dissolve the dry extract. It was observed that 1 mL of a 70:30 (v/v) mixture provided the best results in terms of extraction efficiency and matrix effect (data not shown).

3.1.3. Comparison of the proposed sample treatments

In order to check the effectiveness of the proposed sample treatments, BPs responses (analyte peak area/IS area) obtained from blank

samples spiked at three different concentrations (low-LOQ (see Table 1); medium-50 μg kg^{-1}; and high-250 μg kg^{-1}) were compared, both prior to (BF samples) or following (AF samples) sample treatment were compared. The extraction efficiency ranged from 75 % to 114 % using the QuEChERS approach; while these values were slightly lower when using SUPRAS solvent between 71 % and 103 % (see Table 3). Regarding the evaluation of the matrix effect, calculated as outlined in Section 3.3.3, a significant signal suppression was observed for six BPs (BPA, BPE, BPF, BPM, BPPH and BPS) at the three spiked concentration when employing the SUPRAS approach (see Table 3). This result implies that standard matrix calibration curves should be required for quantifying these compounds by using SUPRAS. In contrast, the QuEChERS approach provided suitable matrix effect values for all studied BPs and spiking levels, indicating that standard in solvent calibration curves could be used to quantify BPs in bee pollen. On the other hand, it must also be considered that the method based on SUPRAS is faster, has fewer steps, and requires less solvent consumption, although due to the instrumentation used, fewer samples can be treated simultaneously. Finally, if we compare the proposed sample treatments (SUPRAS and QuEChERS) with the only existing one (μ-SOA-MSPD; Zhang et al., 2021), it is observed that the performances in terms of recovery percentages are very similar in all cases, although they were slightly better for the QuEChERS. It should be remarked that the effect of the matrix on the ionization of the BPs was not studied in the previous method. On the other hand, the analysis time, simplicity, and consumption of reagents is very similar for the SUPRAS and μ-SOA-MSPD) methodologies, while as previously mentioned, the QuEChERS method has more stages and reagents consumption, although it has the advantage that it minimizes the matrix effect. Thus, it can be concluded that both procedures can be considered as promising alternatives when determining BPs in bee pollen, with the QuEChERS methodology presenting better efficiency when extracting the compounds and avoiding the matrix effect, while the SUPRAS methodology is faster, simpler, and requires less reagent consumption.

3.2. Assessment of the proposed sample treatments sustainability

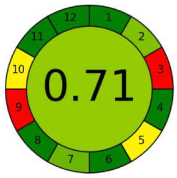
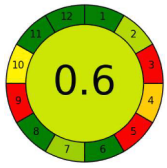
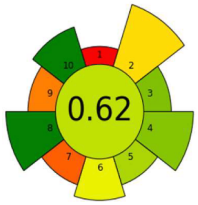
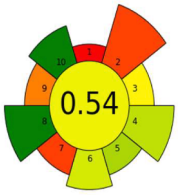
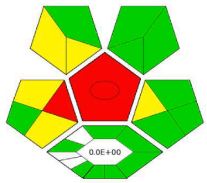
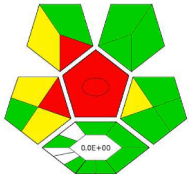
Three commonly employed tools (analytical greenness calculator (AGREE), Pena-Pereira, Wojnowski, & Tobiszewski, 2020; analytical GREEnness (AGREEPrep) metric, Pena-Pereira, Tobiszewski, Wojnowski, & Psillakis, 2022; complex green analytical procedure index (ComplexGAPI), Płotka-Wasylka & Wojnowski, 2021) were used to assess the sustainability of the proposed analytical approaches based on QuEChERS and SUPRAS. AGREE is based on the twelve principles of green analytical chemistry (GAC; Gałuszka et al., 2013), providing a numerical score ranging from 0 to 1. The results are represented in a pictogram on a red-yellow-green color scale, with a method considered "green" when the score is greater than 0.6. AGREEprep is based on ten green sample preparation (GSP) principles (López-Lorente et al., 2022). The results are also based on a numerical score (0–1) and are presented as a pictogram using the same color scales as in AGREE. The main difference between AGREE and AGREEprep lies in the fact that the former evaluates the entire analytical procedure, whereas AGREEprep focuses on sample preparation. The ComplexGAPI metric provides a comprehensive overview for the entire method considers a range of factors, encompassing the final product, reactants/solvents, alignment with a sustainable economic framework, instrumentation, post processing, and purification steps. The results are visualized through hexagons with a color-coded system. Red signifies high environmental concern, yellow indicates a moderate level of concern, and green represents minimal concern. Comparison of the greenness profile of both methodologies is summarized in Table 4. The pictograms obtained in AGREE yielded a central score of 0.71 for SUPRAS, categorizing it as "green". Meanwhile, for QuEChERS methodology, it was on the threshold with a value of 0.60. In both methodologies, the use of UHPLC-MS/MS as an analytical technique and the location of the analytical device (points 5 and 9) have been penalized. In the case of the QuEChERS methodology, there is a penalty for the non-miniaturized sample treatment, even though only 8 mL of solvent is used. In the case of AGREEPrep, slightly lower results were obtained for both methodologies. The weaknesses or areas more penalized were the lack of automation of the sample treatment and the use of UHPLC as the analysis technique. In the case of the QuEChERS methodology, the solvent volume used was also notably penalized. The ComplexGAPI provides a broader perspective. In the SUPRAS case, two red segments were obtained, while in QuEChERS, three were identified. Once again, weaknesses were linked to the instrumentation employed. This metric penalized the requirement for sample treatment, and in the case of QuEChERS, the lack of miniaturization was also noted. The matrix under study is highly complex, and the analytes are typically present at trace levels, requiring a sample treatment approach to achieve these objectives. To attain the desired sensitivity, the UHPLC-MS/MS technique is required. Furthermore, none of the available tools address the energy consumption of UHPLC-MS/MS, for which these techniques are assigned with 1.5 or >1.5 kWh energy utilization per sample. Finally, we decided not to include the previous work (Zhang et al., 2021) in this section, since the comparison would not be carried out under equal conditions. The number of studied compounds was much lower, and the technique used for the analysis (HPLC-FLD) is good enough to determine the compounds and more environmentally friendly than UHPLC-MS/MS, but it did not provide an adequate sensitivity

Table 3
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	SUPRAS						QuEChERS					
	EE			ME			EE			ME		
	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL
BPA	81 ± 10	85 ± 8	82 ± 11	−39 ± 9	−33 ± 5	−36 ± 3	107 ± 6	105 ± 2	104 ± 3	1 ± 8	5 ± 11	2 ± 9
BPAF	92 ± 6	91 ± 9	87 ± 5	0 ± 11	+3 ± 8	−5 ± 9	97 ± 5	92 ± 5	94 ± 4	−6 ± 6	0 ± 7	+5 ± 6
BPAP	92 ± 13	89 ± 9	85 ± 8	−7 ± 10	−10 ± 7	−12 ± 6	90 ± 2	94 ± 7	86 ± 7	4 ± 6	−6 ± 3	−2 ± 3
BPB	82 ± 6	81 ± 2	85 ± 4	−12 ± 4	−13 ± 3	−12 ± 6	101 ± 8	98 ± 7	95 ± 6	+6 ± 4	−2 ± 3	+4 ± 3
BPBP	100 ± 8	96 ± 9	99 ± 8	−1 ± 7	−8 ± 4	−9 ± 4	96 ± 3	101 ± 5	96 ± 4	+1 ± 6	−5 ± 8	−7 ± 9
BPC	81 ± 9	86 ± 10	83 ± 7	−10 ± 9	−15 ± 4	−17 ± 3	92 ± 3	93 ± 2	91 ± 4	−13 ± 4	−9 ± 2	−8 ± 4
BPE	86 ± 3	84 ± 2	82 ± 5	−37 ± 9	−36 ± 5	−31 ± 8	79 ± 2	76 ± 4	78 ± 5	−18 ± 4	−16 ± 6	−17 ± 5
BPF	96 ± 6	90 ± 5	91 ± 2	−40 ± 5	−43 ± 4	−37 ± 6	78 ± 8	86 ± 7	80 ± 7	−15 ± 7	−17 ± 8	−13 ± 7
BPFL	103 ± 12	97 ± 9	96 ± 10	−4 ± 6	−8 ± 7	−11 ± 9	99 ± 11	103 ± 6	97 ± 7	−4 ± 8	−8 ± 7	−11 ± 4
BPM	87 ± 10	91 ± 7	89 ± 9	−24 ± 9	−21 ± 6	−26 ± 8	114 ± 3	106 ± 4	109 ± 6	−5 ± 7	−1 ± 5	−7 ± 8
BPP	84 ± 11	89 ± 7	85 ± 5	−5 ± 9	−8 ± 7	−7 ± 6	95 ± 3	96 ± 5	91 ± 7	−1 ± 5	+2 ± 4	−4 ± 6
BPPH	77 ± 9	82 ± 5	75 ± 7	−39 ± 5	−38 ± 6	−36 ± 8	103 ± 8	99 ± 4	102 ± 8	−8 ± 8	−13 ± 7	−15 ± 9
BPS	71 ± 6	77 ± 2	72 ± 2	−45 ± 6	−38 ± 7	−40 ± 6	79 ± 5	75 ± 7	77 ± 7	−18 ± 6	−14 ± 7	−16 ± 8
BPZ	80 ± 10	83 ± 9	81 ± 6	−1 ± 8	−3 ± 6	−4 ± 9	96 ± 4	92 ± 4	97 ± 5	−6 ± 7	−2 ± 8	−8 ± 5

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

Table 4
Assessment of the proposed sample treatments sustainability using analytical greenness calculator (AGREE), analytical GREEnness (AGREEPrep) metric, and complex green analytical procedure index (ComplexGAPI).

Metric tool	SUPRAS	QuEChERS
AGREE		
AGREEPrep		
ComplexGAPI		

according to the established SMLs.

3.3. Validation of the methods

Validation was performed according to EURACHEM guideline (EURACHEM, 2014). The specific procedures for determining the different validation parameters are summarized in Table 5S.

3.3.1. Selectivity

Selectivity was evaluated by comparing the chromatograms and mass spectra of standards in solvents and blanks of bee pollen. No matrix interferences were observed at the analytes' retention times (see Fig. 2). 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) were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively, and they are summarized in Table 1. As can be seen, lower LODs and LOQs were obtained with QuEChERS methodology (0.3–14.0 μg kg^{-1}) compared to the SUPRAS approach (0.6–22.4 μg kg^{-1}). Those values are much lower than the SMLs established by legislation for BPA and BPS (50 μg kg^{-1}), and that the LODs and LOQs obtained in the previous work (20–80 μg kg^{-1}; Zhang et al., 2021), which demonstrates 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 bee pollen samples spiked at three different concentrations. It was calculated using the following formula: Matrix effect (%) = [(Rmatrix/Rsolvent) − 1] × 100. Results are summarized in Table 3 for both methodologies. Analyte responses at the three levels assayed ranged between −18 % of signal suppression to +5 % of signal enhancement when using the QuEChERS-based method, while a more significant matrix effect was observed for several of the studied BPs with the SUPRAS approach (BPA, BPE, BPF, BPM, BPPH and BPS), for which the signal suppression was quite significant (>20 %; −45 % to +3 %). These results were confirmed by comparing the slope confidence intervals (SCIs) between standards in solvent and standards in matrix extracts (see Table 1). When the QuEChERS methodology was used, an overlap of the SCIs was observed for all BPS, while there were several cases when the SUPRAS methodology was used in which there was no overlap, and which coincided with the compounds already mentioned for which a significant influence of the matrix on their ionization had been observed. Consequently, it can be concluded that the matrix did not significantly affect the BPs signals with the QuEChERS approach. On the contrary, a significant matrix effect was observed for six BPs using the SUPRAS methodology, and this implies that it would be necessary to use standard in matrix calibration curves for quantifying these specific compounds. In this case, a comparison with the previous work in bee pollen samples cannot be made as it was not used a MS detector (Zhang et al., 2021).

3.3.4. Working range

Standard in solvent calibration curves could be used to quantify BPs in all cases, except BPA, BPE, BPF, BPM, BPPH and BPS that should be quantified with the standard in matrix calibration curves when using the SUPRAS approach. 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 250 μg L^{-1} (LOQ (see Table 1), 50, 75, 100, 150, and 250), which corresponds to those between LOQ and 250 μg kg^{-1}. 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 15 % in all cases (data not shown).

3.3.5. Precision studies

Precision was expressed as relative standard deviation (%RSD) and performed concurrently by repeated sample analysis using BF samples, at three different concentrations levels. These experiments took place either on the same day (repeatability) or over three consecutive days (partial reproducibility). %RSD values were consistently lower than 15 % in all cases (see Supplementary Material, Table 6S). In the present study, the %RSD values are slightly higher than those obtained in the previous work (<6%; Zhang et al., 2021), but it should be considered that different detectors were used in each case, which also influence the precision studies.

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. Mean recoveries for the BPs studied ranged in all cases from 71 % to 103 % (SUPRAS) or 75 % to 114 % (QuEChERS), with %RSD values consistently below 15 % in all instances these values (see Table 3). As it has been previously mentioned in Section 3.1.3, the recovery percentages obtained with the proposed methods were comparable to those obtained in the previous work dedicated to the analysis of BPs in bee pollen (83 %–95 %; Zhang et al., 2021).

3.4. Application of the method

The QuEChERS methodology was chosen to determine the residues of BPs in bee pollen due to the lowest LOQ values provided for most compounds, and the absence of a significant matrix effect. Among the fourteen BPs investigated, residues of three (BPM, BPP, and BPS) were detected in fourteen of the samples (see Table 5), and only in one sample (E2; BMP, BPP and BPS; see Supplementary Material, Fig. 2S) was more

Table 5
Results (means of triplicate analyses (μg kg^{-1}); %RSD < 15 % in all cases) of the investigation of the studied BPs in bee pollen. The other BPs under study were below LOD in the samples. LODs and LOQs are summarized in Table 1.

Sample	BPS	BPP	BPM
E1	**6.5**	<LOD	<LOD
E2	<LOD	<LOD	**<LOQ**
E5	**6.5**	**<LOQ**	**<LOQ**
E6	**5.9**	<LOD	<LOD
C1	**5.3**	<LOD	<LOD
C2	**4.6**	<LOD	<LOD
C3	**6.5**	<LOD	<LOD
C4	**6.0**	<LOD	<LOD
C5	**5.4**	<LOD	<LOD
C6	**5.5**	<LOD	<LOD
C7	**5.7**	<LOD	<LOD
C8	**6.4**	<LOD	<LOD
C9	**4.9**	<LOD	<LOD
C12	**<LOQ**	<LOD	<LOD

than one BPs detected. However, only BPS was quantified in twelve of the samples with concentrations lower (4–7 μg kg^{-1}) than the established SML (50 μg kg^{-1}, European Commission, 2011). It can be also concluded that most positive samples were from commercial origin (71 %; 10 out 14), as the number positives in samples from experimental apiaries was much lower (29 %, 4 out of 14). These results are relevant since it is the first time that BPs residues have been detected and quantified in bee pollen samples, as no BPs residues were found in the previous publication (Zhang et al., 2021). It should be also highlighted that the concentrations are very similar in the samples that have been quantified from both origins, and that the prevalence of samples with BPS content in the experimental ones may be tentatively related either to the geographical origin of the samples or to some material/procedure that has been used during their collection, since they have not been treated. On the other hand, when considering the packaging material, residues have been found in both plastic and glass containers, although it is true that most containers were glass, which justifies the largest number of positives found for this material.

3.5. Risk evaluation

Additionally, potential theoretical hazards associated with bee pollen samples containing quantifiable levels of BPS were evaluated (see Supplementary Material, Table 7S). The average concentration of BPS was used to calculate the Estimated Daily Intake (EDI) with the formula: EDI = CI×DI/bw (Isci & Dagdemir, 2024). Here, EDI represents the exposure to BPS per unit of body weight from bee pollen consumption (μg kg^{-1} bw per day), where CI is the average concentration in bee pollen samples (μg kg^{-1}), DI is the recommended daily intake of bee pollen (30 g per day for adults, Sadeghi, Akhlaghi, & Salehi, 2020), and bw is the reference body weight (70 kg). The TDI (Tolerable Daily Intake) estimates the amount of a chemical contaminant that can be ingested daily over a lifetime without significant health risks, considering environmental exposure and dietary intake. The European Food Safety Authority (EFSA) has established a temporary TDI (t-TDI) for BPA at 0.2 ng kg^{-1} bw per day (EFSA, 2023), but no specific value for BPS is available yet. Given that BPS is an analog of BPA, the BPA t-TDI was used as a reference for risk assessment. The Target Hazard Quotient (THQ) was calculated as THQ = EDI/TDI. The Hazard Index (HI), which evaluates the cumulative effect of multiple hazardous elements, is the sum of the THQ values (Cunha, Inácio, Almada, Ferreira, & Fernandes, 2020). However, since only BPS was quantified in this study, the HI value is equivalent to the THQ value. The average EDI values ranged from 1.9 to 2.8 ng kg^{-1} bw per day, with slightly higher values for experimental samples. The THQ and HI values ranged from 9.8 to 14.0, all exceeding the threshold value of 1. These elevated values indicate a significant risk, based on EFSA's limits. Note that these values were calculated using the t-TDI for BPA due to the lack of a specific value for BPS. This highlights the need for further research to establish a t-TDI for BPS and to better understand the risks associated with bee pollen consumption.

4. Conclusions

In this study, an analytical methodology has been developed and validated for the simultaneous determination of fourteen BPs residues in bee pollen. Two efficient sample treatments were proposed, based on the QuEChERS and SUPRAS methodologies, respectively, which present a similar performance and, in some cases, better than the only previous treatment published. QuEChERS methodology presents better efficiency when extracting the compounds and reducing the matrix effect, while the SUPRAS methodology is faster, simpler, and requires less reagent consumption. Chromatographic conditions (UHPLC-MS/MS) were selected from previous and recent work from our group. Additionally, a greenness assessment was conducted for both methodologies following three analytical metrics AGREE, AGREEPrep and Complex GAPI. The results obtained from the three studied metrics were consistent, indicating that the developed methodologies could be considered as "green". SUPRAS demonstrated a slightly better environmental performance than QuEChERS, based on the metrics evaluated, particularly in terms of solvent waste and sample treatment miniaturization. However, both methodologies still present environmental challenges, especially regarding instrumentation and energy consumption. Further improvements in automation and reducing environmental penalties associated with UHPLC-MS/MS could enhance their sustainability profiles. The proposed methods were validated, and the results showed that the analytical performance of both procedures was good. Indeed, LOQs were significantly lower than the SMLs established by the European Commission and the values reported in previous studies. However, the QuEChERS method demonstrated better results in terms of sensitivity (low LODs and LOQs), matrix effect (absence of a significant matrix effect) and trueness (slightly higher recovery percentages). Finally, thirty bee pollen samples from different Spanish regions were analyzed with the QuEChERS-based method, and the results revealed the presence of residues of three (BPM, BPP, and BPS) in twelve of the samples. However, only BPS was quantified in the samples but at concentrations lower than the established SML. Finally, regarding human food safety, the EDI values assessed for BPS in the samples were higher than the oral reference dose recommended by the EFSA. The THQ and HI values were above 1 in all cases, indicating a potential carcinogenic health risk. However, these data were calculated using the established BPA values, due to the lack of a specific value for BPS. This underscores the need for further research in this area.

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CRediT authorship contribution statement

Ana M. Ares: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Lucía Alcaide:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation. **José Bernal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Silvia Valverde:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114955.

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