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Development and validation of a new analytical method for the determination of plasticizers in bee pollen

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ABSTRACT

The determination of plasticizers in bee pollen may be important not only to evaluate the contamination of the hive environment, but also to verify the safety for consumers of this food supplement, since to date it has not been studied. Therefore, a new analytical methodology for the determination of nine plasticizers (phthalate esters: di-(2-ethylhexyl) phthalate, dibutyl phthalate, dimethyl phthalate, diethyl phthalate, benzyl butyl phthalate, and di-*n*-octyl phthalate; diphenyl ethers: 4-bromodiphenyl ether and 4-chlorodiphenyl ether; adipate: bis(2-ethylhexyl) adipate) in bee pollen using gas chromatography-mass spectrometry was developed. An efficient sample treatment (modified QuEChERS, quick, easy, cheap, effective, rugged & safe) is proposed (with average analyte recoveries between 77 % and 104 %) involving extraction with acetonitrile followed by a dispersive solid phase extraction (enhanced matrix removal lipid sorbent). Chromatographic analysis (<21 min) was performed in an Agilent HP-5MS column under programmed temperature conditions, and analyses were performed in selected ion monitoring mode. The method was validated in terms of selectivity, limits of detection (0.2–17.2 μg kg^{-1}) and quantification (0.5–57.5 μg kg^{-1}), linearity, matrix effect, trueness, and precision (relative standard deviation < 15 %). Finally, an analysis of thirty samples from different sources (commercial or experimental apiaries) revealed the presence of residues of five plasticizers in all samples. Quantification was possible in several cases, with overall concentrations ranging from 0.056 to 3.152 mg kg^{-1}. This study not only reports for the first time the presence of some plasticizers in bee pollen, but also corroborates the usefulness of bee pollen as bioindicator of environmental contamination.

1. Introduction

In recent years, there has been a significant increase in plastic production, reaching approximately 400 million tons annually in 2022 [1]. Nevertheless, despite its usefulness and ubiquity, current patterns of plastic consumption and disposal are contributing to substantial pollution in the environment [2]. One notable contributor to this pollution is the use of additives, commonly known as plasticizers, aimed at enhancing the properties of plastics. However, the use of plasticizers raises health-related concerns, as they can reach numerous matrices, including food consumed by humans [3]. Some examples of these additives are phthalate esters (PAEs), adipates, polychlorinated diphenyl esters (PCDEs) and polybrominated diphenyl ethers (PBDEs). PAEs constitute a class of esters derived from phthalic acid and alcohols containing 4–15 carbon atoms that are commonly used as additives in the manufacture of plastics to enhance properties such as flexibility and elasticity of polymers [4]. However, PAEs lack the capacity to form chemical bonds with the polymer structure, and they tend to migrate

Abbreviations: **AF**, samples spiked after sample treatment; **ASE**, accelerated solvent extraction; **BF**, samples spiked before sample treatment; **4-BDE**, 4-bromodiphenyl ether; **4-CDE**, 4-chlorodiphenyl ether; **BBP**, butyl benzyl phthalate; **CIAPA**, center for agroenvironmental and apicultural investigation; **CCC**, column chromatographic clean-up; **DBP**, dibutyl phthalate; **DBP-d_4**, dibutyl phthalate-3,4,5,6-d4 **DEHA,** bis(2-ethylhexyl) adipate; **DEHP**, di(2-ethylhexyl) phthalate; **DEP**, diethyl phthalate; **DLLME**, dispersive liquid–liquid microextraction; **DMP**, dimethyl phthalate; **DNOP**, di-*n*-octyl phthalate; **dSPE**, dispersive solid-phase extraction; **GC–MS**, gas chromatography coupled to mass spectrometry; **IS**, internal standard; **LOD**, limit of detection; **LOQ**, limit of quantification; **ME**, matrix effect; **MSPE**, magnetic solid-phase extraction; **NADES**, natural deep eutectic solvents; **PAEs**, phthalate esters; **PBDEs**, polybrominated diphenyl ethers; **PCDEs**, polychlorinated diphenyl ethers; **QuEChERS**, quick, easy, cheap, effective, rugged & safe; **%R**, recovery percentage; R^2, coefficient of the determination; **%RSD**, relative standard deviation; **SE**, solvent extraction; **SIM**, selected ion monitoring; **SML**, specific migration limit; **SPE**, solid-phase extraction.

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from plastics to environmental components and foods. Thus, humans can be exposed to PAEs by multiple routes, and it has been demonstrated that prolonged exposure to PAEs through food intake can have detrimental effects on human health such as endocrine disruption and reproductive toxicity [5,6]. Some of the most used PAEs are di-(2-ethylhexyl) phthalate (DEHP), which comprises half of the annual production of PAEs, dibutyl phthalate (DBP) and dimethyl phthalate (DMP). Other examples of PAEs include diethyl phthalate (DEP), benzyl butyl phthalate (BBP), or di-*n*-octyl phthalate (DNOP) [7]. Owing to the health risks associated with PAEs exposure, some restrictions have been established on their use in plastic food contact materials, as outlined in the Regulation (EU) No. 10/2011 [8]. This document includes a list of authorized PAEs in food contact materials, and specific migration limits (SMLs) for some of them like DEHP, DBP, and BBP of 1.50, 0.300 and 30.0 mg kg^{-1}, respectively. PAEs for which no SML or other restrictions are provided in this list, such as DEP, DMP, and DNOP, have a generic SML of 60.0 mg kg^{-1}. However, recently, the 16th amendment to Regulation EU 10/2011 on plastic materials and articles intended to come into contact with food was published on July 11, 2023, and it modifies the SMLs for DEHP (1.50 to 0.600 mg kg^{-1}), DBP (0.300 to 0.120 mg kg^{-1}) and BBP (30.0 to 6.00 mg kg^{-1}) [9]. Bis(2-ethylhexyl) adipate (DEHA) emerged as a safer alternative to the most common PAE (DEHP) [10]. While DEHA cannot entirely replace DEHP and other PAEs, it serves as a secondary plasticizer together with other plasticizers to improve low temperature flexibility. Notably, DEHA is primarily used in food contact related applications, constituting over 80 % of its usage in the United States [11]. However, due to potential health risks, a SML of 18.0 mg kg^{-1} was established [8]. Meanwhile, PBDEs and PCDEs are widely used as flame retardants in the production of plastics. They exhibit persistence in various environmental matrices and have the potential to accumulate in organisms through trophic transfer. Indeed, both types of compounds have been already identified in daily food, a noteworthy observation given their associated reproductive toxicity [12,13]. Within the environment, 4-bromodiphenyl ether (4-BDE) stands out as the predominant mono-brominated diphenyl ether, arising from the photodegradation of more complex brominated PBDEs [14]. On the other hand, biotransformation pathways of PCDEs can lead to the formation of 4-chlorodiphenyl ether (4-CDE) [15]. These two diphenyl ethers are not explicitly listed in the first Annex of Regulation (EU) No. 10/2011 [8]. Therefore, a generic SML of 60 mg kg^{-1} should be applied.

This work focuses on the determination of six PAEs (BBP, DBP, DEHP, DEP, DMP, DNOP), one adipate (DEHA), and two diphenyl ethers (4-BDE and 4-CDE) in bee pollen, which is a natural food made by bees from the pollen grains of flowers, along with a small proportion of nectar [16]. Bees explore extensive areas in their pursuit of nectar and pollen, encountering diverse environmental matrices such as air, water, soils, and plants. If these matrices are polluted by PAEs, adipates, or diphenyl ethers, these compounds will ultimately be introduced into the honey bee colony and hive products, like bee pollen. Therefore, the utilization of bee pollen as a bioindicator of environmental contamination has gained attention in recent years [17,18]. Moreover, like any food product, bee pollen could potentially contain plasticizers, since these compounds may migrate from the packaging into this product [19]. Consequently, given the potential for bee pollen to contain traces of plasticizers, it is crucial to develop specific and sensitive analytical methods with low limits of detection (LODs) and quantification (LOQs) for accurately determining them at low concentrations. Moreover, minimizing the contamination of plasticizers due to their widespread presence in the laboratory is necessary in order to ensure the accurate analysis of samples [20].

While no studies for the analysis of these plasticizers in bee pollen have specifically been conducted, PAEs and DEHA have been identified in related bee products, such as honey or royal jelly ([21–27]; see Supplementary Material, Table 1S). In these works, the proposed sample treatments involved a simple solvent extraction (SE), solid phase extraction (SPE), or a dispersive liquid–liquid microextraction (DLLME) followed by gas chromatography coupled to mass spectrometry (GC–MS). However, since bee pollen is a different matrix with other physicochemical properties, such as its insolubility in water, alternative extraction methods must be considered. This is precisely one of the objectives and benefits of this work. Furthermore, PAEs have been found in honey bee wax [28]. Nevertheless, the analysis was a non-targeted one, and included several other compounds. Thus, a QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) sample preparation procedure was employed, lacking specificity for PAEs. Moreover, there are some studies in which diphenyl ethers have been analyzed in bee pollen [29,30]. However, the compounds under examination in these studies differ from those analyzed in our research (4-BDE and 4-CDE). These studies included many other compounds, and non-targeted approaches were employed. Finally, it is important to note that the most employed analytical technique for determining PAEs, adipates, PBDEs and PCDEs (and their metabolites) in foods is GC–MS [31–35], and regarding sample treatment, the prevailing approach is classical SE [35–39]. However, it should be noted that numerous preparation methods can be used to prepare samples for analysis of these compounds in foods, in addition to classical SE. Methods such as liquid-phase microextraction, accelerated solvent extraction (ASE), stir-bar sorptive extraction, SPE, and column chromatographic clean-up (CCC) have been employed in several publications. However, some of these methods (ASE, SPE, CCC) can be considered as expensive, insensitive, and/or time-consuming [40,41]. Therefore, it is not surprising that alternative methods that do not present the aforementioned disadvantages, such as the previously-mentioned QuEChERS [28,42–45], and magnetic solid phase extraction (MSPE) with different particles, such as multi-walled carbon nanotubes magnetized with iron, have been recently used to determine plasticizers in foods [41,42,46,47]. For example, compared to the traditional SPE procedure, MSPE methods present some advantages, including increased effectiveness, time savings, and a less labour-intensive approach [47].

Therefore, the main objective of this study is to introduce a method for the simultaneous determination of nine plasticizers in bee pollen using GC–MS. It is worth noting that, to the best of our knowledge, no studies have proposed an analytical methodology for the detection of all those mentioned compounds in bee pollen, either simultaneously or individually. Thus, this marks the first attempt to monitor these compounds in bee pollen. Another objective of this work is to propose an efficient, simple, cheap, and fast sample treatment applicable. We aim to minimize time, cost, steps, and reagent usage, addressing some of the limitations found in previous studies. Our study also intends to validate the proposed method and analyze experimental and commercial bee pollen samples from different origins (botanical and geographical).

2. Material and methods

2.1. Reagents and materials

PAEs, adipate and diphenyl ethers standards (BBP, DBP, dibutyl phthalate-3,4,5,6-d_4 – DBP-d_4, DEHP, DEP, DMP, DNOP, DEHA, 4-BDE and 4-CDE; see structures in Supplementary Material, Fig. 1S), all of analytical-grade and with purity greater than 98 %, were purchased from Sigma-Aldrich Chemie Gbmh (Steinheim, Germany). It is important to note that two separate standard mixtures were obtained: one containing the six PAEs and DEHA (EPA-506), and another consisting of the six PAEs in addition to 4-BDE and 4-CDE (EPA-8270). All solvents and reagents were of chromatographic/analytical grade. Acetonitrile and methanol were obtained from VWR Prolabo Chemicals (Fontenay-sous-Bois, France), acetone and ethyl acetate were supplied by Carlo Erba Reagents-SA (Milan, Italy), nitric acid was provided by ITW Reagents (Monza, Italy), 1,2-propanediol was purchased from Montplet & Esteban S.A. (Barcelona, Spain), and heptane, methyl *tert*-butyl ether, and choline chloride were from Sigma-Aldrich Chemie Gbmh. 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, all supplied by J.P. Selecta S.A. (Barcelona, Spain), a 5810 R refrigerated bench-top centrifuge from Eppendorf (Hamburg, Germany), a Moulinette chopper device from Moulinex (Paris, France), and PTFE syringe filters (17 mm, 0.2 µm; Branchia, Labbox, Spain) were employed for sample treatment. QuEChERS dispersive solid phase extraction (dSPE) enhanced matrix removal lipid (EMR-Lipid) sorbent was supplied by Agilent Technologies (Folsom, CA, USA), while C_{18} was provided by Supelco (Bellefonte, PA, USA), and Florisil was provided by Alltech Associates, Inc. (Deerfield, IL, USA).

It should be noted that, owing to the common presence of PAEs in the laboratory environment, it is crucial to control their potential presence in the background signal values of blanks [20]. To address this issue, plastic consumables (micropipettes tips) and chemical reagents were newly used/opened, and the absence of any residues of PAEs was verified. Moreover, laboratory glassware underwent a meticulous cleaning process 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 approximately 150 °C for 60 min. Procedural blanks, in which ultrapure water replaced bee pollen, were systematically run between sets of samples to monitor potential abnormal background values. BBP was detected with constant response in all the procedural blanks, and consequently, the blank response of this compound was systematically subtracted in every analysis.

2.2. Preparation of standard solutions

Standard stock solutions (at a concentration of 1000.0 mg L^{-1}) and working solutions for the analyzed compounds were prepared using acetone. It is important to note that two separate stock solutions were prepared according to the obtained standard mixtures (six PAEs and DEHA, EPA-506; six PAEs, 4-BDE and 4-CDE; EPA-8270). Bee pollen samples were spiked with variable amounts of the analytes either before (BF samples) or after (AF samples) sample treatment (see **section 2.3**) to prepare the standard in matrix extracts. The spiking of the samples was done similarly to Fuente-Ballesteros et al. [48]. Representative portions of ground and dried bee pollen were weighed and transferred to a crystallizer. Subsequently, these portions were uniformly spiked with the working solutions. Meanwhile, AF samples were prepared by spiking previously treated bee pollen samples (subjected to the proposed sample treatment) with working standard solutions, which were added to the reconstitution solvent. The internal standard (IS; DBP-d_4) was consistently added at the same concentration (0.100 mg L^{-1}). 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 the studied compounds were extracted in all cases with enough efficiency and precision (see **subsection 3.3.6**), and what was intended to compensate was the variability in the intensity of the MS signals due to differences in ionization efficiency. These samples were used for validation (spiked samples (low, medium, and high) and calibration curves), as well as sample treatment studies. The study involved the preparation of three replicates, each of which was injected three times. Each spiked sample was prepared with a blank sample fortified with three different concentrations of the plasticizers within the linear range. These were as follows: low-LOQ (see Table 1); medium-0.400 mg kg^{-1}; high-2.000 mg kg^{-1}. 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 until the analysis.

2.3. Sample procurement and treatment

2.3.1. Samples

A total of thirty bee pollen samples (n = 30, see Table 2S) were divided into two groups: those obtained from experimental apiaries (n = 15; E1-E15), which were kindly donated by researchers from the Center for Agroenvironmental and Apicultural Investigation (CIAPA, Marchamalo, Guadalajara, Spain); and those of commercial origin (n = 15; C1-C15), which were purchased from local markets in Spain. The botanical origin of the bee pollen samples was confirmed by palynological analysis at CIAPA [49]. 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 [48]. Three replicates (sub-samples) of each bee pollen sample, injected in triplicate, were examined to determine the plasticizers content.

Table 1
Calibration curve data (matrix-matched standards SCI, slope confidence interval; ICI, intercept confidence interval), limits of detection (LOD) and quantification (LOQ), and specific migration limits (SMLs).

Analyte	SCI	ICI	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	SML (mg kg^{-1}; [8,9])
DMP	0.00499 ± 0.00047	0.06134 ± 0.10976	0.995	1.0	3.3	60
DEP	0.00450 ± 0.00037	0.16446 ± 0.45661	0.997	0.3	0.9	60
4-CDE	0.00162 ± 0.00016	0.02837 ± 0.09028	0.997	0.7	2.5	60
4-BDE	0.00164 ± 0.00015	0.02036 ± 0.06479	0.997	0.2	0.8	60
DBP	0.00711 ± 0.00053	0.10033 ± 0.27855	0.997	1.3	4.4	0.12
BBP	0.00398 ± 0.00063	0.17570 ± 0.18033	0.993	0.5	1.7	6
DEHA	0.00566 ± 0.00178	−0.09474 ± 0.50673	0.990	0.2	0.5	18
DEHP	0.00896 ± 0.00045	0.11776 ± 0.10507	0.999	2.9	9.7	0.6
DNOP	0.00402 ± 0.00032	0.03786 ± 0.07544	0.997	17.2	57.5	60

2.3.2. Sample treatment

An efficient sample treatment based on the QuEChERS methodology [50] has been used, but it has been adapted and refined in this study to specifically determine plasticizers in bee pollen. Briefly, 1.000 g of dried and ground bee pollen was weighed in a 10 mL glass centrifuge tube, and 4.00 mL of acetonitrile was added. Then, the tube was then shaken at 1400 rpm for 1 min in a vortex device and placed in an ultrasound bath for 10 min. After that, the mixture was centrifuged at 4000 rpm for 5 min at 5 °C, and the resulting supernatant was collected. Subsequently, 1.000 g of EMR Lipid was added, and the entire process (vortex, ultrasonication, centrifugation) was repeated. Afterward, 1.00 mL of the supernatant was collected and evaporated to dryness under a nitrogen stream at room temperature (≈ 10 min). Finally, the dry extract was reconstituted with 1.00 mL of an IS solution (0.100 mg L^{-1}) in acetone and filtered through a 0.2 µm PTFE filter prior GC–MS analysis. Fig 1S (see Supplementary Material) summarizes the steps of the selected sample treatment.

2.4. GC–MS conditions

An Agilent Technologies (Palo Alto, CA, USA) 7890A gas chromatograph (GC) coupled to an Agilent Technologies 5975C mass spectrometer (MS) equipped with an ALS 7693B autosampler and a MS ChemStation E 01.00.237 software (Agilent Technologies) was employed. The chromatographic column was an Agilent HP-5MS (30 m × 250 µm × 0.25 µm). The GC–MS parameters (programmed temperature conditions; temperatures (injector, transfer line, ion source, and quadrupole), flow rate, injection mode and volume, etc.), which were optimized in a previous and recent work [27], are summarized in Table 2. It should be mentioned that after determining each analyte's retention time via full-scan mode, optimization of SIM mode was

Table 2
GC–MS parameters.

GC parameter	Final setting
Programmed temperature conditions	From 60 °C (1 min) to 125 °C at 25 °C min^{-1}, then increased to 310 °C at 10 °C min^{-1}, and finally kept for 3 min at 310 °C.
Carrier gas	Helium
Transfer line temperature (°C)	310
Injection mode	Splitless
Flow rate (mL/min)	1.2
Injector temperature (°C)	280
Injection volume (μL)	1
MS parameter	**Final setting**
Operating mode	Electron impact
Ionization energy (eV)	70
Scan range (*m/z*)	25–300
Ion source temperature (°C)	230
Quadrupole temperature (°C)	150
Ions (*m/z*)	DMP: 163^{Q,C}, 77^{C} and 92^{C} DEP: 149^{Q,C}, 177^{C} and 176^{C} 4-CDE: 204^{Q,C}, 206^{C} and 141^{C} 4-BDE: 248^{Q,C} and 141^{C} DBP: 149^{Q,C}, 150^{C} and 205^{C} DBP-d$_4$: 153^{Q,C} and 227^{C} BBP: 149^{Q,C}, 91^{C} and 206^{C} DEHA: 129^{Q,C}, 112^{C} and 147^{C} DEHP: 149^{Q,C}, 167^{C} and 279^{C} DNOP: 149^{Q,C}, and 279^{C}

^{Q} Quantification ions; ^{C} Confirmation ions.

conducted to guarantee precise identification and high signal intensity for each analyte. A full scan of the standard solution for each plasticizer was done to obtain its mass spectrum and compare it with the NIST mass spectra library. Molecular ions, highly abundant fragment ions, and distinctive ions were chosen and adjusted to minimize interference and facilitate accurate analyte qualification and quantification. Based on these criteria, specific ions were selected for quantification, while two additional ions were chosen for qualitative evaluation for each plasticizer (see Table 2). Under these optimal GC–MS conditions, all compounds eluted in < 21 min (see Fig. 1). As can be seen, the compounds can be identified/quantified without problems using the ions selected for each of them, even though they would not be separated to the baseline. This fact highlights one of the advantages of using MS detectors: their ability to discriminate between co-eluting compounds by employing different ions for each.

3. Results and discussion

3.1. Optimization of the sample treatment

As most of the analytes studied are PAEs, the optimization of the sample treatment was initially focused on them, and the optimization process of the sample treatment started six PAEs (BBP, DBP, DEHP, DEP, DMP, and DNOP) and two diphenyl ethers (4-BDE and 4-CDE). It should be remarked that the first experiments were conducted with a spiking concentration of 0.250 mg L^{-1} (1.000 mg kg^{-1}), and that the optimization process considered the percentages of recovery (%R) and matrix effect (%ME) of the analytes.

In our effort to develop a sample treatment in line with the principles of green analytical chemistry, an attempt was made to employ natural deep eutectic solvents (NADES). NADES consist of two or more components in solid or liquid states, serving as a hydrogen bond acceptor and a hydrogen bond donor, combined in a specific molar ratio. The preparation process involves the physical mixing of these components

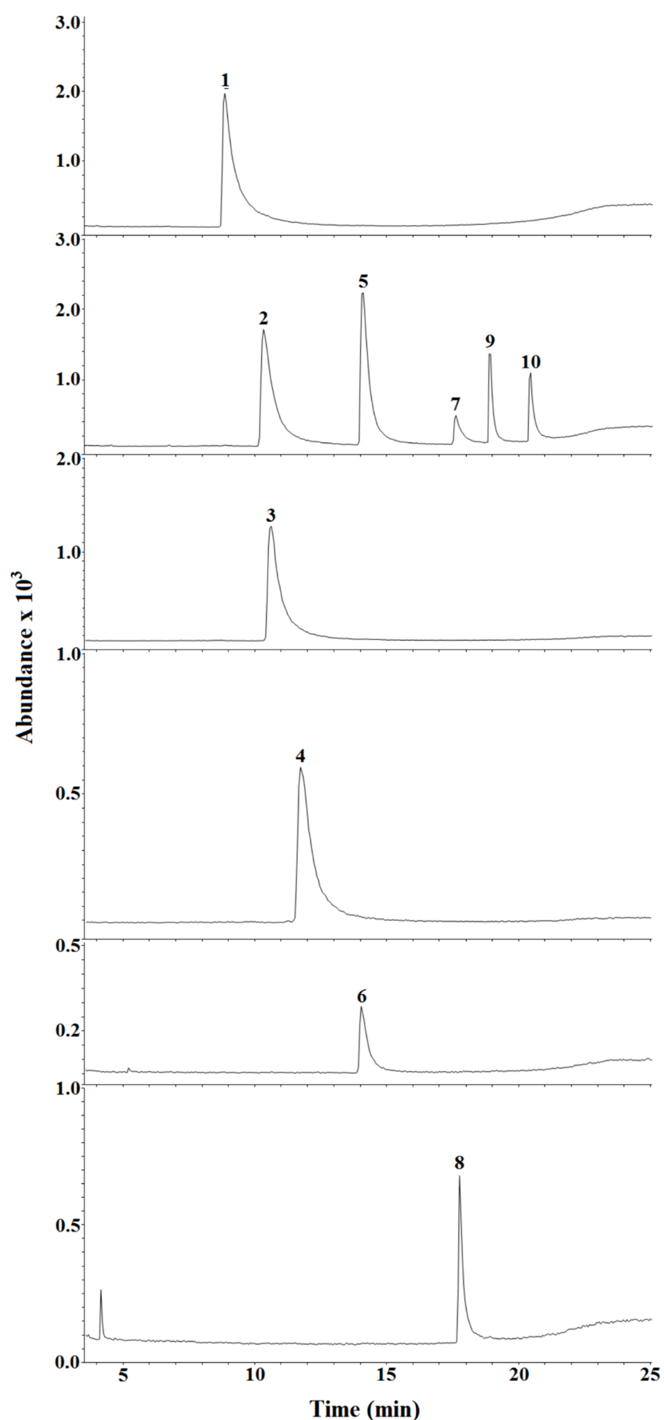

Fig. 1. Representative Gas chromatography-Mass spectrometry (GC–MS) chromatograms (selected ion monitoring mode using the quantification/target ions; see Table 2) obtained from standards in solvent mixture (0.5 mg L^{-1}; IS, 0.1 mg L^{-1}). GC–MS conditions are summarized in section 2.4 and Table 2. **1**, dimethyl phthalate (DMP); **2**, diethyl phthalate (DEP); **3**, 4-chlorodiphenyl ether (4-CDE); **4**, 4-bromodiphenyl ether (4-BDE); **5**, dibutyl phthalate (DBP); **6**, dibutyl phthalate-3,4,5,6-d4 (DBP-d4; internal standard); **7**, butyl benzyl phthalate (BBP); **8**, bis(2-ethylhexyl) adipate (DEHA), **9**, di(2-ethylhexyl) phthalate (DEHP); **10**, di-n-octyl phthalate (DNOP).

under heating and vigorous stirring [51]. Following a recently reported synthesis of NADES for the extraction of PAEs and DEHA, which employed a mixture of choline chloride and 1,2-propanediol [52], two tests were conducted. In the first test, 1.000 g of bee pollen was weighed, and 4 mL of NADES were added, initiating a SE using vortex (1400 rpm,

1 min), ultrasonication (10 min), and centrifugation (4000 rpm, 5 min, 5 °C). Subsequently, the supernatant underwent sequential SE with an ethyl acetate and heptane (1:1, v/v) mixture. After applying vortexing, ultrasonication, and centrifugation under the same conditions mentioned earlier, the organic phase was evaporated under nitrogen stream at room temperature. Finally, the dry extract was reconstituted with 1.00 mL of acetone. In the second test, 2.00 mL of ultrapure water and 3.00 mL of NADES were added to the bee pollen sample, and the same extraction process was repeated, using only ethyl acetate in the SE step. Unfortunately, both tests resulted in low recoveries (<30 %), leading to the decision to discard the use of NADES in our sample treatment. One possible reason that could explain the poor results obtained using NADES is the nature of the matrix under study, since bee pollen is very different from the matrices analyzed in previous studies (tropical fruits [51]; laying hen and goat feed [52]).

Given that PAEs from foods are typically extracted through SE, our next approach consisted of weighing 1.000 g of bee pollen, followed by a SE by using 4 mL of different solvents: ethyl acetate, ethyl acetate and heptane (1:1, v/v) mixture, acetonitrile, and methyl *tert*-butyl ether. These solvents were chosen because they provided promising results in previous analyses conducted by the research group when these compounds were analyzed in honey [27]. The sample underwent a sequential process involving vortex mixing (1400 rpm, 1 min), ultrasonication (10 min), centrifugation (4000 rpm, 5 min, 5 °C), evaporation of the organic phase under an N_2 stream, and reconstitution of the dry residue with acetone. The best results in terms of recovery were achieved with acetonitrile (data not shown). However, despite acceptable recoveries (96 %−125 %), elevated ME were observed (experiment T1; > 200 % in some cases; see Supplementary Material, Table 3S). Therefore, a test involving the addition of sorbents for a clean-up step (dispersive solid-phase extraction, dSPE) was conducted to address the high ME. Three approaches were employed: one with EMR Lipid alone (see Supplementary Material, Table 3S; experiment T2), another with both EMR Lipid and C_{18} (see Supplementary Material, Table 3S; experiment T3), and a third with both EMR Lipid and Florisil together (see Supplementary Material, Table 3S; Experiment T4). In all these tests, 1.000 g of each sorbent was used. The results indicated that, while the ME was not entirely corrected for most compounds, there was a substantial improvement due to the clean-up step (<400 % in all cases). In view of the results of the application of three sorbents, the use of Florisil was excluded from further consideration, and experiments T2 and T3 were replicated at a higher concentration (0.500 mg L^{-1} or 2.000 mg kg^{-1}; see Supplementary Material, Table 3S; experiments T5 and T6, respectively). At this high level of concentration, the results obtained were better for the addition of EMR Lipid alone (experiment T5; %R between 79 % and 96 %). Consequently, the suitability of the extraction with 4.00 mL of acetonitrile and the clean-up step with the addition of EMR Lipid (1.000 g) was checked at three concentration levels (see **section 2.2**). It is important to note that DEHA had not been included up to this point; however, it was at this stage, with three replicates conducted for each concentration level. Although the presence of ME is evident (> ±20 % in most cases), the sample treatment provides acceptable recoveries (77 % − 104 %) for all the analytes at the three concentration levels (see Supplementary Material, Table 3S). Moreover, tests were carried out with different amounts of bee pollen (0.500–1.500 g), larger volumes of acetonitrile (6.00 and 8.00 mL), extended extraction times (15 min), and increased amounts of sorbent (1.500 g). However, these adjustments did not improve the previous ones (data not shown). It should be mentioned that the observed differences in the influence of the ME on the signal of the compounds may be tentatively explained by the different structures of the plasticizers, since they belong to three different groups of compounds. Alternatively, the ME might be because plasticizers are affected by different bee pollen components that could coelute with them without being observed and without affecting their chromatographic separation when working in SIM mode [27]. However, considering that the matrix effect can be effectively addressed by incorporating standard in matrix calibration curves for quantification, and given the good recoveries, the conditions detailed above and summarized in **subsection 2.3.2** were considered definitive.

To sum up, the proposed sample treatment can be considered as a good option to determine plasticizers in bee pollen as it is relatively fast (≈ 35 min), simple (few stages and with common instrumentation), involves little use of reagents (solvents, 5.00 mL; sorbents, 1.000 g), and it can be considered economical. Moreover, recovery values were satisfactory for all the analytes studied, and, although the matrix effect was significant for most compounds, this can be compensated by using matrix-matched calibration curves for quantification. When comparing the proposed sample treatment with previous ones dedicated to analyzing plasticizers in other products from the hive, or those that use QuEChERS-based methods in other foods (see Supplementary Material, Table 1S), it can be concluded that the performance is very similar. It is worth noting that some similar works analyzing plasticizers in bee products [26,28–30] were omitted from Table 1S (see Supplementary Material) because non-targeted analyses were conducted, and/or recoveries, limits of detection (LODs) and limits of quantification (LOQs) were not calculated. The recovery percentages were between 70 % and 120 % in all cases, and plasticizers were usually quantified with matrix-matched calibration curves with only two exceptions [23,28]. The treatment time and the number of stages required are very similar in most cases, being slightly lower in those methodologies based on DLLME [21,22,24]. These methods also use fewer solvents and reagents, compared to QuEChERS-based methodologies [27,42–45], including the proposed method. In contrast, the most classic methodologies (SE [23]; SPE [25]) are the most time-consuming and require the greatest consumption of solvents. However, it should be emphasized that the primary distinction and significant novelty/relevance in this work lies in the fact that it is the first time that a specific sample treatment has been proposed to determine these compounds in bee pollen. Furthermore, the performance of the proposed sample treatment is comparable to those previously published in very different matrices, which demonstrates its effectiveness and usefulness.

3.2. Method validation

Validation was performed according to current legislation [53]. In addition, several of the main elements of uncertainty [54] were considered when optimizing and validating this method (amount of sample used, recovery value of the analytical procedure, precision, and repeatability). The specific procedures for determining the different validation parameters are summarized in Table 5S.

3.2.1. Selectivity

Selectivity was evaluated by comparing the chromatograms and mass spectra of standards in solvents with blanks of bee pollen. No matrix interferences were detected at the retention times of the analytes, except for BBP, which is also present in procedural blanks (data not shown). Additionally, comparable mass spectra were obtained for the standards of plasticizers in both solvents and matrix extracts (see Fig. 2), although certain minor differences in ion intensity were observed and certain low intensity ions appeared only in bee pollen spectra.

3.2.2. Limits of detection and quantification

LODs and LOQs are summarized in Table 1. They ranged from 0.2 to 17.2 μg kg^{-1} and from 0.5 to 57.5 μg kg^{-1}, respectively. Those values are below to the SMLs established by legislation [8,9], and they are similar or even better than those obtained in previous works in other matrices with similar compounds (LODs, 0.01–600 μg kg^{-1}; LOQs, 0.04–1500 μg kg^{-1}; see Supplementary Material, Table 1S). These results demonstrate the excellent sensitivity of the proposed method.

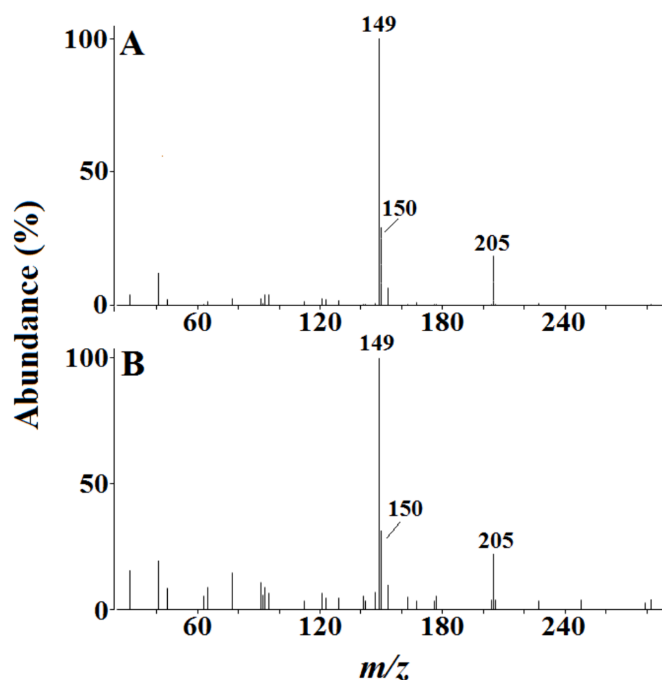

Fig. 2. Mass spectra of dibutyl phthalate (DBP) in (A) standard in solvent, (B) spiked bee pollen sample. Gas chromatography-Mass spectrometry conditions are summarized in **section 2.4** and Table 2.

3.2.3. Matrix effect

To ascertain how the matrix influenced ESI ionization for the compounds, a comparison was made by analyzing the detector responses (analyte peak area/IS area) of standards in solvent and AF samples spiked at three different concentrations. The results showed that there is significant ME in most cases (see Supplementary Material, Table 4S). Nevertheless, this ME can be addressed by using matrix-matched calibration curves for the quantification, as it has been done in most related works (see Supplementary Material, Table 1S). The ME was not significant in only two cases, which involved matrices quite different from bee pollen (mussels [42], honey and royal jelly [23]).

3.2.4. Linearity/Working range

Matrix-matched calibration curves (BF samples) were used to quantify the analytes in bee pollen samples due to the significant ME. 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 0.500 mg L^{-1} (LOQ, 0.025, 0.050, 0.075, 0.100, 0.250 and 0.500 mg L^{-1}), which corresponds to those between LOQ and 2.000 mg kg^{-1}. It should be mentioned that the Breusch-Pagan test [55] was performed to build the calibration model, and the results confirmed that homoscedasticity and independency of residuals are met in all cases (data not shown). Moreover, 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), and the deviation of back-calculation concentration from true concentration was lower than 20 % (data not shown).

3.2.5. Precision

Precision is expressed as the relative standard deviation (%RSD). This was measured through repeated sample analyses using BF samples spiked at three different concentration levels (see **section 2.2**). 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), which fits perfectly with the values reported in previous works (<20 %; see Supplementary Material, Table 1S).

3.2.6. 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 (see **section 2.2**). Mean recoveries for the analytes studied ranged in all cases from 77 % to 104 % (Table 4S), with %RSD values lower than 15 % (data not shown). These results are again comparable to those obtained in the related works included in Table 1S (70 %–120 %; see Supplementary Material).

3.3. Assessment of the applicability of the method

The blue applicability grade index (BAGI) was applied to evaluate the practicality and applicability of the employed analytical methodology [56]. 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 colour 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, a total score exceeding 60.0 is recommended for an analytical method to be considered practical [56]. Therefore, to calculate the BAGI of the proposed method, its main attributes must be considered. The method involved a quantitative, confirmatory, and multi-element analysis of nine plasticizers by GC–MS, with a modified-QuEChERS extraction allowing for complete analysis of 2–4 samples per hour. In addition, common and commercially available reagents were used. The method involved minimal sample (1.000 g of bee pollen per sample), a pre-concentration step, and semi-automated analysis using a GC autosampler. Taking all these factors into account, the method achieved a score

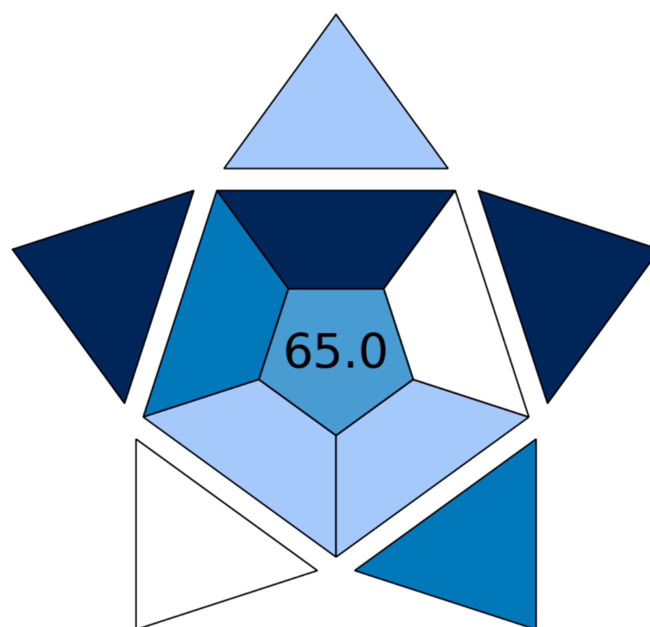

Fig. 3. Blue applicability grade index (BAGI) index pictogram of the proposed analytical method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of 65.0 (see Fig. 3), surpassing the 60-point threshold, which demonstrates its applicability. When comparing the value obtained with the proposed method to those reported in the studies collected in Table 1S (see Supplementary Material), it is evident that the applicability score (BAGI) is comparable with most works. Specifically, the value obtained in the proposed method is equal to or greater than that of previous works, except for two cases (75.0-DLLME [24]; 70.0-QuEChERS [43]). However, it must be highlighted again that this comparison is approximate, since neither the matrices nor the compounds analyzed are the same, which have a great influence when developing the methods.

3.4. Application of the method

The validated method was applied for determining potential analyte residues in thirty bee pollen samples (see **subsection 2.3.1**). IS was added to all samples at the same concentration (0.100 mg L^{-1} or 0.400 mg kg^{-1}). Moreover, sample E10 was diluted (1:2) for the quantification. The results are detailed in Table 7S (see Supplementary Material). Four compounds, two PAEs (DMP and DNOP) and the two diphenyl ethers (4-CDE and 4-BDE) were not detected in any of the samples. In contrast, DBP and DEHA were detected in all samples, while DEP, BBP and DEHP were found in only some of them. DEHA was found to be the only analyte quantified in all samples, followed closely by DBP. Notably, DBP was quantified in every sample from experimental apiaries and nearly all commercial samples, except for one. Despite not being quantified in every sample, BBP exhibited the highest concentrations overall, and in one sample (E10), the concentration value was 2.414 mg kg^{-1}. In contrast, among the quantified analytes, DEHP was found in the lowest concentrations (<0.180 mg kg^{-1}). It should be highlighted that the overall concentrations ranged from 0.056 to 3.152 mg kg^{-1} (see Supplementary Material, Table 3S). Upon comparing commercial samples to those from experimental apiaries, it was observed that a greater number of analytes were both detected and quantified in the experimental samples. The observed disparity can be tentatively explained from the processing methods applied to commercial bee pollen. Fresh bee pollen, containing between 20 % and 30 % water, requires a drying process. However, this drying procedure, which can involve elevated temperatures [57], may lead to a potential partial loss of analytes due to their low boiling points. It is noteworthy that this processing step is not conducted in samples from experimental apiaries. Additionally, residues have been detected in bee pollen from both plastic and glass containers, although most of the containers were glass, which accounts for the higher number of positive findings in this material. Despite this, there is considerable variability in residue concentrations across different containers, with the highest concentrations generally found in glass. However, no clear correlation between container material and residue levels has been established. Regarding the floral origin of the samples, no significant conclusions can be drawn, as most samples are of multifloral origin and the number of samples from other floral sources is insufficient. Future studies should aim to increase the sample size and include bee pollen from various geographical and botanical origins, as well as a broader range of packaging types, to gain a more comprehensive understanding. However, it is important to note that all the analytes in bee pollen samples were found to be significantly below the established SMLs ([8,9]; see Table 1 and Table 7S). This regulatory compliance ensures that there is no discernible risk associated with the consumption of these bee pollen samples in relation to their content of plasticizers.

The occurrence of plasticizer residues, particularly PAEs and adipates, in bee pollen is a novel finding, since to our knowledge, this is the first time that the presence of these compounds has been detected/investigated in this bee product. However, residues of these compounds have been detected in other bee matrices, especially honey (see Supplementary Material, Table 1S). In the case of honey, variable amounts of these compounds have been found, ranging from 0.001 mg kg^{-1} [23] to more than 5.000 mg kg^{-1} [22], and the compounds found in the highest concentration and number of samples were DHP, DEP, DEHA and BBP [21–25,27]. These findings are somewhat consistent with the results obtained in the present study, since the presence of plasticizers in honey may originate from contaminated pollen and nectar [22,27]. When comparing to other food matrices such as those mentioned in Table 1S (mussels, oil, baby food, and capsanthin; see Supplementary Material), a similar trend is observed. The plasticizers DEHP, DEP and BBP are the most frequently detected and are found at the highest concentrations, with variability comparable to that seen in honey (0.001 mg kg^{-1} [45] to 4.930 mg kg^{-1} [44]).

Finally, the identification of contaminant residues associated with plastics in bee pollen underscores the importance of developing analytical methods to ensure the safety of this bee product and preserve human health, since some of them had been classified as endocrine disruptors [22,23,27]. While this issue has been widely studied in different types of food, beverages and food stimulants [35], there is no information regarding bee pollen samples. Moreover, in accordance with the European Regulation, the limitation of 60 mg kg^{-1} for those compounds without a defined SML requires careful consideration.

4. Conclusions

This study introduces a new analytical method combining a modified QuEChERS with GC–MS, that has been successfully developed and validated for the determination of six PAEs, one adipate, and two diphenyl ethers in bee pollen. The method is efficient, simple, fast, and economical, involving SE with acetonitrile and a further dSPE with a EMR-lipid sorbent. With this procedure, good recovery percentages have been achieved for all the compounds, but the matrix effect could not be minimized enough. In addition, the GC–MS method has been specifically developed for this study, and under the proposed conditions, all analytes were eluted in less than 21 min. The proposed method has been validated, and the results showed that the analytical performance of the method was good enough and comparable with related studies. The LODs and LOQs were significantly lower than the established SMLs, and similar or even better than previous works. Therefore, the initial hypothesis presented in the Introduction, underscoring the necessity for selective and sensitive methods in discerning plasticizers in bee pollen, was achieved. The application of this method to thirty samples revealed that five out of the nine analytes were detected, each exhibiting concentration well below the SML. It is important to note that differences were observed between samples of commercial origin and those from experimental apiaries. On the other hand, a relationship between plasticizer content and floral origin or container material could not be established due to the limited diversity of the samples, as most were multifloral and collected in glass containers. Future studies should therefore include a larger and more diverse sample set, analyzing bee pollen with various floral origins and packaging materials using the proposed method. This would allow for a more comprehensive assessment and enable more meaningful conclusions about the potential influences of floral origin and container material on plasticizer content. Lastly, this study represents a significant milestone as the first-ever analytical method for the determination of PAEs, diphenyl ethers, and adipates in bee pollen, either individually (PAEs and diphenyl ethers) or simultaneously (all compounds). Therefore, it serves as the first attempt to systematically monitor these compounds in bee pollen, providing valuable insights to the field.

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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, Project administration, Methodology, Funding acquisition, Conceptualization. **José Bernal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization. **Ana M. Ares:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, 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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.111404.

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