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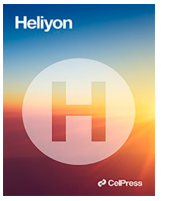

Environmental assessment of PAHs through honey bee colonies – A matrix selection study

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ABSTRACT

The steady conditions of temperature, humidity and air flux within beehives make them a valuable location for conducting environmental monitoring of pollutants such as PAHs. In this context, the selection of an appropriate apicultural matrix plays a key role in these monitoring studies, as it maximizes the information that will be obtained in the analyses while minimizing the inaccurate results. In the present study, three apicultural matrices (honey bees, pollen and propolis) and two passive samplers (APIStrips and silicone wristbands) are compared in terms of the number and total load of PAHs detected in them. Samplings took place in a total of 11 apiaries scattered in Austria, Denmark, and Greece, with analyses performed by GC-MS/MS. Up to 14 different PAHs were identified in silicone wristbands and pollen, whereas the remaining matrices contained a maximum of five contaminants. Naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and pyrene were found to be the most prevalent substances in the environment. Recovery studies were also performed; these suggested that the chemical structure of APIStrips is likely to produce very strong interactions with PAHs, thus hindering the adequate desorption of these substances from their surface. Overall, silicone wristbands placed inside the beehives proved the most suitable matrix for PAH monitoring through honey bee colonies.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) comprise hundreds of chemically associated compounds with a widespread presence and high persistence in the ecosystems. These compounds are formed by at least two fused aromatic rings, and the PAH derivatives (some of which have also been identified in environmental samples) may include other atoms, such as oxygen or nitrogen, in their structure [1,

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2]. Some of the main PAH sources include incomplete combustion during natural or anthropogenic fires [3], automobile exhaust [4,5], tobacco smoke [6], crude oil maturation, or cooking fumes, among others [7]. These compounds have been extensively detected in natural and urban ecosystems, including marine sediments [8,9], air [1,10], cigarette butts [11,12], marine sponges, and fish [13], as well as indoor environments, such as air and dust [14]. Naphthalene (also considered a volatile organic compound, VOC) is one of the most ubiquitous PAHs both in indoor and outdoor systems [11,14–16].

PAHs may produce carcinogenic effects on animals. A recent study reported the relationship between the PAH load in the air or dust and the mutagenicity potency [14]. Humans are constantly exposed to a wide range of PAHs through diet and the environment, and their residues have been detected in biological specimens such as urine and skin [17–19]. Therefore, monitoring studies are essential to ensure acceptable human exposure levels that do not raise health concerns. The use of honey bee colonies as biomonitors for contaminants is a useful approach that allows to simplify the sampling procedures: as honey bees travel long distances during foraging, they are exposed to contaminants from wide areas [20]. These contaminants are then amassed in the different apicultural commodities according to their physicochemical attributes [21], which results in the possibility of obtaining comprehensive information about a given ecosystem with just one sampling spot.

Additionally, the steady conditions within the beehives –e.g., temperature, humidity or air flux– and the protection of the beehive walls favor the reproducibility of the samplings in the context of long-term monitoring studies. Honey bee colonies have been successfully employed for the monitoring of pesticides [22,23], microplastics [24], heavy metals [25] and PAHs, among other groups of contaminants. In 2012, Lambert et al. described the use of honey bees, honey and pollen for the analysis of PAHs in the environment [26]. The lowest contamination levels were found in honey samples, probably due to the high matrix polarity compared to the strong lipophilicity of these contaminants, which results in a reduced migration of PAHs to the honey. Conversely, the most suitable matrix for PAH analysis was found to be honey bees due to their availability within the colony and the higher concentrations found in these samples. These results confirm those reported in a former study by Perugini et al., who compared honey and honey bees for the biomonitoring of environmental PAHs and found larger levels and number of compounds in honey bees compared to honey [27]. Later, in 2017, Kargar et al. compared the analysis of honey bees, propolis, and pine tree leaves [28], identifying the highest PAH concentrations in propolis samples. Moreover, Wei et al. reported the validation of a modified QuEChERS method for the extraction of PAHs in pollen samples to be employed in monitoring studies [29].

There is, however, an increasing trend to replace the traditional active sampling of apicultural matrices with passive sampling approaches. The passive sampling entails a series of advantages, including: (i) minimum human alterations made to the honey bee colonies and no need to kill living bees for the analysis, (ii) constant availability of samples, which results in viability for long-term monitoring studies, and (iii) possibility to simplify the analyses due to the inclusion of only one type of matrix –a unique sampler capable of adsorbing contaminants with a broad spectrum of physicochemical properties. The selection of the most appropriate sampler is a critical step, as the sorbent material should be able to capture all contaminants included in the analytical scope. In this context, various works have already described the suitability of the APIStrip (Adsorb Pesticide In-hive Strip) –a passive sampler based on the sorbent Tenax– for the environmental monitoring of pesticides through honey bee colonies [22,30]. The APIStrips were developed in the framework of the European project INSIGNIA (“Environmental monitoring of pesticide use through honey bees” PP-1-1-2018) and were employed for large-scale monitoring studies in multiple European countries [30]. A newly awarded project, the INSIGNIA-EU (“Preparatory action for monitoring of environmental pollution using honey bees” N° 09.200200/2021/864096/SER/ENV.D.2), relies on these passive samplers to evaluate the environmental presence of pesticides in the 27 countries that form the European Union. Additionally, in 2020, Bullock et al. reported the use of silicone wristbands for the analysis of semiochemicals and honey bee-associated compounds from beehives [31]. Silicone has already been successfully employed for the environmental PAHs monitoring in the form of rubber sheets and strips [32,33]. From a chemical exposure perspective, Anderson et al. assessed in-depth wristband passive samplers for chemicals such as PAHs and volatile organic compounds, expanding their chemical portfolio [34].

The objective of the current work is to assess the viability of apicultural matrices (honey bees, propolis, pollen) and passive samplers (APIStrips, silicone wristbands) in the analysis of 27 PAHs and derivatives through honey bee colonies. The latter possess all the benefits of an appropriate sampler targeting environmental chemicals: beehives can both passively accumulate chemicals due to their widespread presence in the natural environment, but also due to the capacity of bees to fly in a significant radius [35,36], and the consequent transfer of chemicals back to the beehive. Additionally, the inside of the beehives provides steady temperature and humidity conditions (due to thermoregulation and hygroregulation processes within the colony) that contribute to the reproducibility of the sampling conditions [37,38]. In the presented work, the matrices are evaluated in terms of total PAH load and practical sampling aspects –e.g. sample availability within the beehives, harm done to the colonies, and ease of sampling procedure– to determine the most suitable approach.

2. Materials and methods

2.1. Reagents and materials

High-purity PAH standards were obtained from LGC (Teddington, United Kingdom) or Sigma-Aldrich (Steinheim, Germany) and were stored at −30 °C. Individual stock solutions (1000–2000 mg/L) were prepared in acetonitrile and stored in amber screw-capped glass vials in the dark at −20 °C. Individual standard solutions, used for optimization, along with standard-mix solutions, used for calibration, were prepared from the stock standards. The 27 PAHs and derivatives included in the scope of the method were: naphthalene; 1-methylnaphthalene; 2-methylnaphthalene; 1,4-naphthoquinone; 1,4-dimethylnaphthalene; acenaphthene; fluorene; 1-

nitronaphthalene; 9,10-dihydroanthracene; 9-fluorenone; phenanthrene; anthracene; 1-methoxy-4-nitronaphthalene; fluoranthene; pyrene; 9-nitroanthracene; 2-nitro-9-fluorenone; chrysene; benzo[*a*]anthracene; 1-nitropyrene; benzo[*b*]fluoranthene; benzo[k]fluoranthene; benzo[e]pyrene; benzo[*a*]pyrene; indeno [1,2,3-cd]pyrene; dibenzo[ah]anthracene; benzo[ghi]perylene. Internal standards of PAHs (naphthalene-D8, acenaphthene-D10, phenanthrene-D10, pyrene-D10) and lindane-D6 LGC (Teddington, United Kingdom) were also employed.

Optima LC-MS grade water was obtained from Fisher Scientific (Fair Lawn, NJ, USA). LC-MS grade ethyl acetate was acquired from Fluka Analytical (Steinheim, Germany). *n*-Hexane and acetone HPLC-grade grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultra-gradient HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Tenax® TA was purchased from Scharlab (Barcelona, Spain), and silicone wristbands were acquired from ORAKEL (Athens, Greece) and Giftline (Athens, Greece).

2.2. Location and management of the apiaries; sampling dates

Eleven apiaries acting as sampling sites were established in Austria (4 apiaries), Denmark (4 apiaries) and Greece (3 apiaries). The location of these apiaries can be found in the Supplementary material. The rationale for the selection of citizen scientists managing apiaries was based on previous works [23,39]. Briefly, citizen scientists had been regarded as experienced beekeepers well-educated in collecting samples. Through instruction manual, trainings, online meetings, and onsite visits by study coordinators, compliance with the sampling protocols was ensured. Veterinary substances were not applied to the colonies immediately before the onset of the study.

One colony from each apiary was selected for the honey bees, pollen, propolis, APIStrip and wristband sampling, which took place in June 2022. All samples were taken simultaneously from the colonies; the passive samplers –i.e., APIStrips and wristbands– had been placed inside the colonies 14 days before the sampling date to ensure an optimal exposure to the beehive environment (an exposure period already assessed in previous work for APIStrips) [22].

2.3. Sampling and sample treatment

Six different samples were taken from each apiary: three apicultural matrices (honey bees, pollen and propolis) plus three passive

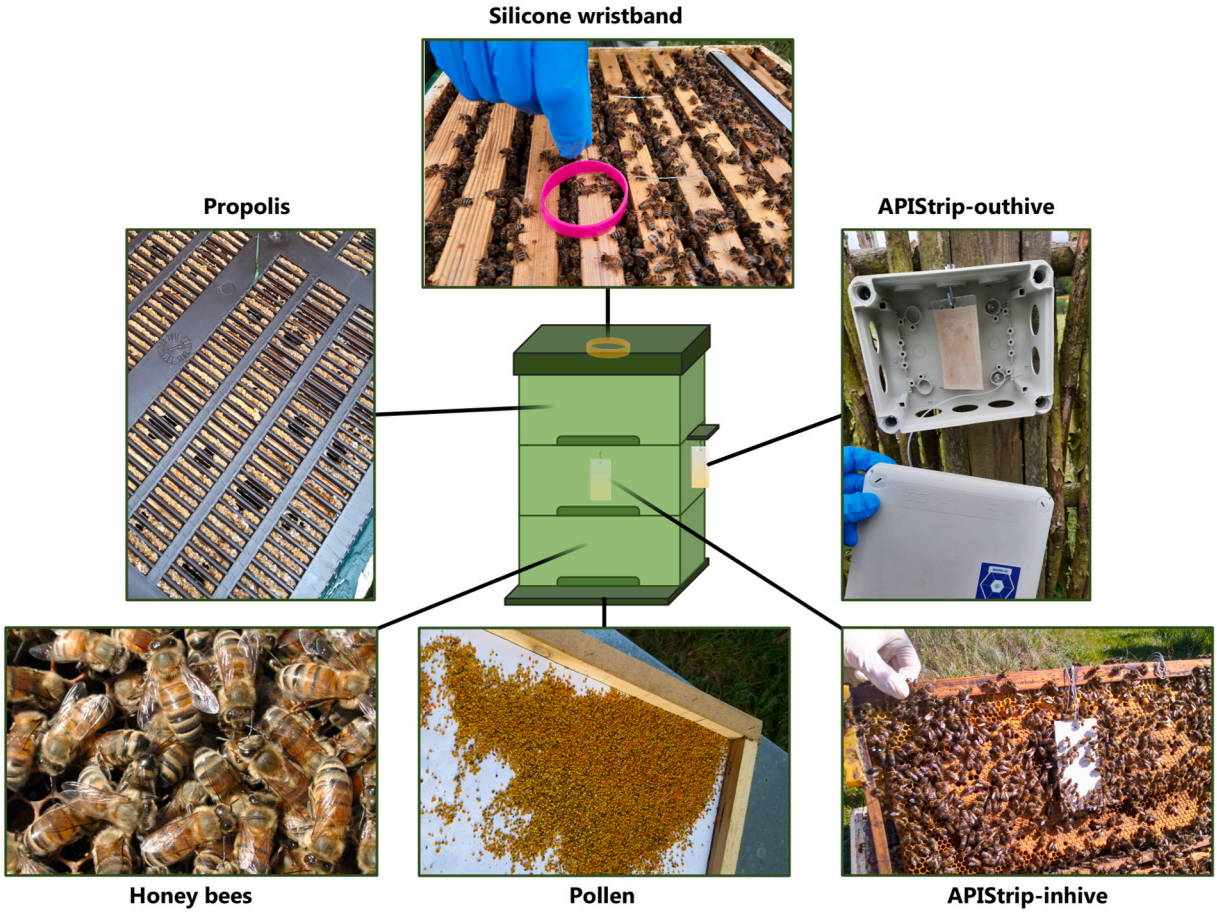


Fig. 1. Matrices sampled in every apiary.

samplers (APIStrips located inside the beehives, APIStrips located outside, and silicone wristbands), as shown in Fig. 1. This resulted in a total of 66 samples: 11 samples per matrix, corresponding to each one of the apiaries.

2.3.1. Active sampling: honey bees, pollen and propolis

Adult honey bees were taken from a brood frame filled with bees in the beehives. The extraction of PAHs residues from the honey bees was performed following a modified QuEChERS method with extra clean-up salts and vigorous agitation, as reported in recently published work [39]. First, the sample (2 g) was weighed in a 50-mL PTFE centrifuge tube and 5 mL of ultrapure water were added. The samples were then shaken manually and let stand for 5 min. Subsequently, 5 mL of acetonitrile were added, and the samples were automatically shaken in a Geno/Grinder® by SPEX® (Metuchen, United States) for 10 min at 1250 rpm. $MgSO_4$, NaCl, and citrate salts (same amounts as in Ref. [17]) were added, and the samples were shaken again in an automatic axial extractor (AGYTAX®, Cirta Lab. S.L., Spain) for 5 min. The samples were centrifuged (4000 rpm) for 5 min, and 2 mL of the supernatant were transferred to a 15-mL PTFE centrifuge tube containing $MgSO_4$, Z-Sep and PSA (same quantities as in Ref. [39]), vortexed and centrifuged (4000 rpm) for 5 min. The eluates were transferred into amber vials and acidified with 10 μL of 5 % formic acid per mL of extract. Overall, the extraction involves a 2.5-fold dilution that was reverted during the preparation of the injection vials. Procedural internal standards were employed to control the extraction performance: naphthalene-D_8, acenaphthene-D_{10}, phenanthrene-D_{10}, and pyrene-D_{10}. Lindane-D_6 was employed as an injection internal standard to check the variations in the injection volume. Matrix-matched calibration curves were constructed as follows: a blank extract was evaporated and reconstituted with the injection solvent containing a mixture of the 27 PAHs and derivatives at 0.5, 1, 5, 10, 50 or 200 μg/L.

The extraction of pollen samples (collected by pollen traps at the beehive entrance over 24 h) was also identical to the previously published procedure regarding beebread [17]. The 2-fold dilution was undone during the vial preparation, analogous to the honey bee samples.

Propolis samples (0.5 g) collected with plastic traps on the top frames of the colony were cut into small pieces using laboratory scissors and fortified with internal standards, mixed with 2 mL *n*-hexane: acetone (1:1, v/v), and subjected to sonication at 20 °C (Elmasonic Select 60, Elma Schmidbauer GmbH, Singen, Germany) for 10 min. Then, 4 mL ACN were added, and the mixture was vortex-mixed (VM-10, Witeg, Wertheim, Germany) for 1 min. 0.5 g NaCl and 1 g $MgSO_4$ were added, the resulting mixture was hand-shaken for 1/2 min, and centrifuged for 10 min (4.500 rpm, 10 °C). The upper layer was transferred to another 15 mL PTFE tube, in which 0.2 g C18, 0.4 g PSA, and 0.2 g Z-Sep$^+$ were sequentially added, followed by vortex-mixing for 1 min. After centrifugation (10 min, 4.500 rpm, 10 °C), the supernatant was evaporated to dryness under vacuum (rotary evaporator, the temperature of the bath not exceeding 25 °C). The dry residue was reconstituted in 1 mL of an equimolar mixture of hexane:acetone, filtered (PTFE syringe filter, 0.22 μm) and injected into the GC-MS/MS system. Before use, PTFE filters were soaked-cleaned with *n*-hexane and left to dry at ambient conditions inside a closed laboratory hood (same soaking-cleaning procedure was applied for the PTFE centrifuge tubes before being used). Surrogate and injection standards were employed for pollen and propolis as well, as described for honey bee analysis.

2.3.2. Passive sampling: APIStrips

The preparation of the APIStrip sampling devices is described in detail in a previous study [22] and a recently published work [39]. APIStrips were placed in two different inside locations in the apiaries: one APIStrip was placed inside the beehives, hanging from the lowest box (in the bee lane between two brood frames); the other APIStrip was placed next to the beehive, hanging inside a small box with holes on the walls to ensure a maximum air flow while protecting the sampler from the rain (Fig. 1).

The desorption procedure of the PAHs from the APIStrips was envisaged from a similar process applied for pesticides desorption [39]. Briefly, it was performed by automatic agitation for 10 min at room temperature in the presence of *n*-hexane as the extraction medium. The injection vials were prepared following the recently published approach [39] and finally reconstituted with 50 μL of the injection solvent (*n*-hexane). Surrogate and injection standards were employed, as in the case of pollen and honey bees.

2.3.3. Passive sampling: silicone wristbands

The silicone wristbands used for sampling were placed inside the beehives. Before their placement in the beehives a clean-up procedure was performed. It entails one silicone wristband that is sequentially extracted (× 5) with a mixture of 50 mL of ethyl acetate/*n*-hexane (1:1, v:v). Each extraction occurs after a minimum of 45 min at 300 revolutions per minute (using a platform shaker). The last extraction is aided by ultrasound sonication at 30 °C for 2 h. The wristbands are positioned in an oven at 150 °C for 3 h. Afterward, the dried wristbands are placed in a glass laboratory desiccator prior to use as a sampler. For ergonomic and cost-effective reasons, the procedure can be upscaled to 5 wristbands that can be cleaned simultaneously using the same organic solvent cleaning sequence eliminating matrix interferences. The organic solvents are collected, distilled, and reused. During dispatch and before field deployment, the wristbands are placed in PTFE transport/storage bags.

After the sampling, each wristband (~4.5 g) was cut in a borosilicate glass bottle containing 5 mL of ethyl acetate and the internal standards (as above) were added. Then, 25 mL of ethyl acetate were added, and the samples were shaken in the platform shaker for 30 min. Then, the sample was sonicated for an additional 30 min. The extract was collected in a round bottom flask. The same extraction process was repeated once, and the overall organic extract was evaporated to dryness under vacuum (rotary evaporator, the temperature of the bath not exceeding 25 °C). The final dried extract was reconstituted in 1 mL of hexane: acetone (1:1, v/v), filtered (PTFE, syringe filter, 0.22 μm) and injected into the GC-MS/MS system. Procedural and injection internal standards were similarly employed as for the other matrices investigated in this study. Silicone wristbands obtained from both companies, after clean-up and extraction, performed equally in terms of matrix interferences (negligible).

2.4. GC-MS/MS analysis and method validation metrics

Two analytical laboratories participated in the sample analysis, which was performed within the framework of the INSIGNIA-EU project. The role of each laboratory in the said project involved the analysis of a specific set of samples and matrices, divided according to their respective expertise. One of the laboratories performed the analysis of honey bees, pollen and APIStrips, whereas the other laboratory processed and analyzed the propolis and wristband samples. Considering that the aim of the present study was to perform a critical matrix comparison for the environmental monitoring of PAHs, ensuring analytical parity was of paramount importance. With this purpose, various precautionary and quality control measures were adopted. Although the method configurations were adapted to each analytical instrument to ensure optimum PAH detection, exhaustive preliminary tests ensured the parity between both instruments for all matrices and analytes included in the present study. With this aim, both laboratories performed simultaneously the validation studies prior to the analysis of real samples, obtaining comparable results in terms of recoveries and limits of quantification. The detailed validation results of both instruments can be found in the *Supplementary information*. Additionally, the presence of interferences was evaluated in both instruments for each matrix-analyte combination: no significant interfering signals that affected the quantification were observed in any case. The results obtained in these preliminary experiments ensure the inter-laboratory reproducibility and verify that any differences in the matrices were not attainable to the use of two independent instruments. To further ensure the comparability of the results, two samples per matrix were exchanged after their analysis and re-analyzed with the other instrument, obtaining the same results in terms of detection of substances and concentration values with a difference lower than 1.2 ng/g.

For the PAH identification, the identification criteria described in the SANTE Document No. 11312/2021 [40] were embraced, emphasizing the compliance of the ion ratio of qualifier and quantifier selected reaction monitoring (SRM) transitions of the samples with those of the calibration standards and the acceptable retention time tolerance (±0.1 min). The instrumental limit of quantification (iLOQ) was defined as the minimum calibration point that allowed the identification of each compound according to the criteria described in the aforementioned document. If one compound was identified according to the quality criteria, but at a concentration lower than its iLOQ, it was reported as "< iLOQ" to indicate that it was detected at trace levels. Two co-eluting pairs of PAHs (benzo[*b*]fluoranthene and benzo[k]fluoranthene, 1-methylnaphthalene and 2-methylnaphthalene) shared transitions and were, therefore, reported as the sum of concentrations for each pair. For accuracy determination, recovery experiments were performed by fortifying all matrices (triplicate spiking) at two concentration levels with an appropriate mixture of PAHs and respective internal standards. Selectivity was assessed after injecting blank control samples (n = 5) of each matrix and comparing their response at the retention time of each analyte with the respective from standard injections (n = 5) at the iLOQ. Selectivity was considered acceptable when the average response of the blank samples was <30 % of the respective response at the iLOQ.

2.4.1. Analysis of honey bees, pollen and APIStrips

The multiresidue analyses were performed in an Agilent Intuvo 9000 GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 7693 autosampler and an Agilent 7010B GC-MS/MS triple quadrupole. Data acquisition and processing was developed by Agilent MassHunter QQQ Acquisition and Quantitative Analysis software version 10.0. Samples were injected using a multimode injector inlet in splitless mode, through an Agilent ultra-inert inlet liner with glass wool frit. The injection volume was 1 μL. The injector temperature was kept at 80 °C during the solvent evaporation stage (0.1 min) and then ramped up to 300 °C at 600 °C/min for 5 min and down to 250 °C at 100 °C/min. Two planar columns (Agilent), HP-5MS UI 15 m length × 0.25 mm i.d. × 0.25 μm film thickness were used, including a mid-column backflush flow chip.

The oven temperature program was as follows: 60 °C for 0.5 min, increased to 170 °C at 80 °C/min and finally up to 310 °C at 20 °C/min (held for 6.5 min). The total run time was 15.38 min with 2.1 additional min for backflushing at 310 °C. The instrument worked at a constant flow (1.28 mL/min column 1, 1.48 mL/min column 2). The system worked in dynamic MRM, acquiring the transitions in a ±0.2 min window from the retention time of each analyte. Helium (99.999 % purity) was used as the carrier and quenching gas, and nitrogen (99.999 % purity) as the collision gas. The collision and quenching gas flows were 1.5 mL/min and 2.25 mL/min, respectively. Both the transfer line and the ion source −operated in electron ionization− were maintained at 280 °C. The quadrupole analyzer temperature was fixed at 150 °C. The solvent delay was 2.6 min.

2.4.2. Analysis of propolis and silicone wristbands

The multiresidue analyses were performed in a Shimadzu Nexis GC 2030 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-6000 autosampler and a Shimadzu GCMS-TQ8040 NX triple quadrupole. Data acquisition and processing were performed by LabSolutions GCMS solution software, version 4.52. Samples were injected using a PTV injector inlet in splitless mode, through a Shimadzu ultra-inert inlet liner with glass wool frit. The injection volume was 2 μL. The injector temperature was kept at 250 °C. A MEGA 5-HT (MEGA S.r.l., Legnano, Italy) column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness) was used.

The oven temperature program was as follows: 50 °C for 1 min, increased to 100 °C at 20 °C/min, ramped linearly to 200 °C at 5 °C/min, and finally up to 280 °C at 20 °C/min (held for 5 min). The total run time was 44.5 min. The instrument worked at a constant flow of 1.6 mL/min, using the MRM mode, acquiring the transitions in a ±0.2 min window from the retention time of each analyte. Helium (99.999 % purity) was used as the carrier gas and argon (99.999 % purity) as the collision gas. The transfer line and the ion source −operated in electron ionization− were maintained at 250 °C and 230 °C, respectively. The detector voltage was set at 0.6 kV (relative to the tuning result). The quadrupole analyzer temperature was fixed at 150 °C. The solvent delay was 1.5 min.

3. Results and discussion

3.1. Recovery studies and iLOQs

Table 1 depicts the average recovery of three replicates per matrix at a concentration of 5 ng/g (wristband, propolis, bees, pollen) or 5 ng/APIStrip (APIStrips) in each one of the matrices incorporated in the present study, as well as their iLOQs. In all cases of recovery assessment, the individual results showed a relative standard deviation (% RSD) lower than 20 %. In most cases, the iLOQ was between 0.5 and 1 ng/g (or ng/APIStrip) with just a few exceptions, such as the derivatives 1-nitronaphthalene and 1-nitropyrene (5 ng/g for most matrices and 5 ng/APIStrip). There were, in general, few remarkable variations in the iLOQ of a given PAH among the matrices, signifying that the variability of these matrices is not probable to affect the detection of PAHs at trace levels within them. Satisfactory recoveries (65–96 %, full data not shown) were also obtained for an appreciable number of analytes (% RSD <18 %) when fortifications were performed at a concentration of 1 ng/g (wristband, propolis, bees, pollen) or 1 ng/APIStrip (APIStrips). To ensure that no bias existed in the matrix comparison due to the use of two different instruments, these experiments were simultaneously performed in both of them, leading to comparable recoveries (Supplementary information, Table S1) with no significant differences (Student's t-test *p*-values higher than 0.05 in the vast majority of cases, for indicative statistics see Fig. S2).

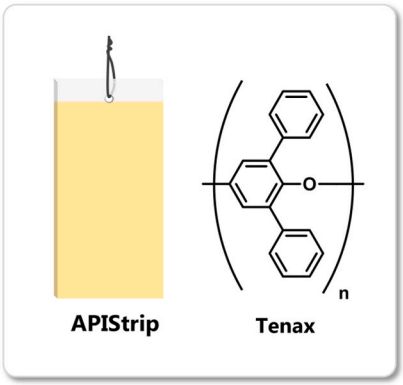
As regards the overall recoveries obtained for PAHs, in most cases, the values were in the range >61–107 % (Table 1). The only exception was found in the APIStrips, in which low to medium recoveries (<60 %) were observed for the larger PAHs –i.e., those having high molecular weight. The reason for this is likely related to the chemical nature of both samplers and PAHs. The APIStrips are made of the organic polymer Tenax, poly(2,6-diphenyl-*p*-phenylene oxide), which contains three aromatic rings in its monomeric structure. Therefore, there is a strong chemical similarity between the APIStrips' sorbent and the PAHs analyzed in this work, which may result in intermolecular π-π interactions between parallel aromatic rings from both PAHs and Tenax (Fig. 2). This spatial disposition is favored due to (i) the abundance of fused aromatic rings (PAH structure) and benzene rings (Tenax polymer); (ii) the rigid structure of PAHs derived from the fused aromatic rings, which results in a planar structure with little to null flexibility [41]; (iii) the relatively low incidence of other functional groups. These intermolecular forces will be stronger with larger PAHs, which contain more fused rings for the π-π interactions with Tenax. Thus, PAHs with high molecular weight can establish chemical interactions with the sorbent which are too strong to allow proper desorption from the APIStrip surface.

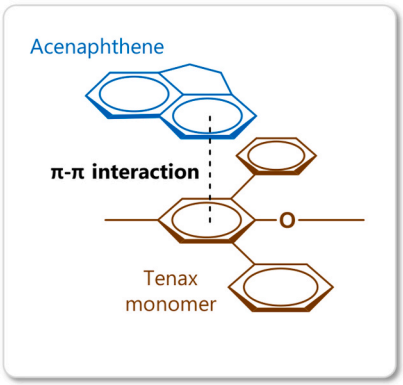
During the extraction method optimization, multiple parameters were tested (including various organic solvents such as acetonitrile, ethyl acetate, *n*-hexane, acetone, and mixtures), shaking strength (1000–1600 rpm), shaking time (5–30 min), use of ultrasound for different amounts of time and temperature (up to 60 °C, according to the solvent possibilities and up to 30 min). However, the recovery values obtained were always similar, with lower recoveries of the large PAHs (**Fig. 2**). These findings are in accordance with

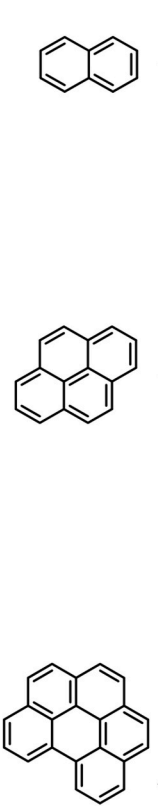
Table 1
Recoveries and iLOQs for PAHs in apicultural matrices and passive samplers.

Name	Recovery (%)					iLOQ (ng/g or ng/APIStrip)				
	Wristband	APIStrip	Propolis	Honey bees	Pollen	Wristband	APIStrip	Propolis	Honey bees	Pollen
Naphthalene	99	101	98	83	78	1	1	0.5	1	1
1-Methylnaphthalene - 2-Methylnaphthalene	84	84	90	85	94	0.5	1	1	1	1
Acenaphthene	96	57	89	105	89	0.5	5	2	1	5
1,4-Dimethylnaphthalene	86	69	81	74	77	0.5	1	2	1	5
1,4-Naphthoquinone	86	79	96	78	74	0.1	0.5	1	0.5	0.5
Fluorene	80	64	91	104	96	1	0.5	1	0.5	0.5
1-Nitronaphthalene[a]	81	80	80	90	84	5	5	5	5	5
Phenanthrene	99	75	94	94	104	0.1	1	1	0.5	1
Anthracene	82	69	89	88	102	0.5	1	2	0.5	1
9-Fluorenone	78	66	80	102	81	0.5	0.5	1	0.5	1
9,10-Dihydroanthracene[a]	79	68	78	94	95	1	1	2	1	1
Fluoranthene	89	66	85	84	99	0.5	0.5	1	0.5	0.5
Pyrene	83	62	79	106	81	0.5	0.5	2	0.5	0.5
1-Methoxy-4-nitronaphthalene[a]	75	54	78	92	88	1	0.5	0.5	0.5	1
9-Nitroanthracene	73	63	76	77	87	2	1	5	1	5
2-Nitro-9-fluorenone[a]	73	51	79	76	101	1	0.5	1	0.5	1
Chrysene	79	62	83	90	97	1	0.5	1	0.5	0.5
Benzo[*a*]anthracene	79	61	76	103	78	1	0.5	1	0.5	0.5
1-Nitropyrene[a]	78	41	82	80	89	1	5	5	5	5
Benzo[*b*]fluoranthene - Benzo[k]fluoranthene	74	54	74	95	79	0.5	0.5	1	0.5	0.5
Benzo[*a*]pyrene	74	40	84	90	85	1	0.5	2	0.5	0.5
Benzo[e]pyrene	76	50	79	105	93	1	0.5	1	0.5	0.5
Indeno [1,2,3-cd]pyrene	72	42	74	94	73	1	0.5	2	0.5	0.5
Benzo[ghi]perylene	71	32	77	74	77	1	0.5	1	0.5	0.5
Dibenzo[ah]anthracene	79	38	77	105	107	1	0.5	2	0.5	0.5

[a] PAH derivative.







Compound	Mol. weight (g/mol)	Rec. (%)
Naphthalene	128.1	101
1-Methylnaphthalene 2-Methylnaphthalene	142.2	84
Acenaphthene	154.2	57
1,4-Dimethylnaphthalene	156.2	69
1,4-Naphthoquinone	158.2	79
Fluorene	166.2	64
1-Nitronaphthalene	173.2	80
Phenanthrene	178.2	75
Anthracene	178.2	69
9-Fluorenone	180.2	66
Fluoranthene	202.3	66
Pyrene	202.3	62
1-Methoxy-4-nitronaphthalene	203.2	54
9-Nitroanthracene	223.2	63
2-Nitro-9-fluorenone	225.2	51
Chrysene	228.3	62
Benzo[a]anthracene	228.3	61
1-Nitropyrene	247.3	41
Benzo[b]fluoranthene Benzo[k]fluoranthene	252.3	54
Benzo[a]pyrene	252.3	40
Benzo[e]pyrene	252.3	50
Indeno[1,2,3-cd]pyrene	276.3	42
Benzo[ghi]perylene	276.3	32
Dibenzo[ah]anthracene	278.4	38

Fig. 2. Chemical structure of poly(2,6-diphenyl-*p*-phenylene oxide) (Tenax) and selected PAHs (naphthalene, fluoranthene, dibenzo[ah]anthracene), plus PAH recoveries from the APIStrip surface employing the selected extraction conditions and example of π-π interaction.

the abovementioned hypothesis: PAHs with a higher number of aromatic rings are bonded too tightly to the APIStrips surface and their desorption is hindered.

On the other hand, silicone wristbands –the other passive sampler evaluated in the study– are made of polydimethylsiloxane (PDMS), with a small monomeric structure that does not contain aromatic rings. This polymer forms weaker bindings with the PAHs and they are easily desorbed from the samplers. This was confirmed during the extraction method optimization: even mild extraction conditions led to recoveries in the range of 71–99 % for all PAHs included in the present study. The analytical method exhibited substantial selectivity since, in all cases, the mean response of each analyte obtained from the blank control samples did not exceed 28 % of the response obtained at the iLOQ.

3.2. PAH detection in apicultural matrices and passive samplers

This study assessed six matrices for environmental PAH monitoring through honey bee colonies. For this purpose, samples were taken from 11 apiaries distributed in Austria, Denmark and Greece, resulting in 66 total samples. These samples included three apicultural matrices (propolis, bees, and pollen), in which PAHs had previously been reported [26–28], as well as two passive sampling devices: APIStrips and silicone wristbands. Two APIStrips were employed in each sampling site: one of them was located inside the beehives (APIStrip-inside), whereas the other APIStrip was placed outside the beehives, attached to the beehive surface and protected from the rain (APIStrip-outside). The use of these two APIStrips allowed the determination of the best sampler location: inside the beehive, there is continuous contact with the honey bees from the colony, whereas outside the beehive, direct contact with bees is minimal.

Considering all six matrices included in the present study, a total of 20 PAHs and derivatives were identified (Table 2). As shown in Table 2 and summarized in Fig. 3, two matrices allowed to detect a significantly higher number of PAHS: wristbands and pollen (14 and 13 compounds detected, respectively). Conversely, APIStrips, propolis, and bees resulted in the identification of only 2 to 5 compounds per matrix. Additionally, all compounds identified in the latter matrices were as well detected in wristbands and/or pollen samples. As none of these matrices provides any information that cannot be found in pollen and/or wristbands, it can be stated that APIStrips, propolis and bees are not the most suitable matrices for PAH monitoring.

Naphthalene and its monomethyl derivatives were identified in all matrices, with the highest frequency of detection corresponding to wristbands (up to 11 positive samples, which corresponds to 100 % wristbands) followed by APIStrips-outside and pollen. Due to its multiple emission sources [15], this compound has been reported in multiple environmental matrices in a large number of studies, and

it is often the most frequently detected PAH [3,11,16]. The average concentrations of naphthalene and the pair 1-methylnaphthalene – 2-methylnaphthalene were also among the highest ones of the set (up to 17.3 ng/g for the monomethyl pair and 13.7 ng/APIStrip for naphthalene). Fluoranthene was also detected in most matrices, the only exception being APIStrip-inhive; the highest concentrations of this compound were identified in wristbands and pollen samples, whereas the concentration accumulated in the remaining matrices was notably lower. It is remarkable that pyrene was detected in the majority of wristband and pollen samples (from 8 to 10 out of 11 samples), whereas it was not identified in any of the other matrices. The common detection of this compound in two of the matrices reveals a widespread presence within the beehives' surroundings, regardless of the apiary location. However, none of the remaining samples allowed to identify this compound. These results further support the idea of APIStrips, propolis and honey bees not being a suitable matrix for PAH analysis.

3.2.1. Apicultural matrices

Propolis is composed of a mixture of phenolic compounds, aromatic acids, essential oils and waxes, among other constituents. It is one of the most complex apicultural matrices due to this considerable number of components and its resin character, which make it necessary to perform specific sample treatment procedures prior to the chemical analysis [42]. However, despite the use of exhaustive clean-up procedures, the matrix complexity may hinder the detection of certain contaminants. In the same context, the analysis of propolis for contaminant monitoring entails a limited representation of the outer environment of the beehive compared to bees and pollen (both having direct contact with the colony surroundings).

Honey bees are directly exposed to PAHs during their foraging activities. However, honey bee samples employed for analysis usually comprise approximately 20 individuals, which makes up a very low proportion of the colony (around 40000 adult honey bees). This may reduce the representativity of the samples –i.e., the findings will depend on the specific individuals employed for the analysis, which may lead to underestimations or overestimations of the actual contaminant presence. In this case, the honey bee sampling led to an underestimation of the PAH content in the apiaries.

The same representativity issues could be, in principle, attributed to pollen samples: bees collect large amounts of pollen, whereas only 2 g are usually employed in the analysis. However, there is a crucial difference between both matrices: whereas not all honey bees perform foraging activities, pollen is always collected from the environment. Considering that PAHs can be transported through the air [2], it is expected that all pollen from a certain environment will be exposed to similar levels of these contaminants. This may result in (i) a higher number of PAHs detected compared to honey bee samples, and (ii) an increased representativity of the pollen analyses compared to honey bees. Therefore, considering its direct contact with the environment outside the beehives, pollen is probably the best apicultural matrix for PAH monitoring.

3.2.2. Passive samplers: APIStrips and silicone wristbands

Considering both types of APIStrips (inhive and outhive), those located inside the beehives only provided detections of naphthalene and its monomethyl derivatives (total 6 detections), whereas the ones located outside the beehives allowed the additional detection of fluoranthene and fluorene (total 28 detections). The different exposures of these APIStrips are probably the cause of these findings: when an APIStrip is placed inside a beehive, honey bees come immediately to try and remove it. This compulsive behavior results in direct, continuous contact APIStrip-bees throughout the sampling time (14 days), and therefore a reduced APIStrip exposure to the air

Table 2
Number of detections and average concentration in ng/g[a] (in brackets) of the identified PAHs.

Compound	Wristband	APIStrip inhive	APIStrip outhive	Propolis	Bees	Pollen
Naphthalene	11 *(4.4)*	3 *(13.7)*	11 *(6.5)*	3 *(1.3)*	4 *(1.0)*	8 *(9.0)*
1-Methylnaphthalene	10 *(17.3)*	3 *(3.1)*	7 *(2.5)*	1 *(5.9)*	1 *(2.0)*	4 *(8.0)*
2-Methylnaphthalene						
Acenaphthene	6 *(2.0)*			1 *(< iLOQ)*		
1,4-Dimethylnaphthalene	8 *(2.0)*					
1,4-Naphthoquinone	4 *(<i LOQ)*					
Fluorene	4 *(2.9)*		4 *(1.1)*			8 *(2.6)*
Phenanthrene	5 *(0.8)*					
Anthracene	6 *(0.8)*			1 *(4.4)*		
9-Fluorenone	7 *(< iLOQ)*					4 *(2.8)*
9,10-Dihydroanthracene	2 *(9.7)*					
Fluoranthene	5 *(9.4)*		6 *(1.1)*	2 *(2.5)*	3 *(1.6)*	11 *(13.8)*
Pyrene	8 *(4.4)*					10 *(6.3)*
2-Nitro-9-fluorenone	1 *(9.9)*					
Chrysene						2 *(1.4)*
Benzo[*a*]anthracene						2 *(2.5)*
Benzo[*b*]fluoranthene	4 *(0.7)*					3 *(0.8)*
Benzo[k]fluoranthene						
Benzo[*a*]pyrene						1 *(1.1)*
Benzo[e]pyrene						2 *(0.8)*
Indeno [1,2,3-cd]pyrene						1 *(0.6)*
Benzo[ghi]perylene						1 *(0.6)*

[a] The concentration of PAHs in APIStrips is reported in ng/APIStrip, and in wristbands, it is ng/g silicone.

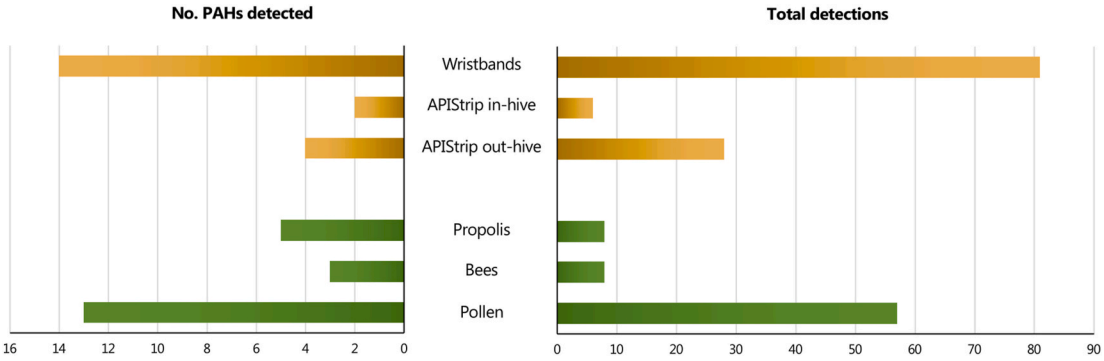


Fig. 3. Number of PAHs and total number of detections in passive samplers (yellow-brown) and apicultural matrices (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

within the beehive. This is something beneficial for pesticide residue analysis, as it ensures that the APIStrips will be exposed to a large amount of pesticides carried out onto the honey bees' bodies and that representative information will be obtained from the subsequent analysis [43]. However, given the small amount of PAH residues accumulated within the honey bees (Table 1), it may reduce the APIStrip-inhive capability to monitor the real environmental levels. Conversely, APIStrips-outhive did not attract the honey bee's attention –i.e., their contact with the ambient air was maximum–. This could explain the differences between the PAH residues detected in APIStrips-inhive and outhive, given that the samplers were the same in both cases.

However, none of the APIStrips (either inhive or outhive) allowed to detect the same PAH levels as the silicone wristbands: the latter sampler resulted in 81 total detections, 289 % more than the APIStrips-outhive. The experimental results obtained support the idea of silicone wristbands being a better passive sampler than APIStrips for PAH sampling. The efficiency of silicone wristbands as PAH passive samplers has been reported by several groups [34,44,45]; hence, this work adds another evidence in this direction and, for the first time, applied to the environmental PAH analysis through honey bee colonies.

In the apiaries involved in the present study, all silicone wristbands were placed inside the beehives. However, they were located in a different area (on top of the frames) than the APIStrips, where they received little attention from the honey bees. Therefore, they were not exposed to intense contact with the colony individuals, as happened with the APIStrips hanging between frames: the wristband exposure to the air within the beehive was more intense than the one of APIStrips-inhive. Therefore, if the silicone wristbands are to be located outside the colony (analogously to the APIStrips-outhive), little differences with the ones inhive are expected. To evaluate these differences, two silicone wristbands were placed simultaneously inside and outside a beehive (located in apiary 1, Greece) and, after 14 days, they were analyzed for PAHs. The results of this study can be found in Table 3: both samplers allowed to detect naphthalene and its monomethyl derivatives, while the wristband outside the beehives contained also low levels of 1,4-dimethylnaphthalene, and the one inside, fluoranthene and benzo[*b*]fluoranthene.

There is no statistical difference between the location (inhive or outhive) of the wristbands in terms of results (p-values obtained after implementing Student's t-test were higher than 0.05, analysis of significance detailed in the Supplementary information, Fig. S3). This can be due to the abovementioned fact that honey bees hesitate to remove the wristbands from the top of the frames, thus ensuring an optimum wristband exposure to the air flux within the beehive. However, the use of silicone wristbands inside the beehives for PAH sampling is herein encouraged for multiple reasons. The most practical aspect concerns the intactness of the samplers and the ease of sampling: whereas the wristbands-inhive can be placed directly in the beehives, where they will be protected from the atmospheric conditions (wind, rain, etc.), the ones located outside need special conditions to ensure their resistance during the exposure period. Therefore, additional materials and preparatory arrangements should be made prior to the samplings. Additionally, the temperature and humidity conditions inside the beehive suffer from lower variations than the environment outside, thus reducing the variables that could affect the homogeneity and representativity of the samplings, especially when multiple locations are to be compared.

3.3. Study limitations

One limitation of this study is the relatively small number of samples analyzed. While the study encompassed a wide range of different matrices, the sample size comprised a total of 66 samples (11 samples per matrix). The findings of this study, as well as the statistical analysis of the data, would have benefited from a larger number of samples to further understand the PAH dynamics in apicultural matrices. Additionally, the use of two GC-MS/MS instruments in different analytical laboratories adds a degree of uncertainty to the results. Although the possible implications of separated analyses have been controlled by the quality control measures before and during the sample analysis, they would have been eliminated if only one instrument had been employed. Lastly, the analytical scope included 27 common PAHs and derivatives, but some widespread PAHs (such as retene or acenaphthylene) were not included in the analyses, with a potential loss of environmental information.

Table 3
PAH residues detected in silicone wristbands located inside and outside the same beehive (ng/g) ± SD.[a].

Compound	Wristband-inhive	Wristband-outhive
Naphthalene	5.2 ± 1.1	3.9 ± 0.8
1-Methylnaphthalene	7.2 ± 1.8	5.4 ± 2.1
2-Methylnaphthalene		
1,4-Dimethylnaphthalene	n.d.[a]	1.1 ± 0.4
Fluoranthene	2.1 ± 0.6	n.d.
Benzo[*b*]fluoranthene	< iLOQ	n.d.

n.d. non-detected.
[a] SD = standard deviation (n = 3).

4. Conclusions

In the studies investigating PAHs monitoring through honey bee colonies, the matrix selected for the analyses determines, to a great extent, the results obtained. The use of propolis, honey bees or APIStrips as samples may lead to important underestimations in the PAH load, whereas silicone wristbands and pollen have been shown to provide a comprehensive and representative image of these substances in the environment. Additionally, silicone wristbands are a passive sampling approach that: (i) minimizes the human alterations produced to the colonies, and (ii) ensures the viability of long-term monitoring studies (as there is no dependence on the existence of pollen collected by the bees at the time of sampling). APIStrips were found to yield low recoveries for PAHs with high molecular weight due to the existence of π-π interactions with the sampler sorbent, Tenax. Overall, the best matrix-location combination for the environmental assessment of PAHs was found to be silicone wristbands placed inside the beehive (as a means to ensure the sampler stability throughout the 14-day sampling period).

CRediT authorship contribution statement

María Murcia-Morales: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Evangelia N. Tzanetou:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. **Guillermo García-Gallego:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization. **Konstantinos M. Kasiotis:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Flemming Vejsnaes:** Resources. **Robert Brodschneider:** Resources. **Fani Hatjina:** Resources. **Kyriaki Machera:** Resources, Supervision. **Jozef J.M. Van der Steen:** Conceptualization, Funding acquisition, Resources, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23564.

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