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An analytical multi-residue approach for the determination of semi-volatile organic pollutants in pine needles

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Abstract

Vegetation (and pine needles in particular) has been widely used as an alternative to other conventional sampling devices to assess the atmospheric presence of semi-volatile organic contaminants (SVOCs). While most analytical procedures developed focus only on one or two chemical classes, this this work intends to establish a multi-component protocol to quantify brominated flame-retardants (BFRs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polynuclear aromatic hydrocarbons (PAHs) and one class of contaminant of emerging concern, the synthetic musks fragrances (SMCs). Pine needles extracts were obtained by ultrasonic solvents extraction (USE), and different cleanup approaches using solid-phase extraction (SPE) employing combinations of sorbents and solvents as well as gel permeation chromatography (GPC) were tested. SPE with alumina followed by GCP yielded the best results, with average recoveries over 80%.

The application of the method under field conditions was proven by the analysis of naturally contaminated samples from 3 sites of different potential exposure (remote, rural and urban). The total detected concentrations ranged from 0.45 to 0.87 ng g^{-1} dry weight (dw) for BFRs, 0.35 to 1.01 ng g^{-1} (dw) for PCBs, 0.36 to 12.2 ng g^{-1} (dw) for HCB, 245.7 to 967.8 ng g^{-1} (dw) for PAHs and 20.7 to 277.5 ng g^{-1} (dw) for SMCs.

This methodology is a viable approach for the simultaneous analysis of five different classes of atmospheric pollutants employing less analytical efforts. Moreover, to the author's best knowledge, this is also the first time vegetation

is employed in the detection of SMCs.

1. Introduction

The continuous release of hazardous chemicals into the atmosphere urges the development of comprehensive and expedite methodologies for their detection and subsequent study. Current emissions may not only comprise new contaminants, with scarce information about occurrence and impact on environmental health available, but also the so-called legacy persistent organic pollutants (POPs). These compounds belong to a variety of chemical classes that were widely used in the past, but have since been, restricted, banned or discontinued, yet they still remain in the environment due to their persistence [1]. Furthermore, their volatility, toxicity, bioaccumulation capacity and resistance to natural breakdown, either by biological, chemical or photochemical reactions, make them prone to long-range atmospheric transport (LRAT) [2] causing an environmental impact on areas far away from their points of emission. In 2004, the Stockholm Convention on Persistent Organic Pollutants (SCPOP) became effective, aiming to ban or restrict the use of POPs [2]. Examples include polychlorinated biphenyls (PCBs), brominated flame- retardants (BFRs), some organochlorine pesticides (OCPs) and, although not a part of the list but with similar properties, polycyclic aromatic hydrocarbons (PAHs). PCBs were used as cooling and dieletric fluids in transformers and capacitors and banned in 1979 in the USA [3] and 1985 in the EU [4]. BFRs, namely polybrominated diphenyl ethers (PBDEs) were widely incorporated as flame retardants in electrical appliances and furniture, but have now been restricted in several states of the USA [5] and in the EU [6]. Some organochlorine pesticides (OCPs) employed in crop protection and pest control, such as hexchlorobenzene (HCB), have also been banned globally under the SCPOP [2]. While BFRs, PCBs and OCPs are synthetic compounds and therefore exclusively of anthropogenic sources, PAHs derive not only from human activities (combustion in traffic, industries and home heating) but also from natural processes associated with fossil fuels (forest fires, volcanic eruptions, etc.) [7]. The list of POPs is periodically reviewed and new contaminants can be added, once their effect on organisms, persistence and LRAT capacity is evaluated. Synthetic musk compounds (SMCs), widely incorporated in personal care and household products, are one of the "emerging" candidates. Used in rather high quantities on a daily basis, their bioaccumulative potential [8,9] and endocrine disrupting action [10,11] allied to their LRAT [12] make them a current issue of concern.

The implementation of atmospheric monitoring plans is essential to assess the properties and behavior of such contaminants. As opposed to other more onerous approaches, monitoring using vegetation avoids previous sampling site set-up and is arguably the best tool for the estimation of the atmospheric contamination

levels at remote or poorly accessible locations [13]. Pine trees proved to be especially suitable, due to their widespread occurrence and the ability to retain lipophilic compounds on their needles, which can remain in the tree for several years [13].

Extraction is an essential step in analytical procedures involving plant matrices and should be able to recover the analytes completely, avoiding the co-extraction of unintended compounds at the same time. The most used extraction technique reported in literature is Soxhlet or Soxtec [14–16] extraction, which offers generally good recoveries, but requires rather large amounts of solvents and is time demanding. Ultrasonic solvent extraction (USE) [14,17,18] and ultrasonic assisted enzymatic digestion (USAED) [19] have been employed as an alternative, using smaller amounts of solvent much shorter extraction times. Pressurized liquid extraction (PLE) [14,20,21] and supercritical fluid extraction (SFE) [22,23] are other alternatives, but require expensive equipment. A subsequent cleanup of the vegetation extracts for multicomponent analysis is often needed and is always challenging step, given the balance between cleanup efficiency and recovery. Solid-phase extraction (SPE) using cartridges or glass columns are broadly employed for pine needle extracts, with silica [24,25], Florisil® [21,26] and alumina [24,26] as the main sorbents. Gel permeation chromatography (GPC), a separation technique based on molecular size [27], is also used, individually [28] or combined with SPE [29].

Our workgroup has previous experience in the development and validation of analytical methodologies to evaluate the levels of PAHs, OCPs and PBDEs in pine needles [13,26,30]. The current study intends to establish an innovative multi-component protocol to extract simultaneously four classes of more "traditional" compounds (BFRs, PCBs, PAHs and OCPs) and, for the first time, SMCs from pine needles. This approach will reduce the workload needed to obtain a comprehensive view of the atmospheric contamination and its deposition in vegetation matrices. To the authors' best knowledge, this is the first time SMCs can be included on a biomonitoring framework using vegetation.

2. Experimental

2.1. *Reagents and materials*

High-purity dichloromethane (DCM), *n*-hexane (Hex) and acetone were supplied by VWR BDH Prolabo (Leuven, Belgium). Florisil® (magnesium silicate, particle size 0.150–0.250 mm), alumina (neutral aluminum oxide 90, particle size 0.063–0.200 mm), silica (silica gel 60, particle size 0.062–0.200 mm) and sodium sulphate were acquired from Merck (Darmstadt, Germany) and activated overnight at 450 ^{o}C. Deactivated alumina was prepared by adding 10% (m/m) of ultrapure water (Fluka Chromasolv, Steinheim, Germany) to the

previously activated alumina and stabilized overnight. S-X3 Bio-Beads® were acquired from Bio-Rad (Amadora, Portugal).

Individual PBDE standards (congeners 28, 47, 85, 99, 100, 153, 154, 183) were bought as individual 50 mg mL^{-1} solutions in isooctane from Sigma–Aldrich (St. Louis, MI, USA). A mix of sixteen PAHs containing naphthalene (Naph), acenaphthylene (Acy), acenaphthene (Ace), fluorine (Fluo), phenanthrene (Phen), anthracene (Ant), fluoranthene (Flt), pyrene (Pyr), benz(a) anthracene (BaA), chrysene (Chry), benzo(b) fluoranthene and benzo(k)fluoranthene (BbF + BkF), benzo(a) pyrene (BaP), indeno(1,2,3-cd) pyrene (IcdP), dibenzo(a,h) anthracene (DahA), and benzo(g,h,i) perylene (BghiP) at 2000 µg/mL in DCM/benzene (1:1), a PCB mix (congeners 28, 52, 101, 138, 153, 180, 209 as 10 µg mL^{-1} in isooctane) and PCB 30 (10 µg mL^{-1} in heptane) as well as musk xylene (MX, 100 µg mL^{-1} in acetonitrile) were also obtained from Sigma–Aldrich. Dr. Ehrenstorfer standards (Augsburg, Germany) supplied a mix of PCBs (congeners 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189, 10 µg mL^{-1} in isooctane), a mix of deuterium labeled PAHs (d-PAHs), containing naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12 and perylene-d12, 10 µg mL^{-1} in hexane and the neat standards of musk ketone (MK), musk ambrette (MA), hexachlorobenzene (HCB) and anthracene-d10. Individual hexabromobenzene (HBB), pentabromotoluene (PBT) and pentabromoethylbenzene (PBEB) standards (each 50 µg mL^{-1} in toluene) and a mix of $^{13}C12$ mass labelled PCB congeners ($^{13}C12$-PCBs) including congeners 28, 52, 101, 118, 138, 153, 180, all at 5 µg mL^{-1} in nonane, were acquired from Wellington laboratories (Guelph, ON, Canada). LGC Standards provided neat standards of cashmeran® (DPMI), celestolide® (ADBI), traseolide® (ATII), phantolide® (AHMI), tonalide® (AHTN), galaxolide® (HHCB) as well as standard solutions of musk moskene (MM) and musk tibetene (MT), both 10 µg mL^{-1} in cyclohexane. All standards and stock solutions were stored in the dark in amber glass vials at 20 °C.

Helium (99.9%) used in the GC–MS system and nitrogen (99.9%) for solvent evaporation were supplied by Air Liquide (Maia, Portugal).

2.2. *Ultrasonic solvent extraction (USE)*

Five grams of pine needles were cut into 1 cm bits, spiked with 10 ng g^{-1} of BFRs, PCBs and HCB and 25 ng g^{-1} of PAHs and SMCs and the same amount of surrogate standards ($^{13}C12$-PCBs and d-PAHs), and extracted with 100 mL of Hex/DCM (1:1) in a 420 W ultrasonic bath (J.P. Selecta, Barcelona, Spain) for 30 min. After solvent cooling, extracts were transferred into pear-shaped flasks and

evaporated to approximately 1 mL in a Büchi R-210 rotary evaporator (Flawil, Switzerland).

2.3. *Cleanup*

Solid phase extraction (SPE) was performed using gravity-fed glass columns packed with 5 g of Florisil®, silica, alumina or deactivated alumina and topped with anhydride sodium sulphate. Prior to sample loading, the columns were conditioned with 50 mL Hex/DCM (1:1). After the sample was transferred into the column, elution was performed with another 50 mL of the same solvent mixture and collected into pear shape flasks for evaporation to near dryness in a rotary evaporator.

Gel permeation chromatography (GPC) was used as an additional cleanup step, following the procedure described by Thomas, et al. [31]. Glass columns equipped with PTFE stopcocks and glass caps were prepared using 6 g of Bio-Beads® S-X3 pre-expanded overnight in Hex/DCM (1:1). Samples were transferred with 3 washings into the GPC columns and eluted with 40 mL of Hex/DCM (1:1), of which the first 15 mL were discarded. Subsequently, the solvent was evaporated to near dryness by rotary evaporation and nitrogen blowdown and reconstituted in 100 μL Hex.

2.4. *Chromatography*

The instrumental quantification of the samples was performed using two different methods. For BFRs, PCBs and HCB, a Varian GC–MS system (Palo Alto, CA, USA), equipped with a Varian 450-GC gas chromatograph, a CP-1177 split/splitless injector, a CP 8410 auto-sampler and a Varian 240-MS ion trap mass spectrometer operated in electron ionization mode (70 eV) with a filament emission current of 50 mA was used. Chromatographic separation was carried out with an Agilent (Santa Clara, CA, USA) CP-Sil 8CB capillary column (50 m 0.25 mm I.D., 0.2 μm film thickness) equipped with a fused silica deactivated retention gap (5 m × 0.25 mm I.D.). Carrier gas was helium at 1 mL min^{-1}. The GC oven temperature started at 110 °C (hold for 1.5 min) then was raised to 150 °C at 20 °C min^{-1}, then to 220 °C at 5 °C min^{-1} (held for 17.5 min) and finally to 300 °C at the same rate and held for 9 min. Injection volume was 1 μL in splitless mode with an injection port temperature of 300 °C. Temperatures of the transfer line, manifold and ion trap were 250 °C, 50 °C and 250 °C, respectively.

For PAHs and SMCs, a Varian 4000 GC/MS (Palo Alto, CA, USA) ion trap mass spectrometer also operated in electron ionization (70 eV) and the same filament current, injector and auto-sampler types was used. Capillary column was an Agilent J&W DB-5 (30 m 0.25 mm, 0.25 μm film thickness) and helium was the

carrier gas (1 mL min^{-1}). The oven temperature program started at 60 ^{o}C for 1 min and was then raised to 175 ^{o}C at 6 ^{o}C min^{-1} (held for 11.11 min) and then to 300 ^{o}C at 5.5 ^{o}C min^{-1} (held for 10 min). The injector was set to 280 ^{o}C and the injection volume was also 1 μL in splitless mode. Transfer line, manifold and ion trap temperatures were the same as above.

In both methods, acquisition was done using time-scheduled selected ion storage (SIS) using the retention time windows and ions shown in Table 1 (BFRs, PCBs and HCB) and Table 2 (PAHs and SMCs). System control, data acquisition and processing were done by Varian MS workstation software (v. 6.9.3). Identification of the target compounds was based on the retention times and the relative abundance of the monitored ions. Quantification was done using internal standards employing $^{13}C_{12}$-PCBs and d-PAHs.

2.5. Operative and storage procedures

The risk of external contaminations is an important issue when leading with this kind of atmospheric pollutants. In particular, the properties and widespread use of SMCs constitute a potential input that needs to be reduced. Consequently, analysts avoided using scented personal care products throughout this study and switched gloves whenever handling different samples. Glassware was also subject to a special cleaning and decontamination procedure. After soaking in a phosphates free detergent solution (Derquim LM03, Panreac, Barcelona, Spain) and rinsing with distilled water and acetone, non-calibrated material was baked-out at 400 ^{o}C for at least one hour. Finally, the containers were rinsed with pure Hex before use.

Photodegradation of the light sensitive compounds was avoided using amber glassware whenever possible. Otherwise, aluminum foil covers were employed. Complete solvent evaporation was also prevented in order to reduce the losses of the target analytes to a minimum. External contaminations were assessed via laboratory blanks, included periodically with extractions. The concentration of the samples were corrected accordingly, whenever needed.

2.6. Naturally contaminated samples

In order to prove the effectiveness of the method in field conditions and the suitability of pine needles to capture SMCs, naturally contaminated needles were collected and analyzed. Being a preliminary assessment, it was decided not to engage in a broad, time-consuming and logistically more expensive sampling campaign, but rather have a few samples representing different exposure patterns (as a function of their location). Three sampling sites in mainland Portugal were chosen. The "Fóia" site is located in a remote mountainous range of

the southern Algarve region of Portugal. Elevation was 838 m a.s.l. and annual precipitation was 700 mm, with an average temperature of 16.8 ºC [32]. The "Benlhevai" sample was collected in the rural inner northern countryside at an elevation of 680 m. Annual precipitation and temperature were 623 mm a.s.l. and 12.8 ºC, respectively [32]. The sampling site designated as "Porto" is located in highly urbanized area in the city of Porto, with an elevation of 123 m a.s.l. and average temperature of 15.9 ºC. Annual accumulated rainfall was 935.3 mm [33].

Two-year-old needles were collected in one piece from P. pinaster species, from the outer bottom branches of the trees and then wrapped in solvent-rinsed aluminum foil, packed in polypropylene freezing bags and stored at -20 ºC. Needle age was accurately identified by the small gaps that are visible in the branches between the shoots from every year. The position of the tree and branches was chosen to allow for the highest exposure to atmosphere (outer branches) but at the same allowing some protection against rainfall and convenient accessibility (bottom branches). Duplicates of each sample were extracted and analyzed together with a procedural blank. Samples collected in the "Porto" site were also used for the method development.

2.7. Moisture content

The moisture content of the pine needles was determined using a procedure described previously [30]. In brief, 5 g of fresh needles were dried at 80 ºC until constant weight. The mass difference corresponds to the amount of water.

3. Results and discussion

3.1. Preliminary tests

A GC/MS method for the simultaneous analysis of all target compounds was initially attempted using a 60 m CP Sil 8CB column. Different oven temperature programs (ranging from 60 to 300 ºC), injector temperatures (250–300 ºC) and injection volumes (1 and 2 μL), filament emission currents (10, 50 and 100 mA) and selected ion storage time schemes were tested in order to achieve optimum chromatographic performance. After several efforts, it became clear that two separate GC/MS programs would deliver the best results and therefore one was developed for BFRs, PCBs and HCB and the other for PAHs and SMCs. At this stage, a 30 m DB-5 column was chosen for the separation of the latter set, as a slightly better performance was obtained, namely for the less volatile PAHs.

As mentioned previously, a thorough cleanup of matrices containing several coextractives such as chlorophylls, lipids, waxes and sugars is essential to obtain a good chromatographic performance. Different cleanup approaches and set-ups were found in literature and, among them, the ones by Thomas et al. [31] and

by Hubert et al. [28] were chosen as the most promising ones for adaptation. Thomas et al. [31] used a two-step cleanup procedure employing silica gel/acid silica followed by GPC to quantify PCBs in vegetation extracts (grass and silage). An identical set-up was tested. 8 g of activated silica were packed together with 8 g of sulphuric acid impregnated silica (2:1 m/m) and samples were eluted with 150 mL of Hex. After solvent evaporation the samples were further subjected to a cleanup by GPC using 6 g of Bio-Beads® S-X3 and elution with Hex/DCM (1:1). For this, the relevant fractions (15–50 mL) were identified by an elution profile, collecting consecutive 5 mL fractions of the eluate. Low recoveries were obtained for PAHs (<29%) and SMCs (<8%), as expected due to the degradation of these compounds in the acid medium. To overcome this, the fractionation of PAHs and SMCs from the other compounds was attempted, but with poor results. Elution profiles of several combinations of sorbents (alumina, Florisil® and silica) and solvents (Hex DCM, Hex/DCM (1:1)) were established collecting 5 mL fractions of eluate but no effective separation was possible, as SMCs and PAHs overlapped with the other compounds. Thus, cleanup with acid-impregnated silica columns was abandoned but nevertheless GPC were tested alone. Hubert et al. [28] obtained good recoveries and chromatographic performance using this approach for PAHs in pine needles extracts. For this, the same columns containing 6 g of Bio-Beads® S-X3 and solvents mentioned above were used, but the extracts were passed through glass columns containing approximately

0.5 g Na_2SO_4 beforehand, in order to remove water. The resulting extracts showed a slight coloration with no waxy deposits, but yielded poor chromatographic performance. Based on these preliminary tests, an optimization of a SPE cleanup methodology followed by GPC was considered necessary.

3.2. *SPE cleanup optimization*

In order to potentiate the best results for the considerable number of target compounds analyzed, the performances of 3 sorbents (alumina, Florisil® and silica), previously activated overnight at 450 °C were compared using glass columns followed by GPC. The amounts of solvents required were established by elution profiles, collecting consecutive 5 mL fractions. Ratola et al.[14] had already tested these three different sorbents for the cleanup of extracts of pine needles and concluded that alumina was the most suitable. However, these tests were only for PAHs and for a different pine species (*P. pinea*) and therefore further testing had to be made to include the other target compounds.

The eluent of the silica assay turned into a viscous green extract after solvent evaporation and was not amenable for GC/MS analysis and therefore discarded as

a possible sorbent. Florisil® rendered low recoveries, on average below 30%, and low reproducibility with RSD occasionally exceeding 100% (data not shown). Alumina, on the other hand, showed the best performance, with high recoveries and good reproducibility as can be seen in Fig. 1. Except for the most volatile compounds (BDE 28, DPMI, Naph) and PBEB, recoveries were above 70% and repeatability with RSDs generally below 10%. Average recoveries ± average RSD were 88±2% for BFRs, 94±4% for PCBs, 80±8% for PAHs and 90±3% for SMCs.

As sorbent activity can play an important role in the cleanup process, a 10% water-deactivated alumina was tested. Recoveries were very similar to the ones of activated alumina: 84±7% for BFRs, 89±4% for PCBs, 75±7% for PAHs and 87 ±8% for SMCs. However, repeatability expressed as RSD of triplicate extracts, was worse, especially for PBEB, HBB and nitromusks of the SMCs class. This is probably due to the difficulty in obtaining a completely homogenous deactivated sorbent but also due to increased matrix interferences, as chromatographic performance was not as good as with activated alumina. So, SPE with activated alumina followed by GPC was ultimately chosen as cleanup protocol.

3.3. *Method validation*

The method developed was validated regarding the linearity ranges, coefficients of determination, LODs and LOQs, recoveries and repeatabilities and the main results are detailed in Table 3. Due to the different levels of occurrence, two calibration levels were chosen. For BFRs, PCBs and HCB, which generally are present at lower levels, a good linear behavior was obtained between 4 and 600 $\mu g\,L^{-1}$, with coefficients of determination (R^2) ranging from 0.991 to 0.999. Due to the lack of data regarding the occurrence of SMCs in vegetation, an identical calibration as for PAHs was chosen. The linearity range was from 10 to 1500 $\mu g\,L^{-1}$ with R^2 between 0.995 and 0.999.

Repeatability and recovery were tested by spiking triplicate samples of needles at two concentration levels. For the lower level, 2 $ng\,g^{-1}$ of BFRs, PCBs and HCB and 5 $ng\,g^{-1}$ of PAHs and SMCs, whereas for the higher levels, 10 $ng\,g^{-1}$ and 25 $ng\,g^{-1}$, respectively. Overall, the recoveries were good, exceeding 70% when considering all chemical classes and both spiking levels. Exceptions were the most volatile compounds of BFRs, PAHs and SMCs (BDE 28, Naph and DPMI) which are more prone to losses during sample handling. Still, recoveries were above 40%. Other compounds showing lower than average recoveries were PBEB, HBB and PCB 126. In these cases, the differences were more significant between both spiking levels, with lower values for the lower concentrations. One possible explanation is that these three compounds elute in a time frame that is more prone to coextraction of unintended chemicals. For all compounds, the repeatability (expressed as the RSD of triplicate extractions) was good, with most

values below 10%.

When dealing with pollutants at residual levels, low LODs and LOQs are essential to take valid conclusion about their occurrence. These parameters were estimated based on a signal-to-noise (S/N) ratio of 3 and 10, respectively. LODs and LOQs in the low pg g^{-1} range were obtained for HCB (LOD 0.4 pg g^{-1}, dw; LOQ 0.4 pg g^{-1}, dw), for PCBs (LODs 0.1–0.5 pg g^{-1}, dw; LOQs 0.4–1.5 pg g^{-1}, dw), but also for BFRs (LODs 0.4–3.3 pg g^{-1}, dw; LOQs 1.2–10.8 pg g^{-1}, dw). For PAHs, LODs and LOQs were lower from for 2–4 ring PAHs (Naph–Chry; LODs 3.4–20.3 pg g^{-1}, dw; LOQs 11.4–65.2 pg g^{-1}, dw) than for the 5–6 ring PAHs (BbF + BkF, BaP, DahA BghiP and IcdP; LODs 69.0–332.6 pg g^{-1}, dw; LOQs 196.7–1016.3 pg g^{-1}, dw). For SMCs, LODs ranged from 3.8 pg g^{-1} (dw) for AHMI and 114.3 pg g^{-1} (dw) for MM.

Taking into account the challenging matrix and multi-residue framework, results are very good, being recoveries and LODs comparable to others reported by authors who developed analytical approaches for only one or two chemical classes. For instance, Ratola et al. [26] employed a two-step SPE cleanup with alumina and Florisil® to quantify the same PBDEs studied here, but with the analysis performed by GC/NCI/MS, which is considered to have a higher sensitivity than the EI equipment [34]. Although the recoveries were slightly higher (from 99% to 138%), the reported LODs were in the same order of magnitude: 11–70 pg g^{-1}, dw. Chen et al. [35] developed a method for the same PBDEs and a different pine species (*P. radiata*) and achieved slightly higher LODs: 44–222 pg g^{-1}, dw. Lavin and Hageman [36] used *P. radiata* needles for the quantification of OCPs and PCBs, comparing PLE combined with GPC to selective PLE with no other cleanup and concluded that both methods perform similarly. Although HCB was not among the studied OCPs, for PCBs the recoveries (58–99%, estimated from chart) and LODs (22–270 pg g^{-1}, fresh weight) were higher than those obtained with our method. PAHs have been extensively studied in pine needles and the LODs found in the current work are comparable to those reported by Gorshkov [37] and Schröter-Kermani et al. [25] for *P. sylvestris* and by Ratola et al. [14] for *P. pinea*. Being the first time that SMCs are analyzed in vegetation, no comparison could be done with a similar matrix. However, the LODs are similar to those reported for sediments [38], which ranged from 30 to 50 pg g^{-1}.

This validation strategy confirmed the proposed method as a perfectly fit multi-residue approach to handle such a complicated matrix as pine needles. However, it was still important to assess its performance when applied to naturally contaminated samples.

3.4. *Naturally contaminated samples*

The results of these assays are reported in Table 4, on a dry-weight basis in order to help the comparison between sites. The moisture levels for all samples of *P. pinaster* needles were very similar, ranging from 54 to 59%.

While for legacy POPs and PAHs the occurrence in pine needles have been reported in literature, this is not the case for SMCs. Although the reduced number of samples does not favor the drawing of definitive conclusions, it is noteworthy that these chemicals show similar levels to PAHs and that being mainly linked to human presence and usage were found in higher concentrations in Fóia (remote) and Benlhevai (rural) and not in the urban area (Porto). Peck and Hornbuckle [39] detected an urban > suburban gradient, which is not seen in our case. Higher levels of SMCs in Benlhevai may be explained by the proximity of a landfill, where household residues are treated. The site in Fóia, on the other hand, may be prone to musks exposure by atmospheric transport. This possibility arises from its specific position overlooking the Algarve coast, a beach-related densely tourist-populated region where personal care products employing SMCs (such as sun-block lotions) are extensively used. The Porto sample was collected in an area of intense vehicular traffic and more shielded from winds, due to nearby high-rise buildings. This may have diminished the needles' exposure to this kind of compounds. The distribution of individual SMCs was similar for all three sites, where no nitromusks were detected. This is probably due to the fact that these compounds were banned (MA, MT, MM), or at least restricted in their use (MX, MK) under the EU Directive 2012/21/EU [40]. In all three sites, HHCB and AHTN were predominant, in line with the 95% quota of these two musks in the EU market [41]. Another prevailing musk is DPMI, the most volatile, hence more prone to atmospheric transport.

Regarding PAHs, urban-stressed Porto shows the highest levels, with a total concentration of 967.8 ng g^{-1} (dw), nearly three times higher than Fóia and Benlhevai, which showed similar levels. Heavy traffic and industrial activity may be responsible for these values and land use gradient. The PAHs fingerprint was also distinct for each site, suggesting the contribution of different sources. For instance, in Fóia the levels of individual PAHs were Phen > Flt > Pyr while for Benlhevai it was Phen > Flt > BaA and for Porto Pyr > Flt > Chry. When compared to literature, levels are within the typically reported ranges and show a similar urban > rural > remote trend [24,25,42,43].

BFRs were detected in all sites at similar total levels (from 0.452 to 0.866 ng g^{-1}, dw), with a urban > rural > remote pattern. The BFRs detected were exclusively BDEs, as the new flame retardants (PBT, PBEB and HBB) were not found. In Fóia and Benlhevai, BDE 28 and BDE 99 were predominant, accounting for more than 70% of the BFRs incidence. Porto showed a slightly different BDE congener profile, with BDE 183 prevailing, but closely followed by BDEs 28 and 47.

Kannan et al. [16] studied the levels of 8 BDEs and found total levels in the same range (0.22–0.70 ng g^{-1}, dw) and a decreasing trend between the city center and the outskirts.

Concentrations in the same order of magnitude (0.353–1.014 ng g^{-1}, dw) were found for PCBs, replicating the same urban > remote gradient found for BFRs. PCB 28 followed by PCB 52 were the most common, although Porto showed a greater variety of detected congeners, reflecting its urban fingerprint, as opposed to sites Fóia and Benlhevai, where only 3 PCBs were detected. The total levels of 22 PCBs found by Kannan et al. [16] were slightly higher (1.56–4.21 ng g^{-1}, dw), while Romanic and Klincic [44] reported similar levels (0.15 and 9.91 ng g^{-1}, dw), with a predominance of PCB 28.

HCB, the only OCP analyzed, showed similar levels for Fóia and Benlhevai, and lower for Porto. Hellstrom [45] analyzed pine needles for organochlorine pesticides in Central and Northern Europe and found comparable incidences, between 0.16 and 10.10 ng g^{-1} (dw). Although no assessment between land uses was made, the author concluded that levels were quite uniform throughout Europe, which is in line with its enormous potential for LRAT.

Even with a reduced number of samples, this field study reinforced the applicability of the proposed multi-residue methodology and the suitability of pine needles to assess the incidence of SMCs. In order to confirm the tendencies or establish new ones, the design of sampling strategies with a wider geographical coverage and time span is strongly needed and should be implemented.

4. Conclusions

The innovative multi-residue methodology validated in this study and based on ultrasonic assisted extraction followed by a two-step cleanup employing activated alumina-SPE and GPC is a viable approach for the simultaneous analysis of BFRs, PCBs, PAHs, OCPs and SMCs in pine needles.

The results found analyzing naturally contaminated samples proved the ability of the method to respond when a field-based strategy is implemented. It was possible to quantify SMCs in vegetation for the first time (only polycyclic musks were detected) and find some preliminary geographical trends. The outcome of this study encourages the future application of this protocol to not only other compounds, but also to other environmental matrices.

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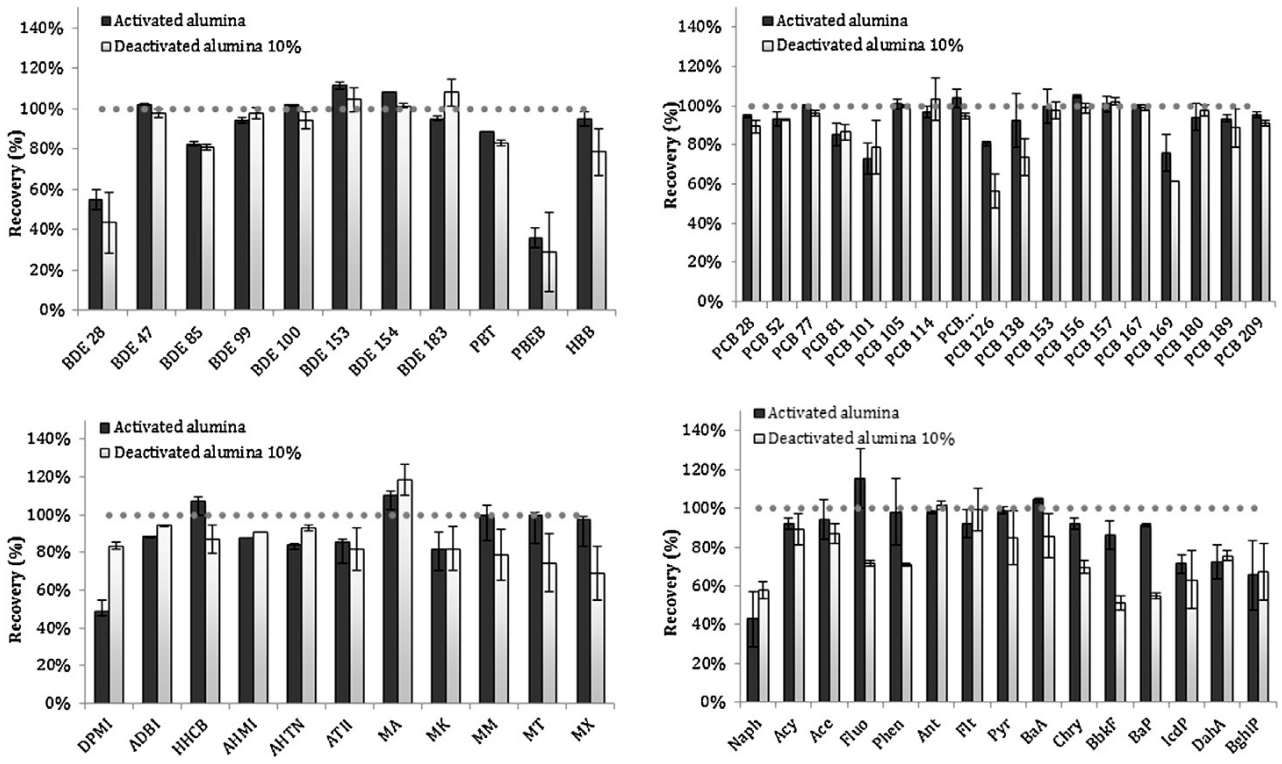

Fig. 1. Recoveries and standard deviation (as error bars) for USE extraction of spiked pine needles with 10 ng g^{-1} of BFRs and PCBs and 25 ng g^{-1} of PAHs and SMCs, using glass columns packed with 5 g of activated or 10% deactivated alumina.

Table 1
GC-MS method parameters for BFRs, PCBs and HCB.

Time segment (min)	Retention time (min)	Target compound	Ions (m/z)
12.00–15.75	12.37	HCB	**284**, 286
	13.04	*PCB 30*	186, 256, **258**
	15.11	*$^{13}C_{12}$-PCB 28*	268, **270**
	15.12	PCB 28	186, 256, **258**
15.75–18.50	16.21	*$^{13}C_{12}$-PCB 52*	302, **306**
	16.33	PCB 52	290, **292**, 294
18.50–40.50	19.60	*$^{13}C_{12}$-PCB 101*	336, **340**
	19.61	PCB 101	324, **326**, 328
	20.82	PCB 77	290, **292**, 294
	21.33	PCB 81	290, **292**, 294
	22.72	*$^{13}C_{12}$-PCB 118*	336, **340**
	22.72	PCB 118 + 123	324, **326**, 328
	22.77	BDE 28	246, **248**
	23.04	PBT	407, 408, **486**, 489
	23.37	PCB 114	324, **326**, 328
	24.10	*$^{13}C_{12}$-PCB 153*	**372**, 374
	24.13	PCB 153	360, **362**
	24.33	PCB 105	324, **326**, 328
	24.51	PBEB	**500**, 504
	26.03	*$^{13}C_{12}$-PCB 138*	370, **374**
	26.06	PCB 138	**360**, 362
	26.73	PCB 126	324, **326**, 328
	28.45	PCB 167	**360**, 362
	29.53	HBB	**551**, 554
	30.53	PCB 156	**360**, 362, 364
	31.11	PCB 157	**360**, 362, 364
	32.43	*$^{13}C_{12}$-PCB 180*	405, **410**
	32.45	PCB 180	392, 394, **396**
	32.51	BDE 47	**484**, 488
	34.92	PCB 169	**360**, 362, 364
	38.88	PCB 189	394, **396**, 398
40.50–51.00	41.35	BDE 100	404, **406**, 408
	43.08	BDE 99	404, **406**, 408
	45.74	PCB 209	**496**, 501
	45.74	BDE 85	404, **406**, 408
	47.10	BDE 154	482, **484**, 486
	48.77	BDE 153	482, **484**, 486
51.00–55.00	53.26	BDE 183	561, **566**, 722, 726

$^{13}C_{12}$-labelled PCBs surrogates in italics well as PCB 30 used as internal standard.
Quantification ions in bold.

Table 2
GC–MS method parameters for PAHs and SMCs.

Time segment (min)	Retention time (min)	Target compound	Ions (m/z)
9.00–15.50	10.12	*Naph-d_8*	**136**
	10.51	Naph	**128**
15.50–20.00	16.38	Acy	**152**
	16.57	*Ace-d_{10}*	**164**
	17.07	Ace	**152**, 153
	17.33	DPMI	163, **191**
	19.10	Fluo	165, **166**
20.00–23.80	21.56	ADBI	173, **229**
	22.54	AHMI	187, **229**
	22.75	*Phen-d_{10}*	**188**
	23.52	Phen	**178**
	23.07	*Ant-d_{10}*	**188**
	23.84	Ant	**178**
23.80–30.00	24.58	MA	**253**
	25.19	ATII	173, **215**
	25.28	HHCB	213, **243**
	25.37	MX	**282**
	25.56	AHTN	159, **243**
	26.16	MM	**263**
	27.56	MT	**251**
	29.00	MK	**279**
30.00–40.00	31.92	Fluo	200, **202**
	32.68	Pyr	200, **202**
40.00–46.00	42.19	BaA	228, **229**
	42.24	*Chry-d_{12}*	**240**
	43.33	Chry	**228**
46.00–52.50	48.52	BbF + BkF	**252**
	49.63	BaP	**252**
	49.88	*Per-d_{12}*	**264**
52.50–57.00	53.00	IcdP	**276**
	53.19	DahA	278, **279**
	54.04	BghiP	**276**

Deuterated PAHs surrogates in italics as well as Ant-d_{10} used as internal standard.
Quantification ions in bold.

Table 3

Method validation parameters: linearity range, coefficient of determination (R^2), limit of detection (LOD), repeatability and recovery (n= 3)

	LOD (pg g^{-1} dw)	LOQ (pg g^{-1} dw)	Repeatability (RSD %)		Recovery (%)			LOD (pg g^{-1} dw)	LOQ (pg g^{-1} dw)	Repeatability (RSD %)		Recovery (%)	
			2 ng g^{-1}	10 ng g^{-1}	2 ng g^{-1}	10 ng g^{-1}				5 ng g^{-1}	25 ng g^{-1}	5 ng g^{-1}	25 ng g^{-1}
BFRs							*SMCs*						
BDE 28	0.4	1.2	13.4	5.1	53	55	DPMI	73.2	206.7	8.9	5.8	48	49
BDE 47	0.7	2.3	5.0	0.5	110	102	ADBI	4.6	15.1	1.7	0.2	85	89
BDE 85	0.8	2.8	0.9	1.1	110	83	HHCB	12.9	41.9	2.8	2.7	80	107
BDE 99	0.8	2.8	0.7	1.3	109	94	AHMI	3.8	12.5	2.2	0.1	84	88
BDE 100	0.7	2.3	2.6	0.4	109	102	AHTN	17.9	57.8	6.5	1.0	82	84
BDE 153	0.7	2.4	6.3	1.7	106	112	ATII	21.8	71.3	4.6	1.2	95	86
BDE 154	0.6	1.8	3.7	0.1	106	108	MA	5.0	11.0	4.3	2.0	99	111
BDE 183	1.1	3.6	6.3	0.9	103	95	MK	54.6	182.0	4.9	8.8	103	82
PBT	0.9	2.9	6.6	0.1	109	89	MM	114.3	348.4	8.4	5.3	102	100
PBEB	1.5	5.1	4.6	4.9	8	36	MT	48.8	162.6	6.2	1.7	104	100
HBB	3.3	10.8	14.7	3.2	18	95	MX	53.0	176.7	1.9	1.6	107	97
PCBs							*PAHs*						
PCB 28	0.2	0.8	4.1	0.5	101	95	Naph	6.1	20.5	47.7	14.3	40	43
PCB 52	0.3	1.2	5.8	3.5	94	93	Acy	17.8	17.7	8.3	3.0	107	92
PCB 77	0.3	0.8	3.0	0.5	93	100	Ace	8.2	27.4	15.4	10.0	83	94
PCB 81	0.3	0.8	9.5	5.9	86	86	Fluo	4.7	15.6	7.1	15.2	113	115
PCB 101	0.4	1.2	7.7	7.7	90	73	Phen	5.6	18.5	8.5	17.2	80	98
PCB 105	0.3	1.1	4.2	2.3	98	101	Ant	4.3	14.2	2.2	0.5	63	98
PCB 114	0.3	1.0	0.5	3.2	98	97	Flt	3.4	11.4	5.2	7.0	58	92
PCB 118 + 123	0.1	0.4	3.1	4.8	85	104	Pyr	5.1	17.1	4.2	1.9	104	99
PCB 126	0.3	1.2	10.1	1.2	35	81	BaA	20.3	65.2	1.0	1.2	106	104
PCB 138	0.4	1.4	4.3	13.7	93	92	Chry	12.9	40.5	4.0	2.9	91	92
PCB 153	0.4	1.4	0.7	8.9	99	100	BbF + BkF	69.0	196.7	3.6	7.4	112	86
PCB 156	0.2	0.8	1.3	0.5	100	105	BaP	261.3	813.0	2.0	0.8	100	91
PCB 157	0.3	0.8	0.9	3.9	107	101	IcdP	304.9	1016.3	2.2	4.8	108	71
PCB 167	0.3	1.0	1.2	1.0	103	100	DahA	203.3	677.5	9.2	8.6	114	72
PCB 169	0.2	0.8	6.8	9.2	101	76	BghiP	332.6	871.1	4.3	17.8	121	66
PCB 180	0.5	1.5	1.9	7.0	103	94							
PCB 189	0.2	0.8	4.2	1.8	111	93	*OCP*						
PCB 209	0.2	0.8	4.7	1.3	113	96	HCB	0.4	1.3	5.3	4.6	79	85

Table 4

Levels of BFRs, PCBs, SMCs, PAHs and HCB in naturally contaminated pine needle samples collected from three sites (Fóia, Benlhevai and Porto). Results in ng g^{-1} dry weight ± SD (mean of duplicate analysis).

	Fóia	Benlhevai	Porto		Fóia	Benlhevai	Porto
BFRs				*SMCs*			
BDE 28	0.177 ± 0.033	0.289 ± 0.011	0.224 ± 0.002	DPMI	72.599 ± 10.878	58.870 ± 1.933	0.215 ± 0.032
BDE 47	0.018 ± 0.001	0.096 ± 0.001	0.176 ± 0.015	ADBI	0.294 ± 0.015	0.275 ± 0.008	0.093 ± 0.010
BDE 85	nd	nd	nd	HHCB	114.172 ± 17.381	216.364 ± 2.805	15.944 ± .370
BDE 99	0.191 ± 0.026	0.139 ± 0.015	0.072 ± 0.009	AHMI	nd	nd	nd
BDE 100	nd	0.024 ± 0.001	0.062 ± 0.002	AHTN	5.587 ± 0.833	2.028 ± 0.147	4.452 ± 0.443
BDE 153	nd	nd	nd	ATII	8.919 ± 0.287	nd	nd
BDE 154	nd	nd	nd	MA	nd	nd	nd
BDE 183	0.066 ± 0.011	0.056 ± 0.009	0.332 ± 0.051	MK	nd	nd	nd
ΣBDEs	0.452	0.604	0.866	MM	nd	nd	nd
PBT	nd	nd	nd	MT	nd	nd	nd
PBEB	nd	nd	nd	MX	nd	nd	nd
HBB	nd	nd	nd	ΣSMCs	201.6	277.5	20.7
PCBs				*PAHs*			
PCB 28	0.199 ± 0.005	0.208 ± 0.019	0.564 ± 0.023	Naph	6.373 ± 1.036	0.776 ± 0.027	1.019 ± 0.139
PCB 52	0.124 ± 0.014	0.205 ± 0.050	0.183 ± 0.034	Acy	3.193 ± 0.231	1.766 ± 0.292	14.799 ± 2.434
PCB 77	nd	nd	nd	Ace	26.682 ± 2.751	10.269 ± 0.241	25.583 ± 0.703
PCB 81	nd	nd	nd	Fluo	11.524 ± 0.941	25.367 ± 1.626	137.351 ± 21.672
PCB 101	nd	nd	nd	Phen	10.602 ± 1.885	112.367 ± 1.067	342.001 ± 17.984
PCB 105	nd	0.089 ± 0.011	0.053 ± 0.005	Ant	1.942 ± 0.222	2.290 ± 0.003	50.273 ± 1.614
PCB 114	nd	nd	0.025 ± 0.001	Flt	49.308 ± 6.331	52.789 ± 0.245	178.852 ± 18.076
PCB 118 + 123	nd	nd	nd	Pyr	62.474 ± 2.970	29.786 ± 0.103	168.091 ± 5.193
PCB 126	nd	nd	nd	BaA	13.007 ± 0.194	36.169 ± 0.685	24.414 ± 3.403
PCB 138	nd	nd	nd	Chry	48.725 ± 1.947	9.343 ± 0.071	14.843 ± 1.796
PCB 153	nd	nd	nd	BbF + BkF	0.587 ± 0.024	0.533 ± 0.033	2.144 ± 1.247
PCB 156	nd	nd	nd	BaP	0.407 ± 0.023	3.628 ± 0.008	0.614 ± 0.083
PCB 157	nd	nd	nd	IcdP	8.542 ± 0.430	11.467 ± 1.589	5.872 ± 0.726
PCB 167	nd	nd	nd	DahA	1.619 ± 0.745	8.332 ± 1.378	nd
PCB 169	nd	nd	nd	BghiP	0.768 ± 0.308	1.031 ± 0.156	1.929 ± 0.051
PCB 180	0.030 ± 0.001	nd	0.135 ± 0.003	ΣPAHs	245.8	305.9	967.8
PCB 189	nd	nd	nd				
PCB 209	nd	nd	0.054 ± 0.007	*OCP*			
ΣPCBs	0.353	0.501	1.014	HCB	12.172 ± 0.947	10.050 ± 0.434	0.362 ± 0.014

nd: not detected.