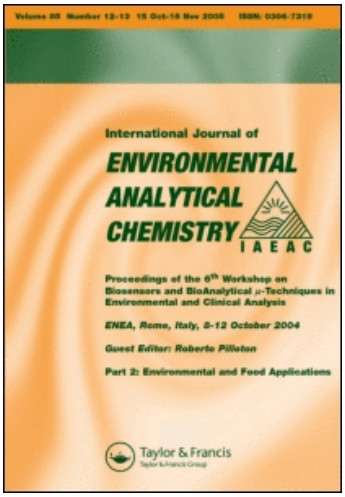

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A clean-up procedure based on a solid-phase extraction column was optimized for determination of polycyclic aromatic hydrocarbons (PAHs) in lichen extracts to remove co-extracted compounds from the matrix in the final extract. Several kinds of solid phases were evaluated: normal phase (-NH_2 and alumina), strong anion exchange and reversed phase. The -NH_2 columns were the most effective by using a packed solid bed of 500 mg. The lichen raw extract was loaded on the column previously conditioned with dichloromethane and hexane. Hexane (0.5 mL) was used as rinsing solvent, and PAHs were quantitatively eluted (80–97%) using 2 mL of hexane–dichloromethane (65–35) as eluting solvent. In these conditions, even the heaviest PAHs were quantitatively eluted. The optimized SPE method provides a short time and low-solvent-consumption sample clean-up compared with other conventional methods based on column chromatography. The analytical procedure, dynamic sonication-assisted extraction, followed by the optimized solid-phase extraction clean-up, was used to determine the 16 EPA priority PAHs from native lichens collected from the Aragon valley in central Pyrenees. The PAH concentrations in lichen samples ranged from 352 to 1654 ng g^{-1}, and the minimum concentration value was established as the regional reference PAH levels in the area.

Keywords: PAHs; Lichens; SPE; Clean-up

1. Introduction

The analysis of organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs) is an important concern in environmental research. The occurrence and emission of these compounds have been substantial in previous centuries because of the abundant use of fuels for industrial applications, heating, transport, and many other purposes. PAHs constitute a wide class of compounds composed of two or more fused aromatic rings. They are formed during incomplete combustion, and the major emission sources to the atmosphere are mainly related to human activities, like domestic wood burning and road traffic. This group of compounds involves potential health risks because of the mutagenic, carcinogenic, and endocrine disrupting effects of some of them [1]. The US

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Environmental Protection Agency (EPA) has identified 16 PAHs as priority pollutants, and they have also been included as persistent organic pollutants (POPs) by the United Nations–European Committee in a global treaty signed in May 2001 in the Stockholm convention for the regulation of POPs [2]. From this point, the interest for the knowledge of those compounds in the environment has increased considerably.

PAHs can be presented in a wide variety of forms and are largely found in water, aerosols, soils, sediments, and biota [3–7]. In the atmosphere, these compounds occur in gaseous forms and adsorbed on particles. They are soluble in fatty and lipid-rich tissues, so they are spread from the air to certain vegetal organisms, such as lichen thalli, moss tissues, or plant leaves, where they are accumulated [8, 9]. Consequently, the bioaccumulation of such compounds in vegetal organisms is an indication of the level of air pollution in a given environmental area where they can be measured [10, 11]. For this purpose, the development of effective analytical procedures of PAH determination in any matrix is of high importance.

The analysis of PAHs has been hardly investigated in previous works [11–14], but the extraction of PAHs from the multiple matrices found in the environment, such as lichens, remains a critical step in the analytical process. Lichens are complex matrices and contain different kinds of organic compounds (fatty acids, alkanes, phenols, chlorophylls, esters, etc.), which could cause interference and introduce errors in the analysis. Some matrix co-extractant compounds, like lipidic compounds and chlorophylls, could significantly reduce the performance of GC-MS due to their accumulation in the injection port, column, and ionization source [15, 16]. For these reasons and considering the low concentration levels of PAHs in lichens (usually ng g^{-1} levels), enrichment and clean-up procedures are usually required prior to the final chromatographic analysis.

Column chromatography has been used as a conventional clean-up procedure using different sorbents as alumina, florisil, or silica-gel [4, 10, 17–20]. However, this technique is usually time-consuming and requires large volumes of eluting solvents, as well as the activation of the selected sorbent and pre- and post-concentration steps. As an alternative, solid-phase extraction (SPE) can significantly reduce solvent consumption and the sample pretreatment. SPE is a popular technique that is used both to preconcentrate components and to clean up matrices for sample analysis. The prepacked cartridges provide users of SPE with a variety of stationary phases to selectively separate and concentrate analytes for detection. SPE columns have been successfully used before for the clean-up and preconcentration of PAHs in different matrices, such as water [3], oil [21], aerosol [5], sediments [14], soils [22], and tobacco smoke [23]. More recently, they have been used with biological tissues [15] and food [24].

In this article, low-solvent-consumption extraction methods, like dynamic sonication-assisted solvent extraction (DSASE) [11], followed by an SPE clean-up step, are presented as a good alternative to obtain a higher efficiency and selectivity in the whole analytical procedure. The final extract is enriched and free from interference, and the sample handling is considerably reduced. The SPE clean-up step is systematically investigated by several factors that would have an influence on SPE performance, like the SPE phase and the elution and rinsing solvents. The clean-up procedure was evaluated in terms of recovery and the lack of interfering compounds in the final extract. The sample handling procedure, including the optimized SPE clean-up step, was successfully used in the analysis of 16 EPA priority PAHs in lichens collected

from the Pyrenees to monitor air pollution in this area and establish a reference PAH level in lichens. The results found are shown and discussed.

2. Experimental

2.1 *Chemicals and standards*

The polycyclic aromatic hydrocarbons naphthalene (Np), acenaphtylene (Acl), acenaphthene (Ace), fluorene (F), anthracene (An), phenanthrene (Ph), dibenzofurane (Db), chrysene (Ch), pyrene (Py), benzo[*a*]pyrene (BaPy), fluoranthene (Fl), benzo[*b*]fluoranthene (BbFl), benzo[*k*]fluoranthene (BkFl), benz[*a*]anthracene (BaAn), dibenz[*ah*]anthracene (dBahA), and benzo[*ghi*]perylene (BghiPe) were supplied as certified standards by the US Environmental Protection Agency (EPA). Acenaphthene d10 from Sigma–Aldrich was used as internal standard in all analyses. The analytical-grade solvents, hexane, methanol, dichloromethane, toluene, and di-ethyl ether, were purchased from Scharlab S.L. (Barcelona). Standard solutions of PAHs and an internal standard solution of acenaphthene d_{10} were prepared in hexane.

The SPE cartridges used were Bond Elut-LRC-SAX and Bond Elut-Al-B, 100 mg from Varian (Walnut Creek, CA); Discovery-NH_2, 100 and 500 mg, Discovery-DPA-6S, 250 mg (Supelco, Bellefonte, PA), Florisil, 100–200 mesh from Aldrich (Deisenhofen, Germany), and anhydrous sodium sulfate from Merck (Darmstadt, Germany).

2.2 *Lichen sampling and sample pretreatment*

Lichen samples of the species *Parmelia sulcata* were collected from the Aragon valley in the central Pyrenees during the spring of 2004. This valley is crossed by a national road, which runs parallel to the Aragon River and passes through four main villages: Castiello de Jaca, Villanua, Canfranc, and Canfranc Station. Lichens were sampled in two different areas in order to compare the PAH content and distribution in lichens from both areas and then establish a reference PAH level in the valley: (1) near the national road passing through the main towns and (2) in natural areas far from the national road and the urban areas. Lichen thalli were collected from trees approximately 1 m above ground level. In the laboratory, lichens were separated from the rest of the bark and other materials like dust, dried at 35°C for 3–4 days, ground in an agate mortar to obtain homogenized samples, and kept at 4°C in the dark until analysis.

2.3 *Dynamic sonication-assisted extraction*

The DSASE procedure used was previously optimized for the determination of PAHs in lichen samples [11]. Briefly, a sample of 0.2 g of lichens was inserted together with silanized glass wool into the 1-mL stainless steel extraction cell using the sandwich technique. A continuous flow of hexane (0.2 mL min^{-1}) was pumped through the sample under sonication for 10 min. A volume of 2 mL of raw extract was collected

in glass vials and kept with the cap on until the clean-up step. The analytical characteristics of the DSASE method can be obtained from [11].

2.4 *Clean-up optimization*

2.4.1 Sample preparation. Spiked raw extracts were used for the optimization of the SPE clean-up as no lichen certified reference material is commercially available. They were prepared as follows: portions of dried lichens from every sampling point were mixed and kept as a representative stock of lichens; samples of 0.2 g of the stock of lichens were extracted using DSASE method as described before, and the 2 mL final extract was spiked with approximately 0.01 g of a standard solution in hexane containing 16 PAHs with a concentration of $15\,\mu g\,g^{-1}$ of each compound. To calculate the PAH percentage recovery, both spiked extracts and blank extracts (non-spiked) were used. The percentage recovery value was calculated as described by Domeño *et al*. [11].

2.4.2 SPE procedure. The clean-up mini-columns were prepared by adding approximately 0.05 g of anhydrous sodium sulfate and 0.05 g of florisil to the top of the commercial SPE cartridges. The cartridges were placed on a Waters manifold (Milford, MA), and 6 mL of dichloromethane and 3 mL of hexane were passed through the bed in order to clean and condition the solid phase, respectively. Once the SPE cartridges were conditioned, the 2-mL raw extract in hexane was loaded on the top, and the vacuum was adjusted in such a way that the flow rate was 1–2 drops per second. The analytes were eluted from the cartridges with solvent once all the raw extract had reached the sorbent bed. The eluted extract was collected in a glass vial and evaporated until 0.5 mL under a gentle nitrogen stream before the analysis.

For the SPE optimization, several kinds of solid phases were tested to remove the maximum amount of co-extracted compounds from the matrix in the final extract: normal phase (-NH_2 and alumina), strong anion exchange (SAX), and reversed phase (DPA). The alumina stationary phase is widely used for the clean-up of extracts containing PAHs from different matrices. It is commonly used in conventional laboratory made columns as well as in commercial cartridges [18–20]. The polymerically bonded aminopropyl phase (-NH_2) was selected because it has been recommended for phenols and plant pigments [25], and the main interfering compounds from the lichen matrix were the chlorophylls. The SAX phase is a polymerically bonded quaternary amine, and it is very similar to the Ambersep 900 OH, a strong basic resin used in a previous work to remove chlorophylls [26]. Finally, the DPA stationary phase is a polyamide resin, which acts as a reversed phase. DPA has been recommended to absorb polar compounds from aqueous and methanolic solutions and it has been suggested as a very useful phase for tannins, chlorophylls, humic acids, and flavanoids among others. This was the reason why it was selected to remove the chlorophylls and vegetal residues from the lichens extract.

Different hexane solutions enriched with a polar solvent were tested as eluting solvent in order to obtain the quantitative PAHs elution using the minimum amount of solvent: hexane, hexane–toluene (75 : 25), hexane-diethyleter (75 : 25) and different dilutions of

hexane–dichloromethane (85 : 15, 75 : 25, and 65 : 35). The percentage of cleaning in the final extract obtained with the optimized SPE cleanup was determined as:

$$\left[\frac{1 - \sum(\text{area of interferences})_{\text{SPE procedure}}}{\sum(\text{area of interferences})_{\text{raw extract}}}\right] \times 100.$$

The clean-up procedure development was evaluated in terms of PAH quantitative recoveries and the lack of interfering compounds in the final extract.

2.4.3 Breakthrough volume. The SPE columns were prepared as in section 2.4.2. For the breakthrough-curve construction, a known volume of a standard solution in hexane containing 16 PAHs with a concentration of approximately $0.1\,\mu g\,g^{-1}$ of each compound (similar concentration to the spiked raw extract concentration) was passed trough the optimum SPE column, collecting the eluted fraction in aliquots of 1 mL. These aliquots were analysed separately by GC-MS, and the breakthrough volume was calculated as the volume of standard solution required to elute a 1% of mass of loaded analyte out of the column [27–29].

2.5 *Analysis*

Analyses to evaluate the relative sample clean-up provided by the different SPE columns were performed using a Thermo Trace Gas Chromatograph and Flame Ionization Detector equipped with a DB5-MS $30\,m \times 0.25\,mm \times 0.25\,\mu m$ capillary column. Helium was used as carrier gas at a flow rate of $1\,mL\,min^{-1}$. The injection in the GC system was performed in splitless mode, with a splitless time of 0.30 min, and the injector temperature was 280°C. The oven temperature was held at 50°C for 2 min, increased to 180°C by a temperature ramp of $30°C\,min^{-1}$, and to 300°C by $8°C\,min^{-1}$ and held for 5 min. The detector temperature was 310°C.

Quantitative analyses, once the SPE clean-up procedure was optimized, were performed using a Hewlett-Packard HP 6890 Series gas chromatograph coupled to a 5973 mass spectrometer. Separations were carried out in a Factorfour VF5-ms $60\,m \times 250\,\mu m \times 0.25\,\mu m$ column. The analysis was performed using helium as carrier gas at $1\,mL\,min^{-1}$. The GC system was equipped with a split/splitless injector operating in the splitless mode with the purge valve open at 0.9 min. The GC oven temperature was held at 50°C for 1 min, increased to 180°C by a temperature ramp of $20°C\,min^{-1}$, ramped at $10°C\,min^{-1}$ to 300°C, and held for 10 min. The injector temperature was 280°C. The MS operating conditions were: EM 1900 eV, transfer line temperature 280°C, and operating SIM (selected ion monitoring) mode, using the following characteristic m/z: naphthalene, 128; acenaphthylene and acenaphthene, 152, 153; dibenzofurane, 168; fluorene, 166; phenanthrene and anthracene, 178; fluoranthene and pyrene, 202; benz[*a*]anthracene and chrysene, 228; benzo[*b*]fluoranthene, benzo[*k*]-fluoranthene and benzo[*a*]pyrene, 252; dibenz[*ah*]anthracene, 278; and benzo[*ghi*]-perylene, 276. Acenaphthene d10, with a characteristic mass of 164, was added to all the standards and sample extracts before the injection as internal standard. Quantitative results of PAHs were based on the area of their peak compared with the internal standard and compared with the PAHs standards.

3. Results and discussion

3.1 *SPE clean-up optimization of DSASE extracts from lichens*

3.1.1 Solid-phase selection. Lichen raw extracts in hexane were passed through every tested column: NH_2, SAX, alumina, and DPA. The retained compounds (target and non-target compounds) were eluted with 1.5 mL of hexane, except for the DPA column, where the raw extract in hexane was evaporated to 0.5 mL and diluted again to 2 mL with methanol, as this phase only accepts aqueous or methanolic solutions. In this case, the elution was performed with 1.5 mL of methanol. Both fractions, the eluted extract containing the non-retained compounds and the 1.5 mL hexane (or methanol) fraction, were collected, evaporated to 0.5 mL and analysed by GC-FID to see which fraction shows the lower presence of interfering compounds.

Prior to the GC-FID analysis, the green colour intensity of the solvent eluted fractions was considered as a visual estimation of the relative clean-up grade. NH_2 and SAX phases provided the more effective visual clean-up because their eluted fractions with hexane turn out completely colourless. An intermediate grade of visual clean-up was obtained using the alumina phase, and no cleaning was performed with the DPA phase, since the methanol eluted fraction kept the same green colour as the raw extract. The GC-FID chromatograms showed that the eluted fraction with hexane (or methanol) gave the maximum relative clean-up in all cases as well as the major presence of target compounds. This means that the PAHs are trapped in the solid phases, and a large amount of interfering compounds are non-retained and therefore removed from the extract. Figure 1 shows the chromatograms corresponding to the eluted fraction with solvent (hexane or methanol) and a chromatogram of the raw extract. As can be seen, the most effective phases for removing interferences were NH_2 and SAX, while the alumina phase showed a lower cleaning efficiency, and in the case of the DPA column, the chromatograms were practically identical to the raw extract. These results fit with the colour observed in every eluted fraction.

From the point of view of the literature, the NH_2 solid phase shows several advantages compared with the SAX phase for organic compounds determination in vegetal extracts:

(1) A previous study showed that the NH_2 solid phase is able to remove some fatty acids that were especially prevalent in green vegetables, like hexadecanoic and octadecanoic, from the raw extract, whereas this was not observed using the SAX phase [16].
(2) The NH_2 solid phase is also indicated in literature for removing phenols and plants pigments.
(3) The NH_2 solid phase combines the normal phase characteristics with the weak cation-exchange mechanism [25].

For these reasons, the NH_2 SPE cartridge was selected to carry out the clean-up procedure.

The amount of NH_2 bed used for the previous solid-phase selection was 100 mg. However, this amount was insufficient to trap all PAHs on the sorbent due to the presence of non-retained PAHs in the eluted extract fraction. Thus, cartridges with higher amounts of bed, 500 mg, were studied in terms of breakthrough volume for the first eluted PAH: naphthalene. To determine the volume of extract at which

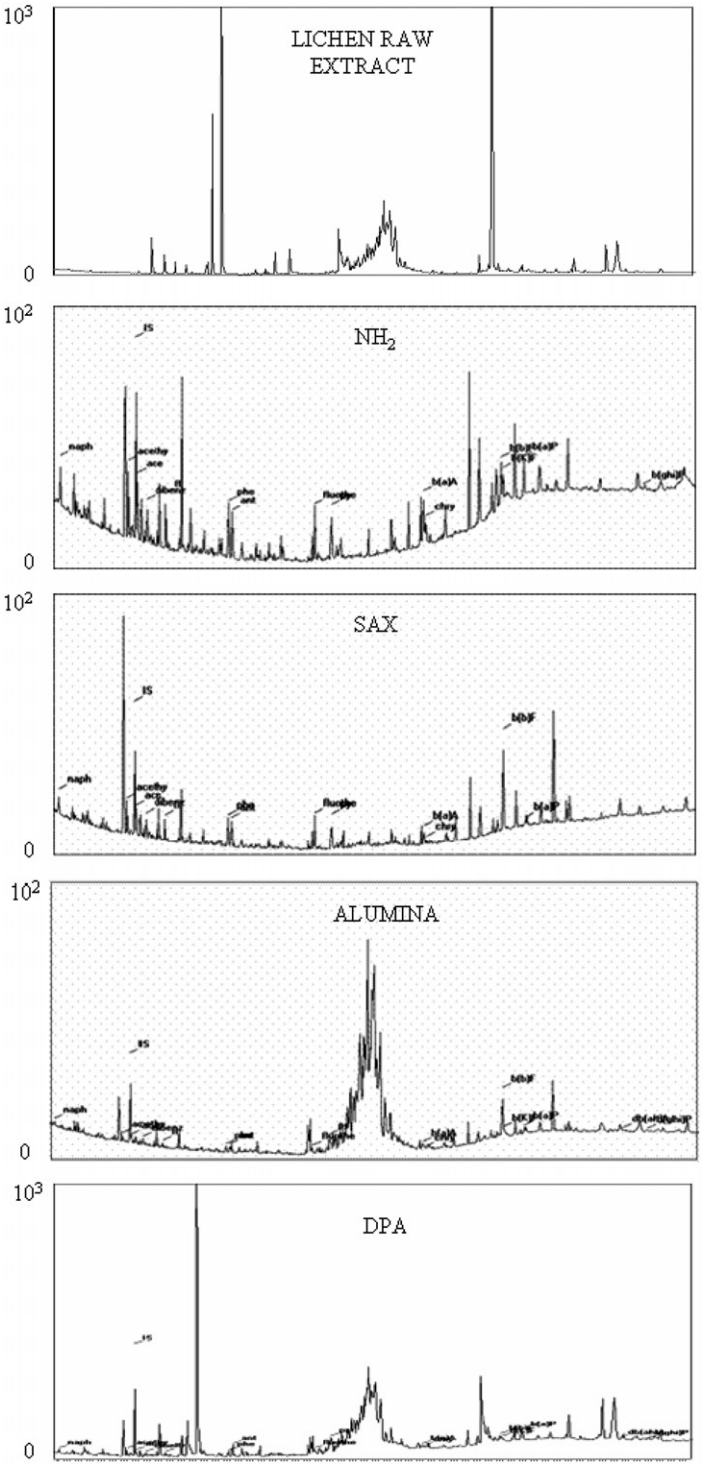

Figure 1. GC-FID chromatograms corresponding to four different SPE eluted fraction with hexane (or methanol) compared with a chromatogram of the raw lichen extract.

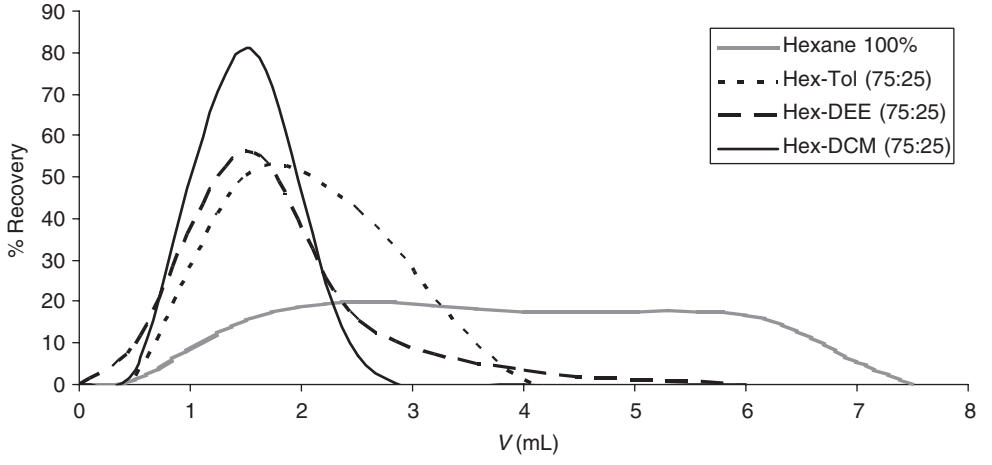

Figure 2. Elution profiles of NH_2 SPE column using hexane, hexane–toluene (75–25), hexane–diethyl ether (75–25) and hexane–dichloromethane (75–25).

naphthalene breakthrough begins, a breakthrough curve was constructed. Using 500-mg NH_2 cartridges, 2.4 mL of sample were required before the elution of naphthalene started. Consequently, 500 mg of NH_2 solid bed was sufficient for the complete PAH retention of native lichen samples (non-spiked).

3.1.2 PAH elution. Once the raw extract was loaded into the SPE cartridge and target analytes were trapped, the next step was the quantitative elution of the PAHs using the minimum amount of solvent. For this purpose, several eluting solvents were tested: hexane, hexane–toluene (75–25), hexane–diethyleter (75–25) and hexane–dichloromethane (75–25). The elution profile was constructed in each case collecting different fractions of solvent, and the recovery of 16 PAHs was determined for every fraction. Figure 2 shows the elution profiles obtained for every solvent. As can be seen, hexane showed the highest amount of eluting solvent; 7.5 mL of hexane was required to elute 76% of total PAHs; and the heaviest compounds, dB[*ah*]A and B[*ghi*]P, were not quantitatively recovered, with recoveries of 63 and 69%, respectively. Nevertheless, a considerable enhancement of the eluting profile was observed using as eluting solvent a solution of hexane and another solvent of higher polarity. The more polar the used solvent was, the lower the amounts of eluting solvent were required to obtain similar recoveries of total PAHs (95.3, 91.2, and 94.2%, respectively). Therefore, the best results were obtained using hexane–dichlorometane, since only 3 mL of eluting solvent was required. For the three cases, all PAHs were quantitatively eluted, even the heaviest ones.

Furthermore, different hexane–dichloromethane proportions, 65–35, 75–25, and 85–15, were used to improve the PAH elution. Two millilitres of hexane–dichloromethane was sufficient to elute 95.1% of the total PAHs. Seven peaks corresponding to co-extracted compounds from the matrix were identified, and their GC signal was evaluated in every collected fraction of hexane. Figure 3 shows the elution of the seven compounds using hexane as eluting solvent. In all cases, the signal

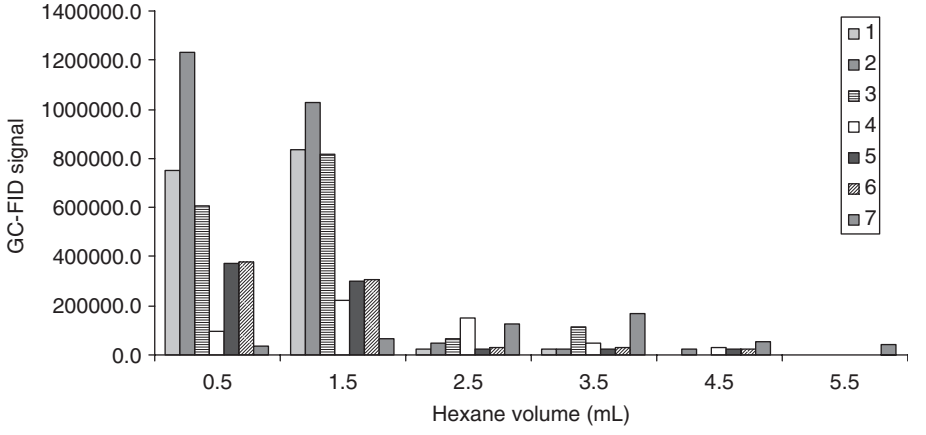

Figure 3. GC signal of seven co-extracted matrix compounds eluted with hexane as eluting solvent.

Table 1. PAH percentage recovery values obtained from approximately 150 ng of PAHs using the optimized SPE clean-up procedure: NH_2 SPE column, 0.5 mL of hexane as rinsing solvent and 2 mL of hexane–dichloromethane (65–35%) as eluting solvent (spiked raw extracts with $n = 5$; blank extracts with $n = 3$).

PAHs	Recovery (%)	RSD (%)
Naphthalene	84.4	7
Acenaphthylene	90.0	0.1
Acenaphthene	89.0	0.2
Dibenzofurane	79.8	0.9
Fluorene	92.3	2
Phenanthrene	95.3	0.2
Anthracene	92.8	0.3
Fluoranthene	97.0	3
Pyrene	92.8	4
Benz[*a*]anthracene	89.6	4
Chrysene	89.4	10
Benzo[*b*]fluoranthene	89.8	2
Benzo[*k*]fluoranthene	95.8	2
Benzo[*a*]pyrene	94.0	1
Dibenz[*Ah*]anthracene	85.2	0.8
Benzo[*Ghi*]perylene	92.7	0.8

of the co-extracted compounds was much lower than in the raw extract chromatogram. As can be seen, most interferences were eluted in the two first fractions (0.5 and 1 mL), but only 1% of total PAHs were eluted in the first 0.5 mL of hexane. Therefore, 0.5 mL of hexane was used as rinsing solvent before the elution of PAHs as an additional step to remove the maximum amount of interference.

The final SPE procedure to determine PAHs in lichen samples was as follows: after conditioning the solid bed, a sample of 2 mL of raw extract was loaded on the SPE column. Hexane (0.5 mL) was used as rinsing solvent to remove a large amount of co-extracted compounds (non-target) retained on the column, and the PAHs (target compounds) were quantitatively eluted from the column using 2 mL of hexane–dichloromethane (65–35) as eluting solvent. The recovery for every PAH is shown

in table 1. As can be seen, the RSD values are very low with the exception of naphthalene and chrysene. This could be attributed to the volatility in the case of naphthalene and the risk of losses in the evaporation step. Chrysene had a broader peak than the other PAHs in the chromatographic conditions, showing a tailing effect which made the interpretation difficult, and so this could increase the RSD. The percentage of cleaning obtained with the optimized SPE cleanup procedure was 98.3%.

The optimized SPE clean-up procedure shows clear advantages compared with other conventional methods, such as the alumina laboratory-made columns used in previous works as a clean-up step [11]. That technique is time-consuming, requires large volumes of eluting solvents, as well as the thermal activation of the sorbent, and usually proves to be inadequate in terms of effective cleaning of the extract. The clean-up step carried out using the final SPE method removes a higher amount of co-extracted compounds from the extract than conventional techniques and considerably reduces the operation time and solvent consumption.

3.2 *Quantitative determination of the 16 PAHs in lichens*

The 16 EPA priority PAHs were determined in native lichen samples, *Parmelia sulcata*, from eight sampling points in the Aragon valley, in the central Pyrenees, using the optimized analytical procedure. Table 2 shows the concentration ($ng\,g^{-1}$) of PAHs found in every lichen sample. The concentration values were calculated as the average of three replicates for every sampling point. Sampling points are shown in figure 4.

The content of 16 EPA priority PAHs in the lichen samples varies from 352 to $1654\,ng\,g^{-1}$. The highest concentration values of these hydrocarbons ($876–1654\,ng\,g^{-1}$) were found in lichens sampled near the national road passing trough the main towns (sampling points 1–4). The lichen samples collected from natural areas far from the road (sampling points 5–8) showed lower concentration values ($352–549\,ng\,g^{-1}$), and the lowest value ($352\,ng\,g^{-1}$) was established as the reference PAH level for the Aragon valley. As can be seen in table 2, the total 16 PAH concentrations expressed as the reference level show considerable differences between lichens collected from the two studied areas.

In all cases, 14–16 PAHs were identified, and so lichens presented an almost full spectrum of analysed PAHs. The most abundant compounds of all 16 PAHs were naphthalene, acenaphthene, phenanthrene, fluoranthene, and pyrene. The best marker for assessing the pollution caused by PAHs is the Fl/Py ratio, shown in table 2, which ranged from 0.95 to 2.22 with a mean of 1.70. These values are very similar to Fl/Py found in soils (1–1.72), lichens (1.92–2.21), mosses (1.35–1.44), and pine root and wood (1.50–1.72) in previous studies carried out in a mountainous area, and indicate a relatively unpolluted environment [8]. The ratio Phe/Ant, also shown in table 2, varies from 3.64 to 8.87 with a mean value of 5.58, which indicates a higher concentration of phenanthrene due to the long exposures of anthracene to sunlight that causes the photochemical degradation of this compound. Phe/Ant ratios lower than 10 and Fl/Py ratios higher than 1 strongly indicate the pyrogenic origin of the PAHs [10].

Table 3 shows the distribution profile according to three-, four-, five-, and six-ring PAHs. Three-ring PAHs were dominant, followed by four-ring PAHs in all samples (67–88%). Lichen samples collected near the road (sampling points 1–4) have lower

Figure 4. Map of the study area and locations of the sampling points: 1, Castiello; 2, Villanúa; 3, Canfranc; 4, Canfranc Station; 5, Camping Canfranc; 6, Fuente del Paco; 7, Villanúa (natural area); 8, Lierde.

Table 2. Concentration of 16 EPA priority PAHs, Σ16 PAHs/reference level and fluoranthene/pyrene (Flu/Py) and phenanthrene/anthracene (Phe/Ant) ratios found in lichen samples.

Sampling point	Sampling point name[a]	Total PAHs ($ng\,g^{-1}$)[b]	Σ16 PAHs/ reference level	Flu/Py	Phe/Ant
1	Castiello	876 ± 19	2.5	1.75	5.36
2	Villanúa	1169 ± 36	3.3	1.64	5.42
3	Canfranc	1654 ± 29	4.7	1.83	6.48
4	Canfranc station	966 ± 60	2.7	1.88	3.72
5	Camping Canfranc	549 ± 33	1.6	2.22	6.72
6	Fuente del Paco	352 ± 28	1.0	1.39	3.64
7	Villanúa (natural area)	484 ± 41	1.4	0.95	4.46
8	Lierde	453 ± 55	1.3	1.94	8.87

[a] For localization of sampling points, see figure 4.
[b] Expresed as average value ($n = 3$).

Table 3. Percentage of PAHs with different number of aromatic rings in lichen samples.

Sampling point	Sampling point name[a]	Three-ring PAHs (%)	Four-ring PAHs (%)	Five-ring PAHs (%)	Six-ring PAHs (%)
1	Castiello	62	19	< 16	< 2
2	Villanúa	63	24	9	3
3	Canfranc	54	29	13	4
4	Canfranc station	56	29	< 12	< 3
5	Camping Canfranc	48	25	18	< 4
6	Fuente del Paco	49	18	18	< 6
7	Villanúa (natural area)	33	33	21	6
8	Lierde	41	26	< 26	7

[a] For localization of sampling points, see figure 4.

percentages of five- and six-ring PAHs (12–19%) than those sampled in natural areas far from traffic-exhaust emissions (22–33%). This could be attributed to the dispersion of fine particles in the atmosphere as well as the joint contribution of other pollution sources by atmospheric transport.

4. Conclusions

Four different solid phases were studied: NH_2, SAX, alumina, and DPA, to develop the SPE clean-up procedure for lichen samples. NH_2 and SAX were found to be the most efficient phases, and the NH_2 SPE phase was selected for cleanup the optimizing procedure. One hundred milligrams of solid bed was found to be insufficient to retain all PAHs due to breakthrough of the analytes, and 500 mg was required to ensure that all PAHs were trapped in the column. A considerable amount of co-extracted matrix compounds was removed from the final extract using 0.5 mL of hexane as rinsing solvent. For the PAH elution, hexane solutions enriched with a polar solvent (toluene, diethyleter, and dichloromethane) were tested. All PAHs were quantitatively eluted

using 2 mL of hexane–dichloromethane (65–35). The optimized SPE method provides a rapid and low-solvent-consumption sample clean-up procedure compared with other conventional methods based on column chromatography and can be applied to other extracts from different matrices that contain chlorophylls or compounds extracted from green vegetables.

Lichen samples collected in the Aragon valley in the central Pyrenees were analysed by the DSASE method and the optimized SPE clean-up procedure. Sixteen EPA priority PAHs were found in lichen samples, and differences in PAH concentrations and profiles were found between lichens collected near the main road and those sampled from natural areas far from traffic-exhaust emissions. In all cases, lichens showed an almost complete spectrum of PAHs.

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