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Proposal of a procedure for the analysis of atmospheric polycyclic aromatic hydrocarbons in mosses

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ABSTRACT

A useful analytical procedure for the analysis of 19 polycyclic aromatic hydrocarbons (PAHs) in moss samples using microwave assisted extraction and programmed temperature vaporization-gas chromatography-tandem mass spectrometry (PTV-GC–MS/MS) determination is proposed. The state of art in PAHs analysis in mosses was reviewed. All the steps of the analysis were optimized regarding not only to the analytical parameters, but also the cost, the total time of analysis and the labour. The method was validated for one moss species used as moss monitor in ambient air, obtaining high recoveries (between 83–108%), low quantitation limits (lower than 2 ng g^{-1}), good intermediate precision (relative standard deviation lower than 10%), uncertainties lower than 20%. Finally, the method was checked for other species, demonstrating its suitability for the analysis of different moss species. For this reason the proposed method can be helpful in air biomonitoring studies.

1. Introduction

Monitoring of polycyclic aromatic hydrocarbons (PAHs) in ambient air is of great interest due to the implications on human health of the presence of those compounds in the atmosphere. The specialized cancer agency of the World Health Organization, the International Agency for Research on Cancer (IARC), classified outdoor air pollution as carcinogenic to humans (Group 1) [1]. PAHs levels can vary considerably in space, and thus it is of great interest the use of sampling tools that are able to assess spatial deposition of PAHs at a local scale. The use of moss as passive accumulators for organic compounds has gained popularity in the last decades because of their usefulness for the large scale monitoring [2]. Directive 2004/107/EC allows the use of alternative sampling methods which it can demonstrate give results equivalent to the reference method to assess spatial deposition of PAHs [3]. The morphological and physiological characteristics of mosses make them excellent tools for biomonitoring [4,5]. The growing interest by using moss as monitors for PAHs sampling makes necessary the development of efficient analytical procedures for the analysis of this kind of samples.

A review of the state of the art in the analysis of PAH in moss can be seen in Table 1. The most common technique for the determination of the PAHs is gas chromatography coupled to mass spectrometry. The most common technique used in the extraction of PAHs from mosses is the Soxhlet extraction. However, it demands large volumes of highly purified organic solvents and long extraction times are need. For these reasons it is interesting the use of alternative techniques that allow a more efficient extraction of the analytes from the matrix by improving the contact of the target compounds with the extraction solvent. By this way a reduction of both the extraction time and the organic solvent consumption is achieved, and also an increase in sample throughput.

In the last years other authors have introduced accelerated solvent extraction (ASE) [4,6–9] or dynamic sonication-assisted solvent extraction (DSASE) [10] as alternative extraction procedures for the analysis of PAHs in moss. Microwave assisted extraction (MAE) has been widely used for the analysis of inorganic elements in moss, but, as far as we know, not for organic compounds.

MAE is more efficient and faster than the traditional liquid-solid procedures, allows the simultaneous extraction of several samples (between 6–12) and is less expensive than ASE. Moreover, the volume of solvent used is about 10 times lower than the required in Soxhlet extraction and also below the required in sonication [11]. The use of MAE for PAHs analysis is very frequent in matrices such as air particulate [12–18], soils [19–21] and sediments [22–26]. Few papers

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Table 1
Review of the state of art in PAH analysis in moss. ACN:acetonitrile, ASE: accelerated solvent extraction, NM: not mentioned.

Compounds	Sample	Extraction	Cean-up	Determ.	Recovery	Sensitivity	Uncertainty/RSD	Ref.
16 PAHs EPA	*Fontinalis antipyretica*	Soxhlet 200 ml ACN24h.	Florisil . Elution 30 ml CAN	HPLC-FLD	60–107%	NM	RSD:20–24%	[35]
13 PAHs	*Dicranum scoparium* *Hypnum cupressiforme* *Thamnobryum alopecurum* *Thuidium tamariscinum*	Soxtec (moss+sodium sulphate+Florisil)	Florisil cartridges 1 g	HPLC-FLD	25–79%	LOQ 3–52 pg instrumental	RSD < 20%	[34]
16 PAHs	*Hylocomium splendens* *Pleurozium scheberi*	ASE, DCM	–	GC–MS	74–96%	LOQ 1-5 ng g^{-1}	U: 10–25%	[6]
17 PAHs	*Hylocomium splendens* *Pleurozium scheberi*	Soxhlet, DCM.	–	GC–MS	74–96%	NM	U: 10–25%	[48]
16 PAHs	*Hypnum cupressiforme*	Sonication 5 g +100 ml H:A (1:1), twice	Silica column	HPLC	65–85%	NM	RSD: 10–15 %	[40]
13 PAHs	*Hypnum cupressiforme* *Isothecium myosuroides*	ASE, H 80 °C, 5 min, 2 cycles	Florisil cartridge 1 g, 8 ml H/DCM (60:40)	HPLC	68–70%	3–52 pg instrumental	RSD: 1–22%	[4,7,49]
11 PAHs	*Fontinalis antipyretica*	Soxhlet 200 ml DCM 16 h	Florisil cartridges	HPLC-FLD	65–78%	NM	NM	[36]
15 PAHs	*Pleurozium scheberi*	Soxhlet 200 ml DCM 16 h	Alumina column, 10 ml DCM	GC–MS	NM	NM	NM	[38]
18 PAHs	*Tortula muralis*	Sonication 5 g 30 min, 100 mL H	No clean up	GC–MS	Average 70%	NM	NM	[50]
16 PAH	*Hypnum plumaeformae*	ASE 5 g, 1500 psi, 100 °C, 2cycles, 5 min DCM:A (1:1)	5 g alumina+5 g florisil+10 g silica, 60 ml DCM GPC: 10 g Biobeads S-X3, 80 ml H:DCM (1:1)	GC–MS	49–99%	MDL:3.3–7.8 ng g^{-1}	RSD:5–8%	[8]
9 PAH	*Hypnum cupressiforme*	Microsoxhlet 3 h immersed in H, and 2 h reflux	–	HPLC-FLD	81–98%	NM	RSD:5.5–24%	[46]
PAHs and OCPs	*Pleurozium scheberi*	ASE 40 °C+120 °C, 3*10 min, H	15 g florisil 160 ml H:DCM (1:1), first 60 ml passed through 3.5 g active florisil 60 ml H:DCM (1:1)	GC–MS	25–78%	NM	NM	[9]
16 PAHs EPA	*Hylocomium splendens* *Scleropodium purum* *Hypnum cupressiforme* *Abietinella abietina*	Soxhlet 5 g, H	PAH soil cartridges 1.5 g. DCM:petroleum ether(1:4)	GC–MS	47–114%	NM	RSD:10–19%	[51,52]
16 PAHs	*Hypnum cupressiforme*	Soxhlet , 8 h DCM. Sulphuric clean up	Florisil column	GC–MS	80–98%	0.3–1 ng g^{-1}	RSD: 3–8%	[37]
16 PAHs	*Leptodon smithii*	Sonication, 3 g, 3*100 ml DCM:A (1:1)	–	GC–MS	NM	LOD 1–3 ng ml^{-1}	NM	[53]
15PAHs 8nPAHs	*Hypnum cupressiforme*	DSASE, 0,2 g, H, 2 ml	0.05 g Florisil+0.5 g NH2-SPE 2 ml H:DCM (65:35)	APGC-Q-TOF-MS	79–98%	instrumental LOD: 7–350 ng g^{-1}	RSD 1.8–17%	[10]
PAHs	*Hypnum cupressiforme*	Soxhlet 5 g, 200 ml DCM.	Silica column	HPLC-UV	NM	NM	NM	[39]
19 PAHs	*Pseudoscleropodium purum*	MAE, 20 ml H: A (90:10)	Dual layer Florisil-Silica (2 g+2 g).	GC–MS/MS	83–108%	MQL: 0.1–1.7 ng g^{-1}	U 8–22%	This work
	Sphagnum sp		5 ml H+15 ml DCM:H (20:80)		56–108%			
	Hypnum cupresiforme				62–112%			

were found about plant samples: pine and spruce needles [27–29], tree leaves [30], tea [31] and cereals [32,33].

Regarding the clean-up, this step is critical in the analysis of complex matrices such as moss. It is very important to achieve an adequate clean-up of the extracts in order to prevent the presence of interferences that make difficult the quantitation of PAHs and also cause damages in the GC–MS system. As can be seen in Table 1, Florisil® is the most popular sorbent [7,9,34–37], and it is mostly used in cartridge disposition. Alumina [27,38] and silica [39,40] are also frequently used, but mainly in glass column disposition, which is laborious and involves large amounts of sorbents and high volume of solvent consumption.

Most of the papers referenced in Table 1 are focussed on the levels of PAHs in moss samples. Nevertheless, the methodological aspects are relegated to the background, and only give few details about the analytical procedure and the performance characteristics of the method. Due to the complexity of the moss matrix, it is important to carefully study all the steps involved in the analytical procedure, in order to ensure reliable results.

After reviewing the state of the art, in this work we study and propose a new procedure for the analysis of 19 PAHs from moss, testing by first time the usefulness of microwaves for the extraction of PAHs from moss. This is the first article that uses MAE to analyse organic compounds in moss. We include in the study some PAHs typically found in air samples, and scarcely determined in moss (for example retene and benzo(e)pyrene). The extraction combined with an appropriate clean up, and the programmed temperature vaporization-gas chromatography-tandem mass spectrometry determination (PTV-GC–MS/MS), allow us to perform a reliable determination of these compounds at low levels. All the steps of the analysis were optimized regarding not only to the analytical parameters, but also the cost, the total time of analysis and the labour, and then the analytical performance characteristics of the method were determined. The moss species *Pseudoscleropodium purum* was selected for the optimization. This is one of the easiest mosses to recognize in the field, and occurs in a wide range of habitats, but especially in unimproved, acidic grassland and heaths, but also in chalk and limestone grassland, on banks, among rocks and on rock ledges [41]. The proposed method also checked for the analysis of other species (*Sphagnum sp* and *Hypnum cupresiforme).*

2. Material and methods

2.1. Standards and reagents

PAH-Mix 45 (10 μg ml^{-1} in cyclohexane containing acenaphthene, acenaphthylene, anthracene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene, benzo[a]pyrene, benzo[e] pyrene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3,c,d]pyrene, naphthalene, perylene, phenanthrene, and pyrene was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Individual standards of benzo[j]fluorathene (10 μg ml^{-1} in cyclohexane), retene (10 μg ml^{-1} in cyclohexane), [$^2H_{12}$] chrysene (chrysene-d_{12}) (10 μg ml^{-1} in cyclohexane), and [$^2H_{10}$] anthracene (anthracene-d_{10}) (100 μg ml^{-1} in cyclohexane) (Internal standard) were also supplied by Dr. Ehrenstorfer GmbH. D-labelled PAH surrogate Cocktail 200 μg ml^{-1} in 50% methylene chloride (D_2, 99.9%) and 50% methanol (D_2, 99%) containing [$^2H_8$] acenaphthylene (acenaphthylene-d_8), [$^2H_{12}$] benzo[a]pyrene (benzo[a]pyrene-d_{12}), [$^2H_{12}$] benzo[g,h,i]perylene (benzo[g,h,i]perylene-d_{12}), [$^2H_{10}$] fluoranthene (fluoranthene-d_{10}), [$^2H_8$] naphthalene (naphthalene -d_8), [$^2H_{10}$] phenanthrene (phenanthrene-d_{10}), and [$^2H_{10}$] pyrene (pyrene-d_{10}) was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

The working standards were prepared as follows: 0.5 μg ml^{-1} of parent PAHs in hexane, 0.2 μg ml^{-1} of surrogate labelled standards (surrogate cocktail + chrysene-d_{12}) in hexane, 0.5 μg ml^{-1} of internal standard (anthracene-d_{10}) in hexane.

Dichloromethane (DCM) and acetone (A) Super Purity grade were supplied by Romil (Cambridge, UK), hexane (H) Unisolv®, for organic trace analysis, was purchased from Merk (Darmstadt, Germany). Ethyl acetate (EA) for instrumental analysis grade was supplied by Panreac (Barcelona, Spain).

Sep-Pack® Vac 3 cc (500 mg) Silica cartridges, Supelclean™ PSA SPE 0.2 g/3 ml and dual layer Supelclean™ Florisil® LC-SI 2 g/2 g 12 ml SPE tubes were supplied by Supelco (Bellefonte, USA). Sep-Pak® Vac 20 cc (5 g) Florisil® cartridges were supplied by Waters. Filtration of supernatant was carried out with 0.6-μm glass fibre filter MN GF-6 (Macherey Nagel, Düren, Germany).

2.2. Sampling

The species selected for the study was the moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch. Moss samples were collected in Galicia (NW Spain), in woods from unpolluted areas distant from urban and industrial areas. Samples were washed with bidistilled water, oven-dried at 45 °C and ground in an ultracentrifuge mill (Retsch ZM100) [42]. Samples from different locations were combined in order to obtain a large homogeneous composite sample used for all the optimizations.

2.3. Extraction and clean up

Briefly, in the optimized procedure 0.5 g of moss samples (with 50 μl of working solution of labelled surrogate standards) were extracted in a microwave oven (Anton Paar Multiwave Graz, Austria) in HF50 reactors, at 80 °C (16 min) using 20 ml of a mixture H:A (90:10) as extracting solvent. The extracts were filtered by 0.6 μm glass fibre filter, and then concentrated to 0.5 ml in a Syncore® Analyst evaporator from Büchi Labortechnik AG (Flawil, Switzerland).

The clean-up was performed using dual layer Supelclean™ Florisil® LC-SI 2 g/2 g 12 ml SPE tubes, previously washed with 10 ml of DCM:H (20:80), using a Visiprep vacuum distribution manifold from Supelco (Bellefonte, PA, USA). The PAHs were eluted with 5 ml of hexane and 15 ml of DCM:H (20:80) mixture. The eluate was concentrated to 0.3 mL in the Syncore® and raised to 1 ml with H. Then 20 μl of internal standard (anthracene-d_{10}) was added. All were transferred to a vial and injected in the PTV-GC–MS-MS for PAH determination.

2.4. Gas chromatography-tandem mass spectrometry

Gas chromatography was performed with a Thermo-Finnigan (Waltham, MA, USA) Trace GC chromatograph equipped with a GC Combi-PAL autosampler (CTC-Analytics, AG, Switzerland), programmed temperature vaporisation injector (PTV), and coupled to an ion trap mass spectrometer (Polaris Q). Xcalibur was the data processor. The system was operated in electron impact mode (EI; 70 eV) and tandem mass spectrometry was the detection mode. The parent and product ions and retention time of PAHs studied are shown in Table 2. Transfer line temperature was set at 300 °C and ion source temperature at 250 °C. Helium (99.9999%) was used as the collision gas at the ion trap chamber, and as carrier gas, under constant flow rate of 1 ml min^{-1}.

The separation was achieved with a DB-XLB column (60 m x 0.25 mm, 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The GC oven temperature program used was: 50 °C (3 min), increased by 4 °C min^{-1} to 325 °C (held for 20 min).

Table 2
Analytical parameters: method detection limits (MDL) method quantitation limits (MQL), accuracy, intermediate precision (IP) and uncertainty. The compounds remarked in italics are the surrogates used for the quantitation of each group of compounds.

	Retention time ($_{min}$)	Parent ion	Product Ion	MDL (ng g^{-1})	MQL (ng g^{-1})	Accuracy(%)	IP (%) (n=7)	Uncertainty (%)
Naphthalene d8	25.79	136	108					
Naphthalene	25.90	128	102	0.385	0.696	99	8.8	10
Acenaphthylene-d8	35.47	160	156					
Acenaphthylene	35.51	152	125	0.809	1.67	90	5.5	18
Acenaphthene	36.59	153	150	0.423	0.971	86	4.0	22
Fluorene	39.90	166	163	0.114	0.262	93	6.0	15
Phenanthrene d10	45.64	188	160					
Phenanthrene	45.79	178	152	0.508	1.02	95	3.5	11
IS anthracene d10	46.07	188	160					
Anthracene	46.19	178	152	0.641	1.29	108	9.9	12
Fluoranthene d10	53.07	212	208					
Fluoranthene	53.21	202	198	0.261	0.597	92	5.1	12
Pyrene d10	54.48	212	208					
Pyrene	54.60	202	198	0.248	0.568	90	3.7	17
Retene	56.04	219	204	0.102	0.214	105	3.2	12
Benz[a]anthracene	62.07	228	224	0.101	0.210	105	5.3	9
Chrysene d12	62.14	240	236					
Chrysene	62.33	228	224	0.099	0.206	100	8.4	9
Benzo[b+j]fluoranthene	68.45	252	248	0.049	0.107	88	2.7	19
Benzo[k]fluoranthene	68.56	252	248	0.074	0.162	92	6.4	10
Benzo[e]pyrene	69.93	252	248	0.099	0.217	83	9.5	18
Benzo[a]pyrene d12	70.11	264	260					
Benzo[a]pyrene	70.24	252	248	0.237	0.520	93	6.1	16
Perylene	70.68	252	248	0.096	0.211	87	12	18
Dibenz[a,h]anthracene	76.14	278	274	0.075	0.151	108	7.3	19
Indeno[1,2,3–cd]pyrene	76.25	276	272	0.360	0.932	99	4.1	8
Benzo[ghi]perylene	78.15	276	272	0.399	1.034	95	6.1	15

IS: Internal standard

A Silcosteel® liner, with 2 mm of inner diameter, glass wool packing for PTV was purchased from Thermo Finnigan (Thermo Electron Corporation, Waltham, USA). The injected volume was 25 μl, the injector programme was started at 55 °C and up at 3 °C s^{-1} until 300 °C (held for 20 min) [43].

2.5. Quantification and quality control

Instrumental and procedural blanks were systematically evaluated (a blank for each batch) and maintained at minimum. Two standard solutions containing all the studied PAHs were injected for each batch for chromatographic control. Anthracene-d_{10} was used as internal standard. Quality control of the complete procedure and quantification was performed using labelled PAHs as surrogate standards.

3. Experimental

3.1. Study of extraction parameters

The extraction method selected in this work to perform the extraction of PAHs from moss was MAE. The initial conditions for the analysis were selected according to our previous studies for the analysis of PAHs from mussel samples [44]. The extraction (0.5 g of moss) was initially made at 130 °C (750 W) for 30 min, using 30 ml of a mixture of H:A (1:1). At these conditions an intense green extract was obtained containing pigments and other matrix interfering compounds that complicate the subsequent clean up step. In order to reduce the coextraction of interfering substances the percentage of A was reduced using a mixture of H:A (90:10), obtaining a less intense green extract. Finally, the extraction volume was reduced to 20 ml, and the extraction temperature, and the extraction time were reduced to 80 °C and 16 min of heating respectively. The power program consists of four repetitions of the sequence 550 W during 3 min followed by 0 W during 1 min, in order to maintain the temperature around 80 °C during all the extraction time. These last conditions are softer than the usual ones for the extraction of PAHs (110 °C 20 min for extraction of PAHs from soils [19], atmospheric particulate [15], or fish [45]), and allow a lower coextraction of interfering substances (such as pigments). A clearer extract was obtained, making easier the purification step, and for this reason these were the conditions finally selected. All these assays were performed simultaneously with the purification step assays in order to obtain the best combination of extraction-purification conditions.

The extraction efficiency (considering only the extraction step) using the selected procedure was satisfactory, with recoveries ranged between 62–105 %.

3.2. Study of the clean up step and matrix effect

The extracts obtained from the MAE are coloured, containing interferences that make quantitation of PAHs more difficult. Moreover the injection of dirty extracts into the gas chromatograph causes a more frequent maintenance shutdown. For these reasons it is very important to achieve an efficient clean up.

The first purification assays were performed for extracts obtained at the initially assayed extraction conditions (30 ml H:A 1:1, at 130 °C, 30 min). Florisil® is the most used sorbent for the clean-up of moss (Table 1). For this reason a cartridge with 5 g of Florisil® was initially selected to perform the first purification assays. Several eluting mixtures were assayed to obtain a colourless eluate with good elution efficiency: 30 ml of a mixture DCM:H (80:20), 30 ml of EA:H (20:80) and 30 ml of DCM:H (65:35). Only with the less polar mixture (DCM:H, 65:35) a colourless eluate was achieved. Nevertheless, bad recoveries were obtained for some of the PAHs at these conditions (34% benzo[a]pyrene d_{12}, < 10% for indeno[1,2,3–cd]pyrene and 0% perylene) . In order to determine if those results are due to an inefficient elution of the compounds or due to other causes, an assay was performed by comparing a moss purified extract spiked before the clean-up, with a moss purified extract spiked after clean up (in both cases 0.15 μg g^{-1} for parent PAHs and 0.02 μg g^{-1} for labelled standards). Low recoveries for

Assay	Extraction	Sorbent	Eluate
A	130ºC, 30min	Florisil® (5g)+Silica (0.5g)	5 ml H + 20 ml DCM:H (65:35)
B		Florisil® (5g)+PSA(0.2g)	20 ml DCM:H (65:35)
C		Florisil® -Silica (2+2g)	15 ml DCM:H (20:80)
D	80ºC, 16 min	Florisil® -Silica (2+2g)	5 ml H + 20 ml DCM:H (20:80)

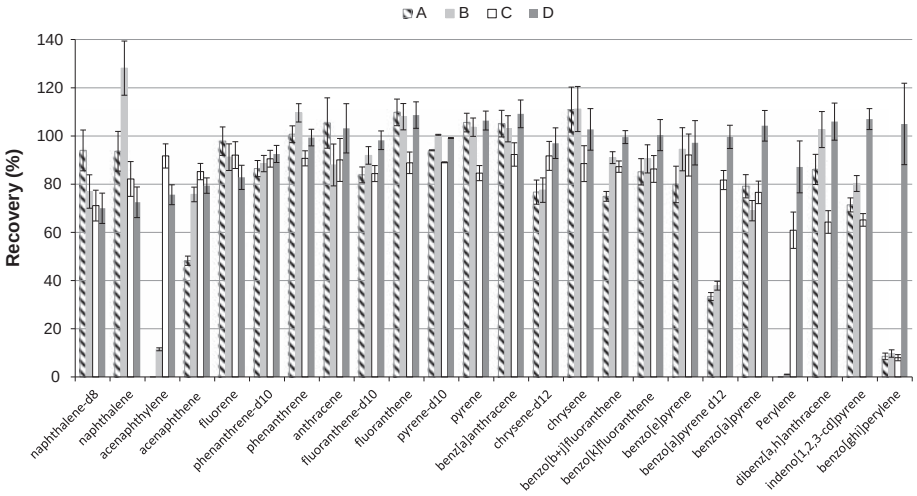

Fig. 1. Purification assays.

the cited compounds were obtained in both experiments, which indicate a matrix effect.

Taking into account the matrix effect, new assays were performed using Florisil® coupled with other sorbents such as PSA (Primary-secondary amine) or Silica (Fig. 1). Assays were performed by spiking (at 0.15 μg g^{-1} level for parent PAHs and 0.02 μg g^{-1} level for labeled PAHs) an extract obtained using a mixture of H:A (90:10). It is important to point out that A, B and C assays were performed at 130 °C during 30 min, whereas assay D was with the extraction conditions finally selected (80 °C, 16 min). As can be seen in the Fig. 1, acenaphtylene, perylene and benzo[ghi]perylene were not recovered (R < 20%) in assays A and B. The overall best results were obtained using a commercial cartridge that contains 2 g of Florisil® and 2 g of Silica, when the sample is extracted at 80 °C, performing the elution with 5 ml of hexane and 15 ml of a mixture DCM:H (20:80). Finally, these conditions were selected.

3.3. Study of the concentration step

Both, the 20 ml MAE extract of H:A (90:10) and the 20 ml eluate (5 ml of H + 15 ml DCM: H 20:80) need to be concentrated to a low volume of solvent (about 0.3 ml) prior to the next steps of the analysis. These concentration steps can be high time consuming when the evaporation is performed using a rotary evaporator or by nitrogen stream. In this work the concentration steps were improved (in comparison with the previous works) by using a Syncore® Analyst Automatic Concentrator where 12 samples were simultaneously concentrated, and therefore the evaporation step time was considerably reduced.

The evaporation conditions for each concentration step (evaporation of the extract and the eluate) were studied in order to achieve high recoveries of the analytes and minimize the evaporation time. The selected conditions for both evaporation steps were: rack temperature: 40 °C, vacuum cover: 50 °C, cooling condenser: -10 °C, cooling rack: 12 °C, vortex speed: 300 rpm. The pressure programs used in each case are shown in Table 3. The recoveries obtained ranged between 75 and 123% for the concentration of H:A extract, and between 60 and 96% for the eluate concentration.

Table 3
Syncore® Analyst evaporator pressure (mbar) conditions.

Concentration of MAE extract			
Step	**Start pressure**	**End pressure**	**Time (min)**
1	500	500	1
2	500	350	1
3	350	350	5
4	350	290	1
5	290	290	10
6	290	210	1
7	210	210	45
Concentration of clean up eluate			
Step	**Start pressure**	**End pressure**	**Time (min)**
1	640	640	1
2	640	540	1
3	540	540	10
4	540	210	1
5	210	210	50

4. Results and discussion

4.1. Selected conditions and analytical method performance characteristics

A summary of the proposed analysis procedure for the determination of PAHs in moss samples can be seen in the scheme of Fig. 2. Comparing the procedure with other procedures reported in literature for the analysis of PAHs in moss samples (Table 1), the overall solvent consumption of the proposed procedure is considerably lower (50 ml) than the typical solvent consumption (higher than 200 ml).

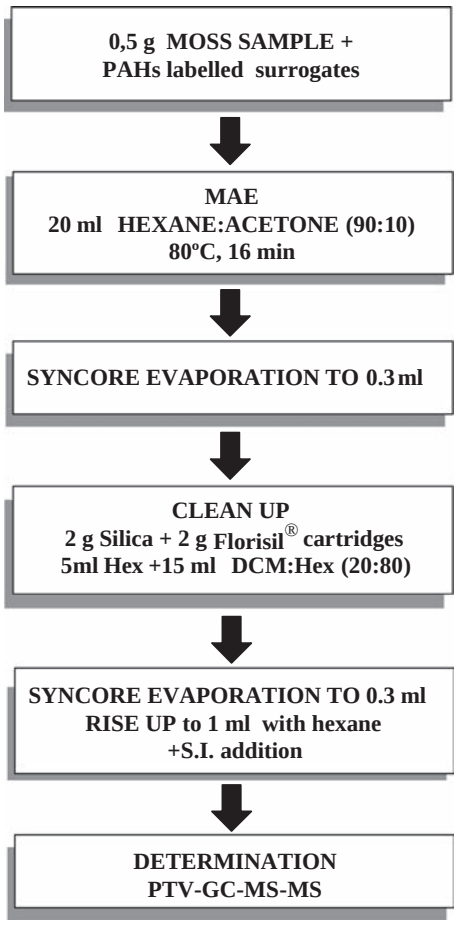

Fig. 2. Scheme of the selected analysis procedure.

There are not certificated reference materials available of PAHs in moss, or similar matrices, so the analytical performance characteristics of the method were determined using spiked moss samples.

Method detection limits (MDL) and method quantification limits (MQL) were calculated as $X_b + 3\,S_b$ and $X_b + 10\,S_b$ respectively, where X_b is the average value and S_b the standard deviation of the background in a moss sample from an unpolluted area (background levels of contaminants) (Table 2). MQL were experimentally verified. The sensitivity achieved is higher than the reported in the literature for this kind of samples (Table 1), in spite of using 10 folds amount of sample. Nevertheless, it should be pointed out that it is difficult to make the comparison because some authors only give instrumental limits, and when method limits are provided, there is not detail about the procedure to determine it.

The precision of the method was evaluated by the determination of the repeatability and the intermediate precision. The repeatability was calculated as within-day relative standard deviation (RSD) of concentrations, using four replicates of spiked moss samples (0.15 μg g^{-1}) analysed with the proposed method during the same day and the same analyst and equipment. The repeatability obtained expressed a RSD was satisfactory for all the PAHs, with values lower than 7%. The intermediate precision of the method was calculated as between-day RSD of concentrations over the course of four weeks (seven replicates). A good intermediate precision, with %RSD lower than 20% in all cases was obtained (Table 2).

Regarding the accuracy, this was determined using the analytical recoveries of spiked samples at 0.15 μg g^{-1} level ($n=7$). Accuracy was calculated quantifying the PAHs with the labelled surrogate standards. The optimized analytical procedure showed good recoveries for all the native PAHs ranged between 83 and 108% (Table 2). The recoveries obtained for the labelled compounds used as surrogates were ranged between 57–88%. These recoveries are comparable to the recoveries found in the literature for analysis of PAHs in moss using different extraction and analysis procedures (Table 1). The most volatile PAH determined in most of the papers is acenaphthylene [9,35,40,46] or only provide recovery data for two representative PAHs [4,35,46]. In the case of MAE extraction of PAHs from other vegetal matrices, Ratola et al. [27] reports recoveries lower than 50% for 5- and 6- ringed PAHs in pine needles using similar extraction power conditions than this work, but a higher volume of solvent (90 ml DCM:H (1:1) in the extraction and 150 ml of solvents in the clean up). Good recoveries are reported by Shen et al. [30] for five PAHs in tree leaves (78–103%), although in this work only anthracene, fluoranthene pyrene, chrysene and benz(a)antracene were determined.

Uncertainty of the analytical method was also estimated on the basis of in-house validation data according to EURACHEM/CITAC [47] guide for all compounds at 0.15 μg g^{-1} level. The main sources of uncertainty were identified and quantified and combined uncertainty (u_c) was calculated as follows:

$$U = k\sqrt{u_1^2 + u_2^2} + |\mu - \bar{x}|$$

where the uncertainties associated with the spiked sample

$$\left(u_1 = C_{sample} * \sqrt{\left(\frac{S_{standard}}{C_{standard}}\right)^2 + \left(\frac{S_{pipette}}{V_{pipette}}\right)^2 + \left(\frac{S_{flask}}{V_{flask}}\right)^2 + \left(\frac{S_{balance}}{m_{standard}}\right)^2}\right),$$

precision ($u_2 = S_{prec}/\sqrt{N}$) and accuracy were taken into account. The coverage factor (k) was 2 for a level of confidence of 95%.

Table 2 shows all values of the relative expanded uncertainty of target compounds at 0.15 μg g^{-1} level. The expanded uncertainty obtained was between 8–22% for all the PAHs, that is comparable to the uncertainty calculated by other authors (Table 1), although most papers only gave RSD values.

4.2. Application of the method to real samples

As an example of the applicability of the proposed procedure to the analysis of *Pseudoscleropodium purum* samples, the results obtained for two samples collected in two different areas of Galicia (NW of Spain) are presented in Table 4. Both samples (sample A and sample B) correspond to unpolluted areas, with very low concentrations of PAHs. In spite of this, the great sensitivity of the method allows the quantitation of most of the PAHs in both samples (Fig. 3). The levels found in both samples are similar than the average values found in a Natural Reserve in Spain in the period 2006–2007 [34].

Some samples of *Sphagnum sp* and *Hypnum cupresiforme* species were also analysed in order to check the suitability of the proposed method for the analysis of different moss species. Both samples were analysed by triplicate un-spiked and also spiked with PAH standard at 0.15 μg g^{-1} level. The recoveries obtained and the relative standard deviations are shown in Table 4 and also the concentration of native PAHs in samples. The recoveries obtained for the heaviest PAHs (indene[1,2,3–cd]pyrene and benzo[ghi]perylene) were lower (56–72%) than the obtained for the *Pseudoscleropodium purum* species (95–99%). Other authors also report lower recoveries for indene[1,2,3–cd]pyrene and benzo[ghi]perylene than the obtained for the light PAHs in *Hypnum cupresiforme* (for example about 60% using Soxtec [34] and between 75 and 80% using DSASE [10]). The good accuracy and precision (RSD lower than 13% in all cases) obtained with this procedure for these moss species demonstrates the suitability of the method not only for the analysis of *Pseudoscleropodium purum* samples, but also for *Sphagnum sp* and *Hypnum cupresiforme* samples.

Table 4
Concentration of PAHs (ng g^{-1}) and surrogate recoveries (%) obtained for two *Pseudoscleropodium purum* moss samples (A and B) analysed with the proposed procedure. Results obtained for the analysis of *Sphagnum sp* and *Hypnum cupresiforme* (n=3) species.

	Pseudoscleropodium purum		***Hypnum cupresiforme***		***Sphagnum sp***	
	Sample A	**Sample B**	**Concentration (ng g^{-1})**	**%Recoveries**	**Concentration (ng g^{-1})**	**%Recoveries**
Naphthalene d8	82%	66%		63		64
Naphthalene	5.1	7.3	6.0	112	8.7	108
Acenaphthylene-d8	97%	94%		76		88
Acenaphthylene	< 1.67	< 1.67	< MQL	90	0.4	79
Acenaphthene	12.2	1.48	24	90	< MQL	91
Fluorene	1.6	2.1	0.7	85	23	83
Phenanthrene d10	91%	84%		88		85
Phenanthrene	7.7	5.5	5.2	99	30	99
Anthracene	9.1	1.3	< MQL	104	8.3	100
Fluoranthene d10	103%	94%		91		94
Fluoranthene	5.8	< 0.6	1.9	82	3.9	82
Pyrene d10	101%	95%		92		94
Pyrene	4.5	2.5	1.5	80	3.2	80
Retene	8.6	1.2	8.9	106	2.4	103
Benz[a]anthracene	3.3	2.6	0.1	96	0.7	100
Chrysene d12	102%	93%		96		91
Chrysene	5.1	0.7	0.6	85	2.2	86
Benzo[b+j]fluoranthene	5.1	0.9	0.5	80	2.2	87
Benzo[k]fluoranthene	3.6	< 0.16	0.1	88	1.2	93
Benzo[e]pyrene	5.6	0.8	0.2	71	1.8	78
Benzo[a]pyrene d12	86%	92%		91		85
Benzo[a]pyrene	4.4	0.8	0.2	81	0.6	91
Perylene	2.7	0.6	0.1	78	0.4	84
Dibenz[a,h]anthracene	2.3	0.3	0.2	93	0.7	95
Indeno[1,2,3–cd]pyrene	3.5	0.9	0.2	72	1.8	70
Benzo[ghi]perylene	5.7	1.1	0.3	62	1.6	56

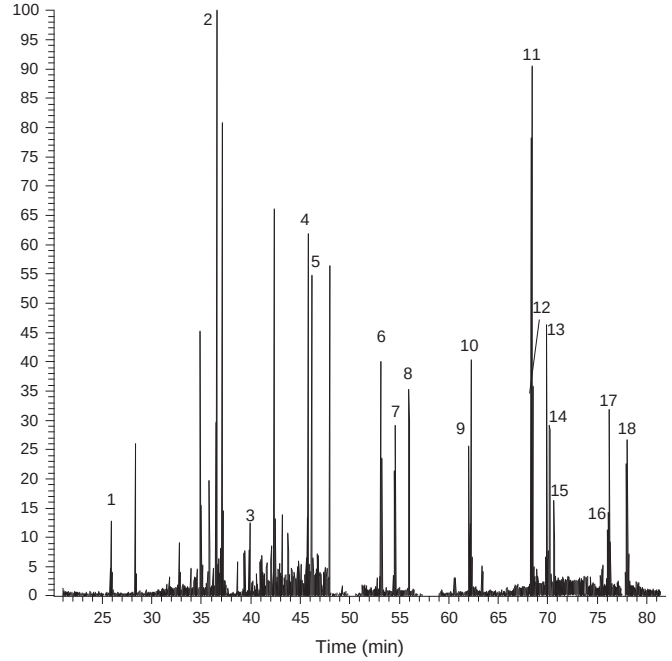

Fig. 3. Chromatogram of a real moss sample (Sample A) analysed by the proposed MAE-SPE-PTV-GC–MS/MS method. 1:naphthalene, 2: acenaphthene, 3: fluorene, 4: phenanthrene, 5: anthracene, 6: fluoranthene, 7: pyrene, 8: retene, 9: benz[a] anthracene, 10: chrysene, 11: benzo[b+j]fluoranthene, 12: benzo[k]fluoranthene, 13: benzo[e]pyrene, 14: benzo[a]pyrene, 15:perylene, 16: dibenz[a,h]anthracene, 17: indeno[1,2,3–cd]pyrene, 18: benzo[ghi]perylene.

5. Conclusions

The main objective of this work has been achieved. A new procedure for the reliable analysis of PAHs from moss samples was proposed. The method involves a microwave assisted extraction, followed by a solid phase extraction clean up, and determination by large volume injection gas chromatography tandem mass spectrometry.

The analytical performance characteristics were satisfactory: good accuracy, with recoveries ranged between 83% and 108% with a satisfactory precision, and uncertainty lower than 20% were obtained. The high sensitivity allowed reducing sample amount (0.5 g), solvent and sorbents consumption of the analysis, improving the previous procedures for this type of samples according with the principles of the green chemistry. The method is also semi-automatic, less laborious, and therefore is faster than the classical procedures. Moreover the use of programmed temperature vaporization for the introduction of samples improves the sensitivity of the method, whereas the detection by means of tandem mass spectrometry improves the selectivity of the procedure.

The method has demonstrated its suitability for the analysis of different moss species and can be helpful for the air biomonitoring studies.

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