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New procedure for selective extraction of polycyclic aromatic hydrocarbons in plants for gas chromatographic–mass spectrometric analysis

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Abstract

A new solid-phase extraction method for the clean-up and the quantitation by GC–MS of regulated polycyclic aromatic hydrocarbons (PAHs) from lettuce was developed and the experimental conditions were optimized. After ultrasonic extraction using toluene and saponification of samples, a clean-up of extracts through solid-phase extraction was performed. Samples were finally analyzed by gas chromatography–mass spectrometry (GC–MS) using an internal deuterated standard. Saponification by KOH in methanol–water (80:20) was successful allowing a good elimination of the interfering chlorophylls from the extracts containing the PAHs. The average recovery of the 16 regulated PAHs was 70, 74, 79 and 89%, respectively, for naphthalene, acenaphthylene, acenaphthene and chrysene and higher than 94% for the others.

Keywords: Solid-phase extraction; Phytoremediation; Polynuclear aromatic hydrocarbons

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants resulting from incomplete combustion or high-temperature pyrolytic processes involving materials containing carbon and hydrogen, and are thus generated whenever fossil fuels or vegetation are burned [1]. Since these compounds are long lasting, poorly degradable pollutants, they accumulate in soil and sediments, surface water, and the atmosphere.

PAHs are hydrophobic compounds of low volatility (with the exception of naphthalene, acenaphthylene and acenaphthene). They are poorly soluble in water (solubility in clear tap water of about 0.001 μg/l) [2,3], but they present a great affinity for soil organic materials. PAHs are potentially carcinogenic [4], and 16 of them have been selected by the US Environmental Protection Agency (EPA) as Constant Decree priority pollutants for regulatory purposes [1].

Contaminated sites have been cleaned up with different physico-chemical as well as biological

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methods, using bacteria, fungi or plants. The object of this bioremediation is to break down, immobilize and/or transform the wastes into beneficial—or at least non-detrimental—constituents. Bioremediation using bacteria or lignolytic fungi has been studied the most extensively. It produces metabolites like phenols or quinones, the behavior in soil and water and the toxicity of which are unknown [5,6]. In contrast, less research has been done on phytoremediation, which uses the remarkable capabilities of plants to remove, contain or render harmless PAH contaminants in soil. Plants can metabolize many xenobiotics by oxidation and conjugation and compartmentalize these products in their tissues. The environmental impact of plant metabolites of xenobiotics and the sink function of the plant global biomass is unknown, but it may play an important role in environmental toxicology [5,7–11].

To assess the penetration of PAHs or their metabolites in the plant, we needed an appropriate method for extracting PAHs from plants. The extraction and the subsequent quantification of PAHs from soil, sewage sludges or water can be achieved with a number of established methods [12–15] including solid-phase extraction (SPE) [3,16–18] and solid-phase micro-extraction (SPME) [19–22]. These methods have not been applied to plants, however, due to the presence of plant pigments, mainly chlorophylls and carotene, which are highly hydrophobic and co-extracted together with the PAHs. A clean-up procedure is thus necessary. Some authors [23] used GPC (gel permeation chromatography) to purify chloroform or hexane–acetone extracts of pollen or spruce needles. This clean-up procedure being rather expensive, we tried to develop another method of purification of the extracts.

In the present study, we used the lettuce (*Lactuca sativa*). This plant was chosen for further studies about PAH metabolism because of its broad leaves and its rapid growth.

2. Experimental

2.1. Instrumentation

An evaporator and a Vac Elut sps 12 vacuum station (Interchim, Asnières, France) were used for solvent evaporation and solid-phase extraction, respectively.

A 320 W ultrasonic bath (Bandelin, Berlin, Germany) was used for PAH extraction by toluene.

Samples were analyzed by GC (Carlo Erba GC8000 series) equipped with a 30 m×0.25 mm I.D. fused-silica capillary column (0.25 μm film thickness, DB-5, Supelco, St. Quentin Fallavier, France) and coupled to a mass spectrometer (Fisons MD 800) operating in the selected ion monitoring (SIM) and electron impact (70 eV) modes. The initial oven temperature was 40 °C (holding time 1 min), and then increased to 160 °C at 18 °C min^{-1} and to 320 °C at 4 °C min^{-1} with a final holding time of 2 min. Injector and transfer lines were heated at 280 °C. Helium was used as carrier gas (30 kPa). Injection was performed with a Fisons autosampler Model CTC A 200S in splitless mode (1/30, split valve closed for 1 min). The SIM program of 12 channels was composed with the molecular ions of PAHs. The injection volume was 1 μl.

2.2. Chemicals

Toluene, methylene chloride, and methanol were purchased from Sigma (St. Quentin Fallavier, France). All solvents were of HPLC grade. KOH and anhydrous Na_2SO_4 were p.a. grade (Prolabo, Paris, France).

An EPA 610 Polynuclear Aromatic Hydrocarbons Mix (16 PAHs, between 100 and 2000 μg/ml in methanol–methylene chloride 1:1) was obtained from Supelco (ref: 48743, St. Quentin Fallavier, France). Deuterated pyrene (d_{10}-pyrene) with a purity of at least 99.5% (Dr. Ehrenstorfer GmbH, Augsburg, Germany) was used as internal standard.

2.3. Ultrasonic extraction and chlorophyll saponification

In practice, commercial lettuces were dried at 100 °C overnight. They were crushed and extracted with 50 ml of toluene for 2 h in an ultrasonic bath (step 1). This extraction method was chosen because Soxhlet extraction is a rather time and solvent consuming method. The toluene was evaporated to dryness in a rotary evaporator at 40 °C (step 2). The residue was then treated with 50 ml of a solution of

KOH (1 mol/l) in different methanol–water mixtures, for 30, 60 or 90 min at about 60 °C, in order to saponify the chlorophylls (step 3). These steps are described in Fig. 1.

2.4. SPE clean-up procedure

The solid-phase extraction was performed on Oasis HLB extraction cartridges (Waters, St. Quentin en Yvelines, France) containing a poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) bonded phase (200 mg, 6.0 ml). These columns were used because their poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) matrix allows π interactions responsible for a good selectivity towards PAHs.

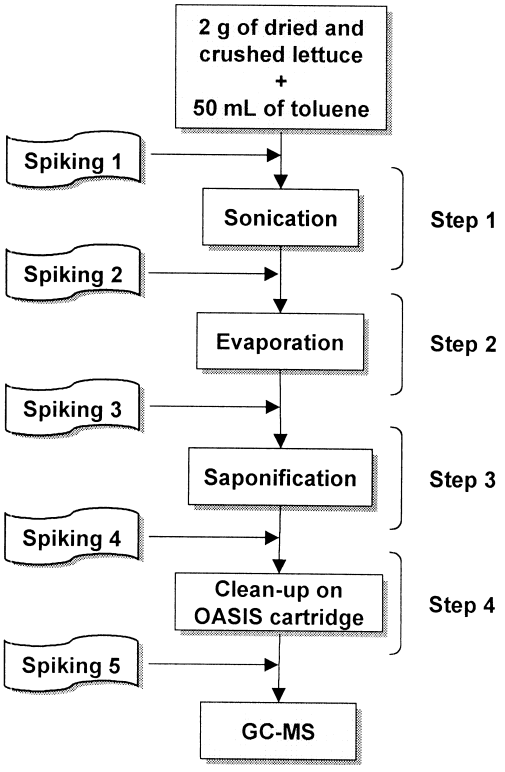

Fig. 1. Different steps of the protocol used for the recovery of PAHs from lettuce. In order to assess the percentage recovery of PAHs at each step of the extraction protocol (step 1, step 2, step 3, step 4), known amounts (about 2 g) of dried and crushed lettuce were spiked at different points in the procedure (spiking 1 to 5) with an appropriate volume of the Supelco PAH mixture (50 μl for 2 g corresponding to 2.5–50 μg of PAHs/g of dried lettuce (ppm)).

The solid-phase extraction cartridges were conditioned with 5 ml of methylene chloride, followed by 5 ml of methanol and then 5 ml of water.

The 50-ml saponified solutions were deposited onto the cartridge, then washed by 10 ml of water to remove interferences. The remaining water in the cartridges was removed by vacuum aspiration. The PAHs were eluted from the sorbent with 8 ml of methylene chloride (step 4), and the extracts dried with anhydrous Na_2SO_4. This volume is necessary to elute PAHs completely. This protocol is schematically described in Fig. 1.

2.5. Samples

As we did not find any information in the literature about PAH penetration in plants, we do not know what concentration levels to expect. We chose to spike 2 g of dried lettuces with 50 μl of EPA PAH standard solution (from Supelco) corresponding to 2.5–50 μg of PAHs/g of dried lettuce (ppm). The results show that the limits of detection are roughly 1000 times lower.

Standard solutions of PAH were obtained by appropriate dilution of PAH kit samples in methylene chloride. The concentrations of the PAH standard solutions used for determining the linearity and detection limits were in the concentration range of 0.005 to 20 μg/ml corresponding to 0.02–80 μg of PAHs/g of dried lettuce (ppm). The same solutions were used to determine the mean recovery for each step of the extraction process. These determinations were performed from calibration curves prepared using standard solutions of PAH in combination with a constant concentration of deuterated pyrene. Relative response (PAH/d-pyrene) was plotted versus PAH concentration and used for the analysis of unknown samples.

3. Results and discussion

3.1. Development of extraction and clean-up

Because of their non-polar nature and high molecular masses, chlorophylls and carotenes, which are present in big quantities in plants, may interfere with PAH analysis. Indeed, they are extracted in large

amounts into the organic solvent. Their presence prevents a simple extraction of PAHs from *Lactuca sativa* and necessitates an additional sample pretreatment step. Since chlorophylls contain two ester functions, they may acquire a hydrophilic character through saponification. In contrast, carotenes remain in the solution and are extracted with PAHs, but the solution is clear enough to avoid contamination of the ion source.

Fig. 2 shows the chromatograms of (a) an extract of a dried and crushed spiked lettuce treated according to the previously described extraction conditions (KOH 1 mol/l in methanol–water 90:10 v/v), (b) a PAH standard mixture and (c) an extract of a dried and crushed non-spiked lettuce treated according to same conditions as in (a). It is worth noting that no interfering peaks appeared with the blank obtained with a non-spiked lettuce. This blank chromatogram being the first of a series, baseline drift was more pronounced than in (a).

In order to assess the percentage recovery of PAHs at each step of the previously described extraction protocol, known amounts (about 2 g) of dried and crushed lettuce were spiked at different points in the procedure with an appropriate volume of the Supelco PAH mixture (50 μl for 2 g corresponding to 2.5–50 μg of PAHs/g of dried lettuce (ppm)) and treated according to the previously described extraction conditions (Fig. 1). For example, to assess the percentage recovery of the saponification step we compared results obtained when samples are spiked before the saponification step (spiking 3) with those spiked after it (spiking 4).

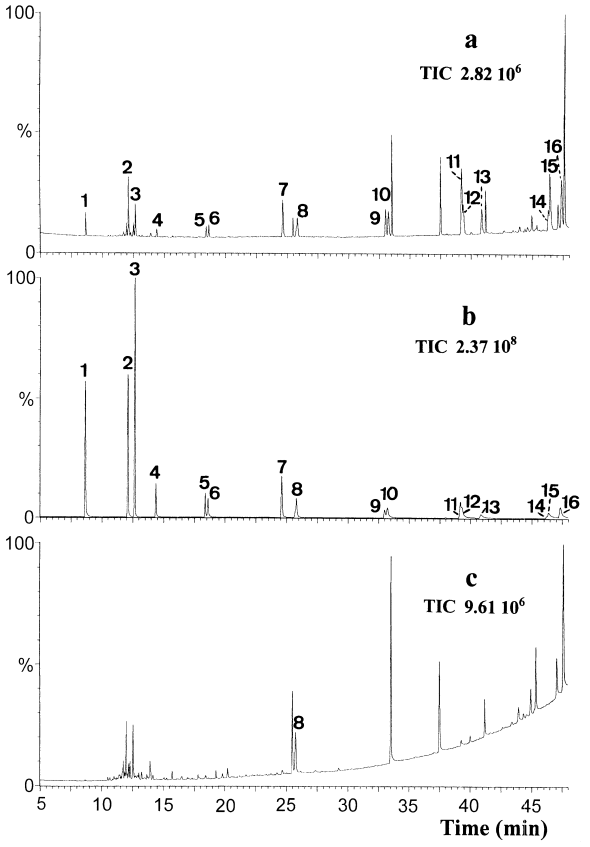

Fig. 2. GC–MS total ion current chromatograms (TIC, corresponding to 100% of scale is indicated on the chromatograms) showing the composition of polycyclic aromatic hydrocarbons in (a) an extract of spiked lettuce (50 μl of Supelco mixture for 2 g of dry lettuce corresponding to 2.5–50 μg of PAHs/g of dried lettuce (ppm) or 0.6–12 ng of PAHS injected); (b) a standard sample (non-diluted Supelco solution of 16 PAHs, between 100 and 2000 μg/ml in methanol–methylene chloride 1/1 corresponding to 100–2000 ng of PAHs injected) and (c) an extract of dried non-spiked lettuce treated in the same conditions as spiked lettuce (blank); peak 8 is due to the added internal standard. PAHs: 1, naphthalene; 2, acenaphthylene; 3, acenaphthene; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, fluoranthene; 8, pyrene+[$^2H_{10}$]pyrene (internal standard); 9, benzo(a)anthracene; 10, chrysene; 11, benzo(b)fluoranthene; 12, benzo(k)fluoranthene; 13, benzo(a)pyrene; 14, indeno(1,2,3,-cd)pyrene; 15, dibenzo(a,h)anthracene; 16, benzo(g,h,i)perylene (injection volume: 1 μl).

3.2. Results and discussion

As we used only spiked lettuces, we chose to dry them at 100 °C overnight to accelerate drying. An alternative would be drying at lower temperature (30–40 °C) for the PAH determination in contaminated lettuces.

In the first assays, a solution of KOH in methanol–water 90:10 (v/v) was used for saponification during 1 h. In this case, if the sample is spiked before the sonication extraction step (spiking 1), we obtained a poor recovery of the first eight PAHs of the EPA list, whereas the remaining ones were completely recovered (Table 1). The same results (Table 1) were obtained if the sample is spiked before or after saponification (spiking 3 and 4),

Table 1
Mean recovery rate±standard deviation (%) of PAHs, from a known amount (about 2 g) of dried and crushed lettuce spiked with 50 μl of Supelco mixture before sonication extraction with toluene (spiking 1), before (spiking 3) and after (spiking 4) saponification with 50 ml KOH (1 mol/l) in methanol–water (90:10 v/v) for 1 h (n=3)

PAH	m/z	Mean recovery rate (%) with spiking before step 1	Mean recovery rate (%) with spiking before step 3	Mean recovery rate (%) with spiking before step 4
Naphthalene	128	14±3	16±2	17±2
Acenaphthylene	152	16±4	13±2	14±1
Acenaphthene	154	22±6	17±2	18±2
Fluorene	166	34±5	32±2	31±2
Phenanthrene	178	38±8	30±2	31±2
Anthracene	178	49±10	44±4	46±3
Fluoranthene	202	65±8	70±3	71±2
Pyrene	202	73±5	75±3	79±3
Benzo(a)anthracene	228	93±5	110±5	114±4
Chrysene	228	82±4	102±4	107±3
Benzo(b)fluoranthene and Benzo(k)fluoranthene	252	81±7	107±4	103±3
Benzo(a)pyrene	252	98±5	109±5	113±4
Benzo(ghi)perylene	276	101±7	110±7	106±9
Dibenzo(ah)anthracene	278	110±9	113±10	111±4
Indeno(1,2,3cd)pyrene	276	107±11	108±11	118±5

proving that the first three steps, extraction, evaporation and saponification (Fig. 1) are not responsible for this low recovery. Thus, the low yield had to be attributed to the cleaning-up on the OASIS cartridge. However, if the high methanol content of the saponification solution led to an insufficient retention of the first eight PAHs of the EPA list, the remaining ones were completely retained on the cartridge. It is worth noting that the same results were achieved with other saponification durations (30 or 90 min) (data not shown), allowing us to conclude that 30 min was sufficient.

In order to increase the efficiency of the PAH cleaning-up step on the OASIS phase, we chose to decrease the methanol percentage in the saponification solution to 80 or 70%, in order to improve the affinity of the first eight PAHs towards the stationary phase. The best results were obtained by adding to the residue 50 ml of KOH (1 mol/l) in methanol–water 80:20 (v/v) (Table 2). Surprisingly, 70% methanol gave less interesting results than 80% due to an unknown compound that under these conditions precipitated in the OASIS column during elution with methylene chloride.

The detection limits of our best extraction method are reported in Table 3. The values were within the range 0.23–39.04 ng of PAH/g of dried lettuce (ppb). The limits are calculated from a chromatogram of a non-spiked lettuce extract by integration of the noise situated at the retention time of the corresponding PAH. The detection limits reported correspond to signal-to-noise ratios of 3. With our extraction method, we obtained thus better detection limits (0.05–10 pg PAH) than the method of Hartmann [16] (150–400 pg PAH). This can be attributed to the lower number of extraction steps due to the better compatibility of our extraction sorbent with the alkaline saponification solution. Whereas in our method we used a poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) phase, Hartmann's Al_2O_3/silica gel columns require previous partitioning of PAHs into hexane to avoid both water and alkaline pH exposure.

As an alternative, we tried to replace the liquid–solid extraction of the saponified solution (KOH in methanol–water, 80:20) with a liquid–liquid extraction with dichloromethane. The extraction recoveries were in the same range (80–100% according to the PAH) in both cases. The liquid–solid extraction method being faster and cheaper (lower solvent

Table 2
Mean recovery rate±standard deviation (%) of PAHs from PAH spiked lettuce samples with 50 μl of the Supelco mixture before saponification (spiking 3) with 50 ml KOH (1 mol/l) in different methanol–water (v/v) ratios for 1 h ($n=3$)

PAH	90:10	80:20	70:30
Naphthalene	16±2	74±4	73±5
Acenaphthylene	13±2	70±3	72±3
Acenaphthene	17±2	79±5	74±2
Fluorene	32±2	94±4	75±7
Phenanthrene	30±2	99±4	76±5
Anthracene	44±4	102±6	77±5
Fluoranthene	70±3	107±8	81±6
Pyrene	75±3	109±5	86±5
Benzo(a)anthracene	110±5	103±5	100±7
Chrysene	102±4	89±4	68±4
Benzo(b)fluoranthene and Benzo(k)fluoranthene	107±4	100±9	74±5
Benzo(a)pyrene	109±5	106±4	86±7
Benzo(ghi)perylene	110±7	109±9	107±10
Dibenzo(ah)anthracene	113±10	100±7	66±7
Indeno(1,2,3cd)pyrene	108±11	108±5	101±9

volumes are needed), we will use it in further studies.

4. Conclusion

In conclusion, we developed a simple, rapid and accurate method to evaluate PAH content in green plants. Our method using saponification and solid-phase cleaning-up steps provides both elimination of chlorophyll from the plant extract and sixfold concentration of PAHs. The conditions leading to the best PAH extraction yields from plants are: (1) saponification solution: KOH 1 mol/l in methanol–water 80:20 (v/v); (2) saponification duration: 30 min; (3) extraction on OASIS HLB cartridges.

Table 3
Detection limits for the EPA 16 PAH mixture

PAH	Limit of detection (ng/ml)	Limit of detection (ng/g of dried lettuce (ppb))
Naphthalene	3.41	13.64
Acenaphthylene	2.65	10.6
Acenaphthene	2.65	10.6
Fluorene	6.72	26.88
Phenanthrene	7.61	30.44
Anthracene	1.09	4.36
Fluoranthene	9.76	39.04
Pyrene	4.64	18.56
Benzo(a)anthracene	1.81	7.24
Chrysene	4.84	19.36
Benzo(b)fluoranthene and Benzo(k)fluoranthene	6.05	24.2
Benzo(a)pyrene	1.92	7.68
Benzo(ghi)perylene	0.45	1.8
Dibenzo(ah)anthracene	0.057	0.228
Indeno(1,2,3cd)pyrene	0.19	0.76

Under these conditions, the extraction recoveries and the detection limits were within the range 70–109% and 0.05–10 pg PAH, respectively.

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