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Comparison of various extraction techniques for the determination of polycyclic aromatic hydrocarbons in worms

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Abstract

Two less laborious extraction methods, viz. (i) a simplified liquid extraction using light petroleum or (ii) microwave-assisted solvent extraction (MASE), for the analysis of polycyclic aromatic hydrocarbons (PAHs) in samples of the compost worm *Eisenia andrei*, were compared with a reference method. After extraction and concentration, analytical methodology consisted of a cleanup of (part) of the extract with high-performance gel permeation chromatography (HPGPC) and instrumental analysis of 15 PAHs with reversed-phase liquid chromatography with fluorescence detection (RPLC–FLD). Comparison of the methods was done by analysing samples with incurred residues ($n=15$, each method) originating from an experiment in which worms were exposed to a soil contaminated with PAHs. Simultaneously, the performance of the total lipid determination of each method was established. Evaluation of the data by means of principal component analysis (PCA) and analysis of variance (ANOVA) revealed that the performance of the light petroleum method for both the extraction of PAHs (concentration range 1–30 ng/g) and lipid content corresponds very well with the reference method. Compared to the reference method, the MASE method yielded somewhat lower concentrations for the less volatile PAHs, e.g., dibenzo[*ah*]anthracene and benzo[*ghi*]perylene and provided a significant higher amount of co-extracted material.

Keywords: Worms; Extraction methods; Principle component analysis; Lipids; Polynuclear aromatic hydrocarbons

1. Introduction

This study compares extraction methods in order to find a more efficient methodology for the determination of polycyclic aromatic hydrocarbons (PAHs) in large series of samples of the compost worms *Eisenia andrei* (oligochaeta) originating from eco-toxicological studies recently performed at our Institute [1]. Methodology should also include the proper determination of the lipid content of worm sample.

Hence, the selected extraction method should be capable to simultaneously extract quantitatively (i) PAHs and (ii) the total lipid content of the samples with incurred residues. Based on our participation in analysing shellfish reference material provided by Quality Assurance Laboratory Performance Studies for Environmental Measurements in Marine Samples (QUASIMEME) [2], the recommended established method of Smedes [3] was selected as the reference method to compare two other distinctly less laborious extraction methods.

The Smedes method is an optimised method for

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the extraction of the total lipid in biological samples using non-chlorinated solvents. In relation to the most reliable existing methodology of Bligh and Dyer [4] the viability of this approach was demonstrated earlier in lipid intercomparison studies [5,6].

The total lipid content of the worm *Eisenia andrei* is about 1%, indicating that a cleanup between co-extracted fatty compounds and residues of PAHs will be necessary. Conventional robust and reliable methodology usually applies fat destruction by means of a saponification step [7–10]. Because of the small amount of sample (max about 1 g) the determination of both PAHs and total lipid content has to be done in the same sample, hence, a non-destructive cleanup will be necessary.

At our laboratory, two efficient cleanup approaches are in use for the determination of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in fatty matrices [11–13]. Either normal-phase liquid chromatography (NPLC) or high-performance gel permeation chromatography (HPGPC) performs automated rapid cleanup. NPLC offers the possibility to inject a large amount of fat (up to 90 mg) [11,12] and, thus, providing in case of sufficient sample (fat) higher sensitivity in comparison to HPGPC (maximum about 2 mg of fat on column). Regarding the available amount of sample, the amount of extracted total lipid will be limited. Because of the somewhat better chromatographic stability and lower evaporation temperature of the mobile phase, HPGPC was selected as cleanup method in this application.

Beside the reference method [3], two less laborious extraction methods were selected based on our experience. The methods are schematically presented in Table 1. The first one, the light petroleum method, is a simplified extraction with a high-speed homogenizer using only two times a volume of light petroleum. The second one is the microwave-assisted solvent extraction (MASE) method. As has been reviewed [14,15], MASE is frequently used for the efficient extraction of PAHs in various types of matrices. In our laboratory this technique has been studied [16] and successfully applied for the efficient extraction of pesticides from soils [17–19]. Therefore, the feasibility of MASE was also included in this study.

An inter-comparison of the three extraction meth-

Table 1
Overview steps extraction methods[a] used for the processing of 1 g worm samples

Step	Light petroleum method	Smedes method [3]	MASE method
1	30 s HSH with 20 ml light petroleum	2 min HSH with 9 ml IPA+10 ml cyclohexane	10 min MASE with 10 ml IPA+10 ml cyclohexane
2	Extract over Na_2SO_4 into KD	Addition of 10 ml of water, 1 min HSH	Extract over Na_2SO_4 into KD
3	30 s HSH with 20 ml light petroleum	Centrifuge, 3 min	Evaporation solvent
4	Evaporation collected solvents to 1 ml volume	Pipette organic layer over Na_2SO_4 into KD	Redissolve in 2 ml of light petroleum
5		Addition of 10 ml IPA-cyclohexane, 1 min HSH	
6		Centrifuge, 3 min	
7		Pipette of organic layer over Na_2SO_4	
8		Evaporation collected solvents	
9		Redissolve in 2 ml light petroleum	

[a] HSH, extraction with high-speed homogenizer; MASE, microwave-assisted solvent extraction; KD, Kuderna Danish apparatus; IPA, isopropyl alcohol.

ods was obtained by analysing worm samples with incurred residues of PAHs. All samples were taken simultaneously after exposure of the worms to a PAH-contaminated soil. Each method was tested with the extraction and cleanup of 15 worm samples followed by the instrumental analysis of the extracts by reversed-phase liquid chromatography with fluorescence detection (RPLC–FLD). Comparison of the methods was performed by a statistical evaluation of the data of all 45 samples.

2. Experimental

2.1. Chemicals and samples

Standard Reference Material (SRM1647d) of the EPA priority pollutant polycyclic aromatic hydrocarbons was obtained from the Standard Reference Materials Program, NIST (Gaithersburg, MD, USA). Each ampoule contains approximately 1.2 ml of an acetonitrile solution with certified values of 16 PAHs. A 1000-fold dilution in acetone–water (40:60, v/v) was prepared for use in chromatographic analysis.

6-Methylchrysene (BCR no. 82) and benzo[*k*]fluoranthene-d_{12} were used as internal standards and were purchased from BCR (Geel, Belgium) and CIL (Massachusetts, USA), respectively. A mixture of the two compounds (ca. 500 ng/ml each) was prepared in acetone. HPLC-grade water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Bedford, MA, USA).

Acetone, acetonitrile, dichloromethane, isopropylalcohol, all HPLC-grade and light petroleum (boiling range, 40–60 °C) were from J.T. Baker (Deventer, The Netherlands); sodium sulphate, *n*-butanol and cyclohexane, all analytical grade were from Merck (Darmstadt, Germany).

2.2. Instrumentation and columns

A high-speed homogenizer Model 17506 of Omni Macro (Waterbury, USA) was used for blending and homogenizing the sample and a Sigma type 2-15 (Salm en Kipp, Breukelen, The Netherlands) for centrifugation of the extract.

MASE was carried out with an MES-1000, 950-W laboratory Microwave Extraction System (CEM, Matthews, NC, USA), equipped with a gas sensor for detecting organic vapors and internal control of either pressure or temperature. At full power it delivers an energy of approximately 950 W at a frequency of 2450 MHz. The system controls simultaneously in up to 12 closed extraction vessels either temperature or pressure.

The instrumental part of the GPC system consisted of a Model 305 LC pump, a Model 321 autosampler and a fraction collector Model FC 204 from Gilson (Villiers-le Bel, France). HPGPC was performed on a 300×7 mm I.D. column packed with 5 μm PLGel (500 Å).

The RPLC–FLD system was from Shimadzu (Kyoto, Japan) and consisted of a Solvent Delivery Module LC-10ADvp, a column oven CTO-1-ASvp, an autosampler SIL-10ADvp, a system controller SCL-10Avp and a spectrofluorometric Detector RF-10Axl with time programmable excitation and emission wavelengths.

A 250×4.6-mm I.D. Vydac PAH C_{18} column (5 μ, 300 Å) with a 20×4-mm I.D. guard column filled with the same material (Vydac, Hesperia, CA, USA) was used for the analytical separation. Both the mixing chamber and the column were placed in an oven at 20 °C.

2.3. Worm samples

Eisenia andrei worms with incurred residues were cultivated in a contaminated clay soil. The soil was characterised by a water content of 15%, a clay content of 28%, a sand content of 46%, a total carbon content of 3.8%, an organic matter content of 5.8% and a pH of 7.3. The concentrations of the individual PAHs ranged between 0.18 and 3.7 mg/kg with a concentration of 23.5 mg/kg for the sum of 15 PAHs.

After an exposure time of 5 days, the animals were recaptured and the guts were emptied by keeping them 24 h on a moist filter. From the collected worms portions of about 1 g were weighted into extraction vessels for further processing.

2.4. Extraction methods (cf. Table 1)

2.4.1. Light petroleum method

A sample of worms of approximately 1 g was placed in a 100-ml centrifuge tube and weighted accurately. After addition of 100 μl of the internal standard mixture 20 ml of light petroleum were added and the mixture was macerated with the homogenizer for 30 s. The organic layer was decanted through a funnel with sodium sulfate to a Kuderna Danish evaporation apparatus. The remaining sample was macerated for another 30 s with 20 ml light petroleum and the extract was added to the first one. After rinsing the sodium sulfate with about 10 ml of light petroleum the total organic layer was evaporated to 1 ml. The total volume of this reduced extract was transferred to a weighed vial.

After injecting 300 μl from this vial into the GPC system, the vial was de-capped and the remaining light petroleum was evaporated. The vial was weighted and the lipid content of the worm sample calculated from the intake.

2.4.2. Smedes (reference) method

One gram of worms was placed in a 100-ml centrifuge tube and weighed accurately. After addition of 100 μl of the internal standard mixture, 9 ml isopropylalcohol and 10 ml cyclohexane were added and the mixture was macerated with the homogenizer for 2 min. Next, 10 ml of water was added and the mixture was macerated again for 1 min. The phases were separated by centrifugation for 10 min at 3000 rpm. The upper organic layer was transferred through a funnel with sodium sulfate to a Kuderna Danish evaporation apparatus by means of a pasteur pipette. In advance, the tube of the Kuderna Danish apparatus together with some boiling chips were pre-weighed for the lipid determination. The remaining part of the sample was macerated again for 1 min with 10 ml of a mixture of iso-propanol–cyclohexane (13:87, v/v). After centrifugation, the upper layer was transferred to the funnel with sodium sulfate and added to the first extract. After rinsing the sodium sulfate with about 5 ml of cyclohexane, the total organic layer was evaporated. The remaining few ml were removed by a stream of nitrogen at about 80 °C.

The tube with the residue was weighed in order to calculate the lipid content of the worm sample from the intake. The lipid residue was dissolved in 2 ml light petroleum and 1 ml was transferred to a vial for clean-up with GPC.

2.4.3. MASE method

A sample of 1 g of worms was transferred to an extraction vessel and weighed accurately. After addition of 100 μl of the internal standard solution and 20 ml of the extraction solvent isopropylalcohol–cyclohexane (50:50, v/v) the worms were extracted by MASE at 115 °C during 10 min at 100% power. After extraction, the vessels were allowed to cool down to room temperature before opening. The extract was transferred through a funnel with sodium sulfate to a Kuderna Danish evaporation apparatus. After rinsing the sodium sulfate with about 5 ml of cyclohexane the organic solvent were evaporated near to dryness. The remaining few ml were removed by a stream of nitrogen at about 80 °C.

The lipid residue was dissolved in 2 ml light petroleum and 1 ml was accurately transferred to a vial for clean-up with GPC. After injecting 300 μl from this vial into the GPC system, the vial was de-capped and the remaining light petroleum was evaporated. The vial was weighed and the lipid content of the worm sample calculated from the intake.

2.5. GPC analysis

The mobile phase, dichloromethane, was set at a flow of 1 ml/min. A volume of 300 μl of the worm extracts was taken from the vial and via a loop 200 μl were injected onto the GPC column. After a cleanup of 10 ml of mobile phase, the PAH-containing fraction of the next 6 ml of dichloromethane was collected in a calibrated tube. After addition of 0.1 ml of *n*-butanol as a holder, the dichloromethane was carefully evaporated assisted by a gentle stream of nitrogen. The residue was re-dissolved in 400 μl acetone and mixed with 600 μl water for the analysis with RPLC–FLD.

2.6. RPLC–FLD analysis

The starting mobile phase consisting of acetonitrile–water (40:60, v/v) was adjusted to a flow-rate of 1 ml/min. A volume of 50 μl of the extract

Table 2
Wavelength programming in fluorescence detection (FLD)

Time (min)	λ_{ex} (nm)	λ_{em} (nm)	Polycyclic aromatic hydrocarbon compounds
0.0	275	325	Naphthalene
19.0	253	333	Acenaphthene, fluorene
22.3	253	373	Phenanthrene, anthracene
25.5	263	420	Fluoranthene, pyrene
29.0	270	382	Benz[*a*]anthracene, chrysene, 6-methylchrysene*
34.5	290	430	Benzo[*b*]fluoranthene, benzo[*k*]fluoranthene-d_{12}^{*}, benzo[*k*]fluoranthene, benzo[*a*]pyrene
40.0	300	400	Dibenzo[*ah*]anthracene, benzo[*ghi*]perylene
42.3	302	506	Indeno[123-*cd*]pyrene

*Internal standard.

obtained after the GPC procedure was injected on the LC column. After 5 min, a linear gradient elution to 100% acetonitrile in 30 min was performed and held for 20 min. Prior to the next injection, the column was reconditioned with the starting mobile phase for 5 min.

The applied wavelength programming for the fluorescence detection of the various PAHs is given in Table 2.

2.7. Statistical methods

Principal component analysis (PCA) [20] is used in order to provide more insight in the data. The analysis results of the compounds are visually represented by using a so-called Box and Whisker plot [20,21]. Before using other statistical methods the normality of the data was examined by using a Kolmogorov–Smirnov test [20]. Analysis of Variance (ANOVA) [20] was used to determine if there are any significant differences between the means of the three methods.

All calculations were performed with the aid of the software package MATLAB 6.1 [22] supplemented with the Statistics Toolbox 3.0 [22] and the PLS_Toolbox 2.1 [23].

3. Results and discussion

3.1. Selection and application of methods

As shown in Table 1, both the light petroleum method and the MASE method require distinct less manual operations in comparison to the reference method of Smedes [3]. The alternative methods and extraction solvents were selected based on our experience with MASE [16–19] and from previous exposure studies carried out at our Institute and dealing with the analysis of various types of organochlorine compounds, e.g., pesticides, polychlorinated biphenyls and chlorobenzenes, in worms.

As mentioned before, non-destructive HPGPC was selected for the automated cleanup of extracts. In this procedure, a crucial step is the solvent switch to be made prior to the RPLC–FLD analysis. The use of a holder, viz. a small volume of *n*-butanol, is necessary in order to avoid severe losses of the more volatile PAHs.

Firstly, the three methods were tested by recovery experiments performed at levels of PAHs ranging from 30 to 80 ng/g. The performances of the three methods were quite similar with recoveries ranging between 65 and 110% and relative standard deviations below 15%.

The next step was the analysis of worm samples with incurred residues (see Section 2). The processing of 15 worm samples originating from the same batch of worms exposed to a contaminated soil tested each extraction method.

The results of these experiments, expressed as the average concentration (ng/g) and standard deviation (ng/g) of each PAH are given in Table 3. The concentrations of naphthalene and acenaphthene were below the limits of detection. Hence, these compounds were excluded from this study.

As shown in Table 3, for the remaining 13 PAHs average concentrations ranged between 0.58 and 27 ng/g. As can be expected from these type of biological samples, standard deviations are relatively

Table 3
Average concentration (Conc.) and standard deviation (SD) of PAHs and extracted matter in worm samples obtained by different extraction methods ($n=15$, each method)

PAH	Light petroleum method		Smedes method*		MASE method	
	Conc. (ng/g)	SD (ng/g)	Conc. (ng/g)	SD (ng/g)	Conc. (ng/g)	SD (ng/g)
Fluorene	1.3	0.45	1.0	0.70	1.4	0.95
Phenanthrene	26	6.1	22	7.0	27	9.7
Anthracene	0.58	0.15	0.67	0.24	0.72	0.31
Fluoranthene	10	2.2	8.2	5.1	11	4.5
Pyrene	13	2.0	13	1.9	12	3.1
Benz[*a*]anthracene	4.7	1.1	6.0	0.88	5.5	2.1
Chrysene	11	0.94	11	1.5	10	3.6
Benzo[*b*]fluoranthene	6.4	0.94	7.1	1.2	6.7	2.4
Benzo[*k*]fluoranthene	3.2	0.41	3.6	0.53	3.5	1.2
Benzo[*a*]pyrene	3.1	0.38	3.7	0.70	3.2	1.8
Dibenzo[*ah*]anthracene	4.1	2.4	3.7	2.2	1.1	1.2
Benzo[*ghi*]perylene	4.9	2.6	4.3	2.1	2.5	1.3
Indeno[123-*cd*]pyrene	1.7	0.30	2.0	0.47	1.9	0.98
Percentage of weight extracted matter (%)	0.86	0.12	1.14	0.58	1.93	0.25

*Reference method [3].

high. Hence, it is difficult to adequately compare the performance of the three methods based on a visual interpretation.

In order to examine whether significant differences exist between the three extraction methods, a statistical evaluation of the data will be necessary.

3.2. Statistical evaluation

The data set consists of 45 independent samples and 13 compounds. The data set displays a large variety in terms of concentrations for different compounds, hence before analysing the data set with a PCA a scaling method had to be used. This scaling was done by performing an autoscaling on the data prior to further calculations. By using autoscaling the mean from each component is subtracted from the measurements and the results are divided by the standard deviation [20].

After performing a PCA on the scaled dataset it was found that PC-1 describes 61.3% and PC-2 15.1% of the total variance present in the data set. Assuming a linear combination of the original variables both samples (scores) and PAHs (loadings) were projected on the first two PCs.

As can be seen in the scoreplot (Fig. 1) no major differences between the three extraction methods are found. Apparently the dispersion between individual analysis results is larger than the systematic difference between the extraction method. A major source for the dispersion of the individual analysis results is a high variability of both fat and PAH content in the worms. This makes it more difficult to establish significant differences between the three methods.

The loadingsplot presented in Fig. 2 indicates the presence of a major difference between dibenzo[*ah*]anthracene and benzo[*ghi*]perylene compared to the other PAHs. A comparison is made between these compounds and two ''normal'' behaving compounds fluorene and anthracene, located on the opposite side in the loadingsplot.

The unscaled dataset is now used for further testing to facilitate the comparison between means. After visual inspection of the results of the Kolmogorov–Smirnov test no evidence of non-normality of the used data was revealed. Box and Whisker plots where made for the four previously selected compounds. The Box and Whisker plot of the results of dibenzo[*ah*]anthracene showed that the median of the MASE method is lower than the medians of the

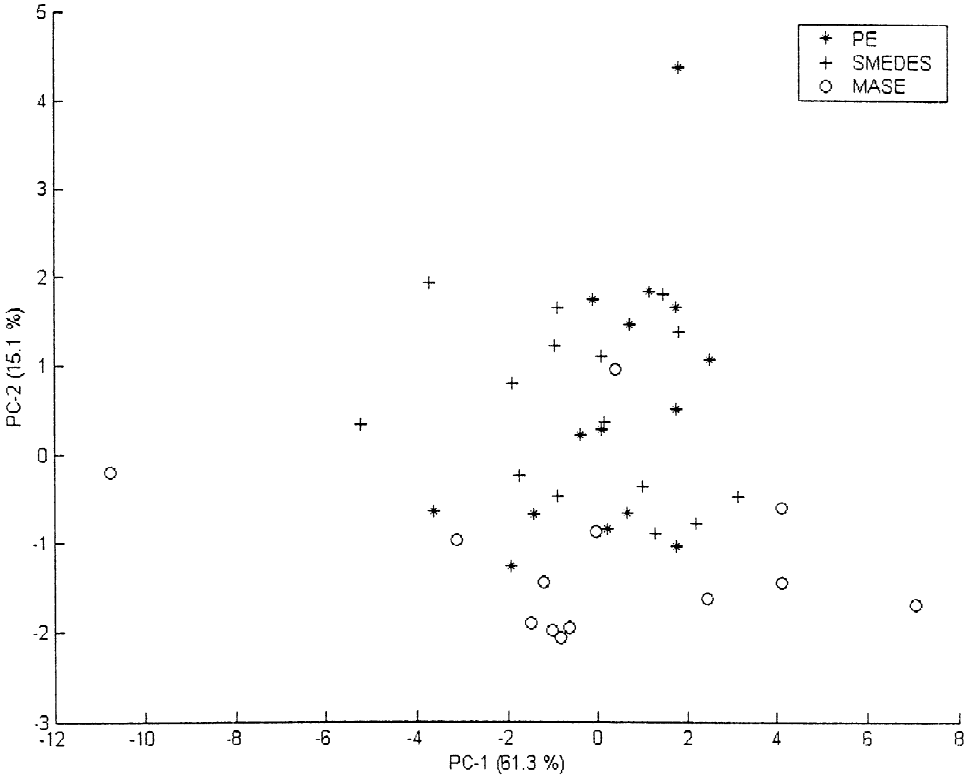

Fig. 1. Scoreplot of the PCA performed on the analysis results of PAHs of the three different methods.

other two methods. To test if this is a significant difference an ANOVA is used. The ANOVA revealed a statistical difference between the means of the three methods. Therefore a multiple comparison between the means was conducted on the results of this compound. The test revealed a significant difference between the mean of the MASE method compared to the means of the other two methods. In this case the mean of the MASE method was significantly lower.

The Box and Whisker plot of the results for benzo[*ghi*]perylene shows that the median of the MASE method is also lower than the medians of the other two methods. After performing an ANOVA no significant differences for the means of the three methods where found.

Of the four selected compounds, fluorene and anthracene are more volatile (lower molecular mass) compounds than dibenzo[*ah*]anthracene and benzo[*ghi*]perylene. This is an indication that the MASE method is somewhat less suitable for extracting the more heavy molecular mass (less volatile) compounds.

The results of the light petroleum method correspond well to the reference Smedes method, rendering for this type of matrix–analyte combination a less time-consuming extraction procedure.

In order to compare the performance of the three methods as regards reliable total lipid extraction, the data of the co-extracted matrix (see Table 3) were also evaluated by using an ANOVA.

However, the MASE method extracts a significantly higher amount of co-extracted materials than the other two methods.

4. Conclusion

In comparison to the reference method of Smedes, two proposed less laborious extraction methods have

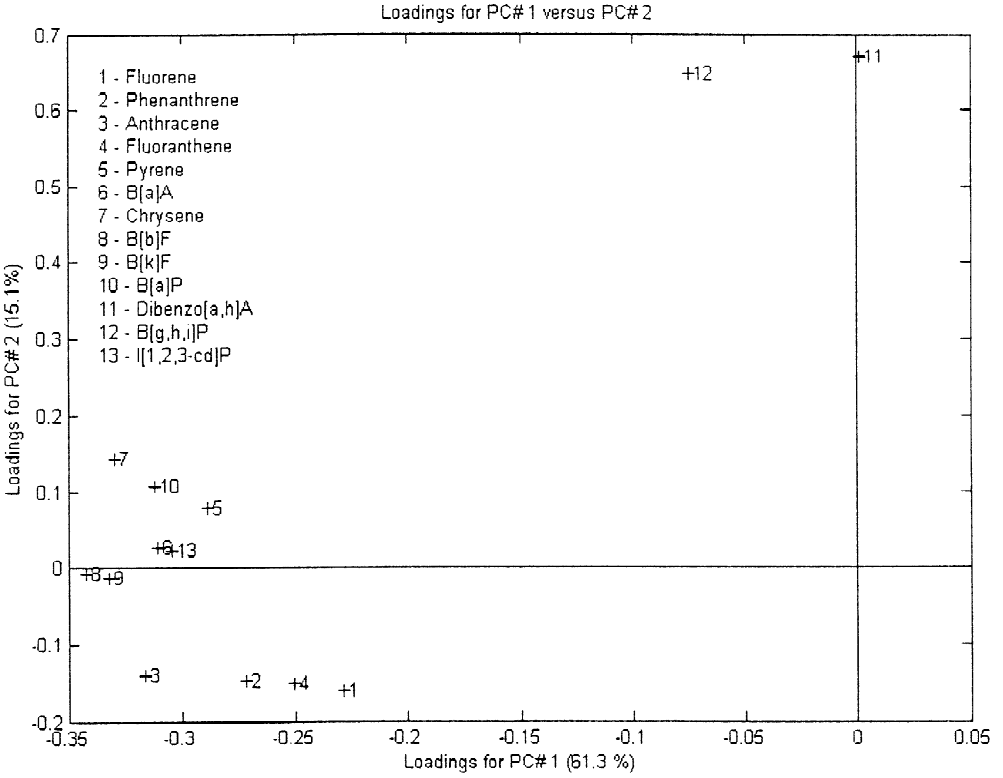

Fig. 2. Loadingsplot of the PCA performed on the analysis results of PAHs of the three different methods.

been tested on their performance for the analysis of polycyclic aromatic hydrocarbons (PAHs) in worm samples. After extraction, high-performance gel permeation chromatography (HPGPC) was applied for automated cleanup and reversed-phase liquid chromatography with fluorescence detection (RPLC–FLD) for instrumental analyses.

Based on the analysis of worm samples with incurred residues and statistical evaluation of the data, it was demonstrated that the alternative method involving two extractions with a volume of light petroleum provided results similar to that of the reference method. The second alternative method involving microwave-assisted solvent extraction (MASE method) resulted in somewhat lower concentrations for the two less volatile PAHs, viz. dibenzo[*ah*]anthracene and benzo[*ghi*]perylene.

As regards the total lipid extraction, the light petroleum method corresponded well with the reference method, while a distinctly higher amount of material was extracted with the MASE method.

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