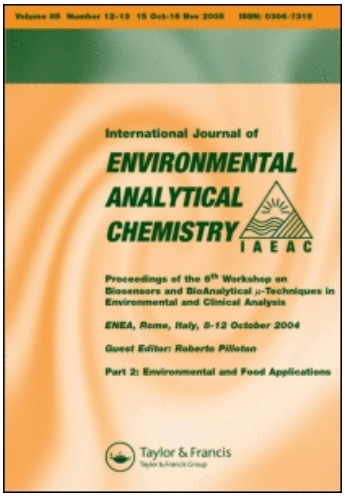

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Supercritical Fluid Extraction of Bivalve Samples for Simultaneous GC-MS Determination of Polychlorobiphenyls and Polycyclic Aromatic Hydrocarbons

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SUPERCRITICAL FLUID EXTRACTION OF BIVALVE SAMPLES FOR SIMULTANEOUS GC-MS DETERMINATION OF POLYCHLOROBIPHENYLS AND POLYCYCLIC AROMATIC HYDROCARBONS

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A method is presented for the simultaneous determination of polychlorobiphenyls and polycyclic aromatic hydrocarbons in bivalve samples. The method implies the fortification of the freeze-dried sample with isotopically labeled internal standards; followed by extraction with supercritical carbon dioxide modified with 3% methanol, then a simple clean-up step on a silicagel packed Pasteur pipette and, finally, determination by gaschomatography-mass spectrometry. The method requires little time and labor compared to traditional methods, uses negligible amounts of solvents and produces little wastes. Sixty different PCB congeners and seven selected PAHs are analyzed. Six replicate analyses were performed on the SRM 2974 certified material and the results are discussed.

Keywords: Supercritical fluid extraction; polychlorobipenyls; polycyclic aromatic hydrocarbons; isotopic dilution; bivalves

INTRODUCTION

The characterization of organic carcinogenic microcontaminants in edible marine biota is of increasing importance in order to assess the risk associated with its inclusion in the human diet. More recently, endocrine disrupting action of organohalogen compounds, including polychlorobiphenyls (PCBs), has been high-

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lighted [1] adding another important reason for the determination of an increasing number of analytes, and especially of organohalogen compounds.

Supercritical fluid extraction (SFE) has the potential for extraction of organic micropollutants from solid matrices with substantial simplification of the clean-up. Bøwadt *et al.* analyzed PCBs in sediment samples [2] with a fast method; similarly, PCB were successfully determinated in fishes and bivalves [3–5]; the main advantages of this technique are the rapidity of extraction and clean-up and the very low procedural blank contribution [6], which allows the analysis of samples from pristine areas like the Antarctic region [7]. Also the determination of polycyclic aromatic hydrocarbons (PAHs) in sea sediments was performed by SFE [8].

PCBs and PAHs are ubiquitous micropollutants, are persistent in the environment, are lipophilic, tend to bioaccumulate and are possible human carcinogens. Monitoring the pollution of aquatic environment often requires the characterization of both classes of micropollutants. In this respect, PCBs and PAHs share some similar chemical features, such as aromaticity, range of both boiling point and polarity, which makes it possible to analyze both classes of compounds with the same analytical process. This would greatly reduce the time and the labor needed for the analysis; if this could be done by SFE, the time and the labor required for the two determinations would be reduced to a small fraction of that needed with traditional methods. The elevated number of determinands can be dealt with by GC-MS, which shows both the sensitivity and the selectivity needed for this analysis. An analytical method for the simultaneous determination of PCBs and PAHs in mollusks based on SFE followed by GC-MS was developed and its performances was evaluated through replicate analyses on a certified reference material (SRM 2974 from NIST).

EXPERIMENTAL

Materials and standards

Freeze-dried mussel tissue -*Mytilus edulis*- (SRM 2974) was obtained from NIST (Gaithersburg).

Standard solutions were prepared as follows: wholly ^{13}C labeled CB congeners were obtained from Cambridge Isotope Laboratories (CIL, Andover, Mass.) as solutions. From the solutions of the pure compounds a mixture (mixCBL, where L stands for labeled) was prepared in 2,2,4-trimethylpentane containing 14 different PCBs in the following relative concentrations (CB 153=1=195.5 ng/mL)

15=0.49; 28=0.51; 52=0.51; 77=0.05; 101=0.49; 105=0.25; 118=0.55; 126=0.01; 138=0.94; 153=1; 169=0.01; 178=0,26; 180=0.57; 202=0.24. Accordingly, from solutions of single compounds obtained from Dr Ehrenstorfer (Augsburg, Germany), a mixture (mix CB) containing 61 different unlabeled congeners in concentrations relative to CB153, ranging from 0.29 (CB 30) to 1 (CB 118, CB 138 and CB153=300 ng/mL) was prepared to be used to determine relative response factors. Volumes ranging from 0.25 to 0.75 mL of each solution were withdrawn, mixed in a volumetric flask and brought to the final volume with fresh solvent. The titre accuracy of this solution is fundamental to the accuracy of the analysis. None of the congeners used for preparing this solution was a certified material.

For PAH analysis, a mixture (mixPAHL) of six wholly deuterated compounds was used as internal standard. Weighed amounts of the crystalline compounds, obtained from Cambridge Isotope Laboratories, were dissolved in toluene to obtain mother solutions. Aliquots were withdrawn and mixed in ratios to benzo[*a*]pyrene (BaP) varying from 0.72 for indeno(1,2,3-*c,d*)pyrene to 1.1 for chrysene (BaP=1=7.7 µg/mL). For the analyses of SRM 2974, mix PAHL was diluted 100 times.

For the determination of the relative response factors (rrf; see later), a mixture (mix PAH) of unlabeled 16 EPA priority PAH, obtained from Supelco (Bellefonte, PA)was used. As neither mix CB nor mix PAH were prepared from certified materials, a second solution of different origin was injected for comparison. A solution from Ultra scientific (North Kingstown, RI) containing 19 congeners was used for CBs, whilst for PAHs, a solution of 5 substances, prepared from crystalline compounds by Dr. E. Menichini of our institute, was used.

Methods

About 50 mg of SRM 2974 lyophilized powder were weighed and 50µl of mix CBL and 10 µl of diluted mix PAHL were added to the sample directly in the extraction thimble; after 1 hour, aluminum oxide, previously washed in a Dionex ASE 200 extractor with dichloromethane at 100°C, was added; the thimble was capped and the extraction began.

A Hewlett-Packard model 7680 T supercritical fluid extractor was used in the following conditions: extraction fluid CO_2 with 3% methanol; density: 0.60 g/mL; pressure 311 bar; flow rate 2 mL/min; chamber temperature 120 °C; extraction time 20 min; extract trap conditions: nozzle temperature 80 °C; trap temperature 75°C, trap packing ODS; fraction output: 2 rinse steps with 1.5 mL *n*-hexane, at a rate of 1.5 mL/min.

The rinsed fractions were then put together, evaporated under a gentle nitrogen stream and further purified on a silica gel packed Pasteur pipette; the silica gel was activated overnight at 170°C, then deactivated with 3% water, kept under n-hexane and slurry packed in a Pasteur pipette; the first 1mL n-hexane fraction was discarded whilst the following 6 mL *n*-hexane/benzene 4/1 v/v were collected.

TABLE I Recovery yields of the internal standards in 6 replicate analyses of SRM 2974, together with the approximate injected amount

Internal Standard	*Recovery Yields*		
	CV	*Mean*	*Injected Amount (pg)*
$T_3CB\ 28\ ^{13}C_{12}$	27%	101%	100
$T_4CB\ 52\ ^{3}C_{12}$	19%	102%	99
$T_4CB\ 77\ ^{13}C_{12}$	16%	98%	9
$P_5CB\ 101\ ^{13}C_{12}$	9%	95%	96
$P_5CB\ 118\ ^{3}C_{12}$	4%	97%	107
$P_5CB\ 105\ ^{13}C_{12}$	8%	95%	49
$H_6CB\ 153\ ^{13}C_{12}$	3%	91%	196
$H_6CB\ 138\ ^{13}C_{12}$	4%	90%	184
$H_7CB\ 178\ ^{13}C_{12}$	5%	92%	51
$H_7CB\ 180\ ^{13}C_{12}$	3%	90%	112
$O_8CB\ 202\ ^{13}C_{12}$	2%	92%	46
Benz[*a*]anthracene D_{12}	17%	89%	15
Chrysene D_{12}	9%	88%	17
Benzo[*k*]fluoranthene D_{12}	14%	88%	11
Benzo[*a*]pyrene D_{12}	11%	86%	15
Indeno[1,2,3-*cd*]pyrene D_{12}	25%	89%	11
Dibenz[*ah*]anthracene D_{14}	27%	89%	13

GC-MS analysis was performed on a HP5989A GC/MS system equipped with a HP Ultra 2 capillary gas chromatographic column (50m – long, 0.2mm – i.d.) and with a high energy dynode. A Programmed Temperature Vaporizer (PTV) injector (SGS, Milan, I) was installed on the HP 5980 °C. The PTV injector allows injection of solutions containing impurities without impairing chromatographic resolution.

The oven temperature program was the following: initial temperature 60°C, for 45 sec, then up to 150°C at 20°C/min, immediately followed by a second ramp at 3.2°C/min up to 270°C, followed again by a third ramp at 5°C/min up to 290°C, at which temperature the run ends after a final isothermal step 75 sec long.

The mass spectrometer was operating using electron impact at 35 eV and in SIM mode. A sequence of 8 groups of masses, each one scanning 19 different masses, each 1 amu wide, for 30 msec each, was used. For PCBs, the two most intense masses of the molecular cluster for both native and isotopically labeled compounds were recorded, whilst for PAHs only the molecular ion was recorded for both native analytes and isotopically labeled standards. Also masses of some chlorinated pesticides were included in the method.

A keeper (*n*-tetradecane from Fluka) was added to the *n*-hexane/benzene eluate, which was then evaporated and taken to the final volume (50 μL) with a solution containing an injection standard ($^{13}C_1$ labeled chlordane, from CIL). An external standard was prepared in the same manner, containing the same volume of the mixtures (mix CBL and mix PAHL) of the isotopically labeled tracers used for spiking the samples and appropriate volumes of mix CB and mix PAH, in order to contain amounts of analytes comparable to the sample. A duplicate injection of this solution was run together -on the same day- with the samples, to assess the recovery yields of the labeled internal standards and to determine the response factors of the native analytes with respect to their internal standard (rrf). This procedure proved to yield accurate results [9]. The recovery yields of the labeled internal standards were checked for quality control reasons only, as correction for losses is intrinsic to the isotope dilution technique.

Two series of 3 complete determinations were carried out on the reference material, at two weeks distance from each other.

RESULTS AND DISCUSSION

The choice of the analytes of Table II may appear unbalanced, as some 60 PCB congeners are searched, whilst only 8 PAHs are determined. The reasons for the latter choice of the determinands is based on the opinion on PAH adopted by the Italian National Advisory Toxicological Committee, which based its risk assessment on seven PAHs [10]. For PCBs the choice is based on both environmental and toxicological criteria. The congeners searched include most of the ones reported to be present in environmental matrices; also included are almost all of the most toxic ones. In this aspect, the two most potent congeners in terms of dioxin-like toxicity (CB 126 and CB 169) are included, but it is not possible, due

to both their very low level and their somewhat higher polarity with respect to the other PCBs, to assess the applicability of the present method to their analysis. The reports on endocrine disrupting ability of PCBs add further relevance to the knowledge of the highest possible number of congeners.

TABLE II Mean concentration (μg/kg) found in 6 replicate analyses of SRM 2974, together with their coefficient of variation and the certified values

Analyte	*CV*	*Mean*	*Certified*	*Analyte*	*CV*	*Mean*	*Certified*
T_3CB 30		<3		H_6CB 146	7%	21.1	
T_3CB 18	17%	25.7	26.8±3.3[a]	H_6CB 153	3%	120.9	145.2±8.8
T_3CB 17	10%	17.6		H_6CB 141		<3	
T_3CB 28	11%	65.4	79±15[a]	H_6CB 137		<3	
T_4CB 54		<3		H_6CB 138+163	4%	131.7	134±10
T_4CB 52	8%	112.6	115±12	H_6CB 128	11%	19.0	22±3.5
T_4CB 49	7%	83.9	88.8±5.7	H_6CB 167	12%	6.1	
T_4CB47+48	9%	58.6		H_6CB 156	24%	7.1	7.4±1
T_4CB 44	8%	76.9	72.7±7.7	H_6CB 157		<3	
T_4CB41+64	15%	51.9		H_7CB 176		<3.5	
T_4CB 74	11%	65.2		H_7CB 187	7%	28.1	34±2.5
T_4CB 70	11%	105.6		H_7CB 183	8%	14.1	16±2.4
T_4CB66+80	13%	94.3	101.4±5.4	H_7CB 174		<3.5	
T_4CB 60	10%	63.4		H_7CB 177	16%	12.2	
T_4CB 81		<3		H_7CB 171	11%	6.7	
T_4CB 77	21%	23.2		H_7CB 172		<3.5	
P_5CB 100		<3		H_7CB 180	14%	13.3	17.1±3.8
P_5CB 95	2%	80.3	83±17	H_7CB 170		<3.5	
P_5CB 91	6%	20.0		H_7CB 189		<3.5	
P_5CB 101	3%	133.5	128±10	O_8CB 202		<3.5	
P_5CB 99	4%	69.9	70.9±4.5	O_8CB 200		<3.5	
P_5CB 97	5%	41.8		O_8CB 201		<3.5	
P_5CB 87	4%	58.3	54±14[a]	O_8CB 203+196		<3.5	
P_5CB 85	6%	29.1		O_8CB 195		<3.5	
P_5CB 110	5%	91.5	127.3±9.4	O_8CB 194		<3.5	

Analyte	*CV*	*Mean*	*Certified*	*Analyte*	*CV*	*Mean*	*Certified*
P_5CB 123		<3					
P_5CB 118	4%	122.9	130.8±5.3	Benz[*a*]anthracene	13%	23.2	32.5±4.8
P_5CB 114		<3		Chrysene	12%	89.2[b]	44.2±2.7
P_5CB 105	6%	46.9	53±3.8	Benzo[*b*]fluoranthene	20%	34.0	46.4±4
P_5CB 126		<3		Benzo[*k*]fluoranthene	8%	17.2	20.2±1
H_6CB 155		<3		Benzo[*a*]pyrene	22%	9.3	15.63±0.8
H_6CB 136	12%	13.5		Indeno[1,2,3-*cd*]pyrene	24%	10.1	14.2+2.8
H_6CB 151	5%	24.1	25.613.6	Dibenz[*ah*]anthracene	41%	3.8[c]	3±0.22[a]
H_6CB 135	6%	18.9		Benzo[*ghi*]perylene	26%	22.6	22±2.3
H_6CB 149	6%	57.9	87.6±3.5				

a. Reference values.
b. Chrysene is coeluting with tryphenylene (certified value: 50.7).
c. Value higher than the limit of detection and lower than the limit of determination.

As already mentioned, a SFE method for the determination of PCB was set up earlier [6]. The extraction conditions of this method, however, were too mild to allow extraction of the more polar PAHs. Generally, the polarity range of the material extracted with supercritical carbon dioxide is narrower than with a conventional organic solvent/extractor couple. In the SFE extraction the solvent strength of the supercritical fluid can be varied by changes in the pressure and to a lesser extent in the temperature, and this property allows the conditions for extraction with a supercritical fluid to be optimized for a given class of analytes. Furthermore, the use of a small percent of an organic solvent in the supercritical fluid (modifier) can be used to widen the polarity interval of the extract. In order to obtain an efficient extraction of the PAHs without losing PCBs, we increased both temperature and pressure of the extraction, and tested extraction both with and without organic modifier (methanol). This yielded a less pure extract, which was no longer ready-to-inject, and required a further clean-up step on a silica gel packed Pasteur pipette. It was however judged that this small increase in labor was outweighed by the time saved for a single analytical procedure and a single GC-MS injection allowing analyses which required previously two different procedures. In fact, the analysis of PCBs was formerly carried out in our laboratory with a concentrated sulfuric acid purification, which destroyed part of the PAHs, while the determination of the latter compounds was based on a laborious modification of the method by Natusch and Tomkins [11] which, in our experience, yielded an incomplete recovery of the PCBs. [12]

There are methods in which the extraction and the clean-up for PCBs and PAHs are carried out together, such as the ones based on size exclusion chromatography [13], but GC-MS determination is carried out separately.

The recovery yields of the isotopically labeled internal standards are reported in Table I together with their coefficients of variation and the amount injected. It is clearly visible that, as usual, injected amounts close to determination limits -as in the case of PAHs- imply higher CV; the recovery values are all higher than 85%, indicating a good extraction efficiency of the method.

The results of the determinations are reported in Table II, together with their coefficients of variation and the certified and reference values.

The agreement between the two data sets, certified and reference values on one side and found values on the other is evident, although there is a slight, systematic, defect on the found results side.

One can consider several reasons to explain this difference. For instance certified values are obtained by different and independent analytical nethods [14] (GC-MS/GC-ECD for PCBs; HPLC-FL/GC-MS for PAHs). When using one single analytical technique, as in this case GC-MS with a single column, a systematic deviation may occur; this happened on the same SRM 2974 to analysts from the same NIST [13] that produced the certified material. The values found in that occasion for PCBs were systematically lower than the certified ones and often lower than the ones found in the present paper. Differences, affecting mainly dispersion of the data, were observed also during certification [14] when the same method was applied at different times.

For PAHs also a systematic negative error in our data, slightly higher than for PCBs is visible; however, the closeness to the determination limit increases the dispersion of the data, as observable from the CV. The agreement among the two sets of data is however still clear. For many of the PAHs, specific chromatographic reasons, besides the low injected amounts, can influence the determination. In Figure 1 the mass-chromatogram of *m/e* 252 is shown, displaying that benzo[*b*]fluoranthene is not resolved from benzo[*j*]fluoranthene, which in turn is not resolved from benzo[*k*]fluoranthene, and that benzo[*a*]pyrene is eluting on the tail of a big peak. Moreover, chrysene is coeluting with triphenylene (certified value: 50.7 μg/kg).

Another possible explanation for the differences observed can be in the sets of unlabeled compounds used in our laboratory for the determination of rrf. The accuracy of our data depends on the accuracy of the solutions used. None of the product used was a certified standard material. The use of other sets of unlabeled compounds was checked for some of our analytes (two CBs and five PAHs); in these cases this had random effect on the agreement with the certified values.

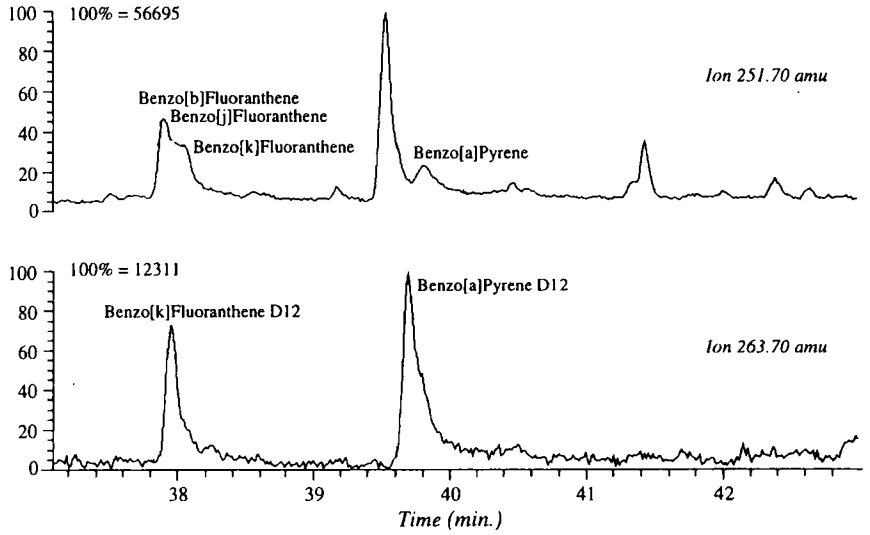

FIGURE 1 Chromatogram of mass 252, native analytes, and of mass 264, deuterated standards

It cannot be ruled out that the systematic underestimation of the data produced by the present method may be due to an incomplete extraction of the native analytes (the isotopically labeled internal standards are completely recovered, as shown by the data in Table II). This possible explanation seems however unlikely or, at least, incomplete. Previous experiments on similar matrices in which the extraction conditions were much milder, yielded data slightly higher than the reference values [6]. Moreover, it seems unlikely that the same slight defect in extraction efficiency may influence exactly in the same way some seventy analytes differing in polarity and in chemical properties.

The determination limits reported in Table II are set at a signal to noise ratio of 5. Some of the analytes, such as CB 141, 137,157,172,176,202,194, were however clearly visible on the chromatogram at S/N lower than 5. Dibenz[*ah*]anthracene is also present at levels lower than the determination level and higher than the detection limit, as suggested by its high coefficient of variation; its found value was, however, reported for comparison with the reference value.

The sensitivity of the method is related to the amount of lyophilized sample analyzed. The SRM 2974 reference material has a very high contamination level; so, a small amount of sample (50mg) was sufficient for the determination, particularly for PCBs. The low amount, however, did not imply problems for the homogeneity of the sample, as shown by the low dispersion of the result on repli-

cate analyses. In the case of low-fat species, such as bivalves, the extractable amount is, in our experience, 500–600mg. With a volume of the final solution of 50μL and an injected volume of 1 μL, this yields limits of detection close to 0.3 μg/kg dry material for tri to hexachlorobiphenyls, 0.35 for hepta to nonachlorobiphenyls and about 0.6 for benzo[a]pyrene. These limits are influenced by numerous factors, such as absolute response of the analyte, the presence of chemical noise (interfering compounds) and chromatographic peak width. The first two factors generally decrease with increasing molecular weight, whilst the third increases, so that sensitivity on high and low chlorinated PCBs is similar.

This method, also without use of organic modifier during extraction, was used in the analyses of mollusks and fish from the Venice lagoon [15,16]; the assessment of its performance was carried out through the evaluation of the recovery yields of the isotopically labeled internal standards and by comparison with the results obtained with different methods. Finally, this method can possibly be used for the determination of some chlorinated pesticides, such as DDE, DDT,DDD and HCB

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