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Evaluation of musk contamination of freshwater fish in Italy by accelerated solvent extraction and gas chromatography with mass spectrometric detection

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Abstract

Musk compounds play an important role as perfuming agents for household chemicals, detergents and cosmetics. It has been demonstrated that the oral absorption pathway of these compounds in humans is significant in the case of contaminated fish. In this study we developed a new extraction procedure, using an accelerated solvent extraction system and a gas chromatography–mass spectrometry detection method, for the determination of 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[*g*]-2-benzopyran, 7-acetyl-1,1,3,4,4,6-hexamethyltetralin, 4-acetyl-1,1-dimethyl-6-*tert*.-butylindan, 6-acetyl-1,1,2,3,3,5-hexamethylindan and 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindan in freshwater fish samples, collected from several Italian rivers and one lake. 6,7-Dihydro-1,1,2,3,3-pentamethyl-4-(5H)-indanon was used as internal standard. The method provides a rapid and highly selective extraction procedure, and is sensitive in determining these musk compounds in freshwater fish samples. This is the first report on the contamination from musk compounds in freshwater fish collected in Italy.

Keywords: Accelerated solvent extraction; Extraction methods; Fish; Food analysis; Musk compounds; Aromatic compounds

1. Introduction

Musk compounds, both of natural and synthetic origin, are widely used as fragrances in consumer products such as cosmetics, toiletries, detergents, soaps, toothpastes and also as flavours in food industry and in fish farming. Tonkinian musk, a natural musk, is also used for pharmaceutical purposes.

On account of their widespread use, musk compounds can be considered ubiquitous throughout the world and can be found in any compartment of the environment: surface water, sewage, sediments and finally fish species living in contaminated rivers and estuaries. According to their lipophilic characteristics and their slow bio-degradation, a bio-concentration trend can be expected. Some studies have shown a bio-accumulation of polycyclic musk compounds in freshwater fish [1,2] and also in human adipose tissue and human milk [3–5]. It has been demonstrated that the oral absorption pathway significantly contributes to human exposure, e.g., in the case of contaminated fish [6] and breast milk [7] as well as dermal absorption and inhalation.

Gas chromatography–electron-capture detection (GC–ECD) and various GC–mass spectrometry

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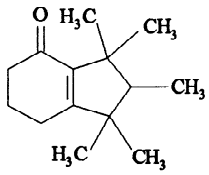
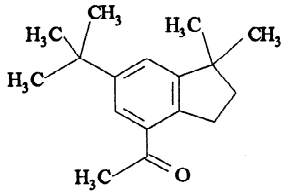
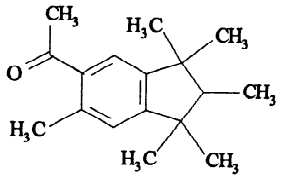
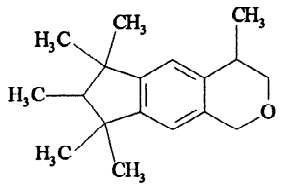
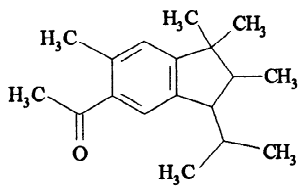
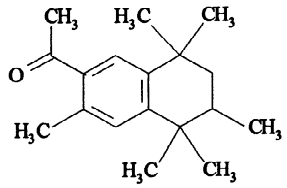
Table 1
Chemical and trade names, CAS numbers and chemical structures of the investigated musk compounds

Chemical name	Trade name	CAS No.	Structure
6,7-Dihydro-1,1,2,3,3-pentamethyl-4-(5H)-indanon (DPMI)	Cashmeran	33704-61-9	
4-Acetyl-1,1-dimethyl-6-*tert*.-butylindan (ADBI)	Celestolide	13171-00-1	
6-Acetyl-1.1.2.3.3.5-hexamethylindan (AHMI)	Phantolide	15323-35-0	
1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8,-hexamethylcyclopenta[*g*]-2-benzopryan (HHCB)	Galaxolide	1222-05-5	
5-Acetyl-1,1,2,6-tetramethyl-3-isopropylindan (ATII)	Traseolide	68140-48-7	
7-Acetyl-1,1,3,4,4,6,-hexamethyltetralin (AHTN)	Tonalide	1506-02-1	

(MS) techniques are currently applied for identification and confirmation analysis of musk compounds. Isolation from various matrices is similar to the classical solvent-based extraction and clean-up methods used for pesticide analysis [8,9]. However, the extraction of musk compounds with such techniques is often time-consuming and can require the use of large amounts of solvents; furthermore it increases a potential for analyte loss.

A new extraction technique, accelerated solvent extraction (ASE), has recently been introduced for the extraction of environmental contaminants [10–13]. Conventional liquid solvents are used to perform extractions at elevated pressures ($1.0\cdot10^4$–$1.4\cdot10^4$ kPa) and temperatures (50–200°C). ASE takes advantage of increases in analyte solubilities occurring at temperatures above the boiling points of commonly used solvents. At the higher temperatures used by ASE, the kinetic processes for the desorption of analytes from the matrix are accelerated compared to conditions when solvents are used at room temperature. Furthermore, high pressure forces the solvent into areas of the matrix that would not normally be contacted by the solvent using atmospheric conditions and maintains the solvents in liquid form at operating temperatures [14].

This paper describes our analytical approach for determining several musk fragrances in fish samples using ASE. GC–MS was chosen as the detection method.

The compounds investigated were the polycyclic musk compounds: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8 hexamethylcyclopenta[*g*]-2-benzopyran (HHCB), 7-acetyl-1,1,3,4,4,6-hexamethyltetralin (AHTN), 4-acetyl-1,1-dimethyl-6-*tert*.-butylindan (ADBI), 6-acetyl-1,1,2,3,3,5-hexamethylindan (AHMI) and 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindan (ATII) (Table 1).

This study demonstrated that ASE can automatically and rapidly extract the polycyclic musk compounds from fish samples with good accuracy and precision and GC–MS permits their highly specific and sensitive detection.

This approach was therefore employed for the determination of polycyclic musk compounds in different species of freshwater fish samples collected from several Italian rivers and one lake. The data achieved in this study, for the first time, provides evidence of contamination from polycyclic musk compounds in Italian aquatic resources and point out the potential health risks associated with eating fish containing residues of these compounds.

2. Experimental

2.1. Chemicals and reagents

Polycyclic musk compounds, Galaxolide 50% in diethylphthalate, Tonalide, Celestolide, Phantolide, Cashmeran, Traseolide, were supplied by Promochem (Wesel, Germany). All solvents used were of analytical grade; ethyl acetate and acetone were purchased from Labscan (Dublin, Ireland), chloroform, hexane, methanol and toluene from J.T. Baker (Deventer, The Netherlands). Diatomaceous earth–Hydromatrix was obtained from Varian (Harbor city, CA, USA).

Alumina (Brockman activity II-III), from Merck (Darmstadt, Germany), was activated at 500°C for 4 h and deactivated with 15% (w/w) water prior to use.

Individual musk standard stock solutions of 400 μg/ml were prepared by dissolving the pure compounds with toluene. Fortification standard solutions (0.12–9.6 μg/ml) and calibration standard solutions (4–320 ng/ml), containing each all the polycyclic musk compounds, were prepared by appropriate dilutions of the stock solutions with toluene. In the fortification and calibration standard solutions, Cashmeran [6,7-dihydro-1,1,2,3,3-pentamethyl-4-(5H)-indanon, DPMI] was used as internal standard at concentration of 80 ng/ml. All the solutions were stored at 4°C and were stable for at least one month.

2.2. Sample collection

All the fish have been taken from the wild and were mostly collected in northern Italy from rivers Piave, Po, Livenza, Ticino, Adige, Torrent Gadera and Lake Sompunt. Other samples were caught from the River Garigliano located in southern Italy.

The examined species were: trout (*Salmo trutta fario* L. and *Salmo trutta lacustris* L.), sheatfish (*Silurus glanis*), crucian carp (*Carassius carassius*),

Italian nose (*Chondrostoma soetta*) and chub (*Leuciscus cephalus*).

After collection, all the samples were immediately preserved in a refrigerated box and frozen at −20°C, within 3 h, until analysis.

2.3. Accelerated solvent extraction

Extraction of fish samples was carried out by using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA). The ASE system was equipped with an auto-sampler carousel and a collection tray that allowed up to 24 separate samples to be extracted sequentially. Stainless steel extraction cells and glass collecting vials with 22 ml and 40 ml volumes, respectively were used. The extraction was performed using a mixture of ethyl acetate–hexane (1:5, v/v) as extraction solvent under the ASE conditions described in Table 2. To obtain a fat-free extracts, 5 g of alumina, deactivated as previously described in Section 2.1, were placed at the outlet end of the ASE cell during the extraction [15].

A 22-ml extraction cell was loaded by inserting a cellulose filter into the cell outlet, followed by 5 g of alumina. After the addition of the alumina, a second cellulose filter was inserted. Alumina, was then conditioned according to the above ASE conditions.

The muscle tissue of the fish was homogenised in a Warring blender and 3 g of the homogenate were mixed with 5 g of Hydromatrix in a mortar with a pestle.

The mixture sample–Hydromatrix was loaded into the ASE cell on top of conditioned alumina and extracted. First the extraction cell was heated to 80°C and filled with the solvent until the pressure reached $1.0 \cdot 10^4$ kPa. After an oven heat-up time of 5 min, under these conditions, two static extractions at constant pressure and temperature of 5 min were employed. After the static period, fresh solvent was introduced to flush the lines and cell, and the extract was collected in the vial. The flush volume amounted to 100% of the extraction cell. The residue solvent in the extraction cell was forced out into the collection vial with pressurised nitrogen.

Table 2
Optimized ASE extraction parameters

Extraction solvent	Ethyl acetate–hexane (1:5, v/v)
Temperature	80°C
Pressure	$1.0 \cdot 10^4$ kPa
Heat time	5 min
Static time	5 min
Flush volume	100%
Purge time	90 s
Static cycles	2

The ASE extract of about 35 ml was evaporated to dryness on a rotary evaporator with a temperature-controlled bath (40°C) and the residue was redissolved in 1 ml of toluene.

2.4. Gas chromatography–mass spectrometry

The GC–MS analysis was performed on a HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) coupled to a HP 5971 mass-selective detector.

Separation was carried out using a Supelcowax 10 fused-silica capillary column (30 m×0.20 mm I.D., 0.20 μm film thickness) (Supelco, Bellefonte, PA, USA). A 1-μl volume was injected in the splitless mode (1 min) at 260°C. The oven temperature program was as follows: initial temperature 80°C (held for 1.5 min), increased at 10°C/min to 160°C, increased at 3°C/min to 260°C, increased at 15°C/min to 270°C and maintained at 270°C for 15 min.

The detector temperature was set at 280°C. Helium was used as the carrier gas at a head pressure of 85 kPa (column flow of 0.52 ml/min).

Electron impact (EI) mass spectra were measured at an acceleration energy of 70 eV. Both identification and quantification were carried out by selected-ion monitoring (SIM) of characteristic ions of each polycyclic musk compound. The most suitable ions (high ion intensity, high mass and low background) were selected to perform the analysis with SIM mode. The ions chosen for the polycyclic musk compound analysis are listed in Table 3.

2.5. Sensitivity and recovery evaluation

In order to evaluate the detection limit and the analytical recovery of the method, fish samples were purchased from the fish market and analysed by GC–MS to verify absence of musk compounds.

To test the limits of detection of the method,

Table 3
Retention time and selected ions for each analyte musk compound

Compound	Peak	Retention time (min)	M_r	$(m/z)^a$
DPMI	1	12.6	206.3	135, 163, <u>191</u>, 206
ABDI	2	17.3	244.4	173, <u>229</u>, 244
AHMI	3	19.6	244.4	187, <u>229</u>, 244
HHCB	4	20.4	258.4	213, <u>243</u>, 258
ATII	5	21.4	258.4	173, <u>215</u>, 258
AHTN	6	22.3	258.4	159, 187, <u>243</u>, 258

[a] Underlined ions correspond to the base peaks used for the quantitative analysis in the GC–MS SIM mode.

GC–MS-SIM analyses were performed on fish muscle tissue blanks spiked with mixtures of polycyclic musk compounds at 0.5, 1, 2 and 5 ng/g.

Standard solution curves obtained by injecting standard mixtures containing each polycyclic musk compounds in the range 4–320 ng/ml and DPMI (I.S.), at a constant concentration of 80 ng/ml, were used to evaluate the analytical recovery. By the comparison of the results obtained by GC–MS-SIM analyses on fish muscle tissue blanks spiked with mixtures of the polycyclic musk compounds at 4, 40 and 80 ng/g and extracted to that of standard solution curves, mean percentages of extraction recovery were estimated. Recovery data were assessed for within-day variation and between-day variation. Duplicate analyses of blank fish samples fortified at the different levels of concentration reported above, were carried out each day for five days.

2.6. Quantitative procedures

Calibration curves were obtained from GC–MS-SIM analyses of extracts of fish muscle tissue blanks spiked with mixtures of the polycyclic musk compounds in the range 4–320 ng/g and with 80 ng/g of

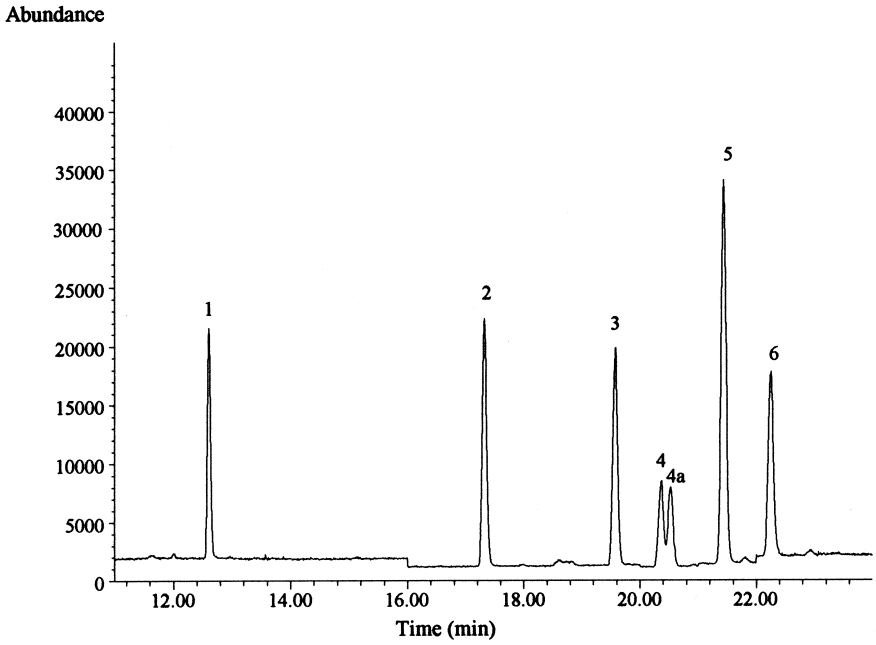


Fig. 1. GC–MS total ion chromatogram of a standard solution (160 ng/ml) of polycyclic musk compounds. Peaks: 1=DPMI (I.S., 80 ng/ml), 2=ADBI, 3=AHMI, 4=HHCB, 4a=HHCB isomer, 5=ATII, 6=AHTN.

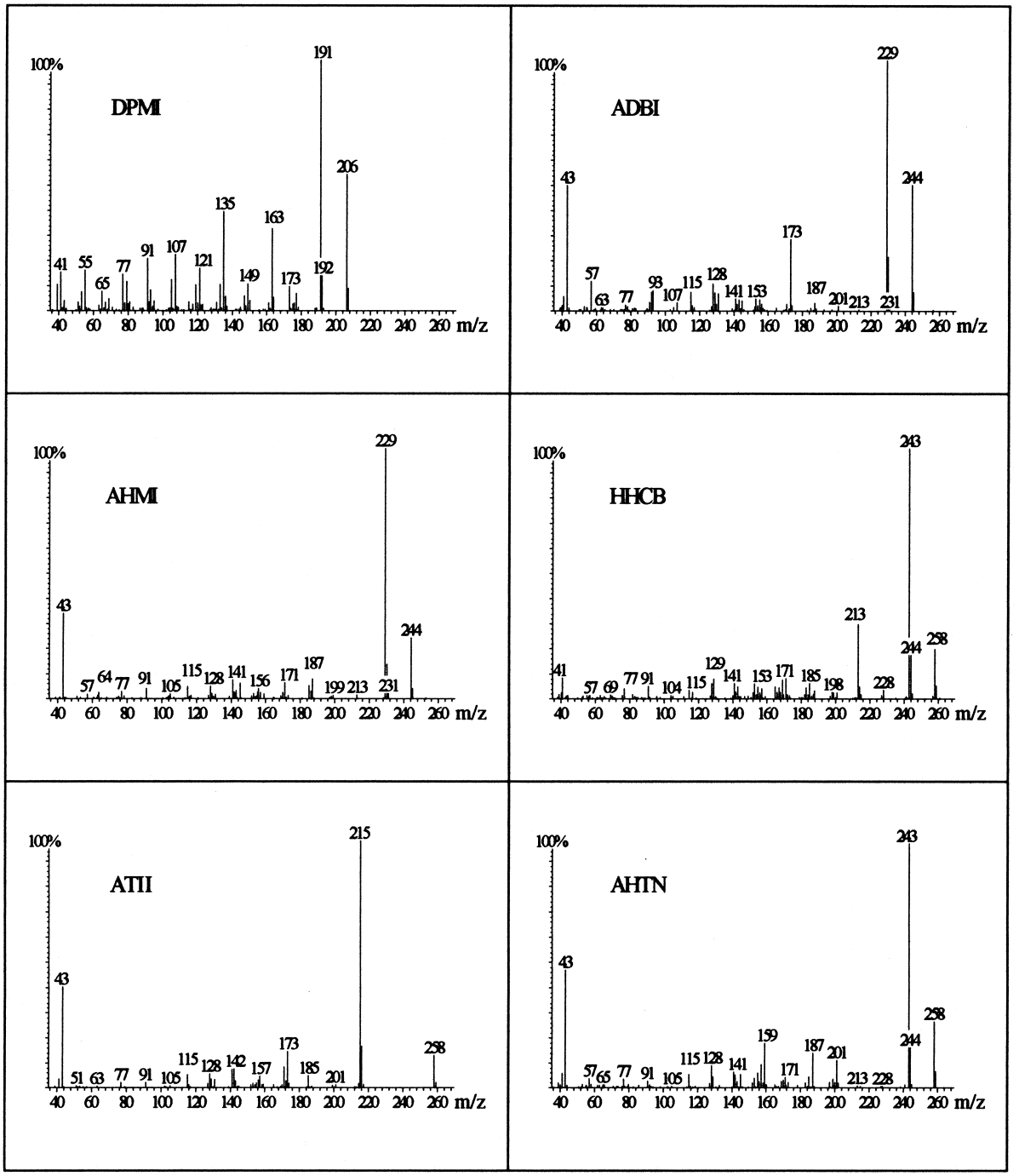


Fig. 2. Electron-impact mass spectra of the polycyclic musk compounds DPMI, ADBI, AHMI, HHCB, ATII, AHTN.

DPMI (I.S.). Estimates of the amounts of polycyclic musk compounds in real samples were extrapolated from these calibration graphs.

2.7. Determination of the lipid content

In order to determine the amount of lipids in fish samples, 5 g of homogenised muscle tissue were extracted with a chloroform–methanol (1:2) mixture according to the Bligh–Dyer method [16].

3. Results and discussion

A total ion chromatogram of a standard mixture of the six substances studied is shown in Fig. 1.

Good chromatographic separation of analytes and

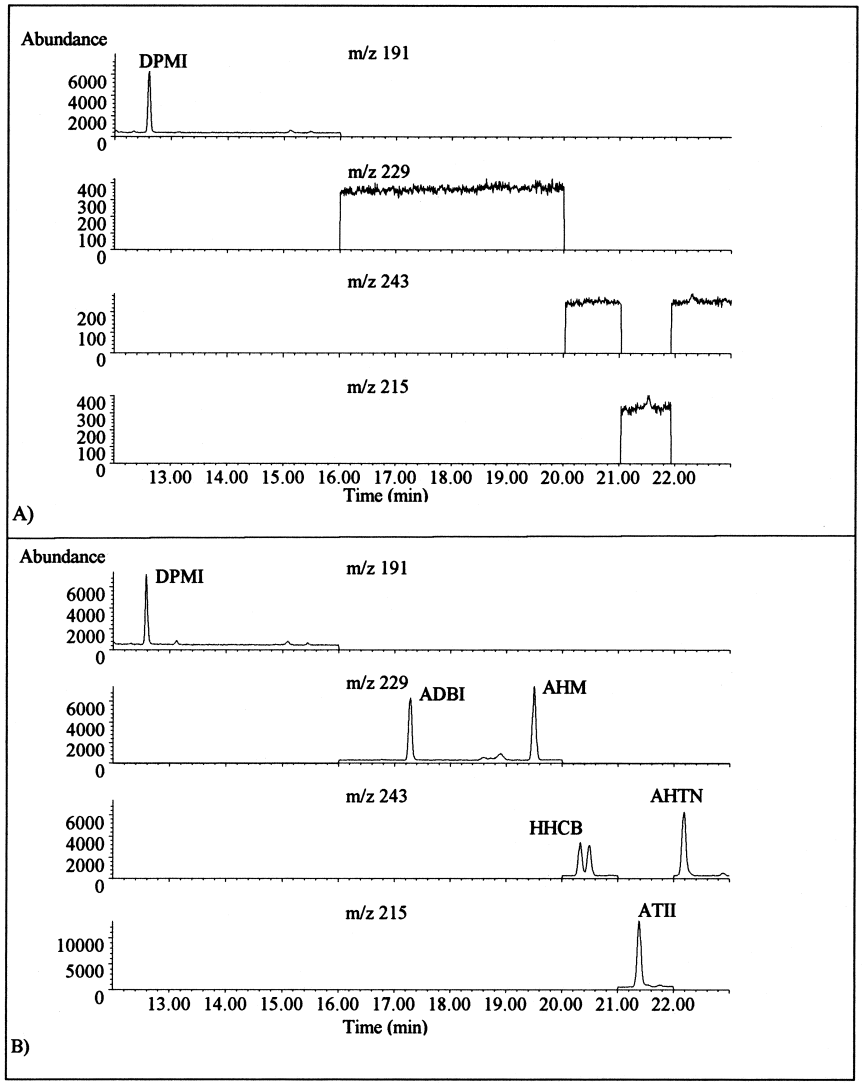


Fig. 3. GC–MS selected-ion monitoring chromatograms of fish muscle tissue blank (A) and fish muscle tissue spiked at 40 ng/g level with ADBI, AHMI, ATII, HHCB and AHTN at the same selected ions (B). DPMI has been added as I.S.> at 80 ng/g. One monitored ion is presented for each compound: m/z 191 (DPMI); m/z 229 (ADBI and AHMI); m/z 215 (ATII); m/z 243 (HHCB and AHTN).

well defined peaks for all compounds were obtained using a Supelcowax 10 fused-silica capillary column under the experimental conditions reported above. Due to its chemical structure Galaxolide occurs in two diastereomeric forms which were separated on the capillary GC column. Since ion intensity ratio of the two isomers was constant, only the peak area of the first isomer was used for quantification.

In order to compensate for any loss during sample preparation and in order to enhance the recoveries, an internal standard calibration was carried out for quantitative analysis of polycyclic musk compounds by GC–MS. Data already reported in previous studies on polycyclic musk compounds indicated that DPMI did not occur as a main contaminant in different environmental and human compartments. On the other hand a preliminary screening analysis demonstrated the absence of this compound in the collected samples. Moreover, although DPMI did not elute very near to the other polycyclic musk compounds, it belongs to the same class of the investigated compounds. DPMI was therefore used as internal standard (peak 1).

The EI mass spectra (70 eV) of the investigated polycyclic musk compounds (Fig. 2) showed several characteristic fragments which can be used for identification and screening analysis.

Fig. 3A shows GC–MS-SIM chromatograms obtained from the blank fish sample and those obtained from the blank fish sample fortified with the different polycyclic musk compounds at 40 ng/g level and I.S. DPMI and monitored at the same ions. No interferences around analyte retention times were observed.

Results of recovery studies are presented in Table 4. Recoveries were satisfactory at each level for all compounds. The highest average recovery was obtained for HHCB (93%), whereas it was lower (75–78%) for ADBI, AHMI, ATII and AHTN. The repeatability was good for all the polycyclic musk compounds at the levels investigated, with relative standard deviations (R.S.D.s) ranging from 4 to 13%. ASE resulted as a potential alternative to the classical solvent-based extraction and clean-up methods and presented the advantage in that sample preparation occurs in a single step avoiding the problems of multiple treatments of sample. The obtained extracts were then clean enough for direct injection to GC–MS in EI mode. In addition analyte diffusion from the sample matrix into the solvent and the overall solvent capacity are increased by elevated temperatures and pressure.

Table 4
Recovery of polycyclic musk compounds from spiked sample

Musk compound	Spike level (ng/g)	Measured[a] concentration (mean±S.D.) (ng/g)	Recovery[a] (%)	R.S.D. (%)
ADBI	4	2.8±0.3	70	11
	40	30.0±1.6	75	5
	80	63.2±2.7	79	4
AHMI	4	3.0±0.3	74	10
	40	32.0±2.0	80	6
	80	64.8±2.7	81	4
HHCB	4	3.1±0.4	77	13
	40	38.7±2.3	97	6
	80	84.0±3.2	105	4
ATII	4	2.8±0.3	70	11
	40	30.8±1.5	77	5
	80	66.4±2.5	83	4
AHTN	4	2.8±0.2	70	7
	40	30.8±1.2	77	4
	80	68.8±2.8	86	4

[a] Each value is the mean of 10 samples (2/day for five days).

The detection limits of the GC–MS-SIM analyses were 0.5 ng/g for ATII, 1.0 ng/g for ADBI, AHMI, AHTN and 2.0 ng/g for HHCB in fish samples, based on a signal-to-noise ratio of at least 3:1.

Relative retention times and relative abundances of confirming ions for each polycyclic musk compound in the spiked and real samples were within 0.5% and 10%, respectively, with respect to the corresponding values of the polycyclic musk standards analysed under the same experimental conditions. This method showed a high degree of specificity since the analyte confirmation is based on retention time, molecular mass (M_r), presence of diagnostic fragments for each analyte and ion ratios. The calibration curves, used for quantification purpose, provided correlation coefficients (r^2) greater than 0.998 for all the analytes.

In order to investigate the presence of polycyclic musk compounds throughout Italy, fish were used as bio-indicators because of their capability to bio-

concentrate lipophilic and persistent chemicals [17] such as polycyclic musk compounds. Furthermore, fish is an important part of the human diet and therefore contributes to bio-accumulation of chemicals in man. Fish were collected from sampling areas along several rivers and one lake which had different types and magnitudes of water pollution. Accumulation of polycyclic musk compounds was evaluated in the muscle tissue of different species of fish.

In Table 5 the concentrations of the investigated polycyclic musk compounds in muscle tissue from 28 fishes are presented, together with their lipid content. HHCB and AHTN were the most frequently detected compounds in the analysed samples. HHCB was present in all the samples at levels ranging from <4 to 47 ng/g. In most samples AHTN was detected (4 to 105 ng/g), whereas the presence of AHMI was demonstrated only in three trout samples from the Piave river at a very low concentration (4–5 ng/g). None of the samples contained detectable traces of ADBI and ATII. Fig. 4 shows the GC–MS-SIM chromatograms of two contaminated samples.

Because of the strong lipophilic properties of polycyclic musk compounds, the lipid content of

Table 5
Concentrations of polycyclic musk compounds in 28 fish samples

Site of collection	Species	Samples	ADBI (ng/g)	AHMI (ng/g)	HHCB (ng/g)	ATII (ng/g)	AHTN (ng/g)	Lipids (%)
River	Trout	A	n.d.	4	29	n.d.	20	4.9
Piave	(*Salmo trutta fario* L.)	B	n.d.	n.d.	9	n.d.	5	3.0
		C	n.d.	n.d.	<4	n.d.	n.d.	1.9
		D	n.d.	n.d.	<4	n.d.	n.d.	2.8
		E	n.d.	5	34	n.d.	24	8.7
		F	n.d.	n.d.	11	n.d.	6	2.9
		G	n.d.	5	47	n.d.	27	8.2
		H	n.d.	n.d.	16	n.d.	8	3.3
River	Crucian carp	A	n.d.	n.d.	5	n.d.	5	0.5
Po	(*Carassius carassius*)	B	n.d.	n.d.	5	n.d.	4	0.4
		C	n.d.	n.d.	5	n.d.	5	0.4
	Sheatfish (*Silurus glanis*)	A	n.d.	n.d.	34	n.d.	105	4.4
River	Trout	A	n.d.	n.d.	6	n.d.	n.d.	2.7
Livenza		B	n.d.	n.d.	5	n.d.	n.d.	3.1
		C	n.d.	n.d.	11	n.d.	n.d.	2.8
		D	n.d.	n.d.	8	n.d.	n.d.	6.4
		E	n.d.	n.d.	6	n.d.	n.d.	2.9
River	Trout	A	n.d.	n.d.	6	n.d.	4	4.1
Ticino		B	n.d.	n.d.	4	n.d.	4	2.2
River Garigilano	Chub (*Leuciscus cephalus*)	A	n.d.	n.d.	5	n.d.	4	1.7
	Crucian carp	A	n.d.	n.d.	9	n.d.	4	0.8
		B	n.d.	n.d.	5	n.d.	4	0.1
River	Italian nose	A	n.d.	n.d.	<4	n.d.	n.d.	0.7
Adige	(*Chondrostoma soetta*)	B	n.d.	n.d.	<4	n.d.	n.d.	1.3
Torrente	Trout	A	n.d.	n.d.	<4	n.d.	n.d.	1.1
Gadera		B	n.d.	n.d.	<4	n.d.	n.d.	1.2
Lake	Trout	A	n.d.	n.d.	<4	n.d.	n.d.	2.3
Sompunt	(*Salmo trutta lacustris* L.)	B	n.d.	n.d.	<4	n.d.	n.d.	1.3

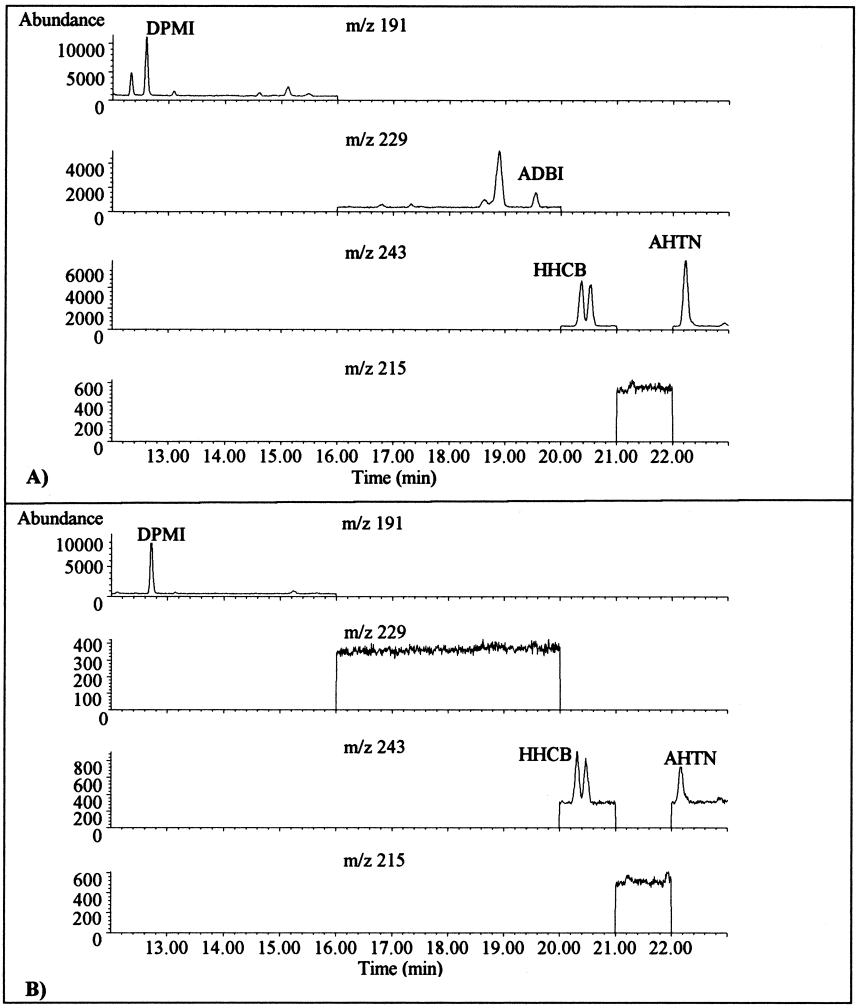


Fig. 4. Selected-ion chromatograms at m/z 191, 229, 243 and 215 obtained by GC–MS analysis of samples collected from the Piave (A) and Ticino (B) rivers.

muscle tissue was considered in the accumulation of these compounds by the analysed fish.

As can be seen in Table 5, in some contaminated fish samples an elevated lipid content corresponds to a high concentration of the polycyclic musk compounds HHCB and AHTN, thus confirming the potential bio-concentration of such lipophilic compounds. Although the lipophilicity of polycyclic musk compounds and their persistence in the environment affect the extent of their accumulation in fish, other factors, such as proximity to source of contamination, species and feeding patterns should be considered too. A wider contamination monitoring program will therefore be carried out by our laboratory in future research.

4. Conclusions

A simple, rapid and accurate method for the determination of polycyclic musk compounds in freshwater fish has been developed using ASE and GC–MS.

Results demonstrated that this analytical approach

compares satisfactorily with traditional methods of analysis of polycyclic musk compounds in this kind of matrix, but also offers many advantages. The extraction procedure is rapid and simple, requiring only the mixing of the sample with a drying agent and transferring the mixture to an extraction cell; one sample can be extracted within 20 min, including washing the solvent line for the next extraction. Furthermore, a selective and single-step extraction and clean-up can be performed with the proper choice of solvent and adsorbent in the extraction cell. The obtained extracts were then clean enough for direct injection to GC–MS in EI mode.

The optimised ASE conditions selected in our study, such as temperature and pressure, significantly reduced solvent usage. Finally GC–MS method detects unambiguously analytes in ASE extracts with a high degree of selectivity even in the presence of matrix components.

This method was used to evaluate the contamination of fish from Italian freshwaters by polycyclic musk compounds. HHCB and AHTN were the most frequently detected compounds in samples and were found at various contamination levels in some species of fish of certain rivers. The presence of AHMI was demonstrated only in few samples and at very low concentrations. None of the samples contained a detectable trace of ADBI and ATII. On the basis of our results a positive correlation of some of these residue levels with lipid content of fish was observed in several contaminated fish. However, further studies are needed to evaluate the effect of other factors, such proximity to source of contamination, species and feeding patterns, on the level of contamination by polycyclic musk compounds.

The method may be further developed for its applicability to the analysis of other matrices where polycyclic musk compounds accumulate, in order to evaluate the presence of these substances not only in food but also in humans.

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