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Closed-loop stripping analysis of synthetic musk compounds from fish tissues with measurement by gas chromatography–mass spectrometry with selected-ion monitoring

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Abstract

Synthetic musk compounds have been found in surface water, fish tissues, and human breast milk. Current techniques for separating these compounds from fish tissues require tedious sample clean-up procedures. A simple method for the determination of synthetic musk compounds in fish tissues has been developed. Closed-loop stripping of saponified fish tissues in a 1-l Wheaton purge-and-trap vessel is used to strip compounds with high vapor pressures such as synthetic musks from the matrix onto a solid sorbent (Abselut Nexus). This technique is useful for screening biological tissues that contain lipids for musk compounds. Analytes are desorbed from the sorbent trap sequentially with polar and nonpolar solvents, concentrated, and directly analyzed by high resolution gas chromatography coupled to a mass spectrometer operating in the selected ion monitoring mode. In this paper, we analyzed two homogenized samples of whole fish tissues with spiked synthetic musk compounds using closed-loop stripping analysis and pressurized liquid extraction (PLE). The analytes were not recovered quantitatively but the extraction yield was sufficiently reproducible for at least semi-quantitative purposes (screening). The method was less expensive to implement and required significantly less sample preparation than the PLE technique.

Keywords: Sample preparation; Extraction methods; Closed-loop stripping analysis; Fish; Environmental analysis; Musk compounds

1. Introduction

Synthetic musk compounds are used as inexpensive fragrance materials for the production of perfumes and also as additives to soap, detergent, shampoo, and even Indian chewing tobacco. Previous reports in the literature have shown that galaxolide, tonalide, musk xylene, and musk ketone, among others in this class of personal care products, are generally present as micro-pollutants in surface water, human breast milk, fish, and human adipose tissues [1–6]. Musk xylene and musk ketone may therefore be considered as suitable indicator chemi-

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cals for the pollution of aquatic ecosystems by domestic and communal sewage [7]. Several extraction techniques employing sonication, liquid–liquid, Soxhlet, or pressurized liquid chromatography (PLE; Dionex trade name ASE, for accelerated solvent extraction) have been used for extracting these compounds from fish tissues [4]. Most of these extraction techniques are not only expensive, they also lack selectivity. Interfering compounds from biological tissues, such as lipids, including cholesterol, are co-extracted. Therefore, after extraction, the daunting task of separating analytes of interest from protein and lipid interferences takes more time than the extraction process itself.

This paper presents an alternate extraction methodology that is simple and produces extracts requiring no additional clean-up. Carp (*Cyprinus carpio*) were chosen to test an alternative methodology because they are bottom dwellers, provide sufficient sample mass for the analytical procedure, and are abundant and easy to catch.

The distribution of chemicals into various fish tissues is believed to be a matter of partitioning of the chemical between water-insoluble (e.g. fats and oils) and water-soluble parts (e.g. blood) [7]. With this in mind, whole fish homogenization was carried out in order to determine the concentration of synthetic musk in the entire fish.

The use of closed-loop stripping of organic compounds is not new. The success of this method is determined by the magnitude of the Henry's law constant of the synthetic musk compound. Henry's law constant provides a measure of a compound's concentration in the gas phase relative to that in the aqueous phase at equilibrium. The general definition of a volatile compound, as given by Lyman et al., is one with Henry's law constant of greater than 7.2×10^{-6} atm l/mole (1 atm=101 325 Pa) [8]. The Henry's law values of synthetic musk compounds presented in Tables 1–3 were estimated using EPI Suite, a computer program, co-developed by the US Environmental Protection Agency (EPA) and the Syracuse Research Corporation (SRC) [9]. The polycyclic musk compounds have values greater than 7.2×10^{-6}, and are therefore volatile by Lyman's definition (Table 1). Tables 2 and 3 show nitro musks and their metabolites to be non-volatile to semi-volatile according to this definition. Previous workers have utilized solvent partition, vapor phase partitioning, and a combination of these methods to measure volatile substances in complex matrices. Grob and Zurcher developed a closed-loop stripping analysis (CLSA) technique for volatile organic compounds in water samples [10]. They used activated pure wood charcoal for trapping the volatile organic compounds. Extracting organic compounds from activated charcoal can potentially result in a greater loss of target analytes of interest due to the non-reversible adsorption between the organic compounds and carbon. McGuire et al. presented a paper about the use of CLSA for detecting odorous compounds in water, at the parts-per-trillion level [11]. Santos et al. also carried out an experiment to determine the aroma of fish by performing static and dynamic head-space extraction of saponified fish tissues [12]. Retzik and Cerra used closed-loop technology to detect volatile compounds by total organic carbon (TOC) analysis [13].

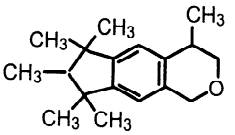
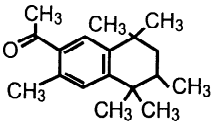
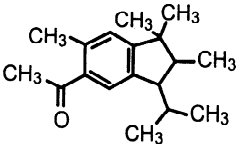
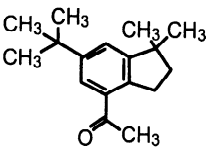
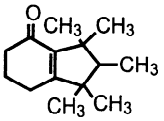
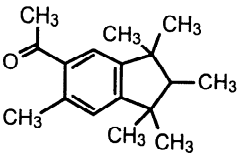
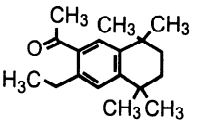
The intent of this paper is to compare the CLSA technique with PLE for the extraction of synthetic musk compounds from two portions of fish tissues using GC–MS as the detection method. Closed-loop stripping of fish tissues involves purging a saponified fish solution with nitrogen gas and trapping the purged analytes on a solid sorbent (Abselut Nexus). The efficient trapping of synthetic musk compounds in the aqueous phase by Abselut Nexus sorbent has been demonstrated elsewhere [14]. However, the low analyte recoveries of this method make it suitable only for screening environmental biological tissues. The method is highly selective, simple, requires no further sample clean-up, and uses only a 50-ml solvent volume.

2. Experimental

2.1. Chemicals and materials

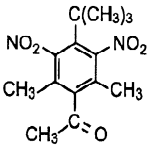
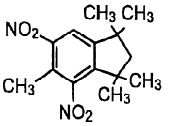
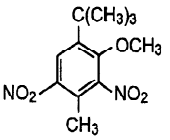
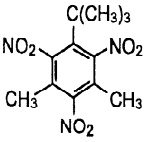
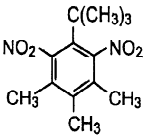
All synthetic musks (polycyclic and nitro) (Tables 1 and 2) were purchased at 99% purity from R.T. Corporation (Laramie, WY, USA), except musk ketone, musk ambrette, and musk xylene, which were provided by the Institute of Food Chemistry,

Table 1
Trade and CAS names, structures, and estimated Henry's law constants for seven polycyclic musks

Trade and CAS names (acronym)	Chemical structure	Henry's law constant[a] (atm l/mol)
Galaxolide, 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-[g]-2-benzopyran (HHCB)		7.56×10^{-4}
Tonalide, 1-(5,6,7,8-Tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthalenyl-ethanone (AHTN)		1.09×10^{-2}
Traseolide 1-[2,3-Dihydro-1,1,2,6-tetramethyl-3-(1-methyl-ethyl)-1H-inden-5-yl]-ethanone (ATII)		1.94×10^{-2}
Celestolide, 1-[6-(1,1-Dimethylethyl)-2,3-dihydro-1,1-dimethyl-1H-inden-4-yl]-ethanone (ADBI)		7.05×10^{-3}
Cashmeran 1,2,3,5,6,7-Hexahydro-1,1,2,3,3-pentamethyl-4H-inden-4-one (DPMI)		1.42×10^{-1}
Phantolide 1-(2,3-Dihydro-1,1,2,3,3,6-hexamethyl-1H-inden-5-yl)-ethanone (AHMI)		7.73×10^{-3}
Versalide, 7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin (AETT)		9.96×10^{-3}

[a] Estimated values derived by EPA and SRC EPI-Suite in Ref. [9].

Table 2
Trade and CAS names, structure, and estimated Henry's law constant for five nitro musks

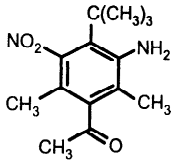
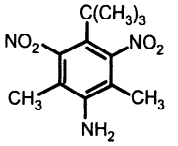
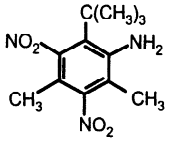
Trade and CAS name (acronym)	Chemical structure	Henry's law constant[a] (atm l/mole)
Musk ketone, 1-*tert*.-butyl-3,5-dimethyl-2,6-dinitro-4-acetyl-benzene (MK)		1.90×10^{-6}
Musk moskene, 4,6-dinitro-1,1,3,3,5-pentamethylindane (MM)		1.54×10^{-4}
Musk ambrette, 2,6-dinitro-3-methoxy-4-*tert*.-butyl toluene (MA)		7.05×10^{-4}
Musk xylene, 1-*tert*.-butyl-3,5-dimethyl-2,4,6-trinitrobenzene (MX)		7.73×10^{-6}
Musk tibetene, 1-*tert*.-butyl-2,6-dinitro-2,4,5-trimethylbenzene (MT)		9.96×10^{-4}

[a] Estimated values derived by U.S. EPA and SRC EPI-Suite™ Ref. [9].

University of Hohenheim (Stuttgart, Germany). Musk versalide (acetylethyltetramethyltetralin, AETT), was provided by Dr. Peter Spencer of the Oregon Health Sciences University (Portland, OR, USA). The surrogate standards, which include a mixture of pentachloronitrobenzene (99.9%), 2,6-dinitrotoluene (99.8%), 2,2′-dinitrobiphenyl (99.9%), and the internal standard, [$^{2}H_{8}$]naphthalene (99.9%), were all at a concentration of 200 μg/ml and were purchased from Absolute Standard (Hamden, CT, USA).

The organic solvents used were 99.9% *n*-hexane and toluene (B&J GC2 grade, Burdick and Jackson, Muskegon, MI, USA). Methylene chloride, ethyl acetate, diethyl ether, light petroleum (b.p. 35 °–60 °C), acetone, methanol (all HPLC grade) and hydrazine hydrate and Raney nickel (slurry) were purchased from Aldrich (Milwaukee, WI, USA). Anhydrous, granular sodium sulfate (Tracepur) was obtained from EM Science (Gibbstown, NJ, USA) and glasswool treated with dimethyldichlorosilane (DMDCS) was purchased from Alltech Associates

Table 3
Trade and CAS names, structures, and estimated Henry's law constants for three nitro musk metabolites

Trade and CAS name (acronym)	Chemical structure	Henry's law constant[a] (atm l/mol)
Amino musk ketone: 2-Amino-1-*tert*.-butyl-3,5-dimethyl-6-nitro-4-acetyl-benzene (2-AMK)		9.30×10^{-8}
4-Amino musk xylene: 4-Amino-1-*tert*.-butyl-3,5-dimethyl-2,6-dinitro-benzene (4-AMX)		3.79×10^{-7}
2-Amino musk xylene: 2-Amino-1-*tert*.-butyl-3,5-dimethyl-4,6-dinitro-benzene (2-AMX)		3.79×10^{-7}

[a] Estimated values derived by EPA and SRC EPI-Suite in Ref. [9].

(Arlington Heights, IL, USA). Diatomaceous earth hydromatrix and polystyrene cross-linked with 50% divinylbenzene and poly(methyl methacrylate) in a polypropylene cartridge (Abselut Nexus) were provided by Varian (Harbor City, CA, USA). One-gram cartridges of amine-functionalized silica-based sorbent (Strata NH_2) were purchased from Phenomenex (Torrance, CA, USA). DMDCS (5%) in toluene was obtained from Supelco (Bellefonte, PA, USA). A Nanopure water system (Barnstead/Thermolyne, Dubuque, IA, USA) provided deionized (DI) water with a resistivity of 17.5 MΩ cm. Isoclean concentrate was used to clean glassware, and was purchased from Isolab (Akron, OH, USA). Non-CFC (chlorofluorocarbons) Biofreezer models 8584 and 8523 (Forma Scientific Division, Marietta, OH, USA), provided ultra-low temperature (−80 °C) for sample storage. A Hobart meat processor was used to grind fish samples and was purchased from Hobart (Troy, OH, USA).

Alumina (Basic, Brockman activity I), from Fisher Scientific (Fair Lawn, NJ, USA), was activated at 480 °C for 24 h and deactivated with 15% (w/w) water just before use.

2.2. Pump and ancillary supplies

A 1-l Wheaton purge-and-trap vessel was purchased from Scientific Instrument Services, USA. A stainless steel bellows vacuum pump/compressor and fittings were purchased from Cole-Parmer (Vernon Hills, IL, USA). A Turbo-Vap II for concentrating samples was purchased from Zymark (Hopkinton, MA, USA). Gel permeation chromatography (GPC) columns for sample clean-up were purchased from Waters (Milford, MA, USA). A Phenogel 10-μm Linear/Mixed guard column 50×7.8 mm for protecting the expensive GPC columns, was purchased from Phenomenex. Fishing nets were purchased locally from a sporting goods store.

2.3. Synthesis of nitro musk metabolites

Musk ketone and musk xylene metabolites (Table

3) were synthesized as previously reported by using hydrazine hydrate as a reducing agent in the presence of a small amount of Raney nickel slurry [15]. Purification and separation of 4-amino musk xylene, 2-amino musk xylene, and 2-amino musk ketone from their respective reaction mixtures was achieved by eluting four preparatory thin-layer chromatography plates (silica gel 60 F_{254}, 2 mm) with a mixture of diethyl ether–light petroleum (60:40, v/v) as described by Zhao and Schwack [16].

2.4. Fish sample collection and preparation

On a monthly basis, for a period of 12 months, eight carp (*Cyprinus carpio*), ca. 2 kg average mass were collected by net from a lake in the southwestern USA, ~200 yards from drinking water intake area of the lake (1 yard=0.9144 m). The fish were transported in a −20 °C cooler to the laboratory (<1 h trip), where they were cleaned with hot tap water and rinsed with DI water. After cleaning, each fish was weighed, measured from mouth to end of tail muscle. The girth was measured at the widest point. Each fish was sectioned with a heavy stainless steel knife and the pieces were ground with a Hobart meat processor which homogenized the fish and produced a fine puree. A sample of the fish, about half of the total homogenate, was transferred into a wide-mouth amber jar and sealed with polypropylene tape before it was stored at −80 °C until extraction. The remaining half was saved in a plastic bag as an archive sample for trace metal analysis. To avoid sample carryover, the stainless steel components of the Hobart meat processing machine were disassembled and washed sequentially with hot tap-water using Iso-clean soap, DI water with 10% acetic acid, and DI water after each fish was processed.

2.5. Closed-loop stripping of synthetic musk compounds from fish tissues

A 25-μl volume of surrogate standard mix (2,6-dinitrotoluene [17], pentachloronitrobenzene [18], and 2,2′-dinitrobiphenyl [19]) at 20 μg/ml was injected into 100 g of homogenized fish tissues in a 1-l Wheaton purge-and-trap vessel. For recovery experiments (see Section 2.7), a 25-μl volume of musk mixture standard was also added to the 100 g fish tissues. Four hundred millilitres of DI water were transferred to the vessel, followed by the addition of 80 ml of 1 *M* sodium hydroxide solution, 50 g sodium sulfate, and a stirrer bar. Fifty millilitres of DI water were added by rinsing the spatula into the purging vessel. The Wheaton vessel was placed on the top of a magnetic stirrer/hotplate with the hotplate turned off and the stirrer turned on. The fish sample was stirred with a stirrer bar, and a pH range of 12.50 to 12.75 was recorded for each sample, using a pH meter (Beckman Coulter model Φ255). A recirculating water bath (Neslab Instruments, model RTE-111 A), set at 50 °C, was allowed to circulate around the vessel through the jacket so that a constant temperature of 50 °C was maintained inside the vessel (Fig. 1). After installing a Nexus cartridge, ultra-pure nitrogen gas was introduced into the system through a stainless steel tee. From a stainless steel bellows vacuum pump/compressor, a 1/8 in. stainless steel tubing was connected to a PTFE tubing which was inserted through the inlet of a Wheaton vessel, and sealed with the aid of an O-ring, to allow for the introduction of nitrogen gas to the sample phase near the bottom of the vessel (1 in.=2.54 cm). To the outlet of the vessel, a short length of 5 mm I.D. flexible PTFE tubing was connected, sealed with the aid of an O-ring, and the end of this tubing was connected to a medical grade polypropylene cartridge containing 6 g of solid-phase material (Abselut Nexus) [14], to allow for the trapping of organic compounds purged out of the saponified fish tissues by a 1.5-l/min flow [10] of nitrogen (maximum capacity of pump). From the gas outlet end of the solid-phase cartridge, a 1/8 in. stainless steel tubing was connected to the recirculating stainless steel bellows vacuum pump/compressor, where bleeding of the system is performed. This set-up allowed the system to recirculate the same nitrogen gas for sparging the aqueous or sample phase, until the operation was terminated after 24 h. The recirculation tubing is rinsed sequentially with 5 ml each of acetone, methanol, and *n*-hexane following each analysis. The versatility of Nexus as adsorbent is exhibited in its high efficiency for

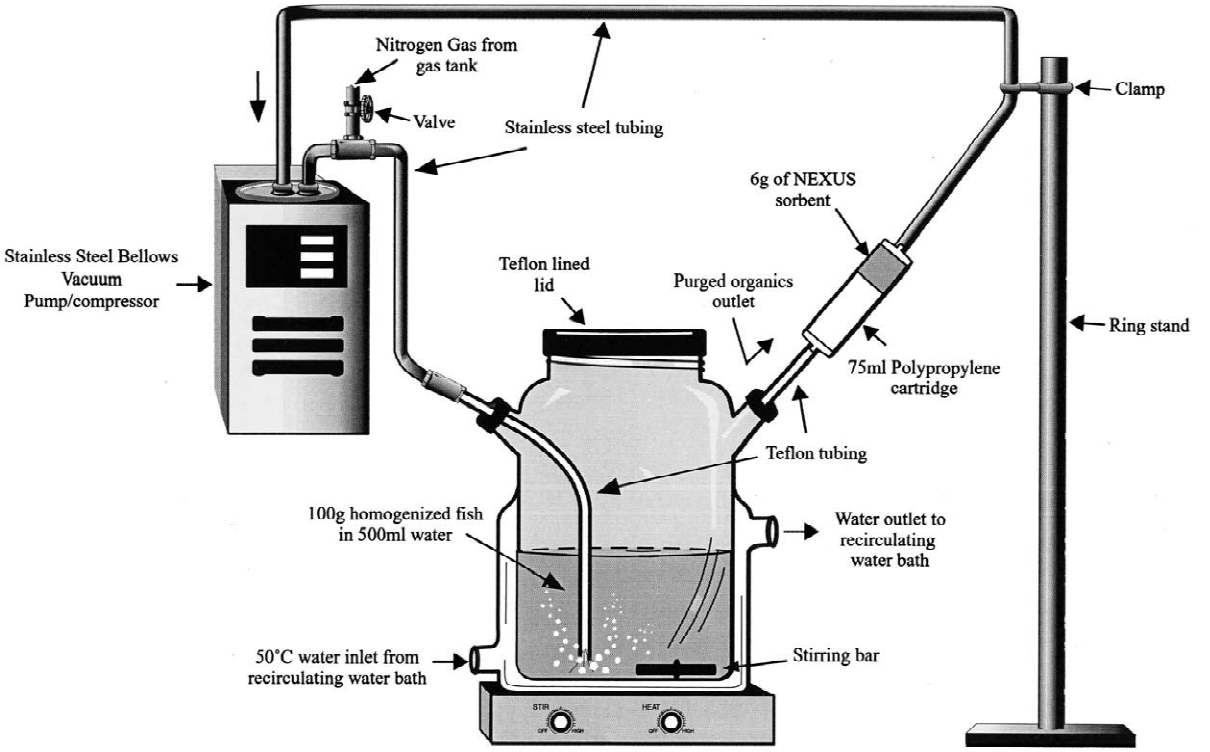


Fig. 1. Closed-loop stripping analysis apparatus.

trapping organic compounds in the aqueous phase [14]. After closed-loop stripping of musk compounds, ~6 g of oil from the saponified 100-g fish sample were suspended on the top of the 500-ml aqueous phase. About half a gram of this oil was cleaned-up with GPC and also analyzed as the other samples. The musk compounds recovered from the 0.5 g of suspended fish oil were below minimum detection limits. However, the concentration of musk compounds will be greater than the method minimum detection limits if all 6 g of oil were analyzed. To confirm the type of oil/lipid suspended on the surface, the oil was then analyzed using a Nicolet model 210 Fourier transform infrared spectrometer. The system was nitrogen purged, background subtracted, and the sample was placed between two NaCl plates and signals were collected at 35 scans/s. The presence of a carbonyl group (1750 cm^{-1}) in the IR spectrum indicated that the oil was a glycerol ester of fatty acids, and the result of incomplete saponification of the fish tissues.

2.6. Solid-phase desorption

The cartridge (Abselut Nexus) containing the trapped organic compounds was detached from the apparatus and the analytes from the sorbents in the cartridge were eluted with successive portions of 25 ml of ethyl acetate and 25 ml of *n*-hexane (approximately five bed volumes of each solvent) using a 12-position Visiprep-DL solid-phase extraction vacuum manifold (Supelco) connected to a GAST vacuum pump (Gast Manufacturing, Benton Harbor, MI, USA). After desorption, 0.5 ml of toluene was added to the eluate as a keeper solvent. The 50-ml eluent was concentrated to a 300 μl volume using a Turbo-Vap II solvent evaporator at 35 °C, under a gentle stream of nitrogen. The 300 μl

Table 4
Concentration and percent spike recoveries of musk compounds in PLE and CLSA of fish tissues

Analyte recovery[a]	PLE analyte conc. (ng/g) (RSD, %)	PLE % spike recovery[b]	CLSA analyte (RSD, %)	CLSA % spike conc. (ng/g)
Musk xylene	2.6	93(5)	1.0	14(3)
Musk ketone	20.1	101(6)	3.0	11(1)
Musk ambrette	<MDL	97(10)	<MDL	15(5)
Musk tibetene	<MDL	102(4)	<MDL	13(3)
Musk moskene	<MDL	94(5)	<MDL	18(6)
Versalide	<MDL	97(1)	<MDL	32(2)
Galaxolide	24.2	96(3)	6.0	23(1)
Phantolide	8.9	110(2)	3.0	28(2)
Cashmeran	<MDL	88(40)	<MDL	29(3)
Celestolide	4.5	96(3)	2.0	32(3)
Traseolide	<MDL	106(5)	<MDL	29(2)
Tonalide	17.8	101(4)	6.0	32(2)
4-Amino musk xylene	6.7	107(6)	1.0	7(5)
2-Amino musk xylene	52.4	92(6)	4.0	6(4)
Amino musk ketone	8.2	104(8)	<MDL	2(2)

[a] Percent recovery of musk compounds from saponified 100 g homogenized whole fish tissues (wet mass) in salted DI water ($n=3$).
[b] Percent recovery of musk compounds from 2.44 g homogenized whole fish tissues (wet mass) in salted DI water ($n=3$).

extract was meticulously concentrated to a little less than 100 μl under a gentle stream of nitrogen, using a Pierce evaporating unit model 18780 (Pierce Reacti-Vap, Rockford, IL, USA). [$^2H_8$]Naphthalene [20], the internal standard, was purchased at a concentration of 200 μg/ml in toluene and 2.5 μl of this solution were added to the extract using a 10-μl gas-tight Hamilton digital syringe. A final extract volume was adjusted to 100 μl and analyzed by GC–MS.

2.7. CLSA recovery experiment

To determine analyte recoveries prior to fish sample analysis, three samples weighing 100 g, 104 g, and 103 g of fish tissues were each placed in a 1-l Wheaton purge-and-trap vessel and were spiked with 25-μl portions of a solution containing 20 μg/ml of each synthetic musk compound and three musk metabolites. The standard was mixed with the fish, using a spatula. Five hundred millilitres of DI water were added to the purge and trap vessel while rinsing the spatula into the vessel. The sample matrix was saponified and purged as described in Section 2.5. The three spiked samples provided the average recoveries for each analyte that are presented in Table 4.

2.8. Sample extraction using the PLE system

An average fish tissue mass of 2.3 g was transferred into a smooth surface mortar that was silanized with DMDCS in toluene. To the fish sample in the mortar, 2.5 μl of a 200 μg/ml concentration of each of the three surrogate standards were added. Eight samples from the same homogenized fish tissue were similarly spiked with the surrogate standards mixture and a 25 μl volume of the musk mixture standard. The musk standard contained 15 musk compounds at a concentration of 20 μg/ml. To each fish sample in the mortar, ~5 g of diatomaceous earth hydromatrix were added and the mixture was homogenized using a silanized pestle until a free flowing powder was achieved.

The extraction of fish samples by PLE has been detailed elsewhere by Draisci et al. [21]. Briefly, a cellulose filter (Dionex) was inserted at the bottom of a 33-ml PLE stainless steel extraction cell, and the homogenate was transferred into the extraction cell using a PTFE-lined funnel. The extraction cell was tapped gently to reduce the air pockets inside the

Table 5
PLE extraction

Cell volume	33 ml
Extraction solvent	Ethyl acetate–*n*-hexane (1:5, v/v)
Temperature	80 °C
Pressure	2000 p.s.i. (1 p.s.i = 6894.76 Pa)
Heat time	5 min
Static time	15 min
Flush volume	100%
Purge time	90 s
Static cycle	2

stainless steel. Another cellulose filter was placed on the top of the sample inside the extraction cell. The remaining space was filled with about 6 g deactivated alumina (Basic, Brockman activity 1). Prior to use, the alumina was activated at 480 °C overnight in a muffle furnace and deactivated with 15% (w/w) water upon achieving room temperature. The stainless steel extraction cells were loaded into the PLE system and extraction was performed under the PLE conditions in Table 5, using pre-mixed solvents, *n*-hexane–ethyl acetate (1:5, v/v), in a PLE reservoir as previously described [21]. While this procedure reduced the lipid content, it did not sufficiently eliminate lipids. This was reflected by the high concentration of lipids removed from the samples during GPC clean-up (see Fig. 2). Each 60-ml PLE extract collected in an amber vial was concentrated to 300 μl using a Turbo Vap II solvent evaporator at 35 °C under a gentle stream of nitrogen. To each

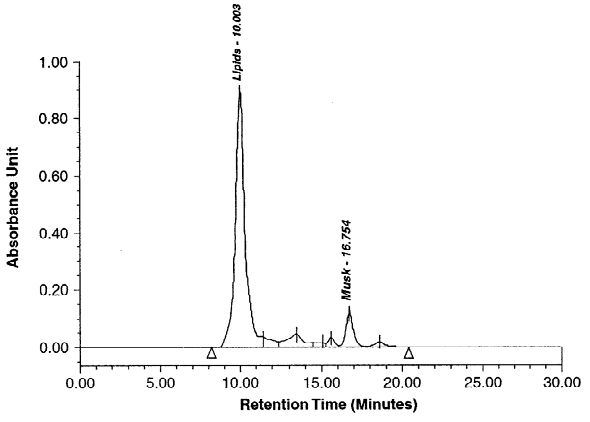


Fig. 2. Gel permeation chromatography showing residual lipids after alumina semi-clean-up of 2.09 g fish sample using PLE.

extract, 2 ml of methylene chloride were added and the volume concentrated to 1 ml for GPC. The solvent exchange to methylene chloride was performed according to the recommendation of the GPC manufacturer.

2.9. Sample clean-up: GPC

In spite of the alumina pre-cleaned procedure performed during fish extraction by PLE, there was a high lipid level in the PLE extracts (Fig. 2). Therefore, purification of the extracts required the use of GPC to eliminate residual lipids from all fish samples. A Waters GPC system was used, equipped with a 515 HPLC pump, a 717 Plus Autosampler, a 2487 dual UV detector, and a Fraction Collector II. The GPC system was fitted with two Envirogel columns in series (300 mm×19 mm and 150 mm×19 mm), preceded by a Phenogel 10-μm Linear/Mixed, 50× 7.8 mm guard column. Prior to sample clean-up, the columns were conditioned with 0.5 l of methylene chloride. To establish collection windows, the instrument was calibrated with a solution containing 1 μg/ml of each of the five nitro musks, seven polycyclic musks, three nitro musk metabolites (Tables 1–3), and three surrogate standards. The HPLC system was operated isocratically with a flow-rate of 5 ml/min. Methylene chloride was used as the mobile phase solvent. All 18 compounds eluted from the columns between 12.5 and 20 min, resulting in a 40-ml collection volume. To the collected volume, 1 ml *n*-hexane was added as a ''keeper'' solvent. This fraction was evaporated to ~1 ml in *n*-hexane for clean-up, using the amino cartridge.

2.10. Amino cartridge treatment

In order to further reduce background interference in the PLE samples, 1 g of 3-aminopropyltriethoxylsilane-treated solid-phase in a 6-ml polypropylene cartridge was used (Strata NH_2). Each Strata NH_2 cartridge was mounted in a Supelco 12-position Visiprep-DL solid-phase extraction vacuum manifold, 1 g alumina (Basic Brockman activity 1) was added, and the cartridge was conditioned sequentially with 5 ml methylene chloride and 5 ml *n*-hexane with a few drops of 2,2,4-trimethylpentane, making

certain that the solvent meniscus was just above the solid-phase within the cartridge. The 1-ml extract in *n*-hexane was quantitatively transferred (using *n*-hexane) into each conditioned Strata NH_2 cartridge and eluted with 8 ml methylene chloride. Approximately 0.5 ml toluene was used to rinse the tube and added to the extract as keeper solvent. Analytes were concentrated as previously described in Section 2.6. The extract volume was adjusted to a little less than 100 μl after which 2.5 μl of [$^2H_8$]naphthalene, the internal standard, were added using a 10-μl gas-tight Hamilton digital syringe. The final extract volume of 100 μl was analyzed by GC–MS, in the selected multiple-ion monitoring mode.

2.11. GC–MS-selected ion monitoring (SIM) analysis

Analytes were quantified relative to [$^2H_8$]naphthalene, using GC–electron impact ionization (EI) MS in the selected ion monitoring mode. Data were acquired with a HP 6890 gas chromatograph equipped with a HP 7683 series automatic sampler coupled to a HP 5973 mass spectrometer (Agilent Technologies, San José, CA, USA), which was operated under the following conditions: EI at 70 eV and 3.94 scans/s were recorded. The transfer line from the GC system to the MS system was maintained at 300 °C, the mass analyzer at 150 °C, and the ion source at 230 °C. Baseline separation between most of the compounds was achieved on a 30 m×0.25 mm I.D., 0.25-μm film thickness, HP-5 MS capillary column (Agilent Technologies) using the following temperature program: 90 °C, 0 min hold, 10 °C/min to 300 °C, and 10 min hold. Helium was the carrier gas with a linear velocity of 37 cm/s measured at 90 °C. Injections of 2 μl were made in the splitless mode using a splitless time of 1 min and an injection port temperature of 250 °C. The ions used for quantitation, identification, and SIM groups are shown in Table 6.

Based on a preliminary analysis of the PLE extracts, a five-level calibration standard mixture was prepared at concentrations of 0.02, 0.05, 0.1, 0.5, and 1.0 ng/μl of musk compounds in toluene, the same solvent used to reconstitute the analytes. Due to the

Table 6
Characteristic ions used for identification and measurement of musk compounds

Compound	Retention time (min)	Quantitation ion	Identification ions	SIM groups
[$^2H_8$]Naphthalene (I.S.)	4.84	136	135, 137	1
2,6-Dinitrotoluene	7.98	165	89, 63	
Cashmeran	8.48	191	192, 206	
Celestolide	10.91	229	244, 173	
Phantolide	11.37	229	244, 187	
Pentachloronitrobenzene (Surr.)	11.55	237	295, 214	
Versalide	11.85	243	244, 258	
Musk ambrette	12.16	253	268, 254	2
Traseolide	12.30	215	216, 173	
Galaxolide	12.35	243	258, 213	
Tonalide	12.45	243	258, 201	
Musk xylene	12.45	282	297, 283	
Musk moskene	12.70	263	278, 264	
Musk tibetene	13.24	251	266, 252	3
Musk ketone	13.64	279	294, 280	
2,2′-Dinitrobiphenyl (Surr.)	14.44	198	168, 139	
2-Amino musk xylene	14.85	267	252, 160	4
Amino musk ketone	15.00	264	249, 215	
4-Amino musk xylene	15.42	252	267, 218	

I.S., internal standard; Surr., surrogate.

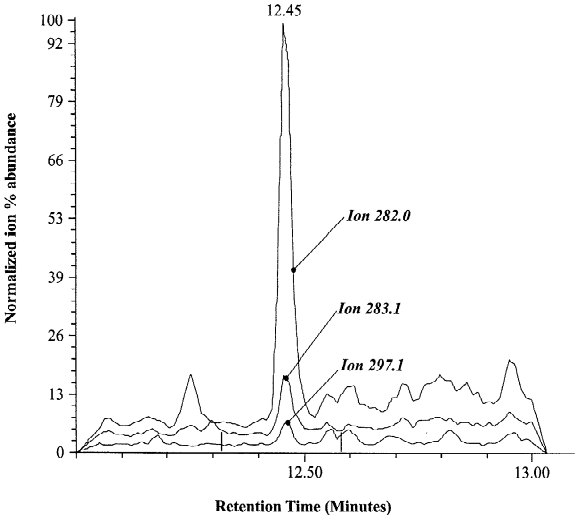


Fig. 3. Positive identification of musk xylene using ion chromatograms at m/z 282.0, 283.1, 297.1, and retention time.

varying concentrations of musk compounds in the extract, another five-level calibration standard mixture was prepared at concentrations of 0.1, 0.5, 1.0, 2.0, and 5.0 ng/μl for the quantification of CLSA and PLE extracts.

Quantitation was performed by the data system software (HP-Chem acquisition software) using the primary ions indicated in Table 6. The EPA test method for evaluating solid waste (Method 3562) was used to calculate the concentrations of each identified analyte in the sample [22]. All compounds found were identified through comparison of their selected ion intensity ratio relative to their base peak for the molecular ion (Fig. 3), or diagnostic fragments for each analyte, and GC retention times of authentic standards. The calibration curves, used for quantification purposes, provided correlation coefficients (r^2) greater than 0.9998 for all synthetic musk compounds. The total ion chromatograms of fish extracts using CLSA and PLE are shown in Figs. 4 and 5.

3. Results and discussion

As a control experiment for CLSA, 500 ml of deionized water were spiked with 25-μl portions of a solution containing 20 μg/ml of each synthetic musk compound and three musk metabolites in methanol. Approximately 50 g of baked (400 °C overnight)

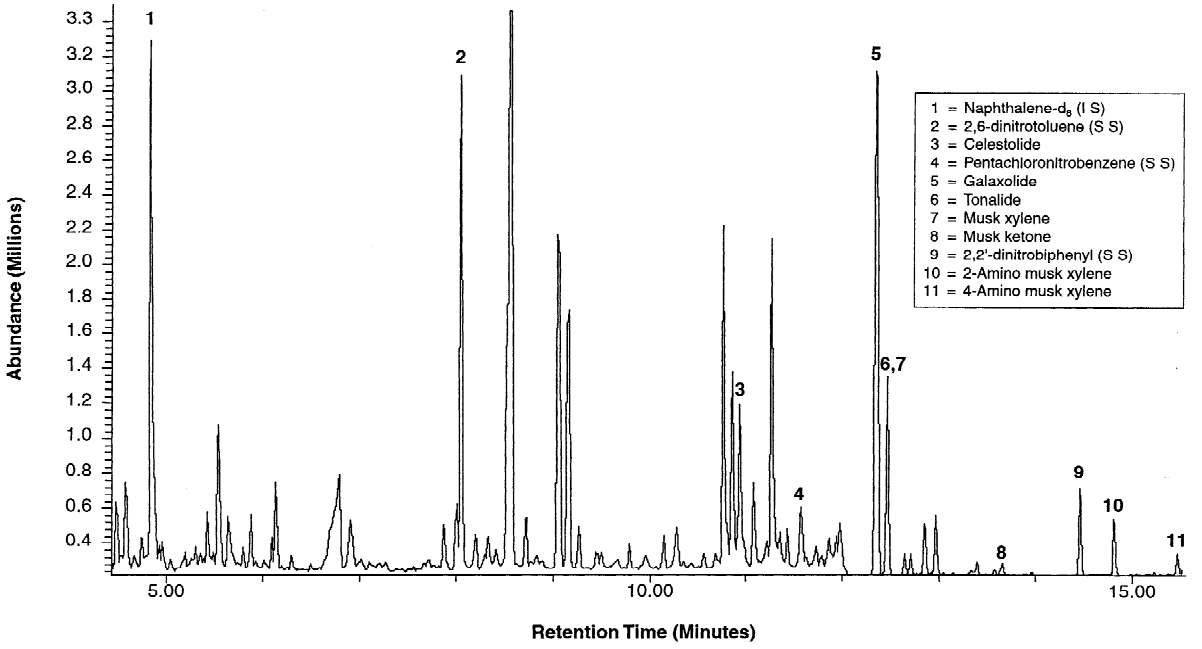


Fig. 4. CLSA–GC–MS chromatogram for synthetic musk compounds in unspiked 100 g fish sample (*Cyprinus carpio*).

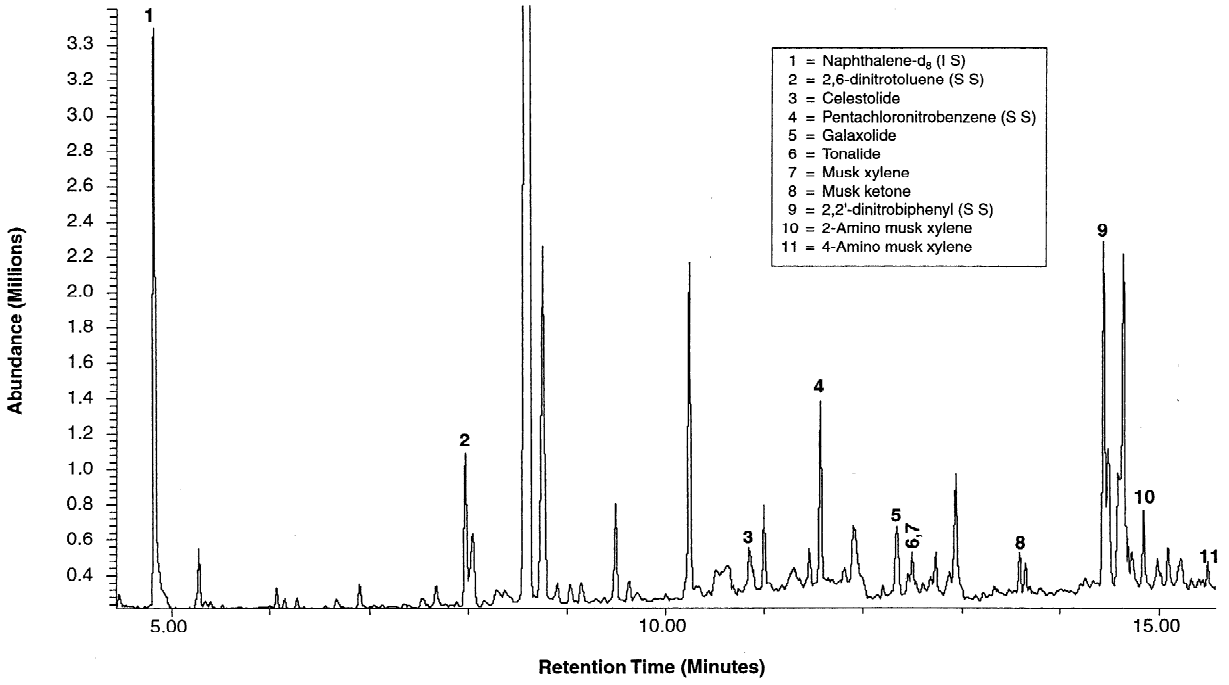


Fig. 5. PLE–GC–MS chromatogram for synthetic musk compounds in unspiked 2.09 g fish sample (*Cyprinus carpio*).

Table 7
Percent recoveries of musk compounds from CLSA of spiked salted DI water ($n=1$)

Analytes	Recovery (%)[a], salted water with base	Recovery (%)[b], salted water without base
Musk xylene	32.6	31.2
Musk ketone	55.6	31.8
Musk ambrette	34.8	37.0
Musk tibetene	39.2	27.8
Musk moskene	30.8	28.2
Versalide	38.6	31.2
Galaxolide	36.8	27.6
Phantolide	40.0	33.0
Cashmeran	40.0	40.0
Celestolide	36.0	31.4
Traseolide	51.4	34.8
Tonalide	44.8	29.8
4-Amino musk xylene	2.8	2.8
2-Amino musk xylene	7.8	16.2
Amino musk ketone	0.2	0.2

[a] Percent analyte recoveries from 500 ml of salted DI water, spiked with 0.5 μg musk compounds with NaOH.

[b] Percent analyte recoveries from 500 ml of salted DI water, spiked with 0.5 μg musk compounds without NaOH.

sodium sulfate were added and allowed to completely dissolve in the 500 ml DI water by shaking vigorously. The solution was subjected to closed-loop stripping for 24 h at 50 °C. The Nexus cartridge was detached and desorbed as previously described in Section 2.6. To ascertain that the base does not affect the analytes of interest, a similar analysis of DI water was also performed with NaOH added to the DI water to obtain a pH of ca. 12.7. The percent of musk compounds recovered from the closed-loop stripping of synthetic musk compounds in salted DI water with and without NaOH, was the same (Table 7). This shows that the addition of NaOH does not affect the recoveries of analytes of interest. However, the analyte recoveries from DI water were higher than those recovered from the saponification of fish tissues. In both cases, this not only suggests that the differences in the Henry's law constants for the individual compounds play a greater role in the recoveries of musk compounds, it also suggests the sorption of musk compounds on solid tissue residue.

3.1. Minimum detection limits

As shown by Curvers et al. [23] in their detailed studies of closed-loop stripping of various organic substances from aqueous solutions, a detection limit in the region of parts per trillion and enrichment factors of up to 5000 can easily be obtained for low polarity compounds with boiling points up to 350 °C. The method detection limits (MDLs) for these analytes were estimated based on a signal-to-noise ratio of at least 3 to 1 ($S/N = 3$) as described by Kaiser [24], and Deming and Morgan [25] using a minimum of two selected ions. In the CLSA method, the MDLs ranged from 0.050 to 0.1 ng/kg (wet mass). The MDLs for synthetic musk compounds extracted with the PLE method ranged from 0.45 to 2.0 ng/kg (wet weight).

3.2. Method optimization

In order to maximize the analytes' concentration in the gaseous phase, the temperature at which saponification was carried out was varied, the sample matrix was agitated by the purging gas as well as the stirrer bar to expose most of the sample surface area to nitrogen gas for efficient analyte transfer into the gaseous phase. Initially, 30 °C was used, followed by 40 °C, 50 °C, and finally 90 °C for 24 h. The analyte recoveries and the magnitude of the background signals were used to determine the optimum temperature for the CLSA. Time was also considered as one of the optimizing variables. During the preliminary analysis of synthetic musk compounds, a purging period of 2 h was found to be inadequate, while 48 h introduced higher backgrounds. A purging period of 24 h was used instead.

Due to the unfavorable Henry's law constants of the amino musk compounds, recovery of these compounds from the saponified solution was very low. The low recovery may also be attributed to sorption of musk compounds on solid tissue residue. The salting out effect as described elsewhere [26] modifies the solubility and related properties of organic compounds in the water. The solubility of the amino compounds was modified by the addition of 50 g sodium sulfate salt to the solution at 50 °C. This resulted in the recovery of 0.2 to 16.2% (Table 7) and 0.2 to 7.8% (Table 7) of the amino musk compounds in salted DI water, and saponified fish tissues, respectively. The amines were not previously detected before the addition of salt. An added benefit of the high concentrations of sodium sulfate and sodium hydroxide in the aqueous phase is that they help check microbial degradation of the homogenate.

3.3. Concentrations of synthetic musk compounds in fish

Examination of the data in Table 4 shows that the percent recoveries of the polycyclic musk compounds in fish using CLSA is on the average, a factor of three lower than that of PLE and sevenfold lower for the heavier nitro musks. The amino musks were barely recovered due to their high solubility in the aqueous phase. The concentration of musk compounds stripped off the saponified fish tissue is a function of the initial concentration of musk compounds in the fish tissues, aqueous phase temperature, and the flow-rate of the stripping gas. As the stripping time and the temperature of the aqueous phase increases, the concentration of analytes recovered is enhanced. The concentration enhancement by the above variables is only limited by the increase in background level. A saponification temperature of 90 °C introduced lipids and higher background level. However, after CLSA extract clean-up, some of the analytes recovered were greater than 76%. Since we were not interested in such a clean-up procedure, a saponification temperature higher than 50 °C was not used.

4. Summary and conclusion

As far as we know, this is the first time Abselut Nexus sorbent has been used as a trap for fragrance materials from saponified fish tissues. CLSA of saponified fish tissues is a simple, rugged, highly reproducible, and relatively inexpensive analytical technique that concentrates synthetic musk compounds from saponified fish tissues into a sorbent bed. Extracts obtained by closed-loop stripping are ideally suited for subsequent gas chromatographic analysis, without further clean-up.

The limiting factor in this method is the high mass of tissues needed for saponification, and the leakage

in the closed-loop connection adaptors, if the adaptors are not properly fitted or tightened. The presence of moisture in the adsorbent cartridge does not reduce the activity of the sorbent. In a previous report, Nexus was shown to effectively strip organic compounds from water [19]. Since musk compounds are mostly hydrophobic, the destruction of fats and protein by a strong base renders synthetic musks amenable to closed-loop stripping. Indeed, closed-loop stripping is a very promising tool for the analysis of these compounds. Since some analytes, as suggested by their small Henry's law constants, require longer stripping periods than others, the recovery of some synthetic musk compounds using this method will be expected to be low. This drawback may be rectified by appropriate recovery calibration. When deuterated standards are used with isotope dilution for recovery correction, CLSA will be a faster and easier technique for analyzing semi-volatile compounds such as synthetic musk compounds and base neutrals. However, perdeuterated synthetic musk compounds are not readily available. Based on the results presented in Table 4, it is clear that CLSA provides a valuable cost-saving semi-quantitative tool for regular bio-monitoring of aquatic species for investigating sewage water contamination.

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References

[1] G. Rimkus, B. Rimkus, Chemosphere 28 (1994) 421.
[2] G.G. Rimkus, M. Wolf, Chemosphere 30 (1995) 641.
[3] G.G. Rimkus, M. Wolf, Chemosphere 33 (1996) 2033.
[4] S.L. Simonich, W.M. Begley, G. Debaere, W.S. Eckoff, Environ. Sci. Technol. 34 (2000) 959.
[5] T. Yamagishi, T. Miyazaki, S. Horii, K. Akiyama, Arch. Environ. Contam. Toxicol. 12 (1983) 83.
[6] T. Yamagishi, T. Miyazaki, S. Horii, S. Kaneko, Bull. Environ. Contam. Toxicol. 26 (1981) 656.
[7] D.R. Branson, G.E. Blau, H.C. Alexander, W.B. Neely, Trans. Am. Fisheries Soc. 4 (1975) 785.
[8] W. Lyman, W. Reehl, D. Rosenblatt, Handbook of Chemical Property Estimation Methods, American Chemical Society, Washington, DC, 1990.
[9] US Environmental Protection Agency (EPA), EPI Suite™ Developed by EPA's Office of Pollution Toxics and Syracuse Research Corporation (SRC), 2000.
[10] K. Grob, F. Zurcher, J. Chromatogr. 117 (1976) 285.
[11] M.J. McGuire, S.W. Krasner, C.J. Hwang, G. Izaguirre, in: Presented at the 8th Annual American Water Works Association Water Quality Technology Conference, Miami Beach, FL, 1980.
[12] G.V. Santos, M.C. Veloso, P.A. Pereira, J.B. Andrade, Am. Lab. 33 (2001) 28.
[13] M. Retzik, R. Cerra, Am. Lab. 34 (2002) 18.
[14] L.I. Osemwengie, S. Steinberg, J. Chromatogr. A 932 (2001) 107.
[15] R. Gatermann, H. Huhnerfuss, G. Rimkus, A. Attar, A. Kettrup, Chemosphere 36 (1998) 2535.
[16] X. Zhao, W. Schwack, Chemosphere 39 (1999) 11.
[17] J. Nair, H. Ohshima, C. Malaveille, M. Friesen, I.K. O'Neil, A. Hautefeuille, H. Bartsch, Food Chem. Toxicol. 24 (1986) 27.
[18] R. Suter-Eichenberger, H. Altorforfer, W. Lichtensteiger, M. Schlumpf, Chemosphere 36 (1998) 2747.
[19] W. Butte, S. Schmidt, A. Schmidt, Chemosphere 38 (1999) 1287.
[20] K. Bester, H. Hühnerfuss, W. Lange, G.G. Rimkus, W. Theobald, Water Res. 32 (1998) 1857.
[21] R. Draisci, C. Marchiafava, E. Ferretti, L. Palleschi, G. Catellani, A. Anastasio, J. Chromatogr. A 814 (1998) 187.
[22] Supercritical Fluid Extraction of Polychlorinated Biphenyls (PCBs) and Organochlorine Pesticides, Test Methods for Evaluating Solid Waste, Method 3562, US Environmental Protection Agency, Washington, DC, 1996.

[23] J. Curvers, T. Noy, C. Cramers, J. Rijks, J. Chromatogr. 289 (1984) 171.
[24] H. Kaiser, Two Papers on the Limit of Detection of a Complete Analytical Procedure, Hafner, New York, 1969.
[25] S.N. Deming, L.S. Morgan, Statistical Analysis of Laboratory Data, University of Houston, Texas, 1995, www.statisticaldesigns.com.
[26] X. Wen-Hui, S. Wan-Ying, M. Donald, Marine Environ. Res. 44 (1997) 429.