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Gas chromatography–mass spectrometry analysis of alkylphenols in cod (*Gadus morhua*) tissues as pentafluorobenzoate derivatives

Sonnich Meier*, Jarle Klungsøyr, Stepan Boitsov, Torunn Eide, Asbjørn Svardal

Institute of Marine Research, P.O. Box 1870 Nordnes, 5817 Bergen, Norway

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Abstract

A highly selective and sensitive method for the determination of 30 *meta*- and *para*-substituted alkylphenols from phenol (C_0) to nonylphenol (C_9) in biota is described. Dichloromethane extracts of spiked cod liver and muscle samples are cleaned up by gel permeation chromatography, derivatised with pentafluorobenzoyl chloride and analysed by gas chromatography–mass spectrometry with negative-ion chemical ionisation. Quantification is done with isotope dilution of five internal standards of different chain length. The detection limits were in the low μg/kg levels. There were encountered problems with background levels of 4-nonylphenol. 4-Nonylphenol isomers were found in a number of plastic and rubber products used in the laboratory.

Keywords: Alkylphenols; Pentafluorobenzoyl; Isotope-dilution method; Cod

1. Introduction

Alkylphenols are some of the most intensively studied substances that have hormone-disrupting effects. Alkylphenols bind to and affect the oestrogen receptors in the same way as 17β-oestradiol, but the response is much weaker [1–4].

An in vitro study showed that oestrogenic effects of alkylphenols depend on both the position (*para* > *meta* > *ortho*) and branching (tertiary > secondary = primary) of the alkyl group. Maximum activity (1000–6000 times less potent than oestradiol) has been found for C_6–C_8 *para*-substituted tertiary alkylphenols, but C_5-, C_4- and C_3-alkylphenols are also "oestrogenic" (10^5–10^7 times less potent than oestradiol) [5].

Virtually all research in this field has dealt with the two long-chain alkylphenols, nonylphenol (C_9) and octylphenol (C_8). These are degradation products of the non-ionic surfactants known as alkylphenol ethoxylates (APEs), which consist of an alkylphenol group, principally nonylphenol (82%), but which also contain octylphenol or dodecylphenol (C_{12}) that are coupled to long ethylene oxide chains [6].

APEs are and have been utilised in a large number of products, including herbicides, paints and industrial cleaning and degreasing agents [7]. APEs form one of the most widely used groups of surfactants in the world, with an annual production of around 500×10^6 kg [8]. In Norway, the use of APEs has been very limited, and has fallen significantly during the 1990s, from 615×10^3 kg in 1995 to 113×10^3 kg in 2000 [9]. A large proportion of the degradation products of APEs end up in the aquatic environment, and nonylphenol and octylphenol have thus been found in a large number of freshwater systems and coastal marine areas all over the world, in concentrations of up to 369 μg/l in particularly highly polluted areas, but typically with values in the low μg/l range (reviewed in [10]).

The use of nonylphenol, octylphenol and their ethoxylates has been forbidden in Norway since January 2002. A number of other European countries are also planning to forbid the use of these substances, which are on the Oslo–Paris Commission's (OSPAR) list of chemicals that ought to be phased out [11].

* Corresponding author. Fax: +47 55238584.
E-mail address: sonnich.meier@imr.no (S. Meier).

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Historically, large quantities of APEs have been used in offshore petroleum production [11]. The use of APEs is now forbidden in the Norwegian sector of the North Sea. Danish and UK authorities are also working on phasing out APEs in their sectors of the North Sea [12]. However, even though the environmental threat from alkylphenols will disappear with the phasing out of APEs, the problems associated with discharges of long-chain alkylphenols from petroleum production will remain.

In addition to being degradation products of APEs, alkylphenols are also a natural component of crude oil [13–15], and as a result of their solubility in water a high proportion will be found in the aqueous phase after water–oil separation and thereby discharged into the sea with the produced water. The alkylphenols are typically found in concentrations of 0.6–10.0 mg/l in produced water. Some 80% of the total consists of the most water-soluble alkylphenols, phenol and cresol (C_1). Of the remaining components, the higher alkylphenols from butyl- to heptylphenols occur in low concentrations of 2–250 μg/l [16–20].

We know very little about the fate of alkylphenols in the marine offshore environment. Kannan et al. [21] traced very low levels of nonylphenol in the sea off Japan (0.002–0.093 ng/l), while a measurement from the North Sea (German Bight) [22] found nonylphenol concentrations in seawater of between 0.7 and 4.4 ng/l and up to 13 μg/kg in offshore sediment samples taken more than 100 km from land. Phenol and alkylphenols (sum C_1–C_4) are found in the sea around offshore installations in the North Sea at concentrations up to 486 ng/l and 140 ng/l, respectively [23]. There is a great need for more knowledge of the effects on the marine environment of discharges into the sea from offshore oil and gas industry, the fate of alkylphenols being of special interest.

The present method has been developed to investigate whether or not fish living around oil installations contains alkylphenols. Our demands to the method include:

(i) Applicability to alkylphenols of different chain lengths, from phenol (C_0) to nonylphenol (C_9).
(ii) Applicability to alkylphenols from both very high (e.g. cod liver, 70% lipid) and low (e.g. cod muscles, 1% lipid) lipid containing samples.
(iii) High sensitivity. Detection limit at low μg/kg level.
(iv) Low total detection limit. The method has to be applicable to analysis of alkylphenols in very small samples (e.g. cod brain, >0.7 g).

Alkylphenols can be analysed with a great variety of chromatographic and electrophoretic methods (reviewed in [24–26]). Most of the methods have been developed for water, sediment and sewage effluent samples. However, the number of studies done on biota has been increasing in the last years. The complexity of alkylphenol analysis of biota samples is clearly reflected by the amount of different approaches employed in all the steps (extraction, clean-up of the extracts, analysis) of the analysis procedure [27–49].

Ahel et al. [50] used a special apparatus developed by Veith and Kiwus [51] for steam-distillation and solvent extraction of alkylphenols in water and sediment samples. This method has also been adapted in various ways for analysis of alkylphenols in biota [28,33,40–42,48]. Other extraction methods are direct solvent extraction with different solvents: dichloromethane (DCM) [27,39], acetonitrile [35,46], methyl *tert*.-butyl ether [29] and acetone–hexane [27,52]); soxhlet extraction with DCM [31,37,43,44]; microwave-assisted solvent extraction (DCM–MeOH) [34]; pressurised fluid extraction [45,47]; matrix solid-phase dispersion [36,38].

Biological samples have a very complex matrix containing a high amount of lipids, proteins, etc. Therefore, purification of the extracts is as a rule necessary. Several different procedures of samples clean-up have been used: preparative reversed-phase HPLC [41], normal-phase HPLC [40,42]; solid-phase extraction (SPE)-NH_2 [34,44,45] or SPE-silica [46]; alumina column [31,33]; silica gel column [37,39]; Florisil column [47]; gel permeation chromatography (GPC) [37,43,53], liquid–liquid partition (acetonitrile–hexane) followed by Florisil column clean-up [35,54]; liquid–liquid partition (acetonitrile–sodium hydroxide) [27,52].

The analysis or detection of alkylphenols in biota have been done by: liquid chromatography with UV detection [28,44], fluorescence detection [36,43,54] and LC–MS [34,46,47]; gas chromatography of underivatised alkylphenols with GC–flame ionization detection (FID) [33], GC–MS [electron impact ionisation (EI)] [29,31,32,35,40–42,52] or with derivatisation to pentafluorobenzoates before GC–electron capture detection (ECD), GC–EI-MS, or GC–MS [negative-ion chemical ionisation (NCI)] [27], pentafluorobenzylation [39,49] or acetylation followed by GC–EI-MS [37].

In the present method the intention was to analyse as many of the individual isomers of alkylphenols as possible. Therefore, gas chromatography was chosen because of the high resolution power. Alkylphenols contain an “active” hydrogen atom and can be converted to nonpolar compounds before GC-analysis for improving their chromatographic performance. Of the many different derivatisation techniques, such as alkylation, arylation, acylation or silylation of alkylphenols, pentafluorobenzoyl derivatisation was chosen since it is an established method for derivatisation of alkylphenols [27,55–62] and it has been found to be the most simple and sensitive method of halogenoacyl derivatisation for GC–ECD [55,56]. GC–ECD was initially intended to be used due to the high sensitivity of this technique. Two different procedures were studied and it was found that an extractive derivatisation with pentafluorobenzoyl chloride in a two phase system ($NaOH/NaHCO_3$ buffer and hexane) gave the best results for our method.

The GC–ECD detection was highly sensitive when analysing standard samples, but the problem with matrix effects in real samples (complex samples such as produced water from oil platforms and biota like cod liver samples) led to the change of the detection technique to GC–MS. Similar

problems with GC–ECD are discussed in [63]. The complexity of alkylphenol composition of oil-related samples [13,15] also made it difficult to choose good internal standards for GC–ECD. Changing to GC–MS made it possible to use isotope-dilution method.

The fragmentation pattern for negative chemical ionisation mode is very simple, the molecular ion is completely dominating and there are only few fragmentation peaks. The NCI analysis has the same high detection selectivity as GC–ECD because only halogenated or other electrophile compounds give rise to high amounts of negative ions. Together with the selection by the selected-ion monitoring (SIM) technique this makes the NCI analysis of pentafluorobenzoyl derivatives an extremely sensitive and selective method, resulting in a detection limit at the low μg/kg when analysing alkylphenols in cod tissue samples.

2. Experimental

2.1. Chemicals and reagents

Pure standards (98–99%) of 44 alkylphenols were from Sigma–Aldrich (Oslo, Norway). Deuterium-labelled alkylphenols {phenol-d_5, *p*-cresol-d_8, 2,4-dimethylphenol-d_3, 4-*n*-propylphenol-d_{12} and 4-*n*-nonylphenol-d_5} were received from C/D/N Isotopes (USA). A standard validation solution of 44 alkylphenols and the internal standard (five deuterium-labelled alkylphenols) were prepared in methanol. All the alkylphenols purchased and used in standard solution are listed in Table 1 in italics. For the GPC elution test the alkylphenols [14 alkylphenols (C_0–C_8)] were dissolved in toluene.

Recovery internal standard (RIS) pentafluorobenzophenone and the derivatisation reagent pentafluorobenzoyl chloride (PFBCI) were from Sigma–Aldrich (Oslo, Norway). All solvents (hexane, toluene, dichloromethane, methanol) were from Merck (Oslo, Norway). All of them were pesticide grade. Standards of pentafluorobenzoyl derivatives of 4-*n*-propylphenol, 4-isopropyl-3-methylphenol and 4-*n*-nonylphenol were synthesised in the laboratory. The purity was controlled by GC–FID to be >99%. Sodium hydroxide (NaOH), sodium hydrogencarbonate ($NaHCO_3$) and sodium sulphate (Na_2SO_4) were purchased from Merck (Oslo, Norway). The water used was purified using Nanopure Ultrapure Water Systems (USA). All glass equipment was washed and heated to 400 °C for 24 h before use.

2.2. Instrumentation

The pentafluorobenzoate derivatives were analysed using GC–ECD and GC–NCI-MS. Fatty acids methyl esters were analysed by GC–FID. Table 2 gives the different instrumental conditions.

2.3. Sample preparation

Samples were dissected by a scalpel immediately after killing the fish and frozen at once in liquid nitrogen. The samples were kept at −80 °C until the analyses were carried out.

Before extraction, samples like muscle, gonad and entrails were thawed and homogenised in Waring commercial blender. Samples with high lipid contents like cod liver (up to 70% lipid) were taken directly from the −80 °C freezer and weighed frozen in the test tubes (these samples give phase separation when thawed and it is then difficult to get homogeneous samples). Small samples like brain, heart and kidney (<1 g) were also weighed directly into the test tubes.

2.4. Liquid extraction

One gram of tissue was weighed into 25 ml test tubes. The samples were spiked with 100 μl methanol solutions of the surrogate internal standards (SIS) (≈40 ng of each of the four deuterium-labelled alkylphenols).

Ten milliliters of DCM were added to the test tube and the samples were homogenised for 1 min at 20,000 rpm using a Virtis Tempest LQ^{2}500 w homogenizer (NY, USA). The homogenisation was repeated twice with 10 ml of DCM in two new 25 ml test tubes. To remove water from the extract, 1–2 g of Na_2SO_4 (activated overnight at 400 °C) was added to all the test tubes. The extracts were filtered through glass filter funnels (Duran, porosity 4) into two new 25 ml test tubes. The total volume of 30 ml was reduced to 4 ml by a gentle N_2 flow at 40 °C on a Turbovap LV evaporator (Zymark, USA) before GPC.

2.5. GPC clean-up

The GPC clean-up was done on systems from Gilson (Gilson 232 autoinjector, injector Gilson 401 dilutor, Gilson 202 fraction collector, Gilson, France) and Pharmacia (LKB 2150 HPLC pump, LKB 2252 LC controller, LKB 2144 fluorescence detector, Pharmacia LKB, Sweden). Two GPC columns from Waters (Envirogel GPC cleanup 300 mm × 19 mm) were used. These GPC columns are often used in series to gain a better separation. We have instead coupled the two columns together using Gilson 232 autoinjector as a switch vent as shown in Fig. 1. This setup permits to separate and discard most of the lipids before the sample enters the second column and thus get a much better clean-up of the samples on the second column. At first, the sample is injected by the autoinjector needle (2 ml sample loop). The elution was performed with DCM at a flow of 5 ml/min. After the injection, the needle is used as a canal for waste from the first column. After 9.4 min the valve is switched and the eluate from the first column is sent consequently to the second column, detector and the fraction collector. The needle is washed with 10 ml DCM and the next injection is prepared and ready 31 min after the start of the first sample's analysis.

Table 1
Name, SIM mass (M_r), retention times and corresponding peak numbers (see Figs. 6 and 8) of all the phenols and the recovery standard

Peak no.	Name	M_r	Retention time (min)
1	Pentafluorobenzophenone (RIS)	272	29.22
2	Phenol-d_5 (SIS)	293	30.49
3	Phenol	288	30.59
4	*p*-Cresol-d_8 (SIS)	309	34.57
5	*o*-Cresol	302	33.49
6	*m*-Cresol	302	34.35
7	*p*-Cresol	302	34.75
8	2,4-Dimethylphenol-d_3 (SIS)	319	37.35
9	Unknown	319	39.16
10	Unknown	319	39.65
11	2-Ethylphenol	316	35.92
12	2,6-Dimethylphenol	316	36.52
13	2,5-Dimethylphenol	316	37.05
14	2,4-Dimethylphenol	316	37.39
15	3-Ethylphenol	316	37.55
16	3,5-Dimethylphenol	316	37.82
17	4-Ethylphenol	316	38.31
18	2,3-Dimethylphenol	316	38.36
19	3,4-Dimethylphenol	316	39.37
20	4-*n*-Propylphenol-d_{12} (SIS)	341	41.37
21	2-Isopropylphenol	330	37.06
22	2-*n*-Propylphenol	330	38.53
23	3-Isopropylphenol	330	39.28
24	2,4,6-Trimethylphenol	330	40.16
25	4-Isopropylphenol	330	40.45
26	3-Ethyl-4-methylphenol	330	40.71
27	2,3,6-Trimethylphenol	330	41.26
28	2,3,5-Trimethylphenol	330	41.59
29	4-*n*-Propylphenol	330	41.63
30	2-*tert*.-Butylphenol	344	39.89
31	3-*tert*.-Butylphenol	344	41.20
32	5-Isopropyl-3-methylphenol	344	42.16
33	4-*tert*.-Butylphenol	344	42.88
34	4-*sec*.-Butylphenol	344	43.55
35	4-Isopropyl-3-methylphenol	344	44.07
36	4-*n*-Butylphenol	344	45.24
37	2-*tert*.-Butyl-4-methylphenol	358	42.84
38	2-*tert*.-Butyl-6-methylphenol	358	42.93
39	4-*tert*.-Butyl-2-methylphenol	358	44.69
40	4-(1,1-Dimethylpropyl)phenol	358	46.56
41	4-*n*-Pentylphenol	358	48.58
42	4-*n*-Nonylphenol-d_4 (SIS)	418	59.58
43	2,6-Diisopropylphenol	372	42.28
44	2,5-Diisopropylphenol	372	44.22
45	2-*tert*.-Butyl-4-ethylphenol	372	45.40
46	4-*n*-Hexylphenol	372	51.86
47	4-(1-Ethyl-1-methylpropyl)-2-methylphenol	386	50.99
48	4-*n*-Heptylphenol	386	55.00
49	4-*tert*.-Octylphenol	400	53.05
50	4-*n*-Octylphenol	400	57.67
51	2-Methyl-4-*tert*.-octylphenol	414	53.86
52	4-*n*-Nonylphenol	414	59.61
NP 1	Tec. NP. 1	414	53.27
NP 2	Tec. NP. 2	414	54.10
NP 3	Tec. NP. 3	414	54.32
NP 4	Tec. NP. 4	414	55.27
NP 5	Tec. NP. 5	414	55.69
NP 6	Tec. NP. 6	414	55.85
NP 7	Tec. NP. 7	414	55.94

Table 1 (*Continued*)

Peak no.	Name	M_r	Retention time (min)
NP 8	Tec. NP. 8	414	56.02
NP 9	Tec. NP. 9	414	56.13
NP 10	Tec. NP. 10	414	56.25
NP 11	Tec. NP. 11	414	56.35
NP 12	Tec. NP. 12	414	56.51
NP 13	Tec. NP. 13	414	56.66
NP 14	Tec. NP. 14	414	56.80
NP 15	Tec. NP. 15	414	56.99
NP 16	Tec. NP. 16	414	57.08
NP 17	Tec. NP. 17	414	57.17
NP 18	Tec. NP. 18	414	57.26
NP 19	Tec. NP. 19	414	57.42

The phenols are sorted according to their molecular mass and after the corresponding internal standard (SIS) that head up each respective group of analytes. RIS is recovery internal standard.

Table 2
Instrumental conditions for GC–MS, GC–FID and GC–ECD analysis

Gas chromatograph	HP 5890 A with flame ionisation detector
Column	25 m × 0.25 mm i.d., 0.2 μm film thickness, CP-WAX 52 CB from Chrompack
Injection	HP-7673A autosampler. 1 μl, splitless (2 min), 280 °C
Oven temperature program	90 °C (2 min)–160 °C (0 min) at 10 °C/min; 160 °C (0 min)–225 °C (12 min) at 3 °C/min; 225 °C (12 min)–240 °C (5 min) at 10 °C/min
Carrier gas	Helium, 1.0 ml/min
Detector temperature	325 °C
Gas chromatograph	HP 6890 with electron-capture detector
Column	60 m × 0.25 mm i.d., 0.25 μm film thickness, DB5 from J&W Scientific (Folsom, CA, USA)
Injection	HP-7673A autosampler. 1 μl, pulse splitless (2 min), 280 °C
Oven temperature program	40 °C (2 min)–110 °C (0 min) at 10 °C/min; 110 °C (0 min)–250 °C (15 min) at 3 °C/min
Carrier gas	Helium, 1.0 ml/min
Detector temperature	320 °C
Detector gas	Nitrogen 60 ml/min
Gas chromatograph	HP 6890 with 5973 mass-selective detector
Column	50 m × 0.2 mm i.d., 0.33 μm film thickness, DB5 MS from J&W Scientific
Injection	HP-7673A autosampler. 1 μl, pulse splitless (50 p.s.i., 2 min); 1 p.s.i. = 6894.76, 250 °C
Oven temperature program	40 °C (2 min)–110 °C (0 min) at 10 °C/min; 110 °C (0 min)–250 °C (0 min) at 3 °C/min; 250 °C (0 min)–300 °C (10 min) at 10 °C/min
Carrier gas	Helium, 1.0 ml/min
Interphase temperature	325 °C (interface), 150 °C (ion-source), 150 °C (quadrupole)
Chemical ionisation	Methane (40%, HP default value)

The alkylphenol fraction was collected at 19.4–28.4 min in two 25 ml glass tubes (22.5 ml in each tube). Following the GPC, the eluates were concentrated on a Turbovap LV evaporator to 4 ml. The contents of one tube was then transferred to the other tube, the first tube washed with 2 ml × 1 ml DCM and the solvent further evaporated down to 1 ml DCM. Since DCM may damage the GC column, 1 ml isooctane was added and the volume reduced to 1 ml to achieve the solvent exchange. It is highly important at this stage that the samples do not evaporate to dryness (see Section 3).

The performance of the GPC columns was controlled by weekly running a standard solution of soya oil (20 mg/ml), 4-*n*-nonylphenol (13 μg/ml) and phenol (11 μg/ml). The area and retention times of each compound were registered continuously. The system was very stable and no significant drift of the retention times has yet been observed after two years of using the same two columns.

2.6. Derivatisation

The optimisation of the derivatisation method is described in detail by Boitsov et al. [64]. The derivatisation was done in the same 25 ml test tubes that the sample was collected in after GPC. Two milliliters of 1 M $NaHCO_3$ and 1 ml of 1 M NaOH were added to the 1 ml of isooctane extract. After 1/2 min shaking (Retsch mixer) 2 ml of hexane and 50 μl of pentaflourobenzoyl chloride (10% solution in toluene) were added and the test tubes were shaken violently for 1 min. The samples were left for 30 min at room temperature. Then 8 ml of 1 M NaOH was added for hydrolysis of the excess of

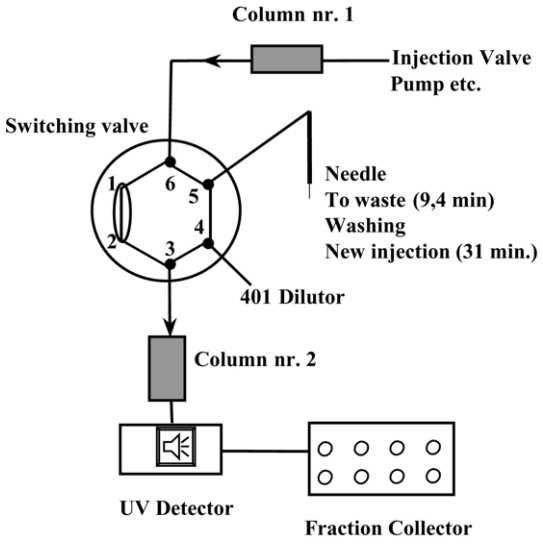

Fig. 1. Schematic setup of the two GPC columns and the switch valve.

derivatisation agent and the samples were put in a refrigerator overnight. The derivatives were extracted with 2 ml × 2 ml of hexane and transferred to GC vials.

The derivatisation step also functions as a secondary clean-up step by saponification of the lipids remaining after GPC. To study the total amount of lipids in the final extract, hexane was evaporated from the extract, fatty acids internal standard (19:0) was added and after methylation the fatty acids methyl esters were analysed on GC–FID. Likewise, after derivatisation the saponificated fatty acids in NaOH fraction were analysed by acidification with HCl, extraction with hexane and transesterfication as described below.

2.7. Determination of elution profile after GPC clean-up

Two test solutions of 14 alkylphenols (C_0–C_8, 0.9–1.4 μg/ml), one with cod liver lipids (151 mg/ml) and one without, were made in DCM. The cod liver lipids were extracted from cod liver by a modified Folch method [65]. These two solutions were run on the GPC clean-up systems and 11 fractions were collected between 5.5 min and 14 min. The first fraction of 17.5 ml was taken at 5.5–9 min and afterwards 10 fractions of 2.5 ml were collected at a rate of two fractions per minute. 0.5 ml from each fraction was taken for lipid analysis and, similarly, 0.1 ml was taken for alkylphenol analysis.

The lipids were analysed by transesterfication of all fatty acids followed by GC–FID analysis of the fatty acids methyl esters according to Joensen et al. [66]. The fatty acids were quantified by using the fatty acid 19:0 as internal standard.

The alkylphenols were converted to their pentafluorobenzoyl derivatives and analysed by GC–ECD. The alkylphenols were quantified by using the pentafluorobenzoate derivative of 4-isopropyl-3-methylphenol added after the derivatisation as a recovery standard. It is only the pentafluorobenzoyl group that contributes to the electron-capture effects of the derivatives and because of that the quantification is corrected for the difference in molecular weight of the target alkylphenols and 4-isopropyl-3-methylphenol (IPMP), the amount of target alkylphenols (X) being calculated from Eq. (1):

$$\mathrm{AM(X)} = \frac{\mathrm{A(X)}}{\mathrm{A(IPMP)}} \times \frac{\mathrm{AM(IPMP)}}{M_\mathrm{r}\mathrm{(IPMP)}} \times M_\mathrm{r}\mathrm{(X)} \quad (1)$$

where A is area from GC–ECD, AM the amount in ng and M_r is molecular mass in g/mol.

The approach of adding an already derivatised internal standard after derivatisation is only used for GC–ECD and only for evaluation of the GPC elution profile. Normally, real internal standards (deuterium-marked alkylphenols) are added before any treatment of the samples and therefore derivatised together with the target compounds, the quantification being done using empirical response factors. Mass spectrometry is then used to uniquely identify the deuterium-labelled materials.

2.8. Method validation procedure

The validation was done with samples of liver (high lipid contents) and muscles (low lipid contents) from Atlantic cod (*Gadus morhua*). The samples were spiked with a standard solution containing 44 alkylphenols. Validation plan is given in Table 3. We studied the linearity of the recovery from approximately 1 μg/kg to 160 μg/kg and the reproducibility at three levels for liver and at one level for muscles. The limit of quantification (LOQ: $Y = Y_\mathrm{B} + 10\mathrm{S.D.}_\mathrm{B}$) and the limit of detection (LOD: $Y = Y_\mathrm{B} + 3\mathrm{S.D.}_\mathrm{B}$), where Y_B is the response of blank sample signal and $\mathrm{S.D.}_\mathrm{B}$ is the standard deviation of the blank samples, were calculated from a triplicate of blank samples. The blank samples have been through all analytical steps (extraction, GPC clean-up, etc.) but contained only pure solvent without any biota.

The analytical variation in each step in the analytical procedure was tested by analysing five replicates (≈10 ng of each of the alkylphenols). We tested the GC–MS performance (analysing the same sample five times), the derivatisation step, the evaporation of the solvent (the standard was added to 20 ml DCM and the solvent volume was reduced

Table 3
Validation plan

Liver (ng)	Muscle (ng)
3 × Blank	
1	1
2.5	2.5
5 × 5	5 × 5
10	10
5 × 20	20
40	40
5 × 80	80
160	160

Spiking of 1 g of cod liver and muscle samples with 44 phenols. The number of replicates and the approximate amount of each phenol are shown.

as described in the method), the GPC clean-up, and the total analysis.

2.9. Testing of 4-nonylphenol (4-NP) contents in plastic and rubber products used for the analysis

All plastic and rubber laboratory equipment used for the analysis was controlled for 4-NP contents. This was done by soaking pieces of plastic/rubber (0.5–1 g) in 2 ml of hot (60 °C) hexane for 2 h, followed by derivatisation and GC–NCI-MS. The test was not quantitative, but a qualitative measurement of whether or not the plastic contains 4-NP. Two different amounts of each sample was extracted.

3. Results and discussion

3.1. GPC clean-up

The chromatograms of elution of lipids and alkylphenols through one or two GPC columns are given in Fig. 2A–C and Fig. 3A and B.

The elution profile of lipids and three representative alkylphenols (4-*n*-octylphenol, 4-*n*-butylphenol and *p*-cresol) (Fig. 4) is established by analysing the different collected fractions shown in Fig. 3A.

GPC systems are very efficient for separating large amounts of lipids from small organic molecules in trace analysis. Most often, "home-packed" glass columns with SX-3 Bio-Beads material are used. These columns can be loaded with higher amounts of lipids (up to 3 g of fat) [67] than the commercial GPC columns (such as Envirogel), but the separation capacity is considerably lower and there is often a need for a secondary clean-up step after GPC to remove the rest of the lipids [68]. Datta et al. [45] examined two different column lengths with SX3-Bio-Beads and found that both failed to separate nonylphenol (NP) and octylphenol (OP) from fish lipids. Bennet and Metcalfe [37] and Snyder et al. [43] used silica gel adsorption chromatography after GPC

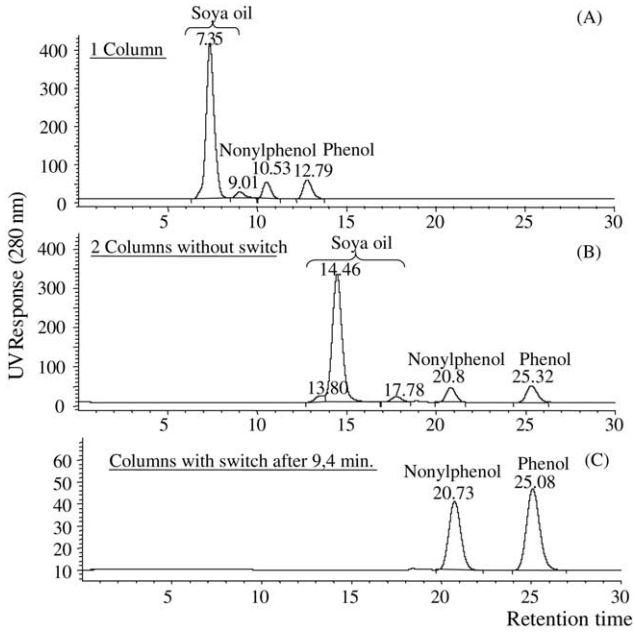

Fig. 2. GPC chromatogram of a DCM standard solution with soya oil (20 mg/ml), 4-*n*-nonylphenol (13 μg/ml) and phenol (11 μg/ml) eluted from one column (A), two columns without switching (B) and two columns with switching from column 1 to column 2 after 9.4 min (C). Time scale in min.

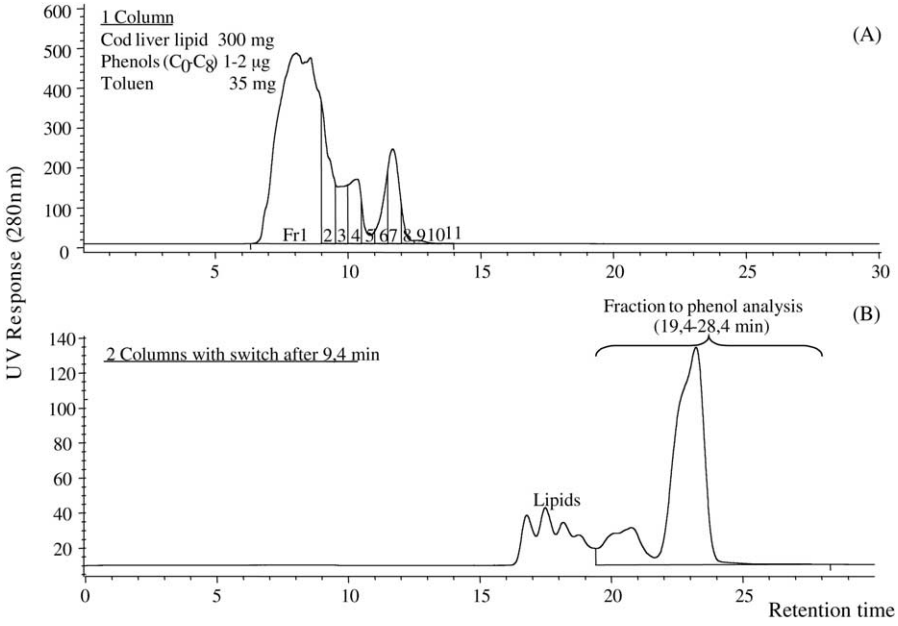

Fig. 3. GPC chromatogram of cod liver lipids and phenol solution. Fractional collection after elution from one column (A) and two columns (B). Note that the big peaks last in both chromatograms are not due to phenols (these are present in concentrations below the UV detection limit) but are due to toluene used as solvent for the spike solutions. Time scale in min.

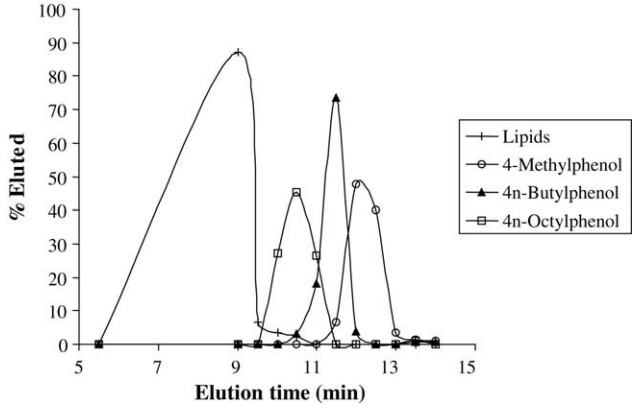

Fig. 4. GPC elution profiles of cod liver lipids (analysed as fatty acid methyl esters) and three representative phenols: *p*-cresol, 4-*n*-butylphenol and 4-*n*-octylphenol, after one column. The results are given as % of total amount.

when cleaning up samples from semipermeable membrane devices (SPMDs), mussels and whole fish homogenates for NP analysis. Dodo and Knight [53] achieved good recovery when analysing a large variety of organic pollutants (including low-mass alkylphenols) from 200 mg of fish lipids by using three polydivinylbenzene columns in series. They report coelution of the target compounds and free fatty acids not causing, however, any interference during GC–MS analysis. The switching between columns used in our method gives a better efficiency compared with having columns coupled in series. Fig. 2 shows good separation capacity between the lipids and alkylphenols after an injection of 40 mg of soya oil and 20 μg of alkylphenols. There is also full baseline separation between the two alkylphenols, nonylphenol (C_9) and phenol. It is important to note that proper switching time is dependent on the lipid matrix. Fig. 5 shows octylphenol as an example. Together with lipids, octylphenol is eluted 0.5 min later and in a broader peak than without lipids.

We found that 300 mg of lipids represented the maximum limit for getting a satisfactory switch point from column No. 1 to No. 2. By discarding the lipid fraction 9.4 min after start, before switching to the other column, only 10% of the orig-

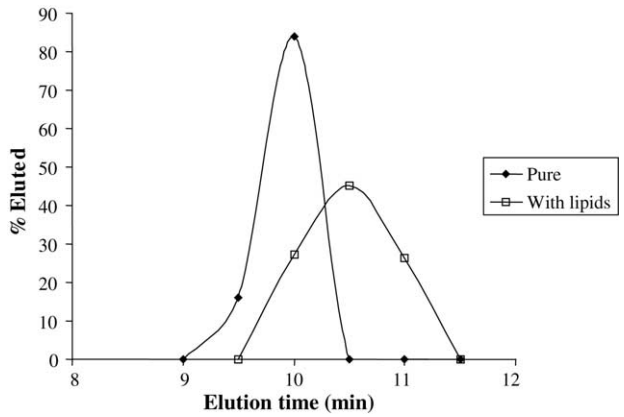

Fig. 5. GPC elution profiles (% of total amount) of 4-*n*-octylphenol with or without lipids.

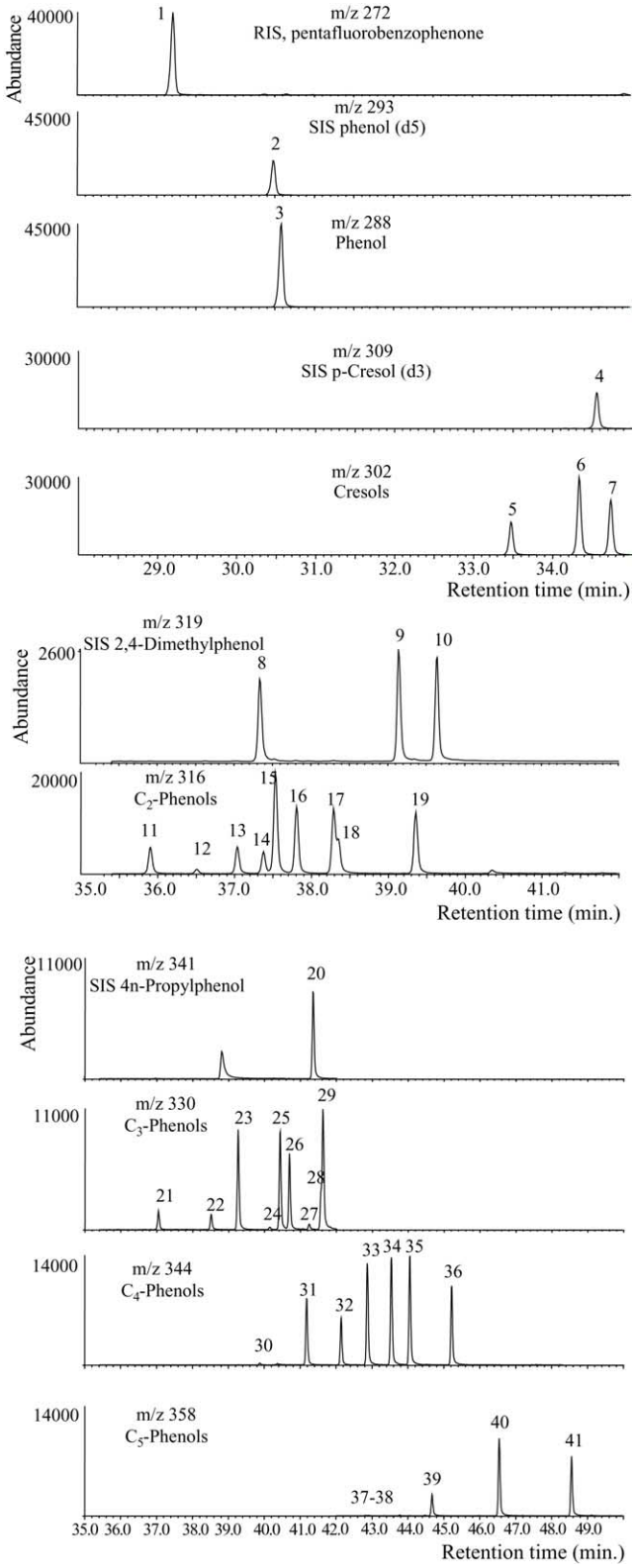

Fig. 6. GC–NCI-MS-SIM of one of the validation samples: 1 g of cod liver spiked with approximately 50 ng of each of the phenols. The numbers over the peaks correspond to the numbers listed in Table 3.

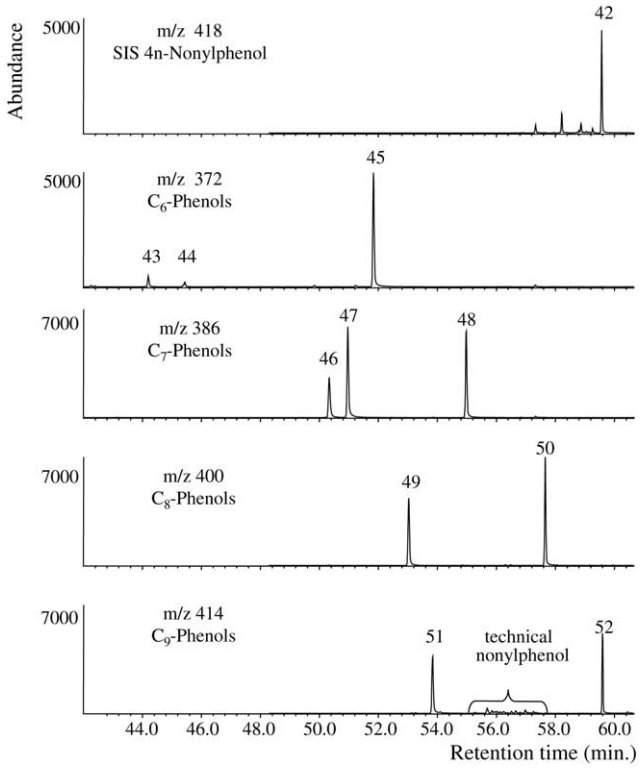

Fig. 6. (*Continued*).

inal lipids (Fig. 3) are let through to column No. 2. This gives a much better sample clean-up than could have been achieved by having the columns in series. In the proposed setup more than 98% of the lipids are removed by GPC. The nature of lipids from the animal tissue sets the limit of how much of the lipids are removed by GPC. Cod liver contains 1% of free fatty acids which have molecular masses (M_r 228–366) lying in the same range as the target compounds and are therefore expected to be eluted in the same fraction. For samples containing 300 mg of lipids this means that approximately 3 mg of lipids in the alkylphenol fraction may be expected. We found 0.7 mg fatty acids eluting in the alkylphenol fraction. The saponification step in the derivatisation procedure removes 20% of these remaining lipids and the final extract only contains 0.5 mg fatty acids. Thus, GPC clean-up and saponification remove more than 99.5% of the original amount of lipids and there was no further need for clean-up before successfully analysing the samples on GC–MS.

Some interference was still experienced during the GC–ECD analysis, but no matrix problems occurred when the samples were run on GC–NCI-MS. The chromatograms in Fig. 6 show how "clean" the samples appear in GC–NCI-MS. Only two large peaks (*m*/*z* 319) and a few small peaks (*m*/*z* 418) were not identified as the alkylphenols added in the spiking experiment, cf. Table 1. Full mass spectra scan of the concentrated samples showed that none of the unknown peaks were derivatives of alkylphenols.

3.2. The analytical variation

Fig. 7 shows the total recovery and the variation in each major analytical step for *p*-cresol, 4-*n*-butylphenol and 4-*n*-octylphenol, calculated from GC–NCI-MS response relatively to RIS (pentafluorobenzophenone). Response factors (RFs) were calculated as the average RF from six replicated derivatisations. The two first bars in Fig. 7, referring to chromatography and derivatisation, therefore represent 100% recovery. Fig. 7 shows how the analytical variation of the recovery grows from R.S.D. < 1.5% for the pure chromatographic performance and R.S.D. < 2.5% for the derivatisation step to R.S.D. between 4.6 and 10.2% for the reproducibility of the recovery in complete analysis. This test reveals that the solvent volume reduction and the GPC clean-up are the most critical steps in the analysis. For *p*-cresol there is a loss of 15% in the volume reduction step, 10% and 8% more are lost in the GPC clean-up and the extraction step respectively. The small molecules (phenol to C_3-alkylphenols) have a lower total recovery as compared to heavier alkylphenols. The total recovery of *p*-cresol is 67%, whereas 4-*n*-butylphenol and 4-*n*-octylphenol have a total recovery close to 90%.

The sample volume reduction was identified as one of the most critical steps of sample preparation. Evaporation to dryness was tested and it was seen that evaporation for only 5 min after drying resulted in a severe loss of AP. Thus, more than 90% of *p*-cresol was lost, while in case of 4-*n*-butylphenol and 4-*n*-octylphenol the loss was approximately 50%. Similar problems with loss of analyte during solvent evaporation are discussed by Gunther et al. [40] and Petrovic et al. [69]. The semi-volatile nature of alkylphenols implies that extreme care should be taken during sample volume reduction, while evaporation to dryness (used in the majority of the methods described in the literature) should if possible be avoided.

The pentafluorobenzoyl derivatisation method is highly reproducible and quantitative down to at least 0.1 ng alkylphenol (smallest total amount tested). The GC–NCI-MS analysis has the same high selectivity in detection as GC–ECD because only halogens and other electrophilic compounds give rise to negative ions. Together with the selectivity obtained with SIM, this makes NCI analysis of pentafluorobenzoyl derivatives an extremely sensitive and selective method with a linear detection from as low as 30 fg/μl to 1200 pg/μl [64].

3.3. Method validation

The recovery and reproducibility for the spiking experiment with both cod liver and cod muscle are given in Table 4 (represented by *p*-cresol, 4-*n*-butylphenol and 4-*n*-octylphenol).

Table 5 shows the limits of quantification (LOQ: $Y = Y_B + 10S.D._B$) and limits of detection (LOD: $Y = Y_B + 3S.D._B$) for 44 alkylphenols as calculated from a triplicate of blank samples. For the alkylphenols that are not found in blank samples the detection limits are defined as

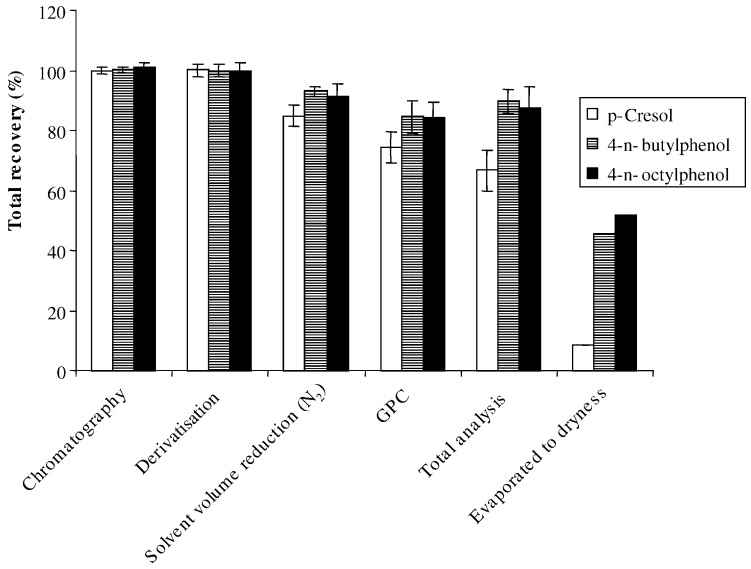

Fig. 7. Total recovery of *p*-cresol, 4-*n*-butylphenol and 4-*n*-octylphenol calculated relative to RIS (pentaflurobenzophenone).

Table 4
Results of the spiking experiment with liver and muscle samples represented by *p*-cresol, 4-*n*-butylphenol, 4-*n*-octylphenol

	Spiked amount (ng)	Liver			Muscle		
		Measured amount (ng)	R.S.D. (%)	Recovery (%)	Measured amount (ng)	R.S.D. (%)	Recovery (%)
p-Cresol							
Blank (3×)	–	3.6 ± 1.2	33.2	–			
	0.7	4.4		644	6.1		906
	1.7	10.1		595	7.3		432
5×	3.4	10.6 ± 0.7	6.4	315	8.7 ± 0.3	3.7	257
	6.8	14.2		211	11.3		167
5×	13.5	23.7 ± 1.5	6.3	175	17.2		127
	27.1	35.2		130	31.9		118
5×	54.1	57.7 ± 1.2	2.2	107	56.5		104
	108.3	109.5		101	107.1		99
4-*n*-Butylphenol							
Blank (3 ×)	–	0.0					
	0.9	0.8		91	1.0		112
	2.2	2.5		115	2.2		100
5×	4.3	4.6 ± 0.2	4.9	107	3.9 ± 0.1	1.7	89
	8.7	8.5		98	7.6		87
5×	17.4	16.3 ± 0.3	1.6	94	17.4		100
	34.8	32.0		92	32.7		94
5×	69.5	80.6 ± 2.7	3.4	116	68.0		98
	139.0	152.4		110	140.1		101
4-*n*-Octylphenol							
Blank (3 ×)	–	0.0					
	1.0	0.7		66	1.2		119
	2.5	2.2		90	2.2		87
5×	5.0	4.6 ± 0.2	3.6	91	4.1 ± 0.1	2.9	81
	10.0	9.1		91	8.3		83
5×	20.0	18.5 ± 0.4	2.2	92	17.0		85
	40.1	35.2		88	32.5		81
5×	80.1	70.9 ± 1.2	1.7	88	69.7		87
	160.3	143.6		90	137.4		86

Table 5
The limits of quantification (LOQs) and the limits of detection (LODs), calculated from a triplicate of blank samples

Peak no.	Compound	LOD (μg/kg) $Y = Y_B + 3S.D._B$	LOQ (μg/kg) $Y = Y_B + 10S.D._B$
3	Phenol	47.50	102.45
5	*o*-Cresol	28.01	69.11
6	*m*-Cresol	4.15	8.91
7	*p*-Cresol	7.24	15.68
11	2-Ethylphenol	0.24	0.70
15	3-Ethylphenol	–	–
17	4-Ethylphenol	–	–
12	2,6-Dimethylphenol	–	–
13	2,5-Dimethylphenol	–	–
14	2,4-Dimethylphenol	–	–
18	2,3-Dimethylphenol	0.39	1.08
16	3,5-Dimethylphenol	–	–
19	3,4-Dimethylphenol	–	–
21	**2-Isopropylphenol**	**0.30**	**0.82**
23	3-Isopropylphenol	0.24	0.65
25	4-Isopropylphenol	0.43	1.27
22	2-*n*-Propylphenol	**0.14**	**0.40**
29	4-*n*-Propylphenol	0.08	0.23
26	3-Ethyl-4-methylphenol	0.07	0.19
24	**2,4,6-Trimethylphenol**	–	–
27	**2,3,6-Trimethylphenol**	–	–
28	**2,3,5-Trimethylphenol**	–	–
30	**2-*tert.*-Butylphenol**	–	–
31	3-*tert.*-Butylphenol	–	–
33	4-*tert.*-Butylphenol	0.55	1.62
34	4-*sec.*-Butylphenol	–	–
32	5-Isopropyl-3-methylphenol	–	–
35	4-Isopropyl-3-methylphenol	–	–
36	4-*n*-Butylphenol	–	–
37	**2-*tert.*-Butyl-4-methylphenol**	–	–
38	**2-*tert.*-Butyl-6-methylphenol**	–	–
39	**4-*tert.*-Butyl-2-methylphenol**	–	–
40	4-(1,1-Dimethylpropyl)phenol	–	–
41	4-*n*-Pentylphenol	–	–
43	**2,6-Diisopropylphenol**	–	–
44	2,5-Diisopropylphenol	–	–
45	**2-*tert.*-Butyl-4-ethylphenol**	–	–
46	*n*-Hexylphenol	–	–
48	4-*n*-Heptylphenol	–	–
47	**4-(1-Ethyl-1-methylpropyl)-2-methylphenol**	–	–
49	4-*tert.*-Octylphenol	2.35	6.35
50	4-*n*-Octylphenol	–	–
51	**2-Methyl-4-*tert.*-octylphenol**	–	–
52	4-*n*-Nonylphenol	2.29	4.72
NP. 1–19	Tec. NP. Val.	10.78	22.72

For the alkylphenols that are not found in blank samples (–) the detection limits are defined as the lowest concentration used in the validation experiment, approximately 1 μg/kg. Peak numbers correspond to the chromatogram in Fig. 8 and Table 3 (*ortho*-substituted alkylphenols $\geq C_3$ are shown in bold).

the lowest concentration used in the validation experiment, approximately 1 μg/kg.

The overall picture shows that there is good recovery and high linearity for the *meta*- and *para*-substituted alkylphenols in both spiked liver and muscle samples. The long chain *ortho*-substituted alkylphenols ($\geq C_3$) have, on the other hand, rather low recovery, while for the most sterically hindered ones like 2,6-diisopropylphenol there is nearly no signal at all. The dimethylphenols and ethylphenols are quantified by an *ortho*-substituted internal standard, 2,4-dimethylphenol-d_3, resulting in good recovery (from 78% to 112%) for the *ortho*-substituted isomers, but giving an overestimation for the *meta*- and *para*-substituted alkylphenols (from 146 to 200%). To avoid this problem, 4-ethylphenol-d_{10} is now also used as internal standard for these isomers.

3.4. Matrix effects of p-nonylphenol in plastic and rubber products

Background contamination and detection of analytes in procedural blanks are recognised problems [37,46,60,70,71]. It seems that some alkylphenols are widely spread in most indoor environments [72] and that it is therefore almost im-

possible not to get some degree of detection of the analytes in procedural blanks.

Phenol and *para*-substituted alkylphenols (*p*-cresol, 4-*tert.*-butylphenol and 4-nonylphenol) are monomers in epoxyacrylic polymers that are intensively used in plastics industry [73]. A number of phenolic compounds, particularly 2,6-di-*tert.*-butyl-4-methylphenol (BHT), are used as antioxidants in plastics [74,75], including nonylphenol as tris(nonylphenyl) phosphite (TNPP), a widely used antioxidant [76]. Nonylphenols are found in poly(vinyl chloride) (PVC) wrapping films, gloves and toys in concentrations of about 530–5500 ppm [77] and in rubber products in concentrations of up to 513 ppm [78]. Relatively large amount of 4-nonylphenol (up to 996 ppm) have also been found in medical PVC tubing [79,80]. Nonylphenols were identified as one of the major potential migrants from plastics and rubber; thermoset, polyester [81]; polystyrene [2]; polyethylene [76]; PVC [76,82,83] and rubber [78]. Phenolic antioxidant compounds are also found to migrate into drinking water from polyethylene pipelines [74]. Therefore, care must be taken when using plastic materials during the analysis (gloves, tubes, stoppers, etc.). Even more important is a good and intensive control of procedural blanks. Detecting trace amounts of alkylphenols in blank samples often sets the limits of the analytical method and makes possible the risk of encountering false positive results.

In our work, 4-NP was found in most of the plastic and rubber products used in the lab, including vinyl gloves, rubber stoppers for glass funnels and plastic tubes used for the nitrogen evaporator (Fig. 8). The most significant finding was the large amount of NP in the soft rubber lining of screw caps (made of Bangalite, Kimble-Kontes, USA) used during the extraction (Fig. 8E). Separate PTEE liners were used inside the screw caps, but leakage may occur and this is believed to be the explanation of finding clusters of 4-NP on two occasions in a series of linearity experiments (only in one sample out of 20 identical ones). We tested a new type of screw caps (made of polyethylene, Kimble-Kontes, USA), but these also contained 4-NP. No cover except aluminium foil is used now for glass tubes before derivatisation is finished. However, despite a significant effort to avoid these problems, we still detect phenol, cresols, 4-*tert.*-butylphenol, 4-*tert.*-octylphenol

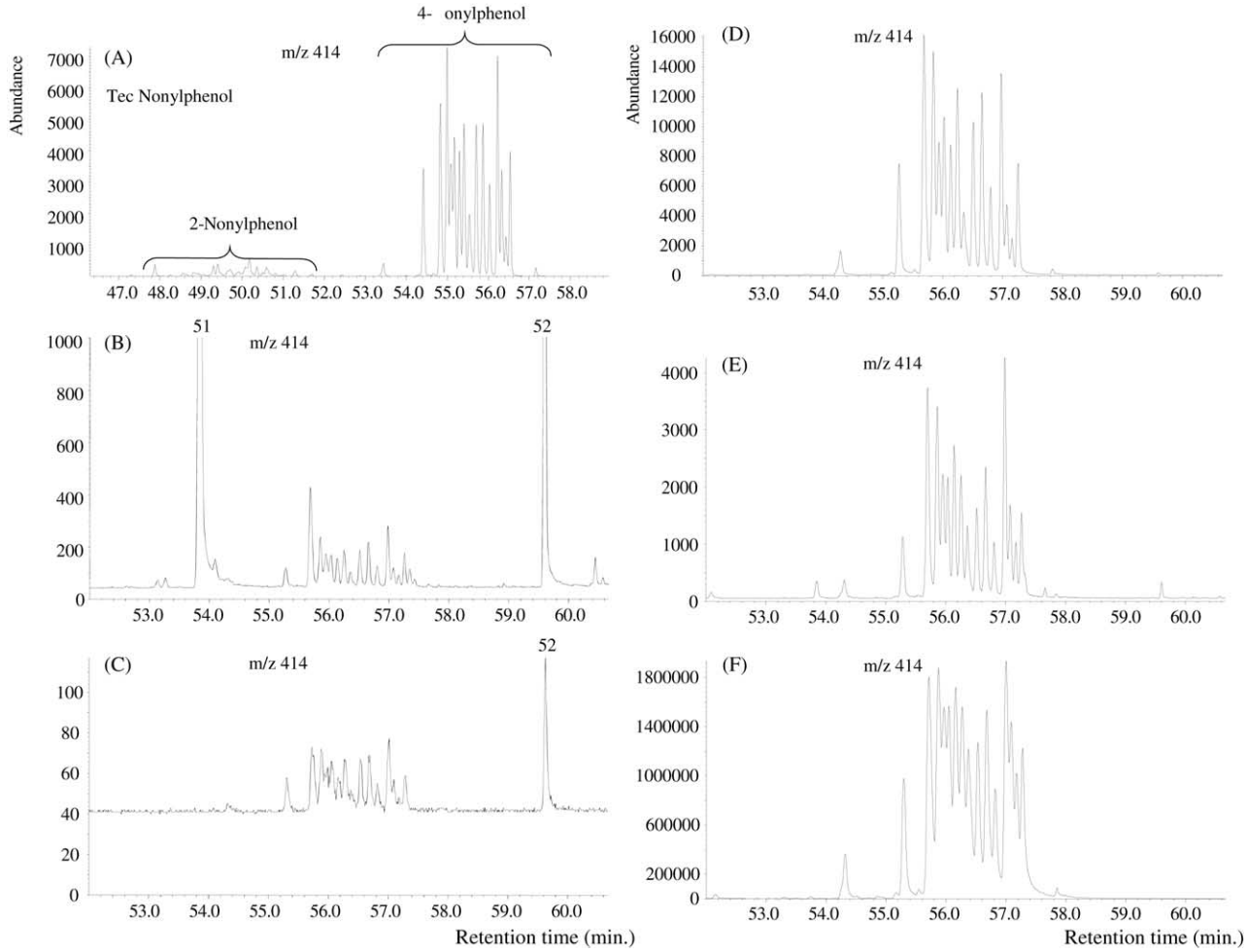

Fig. 8. GC–NCI-MS chromatogram of 4-NP (SIM, *m/z* 414) from: (A) technical nonylphenol; (B) validation samples (enlargement of Fig. 6); (C) blank sample; (D) hexane extract of vinyl gloves; (E) hexane extract of hard plastic from screw caps; (F) hexane extract of soft rubber liner in screw caps.

and 4-NP in blank samples, which results in increasing the LOD for these compounds.

4. Conclusion

A highly sensitive method for the detection of *meta*- and *para*-substituted alkylphenols in fish tissue is described. The method is now used for investigating whether alkylphenols originating from the oil industry are taken up by fish living in the area around the oil installations in the North Sea.

Acknowledgment

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